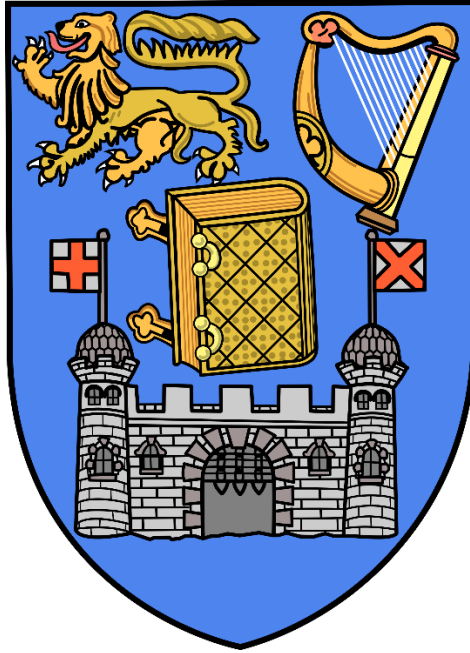


Malonylation of GAPDH in the inflammatory response in macrophages



Thesis submitted to the University of Dublin
for the Degree of Doctor of Philosophy

by

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Declaration

This thesis is submitted by the undersigned. The work herein is entirely my own, with the exception of:

- Figure 3.7: this work was analysed by Steve DeHaro
- Figure 3.13: this experiment was performed by PTM Biolabs
- Figure 3.15A: generated by Alan Nadin
- Figure 4.16 and Figure 4.17: this work was sequenced by George Royal and analysed by Steve DeHaro

Silvia Galván-Peña

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'I won't give up, no I won't give in, until I've reached the end, and then I'll start again' - Zootopia

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Abbreviations

1,3BPG: 1,3 bisphosphoglycerate
2DG: 2-deoxyglucose
2-HG: 2-hydroxyglutarate
3'-UTR: 3' untranslated region
3PG: 3-phosphoglycerate
ACC: acetyl-CoA carboxylase
ACN: acetonitrile
Acox1: acyl-CoA oxidase 1
ACSF3: acyl-CoA synthase family member 3
AMPK: 5' adenosine monophosphate-activated protein kinase
AP1: activating protein
APOL2: apolipoprotein L2
ASC: apoptosis-associated speck-like protein containing CARD
ATP: adenosine triphosphate
BMDMs: bone marrow-derived macrophages
BSA: bovine serum albumin
CBP: p300/CREB binding protein
CCL22: C-C motif chemokine 22
CCR: chemokine receptor
COX2: cyclooxygenase 2
Cp: ceruloplasmin
CPT1: carnitine palmitoyltransferase 1
DAPK1: death associated protein kinase
DCs: dendritic cells
DEPC: diethyl pyrocarbonate
Di: DAPK1 inhibitor
DMEM: Dulbecco's modified eagle medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

dNTPs: deoxyribonucleotide triphosphates

EDC: 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride

EDTA: ethylenediaminetetraacetic

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal regulated kinase

F1,3BP: fructose 1,3 biphosphate

F6P: fructose-6-phosphate

FASN: fatty acid synthase

FBS: fetal bovine serum

FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

G6P: glucose-6-phosphate

GAIT: IFN γ -activated inhibitor of translation

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

GM-CSF: granulocyte-macrophage colony-stimulating factor

HA: heptelidic acid

HIF1 α : hypoxia-inducible factor 1 α

HK: hexokinase

HMG: hydroxymethylglutaryl

IDH: isocitrate dehydrogenase

IFN: interferon

Ig: immunoglobulin

IKK β : I kappa B kinase β

IL: interleukin

iNOS: NO synthase

IP: immunoprecipitation

IRF: interferon regulatory factor

IRG1: immunoresponsive gene protein 1

KO: knockout

LDH: lactate dehydrogenase

LPA: linear polyacrylamide

LPS: lipopolysaccharide

LSD: lysine specific demethylase

MAL: MyD88 adaptor-like protein

malK: malonylated lysine

MCD: malonyl-CoA decarboxylase

MDMs: monocyte-derived macrophages

MLC: myosin light chain

mRNA: messenger RNA

MSD: Meso Scale Discovery

mTORC: mammalian target of rapamycin

MyD88: myeloid differentiation primary response protein 88

NADPH: nicotinamide adenine dinucleotide phosphate

NAG: N-acetylglucosamine

NF- κ B: nuclear factor κ B

NHS: N-hydroxysulfosuccinimide

NLRP3: NLR family pyrin domain containing 3

NO: nitric oxide

NRF2: nuclear factor like 2

NSAP1: NS1 associated protein

P/S: penicillin/streptomycin

PamCSK: Pam₃Cys-Ser-(Lys)₄

PAMPs: pathogen-associated molecular patterns

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PEP: phosphoenolpyruvic acid

PFK: phosphofructokinase

PGC1 β : peroxisome proliferator-activated receptor γ -coactivator 1 β

PGE₂: prostaglandin E2

PHD: prolyl hydroxylase domain

PKM2: pyruvate kinase M2

Poly I:C: polyinosinic:polycytidylic acid

PRR: pattern recognition receptor

PVDF: polyvinylidene fluoride

qPCR: quantitative PCR

R5P: ribose-5-phosphate

RIP: RNA-IP

RNA: ribonucleic acid

ROS: reactive oxygen species

RPML: Rosewell Park Memorial Institute

rRNA: ribosomal RNA

RT-PCR: reverse transcription PCR

SDH: succinate dehydrogenase

SDS-PAGE: sodium dodecyl sulphate – polyacrylamide gel electrophoresis

siRNA: small interfering RNA

SOCS: suppressor of cytokine signalling

SREBP: sterol regulatory element-binding protein

STAT: signal transducer and activation of transcription

succK: succinylated lysine

SUCNR1: succinate receptor

TBS: tris buffer saline

TBTA: tris(benzyltriazolmethyl)amine

TCEP: tris(2-carboxyethyl)phosphine

TFA: trifluoroacetic acid

TGF β : transforming growth factor β

TIR: toll-IL1 resistance

TLR: toll-like receptor

TNF α : tumor necrosis factor α

TRAM: TRIF-related adaptor molecule

TRIF: TIR-domain containing adaptor protein inducing IFN β

tRNA: transfer RNA

UCP2: uncoupling protein 2

VDAC: voltage-gated anion channel

VEGF α : vascular endothelial growth factor α

VLCAD: very long chain acyl-coenzyme A dehydrogenase

ZIPK: zipper-interacting protein kinase

α -KG: α -ketoglutarate

Abstract

Succinylation and malonylation are two recently discovered protein post-translational modifications. They involve the attachment to lysines of metabolism-derived succinyl and malonyl groups respectively, resulting in a change in charge comparable to that of phosphorylation. Given the increasing number of links existing between the metabolic status and the effector function of immune cells, I investigated whether protein succinylation and malonylation might play a role in the activation of macrophages during metabolic reprogramming.

Protein succinylation and malonylation were observed to be induced in murine macrophages following activation with various Toll-Like Receptor (TLR) ligands. I was able to identify via mass spectrometry a wide array of proteins undergoing these post-translational modifications, one of which was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Lipopolysaccharide (LPS) induced malonylation of GAPDH lysine 213, within the catalytic domain of GAPDH. Although it is most widely known for its role as a glycolytic enzyme, GAPDH has blossomed in recent years from its mundane use as an endogenous control into an increasingly interesting multi-functional protein. Through the use of a GAPDH enzymatic activity inhibitor as well as siRNA knockdown, I found that GAPDH activity was needed for the production of cytokines in macrophages, including TNF α , IL6 and IL1 β . However, especially in the case of TNF α , the requirement for GAPDH went beyond its glycolytic activity. Following activation of macrophages with LPS, GAPDH dissociated from TNF α mRNA, enabling its translation. Furthermore, GAPDH enzymatic activity and RNA binding were mutually exclusive, as revealed using heptelidic acid (HA), an inhibitor of enzymatic activity. HA increased the repressory binding of GAPDH to mRNAs, inhibiting TNF α production. A mutant form of GAPDH (GAPDH K213E), which mimicked malonylation was more active enzymatically but less able to bind the mRNA for TNF α . A form that is unable to undergo malonylation, GAPDH K213Q, bound more tightly to the mRNA, but was less enzymatically active.

Overall, these results show that malonylation increased GAPDH enzymatic activity, which is needed for the production of cytokines such as IL1 β and IL10. At the same time, mRNAs bound to GAPDH, including that of TNF α , are released and translated. Therefore, malonylation of GAPDH is an important signal for macrophage activation.

Chapter 1: Introduction

1. Introduction

Innate immunity is one of two arms of the immune system, responsible for starting the inflammatory response, aimed at protecting the host from infection. However, when unregulated, it can also be triggered by host factors and result in autoimmune diseases or chronic inflammation. Understanding the molecular mechanisms responsible for triggering inflammation as well as regulating it is therefore key to the development of effective treatments for inflammatory diseases.

In recent years, the idea of the metabolic status of immune cells controlling their functions has been the focus for much research in immunology. It has been demonstrated that targeting different metabolic enzymes or metabolites can result in effective manipulation of the inflammatory response. In this study, we have identified lysine malonylation, using GAPDH as a substrate, as a new metabolism-derived mechanism used by macrophages to trigger an inflammatory response. We have thus uncovered a new point of communication between metabolic and immune signalling pathways in the cell, that may be used as a target in the development of new anti-inflammatory therapeutics.

1.1 The innate immune system and inflammation

The innate immune system is the first barrier an organism has in place to defend itself from pathogens. The initial detection of an infection is mediated by a set of germline-encoded receptors that are collectively referred to as pattern recognition receptors (PRRs). These comprise a wide range of receptors including Toll-like receptors (TLRs), RIG-like receptors, NOD-like receptors and C-type lectin receptors [1]. Activation of these receptors by pathogen-associated molecular patterns (PAMPs) leads to the production of pro-inflammatory cytokines such as interleukin-1 (IL1), tumor necrosis factor α (TNF α) or interferons (IFNs) among others, which will trigger the initiation of the inflammatory response [2]. The inflammatory response is a very rapid and highly coordinated process that results in the accumulation of immune cells and proteins at the site of infection or tissue damage, the induction of local blood clotting to isolate the infection and ultimately, the repair of the injured tissue. An inflammatory response is usually initiated within hours of the appearance of an infection or wound and is therefore essential for alerting the host and activating downstream systemic defences. A well mounted inflammatory response results in recovery from infection and healing. However, in some cases, this response becomes unregulated and can turn against the host resulting in the development of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease or asthma, among others [3].

1.1.1 Macrophages as key players in inflammation

Macrophages are versatile white blood cells that are part of the first line of defence following tissue injury or infection. They exist in two main forms: as tissue resident macrophages, or as macrophages derived from blood monocytes recruited to the site of infection. Upon activation by PAMPs, they become efficient phagocytic cells responsible for taking up pathogens and cell debris, as well as apoptotic cells [4]. They start by secreting pro-inflammatory mediators such as reactive oxygen species (ROS), nitric oxide (NO), TNF α or IL1, all of which are required for the activation of different anti-microbial mechanisms, including oxidative processes needed for the killing of invading pathogens [5]. They can also produce metalloproteinases which can degrade the extracellular matrix, thus facilitating the recruitment of other pro-inflammatory cells. In addition, they can also activate Th₁ and Th₁₇ as well as other inflammatory cells, through the secretion of chemokines and cytokines such as IL12 and IL23, providing an important link between innate and adaptive immunity [4].

Macrophages are however not only responsible for driving inflammation but also have an important role in its resolution. Once the cause of inflammation is eliminated, the number of pro-inflammatory macrophages diminishes, while the number of macrophages with wound repair and inflammation resolution functions increases. The former are commonly referred to as M1, while the latter as M2 macrophages [6, 7]. M2 macrophages are activated by the cytokines IL4 and IL13, which can be secreted by a variety of cells at the site of infection or tissue damage [8]. They produce growth factors, such as transforming growth factor β (TGF β) that can aid in tissue repair and regeneration, as well as immunoregulatory factors such as the cytokine IL10, that can dampen down inflammation [4]. The balance between M1 and M2 macrophages is tightly regulated, but its alteration can have a major impact on the development of many diseases. For instance, macrophages have been shown to be involved in all stages of cancer, from tumor initiation to progression, through to metastasis [9]. The sustained production of pro-inflammatory cytokines by macrophages is also responsible for many autoimmune disorders. A subset of macrophages has been shown to contribute to the pathogenicity of inflammatory bowel disease through induction of colonic inflammation via the production of TNF α [10]. IFN γ and TNF α -dependent arthritis has also been attributed to the production of IL8 and IL12 by macrophages and dendritic cells (DCs) [11], and macrophage-mediated inflammation has been shown to be a major contributor to axon demyelination in multiple sclerosis [12]. Therefore, progressing our understanding of macrophage biology is a necessity in the fight against many illnesses.

1.1.2 Activation of macrophages through TLRs

TLRs are one of the key mechanisms by which macrophages are activated. They are transmembrane proteins containing a leucine-rich ectodomain responsible for PAMP recognition, and a cytosolic Toll-IL1 resistance (TIR) domain, responsible for triggering downstream signalling pathways [13, 14]. There are ten different TLRs expressed in humans and twelve in mice, with each one detecting distinct PAMPs from a wide range of pathogens including bacteria, viruses, fungi and parasites. TLR2 oligomerizes with TLR1 to recognize triacylated lipopeptides from gram negative bacteria, or with TLR6 to recognize triacylated lipopeptides from gram positive bacteria. TLR5 detects the bacterial product flagellin, whilst TLR4 detects the outer membrane gram negative-derived lipopolysaccharide (LPS) [1]. TLR4 can also recognize fungal-derived mannan groups as well as viral envelope proteins [15, 16]. All the aforementioned TLRs are expressed on the cell surface and as such, are mostly responsible for the detection of microbial membrane components. On the other hand, TLRs 3, 7, 8 and 9 are found within intracellular compartments and are mostly responsible for the detection of nucleic acids. TLR3 recognizes double-stranded RNA, while TLR7 can oligomerize with TLR8 to recognize single-stranded RNA. TLR9 is responsible for the detection of DNA, mostly from viral or bacterial origin, although it can also recognize host DNA [1].

Following PAMP recognition, TLRs recruit a specific set of adaptors that can initiate downstream signalling events leading to the secretion of inflammatory cytokines. TLR4 is the only TLR that recruits all four adaptor proteins: myeloid differentiation primary response protein 88 (Myd88), MyD88 adaptor-like protein (MAL), TIR-domain containing adaptor protein inducing IFN β (TRIF) and TRIF-related adaptor molecule (TRAM) [17]. Following activation, TLR4 can trigger both MyD88-dependent and TRIF-dependent signalling cascades, leading to the activation of a number of transcription factors, including activating protein (AP1), nuclear factor kappa B (NF- κ B) and IFN-regulatory factors (IRFs), which ultimately lead to the transcription of cytokines, type I IFNs, chemokines and anti-microbial peptides [17].

1.2 Metabolic changes in macrophages

It is now well established that the metabolic status of macrophages, as well as other immune cells, is tightly linked to their immune function. Despite a recent surge in immunometabolism studies, early insights into the metabolic status of macrophages date back to more than forty years ago, when Hard showed that activated murine macrophages had higher levels of glycolysis than resting ones [18]. This tight link between metabolism and immune function is exemplified by the clear metabolic differences existing between M1 and M2 macrophages. An M1 macrophage exerts its function within hours to days, as opposed to an M2 macrophage,

which plays a bigger role within the resolution phase and thus has longer term functions. Their metabolism is unsurprisingly a clear reflection of those functions.

In M1 macrophages, aerobic glycolysis is induced upon activation with LPS and IFN γ , which involves an increase in glucose uptake as well as increased levels of glycolytic intermediates. This is required to generate adenosine triphosphate (ATP), needed for many enzymes to function, as well as for the maintenance of the mitochondrial membrane potential, which is required for the cell not to undergo apoptosis [19]. In addition, it also enables cytokine production through the generation of ROS [20]. Furthermore, glycolysis is needed to generate intermediates for the pentose phosphate pathway, which is also induced in M1 macrophages. This pathway is key for the generation of nicotinamide adenine dinucleotide phosphate (NADPH) for the NADPH oxidase, which is important for ROS as well as for NO synthesis [21]. At the same time, the activities of the respiratory chain are attenuated which also allows for ROS production [5]. Altogether, these metabolic events can provide the cell with rapid energy and reducing equivalents, which are required for bactericidal activity (Figure 1.1A).

M2 macrophages on the other hand, activated by the cytokine IL4, have been reported to obtain much of their energy from fatty acid oxidation and oxidative metabolism, which can be sustained for longer. In these macrophages glutamine is needed for the production of α -ketoglutarate (α -KG), which induces transcription of fatty acid oxidation genes through epigenetic reprogramming [22]. Another mechanism by which M2 macrophages are able to induce oxidative metabolism is through 5' adenosine monophosphate-activated protein kinase (AMPK). AMPK is elevated in M2 macrophages, and can increase fatty acid uptake and oxidation in the mitochondria through increased expression of oxidative phosphorylation enzymes, such as peroxisome proliferator-activated receptor γ -coactivator 1 β (PGC1 β) [23]. Other studies have also shown that following activation, M2 macrophages can induce expression of constituents of the electron transport chain that will perform oxidative phosphorylation as well as drive pyruvate into the Krebs cycle [24, 25]. They also induce arginase-1, which results in the production of urea, polyamines and ornithine, all of which are important for the wound healing actions of this macrophage population [26] (Figure 1.1B).

Additionally, recent studies have uncovered that it is not only overall metabolic changes that shape the function of a macrophage, but that metabolites and metabolic enzymes can directly determine a functional output. This is exemplified by the production of the key inflammatory mediator IL1 β . Tannahill *et al* demonstrated that in an LPS-activated macrophage, the Krebs cycle intermediate succinate, accumulates and activates the transcription factor hypoxia-inducible factor 1 α (HIF1 α), which binds to the pro-IL1 β promoter and induces its production [27] (Figure 1.2). This is also enabled by pyruvate kinase M2 (PKM2), which following

macrophage activation with LPS can translocate into the nucleus and directly interact with HIF1 α [28]. Additionally, the pro-form of IL1 β needs to be cleaved by caspase-1, a member of the NLR family pyrin domain containing 3 (NLRP3) inflammasome complex, before being released as mature IL1 β . The activity of the glycolytic enzyme hexokinase-1 is required for both caspase-1-dependent pro-IL1 β cleavage, and thus IL1 β production, as well as NLRP3 activation in response to gram positive bacteria, where hexokinase-1 acts as a cytosolic receptor for N-acetylglucosamine [29, 30]. Another metabolic enzyme that regulates IL1 β production in macrophages is fatty acid synthase (FASN), which is induced by mitochondrial uncoupling protein 2 (UCP2) to activate caspase-1 through NLRP3 [31] (Figure 1.2).

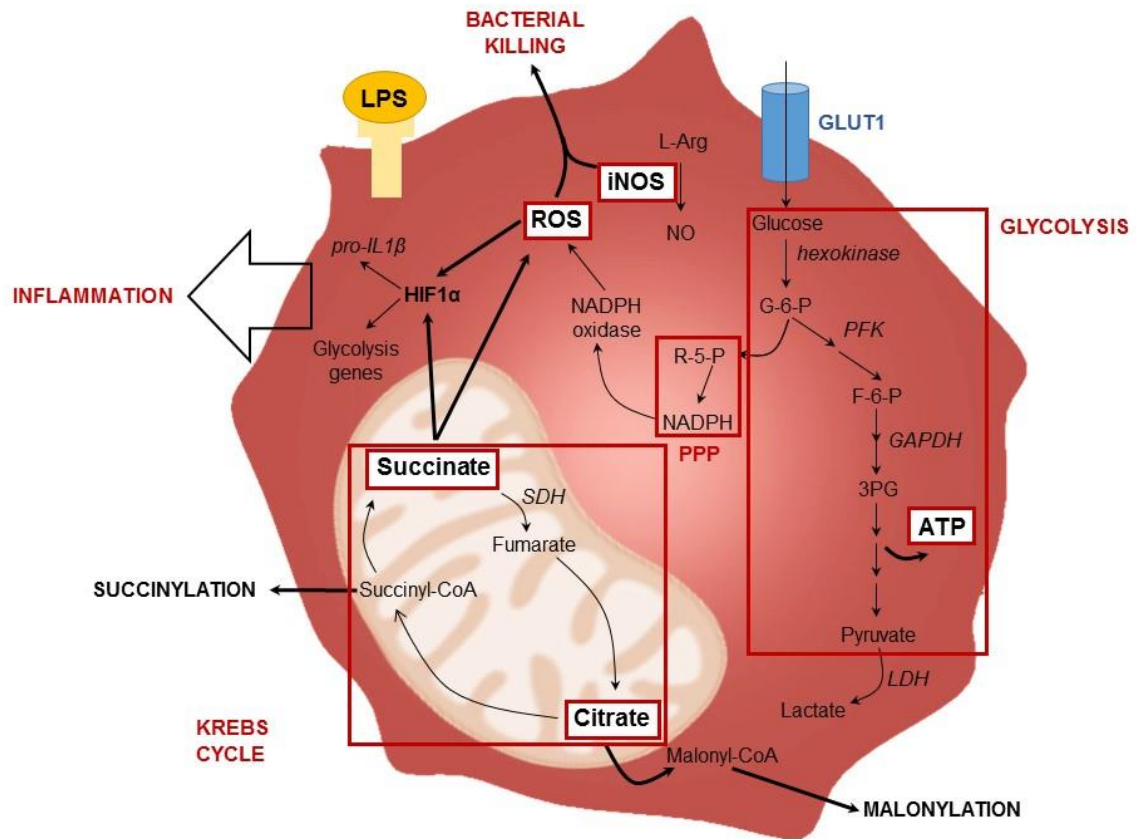
1.2.1 Citrate metabolism

The Krebs cycle in LPS and IFN γ -activated macrophages has been shown to be broken at the level of isocitrate dehydrogenase, the enzyme responsible for the conversion of citrate into α -KG [32]. As a consequence, there is a reduction in α -KG levels as well as a build up of citrate. This α -KG reduction has recently been shown to be enabling of pro-inflammatory cytokine production. In M2 macrophages, α -KG accumulation results in epigenetic reprogramming through Jmjd3 activation, leading to expression of M2-associated genes, while at the same time, suppressing the production of pro-inflammatory cytokines through I kappa B kinase β (IKK β) inhibition [22]. Citrate on the other hand, has been shown to impact the pro-inflammatory phenotype of macrophages in various ways.

Citrate is synthesized in the mitochondria by citrate synthase but it can be exported into the cytosol by the citrate transporter. In the cytosol, it can be converted into oxaloacetate and acetyl-CoA by the ATP-citrate lyase [33]. On the one hand, acetyl-CoA plays a key role in epigenetic modifications, as it is needed for histone acetylation, as well as acetylation of other proteins within the cell. Additionally, acetyl-CoA can be converted into malonyl-CoA by acetyl-CoA carboxylase (ACC), which is required for fatty acid synthesis and thus plays an important role in lipid metabolism [34]. Furthermore, some of the lipids that are synthesized from citrate-derived metabolites include phospholipids, which generate arachidonic acid. Arachidonic acid is then needed for prostaglandins to be synthesized, which act as inflammatory mediators [35]. Oxaloacetate on the other hand, is first reduced to malate and then converted into pyruvate, which can yield NADPH. As previously stated, NADPH is used by the NADPH oxidase to generate ROS, but it is also a cofactor for the NO synthase (iNOS) and thus can also result in NO production [36].

Furthermore, citrate can be converted into cis-aconitate, which in turn is converted into itaconate by the immunoresponsive gene protein 1 (IRG1) enzyme. IRG1 expression is

A.



B.

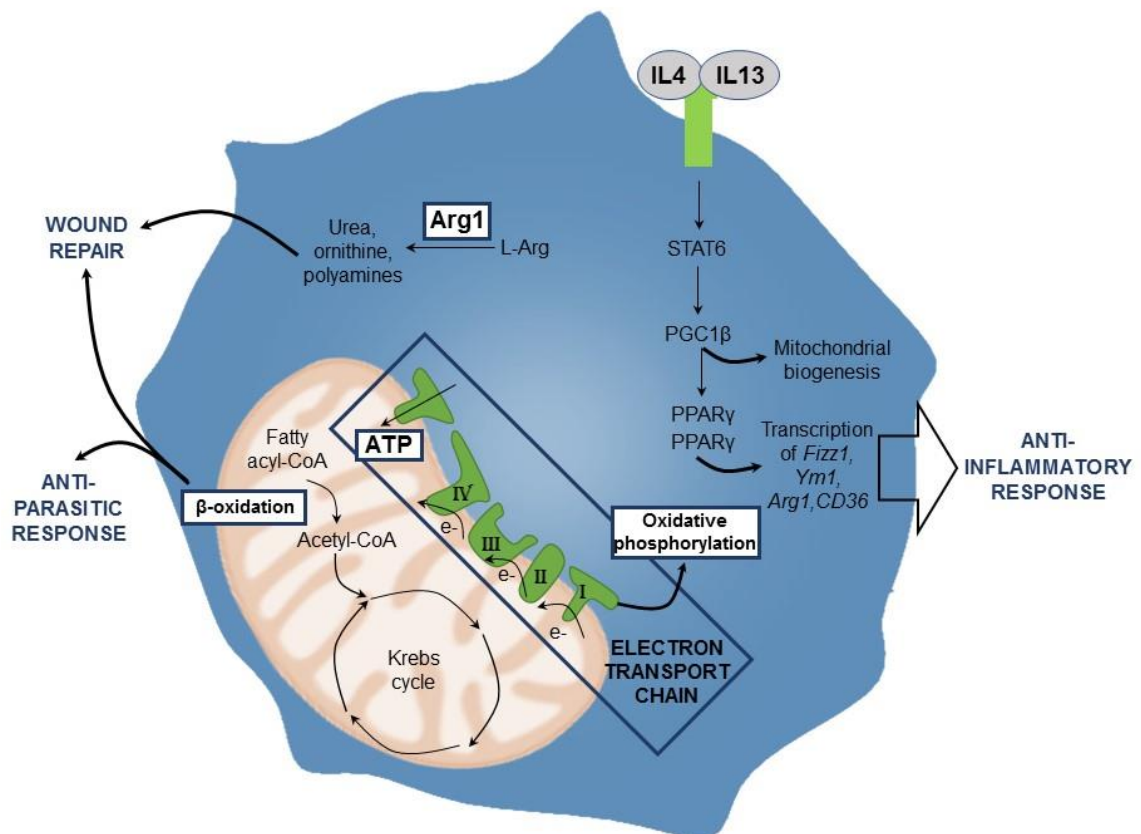


Figure 1.1 Metabolic profile of M1 and M2 macrophages. **A)** LPS-activated macrophages (commonly termed M1 macrophages) induce an aerobic glycolytic program that results in lactate production and increased levels of intermediates of the Krebs cycle. The HIF1 α transcription factor also becomes activated and can drive production of pro-inflammatory cytokines. The key functional consequences are bacterial killing, mostly through the production of ROS and NO, and inflammation, occurring through cytokine production. **B)** IL4-activated macrophages (commonly termed alternatively activated or M2 macrophages) trigger a metabolic program, which includes oxidative phosphorylation as well as fatty acid oxidation, orchestrated by STAT6 and PGC1 β . Arg-1 also drives the production of polyamines and ornithine. The key functional consequences are tissue repair and anti-parasitic responses.

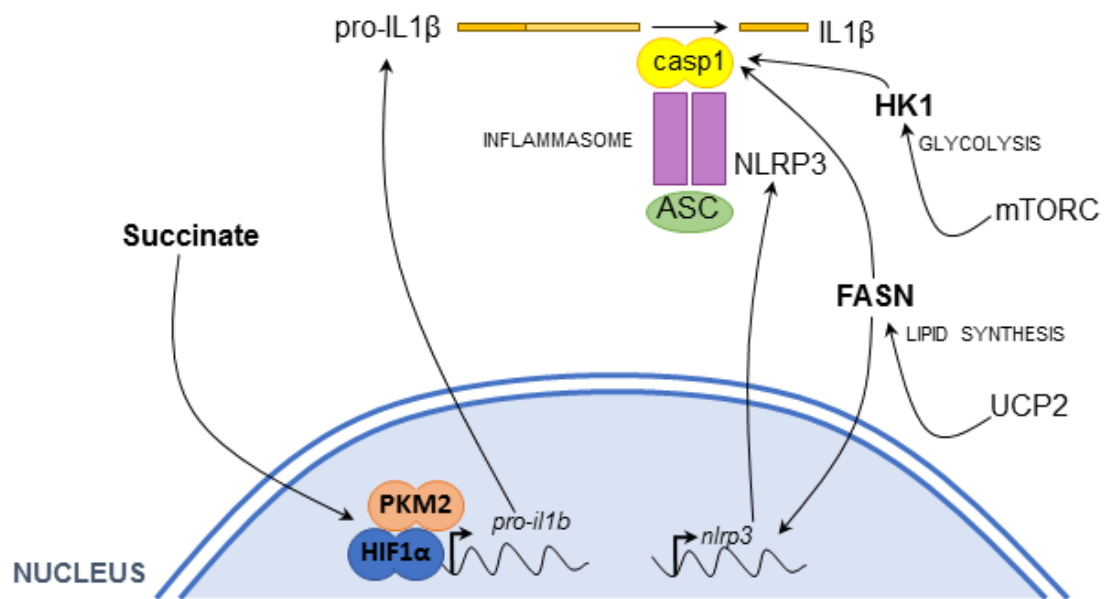


Figure 1.2 Metabolic regulation of inflammasome activation. Various metabolic intermediates and mediators have been shown to regulate the formation of the NLRP3 inflammasome and IL1β production. Succinate and PKM2 can both activate HIF1α to drive transcription of pro-IL1β, while activation of FASN by UCP2 increases NLRP3 transcription as well as caspase-1 processing of pro-IL1β. mTORC1 can also activate HK1 and increase glycolysis, also leading to increase caspase-1 processing of pro-IL1β.

increased in activated macrophages, which leads to an accumulation of itaconate, a metabolite that has been shown to have antimicrobial properties through bacterial isocitrate lyase inhibition [32, 37]. In addition, itaconate has been shown to inhibit LPS-induced cytokine production as well as HIF1 α [38].

1.2.2 Succinate metabolism

Succinate is another Krebs cycle intermediate that has been shown to accumulate in LPS-activated macrophages [27, 32]. It is generated from succinyl-CoA by succinyl-CoA synthase, and can be converted into fumarate by succinate dehydrogenase (SDH). The activity of SDH, which is part of the mitochondrial respiratory chain complex II, has recently been linked to the response against bacteria in macrophages, as well as the production of ROS in an elevated mitochondrial potential setting [20, 39].

One of the key functions of succinate in macrophages appears to be the regulation of cytokine production, boosting pro-inflammatory cytokines, whilst at the same time reducing anti-inflammatory mediators. On the one hand, its oxidation by SDH results in increased ROS production that regulates HIF1 α activity [40]. On the other hand, succinate is also able to directly inhibit prolyl hydroxylase domain (PHD) enzyme activity, which results in stabilization and activation of HIF1 α [27, 41]. This transcription factor plays an important role as one of the key mediator in the adaptation of macrophages to hypoxic conditions, such as those found in inflamed sites. HIF1 α can induce the production of pro-inflammatory cytokines, which includes maintaining the production of pro-IL1 β . It is also responsible for the expression of some of the glycolytic enzymes and glucose transporters [42]. HIF1 α knockout (KO) macrophages also have impaired capacity to clear both gram-positive and gram-negative bacteria, suggesting it also plays a role in bacterial clearance [43].

Succinate can also be secreted and act as a signal on other cells through the GPR91 receptor, also known as the succinate receptor (SUCNR1). This receptor has been shown to have increased expression in LPS-treated macrophages, and appeared to be required for the secretion of IL1 β [44]. Activation of SUCNR1 by succinate leads to calcium mobilization, which can result in NO and prostaglandin E₂ (PGE₂) production [45]. Additionally, in DCs succinate has also been shown to synergize with the TLR3 ligand poly I:C and the TLR7 ligand imiquimod, to increase the production of the pro-inflammatory cytokine TNF α . It can also synergize with LPS to increase production of IL1 β [46]. Furthermore, the secretion of succinate from macrophages, and its detection by SUCNR1, have been shown to be responsible for inflammation in the synovial fluid in a mouse model of rheumatoid arthritis [44].

1.3 Metabolic regulation of post-translational modifications

The key focus of this thesis is post-translational modifications, especially malonylation, as a consequence of metabolic reprogramming in macrophages. Post-translational modifications play an essential role in the regulation of protein activity and function across cellular systems. Despite the different functional impacts of different modifications, they share their origins in metabolic intermediates. For instance, ATP acts as a phosphoryl group donor in phosphorylation, acetyl-CoA is needed for acetylation and nitric oxide is needed for nitrosylation. Furthermore, the last couple of years have seen a significant increase in the discovery of metabolites that result in different post-translational modifications, as shown on Table 1.1. Interestingly, despite the already discussed growing interest in understanding the impact of metabolic rewiring on cellular function, the connection between metabolic changes and post-translational modifications remains a poorly understood area. Recent studies have however found them to be interlinked, especially within the context of epigenetics (Figure 1.3).

Histone lysine acetylation is a key epigenetic modification. It enables increased transcription by changing the positive charge on lysines and thus reducing the tight packaging of chromatin with negatively charged DNA. This results in gene promoter regions becoming readily accessible for transcription factors to bind, which leads to increased transcription [47]. Increasing glucose and acetate levels have been shown to increase intracellular acetyl-CoA levels, which lead to increased histone acetylation [33, 48]. In activated T_{H1} cells, the glycolytic enzyme LDHA is able to maintain glycolysis, which allows citrate to be exported from the mitochondria. Citrate export ensures a steady supply of acetyl-CoA production needed for acetylation of H3K9 at the IFN γ promoter, which results in its increased transcription [48]. In this case, the metabolic status of the cell can directly impact on cytokine production through acetylation. The activity of ATP-citrate lyase, the enzyme responsible for the generation of acetyl-CoA from citrate, has also been found to be needed for histone acetylation to occur [33]. Furthermore, the pyruvate dehydrogenase complex, which can produce acetyl-CoA from pyruvate, has been shown to translocate into the nucleus in a cell cycle-dependent manner. In the nucleus, it can induce transcription of G1-S-associated genes through increased histone acetylation via acetyl-CoA production [49]. Therefore, despite the regulation of histone acetylation by nuclear deacetylases and acetyltransferases, it appears that levels of acetyl-CoA may be the rate-limiting step.

Another key epigenetic post-translational modification is histone lysine methylation. Similarly to histone acetylation, it is regulated by methyltransferases and demethylases, with the latter divided into two main classes: Lysine Specific Demethylases (LSDs) and Jumonji C demethylases (JmjC). JmjC uses the conversion of oxygen and α -KG into succinate, and can

Post-translational modification	Amino acid	Metabolite of origin	Reference
Acetylation	Lysine	Acetyl-CoA	[50]
ADP-ribosylation	Many	ADP-ribose	[51]
Bisphosphoglyceration	Lysine	1,3-bisphosphoglycerate	[52]
Butyrylation	Lysine	Butyryl-CoA	[53]
β -hydroxybutyrylation	Lysine	β -hydroxybutyrate	[54]
Crotonylation	Lysine	Crotonyl-CoA	[55]
Glutarylation	Lysine	Glutaryl-CoA	[56]
Glutathionylation	Cysteine	Glutathione	[57]
Glycosylation	Many	Monosaccharides	[58]
Hypusination	Lysine	Spermidine	[59]
HMGylation	Lysine	HMG-CoA	[60]
Malonylation	Lysine	Malonyl-CoA	[34]
Methylation	Arginine and Lysine	S-adenosyl methionine	[61]
Myristoylation	Glycine	Myristic acid	[62]
Palmitoylation	Cysteine, Serine, Threonine	Palmitic acid	[63, 64]
Phosphorylation	Tyrosine, Threonine, Serine	ATP	[65, 66]
Prenylation	Cysteine	Isoprenoids	[67]
Propionylation	Lysine	Propionyl-CoA	[53]
Polyglutamylation	Glutamate	Glutamate	[68]
S-Nitrosylation	Cysteine	Nitric oxide	[69]
Succination	Cysteine	Fumarate	[70]
Succinylation	Lysine	Succinyl-CoA	[71]

Table 1.1 List of metabolically-derived post-translational modifications

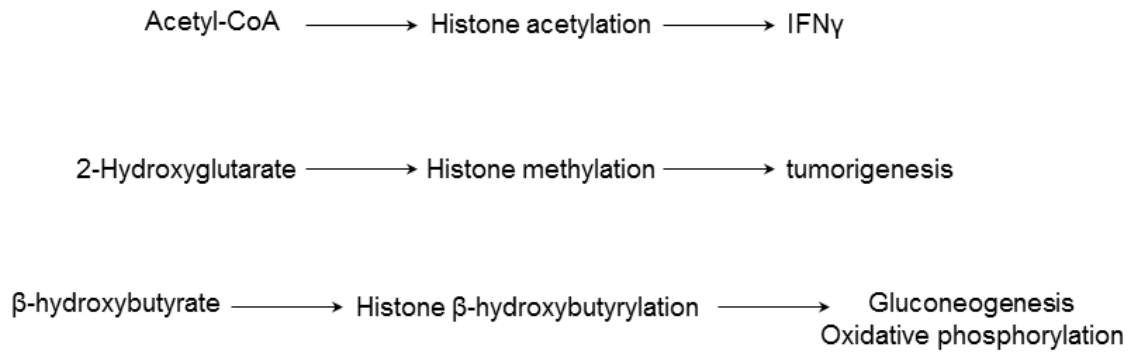


Figure 1.3 Summary of the metabolic impact on cellular function through histone post-translational modifications. Acetyl-CoA levels regulate histone acetylation to increase IFN γ transcription; 2-hydroxyglutarate inhibits histone demethylases and can cause an increase in histone methylation leading to increased tumorigenesis; β -hydroxybutyrate regulates histone β -hydroxybutyrylation and can increase transcription of genes associated with gluconeogenesis and oxidative phosphorylation.

be inhibited through product inhibition by succinate, as well as succinate-derived fumarate [72]. Another metabolite that can also inhibit JmJc demethylases is 2-hydroxyglutarate (2-HG), by competing with α -KG for binding. Many different tumors have been identified as having mutations in isocitrate dehydrogenase (IDH), the enzyme responsible for the generation of α -KG. These mutant enzymes produce 2-HG instead, which by inhibiting the JmJc demethylase KDM4C, inhibits the histone demethylation required for cell differentiation and consequently promote tumorigenesis [73]. Therefore in this instance, tumors can hijack the cell's metabolism impact on histone post-translational modifications to their benefit.

Other more recently identified histone post-translational modifications have also been linked to the metabolic status of the cell. Histone β -hydroxybutyration is derived from the ketone metabolite β -hydroxybutyrate. As a ketone, β -hydroxybutyrate levels are markedly elevated during starvation. This increase results in increased levels of histone β -hydroxybutyration, which triggers transcription of gluconeogenesis and oxidative phosphorylation genes that enable the cell to cope with starvation [54]. Interestingly, β -hydroxybutyrate is also a reported inhibitor of histone deacetylases, and thus increasing concentrations also result in an increase in histone acetylation [74].

Investigations into the link between metabolism and post-translational modifications beyond epigenetics are few, but some do exist. Following bacterial infections, the metabolite acetate has been shown to accumulate in the bloodstream. Uptake of acetate by CD8⁺ T cells results in an increase in acetyl-CoA which leads to increased acetylation of GAPDH. GAPDH acetylation increases the enzyme's activity, which is required for the generation of optimal CD8⁺ T cell memory [75]. Increased GAPDH acetylation has also been reported in response to increased glucose uptake in tumors. This results in increased GAPDH enzymatic activity which supports cell proliferation and tumor growth [76]. Another example of a metabolite impacting on GAPDH function through post-translational modification is the GAPDH substrate 1,3-bisphosphoglycerate. This metabolic intermediate can modify lysines in glycolytic enzymes non-enzymatically given its highly nucleophilic nature. 1,3-bisphosphoglycerate lysine modifications result in inhibition of enzyme activity and thus acts as a feedback mechanism by which cells functioning on high glucose can redirect glycolytic intermediates to alternate metabolic pathways [52].

Finally, in addition to the impact of the metabolic status of the cell on post-translational modifications, there is also evidence to support that post-translational modifications can impact back on metabolism. For instance, most enzymes involved in glycolysis, gluconeogenesis, the Krebs cycle, the urea cycle, fatty acid metabolism and glycogen metabolism have been shown to undergo acetylation, which impacts on metabolic flux through

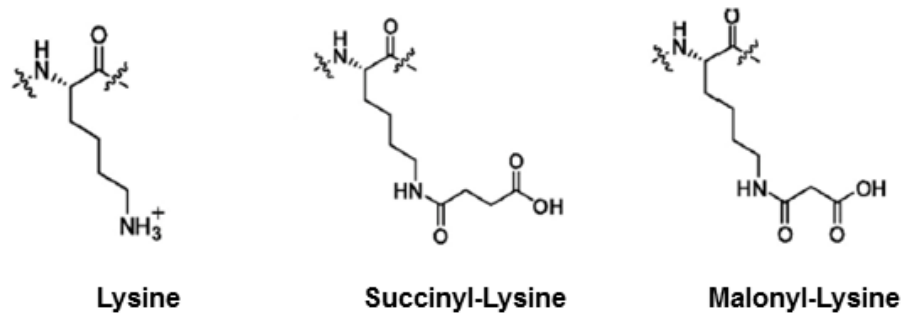
these pathways [77]. The mitochondrial citrate carrier has also been shown to undergo acetylation in the absence of glucose, which increases its activity to enable the export of citrate, which is needed for the generation of NADPH in the absence of glycolysis [78]. Interestingly, this observation is in stark contrast to histone acetylation, which as previously mentioned, has been shown to decrease in the absence of glycolysis. Another example of the impact of post-translational modifications on metabolism is found in trained immunity. Trained immunity refers to the recent observation that cells of the innate immune system are able to remember previous pathogen encounters and mount more efficient responses upon re-encounter, an ability that relies on epigenetic reprogramming [79]. In studies of monocyte immune memory, it's been demonstrated that histone methylation is needed for the induction of glycolysis, which in turn is needed for these cells to mount an efficient innate immune memory response [80].

1.3.1 Lysine succinylation and malonylation and their regulation by Sirt5

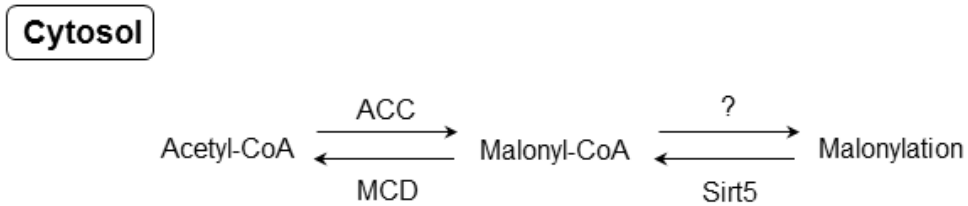
Succinylation and malonylation are recently identified metabolically-derived post-translational modifications affecting lysines. Both modifications are evolutionary conserved and have been identified to be present in several organisms ranging from bacteria to humans. Similar to phosphorylation, they change the charge of the lysine from +1 to -1 under physiological conditions, and would therefore be expected to disrupt electrostatic interactions of lysines with other amino acids. Additionally, the modifications are also of considerable size, with a mass of 100 Da (Figure 1.4A) [34, 81]. The key difference between them is the source of the cofactor required for the modification to occur: succinylation uses succinyl-CoA, while malonylation uses malonyl-CoA [34, 71].

Succinyl-CoA is a Krebs cycle intermediate, synthesized from α -KG by the α -KG dehydrogenase complex in the mitochondria. It can also be converted into succinate by succinyl-CoA synthase. Interestingly, succinyl-CoA has been reported to be at an equilibrium with succinate, which is elevated in activated macrophages. This would suggest that succinyl-CoA levels are elevated as well [82]. Succinylation is of interest here, as one possible consequence of succinate accumulation in LPS-activated macrophages is enhanced succinylation of several metabolic enzymes [27]. Further evidence that this modification can be impacted by the metabolic status of the cell comes from the deletion of α -KG dehydrogenase and succinyl-CoA, which are needed for succinyl-CoA production, causing a significant reduction in overall succinylation levels in yeast cells [83]. Furthermore, inhibition of glycolysis, the Krebs cycle or mitochondrial respiration, all resulted in reduced succinylation levels in the mitochondria, while a hypoxic environment caused an increase. Interestingly, the

A.



B.



C.

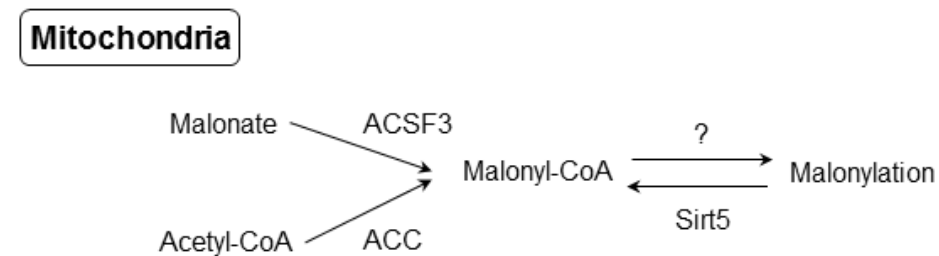


Figure 1.4 Lysine succinylation and malonylation, and generation of malonyl-CoA. **A)** Structure of lysine, succinylated lysine and malonylated lysine. **B)** Generation of malonyl-CoA in the cytosol. **C)** Generation of malonyl-CoA in the mitochondria.

desuccinylase sirt5 relies on NAD⁺ for its activity, yet changes in NAD⁺/NADH ratios did not have any impact on succinylation, thus suggesting succinyl-CoA generation may be the rate-limiting step in the succinylation reaction [84].

Similarly, given that citrate accumulation is another key metabolic feature of LPS-activated macrophages, it is possible that malonylation, occurring in response to malonyl-CoA production may also be another functional consequence of metabolic reprogramming. Malonyl-CoA is derived from citrate through various pathways. Citrate can be converted into acetyl-CoA, which can then be carboxylated and turned into malonyl-CoA by ACC in the cytosol (Figure 1.4B). There are two different ACC isoforms: ACC1 and ACC2. ACC1 is reported to be responsible for the production of malonyl-CoA in tissues with high levels of lipid synthesis and when knocked out in mice, it is embryonically lethal [85]. ACC2 is reported to be mostly expressed in oxidative tissues, where it can inhibit fatty acid oxidation via malonyl-CoA. Malonyl-CoA can bind to and inhibit carnitine palmitoyltransferase 1 (CPT1), which is responsible for transporting fatty acids into the mitochondria for β -oxidation and thus can act as an inhibitor of fatty acid oxidation. ACC2 KO mice don't display embryonic lethality, but are resistant to obesity and diet-induced diabetes [86, 87]. In the mitochondria, malonyl-CoA can be synthesized from malonate by acyl-CoA synthase family member 3 (ACSF3), and it can also be generated through β -oxidation of some dicarboxylic acids (Figure 1.4C) [34, 88, 89]

1.3.1.1 Succinylation

Over the last couple of years, there have been a few studies trying to elucidate the role of lysine succinylation within the cell. All of them have identified succinylation as a regulator of most metabolic pathways in the cell, including the Krebs cycle, ATP synthesis, fatty acid oxidation and the urea cycle (Figure 1.5) [90]. Additionally, it appears that 70% of succinylation substrates are mitochondrial and a high percentage of succinylated sites have also been identified as being acetylated [83]. The functional consequences of this modification are however still not clear.

The enzyme PKM2 has been previously shown to translocate into the nucleus in LPS-activated macrophages, where it can drive IL1 β production through HIF1 α activation [28]. A recent study has shown that succinylation of PKM2 inhibits its activity and acts as a mechanism for this nuclear translocation, which is reported to play a role in the pathogenesis of dextran sulfate sodium-induced colitis in mice [91]. Nevertheless, a second study reports the opposite observation by showing that succinylation of PKM2 boosts its enzymatic activity [92]. However, the activity of different succinylated lysines was assessed in each study, suggesting succinylation may have different effects on the same protein depending on the

location of the modification. Another substrate of succinylation identified is SDH itself. In liver cells from sirt5 KO mice, subunit A of SDH is highly succinylated, which boosts its activity. As SDH is part of complex II of the electron transport chain, desuccinylation of SDH by sirt5 results in reduced levels of oxidative phosphorylation [93]. Another study however, reports opposite results. While SDH is also identified as a substrate of succinylation, they find that liver cells from sirt5 KO mice have impaired respiration through complexes I and II due to impaired SDH enzymatic activity [94]. This observation is supported by a second study also reporting high levels of succinylation within the mitochondria resulting in inhibition of mitochondrial respiration. As previously mentioned, IDH mutations are a common occurrence in many cancers and result in accumulation of 2-hydroxyglutarate. 2-Hydroxyglutarate inhibits SDH and consequently results in succinyl-CoA accumulation. This in turn leads to higher levels of succinylation within the mitochondria, which inhibits mitochondrial respiration at the same time as it promotes apoptosis resistance and aerobic glycolysis [95]. On the other hand, succinylation of IDH itself, appears to inhibit the enzyme's activity [71, 96]. The same occurs with SOD1, an enzyme responsible for balancing ROS levels. When SOD1 is succinylated, its activity is decreased, whilst its desuccinylation by sirt5 results in its activation and subsequent ROS elimination [97]. Finally, succinylation has also been shown to affect histones, such as H3K9, the functional consequences of which remain to be investigated [81, 98].

1.3.1.2 Malonylation

Malonylation is a key focus of this thesis and similarly to succinylation, it has so far been identified as a regulator of metabolic pathways, particularly glycolysis and fatty acid oxidation (Figure 1.5). However, as opposed to succinylation, malonylation is more abundant in the cytosol than in the mitochondria, and it doesn't seem to overlap with acetylation to the same extent that succinylation does [99]. Additionally, while studies on succinylation profiles have so far been limited to sirt5 KO mice, malonylation profiles have been carried out in both sirt5 KO as well as malonyl-CoA decarboxylase (MCD) KO and ACSF3 KO cells [99-101].

MCD is the enzyme responsible for converting malonyl-CoA into acetyl-CoA in the cytosol. MCD KO cells have higher levels of malonylation, which mimics the human rare disease of malonic aciduria, in which patients are born MCD-deficient and consequently also display higher malonylation levels [99]. Colak and colleagues demonstrated that increased malonylation of proteins within the mitochondria resulted in inhibition of fatty acid oxidation, independently of CPT-1. They were also able to show that malonylation of very long chain acyl-coenzyme A dehydrogenase (VLCAD) results in inhibition of its enzymatic activity [99]. Interestingly, ACSF3 KO cells, which have reduced levels of mitochondrial malonylation, also display impaired mitochondrial metabolism, although the impact of malonylation on specific

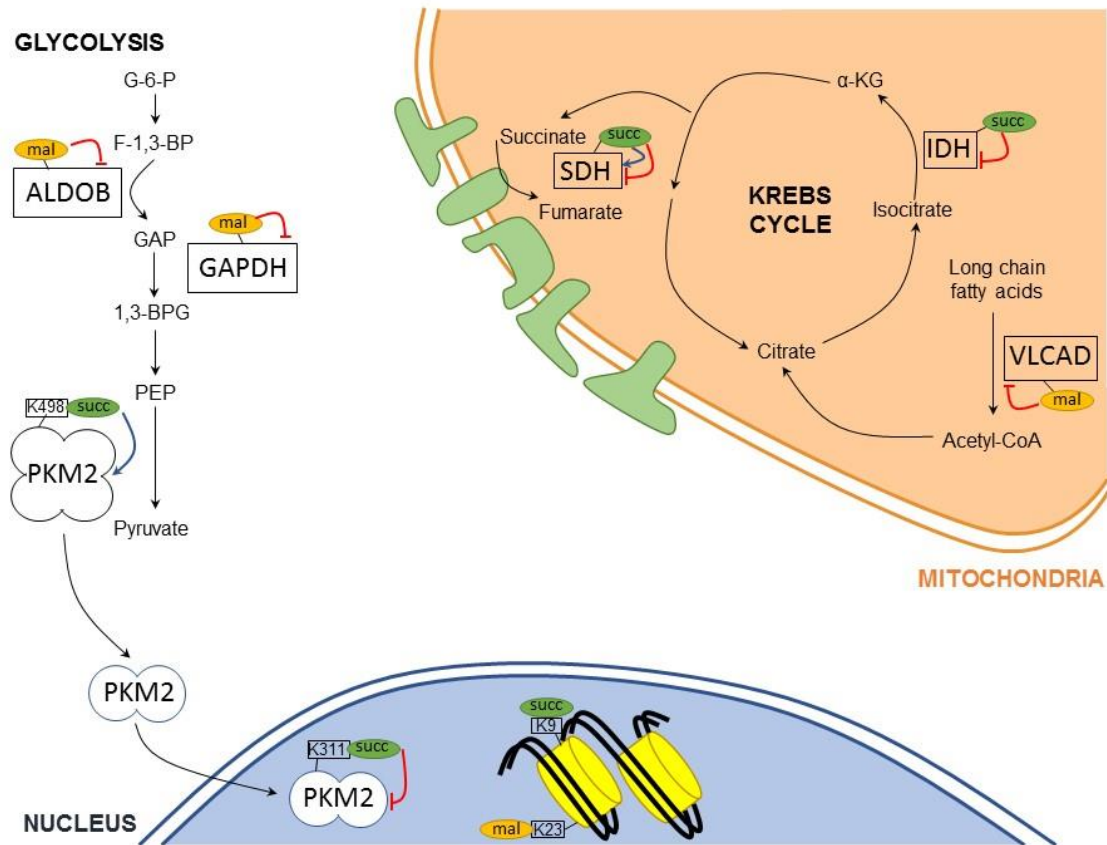


Figure 1.5 Substrates of succinylation and malonylation. The glycolytic enzymes ALDOB and GAPDH have both been identified as undergoing malonylation, which inhibits their enzymatic activity. PKM2 has also been identified as undergoing succinylation, which depending on the site can inhibit or increase its activity. In the nucleus, histone 3 has been identified as undergoing both succinylation and malonylation. In the mitochondria, the β -oxidation enzyme VLCAD has been identified as malonylated while SDH and IDH are both succinylated. They have all been reported to be negatively affected by the modification, with the exception of SDH, the succinylation of which has been shown to both inhibit and boost its activity.

enzymes was not assessed in this particular study [101]. Additionally, investigations in sirt5 KO cells showed that many glycolytic enzymes are malonylated. The impact of malonylation on the glycolytic enzyme GAPDH was assessed and found to be inhibitory of its enzymatic activity [100].

Beyond malonylation-related enzyme knockout studies, higher malonylation levels have also been identified in mouse models of type II diabetes [102]. The substrates identified to be malonylated in these models also involve glycolytic enzymes and enzymes involved in fatty acid metabolism. Particularly, the glycolytic enzyme ALDOB undergoes malonylation, which is shown to block its enzymatic activity [102]. Interestingly, in a mouse model of obesity, malonylation of H3K23 was found to be downregulated, although the functional consequences of this downregulation remain to be investigated [103]. Overall, the evidence obtained thus far would suggest that malonylation has an inhibitory effect on metabolic enzymes.

1.3.1.3 Sirt5

The only identified enzymatic regulator of both succinylation and malonylation to date is sirtuin 5. This enzyme belongs to the sirtuin family of histone deacetylases, which is composed of seven members in mammals, sirtuins 1 – 7. Even though they are known as deacetylases, only sirt1, sirt2 and sirt3 have strong deacetylase activity, with the remaining ones having very weak or non-detectable activity. Sirt5 has very weak deacetylase activity, but can instead remove succinyl and malonyl groups from lysines, using NAD^+ as a cosubstrate and generating nicotinamide and 2'-O-succinyl-ADP-ribose or 2'-O-malonyl-ADP-ribose as a result [81]. Furthermore, it's been more recently identified as capable of catalyzing lysine deglutarylation as well [56]. The role of sirt5 within the cell, apart from its succinylation and malonylation regulatory functions, remains obscure.

Sirt5 expression can be induced by the peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) at the transcriptional level, and it is negatively regulated by AMP-activated protein kinase (AMPK) [104]. It has also been shown to play a role in human non-small cell lung cancer by controlling the expression of the transcription factor nuclear factor like 2 (NRF-2) [105]. However, a metabolic characterization of sirt5 KO mice yielded no significant phenotype other than reduced expression of acyl-CoA oxidase 1 (Acox1), sterol regulatory element-binding protein 1c (SREBP1c) and 2 (SREBP2) under high fat diet. Interestingly, these mice had significantly lower levels of serum amyloid A, an acute phase protein and a marker of inflammation [106]. A more recent study has nonetheless been able to link sirt5 to the inflammatory response in macrophages. Qin and colleagues demonstrated that sirt5, independently of its deacylation activity, is able to sequester Sirt2 and thus prevent the deacetylation of the p65 subunit of NF- κ B. This way, sirt5 enables NF- κ B activation and

the downstream production of cytokines [107]. Sirt5 has also been recently linked to mitochondrial dynamics, with its deletion in murine fibroblasts resulting in mitochondrial fission and fragmentation [108]. Given the recent association between the fusion or fission status of mitochondria dictating the function of T cells through metabolic reprogramming, it is interesting to speculate whether this might be another mechanism whereby sirt5 may be playing a role in inflammation [109].

1.4 Moonlighting properties of glycolytic enzymes

An observation was made in the late 80s that certain proteins, such as GAPDH, have the ability to carry out not just one function within the cell, but multiple unrelated ones. This type of multi-tasking proteins are now referred to as moonlighting proteins [110]. Interestingly, the first one of this type to be identified was the glycolytic enzyme LDH [111]. Although other non-metabolic enzymes have also now been classed as moonlighting proteins, a high percentage remain glycolytic enzymes.

LDH is the enzyme responsible for catalyzing the conversion of pyruvate to lactate in the cytoplasm. LDH can however undergo phosphorylation, which allows it to translocate into the nucleus, where it binds and destabilizes single-stranded DNA [112, 113]. Although it has been hypothesized that this binding may be associated with a potential role for LDH in transcription, this is yet to be confirmed. Furthermore, LDH has also been identified as an RNA-binding protein. It's been shown to specifically bind through its NAD-binding domain to GM-CSF mRNA and aid in its translation [114].

Another example of a moonlighting protein is hexokinase, the first enzyme in glycolysis, responsible for phosphorylating glucose to glucose-6-phosphate. It has been reported that hexokinase uses ATP produced in the mitochondria to carry out this phosphorylation step [115]. Therefore, it is not surprising that hexokinase can be found associated with the mitochondria, bound to the voltage-gated anion channel (VDAC). What may come as a surprise is that this association enables it to play a role in apoptosis. Hexokinase can prevent the recruitment of the apoptotic protein bax to the mitochondria therefore inhibiting the release of cytochrome c and consequently preventing apoptosis [116]. Furthermore, hexokinase has also been recently identified as a receptor of the bacterial peptidoglycan derivative N-acetylglucosamine (NAG). Binding to NAG causes its dissociation from VDAC and acts as an activating signal for the NLRP3 inflammasome, therefore implicating it in the production of IL1 β in macrophages [30].

Pyruvate kinase is another glycolytic enzyme that can also translocate into the nucleus. Although its role in glycolysis finds it converting phosphoenolpyruvic acid to pyruvate in the

cytoplasm, the M2 isoform can also become phosphorylated by extracellular signal-regulated kinase (ERK)1/2, which enables it to carry out multiple different nuclear functions [117]. Some of these functions include activation of the transcription factor stat3, as well as activation of HIF-1 α , leading to pro-IL1 β production [28, 118]. Additionally, it has been shown to interact with suppressor of cytokine signalling 3 (SOCS3), which results in decreased levels of ATP production leading to impaired antigen presentation in dendritic cells [119].

1.4.1 The multifunctional role of GAPDH

GAPDH, the major subject of this thesis, was one of the first moonlighting proteins identified, when it was reported in 1980 by Perucho and colleagues that it could bind DNA [120]. GAPDH is found predominantly in the cytosol as an enzymatically active tetramer that carries out the sixth step in glycolysis, using NAD⁺ as a cofactor for converting glyceraldehyde-3-phosphate into glycerate-1,3-bisphosphate and ATP in the cytosol. However, over the last thirty years, it has been reported to be present not just in the cytosol and the nucleus, but in many other cellular compartments. Consequently, the number of non-glycolytic functions for GAPDH have been on the rise, including regulation of intracellular calcium signalling, DNA repair, RNA-binding, vesicular trafficking and cell death, among others (Figure 1.6) [121].

1.4.1.1 GAPDH and cell death

One of the most predominant roles that have been associated with GAPDH outside of glycolysis across many different types of cells, is a role in cell death. GAPDH becomes S-nitrosylated and acetylated on lysine 225 in the cytosol, which results in inhibition of its enzymatic activity and enables it to bind to the E3 ubiquitin ligase Siah1 and translocate into the nucleus [122-124]. Binding of GAPDH to Siah1 results in Siah1 stabilisation, which allows it to target nuclear proteins for degradation, ultimately causing cell death. Additionally, once in the nucleus, GAPDH is acetylated by the acetyltransferase p300/CREB binding protein (CBP). This in turn stimulates CBP activities, which include induction of cell death mediators, such as p53, further amplifying a cell death cascade [125].

However, GAPDH contributions to cell death go beyond its nuclear translocation. Under stressed conditions, such as serum deprivation or treatment with DNA-damaging agents, GAPDH localizes to the mitochondria where it can interact with VDAC1 and contrary to hexokinase, it can cause the release of cytochrome c as well as apoptosis-inducing factor and thus start an apoptotic signalling cascade [126]. Furthermore, under oxidative stress, cytosolic GAPDH is susceptible to disulfide bond formation within the active site cysteine, resulting in

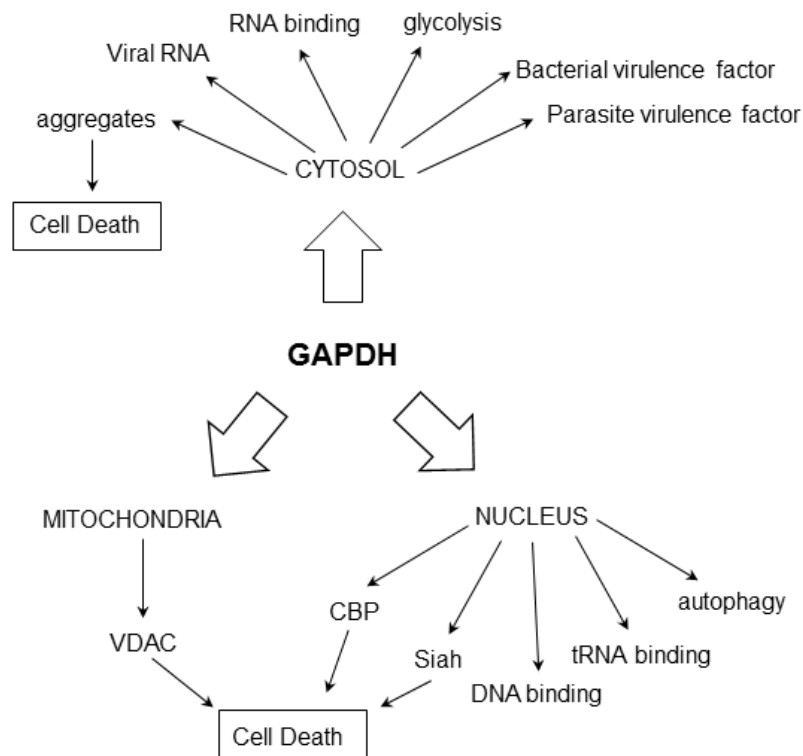


Figure 1.6 Various moonlighting functions of GAPDH. GAPDH has been mainly reported to be present in three different cellular compartments: the cytosol, the mitochondria and the nucleus. In the mitochondria, it can associate with VDAC to induce apoptosis. In the nucleus, it can also induce cell death via binding to Siah and by becoming acetylated by CBP. Additionally, nuclear GAPDH can also bind DNA and tRNA, as well as induce autophagy. Cytosolic GAPDH can also induce cell death through the formation of aggregates following stress signals. It can also bind both host RNA as well as viral RNA and inhibit their translation. Additionally, GAPDH derived from bacteria and parasites is required in many cases for host colonization as well as being implicated in different immune evasion strategies.

amyloid- β peptide, which are a hallmark of Alzheimer's disease. A reduction in the levels of GAPDH also results in reduction of amyloid- β aggregates, thus implicating GAPDH in the pathogenesis of the disease [127]

Interestingly, there are also cases in which rather than inducing cell death, GAPDH has been shown to prevent it. Under standard apoptotic conditions, the mitochondrial outer-membrane becomes permeabilized, which enables the release of cytochrome c, which will lead to caspase activation followed by apoptosis. However, if caspase activation is inhibited following mitochondrial membrane permeabilization, rather than undergoing apoptosis, the cell undergoes a different type of cell death, referred to as caspase-independent cell death. GAPDH has been shown to protect the cells from this type of cell death by increasing glycolysis levels as well as translocating into the nucleus and inducing autophagy [128]. However, the authors fail to explain how both events occur at the same time, since nuclear GAPDH is enzymatically inactive, and cytosolic GAPDH can't induce autophagy. Additionally, these results go against previous reports suggesting that nuclear GAPDH promotes cell death. Therefore, although there is mounting evidence supporting a role for GAPDH in cell death, further studies are required to elucidate this complex network.

1.4.1.2 GAPDH and RNA-binding

Another of GAPDH's extra-glycolytic functions is its RNA-binding activity. This was first reported in 1980, together with its DNA-binding activity by Perucho and colleagues [120]. A further investigation of this observation was carried out in rabbits by Ryazanov five years later, where he hypothesized that GAPDH could bind RNAs in a non-specific manner in polyribosomes, which could act as a means of localizing glycolysis to these organelles [129]. This was proved not to be the case a couple of years later when it was reported that GAPDH could specifically recognize sequence and structural features of transfer RNAs (tRNAs) and could therefore bind to them in the nucleus and export them into the cytosol (Figure 1.7A) [130]. Furthermore, it was also demonstrated that GAPDH in the cytosol could bind AU-rich sequences within the 3'-untranslated region (3'-UTR) of mRNA encoding different cytokines, including IFN γ , GM-CSF and IL2 (Figure 1.7B). The binding was reported to occur through the NAD-binding domain, and could therefore be inhibited by other interactors of this domain, which include not only NAD⁺, but also NADH and ATP (Figure 1.7D). Additionally, the reverse situation also applies as it was demonstrated that RNA-binding can inhibit GAPDH enzymatic activity [131]. Interestingly, in a follow up study, the authors hypothesized that as the sequence and three-dimensional structure of NAD-binding domains across different enzymes is highly conserved, any enzyme that possesses an NAD-binding domain can also display RNA-binding capabilities. However, it is worth noting that even though the NAD-binding domain on its own

was shown to bind RNA, sequence-specificity binding was affected [132]. This would suggest that in the case of GAPDH, the catalytic domain is also involved in binding RNAs. In fact, it's been recently demonstrated that a mutation outside of the NAD-binding region, affecting GAPDH dimer and tetramer formation, prevented RNA-binding. This implies that GAPDH may be binding RNAs as an enzymatically inactive monomer or dimer [133]. Furthermore, the cysteine in the active site has also been implicated in RNA-binding [134]. Therefore, GAPDH RNA-binding capabilities appear to be more complex than originally postulated.

In addition to directly binding RNAs in the cytosol and the nucleus, GAPDH has also been identified as being part of the IFN γ -activated inhibitor of translation (GAIT) complex (Figure 1.7C) [135]. This complex, composed of glutamyl-prolyl-tRNA (EPRS), NS-1 associated protein (NSAP1), ribosomal protein L13a and GAPDH, is a translational regulator of many pro-inflammatory mediators in human myeloid cells [136]. Following activation of cells with IFN- γ , EPRS becomes phosphorylated which enables it to form an inactive complex with NSAP1. L13a also becomes phosphorylated following activation of the death-associated protein kinase 1 (DAPK1)/zipper-interacting protein kinase (ZIPK) cascade, which enables it to form a complex with GAPDH and join EPRS and NSAP1 to form the final active GAIT complex. This complex binds GAIT elements, which are secondary structures containing a stem loop present in the 3'-UTR region of many RNAs, and prevents their translation. Some of said RNAs are those of many pro-inflammatory mediators such as vascular endothelial growth factor (VEGF) DAPK1, C-C motif chemokine 22 (CCL22), chemokine receptors 3, 4, 6 (CCR3, CCR4, CCR6) and apolipoprotein L2 (APOL2) [136, 137]. This complex is also present in murine myeloid cells, where it carries out the same function but as a heterotrimeric complex rather than heteroquaternary, since NSAP1 is missing [137].

The GAIT complex is however not the only report of GAPDH controlling RNAs of pro-inflammatory mediators in immune cells. Chang and colleagues have reported that GAPDH controls IFN γ translation in T cells. The authors demonstrated that in naive T cells, GAPDH is found bound to the AU-rich element in the 3'-UTR of IFN γ mRNA, preventing translation [138]. However, following activation they switch from oxidative phosphorylation to the less energy-efficient glycolytic pathway, for which GAPDH is required, and thus results in the release and subsequent translation of IFN- γ [138]. Additionally, a similar model has been identified in monocytes in which GAPDH appears to bind the 3'-UTR region of TNF α mRNA in resting cells and prevent its translation. However, following activation with LPS, there is an increase in TNF α mRNA translation coupled with an increase in glycolysis, as GAPDH must release the RNA to engage in its glycolytic enzymatic activity [139].

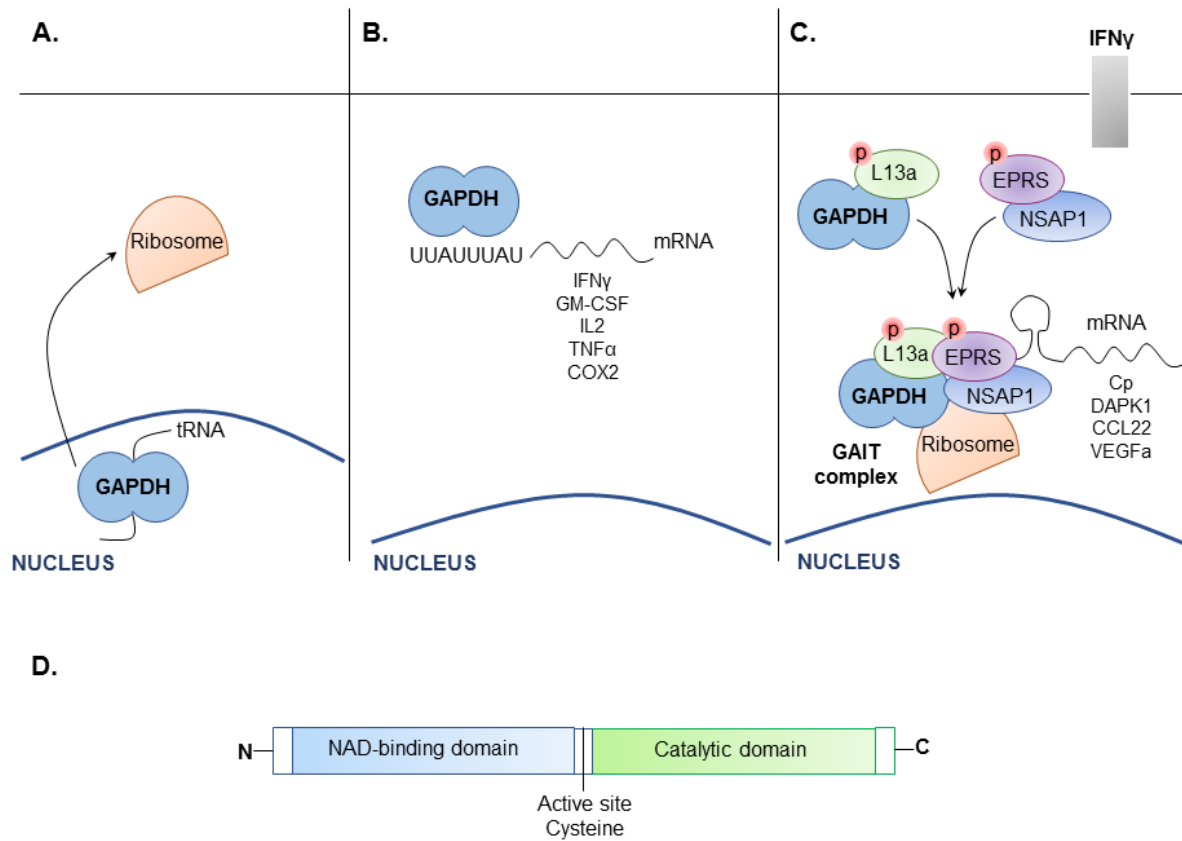


Figure 1.7 Different types of GAPDH RNA binding. **A)** GAPDH can bind tRNAs in the nucleus and export them into cytosol. **B)** GAPDH can bind to the 3'-UTR region of AU-rich containing mRNA in the cytosol and prevent their translation. Many pro-inflammatory mediators, such as IFN γ , GM-CSF, IL2, TNF α or COX2, contain AU-rich sequences within their 3'-UTR. **C)** GAPDH is part of the GAIT complex, which is responsible for repression of translation of GAIT-element containing mRNAs in myeloid cells activated with IFN γ . **D)** Domain structure of GAPDH.

Finally, another interesting aspect of GAPDH RNA-binding capabilities is its ability to bind viral RNAs. This was initially reported twenty years ago, when Schultz and colleagues showed that GAPDH can bind a region within the 5'-UTR of the hepatitis A virus RNA and destabilize it, which they later demonstrate exerts a negative effect on its translation [140, 141]. Furthermore, GAPDH has also been shown to bind to RNA from hepatitis B and C viruses, although the functional consequences of this binding remain to be investigated [142, 143]. In addition to binding to hepatitis viruses, GAPDH has also been shown to bind to human parainfluenza virus type 3 RNA, which results in inhibition of its transcription [144, 145]. It is tempting to speculate whether GAPDH RNA-binding activities not only play a role in the production of inflammatory mediators, but also in anti-viral defense, which is yet to be explored in immune cells.

1.4.1.3 GAPDH as a virulence factor

As mentioned above, mammalian GAPDH has been shown to have a role in viral infections, however, it has also been shown to be a virulence factor in some bacteria and parasites. It was first reported by Pancholi and Fischetti to localize to the cell surface of group A streptococci, where it was later shown to retain its enzymatic activity, while also being able to bind to host cells and prevent phagocytosis [146, 147]. Further studies have also implicated GAPDH in the pathogenesis of other forms of streptococci bacteria. For instance, *Streptococcus pneumoniae* also expresses GAPDH on its surface, where it binds to peptidoglycan and can recruit the first complement protein C1q, but fails to activate the complement system [148]. Furthermore, GAPDH from *Streptococcus pyogenes*, a major sepsis-causing pathogen, can bind complement protein C5a and prevent its chemotactic actions that result in neutrophil recruitment [149]. Thus GAPDH plays a key role in *Streptococcus pyogenes* immune evasion strategies. Additionally, GAPDH from *Streptococcus pyogenes* as well as *Staphylococcus aureus* can be secreted and has been shown to be able to induce apoptosis of murine macrophages [150]. On the other hand, GAPDH secreted by group B streptococci can also manipulate the host immune system by increasing IL10 production, which aids in bacterial colonization [151]. Another study that confirms the important role played by GAPDH in bacterial pathogenesis has shown a vaccine targeting GAPDH to confer protection against streptococci infections in adult mice [152].

However, GAPDH has not only been associated with pathogenesis of gram positive bacteria but also gram negative ones. Enterohemorrhagic and enteropathogenic *Escherichia coli* have been shown to specifically secrete enzymatically active GAPDH through their type III secretion system [153]. Secreted GAPDH is capable of binding to human fibrinogen and plasminogen, which aids in bacterial colonization. Interestingly, non-pathogenic *E. coli* does not secrete

GAPDH [154]. Furthermore, GAPDH has also been implicated in the pathogenesis of the parasite *Haemonchus contortus*. GAPDH secreted by *H. contortus* is reported to bind to complement proteins C1q and C3 and prevent subsequent complement activation [155, 156]. In addition, vaccination of goats with a GAPDH DNA vaccine is reported to result in protection against *H. contortus* infection [157].

Given all of the different examples of the use of GAPDH by pathogens as a means of immune evasion, in addition to bacterial GAPDH displaying RNA-binding activity, it is interesting to speculate whether RNA-binding may be a novel mechanism used by pathogens to evade the immune response [158]

1.6 Aims

The overall aim of this thesis is to examine the role of the metabolically-derived post-translational modifications succinylation and malonylation in macrophage activation. The specific aims are as follows:

- To characterize succinylation and malonylation in activated macrophages and identify possible targets for these modifications.
- To analyse GAPDH as a particular target of these modifications, involving identification of modified lysines.
- To examine the effects of the GAPDH enzymatic inhibitor heptelidic acid on LPS action and GAPDH function in macrophages.
- To examine GAPDH as an mRNA binding protein and its potential involvement in the regulation of translation in LPS-activated macrophages.
- To investigate the link between GAPDH post-translational modifications and its identified functions in macrophages.

Overall I have found a role for GAPDH malonylation in LPS signalling, important for the translation of critical LPS-induced mediators, especially TNF α . This novel finding reveals a hitherto unknown mechanism in LPS signalling that regulates the expression of central pro-inflammatory mediators implicated in many inflammatory diseases. These results also further emphasize the importance of metabolic reprogramming in macrophage activation.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell culture

2.1.1.1 Cell culture reagents

RPMI (Rosewell Park Memorial Institute) 1640 medium with GlutaMAX-I and high glucose DMEM (Dulbecco's Modified Eagle Medium) with GlutaMAX-I were obtained from Gibco. Fetal bovine serum (FBS) was also from Gibco. Penicillin/Streptomycin and Trypsin were obtained from Sigma Aldrich. TripleE was obtained from Gibco.

2.1.1.2 Cell lines

RAW264.7, L929 and HEK293T cells were obtained from ECACC. Immortalized BMDMs were a kind gift from Prof Douglas Golenbock, University of Massachusetts Medical School, USA.

2.1.1.3 Primary cells

Wild-type mice used to produce BMDMs were bought from Harlan UK. Legs from DAPK1 KO mice were a kind gift from Dr Adi Kimchi, Weizmann Institute of Science, Israel. Legs from sirt5 KO mice were kindly provided by Johan Auwerx, EPFL, Lausanne. All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

Human blood used for the generation of monocyte-derived macrophages was obtained from the GSK Stevenage blood donation unit. The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.

2.1.1.4 Stimuli

Ultrapure rough LPS (from *E.Coli*, serotype EH100) was purchased from Alexis. Pam₃Cys-Ser-(Lys)₄ (PamCSK) was obtained from Calbiochem. R848 and low molecular weight poly I:C were from Invivogen. Malonyl coenzyme A tetralithium salt was from Sigma.

2.1.1.5 Inhibitors

Cyclohexamide was purchased from Alexis. Heptelidic acid (HA) was from Abcam. 2-deoxyglucose (2DG) was purchased from Sigma and DAPK1 inhibitor (CAS 315694-89-4) was from Merck Millipore.

2.1.2 Western blotting

2.1.2.1 Western blotting reagents

4-12% Bis-Tris NuPAGE gels, NuPAGE MOPS running buffer, 20X transfer buffer and iBlot gel transfer nitrocellulose or polyvinylidenedifluoride (PVDF) stacks were all purchased from Invitrogen. Broad range pre-stained protein marker was purchased from New England BioLabs. 20X LumiGlo Chemiluminescent reagent was from Cell Signalling and Restore PLUS Western Blot stripping buffer was from Thermo Scientific. LiCOR blocking solution was obtained from LiCOR Bioscience. Peptides used for specificity testing of the succinyl-lysine and malonyl-lysine antibodies were synthesized using JPT Peptide Technologies.

2.1.2.2 Western blotting antibodies

Succinyl-lysine and malonyl-lysine antibodies were obtained from PTM Biolabs. DAPK1, Sirt5 and pMLC (Thr18/Ser19) antibodies were from Cell Signalling. GAPDH antibody was from Merck Millipore. IL-1 β antibody was from R&D systems. β -actin, mouse IgG and rabbit IgG were purchased from Sigma Aldrich. Secondary horseradish peroxidase conjugated anti-mouse IgG, anti-rabbit IgG and anti-goat IgG were from Jackson ImmunoResearch Inc. Secondary donkey anti-goat 800 CW, anti-mouse 680 CW and anti-rabbit 800 CW antibodies were purchased from LiCOR Biosciences.

2.1.3 Immunoprecipitation reagents

Protein A/G PLUS Agarose beads were purchased from Santa Cruz. DEPC-treated water used to make up buffers used in RNA immunoprecipitation was obtained from Ambion. RNase inhibitors were purchased from Thermo Scientific. The rest of the materials were all obtained from Sigma Aldrich.

2.1.4 Cu(I)-catalysed click chemistry reagents

The MalAMyne probe was kindly synthesized by Alan Nadin, NCE Molecular Tools Group, GlaxoSmithKline, Stevenage, UK. Biotin azide and high capacity streptavidin agarose beads were obtained from Thermo Scientific. The rest of the materials were all obtained from Sigma Aldrich.

2.1.5 RNA extraction and PCR reagents

RNeasy mini RNA purification kits, RNase-free water and the QIAzol lysis reagent were purchased from QIAGEN. Linear polyacrylamide (LPA) was purchased from Thermo Scientific. Reverse-transcription reagents, PCR plates, Taqman probes and Taqman gene expression mastermix were from Applied Biosystems. SYBR primers were synthesized by Eurofins Genomics. The KAPA SYBR Fast qPCR master mix was obtained from KAPA Biosystems.

2.1.6 Luminex reagents

96 well filter plates were obtained from Millipore. The beads were purchased from Thermo Fisher. Antibodies were purchased from R&D. Streptavidin-PE was obtained from BD Biosciences and sheath fluid was from Life Technologies

2.1.7 Transfection and plasmid reagents

Lipofectamine 2000 and Lipofectamine RNAiMax were obtained from Invitrogen. Plasmid purification maxiprep and miniprep kits were purchased from Qiagen. The luciferase psiCHECK2 vector was obtained from Promega and the GAPDH-FLAG-myc plasmid was obtained from Addgene. The TNFa-3'UTR and DAPK1-3'UTR plasmids as well as the mutant versions of GAPDH K213 were generated. 5X passive lysis buffer was purchased from Promega. Coelentrazine was from Biotium. D-Luciferin was purchased Biosynth. Ladders for agarose gels were purchased from Biotium. DH5 α cells were purchased from Invitrogen and the QuickChange mutagenesis kit was obtained from Agilent.

2.1.8 Miscellaneous reagents

General laboratory chemicals were purchased from Sigma Aldrich with the exception of: murine TNFa, IL1 β , IL6, and RANTES ELISA DuoSets and TMB substrate solution were purchased from R&D Systems; MSD plates and reagents were purchased from Meso Scale Discovery; Malonyl-CoA ELISA was purchased from Cusabio; Cytotox96 $^{\circledR}$ non-radioactive cytotoxicity assay and sequence-grade trypsin were purchased from Promega; Instant Blue coomassie stain was obtained from Expedeon; colorimetric GAPDH enzymatic assay was obtained from Abcam; XF assay buffer, seahorse cell culture plates, utility plates and calibrant solution were purchased from Seahorse Bioscience; GAPDH and control siRNAs were obtained from Ambion; CD14 microbeads were purchased from MACS Miltenyi Biotech; TruSeq Stranded total RNA library prep kit was obtained from Illumina; BCA protein assay kit was purchased from Thermo Scientific.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Growth and maintenance of cells lines

iBMDM, RAW264.7 and HEK293T cell lines were cultured in DMEM medium containing 10% FBS and 1% P/S. L929 cells were cultured in RPMI medium containing 10% FBS and 1% P/S. Cells were maintained in an incubator at 37°C with a humidified atmosphere of 5% CO₂.

For continuing cell culture, all cell lines were seeded at $1 - 5 \times 10^5$ per mL and subcultured twice a week. L929 and HEK293T cells were washed with phosphate-buffered saline (PBS) followed by incubation with 10 mL trypsin-ethylenediaminetetraacetic acid (EDTA) or TripLE for 5 – 10 minutes in order to lift the cells off the flask. The medium coming off the L929 would be pooled and filtered and used as the source of MCSF for the generation of bone marrow-derived macrophages (BMDMs). iBMDM and RAW264.7 cells were removed from the culturing flask using a cell scraper. Cells were centrifuged at $435 \times g$ for 5 minutes before resuspending the pellet in fresh media. 20 µL of cell suspension was removed and used to establish cell number and cell viability using a 1:1 ratio of the dye Trypan Blue. 10 µL of this solution is applied to a haemocytometre. The dye is excluded from healthy cells but is taken up by non-viable cells, which appear as blue-violet under the light microscope. The following equation was used to find the number of cells/mL:

$$\text{Cell count} \times \text{dilution factor} \times 10^4 = \text{number of cells/mL}$$

2.2.1.2 Cryo-preservation of cells

Cells were cultured in a T175 flask until 80 – 90% confluent and then harvested. They were centrifuged at $453 \times g$ for 5 minutes and the pellet resuspended in 4 mL of 90% FBS and 10% DMSO. 1 mL aliquots of the cell suspension were prepared in 1.5 mL cryovials and incubated for 24 hours in a cryo-freezing container at -80°C before being transferred to liquid nitrogen for long term storage.

2.2.1.3 Generation of mouse bone marrow-derived macrophages

The bone marrow was harvested in a laminar flow hood following the euthanization of C57BL/6 mice in a CO₂ chamber. The abdomen and hind legs were sprayed with 70% ethanol and an incision was made in the midline of the abdomen and the skin was pulled down to expose the hind legs. The legs were separated from the body by cutting around the hip joint and they were placed in a 10 cm dish containing 10 mL DMEM cell culture media. The muscle was removed from around the femur and tibia. Following separation of the bones at the knee joint, the bone

marrow was flushed out into a 50 mL tube using a 20 mL syringe filled with PBS and a 23 gauge needle that was inserted into the bone cavity. After flushing all the bones, the cells were resuspended by pipetting up and down using a 10 mL pipette to bring the cells into single cell suspension. The cells were then centrifuged at 453 x g for 5 minutes and resuspended in 4 mL red blood lysis buffer for 5 minutes. The reaction was stopped by adding 30 mL of media followed by the filtration of the cells using a cell strainer. Cells were centrifuged again at 453 x g for 5 minutes and resuspended in 40 mL of DMEM (4.5mg/L glucose) containing 10% FBS, 1% P/S and 20% MCSF. Cells were seeded in non-coated tissue culture sterile 10 cm dishes at a volume of 10 mL/dish. They were incubated at 37°C for 7 days.

After the incubation period, they were lifted off the plate by adding cold PBS followed by the use of a cell scraper. They are then counted as stated previously, or using a Nucleocounter, and plated as required by the experiment.

2.2.1.4 Generation of human blood monocyte-derived macrophages

Leukodepletion filters (cones) from platelet apheresis were bought by GSK and used for PBMC generation. Two falcon tubes were prepared per cone with 15 mL of histopaque. The top and bottom of each cone was cut while placing the cone on top of the 50 mL falcon tube. A 10 mL syringe and a 23 gauge needle were used to gently flush the cone with 10 mL of filtered Miltenyi buffer (0.5% BSA, 0.2% EDTA in PBS). The falcon was then topped up to 40 mL with PBS. Slowly, 20 mL of blood were layered on top of the histopaque. The falcons were then centrifuged at 800 x g for 20 minutes with no brake at room temperature.

The PBMC layer was collected with a pipette and transferred to a new 50 mL falcon tube (two tubes per cone). Each falcon was topped up to 50 mL with PBS and centrifuged at 400 x g for 10 minutes at room temperature. The supernatant was discarded and both pellets resuspended in a total of 50 mL PBS per falcon tube, followed by a centrifugation step at 300 x g for 10 minutes at room temperature.

The supernatant was discarded and the pellets resuspended in 10mL of ice-cold Miltenyi buffer and 500 µL of MACS CD14 beads. They were left to incubate at 4°C for 15 minutes. They were then topped up to 50 mL with Miltenyi buffer, mixed and centrifuged for 5 minutes at 1,500 rpm. The supernatant was removed and the remainings resuspended in 5 mL Miltenyi buffer.

Two LS columns were set up per cone in a midi MACS separator and washed by adding 3 mL of Miltenyi buffer. The cells were then added to the column through a 30 µm pre-separation

filter to ensure only a single-cell suspension would enter the column. The column was washed with 3 mL of Miltenyi buffer three times.

The column was removed from the separator and placed on top of a collection tube. 5 mL of Miltenyi buffer were added and a plunger inserted to flush out the cells, which were then centrifuged at 1,300 rpm for 5 minutes.

The pellet was resuspended in 20 mL of RPMI supplemented with 10% FBS, 1% Pen/Strep and 1% Glutamine. 50 μ L of cells were then taken out and added to 450 μ L of PBS for counting on a nucleocounter. All cells were then made up to 1×10^6 cells/mL and recombinant M-CSF added to give a final concentration of 100 ng/mL. Cells were seeded at the concentration and in the plates required by the experiment, and were left to differentiate for 7 days.

2.2.2 Western blotting

2.2.2.1 Preparation of cell lysates

Cells for lysis were seeded and stimulated at 1×10^6 cells/mL in 12 well plates. Before lysis, cells were washed with cold PBS. Cell lysates were prepared by direct lysis in 50 - 100 μ L/well of 5X sample buffer (10% (v/v) glycerol, 2% (w/v) SDS, 200 μ g/mL bromophenol blue, 125 mM Tris pH 6.8) and 10% DTT that was added just before used. Samples were then moved to microcentrifuge tubes and boiled for 5 minutes at 95°C.

2.2.2.2 Sodium Dodecyl Sulphate – Polyacrylamide gel electrophoresis (SDS-PAGE)

Gels were prepared in BioRad® apparatus. A 12% resolving gel is prepared and poured first. To make four gels, 30 mL were prepared as follows: 10 mL H₂O, 12 mL 30% (v/v) acrylamide mix (29.2% acrylamide and 0.8% N,N'-methylene-bis-acrylamide), 7.6 mL 1.5M Tris-HCl (pH 8.8), 300 μ L 10% (w/v) ammonium persulphate and 12 μ L N,N,N',N'-tetramethylethylenediamine (TEMED). After the resolving gel was set, the stacking gel was poured on top. For four gels, 10 mL were prepared as follows: 6.8 mL H₂O, 1.7 mL 30% (v/v) acrylamide mix, 1.25 mL 1M Tris-HCl (pH 6.8), 100 μ L 10% (w/v) SDS, 100 μ L 10% (w/v) ammonium persulphate and 10 μ L TEMED. The gels were then placed in a BioRad® gel running apparatus containing running buffer (1X made from 10X stock which is prepared as follows: 30.3 g 25 mM Tris, 144 g 192 mM glycine, 10 g 0.1% SDS and made up to 1 L with distilled H₂O). 10 – 20 μ L/well of protein sample and 10 μ L/gel of molecular weight markers were added. The gels were run using a constant current of 25 mA per gel, until the dye front from the sample buffer ran off the end of the gel – approximately 1 hour.

2.2.2.3 Electrophoretic transfer of proteins

The resolved proteins were transferred to an immobilon PVDF membrane using a wet transfer system. A piece of PVDF membrane was soaked in methanol to activate it. All other components were soaked before use in transfer buffer (25 mM Tris pH 8, 0.2 M glycine, 20% methanol). The gel was placed on two sheets of filter paper and a sponge, which were overlaid with the membrane. Two more sheets of filter paper were placed on top, followed by a second sponge. The entire assembly was placed in a cassette, the chamber was filled with transfer buffer and a constant current of 200 mA was applied for 2 hours.

2.2.2.4 Blocking of non-specific binding sites

Following protein transfer, non-specific binding of proteins to the membrane was prevented by incubating the membrane for 1 hour at room temperature in 5% non-fat dried milk powder (Marvel) or bovine serum albumin (BSA, w/v) in 0.01% (v/v) Tris Buffer Saline (TBS)-Tween20®. TBS-Tween20® was prepared from a 10X stock which was made up as follows: 12.11 g Tris, 87.6 g NaCl, 10 mL Tween20® and made up to 1 L in distilled water.

2.2.2.5 Antibody probing

Following blocking, the membranes were incubated with the antibody to the protein of interest diluted in 5% Marvel or BSA (**Table 2.1**). The rotating incubation was carried out either overnight at 4°C or for 2 hours at room temperature. After the incubation, the membranes were washed three times in 0.1% (v/v) TBS-Tween20® for at least 10 minutes at room temperature. They were then incubated for an hour at room temperature with the corresponding IgG horseradish peroxidase conjugated antibody (**Table 2.1**).

Table 2.1 Antibodies used in western blotting

Primary Ab	Dilution factor	Secondary Ab	Dilution factor
Succinyl-lysine	1 : 1,000	Anti-mouse IgG (H+L)	1 : 2,000
Malonyl-lysine	1 : 1,000	Anti-rabbit IgG (H+L)	1 : 2,000
β-actin	1 : 10,000	Anti-mouse IgG (H+L)	1 : 2,000
IL-1β	1 : 1,000	Anti-goat IgG (H+L)	1 : 2,000
GAPDH	1 : 1,000	Anti-mouse IgG (H+L)	1 : 2,000
DAPK1	1 : 1,000	Anti-rabbit IgG (H+L)	1 : 2,000
Sirt5	1 : 1,000	Anti-rabbit IgG (H+L)	1 : 2,000
Phospho-MLC	1 : 1,000	Anti-rabbit IgG (H+L)	1 : 2,000
FLAG	1 : 1,000	Anti-rabbit IgG (H+L)	1 : 2,000

myc	1 : 2,000	Anti-mouse IgG (H+L)	1 : 2,000
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The membranes were then washed again three times in 0.1% (v/v) TBS-Tween20® for at least 10 minutes at room temperature. Chemiluminescent substrate was then added to the surface of the membranes for 10 seconds to 5 minutes. The chemiluminescent substrate was prepared according to manufacturer's instructions (Cell Signalling). The membranes were placed in a cassette together with a scientific imaging film. The film was developed and the position of the antibodies visualised.

2.2.2.6 Testing of antibody specificity

To test the specificity of the anti-succinyllysine and anti-malonyllysine antibodies, each antibody (1 µg/mL) was incubated in 4 mL of 5% BSA overnight at 4°C on a shaking platform with either no peptide or 2 µg/mL of a peptide containing a succinylated lysine or a malonylated lysine (**Table 2.2**). The following day, they were centrifuged at 4°C at 5590 x g for 15 minutes to precipitate immune complexes. The supernatant was then used to probe the membranes.

Table 2.2 Peptides used for antibody-specificity testing

Peptide	Sequence	Concentration
Succinylated	H-FHSQ-Lys(Succ)-YSPVY-OH	1.5 mg/mL
Malonylated	H-FHSQ-Lys(Mal)-YSPVY-OH	1 mg/mL

2.2.2.7 Stripping and reprobing of PVDF membrane

After development of the blot, the membrane was washed twice in 0.1% TBS-Tween20® and incubated for 10 minutes in Restore™ PLUS Western Blot stripping buffer. Following stripping, the membrane was washed in 0.1% TBS-Tween20® and blocked in 5% Marvel in 0.01% TBS-Tween20® for 20 minutes at room temperature. All incubations and washing steps were carried out on a shaking platform.

At this stage, the membranes were either re-probed with antibodies, or dried and stored at room temperature.

2.2.2.8 Invitrogen western blotting system

Pre-cast 4-12% Bis-Tris NuPAGE gels were used. The gels were placed in an Invitrogen gel running apparatus containing running buffer (1X made from a 20X stock of MOPS SDS Running buffer, made up to 1 L in distilled water). 10 – 20 µL/well of protein sample and 8

μL/gel of molecular weight marker were added. The gels were run using a constant current of 200 V per box, until the dye front from the sample buffer ran off the end of the gel – 45 to 60 minutes.

The resolved proteins were transferred to a PVDF or nitrocellulose membrane using the iBlot2 system. Regular stacks were used and the transfer was performed using the P0 pre-set program (20 V for 1 minute, 23 V for 4 minutes, 25 V for 2 minutes). Following protein transfer, non-specific binding of proteins to the membrane was prevented by incubating the membrane for 1 hour at room temperature with LiCOR Odyssey Blocking Buffer (PBS). Following blocking, the membranes were incubated with the antibody to the protein of interest diluted in the LiCOR buffer in the same manner as previously described. After the incubation, the membranes were washed three times with PBS for at least 10 minutes at room temperature. The membranes were then incubated for 2 hours at room temperature in the dark with secondary 800 CW or 680 CW LiCOR antibodies. The membranes were then washed again three times in PBS for at least 10 minutes at room temperature. The membranes were visualised using an Odyssey scanner.

2.2.3 Protein immunoprecipitation

Cell lines were seeded at 0.4×10^6 cells/mL and primary cells at 1×10^6 cells/mL in 10 cm dishes and left at 37°C, 5% CO₂ overnight. Following incubation with the corresponding stimulus, the media was removed and cells were washed twice with ice-cold PBS. Cells were then lysed and harvested in 800 μL of low stringency lysis buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 0.5% (v/v) NP-40) with freshly added protease inhibitors (1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/mL Leupeptin, 11.5 μg/mL aprotinin and 1 mM sodium orthovanadate), transferred to pre-cooled microcentrifuge tubes and left on ice for 10 minutes. Lysates were centrifuged at 1610 x g for 10 minutes at 4°C. 50 μL lysate was removed, mixed with 20 μL of 5X sample buffer and saved for further SDS-PAGE and western blot analysis as previously described.

2.2.3.1 GAPDH and succinyl-lysine immunoprecipitation

The lysates were pre-cleared for 30 minutes with 15 μL Protein A/G Plus agarose beads in a rotator at 4°C. Prior to incubation with the lysates, 3 μg of GAPDH antibody, 10 μg succinyl-lysine antibody or the corresponding control antibody, were incubated with 30 μL Protein A/G Plus agarose beads overnight, rotating at 4°C. The pre-cleared lysates and the pre-coupled beads were centrifuged at 80 x g for 3 minutes at 4°C. The pre-coupled beads were washed by adding 1 mL of low stringency buffer, centrifuging at 80 x g and removing all the liquid. They were washed three times and then reconstituted in 15 μL of low stringency buffer. The

pre-cleared lysates on the other hand, were transferred to new pre-cooled microcentrifuge tubes to which 30 μ L of pre-coupled antibodies/beads were added and incubated for 2 to 4 hours rolling at 4°C. The lysates with beads were centrifuged at 80 x g for 3 minutes at 4°C, the supernatant was removed and the beads were washed three times as previously described. The immune complexes were eluted by adding 30 μ L of 5X sample buffer and boiling samples for 5 minutes at 95°C. Further analysis was carried out by SDS-PAGE and western blotting.

2.2.3.2 Malonyl-lysine immunoprecipitation

10 μ g of the corresponding antibody (malonyl-lysine or rabbit IgG) were added to each lysate and were left rotating at 4°C for 12 hours overnight. 200 μ L of A/G protein beads were washed with low stringency lysis buffer three times as previously described. They were resuspended in 100 μ L of low stringency lysis buffer and 50 μ L added to each lysate. Lysates were left rolling for 2 hours at room temperature. They were then centrifuged at 3,500 x g for 1 minute and the liquid removed. The beads were washed three times followed by snap-freezing in liquid nitrogen and storage in -80°C. When ready for use, immune complexes were eluted by adding 20 μ L of 5X sample buffer and boiling samples for 5 minutes at 95°C. Further analysis was carried out by SDS-PAGE and western blotting.

2.2.3.3 Myc immunoprecipitation

50 μ L of A/G beads were prepared per condition and washed by adding 1 mL of low stringency buffer, centrifuging at 80 x g and removing all the liquid. They were washed three times and then reconstituted in 25 μ L of low stringency buffer. The washed beads were added to the lysates together with 5 μ g of anti-myc antibody and the samples were incubated for 5 hours rolling at 4°C. The lysates with beads were centrifuged at 80 x g for 3 minutes at 4°C, the supernatant was removed and the beads were washed three times as previously described. The immune complexes were eluted by adding 30 μ L of 5X sample buffer and boiling samples for 5 minutes at 95°C. Further analysis was carried out by SDS-PAGE and western blotting.

2.2.4 BCA assay

A Pierce™ BCA protein assay kit was used to calculate the amount of protein and normalise it among samples prior to immunoprecipitation. 25 μ L of a 1:2 and a 1:5 dilution of samples were added to a 96 well plate in triplicate. A standard curve was prepared following manufacturer's instructions (Thermo Fisher Scientific). The BCA working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B, and 200 μ L added to each well. The plate was thoroughly mixed, and was incubated at 37°C for 30 minutes in

the dark. The absorbance of the samples at 562 nm was measured using a spectrophotometre and the protein concentration of the samples calculated based on the standard curve.

2.2.5 Identification of malonylated proteins by liquid chromatography – mass spectrometry (LC/MS)

For the identification of malonylated proteins, malonyl-lysine immunoprecipitations were performed as previously described, and were separated on 4 – 12% Bis-Tris Bolt gels. Gels were stained in 20 mL of Instant Blue Coomassie solution overnight, and were then washed with distilled water until gel background was clear.

2.2.5.1 Trypsin in-gel digestion

The selected bands were excised with a clean scalpel, chopped into small cubes and transferred into clean eppendorfs. They were washed with 150 μ L water for 5 minutes on an orbital shaker, the tube spun and the liquid removed with a long gel loading pipette tip. 50 μ L of acetonitrile (ACN) was added for 10 – 15 minutes to allow the gel pieces to shrink. The ACN was removed with a gel loading tip and the tubes left open to dry for 10 minutes.

The gel pieces were then swollen in 50 μ L 1.5 mg/mL DTT, 0.1 M ammonium bicarbonate for 30 minutes at 56°C to reduce the protein. Following centrifugation, liquid was removed and 50 μ L ACN added to shrink the gel pieces. The ACN was removed with a gel loading tip and the tubes left open to dry for 10 minutes.

The gel pieces were then swollen in 50 μ L 10 mg/mL iodoacetamide, 0.1 M ammonium bicarbonate for 20 minutes in the dark, to derivatise the cysteine residues in the protein. Liquid was then removed and the gel pieces washed with 200 μ L 0.1 M ammonium bicarbonate for 15 minutes on an orbital shaker. Following centrifugation, liquid was removed and 50 μ L ACN added to shrink the gel pieces. The ACN was removed with a gel loading tip and the tubes left open to dry for 20 minutes.

The dry gel pieces were then swollen in 15 μ L digestion buffer (50 mM ammonium bicarbonate, 5 mM calcium chloride) containing 100 ng/ μ L trypsin for 45 minutes on ice. The sample was then digested overnight at 37°C. The next day, the samples were first centrifuged followed by the addition of 15 μ L of 1% trifluoroacetic acid (TFA). They were left at room temperature for 1 hour.

2.2.5.2 LC/MS analysis

Peptides were injected onto a C18 spray tip and analysed by QExactive™ Orbitrap mass spectrometer. The raw data was searched in Mascot. Trypsin was specified as the cleavage

enzyme, allowing up to one missing cleavage. Carbamidomethylation on cysteines was specified as a fixed modification.

2.2.6 Identification of post-translational modifications by LC/MS

Mass spectrometry of GAPDH immunoprecipitated samples was performed by PTM Biolabs in Zhejiang, China.

2.2.6.1 Trypsin in-gel digestion

The protein samples were first separated on a 12% bis-tris polyacrylamide gel and stained with coomassie blue. The bands corresponding to GAPDH were cut and destained in 50 mM Ambic in 50% (v/v) ACN until clear. Gel pieces were dehydrated with 100 μ L of 100% ACN for 5 minutes followed by rehydration in 10 mM DTT at 5°C for 60 minutes. Gel pieces were dehydrated again, but then rehydrated with 55 mM iodoacetamide and left at room temperature in the dark for 45 minutes. The samples were dehydrated again and then rehydrated with 10 ng/ μ L trypsin on ice for 1 hour. The samples were then digested with trypsin at 37°C overnight, and the peptides extracted with 50% ACN/5% formaldehyde, followed by 100% ACN.

2.2.6.2 LC/MS analysis

Peptides were dissolved in 0.1% formaldehyde and loaded onto a reversed-phased column packed with 3 μ m C18 beads and eluted with a linear gradient of 6 – 20% solvent B (0.1% formaldehyde in 98% ACN) for 26 minutes and 20 – 35% solvent B for 10 minutes at a constant flow rate of 300 nL/min on an EASY-nLC 1000 UPLC system. The resulting peptides were analysed by QExactive™ Orbitrap mass spectrometre. Trypsin was specified as cleavage enzyme, allowing up to four miscleavages. Carbamidomethylation on cysteine was specified as fixed modification and oxidation on methionine, succinylation, acetylation, malonylation or crotonylation on lysine, phosphorylation on serine, threonine or tyrosine, were specified as variable modifications.

2.2.7 MalAMyne Cu(I)-catalysed click chemistry

2.2.7.1 Labelling of cells with MalAMyne

Cells were seeded at 1×10^6 cells/mL in 10 cm dishes and left at 37°C, 5% CO₂ overnight. Following incubation with the corresponding stimulus, cells were treated with 10 μ M MalAMyne and the corresponding volume of DMSO for 2 hours. Following labelling, cells were washed with cold PBS twice and were then lysed in lysis buffer (1% NP40, 150 mM NaCl, 50 mM HEPES, 2 mM MgCl₂, 20% glycerol, pH 7.4) containing 1X protease inhibitor mix. Lysates

were centrifuged at 16,000 x g for 15 minutes at 4°C, and the supernatant collected. Protein concentration was determined using BCA assay, as previously described.

2.2.7.2 Cu(I)-catalysed biotin click chemistry

To the cell lysates, 50 µM of cleavable biotin azide was added, followed by 1 mM tris(2-carboxyethyl)phosphine (TCEP), 100 µM tris(benzyltriazolmethyl)amine (TBTA) and 1 mM CuSO₄ to initiate the reaction. The reactions were left rolling for one and a half hours at room temperature. The click chemistry reaction was stopped by adding 4 mL of ice-cold acetone to precipitate proteins, and the samples stored at -20°C overnight.

2.2.7.3 Streptavidin affinity enrichment of biotinylated proteins

Samples were centrifuged at 3,500 x g for 5 minutes at 4°C. The liquid was removed and the samples were washed with ice-cold methanol twice and left to dry for 10 minutes, and then dissolved in PBS with 4% SDS, 20 mM EDTA and 10% glycerol. The samples were then diluted 1 in 8 with PBS to give a final concentration of SDS of 0.5%. 50 µL of high capacity streptavidin agarose beads were added to bind to the biotinylated proteins and left rotating for one and a half hours at room temperature. The beads were then centrifuged at 6,000 x g for 1 minute, followed by washing first with wash buffer 1 (PBS, 0.2% SDS), then wash buffer 2 (6 M Urea in PBS, 0.1% SDS), and finally wash buffer 3 (250 mM NH₄HCO₃, 0.05% SDS). Proteins were eluted by incubating with 100 µL 25 mM Na₂S₂O₄, 250 mM NH₄HCO₃ and 0.05% SDS for 1 hour. The eluted proteins were dried down by centrifuging on a SpeedVac for 1 hour. The dried proteins were resuspended in LDS sample loading buffer with 50 mM DTT, and analysed via western blotting as previously described.

2.2.8 RNA analysis

2.2.8.1 RNA extraction using RNeasy kit

Cells were rinsed in ice-cold PBS prior to the addition of 350 µL RLT lysis buffer (QIAGEN). The cell lysates were then frozen and thawed prior to the extraction of the RNA using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. The RNA was quantified using a Nanodrop 2000 micro-volume UV-visible spectrophotometre and normalised using nuclease-free water.

2.2.8.2 RNA extraction using QIAzol

1 mL of QIAzol lysis reagent was added to each sample and frozen at -20°C or left at room temperature for approximately 5 minutes. 300 µL chloroform was then added, followed by shaking of the samples vigorously for 15 seconds. They were then placed at room temperature

for 2 – 3 minutes followed by a centrifugation step at 12,000 x g at 4°C for 15 minutes. The upper aqueous phase was transferred into a new eppendorf with 500 µL of isopropanol and 3 µL of LPA, which was used as an RNA carrier. The samples were next vortexed for 10 seconds and frozen at -80°C for a couple of hours or overnight. They were then defrosted at room temperature and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatants were discarded and the pellet washed with 200 µL of 75% ethanol. Samples were centrifuged at 7,500 x g for 5 minutes at 4°C. They were then washed for a second time with 50µL of 75% ethanol. The supernatants were removed and the pellet was resuspended in 20 µL of RNase-free water. The quality of the RNA was analysed using a Nanodrop 2000 micro-volume UV-visible spectrophotometre.

2.2.8.3 Reverse Transcription PCR (RT-PCR)

RT-PCR was carried out on RNA to create cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). The following reaction mix was prepared for each sample:

- 2 µL 10X RT Buffer
- 1 µL 100 mM deoxyribonucleotide triphosphates (dNTPs)
- 1 µL reverse transcriptase
- 2 µL random primers
- 4 µL nuclease free water
- 10 µL RNA

The following parametres were used on a thermal cycler:

- 25°C for 10 minutes
- 37°C for 2 hours.
- 85°C for 15 minutes
- 4°C ∞

The cDNA was either used directly for further experiments or stored at -20°C for later use.

2.2.8.4 Real Time quantitative PCR using Taqman® gene expression assays

FAM labelled primers were designed by Applied Biosystems. The following reaction mix was prepared for each quantitative PCR sample:

- 5.5 µL Taqman gene expression mastermix
- 0.55 µL gene specific primer
- 1.95 µL DEPC-treated water
- 2 µL cDNA

The following parameters were used on an Applied Biosystems 7900 Fast system:

40 cycles of 95°C for 5 seconds, followed by 60°C for 30 seconds.

2.2.8.5 Design and validation of Sybr primers

SYBR primers (**Table 2.3**) were designed using the online primer design tool IDT PrimerQuest. The UCSC genome browser was used to make sure the primers would span an exon-exon border so as to distinguish between genomic DNA and spliced mRNA, and that they would amplify a product of the right size. Primer-BLAST was used to test the specificity of the primers.

Table 2.3 SYBR primer sequences.

Target	Sense primer sequence	Antisense primer sequence
m18S	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
mGAPDH	TGTGTCCGTGGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT
mBeta actin	CACAGAGCCTCGCCTTT	GAGCGCGGCGATATCAT
mIL1 β	TGGCAACTGTTCTG	GGAAGCAGCCCTTCATCTTT
mIL6	ACACATGTTCTCTGGGAAATCGT	AAGTGCATCATCGTTGTTCATACA
mIL10	AGGCGCTGTCATCGATTT	CACCTTGGTCTTGGAGCTTAT
mIL12	TGTCCTCAGAAGCTAACCA	GTCCAGTCCACCTCTACAA
mTNF α	GCCTCTTCTCATTCCTGCTT	TGGGAACCTTCTCATCCCTTTG
mCXCL1	CCGAAGTCATAGCCACACTCAA	AATTTTCTGAACCAAGGGAGCTT
mIFN β	GCTCCTGGAGCAGCTGAATG	CGTCATCTCCATAGGGATCTTGA
mPTGS2	CGGACTGGATTCTATGGTGAAA	CTTGAAGTGGGGTCAGGATGTAG
mHIF1 α	CCCATTCTCATCCGTCAAA	CGGCTCATAACCCATCAACT
mNOS2	GGAATCTTGGAGCGAGTTGT	CCTCTTGTCTTTGACCCAGTAG
mIL1 α	GCTTGAGTCGGCAAAGAAATC	GAGAGATGGTCAATGGCAGAA
mNLRP3	TGTGTGGATCTTTGCTGCGA	ACCCATCCACTCTTCTTCAAGGCT
mDAK1	TGATGACCAGTCCACCAAAG	GAAGTACACAGGGTTTCCAGAG

2.2.8.6 Real Time quantitative PCR using SYBR green

The following reaction mix was prepared for each sample:

- 5 μ L 2X KAPA SYBR fast master mix
- 0.2 μ L 10 μ M gene specific primers (forward and reverse)
- 2.8 μ L DEPC-treated water
- 2 μ L cDNA

Additionally, a four-point standard curve was run using a pool of murine cDNA the first two times a new primer was used, to test for the presence of a single PCR product and acceptable primer efficiency. The same parameters were used as for Taqman®, with the addition of a final dissociation stage.

2.2.8.7 Data analysis

The $\Delta\Delta C_t$ method was used to calculate the relative expression values. The ΔC_t value of the control sample was subtracted from the ΔC_t value of each test sample. The resulting $\Delta\Delta C_t$ value was normalised using $2^{(\Delta\Delta C_t)}$. The effects of loading and RNA normalisation were then corrected in each sample by dividing the value of the gene probe by the value of the endogenous control. When more than one endogenous control was used, the geometric mean of all controls was used. The final results represent the fold change in expression of a test sample relative to the control sample.

2.2.9 Enzyme linked immunosorbent Assay (ELISA)

Supernatants were screened for murine TNF α , IL6, IL10 and RANTES production using ELISA kits according to manufacturer's instructions (R&D Systems). The optical density values were measured at 450 nm and concentration was calculated using a standard curve.

2.2.10 Meso Scale Discovery (MSD) assay

Supernatants were screened for human IFN γ , IL2, IL6, IL8, IL10, IL12p70, TNF α and GM-CSF production using a 9-plex MULTI-SPOT 96 well MSD following manufacturer's instructions. Supernatants were also screened for murine IL6, IL10, IL12p70 and TNF α production using an 8-plex MULTI-SPOT 96 well MSD following manufacturer's instructions. Plates were read and analysed on the MSD Sector® Imager.

2.2.11 Luminex cytokine assay

2.2.11.1 Antibody-bead pre-coupling

A vial of beads (1.25×10^7 beads) was vortexed and sonicated prior to being transferred to an eppendorf. They were washed with 500 μ L of triton-containing activation buffer (0.1 M sodium phosphate pH 6.1, 0.025% triton) and centrifuged for 1 minute at 10,000 x g. The liquid was removed, and the same washing step was repeated, followed by the addition of 320 μ L of activation buffer (0.1 M sodium phosphate pH 6.1). The beads were vortexed and sonicated, and 40 μ L of N-hydroxysulfosuccinimide (NHS) at 11 mg/mL and 40 μ L 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) at 75 mg/mL were added. The eppendorfs were wrapped in foil and incubated for 1 hour at room temperature on a rotor. The beads were then washed with 500 μ L of triton-containing coupling buffer (5 mM sodium acetate pH 5.0, 0.025% triton) and centrifuged for 1 minute at 10,000 x g. The same liquid was removed, and the same washing step was repeated, followed by the addition of 1 mL of the corresponding antibody diluted in coupling buffer (5 mM sodium acetate pH 5.0) to 50 μ g/mL. Following vortexing, the eppendorfs were wrapped in foil and incubated at room temperature on a rotor. The liquid was then removed and the beads washed with 500 μ L of wash buffer (50 mM Na_2HPO_4 , 150 mM NaCl, 0.025% triton) and centrifuged for 1 minute at 10,000 x g. The liquid was removed, and the same washing step was repeated. The beads were then resuspended in 1 mL of storage buffer (50 mM Na_2HPO_4 , 150 mM NaCl, 0.025% triton, 10 mg/mL BSA) and stored at 4°C.

2.2.11.2 Luminex assay

A 96-well filter plate was pre-wetted by adding 50 μ L of luminex assay buffer per well for five minutes. The buffer was then removed from the wells via vacuum filtration. A combined suspension of capture antibody-coupled beads corresponding to the cytokines to be used, was prepared in luminex buffer at a concentration of 1×10^5 per mL for each bead set, by carrying out a 1 in 100 dilution of the original stock.

An 11-point standard curve was prepared with a starting concentration of 10,000 pg/mL, using 3-fold dilutions in luminex assay buffer (60 μ L of standards added to 120 μ L buffer). 50 μ L of the standard or sample was added to each well in triplicate, followed by the addition of 50 μ L of the combined bead suspension to each well. The plates were shaken at 700 rpm for 2 to 5 minutes and incubated overnight at 4°C in the dark. The next day the beads were washed twice with 100 μ L of luminex buffer and the liquid removed via vacuum filtration. 50 μ L per well of the corresponding biotinylated detection antibodies were added per well at a final concentration of 500 ng/mL in luminex buffer, from a stock concentration of 500 μ g/mL. The

plate was left shaking initially at 1,200 rpm in the dark for 2-5 minutes, and then at 700 rpm for 1 hour. The beads were then washed twice with 100 μ L of luminex buffer. Liquid was removed via vacuum filtration. 50 μ L of streptavidin-PE was added to each well at a final concentration of 250 ng/mL in luminex buffer and the plate was shaken at 700 rpm in the dark for 30 minutes. The beads were then washed twice with 100 μ L of luminex buffer and the liquid was removed via vacuum filtration. 120 μ L of sheath fluid was added per well and the plate shaken at 1,200 rpm for one minute and immediately read on the Luminex xMAP (xPLORE software), with bead count set to 100. Standard curves were generated and sample values determined by manufacturer's software.

2.2.12 Malonyl-CoA ELISA

The supernatants were removed from the cells, and they were lysed by being stored at -80°C in 1 mL of PBS, followed by three freeze-thaw cycles at -20°C. Lysates were assayed for production of malonyl-CoA using a malonyl-CoA ELISA following manufacturer's instructions (Cusabio). The optical density values were measured at 450 nm and 570 nm, with readings at 570 nm subtracted from those at 450 nm to account for optical imperfections in the plate. Malonyl-CoA concentration was calculated using the standard curve.

2.2.13 GAPDH enzymatic assay

The medium was removed from the cells and they were washed twice with ice-cold PBS. 100 μ L of cell lysis buffer was added per well and the plate was incubated at 4°C on a rocking platform for 20 minutes to allow cell lysis to occur. The lysates were then centrifuged at 14,000 x g for 4 minutes at 4°C. The supernatants were transferred to fresh eppendorfs and frozen at -80°C until required.

2.2.13.1 Enzymatic assay as absorbance versus time

When the enzymatic activity was to be represented as a plot of absorbance versus time, the following reaction mixture was used per sample:

- 121.25 μ L of GAPDH assay buffer
- 10 μ L of 100 mM 3-phosphoglyceric acid
- 5 μ L of 100 mM L-cysteine
- 2.5 μ L of 7 mM β -NADH
- 5 μ L of 34 mM ATP
- 1.25 μ L of 3-phosphoglycerate kinase (400 Units/mL)

The assay mixture was prepared for each sample and transferred to a 96 well plate. The initial absorbance was measured at 340 nm. 5 µL of each sample was added to each well. Five different GAPDH standards ranging from 0.2 to 1 Units/mL were also generated from a 200 Units/mL stock and included in the assay as a positive control. Once the samples and the standards were added to the plate and mixed, the rate of NADH oxidation was measured, which is proportional to the reduction in absorbance at 340 nm over time. Data was collected every 3 minutes over the course of 12 minutes.

2.2.13.2 Enzymatic assay as absorbance versus substrate concentration

When the enzymatic activity was to be represented as a plot of absorbance versus substrate concentration (NADH), the same reaction mixture as previously stated was used except for the β-NADH additions. Three different concentrations of β-NADH were used: 60 µM, 120 µM and 240 µM. The overall volume was kept at 145 µL of assay mixture by adjusting the volume of GAPDH assay buffer. Absorbance values at 340 nm were measured every 2 minutes over the course of 10 minutes.

2.2.13.3 Colorimetric GAPDH enzymatic assay

GAPDH enzymatic activity was measured from lysates following manufacturer's instructions (Abcam). The absorbance was measured at 450 nm every 2 – 3 minutes for 12 minutes at 37°C. GAPDH enzymatic activity was calculated as per below:

$$\text{GAPDH enzymatic activity} = \left(\frac{B}{\Delta T \times V} \right) \times D = \text{nmol/min/}\mu\text{L} = \text{mU/}\mu\text{L}$$

ΔT = reaction time (minutes)

V = original sample volume added into the reaction well (µL)

D = sample dilution factor

B = amount of NADH, calculated as:

$$\Delta A_{450} = (A_2 - A_{2B}) - (A_1 - A_{1B})$$

where A_1 is the sample reading at time 1, and A_{1B} is the control at time 1. ΔA_{450} is used to obtain B , the nmol of NADH generated by GAPDH during the reaction time.

2.2.14 Cytotoxicity assay

A Cytotox96® non-radioactive cytotoxicity assay kit was used to determine LDH release from the cells. As positive control, maximum LDH release was determined by the addition of a 10X

Lysis solution (9% (v/v) Triton X-100) to control cells for 1 hour prior to harvesting. Following stimulations, supernatants were harvested from the cells and 50 μ L added per well to a flat-bottomed 96 well plate. The media used to carry out the experiment is used as a negative control. 50 μ L of the substrate mix were added to each well and incubated for 30 minutes at room temperature in the dark. The reaction was stopped using 50 μ L of 1 M acetic acid and the optical density of each well was determined at 490 nm using an ELISA plate reader. The percentage of cytotoxicity was determined by comparison of the OD values of the experimental supernatants to the maximum LDH controls (100% cytotoxicity).

2.2.15 RNA-immunoprecipitation (RIP)

Cells were seeded at 1×10^6 cells/mL in 15 cm dishes. Once the corresponding treatments were carried out, they were washed with cold PBS. They were then fixed with 15 mL of 1% formaldehyde for 15 minutes at room temperature followed by 3 mL of 1 M glycine for 5 minutes. The cells were then washed prior to being lysed with 1 mL per dish of freshly made polysome lysis buffer (0.1 M HEPES, 0.1 M KCl, 5 mM $MgCl_2$, 0.5% (v/v) NP40, 1 mM DTT) to which 1X protein inhibitor cocktail was added. Additionally, to 10 mL of polysome lysis buffer, 20 μ L of vanadyl ribonucleoside complexes as well as 5 μ L RNase inhibitors were added. The cells were scraped and the samples transferred to pre-cooled eppendorfs and left on ice for 5 – 10 minutes to allow lysis to occur. They were then centrifuged at $10,000 \times g$ for 15 minutes at 4°C.

2.2.15.1 Immunoprecipitation

100 μ L of A/G beads were prepared per sample by washing with 500 μ L of NT2 buffer (50 mM TrisHCl pH 7.4, 0.3 M NaCl, 5 mM $MgCl_2$, 0.01% NP40) and centrifuging at $100 \times g$ for 2.5 minutes. The liquid was removed and the same washing step repeated two more times. Beads were then resuspended in 50 μ L of NT2 buffer and added to the lysates, together with 10 μ g of the corresponding antibody (GAPDH or control IgG). The samples were left rolling at 4°C for 6 hours. They were then centrifuged at $10,000 \times g$ for 30 seconds at 4°C and then washed as previously described with 500 μ L of freshly made polysome lysis buffer.

2.2.15.2 RNA isolation

After the last wash, the supernatant was aspirated carefully and as much as possible removed. 85 μ L of protein-RNA elution buffer (0.1 M TrisHCl pH 8, 0.01 M EDTA, 1% SDS) was added to each sample and they were incubated at 37°C for 10 minutes. This was followed by a two minute centrifugation step at $604 \times g$. The samples were incubated again at 37°C for 10

minutes, followed by the same centrifugation step. 5 µL of eluate were removed at this stage for western blot analysis.

3 µL of 5 M NaCl and 20 µg of proteinase K were added to 80 µL of eluates. The samples were then incubated at 42°C for 1 hour in order to digest cross-linked polypeptides, followed by another hour incubation at 65°C to reverse formaldehyde cross-links. The RNA was then extracted using the Qiazol method previously described. This was followed by a reverse transcription and quantitative PCR reaction, both of which have also been previously described. Relative enrichment was calculated by dividing the signal of the target gene in the GAPDH IP over that of the IgG control.

2.2.16 RIP-Seq

2.2.16.1 Immunoprecipitation and RNA isolation

BMDMs were seeded at 1×10^6 cells/mL in 15 cm dishes in duplicate. Immunoprecipitation and protein-RNA elution were performed as previously described. Prior to RNA extraction by the addition of Trizol, the eluates corresponding to the same sample were pooled. RNA isolation was performed using phenol-chloroform as previously described.

The quality and quantity of the RNA was first checked on a Nanodrop 2000 micro-volume UV-visible spectrophotometre. The RNA was then run on a high sensitivity RNA ScreenTape kit on the Tapestation following manufacturer's instructions (Agilent Technologies). The RNA integrity value (RIN) of the samples generated by the Tapestation cannot be used, as it uses an algorithm that looks for 18S and 28S peaks in the samples, and given that GAPDH doesn't bind any of those RNAs, they should not be present in the samples. It can however be used to visualise the RNA on the gels, as another means of estimating quantity.

2.2.16.2 Library generation

The library was generated using the TruSeq® Stranded Total RNA LT kit, low sample protocol.

2.2.16.2.1 Ribo-Zero deplete and fragment RNA

10 µL of RNA were added to a 96 well 0.3 mL PCR plate, followed by 5 µL of rRNA binding buffer and 5 µL of rRNA removal mix. The entire volume of each well was mixed six times, and the plate placed on a thermal cycler where an RNA denaturation program (100°C pre-heat lid, 68°C for 5 minutes) was run. The plate was then left to incubate at room temperature for a minute, and the contents then transferred to a new plate.

rRNA removal beads were vortexed and 35 μ L added to each well. The samples were thoroughly mixed twenty times, and the plate left to incubate at room temperature for a minute. The plate was then placed on a magnetic stand, and after a minute, 60 μ L of samples were transferred to a new plate, to which 110 μ L of RNAClean XP beads were added. The samples were mixed ten times and the plate was left for 15 minutes at room temperature, followed by a 5 minute incubation on a magnetic stand. The liquid was then discarded and the beads were washed with 200 μ L 70% ethanol for 30 seconds and then removed. They were left to dry for 15 minutes and were then removed from the magnetic stand. 11 μ L of elution buffer were added, and everything mixed ten times. The plate was then left to incubate first at room temperature for two minutes, and then on the magnetic stand for five minutes. 8.5 μ L of sample were transferred to a new plate, to which 8.5 μ L of Elute, Prime, Fragment high mix were added. The plate was placed on a thermal cycler and the Elution-Fragment-Prime program (100°C pre-heat lid, 94°C for 8 minutes, 4°C ∞) was run.

2.2.16.2.2 cDNA synthesis

50 μ L of SuperScript II (Invitrogen) were first added to the first strand synthesis Act D mix, and then 8 μ L of the SuperScript/Act D mix were added to the samples, which were mixed six times. The plate was placed on a thermal cycler and the first strand synthesis program was run with the following parameters and a lid pre-heated at 100°C:

- 25°C for 10 minutes
- 42°C for 15 minutes
- 70°C for 15 minutes
- 4°C ∞

5 μ L resuspension buffer and 20 μ L second strand marking master mix were added to each well of the PCR plate and the contents were mixed six times. The plate was then placed on the thermal cycler and incubated at 16°C. After 1 hour the plate was removed and left to reach room temperature.

AMPure XP beads were vortexed and 90 μ L added to the samples, which were thoroughly mixed ten times. The plate was left at room temperature for 15 minutes, followed by a 5 minute incubation on a magnetic stand. 135 μ L of liquid was discarded, and the beads were washed with 200 μ L 80% ethanol for 30 seconds. The ethanol was discarded and the same washing step was repeated once more. The plate was then left at room temperature for 15 minutes to dry and it was then removed from the magnetic stand. 17.5 μ L of resuspension buffer were added, and the plate left at room temperature for 2 minutes, followed by a 5 minute incubation

on a magnetic stand. While on the magnetic stand, 15 µL of cDNA samples were transferred to a new PCR plate, and stored overnight at -20°C

2.2.16.2.3 3' ends adenylation and adapter ligation

Samples were defrosted and 2.5 µL resuspension buffer and 12.5 µL A-tailing mix were added. The samples were mixed ten times, and the plate was placed on the thermal cycler and the ATAIL70 program was run with the following parameters and a lid pre-heated at 100°C:

- 37°C for 30 minutes
- 70°C for 5 minutes
- 4°C ∞

A randomization scheme was generated for the assignment of RNA adapter indexes across two sequencing lanes. The plate was removed from the thermal cycler and 2.5 µL resuspension buffer, 2.5 µL ligation mix and 2.5 µL of the corresponding RNA adapter index were added to each sample. They were thoroughly mixed ten times and placed on the thermal cycler where they were incubated at 30°C. After 10 minutes, the plate was removed and 5 µL of stop ligation buffer were added.

AMPure XP beads were vortexed and 42 µL added to the samples, which were thoroughly mixed ten times. The plate was left at room temperature for 15 minutes, followed by a 5 minute incubation on a magnetic stand. 79.5 µL of liquid was discarded, and the beads were washed with 80% ethanol twice as previously described. The plate was then left at room temperature for 15 minutes to dry and it was then removed from the magnetic stand. 52.5 µL of resuspension buffer were added, and the plate left at room temperature for 2 minutes, followed by a 5 minute incubation on a magnetic stand. While on the magnetic stand, 50 µL of samples were transferred to a new PCR plate. The same methodology was repeated once more, this time adding 50 µL AMPure XP beads, discarding 95 µL of liquid and resuspending the beads in 22.5 µL resuspension buffer. At the end, 20 µL of samples were transferred to a new PCR plate.

2.2.16.2.4 DNA fragments enrichment

5 µL PCR primer cocktail and 25 µL PCR mastermix were added to the plate, and the samples were thoroughly mixed ten times. The plate was placed on the thermal cycler and the PCR program was run with the following parameters and a lid pre-heated at 100°C:

- 98°C for 30 seconds
- 15 cycles: 98°C for 10 seconds
60°C for 30 seconds
72°C for 30 seconds
- 72°C for 5 minutes
- 4°C ∞

AMPure XP beads were vortexed and 50 µL added to the samples, which were thoroughly mixed ten times. The plate was left at room temperature for 15 minutes, followed by a 5 minute incubation on a magnetic stand. 95 µL of liquid was discarded, and the beads were washed with 80% ethanol twice as previously described. The plate was then left at room temperature for 15 minutes to dry and it was then removed from the magnetic stand. 32.5 µL of resuspension buffer were added, and the plate left at room temperature for 2 minutes, followed by a 5 minute incubation on a magnetic stand. While on the magnetic stand, 30 µL of samples were transferred to a new PCR plate.

2.2.16.3 Library quality check and quantification

2 µL of library prep were loaded on an Agilent DNA 1000 kit and run on a Bioanalyser following manufacturer's instructions (Agilent Technologies). The detection of a band between 200 and 300 bp was used to determine the success of the library generation. The library was then quantified by George Royal using qPCR and all samples normalised to 10 nM for sequencing.

2.2.16.4 Sequencing and analysis

The cBot Cluster Generation System was first used to generate DNA templates for sequencing through bridge amplification. Samples were then sequenced on the HiSeq 1500 (indexed paired end run). Sequencing results were analysed by Steve DeHaro.

2.2.17 Bacterial work

2.2.17.1 Bacterial transformation

Competent DH5α *Escherichia Coli* (*E. Coli*) were transformed with 0.5 – 1 µg of DNA by heat shock for 45 seconds at 42°C, following a 20 minute incubation of the the DNA or plasmids on ice. After heat shock, the bacteria were placed on ice for 2 minutes. Room temperature SOCS media was then added and incubated at 37°C for 1 – 2 hours. Cultures were then spread on agar plates containing the corresponding antibiotic, and incubated overnight at 37°C.

2.2.17.2 Bacterial culture for minipreps and maxipreps

Single bacterial colonies of *E. Coli* were picked and grown in Lysogeny Broth (LB) media containing the corresponding antibiotic overnight in a shaking incubator at 37°C. For maxipreps, 100 mL of culture were pelleted and the plasmids isolated following manufacturer's instructions (Endofree plasmid maxi kit, QIAGEN). For minipreps, 3 mL of culture were used and the plasmids isolated following manufacturer's instructions (Endofree plasmid mini kit, QIAGEN).

2.2.18 Site-directed mutagenesis

2.2.18.1 Mutagenesis reaction

Mutagenesis primers were designed with the QuickChange Primer Design Tool (**Table 2.4**), and site-directed mutagenesis was performed using the QuickChange Lightning kit.

Table 2.4 Mutagenesis primers

Mutant primer	Sequence
K213Q forward primer	CTACTGGTGCTGCCCAGGCTGTGGGCAAGG
K213Q reverse primer	GATGACCACGACGGGTCCGACACCCGTTCC
K213E forward primer	CTACTGGTGCTGCCGAGGCTGTGGGCAAGG
K213E reverse primer	GATGACCACGACGGCTCCGACACCCGTTCC

The following 20 µL control reaction was first set up:

- 2 µL of 10X reaction buffer
- 0.8 µL (10 ng) of pWhiteScript 4.5 Kb control plasmid
- 0.5 µL (125 ng) of oligonucleotide control primer #1
- 0.5 µL (125 ng) of oligonucleotide control primer #2
- 0.4 µL dNTP mix
- 15.8 µL water

The following mutagenesis reactions using the hGAPDH-DKK-myc plasmid were set up:

- 40, 80 or 160 ng of plasmid
- 0.5 µL (100 µM) forward primer
- 0.5 µL (100 µM) reverse primer
- 2 µL 10X reaction buffer
- 0.4 µL dNTP mix

All reactions were made up to 20 µL with water. Directly prior to the PCR reaction, 0.5 µL of Pfu Ultra was added to each reaction. The following cycling parameters were used:

- Stage 1: 95°C – 30 seconds
- Stage 2: 95°C – 30 seconds
 - For K213Q: 62°C – 1 minute
 - For K213E: 55°C – 1 minute
 - 68°C – 8 minutes
- Stage 3: 68°C – 15 minutes
- Stage 4: 4°C - ∞

2.2.18.2 Digestion of amplification products and transformation

0.5 µL of the restriction enzyme Dpn I were added to each reaction. The samples were mixed and then incubated at 37°C for 2 hours so as to digest the parental DNA.

XL-1 Blue Supercompetent bacteria were defrosted on ice, and 4µL of each mutagenesis reaction was added to 20 µL of bacteria in pre-chilled eppendorfs. Bacteria were transformed and grown, and plasmids isolated as previously described.

2.2.18.3 Sequencing

The sequencing primers shown in **Table 2.5** were used. 20 µL reactions were set up as follows in a sequencing 96 well plate:

- 1 µL of primer (100µM stock)
- 1 µg of DNA
- water up to 20 µL

Sequencing was performed in-house in GSK. Sequencing results were analysed using SeqMan Pro, from the DNASTAR Lasergene 12 Core Suite package.

Table 2.5 Sequencing primers

Primer name	Primer sequence
VP1.5	GGACTTTCCAAAATGTCG
XL39	ATTAGGACAAGGCTGGTGGG
EcoRI	GAATTCGTCGACTGGATC

2.2.19 Transfection

2.2.19.1 Plasmid transfection

HEK293T cells were seeded at 0.5×10^6 cells/mL in antibiotic-free media, in 10 cm plates. 20 µg of hGAPDH-DKK-myc (WT), hGAPDH-K213Q and hGAPDH-K213E were added to 500 µL of Optimem, while 80 µL of Lipofectamine 2000 were added to 500 µL of Optimem. All reactions were left to incubate at room temperature. After 10 minutes, the lipofectamine/optimem mixture was added dropwise to each of the DNA/optimem mixtures, and incubated at room temperature. After 20 minutes, 1 mL of each reaction was added per plate. The media was removed from the cells the next day and replenished with fresh media.

2.2.19.2 siRNA transfection

GAPDH siRNAs #s234321 and #s103461 together with a silencer® select negative control (4390843) from Ambion were used. BMDMs were seeded at 0.5×10^6 cells/mL in 12 well plates. The following reactions were prepared per well: 0.25 µL of 10 nM siRNA were added to 49.75 µL of Optimem, while 2.5 µL of Lipofectamine RNAiMax were added to 47.5 µL, and both reactions were left to incubate at room temperature. After 10 minutes, the lipofectamine/Optimem mixture was added dropwise to the siRNA/Optimem mixture, and was left for another 10 minutes at room temperature. The media on each well was replaced by 450 µL Optimem prior to the addition of 50 µL of the transfection mix. The media was removed from the cells the next day and replenished with fresh media.

2.2.20 Agarose gel electrophoresis

Agarose gels (0.8 – 1.5%) were prepared by boiling agarose in 1X Tris-acetate-EDTA (TAE) until completely dissolved. The mixture was allowed to cool down slightly and Nancy-520 was added at a final dilution of 1:20,000. The gel was poured into the chamber and left to set. Agarose gel loading buffer was added to the DNA samples to a final concentration of 1X and samples were loaded onto the gel together with the ladders Hyperladder I or IV (Bioline). Bands were separated at a constant voltage of 80 – 150 V and visualised on a UV transilluminator or gel imaging system.

2.2.21 Cloning

2.2.21.1 Genomic amplification

The primers shown in **Table 2.6** were designed for the amplification of the 3'UTR regions of TNFα and DAPK1 from the murine genome.

Table 2.6 3'UTR amplification primers

Primer name	Primer sequence
DAPK1 3'UTR forward primer	TCTCGAGGTGGGCAGCCTTGGCTT
DAPK1 3'UTR reverse primer	TAAACGGTTGCTTATTGCGCGGCCGCG
TNF α 3'UTR forward primer	TCTCGAGGTAGAGACTCCCATGCTT
TNF α 3'UTR reverse primer	GAATTCTAAATCAGTGTGCGGCCGCG

The following amplification reactions were set up using the KAPA HiFi Hotstart ReadyMix:

- 5 μ L HiFi mastermix
- 0.3 μ L primers
- 2 μ L DNA
- 2.7 μ L water

The following cycling parametres were used:

- 95°C – 20 seconds
- 40 cycles: 95°C – 3 seconds
60°C – 30 seconds
- 95°C – 15 seconds
- 60°C – 1 minute
- 95°C – 15 seconds
- 60°C – 15 seconds

A second amplification reaction was then set up:

- 15 μ L HiFi mastermix
- 0.9 μ L primers
- 6 μ L DNA from first amplification reaction
- 8.1 μ L water

2.2.21.2 DNA purification and digestion

PCR products were separated by gel electrophoresis as previously described and the corresponding bands were cut out. The DNA was then gel purified using the Wizard SV and PCR Clean Up system following manufacturer's instructions (Promega). DNA was eluted in 30 μ L nuclease-free water.

The following digestion reactions were then set up with the gel-purified inserts as well as the psiCHECK-2 plasmid:

- 500 ng DNA
- 1 μ L XhoI restriction enzyme
- 1 μ L NotI restriction enzyme
- 2 μ L CutSmart Buffer

Reactions were made up to 20 μ L in water, and were incubated at 37°C for 2 to 3 hours. The digested DNA fragments and plasmid were then dephosphorylated by incubating with 1 μ L calf-intestinal alkaline phosphatase (CIP) for another 30 minutes at 37°C.

2.2.21.3 Ligation reaction

The following ligation reactions were set up:

- 500 ng plasmid DNA
- 500 ng insert DNA
- 2 μ L T4 DNA ligase
- 2 μ L ligase buffer

Reactions were left incubating at 16°C overnight with mild shaking. A separate reaction was also set up containing plasmid DNA but without any insert DNA as a negative control. Bacteria were transformed using 5 μ L of ligation reactions as previously described. Single colonies were picked, grown, and plasmids subsequently isolated by miniprep. Plasmids were digested as previously described and run on an agarose gel to detect the presence of the insert. Those that appeared to contain the inserts were then subsequently sequenced for confirmation.

2.2.22 Luciferase assay

HEK293T cells were seeded at 0.2×10^6 cells/mL in antibiotic-free media, with 100 μ L added to each well of a 96 well plate. 0.5 μ g of psiCHECK2 (empty vector), TNF α -3'UTR, DAPK1-3'UTR were added to 25 μ L of Optimem, while 16 μ L of Lipofectamine 2000 were added to 100 μ L of Optimem. All reactions were left to incubate at room temperature. After 10 minutes, 25 μ L of the lipofectamine/optimem mixture were added dropwise to each of the DNA/optimem mixtures, and incubated at room temperature. After 20 minutes, 10 μ L of each reaction was added per well in triplicate, transfecting a final amount of plasmid of 100 ng per well. The media was removed from the cells the next day and replenished with fresh media.

48 hours post-transfection cells were lysed with 1X passive lysis buffer. 40 μ L of coelenterazine solution (1 μ g/mL) was added to 20 μ L of lysate and read immediately on a

luminometre to measure the renilla luciferase activity. A 1X solution of luciferase mix was generated by diluting a 2X solution (20 mM Tricine; 2.67 mM MgSO₄ 7H₂O; 0.1 mM EDTA; 33.3 mM DTT; 530 µM ATP; 270 µM acetylCoA; 470 µM D-Luciferin; 5 mM NaOH; 267 µM magnesium carbonate hydroxide) in water. 40 µL of 1X luciferase mix was added to 20 µL of lysate and the plate read immediately to measure the firefly luciferase activity. For analysis, the renilla activity was normalised to the firefly reading.

2.2.23 Seahorse analysis of oxygen consumption

Cells were seeded at 0.2×10^6 cells/well in a seahorse plate in a volume of 250 µL. One well per row in the culture plate was used as a negative control for background measurements and contained only medium without any cells. The day before stimulation of the cells was completed, a utility plate containing calibrant solution (1 mL/well) was placed in a CO₂-free incubator at 37°C overnight, together with a plate containing the injector ports and probes. On the day of the seahorse assay, seahorse medium supplemented with glucose (25 mM) was prepared. The pH of the medium was adjusted to 7.4 and then filter-sterilised. This medium was then used to replace the one present on the cells (500 µL/well), and the plate was placed in a CO₂-free incubator for at least 30 minutes.

The required inhibitors were prepared in glucose-supplemented XF Assay buffer (with the exception of 2-DG, which was prepared in non-supplemented media) as illustrated in **Table 2.6**.

Table 2.6. Inhibitor dilutions for Seahorse Assay

Inhibitor	Medium volume (µL)	Inhibitor volume (µL)	Port
Oligomycin	1984	16	A
FCCP	1991	9	B
2-DG	990	1010	C
Rotenone	1980	2.2	D
Antinomycin		18	

Inhibitors (70 µL) were added to the appropriate port of the injector plate as indicated in table 4. This plate, together with the utility plate was run on the Seahorse XF24 analyser for calibration. Once complete, the utility plate was replaced with the cell culture plate and oxygen consumption measured.

2.2.24 FLAG affinity chromatography

HEK293T cells were seeded at 1×10^6 cells/mL in 10 cm dishes and were transfected with hGAPDH and the various mutant variants using Lipofectamine 2000 as previously described. 48 hours post-transfection the media was removed and the cells were washed with PBS twice. The cells were then lysed with 1 mL of lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X100) with freshly added 1X Protease Inhibitor cocktail. Cells were scraped and centrifuged for 10 minutes at $12,000 \times g$ at 4°C .

2.2.24.1 Resin preparation

The empty columns were washed twice with TBS (50 mM TrisHCl, 150 mM NaCl, pH 7.4). 1 mL of resuspended ANTI-FLAG® M2 affinity resin was then transferred onto each column. The gel was first allowed to drain, and it was then washed once with TBS, followed by three washes of 1 mL glycine HCl, pH 3.5. The packed column was then washed with 1 mL of TBS five times. A small amount of TBS was left at the top of the column until ready for use.

2.2.24.2 Column chromatography and elution

The lysates were loaded onto the column and were left to go through under gravity flow. They were collected and were run through the column again 5 - 10 times. The column was then washed with 1 mL of TBS 15 – 20 minutes. Bound FLAG was eluted off the column using 4 mL of a 100 $\mu\text{g/mL}$ solution of 3X FLAG peptide in TBS.

2.2.25 Statistical analysis

Statistical tests were performed on GraphPad prism. A value of $p < 0.05$ was considered statistically significant.

Chapter 3: Results

Investigating the role of succinylation and malonylation in macrophages

3.1 Introduction

Post-translational modifications (PTMs) are key to expanding the functional diversity of proteins. Many metabolite intermediates are used as substrates in PTMs, including acetylation, propionylation, myristoylation, palmitoylation, butyrylation, succinylation, malonylation, glutarylation or crotonylation, among others. Despite several studies recently highlighting the role of metabolic reprogramming in determining the function of immune cells, the regulation of metabolism by PTMs, as well as how PTMs may be regulating metabolism, remains a poorly understood area. Studies have shown that during high energy conditions, histone acetylation can directly alter the cell's metabolism by triggering the selective expression of genes regulating lipogenesis and cellular proliferation [159, 160]. On the other hand, the concentration of metabolites in the liver has been shown to influence the acetylation status of metabolic enzymes, which directly impacted on the TCA cycle, the urea cycle and the gluconeogenesis pathway [77]. Both studies suggest that PTMs could be the key central players executing the metabolic rewiring of cells.

Macrophages have been a particular focus in metabolic reprogramming studies. Pro-inflammatory macrophages, such as those activated by the gram negative bacterial product LPS, are highly glycolytic with a disrupted TCA cycle. In these macrophages, there are two particular metabolites that have been shown to accumulate: citrate and succinate. This is of interest to this study as the succinate precursor succinyl-CoA, and the citrate-derived metabolite malonyl-CoA, are responsible for the recently identified and poorly understood PTMs succinylation and malonylation, respectively [83, 101]. Both modifications have been shown to affect various metabolic enzymes. However, studies are limited to cell lines or the liver, and no function has yet been attributed beyond metabolism. Moreover, their regulation also remains poorly understood. Sirt5 is the sole enzyme identified to date capable of regulating these modifications by acting as a desuccinylase and a demalonylase [81]. There are studies that have demonstrated that these modifications can occur non-enzymatically *in vitro* [161]. However, while non-enzymatic malonylation or succinylation reactions have been found to be favourable in the mitochondrial environment, where coincidentally sirt5 is also predominantly found, the reactions are not favourable in the cytosol [162]. Therefore the question of how malonylation and succinylation may occur in the cell remains.

The present study aims to investigate the role of succinylation and malonylation in macrophages. Here we have shown that both modifications can be induced following activation of macrophages with various different TLR ligands. We have also for the first time characterized the malonylome in macrophages and validated the glycolytic enzyme GAPDH as undergoing malonylation following macrophage activation. Finally, we have shown that the

malonylation related metabolites, citrate and malonyl-CoA, can alter cytokine production in response to LPS.

3.2 Characterizing the succinylation and malonylation profiles of activated macrophages

3.2.1 LPS increases protein succinylation

The effect of LPS on protein succinylation levels in macrophage cell lines was first examined. In agreement with a previous report by Tannahill *et al* (2013), an increase in succinylation was observed [27] (Figure 3.1). This increase occurs in RAW264.7 cells at 30, 40 and 46 kDa, after 12 hours of LPS stimulation (lane 4) and is still observed after 24 hours (lane 5) (Figure 3.1A). Immortalized BMDMs (iBMDMs) were also tested with three different doses of LPS at 6 and 24 hours (Figure 3.1B). Although an increase in succinylation was also observed, it was only detected at 35 kDa. In addition, it was detected earlier, after 6 hours of LPS stimulation, despite requiring 10 ng/mL of LPS (lane 5) and 100 ng/mL at 24 hours (lane 9). Interestingly, succinylation levels do not appear to correlate with pro-IL1 β levels, even less so in iBMDMs than in the RAW264.7 cells. In fact, in the case of iBMDMs, when succinylation levels increased, pro-IL1 β levels decreased (lanes 5 and 6). Furthermore, barely any pro-IL1 β was detected after 24 hours of treatment with LPS. This, combined with the increase in succinylation requiring a higher dose of LPS at this time, would suggest that the iBMDMs may lose sensitivity to LPS after 24 hours.

Next, the effect of LPS on protein succinylation in primary macrophages was investigated. An LPS time course was performed in BMDMs, where a similar increase in succinylation as previously described was observed (Figure 3.2). This increase occurs at 40 and 46 kDa after 6 hours of LPS stimulation (Figure 3.2A, lane 3) and is still observed after 24 hours (lane 5).

In an attempt to demonstrate that the bands observed to increase with LPS correspond to succinylated lysine (succ-K) peptides, the antibody used for their detection was incubated beforehand with a peptide containing succ-K, or malonylated lysine (mal-K) (Figure 3.2B). Prior incubation with succ-K peptides was shown to abolish the detection of bands (middle panel), while prior incubation with mal-K peptides (lower panel) still resulted in the detection of LPS-induced bands at 40 and 46 kDa. This would suggest that the succ-K antibody is specific in the detection of succinylation.

3.2.2 Other TLR ligands can also increase protein succinylation

Following confirmation of the effect of LPS on succinylation, we next examined whether other TLR ligands could also exert similar effects. The TLR7/8, TLR3 and TLR2/1 agonists R848, poly I:C and Pam3CSK respectively, can also increase succinylation levels in BMDMs (Figure 3.3).

R848-induced succinylation was observed only at 58 kDa and after 12 hours of stimulation (Figure 3.3A, lanes 7 – 12). It was also especially visible at the highest dose of R848, 5 µg/mL (lane 12), although 1 µg/mL could also induce as much succinylation after 24 hours (lane 11). Poly I:C on the other hand, was shown to increase succinylation levels at a wide range of protein sizes: 50, 38, 30 and 20 kDa (Figure 3.3B). However, this effect, although the most varied, could only be observed after 24 hours of stimulation (lanes 7 – 9). Finally, Pam3CSK could also increase protein succinylation after 10 hours of stimulation at 70 and 90 kDa (Figure 3.3C). This effect was only observed with the highest dose of Pam3CSK at 90 kDa (lanes 9 and 12), however the lowest dose of 1 µg/mL could also induce succinylation at 24 hours (lane 10).

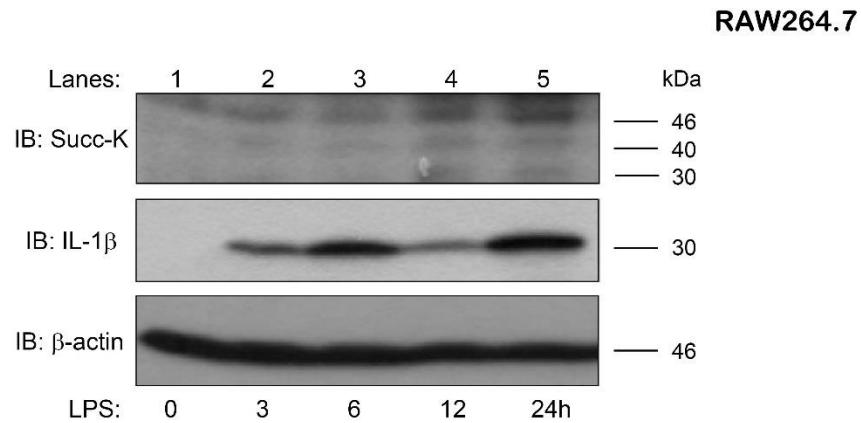
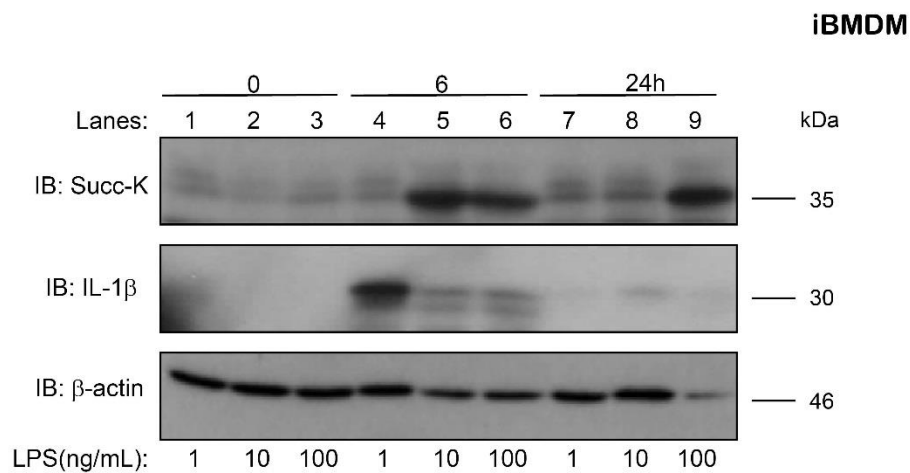
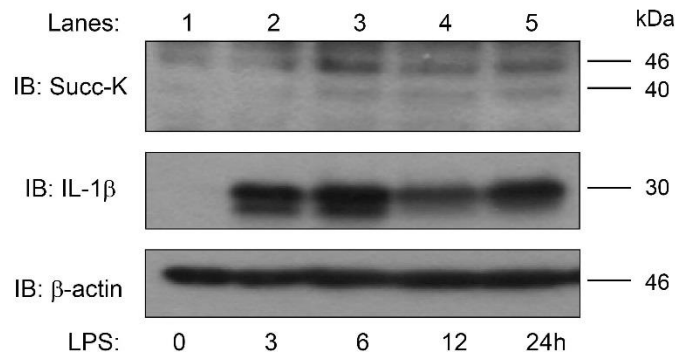
A.**B.**

Figure 3.1 LPS drives overall protein succinylation in macrophage cell lines. RAW264.7 cells (**A**) and iBMDMs (**B**) were seeded at 0.4×10^6 cells/mL in 12 well plates and left at 37°C, 5% CO₂ overnight. Cells were left unstimulated, or were stimulated with LPS (100ng/mL) for 3, 6, 12 and 24 hours (**A**) or with LPS (1, 10 and 100 ng/mL) for 6 and 24 hours (**B**). Cells were lysed in sample loading buffer and separated via SDS-PAGE electrophoresis. Succinyl-lysine (Succ-K), IL-1 β and β -actin expression was analysed by western blot. Results are representative of three independent experiments.

A.



B.

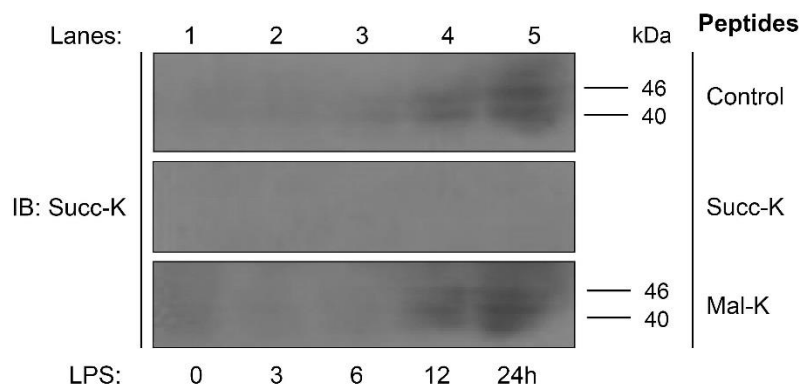
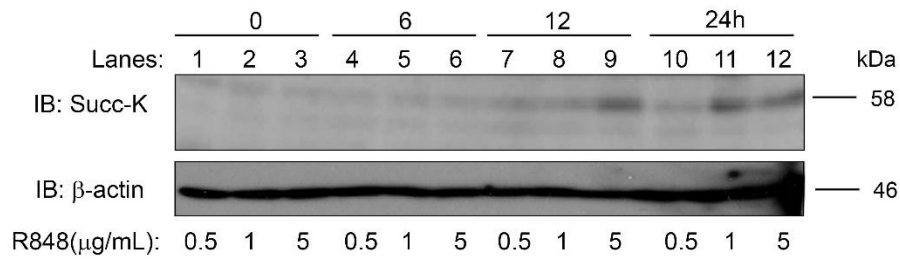
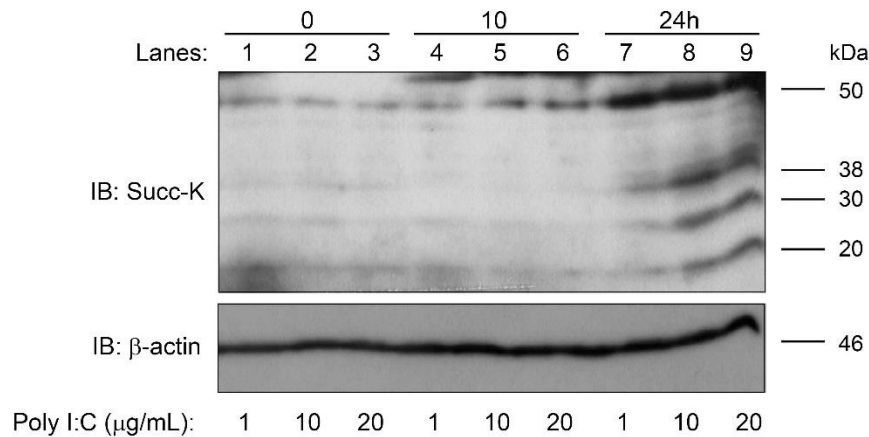


Figure 3.2 LPS drives overall protein succinylation in BMDMs. BMDMs were seeded at 1×10^6 cells/mL in a 12 well plate and incubated overnight at 37°C, 5% CO₂. The following day, cells were left untreated or were treated with LPS (100 ng/mL) for 3, 6, 12 and 24 hours. Cells were lysed using samples loading buffer and separated via SDS-PAGE electrophoresis. **A)** Lysine succinylation levels were analysed by western blotting. Results are representative of six independent experiments. **B)** The succ-K antibody was pre-incubated with either a succ-K peptide, a malonylated-lysine (mal-K) peptide or no peptide, prior to its use in the analysis of succ-K levels via western blotting. Results are representative of two independent experiments.

A.



B.



C.

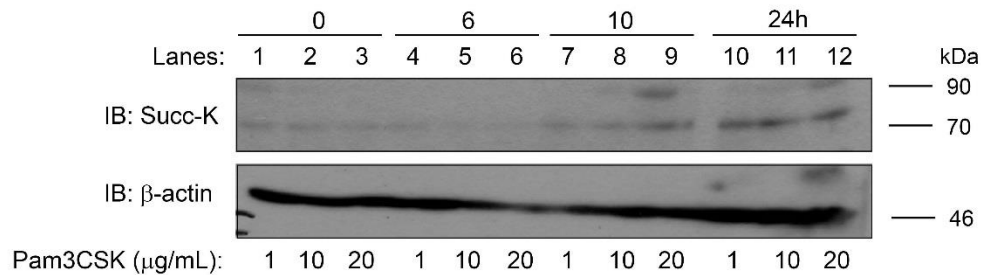


Figure 3.3 R848, poly I:C and Pam3CSK drive overall protein succinylation in BMDMs.

Cells were seeded at 1×10^6 cells/mL in 12 well plates and left at 37°C, 5% CO₂ overnight. **A)** Cells were left unstimulated or were stimulated with R848 (0.5, 1, 5μg/mL) for 6, 12 and 24 hours. **B)** Cells were left unstimulated or were stimulated with poly I:C (1, 10, 20 μg/mL) for 10 and 24 hours. **C)** Cells were left unstimulated or were stimulated with pam3CSK (1, 10, 20 μg/mL) for 6, 10 and 24 hours. All cells were lysed in sample loading buffer and separated via SDS-PAGE electrophoresis. Succ-K and β-actin levels were measured via western blotting. Results are representative of three independent experiments.

3.2.3 LPS and R848 increase protein malonylation

We next investigated whether activation of macrophages through TLRs could also increase protein malonylation. Figure 3.4A illustrates an increase in malonylation at 40 and 46 kDa in BMDMs following activation with LPS. This increase was observed after 6 hours of LPS stimulation and is present even at the lowest concentration of LPS, 1 ng/mL (lanes 4 – 6).

Similarly to that shown for succinylation (Figure 3.2B), the specificity of the mal-K antibody was tested by incubation, prior to its use in western blotting, with a peptide containing mal-K or succ-K (Figure 3.4B). Prior incubation with mal-K peptides was shown to abolish the detection of bands (middle panel), while prior incubation with succ-K peptides (lower panel) still resulted in the detection of LPS-induced bands at 40 and 46 kDa. This would suggest that the mal-K antibody is specific in the detection of malonylation.

Next we examined whether a different TLR ligand, such as R848, could also increase malonylation levels. BMDMs treated with R848 showed increased levels of malonylation, particularly at 30 kDa after 24 hours (Figure 3.5, lanes 10 – 12). An increase was also observed at 50 kDa after 6 hours of stimulation (lanes 7 – 9), and even at 3 hours at the highest R848 dose, 5 µg/mL (lane 6). As opposed to the results observed when investigating succinylation levels in R848-treated BMDMs (Figure 3.3A), which required of the highest R848 dose to induce succinylation, the lowest dose of R848 used appeared sufficient to induce malonylation (Figure 3.5, lanes 7 and 10). Also, while the increase in succinylation was observed to occur at 58 kDa, the increase in malonylation was observed to occur at 30 and 50 kDa.

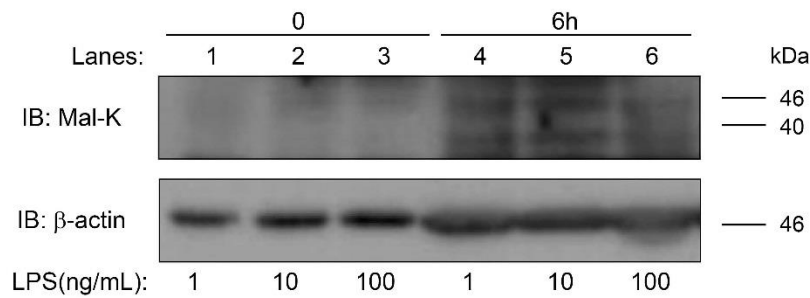
3.2.4 Identification of LPS-induced malonylated proteins

Next we proceeded to identify which proteins within BMDMs were undergoing malonylation following treatment with LPS. In agreement with previous western blotting results, there was a clear increase in malonylated proteins in the lane corresponding to the LPS-treated malonyl-lysine immunoprecipitated sample (IP LPS) (Figure 3.6). The range of proteins undergoing malonylation however ranged from 20 kDa to over 100 kDa. Five different sections of the gel corresponding to different molecular weights were cut out and were analysed by mass spectrometry. Nearly 100 different proteins were identified as undergoing malonylation on the basis of increased peptide number in the IP LPS sample (Table 3.1). Interestingly, there was also a small group of proteins which were identified as undergoing demalonylation following treatment with LPS (Table 3.2), which includes two histone proteins. There weren't any histone proteins identified as undergoing malonylation with LPS. There was also another small group

of proteins that appeared to be basally malonylated and were unaffected by treatment with LPS (Table 3.3).

To gain further insight into the LPS-induced malonylated proteins we next identified the associated functions of each protein through gene ontology (GO) terms. Surprisingly, we found that nearly 60% of proteins were associated with RNA binding, followed by cell differentiation and innate immune responses (Figure 3.7). This is interesting given that previous reports in which malonylated proteins have been characterized have found the top associated function to be metabolism [99, 100, 102]. Although not the top function, we also have identified metabolism as represented in 30% of LPS-induced malonylated proteins, specifically fatty acid metabolism and carbohydrate metabolism.

A.



B.

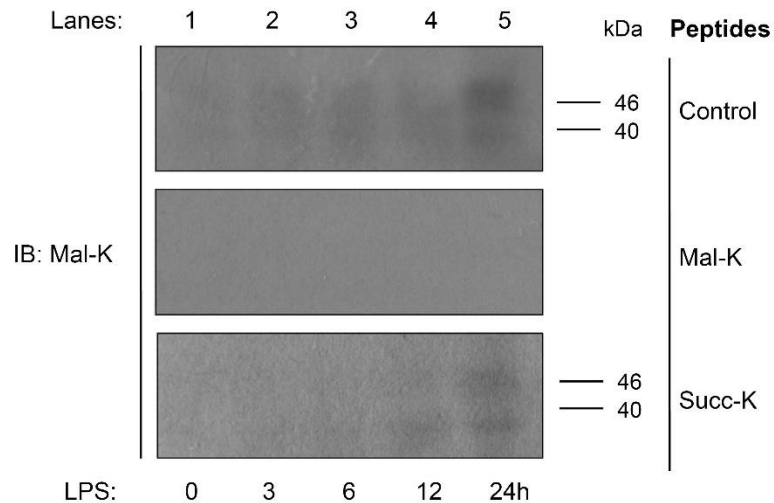


Figure 3.4 LPS drives overall protein malonylation in BMDMs. BMDMs were seeded at 1×10^6 cells/mL in a 12 well plate and left overnight at 37°C, 5% CO₂. **A)** Cells were left untreated or were treated with LPS (1, 10, 100 ng/mL) for 6 hours. Cells were lysed using sample loading buffer and separated via SDS-PAGE electrophoresis. Lysine malonylation levels were analysed by western blotting. Results are representative of three independent experiments. **B)** Cells were left untreated or treated with LPS (100 ng/mL) for 3, 6, 12 and 24 hours. Cells were lysed using sample loading buffer and separated via SDS-PAGE electrophoresis. The mal-K antibody was pre-incubated with either a succ-K peptide, a mal-K peptide, or no peptide, prior to its use in the analysis of mal-K levels via western blotting. Results are representative of two independent experiments.

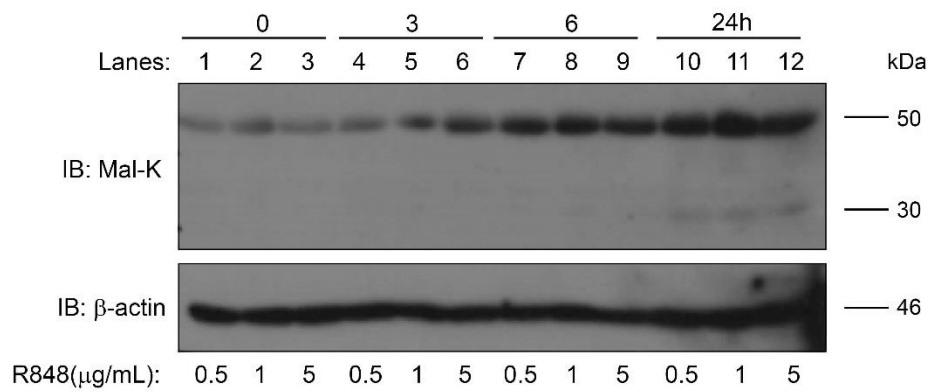


Figure 3.5 R848 drives overall protein malonylation in BMDMs. Cells were seeded at 1×10^6 cells/mL in a 12 well plate and left overnight at 37°C, 5% CO₂. Cells were left untreated or were treated with R848 (0.5, 1, 5 μg/mL) for 3, 6 and 24 hours. Cells were lysed in sample loading buffer and separated via SDS-PAGE electrophoresis. Mal-K and β-actin expression was analysed via western blotting. Results are representative of two independent experiments.

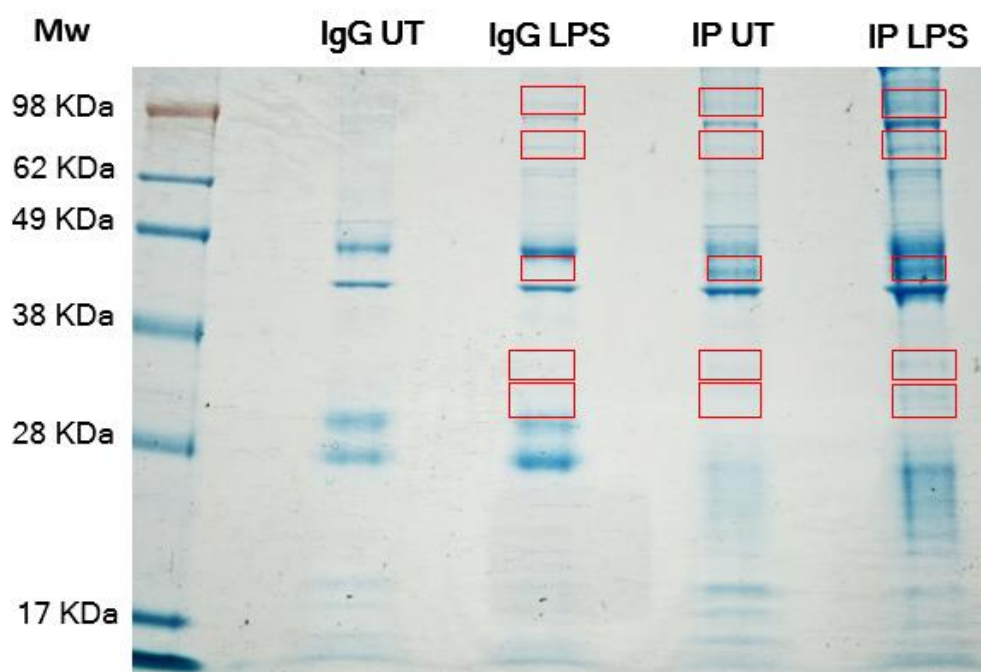


Figure 3.6 Immunoprecipitation of malonylated proteins in BMDMs. 10^7 cells were seeded per condition in 10 cm dishes, and left overnight at 37°C, 5% CO₂. Cells were unstimulated (UT) or were stimulated with LPS (100 ng/mL) for 24 hours, followed by lysis with low stringency lysis buffer. Following protein quantification and normalization using a BCA assay, lysates were used for immunoprecipitation (IP) with a control antibody (IgG) or a malonyl-lysine antibody rotating overnight at 4°C. After 12 – 16 hours, A/G beads were added to the antibody-containing lysates and left rotating at room temperature for 2 hours. Samples were washed three times with low stringency lysis buffer and resuspended in 25µL sample loading buffer. 15µL from three separate experiments were pooled to separate 45µL of protein sample via SDS-PAGE electrophoresis. The gel was stained overnight with coomassie stain, and following washing with water, a selection of bands were cut out and in-gel digested using trypsin. Results are representative of three independent experiments.

Rho GTPase-activating protein 17	Coatomer subunit epsilon	LDHA
Spermatogenesis associated protein 5	60S ribosomal protein L6	C1q subunit B
Rho GTPase-activating protein 5	Succinate CoA ligase	Emerin
Signal transducer and activator of transcription 1	SRSF protein kinase 1	eIF2
Dehydrogenase/reductase SDR family member	Asparagine-tRNA ligase	IL1 β
IFN-induced very large GTPase 1	NADH dehydrogenase 1 α	Annexin A1
Nascent polypeptide associated complex	Mitochondrial glutamate carrier 1	Sideroflexin 1
NFX1-type zinc finger containing protein 1	Poly[ADP ribose] polymerase 14	Sideroflexin 3
E3 ubiquitin protein ligase RNF213	Ribosome binding protein 1	GAPDH
Ubiquitin-like protein ISG15	Coatomer subunit γ 1	IRG1
DENN domain containing protein 4B	Schlafen family member 5	DDX3
Transcriptional activator protein Pur-beta	Elongation factor Tu	Endoplasmin
Tyrosine protein kinase Fes/Fps	Transmembrane protein 43	CD64
Anaphase promoting complex subunit 1	28S ribosomal protein S35	Rabankyrin
IFN-induced helicase C domain-containing protein	2'-5'-oligoadenylate synthase 1A	MAPK1
Bifunctional glutamate/proline tRNA ligase	Ser/Arg-rich splicing factor 5	eIF3
DNA replication licensing factor MCM4	N-terminal kinase-like protein	TRIM25
Choline phosphate cytidyltransferase A	Ser/Thr phosphatase PGAM5	TRIM28
Putative RNA-binding protein Luc7-like 2	Poly[ADP ribose] polymerase 9	Calumenin
26S proteasome non ATPase regulatory subunit 7	28S ribosomal protein S22	Sorting nexin 9
Zinc-finger CCCH-type antiviral protein 1	2-oxoglutarate dehydrogenase	N-myc interactor
Ras-associated domain-containing protein 4	Formin-like protein 1	CYFP1
Cytochrome b-c1 complex subunit 2	Deubiquitinating protein VCIP35	Protooncogene vav
Vacuolar protein sorting associated protein 33B	Ser/Thr protein kinase MRCK β	
Glutamyl-peptide cyclotransferase-like protein	Extended synaptotagmin-1	

Table 3.1 LPS-induced malonylated proteins in BMDMs. Proteins identified as undergoing LPS-induced malonylation from a malonyl-lysine immunoprecipitation in BMDMs analysed on a Q Exactive® mass spectrometre and searched in Mascot.

WASH complex subunit strumpellin	Myosin 9
Molybdenum cofactor sulfurase	Unconventional myosin If
Ribosomal RNA processing protein 36 homolog	Histone H1.5
Minor histocompatibility protein HA1	Regucalcin
Hemoglobin subunit α	Histone H1.4
Aspartyl/aspariginyl β -hydroxylase	Annexin A2
Polypyrimidine tract-binding protein	AMPK γ
Hepatoma-derived growth factor-related protein 2	60S ribosomal protein L4
DNA replication licensing factor MCM3	Replication factor C

Table 3.2 LPS demalonylated proteins in BMDMs. Proteins identified as undergoing LPS-induced demalonylation from a malonyl-lysine immunoprecipitation in BMDMs analysed on a Q Exactive® mass spectrometre and searched in Mascot.

2,4-dienoylCoA reductase	LIM and SH3 domain protein 1
ER membrane protein complex subunit 2	Elongation factor 2
Electron transfer flavoprotein subunit α	Macrosialin (CD68)
Glutathione S transferase Mu1	Vacuolar sorting associated protein 53
Leupaxin	WASH complex subunit SWIP
Dedicator of cytokinesis protein 11	Lysosomal alpha mannosidase

Table 3.3 Malonylated proteins unaffected by LPS in BMDMs. Proteins identified equally in both untreated and LPS-treated samples from a malonyl-lysine immunoprecipitation in BMDMs analysed on a Q Exactive® mass spectrometre and searched in Mascot.

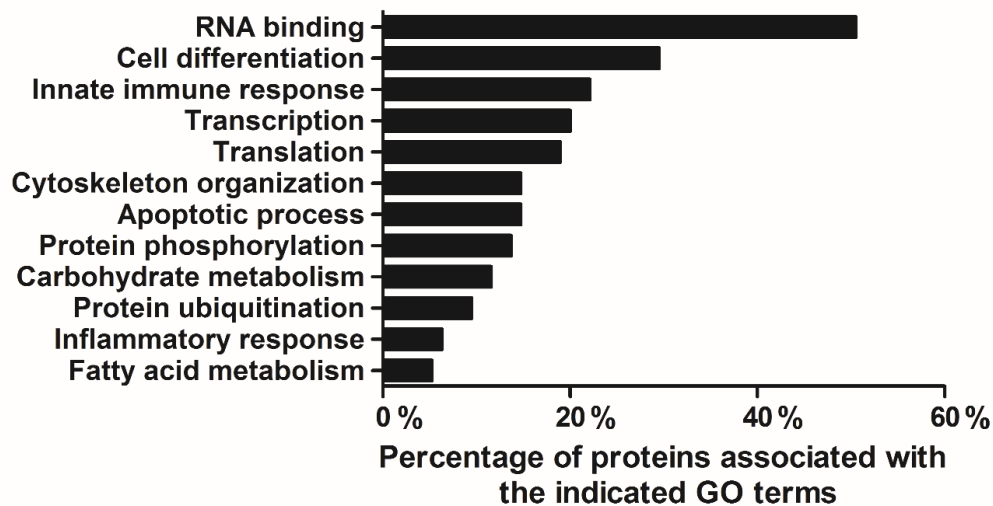


Figure 3.7 Categories associated with LPS-malonylated proteins in BMDMs. The LPS-malonylated proteins identified from LC-MS were annotated using Uniprot. The gene ontology (GO) terms that were associated with the highest numbers of proteins are depicted as percentage of the total number of proteins identified.

3.3 Characterizing the succinylation and malonylation of GAPDH

3.3.1 LPS induces GAPDH succinylation

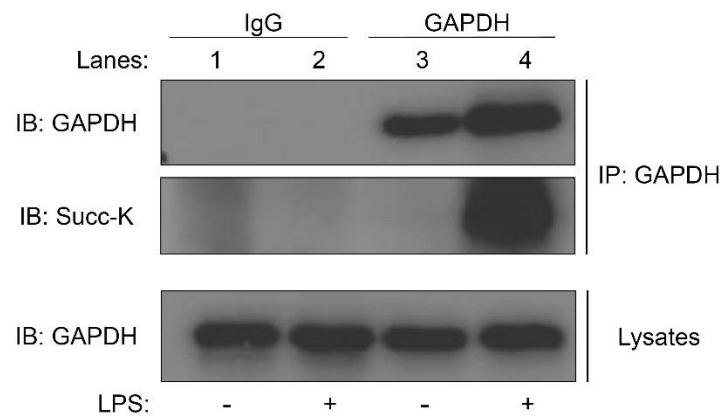
Previously published results had identified GAPDH by mass spectrometry as one of the substrates of succinylation in macrophages [27]. We proceeded to validate the succinylation of GAPDH by immunoprecipitating it from iBMDMs either unstimulated or treated with LPS. Western blot analysis was then used to test for succ-K in the immunoprecipitated samples. In agreement with Tannahill *et al*, GAPDH was observed to be succinylated following LPS treatment (lane 4, Figure 3.8A). To further confirm these results, the reverse experiment was carried out in which a succ-K antibody was used to immunoprecipitate succinylated peptides. Western blot analysis showed that GAPDH was only present in the LPS-treated immunoprecipitated sample (lane 4, Figure 3.8B).

Next, the same GAPDH immunoprecipitation experiment was performed in BMDMs, where a similar increase in succinylation was observed after treatment with LPS for 24 hours (lane 4, Figure 3.9). An LPS time course was then carried out to further characterize the LPS effect on GAPDH succinylation. Results showed that in iBMDMs, GAPDH only becomes succinylated after 24 hours of LPS treatment, and remains succinylated after 48 hours (lanes 7 and 8, Figure 3.10).

3.3.2 R848 is unable to induce succinylation of GAPDH

Given that other TLR agonists besides LPS had been shown to increase succinylation levels, we next examined whether the TLR7/8 agonist, R848, would also affect GAPDH succinylation using the same immunoprecipitation approach as previously described. However, R848 did not appear to have any effect on GAPDH succinylation in iBMDMs (lane 4, Figure 3.11). It is nevertheless important to note that when BMDMs were treated with R848, as previously shown in Figure 3.3A, only proteins of 58 kDa were observed to be succinylated, whilst GAPDH is 37 kDa.

A.



B.

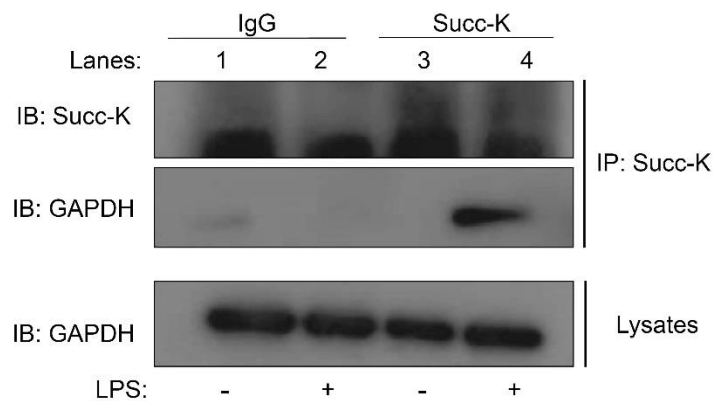


Figure 3.8 LPS induces GAPDH succinylation in iBMDMs. Cells were seeded at 0.6×10^6 cells/mL in 10 cm dishes and left at 37°C, 5% CO₂ overnight. Cells were left untreated or treated with LPS (100 ng/mL) for 24 hours, followed by lysis with low stringency lysis buffer. 50µL of lysate was kept for analysis while the remaining lysate was pre-cleared with proteins A/G beads for 30 minutes. Lysates were used for IP with a control antibody (IgG), a GAPDH antibody (**A**) or a succ-K antibody (**B**) coupled to protein A/G beads. Samples were washed three times with low stringency lysis buffer and resuspended in 35µL sample loading buffer. Samples were separated via SDS-PAGE electrophoresis and analysed by western blotting using anti-GAPDH or anti-succ-K antibodies. Results are representative of five independent experiments (**A**) and two independent experiments (**B**).

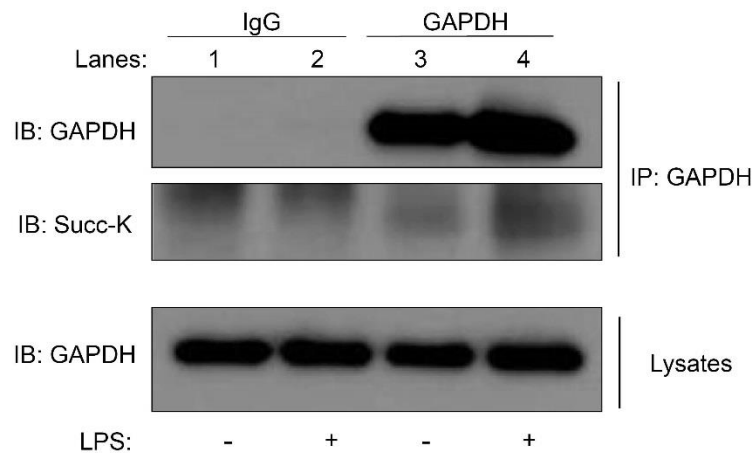


Figure 3.9 LPS induces GAPDH succinylation in BMDMs. Cells were seeded at 1×10^6 cells/mL in 10 cm dishes and were left overnight at 37°C, 5% CO₂. Cells were left unstimulated or treated with LPS (100 ng/mL) for 24 hours, followed by lysis with low stringency lysis buffer. 50 µL of lysate was kept for analysis while the remaining lysate was pre-cleared with protein A/G beads for 30 minutes. Lysates were used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed three times with low stringency lysis buffer and resuspended in 35 µL sample loading buffer. Samples were separated via SDS-PAGE electrophoresis and analysed by western blotting using anti-GAPDH or anti-succ-K antibodies. Results are representative of three independent experiments.

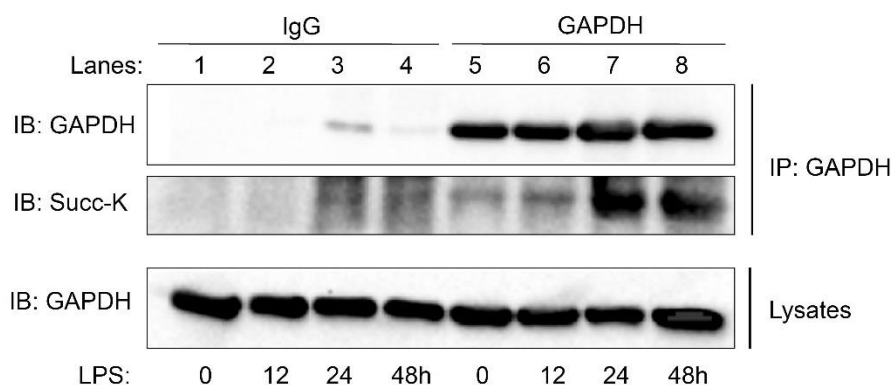


Figure 3.10 LPS induces GAPDH succinylation only after 24 hours in iBMDMs. Cells were seeded at 0.6×10^6 cells/mL in 10 cm dishes and left overnight at 37°C, 5% CO₂. Cells were left unstimulated or treated with LPS (100 ng/mL) for 12, 24 and 48 hours, followed by lysis with low stringency lysis buffer. 50 µL of lysate was kept for analysis while the remaining lysate was pre-cleared with protein A/G beads for 30 minutes. Lysates were used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed three times with low stringency lysis buffer and resuspended in 35 µL sample loading buffer. Samples were separated via SDS-PAGE electrophoresis and analysed by western blotting using anti-GAPDH or anti-succ-K antibodies. Results are representative of three independent experiments.

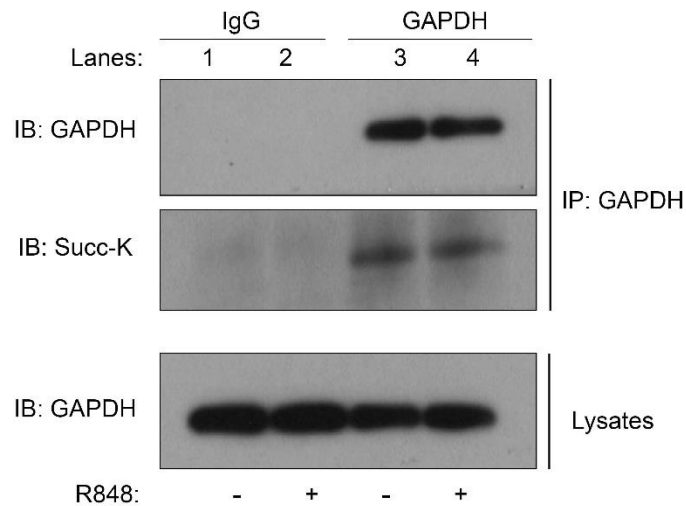


Figure 3.11 R848 is unable to induce GAPDH succinylation in iBMDMs. Cells were seeded at 0.6×10^6 cells/mL in 10 cm dishes and were left at 37°C, 5% CO₂ overnight. Cells were left unstimulated or were treated with R848 (5 µg/mL) for 24 hours, followed by lysis with low stringency lysis buffer. 50 µL of lysate was kept for analysis while the remaining lysate was pre-cleared with protein A/G beads for 30 minutes. Lysates were used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed three times with low stringency lysis buffer and resuspended in 35 µL sample loading buffer. Samples were separated via SDS-PAGE electrophoresis and analysed by western blotting using anti-GAPDH or anti-succ-K antibodies. Results are representative of three independent experiments.

3.3.3 LPS induces GAPDH malonylation at lysine 213

Having also identified GAPDH as one of the proteins undergoing malonylation in our initial screen (Table 3.1), we proceeded to examine this result in more detail. An antibody was used to immunoprecipitate GAPDH from BMDMs unstimulated or treated with LPS for 24 hours. Western blotting was then used to test for the expression of mal-K in the immunoprecipitated samples which, in agreement with previous results, showed GAPDH malonylation following 24 hours of LPS treatment (lane 4, Figure 3.12).

Following the observation that GAPDH could undergo both succinylation and malonylation, we proceeded to investigate which amino acids within the protein undergo post-translational modification. To this effect, GAPDH was immunoprecipitated from both unstimulated and LPS-treated macrophages. PTM Biolabs carried out further processing of the samples in preparation for liquid chromatography mass spectrometry (LC/MS). The samples sent for analysis are shown in Figure 3.13A. Following the analysis of the samples on the Q Exactive® mass spectrometer, no succinylation was detected neither in the unstimulated sample, nor in the LPS-treated (Figure 3.13B). Malonylation on the other hand, was detected on one lysine in the untreated sample (K192), and on two lysines in the LPS-treated sample (K192 and K213), thus providing further evidence for the LPS-induced malonylation of GAPDH. Interestingly, both K192 and K213 were also observed to be acetylated. A third acetylated lysine (K332) was also detected in the untreated sample in the carboxy terminus, suggesting that LPS causes deacetylation of GAPDH. Furthermore, a phosphorylated threonine (pT151), located beside the active site of the enzyme, was identified as present in the LPS-treated sample only. It is interesting to note that all post-translational modifications identified, with the exception of phosphorylation, are present in the catalytic domain of GAPDH (Figure 3.13B).

Further insight into the only LPS-induced malonylated site identified, K213, revealed that it is located in the exterior of an α -helix and in close proximity with the enzyme's active site (Figure 3.14A). In addition, it is also located in the dimer interface region of the enzyme [133], as well as being conserved across species (Figure 3.14B). All in all, this would suggest that it has an important role to play in the function of GAPDH.

Up to this point, we had collected evidence from western blotting, as well as GAPDH and malonyl-lysine mass spectrometry, showing LPS-induced malonylation of GAPDH. However, all results relied on the use of a mal-K antibody. Therefore, we next proceeded to investigate LPS-induced GAPDH malonylation through an antibody-independent method. To that effect, we used a chemical malonylation probe, malAMyne [163] (Figure 3.15A). MalAMyne was designed by X. Bao and colleagues as a malonate analog, building on the premise that isotopic sodium malonate has previously been used in cell culture to enhance malonylation [163].

Unstimulated and LPS-treated BMDMs were labelled with MalAMyne, which was then coupled to biotin through copper-catalysed click chemistry. The biotin-labelled malonylated proteins were immunoprecipitated using streptavidin and the presence of GAPDH assessed via western blotting. In agreement with previous results, GAPDH could only be detected in the malAMyne-labelled samples that had been treated with LPS (lane 4, Figure 3.15).

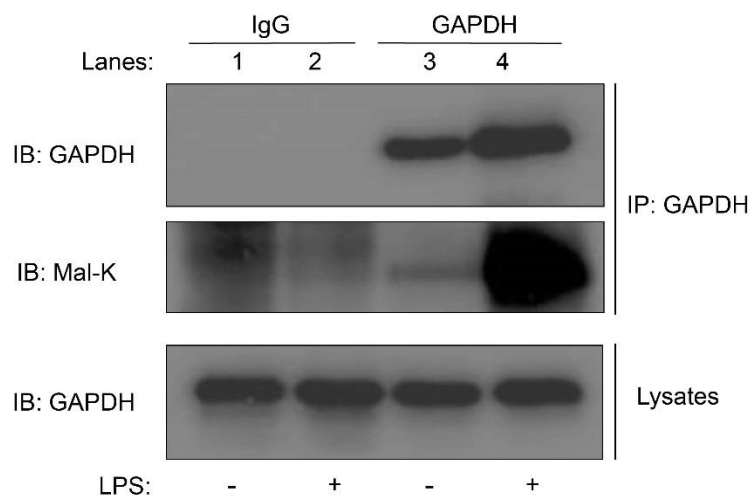
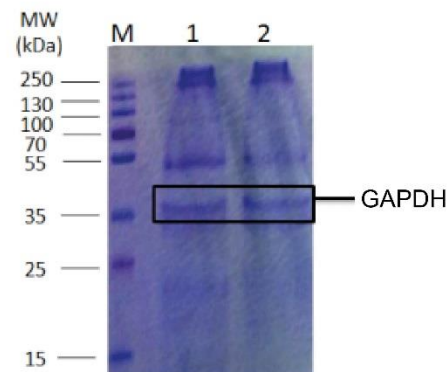


Figure 3.12 LPS induces GAPDH malonylation in BMDMs. Cells were seeded at 0.4×10^6 cells/mL in 10 cm dishes and were left overnight at 37°C, 5% CO₂. Cells were left unstimulated or treated with LPS (100 ng/mL) for 24 hours, followed by lysis with low stringency lysis buffer. 50 µL of lysate was kept for analysis while the remaining lysate was pre-cleared with protein A/G beads for 30 minutes. Lysates were used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed three times with low stringency lysis buffer and resuspended in 35 µL sample loading buffer. Samples were separated via SDS-PAGE electrophoresis and analysed by western blotting using anti-GAPDH or anti-mal-K antibodies. Results are representative of three independent experiments.

A.



B.

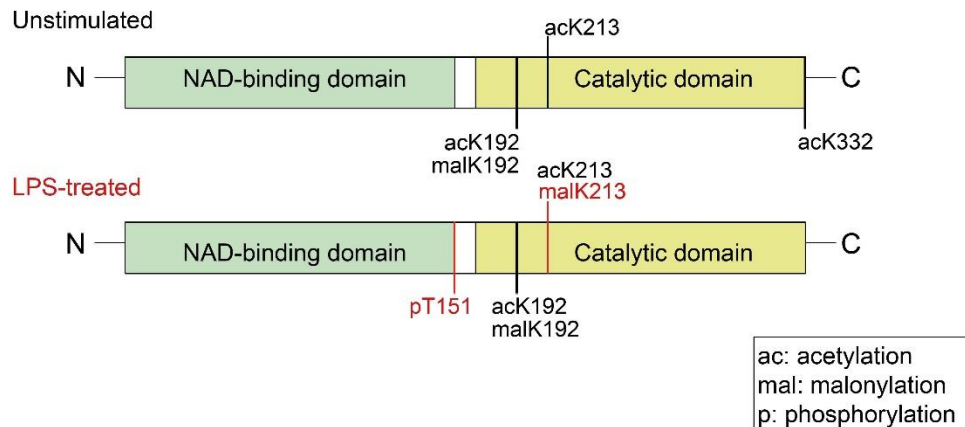
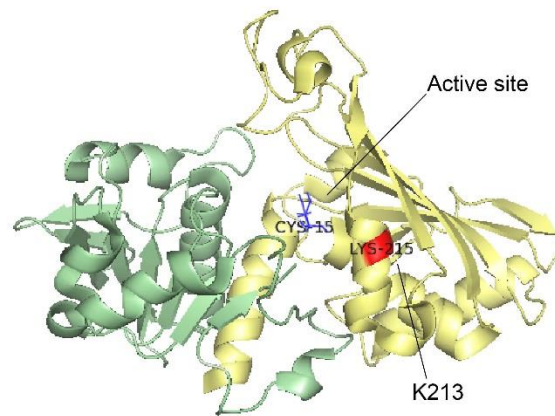


Figure 3.13 GAPDH has one malonylated site in unstimulated macrophages and 2 sites in LPS-treated macrophages. Cells were seeded at 1×10^6 cells/mL in 10 cm dishes and were left overnight at 37°C, 5% CO₂. Cells were left unstimulated or were treated with LPS (100 ng/mL) in triplicate for 24 hours, followed by lysis with low stringency lysis buffer. 50 µL of lysate was kept for analysis while the remaining lysate was pre-cleared with protein A/G beads for 30 minutes. Lysates were used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed three times with low stringency lysis buffer and snap-frozen. **A)** Immunoprecipitated unstimulated (1) and LPS-treated (2) samples were separated on a polyacrylamide gel and stained with coomassie blue. The 37 kDa bands were cut out and in-gel digested using trypsin. **B)** Post-translational modifications identified by LC-MS.

A.

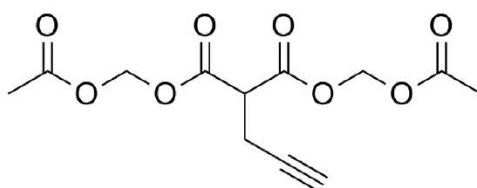


B.

	K213	
Human	KLWRDGRGAAQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPNVSVV	241
Mouse	KLWRDGRGAAQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPNVSVV	241
Rat	KLWRDGRGALQNIIPASTGAAKAVGKVIPELDGKLTGMAFRVPTANVSVV	243
C. Elegans	KLWRDGRGAGQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPDVSVV	249
E.Coli	KDLRASRAAAENIIPHTTGAAKAIGLVIPELSGKLGHAQRVPVKTGSVT	241

Figure 3.14 Structure of GAPDH and conservation of lysine K213 across different species. **A)** Structure of human GAPDH depicted using Pymol. **B)** 50-amino acid sequences from five different species containing GAPDH K213 were aligned using the Basic Local Alignment Search Tool.

A.



B.

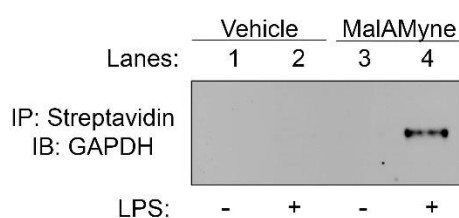


Figure 3.15 MalAMyne labels malonylated lysines and shows LPS-induced malonylation of GAPDH in BMDMs. A) Structure of MalAMyne. **B)** Cells were seeded at 1×10^6 cells/mL in 10 cm dishes and left at 37°C , 5% CO_2 overnight. They were left unstimulated or treated with LPS (100 ng/mL) for 24 hours, followed by labelling with 10 μM MalAMyne or DMSO vehicle for 2 hours. Cells were lysed using HEPES lysis buffer and protein concentration measured and normalised to 1 mg/mL using a BCA assay. Cu (I)-catalysed click chemistry was used to tag MalAMyne with 50 μM biotin, followed by precipitation of proteins using acetone and a -20°C overnight incubation. Streptavidin was used for precipitating of biotin-tagged proteins for 1.5 hours and samples were washed three times with decreasing SDS-containing buffers. Samples were eluted for 1 hour by incubating with an SDS-containing elution buffer. The eluted proteins were dried down with SpeedVac and resuspended in 30 μL LDS sample loading buffer. Samples were separated via SDS-PAGE electrophoresis and analysed by western blotting using an anti-GAPDH antibody. Results are representative of three independent experiments.

3.4 Investigating the regulation of LPS-induced malonylation

3.4.1 LPS increases malonyl-CoA levels

Very little is known as to how malonylation occurs and how it is regulated. Given that malonylation has been shown to be generated from malonyl-CoA [99, 101], we proceeded to investigate the effects of LPS on malonyl-CoA generation by macrophages. A significant 2-fold increase in malonyl-CoA levels was observed following 24 hours of LPS treatment (Figure 3.16). Interestingly, no increase in malonyl-CoA production was observed after 6 hours of LPS, even though malonylation has been shown to occur in response to LPS at that time point (Figure 3.4A)

3.4.2 Malonyl-CoA boosts IL10 production, while reducing TNF α and pro-IL1 β in response to LPS

Next we proceeded to investigate the effects of malonyl-CoA itself in LPS-treated macrophages. BMDMs pre-treated with malonyl-CoA showed decreased levels of the pro-inflammatory cytokines pro-IL1 β and TNF α in a dose-response manner (Figure 3.17A and Figure 3.17C). On the other hand, the pro-resolution cytokine IL10 was increased, although the effect was only observed at 5 mM and was lost at the 10 mM dose (Figure 3.17B). Interestingly, malonyl-CoA treatment on its own was also observed to drive cytokine production (Figure 3.17).

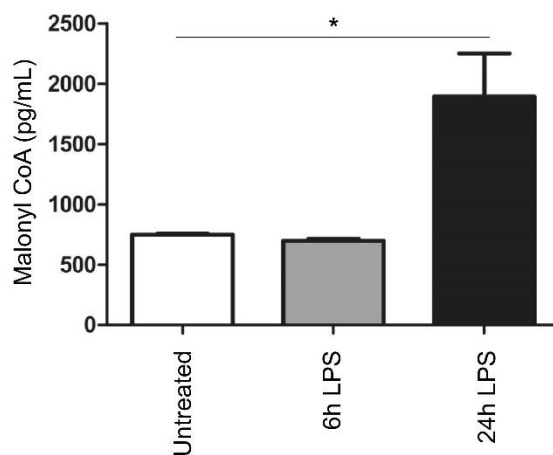
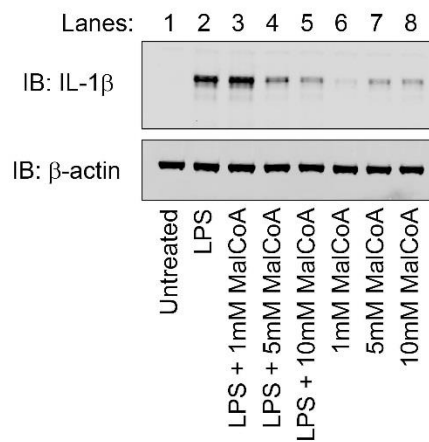
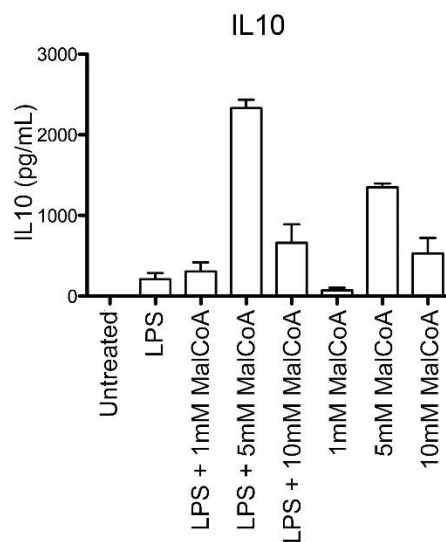


Figure 3.16 LPS increases malonyl-CoA levels in BMDMs. Cells were seeded at 1×10^6 cells/mL in 6 well plates and left at 37°C, 5% CO₂ overnight. Cells were left untreated or treated with LPS (100 ng/mL) for 6 and 24 hours. They were lysed by repeated freeze-thaw cycles at -20°C and -80°C degrees in PBS. Malonyl-CoA levels were measured by ELISA. Results are expressed as mean + SEM (* $p < 0.05$, student's unpaired t test, $n=3$).

A.



B.



C.

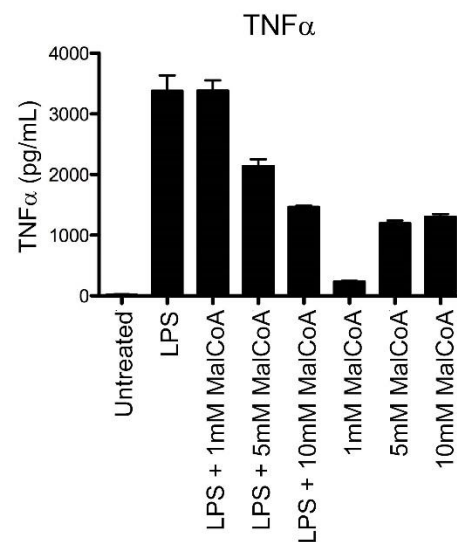


Figure 3.17 Treatment with malonyl-CoA reduces pro-inflammatory cytokines and boosts IL10 production in response to LPS in BMDMs. Cells were seeded at 1×10^6 cells/mL in 12 well plates and left at 37°C, 5% CO₂ overnight. Medium was removed from the cells and replaced with medium alone or medium containing 1, 5 or 10 mM malonyl-CoA for 2 hours, followed by 6 hours LPS (100 ng/mL). **A)** Cells were lysed using sample loading buffer and separated via SDS-PAGE electrophoresis. Pro-IL-1 β and β -actin expression was measured by western blotting. Results are representative of three independent experiments. Supernatants were analysed by ELISA for IL10 **(B)** and TNF α **(C)** production. Results are expressed as mean + SD (n=3).

3.4.3 LPS modulates sirt5 expression levels

Sirt5 is the only enzyme identified to date capable of regulating malonylation through its demalonylase activity [81]. Based on our observation of LPS treatment increasing malonylation levels, we wondered whether LPS could affect sirt5 expression. LPS was shown to decrease sirt5 mRNA expression by more than half after 6 hours of treatment (Figure 3.18A). The effect was however transient, with sirt5 levels restored at 12 hours. At the protein level, while there is no effect after 6 hours of treatment with LPS (lane 3, Figure 3.18B), there appears to be a small decrease after 3 and 24 hours (lanes 2 and 4). Therefore, LPS might be transiently regulating the levels of sirt5 in macrophages.

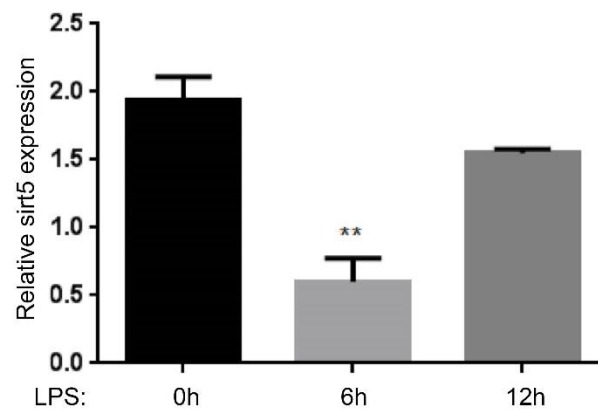
3.4.4 Sirt5 KO macrophages have impaired cytokine production in response to LPS

Sirt5 KO mice have higher basal levels of succinylation and malonylation than wild type mice [106]. However, based on the previously described observation of succinylation and malonylation events occurring in response to LPS treatment, I proceeded to investigate how bone marrow-derived macrophages from these Sirt5-deficient mice would respond to LPS. After 6 hours of LPS, Sirt5 KO produce significantly lower levels of the pro-inflammatory cytokines IL6 and TNF α , while no difference was observed on the production of the pro-resolution cytokine IL10 (Figure 3.19A). After 24 hours of LPS treatment however, IL10 levels are significantly higher in the Sirt5 KO macrophages (Figure 3.19B). Furthermore, pro-IL1 β expression is also lower in Sirt5 KO macrophages at the protein level. At the mRNA level however, there isn't a statistical significant difference (Figure 3.20).

3.4.5 Blocking of transcription prevents the induction of malonylation by LPS

The role of transcription in the induction of malonylation in LPS-activated BMDMs was next examined. Both 1 and 10 μ g/mL of cycloheximide were observed to completely abrogate malonylation (lanes 4 and 5, Figure 3.21). This result would suggest that *de novo* protein synthesis is required for the LPS induction of malonylation in macrophages.

A.



B.

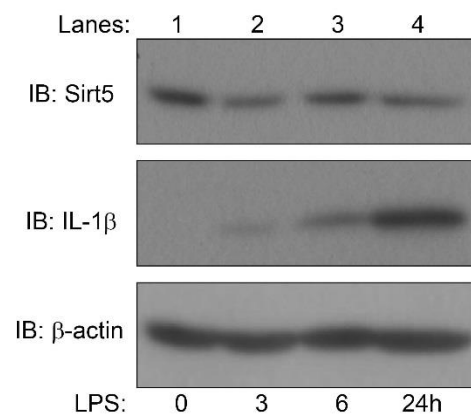


Figure 3.18 LPS modulates sirt5 expression levels. BMDMs were seeded at 1×10^6 cells/mL in a 12 well plate and left overnight at 37°C, 5% CO₂. **A)** Cells were left unstimulated or were stimulated with LPS (100 ng/mL) for 6 and 12 hours. RNA was extracted and normalised, followed by a reverse transcription reaction. Mean sirt5 expression normalised to 18S + SEM from three independent experiments carried out in triplicates is shown (**p < 0.01, student's unpaired t test). **B)** Cells were left unstimulated or were stimulated with LPS (100 ng/mL) for 3, 6 and 24 hours. Cells were lysed using sample loading buffer and separated via SDS-PAGE electrophoresis. Sirt5, pro-IL-1β and β-actin expression was measured by western blotting. Results are representative of six independent experiments.

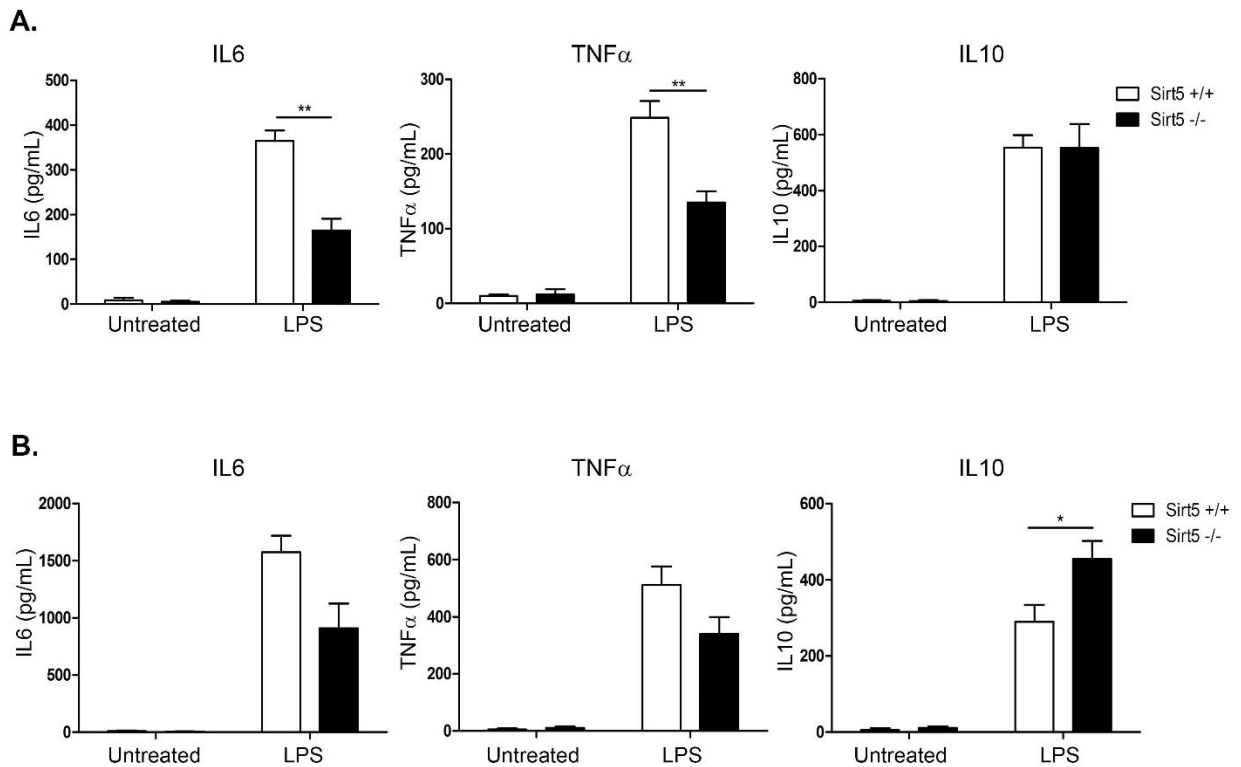
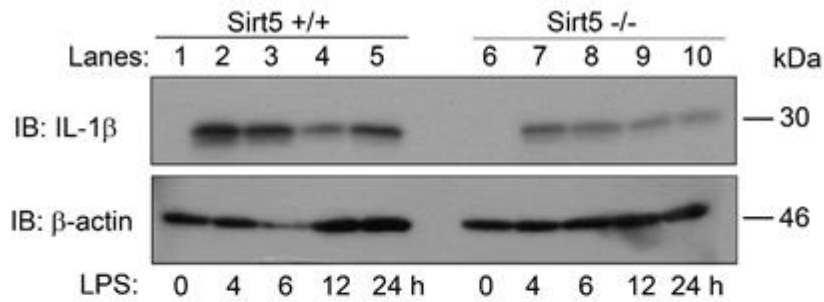


Figure 3.19 Sirt5 KO BMDMs have impaired cytokine production in response to LPS. BMDMs were seeded at 1×10^6 cells/mL in 12 well plates and left at 37°C, 5% CO₂ overnight. Cells were left unstimulated or were treated with LPS (100 ng/mL) for 6 hours (**A**) or 24 hours (**B**). Supernatants were analysed by ELISA for IL6, TNF α and IL10 production. The results are expressed as mean + SEM of four independent experiments, each carried out in triplicate (n=4; *p < 0.05, **p < 0.01, ANOVA).

A.



B.

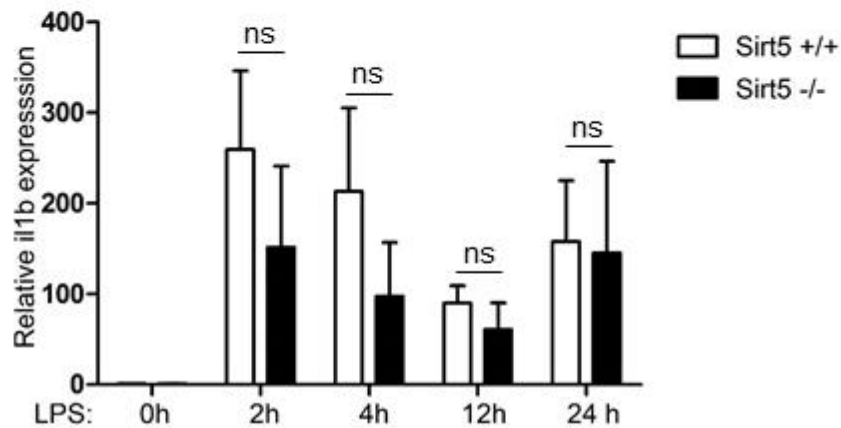


Figure 3.20 Sirt5 KO BMDMs have lower levels of pro-IL1β. BMDM cells were seeded at 1×10^6 cells/mL in 12 well plates and left to incubate at 37°C, 5% CO₂ overnight. **A)** Cells were left unstimulated or were treated with LPS (100 ng/mL) for 4, 6, 12 and 24 hours. Cells were lysed in sample loading buffer and separated via SDS-PAGE electrophoresis. Pro-IL1β and β-actin expression was measured by western blotting. Results representative of two independent experiments. **B)** Cells were left unstimulated or were treated with LPS (100 ng/mL) for 2, 4, 12 and 24 hours. RNA was extracted and normalised, followed by a reverse transcription reaction. Mean sirt5 expression normalised to 18S expression + SEM from three independent experiments, each carried out in triplicates is shown (ns, not-significant; student's unpaired t test).

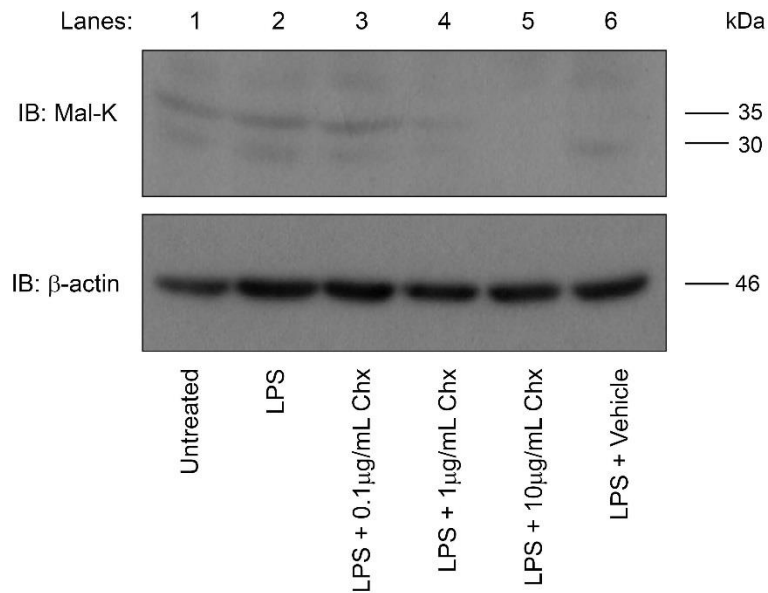


Figure 3.21 Cyclohexamide blocks induction of malonylation by LPS in BMDMs. Cells were seeded at 1×10^6 cells/mL in a 12 well plate and left at 37°C, 5% CO₂ overnight. The media was removed from the cells and replaced with serum free media alone or with media containing vehicle (ethanol), 0.1, 1 or 10 μ g/mL for 30 minutes followed by 100 ng/mL LPS for 6 hours. Samples were lysed in sample loading buffer and separated via SDS-PAGE electrophoresis. Mal-K and β -actin levels were measured via western blotting. Results are representative of two independent experiments.

3.5 Discussion

The first aim of this project was to investigate whether succinylation and malonylation could be playing a role in the inflammatory response in macrophages. A previous report had shown that overall levels of succinylation in macrophages increase following stimulation with the TLR4 agonist LPS [27]. I was able to confirm this result, not only in primary macrophages, but also in two macrophage cell lines: iBMDMs and RAW264.7 cells. The succinylation patterns observed were highly similar between BMDMs and RAW264.7, with proteins of similar molecular weights modified at the same time, following stimulation with the same doses of LPS. In the case of iBMDMs however, succinylation was only observed to increase at one particular molecular weight, which hadn't been detected in the other two cell types. Furthermore, the highest dose of LPS was required to observe increased succinylation in iBMDMs, suggesting these cells have a higher tolerance for TLR4 stimulation, and that they might not be a good model for succinylation investigations overall.

Activation of macrophages with TLR1/2, TLR3 and TLR7/8 ligands also resulted in an increase in the overall levels of succinylation. Interestingly, proteins of different molecular weights became succinylated depending on the TLR ligand used. For instance, activation of macrophages through TLR3 was observed to result in succinylation of proteins ranging from 20 to 50 kDa, while activation via TLR1/2 resulted in an increase in succinylation of proteins ranging from 70 to 90 kDa. TLR3 is located in endosomes and signals through the adaptor protein TRIF, while TLR1/2 can be found on the membrane and signals through the adaptor protein MyD88, and it may be these differences that account for the differences in the range of proteins undergoing succinylation. However, TLR4 can also be found on the membrane and can signal through MyD88, and yet there are still differences in the succinylation profiles when compared to TLR1/2, therefore dissimilarities in the signalling cascades can't be the sole explanation. Additional work using mass spectrometry to characterize the identity of the succinylation substrates would be needed before we are able to draw any further conclusions.

Next I proceeded to characterize the malonylation response in macrophages. No significant differences in malonylation were observed when compared to succinylation. Malonylation, much like succinylation, was inducible following 6 hours of LPS treatment (TLR4) and 24 hours of R848 (TLR7/8). While antibody specificity was a concern, this was ruled out through a competition experiment using succinylated and malonylated peptides, which suggested both succinylation and malonylation antibodies are specific. It is also important to note, that despite their similarities, the sources for succinylation and malonylation are very different. Succinyl-CoA is produced via the TCA cycle in the mitochondria, and although cytosolic proteins have been reported to undergo succinylation, there is no known mechanism as to how the succinyl-

CoA could exit the mitochondria. Malonyl-CoA on the other hand, is derived from citrate, and can be produced both inside the mitochondria as well as in the cytosol. Therefore the modification might exert similar effects on proteins, but may be restricted by different cellular compartments.

To further characterize the succinylation and malonylation response in activated macrophages, I next sought to identify the modified substrates. A preliminary mass spectrometry screen of succinylated peptides in macrophages activated with LPS had already been performed by Tannahill *et al* (2013) [27]. Previous studies that had characterized the malonylome in malonic aciduria, type 2 diabetes and mouse livers had all identified metabolic pathways, especially glycolysis, the TCA cycle and fatty acid oxidation, as the top and main functions associated with malonylated proteins [99]. However, no such study had been performed in macrophages, thus we proceeded to identify the malonylation substrates in activated macrophages via immunoprecipitation using an anti-malonyl-lysine antibody. Nearly 100 proteins were found to undergo LPS-induced malonylation. Interestingly, while enzymes involved in carbohydrate and fatty acid metabolism were identified, the top function associated with LPS-induced malonylated proteins was found to be RNA binding. It's worth considering that ribosomal proteins, a lot of which function as RNA-binding proteins, are highly abundant in the cell and could result in false positives in these types of studies. However, a significant proportion of the RNA-binding proteins identified aren't ribosomal proteins, but proteins involved in viral defense, such as DDX3X, interferon-induced helicase C domain-containing protein 1 (MDA5), TRIM28 or ADP-ribosyltransferase diphtheria toxin-like 13 (ARTD13) among others. Furthermore, while none of the other malonylome studies in mammalian cells identified RNA-binding proteins as malonylation substrates, they were identified as malonylation substrates in a study in *E. Coli*, suggesting this could be an evolutionarily conserved mechanism [164]. Another interesting observation is that a small number of proteins were detected to be demalonylated following macrophage activation, including the histone 1 variants H1.4 and H1.5. Interestingly, a study in a mouse model of obesity has also reported a downregulation in histone malonylation [103]. Histone malonylation has also been identified in various cell lines, although given the lack of functional studies, this remains a promising but completely unexplored area [98].

One of the proteins identified in both succinylation and malonylation mass spectrometry studies was GAPDH. GAPDH is responsible for catalyzing the sixth step in glycolysis, but it is also an RNA-binding protein, which led us to believe it would be a good candidate to use when investigating the functional consequences of LPS-induced succinylation and malonylation. First, I proceeded to validate the mass spectrometry results by immunoprecipitating GAPDH and blotting for succinyl-lysine, as well as vice versa. Interestingly, even though an increase

in overall succinylation levels in macrophages was observed after six hours of LPS stimulation, succinylation of GAPDH doesn't appear to occur until after 24 hours of LPS treatment. Furthermore, the TLR7/8 ligand R848 is capable of inducing succinylation and yet it can't induce succinylation of GAPDH. However, it's worth noting that only 58 kDa proteins were observed to undergo succinylation following treatment with R848, while GAPDH is 37 kDa. These results would suggest lysine succinylation can't solely rely on fluctuating succinyl-CoA levels and sirt5 but rather on a more complex enzyme regulatory network like most other post-translational modifications.

I was also able to validate malonylation of GAPDH following 24-hour treatment with LPS, both through GAPDH immunoprecipitation as well as using a malonylation chemical probe. I proceeded to characterize which lysines in GAPDH undergo succinylation and malonylation. Surprisingly, succinylation was not detected in any lysine, not even lysines that had previously been reported in other cell types to be succinylated in GAPDH basally [71]. This might be due to the fact that samples weren't freshly analysed, which combined with the possibility that the modification might be unstable following cell lysis, might have resulted in its degradation. However, it was still possible to detect GAPDH malonylation, which is structurally similar, and thus as likely to be unstable following cell lysis. It is also worth noting that all the succinylation data obtained thus far has been through the use of an anti-succinyl-lysine antibody. It is possible that despite having tested the antibody specificity *in vitro* and having found it to be specific, a certain degree of cross-reactivity may still occur. Nonetheless, the experiment would have to be repeated on fresh samples before succinylation of GAPDH following LPS treatment can be completely ruled out.

Lysine 213 (K213) was detected as malonylated only in the sample prepared from LPS-treated macrophages. K213 is located in the catalytic domain of GAPDH and is highly conserved across different species. It's also located at relative close proximity to the enzyme's active site, which overall suggests it may have an important role to play in GAPDH function. Interestingly, K213 was also found to be acetylated in the untreated sample. Furthermore, while there was a total of three different lysines detected as acetylated in GAPDH from untreated macrophages, only two were detected following LPS treatment. On the other hand, only one lysine in GAPDH was detected as malonylated in resting macrophages, while two were detected following LPS treatment. Therefore, LPS seems to cause GAPDH deacetylation while causing malonylation. A previous report has shown that in obese and type 2 diabetic db/db mice, GAPDH has increased levels of malonylation, while a separate study demonstrated GAPDH has decreased acetylation levels in the same model, supporting our observation [102, 165]. Acetylation is smaller than malonylation and neutralises the positive charge of the lysine, while malonylation goes one step further and converts it into a negative

charge. It is therefore interesting to speculate, whether the cell uses a switch from acetylation to malonylation as a means to turn proteins more reactive and alter their conformation as a means to activate them.

I next sought to investigate the mechanism of LPS-induced malonylation. The metabolite malonyl-CoA has been previously shown to be needed for malonylation to occur, and we found malonyl-CoA levels to be elevated in macrophages treated with LPS for 24 hours. We did not however find an increase in malonyl-CoA after 6 hours of LPS, despite having observed LPS-induced malonylation to occur at that same time point. This may be due to assay limitations, or it might suggest that although needed for the malonylation to occur, malonyl-CoA may not be the rate-limiting step. The latter is also the case in phosphorylation, where the phosphoryl donor ATP is typically not limiting [166]. Interestingly, while Kulkarni and colleagues have recently found malonyl-CoA alone to be capable of malonylating proteins *in vitro* to inhibit their activity, another report by Wagner and colleagues identifies malonyl-CoA as having poor reactivity within the cytoplasm of cells and consequently being incapable of malonylating on its own [60, 161]. I examined the effects of the pre-treatment of cells with malonyl-CoA prior to LPS, which was found to increase IL10 production while reducing pro-IL1 β and TNF α . However, further work is required to determine whether the effect observed is due to increased malonylation within the cells, or due to unknown effects of malonyl-CoA on its own.

Having briefly examined the role of malonyl-CoA in LPS-induced malonylation, I next proceeded to investigate the role of sirt5, the only demalonylase identified to date [81]. A transient downregulation of sirt5 mRNA was observed after 6 hours, however there was only a small fluctuating effect at the protein level. This small effect is unlikely to be solely responsible for the significant increase in both succinylation and malonylation observed in macrophages. There is however, the yet unexplored possibility that LPS might be directly affecting its enzymatic activity rather than its expression. Either way, sirt5 remains a useful tool for studying the functional consequences of the modification. To this effect, BMDMs derived from sirt5-deficient mice were used. Previous reports had been unable to detect any altered metabolism or other significant phenotype in these mice [106]. Nonetheless, I found that following treatment with LPS, sirt5-deficient macrophages produced reduced levels of the pro-inflammatory cytokines IL6, pro-IL1 β and TNF α , while at the same time producing higher levels of the pro-resolution cytokine IL10. While initially tempting to link this observation to increased succinylation and malonylation levels in these cells, a recent report has identified a new role for sirt5 in the regulation of cytokine production in macrophages independently of its demalonylase and desuccinylase activity. Sirt5 was shown to sequester the deacetylase sirt2, enabling acetylation of p65 and subsequent NF- κ B activation. Sirt5 deficiency resulted in reduced TNF α and IL6 production, in agreement with our observation [107]. Interestingly,

while the authors didn't measure IL10 production, they did measure ERK activation and found it to be unaffected by the absence of sirt5 [107]. Both NF- κ B and ERK are the two main transcription factors responsible for the production of IL10 in macrophages [167], therefore this novel sirt5 function still doesn't explain the increased IL10 production I have shown in sirt5 KO macrophages. Interestingly, this observation correlates with the previously mentioned increased IL10 production observed following pre-treatment of cells with malonyl-CoA. It is interesting to speculate whether increased malonylation levels may somehow be regulating IL10 production. The question of how malonylation occurs however remains. While fluctuating malonyl-CoA levels might be an option, as previously discussed, it appears unlikely to be the sole origin. In addition, I have shown that the use of a transcriptional inhibitor resulted in complete abrogation of the modification, which would suggest malonylation requires of a group or a single protein to be synthesized, the identity of which remains to be determined.

Overall, I have here characterized for the first time, lysine succinylation and malonylation occurring in activated macrophages. I have also found GAPDH to be a substrate of malonylation occurring specifically in response to LPS, the functional consequences of which will next be explored.

Chapter 4: Results

Investigating the role of GAPDH in macrophages

4.1 Introduction

Having characterized GAPDH as a substrate of malonylation in LPS-activated macrophages, I next sought to investigate the functional consequences of the modification. GAPDH is responsible for catalyzing the sixth step in glycolysis, a metabolic pathway that has been shown to be induced in many different immune cells, including macrophages, dendritic cells, natural killer cells and effector T and B cells [168]. Inhibition of glycolysis using 2DG, an inhibitor responsible for targetting the first step in the pathway, has been shown to reduce cytokine production in both macrophages and DCs as well as changing the phenotype of T cells from Th₁₇ to T_{regs} [27, 169, 170]. Furthermore, GAPDH has functions that go beyond glycolysis. Some of these include DNA repair, vesicular trafficking, cell death, RNA-binding and regulation of intracellular calcium signalling, among others [121].

Investigations into the role of GAPDH in the immune response however, both independently and within glycolysis, have been sparse. GAPDH has been shown to regulate IFN γ production in T cells through binding to its mRNA 3'-UTR region, and it has also been suggested to regulate TNF α production in monocytes in a similar manner [138, 139]. In CD8⁺ T cells, GAPDH enzymatic activity, regulated by acetylation, appears to be required for the generation of effective memory cells [75]. In macrophages in particular, GAPDH has only been studied in the context of the GAIT complex, which is responsible for binding to GAIT elements present in the 3'UTR of inflammation-related transcripts and regulating their translation [136]. However, the role of GAPDH within the complex isn't clear. In addition, whether the GAIT complex functions only in IFN γ -activated cells or can also play a role in LPS-activated macrophages is yet to be determined.

Mechanistic insights into GAPDH functions are also lacking. With the exception of cancer cells, GAPDH protein expression doesn't usually fluctuate, which is one of the reasons it's become a preferred control in experiments by many. This leaves cellular localization and post-translational modifications as the two main candidates in the regulation of its activity. In macrophages, GAPDH has been shown to translocate into the nucleus as a response to cellular stress, where it engages in DNA repair, transcription or cell death among other activities [123]. GAPDH nuclear translocation has however been shown to be driven by its nitrosylation or by β -N-acetylglucosamine glycosylation, thus suggesting that ultimately it comes down to post-translational modifications to enable GAPDH activities [123, 171]. This brings us back to our original observation of GAPDH malonylation following macrophage activation with LPS.

In this chapter I demonstrate that both GAPDH enzymatic activity and RNA binding are linked and are both needed for the production of cytokines in LPS-activated macrophages. I have

also for the first time characterized the RNA binding scope of GAPDH and shown that it can regulate the production of TNF α as well as DAPK1. Finally, I have found that malonylation of GAPDH increases its enzymatic activity at the same time as it enables the release of bound RNAs which are then translated.

4.2 Investigating the role of GAPDH enzymatic activity in cytokine production in macrophages

4.2.1 LPS increases GAPDH enzymatic activity

It is widely accepted that macrophages have increased glycolytic flux following activation by LPS [24], which led me to first investigate the effects of LPS on GAPDH enzymatic activity (Figure 4.1). LPS was observed to have a small effect in boosting GAPDH enzymatic activity after 6 hours of stimulation (Figure 4.2). After 24 hours however, the enzymatic rate had doubled compared to the basal rate, thus showing that LPS can increase GAPDH enzymatic activity.

4.2.2 Inhibition of GAPDH enzymatic activity blocks cytokine production

To further examine the role of GAPDH enzymatic activity in macrophage activation, the effects of the GAPDH inhibitor Heptelidic Acid (HA) were next examined. First, its efficiency at blocking GAPDH enzymatic activity was investigated, and it was found to completely abrogate enzymatic activity at a 10 μ M dose in BMDMs (Figure 4.3A). This effect was confirmed to not be due to toxicity by measuring LDH release from the cells. After 24 hours of incubation, doses as high as 20 μ M did not show a significant increase in the release of LDH. However, doses higher than 20 μ M did appear to be toxic, with a percentage of LDH release of over 50% (Figure 4.3B).

Next, I proceeded to test the effect of the inhibitor on LPS-induced cytokine production. BMDMs were pre-treated with HA for 2 hours, followed by treatment with LPS for 24 hours. 10 μ M pre-treatment of HA was shown to completely abrogate the induction of both pro-IL1 β mRNA (Figure 4.4A) as well as protein (Figure 4.4B). IL6 (Figure 4.5A), IL10 (Figure 4.5B) and TNF α (Figure 4.5D) were also found to be inhibited by more than 50% by 5 μ M and 10 μ M of HA, while 1 μ M was found to have no effect. No significant effect was observed on IL12p70 production (Figure 4.5C). Both IL6 and IL10, much like pro-IL1 β , were also inhibited by HA at the mRNA level (Figure 4.6A and Figure 4.6B). Strikingly, no effect was observed on TNF α mRNA expression (Figure 4.6D). In addition, the opposite effect was observed on IL12 mRNA compared to protein, with HA boosting IL12 mRNA expression, especially at 10 μ M (Figure 4.6C). This same effect was also observed with CXCL1 expression (Figure 4.6E). In the case of IFN β , LPS didn't appear to induce its expression, and neither did 10 μ M HA alone, however pre-treatment with 10 μ M HA followed by LPS treatment did cause an increase (Figure 4.6F). Overall, the cytokine data obtained up to this point, suggests GAPDH enzymatic activity plays a role in cytokine production following activation of macrophages by LPS.

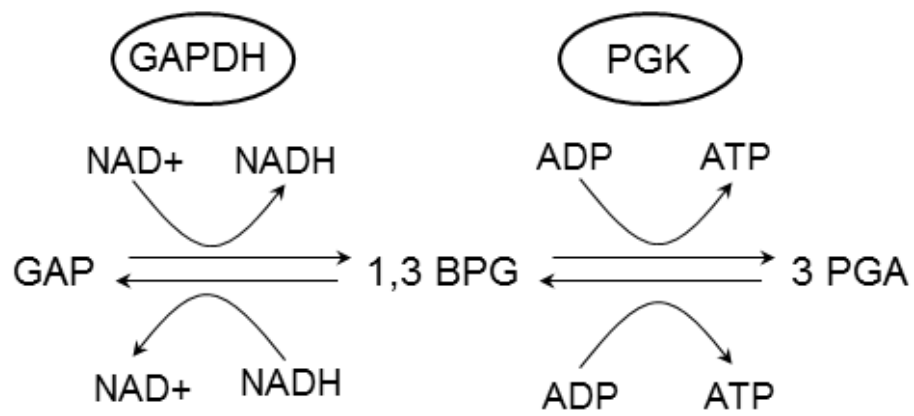
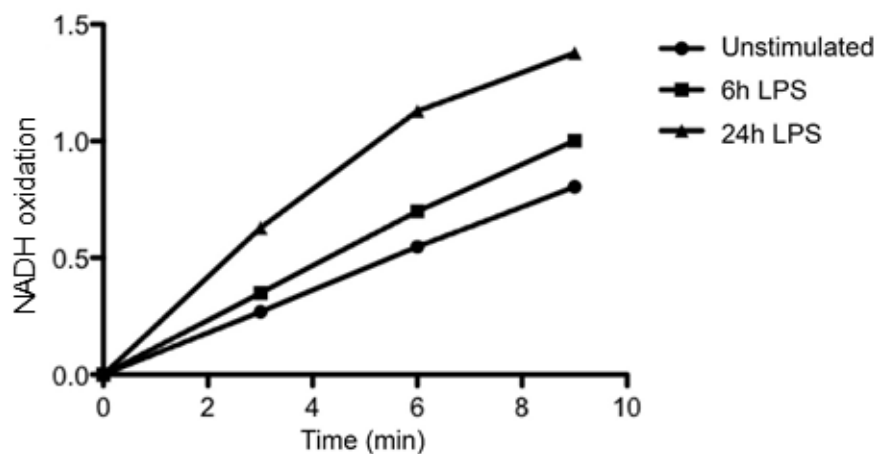


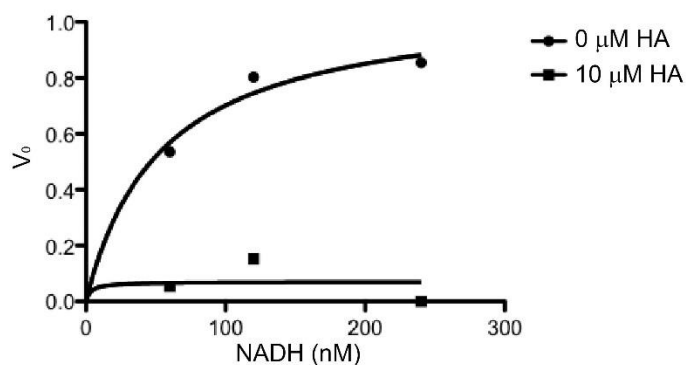
Figure 4.1 GAPDH enzymatic reaction



Treatments	Enzymatic rate (Δ abs/min)
Unstimulated	0.09 ± 0.01
6h LPS	0.11 ± 0.03
24h LPS	0.16 ± 0.02

Figure 4.2 GAPDH enzymatic activity is boosted with LPS. BMDMs were seeded at 0.5×10^6 cells/mL and left at 37°C, 5% CO₂ overnight. Cells were left unstimulated or they were stimulated with LPS (100 ng/mL) for 6 or 24 hours. Cells were lysed and lysates used to measure GAPDH enzymatic activity by measuring the absorbance of the samples at 340 nm in the presence of 3-PGA, 3-PGK, ATP, Cysteine and NADH at different times, which corresponds to the rate of NADH oxidation. Values are expressed as mean $\pm$ SEM of three independent experiments each carried out in triplicates.

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B.

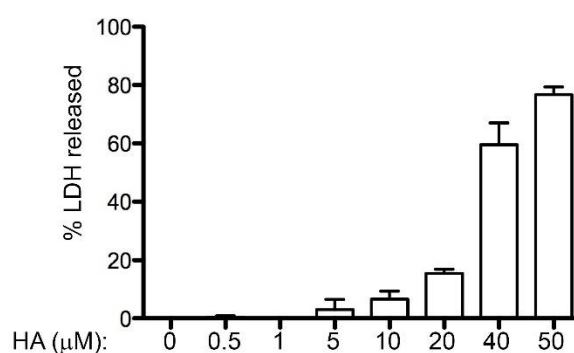
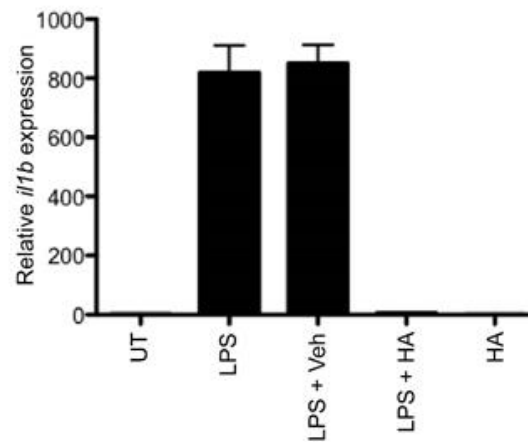


Figure 4.3 Heptelidic acid inhibits GAPDH enzymatic activity in macrophages and is not toxic. BMDMs were seeded at 0.5×10^6 cells/mL and left at 37°C, 5% CO₂ overnight. **A)** Medium was replaced by serum free medium alone or with medium containing 10 μM heptelidic acid (HA). Cells were lysed and the lysates used to measure GAPDH enzymatic activity by measuring NADH oxidation. Absorbance at 340 nm of the samples in the presence of 3-PGA, 3-PGK, ATP, cysteine and increasing concentrations of NADH after 6 minutes was recorded. Values are expressed as mean $\pm$ SD. Results are representative of two independent experiments each carried out in triplicates (V_0 = initial reaction rate). **B)** Medium was replaced by serum free medium alone or with medium containing various concentrations of HA for 24 hours. Supernatants were analysed for LDH release by Cytotox® assay. The level of cell death is expressed as percentage of that of control cells treated with a lysis solution. Values are expressed as mean $\pm$ SEM of three independent experiment, each carried out in triplicate.

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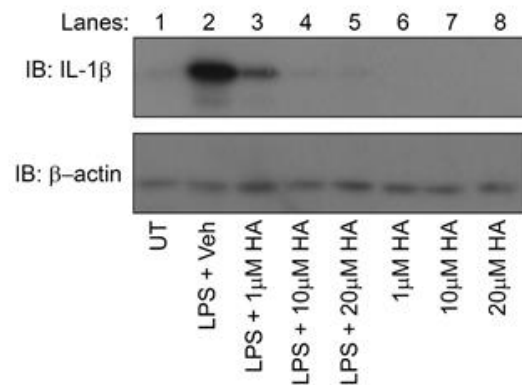


Figure 4.4 Heptelidic acid blocks pro-IL-1 β expression in BMDMs. BMDMs were seeded at 1×10^6 cells/mL in a 12 well plate and left at 37°C, 5% CO₂ overnight. **A)** The following day the medium was removed from the cells and replaced with serum free medium alone or with medium containing vehicle (DMSO, Veh), 10 μ M HA for 2 hours followed by 100 ng/mL of LPS for 24 hours. RNA was extracted and normalised, followed by a reverse transcription reaction. IL-1 β and 18S mRNA were quantified by qPCR using SYBR primers. Results are represented with the mean *il1b* expression normalised to 18S + SEM of three independent experiments, each carried out in triplicate determinations. **B)** The medium was removed from the cells and replaced with serum free medium alone or with medium containing 1, 10 or 20 μ M HA for 2 hours, followed by 100 ng/mL of LPS for 24 hours. Cells were lysed using sample loading buffer and separated via SDS-PAGE electrophoresis. Pro-IL-1 β and β -actin expression was measured by western blotting. Results are representative of five independent experiments.

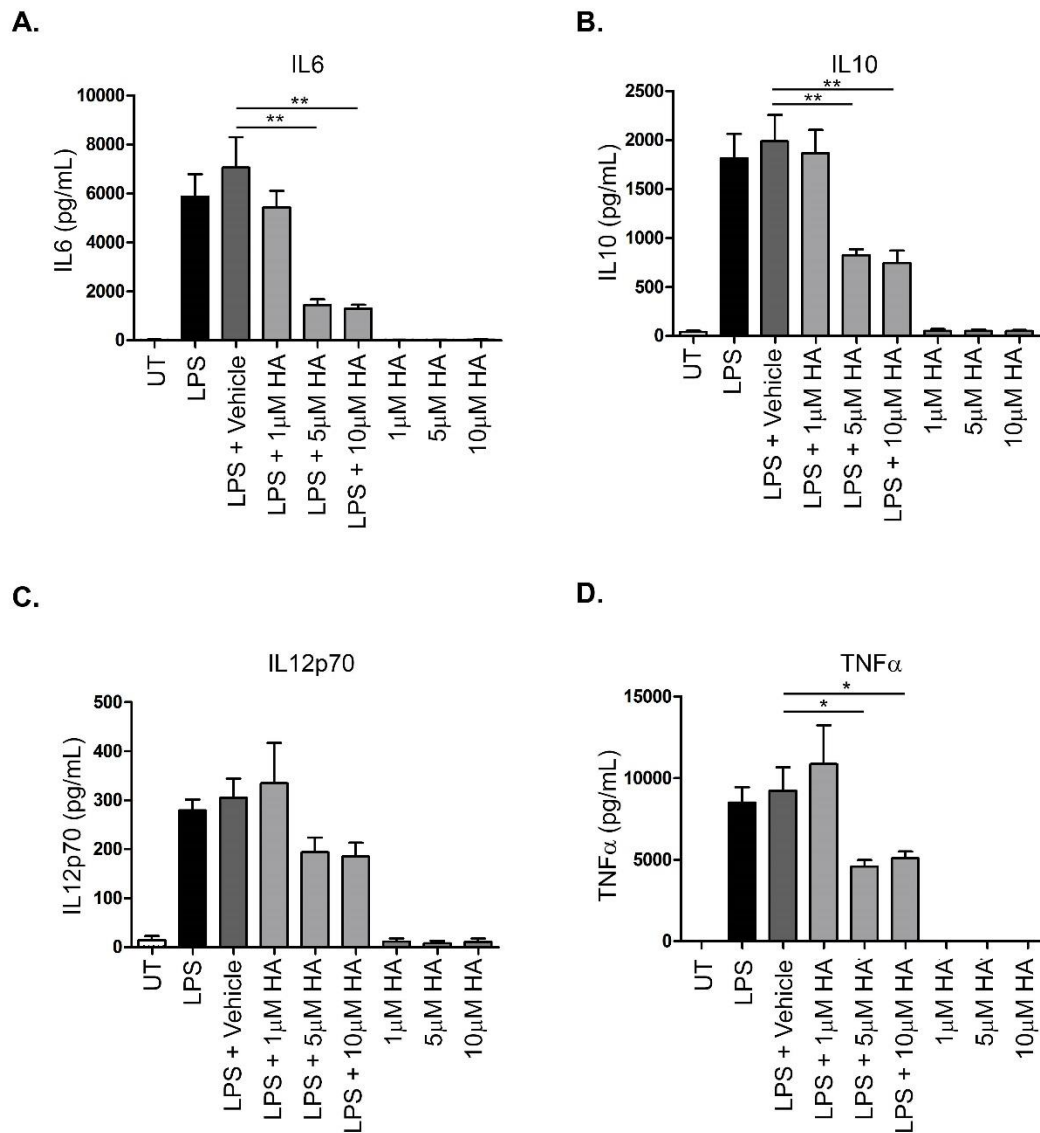
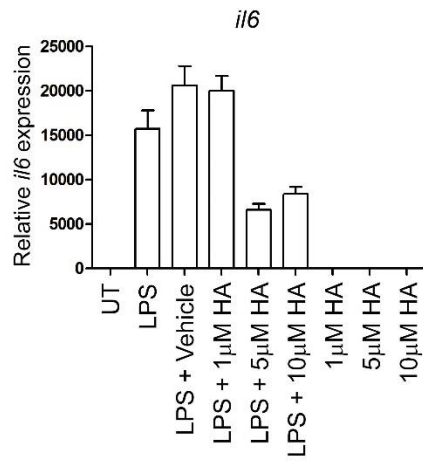
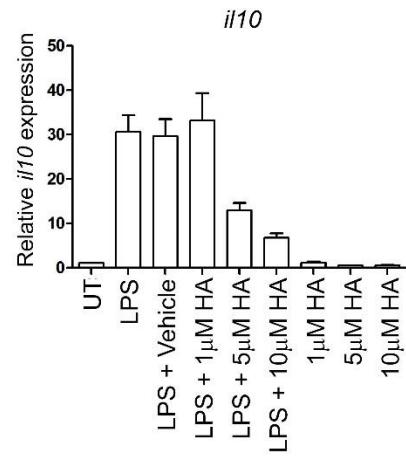


Figure 4.5 Heptelidic acid blocks IL6, IL10 and TNFα production in BMDMs. BMDMs were seeded at 1×10^6 cells/mL in 12 well plates and left at 37°C, 5% CO₂ overnight. The following day the medium was removed from the cells and replaced with serum free medium alone or with medium containing vehicle (DMSO), 1, 5 or 10 μM HA for 2 hours followed by 100 ng/mL LPS for 6 hours. Supernatants were analysed by MSD for **A)** IL6, **B)** IL10, **C)** IL12p70 and **D)** TNFα. The results are expressed as mean + SEM of four independent experiments, each carried out in triplicate determinations. (*p<0.05, **p<0.01, student's unpaired t test)

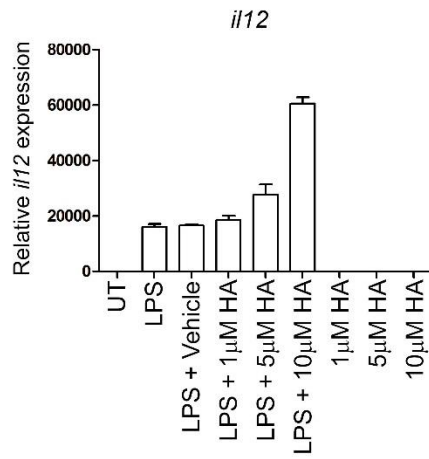
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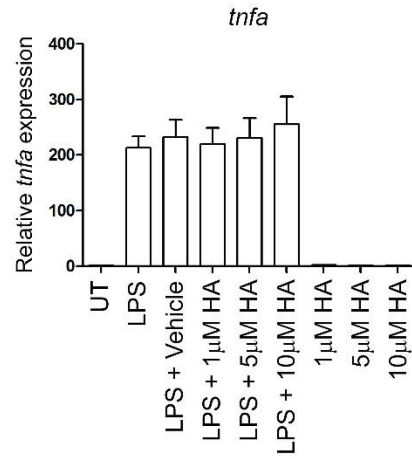
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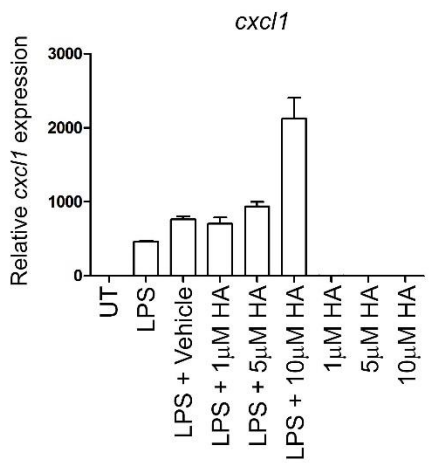
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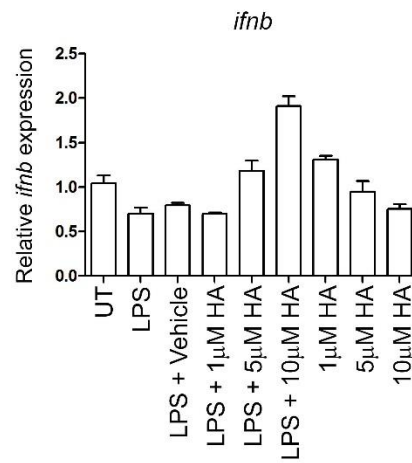


Figure 4.6 Heptelidic acid alters cytokine mRNA expression. BMDMs were seeded at 1×10^6 cells/mL in 12 well plates and left overnight at 37°C, 5% CO₂. The following day the medium was removed from the cells and was replaced with medium alone or medium containing vehicle (DMSO), 1, 5 or 10 µM HA for 2 hours followed by 100 ng/mL LPS for 6 hours. RNA was extracted and normalised, followed by a reverse transcription reaction. **A)** IL6, **B)** IL10, **C)** IL12, **D)** TNFα, **E)** CXCL1 and **F)** IFNβ mRNA were quantified by qPCR using SYBR primers. Results are represented with the mean cytokine expression normalised to β-actin + SD. Results are representative of three independent experiments, each carried out in triplicate.

4.2.3 Inhibiting glycolysis through 2DG has different effects to GAPDH inhibition alone

Given the differences observed in cytokine production when inhibiting GAPDH using HA, I proceeded to compare its effects with the glycolytic inhibitor 2DG. 2DG inhibits further upstream than HA, blocking hexokinase, the first enzyme in the pathway [172]. 2DG has previously been identified to block pro-IL1 β production in macrophages [27], therefore I started by comparing the effects of both inhibitors on IL1 β transcription. HA was found to inhibit IL1 β transcription to the same extent as 2DG, but at much lower doses (Figure 4.7A). The same inhibitory effect was also observed on IL10 transcription (Figure 4.7D). Interestingly, this was not the case for TNF α and IL6 transcription, both of which were boosted by 2DG but not HA (Figure 4.7B and Figure 4.7C). Similar differences were observed between both inhibitors on LPS-induced cytokine production at the protein level. While HA could inhibit the production of IL6, IL10 and TNF α , 2DG was only found to inhibit IL10 (Figure 4.8B), with no significant effects observed on IL6, TNF α or IL12 (Figure 4.8).

Next I proceeded to examine the effects of both HA and 2DG side by side on human macrophages derived from circulating CD14⁺ monocytes (MDMs). Human macrophages were pre-treated with 10 μ M HA or 10 mM 2DG for 2 hours, followed by 6 hours of LPS stimulation. IL10 production following LPS treatment was the only cytokine found to be significantly inhibited by both HA and 2DG (Figures 4.9E). IL6 production was found to be reduced by HA, while IL12p70 was reduced by 2DG (Figure 4.9C and 4.9F). Similar non-significant trends were also observed with other cytokines, although it is important to note that only a maximum of five donors were used in the experiments, and that increasing the donor number is likely to increase the statistical significance of the experiment.

Overall the results of examining LPS-induced cytokine production following glycolysis inhibition through 2DG or HA are complex. A clear conclusion emerges however, and that is that the inhibition in the production of certain cytokines by HA can't solely be attributed to the inhibition of ATP production from glycolysis. Furthermore, it is interesting to compare the results obtained from murine macrophages versus human ones. While both inhibitors did have the same effects on the production of certain cytokines in both species, as observed for IL10, there are other cytokines, like IL6 and IL12, that were affected differently by 2DG in mouse cells when compared to humans (Table 4.1). In the case of IL12p70 production for instance, 2DG significantly reduced production levels in MDMs, while having no effect on BMDMs.

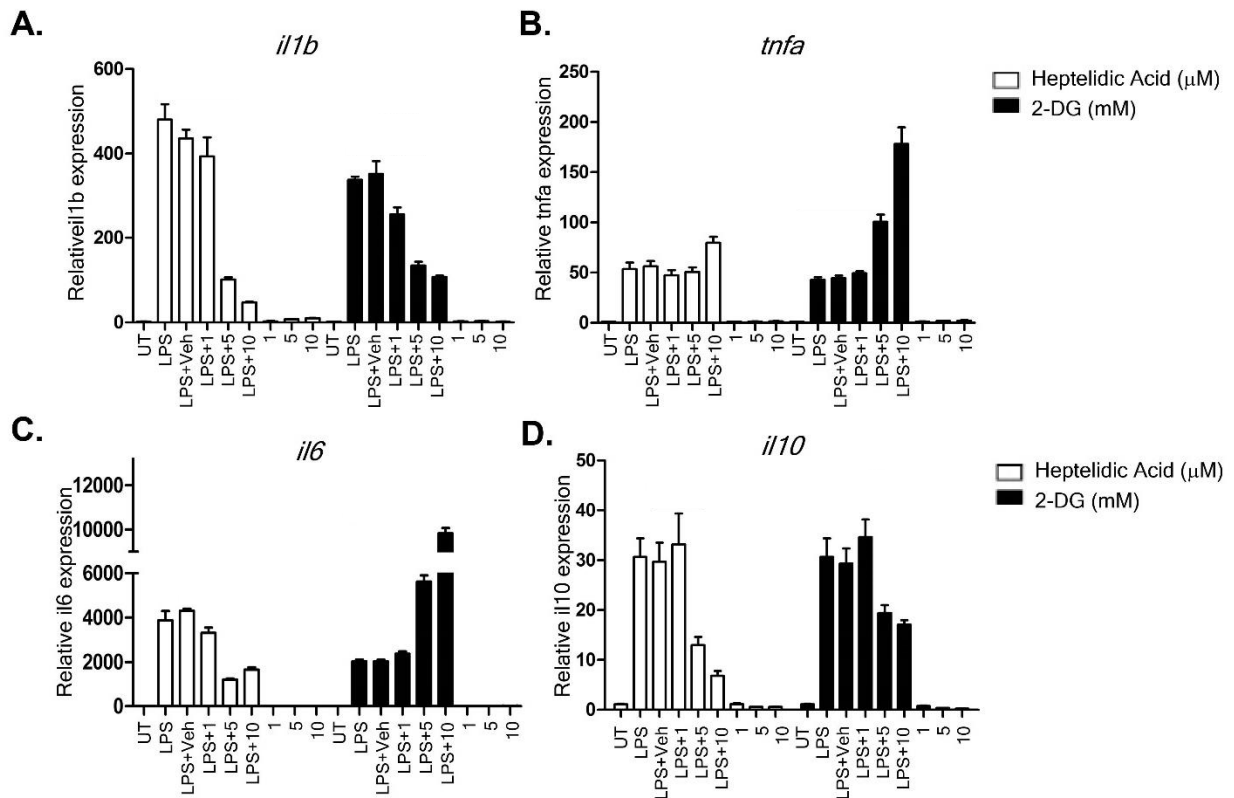


Figure 4.7 HA has the same effect as 2DG on IL1 β and IL10 transcription, but not TNF α and IL6 in BMDMs. BMDMs were seeded at 1×10^6 cells/mL in 12 well plates and left overnight to incubate at 37°C, 5% CO₂. The following day the medium was removed from the cells and replaced with serum free medium alone or with medium containing vehicle (water for 2DG, DMSO for HA), 1, 5 or 10 μ M HA, or 1, 5 or 10 mM 2DG for 2 hours. Cells were then treated with 100 ng/mL LPS for 6 hours. RNA was extracted and normalised, followed by a reverse transcription reaction. **A)** IL1 β , **B)** TNF α , **C)** IL6 and **D)** IL10 mRNA were quantified by qPCR using SYBR primers. Results are represented with the mean cytokine expression normalised to the geometrical mean of *gapdh*, *18S* and *actb* + SD, representative of three independent experiments, each carried out in triplicate.

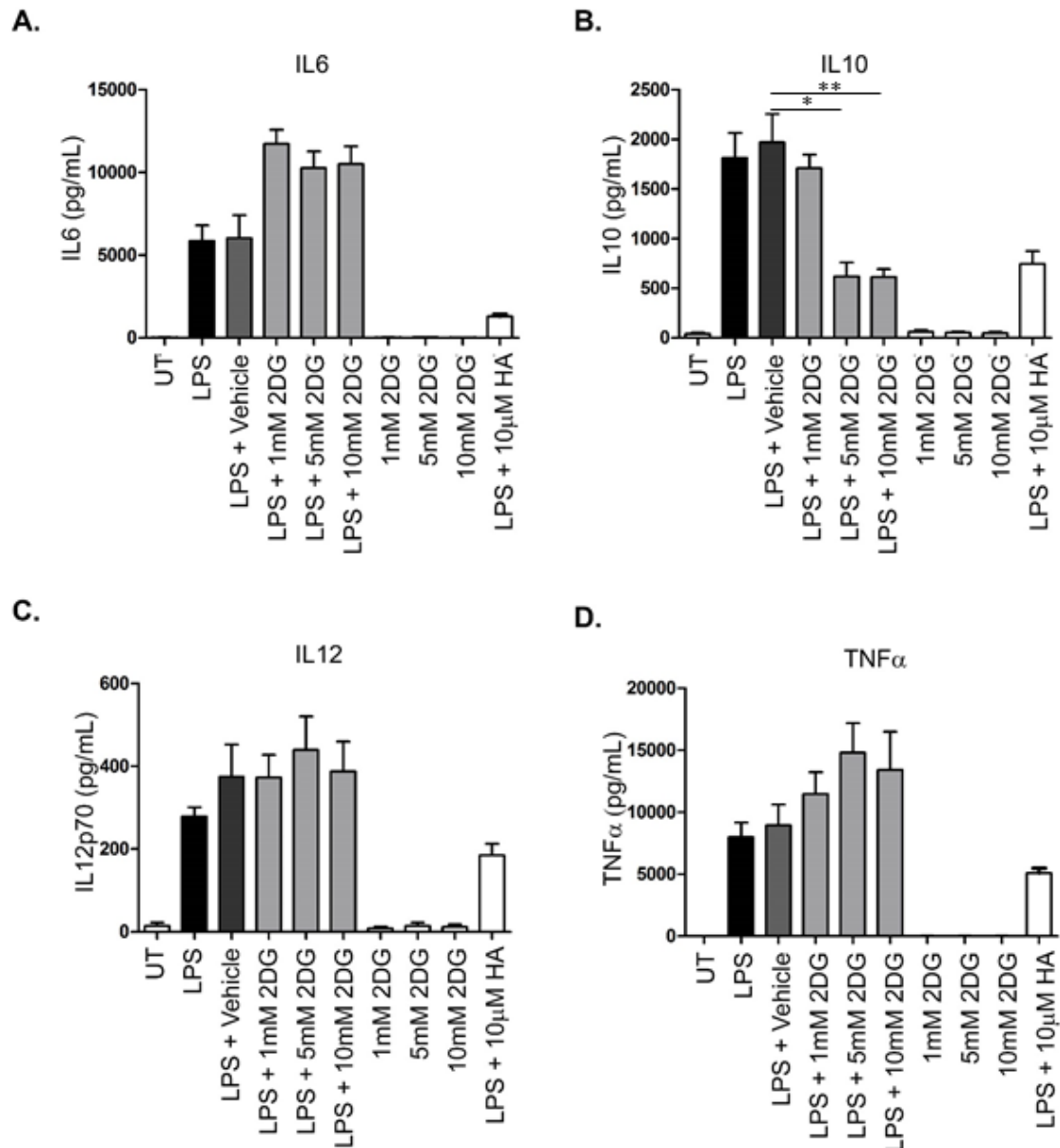


Figure 4.8 HA has the same effect as 2DG on IL10 production but not other cytokines in BMDMs. BMDMs were seeded at 1×10^6 cells/mL in 12 well plates and left at 37°C, 5% CO₂ overnight. The following day the medium was removed from the cells and replaced with serum free medium alone or with medium containing vehicle (water), 1, 5 or 10 mM 2DG for 2 hours. Some cells were also treated with 10 μ M HA for 2 hours. Cells were then treated with 100 ng/mL LPS for 6 hours. Supernatants were analysed by MSD for **A)** IL6, **B)** IL10, **C)** IL12p70 and **D)** TNF α . The results are expressed as mean + SEM of four independent experiments, each carried out in triplicate determinations (*p < 0.05, **p < 0.01, student's unpaired t test).

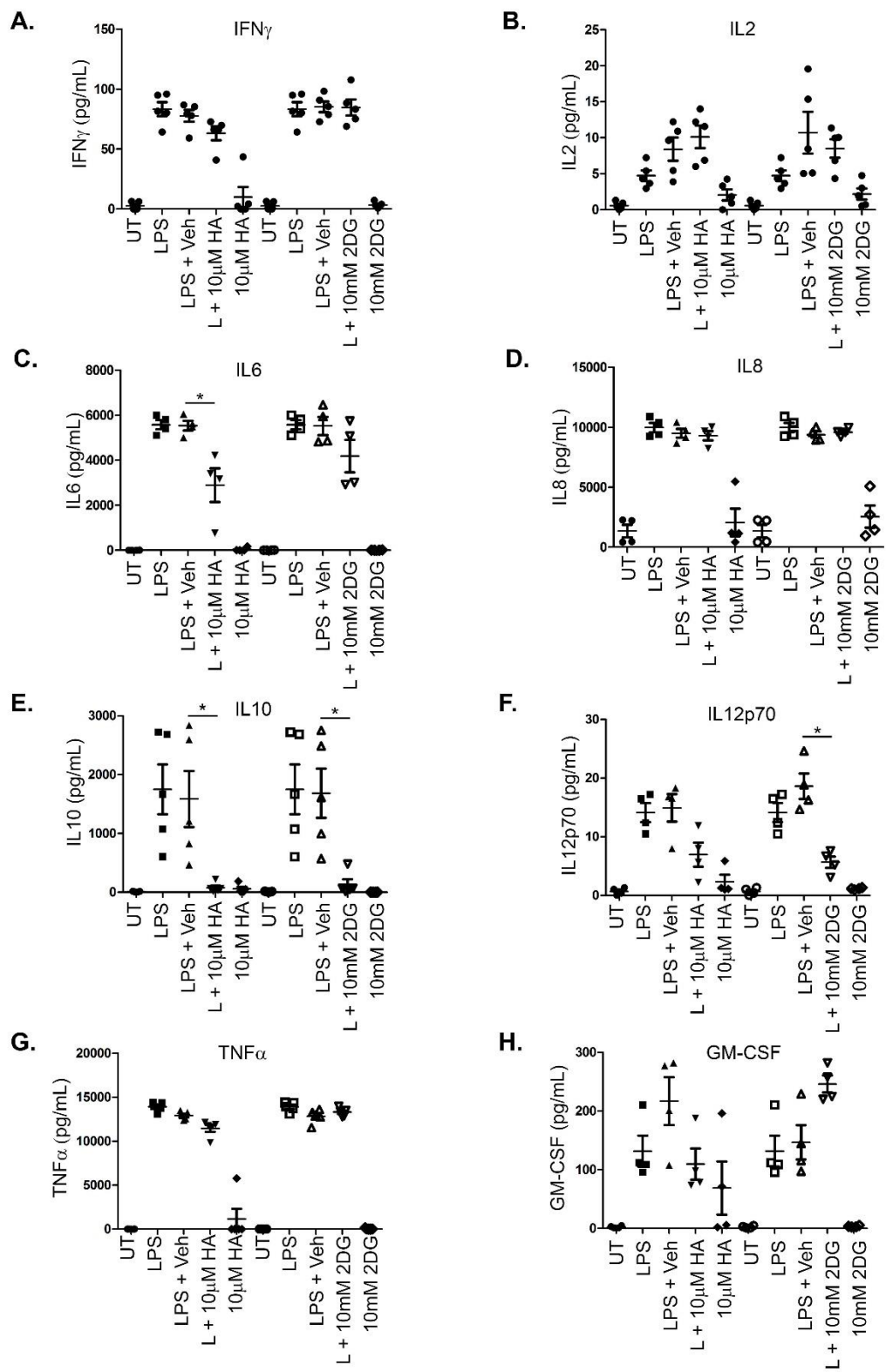


Figure 4.9 HA and 2DG alter cytokine production in human monocyte-derived macrophages. CD14⁺ monocytes were seeded at 0.5×10^6 cells/mL with MCSF for 7 days at 37°C, 5% CO₂. The following day the medium was replaced with serum free medium alone or with medium containing vehicle, 10 µM HA or 10 mM 2DG for 2 hours followed by 100 ng/mL LPS for 6 hours. Supernatants were analysed by MSD for **A)** IFN γ , **B)** IL2, **C)** IL6, **D)** IL8, **E)** IL10, **F)** IL12p70, **G)** TNF α and **H)** GM-CSF. Results of five donors shown + SEM (*p<0.05, student's paired t test).

	BMDMs		MDMs	
	HA	2DG	HA	2DG
IL6	↓	no effect	↓	no effect
IL10	↓	↓	↓	↓
IL12	no effect	no effect	no effect	↓
TNFα	↓	no effect	no effect	no effect
IL1β	↓	↓	-	-

Table 4.1 Summary of cytokine results obtained from pre-treatment of human (MDMs) versus mouse (BMDMs) LPS-activated macrophages with HA or 2DG.

4.2.4 GAPDH knockdown boosts TNF α production while reducing IL10, IL6 and pro-IL1 β

Next I proceeded to investigate the effects of knocking down GAPDH in BMDMs on LPS-induced cytokine production. GAPDH was first knockdown by over 50% using two independent siRNAs, when compared to a scramble siRNA control (Figure 4.10, upper panel, lanes 5 and 6). Pro-IL1 β production was also measured via western blotting in the same samples and found to be reduced following LPS stimulation, to the same extent as the knockdown (Figure 4.10, middle panel, lanes 5 and 6). This result is in agreement with GAPDH inhibition using HA, which also showed a reduction in pro-IL1 β production (Figure 4.4). IL10 and IL6 were also measured in the supernatants, and were found to be significantly reduced with both siRNAs (Figures 4.11B and 4.11C). Interestingly, the opposite effect was observed on TNF α production, which was boosted (Figure 4.11A). This is not only in contrast to the other cytokines measured, but also to the inhibition of GAPDH enzymatic activity using HA, which was observed to reduce TNF α production (Figure 4.5D). The levels of the anti-viral cytokine RANTES were also measured and were found to be unaffected by GAPDH knockdown (Figure 4.9D).

A key result that emerges from the various approaches used to study GAPDH activity thus far is TNF α production. First, 2DG does not block induction of TNF α in response to LPS (Figure 4.8D). HA however inhibits the induction of TNF α protein (Figure 4.5D), but not its mRNA (Figure 4.6D). Furthermore, knockdown of GAPDH boosts TNF α production (Figure 4.11A, Table 4.2). These results together indicate that glycolysis is not involved in TNF α production, as previously shown [27], but point towards GAPDH having a post-transcriptional role in TNF α production.

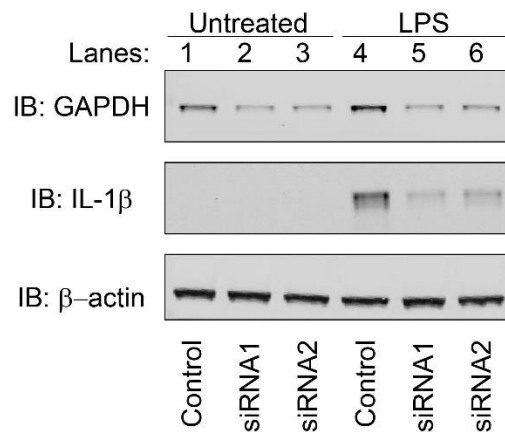


Figure 4.10 GAPDH knockdown reduces pro-IL-1β in BMDMs. BMDMs were seeded at 0.5×10^6 cells/mL in 12 well plates and left at 37°C, 5% CO₂. Medium was replaced with Optimem and cells were transfected with 10 nM of a negative control siRNA, a GAPDH siRNA1 or a GAPDH siRNA2. Optimem was replaced with medium 12 – 24 hours post-transfection and cells were treated with 100 ng/mL LPS 48 hours post-transfection. Cells were lysed using sample loading buffer 24 hours later and samples were separated via SDS-PAGE electrophoresis. GAPDH, pro-IL-1β and β-actin expression was measured by western blotting. Results are representative of three independent experiments.

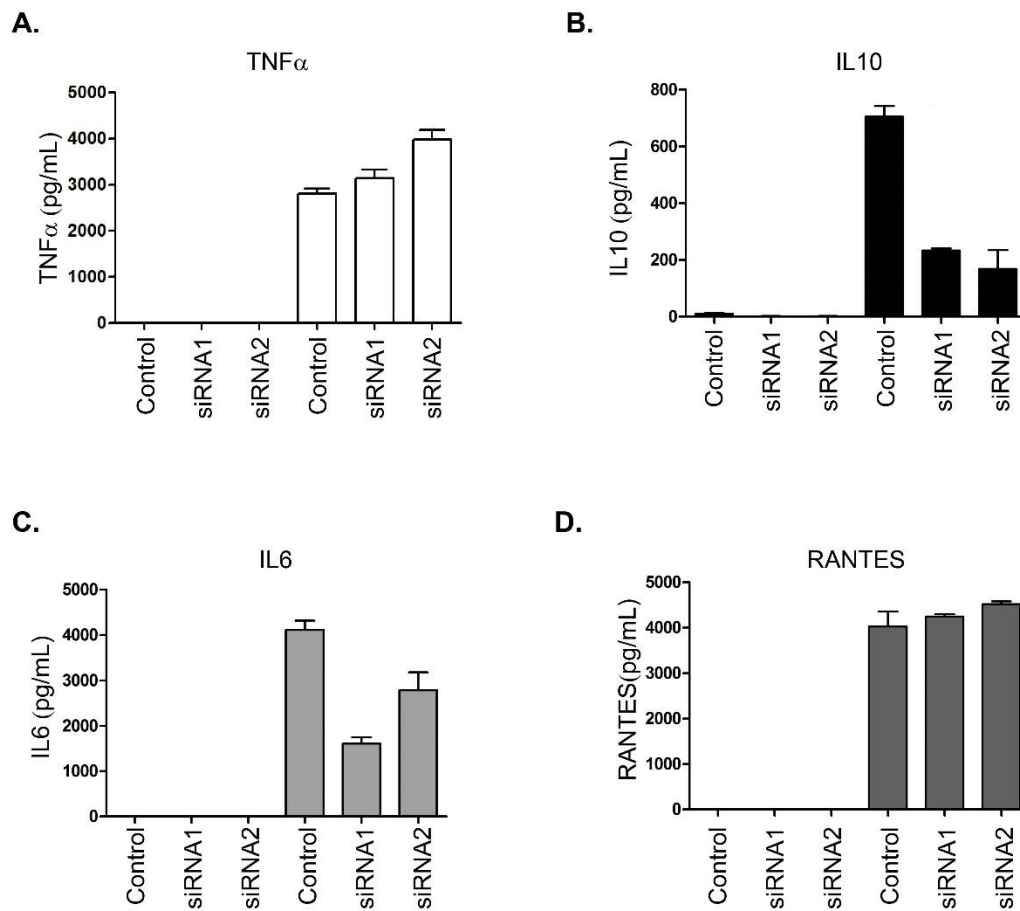


Figure 4.11 GAPDH knockdown boosts TNF α and reduces IL10 and IL6 production in BMDMs. BMDMs were seeded at 0.5×10^6 cells/mL in 12 well plates and left at 37°C, 5% CO₂. Medium was replaced with Optimem and cells were transfected with 10 nM of a negative control siRNA, a GAPDH siRNA1 or a GAPDH siRNA2. Optimem was replaced with medium 12 – 24 hours post-transfection and cells were treated with 100 ng/mL LPS 48 hours post-transfection. Supernatants were harvested 24 hours later and they were analysed by ELISA for **A)** TNF α , **B)** IL10, **C)** IL6 and **D)** RANTES production. Results are expressed as mean + SD and are representative of three independent experiments, each carried out in triplicate.

	IL1 β		TNF α		IL6		IL10	
	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA
2DG	↓	↓	no effect	↑	no effect	↑	↓	↓
HA	↓	↓	↓	no effect	↓	↓	↓	↓
GAPDH KD	↓	-	↑	-	↓	-	↓	-

Table 4.2 Summary of cytokine results obtained when using the inhibitors 2DG and HA, as well as knocking down GAPDH.

4.3 Characterizing GAPDH RNA-binding activities

4.3.1 GAPDH binds TNF α and DAPK1 mRNA and releases them following treatment with LPS

Previous results would suggest that the role of GAPDH in regulating cytokine production in macrophages goes beyond its glycolytic enzymatic activity. GAPDH is also an RNA-binding protein and has been reported to bind, among others, to the 3'-UTR of mRNAs encoding the inflammatory mediators TNF α and COX2 [133, 173]. As shown in Figure 4.12, GAPDH was found to bind to mRNAs for TNF α and COX2 in RAW264.7 cells, as revealed by enhanced binding detected in the immunoprecipitated sample over the IgG control. Interestingly, upon stimulation with LPS for 24 hours, the RNAs did not appear to be bound to GAPDH any longer. The same experiment was repeated in BMDMs, where the same result was observed with TNF α mRNA bound by GAPDH and released following LPS activation (Figure 4.13). COX2 mRNA on the other hand, while initially found to bind to GAPDH, this effect was not reproducible. Therefore, COX2 mRNA seems to overall not be regulated by GAPDH in primary macrophages (Figure 4.13).

Next we proceeded to investigate whether GAPDH could bind other mRNAs. To this effect, primers were designed for different inflammatory mediators known to carry AU-rich or GAIT elements in their 3'-UTR, which are the sequences that GAPDH has been reported to bind. GAPDH was immunoprecipitated from BMDMs and bound RNAs were analysed by qPCR. DAPK1 was found to have a 50 fold increase of RNA bound to GAPDH when compared to the IgG control (Figure 4.14). Furthermore, and as seen previously with TNF α , DAPK1 mRNA was also released following stimulation with LPS. This effect was examined further by repeating the RNA-IP in RAW264.7 cells, in which a similar effect was observed (Figure 4.15A). Furthermore, the RNA binding was studied under two different times of LPS stimulation. A reduction in the presence of DAPK1 mRNA bound to GAPDH versus bound to the control IgG after 6 hours of LPS stimulation was observed, although the binding was not completely abrogated until after 24 hours of LPS stimulation (Figure 4.15B).

4.3.2 Identifying GAPDH RNA-binders through RNA-IP sequencing (RIP-seq)

While manual selection of potential binders enabled us to find DAPK1 as a novel RNA-binder, we next sought to use an unbiased and much more thorough approach to search for novel GAPDH RNA targets. To that effect, we designed a RIP-Seq experiment, in which following GAPDH RNA-IP, a cDNA library was generated and sequenced. Various interesting observations were noted, starting with having found as many genes to bind GAPDH from LPS-treated macrophages as GAPDH from untreated ones (Figure 4.16). The associated functions

of the RNAs identified under each condition however differ greatly. The top ten RNAs identified to bind GAPDH from resting macrophages, including CDCA5, MCM10, E2F6, UHRF1 and MLH3, are mostly coding for proteins involved in cell proliferation and DNA replication and repair (Table 4.3). However, the top RNAs identified to bind GAPDH from LPS-treated macrophages, including Gm12346, Gm13216, Gm15590 and Gm26586, are mostly non-coding RNAs (Table 4.4). Furthermore, TNF α was still identified as binding to GAPDH only in resting macrophages (Figure 4.17A). Interestingly, despite previous observations from RNA-IPs, neither COX2 nor DAPK1 were detected to bind GAPDH in this experiment (Figure 4.17B and Figure 4.17C). Overall, further work is required to validate this results, but they would indicate that GAPDH RNA-binding activity is much more complex than what has been reported thus far.

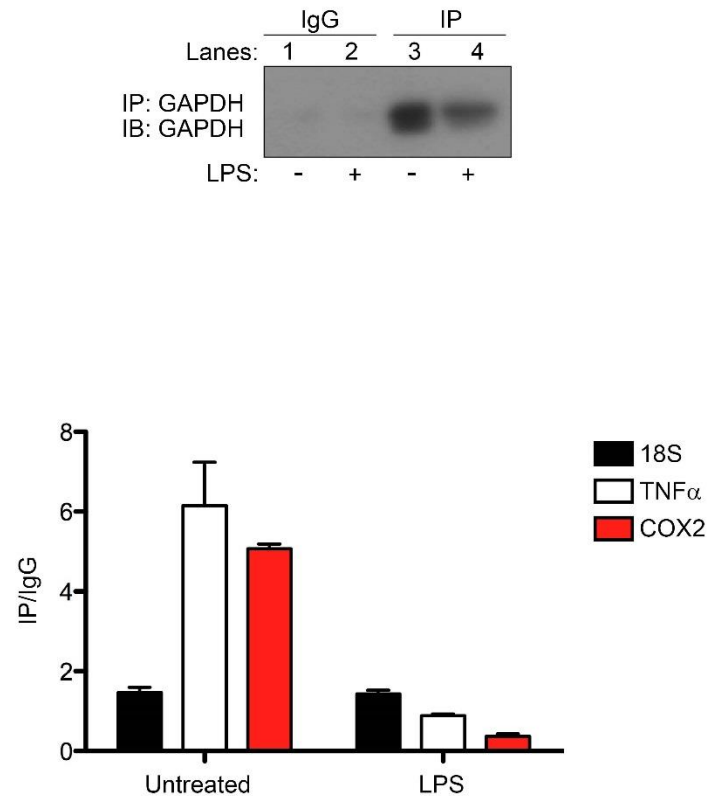
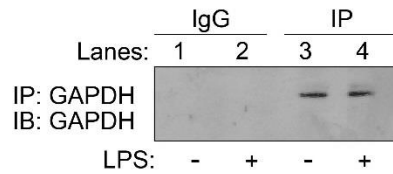
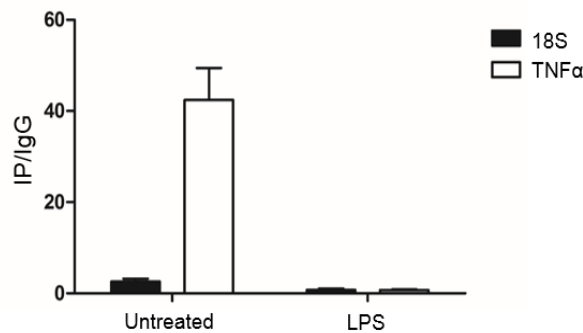


Figure 4.12 GAPDH binds to TNF α and COX2 mRNA in RAW264.7 macrophages. RAW264.7 cells were seeded in 10 cm dishes at 0.8×10^6 cells/mL and left overnight at 37°C. Cells were left unstimulated or were stimulated with LPS (100 ng/mL) for 24 hours and then fixed. They were then lysed using polysome lysis buffer and pre-cleared with protein A/G beads and yeast RNA for 30 minutes. Lysates were used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed five times with polysome lysis buffer and were resuspended in protein-RNA elution buffer. **A)** 30 μ L of sample was kept for western blot analysis using a GAPDH antibody. **B)** The rest of the sample was used for purification of RNA using QIAzol, followed by a reverse transcription reaction. TNF α , COX2 and 18S mRNA were quantified by qPCR using SYBR primers. Results shown correspond to the mean expression of the IP samples folded over the IgG samples + SD representative of three independent experiments, each carried out in triplicate.

A.



B.



C.

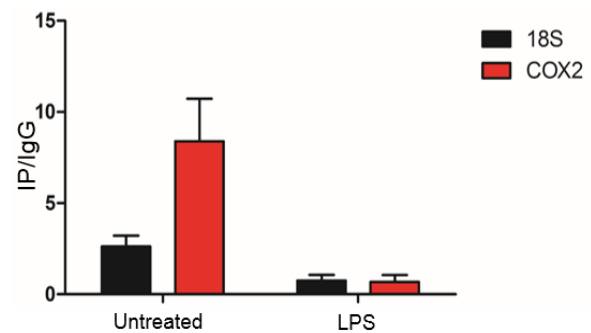


Figure 4.13 GAPDH binds to TNFα mRNA in BMDMs and COX2 to a lesser extent. BMDMs were seeded in 10 cm dishes at 1×10^6 cells/mL and left overnight at 37°C. Cells were left unstimulated or were stimulated with LPS (100 ng/mL) for 24 hours and then fixed. They were then lysed using polysome lysis buffer and pre-cleared with protein A/G beads and yeast RNA for 30 minutes. Lysates were used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed five times with polysome lysis buffer and were resuspended in protein-RNA elution buffer. **A)** 30 μL of sample was kept for western blot analysis using a GAPDH antibody. The rest of the sample was used for purification of RNA using QIAzol, followed by a reverse transcription reaction. **B)** TNFα and 18S mRNA were quantified by qPCR using SYBR primers. Results shown correspond to the mean expression of the IP samples folded over the IgG samples + SD representative of three independent experiments, each carried out in triplicate. **C)** COX2 and 18S mRNA were quantified by qPCR using SYBR primers. Results shown correspond to the mean expression of the IP samples folded over the IgG samples + SD of one experiment, carried out in triplicate.

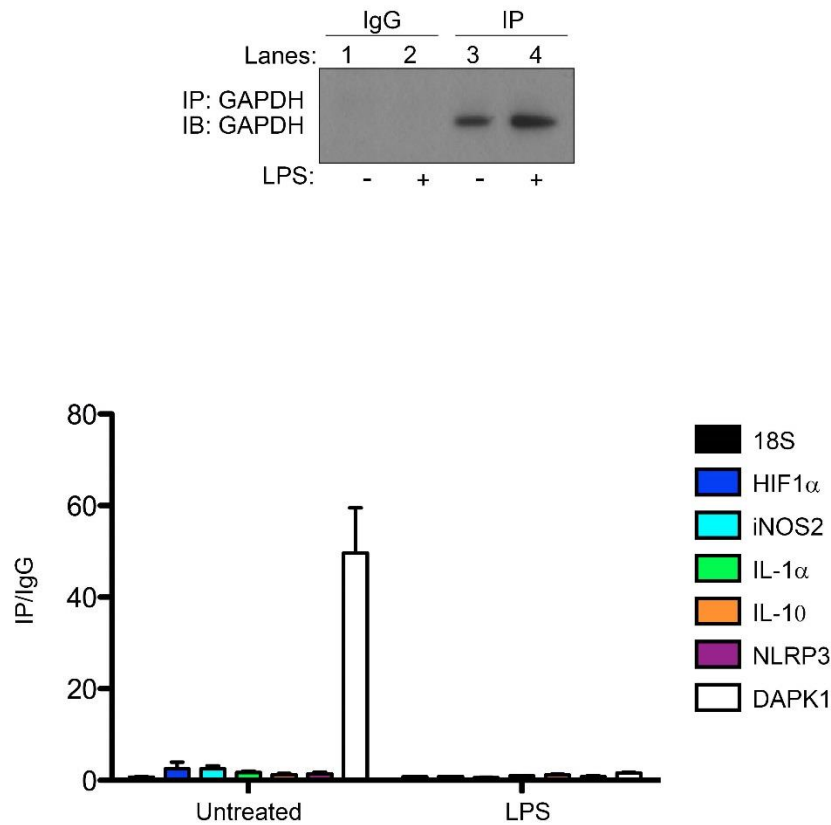
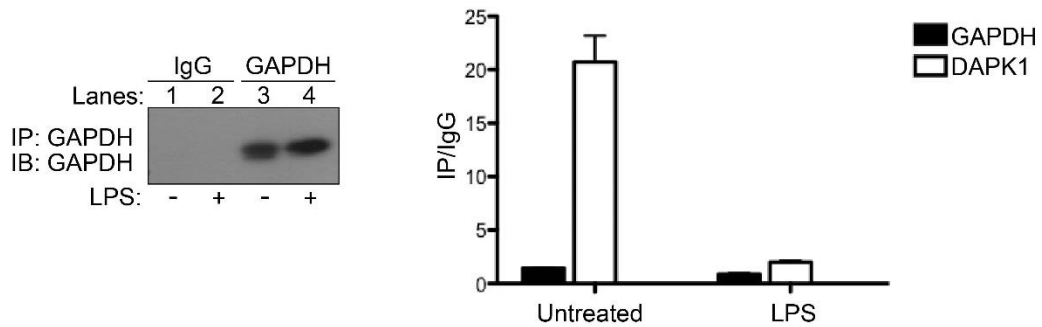


Figure 4.14 Screen of potential binding mRNA for GAPDH in BMDMs. BMDMs were seeded in 10 cm dishes at 1×10^6 cells/mL and left overnight at 37°C , 5% CO_2 . Cells were left unstimulated or were stimulated with LPS (100 ng/mL) for 24 hours and then fixed. They were then lysed using polysome lysis buffer and pre-cleared with protein A/G beads and yeast RNA for 30 minutes. Lysates were used for IP with a control antibody or a GAPDH antibody coupled to protein A/G beads. Samples were washed five times with polysome lysis buffer and were resuspended in protein-RNA elution buffer. **A)** 30 μL of sample was kept for western blot analysis using a GAPDH antibody. **B)** The rest of the sample was used for purification of RNA using QIAzol, followed by a reverse transcription reaction. HIF1 α , iNOS, IL-1 α , IL10, NLRP3, DAPK1 and 18S mRNA were quantified by qPCR using SYBR primers. Results shown correspond to the mean expression of the IP samples folded over the IgG samples + SD of triplicate determinations.

A.



B.

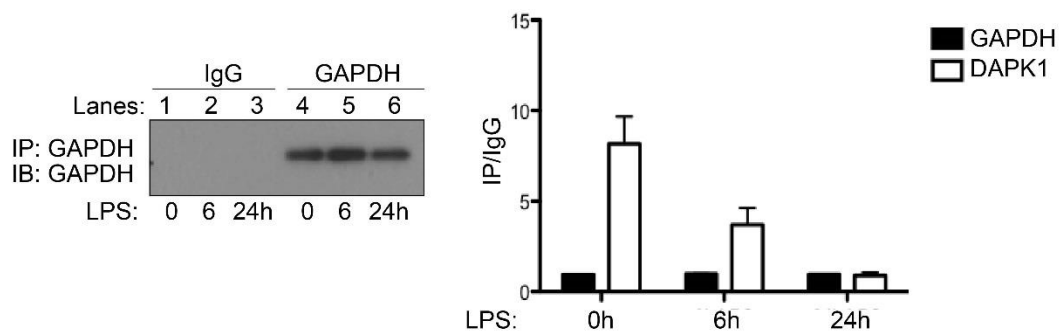


Figure 4.15 GAPDH binds to DAPK1 mRNA in resting macrophages and releases it following LPS treatment. RAW264.7 cells were seeded in 10 cm dishes at 0.8×10^6 cells/mL and left overnight at 37°C, 5% CO₂. Cells were left unstimulated or were stimulated with LPS (100 ng/mL) for only 24 hours (**A**) or for 6 and 24 hours (**B**). Cells were then fixed and lysed using polysome lysis buffer followed by pre-clearance with protein A/G beads. Samples were washed five times with polysome lysis buffer and resuspended in protein-RNA elution buffer. 30 μ L of sample was kept for western blot analysis using a GAPDH antibody (**A**, **B**, first panel). The rest of the sample was used for purification of RNA using QIAzol, followed by a reverse transcription reaction. DAPK1 and GAPDH mRNA were quantified by qPCR using SYBR primers. Results shown correspond to the mean expression of the IP samples folded over the IgG samples + SD of triplicate determinations. Results are representative of five independent experiments.

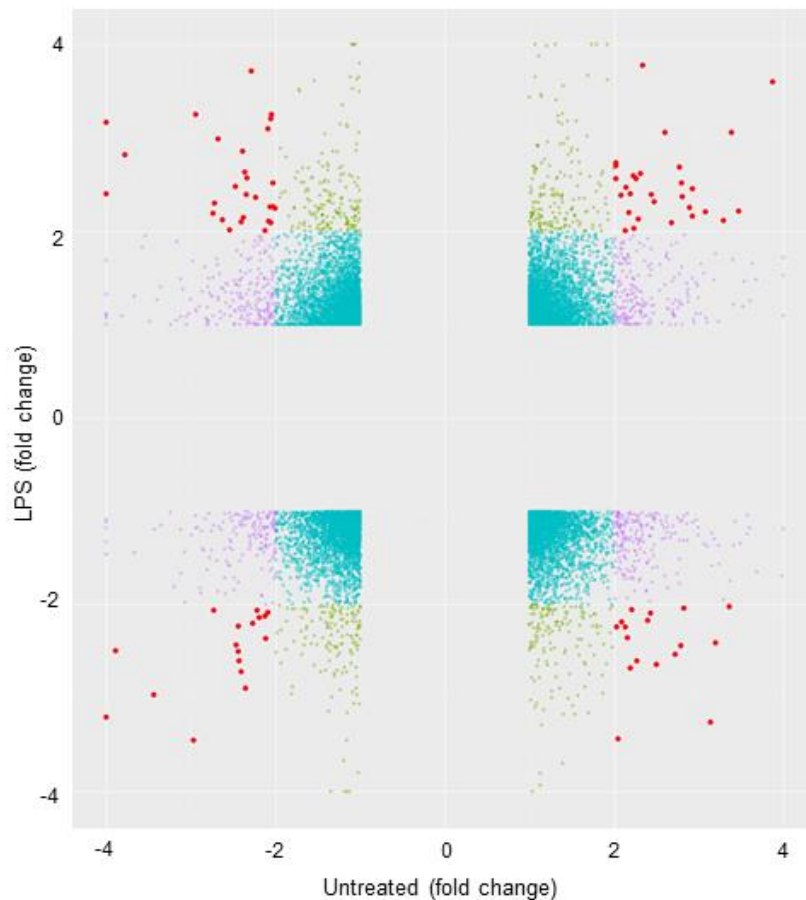


Figure 4.16 Dot representation of the genes identified in GAPDH RIPSeq. BMDMs were seeded in 15 cm dishes at 1×10^6 cells/mL and left overnight at 37°C. Cells were left unstimulated or were stimulated with LPS (100 ng/mL) for 24 hours in duplicates and then fixed. They were then lysed using polysome lysis buffer and lysates used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed five times with polysome lysis buffer and were resuspended in protein-RNA elution buffer. RNA was extracted using phenol-chloroform. A cDNA indexed library was generated and sequenced on HiSeq. The number of reads obtained in the IP samples was compared to the number of reads in the IgG control sample to yield a fold change value. Each gene identified is depicted as a dot (n=4).

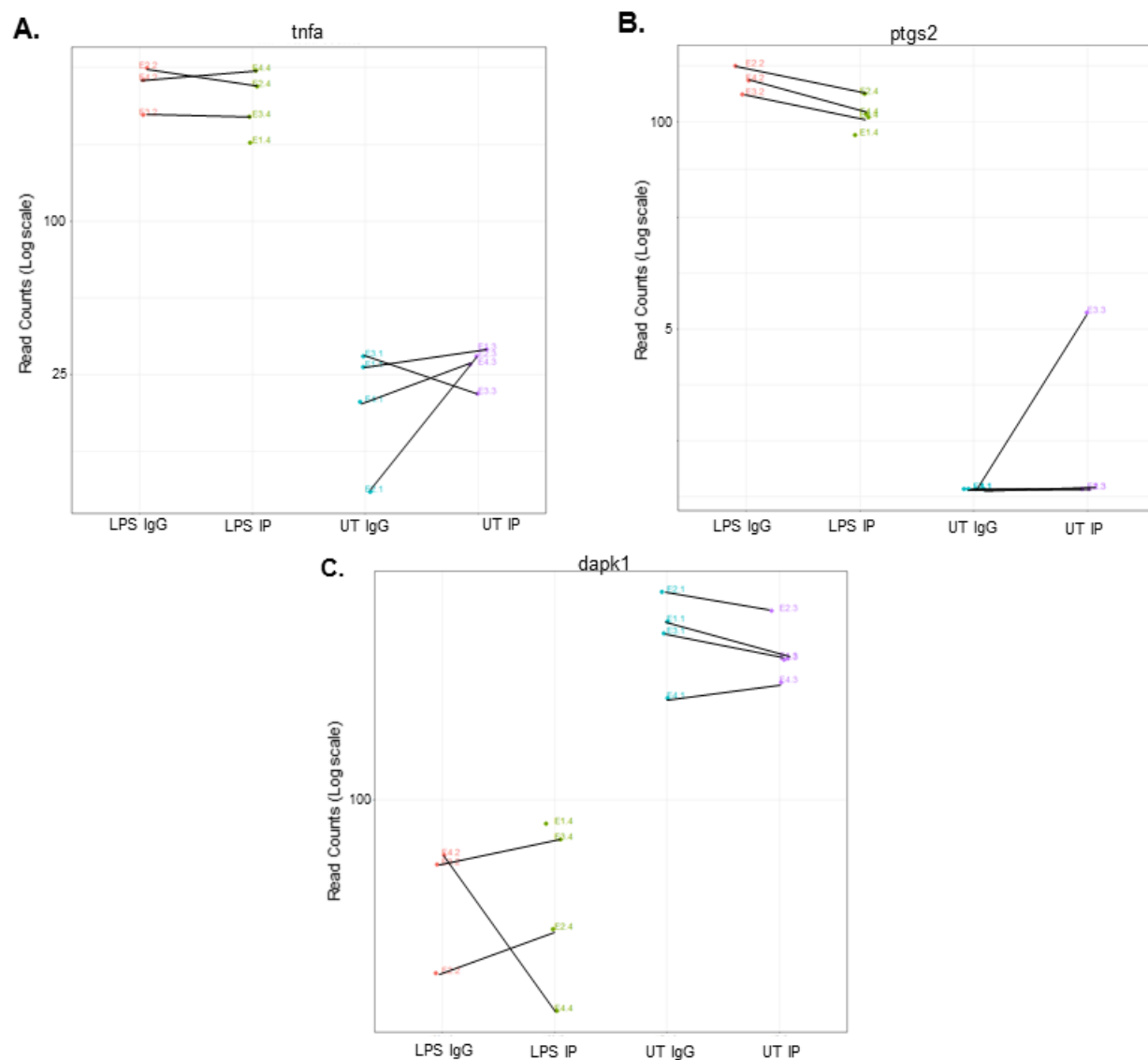


Figure 4.17 TNF α , COX2 and DAPK1 sequencing results. The read counts obtained for each gene from each sample in the four experiments are depicted.

Gene_Symbol	Fold_Change	Log2_Fold_Change	pvalue
Snord49a	8.594583515	3.10342773	1.75E-05
Fkbp9	7.888018142	2.97966287	8.73E-07
Cdca5	4.729399522	2.24165702	0.0023965
Mcm10	3.605033428	1.850012637	0.0048043
Hells	3.57096421	1.836313675	0.0004794
E2f6	3.410441621	1.769958567	0.0013255
Uhrf1	3.159526112	1.659708189	0.000803
Emc2	2.707521893	1.436973004	0.0046622
Slx4ip	2.266655469	1.180565119	0.0068502
Mlh3	2.213340854	1.146225643	0.0005398
Adgb	2.038103754	1.027227497	0.0321075
Fam114a1	1.816702943	0.861322537	0.0022222
Cp	1.793931143	0.843124516	0.0258949
ENSMUSG00000089832	1.782019449	0.833513082	0.0152852
Rad23a	1.76201557	0.817226673	0.0176584
Zfp738	1.742004637	0.800748464	0.0325668
Txndc12	1.729836139	0.790635383	0.0103975
Ilf2	1.728846273	0.789809592	0.0262043
Ppfia1	1.720328742	0.78268428	0.0007026
Ybx3	1.709563021	0.773627607	0.0452311
Stard4	1.689181295	0.756324177	0.0441783
Cdc25a	1.671552918	0.741189029	0.0382979
Adnp2	1.661529417	0.732511836	0.0357491
Ubash3b	1.635626426	0.709843277	0.0134712
Ccdc134	1.632729393	0.7072857	0.0481057
Pxk	1.624192646	0.699722761	0.0046712
Rpl11	1.622235473	0.697983247	0.0262039
Zfp568	1.621066494	0.69694327	0.0400199
Vdac3	1.616754906	0.693100988	0.0410909
ENSMUSG00000103233	1.609549877	0.686657284	0.0340615
Utp14a	1.597671919	0.675971182	0.0391751
Utp14b	1.594554755	0.673153639	0.0412289
Wdr62	1.594143696	0.672781679	0.037615
Sap30	1.571639479	0.652270313	0.0492829
Tcp11l1	1.571435648	0.652083193	0.0037712
Dnaja1	1.555221137	0.637119732	0.007693
Rab11a	1.537378564	0.620472458	0.0425828
Tti1	1.532043798	0.615457542	0.0231283
Nde1	1.519896016	0.603972625	0.0371258
Nucks1	1.513634047	0.598016446	0.0281704
Slc35f5	1.49199947	0.577247023	0.0327615

Table 4.3 Top 40 genes enriched in GAPDH IP from untreated BMDMs.

Gene_Symbol	Fold_Change	Log2_Fold_change	pvalue
Gm12346	4.97904578	2.31586928	0.00328
Zfp764	4.791880186	2.260591836	9.15E-05
Gm13216	4.689796326	2.229525269	0.003679
ENSMUSG00000089960	4.655702794	2.218998964	0.004382
Gm15590	4.617264167	2.207038275	0.003025
Gm26586	4.324317597	2.112472485	0.004714
Rny1	3.984025809	1.994226994	0.000674
Brsk2	3.801128727	1.926427884	0.001469
Mag	3.777030382	1.917252389	0.004359
Zfp874b	3.654182176	1.86954856	0.001668
Rny3	3.403077958	1.766840201	0.001905
Trim47	2.703238318	1.434688706	0.002442
Ankmy2	2.692251914	1.428813409	0.002373
Rn7s6	2.597483512	1.377114592	0.003867
Gm14461	2.584338788	1.369795209	0.033725
Armc10	2.570719721	1.362172325	0.003957
Gm24514	2.340280806	1.226681647	0.00986
mt-Co2	2.331378352	1.221183154	0.010153
Ntan1	2.233371381	1.159223173	0.012955
Fam3c	2.20179835	1.138682347	0.005374
Prune2	2.198609519	1.136591399	0.01211
Eef2k	2.152935917	1.106305378	0.03229
Slc9a7	2.152330407	1.105899565	0.014791
Rn7s1	2.149343885	1.103896325	0.024882
Gm26461	2.127553272	1.089195256	0.018162
Wdr47	2.03930973	1.028080908	0.024642
Prepl	2.033693453	1.024102232	0.032593
Fth1	1.987404539	0.990885565	0.001273
Rn7s2	1.941270861	0.957001428	0.04364
Ctnnbl1	1.932613968	0.950553494	0.009384
Akap7	1.918533284	0.940003794	0.032113
Snx32	1.900062091	0.926046564	0.010503
Btbd2	1.871109478	0.903893973	0.027615
Hinfp	1.870896343	0.903729628	0.012398
Homer3	1.862936365	0.897578395	0.01082
Hras	1.822512521	0.865928726	0.039049
Snpc1	1.817607969	0.862041065	0.041214
Fam174a	1.77766808	0.829985974	0.013136
Eif2b5	1.757161425	0.813246733	0.022282
Cyc1	1.748423883	0.80605499	0.012946
Zfp62	1.733780026	0.793920868	0.016983

Table 4.4 Top 40 genes enriched in GAPDH IP from LPS-treated BMDMs

4.3.3 TNF α and DAPK1 are post-transcriptionally regulated

We next proceeded to further examine the functional consequences of GAPDH binding to TNF α and DAPK1 mRNA. Given previous reports of GAPDH binding the 3'-UTR region of mRNAs, luciferase constructs were generated expressing the DAPK1-3'UTR and TNF α -3'UTR regions. They were transfected into HEK293T cells and the luciferase activity measured. Both regions were found to be significantly repressed when compared to the empty vector (EV) (Figure 4.18). While the TNF α 3'-UTR is known to be bound by many repressors [174], it is the first time the DAPK1-3'UTR promoter has been shown to be repressed. Furthermore, both mRNA and protein expression following LPS stimulation in BMDMs were examined. LPS was found to reduce DAPK1 mRNA expression as early as 4 hours, an effect which was maintained up until 24 hours of stimulation (Figure 4.19C). At the protein level however, DAPK1 expression was induced especially after 24 hours of LPS stimulation (Figure 4.19D). A similar disconnect was observed with TNF α expression. Unlike DAPK1, which had the highest mRNA expression basally, TNF α mRNA was induced by LPS after 4 hours of stimulation (Figure 4.19A). Nevertheless, this initial induction was rapidly reduced over time, with less than 25% expression after 24 hours. At the protein level however, TNF α expression showed an over 4 fold increase after 24 hours of LPS when compared to 4 hours of stimulation, showing the same disconnect between mRNA and protein expression as observed with DAPK1 (Figure 4.19B). Overall, these results demonstrate that both TNF α and DAPK1 are post-transcriptionally regulated and point towards a role for GAPDH as a translational repressor.

4.3.4 Inhibiting GAPDH enzymatic activity boosts its RNA-binding to TNF α and DAPK1

There is very little information available on the mechanism and the regulation of GAPDH RNA-binding activities. It has however been reported to occur through its NAD⁺ binding domain [131, 132], which led us to hypothesize that using an inhibitor to block GAPDH enzymatic activity, which requires a functional NAD⁺ binding domain, might alter its RNA binding activity. Treatment of BMDMs with 10 μ M HA prior to stimulation with LPS for 24 hours, resulted in increased binding to both DAPK1 and TNF α mRNAs, when compared to binding in the untreated samples (Figure 4.20). As previously observed, there was no fold increase following 24 hours of LPS stimulation, and interestingly, neither was there with the inhibitor alone. It is also interesting to note that immunoprecipitation of GAPDH following fixing with formaldehyde resulted in a double band, which was not present when samples were pre-treated with HA, either with or without LPS (Figure 4.20A).

When the results of these RNA-IPs are compared to TNF α and DAPK1 protein expression, the increased binding of GAPDH to TNF α mRNA in the presence of HA corresponds to an inhibition in TNF α production (Figure 4.5D). At the same time, increased binding to DAPK1 mRNA also corresponds to an inhibition in DAPK1 protein expression in the presence of LPS and HA (Figure 4.21, lane 4). It is worth noting that in this case, there is not a strong induction of DAPK1 by LPS alone due to the length of the stimulation being 6 hours and not 24, as previously shown.

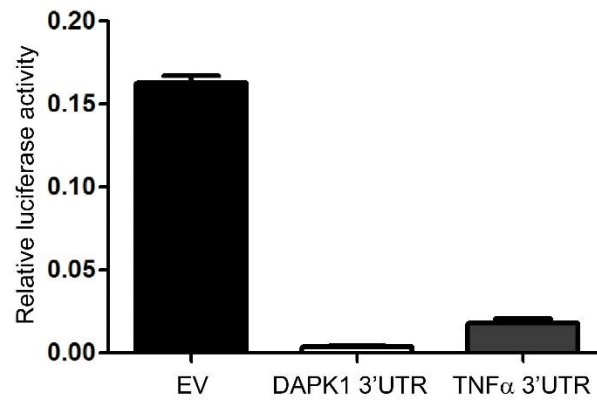


Figure 4.18 The 3'UTR promoter activities of DAPK1 and TNF α are inhibited. HEK293T cells were seeded at 0.4×10^6 cells/mL in 96 well plates and were left at 37°C, 5% CO₂ overnight. Cells were transfected with luciferase reporter constructs for 48 hours and then lysed with passive lysis buffer. Luciferase activity was measured and the renilla luciferase activity normalized to firefly luciferase activity. Results shown correspond to the mean expression + SD, representative of three independent experiments, each carried out in triplicate.

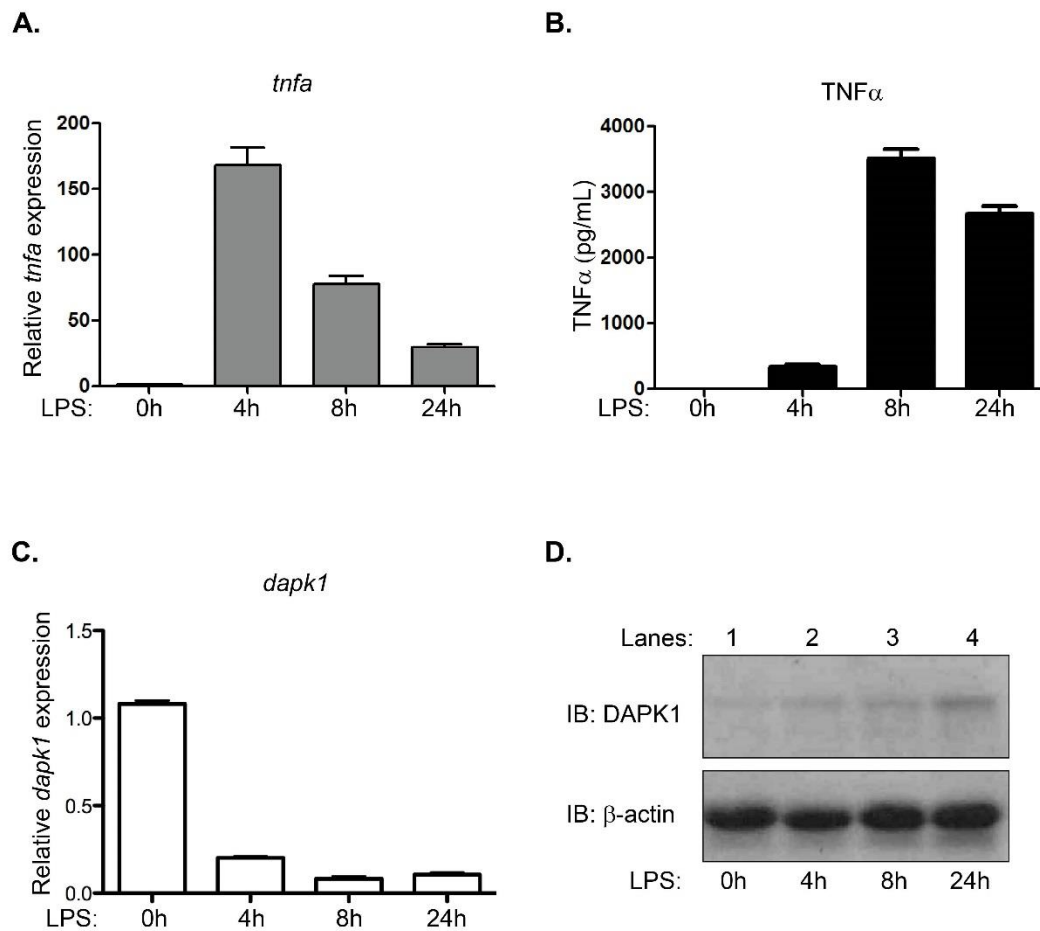
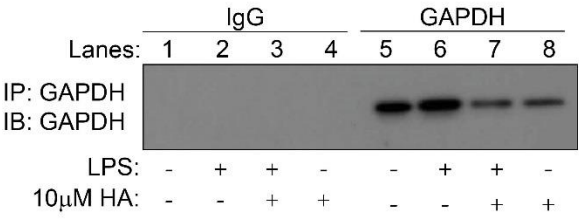
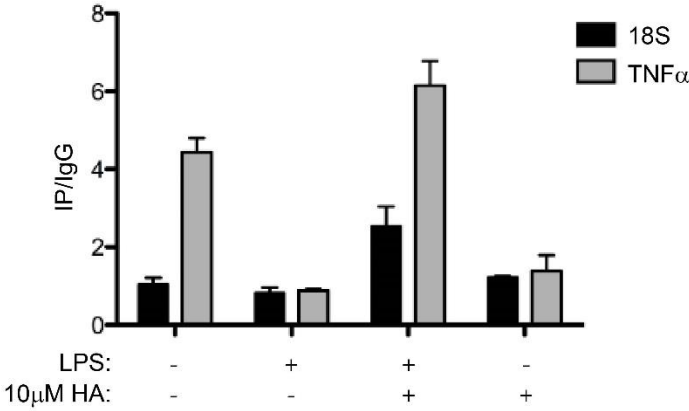


Figure 4.19 $TNF\alpha$ and DAPK1 are post-transcriptionally regulated. BMDMs were seeded at 0.5×10^6 cells/mL in 12 well plates and were left at 37°C , 5% CO_2 overnight. Cells were treated with LPS (100 ng/mL) for 4, 8 or 24 hours. **A, C**) RNA was extracted and normalised, followed by a reverse transcription reaction. $TNF\alpha$ and DAPK1 mRNA were quantified by qPCR using SYBR primers. Results are represented as the mean expression normalised to the geometrical mean of *gapdh*, *18S* and *actb* + SD, representative of three independent experiments, each carried out in triplicate. **B**) Supernatants were assayed by ELISA for $TNF\alpha$ production. Results are expressed as mean + SD of triplicate determinations, representative of three independent experiments. **D**) Cells were lysed using sample loading buffer and were separated using SDS-PAGE electrophoresis. DAPK1 and β -actin expression was analysed by western blotting. Results are representative of five independent experiments.

A.



B.



C.

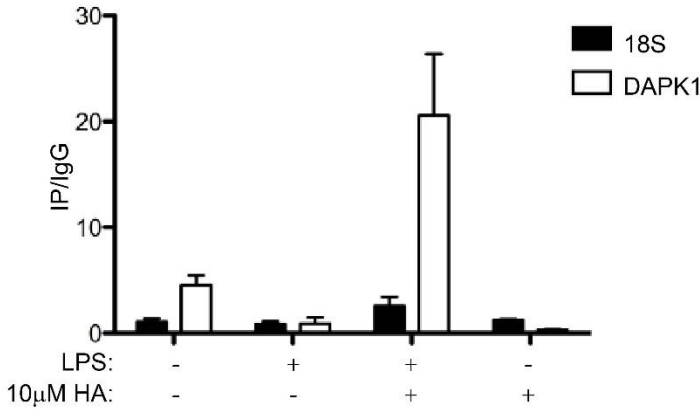


Figure 4.20 Heptelidic acid boosts binding of DAPK1 and TNF α mRNA to GAPDH.

BMDMs were seeded in 10 cm dishes at 1×10^6 cells/mL and left overnight at 37°C, 5% CO₂. The following day the medium was removed from the cells and replaced with medium alone or 10 μ M HA for 2 hours followed by 100 ng/mL of LPS for 24 hours. Cells were then fixed and lysed using polysome lysis buffer followed by pre-clearance with protein A/G beads and yeast RNA for 30 minutes. Lysates were used for IP with a control antibody (IgG) or a GAPDH antibody coupled to protein A/G beads. Samples were washed five times with polysome lysis buffer and resuspended in protein-RNA elution buffer. **A)** 30 μ L of sample was kept for western blotting using GAPDH antibody. **B,C)** The rest of the sample was used for purification of RNA using QIAzol, followed by a reverse transcription reaction. TNF α (**B**), DAPK1 (**C**) and 18S mRNA were quantified by qPCR using SYBR primers. Results are shown as the mean expression of the IP samples folded over the IgG samples + SD. Results are representative of three independent experiments, each carried out in triplicate.

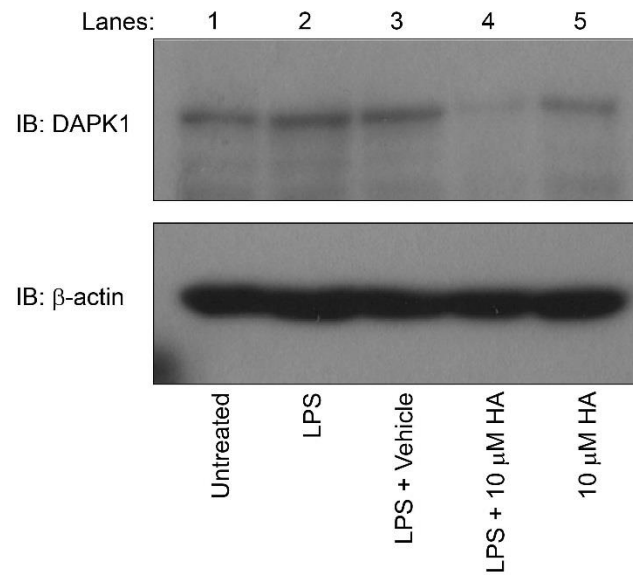


Figure 4.21 Heptelidic acid blocks DAPK1 protein expression in BMDMs. Cells were seeded at 1×10^6 cells/mL in a 12 well plate and left overnight at 37°C, 5% CO₂. The following day the medium was removed from the cells and replaced with medium alone or 10 μM HA for 2 hours followed by 100 ng/mL of LPS for 6 hours. Cells were lysed using sample loading buffer and separated via SDS-PAGE electrophoresis. DAPK1 and β-actin expression was analysed by western blotting. Results are representative of five independent experiments.

4.4 Investigating the role of DAPK1 in cytokine production

4.4.1 DAPK1 does not appear to be involved in LPS-induced cytokine production

The role of DAPK1 in inflammation and especially, in macrophages, remains largely unknown. It has been previously identified as participating in the RNA-binding GAIT complex formation in myeloid cells, as well as involved in IFN γ -induced cell death and as a tumor suppressor in other cell types [175-177]. Having previously shown that DAPK1 expression could be induced by LPS, we next wanted to investigate whether there was a role for DAPK1 in cytokine production in BMDMs. To this effect a DAPK1 inhibitor was used. First, the capacity of the inhibitor to block DAPK1 activity was tested. BMDMs were pre-treated with the inhibitor (Di) for 1 hour followed by LPS stimulation for 30 minutes. The phosphorylation of the myosin light chain (MLC) protein, which is a substrate of DAPK1 kinase activity, appeared to be induced by LPS, and blocked by Di, suggesting the inhibitor is successfully blocking DAPK1 (Figure 4.22A, lanes 2 and 3). Next we proceeded to examine the effect of the inhibitor on the production of IL10, IL6 and TNF α following 24 hour LPS stimulation, corresponding to the induction of DAPK1 by LPS. There was no significant effect observed in the production of any of the cytokines, although a non-significant increase in IL10 production was detected, even in the presence of the inhibitor alone (Figures 4.22B, 4.22C and 4.22D).

The same experiment was repeated using DAPK1 KO BMDMs instead of a DAPK1 inhibitor, and similar results were obtained. There was a small boost in IL10 production observed in DAPK1 KO compared to WT BMDMs (Figure 4.23B), as well as a small decrease in TNF α (Figure 4.23C) and pro-IL1 β production (Figure 4.23D). No differences were observed in IL6 production (Figure 4.23A). However, differences observed were of less than 20% and non-significant, and although these experiments are preliminary and need to be repeated, they would point towards DAPK1 not having a role in LPS-induced cytokine production.

4.4.2 Inhibition of DAPK1 reduces glycolysis in macrophages

Given previous reports showing that DAPK1 could increase glycolysis through activation of PKM2 [178], we next proceeded to investigate the effects of inhibiting DAPK1 on the glycolytic flux. Treatment of BMDMs with Di prior to LPS stimulation resulted in a reduction of LPS-induced glycolysis as determined by the proton production rate measured by Seahorse (Figure 4.24). This would suggest that DAPK1 may have a role to play in LPS-induced glycolysis in macrophages. This is interesting given that glycolysis is needed for cytokine production and yet no significant effects on cytokine production were observed when using the inhibitor. It may be the case that other processes, such as HIF1 α activation, may have a more important role than DAPK1 in the induction of glycolysis.

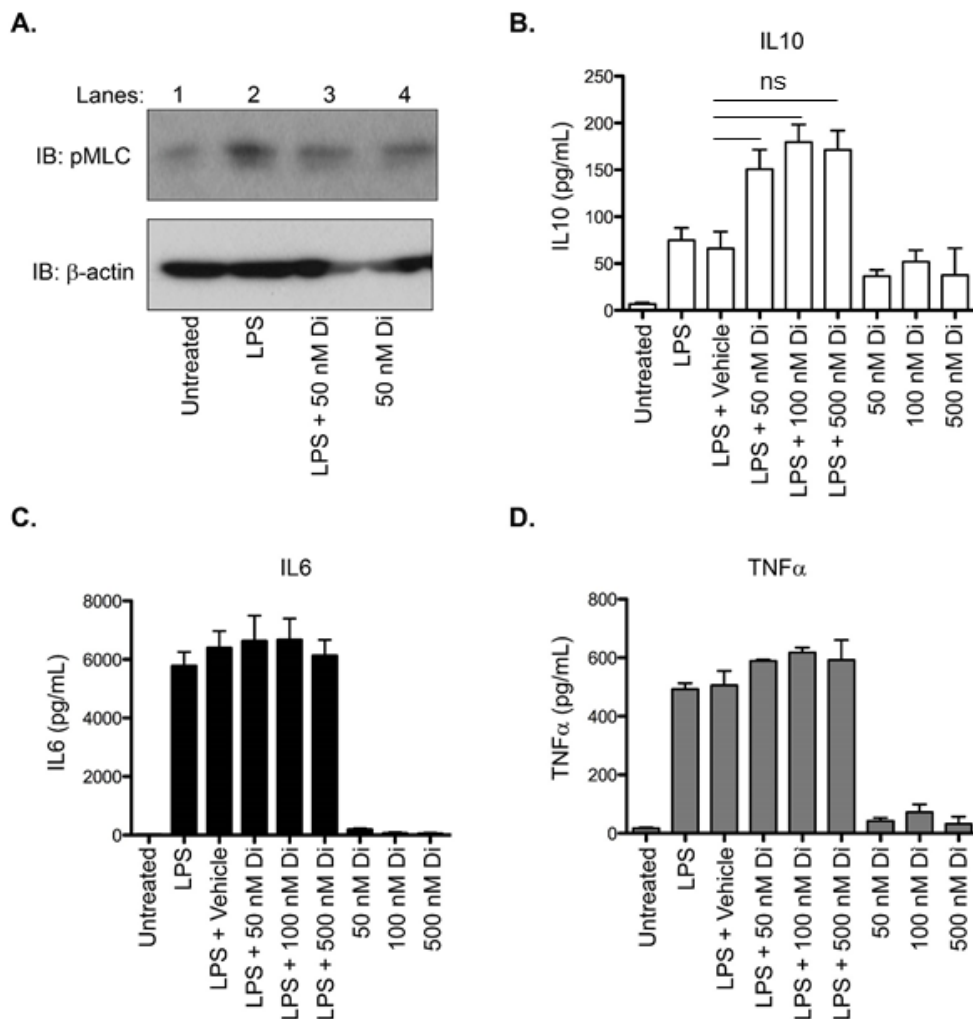


Figure 4.22 Inhibition of DAPK1 doesn't have a significant effect on cytokine production in BMDMs. Cells were seeded at 1×10^6 cells/mL in a 12 well plate and left at 37°C, 5% CO₂. **A)** The following day the medium was removed from the cells and replaced with medium alone or 50 nM DAPK1 inhibitor (Di) for 1 hour followed by 100 ng/mL of LPS for 30 minutes. Cells were lysed using sample loading buffer and separated via SDS-PAGE electrophoresis. Phospho-myosin light chain (pMLC) and β-actin expression was analysed by western blotting. Results are representative of two independent experiments. **B, C, D)** The medium was removed from the cells and replaced with medium alone or with medium containing vehicle (DMSO), 50, 100 or 200 nM Di for 1 hour followed by 100 ng/mL of LPS for 24 hours. Supernatants were analysed by ELISA for IL10 (**B**), IL6 (**C**), and TNFα (**D**) production. The results are expressed as mean + SEM of five independent experiments, each carried out in triplicate (ns, not significant).

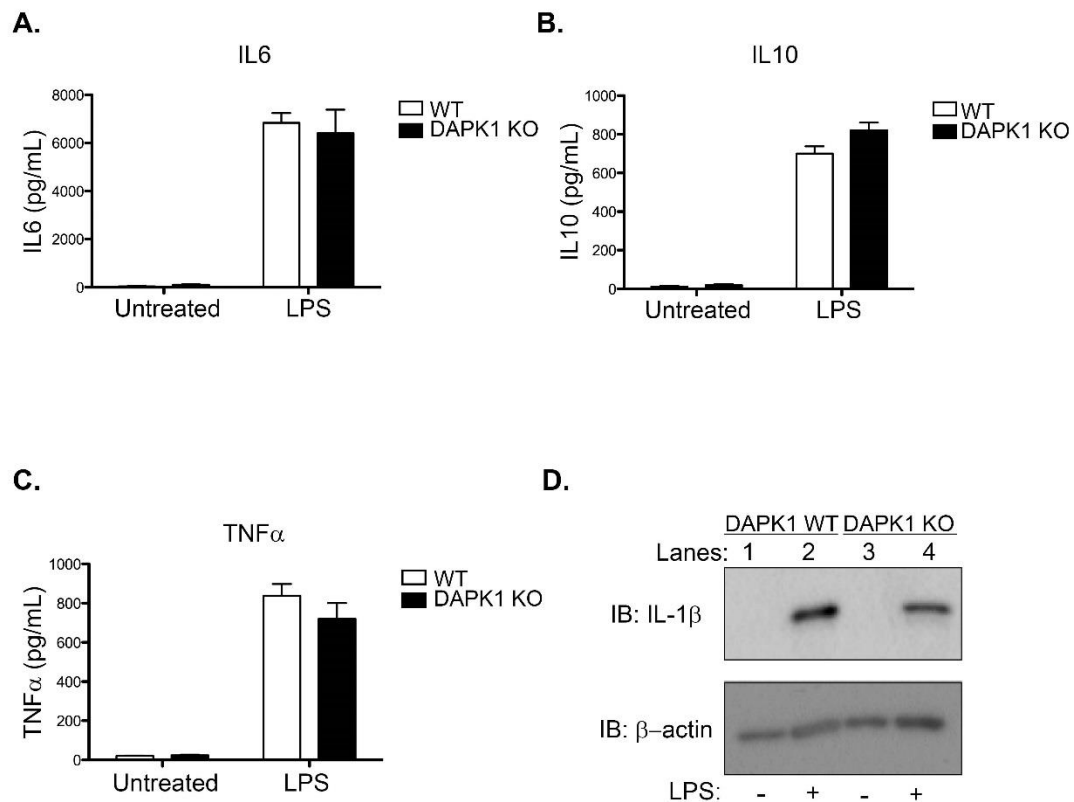


Figure 4.23 DAPK1 KO BMDMs do not appear to have a strong phenotype in response to LPS. BMDMs were seeded at 0.5×10^6 cells/mL in a 96 well plate and left at 37°C, 5% CO₂ overnight. Cells were left unstimulated or were stimulated with LPS (100 ng/mL) for 24 hours. Supernatants were analysed by ELISA for IL6, (A), IL10 (B) and TNF α (C) production. Results are expressed as mean + SD of two experiments, each carried out in triplicate. D) Cells were lysed using sample loading buffer and were separated via SDS-PAGE electrophoresis. Pro-IL-1 β and β -actin expression was analysed by western blotting. Results are representative of two independent experiments.

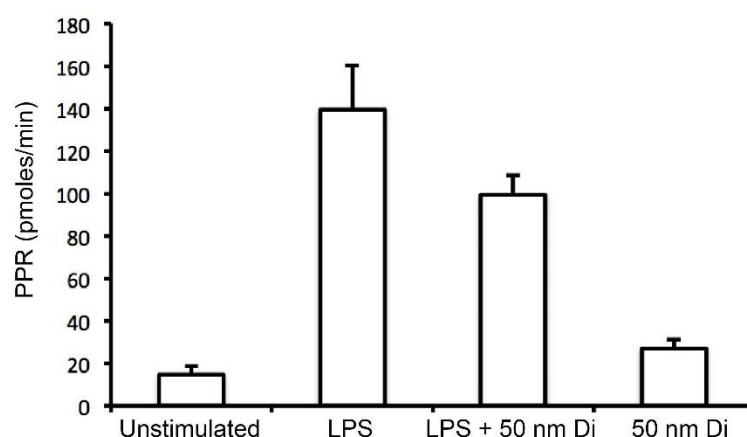


Figure 4.24 Inhibition of DAPK1 reduces glycolysis in BMDMs. Cells were seeded at 0.2×10^6 cells/mL in a seahorse plate and left overnight at 37°C, 5% CO₂ overnight. The following day they were left untreated or were treated with 50 nM Di for 1 hour followed by LPS (100 ng/mL) for 24 hours. The medium was removed from the cells and was replaced with glucose-supplemented XF assay buffer and the plate was placed in a CO₂-free incubator for at least 30 minutes, prior to the analysis of the proton production rate (PPR) using a Seahorse XF-24 analyser. The results are expressed as the mean + SD of a representative experiment carried out with five replicate determinations.

4.5 Investigating the functional consequences of GAPDH malonylation

4.5.1 GAPDH expression is not affected by LPS

I have shown that GAPDH plays a role in LPS-induced cytokine production in macrophages both through RNA-binding and its enzymatic activity. I next sought to investigate the mechanisms by which LPS might be regulating these functions. GAPDH protein expression was found to be unaffected following stimulation with up to 24 hours of LPS. This would indicate that increased protein expression is not the mechanism by which LPS is regulating GAPDH functions (Figure 4.25).

4.5.2 The GAPDH malonylation mimic mutant exhibits increased enzymatic activity and impaired RNA-binding

Having initially characterised LPS-induced malonylation of GAPDH, I next examined whether this could be the post-translational mechanism regulating its function. To this effect, two different K213 mutants were generated through site-directed mutagenesis. On the one hand, a GAPDH K213 glutamine mutant (K213Q) was used to mimic acetylation, which is the state in which K213 was identified in resting macrophages (Figure 3.13B). On the other hand, a K213 glutamate mutant (K213E) was used to mimic malonylation, as although smaller in size, it reproduces the negative charge caused by the modification. Both mutants together with a wild-type (WT) version of GAPDH were first overexpressed in HEK293T cells (Figure 4.26A). They were then affinity purified using their FLAG tag (Figure 4.26B) and their enzymatic activity was measured. The malonylation mimic K213E was found to have the highest enzymatic activity, with a more than two-fold increase over the acetylation mimic K213Q (Figure 4.26C). This would point towards GAPDH malonylation resulting in increased enzymatic activity.

I next proceeded to examine the RNA-binding capacity of the mutants. All three GAPDH variants were overexpressed in HEK293T cells, immunoprecipitated via their myc tag and their capacity to bind to TNF α mRNA examined by qPCR (Figure 4.27). In this case, the K213Q mutant was found to have the highest level of binding to TNF α mRNA, with more than five times the binding of either the WT or the K213E mutant. Furthermore, RNA binding activity appeared to be completely abrogated in the malonylation mimic K213E. These results are in agreement with previous reports of GAPDH RNA binding and enzymatic activities being mutually exclusive [132], and would suggest that GAPDH malonylation can thus regulate both activities. Overall, these results indicate that malonylation of GAPDH can cause it to dissociate from TNF α mRNA, enabling its translation while at the same time promoting glycolysis.

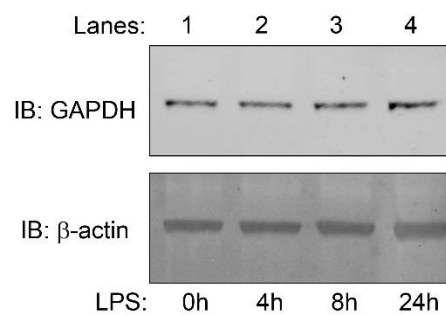


Figure 4.25 Activation of macrophages with LPS doesn't alter GAPDH protein expression. BMDMs were seeded at 0.5×10^6 cells/mL in 12 well plates and were left at 37°C, 5% CO₂ overnight. Cells were treated with LPS (100 ng/mL) for 4, 8 or 24 hours and then were lysed using sample loading buffer. They were separated using SDS-PAGE electrophoresis. GAPDH and β-actin expression was analysed by western blotting. Results are representative of five independent experiments.

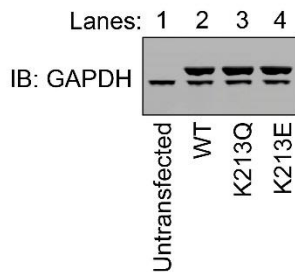
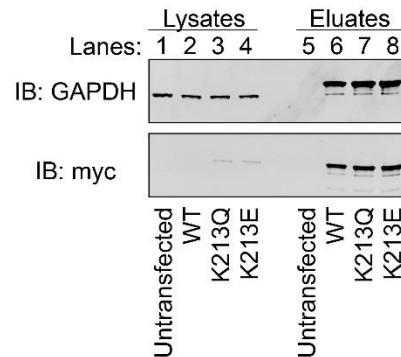
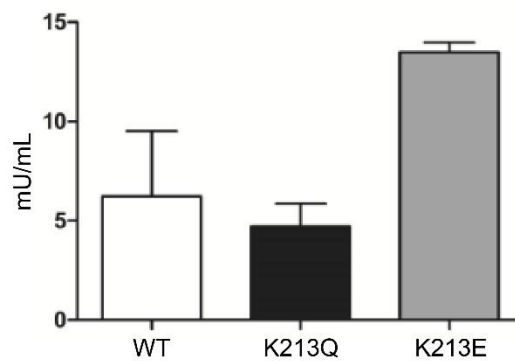
A.**B.****C.**

Figure 4.26 An affinity purified malonylation mimick mutant of GAPDH K213 shows increased enzymatic activity. HEK293T cells were seeded at 0.4×10^6 cells/mL in 10 cm dishes and left at 37°C, 5% CO₂ overnight. The medium was replaced and the cells transfected with a myc-FLAG vector expressing WT, K213Q or K213E GAPDH. 48 hours – post-transfection the cells were lysed using a Tris-based lysis buffer. **A)** 30 µL of lysate was kept for western blot analysis using a GAPDH antibody. **B)** The remaining lysate was put through a column packed with FLAG M2 affinity gel, followed by eight washes. FLAG-GAPDH was eluted off the column using 3X FLAG peptide. Both the run-through sample (lysates) and the eluted proteins were separated via SDS-PAGE and GAPDH and myc expression analysed via western blotting. Results are representative of two independent experiments. **C)** GAPDH activity was assayed by measuring NADH production at 340 nm. Results shown represent the mean + SD representative of four independent experiments, each carried out in triplicate.

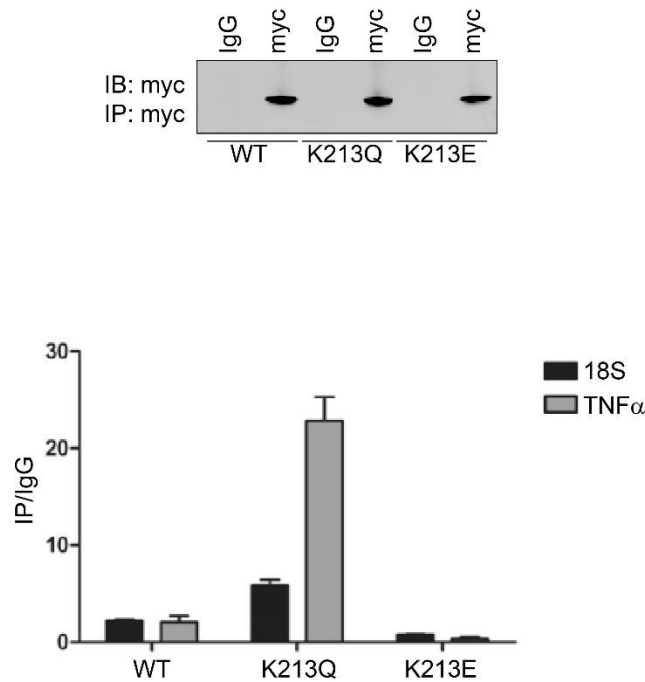


Figure 4.27 A malonylation mimick mutant of GAPDH K213 can't bind TNFα mRNA.

HEK293T cells were seeded at 0.4×10^6 cells/mL in 15 cm dishes and left at 37°C, 5% CO₂ overnight. The next day cells were transfected with a myc-FLAG vector expressing WT, K213Q or K213E GAPDH. 48 hours post-transfection, cells were fixed and then lysed using polysome lysis buffer. Lysates were used for IP with a control antibody (IgG) or an anti-myc antibody coupled to A/G beads. Samples were washed five times with polysome lysis buffer and were resuspended in protein-RNA elution buffer. **A)** 20 μL of sample was kept for western blot analysis using an anti-myc antibody. **B)** The rest of the sample was used for purification of RNA using QIAzol, followed by a reverse transcription reaction. TNFα and 18S mRNA were quantified by qPCR using Taqman primers. Results shown correspond to the mean expression of the IP samples folded over the IgG samples + SD, representative of three independent experiments, each carried out in triplicate.

4.6 Discussion

Having characterized the malonylation of GAPDH in activated macrophages, I next sought to investigate the functional consequences of this modification. However, while it has been reported numerous times that activated macrophages display increased glycolysis, the role of GAPDH itself has never been studied. Therefore, I first aimed to identify the role of GAPDH within these cells.

A recent report by Liberti and colleagues identified GAPDH as the enzyme controlling the rate of glycolysis in cells undergoing Warburg metabolism, as is the case of activated macrophages [179]. In support of this, and combined with previous studies reporting increased glycolytic flux [24, 27], we found GAPDH enzymatic activity to be increased following treatment with LPS. I sought to investigate the impact of this observation on LPS-induced cytokine production by using the GAPDH inhibitor heptelidic acid [180]. Pro-IL1 β , IL10 and IL6 production was found to be significantly reduced following inhibition of GAPDH, at the transcriptional as well as the protein level. This is in agreement with previous reports that demonstrate all of these cytokines rely on glycolysis for their production [20, 27, 181, 182]. Another glycolytic inhibitor, 2DG, was used along heptelidic acid as a control for glycolysis inhibition. Interestingly, while 2DG has the same effects as heptelidic acid on pro-IL1 β and IL10 production, it has the opposite effect on IL6, causing a boost in its production. This boost in IL6 with 2DG has also been previously reported [27, 183]. In fact, Suzuki and colleagues report IL6 as one of the highest upregulated genes following 2DG treatment in macrophages [183]. 2DG inhibits at the first step in glycolysis, through competitive inhibition of hexokinase, while heptelidic acid inhibits at the sixth step. This would suggest that either hexokinase itself, or any of the other intermediates involved in the first five steps of glycolysis may be having a direct effect on IL6 production.

The other two cytokines examined, IL12 and TNF α were also found to be inhibited by heptelidic acid, although only at the protein level. TNF α transcription was unaffected by heptelidic acid, and as reported previously, 2DG also failed to inhibit its production [27]. This would suggest that the effects of heptelidic acid on TNF α are post-transcriptional and likely to go beyond the role of GAPDH in glycolysis. IL12 production was also unaffected by 2DG, however its transcription was significantly increased. This increase in transcription was also observed for CXCL1 as well as IFN β . Interestingly, heptelidic acid-derived compounds have previously been identified as inhibitors of DNA polymerases [184]. While the activity of the only mouse polymerase tested in the study was unaffected by heptelidic acid, the activity of rat polymerase β and human polymerase λ were both inhibited by heptelidic acid. Therefore a possibility may exist where other murine DNA polymerases may be affected by heptelidic acid, thus causing the observed cytokine transcriptional effects.

There are reports demonstrating a link between glycolysis and IL12 production in DCs, although they have strictly relied on the use of 2DG [170, 185]. Interestingly, when the same experiment performed on BMDMs was performed in human monocyte-derived macrophages, 2DG did significantly reduce IL12 production. Overall, the lack of studies in the metabolic reprogramming of human macrophages as well as the differences with murine macrophages, make these results hard to interpret. Nevertheless they definitely point towards striking metabolic differences between the two, that go beyond the identified differences in arginine and NO metabolism [186]. Furthermore, it is clear that GAPDH is playing a role in the LPS-induced cytokine response in macrophages. However, given the striking differences observed with 2DG in some cases, it is unlikely that the effects observed on all cytokines are solely due to its role in glycolysis.

In an attempt to further clarify the role of GAPDH in LPS-induced cytokine production, I next proceeded to knock it down. Reduced levels of GAPDH resulted in a reduction in pro-IL1 β , IL10 and IL6 production. This correlates with the effects observed following inhibition of its enzymatic activity using heptelidic acid, and thus would suggest that GAPDH enzymatic activity is needed for the production of these cytokines in macrophages. The production of TNF α on the other hand, was boosted following GAPDH knockdown. Given that the opposite results are observed when inhibiting GAPDH enzymatic activity, it was felt that the regulation of TNF α production by GAPDH would go beyond its glycolytic activity.

One of GAPDH many roles includes RNA binding. A report by Chang and colleagues has recently demonstrated that GAPDH controls the production of interferon- γ in T cells by binding to its 3'-UTR [138]. It has also been reported that GAPDH can bind to TNF α mRNA [133, 139], which led us to investigate whether this could be the mechanism by which GAPDH could be regulating TNF α production in macrophages. GAPDH in resting macrophages was found to bind to TNF α mRNA, as well as the pro-inflammatory mediator COX2, as reported previously [173]. Interestingly, following LPS treatment, binding to these RNAs was completely abrogated.

There are two ways in which GAPDH is reported to bind RNAs: by directly binding to AU-rich elements in the 3'-UTR region of the RNAs or through formation of the GAIT complex, which binds to RNAs containing GAIT elements [131, 136]. We therefore explored the possibility of GAPDH binding to other unreported RNAs in macrophages, first by selecting some transcripts known to contain either AU-rich or GAIT elements. From the selected targets, GAPDH was found to strongly bind to the GAIT-element-containing DAPK1 RNA. In a similar manner to that observed for TNF α and COX2, this binding was found to occur in resting macrophages, but not following LPS treatment. Interestingly, even though GAPDH is part of the GAIT

complex, its role within the complex is not to directly bind RNAs [136]. It might be possible that the binding of DAPK1 observed is indirect, and occurring through other proteins in the GAIT complex that have been immunoprecipitated with GAPDH. However, it is worth noting that the GAIT complex is only reported to form following treatment of myeloid cells with IFN γ , which is not the case in this instance. Therefore, this observation would suggest that either the GAIT complex can be induced by stimuli other than IFN γ , such as LPS, or that DAPK1 may contain an unreported AU-rich element to which GAPDH may be directly binding. Another possibility could also be that there are other unreported RNA motifs that GAPDH may be able to bind to.

I next sought to investigate the functional consequences of GAPDH RNA-binding. Previous reports have suggested that binding of GAPDH to TNF α and COX2 3'-UTR has a repressive effect on protein production [139, 173], while nothing is known about DAPK1 binding. The activity of TNF α and DAPK1 3'-UTRs was assessed and was found to be inhibited. In addition, high mRNA expression was found to not correlate with high protein production for both TNF α and DAPK1. Furthermore, at the time of GAPDH binding, protein expression was found to be very low, while the release of bound RNA by GAPDH correlates with protein production. In an attempt to implicate GAPDH directly we used the inhibitor heptelidic acid. With previous reports having shown that GAPDH enzymatic activity and RNA-binding are mutually exclusive [132], we hypothesized that by blocking the enzyme's activity, we would interfere with its RNA binding. As predicted, pre-treatment with heptelidic acid prior to LPS, resulted in increase binding of GAPDH to both TNF α and DAPK1 RNA. In addition, this also resulted in a reduction in protein expression, thus further indicating that GAPDH binding to these RNAs is preventing their translation. Furthermore, this observation explains why knockdown of GAPDH resulted in increased TNF α production: reduced GAPDH levels result in reduced repressive RNA-binding, leading to increased mRNA translation.

The role of TNF α in activated macrophages and inflammation is widely known [187]. On the other hand, while the role of DAPK1 has extensively been studied in cancer, its role in inflammation and especially in macrophages, remains obscure. Much like GAPDH itself, DAPK1 is a multifunctional protein, originally identified as a serine/threonine kinase involved in IFN- γ -induced cell death [176]. However, DAPK1 implication in cell death was later shown to not just be limited to IFN- γ -induced but to also be part of TGF- β or TNF α -induced apoptosis, as well as other caspase-independent forms of cell death in many different cell types [188]. Further characterization of the protein also indentified a calcium-activated calmodulin domain, as well as a death domain and a cytoskeletal binding domain, the functions of which haven't yet been well characterized [189]. In order to evaluate the impact of GAPDH RNA-binding activities on macrophage function, we sought to investigate the role of DAPK1 in LPS-activated macrophages. Having shown that LPS could induce its expression, we next

evaluated the effects of inhibiting its kinase activity. There was a small boost in IL10 production, while no effects on IL6 and TNF α were observed. However, the DAPK1 inhibitor used, only inhibits DAPK1 kinase activity, and many of DAPK1 cellular functions have been shown to be independent of this activity [190]. Therefore, I proceeded to validate these results in macrophages derived from DAPK1 KO mice. Preliminary results showed that these macrophages produced slightly more IL10 and slightly less TNF α , although these effects were non-significant. In addition, they also appeared to produce reduced levels of pro-IL1 β . These results are however preliminary and need to be repeated to be confirmed. Nonetheless, there are already published reports of DAPK1 downregulating TNF α as well as interfering with NF- κ B activity in other systems [191]. Interestingly, DAPK1 has also been reported to boost glycolysis, which is a known driver of inflammation in macrophages. It was initially reported to be able to do so, through binding to and activating PKM2 [178]. A more recent report has however shown that activation of PKM2 results in glycolysis inhibition, and as a consequence, inhibition of the inflammatory profile in macrophages [28]. We proceeded to further investigate the role of DAPK1 in glycolysis in macrophages and found that inhibiting DAPK1 kinase activity resulted in a reduction in LPS-induced glycolysis. This observation would support the reduction of pro-inflammatory cytokines observed in DAPK1 KO macrophages, however it questions the link between DAPK1, PKM2 and glycolysis. If DAPK1 were a PKM2 activator, inhibiting DAPK1 activity would result in reduced PKM2 activity and an increase in glycolysis as a result. Therefore, the interaction between PKM2 and DAPK1, and the DAPK1-dependent increase in glycolysis reported by Mor and colleagues, are likely to be independent of each other. Overall, while further validation of these studies into the role of DAPK1 is still needed, these preliminary observations might be indicative of DAPK1 playing a role in the inflammatory response in macrophages.

Our evidence thus far indicates that LPS can regulate GAPDH RNA-binding as a means of amplifying the inflammatory response, by enabling the translation of pro-inflammatory mediators. Many pro-inflammatory mediators are known to be post-transcriptionally regulated, a regulation that in most cases occurs through AU-rich elements [192]. This led us to question whether GAPDH may be regulating inflammation through binding to other pro-inflammatory RNAs beyond TNF α and DAPK1. To this effect, a RIP-Seq experiment was designed, in which following GAPDH immunoprecipitation, all bound RNAs were sequenced and identified, as opposed to analysed via qPCR. Interestingly, despite our previous observation of LPS abrogating RNA-binding to TNF α and DAPK1 mRNA, similar numbers of RNAs were found to bind to GAPDH from untreated samples compared to LPS-treated. This would suggest that the scope of GAPDH RNA-binding in macrophages is likely to go beyond simply regulating translation of AU-rich pro-inflammatory RNAs. It is also worth noting that GAPDH binding does

not always lead to translation repression. There are previous examples, such as that of the mRNA of colony stimulating factor 1 (CSF1), in which GAPDH binding stabilizes the mRNA and enables its translation [193, 194].

Another interesting observation is that despite similar numbers of RNA binding GAPDH under both conditions, the identities of the RNAs bound in each are very different. Surprisingly, not many genes were identified that had a function associated with inflammation, with the exception of *Cp*. *Cp* is the gene corresponding to ceruloplasmin, an acute phase protein and a known marker of inflammation [195]. Ceruloplasmin mRNA, much like that of TNF α and DAPK1, was found to bind in GAPDH from resting macrophages, while binding in the LPS-treated samples was not detected. Interestingly, rather than an inflammatory role, ceruloplasmin produced by macrophages has been shown to have a protective role in a mouse model of inflammatory bowel disease (IBD) [196]. Even more interesting however, is the fact that ceruloplasmin was one of the first transcripts to be identified as containing a GAIT element. Its expression, as that of DAPK1, is thus known to be regulated by the GAIT complex [197]. Although further experiments are required to confirm this result, it yet again poses a conundrum as to what the role of the GAIT complex is in an LPS-treated macrophage, as well as the role of GAPDH within the complex.

Nevertheless, as previously stated, the majority of genes identified did not have an associated inflammatory function. Interestingly, an initial analysis of the top genes identified to be binding GAPDH in resting macrophages, led to one common element: a role in tumor pathogenesis. Both *Fkbp9* and *Hells*, which encode for peptidyl prolyl cis-trans isomerase FKBP9 and lymphoid specific helicase respectively, have been linked to leukaemia. While *Fkbp9* has been found to be a marker of megakaryoblastic leukaemia, deletion of *Hells* in a mouse model leads to the spontaneous development of the disease [198, 199]. *Cdca5*, which was found to be the third most enriched RNA in the untreated samples, is linked to poor prognosis of urothelial carcinomas [200]. *E2f6* on the other hand, has been linked to the tumorigenesis of breast cancer through its regulation of BRAC1 expression [201, 202]. Some of the other genes that have been found at the top of the list of GAPDH-binding RNAs in resting macrophages, and are also associated with cancer, include *Uhrf1*, *Mlh3* and *Adgb*. *Mlh3* mutations are a common occurrence in patients suffering from hereditary nonpolyposis colorectal cancer, while *Adgb* has been linked to the growth of glioma cell lines [203, 204]. *Uhrf1* on the other hand, has been linked to tumorigenesis of many different types of cancer. In addition, its ability to repair DNA has also been associated with drug resistance exhibited by tumors where it is found overexpressed [205]. Further validation is still required before we are able to confirm that GAPDH is binding to these genes in resting macrophages, as well as the functional consequences of the binding. Furthermore, while none of the aforementioned genes have

associated functions within macrophages, it's worth noting that GAPDH is highly expressed in many different types of cancer, and the function of this overexpression is not well understood [206].

RNAs identified as binding to GAPDH following LPS treatment on the other hand, pose a very different picture. Surprisingly, a significant proportion of those RNAs identified are non-coding RNAs, with half of the top ten genes corresponding to pseudogenes of unknown functions. While these results are yet to be validated, they could prove very interesting if demonstrated to be true. On the one hand, over 90% of the mouse and human genome is composed of non-coding DNA, a lot of which in turn gives rise to non-coding RNAs, many of which are considered junk left by evolution [207]. There is a possibility that GAPDH isn't actively binding RNAs following activation of macrophages with LPS, but given the high proportion of 'junk RNA' present in the cell, and given that the RIPSeq methodology involves fixing of the cells, it might lead to false-positives without any biological significance. However, on the other hand, there is an increasing number of reports of pseudogenes and other non-coding RNAs having important cellular functions. A recent report in mouse embryonic fibroblasts has identified a pseudogene of Rps15a, Lethes, to be induced following activation of cells with TNF α . Lethes can bind to RelA and consequently inhibit NF- κ B [208]. Another report has demonstrated induction of the long noncoding RNA (lncRNA)-Cox2 in activated macrophages, which can directly repress or trigger the induction of various immune genes, thus directly impacting on macrophage function [209]. Therefore, there is a possibility that some of the noncoding genes identified as binding LPS-treated GAPDH may have an important functional role to play. However, this remains hypothetical until further work is carried out, as if this were true, it would question the validity of the findings that GAPDH enzymatic activity and RNA-binding are mutually exclusive.

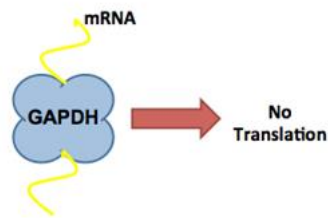
Up to this point, we had been able to identify on the one hand, an increase in GAPDH enzymatic activity following activation of macrophages with LPS, which is needed for the production of certain cytokines such as pro-IL1 β , IL6 and IL10. On the other hand, a decrease in the RNA-binding capacity of GAPDH under the same conditions, which enables the release of the pro-inflammatory mediators TNF α and DAPK1. Having identified GAPDH as a central player in the regulation of inflammation in macrophages, we were now able to investigate the impact of the previously identified LPS-induced malonylation on the newly identified GAPDH functions. To this effect, we generated a malonylation mimic mutant for the LPS-induced malonylated K213, as well as an acetylation mimic of the same site. Measuring of the enzymatic activity as well as the RNA-binding of both mutants and a wild-type control, demonstrated that the K213 malonylation mimic has increased enzymatic activity, while being unable to bind TNF α mRNA. The K213 acetylation mimic, which mirrors the state of GAPDH

in resting macrophages, was found to have a much higher RNA-binding capacity, while displaying reduced enzymatic activity when compared to the malonylation mimic. Therefore, this data would suggest that LPS-induced malonylation of K213 increases GAPDH enzymatic activity, while at the same time, reducing its RNA-binding capacity.

It is interesting to speculate what the mechanism by which malonylation can induce this enzymatic versus RNA-binding switch may be. One possibility could be that K213 malonylation is able to interfere with GAPDH tetramer formation, as it is only a couple of amino acids away from both the dimer and tetramer interface [133]. Interestingly, GAPDH dimers have been suggested to be both enzymatically inactive and unable to bind RNAs, despite retaining the capacity to bind NAD^+ [133, 210]. It is therefore unlikely that K213 malonylation can cause the complete conversion from the tetrameric to the dimeric GAPDH form, which is also a very slow and unfavourable reaction [211]. However, rather than a complete disruption, it may still be able to subtly disrupt the tetramer formation, which has been previously reported by White and colleagues to affect binding of GAPDH to the $\text{TNF}\alpha$ RNA [133].

The present study thus suggests that in an activated macrophage, citrate accumulation leads to malonyl-CoA production, which in turn can be used for the malonylation of a wide array of proteins. The glycolytic enzyme GAPDH, is one of the identified LPS-induced malonylated substrates, and undergoes malonylation of a specific lysine within its catalytic domain. This results, on the one hand, in a decrease in its repressory RNA-binding capacity, enabling the translation of previously sequestered pro-inflammatory mRNAs, such as that of $\text{TNF}\alpha$. On the other hand, it results in the increase of its enzymatic activity, which leads to increased glycolytic flux needed for the production of other pro-inflammatory cytokines such as pro-IL1 β and IL6. We have therefore identified malonylation as a novel mechanism by which macrophages can control the production of pro-inflammatory cytokines through GAPDH.

A. Resting macrophage



B. Activated macrophage

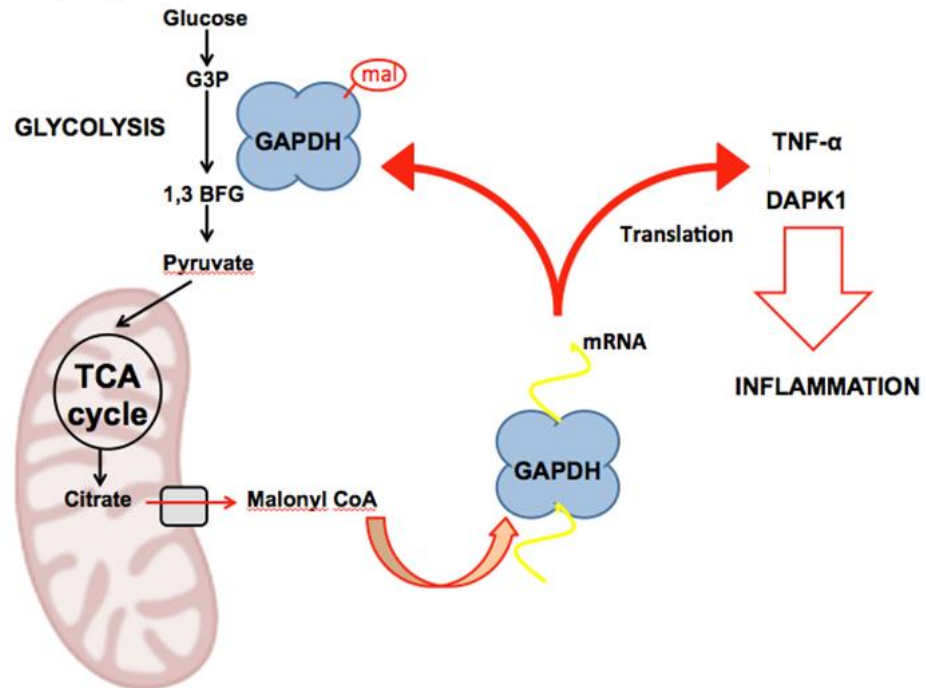


Figure 4.28 Model of GAPDH malonylation in macrophages. A) In resting macrophages, GAPDH binds to the 3'-UTR of various pro-inflammatory mediators, including TNF α and DAPK1, and prevents their translation. **B)** Following activation of macrophages with LPS, there is an accumulation of citrate that can be converted into malonyl-CoA, which in turn can mediate lysine malonylation. One of the substrates of this modification is GAPDH, which after undergoing malonylation, releases the RNAs which can now be translated. At the same time, GAPDH enzymatic activity increases, which allows for an increased glycolytic flux, required for the activated macrophage to carry out its pro-inflammatory functions.

Chapter 5: Final discussion

5.1 Malonylation model

In this study, I have identified LPS-induced malonylation as an activating signal for GAPDH, which results in an increase in its enzymatic activity while reducing its RNA-binding capacity. This is in contrast to other malonylated substrates previously identified in the literature, in all of which malonylation has been identified as a negative regulator, reducing the activity of the modified enzymes. In MCD KO fibroblasts, very-long-chain acyl-CoA dehydrogenase (VLCAD) as well as long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) have been identified as hypermalonylated, which significantly reduces their enzymatic activity [99]. In SIRT5 KO fibroblasts, GAPDH has also been identified as hypermalonylated, while ALDOB has been found to be hypermalonylated in a mouse model of type 2 diabetes. In both cases, malonylation was also found to negatively affect enzymatic activity [100, 102]. However, all the previously examined malonylation substrates have been studied under basal conditions or in KO models. Nonetheless, there are no reports to date of any signal or condition under which Sirt5 or MCD are absent or downregulated, thus questioning the physiological significance of the findings. Interestingly, spontaneous malonylation based on malonyl-CoA reactivity has also been found to be inhibitory [161].

Another striking difference between our LPS-induced malonylation findings and those previously reported, is the difference in malonylation substrates. All previous studies only identified metabolic enzymes as being malonylated, with the exception of *E. Coli*, while LPS-induced malonylation appears to impact many different cellular pathways. These differences led us to believe there might be two different types of malonylation occurring in the cell: spontaneous malonylation and signal-induced malonylation. In a similar manner as that reported to occur with the GAPDH substrate 1,3-bisphosphoglycerate [52], malonyl-CoA may be spontaneously modifying metabolic enzymes through malonylation as a negative feedback mechanism. This spontaneous malonylation could be especially relevant within the mitochondria, where unlike the cytosol, the conditions for this spontaneous reaction have been found to be favourable. Once the generation of malonyl-CoA from acetyl-CoA reaches a certain threshold, it may result in the malonylation of enzymes that lead to malonyl-CoA generation, such as those involved in the Krebs cycle and fatty acid oxidation. This modification would inhibit their activity, and thus act as a signal to cease malonyl-CoA production. Sirt5, which is found most abundantly in the mitochondria, can act to remove the malonylation and return the mitochondria to a steady generation of malonyl-CoA when needed. This spontaneous malonylation model could help explain why most malonylation studies have found mitochondrial respiration and fatty acid metabolism as especially affected by increasing malonylation levels, as well as only metabolic enzymes as substrates [99, 101].

Signal-induced malonylation on the other hand, such as that we have observed in LPS-activated macrophages, is likely to be much more similar to previously reported enzyme-mediated acetylation and phosphorylation, and be tightly regulated enzymatically as well as impact a wide variety of substrates. Signalling modifications are evolutionarily conserved, which would explain why the scope of malonylated substrates in LPS-activated macrophages is much more similar to that observed in bacteria than in the other metabolic models.

5.2 The role of GAPDH and malonylation in cancer

GAPDH has been reported to be upregulated in numerous different types of tumors. A more than 100% increase in expression has been identified in lung adenocarcinoma biopsies [212]. Other than being upregulated, it has also been linked to metastasis in renal cell carcinomas as well as colon adenocarcinomas [213, 214]. Furthermore, GAPDH expression has been found to correlate with the pathological grading of human gliomas [206]. This increase in GAPDH expression is generally assumed to be linked to the reliance of cancer cells on glycolysis, otherwise known as the 'Warburg effect' [215]. However, GAPDH RNA-binding activity has also been identified as contributing to the metastatic properties of ovarian cancer. In a human ovarian cancer cell line GAPDH was found to bind to CSF1 mRNA and prevent its decay, leading to CSF1 overexpression. CSF1 in turn, is responsible for the increased invasive capacity of the cells and is found in high levels in the metastatic versions of the cancer [193]. Interestingly, TNF α , as the name would suggest, has also been implicated in cancer. Although originally identified as having anti-tumor activity, it's also been found to promote tumorigenesis of certain tumors. Whether detrimental or beneficial, its expression is found to be elevated in numerous different types of tumors, including chronic lymphocytic leukaemia, ovarian and prostate cancer [216-218]. Based on our model, the increased GAPDH expression in many tumors, combined with the increased glycolytic flux, would result in less repressive RNA-binding. Furthermore, given our observation of GAPDH binding primarily RNAs with a role in tumorigenesis, it's worth speculating whether the main contribution of GAPDH to tumor development is actually through its glycolytic activity, or its RNA-binding, or both. Interestingly, in support of the importance of GAPDH in this model, a recent study has shown that treatment with heptelidic acid in a mouse model of breast cancer can significantly reduce tumor growth [179].

The role of malonylation in tumor development has not yet been investigated. However, inhibition of malonyl-CoA decarboxylase, and thus malonyl-CoA accumulation, has been reported to be toxic to human breast cancer cells [219]. Furthermore, another study has demonstrated that deletion of malonyl-CoA decarboxylase significantly reduces the growth of

leukaemia and renal cancer cell lines [220]. Therefore, further studies into the role of malonylation in tumors is bound to produce promising results.

5.3 The role of GAPDH and malonylation in obesity and diabetes

While the only recent discovery of malonylation means there are yet not many studies on its impact in disease, it has been linked to diabetes. In a mouse model of type 2 diabetes, malonylation levels were found to be elevated. Furthermore, one of the proteins identified as undergoing increased malonylation included GAPDH, with K213 specifically also found malonylated at higher numbers in diabetic mice [102]. Furthermore, mice deficient in the ACC2 enzyme, and thus deficient in cytosolic malonyl-CoA, have been found to be resistant to high-fat diet-induced obesity and diabetes. In addition, these mice also display increasing fatty acid oxidation rates [87]. While the mechanism behind this phenotype has not yet been uncovered, it's tempting to speculate that it is due to reduced malonylation levels. The aforementioned link between high malonylation levels and type 2 diabetes, combined with malonylation having previously been linked to fatty acid oxidation inhibition, may suggest that the mechanism behind the observed ACC2 KO phenotype might be reduced malonylation levels.

Pro-inflammatory macrophages have been shown to be mostly responsible for the chronic inflammation found in obesity, and more importantly, for the production of pro-inflammatory cytokines within adipose tissue [221]. Interestingly, macrophage-derived TNF α has been shown to interfere with insulin signalling, with an absence of TNF α resulting in increased insulin sensitivity [222, 223]. Furthermore, TNF α has been shown to be elevated in patients suffering from type 2 diabetes, and its exogenous administration into mice causes insulin resistance [224, 225]. The mechanism behind many of these observations might be an increase in GAPDH malonylation resulting in increased TNF α production. Targetting GAPDH malonylation therapeutically could prove to be a beneficial way of targeting increased TNF α levels and thus improving insulin sensitivity in diabetic patients. The same might apply to other diseases such as rheumatoid arthritis or Crohn's disease where TNF α is a key therapeutic target.

Overall, my study has revealed a new signal in metabolic reprogramming in macrophages: malonylation of GAPDH leading to TNF α production. Further work might reveal the potential of targetting this process to treat cancer as well as inflammatory diseases.

Chapter 6: References

1. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation*. Cell, 2010. **140**(6): p. 805-20.
2. Barton, G.M., *A calculated response: control of inflammation by the innate immune system*. J Clin Invest, 2008. **118**(2): p. 413-20.
3. Han, J. and R.J. Ulevitch, *Limiting inflammatory responses during activation of innate immunity*. Nat Immunol, 2005. **6**(12): p. 1198-205.
4. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets*. Nat Rev Immunol, 2011. **11**(11): p. 723-37.
5. West, A.P., et al., *TLR signalling augments macrophage bactericidal activity through mitochondrial ROS*. Nature, 2011. **472**(7344): p. 476-80.
6. Mills, C.D., et al., *M-1/M-2 macrophages and the Th1/Th2 paradigm*. J Immunol, 2000. **164**(12): p. 6166-73.
7. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment*. F1000Prime Rep, 2014. **6**: p. 13.
8. Jenkins, S.J., et al., *Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation*. Science, 2011. **332**(6035): p. 1284-8.
9. Qian, B.Z. and J.W. Pollard, *Macrophage diversity enhances tumor progression and metastasis*. Cell, 2010. **141**(1): p. 39-51.
10. Platt, A.M., et al., *An independent subset of TLR expressing CCR2-dependent macrophages promotes colonic inflammation*. J Immunol, 2010. **184**(12): p. 6843-54.
11. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease*. Nature, 2013. **496**(7446): p. 445-55.
12. Ponomarev, E.D., et al., *MicroRNA-124 promotes microglia quiescence and suppresses EAE by deactivating macrophages via the C/EBP-alpha-PU.1 pathway*. Nat Med, 2011. **17**(1): p. 64-70.
13. Slack, J.L., et al., *Identification of two major sites in the type I interleukin-1 receptor cytoplasmic region responsible for coupling to pro-inflammatory signaling pathways*. J Biol Chem, 2000. **275**(7): p. 4670-8.
14. Bell, J.K., et al., *Leucine-rich repeats and pathogen recognition in Toll-like receptors*. Trends Immunol, 2003. **24**(10): p. 528-33.
15. Kurt-Jones, E.A., et al., *Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus*. Nat Immunol, 2000. **1**(5): p. 398-401.
16. Tada, H., et al., *Saccharomyces cerevisiae- and Candida albicans-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and Toll-like receptor 4-dependent manner*. Microbiol Immunol, 2002. **46**(7): p. 503-12.
17. Akira, S. and K. Takeda, *Toll-like receptor signalling*. Nat Rev Immunol, 2004. **4**(7): p. 499-511.
18. Hard, G.C., *Some biochemical aspects of the immune macrophage*. Br J Exp Pathol, 1970. **51**(1): p. 97-105.
19. Garedew, A., S.O. Henderson, and S. Moncada, *Activated macrophages utilize glycolytic ATP to maintain mitochondrial membrane potential and prevent apoptotic cell death*. Cell Death Differ, 2010. **17**(10): p. 1540-50.
20. Mills, E.L., et al., *Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages*. Cell, 2016. **167**(2): p. 457-470 e13.
21. Aktan, F., *iNOS-mediated nitric oxide production and its regulation*. Life Sci, 2004. **75**(6): p. 639-53.
22. Liu, P.S., et al., *alpha-ketoglutarate orchestrates macrophage activation through metabolic and epigenetic reprogramming*. Nat Immunol, 2017. **18**(9): p. 985-994.
23. O'Neill, L.A. and D.G. Hardie, *Metabolism of inflammation limited by AMPK and pseudo-starvation*. Nature, 2013. **493**(7432): p. 346-55.

24. Rodriguez-Prados, J.C., et al., *Substrate fate in activated macrophages: a comparison between innate, classic, and alternative activation*. J Immunol, 2010. **185**(1): p. 605-14.
25. Vats, D., et al., *Oxidative metabolism and PGC-1 β attenuate macrophage-mediated inflammation*. Cell Metab, 2006. **4**(1): p. 13-24.
26. Corraliza, I.M., et al., *Arginase induction by suppressors of nitric oxide synthesis (IL-4, IL-10 and PGE2) in murine bone-marrow-derived macrophages*. Biochem Biophys Res Commun, 1995. **206**(2): p. 667-73.
27. Tannahill, G.M., et al., *Succinate is an inflammatory signal that induces IL-1 β through HIF-1 α* . Nature, 2013. **496**(7444): p. 238-42.
28. Palsson-McDermott, E.M., et al., *Pyruvate kinase M2 regulates Hif-1 α activity and IL-1 β induction and is a critical determinant of the warburg effect in LPS-activated macrophages*. Cell Metab, 2015. **21**(1): p. 65-80.
29. Moon, J.S., et al., *mTORC1-Induced HK1-Dependent Glycolysis Regulates NLRP3 Inflammasome Activation*. Cell Rep, 2015. **12**(1): p. 102-115.
30. Wolf, A.J., et al., *Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan*. Cell, 2016. **166**(3): p. 624-636.
31. Moon, J.S., et al., *UCP2-induced fatty acid synthase promotes NLRP3 inflammasome activation during sepsis*. J Clin Invest, 2015. **125**(2): p. 665-80.
32. Jha, A.K., et al., *Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization*. Immunity, 2015. **42**(3): p. 419-30.
33. Wellen, K.E., et al., *ATP-citrate lyase links cellular metabolism to histone acetylation*. Science, 2009. **324**(5930): p. 1076-80.
34. Peng, C., et al., *The first identification of lysine malonylation substrates and its regulatory enzyme*. Mol Cell Proteomics, 2011. **10**(12): p. M111 012658.
35. Infantino, V., et al., *The mitochondrial citrate carrier: a new player in inflammation*. Biochem J, 2011. **438**(3): p. 433-6.
36. Iacobazzi, V. and V. Infantino, *Citrate--new functions for an old metabolite*. Biol Chem, 2014. **395**(4): p. 387-99.
37. Michelucci, A., et al., *Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production*. Proc Natl Acad Sci U S A, 2013. **110**(19): p. 7820-5.
38. Lampropoulou, V., et al., *Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation*. Cell Metab, 2016. **24**(1): p. 158-66.
39. Garaude, J., et al., *Mitochondrial respiratory-chain adaptations in macrophages contribute to antibacterial host defense*. Nat Immunol, 2016. **17**(9): p. 1037-1045.
40. Hamanaka, R.B., et al., *The Mitochondrial Respiratory Chain Is Required for Organismal Adaptation to Hypoxia*. Cell Rep, 2016. **15**(3): p. 451-459.
41. Selak, M.A., et al., *Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF- α prolyl hydroxylase*. Cancer Cell, 2005. **7**(1): p. 77-85.
42. Fang, H.Y., et al., *Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia*. Blood, 2009. **114**(4): p. 844-59.
43. Peyssonnaud, C., et al., *HIF-1 α expression regulates the bactericidal capacity of phagocytes*. J Clin Invest, 2005. **115**(7): p. 1806-15.
44. Littlewood-Evans, A., et al., *GPR91 senses extracellular succinate released from inflammatory macrophages and exacerbates rheumatoid arthritis*. J Exp Med, 2016. **213**(9): p. 1655-62.
45. Robben, J.H., et al., *Localization of the succinate receptor in the distal nephron and its signaling in polarized MDCK cells*. Kidney Int, 2009. **76**(12): p. 1258-67.
46. Rubic, T., et al., *Triggering the succinate receptor GPR91 on dendritic cells enhances immunity*. Nat Immunol, 2008. **9**(11): p. 1261-9.
47. Sabari, B.R., et al., *Metabolic regulation of gene expression through histone acylations*. Nat Rev Mol Cell Biol, 2017. **18**(2): p. 90-101.

48. Peng, M., et al., *Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism*. Science, 2016. **354**(6311): p. 481-484.
49. Sutendra, G., et al., *A nuclear pyruvate dehydrogenase complex is important for the generation of acetyl-CoA and histone acetylation*. Cell, 2014. **158**(1): p. 84-97.
50. Allfrey, V.G., R. Faulkner, and A.E. Mirsky, *Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis*. Proc Natl Acad Sci U S A, 1964. **51**: p. 786-94.
51. Honjo, T., Y. Nishizuka, and O. Hayaishi, *Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis*. J Biol Chem, 1968. **243**(12): p. 3553-5.
52. Moellering, R.E. and B.F. Cravatt, *Functional lysine modification by an intrinsically reactive primary glycolytic metabolite*. Science, 2013. **341**(6145): p. 549-53.
53. Chen, Y., et al., *Lysine propionylation and butyrylation are novel post-translational modifications in histones*. Mol Cell Proteomics, 2007. **6**(5): p. 812-9.
54. Xie, Z., et al., *Metabolic Regulation of Gene Expression by Histone Lysine beta-Hydroxybutyrylation*. Mol Cell, 2016. **62**(2): p. 194-206.
55. Tan, M., et al., *Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification*. Cell, 2011. **146**(6): p. 1016-28.
56. Tan, M., et al., *Lysine glutarylation is a protein posttranslational modification regulated by SIRT5*. Cell Metab, 2014. **19**(4): p. 605-17.
57. Sies, H., *Glutathione and its role in cellular functions*. Free Radic Biol Med, 1999. **27**(9-10): p. 916-21.
58. Meezan, E., et al., *Comparative studies on the carbohydrate-containing membrane components of normal and virus-transformed mouse fibroblasts. II. Separation of glycoproteins and glycopeptides by sephadex chromatography*. Biochemistry, 1969. **8**(6): p. 2518-24.
59. Cooper, H.L., et al., *Identification of the hypusine-containing protein hy+ as translation initiation factor eIF-4D*. Proc Natl Acad Sci U S A, 1983. **80**(7): p. 1854-7.
60. Wagner, G.R., et al., *A Class of Reactive Acyl-CoA Species Reveals the Non-enzymatic Origins of Protein Acylation*. Cell Metab, 2017. **25**(4): p. 823-837 e8.
61. Chiang, P.K., et al., *S-Adenosylmethionine and methylation*. FASEB J, 1996. **10**(4): p. 471-80.
62. Berthiaume, L., et al., *Regulation of enzymatic activity by active site fatty acylation. A new role for long chain fatty acid acylation of proteins*. J Biol Chem, 1994. **269**(9): p. 6498-505.
63. Schmidt, M.F. and M.J. Schlesinger, *Fatty acid binding to vesicular stomatitis virus glycoprotein: a new type of post-translational modification of the viral glycoprotein*. Cell, 1979. **17**(4): p. 813-9.
64. Schlesinger, M.J., A.I. Magee, and M.F. Schmidt, *Fatty acid acylation of proteins in cultured cells*. J Biol Chem, 1980. **255**(21): p. 10021-4.
65. Fischer, E.H. and E.G. Krebs, *Conversion of phosphorylase b to phosphorylase a in muscle extracts*. J Biol Chem, 1955. **216**(1): p. 121-32.
66. Krebs, E.G. and E.H. Fischer, *The phosphorylase b to a converting enzyme of rabbit skeletal muscle*. Biochim Biophys Acta, 1956. **20**(1): p. 150-7.
67. Rilling, H.C., et al., *Prenylated proteins: the structure of the isoprenoid group*. Science, 1990. **247**(4940): p. 318-20.
68. Edde, B., et al., *Posttranslational glutamylation of alpha-tubulin*. Science, 1990. **247**(4938): p. 83-5.
69. Stamler, J.S., et al., *S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds*. Proc Natl Acad Sci U S A, 1992. **89**(1): p. 444-8.
70. Alderson, N.L., et al., *S-(2-Succinyl)cysteine: a novel chemical modification of tissue proteins by a Krebs cycle intermediate*. Arch Biochem Biophys, 2006. **450**(1): p. 1-8.

71. Zhang, Z., et al., *Identification of lysine succinylation as a new post-translational modification*. Nat Chem Biol, 2011. **7**(1): p. 58-63.
72. Xiao, M., et al., *Inhibition of alpha-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors*. Genes Dev, 2012. **26**(12): p. 1326-38.
73. Lu, C., et al., *IDH mutation impairs histone demethylation and results in a block to cell differentiation*. Nature, 2012. **483**(7390): p. 474-8.
74. Shimazu, T., et al., *Suppression of oxidative stress by beta-hydroxybutyrate, an endogenous histone deacetylase inhibitor*. Science, 2013. **339**(6116): p. 211-4.
75. Balmer, M.L., et al., *Memory CD8(+) T Cells Require Increased Concentrations of Acetate Induced by Stress for Optimal Function*. Immunity, 2016. **44**(6): p. 1312-24.
76. Li, T., et al., *Glyceraldehyde-3-phosphate dehydrogenase is activated by lysine 254 acetylation in response to glucose signal*. J Biol Chem, 2014. **289**(6): p. 3775-85.
77. Zhao, S., et al., *Regulation of cellular metabolism by protein lysine acetylation*. Science, 2010. **327**(5968): p. 1000-4.
78. Palmieri, E.M., et al., *Acetylation of human mitochondrial citrate carrier modulates mitochondrial citrate/malate exchange activity to sustain NADPH production during macrophage activation*. Biochim Biophys Acta, 2015. **1847**(8): p. 729-38.
79. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease*. Science, 2016. **352**(6284): p. aaf1098.
80. Cheng, S.C., et al., *mTOR- and HIF-1alpha-mediated aerobic glycolysis as metabolic basis for trained immunity*. Science, 2014. **345**(6204): p. 1250684.
81. Du, J., et al., *Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase*. Science, 2011. **334**(6057): p. 806-9.
82. Cox, M.a.N., D.L., *Lehninger Principles of Biochemistry*. 2008: Palgrave Macmillan.
83. Weinert, B.T., et al., *Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation*. Cell Rep, 2013. **4**(4): p. 842-51.
84. Chen, H., et al., *Mild metabolic perturbations alter succinylation of mitochondrial proteins*. J Neurosci Res, 2017. **95**(11): p. 2244-2252.
85. Abu-Elheiga, L., et al., *Mutant mice lacking acetyl-CoA carboxylase 1 are embryonically lethal*. Proc Natl Acad Sci U S A, 2005. **102**(34): p. 12011-6.
86. Abu-Elheiga, L., et al., *Continuous fatty acid oxidation and reduced fat storage in mice lacking acetyl-CoA carboxylase 2*. Science, 2001. **291**(5513): p. 2613-6.
87. Abu-Elheiga, L., et al., *Acetyl-CoA carboxylase 2 mutant mice are protected against obesity and diabetes induced by high-fat/high-carbohydrate diets*. Proc Natl Acad Sci U S A, 2003. **100**(18): p. 10207-12.
88. Saggerson, D., *Malonyl-CoA, a key signaling molecule in mammalian cells*. Annu Rev Nutr, 2008. **28**: p. 253-72.
89. Witkowski, A., J. Thweatt, and S. Smith, *Mammalian ACSF3 protein is a malonyl-CoA synthetase that supplies the chain extender units for mitochondrial fatty acid synthesis*. J Biol Chem, 2011. **286**(39): p. 33729-36.
90. Hirschey, M.D. and Y. Zhao, *Metabolic Regulation by Lysine Malonylation, Succinylation, and Glutarylation*. Mol Cell Proteomics, 2015. **14**(9): p. 2308-15.
91. Wang, F., et al., *SIRT5 Desuccinylates and Activates Pyruvate Kinase M2 to Block Macrophage IL-1beta Production and to Prevent DSS-Induced Colitis in Mice*. Cell Rep, 2017. **19**(11): p. 2331-2344.
92. Xiangyun, Y., et al., *Desuccinylation of pyruvate kinase M2 by SIRT5 contributes to antioxidant response and tumor growth*. Oncotarget, 2017. **8**(4): p. 6984-6993.
93. Park, J., et al., *SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways*. Mol Cell, 2013. **50**(6): p. 919-30.

94. Zhang, Y., et al., *Lysine desuccinylase SIRT5 binds to cardiolipin and regulates the electron transport chain*. J Biol Chem, 2017. **292**(24): p. 10239-10249.
95. Li, F., et al., *NADP(+)-IDH Mutations Promote Hypersuccinylation that Impairs Mitochondria Respiration and Induces Apoptosis Resistance*. Mol Cell, 2015. **60**(4): p. 661-75.
96. Zhou, L., et al., *SIRT5 promotes IDH2 desuccinylation and G6PD deglutarylation to enhance cellular antioxidant defense*. EMBO Rep, 2016. **17**(6): p. 811-22.
97. Lin, Z.F., et al., *SIRT5 desuccinylates and activates SOD1 to eliminate ROS*. Biochem Biophys Res Commun, 2013. **441**(1): p. 191-5.
98. Xie, Z., et al., *Lysine succinylation and lysine malonylation in histones*. Mol Cell Proteomics, 2012. **11**(5): p. 100-7.
99. Colak, G., et al., *Proteomic and Biochemical Studies of Lysine Malonylation Suggest Its Malonic Aciduria-associated Regulatory Role in Mitochondrial Function and Fatty Acid Oxidation*. Mol Cell Proteomics, 2015. **14**(11): p. 3056-71.
100. Nishida, Y., et al., *SIRT5 Regulates both Cytosolic and Mitochondrial Protein Malonylation with Glycolysis as a Major Target*. Mol Cell, 2015. **59**(2): p. 321-32.
101. Bowman, C.E., et al., *The Mammalian Malonyl-CoA Synthetase ACSF3 Is Required for Mitochondrial Protein Malonylation and Metabolic Efficiency*. Cell Chem Biol, 2017. **24**(6): p. 673-684 e4.
102. Du, Y., et al., *Lysine malonylation is elevated in type 2 diabetic mouse models and enriched in metabolic associated proteins*. Mol Cell Proteomics, 2015. **14**(1): p. 227-36.
103. Nie, L., et al., *The Landscape of Histone Modifications in a High-Fat Diet-Induced Obese (DIO) Mouse Model*. Mol Cell Proteomics, 2017. **16**(7): p. 1324-1334.
104. Buler, M., et al., *SIRT5 is under the control of PGC-1alpha and AMPK and is involved in regulation of mitochondrial energy metabolism*. FASEB J, 2014. **28**(7): p. 3225-37.
105. Lu, W., et al., *SIRT5 facilitates cancer cell growth and drug resistance in non-small cell lung cancer*. Tumour Biol, 2014. **35**(11): p. 10699-705.
106. Yu, J., et al., *Metabolic characterization of a Sirt5 deficient mouse model*. Sci Rep, 2013. **3**: p. 2806.
107. Qin, K., et al., *NAD⁺ dependent deacetylase Sirtuin 5 rescues the innate inflammatory response of endotoxin tolerant macrophages by promoting acetylation of p65*. J Autoimmun, 2017. **81**: p. 120-129.
108. Guedouari, H., et al., *Sirtuin 5 protects mitochondria from fragmentation and degradation during starvation*. Biochim Biophys Acta, 2017. **1864**(1): p. 169-176.
109. Buck, M.D., et al., *Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming*. Cell, 2016. **166**(1): p. 63-76.
110. Jeffery, C.J., *Moonlighting proteins*. Trends Biochem Sci, 1999. **24**(1): p. 8-11.
111. Hendriks, W., et al., *Duck lens epsilon-crystallin and lactate dehydrogenase B4 are identical: a single-copy gene product with two distinct functions*. Proc Natl Acad Sci U S A, 1988. **85**(19): p. 7114-8.
112. Williams, K.R., S. Reddigari, and G.L. Patel, *Identification of a nucleic acid helix-destabilizing protein from rat liver as lactate dehydrogenase-5*. Proc Natl Acad Sci U S A, 1985. **82**(16): p. 5260-4.
113. Zhong, X.H. and B.D. Howard, *Phosphotyrosine-containing lactate dehydrogenase is restricted to the nuclei of PC12 pheochromocytoma cells*. Mol Cell Biol, 1990. **10**(2): p. 770-6.
114. Pioli, P.A., et al., *Lactate dehydrogenase is an AU-rich element-binding protein that directly interacts with AUF1*. J Biol Chem, 2002. **277**(38): p. 35738-45.
115. Golshani-Hebroni, S.G. and S.P. Bessman, *Hexokinase binding to mitochondria: a basis for proliferative energy metabolism*. J Bioenerg Biomembr, 1997. **29**(4): p. 331-8.
116. Gottlob, K., et al., *Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase*. Genes Dev, 2001. **15**(11): p. 1406-18.

117. Yang, W., et al., *ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect*. Nat Cell Biol, 2012. **14**(12): p. 1295-304.
118. Gao, X., et al., *Pyruvate kinase M2 regulates gene transcription by acting as a protein kinase*. Mol Cell, 2012. **45**(5): p. 598-609.
119. Zhang, Z., et al., *Antigen presentation by dendritic cells in tumors is disrupted by altered metabolism that involves pyruvate kinase M2 and its interaction with SOCS3*. Cancer Res, 2010. **70**(1): p. 89-98.
120. Perucho, M., J. Salas, and M.L. Salas, *Study of the interaction of glyceraldehyde-3-phosphate dehydrogenase with DNA*. Biochim Biophys Acta, 1980. **606**(2): p. 181-95.
121. Tristan, C., et al., *The diverse functions of GAPDH: views from different subcellular compartments*. Cell Signal, 2011. **23**(2): p. 317-23.
122. Ventura, M., et al., *Nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase is regulated by acetylation*. Int J Biochem Cell Biol, 2010. **42**(10): p. 1672-80.
123. Hara, M.R., et al., *S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding*. Nat Cell Biol, 2005. **7**(7): p. 665-74.
124. Hara, M.R., M.B. Cascio, and A. Sawa, *GAPDH as a sensor of NO stress*. Biochim Biophys Acta, 2006. **1762**(5): p. 502-9.
125. Sen, N., et al., *Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis*. Nat Cell Biol, 2008. **10**(7): p. 866-73.
126. Tarze, A., et al., *GAPDH, a novel regulator of the pro-apoptotic mitochondrial membrane permeabilization*. Oncogene, 2007. **26**(18): p. 2606-20.
127. Itakura, M., et al., *Glyceraldehyde-3-phosphate Dehydrogenase Aggregates Accelerate Amyloid-beta Amyloidogenesis in Alzheimer Disease*. J Biol Chem, 2015. **290**(43): p. 26072-87.
128. Colell, A., et al., *GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation*. Cell, 2007. **129**(5): p. 983-97.
129. Ryazanov, A.G., *Glyceraldehyde-3-phosphate dehydrogenase is one of the three major RNA-binding proteins of rabbit reticulocytes*. FEBS Lett, 1985. **192**(1): p. 131-4.
130. Singh, R. and M.R. Green, *Sequence-specific binding of transfer RNA by glyceraldehyde-3-phosphate dehydrogenase*. Science, 1993. **259**(5093): p. 365-8.
131. Nagy, E. and W.F. Rigby, *Glyceraldehyde-3-phosphate dehydrogenase selectively binds AU-rich RNA in the NAD(+)-binding region (Rossmann fold)*. J Biol Chem, 1995. **270**(6): p. 2755-63.
132. Nagy, E., et al., *Identification of the NAD(+)-binding fold of glyceraldehyde-3-phosphate dehydrogenase as a novel RNA-binding domain*. Biochem Biophys Res Commun, 2000. **275**(2): p. 253-60.
133. White, M.R., et al., *A dimer interface mutation in glyceraldehyde 3-phosphate dehydrogenase regulates its binding to AU-rich RNA*. J Biol Chem, 2015. **290**(7): p. 4129.
134. Rodriguez-Pascual, F., et al., *Glyceraldehyde-3-phosphate dehydrogenase regulates endothelin-1 expression by a novel, redox-sensitive mechanism involving mRNA stability*. Mol Cell Biol, 2008. **28**(23): p. 7139-55.
135. Sampath, P., et al., *Noncanonical function of glutamyl-prolyl-tRNA synthetase: gene-specific silencing of translation*. Cell, 2004. **119**(2): p. 195-208.
136. Mukhopadhyay, R., et al., *The GAIT system: a gatekeeper of inflammatory gene expression*. Trends Biochem Sci, 2009. **34**(7): p. 324-31.
137. Arif, A., et al., *Heterotrimeric GAIT complex drives transcript-selective translation inhibition in murine macrophages*. Mol Cell Biol, 2012. **32**(24): p. 5046-55.
138. Chang, C.H., et al., *Posttranscriptional control of T cell effector function by aerobic glycolysis*. Cell, 2013. **153**(6): p. 1239-51.

139. Millet, P., et al., *GAPDH Binding to TNF-alpha mRNA Contributes to Posttranscriptional Repression in Monocytes: A Novel Mechanism of Communication between Inflammation and Metabolism*. J Immunol, 2016. **196**(6): p. 2541-51.
140. Schultz, D.E., C.C. Hardin, and S.M. Lemon, *Specific interaction of glyceraldehyde 3-phosphate dehydrogenase with the 5'-nontranslated RNA of hepatitis A virus*. J Biol Chem, 1996. **271**(24): p. 14134-42.
141. Yi, M., D.E. Schultz, and S.M. Lemon, *Functional significance of the interaction of hepatitis A virus RNA with glyceraldehyde 3-phosphate dehydrogenase (GAPDH): opposing effects of GAPDH and polypyrimidine tract binding protein on internal ribosome entry site function*. J Virol, 2000. **74**(14): p. 6459-68.
142. Zang, W.Q., et al., *Identification of glyceraldehyde-3-phosphate dehydrogenase as a cellular protein that binds to the hepatitis B virus posttranscriptional regulatory element*. Virology, 1998. **248**(1): p. 46-52.
143. Petrik, J., H. Parker, and G.J. Alexander, *Human hepatic glyceraldehyde-3-phosphate dehydrogenase binds to the poly(U) tract of the 3' non-coding region of hepatitis C virus genomic RNA*. J Gen Virol, 1999. **80** (Pt 12): p. 3109-13.
144. Choudhary, S., B.P. De, and A.K. Banerjee, *Specific phosphorylated forms of glyceraldehyde 3-phosphate dehydrogenase associate with human parainfluenza virus type 3 and inhibit viral transcription in vitro*. J Virol, 2000. **74**(8): p. 3634-41.
145. De, B.P., et al., *Specific interaction in vitro and in vivo of glyceraldehyde-3-phosphate dehydrogenase and LA protein with cis-acting RNAs of human parainfluenza virus type 3*. J Biol Chem, 1996. **271**(40): p. 24728-35.
146. Pancholi, V. and V.A. Fischetti, *A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity*. J Exp Med, 1992. **176**(2): p. 415-26.
147. Boel, G., H. Jin, and V. Pancholi, *Inhibition of cell surface export of group A streptococcal anchorless surface dehydrogenase affects bacterial adherence and antiphagocytic properties*. Infect Immun, 2005. **73**(10): p. 6237-48.
148. Terrasse, R., et al., *Streptococcus pneumoniae GAPDH Is Released by Cell Lysis and Interacts with Peptidoglycan*. PLoS One, 2015. **10**(4): p. e0125377.
149. Terao, Y., et al., *Multifunctional glyceraldehyde-3-phosphate dehydrogenase of Streptococcus pyogenes is essential for evasion from neutrophils*. J Biol Chem, 2006. **281**(20): p. 14215-23.
150. Oliveira, L., et al., *Group B streptococcus GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages*. PLoS One, 2012. **7**(1): p. e29963.
151. Madureira, P., et al., *Inhibition of IL-10 production by maternal antibodies against Group B Streptococcus GAPDH confers immunity to offspring by favoring neutrophil recruitment*. PLoS Pathog, 2011. **7**(11): p. e1002363.
152. Alves, J., et al., *A Safe and Stable Neonatal Vaccine Targeting GAPDH Confers Protection against Group B Streptococcus Infections in Adult Susceptible Mice*. PLoS One, 2015. **10**(12): p. e0144196.
153. Aguilera, L., et al., *Secretion of the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase by the LEE-encoded type III secretion system in enteropathogenic Escherichia coli*. Int J Biochem Cell Biol, 2012. **44**(6): p. 955-62.
154. Egea, L., et al., *Role of secreted glyceraldehyde-3-phosphate dehydrogenase in the infection mechanism of enterohemorrhagic and enteropathogenic Escherichia coli: interaction of the extracellular enzyme with human plasminogen and fibrinogen*. Int J Biochem Cell Biol, 2007. **39**(6): p. 1190-203.

155. Sahoo, S., et al., *Glyceraldehyde-3-phosphate dehydrogenase of the parasitic nematode Haemonchus contortus binds to complement C3 and inhibits its activity*. Parasite Immunol, 2013. **35**(12): p. 457-67.
156. Vedamurthy, G.V., et al., *The N-terminal segment of glyceraldehyde-3-phosphate dehydrogenase of Haemonchus contortus interacts with complements C1q and C3*. Parasite Immunol, 2015. **37**(11): p. 568-78.
157. Han, K., et al., *Vaccination of goats with glyceraldehyde-3-phosphate dehydrogenase DNA vaccine induced partial protection against Haemonchus contortus*. Vet Immunol Immunopathol, 2012. **149**(3-4): p. 177-85.
158. Evguenieva-Hackenberg, E., E. Schiltz, and G. Klug, *Dehydrogenases from all three domains of life cleave RNA*. J Biol Chem, 2002. **277**(48): p. 46145-50.
159. Cai, L., et al., *Acetyl-CoA induces cell growth and proliferation by promoting the acetylation of histones at growth genes*. Mol Cell, 2011. **42**(4): p. 426-37.
160. Lee, J.V., et al., *Akt-dependent metabolic reprogramming regulates tumor cell histone acetylation*. Cell Metab, 2014. **20**(2): p. 306-319.
161. Kulkarni, R.A., et al., *Discovering Targets of Non-enzymatic Acylation by Thioester Reactivity Profiling*. Cell Chem Biol, 2017. **24**(2): p. 231-242.
162. Wagner, G.R. and R.M. Payne, *Widespread and enzyme-independent Nepsilon-acetylation and Nepsilon-succinylation of proteins in the chemical conditions of the mitochondrial matrix*. J Biol Chem, 2013. **288**(40): p. 29036-45.
163. Bao, X., et al., *A chemical probe for lysine malonylation*. Angew Chem Int Ed Engl, 2013. **52**(18): p. 4883-6.
164. Qian, L., et al., *Global Profiling of Protein Lysine Malonylation in Escherichia coli Reveals Its Role in Energy Metabolism*. J Proteome Res, 2016. **15**(6): p. 2060-71.
165. Bond, S.T., et al., *Lysine post-translational modification of glyceraldehyde-3-phosphate dehydrogenase regulates hepatic and systemic metabolism*. FASEB J, 2017. **31**(6): p. 2592-2602.
166. Wang, Z. and P.A. Cole, *Catalytic mechanisms and regulation of protein kinases*. Methods Enzymol, 2014. **548**: p. 1-21.
167. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells*. Nat Rev Immunol, 2010. **10**(3): p. 170-81.
168. O'Neill, L.A., R.J. Kishton, and J. Rathmell, *A guide to immunometabolism for immunologists*. Nat Rev Immunol, 2016. **16**(9): p. 553-65.
169. Shi, L.Z., et al., *HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells*. J Exp Med, 2011. **208**(7): p. 1367-76.
170. Everts, B., et al., *TLR-driven early glycolytic reprogramming via the kinases TBK1- IKKvarepsilon supports the anabolic demands of dendritic cell activation*. Nat Immunol, 2014. **15**(4): p. 323-32.
171. Park, J., et al., *O-GlcNAcylation disrupts glyceraldehyde-3-phosphate dehydrogenase homo-tetramer formation and mediates its nuclear translocation*. Biochim Biophys Acta, 2009. **1794**(2): p. 254-62.
172. Combs, D.J., et al., *Glycolytic inhibition by 2-deoxyglucose reduces hyperglycemia-associated mortality and morbidity in the ischemic rat*. Stroke, 1986. **17**(5): p. 989-94.
173. Ikeda, Y., et al., *Glyceraldehyde-3-phosphate dehydrogenase regulates cyclooxygenase-2 expression by targeting mRNA stability*. Arch Biochem Biophys, 2012. **528**(2): p. 141-7.
174. Clark, A., *Post-transcriptional regulation of pro-inflammatory gene expression*. Arthritis Res, 2000. **2**(3): p. 172-4.
175. Mukhopadhyay, R., et al., *DAPK-ZIPK-L13a axis constitutes a negative-feedback module regulating inflammatory gene expression*. Mol Cell, 2008. **32**(3): p. 371-82.

176. Deiss, L.P., et al., *Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death*. Genes Dev, 1995. **9**(1): p. 15-30.
177. Michie, A.M., et al., *Death-associated protein kinase (DAPK) and signal transduction: regulation in cancer*. FEBS J, 2010. **277**(1): p. 74-80.
178. Mor, I., et al., *Death-associated protein kinase increases glycolytic rate through binding and activation of pyruvate kinase*. Oncogene, 2012. **31**(6): p. 683-93.
179. Liberti, M.V., et al., *A Predictive Model for Selective Targeting of the Warburg Effect through GAPDH Inhibition with a Natural Product*. Cell Metab, 2017. **26**(4): p. 648-659 e8.
180. Endo, A., et al., *Specific inhibition of glyceraldehyde-3-phosphate dehydrogenase by koniginic acid (heptelidic acid)*. J Antibiot (Tokyo), 1985. **38**(7): p. 920-5.
181. Shirai, T., et al., *The glycolytic enzyme PKM2 bridges metabolic and inflammatory dysfunction in coronary artery disease*. J Exp Med, 2016. **213**(3): p. 337-54.
182. Chiba, S., et al., *Glycolysis regulates LPS-induced cytokine production in M2 polarized human macrophages*. Immunol Lett, 2017. **183**: p. 17-23.
183. Suzuki, H., et al., *Glycolytic pathway affects differentiation of human monocytes to regulatory macrophages*. Immunol Lett, 2016. **176**: p. 18-27.
184. Yamaguchi, Y., et al., *Novel terpenoids, trichoderonic acids A and B isolated from Trichoderma virens, are selective inhibitors of family X DNA polymerases*. Biosci Biotechnol Biochem, 2010. **74**(4): p. 793-801.
185. Danova, K., et al., *NF-kappaB, p38 MAPK, ERK1/2, mTOR, STAT3 and increased glycolysis regulate stability of paricalcitol/dexamethasone-generated tolerogenic dendritic cells in the inflammatory environment*. Oncotarget, 2015. **6**(16): p. 14123-38.
186. Galvan-Pena, S. and L.A. O'Neill, *Metabolic reprogramming in macrophage polarization*. Front Immunol, 2014. **5**: p. 420.
187. Bradley, J.R., *TNF-mediated inflammatory disease*. J Pathol, 2008. **214**(2): p. 149-60.
188. Bialik, S. and A. Kimchi, *DAP-kinase as a target for drug design in cancer and diseases associated with accelerated cell death*. Semin Cancer Biol, 2004. **14**(4): p. 283-94.
189. Lin, Y., T.R. Hupp, and C. Stevens, *Death-associated protein kinase (DAPK) and signal transduction: additional roles beyond cell death*. FEBS J, 2010. **277**(1): p. 48-57.
190. Chuang, Y.T., et al., *Tumor suppressor death-associated protein kinase is required for full IL-1beta production*. Blood, 2011. **117**(3): p. 960-70.
191. Yoo, H.J., et al., *DAPk1 inhibits NF-kappaB activation through TNF-alpha and INF-gamma-induced apoptosis*. Cell Signal, 2012. **24**(7): p. 1471-7.
192. Anderson, P., *Post-transcriptional regulons coordinate the initiation and resolution of inflammation*. Nat Rev Immunol, 2010. **10**(1): p. 24-35.
193. Bonafe, N., et al., *Glyceraldehyde-3-phosphate dehydrogenase binds to the AU-Rich 3' untranslated region of colony-stimulating factor-1 (CSF-1) messenger RNA in human ovarian cancer cells: possible role in CSF-1 posttranscriptional regulation and tumor phenotype*. Cancer Res, 2005. **65**(9): p. 3762-71.
194. Zhou, Y., et al., *The multifunctional protein glyceraldehyde-3-phosphate dehydrogenase is both regulated and controls colony-stimulating factor-1 messenger RNA stability in ovarian cancer*. Mol Cancer Res, 2008. **6**(8): p. 1375-84.
195. Deshmukh, V.K., et al., *Role of ceruloplasmin in inflammation: increased serum ceruloplasmin levels during inflammatory conditions and its possible relationship with anti-inflammatory agents*. Pharmacol Res Commun, 1985. **17**(7): p. 633-42.
196. Bakhautdin, B., et al., *Protective role of macrophage-derived ceruloplasmin in inflammatory bowel disease*. Gut, 2013. **62**(2): p. 209-19.
197. Sampath, P., et al., *Transcript-selective translational silencing by gamma interferon is directed by a novel structural element in the ceruloplasmin mRNA 3' untranslated region*. Mol Cell Biol, 2003. **23**(5): p. 1509-19.

198. Fan, T., et al., *DNA hypomethylation caused by Lsh deletion promotes erythroleukemia development*. Epigenetics, 2008. **3**(3): p. 134-42.
199. Voisin, V., et al., *Gene profiling of the erythro- and megakaryoblastic leukaemias induced by the Graffi murine retrovirus*. BMC Med Genomics, 2010. **3**: p. 2.
200. Chang, I.W., et al., *CDCA5 overexpression is an indicator of poor prognosis in patients with urothelial carcinomas of the upper urinary tract and urinary bladder*. Am J Transl Res, 2015. **7**(4): p. 710-22.
201. Tang, H., et al., *miR-185 suppresses tumor proliferation by directly targeting E2F6 and DNMT1 and indirectly upregulating BRCA1 in triple-negative breast cancer*. Mol Cancer Ther, 2014. **13**(12): p. 3185-97.
202. Oberley, M.J., D.R. Inman, and P.J. Farnham, *E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9*. J Biol Chem, 2003. **278**(43): p. 42466-76.
203. Korhonen, M.K., E. Vuorenmaa, and M. Nystrom, *The first functional study of MLH3 mutations found in cancer patients*. Genes Chromosomes Cancer, 2008. **47**(9): p. 803-9.
204. Huang, B., et al., *Androglobin knockdown inhibits growth of glioma cell lines*. Int J Clin Exp Pathol, 2014. **7**(5): p. 2179-84.
205. Sidhu, H. and N. Capalash, *UHRF1: The key regulator of epigenetics and molecular target for cancer therapeutics*. Tumour Biol, 2017. **39**(2): p. 1010428317692205.
206. Guo, C., S. Liu, and M.Z. Sun, *Novel insight into the role of GAPDH playing in tumor*. Clin Transl Oncol, 2013. **15**(3): p. 167-72.
207. Palazzo, A.F. and E.S. Lee, *Non-coding RNA: what is functional and what is junk?* Front Genet, 2015. **6**: p. 2.
208. Rapicavoli, N.A., et al., *A mammalian pseudogene lncRNA at the interface of inflammation and anti-inflammatory therapeutics*. Elife, 2013. **2**: p. e00762.
209. Carpenter, S., et al., *A long noncoding RNA mediates both activation and repression of immune response genes*. Science, 2013. **341**(6147): p. 789-92.
210. Roitel, O., E. Sergienko, and G. Branlant, *Dimers generated from tetrameric phosphorylating glyceraldehyde-3-phosphate dehydrogenase from Bacillus stearothermophilus are inactive but exhibit cooperativity in NAD binding*. Biochemistry, 1999. **38**(49): p. 16084-91.
211. Polyakova, O.V., et al., *Misfolded forms of glyceraldehyde-3-phosphate dehydrogenase interact with GroEL and inhibit chaperonin-assisted folding of the wild-type enzyme*. Protein Sci, 2005. **14**(4): p. 921-8.
212. Cuezva, J.M., et al., *The bioenergetic signature of lung adenocarcinomas is a molecular marker of cancer diagnosis and prognosis*. Carcinogenesis, 2004. **25**(7): p. 1157-63.
213. Epner, D.E., et al., *Association of glyceraldehyde-3-phosphate dehydrogenase expression with cell motility and metastatic potential of rat prostatic adenocarcinoma*. Cancer Res, 1993. **53**(9): p. 1995-7.
214. Schek, N., B.L. Hall, and O.J. Finn, *Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in human pancreatic adenocarcinoma*. Cancer Res, 1988. **48**(22): p. 6354-9.
215. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, *Understanding the Warburg effect: the metabolic requirements of cell proliferation*. Science, 2009. **324**(5930): p. 1029-33.
216. Ferrajoli, A., et al., *The clinical significance of tumor necrosis factor-alpha plasma level in patients having chronic lymphocytic leukemia*. Blood, 2002. **100**(4): p. 1215-9.
217. Szlosarek, P.W., et al., *Expression and regulation of tumor necrosis factor alpha in normal and malignant ovarian epithelium*. Mol Cancer Ther, 2006. **5**(2): p. 382-90.
218. Michalaki, V., et al., *Serum levels of IL-6 and TNF-alpha correlate with clinicopathological features and patient survival in patients with prostate cancer*. Br J Cancer, 2004. **90**(12): p. 2312-6.
219. Zhou, W., et al., *Malonyl-CoA decarboxylase inhibition is selectively cytotoxic to human breast cancer cells*. Oncogene, 2009. **28**(33): p. 2979-87.

- 220. Yizhak, K., et al., *Phenotype-based cell-specific metabolic modeling reveals metabolic liabilities of cancer*. Elife, 2014. **3**.
- 221. Surmi, B.K. and A.H. Hasty, *Macrophage infiltration into adipose tissue: initiation, propagation and remodeling*. Future Lipidol, 2008. **3**(5): p. 545-556.
- 222. De Taeye, B.M., et al., *Macrophage TNF-alpha contributes to insulin resistance and hepatic steatosis in diet-induced obesity*. Am J Physiol Endocrinol Metab, 2007. **293**(3): p. E713-25.
- 223. Suganami, T., J. Nishida, and Y. Ogawa, *A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha*. Arterioscler Thromb Vasc Biol, 2005. **25**(10): p. 2062-8.
- 224. Swaroop, J.J., D. Rajarajeswari, and J.N. Naidu, *Association of TNF-alpha with insulin resistance in type 2 diabetes mellitus*. Indian J Med Res, 2012. **135**: p. 127-30.
- 225. Moller, D.E., *Potential role of TNF-alpha in the pathogenesis of insulin resistance and type 2 diabetes*. Trends Endocrinol Metab, 2000. **11**(6): p. 212-7.

Chapter 7: Appendix

7.1 Extended RIPSeq data

Differentially expressed genes - UT_IP - UT_IgG				
Gene_Symbol	Fold_Change	pvalue	Log2_FC	lfcSE
Snord49a	8.594583515	1.75E-05	3.10342773	0.722651076
Fkbp9	7.888018142	8.73E-07	2.97966287	0.605844509
Cdca5	4.729399522	0.002396494	2.24165702	0.738331182
Mcm10	3.605033428	0.004804318	1.850012637	0.656063202
Hells	3.57096421	0.000479386	1.836313675	0.525860317
E2f6	3.410441621	0.001325459	1.769958567	0.551318589
Uhrf1	3.159526112	0.000802977	1.659708189	0.495174139
Emc2	2.707521893	0.004662229	1.436973004	0.507855874
Slx4ip	2.266655469	0.006850177	1.180565119	0.436592858
MIh3	2.213340854	0.000539812	1.146225643	0.331261727
Adgb	2.038103754	0.032107489	1.027227497	0.479325249
Procr	2.010070172	0.061143663	1.007245867	0.537927905
Fam114a1	1.816702943	0.002222237	0.861322537	0.281588165
Cp	1.793931143	0.02589494	0.843124516	0.37845887
ENSMUSG00000089832	1.782019449	0.0152852	0.833513082	0.343638763
Rad23a	1.76201557	0.017658365	0.817226673	0.344428281
Vma21	1.757170847	0.121640162	0.813254469	0.525383367
Zfp738	1.742004637	0.032566794	0.800748464	0.374639543
Txndc12	1.729836139	0.010397549	0.790635383	0.308562322
Ilf2	1.728846273	0.026204254	0.789809592	0.355262594
Ppfia1	1.720328742	0.000702553	0.78268428	0.230977016
Ybx3	1.709563021	0.045231129	0.773627607	0.386331152
Stard4	1.689181295	0.044178257	0.756324177	0.375832761
Slc7a11	1.672565708	0.206988338	0.742062889	0.588056673
Cdc25a	1.671552918	0.038297908	0.741189029	0.357776805
Adnp2	1.661529417	0.035749083	0.732511836	0.348853377
Ubash3b	1.635626426	0.013471208	0.709843277	0.287262313
Bbs10	1.635044265	0.096121036	0.709329694	0.426290896
Ccdc134	1.632729393	0.048105723	0.7072857	0.357859635
Elac1	1.632536067	0.070757503	0.707114865	0.391313159
Phldb1	1.627822473	0.089184944	0.702943371	0.413565948
Pxk	1.624192646	0.004671191	0.699722761	0.247350172
Rpl11	1.622235473	0.026203869	0.697983247	0.313957573
Zfp568	1.621066494	0.040019912	0.69694327	0.339385714
Vdac3	1.616754906	0.041090886	0.693100988	0.339320962
Endov	1.616550119	0.072203194	0.692918237	0.385418195
Rbms2	1.610246843	0.062481569	0.687281864	0.368938526
ENSMUSG00000103233	1.609549877	0.034061504	0.686657284	0.323995379
Utp14a	1.597671919	0.039175069	0.675971182	0.327768515
Ndufc2	1.596652876	0.07162177	0.675050694	0.374712813
Utp14b	1.594554755	0.041228894	0.673153639	0.329779938

Wdr62	1.594143696	0.037614976	0.672781679	0.32360429
Gpaa1	1.589804629	0.05490636	0.668849483	0.348428703
Zfp830	1.576733799	0.052998557	0.65693911	0.339515227
Sap30	1.571639479	0.049282861	0.652270313	0.331752327
Tcp11l1	1.571435648	0.003771243	0.652083193	0.22511335
Carm1	1.56832526	0.069543335	0.649224796	0.357724522
ENSMUSG00000096768	1.563558333	0.098573661	0.644833044	0.390380101
Ing5	1.561677613	0.101133235	0.64309666	0.392279366
Dnaja1	1.555221137	0.007692959	0.637119732	0.2390463
Stim2	1.550112972	0.053248455	0.632373363	0.327162914
Cxcl2	1.548617692	0.178667412	0.63098103	0.469176432
March5	1.546477116	0.064693102	0.628985486	0.340475633
Cit	1.540242316	0.198704615	0.623157339	0.484852668
Rab11a	1.537378564	0.04258281	0.620472458	0.305986235
Ppp2r2d	1.535695356	0.06886797	0.61889205	0.340184232
Fmn12	1.533356871	0.119083679	0.616693506	0.395663503
Tti1	1.532043798	0.023128324	0.615457542	0.270970504
Al837181	1.53121532	0.095325973	0.614677169	0.368522895
Nrf1	1.526426455	0.07594396	0.61015808	0.343805065
Trip10	1.525368652	0.0551825	0.609157956	0.317693939
Nde1	1.519896016	0.037125824	0.603972625	0.289761603
ENSMUSG00000092702	1.518840622	0.17642381	0.60297049	0.446034097
Hinfp	1.51751171	0.079794419	0.601707649	0.343463935
Cdc123	1.516240239	0.089250062	0.600498358	0.353365912
Sdf2l1	1.514768574	0.115758712	0.599097396	0.380904649
Nucks1	1.513634047	0.028170447	0.598016446	0.2724567
Odf2l	1.512233256	0.060259216	0.596680686	0.317570476
Rrn3	1.506255948	0.062430389	0.590966939	0.317173945
Tbc1d32	1.506098945	0.052014256	0.590816553	0.304071933
Gas5	1.500870702	0.06556754	0.585799696	0.318132746
Otud6b	1.500457994	0.081175152	0.585402931	0.335684547
Gxylt1	1.498339926	0.052289854	0.583364962	0.300588973
Gngt2	1.496180164	0.079084564	0.581283909	0.331023038
Slc35f5	1.49199947	0.032761524	0.577247023	0.270374109
Rnps1	1.487878354	0.036924088	0.573256579	0.274732059
ENSMUSG00000102964	1.486832603	0.159133016	0.572242229	0.406423934
6430548M08Rik	1.486187626	0.050593257	0.571616263	0.292399743
ENSMUSG00000107390	1.485012608	0.059454581	0.57047518	0.302668775
Tbl1x	1.482041813	0.006735047	0.567586151	0.209467001
Eif4a3	1.480759328	0.132228309	0.566337174	0.376210629
Tmem165	1.480429531	0.132606312	0.56601582	0.376364547
Sema4c	1.480147515	0.142017093	0.565740965	0.38529792
Fam172a	1.479057135	0.06169493	0.564677784	0.30221207
Tgm2	1.475642224	0.074104708	0.561342976	0.314307702
Proser1	1.474646336	0.022301938	0.560368995	0.245218138

Slc28a2	1.473531969	0.012363683	0.559278361	0.223569107
Atpif1	1.472122269	0.098655405	0.557897501	0.337831527
Arcn1	1.470876813	0.047635239	0.556676425	0.281063388
Tank	1.469913342	0.036986685	0.555731104	0.266421291
Nol6	1.469784495	0.033747587	0.555604637	0.261698102
ENSMUSG00000096954	1.46870326	0.173969382	0.55454294	0.407884293
Scd1	1.468529205	0.284473549	0.554371957	0.517947855
Spryd3	1.468150703	0.083290301	0.554000065	0.319878831
Slc27a4	1.463754761	0.030383133	0.549673863	0.253883958
1700037H04Rik	1.462862592	0.131470206	0.548794263	0.363842414
Sept9	1.461962969	0.019123582	0.547906769	0.233837093
Vrk2	1.460260115	0.115260143	0.546225378	0.346813292
Rab22a	1.458673591	0.020093487	0.544657086	0.234301664
Atp5d	1.453620996	0.03368051	0.539651163	0.254087881
Ldlrad3	1.45016279	0.080917945	0.536214861	0.307218742
Trim24	1.45008976	0.113837029	0.536142206	0.339077408
Lrpap1	1.448082095	0.017033506	0.534143394	0.223867159
Dhx58	1.447251941	0.057684201	0.533316092	0.280975105
Asb7	1.444503581	0.119726285	0.53057378	0.341002486
Prpf38a	1.443385273	0.171688927	0.52945644	0.387370194
Keap1	1.442204175	0.105057596	0.528275424	0.325932227
Nom1	1.439819905	0.055451417	0.525888368	0.274569129
Cacna1d	1.437768828	0.080139551	0.52383173	0.299353468
Cep192	1.437016758	0.016721378	0.523076886	0.218606571
ENSMUSG00000099250	1.436868276	0.239501353	0.522927809	0.444578016
Tmem38b	1.434609078	0.032048428	0.520657665	0.242866027
Cd302	1.434593271	0.114171513	0.520641768	0.329578977
Ttll3	1.433967527	0.178019247	0.520012353	0.386086948
Tmem167	1.432843453	0.11921169	0.518880995	0.33302356
ENSMUSG00000108752	1.43216128	0.092973368	0.518193968	0.308463938
Spn	1.431218523	0.188660686	0.517243964	0.393475624
Dhdh	1.427288001	0.090791314	0.513276474	0.303491529
Cdk20	1.426851699	0.122232757	0.512835395	0.331831471
Wbp5	1.42559901	0.140515977	0.51156824	0.347091291
Smndc1	1.424323212	0.199328384	0.510276564	0.397576577
Riok1	1.421746671	0.167353839	0.507664426	0.367671217
Zfp598	1.421601354	0.016061506	0.507516961	0.210805324
Bmi1	1.420829181	0.156605964	0.506733117	0.357716025
Zfp84	1.419937536	0.183941532	0.505827466	0.380688793
Eps8l1	1.418806783	0.150373658	0.504678132	0.350906416
Gng5	1.418118738	0.152680263	0.503978333	0.352400838
ENSMUSG00000092746	1.418112702	0.259899968	0.503972193	0.447328111
Mapkapk3	1.416667472	0.018391167	0.50250116	0.213136716
ENSMUSG00000092805	1.416214635	0.245073037	0.502039931	0.431899471
Fbf1	1.416202276	0.07033168	0.50202734	0.277398636

Kdm4b	1.415315257	0.065718268	0.501123445	0.272299314
Traf1	1.414791834	0.1026838	0.500589797	0.306737793
Dgcr2	1.412869679	0.147950745	0.4986284	0.344640373
Irgq	1.412349405	0.036145142	0.498097045	0.237722145
Tbrg1	1.407057271	0.207494636	0.492681051	0.39086641
Zfp386	1.406073538	0.224354042	0.49167205	0.404657832
Fmr1	1.405828998	0.205746836	0.491421119	0.388368549
Braf	1.405252354	0.049641672	0.490829232	0.250035427
Zfp687	1.402540207	0.032671622	0.48804213	0.228473917
Cds1	1.401145363	0.1414704	0.486606637	0.330948966
Snx15	1.400708087	0.124185082	0.486156323	0.316211142
Tmem167b	1.40016232	0.136464541	0.485594088	0.326101959
Pom121	1.397313941	0.088568074	0.482656194	0.283415269
Taf15	1.396696728	0.134316521	0.482018794	0.321926408
Hsf1	1.395362304	0.212404103	0.480639764	0.385442428
Dgat1	1.394070938	0.169603211	0.479303975	0.34897051
Map1lc3b	1.39398993	0.061993283	0.479220139	0.256769489
Ppp1r2	1.393799258	0.108989739	0.479022792	0.298875451
Srp68	1.393692478	0.221460704	0.478912261	0.391699179
2510039O18Rik	1.392373128	0.057896089	0.477545876	0.251805924
Ltn1	1.392185133	0.060488358	0.477351074	0.254286571
Npat	1.3920017	0.132942863	0.477160974	0.317557408
Cmas	1.391952346	0.136288096	0.477109821	0.320260127
Slc37a3	1.390555554	0.122960877	0.475661383	0.308377622
Setd3	1.38969627	0.01903707	0.474769604	0.202477285
Mbd5	1.38958287	0.063015414	0.474651875	0.255315091
Sh2b2	1.388141966	0.243815555	0.473155121	0.405966548
Angel2	1.385481375	0.091425103	0.470387317	0.278677404
Zfp628	1.384916755	0.120407836	0.469799261	0.302498319
Ap1s1	1.384773727	0.099745472	0.469650258	0.285312828
Icosl	1.383015405	0.123487216	0.467817227	0.303718231
Ccdc127	1.382498358	0.223222605	0.467277767	0.383642353
Nfe2l1	1.381481267	0.003742412	0.466215999	0.160814309
Scap	1.381409027	0.080671211	0.466140556	0.266853267
Def8	1.379113596	0.11421743	0.463741295	0.293596827
Timm44	1.379067883	0.105470566	0.463693474	0.286426493
Glb1l	1.378404285	0.185331798	0.462999091	0.349561017
Klhl17	1.377855979	0.138282053	0.462425097	0.311981645
Cog2	1.37759247	0.049031959	0.462149162	0.234794469
Slc25a19	1.377541409	0.078727155	0.462095687	0.262835177
Wwc2	1.376606154	0.051064797	0.461115864	0.236356363
Stxbp1	1.376059871	0.147058439	0.460543241	0.317615966
ENSMUSG00000109498	1.373950696	0.139114565	0.458330235	0.30987179
Rab8a	1.373713295	0.156925817	0.458080934	0.323620775
Dcp1a	1.373360189	0.130441172	0.457710048	0.302645336

Vimp	1.373274238	0.036258017	0.457619755	0.218536264
Klhl2	1.371705429	0.239180727	0.455970699	0.387388576
Tmed8	1.370588879	0.153738286	0.454795886	0.318830425
Tecpr2	1.37048615	0.064409503	0.454687749	0.245865584
Ifi27l2a	1.370386655	0.188630281	0.454583007	0.345784664
Itgam	1.368416227	0.011740952	0.452507117	0.179578003
Fez2	1.368092973	0.139421409	0.452166277	0.305941716
Gprasp1	1.367298401	0.127045371	0.451328133	0.295786566
Ccny	1.367125717	0.035367804	0.451145915	0.214410529
Slc39a13	1.365393942	0.09556504	0.449317256	0.269577618
Ppp1r15b	1.364057949	0.023156454	0.447904936	0.197241659
Ccdc86	1.363217901	0.193428017	0.447016185	0.343725136
D230025D16Rik	1.363099538	0.196415445	0.446890917	0.345935873
Rnf103	1.362085045	0.118107793	0.445816785	0.285274293
Celf3	1.362003157	0.267644609	0.445730047	0.402100865
ENSMUSG00000084796	1.358416704	0.228695743	0.441926105	0.367132002
Gbp2	1.356862459	0.290408199	0.440274487	0.416442891
Mta1	1.356822618	0.057344343	0.440232125	0.231618599
Cbx5	1.356732748	0.227988802	0.440136564	0.365090157
Mtmr9	1.355637037	0.174085878	0.438970958	0.322964734
Zbtb18	1.355609166	0.089834436	0.438941297	0.258768271
Ankrd28	1.354983809	0.067048843	0.438275612	0.239318783
Slit1	1.354559828	0.174856193	0.437824115	0.322697314
Mterf3	1.353487517	0.182665565	0.436681582	0.327693259
Exoc7	1.353210207	0.084866042	0.436385964	0.253252742
Orai3	1.353175623	0.148850331	0.436349093	0.302263654
Snf8	1.35306077	0.199330831	0.436226637	0.339883211
Ercc6	1.353046473	0.072944596	0.436211393	0.243261734
Luzp1	1.352945533	0.099910814	0.43610376	0.26506256
Gcc2	1.352751615	0.063250388	0.435896963	0.234677808
Inpp5a	1.352577946	0.212154755	0.435711736	0.349222493
Emc8	1.351705249	0.151270888	0.434780594	0.302970982
Fam65a	1.351439653	0.194290286	0.434497092	0.334745737
Letm1	1.350247451	0.225211458	0.433223824	0.357213594
Spire1	1.350074328	0.187289146	0.433038837	0.328397815
Anp32e	1.349756295	0.247977157	0.432698945	0.374541412
Sqle	1.34963845	0.18024843	0.432572981	0.322817943
Ghitm	1.347523157	0.115184497	0.430310066	0.273158667
Mus81	1.347207655	0.197400726	0.429972241	0.333572361
Sulf2	1.346339126	0.150351702	0.429041852	0.298299899
Trpm2	1.346201927	0.12438672	0.428894827	0.279115982
Zdhhc6	1.34573461	0.14838329	0.428393927	0.296411898
Samd4	1.34527974	0.17539	0.4279062	0.315777775
Golt1b	1.344483487	0.269042652	0.427052036	0.386378052
Gsk3a	1.344424519	0.195282617	0.426988759	0.329693289

U2af1	1.343917522	0.143361223	0.426444601	0.291409101
Herc6	1.343770702	0.114679237	0.426286981	0.270228507
Med25	1.342883081	0.16424861	0.425333701	0.305792292
Ddx50	1.342382706	0.134235166	0.424796035	0.283649712
Tes	1.341565693	0.1698447	0.423917702	0.308819683
Zdhhc1	1.341070585	0.179802151	0.423385173	0.315637685
Fgd6	1.340799984	0.05509634	0.423094037	0.220577952
Top2b	1.340632463	0.029051653	0.422913774	0.193749632
Slc35b3	1.34039574	0.244205545	0.422659006	0.36294097
Dld	1.340379588	0.155605568	0.422641621	0.297633423
Rnf146	1.340308703	0.237510668	0.422565324	0.357732782
Gnas	1.340293078	0.275972059	0.422548505	0.387868267
Slc30a1	1.339219203	0.250402502	0.42139212	0.36662805
Surf1	1.337762318	0.215333373	0.419821813	0.338830282
Smox	1.336872273	0.197296405	0.418861635	0.324877111
Trim37	1.33676415	0.183299985	0.418744948	0.314688999
Zfp219	1.33615965	0.080218973	0.418092397	0.238989566
Rps26	1.335289301	0.162716187	0.417152347	0.298820935
Decr2	1.334587472	0.258438922	0.416393867	0.36846128
Gpatch1	1.33434824	0.21461069	0.416135232	0.335326122
ENSMUSG00000102976	1.333866407	0.246125469	0.415614181	0.358346249
Atp10d	1.333481066	0.197089733	0.415197341	0.321886484
Dnm1l	1.333294773	0.082878086	0.414995776	0.239297476
Pitpnc1	1.332878365	0.033019353	0.41454513	0.194453427
Tmem176b	1.332359659	0.130535549	0.413983578	0.273799863
Cgnl1	1.331504038	0.085970247	0.413056804	0.240562057
Rdh13	1.330891858	0.236262303	0.412393349	0.348193167
Impdh1	1.330405323	0.193541441	0.411865846	0.316777554
Mettl17	1.328544391	0.200895026	0.409846433	0.320441428
Hsp90aa1	1.326973543	0.123955738	0.408139607	0.265304812
Wdr70	1.32616589	0.22866829	0.407261253	0.338314071
Bri3	1.326032447	0.102905302	0.407116077	0.249622195
Ranbp1	1.325111935	0.239365116	0.406114232	0.345166416
Aifm2	1.325056586	0.195785689	0.406053971	0.313881943
Zfp729a	1.323943309	0.212832836	0.404841347	0.324961413
Ssrp1	1.323387792	0.155203811	0.404235876	0.284395041
Irf1	1.322946978	0.054266679	0.403755242	0.209775483
Rnase4	1.322403873	0.157769853	0.403162855	0.28540241
St3gal3	1.321439266	0.290494511	0.402110119	0.380412379
Brms1	1.32066939	0.226746992	0.401269355	0.331961996
Pip4k2c	1.319798226	0.203606323	0.400317384	0.314877333
Tut1	1.319418327	0.146388065	0.399902049	0.275337179
Cstf2t	1.318963655	0.142687815	0.399404811	0.272472266
Rala	1.318751384	0.143270859	0.399172608	0.272711309
Zfp629	1.318097651	0.119939785	0.398457256	0.25623826

Atmin	1.318097103	0.263237029	0.398456657	0.356155079
Ttf1	1.317667317	0.124691861	0.397986166	0.259211133
Cdc27	1.317651192	0.194963727	0.397968511	0.307066382
Srp72	1.317347138	0.064588243	0.397635565	0.215159411
Sms	1.316949795	0.253154109	0.397200348	0.347591464
Plekhhg3	1.316565387	0.089319857	0.396779175	0.23353738
Esco1	1.316355054	0.169847439	0.396548673	0.288883481
Mpnd	1.316026284	0.197932368	0.396188303	0.307727509
Ppp1r13b	1.314254215	0.146433636	0.394244362	0.271472445
Larp4b	1.314037024	0.014914038	0.394005925	0.161845322
Npm1	1.313081601	0.152870091	0.392956575	0.274897294
Zfp664	1.312917131	0.271844372	0.392775859	0.35745076
Pcmt1	1.312535433	0.175486185	0.39235637	0.289607929
Ssbp4	1.311126848	0.103249242	0.390807269	0.239861872
Hif1a	1.310877289	0.062278615	0.390532642	0.209478727
Ino80e	1.310480113	0.172850339	0.39009546	0.286182131
Tars2	1.309940999	0.106663208	0.389501833	0.241420622
Tra2a	1.30947698	0.026328224	0.388990698	0.175115663
Ube4b	1.309427175	0.077581734	0.388935825	0.220373589
Dcun1d3	1.308204812	0.269671603	0.387588426	0.351133999
Xpo6	1.30807545	0.005317896	0.387445759	0.139012997
Mettl16	1.307704298	0.259792092	0.387036352	0.343457542
Jtb	1.307507807	0.282946028	0.38681956	0.36025949
Prdm15	1.307408848	0.222728492	0.386710366	0.317156413
Usp38	1.306890556	0.240375022	0.386138329	0.328894296
Cdca4	1.306002962	0.297319821	0.385158169	0.369563283
Set	1.305646008	0.19113494	0.3847638	0.294335416
Hibadh	1.305308749	0.22809866	0.384391093	0.318925004
Fam192a	1.30497096	0.250447907	0.384017702	0.33414283
Zscan29	1.304885325	0.214789705	0.383923026	0.309490003
Vps35	1.304738677	0.122288606	0.383760882	0.248350596
1600014C10Rik	1.304473466	0.276498004	0.383467599	0.352380588
Plxdc1	1.304400805	0.110161686	0.383387237	0.239996994
Lima1	1.304191372	0.044421259	0.383155581	0.190615769
Tmcc1	1.303865661	0.091935749	0.382795235	0.227141265
C330007P06Rik	1.303691828	0.218461537	0.38260288	0.310900184
Rexo4	1.302986975	0.139246449	0.381822663	0.258232035
Rpl37	1.302938224	0.205140914	0.381768683	0.301307473
Wapl	1.302655465	0.023617503	0.38145556	0.168539912
Fbxo21	1.302563743	0.154493115	0.381353975	0.26783506
Inpp5b	1.30157209	0.203302944	0.38025522	0.298896367
Mamdc2	1.30155207	0.243139413	0.38023303	0.325771881
Sec62	1.301336105	0.245096182	0.379993625	0.326920399
2410089E03Rik	1.301226892	0.032251232	0.379872543	0.177404184
Rnf7	1.300974028	0.286664007	0.379592162	0.356267865

Mfn1	1.300794558	0.204835008	0.379393127	0.299230423
Hnrnpc	1.300402793	0.135645773	0.37895856	0.253959014
Ncbp1	1.300273053	0.17136938	0.378814616	0.276948327
Dcaf10	1.300185022	0.28440645	0.37871694	0.353784669
Cdyl2	1.299846969	0.259565596	0.378341784	0.335582391
Ddrgk1	1.299392614	0.258577795	0.37783741	0.334440721
Eif1b	1.29926914	0.214623872	0.377700312	0.304363613
Iscu	1.29920573	0.283869002	0.377629901	0.352375701
Crip1	1.298185671	0.291806229	0.376496737	0.357150767
Ppp2r5e	1.297900843	0.191509583	0.376180169	0.288012075
Mvb12b	1.297670015	0.22357156	0.375923566	0.30887186
Fto	1.297374125	0.11756885	0.375594571	0.239987335
Eif4e	1.297188304	0.232510909	0.375387922	0.314417419
Anp32b	1.2963711	0.258165568	0.374478764	0.331180972
Zfp871	1.295124443	0.238637792	0.373090727	0.316608295
Eif3a	1.294458367	0.042788546	0.372348565	0.183806108
Zfp622	1.293889851	0.170650228	0.371714806	0.271301409
ENSMUSG00000086507	1.293709772	0.25606733	0.371514003	0.327113173
Got2	1.293339835	0.170130862	0.371101405	0.270524772
Eif2ak3	1.293139105	0.205054689	0.370877476	0.292655977
Rnf157	1.292618121	0.255137297	0.370296122	0.325403537
S100a1	1.291203399	0.220381301	0.368716282	0.300866435
Mier2	1.291143524	0.16338444	0.36864938	0.26449635
Hnrnpd	1.291059135	0.11907291	0.368555082	0.236453829
Nop56	1.289101647	0.182571823	0.366366027	0.274868375
Xrn2	1.289045886	0.073144945	0.366303621	0.204419033
Map2k7	1.289015331	0.262593329	0.366269422	0.326943757
Cox5b	1.288730349	0.269598752	0.365950429	0.331480724
Ythdf3	1.288588805	0.078154984	0.365791967	0.207660179
Cnppd1	1.286977111	0.042266729	0.363986396	0.179225567
Lrrc8c	1.286754507	0.238804509	0.363736836	0.308780099
Emsy	1.286133673	0.085557197	0.363040595	0.21115432
Ppm1g	1.285691094	0.200415163	0.362544056	0.283155772
Lmbrd2	1.28373504	0.209663324	0.360347465	0.287245784
Ptgs2	1.283572445	0.639934322	0.360164725	0.769927187
Rfwd3	1.283569986	0.193691699	0.360161961	0.27710408
Alyref	1.282167319	0.290494731	0.358584542	0.339235584
Mov10	1.281878794	0.101608397	0.358259857	0.218837347
Srek1	1.280368618	0.071387015	0.356559222	0.197758021
Dag1	1.279999891	0.061495296	0.356143688	0.190459587
Eno2	1.279943709	0.228730947	0.356080363	0.295837661
Prdx4	1.279827396	0.261391211	0.355949254	0.316932069
Cad	1.279034411	0.263545665	0.355055079	0.317566386
Cnot4	1.278867495	0.077859087	0.354866793	0.20125773
Irak3	1.278423781	0.027919607	0.35436615	0.161191717

Il2rg	1.278134196	0.274501548	0.354039318	0.323987938
Cep95	1.277741606	0.203076279	0.353596113	0.277801807
Foxk2	1.277182966	0.183516541	0.352965217	0.265386333
Abcf2	1.277098273	0.089457958	0.352869545	0.207782511
Pibf1	1.276933546	0.104786142	0.352683446	0.217426617
Epc1	1.276174403	0.145501908	0.351825502	0.241704004
ENSMUSG00000108365	1.275571225	0.1414975	0.351143458	0.238834548
Epm2aip1	1.275426204	0.20656435	0.350979427	0.277878248
Hlx	1.275379786	0.232737227	0.35092692	0.294071814
Pias4	1.275272209	0.183191118	0.350805226	0.263566427
Wbp4	1.275050371	0.218412451	0.350554242	0.28482734
Fam160b1	1.274312678	0.104348723	0.349719315	0.215327577
Trim14	1.274098996	0.143571216	0.349477378	0.238939183
Dda1	1.273851997	0.288560929	0.349197667	0.329033247
Polr3c	1.273183069	0.108752572	0.348439877	0.21725551
Sepn1	1.272171542	0.208111474	0.34729322	0.275897689
Txn1	1.272100809	0.259847737	0.347213004	0.308154133
Naa60	1.271945366	0.232439645	0.347036703	0.290626658
Rad9a	1.271411595	0.28802819	0.346431151	0.326065991
Ppp5c	1.271370931	0.197573214	0.346385007	0.268828847
Pogk	1.271329368	0.274928775	0.346337843	0.317222421
Nsa2	1.271044498	0.279388146	0.346014538	0.319881842
Trappc13	1.270937479	0.145276772	0.345893062	0.237495539
Rnf19b	1.270920264	0.105327351	0.34587352	0.213560498
Sort1	1.26919547	0.246961136	0.343914278	0.297050905
Antxr2	1.268567344	0.059007169	0.34320011	0.181766248
Otud4	1.267075478	0.066792072	0.341502466	0.18630077
Pde4a	1.266952843	0.171376337	0.341362828	0.249571679
Tpra1	1.26675317	0.085954369	0.341135439	0.198665386
Bbs9	1.266025166	0.217666312	0.340306083	0.276052774
Armc5	1.263776552	0.228299568	0.337741403	0.280341328
Baz1a	1.261969456	0.204531233	0.335676993	0.264573578
Hnrnpu	1.261111575	0.244772864	0.334695921	0.287752148
Dedd2	1.260721305	0.277252055	0.334249389	0.307634941
Hnrnpnr	1.260719488	0.144576571	0.33424731	0.229099967
Gpkow	1.260716501	0.264952557	0.334243892	0.29983439
Hs6st1	1.260701082	0.223732073	0.334226247	0.274707101
Plcg1	1.26050312	0.254586998	0.33399969	0.293167719
Ptges3	1.260352007	0.276910557	0.333826724	0.307027599
Rfc2	1.260015794	0.221759039	0.333441818	0.272896003
Bin3	1.258880131	0.166508426	0.332140918	0.240071142
Pfdn5	1.258283148	0.250300618	0.331456604	0.288318527
Nck2	1.257879249	0.22242739	0.330993436	0.271284106
Pelp1	1.257787654	0.262399	0.330888379	0.295241261
Narfl	1.257546337	0.257774361	0.33061156	0.292145561

0610010F05Rik	1.257459392	0.233557353	0.330511811	0.277450704
Pigs	1.256714405	0.113932785	0.329656827	0.20854317
Ppp1r12b	1.256406997	0.159786518	0.329303883	0.23424829
Preb	1.256019071	0.096681598	0.32885837	0.197969279
Nemf	1.255899305	0.056895968	0.328720797	0.172637579
Txndc9	1.255569009	0.173439708	0.328341325	0.241208558
Elp2	1.255568699	0.209861254	0.328340968	0.26184596
Aldh3b1	1.255101527	0.129981612	0.327804071	0.2164903
Psmf1	1.254776521	0.291947612	0.327430439	0.310696717
Calhm2	1.254650962	0.223408508	0.327286069	0.268814916
Dhx33	1.254576674	0.230140755	0.327200645	0.272668032
Ddx10	1.254427736	0.273049767	0.327029363	0.29836641
Map2k2	1.254410047	0.082700376	0.32700902	0.188453024
Ubtg	1.253970897	0.07384012	0.326503865	0.182649005
Zfp180	1.253689816	0.272633452	0.326180445	0.297333678
Otub1	1.253386771	0.208096529	0.325831671	0.258839637
Wars	1.252905804	0.21024521	0.325277953	0.259621814
Hspbp1	1.252350873	0.238888369	0.324638821	0.275638589
Usp15	1.251136592	0.107967993	0.323239303	0.201095269
Ccdc88a	1.250899138	0.101833751	0.322965468	0.19740829
Arid3b	1.250573552	0.259244894	0.322589912	0.285938927
Dhx8	1.248654272	0.136737914	0.320374078	0.215298038
Cog5	1.248593595	0.251155909	0.320303971	0.279120739
Ecd	1.247877402	0.260251102	0.319476203	0.283777597
Mtmr4	1.247582498	0.168167134	0.319135219	0.231573367
St13	1.247469412	0.152129061	0.319004441	0.222760441
Cotl1	1.247256915	0.087422045	0.318758668	0.186500621
Slc11a2	1.247137742	0.223025441	0.318620814	0.261481251
Ppp2r5d	1.247022663	0.249094829	0.318487684	0.276332895
Lrch3	1.24626593	0.127465188	0.317611946	0.208382962
Usp33	1.245853671	0.126563314	0.31713463	0.20757662
Mrpl24	1.244646976	0.239444103	0.315736603	0.268397375
Rangap1	1.244605137	0.27261855	0.315688106	0.287760318
ENSMUSG00000106636	1.24448237	0.298293203	0.315545793	0.303380094
Ehmt1	1.244328285	0.069918004	0.315367154	0.174001286
Map2k5	1.244006907	0.131281801	0.314994496	0.208734675
Sec24d	1.243602304	0.282260207	0.314525194	0.292511964
Atg16l2	1.243182087	0.249999837	0.314037621	0.27299316
Atg4b	1.241849531	0.223112802	0.31249038	0.256498628
ENSMUSG00000092558	1.241816751	0.242944623	0.312452298	0.267588812
Wdr24	1.241357287	0.2351313	0.311918411	0.262724723
Tnf	1.24126814	0.26437375	0.311814802	0.279375646
Cdipt	1.240955823	0.12278067	0.311451757	0.201821175
Limk1	1.240760278	0.271898297	0.311224406	0.283265665
ENSMUSG00000108394	1.240386496	0.298690298	0.310789724	0.299053059

Gnl1	1.238810756	0.169246699	0.308955814	0.224755889
Phf23	1.238779778	0.250655113	0.308919737	0.268915908
Tbc1d1	1.237394814	0.099023658	0.307305893	0.186290541
Smad5	1.237393186	0.291718525	0.307303994	0.291460456
Mgat2	1.237377236	0.19448035	0.307285398	0.236840048
Tor1a	1.236747389	0.258132016	0.306550853	0.271087881
Unk	1.236599081	0.129513572	0.306377839	0.202093152
Kcnj2	1.236159199	0.186750615	0.305864553	0.231671195
Clasrp	1.234466233	0.282557561	0.303887375	0.282793334
Abi1	1.233880152	0.060215108	0.30320227	0.161345152
Mapk1	1.23333041	0.170813186	0.30255935	0.220911456
Skiv2l	1.23320303	0.17417648	0.302410339	0.22253961
Prr14	1.232410037	0.209421653	0.301482337	0.240194859
Senp5	1.231636868	0.213793119	0.300576959	0.241776292
Dek	1.231346192	0.245064222	0.300236432	0.2582853
Tle3	1.231215647	0.105274998	0.300083472	0.18525945
Mapk3	1.230572793	0.075903873	0.299330001	0.168640064
Uspl1	1.230392534	0.283699813	0.299118654	0.279016878
Lrba	1.230149381	0.270258961	0.298833517	0.271059072
Fbxw2	1.229625549	0.227551181	0.298219046	0.24713788
Shisa5	1.229418843	0.086747576	0.297976502	0.173969426
Galnt6	1.229302679	0.129601158	0.297840179	0.196506423
Mtpn	1.229017116	0.159144708	0.297505008	0.211303082
Prkag2	1.228257297	0.221570379	0.29661281	0.242655256
Eif4a1	1.227440731	0.120663753	0.295653364	0.190499086
Pnrc2	1.227434335	0.218517681	0.295645846	0.240268853
Scarf1	1.225296854	0.248104985	0.293131315	0.253801159
Idh3g	1.225286053	0.26235684	0.293118598	0.261517371
Cope	1.225071808	0.178248835	0.292866315	0.217555794
Ccz1	1.22397994	0.273582352	0.291579914	0.266319506
Rnf123	1.223544139	0.191399272	0.291066148	0.222791497
Hnrnpa1	1.223373079	0.247512152	0.290864435	0.251522911
Mast4	1.223173664	0.204841857	0.290629249	0.229225103
Zbtb1	1.222921417	0.24835719	0.290331702	0.25151124
Pnp	1.222405509	0.207492137	0.28972295	0.229849203
Parg	1.221368204	0.138844529	0.288498193	0.194917038
Nfkbia	1.221146118	0.183152183	0.288235838	0.216537641
Pcf11	1.220999788	0.192514746	0.288062949	0.221046882
Dram1	1.220821773	0.141000802	0.287852598	0.19554216
Mcm3ap	1.220786088	0.171397375	0.287810427	0.210429649
Sirt7	1.220392076	0.141400344	0.287344717	0.195393346
Card9	1.220127311	0.230528895	0.287031689	0.239393056
Nbas	1.21956688	0.145042149	0.286368877	0.196510653
Trps1	1.219326431	0.109090236	0.286084407	0.178546574
Kif1b	1.21897229	0.102767381	0.28566533	0.175084777

Rer1	1.218631691	0.215125811	0.285262164	0.230125585
Gnpat	1.217771696	0.290136584	0.284243686	0.268706527
Pepd	1.217544089	0.279428523	0.283974016	0.262548968
Tnfrsf11a	1.217516724	0.163677059	0.28394159	0.203862306
Gabpb2	1.217058824	0.251992333	0.2833989	0.247396827
Zhx1	1.216796331	0.263193985	0.283087708	0.253011296
Supt16	1.216587783	0.268569155	0.282840422	0.255648695
Phc2	1.215367968	0.22037824	0.281393174	0.229610689
Atxn7l3	1.214897722	0.106523582	0.280834863	0.173997385
Rabgap1	1.214789274	0.281776169	0.280706076	0.260797356
Cnbp	1.214345857	0.282532072	0.280179373	0.260717193
Slc39a3	1.213383882		0.279036052	0.340169518
Hk3	1.212287823	0.10641031	0.277732266	0.172019421
Sik2	1.212276831	0.226458445	0.277719184	0.229608755
Senp3	1.212118578	0.233430532	0.277530841	0.232912225
Derl2	1.212112121	0.284563132	0.277523155	0.259337184
Srpkl	1.21211002	0.158785288	0.277520654	0.196939268
Zc3h4	1.210060613	0.122436347	0.275079315	0.17808778
Snx30	1.209966089	0.07504464	0.274966614	0.154459016
ENSMUSG00000091562	1.209630161	0.264636081	0.274566017	0.246137103
Pkp4	1.209123203	0.290982419	0.273961255	0.259440644
Fer	1.20879627	0.232085257	0.273571114	0.228928933
Mfsd12	1.207586234	0.092853553	0.272126216	0.161928464
Hivep3	1.206620362	0.247173759	0.270971834	0.234153254
Bms1	1.206470111	0.239383679	0.270792174	0.230161984
Agtpbp1	1.205997325	0.290845265	0.270226707	0.255831297
Axin1	1.205658391	0.231302458	0.269821195	0.225412553
Wbp1l	1.205274452	0.124919482	0.269361699	0.175543063
Lrrc8d	1.205120542	0.226261542	0.269177459	0.222452397
9930021J03Rik	1.205026901	0.27071411	0.269065353	0.244289615
Pex5	1.204931173	0.249548555	0.268950741	0.233576392
Sdha	1.204543111	0.246000047	0.268486028	0.231429578
Rps2	1.202300483	0.269236937	0.265797504	0.240579588
Pmm2	1.202064152	0.29133077	0.265513892	0.251622621
Psmd1	1.201529025	0.203212043	0.264871499	0.208158101
Sesn1	1.200976935	0.08855009	0.264208445	0.155134198
Wipi2	1.200749197	0.198522369	0.263934844	0.205273315

Table 7.1 List of RNAs binding to GAPDH in untreated macrophages. Data shown includes the genes with a higher than 1.2 fold increase in sequencing reads in the immunoprecipitated sample compared to the IgG control.

Differentially expressed genes - LPS_IP - LPS_IgG				
Gene_Symbol	Fold_Change	pvalue	Log2_FC	lfcSE
ENSMUSG00000083899	4.97904578	0.003279599	2.31586928	0.787646797
Zfp764	4.791880186	9.15E-05	2.260591836	0.577856224
Gm13216	4.689796326	0.003678552	2.229525269	0.767614081
ENSMUSG00000089960	4.655702794	0.00438208	2.218998964	0.77879784
Gm15590	4.617264167	0.003025118	2.207038275	0.744319987
ENSMUSG00000097472	4.324317597	0.004714212	2.112472485	0.747529179
Rny1	3.984025809	0.000673794	1.994226994	0.586532806
Brsk2	3.801128727	0.001468704	1.926427884	0.605643088
Mag	3.777030382	0.004359496	1.917252389	0.67250653
Zfp874b	3.654182176	0.001668059	1.86954856	0.594690077
Rny3	3.403077958	0.001904685	1.766840201	0.56908461
Trim47	2.703238318	0.002441834	1.434688706	0.473423115
Ankmy2	2.692251914	0.002373005	1.428813409	0.470146423
ENSMUSG00000092746	2.597483512	0.003866999	1.377114592	0.476706157
Gm14461	2.584338788	0.03372539	1.369795209	0.645113528
Armc10	2.570719721	0.003956698	1.362172325	0.472714943
ENSMUSG00000092702	2.340280806	0.009860406	1.226681647	0.475331512
mt-Co2	2.331378352	0.010152746	1.221183154	0.475060752
Ntan1	2.233371381	0.012954895	1.159223173	0.466486915
Fam3c	2.20179835	0.005373762	1.138682347	0.40904902
Prune2	2.198609519	0.012109941	1.136591399	0.453019395
Adcy3	2.188898514	0.0618555	1.130205067	0.605251877
Ephx1	2.156094006	0.08823609	1.108420081	0.650184931
Eef2k	2.152935917	0.03229014	1.106305378	0.516771855
Slc9a7	2.152330407	0.014790628	1.105899565	0.453708821
ENSMUSG00000099021	2.149343885	0.02488187	1.103896325	0.492100871
ENSMUSG00000092805	2.127553272	0.018162231	1.089195256	0.461075229
Wdr47	2.03930973	0.024641714	1.028080908	0.45754027
Prepl	2.033693453	0.032592541	1.024102232	0.479209225
Hist1h1d	2.010369515	0.061802467	1.0074607	0.539409521
Fth1	1.987404539	0.001272825	0.990885565	0.307533839
Slit1	1.958977411	0.057038645	0.970100762	0.509770541
ENSMUSG00000099250	1.941270861	0.043639522	0.957001428	0.474341492
Slc25a13	1.93814963	0.090840742	0.954679955	0.564572206
Ctnnbl1	1.932613968	0.009384034	0.950553494	0.365915986
Akap7	1.918533284	0.032113232	0.940003794	0.438639535
Snx32	1.900062091	0.010503441	0.926046564	0.361906705
Rab33b	1.899254307	0.054123099	0.925433093	0.480532266
Btbd2	1.871109478	0.027614647	0.903893973	0.410353966
Hinfp	1.870896343	0.012397694	0.903729628	0.361402559
Homer3	1.862936365	0.010820243	0.897578395	0.352205448
Hras	1.822512521	0.039049122	0.865928726	0.419606429

Snapc1	1.817607969	0.04121361	0.862041065	0.422284611
ENSMUSG00000095280	1.778315041	0.148432155	0.83051093	0.574711613
Fam174a	1.77766808	0.013136441	0.829985974	0.334665182
Acd	1.775611188	0.061155553	0.828315704	0.442389105
ENSMUSG00000089647	1.767723643	0.070366532	0.821892749	0.454198821
Eif2b5	1.757161425	0.022281512	0.813246733	0.355823449
Kifc2	1.754804724	0.085548331	0.811310495	0.471866958
Cyc1	1.748423883	0.012945553	0.80605499	0.324333799
Armt1	1.738363228	0.075004028	0.797729562	0.448051994
Zfp62	1.733780026	0.016983051	0.793920868	0.33259158
Ighmbp2	1.726795117	0.023967386	0.788096918	0.349078175
Slc35c1	1.726505648	0.05355467	0.787855054	0.408125984
Zfp777	1.725099181	0.095828677	0.786679309	0.472360463
Arhgap6	1.723901116	0.121586686	0.785677023	0.507494801
Pebp1	1.723895943	0.084201762	0.785672694	0.454984982
Rpl36	1.722481887	0.011986471	0.784488812	0.312229089
Cenpa	1.716805214	0.15540717	0.779726363	0.548836742
Tacc3	1.714103185	0.152325774	0.777453959	0.543155816
Arl6ip4	1.708630171	0.08560638	0.772840163	0.449575585
Myeov2	1.708212351	0.023596637	0.77248733	0.341259825
0610009O20Rik	1.707836362	0.109871063	0.772169749	0.482976492
Nxpe5	1.700956396	0.049524814	0.766346158	0.390187576
BC003965	1.698321544	0.071220268	0.764109631	0.423547563
Lin54	1.694877496	0.056652205	0.761181001	0.39936367
Klhl21	1.689014805	0.082710617	0.756181974	0.435796938
Cd300a	1.681468595	0.127905262	0.749721834	0.492455799
Leo1	1.676394844	0.064788606	0.745361989	0.403615287
Aifm1	1.666134472	0.087355401	0.736504844	0.430826516
Rrs1	1.661414231	0.111793827	0.732411817	0.460583694
Tmem18	1.660461167	0.066017028	0.731583983	0.397965939
Zfp367	1.655694989	0.079282461	0.727436925	0.414525942
Anxa9	1.654005147	0.124143189	0.725963724	0.47213673
Cdc123	1.65327635	0.067504852	0.725327896	0.396723033
Ankrd13d	1.648655934	0.02618867	0.721290347	0.324408352
Gopc	1.644609898	0.072138257	0.717745417	0.399136704
ENSMUSG00000098055	1.644308861	0.092948912	0.717481315	0.427061232
Gm10800	1.64123906	0.236545076	0.714785395	0.603873938
AU022252	1.640842841	0.058339107	0.714437065	0.377381836
Txndc17	1.639136635	0.032031843	0.712936119	0.332524155
Lig1	1.637842192	0.117034192	0.711796359	0.454141616
Ndufb8	1.635517175	0.133054613	0.709746909	0.472482792
Irs2	1.634693755	0.193907857	0.709020385	0.545775993
Gm10801	1.63177968	0.261380808	0.706446281	0.628995834
Bcat2	1.628506665	0.102541187	0.703549625	0.430923145
Ftl1	1.627768865	0.009412315	0.702895859	0.27068777

Snord118	1.623770153	0.213715001	0.699347431	0.562440951
ENSMUSG00000090031	1.623636285	0.063680079	0.699228487	0.377061875
Ppid	1.623510676	0.018690551	0.699116871	0.297288308
ENSMUSG00000097429	1.615288904	0.054193006	0.691792222	0.35931833
Suv420h2	1.612287956	0.043530114	0.689109434	0.341382002
Clec7a	1.612069416	0.162631627	0.688913868	0.49339403
Mrgbp	1.611390993	0.078390081	0.688306597	0.391060063
Gm12164	1.611121069	0.100215091	0.68806491	0.418578895
Al413582	1.611080651	0.155557458	0.688028717	0.484468401
Sprtn	1.606420684	0.124952622	0.683849751	0.445704146
Adamtsl4	1.605109647	0.134233401	0.682671853	0.455839463
Prmt2	1.604991388	0.072331009	0.682565556	0.379830008
Fancg	1.598355046	0.129436111	0.676587912	0.446201179
Adgrl2	1.597109172	0.004627843	0.675462933	0.238522902
ENSMUSG00000099375	1.594333829	0.062835649	0.672953739	0.361734502
1810043G02Rik	1.591103389	0.081183805	0.670027584	0.384221351
Lamtor1	1.591068059	0.007837676	0.669995549	0.2519743
Rpl11	1.58305035	0.049734847	0.662707142	0.337730352
Jmjd4	1.580609488	0.126412436	0.660480973	0.432137888
Rps26	1.577401925	0.038547825	0.657550308	0.317813531
Mre11a	1.573165053	0.060855277	0.653670043	0.34870855
Tmem242	1.570761811	0.214859264	0.651464428	0.52524152
Sigmar1	1.570002357	0.117554611	0.650766725	0.415793316
Mroh2a	1.567406939	0.087426235	0.648379789	0.379361714
Cadm1	1.566633264	0.030612302	0.647667496	0.299558125
Idh2	1.565557771	0.091450981	0.646676747	0.383149335
Rps21	1.561992295	0.146112347	0.643387337	0.442676986
Pdcl	1.559666382	0.059378607	0.641237465	0.340110536
Idnk	1.558056413	0.067365658	0.639747471	0.349736575
Tceb2	1.555118312	0.087726387	0.637024344	0.373071027
Ndufv2	1.554999154	0.09091244	0.636913796	0.3767374
Prpsap1	1.551535618	0.042682651	0.633696817	0.31265845
Msantd2	1.550286939	0.087764321	0.632535265	0.370486326
Aip	1.550205443	0.105079336	0.632459424	0.390235448
Psma4	1.547891562	0.207536241	0.630304407	0.500095094
Mcm3	1.547664539	0.144082387	0.630092797	0.431347024
Pdp1	1.546648192	0.123340221	0.629145071	0.408296161
Phf5a	1.545207796	0.109613242	0.627800861	0.392391743
Igfbp4	1.54441764	0.038277644	0.627062937	0.302655664
Zdhc7	1.543829191	0.03326564	0.626513142	0.294294794
Ap1m1	1.538517426	0.012015662	0.621540784	0.247459784
Atf5	1.538335506	0.142756566	0.621370185	0.423969062
Bcar3	1.536897661	0.085041998	0.620021102	0.360026945
ENSMUSG00000087881	1.535796949	0.121174144	0.618987487	0.39938179
Rpl23a	1.53343319	0.046338584	0.616765311	0.309575131

Cuedc2	1.530193673	0.013947287	0.613714263	0.249617559
Rxrb	1.528989489	0.042886351	0.612578489	0.302535515
Gngt2	1.528944312	0.040132052	0.612535861	0.298450446
Ypel2	1.526900079	0.127677467	0.610605655	0.400837469
Rps12	1.526579226	0.042030519	0.610302464	0.300165802
Tfdp1	1.520173488	0.089497295	0.604235979	0.355839956
Tpt1	1.519284902	0.022871664	0.603392434	0.265160823
Tada3	1.517129463	0.152578382	0.601344202	0.420378372
Rell1	1.516878741	0.074688903	0.601105761	0.337250816
Sh2b2	1.515386873	0.141513144	0.599686156	0.407899956
Usp39	1.515226844	0.09061161	0.599533795	0.35429665
Usp21	1.514907396	0.045783327	0.599229607	0.300006995
Prkci	1.513123012	0.089706363	0.597529279	0.352119828
Fam53b	1.512817984	0.005797735	0.597238419	0.216468638
Cep95	1.511425625	0.07648767	0.595909988	0.336398753
ENSMUSG00000105071	1.511338115	0.19901071	0.595826455	0.463903741
Rps16	1.510056112	0.140812492	0.59460216	0.403729913
Hagh	1.50736308	0.183295129	0.592026963	0.444906399
3110043O21Rik	1.50412661	0.066177278	0.588926012	0.320552624
Tsg101	1.500792667	0.071602471	0.585724684	0.325106861
Rb1cc1	1.500514894	0.040737596	0.585457639	0.286120738
Prdm15	1.500422309	0.076971512	0.585368619	0.330990696
Srpk2	1.498806871	0.041143257	0.583814496	0.2858916
Zfp738	1.496219084	0.167084378	0.581321438	0.420749494
Asb8	1.495977516	0.096189773	0.581088492	0.349293049
Zfp110	1.494367437	0.1048589	0.579534924	0.357353649
Atg5	1.491224063	0.065586368	0.576497045	0.313102575
Rpl36a	1.490491539	0.076634433	0.575788186	0.325201761
Pum3	1.490419313	0.164281633	0.575718274	0.413943199
Dynll2	1.490028169	0.108890139	0.575339605	0.358869142
Otud6b	1.489039061	0.097321587	0.5743816	0.346434664
Ppard	1.485697178	0.156576901	0.571140089	0.403154299
Tmem243	1.485099591	0.091887154	0.570559682	0.338505439
Rnf166	1.484863266	0.080171797	0.570330086	0.325960623
Acadsb	1.48482089	0.082491907	0.570288913	0.328430451
Narfl	1.482943192	0.073581163	0.568463333	0.317717681
Tef	1.478277864	0.017270079	0.563917471	0.236849868
Rps28	1.476655204	0.049052812	0.562332999	0.285719098
Lrif1	1.476383084	0.155695053	0.562067113	0.395905567
Rplp0	1.475741554	0.027747245	0.561440085	0.255102459
H2-M3	1.475523653	0.106992537	0.561227048	0.348186104
Ercc2	1.473614997	0.034970598	0.559359649	0.265262957
Trap1	1.47309054	0.043939337	0.558846104	0.277388539
Srprb	1.473070241	0.244749724	0.558826224	0.480422827
Rpl29	1.473036794	0.130025113	0.558793467	0.369083494

Rcan3	1.472013169	0.072840336	0.557790579	0.310949552
Asah2	1.471607631	0.225089201	0.557393063	0.459475881
ENSMUSG00000091537	1.471403228	0.23459479	0.557192661	0.468778312
ENSMUSG00000090862	1.471331687	0.071152314	0.557122515	0.308739952
H2-T22	1.469799098	0.083965597	0.555618971	0.321515271
Ndfip1	1.469462524	0.078051711	0.555288566	0.315128129
Dnajc27	1.468162049	0.253634976	0.554011215	0.485308614
Nadsyn1	1.468064215	0.217155729	0.553915075	0.448831644
Sulf2	1.465719445	0.281357883	0.551608982	0.512041498
Cdc34	1.465484089	0.092136781	0.551377305	0.32737604
Zbed4	1.465478867	0.209719543	0.551372164	0.439572593
ENSMUSG00000102153	1.464326429	0.242020688	0.550237196	0.470307898
ENSMUSG00000103845	1.464293606	0.050516212	0.550204858	0.281353145
Tuba1b	1.463462901	0.075644964	0.549386174	0.309246102
Taf15	1.460752736	0.098129707	0.546711991	0.330541452
Gtpbp8	1.460690664	0.191063925	0.546650686	0.418108235
Rnf121	1.460490931	0.178389815	0.546453399	0.406064857
Rpl39	1.459317663	0.136664301	0.545293962	0.366380129
Zcchc17	1.459270214	0.220673807	0.545247052	0.445194712
Ddx18	1.459173872	0.12571078	0.545151802	0.35602048
Ccl6	1.457263573	0.094930934	0.543261839	0.325317882
Gmpr2	1.456854684	0.104192025	0.542856981	0.334094263
Itpril1	1.456336239	0.106220053	0.542343484	0.335729358
Vps4a	1.456239642	0.057288265	0.542247788	0.285227695
Entpd6	1.456191317	0.293073406	0.542199912	0.515691664
Rps17	1.455861416	0.124258354	0.541873032	0.352519653
Ip6k2	1.454602836	0.14834742	0.540625294	0.374033325
Nupr1	1.453799836	0.112618175	0.539828648	0.340256542
Polk	1.453362467	0.144327541	0.539394555	0.369482822
Phf1	1.452847492	0.093704251	0.538883268	0.321496152
Bsdcl1	1.452528963	0.035098133	0.538566931	0.255581196
Pnpla7	1.452249661	0.000909722	0.538289493	0.162279416
Mrc1	1.451797462	0.041334672	0.5378402	0.263626843
Rars2	1.451199661	0.221222151	0.537246024	0.439183115
Chek2	1.449928356	0.268091015	0.535981616	0.483969664
Slc7a7	1.449404197	0.133301257	0.535459977	0.356685595
Rpl35	1.449151797	0.051154358	0.535208724	0.274440312
Spsb2	1.448233985	0.136433251	0.534294712	0.358778353
Elac2	1.447673147	0.094282342	0.53373591	0.318985687
Dcaf15	1.44744205	0.255934259	0.533505589	0.469613169
Serinc5	1.447181431	0.119295249	0.533245802	0.342320486
Gas5	1.446922374	0.095929224	0.532987525	0.320128497
ENSMUSG00000107881	1.445173363	0.293698104	0.531242568	0.505924333
Cenpt	1.443041043	0.259168858	0.529112334	0.468922492
Zfp12	1.442502241	0.159054008	0.52857356	0.375337969

Atic	1.4411959	0.134839086	0.527266452	0.352618472
Casp1	1.440936539	0.035325194	0.527006799	0.250405813
Ccdc137	1.440888853	0.241363785	0.526959054	0.449783101
Ddx49	1.440730271	0.192289584	0.526800264	0.404038818
St3gal5	1.440407634	0.141876699	0.526477151	0.358431021
Pkig	1.44010998	0.225391797	0.526178994	0.434028254
Ddx47	1.437849878	0.076555041	0.523913056	0.295823255
Rpl32	1.437761738	0.072737463	0.523824616	0.291909755
Ube2q2	1.437601866	0.077999149	0.523664187	0.297128705
Ppcdc	1.437582245	0.227386001	0.523644495	0.433796616
ENSMUSG00000093674	1.437542851	0.022111815	0.523604962	0.228804157
Aars2	1.436403087	0.218437139	0.522460659	0.424525056
Acbd4	1.435082192	0.261642385	0.521133367	0.464254029
Arhgap24	1.43444078	0.200033174	0.520488409	0.406169216
Sms	1.431222984	0.202106172	0.51724846	0.405502531
1810058I24Rik	1.429809018	0.141201109	0.515822456	0.350581745
Tshz1	1.428960627	0.213139992	0.514966165	0.413634891
ENSMUSG00000098112	1.428130509	0.019183586	0.514127825	0.219530353
Bcl2l13	1.427672376	0.159801326	0.513664946	0.365405355
Fos	1.427309856	0.264609578	0.513298565	0.460125453
Rps29	1.427250732	0.122544297	0.513238802	0.332369416
Kctd20	1.426391802	0.04157396	0.512370317	0.251438033
Mbd3	1.426058497	0.139893785	0.512033162	0.346862067
Rpl35a	1.425177267	0.160315703	0.511141376	0.364058069
Rps24	1.424752109	0.0255639	0.510710929	0.228733896
Zfp24	1.423781755	0.272455276	0.509728019	0.464476043
Pdlim2	1.423177794	0.287301482	0.509115905	0.478465374
Pou2f1	1.420242788	0.123343003	0.506137577	0.328470446
Nlrc3	1.420103403	0.269494516	0.505995981	0.458235315
ENSMUSG00000082286	1.419553142	0.102870251	0.505436858	0.309875772
Rpl18a	1.418346549	0.096191852	0.504210074	0.303083227
Ndufa13	1.417754918	0.101125278	0.503608161	0.307186298
ENSMUSG00000104060	1.417686657	0.193209165	0.503538697	0.386996898
Dhx16	1.417206955	0.073010485	0.503050451	0.280600307
Gpr162	1.416788958	0.179973963	0.502624873	0.374859543
Spag7	1.415172223	0.184612265	0.500977637	0.377615565
Vezt	1.415025967	0.033911652	0.500828528	0.236115062
Bri3	1.413975816	0.083312133	0.499757445	0.288579647
Atad2	1.413912763	0.199426645	0.49969311	0.389415724
Ntpcr	1.412499637	0.218250411	0.498250496	0.404688945
Akap8l	1.41226444	0.068593879	0.498010252	0.273468919
D8Ertd738e	1.410701723	0.103800799	0.496412979	0.305165633
Zdhhc6	1.409871152	0.095373359	0.49556332	0.29715202
Pigv	1.409847148	0.155828308	0.495538758	0.349157196
Baiap2l1	1.409667528	0.280408652	0.495354941	0.458916174

Vps72	1.409611705	0.218132369	0.495297809	0.40218757
Anxa3	1.409585784	0.069134418	0.495271279	0.272495344
Gid4	1.409395444	0.247093495	0.495076455	0.427734786
Atp5g2	1.407771895	0.287465347	0.49341359	0.463866061
Hdac9	1.407755382	0.294139798	0.493396666	0.470312208
Rpl10a	1.406247451	0.028998858	0.491850481	0.225257623
Mta1	1.405773863	0.058467649	0.491364537	0.259682375
6030458C11Rik	1.40551134	0.141709513	0.491095093	0.334202278
Gps1	1.404720891	0.15949141	0.490283505	0.348513674
Cuedc1	1.404577185	0.175070303	0.490135907	0.361432976
Fmr1	1.404406563	0.247514805	0.489960644	0.423692292
Creb3	1.403975977	0.157455748	0.48951825	0.346272207
Calm2	1.402465187	0.070242601	0.48796496	0.26954281
Dync2h1	1.402033905	0.211395741	0.487521238	0.390099157
Atf6b	1.400791718	0.029686899	0.486242459	0.223637599
Mbd1	1.400755825	0.030972914	0.486205492	0.225364266
S1pr1	1.400651332	0.150588019	0.486097866	0.338165018
ENSMUSG00000094936	1.400228651	0.220007938	0.485662432	0.395971997
Ybx1	1.399835913	0.021811066	0.485257727	0.211566456
Papss1	1.398715874	0.154459824	0.484102932	0.339970936
Rpl37a	1.398200987	0.060468285	0.483571758	0.257580243
Zfp626	1.39804888	0.196271658	0.483414802	0.374088578
Pak1	1.397774525	0.146370792	0.483131659	0.332627491
Lxn	1.39758111	0.26369516	0.482932014	0.432076614
Pgrmc2	1.396313278	0.257006938	0.481622662	0.424901007
Gm17068	1.39525606	0.203026519	0.480529913	0.377485413
Dcp1b	1.395169604	0.212134685	0.480440514	0.385055653
Rplp1	1.395105435	0.046413601	0.480374158	0.241198617
Stk17b	1.394950748	0.178430448	0.480214185	0.356876459
Zfp329	1.394836487	0.187010114	0.480096009	0.363853608
Tmem245	1.394261187	0.090112792	0.479500846	0.282924232
Hdac8	1.392829959	0.260936755	0.47801914	0.425215912
Rps20	1.39242585	0.042974541	0.477600503	0.235973601
Tesk1	1.391745234	0.107778157	0.476895143	0.29652856
Camta1	1.391308208	0.201311608	0.476442047	0.372854299
5430427O19Rik	1.390819808	0.184787563	0.475935519	0.358883128
Mpi	1.390651336	0.215074253	0.475760753	0.383760731
Adgrg6	1.390502116	0.144124699	0.47560594	0.325623231
Adra1a	1.390415469	0.282670079	0.475516038	0.44261202
Rpl31	1.389740051	0.11967875	0.474815054	0.305126863
Nckap5l	1.389487702	0.1985231	0.474553066	0.369080636
Pdp2	1.388765539	0.273236274	0.473803054	0.432444032
Fastk	1.388665387	0.089766597	0.473699009	0.279199917
Ttll12	1.388613673	0.269366998	0.473645282	0.428823973
D11Wsu47e	1.387773707	0.178053825	0.472772338	0.351041232

Diexf	1.387381536	0.212379575	0.47236459	0.378785946
BC100451	1.386038049	0.260795984	0.470966862	0.418818974
Zbtb48	1.386008225	0.201009407	0.470935819	0.368298123
Guf1	1.385040271	0.21585141	0.469927924	0.379698298
Gpaa1	1.384960306	0.208532723	0.469844628	0.373601126
Sucla2	1.384771591	0.220035238	0.469648033	0.382937745
Sesn2	1.384721449	0.164560748	0.469595793	0.337864208
Lta4h	1.383004079	0.13069167	0.467805411	0.30952204
Ercc3	1.38268757	0.200894718	0.467475204	0.365498653
Rps5	1.38197222	0.140252384	0.466728615	0.31645797
Serbp1	1.381341499	0.077324745	0.46607003	0.263849469
Ppp2r5d	1.380855778	0.124716015	0.465562647	0.303243591
Gle1	1.380284366	0.232859624	0.464965521	0.389736647
Sp2	1.379256141	0.254054673	0.463890404	0.406722986
Fam188a	1.379050873	0.184444344	0.463675679	0.349365275
Gng5	1.377820073	0.209301383	0.462387501	0.368292822
Fzr1	1.37772414	0.083977705	0.462287048	0.267518143
Commd9	1.377641152	0.287524098	0.462200144	0.434574763
Tmem161b	1.377198346	0.140373063	0.461736353	0.313168306
Dffa	1.376997258	0.177041106	0.461525687	0.341890713
Apba3	1.376722917	0.138765257	0.461238227	0.31156224
Unc45a	1.376544141	0.175791972	0.461050873	0.34055407
Rps11	1.375778936	0.1934636	0.460248672	0.353928293
Gcc1	1.375222559	0.137750803	0.459665116	0.309701657
St3gal2	1.374200851	0.179338181	0.458592881	0.341520999
Eif1b	1.373741474	0.151078013	0.458110527	0.319077552
Actn1	1.372991224	0.037712284	0.457322404	0.220081518
Vps51	1.372480583	0.21508071	0.45678574	0.368460189
Polrmt	1.372318973	0.165417875	0.456615851	0.329192546
Ptov1	1.3722027	0.048363749	0.45649361	0.231234555
Tk2	1.371890368	0.123078854	0.456165196	0.295831128
Stau1	1.371506766	0.064716608	0.455761739	0.246729697
Rpl15	1.371428231	0.096303701	0.455679125	0.274003108
Ptger2	1.371189236	0.188259636	0.455427689	0.346136737
Slc17a5	1.370391666	0.140928499	0.454588283	0.30875179
Srsf7	1.369975544	0.165202594	0.454150139	0.327248252
Uhrf1bp1	1.367117159	0.118448675	0.451136884	0.288946138
Inip	1.36648558	0.259762103	0.450470236	0.399723857
Fbxw4	1.366404543	0.206883907	0.450384677	0.356830691
Dedd2	1.36616079	0.207907036	0.450127291	0.357430702
Rps23	1.365096411	0.114144004	0.449002846	0.284208183
Atm	1.364458106	0.094953536	0.448328099	0.268487704
Tut1	1.363911132	0.137352654	0.447749646	0.301368378
Tial1	1.363807922	0.048828348	0.44764047	0.227218578
Dhdds	1.363292082	0.160470752	0.447094689	0.318559211

Uqcc1	1.362303472	0.203228441	0.446048119	0.35055451
Commd3	1.361882112	0.207408489	0.445601825	0.353449293
Hmox2	1.361384034	0.272677998	0.445074095	0.405750293
Spata2	1.360774435	0.265661618	0.444427943	0.399267225
Mmadhc	1.360731607	0.264688544	0.444382536	0.398414351
Smg6	1.360024584	0.101293048	0.44363273	0.270736071
Rpl23	1.359844116	0.099988927	0.44344128	0.269584357
Ap5s1	1.3597647	0.242337183	0.443357023	0.379208277
Rab11b	1.359698506	0.097404951	0.44328679	0.267432245
Sri	1.35966689	0.156117103	0.443253244	0.31253482
Stat5a	1.357914722	0.168161662	0.44139288	0.320282797
Abca9	1.357290192	0.128414696	0.440729205	0.289880345
Rpl12	1.356391278	0.064238307	0.439773413	0.237648264
Rpl28	1.3559953	0.148861462	0.439352178	0.30435226
Rad17	1.355499055	0.187009718	0.438824107	0.332574293
H2afj	1.355488244	0.287010905	0.438812601	0.412146021
Cbx5	1.355475777	0.24942912	0.438799331	0.380989142
Psmc2	1.355332555	0.11434396	0.438646886	0.277806496
Narf	1.354728419	0.280834183	0.438003665	0.406142851
Ssbp4	1.354681159	0.079638516	0.437953336	0.249861078
Itgb5	1.354543795	0.084794055	0.437807039	0.254018735
Vapb	1.35425635	0.082565957	0.437500855	0.252018364
Ago4	1.353605992	0.191191856	0.43680786	0.33419077
Gramd4	1.353520048	0.247897804	0.436716256	0.377955347
Orai3	1.353183774	0.20753845	0.436357783	0.346215963
Drap1	1.353163745	0.15102364	0.436336429	0.30387129
Ddx27	1.351917582	0.23298976	0.435007202	0.364726988
Ahdcl1	1.351827954	0.144054461	0.434911553	0.297709704
Hadh	1.350433561	0.299245574	0.433422664	0.417534401
Xab2	1.35032019	0.067563	0.433301542	0.237047473
Parvg	1.350248498	0.266205731	0.433224943	0.389645847
Ttc37	1.35016955	0.116966905	0.433140588	0.276302364
Tmem68	1.350104992	0.263101546	0.433071604	0.386985339
Strn4	1.349685006	0.044118598	0.432622746	0.214918456
Cdc26	1.34922281	0.122695323	0.432128614	0.279956096
Rab3il1	1.347647861	0.045902509	0.430443571	0.215621906
Nup160	1.347594794	0.203720991	0.43038676	0.338614847
Zbtb11	1.347284955	0.212460648	0.430055017	0.344919339
Cnot7	1.347058982	0.163924441	0.429813022	0.308775219
Npm1	1.346650135	0.149374741	0.429375082	0.29781655
ENSMUSG00000090386	1.346498283	0.0689266	0.429212391	0.235973531
Wfdc17	1.346213395	0.227233252	0.428907117	0.355197695
Tti1	1.344869902	0.151006422	0.427466618	0.297681676
Meaf6	1.343218364	0.210975759	0.42569386	0.34031364
Ccdc171	1.343148667	0.255936333	0.425618999	0.374648669

St6galnac4	1.342853751	0.216819037	0.425302191	0.344365652
Mterf3	1.342758076	0.225454265	0.425199399	0.350780643
Rps18	1.341954762	0.07309738	0.424336039	0.236765307
Cyp27a1	1.341490777	0.193422929	0.423837136	0.325898297
Ypel5	1.340849955	0.189866908	0.423147805	0.322773868
Zw10	1.340096331	0.191500391	0.422336711	0.323343878
Dhrs1	1.339882327	0.129938009	0.422106303	0.278738277
Rpl19	1.339568112	0.026268193	0.421767938	0.189795447
Stard8	1.339349482	0.028962043	0.421532458	0.193009121
Serinc3	1.338578421	0.052438741	0.420701662	0.216910829
ENSMUSG00000102976	1.337723477	0.262411773	0.419779925	0.374566409
Fis1	1.337706456	0.165465206	0.419761568	0.302656695
Ddx23	1.337451223	0.085193528	0.419486277	0.243700935
Eif3k	1.336341465	0.138033783	0.418288696	0.282026679
Ugcg	1.336134518	0.196669942	0.418065261	0.323806163
Zdhhc17	1.335967877	0.162643292	0.417885318	0.299294082
Mbtps2	1.334837489	0.219029508	0.41666411	0.338995821
Mrpl15	1.334830428	0.285922699	0.416656478	0.390453598
Rpsa	1.334341187	0.021858424	0.416127607	0.18149169
Trmt1	1.333359748	0.119289023	0.41506608	0.266449782
Usf2	1.332392692	0.177432698	0.414019347	0.306976146
Gpcpd1	1.331065488	0.132793599	0.412581553	0.27447317
ENSMUSG00000109536	1.330777545	0.175993972	0.412269428	0.304664168
Micu1	1.330385979	0.109692008	0.411844869	0.257470789
Trpv4	1.33014493	0.289983856	0.411583447	0.38896254
Ccl9	1.33003742	0.074223797	0.411466836	0.230483759
Zgpat	1.329889022	0.108013604	0.411305859	0.255916798
Utp14b	1.329738877	0.261026244	0.411142969	0.3657957
Bag1	1.32973461	0.239165579	0.411138339	0.349288147
Hvcn1	1.32950251	0.191588104	0.410886501	0.314639653
Dnajc8	1.329235399	0.151306449	0.410596619	0.286143589
Slc26a6	1.328947113	0.180314287	0.410283692	0.306230314
Ube2b	1.325761369	0.175909747	0.406821121	0.30057932
Rsb1l1	1.325267304	0.170079009	0.406283378	0.296135706
Arid5a	1.32438036	0.124810241	0.405317521	0.264068999
Rpl7a	1.323464055	0.139055459	0.404319012	0.273314547
Plekhf2	1.323356799	0.234136065	0.404202089	0.339730747
Mia3	1.323161295	0.05400783	0.403988939	0.209671185
Fcgrt	1.322447683	0.205939765	0.403210649	0.318791703
Rbm41	1.322034914	0.295035839	0.402760278	0.384629531
Ahcy1	1.320980673	0.119217848	0.401609359	0.257761625
Gm24146	1.320410236	0.149127582	0.400986227	0.277956955
Lrrc14	1.320311896	0.290957926	0.400878776	0.379611958
Fam96a	1.320123375	0.234508618	0.400672766	0.337032645
Uqcrh	1.320063207	0.145451363	0.400607011	0.275182333

Hnrnpab	1.31980935	0.261129572	0.400329544	0.356252135
Fuca1	1.31892114	0.043470954	0.399358307	0.19778472
Ubap1	1.318722937	0.08896891	0.399141487	0.234670123
Aup1	1.318379652	0.060877797	0.398765881	0.212745261
Wdr12	1.315765936	0.233513974	0.395902868	0.332312899
Gas6	1.315712388	0.125104432	0.395844153	0.258098167
Srcap	1.314913346	0.083784853	0.394967727	0.228419193
Fto	1.314667119	0.126402773	0.394697548	0.258235173
Rps2	1.314482287	0.123222884	0.394494702	0.255935059
Tubb6	1.314292587	0.258022199	0.394286483	0.348593492
Vps35	1.313664442	0.141856318	0.393596805	0.26795102
Cog2	1.313615401	0.130561035	0.393542946	0.260298113
Rpl13	1.313115608	0.072203466	0.392993938	0.21859312
Lmf1	1.312536616	0.132652334	0.392357671	0.260923917
Brat1	1.31168898	0.280237748	0.391425677	0.362503209
Fbxl18	1.311456455	0.197144733	0.391169906	0.303296178
Atp5e	1.311258973	0.273447346	0.390952645	0.356982812
Rpl17	1.311000612	0.046119799	0.390668359	0.19589294
Plekhg2	1.310690842	0.194693742	0.390327432	0.300988548
Atp5j	1.309342336	0.236561646	0.388842348	0.328518269
Zfp655	1.309202064	0.265433187	0.388687782	0.349024279
Tbc1d17	1.308843625	0.07959735	0.38829274	0.22149842
Hsd17b4	1.308672952	0.066775015	0.388104602	0.21171051
Usf1	1.30839252	0.06221316	0.387795417	0.207958456
Chtf8	1.305963951	0.280784277	0.385115075	0.357064382
Rps4x	1.30519269	0.242758592	0.384262812	0.328958496
Hdac6	1.304394114	0.25982188	0.383379836	0.340234005
Smurf2	1.304221075	0.255195874	0.383188437	0.336774372
Atxn1	1.304043747	0.054904398	0.382992269	0.19951338
Gm15922	1.30402925	0.280890059	0.38297623	0.355159415
Rpl36a1	1.30195054	0.155279907	0.380674643	0.26786818
Ncbp3	1.30150152	0.156394819	0.380176996	0.268240044
Dnmt1	1.300554447	0.07298546	0.379126799	0.211457543
ENSMUSG00000102336	1.300174641	0.130499706	0.378705421	0.250444307
Sdf2l1	1.299526527	0.284620715	0.377986083	0.353259053
Atp6v1f	1.298832922	0.178115554	0.377215859	0.280128824
Tecr	1.298592798	0.099412309	0.376949113	0.228771592
Arfgap2	1.298201771	0.242206091	0.376514629	0.32194759
Hadha	1.297547892	0.115959615	0.375787788	0.239056699
Hs2st1	1.296510099	0.294920275	0.374633444	0.357683248
Vps9d1	1.296504987	0.152079822	0.374627756	0.261570707
Actr1a	1.296454618	0.033595168	0.374571706	0.176277543
Pja2	1.296305601	0.170439465	0.374405871	0.273130841
Hars	1.295744108	0.280306023	0.373780833	0.346211288
Mier1	1.29560038	0.110227081	0.373620797	0.233926272

Tomm22	1.294757767	0.152717843	0.372682213	0.260617455
ENSMUSG00000106636	1.294401222	0.250643808	0.372284875	0.32406779
Mrpl45	1.294233622	0.223398601	0.372098061	0.305614501
Zer1	1.293955314	0.239105933	0.371787795	0.315817256
Ppox	1.293868851	0.222794069	0.371691391	0.304881973
Txndc5	1.293064487	0.117935879	0.370794227	0.237157069
Micall2	1.292736408	0.221189705	0.370428137	0.302793036
Psmc8	1.292640713	0.085676285	0.370321337	0.21547096
Farsb	1.291004538	0.209420607	0.368494072	0.293583297
Wrn	1.290238912	0.116705716	0.367638233	0.234350752
Gabpa	1.289466842	0.240564043	0.366774676	0.312526848
Cdyl2	1.289442805	0.281774446	0.366747782	0.340735444
Rps19	1.288004626	0.060984467	0.365137775	0.194884824
Zscan20	1.287939837	0.293187656	0.365065203	0.347299296
Zranb2	1.287425897	0.152055843	0.364489395	0.254477058
Ube2f	1.286382399	0.206792696	0.363319571	0.287792946
Slu7	1.286071074	0.220052682	0.362970375	0.295966987
Zbtb38	1.284714296	0.222472595	0.361447558	0.296273436
Rpl21	1.28461516	0.223151798	0.361336227	0.296617311
Slc31a1	1.28393048	0.050696558	0.360567088	0.18452396
Ttyh2	1.283899598	0.17793567	0.360532387	0.267628325
Rpap1	1.283779336	0.218742653	0.360397244	0.293034865
Sh3glb2	1.28334341	0.161085399	0.359907273	0.256814228
Slc4a2	1.282906833	0.089287117	0.359416403	0.211524661
Naca	1.282603159	0.212525397	0.359074866	0.288031526
Sepp1	1.281006234	0.229600576	0.357277497	0.297387172
Swap70	1.280168481	0.079051494	0.356333693	0.202898531
Suco	1.278812224	0.150526404	0.35480444	0.246790505
Arhgef1	1.278675878	0.08187009	0.354650613	0.203829577
Taf4a	1.278486177	0.221411453	0.354436563	0.289860423
Gps2	1.278022369	0.273663866	0.353913088	0.323307506
Ctdnep1	1.27697999	0.155590322	0.352735918	0.248395146
Kcmf1	1.275485843	0.210156664	0.351046886	0.280134982
Atp5c1	1.274791766	0.243175222	0.350261606	0.300116097
Rpl27a	1.274161783	0.157701812	0.349548472	0.247407819
Dhx37	1.273845615	0.230829571	0.349190439	0.291423181
Dek	1.272639636	0.210658003	0.347823959	0.27786834
Cog4	1.272571372	0.170716219	0.347746572	0.253847016
Atp5h	1.272461437	0.156990586	0.347621935	0.245623077
Pias2	1.271722256	0.153495927	0.34678362	0.242966242
Rdx	1.27153353	0.225901645	0.346569506	0.286188411
Ubxn7	1.270971521	0.2472408	0.345931704	0.298970275
Btbd7	1.270906215	0.241421962	0.345857572	0.295241388
Wbp4	1.270046288	0.260108465	0.344881079	0.306252038
Gpsm3	1.269837238	0.130815668	0.344643591	0.228105861

Ssh1	1.26839232	0.14767071	0.343001047	0.236910564
Slc38a7	1.267118355	0.148219475	0.341551286	0.236228828
Htati2	1.265260063	0.292626072	0.339433948	0.322539893
Rpl18	1.264908557	0.117124439	0.339033093	0.216363826
Tk1	1.26462218	0.235289659	0.338706428	0.285384422
Maged1	1.263723078	0.232152631	0.337680357	0.28261741
Rpl8	1.263670988	0.065711764	0.33762089	0.183451249
Tmsb10	1.263608817	0.238936621	0.337549909	0.286630353
Skiv2l	1.263253268	0.168496197	0.337143912	0.244830212
Atg13	1.262565736	0.201924152	0.336358504	0.263585512
Ero1lb	1.262431636	0.286430153	0.336205264	0.315393815
Urm1	1.261071145	0.29082281	0.33464967	0.316807599
Ubb	1.260400844	0.11693265	0.333882625	0.212965325
Paip2	1.260251873	0.289562086	0.333712098	0.315095389
Rock2	1.260188795	0.128796908	0.333639887	0.219664197
Comt	1.26000381	0.257700333	0.333428096	0.294588576
H3f3a	1.259963888	0.250290261	0.333382385	0.289987333
Slc36a1	1.25976897	0.06134804	0.333159181	0.178066799
Psmd12	1.258475309	0.280774081	0.331676911	0.307511985
Scamp3	1.258405404	0.268832095	0.331596771	0.299882377
Ccz1	1.257335328	0.23850936	0.330369464	0.280277957
Rabl6	1.256286527	0.164202578	0.329165544	0.236626681
Pced1a	1.255594904	0.274362235	0.328371079	0.300411236
Amn1	1.25553144	0.278500389	0.328298156	0.302943527
Snap23	1.254983358	0.156980497	0.327668233	0.231518538
Mtch1	1.252733608	0.175254757	0.32507966	0.239820719
Rnf31	1.251773056	0.118549555	0.323973029	0.207556519
Rpl38	1.251579039	0.170371422	0.323749402	0.236139114
Ubr1	1.251109834	0.164810794	0.323208449	0.232679372
Nacc2	1.25101289	0.258322928	0.323096655	0.285834199
Emc3	1.250712246	0.151906792	0.322749904	0.225253653
Rufy1	1.249743295	0.268424837	0.321631787	0.290623097
Calm3	1.249652558	0.143529736	0.321527037	0.219806644
Cstf1	1.249562161	0.280782261	0.321422671	0.298009904
Trnt1	1.249497665	0.288518874	0.321348205	0.302765544
Nt5dc3	1.248686504	0.293903356	0.320411318	0.305270751
Rps15a	1.248282872	0.189787167	0.319944899	0.244007555
Dcaf11	1.246826684	0.245985099	0.318260937	0.274325869
Ept1	1.246609664	0.297698707	0.318009802	0.305373124
Myd88	1.246497063	0.292910778	0.317879483	0.302236451
Bclaf1	1.246434438	0.119050599	0.317807	0.203883063
Nom1	1.24596735	0.293551582	0.317266263	0.302054129
Ssb	1.245806656	0.224534139	0.317080185	0.261065993
Rnf13	1.244961241	0.297413874	0.316100828	0.303361098
Trim3	1.244321643	0.226076158	0.315359454	0.26051386

ENSMUSG00000090673	1.244266942	0.198500853	0.315296032	0.245207334
Thra	1.243803593	0.287671284	0.314758689	0.29603616
Snrk	1.243374604	0.282251697	0.314261018	0.292261109
Tmsb4x	1.242387148	0.087761309	0.313114811	0.183394762
Ctdspl2	1.241973538	0.299311274	0.312634435	0.301214935
Zufsp	1.23921807	0.257230317	0.309430087	0.273116094
Flcn	1.238856375	0.153868638	0.30900894	0.216696467
Rpl13a	1.238330934	0.138459704	0.308396915	0.208158096
Cct5	1.237418774	0.171915863	0.307333828	0.224976039
Mtmr6	1.235935342	0.163963607	0.305603271	0.219564043
Nfatc2	1.235570074	0.160076601	0.305176834	0.217236473
Arsa	1.234457357	0.28873956	0.303877002	0.28643572
ENSMUSG00000091896	1.234323667	0.15603876	0.303720751	0.214110885
Luc7l	1.233985634	0.149401726	0.303325599	0.210401997
Cd163	1.233743787	0.248470456	0.30304282	0.262585605
Cog8	1.23358793	0.299575588	0.302860554	0.2919577
Acox1	1.232857798	0.133529326	0.302006404	0.201293517
Fubp3	1.231436378	0.238061702	0.300342094	0.254560546
Usp35	1.231239569	0.140143564	0.300111502	0.203430032
Brpf3	1.230509169	0.221712208	0.299255408	0.244892286
Klhl24	1.229576446	0.224647363	0.298161434	0.245549331
Vapa	1.229123572	0.238600648	0.297629967	0.252551606
Gdi1	1.228961631	0.17067903	0.297439875	0.217105402
Fem1b	1.228717383	0.207632445	0.29715312	0.235816655
Sec24b	1.228321637	0.217493179	0.296688381	0.240579984
Sdha	1.22794758	0.240974981	0.296248975	0.252652883
Crybg3	1.22765965	0.235061142	0.295910651	0.249204251
Rcsd1	1.226410517	0.225977223	0.294441973	0.243182396
Hps3	1.226342403	0.171681305	0.294361846	0.215362339
Mtss1	1.226203321	0.215806	0.294198217	0.23768648
Paxip1	1.225165515	0.266872735	0.292976665	0.263873259
Daglb	1.224935492	0.185311093	0.292705775	0.220980367
Rpl9	1.224167585	0.199119975	0.291801072	0.227248283
Qars	1.2234487	0.191081798	0.290953609	0.222546139
Capzb	1.222809363	0.083194791	0.290199504	0.167508872
Srek1	1.222244234	0.18495164	0.289532599	0.218406029
ENSMUSG00000103593	1.222108454	0.287605634	0.28937232	0.272122764
Tpcn2	1.221885613	0.142017525	0.289109233	0.196898075
Ankrd40	1.221739265	0.27638161	0.288936428	0.265448547
Sec14l1	1.221567483	0.139529178	0.288733565	0.195414186
Nrd1	1.221543767	0.059482291	0.288705555	0.153191008
Exoc5	1.221320494	0.245675488	0.288441836	0.248460256
Arpc1b	1.220549759	0.110493957	0.287531112	0.180159949
Cdk12	1.22035936	0.133560862	0.287306042	0.191510968
Nup155	1.219633308	0.258683996	0.286447456	0.253603955

Uvrag	1.216863829	0.131654048	0.283167735	0.18782543
Pgap2	1.216762679	0.216293508	0.283047808	0.228920817
Api5	1.216510308	0.21351156	0.282748545	0.227295783
Cdc37	1.216436059	0.265307226	0.282660488	0.253749616
Cnpy3	1.216199694	0.280430411	0.282380131	0.261619823
Atp6v0a2	1.216155548	0.282044177	0.282327763	0.262450154
Maz	1.215977667	0.220541107	0.282116732	0.230282352
Cox7a2l	1.215436356	0.113714633	0.281474351	0.177955157
Rps6ka3	1.215299466	0.187890276	0.281311857	0.213625491
ENSMUSG00000090112	1.214997718	0.153462853	0.280953604	0.196828071
Sin3b	1.21471681	0.267320533	0.280620014	0.252980797
Psmc1	1.214637703	0.240296592	0.280526057	0.238898926
Rab11fip5	1.214447683	0.047023567	0.280300342	0.141131893
Chpf2	1.214351672	0.2208184	0.280186282	0.22884402
Cand1	1.21428442	0.210275173	0.280106382	0.223582645
Pkd2	1.214037521	0.228541677	0.27981301	0.232378911
Rps15	1.212926686	0.202345179	0.278492351	0.218442767
Cat	1.212394786	0.171939211	0.277859553	0.20341121
Rftn1	1.21227992	0.151664579	0.27772286	0.193713746
Tob2	1.212272923	0.134424243	0.277714534	0.185528808
Mta3	1.211410527	0.212661749	0.276687853	0.222011051
Rps14	1.210163394	0.168189723	0.27520185	0.199704699
ENSMUSG00000090733	1.210045391	0.223305716	0.275061167	0.225870072
Mbtps1	1.208942961	0.137053641	0.273746179	0.184111126
Foxk1	1.207903042	0.259914536	0.272504654	0.24188382
Snx3	1.206593608	0.268405473	0.270939845	0.244808487
F630028O10Rik	1.206592883	0.29283043	0.270938978	0.257563036
Pafah1b1	1.206269933	0.1330244	0.270552782	0.180094586
Flii	1.206175907	0.119973987	0.270440324	0.173929717
Trmt1l	1.205008531	0.296401001	0.26904336	0.257659347
Ubtg	1.204768829	0.180556973	0.268756348	0.200707918
Cluh	1.202797031	0.191432567	0.266393212	0.20392132
Ssh2	1.202519973	0.254731765	0.266060856	0.233605689
Ulk1	1.202443515	0.2095749	0.265969125	0.211972313
Arrb2	1.200696905	0.235391642	0.263872014	0.222379497

Table 7.2 List of RNAs binding to GAPDH in LPS-treated macrophages. Data shown includes the genes with a higher than 1.2 fold increase in sequencing reads in the immunoprecipitated sample compared to the IgG control.

7.2 Conference abstracts

Metabolism and Immunity: a rediscovered frontier – Dublin, Ireland May 2014

Poster presentation:

The potential of succinylation as a new inflammatory signal

Silvia Galvan-Pena and Luke A.J. O'Neill

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Succinylation is a recently uncovered protein post-translational modification. It involves the attachment of a succinyl group to lysines, resulting in an extra 100 Da mass, as well as a change in charge comparable to that caused by protein phosphorylation. It has been reported to be present in a wide range of organisms ranging from bacteria to humans and although thousands of proteins have been identified as being succinylated basally, the modification on these proteins is limited to only a few lysines. The enzyme responsible for succinylation has not yet been uncovered, but succinylation levels have been shown to be dependent on succinyl-CoA concentration.

The discovery of succinylation was closely followed by the discovery of the role played by succinate in inflammation. Succinate, which is a metabolite derived from the Krebs cycle, can drive production of the inflammatory cytokine interleukin-1 β through the transcription factor HIF1 α . These two discoveries led us to investigate the potential role succinylation may play in the inflammatory response.

We have been able to show that protein succinylation can be induced in murine macrophages following activation with various toll-like receptor ligands. In addition, LPS can specifically increase succinylation of the glycolytic enzyme GAPDH in macrophages, and this increase in succinylation coincides with a doubling in its enzymatic activity. We then tested the effect of LPS on bone-marrow derived macrophages (BMDMs) from SIRT5-deficient mice. SIRT5 is the only desuccinylase known up to this date. In response to LPS, these BMDMs produce significantly lower levels of inflammatory cytokines, and higher levels of the cytokine IL10. Additionally, LPS decreases transcript levels of SIRT5.

Overall, our results suggest that succinylation may be an important signal for LPS in macrophages, although the exact functional consequences for this process requires further work.

Poster presentation:

Lysine Malonylation as a New Inflammatory Signal

Silvia Galvan-Pena and Luke A.J. O'Neill

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Malonylation is a recently uncovered protein post-translational modification. It involves the attachment of a malonyl group to lysines, resulting in an extra 86 Da mass, as well as a change in charge comparable to that of phosphorylation. It has been reported to be present in *E. Coli* as well as in various mammalian cell lines and tissues, with over three hundred proteins identified as having at least one malonylated lysine. The enzyme responsible for malonylation has not yet been uncovered, but it seems like malonylation levels are dependent on malonyl-CoA concentration.

The discovery of lysine malonylation took place during a time in which immunometabolism became an emerging field. Studies into the metabolism of immune cells have recently highlighted the tight link existing between the metabolic status and the function of these cells. For instance, citrate, from which malonyl CoA is derived, has been associated with the production of prostaglandins, reactive-oxygen species and nitric oxide, all of which are produced by macrophages following activation. This led us to investigate whether protein malonylation might be a mechanism by which the cell's metabolism can control the inflammatory response.

We have been able to show that protein malonylation can be induced in murine macrophages following activation with various toll-like receptor ligands. In addition, the TLR4 ligand, LPS, can specifically increase malonylation of the glycolytic enzyme GAPDH in macrophages. Through mass-spec analysis we identified a single malonylated lysine present in GAPDH in resting macrophages, while two were identified following activation with LPS. Interestingly, GAPDH enzymatic activity increases under the same conditions. Furthermore, GAPDH functions are known to go beyond its role in glycolysis; for instance, as part of the GAIT complex, GAPDH is capable of binding to the 3'-UTR region of certain mRNAs, such as that of the death-associated protein kinase (DAPK) and inhibit their translation. Our data would suggest that following activation, GAPDH releases DAPK mRNA. The potential post-translational modifications mediating these variable functions remain unknown up to this point.

Overall, our results suggest that protein malonylation is an important signal for LPS signaling in macrophages, that may be responsible for controlling the functions of the glycolytic enzyme GAPDH following macrophage activation.

Oral presentation:

GAPDH: a new player in the inflammatory response in macrophages

Silvia Galvan-Pena and Luke A.J. O'Neill

School of Biochemistry and Immunology, TBSI, Trinity College Dublin, Dublin, Ireland

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is most widely known for its role as the sixth glycolytic enzyme. However in recent years it has blossomed from its mundane use as an endogenous control in western blots into an increasingly interesting multi-functional protein. It has been implicated in cell death, DNA repair, and membrane fusion and transport. It is also part of the gamma interferon inhibitor of translation (GAIT) complex where it is capable of binding to the 3'-UTR region of certain mRNAs and prevent their translation.

Studies into the metabolism of immune cells have also recently highlighted the tight link existing between the metabolic status and the function of these cells. This led us to investigate whether GAPDH might be playing a role in the inflammatory response in macrophages.

We have been able to show that in murine macrophages, GAPDH is required for cytokine production as blocking of its enzymatic results in decreased production of TNF α , IL6, IL10 and pro-IL1 β . Additionally, in a resting macrophage, GAPDH appears to control expression of the death-associated protein kinase 1 (DAPK1), as well as COX2 and TNF α , by binding to their mRNA and preventing its translation. Binding of GAPDH to DAPK1 mRNA can be reversed following activation of macrophages with LPS, allowing for the RNA to be translated. At the same time as the RNA is released, GAPDH enzymatic activity is boosted. We have evidence to suggest that this effect is enabled by the malonylation of a lysine within the NAD-binding domain. Furthermore, following its translation, DAPK1 appears to play a role in blocking IL-10 production, as inhibition of its kinase activity results in a boost in the secretion of this cytokine.

Overall, these results place GAPDH as a novel direct regulator of inflammation in macrophages.

Poster presentation:

GAPDH: a new player in the inflammatory response in macrophages

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Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is most widely known for its role as a glycolytic enzyme. However in recent years it has blossomed from its mundane use as an endogenous control into an increasingly interesting multi-functional protein. It has been implicated in cell death and it is also part of the gamma interferon inhibitor of translation (GAIT) complex where it binds to the 3'-UTR region of certain mRNAs and prevents their translation. This led us to investigate whether there is a role for GAPDH in the inflammatory response in macrophages. We have been able to show that in macrophages, GAPDH is required for cytokine production as blocking of its enzymatic results in decreased production of TNF α , IL6, IL10 and pro-IL1 β . Additionally, in a resting macrophage, GAPDH appears to control expression of the death-associated protein kinase 1 (DAPK1), as well as COX2 and TNF α , by binding to their mRNA and preventing their translation. Binding of GAPDH to DAPK1 mRNA can be reversed following activation of macrophages with LPS, allowing for the RNA to be translated. At the same time as the RNA is released, GAPDH enzymatic activity is boosted. We have evidence to suggest that this effect is enabled by the malonylation of a lysine within the NAD-binding domain. Furthermore, we have also observed that not only GAPDH undergoes malonylation in activated macrophages but many other proteins within the cell. Overall, these results place GAPDH as a novel direct regulator of inflammation in macrophages.

Oral presentation:

Malonylation as a novel inflammatory signal in macrophages

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Malonylation is a recently uncovered protein post-translational modification. It involves the attachment of a malonyl-CoA-derived malonyl group to lysines, resulting in an extra 86 Da mass, as well as a change in charge comparable to that of phosphorylation. Given the increasing number of links existing between the metabolic status and the function of immune cells, it led us to investigate whether protein malonylation might be a new mechanism by which the cell's metabolism can control immune responses.

We have been able to show that protein malonylation can be induced in macrophages following activation. We have also been able to identify via mass spectrometry a wide array of proteins undergoing this post-translational modification, one of which is glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Although it is most widely known for its role as a glycolytic enzyme, GAPDH has blossomed in recent years from its mundane use as an endogenous control into an increasingly interesting multi-functional protein. Through the use of a GAPDH enzymatic activity inhibitor, and siRNA knockdown, we have shown that GAPDH activity is needed for the production of cytokines in macrophages, including TNF α , IL6 and IL1 β . However, especially in the case of TNF α , the requirement for GAPDH goes beyond its glycolytic activity. GAPDH can bind TNF α mRNA, as well as other pro-inflammatory mRNAs, and block their translation. Following activation of macrophages with lipopolysaccharide (LPS), GAPDH can release these mRNAs and enable their translation.

Our data would suggest that there is a specific lysine within GAPDH catalytic domain that undergoes LPS-induced malonylation. By generating a malonylation-mimic mutant of this lysine, GAPDH activity is boosted while its binding to TNF α mRNA is inhibited. Overall, our results suggest that malonylation has important signalling functions in macrophages and can act as a key regulator of cytokine production and inflammation via GAPDH.

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