

**Effect of small molecule
modulators of Complex III
of the electron transport chain on
cytokine production in macrophages**



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Declaration of Authorship

This thesis is submitted by the undersigned to the University of Dublin for the examination of Doctor of Philosophy. I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and is entirely my own work except for the following figures:

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Acknowledgements

This is (almost) the end!

Monday the 30th of September 2019 was my first day in the lab here in TBSI. It looks like yesterday to me but, apparently, 4 years went by in the quickest way possible. And today, on a Friday, the 29th of September 2023, I submit for the first time my PhD thesis. Three pages will not be able to contain all the things I would like to write down in this moment..

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Abbreviations

AA	Antimycin A
ADP	Adenosine diphosphate
AIM2	Absent in melanoma 2
AMPK	AMP-activated protein kinase
ANT	Adenine nucleotide translocase
AOX	Alternative OXidase
APC	Antigen processing cell
AP-1	Activator protein 1
APS	Ammonium Persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BDNF	Brain derived neurotrophic factor
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
CAPS	Cryopyrin-associated periodic syndrome
CARD	Caspase-recruitment domain
CCL	CC chemokine ligand
CCCP	Carbonyl cyanide m-clorophenyl hydrazone
CD	Chron's disease
CNS	Central nervous system
COX	Cyclooxygenase
CPT-1	Carnitin palmitoyl transferase
CREB	cAMP response element-binding protein
DAMP	Damage-associated molecular pattern
DC	Dendritic cells
DMEM	Dulbecco's modified eagle medium

DMM	Dimethyl maleate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DRP1	Dynamin-related protein
EAE	External autoimmune encephalomyelitis
ECAR	Extracellular acidification rate
ECSIT	Evolutionarily conserved signaling intermediate in Toll pathway
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N, N, N', N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Endothelial mesenchymal transition
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
FCAS	Familial cold autoinflammatory syndrome
FCCP	Carbonyl cyanide-p-trifluoromethoxy phenylhydrazone
FCS	Fetal calf serum
FET	Forward electron transport
FFA	Free fatty acid
FGF	Fibroblast growth factor
FMN	Flavin mononucleotide
FPKM	Fragments per kilobase million
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF15	Growth differentiation factor 15
GDP	Guanosine diphosphate
GLUT	Glucose transporter
GSDMD	Gasdermin D
GSK3	Glycogen synthase kinase
GTP	Guanosine triphosphate
HCC	Hepatocellular carcinoma

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia-inducible factor
HNE	Hydroxynonenal
IDH	Isocitrate dehydrogenase
IDO1	Indoleamine-2,3-dioxygenase 1
IFN	Interferon
IL	Interleukin
LDH	Lactate dehydrogenase
LRR	Leucine rich repeat
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MIP-1	Macrophage inflammatory protein
MS	Multiple sclerosis
MSU	Monosodium urate
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial ROS
MAPK	Mitogen-activated protein kinase
MDSCs	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MIP-2	Macrophage inflammatory protein-2
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWS	Muckle-wells syndrome
MYD88	Myeloid differentiation primary response 88
MYX	Myxothiazol
NAC	N-acetyl Cysteine
NADH	Nicotinamide adenine nucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NEK	NIMA-related kinase

NET	Neutrophil extracellular trap
NFAM1	NFAT activating protein with ITAM motif 1
NF-AT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor kappa B
NK	Natural killer
NLR	NOD-like receptors
NLRC4	NOD family CARD domain-containing protein 4
NLRP3	NOD-, LRR-, and pyrin domain-containing protein 3
NO	Nitric oxide
NOMID	Neonatal onset multisystem inflammatory disease
NQO1	NADPH quinone dehydrogenase
NRF2	Nuclear factor erythroid derived 2-like 2
OCR	Oxygen consumption rate
ODN	Oligodeoxynucleotides
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular pattern
PARP	Poly ADP-ribose polymerase
PBMCs	Peripheral mononuclear blood cells
PBS	Phosphate buffered saline
PCr	Phosphocreatine
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PD-1	Programmed death 1
PECs	Peritoneal exudate cells
PGE	Prostaglandin E2
PHD	Prolyl hydroxylase
PI3K	Phosphoinositide 3 kinase
PINK1	PTEN-induced kinase 1
PKM2	Pyruvate kinase M2
PMP	Plasma membrane permeabilizer
PMSF	Phenylmethylsulfonyl fluoride
PRR	Pattern recognition receptor

PTEN	Phosphatase and intensin homolog
PTM	Post-translational modification
PVDF	Polyvinylidene difluoride
PYD	Pyrin domain
RA	Rheumatoid arthritis
RET	Reverse electron transport
RISP	Rieske iron sulfur protein
ROS	Reactive oxygen species
SC	Supercomplex
SDH	Succinate dehydrogenase
SDS	Sodium dodecyl sulfate
SLE	Systemic Lupus Erythematosus
SIQEL	Suppressor of site I Q and III Q _O Electron Leak
SNP	Single nucleotide polymorphism
SS	Sjogren's syndrome
STAT	Signal transducer and activator of transcription
TAM	Tumor associated macrophages
TCA	Tricarboxylic acid
TEMED	N, N, N', N'-Tetramethylethylenediamine
TFEA	Transcription factor enrichment analysis
TGF	Transforming growth factor
TIL	Tumour infiltrating lymphocytes
TLR	Toll-like receptor
TME	Tumour micro environment
TNF	Tumour necrosis factor
TPP	Triphenylphosphonium
UCP	Uncoupling protein
UQCRH	Ubiquinol-cytochrome c reductase hinge protein
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand factor
2-DG	2-deoxy-glucose

Abstract

Innate immune cells promote inflammation when activated by Pathogen-associated molecular patterns (PAMPs) and initiate the immune response to stress and microbial invasion. When macrophages are primed with the Gram-negative bacterial product Lipopolysaccharide (LPS), they show an M1 pro-inflammatory phenotype and aberrant macrophage activation can contribute to the pathogenesis of inflammatory diseases.

M1 macrophages also undergo profound metabolic changes, with glycolysis being elevated and a disturbance in mitochondrial metabolism, leading to an elevation in such metabolites as succinate and itaconate. The role of the electron transport chain (ETC) in shaping the macrophage immune response has been less studied.

M1 macrophages release multiple cytokines including the pro-inflammatory cytokines Interleukin(IL)-1 β and Tumour Necrosis Factor (TNF)- α and the immunomodulatory cytokine Interleukin (IL)-10. In this thesis, I have explored the link between the ETC and the innate immune response, with a focus on the mechanisms driving cytokine production. Novel specific inhibitors of Complex I and Complex III of the ETC termed Suppressors of site I Q and III Q_o Electron Leak (S1QELs and S3QELs respectively) have been assessed. These compounds inhibit Reactive oxygen species (ROS) production without affecting basal cellular respiration. S1QEL 1.1 and S3QEL 1.2 blocked IL-1 β expression at the translational level in LPS-treated macrophages. They also suppressed Hypoxia-Inducible Factor (HIF)-1 α activation but they failed to repress NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome activation. Moreover, S3QEL 1.2 slightly affected respiration and lowered ROS production in activated macrophages and importantly inhibited LPS and CpG-induced IL-10 release in macrophages *in vitro* but also *in vivo*. Macrophages from transgenic mice expressing Alternative Oxidase (AOX), which diverts electrons from Complex III lowering ROS, also had less IL-10 in response to LPS. Further analysis revealed that IL-10 downregulation might involve inhibition of Activator protein (AP)-1 activation but not CREB activation. The effect on AP-1 and AP-1-dependent genes was shown by RNAseq analysis, which also revealed that S3QEL 1.2 and

Myxothiazol (which inhibits Complex III activity) also regulated the TNF- α pathway in a positive way likely via the negative regulation of IL-10. Subsequent studies in the B16F10 melanoma model showed that S3QEL 1.2 improved survival and decreased tumour growth in mice, particularly in combination with CpG DNA.

These results provide evidence for ROS derived from Complex III in the regulation of cytokine production, particularly IL-10, in macrophages and possibly in tumour-mediated immune evasion. This work suggests the potential of S3QELs as mitochondria-targeted therapies in cancer.

1. Introduction

1.1 General introduction

1.1.1 The innate immune system and macrophages

The immune system represents the body's defence against stress and pathogen invasion. It is composed of two arms, the innate immune system and the adaptive immune system. The innate immune system is the first line of defence which develops acutely and is characterized by several cell types such as macrophages and neutrophils. These cells recognize specific structural components of pathogens through germline-encoded receptors called Pattern recognition receptors (PRRs). Adaptive immunity is the second phase which occurs during an infection. It takes more time to develop and involves antigen-specific cells, such as T and B lymphocytes.

Macrophages are one of the most important cell types of the innate immune response. They are myeloid cells derived from the proliferation of hematopoietic stem cells in the bone marrow. Monocytes from the bone marrow migrate to target tissues depending on the stimulus they receive and then differentiate into mature macrophages (1). Some populations of macrophages are classified as 'tissue-resident' macrophages because they are derived from embryonically pre-established progenitors. These cells occupy specific niches and show different structures and functionalities depending on the microenvironment where they reside (2).

Macrophages are able to identify pathogens thanks to PRRs which recognize two classes of molecules: pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens, and damage-associated molecular patterns (DAMPs), which are associated with components of host cells that are released during cell damage or death. Toll-like Receptors (TLRs) are an important class of PRRs. TLR4 is the receptor for Lipopolysaccharide (LPS), a key component of the cell wall of Gram-negative bacteria.

When activated, macrophages undergo polarisation depending on the type of stimulus they receive: M1 macrophages are considered 'pro-inflammatory' and

their activation is induced by the recognition of microbial cues and proinflammatory Th1-type cytokines such as Interferon gamma (IFN- γ) (3). Through the production of cytokines such as Interleukin IL-1 β and TNF- α , M1 macrophages are essential for the development of an acute phase response and promoting pathogen clearance but can also cause tissue damage when activated aberrantly. In contrast, the Th2-type cytokines IL-4 and IL-13 promote M2 macrophage polarisation (4). M2 macrophages produce IL-10 and Transforming Growth Factor (TGF)- β , which are anti-inflammatory cytokines that promote tissue repair and restrict tissue damage. Nowadays, the current M1 vs M2 classification system is considered an oversimplification and does not clearly explain the great heterogeneity of this type of cell. It is understood that a balance between the two is required to promote pathogen clearance while reducing unwanted autoinflammation.

1.2 Cytokine production by macrophages

When macrophages are activated, they exert different functions which include phagocytosis of microorganisms and extraneous particles, presentation of antigens to T cells and cytokine production. This last action is of high relevance for recruiting specific cells to the site of inflammation and for modifying their activity.

1.2.1 IL-1 β production in macrophages and its regulation by the transcription factor HIF-1 α

IL-1 β is an important pro-inflammatory cytokine produced in inflamed macrophages when they are stimulated by LPS (5) and other PAMPs such as poly(I:C) (5). It is a particular focus of this thesis, especially its regulation by HIF-1 α and the NLRP3 inflammasome.

HIF-1 α is responsible for IL-1 β regulation during hypoxia. Hypoxia consists of a low percentage of O₂ (2-6%) and activates defence mechanisms in the damaged cell, including hypertrophy, hyperplasia and ROS production. In the situation of

normoxia, HIF-1 α , the regulatory subunit of HIF-1, is continuously hydroxylated on Proline by PHDs (Prolyl Hydroxylases) and their usage of O₂ for giving OH groups. When hydroxylated, HIF-1 α is redirected to the proteasome and degraded. In the situation of hypoxia, O₂ is not available to participate to the hydroxylation reaction and HIF-1 α , avoiding proteasome-dependent degradation, can move to the nucleus, dimerize with HIF-1 β and activate the transcription of specific genes such as IL-1 β , IL-10, Vascular endothelial growth factor (VEGF)-A, Lactate dehydrogenase (LDH)-A, Pyruvate kinase M2 (PKM2), Glucose Transporter (GLUT)1 and hexokinase (6). During hypoxia conditions, mitochondrial ROS are critically involved in the stabilisation of HIF-1 α , as suggested by ROS sensitive probes becoming more responsive in presence of low oxygen levels (7); LPS mimics hypoxia conditions when macrophages are activated.

Mitochondrial complex I has also been indicated as a regulator of IL-1 β production (8). In their work, Mills et al. showed that rotenone, a complex I inhibitor, decreased LPS-induced ROS generation in bone marrow-derived macrophages (BMDMs) and led to diminished IL-1 β expression. AOX-expressing mice, where electrons are diverted from the electron transport chain (ETC) and do not cause oxidative stress, had lower amounts of LPS-generated ROS and IL-1 β . Furthermore, the use of antioxidants such as MitoTEMPO, N-acetyl cysteine (NAC) and MitoQ also impaired the induction of HIF-1 α and IL-1 β after LPS priming. Overall, it was concluded that reverse electron transport (RET) from Complex I was driving ROS production, promoting IL-1 β transcription via HIF-1 in TLR4-activated macrophages (8).

1.2.2 NLRP3 inflammasome

NLRP3 is a member of the NOD-like receptors (NLRs) family of PRRs. They are structurally characterized by the presence of a central nucleotide-binding and oligomerisation (NACHT) domain flanked by a C-terminal leucine rich repeat (LRR) domain and an N-terminal caspase-recruitment (CARD) or pyrin (PYD) domain (9). These components associate in order to form large intracellular protein complexes called inflammasomes which activate caspase-1 as an effector molecule to cleave

pro-IL-1 β and pro-IL-18 into their active forms prior to their release from the cell (10). Caspase-1, in addition to cleavage of these cytokines, also cleaves the protein gasdermin D (GSDMD) into its active n-terminal subunit, which inserts into the cell membrane and initiates a programmed form of inflammatory cell death named pyroptosis (11, 12). Inflammasomes respond rapidly to general clues of cellular stress and damage and their activation usually requires two signals: a priming signal (signal 1) in order to induce transcriptional upregulation of pro-IL-1 β and pro-IL-18, followed by a second activation signal (signal 2) which differs depending on the specific inflammasome (13).

The NLRP3 inflammasome complex consists of the central NLRP3 protein, the adaptor protein Apoptosis-associated speck-like protein containing a CARD domain (ASC) and the effector protein pro-caspase 1. NLRP3 itself consists of a LRR motif, a nucleotide-binding oligomerisation domain (NOD) and a PYD domain which interacts with the PYD domain of ASC (14, 15). Similarly, the CARD of ASC interacts with the CARD domain of pro-caspase 1, which autocatalytically cleaves to generate the active intermediate subunits p10 and p33. Further processing then generates the subunits p10 and p20 (15). Upon inflammasome activation, the active caspase-1 subunits carry out the effector functions of the inflammasome complex. The mitotic kinase NIMA-related protein kinase (NEK) 7 is another component of the NLRP3 inflammasome complex, and is required for NLRP3 oligomerisation to occur (16).

The NLRP3 inflammasome is the most well-characterised of the inflammasomes but there is still no unifying mechanism for its activation. The main issue regarding NLRP3 activation is that it may be triggered by a wide range of different stimuli, in contrast to most other inflammasomes which respond to more specific triggers.

Potassium efflux is a common mechanism for various triggers of inflammasome activation including Adenosine triphosphate (ATP), Nigericin, pore-forming toxins and the phagocytosis of particulate matter (17, 18). The presence of extracellular ATP is usually indicative of dying cells and activates the NLRP3 inflammasome in neighbouring cells by binding to the P2X7 channel, which opens to allow potassium efflux from the cell. Potassium efflux and subsequent inflammasome activation can

simply be achieved by incubating cells in hypotonic solutions (19), demonstrating that it is sufficient for NLRP3 activation.

Phagocytosis of particulates including crystalline material (monosodium urate (MSU) crystals) (20) and protein aggregates (e.g., amyloid- β (21)) induces cathepsin-dependent lysosomal damage and subsequent K^+ -dependent inflammasome activation (21). This is a characteristic underlying mechanism for pathological conditions. For example, MSU crystals are associated with gout (20), and amyloid- β is associated with Alzheimer's disease (21).

K^+ efflux is nowadays one of the most well-established mechanisms for NLRP3 inflammasome activation, but more studies have to be done in order to better define its dynamics and how it can regulate the concentration gradient of other ions. Similar to this, other stimuli involving ions movement through cellular bilayers could be of great interest in this context. Moreover, *Trans*-Golgi disassembly has been proposed as a unifying mechanism for NLRP3 activation downstream of potassium efflux and other triggers (22).

The regulatory mechanisms for IL-1 β are depicted in **figure 1.1**.

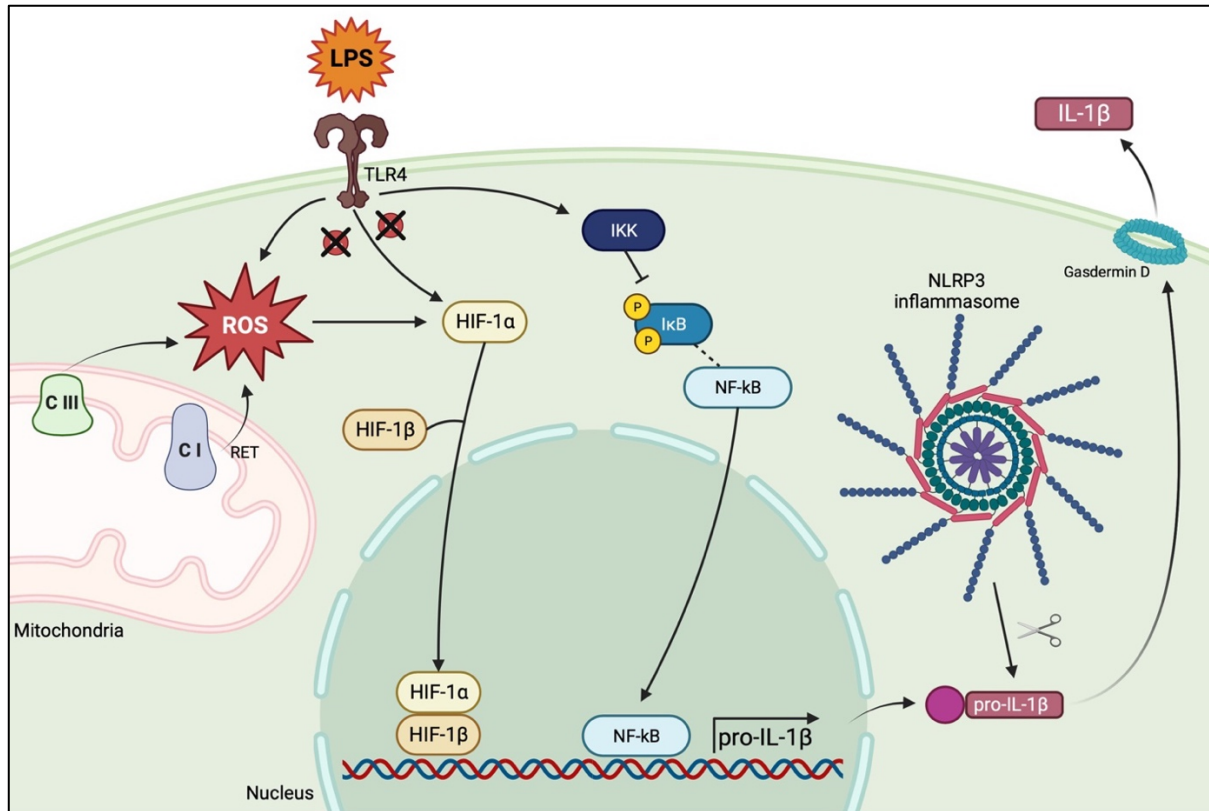


Figure 1.1 Production of IL-1 β following NLRP3 inflammasome activation.

LPS binding to TLR4 mimics hypoxia conditions, decreases O₂ presence and generates ROS production from mitochondrial Complex I, through RET, and Complex III. ROS stabilises HIF-1 α , which can dimerise with HIF-1 β and move to the nucleus to start the transcription of pro-IL-1 β . Furthermore, LPS can drive IKK-mediated phosphorylation of Ser residues on I κ B, which detaches from NF- κ B. NF- κ B then translocates to the nucleus to drive pro-IL-1 β . The inactive form of IL-1 β is successively cleaved into its active form by the NLRP3 inflammasome. Finally, IL-1 β can exit the cell through Gasdermin D pores.

1.2.3 IL-10 production by macrophages

IL-10 is another cytokine of particular interest in this thesis. It is a dimer consisting of 2 α -helix chains, IL-10 α and IL-10 β and it is produced as an anti-inflammatory and pro-fibrotic cytokine, together with TGF- β . It is encoded by the IL-10 gene on chromosome 1 in humans and it is responsible for dampening the activation of the immune system and exerts negative feedback action in the context of infection. Both cells of the innate and adaptive immune response produce it and those include macrophages, neutrophils, eosinophils, natural killer (NK) cells, Th1, Th2 and Th17 cells, regulatory T cells and B cells (23).

The receptor for IL-10 is a 110 kDa transmembrane glycoprotein formed by two chains (IL-10R1 and IL-10R2), which belong to class II of the cytokine receptor family, which also include receptor for interferons (IFN)s. The R1 chains of IL-10 receptors are the high-affinity chains for ligand binding, while the R2 chains have weak or no direct binding to the ligands (24). Only during the last few years, IL-10R2 has been shown to bind to newly discovered cytokines such as IL-26, IL-28 and IL-29 (25).

IL-10 biological effects consist of regulation of the expression of pro-inflammatory immune mediators (26), phagocytosis, antigen presentation, cellular programs of cell death and costimulation. It also plays a role in the differentiation of specific subsets of mast cells, T and B cells.

Because of its suppressive function on various immune cells, IL-10 and its downstream signalling is involved in the pathogenesis of several autoimmune diseases such as Systemic Lupus Erythematosus (SLE), Crohn's Disease (CD), Rheumatoid Arthritis (RA), Multiple Sclerosis (MS), psoriasis and Sjogren's syndrome (SS) (27). In SLE, excessive activation of Th2 cells directly correlates with disease progression and patients have increased levels of IL-10 mRNA and protein (28). Moreover, Single Nucleotide Polymorphisms (SNPs) in the IL-10 gene can increase susceptibility to develop the disease (29).

IL-10 mRNA levels are elevated in synovial biopsies from RA patients (30). In MS however, IL-10 induces cytotoxic T cells to cause demyelination in the Central Nervous System (CNS) (31). In psoriasis, high IL-10 levels can be detected in the biopsies from patients (32).

IL-10 can regulate IL-2, IL-6, IFN- γ and IL-12 (33) and repress TNF- α production (34). IL-10 inhibits all the inflammatory effects caused by LPS on macrophages such as mitochondrial dysfunction, ROS boost and downregulation of oxidative phosphorylation (OXPHOS), thus promoting mitochondrial fitness and autophagy. Moreover, IL-10 impairs the promotion of vasodilation and of programmed cell death induced by TNF- α and drives the recruitment of tolerogenic cells from adaptive immunity during organ transplantation (35).

IL-10 also represents an essential signal for survival and cell function (36). For maintaining intestinal homeostasis, CD4⁺ T regulatory type 1 (T_R1) cells produce huge amounts of IL-10 and express IL-10R1. Both *in vivo* and *in vitro*, T_R1 cells respond to IL-10, accompanied by the phosphorylation of the transcription factor signal transducer and activator of transcription (STAT) 3. For this reason, T_R1 cells are considered in clinical trials as T-cell therapy for patients with inflammatory bowel disease (36).

During helminth infection, BMDMs can rapidly produce huge quantities of IL-10. The internalisation of vesicles containing *S. Mansoni* by macrophages provoke ERK1/2, p38 and cAMP response element-binding protein (CREB) phosphorylation through TLR2/4 engagement. Increased IL-10 and decreased IL-12 production were also detected in resident skin macrophages exposed to the parasite (37).

Apart from TLR4, other TLRs can drive IL-10 production in different settings (38). TLR3 is an intracellular sensor for viral and host dsRNA which has an immunomodulatory role during *M. Tuberculosis* infection. The study published by Ge et al. (39) showed that peritoneal macrophages from TLR3^{-/-} mice infected with *Bacillus Calmette-Guérin* (BCG) had lower IL-10 and higher IL-12 expression 3 weeks after infection. The treatment of infected macrophages with a Phosphatidil-

inositol-3 kinase (PI3K) inhibitor exacerbated the same effects induced by the bacteria, demonstrating a clear mechanism driving IL-10 production. As a downstream consequence, TLR3^{-/-} infected mice had higher IFN- γ in the lungs and higher populations of CD4⁺IFN γ ⁺ Th1 cells, which contained *Mtb* burden (39).

TLR9 and TLR8 sense viral DNA containing CpG unmethylated motifs and uridine-rich ssRNA, respectively. Triggering the activation of both of them has been shown to induce IL-10 production in microglia (40), peritoneal and spleen cells (41) and in human peripheral blood mononuclear cells (PBMCs) (42, 43).

Also TLR7, which is another intracellular sensor for ssRNA, can drive IL-10 production in human monocyte-derived macrophages and dendritic cells (44).

Of major relevance to this thesis, IL-10 has also been shown to inhibit anti-tumour immunity. The blockade of IL-10 in the tumour microenvironment leads to a functional change in the immune populations fighting the cancer cells, such as Th1 and Th2 lymphocytes and conventional and plasmacytoid dendritic cells. IL-10 blockade increases vaccine-induced antigen-specific CD8⁺ T cell responses (45).

Tumour antigen-specific CD8⁺ T cells isolated from the peripheral blood of melanoma patients have upregulated IL-10R along with programmed cell death protein (PD)-1 upon TCR activation *in vitro*. IL-10 impedes the expansion of the tumour antigen-specific CD8⁺ T cells upon antigen stimulation, while IL-10R and PD-1 blockade increase the expansion and functions of the tumour antigen-specific CD8⁺ T cells. CD8⁺ tumour-infiltrating lymphocytes (TILs) present in metastatic melanoma co-upregulate IL-10R and PD-1. The blockade of these two factors promotes the proliferation of CD8⁺ TILs (46) and anti-tumour immunity.

Furthermore, the use of antibodies against IL-10 is well established for people experiencing pathogen infection. For example, the targeting of IL-10 improved PBMCs immune activity and responsiveness against infection in humans affected by visceral Leishmaniasis (47), tuberculosis, leprosy and schistosomiasis (48).

By contrast, systemic administration of recombinant IL-10 has been proved effective in clinical trials with patients affected by autoimmune diseases such as

Chron's diseases (CD), Rheumatoid arthritis (RA) and psoriasis. IL-10 treatment in people affected by psoriasis led to the downregulation of pro-inflammatory cytokines like IL-1 β , TNF- α and IL-12p40 from PBMCs (49).

Possible TLR-dependent mechanisms promoting IL-10 production are summarised in **Figure 1.2** whilst **figure 1.3** depicts therapeutic strategies involving IL-10.

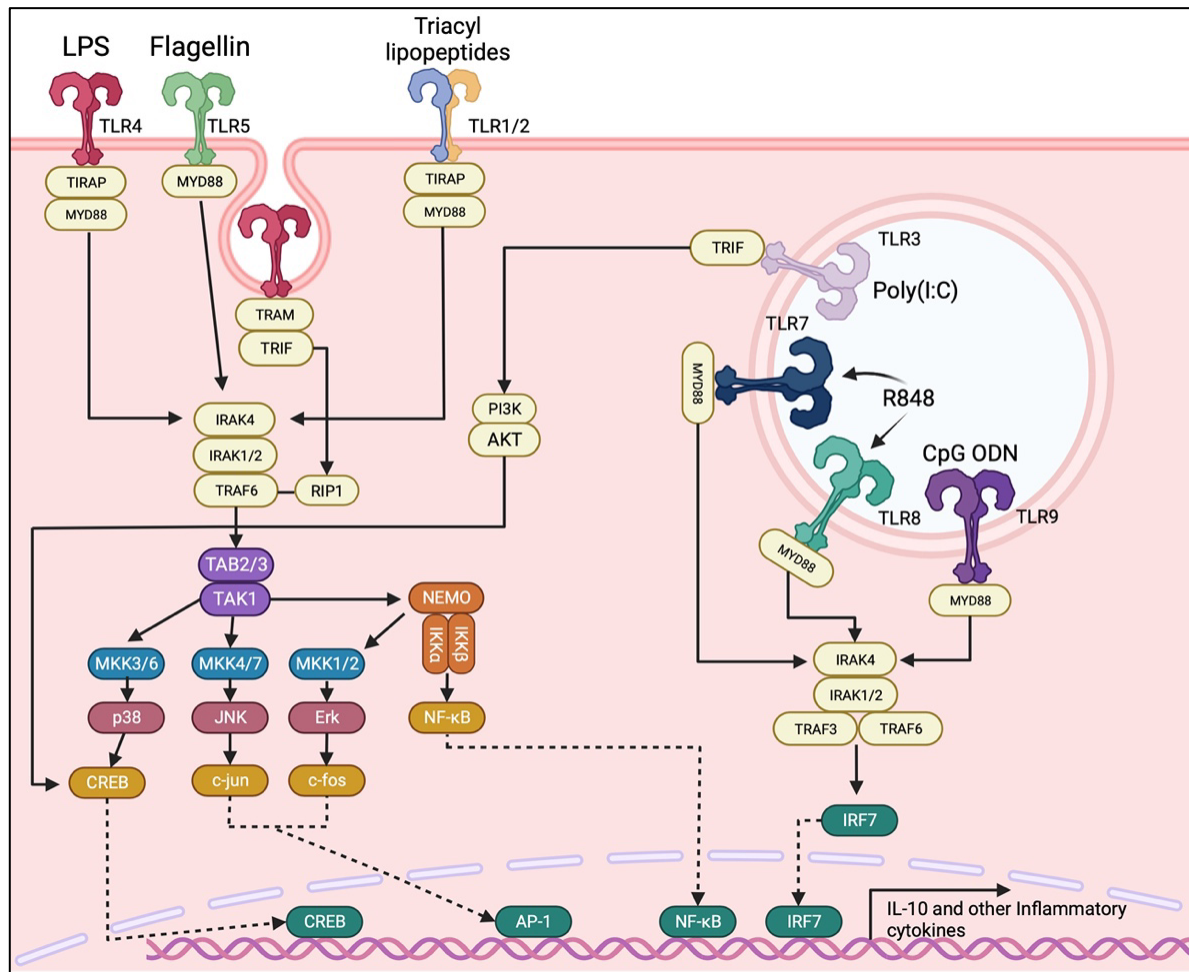


Figure 1.2 Intracellular signalling pathways driving IL-10 production in cells.

The extracellular TLR4, TLR5 and the dimer TLR1/2 bind to LPS, flagellin and triacyl lipopeptides, respectively. Through the adaptor proteins Myd88 and TIRAP, they activate IRAK4 and TRAF6, which induce TAK1 activation. This last protein then phosphorylates MAPKs, which allow the translocation to the nucleus of transcription factors such as CREB, AP-1 and NF-κB and their binding to the IL-10 gene promoter. The intracellular TLR3 binds to poly(I:C) and activate the PI3K/Akt pathway which activates CREB transcription factors. Also the intracellular TLR7, TLR8 and TLR9 bind to R848 and CpG ODN respectively and can drive IL-10 production most likely via similar Myd88 mechanisms.

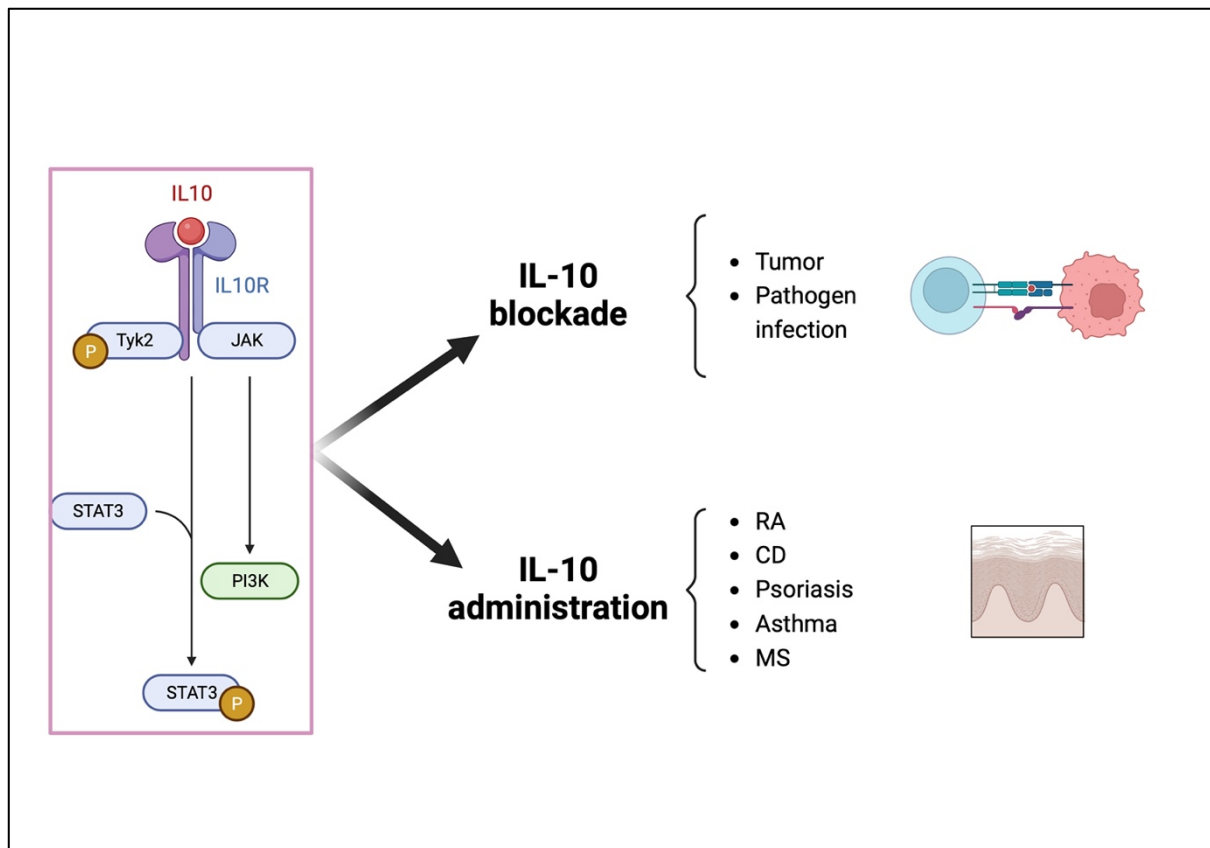


Figure 1.3 Therapeutic strategies involving IL-10.

IL-10 blockade represents one of the main immunotherapies which is undergoing development in the last decade. Antibodies against IL-10 are also used as therapy in people experiencing pathogen infections. Moreover, IL-10 is administered to patients affected by autoimmune diseases such as Rheumatoid Arthritis (RA), Chron's disease (CD), psoriasis, Multiple Sclerosis (MS). IL-10 administration is also considered in the treatment of asthma.

1.2.4 TNF- α and IL-6 cytokines

TNF- α is a homotrimer which is produced by primed phagocytes, NK and T cells and which binds to its type 1 and type 2 receptors (TNFR1 and TNFR2) (50). It triggers NF- κ B (51) and MAPK (52) pathways that could finally lead to amplification of the inflammatory response and cell death in the form of apoptosis, pyroptosis or necroptosis (53).

IL-6 is a pleiotropic cytokine formed by a bundle of 4 α -helices and binds to its receptor IL-6R α on the surface of macrophages, neutrophils, lymphocytes and hepatocytes, driving a JAK-STAT1/3-dependent signalling pathway (54, 55). It promotes vasodilation, synthesis of acute phase proteins, differentiation of T cells and other main processes involved in the host defence against infections and stress (56).

Therapies inhibiting TNF- α and IL-6 have been developed and are efficacious in diseases such as rheumatoid arthritis (RA) and in the case of IL-6 blockade in COVID19 (57, 58, 59).

1.3 Innate immunity and metabolic reprogramming

This thesis concerns an analysis of the role of mitochondrial metabolism, specifically complex I and complex III in the ETC and in cytokine production by macrophages.

During the last decade “immunometabolism” has become one of the most innovative and attractive branches of immunology. Today we refer to immunometabolism as the study of metabolic pathways in immune cells and how they change in response to exogenous and endogenous stress triggers. Previous findings have shown that B and T lymphocytes, dendritic cells (DCs), NK cells, eosinophils and macrophages face modulation of their anabolic and catabolic

processes in order to generate ATP for their energy needs in protection from microorganisms (60). Immune cell functionality can change depending on the type of cell considered, the nature and size of the stimulus, the exposure to it, the microenvironment where the cells reside and the metabolic pathway which they engage.

The role of mitochondria in immunometabolism has been a major area of interest. This project concerns the ETC in macrophage mitochondria. The molecules derived from glucose and also amino acid catabolism are used in the tricarboxylic acid (TCA) cycle, also known as Krebs cycle, are summarised in **figure 1.4** and **figure 1.5** and generate substrates to feed OXPHOS and ETC (61).

Apart from cytokine production, macrophages also release arachidonic acid-derived eicosanoids, phagocytose apoptotic cells, present antigens to T cells and promote their clonal expansion, and release chemoattractant molecules to recruit other immune cells to the site of infection (62). To enable these processes, there are numerous metabolic pathways which may be engaged by macrophages varying from glycolysis, which involves the conversion of glucose imported into the cell to lactate, pyruvate, or to feed the TCA cycle. Furthermore, the pentose-phosphate pathway diverts intermediates from glycolysis towards nucleotide and amino acid production, while fatty acid synthesis and β -oxidation are two complementary tracks with the target of restoring short and long-chain lipids and cholesterol balance. Finally, *de novo* synthesis of amino acid, glutamine and tryptophan can occur from the TCA cycle (63).

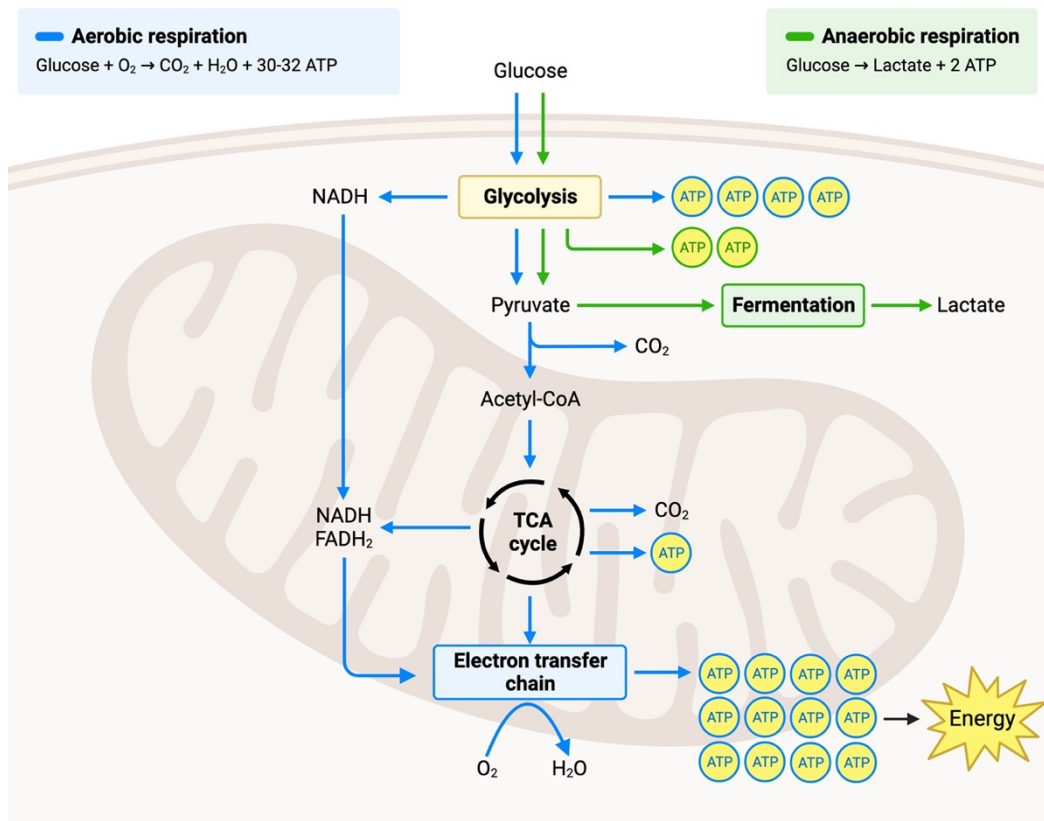


Figure 1.4 . Respiration in mammalian cells.

When glucose enters the cell, the process of glycolysis starts in the cytosol. Glycolysis is part of both aerobic and anaerobic respiration and generates 2 molecules of ATP, NADH and pyruvate as final products. Pyruvate is converted to lactate in anaerobic conditions (fermentation) or enters the mitochondria where it is converted to Acetyl-CoA. Acetyl-CoA takes then part to the TCA cycle, which is followed by the process of OXPHOS. The molecules of NADH generated from glycolysis and Krebs cycle are used as electron donors together with oxygen during the electron transfer. OXPHOS generates energy (in the form of 30-32 molecules of ATP) and water as final products.

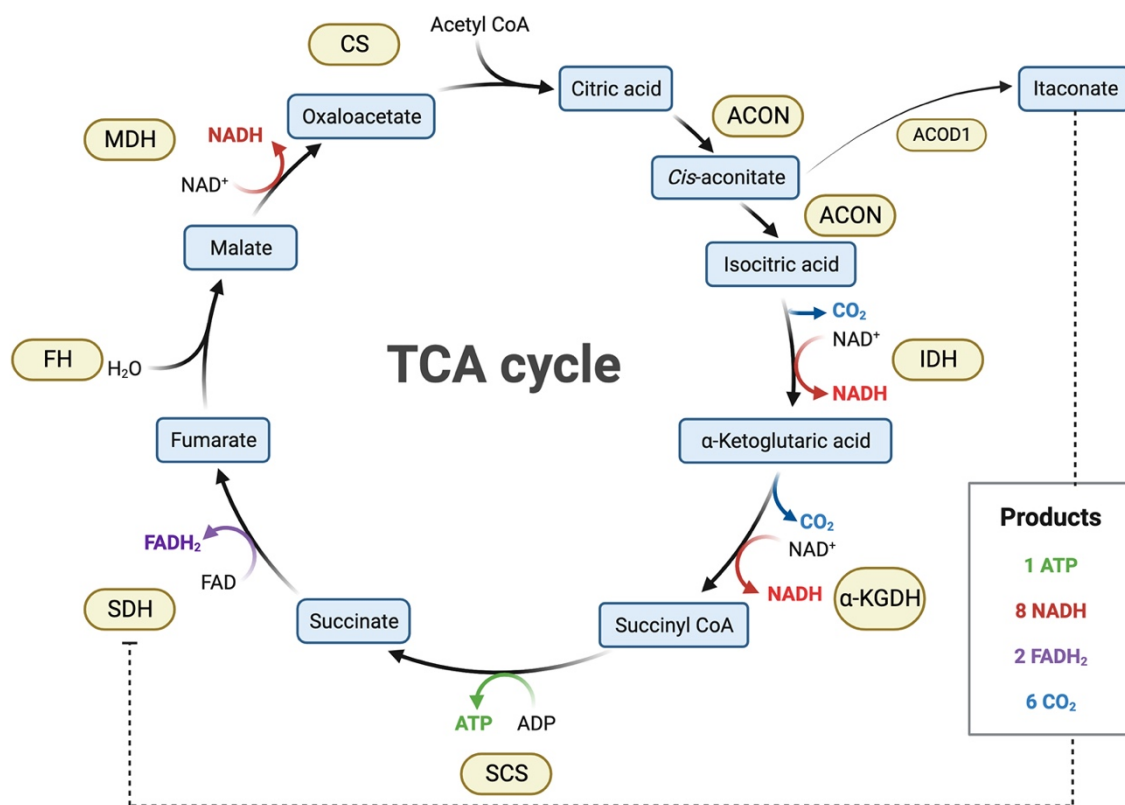


Figure 1.5 . TCA cycle revisited.

Acetyl-CoA enters the mitochondria and is condensed with oxaloacetate into citric acid by the enzyme citrate synthase. Citrate is then isomerised to isocitric acid by aconitase and through the intermediate *cis*-aconitate. Itaconate is generated from *cis*-aconitate by the enzyme aconitate decarboxylase 1. Isocitrate dehydrogenase transforms isocitric acid into α -ketoglutaric acid through NAD^+ reduction and CO_2 generation. α -ketoglutaric acid is converted to succinyl-CoA by α -ketoglutaric acid dehydrogenase, reducing NAD^+ and generating CO_2 . Succinyl-CoA becomes succinate through ATP production by succinyl-CoA synthetase. Succinate is then oxidized to fumarate by succinate dehydrogenase and FADH_2 is produced during this reaction. Fumarate is hydrated to malate by fumarate hydratase. Finally malate becomes oxaloacetate again thank to the enzyme malate dehydrogenase and another molecule of NADH is formed. Oxaloacetate is ready to start a new reaction with acetyl-CoA. Itaconate can inhibit succinate dehydrogenase activity.

1.3.1 The electron transport chain driving oxidative phosphorylation

As depicted in **figure 1.4** and **figure 1.5**, OXPHOS represents the major mechanism for ATP production before glycolysis in aerobic organisms. This process involves the inner membrane of mitochondria, where the ETC through different complexes generates a proton gradient able to finally synthesize ATP, as illustrated in **figure 1.6**. These redox reactions are aimed at transporting electrons from donor molecules such as FADH_2 and NADH to the final electron acceptor, the O_2 molecule. Four complexes are located along the inner mitochondrial membrane: complex I (NADH dehydrogenase), complex II (Succinate dehydrogenase, SDH), complex III (Cytochrome C reductase) and complex IV (Cytochrome C oxidase). Through the first complex, NADH is oxidized and gives electrons to flavin mononucleotide (FMN), that becomes FMNH_2 . FMNH_2 is then oxidized again and transfers electrons to complex I, that is then able to reduce Coenzyme Q, which becomes QH_2 . During these reactions, four protons are pumped out from the mitochondrial matrix. Complex II is an enzyme involved in the TCA cycle and catalyses the oxidation of succinate into fumarate. Complex III, a key focus of this thesis, is responsible for the electron transport from QH_2 , which when oxidized again becomes Q, to cytochrome b and cytochrome c, while four protons exit the mitochondrial cytoplasm. The last step of the ETC is represented by the oxidation of cytochrome c and the reduction of O_2 through electron transport to cytochrome a and cytochrome a_3 in Complex IV; this process brings two other protons from the mitochondrial matrix to the intermembrane space (61).

The proton gradient that has been formed across the mitochondrial membrane is used as an electrochemical potential to generate ATP with the enzyme ATP synthase from ADP and inorganic phosphate (61). As a final result, all the protons return to the mitochondrial matrix via the ATP synthase. The process is outlined in **figure 1.6**.

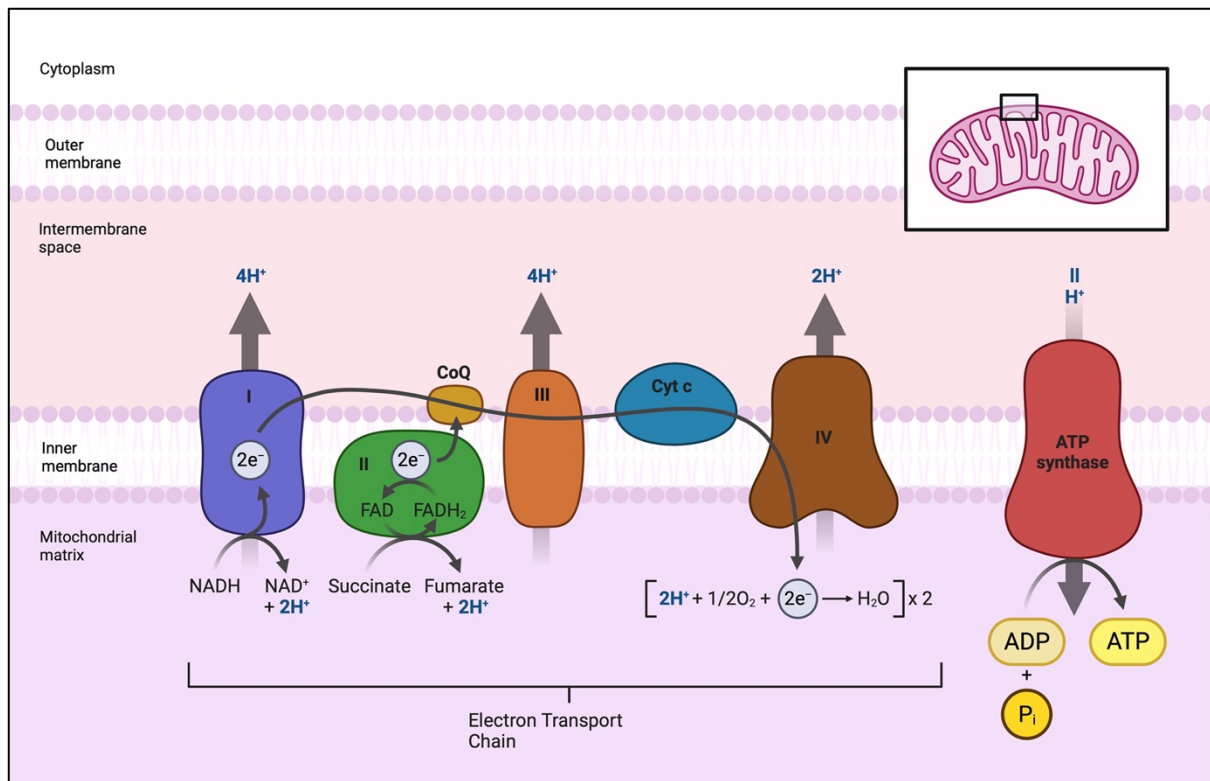


Figure 1.6 Mitochondrial electron transport chain.

Oxidized NADH transfer electrons to complex I, that is then able to reduce Coenzyme Q. During these reactions, four protons are pumped out from the mitochondrial matrix. Reduced CoQ transfers electrons to complex III, which then negatively charges cytochrome c, while four protons exit the mitochondrial cytoplasm. Next, cytochrome c transfers electrons to complex IV, reducing O₂. Finally, all the protons in the intermembrane space return to the matrix through complex V, which generates ATP from ADP and inorganic phosphate (P_i).

The correct flow of electrons through all the mitochondrial complexes, from complex I to complex IV, is called forward electron transfer (FET), while RET is produced when electrons from ubiquinol are transferred back to respiratory complex I, reducing NAD^+ to NADH. RET is a major source of ROS within a cell, generating free radicals including superoxide anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot\text{OH}$) and hydroperoxyl radical ($\text{HO}_2^{\cdot}$) (64). In turn, $\text{O}_2^{\cdot-}$ can be dismutated to H_2O_2 , which is stable and can diffuse through cellular bilayers (64). $\text{O}_2^{\cdot-}$ production from the ETC depends on several factors like membrane potential, electron flux, O_2 presence and concentration of the several substrates feeding the respiratory chain and ATPase activity (64, 65). $\text{O}_2^{\cdot-}$ can be generated from the FMN cofactor and the Q binding site at Complex I and from the site of QH_2 oxidation at Complex III. Also Complex II can generate ROS at the flavin site, but the level produced can be considered physiologically low (66). The ROS that are generated can behave as signalling molecules and, if in excess, damage cellular proteins and lipids through electrophilic peroxidation, thus leading to cell dysfunction and death (67).

A part of the electrochemical potential is dissipated through processes called basal and inducible proton leak, electron leak and electron slip. Protons can directly escape from the inner mitochondrial space by diffusion through it, a process named proton leak. Basal proton leak can happen via uncoupling proteins (UCPs) and adenine nucleotide translocases (ANTs), whose expression is regulated by inner membrane hyperpolarization and ROS levels. In addition to that, the mitochondrial ATP-sensitive potassium channel ($\text{mitoK}_{\text{ATP}}$) mediates uncoupling in response to superoxide and H_2O_2 concentrations (65).

Induced electron leak is finely regulated and catalysed only by uncoupling proteins (UCPs), of which there are five (UCP1-5) and are present as dimers in the inner mitochondrial membrane. ATP, ADP, GTP and GDP can inhibit UCPs while fatty acids can activate them (66).

Electron slip consists of the transfer of electrons through the respiratory complexes without proton pumping and leads to an excessive increase in oxygen consumption in the presence of high protonmotive force (68).

Compounds which are referred to as 'uncouplers' inhibit the normal electron flux through the complexes and lead to OXPHOS defects (68). Besides the common mitochondrial uncouplers such as UCPs and ANTs, natural and synthetic uncouplers are used to study the cellular response to mitochondrial respiration dysfunction in living cells. Free fatty acids (FFAs), the thyroid hormone T3 and capsaicins are well known to uncouple mitochondria in a direct or indirect manner, thus activating several UCPs. Artificial uncouplers like Carbonyl cyanide p-trifluoromethoxyphenyl hydrazone (FCCP) and carbonylcyanide-3-chlorophenylhydrazone (CCCP) can easily traffic across biological membranes and allow the proton flow across the membrane, due to their hydrophobic nature (68).

1.3.2 Reprogramming in mitochondria of immune cells

Mitochondria are dynamic organelles, which undergo modification of their functional and structural properties when immune cells are activated by various stimuli. These modifications can include disturbance of respiration, fission and fusion dynamics, change in microtubule arrangement or in the expression of each respiratory subunit. One newly discovered role for them involves also the release of mitochondrial DNA which acts as signalling molecule. Importantly in response to LPS, in macrophages there is a decrease in OXPHOS and the two breakpoints of the Krebs cycle (69) at succinate dehydrogenase (SDH) level, leading to succinate accumulation, and at isocitrate dehydrogenase (IDH), leading to citrate accumulation (**figure 1.7**). Mitochondria also show a smaller size, fragmentation, dispersion into the cytosol and decreased mitophagy in activated macrophages and microglia (70, 71). This last phenomenon explains the LPS-induced fragmentation and Drp1-dependent fission of mitochondria (72). The same fission outcome could be reached with other TLR ligands such as poly(I:C), together with the suppression of the canonical fusion mediators *Mfn1/Mfn2* and *Fam73b*. (73).

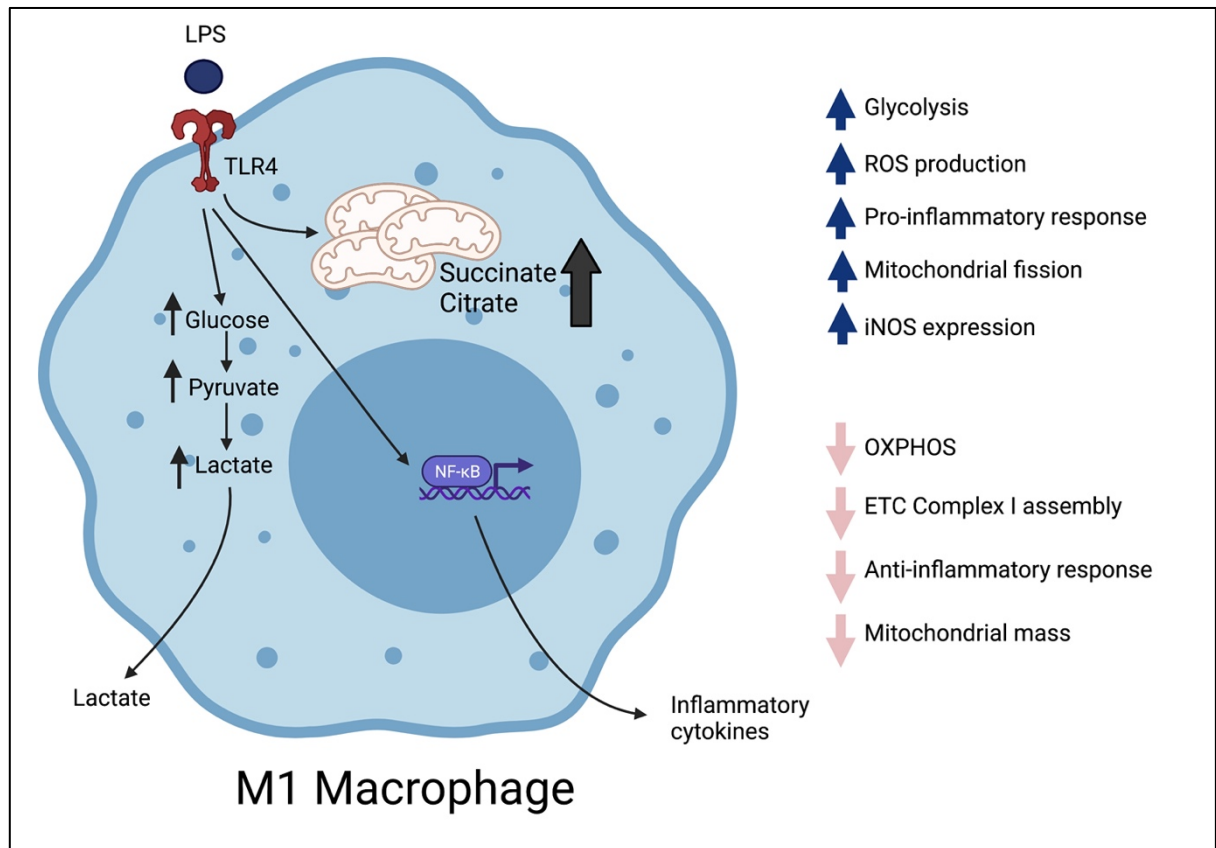


Figure 1.7 Mitochondria and electron transport chain involvement in macrophage metabolic reprogramming.

When macrophages are activated by LPS through TLR4, they undergo several metabolic changes. Pro-inflammatory M1 macrophages show a decrease in OXPHOS and a boost in glycolysis. The metabolites of the TCA cycle citrate and succinate are accumulated. The expression of inducible Nitric Oxide Synthase (iNOS) is increased, and mitochondrial fission is induced, together with a strong ROS generation. ETC Complex I assembly is dampened and there is a decrease in mitochondrial mass (74).

Mitophagy, the selective process for eliminating damaged mitochondria by autophagy, is finely regulated during activation of macrophages: LPS/IFN- γ stimulation increases mitochondrial ROS production and inhibits Parkin, PINK1 and DJ-1-dependent mitophagy. BMDMs from mice lacking STAT1 protein were not affected, suggesting a role for STAT1 in regulating mitophagy. Indeed, researchers revealed that STAT1 regulates caspases 1 and 11, which are induced by LPS and are involved in the inhibition of mitophagy by decreasing PINK1 protein levels. RNA silencing of caspase 1 or 11 improved mitophagy, having the same outcome as STAT1-silencing (75). *In vivo*, the administration of the uncoupler 2,4-DNP, which promotes mitophagy, reversed the LPS-induced inhibition of mitophagy in peritoneal macrophages (75).

When activated macrophages show a decreased mitochondrial membrane potential, mitochondrial dysfunction and a less controlled mitophagy, they can be rescued by immunoregulatory cytokines such as IL-10 (76). Additionally, also mitochondria-dependent metabolic pathways can be affected if mitochondria are dysfunctional. For example, PAMPs trigger fatty acid synthesis and diminish fatty acid oxidation by downregulation of the mitochondrial transporter Carnitine Palmitoyltransferase 1 (CPT-1) (74, 77).

Naïve CD8⁺ T cells have small and fragmented mitochondria and increased OXPHOS while effector T cells have increased mitochondrial fission which accompanies glycolytic activity. Memory CD8⁺ T cells show increased mitochondrial fusion and mass and their generation is promoted if Dynamin-related protein (Drp) 1 is knocked down and OPA1 (mitochondrial dynamin function like GTPase) is overexpressed (78).

LPS stimulation leads to an elevated mitochondrial membrane potential ($\Delta\Psi_m$), which together with enhanced SDH oxidation of succinate leads to the accumulation of mtROS (74). MtROS are used by macrophages to exert bactericidal activity (79), as shown by West et al. Their work is focused on the engagement of TLR1, TLR2 and TLR4 in macrophages and the subsequent recruitment of tumor necrosis factor receptor-associated factor (TRAF) 6 to

mitochondria, where it binds to Evolutionarily conserved signalling intermediate in Toll pathway (ECSIT), a complex-I associated protein, promoting its ubiquitination. Both TRAF6 and ECSIT knockdown in LPS-activated BMDMs lead to a decrease in cellular and mitochondrial ROS production (80, 81).

Importantly for this thesis, recent data support the hypothesis that innate sensing of microbial cues leads to the assembly of the ETC complexes in supercomplexes (SCs) to adapt the electron flow to the substrates available. Upon TLR engagement, less SCs including Complex I are formed in macrophages and there is an increase of the free form of Complex III, while the activity of Complex II and glycerol 3-phosphate dehydrogenase are increased, raising the usage of FADH₂ (82).

1.3.3 Mitochondrial Complex I and III involvement in the immune response

Both Complex I and Complex III represent fundamental cores of the ETC and have been demonstrated to play a role in shaping the behaviour of immune cells.

As mentioned above, in LPS-activated macrophages, Complex I is required for the production of IL-1 β (83). Kelly et al. showed that rotenone, a Complex I inhibitor, inhibited the pro-form of the inflammatory cytokine and boosted IL-10 expression. Metformin, a treatment for type II diabetes and well known inhibitor of complex I, had the same effects as rotenone and blocked complex I activity and ROS production, suggesting a novel role for the drug in immune-mediated pathologies (83). The suppression of Complex I-derived ROS lead to the same effect on IL-1 β in LPS-activated macrophages (8).

In T lymphocytes, complex I has been shown to be necessary for the proliferation of T cells and their differentiation into Th₁, Th₂ and Th₁₇ cells (84). Rotenone treatment of CD4⁺ T cells cultured in Th₁ conditions decreased the ATP/AMP ratio and increased the NADH/NAD⁺ ratio, lowered the cellular levels of aspartate and blocked their proliferation. Aspartate supplementation of rotenone-treated T cells ameliorated their proliferation (85). Furthermore, CD8⁺ T cells treated with rotenone displayed lower proliferation and expression of CD69, which is a marker

of T-cell receptor (TCR) stimulation. The inhibition of complex I suppressed H₂O₂ generation, calcium flux and ERK1/2 phosphorylation, all signal transducers for cell growth (86). Also, IFN- γ , TNF- α and degranulation levels were negatively affected by rotenone (86).

Complex III is a major focus of this thesis. It is responsible for cellular survival in health and pathological conditions through several mechanisms. A recent work from Kallijarvi J. et al (*bioRxiv*, 2023) shows that mice with respiratory complex III deficiency display nuclear DNA damage, cell cycle arrest, aberrant mitoses and cellular senescence in the affected organs such as liver and kidney. Complex III deficiency triggered pre-symptomatic cancer-like c-MYC upregulation followed by excessive anabolic metabolism and boosted cell proliferation against lack of energy and biosynthetic precursors.

The role of complex III in activation of immune cells and mediation of the inflammatory response has not been thoroughly studied and is not well understood.

However, a paper from Chandel and colleagues (87) establishes mitochondrial Complex III as essential for regulatory T cell function. KO mice for the iron-sulfur protein subunit of Complex III showed decreased Treg function and resulted in the development of inflammatory diseases. The use of myxothiazol for the inhibition of ROS production by Complex III in macrophages resulted in decreased expression of costimulatory molecule CD80, Nitric oxide synthase (NOS) 2, pro-IL-1 β , IL-6 and TNF- α , indicating a role in these responses (87). Furthermore, the engagement of TLR4 by LPS and TLR3 by Poly(I:C) induced the remodelling of ETC complexes, thus producing mitochondrial ROS and sustaining the pro-inflammatory and anti-viral response. When BMDMs were treated with antimycin A and cyanide, Complex III and Complex IV inhibitors, respectively, pro-inflammatory cytokines such as IFN- β , CXCL10, TNF- α were decreased (88).

In contrast, under normal conditions, without any exogenous trigger such as LPS activating the cells, the use of Complex III inhibitors leads to an enhancement of ROS production by mitochondrial dysfunction and to an amplification of the inflammatory response. As shown by Vaamonde-García et al (89), pre-treatment

with antimycin A or oligomycin, which inhibit complex III and complex V, respectively, triggered a strong IL-1 β -induced IL-8 response.

Abraham et al. (90) investigated the role of Complex III in neutrophils during lung injury. The inhibition of complex III by myxothiazol and antimycin A provoked a boost in ROS production, which could be reversed by the use of pegylated catalase in both cases. Myxothiazol and Antimycin A had the same effect in mice lacking the p47^{phox} subunit of NADPH oxidase, highlighting the complete dependence on Complex III for the redox process. Treatment of LPS-stimulated neutrophils with the two inhibitors leads to downregulation of TNF- α and Macrophage inflammatory protein (MIP-2) and accumulation of NF- κ B in the cytosol through inhibition of its nuclear translocation. Antimycin A also inhibited LPS-induced I κ B- α degradation, which is the process contributing to NF- κ B translocation into the nucleus. The use of pegylated catalase restored the inflammatory phenotype of neutrophils and demonstrated that the mechanism is H₂O₂ dependent (90).

Moreover, Complex III derived ROS are essential for the activation of antigen-specific T cells. Another work by Chandel et al showed that mice lacking the *Uqcrrf1* gene, that normally encodes the Rieske Iron Sulfur Protein (RISP), a complex III subunit that is required for transfer of electrons downstream of complex III, had less peripheral CD8⁺ and CD4⁺T cells. *Uqcrrf1*^{-/-} CD4⁺ T cells failed to induce IL-2 and had lower expression of activation markers CD69 and CD25. Subsequently, these CD4⁺ and CD8⁺ T cells without the RISP complex had a decrease in mitochondrial ROS production but normal respiration and glycolytic metabolism were not affected. The whole *in vivo* expansion of the two cell subsets was also affected in the knockout mice (91).

1.3.4 ROS, mitochondria and NLRP3

ROS generated at mitochondrial sites can become signalling molecules if produced in excessive amounts.

As discussed above, while NLRP3 inflammasome activation mechanisms are still unclear, previous findings have demonstrated that ROS may act as one of its triggers (92). For example, inflammasome activation in response to ATP may be contributed to by Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived ROS (88). Certain triggers of NLRP3 activation, such as the TLR7 ligand imiquimod, have been shown to drive NLRP3 activation in a ROS-dependent manner (93). Interestingly, the Complex I and III inhibitors rotenone and antimycin A also drove NLRP3 activation through the generation of mitochondrial ROS (mtROS) (94). Moreover, ATP induces mitochondrial dysfunction, resulting in a release of oxidized mitochondrial DNA (mtDNA) into the cytosol, where it binds to and activates the NLRP3 inflammasome (95). The engagement of TLRs activates mtDNA synthesis through a MYD88/TRIF-IRF1-CMPK2 axis and the oxidized fragments of these nucleotides directly associate with NLRP3, triggering its activation (95). Defects in autophagy result in reduced mitochondrial clearance and subsequently, in inflammasome activation (96). In addition, structural components of mitochondria, such as the lipid cardiolipin, which is usually present in the inner mitochondrial membrane, translocates to the outer membrane upon mitochondrial disruption and directly interacts with NLRP3, thereby promoting IL-1 β processing (97).

A recent paper by Chandel et al. (98) confirmed the link between the ETC and NLRP3 inflammasome activation but excluded a ROS-dependent mechanism for it. The researchers used inhibitors of Complex I, II, III and V, which all blocked caspase-1 activation and IL-1 β protein expression in BMDMs. BMDMs from transgenic mice lacking Complex I and III and, subsequently, ROS production, did not recapitulate the same effects of the inhibitors, suggesting a ROS-independent regulation of NLRP3 inflammasome. Metabolomics analysis revealed that all the inhibitors decreased the LPS-induced production of the metabolite phosphocreatine (PCr), which is generated from creatine and ATP in mitochondria. Phosphocreatine is then transported to the cytosol where it generates ATP, which binds NLRP3 leading to inflammasome activation. BMDMs treated with cyclocreatine, a creatine analog that depletes phosphocreatine, showed diminished cleaved caspase-1 and secreted IL-1 β levels after LPS and ATP

stimulation. *In vivo*, administration of cyclocreatine also lowered IL-1 β levels in serum (98).

The mitochondria-related mechanisms which regulate the NLRP3 inflammasome are depicted in **figure 1.8**.

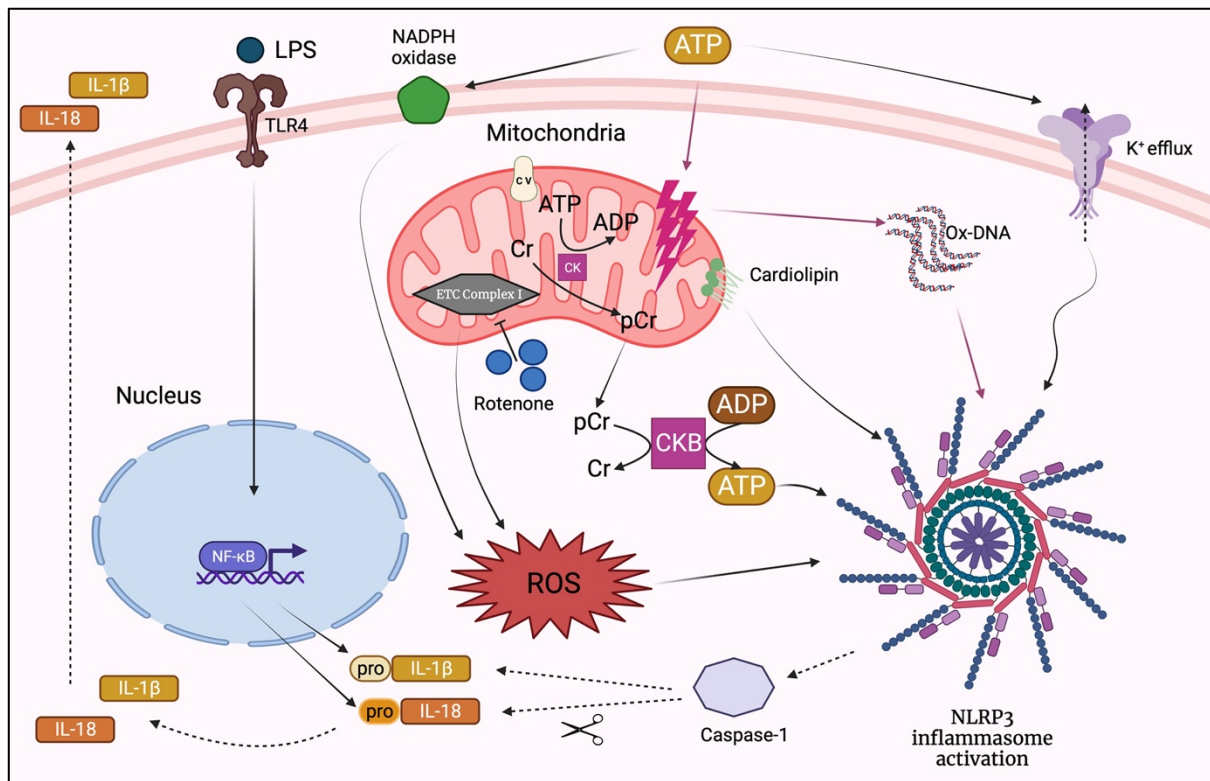


Figure 1.8. Mitochondrial signalling and NLRP3 inflammasome.

LPS (signal 1) activates TLR4 and induces NF-κB-driven transcription of specific inflammatory genes. NLRP3 inflammasome can receive several stimuli from mitochondria in order to be activated. ATP (signal 2) activates P2X7 channels on the phospholipidic bilayer triggering K⁺ ions efflux from the cell. ATP also induces NADPH oxidase-dependent ROS production. ATP leads to mitochondria dysfunction and promotes the release of oxidized-mitochondrial DNA which activates NLRP3. Cardiolipin, which usually is present along the mitochondrial inner membrane, can move to the external bilayer and directly interact with NLRP3 protein. Indeed, the inhibition of Complex I of the ETC by rotenone induces ROS generation, which in turn activates the inflammasome. Moreover, mitochondria-derived ATP phosphorylates creatine (Cr) to phosphocreatine (pCr), releasing ADP. pCr moves to the cytosol where it is converted by creatine kinase B (CKB) to Cr. The inorganic phosphate derived from this last phosphorylation is used to generate ATP from cytosolic ADP. Cytosolic ATP is then another factor which can trigger NLRP3 inflammasome via direct binding. Activated NLRP3 inflammasome cleaves inflammatory pro-IL-1β and pro-IL-18 into their active forms, which are released outside of the cell.

These findings indicate that mitochondrial dysfunction and the accompanied release of mitochondrial reactive oxygen species (mtROS) is thought to be required for inflammasome activation in a process in which potassium efflux may not be involved. Indeed, inflammasome activation due to mitochondrial disruption can be blocked using antioxidants and by the activation of the antioxidant transcription factor Nrf2 (99), indicating that mtROS are important triggers of inflammasome activation (100).

1.3.5 IL-10 regulation and its redox-dependent mechanism

IL-10 *per se* is responsible for ROS dampening and mitochondria rescuing, as exerting a negative feedback loop. For example, as shown by Medhazitov et al., IL-10 can revert the M1 glycolytic phenotype acquired by macrophages when activated by LPS, thus decreasing glucose uptake and targeting mTOR. This last inhibition suggest mitophagy as the fundamental process through which immune cells eliminate dysfunctional mitochondria (76).

IL-10 has been also shown to decrease ROS-dependent NF- κ B activation in LPS-activated RAW macrophages. In particular, IL-10 blocked NF- κ B mRNA expression and inhibited I κ B- α degradation. The use of ROS scavenger (catalase and sodium formate) before LPS stimulation mimicked the same effects as IL-10, thus suggesting a ROS-modulating action of IL-10 (101).

IL-10 appeared to prevent neurodegeneration after intracranial injection of LPS. Activated IL-10^{-/-} microglial cells had decreased levels of NLRP3, pro-Casp1, pro-IL-1 β and both their cleaved forms, compared to WT microglia. Together with that, microglia lacking IL-10 produced higher amount of ROS, which were dampened by pre-treatment with IL-10 (102).

Additionally, the study published by McCoy et al. in 2021 (103) shows that exogenous IL-10 treatment of LPS-activated macrophages enhanced the expression of mitochondrial arginase 2, an arginase protein which catalyses the hydrolysis of arginine to ornithine and urea, in a STAT3-dependent manner (103).

IL-10 administration also significantly decreased LPS-induced mitochondrial fission and restored OXPHOS in IL10^{-/-} BMDMs primed with LPS. This last effect was completely abolished in Arg2^{-/-} cells. LPS+IL-10 macrophages also showed accumulation of fumarate and malate metabolites, as a result of Arg2-dependent upregulation of Complex II activity. Finally, spleens from Arg2^{-/-} had a higher expression of HIF- α and IL-1 β , confirming an IL-10-linked anti-inflammatory role of mitochondrial arginase 2 (103).

Importantly, IL-10 appears to be induced by ROS as an anti-oxidant adaptive response. In the work published in 2010 by Ivashkiv et al., ROS production is considered an essential requirement to promote IL-10 release from zymosan-activated dendritic cells together with the triggering of calcium-dependent intracellular pathways such as Ca⁺⁺/Calmodulin pathway, ERK and CREB phosphorylation (104).

Moreover, in an *in vivo* model of non-infectious sepsis induced by endotoxin, mice lacking the plasma protein p47 of NADPH oxidase showed increased pulmonary inflammation and the development of peritoneal macrophages with pro-inflammatory characteristics because IL-10 production was decreased due to the absence of NOX-produced ROS. The mechanism involved Akt and GSK-3 β because p47^{nox-/-} showed a decrease in the activation of these two kinases. The restoration of immune defences and resolution of lung injury were achieved when p47-deficient mice were administered rIL-10 (105).

Because ROS can induce IL-10 production, naturally occurring antioxidants were reported to suppress its release. For example, PBMCs treated with N-acetyl cysteine displayed downregulation of IL-10 (106). In addition, the recent work from Suzuki et al. established a link between the anti-oxidant enzyme NQO1 (NAD(P)H quinone oxidoreductase 1) and IL-10 expression in T lymphocytes. T cells lacking NQO1 showed increased expression of IL-10, which was responsible for the suppression of IL-17 production and, subsequently, Th17 cell differentiation. NQO1-depleted Th17 cells had higher levels of ROS, which boosted IL-10

expression. Furthermore, NQO1 deficiency ameliorated the symptoms of a Th17-dependent autoimmune encephalomyelitis *in vivo* (107). The interface between IL-10 and ROS is depicted in **figure 1.9**.

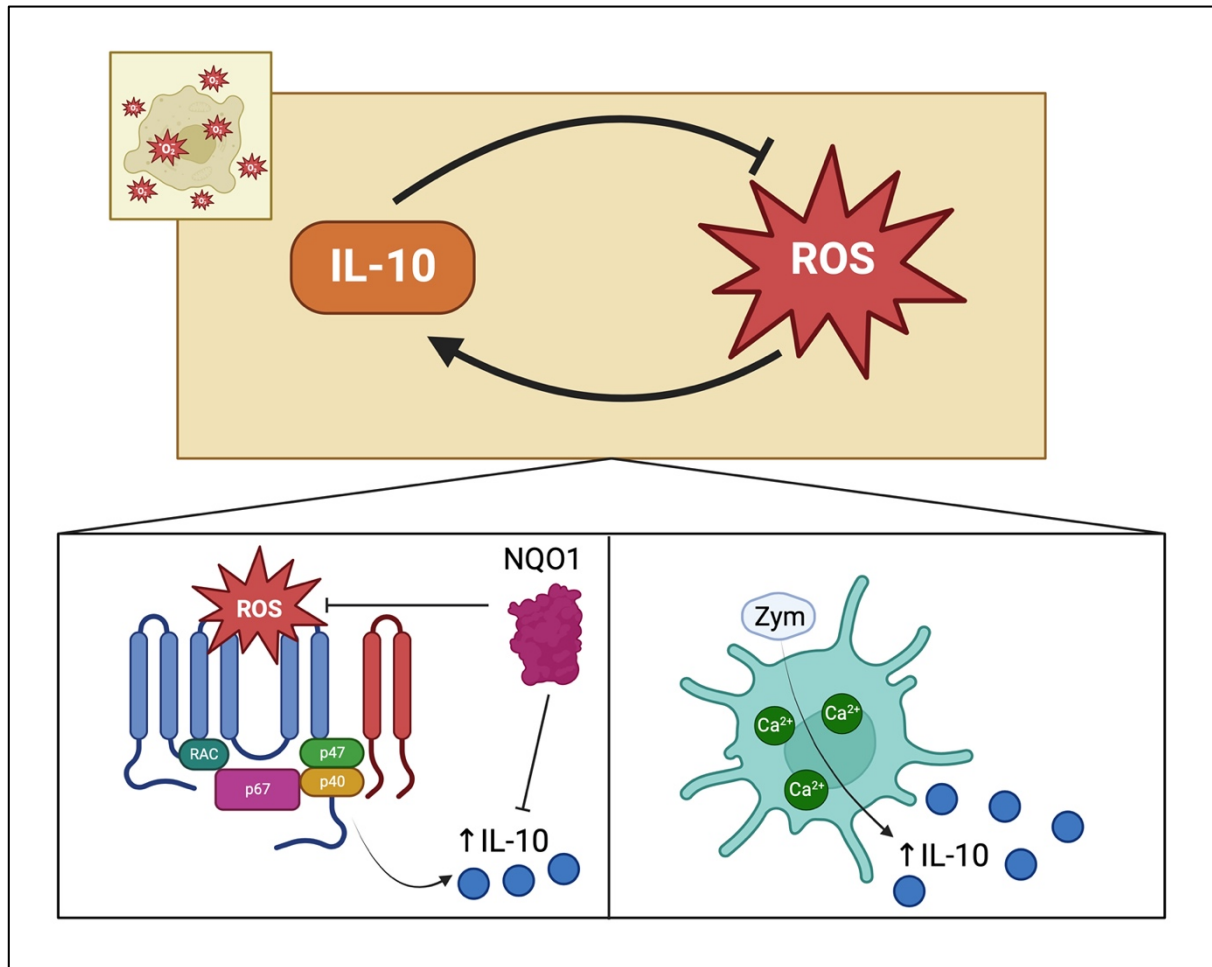


Figure 1.9. Redox-dependent regulation of IL-10.

IL-10 and ROS participate in a mutual regulation loop in cells when there is perturbation of redox balance. Examples of this cross-talk are LPS-activated macrophages, whose IL-10 generation impairs mitochondrial ROS, targets mTOR and inhibits glucose uptake, glycolysis and NF- κ B activation. Secondly, Zymosan-activated dendritic cells show upregulated IL-10 production through a Ca⁺⁺ and ROS-dependent mechanism. Moreover, also NQO1 and NADPH oxidase can regulate IL-10 expression in T cells.

1.3.6 The SIQEL family as new potential ROS inhibitors

In this thesis, I aimed at inhibiting ROS generation and Complex I and III activity from mitochondria in macrophages.

Previous studies have identified at least 11 sites within mammalian mitochondria to produce $O_2^{\cdot-}$ and H_2O_2 at a high rate during cellular respiration and electron transport (108). These sites can be divided into two sub-sets according to the flavin group (F) or the ubiquinone-binding site (Q) they contain. This second category operates at the redox potential of the ubiquinol/ubiquinone pool and consists of the ubiquinone-binding sites of mitochondrial complex I (I_Q), complex III (III_{QO}), glycerol-3-phosphate dehydrogenase (G_Q) and dihydroorotate dehydrogenase (D_Q) (108). The ubiquinone-binding sites on Complex I and Complex III account for the 75% of $O_2^{\cdot-}$ and H_2O_2 release in C2C12 myotubes and rat skeletal muscle mitochondria (109), thus representing a crucial step for ROS production.

Suppressors of site I_Q and III_{QO} electron leak (S1QELs and S3QELs) are low-molecular-weight chemicals synthesized by Martin Brand and his team thanks to specific high throughput screening assays in isolated mitochondria of approximately 20,000 compounds. S1QEL (**Figure 1.10**) specifically binds to the quinone site on Complex I while S3QEL (**Figure 1.11**) binds to the quinone site Q_O on Complex III (**Figure 1.12**). These are new inhibitors of Complex I and Complex III of ETC and their mechanism of action is not fully understood. The novelty resides in their ability to inhibit respiratory complexes in a different way compared to the already well-established inhibitors (such as rotenone and piericidin A) and non-specific mitochondria-targeted antioxidants such as mitoQ, mitoVitE and mitoTEMPO (108). Importantly, their novel role consists in the inhibition of ROS production without affecting FET and basal respiration (108). Indeed, they do not participate in redox reactions and do not require redox recycling, so they remain highly potent. Finally, due to their high selectivity, other important sites of redox signalling are unaffected (108).

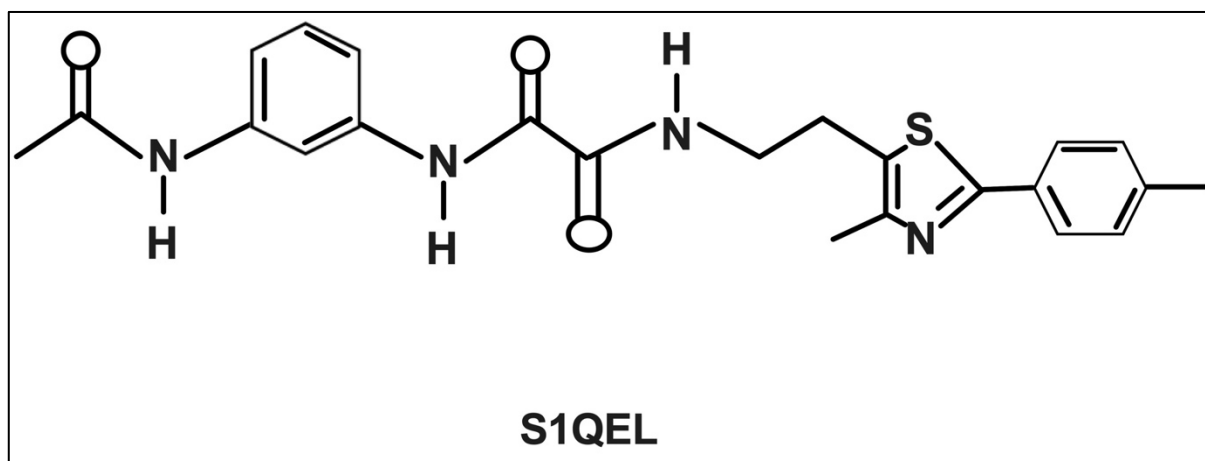


Figure 1.10. S1QEL structure.

The formal name of S1QEL is N¹-(3acetamidophenyl)-N²-(2-(4-methyl-2-(*p*-tolyl)thiazol-5-yl)ethyl)oxalamide. Molecular formula: C₂₃H₂₄N₄O₃S.

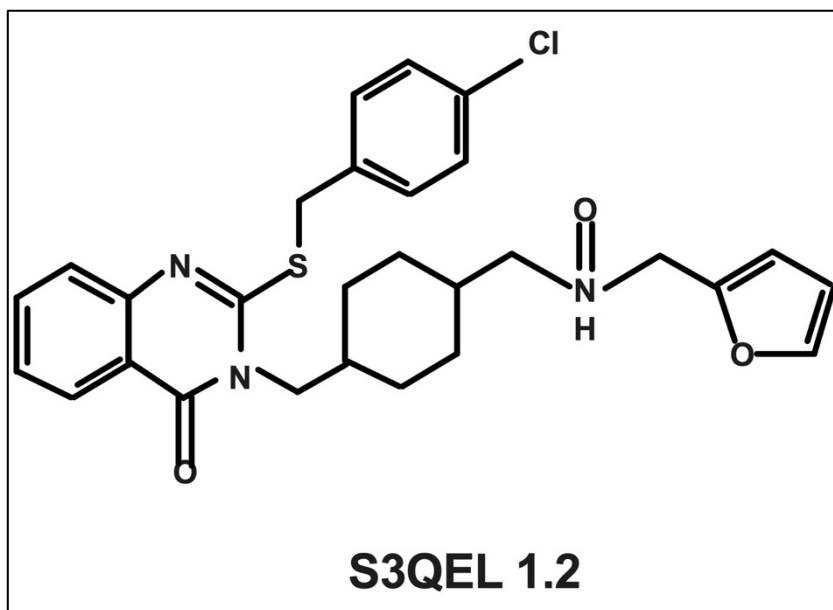


Figure 1.11. S3QEL 1.2 structure.

The formal name of S3QEL is 1-(3,4-dimethylphenyl)-N,N-dipropyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine. Molecular formula: $C_{19}H_{25}N_5$.

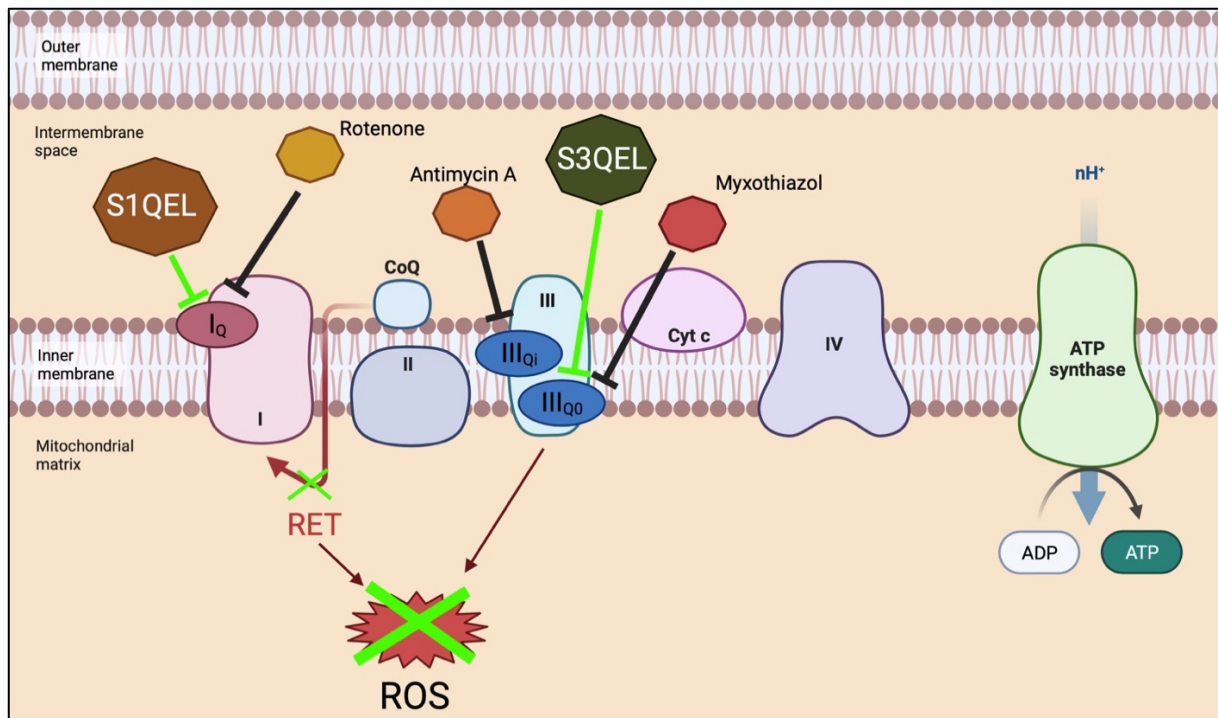


Figure 1.12. Mechanism of action of S1QEL and S3QEL.

Complex I (through RET) and Complex III of the ETC account for 75% of $O_2^{\cdot-}$ and H_2O_2 production in mammalian mitochondria. S1QEL 1 binds to the quinone site Q on Complex I and inhibits ROS production without affecting basal respiration. S3QEL binds to the quinone site Q_0 on Complex III and inhibits superoxide and hydrogen peroxide production. Both compounds suppress mitochondrial ROS generation. Rotenone binds to the same quinone site on Complex I, while Antimycin A binds to the quinone site Q_i on Complex III and Myxothiazol binds to the quinone site Q_0 on Complex III.

The effect of S1QEL and S3QEL have been elucidated in several models. During ischemia-reperfusion injury, S1QEL improved post-ischemic recovery of cardiac function and decreased infarct size in the Langendorff-perfused mouse heart model (110). S1QEL and S3QEL protected embryonic cardiomyocyte H9C2 cells treated with tunicamycin, used to induce endoplasmic reticulum (ER) stress and apoptosis against caspase 3 and 7 cleavage (110). S3QEL had the same effect in a rat INS-1 insulinoma cell line (111). In a murine alveolar macrophage cell line incubated with particulate matter, S3QELs significantly ameliorated the increase in ROS generation and inhibited IL-6 production (112). Activation of plasmacytoid dendritic cells (pDCs) causes a boost in mitochondrial oxidant production and this increase was inhibited by incubation with S3QEL. Concurrently, treatment with S3QEL suppressed the cross-presentation capacity of activated pDCs to elicit a clonal CD8⁺ T cell expansion response (113).

1.3.7 Myxothiazol and Antimycin A, which target mitochondrial complexes, are context-dependent modulators of the immune response

Together with SIQELs, I also used Myxothiazol and Antimycin A while performing my experiments.

Myxothiazol (**Figure 1.13**) is a common and reversible inhibitor for Complex-III activity and binds to the site Q_o for the quinone on Complex III, on the outer side of the mitochondrial membrane. Antimycin A (**Figure 1.14**) binds to the inner site for the quinone binding on Complex III, called Q_i. Both compounds are synthetically produced and completely shut down respiration from the complex because they block the exchange of electrons between the quinone and the flavin protein. This exchange lead to a disruption of the Q-cycle and of the turnover of quinone substrate. The inhibitors also promote the increased production of ROS, especially O₂^{•-}. This is the reason why they are considered pro-oxidant and toxic to the cells. Despite this, their role appears to be context-dependent because they showed novel effects when immune cells are activated. Also, time of exposure and concentrations of the two compounds can have opposite effects in the same cell type.

Antimycin A and Myxothiazol have a plethora of effects in tissue-resident macrophage population. Antimycin A augmented ROS production in primary microglia cells and BV-2 cell line and enhanced IL-1 β , TNF- α , IL-12 and IL-6 production through activation of MAPK pathways. Cytokine production was blocked by treatment with the anti-oxidant mitoTEMPO (114).

Inhibition of Complex III activity by Myxothiazol and antimycin A reduced LPS-induced nuclear accumulation of NF- κ B, and the production of TNF- α and MIP-2 in neutrophils. This mechanism appeared to be H₂O₂-dependent. *In vivo*, in the context of LPS-induced lung injury, myxothiazol diminished neutrophil accumulation in the lung and TNF- α , IL-6 and KC presence in lung homogenates (90).

1 hour of Myxothiazol treatment reduced Poly (ADP-ribose) Polymerase (PARP) activity, production of mitochondrial ROS and upregulation of *Nampt* mRNA in macrophages stimulated for 1 hour with IFN- γ together with LPS (115).

Inhibition of Complex III with Antimycin A in macrophages significantly reduced CXCL10 and TNF- α production following both LPS and Poly I:C stimulation (88). In the same paper, S3QEL decreased TNF- α and IFN- β in LPS and poly I:C-primed BMMs (88).

In another study, human neutrophils isolated from healthy donors were inoculated with *S. Aureus* and their activities of engulfment, phagocytosis and Neutrophils Extracellular Traps (NET)osis of extracellular organisms were observed (116). Mitochondria appeared to actively participate in microbial sensing and phagocytosis because their fragments were found in the material expelled during the bacterial killing process. To analyse the involvement of healthy and working mitochondria, neutrophils were treated with antimycin A, Myxothiazol and other inhibitors of the ETC before the incubation with *S. Aureus*. Myxothiazol pre-treatment increased the amount of engulfed *S. Aureus* while antimycin A pre-treatment decreased the amount of NETs; ROS generated by activated neutrophils

were abolished after the pre-treatments. Finally, Myxothiazol and antimycin A-treated cells diminished bacterial killing capacity and the same inhibition was seen with mitoTEMPO pre-treatment, suggesting a ROS-dependent modulation of neutrophil behaviour (116).

In human PBMCs, Antimycin A treatment inhibited IL-6 production, promoting a reduction in more than a half of the participants. 85-100% of individuals showed a decrease in TNF- α too after the treatment with the inhibitor (117).

The positive and negative effects caused by S1QEL 1.1, S3QEL 1.2, Antimycin A and Myxothiazol are summarised in **table 1.1**.

Compound	Negative effect	Positive effect	Effect on ROS
S1QEL 1.1	↓ infarct size	↑ post-ischemic recovery; protection from ER stress and apoptosis	↓
S3QEL 1.2	↓ IL-6, TNF- α , IFN- β	Protection from ER stress and apoptosis; CD8+ T cell response	↓
AA	↓ IFN- β , CXCL-10, TNF- α , MIP-2, IL-6	↑ IL-8	↑
MYX	↓ CD80, NOS2, pro-IL-1 β , IL-6, TNF- α , MIP-2	↑ response against bacterial infections	↑ ↓

Table 1.1. Differences and similarities among S1QEL, S3QEL, AA and MYX in immunity.

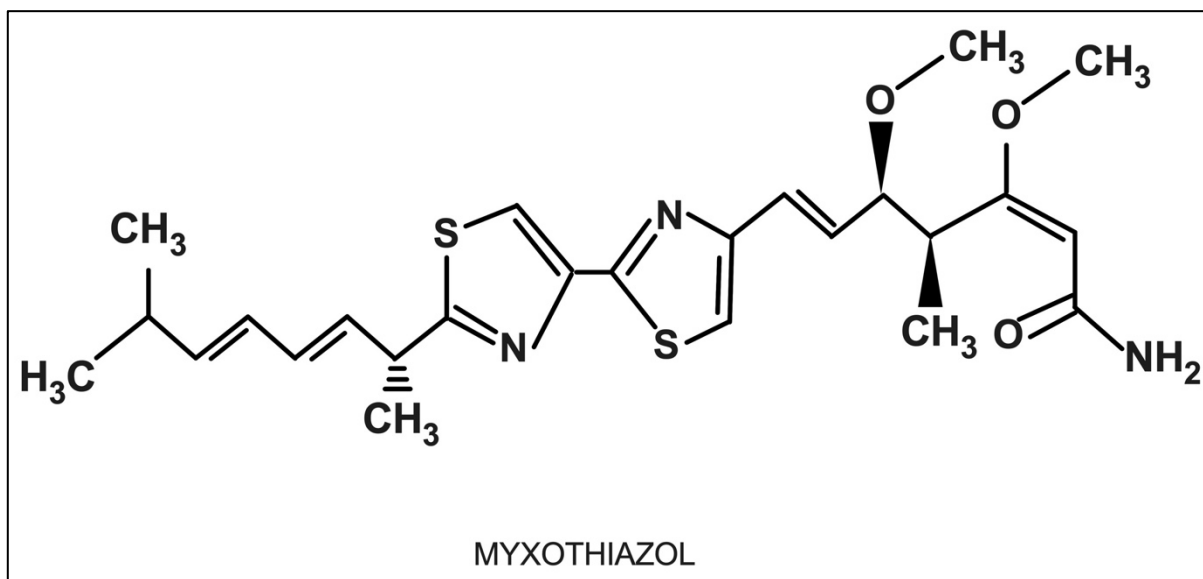


Figure 1.13. Myxothiazol structure.

The formal name of MYX is (2E,4R,5S,6E)-3,5-dimethoxy-4-methyl-7-[2-[2-[(2S,3E,5E)-7-methylocta-3,5-dien-2-yl]-1,3-thiazol-4-yl]-1,3-thiazol-4-yl]hepta-2,6-dienamide. Molecular formula: $C_{25}H_{33}N_3O_3S_2$.

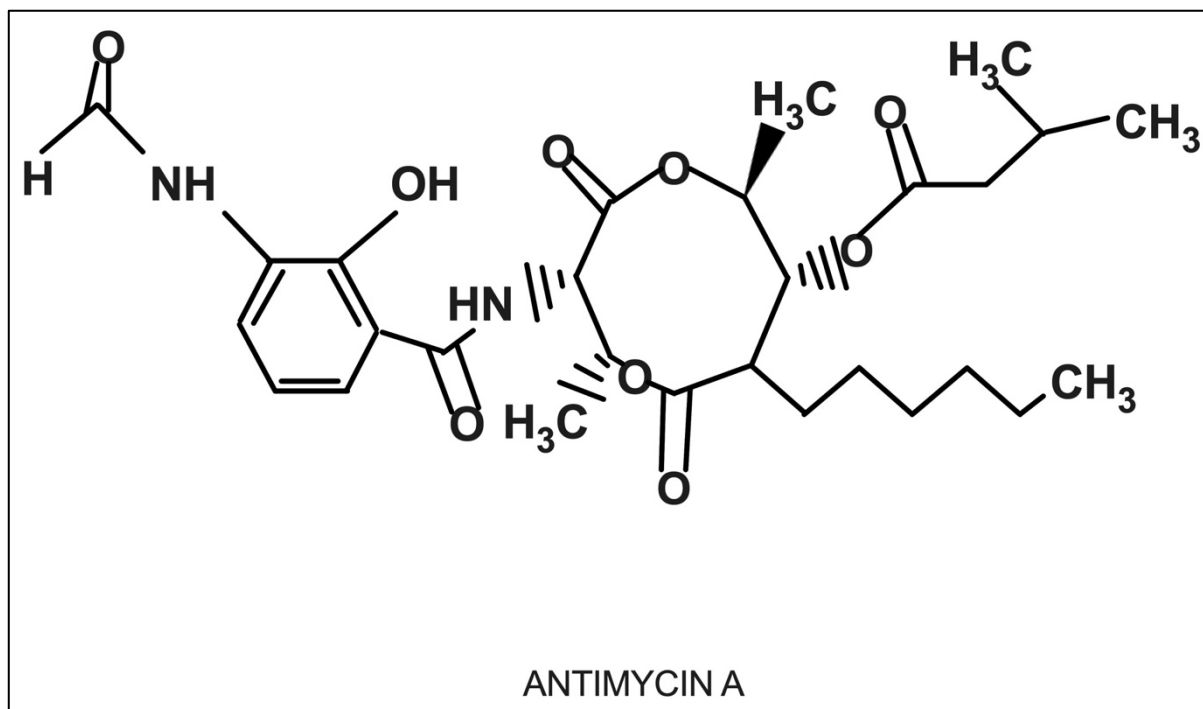


Figure 1.14. Antimycin A structure.

The formal name of AA is (2R, 3S, 6S, 7R, 8R)-3-(3-Formamido-2-hydroxybenzamido)-8-hexyl-2,6-dimethyl-4,9-dioxo-1,5-dioxonan-7-yl 3-methylbutanoate. Molecular formula: $C_{28}H_{40}N_2O_9$.

1.4 Aims

My project focuses on further analysis of the role of Complex I and Complex III in macrophage activation, using S1QEL 1.1 and S3QEL 1.2 as key tool compounds which inhibit ROS production by Complex I and Complex III. I also used Antimycin A and Myxothiazol as inhibitors of respiratory redox chain activity and ROS generation.

I hypothesize that these inhibitors could dampen inflammation and inflammasome activation through the blocking of ROS production along the respiratory chain. I also hypothesize that they would behave as anti-inflammatory compounds through the modulation of specific signalling pathways and cytokine production.

The specific aims are as follows:

- To analyse the impact of the specific compounds inhibiting Complex I and Complex III on macrophage respiration and viability
- To define whether ETC inhibitors can specifically block NLRP3 inflammasome activation
- To investigate if S1QEL 1.1, S3QEL 1.2 and Myxothiazol can inhibit transcription and protein expression of cytokines, with particular attention to IL-1 β , IL-10 and related-pathways
- To confirm that the effects that I have seen *in vitro* can be reproduced in mouse *in vivo* models, especially pathologically relevant ones such as cancer models

This project improves our understanding of mitochondrial biology and metabolism and might help in the characterisation of possible mitochondria-targeted inhibitors with immunomodulatory properties.

2. Materials and methods

2.1 Materials

2.1.1 Buffers

Buffers used for this project are listed in **table 2.1**.

Name	Composition
ELISA wash buffer	Tween-20 [0.05%], PBS
FACS buffer	Sodium azide [0.1%], BSA [0.1%], FCS [1.5%], PBS
PBS (10X)	NaCl [1.45M], NaH ₂ PO ₄ [39mM], NaH ₂ PO ₄ [22.7M]
Sample lysis buffer	Tris pH 6.8 [0.125M], glycerol [10%], SDS [0.02%], DTT [5%]
SDS PAGE running buffer (10X)	Tris [25mM], glycine [192mM], SDS [0.1%]
SDS PAGE transfer buffer (10X)	Tris [0.25M], glycine [1.9M], SDS [35mM]
Tris-buffered saline tween (TBST) (10X)	Tris (12.11g), NaCl (87.6g), tween 20 (10mL), distilled H ₂ O to 1L

Table 2.1. List of buffers used in this project.

2.1.2 Cell culture reagents

DMEM, RPMI, T175 flasks and 10cm cell culture dishes were purchased from Thermo Fisher Scientific (Waltham, USA). FCS was purchased from Biosera (Nuaille, France). Penicillin-streptomycin, trypsin-EDTA and red cell lysis buffer were purchased from Sigma Aldrich (St Louis, USA). L929 supernatant was obtained by removing the supernatant from L929 cells at 60-70% confluency. Lymphoprep was purchased from Stemcell Technologies (Cambridge, UK).

2.1.3 Cell lines and primary cells

L929 cell line was purchased from Sigma Aldrich. HEK293T and B16F10 cell line were purchased from ATCC (Manassas, USA). BMDMs were isolated from the tibia and femur of 6-10-week-old C57BL/6 mice which were purchased from Harlan Laboratories (Bicester, UK). AOX mice were kindly provided by Prof. Mike Murphy (University of Cambridge, UK). All experiments were carried out with prior ethical approval from Trinity College Dublin Animal Research Ethics Committee under the relevant project and individual authorisations, granted by the Health Products Regulatory Authority (HPRA).

2.1.4 Stimulants and metabolites

LPS from *Escherichia coli*, serotype EH100, was purchased from Enzo Life Sciences (Farmingdale, USA). ATP disodium salt was purchased from Sigma Aldrich. Nigericin, poly (dA:DT) were all purchased from Invivogen (San Diego, USA). Dimethyl sulfoxide (DMSO), MYX, AA and MitoTEMPO were purchased from Sigma Aldrich. MitoQ was purchased from Abcam. S1QEL 1.1 and S3QEL 1.2, together with S1QEL 1.2, S1QEL 2.1 and S3QEL 3 were synthesised and kindly provided by Prof. Martin D. Brand (Buck Institute for Aging, San Francisco, CA, USA).

2.1.5 Antibodies

Anti-mouse IL-1 β , HIF-1 α , α -Tub, phospho-C-fos, total C-fos, phospho-C-jun, total C-jun, phospho-CREB, total CREB and GAPDH antibodies were purchased from Cell Signalling Technology (Danvers, USA). Anti-mouse β -actin antibody was purchased from Sigma Aldrich. Total OXPHOS rodent WB antibody cocktail was purchased from Abcam. Anti-mouse caspase-1 antibody was purchased from AdipoGen Life Sciences (Liestal, Switzerland). Anti-mouse AOX antibody was a gift of Prof. Mike Murphy's lab in university of Cambridge, UK. Horseradish peroxidase (HRP)-conjugated anti-mouse, anti-goat, anti-rabbit and anti-rat immunoglobulin G (IgG) antibodies were purchased from Jackson

Immunoresearch (West Grove, USA). Unconjugated anti-mouse IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

2.1.6 Western blotting reagents

The gel running system was purchased from BioRad (Berkeley, USA). HEPES, triton X-100, aprotinin, sodium orthovanadate, PMSF, benzonase nuclease, sodium dodecyl sulphate (SDS), N,N,N',N'-Tetramethylethylenediamine (TEMED), ammonium persulphate (APS), tris base, hydrochloric acid and acrylamide were all purchased from Sigma Aldrich. StrataClean Resin was purchased from Agilent Technologies (Santa Clara, USA). Spectra BR protein ladder and disuccinimidyl suberate (DSS) were purchased from Thermo Fisher Scientific. PVDF transfer membrane was purchased from Merck Millipore (Burlington, USA). Marvel powdered milk was purchased from Tesco. WesternBright ECL spray was purchased from Advansta (San Jose, USA). The system used for protein visualisation was a ChemiDoc™ MP Imaging System from BioRad.

2.1.7 RT-qPCR reagents and primers

Primers were designed in house and purchased from Eurofins Genomics (Ebersberg, Germany). Primer sequences are given in **table 2.2**. RNA extraction kit, reverse transcription kit, 8-strip PCR tubes, caps, plate seals, SYBR green master mix, and 96-well qPCR plates were all purchased from Applied Biosystems (Foster City, USA). Amplification grade DNase I was purchased from Thermo Fisher Scientific. cDNA synthesis was performed on a Veriti 96-well fast thermal cycler from Applied Biosystems, and qPCR was performed on a 7500 fast real-time PCR system, also from Applied Biosystems.

Target gene	Forward Primer	Reverse Primer
<i>IL-1β</i>	GAGGACATGAGCACCTTCTTT	GCCTGTAGTGCAGTTGTCTAA
<i>TNF-α</i>	GCCTCTTCTCATTCCTGCTT	TGGGAAGTTCTCATCCCTTTG
<i>IL-10</i>	AGGCGCTGTCATCGATTT	CACCTTGGTCTTGGAGCTTAT
<i>IL-6</i>	CCACAGTCCTTCAGAGAGATAC A	CCTTCTGTGACTCCAGCTTAT C
<i>RPS18</i>	GCGAGTACTCAACACCAACA	CCTCAACACCACATGAGCATA
<i>C-fos</i>	GGGAATGGTGAAGACCGTGTC A	GCAGCCATCTTATTCCGTTCC C
<i>C-jun</i>	CAGTCCAGCAATGGGCACATC A	GGAAGCGTGTTCTGGCTATGC A

Table 2.2. List of murine primers used in this project.

2.1.8 ELISA Reagents

Mouse IL-1 β , IL-10, IL-6, TNF- α duoset ELISA kits were purchased from R&D Systems (Minneapolis, USA). TMB substrate reagent set was purchased from BD Biosciences. Mouse Quantikine IL-10 ELISA kit was purchased from R&D Systems (Minneapolis, USA). Nunc™ 96-well ELISA plates were purchased from Thermo Fisher Scientific. Plates were read on a FLUOstar OPTIMA from BMG Labtech (Ortenberg, Germany).

2.1.9 Seahorse Reagents

XFe96 cell plates and cartridges were purchased from Agilent. XF DMEM Medium, pH 7.4, XF Plasma Membrane Permeabilizer and XF Cell Mitostress (**table 2.3**) and Glycostress (**table 2.4**) test kits were purchased from Agilent. The analysis was performed on a Seahorse XFe96 Analyzer from Agilent Technologies.

Compound	Final concentration in the well	Volume in the port	Final volume in the well
Oligomycin	1 μ M	20 μ L	200 μ L
FCCP	1 μ M	22 μ L	200 μ L
Rotenone/Antimycin A	0.5 μ M	25 μ L	200 μ L

Table 2.3. List of compounds used for Mito Stress test in this project.

Compound	Final concentration in the well	Volume in the port	Final volume in the well
Glucose	10mM	20 μ L	200 μ L
Oligomycin	1 μ M	22 μ L	200 μ L
2-DG	50mM	25 μ L	200 μ L

Table 2.4. List of compounds used for Glycolysis Stress test in this project.

2.1.10 Complex-III specific activity assay

XFe96 cell plates and cartridges were purchased from Agilent. Mitochondrial Assay Solution was used to resuspend the cells and was made as following **table 2.5**. Cells were injected with duroquinone, ADP disodium salt, Oligomycin and Antimycin A, all purchased by Sigma (**table 2.6**). The analysis was performed on a Seahorse XFe96 Analyzer from Agilent Technologies.

Reagent	3X MAS	Amount for 1L of 3X MAS
Mannitol	660 mM	120.23g
Sucrose	210 mM	71.88g
KH ₂ PO ₄	30 mM	4.08g
MgCl ₂	15 mM	15mL of 1.0M solution
HEPES	6 mM	6mL of 1.0M solution
EGTA	3 mM	12mL of 0.25mM solution
XF PMP	1 nM	100μL of 10μM solution

Table 2.5. Mitochondrial Assay Solution recipe.

Compound	Final concentration in the well	Volume in the port	Final volume in the well
ADP	1 mM	20μL	200μL
Duroquinol	0.5 mM	20μL	200μL
Oligomycin	15 μm	22μL	200μL
Antimycin A	0.02 mM	25μL	200μL

Table 2.6. List of compounds used for Complex III specific activity assay.

2.9.11 Amplex Red assay for H₂O₂ detection

96 wells black plates with clear bottom were purchased from Eppendorf. Krebs-Ringer Phosphate Glucose (KRPB) buffer was used to resuspend the cells and was made as following **table 2.7**. Amplex Red reagent was purchased from Invitrogen. The reading was performed on a SpectraMax Gemini microplate reader.

Compound	Amount for 50 mL
NaCl	0.42g
NaH ₂ PO ₄	0.034g
KCl	0.018g
CaCl ₂	0.003g
MgSO ₄	0.015g
Glucose	0.05g
XF PMP	5 µL

Table 2.7. KRPB buffer recipe.

2.2 Methods

2.2.1 Bone-marrow derived macrophage generation

6-10-week old C57BL/6OlaHsd were euthanised in a CO₂ chamber, and death was confirmed by cervical dislocation. Bone marrow was subsequently harvested from the tibia and femur in a laminar flow hood. The abdomen and hind legs of the mouse were sprayed with 70% ethanol and a small incision was made in the midline of the abdomen, allowing the skin to be pulled down to expose the legs. By cutting around the hip joint, the legs were separated from the body and placed in a 10cm dish containing DMEM. The muscles and fat were subsequently removed from the legs, and the tibia and femur were separated from each other. The bones were cut open at each side, and bone marrow was flushed into a 50ml Falcon™ tube using a 25-gauge needle attached to a 25ml syringe containing DMEM. The bone marrow was centrifuged at 453 x g for 5 minutes and supernatant was removed. Cells were resuspended in 3ml of red cell lysis buffer for 3 minutes before again being supplemented with 7ml DMEM and centrifuged at 453 x g for 5 minutes. Supernatant was removed and cells were resuspended in 30ml DMEM containing L929 supernatant [10%], FCS [10%], and penicillin/streptomycin [1%]. Cells were seeded in non-tissue culture-coated

10cm dishes at a volume of 10ml per dish. Cells were incubated for 6 days at 37 Celsius degrees and 5% CO₂. Following this incubation period, the medium was replaced by cold PBS and cells were scraped off the plates using a cell scraper. Cells were counted by haemocytometer and plated at either 0.5 or 1 x 10⁶ cells/well in DMEM containing L929 supernatant [10%], FCS [10%] and penicillin-streptomycin [1%].

2.2.2 *In vivo* experiments

4-6-week old C57BL/6OlaHsd were pre-treated intraperitoneally with Myxothiazol, S3QEL 1.2 or DMSO vehicle control at the final concentration of 1mg/kg (1:10 in PBS) for 2 hours and then injected intraperitoneally with LPS or PBS vehicle control at the final concentration of 2,5mg/kg for 2 hours. After 4 hours, mice were sacrificed and spleens, livers were collected. Also, blood from the eye and peritoneal exudate cells (PECs) were harvested using 10mL PBS and a syringe. Blood was pelleted at 4 degrees for 10 minutes and then the supernatant (serum) was kept at -80 degrees for further analysis by ELISA. PECs were pelleted and lysed in RNA lysis buffer (Invitrogen) for further analysis by qPCR.

2.2.2.1 B16-F10 *in vivo* experiment

Mice were injected subcutaneously with 3.5x10⁵ B16-F10 cells on the right flank which has been previously shaved. Tumour growth was measured daily using a digital callipers and tumour volume was calculated as (Length x (Width)²)/2. Upon reaching 30-660mm³, mice were randomly assigned to experimental treatment groups, and received intratumoral injections of PBS or CpG ODN 1826 (50 mg/kg each treatment), with treatments repeated 4 days later on day 4 and on day 8 for a total of 3 intratumoral treatments. In one experiment two groups of mice received 1 mg/kg of S3QEL 1.2 via the intraperitoneal route every two days from one day prior to the first intratumoral treatment until day 19. Tumour growth was monitored daily. Mice were culled upon reaching the predetermined humane endpoint of tumour diameter ≥ 15mm or in the case of ulceration with survival analysed using Kaplan-Meier analysis.

2.2.3 Inflammasome assay

2.2.3.1 NLRP3 inflammasome

BMDMs were plated in DMEM with FCS [10%] and penicillin-streptomycin in 12-well cell culture plates, at a density of $0.5-1 \times 10^6$ cells/ml. Cells were left overnight to adhere and treated the following day with LPS [100ng/ml] for 3 hours. Medium was removed and replaced with serum and antibiotic-free DMEM and treated with compounds as required for 45 minutes. Cells were subsequently treated with nigericin [10 μ M] for 45 minutes, after which the cell supernatants were removed and stored, and cells were lysed in sample lysis buffer and also stored.

2.2.3.2 Cytotoxicity assay

The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) was used to quantify lactate dehydrogenase (LDH) release from cells as a measure of cell death in BMDMs following inflammasome stimulation. Freshly harvested supernatants were used in this assay. 50 μ l of each supernatant was added to 50 μ l Cytotox 96 Reagent and incubated in the dark at room temperature for 30 minutes. 50 μ l acetic acid was added to stop the reaction, and the absorbance at 492nm was measured using a FLUOstar OPTIMA reader. 100 μ l total lysis solution was added to untreated cells 30 minutes before harvesting and served as a maximum LDH release control. Medium alone was also used to correct for background absorbance.

2.2.4 Western blot

2.2.4.1 Cell lysis and protein denaturation

Supernatant was harvested from cells following stimulation and 30-50 μ l lysis buffer was added to each well of cells. The plate was gently agitated to ensure maximum coverage of each well with lysis buffer. Lysates were then transferred to 1.5ml microcentrifuge tubes and heated to 95 Celsius degrees for 5 minutes to denature the proteins. Protein samples were stored at -20°C until further use.

2.2.4.2 Concentrating supernatants for western blot

5µl of Strataclean Resin was added to 500µl of supernatant before vortexing for 1 minute. Supernatants were then centrifuged at 210 x g for 2 minutes at 4°C. Supernatants were removed and discarded, and the remaining pellet was suspended in 30µl of lysis buffer. Samples were stored at -20 Celsius degrees until needed.

2.2.4.3 SDS-PAGE

SDS polyacrylamide gel electrophoresis was used to resolve proteins by molecular weight. Samples were boiled at 95 Celsius degrees for 5 minutes prior to loading into a 5% stacking gel. The percentage resolving gel depended on the molecular weight of the given protein. Gel compositions are given in **table 2.2**. The BioRad gel running system was used, whereby the gels were secured in electrodes which were placed into a tetra cell and covered with SDS-PAGE running buffer. Samples were passed through the stacking and resolving gel at 80V using the molecular weight marker as a reference.

Reagent	8% Resolving Gel	10% Resolving Gel	12% Resolving Gel	Stacking Gel
dH ₂ O	3.5mL	3mL	2.5mL	2mL
30% Acrylamide Mix	2mL	2.5mL	3mL	0.5mL
Tris-HCl pH 6.8 [1M]	-	-	-	375µL
Tris-HCl pH 8.8 [1.5M]	1.9mL	1.9mL	1.9mL	-
10% SDS	75µL	75µL	75µL	30µL
10% APS	75µL	75µL	75µL	30µL
TEMED	3µL	3µL	3µL	3µL

Table 2.8. Composition of gels used for SDS PAGE.

2.2.4.4 Electrophoretic transfer of proteins

The BioRad wet transfer system was used for the electrophoretic transfer of proteins. A gel and membrane sandwich was assembled: a sponge soaked in buffer was placed onto the cathode side of the sandwich cassette, followed by 2 soaked pieces of rectangular filter paper, the gel, a PVDF membrane (activated in methanol), 2 pieces of filter paper and a sponge. The cassette was closed and firmly wedged into the transfer cell, which was filled with transfer buffer. The transfer was run at 200mA per box for 2 hours, or at 60mA overnight.

2.2.4.5 Incubation with antibody and protein visualisation

Following transfer, the PVDF membrane was removed from the transfer cell and incubated in milk powder [5% in TBST] for 1 hour to prevent non-specific antibody binding to the membrane. The membrane was then incubated in primary antibody (diluted in 5% milk powder) rolling overnight at 4°C. The following morning, the membrane was washed three times in TBST for 10 minutes per wash prior to a 1-hour incubation with secondary antibody (diluted in 5% milk powder) at room temperature. The membrane was again washed three times in TBST as previously performed. For phospho-antibodies, 5% milk powder was replaced by 5% BSA (also in TBST). Prior to visualisation, the membrane was immersed in WesternBright ECL Spray. Protein visualisation took place on a ChemiDoc MP™ Imaging System, and both chemiluminescent and white light images were taken.

2.2.5 RT-qPCR

2.2.5.1 RNA extraction

RNA extraction from cells was carried out using a RNA extraction kit according to the manufacturer's instructions. BMDMs were treated as required, and following treatment were instantly lysed in 350µl RNA lysis buffer. Samples were either processed further or stored at - 20°C for later use. Isolated RNA was quantified using a NanoDrop 2000 spectrophotometer, and RNA concentration was normalised to the lowest concentration across all samples with RNase-free water.

If necessary, samples were DNase treated after quantification using DNase I according to the manufacturer's instructions.

2.2.5.2 Reverse transcription

Isolated RNA samples were normalised and converted into cDNA using the reverse transcription kit according to manufacturer's instructions. 10µl of RNA (at a maximum concentration of 100ng/µl) was added to 10µl of reverse transcription master mix to complete the reaction mixture. The contents of the master mix are given in **table 2.4**. The reaction was performed in 8-strip PCR tubes by a thermal cycler using the following steps: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes followed by holding at 4°C. The resulting cDNA was diluted 1 in 5 in RNase-free water and stored at -20°C until further use.

Reagent	Volume per sample (µL)
10X RT Buffer	2
10X Random primers	2
25X Deoxyribonucleotide triphosphates (dNTPs)	1
Multiscribe reverse transcriptase enzyme	1
dH ₂ O	4

Table 2.9. List of components of reverse transcription mix.

2.2.5.3 qPCR

Real-time quantitative PCR was performed on the cDNA generated in the previous step, using primers designed in-house as listed in **table 2.2**. 4.7µl of cDNA was added to 5.3µl of qPCR master mix, the constituents of which are listed in **table 2.5**. The reaction was performed in a 96-well qPCR plate by a 7500 Fast Real-Time PCR machine, and a melt curve was also generated for all primers which were run for the first time. Relative expression (2^{-DDCT}) was calculated from the C_T values for each sample and gene of interest.

Reagent	Volume (μL) per sample
SYBR Green master mix	5
Primers	0.3
dH ₂ O	0.7

Table 2.10. List of components of SYBR Green mix.

2.2.6 RNA sequencing

BMDMs (3 independent mice) were treated as indicated and RNA was extracted as previously detailed. mRNA was extracted from total RNA using poly-T-oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on the NovaSeq 6000 S4 (Illumina).

2.2.7 ELISA

DuoSet and Quantikine ELISA kits were carried out to the manufacturer's instructions with appropriately diluted cell supernatants. 50μl TMB substrate solution was added to form the blue solution product, and 25μl hydrochloric acid [1M] was added to stop the reaction once a colour gradient had been established in the standards. Absorbance at 450nm was then quantified using a FLUOstar Optima plate reader. Corrected absorbance values were calculated by subtracting the background absorbance, and cytokine concentrations were subsequently obtained by extrapolation from a standard curve plotted on GraphPad Prism 7.0.

2.2.8 Metabolic flux analysis

A Seahorse XF-96e extracellular flux analyser (Seahorse Bioscience, Agilent Technologies) was used to define OCRs and ECARs. For monitoring oxidative phosphorylation and OCR, cells were resuspended in XF DMEM pH 7.4 supplemented with pyruvate, glutamine and glucose. Mitochondrial perturbation experiments were carried out by sequential addition of oligomycin, FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) and rotenone, according to

manufacturer's instructions. For monitoring glycolysis and ECAR, For monitoring the OCR of only intact mitochondria, cells were resuspended in XF DMEM pH 7.4 supplemented with glucose. Perturbation experiments were carried out by sequential addition of glucose, oligomycin and 2-deoxyglucose, according to manufacturer's instructions. Cells were resuspended in MAS buffer (70 mM sucrose, 220 mM mannitol, 10 mM KH₂PO₄, 5 mM MgCl₂, 2 mM HEPES and 1 mM EGTA), then treated with the XF plasma membrane permeabilizer (Seahorse, Agilent Technologies), which was followed by addition of 0.5 mM duroquinol, 1 mM ADP, 15 μM oligomycin and 0.02 mM antimycin A for monitoring complex III-driven respiration. In this case, the assay includes a cycle of mix for 2', wait for 2' and measure for 3' (repeated 3 times), Duroquinol and ADP injections, followed by the same cycle repeated two times, then Oligomycin injection, followed by the same cycle repeated two times and Antimycin A injection, followed by the same cycle repeated two times. Changes in OCR on substrate addition were calculated relative to the pre injection rate.

2.2.9 MTT assay

BMDMs as 0.1×10^6 cells were seeded in each well of a 96-wells F-bottom plate in duplicates or triplicates and treated accordingly. MTT was purchased from Sigma and reconstituted in PBS to a concentration of 1 mg/ml. On each plate three wells had no cells but medium only to serve as a blank. At the end of the treatment, cells were washed with 100 μL of PBS per well and 100 μL of MTT solution were added in each well. The plate was incubated for 2 hours at 37 degrees wrapped in tin foil. At the end of the incubation, the MTT solution was discarded and the crystals were dissolved in 100 μL of DMSO solution in each well. The plate was incubated for 20 minutes at 37 degrees in the dark and read on a spectrophotometer at 595 nm of wavelength. Data were normalised to untreated and unstimulated cells.

2.2.10 Amplex Red Assay for H₂O₂ production measurement

BMDMs were seeded in a 96 wells plate with black opaque inserts with concentration of 0.1×10^5 cells per well, in duplicate. Cells were treated accordingly

and stimulated with LPS for 48 hours (200 ng/mL), with MitoTEMPO for 48 hours (5mM) for 48 hours. During the last 24 hours of stimulation, cells were treated with S3QEL 1.2 (25 μ M) and Myxothiazol (12,5 μ M). On the day of the assay, media was changed to KRPG Buffer and cells were re-stimulated for the length of the assay. H₂O₂ standard was prepared as per manufacturer's instructions. 50 μ L of 100 μ M Amplex Red and 0.2 U/mL HRP working solution were added to the wells of the H₂O₂ standard. 100 μ L of 50 μ M Amplex Red and 0.1 U/mL HRP working solution were added to the wells containing the samples. Fluorescence with excitation between 450 and 490 nm and emission of 590 nm was read at the beginning of the reaction, after 30 minutes and then every hour for maximum 3 hours. Fluorescence of the samples was extrapolated from the H₂O₂ standard curve and compared to the untreated sample.

2.2.11 Statistics

Statistical analysis was carried out using GraphPad Prism 10.0. Statistical significance was calculated using a one-way analysis of variance (ANOVA) followed by t-test with multiple comparisons test, unless otherwise stated. Results were considered significant at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).. For the *in vivo* experiments carried out with AOX mice, statistical significance was calculated using two-ways analysis of variance (ANOVA) followed by t-test with multiple comparisons test. Results were considered significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). For the *in vivo* experiments carried out with B16-F10 injected mice, statistical significance was calculated using two-way analysis of variance (ANOVA) followed by Dunnett t-test. Results were considered significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Statistical significance for survival analysis was determined by Mantel-Cox test.

3. Effects of SIQELs on cytokine production and inflammasome activation in macrophages

3.1 SIQELs and modulation of inflammatory cytokines

I first studied the effect of S1QEL 1.1 and S3QEL 1.2 on cytokine production by macrophages.

As can be seen in **figure 3.1**, LPS induced the mRNA expression of IL-1 β , IL-6 and TNF- α . The pre-treatment with S1QEL 1.1 for 90 minutes at all concentrations did not affect induction of IL-1 β (A), IL-6 (B) or TNF- α (C) mRNA in response to LPS.

Figure 3.2 shows a 3 hours pre-incubation with S1QEL 1.1 before LPS priming. Here, LPS induced again the mRNA expression of IL-1 β , IL-6 and TNF- α . S1QEL 1.1 also had no significant effect on IL-6 (B) or TNF- α (C) expression at any concentrations. S1QEL 1.1 at 3 μ M and 10 μ M however slightly decreased IL-1 β production (A).

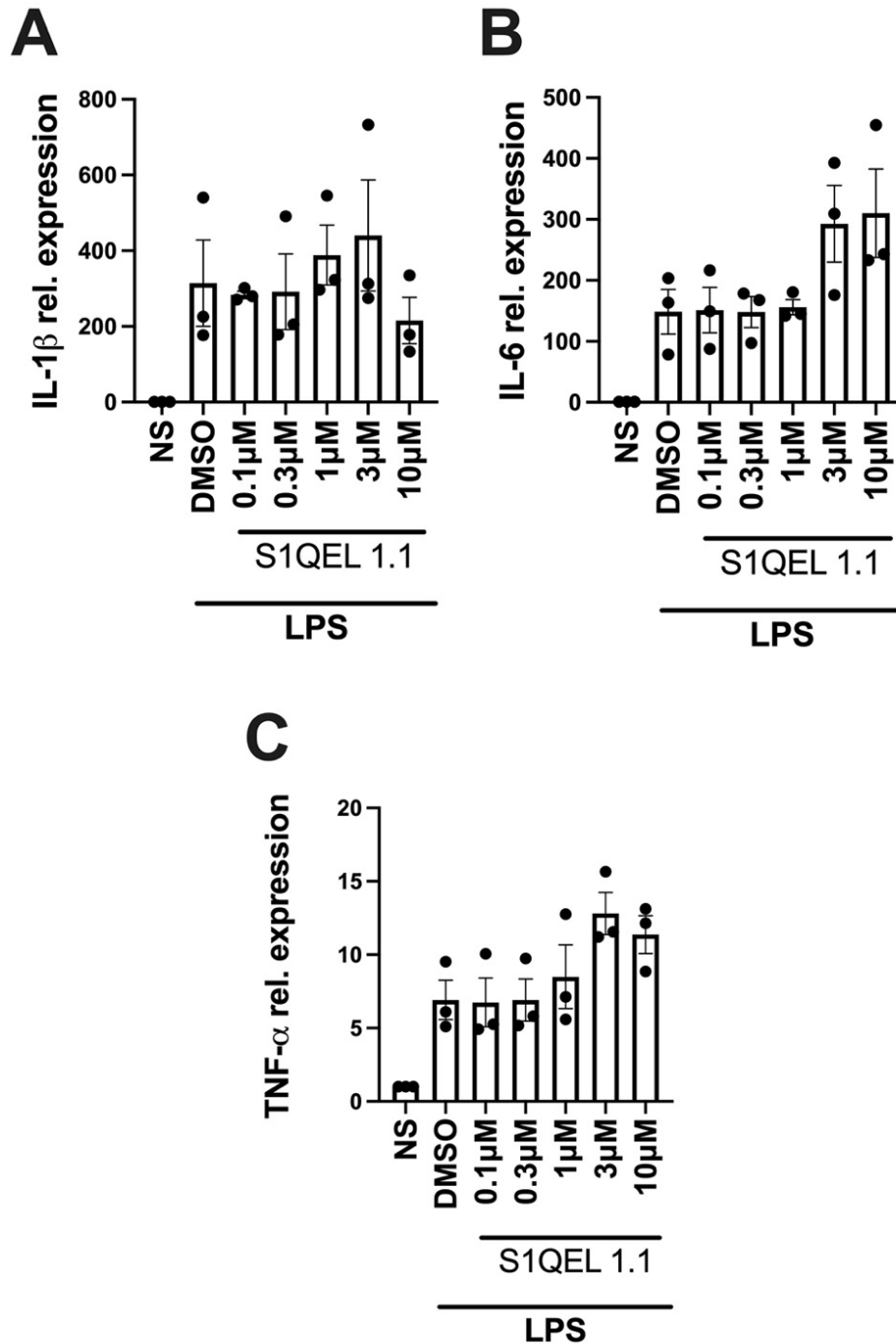


Figure 3.1 S1QEL 1.1 does not affect the expression of IL-1 β , TNF- α or IL-6.

BMDMs (1×10^6 cells) were pre-treated with DMSO vehicle or increasing concentrations of S1QEL 1.1 for 90 minutes, after which they were stimulated with LPS (100ng/mL) for 24 hours. After stimulation, RNA was isolated from cell lysates and analyzed by RT-qPCR for the expression of IL-1 β (A), TNF- α (B) and IL-6 (C). The data shown are fold increase over RPS18 control and are represented as the mean $\pm$ SEM for $n=3$ from three independent experiments

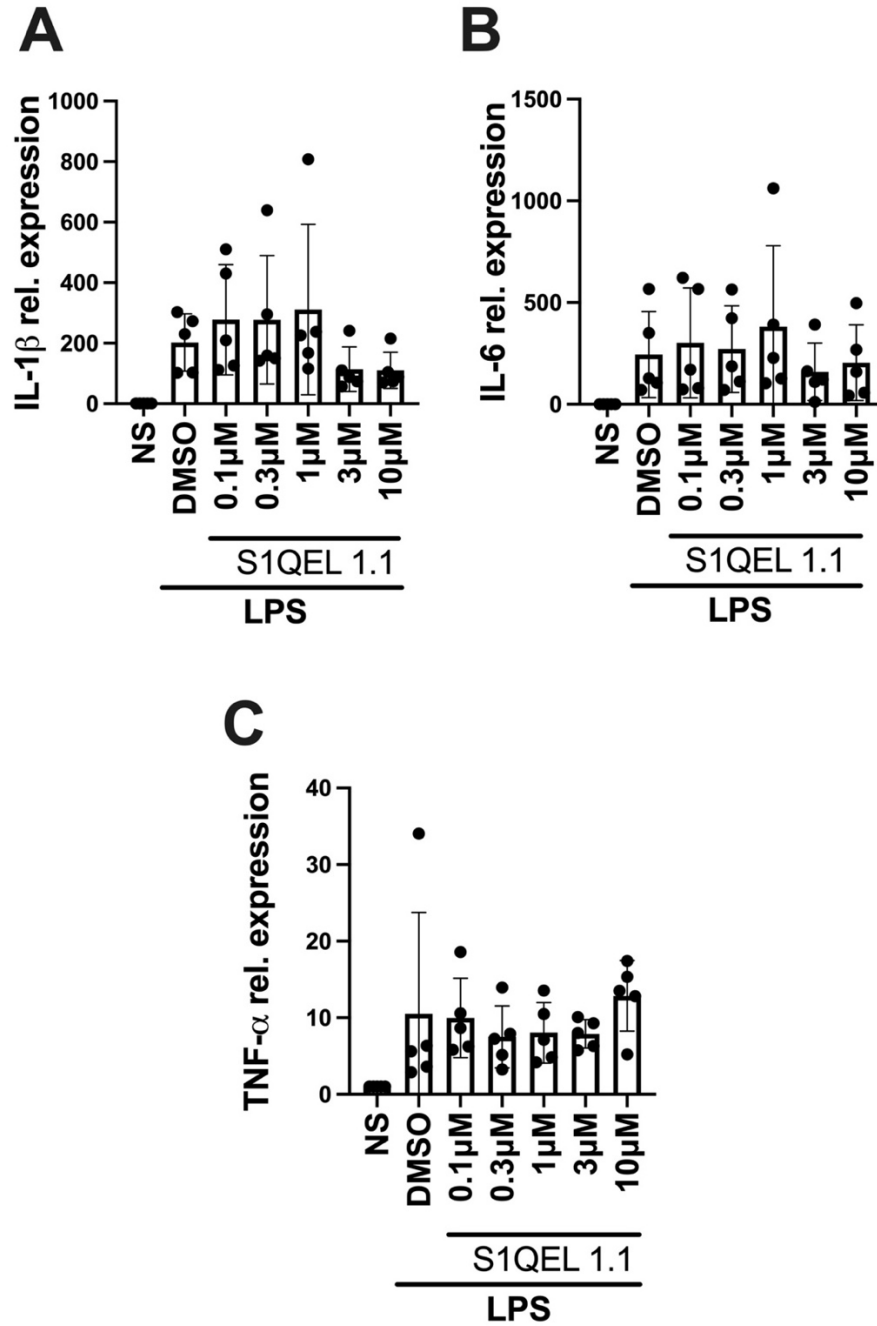


Figure 3.2 Effect of 3 hours pre-treatment with S1QEL 1.1 on the expression of IL-1 β , TNF- α and IL-6.

BMDMs (1×10^6 cells) were pre-treated with DMSO and S1QEL 1.1 for 3 hours, after which they were stimulated with LPS (100ng/mL) for 24 hours. After stimulation, RNA was isolated from cell lysates and analyzed by RT-qPCR for the expression of IL-1 β (A), TNF- α (B) and IL-6 (C). The data shown are fold increase over RPS18 control and are the mean $\pm$ SD for $n=5$ from two independent experiments.

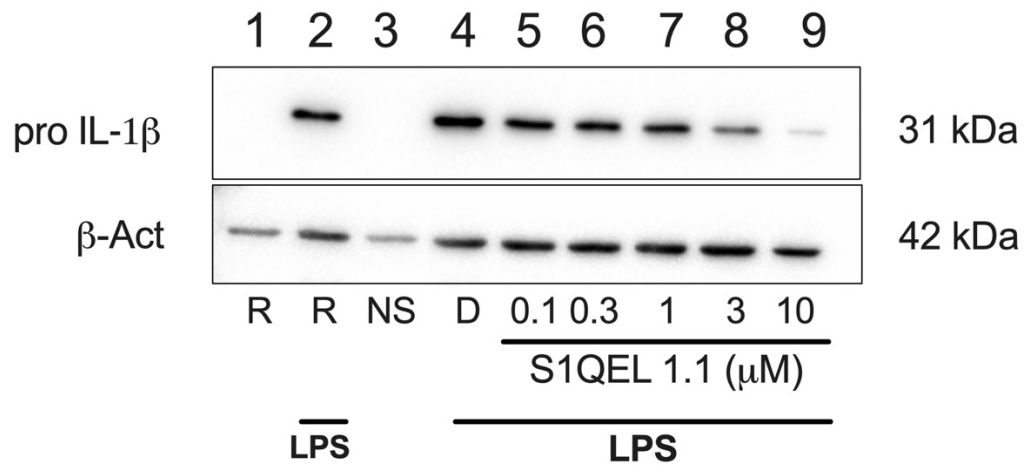
I next investigated the effect of S1QEL 1.1 on protein expression of pro-IL-1 β .

As can be seen in **figure 3.3**, I compared S1QEL 1.1 to one of the most common inhibitors of Complex I-generated ROS, rotenone.

Rotenone alone did not induce pro-IL-1 β (lane 1, upper panel), while there was a minor decrease in LPS-induced pro-IL-1 β expression compared to the DMSO vehicle treated control (compare lane 2 to lane 4, upper panel). There was a minor difference between the DMSO vehicle control and S1QEL 1.1 at low concentrations (0.1-1 μ M) (compare lane 4 to lanes 5-7 of the upper panel) and S1QEL 1.1 inhibited pro-IL-1 β levels at 3 and 10 μ M (compare lanes 8-9 to lane 4 of the upper panel).

Because HIF-1 α is a transcription factor responsible for IL-1 β transcription (118), I next examined the effect of S1QEL on HIF-1 α protein levels. As can be seen in **figure 3.4**, S1QEL 1.1 decreased HIF-1 α expression at high concentrations (compare lanes 7-8 to lane 2), with a boost occurring at 0.1-1 μ M (compare lanes 4-6 to lane 3).

A



B

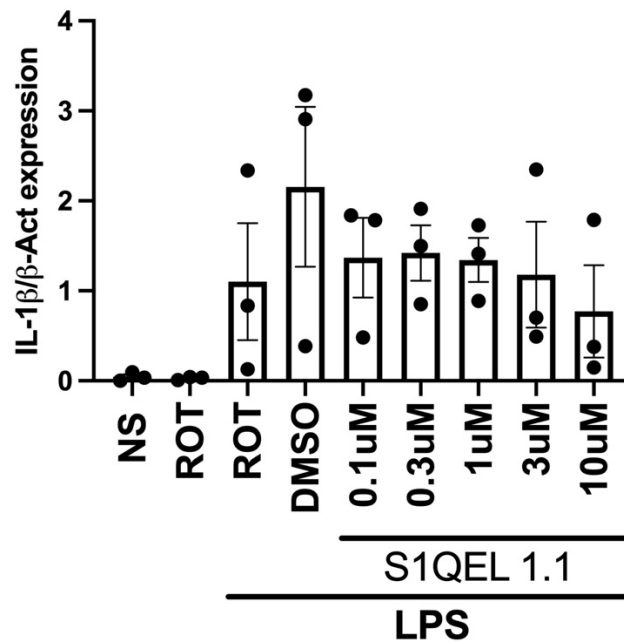


Figure 3.3 S1QEL 1.1 pre-treatment for 3 hours shows a trend in inhibition of pro IL-1 β at high concentrations.

(A) BMDMs (0.5×10^6) were pre-treated for 3 hours with DMSO (vehicle control), Rotenone (positive control) ($0.5 \mu\text{M}$) and S1QEL1.1 (0.1 – $10 \mu\text{M}$). They were then stimulated for 24 hours with LPS (100 ng/mL), before whole cell lysates were harvested and analyzed by western blot for the expression of pro IL-1 β and β -actin. Results shown are representative of three separate experiments. (B) Densitometry of 3 independent experiments. Data are shown as mean $\pm$ SEM for $n=3$ from three independent experiments.

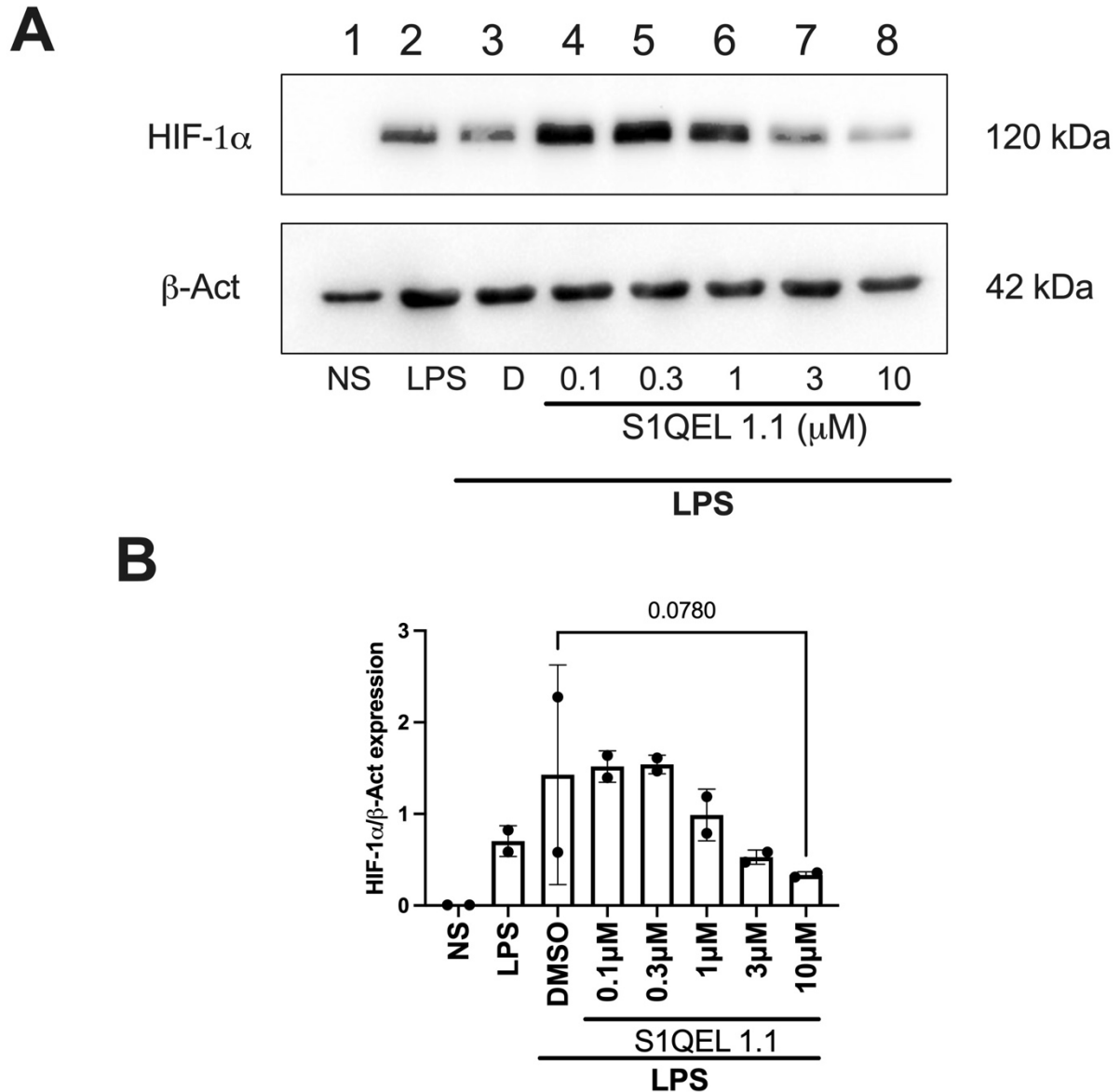


Figure 3.4 S1QEL 1.1 boosts HIF-1α expression at low concentrations while suppressing it at high concentrations.

BMDMs (0.5×10^6) were pre-treated for 3 hours with DMSO (vehicle control) and S1QEL1.1 (0.1-10 μM). They were then stimulated for 24 hours with LPS (100 ng/mL) before whole cell lysates were harvested and analyzed by western blot for the expression of HIF-1α and β-actin (A). Results shown are representative of two separate experiments. (B) Densitometry of 2 independent experiments. Data are shown as mean $\pm$ SD for $n=2$ from two independent experiments.

Having examined the role of Complex I in the regulation of pro-inflammatory cytokine response in macrophages, I focused on the involvement of Complex III.

As can be seen in **figure 3.5**, LPS stimulation boosted the expression of the mRNA of the pro-inflammatory cytokines. The 3 hours pre-treatment with S3QEL 1.2 had no effect on LPS-induced IL-1 β (A) or IL-6 (B) mRNA expression but at 3 μ M and 10 μ M there was an increase in TNF- α production (C).

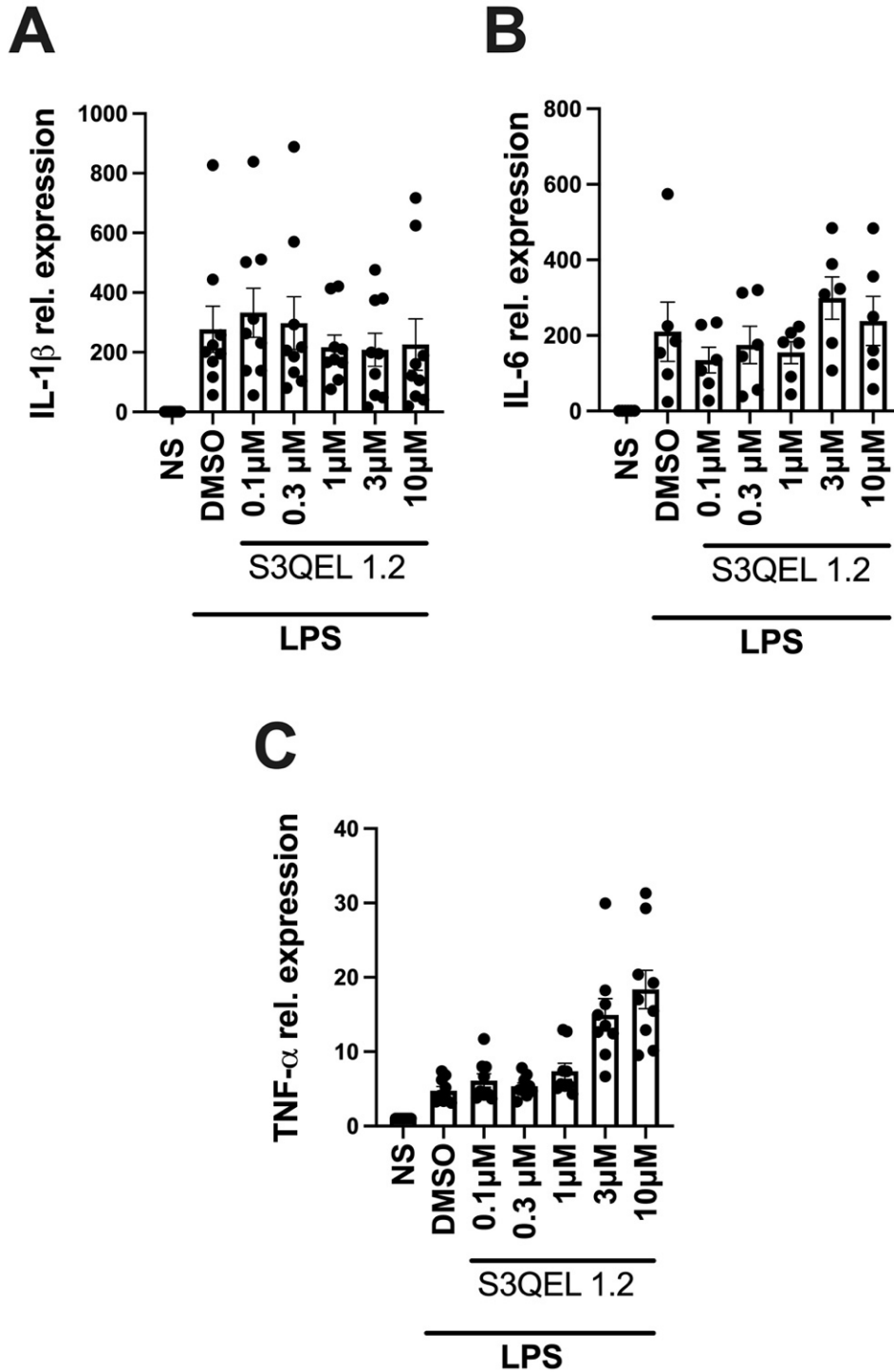


Figure 3.5 Effect of 3 hours pre-treatment with S3QEL 1.2 on the expression of IL-1 β , TNF- α and IL-6.

BMDMs (1×10^6 cells) were pre-treated with DMSO, S3QEL 1.2 (0.1-10 μ M) for 3 hours, after which they were stimulated with LPS (100ng/mL) for 24 hours. After stimulation, RNA was isolated from cell lysates and analyzed by RT-qPCR for the expression of IL-1 β (A), TNF- α (B) and IL-6 (C). The data shown are fold increase over RPS18 control and are the mean $\pm$ SEM for n=9 from three independent experiments.

I next tested the effect of S3QEL 1.2 on pro-IL-1 β and HIF-1 α protein production. **Figure 3.6** shows the decrease of pro-IL-1 β after the pretreatment with S3QEL at high concentrations (3 and 10 μ M) (middle panel, compare lane 2 to lanes 6 and 7) and Myxothiazol (middle panel, compare lane 2 to lane 9) and the same result was confirmed by densitometry.

Both S3QEL 1.2 and Myxothiazol also inhibited HIF-1 α production (lower panel, compare lane 2 to lane 6 and 7 and compare lane 2 to lane 9) (**figure 3.6**). Also, Antimycin A, another inhibitor for Complex III, decreased the protein expression levels of pro-IL-1 β and HIF-1 α (compare lane 2 to lane 8 in the middle and upper panel) (**figure 3.6**).

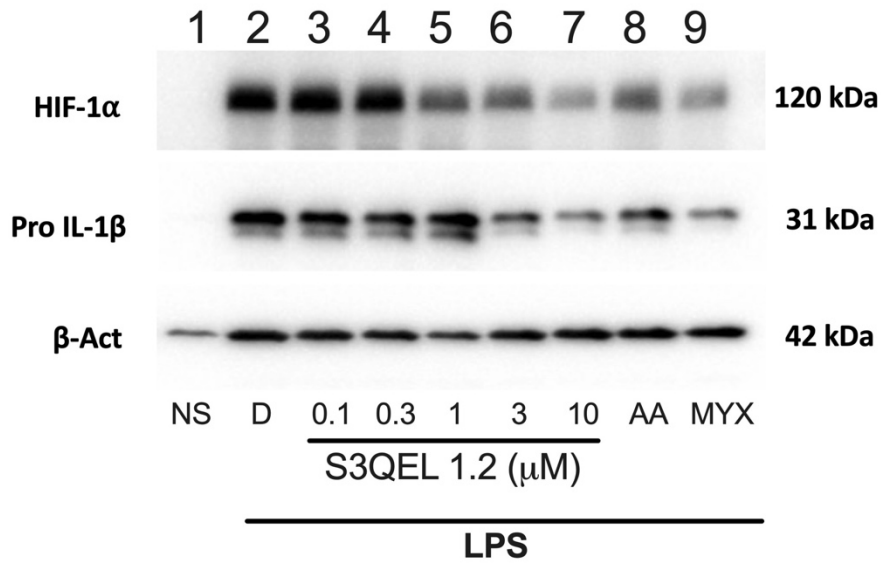
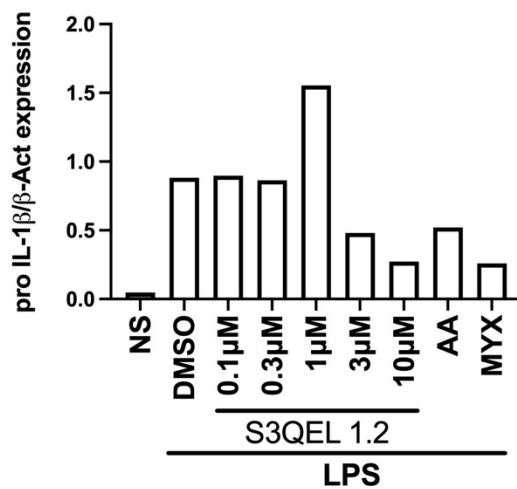
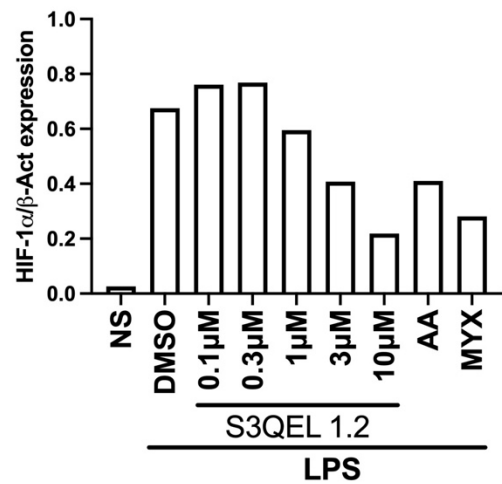
A**B****C**

Figure 3.6 S3QEL 1.2 suppresses HIF-1α pro-IL-1β expression at high concentrations.

BMDMs (0.5×10^6) were pre-treated for 3 hours with DMSO (vehicle control), MYX (2 μM), Antimycin A (5 μM) and S3QEL 1.2 (0.1-10 μM). They were then stimulated for 24 hours with LPS (100 ng/mL), before whole cell lysates were harvested and analyzed by western blot for the expression of HIF-1α, pro-IL-1β and β-actin (A) with densitometry in (B) and (C). The results shown are representative of one set of samples.

After obtaining these results, I sought to understand if also the mRNA levels of HIF-1 α were following the trend of the protein levels. I also used other ETC inhibitors, such as Antimycin A and Myxothiazol as internal controls. Unexpectedly, the mRNA levels of HIF-1 α were not significantly affected by any treatment (panel A and B, **figure 3.7**).

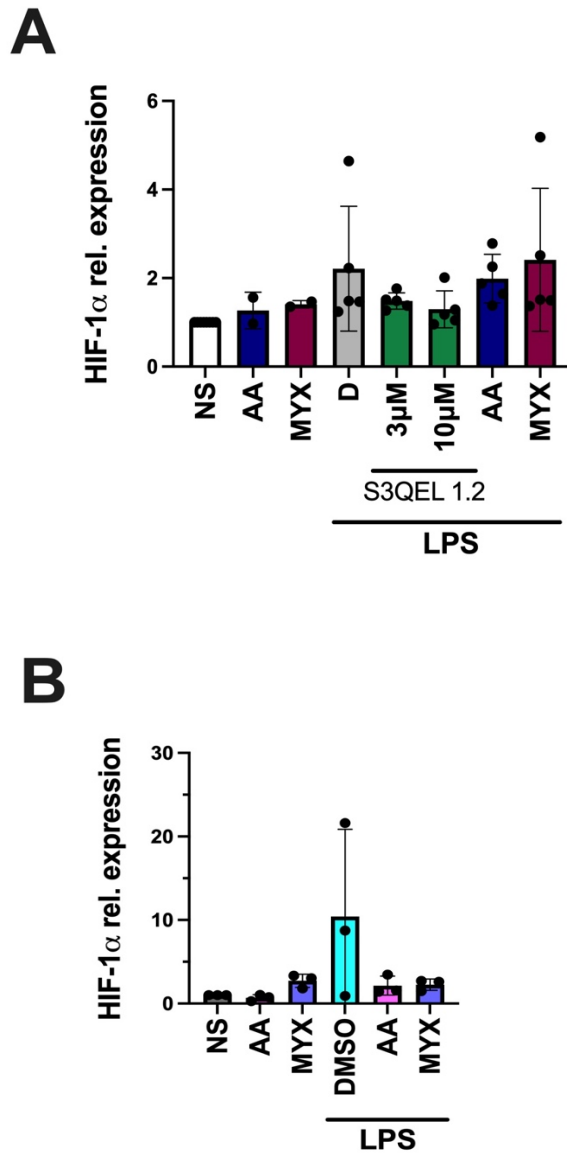


Figure 3.7 S3QEL 1.2 and Myxothiazol do not change mRNA expression of HIF-1 α .

(A and B) BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL 1.2 (10 μ M), Antimycin A (5 μ M) and Myxothiazol (2 μ M) and then stimulated for 24 hours with LPS (100ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-1 β , HIF-1 α and RPS18. Data are mean $\pm$ SD for n=3-5 from two independent experiments.

3.2 SIQELs and NLRP3 inflammasome activation

Next, I examined the effect of SIQELs on NLRP3 activation. As can be seen in **figure 3.8**, treatment of BMDMs with S1QEL 1.1 had no effect on LDH release (A) or IL-1 β production (B) following NLRP3 inflammasome activation with Nigericin.

I also tested the effect of S3QEL on NLRP3 activation. As can be seen in **figure 3.9**, S3QEL 1.2 did not block LDH release (A) or IL-1 β release (B) following NLRP3 inflammasome activation with Nigericin, while the well-known NLRP3 inflammasome inhibitor MCC950 was clearly inhibitory. However, IL-1 β release and LDH release were increased at high concentrations.

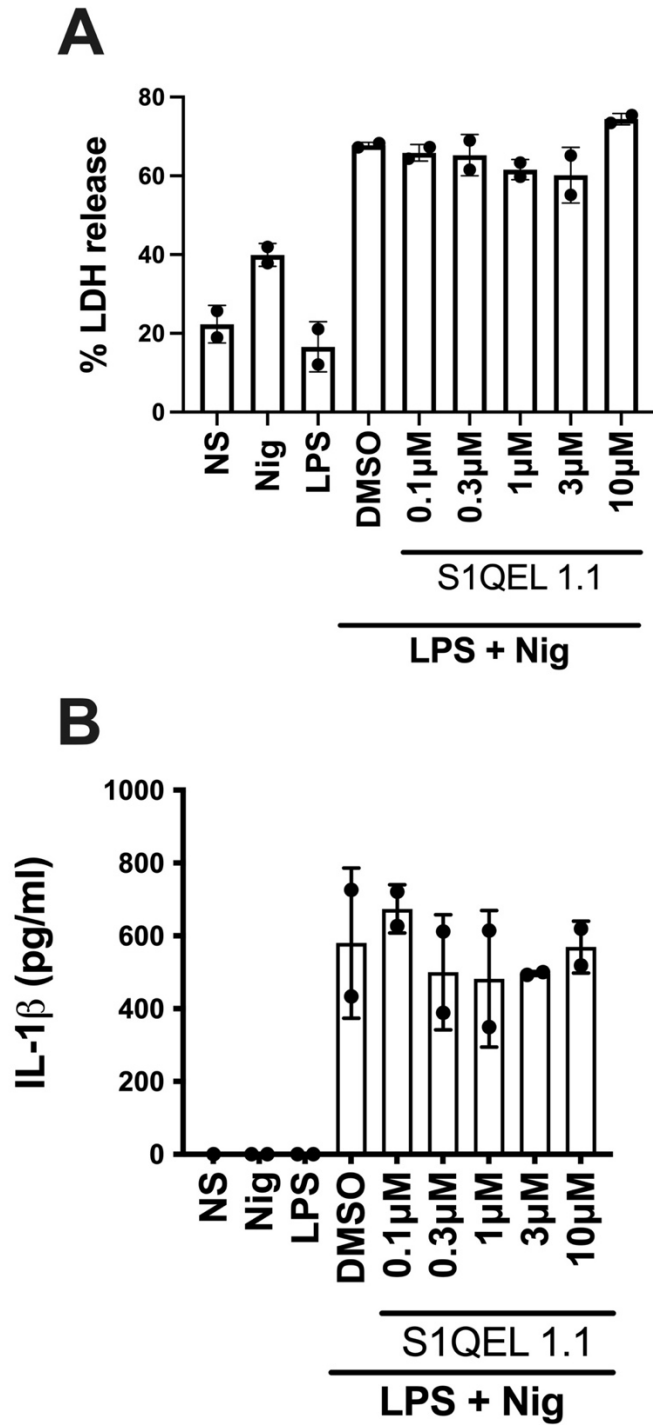


Figure 3.8 Effect of S1QEL 1.1 on NLRP3 activation.

BMDMs (0.5×10^6 cells) were treated for three hours with LPS (100ng/mL). Medium was replaced with serum-free media and cells were treated for 45 minutes with DMSO or increasing concentrations of S1QEL 1.1. 10µM nigericin was added for 45 minutes as a signal 2 activator, after which supernatants were harvested and LDH release (A) and IL-1β concentration by ELISA (B) were quantified. LDH release is expressed as a percentage of total lysis positive control. Data are mean $\pm$ SD for n=2 from two independent experiments.

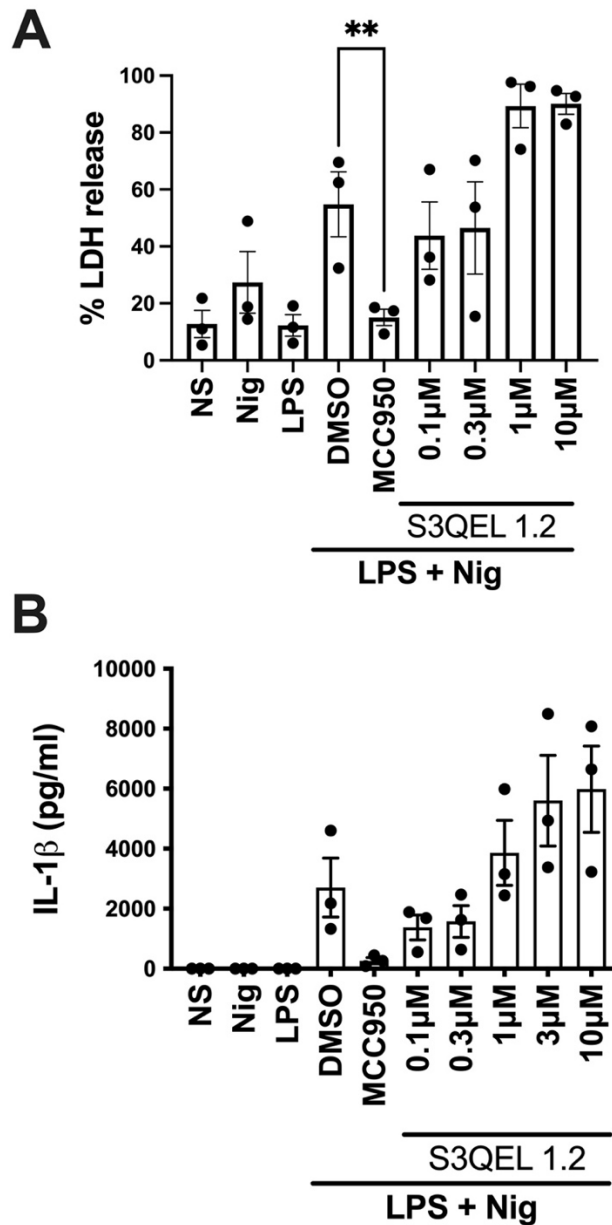


Figure 3.9 S3QEL 1.2 post-treatment does not affect NLRP3 inflammasome activation.

BMDMs (0.5×10^6 cells) were treated for three hours with LPS [100ng/mL]. Medium was replaced with serum-free media and cells were treated for 45 minutes with DMSO, MCC950 or increasing concentrations of S3QEL 1.2. 10μM nigericin was added for 45 minutes as a signal 2 activator, after which supernatants were harvested and LDH release (A) and IL-1β concentration by ELISA (B) were quantified. LDH release is expressed as a percentage of total lysis positive control. The data shown are the mean ± SEM for n=3 from three independent experiments. Differences were considered statistically significant at **p<0.01.

3.3 Discussion

Mitochondria are involved in PAMPs and PRR-mediated immune signalling in innate and adaptive immune cells (119) and participate to the release of immunomodulatory cytokines into the extracellular environment, especially through ROS generation (120). The rationale for examining the role of ROS in inflammasome activation and IL-1 β production originated from the need to link redox stress and the inflammatory response in LPS-activated macrophages. Several work already highlighted the connection between mitochondria and inflammasome activation (121), but the findings did not show specific evidence for mitochondrial ROS and dysfunction.

S1QEL 1.1 and S3QEL 1.2 represent a new class of specific inhibitors of ROS production at Complex I and Complex III of the ETC and their use has strong advantages over the classic and non-specific mitochondria-targeted anti-oxidants (108). They do not affect basal respiration and do not undergo redox recycling because they do not participate in redox reactions, so their amount remains unchanged in cells. Indeed, they do not alter the mitochondrial membrane potential. These properties led me to verify their effects in macrophages and to use them to dissect ROS-sensitive processes such as activation of the inflammasome (98). The involvement of ROS in the stabilisation of HIF-1 α was already established (122) and was confirmed with the use of SIQELs in tumour cells (111) but there is no great evidence for SIQELs use in immune cells, such as activated macrophages.

I therefore decided to assess the changes in cytokine production when macrophages are highly glycolytic and undergo ETC redox state remodelling.

The concentrations of S1QEL 1.1 and S3QEL 1.2 used in the experiments of this project have been suggested by the researchers who discovered the compound and vary from 0.1 to 10 μ M without exceeding the latter because of the possibility of running into ETC inhibition.

Aware of the connection between mitochondrial ROS and NLRP3 inflammasome activation, I first examined Complex I and Complex III-derived ROS in regulating macrophage cytokine profile. S1QEL 1.1 and S3QEL 1.2 blocked pro-IL-1 β protein

at high concentrations while low concentrations might increase it. This might be explained by low concentrations of SIQELs increasing ROS generation through the indirect disturbance of the electron flow. Furthermore, ROS could represent a way to stabilise pro-IL-1 β protein by avoiding its ubiquitylation (123). Although different incubation times may be required for the compounds to enter the cell and bind to the quinone site on Complex I and Complex III in mitochondria, SIQELs have clear effects when cells are treated for a short time.

Likewise, HIF-1 α levels were boosted at lower concentrations but inhibited at 10 μ M by both S1QEL 1.1 and S3QEL 1.2, similar to the effect of other ETC inhibitors like AA and MYX. The mechanisms underlying the HIF-1 α regulation by ROS are not completely understood (124). There is evidence that superoxide generated from site III_{Qo} is essential for the stabilisation of HIF-1 α (125), but the same result could be due to the secondary effects of inhibiting electron flow, like the interruption of ATP supply and alterations in levels of metabolites such as succinate. Treatment of HEK-293 cells with S3QELs inhibited the accumulation of HIF-1 α (126) and this particular result fits with my own findings. Superoxide anion production by Complex I and Complex III of the ETC might be activating specific signalling pathways regulating the transcription of IL-1 β and relying on HIF-1 α stabilisation. There could be redundancy between Complex I and Complex III in the regulation of ROS, HIF-1 α and IL-1 β . Mechanistically, SIQEL-mediated inhibition of superoxide production may be decreasing HIF-1 α protein levels, which leads to a consequent decrease in levels of IL-1 β mRNA and protein. The two experiments in **figure 3.7** show no effect of ETC inhibitors on HIF-1 α mRNA. Previous literature has demonstrated that HIF-1 α mRNA and protein levels could have opposite expression (127, 128). This is because HIF-1 α can be regulated by many transcriptional (promotion or repression of transcription by transcription factors), post-transcriptional (splicing and mRNA export from the nucleus) and post-translational modification (such as the ones led by hypoxia and PHDs-dependent activity) (129, 130).

The S1QEL and S3QEL-mediated decrease in the protein levels of HIF-1 α suggest a subsequent decrease in the mRNA and protein levels of IL-1 β . My findings show that S1QEL 1.1 and S3QEL 1.2 had no effect on IL-1 β mRNA but only on IL-1 β

protein, which will require further investigation given the role of HIF-1 α in regulating IL-1 β transcription.

S1QEL 1.1 and S3QEL 1.2, at high concentrations, did not decrease the mRNA levels of IL-6, TNF- α and IL-1 β . Interestingly, S1QEL 1.1 and S3QEL 1.2 at 3 μ M and 10 μ M boosted TNF- α mRNA production. This may indicate that high doses trigger ROS generation through mitochondrial ETC dysfunction and this may lead to a TNF- α mediated pro-inflammatory response. This last finding gave us the idea to investigate SIQELs regulating IL-10 because IL-10 participates into a cross-talk with TNF- α . This will be the subject of next chapter.

Although I demonstrated that SIQELs have regulatory effects on LPS-induced pro-IL-1 β expression (signal 1), I found no effect on NLRP3 inflammasome activation (signal 2). Treatment with S1QEL 1.1 slightly increased cell death when administered to cells before signal 2 as assessed by LDH release and this indicates that it could behave as another signal 2 driver, possibly by contributing to potassium efflux from the cell. The inhibition of ROS at the quinone site on Complex I and inhibition of O₂⁻ produced by Complex III may induce generation of ROS from other sources (like NADPH oxidases), which can then activate the NLRP3 inflammasome and lead to cell death by pyroptosis. Moreover, treatment with S3QEL 1.2 increased IL-1 β release and cell death in a dose-dependent manner, showing an opposite trend compared to the positive control MCC950, which was decreasing both readouts. These conclusions indicate that high concentration of SIQELs could cause pyroptotic death in combination with LPS and nigericin.

More insight into the topic of redox-sensitive inflammasome activation was given by Chandel et al. in their work published in 2022 (98). The researchers showed that the inhibition of the ETC at the levels of Complex I, III and IV prevents NLRP3 inflammasome activation but in a ROS-independent manner. The real mechanism was explained through the phosphocreatine (PCr) intermediary: metabolomics analysis showed that phosphocreatine was the common metabolite which was downregulated after ETC inhibition. Phosphocreatine is generated by creatine and ATP in mitochondria through the action of the enzyme creatine kinase (CKMT2); after that, it can exit the mitochondria and be converted again into creatine and P_i.

by cytosolic creatine kinase (CKB), thus recreating cytosolic ATP from ADP and inorganic phosphate. The treatment of activated macrophages with cyclocreatine (cyCr), a creatine analogue, inhibited further ATP production and diminished NLRP3 inflammasome activation which requires ATP binding, and subsequent IL-1 β production.

The fact that the use of SIQELs in my system did not decrease IL-1 β production, cell death by pyroptosis and Caspase-1 cleavage suggest that the activation of NLRP3 inflammasome might be independent of ROS generation by Complex I and III and instead might involve ATP and phosphocreatine. Additionally, in a paper published by Hornung et al. (131), the authors affirm that ROS scavengers can affect NLRP3 inflammasome priming but not activation. They used ROS inhibitors (NAC and DPI) treatment before and after LPS priming and found that IL-1 β release and NLRP3 activation were decreased in macrophages only when DPI and NAC were added before and not after LPS. This could explain the selective effect of ROS only on the priming of NLRP3, while its direct activation is not affected. This selectivity might be related to the necessity of a priming signal for NLRP3 expression, considering that its expression is almost poor in non-activated macrophages. On the contrary, AIM2 and NLRC4 inflammasomes are constitutively expressed in unprimed macrophages and, subsequently, they are insensitive to ROS inhibition (131). Furthermore, it is possible that other processes can change ATP presence in the cell and regulate the NLRP3 inflammasome. Of great interest is the release of mitochondrial DNA from damaged organelles and the subsequent trigger of the nucleic acid sensing cGAS-STING pathway. Thereafter, other studies need to be performed to specifically exclude or include these signals as component in the regulation of the inflammasome activation.

Overall, here I have found a possible role for Complex I and Complex III-derived ROS in HIF-1 α activation and pro IL-1 β production but more importantly a positive role for Complex III-derived ROS on TNF- α production. This effect will be explored further in the next chapter.

4. Mitochondrial Complex III-dependent gene expression in activated macrophages

4.1 Characterisation of S3QEL 1.2 and MYX in macrophages

I next focused my attention on mitochondrial complex III using the Complex III inhibitors S3QEL 1.2 and myxothiazol. This was due to the poor characterisation of complex III-dependent regulation of the immune response in macrophages.

Since there are only limited studies on Complex III inhibition, and in particular on S3QEL 1.2 in macrophages, I first investigated the effects of these small molecule modulators on general cellular activity and viability.

4.1.1 S3QEL 1.2 and Myxothiazol do not affect macrophage cell viability

In LPS-treated BMDMs, as can be seen in **figure 4.1**, the inhibitors did not affect cell viability, as assessed in an MTT assay.

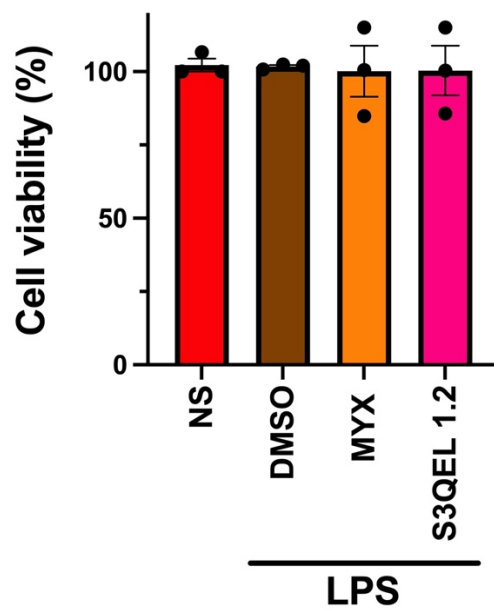


Figure 4.1. S3QEL 1.2 and Myxothiazol do not affect cell viability.

BMDMs (0.1×10^6 cells) were treated for 3 hours with S3QEL 1.2 ($10 \mu\text{M}$) or Myxothiazol (500 nM) and then stimulated for 4 hours with LPS (100 ng/mL). The viability of cells was assessed by MTT assay. Bar diagram representing the percentage of viable BMDMs in different treatment groups (triplicates for each group). The untreated sample (positive control) is considered as 100% viable while the other conditions are related to the untreated sample. Data are mean $\pm$ SEM for $n=3$ from three independent experiments.

4.1.2 S3QEL 1.2 and Myxothiazol do not affect mitochondrial respiratory complexes expression

I next investigated if the inhibitors were changing the protein expression of the ETC complexes. As can be seen in **figure 4.2** the protein levels of the complexes I-V were not significantly affected by the inhibitors (panel A is showing the protein levels and panel B is showing densitometry).

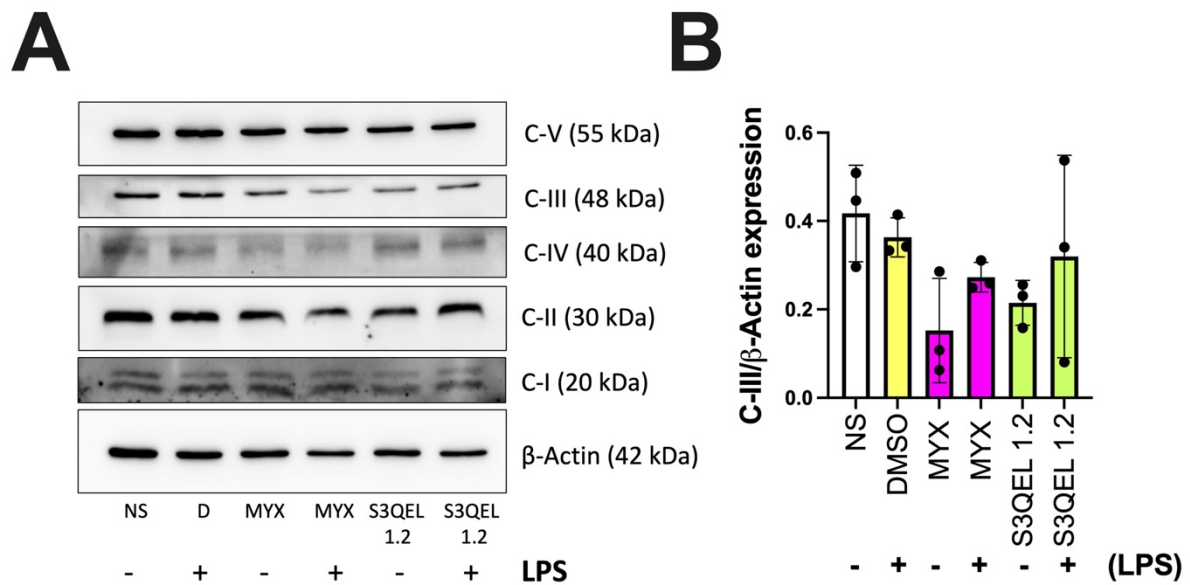


Figure 4.2. S3QEL 1.2 and Myxothiazol do not affect the expression of respiratory complexes.

BMBDs (1×10^6 cells) were treated for 3 hours with DMSO, S3QEL 1.2 ($10 \mu\text{M}$) and Myxothiazol (500 nM) and then stimulated for 4 hours with LPS (100 ng/mL). After that, cell lysates were harvested and analysed by western blot for the expression of (A) Complex I, II, III, IV and V. Blots is presented from one mouse. (B) Expression of Complex III was quantified by densitometry. Data are mean $\pm$ SD for $n=3$ from three independent experiments.

4.1.3 S3QEL 1.2 and Myxothiazol affect OXPHOS in different ways

I next examined OXPHOS in BMDMs by Seahorse analysis after S3QEL 1.2 and myxothiazol treatment and LPS triggering. As expected, OXPHOS significantly decreased when BMDMs were stimulated with LPS (**figure 4.3A**), explaining the M1 phenotype and the switch towards glycolytic activity of pro-inflammatory macrophages activated by LPS. Myxothiazol treatment completely inhibited OXPHOS, both in normal and LPS-stimulated conditions (**figure 4.3B and C**), as expected. S3QEL 1.2 slightly diminished respiration in control samples, still keeping OXPHOS to normal levels while it slightly increased the oxidative process in the presence of LPS, having overall an unchanging influence on the oxidative metabolism of BMDMs (**figure 4.3B and C**).

A

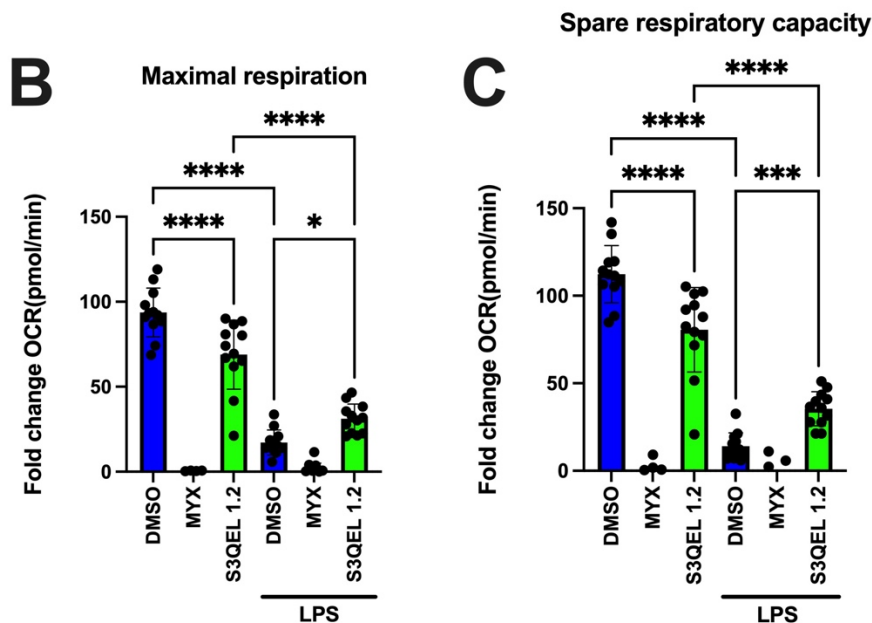
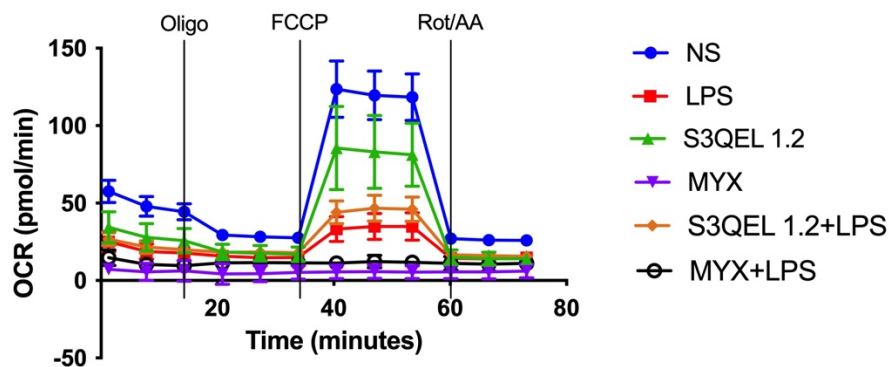


Figure 4.3. Oxidative phosphorylation is affected by S3QEL 1.2 and Myxothiazol.

BMDMs (0.1×10^6 cells/well) were treated for 3 hours with DMSO, S3QEL 1.2 ($10 \mu\text{M}$) and Myxothiazol (500 nM) and then stimulated for 24 hours with LPS (100 ng/mL). Real-time changes in OCR were measured by Seahorse XF analysis. (A) Representative OCR trace from one of three independent experiments, each experiment being performed with 12 technical replicates. Lines indicate injection times of oligomycin ($1 \mu\text{M}$), FCCP ($1 \mu\text{M}$), rotenone ($1 \mu\text{M}$) and antimycin A ($2 \mu\text{M}$). Maximal respiration (B) and spare respiratory capacity (C) rates were calculated and displayed as bar charts. Data are mean $\pm$ SD from one of three independent experiments. *P*-values presented as calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at $*p < 0.05$, $****p < 0.0001$.

4.1.4 Glycolysis is upregulated in response to S3QEL 1.2 and Myxothiazol

I next examined if glycolysis was affected by the same compounds. **Figure 4.4 (A, B and C)** clearly shows that S3QEL 1.2 and MYX both upregulated glycolysis compared to the untreated control in macrophages not activated by LPS. Moreover, Oligomycin did not have a boosting effect on glycolysis in the MYX-treated condition because OXPHOS was already shut down by Myxothiazol itself.

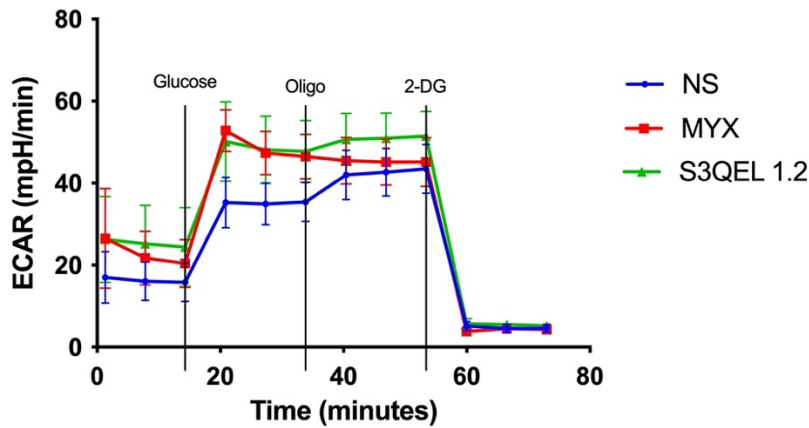
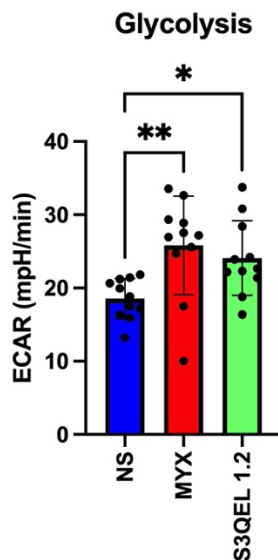
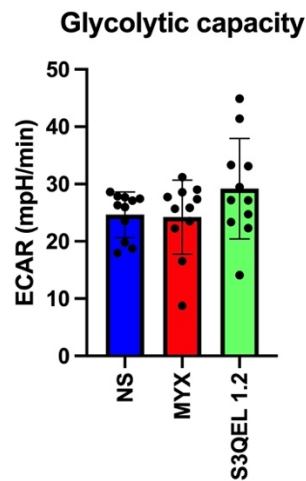
A**B****C**

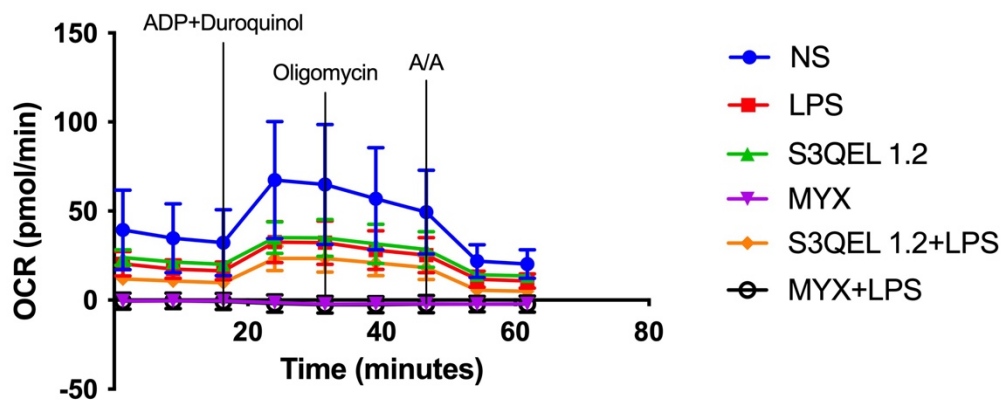
Figure 4.4. Glycolysis is upregulated by S3QEL 1.2 and Myxothiazol in basal conditions.

BMDMs (1.5×10^5 cells/well) were treated for 3 hours with DMSO, S3QEL 1.2 ($10 \mu\text{M}$) and Myxothiazol (500nM). Real-time changes in ECAR were measured by Seahorse XF analysis. (A) Representative ECAR trace from one of three independent experiments, each experiment being performed with 11 technical replicates. Lines indicate injection times of glucose (10 mM), oligomycin ($1 \mu\text{M}$), 2-DG (50 mM). Glycolysis (B) and Glycolytic capacity (C) rates were calculated and displayed as bar charts. Data are mean $\pm$ SD from one of three independent experiments. *P*-values presented as calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$.

4.1.5 S3QEL 1.2 and Myxothiazol affect Complex-III specific activity in different ways

I next examined Complex III specific respiration. I did this by giving specific substrates to macrophages for boosting only Complex III activity and I examined it by Seahorse analysis after S3QEL 1.2 and myxothiazol treatment and LPS triggering. As expected, Complex III respiration significantly decreased when BMDMs were stimulated with LPS (**figure 4.5A**), mirroring the dampening of OXPHOS in the M1 phenotype. Myxothiazol treatment completely inhibited Complex III specific respiration, both in normal and LPS-stimulated conditions (**figure 4.5A**), as expected. S3QEL 1.2 did not disrupt the process in the presence and absence of LPS, consistent with S3QEL 1.2 not inhibiting complex III activity in the ETC (**figure 4.5B**).

A



B

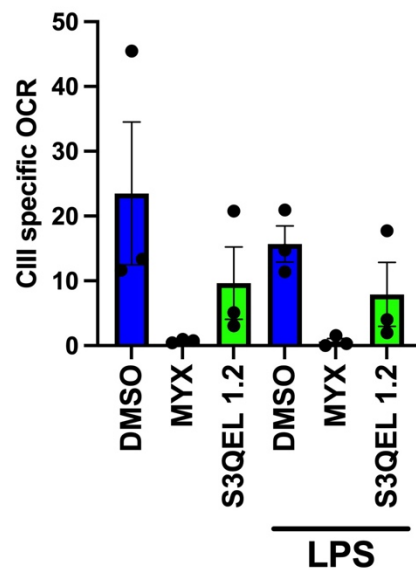


Figure 4.5. Effect on Complex III specific respiration by S3QEL 1.2 and Myxothiazol.

BMDMs (0.1×10^6 cells/well) were treated for 3 hours with DMSO, S3QEL 1.2 ($10 \mu\text{M}$) and Myxothiazol (500 nM) and then stimulated for 24 hours with LPS (100 ng/mL). Real-time changes in OCR were measured by Seahorse XF analysis. (A) Representative OCR trace from one of three independent experiments, each experiment being performed with 12 technical replicates. Lines indicate injection times of ADP (1 mM), Duroquinone (0.5 mM), Oligomycin ($15 \mu\text{M}$) and antimycin A ($20 \mu\text{M}$). (B) C III specific OCR was calculated as OCR after first injection – OCR after last injection and displayed as bar chart. Data are mean $\pm$ SEM for $n=3$ from three independent experiments.

4.1.6 Myxothiazol and S3QEL 1.2 decrease ROS production in murine macrophages

As previously shown (108, 111, 132), S3QEL 1.2 specifically decreases superoxide production from mitochondrial Complex III (111). Myxothiazol has also been shown to decrease LPS-induced superoxide production (88). I therefore measured the H₂O₂ production as an indicator of ROS in cells treated with S3QEL 1.2 and MYX.

As shown in **figure 4.6**, MYX and LPS alone boosted H₂O₂ generation in macrophages while S3QEL 1.2 and the mitochondrial antioxidant MitoT alone did not. The LPS-induced boost was slightly decreased by pre-treatment with S3QEL 1.2, MYX and MitoT.

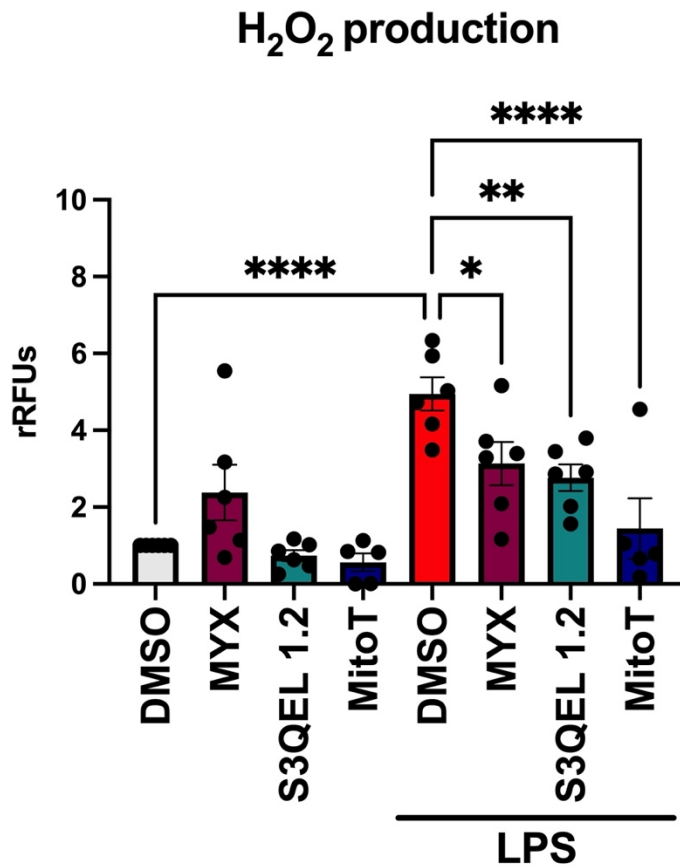


Figure 4.6. S3QEL 1.2 and Myxothiazol slightly decrease LPS-induced H₂O₂ production in BMDMs.

BMDMs (0.1×10^6 cells/well) were treated for 24 hours with DMSO, S3QEL 1.2 ($10 \mu\text{M}$) and Myxothiazol (500 nM) or for 48 hours with MitoTEMPO (5 mM) then stimulated for 48 hours with LPS (200 ng/mL). After that, cell production of H₂O₂ was detected by the Amplex Red assay. Data are mean $\pm$ SEM for $n=3$ from three independent experiments. $**p < 0.01$ as calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. RFUs = relative fluorescence units.

4.2 The inhibition of mitochondrial Complex III decreases IL-10 production from macrophages

4.2.1 S3QEL 1.2 at high concentrations decreases IL-10 expression and increases TNF- α expression

I next examined effect of S3QEL 1.2 on the anti-inflammatory cytokine IL-10.

As shown in **figure 4.7**, S3QEL 1.2 significantly decreased IL-10 expression and as shown in the previous chapter, boosted TNF- α production, both at the mRNA level (A and B) and protein level (C and D) due to IL-10 differences.

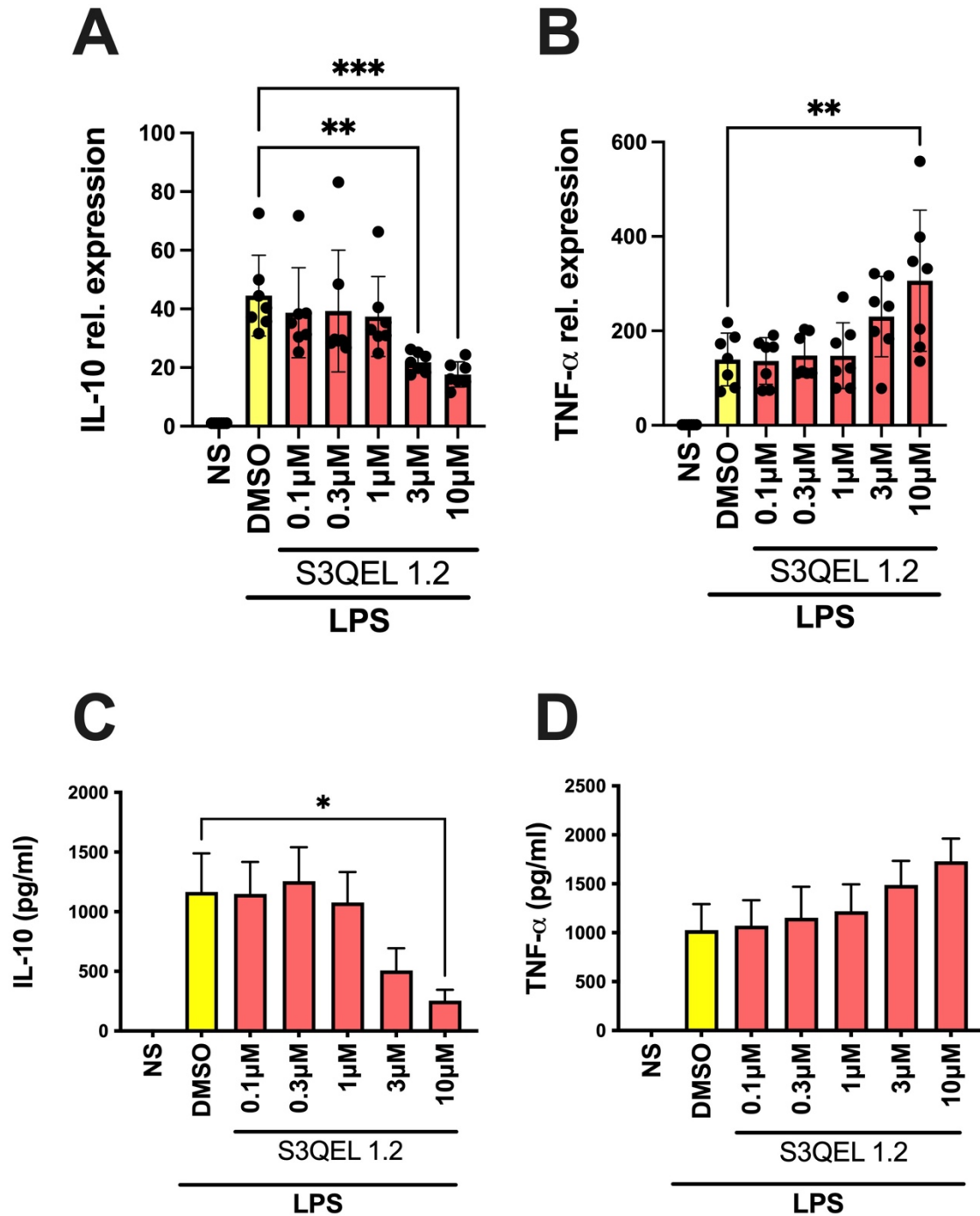


Figure 4.7. S3QEL 1.2 decreases IL-10 and increases TNF-α expression.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL 1.2 (10 μM) and then stimulated for 4 hours with LPS (100 ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10, TNF-α with RPS18 as a housekeeping control (A and B). IL-10 and TNF-α concentrations in supernatants were analyzed by ELISA (C and D). Data are mean $\pm$ SEM for $n=7$ from three independent experiments. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

4.2.2 Myxothiazol decreases IL-10 release

The same experiment was performed after myxothiazol treatment. As before, myxothiazol significantly decreased IL-10 production by ELISA (panel C) but there was a slight effect on the mRNA levels (panel A) (**figure 4.8**).

TNF- α levels did not change after myxothiazol treatment (**figure 4.8B and D**).

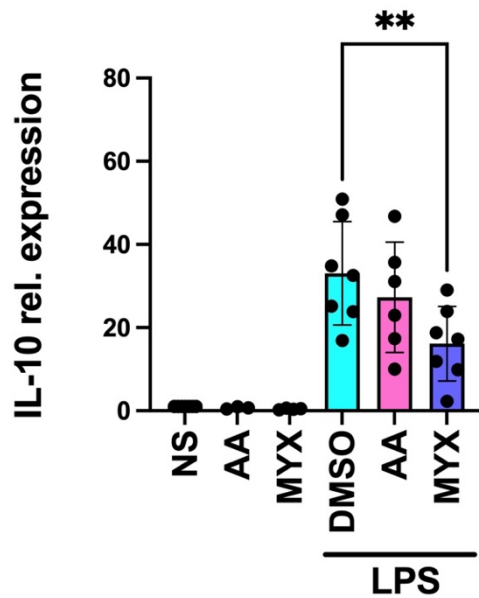
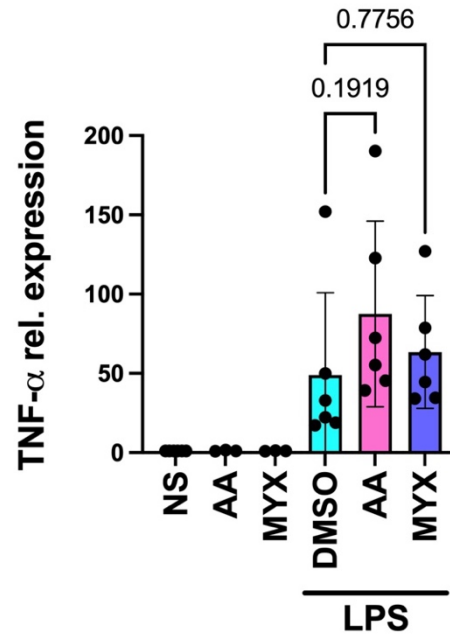
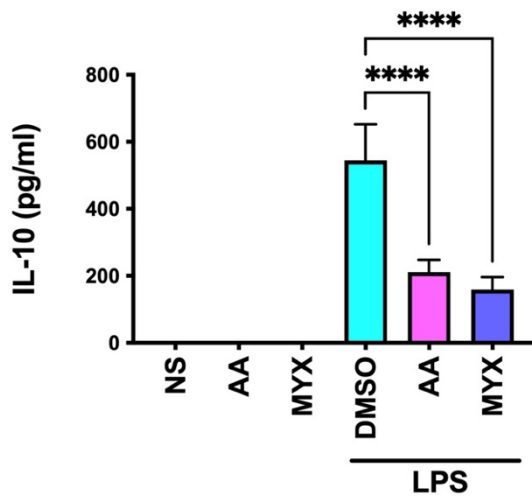
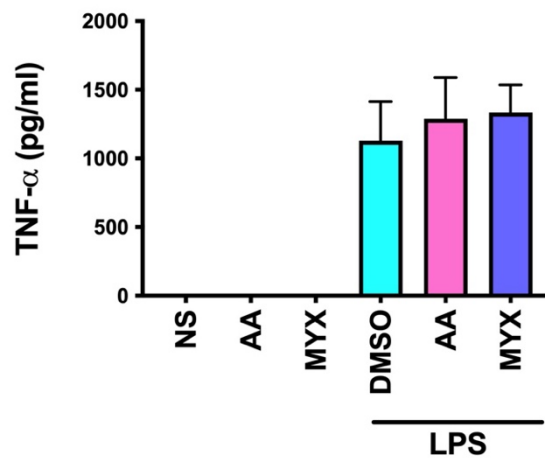
A**B****C****D**

Figure 4.8. Myxothiazol decreases IL-10 and does not changes TNF-α expression.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL (10 μ M) and Myxothiazol (500nM) and then stimulated for 4 hours with LPS (100ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10, TNF- α with RPS18 as a housekeeping control (A and B). IL-10 and TNF- α concentrations in supernatants were analyzed by ELISA (C and D). Data are mean $\pm$ SEM for $n=6-7$ from three independent experiments. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at **** $p < 0.0001$, ** $p < 0.01$.

4.2.3 Bulk RNAseq analysis on unstimulated and activated macrophages after S3QEL 1.2 and MYX pre-treatment

Given the results shown in **figure 4.7** and **figure 4.8** regarding the downregulation of IL-10 and upregulation of TNF- α after exposure to Complex III inhibitors, I decided to confirm my results and obtain a deeper insight into global transcriptional changes. The samples of BMDMs were treated as for previous protocols and mRNA expression analysis was performed confirming repression of IL-10 (panel A) and increased TNF- α (panel B) production (**figure 4.9**).

The volcano plot shown in **figure 4.10** represents the highest upregulated and downregulated genes in the samples analyzed in **figure 4.9**, in a comparison between unstimulated and LPS-activated macrophages.

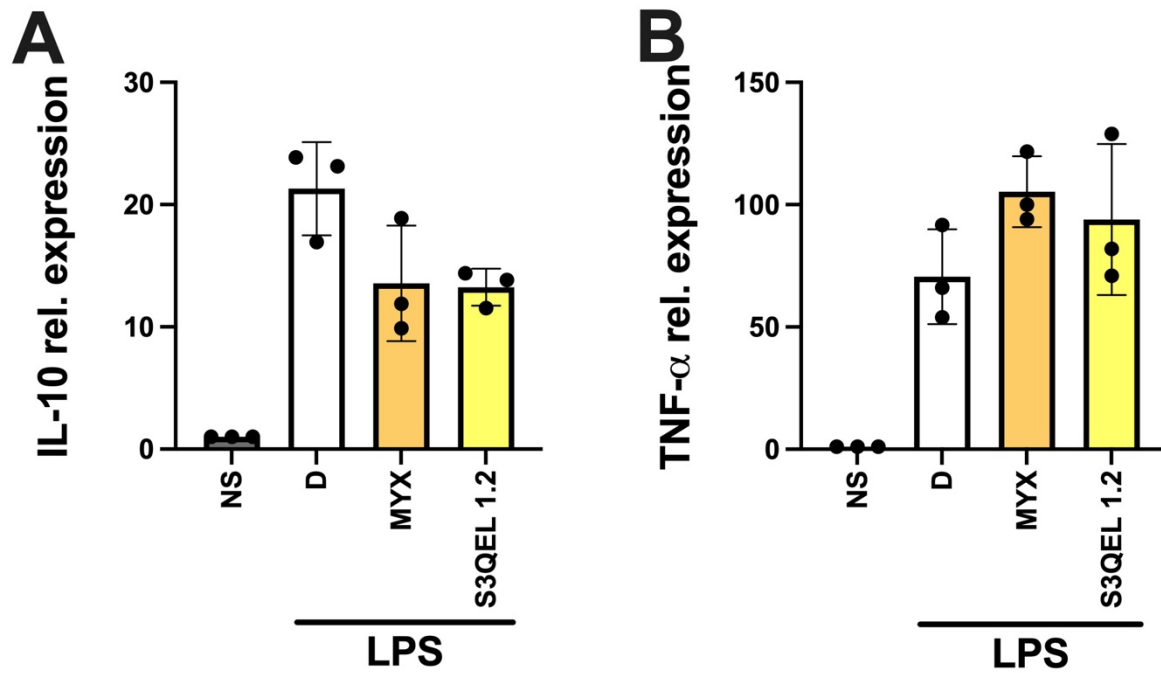


Figure 4.9. Myxothiazol and S3QEL 1.2 decrease IL-10 and increase TNF- α mRNA expression.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL 1.2 (10 μ M) and Myxothiazol (500nM) and then stimulated for 4 hours with LPS (100ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10, TNF- α with RPS18 as a housekeeping control (A and B). Data are mean $\pm$ SD for $n=3$, where one representative experiment is shown.

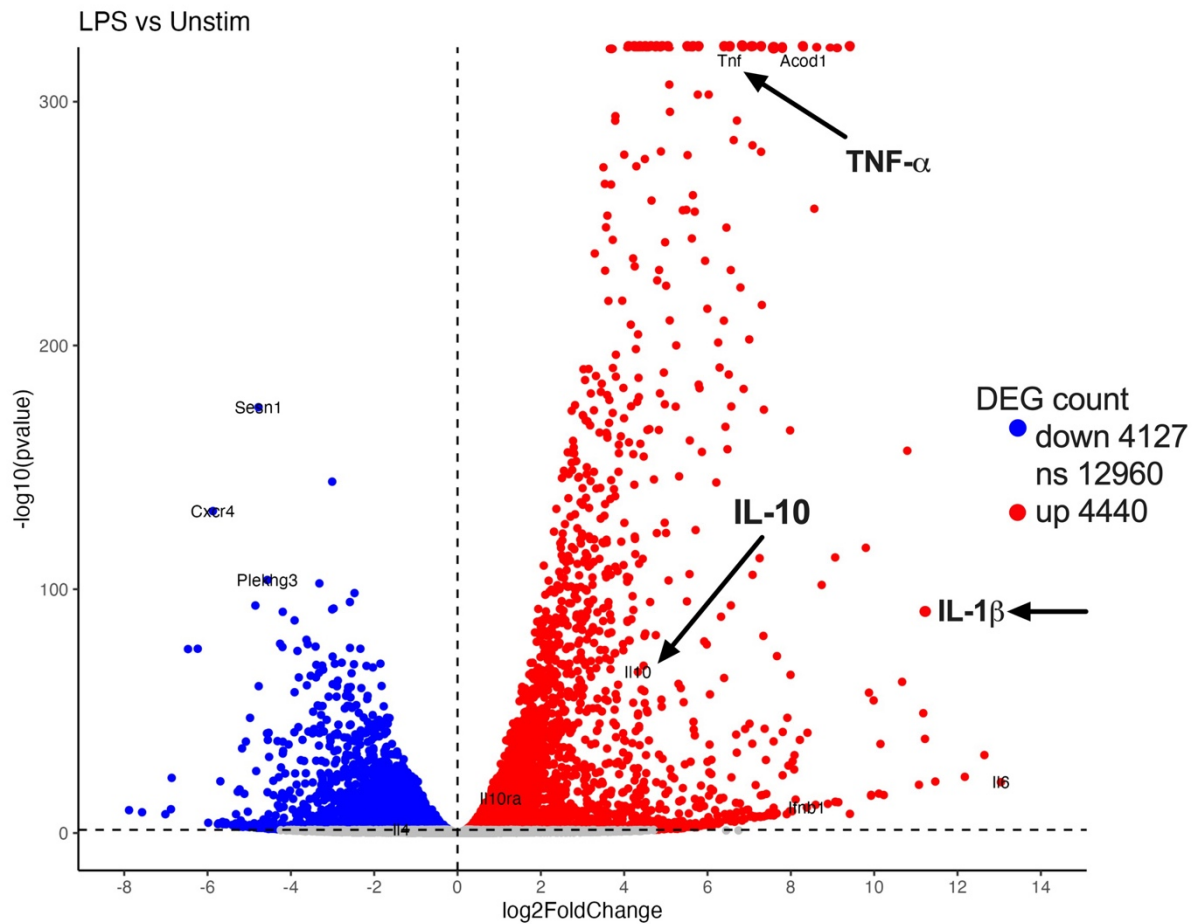


Figure 4.10. The most upregulated genes in LPS-treated BMDMs.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO and then stimulated for 4 hours with LPS (100ng/mL). Data are from $n=3$ from one independent experiment. After that, cell lysates were harvested, quantified and RNAseq was performed. Volcano plot representing the differentially upregulated genes with p value < 0.05 in LPS-treated sample. Each dot represents one gene (red dots represent upregulated genes while blue dots represent downregulated genes), $\log_2FC$ indicates the mean expression level of each gene.

Specific genes were upregulated after LPS challenge and notable genes were *Acod1* (which synthesizes itaconate), *IL-6*, *IL-1 β* , *TNF- α* , *IFN- β 1* and, as expected, *IL-10* and its receptors *IL-10R1* and *IL-10R2*. On the other hand, a smaller number of genes were downregulated by LPS and included zinc finger proteins (*Zfp937*, *Zfp72*, *Zfp128*, *Zfp668* and *Zfp280d*), matrix metalloproteinases *Mmp11* and membrane transport proteins (SLC-solute carriers) such as *Slc48a1*, *Slc35e2*, *Slc25a32*.

After pre-treatment with S3QEL 1.2, IL-10 appeared as one of the most downregulated genes (p value = 0.181, log2FC = -0.51) (**figure 4.11**), while TNF- α as one of the most upregulated. The same happened after pre-treatment with MYX (**figure 4.12**), which was decreasing the gene expression levels of IL-10, while it was increasing the gene expression levels of TNF- α . The more detailed findings are showed in **figure 4.13** and **figure 4.14** as considering fragment per kilobase of transcript sequence per million base pairs sequenced (FPKM).

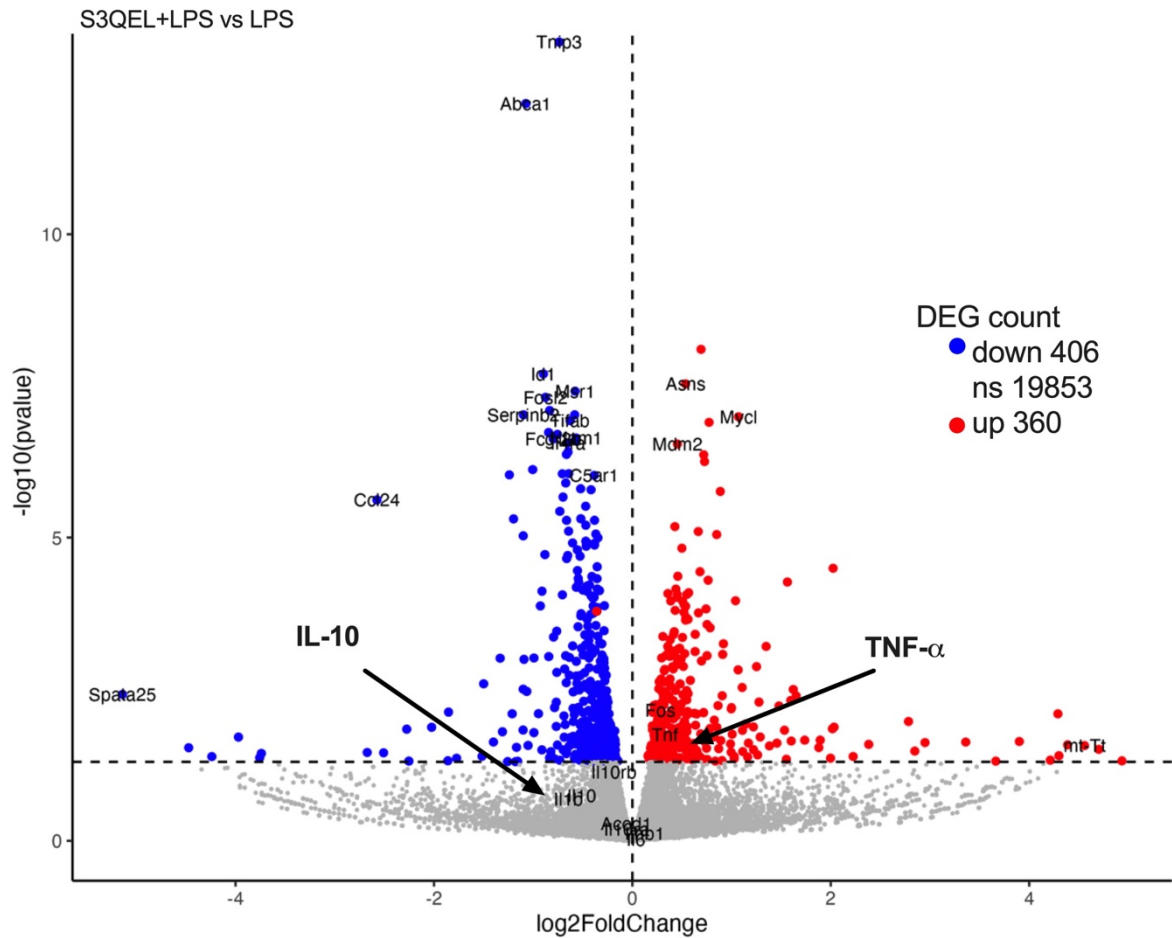


Figure 4.11. The most upregulated and downregulated genes in LPS-activated BMDMs after S3QEL 1.2 pre-treatment.

BMDMs (0.5×10^6 cells) were treated for 3 hours with S3QEL 1.2 (10 μM) and then stimulated for 4 hours with LPS (100ng/mL). Data are from $n=3$ from one independent experiment. After that, cell lysates were harvested, quantified and RNAseq was performed. Volcano plot representing the differentially upregulated genes with p value < 0.05 in LPS-treated sample. Each dot represents one gene (red dots represent upregulated genes while blue dots represent downregulated genes), $\log_2\text{FC}$ indicates the mean expression level of each gene.

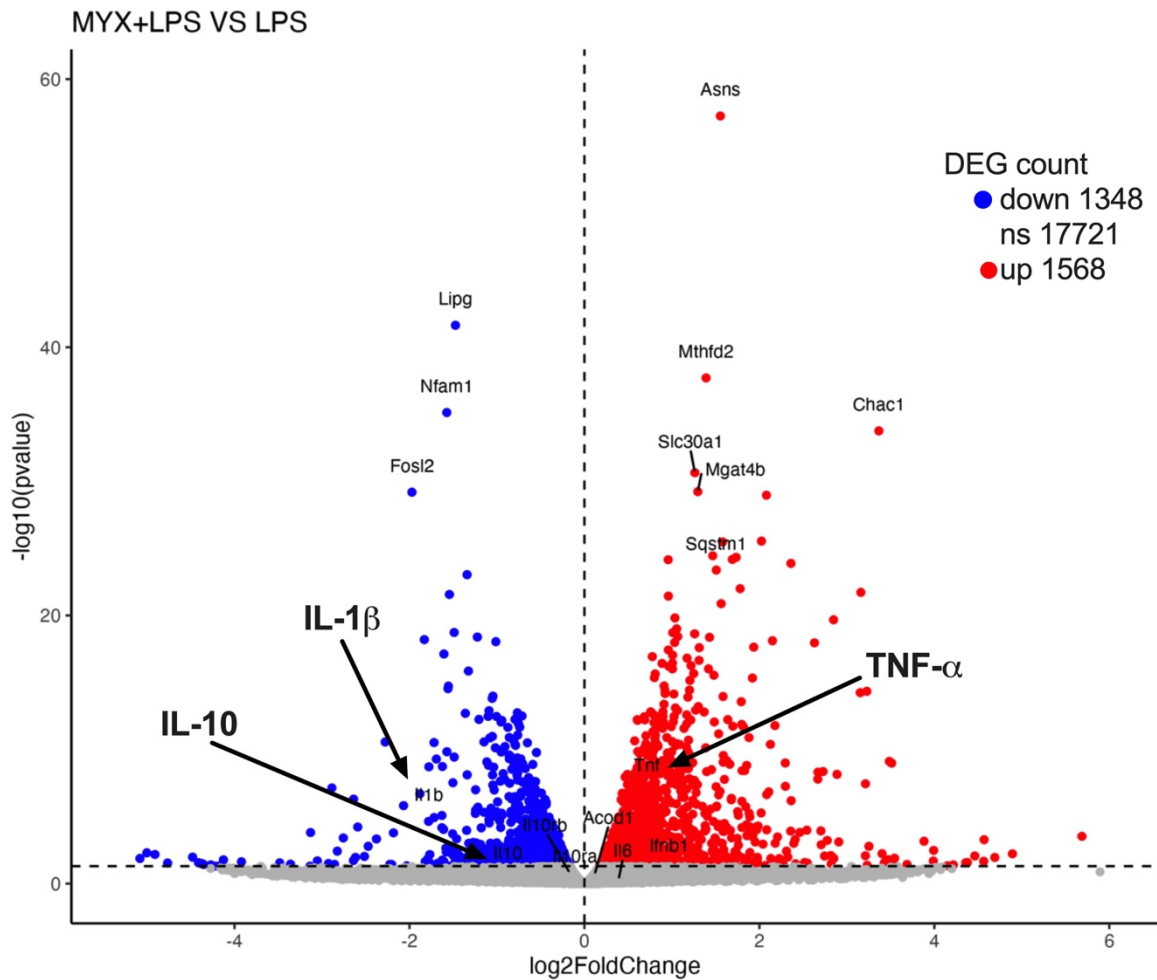


Figure 4.12. The most upregulated and downregulated genes in LPS-activated BMDMs after MYX pre-treatment.

BMDMs (0.5×10^6 cells) were treated for 3 hours with MYX (500 nM) and then stimulated for 4 hours with LPS (100ng/mL). Data are from $n=3$ from one independent experiment. After that, cell lysates were harvested, quantified and RNAseq was performed. Volcano plot representing the differentially upregulated genes with p value < 0.05 in LPS-treated sample. Each dot represents one gene (red dots represent upregulated genes while blue dots represent downregulated genes), $\log_2FC$ indicates the mean expression level of each gene.

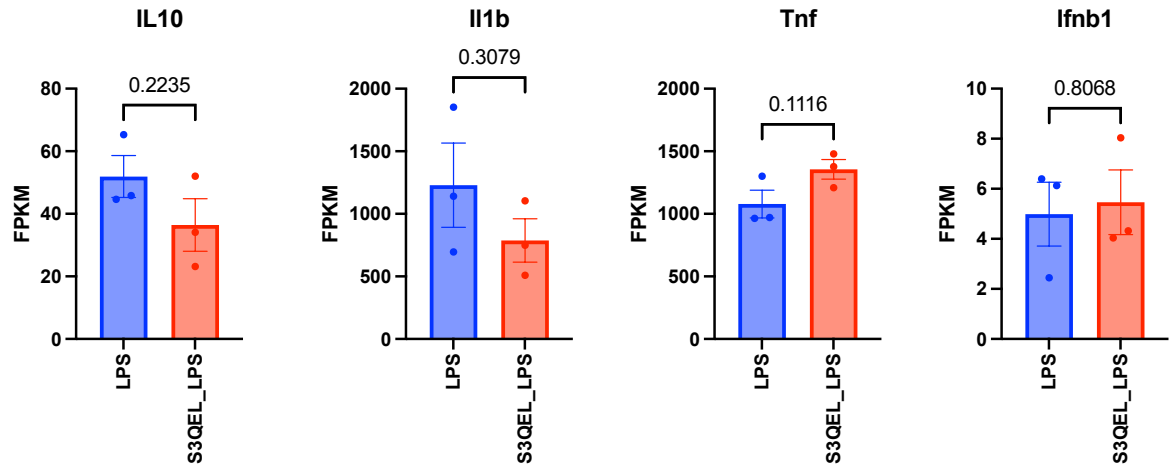


Figure 4.13. FPKM representation of differentially expressed genes in LPS-activated BMDMs after S3QEL 1.2 pre-treatment.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL 1.2 ($10 \mu\text{M}$) and then stimulated for 4 hours with LPS (100 ng/mL). Data are from $n=3$ from one experiment. After that, cell lysates were harvested, quantified and RNAseq was performed. Bar graphs showing the FPKM (Fragment Per Kilobase of transcript per Million mapped reads) values of our specific genes of interest. Data were calculated using unpaired t test.

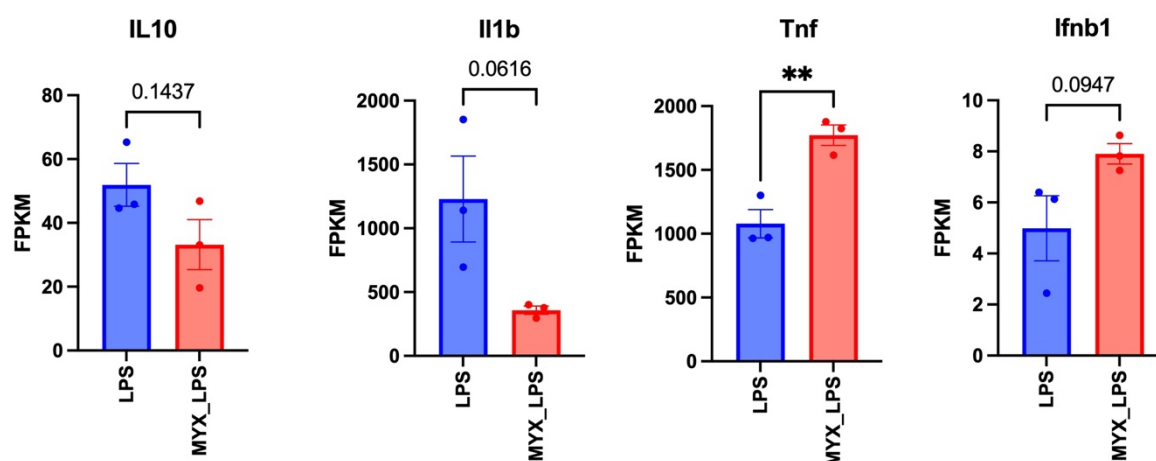


Figure 4.14. FPKM representation of differentially expresses genes in LPS-activated BMDMs after MYX pre-treatment.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO and Myxothiazol (500nM) and then stimulated for 4 hours with LPS (100ng/mL). Data are from $n=3$ from one experiment. After that, cell lysates were harvested, quantified and RNAseq was performed. Bar graphs showing the FPKM (Fragment Per Kilobase of transcript per Million mapped reads) values of our specific genes of interest. Data were calculated using unpaired t test. Differences were considered statistically significant at $**p < 0.01$.

In total, the commonly differentially expressed genes between S3QEL+LPSvsLPS and MYX+LPSvsLPS samples were 146 (adj p val<0.1).

Enrichment analysis of the differential expressed genes was performed to infer the biological process, cellular component and molecular function correlated to gene expression. When BMDMs were primed with LPS alone, they showed an overexpression of genes involved in the defense response against viruses and bacteria (**figure 4.15**), as expected. The LPS stimulation also triggered the expression of genes responsible for the generation of ROS and their dependent changes in metabolism, confirming the strong correlation between LPS-mediated inflammation and oxidative stress (133).

In particular, the inhibition of Complex III by MYX appeared to significantly downregulate the expression of genes involved in myeloid leukocyte activation and migration, together with genes related to regulation of defense response, growth factors and cytokine binding to receptors and their subsequent transmembrane signaling activity (**figure 4.16**).

The inhibition of Complex III-derived ROS generation by S3QEL 1.2 also significantly downregulated the expression of genes involved in defense response and cytokine signaling, with focus on genes involved in the phosphorylation and binding of a range of proteins (kinases and phosphatases) and without a specific lineage commitment (**figure 4.17**).

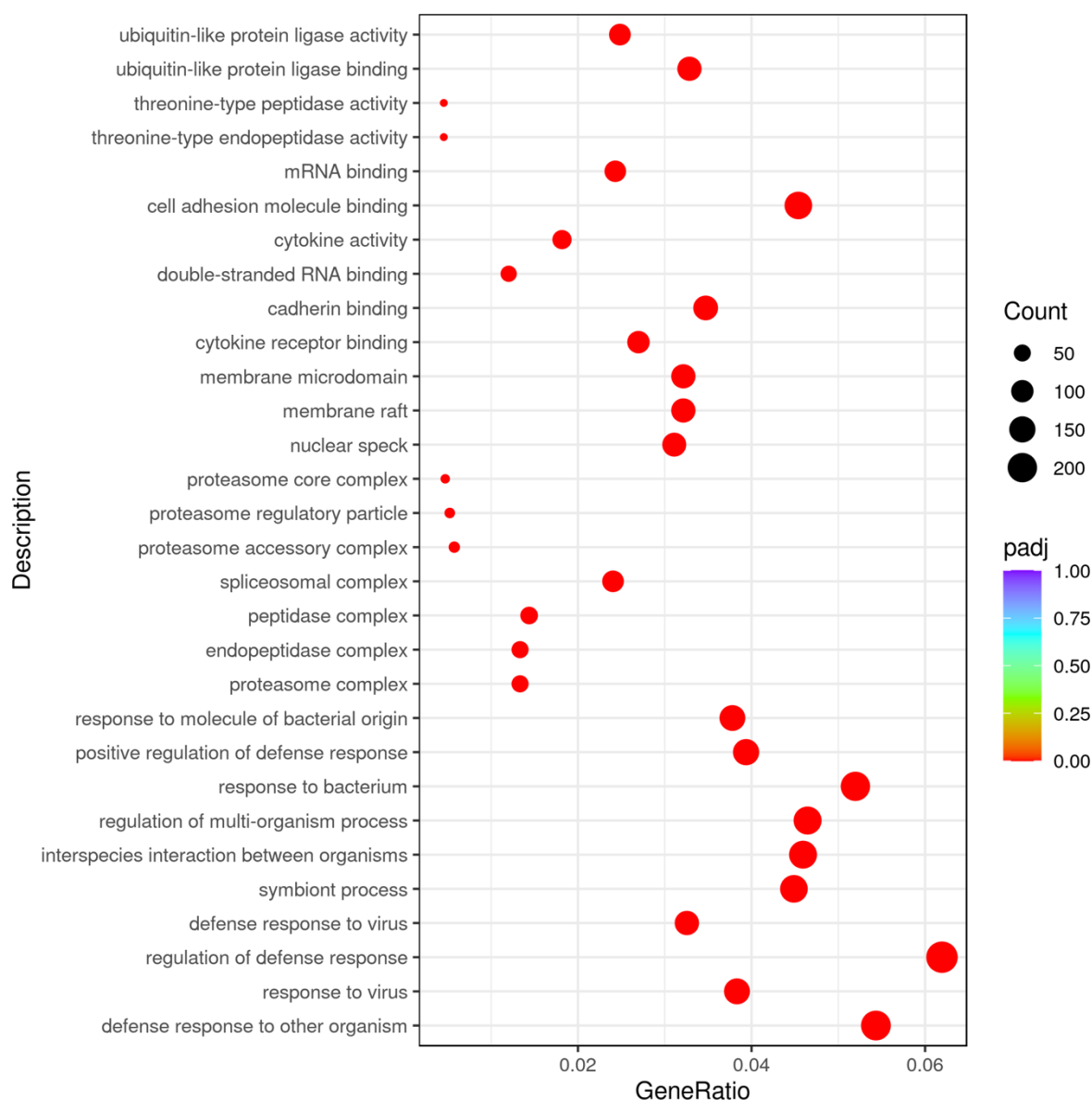


Figure 4.15. Gene set enrichment analysis of UNST vs LPS treated BMDMs.

GOdot plot representing the enrichment analysis of the differentially upregulated genes when comparing unstimulated and LPS-primed BMDMs. The most significant 30 Gene ontology terms were selected to display. X axis represents the ratio of the number of differentially expressed genes linked with the GO term to the total number of differentially expressed genes, Y axis represents the GO terms. The size of each point represents the number of genes annotated to a specific GO term and the color from red to purple represents the significant level of the enrichment. GO terms with padj < 0.05 are representing significant enrichment. Data are from n=3 from one independent experiment; LPS 4h.

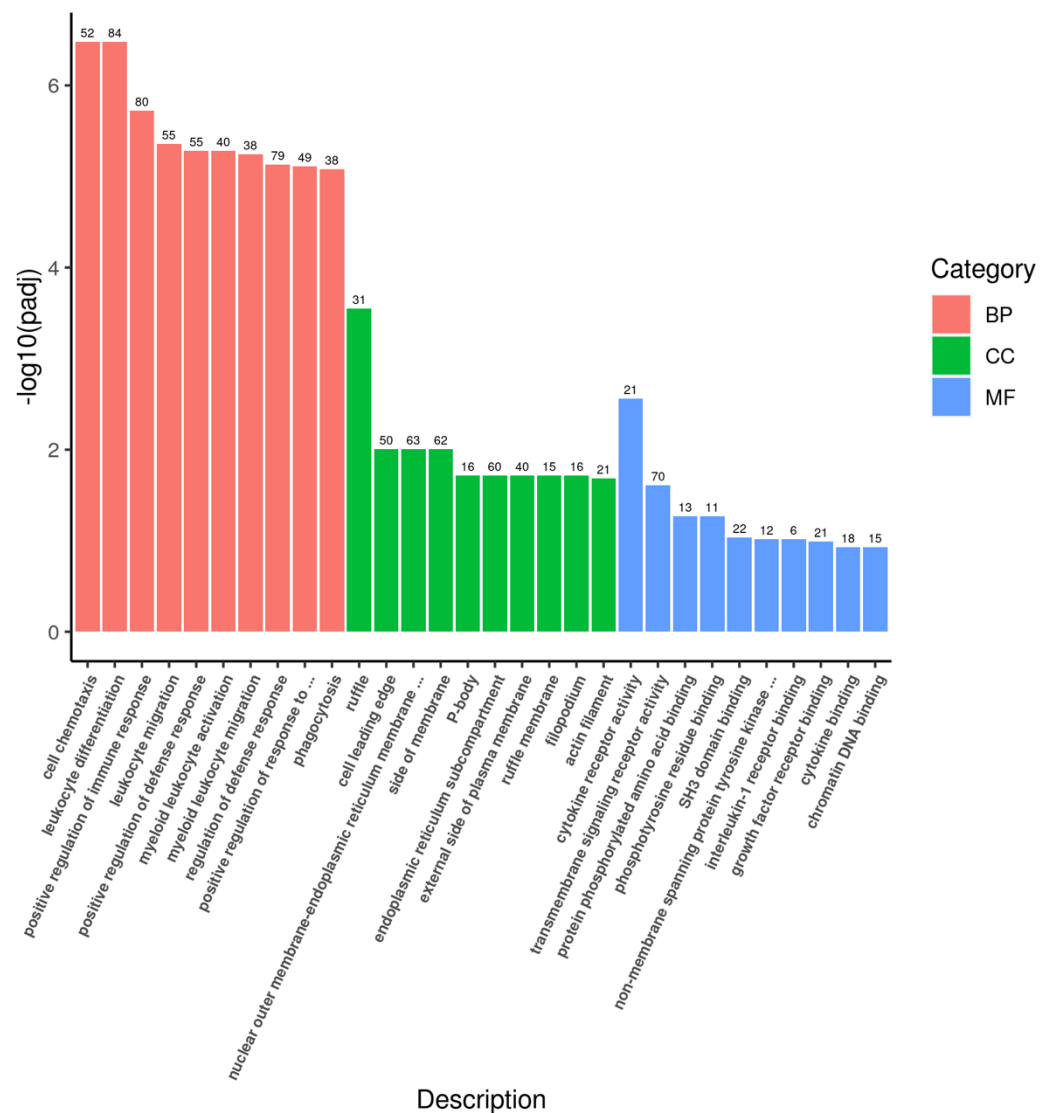


Figure 4.16. Gene set enrichment analysis of differentially downregulated genes between LPS vs MYX+LPS treated BMDMs.

GObar plot representing the enrichment analysis of the differentially upregulated genes when comparing unstimulated and LPS-primed BMDMs. The most significant 30 Gene ontology terms were selected to display. X axis represents the GO term, Y axis represents the significance level of the GO term enrichment. Higher values correspond to higher significance. The different colors represent the GO enrichment subclasses of BP (biological process), CC (cellular component) and MF (molecular function). GO terms with $\text{padj} < 0.05$ are significant enrichment. Data are from $n=3$ from one independent experiment; LPS 4h.

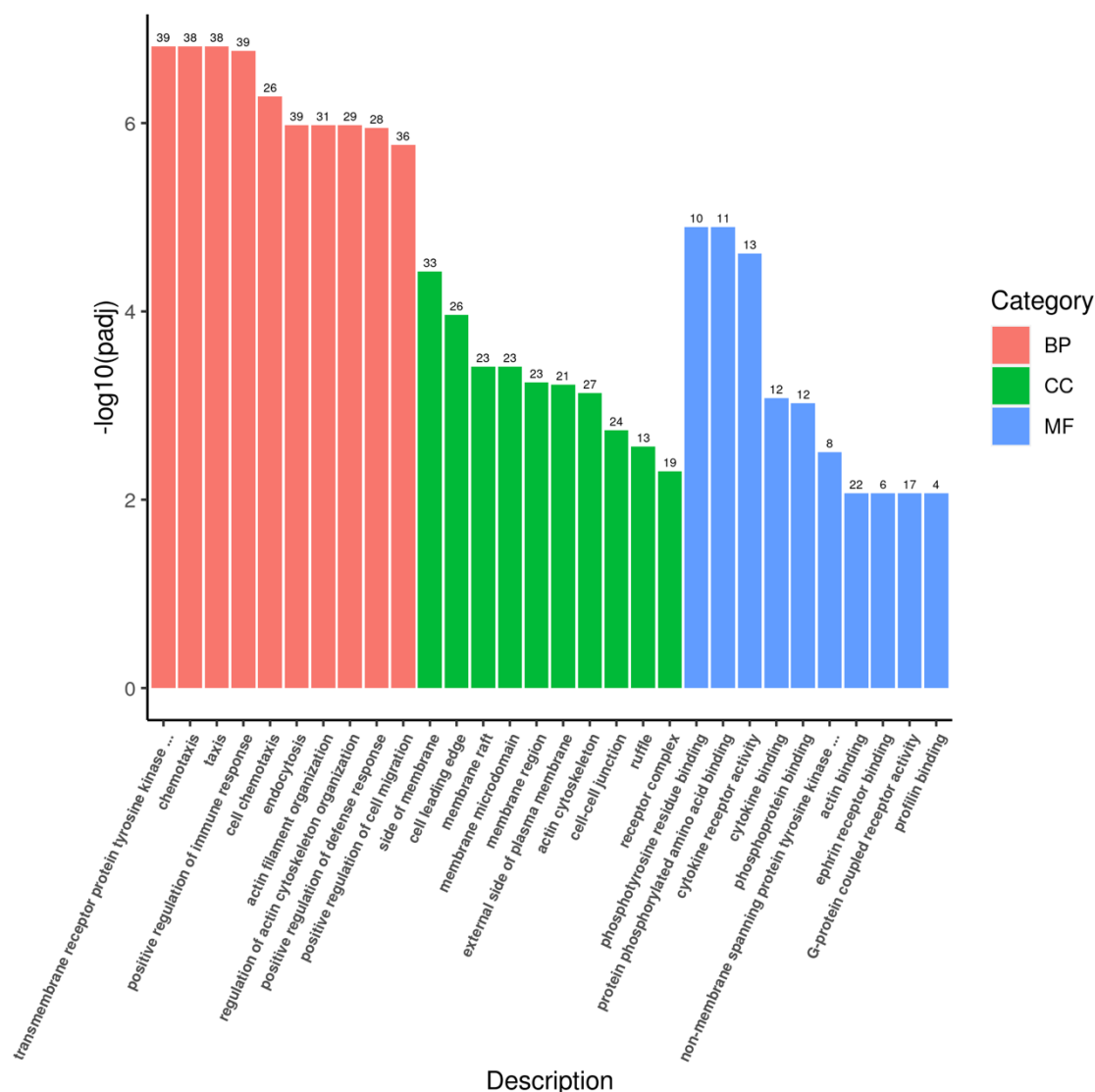


Figure 4.17. Gene set enrichment analysis of differentially downregulated genes between LPS vs S3QEL 1.2+LPS treated BMDMs.

GObar plot representing the enrichment analysis of the differentially upregulated genes when comparing unstimulated and LPS-primed BMDMs. The most significant 30 Gene ontology terms were selected to display. X axis represents the GO term, Y axis represents the significance level of the GO term enrichment. Higher values correspond to higher significance. The different colors represent the GO enrichment subclasses of BP (biological process), CC (cellular component) and MF (molecular function). GO terms with $p_{adj} < 0.05$ are significant enrichment. Data are from $n=3$ from one independent experiment; LPS 4h).

After this general step of the analysis, I focused my attention on the differentially downregulated genes after both S3QEL 1.2 and MYX pretreatments which were following the same trend as IL-10 expression. The list of the most downregulated genes is visualized as a heatmap in **figure 4.18**. Some of the genes affected were part of the signaling pathways involving interferons, proteases, and metallopeptidases, as assessed later by gene set enrichment analysis tools. My interest was caught by *Nfam1* (protein activating Ca^{++} -dependent NF-AT signaling) because of its importance in regulating the homeostasis of innate immune cells (134), *Ifnar1* and 2 (IFNs are negatively regulating IL-10 in some contexts (40)), *Ccl12* (involved in monocyte chemotaxis) and *CD14* (marker for monocytes and macrophages) because of their high presence in macrophages in basal conditions. Some protooncogenes such as *Hck* (shown in the figure) and the transcription factor *Ets2* (shown in the figure) could also be relevant for understanding if macrophage Complex III modulates some processes related to tumor progression, because of their necessity to polarize macrophages towards a tumour-promoting alternatively activated phenotype (135).

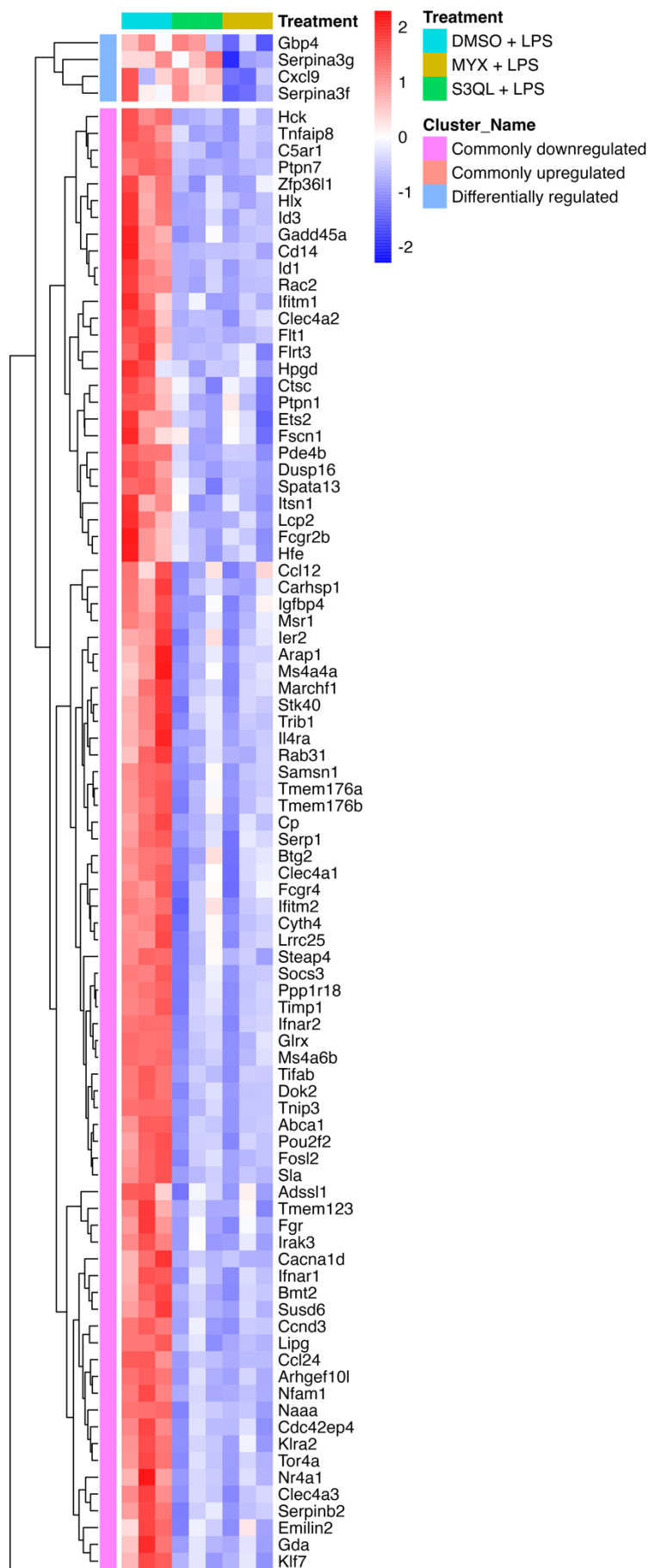


Figure 4.18. Heatmap representing the commonly downregulated genes after S3QEL 1.2 and MYX treatment.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO and Myxothiazol (500nM) and then stimulated for 4 hours with LPS (100ng/mL). Data are from n=3 from one experiment; LPS 4h. After that, cell lysates were harvested, quantified and RNAseq was performed. Heatmap of significantly downregulated genes with adjusted p value < 0.1. Normalized counts were converted to Z-score [(normalized counts-mean)/St Dev]. The original image was modified for visualization purposes.

Among the signaling events commonly downregulated by S3QEL 1.2 and MYX, IL-10 signaling pathway was in the top 20 (**figure 4.19**). Moreover, the ROS and hypoxia-related genes appeared in the list of the top 25 downregulated genes together with TNF- α signaling (**figure 4.20**). In this last case, the value came up in the analysis for the strong downregulation of TNFAIP8, which is a protein induced by TNF- α and suppressing TNF- α -mediated apoptosis through caspase 8 inhibition (136). Conversely, TNF- α and NF- κ B signaling pathway appeared among the 20 most upregulated pathways by both S3QEL 1.2 and MYX (**figure 4.21**). A detailed heatmap showing the differentially upregulated genes is represented in **figure 4.22**. Some of the upregulated genes belong to the haemostasis and platelet adhesion processes (Von Willebrand Factor, *Vwf*) and to metabolism (Growth differentiation factor 15, *Gdf15*). Other ones of major interest were *MSR1* (Macrophage scavenger receptor 1) and *Glrx* (glutaredoxin), because of its function in protecting from oxidative stress.

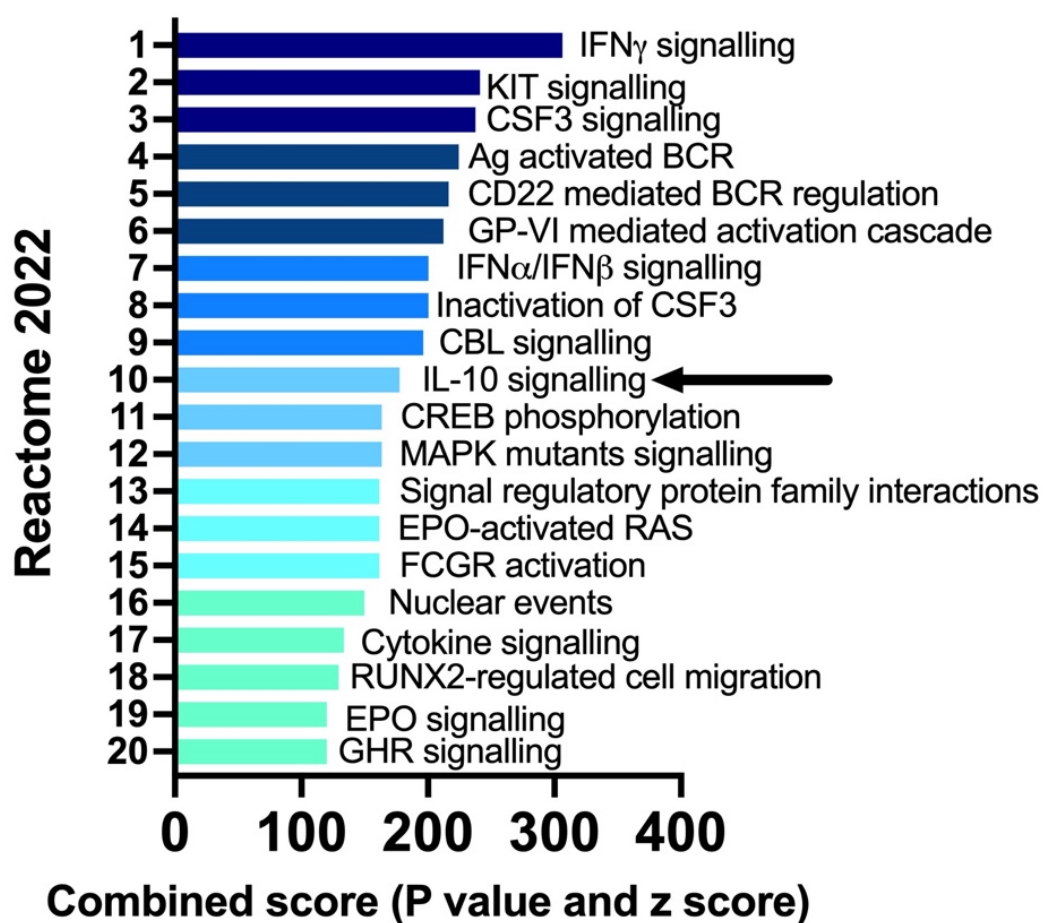


Figure 4.19. Reactome pathway analysis for commonly downregulated genes by S3QEL 1.2 and MYX.

Overrepresentation analysis of RNAseq data of BMDMs pretreated with S3QEL 1.2 or MYX (n=3 from one independent experiment; LPS 4h). Reactome pathway enrichment analysis of differentially expressed genes. The top 20 downregulated pathways are represented on the y axis while the x axis represent the combined score.

Downregulated with both MYX and S3QEL

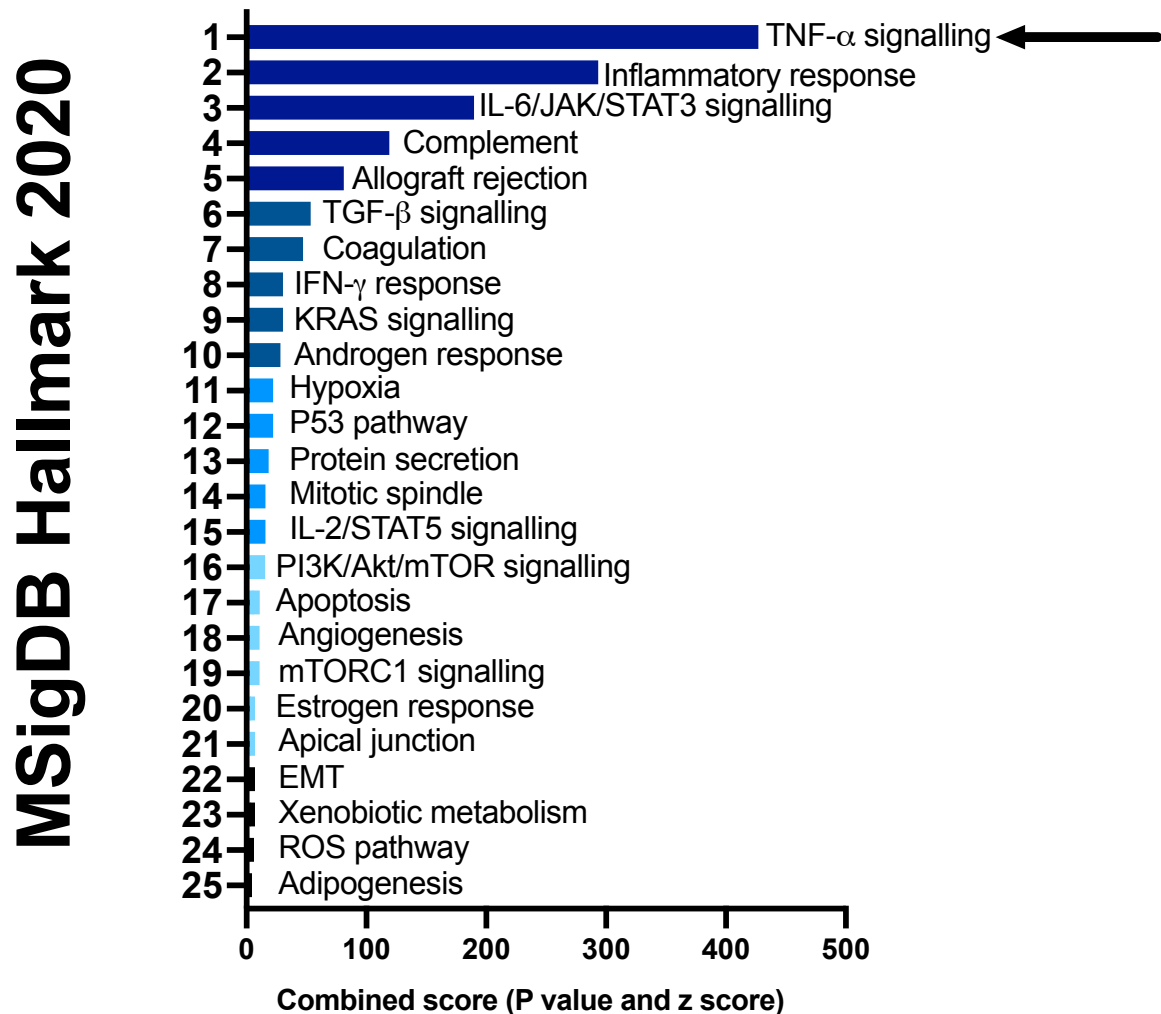


Figure 4.20. Molecular signature database analysis for commonly downregulated genes by S3QEL 1.2 and MYX.

Overrepresentation analysis of RNAseq data of BMDMs pretreated with S3QEL 1.2 or MYX (n=3 from one independent experiment; LPS 4h). Molecular signature enrichment analysis of differentially expressed genes. The top 25 downregulated processes are represented on the y axis while the x axis represent the combined score.

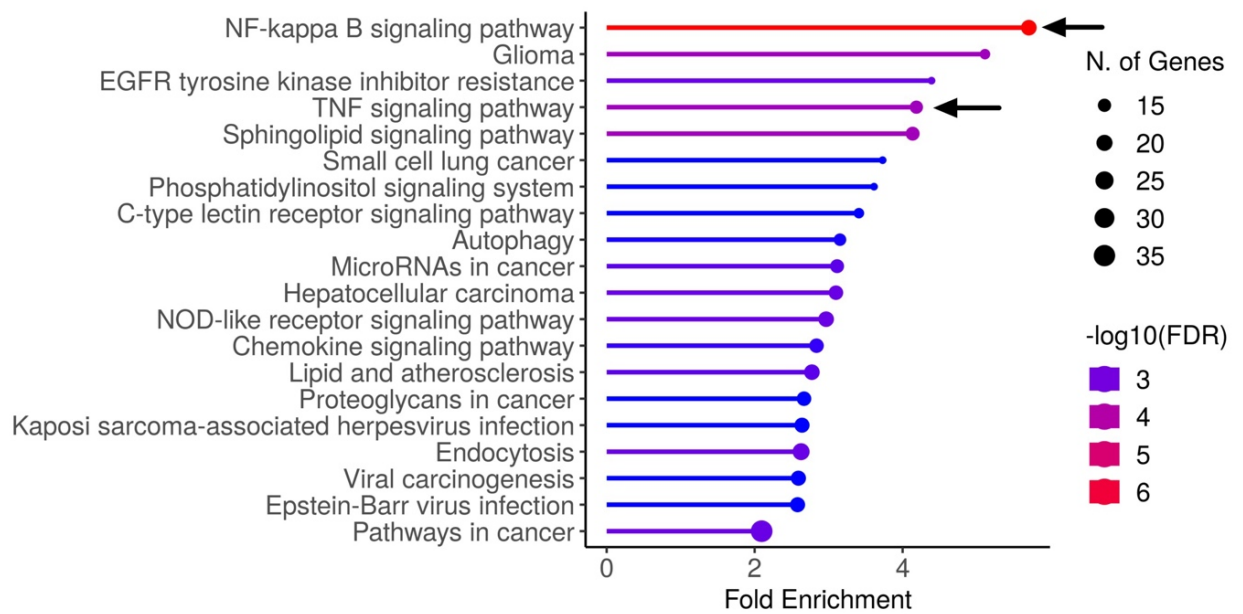


Figure 4.21 GSE analysis of the differentially upregulated genes after S3QEL 1.2 and MYX pre-treatment and LPS stimulation.

Lollipop graph representing RNAseq data of BMDMs pretreated with S3QEL 1.2 or MYX (n=3 from one independent experiment; LPS 4h). GSEA of differentially upregulated pathways. The top 20 upregulated pathways are represented on the y axis while the x axis represent the fold enrichment. The size of the dots represents the number of genes in the pathway and the color of the lollipop represent the FDR (false discovery rate).

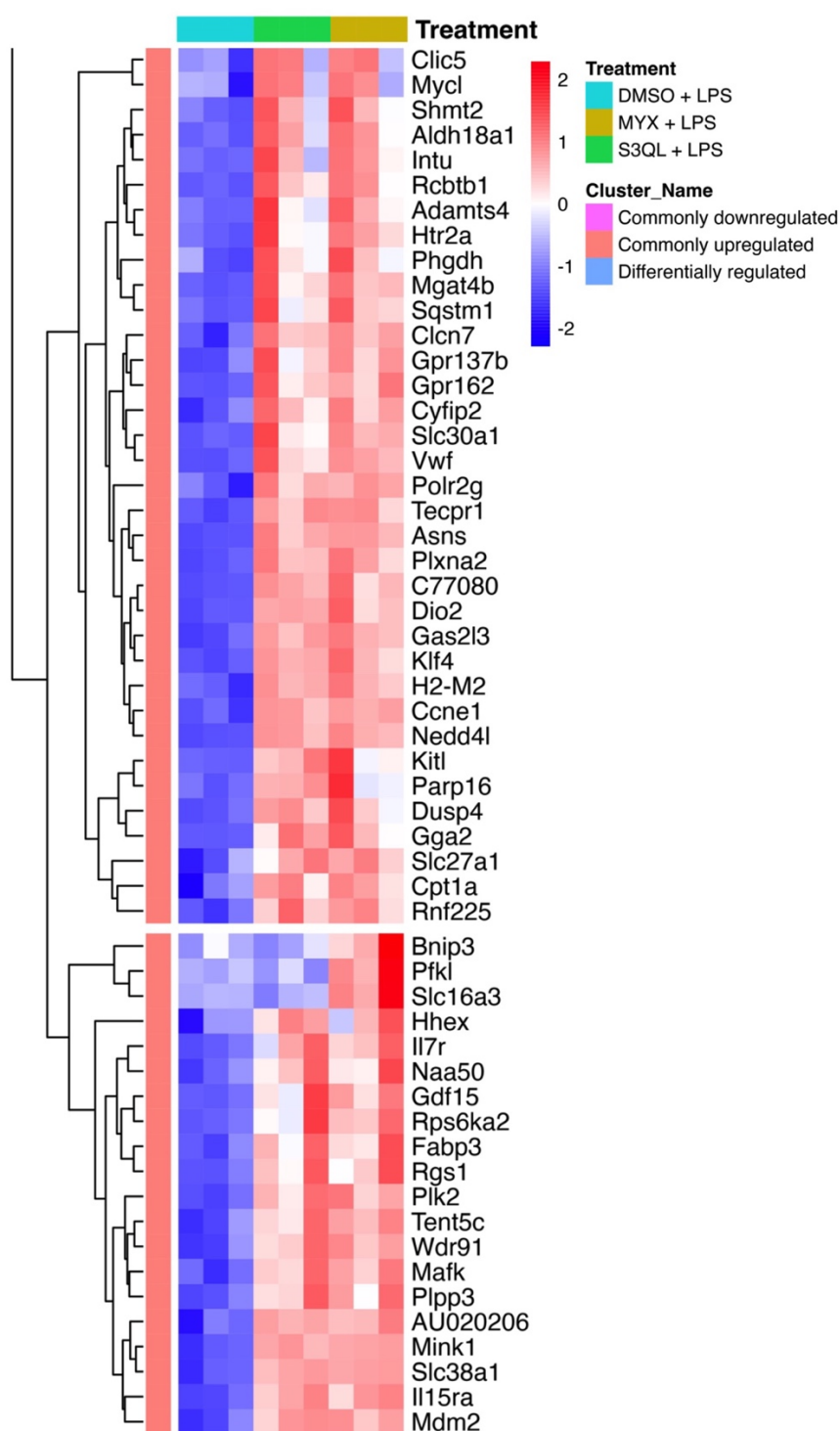


Figure 4.22 Heatmap representing the commonly upregulated genes after S3QEL 1.2 and MYX treatment.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL 1.2 ($10 \mu\text{M}$) and Myxothiazol (500nM) and then stimulated for 4 hours with LPS (100ng/mL). Data are from $n=3$ from one experiment; LPS 4h. After that, cell lysates were harvested, quantified and RNAseq was performed. Heatmap of significantly upregulated genes with adjusted p value < 0.1 . Normalized counts were converted to Z-score $[(\text{normalized counts} - \text{mean}) / \text{St Dev}]$. The original image was modified for visualization purposes.

I also interrogated the RNAseq database about transcription factors whose expression was differentially downregulated after Complex III inhibition to help elucidate the mechanism driving IL-10 suppression. Transcription factor enrichment analysis revealed that Fosl2, a subunit of the transcription factor AP-1, was strongly downregulated after S3QEL 1.2 and MYX treatment in activated BMDMs (**figure 4.23**). The same finding was also suggested by the overall downregulation of many genes which are targets of AP-1 (**figure 4.24**).

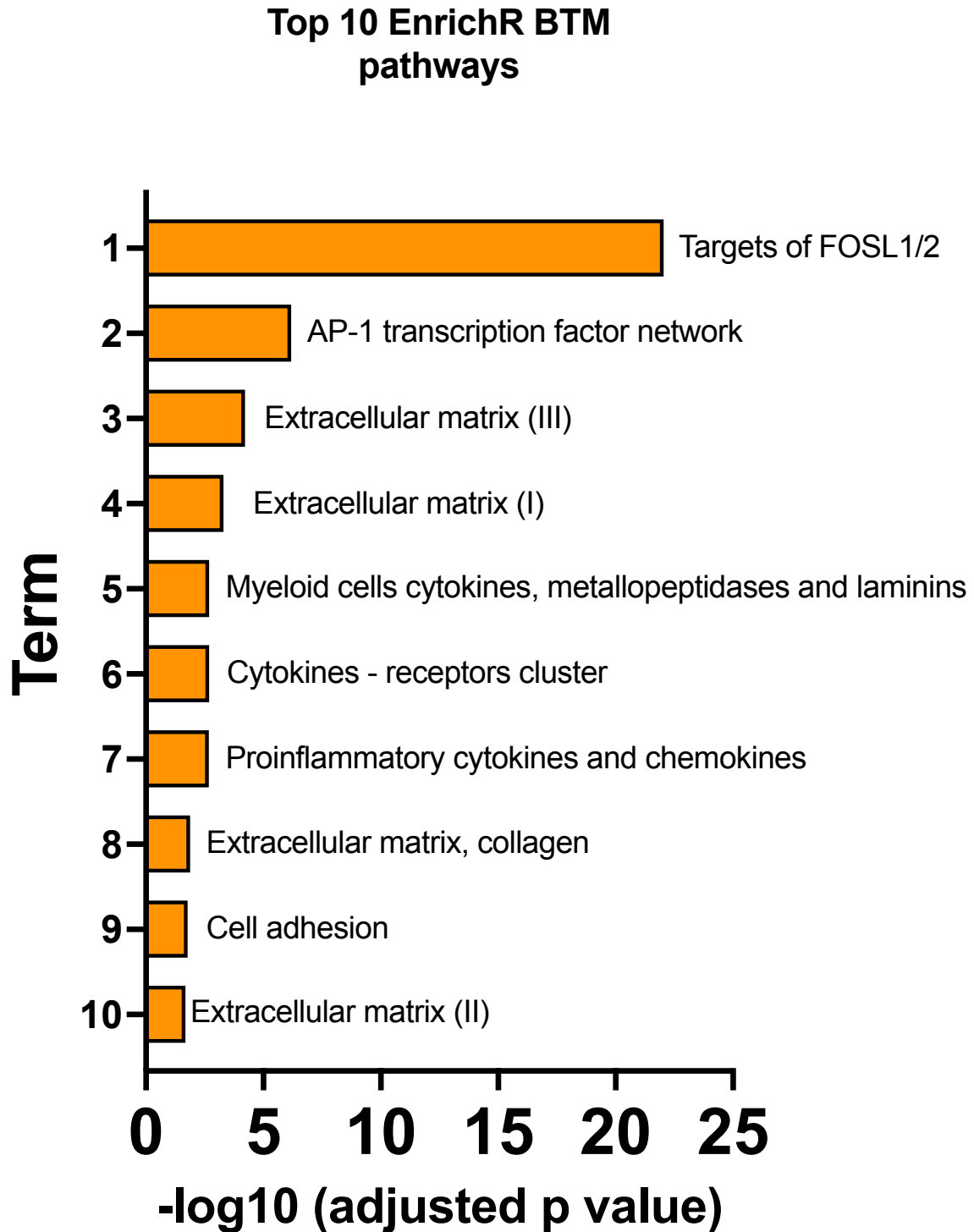


Figure 4.23. Pathway enrichment analysis for commonly downregulated genes by S3QEL 1.2 and MYX.

Overrepresentation analysis of RNAseq data of BMDMs pretreated with S3QEL 1.2 or MYX (data are from n=3 from one independent experiment; LPS 4h). EnrichR BTM pathways analysis of differentially downregulated genes. The top 10 downregulated terms are represented on the y axis while the x axis represent the adjusted p value.

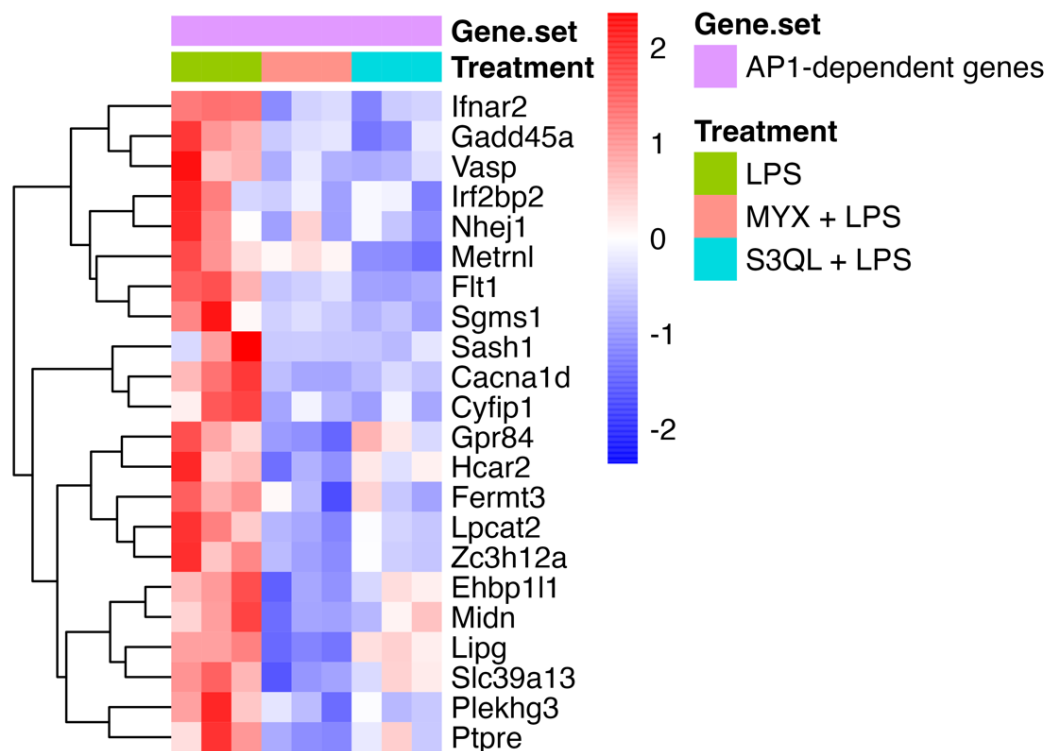


Figure 4.24. Heatmap representing the commonly downregulated AP-1 target genes after S3QEL 1.2 and MYX treatment.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL 1.2 ($10 \mu\text{M}$) and Myxothiazol (500 nM) and then stimulated for 4 hours with LPS (100 ng/mL). Data are from $n=3$ from one independent experiment. After that, cell lysates were harvested, quantified and RNAseq was performed. Heatmap of significantly downregulated AP-1 target genes with adjusted p value < 0.05 . Normalized counts were converted to Z-score [(normalized counts-mean)/St Dev].

Extrapolating more the most differentially downregulated genes after MYX and S3QEL 1.2 pre-treatments from the RNAseq database and performing on them transcription factor enrichment analysis (TFEA), I got the confirmation that both JunB (ranked #38) and FosB (ranked #49) were included in the top 50 transcription factors regulating the expression of the genes which were inhibited after MYX and S3QEL 1.2 treatments. JunB controlled the expression of 81 genes while FosB controlled the expression of 41 genes.

Overall therefore, based on the RNAseq analysis, inhibition of JunB and FosB could be important for the inhibitory effect of S3QEL 1.2 on IL-10 production. S3QEL 1.2 can repress AP-1 transcriptionally, which might be an important aspect of its ability to inhibit IL-10 induction.

4.2.4 IL-10 downregulation is Complex I-derived ROS independent

Having shown that S3QEL 1.2 blocks IL-10 induction, I also tested S1QEL 1.1. There was no effect on IL-10 mRNA (A) and released form (D) and a slight boost in TNF- α mRNA (B) and secreted form (E) at the highest concentration tested (10 μ M) (**figure 4.25**), with no effect on IL-1 β (**figure 4.25C**).

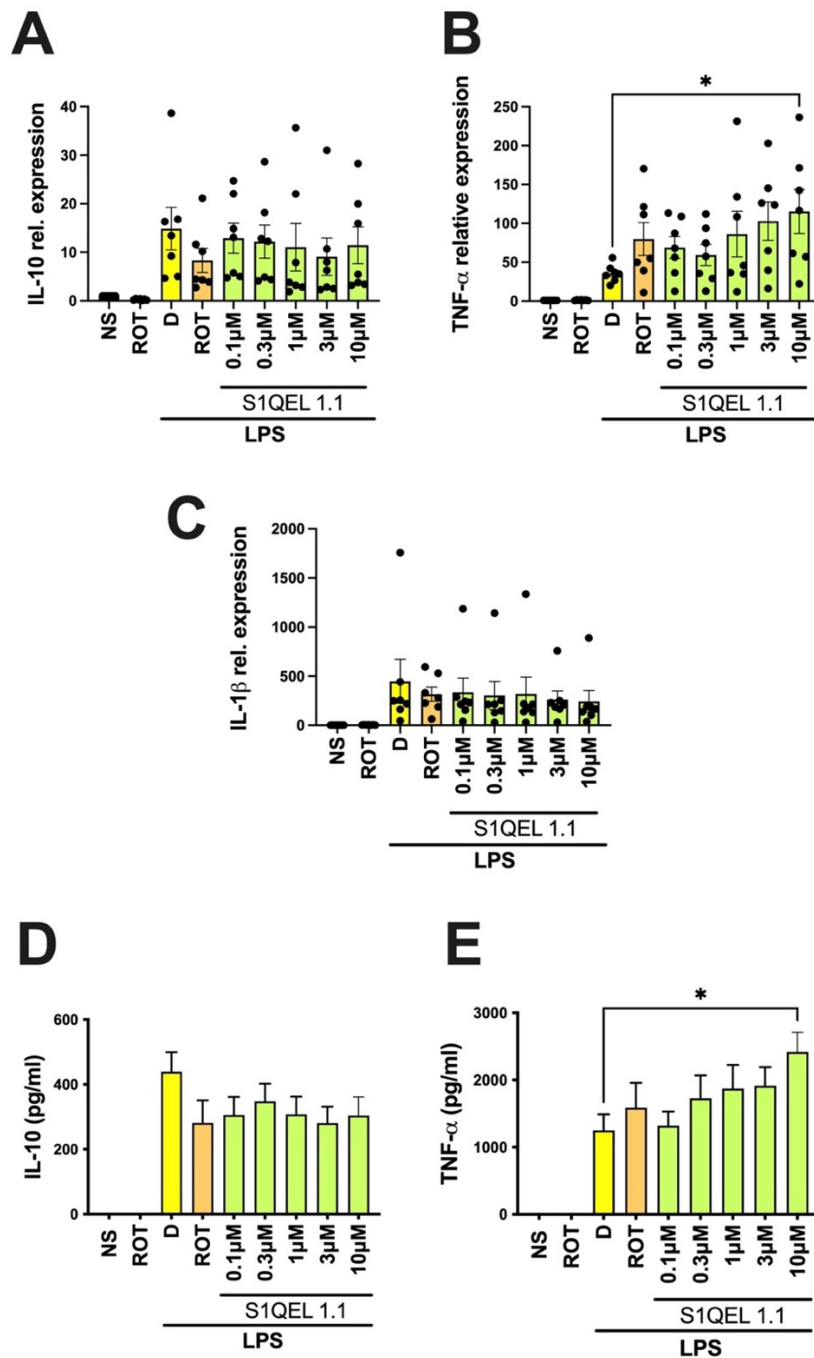


Figure 4.25. IL-10 downregulation is not respiratory Complex-I dependent.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S1QEL 1.1 (10μM) and Rotenone (5μM) and then stimulated for 4 hours with LPS (100ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10, IL-1β, TNF-α with RPS18 as a housekeeping control (A, B and C). IL-10 and TNF-α concentrations in supernatants were analyzed by ELISA (D and E). Data are presented as mean ± SEM for n=7 from three independent experiments. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at *p<0.05.

4.2.5 MitoQ and MitoTEMPO-related regulation of cytokine production

I next tested the mitochondrial targeted antioxidants Mitoquinone (MitoQ) and MitoTEMPO.

Figure 4.26 indicates that high concentration of MitoQ decreased IL-10 mRNA (A), in a non-significant manner, and release (D) from LPS-activated macrophages, together with a decrease in TNF- α mRNA (B) and released form (E). Also, IL-1 β induced by LPS was downregulated at the transcriptional level (C). MitoTEMPO did not have any effect however, probably due to its high concentration.

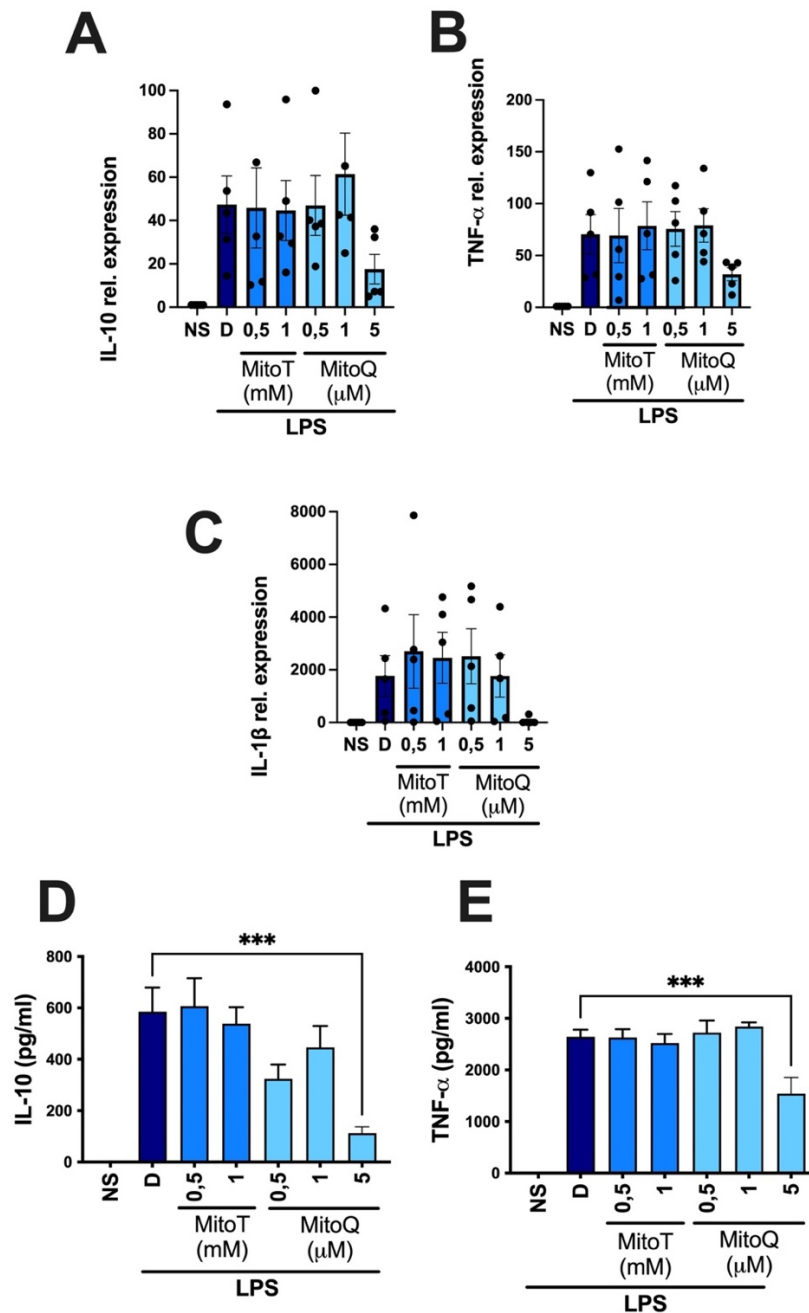


Figure 4.26. Mitochondria-targeted antioxidants modulate IL-10 production in activated macrophages.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, MitoTEMPO (0.5 and 1mM) and MitoQ (0.5, 1 and 5 μ M) and then stimulated for 4 hours with LPS (100ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10, IL-1 β , TNF- α with RPS18 as a housekeeping control (A, B and C). IL-10 and TNF- α concentrations in supernatants were analyzed by ELISA (D and E). Data are mean $\pm$ SEM for n=5-6 from three independent experiments. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at ***p<0.001.

4.2.6 A different chemical backbone of S3QEL has the same effect on IL-10 expression

I next tested another S3QEL compound, called S3QEL 2.2, on macrophage cytokine production. S3QEL 2.2 has a different chemical backbone compared to S3QEL 1.2 but it is still able to bind to the same site for the quinone on Complex III and have the same effects as S3QEL 1.2, inhibiting Complex-III derived ROS. S3QEL 2.2 showed a trend in decreasing LPS-induced IL-10 mRNA (A) and release (C) but in a less extent compared to S3QEL 1.2, as can be seen in **figure 4.27**. S3QEL 2.2 did not have effect on TNF- α production, both at the mRNA (B) and released protein (D) level.

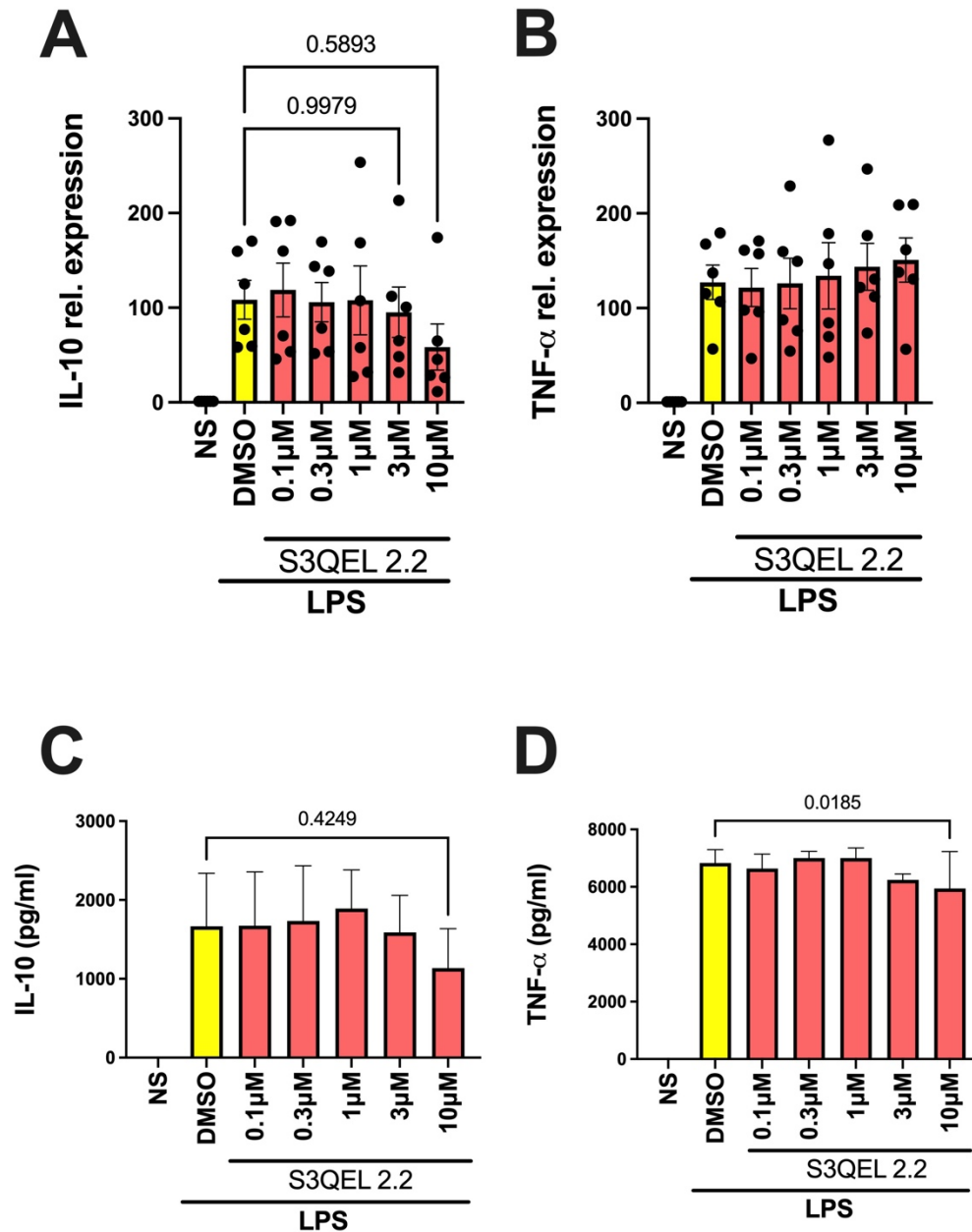


Figure 4.27. Different backbones of S3QEL regulate IL-10 and TNF-α in a consistent manner.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL 2.2 (10 μM) and then stimulated for 4 hours with LPS (100 ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10, IL-1β, TNF-α with RPS18 as a housekeeping control (A and B). IL-10 and TNF-α concentrations in supernatants were analyzed by ELISA (C and D). Data are mean $\pm$ SEM for $n=6$ from three independent experiments. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test.

4.2.7 IL-10 expression in AOX mice shows regulation

After having established that Complex III-derived ROS was likely to be required for IL-10 production using S3QEL, I next examined BMDMs from AOX-expressing mice. AOX stands for Alternative Oxidase, an enzyme expressed by tunicates like *Ciona Intestinalis* and which encompasses Complex III and Complex IV of the electron transport chain. AOX is not present in mammals and can allow normal electron flow, from ubiquinol to oxygen, without proton pumping. This process favors basal respiration of the cells and inhibits ROS generation in mitochondria.

As shown in **figure 4.28**, AOX could be detected in BMDMs from mice expressing one copy of AOX (het) or both copies (hom). LPS had no effect on AOX expression. IL-10 mRNA expression and release did not change between different genotypes after short stimulation with LPS (**figure 4.29A and C**), but it was downregulated in AOX-expressing mice after 12 hours of LPS, both at the transcriptional and translational level (**figure 4.30A and B**). This however is a single experiment and needs to be repeated. AOX expressing mice show no difference in TNF- α expression, compared to WT mice (**figure 4.29B and D**).

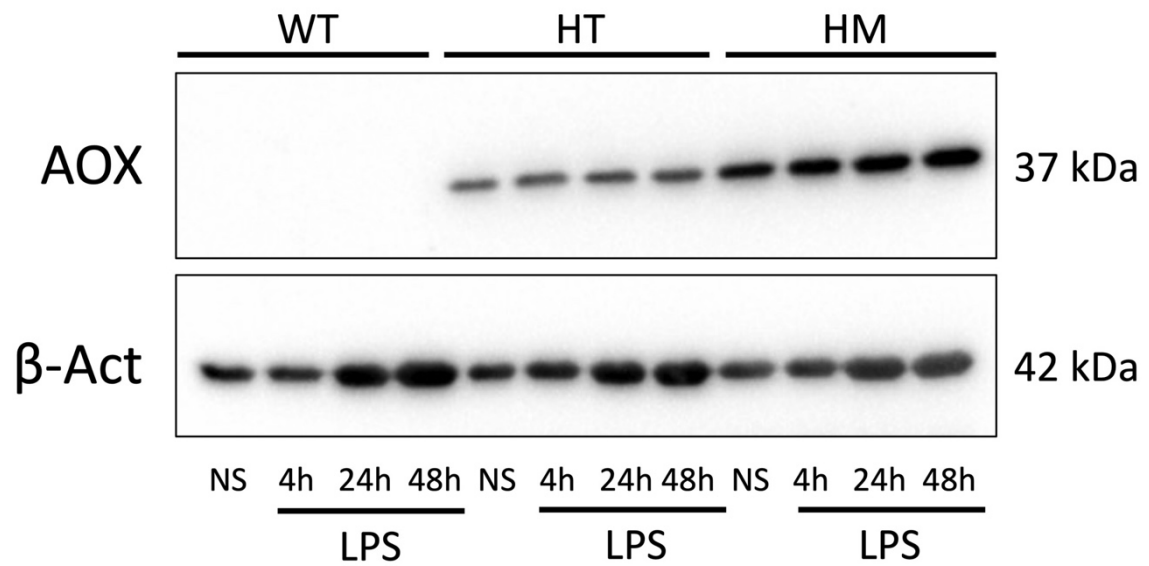


Figure 4.28. AOX expression in WT and AOX-expressing mice did not change upon LPS stimulation.

BMDMs (0.5×10^6 cells) isolated from WT and AOX expressing mice (homozygous and heterozygous) were stimulated for 4, 24 and 48 hours with LPS (100ng/mL). After that, cell lysates were harvested, and protein levels were analysed by WB for the expression of AOX and GAPDH. Blots are representative of three independent experiments.

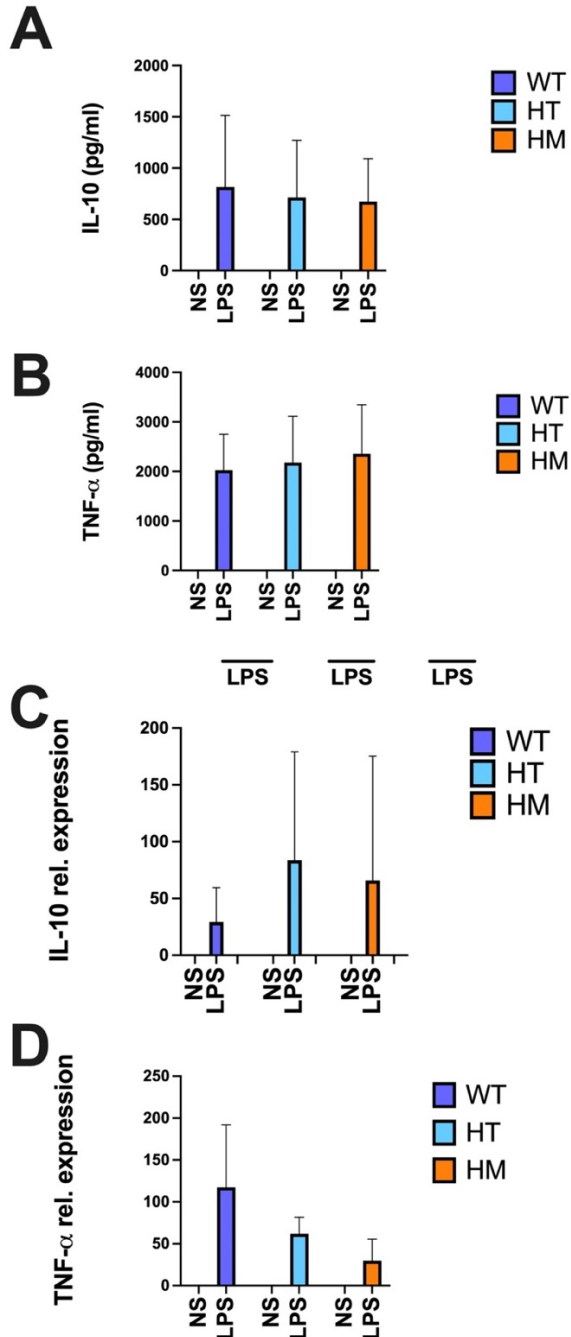


Figure 4.29. AOX mice do not show downregulated IL-10 expression after 4 hours of LPS.

BMDMs (0.5×10^6 cells) from WT and AOX expressing mice (homozygous (HM) and heterozygous (HT)) were stimulated for 4 hours with LPS (100ng/mL). After that, IL-10 and TNF- α concentrations in supernatants were analyzed by ELISA (A and B) and cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10, TNF- α with RPS18 as a housekeeping control (Rps18) (C and D). IL-10 and TNF- α concentrations in supernatants were analyzed by ELISA. Data are mean $\pm$ SD for n=4 from two independent experiments.

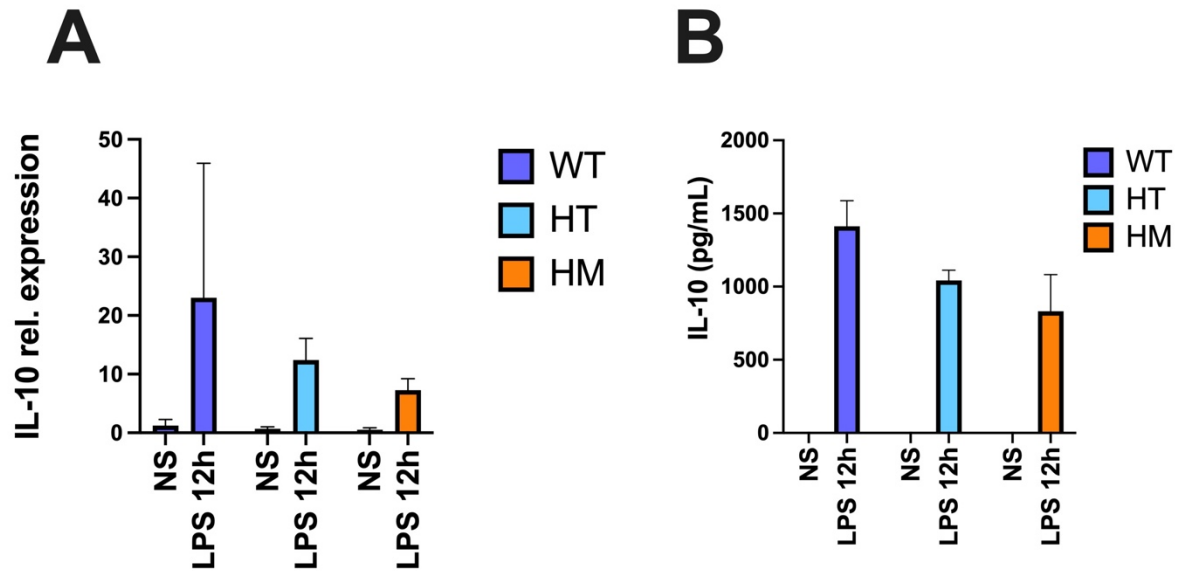


Figure 4.30. AOX mice show downregulated IL-10 expression after 12 hours of LPS.

BMDMs (0.5×10^6 cells) from WT and AOX expressing mice (homozygous (HM) and heterozygous (HT)) were stimulated for 12 hours with LPS (100ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10 with RPS18 as a housekeeping control (A). IL-10 concentration in supernatants was analyzed by ELISA (B). Data are mean $\pm$ spread for $n=2$, where one representative experiment is shown.

4.2.8 IL-10 decrease does not involve CREB activation

The transcription factor CREB has also been shown to regulate IL-10 transcription (137). I therefore next examined CREB phosphorylation. As can be seen in **figure 4.31**, LPS induced CREB phosphorylation at 5 to 30 minutes (A and B, upper panel, lanes 2-3, 8-9, 3-4 and 9-10). There was no difference in CREB phosphorylation between the control and the MYX-treated sample (A, compare lanes 2-3 to 8-9 in the upper panel). The same was observed between the control and the S3QEL 1.2-treated sample (B, compare lanes 3-4 to 9-10 in the upper panel). The levels of total CREB decreased when the levels of phospho-CREB increased (A and B, middle panels, lanes 2-3, 8-9, 3-4, 9-10). Panel C and D show densitometry of the experiments.

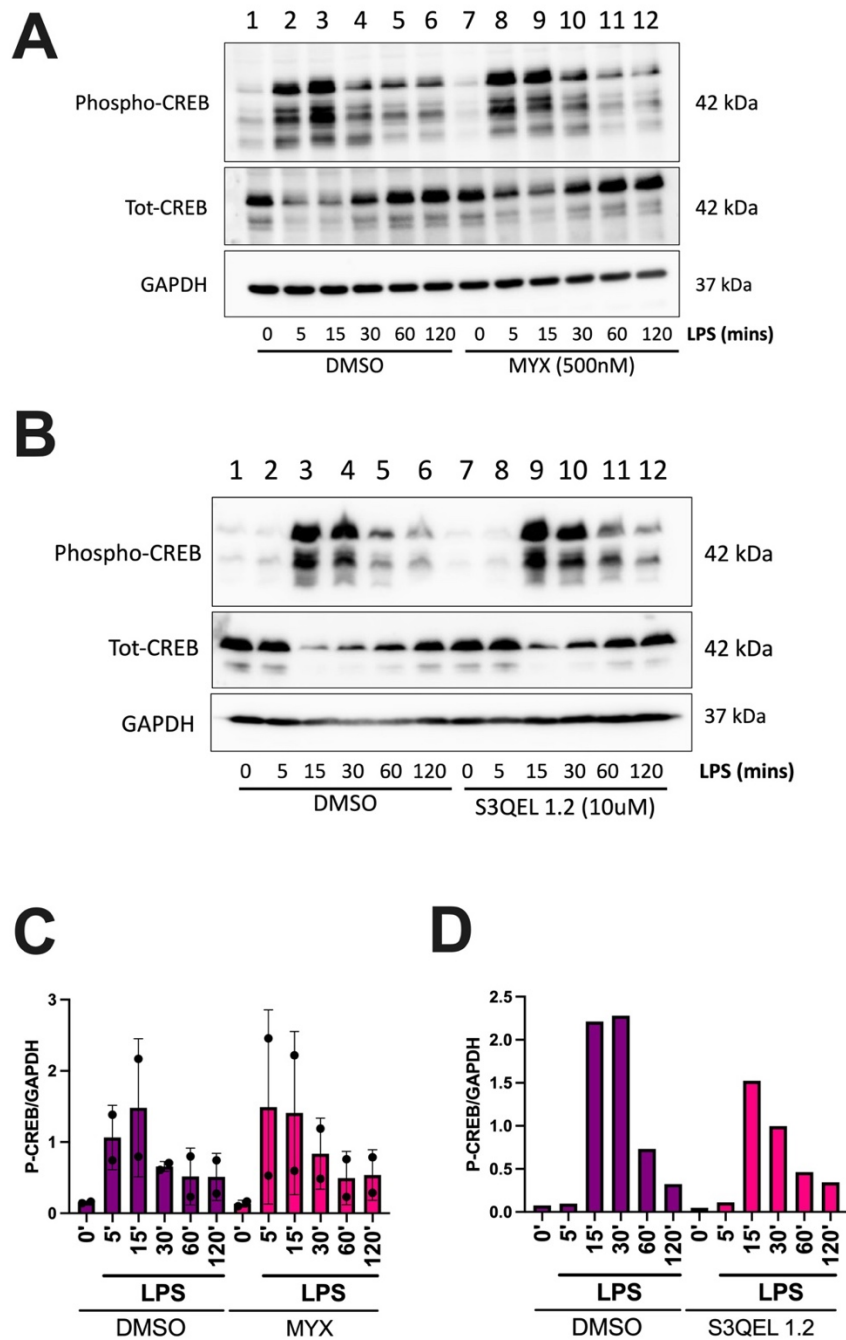


Figure 4.31. IL-10 downregulation by S3QEL 1.2 and Myxothiazol is not a CREB-dependent mechanism.

BMDMs (0.5×10^6 cells) were treated for 1 hour with DMSO, S3QEL 1.2 (10 μ M) and Myxothiazol (500nM) and then stimulated for 5, 15, 30, 60, 120 minutes with LPS (100ng/mL). After that, cell lysates were harvested and protein levels were analysed by western blot for the expression of p-CREB, CREB and GAPDH. Blots are representative of one independent experiment from n=1 or n=2. Blots were also quantified by densitometry. Data are expressed as mean $\pm$ spread for n=2, where one representative experiment is shown.

4.2.9 S3QEL 1.2 and Myxothiazol decrease LPS-driven phosphorylation of C-Fos and C-Jun transcription factors

I next returned to AP-1.

Figure 4.32A-B and **4.33A-B** depicts the phosphorylation of C-Fos and C-Jun, especially at 60 and 120 minutes after LPS for the first one and at 15 and 30 minutes of LPS for the second one (lanes 5-6 and 3-4 of the upper panels). Phosphorylated C-Jun is slightly expressed in basal conditions and it increases its expression after LPS stimulation (lanes 1 to 4 of B panels in **figure 4.32** and **4.33**). S3QEL 1.2 treatment clearly decreased the levels of phospho-C-Fos (**figure 4.32A**, lanes 7 to 12 of the upper panel) and phospho-C-Jun (**figure 4.32B**, lanes 7 to 12 of the upper panel). The panels C and D represent densitometry. Myxothiazol-induced decrease in the phosphorylation of C-Fos (**figure 4.33A**, lanes 7 to 12, upper panel) is probably due to the decrease in C-Fos total levels (**figure 4.33A**, lanes 7 to 12, middle panel). The same effect was observed on the phosphorylated and total levels of C-Jun (**figure 4.33B**, lanes 7 to 12, upper and middle panels). Blots were therefore quantified by densitometry in **figure 4.33C and D**.

I also assessed the mRNA levels of C-Fos and C-Jun in the presence and absence of serum in the media with the aim of not having a serum-dependent induction of AP-1 transcription after LPS treatment. **Figure 4.34** and **figure 4.35** both show that total mRNA levels of C-Fos and C-Jun were decreased with Myxothiazol or S3QEL 1.2 pre-treatment, respectively, in the presence of supplemented media (panels A and B) or serum-free DMEM (panels C and D).

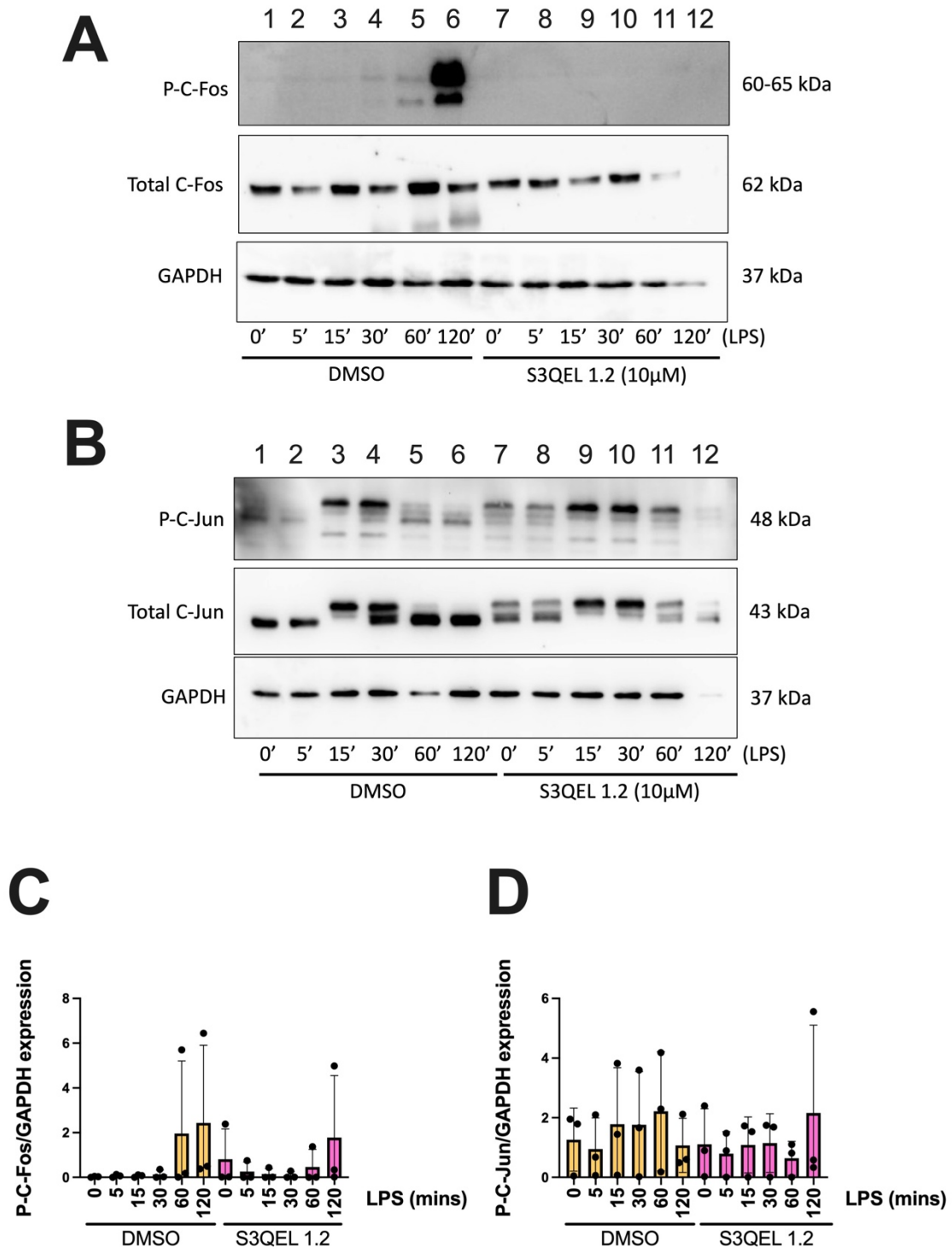


Figure 4.32. S3QEL 1.2 pre-treatment downregulates c-fos and c-jun protein levels.

BMDMs (0.5×10^6 cells) were treated for 1 hour with DMSO, S3QEL 1.2 (10µM) and then stimulated for 5, 15, 30, 60, 120 minutes with LPS (100ng/mL). After that, cell lysates were harvested and protein levels were analysed by western blot for the expression of p-C-Fos, p-C-Jun, C-Fos, C-Jun and GAPDH. Blots are representative of one of three independent experiments. Blots were also quantified by densitometry. Data are mean $\pm$ SEM for $n=3$ from three independent experiments.

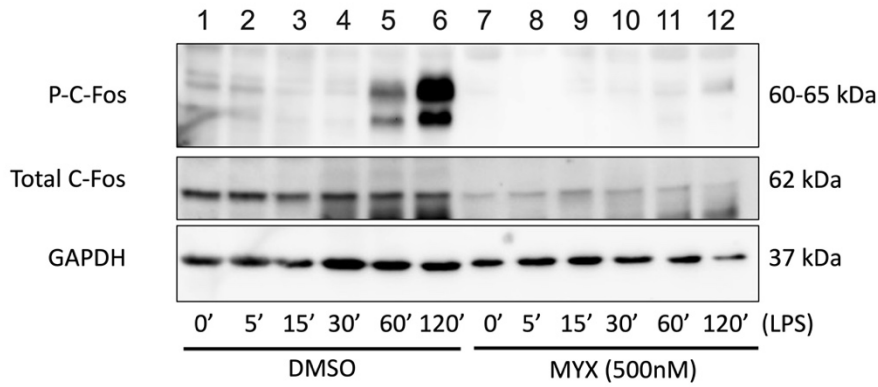
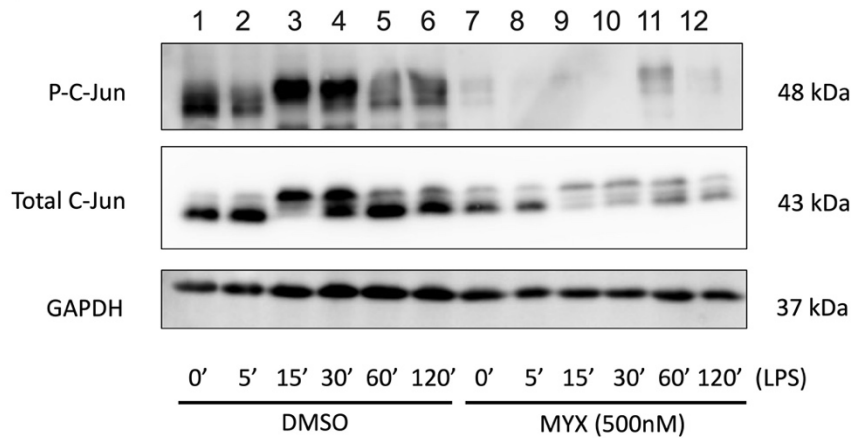
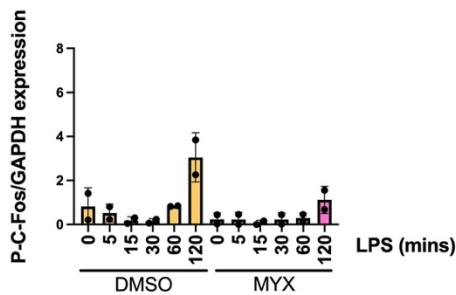
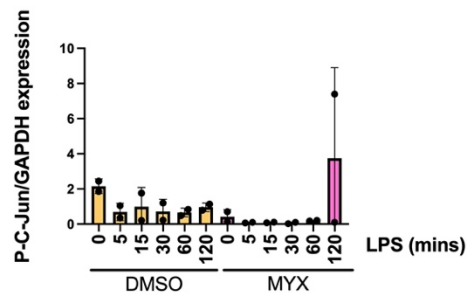
A**B****C****D**

Figure 4.33. Myxothiazol pre-treatment downregulates c-fos and c-jun protein levels.

BMDMs (0.5×10^6 cells) were treated for 1 hour with DMSO, MYX (500 nM) and then stimulated for 5, 15, 30, 60, 120 minutes with LPS (100ng/mL). After that, cell lysates were harvested and protein levels were analysed by western blot for the expression of p-C-Fos, p-C-Jun, C-Fos, C-Jun and GAPDH. Blots are representative of three independent experiments. Blots were also quantified by densitometry. Data are mean $\pm$ SEM for $n=2$ from two independent experiments.

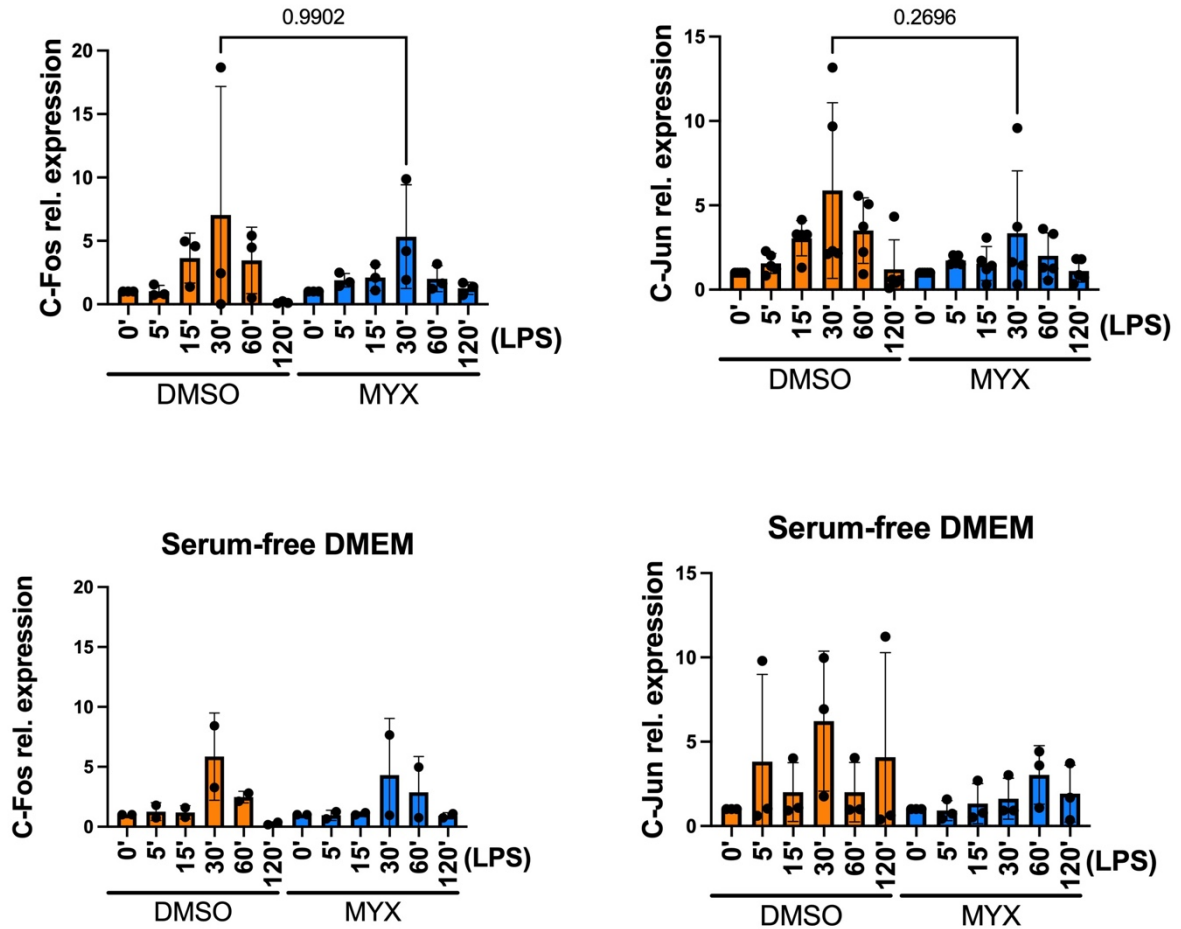


Figure 4.34. C-Fos and C-Jun mRNA levels are decreased after Myxothiazol pre-treatment.

BMDMs (0.5×10^6 cells) were treated for 1 hour with DMSO and Myxothiazol (500nM) and then stimulated for 5, 15, 30, 60, 120 minutes with LPS (100ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of C-Fos, C-Jun with RPS18 as a housekeeping control. Data are mean $\pm$ SEM for $n=2-3$ from three independent experiments for the serum-free DMEM samples and for $n=3-4$ from three independent experiments for the supplemented DMEM samples. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test.

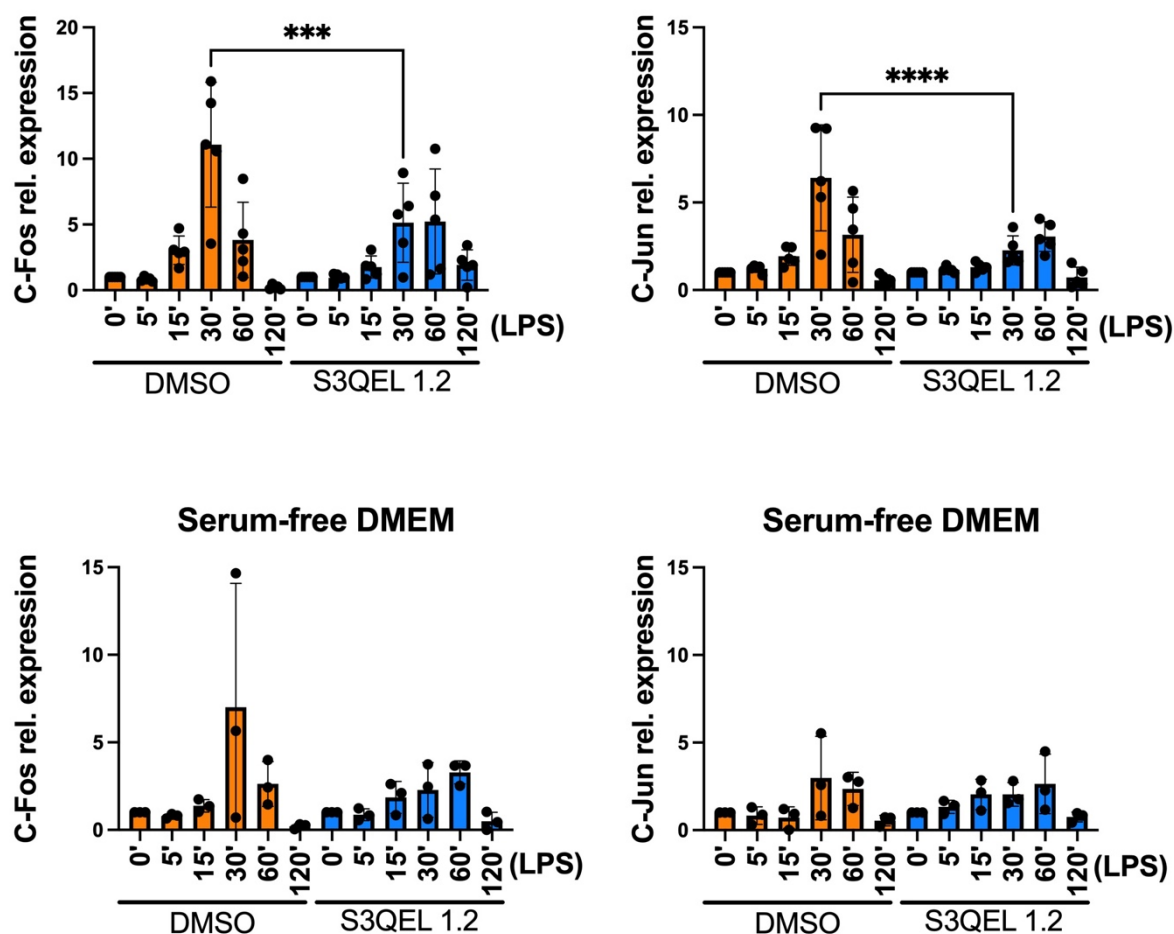


Figure 4.35. C-Fos and C-Jun mRNA levels are decreased after S3QEL 1.2 pre-treatment.

BMDMs (0.5×10^6 cells) were treated for 1 hour with DMSO and S3QEL 1.2 (10 μ M) and then stimulated for 5, 15, 30, 60, 120 minutes with LPS (100ng/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of C-Fos, C-Jun with RPS18 as a housekeeping control. Data are mean $\pm$ SEM for $n=3$ from three independent experiments for the serum-free DMEM samples and for $n=5$ from three independent experiments for the supplemented DMEM samples. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at *** $p < 0.001$, **** $p < 0.0001$.

4.2.10 Myxothiazol slightly decreases IL-10 in serum and in peritoneal exudate cells *in vivo*

Having elucidated the effects that MYX and S3QEL 1.2 can have on the expression of cytokines produced during LPS stimulation *in vitro*, I next tested their effects in an *in vivo* model involving LPS-induced local inflammation in the peritoneum.

Figure 4.36 shows that intraperitoneal injection of LPS induced the expression of IL-10 (A) in the serum and in the peritoneal exudate cells (B), together with IL-6 (C), while there was no induction of IL-1 β (D) and TNF- α (E). Myxothiazol injection decreased in a non-significant manner the IL-10 induction in serum and in PECs and it did not have an effect on the IL-6 induction in cells recruited to the peritoneum.

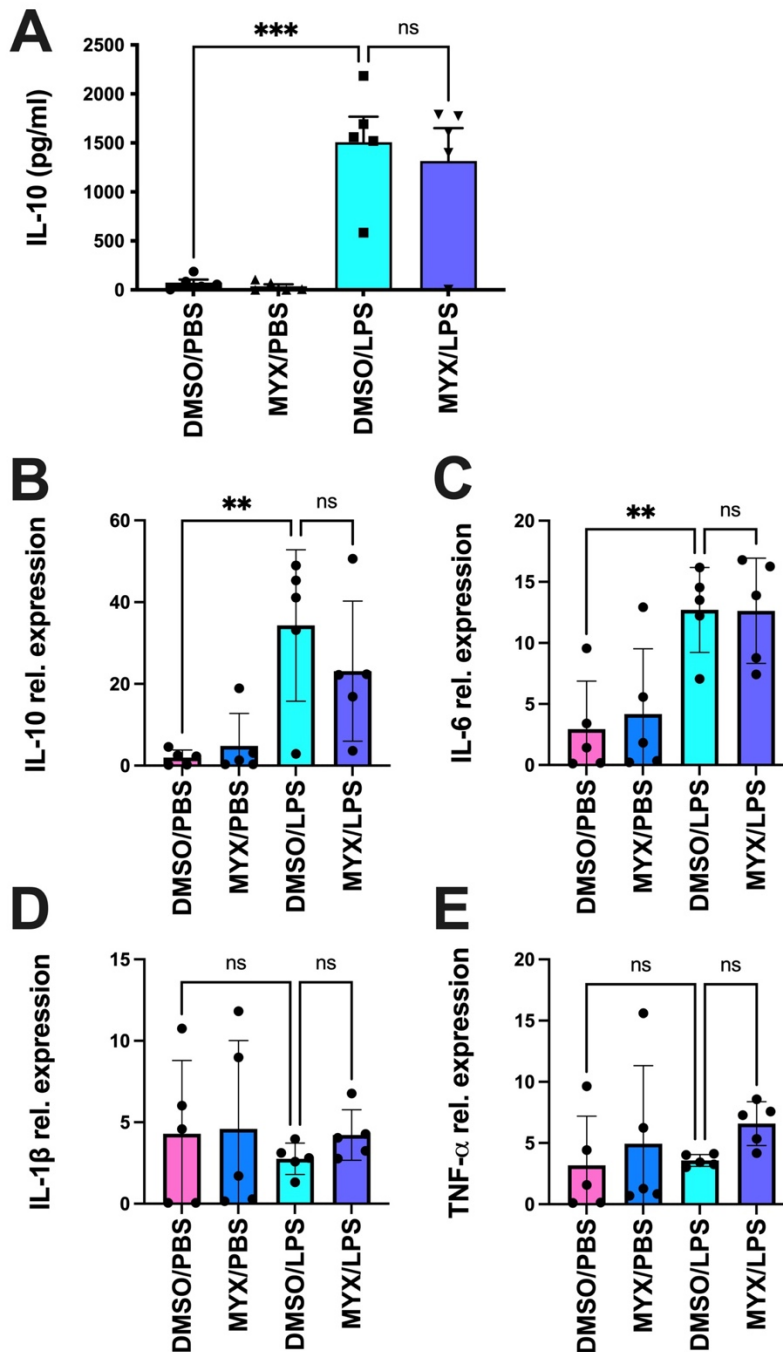


Figure 4.36. Myxothiazol slightly decreases LPS-induced IL-10 in vivo.

6-week-old female C57/BL6OlaHsd mice were injected intraperitoneally with a mixture of Myxothiazol (1 mg/kg) or vehicle for 1 hour and LPS (2,5 mg/kg) or PBS for 2 hours. Mice were subsequently euthanized and IL-10 concentration in serum was analyzed by ELISA. Peritoneal exudate cells were harvested from the peritoneal lavage and lysed for measuring the expression of IL-10, IL-6, IL-1 β , TNF- α with RPS18 as a housekeeping control by RT-qPCR. Data mean $\pm$ SEM for n=5 per group from one independent experiment. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at **p<0.01, ***p<0.001.

4.2.11 S3QEL 1.2 suppresses IL-10 in serum and in peritoneal exudate cells *in vivo*

I next tested S3QEL 1.2 *in vivo*. As it can be seen in **figure 4.37**, intraperitoneal injection of LPS induced IL-10 (B) and IL-6 (C) after 2 hours, while it did not induce IL-1 β (D) nor TNF- α (E) in the peritoneum, as detected by qPCR performed on peritoneal exudate cells. The release of IL-10 was blocked by the peritoneal injection with S3QEL, both in serum (A) and in PECs (B).

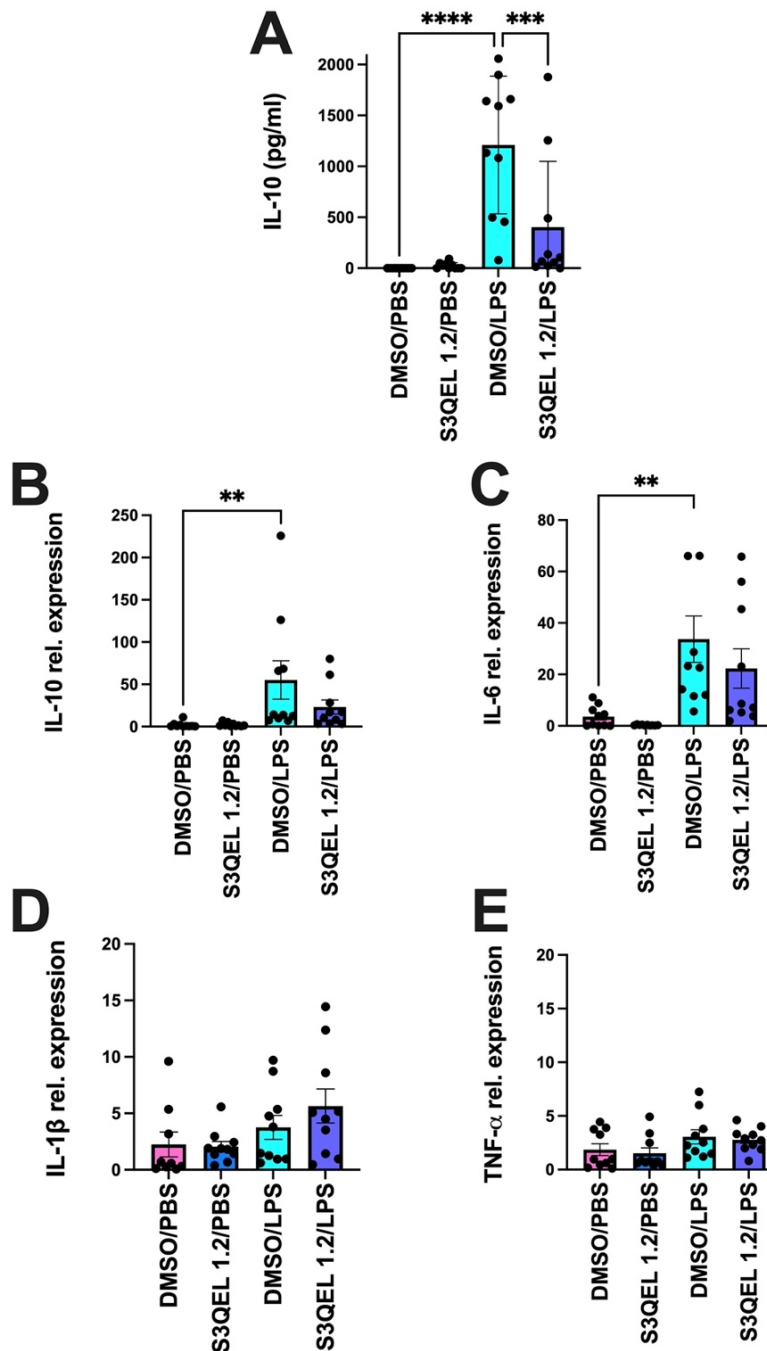


Figure 4.37. S3QEL 1.2 blocks LPS-induced IL-10 and IL-6 release in vivo.

6-week-old female C57/BL6OlaHsd mice were injected intraperitoneally with a mixture of S3QEL 1.2 (1 mg/kg) or vehicle for 2 hours and LPS (2,5 mg/kg) or PBS for 2 hours. Mice were subsequently euthanized and IL-10 concentration in serum was analyzed by ELISA (A). Peritoneal exudate cells were harvested from the peritoneal lavage and lysed for measuring the expression of IL-10, IL-6, IL-1 β , TNF- α with RPS18 as a housekeeping control by RT-qPCR (B, C, D and E). Data mean $\pm$ SEM for $n=10$ per group from two independent experiments. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at **** $p<0.0001$, *** $p<0.001$.

4.3 Complex III-dependent IL-10 regulation is necessary for tumour-induced immune evasion

To deepen the knowledge of the role of Complex III in a more relevant *in vivo* setting and to explore the potential of S3QEL 1.2 as an innovative therapeutic tool, I decided to investigate its effect in tumor development in mice. Having shown that S3QEL 1.2 and myxothiazol decrease IL-10 expression and since IL-10 has been shown to promote immunosuppression in tumors such as melanoma (138, 139), I chose the B16F10 melanoma model.

Mice received S3QEL 1.2 every two days of the trial and the immunotherapy tool CpG, which is a TLR9 ligand, as a positive control (**figure 4.38A**). As can be seen in **figure 4.38**, S3QEL 1.2 alone slowed melanoma growth and in a more significant manner in combination with CpG, as compared to the PBS treated mice (C). Overall, the mice administered with the combination of S3QEL 1.2 and CpG survived longer (B), followed by CpG alone treated mice and S3QEL 1.2 alone treated mice.

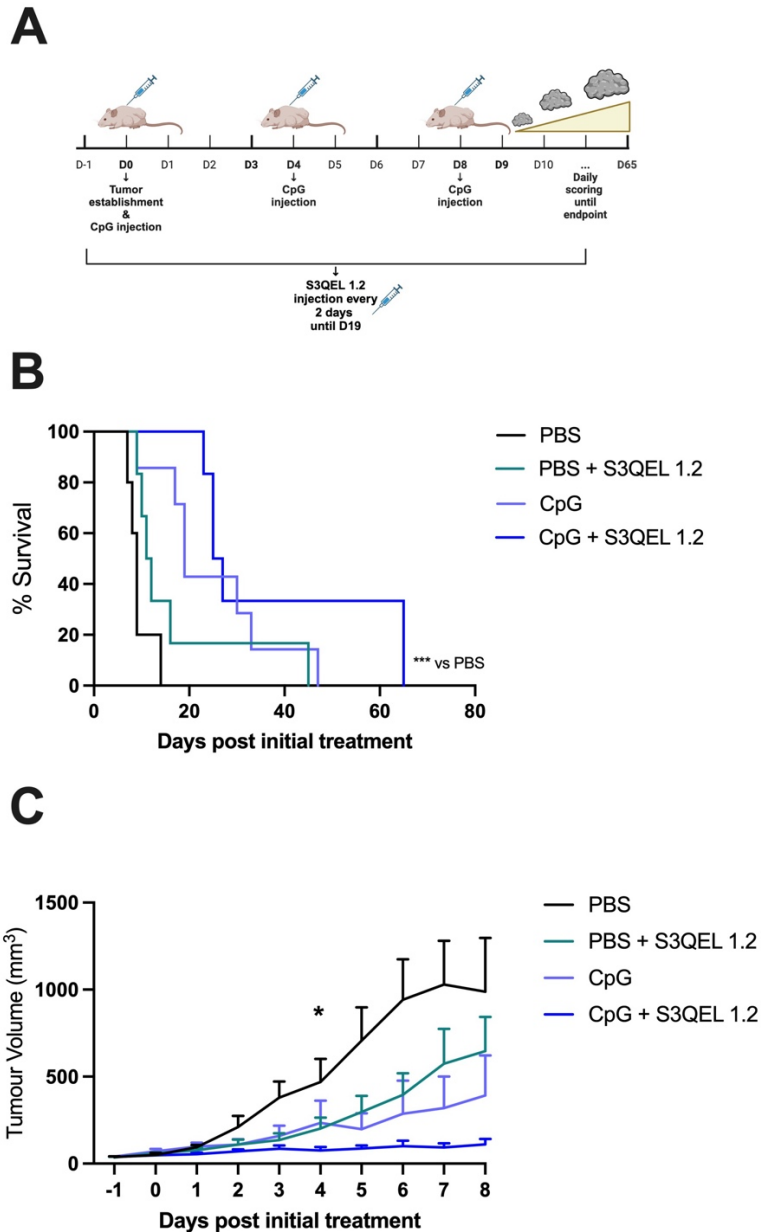


Figure 4.38. S3QEL 1.2 co-treatment with CpG ameliorates overall survival and inhibit tumor growth after melanoma establishment in mice.

3×10^5 B16F10 cells in exponential growth phase were implanted subcutaneously in a volume of 100 μ L in C57/BL6J mice. Mice were monitored daily; once the tumours became palpable the diameter of the lesion was measured using a callipers and tumour volume in mm³ was recorded. Animals were humanely euthanised when the lesion reached 15mm³ or if necrosis was noted. (A) In vivo protocol schematic. (B) Kaplan-Meier curve of up to day 65 (end of the experiment). (C) Tumor growth curves relative to the mean of tumor volume from Day (-1) to Day (8). (A-C) N=5 for PBS group, n=6 for S3QEL 1.2 group, n=7 for CpG group and n=6 for S3QEL 1.2+CpG group. Statistical significance for survival analysis was determined by Mantel-Cox test. Statistical significance for tumor growth analysis was determined by two-way ANOVA with Dunnett's multiple comparison, asterisks are representative of p values ***<0.001 in the group's comparison.

4.3.1 S3QEL 1.2 effect on CpG activated macrophages

I assessed if S3QEL 1.2 would inhibit IL-10 induction by CpG, which is a ligand for TLR9. As shown in **figure 4.39**, S3QEL 1.2 decreased CpG-induced IL-10 mRNA (top left panel) and protein (top right panel) expression. Induction of IL-1 β mRNA was also inhibited by S3QEL 1.2 and Myxothiazol pre-treatment (bottom panel).

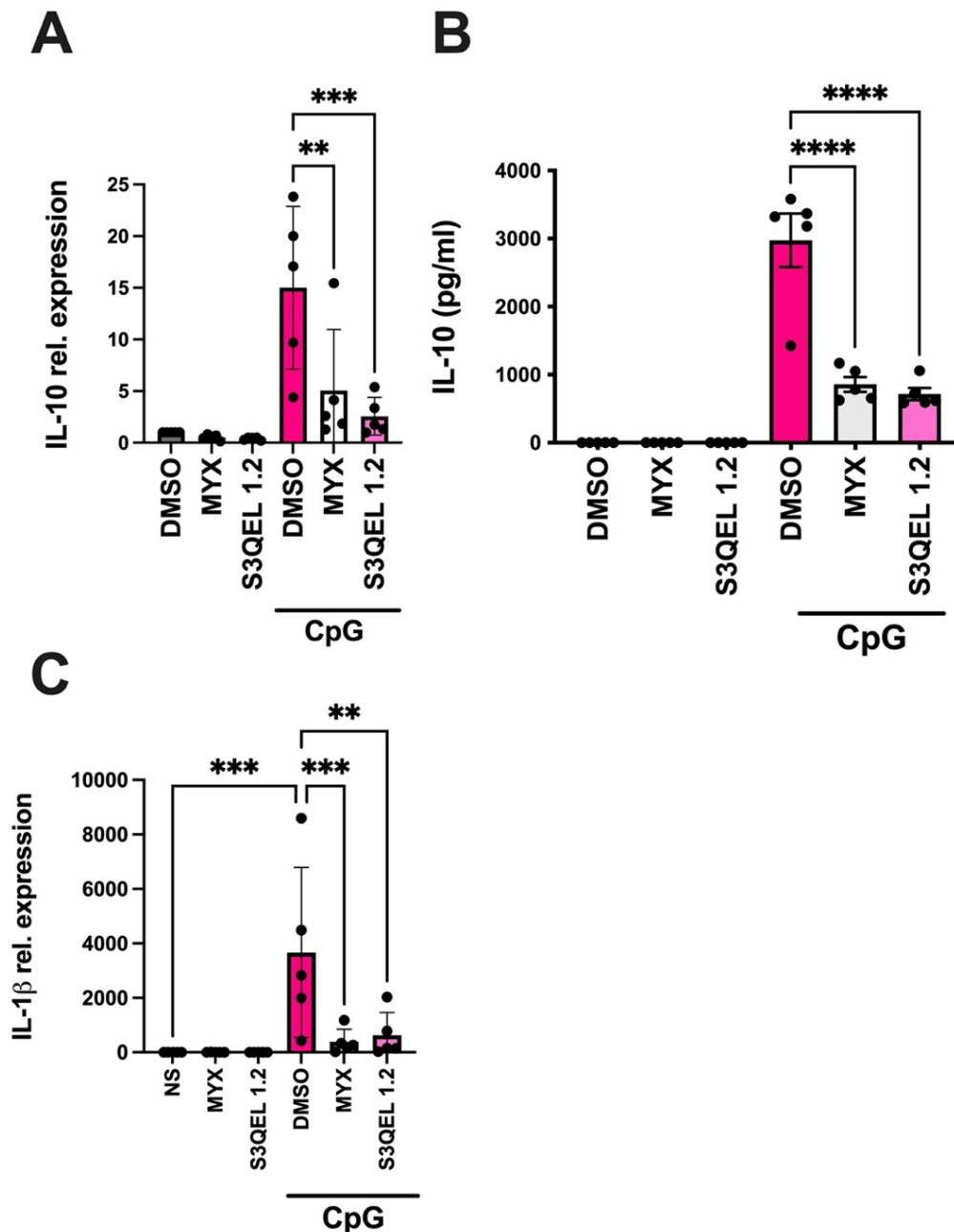


Figure 4.39. S3QEL 1.2 and myxothiazol decrease IL-1 β and IL-10 production in CpG-activated macrophages.

BMDMs (0.5×10^6 cells) were treated for 3 hours with DMSO, S3QEL 1.2 (10 μ M) and myxothiazol (500nm) then stimulated for 4 hours with CpG (1 μ g/mL). After that, cell lysates were harvested and analysed by RT-qPCR for the expression of IL-10, IL-1 β with RPS18 as a housekeeping control. IL-10 concentration in supernatants were analyzed by ELISA. Data are presented as mean $\pm$ SEM for $n=5$ from three independent experiments. Data were calculated using a one-way ANOVA followed by Sidak's multiple comparisons test. Differences were considered statistically significant at **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$.

4.3.2 S3QEL 1.2 effect on tumor cells

I also investigated the effect of S3QEL 1.2 on tumor cells *in vitro*.

As shown in **figure 4.40**, MYX at any concentration totally blocked the proliferation of B16F10 melanoma cells, suggesting Complex III and respiration are essential for cell survival. Also, S3QEL 1.2 blocked B16F10 cell proliferation but only at the concentration of 10 μ M.

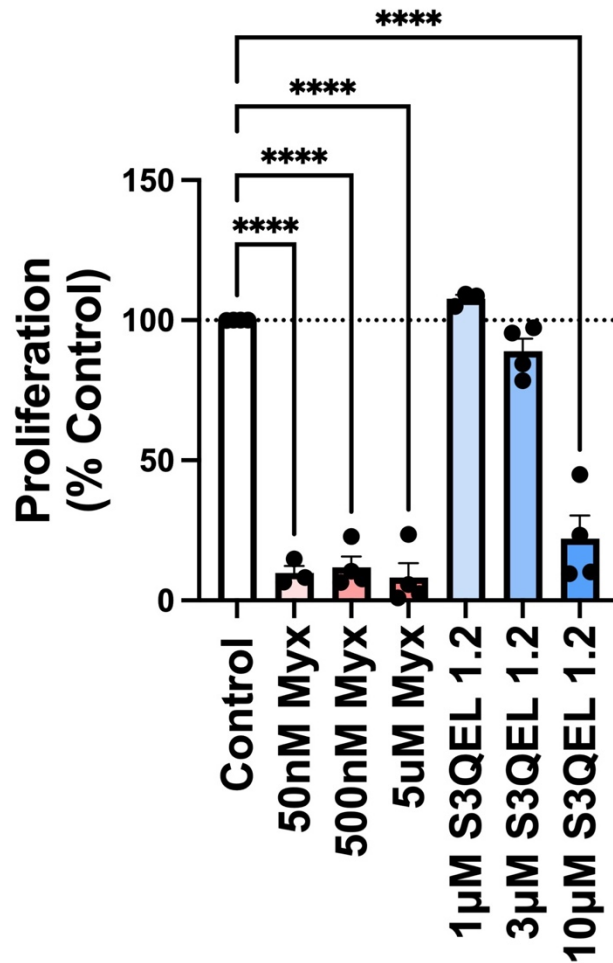


Figure 4.40. S3QEL 1.2 and MYX can block melanoma cells proliferation.

B16F10 melanoma cells at 1000 cells/well concentration were treated with DMSO, MYX (50nM, 500nM, 5μM) or S3QEL 1.2 (1, 3 and 10μM) and allowed proliferating for 2-3 days. Quantification of cells coloured with crystal violet dye. Data are expressed as mean ± SEM (n=4-7 technical replicates from three independent experiments). ****p<0.0001 as calculated using a one-way ANOVA followed by Sidak's multiple comparisons test.

4.4 Discussion

In this chapter I have investigated the role that respiratory Complex III can have in the regulation of transcription and secretion of IL-10. Few studies have investigated the role of mitochondria in IL-10 induction. Mitochondria have been demonstrated to boost IFN- β production by mtRNA release (140) and to change IL-1 β expression by redox-dependent and independent mechanisms, also involving inflammasome activation (94). Previous research on the link between the ETC and IL-10 is based on few examples and one of them was recently published by Simarro et al. (141). In their paper, the researchers showed that the inhibition of succinate dehydrogenase (mitochondrial Complex II) with dimethyl malonate (DMM) decreased IL-10 production in LPS-activated BMDMs and RAW 264.7 macrophages and the same result was achieved in knockout cells for SDHA and SDHB subunits (141). This is in contrast with previous findings showing IL-10 increases after DMM-induced SDH inhibition (8). These two contradictory results suggest that DMM might not be a great tool to study complex II involvement.

I focused my attention on Complex III because it is a complex whose full function needs to be elucidated. Complex I and Complex III are both sources of superoxide generation and mitochondrial ROS. Respiratory Complex I has already been studied in innate immune cells and it is responsible for regulation of IL-1 β and IL-10 production (8). Considering that Complex III is another source of ROS, it might be involved in the same way as Complex I in the regulation of cytokine production, particularly as it releases ROS in the cytosol (126). I also thought that new insights might be made into the ways these two complexes are similar but also about what they do differently and what this can mean in the context of their therapeutic targeting.

Of interest is that the complete deletion of Complex I or Complex III is lethal for the organism and leads to serious illnesses because of the impairment of OXPHOS (142, 143), the major source of energy in living organisms. Indeed, many models to evaluate the two complexes have been created and include conditional knockout

of specific subunits of the complexes, which do not repress the total activity of the complex and can make possible their study (144, 145).

The development of new and more specific technologies has also allowed the discovery of small molecules inhibiting only the production of ROS from the complexes, to gain deeper insight into the understanding of physical and chemical properties of the mitochondrial machinery (146).

Complex III biochemistry and functionality has been studied in several tissues like liver, muscle, heart, brain but almost nothing about its profile in immune cells has been reported, comparing the number of papers published on each topic. Complex III activity can be subject to change under certain circumstances like pathological states and senescence. As an example, aging decreases OXPHOS and Complex III activity in cardiac mitochondria located among the myofibers (147). Hoppel et al. found that the ageing effect was localised to the cytochrome c binding site (147).

Moreover, some mitochondrial disorders can be due to the lack or malfunction of specific subunits of Complex III. For example, one mitochondrial disorder is associated with two-exon deletion in the human *UQCRH* gene, which encodes for the Ubiquinol cytochrome c reductase hinge protein. UQCRH protein allows electron transfer between cytochrome c_1 and c of Complex III. In mice, knockout of UQCRH lead to elevated glucose levels, growth difficulties and early death. From the cardiovascular point of view, hearts from KO mice appeared smaller and with higher contractile dysfunction. Indeed, mitochondrial respiratory capacity was decreased but ROS production was not affected in the mice lacking the subunit (148).

4.4.1 Complex III regulates IL-10 expression in activated macrophages

After having examined IL-1 β production by macrophages, I decided to focus my attention on IL-10. Previous studies established IL-10 production as a classical feature of M2 macrophages but M1 macrophages also produce it as a negative feedback signal to dampen inflammatory response (149). Evidence of a role for IL-10 in the maintenance of the metabolic equilibrium of M1 macrophages was given by Mc Vicar et al (149). The treatment of LPS-activated macrophages with 2-DG, an inhibitor of glycolysis or the stimulation of BMDMs with LPS in decreasing concentrations of glucose provoked a decrease in IL-10 production. After 2-DG treatment, the levels of phosphorylated AMPK were elevated and the levels of mTOR were decreased, contributing to the repression of IL-10 expression. This directly correlated IL-10 regulation to ATP sensing through the activity of AMPK and the metabolic switch of macrophages towards glycolysis. Moreover, when LPS activated macrophages were incubated with exogenous IL-10 overnight, NO levels were decreased and OXPHOS was rescued, suggesting NO as a regulator of metabolic commitment of macrophages, together with IL-10 (149).

To test the toxicity of the compounds, I performed the MTT assay, which consists in the detection of cell viability through visualization of SDH metabolic activity. No effect of the inhibitors on viability was observed, suggesting that the perturbation of Complex I or Complex III does not interfere with Complex II activity and cellular respiration still happens. Together with this, the protein levels of the five complexes constituting the ETC are also not affected by the inhibitors but endure reduction after LPS treatment, an event that is expected according to previous findings (150).

The analysis of macrophage OXPHOS and Complex III specific activity shows the same trend in every treatment condition. MYX completely blocked respiration and, together with that, the activity of Complex III in a greater manner than S3QEL 1.2, which slightly decreases both OXPHOS and Complex III specific activity. After S3QEL 1.2 pre-treatment, cells are still able to perform aerobic respiration and to oxidize coenzyme Q. LPS is decreasing OXPHOS and C-III activity, as expected.

There are existing more specific assays to evaluate complex III activity but some of them require the isolation of a large number of mitochondria, which are not possible to achieve when using immune cells and not tissues or organs. I have not investigated the effect of S3QEL 1.2 and MYX on membrane potential for lack of time, but my hypothesis is that S3QEL 1.2 will not affect it, as described in previous studies (111). On the contrary, MYX has already been shown to collapse mitochondrial membrane potential (151).

When ETC is unable to function properly, the cells start using alternative processes to create energy and compensate for the loss of OXPHOS. As expected, the inhibition of respiration by MYX augmented glycolysis. Indeed, also S3QEL 1.2 pre-treatment increased glycolysis. Within the experiment itself, oligomycin did not increase glycolysis and had no effect on ETC inhibition because the chain had already been affected by MYX. I did not check the effects of the combination of MYX/S3QEL 1.2 and LPS on glycolysis but I would expect an LPS-induced boost in glycolysis as described previously (152) and a S3QEL 1.2 and MYX-related boost of the LPS-upregulated glycolysis.

LPS is an important driver of ROS in macrophages (153) but the various cell lines, human and murine, require different times of LPS exposure and different concentrations of the PAMP to strongly induce oxidative stress. Analysis of ROS production by flow cytometry is not ideal because ROS are generated and dissipated very quickly within the cell. I used instead a fluorescence-based assay which was able to detect ROS produced as soon as the substrate is added to the cells. I confirmed the antioxidant properties of S3QEL 1.2, MYX and MitoTEMPO in decreasing LPS-derived ROS levels.

The use of S3QEL 1.2 in my system highlights the importance of mitochondrial ROS generated at Complex III in the specific modulation of IL-10 expression in BMDMs. Together with that, the use of Myxothiazol, which shuts down the activity of the complex, lead to the same outcome. S3QEL 1.2 and MYX had the same effect on IL-10 expression after activation of macrophages with CpG, a TLR9 ligand. This suggests that ROS are responsible for IL-10 regulation regardless of the TLR pathway engaged.

The experiments where I used rotenone and S1QEL 1.1 as pretreatment before stimulation of macrophages excluded Complex I activity from the regulation of IL-10. Different chemical structures of S3QEL (in this case, S3QEL 2.2) recapitulated the same effect on IL-10, although in a non-significant manner, as S3QEL 1.2.

I also found that TNF- α expression after Complex III inhibition was regulated and I found an increase in it, as expected. These results can state that TNF- α upregulation is likely due to IL-10 downregulation. This is because IL-10 and TNF- α participate in a mutual inhibitory crosstalk where IL-10 represses pro-inflammatory cytokines including TNF- α , which in turn restrains IL-10 expression (154, 155, 156).

The experiment of RNA sequencing that I performed was helpful in confirming the finding on IL-10. Firstly, the analysis showed the upregulation of pathways involved in the response against viruses and bacteria and relative cytokine production in the LPS-treated samples. As expected, LPS alone boosted the expression of specific interleukins and their receptors, with a particular focus on IL-1 β , TNF- α , ACOD1, IFN- β , IL-6 and IL-10. After Complex III inhibition, a plethora of genes were commonly differentially expressed by both Myxothiazol and S3QEL (146 genes). The differentially upregulated genes belonged to NF- κ B, TNF- α , NOD-like receptor and sphingolipid signaling. This confirms the effect of S3QEL 1.2 and MYX on LPS-induced TNF- α production *in vitro*. The heatmaps generated showing the genes regulated in the same way by S3QEL 1.2 and Myxothiazol gave a more comprehensive understanding of the role of the ETC role in shaping immune cell behavior. Some examples are not only linked to cytokine production: the upregulation of VWF (Von Willebrand factor), a glycoprotein fundamental in primary hemostasis, can suggest a link between inflammation and coagulation and could explain a role for ROS in controlling thrombosis. The upregulation of Growth Differentiating Factor 15 (GDF15), responsible for reduced food intake and weight loss in mice and which seems to have anti-inflammatory effects (157), is interesting because ROS have already been shown to regulate hypothalamic neurons and

control satiety and hunger behavior (158). This proposes S3QEL 1.2 as a candidate compound to be studied in obesity research.

From the analysis, I noticed that the two inhibitors differentially downregulated some protooncogenes such as HCK and ETS2, metalloproteinase inhibitors such as TIMP3, cathepsins such as CTSC and the chemokines CCL-12, CCL-22 and CCL-24, all factors produced by macrophages and involved in tumor development, degradation of extracellular matrix, vasodilation and monocyte recruitment, respectively (159, 160). This would help to explain the efficacy of S3QEL 1.2 in lowering melanoma progression *in vivo* but further studies are necessary to confirm this. For now, I hypothesize that S3QEL 1.2 effect on the tumor is mainly IL-10-dependent.

The RNAseq data about downregulated genes gathered useful information about ROS and their role as signaling molecules. ROS appeared to regulate the expression of macrophage scavenger receptor 1 (MSR1), which has already been shown to be downregulated after treatment with oxygen radical scavengers sodium benzoate and dimethylthiourea (161). Another redox-sensitive target is glutaredoxin (Glr), responsible for deglutathionylation and restoration of glutathione (GSH) levels (162), whose mRNA levels were dampened by S3QEL 1.2 and MYX.

Finally, the transcription factor ETS2 was identified among the most downregulated genes after complex III repression. Previous works have indicated that ETS2 and AP-1 can cooperate and bind to DNA at the same time (163) and hinder the expression of pro-inflammatory cytokines such as TNF- α (164) in mouse primary peritoneal macrophages. This could be the mechanism underlying the upregulation of TNF- α in my samples.

Antioxidants that target mitochondria gave the same outcome as S3QEL 1.2 and Myxothiazol in blocking IL-10 production, thus suggesting that the mechanism driving IL-10 secretion indeed could be ROS-dependent.

MitoQ is a derivative of coenzyme Q, in which coenzyme Q is linked to triphenylphosphonium (TPP), a lipophilic cation that allows the passage through biological membranes and its accumulation within mitochondria. MitoQ is a reliable and feasible tool to investigate mitochondrial ROS involvement in several biological processes and signaling pathways. It has been shown to revert LPS-induced lipid biogenesis in epithelial cells (165), attenuating liver inflammation and fibrosis in cirrhosis (166), and ameliorating experimental mouse colitis (167), thus blocking NLRP3 inflammasome activation.

MitoTEMPO is the combination of the antioxidant compound piperidine nitroxide and the lipophilic cation triphenylphosphonium (TPP) and acts as scavenger of mitochondrial superoxide. It has been shown to attenuate oxidative stress and mitochondrial dysfunction in numerous cell types (168). It is responsible of dampening necrosis and apoptosis of immune cells infected by Influenza A virus (169), decreasing ROS-mediated IL-1 β generation during rhinitis (170) and redox stress in endothelial cells infected by Dengue virus (171).

IL-10 is downregulated by general mitochondrial antioxidants but not Complex I targeted antioxidants, and this clearly suggests that Complex-III derived ROS is the key regulator of IL-10 expression.

The same conclusion can be reached using pro-oxidant compounds such as MitoPQ (172), a mitochondrion targeted paraquat, and menadione (173). So far, no pro-oxidant compound specific for mitochondrial Complex III is existing, while MitoPQ induces superoxide production at the Complex I site. Pretreatments with MitoPQ or menadione should probably boost IL-10 expression in activated macrophages.

The decrease in IL-10 transcription and release was not recapitulated in a transgenic *in vitro* model represented by mice expressing the protein AOX, after 4 hours of LPS stimulation. However, IL-10 expression was decreased in AOX expressing macrophages after 12 hours of LPS stimulation, but other independent experiments are necessary to confirm this. AOX diverts electrons away from Complex III and should reduce ROS production. My data on AOX BMDMs could

suggest that a longer exposure to LPS is necessary to decrease IL-10 because of the different mouse model used. Moreover, future experiments with the use of a conditional knock out model for Complex III by ablation of the Rieske iron-sulfur protein (RISP) will emphasize the specificity of Complex III in regulating IL-10.

A lot of different pathways could be involved in IL-10 regulation and a major part of them can be redox-sensitive, which means that effector proteins or transcription factors of the pathway can directly or indirectly be modified by ROS (174).

The analysis of the RNAseq database through transcription factor enrichment tools revealed that *Fosl2* and *Fosl2*-target genes were significantly decreased after S3QEL 1.2 and MYX treatment. *Fosl2* is a subunit of the transcription factor AP-1 (175) and can form heterodimers with proteins of the JUN family, move to the nucleus and activate the transcription of specific genes. I then blotted for c-Fos and c-Jun and, as expected, Complex III inhibition had a direct effect on AP-1. Particularly, there was a significant decrease in the levels of c-Fos while there was a smaller effect in the reduction of the levels of C-Jun, as was also found from the analysis of mRNA expression. These findings underline the induction of IL-10 by ROS derived from Complex III as an AP-1-dependent process with ROS somehow targeting AP-1. Further studies are needed to understand exactly where ROS are targeting the AP-1 pathway. Earlier publications have shown how peroxiredoxins, which are scaffold proteins involved in AP-1 activation, can be covalently modified by redox messengers (176) and it would be interesting to investigate that in my project.

4.4.2 In vivo studies for the evaluation of S3QEL 1.2 efficacy

IL-10 production can be high *in vivo* in context of tumor development and parasite infection. For the study of IL-10 regulation *in vivo*, I performed a model of LPS-induced local inflammation. So far, there is no *in vivo* protocol for S3QEL 1.2 administration in mice. Only one paper was published about the *in vivo* use of S3QEL 1.2 and it was supplemented to mice through water and not through intraperitoneal injections (177). I performed my *in vivo* experiments taking into account the same concentration of S3QEL 1.2 I was using for my *in vitro*

experiments and translating it into quantity per mouse. Also, I used my compound at the same concentration that previous researchers used for intraperitoneal injection of ETC modulators and mitochondria targeted antioxidants such as mitoTEMPOL and mitoTEMPO (178, 179).

In this first model using LPS, the treatment with S3QEL 1.2 significantly decreased IL-10 levels in the serum confirming the effect seen *in vitro*. I also tried to analyze ROS production in monocytes but failed to do so. A state of the art about measuring oxidative species *in vivo* has been given by Murphy and collaborators in 2022 (180). They affirm how measuring ROS production *in vivo* is considered a difficult, if not impossible, challenge. Various methods, including EPR, PET, bioluminescent approaches and genetically encoded biosensor have been just established but need to be improved (180).

After having found that S3QEL 1.2 was decreasing LPS-induced IL-10 in the serum of mice, I decided to investigate IL-10 in a more complex *in vivo* model involving the B16F10 melanoma cells. Melanoma is one of the highest IL-10-inducer cancer type. IL-10 can dampen the inflammatory response of local and systemic innate and adaptive immune cells against the cancer cells (**Figure 4.41**). *In situ*, invasive and metastatic melanomas extinguish the activation of immune cells surrounding the mass because tumour-associated T lymphocytes, dendritic cells and macrophages upregulate IL-10R (139). In mice, the melanoma model B16F10 achieves the dampening of anti-tumour immunity through an IL-10-dependent mechanism and the involvement of B cells. In the first case, induced-secretion of IL-10 in melanoma cells showed increased tumour growth, angiogenesis and proliferation *in vivo*, dampened expression of MHC class I and II molecules and upregulated percentage of PCNA (proliferating cell nuclear antigen)+ tumour cells (138). In the second case, the amount of tumour-infiltrating Bregs significantly increased in B cell-specific PTEN-deficient mice and the subset negatively regulated anti-melanoma immunity by producing IL-10 and reducing T helper 1 type cytokine production in tumour-infiltrating CD8⁺ T cells (181). The adoptive transfer of B1a Bregs from WT mice completely shut down the immune response against the tumour and allowed mass growth while adoptive transfer of B1a Breg

cells from IL10^{-/-} mice did not, suggesting a role for IL-10 in tumour-mediated immune suppression (181).

IL10^{-/-} mice survived longer and showed higher tumour rejection compared to the WT in a bladder tumour (MB49) in mice analysing the tumour-specific type 1 interferon immune response (182). IL-10 is a mark of suppressive DCs in cancer for its ability to induce inhibitory ligands, such as PD-L1, and suppress antigen presentation. For this reason, antibodies neutralising DC-derived IL-10 are being used as adjuvants for antitumor vaccination to enhance immune response against melanoma, colorectal carcinoma and cervical carcinoma in mice (183).

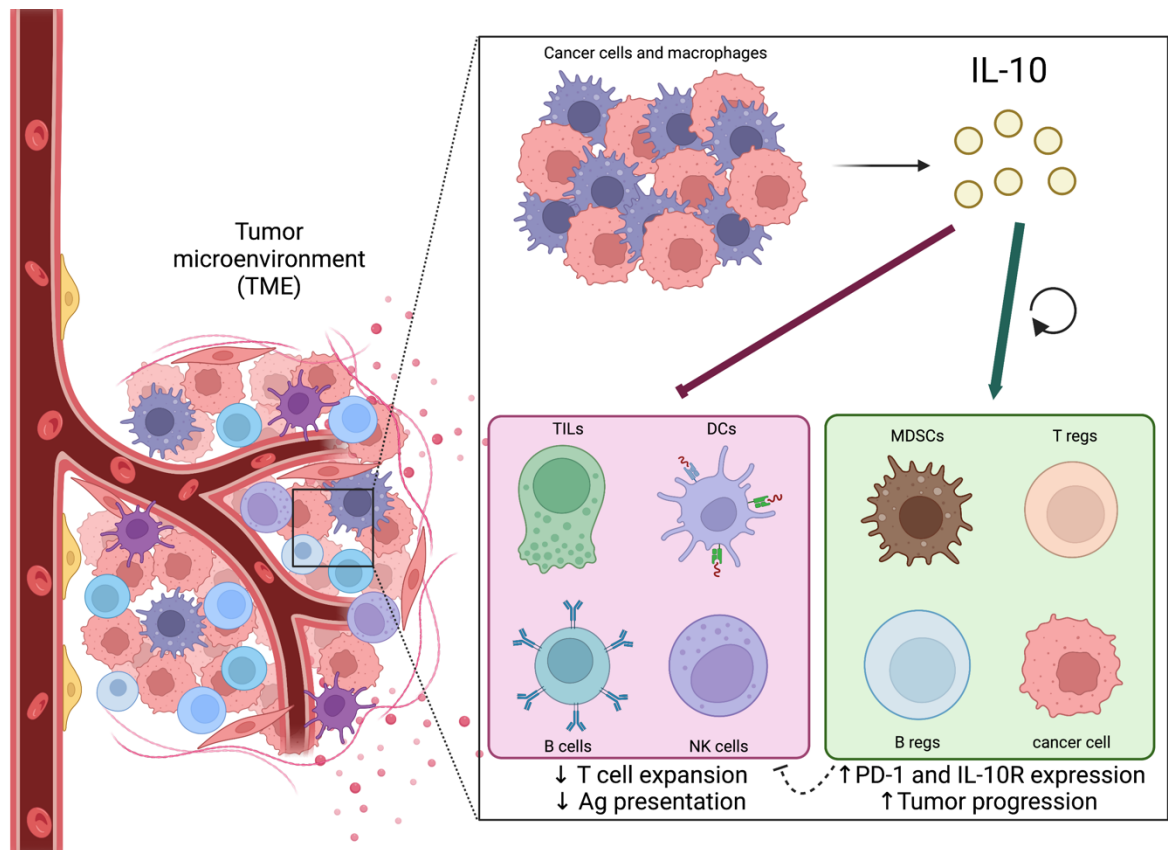


Figure 4.41 IL-10 effects in the tumour microenvironment.

Macrophages and cancer cells within the tumour microenvironment produce IL-10 which has two opposite functions. The first one inhibits the development and anti-tumour activity of TILs, DCs, B cells and NK cells. The second one promotes the functions of immunoregulatory cells like MDSCs, T and B regs and allows tumour progression.

Treatment with S3QEL 1.2 improved survival of the mice and decreased tumor growth. The combination of S3QEL 1.2 and the immunotherapy tool CpG proved even more effective. Macrophages contribute in different ways to the TME sustenance and to tumor proliferation, so the S3QEL 1.2 effect could not only be specifically related to IL-10 expression but also regulate other aspects of TAMs. To investigate this, co-cultures of S3QEL 1.2-treated macrophages and tumor cells or S3QEL 1.2-treated macrophages and lymphocytes will elucidate the interplay between innate and adaptive immunity in the TME and how macrophages can modulate cytotoxic activity of T cells through IL-10.

Proliferation assays conducted on melanoma cells showed that MYX at any concentration and S3QEL 1.2 at high concentration completely blocked tumor proliferation, probably because tumor cells strongly rely on metabolism for their expansion, despite being mostly glycolytic. For this reason, more studies are needed to understand S3QEL 1.2 contribution to tumor cells repression *in vitro* and *in vivo*. Further experiments on B16F10 cells could focus on the expression of pro-angiogenetic factors, ROS production and chemokines for the recruiting of immune cells.

In summary, I have demonstrated that Complex III causes downregulation of IL-10 expression, if inhibited. There are two mechanisms related to this aspect: the first one can be linked to the activity of the complex while the second one can be ROS-dependent. Moreover, I also showed evidence that the transcription factor AP-1 might be involved in IL-10 regulation here. Finally, Complex III could represent a potential target in cancer immunotherapy.

5. Final discussion and future perspectives

5.1 Unveiling the link between mitochondria and immunotolerance

This work explored the immunomodulatory potential of mitochondrial ETC inhibitors. The mechanism uncovered here can help to better understand how respiratory complexes shape the biology of immune cell activation and response. In this project, IL-10 regulation was the main finding, but other cytokines and chemokines could undergo a similar modulation. Additionally, in this study I proposed S3QEL 1.2 as a promising tool with possible therapeutic potential.

For twenty years macrophages have been classified as M1 and M2 subtypes and their energy production has always been labeled by upregulated glycolysis and oxidative phosphorylation, respectively (184). Similar to this, also the plethora of cytokines they produce in pathological conditions have always been associated to a specific M1 or M2 profile (185). Nevertheless, macrophage functionality is very plastic and heterogeneous. This project started with a focus on the contribution of oxidative phosphorylation to cytokine production in LPS-activated (and so M1-polarised) macrophages because, although the cells rely on glycolysis when activated by TLR4 engagement, the ETC still maintains its activity and contribute to shaping their immune response. IL-10 is one of the highly expressed cytokines when macrophages are switched on, together with IL-1 β , IL-6 and TNF- α (186); considering this, also M1 macrophages can drive anti-inflammatory response as a negative feedback loop.

It is known that mitochondria can participate to intracellular signaling in innate and adaptive immunity (187) through different ways, especially mitochondrial ROS production. Oxidative stress has been studied extensively, thus focusing the attention on the specific source of ROS to develop finely targeted approaches. Complex III plays a role in immune function of T regs and can drive tumoral progression and senescence (87), but nothing is known about how macrophages rely on its activity during the development of inflammation. This was the reason why I investigated its contribution to the response of macrophages. The findings in

this thesis have in part explained that the anti-inflammatory immune response of primed macrophages could be redox-sensitive and could rely on Complex III for its development. A better comprehension of Complex III immunomodulatory properties will represent the foundations for the use of mitochondria-targeted compounds in inflammatory and metabolic diseases.

Importantly, the timing of the PAMP stimulation can have different effects on the intracellular signaling pathways driving ROS generation and IL-10 production, and the permanence of the ETC inhibitors into the cells can affect these processes in different ways. Previous studies highlighted the transient generation of superoxide anion and the speed it can cross biological membranes, start multiple signaling cascades and oxidize proteins and lipids. Further proteomic analysis and the examination of the CoQ pool oxidation state of activated macrophages would help trace the destiny of ROS within the cell.

In this study, ROS derived from Complex III is driving IL-10 production in LPS-activated macrophages, in a mechanism involving AP-1, as depicted in **figure 5.1**.

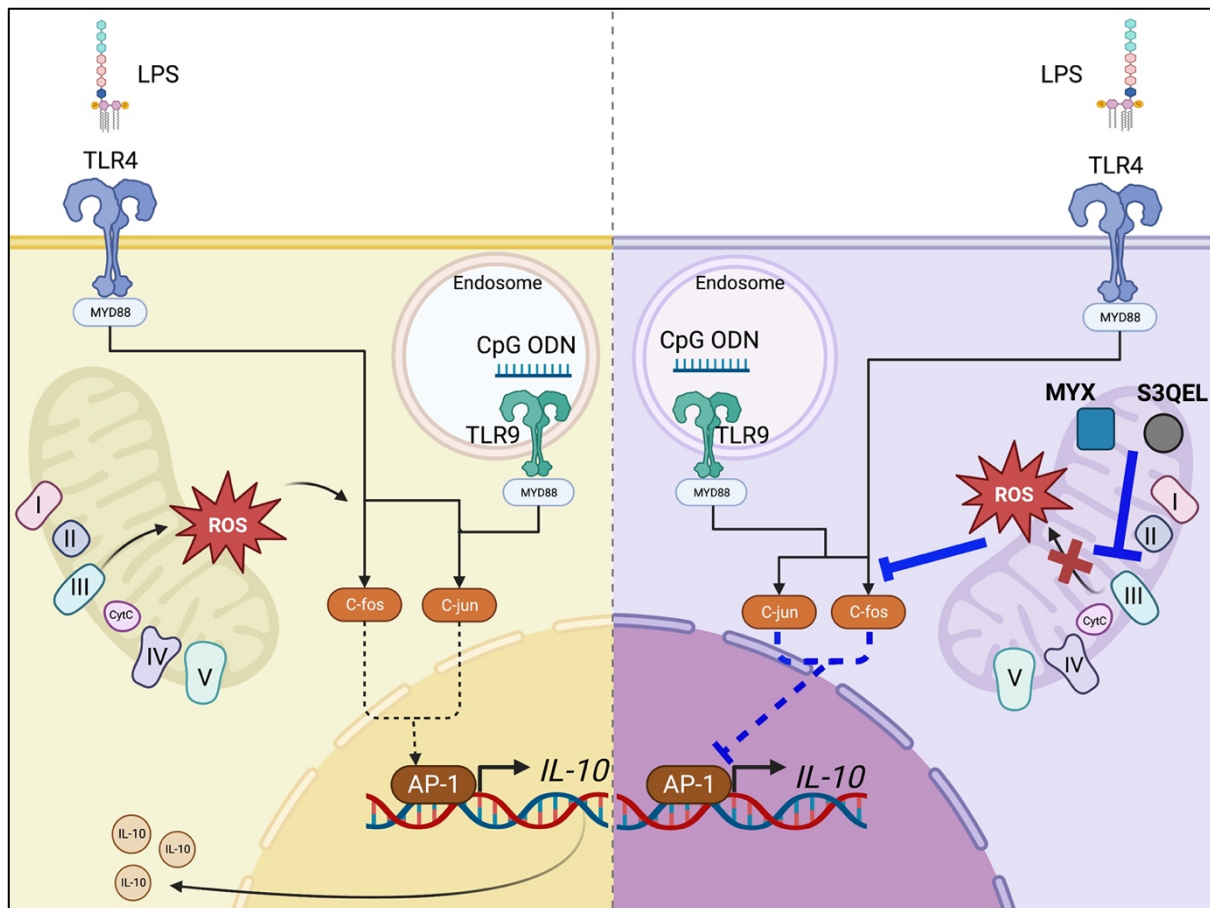


Figure 5.1 IL-10 requires ROS production from Complex III which promotes AP-1.

MYX and S3QEL 1.2 inhibit the activity and ROS generation from Complex III, repressing IL-10 production in macrophages activated by LPS and CpG. MYX and S3QEL decrease the mRNA and protein levels of c-fos and c-jun, which together dimerize to form the transcription factor AP-1. In the presence of Complex III inhibitors, AP-1 is repressed, leading to a decrease IL-10 transcription.

MYX: Myxothiazol
 S3QEL: S3QEL 1.2
 ROS: reactive oxygen species
 IL-10: interleukin 10
 LPS: lipopolysaccharide
 CpG (ODN): CpG oligodeoxynucleotides
 AP-1: Activator protein 1

5.2 Mitochondrial diseases and cancer progression – an issue for energy demand

Mitochondria have always only been considered a source of energy for the cells but most publications in the last two decades have established novel functions for them as drivers of intracellular signaling cascades (187). From the release of oxidized mitochondrial DNA to MAVS aggregation and from the production of ROS to the generation of immunomodulatory byproducts, mitochondria can shape the functionality of cells in many ways. Understanding the participation of these processes into the pathogenesis of several diseases will help the manufacturing of new mitochondria-targeted therapies.

Cancer represents one of the leading cause of death worldwide with 19 million people newly diagnosed every year and 10 millions of deaths since 2020 (WHO data). Each type of tumour appears with diverse differentiation stage, prognosis and therapies. Initially, cancer arises as an abnormal growth of cell mass and number, which starts transforming into malignant cells in a multi-step process. The causes can be genetic (mutations in gene coding for proteins involved in proliferation, respiration and protection from DNA damage) and external (tobacco consumption, unhealthy lifestyle, exposure to chemical hazard and some virus infections). The tumour becomes aggressive when it invades the tissue around it, starts flowing into the blood and colonising new tissues, generating metastasis. Many therapies are nowadays used to eliminate or reduce tumour burden and its related metastasis: usually chemotherapy and radiotherapy are aimed at targeting only cancer cells but unfortunately, healthy cells are not excluded from the damage, resulting in poor recovery during the post-chemotherapy period. Moreover, the most aggressive cancer types are characterised by an heterogeneous population of cells, including undifferentiated cells and stem progenitors, which do not express targets for the validated therapies, can still proliferate and mutate again to gain resistance against chemotherapy. 'Hot tumours' trigger a huge immune response, stimulating T cells through the

expression of tumour-specific antigens and recruiting other immune infiltrating cells which can process these antigens and drive tumour-related inflammation. These tumours include bladder cancer, melanoma, kidney and liver cancer and non-small cell lung cancer. Since the huge engagement of immune cells, these subsets of cancer can respond to immunotherapy. Several approaches have been developed in the field of immuno-oncology, with the aim of boosting immune cell defence against cancer cells and decrease the tumour-mediated mechanisms of immunotolerance. Checkpoint inhibitors represent immunotherapy drugs which target the expression of receptors on the cell surface of T cells and tumour cells that in normal conditions induce the shutdown of immune cells. Checkpoint inhibitors have been developed against CTLA-4, PD-1 and its ligand PD-L1 (188). Synthetic oligodeoxynucleotides contain unmethylated CG dinucleotides which can stimulate the immune response of NK cells, T cells, B cells, monocytes, macrophages and dendritic cells because ligand of TLR9. Animal models suggest that CpG ODN may be more useful as a component of multiagent therapy for cancer rather than as a single agent. Promising combinations include CpG ODN with monoclonal antibody such as rituximab and monoclonal antibodies against CD20 (189). Activators for the cGAS/STING pathway are recently representing an innovative tool for immune therapeutics and are showing great results in pre-clinical works. The Cytosolic DNA sensing, the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway is activating populations of T cells and NK cells through the production of type I IFN response (190).

This state of the art explained how both mitochondrial disease and tumour have one thing in common: the perturbation of metabolic activity and respiration of cells, which then is followed by collateral consequences also regarding other biological processes. The lack of optimal therapies drives the urgent need for new ideas to control mitochondria homeostasis in pathology. In this context, my findings could help the development of new and more specific treatments for disease where mitochondrial activity and metabolism are hyperactivated.

The results shown here have been produced using murine models. For this reason, investigation of IL-10 production by human macrophages and its reliance on Complex III could help in deciphering mitochondrial control of immunity in different

species. The human immune phenotypes could differ from the murine one (191). As an example, different time exposure to LPS is required to activate human macrophages and to induce IL-10 (192, 193). Subsequently, Complex III inhibition-induced effects in humans could be achieved with different concentrations of S3QEL and MYX from the one used in this thesis.

IL-10 dampening is one of the most common mechanisms of evasion of immune surveillance mediated by cancer cells. Melanoma-induced immune evasion could directly rely on IL-10 presence in the tumor microenvironment (TME). Future examination of IL-10 expression in serum and flow cytometry analysis of activation markers in samples from the tumor mass and lymph nodes of the mice participating in the survival trial would help characterize the specific subsets of cells infiltrating the TME. This last point could be also better elucidated by *in vitro* co-cultures of melanoma cells and immune cells or by co-cultures of macrophages and lymphocytes and help to dissect the specificity of immune cell interactions.

5.3: Towards novel anti-cancer therapies targeting mitochondria – what is their clinical relevance?

Cancer cells are characterized by an exaggerated glucose consumption and production of lactate by aerobic glycolysis, a phenomenon called the Warburg effect. Along with their growth, they require more and more oxygen to accomplish aerobic glycolysis, thus inducing angiogenesis, characterized by more blood vessels reaching the tumor and upregulated VEGF signaling for augmented vasodilation. The massive usage of oxygen and glucose and the special requirement of mitochondria by cancer cells made tumor metabolism an interesting topic for the development of new therapeutic strategies. Mitochondrial inhibitors can be used alone as a direct treatment of cancer disease or in combination with

first line chemotherapy and radiotherapy, according to their *in vitro* effectiveness. As regarding the first class, few of them have been involved in clinical trials of various phases but the high occurrence of side effects and low tolerability due to off-target effects led to a re-estimation of optimal doses and pharmacodynamics (194). For example, IACS-010579 is a highly potent and selective inhibitor of respiratory Complex I (195). Mechanistically, it binds to the ND1 subunit at the entrance to the quinone-binding channel and has been shown to reduce cancer cell growth and viability, induce cancer cell apoptosis and extend patients survival when used in several models of cancer such as lung cancer, brain cancer and acute myeloid leukemia (AML). For these reasons, IACS-010759 started as a novel therapeutic tool in two Phase-I clinical trials in patients affected by relapsed/refractory AML and solid tumors in 2023 (196). The work led by Yap et al. described the story of IACS-010759 and its final stop on clinical trials because of huge toxicities, including extreme lactic acidosis and neurotoxicity. Lactic acidosis was due to the switch of cancer cell metabolism to glycolysis after the inhibition of oxidative phosphorylation by IACS-010759 and a bigger amount of pyruvate converted into lactate. Moreover, IACS-010759 administration was associated with 50% rate of neurotoxic side effects in all patients and the clinical response was marginal, with only one patient with prostate cancer out of 23 showing a partial clinical response (196).

About the second class of inhibitors of mitochondrial complexes, metformin has been successfully used as a co-treatment in a variety of tumor trials. Metformin, initially used as a drug for type II diabetes, inhibits mitochondrial Complex I and improves insulin sensitivity (197). People treated with metformin show a decrease in cancer diagnoses and better cancer prognosis and it is possible that anti-tumor effects of metformin are due to its antidiabetic activity (198). Moreover, Metformin has been shown to reduce cancer cells tumorigenesis *in vitro* through the targeting of complex I (199). For these reasons, metformin had been used and showed efficacy in combinatorial treatment for cancer. An interesting example is the one given by Tsakiridis et al in 2021: metformin was used in combination with salicylate, a chemical compound derived from salicylic acid from plants, in prostate cancer cells and xenografts. The two treatments together inhibited the proliferation of tumor cells and ameliorated their response to radiotherapy at clinically relevant

doses. These effects were due to the suppression of HIF-1 α and AMPK pathways and to the inhibition of oxygen consumption from cancer cells (197). Similar effects and mechanism of action of metformin have been reported in prostate cancer cells and breast cancer cells (200).

For now, these are only two examples of how mitochondria metabolism can be considered as a possible target for stopping tumorigenesis and improving chemotherapy and radiotherapy approaches. The results until now are not enough to guarantee a safe use of mitochondrial inhibitors in clinical practice and further studies must be pursued to obtain more information about side effects and bioavailability of the drug in the intracellular and intramitochondrial space. Moreover, the contribution of the immune system to tumor progression and metastasis needs to be more elucidated and kept in consideration for new anti-tumor therapies. However, the data I generated using S3QEL 1.2 might well pave the way towards the development of ROS inhibitors as an unconventional option for the treatment of cancer. In conclusion, I hope that the findings presented in this thesis may expand the knowledge on mitochondrial function in immunity and inspire other researchers to deeply consider their role when dealing with oncologic and autoimmune diseases.

6. References

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7. Publications

COVID-19

SARS-CoV-2 targets MAVS for immune evasion

A new study shows that the SARS-CoV-2 nucleocapsid protein represses the antiviral type I interferon response through direct interaction with the signalling adaptor protein MAVS. Targeting this process might be a useful therapeutic strategy to boost immunity against COVID-19.

Alessia Zotta, Alexander Hooftman and Luke A. J. O'Neill

COVID-19 is a respiratory disease caused by SARS-CoV-2 and remains a major global public health issue despite recent therapeutic advances and the approval of several vaccines for use¹. Many studies have focused on how the immune system responds to infection with SARS-CoV-2 (ref. ²). The induction of antiviral type I interferons (IFNs) is a critical response and is driven by the sensing of viral single-stranded RNA, most likely by RNA receptors such as RIG-I³. SARS-CoV-2 RNA is contained within the viral particle that is composed of four structural proteins: the spike glycoprotein (S), the membrane protein (M), the ion channel envelope protein (E) and the nucleocapsid protein (NP), which are involved in viral invasion and replication⁴. The S protein binds to the angiotensin-converting enzyme 2 (ACE2) receptor on the respiratory epithelium⁵ and is responsible for the entry of the virus into host cells. The NP is involved in the packaging of RNA into new virions. Whether NP has other roles is not known. In this issue of *Nature Cell Biology*, Wang et al.⁶ report that NP can inhibit the key signalling adaptor mitochondrial antiviral-signalling protein (MAVS), which is activated by RIG-I, limiting the host defence response to the virus. They demonstrate that this process can be blocked with a peptide, raising the prospect of a novel therapy to boost immunity and limit pathology in COVID-19 (Fig. 1).

The study began with the demonstration that NP can undergo phase separation and form micro-sized droplets that can co-localise with viral RNA in infected HeLa cells. The dimerisation domain (DD) in NP was found to be essential for droplet formation and RNA binding.

The effect of NP on the antiviral response was examined next. The authors studied IFNs, as well as RIG-I and MAVS⁷. Transfection of HEK293 cells with NP completely blocked the expression of IFN- β in response to Sendai virus (SeV) and vesicular stomatitis virus (VSV) infection, an effect requiring the DD. NP expression also inhibited the induction of IFN- β by

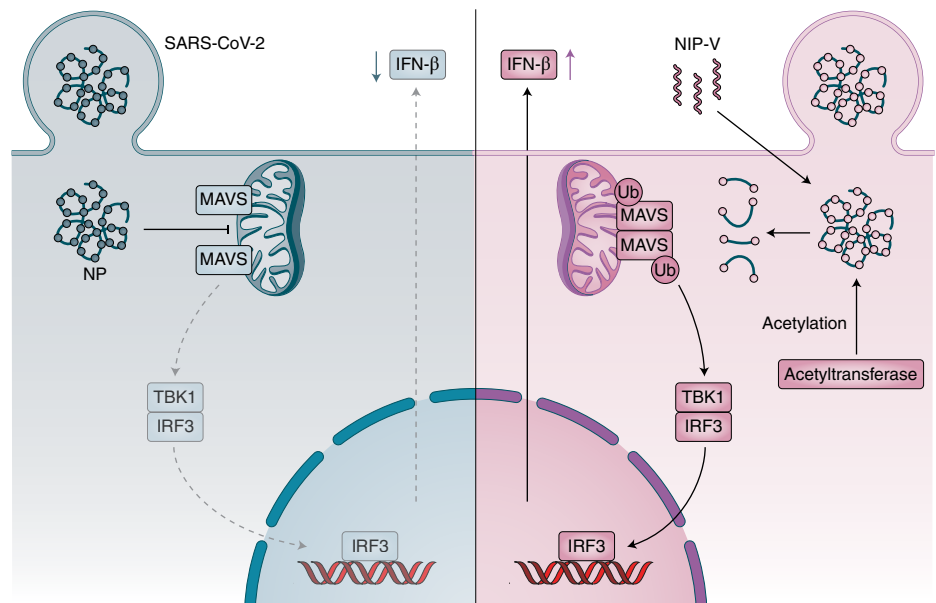


Fig. 1 | SARS-CoV-2 nucleocapsid protein (NP) represses MAVS signalling and IFN- β release.

SARS-CoV-2 NP inhibits polyubiquitination and aggregation of MAVS, repressing the downstream TBK1-IRF3-mediated upregulation and release of IFN- β (left panel). Targeting of NP by host acetyltransferases or interfering peptides such as NIP-V disrupts NP droplet formation, preventing NP-mediated repression of MAVS. This facilitates release of IFN- β , thereby promoting antiviral immunity and viral clearance (right panel).

5'-pppRNA (which acts via RIG-I) and Poly (I:C) (which like double-stranded RNA is detected by TLR3). Mice infected with genetically engineered recombinant VSV containing NP lacking the DD exhibited lower viral load in lungs, liver and spleen compared to VSV containing intact NP (NP-VSV). This was likely due to NP limiting the induction of IFN- β via its DD domain, promoting viral replication.

The authors then examined the signalling pathway driven by RIG-I, which involves the protein kinases TBK1 and IKK ϵ and the transcription factor they activate, IRF3, which regulates IFN transcription. Each of the kinases becomes phosphorylated and then activated; they phosphorylate IRF3, which then dimerises and translocates to the nucleus^{7,8}. NP transfection was shown to reduce phosphorylation of TBK1, IKK ϵ

and IRF3, as well as IRF3 dimerisation and nuclear translocation induced by SeV. These inhibitory effects also required the DD.

MAVS is the key signal for activation of TBK1 and IKK ϵ , and this was examined next. MAVS is known to undergo K63-linked polyubiquitination by the E3 ligase TRIM31, leading to its aggregation and recruitment of TRAF3, TRADD and TRAF6 (ref. ⁹). Co-immunoprecipitation experiments showed a direct interaction between NP and MAVS. The CARD and proline-rich region of MAVS were required for the interaction. NP was shown to disrupt the interaction between MAVS and RIG-I, as well as the interaction between RIG-I and TRIM31. MAVS was also found to undergo phase separation and form micro-sized droplets, a process which was disrupted by NP. NP also blocked K63-linked

polyubiquitination of MAVS by TRIM31, impairing TBK1 and IKK ϵ recruitment to MAVS.

These findings led the authors to examine whether the infected cells might somehow target NP. They demonstrated that NP undergoes acetylation at K375, a residue next to the DD that is conserved in the NP of the related viruses SARS-CoV and MERS-CoV. Acetylation of this amino acid by the host acetyltransferase CREB-binding protein inhibited NP phase transition and prevented NP from blocking MAVS signalling and the IFN- β response. Infected cells therefore appear to possess a mechanism to limit the inhibitory effect of NP, allowing them to counter the infection.

RNA viruses like SARS-CoV-2 have a high mutation rate generating viral variants¹⁰. The authors next characterised some of the reported SARS-CoV-2 variants that exhibited mutations in the NP, including K375Q, K375N and K375E. These changes mimic acetylation of K375 and attenuate phase separation and dimerisation of the NP, impeding the ability of these variant NPs to suppress MAVS aggregation and limit IFN- β production. Mice infected with recombinant VSV expressing these NP variants exhibited lower viral loads compared with those infected with NP-VSV, implying that these variants are less virulent because of the alteration in NP.

Finally, the authors designed interfering peptides targeting the regions of the NP DD involved in the interaction with MAVS. One particular peptide, termed NIP-V, was shown to suppress droplet formation of NP and viral load in vitro and in vivo

and boosted IFN- β signalling in response to NP-VSV infection. Humanised ACE2 transgenic mice were injected with NIP-V and infected with SARS-CoV-2. The peptide led to an elevated antiviral response, as characterised by increased *Ifnb1* and *Isg56* expression in lungs and lower SARS-CoV-2 genomic RNA levels in spleen and liver. The severe lung damage associated with SARS-CoV-2 infection was also reduced by administration of NIP-V in this model.

This work therefore provides a mechanism for immune evasion by SARS-CoV-2. This could be a major mechanism whereby SARS-CoV-2 limits IFN production, as the virus is known to induce low levels of IFNs both in vitro and in vivo¹⁰. The DD in the NP protein promotes liquid phase interaction with MAVS, repressing the IFN response in infected cells. Acetylation of NP by host acetyltransferases or its targeting by interfering peptides boosts anti-viral immunity.

There is complex biochemistry at play here, with the prion-like aggregation of MAVS being controlled by the liquid-liquid phase separation of the SARS-CoV-2 NP. A key question therefore is whether this process would be amenable to therapeutic targeting, most likely with small molecules that will mimic the peptide approach, or perhaps with modified peptides. Information on the control of acetylation of NP might provide additional insights which could lend themselves to other therapeutic approaches. Further investigation is required to study whether this effect of NP is a major mechanism of immune evasion by SARS-CoV-2 relative to other

proteins in SARS-CoV-2. The work might inform studies into other viruses and how they might target MAVS as an immune evasion strategy. There is also interesting information on some of the variants in SARS-CoV-2, in this case variants that are less virulent, which might be useful in the effort to understand the evolutionary trajectory of SARS-CoV-2. This study reveals an elegant mechanism of immune targeting by SARS-CoV-2 which could well lead to a whole new approach to boosting anti-viral immunity against COVID-19. $\square$

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Competing interests

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4-Octyl-itaconate and dimethyl fumarate inhibit COX2 expression and prostaglandin production in macrophages

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Abstract

Prostaglandins (PGs) are important proinflammatory lipid mediators, the significance of which is highlighted by the widespread and efficacious use of non-steroidal anti-inflammatory drugs (NSAIDs) in the treatment of inflammation. 4-Octyl itaconate (4-OI), a derivative of the Krebs cycle-derived metabolite itaconate, has recently garnered much interest as an anti-inflammatory agent. Here we show that 4-OI limits PG production in macrophages stimulated with the Toll-like receptor 1/2 (TLR1/2) ligand Pam3CSK4. This decrease in PG secretion is due to a robust suppression of COX2 expression by 4-OI, with both mRNA and protein levels decreased. Dimethyl fumarate (DMF), a fumarate derivative used in the treatment of multiple sclerosis (MS), with properties similar to itaconate, replicated the phenotype observed with 4-OI. We also demonstrate that the decrease in COX2 expression and inhibition of downstream prostaglandin production occurs in an NRF2-independent manner. Our findings provide a new insight into the potential of 4-OI as an anti-inflammatory agent and also identifies a novel anti-inflammatory function of DMF.

Introduction

Prostaglandins are key lipid mediators, which exert a wide variety of physiological roles. Their synthesis begins with the release of arachidonic acid by cytoplasmic phospholipase A2 (cPLA2) from membrane phospholipids, after which it is converted to prostaglandin H₂ (PGH₂) by the cyclooxygenase enzymes. COX1 is constitutively and ubiquitously expressed and is known to play several homeostatic roles, while COX2 is inducible by inflammatory stimuli including Toll-like receptor (TLR) ligands and cytokines. PGH₂ can then be converted into various prostaglandins (PGs) and thromboxanes by a range of synthase enzymes (1).

The widespread and effective use of NSAIDs, which inhibit COX enzymes, highlights the clinical importance of blocking prostaglandin production in inflammation (2). The proinflammatory effects of PGE₂ in particular are well characterised. The capacity of PGE₂ to induce a wide range of physiological and often pathological phenotypes is largely due to its binding to four different receptors (EP1-4), which vary in their tissue expression

and downstream signal transduction pathways (3). PGE₂ has been shown to activate mast cells (4), T helper 1 (Th1) cells (5, 6) and Th17 cells (7–9). PGE₂ has been implicated in inflammatory diseases such as psoriasis (9) and rheumatoid arthritis (10), in pain responses (11, 12) and recently in aging (13). However PGE₂ has also been shown to exert anti-inflammatory functions, particularly in the environment of the lung (14–16). Another prostaglandin, PGD₂, has been reported to contribute to the allergic response. Engagement of PGD₂ with its receptors facilitates chemotaxis and activation of eosinophils and Th2 cells during allergic disease (17–19). Thromboxanes, which are also downstream of COX activity, promote vasoconstriction and platelet aggregation (20).

Itaconate is a metabolite that has emerged in recent years as an important immunomodulator (21). It is synthesised via the decarboxylation of the Krebs cycle intermediate *cis*-aconitate by the enzyme aconitate decarboxylase 1 (ACOD1, also known as IRG1), encoded by *immune responsive gene 1* (*Irg1*) (22). The expression of IRG1 is predominantly restricted to macrophages and several other immune cell types and is markedly upregulated upon stimulation with TLR ligands (23), thereby leading to an increase in intracellular itaconate. 4-Octyl itaconate (4-OI) is a cell-permeable derivative that is commonly used in the study of itaconate and has been shown to be converted into itaconate intracellularly in macrophages (24).

An important aspect of itaconate biology is that both the endogenous metabolite and 4-OI have been shown to function as cysteine modifiers (25). This cysteine alkylation was originally termed 2,3-dicarboxypropylation and is also referred to as itaconation. This post-translational modification (PTM) is the basis of many of the anti-inflammatory functions associated with itaconate and 4-OI (24, 26–30). 4-OI has been shown to modify cysteine residues on KEAP1 (25), which functions as a negative regulator of the master antioxidant transcription factor NRF2. Modifications of these cysteine residues cause KEAP1 to be degraded, which liberates NRF2 and permits translocation to the nucleus (31), where NRF2 induces transcription of antioxidant genes (32) and inhibits transcription of certain pro-inflammatory cytokines (33). The ability of 4-OI to activate NRF2 has been implicated in several of its anti-inflammatory and protective functions (25, 34–38).

Dimethyl fumarate (DMF) is a derivative of another Krebs cycle metabolite, fumarate, that is clinically approved for the treatment of multiple sclerosis (MS) (39). Like 4-OI, DMF is also a potent cysteine modifier (cysteine alkylation by fumarate is termed succination) (40), and DMF shares some of the same targets as 4-OI, such as GAPDH (27, 41), gasdermin D (28, 30, 42) and importantly KEAP1 (25, 43), meaning that DMF is also a potent NRF2 activator. Therefore these two metabolite derivatives often have similar effects on biological pathways.

The effect of itaconate and its derivatives on PG production has not been studied to date. Here we show that 4-OI greatly reduces PG production in pro-inflammatory macrophages through transcriptional suppression of COX2. We demonstrate that PG production and COX2 transcription is unchanged by the deletion of IRG1, indicating a difference with endogenous itaconate. However, DMF replicates the decrease in COX2 expression and PG secretion observed with 4-OI. Finally we show that 4-OI and DMF reduce COX2 expression

in an NRF2-independent manner. We have therefore uncovered a novel anti-inflammatory role of 4-OI and DMF in macrophages.

Materials and Methods

Reagents

4-Octyl itaconate was initially supplied by Professor Richard Hartley and results were later confirmed with commercially available 4-OI (Sigma Aldrich). Pam3CSK4, dimethyl fumarate, diethyl maleate, indomethacin and NS-398 (Sigma Aldrich) were also used. Antibodies used were anti- β -actin (Sigma Aldrich), anti-COX2 (Abcam), anti-phospho-cPLA2 (Ser505), anti-cPLA2, anti-NRF2, anti-KEAP1, anti-ATF4, anti-phospho-NF- κ B p65 (Ser536), anti-NF- κ B p65, anti-phospho-p38 MAPK (Thr180/Tyr182), anti-p38 MAPK, anti-phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr 204) and anti-p44/42 MAPK (Erk1/2) (Cell Signaling). Anti-mouse IgG and anti-rabbit IgG secondary horseradish peroxidase-conjugated antibodies (Jackson ImmunoResearch) were also used. A PGE₂ ELISA kit was used (Enzo Life Sciences). The Silencer Select siRNAs against NRF2 (s70522), ATF4 (s62689) and Anxa1 (s69299), as well as the Silencer Select negative control, were used (ThermoFisher Scientific).

Mice and BMDM generation

Bone marrow-derived macrophages (BMDMs) were isolated from C57BL/6J mice (Harlan UK). Legs from NRF2 knockout, KEAP1 knockdown and matched wild type mice were kindly provided by Professor Albeena Dinakova-Kostova (University of Dundee). The animals were housed under specific pathogen-free conditions in keeping with Irish and European Union regulations. All experiments carried out required prior ethical approval by Trinity College Dublin Animal Research Ethics Committee and Health Products Regulatory Authority. Mice were euthanised in a carbon dioxide chamber, after which cervical dislocation was used to confirm death. The ends of the tibia, femur and hip bones were cut and the bone marrow was flushed. The cells were then differentiated in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) foetal calf serum (FCS), 1% penicillin/streptomycin and 20% L929 supernatant for six days. After this time the macrophages were counted and replated for use in experiments.

Human PBMC isolation

Thirty mL of whole blood was layered onto 20 mL Lymphoprep (Stemcell Technologies) in a 50 mL conical tube and centrifuged for 20 min at 2,000 rpm with no brake. The peripheral blood mononuclear cells (PBMCs) were then isolated from the middle layer and washed twice in PBS. PBMCs were cultured in RPMI supplemented with 10% (vol/vol) FCS and 1% penicillin/streptomycin for use in experiments.

siRNA transfection

The media on the cells was replaced with 500 μ L DMEM containing no FCS or penicillin/streptomycin. The required amount of Lipofectamine RNAiMAX transfection reagent (Thermo Fischer Scientific) and the siRNAs were diluted in DMEM containing no FCS or penicillin/streptomycin and pre-incubated together for 15 minutes. 500 μ L of this mix was

then added to each well so that the final dilution of Lipofectamine RNAiMAX was 5 μ L/mL and the final concentration of siRNA was 50 nM.

Western blotting

Cells were lysed in sample buffer [0.125 M Tris pH 6.8, 10% (vol/vol) glycerol, 0.02% SDS] and subsequently incubated at 95°C for a duration of five minutes. The samples were resolved on SDS-polyacrylamide gels, alongside the Spectra BR protein ladder (ThermoFisher Scientific) so that proteins could be identified by molecular weight. The protein was then transferred to polyvinylidene fluoride membrane and membranes were blocked for one hour in 5% (w/v) dried milk in Tris-buffered saline/Tween (TBST). The blots were then incubated overnight at 4°C with the primary antibody. Following incubation for one hour with the secondary antibody, as well as three washes in TBST before and after secondary antibody, the blots were developed using chemiluminescent substrate (ThermoFisher Scientific).

Real time PCR

Cells were lysed and the RNA was extracted using the PureLink RNA minikit (Ambion). cDNA was subsequently prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to manufacturer's instructions. Real-time quantitative PCR (qPCR) was then performed with the resulting cDNA using a 7500 Fast Real-Time PCR System with PowerUp SYBR Green Master Mix (Applied Biosystems). All genes were normalised to *Rps18* expression for mouse or *RPS13* for human. The sequences of the primer pairs for murine genes that were used are as follows; *Rps18*, 5'-GGA TGT GAA GGA TGG GAA GT-3' (forward) and 5'-CCC TCT ATG GGC TCG AAT TT-3' (reverse); *Ptgs2*, 5'-CGG ACT GGA TTC TAT GGT GAA A-3' (forward) and 5'-CTT GAA GTG GGT CAG GAT GTA G-3' (reverse); *Ptges*, 5'-GGA AGA AGG CTT TTG CCA ACC-3' (forward) and 5'-CGA AGC CGA GGA AGA GGA AA-3' (reverse); *Nqo1*, 5'-GCT GCA GAC CTG GTG ATA TT-3' (forward) and 5'-ACT CTC TCA AAC CAG CCT TT-3' (reverse); *Il1b*, 5'-GGA AGC AGC CCT TCA TCT TT-3' (forward) and 5'-TGG CAA CTG TTC CTG AAC TC-3' (reverse); *Il6*, 5'-CCA CAG TCC TTC AGA GAG ATA CA-3' (forward) and 5'-CCT TCT GTG ACT CCA GCT TAT C-3' (reverse); *Tnf*, 5'-GCC TCT TCT CAT TCC TGC TT-3' (forward) and 5'-TGG GAA CTT CTC ATC CCT TTG-3' (reverse); *Il10*, 5'-AGG CGC TGT CAT CGA TTT-3' (forward) and 5'-CAC CTT GGT CTT GGA GCT T-3' (reverse); *Tgfb1*, 5'-CGA AGC GGA CTA CTA TGC TAA A-3' (forward) and 5'-TCC CGA ATG TCT GAC GTA TTG-3' (reverse); *Hmox1*, 5'-CCT CAC AGA TGG CGT CAC TT-3' (forward) and 5'-GCT GAT CTG GGG TTT CCC TC-3' (reverse); *Nos2*, 5'-TTC ACC CAG TTG TGC ATC GAC CTA-3' (forward) and 5'-TCC ATG GTC ACC TCC AAC ACA AGA-3' (reverse); *Ccl2*, 5'-GTT GGC TCA GCC AGA TGC A-3' (forward) and 5'-AGC CTA CTC ATT GGG ATC ATC TTG-3' (reverse); *Cd86*, 5'-TCT CCA CGG AAA CAG CAT CT-3' (forward) and 5'-CTT ACG GAA GCA CCC ATG AT-3' (reverse). The sequences of the primer pairs for human genes that were used are as follows; *RPS13*, 5'-TCA CCG TTT GGC TCG ATA TT-3' (forward) and 5'-GGC AGA GGC TGT AGA TGA TT-3' (reverse); *PTGS2*, 5'-TGC GCC TTT TCA AGG ATG GA-3' (forward) and 5'-CCC CAC AGC AAA CCG TAG AT-3' (reverse); *IL1B*, 5'-AGC TGA TGG CCC TAA ACA GA-3' (forward) and 5'-TGT CCA TGG CCA CAA CAA CTG

A-3' (reverse); *IL6*, 5'-TCT GGA TTC AAT GAG GAG ACT TG-3' (forward) and 5'-CTC AAA TCT GTT CTG GAG GTA CT-3' (reverse); *TNF*, 5'-CCA GGG ACC TCT CTC TAA TCA-3' (forward) and 5'-TCA GCT TGA GGG TTT GCT AC-3' (reverse); *IL10*, 5'-CTG TCA TCG ATT TCT TCC CTG T-3' (forward) and 5'-TGC CTT TCT CTT GGA GCT TAT T-3' (reverse); *TGFB1*, 5'-CTG CAC TAT TCC TTT GCC C-3' (forward) and 5'-TCT TCT TCA CTA TCC CCC AC-3' (reverse); *HMOX1*, 5'-CCC AGC CCT ACA CCC GCT AC-3' (forward) and 5'-GGT GGC ACT GGC AAT GTT GG-3' (reverse).

ELISA

Cell supernatants were harvested and PG concentrations were quantified using an ELISA kit for PGE₂ (Enzo Life Sciences), according to the manufacturer's instructions. This item is sold as a PGE₂-specific ELISA kit, however, our data indicated that other COX-derived oxylipins were also likely to be detected with this kit. Therefore we refer to results obtained using this ELISA kit as measuring PGs instead of PGE₂.

Oxylipin analysis

Immediately upon harvesting, cell supernatants were snap frozen in liquid nitrogen. Samples were spiked with 2.1-2.9ng of PGE₂-d4, PGD₂-d4, 20-HETE-d6 and TXB₂-d4 standards (Cayman Chemical). Lipids were extracted by adding a 2.5 ml solvent mixture (1 M acetic acid/isopropanol/hexane; 2:20:30, v/v/v) to 1 ml supernatants in a glass extraction vial and vortexed for 30 sec. 2.5ml hexane was added to samples and after vortexing for 30 seconds, tubes were centrifuged (500 g for 5 min at 4 °C) to recover lipids in the upper hexane layer (aqueous phase), which was transferred to a clean tube. Aqueous samples were re-extracted as above by addition of 2.5 ml hexane, and upper layers were combined. Lipid extraction from the lower aqueous layer was then completed according to the Bligh and Dyer technique. Specifically, 3.75ml of a 2:1 ratio of methanol:chloroform was added followed by vortexing for 30 secs. Subsequent additions of 1.25ml chloroform and 1.25ml water were followed with a vortexing step for 30 seconds, and the lower layer was recovered following centrifugation as above and combined with the upper layers from the first stage of extraction. Solvent was dried under vacuum and lipid extract was reconstituted in 100µl HPLC grade methanol. Lipids were separated by liquid chromatography (LC) using a gradient of 30-100% B over 20 minutes (A: Water:Mob B 95:5 + 0.1% Acetic Acid, B: Acetonitrile: Methanol – 80:15 + 0.1% Acetic Acid) on an Eclipse Plus C18 Column (Agilent), and analysed on a Sciex QTRAP® 6500 LC-MS/MS system. Source conditions: TEM 475°C, IS -4500, GS1 60, GS2 60, CUR 35. Lipids were detected using MRM monitoring with the following parent to daughter ion transitions: PGE₂ and PGD₂ [M-H]- 351.2/271.1, 15-deoxy-PGJ2 [M-H]- 315.2/271.1, TXB₂ [M-H]- 369.2/169.1. Deuterated internal standards were monitored using precursor to product ions transitions of: TXB₂-d4 [M-H]- 373.2/173.1, PGE₂-d4 and PGD₂-d4 [M-H]-355.2/275.1, and 20-HETE-d6 [M-H]- 325.2/281.1. Chromatographic peaks were integrated using Multiquant 3.0.2 software (Sciex). The criteria for assigning a peak was signal:noise of at least 5:1 and with at least 7 points across a peak. The ratio of analyte peak areas to internal standard was taken and lipids quantified using a standard curve made up and run at the same time as the samples.

Statistical Analysis

Statistical significance was established by the one-way or two-way ANOVA methods as indicated in the figure legends. Data are expressed as mean $\pm$ standard error of the mean. Significance was designated as follows: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$. GraphPad Prism version 9 software was used for statistical analysis.

Results

4-OI inhibits Pam3CSK4-induced PG and thromboxane secretion by macrophages

In order to investigate if 4-OI might play a role in modulation of prostaglandins, we treated murine BMDMs with 4-OI prior to stimulation with the TLR1/2 agonist Pam3CSK4 for 24 hours, which strongly induces PGs. We first used a PGE₂ ELISA and observed a strong upregulation of cell supernatant PGs by Pam3CSK4, which was inhibited by 4-OI at concentrations as low as 25 μ M (Figure 1A). 4-OI also reduced Pam3CSK4-induced PG secretion by human PBMCs (Figure 1B). To validate this result, we performed a lipidomic screen of oxylipins in BMDMs using quantitative tandem mass spectrometry (LC/MS/MS). Again, stimulation with Pam3CSK4 lead to a robust increase in PGE₂ that was blocked by 4-OI pretreatment (Figure 1C), however the concentrations were notably lower than those measured by ELISA (Figure 1A). This is likely because of antibody-based measurements for lipid measurements displaying lower specificity and reporting on COX-derived oxylipins more broadly. From the LC/MS/MS screen we also observed that 4-OI potentially inhibited Pam3CSK4-induced increases in PGD₂ (Figure 1D), 15-deoxy-PGJ₂ (Figure 1E) and thromboxane B₂ (TXB₂) (Figure 1F), in addition to PGE₂. Therefore 4-OI was inhibiting all oxylipins downstream of COX activity. As confirmation that the ELISA was detecting COX-derived PGs in general, we found that the COX inhibitor indomethacin completely inhibited the Pam3CSK4-induced lipids from BMDMs detected by the ELISA (Figure 1G). In addition, we used the COX2-specific inhibitor NS-398, which also inhibited all Pam3CSK4-induced lipids detected by the ELISA (Figure 1H), indicating that PG secretion from BMDMs is almost entirely dependent on COX2.

4-OI suppresses Pam3CSK4-induced COX2 expression

As we had observed that 4-OI suppressed all detected oxylipins downstream of COX activity, we next investigated if 4-OI had an effect on COX2 expression. COX2 is potentially upregulated by TLR ligands such as Pam3CSK4, as seen by upregulation of *Ptgs2* transcript, the gene that encodes COX2, as well as strong boost in COX2 protein levels. 200 μ M 4-OI potentially blocked Pam3CSK4-induced *Ptgs2* levels at four hours and eight hours, the timepoints with the greatest induction of *Ptgs2* (Figure 2A). 4-OI also greatly decreased COX2 protein levels at both six hours and 24 hours (Figure 2B). Concentrations as low as 25 μ M 4-OI reduced COX2 protein at 24 hours (Figure 2C), while concentrations 50 μ M or higher decreased *Ptgs2* transcript at six hours (Figure 2D). 4-OI also reduced transcript levels of *PTGS2* in human PBMCs (Figure 2E). The reduction of COX2 expression with 4-OI is likely the reason that 4-OI inhibits PG secretion, as COX2 is often referred to as the rate-limiting enzyme for PG synthesis. 4-OI did not alter the phosphorylation levels of cPLA₂, nor did it affect total cPLA₂ (Figure 2F). 4-OI also had no significant effect on the mRNA levels of *Ptges* (Figure 2E), the gene that encodes prostaglandin E2 synthase

(PGES), the enzyme that catalyses conversion of PGH₂ to PGE₂. Therefore, on the PGE₂ biosynthetic pathway 4-OI seems to specifically suppress COX2 expression, which leads to a decrease in downstream PG production.

As has been previously reported (25, 27), we observed that 4-OI modulated the mRNA expression of several cytokine genes in BMDMs and PBMCs that were induced by Pam3CSK4 stimulation (Supplemental Figure 1A-E, J-N), such as yielding a reduction in *Il1b*, *Il6* and *Il10* transcripts. 4-OI also reduced mRNA levels of *Nos2*, which encodes nitric oxide synthase (Supplemental Figure 1G), and the chemokine *Ccl2* (Supplemental Figure 1H). As expected, 4-OI increased transcript levels of the NRF2-dependent gene *Hmox1* in both BMDMs and PBMCs (Supplemental Figure 1F and O respectively).

Endogenous itaconate does not affect COX2 expression or PG production

We next tested if endogenous itaconate, as well as the derivatised 4-OI, would impact COX2 expression and PG synthesis. For this we used BMDMs lacking *Irg1*, the gene that encodes the enzyme responsible for itaconate synthesis. However, when *Ptgs2* transcript levels between *Irg1*^{+/+} BMDMs and *Irg1*^{-/-} BMDMs are compared, there is no difference in COX2 induction by Pam3CSK4 (Figure 3A). In addition, there were no changes in the COX-derived oxylipins PGE₂ (Figure 3B), PGD₂ (Figure 3C), 15-deoxy-PGJ₂ (Figure 3D) and TXB₂ (Figure 3E) when measured by tandem mass spectrometry. This shows that endogenous itaconate does not affect COX2 expression or PG production.

DMF also reduces Pam3CSK4-induced COX2 expression and PG production

We next tested whether DMF might have similar effects on COX2 and PG production to 4-OI. Pretreatment of BMDMs with concentrations as low as 5 µM of DMF decreased levels of *Ptgs2* transcript (Figure 4A). DMF significantly downregulated *Ptgs2* mRNA levels at four hours and at eight hours (Figure 4B), similar to 4-OI. COX2 protein levels were also attenuated by DMF, using concentrations as low as 5 µM (Figure 4C). DMF also decreased Pam3CSK4-induced PG secretion by BMDMs (Figure 4D). Given that 4-OI and DMF are both potent cysteine modifiers and have very similar effects regarding COX2 expression and PG synthesis, it is likely that they are acting through a shared mechanism.

The 4-OI- and DMF-induced suppression of COX2 and PGs is NRF2-independent

4-OI and DMF are both known to be potent NRF2 activators through their ability to modify crucial cysteine residues on KEAP1, a negative regulator of NRF2. We also tested if another NRF2 activating compound, diethyl maleate, DEM, (44) would also modulate COX2. Indeed pretreatment of BMDMs with DEM resulted in decreased COX2 protein levels (Supplemental Figure 2A) and transcription (Supplemental Figure 2B), in addition to suppression of PG secretion (Supplemental Figure 2C). This indicated that perhaps NRF2 was somehow involved in the modulation of COX2 and PGs. To test this, we used BMDMs isolated from wild-type, NRF2 knockout and KEAP1 knockdown mice. As expected, expression of the NRF2-dependent gene *Nqo1* was completely ablated in the NRF2 knockout BMDMs whereas the KEAP1 knockdown BMDMs displayed enhanced *Nqo1* transcription compared to wild type cells (Figure 5A and B, Supplemental Figure 2D). Both 4-OI and DMF maintained the capacity to suppress *Ptgs2* transcription in

NRF2 knockout and KEAP1 knockdown BMDMs (Figure 5C and D respectively). 4-OI and DMF also reduced COX2 protein levels in NRF2 knockout and KEAP1 knockdown BMDMs (Figure 5E and F respectively). NRF2 knockdown using siRNA also did not abolish the capacity of 4-OI to reduce COX2 expression (Supplemental Figure 3A). Furthermore, 4-OI and DMF both blocked PG production in all genotypes (Figure 5G and H respectively). It is also worth noting that cells lacking NRF2 have lower levels of COX2 and PGs, whereas the KEAP1 knockdown cells, which exhibit augmented NRF2 activation, display elevated COX2 expression and PG production (Figure 5E-H). Interestingly, DEM, which is considered to be a well characterised NRF2 activator, could also still block *Ptgs2* transcription in NRF2 knockout and KEAP1 knockdown BMDMs (Supplemental Figure 2E), indicating that its ability to reduce COX2 is separate to its NRF2-activating function. We also observed that 4-OI had no effect on NF- κ B p65 phosphorylation, p38 phosphorylation or ERK phosphorylation (Supplemental Figure 3B), all of which are signalling pathways that are known to modulate *ptgs2* transcription. We also investigated if ATF4 was involved in the observed effect, given that ATF4 was recently shown to bind directly to the *ptgs2* promoter and induce its transcription (45). However, 4-OI actually increased ATF4 expression and ATF4 silencing had no effect on the inhibition of COX2 by 4-OI (Supplemental Figure 3C). We also tested if the effect of 4-OI on COX2 might be dependent on annexin A1, which has been shown to be modified by itaconate and itaconate derivatives (25, 28, 29) and is known to function as a negative regulator of cPLA2, which catalyses the first step of PG biosynthesis. However, silencing annexin A1 did not alter the inhibition of PG production by 4-OI (Supplemental Figure 3D). These results indicate that the capacity of 4-OI and DMF to suppress COX2 expression and downstream PG production is independent of NRF2 activation and other known signals that regulate COX2.

Discussion

The itaconate derivative 4-OI has recently garnered much attention as an immunomodulator. The findings of this study suggest a role for 4-OI in the inhibition of PG production in macrophages activated with the TLR1/2 ligand Pam3CSK4. We show that 4-OI potentially reduces several COX-derived oxylipins and provide evidence that 4-OI suppresses COX2 transcription. We demonstrate that while endogenous itaconate derived from IRG1 activity does not affect COX2 levels, DMF attenuated COX2 expression and PG secretion in a similar manner to 4-OI. This implies that a cysteine modification may be involved but we also provide evidence that suggests that the effect of 4-OI and DMF on PG production is not via KEAP1 degradation and NRF2 activation. Further work is required in order to fully elucidate the mechanism by which 4-OI and DMF impair COX2 transcription.

Our knowledge of how Krebs cycle activity impacts inflammation has been rapidly expanding over the last number of years. Some Krebs cycle intermediates are known to exert proinflammatory actions, such as succinate which has been shown to stabilise HIF-1 α , thereby upregulating IL-1 β transcription (46). Succinate has also been reported to exacerbate certain inflammatory diseases, such as arthritis (47) and type 2 diabetes (48). Another Krebs cycle intermediate, citrate, has also been shown to alter the inflammatory response. The mitochondrial export, in addition to the breakdown of citrate, have been reported to be essential for nitric oxide and prostaglandin production (49, 50). Several anti-inflammatory

roles have recently been described for both itaconate (25, 30, 51) and fumarate (41, 42) and here we report a novel function for derivatives of these two Krebs cycle metabolites in macrophages.

This work also highlights the importance of bearing in mind that metabolite derivatives do not always truly represent the action of the corresponding endogenous metabolites. In the case of 4-OI, the use of this derivative replicates the biological effects of endogenous itaconate in a number of cases such as the inactivation of the NLRP3 inflammasome (24) and impairment of glycolysis (27). However there are also incidences where 4-OI and endogenous itaconate differ in their effects, such as type I interferon (IFN) production, which is boosted by endogenous itaconate (52) but inhibited by 4-OI (25). While itaconate has also been shown to modify proteins in the same manner as 4-OI, the targets are not always the same. Our previous study (25) found that, while there were some overlapping proteins modified by both 4-OI and endogenous itaconate, many targets were mutually exclusive. This could potentially be due to differences in electrophilicity between itaconate and 4-OI.

Although DMF is known to be a potent NRF2 activator (43, 53), NRF2-independent anti-inflammatory functions of DMF have begun to emerge, such as the impairment of glycolysis through GAPDH succination (41) and inhibition of pyroptosis via gasdermin D (42). It is also true for 4-OI that a number of its currently known anti-inflammatory effects are NRF2-dependent (25, 34, 35), although others are not (24, 27, 30, 51). Interestingly, DEM, which is predominantly used as an experimental tool to pharmacologically activate NRF2, also suppressed COX2 expression in an NRF2-independent manner. As DEM has been shown to modify cysteines on KEAP1 in a similar way to 4-OI and DMF (44), perhaps DEM also possesses the capacity to modify other reactive cysteines in the cell with further reaching implications. An observation that adds complexity to this system is that 15-deoxy- $\Delta^{12,14}$ -PGJ2, which is downstream of COX activity and therefore would be reduced by 4-OI, has been shown to activate NRF2 (54). Nonetheless, our data reveals another NRF2-independent anti-inflammatory role for DMF, 4-OI and even DEM.

There is also the possibility that the effect of 4-OI on COX2 and prostaglandins will contribute to the attenuation of other inflammatory markers. For example, macrophages express EP receptors (55) and therefore PGE₂ can signal in an autocrine manner. We have previously shown that endogenous PGE₂ acting via the EP2 receptor is required for induction of pro-IL-1 β (56). Hence the inhibition of PGE₂ secretion from the macrophage and subsequent binding to EP2 could potentially contribute to the 4-OI-induced decrease in pro-IL-1 β that has been reported (25). PGE₂ has also been shown to augment IL-6 production in macrophages (57, 58), another cytokine that is blocked by 4-OI treatment (25). Therefore inhibition of PGE₂ by 4-OI could potentially be a factor in the downregulation of IL-6. Treatment of macrophages with PGE₂ was shown to boost production of IL-10 in macrophages (59), an anti-inflammatory cytokine that is also downregulated by 4-OI. It is also conceivable that a reduction in PGs secreted by macrophages could contribute to the anti-inflammatory effects of 4-OI during *in vivo* models (24, 25, 27). Conversely, the capacity of 4-OI to attenuate the production of other proinflammatory mediators could

potentially affect the induction of COX2. For example, IL-1 β and nitric oxide, both of which are inhibited by 4-OI (25), have been reported to contribute to COX2 induction (60, 61).

Our results therefore identify 4-OI and DMF as inhibitors of prostaglandin synthesis in macrophages. This work further highlights the potential of 4-OI as a therapeutic anti-inflammatory treatment and supplements the current knowledge of the anti-inflammatory effects of DMF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points

- 4-OI reduces COX2 expression and inhibits prostaglandin production in macrophages
- DMF also attenuates COX2 expression in macrophages and decreases prostaglandins
- The 4-OI- and DMF-induced decrease in COX2 and prostaglandins is NRF2-independent

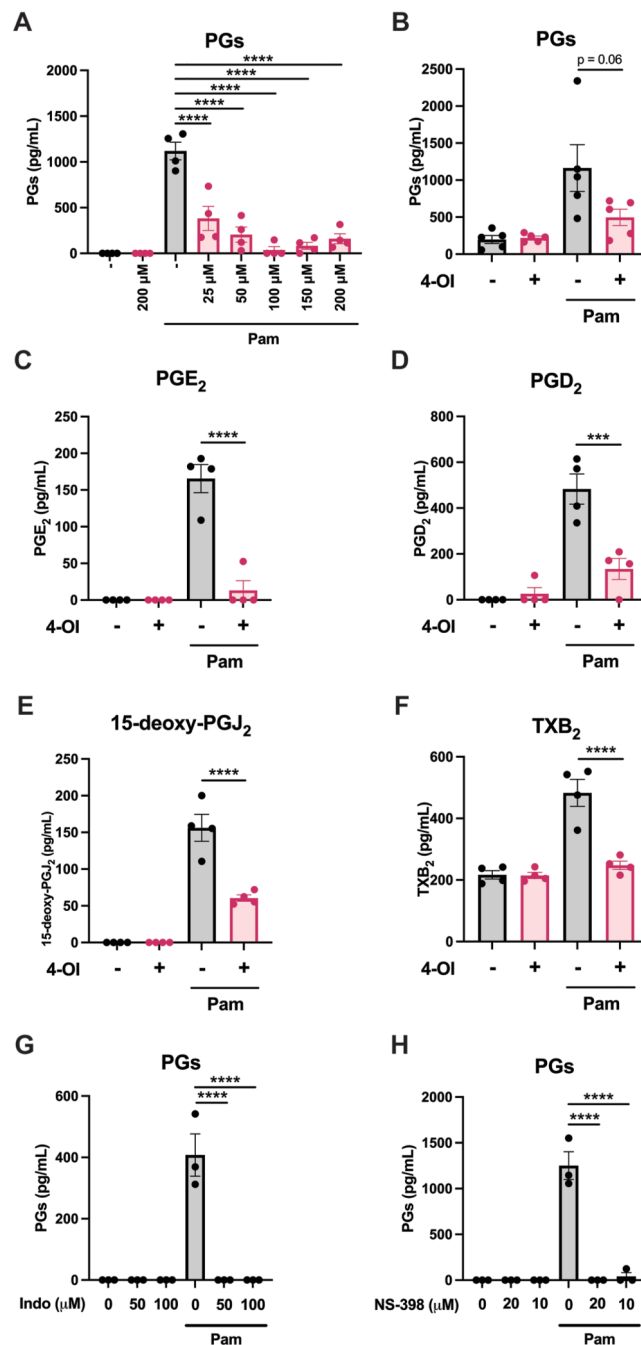


Figure 1. 4-OI inhibits Pam3CSK4-induced prostaglandin production.

(A) BMDMs were pretreated with various concentrations of 4-OI (25-200 μ M) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA (n=4). (B) Human PBMCs were pretreated with 200 μ M 4-OI for two hours prior to stimulation with Pam3CSK4 (1 μ g/mL) for 24 hours. Supernatants were analysed for PG concentration by ELISA (n=5). (C-F) BMDMs were pretreated with 200 μ M 4-OI for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. Supernatants were subsequently

analysed by tandem mass spectrometry in order to determine (C) PGE₂, (D) PGD₂, (E) 15-deoxy-PGJ₂ and (F) TXB₂ concentrations (n=4). (G) BMDMs were pretreated with 50 μ M or 100 μ M indomethacin for one hour prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA (n=3). (H) BMDMs were pretreated with 10 μ M or 20 μ M NS-398 for one hour prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA (n=3). Data are mean $\pm$ S.E.M. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.

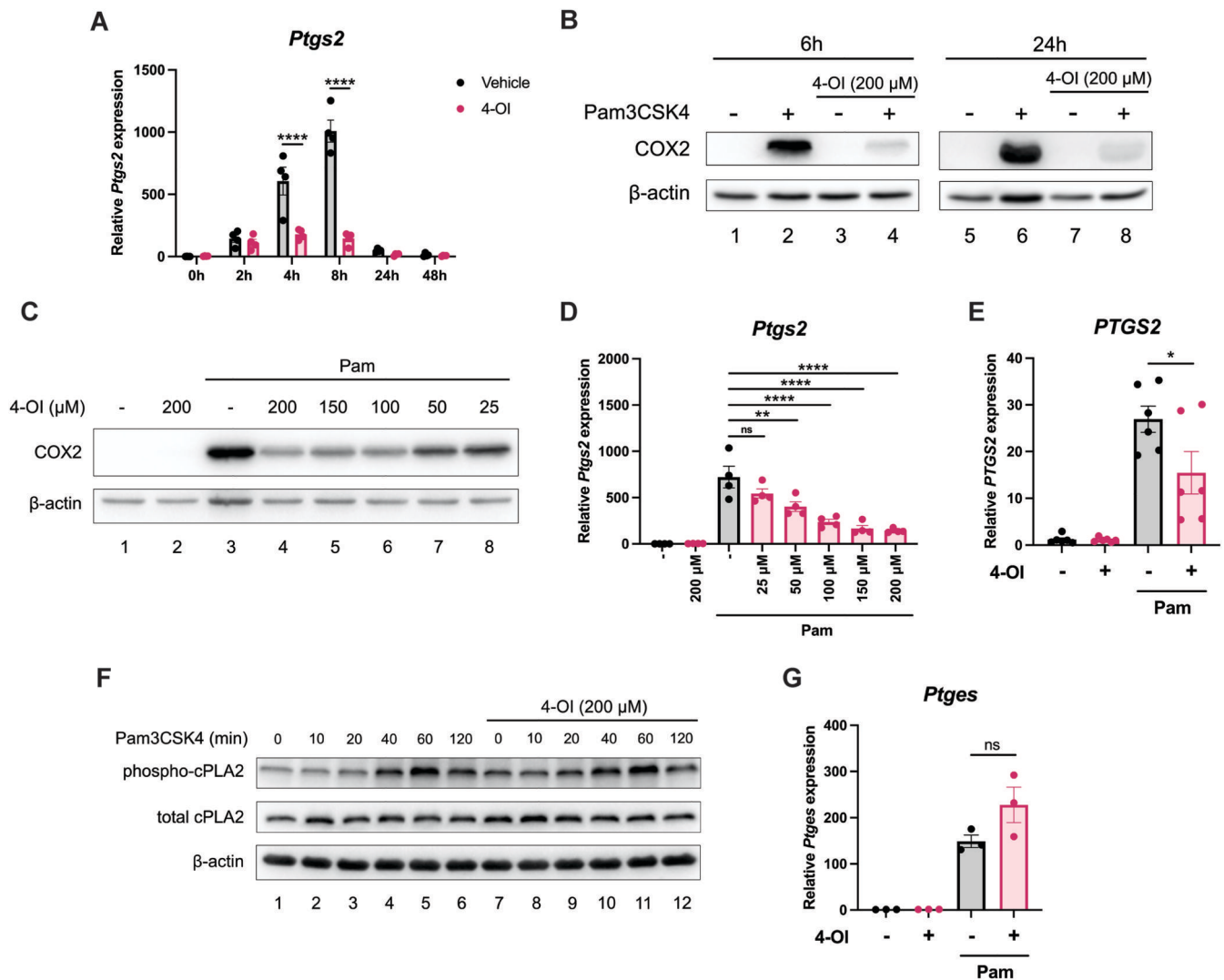


Figure 2. 4-OI inhibits Pam3CSK4-induced COX2 expression.

(A) BMDMs were pretreated with 200 μ M 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for 2, 4, 8, 24 or 48 hours. The cells were lysed, mRNA was extracted and *Ptgs2* expression was measured by qPCR (n=4). (B) BMDMs were pretreated with 200 μ M 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for 6 hours or 24 hours. COX2 expression was analysed by Western blotting (n=6). (C) BMDMs were pretreated with various concentrations of 4-OI (25-200 μ M) prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. COX2 expression was analysed by Western blotting (n=4). (D) BMDMs were pretreated with various concentrations of 4-OI (25-200 μ M) prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. After cell lysis, mRNA was extracted and *Ptgs2* expression was measured by qPCR (n=4). (E) PBMCs were pretreated with 200 μ M 4-OI for two hours prior to stimulation with Pam3CSK4 (1 μ g/mL) for six hours. The cells were then lysed, mRNA was extracted and *PTGS2* expression was measured by qPCR (n=6). (F) BMDMs were pretreated with 200 μ M 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for various timepoints (10-120 minutes). Phospho-cPLA2 and total cPLA2

expression was analysed by Western blotting (n=3). (G) BMDMs were pretreated with 200 μ M 4-OI prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. After cell lysis, mRNA was extracted and *Ptges* expression was measured by qPCR (n=3). Data are mean $\pm$ S.E.M. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001, ns = non-significant, by one-way ANOVA.

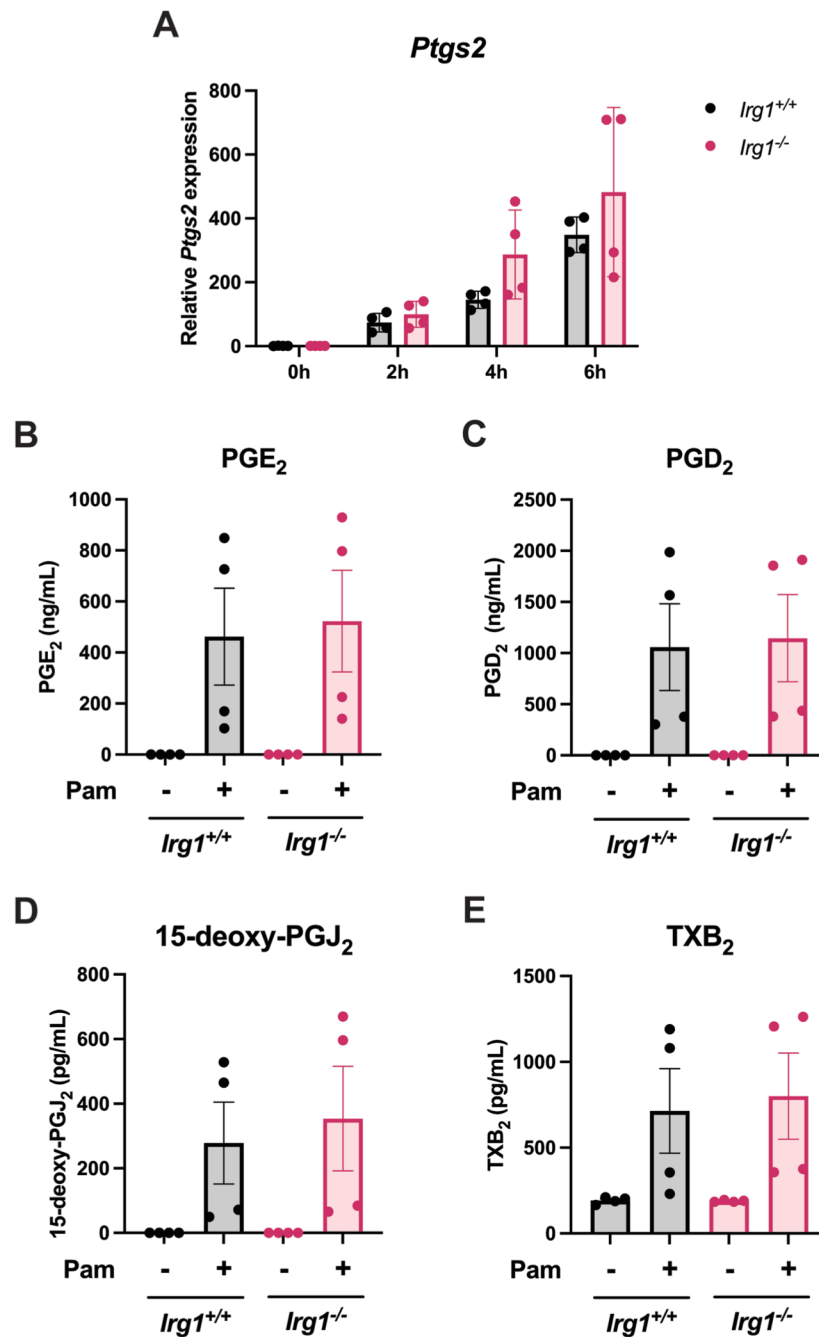


Figure 3. Endogenous itaconate does not affect COX2 expression or prostaglandin production. (A) BMDMs from *Irg1*^{+/+} and *Irg1*^{-/-} mice were stimulated with Pam3CSK4 (100 ng/mL) for two, four or six hours. The cells were lysed and mRNA extracted in order to quantify *Ptgs2* by qPCR (n=4). (B-E) BMDMs from *Irg1*^{+/+} and *Irg1*^{-/-} mice were stimulated with Pam3CSK4 (100 ng/mL) for 24 hours. The cell supernatants were then analysed by tandem mass spectrometry in order to determine (B) *PGE*₂, (C) *PGD*₂, (D) 15-deoxy-PGJ₂ and (E) TXB₂ concentrations (n=4). Data are mean ± S.E.M. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA, or two-way ANOVA for (A).

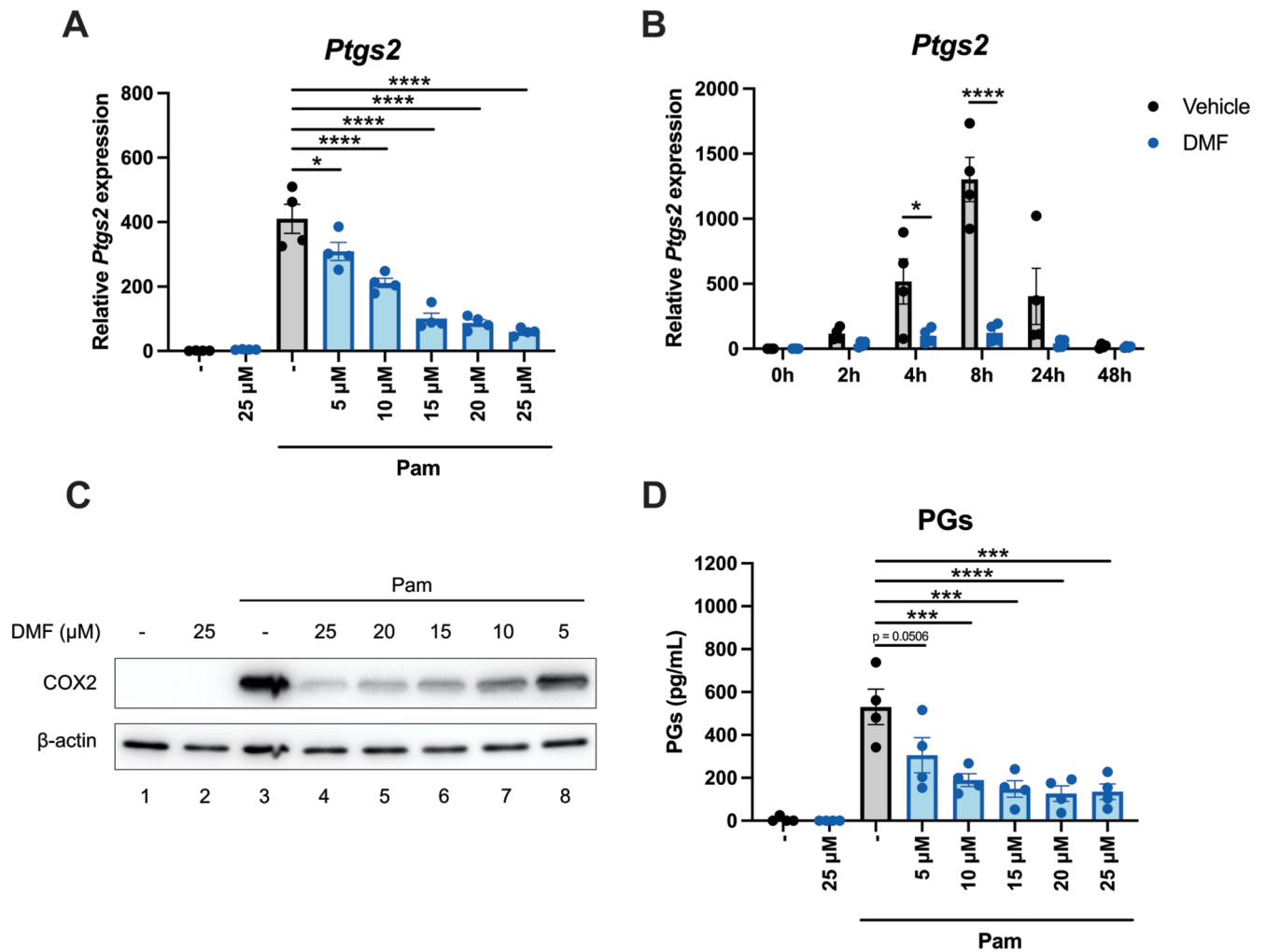


Figure 4. DMF decreases COX2 expression and prostaglandin production.

(A) BMDMs were pretreated with various concentrations of DMF (5-25 μ M) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for four hours. After cell lysis, mRNA was extracted and *Ptgs2* levels were quantified by qPCR (n=4). (B) BMDMs were pretreated with 25 μ M DMF for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 2, 4, 8, 24 or 48 hours. After cell lysis, mRNA was extracted and *Ptgs2* levels were quantified by qPCR (n=4). (C) BMDMs were pretreated with various concentrations of DMF (5-25 μ M) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. COX2 expression was analysed by Western blotting (n=4). (D) BMDMs were pretreated with various concentrations of DMF (5-25 μ M) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. The PG concentrations in the resulting supernatants were subsequently quantified by ELISA (n=4). Data are mean $\pm$ S.E.M. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.

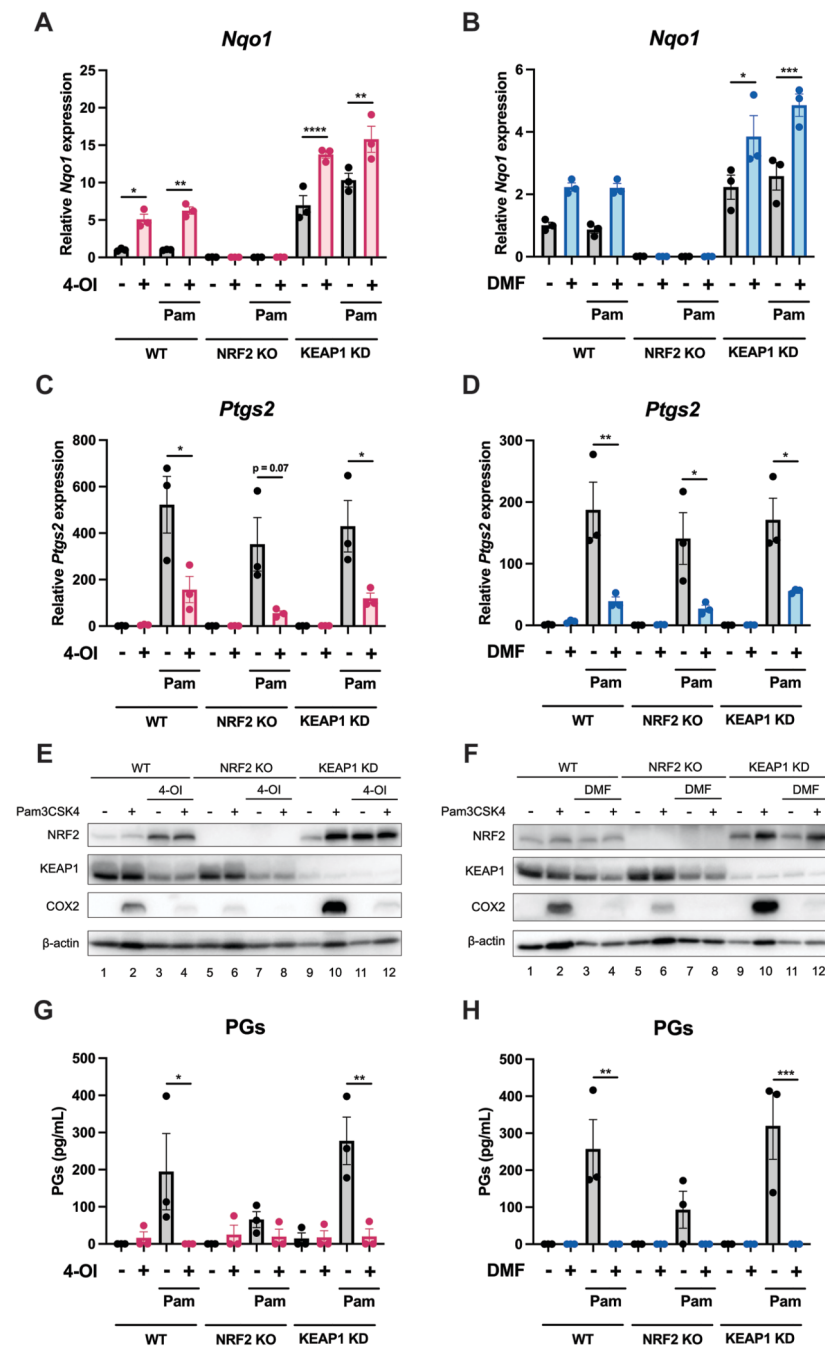


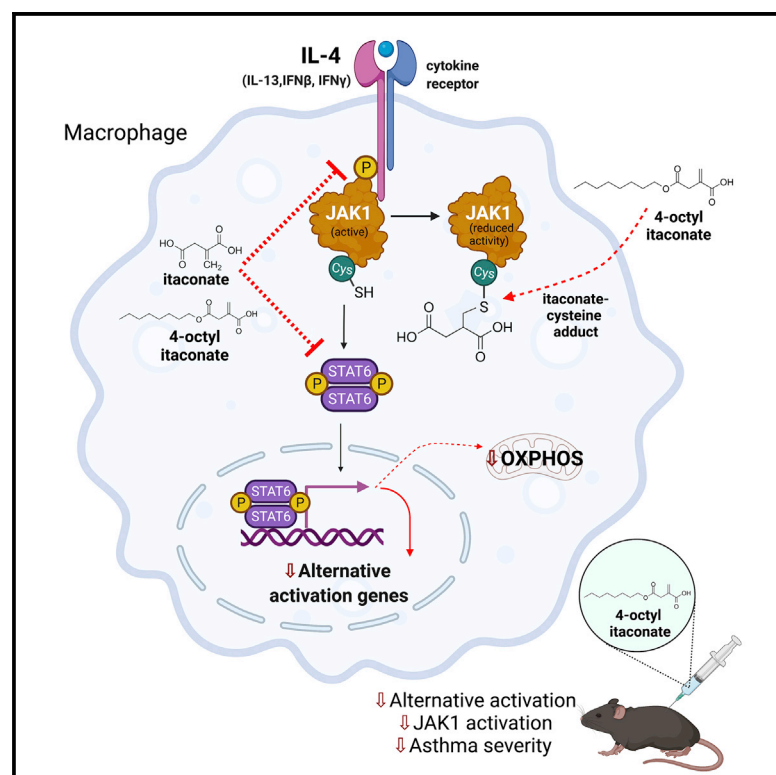
Figure 5. The capacity of 4-OI and DMF to reduce COX2 expression and prostaglandin production is not NRF2-dependent.

(A-D) BMDMs from wild-type, NRF2 knockout and KEAP1 knockdown mice were pretreated with 200 μ M 4-OI (A and C) or 25 μ M DMF (B and D) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for six hours. The cells were lysed, mRNA was extracted and *Nqo1* expression (A and B) and *Ptgs2* expression (C and D) were quantified by qPCR (n=3). (E-H) BMDMs from wild-type, NRF2 knockout and KEAP1 knockdown mice were pretreated with 200 μ M 4-OI (E and G) or 25 μ M DMF (F and H) for two hours prior to stimulation with Pam3CSK4 (100 ng/mL) for 24 hours. COX2 expression

was analysed by Western blotting (E and F) (n=3). The supernatants were analysed for PG concentration by ELISA (G and H) (n=3). Data are mean $\pm$ S.E.M. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001 by one-way ANOVA.

Itaconate and itaconate derivatives target JAK1 to suppress alternative activation of macrophages

Graphical abstract



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In brief

The wide-reaching roles of the immunomodulatory metabolite itaconate are incompletely understood. Here, Runtsch et al. demonstrate that itaconate and derivatives directly modify JAK1 and are JAK1 inhibitors during macrophage alternative activation. These discoveries provide a novel basis for itaconate and derivatives as therapies in type-2-driven diseases, including asthma.

Highlights

- Itaconate reduces M2 macrophage polarization, JAK1/STAT6 activation, and OXPHOS
- Itaconate derivative OI is a JAK1 inhibitor in type 1 and 2 cytokine pathways
- Cysteine residues on JAK1 are directly modified by itaconate derivatives
- Itaconate derivative OI inhibits JAK phosphorylation and asthma severity *in vivo*



Article

Itaconate and itaconate derivatives target JAK1 to suppress alternative activation of macrophages

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SUMMARY

The Krebs cycle-derived metabolite itaconate and its derivatives suppress the inflammatory response in pro-inflammatory “M1” macrophages. However, alternatively activated “M2” macrophages can take up itaconate. We therefore examined the effect of itaconate and 4-octyl itaconate (OI) on M2 macrophage activation. We demonstrate that itaconate and OI inhibit M2 polarization and metabolic remodeling. Examination of IL-4 signaling revealed inhibition of JAK1 and STAT6 phosphorylation by both itaconate and OI. JAK1 activation was also inhibited by OI in response to IL-13, interferon- β , and interferon- γ in macrophages and in T helper 2 (Th2) cells. Importantly, JAK1 was directly modified by itaconate derivatives at multiple residues, including cysteines 715, 816, 943, and 1130. Itaconate and OI also inhibited JAK1 kinase activity. Finally, OI treatment suppressed M2 macrophage polarization and JAK1 phosphorylation *in vivo*. We therefore identify itaconate and OI as JAK1 inhibitors, suggesting a new strategy to inhibit JAK1 in M2 macrophage-driven diseases.

INTRODUCTION

Itaconate has recently emerged as an important regulator of immunity and inflammation, with studies highlighting its immunomodulatory effects in multiple contexts (Hooftman and O'Neill, 2019). It is one of the most highly upregulated metabolites during pro-inflammatory (M1) activation of macrophages (Hooftman and O'Neill, 2019; Lampropoulou et al., 2016; Mills et al., 2018; O'Neill and Artyomov, 2019; Weiss et al., 2018). M1 macrophages activated by lipopolysaccharide (LPS), viral components, or interferon gamma (IFN- γ) express high levels of the gene *Irg1*, also known as *Acod1*, which encodes the enzyme aconitate decarboxylase (CAD) (Hooftman and O'Neill, 2019; Lampropoulou et al., 2016; Mills et al., 2018). This enzyme

uses the Krebs cycle intermediate *cis*-aconitate as a substrate and converts it into itaconate, and in this way, itaconate accumulates intracellularly and extracellularly (Lampropoulou et al., 2016). In macrophages, itaconate and itaconate derivatives have been shown to inhibit the Krebs cycle enzyme succinate dehydrogenase (SDH) and thereby block reactive oxygen species (ROS) production from complex I, leading to inhibition of hypoxia-inducible factor (HIF)-1 α activity and interleukin (IL)-1 β production (Lampropoulou et al., 2016; Mills et al., 2018). Itaconate and derivative compounds have also been reported to activate nuclear factor erythroid 2-related factor 2 (NRF2), leading to a boost in the antioxidant response, and to drive activating transcription factor 3 (ATF3) expression, which limits production of cytokines such as IL-6 (Bambouskova et al., 2018; Mills et al.,



2018). Additionally, itaconate has recently been shown to inhibit the NLR family pyrin domain containing 3 (NLRP3) inflammasome (Hooftman et al., 2020). Overall, evidence so far indicates that itaconate is an immunomodulatory metabolite whose targets are still being uncovered.

Itaconate and its derivatives are potent cysteine modifiers, due to its nature as an α,β -unsaturated dicarboxylic acid (Hooftman and O'Neill, 2019; Hooftman et al., 2020; Mills et al., 2018; Qin et al., 2019, 2020). This is similar to fumarate and its derivative dimethyl fumarate (DMF), which also modify cysteines, with DMF being an approved treatment for multiple sclerosis and psoriasis (Blewett et al., 2016). Itaconate and derivatives are electrophilic, and can react with thiol groups with varying electrophilicity (Bambouskova et al., 2018; Li et al., 2020). This is an alkylation reaction and is termed 2,3-dicarboxypropylation or itaconation (Hooftman and O'Neill, 2019; Mills et al., 2018; Qin et al., 2020; Zhang et al., 2021). Many studies of itaconate involve the use of the derivative compound 4-octyl itaconate (OI) because OI has enhanced cell permeability compared with itaconic acid itself, similar thiol reactivity to itaconate versus other derivatives, and is converted into itaconate intracellularly (Hooftman and O'Neill, 2019; Hooftman et al., 2020; Li et al., 2020). This compound and similar itaconate derivatives show therapeutic promise in a number of inflammatory and infectious disease models, including pulmonary fibrosis, multiple sclerosis, sepsis, SARS-CoV-2 infection, and cardiovascular disease (Hooftman and O'Neill, 2019; Jaiswal et al., 2021; Kuo et al., 2020; Mills et al., 2018; Ogger et al., 2020; Olagnier et al., 2020; Yang et al., 2020). These investigations have highlighted the potential utility of itaconic acid, OI, or similar derivatives as potential therapies for the treatment of inflammatory diseases.

While many studies have explored the roles of itaconate and derivatives in pro-inflammatory, M1 macrophages, little is known about the effects this metabolite has in other macrophage subtypes. Macrophage polarization is a diverse and complex spectrum, with many macrophages switching or adopting mixed phenotypes depending on the cytokine milieu and environmental cues (Italiani and Boraschi, 2014; Martinez and Gordon, 2014; Sica and Mantovani, 2012). In general, so-called M1 polarization gives rise to pro-inflammatory responses via enhanced glycolysis, while M2, or alternatively activated, macrophages undergo enhanced oxidative phosphorylation (OXPHOS). M2 macrophages (specifically M2a) are activated by the cytokines IL-4 and IL-13, which signal through the Janus kinase 1 (JAK1) and Signal transducer and activator of transcription 6 (STAT6) pathway. M2 macrophages express signature genes such as peroxisome proliferator-activated receptor γ (*Pparg*), mannose receptor (*Mrc1*/CD206), arginase (*Arg1*), galactose-binding lectin (*Clec10a*/CD301), and resistin-like alpha (*Retnla*/*Fizz1*), which promote wound healing and tissue remodeling (Langston et al., 2017; Viola et al., 2019). Metabolically, alternatively activated macrophages utilize glucose, glutamine, and fatty acids as fuel sources to feed OXPHOS, which is required for their polarization and function (Langston et al., 2017; Viola et al., 2019; Wang et al., 2018). While the current paradigm of M2 macrophages is that they are more anti-inflammatory, they also play a role in promoting certain immunologic diseases. For example, type 2 inflammatory responses characteristic of M2 macrophages and T

helper 2 (Th2) cells are found in lung pathologies such as asthma, allergy, and fibrosis (Chávez-Galán et al., 2015).

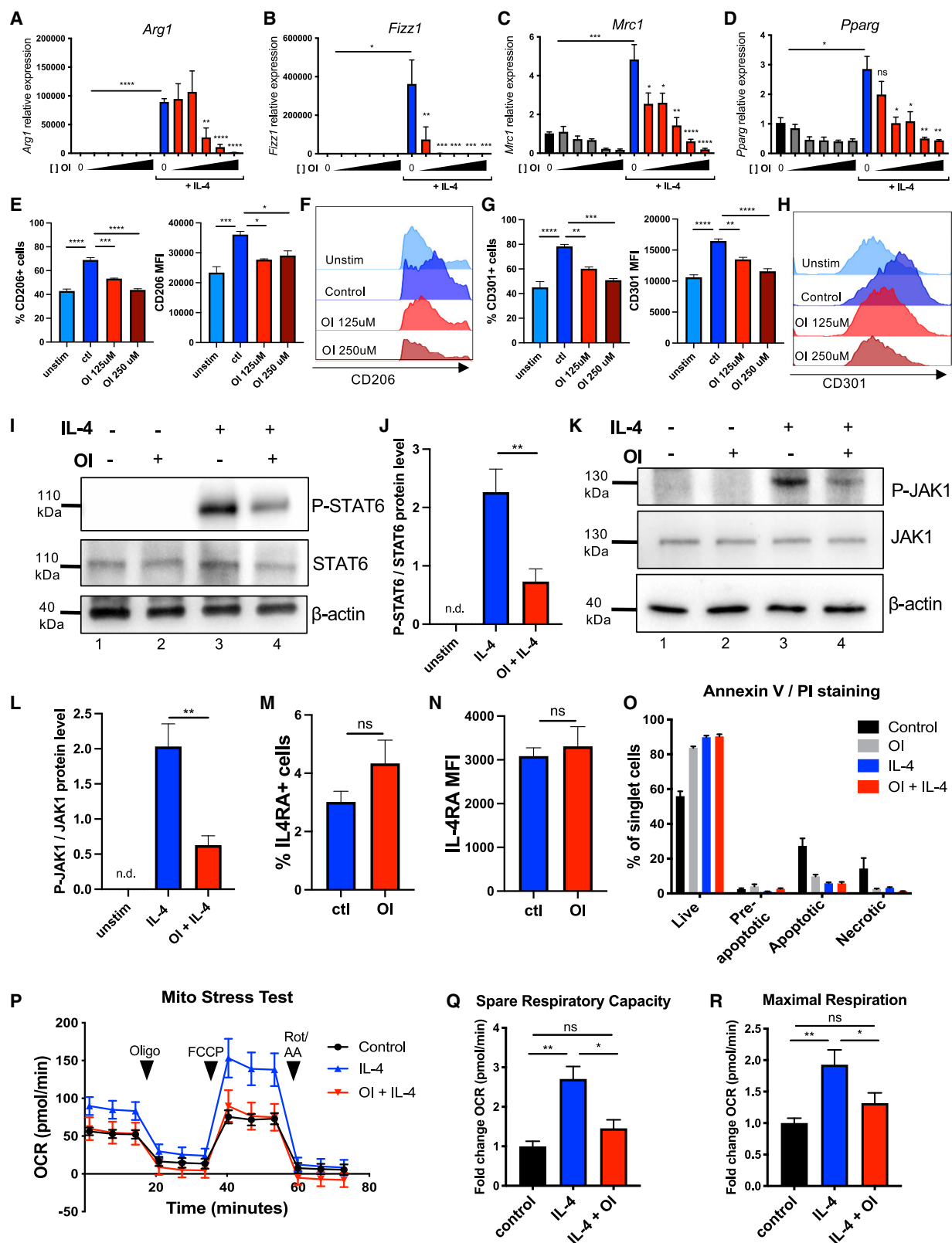
Recently, Nelson and colleagues found that loss of PPAR γ resulted in enhanced IRG1 and itaconate levels, demonstrating that this important M2-promoting regulator is acting as a brake on itaconate, which might therefore be an M2 macrophage inhibitor (Nelson et al., 2018; O'Neill and Artyomov, 2019). In addition, the M2-promoting microRNA miR-93 targets IRF9, which in turn represses IRG1 and itaconate levels during macrophage alternative activation (Ganta et al., 2017; O'Neill and Artyomov, 2019). Both of these studies show that M2 macrophages have mechanisms in place to repress IRG1, suggesting that itaconate may limit alternative activation and must be attenuated for M2 polarization. Intriguingly, M2 macrophages have also been shown to take up itaconate (Puchalska et al., 2018). We therefore assessed the impact of itaconate on alternatively activated M2 macrophages, to understand how their polarization might be affected in the presence of this metabolite. Our results indicate that itaconate and its derivative OI block M2 polarization via inhibition of the JAK1/STAT6 signaling pathway downstream of IL-4. By directly targeting JAK1, itaconate and OI are thus identified as JAK1 inhibitors, which could ultimately have utility in the treatment of type 2 immune-mediated diseases, such as allergy, asthma, and fibrosis.

RESULTS

Itaconate derivative 4-octyl itaconate blocks M2 polarization

We first investigated the effect of OI on IL-4-activated "M2" murine bone-marrow-derived macrophages (BMDMs). OI reduced the IL-4-induced expression of the M2-signature genes *Arg1*, *Fizz1*, *Mrc1*, and *Pparg* in a dose-dependent manner in BMDMs (Figures 1A–1D). There was also a significant reduction in both the percentage and mean fluorescence intensity (MFI) of the M2 surface markers CD206 (mannose receptor) (Figures 1E and 1F) and CD301 (C-type lectin domain family 10 member A) (Figures 1G and 1H) with OI treatment, compared with control.

One of the key "master" transcription factors activated in M2 macrophages is STAT6, which is required for the alternative activation program. We also observed a decrease in phosphorylated STAT6 in BMDMs treated with OI and IL-4 compared with those treated with IL-4 alone for 24 h (Figure 1I, lane 3 versus 4, and Figure 1J). OI repressed STAT6 phosphorylation at early time points up to 30 min post-IL-4 stimulation (Figure S1A, lanes 2 versus 3, 4 versus 5, and 6 versus 7). Upstream of STAT6 activation, and immediately downstream of IL-4 receptor stimulation, the tyrosine kinase JAK1 is phosphorylated, which is also required for M2 macrophage activation (Byles et al., 2013; Chawla, 2010). OI blocked phosphorylation of JAK1 after 1 h of IL-4 treatment (Figure 1K, lane 3 versus 4, and Figure 1L), and also at earlier times (5 and 10 min after IL-4 stimulation) (Figure S1B, lane 3 versus 4, 5 versus 6), indicating that OI-mediated inhibition of the M2 program happens early in the IL-4/JAK1/STAT6 signaling cascade. Reduction of phospho-JAK1 levels by OI was maintained at time points up to 24 h (data not shown). We next examined signaling further upstream, measuring expression of the cytokine receptor subunit IL-4 receptor alpha (IL4RA), which IL-4 signals through to activate JAK1 and



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STAT6. Flow cytometric analysis of IL4RA surface expression on IL-4-treated BMDMs showed no change in the percentage or per-cell basis expression of this protein, comparing OI-treated cells to control (Figures 1M and 1N). These data suggest that OI inhibits M2 macrophage activation by blocking JAK1 phosphorylation.

OI treatment also inhibited Suppressor of cytokine signaling 1 (Socs1), another M2 marker in the JAK1/STAT6 signaling pathway (Langston et al., 2017; Viola et al., 2019) (Figure S1C, lane 2 versus 3), further indicating inhibition of IL-4 signaling. Because our results show inhibition of many of the key components of the M2 macrophage program, we measured cell death during IL-4 and OI treatment to test if the inhibitory effects of OI were due to toxicity. Annexin V and propidium iodide (PI) staining showed that OI does not induce apoptosis or necrosis in unstimulated and IL-4-activated macrophages (Figures 1O and S1G). Taken together, these data indicate an inhibitory effect of OI on early events in IL-4 signaling.

Since OI is known to activate Nrf2 (Mills et al., 2018), we measured activation of this transcription factor as a positive control to ensure efficacy of OI in this model. Our results show that treatment with OI resulted in the induction of Nrf2 protein in both M0 and M2 BMDMs (Figure S1D, lane 3 and 4). Nrf2 downstream target genes *Gclm*, *Nqo1*, and *Hmox1* also had enhanced expression upon addition of increasing doses of OI in both control and IL-4-stimulated BMDMs (Figure S1E). These results confirm that OI is not repressing gene expression globally in M2 macrophages. However, to examine OI's dependency on Nrf2 in this context, OI was tested in Nrf2-null (*Nfe2l2*^{-/-}) M2 macrophages. OI significantly inhibited expression of *Arg1*, *Fizz1*, and *Pparg* upon IL-4 stimulation in *Nfe2l2*^{-/-} BMDMs,

similar to wild-type (WT) macrophages (Figure S1F). These results demonstrate that Nrf2 is not required for the inhibitory effect of OI on M2 macrophage activation.

Next, we examined the effect of OI on metabolic reprogramming in M2 macrophages, which require OXPHOS for their polarization (Langston et al., 2017; Viola et al., 2019). Consistent with previous studies, treatment of BMDMs with IL-4 increased OXPHOS, as measured by oxygen consumption rate (OCR), compared with unstimulated macrophages (Figure 1P). Importantly, this response was inhibited in OI-treated M2 macrophages (Figure 1P). OI treatment also reduced spare respiratory capacity and maximal respiration levels in response to IL-4 compared with control (Figures 1Q and 1R). OI had no significant effect on glycolysis in IL-4-treated BMDMs (Figure S2A), nor on basal OXPHOS and glycolysis in unpolarized, control macrophages (Figures S2B and S2C).

Because OXPHOS was inhibited by OI in M2 macrophages, we tested if supplementation of the TCA cycle with alpha-ketoglutarate (α KG) could rescue OI's inhibitory effects on M2 gene expression. M2 macrophages utilize glutamine as a fuel source (Jha et al., 2015; Wang et al., 2018), which during glutaminolysis is metabolized into α KG and supplements the TCA cycle, and this process is important for the M2 metabolic program and function (Liu et al., 2017). Itaconate has previously been shown to alter glutamine metabolism in other cell types (Cordes et al., 2020). To test if OI was blocking M2 activation by affecting glutamine metabolism or the TCA cycle directly, IL-4-activated BMDMs were treated with a cell-permeable version of α KG, DM- α KG, to directly supplement the TCA cycle (Liu et al., 2017). As these cells were not glutamine starved, there were no changes in expression of the M2 genes *Arg1*, *Fizz1*, and

Figure 1. Itaconate derivative OI blocks M2 polarization

(A–D) BMDMs were pretreated with DMSO (vehicle control) or increasing concentrations of OI for 2 h, then stimulated with media or IL-4 (20 ng/mL) for 24 h. The black triangle represents increasing OI concentrations ([] OI) starting from the left: 31.25, 62.5, 125, 250, and 500 μ M. qRT-PCR results showing mRNA expression of *Arg1* (A), *Fizz1* (B), *Mrc1* (C), and *Pparg* (D) normalized to 18S. n = 6, 2 experimental repeats.

(E) BMDMs were pretreated with DMSO (unstim, ctrl) or 125 or 250 μ M OI for 2 h, then stimulated with media (unstim) or IL-4 (ctrl, OI 125 μ M, 250 μ M) for 24 h. Flow cytometry data for the M2 surface marker CD206 (mannose receptor), showing percentage of CD206⁺ BMDMs (left) and mean fluorescence intensity (MFI) of CD206 (right) (n = 6, showing one representative experiment of 2 repeats).

(F) Flow cytometry histograms of one representative mouse from (E); CD206 fluorescence expression in BMDMs treated with the indicated label.

(G) BMDMs were treated as in (E). Flow cytometry data for the M2 surface marker CD301 (CLEC10a), showing percentage of CD301⁺ BMDMs (left) and mean fluorescence intensity (MFI) of CD301 (right) (n = 6, showing one representative experiment of 2 repeats).

(H) Flow cytometry histograms of one representative mouse from (G); CD301 fluorescence expression in BMDMs treated with the indicated label.

(I) WT BMDMs were pretreated with OI for 2 h, then stimulated with IL-4 for 24 h. Western blot performed on cell lysates for phosphorylated STAT6 (Tyr641), total STAT6, and β -actin housekeeping control. Showing one representative blot of n = 5.

(J) Western blot densitometry values of phosphorylated STAT6 (Tyr641) normalized to total STAT6 protein in BMDMs treated with the indicated label (n = 5).

(K) WT BMDMs were pretreated with OI for 2 h, then stimulated with IL-4 for 1 h. Western blot performed on cell lysates for phosphorylated JAK1 (Tyr1034/1035), total JAK1, and β -actin housekeeping control. Showing one representative blot of n = 5.

(L) Western blot densitometry values of phosphorylated JAK1 (Tyr1034/1035) normalized to total JAK1 protein in BMDMs treated with the indicated label (n = 5).

(M) WT BMDMs were pretreated with OI for 2 h, then stimulated with IL-4 for 24 h. Flow cytometry was performed on cells treated with IL-4 alone (ctrl) or OI + IL-4, stained for the surface marker IL-4R α (CD124). Data show percentage of IL-4R α ⁺ BMDMs.

(N) Mean fluorescence intensity (MFI) of IL-4R α on BMDMs (n = 6, 2 experimental repeats).

(O) WT BMDMs were pretreated with OI for 2 h, then stimulated with IL-4 for 24 h, and flow cytometry was performed on cells stained for annexin V surface expression and the DNA dye propidium iodide (PI). Percentage of singlet cells that are live (Annexin V⁻ PI⁻), pre- or early apoptotic (Annexin V⁺ PI⁻), apoptotic (Annexin V⁺ PI⁺), and necrotic (Annexin V⁻ PI⁺) (n = 3). Representative flow plots can be found in Figure S1G.

(P) WT BMDMs were pretreated with OI for 2 h, then stimulated with IL-4 for 24 h. Seahorse Mito Stress Test showing oxygen consumption rate (OCR) of cells over time after addition of oligomycin (oligo), carbonyl cyanide-4-phenylhydrazone (FCCP), and rotenone/antimycin A (Rot/AA), in unstimulated cells (control), IL-4 alone, or OI + IL-4 (n = 8, 3 experimental repeats).

(Q) Spare respiratory capacity of BMDMs that were unstimulated (control), treated with IL-4 alone, or OI + IL-4, as measured by Seahorse Mito Stress test.

(R) Maximal respiration of BMDMs that were unstimulated (control), treated with IL-4 alone, or OI + IL-4, as measured by Seahorse Mito Stress test. Data are displayed as mean $\pm$ SEM. Differences were considered statistically significant at *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, and ****p $\leq$ 0.0001. See also Figures S1 and S2.

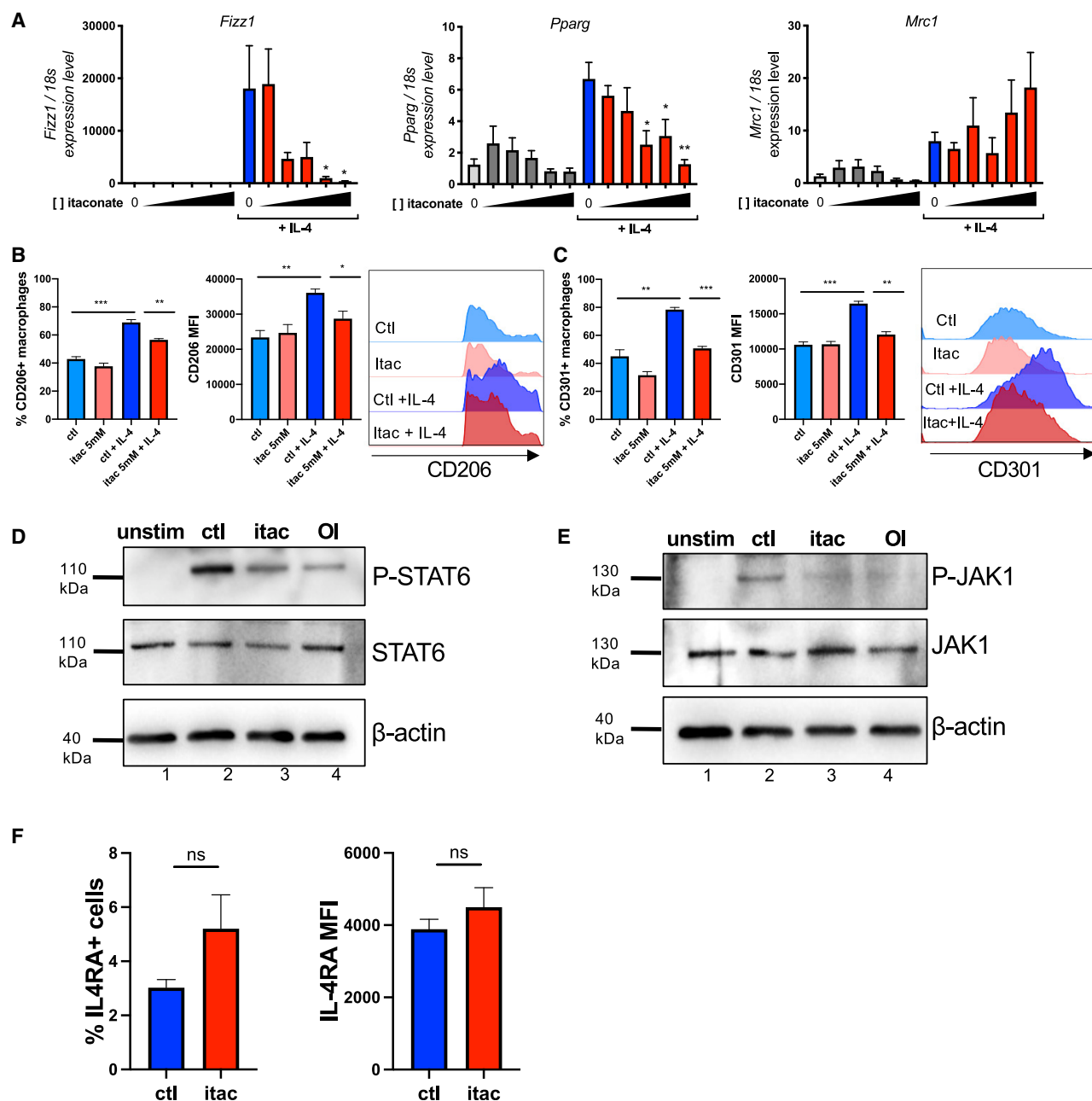


Figure 2. Itaconate blocks M2 polarization similarly to OI

(A) BMDMs were pretreated with media (0 mM itaconate) or increasing concentrations of itaconate for 2 h, then stimulated with media (ctl) or IL-4 for 24 h. The triangle represents increasing itaconate concentrations ([I]) starting from the left: 0.31, 0.63, 1.25, 2.5, and 5 mM. qRT-PCR results showing mRNA expression of M2 genes *Fizz1*, *Pparg*, and *Mrc1*, all normalized to 18S (n = 6, 2 experimental repeats).

(B and C) BMDMs were pretreated with media (ctl) or 5-mM itaconate for 2 h, then stimulated with media or IL-4 for 24 h. (B) Flow cytometry data for the M2 surface marker CD206 showing percentage of CD206⁺ BMDMs (left) and mean fluorescence intensity (MFI) of CD206 (middle) (n = 6, showing one representative experiment of 2 repeats). Right: flow cytometry histograms of one representative mouse; CD206 fluorescence expression in BMDMs treated with the indicated label.

(C) Flow cytometry data for the M2 surface marker CD301 showing percentage of CD301⁺ BMDMs (left) and mean fluorescence intensity of CD301 (middle) (n = 6, showing one representative experiment of 2 repeats). Right: flow cytometry histograms of one representative mouse; CD301 fluorescence expression in BMDMs treated with the indicated label.

(D and E) WT BMDMs were pretreated with itaconate (itac) or OI for 2 h, then stimulated with IL-4 for 24 h.

(D) Western blot performed on cell lysates for phosphorylated STAT6 (Tyr641), total STAT6, and β-actin housekeeping control. Showing one representative blot of n = 3.

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Mrc1 comparing DM- α KG plus IL-4-treated cells with IL-4 treatment alone (Figure S2D). Notably, the addition of DM- α KG did not rescue the suppression of these M2 genes observed during OI treatment (Figure S2D), suggesting that the inhibitory effects of this itaconate derivative are not mediated through direct TCA cycle inhibition or altered glutamine metabolism. These data altogether demonstrate that OI inhibits IL-4-induced M2 gene expression, JAK1/STAT6 signaling, and metabolic reprogramming, with inhibition likely to be occurring at the level of JAK1 phosphorylation.

Itaconate blocks M2 polarization

We then tested whether underivatized itaconic acid could recapitulate the inhibitory effects of OI in macrophage alternative activation. Treatment of BMDMs with increasing concentrations of itaconate at a physiologic pH (~ 7.4 , to rule out any pH-dependent effects) resulted in a decrease in IL-4-induced *Fizz1* and *Pparg* expression, while *Mrc1* (mannose receptor) mRNA expression was not significantly affected (Figure 2A). Despite mannose receptor mRNA being unaffected, surface protein expression of the mannose receptor, or CD206, was decreased in itaconate-treated M2 macrophages (Figure 2B). Itaconate treatment also decreased the percentage of CD301⁺ cells and CD301 MFI, compared with control M2 macrophages (Figure 2C). Similar to OI, itaconate inhibited IL-4-induced STAT6 phosphorylation (Figure 2D, lane 2 versus 3 and 4). Further, phosphorylation of JAK1 was inhibited by itaconate as well as OI upon IL-4 stimulation (Figure 2E, lane 2 versus 3 and 4), while IL4RA surface protein expression was not affected (Figure 2F). These data altogether indicate that itaconic acid itself can inhibit M2 macrophage polarization in a similar manner to OI.

OI suppresses activation of JAK1 in macrophages and Th2 cells

To further probe the mechanism by which OI inhibits the M2 macrophage program, we examined JAK1 activation in more detail, as it was the earliest upstream component inhibited by OI during IL-4 activation. IL-13 uses a similar cytokine signaling pathway to IL-4, including activation of JAK1 through IL4RA and IL13RA heterodimers (Jiang et al., 2000; Junttila, 2018). OI inhibited the IL-13-induced expression of M2-signature genes *Arg1*, *Mrc1*, and *Pparg* (Figure 3A). Underivatized itaconate also repressed induction of *Mrc1* and *Pparg* upon IL-13 stimulation, but not *Arg1* (Figure 3A). Importantly, OI treatment reduced JAK1 phosphorylation during IL-13 stimulation (Figures 3B and 3C), consistent with our earlier results with IL-4. Thus, OI suppresses M2 macrophage effector genes and JAK1 phosphorylation, both through IL-4 and IL-13 stimulation.

JAK1 is activated by multiple other cytokine receptors, including the interferon- α/β receptor (IFNAR). The effect of OI on JAK1 activation was therefore tested in type-I interferon (IFN)-stimulated macrophages. OI treatment resulted in a

decrease of phosphorylated JAK1 levels in BMDMs stimulated with the cytokine IFN- β (Figures 3D and 3E). IFNAR stimulation by IFN- β activates STAT1 downstream of JAK1, and we found a decrease in STAT1 phosphorylation upon OI treatment compared with control IFN- β -stimulated cells (Figures 3F and 3G). Phosphorylated JAK1 was also reduced in BMDMs stimulated with interferon gamma (IFN- γ), another cytokine that utilizes JAK1 for signaling during M1 polarization (Figures S3B and S3C). These data provide further evidence for inhibition of JAK1 activation by OI upon cytokine signaling and suggest that OI inhibits JAK1 phosphorylation broadly, even outside of M2 polarization.

We also tested the effect of OI in another immune cell type that utilizes JAK1 for signaling and activation, T helper 2 subset (Th2) CD4⁺ T cells (Ashino et al., 2014). Resting murine CD4⁺ T cells were activated with α CD3/CD28 and stimulated with IL-4 to skew these cells to the Th2 subset. OI dose-dependently inhibited expression of the Th2 genes *Il13*, *Il5*, and *Pparg* (Figure 3H), while underivatized itaconate inhibited *Il5* and *Il13* mRNA expression (Figure S3D). JAK1 phosphorylation was also inhibited by OI in a dose-dependent manner in Th2 cells (Figure 3I, lanes 3 and 4 versus lane 2, and Figure 3J). Further, intracellular cytokine staining revealed inhibition of IL-13 production by Th2 cells upon treatment with OI (Figures 3K and 3L). Taken together, these data suggest that JAK1 is a key cellular signaling component inhibited by the itaconate derivative OI.

JAK1 is directly modified by itaconate derivatives

The inhibition of JAK1 phosphorylation in IL-4, IL-13, IFN-stimulated macrophages, and Th2 cells by OI and itaconate suggests a direct effect on the JAK1 protein. We therefore next assayed JAK1 enzymatic activity. Both itaconate and OI reduced JAK1 kinase activity by $\sim 50\%$ at 50 μ M, demonstrating some direct inhibition on the enzyme (Figure 4A). The JAK1 inhibitors ruxolitinib, tofacitinib, and staurosporine were included as positive controls. Because itaconate and its derivatives can modify cysteine residues on many proteins and alter their function (Mills et al., 2018; Qin et al., 2019, 2020), we hypothesized that cysteines on JAK1 might be directly modified by itaconate. Suppression of JAK1 phosphorylation was maintained after OI was washed from cells prior to IL-4 treatment, suggesting a stable, covalent modification (Figures S4A and S4B).

In a recent screen, JAK1 was suggested to be modified by an itaconate bioorthogonal probe termed ITalk (Qin et al., 2020). To confirm JAK1 modification by itaconate, we overexpressed rat JAK1 in HEK293T cells, and then treated these cells with the ITalk probe. We observed an enrichment in ITalk labeling upon immunoprecipitation of JAK1, demonstrating JAK1 modification (Figure 4B). We then performed LC-MS/MS analysis and found 2,3-dicarboxypropylation of four key cysteine residues on JAK1: C715, C816, C943, and C1130 (Figures 4C and S4C). To confirm this finding in human macrophages, we profiled for

(E) Western blot performed on cell lysates for phosphorylated JAK1 (Tyr1034/1035), total JAK1, and β -actin housekeeping control. Showing one representative blot of $n = 3$.

(F) WT BMDMs were pretreated with 5 mM itaconate for 2 h, then stimulated with IL-4 for 24 h. Flow cytometry was performed on cells treated with IL-4 alone (ctl) or itaconate + IL-4 (itac), stained for the surface marker IL-4R α (CD124). Left: data show percentage of IL-4R α ⁺ BMDMs. Right: mean fluorescence intensity (MFI) of IL-4R α on BMDMs ($n = 6$, 2 experimental repeats). Data are displayed as mean $\pm$ SEM. Differences were considered statistically significant at * $p \leq 0.05$. ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p < 0.0001$.

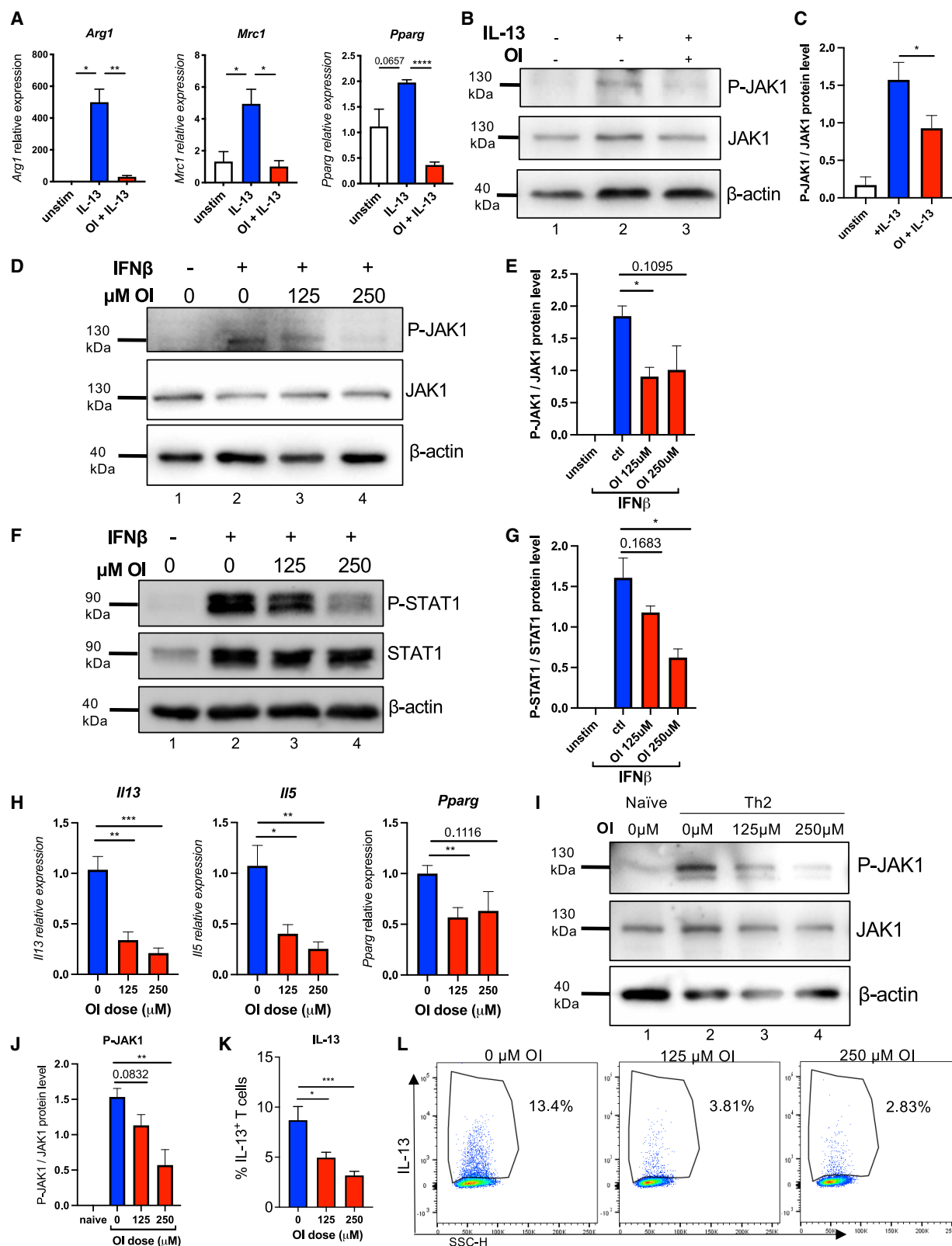


Figure 3. OI blocks JAK1 phosphorylation during IL-13, IFN-β, and Th2 stimulation

(A) BMDMs were pretreated with DMSO (unstim and IL-13) or 250 μM OI for 2 h, then stimulated with media (unstim) or IL-13 (20 ng/mL) for 24 h. qRT-PCR results showing mRNA expression of M2 genes *Arg*, *Mrc1*, and *Pparg*, all normalized to *18s* (n = 3).

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proteins modified by OI in peripheral blood mononuclear cell (PBMC)-derived primary macrophages and found JAK1 among the top enriched proteins, with KEAP1 as a positive control (Figure S4D). Next, we performed iodoacetamide-desthiobiotin (IA-DTB)-based competitive cysteine profiling in the human macrophage cell line THP1 during OI treatment (Kuljanin et al., 2021). Among the top 10 cysteines significantly modified by OI was C817 on JAK1 (Figures 4D and 4E), corresponding to C816 on rat JAK1 observed with the ITalk probe (Figure S4E). As a positive control, C288 on KEAP1 was found to be significantly modified by OI at 125 and 250 μ M (Figure 4D), which has previously been shown to be alkylated by OI (Mills et al., 2018), although KEAP1 is not required for M2 macrophage polarization (Kobayashi et al., 2016). 2,3-dicarboxypropylation of C716 and C944 were also detected, equating to the observed modification on rat C715 and C943; however, the competition ratio was not highly significant compared with control (Figure 4E). Additional OI-modified cysteines on human JAK1 were also observed but were not significant versus DMSO control (Figure 4E). Taken together, these results demonstrate that itaconate derivatives ITalk and OI directly modify C816/C817 on rodent and human JAK1, in addition to modification of C715/C716, C943/C944, and C1130/C1131 by the ITalk probe (Figures 4F and 4G). These cysteines are located within the pseudokinase and kinase domains of JAK1 (Figure 4F), which are key domains for JAK1 phosphorylation and signaling activity. These residues are highlighted within a generated 3D model of the JH1-JH2 domains of human JAK1, and additionally, showing itaconate covalently docked to C817 (Figure 4G). Amino acid sequence alignment of the four Janus kinase proteins, JAK1, JAK2, JAK3, and TYK2, revealed that a cysteine residue at amino acid 816 is unique to murine JAK1, while human TYK2 also contains a cysteine residue at the site corresponding to C817 in human JAK1 (Figure 4H). No other Janus Kinases were significantly modified by OI or ITalk in our cysteine profiling screen (data not shown) (Qin et al., 2020). Thus, JAK inhibition by OI is likely to be specific to JAK1, which is modified by this itaconate derivative at cysteine 816/817. Altogether, our data show that the inhibitory

role of the itaconate derivative OI in macrophage alternative activation is likely through direct cysteine modification of JAK1 and inhibition of its kinase activity, suggesting that this itaconate derivative is a direct JAK1 inhibitor.

OI inhibits M2 macrophages and JAK1 activation *in vivo*

Finally, we tested whether OI has similar M2-inhibitory effects *in vivo*. We first utilized the IL-4-anti-IL-4 mAB complex (IL-4c) murine model, comparing mice that were injected with OI to vehicle control. As expected, treatment of mice with IL-4c resulted in enhanced expression of M2 genes in peritoneal exudate cells (PECs), as measured by mRNA expression of *Arg1*, *Fizz1*, *Ym1*, *Mrc1*, and *Pparg* (Figures 5A–5E). IL-4c-treated mice that were injected with OI showed decreased *Arg1* (Figure 5A), *Fizz1* (Figure 5B), and *Ym1* (Figure 5C) mRNA expression compared with controls, with a more minor repression of *Mrc1* (Figure 5D) and *Pparg* (Figure 5E).

IL-4c treatment also resulted in an increase in phospho-STAT6 (Figure 5G) and phospho-JAK1 (Figure 5I) in PECs. Similar to the *in vitro* results, OI treatment decreased phosphorylated STAT6 during IL-4c treatment (Figures 5F and 5G). Importantly, phospho-JAK1 levels were reduced in PECs from mice treated with OI (Figures 5H and 5I).

Next, we performed a previously established murine model of severe steroid-resistant asthma using ovalbumin (Ova) and *Chlamydia muridarum* (Cmu), a natural mouse respiratory pathogen (Horvat et al., 2007; Kim et al., 2017b; Wadhwa et al., 2019). Mice treated with Ova and Cmu develop airway inflammation, infiltration of immune cells, and other features that represent that of asthma and allergic airway diseases, and they do not respond to steroid treatment with dexamethasone (DEX) (Wadhwa et al., 2019). As expected, WT mice treated with Ova/Cmu had enhanced airway resistance (Rn) compared with sham-treated, control animals (Figures 5J and 5K). Treatment with DEX did not improve Rn, but importantly, OI significantly reduced Rn in Ova/Cmu-treated mice (Figures 5J and 5K). Ova/Cmu-treated animals and those also given Ova/Cmu plus DEX had increased macrophage infiltration into the

(B) Western blot performed on cell lysates for phosphorylated JAK1 (Tyr1034/1035), total JAK1, and β -actin housekeeping control after BMDMs were pretreated with DMSO or 250 μ M OI for 2 h, then stimulated with IL-13 (20 ng/mL) for 1 h. Showing one representative blot of $n = 7$.

(C) Western blot densitometry values of phosphorylated JAK1 normalized to total JAK1 protein in BMDMs treated with the indicated label ($n = 7$).

(D) Western blot performed on cell lysates for phosphorylated JAK1 (Tyr1034/1035), total JAK1, and β -actin housekeeping control after BMDMs were pretreated with DMSO (0 μ M) or 125 or 250 μ M OI for 2 h, then stimulated with IFN- β (20 ng/mL) for 1 h.

(E) Western blot densitometry values of phosphorylated JAK1 (Tyr1034/1035) normalized to total JAK1 protein in BMDMs treated with the indicated label ($n = 3$, 2 experimental repeats).

(F) Western blot performed on cell lysates for phosphorylated STAT1, total STAT1, and β -actin housekeeping control after BMDMs were pretreated with DMSO (0 μ M) or 125 or 250 μ M OI for 2 h, then stimulated with IFN- β (20 ng/mL) for 24 h.

(G) Western blot densitometry values of phosphorylated STAT1 normalized to total STAT1 protein in BMDMs treated with the indicated label ($n = 3$, 2 experimental repeats).

(H) Murine splenic resting CD4⁺ T cells were treated with DMSO (0 μ M OI) or 125 or 250 μ M OI and activated with α CD3/CD28 and skewed to Th2 with IL-4 and α -IFN- γ for 72 h. qRT-PCR results showing mRNA expression of *Il13*, *Il5*, and *Pparg*, all normalized to *Tbp* ($n = 5$, 2 experimental repeats).

(I) Resting CD4⁺ T cells were treated with DMSO (0 μ M OI) or 125 or 250 μ M OI and activated and skewed to Th2 for 24 h. Western blot performed on cell lysates for phosphorylated JAK1 (Tyr1034/1035), total JAK1, and β -actin housekeeping control. Showing one representative blot of $n = 4$.

(J) Western blot densitometry values of phosphorylated JAK1 (Tyr1034/1035) normalized to total JAK1 protein in BMDMs treated with the indicated label ($n = 4$, 3 experimental repeats).

(K) Resting CD4⁺ T cells were treated with DMSO (0 μ M OI) or 125 or 250 μ M OI and activated and skewed to Th2 for 72 h. Intracellular staining of IL-13, measured by flow cytometry, showing percentage of cells producing IL-13 ($n = 6$, 2 experimental repeats).

(L) Representative flow plot from one mouse showing IL-13 expression in CD4⁺ Th2 cells treated with the indicated concentration of OI.

Data are displayed as mean $\pm$ SEM. Differences were considered statistically significant at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p < 0.0001$. See also Figures S2 and S3.

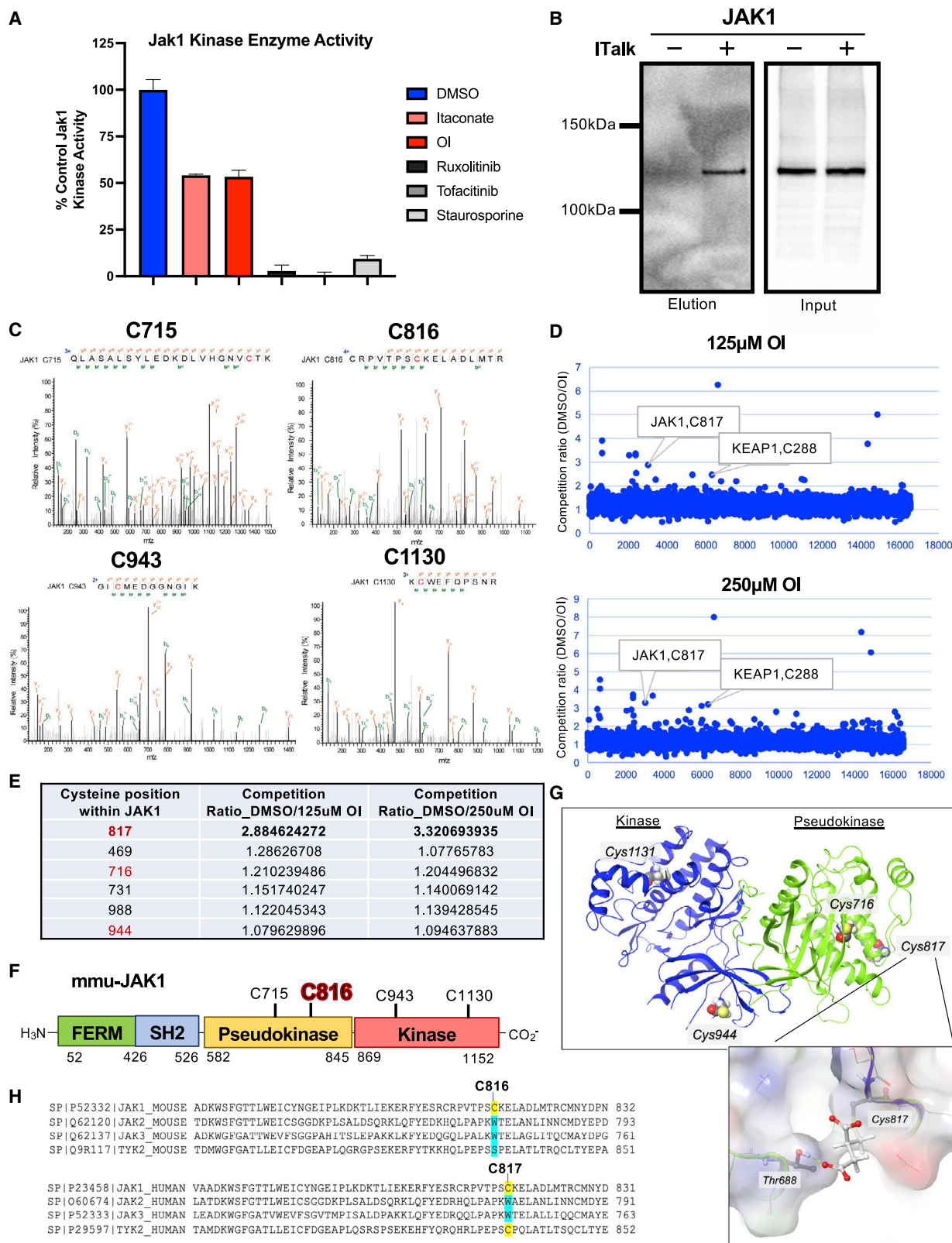


Figure 4. JAK1 is directly modified by itaconate derivatives

(A) JAK1 kinase activity was measured after pre-incubating recombinant JAK1 for 1 h with 50 μ M DMSO (control), itaconate, OI, ruxolitinib, tofacitinib, or staurosporine. Data show kinase activity as percent relative to DMSO control.

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bronchoalveolar lavage fluid (BALF), while OI-treated mice showed significantly reduced macrophage levels in the BALF (Figure 5L). Furthermore, mRNA expression of M2-signature genes was increased in the lungs of Ova/Cmu and Ova/Cmu/DEX-treated groups, while OI repressed *Arg1*, *Fizz1*, *Ym1*, and *Mrc1* expression (Figures 5M–5P). Finally, OI-treated mice had decreased JAK1 activation in lung tissue, compared with Ova/Cmu and Ova/Cmu/DEX. Notably, JAK1 phosphorylation levels were reduced in the lung tissue from OI-treated mice compared with Ova/Cmu alone (Figures 5Q and 5R). These results confirm our *in vitro* findings and show that OI inhibits the IL-4 signaling pathway, M2 polarization, and JAK1 activation in two different *in vivo* models. Taken together, our data point to itaconate and OI as inhibitors of JAK1 *in vitro* and *in vivo*.

DISCUSSION

Our data demonstrate that itaconate and OI inhibit phosphorylation of JAK1, identifying another mechanism for the immunomodulatory effects of itaconate. JAK inhibitors, or “jakinibs,” are effective therapies in a multitude of inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, and atopic dermatitis (Schwartz et al., 2017). JAKs have been shown to be sensitive to redox regulation and cysteine modification, in which the cellular redox state affects the activation of JAK proteins and thus the immunologic status of the cell (Duhé, 2013; Smith et al., 2012). JAK3 has also been shown to be covalently modified by some jakinibs, including ritlecitinib, at Cys909 (Forster et al., 2017; Goedken et al., 2015). Specific cysteine residues on JAK2 have also been found to be redox-sensitive, with these amino acids affecting the catalytic activity of the enzyme (Smith et al., 2012). Interestingly, one of these cysteines that regulates the redox-sensitive switch in JAK2, C917 (Duhé, 2013; Smith et al., 2012), aligns with the homologous residue C943 on JAK1, found to be modified by the itaconate derivative ITalk in our study. As a known cysteine modifier, itaconate could well form the basis for a new class of JAK1 inhibitors.

OI inhibited JAK1 phosphorylation in IL-4-, IL-13-, and type-I and II-IFN-activated macrophages, as well as in Th2 cells. All of

these cytokines and cell types utilize JAK1 for downstream signaling. Chemoproteomic profiling of the itaconate modification 2,3-dicarboxypropylation (or “itaconation”) in macrophages found that JAK1 is modified by the itaconate derivative ITalk (Qin et al., 2020) and our data confirm this finding with OI, in both a human macrophage cell line and primary human macrophages. We observed JAK1 modification at four key cysteine residues in rat JAK1, C715, C816, C943, and C1130, and one conserved cysteine in human JAK1, C817. JAK1 kinase activity was inhibited by OI and itaconate, most likely via modification of these cysteine residues. C816/817 and C715 are located in the pseudokinase domain of JAK1, while C943 and C1130 are within the kinase domain, and with 3D modeling, we speculate that the 2,3-dicarboxypropylation of JAK1 somehow disrupts the protein’s structure to reduce kinase activity (Duhé, 2013). It is also possible that the JAK1 modification by itaconate derivatives interrupts the interaction of this tyrosine kinase with the IL-4 receptor or other accessory proteins that are required for JAK1 phosphorylation.

Itaconate and its derivatives have previously been shown to have anti-inflammatory effects during M1 macrophage activation. M1 and M2 macrophages are often viewed as a “yin and yang” dichotomy of inflammatory states, with M2 alternative activation often cited as being anti-inflammatory. Our results show that itaconate represses macrophage activation in general, rather than promoting a particular polarization state. Indeed, JAK1 is a tyrosine kinase that is commonly used by many cytokine receptors, including IL-4, IL-13, IL-2, and type-I and -II interferons. Thus, itaconate’s inhibition of JAK1 phosphorylation might reduce activation of any cytokine pathway that uses JAK1 for activation and polarization. The ability of OI to inhibit glycolytic activation of pro-inflammatory “M1” macrophages (Liao et al., 2019) and IL-4-stimulated “M2” macrophages (our results) suggests that any macrophage on the “M1-M2” spectrum would have reduced function in a disease setting driven by activated macrophages. Many *in vivo* and clinical disease states contain a considerable amount of heterogeneity within the “M1-M2” spectrum (Chimal-Ramírez et al., 2016; Röszer, 2015; Sica and Mantovani,

(B) HEK293T cells were transfected with a rat JAK1-FLAG overexpression plasmid for 24 h and then treated with 100 μ M ITalk or DMSO for 12 h. The samples before and after enrichment were analyzed by anti-FLAG immunoblotting.

(C) LC-MS/MS spectra of ITalk modifications on rat JAK1 following 2-h treatment with ITalk itaconate probe, showing 2,3-dicarboxypropylation of cysteines C715, C816, C943, and C1130 ($n = 3$).

(D) THP1 cells were pretreated with DMSO control or 125 or 250 μ M OI for 16 h, before treatment with IA-DTB, cell lysis, and measurement of modified cysteines via LC-MS. Competition ratio of modified cysteines in DMSO control compared with OI treatment, with each blue dot representing a specific cysteine on a protein. JAK1 C817 and KEAP1 C288 are highlighted among the top cysteines significantly modified over control. Showing 1 representative plot of 3 independent experiments.

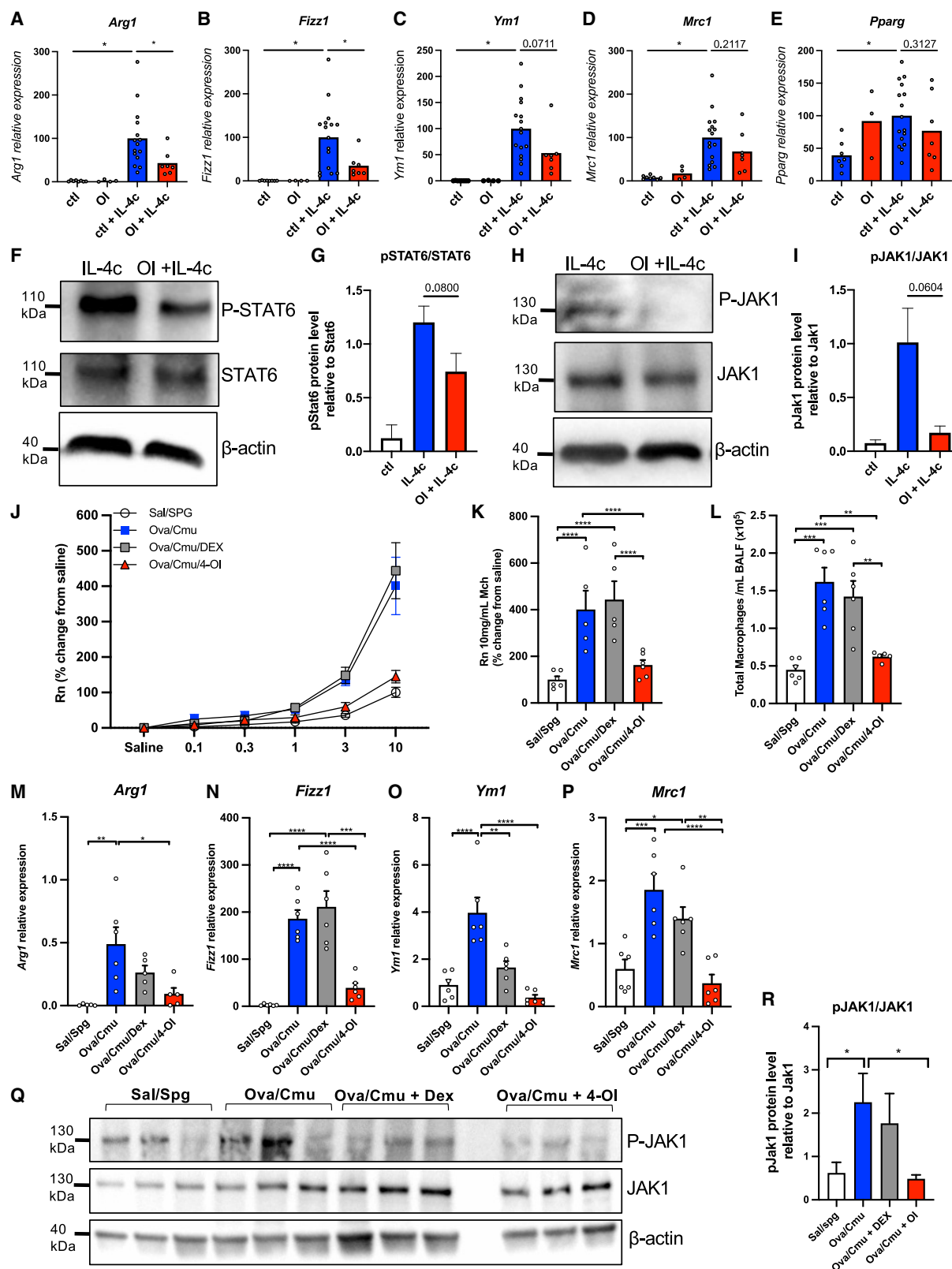
(E) Raw competition ratio values of detected cysteines within JAK1 showing 2,3-dicarboxypropylation with 125 or 250 μ M OI treatment. C817 is shown in bold because of its high competition ratios over DMSO control, indicating significant enrichment. Cysteines in red are those that overlap with residues modified by ITalk in rat JAK1. Showing representative data of 3 independent experiments.

(F) Schematic of murine JAK1 protein, showing amino acid positions of the FERM, SH2, pseudokinase, and kinase domains, as well as positions of itaconate-modified cysteines within JAK1, with C816 (human 817) highlighted.

(G) 3D structure of JH1-JH2 (pseudokinase and kinase) domains of human JAK1 with Cys716, Cys817, Cys994, and Cys1131 highlighted in spheres (upper). Itaconate covalently docked to Cys817. Thr688 is proximal and potentially engages in a hydrogen-bond interaction with a carbonyl group from itaconate (lower).

(H) Uniprot amino acid sequence alignment of mouse JAK1, JAK2, JAK3, and TYK2, showing a cysteine residue at site 816 in JAK1, tryptophan at the homologous sites in JAK2 and JAK3, and a serine in TYK2 (top). Uniprot amino acid sequence alignment of human JAK1, JAK2, JAK3, and TYK2, showing a cysteine residue at site 817 in JAK1, tryptophan at the homologous sites in JAK2 and JAK3, and a cysteine in TYK2 (bottom).

Data are displayed as mean $\pm$ SEM. Differences were considered statistically significant at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p < 0.0001$. See also Figure S4.



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2012). Thus, we hypothesize that itaconate derivatives are an effective therapy in many disease models because of their generally inhibitory properties in macrophages. In the context of cancer, itaconate has been shown to have tumor-promoting properties (Weiss et al., 2018). Our data demonstrate that itaconate can inhibit M2 macrophages, suggesting that it might inhibit activation of tumor-associated macrophages (TAMs), which indeed have M2 properties and promote tumor growth (Mantovani et al., 2002). Thus, further work is still needed to differentiate the effect itaconate and derivatives have in TAMs and other resident and infiltrating myeloid cells, versus their direct role in tumor cells which has been previously investigated (Weiss et al., 2018).

In a recent paper by Willenborg et al., the authors demonstrate that in a model of wound healing, early-stage macrophages are glycolytic, promoting angiogenesis and inflammation. However, late-stage macrophages, activated by IL-4 and IL-13, drive OXPHOS, including the mitochondrial unfolded protein response, which in turn leads to mitohormesis and tissue repair (Willenborg et al., 2021). While this process is dependent upon cytokine signaling through IL-4R α , whether JAK1 directly regulates mitochondrial dynamics and metabolic remodeling requires further investigation. Since itaconate attenuates IL-4 and IL-13 signaling via JAK1, this metabolite would be expected to limit OXPHOS in that context as well, potentially modulating the repair process. Thus, it may be important to attenuate itaconate production for macrophages to appropriately switch to an “M2-like” late-stage metabolic state during wound healing. The overall broader metabolic consequences of inhibiting IL-4 and IL-13 signaling in macrophages requires further investigation.

Our results are important in considering how extracellular itaconate might target M2 macrophages. Mining of publicly available data showed that IL-4-activated macrophages have STAT6- and PPAR γ -dependent mechanisms in place to repress *Irg1* (Szanto et al., 2010), suggesting that itaconate is indeed inhibitory for M2 polarization. How *Irg1* is regulated in M2 macrophages requires further work. We performed experiments with OI and underivatized itaconate in parallel and observed similar effects between the two compounds on M2 macrophage activation. Some effects were less potent with itaconate than with OI, and it should

be noted that experiments with itaconate were performed at higher concentrations in the mM range (similar to what is found in an LPS-stimulated macrophage) (Hooftman et al., 2020; Mills et al., 2018), while OI is immunomodulatory at concentrations in the μ M range. Even so, it is clear that both compounds have a strong effect on the IL-4 cytokine signaling pathway in repressing phosphorylation of JAK1 and STAT6. We have recently confirmed that OI is effectively converted into itaconate in macrophages, even in the absence of LPS (Hooftman et al., 2020). This supports the use of the cell-permeable derivative OI to study the roles of itaconate.

While underivatized itaconate is important for understanding the physiologic relevance of this metabolite, OI also represents a pharmacological compound that mimics itaconate more potently. This is similar to the Krebs-cycle-derivative compound, DMF, which is a methyl ester of fumarate. DMF has been useful in understanding biochemical mechanisms of endogenous fumarate, while also being potentially immunomodulatory and clinically approved to treat inflammatory diseases (Kornberg et al., 2018; Ryan et al., 2019). Itaconate derivatives may prove to be potent and safe pharmacological agents that might be used to treat inflammatory diseases, including those driven by alternative macrophage activation, such as asthma, allergy, and fibrosis.

Limitations of study

Our study shows that OI and itaconate repress activation of JAK1 most likely via modification of cysteine residues on JAK1. While these results show an association between the cysteine modifications and reduction in JAK1 phosphorylation, the evidence linking these two events is somewhat indirect. Further, modifications of JAK1 by endogenous itaconate could be examined in LPS-stimulated macrophages comparing WT and cells lacking *Irg1/Acod1*. The physiological relevance of endogenous itaconate in M2 macrophages could be further explored. M2 (IL-4) macrophages do not produce itaconate, but whether M1 macrophages or other itaconate-producing cells can communicate with M2 or similar cell types to repress their activation *in vivo* is an interesting concept that requires further study. One recent publication showed that IL-33 activated macrophages have a GATA3-dependent but IL-4-independent induction of itaconate (Faas et al., 2021), suggesting

Figure 5. OI inhibits M2 macrophages and JAK1 activation *in vivo*

(A–E) WT mice were i.p. injected with 50 mg/kg OI on days 0 and 2, followed by IL-4c i.p. injection 3 h later on days 0 and 2. Peritoneal exudate cells (PECs) were collected on day 4. qRT-PCR results showing mRNA expression of *Arg1* (A), *Fizz1* (B), *Ym1* (C), *Mrc1* (D), and *Pparg* (E) normalized to *18s*. n = 8 (ctl), 8 (OI), 16 (ctl + IL-4c), and 7 (OI + IL-4c).

(F–I) WT mice were i.p. injected with 50 mg/kg OI, followed by IL-4c i.p. injection 3 h later. PECs were collected after 5 h IL-4c treatment.

(F) Representative western blot performed on cell lysates for phosphorylated STAT6, STAT6, and β -actin housekeeping control.

(G) Densitometry values of pSTAT6 western blot bands normalized to total STAT6 (n = 6).

(H) Western blot performed on cell lysates for phosphorylated JAK1, JAK1, and β -actin housekeeping control.

(I) Densitometry values of pJAK1 western blot bands normalized to total JAK1 (n = 3).

(J) WT mice were treated with sham control (Sal/SPG), ovalbumin (Ova), and *Chlamydia muridarum* (Cmu) to induce severe steroid-resistant asthma, or Ova/Cmu plus dexamethasone (DEX), or Ova/Cmu plus OI. Airway hyperresponsiveness in terms of airway resistance (Rn) in response to increasing doses of methacholine (Mch) (n = 6).

(K) Airway resistance in response to 10 mg/mL Mch.

(L) Number of macrophages in bronchoalveolar lavage fluid (BALF).

(M–P) mRNA expression in lung tissue after asthma induction of (M) *Arg1*, (N) *Fizz1*, (O) *Ym1*, and (P) *Mrc1* (n = 6).

(Q) Western blot performed on lung tissue lysates for pJAK1, total JAK1, and β -actin housekeeping control following asthma induction.

(R) Western blot densitometry values of pJAK1 normalized to total JAK1 from lung tissue lysates after asthma treatment (n = 6). Data are displayed as mean $\pm$ SEM. Differences were considered statistically significant at *p $\leq$ 0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001, and ****p $\leq$ 0.0001.

specificity of endogenous itaconate induction in macrophage polarization states. Thus, future work is needed to differentiate roles of itaconate endogenously versus exogenously in cell-cell communication.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cmet.2022.02.002>.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.C. Runtsch and L.A.J.O.; methodology, M.C. Runtsch, S.A., A.H., and L.A.J.O.; validation, S.A., A.H., Y. Zhang, Y. Zheng, J.S.S., M.C. Ruzek, R.S.M., A.Z., C.G.P., A.W., and R.C.; formal analysis, M.C. Runtsch, S.A., R.W., Y. Zhang, and Y. Zheng; investigation, M.C. Runtsch,

S.A., A.H., R.W., Y. Zhang, Y. Zheng, J.S.S., M.C. Ruzek, M.A.A., A.F.M., R.S.M., A.Z., C.G.P., A.W., R.C., E.H., P.G.F., R.J., R.Y.K., K.D., A.C.B., and J.C.H.; resources, Y. Zheng, J.S.S., M.C. Ruzek, M.A.A., P.G.F., P.M.H., C.W., and L.A.J.O.; writing – original draft, M.C. Runtsch and L.A.J.O.; writing – review & editing, M.C. Runtsch, S.A., A.H., M.C. Ruzek, and L.A.J.O.; visualization, M.C. Runtsch, S.A., A.H., Y. Zheng, and M.A.A.; supervision, M.C. Runtsch, M.C. Ruzek, P.G.F., P.M.H., C.W., and L.A.J.O.; funding acquisition, L.A.J.O.

DECLARATION OF INTERESTS

Y. Zheng, J.S.S., M.C. Ruzek, and M.A.A. are employees and shareholders of AbbVie. All other authors declare no competing interests related to this manuscript.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community. While citing references scientifically relevant for this work, we also actively worked to promote gender balance in our reference list. The author list of this paper includes contributors from the location where the research was conducted who participated in the data collection, design, analysis, and/or interpretation of the work.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti- β -actin	Sigma	Cat# A5316; RRID: AB_476743
Anti-NRF2	Cell Signaling	Cat# 12721S; RRID: AB_2715528
Anti-Phospho-Stat6 (Tyr641)	Cell Signaling	Cat# 56554S; RRID: AB_2799514
Anti-Stat6	Cell Signaling	Cat# 9362S; RRID: AB_2271211
Anti-Phospho-Jak1 (Tyr1034/1035)	Cell Signaling	Cat# 3331S; RRID: AB_2265057
Anti-Jak1	Cell Signaling	Cat# 3332S; RRID: AB_2128499
Anti-SOCS1 (A156)	Cell Signaling	Cat# 3950T
Anti-Phospho-Stat1 (Tyr701) (58D6)	Cell Signaling	Cat# 9167S; RRID: AB_561284
Anti-Stat1	Cell Signaling	Cat# 9172S; RRID: AB_2198300
Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block)	Biolegend	Cat # 553141; RRID: AB_394656
PE anti-mouse CD206 (MMR)	Biolegend	Cat# 141706; Clone C068C2
APC anti-mouse CD301 (MGL1/MGL2)	Biolegend	Cat# 145708; Clone LOM-14
PE anti-mouse CD124 (IL-4R α)	Biolegend	Cat# 144804; Clone I015F8
PerCP/Cyanine5.5 anti-mouse F4/80	Biolegend	Cat# 123128; Clone BM8
PE anti-mouse IL-13	Biolegend	Cat# 159403; Clone W17010B
nVivoMAb anti-mouse IL-4	Bio X Cell	Cat# BE0045; Clone 11B11
Hamster anti-mouse CD3 (clone 145-2C11)	BD Biosciences	Cat #: 553057; RRID: AB_394590
Hamster anti-mouse CD28 (clone 37.51)	Tonbo Biosciences	Cat# 70-0281; RRID: AB_2621492
Rat anti-mouse IFN- γ (clone XMG1.2)	BD Biosciences	Cat# 554408; RRID: AB_395373
Phospho-tyrosine-specific antibody PT66K	Cisbio	Cat# 61T66KLA and 61T66KLB
ANTI-FLAG M2 antibody	Sigma Aldrich	Cat# F3165
Bacterial and virus strains		
<i>C. muridarum</i> (Cmu) (VR-123)	ATCC	Cat# ATCC CRL-1696
Biological samples		
Blood samples from healthy donors	School of Biochemistry and Immunology, TCD	N/A
Chemicals, peptides, and recombinant proteins		
4-Octyl Itaconate	Professor Richard Hartley, University of Glasgow	N/A
Itaconic Acid	Sigma	Cat# I29204
Lymphoprep	StemCell Technologies	Cat# 07861
Recombinant Human M-CSF	Peptotech	Cat# 300-25
Pacific Blue Annexin V Apoptosis Detection Kit with PI	Biolegend	Cat# 640928
CD62L MicroBeads, mouse	Miltenyi Biotec	Cat# 130-049-701
WesternBright ECL	Advanta	Cat# K-12049-D50
PMA	Sigma Aldrich	Cat# P8139; CAS:16561-29-8
Ionomycin	Sigma Aldrich	Cat# I0634; CAS: 56092-82-1
Brefeldin A	Sigma Aldrich	Cat# B7651; CAS: 20350-15-6
Fixation Buffer	BioLegend	Cat# 420801
Intracellular Staining Permeabilization Wash Buffer (10X)	BioLegend	Cat# 421002
(2-Hydroxypropyl)- β -cyclodextrin	Sigma Aldrich	Cat# H107; CAS: 128446-35-5
Recombinant Mouse IL-4 (carrier-free)	Biolegend	Cat# 574306
Recombinant Mouse IL-13 (carrier-free)	Biolegend	Cat# 575904
Recombinant Mouse IFN- β 1 (carrier-free)	Biolegend	Cat# 581304

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant Mouse IFN- γ (carrier-free)	Biolegend	Cat# 575306
Dimethyl 2-oxoglutarate (DM- α KG)	Sigma-Aldrich	Cat# 349631
Recombinant Murine IL-4	Peptotech	Cat# 214-14
Ovalbumin (323-339)	Sigma-Aldrich	Cat# O1641
Alhydrogel adjuvant 2%	Jomar Life Research	Cat# vac-alu-250
Dexamethasone	Sigma Aldrich	Cat# D4902-500MG; CAS 50-02-2
4-Octyl Itaconate	Sigma Aldrich	Cat# SML2338-25MG; CAS 3133-16-2
Halt Protease Inhibitor Cocktail (100X)	Thermo Fisher Scientific	Cat# 78430
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Fisher Scientific	Cat# 34095
Seahorse XF Medium	Agilent	Cat# 103575
JAK1 Kinase Domain	AbbVie	N/A
Tofacitinib	AbbVie	N/A
Ruxolitinib	AbbVie	N/A
Staurosporine	AbbVie	N/A
Streptavidin-Allophycocyanin	Aglient (Formerly ProZyme)	PJ27S
ATP	Promega	Cat# P1132
Lipofectamine 2000	ThermoFisher	Cat# 11668030
Itaconate-alkyne (ITalk)	Professor Chu Wang, Peking University	Qin et al. (2020)
TBTA ligand	Strem	Cat# 07-3215
Biotin-Azide	ChemPep	Cat# 271604
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)	Sigma Aldrich	Cat# 4706
Streptavidin-agarose beads	Thermo Fisher Scientific	Cat# 20353
CD14 MicroBeads, human	Miltenyi Biotec	Cat# 130-050-201
Pierce Streptavidin Plus Ultralink beads	Thermo Fisher Scientific	Cat# PI53116
Critical commercial assays		
PureLink RNA Mini Kit	Invitrogen	Cat# 12183025
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems	Cat# 4368814
PowerUp SYBR Green Master Mix	Applied Biosystems	Cat# A25743
Seahorse XF Cell Mito Stress Test Kit	Agilent	Cat# 103015-100
CD4+ T Cell Isolation Kit, mouse	Miltenyi Biotec	Cat# 130-104-454
Seahorse XFe96 FluxPak	Agilent	Cat# 102416-100
Pierce High pH Reversed-Phase Peptide Fractionation Kit	Thermo Fisher Scientific	Cat# 84868
Experimental models: Cell lines		
Human: HEK293T cells	Sigma	Cat# 12022001
Human: THP-1 cells	ATCC	Cat# TIB-202
Experimental models: Organisms/strains		
Mouse: C57BL/6JOLaHsd	Harlan UK	N/A
Mouse: BALB/c	Phil Hansbro Laboratory, University of Newcastle	N/A
Mouse: NFE2L2 ^{-/-}	Albena Dinkova-Kostova Lab, University of Dundee	N/A
Oligonucleotides		
18s Primer: F (5'-3'): GTAACCCGTTGAACCCC ATT; R (5'-3'): CCATCCAATCGGTAGTAGGG	This paper	N/A
Fizz1 Primer: F (5'-3'): ACCTTTCCTGAGATTCTGC CCC; R (5'-3'): CAGTGGTCCAGTCAACGAGTAAGC	This paper	N/A
Arg1 Primer: F (5'-3'): TGACATCAACACTCCCCTGA CAAC; R (5'-3'): GCCTTTTCTTCTTCCCAGCAG	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Mrc1</i> Primer: F (5'-3'): TCTTTTACGAGAAGTTGGG GTCAG; R (5'-3'): ATCATTCGGTTCACCAGAGGG	This paper	N/A
<i>Pparg</i> Primer: F (5'-3'): ACGATCTGCCTGAGGT CTGT; R (5'-3'): CATCGAGGACATCCAAGACA	This paper	N/A
<i>Ym1</i> Primer: F (5'-3'): GGCTACACTGGAGAAAAT AGTC CCC; R (5'-3'): CCAACCCACTCATTACCCTGATAG	This paper	N/A
<i>Tbp</i> Primer: F (5'-3'): CCGTGAATCTTGGCTGTA AAC; R (5'-3'): TGTCCGTGGCTCTCTTATTC	This paper	N/A
<i>Il13</i> Primer: F (5'-3'): GCTGAGCAACATCACAC AAG; R (5'-3'): AATCCAGGGCTACACAGAAC	This paper	N/A
<i>Il5</i> Primer: F (5'-3'): GCTTCCTGTCCCTACTCATAAA; R (5'-3'): CCCACGGACAGTTTGATTCT	This paper	N/A
Recombinant DNA		
JAK1 cDNA ORF Clone, Rat, C-DYKDDDDK (Flag) tag	Sino Biological	RG81640-CF
Software and algorithms		
Image Lab (Version 6.1)	Bio-Rad	https://www.bio-rad.com/
FlowJo v 10.7	FlowJo	https://www.flowjo.com/
Graphpad Prism 9.0	Graphpad	https://www.graphpad.com/
Seahorse Wave Controller Software (version 2.4.2)	Agilent	https://www.agilent.com/
ImageJ	NIH	https://imagej.nih.gov/ij/
ProLuCID	Xu et al. (2015)	http://fields.scripps.edu/yates/wp/?page_id=821
pFind	Chi et al. (2018)	http://pfind.ict.ac.cn/software/pFind3/index.html
MaxQuant 1.6.15.0	Max Planck Institute of Biochemistry	https://www.maxquant.org/
MAESTRO	Schrödinger	https://www.schrodinger.com/products/maestro
CovDock	Schrödinger	https://www.schrodinger.com/science-articles/covdock
Other		
UltiMate 3000 Nano-LC system	Thermo Fisher Scientific	Cat# ULTIM3000RSLCNANO
FlexiVent apparatus (FX1 System)	SCIREQ	https://www.scireq.com/flexivent/

RESOURCE AVAILABILITY**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Professor Luke A.J. O'Neill (laoneill@tcd.ie).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS**Mice**

6-8 weeks old C57Bl/6J female mice (Harlan UK) were used to isolate murine bone marrow- derived macrophages and splenic T cells. Mice were bred and housed in the Comparative Medicine Unit (CMU) in Trinity Biomedical Sciences Institute (TBSI) (Trinity

College Dublin, TCD, Ireland). All mice were maintained under specific pathogen-free conditions according to Irish and European Union regulations. All the procedures involving experiments on animals have been approved by the Health Products Regulatory Authority (HPRA, Ireland), and were conducted according to Directive 2010/63/EU of the European Parliament and Council on the protection of animals used for scientific purposes. Legs of Nfe2l2-/- (NRF2-null) and matched WT mice were kindly donated by Professor Albena Dinkova-Kostova and Elena Knatko at University of Dundee, Scotland.

Murine bone marrow-derived macrophage (BMDM) culture

Bone marrow was obtained by flushing DMEM media through the femur, tibia, and hip bone of mice with a 25-gauge needle. The bone marrow was subsequently resuspended in red cell lysis buffer (Sigma) for 3 minutes before being centrifuged, resuspended, and passed through a 70 μ m cell strainer. Cells were plated in 10 cm petri dishes and in DMEM media containing L-glutamine (Gibco) supplemented with FCS (10%) (Sigma-Aldrich), penicillin-streptomycin (1%) (Sigma-Aldrich) and L929 supernatant (10%) and cultured at 37°C in a 5% CO₂ incubator for 6 days. On day 6, cells were scraped, resuspended in DMEM complete media, and seeded into multiwell plates at 5x10⁵ or 1x10⁶ cells/mL.

Macrophage activation

BMDMs were seeded in 12-well plates and left overnight at 37°C in 5% CO₂ incubator. 1 hour prior to treatment, BMDMs were resuspended in serum-free DMEM media. Cells were treated with recombinant mouse IL-4 (20 ng/mL, Biolegend) for 5 min to 24h, as noted in the figure legends/results. For IL-13 stimulations of BMDMs, 20 ng/mL recombinant mouse IL-13 was used (Biolegend). Recombinant mouse IFN- β 1 (Biolegend) and IFN- γ (Biolegend) were used at 20 ng/mL.

Treatment of cells with OI and itaconate

4-octyl itaconate (OI) was originally synthesized and kindly provided by the laboratory of Professor Richard Hartley, University of Glasgow, Scotland. Experimental observations were confirmed using OI purchased from commercial suppliers (Fluorochem and Sigma). OI was prepared at a concentration of 250 mM in DMSO and used at a working concentration of 250 μ M, unless otherwise noted. For all *in vitro* experiments with OI, DMSO was used as a vehicle control. Itaconic acid (Sigma-Aldrich) was prepared in DMEM media (Gibco) at a concentration of 5 mM, and pH was brought to 7.4 using 1 M NaOH. Both control and itaconate media were then sterilized using a 0.2 μ m filter and added directly to BMDMs. BMDMs were pretreated with OI or itaconate for 2 hours before cytokine stimulation. For dimethyl 2-oxoglutarate (DM- α KG, Sigma-Aldrich 349631), cells were treated at a concentration of 1 mM.

T Cell isolation and culture

Murine resting T cells were purified from total mouse splenocytes by magnetic cell sorting with a CD4⁺ T Cell Isolation Kit (Miltenyi), followed by incubation with CD62L microbeads, all according to manufacturer's instructions (all reagents from Miltenyi Biotec). Cells were stimulated *in vitro* with plate-bound anti-CD3 and anti-CD28 antibodies (1 mg/ml and 2 mg/ml, respectively; BD Biosciences) + 40 ng/ml IL-4 (Biolegend) + 5 μ g/ml anti-mouse IFN- γ antibody (BD Biosciences) in RPMI 1640 containing L-glutamine (Gibco), supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), 55 mM 2-mercaptoethanol (Gibco) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich). For experiments with OI, cells were pre-incubated at 37°C for 30 min with OI at 125 μ M or 250 μ M, or equal amounts of DMSO (control condition) before activation. For experiments with itaconate, cells were pre-incubated at 37°C for 30 min with 2.5 mM or 5 mM itaconate pH 7.4.

In vivo IL-4 complex murine model

IL-4-anti-IL-4 mAb complex (IL-4c) was prepared as described previously by Finkelman and colleagues (Finkelman et al., 1993). Briefly, 5 μ g of recombinant murine IL-4 (PeproTech) was incubated with 25 μ g 11B11 (Bio X Cell) for 10 minutes on ice to allow for antibody-cytokine complex to form, then diluted in PBS. Female, 6-8 weeks old, C57BL/6 WT mice were then intraperitoneally injected with OI 50 mg/kg dissolved in PBS/40% cyclodextrin or PBS/cyclodextrin alone (vehicle control) for 3 hours. Mice were then injected with IL-4c or PBS (control). For JAK1/STAT6 Western blot analysis, PECs were collected 5 and 24 hours after IL-4c injection, counted, and lysed in Laemmli buffer containing 10mM Na₃VO₄. We determined that JAK1/STAT6 phosphorylation was highest in WT control mice 5 hours after IL-4c injection, and thus collected PECs from OI and control-treated mice using the 5-hour time point. For mRNA analysis, OI and IL-4c injections were repeated on day 2, and PECs were collected on day 4. PECs were pelleted, red blood cell-lysed, and lysed in RNA lysis buffer (Invitrogen).

Experimental asthma infection murine model

Murine models of ovalbumin (Ova)-induced allergic airway disease and Chlamydia respiratory infections were combined to induce severe steroid-resistant allergic airway disease as previously described (Essilfie et al., 2015; Kim et al., 2017a, 2017b). Briefly, female wild-type BALB/c mice, 6-8 weeks old, were sensitized to Ova (50 μ g intraperitoneal [i.p.] injection, Sigma-Aldrich, Sydney, Australia) in Th2-inducing adjuvant aluminium hydroxide (Alhydrogel, 2%, Jomar) in sterile saline (200 μ l). They were subsequently challenged intranasally (i.n.) with Ova (10 μ g/50 μ L sterile saline) on day 12-13 and again on day 33-34. Mice were then inoculated under isoflurane anesthesia on day 14 i.n. with *C. muridarum* (Cmu) (ATCC VR-123), 100 inclusion-forming units, in 30 μ L sucrose phosphate glutamate [SPG] buffer. Sham-sensitized controls received saline sensitization with Alhydrogel and the subsequent Ova challenges. AAD was

characterized on day 35. Some mice were treated with dexamethasone (DEX) (2mg/kg, Sigma-Aldrich) i.n. or 4-octyl itaconate (OI) (5mg/kg i.n.) on day 32-34.

Lung function (airway resistance or R_n) was measured by anesthetizing mice with ketamine (100mg/kg) and xylazine (10mg/kg, Troy Laboratories, Smithfield, Australia) and cannulating their tracheas (tracheostomy with ligation). FlexiVent apparatus (FX1 System, SCIREQ, Montreal, Canada) was used to assess airway-specific resistance (R_n , tidal volume of 8mL/kg at a respiratory rate of 450 breaths/min) in response to increasing doses of nebulized methacholine (Sigma-Aldrich). Assessments were performed at least three times per dose of saline/methacholine and the average calculated.

Mice were sacrificed on day 35, and their lungs were flash-frozen. Lung tissue was then used for subsequent RNA extraction or protein isolation for qPCR and immunoblot analysis, respectively. Macrophage counts were obtained from May Grunwald Giemsa stained bronchoalveolar lavage fluid cells using a light microscope (Essilfie et al., 2015; Kim et al., 2017a, 2017b).

METHOD DETAILS

RNA extraction and qPCR

Media supernatant was removed, cells were washed with PBS and lysed in 350 μ L RNA lysis buffer (Invitrogen). One volume 70% EtOH was added before transferring to Invitrogen RNA spin columns. The purification was followed as per manufacturer's instructions (Invitrogen PureLink RNA kit, ThermoFisher). Eluted RNA was quantified using a Nanodrop 2000 spectrophotometer and each RNA sample was diluted to the lowest yield before reverse-transcriptase PCR. cDNA was prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to manufacturer's instructions. qRT-PCR was then performed using the PowerUp SYBR Green Master on a 7500 Fast thermocycler (Applied Biosystems), and Ct values were converted to $2^{(-\Delta\Delta Ct)}$ using the Ct of the housekeeping gene *18s* (BMDMs) or *Tbp* ($CD4^+$ T cells).

Western blotting

Media supernatant was removed, and cells were lysed in 5x Laemmli Sample Buffer containing 10mM Na_3VO_4 or HALT Protease Inhibitor Cocktail (Thermo Scientific) and heated at 95°C for 5 minutes. Protein samples were resolved on SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane via wet transfer. Membranes were probed with primary antibodies at a 1:1000 dilution (or 1:10000 for β -actin) and secondary HRP-conjugated antibodies at a 1:2000 dilution. Membranes were visualized using WesternBright ECL HRP substrate (Advansta) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) on a GelDoc system (Bio-Rad). Images were analyzed with ImageLab (Bio-Rad) and ImageJ software. β -actin was used as reference housekeeping protein.

Flow cytometry

BMDM media supernatant was removed, cells were washed with PBS and then removed using a cell scraper. Macrophages were then treated with Fc γ -blocking antibody anti-mouse CD16/32 (Biolegend, clone 93) for 10 minutes, before they were stained with fluorochrome-conjugated antibodies for surface proteins in FACS buffer. The antibodies used were: PerCP/Cy5.5 anti-mouse F4/80 1:1000 (clone BM8, Biolegend), APC anti-mouse CD301 (MGL1/MGL2) 1:250 (clone LOM-14, Biolegend), PE anti-mouse CD206 1:250 (clone C068C2, Biolegend), PE anti-mouse CD124 (IL-4Ra) 1:250 (clone I015F8, Biolegend). To analyze BMDM expression of M2 surface markers, cells were first gated on F4/80 $^+$ followed by CD206, CD301, or CD124. For annexin V/PI staining to measure cell death, cells were scraped and washed with PBS, then resuspended in cold annexin V binding buffer (Biolegend). Anti-annexin V Pacific Blue (Biolegend) and propidium iodide (PI) (Invitrogen) were added according to manufacturer's instructions (Annexin V Apoptosis Detection Kit, Biolegend). Cells were incubated for 15 minutes, then additional annexin V binding buffer was added, and cells were run on the flow cytometer. For intracellular cytokine staining in $CD4^+$ T cells, cells were collected, washed, and re-stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) + 1 μ g/ml ionomycin + 10 μ g/ml brefeldin A (all from Sigma Aldrich). After 4 hours, cells were washed, fixed/permeabilized with fixation and permeabilization buffers (BioLegend) and stained with anti-mouse IL-13 (Biolegend). All samples were analyzed on a FACS Canto II Cell analyzer (BD Biosciences). Analysis of acquired data was performed with the FlowJo software (FlowJo LLC).

Seahorse metabolic assays

Mito Stress Tests were performed to measure oxygen consumption rate (OCR) of BMDMs using the Agilent Seahorse XF Cell Mito Stress Test Kit and system, according to manufacturer's instructions. Briefly, 50,000 or 100,000 cells per well were seeded overnight in a 96-well XF Cell Culture Microplate (Agilent) in DMEM medium containing DMEM media containing L-glutamine (Gibco) supplemented with FCS (10%) (Sigma-Aldrich), penicillin-streptomycin (1%) (Sigma-Aldrich). The morning of the assay, DMEM was removed, and cells were washed twice with Seahorse XF Base Medium (Agilent). XF Base medium containing 25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate was then added and cells were left in a CO $_2$ -free incubator for 1 h prior to the assay. OCR was measured on a Seahorse XF Analyser (Agilent). OCR was measured at three timepoints each, in basal conditions and following the addition of oligomycin (1.0 μ M), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 1.0 μ M), and rotenone/antimycin A (both at 0.5 μ M) (all from Agilent Seahorse XF Cell Mito Stress Test Kit), to determine maximum respiration and spare respiratory capacity of tested cells. Data was analyzed using the Seahorse XF software.

JAK1 kinase enzymatic activity assay

Jak1 kinase activity was measured biochemically using a kinase domain construct from an AbbVie in-house preparation. The reaction buffer contains 50mM MOPSO, pH 6.5, 10 mM MgCl₂, 2 mM MnCl₂, 0.1% BSA, 0.1 mM Na₃VO₄, and 2.5 mM DTT. A mixture containing final concentrations of 2 μM biotinylated-Fibroblast Growth Factor Receptor (bio-FGFR) peptide substrate (LCBiot-(Ahx)AEEYFFLFA-amide) (New England Peptide), 3.1 nM Jak1 kinase domain, 5% DMSO (Sigma) and a dose response of itaconate, OI (Sigma), tofacitinib, ruxolitinib, and staurosporine (AbbVie) was pre-incubated for one-hour at room temperature. The reaction was initiated with 1 μM final concentration of ATP (Promega) and incubated for one hour at room temperature. The reaction was quenched using 10 μL of 0.5 M EDTA, pH 7.5. Phosphorylated bio-FGFR was detected using a revelation buffer of 50mM HEPES, 400mM KF, 0.01% Tween-20, 0.1% BSA (all Sigma) supplemented with 11.3 ng/well phospho-tyrosine-specific antibody PT66K (Cisbio) and 78 ng/well Phycolink Streptavidin-APC (SAXL) (Prozyme). The reaction plate was stored at 4°C overnight and read on a PHERAstar plate reader using FRET settings with an excitation wavelength of 337nm and emission wavelengths of 620nm and 665nm.

Validation of ITalk labeling of JAK1

HEK293T cells were grown to 50% confluence in 15 cm dishes, then transfected with PCMV3-ORF-FLAG-JAK1 plasmid (Sino Biological) using Lipofectamine2000 for 24 hours, according to manufacturer's instructions. Subsequently, the cells were treated with 100 μM ITalk or DMSO for 12 hours. The cells were washed with PBS three times and centrifuged at 1000 rpm for 3 min. The cell pellets were stored at -80°C. Cell pellets were then resuspended in 1 ml ice-cold PBS containing EDTA-free Pierce Halt protease inhibitor cocktail. The cells were lysed by sonication on ice and lysates were collected by centrifugation (20,000 g, 30 min) at 4°C to remove debris. Protein concentration was determined by using a BCA protein assay kit. 50 μl lysate (2 mg/ml) was saved for the input portion, and the remaining portion (950 μl lysate, 2 mg/mL) was mixed with 1 mM CuSO₄, 100 μM TBTA ligand (Strem, 07-3215), 100 μM Biotin-Azide (ChemPep, 271604) and 1 mM TCEP (Sigma Aldrich, C4706) for 1 hour at room temperature. The resulting click-labeled lysates were centrifuged at 8000 g for 5 min at 4°C and washed twice with 1 mL cold methanol. The proteins were resuspended in 1 mL PBS containing 1.2% SDS via sonication and heating (5 min, 90°C). Samples were then diluted with 5 mL of PBS for a final SDS concentration of 0.2%. The solutions were then incubated with 100 μl of streptavidin-agarose beads (Thermo Fisher Scientific, 20353) for 3 hours at 29°C, followed by washing with 5 mL PBS three times, and 5 mL distilled water three times. 40 μl loading buffer was added in the resulting beads and heated for 10 min (95°C), centrifuged at 2000 g for 3 min and supernatant was collected for elution input. The input and elution were resolved on 6% SDS-PAGE gels, and JAK1 was detected via Western Blot using monoclonal anti-flag M2 antibody (Sigma, F3165).

Identification of JAK1 modification sites by ITalk

HEK293T cells were grown to 70% confluence in 15 cm dishes, then transfected with PCMV3-ORF-Flagged-JAK1 plasmids using Lipofactamine 2000 for 24 hours. Cells were then washed with PBS three times, centrifuged at 1000 rpm for 3 min and stored at -80 °C. The cell pellets were lysed via sonication in 1 mL ice-cold PBS containing EDTA-free Pierce Halt protease inhibitor cocktail. Cell lysates were collected by centrifugation (20,000 g, 30 min) at 4 °C to remove the debris. Protein concentration was determined via BCA protein assay. 1 mL cell lysates (2 mg/mL) were incubated with 1 mM ITalk in 37 °C for 2 hours, then resulting lysates were precipitated with 4 mL methanol, 1 mL chloroform and 3 mL Milli-Q water. The precipitated proteins were centrifuged at 4000 g for 10 min at 4 °C and washed twice with 500 μL cold methanol and resuspended in 1 mL PBS containing 0.4% SDS. 1 mL cell lysates (2 mg/mL) were then reacted with 1 mM CuSO₄, 100 μM TBTA ligand (Strem, 07-3215), 1 mM TCEP (Sigma Aldrich, C4706) and 100 μM acid-cleavable azide-biotin (DADPS Biotin Azide, cat. no. 1330-5) for 1 h at room temperature. The resulting click-labeled lysates were precipitated by 4 mL methanol, 1 mL chloroform and 3 mL Milli-Q water. The precipitated proteins were centrifuged at 4000 g for 10 min at 4 °C, washed twice with 500 μL cold methanol, and then resuspended in 1 mL PBS containing 1.2% SDS. 100 μL streptavidin beads (Thermo Fisher Scientific) were washed three times with 1 mL PBS and resuspended in 5 mL PBS, which was added to the protein solution. The beads were incubated with the protein solution for 4 h at 29°C, then washed with 5 mL PBS three times, then 5 mL distilled water three times. The resulting beads were resuspended in 500 μL PBS containing 6 M urea, incubated in 10 mM DTT at 37 °C for 30 min, and added with 20 mM iodoacetamide for 30 min at 35 °C in the dark. The beads were then collected by centrifugation and resuspended in 200 μL PBS containing 2M urea, 1 mM CaCl₂ and 10 ng/μL trypsin (Promega). Trypsin digestion was performed at 37 °C with rotation overnight and the beads were washed with 200 μL distilled water three times. Release of the modified peptides from the beads was carried out by incubating the beads with 200 μL of 2% formic acid/water for 1 h with gentle rotation in 25 °C. After centrifugation, the supernatant was collected. Then the cleavage process was repeated, and supernatant was combined. In addition, the beads were washed with 50% acetonitrile/water containing 1% formic acid (400 μL), and the washes were combined with the supernatant to form the cleavage fraction. Sample was dried in a vacuum centrifuge and stored at -30 °C until LC-MS/MS analysis.

Data analysis of JAK1 modification by ITalk

To identify the modification sites, LC-MS/MS data was analyzed using ProLuCID (Xu et al., 2015) with static modification of cysteine (+57.0215 Da) as well as variable modifications of +325.16378 Da on cysteine. The MS/MS spectra of the peptide containing ITalk modification were generated using the pFind software (Chi et al., 2018).

Profiling of OI modification in human macrophages

Human PBMCs were isolated from human blood using Lymphoprep (Axis-Shield). Whole blood (30 ml) was layered on 20 ml Lymphoprep and spun for 20 min at 2,000 r.p.m. with no brake on. The PBMC layer was isolated and CD14⁺ monocytes were sorted using magnetic-activated cell sorting (MACS) CD14 beads. Cells were plated at 1×10^6 cells ml⁻¹ in RPMI supplemented with 10% (v/v) FCS, 2 mM L-glutamine, and 1% penicillin/streptomycin solution containing M-CSF (50 ng ml⁻¹) and maintained at 37 °C, 5% CO₂ for 6 days, to allow differentiation into macrophages. The macrophages were pre-incubated with DMSO or 125 μM OI for 2 hr prior to incubation with 150 μM ITalk for 2 hr. The cells were trypsinized and pelleted. The cells were lysed in 0.5 mL ice-cold PBS buffer containing EDTA-free Pierce Halt protease inhibitor cocktail with sonication. The cell lysates were collected by centrifugation (20,000 g, 30 min) at 4 °C to remove the debris. The protein concentration was determined by using the BCA protein assay kit. 500 μg cell lysates were reacted with 1 mM CuSO₄, 100 μM TBTA ligand, 100 μM azide-biotin, and 1 mM TCEP for 1 h at room temperature. The resulting click-labeled lysates were precipitated by methanol/chloroform and centrifuged at 1400 g for 20 min at 4 °C and washed twice with cold methanol. The proteins were re-solubilized with 6 M urea containing 1% SDS, incubated with 10 mM DTT at 65 °C for 15 min, followed by addition of 20 mM iodoacetamide for 30 min at room temperature in the dark. 100 μL Pierce streptavidin plus ultralink beads (Thermo Fisher Scientific) were washed for three times with 1 mL PBS, and resuspended in 5 mL PBS, which was added to the protein solution. The resulting solution was incubated overnight at 4 °C, followed by washing with 5 mL PBS containing 1% SDS for three times, 5 mL PBS for three times, and 5 mL distilled water for three times. The beads were then collected by centrifugation and resuspended in 200 μL 100 mM TEAB buffer (pH 8.5). 0.5 μg of trypsin/Lys-C was added to the beads for overnight digestion at 37 °C with shaking at 500 rpm. TMT-duplex reagents (0.2 mg in each reagent) were resuspended in dry acetonitrile, added to the digested samples, and incubated for 1 hr at room temperature with shaking at 500 rpm. Afterwards, the TMT labeling reaction was terminated by adding 50% hydroxylamine (0.3% final concentration) to the mixture and incubate for 15 min. The labeled peptide samples were dried in speedvac and resuspended in 300 μL 0.1% TFA and subjected to Pierce High pH Reversed-Phase Peptide Fractionation Kit following manufacturer's protocol.

Quantitative profiling of OI modified cysteinome

THP1 cells were seeded into 6 cm petri dishes (4 million per dish) in 5 ml media, and treated with DMSO, 125 μM OI or 250 μM OI, respectively, for 16 hours in a cell culture incubator. 10 dishes of cells were treated (4 dishes with DMSO, 3 dishes with 125 μM OI and 3 dishes with 250 μM OI) in each replicate experiment for utilizing TMT-10plex isobaric label reagent. Cells were harvested and washed with PBS twice. Each cell pellet was lysed on ice with 100 μL of 0.5% CHAPS buffer containing 1x EDTA-free Pierce Halt protease inhibitor cocktail and 1x benzonase for 30 min. Lysate was collected by centrifugation (20,000 g, 15 min) at 4 °C to remove debris. Protein concentration was determined using a BCA protein assay kit. Lysates (0.4 mg from each dish, 4 mg/ml) were then incubated with 500 μM of IA-DTB for 1 hour, followed by 5 mM DTT for 30 min, and 20 mM iodoacetamide for 30 min at room temperature in the dark. The resulting lysate was precipitated by 400 μL methanol, 100 μL chloroform, and 300 μL water. The precipitated protein was centrifuged at 4000 g for 10 min at 4 °C, washed twice with 500 μL methanol with sonicating briefly each time to break up the protein pellet. Washed protein pellet was resuspended in 200 μL of 0.2 M EPPS buffer (pH 8.5) with brief sonication and digested with 8 μg of trypsin/Lys-C overnight at 37 °C shaking at 500 rpm. TMT-10plex reagents (0.8 mg in each reagent) were resuspended in dry acetonitrile, added to the digested samples, and incubated for 1 hr in a thermomixer at room temperature with shaking at 500 rpm. Afterwards, the TMT labeling reaction was terminated by adding 50% hydroxylamine (0.3% final concentration) to the mixture and incubate for 15 min. The labeled peptide samples were dried in a speedvac. 600 μL of 50% streptavidin resin slurry was washed with 10 mL PBS three times. Dried peptides were resuspended in 1 mL PBS and pooled together to incubate with the streptavidin resin overnight at 4 °C. Resin was washed three times each with PBS, followed by PBS containing 0.1% SDS, and water to remove non-specific binding peptides from the resin. Resin-bound peptides were eluted by incubating the resin with 500 μL of elution buffer (50% acetonitrile + 0.1% TFA) in a Thermomixer shaking at 1000 rpm for 10 min. Resin was centrifuged to collect supernatant. Repeat elution with 200 μL of elution buffer and combined supernatant was dried in speedvac. The dried peptide was resuspended in 300 μL 0.1% TFA and subjected to Pierce High pH Reversed-Phase Peptide Fractionation Kit following manufacturer's protocol.

Fractionated peptides were reconstituted in 20 μL 0.1% formic acid. Samples were then analyzed by LC-MS/MS on a Fusion Lumos mass spectrometry. Around 0.5 μg of total peptides from each fraction were analyzed on an UltiMate 3000 nano-LC system (Thermo) coupled via a 25-cm C18 column (EASY-Spray, Thermo). Peptides were separated at a flow rate of 250 nL min⁻¹ with a linear 110 min gradient from 2% to 35% solvent B (100% acetonitrile, 0.1% formic acid). Each sample was run for 120 min, including sample loading and column equilibrating times. Data were acquired in data-dependent mode. MS1 spectra were measured with a resolution of 120,000, an automatic gain control target of 4e5, maximum injection time 50 ms, and a mass range from 300 to 1800 m/z. MS2 spectra were collected using top-speed mass spec method within 3 s of cycle time at a resolution of 50,000, an automatic gain control target of 8e5, an isolation window of 1.0 m/z and a normalized collision energy of 38.

All raw data were analyzed with MaxQuant software version 1.6.15.0. Spectral searches were performed using 10plex TMT as the isobaric labels. Carbamidomethylation of cysteine residues was set as fixed modification, while oxidation of methionine residues, acetylation of protein N-term and IA-DTB on cysteine residues were set as variable modifications. Competition ratio was calculated by dividing the TMT reporter ion intensities from control channel (DMSO) by the OI treated channel.

JAK1 structural modeling

The model of the JH1-JH2 domains of JAK1 was created by superimposing coordinates for the JAK1 JH1 (PDB: 5WO4) ([Siu et al., 2017](#)) and JH2 (PDB: 4L00) ([Toms et al., 2013](#)) domains onto a previously solved JAK family JH1-JH2 structure (PDB: 4OLI) ([Lupardus et al., 2014](#)). The overall complex was created and minimized in the program MAESTRO (Schrödinger Release 2021-2: Maestro, Schrödinger, LLC, New York, NY, 2021). 4 cysteine residues were highlighted in spheres. The model of itaconate covalently bound to Cys817 was created using CovDock in Schrödinger Release 2021-2 ([Zhu et al., 2014](#)).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were carried out using GraphPad Prism 9 (GraphPad) software. A Student's t test was used to compare two groups affected by a single variable, whereas a one-way ANOVA followed by Sidak's multiple comparisons test was used to compare multiple groups. Details of the n number (representing biological replicates) can be found in the appropriate figure legend. Data are displayed as mean $\pm$ SEM or as individual data points. Differences were considered statistically significant at P values of $p \leq 0.05$. ** signifies a P value of ≤ 0.01 , *** signifies a P value of ≤ 0.001 , and **** signifies a P value of < 0.0001 .

Is Citrate A Critical Signal in Immunity and Inflammation?

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Abstract

When immune cells are activated, they undergo metabolic change in order to have sufficient energy to function effectively. The Krebs cycle is one of the most important pathways involved in this response and citrate, a critical component of this pathway, regulates carbohydrate and lipid metabolism. Recently, citrate has also emerged as a key immunometabolite, exerting specific effects on immunity and inflammation. Citrate has been shown to be involved in the production of prostaglandins and nitric oxide, and via the generation of malonyl-CoA and acetyl-CoA in the cytosol, to impact cytokine production. Citrate can also generate itaconate via aconitate, which promotes an anti-inflammatory response. All these findings support citrate as a crucial signal for immune cell activation and function.

In this review we will describe the various ways through which citrate participates in immune cell behaviour and speculate on the targeting of citrate as a possible novel therapeutic approach for immune and inflammatory diseases.

Keywords: Citrate; Krebs cycle; immunometabolism; itaconate; inflammation; ATP-citrate lyase

Introduction

Citrate, or citric acid, is one of the most common acids in plants and a metabolic product in aerobic organisms. Beyond its reputation for its anticoagulant activity, first described in the early 1990s [1], citrate is well known as a master metabolite in living cells.

Citrate is one of the most important intermediates in Krebs Cycle (also called the citric acid cycle) and has several roles depending on whether it remains inside mitochondria or is released into the cytosolic compartment [2].

It is a fundamental intermediate in aerobic nutrient-derived energy production and it was subsequently shown to link carbohydrate metabolism to fatty acid metabolism [3].

Much more recently, citrate is generating much interest in the areas of immunometabolism and immune cell activation [4]. Here, we explore the role of citrate in inflammation and how it can modulate immune cell responses via multiple mechanisms. In many ways, citrate

has been reborn as a metabolite critical for the immune system.

Krebs cycle

Krebs cycle, elucidated by Sir Hans Krebs, is a bioenergetic pathway occurring in the mitochondrial matrix of all eukaryotic organisms. It is an essential process for energy generation from diet carbon-based nutrients. Krebs was studying how bird muscle tissue takes up oxygen very quickly in the presence of carbohydrates and hypothesized that carbohydrate metabolism should occur through a cyclic series of reactions [5].

Glucose and lipid catabolism-derived acetyl-CoA is redirected to the Krebs cycle in order to generate ATP and redox coenzymes. These redox cofactors will be then used to produce the energy necessary for the cell through the mitochondrial electron transport chain, during a process known as oxidative phosphorylation (OXPHOS).

We now know however that Krebs cycle rewiring occurs in activated immune cells and is pivotal for protection

against viral and bacterial infection. This process, known as metabolic reprogramming [6], is essential for the production of energy and metabolites that can also act as immune signalling molecules.

Early metabolomic studies on macrophages activated with the Gram negative bacterial product LPS revealed that Krebs cycle undergoes fragmentation at two important points; these breaks lead to the withdrawal of metabolic intermediates from the cycle [3,7,8]. A breakpoint at SDH (succinate dehydrogenase) leads to succinate accumulation which promotes HIF1 α stabilization and boosts the expression of inflammatory genes such as that encoding IL1- β .

Succinate oxidation via SDH however can also boost HIF1- α via reactive oxygen species (ROS) generated by mitochondrial complex I, indicating complexity in the role of succinate regarding HIF1- α [3]. The second breakpoint occurs at the level of isocitrate dehydrogenase (IDH), which is responsible for the conversion of isocitrate into α -ketoglutarate [9]. This block will lead to citrate accumulation which has various downstream consequences for inflammatory cell activation.

Krebs cycle as the only source of endogenous citrate has been considered so far and if microorganism infections could somehow boost or impair citrate uptake from the extracellular environment is a question that still need to be answered.

Citrate as an immunomodulator: the role of CIC

Few studies have actually received attention about the role of citrate during viral and bacterial infections in animal cells. Citrate salts were previously used to protect plants against phytopathogens while reducing the use of pesticides. For example, citrate has been shown to protect lettuce from four different species of *Shigella* [10]. Important evidence of a role for citrate in blocking microbial infection in mammals has also been indicated in a study by Hojo et al., who found that oral bacteria such as *Fusobacterium Nucleatum*, *Streptococcus Mutans* and *Streptococcus Pneumoniae* are susceptible to sodium citrate, although the underlying mechanisms have not been defined yet [11]. Citrate also inhibits the growth of the Gram negative bacteria *Arcobacter butzleri* [12] thanks to its acid pH and has antibacterial activity also against Gram positive *Staphylococci*, including MRSA strains [13].

As regarding citrate role during viral infection, the iron salt ferric ammonium citrate (FAC) blocked Influenza A virus, ZIKA virus, HIV virus and Enterovirus 71 (EV71) infections [14]. *In vitro*, in A549 human lung epithelial cell line and in human monocytic THP-1 macrophages,

FAC inhibits IAV PR8 infection and virus-induced IL1- β production [14]. *In vivo*, mice treated with FAC after IAV PR8 airways injection significantly blocked viral replication dampening IL1- β , IFN- β , CCL2, IL-6 and TNF- α expression, decreasing the number of migrated neutrophils to the lungs and lengthening mice survival [14]. FAC treatment had a significant effect also on HIV-1 through the inhibition of infection in human PBMC-differentiated dendritic cells and the related virus-induced immune response mediated by IL-6, IFN- β and IL1- β [14]. Moreover, Vero cells infected with Zika virus showed low viral RNA 72 hours post FAC treatment while mice injected with the same virus displayed decreased weight loss and longer survival upon ferric citrate supplement [14]. Interestingly, FAC decreased EV71 replication in THP-1 macrophages, thus leading to inhibition of NLRP3 inflammasome-dependent Caspase-1 and IL1- β release [14]. Indeed, FAC impaired the induction of the oligomerization of NLRP3 inflammasome adapter protein ASC [14].

Regarding the immunomodulatory role of citrate, most of the work has been carried out on macrophages specifically exploring the effect of LPS. In THP-1 cells at high concentrations, when calcium availability was limiting due to sequestration by citrate chelation, citrate inhibited TNF- α and IL-8 mRNA production following LPS stimulation [15]. However, when calcium availability was not limiting, as can be seen with low citrate concentrations, citrate augmented TNF- α and IL-8 mRNA production [15]. Indeed, citrate treatment promoted histone H3 and H4 acetylation because of acetyl-CoA production by ATP citrate lyase (ACLY) (Figure 1). H3 lysine 9 acetylation, H4 lysine 8 acetylation and H4 lysine 12 acetylation were augmented significantly in THP-1 cells incubated with citrate compared to controls at both the TNF- α and IL-8 promoter regions [15]. This demonstrates how citrate drives inflammatory gene expression via histone acetylation. Ji-Sook et al. described the use of calcium citrate, the calcium salt of citric acid, on the mouse macrophage cell line RAW 264.7 cells [16]. Calcium citrate strongly reduced LPS-induced ROS generation and rescued the activity of antioxidant defence enzymes such as Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Figure 2) [16]. Exposure to LPS enhanced NO production and IL-6, IL-1 β and TNF- α expression, which were all suppressed by calcium citrate treatment. NF- κ B, iNOS and COX-2 expression were augmented after LPS immune cells activation and then significantly inhibited by calcium citrate (Figure 2) [16]. These early studies highlighted the difficulties of understanding the role of citrate in macrophages.

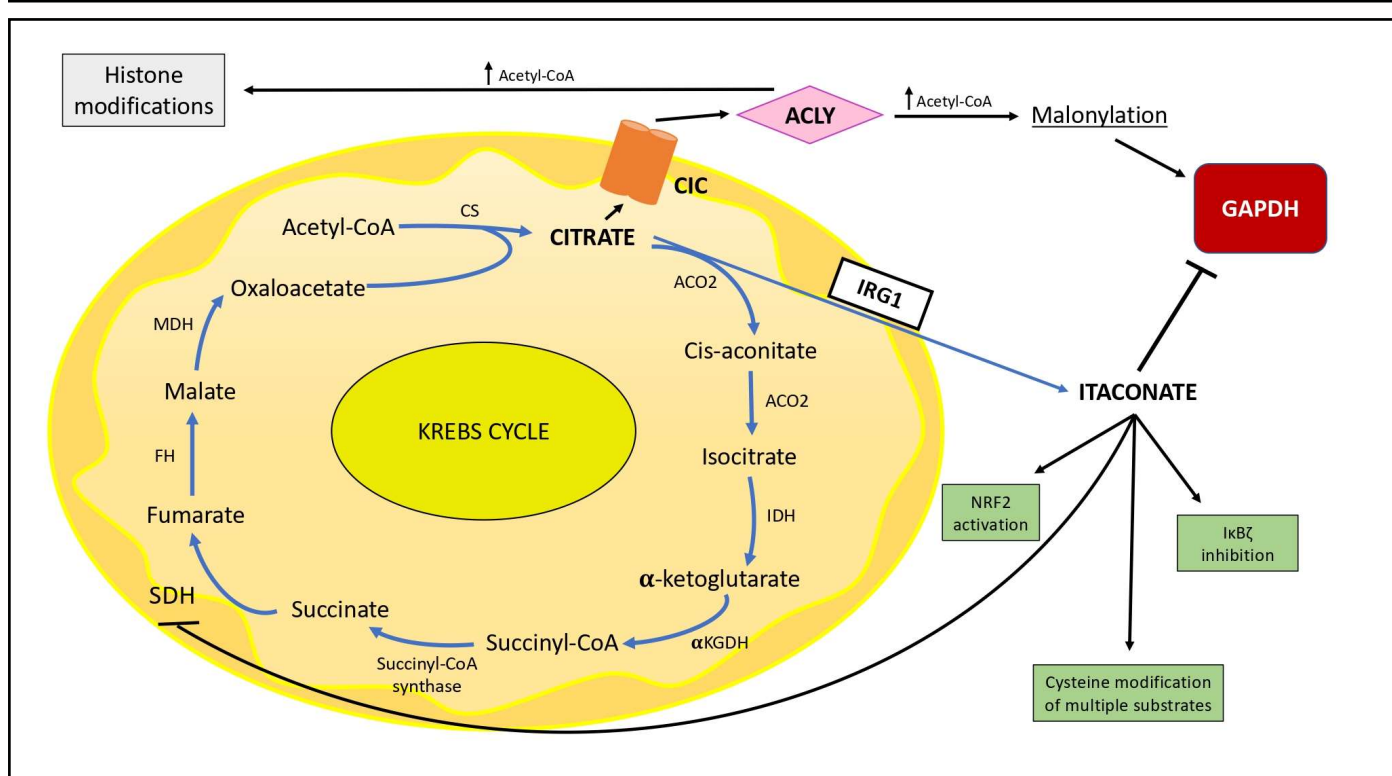


Figure 1: The multiple roles of citrate in inflammatory macrophages. Citrate accumulates during Krebs cycle and exits mitochondria through its transporter CIC. In the cytosol, ACLY converts citrate into oxaloacetate and acetyl-CoA. Acetyl-CoA leads to both histone modifications and malonylation, which modulates the glycolytic enzyme GAPDH. Citrate generates itaconate through IRG1 decarboxylase. Outside mitochondria, itaconate inhibits GAPDH and SDH activity and drives Nrf2 activation and IκBζ inhibition. Itaconate also modifies multiple target proteins on Cysteine (ex. LDH-A and GAPDH) which might also contribute to its anti-inflammatory effect.

The first study clearly pointing to a role for citrate in macrophage activation was published by Infantino et al. [17] in 2011 and showed for the first time citrate carrier (CIC or also called SLC25A1) involvement in inflammation. CIC transports citrate from the mitochondria in exchange for cytosolic malate. Infantino demonstrated that CIC makes citrate available for fatty acid-dependent generation of lipid inflammatory modulators like prostaglandins (PGs) [17]. CIC mRNA levels were increased in LPS-stimulated macrophages while CIC silencing or inhibition in activated macrophages led to a significant decrease in the amount of NO, PGE2 and ROS. An *in silico* analysis for the human CIC gene promoter found two NF-κB-responsive elements [17]. All these outcomes propose CIC as a NF-κB target gene required for LPS-driven inflammation through citrate pool availability.

The key role of CIC was confirmed in human macrophage cell line U937 treated with TNFα and IFN-γ alone or in combination showing an upregulation of CIC mRNA [18]. Inhibition of IκB kinase, whose activity is required for NF-κB function, or the use of p50-specific antibody reduced CIC mRNA and protein levels after TNFα stimulation [18]. This indicated a NF-κB dependent mechanism for TNFα-

driven citrate carrier induction. Concurrently, the use of a STAT inhibitor strongly decreased CIC mRNA and protein compared to cells only treated with IFN-γ and showed that CIC is boosted by IFN-γ through a STAT-dependent mechanism [18]. In CIC-silenced cells induced with TNF-α and IFN-γ, alone or in combination, there was a prominent decrease in PGE2, NO and ROS levels (Figure 2) [18]. This work displays how CIC is responsible for pro-inflammatory mediator production after cytokine-mediated activation of macrophages.

A recent publication by Avantaggiati et al. [19] might shed light on a possible mechanism for citrate contribution to inflammatory disease pathogenesis. The role of mitochondrial citrate carrier CIC was examined in non-alcoholic fatty liver disease (NAFLD) and the development of inflammatory steatohepatitis (NASH). Impairment of CIC by an inhibitor called CTPI-2, which competitively binds to its citrate-binding pocket, or complete knockout (KO) of the *CIC* gene lead to lowering of IL-6 and monocytes chemoattractant protein-1 (MCP1) levels and increased IL-10 and IL-4 [19]. Liver tissue resident macrophages, also known as Kupffer cells, were highly increased following a high fat diet (HFD) in mice and the positive

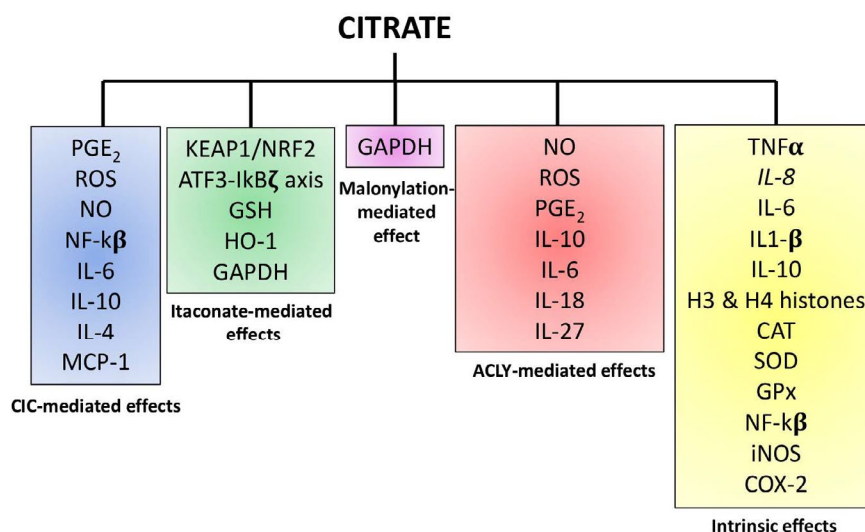


Figure 2: Targets for citrate in immunity and inflammation. Citrate withdrawal from the Krebs cycle and its exit from the mitochondria allows it to exert several effects in the cytosol. These could be citrate-intrinsic effects or indirectly mediated by citric acid.

Citrate plays an immunomodulatory role through the IRG1-dependent production of itaconate and its transmembrane carrier CIC. Its availability in the cytosol provides acetyl-CoA pool for ACLY-mediated and malonylation-mediated regulation of genes and proteins involved in inflammation signaling and oxidative stress development, such as IL1-β, COX-2, NO and PGE2.

trend was reversed by the use of CTPI-2. CIC inhibition significantly blocked inflammatory M1 macrophage polarization and tissue infiltration, as evidenced by TNF-α and iNOS expression [19]. Alternative pathways that could compensate for citrate deficiency involve isocitrate dehydrogenases 1 and 2 (IDH 1 and 2) and acetyl-CoA synthetases (ACSS 1 and 2), that synthesize acetyl-CoA from acetate. CTPI-2 reduced both of these enzymes and increased mRNA and protein expression of SLC13A5, a transporter which takes up citrate from the extracellular environment [19]. This showed that extracellular citrate can be the only source of cytosolic acetyl-coA in absence of CIC.

CIC is thus essential for the role of citrate as a signal in immune cells and, hence, it could be a candidate target for treatment of immune diseases. Counteracting CIC activity through CIC inhibitors or CIC silencing could induce an anti-inflammatory and resolution response while dampening local inflammation development.

Citrate and ACLY

Beyond citrate transport, another process that has been shown to be important for citrate in inflammation involves the enzyme ATP-dependent citrate lyase (ACLY). ACLY promotes citrate conversion to acetyl-CoA and

oxaloacetate outside mitochondria. It drives acetyl-CoA pool availability and sustains cell activation in innate and adaptive immunity (Figure 1) [3]. The Infantino study highlighted the importance of citrate export from the mitochondria for its immunomodulatory effects. This has been confirmed in studies on ACLY which has been implicated in LPS-induced epigenetic changes [20]. The resulting acetyl-CoA can feed the mevalonate pathway for cholesterol formation, promote fatty acid synthesis for membrane biogenesis or be a substrate for acetylation of proteins including histones, thus participating in epigenetic modifications [3,20]. ACLY guides acetylation in Toll like receptor (TLR)-dependent gene expression via acetyl-CoA derived from citrate [19].

It is well known that genes encoding for inflammatory mediators are epigenetically regulated via acetylation and methylation in their promoter regions and on histones [21-23], thus driving silencing or activation of transcription. The Infantino et al. study had underlined the indispensable role of ACLY in macrophages activation, during which LPS, IFNγ and TNFα up-regulate ACLY protein and mRNA expression [24]. It has also been shown that the inhibition of ACLY by radicicol decreased NO, ROS and PGE2 production in activated THP- 1 cells (Figure 2) [24], pointing to its role in modulating inflammatory mediator production.

ACLY induction during macrophage activation was confirmed in 2019 by Lauterbach et al., who examined the LPS-TLR4 axis in macrophage metabolism and histones acetylation. They found that LPS increased the extracellular acidification rate (ECAR) in bone marrow-derived macrophages within 2 hours after stimulation, thus increasing glycolysis [25]. LPS signalling resulted in increased maximal respiration in macrophages when measured 8 hours after LPS exposure. In contrast, LPS activation of BMDMs for 24 hours led to decreased basal OXPHOS as measured by oxygen consumption rate (OCR) [25]. These data suggested that mitochondrial metabolism reacts rapidly to TLR4 signalling and that the early response, relevant for gene transcription, differed from that obtained after 24 hours of LPS stimulation [25].

LPS increased CXCL1, IL-6, IL-1 β expression and synthesis of some important metabolites of the Krebs cycle such as succinate, lactate and citrate. The change in citrate encouraged them to analyse ACLY expression, which was found to be induced through a MyD88 and TRIF-dependent mechanism [25]. RNA-seq of LPS-stimulated BMDMs treated with ACLY inhibitor showed increased mRNA expression of IL-10 and IL-1R antagonist (IL-1RA) and decreased mRNA expression of IL-6, IL-18, IL-27, identified as ACLY target genes regulated by histone modification (Figure 2) [25].

ACLY can be in turn regulated by acetylation [26] and phosphorylation by Akt [27]. The latter is part of IL-4 mediated M2 macrophages activation. IL-4 treatment of BMDMs increased glucose uptake and glycolysis and induced expression of M2 genes *Arg1*, *Retnla*, *Mgl2* [27]. The use of an Akt inhibitor reduced *Arg1*, *Retnla* and *Mgl2* induction, suggesting an Akt-dependent mechanism for M2 polarization [27]. To understand how Akt regulates M2 genes, histones acetylation in IL-4 treated macrophages demonstrated that IL-4-R axis enhanced acetylation of H3 and H4 histones while the Akt inhibitor strongly decreased it. IL-4 stimulation promoted the phosphorylation of ACLY by Akt, thus leading to M2 gene expression [27]. Indeed, ACLY inhibition abolished Akt-dependent M2 polarization, identifying ACLY as a master regulator for M2 activation.

Another study by Dekoter et al. revealed a new role for ACLY during macrophage differentiation. Myeloid progenitor cells differentiate into macrophages and upregulate the transcriptional factor PU.1, essential for their development [28]. The induction of PU.1 expression in BN cells, myeloid precursor cells lacking PU.1 factor, downregulated mRNA levels of ACLY and reduced cell cycle progression. The same downregulation was observed also in M-CSF-dependent differentiation of bone marrow cells [28]. ACLY inhibitor treatment on cultured BN cells

abolished cell proliferation, indicating ACLY as the enzyme involved in cell cycle promotion. These outcomes led to study on whether acetate or acetyl-CoA supplementation could rescue cell cycle progression. Both metabolites increased cell cycle progression of BN cells and suggested that ACLY-dependent synthesis of acetyl-CoA is required for macrophage maturation [28].

Apart from macrophages, ACLY expression has been detected in T and B lymphocytes. Bacterial LPS induces ACLY expression and enzymatic activity in naïve B lymphocytes, thus driving their differentiation into Ig-secreting plasma cells through the support of glucose-dependent *de novo* lipogenesis aimed at expanding the lymphocyte endomembrane network [29]. Moreover, ACLY phosphorylation on Ser455 by Akt in T lymphocytes is induced by IL-2 and promotes acetylation of cell cycle regulating genes thereby driving to CD4⁺ T cells proliferation [30].

All these studies point to ACLY as a key driver for innate and adaptive immune cell activation via its role in promoting acetylation. Given the role of ACLY in immune cells, its inhibition might have potential as an anti-inflammatory therapeutic. *In vivo* studies are necessary to thoroughly characterize this enzyme and assess if its expression could be modulated by citrate itself. We know from previous literature that homozygous *Acly* knockout mice died early in development. So heterozygous mice for ACLY can be a suitable model for studying its therapeutic potential.

Post-translational modifications caused by citrate

Post-translational modifications (PTMs) are reversible modifications of specific aminoacids that can change the tertiary structure and consequent function of proteins. Acetyl-CoA provokes acetylation, a PTM that prominently occurs on lysine on histones [4]. Histones can also receive an acetyl group from substrates such as malonyl-CoA, succinyl-CoA, crotonyl-CoA, glutaryl-CoA, to as lysine acylation [31].

Apart from acetylation, malonylation is another post-translational modification which might regulate protein function [32]. This also depends on citrate, since malonyl-CoA is generated from acetyl-CoA, which as we saw in the preceding section comes from citrate (Figure 1). Malonyl-CoA is generated by acetyl-CoA carboxylase ACC1 (of which there are cytosolic and mitochondrial isoforms), while the reverse reaction is catalysed by malonyl-CoA decarboxylase (MCD) [33]. The major role of malonyl-CoA is as an intermediate in *de novo* fatty acid synthesis and elongation [34]. It blocks β -oxidation of fatty acids through allosteric inhibition of carnitine-palmitoyl

transferase (CPT1) [35-37]. Lysine residues can however be modified by malonyl-CoA [32]. This makes Lysine residues negatively charged and SIRT5 can control this process by acting as a demalonylase [38-40].

The first report of malonylation as an inflammatory signal was shown in LPS treated BMDMs where it increased expression of ACC1 and mitochondrial malonyl-CoA synthetase 3 (ACSF3) [41]. Boosting malonyl-CoA production led to malonylation of many proteins and in particular of the glycolytic enzyme GAPDH protein on Lys 213 [41]. GAPDH is required for the induction of cytokines such as IL1- β and IL-6, but a more relevant role shows up in TNF- α modulation [41]. GAPDH has RNA-binding activity and binds to AU-rich elements in TNF- α mRNA in resting macrophages, thus blocking its translation [41]. LPS stimulation induces GAPDH malonylation leading to its dissociation from TNF- α mRNA, so that TNF α can be translated [41]. This suggests that inhibiting ACC1 (which also has a role in Th1 cell development) or ACSF3 (which also is toxic to mitochondria) could have anti-inflammatory potential.

It remains unknown whether malonylation affects other proteins involved in inflammatory signalling, apart from GAPDH. It is also unknown whether malonylation occurs when other inflammatory stimuli, such as TNF- α and IFN- γ , activate immune cells. ACC1 is one of the enzymes making malonyl-CoA available for fatty acid biosynthesis, an event known to be pro-inflammatory. Further studies should address whether ACC1 inhibition might modulate malonylation and consequently inflammatory response.

Upregulation of SIRT1 function might decrease citrate-mediated malonylation of proteins but specific enzyme-amino acid interactions have to be sharply identified.

Citrate as a regulator of itaconate

A final recent aspect concerning citrate is its role in generating itaconate, which is derived from decarboxylation of cis-aconitate, the intermediate product of citrate conversion to isocitrate by aconitase (ACO2) during the second step of Krebs cycle [42]. The shift in Krebs cycle towards itaconate production is a prominent consequence of LPS-dependent activation in macrophages [43]. The enzyme responsible for itaconate generation, cis-aconitate decarboxylase, is encoded by the *IRG1* gene, first discovered in 1995 [44].

The first studies about itaconate action focus on its anti-microbial and growth inhibition activity towards various microorganisms [45-47]. A key target of itaconate was isocitrate lyase (ICL), the enzyme responsible for the cleavage of isocitrate into succinate and glyoxylate during the first step of the glyoxylate shunt, a process not present

in mammals [46]. Itaconate was then shown to have a role in mammalian cells and in particular to exert an anti-inflammatory effect. Itaconate production is induced in macrophages by LPS and IFN γ [48] and one of its targets is succinate dehydrogenase (SDH). This inhibits ROS generation from complex I, lowering IL1- β production in BMDMs [48].

Succinate levels in macrophages are strictly linked to itaconic acid metabolism. Itaconate reduces ROS/RNS (reactive nitrogen species) and improves neurological function in a model of ischemia-reperfusion confirming its role as a ROS regulator [49].

Another mechanism of action for itaconate is modulation of the transcriptional factor Nuclear factor erythroid 2-related factor 2 (NRF2) [50] (Figure 1), essential in driving anti-oxidant and anti-inflammatory responses [51], which in physiological conditions is controlled by Kelch-like ECH-associated protein 1 (KEAP1) [52]. A new post-translational modification – called 2,3-dicarboxypropylation – occurs on cysteines of KEAP1 in human and murine macrophages treated with the itaconate derivative 4-Octyl Itaconate (4-OI) [50]. This specific change inactivates KEAP1 making NRF2 free to translocate to the nucleus and induce transcription of anti-oxidant enzymes such as heme-oxygenase (HO-1) and glutathione (GSH) and repression of pro-inflammatory cytokines [50]. Endogenous itaconate is likely to regulate Nrf2 since this response was reduced in IRG1-deficient macrophages [52,53]. Multiple targets for 2,3-dicarboxypropylation were identified, notably of enzymes in glycolysis [50,54] but also inflammatory proteins such as NLRP3 and Gasdermins [55].

4-OI also modifies and inhibits GAPDH in LPS-stimulated RAW264.7 macrophages [56] (Figure 1), further indicating that GAPDH is a target for citrate-derived modification, as shown for malonylation (Figure 2). 4-OI alkylation of GAPDH on Cys22 via Michael addition inhibited its activity, hindering IL-1 β and iNOS expression [56].

Furthermore, dimethyl itaconate (DMI) abolishes LPS-dependent induction of I κ B ζ through ATF3 transcriptional factor (Figure 1) and blocks this axis responsible for secondary transcriptional responses in activated BMDMs [57]. DMI-treated bone marrow-derived macrophages (BMDMs) displayed upregulation of Nrf2 and its target genes *Hmox1*, *Nqo1* and *Gclm* [57]. DMI structure was found to act as an electrophile in Michael reactions and trigger electrophilic stress. Usually, electrophilic stress affects the glutathione (GSH) pool. DMI treatment decreased GSH intracellular concentration and increased ROS cell production [57]. The transcription factor I κ B ζ regulates secondary transcriptional response to TLR activation. DMI treatment totally abolished the LPS-

associated induction of I κ B ζ protein and inhibited its target genes, but did not change I κ B ζ mRNA expression, meaning that it has to be affected at the post-transcriptional level [57]. Indeed, electrophilic stress induced by DMI presence increased phosphorylation of macrophage eIF2 α and consequently the inhibition of I κ B ζ translation [57]. RNA-seq of DMI-treated WT and Nrf2 $^{-/-}$ BMDMs showed an upregulation of ATF3 transcriptional factor. *Atf3* $^{-/-}$ cells restored I κ B ζ protein levels upon DMI treatment and significantly increased IL-6 production [57]. Complete deficiency for *Atf3* in macrophages failed to increase eIF2 α phosphorylation after DMI treatment, thus confirming the ATF3-dependent effect of DMI on I κ B ζ regulation [57].

These studies reveal citrate as a precursor for itaconate production and its several actions as a crucial immunomodulatory metabolite. Itaconate could be a potential therapeutic treatment for inflammatory disease in the future. Other studies are needed to assess the existence of a cell surface receptor for itaconate and to know if some of the effects previously reported could be receptor-mediated effects. Moreover, itaconate could even boost a positive or negative feedback loop on citrate production and transport. Overexpression of *Irg1*, the enzyme generating itaconate, could lead to a rise in itaconate production and a subsequent activation of cellular anti-oxidant and anti-inflammatory response.

Concluding Remarks

In this review, we have discussed the role of citrate as a critical signal in immunity and inflammation. LPS-induced metabolic reprogramming in immune cells drives citrate pool availability and CIC and ACLY expression. A breakpoint across Krebs cycle leads to citrate withdrawal from the cycle and production of itaconate, an anti-inflammatory metabolite.

Outside mitochondria, ACLY generates acetyl-CoA which leads to histone modification and malonylation of proteins. Thus, citrate represents a key metabolite in various effector response in immune cells, although its mechanisms of action need to be further defined.

Here, we consider citrate as a Krebs cycle intermediate and we do not know how inflammatory triggers would modulate its uptake from the extracellular environment. Citrate-dependent modulation of its carrier for extracellular uptake is a subject that would be fascinating to explore.

Citrate pathway investigation in tissue resident immune cells such as tissue resident macrophages and DCs could unveil how tissue specific microenvironment drive citrate production and would be helpful to gain further insight into leukocytes characterization and study of local inflammatory response.

Despite its clear immunomodulatory effects, further studies have to be carried out in order to understand which will be citrate impact on other fundamental immunometabolites production in mitochondria and if it could in turn impair Krebs cycle.

The use of a citrate synthase knockout would stop citrate production and prevent all these citrate-mediated mechanisms, potentially confirming its role in inflammation. Although this would also impair Krebs cycle. Additional *in vivo* models would be useful to discover how the citrate pathway can be used as a therapeutic target for inflammatory diseases. Studies into some variations of the *CIC* gene and susceptibility to inflammatory disease might prove informative.

We can look forward to further insights into the role of citrate and its metabolism in immunity and inflammation in both health and disease.

Authors Contribution

AZ and LAO'N wrote the manuscript. ZZ corrected the draft and gave advice on the manuscript. LAO'N supervised and edited the manuscript.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dimethyl fumarate and 4-octyl itaconate are anticoagulants that suppress Tissue Factor in macrophages via inhibition of Type I Interferon

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Excessive inflammation-associated coagulation is a feature of infectious diseases, occurring in such conditions as bacterial sepsis and COVID-19. It can lead to disseminated intravascular coagulation, one of the leading causes of mortality worldwide. Recently, type I interferon (IFN) signaling has been shown to be required for tissue factor (TF; gene name *F3*) release from macrophages, a critical initiator of coagulation, providing an important mechanistic link between innate immunity and coagulation. The mechanism of release involves type I IFN-induced caspase-11 which promotes macrophage pyroptosis. Here we find that *F3* is a type I IFN-stimulated gene. Furthermore, *F3* induction by lipopolysaccharide (LPS) is inhibited by the anti-inflammatory agents dimethyl fumarate (DMF) and 4-octyl itaconate (4-OI). Mechanistically, inhibition of *F3* by DMF and 4-OI involves suppression of *Ifnb1* expression. Additionally, they block type I IFN- and caspase-11-mediated macrophage pyroptosis, and subsequent TF release. Thereby, DMF and 4-OI inhibit TF-dependent thrombin generation. In vivo, DMF and 4-OI suppress TF-dependent thrombin generation, pulmonary thromboinflammation, and lethality induced by LPS, *E. coli*, and *S. aureus*, with 4-OI additionally attenuating inflammation-associated coagulation in a model of SARS-CoV-2 infection. Our results identify the clinically approved drug DMF and the pre-clinical tool compound 4-OI as anticoagulants that inhibit TF-mediated coagulopathy via inhibition of the macrophage type I IFN-TF axis.

Inflammation and coagulation are evolutionarily conserved host defence mechanisms that maintain hemostasis by rapidly forming blood clots in response to infection, thereby preventing dissemination of the invading pathogen¹. Excessive activation of the coagulation cascade is intrinsically linked to increased activation of innate immune pathways and can lead to thrombosis, a pathological deviation from hemostasis². Thrombosis is an integral part of inflammation, and this interplay between innate immunity and coagulation can lead to disseminated intravascular coagulation (DIC), an infection-induced life-

threatening illness characterized by organ dysfunction often accompanied by coagulopathy, and the leading cause of death in intensive care units^{3–5}.

Tissue factor (TF; also known as coagulation factor 3 (gene name *F3*)) is the primary initiator of trauma-induced coagulation⁶. TF is essential for survival via rapid formation of blood clots. Its deletion is fatal in mice^{7–9}. Under normal conditions, TF is expressed at low, basal levels in complex with coagulation factor VII on the membrane of circulating immune cells, such as macrophages, and cells in the blood

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vessel wall^{10,11}. This TF:FVIIa complex serves as the key regulator of physiological hemostasis, triggering local coagulation cascade activation which ultimately leads to thrombin generation and blood clot formation. However, under inflammatory conditions such as exposure to lipopolysaccharide (LPS) from gram-negative bacteria, which account for approximately 60% of all sepsis cases¹², *F3* is rapidly induced¹³ and its procoagulant activity is increased up to 100-fold^{14,15}. Recently, LPS-mediated type I interferon (IFN) and non-canonical inflammasome signaling, which involves caspase-11 or its human homolog caspase-4, have been shown to be critical for TF release from immune cells via pyroptosis, a proinflammatory, lytic form of cell death^{16,17}. Extracellular LPS induces caspase-11 via type I IFN production. Cytosolic LPS then binds and activates caspase-11, which in turn cleaves gasdermin D (GSDMD), leading to pyroptosis^{18–21}. Procoagulant TF is thereby released on extracellular vesicles through pyroptotic pores in the macrophage cell membrane^{22–24} which can lead to aberrant thrombin generation and pathological thrombosis. In addition to its primary function in blood clotting, excessive thrombin generation can trigger detrimental feed-forward inflammation via activation of protease-activated receptors (PARs), which can synergize with Toll-like receptors (TLRs) to induce proinflammatory cytokines and IFN- β ²⁵. Thrombin can also cleave and activate the proinflammatory cytokine IL-1 α ²⁶. Collectively, this process is termed thromboinflammation²⁷.

The immunomodulatory agents dimethyl fumarate (DMF), which is clinically approved for the treatment of multiple sclerosis and psoriasis, and the related compound 4-octyl itaconate (4-OI), are potentially anti-inflammatory^{28–31}. Both are based on endogenous metabolites. DMF is a derivative of the tricarboxylic (TCA) cycle intermediate fumarate, and 4-OI is a derivative of itaconate, which is synthesized in macrophages from the TCA cycle intermediate aconitate via the enzyme ACOD1. 4-OI and itaconate have been shown to have a range of anti-inflammatory effects, including activation of the transcription factor NRF2²⁹, and inhibition of NLRP3³⁰, glycolysis³², TET DNA dioxygenases³³, and the kinase JAK1³⁴. Some of these effects such as NRF2 activation³⁵ and inhibition of glycolysis²⁸, NLRP3³¹, and JAK1³⁶ are shared with DMF. Both DMF and 4-OI have also been shown to inhibit IFN- β production^{29,37}.

We have therefore examined whether DMF and 4-OI might block inflammation-associated coagulation via inhibition of type I IFN signaling and the release of TF. We found that *F3* is a type I IFN-stimulated gene (ISG), and inhibition of *F3* induction by DMF and 4-OI is likely to involve suppression of *Ifnb1*. DMF and 4-OI also block type I IFN- and caspase-11-driven macrophage pyroptosis, thereby inhibiting the release of procoagulant TF, leading to suppression of inflammation-associated coagulation in vivo in response to LPS, *E. coli*, *S. aureus*, and SARS-CoV-2. DMF and 4-OI are therefore identified as anticoagulants that inhibit the macrophage type I IFN-TF axis with potential to limit infection-associated coagulopathy.

Results

DMF and 4-OI inhibit LPS-mediated *F3* induction and TF-dependent thrombin generation in macrophages

Recent evidence has identified macrophages as important contributors to the total pool of TF available for the initiation of blood clotting^{6,22}. Using publicly available data³⁸, we first confirmed that macrophages are a major expressor of *F3* mRNA (Supplementary Fig. 1), supporting the use of macrophages as an appropriate cell type for this study. Differential gene expression of LPS-stimulated mouse bone marrow-derived macrophages (BMDMs) revealed that DMF suppressed induction of *F3* in addition to *F7* (Fig. 1a), suggesting that DMF may inhibit TF:FVII(a) complex formation. DMF also inhibited induction of *F10* and *F2r* (PARI) which indicated that DMF may be a potent inhibitor of the extrinsic pathway of coagulation as well as downstream thrombin signaling. Furthermore, expression of *Casp4/11* and *Gsdmd*, which are required for non-canonical inflammasome-

mediated pyroptosis, were also reduced (Fig. 1a). We, therefore, confirmed by PCR that DMF and 4-OI downregulated LPS-induced mRNA expression of *F3* in both BMDMs and human peripheral blood mononuclear cells (PBMCs) (Fig. 1b, c), in addition to suppressing TF protein levels in BMDMs (Fig. 1d, e). We next assessed the effect of DMF and 4-OI on TF-dependent thrombin generation, a functional readout of TF procoagulant activity. Thrombin generation was assessed using FXII-deficient plasma to examine the effect of DMF and 4-OI on TF-dependent, and therefore FXII-independent, thrombin generation³⁹. After stimulating BMDMs with LPS, DMF and 4-OI both significantly increased TF-dependent thrombin generation lagtime in normal platelet-poor plasma (Fig. 1f–h), indicating functional inhibition of thrombin generation via suppression of *F3*.

F3 is a type I IFN-stimulated gene with JAK-STAT-dependency

To assess if DMF and 4-OI regulate type I IFN in the context of coagulation, we first examined their effect on LPS-induced type I IFN production. Consistent with previous studies^{29,37}, DMF and 4-OI blocked LPS-mediated transcriptional induction of *Ifnb1* (Fig. 2a) and subsequent IFN- β release from BMDMs (Fig. 2b). We next hypothesized that *F3* is induced directly via type I IFN signaling, and found that LPS-mediated induction of *F3* is significantly decreased in BMDMs from *Ifnar*^{-/-} mice (Fig. 2c), which do not express the type I IFN receptor. Furthermore, IFN- β itself induced *F3* after 4 h, which was transient (Fig. 2d), indicating that *F3* is an ISG. IFN- β -induced *F3* was inhibited by DMF and 4-OI (Fig. 2e).

Both DMF and 4-OI exert their anti-inflammatory effects in part via NRF2 activation^{29,35}, and NRF2 activation has recently been shown to inhibit type I IFN signaling⁴⁰. We confirmed that DMF and 4-OI drive the NRF2-dependent genes *Gclm* and *Hmox1* (Supplementary Fig. 2a, b). However, knockdown of NRF2 in BMDMs did not alter the suppression of LPS-induced *F3* by DMF and 4-OI (Supplementary Fig. 2c). We confirmed the knockdown in Supplementary Fig. 2d, whereby *Gclm* expression was decreased in the NRF2-deficient cells. This suggests that the mechanism of inhibition of *F3* by DMF and 4-OI is NRF2-independent.

Type I IFNs act upon their receptor, IFNAR, to induce hundreds of ISGs via the JAK-STAT signaling pathway⁴¹. We next explored this component of the type I IFN pathway further and found that LPS-induced *F3* was blocked by baricitinib (Fig. 2f), a specific JAK1/2 inhibitor that limits type I IFN signaling. It has recently been shown that JAK1 is modified and inhibited by both DMF and 4-OI^{34,36}. Knockdown of JAK1 significantly reduced LPS-mediated *F3* induction (Fig. 2g; knockdown confirmed in Supplementary Fig. 3a), further indicating a JAK1-dependency for *F3* induction. We next analyzed transcription factor binding sites in the region of the *F3* gene promoter using the publicly available Interferome database⁴² which predicted STAT-binding sites in the promoter of the *F3* gene (Fig. 2h; coordinates in Supplementary Table 1). We verified these findings using the ChIP-Atlas enrichment analysis tool⁴³ which also predicted STAT- and IRF-binding sites in the downstream region of the human *F3* promoter (Fig. 2i), further suggesting a type I IFN- and STAT-dependency for *F3*. The inhibition by DMF and 4-OI of *F3* induction in response to LPS would therefore appear to involve suppression of type I IFN production and signaling via JAK-STATs.

DMF and 4-OI inhibit caspase-11-mediated pyroptosis, suppressing TF release from pyroptotic macrophages and subsequent thrombin generation

We next examined whether DMF and 4-OI might block the release of TF by inhibiting type I IFN- and caspase-11-mediated pyroptosis. As caspase-11 is an ISG⁴⁴ (as confirmed in Supplementary Fig. 3b), DMF and 4-OI dose-dependently blocked LPS-induced *Casp11* expression in BMDMs (Fig. 2j; Supplementary Fig. 3c, d). DMF and 4-OI also inhibited induction of the ISGs *Isg15* and *Usp18* (Supplementary Fig. 3e, f),

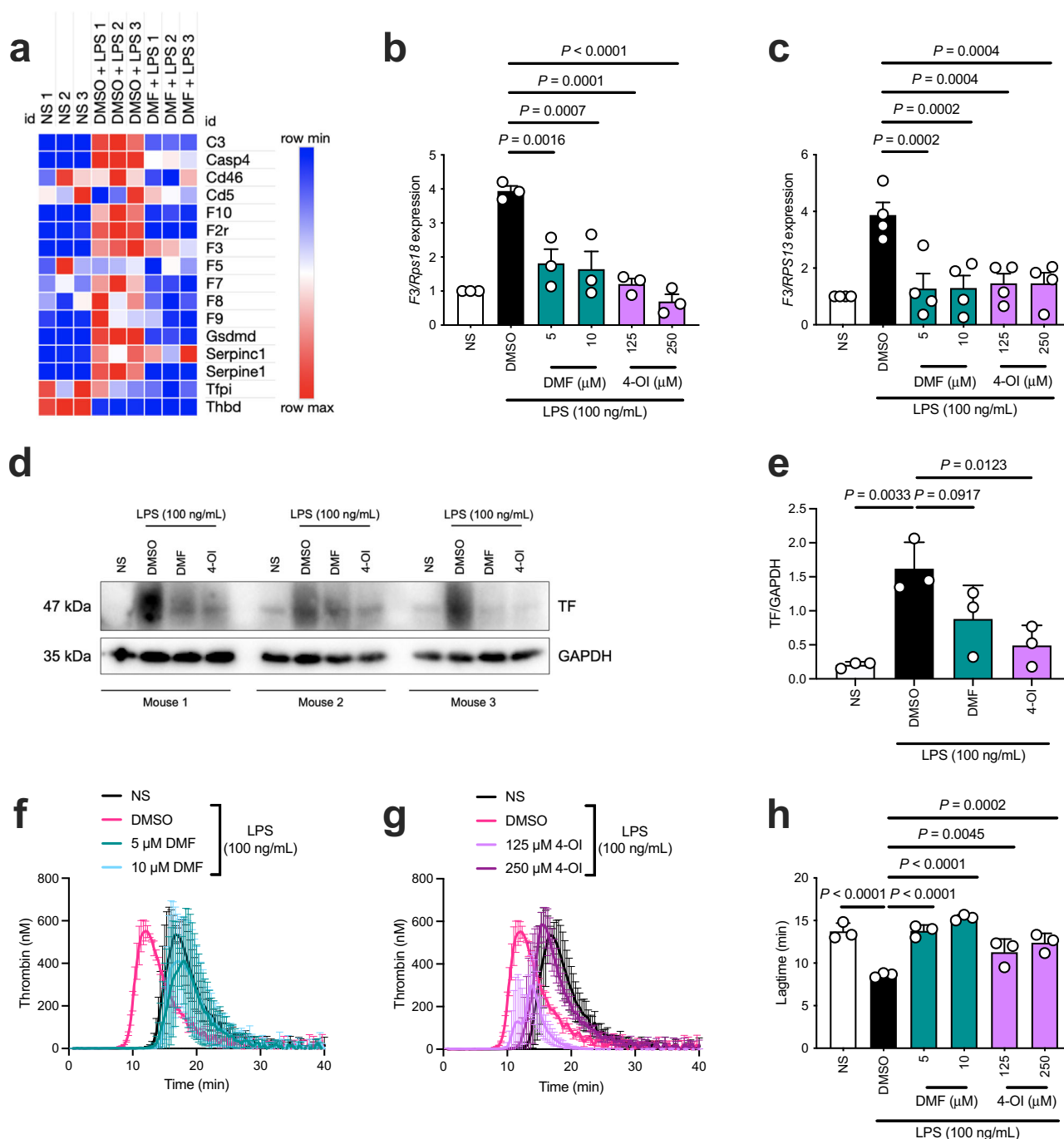
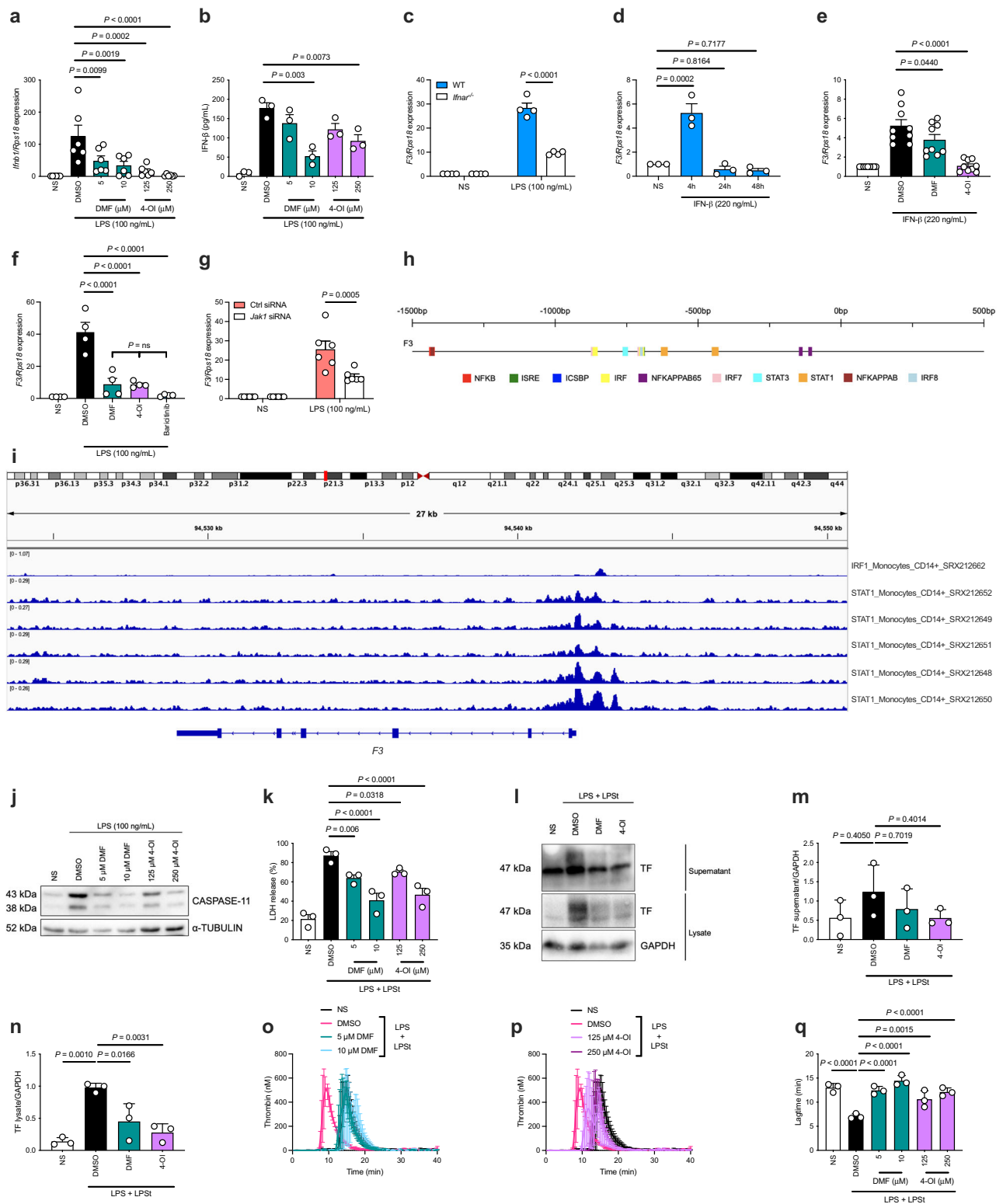


Fig. 1 | DMF and 4-OI inhibit LPS-mediated *F3* induction and TF-dependent thrombin generation in macrophages. **a** Heatmap from RNA sequencing of selected inflammation-associated coagulation genes in LPS-stimulated mouse macrophages (BMDMs) pre-treated (3 h) with DMF compared to DMSO control prior to LPS stimulation (4 h). **b** BMDMs (n = 3) and **c** PBMCs (n = 4) were pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS stimulation (3 h) and harvesting cell lysates. Quantification of *F3* mRNA by qRT-PCR in **b** BMDMs (DMSO–250 μ M 4-OI, $P = 0.000178$) and **c** PBMCs. **d** Western blot and **e** densitometry analysis of TF in BMDM cell lysates pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS

stimulation (3 h), with GAPDH as loading control. **f–h** BMDMs were pre-treated with DMSO, **f** DMF, or **g** 4-OI (1 h) before LPS priming (3 h). Thrombin generation was measured in BMDMs in situ in plate wells using FXII-deficient plasma. **h** Lagtime represents time-to-clot formation. NS–DMSO, $P = 0.000015$; DMSO–5 μ M DMF, $P = 0.00001$; DMSO–10 μ M DMF, $P = 0.0000006$. Data from **b**, **c** are mean $\pm$ SEM from 3, 4 independent experiments. Blot from **d** is three mice from three independent experiments. Data from **e–h** are mean $\pm$ SD from three independent experiments. P values calculated one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.

indicating a broad reduction in type I IFN signaling. Detection of cytosolic LPS activates caspase-11, which in turn cleaves GSDMD to form pyroptotic pores^{20,21}. We therefore next assessed caspase-11-mediated pyroptosis. BMDMs were primed with LPS and subsequently transfected with LPS (which delivers LPS to the cytosol to activate caspase-11⁴⁵). Pre-treatment, but not post-treatment, of BMDMs

(Supplementary Fig. 3g) with DMF and 4-OI dose-dependently blocked pyroptosis, as measured by LDH release (Fig. 2k) and PI staining (Supplementary Fig. 3h), consistent with induction of *Ifnb1* and subsequent *Casp11* as being the process being targeted here. Caspase-11- and pyroptosis-mediated TF release (assessed by release into the supernatant) was also suppressed by DMF and 4-OI (Fig. 2l–n),



resulting in decreased TF procoagulant activity and TF-dependent thrombin generation after activation of the non-canonical inflammasome, shown in Fig. 2o for DMF, Fig. 2p for 4-OI, and lagtime in Fig. 2q.

Therapeutic treatment with DMF and 4-OI suppresses LPS-induced thrombin generation in vivo, improving survival in mice
Following our findings that DMF and 4-OI block the induction and release of TF in response to LPS, thereby blocking thrombin generation in vitro, we next investigated the effects of DMF and 4-OI in vivo in

a systemic model of LPS-induced inflammation and coagulopathy. Intraperitoneal LPS injection significantly induced thrombin generation in mice (Fig. 3a–f). Therapeutic administration (2 h after LPS injection) revealed that DMF (Fig. 3a) and 4-OI (Fig. 3b) significantly inhibited thrombin generation in vivo, as did baricitinib (Fig. 3c) and the specific anti-TF antibody, 1H1, which blocks TF:FVIIa assembly⁴⁶ (Fig. 3d). These data are quantified in Fig. 3e, f. Collectively, this demonstrates the multiple points of TF control as the compounds used target three separate stages of TF activity: F3 induction and

Fig. 2 | *F3* is a type I IFN-stimulated gene with JAK-STAT-dependency, and DMF and 4-OI suppress TF release from macrophages via inhibition of type I IFN- and caspase-11-mediated pyroptosis. **a** BMDMs were pre-treated with DMSO, DMF, or 4-OI (3 h) before LPS priming (16 h) and harvesting cell lysates ($n = 6$). *Ifnb1* mRNA was quantified by qRT-PCR. DMSO–250 μ M 4-OI, $P = 0.00004$. **b** BMDMs were pre-treated with DMSO, DMF, or 4-OI (3 h) prior to LPS priming (6 h). IFN- β in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA) ($n = 3$). **c** BMDMs from WT and *Ifnar^{-/-}* mice ($n = 4$) were stimulated with LPS (3 h). *F3* mRNA was quantified by qRT-PCR. LPS 3 h, WT–KO, $P = 0.0000000488$. **d** BMDMs were stimulated with recombinant mouse IFN- β for a timecourse as indicated ($n = 3$). *F3* mRNA was quantified by qRT-PCR. **e** BMDMs pre-treated with DMSO, DMF, or 4-OI (1 h) before stimulation with recombinant mouse IFN- β (4 h) and harvesting cell lysates ($n = 9$). *F3* mRNA was quantified by qRT-PCR. DMSO–4-OI, $P = 0.00000018$. **f** BMDMs ($n = 4$) were pre-treated with DMSO, DMF, 4-OI, or baricitinib (1 h) before LPS stimulation (3 h). *F3* mRNA was quantified by qRT-PCR. DMSO–DMF, $P = 0.0000144$; DMSO–4-OI, $P = 0.0000133$; DMSO–baricitinib, $P = 0.00000134$. **g** BMDMs ($n = 6$) were transfected with Ctrl or *Jak1* siRNA and stimulated with LPS (3 h). *F3* mRNA was quantified by qRT-PCR. **h** Predicted transcription factor sites in the promoter of the *F3* gene via the Interferome database⁴². Data presented shows the predicted location of transcription factors from the region spanning –1500 bp to +500 bp from the start site of the *F3* gene promoter. **i** The location of STAT1- and IRF1-transcription factor sites in the promoter of the

human *F3* gene, analyzed from enrichment analysis of 6 independent experiments from publicly available data using the ChIP-Atlas⁴³. **j** Representative western blot of CASPASE-11 in BMDM cell lysates pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS stimulation (3 h), with α -TUBULIN as loading control. Blot is representative of three independent experiments. **k** BMDMs ($n = 3$) were pre-treated with DMSO, DMF, or 4-OI (1 h) before priming with LPS (3 h) and LPS transfection (16 h). Pyroptosis is represented as percentage cell death measured by LDH release in BMDM supernatants. DMSO–10 μ M DMF, $P = 0.0000247$; DMSO–250 μ M 4-OI, $P = 0.000084$. **l** Representative western blot of TF in BMDMs pre-treated with DMSO, DMF, or 4-OI (1 h) prior to LPS priming (3 h) and LPS transfection (16 h). Blots are representative of three independent experiments. Densitometry analysis of TF in BMDM (**m**) supernatants and **n** cell lysates, with GAPDH as loading control. **o–q** BMDMs were pre-treated with DMSO, **o** DMF, or **p** 4-OI (1 h) before priming with LPS (3 h) and LPS transfection (16 h). Thrombin generation was measured in BMDMs in situ in plate wells using FXII-deficient plasma. **q** Lagtime represents time-to-clot formation. NS–DMSO, $P = 0.000017$; DMSO–5 μ M DMF, $P = 0.000044$; DMSO–10 μ M DMF, $P = 0.0000019$; DMSO–250 μ M 4-OI, $P = 0.00007985$. Data from **a–g**, **k** are mean $\pm$ SEM from 3–6 independent experiments. Data from **m–q** are mean $\pm$ SD from 3 independent experiments. P values calculated using two-tailed Student's t test for paired comparisons or one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.

suppression of pyroptosis via inhibition of type I IFN (DMF and 4-OI), downstream JAK1 activity (baricitinib), and extrinsic-mediated coagulation via inhibition of TF:FVIIa (1H1).

We next assessed the effects of DMF and 4-OI in a model of LPS-induced lethality. We challenged mice intraperitoneally with a lethal dose of LPS for 24 h, before treating therapeutically with DMF or 4-OI. We also included a mouse group that received heparin as a control anticoagulant and caspase-11 inhibitor⁴⁷. All DMF- and heparin-treated mice survived a lethal dose of LPS, with 4-OI reducing mortality by 80% compared with PBS-treated mice (Fig. 3g). Clinical scores of mice (assessing weight loss, activity level, eye closure, appearance of fur and posture) highlighted the protective roles of DMF and 4-OI when administered 24 h after LPS compared with PBS-treated mice (Fig. 3h). This is highlighted by the divergence in clinical scores of DMF- and 4-OI-treated mice compared with PBS-treated mice when the treatments were administered 24 h after LPS injection (Fig. 3h). Prophylactic administration of DMF and 4-OI also improved survival, with all DMF- and 4-OI-treated mice surviving a lethal dose of LPS, as did all *Casp11^{-/-}* mice (Supplementary Fig. 4a), thus supporting the critical role played by caspase-11 in LPS-induced sepsis and coagulopathy. Clinical scoring of mice further showed the protective effects of DMF and 4-OI (Supplementary Fig. 4b). Taken together, these results indicate that DMF and 4-OI can protect against LPS-induced lethality, with inhibition of type I IFN- and TF-mediated thrombin generation likely to be a critical aspect of their protective effects.

DMF and 4-OI inhibit TF release and TF-mediated thrombin generation following infection with *E. coli* and *S. aureus*

Having shown an inhibitory effect against LPS, we next turned to infectious agents. *Escherichia coli* (*E. coli*) infection represents a clinically relevant model of TLR4, gram-negative bacterial-mediated coagulation. Consistent with our LPS data, DMF significantly inhibited, with 4-OI non-significantly reducing, *E. coli*-induced thrombin generation in vivo (Fig. 3i–k).

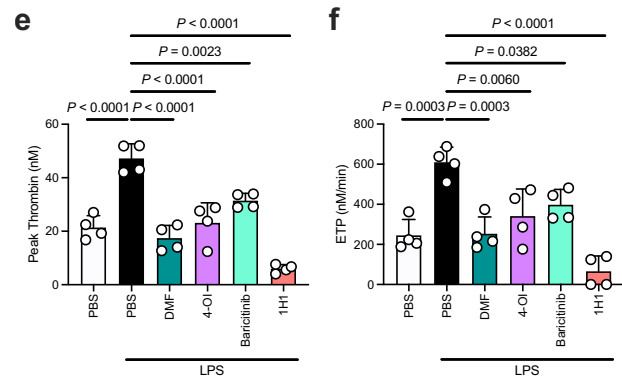
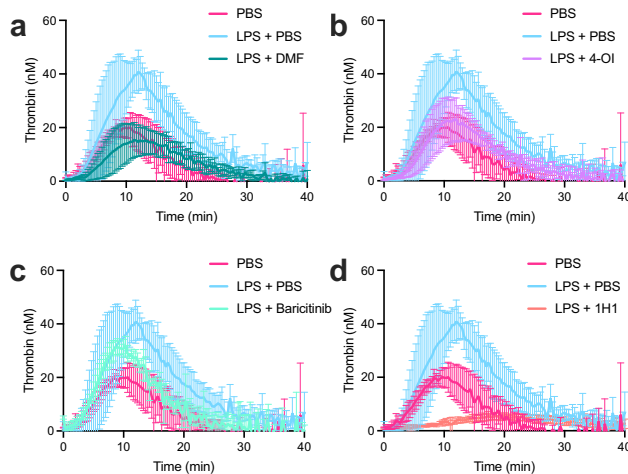
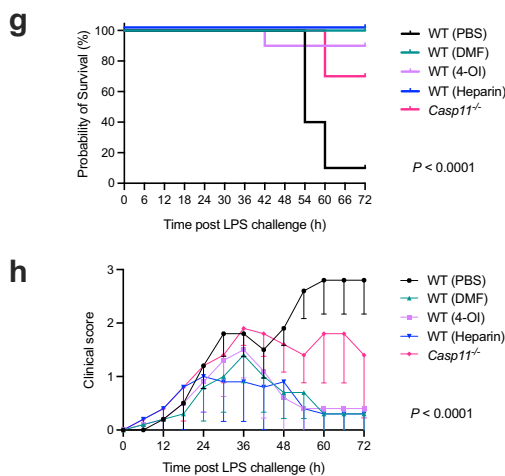
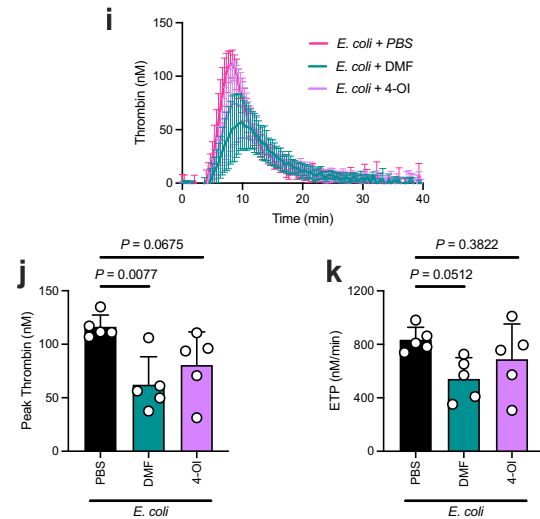
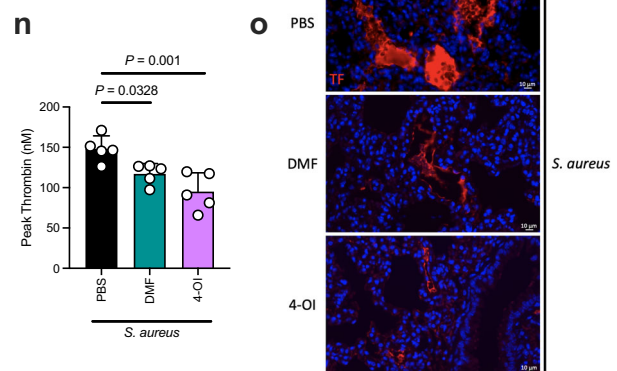
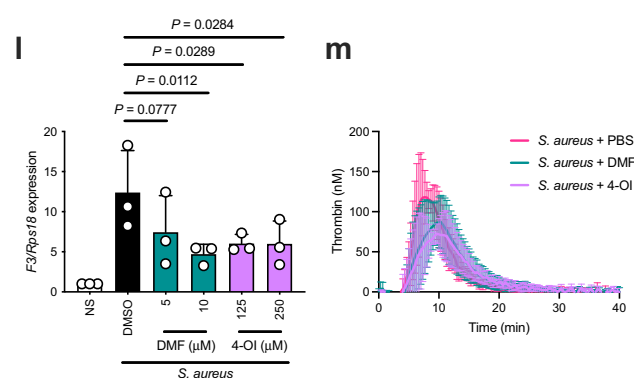
We next assessed whether the anticoagulant effects of DMF and 4-OI could extend beyond gram-negative bacterial infection. We thus tested if DMF and 4-OI could inhibit coagulopathy induced by two different strains of the gram-positive bacterium *Staphylococcus aureus* (*S. aureus*), which is driven by *F3* induction and TF procoagulant activity⁴⁸. We first demonstrated inhibition of *F3* induction by DMF and 4-OI in vitro when BMDMs were infected with *S. aureus* (Fig. 3l). In vivo, DMF and 4-OI both reduced thrombin generation following intraperitoneal injection with *S. aureus* (Fig. 3m, n). Consistent with this

reduction in TF-dependent thrombin generation, DMF and 4-OI markedly reduced TF deposition in the lungs of intraperitoneally-infected mice (Fig. 3o). DMF and 4-OI also reduced thrombin generation following intravenous injection of *S. aureus* (Supplementary Fig. 5a–c). Histological analysis showed decreased TF expression in the lungs of DMF- and 4-OI-treated mice in this model compared with PBS-treated mice (Supplementary Fig. 5d), demonstrating inhibition of *S. aureus*-driven coagulopathy via different modes of infection. As *S. aureus*-mediated coagulopathy occurs in a caspase-11-independent manner⁴⁹, this finding highlights the importance of the inhibition by DMF and 4-OI on *F3* induction and subsequent TF procoagulant activity in blocking aberrant thrombin generation in bacterial-driven sepsis.

4-OI suppresses lung inflammation with associated coagulopathy in mice after SARS-CoV-2 infection

Having established that DMF and 4-OI potentially inhibit type I IFN- and TF-mediated thrombin generation in vivo following bacterial infection, we next assessed the broader anticoagulant effects of these compounds in a model of viral-induced coagulopathy. TF is a key contributor to viral-associated coagulation⁵⁰, so we next tested the effects of DMF and 4-OI on the prothrombotic effect of the viral dsRNA mimetic, poly(I:C). Both DMF and 4-OI inhibited *F3* induction in BMDMs in vitro after stimulation with poly(I:C) (Fig. 4a). Consistent with our LPS data, poly(I:C) stimulation also induced *Casp11*, which was potentially inhibited by DMF and 4-OI (Fig. 4b).

Coagulopathy is a hallmark of COVID-19-associated pathology, and dysregulated immunothrombosis is a critical driver of this aberrant state of coagulation^{51–54}. Inflammasome activation⁵⁵ (in particular via caspase-11 activation⁵⁶) and TF expression^{57,58} and procoagulant activity^{59,60} have been shown to exacerbate SARS-CoV-2-associated immunothrombosis and coagulopathy. Recent evidence indicates a prothrombotic shift in the phenotype of monocytes following COVID-19 infection⁶¹. Furthermore, dysregulated type I IFN signaling is a critical mediator of COVID-19-associated pathology^{62–64}. We therefore tested 4-OI in a mouse model of SARS-CoV-2 infection and assessed broader anticoagulant effects. SARS-CoV-2 infection in mice induced extensive lung inflammation and an increase in the presence of inflammatory cells in the parenchyma (Fig. 4c; lower left-hand quadrant). 4-OI treatment reduced lung inflammation and infiltration of inflammatory cells (Fig. 4c; lower right-hand quadrant). Diffuse pulmonary damage and coagulopathy following SARS-CoV-2 infection is characterized by elevated lung von Willebrand Factor (vWF)

LPS intraperitoneal injection murine model**LPS intraperitoneal injection lethality murine model*****E. coli* intraperitoneal injection murine model*****S. aureus* intraperitoneal injection murine model**

expression⁶⁵ and extensive intraalveolar fibrin deposition⁵¹. Following SARS-CoV-2 infection, we found a significant elevation of vWF and fibrinogen/fibrin surrounding the parenchyma, blood vessels, and airways in the lungs (Fig. 4d–g; lower left-hand quadrant). Mice that received 4-OI had significantly lower levels of both vWF and fibrinogen/fibrin (Fig. 4d–g; lower right-hand quadrant), which was indistinguishable from mock-infected mice. Increased collagen deposition indicates dysregulated tissue repair in the lungs, and SARS-CoV-2-infected mice also displayed elevated collagen deposition (Fig. 4, h; lower left-hand quadrant), a marker of lung damage induced by

inflammation-associated coagulation⁶⁶. There was a significant reduction in collagen deposition in the lungs of 4-OI-treated mice (Fig. 4h, i; lower right-hand quadrant).

4-OI treatment also reduced total numbers of neutrophils (Fig. 4j), lymphocytes (Fig. 4k), and leukocytes (Fig. 4l) in the bronchoalveolar lavage fluid (BALF) of SARS-CoV-2-infected mice. This is notable as neutrophils are a significant contributor to SARS-CoV-2-associated inflammation-driven coagulopathy^{67,68}. 4-OI also significantly reduced infectious viral load in the BALF (Fig. 4m) and lung homogenates (Fig. 4n) of infected mice, indicating an anti-viral effect of 4-OI,

Fig. 3 | Therapeutic treatment with DMF and 4-OI suppresses thrombin generation in vivo induced by LPS, *E. coli*, and *S. aureus*, improving survival in mice. **a–f** Mice were intraperitoneally injected with 1 mg/kg LPS (2 h) followed by treatment with PBS, **a** 50 mg/kg DMF, **b** 50 mg/kg 4-OI, **c** 50 mg/kg baricitinib, or **d** 15 mg/kg 1H1 anti-TF antibody (for a further 4 h) via intraperitoneal injection. Citrated plasma was harvested and thrombin generation was assessed ($n = 4$ per group). Thrombin generation in **a–d** is compared relative to PBS-treated mice $\pm$ LPS challenge. **e** Peak thrombin in mouse citrated plasma treated as in **a–d**. PBS–LPS + PBS, $P = 0.00000637$; LPS + PBS–LPS + DMF, $P = 0.0000008$; LPS + PBS–LPS + 4-OI, $P = 0.0000167$; LPS + PBS–LPS + 1H1, $P = 0.000000055$. **f** Total thrombin generation (ETP = endogenous thrombin potential) in mouse citrated plasma treated as in **a–d**. LPS + PBS–LPS + 1H1, $P = 0.00000135$. **g** Kaplan–Meier survival curve of mice injected intraperitoneally with 15 mg/kg LPS (24 h) followed by treatment with PBS, 50 mg/kg DMF, 50 mg/kg 4-OI, or 200 IU/kg heparin (for a further 48 h) ($n = 10$ per group). LPS + PBS–treatment groups, $P = 0.0000003$. **h** Mice from **g** were scored clinically (assessing weight loss, activity level, eye closure, and appearance of fur and posture) every 6 h for the duration of the 72 h experiment. LPS + PBS–LPS + DMF, $P = 0.00000001997$. Data points indicate individual mice in **g**, **h**. For **g**, **h** Mantel–Cox survival analysis was performed.

i Mice were intraperitoneally injected with 1×10^7 CFU *E. coli* (CFT073) and co-treated with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (6 h), followed by supplemental treatment via intraperitoneal injection with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (for a further 18 h). Citrated plasma was harvested and thrombin generation was assessed ($n = 5$ per group). **j** Peak and **k** total thrombin generation in mouse citrated plasma treated as in **i** ($n = 5$ per group). **l** BMDMs were pre-treated with DMSO, DMF, or 4-OI (1 h) before infection with 5×10^6 CFU *S. aureus* (USA300-LAC) (3 h) and harvesting cell lysates. *F3* mRNA was quantified by qRT-PCR. **m** Mice were intraperitoneally injected with 5×10^8 CFU *S. aureus* (PS80) and co-treated with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (6 h), followed by supplemental treatment via intraperitoneal injection with PBS, 50 mg/kg DMF, or 50 mg/kg 4-OI (for a further 18 h). Citrated plasma was harvested and thrombin generation was assessed ($n = 5$ per group). **n** Peak thrombin generation in mouse citrated plasma treated as in **m**. **o** TF-positive regions (red) in the lungs of mice treated as in **m**. Representative lung tissue sections are shown. Magnification $\times 20$. Scale bar = 10 μ m. Data from **a–k** and **m**, **n** are mean $\pm$ SD. Data from **l** are mean $\pm$ SEM from three independent in vitro experiments. P values were calculated using a two-tailed Student's t test for paired comparisons or one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.

consistent with previous reports^{69,70}. Whilst SARS-CoV-2-infected mice exhibited significant weight loss, the weight loss of 4-OI-treated mice following SARS-CoV-2 infection was not significantly different compared with mock-infected mice (Supplementary Fig. 6a). Clinical scoring of mice (assessing weight loss, activity level, eye closure, appearance of fur and posture) highlighted the protective role of 4-OI in this model (Fig. 4o). At 6 days post infection, *Ifnb1* expression was significantly attenuated by 4-OI (Fig. 4p), providing further indication that suppressing type I IFN signaling is critical in reducing TF-mediated coagulopathy in COVID-19. Notably, 4-OI significantly increased *Nfe2l2* expression after SARS-CoV-2 infection (Fig. 4q). Although NRF2 knockdown did not directly alter LPS-induced *F3* expression (previously shown in Supplementary Fig. 2c), NRF2 activation by 4-OI may contribute to decreased type I IFN signaling^{40,69} by an as-yet-unknown mechanism and therefore decrease coagulopathy in COVID-19. Furthermore, 4-OI limited SARS-CoV-2-induced expression of the proinflammatory genes *Ifng* and *Il1b* (Supplementary Fig. 6a, b), indicating a broad reduction in inflammation upon 4-OI treatment. Finally, *F3* expression was elevated in PBMCs from SARS-CoV-2-infected patients compared with healthy controls (Fig. 4r), in addition to *CASP4* (Fig. 4s), confirming the procoagulant genotype associated with COVID-19.

Discussion

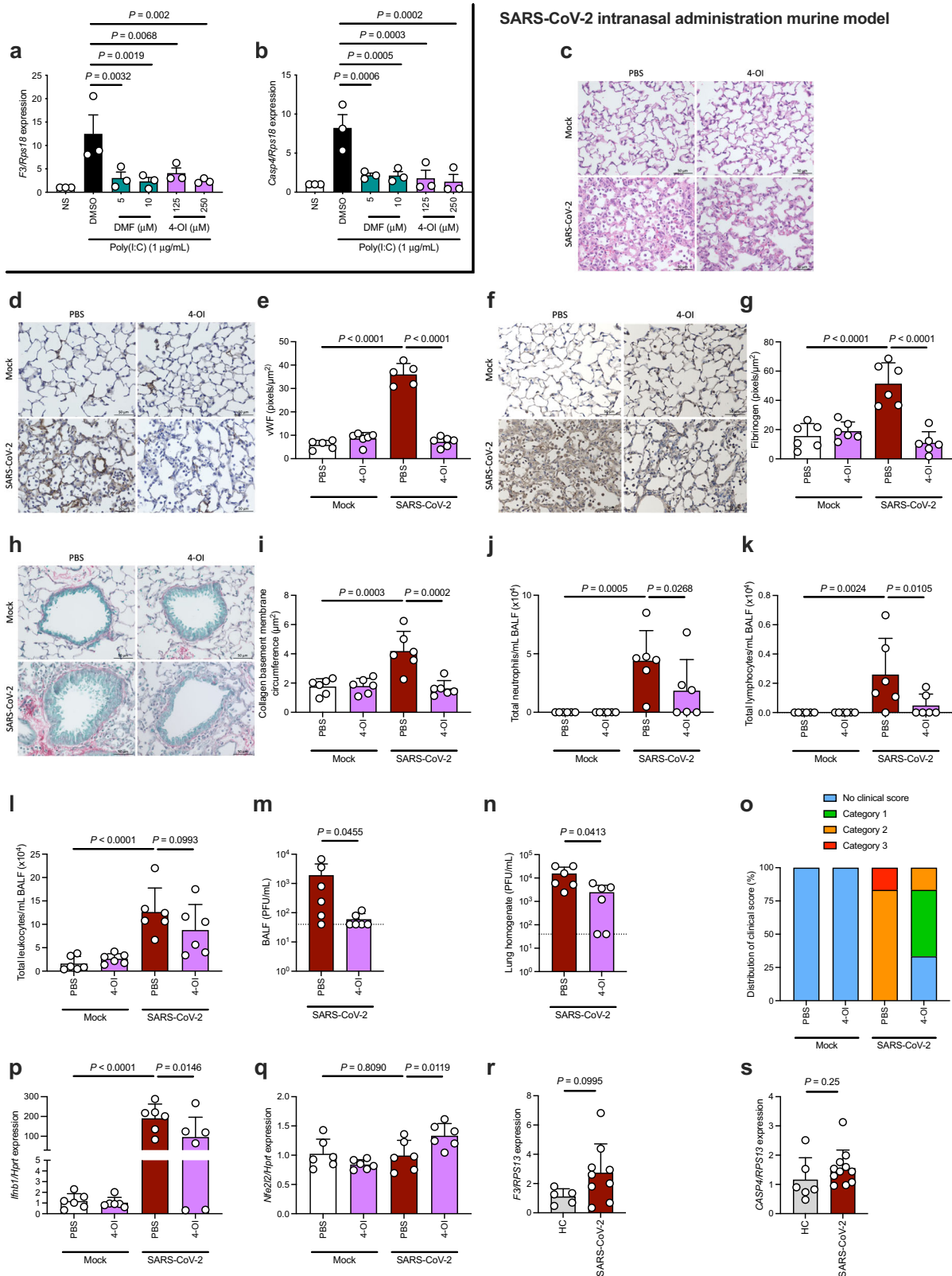
TF mediates hemostasis upon vessel injury, but infection and other proinflammatory stimuli can trigger rapid *F3* induction in macrophages to provoke aberrant coagulation, with inflammation-associated coagulation an underlying driver of sepsis-associated coagulopathies. In particular, it has recently been shown that type I IFN signaling is a critical mediator of coagulation¹⁷. Type I IFN induction facilitates the increased expression of caspase-11, which is activated and cleaved upon detection of cytosolic LPS, leading to the formation of pyroptotic pores in macrophages^{18–21}. Procoagulant TF is released from macrophages on extracellular vesicles through these pyroptotic pores, initiating rapid, pathological blood clotting^{22–24}.

Whilst current anticoagulant therapies are effective, they are associated with an increased bleeding risk for patients^{27,71}. This bleeding is significantly enhanced in septic patients and can manifest as life-threatening bleeds such as hemorrhagic stroke⁷². Thus, developing anticoagulants without an associated bleeding risk is a key goal for the treatment of coagulopathies⁷³. Notably, no specific TF inhibitor has been clinically approved for anticoagulation in humans due to the essential role of TF in maintaining hemostasis and the associated bleeding risk of solely targeting the TF:FVIIa complex. Furthermore, it was recently shown that the anticoagulant heparin does not improve the likelihood of survival-to-hospital-discharge when administered to

critically ill COVID-19 patients⁷⁴, highlighting the urgent need to counteract the innate immune signaling component of coagulopathies and develop broad-spectrum treatments for coagulopathies associated with excessive inflammation.

This recognition of the role of innate immune signaling as a key contributor to pathological thrombosis has led to a recent surge in assessing clinically approved anti-inflammatory therapies as potential anticoagulants^{75–78}. The clinically approved drug DMF and the pre-clinical tool compound 4-OI are potently anti-inflammatory agents and share multiple targets, including NRF2 activation^{29,35}, and inhibition of glycolysis^{28,32} and NLRP3 inflammasome activation^{30,31}. Furthermore, they also inhibit type I IFN induction^{29,37} which contributes to aberrant coagulation. Therefore, we hypothesized that DMF and 4-OI might regulate coagulation via modulation of the type I IFN-TF axis in macrophages.

In our study, we have identified DMF and 4-OI as inhibitors of inflammation-associated coagulation and report for the first time their anticoagulant effects via suppression of the type I IFN-TF axis in macrophages (Supplementary Fig. 7), which has been implicated in aberrant coagulation¹⁷. Mechanistically, we have found that *F3* is an ISG with predicted STAT-binding sites in the *F3* gene promoter, and that DMF and 4-OI likely inhibit *F3* induction at least in part via suppression of *Ifnb1*. Additionally, they block caspase-11-mediated release of TF via suppression of macrophage pyroptosis, again most likely as a consequence of inhibiting type I IFN production with a subsequent decrease in caspase-11 expression. DMF and 4-OI protected against aberrant TF-mediated thrombin generation in vivo in models of bacterial- (both gram-negative and gram-positive) and viral infection and via multiple routes of administration. In particular, our results showing reduced deposition of TF in the lungs with DMF and 4-OI treatment might be particularly relevant in acute respiratory distress syndrome, a leading cause of death in bacterial sepsis due to wholesale destruction of the lung endothelial barrier induced by excessive inflammation and coagulation⁷⁹. 4-OI also reduced deposition of fibrinogen/fibrin in the lungs of mice following SARS-CoV-2 infection. This is likely due to inhibition of excessive TF-dependent thrombin generation, which cleaves fibrinogen into fibrin. The presence of persistent fibrin amyloid microclots has been linked with the long-term debilitating effects of long COVID⁸⁰, and furthermore, elevated type I IFN production has been detected at least 8 months after the onset of COVID-19 infection⁸¹. This suggests potential utility for 4-OI and inhibitors of the type I IFN-TF axis in long COVID. Furthermore, vWF deposition, which was blocked by 4-OI, has recently been shown to activate proinflammatory signaling in macrophages⁸². This suggests that, in addition to its primary anti-inflammatory and anticoagulant properties, 4-OI might also inhibit downstream amplification of thromboinflammation.



We also found that 4-OI reduces viral titers following SARS-CoV-2 infection. Emerging evidence points to itaconate derivatives including 4-OI as being anti-viral. For example, 4-OI attenuates pathology associated with HSV-1 and -2, vaccinia, and zika viruses⁶⁹ as well as influenza A⁷⁰. It has recently been shown that thrombin can directly cleave the SARS-CoV-2 spike protein, augmenting viral entry into the lungs⁸³. This suggests a broader anti-viral effect of

therapeutic thrombin inhibition in the treatment of COVID-19, as this will likely suppress pathological type I IFN production (as a result of reduced viral uptake) as well as thromboinflammation. This also might be a critical downstream effect of the inhibition of TF-mediated thrombin generation by 4-OI following SARS-CoV-2 infection and may explain the reduction in viral titers following 4-OI treatment.

Fig. 4 | 4-OI suppresses lung inflammation with associated coagulopathy after SARS-CoV-2 infection. **a, b** BMDMs were pre-treated with DMSO, DMF, or 4-OI (1 h) before poly(I:C) stimulation (3 h) and harvesting cell lysates. **a** *F3* and **b** *Casp4* (caspase-11) mRNA was quantified by qRT-PCR. **c** Representative images of H&E staining examining histological changes in lung structure and inflammatory cell infiltration in the primary airways, parenchyma, and vasculature from male K18-hACE2 mice infected with 10^3 PFU SARS-CoV-2 (Wuhan isolate; VIC01/2020) or mock infection and intranasally treated with PBS or 4-OI (10 mg/kg) from 1 day post infection, with daily treatments continuing until the termination of the experiment at day 6 post infection ($n = 5$ –6 per group). **d** Representative images and **e** quantification of vWF staining in lung sections from mice treated as in **c** ($n = 5$ –6 per group). Mock+PBS–SARS-CoV-2 + PBS, $P = 0.00000000002$; SARS-CoV-2 + PBS–SARS-CoV-2 + 4-OI, $P = 0.00000000002$. **f** Representative images and **g** quantification of fibrinogen staining in lung sections from mice treated as in **c** ($n = 5$, 6 per group). Mock+PBS–SARS-CoV-2 + PBS, $P = 0.000016$; SARS-CoV-2 + PBS–SARS-CoV-2 + 4-OI, $P = 0.00002887$. **h** Representative images and **i** quantification of collagen deposition in lung sections from mice treated as in **c**. Total numbers of **j** neutrophils, **k** lymphocytes, and **l** leukocytes (Mock+PBS–SARS-

CoV-2 + PBS, $P = 0.000078$) in the bronchoalveolar lavage fluid (BALF) of mice treated as in **c**. Viral titers in the **m** BALF and in **n** lung homogenates from SARS-CoV-2-infected mice treated as in **c**, detected using plaque assays. $n = 5$, 6 per group for **i–n**. **o** Clinical scoring of mice (assessed for weight loss, activity level, eye closure, and appearance of fur and posture) treated as in **c**. Clinical scoring of mice assigned SARS-CoV-2-infected mice to both categories 2 and 3 (83% to category 2, 17% mice to category 3), while SARS-CoV-2-infected mice that received therapeutic administration of 4-OI were dispersed across no clinical score, category 1, and category 2 groups (33% mice to no clinical score, 50% mice to category 1, and 17% mice to category 2). Quantification of **p** *Ifnb1* (Mock+PBS–SARS-CoV-2 + PBS, $P = 0.00003$) and **q** *Nfe2l2* mRNA by qRT-PCR in PBMCs of mice treated as in **c** ($n = 5$, 6 per group). Human PBMCs from healthy controls (HC) ($n = 5$, 6) and SARS-CoV-2-infected patients ($n = 9$ –11) were assessed for quantification of **r** *F3* and **s** *CASP4* mRNA by qRT-PCR. Data from **a**, **b** and **r**, **s** are mean $\pm$ SEM from three independent in vitro experiments. Data from **e**, **g**, **i–n**, and **p**, **q** are mean $\pm$ SD. Scale bar for **c**, **d**, **f**, and **h** = 50 μ m. P values calculated using two-tailed Student's t test for paired comparisons or one-way ANOVA for multiple comparisons. Source data are provided as a Source Data file.

Collectively, our work supports recent clinical trials testing anti-inflammatory compounds as potential inhibitors of inflammation-associated coagulation^{75–78}. There is an extensive crosstalk between inflammation and coagulation and therefore the broad reduction in inflammation exerted by DMF and 4-OI may contribute to their anticoagulant properties. However, our study identifies a targetable pathway (the type I IFN-TF axis) in thromboinflammation and our work supports redeploying the clinically approved DMF in clinical trials for inflammation-associated coagulopathies, in particular for autoimmune and thromboinflammatory diseases including systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS), which are characterized by excessive type I IFN production and are associated with significantly greater risk of developing thrombotic cardiovascular disease^{84,85}. This is likely due in part to significantly elevated expression of *F3* mRNA in PBMCs from patients with SLE⁸⁶ and APS^{87,88}, which correlates with risk of vascular disease. Our work also supports the future development of 4-OI-based compounds as a new class of anticoagulants that inhibit inflammation-associated coagulation. For example, DMF and 4-OI-based compounds may have utility in sepsis-associated DIC, which is exacerbated by increased thrombin levels in patients⁷².

In summary, these anticoagulant effects that we describe add to the well-characterized anti-inflammatory properties of DMF and 4-OI, further supporting their use in infectious diseases where both inflammation and coagulation are key pathologic features.

Methods

Animal details

All mice were on a C57BL/6J OlaHsd background unless stated below. Wild-type (WT) mice were bred in-house. *Caspase-11*^{−/−} mice on the C57BL/6J background were backcrossed onto the C57BL/6J background for another 8 generations. Heterozygous breeding pairs were used to generate wild-type and *Caspase-11*^{−/−} littermates, which were used for all experiments described. *Ifnar*^{−/−} mice were also generated on the C57BL/6J background and age- and sex-matched WT mice were used in all experiments described. Experiments were performed with 6-to-12-week-old male and female mice bred under specific pathogen-free conditions, under license and approval of the local animal research ethics committee (Health Products Regulatory Authority) and European Union regulations. In vivo models were performed with 6-12-week-old C57BL/6J OlaHsd mice and littermates were randomly assigned to experimental groups. All animal procedures were ethically approved by the Trinity College Dublin Animal Research Ethics Committee prior to experimentation, and conformed with the Directive 2010/63/EU of the European Parliament.

Generation of murine BMDMs

6–12-week-old mice were euthanized in a CO₂ chamber and death was confirmed by cervical dislocation. Bone marrow was subsequently harvested from the tibia, femur, and ilium. For monocyte/macrophage isolation, cells were differentiated in DMEM containing L929 supernatant (10%), fetal calf serum (FCS) (10%), and penicillin/streptomycin (1%) for 6 days, after which cells were counted and plated at 0.5×10^6 cells/mL unless otherwise stated.

Isolation of human PBMCs

Human blood samples from healthy donors and SARS-CoV-2-infected patients were collected and processed at the School of Biochemistry and Immunology in TBSI (TCD) or the St James's, Tallaght University Hospital, Trinity Alliance for Research (STTAR) Bioresource, St. James's Hospital, Tallaght University Hospital, and Trinity Translational Medicine Institute (TCD) (for PBMCs isolated from SARS-CoV-2-infected patients). Blood samples were obtained anonymously and written informed consent for the use of blood for research purposes was obtained from the donors. All the procedures involving experiments on human samples were approved by the School of Biochemistry and Immunology Research Ethics Committee (TCD). The collection of human samples for STTAR was approved by the National Research Ethics Committee. Experiments were conducted according to the TCD guide on good research practice, which follows the guidelines detailed in the National Institutes of Health Belmont Report (1978) and the Declaration of Helsinki. 30 mL whole blood was layered on 20 mL Lymphoprep (StemCell Technologies, Inc.), followed by centrifugation for 20 min at $400 \times g$ with the brake off, after which the upper plasma layer was removed and discarded. The layer of mononuclear cells at the plasma-density gradient medium interface was retained, and 20 mL PBS was added. Cells were centrifuged for 8 min at $300 \times g$ and the resulting supernatant was removed and discarded. The remaining pellet of mononuclear cells was resuspended, counted and plated at 1×10^6 cells/mL in RPMI supplemented with FCS (10%) and penicillin-streptomycin (1%).

Reagents

Dimethyl fumarate (DMF) (242926) and 4-octyl itaconate (4-OI) (SML2338) were dissolved in dimethyl sulfoxide (DMSO) (D8418), all purchased from Sigma. Baricitinib (7222) was purchased from R&D and also dissolved in DMSO. Recombinant mouse IFN- β was purchased from BioLegend (581304). High molecular weight poly(I:C) (tlrl-pic) was purchased from Invivogen. LPS from *E. coli* (ALX-581-010-L002) was purchased from Enzo Life Sciences for in vitro experiments. LPS from *E. coli* (L4524) and heparin (SRE-0027) were purchased from Sigma for in vivo experiments. 1H1 anti-TF antibody was a kind gift

Table 1 | Mouse and human primer sequences for quantitative real-time PCR

Mouse primers	
Primer	Sequence (5'–3')
Casp4_F	GGTGGTGAAAGAGGAGCTTAC
Casp4_R	CCAGGAATGTGCTGTCTGAT
F3_F	ACCCAAACCCACCAACTATAC
F3_R	GGTCACATCCTTCACGATCTC
Gclm_F	TGGAGTTCCTCAATCAGCCC
Gclm_R	TGCATGGGACATGGTGCATT
Hmox1_F	CCTCAGATGGCGTCACTT
Hmox1_R	GCTGATCTGGGGTTTCCCTC
Ifnb1_F	ATGGTGGTCCGAGCAGAGAT
Ifnb1_R	CCACCACTATTCTGAGGCA
Isg15_F	GCCAGAAGCAGACTCCTTAAT
Isg15_R	ACACCAAGAAATCGTTACCC
Rps18_F	ATGTGAAGGATGGGAAGTACAG
Rps18_R	CCCTCTATGGGCTCGAATTT
Usp18_F	TCAGTGCCTGCAGAAATACA
Usp18_R	TCCGTGATCTGGTCTTAGT

SARS-CoV-2 primers	
Primer	Sequence (5'–3')
Hprt_F	AGGCCAGACTTTGTTGGATTGAA
Hprt_R	CAACTTGCCTCATCTTAGGCTTT
Ifnb1_F	AACTCCACCAGCAGACAGTG
Ifnb1_R	GGTACCTTTGCACCTCCAG
Ifng_F	GAGGTCAACAACCCACAGGT
Ifng_R	GGGACAATCTCTCCCCACC
Il1b_F	GAGGACATGAGCACCTTCTTT
Il1b_R	GCCTGTAGTGACAGTTGTCTAA
Nfe2l2_F	GGACATGGAGCAAGTTTGGC
Nfe2l2_R	CCAGCGAGGAGATCGATGAG

Human primers	
Primer	Sequence (5'–3')
CASP4_F	GCTCTTCAACGCCACACAAC
CASP4_R	GGTGGGCATTGAGCTTTGG
F3_F	CGCTTCAGGCACTACAAATAC
F3_R	GACTTGATTGACGGTTTGG
RPS13_F	TCACCGTTTGGCTCGATATT
RPS13_R	GGCAGAGGCTGTAGATGATT

from Dr Helen Bettencourt (Genentech, Inc. South San Francisco, CA)⁴⁶. Cells were transfected (to activate caspase-11) using FuGENE HD (Promega).

Non-canonical inflammasome assay

Cells were treated with DMF (5–10 μ M) and 4-OI (125–250 μ M) for 1 h before being primed with LPS (100 ng/mL) for 3 h, after which the medium was replaced and 2 mg LPS was transfected using FuGENE HD (Promega) overnight (16 h) in serum-free media in order to activate the inflammasome by cleaving and activating caspase-11¹⁹.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using the PureLink RNA Mini Kit (Invitrogen) and quantified using a Nanodrop 2000 UV-visible spectrophotometer. cDNA was prepared using 20–100 ng/ μ L total RNA by a reverse transcription-polymerase chain reaction (RT-PCR) using a high-

capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed with an ABI 7500 Fast real-time PCR system (Applied Biosystem) on cDNA using SYBR Green (Invitrogen). Data were normalized to murine *Rps18* or *Hprt* and human *RPS13* as endogenous controls, and mRNA expression fold change relative to controls was calculated using the $2^{-\Delta\Delta C_t}$ method. All fold changes are expressed normalized to the untreated control. Table 1 lists the primer sequences that were used:

RNA silencing (siRNA)

Pre-designed mouse-specific silencer select siRNAs for *Jak1* (s68537; Ref Seq NM_146145; sense sequence: CACUGAUUGUCCACAAUAUTT; antisense sequence: AUAUUGUGGACAAUCAGUGGG), *Nrf2* (s70522; Ref Seq NM_010902; sense sequence: CAGGAGAGGUAAGAAUAAATT; antisense sequence: UUUUUAUCCUCCUCUGCG), and negative control (4390843) were ordered from Thermo Fisher. Cells were transfected with 50 nM siRNA using 5 μ L lipofectamine RNAiMAX according to manufacturer's instructions (Thermo Fisher). Cells were transfected in medium without serum and antibiotics which was replaced with complete medium 8 h later. Cells were subsequently left for a further 12 h prior to treatment.

RNA sequencing

BMDMs (3 independent mice) were treated as indicated and RNA was extracted as previously detailed. mRNA was extracted from total RNA using poly-T-oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by the second strand cDNA synthesis. The library was checked with Qubit and RT-PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on the NovaSeq 6000 S4 (Illumina).

ELISA

A DuoSet ELISA kit for IFN- β (R&D) in the supernatant of BMDMs was purchased and carried out according to the manufacturer's instructions with cell supernatants added to each plate in duplicate or triplicate. Absorbance was read at 450 nm and quantified using a FLUOstar Optima plate reader. Corrected absorbance values were calculated by subtracting the background absorbance and cytokine concentrations were subsequently obtained by extrapolation from a standard curve plotted on GraphPad Prism 9.0.

Western blotting

Supernatant was removed from cells following stimulation and lysates were harvested in 30–50 μ L lysis buffer (0.125 M Tris pH 6.8, 10% glycerol, 0.02% SDS, 5% DTT). Lysates were subsequently heated to 95 °C for 5 min to denature proteins. To concentrate supernatants for western blot, 5 μ L Strataclean Resin (Agilent) was added to 500 μ L of supernatant and vortexed for 1 min. Supernatants were then centrifuged at 210 $\times$ g for 2 min at 4 °C. Supernatants were removed and discarded, and the remaining pellet was resuspended in 30 μ L lysis buffer. SDS-PAGE was used to resolve proteins by molecular weight. Samples were boiled at 95 °C for 5 min prior to loading into a 5% stacking gel. The percentage resolving gel (8–12%) depended on the molecular weight of the given protein. The Bio-Rad gel running system was used to resolve proteins and the Bio-Rad wet transfer system was used for the electrophoretic transfer of proteins onto PVDF membrane. Following transfer, the membrane was incubated in blocking solution (5% milk powder or 5% BSA in TBST) for 1 h and subsequently incubated in primary antibody (5% milk powder or 5% BSA in TBST) rolling overnight at 4 °C. The membrane was incubated for 1 h with secondary antibody (diluted in 5% milk powder or 5% BSA in TBST) at room temperature. Prior to visualization, the membrane was immersed in WesternBright ECL Spray (Advansta). Proteins were

visualized on a ChemiDoc MPTM Imaging System (Bio-Rad), and both chemiluminescent and white light images were taken. Quantification of western blot images was performed using Image Lab Software (Bio-Rad). Adjusted band volume was calculated for each band and for each experimental condition this was presented as target protein/house-keeping protein. Uncropped and unprocessed blots are available as source data in the Source Data file.

The following antibodies were used for western blotting: mouse-reactive anti-rat CASPASE-11 (14340), anti-rabbit GAPDH (2118), anti-rabbit JAK1 (3344), anti-rabbit α -TUBULIN (2144), and anti-rabbit TF (44861) were purchased from Cell Signaling. Working dilutions of primary antibodies for western blotting were 1:1000. Horseradish peroxidase (HRP)-conjugated anti-rat (112-035-003) and anti-rabbit (111-035-003) IgG antibodies (both 1:2500) were purchased from Jackson ImmunoResearch. All antibodies have been validated for mouse reactivity and for western blotting.

LDH assay

The CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) was used to quantify lactate dehydrogenase (LDH) release from cells as a measure of pyroptotic cell death in BMDMs following non-canonical inflammasome stimulation. Freshly harvested supernatants were used in this assay. 25 μ L of each supernatant was added to 25 μ L CytoTox 96 Reagent and incubated in the dark at room temperature for 30 min. 12.5 μ L acetic acid was added to stop the reaction, and the absorbance at 492 nm was measured using a FLUOstar optima. 100 μ L total lysis solution was added to untreated cells 30 min before harvesting and served as a maximum LDH release control. Medium alone was used to correct for background absorbance.

Live-cell imaging

Pyroptosis was visualized by adding propidium iodide (PI) (R37169, Invitrogen) (1 drop/ 1×10^6 cells/mL) at time of transfection and imaged using the IncuCyte S3 Live-Cell Analysis System (Essen BioScience). PI is not permeant to live cells; pyroptotic dead cells stained red. Images and movies were saved in tiff format, exported, and analyzed in ImageJ (NIH).

LPS (endotoxin)-induced model of inflammation with associated coagulopathy

6-week-old female mice were used, and littermates were randomly assigned to experimental groups. Compounds were resuspended in 10% DMSO followed by 90% cyclodextrin/PBS (40% w/v). For therapeutic assessment, mice were challenged with intraperitoneally (i.p.) injected LPS (1 mg/kg) for 2 h before i.p. injection of DMF (50 mg/kg), 4-OI (50 mg/kg), baricitinib (50 mg/kg), IH1 anti-TF antibody (15 mg/kg), or vehicle (PBS in 40% cyclodextrin) for 4 h. Mice were euthanized in a CO₂ chamber and lungs were harvested and whole blood samples were collected in 3.2% sodium citrate-coated tubes to prevent coagulation. For platelet-poor plasma (PPP) isolation, whole blood was spun at 2000 rpm at 4 °C for 15 min, and plasma (the top, liquid layer of the buffy coat) was used fresh. For the survival trial, mice were challenged i.p. with LPS (15 mg/kg) for 24 h before treatment i.p. with DMF (50 mg/kg), 4-OI (50 mg/kg), or vehicle (PBS in 40% cyclodextrin) for 48 h. Heparin (200 IU/kg) was injected subcutaneously 24 h after LPS injection. Mice were assessed every 6 h for 72 h post LPS challenge, and graded clinically from 0 to 3 (assessing weight loss, activity level, eye closure, and appearance of fur and posture), with 0 representing healthy and 3 reaching humane endpoint. Mice reaching humane endpoint were euthanized in a CO₂ chamber.

Staphylococcus aureus-systemic infection model with coagulopathy

For in vitro infection with *S. aureus*, BMDMs were treated with DMF (5–10 μ M) or 4-OI (125–250 μ M) for 1 h before being primed with

5×10^6 CFU LAC for 3 h prior to harvesting cell lysates. For the community-acquired MRSA strain *S. aureus* USA300-LAC in vivo, mice were co-treated via intravenous injection with 5×10^7 CFU LAC $\pm$ DMF (50 mg/kg), 4-OI (50 mg/kg), or PBS in 40% cyclodextrin for 6 h before harvesting as per our LPS (endotoxin)-induced septic shock model. For *S. aureus* PS80-strain, mice were co-treated i.p. with 5×10^8 CFU PS80 $\pm$ DMF (50 mg/kg), 4-OI (50 mg/kg), or PBS in 40% cyclodextrin for 6 h before a second dose of inhibitor and harvesting after 18 h.

E. coli-induced systemic infection model with coagulopathy

Mice were co-treated i.p. with 1×10^7 CFU CFT073 *E. coli* $\pm$ DMF (50 mg/kg), 4-OI (50 mg/kg), or PBS in 40% cyclodextrin for 6 h before a second dose of inhibitor and harvesting after 18 h.

SARS-CoV-2 model of inflammation with associated coagulopathy

Heterozygous male K18-hACE2 c57BL/6J mice (strain: 2B6.Cg-Tg(K18-ACE2)2Prln/J) were obtained from The Jackson Laboratory. Animals were housed in groups and fed standard chow diets. Mice were administered 10^3 PFU SARS-CoV-2 (Wuhan isolate; VIC01/2020) via intranasal administration and intranasally treated with 4-OI (10 mg/kg) from 1 day post infection, with daily treatments continuing until the termination of the experiment at day 6 post infection. Numbers of total neutrophils, lymphocytes, and leukocytes were assessed in the bronchoalveolar lavage fluid (BALF) and lung homogenates of mice. A plaque assay was utilized to assess viral titers from SARS-CoV-2-infected mice. Collagen deposition was quantified using Sirius Red-stained lung histological sections, with the area surrounding the primary airways (red) enumerated for each mouse. PBMCs were isolated from whole blood for assessment of mRNA expression. Clinical scoring of mice assessed weight loss, activity level, eye closure, and appearance of fur and posture. Mice were graded clinically with increasing severity in four categories, from 0 to 3.

Cell-based thrombin generation assay

Cells were seeded onto a 96-well plate and left overnight to adhere. Following the described cell stimulations, supernatants were removed and cells were washed once with PBS. MP-reagent (Thrombinoscope) was used as a source of phospholipids in 80 μ L FXII-deficient plasma (Haemtech). Thrombin generation of cells in situ in the plate was measured by comparing experimental wells against thrombin activity of a calibrator, as recently described³⁹. As BMDM TGA was carried out with cells in situ, changes in thrombin generation will most likely be as a result of surface-bound TF rather than TF released from the surface. The reaction was initiated with 20 μ L FluCa-kit (0.42 mM fluorometric substrate, 16.67 nM CaCl₂). Fluorescence was quantified using Thrombinoscope software on the Fluoroskan for 60 min. Data was analyzed on GraphPad Prism 9.0.

Plasma thrombin generation assay

Plasma was diluted 1:3 for all experiments described. MP-reagent which contains phospholipids was added to wells to allow coagulation complex formation. Mouse thrombin converts the fluorogenic substrate at a rate which is 20% lower than human thrombin, therefore to correct for this discrepancy, calibrator activity was set to 20% higher in the Thrombinoscope settings. The temperature of the Fluoroskan was set to 33 °C. 20 μ L plasma and 20 μ L of MP-reagent were added to wells and the reaction was initiated with 20 μ L FluCa-kit (0.42 mM fluorometric substrate, 8.2 nM CaCl₂). Fluorescence was quantified using Thrombinoscope software on the Fluoroskan for 60 min. Data was analyzed on GraphPad Prism 9.0.

Histology

The superior, middle, and inferior lobes of right lungs were rapidly removed from mice, fixed with 4% paraformaldehyde in PBS, and

embedded in paraffin using an automatic tissue processor (Leica Microsystems). 3 μ m-thick lung sections were deparaffinized in xylene and rehydrated through graded ethanol washes. The antigen retrieval was performed in Tris-EDTA buffer (10 mM Tris pH 9.0, 1 mM EDTA) for 30 min at 95 °C followed by incubation with proteinase K for 5 min at room temperature. The lung tissue sections were blocked with 10% BSA in TBS for 1 h at room temperature and then incubated overnight at 4 °C with either a rabbit anti-fibrinogen/fibrin (cat. no.: A 0080; Dako, Gostrup, Denmark), a rabbit anti-von Willebrand factor (VWF; cat. no.: A 0082; Dako), or a rabbit anti-Tissue Factor antibody (Novus Bio; NBP2-15139). Afterwards, the lung tissue sections were extensively washed with TBS and incubated with a secondary antibody labeled with Alexa Fluor™ 555 (Thermo-Fisher Scientific; Waltham, MA) for 1 h at room temperature. Finally, the slides were embedded in Vectashield Mounting Medium with DAPI (Vector Laboratories Inc., Burlingame, CA). Images were taken with a Leica DM 6000 microscope (Leica, Wetzlar, Germany) using a 20x objective and processed using Leica Application Suite Advanced Fluorescence (LAS AF) software. Alternatively, antigen detection was performed using a Zytochem-Plus AP Polymer Kit in accordance with the manufacturer's instructions (Zymed Systems, Berlin, Germany) and the slides were scanned with a Miramax slide scanner (Zeiss, Oberkochen Germany).

Quantification, statistical analysis, and reproducibility

Details of all statistical analyses performed can be found in the figure legends. Data were expressed as mean $\pm$ standard error of the mean (SEM) unless stated otherwise. *P* values were calculated using two-tailed Student's *t* test for pairwise comparison of variables and one-way ANOVA for multiple comparison of variables. A Sidak's or Tukey's multiple comparisons test was used as a post-test when performing an ANOVA. A Mantel–Cox test was used for log-rank analysis of the survival Kaplan–Meier curve. A confidence interval of 95% was used for all statistical tests. For statistical significance, exact *P* values are included with each panel, and for *P* values < 0.0001, exact *P* values are included in the figure legends. Sample sizes were determined on the basis of previous experiments using similar methodologies. All depicted data points are biological replicates taken from distinct samples. Each figure consists of a minimum of three independent experiments from multiple biological replicates unless stated otherwise in the figure legends. *n* = the number of animals or the number of independent experiments with primary BMDMs. For in vivo studies, age- and sex-matched mice were randomly assigned to treatment groups, unless otherwise stated.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

RNA sequencing data (<https://doi.org/10.5061/dryad.6wwpzgn28>) is available via the Dryad Data Platform. Data generated from the Immunological Genome Project (ImmGen) bulk-population RNA-seq database³⁸, Interferome v2.0⁴², and the ChIP-Atlas enrichment analysis tool⁴³ are publicly available. Source data are provided with this paper in the Supplementary Information. Source data are provided with this paper.

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T.A.J.R., Z.Z., and L.A.J.O'N. conceptualized the project. T.A.J.R. was the lead experimentalist and designed and performed in vitro and in vivo experiments, analyzed and interpreted data, and wrote the manuscript. A.H. conducted in vitro experiments, analyzed and interpreted data, assisted with specimen monitoring, and provided critical input. A.M.R. conducted in vitro experiments, performed thrombin generation assays, and analyzed and interpreted data. M.D.J. performed in vivo experiments and analyzed and interpreted data. E.C.O'B. performed in vivo experiments. J.E.T.-K., M.M.W., E.A.D., S.M., and N.G.H. assisted with in vivo experiments. H.J.W., C.G.P., and A.Z. assisted with specimen monitoring. P.S., F.S., and C.N. performed histological analysis. E.G.V. performed in vitro experiments. L.O'D., S.G., A.L., J.D., C.N.C. and N.C. provided PBMCs from COVID-19-infected patients. M.C., P.G.F., and K.H.G.M. provided helpful discussions. G.M. advised on live-cell imaging and assisted with the preparation of samples for histological analysis. E.M.C. supplied Caspase-11^{-/-} mice. J.S.O'D., P.M.H., and R.M.M. provided critical input. P.J.H. assisted with and advised on transcription factor promoter analyses. M.W. performed and advised on histological analysis. R.J.S.P. assisted with experimental design and provided critical input. Z.Z. designed experiments and oversaw a portion of the research project. L.A.J.O'N. obtained funding and oversaw the research project.

Competing interests

The authors declare no competing interests.

Additional information

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The metabolic function of pyruvate kinase M2 regulates reactive oxygen species production and microbial killing by neutrophils

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Neutrophils rely predominantly on glycolytic metabolism for their biological functions, including reactive oxygen species (ROS) production. Although pyruvate kinase M2 (PKM2) is a glycolytic enzyme known to be involved in metabolic reprogramming and gene transcription in many immune cell types, its role in neutrophils remains poorly understood. Here, we report that PKM2 regulates ROS production and microbial killing by neutrophils. Zymosan-activated neutrophils showed increased cytoplasmic expression of PKM2. Pharmacological inhibition or genetic deficiency of PKM2 in neutrophils reduced ROS production and *Staphylococcus aureus* killing in vitro. In addition, this also resulted in phosphoenolpyruvate (PEP) accumulation and decreased dihydroxyacetone phosphate (DHAP) production, which is required for de novo synthesis of diacylglycerol (DAG) from glycolysis. In vivo, PKM2 deficiency in myeloid cells impaired the control of infection with *Staphylococcus aureus*. Our results fill the gap in the current knowledge of the importance of lower glycolysis for ROS production in neutrophils, highlighting the role of PKM2 in regulating the DHAP and DAG synthesis to promote ROS production in neutrophils.

Neutrophils are the most abundant leukocytes in human blood and are the first immune cell type recruited into the site of infection¹. Once activated, neutrophils produce a large amount of superoxide, the precursor of hydrogen peroxide and other reactive oxygen species (ROS), through the activation of the nicotinamide adenine

dinucleotide phosphate (NADPH) oxidase complex. Defects in ROS production impair pathogen elimination by neutrophils leading to recurring infections, as seen in patients with chronic granulomatous disease². The NADPH oxidase is an enzymatic complex composed of two membrane-bound (gp91-phox, p22-phox) and three cytosolic

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(p47-phox, p40-phox, p67-phox) subunits. The activation of NADPH oxidase requires the phosphorylation of these subunits, mainly performed by protein kinase C (PKC), which is activated by diacylglycerol (DAG) in a calcium-dependent manner. DAG is produced from the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C (PLC), which is activated by a wide variety of pro-inflammatory stimuli that signal mainly through G protein-coupled receptors (GPCRs)³. In addition, neutrophil metabolism reprogramming is essential for ROS production⁴⁻⁶.

Neutrophil metabolism relies mainly on glycolysis rather than oxidative phosphorylation (OXPHOS) for energy production since they harbour fewer mitochondria than other immune cells⁷⁻⁹. Interestingly, the inhibition of glycolysis using 2-deoxyglucose (2-DG) severely impairs phagocytosis, ROS production, and bacterial killing by neutrophils^{4,5}. In this context, it is well-known that glycolysis fuels the pentose phosphate pathway (PPP) that utilises the glycolytic intermediate glucose-6-phosphate (G6P) to generate NADPH, essential for NADPH oxidase-dependent ROS production⁶. The importance of the PPP for neutrophil activation is observed in patients with glucose-6-phosphate dehydrogenase (G6PD) syndrome with recurrent infections associated with less ROS production by neutrophils¹⁰. Furthermore, Rossi and colleagues¹¹ showed that de novo synthesis of DAG from glucose represents a substantial source of DAG in activated neutrophils. In this pathway, fructose-1,6-bisphosphate (F1,6BP) is converted into glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) by fructose bisphosphate aldolase and the balance between GAP and DHAP is regulated by triosephosphate isomerase (TPI). DHAP is further converted to glycerol-3-phosphate (G3P) by glycerol-3-phosphate dehydrogenase. G3P is metabolised to phosphatidic acid and, subsequently, to DAG by phosphatidate phosphohydrolase¹². However, whether DAG produced from glycolysis is important for ROS production is unknown.

The importance of the last steps of glycolysis for ROS production is less understood. The pyruvate kinase (PK) is a rate-limiting glycolytic enzyme that catalyses the last step of glycolysis, converting phosphoenolpyruvate (PEP) to pyruvate¹³. There are four PK isoforms: PKL, PKR, PKM1, and PKM2. PKL and PKR are expressed in the liver and erythrocytes, respectively, whereas PKM1 and PKM2 are expressed in many cell types, including neutrophils¹⁴. Particularly, PKM2 can be activated allosterically by endogenous regulators such as F1,6BP, some amino acids, and small synthetic molecules such as TEPP-46 that affect its PEP-binding affinities. In the absence of these activators, PKM2 assumes a dimeric or monomeric form, resulting in the accumulation of glycolytic intermediates. While dimeric PKM2 can translocate to the nucleus to function as a coactivator of transcriptional factors, regulating genes important for cell proliferation and glycolysis, allosterically activated tetrameric PKM2 has a high glycolytic capacity¹⁵. Although several reports have recently shown the importance of PKM2 in regulating the metabolic reprogramming and the immune function of macrophages and lymphocytes¹⁶⁻¹⁸, the role of PKM2 in the cellular metabolism and transcriptional activity in neutrophils remains poorly characterised.

Here, we show that ROS production in neutrophils is dependent on glycolytic flux. In this context, PKM2 regulates the DHAP and DAG levels, which activates PKC and, therefore, the NADPH oxidase complex to promote ROS production and killing activity in neutrophils. Our results suggest a role for PKM2 in neutrophil activation and highlight the importance of PKM2 in host defence and as a potential therapeutic target in neutrophil-mediated diseases.

Results

PKM2 expression increases in the cytoplasm of activated neutrophils

We initially compared the expression of *Pkm2* between murine neutrophils and other myeloid cells by analysing data from the

Immunological Genome Project¹⁴. We found that *Pkm2* expression is higher in neutrophils (NØ) compared to macrophages (MØ), basophils (BØ), or eosinophils (EØ) (Fig. 1a). In addition, we found that thioglycolate-elicited peritoneal neutrophils expressed higher amounts of *Pkm2* than bone marrow (BM) neutrophils, indicating that *Pkm2* expression increases in activated neutrophils (Fig. 1a). To investigate the PKM2 protein expression pattern, wild-type and PKM2-deficient neutrophils were isolated from the bone marrow of *Pkm2*^{fl/fl} and *Pkm2*^{Δ,yz2} mice, respectively (Supplementary Fig. 1), then activated with serum-opsonized zymosan (Zy/op) for 6 h. We found that PKM2 is constitutively expressed in control (Ctrl) neutrophils and increased when neutrophils were activated with Zy/op (Fig. 1b). Of note, PKM2-deficient neutrophils showed very low levels of PKM2 protein expression (Fig. 1b).

PKM2 exists in different conformations as a monomer, dimer, and tetramer. The stabilised tetrameric form has high glycolytic activity, converting PEP to pyruvate, and is located in the cytoplasm. The dimeric form has low glycolytic activity but can translocate into the nucleus, acting as a nuclear transcriptional coactivator regulating gene expression¹⁵. We found an increase in the PKM2-tetrameric form but not in the PKM2-dimeric form in Zy/op-activated neutrophils (Fig. 1c). Accordingly, using confocal microscopy, we found that PKM2 is primarily located in the cytoplasm of neutrophils (Fig. 1d). We also evaluated PKM2 expression in human neutrophils. Neutrophils were isolated from the blood of three healthy subjects and activated with Zy/op. Consistent with the data observed in murine neutrophils, human neutrophils also showed a constitutive basal expression of PKM2, increasing when neutrophils were activated with Zy/op for 6 h (Fig. 1e). Moreover, the confocal analysis showed that PKM2 was also mainly expressed in the cytoplasm of human neutrophils (Fig. 1f). Together these results show that PKM2 expression is upregulated in Zy/op-activated neutrophils and is localised mainly in the cytoplasm.

PKM2 is required for ROS production and killing capacity by neutrophils

We then conducted a series of experiments to evaluate the role of PKM2 in neutrophils' functions. We first performed flow cytometry analysis to evaluate phagocytosis in wild-type and PKM2-deficient neutrophils activated with Zy/op-FITC. We found that PKM2 did not affect the phagocytic activity of neutrophils since the percentage of neutrophils bearing Zy/op-FITC and the amount of phagocytosed Zy/op-FITC was similar between wild-type and PKM2-deficient neutrophils (Fig. 2a). Also, there was no difference in the production of neutrophil extracellular traps (NETs) by PKM2-deficient neutrophils (Supplementary Fig. 2a). We next evaluated real-time ROS production. PKM2-deficient neutrophils produced a lower amount of total ROS and superoxide than wild-type neutrophils when activated with Zy/op (Fig. 2b and Supplementary Fig. 2b). Consistently, ROS production was also impaired when PKM2-deficient neutrophils were exposed to *Staphylococcus aureus* (*S. aureus*) (Fig. 2c).

ROS is an important tool for the microbicidal activity of neutrophils¹⁹. Thus, we next determined the killing activity of neutrophils exposed to *S. aureus* and found that it was impaired in PKM2-deficient neutrophils (Fig. 2d). To extend the evaluation of the role of PKM2 to human neutrophils, we isolated cells from the blood of four healthy subjects and pre-treated them with oxalate, a non-selective pyruvate kinase activity inhibitor²⁰, for 1 h before the activation with Zy/op. We used oxalate because there is no selective inhibitor of PKM2 enzymatic activity. We also pre-treated human neutrophils with TEPP-46, which activates PKM2 in the tetrameric form, maintaining PKM2 in the glycolytic pathway and impairing its migration into the nucleus²¹. The inhibition of PK with oxalate decreased ROS production (Fig. 2e). In contrast, activation of PKM2 with TEPP-46 increased ROS production, although this effect varies for each subject (Fig. 2f).

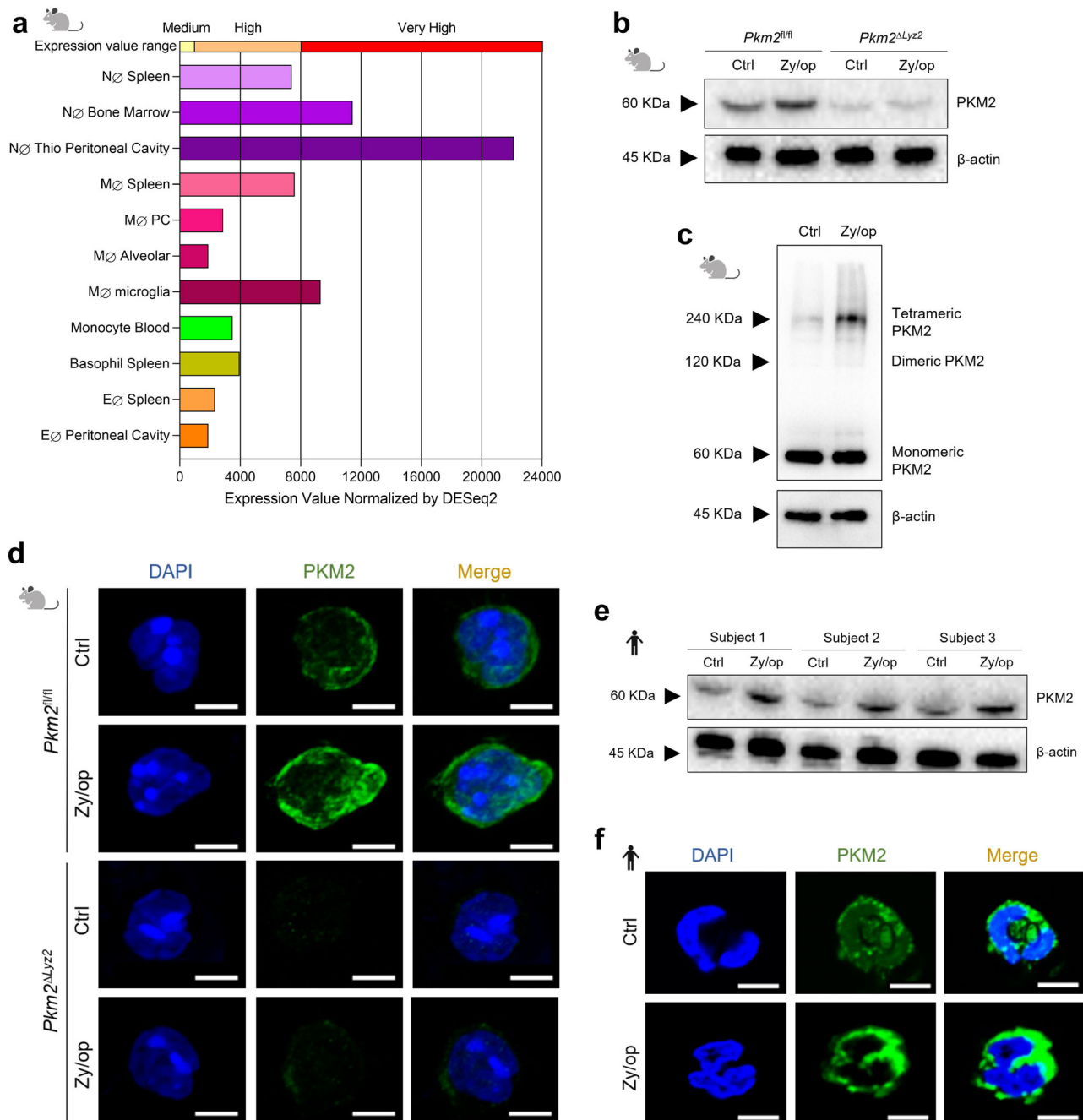


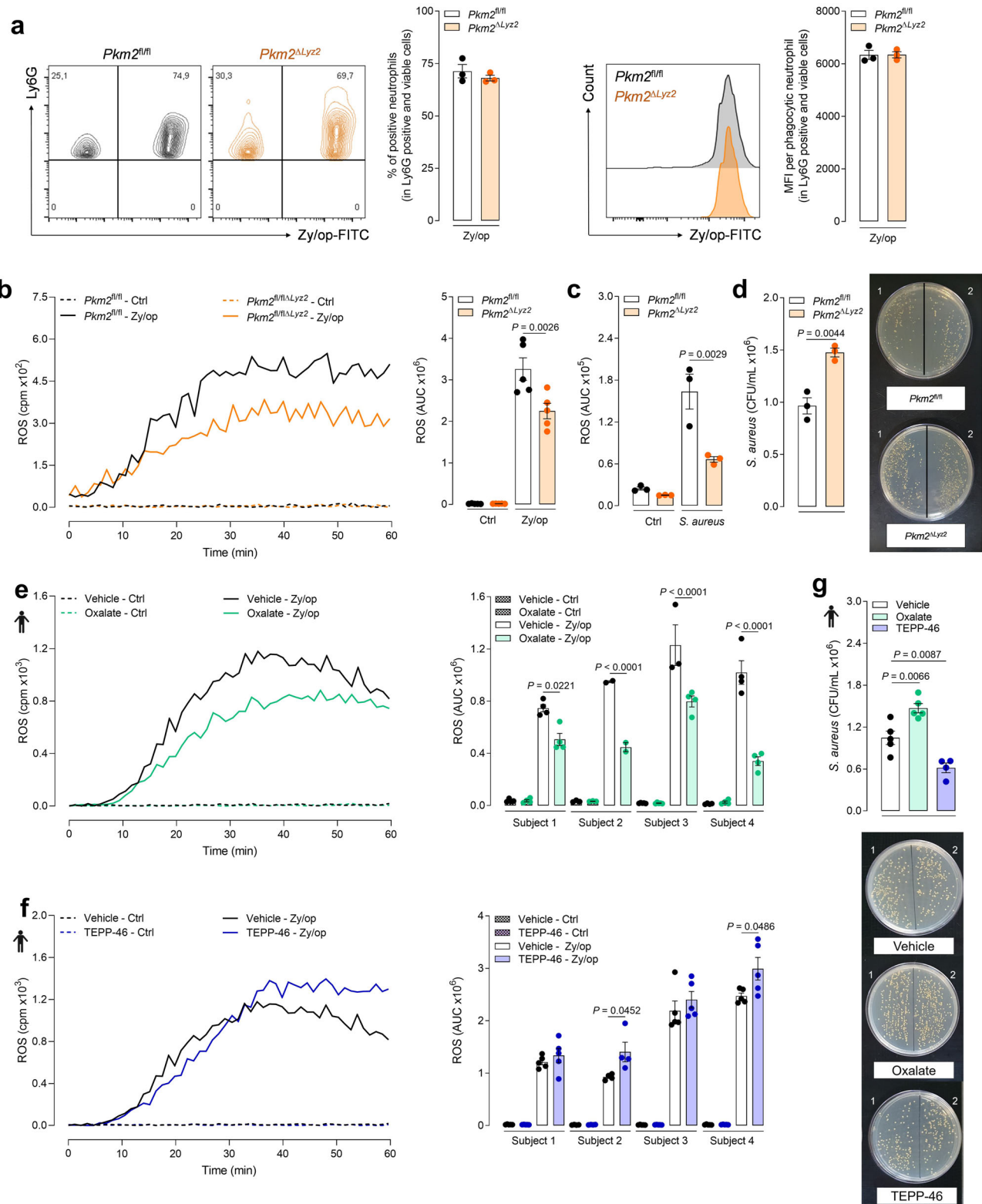
Fig. 1 | PKM2 expression increases in activated neutrophils. **a** *Pkm2* expression analysis in myeloid cells from the Immunological Genome Project (ImmGen) bulk-population RNA-seq database¹⁴. **b** Immunoblot analysis of PKM2 expression in BM-isolated neutrophils from wild-type (*Pkm2^{fl/m}*) and PKM2-deficient (*Pkm2^{ΔLyz2}*) mice. Cells were activated with Zy/op (100 μ g/mL) or medium only (Ctrl) for 6 h. β -actin was used as the loading control. Representative of three independent experiments. **c** Immunoblot analysis of PKM2 conformational states in murine neutrophils after activation with Zy/op for 6 h using DSS cross-linking assay. Representative of two independent experiments. **d** Confocal immunofluorescence analysis of Zy/op-

activated murine neutrophils stained for PKM2 (green) and DAPI (blue). The scale bar indicates 5 μ m. Representative of three independent experiments. **e** Immunoblot analysis of PKM2 expression in blood-isolated neutrophils from three healthy volunteers. Cells were activated with Zy/op for 6 h. β -actin was used as the loading control. Representative of three independent experiments. **f** Confocal immunofluorescence analysis of Zy/op-activated human neutrophils stained for PKM2 (green) and DAPI (blue). The scale bar indicates 5 μ m. Representative of three independent experiments. Clip art provided by Biorender. Source data are provided in the Source data file.

Accordingly, killing activity by human neutrophils was impaired by oxalate and enhanced by TEPP-46 (Fig. 2g).

In addition to neutrophils, macrophages are also important phagocytic cells that produce ROS and contribute to bacterial killing. To verify whether our findings in neutrophils can also be extended to macrophages, we activated PKM2-deficient bone marrow-derived macrophages (BMDMs) with Zy/op (Supplementary Fig. 3a, b) or *S.*

aureus (Supplementary Fig. 3c–e). Similar to neutrophils, PKM2 deficiency did not affect phagocytosis in BMDMs (Supplementary Fig. 3a, c). However, ROS production was actually slightly increased in BMDMs (Supplementary Fig. 3b, d). Despite this, there was no difference in the killing capacity of PKM2-deficient BMDMs (Supplementary Fig. 3e). Thus, PKM2 regulates ROS production and, consequently, killing activity in neutrophils but not in macrophages.



Aerobic glycolysis is essential for ROS production in neutrophils

The NADPH oxidase complex catalyses superoxide production, the primary source of ROS in neutrophils, a process that requires a large amount of NADPH². Among the different pathways contributing to NADPH production, the activation of PPP is crucial to trigger oxidative burst in neutrophils⁹. Since ROS production was impaired in murine and human neutrophils in the absence of glucose (Supplementary Fig. 4a), we next evaluated the relationship between glycolytic activity

and ROS production. To this end, we performed a kinetic experiment using two simultaneous assays, ROS production by chemiluminescence and glycolysis stress test using the Seahorse XF96 Analyzer (Agilent Technologies), plotting the results in the same graph for comparison (Fig. 3a). Murine neutrophils in glucose-free media were stimulated with Zy/op just before starting the assays. For both assays, glucose was added at 18 min after initiation. At 36 min, rotenone and antimycin A (Rot/AA), complex I and complex III inhibitors,

Fig. 2 | PKM2 deficiency impairs ROS production and microbial killing by neutrophils. **a** Phagocytosis of Zy/op-FITC (100 μ g/mL) by wild-type (*Pkm2^{fl/fl}*) and PKM2-deficient (*Pkm2^{Δ1/2}*) neutrophils. Representative dot plots, histograms and bar graphs showing the frequency and the median fluorescence intensity (MFI) of phagocytic cells. Gating strategies are shown in Supplementary Fig. 5a. *n* = 3 mice per group, representative of two independent experiments. **b** Kinetic measurement of ROS production by wild-type and PKM2-deficient neutrophils activated with Zy/op. Representative time-response graph and area under the curve (AUC) graph bar. *n* = 5 mice per group, representative of five independent experiments. **c** ROS production by wild-type and PKM2-deficient neutrophils exposed to *S. aureus* (2×10^6) opsonized with serum. *n* = 3 mice per group, representative of two independent experiments. **d** Quantification of viable *S. aureus* recovered from lysates of wild-

type and PKM2-deficient neutrophils after 2 h. *n* = 3 mice per group, representative of three independent experiments. **e** ROS production by human neutrophils pre-treated with oxalate (3 mM) for 1 h and then activated with Zy/op. Representative time-response graph and AUC graph bar. *n* = 4 donors in four independent experiments. **f** ROS production by human neutrophils pre-treated with TEPP-46 (30 μ M) for 1 h and then activated with Zy/op. Representative time-response graph and AUC graph bar. *n* = 4 donors in four independent experiments. **g** Number of viable *S. aureus* recovered after 2 h from lysates of human neutrophils pre-treated with oxalate or TEPP-46 for 1 h. *n* = 5 donors in three independent experiments. Error bars are mean $\pm$ SEM. *p* values were determined by two-tailed unpaired Student's *t*-test (**a**, **d**) or one-way ANOVA followed by Tukey's post hoc test (**b**, **c**, **e**–**g**). Clip art provided by Biorender. Source data are provided in the Source data file.

respectively, were added to shut down mitochondrial respiration; and, at 54 min, 2-DG was added to inhibit glycolysis. Figure 3b shows ECAR (extracellular acidification rate, blue line) and ROS production (black line). We found that Zy/op could not increase ECAR or ROS production without glucose. Interestingly, the addition of glucose increased ECAR, while Rot/AA had no effect, and 2-DG reduced ECAR to basal levels (blue line) (Fig. 3b). In parallel, we observed that ROS production increased when glucose was added with a slight decrease upon adding Rot/AA, but it was completely abolished when 2-DG was added. Together, these results show that glycolytic metabolism is crucial for ROS production by activated neutrophils. Since activated neutrophils increase oxygen consumption to produce ROS²², we next assessed the correlation between OCR (oxygen consumption rate), glucose metabolism, and ROS production (Fig. 3c). As shown in the red line, Zy/op was unable to increase OCR in the absence of glucose; however, OCR increased when glucose was added, slightly decreased when Rot/AA was added and returned to basal levels with the addition of 2-DG. Together with ROS results (black line), this highlights that while a portion of oxygen consumption and ROS production is due to mitochondrial respiration, both oxygen consumption and ROS production are dependent on glycolysis.

We then conducted a set of experiments targeting different steps of the glycolytic pathway to elucidate the importance of each one for regulating ROS production (Fig. 3d). First, wild-type and PKM2-deficient neutrophils were treated with the PPP inhibitor 6-AN (6-aminonicotinamide). We observed that while PKM2 deficiency partially reduced ROS production, 6-AN treatment resulted in a massive impairment in ROS production regardless of PKM2 presence (Fig. 3e), highlighting the well-known importance of PPP for this function⁹. To further explore whether PKM2-deficiency could impair PPP activation, thus leading to a decreased ROS production, we evaluated the NADPH and NADP concentrations in Zy/op-activated neutrophils, but no differences were found (Fig. 3f). We then examined the expression of some NADPH oxidase subunits. There were no differences in the gene expression of *gp91-phox* or protein expression of gp91-phox and p47-phox between wild-type and PKM2-deficient neutrophils (Fig. 3g, h). It has been shown that activated neutrophils have increased glutathione turnover, which may be consumed when ROS is produced²³. Glutathione is an antioxidant molecule present at high concentrations in neutrophils, and the ratio between its reduced (GSH) and oxidised (GSSG) forms can be used to measure intracellular oxidative balance. However, PKM2 deficiency did not alter the concentration of total glutathione or GSH/GSSG ratio in neutrophils (Supplementary Fig. 4b). Second, we treated murine and human neutrophils with 2-DG to inhibit hexokinase (HK), 3PO to inhibit PFKFB3 and oxalate to inhibit PK, at doses that showed comparable inhibition of lactate production. As shown in Fig. 3i, the inhibition of glycolysis at HK, PFKFB3, or PK decreased ROS production. Altogether these results show that different glycolytic steps are important for ROS production; however, PKM2 regulates ROS production by a PPP-independent mechanism.

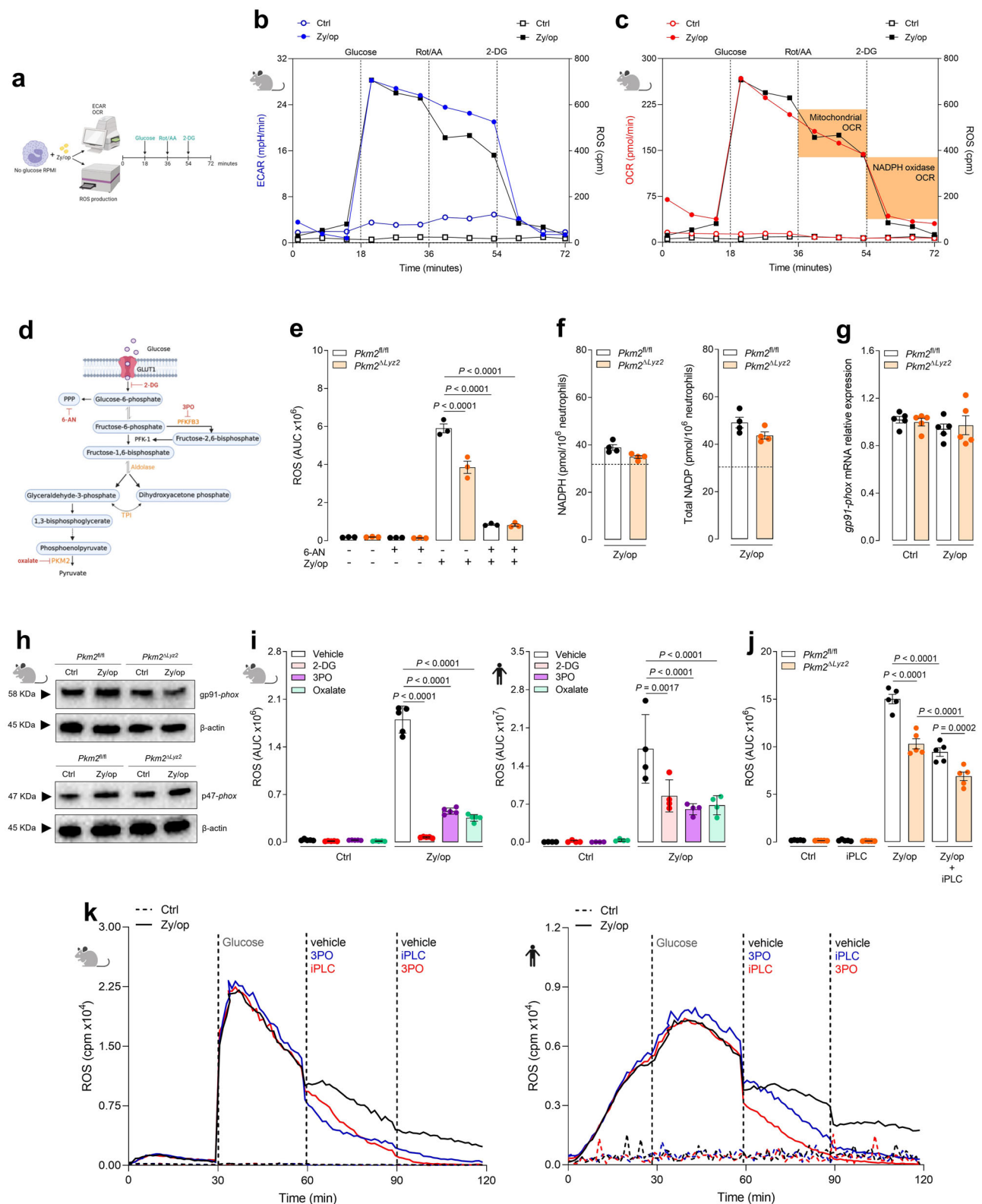
Besides being dependent on glycolysis, ROS production also depends on PLC activation, which results in calcium release, DAG

production, and, consequently, PKC activation²⁴. We performed two experiments to better understand the role of the PLC-dependent and the glucose-dependent pathways in ROS production. In the first one, wild-type and PKM2-deficient neutrophils were pre-treated with a PLC inhibitor (iPLC) for 1 h before being stimulated with Zy/op. PLC inhibition decreased ROS production in both cells, but it had an additive effect in PKM2-deficient neutrophils (Fig. 3j), suggesting that PKM2 regulates ROS production independent of PLC. In the second one, murine or human neutrophils were activated with Zy/op without glucose (Fig. 3k). After 30 min, glucose was added to the media, and an increase in ROS production was observed. After 30 min, we added vehicle (black line), 3PO (blue line), or iPLC (red line) and found that both inhibitors decreased ROS production, showing that both pathways are important for this function. Finally, after 30 min, we added vehicle (black line), iPLC (blue line) or 3PO (red line) and found that, in both cases, ROS production decreased to basal levels, showing that PLC-dependent and glucose-dependent pathways complement each other for ROS production. Together, these results suggest that PKM2-mediated glycolysis and PLC pathway are independently required for ROS production in activated neutrophils.

The metabolic activity of PKM2 regulates ROS production in neutrophils

Because PKM2, acting as a transcriptional coactivator, has been reported to have an important role in the expression of glycolytic genes and induction of glycolytic flux²⁵, we next investigated whether PKM2 deficiency affects the expression of glucose transporter 1 (GLUT1) or glucose uptake (evaluated by flow cytometer analysis using 2-NBDG) in activated neutrophils. As expected, we found an upregulation of GLUT1 expression (Fig. 4a) and glucose uptake (Fig. 4b) in Zy/op-activated neutrophils compared to control neutrophils. However, PKM2 deficiency did not affect these parameters (Fig. 4a, b). We then evaluated total pyruvate kinase activity (including PKM1 and PKM2) and found a significant reduction in PKM2-deficient neutrophils activated with Zy/op (Fig. 4c). In accordance, Zy/op-activated control neutrophils showed increased pyruvate (Fig. 4d) and lactate (Fig. 4e) production when compared to PKM2-deficient neutrophils. In line, oxalate-treated human neutrophils showed a decreased lactate production (Fig. 4e). Despite mitochondrial ROS production being increased in PKM2-deficient neutrophils (Supplementary Fig. 5a), no mitochondrial dysfunction was observed in these cells, as evaluated by MitoTracker Green and Red (Supplementary Fig. 5b). Taken together, these results show that PKM2 is required for the canonical metabolic function (pyruvate kinase activity) rather than transcriptional control activity in activated neutrophils.

To further explore the metabolic changes regulated by PKM2, we measured the redistribution of glucose metabolism flux during neutrophil activation by a tracing study with ¹³C-glucose. Zy/op-activated wild-type neutrophils showed an increase in glycolysis metabolites, including G6P, F1,6BP, pyruvate, and lactate, consistent with the activation of the glycolytic pathway. Importantly, DHAP and G3P, the precursors for DAG de novo synthesis from glucose, were also



increased (Fig. 4f). We then evaluated the glucose metabolism flux in PKM2-deficient neutrophils and found that, as in wild-type neutrophils, F1,6BP production was also increased in PKM2-deficient neutrophils activated with Zy/op. However, pyruvate and lactate, in addition to DHAP and G3P, were all decreased (Fig. 4f and Supplementary Fig. 6a, b). Importantly, PEP was hugely accumulated in PKM2-deficient neutrophils. To better understand the direction of the glycolytic flux in neutrophils (i.e., whether it favours lactate or DAG production), we

evaluated the GAP and DHAP concentrations in Zy/op-activated murine neutrophils. Both metabolites were boosted, confirming that both pathways were activated during neutrophil activation (Supplementary Fig. 6c, d). In addition, we found that GAP accumulates, and the production of DHAP is impaired when PKM2 is inhibited by oxalate (Supplementary Fig. 6c, d). These data indicate that PKM2 activity regulates the DHAP pathway leading to de novo synthesis of DAG from glucose.

Fig. 3 | Aerobic glycolysis is essential for ROS production in activated neutrophils. **a** Schematic representation of the two simultaneous assays pooled together in the graphs of Fig. 3b, c. **b** Kinetic profile of ECAR and ROS and c, OCR and ROS production in neutrophils activated with Zy/op (100 μ g/mL). $n = 5$ mice per group, representative of two independent experiments. **d** Schematic of the steps where the drugs inhibit glycolysis. **e** ROS production by neutrophils pre-treated with 6-AN (3 mM) for 1 h and then activated with Zy/op for 1 h. $n = 3$ mice per group, representative of three independent experiments. **f** NADPH and total NADP production by neutrophils activated with Zy/op for 30 min. $n = 4$ mice per group, representative of two independent experiments. **g** *gp91-phox* mRNA expression normalised to *Gapdh* in neutrophils activated with Zy/op for 3 h. $n = 5$ mice per group, representative of two independent experiments. **h** Immunoblot analysis of *gp91-phox* and *p47-phox* expression in neutrophils from wild-type and PKM2-deficient mice. β -actin was used as the loading control. Representative of two

independent experiments. **i** ROS production by wild-type murine (left) or human (right) neutrophils pre-treated with 2-DG (3 mM), 3PO (10 μ M), or oxalate (3 mM) for 1 h, then activated with Zy/op for 1 h. $n = 5$ mice per group in five independent experiments and $n = 4$ donors in four independent experiments, respectively. **j** ROS production by neutrophils pre-treated with a PLC inhibitor (iPLC, 1 μ M) for 1 h, then activated with Zy/op for 1 h. $n = 5$ mice per group, representative of two independent experiments. **k** Representative real-time ROS production in wild-type and human neutrophils activated with Zy/op. ROS production was measured in response to glucose, 3PO, and iPLC at the indicated time points. $n = 3$ mice per group, representative of two independent experiments and $n = 4$ donors in four independent experiments, respectively. Error bars are mean $\pm$ SEM. p values were determined by two-tailed unpaired Student's t -test (**f**) or one-way ANOVA followed by Tukey's post hoc test (**b**, **c**, **e**, **g**, **i**–**k**). Clip art provided by Biorender. Source data are provided in the Source data file.

Phosphoenolpyruvate accumulation reduces ROS production in neutrophils

To elucidate whether the DHAP pathway that leads to DAG production from glucose is important for ROS production, we pre-treated murine neutrophils with 5-pentadecylresorcinol (5-PDR), an inhibitor of glycerol-3-phosphate dehydrogenase (GDP), or propranolol, an inhibitor of phosphatidate phosphohydrolase (Fig. 5a). We found that both inhibitors impaired ROS production in Zy/op-activated neutrophils (Fig. 5b). These results, together with the glucose tracing analysis, indicate that PKM2 deficiency or inhibition could decrease ROS production by downregulating DHAP and G3P production and, consequently, DAG synthesis from glucose, thus decreasing NADPH oxidase complex activation. In fact, PKM2-deficient neutrophils activated with Zy/op had reduced phosphorylation of *p47-phox*, indicating an impaired activation of the NADPH oxidase complex (Fig. 5c). In addition, neutrophil activation with PMA – a PKC direct activator – bypassed the PKM2 deficiency role in ROS production (Fig. 5d), showing that the impairment in ROS production in PKM2-deficient neutrophils is due to an upstream event for PKC activation.

To further explore the importance of DAG on ROS production, we examined whether 1-oleoyl-2-acetyl-sn-glycerol (OAG), a synthetic DAG analogue, would rescue ROS production in PKM2-deficient neutrophils or oxalate-treated human neutrophils. We found that OAG restored ROS production Zy/op-activated neutrophils in both cases (Fig. 5e). Together, these data indicate that PKM2 regulates ROS production in activated neutrophils by modulating DAG de novo synthesis from glucose. In this way, two independent pathways – through PLC activation and de novo synthesis from glucose – are responsible for maintaining DAG levels in neutrophils, which is important for PKC and NADPH oxidase activation.

Grüning and collaborators²⁶ showed that a decrease in pyruvate kinase activity leads to PEP accumulation in yeast, inhibiting the enzyme TPI by binding to its catalytic pocket. Accordingly, we also found that PEP accumulates in PKM2-deficient neutrophils (Fig. 4f). TPI interconverts GAP and DHAP²⁷, and PEP competes with GAP for binding to TPI²⁶. We then hypothesised that PEP accumulates and inhibits TPI activity when PKM2 is absent or inhibited in neutrophils. This would decrease DHAP accumulation and, consequently, DAG production, impairing ROS production. To further explore this hypothesis, we treated wild-type neutrophils with PEP for 1 h prior to the activation with Zy/op and found that ROS production was impaired (Fig. 5f). In addition, DHAP (Fig. 5g) and G3P (Fig. 5h) concentrations and TPI activity (Fig. 5i) were also reduced. Furthermore, PEP and oxalate treatment decreased phosphorylation of *p47-phox*, which indicates a decreased NADPH oxidase complex activation (Fig. 5j). We then evaluated TPI activity in PKM2-deficient neutrophils and oxalate-treated human neutrophils. We found that the absence or inhibition of PKM2 decreased TPI activity (Fig. 5k). To further assess TPI importance for ROS production, we utilised CRISPR/Cas9-based editing to disrupt *Pkm2* and *Tpi* (Supplementary Fig. 7a–c) in NB4 cells, a promyelocytic

leukaemia cell line that differentiates to mature neutrophils upon exposure to all-trans retinoic acid (ATRA)²⁸. We found that ROS production was impaired in the absence of PKM2 and TPI (Fig. 5l). Together, these results show that PKM2 deficiency reduces ROS production in activated neutrophils by increasing PEP accumulation, which inhibits TPI activity and DHAP metabolism, impairing NADPH oxidase activation and ROS production (Fig. 5m). In this way, PKM2 regulates the DHAP-DAG-PKC-NADPH oxidase axis to promote ROS production in neutrophils.

PKM2 is important for *S. aureus* killing in vivo

S. aureus is a Gram-positive bacterium that is a common pathogen responsible for a skin infection²⁹. A hallmark of *S. aureus* infections is neutrophil recruitment and abscess formation, which is required to eliminate the pathogen^{30,31}. To determine the importance of PKM2 in vivo, we used two different mouse models: peritonitis and cutaneous infection with *S. aureus*³² in *Pkm2*^{fl/fl} and *Pkm2* ^{Δ lyz2} mice and evaluated the infection progression. For the first model, we inoculated mice with *S. aureus* via intraperitoneal (i.p.) injection, and the animals were assessed for 18 h (Fig. 6a). We found that *Pkm2* ^{Δ lyz2} mice were more susceptible to *S. aureus* infection than *Pkm2*^{fl/fl} mice, showing higher bacterial loads in the blood (Fig. 6b) and in the peritoneal exudate (Fig. 6c). Despite that, neutrophils (Fig. 6d) and mononuclear cells (Fig. 6e) counts were unchanged. In addition, IL-6 was increased in the plasma (Fig. 6f) of *Pkm2* ^{Δ lyz2} mice, but no differences were found in CXCL2 (Fig. 6g) and CCL2 (Fig. 6h) levels in the exudate.

It has been shown that mice inoculated intracutaneously with high doses of *S. aureus* display clinical and histopathological signs of local infection and inflammation and massive neutrophil recruitment within 48 h³³. In this way, for the second model, we performed two 3 mm punches through the dermis in the back of shaved mice and inoculated the wound with *S. aureus* (Fig. 6i). The wound lesion sizes of anaesthetised mice were evaluated for 11 days and determined by measuring the total lesion size (square centimeters). We found that sham *Pkm2*^{fl/fl} and *Pkm2* ^{Δ lyz2} mice had the same wound healing timing, showing no difference in the cicatrization processes in mice that do not express PKM2 in myeloid cells (Fig. 6f). However, when inoculated with *S. aureus*, *Pkm2* ^{Δ lyz2} mice had larger wounds than *Pkm2*^{fl/fl} mice in the first four days of infection (Fig. 6j, k). Moreover, compared to *Pkm2*^{fl/fl}, the wound from *Pkm2* ^{Δ lyz2} had more colony-forming units (CFU) of *S. aureus* at 48 h of inoculation despite having the same number of neutrophils at later time points (Fig. 6l, m). In addition, there was no difference in the concentrations of inflammatory cytokines CXCL1 (Fig. 6n), TNF- α (Fig. 6o), and IL-6 (Fig. 6p) in the wound tissue homogenates. Altogether these results show that PKM2 is important for controlling *S. aureus* infection in vivo.

Discussion

In the past few years, the role of metabolic reprogramming as a key immuno-regulatory phenomenon has been investigated by its

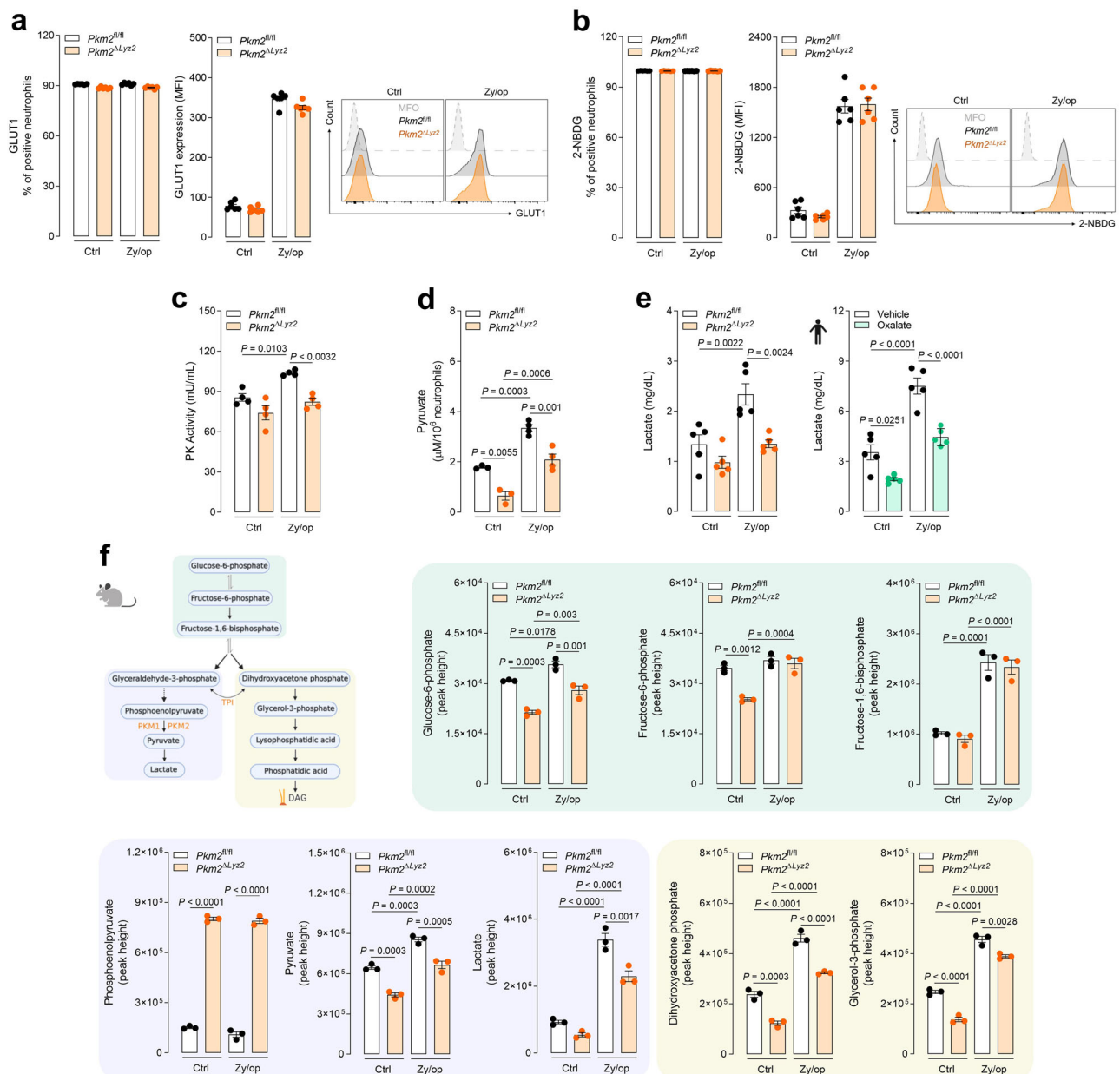


Fig. 4 | Metabolic activity of PKM2 regulates ROS production in neutrophils.

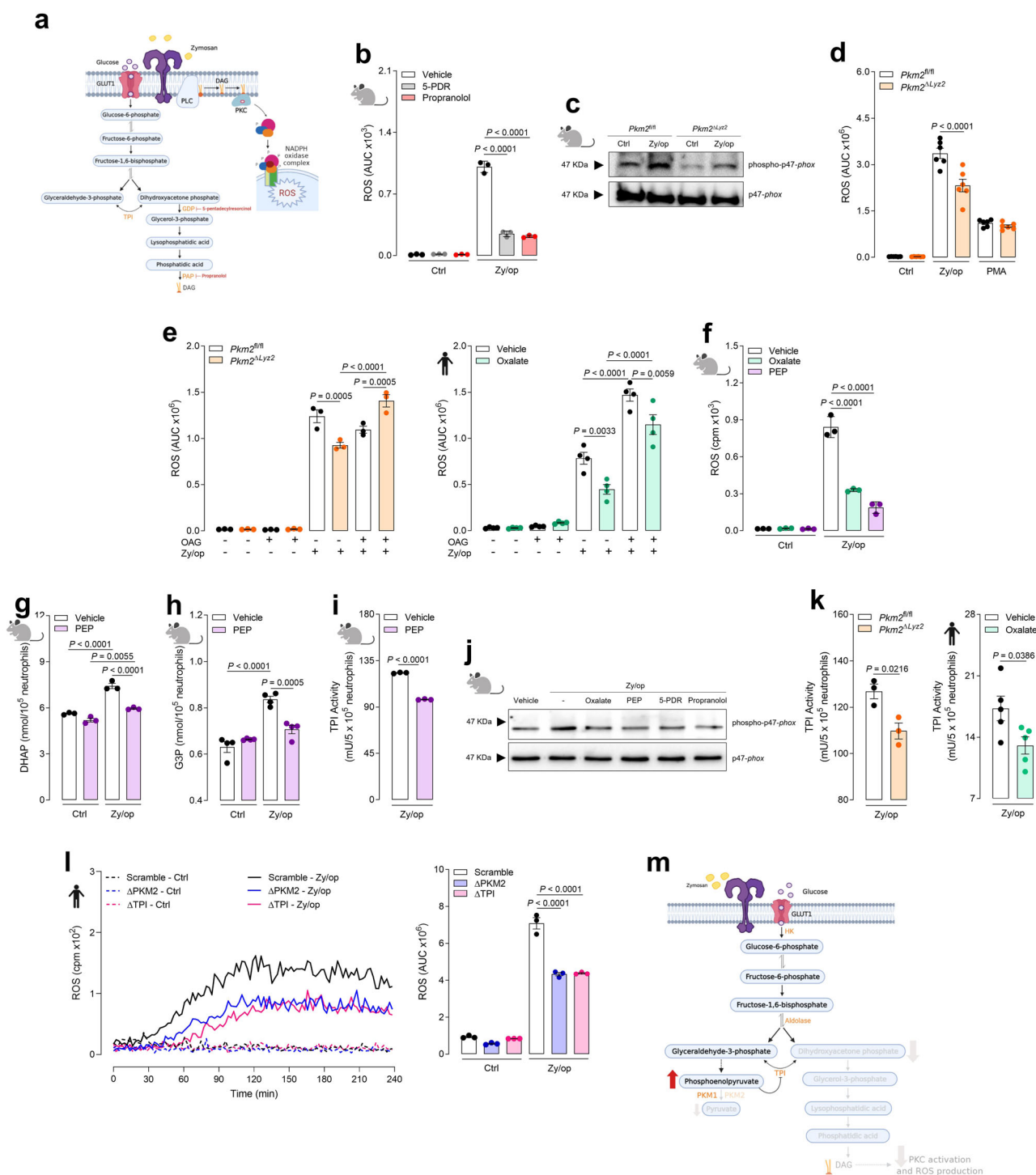
a GLUT1 expression in wild-type ($Pkm2^{fl/fl}$) and PKM2-deficient ($Pkm2^{\Delta Lyz2}$) activated with Zy/op (100 μ M) for 1 h. Representative histogram and graph bar showing the GLUT1 frequency and median fluorescence intensity (MFI). Gating strategies are shown in Supplementary Fig. 5a. $n = 6$ mice per group in two independent experiments. **b** Glucose uptake in wild-type and PKM2-deficient neutrophils activated with Zy/op for 30 min. Representative histogram and graph bar showing frequency and MFI for 2-NBDG. Gating strategies are shown in Supplementary Fig. 5a. $n = 6$ mice per group in two independent experiments. **c** Pyruvate Kinase (PK) activity in neutrophils activated with Zy/op for 1 h. $n = 5$ mice per group in two independent experiments. **d** Pyruvate production by neutrophils activated with Zy/op for 1 h.

$n = 4$ mice per group in two independent experiments. **e** Lactate production by wild-type and PKM2-deficient mouse neutrophils and by human neutrophils pre-treated with oxalate (3 mM) for 1 h and activated with Zy/op for 1 h. $n = 5$ mice per group in two independent experiments and $n = 5$ donors in five independent experiments. **f** Glycolytic metabolites from 13 C-glucose tracing in wild-type and PKM2-deficient neutrophils activated with Zy/op for 1 h. The y-axis refers to the height of the chromatography peak obtained by LC-MS. $n = 3$ mice per group in three independent experiments. Error bars are mean $\pm$ SEM. p values were determined by one-way ANOVA followed by Tukey's post hoc test. Clip art provided by Biorender. Source data are provided in the Source data file.

influence on cell differentiation, function, and fate³⁴. In this context, the enzyme PKM2 has been implicated as a critical regulator of aerobic glycolysis and recently has generated significant interest due to its upregulation in activated immune cells¹⁵. Of note, it was shown that in macrophages and lymphocytes, PKM2 has a major transcriptional role, migrating to the nucleus and regulating gene expression^{16–18}. Conversely, we show here that PKM2 is mainly expressed in the cytoplasm of neutrophils. Its expression in the tetrameric form is upregulated during neutrophil activation, indicating

that PKM2's primary role is on the glycolytic pathway in activated neutrophils.

Neutrophils are rapidly recruited to tissues to phagocytose and eliminate invading pathogens via antimicrobial actions, especially ROS production^{2,35}. It is worth noting that neutrophils recognise both Zymosan and *S. aureus* by TLR2, which bind to ligands with very diverse structures such as lipoproteins/lipopeptides, peptidoglycan, glycopolymers, and proteins³⁶. Here, we showed that while PKM2 was not important for the phagocytosis process, it regulates ROS



production and, consequently, the killing capacity in neutrophils. It is already known that ROS production is dependent on glycolysis⁷, but until now, this was mainly associated with the PPP activation and the balance of NADPH/NADP in neutrophils^{6,10,37}. Surprisingly, we found that, in the absence of PKM2, ROS production was reduced without impairments in NADPH/NADP balance, while PK activity and lactate production was decreased.

We found a difference between murine and human neutrophil responses during our experiments utilising glucose-free media. The absence of glucose almost totally impaired ROS production in murine neutrophils and reduced it by 50% in human neutrophils. In this regard, it is interesting to note that neutrophils contain glycogen within granules, which is important to their function and

survival. Furthermore, human neutrophils contain more glycogen than murine neutrophils³⁸, which could explain the more drastic effect of glucose starvation in ROS production of murine neutrophils. Moreover, we found that ROS production is associated with increased glycolytic rate and the inhibition of glycolysis in different steps of this pathway impaired ROS production. Previously, the canonical pathway for ROS production was not thought to involve the direct activation of the glycolytic pathway, and it has been shown that glycolysis provides the NADPH used during NADPH oxidase activation for ROS production³⁹. However, our results highlight that PEP, a glycolytic metabolite at PKM2 enzymatic reaction, is involved in regulating ROS production in activated neutrophils.

Fig. 5 | PKM2 modulates ROS production in neutrophils by a TPI-dependent mechanism. **a** Schematic of glycolysis inhibition by various drugs. **b** ROS production by neutrophils treated with 5-PDR (30 μ M) or propranolol (30 μ M) for 1 h and activated with Zy/op for 1 h. $n = 3$ mice per group, representative of three independent experiments. **c** Immunoblot analysis of p47-phox phosphorylation in wild-type (*Pkm2^{fl/fl}*) and PKM2-deficient (*Pkm2^{ΔLyz2}*) neutrophils activated with Zy/op for 30 min. Representative of two independent experiments. **d** ROS production by neutrophils activated with Zy/op or PMA (50 nM) for 1 h. $n = 6$ mice per group, representative of three independent experiments. **e** ROS production by murine and human neutrophils pre-treated with oxalate (3 mM) for 1 h and activated with Zy/op for 1 h in the presence of OAG (10 μ M). $n = 3$ mice per group in three independent experiments and $n = 4$ donors in four independent experiments, respectively. **f** ROS production by neutrophils pre-treated with oxalate or PEP (1 mM) for 1 h and activated with Zy/op for 1 h. $n = 3$ mice per group, representative of three independent experiments. **g** DHAP production by neutrophils pre-treated with PEP for 1 h and activated with Zy/op for 1 h. $n = 3$ mice per group, representative of two

independent experiments. **h** G3P production by neutrophils pre-treated with PEP and activated with Zy/op. $n = 4$ mice per group, representative of two independent experiments. **i** TPI activity in neutrophils pre-treated with PEP and activated with Zy/op. $n = 3$ mice per group, representative of two independent experiments. **j** Immunoblot analysis of p47-phox phosphorylation in wild-type neutrophils pre-treated with oxalate, PEP, 5-PDR or propranolol for 1 h and activated with Zy/op for 30 min. Representative of two independent experiments. **k** TPI activity in murine and in human neutrophils pre-treated with oxalate for 1 h and activated with Zy/op for 1 h. $n = 3$ mice per group (representative of two independent experiments) and $n = 5$ donors (representative of five pooled independent experiments). **l** ROS production by scramble, PKM2-(Δ PKM2) or TPI-(Δ TPI) deficient NB4 cells activated with Zy/op for 1 h. $n = 3$ independent experiments. **m** Working hypothesis. Error bars are mean $\pm$ SEM. p values were determined by two-tailed unpaired Student's t -test (**l**, **k**) or one-way ANOVA followed by Tukey's post hoc test (**b**, **d–h**, **l**). Clip art provided by Biorender. Source data are provided in the Source data file.

DAG is an important PKC activator and can be produced from glucose in neutrophils in a pathway dependent on DHAP metabolism¹¹. However, if DAG produced from glucose/DHAP metabolism contributes to ROS production is unknown. We found that the inhibition of DAG production from glycolysis by blocking the glycerol-3-phosphate dehydrogenase, which converts DHAP into G3P, and the phosphatidate phosphohydrolase, which converts phosphatidic acid into DAG, impaired ROS production. In addition, as demonstrated by others³⁸, we found here that neutrophil activation increased DHAP production. However, DHAP and G3P concentrations were decreased in PKM2-deficient and oxalate-treated neutrophils, indicating that PKM2 somehow regulates the DHAP downstream pathway. As aforementioned, this pathway is important for de novo synthesis of DAG. In fact, the treatment of neutrophils with OAG restored ROS production in PKM2-deficient and oxalate-treated neutrophils, suggesting that ROS production is impaired in these cells because DAG production from glucose is decreased.

It has been shown that a decrease of pyruvate kinase activity leads to PEP accumulation, which can inhibit the activity of triose phosphate isomerase (TPI) by binding to its catalytic pocket²⁶. TPI has high catalytic efficiency and enhances the movement of a single proton to constantly interconvert DHAP and GAP, where the equilibrium favours the synthesis of DHAP^{40,41}. Here we found a decrease in TPI activity in PKM2-deficient and oxalate-treated neutrophils. We hypothesised that PKM2 modulates TPI activity by regulating PEP concentration, which would impact on DHAP concentration and, ultimately, ROS production, explaining, at least in part, our results. Indeed, when neutrophils were treated with PEP, there was a decrease in ROS production, DHAP, and G3P concentrations. Conversely, it has been shown that the low TPI activity increases DHAP concentration in erythrocytes⁴² and yeast²⁶ and mediates an increase in PPP metabolites concentrations and oxidant resistance in yeast²⁶. However, PKM2-deficient neutrophils had normal glutathione concentrations, suggesting that, in contrast to what is seen in other cell types, the low TPI activity did not alter the PPP activation in neutrophils. Thus, further studies are needed to understand the role of TPI in neutrophil function.

Finally, we showed that PKM2 is important for early infection resolution in vivo in two different *S. aureus* infection models, peritonitis and skin infection, where a massive neutrophil infiltration is observed in the first hours of infection⁴³. The importance of glycolysis for *S. aureus* killing by neutrophils was shown by Boxer and collaborators⁵, where neutrophils treated with 2-DG had impaired killing capacity. Furthermore, Burge and collaborators⁴⁴ showed that a patient with recurrent *S. aureus* infections had a defect in PK activity. In both models we evaluated, there was a higher number of *S. aureus* CFU despite having the same number of neutrophils in the peritoneum and the wound. It is known that defects in neutrophil functions, including ROS production, chemotaxis, and granules disorders impair *S. aureus*

elimination and infection resolution⁴³. Together with our in vitro findings, these results indicate that PKM2 regulation of ROS production in neutrophils is important for bacterial elimination in vivo.

However, because we used *Lyz2^{Cre}* PKM2^{fl/fl} mice, we cannot exclude that PKM2 plays additional roles in macrophages that could account for controlling *S. aureus* infection in vivo.

Our results reveal a new role of PKM2 that regulates the DHAP-DAG-PKC-NADPH oxidase axis and, at least in part, ROS production in neutrophils by impairing PEP accumulation. These findings fill the gaps in the current knowledge of the importance of the glycolytic pathway for ROS production and provide insight into the mechanisms underlying the role of PKM2 in neutrophil metabolism and function.

Methods

Animals

C57BL/6 wild-type mice were purchased from Charles River. *Lyz2^{Cre}* (strain# 004781) and *PKM2^{fl/fl}* (strain# 024048) mice were obtained from Jackson Laboratories. Myeloid cell-*(Pkm2^{ΔLyz2})* specific-*Pkm2*-deficient mice were generated by crossing the *Pkm2^{fl/fl}* mice with *Lyz2^{Cre}* mice on a C57BL/6 background. *Pkm2^{fl/fl}* mice without *Lyz2^{Cre}* gene were used as controls for all experiments. All experiments were carried out with 6–7-week-old female and male littermate mice. The protocols used for animal experimentation were approved by the Animal Welfare Committee of the Ribeirão Preto Medical School, University of São Paulo (protocol number: 143/2017). Animals were bred and maintained under specific pathogen-free conditions (12/12-h light/dark cycle, 55 $\pm$ 5% humidity, 21–23 °C) at the animal facility of the Ribeirão Preto Medical School, University of São Paulo. Mice were randomly assigned to experimental groups. Mice were euthanized with Xylazine/Ketamine (10 mg/kg / 80 mg/kg) administrated intraperitoneally (i.p.).

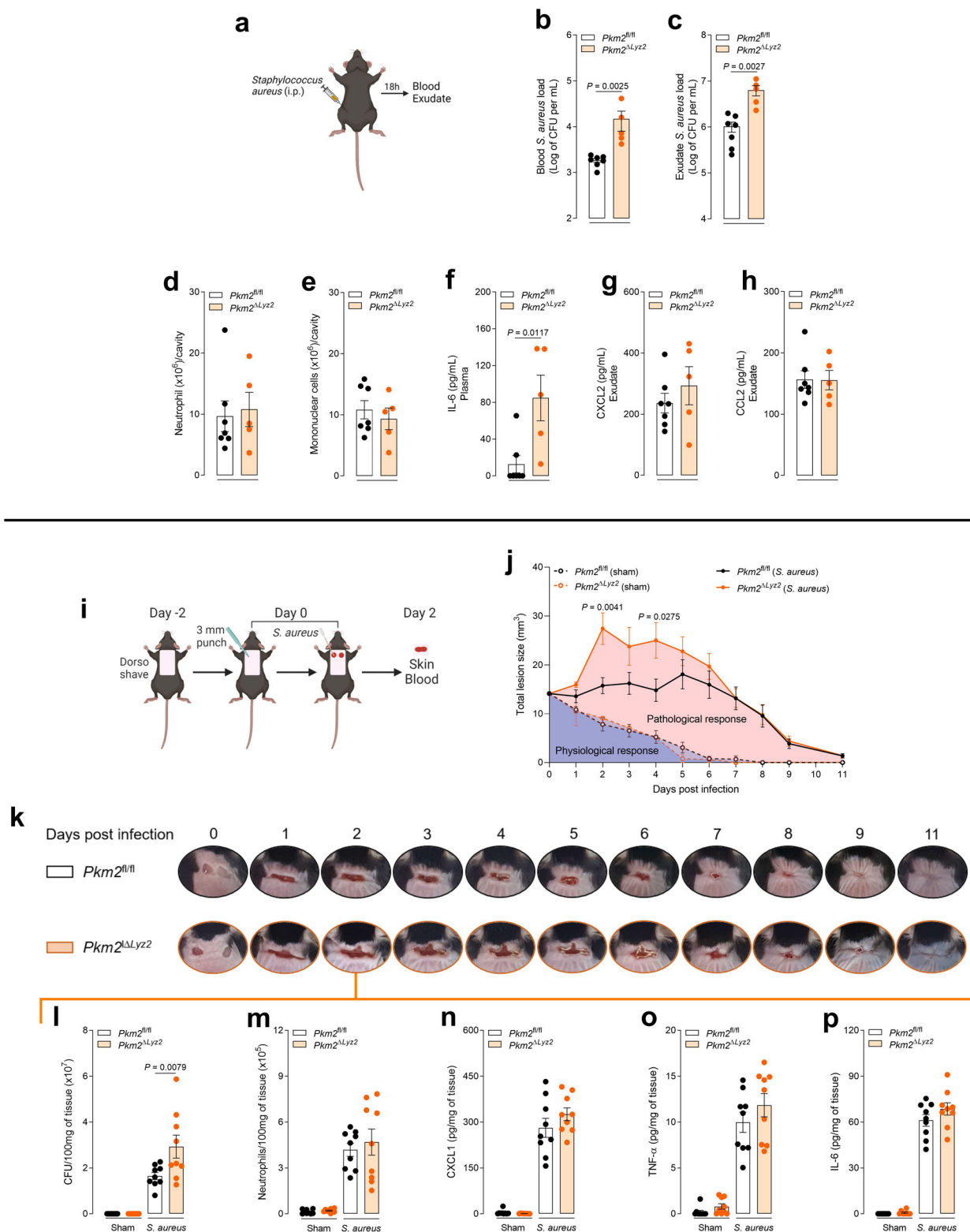
Human blood samples

Thirty healthy donors of both sexes with ages between 18 and 40 years were included in this study. All recruited volunteers provided written informed consent form. The exclusion criteria were smoking, obesity, diabetes, pregnancy and anti-inflammatory use. The study was approved by the Human Subjects Institutional Committee of the Ribeirão Preto Medical School, Brazil (License number: 31033519.4.0000.5440).

Neutrophil isolation and culture

Murine neutrophils were obtained from mouse bone marrow. Briefly, mouse femurs and tibias were removed and flushed with phosphate buffer solution (PBS, Corning) and then isolated by immunomagnetic negative selection with the Neutrophil Isolation Kit, following manufacturer's instructions (Miltenyi Biotec, 130-097-658).

Human neutrophils were obtained from peripheral blood by Percoll (GE Healthcare, 17-0891-01) density gradient⁴⁵. Briefly, whole



blood was collected into Vacutainer® Citrate Tubes with 3.2% buffered sodium citrate solution as anticoagulant (BD Bioscience). Blood was laid on top of a 4-layer Percoll gradient (72, 63, 54 and 45% in PBS) and then centrifuged at $650 \times g$, 30 min, at 20°C . Neutrophils were recovered at the interface of the 63–72% fractions. The remaining erythrocytes were lysed (1X RBC Lysis Buffer, Thermo, 00-4333-57).

Both murine and human neutrophils were resuspended in Hank's balanced saline solution (HBSS, Corning, 21-020-CM) containing 2% of

bovine serum albumin (BSA) and calcium. Cell suspensions contained >95% of viable neutrophils, as established by flow cytometry.

Bone marrow-derived macrophages (BMDM) isolation and culture

BMDMs were obtained using L929 cell-conditioned medium, as previously described⁴⁶. Briefly, bone marrow cells were obtained by flushing both mice femurs and tibias with RPMI-1640 culture media (Corning, 15-040-CV). After centrifugation, cells were resuspended in

Fig. 6 | PKM2-deficiency in myeloid cells impairs host defence against *S. aureus* infection. **a** Schematic protocol of peritonitis infection with *S. aureus*. **b** Bacterial load in the blood 18 h after challenge in *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice. *n* = 7 and 5 mice, respectively. Representative of two independent experiments. **c** Bacterial load in the exudate 18 h after challenge in *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice. *n* = 7 and 5 mice, respectively. Representative of two independent experiments. **d** Neutrophil cell counts 18 h after challenge in *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice. *n* = 7 and 5 mice, respectively. Representative of two independent experiments. **e** Mononuclear cell counts 18 h after challenge in *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice. *n* = 7 and 5 mice, respectively. Representative of two independent experiments. **f–h** Concentration of IL-6, CXCL2, and CCL2 in the plasma and exudate 18 h after *S. aureus* challenge in *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice. *n* = 7 and 5 mice, respectively. Representative of two independent experiments. **i** Schematic protocol of localised cutaneous infection

with *S. aureus*. **j** Mean of total lesion size (cm²) ± the standard error of the mean (SEM) evaluated for 11 days. *n* = 10 mice. Representative of two independent experiments. **k** Representative pictures of the dorsal skin lesions in the back of *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice evaluated for 11 days. **l** Bacterial load in the wound two days after challenge in *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice. *n* = 9 mice. Representative of two independent experiments. **m** Myeloperoxidase activity evaluated in the wound two days after *S. aureus* challenge in *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice. *n* = 9 mice. Representative of two independent experiments. **n–p** Concentration of CXCL1, TNF-α and IL-6 in the wound two days after *S. aureus* challenge in *Pkm2*^{fl/fl} and *Pkm2*^{Δlyz2} mice. *n* = 9 mice. Representative of two independent experiments. Error bars are mean ± SEM. *p* values were determined by one-way ANOVA followed by Tukey's post hoc test. Clip art provided by Biorender. Source data are provided in the Source data file.

RPMI-1640 containing 20% L929, 10% foetal bovine serum (FBS, GE Life Sciences, SV30160.03), L-glutamine (2 mM, Sigma, G7513), penicillin (100 U/mL, Sigma, P4333), and fungizone (2.5 μg/mL, Gibco, 15-290-018) and seeded for 7 days. After the end of this period, BMDMs were harvested for subsequent experiments.

CRISPR/Cas9 gene editing in NB4 cell line

NB4 cell line (ATCC, ACC-207) was cultured in RPMI-1640 culture medium supplemented with 10% FBS, penicillin (100 U/ml), and L-glutamine (2 mM) at 37 °C and 5% CO₂. For the generation of NB4 deficient for PKM2 and TPI, we used the CRISPR/Cas9 plasmid PXL (plasmid # 75349, Addgene). For the selection of the gene region to be targeted by the Cas9 endonuclease, we utilised Benchling (Biology Software), selecting the exon 10 of the coding sequence of the PKM2 gene or exon 3 of the coding sequence of TPI, all responsible for the production of their respective protein. Succinctly, based on those sequences, guide RNAs were selected using CRISPOR⁴⁷. The oligonucleotides synthesised carrying the targeting sequence of PKM2 were 5'-caccATTGAGGAACCTCCGCC-3' and 5'-aacGGCGGGGAGTTCTCAAT-3'. For TPI 5'-acaccGCAAAGACTGCGGAGCCACGTG-3' and 5'-aaaacACGTGGCTCCGAGTCTTTGCG-3' (Exxtend). The oligos were then cloned into the PXL vector (Addgene, 75349)⁴⁸. Briefly, *E. coli* DH5-α chemo-competent bacteria were transformed with the plasmid, and on the next day, the plasmids were purified by the method of MiniPrep, following the manufacturer's instructions (Qiagen-Hilden, 27104). NB4 cells (2 × 10⁶ cells) were resuspended in 100 μl of Cell Line Nucleofector Solution V buffer. To this solution was added 2 μg of the cloned PXL plasmid. This mixture was transferred to cuvettes, and electroporation was performed using the X-001 (Nucleofector I Device) protocol. After transfection, 500 μl of RPMI-1640 supplemented with 10% FBS was immediately added, and the cells were plated. After 48 h, NB4 cells were differentiated into granulocytes with 1 μM ATRA (Sigma-Aldrich) for 5 days. Granulocytic differentiation was evaluated by cell morphology.

Mouse model of skin wound infection

The mouse model of skin wound infection was done as described by Guo and collaborators³³, with modifications. Briefly, mice were anaesthetised by inhalation administration of isoflurane (1–3%), and their posterior backs were shaved. On the following day, two full-thickness wounds were made through the dermis using a 3 mm biopsy punch. The wounds were subsequently inoculated with *Staphylococcus aureus* (ATCC, Strain 6538, 1 × 10⁸ CFU per 7.5 μl of PBS in each wound) using a micropipette. Wounds of sham mice were not inoculated. All mice were given analgesics (12.5 mg.kg⁻¹, tramadol, Agener União, subcutaneous) beginning 30 min before making the wounds and then every 12 h up to day 3. Measurements of total lesion size (square centimetres) were made daily.

Wound healing was evaluated for 11 days. For wound analysis, biopsy skin specimens were obtained from the lesions of infected

mice. Biopsy of healthy skin from the same mouse was used as a control. Tissues were weighed before being homogenised in 500 μl PBS. Recovered bacteria were counted by plating serial dilutions onto Mueller-Hinton agar (Difco, 225250) plates. Samples were centrifuged, and supernatants were used for cytokines measurements. The pellet was used for MPO activity assay by measuring the change in OD at 450 nm using tetramethylbenzidine⁴⁹. This assay was used as a quantitative measurement of neutrophil migration and accumulation in the wound.

Mouse model of peritonitis infection

Mice were anaesthetised by inhalation administration of isoflurane (1–3%) and inoculated with *S. aureus* (1 × 10⁸ CFU) intraperitoneally (i.p.). After 18 h, mice were euthanised, and exudate and blood were obtained. Recovered bacteria were counted by plating serial dilutions onto Mueller-Hinton agar plates. Samples were centrifuged and supernatants were used for cytokines measurements.

Zymosan opsonisation

For Zymosan opsonisation, 10 mg of zymosan (Sigma, Z4250) was washed in PBS and then incubated with murine or human serum diluted twice in PBS at 37 °C for 30 min. Zymosan opsonised (Zy/op) was washed twice, resuspended in PBS and stored at -20 °C. For phagocytosis experiments, Zymosan was first labelled with Fluorescein isothiocyanate isomer I (FITC, Sigma, F4274) and then incubated with serum (Zy-FITC/op).

Bacteria

Staphylococcus aureus was obtained from the American Type Culture Collection (ATCC, USA, Strain 6538). Bacteria were cultivated from frozen stocks for 24 h at 37 °C in Brain Heart Infusion Agar (Oxoid, CMI135). Bacteria suspension was prepared in sterile NaCl 0.9% and the calculations were estimated by measuring the OD of solutions at 600 nm. CFU were verified by plating serial dilutions onto Mueller-Hinton agar plates.

Western blotting

For standard western blot protocols, neutrophils (3 × 10⁶) were activated with Zy/op (100 μg/mL) at 37 °C and 5% CO₂. After 6 h, cells were harvested in boiling Laemmli Sample Buffer (Biorad, 161-0737), followed by heating at 95 °C for 5 min. For crosslink assay, neutrophils were incubated with 500 μM disuccinimidyl suberate (Sigma-Aldrich, S1885) for 30 min, and then cell lysates were prepared as described above. Protein samples were resolved on SDS-PAGE gels and transferred onto a nitrocellulose membrane (GE Healthcare). Membranes were blocked with 5% (wt/vol) non-fat milk (Cell Signalling) in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated overnight at 4 °C with 1:1000 dilutions of primary antibodies against PKM2 (Cell signalling, Cat# 4053 P), β-actin (Cell Signalling, Cat# 3700 S), gp91-phox (BD

Transductions, Cat# 611414), p47-*phox* (Millipore, Cat# 07-500) and phospho-p47-*phox* (Invitrogen, Cat# PA5-36863). Membranes were repeatedly washed with TBST and incubated for 2 h with the appropriate HRP-conjugated secondary antibody (1:5000 dilution; Sigma-Aldrich, anti-rabbit Cat# A0545; anti-mouse Cat# A9044). Immunoreactivity was detected using the ECL prime reagent (GE Healthcare, RPN2236), and then the chemiluminescence signal was recorded on the ChemiDoc XRS Imager (Bio-Rad Laboratories). Data were analysed with Image Lab software (Bio-Rad Laboratories). Total β -actin levels were used as a loading control.

Confocal microscopy

Neutrophils (8×10^4) were incubated on poly-L-lysine-coated coverslips for 30 min and activated with Zy/op (100 μ g/mL) for 6 h. After this time cells were fixed (2% PFA), permeabilised and stained with anti-PKM2 (1:200, Cell signalling, Cat# 4053P) overnight. After incubation with a secondary antibody (1:800, AlexaFluor 488, Abcam, Cat# ab150065), coverslips were washed in PBS and mounted onto microscope slides using a DAPI-containing mounting medium (Vector Laboratories, H-1200-10). Images were acquired by Axio Observer combined with LSM 800 microscope (Carl Zeiss) and DMI6000B microscope (Leica Microsystems) and analysed with Fiji/ImageJ.

Phagocytosis

Neutrophils (0.3×10^6) were activated with Zy-FITC/op (100 μ g/mL) for 20 min at 37 °C and then washed with PBS. Fluorescence of the samples was measured in a flow cytometer (FACSVerse™, BD Biosciences) before and after the addition of the quenching solution (0.4% Trypan blue in PBS citrate, pH 4.4), as described by Nuutila and collaborators⁵⁰, with modifications. Ten thousand cells were analysed.

ROS production

ROS production was measured by luminol-dependent chemiluminescence (CL) assay as described by Kanashiro and collaborators⁵¹. Briefly, neutrophils (0.2×10^6) were pre-treated (when indicated) for 1 h with oxalate (3 mM, Sigma, 71800), TEPP-46 (30 μ M, Millipore, 5054870001), 2-Deoxy-D-glucose (2-DG, 3 mM, Sigma, D8375), 3PO (10 μ M, Calbiochem, 525330), phosphoenolpyruvate (PEP, 1 mM, Sigma, P0564), 5-pentadecylresorcinol (5-PDR, 30 μ M, Sigma, 91822) or propranolol (30 μ M, Sigma, P0884). Neutrophils were then activated with Zy/op (100 μ g/mL) or *S. aureus* (MOI = 3) in the presence of luminol (Sigma, A8511) 10^{-4} M or lucigenin (Sigma, M8010). The reaction was monitored in a luminometer (FlexStation 3 Multi-Mode Microplate Reader, Molecular Devices, San Jose, CA) for 1 h, at 37 °C, and the results were expressed as the area under the time-course CL curve (AUC).

Killing assays

Neutrophils (1×10^6) were exposed to *S. aureus* (3×10^6) opsonised with serum (MOI = 3) for 2 h, at 37 °C and 5% CO₂. For human samples, neutrophils were pre-treated with oxalate (3 mM) or TEPP-46 (30 μ M) for 1 h before being exposed to *S. aureus*. Cells were centrifuged and the supernatant discarded. Neutrophils were lysed by adding distilled H₂O. The lysates were serially diluted in PBS and plated on Mueller-Hinton agar plates and incubated overnight at 37 °C. The results were expressed by CFU per mL.

Neutrophil extracellular trap assay

This procedure was performed as previously described⁴⁵. Briefly, plasma or culture supernatant was added to a 96-well clear-bottom black plate coated with anti-MPO antibody (5 μ g/mL, Thermo Fisher Scientific, PA5-16672) and incubated overnight at 4 °C. The amount of DNA bound to MPO was quantified using the Quant-iT™ PicoGreen® kit (Invitrogen, P11496). The fluorescence intensity (excitation at 488 nm and emission at 525 nm) was quantified by a FlexStation 3 Microplate Reader (Molecular Devices, CA, USA).

Biochemical parameters

Neutrophils (0.2×10^6) were activated with Zy/op (100 μ g/mL) for 6 h, at 37 °C and 5% CO₂. Lactate concentration in cultured supernatant was measured with the Enzymatic Lactate Kit (Labtest, 138).

For Dihydroxyacetone Phosphate, Glycerol-3-Phosphate, Triose Phosphate Isomerase, NADP/NDPH, glutathione (GSH/GSSG/Total), Pyruvate Kinase activity and Pyruvate analysis, we used commercial kits. Briefly, murine or human neutrophils (1×10^6) were activated with Zy/op (100 μ g/mL) for 1 h, at 37 °C and 5% CO₂. For human samples, neutrophils were pre-treated for 1 h with oxalate (3 mM) before being activated with Zy/op. Samples were centrifuged and prepared according to manufacturer's instructions. The following kits were used: PicoProbe™ Dihydroxyacetone Phosphate (DHAP) Fluorometric Assay Kit, Glycerol-3-Phosphate (G3P) Colorimetric Assay Kit, Triose Phosphate Isomerase (TPI) Activity Colorimetric Assay Kit, NAD/NADH Quantitation Colorimetric Kit, Glutathione (GSH/GSSG/Total) Fluorometric Assay Kit and Pyruvate Kinase Activity Colorimetric/Fluorometric Assay kit (Biovision, K673, K641, K670, K337 and K709, respectively); and Pyruvate Assay kit (Cayman, 700470).

Glucose tracing analysis

Neutrophils were activated with Zy/op at 37 °C for 1 h in the presence of D-(+)-Glucose-¹³C₆ (Cayman, 26707) under slow rotation. Samples were centrifuged and pellets were extracted with 200 μ L of methanol/water (8:2 v:v), and centrifuged at 21,000 $\times$ g, 4 °C, for 10 min (Boeco M-240R, Germany). The supernatant (100 μ L) was evaporated up to dryness (Analytica Christ RVC2-18, Sao Paulo, Brazil) and the residue was reconstituted in 80 μ L of water. The analysis was performed using an Acquity UPLC-TQD MS/MS System (Waters Corp., Massachusetts, EUA) and metabolites were separated on a Kinetex F5 column 50 mm $\times$ 2.1 mm, 1.7 μ m (Phenomenex, California, EUA) with a pre-column of the same material, maintained at 40 °C. The mobile phase was composed by solvent A (10 mM tributylamine and 15 mM acetic acid in water) and B (methanol and water, 8:2 v:v). The elution program was as follows: 1% B (initial), 1–10% B (3 min), 10–90% B (4 min), 1% B (4.1 min), 1% B (6.5 min). The flow rate was 0.4 mL.min⁻¹, the injection volume was 5 μ L in full loop mode, and the autosampler temperature was set to 15 °C. The mass spectrometry (MS) was operated in the negative ion and selected ion recording (SIR) mode using an electrospray voltage of 2.5 kV, source temperature of 150 °C and desolvation temperature of 500 °C. Nitrogen was used as desolvation gas (1000 L h⁻¹) and as a cone gas (50 L h⁻¹). Identification of isotopically labelled metabolites was based on its unit resolution *m/z* and retention time corresponding to unlabelled metabolites determined with chemical standards (Sigma). The analyses were performed using MassLynx V 4.1 Software (Waters Corp.) and the abundance of labelled glycolytic intermediates was determined by the peak height.

Flow cytometry analysis

Neutrophils (0.3×10^6) were activated with Zy/op (100 μ g/mL) for 1 h, at 37 °C and 5% CO₂. Neutrophils were washed in PBS and incubated with MitoSOX (5 μ M, Thermo, M36008) or Mitotracker Red (50 nM, Thermo, M7512) and Green (100 nM, Thermo, M7514) for 30 min. Samples were washed twice in PBS and immediately analysed by flow cytometry.

For glucose uptake assay, neutrophils (0.5×10^6) in glucose-free RPMI medium supplemented with the fluorescent glucose analogue 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG, Invitrogen, 30 μ M, N13195) were activated with Zy/op (100 μ g/mL) for 30 min, at 37 °C and 5% CO₂. Neutrophils were washed twice in PBS and immediately analysed by flow cytometry.

To evaluate the expression of surface antigens, neutrophils were incubated with specific antibodies to GLUT1 (1:200, Abcam, Cat# ab209449), Ly6G (1:200, BD Bioscience, Cat# 560599) CD15 (1:100, BD,

Cat# 562370) or CD11b (1:200, Biolegend, Cat# 101212) or the appropriate isotype controls for 1 h. Viable cells were assessed by incubating cells with Fixable Viability Dye (Thermo).

The fluorescence of samples was measured in a flow cytometer (FACSVerse™, BD Biosciences) and analysed using FlowJo software (Tree Star). Ten thousand cells were analysed⁵².

Analysis of ECAR and OCR with the Seahorse XF Platform

To evaluate the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR), neutrophils (0.1×10^6) in Seahorse XF RPMI Medium without glucose (Agilent) were seeded in a 96-well XF Cell Culture Microplate (Agilent) pre-coated with poly-L-lysine. Cells were left to adhere for one hour at 37 °C without CO₂. Neutrophils were activated with Zy/Op (100 µg/mL), and ECAR and OCR were then measured on a Seahorse XF Analyser (Agilent) in basal conditions, and upon the sequential addition of glucose (5.56 mM), Rotenone/Antimycin (5 µM), and 2-deoxyglucose (2-DG; 50 mM).

Quantification and statistical analysis. Prism 9.2.0 software (GraphPad) was used for data analysis. Comparisons for two groups were carried out using unpaired two-tailed Student's *t* tests, and one-way ANOVA with Tukey's post hoc test for multiple comparisons. Comparisons for the time course of skin infection were performed using two-way ANOVA with Bonferroni's post hoc test. Data are represented as mean ± SEM or bacterial load as a median. Statistical significance: **p* < 0.05.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files). Data sharing not applicable to this article as no datasets were generated or analysed during the current study. Source data are provided with this paper.

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Author contributions

J.E.T.-K. planned and performed experiments, analysed data, and wrote the manuscript. J.C.A.-F. planned experiments, analysed data, wrote the manuscript, and supervised the project. L.A.J.O., F.Q.C., T.M.C., and N.P.L. planned and analysed experiments. C.H.H., C.M.S., D.C.N., G.A.P., T.V.M., L.E.A.D., F.P.V., P.R.V., E.A.D., A.Z., T.A.J.R., F. Y. S. and R.M.S. performed and analysed experiments.

Competing interests

The authors declare no competing interests.

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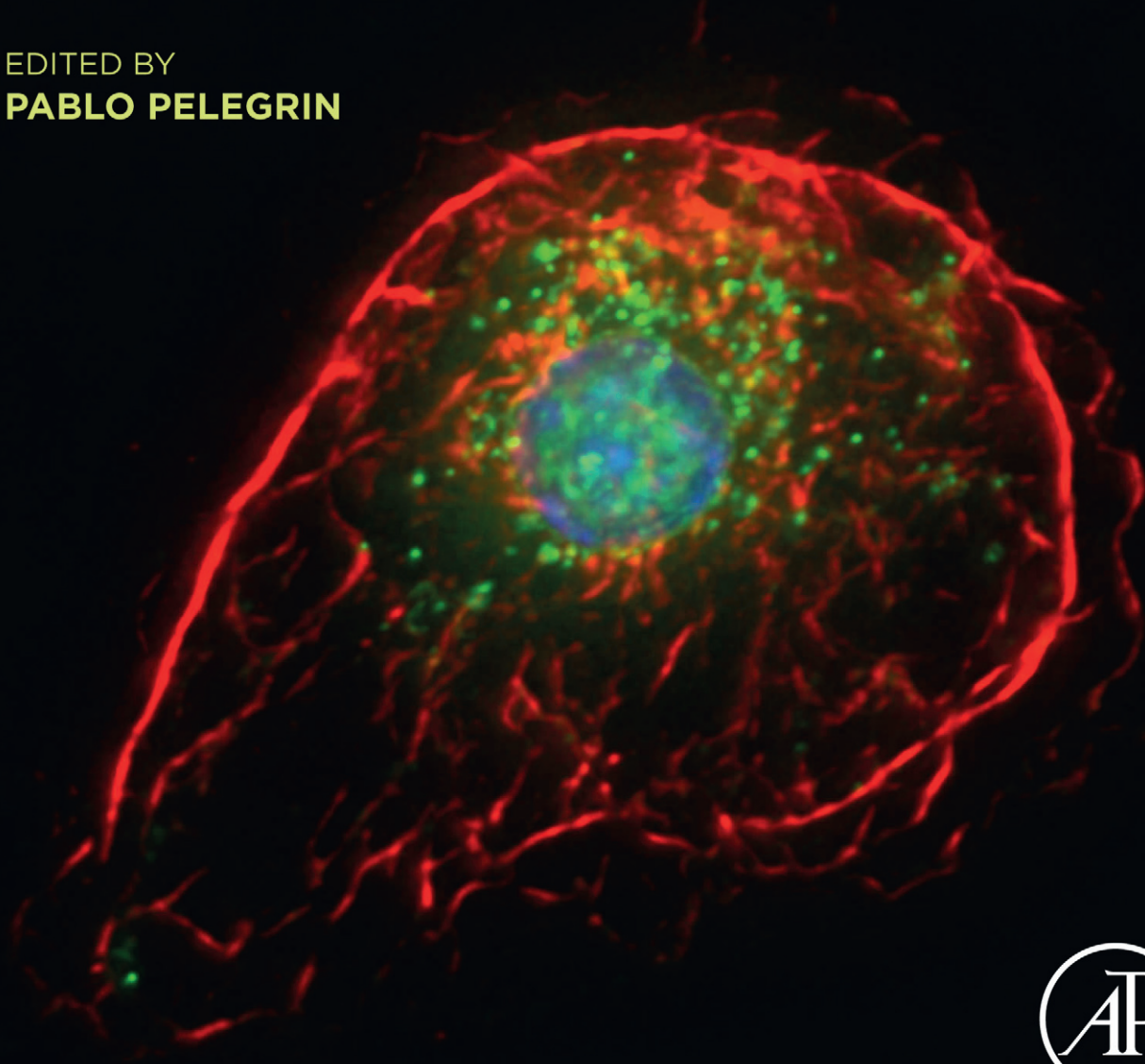
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INFLAMMASOME BIOLOGY

FUNDAMENTALS, ROLE IN DISEASE STATES,
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Therapeutic opportunities targeting the NLRP3 inflammasome

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1. Inflammasomes as therapeutic targets

As the best characterized inflammasome, nucleotide-binding oligomerization domain-like receptor-family, pyrin domain-containing 3 (NLRP3) has been the focal point in the development of novel therapeutics to target inflammasome activation. NLRP3 is a highly attractive drug target due to its implication in the pathogenesis of a number of diseases. These diseases include both monogenic NLRP3-driven disorders, such as Cryopyrin-Associated Periodic Syndromes (CAPS), as well as more complex inflammatory disorders. These have been discussed in previous chapters. Interleukin (IL)-1 has been a target for virtually every pharmaceutical company since the mid-1980s, but blocking IL-1 in the clinic has been limited to rare diseases such as CAPS or Still's disease [1]. NLRP3 is a more attractive target since inhibiting it will also impact on IL-18 production and pyroptosis which might result in a better antiinflammatory effect. As our understanding of the molecular mechanisms underlying NLRP3 activation improves, so does our capability to design novel specific pharmacological inhibitors of the NLRP3 pathway. As such, several NLRP3 inhibitors have been reported to date and are at varying stages of clinical development. Due to the complexity of inflammasome activation, there are various steps along the inflammasome pathway which may potentially be targeted therapeutically. This chapter will highlight these potential therapeutic opportunities, and provide a framework for the development of future therapeutics which target the inflammasome.

2. Targeting NLRP3 priming

Transcriptional upregulation of NLRP3 and pro-IL-1 β occurs following recognition of pathogen- or damage-associated molecular patterns (PAMPs or DAMPs, respectively) by pattern recognition receptors (PRRs) and is followed by downstream signaling which culminates in the activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). One potential means of therapeutically targeting the inflammasome is to suppress transcriptional upregulation of NLRP3 and pro-IL-1 β by blocking specific components of the Toll-like receptor (TLR) signaling pathway. Interleukin 1 receptor-associated kinase 4 (IRAK4) is a protein kinase which is critical for myeloid differentiation primary response 88 (MyD88)-dependent signaling downstream of

TLR4 and has emerged as a potential target in the treatment of inflammatory diseases [2]. Inhibition of IRAK4 would in principle inhibit TLR4-induced NLRP3 and pro-IL-1 β accumulation, thereby preventing inflammasome activation. Indeed, IRAK4 inhibitors have proved efficacious in various murine models of inflammatory disease, including the NLRP3-driven monosodium urate (MSU)-induced gout model [3]. However, as inflammasome priming may occur through TNF-mediated signaling which would not involve IRAK4, inhibition of IRAK4 may not be effective in all inflammatory situations. Furthermore, TLR4 signaling is key to mounting an innate immune response to gram-negative bacteria, and inhibition of this pathway may render the host susceptible to bacterial infections.

In addition to upregulating NLRP3 transcriptionally, priming also leads to specific posttranslational modifications of NLRP3 which license subsequent NLRP3 activation. Posttranslational modifications involve the enzymatic addition or removal of functional groups from a protein, which may influence protein function. Key modifications of NLRP3 upon priming include deubiquitylation [4], phosphorylation [5], and dephosphorylation [6] (on different amino acids), without which subsequent NLRP3 activation is prevented. Potential therapies to suppress inflammasome activation may therefore be focused on interfering with posttranslational modification of NLRP3. For example, bile acids and prostaglandin E₂ can suppress NLRP3 activation by inducing protein kinase A-mediated phosphorylation of NLRP3 [7]. Alternatively, deubiquitinase inhibitors have also been investigated as a potential cancer therapy and may be considered in the treatment of NLRP3-driven diseases [8]. However, targeting the ubiquitin system could also have the disadvantage of not being specific to NLRP3 and may therefore suppress host defense. The various means of targeting NLRP3 priming are displayed in Fig. 35.1.

3. Targeting NLRP3 activation

A number of small molecule NLRP3 inhibitors have been described which target NLRP3 activation directly. As these inhibitors do not affect transcriptional activation of NLRP3 and pro-IL-1 β and do not affect the production of IL-1 β from other inflammasomes, they are considered to be more specific and allow for compensatory IL-1 β production during infection. Inhibiting NLRP3 is therefore considered a safer option than blocking IL-1 β itself [9]. However, as many of these inhibitors emerged from phenotypic screens, there may be limited data regarding their mechanisms of action and they could have off-target effects.

One approach to inhibiting NLRP3 inflammasome activation would be to block the signal for inflammasome activation. The P2X purinoceptor 7 (P2X₇) receptor is a receptor for ATP which, together with the potassium two pore domain channel subfamily K⁺ member 2 (TWIK2), regulates potassium efflux from the cell in response to ATP binding [10]. Small molecule inhibitors of P2X₇ would theoretically prevent potassium efflux from occurring and in this way prevent inflammasome activation. However, clinical trials have demonstrated these inhibitors to be ineffective in the treatment of rheumatoid arthritis [11], but they are yet to be tested in canonical NLRP3-driven diseases such as gout or CAPS. The type 2 diabetes drug glyburide also blocks potassium transport across ATP-sensitive K⁺ channels in pancreatic beta cells, but this is not thought to be required for its ability to block NLRP3 inflammasome activation [12]. Although glyburide provided early evidence that NLRP3 was a druggable target, it requires high doses in vivo and has an as yet undetermined mechanism of action [13].

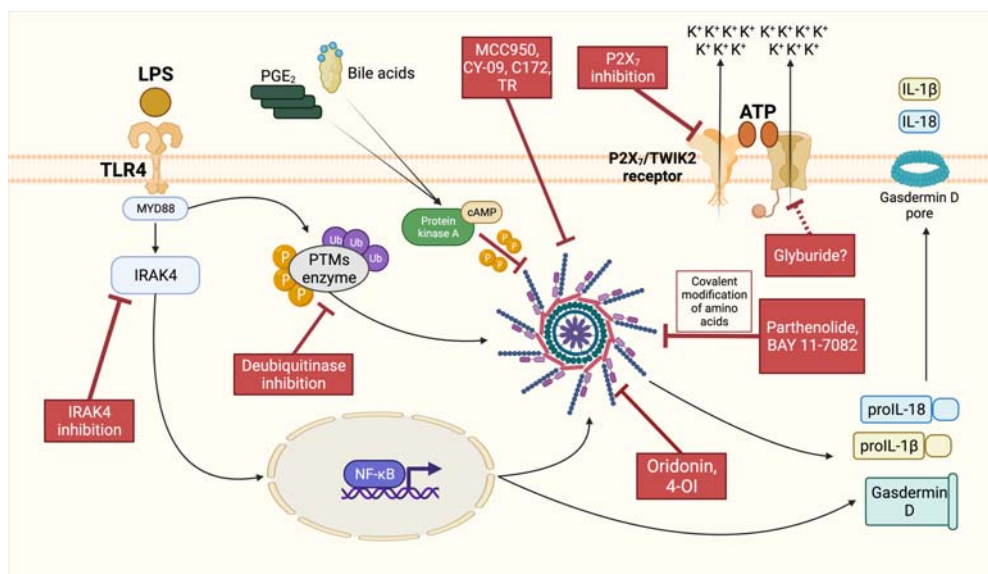


FIGURE 35.1 Targeting NLRP3 inflammasome priming and activation.

cAMP, cyclic adenosine monophosphate; *LPS*, lipopolysaccharide; *MYD88*, myeloid differentiation primary response 88; *NF-κB*, nuclear factor kappa-light-chain-enhancer of activated B cells; *P*, inorganic phosphate; *P2X7*, purinoceptor 7; *TWIK2*, potassium two pore domain channel subfamily K member; *Ub*, ubiquitin. Interleukin-1 receptor–associated kinase 4 (IRAK4) inhibition could block the induction of NLRP3 and proIL-1β transcription by TLR4. Posttranslational modifications (PTMs) of NLRP3 include deubiquitylation, phosphorylation, and dephosphorylation and are all mechanisms of regulating NLRP3 activation. Deubiquitinase inhibition and the phosphorylation of NLRP3 triggered by prostaglandin E2 both prevent inflammasome initiation. MCC950, CY-09, and C172 all inhibit the ATPase activity of NLRP3, while tranilast (TR) binds directly to NLRP3 and prevents its self-oligomerization. 4-OI (4-octyl itaconate), oridonin, parthenolide, and BAY-117082 are compounds which induce covalent modification of specific cysteines on NLRP3, thereby preventing inflammasome activation. P2X7 inhibitors modulate ATP (adenosine triphosphate)-dependent K⁺ (potassium) channel opening, thereby targeting signal 2 and preventing NLRP3 activation.

Rational design of specific NLRP3 inhibitors was limited due to the lack of high resolution crystal structures of the protein. Many of these inhibitors were preexisting compounds which have later been identified as NLRP3 inhibitors. One such example is the sulfonyleurea-containing compound MCC950, also known as CRID3 and CP-456,773. MCC950 was initially recognized as an inhibitor of IL-1β processing in 2001 [14] and was trialed by Pfizer as a treatment for rheumatoid arthritis, but development was halted following phase 1b testing. 14 years after the initial discovery of the Pfizer compound, MCC950 was shown to be a potent and specific inhibitor of NLRP3 inflammasome activation [15]. However, the mechanistic basis of its inhibition has only recently been elucidated [16,17].

MCC950, as well as various other NLRP3 inhibitors, targets and binds to NLRP3 directly [16,17]. Direct binding to NLRP3 blocks its ATPase activity and prevents NLRP3 self-oligomerization, a

process which requires ATP hydrolysis, thereby preventing inflammasome assembly and activation. C172 and CY-09, in addition to MCC950, are compounds which target NLRP3 in this manner [18]. OLT1177, also known as dapansutrile, is a β -sulfonyl nitrile molecule which blocks NLRP3 activation at nanomolar concentrations, much like MCC950. Although OLT1177 also reduces ATPase activity of NLRP3 [19], its mechanism of action is less well elucidated. NLRP3 oligomerization may also be inhibited without any effect on its ATPase activity, as demonstrated by the tryptophan metabolite analogue Tranilast [20].

Covalent modification of specific amino acids on NLRP3 may also regulate its activity. Oridonin, the active ingredient in a Chinese herbal medicine used to treat inflammatory diseases, and the metabolite derivative 4-octyl itaconate (4-OI) modify separate cysteines on NLRP3 which blocks its interaction with never in mitosis kinase 7 (NEK7) [21,22]. The electrophilic compounds parthenolide and BAY 11-7082 also form covalent adducts with NLRP3 to block inflammasome activation [23]. However, covalent inhibitors of NLRP3 are likely to be nonspecific owing to their ability to modify other reactive cysteine residues. The various inhibitors of NLRP3 activation are displayed in Fig. 35.1.

4. Targeting ASC, caspase-1, and gasdermin D

The apoptosis speck-like protein with a caspase activator and recruitment domain (ASC), the adaptor protein in the inflammasome complex, links NLRP3 to caspase-1 and oligomerizes into dense multimeric “specks” upon activation. Preventing ASC oligomerization would in theory be a means of blocking inflammasome activation. However, since ASC is shared by most other inflammasomes—NLRC4 and NLRP1b may be activated in an ASC-independent manner [24]—targeting of ASC would be a relatively nonspecific way of blocking NLRP3 activation. Furthermore, the molecular mechanisms underlying ASC oligomerization are incompletely understood, which increases the difficulty of designing compounds to target ASC [25].

Targeting of caspase-1 is similarly nonspecific, as caspase-1 is the effector protein which all inflammasomes, excluding the noncanonical inflammasome, converge upon. Blocking caspase-1 would prevent caspase-1-mediated cleavage of IL-1 β and IL-18, as well as cleavage and activation of the pyroptosis executioner gasdermin D. Various caspase-1 inhibitors have been described, two of which, VX-740 and VX-765, reached phase II clinical trials for the treatment of epilepsy and psoriasis, respectively [26,27]. However, these compounds were not taken further due to concerns around hepatotoxicity.

Gasdermin D has recently emerged as a target in the NLRP3 pathway. Although initially described as the pyroptosis executioner, the oligomerization of the N-terminal fragment of gasdermin D also provides a pathway for IL-1 β to be released from the cell [28]. Hence, the targeting of gasdermin D would block both pyroptosis and cytokine release. Gasdermin D, like NLRP3, appears to be susceptible to covalent modification, as the FDA-approved drugs dimethyl fumarate and disulfiram were found to modify the same amino acid (Cys192) to block gasdermin D pore formation [29,30]. Due to their demonstrated safety profiles, it is possible that one or both of these drugs may be repurposed as NLRP3 inflammasome inhibitors. Once again, however, the nature of these drugs as covalent cysteine-modifiers would indicate that they may be targeting numerous proteins in addition to gasdermin D. A specific gasdermin D inhibitor has yet to be reported. The various approaches to targeting ASC, caspase-1, and gasdermin D are displayed in Fig. 35.2.

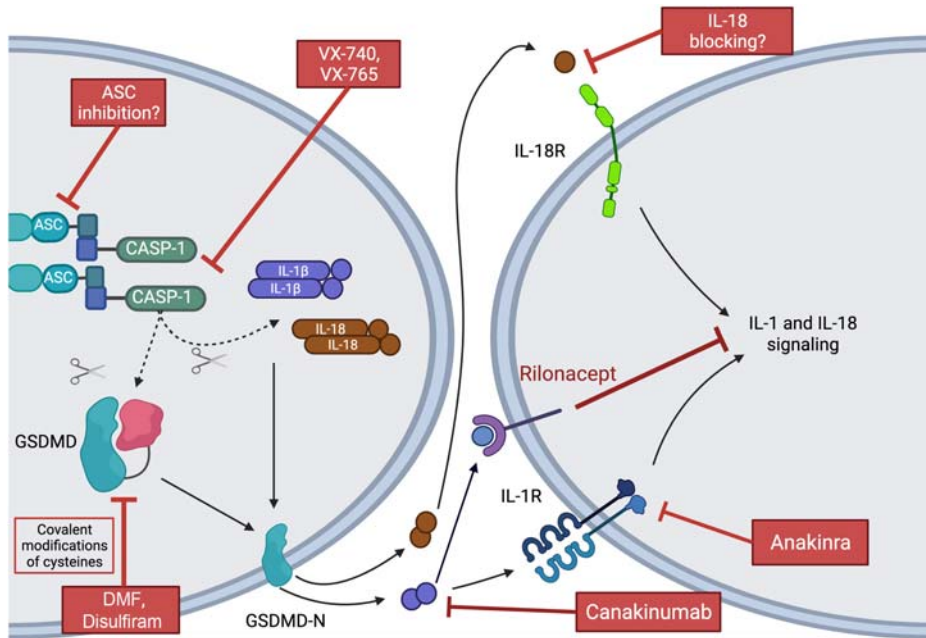


FIGURE 35.2 Targeting NLRP3 effector functions.

GSDMD, gasdermin D.

Apoptosis-associated speck-like protein containing a CARD domain (ASC) oligomerization inhibition, Caspase-1 (CASP1) inhibition by VX-740 and VX-765, and gasdermin D inhibition by dimethyl fumarate (DMF) and disulfiram have been demonstrated to prevent inflammasome activation. IL-1 β and IL-18 are released via gasdermin D pores and can initiate signaling by binding to their cognate receptor on neighboring cells. The IL-1 receptor antagonist anakinra, the IL-1 β neutralizing antibody canakinumab and the soluble decoy receptor for IL-1 α and IL-1 β rilonacept have been shown to block IL-1 signaling. Anti-IL-18 blocking antibodies reduce inflammation in models of Crohn's disease and atopic dermatitis.

5. Targeting downstream effector functions

IL-1 β signaling, following its inflammasome-dependent release, is one of the effector functions which may be targeted downstream of inflammasome activation. Three therapeutics exist which target IL-1 β signaling: the IL-1 receptor antagonist anakinra, the IL-1 β neutralizing antibody canakinumab, and the soluble decoy receptor for IL-1 α and IL-1 β rilonacept. These therapeutics provide significant clinical benefit for the treatment of NLRP3-driven diseases such as CAPS. Both IL-1 α and IL-1 β have been implicated in a broad range of inflammatory diseases [31], and there is hope that new indications may be a possibility for anti-IL-1 therapy. Canakinumab is the preferred treatment option due to its longer plasma half-life, and was studied in the CANTOS clinical trial, the first large-scale trial to assess the efficacy of anti-IL-1 therapy in cardiovascular disease. Results from the CANTOS trial demonstrated the efficacy of IL-1 blockade in reducing the rate of cardiovascular events and, unexpectedly, lung

cancer incidence when compared to placebo [32,33]. The CANTOS trial has therefore raised hopes that anti-IL-1 therapy may be effective in the treatment of diseases that were previously not considered to be classically IL-1-driven. However, there are still multiple issues associated with therapeutic targeting of IL-1. Anakinra, canakinumab, and rilonacept are all administered by subcutaneous injection which may cause discomfort for patients, especially in the case of anakinra which requires daily injections [25]. Furthermore, an increased risk of infection was observed with canakinumab treatment in the CANTOS trial [32,33], which may be expected given these therapeutics target IL-1 rather than specifically NLRP3-driven IL-1. There are also concerns that these protein-based therapeutics do not penetrate the blood–brain barrier, which would be an obstacle to their use in IL-1–driven neuroinflammatory diseases such as Alzheimer’s disease [34].

The other downstream effector functions of inflammasome activation are IL-18 release and pyroptosis, which would be left unaffected by IL-1 blockade. Therefore, diseases where the pathology are mainly driven by these effector functions would not be effectively treated by anti-IL-1 therapy. An anti-IL-18 blocking antibody recently completed a phase IIb trial for the treatment of Crohn’s disease (NCT03681067) and will also undergo trials for the treatment of atopic dermatitis (NCT04975438). IL-18 signaling drives the breakdown of epithelial barrier integrity, and its deletion or blockade has been demonstrated to confer protection in murine models of colitis [35]. The various approaches targeting downstream effector functions are displayed in Fig. 35.2.

6. NLRP3 agonism

Given the immunostimulatory consequences of NLRP3 activation, NLRP3 agonism may be desirable in the development of vaccine adjuvants and in cancer immunotherapy. Several particulate adjuvants which are commonly used to enhance vaccine responses, including alum, have been demonstrated to act as NLRP3 agonists through the lysosomal pathway of NLRP3 activation [36]. This is not thought to contribute to the production of antigen-specific antibodies following vaccination, but rather promotes antigen-specific T-cell responses [37]. It is unclear whether NLRP3 activation in this context contributes to long-term immunological memory following vaccination. If so, there would be a basis for the inclusion of NLRP3 agonists in future vaccine formulations.

Cancer immunotherapy revolves around the principle of altering the immunosuppressive tumor environment in order to facilitate immune responses against tumor cells. Several aspects of NLRP3 activation may potentially be harnessed to complement conventional anticancer therapeutics. For example, selective induction of gasdermin-dependent pyroptosis in cancer cells using a biorthogonal probe has been demonstrated to aid tumor regression, and this was dependent upon T-cell–mediated immune responses [38]. T-cell responses are critical to the success of checkpoint inhibitors, and these may be boosted by the immunogenic cell death caused by co-administered chemotherapeutics [39]. The inflammasome-dependent release of proinflammatory cytokines would also serve to augment immune responses during immunotherapy. However, results from the CANTOS trial, which showed anti-IL-1 therapy to reduce lung cancer incidence, would indicate that IL-1 signaling may in fact promote tumor growth. It is thought that chronic, prolonged IL-1 production in the tumor microenvironment may promote the recruitment of myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) [40]. Furthermore, NLRP3 activation in macrophages has been demonstrated to suppress T helper cell 1 (T_H1) and cytotoxic CD8⁺ cell activation, thereby suppressing antitumor adaptive immune responses [41].

7. Conclusions

Clearly the next exciting prospect therapeutically remains to inhibit NLRP3 directly and with high specificity. A trial is currently ongoing in COVID-19 with dapansutride (NCT04540120) which was estimated to report in June 2022. Novartis are running trials with their NLRP3 inhibitor DFV890 in FCAS (NCT04868968) and knee osteoarthritis (NCT04886258). However, they recently completed a trial in COVID-19 pneumonia (NCT04382053) which failed to show any benefit. The NLRP3 inhibitor inzomelid, derived from its parent molecule MCC950, has also completed phase 1 trials (NCT04015076) prior to its acquisition by Roche. We therefore await further clinical trials on NLRP3 inhibitors which are bound to emerge in the coming years, remaining optimistic of substantial clinical benefits across multiple indications.

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