Characterising the role of SIRT1 in macrophage function during bile acid metabolism disturbances

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Abstract

Inflammation is the hallmark of cholestasis. Macrophages promote the inflammatory response however their precise role or how metabolism may control their function during cholestasis is enigmatic. Silent information regulator type 1 (SIRT1) is a master metabolic regulator and metabolism underpins macrophage activation, thus we here aim to characterise its role in macrophage activation during cholestasis. Also, while cholestasis associates with changes in the intestinal microbiome composition, its impact in the pathogenesis of the disease is undefined.

Here, we used conventionalised germ free animals and induced cholestasis with α-naphthylisothiocyanate. Also, we used endotoxin to induce liver inflammation in wild type and SIRT1 overexpressing animals. We performed transfer of WT and SIRT1 overexpressing myeloid cells and induced cholestasis by bile duct ligation (BDL). Lastly, we subjected myeloid-cell specific SIRT1 knockout mice to BDL.

We demonstrated that the microbiome synergises with bile acids to promote liver damage and macrophage recruitment during cholestasis.

Also, we identified that SIRT1 overexpression promotes liver inflammation in response to endotoxin that associated with increased inflammasome activation *in vivo*. Mechanistically, macrophage SIRT1 overexpression associated with the activation of the mTOR pathway and metabolic rewiring, as well as increased inflammasome activation and delayed autophagy after endotoxin.

During cholestasis, the overexpression of SIRT1 associated with increased inflammation and the transfer of myeloid SIRT1 overexpressing cells led to increased liver injury and fibrosis, indicating that myeloid SIRT1 contributes to the progression of cholestasis.

Our results suggested that myeloid SIRT1 could be a therapeutic target during cholestasis, therefore we investigated myeloid SIRT1 depletion in response to BDL. Interestingly, we discovered that myeloid SIRT1 depletion contributed to the pathogenesis of cholestasis.

Overall, we identify the role for SIRT1 in macrophage activation during inflammatory liver disease. Our findings point to the modulation, but not complete ablation, of SIRT1 in myeloid cells to preserve liver health.

Contents

Abbreviations

ADT- adenosine diphosphate

ATP- adenosine triphosphate

ACO2- aconitase 2

ALD- alcoholic liver disease

ALF- alcoholic fatty liver

ALT- alanine aminotransferase

ALP- alkaline phosphatase

AMPK- Adenosine monophosphate activated protein kinase

ANIT- α-naphthylisothiocyanate

ANOVA- analysis of variance

Akt- Akt serine/threonine kinase 1

Arg1- Arginase 1

AP1- activator protein 1

ASC- apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain

ASH- alcoholic steatohepatitis

AST- aspartate aminotransferase

ATG- autophagy gene

BAC- bacterial artificial chromosome

BDL- bile duct ligation

BMDM- bone marrow derived macrophages

BSEP- bile salt export pump

BSH- bile salt hydrolase

CA- cholic acid

CCL- CC motif chemokine ligand

CCL_4- carbon tetrachloride

CCR - CC motif chemokine receptor 2

CD- cluster of differentiation

CDCA- chenodeoxycholic acid

CHAPS -[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate

Clec4F- C-type lectin domain family 4, member f

CK19 – cytokeratin 19

CREB- cyclic adenosine monophosphate response element binding protein

Cyp2c70- sterol 6b-hydroxylase

Cyp7A1- 7-α-hydroxylase cholesterol

CSFR1- colony stimulating factor receptor 1

CXCL- C-X-C Motif ligand

DAMPs- damage associated molecular patterns

DCA- deoxycholic acid

DDC- 3,5-diethoxycarbonyl-1,4-dihydrocollidine

DNA- deoxyribonucleic acid

DTT- dithiothreitol

ECM- extracellular matrix

EDTA- ethylenediaminetetraacetic acid

FBS- foetal bovine serum

FXR- farnesoid X receptor

GalN- galactosamine

G-glycine

GAPDH- glyceraldehyde 3- phosphate dehydrogenase

GCA- glycocholic acid

GF- germ free

GM-CSF- granulocyte-macrophage colony stimulating factor

GMP- granulocyte-macrophage progenitor

Gst- glutathione-S-transferase

Gxp- glutathione peroxidase

H and E- haematoxylin and eosin

HEK- human embryonic kidney

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF1α- hypoxia inducible factor 1 α

HPC- hepatic progenitor cells

HRP- horseradish peroxidase

HSC- hepatic stellate cells

HSEC – human hepatic sinusoidal endothelial cells

IDH- isocitrate dehydrogenase

IFN- interferon

IL- interleukin

IKK- inhibitor of nuclear factor κ B kinase complex

i.p.- intraperitoneal

IRAK- interleukin 1 receptor associated kinase

JNK- c-Jun terminal kinase

K- lysine

KDH- α-ketoglutarate dehydrogenase

LC3- light chain 3

LCA- lithocholic acid

LC-MS/MS- liquid chromatography with tandem mass spectrometry

LKB1- liver kinase B1

LSEC- liver sinusoidal endothelial cells

LPS- lipopolysaccharide

Ly6C- lymphocyte antigen 6 complex C1

MAPK- mitogen activated protein kinase

MCA- murocholic acid

M-CSF- Macrophage colony stimulating factor

MD2- myeloid differentiation factor 2

MDP- monocyte-macrophage dendritic cell progenitor

Mdr- multidrug resistance protein

MEM- Minimum Essential Eagle media

MKK- mitogen activated protein kinase kinase

M(LPS)- macrophages activated by LPS

MMP- matrix metalloprotease

MMPs- matrix metalloproteases

Mrp- multidrug resistance related prot

MS- mass spectrometry

mTOR- mammalian target of rapamycin

mTORC1- mammalian target of rapamycin complex 1

MyD88- myeloid differentiation primary response 88

NaCl- sodium chloride

NAFLD- non-alcoholic fatty liver disease

NAD^+ - nicotinamide adenine dinucleotide

NASH- non-alcoholic steatohepatitis

NFκB- nuclear factor κ light chain enhancer of activated B cells

NK- natural killer

Nlrp3- NOD-, LRR- and pyrin domain- containing protein 3

NorUDCA- norursodeoxycholic acid

NO- nitric oxide

NOS2- nitric oxide synthase 2

NOD- no obese diabetic

NTCP- Na^+- taurocholate co-transporting polypeptide

O-A-ADP-ribose- O-Acetyl-ADP-ribose

OATP- organic anion transporting polypeptides

OCA- obeticholic acid

OXPHOS- oxidative phosphorylation

p- phosphorylated

PAMPs- pathogen associated molecular patterns

PAPR1- poly adenosine diphosphate ribose polymerase 1

PBC- primary biliary cholangitis

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PDGF- platelet derived growth factor

PDHC- pyruvate dehydrogenase complex

PE- phosphatidylethanolamine

PGC1α- peroxisome proliferator-activated receptor-gamma coactivator 1 α

PI3K- class III phosphatidylinositol-3-kinase

PIPES- piperazine-N,N'-bis(2-ethanesulfonic acid)

PMSF- phenylmethylsulphonyl fluoride

PPARγ- peroxisome proliferator activated receptor γ

RET- reverse electron transport

ROS- reactive oxygen species

PRR- pattern recognition receptor

PSC- primary sclerosing cholangitis

PTEN- phosphatase and tensin homolog

qPCR- quantitative polymerase chain reaction

RPMI- Roswell Park Memorial Institute

RIPA- radio immunoprecipitation assay

RNA- ribonucleic acid

S6- ribosomal protein S6

S6K1- ribosomal protein S6 kinase beta 1

SDH- succinate dehydrogenase

SDS- sodium dodecyl sulphate

SIRT1 - silent information regulator type 1, sirtuin 1

SMA – α smooth muscle actin

SPF- specific pathogen free

SUCNR1- succinate receptor 1

T-taurine

TAK1- tumour growth factor β activated kinase 1

TBS- tris buffered saline

TCA- taurocholic acid

TGFβ- tumour growth factor β

TIRAP- toll-interleukin 1 receptor domain-containing adaptor protein

TLC- taurolithocholic acid

TNF- tumour necrosis factor

TRAF6- TNF associated factor 6

TRAIL- tumour necrosis factor related apoptosis inducing ligand

Tris-HCL- Tris(hydroxymethyl)aminomethane hydrochloride

TLR- toll like receptor

TSC2- tuberous sclerosis complex 2

TWEAK- tumour necrosis factor like weak inducer of apoptosis

UDCA- ursodeoxycholic acid

UDT- uridine diphosphate

UGT- uridine diphosphate-glucuronosyltransferase

ULK1- UNC-51-like kinase 1

UTP- uridine triphosphate

Wnt- wingless/integrated

WT- wild type

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Chapter 1 – Introduction

1.1 Overview of the liver

1.1.1 The liver

The liver is the largest vital organ in the body with over 500 functions, including metabolism and immunity. The main metabolic functions of the liver include maintaining glucose homeostasis, gluconeogenesis, glycogenolysis, cholesterol, fatty acid and bile acid synthesis as well as drug detoxification (Lieberman et al., 2018). The liver is also responsible for vitamin storage and the production of fibrinogen and coagulation factors to aid immunity (Radu-Ionita et al., 2020). The liver is located in the right upper abdomen of the body and receives its blood supply through the hepatic artery and portal vein.

1.1.2 Hepatocytes

Hepatocytes are the main cell type in the liver and comprise 85% of liver cells. They carry out most of the liver's functions including bile acid synthesis and unique metabolic functions. For example, hepatocytes are the only cells of the liver able to process the galactose sugar (Lieberman et al., 2018).

In addition to this, hepatocytes have a crucial role in innate immunity. Hepatocytes produce lipopolysaccharide (LPS) binding protein which is essential to initiate the immune response to endotoxin (Z. Zhou et al., 2016). Hepatocytes produce the chemokines to recruit monocytes to the liver to initiate the inflammatory response (Z. Zhou et al., 2016). During liver injury, hepatocyte cell death can occur and dying hepatocytes then act as damage associated molecular patterns (DAMPs) which activate innate immune cells to promote a pro-inflammatory response (Canbay et al., 2003).

1.1.3 Cholangiocytes

Cholangiocytes comprise up to 3-5% of the liver's cell pool (Lieberman et al., 2018). Cholangiocytes are biliary epithelial cells that line the bile ducts to form the biliary tree and contribute to the composition of bile by secreting bicarbonate (Maroni et al., 2015). During homeostasis, the main function of cholangiocytes is to regulate bile flow however they contribute to anti-microbial defence by secreting immunoglobulin A and other anti-microbial peptides into bile (Banales et al., 2019).

1.1.4 Hepatic stellate cells

Hepatic stellate cells (HSC) make up 10% of liver resident cells and their primary function is vitamin A storage (Radu-Ionita et al., 2020). In a healthy liver, HSC exist in a quiescent state in the space of Disse. Upon liver injury, HSC activation occurs and the cells differentiate into hepatic myofibroblasts which function to produce extracellular matrix (ECM), the main source of collagen in the fibrotic liver (Mederacke et al., 2013). During disease, a range of signals can promote HSC activation including bacterial products from a "leaky gut", DAMPs from dying hepatocytes and cytokines and chemokines from immune cells and cholangiocytes (Trautwein et al., 2015).

1.1.5 Liver sinusoidal endothelial cells

Termed liver sinusoidal endothelial cells (LSEC) in animals and human hepatic sinusoidal endothelial cells (HSEC) in humans, LSEC comprise 3% of the liver's cell pool and function to line the blood vessels of the liver (Radu-Ionita et al. 2020).

1.2 Bile acid synthesis

One of the key functions of the liver is the production and secretion of bile. Bile is secreted by hepatocytes and is comprised of 95% water which dissolves soluble molecules including bile acids, cholesterol, bilirubin and amino acids (Boyer, 2013). While the main function of bile is the emulsification of dietary fats to aid their absorption in the intestine, bile also has antimicrobial properties and helps prevent microbial overgrowth in the intestine (Urdaneta & Casadesús, 2017).

Bile acids are amphiphilic molecules which are synthesised from cholesterol. Primary bile acids are produced in the liver by hepatocytes and secondary bile acids are made by the modification of primary bile acids by the microbiome in the intestine (Šarenac & Mikov, 2018). In humans, there are two primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA). Mice contain four primary bile acids CA, CDCA and α-muricholic acid (MCA) and β-MCA.

There are two pathways which catabolise cholesterol to bile acids. The classic bile acid synthesis pathway occurs in the liver and the alternative bile acid synthesis pathway which can take place in macrophages, adrenal glands, the liver and the brain (T. Li & Chiang, 2015). 90% of primary bile acids are produced using the classic bile acid synthesis pathway in humans.

Classic bile acid synthesis begins when cholesterol is converted to 7-α-hydrocholesterol by the enzyme 7-α-hydroxylase cholesterol (Cyp7A1). A series of oxidation-reduction reactions take place to produce CA and CDCA. The alternative pathway uses the enzyme Cyp27A1 to convert

cholesterol to 27-hydroxycholesterol. The bile acid intermediates formed by extra-hepatic tissues utilising the alternative pathway are then transported to the liver to produce CA and CDCA.

In mice, CDCA can be converted to αMCA by the enzyme sterol 6b-hydroxylase (Cyp2c70) and α-MCA can form an isomer, β-MCA. CA and CDCA can undergo amino acid conjugation to taurine (T) and glycine (G) to form taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid and glycochenodeoxycholic acid. As well as amino acid conjugation, bile acids can also have sulphur groups added by bile acid sulfotransferases and they can undergo glucuronide conjugation by uridine diphosphate (UDT)-glucuronosyltransferases (Wagner et al., 2005). Conjugation increases bile acid solubility and therefore reduces their toxicity.

Cholangiocytes line the bile ducts to form the biliary tree and contribute to the composition of bile by secreting bicarbonate (Maroni et al., 2015). Conjugated primary bile acids are secreted out of hepatocytes into bile, by active transport, using the bile salt export pump (BSEP) and bile travels down the biliary tree into the gall bladder for storage. Bile is released postprandially from the gall bladder into the intestine. In the ileum and colon, bile acids become deconjugated by the microbiome's enzymes bacterial bile salt hydrolases (BSH) to form free bile acids. Secondary bile acids are formed in the gut from primary bile acids by bacterial 7α dehydroxylase, which removes a hydroxyl group from free primary bile acids. CA forms deoxycholic acid (DCA) and CDCA forms lithocholic acid (LCA). Secondary bile acids are more hydrophobic and more toxic than primary bile acids. 5% of the total bile acid pool is lost through excretion in urine and faeces and 95% of the bile acid pool is reabsorbed back into the portal circulation in the ileum (Chiang & Ferrell, 2018). Hepatocytes uptake bile acids back into the liver from the portal circulation using the transporters Na^+-taurocholate co-transporting polypeptide (NTCP) and organic anion transporting polypeptides (OATPs) (T. Li & Chiang, 2015).

The nuclear receptor farnesoid X receptor (FXR) is the key negative regulator of bile acid metabolism. FXR is activated by CDCA and functions to repress the transcription of Cyp7A1 (Fiorucci et al., 2018). The regulation of FXR signaling is a dynamic process where FXR is activated by acetylation by p300 (Fang et al., 2008) and deactivated by deacetylation by the metabolic regulator silent information regulator type 1 (SIRT1, sirtuin 1) (Kemper et al., 2009).

As well as having a key role in digestion, bile acids act as key signaling molecules in the innate immune response by activating bile acid receptors on macrophages (Fiorucci et al., 2018). Some studies identified that bile acids promote the anti-inflammatory function of macrophages (Haselow et al., 2013; Wammers et al., 2018) while others described that bile acids promote the pro-inflammatory response through the activation of the Nlrp3 inflammasome (Gong et al., 2016; Hao et al., 2017). Bile acids and macrophages will be discussed in more detail in section 1.7.2.

1.3 Liver immune cells

1.3.1 Non-macrophage liver immune cells

As an important organ for immunity, the liver contains a small percentage of all types of immune cells. Natural killer (NK) cells comprise up to 50% of the liver's lymphoid cell pool (Mikulak et al., 2019). NK cells are innate lymphoid cells which function to lyse damaged or infected cells and produce cytokines to aid the immune response (Vivier et al., 2008).

T lymphocyte cell mature in the thymus and are involved in the adaptive immune response. There exists a range of T cells: naïve T cells, helper T cells, regulatory T cells, effector T cells, natural killer T cells and memory T cells. Liver T cells are localised in the vasculature and the around the portal ducts and comprise up to 23% of the liver's lymphocytes (Bogdanos et al., 2013).

B lymphocytes are cells of the adaptive immune system which develop in the bone marrow. Their main functions are antibody production and immunological memory (Cargill & Culver, 2021).

Neutrophils are granulocytes which function to promote the innate immune response by releasing pro-inflammatory cytokines and performing phagocytosis (K. Liu et al., 2021).

Dendritic cells are a type of antigen presenting cell found around the portal triads which function to phagocytose and present antigens to B and T cells to maintain immune tolerance in the liver (Lau & Thomson, 2003).

1.3.2 Kupffer cells

Macrophages are the most abundant immune cells in the liver and comprise 2% of the liver's volume. Although this seems like a small cell number compared to the non-immune cells of the liver, the liver contains 90% of the tissue resident macrophages in the body (Bilzer et al., 2006). Liver macrophages are comprised of several populations.

Liver resident macrophages are named Kupffer cells after Karl Wilhelm von Kupffer who first described them (Wake, 2004). In the developing foetus, Kupffer cells originate from macrophage colony stimulating factor receptor 1 positive ($CSFR1^{+}$) yolk sac erythromyeloid progenitors which migrate to the foetal liver and differentiate into a self-renewing liver resident macrophage population (Guillot & Tacke, 2019). Interestingly, Scott and colleagues have demonstrated that bone marrow derived monocytes can also migrate to the liver and differentiate into Kupffer cells if the niche becomes available during liver damage (Scott et al., 2016). However, while functionally similar, these cells are a distinct population with different transcriptomic profiles to yolk sac derived Kupffer cells (Beattie et al., 2016).

Kupffer cells are localised in the sinusoidal lumen of the liver and function to remove gut derived pathogens from portal circulation (Jenne & Kubes, 2013). Kupffer cells possess endotoxin tolerance due to exposure to gut-derived bacterial products. Therefore, exposure of Kupffer cells to the bacterial endotoxin LPS does not initiate an immune response during homeostasis (Bilzer et al., 2006). This is a mechanism to avoid a constant state of inflammation in the liver. To facilitate the liver's immune surveillance functions, all liver resident cells possess pattern recognition receptors (PRRs) such as toll like receptors (TLRs) and Kupffer cells express a range of receptors including PRRs and complement receptors (Krenkel & Tacke, 2017). The disruption of the liver's tightly controlled immune surveillance system can lead to a dysregulated inflammatory response which contributes to chronic liver disease (Strnad et al., 2017).

Additionally, Kupffer cells maintain cholesterol, iron and bilirubin metabolism (Gordon & Plüddemann, 2017) and dispose of aged platelets from the bloodstream (Deppermann et al., 2020).

During health, the liver macrophage pool is comprised primarily of Kupffer cells and a small population of monocyte-derived macrophages termed liver capsular macrophages (Sierro et al., 2017). During disease, Kupffer cells secrete chemokines to recruit monocytes from the bone marrow which can differentiate into a distinct macrophage population when they reach the liver (Elsegood et al., 2015). Additionally, during disease, mature peritoneal macrophages can infiltrate the liver and they have been described to aid tissue repair (J. Wang & Kubes, 2016). Liver cell percentages are detailed in table 1.

Table 1. Liver cell percentages

Liver cell	Percentage (%)
Hepatocyte	80-85
Cholangiocyte	3-5
Hepatic stellate cell	10
LSEC	3
Macrophages (Kupffer cells)	2
Other immune cells	1

1.3.3 Monocyte-derived macrophages

Monocyte-derived macrophages are a distinct population from tissue-resident macrophages. Monocyte-derived macrophages are cells of the mononuclear phagocyte system which develop in the bone marrow from haematopoietic stem cells. The common myeloid progenitor differentiates into the granulocyte-macrophage progenitor (GMP) (Italiani & Boraschi,

2014). GMP then forms the monocyte-macrophage dendritic cell progenitor (MDP) (Fogg D, Sibon C, Miled C, Jung S,Aucouturier, Dan R. Littman, Ana Cumano, 2006). The MDP then gives rise to the common monocyte progenitor, a population which gives rise to circulating monocytes (Hettinger et al., 2013). Macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) stimulate mononuclear phagocyte development into macrophages via the CSFR1 (Italiani & Boraschi, 2014).

Monocytes patrol the bloodstream and infiltrate the liver in response to injury. Monocytes are recruited from the bone marrow by Kupffer cell and cholangiocyte secretion of pro-inflammatory chemokines and differentiate into macrophages in the liver (C. Shi & Pamer, 2011). These bone marrow derived macrophages have a Lymphocyte antigen 6 complex C1 (Ly6C) high pro-inflammatory phenotype and contribute to inflammation and fibrosis by secreting pro-fibrotic tumour growth factor β (TGF-β) (Karlmark et al., 2009). Whilst during homeostasis, Kupffer cells are the most abundant liver macrophage population, in an inflamed liver, monocyte-derived macrophages are the most abundant macrophage population (Guillot & Tacke, 2019).

Since Kupffer cells and monocyte-derived macrophages originate from different lineages, they express differential cell surface markers (table 2). Cluster of differentiation molecule 11 B (CD11b) is an integrin involved in cell adhesion (Solovjov et al., 2005). Kupffer cells and monocyte-derived macrophages both express CD11b on their cell surface. F4/80 was first described as a murine macrophage marker in 1981 and is thought to have a role in signaling and adhesion (Gordon et al., 2011). F4/80 is highly expressed by Kupffer cells and presents intermediate expression on monocyte-derived macrophages (Krenkel & Tacke, 2017). Ly6C is a marker of monocyte-derived macrophages which originated from Ly6C positive circulating monocytes (Yona et al., 2013). Kupffer cells do not express Ly6C. Recently, C-type lectin domain family 4, member f (Clec4F) has been confirmed as a Kupffer cell specific marker (Scott et al., 2016).

Table 2. Liver macrophage markers

Cell surface marker	Kupffer cell	Monocyte-derived macrophage
CD11b	$CD11b^{+}$	$CD11b^{+}$
F4/80	$F4/80^{+}$	$F4/80^{+/-}$
Ly6C	$Ly6C^{-}$	$Ly6C^{+}$
Clec4F	$Clec4F^{+}$	$Clec4F^{-}$

1.4 The origin and function of macrophages

1.4.1 Introduction to macrophages

Inflammation is a physiological response to a pathological stimulus which functions to restore homeostasis. The inflammatory response comprises of three stages: onset, resolution and adapted homeostasis (Sugimoto et al., 2016). During the onset stage, the pro-inflammatory cascade is initiated by pro-inflammatory cytokines. During the resolution phase, anti-inflammatory cytokines end the inflammatory process and adaptive homeostasis is the repopulation of inflamed tissue with alternative cell types with the aim of restoring tissue homeostasis (Sugimoto et al., 2019).

The innate immune response is the body's first line of defence against invading pathogens. Macrophages are defined as the main phagocyte population of the body because macrophages were first discovered by Elie Metchnikoff in the 1900s when he observed a population of white blood cells uptaking particles in the digestive tracts of starfish larvae (Gordon, 2016). Phagocytosis is a process by which macrophages engulf large particles of up to 0.5µm into a plasma membrane envelope in order to eliminate invading pathogens (Rosales & Uribe-Querol, 2017). Phagocytosis begins when macrophages sense pathogen associated molecular patterns (PAMPs) with their PRRs. This activates an ingestion and internalisation of the large particle, caused by polarization of actin molecules. Actin is then shed and a phagosome is formed. The engulfed pathogen is degraded within the phagosome (Aderem & Underhill, 1999).

In addition to phagocytosis, macrophages have two other key functions in the innate immune response, cytokine production and antigen presentation. Cytokines are produced by macrophages in response to pro-inflammatory stimuli such as bacterial endotoxin, as described below. Macrophages initiate the adaptive immune response by presenting the antigens of ingested peptides to T cells (Muntjewerff et al., 2020).

1.4.2 Macrophage response to endotoxin

The inflammatory response of macrophages to the bacterial endotoxin LPS has been well characterised and is a good example of how macrophages promote inflammation. LPS is a cell wall component of gram-negative bacteria which is known to promote macrophage activation and inflammation. LPS was originally described by Richard Pfeiffer in his studies of bacterial toxins from *Vibrio cholerae* (Hamesch et al., 2015). LPS is a glycolipid which functions to provide structural support in the outer membrane of the bacterial cell envelope and provides gram-negative bacteria with resistance to bile acids (Bertani & Ruiz, 2018) .

LPS is a PAMP which is recognised by macrophages to initiate an innate immune response. TLR4 and myeloid differentiation factor 2 (MD2) recognise LPS and recruit myeloid differentiation primary response 88 (MyD88) to the TLR4 site. The interaction between TLR4 and MyD88 is modulated by toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP) (Pålsson-McDermott & O'Neill, 2004). MyD88 activates interleukin 1 (IL-1) Receptor associated kinase (IRAK)-4 and starts a signaling cascade. IRAK-4 activates IRAK-1 and IRAK-2. The IRAK complex activates tumour necrosis factor associated factor 6 (TRAF-6) which aids the recruitment of tumour growth factor β activated kinase 1 (TAK1) complex. The TAK1 complex has a range of targets, all of which promote the expression of pro-inflammatory cytokines. The TAK1 complex phosphorylates inhibitor of nuclear factor (NF) κ B (IκB) kinase complex (IKK), mitogen activated protein kinase kinase 6 (MKK6) and MKK7. MKK's phosphorylate p38 mitogen activated protein kinase (MAPK) (p38) and c-Jun terminal kinase (JNK), respectively. The IKK complex phosphorylates IκB subunit α (IκBα) (O'Neill et al., 2013). The activation of these three proteins leads to the activation of the transcription factors cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), activator protein 1 (AP-1) and nuclear factor κ light chain enhancer of activated B cells (NFκB), their nuclear translocation and transcription of pro-inflammatory cytokine genes (figure 1.1).

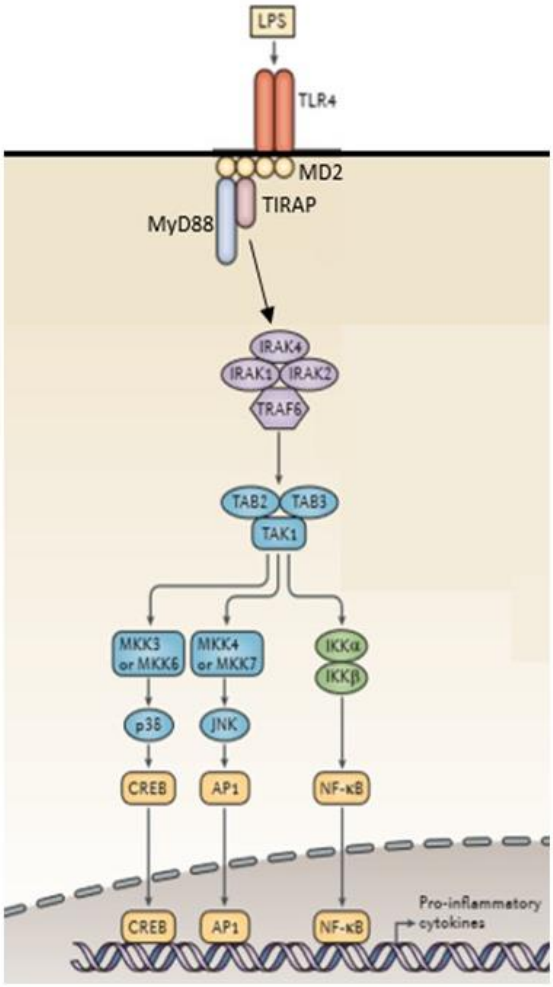

Figure 1.1 TLR4 signaling pathway. *LPS from gram negative bacteria is recognised by PRR TLR4 and MD2. MyD88 is recruited to the TLR4 site and this interaction is modulated by TIRAP. MyD88 activates the IRAK complex. The IRAK complex activates TRAF-6. TRAF-6 recruits the TAK1 complex. TAK1 phosphorylates MKK6, MKK7 and IKK. These proteins phosphorylate p38, JNK and IκBα. This leads to the activation of the transcription factors CREB, AP-1 and NFκB and the transcription of pro-inflammatory cytokine genes. Taken from O'Neill et al., 2013.*

Additionally, LPS causes the activation of the NOD-, LRR- and pyrin domain- containing protein 3 (Nlrp3) inflammasome in macrophages (Broz & Dixit, 2016). Inflammasomes are multi-protein complexes which respond to a range of stimuli by upregulating the production of pro-inflammatory cytokines via the activation of pro-inflammatory caspases (Broz & Dixit, 2016). The Nlrp3 inflammasome is the most widely studied inflammasome with described roles in the pathogenesis of liver diseases (Szabo & Petrasek, 2015). The Nlrp3 inflammasome is comprised of a sensor protein, an adaptor protein apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and the effector protein, caspase 1. The first stage of inflammasome activation is the priming stage where TLR4 sensing of PAMPs initiates the

transcription of Nlrp3 and IL1β precursors. Next, upon sensing a secondary PAMP or DAMP signal, the Nlrp3 sensor oligomerises and recruits ASC. ASC then assembles into filaments and recruits caspase 1, promoting its self-cleavage and activation. Activated caspase 1 then cleaves pro-IL1β allowing the release of the pro-inflammatory cytokine (Swanson et al., 2019) (figure 1.2).

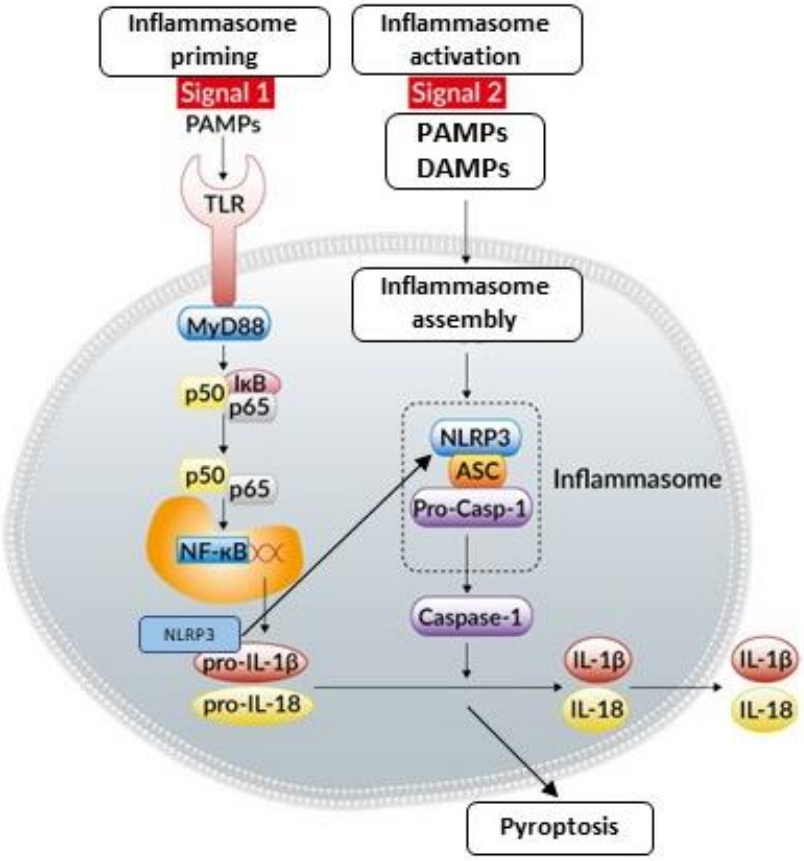

Figure 1.2 Nlrp3 inflammasome assembly. *Inflammasome priming occurs when TLRs encounter PAMPs such as LPS. This results in the nuclear translocation of NFκB and the transcription of Nlrp3 and pro-IL1β. Upon secondary encounter with PAMPs or DAMPs, Nlrp3 undergoes dimerization and inflammasome assembly occurs into the Nlrp3 inflammasome complex which is comprised of Nlrp3, ASC and caspase 1. Activated caspase 1 then cleaves and releases IL1β and promotes cell death by pyroptosis. Adapted from https://www.invivogen.com/alum.*

1.4.3 Cytokines, chemokines and receptors in macrophage function

First described in the 1970s, cytokines are low molecular weight signaling molecules which include interleukins (IL), interferons (IFN), the tumour necrosis factor family (TNF) and chemokines (Dinarello, 2007). Depending on the stimuli, macrophages can secrete a range of pro or anti-inflammatory cytokines.

The IL1 family of interleukins was originally thought to be secreted only by macrophages (Dinarello, 2007). IL1β is produced by the Nlrp3 inflammasome and is a key pro-inflammatory cytokine in the innate immune response which initiates inflammation by binding to the IL1 receptor (Swanson et al., 2019).

IL6 is a pro-inflammatory cytokine secreted mainly by monocytes and macrophages in response to most infections and inflammatory stimuli. IL6 can be produced by a range of other cell types including endothelial cells, fibroblasts, B and T lymphocytes (Tanaka et al., 2014). IL6 binds to the IL6 receptor and initiates pro-inflammatory signaling (Garbers et al., 2018).

TNFα is predominantly produced by monocytes and macrophages but can be secreted in smaller quantities by B and T cells, natural killer cells and fibroblasts (Holbrook et al., 2019). TNFα is another pro-inflammatory cytokine which binds to TNF receptors 1 and 2 and is involved in a range of processes including pro-inflammatory signaling and induction of cell death pathways (Wajant & Siegmund, 2019).

IFNγ is produced by T and NK cells and is responsible for pro-inflammatory polarization of macrophages (Ivashkiv, 2018). CC motif chemokine ligand 2 (CCL2) is a chemokine which is responsible for the recruitment of monocytes to a site of inflammation by chemotaxis through the activation of its receptor CC motif chemokine receptor (CCR2). CCL2 can be produced by a variety of cells including macrophages, fibroblasts and endothelial cells in response to stimulation with pro-inflammatory cytokines such as interleukins and TNFα (Gschwandtner et al., 2019).

Monocytes are recruited into the injured liver by chemokines, such as CCL2, which are secreted by Kupffer cells and other liver cells during the inflammatory response. When monocytes sense chemokines with their chemokine receptors, they undergo chemotaxis to reach the liver.

Chemokine receptors are classed as G protein coupled receptors. There are two main types of chemokine receptors, the CC class and the CXC class. The discovery of the first chemokine receptor, CXCR1, was published in Science in 1991 (Holmes et al., 1991).

CCR1 is expressed by monocytes, neutrophils and leukocytes and it responds to the chemokines CCL3, CCL5 and CCL7 (Vaddi et al., 1997).

CCR2 is the key receptor of CCL2, but can also bind the chemokines CCL8 and CCL13 (Proudfoot, 2002). CCR2 is mainly expressed by monocyte-derived macrophages but can also be expressed by some types of T cells (Gschwandtner et al., 2019).

CCR5 is the primary receptor of CCL5 and can also bind CCL3 and CCL4 (Proudfoot, 2002) and is predominantly expressed on monocytes and T cells (Vangelista & Vento, 2018).

CX3CR1 is the receptor for the chemokine CX3CL1 which is highly expressed on monocyte-derived macrophages and is not present on Kupffer cells (Meghraoui-Kheddar et al., 2020).

1.4.4 Macrophage polarization

When macrophages are activated by LPS they undergo polarization into a pro-inflammatory state. Twenty years ago, Charles Mills and colleagues described the M1 (pro-inflammatory) and M2 (anti-inflammatory) system for naming macrophage subtypes, based on the ability of macrophages to metabolise arginine by inducible nitric oxide synthase (NOS2) and Arginase 1 (Arg1) (Mills et al., 2000). M1 macrophages, activated by LPS, secrete pro-inflammatory cytokines such as TNFα, IL1β and IL6 and chemokines, such as CCL2, as well as nitric oxide (NO). M2 macrophages, activated by interleukin 4 (IL4), secrete a variety of growth factors and anti-inflammatory cytokines such as transforming growth factor β (TGF-β), vascular endothelial growth factor α, insulin like growth factor 1, interleukin 10 (IL10) and IL4, and they express the cell surface receptors programmed cell death ligands 1 and 2 (Wynn & Vannella, 2016).

The M1/M2 nomenclature has been described as an oversimplification as it has now become apparent that macrophages can rapidly switch between activation states (Murray et al., 2014). In this thesis we will refer to pro-inflammatory macrophages activated by LPS as M(LPS) as suggested by Murray and colleagues.

1.4.5 Macrophage metabolism

Metabolic reprograming of macrophages underpins their activation into pro- or anti-inflammatory states, with pro-inflammatory macrophages relying primarily on aerobic glycolysis and anti-inflammatory macrophages utilising oxidative phosphorylation (OXPHOS) (Van den Bossche et al., 2017).

M(LPS) macrophages require a rapid proliferative burst to initiate inflammation. Inflammation is a bioenergetically expensive process which requires rapid production of pro-inflammatory cytokines. Although glycolysis only generates two molecules of adenosine triphosphate (ATP) per cycle, it can be initiated quickly and enzymes of glycolysis have moonlighting functions which aid the pro-inflammatory functions of macrophages (Van den Bossche et al., 2017) (figure 1.3). During aerobic glycolysis, lactate is produced and can be used by the cells as an additional carbon source for energy or biomass generation. Aerobic glycolysis is a preferential metabolic pathway for some proliferating immune cells (Vander Heiden et al., 2009).

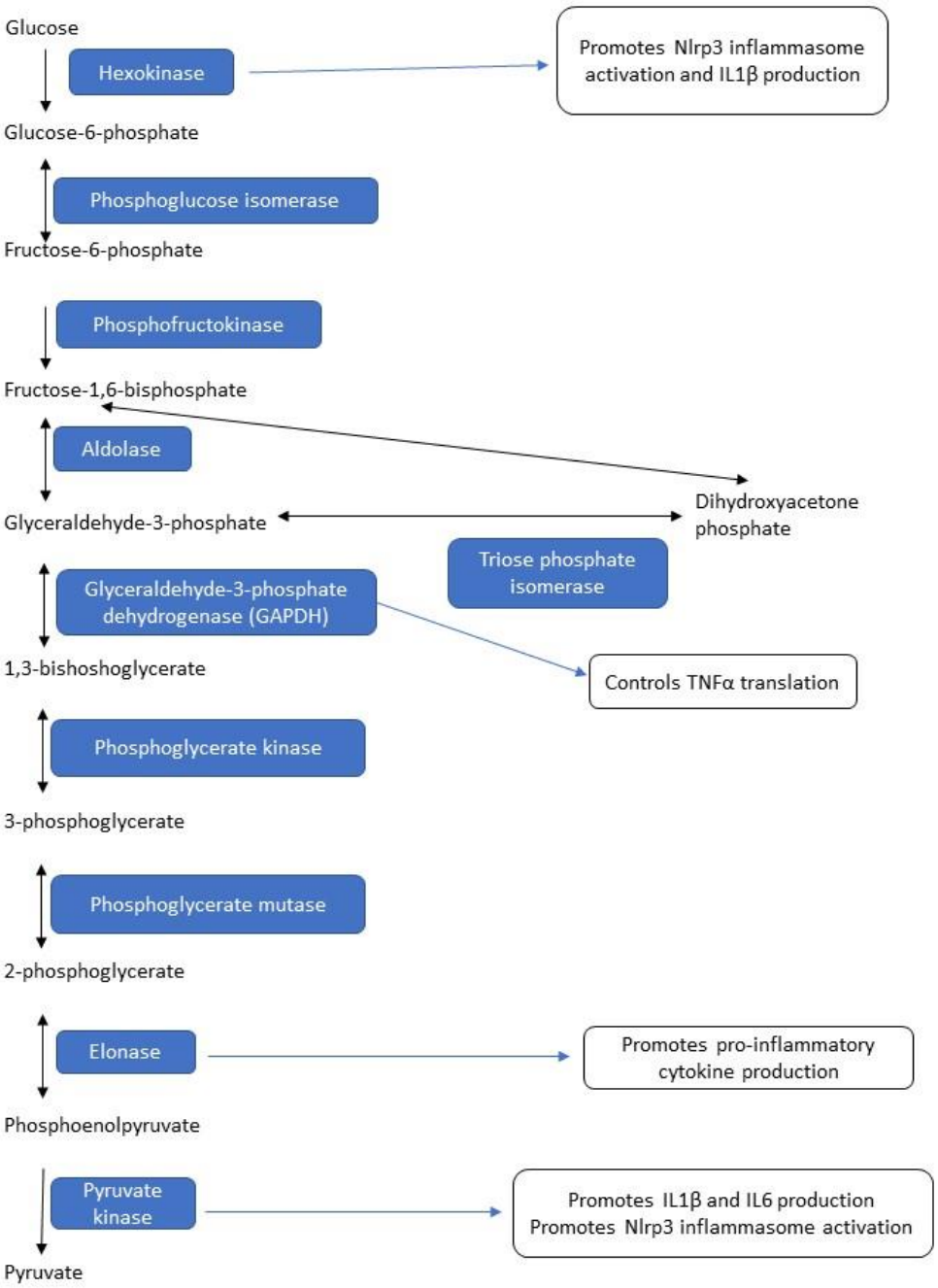

Figure 1.3 Glycolysis. *Enzymes of glycolysis promote pro-inflammatory macrophage phenotype. Hexokinase promotes Nlrp3 inflammasome activation and IL1ϐ production. Glyceraldehyde 3- phosphate dehydrogenase (GAPDH) controls TNFα translation. Elonase promotes pro-inflammatory cytokine production. Pyruvate kinase promotes pro-inflammatory cytokine production and Nlrp3 inflammasome activation. Adapted from Van den Bossche et al. 2017.*

While M(LPS) rely primarily on glycolysis for their metabolism, their Krebs cycle is disrupted (figure 1.4). The Krebs cycle breaks generate metabolites that are essential to support the pro-inflammatory response. The first Krebs cycle break point occurs at the enzyme isocitrate

dehydrogenase (IDH), during the conversion of isocitrate to α-ketoglutarate and results in the accumulation of citrate (Jha et al., 2015). The result of this break has been proposed to be the redirection of the Krebs cycle flux to the production of itaconate, a key metabolite for anti-inflammatory macrophage reprogramming (O'Neill & Artyomov, 2019). Very recently, this view has been challenged by Palmieri and colleagues who proposed that the first Krebs cycle break instead occurs at the previous step of the Krebs cycle, during the conversion of citrate to isocitrate, at the enzyme aconitase 2 (ACO2) instead of IDH (Palmieri et al., 2020). Both proposed breaks result in the accumulation of citrate. Citrate is used by M(LPS) for NO production, lipogenesis (Van den Bossche et al., 2017) and to replenish the acetyl co enzyme A (acetyl co-A) pool for acetylation (Lauterbach et al., 2019).

The second Krebs cycle break point occurs at succinate dehydrogenase (SDH), during the conversion of succinate to fumarate. The break was first identified in M(LPS) by the O'Neill group, in a study where they described that succinate was essential for HIF1α dependent IL1β production (Tannahill et al., 2013). Additionally, succinate has been shown to promote the production of mitochondrial reactive oxygen species (ROS) and inhibit the production of anti-inflammatory cytokines (Zas & Neill, 2020). During the pro-inflammatory response, accumulating succinate can activate the receptor succinate receptor 1 (SUCNR1). This activation has been shown to increase IL1β production in pro-inflammatory macrophages (Littlewood-Evans et al., 2016). More recently, SUCNR1 depletion was shown to promote pro-inflammatory macrophage polarization and a novel role was discovered for succinate in stimulating the production of pro-inflammatory cytokines (Keiran et al., 2019).

Recently, two novel break points in the Krebs cycle were identified, one at the entry point of the Krebs cycle at the pyruvate dehydrogenase complex (PDHC) and the second one at α-ketoglutarate dehydrogenase (KDH) (Seim et al., 2019). While the break points at IDH and SDH occur early (up to 24 hours) in response to LPS stimulation, the breaks at PDHC and KDH have been described to occur later, from 48 hours of LPS and IFN γ stimulation (Seim et al., 2019). These later Krebs cycle breaks allow the downregulation of hypoxia inducible factor 1 α (HIF1α) activity by pro-inflammatory macrophages (Seim et al., 2019).

In summary, metabolism underpins macrophage activation states.

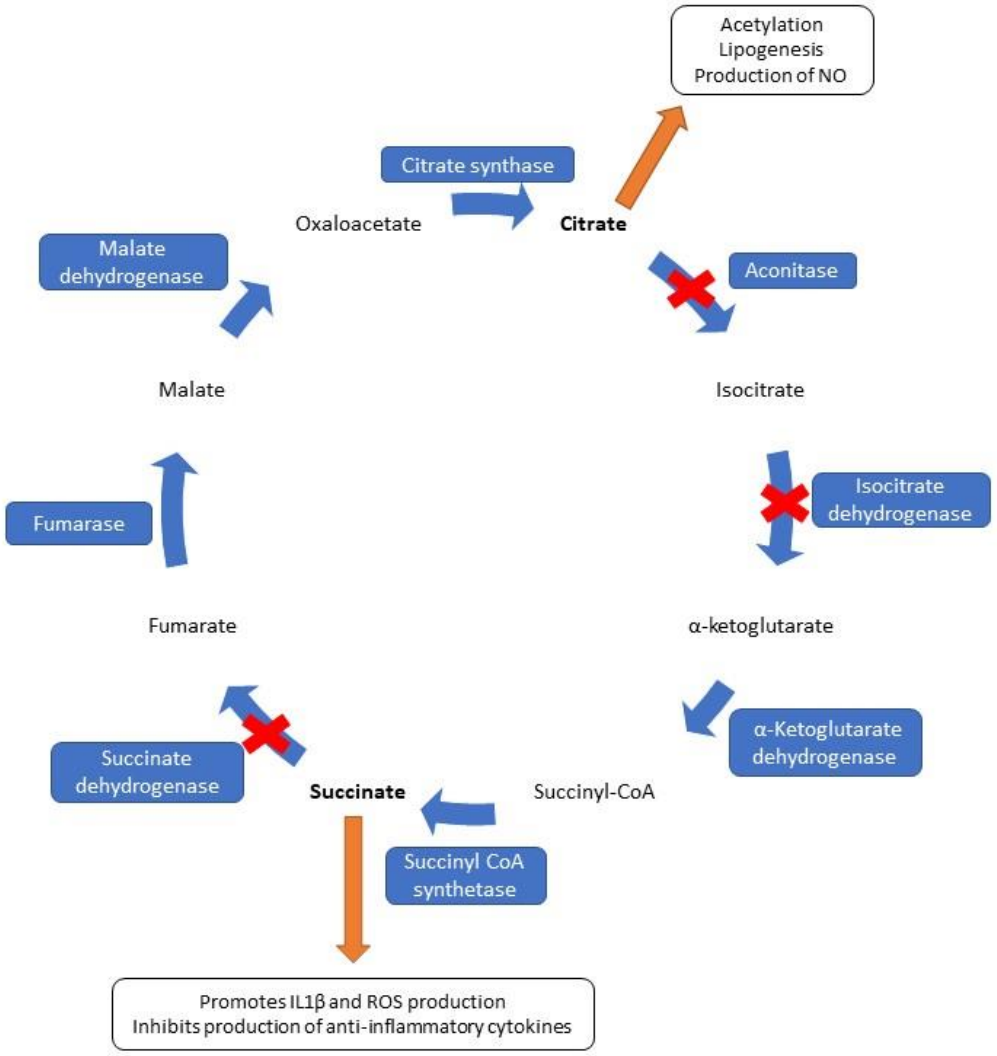

Figure 1.4 Krebs cycle. *Krebs cycle breaks in pro-inflammatory macrophages underpin their phenotype. The first Krebs cycle break, proposed at aconitase and IDH by two different groups, results in the accumulation of citrate for acetylation, lipid and NO production. The second Krebs cycle break occurs at the enzyme SDH and produces succinate to promote IL1β and ROS production and inhibit anti-inflammatory cytokine production by macrophages.*

1.4.6 Macrophage autophagy

Translated from Greek as "self-eating", autophagy is a cellular process whereby large proteins and organelles are engulfed within lysosomes and degraded (Yang & Klionsky, 2010). During starvation, for example, the cell can survive by using autophagy to recycle energy from organelles for nutrients. The pathway is also used to maintain cellular homeostasis by removing misfolded proteins and damaged organelles which would otherwise cause stress to the cell (Codogno et al., 2011). Autophagy comprises of four main stages: initiation, elongation of the

autophagosome, closure of the autophagosome and fusion of the autophagosome with the lysosome (figure 1.5) (Vural & Kehrl, 2014). Phagophore assembly begins during the initiation stage.

During starvation, nicotinamide adenine dinucleotide (NAD^+) levels are high and this leads to the activation of SIRT1, an NAD^+ dependent protein and histone deacetylase (HDAC). SIRT1 causes the inhibition of mammalian target of rapamycin (mTOR) (Ghosh et al., 2010). Adenosine monophosphate (AMP) activated protein kinase (AMPK) is also involved in the inhibition of mTOR and in the phosphorylation of the UNC-51-like kinase 1 (ULK1) complex (J. Kim et al., 2011). Inhibition of mTOR and phosphorylation of ULK1 by AMPK allow it to translocate from the cytosol to the endoplasmic reticulum and begin recruiting autophagy proteins, namely the class III phosphatidylinositol-3-OH kinase (PI(3)K) complex (Levine et al., 2011). The elongation of the autophagosome occurs next, which is dependent on two ubiquitin- like conjugation systems, the autophagy gene (ATG) complex ATG5-ATG12-ATG16L and the lipid modification of light chain 3 (LC3) with phosphatidylethanolamine (PE). The lipidation of LC3 with PE allows the autophagosome to close and fuse with the lysosome. The newly formed autolysosome then digests its cargo, aided by lysosomal hydrolytic enzymes and a low pH of 4.5 to 5 (C.-Y. Lim & Zoncu, 2016).

Autophagy is one of the host's innate immune responses to infection. Autophagy induced by PAMPs and DAMPs leads to lysosomal degradation of invading bacteria (Bah & Vergne, 2017). Autophagy controls the inflammatory response by degrading inflammasomes in autophagosomes to aid the resolution of inflammation (C. S. Shi et al., 2012). In summary, autophagy is a key process essential for macrophage function which plays a key role in the resolution of the inflammatory response, as autophagy promotes anti-inflammatory macrophage polarization (Germic et al., 2019; M. Y. Wu & Lu, 2019).

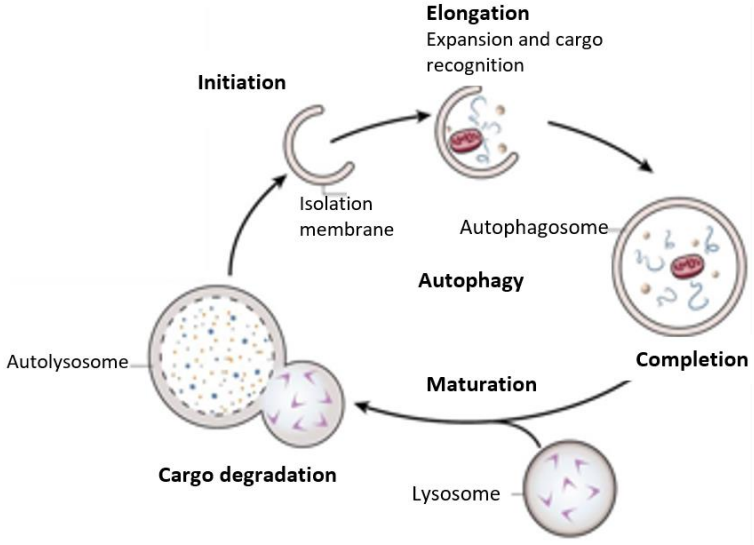

Figure 1.5 The autophagy pathway. *The initiation, elongation, closure of the autophagosome and the degradation of cargo by the autolysosome. Taken from Levine, Mizushima et al. 2011.*

1.5 Gut/liver axis

The gut/liver axis is the two-directional crosstalk between the intestine and the liver, which takes place through the biliary tract, portal vein and systemic circulation (Tripathi et al., 2018). The intestine allows the absorption of nutrients and water while acting as a barrier against harmful toxins and pathogens. The intestinal barrier is comprised of three main layers: the mucus layer, the epithelial layer and the lamina propria (König et al., 2016). There are three paracellular epithelial permeability pathways, which allow the movement of water and nutrients across the intestinal barrier whilst preserving barrier function, the pore pathway, the leak pathway and the unrestricted pathway (Odenwald & Turner, 2017). Intestinal permeability is controlled by tight junction (TJ) proteins, which function to maintain contacts between intestinal epithelial cells (Turner, 2009). The TJ complex includes the TJ (zona occludens) and the adherens junction (zona adherens). TJ proteins include occludin and claudin, and adherens junctions include cadherin proteins such as epithelial cadherin (E-cadherin) (Odenwald & Turner, 2017). The intestinal microbiome is the largest collection of bacteria, bacteriophages, archaea, fungi and viruses in the body and these micro-organisms have a symbiotic relationship with the host (Fan & Pedersen, 2021). A diverse microbiome composition is essential to maintain intestinal health as microbial metabolites can strengthen TJ and prevent the overgrowth of pathogenic bacteria in the intestine (Tripathi et al., 2018).

The gut/liver axis is vital for maintaining liver and intestinal health during homeostasis. Bile is secreted from the gall bladder to the duodenum postprandially and the intestinal microbiome's bacteria contain BSH which modify bile acids as discussed above. The presence of bile acids in the intestine is beneficial because bile is bactericidal and its presence regulates the composition of the microbiome by preventing microbial overgrowth in the intestine (Urdaneta & Casadesús, 2017).

The gut/vascular barrier functions to prevent the intestinal microbiome from reaching the liver during health (Spadoni et al., 2015). If any intestinal products reach the liver, Kupffer cells perform immune surveillance functions and filter portal vein blood to remove them. The concentrations of LPS in portal blood reduce 100 fold after passing through the liver and being transported into the circulation (Jenne & Kubes, 2013).

As well as having a key role in the liver, macrophages are essential in the maintenance of intestinal barrier integrity (Mowat & Bain, 2011). The intestine's largest resident macrophage population is localised to the lamina propria and this unique position allows them to catch bacteria at the epithelial barrier and remove apoptotic intestinal epithelial cells (Bain & Schridde, 2018). The intestinal macrophage pool is maintained throughout life by the recruitment of monocyte-derived macrophages (Bain et al., 2014). To maintain a healthy intestinal barrier, intestinal macrophages

maintain tolerance to microbes and food allergens therefore while intestinal macrophages are highly bactericidal and phagocytic, they produce anti-inflammatory cytokines IL10 and TGFβ and their TLRs are hyporesponsive and do not initiate inflammation in response to endotoxin (Bain & Schridde, 2018).

In summary, the gut/liver axis contributes to whole body health by preventing intestinal products reaching the liver and the disruption of the intestinal barrier is often associated with chronic liver diseases.

1.6 Chronic liver disease

1.6.1 Overview of chronic liver disease

Chronic liver diseases are a leading cause of disability (Paik et al., 2021) and cause 2 million deaths per year worldwide (Asrani et al., 2019). Chronic liver diseases are defined as liver conditions that last for at least six months and involve cycles of liver destruction and regeneration which result in fibrosis.

Currently, the most common type of liver disease in the west is non-alcoholic fatty liver disease (NALFD). NALFD affects 25% of adults worldwide (Asrani et al., 2019). Alcohol associated liver diseases account for 5.9% of all deaths and 50% of cirrhosis deaths worldwide (Seitz et al., 2018). Hepatitis B is the main cause of chronic liver disease in Asia and affects 3.5% of the world's population despite the progress made with vaccinations (Asrani et al., 2019). Cholestatic liver diseases are rare diseases which affect 1/10000 people worldwide, with a higher incidence in the west (X. Jiang & Karlsen, 2017).

Treatments for chronic liver diseases are limited and the main treatment option for most patients is liver transplantation which is limited by the shortage of suitable donors and further delayed by Coronavirus disease 2019 (Merola et al., 2021).

1.6.2 Macrophages in chronic liver disease

1.6.2.1 Overview of macrophages in chronic liver disease

Inflammation is the hallmark of all chronic liver diseases and macrophages promote the pathological cycles of tissue destruction and repair which can lead to fibrosis, cirrhosis, hepatocellular carcinoma and loss of liver function (Singanayagam & Triantafyllou, 2021).

Macrophages contribute to the pathogenesis of all chronic liver diseases because inflammation is a hallmark of all chronic liver diseases and macrophages drive inflammation (Krenkel & Tacke, 2017). Upon liver damage, Kupffer cells recruit monocyte-derived macrophages

from the bone marrow via the production of pro-inflammatory chemokines. Both resident and infiltrating macrophages then contribute to cycles of inflammation and repair in the liver, which ultimately results in fibrosis of the organ and loss of function (Pellicoro et al., 2014). Below we discuss the role of macrophages in chronic liver diseases.

1.6.2.2 Macrophages in non-alcoholic fatty liver disease

NAFLD can progress to non-alcoholic steatohepatitis (NASH) which is the primary cause of liver transplantation in the west (Merola et al., 2021). NAFLD is characterised by the accumulation of fat in hepatocytes due to obesity, not alcohol consumption (Kazankov et al., 2019). Many studies support the pathological role of Kupffer cells in NAFLD pathogenesis. Kupffer cell depletion protected mice from high fat diet induced hepatic steatosis (W. Huang et al., 2010) through the reduction of TLR4 expression (Rivera et al., 2007) as well as the reduction of the pro-inflammatory cytokine IL1β (Stienstra et al., 2010) and TNFα mediated monocyte-derived macrophage recruitment (Tosello-Trampont et al., 2012). In NASH patients and murine models, Kupffer cells have been described to surround dying hepatocytes in lipid droplets, termed hepatic crown-like structures (Itoh et al., 2013). The formation of these Kupffer cell structures contributes to the development of liver fibrosis during NASH (Itoh et al., 2017; Kanamori et al., 2021).

As with all chronic liver diseases, distinct populations of liver macrophages comprising of Kupffer cells and recruited monocyte-derived macrophages have also been identified in a mouse model of NAFLD (Morinaga et al., 2015). While Kupffer cells are important initiators of inflammation in NAFLD, monocyte-derived macrophages are the main drivers of inflammation and pathogenesis. NAFLD are associated with obesity and obese mice recruited monocyte-derived macrophages to their livers via CCR2 receptor signaling and these recruited macrophages promoted the pathogenesis of hepatic steatosis (Obstfeld et al., 2010). Monocyte-derived macrophages were the first infiltrating immune cells at the site of portal tracts of NAFLD patients, these macrophages then promoted ductular reaction through the production of pro-inflammatory cytokines TNFα and IL1β (Gadd et al., 2014). Several studies showed that the inhibition on monocyte recruitment either by genetic depletion of the CCR2 receptor (Miura et al., 2012; Weisberg et al., 2006) or with pharmacological CCR2 inhibitors (Baeck et al., 2012; Krenkel et al., 2018; Lefebvre et al., 2016) reduced hepatic steatosis, hepatic inflammation and fibrosis in murine NAFLD and NASH models. These exciting findings led to several clinical trials for CCR2 inhibition for the treatment of NAFLD including the CCR2/CCR5 inhibitor cenicriviroc which showed promising results as NASH patients showed improvement in liver fibrosis compared to the placebo group (Friedman et al., 2018). Unfortunately, cenicriviroc did not pass the Phase 3 clinical trials due to a lack of efficacy and the

drug will no longer be trialled as a NASH therapeutic (https://clinicaltrials.gov/ct2/show/NCT03028740). However, cenicriviroc still has potential as a therapeutic for other chronic liver diseases.

1.6.2.3 Macrophages in alcoholic fatty liver disease

Alcoholic fatty liver (AFL) disease is defined as the accumulation of fat in hepatocytes which develops due to chronic alcohol (ethanol) consumption (Seitz et al., 2018). AFL disease can progress to alcoholic steatohepatitis (ASH), alcoholic hepatitis, alcoholic cirrhosis and hepatocellular cancer. Alcoholic liver diseases (ALD) are associated with a leaky gut because chronic alcohol consumption leads to the destruction of TJs between intestinal epithelial cells, allowing the translocation of bacterial products to the liver (Szabo, 2015).

Ethanol sensitises Kupffer cells to gut-derived LPS and during ALD Kupffer cells secrete pro-inflammatory cytokines in response to endotoxin (Bala et al., 2017; Enomoto et al., 2001). The genetic or pharmacological Kupffer cell polarization to an anti-inflammatory phenotype limited liver injury in mouse models of both, ALD and NAFLD as anti-inflammatory macrophage IL10 production induced the apoptosis of pro-inflammatory macrophages (Wan et al., 2014). Kupffer cells are essential for the development of ALD as Kupffer cell depletion with gadolinium chloride protected rats from ethanol-induced liver damage (Adachi et al., 1994; Koop et al., 1997). Monocyte-derived macrophages also infiltrate the liver in response to ethanol and contribute to liver damage (M. Wang et al., 2014), especially in female mice (Alharshawi et al., 2021).

1.6.2.4 Macrophages in fibrosis

Hepatic fibrosis is the dysregulated wound healing response of the liver and is a common endpoint of many chronic liver diseases including NASH, ASH and cholestasis, which can lead to liver cirrhosis and, ultimately, loss of function.

Fibrosis is defined as the accumulation of ECM and activated HSC are the main ECM producing cell type in the fibrotic liver (Trautwein et al., 2015). During chronic liver disease, HSC are activated by a range of signals from damaged hepatocytes, cholangiocytes and macrophages (Karlsen et al., 2017).

Macrophages act as a double-edged sword in the process of fibrosis as pro-inflammatory macrophages produce pro-inflammatory cytokines which drive fibrogenesis however anti-inflammatory macrophages contribute to the resolution of fibrosis by promoting the degradation of collagen fibres in the liver (figure 1.6) (Wynn & Vannella, 2016).

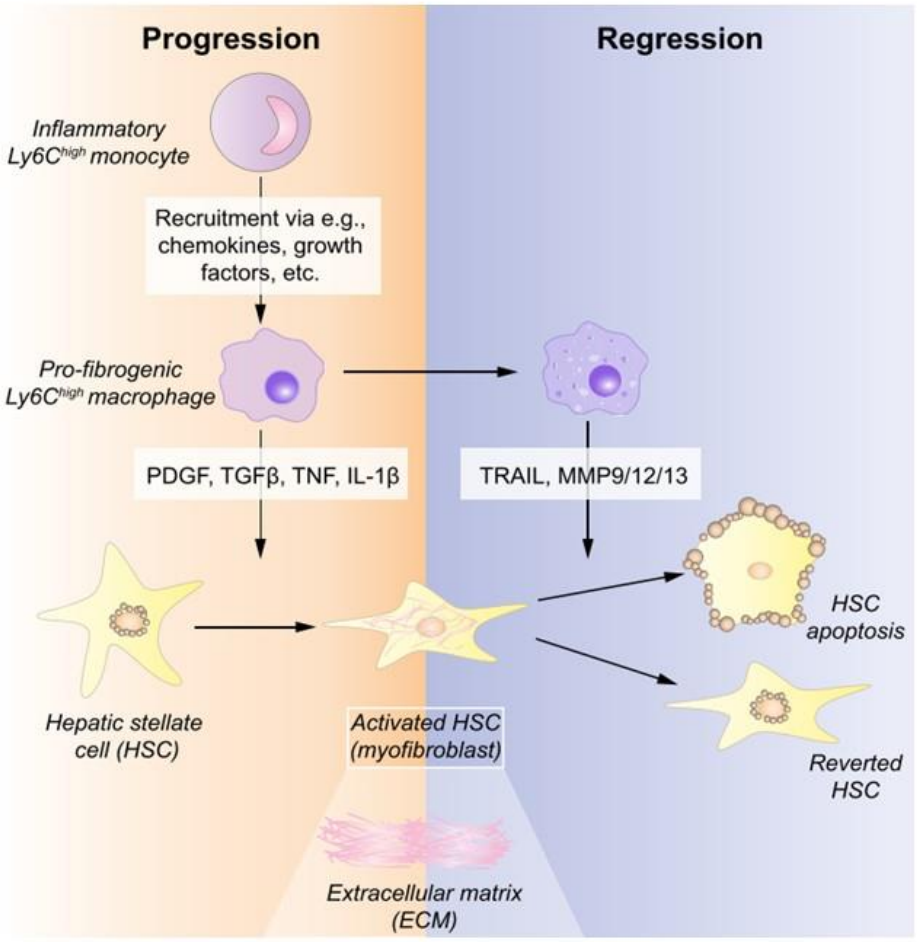

Figure 1.6 The role of macrophages in fibrosis*. Macrophages promote the progression and regression of fibrosis in the liver. Pro-inflammatory macrophages produce cytokines which activate HSC differentiation into ECM producing myofibroblasts. Macrophages can also promote the resolution of fibrosis. Macrophages secrete MMPs which degrade ECM and macrophages promote HSC apoptosis. Taken from Trautwein et al. 2015.*

Pro-inflammatory macrophages are essential for the initiation of fibrosis (Kisseleva & Brenner, 2021). Upon liver injury, Kupffer cells and monocyte-derived macrophages promote HSC activation, differentiation into myofibroblasts and the deposition of ECM through two cytokines TGFβ and platelet derived growth factor (PDGF) (Trautwein et al., 2015). Intestinal-derived bacterial endotoxin has also been shown to promote fibrosis in the liver through macrophage activation. TLR4 signaling in response to LPS has been shown to promote fibrogenesis through the interplay between Kupffer cells and HSC (Seki et al., 2007).

Several pre-clinical studies have elucidated the detrimental role of macrophages in liver fibrosis. Using bile duct ligation (BDL) to induce fibrosis in a spontaneous TLR4 mutant mouse

model, Seki et al. demonstrated that TLR4 signaling sensitised HSC to Kupffer cell TGFβ production (Seki et al., 2007). Also, the chemical depletion of macrophages protected rats (Ide et al., 2005) and mice from liver fibrosis (Best et al., 2016; Guicciardi et al., 2018). Additionally, macrophages promoted myofibroblast survival in a carbon tetrachloride (CCL_4) model of liver fibrosis though the secretion of pro-inflammatory cytokines TNFα and IL1β (Pradere et al., 2013).

However, macrophages also promote the resolution of fibrosis and one of the ways in which macrophages contribute to this process is through their phagocytic function. The uptake of apoptotic hepatocytes by Kupffer cells promoted the secretion of TNFα and Kupffer cell depletion with gadolinium chloride reduced fibrogenesis in mice in response to BDL (Canbay et al., 2003). The BALB/cJ mouse strain has previously been identified to be highly susceptible to fibrosis while the FVB stain was resistant to fibrosis in response to CCL_4 treatment (Hillebrandt et al., 2002). Recently, the Popov group investigated the macrophage function of those two mouse strains in fibrosis using the acetamide administration model and they found that the macrophages of the fibrosis susceptible BALB/cJ strain were defective in phagocytosis, therefore they had a delayed clearance of necrotic bodies and increased macrophage infiltrates in the liver (An et al., 2020). Earlier work showed that phagocytosis of cellular debris by pro-fibrogenic $Ly6C^{+}$ macrophages re-polarized these cells to a $Ly6C^{Low}$ restorative phenotype, which had a significant increase in matrix metalloprotease (MMP) 12 expression (Ramachandran et al., 2012). The uptake of dying cells allows pro-inflammatory macrophages to undergo a phenotypic switch from pro-inflammatory to anti-inflammatory cells (Pellicoro et al., 2014) and anti-inflammatory macrophages promote ECM degradation by secreting matrix metalloproteases (MMPs) however HSC produce tissue inhibitors of metalloproteases (TIMPs) which inhibit the MMPs in the fibrotic liver (Wynn & Vannella, 2016).

Some MMPs play a key role in the regulation of fibrosis as they are essential for ECM digestion, for example MMP12 digests elastin (Pellicoro et al., 2012) and MMP13 breaks down collagen type 1 (Fallowfield et al., 2007) which contributes to the resolution of fibrosis. However, the expression of MMP9 by portal monocyte-derived macrophage infiltrates promoted fibrosis through enhanced ECM remodelling (Gadd et al., 2013).

Interestingly, fibrosis can be reversed and liver architecture can be restored (Ramachandran & Iredale, 2009) if the signal that is driving the liver damage is removed and the wound healing processes in the liver complete (Wynn & Barron, 2010). This has been observed in the clinic in NASH patients who lost weight (Dixon et al., 2004), ASH patients who abstained from alcohol (Parés et al., 1986), hepatitis C patients who received treatment for the virus (Poynard et al., 2002) and cholestatic patients who underwent biliary drainage (Hammel et al., 2001). The reversal of fibrosis, and possibly cirrhosis, holds tremendous promise as a future therapeutic option

for chronic liver diseases because currently, the only treatment option for end stage liver disease is transplantation (Singanayagam & Triantafyllou, 2021).

In conclusion, macrophages are essential drivers of HSC activation and collagen deposition which leads to liver fibrosis. However, macrophages are also essential for the resolution of fibrosis and the restoration of healthy liver architecture. The regression of fibrosis shows tremendous therapeutic promise for the treatment of chronic liver diseases and macrophage therapy provides an exciting therapeutic target to drive the regression of fibrosis in patients.

1.6.2.5 Macrophage therapy

As mentioned above in section 1.6.1, the main treatment option for chronic liver diseases is liver transplantation which is limited by the shortage of donors and transplant rejection, therefore new therapies are under development and some of these therapies focus on macrophages (Sato et al., 2020).

Macrophage transplantation has been under development for a decade and shows tremendous promise in the treatment of fibrosis in a range of pre-clinical (Lewis et al., 2020; Thomas et al., 2011) and clinical models (Moroni et al., 2019). Ma and colleagues showed that the transplantation of pro-inflammatory, but not anti-inflammatory or unpolarized, bone marrow derived macrophages (BMDM) to mice after BDL or CCL_4 treatment resulted in reduced fibrosis due to increased recruitment of restorative $Ly6C^{Low}$ monocytes (Ma et al., 2017). Good Manufacturing Practice autologous macrophage transplantation therapy for the treatment of liver fibrosis has been successfully developed (Fraser et al., 2017; Moore et al., 2015), trialled and well tolerated in patients with fibrosis, highlighting the potential of macrophage therapy for disease treatment (Moroni et al., 2019). Most studies that have been carried out to date were done using the re-infusion of BMDM. Luo and colleagues described that bone marrow transplantation upregulated MMP13 expression by anti-inflammatory macrophages, and inhibited pro-inflammatory macrophage polarization, in a model of liver fibrosis induced by CCL_4 (Luo et al., 2019). Most recently, Li and He described that *ex-vivo* expanded Kupffer cells protected a CCL_4-induced mouse model from liver fibrosis through the upregulation of MMPs (W. Li & He, 2021).

The re-polarization of macrophages has also been investigated in murine models of cancer. Recently, a novel technology termed the cellular “backpack” has been described where transplantation of macrophages with the IFNγ cell adhesive “backpacks” was shown to re-polarize anti-inflammatory tumour associated macrophages to the pro-inflammatory state in a murine model of breast cancer (Iv et al., 2020). Additionally, pro-inflammatory chimeric antigen receptor

macrophages were used to re-polarize anti-inflammatory macrophages to pro-inflammatory in a range of cancer models (Klichinsky et al., 2020).

In summary, macrophages are an exciting therapeutic target for the treatment of chronic liver diseases and other diseases where macrophages underpin the disease pathogenesis.

1.7 Cholestasis

1.7.1 Introduction to cholestasis

Cholestasis is defined as the loss of bile flow from the liver to the duodenum. Cholestasis, a common feature of many liver diseases, includes a broad range of hepatobiliary disorders such as primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC), progressive familial intrahepatic cholestasis, biliary atresia in children and intrahepatic cholestasis of pregnancy in women (Jüngst et al., 2013). Cholestatic diseases are often asymptomatic at the early stages and liver damage can progress undetected for many years (Jüngst et al., 2013). The limited symptoms of cholestasis can include jaundice, itchy skin, dark urine and pale stool (Eaton et al., 2013).

PBC and PSC are the main cholestatic disorders in adults. They are considered rare diseases, each affecting 1 in 10000 people (Karlsen et al., 2017). Cholestatic patients are at increased risk of cholangiocarcinoma and hepatocellular carcinoma (Eaton et al., 2013). PBC and PSC closely associate with inflammatory bowel diseases (Liberal et al., 2020; Ostrowski et al., 2019) which increases risk of colorectal cancer in these patients (Karlsen et al., 2017). Despite the rarity of the diseases, further research is urgently needed as therapeutic options are limited to the therapeutic bile acids ursodeoxycholic acid (UDCA) and obeticholic acid (OCA) (Gerussi et al., 2020) however these treatments have limited success; 40% of PSC patients require liver transplantation and the disease will recur in 25% of the transplant recipients (Lazaridis & LaRusso, 2016).

PSC and PBC are both classed as autoimmune disorders (Gulamhusein & Hirschfield, 2020; X. Jiang & Karlsen, 2017). PBC is characterised by the production of anti-mitochondrial antibodies which target cholangiocytes and cause bile duct destruction (Gulamhusein & Hirschfield, 2020). While the initiating events of the diseases are currently unknown, a combination of genetic and environmental factors are thought to promote bile duct injury, which then leads to liver inflammation, which drives liver destruction during cholestasis, and the disease can progress to fibrosis, cirrhosis and ultimately loss of function (Eaton et al., 2013).

The pathogenesis of cholestasis begins when cholangiocytes, which do not normally proliferate, become activated due to unknown stimuli (figure 1.7). Ductular reaction then leads to bile duct loss (Sato et al., 2019). The destruction of bile ducts leads to accumulation of bile acids in

the liver, which causes hepatocyte cell death due to a toxic bile acid concentration (Fickert & Wagner, 2017). Dying hepatocytes cause Kupffer cell activation (Canbay et al., 2003) and monocyte recruitment from the bone marrow to the liver, where the monocytes differentiate into macrophages and promote further inflammation which contributes to the pathogenesis of cholestasis (Guicciardi et al., 2018).

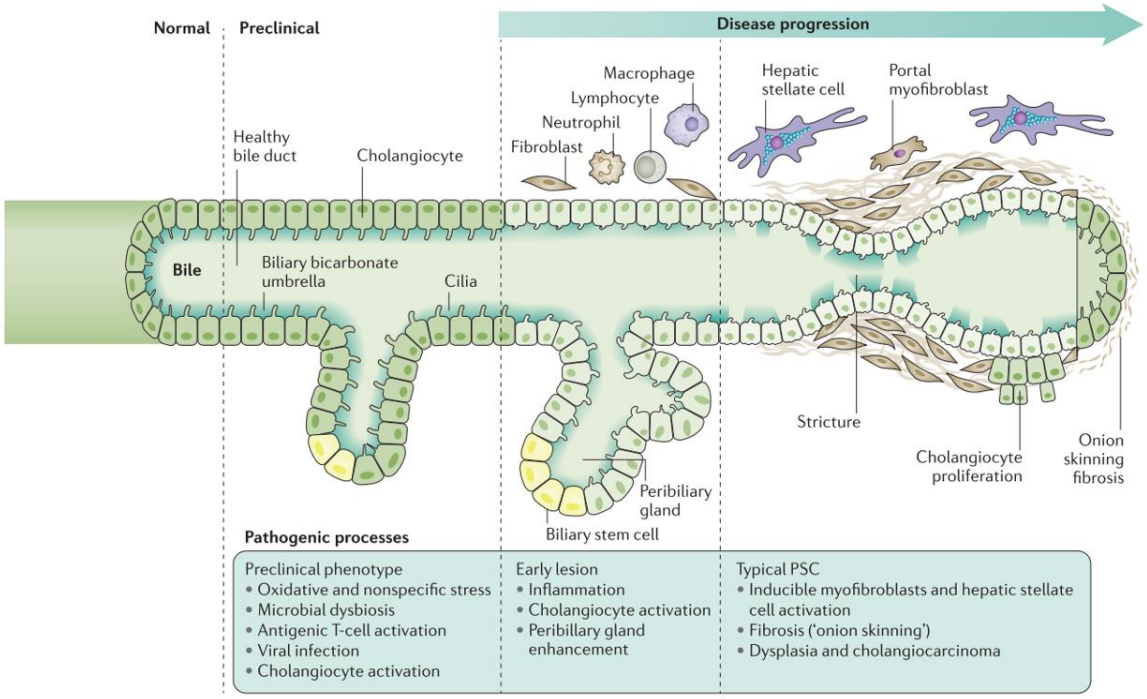

Figure 1.7 Bile duct destruction in PSC. *Cholestasis begins when cholangiocytes become activated by unknown stimuli. Cholangiocyte proliferation leads to bile duct loss, bile acid accumulation in the liver and inflammation. As the disease progresses, HSC activation leads to fibrosis and loss of liver function. Taken from Jiang and Karlsen 2017.*

The lack of bile flow from the liver to the duodenum causes intestinal dysbiosis and translocation of bacterial products to the liver via the portal vein (Urdaneta & Casadesús, 2017). Bacterial products in the liver promote Kupffer cell and monocyte-derived macrophage activation, inflammation and further liver damage. HSC cells try to repair the liver by upregulating collagen deposition, which results in fibrosis and loss of liver function (Trautwein et al., 2015). The role of macrophages in cholestasis is discussed below.

1.7.2 Macrophages in cholestasis

During cholestasis, bile acids do not flow out of the liver and they can reach a concentration of up to 2mM in the liver of cholestatic mice (Jansen et al., 2017). At high concentrations, bile acids are toxic to hepatocytes and, during cholestasis, hepatocytes die by apoptosis and necrosis and are phagocytosed by Kupffer cells (Canbay et al., 2003). This engulfment of apoptotic hepatocytes stimulates Fas ligand and TNFα production by Kupffer cells, which results in inflammation and fibrogenesis (Canbay et al., 2003). Additionally, bile acids have been described to act as DAMPs which can synergise with LPS to activate the inflammasome during cholestasis (Hao et al., 2017). Interestingly, bile acids have also been described to promote the anti-inflammatory functions of macrophages. Bile acids have been described to inhibit the activation of the Nlrp3 inflammasome through Takeda G-protein-coupled receptor 5 activation (Guo et al., 2016) and to promote anti-inflammatory macrophage polarization by upregulating the production of anti-inflammatory cytokines and reducing phagocytic activity (Haselow et al., 2013). Additionally, Wammers and colleagues described that the pre-treatment of primary human macrophages with the bile acid taurolithocholic acid (TLC) attenuated the pro-inflammatory phenotype of LPS stimulation, supporting the anti-inflammatory effects of bile acids on macrophage function (Wammers et al., 2018).

As described above, Kupffer cells provide the first line of defence against PAMPs and DAMPs during cholestasis. The approach of Kupffer cell depletion has been deployed in several animal studies of cholestasis. While some groups demonstrated that depletion of Kupffer cells protected rats from cholestasis induced by BDL (Zandieh et al., 2011) and sepsis (Sturm et al., 2005), others found that Kupffer cells protected the liver from cholestasis via an IL6 mediated mechanism (Gehring et al., 2006) and contributed to liver regeneration after BDL by a mechanism involving Akt serine/threonine kinase 1 (Akt) activation (Osawa et al., 2010). In a study of cholestasis induced by 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) feeding, Jemail and colleagues described that Kupffer cell depletion by liposomal clodronate was beneficial in the early stages of the disease but detrimental in the later stages of the disease (Jemail et al., 2018). Kupffer cell IL10 secretion resulted in impaired bacterial clearance after BDL surgery (Abe et al., 2004). IL17 signaling by Kupffer cells promoted fibrosis in response to BDL or CCL_4 induced fibrosis (Meng et al., 2012). While Kupffer cells have a key role in the pathogenesis of cholestatic disease (Sato et al., 2016), recent studies have elucidated that monocyte-derived macrophages, which infiltrate the liver during injury, are the key drivers of inflammation, fibrosis and loss of liver function (Triantafyllou et al., 2018).

Pro-inflammatory macrophage infiltrates were found in the livers of PSC patients and murine models of cholestasis, inhibition of CCR2 signaling reduced macrophage recruitment to the liver and this correlated with reduced fibrosis during PSC (Guicciardi et al., 2018). Three distinct pathogenic populations of monocyte-derived macrophages were recently identified in cholestatic children (Id et al., 2021). Most recently, monocyte-derived macrophages were found to promote fibrogenesis in a novel mouse model of cholestasis (Guillot et al., 2021). The inhibitor of monocyte recruitment cenicriviroc protected mice from cholestatic liver injury in response to BDL (D. Yu et al., 2018). Cenicriviroc was recently trialled by PSC patients, was well tolerated, and the cholestatic serum marker alkaline phosphatase (ALP) was reduced (Eksteen et al., 2021). This exciting development in the treatment of PSC highlights the important pathological role for monocyte-derived macrophages in cholestatic disease progression.

1.7.3 Gut/liver axis and macrophages in cholestasis

The absence of bactericidal bile acids in the intestine during cholestasis causes microbial overgrowth, changes in the intestinal microbiome composition (Iwasawa et al., 2017; Kummen et al., 2017; Quraishi et al., 2017) and increased intestinal permeability, therefore cholestatic disease is associated with a leaky gut where bacterial products translocate to the liver and cause further inflammation (figure 1.8) (Mattner, 2016).

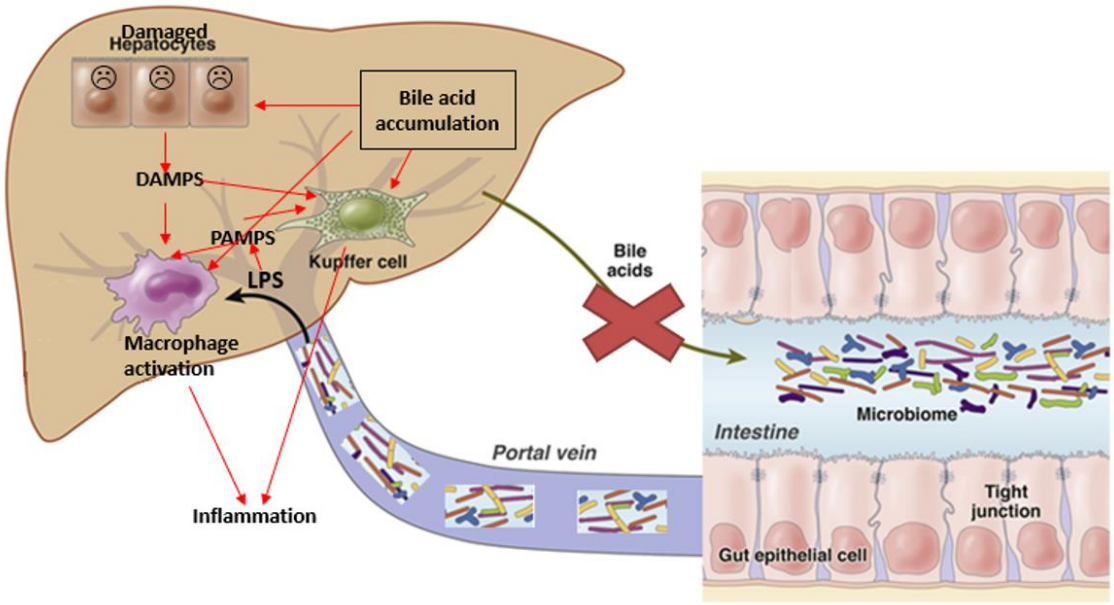

***Figure 1.8 The gut/liver axis during cholestasis**. Cholestasis can cause disturbances in the intestine and in the composition of the microbiome. Bacterial overgrowth in the intestine and bacterial translocation to the liver can occur during cholestasis. This aggravates inflammation and contributes to a worse outcome during disease. Adapted from Szabo, 2015.*

In response to intestinal injury, lamina propria resident macrophages produce CCL8 which recruits monocyte-derived macrophages (Asano et al., 2015) which infiltrate to an inflamed intestine and, unlike intestinal resident macrophages, they have a pro-inflammatory phenotype and secrete pro-inflammatory cytokines such as TNFα and IL1β, and produce NO (Ruder & Becker, 2020). TNFα has been described to promote intestinal permeability in an *in vitro* model (Söderholm et al., 2004). TNFα knockout mice preserved their intestinal barrier function into old age, which was disrupted in wild type (WT) mice due to macrophage dysfunction and intestinal dysbiosis (Thevaranjan et al., 2017). We and others have shown that the Nlrp3 inflammasome, which functions to produce IL1β, promotes TJ loss in the intestine in mouse models of cholestasis (Isaacs-Ten et al., 2020; Liao et al., 2019). Additionally, some commensal bacteria have been described to promote Nlrp3 inflammasome activation, intestinal inflammation and permeability in mouse models of colitis (Seo et al., 2015).

PSC is often associated with inflammatory bowel diseases such as ulcerative colitis and Chron's disease and PSC patients have an increased risk of developing colorectal cancers (Karlsen et al., 2017). Recent sequencing studies have identified distinct profiles of the intestinal microbiome of PSC patients (Kummen et al., 2017; Sabino et al., 2016). Remarkably, the intestinal microbiome profile of PBC patients was partially normalised by treatment with the therapeutic bile acid UDCA, highlighting the importance of the crosstalk between the gut and the liver during health and disease (Tang et al., 2018). Increased intestinal permeability has been described in PBC patients (Di Leo et al., 2003; Feld et al., 2006) and recent pre-clinical studies have identified that another bile acid therapeutic, OCA, restored the integrity of the intestinal barrier, by restoring TJ protein expression, and reduced bacterial translocation to the liver (Úbeda et al., 2016). Interestingly, mutations in the tight junction protein 2 (TJP2) gene, which disrupt TJ structure, are associated with progressive familial intrahepatic cholestasis (Sambrotta et al., 2014).

1.7.4 Macrophages and hepatocytes in cholestasis

During cholestasis, bile acids accumulate and their increased concentration kills hepatocytes in the liver (Fickert & Wagner, 2017) which act as DAMPs and activate Kupffer cells to promote a pro-inflammatory response (Canbay et al., 2003) and recruit monocyte-derived macrophages which promote further inflammation (Guicciardi et al., 2018). As described above, chronic liver diseases are often associated with increased intestinal permeability where bacterial products reach the liver via the portal vein (Wiest et al., 2017) and the translocating PAMPs cause

hepatocyte cell death (Z. Zhou et al., 2016) in the liver using two pathways; apoptosis and pyroptosis.

Caspases are cysteine proteases actively involved in both cell death pathways, apoptosis and pyroptosis (Kolb et al., 2017). Apoptosis is the most common form of cell death in the body where DAMPs are degraded and an inflammatory response is not initiated (Taylor et al., 2008). Caspase 3 is an enzyme essential for apoptosis. When hepatocytes sense LPS they initiate a proteolytic cascade, which results in caspase 3 cleavage and activation. Cleaved caspase 3 then cleaves and activates poly (ADP ribose) polymerase 1 (PAPR1) which allows apoptosis to proceed (Taylor et al., 2008). Macrophages engulf these apoptotic cells by efferocytosis (Kourtzelis et al., 2020). Efficient efferocytosis allows the resolution of the immune response and promotes anti-inflammatory macrophage polarization (Ramachandran et al., 2012).

Pyroptosis is a form of lytic cell death driven by the Nlrp3 inflammasome (Opdenbosch & Lamkanfi, 2019) that causes the release of DAMPs into the liver which are then sensed by macrophages contributing to further inflammation and liver damage in response to LPS (Kolb et al., 2017).

1.7.5 Macrophages and ductular reaction in cholestasis

Liver cells exist in a niche and crosstalk to initiate pathogenic reactions in the liver. The bile ducts are lined with cholangiocytes and contain a small population of liver resident stem cells termed hepatic progenitor cells (HPC) in the canals of Hering (Banales et al., 2019). In response to liver injury, cholangiocytes try to repair the damaged bile ducts and the repair processes is termed ductular reaction which can lead to bile duct loss called ductopenia (Pinto et al., 2018). Ductular reaction is a common pathological feature of cholestatic liver diseases (Sato et al., 2019).

Upon liver injury, the liver forms a HPC niche which is comprised of liver resident stem cells termed oval cells in mice and HPC in humans, monocyte-derive macrophages, the ECM component laminin and myofibroblasts (Lorenzini et al., 2010). Upon liver injury, cholangiocytes and HPC recruit monocyte-derived macrophages to form the HPC niche by secreting the chemokine CCL2 (Lukacs-Kornek & Lammert, 2017). Additionally, cholangiocytes secrete chemokine C-X-C Motif ligand 10 (CXCL10) which functions to recruit monocyte-derived macrophages (Kaffe et al., 2018). CXCL10, CXCL1 and CXCL12 then stimulate the secretion of TNFα, IL1β (Gadd et al., 2014) and TGFβ by macrophages which then contribute to the development of ductular reaction and periportal fibrosis (Locatelli et al., 2016).

Macrophages then interact with HPC and cholangiocytes to contribute to the development of ductular reaction in the liver but also can attenuate the progression of ductular reaction (Sato et

al., 2019). Recently, the ablation of the tumour necrosis factor related apoptosis inducing ligand (TRAIL) receptor in the cholestatic (multidrug resistance protein 2) $Mdr2^{-/-}$ mouse model resulted in increased macrophage infiltration which associated with increased ductular reaction and fibrosis compared to the $Mdr2^{-/-}$ mice (Krishnan et al., 2020). Macrophage transfer alone, without any liver injury stimuli, induced ductular reaction through increased secretion of the cytokine TNF like weak inducer of apoptosis (TWEAK) (Bird et al., 2013). Macrophage depletion attenuated ductular reaction in a murine model of cholestasis (Best et al., 2016). Macrophage-derived (Wingless and Int-1) Wnt proteins have been described to attenuate ductular reaction and fibrosis in models of chronic liver injury as the monocyte-specific depletion of Wnt has been shown to promote the pathogenesis of cholestasis in two independent studies (Irvine et al., 2015; A. Jiang et al., 2019). Macrophages promoted ductopenia by inducing cholangiocyte apoptosis (Alabraba et al., 2008).

In summary, macrophages promote ductular reaction during cholestasis and this leads to bile duct loss which is detrimental to liver function.

1.7.6 Mouse models of cholestasis

Bile disorders have fascinated researches in the field of hepatology for many years and therefore a variety of pre-clinical models have been developed to study cholestasis, which are discussed below.

Bile duct ligation (BDL): BDL is one of the most popular models of cholestasis and has been employed by scientists to study the flux of bile in a range of animals for centuries. The first recorded BDL experiments were performed in cats in 1687 by Marcello Malpighi, who identified that bile can flow into the duodenum from the liver independently of the gall bladder (Cameron & Oakley, 1932). A modern version of the BDL protocol, where the common bile duct was surgically tied with two knots, was established in rats nearly 40 years ago (Kountouras et al., 1984). The authors established that early time points after BDL surgery (five to ten days) correlated with clinical signs of extrahepatic biliary obstruction, such as immune cell infiltration and marginal bile duct proliferation, thereby first establishing BDL as an accurate model of obstructive cholestasis (Kountouras et al., 1984).

The procedure has since been modified for smaller rodents and is widely used in mice to study bile acid accumulation in the liver. The cholestatic disease phenotype is rapidly established after BDL surgery and can be characterised by the increase of bile acids in the liver and the serum and a decrease in the faeces. Clinical features of disease progression include immune cell infiltration to the liver and liver inflammation, biliary fibrosis, ductular reaction and subsequent liver damage which can be determined with serum transaminase and histology analysis (Tag et al., 2015).

3,5-Diethoxycarbonyl-1,4-Dihydrocollidine Diet (DDC): cholestasis can also be induced chemically. One model of chemically induced cholestasis is feeding with a diet containing 0.1% DDC. DDC is porphyrinogenic and causes cholestasis by blocking small bile ducts with porphyrin plugs, leading to ductular obstruction and subsequent accumulation of bile in the liver (Sta & Leclercq, 2015). The features of cholestasis are ductular reaction, immune cell infiltration and "onion skin-type" fibrosis (Fickert et al., 2007). This "onion skin-type" phenotype of fibrosis is often observed in PSC patients, therefore DDC feeding is a widely accepted pre-clinical model of PSC (Fickert et al., 2014).

α-naphthylisothiocyanate (ANIT): ANIT is a chemical which causes intrahepatic cholestasis. ANIT is transported into hepatocytes where it is conjugated to glutathione and transported to bile acid transporters (Dietrich et al., 2001). Once in bile, ANIT is deconjugated from glutathione and free ANIT damages cholangiocytes, causes hepatocyte necrosis and therefore leads to intrahepatic cholestasis (Mariotti et al., 2019).

Multidrug resistance protein 2 (Mdr2) knockout mouse: the Mdr2 knockout mouse is a genetically modified mouse model of PSC where the mice spontaneously develop biliary inflammation and fibrosis due to the lack of phospholipid transport into bile, which causes a high concentration of unconjugated bile acids in the bile of these animals, resulting in cholangiocyte damage and subsequent cholestasis (Fickert et al., 2014).

Other popular models of cholestasis include 1% LCA feeding (Fickert et al., 2006), the genetically modified non-obese diabetic mice (NOD) NOD.c3c4 which spontaneously develop bile duct inflammation that resembles human PBC (Pollheimer & Fickert, 2015) and rhesus rotavirus type A infection of mice in the timeframe of the first two postnatal days to mimic children's biliary atresia (Mariotti et al., 2019).

In conclusion, work using pre-clinical models has been invaluable in generating insight into the pathogenesis of cholestasis. Using mouse models of cholestasis, the Beraza group has recently identified that SIRT1 overexpression is detrimental during cholestasis (Blokker et al., 2018), which is discussed below.

1.8 SIRT1

1.8.1 SIRT1 overview

SIRT1 is the mammalian homolog of the yeast protein Silent information regulator 2 (Sir2). The SIRT1 protein is encoded by the *SIRT1* gene which is found on chromosome 10 in humans and mice. Human *SIRT1* contains 11 exons and mouse *Sirt1* contains 9 exons. SIRT1 translates to 747

amino acids in humans and 737 amino acids in mice. The molecular weight of SIRT1 is 120kDa and it localises to the nucleus however it has been described to shuttle to the cytoplasm in some cell types (Tanno et al., 2007).

SIRT1 is an NAD^+ dependent protein and HDAC which functions to remove lysine (K) residues (figure 1.9). When SIRT1 removes acetyl groups from proteins or histones, it generates the metabolite O-Acetyl-ADP-ribose (O-A-ADP-ribose) (Tong & Denu, 2010). To date, SIRT1 has been described to have the most targets for deacetylation than any other HDAC and it is involved in many cellular processes (Narita et al., 2018).

Acetylated Lysine + $NAD^+ \rightarrow^{SIRT1} \rightarrow$ Deacetylated lysine + Nicotinamide + O-A-ADP-ribose

Figure 1.9 The deacetylation function of SIRT1. *SIRT1 removes acetyl groups from lysine residues to produce O-Acetyl-ADP-ribose*

Studies in the late 1990s identified that Sir2 promoted longevity in yeast (Kaeberlein et al., 1999) and a subsequent study from the same group identified that NAD^+ is essential for the deacetylation function of Sir2, first describing that Sir2 is activated during low energy states and proposing that this protein is responsible for the beneficial effects of caloric restriction on lifespan extension (Imai et al., 2000). Studies of its effect on the lifespan of other organisms followed. As proposed by the Guarente group, Sir2 was found to increase lifespan of worms (Tissenbaum & Guarente, 2001) and flies through mechanisms involving caloric restriction (Rogina & Helfand, 2004). These findings were later challenged by other groups and the effects in *C. elegans* were found to be an off-target effect of the genetic background of the Sir2 overexpressing strain and in flies, caloric restriction was found to increase lifespan independently of SIRT1 (Burnett et al., 2011). In rats, caloric restriction from weaning to 12 months of age was found to upregulate SIRT1 expression in the liver, brain, visceral fat and kidney tissue (Cohen et al., 2004). This report by the Sinclair group was the first to describe SIRT1 upregulation in response to caloric restriction in mammals and the authors also described that SIRT1 inhibited apoptosis and therefore promoted human embryonic kidney (HEK) 293 cell survival *in vitro* (Cohen et al., 2004). Caloric restriction in mammals is associated with an upregulation of SIRT1 expression in many cell types (Cantó & Auwerx, 2009) however SIRT1 overexpression in mice did not enhance the beneficial effects of caloric restriction (Boutant et al., 2016) highlighting the complexity of SIRT1 in metabolic processes.

Other studies using SIRT1 overexpressing animals found that constitutive SIRT1 overexpression ($SIRT^{oe}$) did not increase lifespan in response to a standard chow diet, however in response to high fat diet feeding $SIRT^{oe}$ animals exhibited healthier ageing than WT controls as

SIRT^{oe} mice had a decreased incidence of spontaneous cancers, less osteoporosis and improved glucose tolerance (Herranz et al., 2010). Additionally, SIRT^{oe} mice were protected from high-fat diet induced hepatic steatosis and insulin resistance (Pfluger et al., 2008). Interestingly, brain specific SIRT1 overexpression promoted youthful physiology and longevity in mice at 20 months, no differences were observed compared to WT controls at younger ages (Satoh et al., 2013).

Since SIRT1 was associated with the beneficial effects of caloric restriction, studies on the role of SIRT1 in metabolic processes followed and are discussed below.

1.8.2 SIRT1 and metabolism

SIRT1 has been described as an important metabolic regulator implicated in many processes including glucose, fatty acid, cholesterol and bile acid metabolism (Chang & Guarente, 2014).

SIRT1 has been described to promote gluconeogenesis through the deacetylation and activation of the transcription factor forkhead box O1 and its target genes in hepatocytes (Frescas et al., 2005). In agreement with the upregulation of gluconeogenesis, SIRT1 was found to repress the opposing pathway of gluconeogenesis, glycolysis. SIRT1 was found to deacetylate and deactivate phosphoglycerate mutase 1, an enzyme in the glycolysis pathway, and inhibit glycolysis (Hallows et al., 2012). Lim and colleagues described that deacetylation of HIF1α by SIRT1 at K674 prevents the binding of the acetylase p300 and represses the transcription of glycolytic genes (J.-H. Lim et al., 2010). However, later reports showed that deacetylation by SIRT1 stabilised HIF1α and its target genes (Laemmle et al., 2012), including the glucose transporter GLUT1, hence SIRT1 promoted glycolysis during hypoxia (Joo et al., 2015).

SIRT1 has been described to promote mitochondrial biogenesis, in liver extracts and cultured hepatocytes, through deacetylation of peroxisome proliferator-activated receptor-gamma coactivator α (PGC1α) (Rodgers et al., 2005) and mitochondrial respiration, in the model cell line HEK293, through deacetylation of CCAAT/enhancer-binding protein α (Zaini et al., 2018). Most recently, SIRT1 was found to promote mitochondrial biogenesis and respiration in adipocytes, again via a mechanism involving PGC1α (Majeed et al., 2021).

SIRT1 enhanced fatty acid synthesis through deacetylation of acetyl CoA synthetase (Hallows et al., 2006). However, SIRT1 promoted fat mobilisation in cultured adipocytes and in mouse white adipose tissue as SIRT1 bound to and inactivated peroxisome proliferator activated receptor γ (PPARγ) and repressed the transcription of genes that promote fat storage (Picard et al., 2004). SIRT1 has a role in hepatic fat and cholesterol metabolism through deacetylation and activation of liver X receptor, a key regulator of cholesterol metabolism (X. Li et al., 2007).

As mentioned in section 1.2, SIRT1 regulates bile acid metabolism through the deacetylation, and deactivation, of FXR (Kemper et al., 2009). SIRT1 has also been described to upregulate FXR activity indirectly, through hepatocyte nuclear factor 1 α, and hepatocyte-specific SIRT1 depletion promoted gall stone formation in mice due to the loss of FXR signaling (Purushotham et al., 2012).

To date, while SIRT1 has been described as an important metabolic regulator, involved in a range of various metabolic processes, the role of SIRT1 in macrophage metabolism has not been elucidated.

1.8.3 SIRT1 and autophagy

As an important energy sensing molecule, SIRT1 has been described to promote several stages of the autophagy process (figure 1.10) (Lee, 2019). SIRT1 promotes autophagy initiation through the activation of AMPK and inhibition of mTOR, as described in section 1.4.6 above. SIRT1 has been described to form a complex with essential components of the autophagy machinery, Atg5, Atg7 and LC3 (Lee et al., 2008). The deacetylation of Beclin 1, a component of the PI(3)K complex, by SIRT1 has also been described to promote autophagosome formation (Sun et al., 2015). Additionally, SIRT1 deacetylated nuclear LC3 allowing it to be transported to the cytoplasm to form autophagosomes during starvation (R. Huang, Xu, Lippincott-Schwartz, et al., 2015). Most recently SIRT1 was described to deacetylate p62, a key protein for autophagosomal degradation, which prevented the degradation of p62 and triggered p62 mediated mTOR activation (L. Feng et al., 2021).

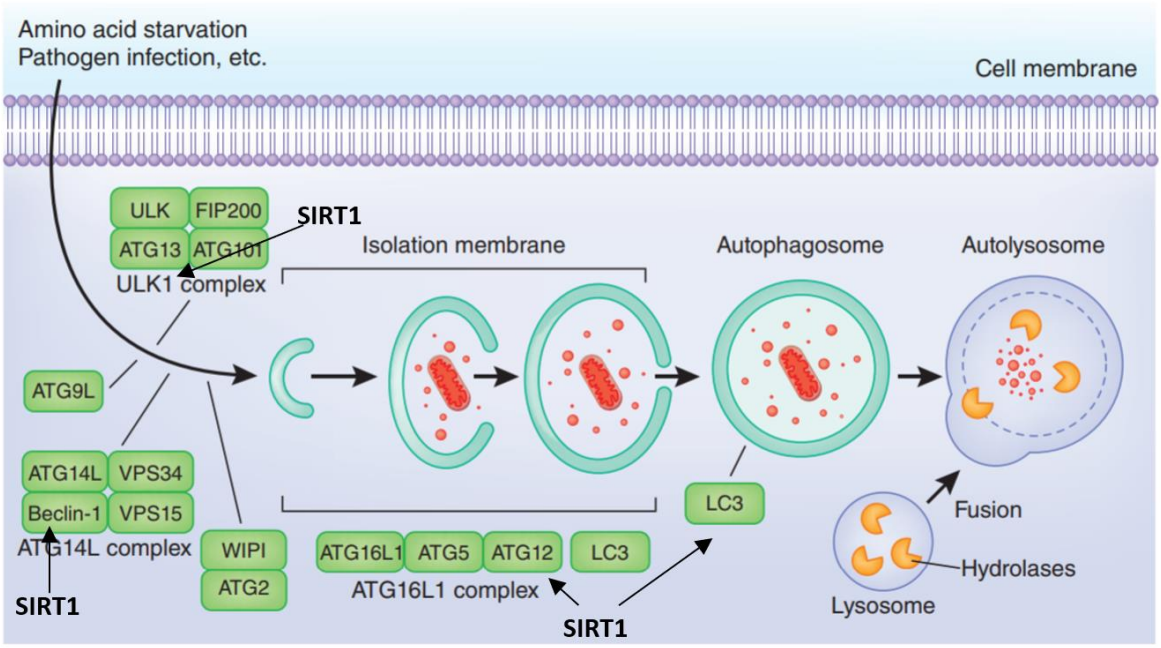

Figure 1.10 SIRT1 and autophagy. *SIRT1 promotes autophagy initiation by promoting AMPK phosphorylation and activation. Activated AMPK phosphorylates ULK1 and allows the formation of the ULK1 complex. Additionally, SIRT1 represses mTOR and represses its inhibitory phosphorylations of ULK1. SIRT1 promotes autophagosome formation by deacetylation of Beclin 1. SIRT1 deacetylates LC3 and allows it to form autophagosomes. Taken from Shibutani et al. 2015.*

Taken together, SIRT1 is involved in many stages of autophagy, from initiation to autophagosome formation and degradation of cargo. Some of the functions of SIRT1 in the autophagy process rely on the interactions between SIRT1 and mTOR, which are discussed in depth below.

1.8.4 SIRT1 and mTOR

Activated during high energy states, the mTOR pathway is involved in many processes including anabolism, inflammation and the activation of the Nlrp3 inflammasome (Linke et al., 2017). In macrophages, mTOR signaling can be initiated by PAMPs binding to TLRs and results in the upregulation of translation (Weichhart et al., 2015). Increased translation is required for the rapid production of pro-inflammatory cytokines needed to sustain the pro-inflammatory response (Lelouard et al., 2007).

The interplay between mTOR and SIRT1 is complex as SIRT1 is active during food scarcity and is involved in catabolic processes, and mTOR is active during periods of amino acid abundance and is known to promote anabolism. To ensure that energy is not wasted for unnecessary biomass synthesis in times of starvation, SIRT1 directly inhibits mTOR by interacting with tuberous sclerosis complex 2 (TSC2) (Ghosh et al., 2010). However, SIRT1 has been described to activate several other components of the mTOR pathway (figure 1.11). Phosphatase and tensin homolog (PTEN) is a phosphatase which dephosphorylates phosphatidylinositol 3-kinase (PI3K), which inhibits mTOR signalling by phosphorylating Akt (Manning et al., 2002). SIRT1 has been described to deacetylate PTEN at K402 (Ikenoue et al., 2008) and inhibit its activity, therefore SIRT1 could indirectly promote the initiation of the mTOR pathway. SIRT1 has been described to deacetylate Akt at K14 and K20 and PDK1 at K495 and K543 (Sundaresan et al., 2011), these deacetylations promote the activation of Akt. Activated Akt phosphorylates TSC2 and this phosphorylation deactivates its function, which is inhibiting mTOR (Manning et al., 2002). Through the deacetylation of Akt and PDK1, SIRT1 could be bypassing its inhibition of TSC2. When mTOR is activated by Akt, it binds to and activates S6 kinase 1 (S6K1) which then phosphorylates and activates its target ribosomal protein S6 (S6) to initiate translation (Weichhart et al., 2015). The deacetylation of S6K1 by SIRT1 at the C terminal

region is essential to allow for the mammalian target of rapamycin complex 1 (mTORC1), a component of the mTOR complex, phosphorylation at T398 (Hong et al., 2014) and in this way, SIRT1 also promotes the activity of the mTOR pathway. Interestingly, the mechanism where SIRT1 deacetylates S6K1, facilitates its phosphorylation by mTORC1 and promotes mTOR mediated anabolism was found to promote the self-renewal of intestinal stem cells in response to caloric restriction (Igarashi & Guarente, 2016). Most recently, SIRT1 and mTOR promoted muscle growth in a rat model of muscle hypertrophy (Gombos et al., 2021). Additionally, the activation of mTOR by SIRT1 upregulated the production of pro-inflammatory cytokines and promoted tissue injury in a mouse model of endotoxin induced lung injury (J. Huang et al., 2017). The role of SIRT1 in inflammation is discussed in the next section.

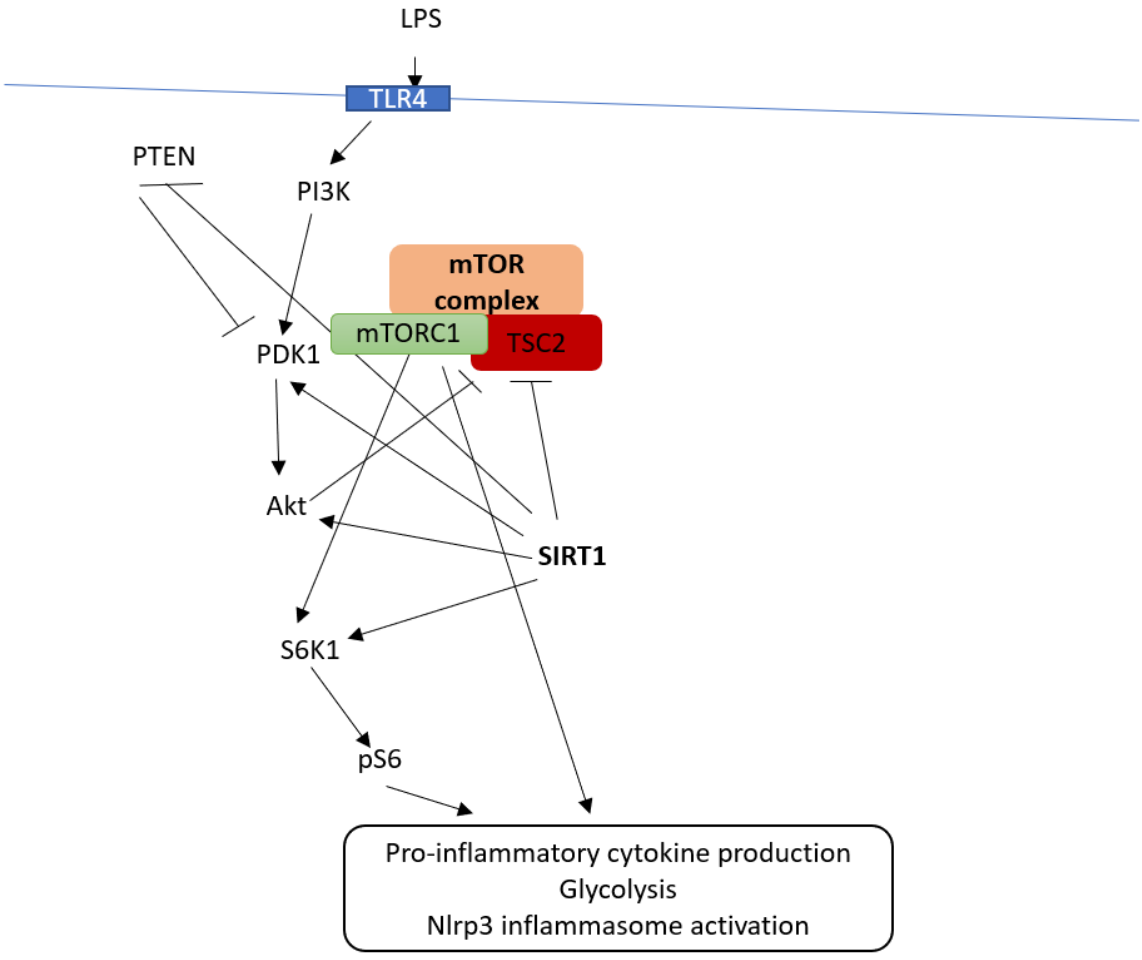

Figure 1.11 SIRT1 and mTOR. *SIRT1 interacts with several components of the mTOR pathway. SIRT1 inactivates mTOR by interacting with TSC2. SIRT1 promotes the activity of the mTOR pathway by interacting with several components of the pathway. SIRT1 inactivates PTEN and allows the activation of PDK1. SIRT1 deacetylates and activates PDK1 and Akt. SIRT1 deacetylates and activates S6K1.*

1.8.5 SIRT1 and inflammation

The role of SIRT1 in inflammation is multifaceted and investigations of SIRT1 in inflammation have generated conflicting reports. While some studies support the anti-inflammatory function of SIRT1 (D. H. Kim et al., 2019; Naito et al., 2007; Park et al., 2017; Roh et al., 2015) we and others have highlighted a detrimental role for SIRT1 in the context of inflammatory diseases (Blokker et al., 2018; J. Huang et al., 2017; Legutko et al., 2011; Smith et al., 2019; Woo et al., 2016). These studies are summarised in table 4.

The anti-inflammatory properties of SIRT1 have been linked to its beneficial effects in promoting healthy ageing (T. F. Liu & McCall, 2013). In 2004, Yeung and colleagues published a mechanistic study where they described that SIRT1 deacetylates the RelA/p65 subunit of the transcription factor NFκB at K310 and thus inhibits the nuclear translocation of NFκB which enables the transcription of pro-inflammatory cytokine genes (Yeung et al., 2004). This mechanism has been applied to propose an anti-inflammatory role for SIRT1 in immune cells, including macrophages (T. F. Liu & McCall, 2013). Interestingly, Yeung et al. did not use macrophages in their study but lung cancer cell lines which had a high endogenous expression level of SIRT1 as well as HEK293 cells which they transiently transfected with a SIRT1 overexpressing plasmid (Yeung et al., 2004).

In a further study, this mechanism was applied to explain the reduction of pro-inflammatory cytokine production when SIRT1 expression was pharmacologically activated with resveratrol in BMDM (Nakamura et al., 2017) however, the activation of SIRT1 by resveratrol has been identified as unspecific and resveratrol was found to activate AMPK independently of SIRT1 (Beher et al., 2009; Pacholec et al., 2010) therefore the reduction of inflammation in BMDM observed by Nakamura and colleagues could be independent of SIRT1.

In accord with the mechanism proposed by Yeung et al. (Yeung et al., 2004), overexpression of SIRT1 in BMDM extracted from $SIRT^{oe}$ animals promoted anti-inflammatory polarization (Park et al., 2017) while BMDM extracted from myeloid cell specific SIRT1 knockout mice ($SIRT^{mye-/-}$) showed hyperacetylation of NFκB which led to its hyperactivation and inflammation (Schug et al., 2010). Knockdown of SIRT1 with shRNA in immortalised macrophage cell lines also resulted in decreased pro-inflammatory cytokine production upon treatment with ethanol and LPS (Shen et al., 2009).

Jia and colleagues uncovered an additional anti-inflammatory function of SIRT1 using *in vitro* work, where they described that pre-treatment of WT mouse peritoneal macrophages with the SIRT1 activator SRT1720 reduced pro-inflammatory cytokine production (Jia et al., 2017). However, SRT1720 is unspecific and has over 100 other targets of activation apart from SIRT1 (Pacholec et al., 2010). In their study, Jia et al. performed mechanistic experiments in the immortalised macrophage cell line RAW264.7 treated with the SIRT1 activator EX527 and showed

that SIRT1 deacetylates and deactivates IFN regulatory factor 8 (IRF8) gene expression, explaining their decrease in inflammation observed in peritoneal macrophages (Jia et al., 2017).

Others found that transfection of a SIRT1 overexpressing plasmid into the RAW264.7 cell line promoted pro-inflammatory macrophage polarization (B. Zhou et al., 2019), highlighting the complex role of SIRT1 in macrophage function.

The various *in vitro* studies are summarised in table 3 below.

Table 3. A summary of cell line studies investigating the role of SIRT1 in inflammation

Study findings	Effect on inflammation	Cell type	Limitations	References
SIRT1 deacetylates and deactivates NFκB	Anti-inflammatory	Transfected HEK293 and lung cancer cell lines	Transient transfection, not immune cells	(Yeung et al., 2004)
Upregulation of SIRT1 expression reduced pro-inflammatory cytokine production	Anti-inflammatory	BMDM treated with resveratrol and LPS for 6 hours	Resveratrol is an unspecific SIRT1 activator	(Nakamura et al., 2017)
Overexpression of SIRT1 reduces pro-inflammatory cytokine production and promotes pro-inflammatory macrophage polarization	Anti-inflammatory	BMDM from $SIRT^{oe}$ mice treated with LPS for 12 hours	Quantification of gene expression of pro-inflammatory cytokines is recommended at earlier time points	(Park et al., 2017)
SIRT1 knockout promotes hyperinflammation	Anti-inflammatory	BMDM from $SIRT^{mye-/-}$ treated with TNFα		(Schug et al., 2010)
SIRT1 knockdown promotes TNFα production	Anti-inflammatory	Rat Kupffer cell line 1 (RKC1) transfected with a SIRT1	Transient transfection	(Shen et al., 2009)

		knockdown plasmid		
SIRT1 deacetylates and deactivates IRF8	Anti-inflammatory	Pharmacological activators and inhibitors in RAW264.7 and WT mouse peritoneal macrophages	SRT1720 is an unspecific activator of SIRT1	(Jia et al., 2017)
SIRT1 overexpression promotes pro-inflammatory cytokine expression	Pro-inflammatory	Transfection of SIRT1 overexpressing plasmid into RAW264.7	Transient transfection	(B. Zhou et al., 2019)

Since SIRT1 has been described as an important inflammatory regulator, a number of studies have focused on the role of SIRT1 in macrophage function, as described above, and, additionally, Imperatore and colleagues found that SIRT1 promoted macrophage self-renewal by regulating G1 to S phase cell cycle progression (Imperatore et al., 2017). Interestingly, despite the research into macrophage SIRT1, no interplay between SIRT1 and the Nlrp3 inflammasome has been identified to date in macrophages. However, several groups have identified that SIRT1 functions as an inhibitor of inflammasome activation in other cell types such as hepatocytes (M. Zhou et al., 2018), vascular endothelial cells (Y. Li et al., 2017) and in lung tissue (Peng et al., 2018).

Despite the *in vitro* evidence supporting the anti-inflammatory functions of SIRT1, the modulation of SIRT1 in animal models of inflammatory diseases has generated conflicting reports. Myeloid-specific SIRT1 depletion promoted liver fibrosis in response to high fat diet feeding and the authors correlated the increased inflammation with the absence of SIRT1 in macrophages, and therefore increased NFκB activity (Roh et al., 2015), agreeing with the established function of SIRT1 to repress NFκB mediated inflammation (Yeung et al., 2004). In accord with this, more recently, SIRT1 depletion by CRISPR-Cas9 in zebrafish resulted in chronic inflammation and reduced lifespan (D. H. Kim et al., 2019). In the study by Park and colleagues, $SIRT^{oe}$ mice were protected from rheumatoid arthritis due to a reduction in the inflammatory response (Park et al., 2017).

Conversely, another group described that $SIRT^{mye-/-}$ mice were protected from collagen induced rheumatoid arthritis and inflammation, suggesting a pro-inflammatory role for SIRT1 (Woo et al., 2016). Supporting the pro-inflammatory function of SIRT1, SIRT1 was found to promote

inflammation in lung dendritic cells in a model of bronchial asthma (Legutko et al., 2011) and lung inflammation in the context of LPS induced lung injury (J. Huang et al., 2017). Additionally, our group has recently described that SIRT1 overexpression promoted macrophage recruitment and inflammatory liver damage in murine models of cholestasis (Blokker et al., 2018) and the McCall group described that SIRT1 promoted inflammation in the context of cardiac sepsis (Smith et al., 2019). *In vivo* studies investigating SIRT1 in inflammation are summarised below in table 4.

Taken together, the role of SIRT1 in inflammation requires further investigation.

Table 4. Summary of in vivo studies investigating the role of SIRT1 in inflammation

Study findings	Effect on inflammation	Disease model	References
Myeloid SIRT1 depletion promoted liver damage in response to high fat diet	Anti-inflammatory	$SIRT^{mye-/-}$ mice fed a high fat diet	(Roh et al., 2015)
SIRT1 depletion resulted in chronic inflammation	Anti-inflammatory	CRISPR-Cas9 deletion of SIRT1 in zebrafish	(D. H. Kim et al., 2019)
SIRT1 overexpression protected mice from arthritis	Anti-inflammatory	$SIRT^{oe}$ mice used for a model of rheumatoid arthritis	(Park et al., 2017)
Myeloid SIRT1 depletion protected mice from rheumatoid arthritis	Pro-inflammatory	$SIRT^{mye-/-}$ mice used for a model of rheumatoid arthritis	(Woo et al., 2016)
Treatment of mice with SIRT1 inhibitors reduced bronchial asthma pathogenesis	Pro-inflammatory	Bronchial asthma model in mice, pre-treated with two SIRT1 inhibitors, sirtinol and cambinol	(Legutko et al., 2011)
SIRT1 promotes lung inflammation in response to LPS	Pro-inflammatory	SIRT1 inhibited with EX-527 and acute lung injury induced by LPS	(J. Huang et al., 2017)
SIRT1 promoted inflammation in cardiac sepsis	Pro-inflammatory	Cardiac inflammation alleviated in a Caecal ligation and puncture model of sepsis in response to SIRT1	(Smith et al., 2019)

		inhibition with EX-527	
SIRT1 overexpression promoted inflammation in models of cholestasis	Pro-inflammatory	BDL and DDC diet on $SIRT^{oe}$ mice	(Blokker et al., 2018)

1.8.6 SIRT1 and liver disease

The role of SIRT1 has been intensively studied in the context of liver disease, showing differential functions depending on the disease context. As mentioned above, the Serrano group described that SIRT1 overexpression protected the mouse liver from hepatic steatosis and metabolic syndrome associated liver cancer in response to high fat diet feeding (Herranz et al., 2010; Pfluger et al., 2008). In accord with this, other groups described that hepatocyte-specific SIRT1 depletion resulted in increased hepatic steatosis (Y. Li et al., 2014; Purushotham et al., 2009). However, other colleagues have found that SIRT1 expression is upregulated in hepatocellular carcinoma and cholangiocarcinoma in human livers (Al-Bahrani et al., 2015; García-Rodríguez et al., 2014; Jang et al., 2012; X. Liu et al., 2020; Pant et al., 2021; B. Zhou et al., 2019).

SIRT1 upregulation contributes to hepatocellular carcinoma tumorigenesis, drives hepatocarcinogenesis through the inhibition of fat mass and obesity associated protein (X. Liu et al., 2020) and hepatocellular carcinoma formation by deacetylation of p62 and inhibition of autophagy (L. Feng et al., 2021). Most recently, SIRT1 overexpression was found to promote cholangiocarcinoma via mechanisms involving cilia loss on cholangiocytes (Pant et al., 2021). Accordingly, SIRT1 silencing was found to suppress tumour growth (Chen et al., 2011; S. Portmann et al., 2013).

Previous work led by Beraza and colleagues used SIRT1 overexpressing mice in a study of liver regeneration (García-Rodríguez et al., 2014) using partial hepatectomy, a procedure where 2/3 of the liver is surgically removed, they showed that the persistent deacetylation of the bile acid receptor FXR by SIRT1 caused dysregulation of bile acid homeostasis which contributed to hepatocellular carcinoma.

1.8.7 SIRT1 in cholestasis

The studies on the role of SIRT1 in cholestasis to date have focused on its function in the regulation of bile acid metabolism through the deacetylation of the bile acid receptor FXR (Blokker et al., 2018; Kulkarni et al., 2016; Purushotham et al., 2012).

The nuclear receptor FXR is the key negative regulator of bile acid metabolism which is activated by bile acids and functions to repress the transcription of the enzymes which promote

bile acid synthesis from cholesterol (Fiorucci et al., 2018). The regulation of FXR signaling is a dynamic process, where FXR is activated by acetylation by p300 (Fang et al., 2008) and deactivated by deacetylation by SIRT1 (Kemper et al., 2009), which must be tightly controlled to maintain signaling. Constitutive acetylation of FXR causes decreased activity and persistent deacetylation by SIRT1 leads to the proteasomal degradation of FXR (Kemper et al., 2009) and its loss can lead to unregulated bile acid synthesis, accumulation of bile acids in the liver, and, ultimately, cholestasis, as was shown by Purushotham and colleagues when they demonstrated that hepatocyte-specific SIRT1 depletion causes gallstone formation due to the loss of FXR signaling in the liver (Purushotham et al., 2012).

Several pre-clinical studies found that pharmacological activators of SIRT1 are protective in cholic acid feeding (Kulkarni et al., 2016), ANIT administration (L. Yu et al., 2017; Zhao et al., 2019) and poly I:C injection (Y. Li et al., 2020) models of cholestasis through the restoration of FXR signaling which is inhibited during cholestasis (Kulkarni et al., 2016; L. Yu et al., 2017; Zhao et al., 2019). However, SRT1720, the SIRT1 activator used by Kulkarni, Yu and Li (Kulkarni et al., 2016; Y. Li et al., 2020; L. Yu et al., 2017) was proven to not be a specific SIRT1 activator (Pacholec et al., 2010) and celastrol, used by Zhao and colleagues is also known to activate many other molecules such as mTOR, JNK, NFκB and p53 (J. Shi et al., 2020) therefore the beneficial effects described in these studies could have occurred independently of SIRT1 activation.

Recent work from the Beraza laboratory identified a detrimental role for SIRT1 in cholestasis (Blokker et al., 2018). We showed that SIRT1 expression was upregulated in liver tissue of patients from two diseases of obstructive cholestasis, PSC and PBC and in the pre-clinical model of obstructive cholestasis, BDL, in agreement with what was reported by the Boyer group in mouse liver in response to BDL (Kulkarni et al., 2016). To investigate the biological relevance of SIRT1 upregulation during obstructive cholestasis, we performed BDL and fed a 0.1% DDC diet to mice which constitutively overexpress SIRT1. We described that SIRT1 overexpression had a detrimental effect in the cholestatic liver as SIRT1 overexpressing mice had increased inflammation characterised by increased macrophage infiltration and pro-inflammatory cytokine production in the organ (Blokker et al., 2018).

$SIRT^{oe}$ mice had increased accumulation of bile acids in the liver due to increased bile acid synthesis which occurred in these mice because SIRT1 promoted the degradation of FXR, and in line with this increased bile acid load in the $SIRT^{oe}$ livers, we saw increased liver damage, inflammation and fibrosis in these animals (Blokker et al., 2018).

To investigate whether the modulation of liver SIRT1 could be used as a therapeutic approach to treat cholestasis, we characterised the effect of hepatocyte specific SIRT1 depletion in

our models of obstructive cholestasis. We found that depleting SIRT1 from hepatocytes protected the hepatocytes from apoptotic cell death in response to BDL *in vivo* and bile acids *in vitro* (Blokker et al., 2018). This decrease in apoptosis provided transient protection from cholestasis three days after BDL, which was lost by day seven of the procedure. There were no differences observed in liver inflammation or macrophage recruitment between WT and hepatocyte-SIRT1 knockout mice after BDL or DDC feeding (Blokker et al., 2018).

In agreement with previous work, we showed that SIRT1 is essential to maintain FXR signaling (Kulkarni et al., 2016; L. Yu et al., 2017; Zhao et al., 2019), the loss of which promotes pathogenesis during cholestasis. Previous work by Beraza and colleagues had shown that the therapeutic UDCA homologue 24-norursodeoxycholic acid (NorUDCA) lowered SIRT1 expression of $SIRT^{oe}$ animals to the SIRT1 level of WT animals and exhibited beneficial effects in response to partial hepatectomy via the restoration of FXR signaling (García-Rodríguez et al., 2014). Thus, we investigated the effect of NorUDCA on cholestatic $SIRT^{oe}$ animals and found that it lowered, but not ablated, SIRT1 expression in $SIRT^{oe}$ mice after BDL and this resulted in decreased inflammation and liver damage. Therefore, we concluded that the restoration of SIRT1 expression to a moderate amount, not complete inhibition, is required to protect the liver from bile acid induced damage (Blokker et al., 2018).

Overall, our work showed that, to preserve liver health, SIRT1 expression must be finely tuned in the liver as overexpression or hepatocyte-specific depletion of SIRT1 led to liver damage during cholestasis (Blokker et al., 2018). However, we did not investigate the biological relevance of SIRT1 overexpression in macrophage function during cholestatic liver diseases, which is the focus of this thesis.

1.9 Thesis hypothesis and aims

1.9.1 Hypothesis

In this thesis, we hypothesise that SIRT1 overexpression promotes inflammation by controlling macrophage activation via modulating macrophage metabolism and autophagy. The overall aim of this thesis is to characterise the role of SIRT1 in macrophage function in the liver in response to cholestasis and bacterial endotoxin.

1.9.2 Specific aims of this thesis

1. Determine that presence of the microbiome associates with increased liver damage and macrophage infiltration in response to cholestatic liver injury

2. Characterise the effect of SIRT1 overexpression in macrophage function in response to endotoxin challenge
3. Define the role of myeloid SIRT1 overexpression in the pathogenesis of cholestasis
4. Define the role of myeloid cell specific SIRT1 depletion during cholestasis

Chapter 2 - Materials and methods

All experimental procedures were performed by the PhD candidate unless otherwise stated.

2.1 Animal techniques

2.1.1 Animals

All experimental procedures were performed in 8-12 week old male mice at the Disease Modelling Unit (University of East Anglia, UK). All experiments were approved by the Animal Welfare and Ethical Review Body (AWERB, University of East Anglia, Norwich, UK). All procedures were carried out following the guidelines of the National Academy of Sciences (National Institutes of Health, publication 86-23, revised 1985) and were performed within the provisions of the Animals (Scientific Procedures) Act 1986 (ASPA) and the LASA Guiding Principles for Preparing for Undertaking Aseptic Surgery (2010) under UK Home Office approval (70/8929). All animals were maintained at the Disease Modelling Unit (University of East Anglia, UK) and were fed the standard chow diet.

Germ free (GF) mice were of a C57/B6J background and were maintained in the GF facility at the Disease Modelling Unit (University of East Anglia, UK). GF mice were conventionalised with the microbiome from specific pathogen free (SPF) WT mice by oral gavage of SPF WT faecal matter three weeks prior to experimentation (GF+WT). Oral gavage was performed by Dr A Goldson or Dr A Brion.

SIRT1 overexpressing mice ($SIRT^{oe}$) were generated on a C57/B6J background as previously described (Herranz et al., 2010). Briefly, the murine *Sirt1* gene was cloned into a Bacterial Artificial Chromosome (BAC) vector (Pfluger et al., 2008). The mice were kindly provided by Professor Manuel Serrano (Spanish National Cancer Research Centre; CNIO, Spain).

Myeloid lineage-specific SIRT1 KO ($SIRT^{mye-/-}$) animals were generated on a C57/B6J background by crossing mice containing the Lysozyme Cre promoter (a generous gift from Professor Tom Wileman) with mice containing floxed sites flanking exon four of the SIRT1 gene (B6;129-Sirt1tmYgu/J; Jackson laboratories). Animals carrying the loxP-flanked SIRT1 alleles ($SIRT^{mye+/+}$) were used as WT littermate controls compared to LysMCre-SIR1 floxed mice.

2.1.2 Genotyping

2.1.2.1 Genomic DNA extraction

Genomic DNA was extracted from ear notches. Ear notches from mice were incubated with shaking at 56°C overnight in 750µL of Solution A (50mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8, 100mM ethylenediaminetetraacetic acid (EDTA) pH 8, 100mM sodium chloride (NaCl), 1% sodium dodecyl sulphate (SDS)) and 10µL of 20mg/mL Proteinase K (Merck). The following day, samples were centrifuged for 10 minutes at 12000 x rpm at room temperature. Supernatant containing DNA was mixed with an equal volume of molecular grade isopropanol (Merck) by vortexing. DNA was precipitated by centrifugation at 12000 x rpm at 4°C for 10 minutes. Pellet was washed twice with molecular grade 70% ethanol by centrifugation at 12000 x rpm at 4°C for 10 minutes, air dried and resuspended in 150µL of molecular grade water (Merck).

2.1.2.2 Genotyping of SIRT^{oe} mice

SIRT^{oe} mice were genotyped by quantitate polymerase chain reaction (qPCR). The master mix contained 1x TaqMan Universal PCR master mix (Applied Biosystems), 0.4µM SIRT1 TGM forward primer, 0.4µM SIRT1 TGM reverse primer, 0.2µM SIRT1 probe, 0.4µM Actin forward primer, 0.4µM Actin reverse primer, 0.2µM Actin probe (sequences below in table 5). 10ng of genomic DNA was amplified using a Vii7 cycler (Applied Biosystems). The polymerase chain reaction (PCR) cycling conditions were 95°C for 10 minutes, 40 cycles of (95°C for 30 seconds, 60°C for 1 minute), 4°C hold. The number of SIRT1 copies was determined in Excel using the following equation:

2*2^(-deltaCt) where DeltaCt= VIC_Ct (SIRT) – FAM_Ct (Actin)

Table 5. Primers and probes used for genotyping SIRT^{oe} mice

Name	Sequence
MGB_Actin B_TGM_probe (6FAM)	GTACACAGTATTGGGAACC
MGB_SIRT1_TGM_probe (VIC)	ATCGTTACCCAATCTG
SIRT1_TGM_F1	TGGCGAGCTGGATGATGAG
SIRT1_TGM_R1	GCCGTATTGGTCCATCCATCTTGAGA
TGM_ActinB _F1	GACCACGTTCATAAGCACTTGTTG
TGM_ActinB _R1	AAGACCCAGAGGCCATTGAG

2.1.2.3 Genotyping of SIRT$^{mye-/-}$ mice

SIRT$^{mye-/-}$ were genotyped using PCR followed by agarose gel electrophoresis. The master mix contained 1x mi-Hot Taq mix (Metabion) 20pmol/µL forward primer, 20pmol/µL reverse primer (sequences in table 6 below), 0.5µL genomic DNA. The PCR cycling conditions were 94°C for 2

minutes, 35 cycles of (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds), a final extension step at 72°C for 7 minutes and a hold stage at 4°C. The PCR product was mixed with blue loading dye (NEB) and was loaded on a 1% agarose gel. The expected band size of the mice carrying two copies of the SIRT1 floxed gene was 750bp. For mice containing the Lysozyme cre promoter, the expected band was 700bp and for WT mice the expected band was 350bp.

Table 6. Primers used for genotyping SIRT$^{mye-/-}$ mice

Primer name	Sequence
oIMR7909 (SIRT Floxed forward)	GGT TGA CTT AGG TCT TGT CTG
oIMR7912 (SIRT Floxed reverse)	CGT CCC TTG TAA TGT TTC CC
oIMR3066 (Lyz2 mutant)	CCC AGA AAT GCG AGA TTA CG
oIMR3067 (Lyz2 common)	CTT GGG CTG CCA GAA TTT CTC
oIMR3068 (Lyz2 wild type)	TTA CAG TCG GCC AGG CTG AC

2.1.3 Induction of cholestasis *in vivo*

In SPF mice, cholestasis was induced by ligating the common bile duct under anaesthesia (Tag et al., 2015). BDL was performed by Dr N. Beraza on 8-12 week old male mice. Mice were sacrificed by cardiac puncture under terminal anaesthesia 7 days after the procedure.

In GF mice and GF+WT, cholestasis was induced by administration of 100mg/kg ANIT by oral gavage 48 hours prior to sacrifice. For the vehicle, corn oil was used for oral gavage 48 hours before the sacrifice. Administration was carried out by Dr A Goldson or Dr A Brion. Mice were sacrificed by cardiac puncture under terminal anaesthesia.

2.1.4 Septic liver injury

Septic liver injury was induced by intraperitoneal (i.p) injection of lipopolysaccharide (LPS) (Escherichia coli E055:B5, Merck) at a dose of 20mg/kg of body weight for up to 14 hours or by intraperitoneal administration of 35mg/kg LPS and 700mg/kg D-galactosamine (Merck) (LPS/GalN) for up to 6 hours.

2.1.5 PepCboy allograft model

To assess the effect of SIRT1 overexpression on macrophage function in cholestasis, peptidase C variant B (PepCboy) mice were transplanted with bone marrow from WT or SIRT^{oe} animals. Transplantation was carried out by Dr S. Rushworth's laboratory at the University of East Anglia (Norwich, UK). The haematopoietic stem cell population of the PepCboy mice was depleted using 25mg/kg/day of busulfan for 3 days prior to transplantation. Bone marrow of WT or SIRT^{oe}

animals was isolated and 2 x 10^5 cells were injected into the tail vein of PepCboy animals. Transplanted mice were termed PEPC+WT and PEPC+SIRT^{oe}. Four weeks after engraftment BDL was performed by Dr N. Beraza.

2.1.6 Tissue collection

Mice were sacrificed under non-recovery anaesthesia and blood was collected by cardiac puncture. Mouse tissues were collected and were snap frozen in liquid nitrogen for molecular biology analysis, fixed in 10% formalin (Merck) for histology analysis or kept on ice in phosphate buffered saline (PBS) supplemented with 2% foetal bovine serum (FBS) until processing for flow cytometry analysis.

2.2 Histology and imaging techniques

2.2.1 Tissue processing for histology

Formalin fixed liver tissues were sequentially dehydrated using the Leica tissue processor following the manufacturer's instructions. Dehydrated livers were embedded in paraffin and sectioned at a thickness of 4µm using a Leica microtome.

2.2.2 Deparaffinisation of liver sections

Liver sections were deparaffinised for 10 minutes in histoclear (Merck) and were hydrated sequentially in 100%, 80% and 70% ethanol for 2 minutes. Samples were then hydrated for 5 minutes in distilled water.

2.2.3 Haematoxylin and Eosin staining

Haematoxylin and eosin (H and E) staining was carried out for the assessment of liver parenchymal cells. Haematoxylin stains nuclei blue and eosin stains the cytoplasm pink. Immune cell infiltrates and necrotic areas can be detected using this staining method (B. Portmann & Zen, 2012). After deparaffinisation as described above, liver samples were stained in haematoxylin for 5 minutes and rinsed for 5 minutes in running water. Samples were incubated for 15 seconds in 1% hydrochloric acid diluted in 70% ethanol and rinsed in distilled water. Tissues were then stained in eosin for 30 seconds. Samples were then dehydrated sequentially for 2 minutes in 70%, 80% and 100% ethanol and histoclear (Merck) for 10 minutes prior to mounting in DXP mounting solution (DXP).

2.2.4 Sirius red staining

Sirius red staining was performed for the visualisation of collagen deposition during bile acid metabolism disturbances in the liver. Sirius red stains type I collagen fibres red and the rest of the cells a pale yellow (Junqueira et al., 1979). Liver sections were deparaffinised as described above. Samples were stained in 0.01% Fast green FCF solution (104022, Merk) prepared in saturated picric aqueous solution for 15 minutes. Samples were then stained in 0.04% Fast green FCF/0.1% Sirius red in saturated picric aqueous solution for 15 minutes. Liver sections were then dehydrated in 100% ethanol for 5 minutes, histoclear for 10 minutes and were mounted using DXP mounting solution.

2.2.5 Cytokeratin 19 immunohistochemistry

Ductular reaction can be visualised in a sample from a cholestatic liver by immunohistochemical staining for the cholangiocyte marker cytokeratin 19 (CK19) (Pollheimer et al., 2014). Liver sections were deparaffinised as described above. Endogenous peroxide activity was blocked using 3% hydrogen peroxide (Merck) in methanol (Fisher scientific) for 10 minutes at room temperature. Slides were washed three times in PBS. Antigen retrieval was performed by heating the slides in a microwave for 10 minutes in 1mM EDTA solution. Slides were cooled and washed three times in PBS. Non-specific antibody binding was blocked using blocking buffer (10% goat serum (Merck), 0.1% Triton X100 (Merck), PBS) for 30 minutes at room temperature. Slides were incubated with CK19 primary antibody (TROMA III, Developmental Studies Hybridoma Bank, University of Iowa), diluted 1:200 in antibody diluent (Dako) overnight at 4°C in a humid chamber. Slides were washed three times in PBS and incubated for 60 minutes at room temperature with a 1:200 dilution of anti-rat secondary horseradish peroxidase (HRP) conjugated antibody (Abcam) diluted in antibody diluent (Dako). Slides were washed three times in PBS and developed with chromogen (Dako) solution. Slides were counterstained with haematoxylin and dehydrated as described above in 2.2.3 and mounted in DXP mountant.

2.2.6 Alpha smooth muscle actin immunofluorescence

α-smooth muscle actin (α-SMA) is a marker of hepatic stellate cells which have differentiated into collagen-producing myofibroblasts (Carpino et al., 2005). α-SMA was detected in liver tissue by immunofluorescence staining. Liver sections were dewaxed as described above and washed once with PBS. Antigens were retrieved by microwaving the slides for 10 minutes in sodium citrate buffer (0.053% trisodium citrate dihydrate and 0.17% citric acid, pH 6). Slides were cooled and washed with PBS three times. Endogenous peroxidase was blocked using 3% hydrogen

peroxide in PBS at room temperature. Slides were washed three times with PBS and blocked in blocking buffer (5% goat serum in PBS). α-SMA Cy3 conjugated primary antibody (Merck), 1:200 dilution in antibody diluent (Dako), was added to the samples overnight in a humid chamber at 4°C. Slides were washed in PBS and were mounted in Vectashield antifade mounting media with DAPI (Vector Laboratories).

2.2.7 Imaging and image analysis

H and E, Sirius red and CK19 samples were imaged using the Olympus BX60 microscope using the 4x objective. α-SMA slides were imaged using the Zeiss Axio Imager M2 fluorescent microscope using the 10x objective. 5-10 fields per sample were imaged and analysed. Image analysis was performed using the Fiji software (ImageJ) and is represented as the percentage of stained area relative to total area per field.

2.3 Flow Cytometry

Immune cells were isolated from mouse liver. The right lateral lobe was removed and placed on ice in 2% FBS/PBS until isolation. The liver was digested in 0.25mg/ml collagenase D (Roche) at 37°C for 30 minutes. The tissue was homogenised by passing it through a 3mL syringe. The homogenate was passed through a 70µM cell strainer. Samples were centrifuged at 1300 x rotations per minute (rpm) at 4°C for 10 minutes. The pellet was resuspended in 35% Percol (GE) in 2% FBS/PBS and the samples were centrifuged at 1700 x rpm for 40 minutes at room temperature. Pellet was resuspended in red blood cell lysis buffer (Biolegend) for 3 minutes. Cells were washed with 2% FBS/PBS and centrifuged at 1300 x rpm for 10 minutes at 4°C. Cells were stained using staining solution (2% goat serum (Merck), 2% human serum (Merck), 2% mouse serum (Merck), 2% bovine serum albumin (Merck) in PBS), using the following antibodies at a 1:200 dilution: CD45-APC-Cy7 (BD), CD11b-PE (BD), F4/80-FITC (Myltenyi), and Ly6C-Pacific blue (MACS) for at least 30 minutes. Cells were washed with 2% FBS/PBS and centrifuged at 1300 x rpm for 10 minutes at 4°C. Flow cytometry was carried out using BD LSRFortessa. Analysis was performed using FlowJo software.

2.4 Bile acid extraction

Bile acids were extracted from the liver and serum of cholestatic animals after BDL. For bile acid extraction from liver, 25mg of liver tissue was homogenised in 500µL of 90% methanol in the Precellys®24 homogeniser, using zirconium oxide beads. For bile acid extraction from serum, 15µL

of serum added to 485µL of 90% methanol and mixed by vortexing. Samples were then centrifuged at 12000 x rpm for 10 minutes. The pellets were discarded and 25µL of internal standard was added to the samples. Sample clean-up was performed using the Oasis PRiME HLB µElution Plate (Waters). Mass spectrometry (MS) was performed by Dr M Philo using the Agilent 1260 binary HPLC coupled to an AB Sciex 4000 QTrap triple quadrupole mass spectrometer as previously described in (Isaacs-Ten et al., 2020).

2.5 *In vitro* studies

2.5.1 Bone marrow cell isolation

Bone marrow is a site of haematopoiesis and contains monocytes which can be differentiated into macrophages. Male WT and SIRT^{oe} mice were sacrificed and femur bones were collected for the isolation of bone marrow cells. To obtain the bone marrow, bones were flushed with Roswell Park Memorial Institute (RPMI) medium (Merck) with 10% FBS (Merck) and 50 units/ml Penicillin and 50 units/ml Streptomycin (Merck) onto a cell strainer. The bone marrow cell suspension was centrifuged at 1000 x rpm for 5 minutes. Cells were resuspended complete RPMI and 30ng/ml macrophage colony stimulating factor (M-CFS). Cells were incubated at 37°C in a 5% carbon dioxide environment for four days. The media was replaced with fresh RPMI and M-CFS and the cells were incubated for a further three days. Macrophages were collected and seeded for experiments.

2.5.2 Bone marrow experiments

BMDM were seeded at 1x10^6 per well in 6 well plates and were incubated at 37°C in a 5% carbon dioxide environment overnight. Cells were serum starved for at least four hours before treatment with 100ng/ml LPS and/or 100µM of the bile acids CDCA (Merck) or DCA (Merck) for up to 24 hours.

For immunofluorescence imaging, BMDM were grown on glass coverslips and were fixed with ice cold 30% acetone 70% methanol for 15 minutes at 4°C. Cells were washed and blocked with 5% goat serum, 0.01% Triton X-100, 1% BSA in PBS. Cells were incubated overnight with α-p65 (Cell Signaling Technology) in 1% BSA in PBS. Cells were washed and incubated for 1 hour with goat anti-rabbit Alexa Fluor 568 secondary (ThermoFisher Scientific) in 1% BSA in PBS. Cells were washed and mounted using Vectashield Antifade mounting medium with DAPI (Vector). Slides were imaged using the Zeiss Axio Imager M2 microscope using the 40x objective.

2.5.3 Phagocytosis assay

BMDM were seeded on cover slips in 24 well plates at 40000 cells per well and were incubated at 37°C in a 5% carbon dioxide environment overnight. Cells were treated with zymosan A Alexa Fluor 594, beads according to the manufacturer's instructions (ThermoFisher Scientific). Cells were fixed in ice cold methanol for 7 minutes, washed three times with PBS and blocked for 30 minutes using blocking buffer (5% goat serum, 0.3% Triton X-100 in PBS). Cells were stained overnight at 4°C with LC3 A/B antibody (Cell Signaling) at a 1:500 dilution. Cells were washed three times in PBS. Cells were stained with a goat anti-rabbit Alexa Fluor 488 secondary (ThermoFisher Scientific) at a 1:1000 dilution for 1 hour at room temperature. Cells were washed three times in PBS and mounted using Vectashield Antifade mounting medium with DAPI (Vector). Slides were imaged using the Zeiss Axio Imager M2 microscope and the 100x objective.

2.5.4 Krebs cycle metabolite extraction

10x10^6 bone marrow cells were seeded in 10 cm dishes to differentiate as described above. After differentiation, macrophages were serum starved for four hours and treated with 100ng/mL LPS for three and six hours. Cells were washed twice with ice cold 0.9% NaCl and detached by scraping. Cells were centrifuged at 1200 x g for 10 minutes at 4°C. The pellet was resuspended in 500µL of 0.3mM perchloric acid and incubated on ice for 10 minutes. Samples were centrifuged at 12000 x g for 10 minutes and supernatant was used for Krebs cycle metabolite analysis by liquid chromatography with tandem mass spectrometry (LC-MS/MS) using the Agilent 1200 series LC 6490 Triple Quad LC-MS mass spectrometer (Agilent Technologies, CA, US). LC-MS/MS was performed by Dr S. Saha as described in (Al Kadhi et al., 2017).

2.5.5 Hepatocyte extraction and culture

Hepatocytes are the most abundant cell type in the liver and comprise 85% of the liver's cells. Hepatocytes were isolated from GF and GF+WT mice by perfusion of the liver through the portal vein and further digestion with collagenase I (Worthington). Perfusion was performed by Dr N. Beraza. Hepatocytes were washed, pelleted by centrifugation at 1000 x rpm for 5 minutes, and plated at a density of 500000 cells per well of a 6 well dish in 6 well plates pre-coated with rat collagen type I (BD Bioscience) in Minimum Essential Eagle media (MEM), supplemented with 10% FBS, 50 units/ml Penicillin and 50 units/ml Streptomycin (Merck), and 2mM Glutamine (Merck). Cells were left to attach for two hours. Cells were washed in PBS and the media was changed to serum free MEM with 50 units/ml Penicillin and 50 units/ml Streptomycin (Merck), and 2mM

Glutamine (Merck). The following morning, cells were treated with 125µM CDCA, 125µM DCA, 250µM GCA (Merck), 500µM TCA (Merck) for up to four hours.

2.6 Molecular Biology Techniques

2.6.1 RNA extraction

RNA was extracted from snap frozen liver tissues. Snap frozen tissues were homogenised in Qiazol lysis reagent (Qiagen) in a Precellys®24 homogeniser, using zirconium oxide beads. Chloroform (Merck) was added to induce phase separation and samples were centrifuged at 12000 x rpm for 10 minutes, in a pre-chilled centrifuge. The aqueous phase containing RNA was collected and RNA was precipitated using isopropanol. To obtain an RNA pellet, samples were centrifuged at 12000 x rpm for 10 minutes, at 4°C. The RNA pellet was washed twice in 70% ethanol, by centrifugation at 12000 x rpm for 10 minutes at 4°C. Pellets were air dried and resuspended in an appropriate amount of RNase free water.

RNA from cells was extracted as above, omitting the homogenisation step.

2.6.2 Reverse transcription

1µg of RNA was treated with DNase I (Roche) according to the manufacturer's instructions. Following DNase treatment, cDNA synthesis was performed using M-MLV reverse transcriptase (Invitrogen), following the manufacturer's instructions.

2.6.3 Gene expression analysis by quantitative polymerase chain reaction (qPCR)

Synthesised cDNA, diluted 1/20 with water, was used for qPCR to analyse the expression of various genes (table 7). SYBR Select Master Mix (Applied Biosystems) was used according to the manufacturer's instructions. The PCR cycling conditions were 95°C for 3 minutes, 40 cycles of (95°C for 15 seconds, 60°C for one minute), followed by a melt curve.

Table 7. Primers used for qPCR

Primer name	Forward sequence	Reverse sequence
Oatp	CCTTTGTTTAGCCCTGTCACAC	ATGGGTCCAACAAGCTTTGC
Ntcp	GGTAAAACAGCATGCCAGCG	CCCATGAGAACAACGCCAGA
Mrp2	AGAAGTGCCCTGGAAATCACG	ACACAACGAACACCTGCTTG
Mrp3	ATGCGGGACTTGCCTAGATG	GTGGCTTTGAACTGGCTGTG
Mrp4	GGTTGGAATTGTGGGCAGAA	TCGTCCGTGTGCTCATTCAA
Mdr2	GATGGATCTTGAGGACAGCGA	GAGCTATGGCCATGAGGGTG
BSEP	CTCCTGTGCTTGGCACATCA	ATCGCCGTCATGTCACAAGG
Cyp2b10	TCCAGGGCTCCAAGGCATGT	ACAGAGTCCATTAGCACAGATCCCA

Cyp3a11	ACCTGGGTGCTCCTAGCAATC	AAGGAGAGGCTTTGACCATC
Cyp2c70	AGTATGGCCCTGTGTTTACTGT	GCCTTGGCTGGTTCTACTGAG
Ugt1a1	CCTTCTGTTGTGTGTTCGG	CCGTCCAAGTTCCAACCAAAG
Ugt1a2	TGATGTGATCTTAACAGACCCCA	GTCAGAAAGCCTTGTGAGTAGG
Gst3	GAATGGAGCCTATCCGGTGG	GCATGGCGGTACAAGCCTTT
Gxp1	CCACCGTGTATGCCTTCTCC	AGAGAGACGCGACATTCTCAAT
Gst4	ACTTTAATGGCAGGGGACGG	CAGCGAGGTAGCTGAGGATG
TLR4	GCCTTTCAGGGAATTAAGCTCC	AGATCAACCGATGGACGTGTAA
Nlrp3	ACACGAGTCCTGGTGACTTTG	GGGCTTAGGTCCACACAGAAA
Caspase 1	GGCACATTTCCAGGACTGACTG	GCAAGACGTGTACGAGTGGTTG
IL1β	GAAATGCCACCTTTGACAGTG	TGGATGCTCTCATCAGGACAG
TNFα	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL6	TACCACTTCACAAGTCGGACCG	CTGCAAGTGCATCATCGTTGTTC
IFNγ	CTTCAGCAACAGCAAGGC	CGAATCAGCAGGGACTCC
CCL2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
NOS2	TGCGACAGCAGGAAGGCAGC	CTGGCTCGCTTTGCCACGGA
CCR1	GCCAAAAGACTGCTGTAAGAGCC	GCTTTGAAGCCTCCTATGCTGC
CCR2	GCTGTGTTTGCCTCTCTCTACCAG	CAAGTAGAGGCAGGATCAGGCT
CCR5	GTCAGAACGGTCAACTTTGGG	GTGGGAAAACGAGGACTGCA
CX3CR1	GAGCATCACTGACATCTACCTCC	AGAAGGCAGTCGTGAGCTTGCA
SIRT1	CCAGACCCTCAAGCCATGTT	GACAACGAGACGGCTGGAA
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGATGATGTTC
TBP	GAAGCTGCGGTACATTCCAG	CCCCTTGTACCCTTCACCAAAT
HPRT1	GTTGGGCTTACCTCACTGCT	TAATCACGACGCTGGGACTG

2.7 Protein analysis techniques

2.7.1 Whole cell extract preparation

BMDM cells were washed in ice cold PBS and were scraped in radio immunoprecipitation assay (RIPA) buffer (50mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) pH 8, 150mM sodium chloride (NaCl), 2mM ethylenediaminetetraacetic acid (EDTA), 1% Igepal 630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1mM phenylmethylsulphonyl fluoride (PMSF) and PhosStop tablets (Merck)). Lysed cells were centrifuged for 10 minutes at 12000 x rpm in a pre-chilled centrifuge. Supernatants were collected and used for protein analysis.

Snap frozen mouse tissues were homogenised the appropriate lysis buffer in a Precellys®24 homogeniser, using zirconium oxide beads. Homogenised samples were centrifuged at 12000 x rpm for 10 minutes at 4°C. The protein containing supernatant was collected and stored at -20°C. For western blotting, liver tissue was lysed in RIPA buffer. For caspase 3 assay, liver tissue was lysed in Caspase 3 buffer (10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 0.1% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 2mM EDTA, 5mM dithiothreitol (DTT)).

Protein concentration was determined using a Bradford assay (Bio-Rad) following the manufacturer's instructions.

2.7.2 Western Blotting

Western blotting was used for protein analysis. Proteins were denatured by heating at 95°C with Laemmli sample buffer and 2-mercaptoethanol reducing agent. The Bio-Rad Mini Protean PAGE system was used to resolve 8-15% acrylamide gels. Electrophoresis was carried out at 100V for one hour in running buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 0.1% SDS). After electrophoresis, resolved proteins were transferred from the gel onto a 0.2µm nitrocellulose membrane using the Bio-Rad wet transfer system. The transfer was carried out for two hours at 0.5mA in transfer buffer (25mM Tris-HCl pH 8.3, 192mM glycine, 20% methanol). Membranes were stained with ponceau solution to verify transfer efficiency. Membranes were blocked with blocking solution (tris buffered saline (TBS) and 0.1% tween-20 (TBS-T), 5% non-fat dry milk, 1% BSA) for 30 minutes at room temperature. Membranes were washed for five minutes in TBS-T. Membranes were incubated with appropriate primary antibodies at 4°C overnight, with rotation. Membranes were probed with interleukin 1β (Santa Cruz Biotechnologies sc-32294), Caspase 1 (2225), PARP1 (9542), phosphorylated (p)-pS6 (S235,236) (4857), pAMPK (T172) (2535), LC3 A/B (2775S), SIRT1 (D1D7) (Cell Signaling Technologies), pJNK (Thr 183/Tyr 185) (Thermofisher scientific 44682G). β-actin, GAPDH (ab8245), or α-Tubulin (ab18251) (Abcam) were used as loading controls. After the incubation, membranes were washed four times with TBS-T and were incubated with anti-rabbit IgG-HRP-linked or anti-mouse IgG-HRP linked secondary antibodies (mouse 7076S) (rabbit 7074S) (Cell Signaling Technologies) for one hour. Membranes were imaged using a chemiluminescent substrate for HRP detection (Bio-Rad Clarity) using the Bio-Rad Chemidoc and images were analysed using Image Lab software (Bio-Rad).

2.8 Biochemical assays

2.8.1 Serum transaminase quantification

Cholestasis is usually diagnosed during routine blood tests. Serum levels of the enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT) and ALP, bilirubin are often elevated (Chapman et al., 2019). The function of AST is to convert aspartate and α-ketoglutarate to oxaloacetate and glutamate (Lieberman et al., 2018). The liver and skeletal muscle contain the highest levels of AST, however it is expressed in all tissues of the body apart from bone (Washington & Van Hoosier, 2012). Elevated AST levels serve as a marker for liver injury, however due to its expression in other cell types ALT is used as a more specific marker of liver damage. ALT catalyses the conversion of alanine and α-ketoglutarate to pyruvate and glutamate (Lieberman et al., 2018). ALT is used as a specific marker for hepatocyte damage because it is most highly expressed in hepatocytes, although it is also found in the intestinal epithelium and skeletal muscle (Radu-Ionita

et al., 2020). Both serum transaminases indicate liver inflammation and hepatocyte cell death by necrosis and they are often elevated in response to liver damage regardless of aetiology (Radu-Ionita et al., 2020). ALP is a phosphatase highly expressed in the liver and bone. It is widely used as an indirect marker of cholestatic liver injury (Chapman et al., 2019). ALP is a marker for bile acid retention in the liver. One of the functions of ALP is to maintain an alkaline pH in bile. The destruction of bile ducts and accumulation of bile acids during cholestasis leads to increased ALP synthesis by hepatocytes and ALP is secreted into the bloodstream (Poupon, 2015). Bilirubin is the end product of haemoglobin metabolism (Lieberman et al., 2018). Bilirubin is transported to the liver via the bloodstream, where it is usually conjugated to bilirubin diglucuronide and excreted in bile into the intestine where it is broken down by the microbiome and excreted in faeces. During cholestasis, when bile accumulates in the liver, bilirubin is instead secreted into the bloodstream where it is excreted through the urine. Elevated bilirubin in the serum is a direct indicator of cholestasis (Fevery, 2008). Mouse serum was obtained by centrifuging mouse blood for at least one hour at 3000 x rpm at 4°C. The supernatant was taken as the serum. AST, ALT, ALP and bilirubin levels were measured in mouse serum using the Randox analyser (Daytona) following the manufacturer's instructions.

2.8.2 Caspase 3 assay

Apoptotic cell death was determined from snap-frozen liver tissue or cultured hepatocytes using a fluorescently-labelled substrate to quantify Caspase-3 activity. Cell lysate was prepared as described in 2.6.2. A reaction master mix was prepared using 2.5µL of Caspase-3 substrate Ac-DEVD-AFC (Enzo), 20µL of 25x caspase 3 reaction buffer (250mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 50mM EDTA, 2.5% CHAPS, 125Mm DTT), 70µL liver protein or 30µL hepatocyte cell protein and water up to 500µL. Caspase 3 activity was measured at 0 hours, 1 hour and 2 hours using an Optima spectrophotometer. Excitation was measured at 380nm and emission at 510nm.

2.9 Graphical art

Graphical art for figures 4.10 and 7.1 was obtained from BioRender (https://biorender.com) and for figures 5.3 and 6.1 was obtained from SMART Servier Medical Art (https://smart.servier.com).

2.10 Statistical analysis

Data are expressed as mean ± standard error of the mean. Statistical significance was determined using two-way analysis of variance (ANOVA) followed by Bonferroni's post-test, or Student's t test, as appropriate, using GraphPad Prism software.

Chapter 3 – Determining that presence of the microbiome associates with increased liver damage and macrophage infiltration in response to cholestatic liver injury

This chapter is an adaptation of work that has been published in Hepatology and appears with permission.

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3.1 Introduction and aims

The microbiome is defined as the collection of bacteria, bacteriophages, archaea, fungi and viruses that reside in all surfaces and cavities of the body and have a symbiotic relationship with the host. The intestinal microbiome is the best characterised microbiome to date (Fan & Pedersen, 2021). Changes in the intestinal microbiome are associated with many metabolic diseases including chronic and cholestatic liver diseases (Kummen & Hov, 2019). The liver is in constant crosstalk with the microbiome via the gut/liver axis which utilises the portal vein and the biliary system (Tripathi et al., 2018).

The main function of the liver is the production of bile and the secretion of bile acids into the intestine to aid digestion. Primary bile acids, CDCA and CA, are synthesized in hepatocytes from cholesterol by the rate limiting enzyme Cyp7a1. Primary bile acids are highly toxic and they undergo conjugation with various compounds to decrease their toxicity and increase water solubility that allows export out of the liver. In mice, CDCA undergoes phase I detoxification to allow for conjugation reactions by phase II enzymes.

Phase I detoxification enzyme Cyp2c70 converts CDCA to αMCA (Takahashi et al., 2016). Additionally, Cyp2b10 and Cyp3a11 can perform this step, however Cyp3a11 is not essential for this conversion (Wahlström et al., 2017). Phase II detoxification enzymes include UDT-glucuronosyltransferases such as UDT-glucuronosyltransferases 1a1 and 1a2 (Ugt1a1) and (Ugt1a2) which perform glucuronide conjugation (Wagner et al., 2005). Bile acids can also be conjugated to glutathione by the enzymes glutathione-S-transferase 3 (Gst3) and glutathione-S-transferase 4

(Gst4). Glutathione peroxidase 1 (Gxp1) functions to reduce hydrogen peroxide to water in a glutathione dependent manner (Lubos et al., 2011).

Once conjugated, water soluble bile acids are exported across the hepatocyte canalicular membrane using the transporter BSEP and phospholipids are transported into bile via Mdr2 (Boyer & Soroka, 2021). Bile acids and bilirubin that are conjugated to glucuronides or glutathione are exported into bile using multidrug resistance related protein 2 (Mrp2) (Chiang & Ferrell, 2018). Multidrug resistance related protein 3 (Mrp3) also transports glutathione and glucuronide conjugated products and multidrug resistance related protein 4 (Mrp4) transports glutathione conjugates as well as TCA and GCA (Jansen et al., 2012). Bile is stored in the gall bladder and is released into the duodenum after a meal.

Bile acids are deconjugated to form free bile acids, in the ileum and colon by BSH of the microbiome. In the intestine, bile acids maintain a healthy microbiome composition and prevent bacterial overgrowth (Urdaneta & Casadesús, 2017). The microbiome metabolises primary bile acids to form secondary bile acids which are transported back to the liver via the portal vein. Hepatocytes uptake these recycled bile acids using the transporters Oatp and Ntcp (Boyer, 2013). Bile acid transporters are shown in figure 3.1.

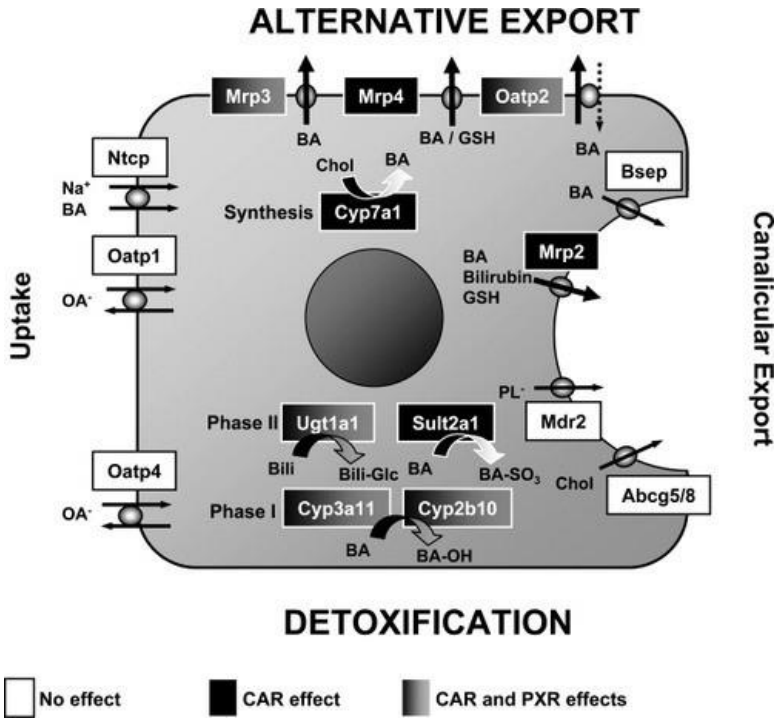


Figure 3.1 Bile acid transport. *Transporters involved in bile acid uptake, alternative export and canalicular export. Taken from Wagner et al., 2005.*

During cholestasis, the accumulation of the high concentration of bile acids causes hepatocyte cell death by apoptosis (Fickert & Wagner, 2017). These dying hepatocytes release DAMPs which activate the pro-inflammatory signature of Kupffer cells and recruitment of monocyte-derived macrophages to the injured liver (Canbay et al., 2003). Hepatocytes possess TLRs and can respond to bacterial endotoxin that is carried to the liver by the portal vein by producing LPS binding protein and the soluble form of cluster of differentiation 14 (Z. Zhou et al., 2016). However, mice are resistant to high doses of endotoxin and treatment with endotoxin alone is usually not enough to induce hepatocyte cell death (Hamesch et al., 2015).

The aim of this chapter is to demonstrate that the microbiome promotes macrophage recruitment to the liver in response to cholestatic liver injury and sensitises the liver to bile acid induced cell death.

3.2 Results

3.2.1 The absence of the microbiome reduces liver injury and macrophage recruitment during cholestasis induced by ANIT

GF mice are defined as anexic, where they are completely free of any living microorganisms (TREXLER & REYNOLDS, 1957). GF animals have been widely used to study the effects of the microbiome since the 1950s as they can be colonised with a defined microbiome or microorganism of choice (Kennedy et al., 2018).

The aim of our study was to characterise the role of the microbiome in cholestatic liver disease therefore we conventionalised GF mice with the microbiome of WT SPF animals for three weeks and used pure GF mice as the microbiome free controls. After colonisation, we induced cholestatic liver injury by oral gavage with ANIT and we sacrificed the animals 48 hours after the treatment. We chose this method of inducing cholestasis as ANIT could be conveniently administered by oral gavage under GF conditions without compromising the GF environment.

Firstly, to characterise the liver damage of the GF and GF+WT animals after ANIT treatment we measured serum transaminase levels, ALT and AST and serum markers of cholestasis, ALP and bilirubin. Interestingly, we found a statistically significant increase in ALT and AST (figure 3.2). These transaminases are produced by hepatocytes in response to liver injury and their elevation indicated severe liver damage in GF+WT mice compared to GF mice in response to ANIT induced cholestasis. Additionally, GF+WT mice had more severe cholestatic liver injury as evidenced by significantly increased ALP and bilirubin levels in their serum compared to GF mice (figure 3.2). This was a

fascinating discovery as these results showed that the presence of the microbiome associated with severe liver damage during cholestatic liver injury.

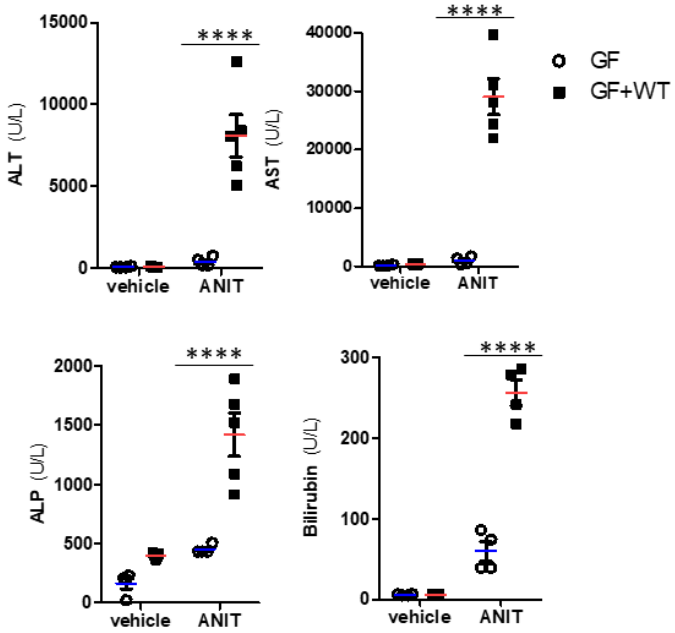


Figure 3.2 GF mice are protected from ANIT-induced cholestatic liver injury. *Levels of serum transaminases and cholestatic markers are significantly elevated in GF+WT mice following ANIT treatment for 48 hours. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 (GF vs GF+WT). Error bars represent standard error of the mean (n=5). This figure has been adapted with permission from one published by Isaacs-Ten et al., 2020.*

In response to liver injury, Kupffer cells secrete chemokines which promote monocyte recruitment to the liver where monocytes differentiate into macrophages and promote inflammation and further tissue damage (Krenkel & Tacke, 2017). To study the effect of the microbiome on macrophage recruitment to the liver during cholestasis, we quantified the macrophages in the livers of the GF and GF+WT animals, treated with ANIT, using flow cytometry (figure 3.3A and B). GF mice had no infiltration of monocyte-derived macrophages when treated with ANIT compared to untreated GF mice (figures 3.3A and B) while conventionalised GF+WT mice displayed significantly increased liver macrophage infiltration compared to ANIT treated GF animals.

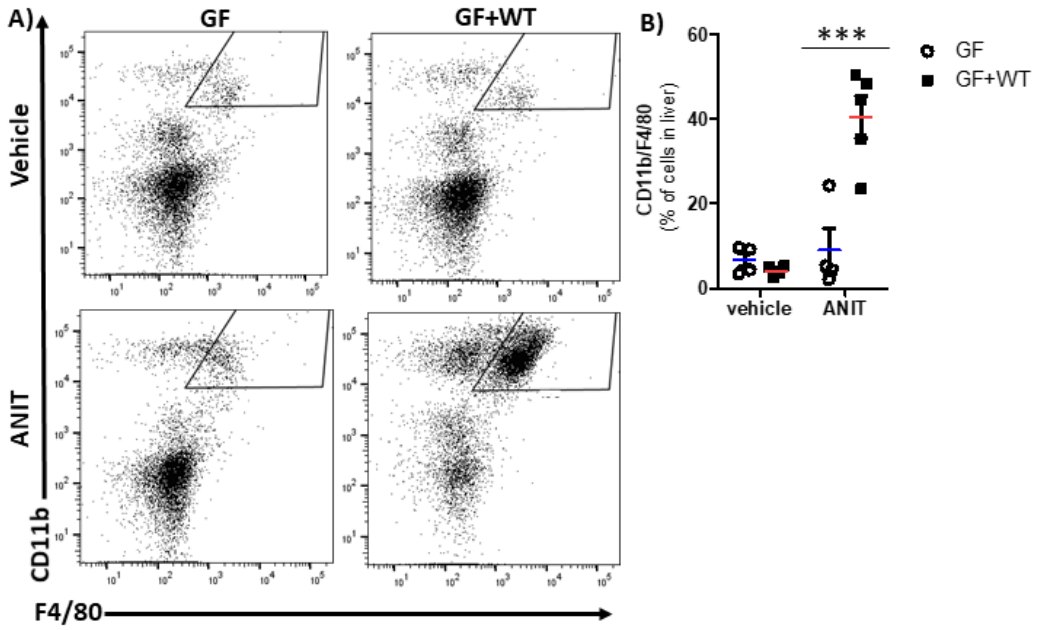


Figure 3.3 GF mice are protected from inflammation in response to cholestatic liver injury. *The absence of the microbiome protects GF mice from macrophage infiltration in response to liver injury by ANIT. A) FACS analysis of liver isolated immune cells. B) Quantification of liver isolated macrophages. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 (GF vs GF+WT). Error bars represent standard error of the mean (n=5). This figure has been adapted with permission from one published by Isaacs-Ten et al., 2020.*

In summary, our results show that the presence of the microbiome associates with increased liver damage and macrophage infiltration in response to cholestatic liver injury.

3.2.2 Bile acid accumulation (cholestasis) occurs in the liver after ANIT treatment in the absence of the microbiome

Our results demonstrate that the presence of the microbiome promoted liver damage and macrophage infiltration during cholestasis. Cholestasis is defined as the accumulation of bile acids in the liver, where the high concentration of bile acids in the liver causes hepatocyte cell death (Fickert & Wagner, 2017). Therefore, the lack of liver injury in ANIT treated GF animals could be due to a lack of the accumulation of bile acids in the liver or in the differential composition of the bile acid pool in GF animals. A differential composition of the bile acid pool could arise due to the lack of the microbiome in GF animals as BSH enzymes, produced by the intestinal microbiome, modify primary bile acids into secondary bile acids and transport them back to the liver (Chiang & Ferrell, 2018). Therefore, a differential composition of the bile acid pool could occur in GF animals where the bile acids of GF mice could be less hydrophobic and therefore less hepatotoxic.

Therefore, the Beraza group analysed the composition of the liver bile acid pool in the animals using mass spectrometry. There was comparable accumulation of bile acids in the livers of cholestatic GF and GF+WT animals, however, there were differences in bile acid pool composition due to the absence of the microbiome in GF animals (Isaacs-Ten et al., 2020). Since the bile acid accumulation in the liver was comparable between both the GF and GF+WT animals, we investigated bile acid metabolism, because the liver protection that we observed in the GF animals could be a result of differential bile acid metabolism and not the presence of the microbiome.

Next, we analysed the gene expression of hepatic transporters in liver tissue of the mice and found a reduction of uptake transporters Oatp and Ntcp in response to ANIT induced cholestasis (figure 3.4). At basal conditions, the phase I enzymes Cyp2b10 and Cyp3a11 were elevated in GF+WT mice and Cyp2c70 expression was reduced compared to GF (figure 3.5). After ANIT administration, we did not see any statistically significant differences in alternative or canalicular bile acid transporters while phase I, phase II and GSSH enzymes were reduced and reached comparable levels between the GF and GF+WT groups (figure 3.5).

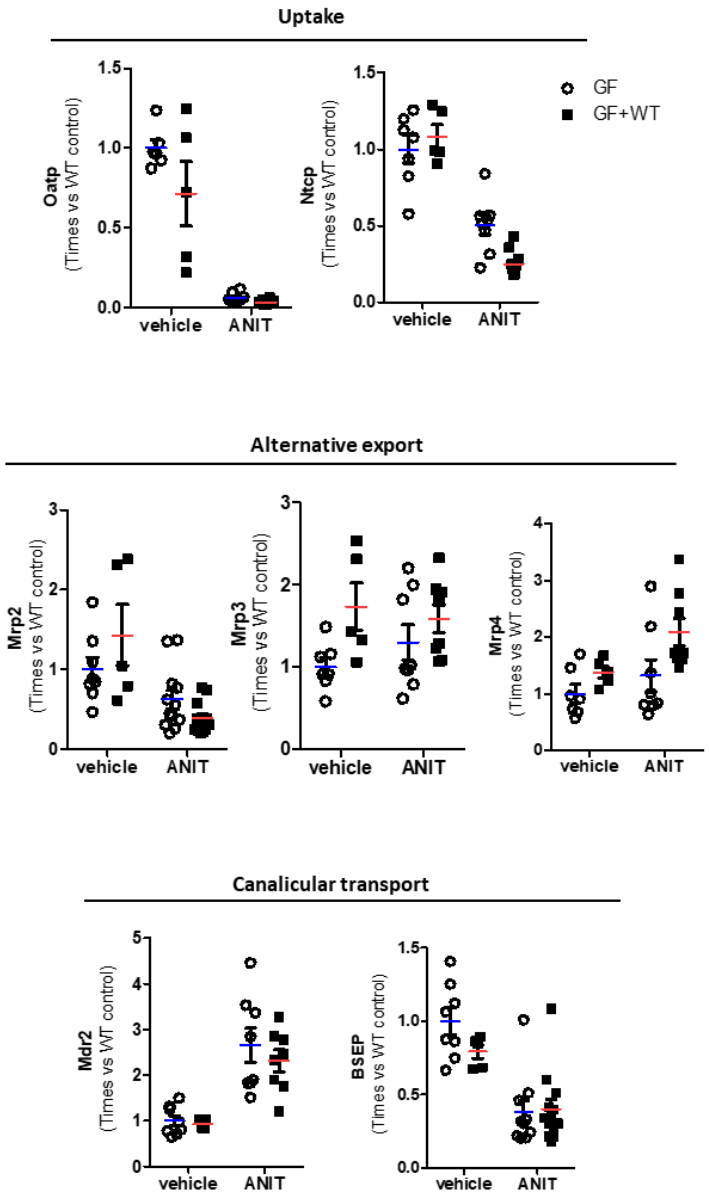


Figure 3.4 GF and GF+WT mice show differences in bile acid transport 48 hours after ANIT treatment. *Gene expression analysis of bile acid transporters shows an increase in Mrp3, and a decrease in BSEP expression in GF+WT animals at basal conditions, compared to GF animals, however the results did not reach statistical significance. Upon cholestasis induction with ANIT, there is a decrease in Ntcp and Mdr2 in GF+WT animals compared to GF animals, however the results did not reach statistical significance. This figure has been adapted with permission from one published by Isaacs-Ten et al., 2020.*

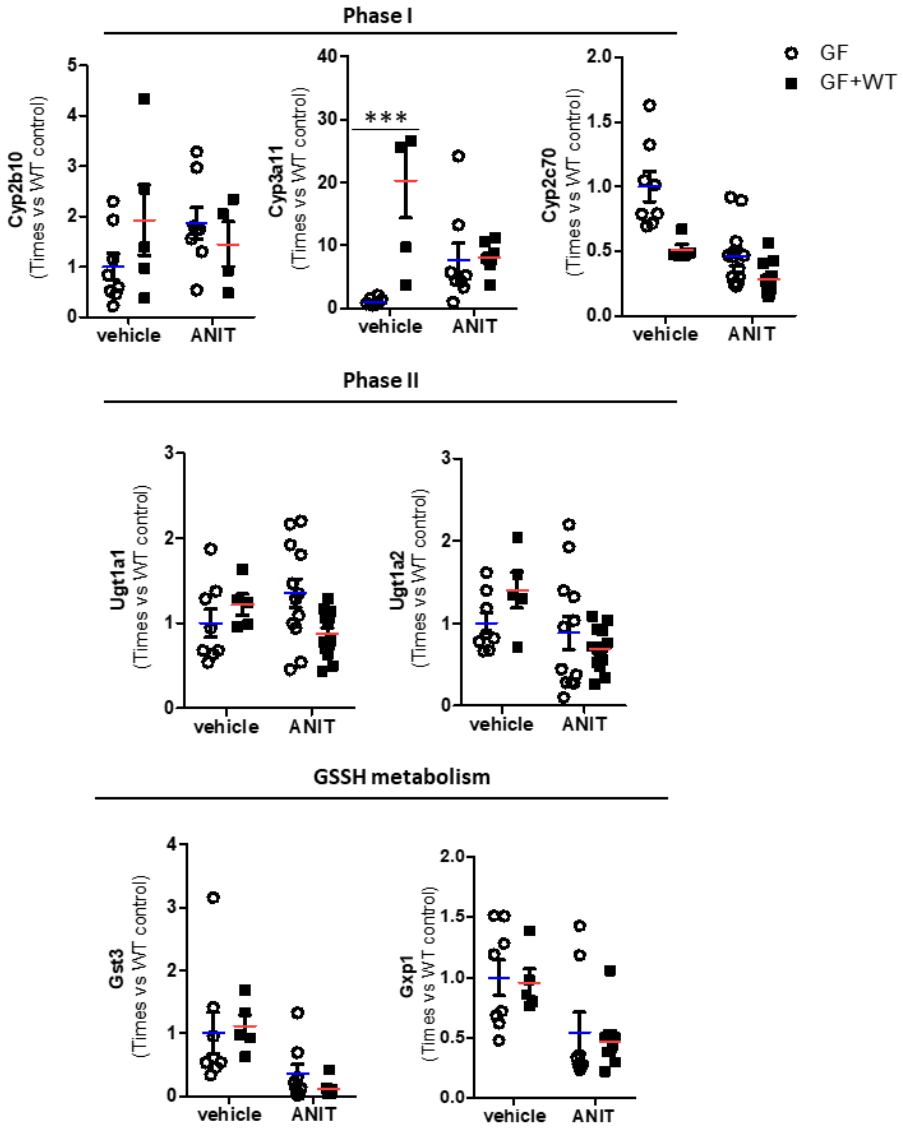


Figure 3.5 GF and GF+WT mice show differences hepatic metabolism 48 hours after ANIT treatment. *Gene expression analysis of phase I hepatic metabolism shows a significant increase in Cyp3a11 expression after colonisation with the microbiome. Additionally, at basal conditions, expression of Cyp2b10 is elevated in GF+WT animals compared to GF, while Cyp2c70 expression is reduced, however, these results did not reach statistical significance. Gene expression analysis of phase II hepatic detoxification and GSSH metabolism shows a reduction of Ugt1a1 and Ugt1a2, as well as Gst3 and Gxp1, in GF+WT animals in response to ANIT treatment compared to GF mice, however the results did not reach statistical significance. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 (GF vs GF+WT). Error bars represent standard error of the mean (n=5). This figure has been adapted with permission from one published by Isaacs-Ten et al., 2020.*

In conclusion, while the presence of the microbiome affects bile acid composition and bile acid transporter expression, bile acid accumulation still occurs in the liver in the absence of the microbiome.

3.2.3 Hepatocytes from GF mice are protected from bile acid-induced cell death

Our results showed that GF mice are protected from liver damage in response to cholestasis, and colonised GF+WT animals showed severe liver injury, despite an equal bile acid load in the livers of both experimental groups. Therefore, we hypothesised that bacterial products sensitise the liver to bile acid-induced cell death. To confirm our hypothesis, we isolated hepatocytes from livers of healthy GF and GF+WT animals and treated the hepatocytes with a range of primary and secondary bile acids. We chose this experimental design to mimic the cholestatic liver environment *in vitro*. We observed increased cell death in cultured GF+WT hepatocytes in response to bile acid treatment with CDCA, DCA and GCA compared to GF hepatocytes (figure 3.6A). We confirmed our microscopic observations by measuring caspase 3 activity in hepatocyte cell lysates and found a significant increase in apoptotic cell death in GF+WT hepatocytes in response to treatment with CDCA, DCA, GCA and TCA (figure 3.6B). In particular, we saw profuse apoptotic cell death in CDCA and DCA treated cells where the apoptotic activity of GF+WT hepatocytes was three times higher than that of GF hepatocytes (figure 3.6B).

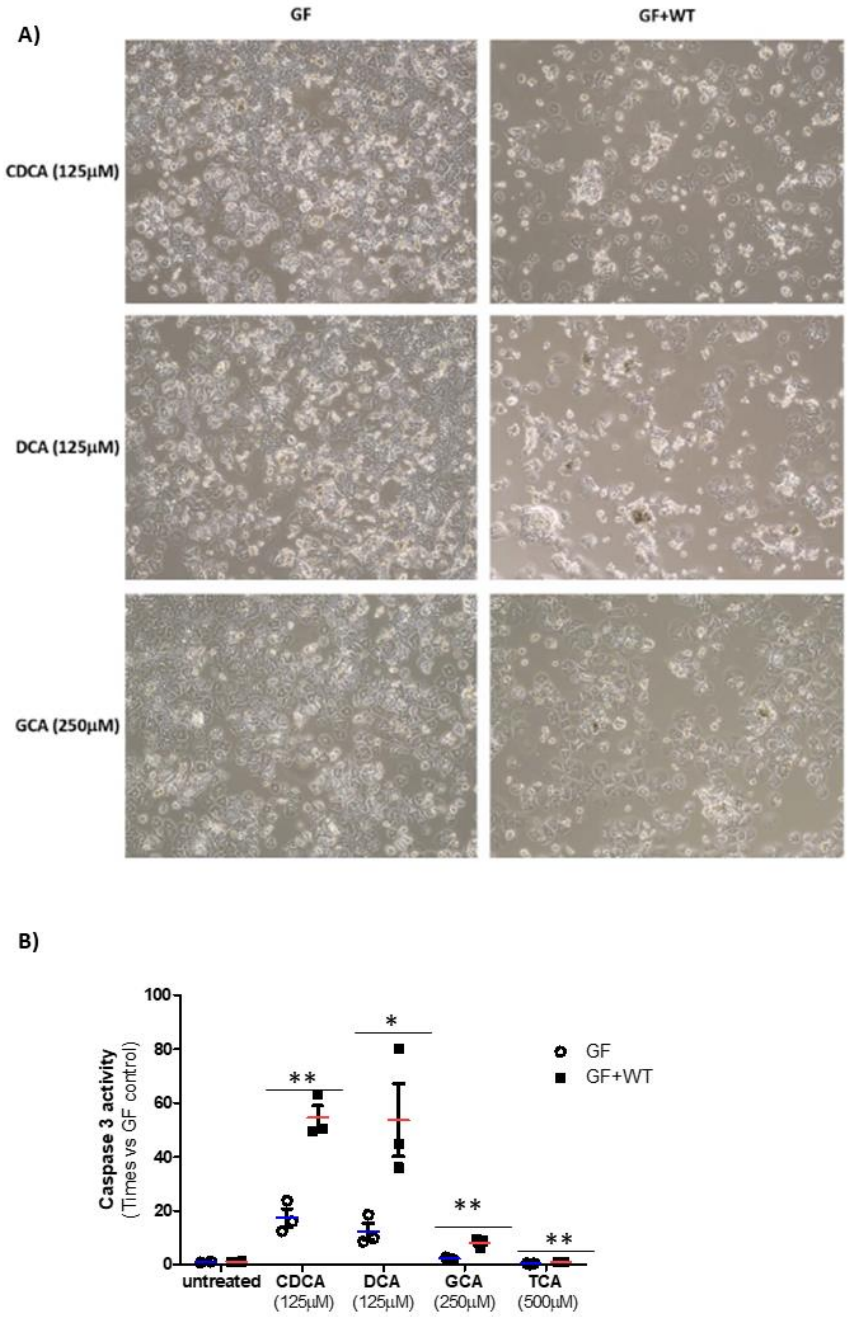


Figure 3.6 Hepatocytes isolated from GF+WT mice have more apoptotic cell death in response to bile acids. *A) Light microscopy imaging shows increased cell death in GF+WT hepatocytes in response to bile acid treatment. B) Caspase 3 analysis shows increased apoptotic cell death in GF+WT hepatocytes in response to bile acids. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 (GF vs GF+WT). Error bars represent standard error of the mean (n=3). Results show representative data of three independent experiments. Part A of this figure has also been published in Isaacs-Ten et al., 2020 and appears here with permission. Part B of this figure has been adapted with permission from one published by Isaacs-Ten et al., 2020.*

The higher cell death we observed in GF+WT hepatocytes compared to GF hepatocytes could be a result of impaired bile acid detoxification, therefore we checked the gene expression of bile acid transporters and detoxification enzymes during homeostasis (figure 3.7). We found comparable levels of bile acid transporters between the genotypes. We saw a significant increase in phase I detoxification enzyme Cyp3a11 and a reduction in Cyp2c70, which was consistent with the expression of this enzyme in the whole liver after colonisation with the microbiome.

Our results suggest that the presence of bacterial endotoxin sensitises hepatocytes to bile acid induced cell death.

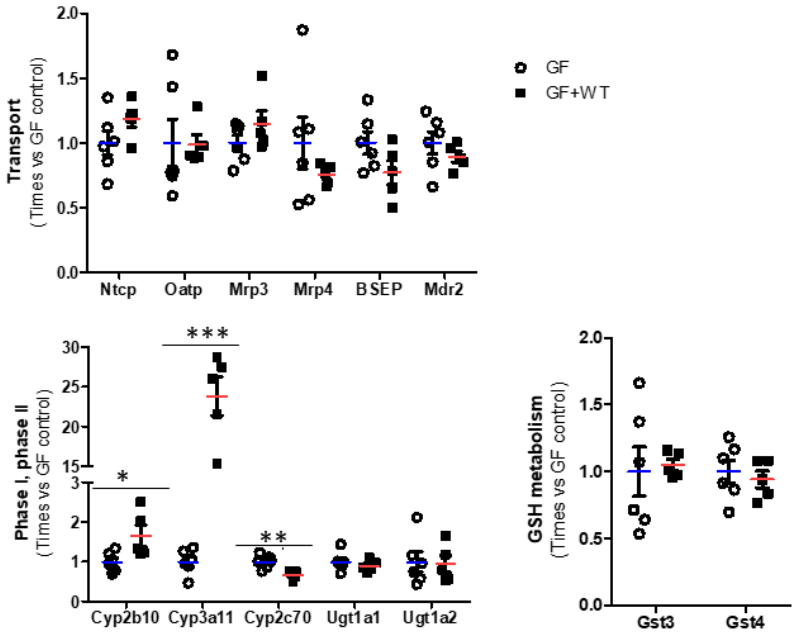


Figure 3.7 Bile acid transporter and hepatic metabolism gene expression in GF and GF+WT hepatocytes at basal conditions. *Gene expression of the hepatic transporters Oatp and Bsep is reduced in hepatocytes isolated from GF+WT animals, and Mrp3 expression is increased, however these results do not reach statistical significance. There was a statistically significant increase in the phase I detoxification enzymes Cyp2b10 and Cyp3a11, and a decrease in Cyp2c70 in GF+WT hepatocytes compared to GF hepatocytes. There were comparable levels of GSH metabolism enzymes Gst3 and Gst4 in hepatocytes from both animals. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 (GF vs GF+LPS). Error bars represent standard error of the mean (n=3). Results show representative data of three independent experiments. This figure has been adapted with permission from one published by Isaacs-Ten et al., 2020.*

3.2.4 Bacterial endotoxin sensitises hepatocytes from GF mice to bile acid-induced cell death

To confirm our hypothesis that microbiome-derived products sensitise the liver to bile-acid induced cell death, we pre-treated hepatocytes from healthy GF animals with 100ng/mL LPS for two hours prior to bile acid treatment. Quantification of caspase 3 activity in hepatocyte cell lysates (figure 3.8A) showed profuse apoptotic cell death in GF+LPS hepatocytes in response to treatment with bile acids, particularly with CDCA and DCA where caspase 3 activity was up to four times higher than in GF hepatocytes (figure 3.8A). These results confirmed that LPS sensitised GF hepatocytes to apoptosis induced by bile acids.

Interestingly, LPS treatment had limited effect on bile acid detoxification of GF hepatocytes. The gene expression of the bile acid transporters Mrp4 and BSEP was reduced with LPS treatment, but not significantly (figure 3.9B). Interestingly, the expression of Cyp3a11 was significantly reduced in response to LPS treatment (figure 3.9B).

These *in vitro* experiments show that bacterial endotoxin sensitises hepatocytes to bile acid induced cell death.

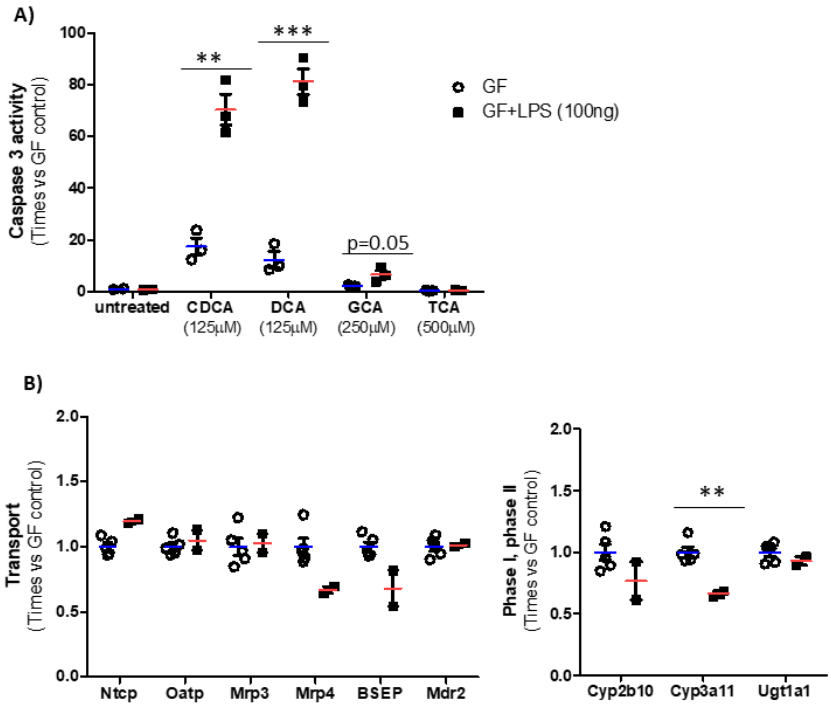


Figure 3.8 Bacterial endotoxin sensitises hepatocytes to bile acid induced cell death. *A) Caspase 3 activity in GF hepatocytes in response to bile acids, with and without LPS pre-treatment. B) Bile acid transporters and hepatic metabolism gene expression in GF hepatocytes with and without LPS treatment. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 (GF vs GF+LPS). Error bars represent standard error of the mean (n=3). Results show representative data of three independent experiments. This figure has been adapted with permission from one published by Isaacs-Ten et al., 2020.*

3.3. Discussion

In this chapter, we have demonstrated that the presence of the microbiome sensitises hepatocytes to bile acid induced cell death and promotes macrophage recruitment to the liver in response to cholestatic liver injury.

Our work provides a valuable contribution to the field because previous studies of cholestasis on GF mice generated conflicting results. Re-derivation of a genetic mouse model of cholestasis ($Mdr2^{-/-}$) into a GF environment led to increased liver damage and fibrosis compared to age-matched $Mdr2^{-/-}$ mice housed under SPF conditions (Tabibian et al., 2016). However, when a mouse model of spontaneous bile duct inflammation (NOD.c3c4) was re-derived under GF conditions, the absence of the microbiome did not lead to inflammatory biliary disease, which is observed when the NOD.c3c4 mice are conventionally housed (Schrumpf et al., 2017). We have shown that the absence of the microbiome protected mice from ANIT induced liver injury and associated with a lack of monocyte-derived macrophage recruitment to the liver. Further work from the Beraza laboratory demonstrated that ANIT-induced liver damage was significantly reduced in GF+WT mice upon macrophage depletion with liposomal clodronate, highlighting the key role of macrophages in the pathogenesis of cholestatic liver disease (Isaacs-Ten et al., 2020). Importantly, we showed that macrophages contributed to increased intestinal permeability in GF+WT and GF+WT+ANIT mice, which was restored in GF+WT+ANIT mice with clodronate treatment (Isaacs-Ten et al., 2020).

We propose a novel mechanism for the role of the microbiome and macrophages in liver damage during cholestatic liver disease. During cholestasis, there are changes in the composition of the intestinal microbiome due to the lack of bile flow to the intestine and here macrophages promote inflammation which results in loss of intestinal barrier integrity and the translocation of bacterial products to the liver. In the cholestatic liver, translocated bacterial endotoxin synergises with accumulating bile acids to promote hepatocyte cell death and hence, liver injury. Monocyte-derived macrophage recruitment to the liver then occurs and these infiltrating liver macrophages promote Nlrp3 inflammasome activation, inflammation and further liver damage (Isaacs-Ten et al., 2020). We demonstrated that the Nlrp3 inflammasome in macrophages promoted intestinal permeability during cholestasis, and hence the pathogenesis of cholestatic liver disease as described above, by contributing to the loss of intestinal TJ proteins (Isaacs-Ten et al., 2020). Recent work from the Trautwein group showed that the Nlrp3 inflammasome is activated in livers of PSC patients and in the gut/liver axis of $Mdr2^{-/-}$ mice, and that in mice this correlated with a loss of

intestinal barrier integrity (Liao et al., 2019). Using a specific Nlrp3 inhibitor, MCC950, we showed a key role for the Nlrp3 inflammasome in regulating intestinal barrier integrity.

In summary, we described a novel detrimental role for macrophages in the gut/liver axis during cholestasis.

Chapter 4- Characterising the role of SIRT1 in macrophage function in response to endotoxin

4.1 Introduction and aims

Increased intestinal permeability is a common pathological feature in cholestatic liver diseases (Di Leo et al., 2003; Feld et al., 2006). We and others have described that increased intestinal permeability during cholestasis contributes to the pathogenesis of cholestasis via mechanisms involving the Nlrp3 inflammasome (Isaacs-Ten et al., 2020; Liao et al., 2019). When gut-derived pathogens breach the intestinal barrier, microbial products reach the liver and activate liver macrophages which promote inflammation via mechanisms involving the Nlrp3 inflammasome (Swanson et al., 2019). Recruitment of bone marrow derived monocytes to the liver then occurs and the resulting inflammatory response causes increased liver damage, fibrosis and loss of liver function (Guicciardi et al., 2018). We have previously described that SIRT1 overexpression promotes liver damage and macrophage recruitment and inflammation in experimental models of cholestasis (Blokker et al., 2018). In accord with this, our group has found that SIRT1 overexpressing animals have increased intestinal permeability during cholestasis (N. Beraza, personal communication).

SIRT1 is an important metabolic regulator and metabolic changes in macrophages underpin their activation states. We hypothesise that SIRT1 overexpression promotes inflammation in response to LPS by rewiring macrophage metabolism. The aim of this chapter is to characterise the role of SIRT1 overexpression in macrophage function in response to LPS using a combination of *in vivo* and *in vitro* approaches.

4. 2 Results

4.2.1 SIRT1 overexpression promotes liver damage and inflammation in response to LPS challenge *in vivo*

4.2.1.1 SIRT1 overexpression promotes liver damage and inflammation in response to LPS/GalN treatment

To study the role of macrophage SIRT1 overexpression in the liver in response to endotoxin, we used a mouse model of acute hepatoxicity and administered LPS/GalN intraperitoneally (i.p). This model combines the use of LPS with galactosamine (GalN), a chemical that causes hepatocyte cell death and sensitises the liver to LPS-induced injury. The Leloir pathway is a process used for the breakdown of galactose which is performed exclusively by hepatocytes (Lieberman et al., 2018) and

GalN is a competitive inhibitor of the enzyme responsible for the final stage of the Leloir pathway, UDP-galactose-4-epimirase (Korneev, 2019). The inhibition of the final stage of galactose metabolism leads to the build-up of UDP-galactosamine in hepatocytes, that cannot be metabolised further and its accumulation causes a depletion of uridine triphosphate (UTP) which is essential for transcription and, as a result of GalN administration, the process is inhibited and hepatocytes die by apoptosis (Y. H. Wu et al., 2014). Macrophages are essential to the LPS/GalN response and this model has been widely used for many years to study the role of macrophages in the liver in response to sepsis (Freudenberg & Galanos, 1988).

Six hours after LPS/GalN administration, $SIRT^{oe}$ mice showed increased serum markers of liver damage compared to WT mice as showed by a statistically significant increase in AST activity, and while ALT levels were increased in $SIRT^{oe}$ animals the data did not reach statistical significance (figure 4.1A). H and E staining of liver sections supported the transaminase data and indicated increased liver damage in $SIRT^{oe}$ animals compared to WT, as evidenced by abundant red blood cell infiltration in the livers of $SIRT^{oe}$ mice six hours after LPS/GalN administration, which was not present in WT animals (figure 4.1B).

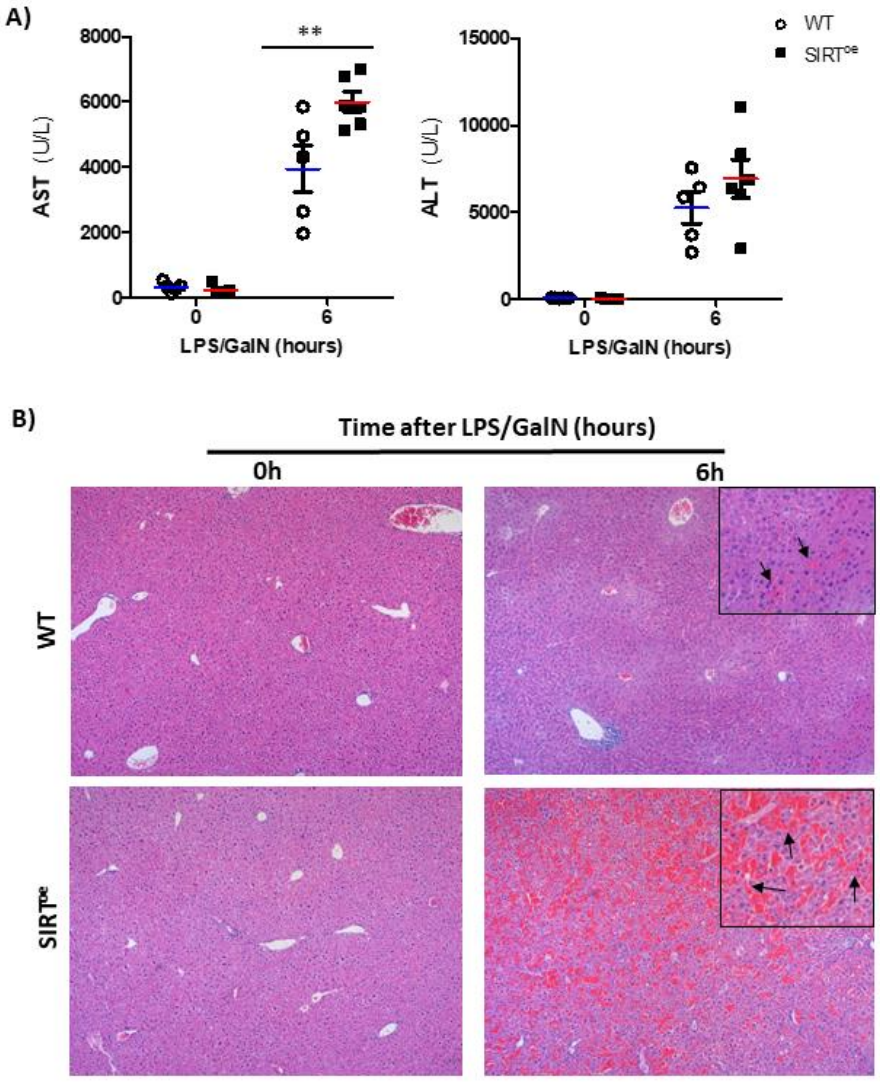


Figure 4.1 SIRT1 overexpression promotes increased liver damage in response to LPS/GalN challenge. *A) serum transaminase levels are elevated in SIRT^{oe} mice compared to WT mice 6 hours after LPS/GalN administration. B) H and E staining shows increased liver damage, as seen by increased red blood cell infiltration, in SIRT^{oe} mice compared to WT mice 6 hours after LPS/GalN administration. Arrows show red blood cell infiltration. Images were taken using the 4x magnification. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 (SIRT^{oe} vs WT littermate). Error bars represent standard error of the mean (n=6).*

Additionally, we found increased apoptotic cell death in the livers of SIRT^{oe} animals compared to WT mice as is shown by an increase in caspase 3 activity (figures 4.2A) and PARP1 cleavage (figure 4.2B) six hours after LPS/GalN injection.

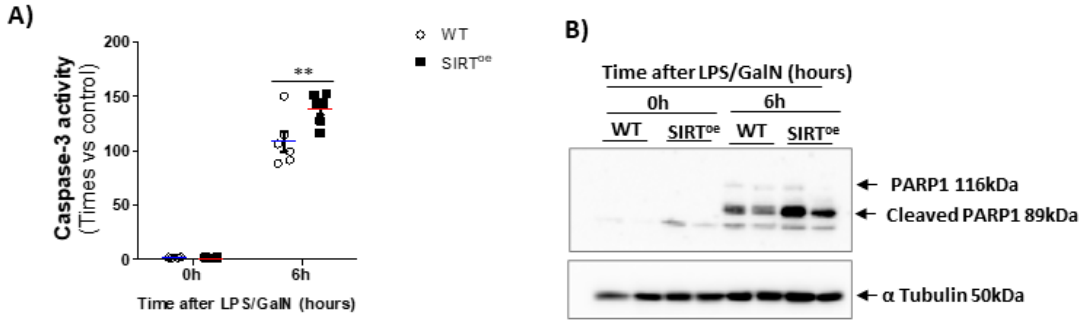


Figure 4.2 SIRT1 overexpression promotes increased liver apoptosis in response to LPS/GalN challenge*. A) Caspase 3 activity is increased SIRT^{oe} mice compared to WT mice 6 hours after LPS/GalN administration. B) western blotting shows increased PARP1 cleavage in SIRT^{oe} mice 6 hours after LPS/GalN administration. Wester blot is representative of n=6 mice. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 (SIRT^{oe} vs WT littermate). Error bars represent standard error of the mean (n=6).*

As well as apoptosis, during LPS/GalN induced liver injury cell death by pyroptosis can occur. Hepatocyte pyroptosis occurs due to increased Nlrp3 inflammasome activation in macrophages (Szabo & Petrasek, 2015). We analysed the gene expression of markers of Nlrp3 inflammasome priming (TLR4 and Nlrp3) and inflammasome activation (Caspase 1, IL1β) in WT and SIRT^{oe} mice and found that SIRT^{oe} mice have increased inflammasome activation compared to WT animals (figure 4.3A). At the protein level, SIRT^{oe} mice have increased levels of IL1β in the liver after LPS/GalN treatment (figure 4.3B) confirming the gene expression results.

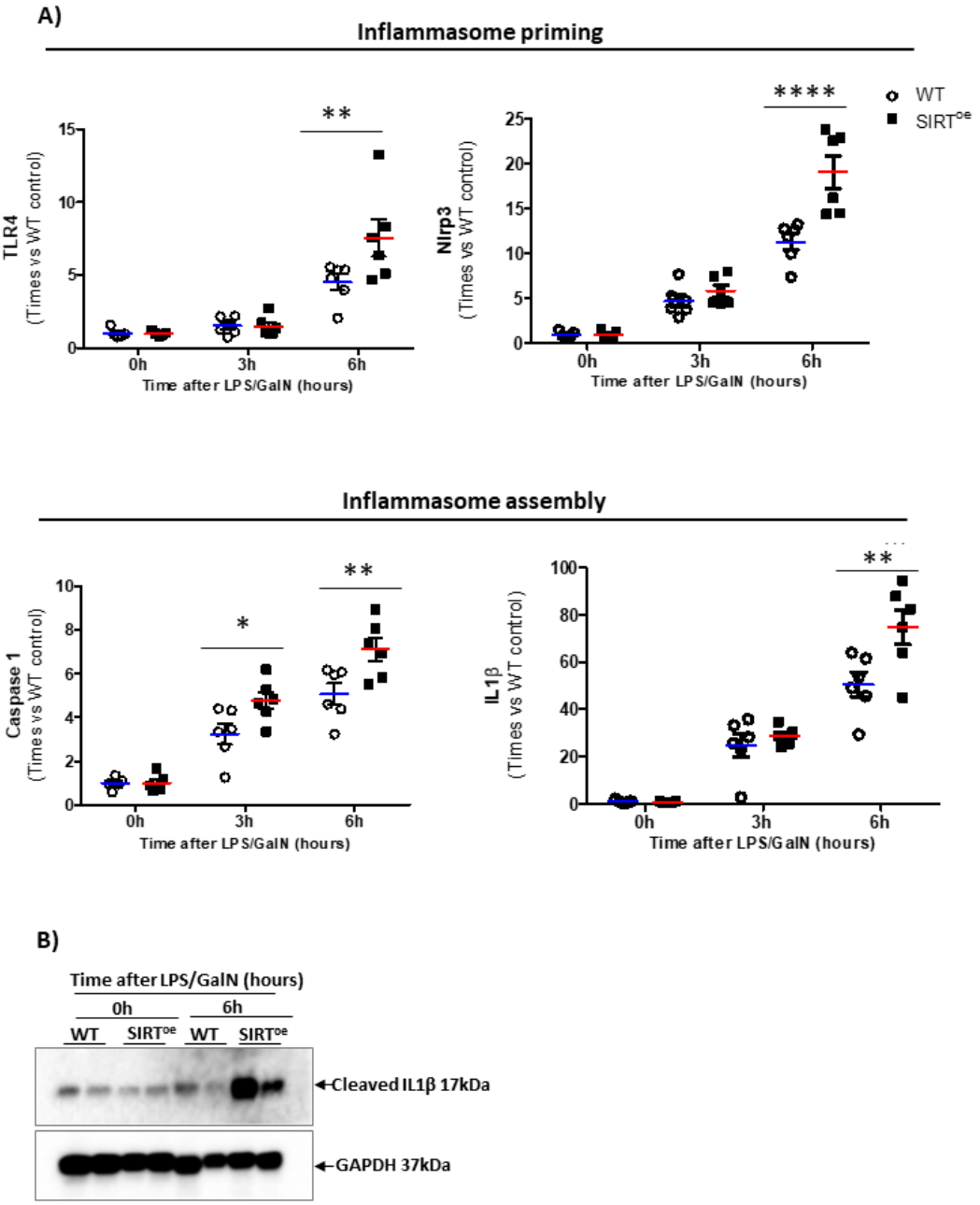


Figure 4.3 SIRT1 overexpression promotes increased Nlrp3 inflammasome activation in the liver in response to LPS/GalN challenge. *A) mRNA from livers from mice was analysed by qPCR. Gene expression of Nlrp3 inflammasome priming markers TLR4 and Nlrp3, and inflammasome activation markers Caspase 1 ẞ is significantly higher in SIRT^{oe} mice compared to WT mice 3 hours and 6 hours post IL1ẞ administration and IL1ẞ is significantly higher in SIRT^{oe} mice compared to WT mice 6 hours after LPS/GalN administration. B) IL1ẞ production was analysed by western blotting of liver protein extracts. Liver extracts from SIRT^{oe} mice have more cleaved IL1ẞ protein than WT mice 6 hours after LPS/GalN treatment. Western blot shows representative results of n=6 mice. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 **** P ≤ 0.0001 (SIRT^{oe} vs WT littermate). Error bars represent standard error of the mean (n=6).*

The mRNA levels of the pro-inflammatory cytokine TNFα were increased in SIRT^{oe} mice compared to WT (figure 4.4). The levels of the pro-inflammatory cytokines IL6 and IFNγ were increased in SIRT^{oe} mice six hours after LPS/GalN injection but did not reach statistical significance (figure 4.4). The levels of the pro-inflammatory markers NOS2 and CCL2 were comparable in both genotypes (figure 4.4).

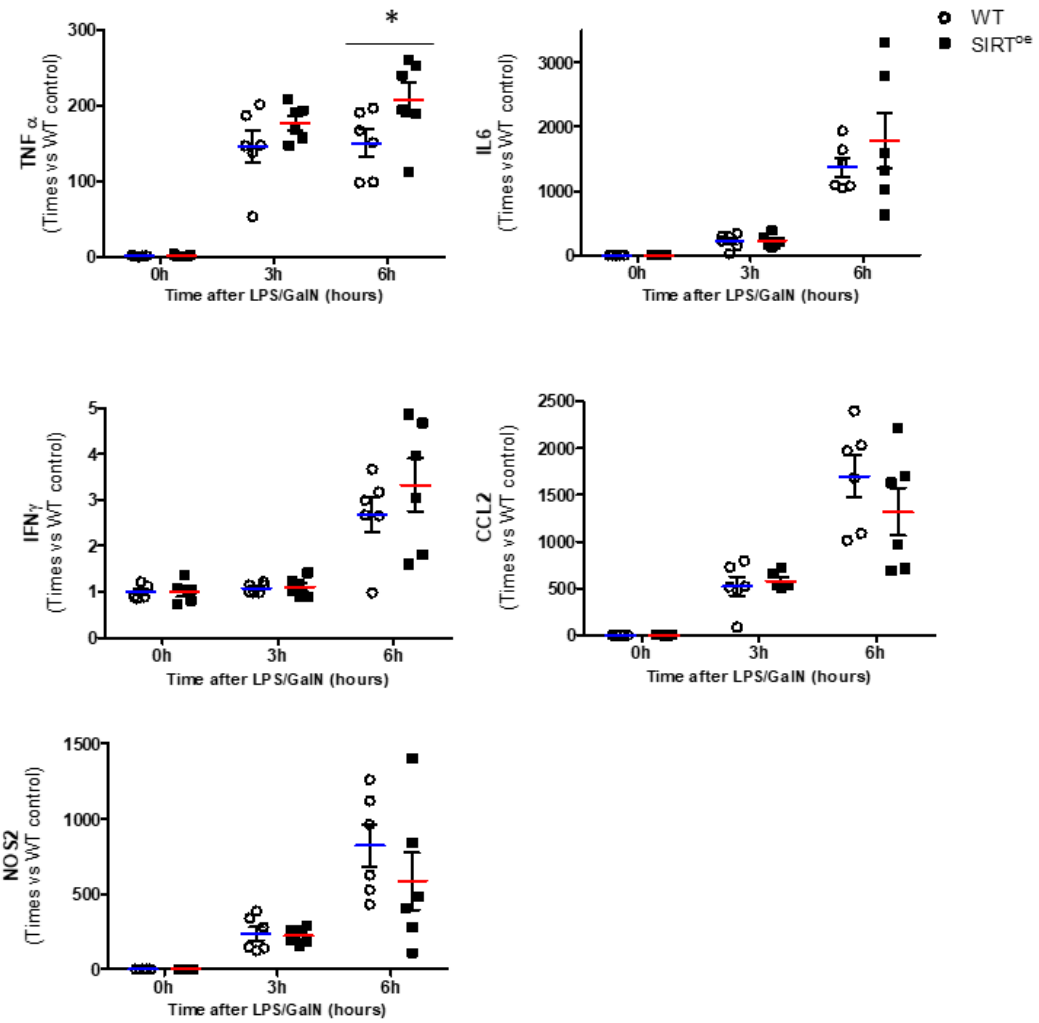


Figure 4.4 SIRT1 overexpression promotes inflammation in the liver in response to LPS/GalN challenge. *A) mRNA from livers from mice was analysed by qPCR. Gene expression of TNFα is significantly higher in SIRT^{oe} mice compared to WT mice 6 hours after LPS/GalN administration. Gene expression of IL6 and IFNγ is increased in SIRT^{oe} mice 6 hours post LPS/GalN administration but did not reach statistical significance. Gene expression of the chemokines CCL2 and NOS2 is comparable between both genotypes 3 hours and 6 hours after LPS/GalN administration. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 (SIRT^{oe} vs WT littermate). Error bars represent standard error of the mean (n=6).*

Additionally, the livers of SIRT^{oe} mice had increased levels of phosphorylated JNK, a transcription factor involved in the pro-inflammatory response, apoptosis and Nlrp3 inflammasome activation (Dhanasekaran & Premkumar Reddy, 2017) (figure 4.5A). The mTOR complex is involved in the induction of the inflammatory response. When macrophages encounter pro-inflammatory stimuli such as endotoxin, they require a rapid increase in the translation of pro-inflammatory cytokines. The mTOR complex is responsible for the initiation of this translation (Lelouard et al., 2007) and is involved in Nlrp3 inflammasome activation (Moon et al., 2015). The phosphorylation of ribosomal protein S6 by S6K1 is a downstream event of the mTOR signaling cascade, indicating increased mTOR pathway activity (Hong et al., 2014). The livers of SIRT^{oe} mice had increased levels of phosphorylated ribosomal protein S6 (pS6) (figure 4.5B).

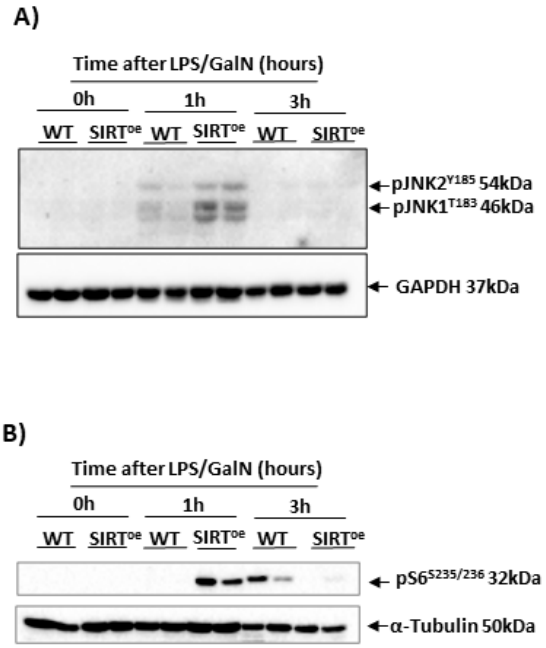


Figure 4.5 SIRT1 overexpression increased JNK and S6 phosphorylation in liver in response to LPS/GalN challenge. *A) Liver protein extracts from SIRT^{oe} mice showed increased levels of JNK phosphorylation compared to WT mice 1 hour after LPS/GalN challenge. B) Liver protein extracts from SIRT^{oe} mice showed increased levels of S6 phosphorylation compared to WT mice 3 hours after LPS/GalN administration. Western blots show representative results of n=6 mice.*

Interestingly, FACS analysis of liver isolated immune cells revealed that both genotypes had comparable macrophage numbers (figure 4.6) indicating that the increased liver damage and inflammation we observed was driven by increased macrophage activation in response to LPS/GalN in SIRT^{oe} mice and not by increased macrophage recruitment to the liver.

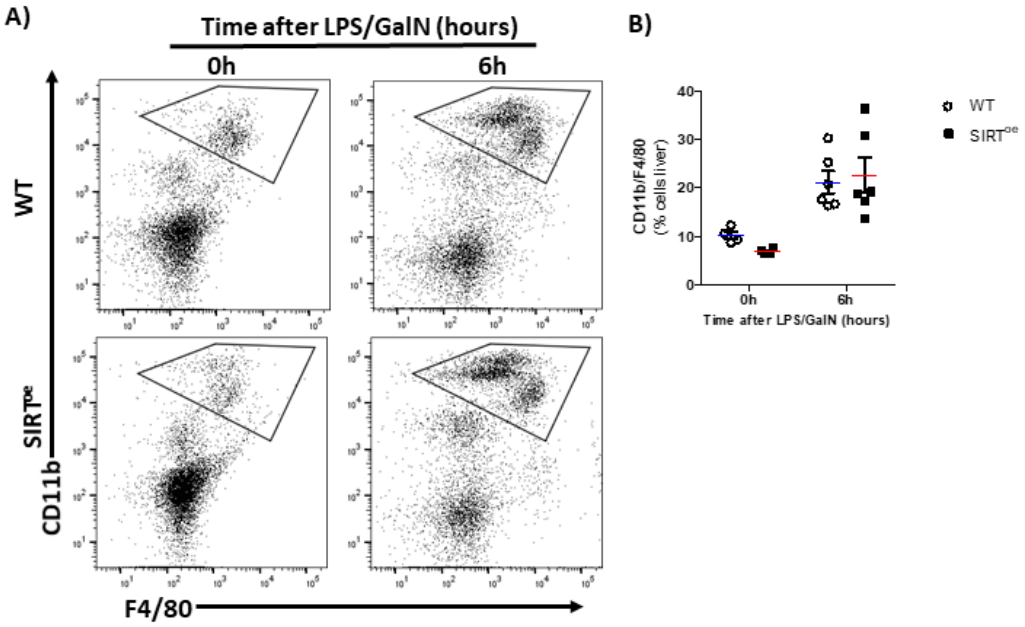


Figure 4.6 SIRT1 overexpression has no effect on liver macrophage recruitment to response to LPS/GalN challenge. *A) FACS analysis on immune cells isolated from the liver shows comparable macrophage numbers in both genotypes 6 hours after LPS/GalN administration B) Quantification of FACS analysis. Error bars represent standard error of the mean (n=at least 4 mice).*

In conclusion, here we show that SIRT^{oe} animals have increased liver damage and inflammation in response to LPS/GalN treatment and this associated with increased activation of the Nlrp3 inflammasome.

4.2.1.2 SIRT1 overexpression promotes liver damage and inflammation in response to LPS treatment

To confirm our findings of the LPS/GalN study, we used an additional sepsis model. We i.p. injected WT and SIRT^{oe} mice with LPS for up to 14 hours. Injection with LPS is a model of direct LPS toxicity. We found that SIRT1 gene expression increased in the livers of WT animals in response to LPS treatment, indicating that SIRT1 is important in the liver's response to LPS (figure 4.7A). In accord with our LPS/GalN data, SIRT^{oe} animals had increased liver damage as evidenced by increased ALT and AST activity (figure 4.7B) and increased apoptosis as shown by increased caspase 3 activity in SIRT^{oe} animals (figure 4.7C).

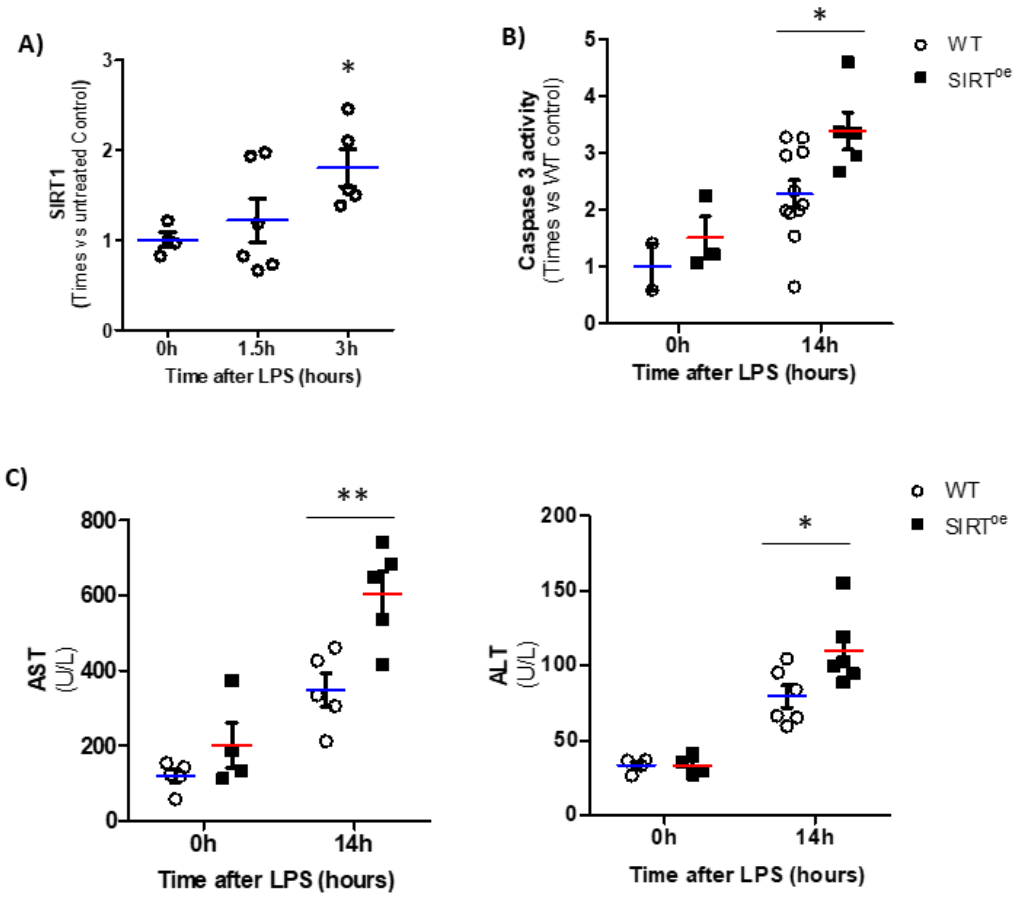


Figure 4.7 SIRT1 overexpression promotes liver damage and inflammation in response to LPS challenge. *A) SIRT1 is upregulated in liver tissue of mice 3 hours after LPS challenge. SIRT1 overexpression promotes liver damage B) and apoptosis C) serum transaminases 14 hours after LPS challenge. Significance of A was determined using unpaired T test * P ≤ 0.05 (WT untreated vs LPS treated) (n= at least 4 mice) Significance of figures B and C was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 (SIRT^{oe} vs WT littermate). Error bars represent standard error of the mean (n=at least 4 mice).*

At the gene expression level, SIRT^{oe} mice had increased levels of the pro-inflammatory cytokines IL1β, TNFα and the chemokine CCL2 in the liver at 1.5 hours, which decreased by 3 hours in all three genes and reached statistical significance in CCL2 expression (figure 4.8A), which suggests that the pro-inflammatory gene transcription occurred faster in SIRT^{oe} animals. The mRNA levels of NOS2 were comparable between both genotypes at both timepoints (figure 4.8A). Additionally, at the protein level, SIRT^{oe} mice had increased IL1β in the liver compared to WT animals (figure 4.8B). The LPS study supports our LPS/GalN findings. The data suggest that liver macrophages of SIRT^{oe} mice are more active in response to LPS than the macrophages of WT animals. This is supported by FACS data which showed comparable numbers of macrophages in the livers of both genotypes at 14 hours of LPS challenge (figure 4.9).

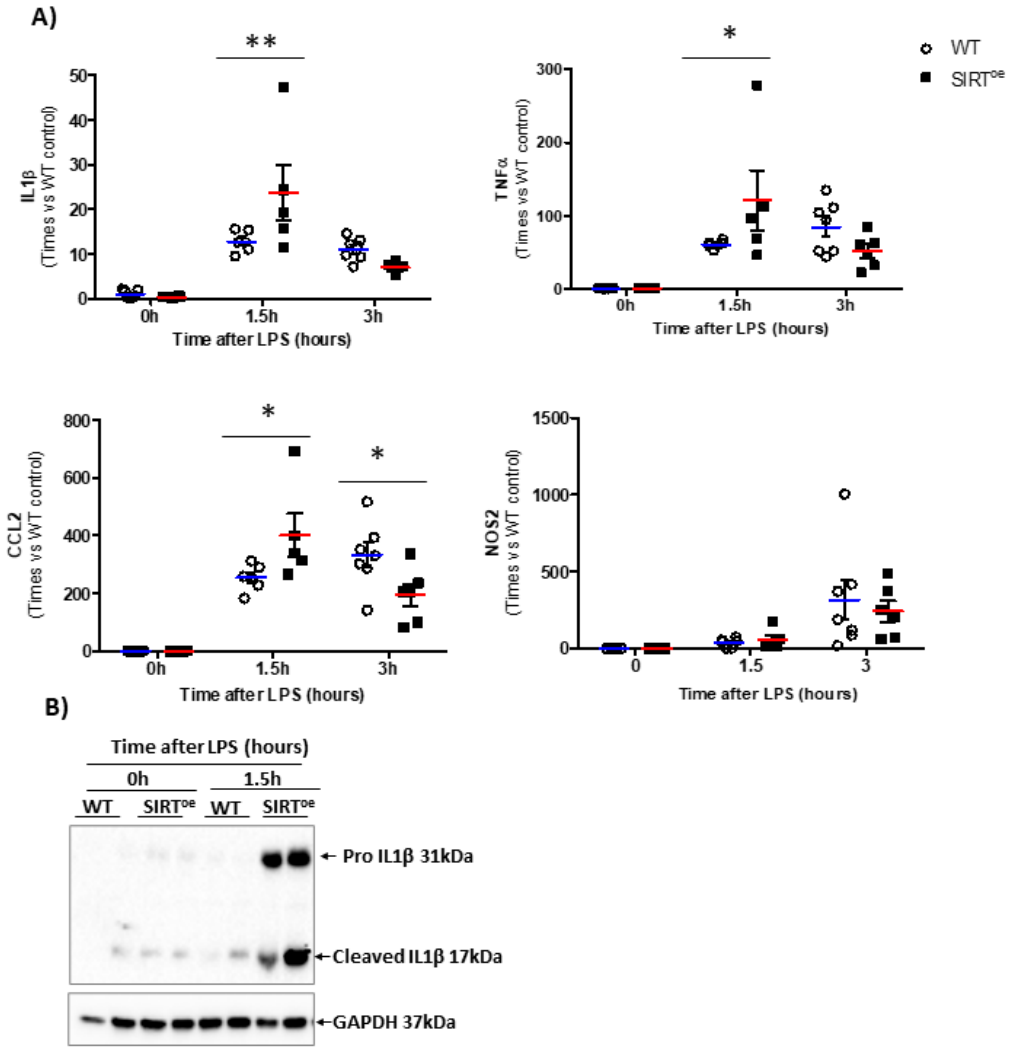


Figure 4.8 SIRT1 overexpression promotes liver damage and inflammation in response to LPS*. A) mRNA from livers from mice was analysed by qPCR. Gene expression of pro-inflammatory cytokines is significantly higher in SIRT^{oe} mice compared to WT mice. IL1ϐ and TNFα expression is significantly increased 1.5 hours after LPS challenge. CCL2 expression is significantly increased 1.5 hours and significantly decreased 3 hours after LPS challenge. B) SIRT1 overexpression in the liver promotes Nlrp3 inflammasome activation as seen by increased levels of cleaved IL1ϐ 1.5 hours after LPS administration. Western blot shows representative results of n=6 mice. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 (SIRT^{oe} vs WT littermate). Error bars represent standard error of the mean (n=at least 4 mice).*

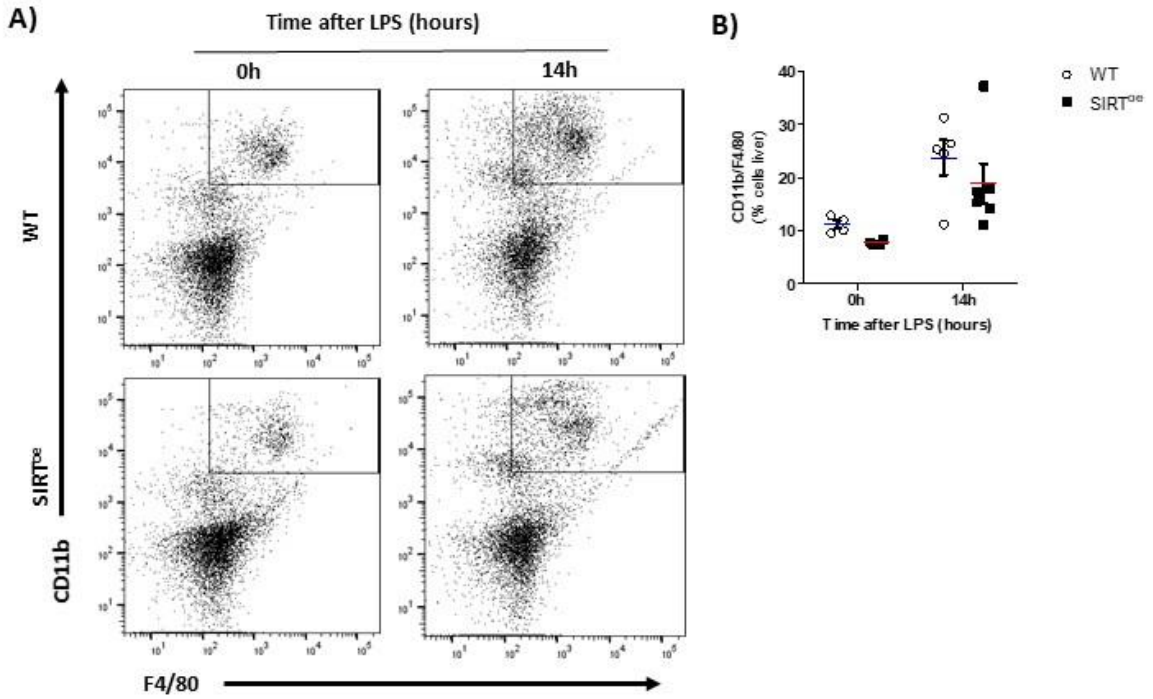


Figure 4.9 SIRT1 overexpression has no effect on liver macrophage recruitment to response to LPS challenge. *A) FACS analysis on immune cells isolated from the liver shows comparable macrophage numbers in both genotypes 14 hours after LPS administration B) Quantification of FACS analysis. Error bars represent standard error of the mean (n=at least 4 mice).*

In conclusion, this study supports the LPS/GalN findings. Using LPS alone, here we show increased liver damage, inflammation and Nlrp3 inflammasome activation in SIRT^{oe} animals.

4.2.2 Macrophage SIRT1 overexpression associates with pro-inflammatory polarization and Nlrp3 inflammasome activation in response to LPS *in vitro*

4.2.2.1 SIRT1 overexpression in BMDM correlates with increased inflammation and Nlrp3 inflammasome activation in response to LPS challenge

In this chapter we have shown that SIRT^{oe} animals have increased liver damage and inflammasome activation in response to LPS challenge, using two models of i.p. injection with LPS/GalN and LPS only.

The liver has the most macrophages out of any solid organ of the body but despite this macrophages comprise a small percentage of the liver's total cell pool as 90% of the liver's cells are hepatocytes (Krenkel & Tacke, 2017). Our SIRT^{oe} animals constitutively overexpress SIRT1 and we have previously described that SIRT1 overexpression promotes hepatocyte apoptosis *in vivo* in

response to cholestasis induced by BDL and *in vitro*, SIRT1 overexpression sensitised hepatocytes to apoptosis in response to bile acid treatment (Blokker et al., 2018). Therefore SIRT1 overexpression in hepatocytes could also sensitise hepatocytes to apoptosis in response to endotoxin. To determine whether this phenotype of SIRT^{oe} animals was driven by macrophage-mediated inflammation and not a result of increased hepatocyte apoptosis, we performed bone marrow derived macrophage (BMDM) experiments and challenged BMDM from WT and SIRT^{oe} mice with LPS *in vitro*.

The bone marrow is a site of haematopoiesis which contains myeloid cell progenitors that can be differentiated into macrophages. The bone marrow of WT and SIRT^{oe} animals was isolated from the femur and tibia and was plated with M-CSF for seven days to stimulate the differentiation of macrophages (figure 4.10). Cells were then seeded for experiments and stimulated with 100ng/ml of LPS for up to 24 hours.

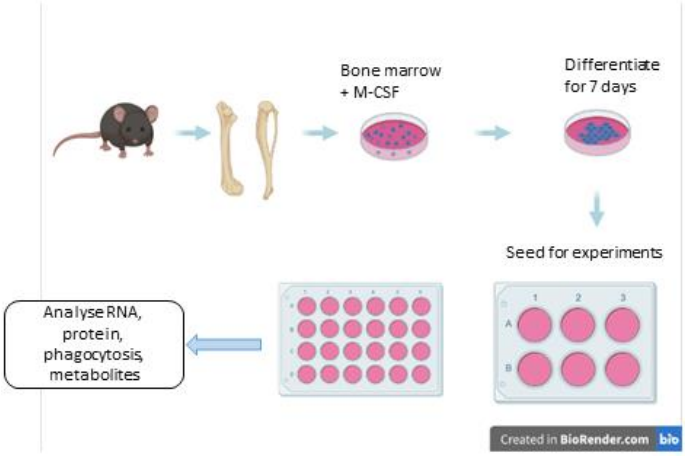


Figure 4.10 Schematic representation of bone marrow derived macrophage (BMDM) differentiation. *Bone marrow was flushed from the femur and tibia of mice. Cells were plated with M-CSF and left to differentiate for 7 days. Macrophages were then collected and seeded for experiments.*

The expression of SIRT1 mRNA was upregulated in WT BMDM in response to LPS (figure 4.11). This correlated with the result of figure 4.7A, where SIRT1 expression was upregulated in the livers of WT mice in response to LPS challenge. BMDM from SIRT^{oe} mice showed an increased pro-inflammatory phenotype at the mRNA level with increased gene expression of the pro-inflammatory cytokines TNFα, IL6 and NOS2 in response to LPS treatment (figure 4.12). In accord with the LPS/GalN study, BMDM from SIRT^{oe} macrophages showed increased gene expression levels

of Nlrp3 and IL1β (figure 4.12). The *in vitro* results show that SIRT1 overexpression promotes a pro-inflammatory phenotype in macrophages.

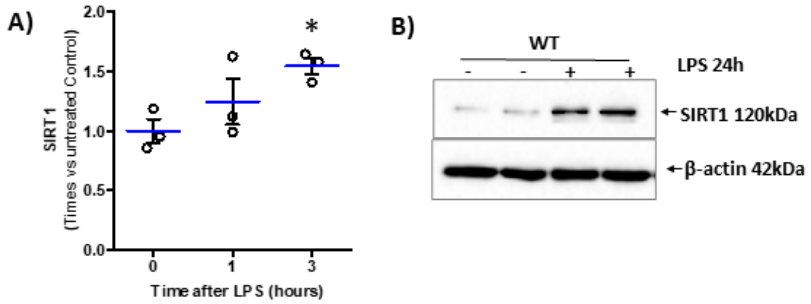


***Figure 4.11 SIRT1 expression is upregulated in WT BMDM in response to LPS**. A) WT BMDM upregulate SIRT1 gene expression 3 hours after LPS treatment. Significance was determined by Student's t test. Error bars represent standard error of the mean (n=3). * P ≤ 0.05. Data is representative of three independent experiments. B) WT BMDM upregulate SIRT1 expression in response to 24 hours of LPS challenge. Western blot is a representative image of three independent experiments.*

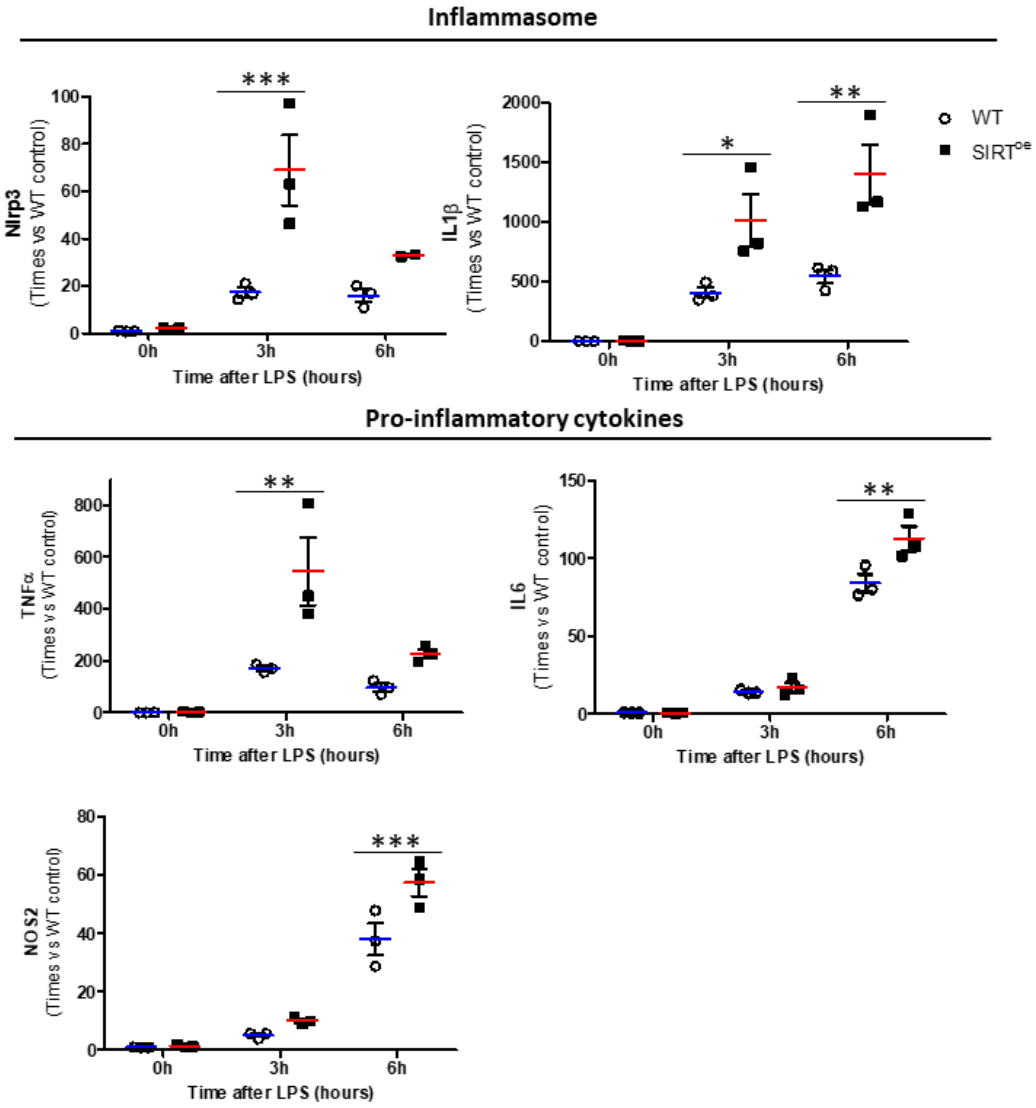


Figure 4.12 SIRT1 overexpression in BMDM promotes a pro-inflammatory phenotype in response to LPS. *Gene expression of pro inflammatory cytokines is significantly increased in macrophages from SIRT^{oe} mice upon LPS challenge. Nlrp3 gene expression is increased 3 hours after LPS challenge. IL1ϐ is increased 6 hours after LPS challenge. These genes indicate increased activation of the Nlrp3 inflammasome in BMDM from SIRT^{oe} mice. TNFα is increased 3 hours after LPS challenge. IL6 and NOS2 are increased 6 hours after LPS challenge. Significance was determined by 2-way ANOVA with Bonferroni post test. Error bars represent standard error of the mean (n=3). * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 (SIRToe vs WT littermate). Data is representative of three independent experiments.*

Our *in vitro* data support our *in vivo* observations where we showed that SIRT1 overexpression promotes liver damage in response to LPS treatment.

4.2.2.2 Inflammation in SIRT1 overexpressing macrophages is independent of NFκB

After establishing that SIRT1 overexpression in macrophages promotes a pro-inflammatory phenotype in response to LPS we continued *in vitro* studies to investigate mechanisms which could underpin this function.

The pro-inflammatory phenotype of BMDM from SIRT^{oe} mice described above supports some findings (B. Zhou et al., 2019) but not others, as some authors (Schug et al., 2010; Shen et al., 2009; Yeung et al., 2004; Yoshizaki et al., 2009) established that SIRT1 promotes an anti-inflammatory phenotype in macrophages due to the deacetylation function of SIRT1 on the RelA/p65 subunit of NFκB. The translocation of NFκB from the cytoplasm to the nucleus in response to endotoxin, promotes the transcription of pro-inflammatory cytokine genes (O'Neill et al., 2013). To understand the pro-inflammatory phenotype of SIRT^{oe} macrophages, we investigated NFκB localisation in WT and SIRT^{oe} BMDM in response to LPS treatment using p65 immunofluorescence microscopy. Here we show that nuclear translocation of p65 in SIRT^{oe} BMDM is delayed in response to LPS treatment compared to WT BMDM (figure 4.13), suggesting that the pro-inflammatory phenotype that we observed is independent of NFκB. These findings were confirmed by further immunofluorescence experiments from the Beraza laboratory (N. Beraza, personal communication).

Activation of the inflammasome can be promoted by phosphorylation of Nlrp3 by activated JNK (Song et al., 2017). We found that phosphorylation of JNK in response to LPS occurred earlier in SIRT^{oe} BMDM compared to WT (figure 4.14). We hypothesise that increased JNK activity could be one way that SIRT1 overexpression promotes inflammasome activation.

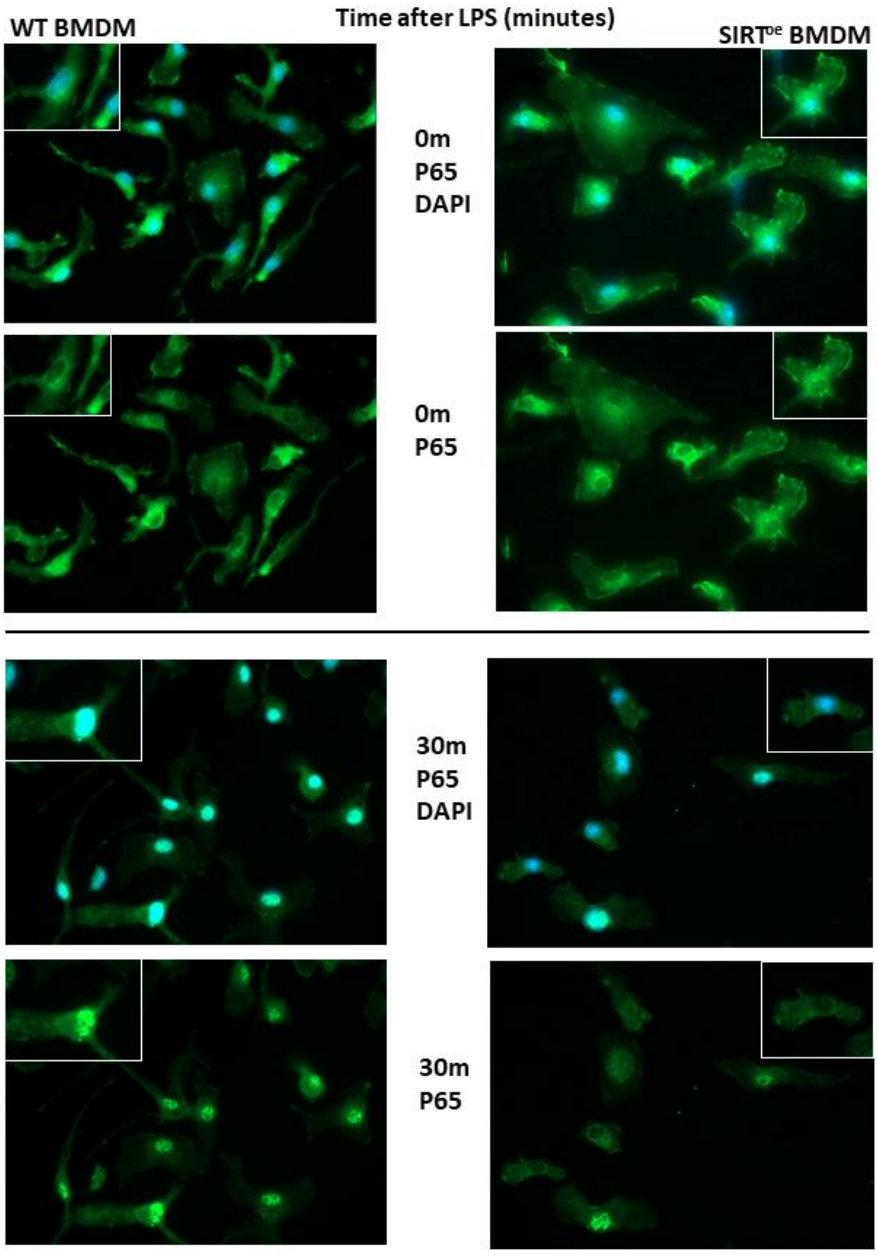


Figure 4.13 The pro-inflammatory phenotype of BMDM from SIRT1 overexpressing mice is independent of NFκB. *Immunofluorescence staining of P65 (green) and DAPI (blue) shows that nuclear translocation of p65 is delayed in SIRT1 overexpressing BMDM compared to WT 30 minutes after LPS treatment. Images are representative of two independent experiments and were taken using the 40x magnification.*

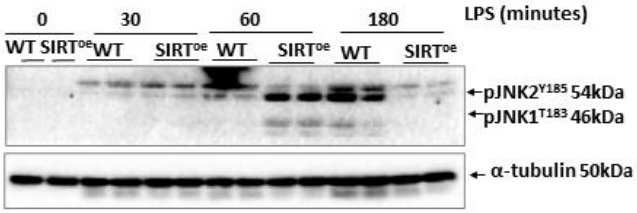


Figure 4.14 JNK signaling is upregulated in BMDM from SIRT1 overexpressing mice in response to LPS. *Phosphorylation of JNK occurs earlier in SIRT1 overexpressing BMDM than WT in response to LPS. JNK phosphorylation in SIRT^{oe} mice occurs 60 minutes after LPS treatment. Image is representative of three independent experiments.*

Additionally, the activation of the Nlrp3 inflammasome can be promoted by mTOR (Moon et al., 2015). When the mTOR pathway is active, increased S6K activity can result in increased S6 phosphorylation. We checked for levels of pS6 protein in BMDM from WT and SIRT^{oe} mice in response to LPS treatment and found that in SIRT^{oe} mice, phosphorylation of S6 occurred earlier than in WT BMDM (figure 4.15).

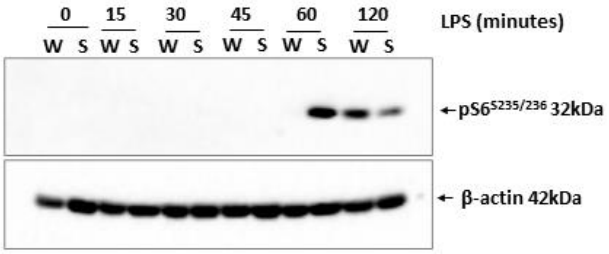


Figure 4.15 SIRT1 overexpression in BMDM promotes S6 phosphorylation in response to LPS*. There are increased levels of pS6 in from SIRT^{oe} BMDM compared to WT in response to LPS treatment. Western blots are representative of three independent experiments. (W-WT) (S-SIRT^{oe}).*

Overall, our *in vitro* results support previous findings that NFκB activity is reduced in the context of SIRT1 overexpression (Yeung et al., 2004), however we found that the nuclear translocation of NFκB in SIRT^{oe} cells is reduced and not completely inhibited as previously described by Yeung and colleagues. Also, our results suggest that increased mTOR activity could result in increased inflammation in SIRT^{oe} macrophages (Weichhart et al., 2015).

4.2.2.3 Autophagy is defective in SIRT1 overexpressing macrophages

The mTOR pathway is involved in many processes including the inhibition of autophagy (J. Kim et al., 2011). The activation of the Nlrp3 inflammasome can also occur when autophagy is impaired (C. S. Shi et al., 2012). Thus, we investigated autophagy in BMDM from WT and SIRT^{oe} mice in response to LPS treatment.

Autophagy is a cellular process where damaged organelles are degraded within low pH structures termed autolysosomes (Yang & Klionsky, 2010). The progression of autophagy, termed autophagy flux, can be investigated experimentally by inhibiting the fusion of autophagosomes and

lysosomes and investigating the accumulation of LC3I and its PE lipidated form LC3 II. Ammonium chloride and leupeptin are inhibitors of autophagosome fusion with lysosomes and allow the visualisation of the accumulation of LC3 I and LC3 II by western blotting (Mizushima & Yoshimori, 2007). BMDM from SIRT^{oe} mice had less LC3 II and more LC3 I in the presence of LPS, ammonium chloride and leupeptin, to stop the autophagy flux suggesting that either autophagosome fusion does not take place in BMDM from SIRT^{oe} mice, or that autophagosome fusion occurs much later (figure 4.16).

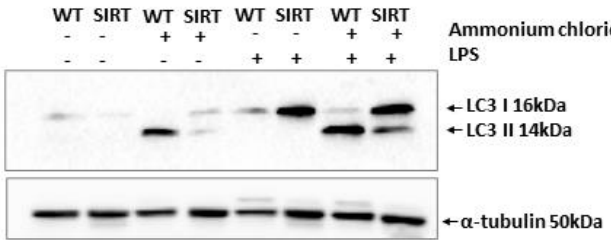


Figure 4.16 Autophagosome formation is disrupted in BMDM from SIRT^{oe} mice as seen by reduced levels of LC3 II. *Western blotting showing reduced levels of LC3 II in BMDM from SIRT^{oe} mice. Cells were treated with ammonium chloride and leupeptin, and, or LPS, for 3 hours. Image is representative of three independent experiments.*

These findings were surprising as SIRT1 has been described to promote autophagy. One of the ways in which SIRT1 initiates autophagy is via the activation of liver kinase B1 (LKB1), which phosphorylates and activates AMPK (F. Lan et al., 2008), which then intiates autophagy. Previously, BMDM from SIRT^{oe} mice were described to have increased basal levels of pAMPK (Park et al., 2017) and, our BMDM from SIRT^{oe} mice had increased levels of pAMPK compared to WT BMDM as basally and in response to LPS treatment, AMPK phosphorylation occurred earlier in SIRT^{oe} BMDM (figure 4.17), suggesting that autophagy initiation appeared to proceed at an increased rate in SIRT^{oe} BMDM than in WT.

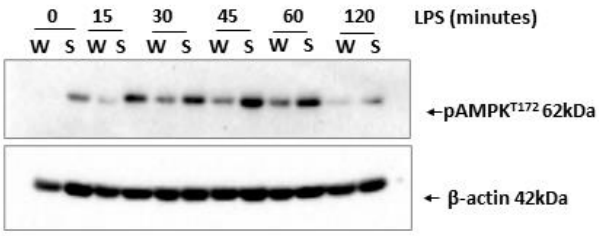


Figure 4.17 SIRT1 overexpression in BMDM promotes AMPK phosphorylation in response to LPS. *There are increased levels of pAMPK in from SIRT^{oe} BMDM compared to WT in response to LPS treatment. Western blots are representative of three independent experiments. (W-WT) (S-SIRT^{oe}).*

Autophagy is linked to phagocytosis, a key process in macrophage function. When pro-inflammatory macrophages perform phagocytosis, they are reprogrammed to an anti-inflammatory phenotype and the production of anti-inflammatory cytokines begins and pro-inflammatory effectors decreases (Freire-de-Lima et al., 2006; Johann et al., 2006; Ramachandran et al., 2012). Zymosan is a yeast preparation which is widely used to study phagocytosis (Underhill, 2003). Next, we investigated phagocytosis using BMDM from WT and SIRT^{oe} mice and zymosan beads (figure 4.25). We found that although BMDM from SIRT^{oe} mice were able to take up the zymosan beads, they did this significantly slower than WT macrophages (figure 4.18A). The pro-inflammatory phenotype that we have described in SIRT^{oe} mice could be caused by delayed phagocytic capacity of macrophages (Freire-de-Lima et al., 2006; Johann et al., 2006; Ramachandran et al., 2012). Since the phagocytosis of the SIRT^{oe} macrophages is delayed, there could be a delay in the switch of pro-inflammatory macrophages to the anti-inflammatory phenotype.

These *in vitro* experiments suggest that SIRT1 overexpression in BMDM results in impaired autophagosome and lysosome fusion and this correlates with delayed phagocytic function of SIRT^{oe} BMDM.

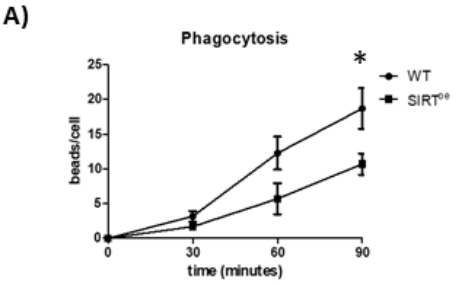
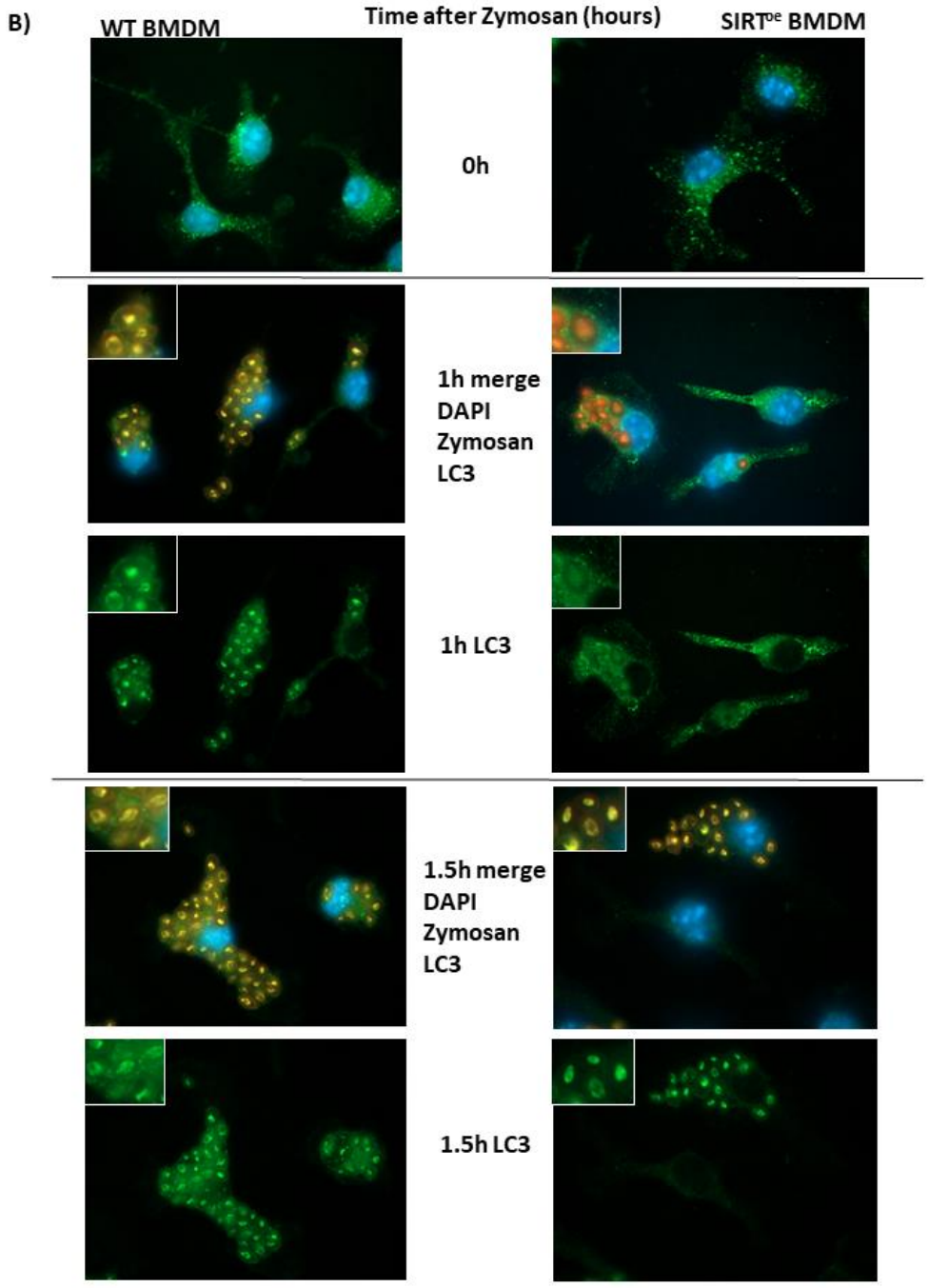


Figure 4.18 SIRT1 overexpression correlates with decreased phagocytic activity in BMDM. *A) Count of the total number of beads phagocytosed by BMDM. B) BMDM from WT mice took up zymosan beads faster than SIRT1 overexpressing macrophages. Zymosan (red) LC3 (green) DAPI (blue), 100x magnification. Data is representative of three independent experiments.*



4.2.2.4 SIRT1 overexpression promotes aerobic glycolysis and Krebs cycle breaks in BMDM in response to LPS

In this chapter we have described that macrophages from SIRT^{oe} mice are more activated than macrophages from WT mice and exhibit pro-inflammatory polarization characteristics in response to LPS challenge, including Nlrp3 inflammasome activation, impaired autophagy and reduced phagocytic activity. Metabolic states underpin macrophage activation and a broken Krebs cycle has been described to drive pro-inflammatory macrophage polarization (Ryan & O'Neill, 2020).

Next, to investigate the metabolic states of WT and SIRT^{oe} macrophages in response to LPS stimulation, we performed metabolite analysis of BMDM form WT and SIRT^{oe} mice in response to LPS stimulation for up to six hours using LC-MS/MS. We identified increased lactate levels in SIRT^{oe} BMDM (figure 4.19), indicating an increased aerobic glycolysis phenotype, characteristic of M(LPS) macrophages (Nonnenmacher & Hiller, 2018). We identified that BMDM from SIRT^{oe} mice had a significantly greater accumulation of citrate and succinate than WT BMDM (figures 4.19), two metabolites known to be accumulated in M(LPS) macrophages as a result of Krebs cycle breaks in response to LPS (Jha et al., 2015; Palmieri et al., 2020; Tannahill et al., 2013). These findings were confirmed by external collaborators who performed ^{13}C glucose tracing on BMDM isolated from WT and SIRT^{oe} in response to LPS and measured Krebs cycle metabolites using gas chromatography coupled with MS (K. Hiller, personal communication).

In summary, using metabolomic extraction of Krebs cycle metabolites, we identified that SIRT1 overexpression in macrophages promotes metabolic rewiring towards a pro-inflammatory metabolic state.

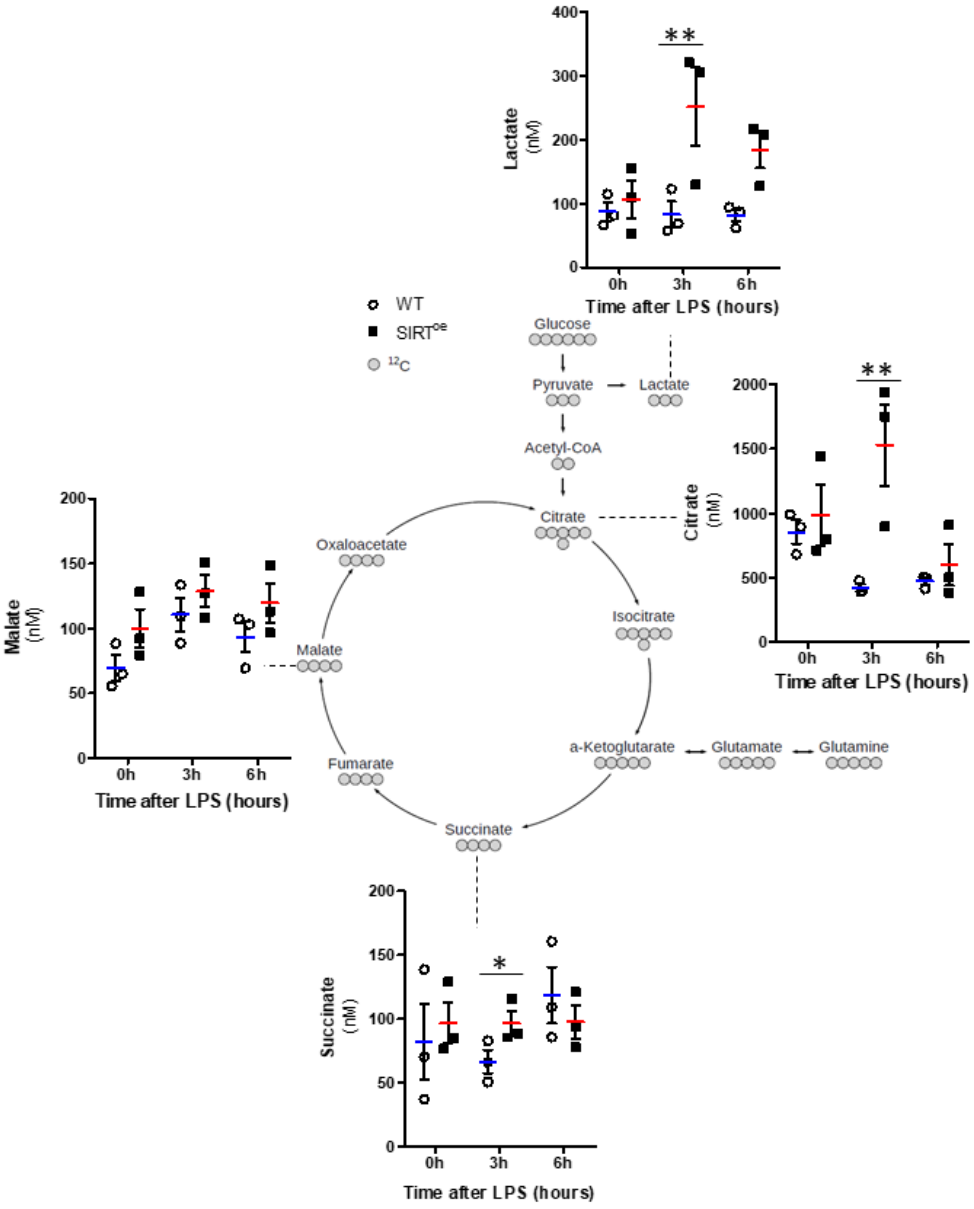


Figure 4.19 SIRT1 overexpression correlates with increased lactate, citrate and succinate production in BMDM. *Krebs cycle metabolite levels were determined in WT and SIRT^{oe} mice in response to 3 and 6 hours of LPS treatment using LC-MS/MS. SIRT1 overexpression in BMDM correlated with increased accumulation of lactate, citrate and succinate. Significance was determined by 2-way ANOVA with Bonferroni post test. Error bars represent standard error of the mean (n=3). * P ≤ 0.05 ** P ≤0.01 (SIRToe vs WT littermate). Data is representative of two independent experiments*.

4.3 Discussion

In this chapter we have demonstrated that SIRT1 overexpression in macrophages promotes macrophage activation in response to bacterial endotoxin. *In vivo*, we defined that constitutive SIRT1 overexpression contributes to liver damage and inflammation in response to LPS challenge.

In vitro, we established that SIRT1 overexpression promoted macrophage activation that associated to metabolic changes.

We found that SIRT1 overexpression correlated with increased liver damage and Nlrp3 inflammasome activation in response to endotoxin. Conversely, several other groups found that pharmacological SIRT1 activators protected rats (Farghali et al., 2019) and mice (D. Zhou et al., 2021) from LPS/GalN induced liver injury, highlighting the multifaceted role of SIRT1 in inflammation. The $SIRT^{oe}$ mice used in our study have previously been well characterised and the BAC system of SIRT1 overexpression was found to have no unspecific effects (Pfluger et al., 2008) therefore our mice which overexpress SIRT1 are more biologically relevant than pharmacological activators as common SIRT1 activators have been identified as being unspecific where resveratrol was shown to activate AMPK independently of SIRT1 (Beher et al., 2009) and a selection of common SIRT1 activators including SRT1720 and resveratrol were shown to affect over 100 other pathways including a range of enzymes, receptors and ion channels (Pacholec et al., 2010). While ours is the first study to use $SIRT^{oe}$ animals to study SIRT1 in response to endotoxin in the liver, another group used hepatocyte-specific SIRT1 knockout mice and LPS/GalN administration to investigate the role of SIRT1 in septic liver failure (Cui et al., 2016). Interestingly, these authors found that hepatocyte-specific SIRT1 depletion and pharmacological inhibition of SIRT1 with nicotinamide protected mice from septic liver damage in response to LPS/GalN and LPS/TNFα induced liver injury (Cui et al., 2016). The work of Cui and colleagues supports the detrimental role of SIRT1 in the liver which has been described by several studies (Blokker et al., 2018; Chen et al., 2011; L. Feng et al., 2021; García-Rodríguez et al., 2014).

While macrophages are responsible for inflammation in the liver in response to LPS, arguably, in accord with Cui et al., the increased liver damage described in SIRT1 overexpressing animals in response to endotoxin in this thesis could be a result of SIRT1 overexpression sensitising hepatocytes to LPS induced apoptosis. Our mice constitutively overexpress SIRT1, therefore, to confirm that the increased pro-inflammatory response of $SIRT^{oe}$ macrophages in response to LPS drove the increased liver inflammation in these animals *in vivo*, we isolated BMDM and treated the cells with LPS *in vitro*. Our *in vitro* results showed that SIRT1 overexpression promoted macrophage activation in response to LPS. In summary, our *in vivo* and *in vitro* data support that SIRT1 overexpression promotes inflammation in mouse livers and mouse BMDM.

Our *in vitro* data showed that SIRT1 overexpression in BMDM promotes the gene expression of pro-inflammatory cytokines in response to LPS. The finding that SIRT1 overexpression promoted a pro-inflammatory macrophage phenotype in response to endotoxin was in accord with Zhou et al.'s study of SIRT1 overexpression using SIRT1 transfection in the immortalised RAW 264.7

cell line (B. Zhou et al., 2019). We have also observed that transfection of SIRT1 into RAW 264.7 promotes a pro-inflammatory phenotype in response to LPS challenge (data not shown). Interestingly, Park and colleagues observed that BMDM from SIRT^{oe} mice showed a reduction of pro-inflammatory cytokine gene expression 12 hours after LPS treatment (Park et al., 2017). Investigation of gene expression of early inflammatory response cytokines such as TNFα, IL1β and CCL2 is advised to be observed at earlier timepoints as these genes are downregulated with prolonged LPS exposure (Lauterbach et al., 2019). The significant upregulation of pro-inflammatory cytokine genes of BMDM isolated from SIRT^{oe} animals which we describe in this chapter was observed at up to six hours of LPS exposure. It is likely that as BMDM from SIRT^{oe} mice upregulated the expression of pro-inflammatory cytokine genes earlier than BMDM from WT mice Park et al. could have missed this response if they investigated the gene expression only at 12 hours post LPS stimulation (Park et al., 2017).

However, various groups demonstrated that SIRT1 inhibits inflammation in macrophages by deacetylating the RelA subunit of NFκB, thus causing the transcription factor's inhibition (Schug et al., 2010; Yeung et al., 2004). In their mechanistic study, Schug and colleagues used a combination of immortalised lung cancer cell lines which had a high expression level of SIRT1 and transfection of SIRT1 into HEK293 cells to elucidate how SIRT1 deacetylates K310 of NFκB (Yeung et al., 2004). Yeung et al. defined that this deacetylation of NFκB by SIRT1 stops the translocation of the pro-inflammatory transcription factor from the cytoplasm to the nucleus and hence the transcription of pro-inflammatory cytokine genes (Yeung et al., 2004). Subsequent studies applied this mechanism to explain the anti-inflammatory effect of SIRT1 on macrophage function observed when SIRT1 was knocked down in macrophage cell lines (Shen et al., 2009). A subsequent study from the Li group showed that the knockout of SIRT1 from cells of the myeloid lineage resulted in hyperacetylation of NFκB in BMDM (Schug et al., 2010), supporting the mechanism of Yeung and colleagues (Yeung et al., 2004) however, this study showed the effect of SIRT1 macrophage knockout and not overexpression on NFκB function. Here we show that the nuclear translocation of NFκB is delayed in BMDM from SIRT^{oe} animals in response to LPS. These findings were further confirmed by the Beraza laboratory (N. Beraza, personal communication). Our study is the first to show the effect of SIRT1 on NFκB in a constitutively overexpressing primary murine macrophage cell type, therefore our work is more biologically relevant than previous studies which used transfection into epithelial cell lines to study SIRT1 overexpression (Yeung et al., 2004). While immortalised cell lines have been a useful tool for biological studies, it is well established that immortalised cell lines undergo continuous cell cycling which can lead to undefined modifications therefore primary cells are an *in vitro* model that is more similar to the organism (Assouvie et al.,

2018). HEK293 cells, for example, while described as kidney epithelial cells, have been described as being more close to a neuronal cell type (Shaw et al., 2002). Despite the differences in the experimental design and findings, our work where we describe that NFκB nuclear translocation is not inhibited by SIRT1 overexpression, as demonstrated by Yeung et al. (Yeung et al., 2004), but delayed, still suggests that the pro-inflammatory phenotype we observe in $SIRT^{oe}$ macrophages is independent of NFκB.

Although NFκB has been described to promote the transcription of Nlrp3 during the priming step of inflammasome activation, in our $SIRT^{oe}$ macrophages where nuclear NFκB translocation is delayed, there are other pathways which could promote Nlrp3 inflammasome activation, including phosphorylation of the inflammasome by JNK (Swanson et al., 2019). In response to LPS, JNK is phosphorylated and activated by TAK1 (O'Neill et al., 2013). One of the ways in which pJNK contributes to inflammation is by phosphorylation and activation of components of the Nlrp3 inflammasome (Hara et al., 2013; Song et al., 2017). Phosphorylation of Nlrp3 at S194 by JNK is essential to initiate the priming step of Nlrp3 inflammasome assembly in response to LPS (Song et al., 2017). Additionally, JNK has been described to phosphorylate the ASC component and this phosphorylation by JNK facilitates Nlrp3 inflammasome assembly (Hara et al., 2013). In both, our *in vivo* and *in vitro* models we found increased protein levels of pJNK one hour after LPS stimulation in livers and BMDM of $SIRT^{oe}$ mice, respectively. Phosphorylation of JNK occurred earlier in $SIRT^{oe}$ than in WT animals. A homolog of SIRT1, SIRT2, has been described to promote the phosphorylation and activation of JNK by deacetylation of K153 (Sarikhani et al., 2018). In our model of SIRT1 overexpression, SIRT1 may have a similar effect and may be promoting the upregulation of inflammasome activity through its increased phosphorylation by JNK.

While increased phosphorylation of inflammasome components by JNK could be one mechanism by which SIRT1 overexpression in macrophages may contribute to increased Nlrp3 inflammasome activation, another mechanism which contributes to IL1β production by the Nlrp3 inflammasome is a defective autophagy pathway (C. S. Shi et al., 2012).

Autophagy is key to the resolution of inflammation, one of the ways in which autophagy contributes to this is by the degradation of inflammasomes in autophagosomes (C. S. Shi et al., 2012). The impaired autophagosome formation of $SIRT^{oe}$ macrophages could be one explanation of increased inflammasome activation which we have seen in our models. Our data suggest that SIRT1 overexpression prevents autophagosomal fusion. Therefore, there could be a lack of Nlrp3 inflammasome degradation in macrophages of $SIRT^{oe}$ animals, which would be operating normally in WT mice. This could lead to the increased IL1β production, inflammatory liver damage and

pyroptosis that we observe *in vivo* in LPS treated SIRT^{oe} mice. This is supported by studies which found that autophagy is impaired during severe sepsis (Y. Feng et al., 2019).

In this chapter we show that macrophages from SIRT^{oe} animals have defective autophagosome formation, as evidenced by decreased LC3 I to LC3 II conversion in the presence of inhibitors of autophagosome fusion in response to LPS (Mizushima & Yoshimori, 2007). These findings were further confirmed by other colleagues from the Beraza group who further demonstrated that autophagy is impaired, as they described that, the adaptor protein p62, a marker of failed cargo degradation due to impaired autophagy (Katsuragi et al., 2015), accumulated in SIRT^{oe} BMDM in response to LPS treatment (N. Beraza, personal communication). Although SIRT1 has been described to promote autophagy initiation, autophagosome maturation and autophagosome formation (R. Huang, Xu, Wan, et al., 2015; Lee et al., 2008; Sun et al., 2015), our findings support recent work by Feng and colleagues who described that SIRT1 deacetylates p62 at K295 and this deacetylation promotes p62 accumulation and mTOR activation in a mouse model of hepatocellular carcinoma (L. Feng et al., 2021).

One of the ways which SIRT1 has been described to promote autophagy initiation is through its activation of AMPK during low energy states. SIRT1 deacetylates LKB1 which becomes activated and phosphorylates and activates AMPK (F. Lan et al., 2008), pAMPK then phosphorylates and activates the autophagy initiator ULK1 at S317 and S555 (J. Kim et al., 2011). In accord with this, we found that BMDM from SIRT^{oe} animals had increased levels of pAMPK basally, as described previously (Park et al., 2017), and in response to LPS however, autophagosome fusion appeared reduced and autophagy defective. mTOR is a protein complex which is known to inhibit autophagy (J. Kim et al., 2011). Below we discuss how SIRT1 overexpression could be inhibiting autophagy through mTOR activation.

The literature describing the role of SIRT1 in phagocytosis has been contradictory to date. Crotty Alexander and colleagues described that pharmacological activation of SIRT1 with resveratrol or its knockout from primary murine macrophages of SIRT$^{mye-/-}$ animals had no effect on phagocytosis (Crotty Alexander et al., 2013). Conversely, Zhang et al. showed that SIRT1 overexpression in peritoneal macrophages promoted their phagocytic function (R. Zhang et al., 2010). Most recently, in agreement with Zhang and colleagues, SIRT1 overexpression promoted the ability of endometrial microvascular endothelial cells to phagocytose platelets (Y. Lan et al., 2021). It is interesting to note that the studies of Zhang and Lan used *in vitro* transfection to overexpress SIRT1 in their cells. Our model of differentiating BMDM from SIRT^{oe} mice is more biologically relevant to study SIRT1 overexpression *in vitro* than transfection as transfection may introduce off target effects (Jacobsen et al., 2009) while tissues and cells of SIRT^{oe} animals have been well

characterised by the Serrano group and the BAC insert was only found to upregulate SIRT1 expression without any off target effects (Pfluger et al., 2008). Knockout of the SIRT1 homolog, SIRT2, has been described to promote phagocytosis in BMDM and the authors hypothesized that SIRT2 may regulate phagocytosis via mechanisms involving autophagy (Ciarlo et al., 2017). In our model of macrophage SIRT1 overexpression, SIRT1 may delay phagocytosis through delayed phagosome formation because autophagy is delayed in SIRT^{oe} BMDM through mechanisms involving mTOR, as discussed below.

Being an important metabolic regulator, SIRT1 has also been described to interact with the mTOR complex (Ghosh et al., 2010) and components of the mTOR pathway (Hong et al., 2014; Ikenoue et al., 2008; Sundaresan et al., 2011). mTOR is a known inhibitor of autophagy (J. Kim et al., 2011), and interestingly, the Beraza group identified that SIRT^{oe} BMDM have increased phosphorylation of the ULK1 complex at S757, which is phosphorylated by mTOR (N. Beraza, personal communication). SIRT1 has been described to promote the activation of the mTOR pathway through interactions with mTOR inhibitors (Ikenoue et al., 2008) and activators (Hong et al., 2014; Sundaresan et al., 2011). Responding to LPS challenge is a bioenergetically expensive process which requires rapid production of pro-inflammatory cytokines (Lelouard et al., 2007). mTOR promotes translation which is essential for production of pro-inflammatory mediators during early inflammatory response by macrophages (Weichhart et al., 2015). Interestingly, SIRT1 was found to promote inflammation in the lungs in response to i.p. administration of LPS through a mechanism involving the activation of mTOR and its downstream target 4E-BP1 (J. Huang et al., 2017). Phosphorylated S6 is a downstream target of the mTOR pathway and, in agreement with SIRT1 promoting mTOR pathway activation, we found that SIRT1 overexpression promoted earlier S6 phosphorylation in BMDM in response to LPS and in livers of LPS/GalN treated mice. S6 is phosphorylated by S6K1 and SIRT1 has been described to deacetylate S6K1 and promote its activity (Hong et al., 2014). In this thesis we did not identify whether SIRT1 overexpression promotes S6 phosphorylation through the increased activity of S6K1 or upstream regulators of the mTOR pathway while this could be done by performing further mechanistic studies, as discussed in future work.

mTORC1 has been described to promote Nlrp3 inflammasome activation by upregulating macrophage glycolysis (Moon et al., 2015). Our data suggest that SIRT1 overexpression in macrophages contributes to liver damage via the Nlrp3 inflammasome, which is likely activated by SIRT1 promoting the activation of the mTOR pathway. This has been confirmed by work from the Beraza laboratory, using the mTOR pathway inhibitor rapamycin the group identified that IL1β production in SIRT^{oe} BMDM is mTORC1 dependent (N. Beraza, personal communication). As well as

inhibiting the initiation of autophagy, mTOR has been described to inhibit autophagosome maturation (Y. M. Kim et al., 2015) and lysosome biogenesis (J. Zhou et al., 2013). The activation of mTOR by SIRT1 in this context may be contributing to the inhibition of autophagosome maturation or the inhibition of the fusion of autophagosomes and lysosomes, and hence decreased phagocytic capacity which we see in SIRT^{oe} macrophages. This could be contributing to the pro-inflammatory phenotype of these cells. The increase of AMPK activity could be a compensatory response by SIRT^{oe} macrophages to try and restore autophagy flux in these cells.

Metabolism is the key process of any living cell and is known to regulate the mTOR pathway and autophagy. In the last decade metabolism has been identified as the key process which underpins the plasticity of the activation states of all immune cells, including macrophages (Van den Bossche et al., 2017). We found increased lactate production in BMDM from SIRT^{oe} mice compared to WT mice in response to LPS treatment. While mTOR has been described to promote glycolysis through the activation of the transcription factor HIF1α, and increased mTOR mediated glycolysis could explain the pro-inflammatory phenotype of SIRT1 overexpressing macrophages, the Krebs cycle has been described to underpin macrophage polarization states (Ryan & O'Neill, 2020). Pro-inflammatory macrophages primarily utilise aerobic glycolysis for their bioenergetic needs (Nonnenmacher & Hiller, 2018). The Krebs cycle of pro-inflammatory macrophages is disrupted and the accumulating metabolites are utilised to support the pro-inflammatory response (Ryan et al., 2019).

The first Krebs cycle break occurs at the enzyme IDH and results in the accumulation of citrate (Jha et al., 2015). This view has recently been challenged by Palmieri and colleagues who propose that the first Krebs cycle break instead occurs at the previous step of the Krebs cycle at the enzyme aconitase (Palmieri et al., 2020), however both breaks result in the accumulation of citrate.

The second Krebs cycle break is found at SDH and results in the accumulation of succinate, a metabolite essential for IL1β production (Tannahill et al., 2013) and the production of mitochondrial ROS which inhibit the production of anti-inflammatory cytokines and contribute to Nlrp3 inflammasome activation (Zas & Neill, 2020). We identified that the two Krebs cycle breaks at IDH and SDH were exaggerated in SIRT^{oe} macrophages, leading to significantly greater accumulation of citrate and succinate in these cells in response to LPS. These findings were confirmed by external collaborators who used a different method of metabolite analysis (K. Hiller, personal communication). Interestingly, succinate accumulation has been found to promote Nlrp3 inflammasome activation and IL1β production through increased HIF1α activity (Y. Li et al., 2016). The increased succinate accumulation in SIRT^{oe} macrophages could activate the Nlrp3 inflammasome via this HIF1α mediated mechanism. Additionally, succinate can accumulate in

macrophages when fumarate is utilised for reverse electron transport (RET) during the pro-inflammatory response (Murphy & O'neill, 2018). ROS produced by RET can act as an activation signal for Nlrp3 inflammasome assembly (Hughes & O'Neill, 2018). In SIRT^{oe} macrophages, ROS generated in this way could contribute to the increased Nlrp3 inflammasome activation and subsequent inflammation which we described in this chapter. The metabolomics data strongly support the pro-inflammatory phenotype of SIRT1 overexpressing macrophages in response to LPS challenge and suggest that succinate accumulation could be a potential mechanism for Nlrp3 inflammasome activation in the context of SIRT1 overexpression in macrophages.

Our discovery that SIRT1 overexpression correlates with a disrupted Krebs cycle is fascinating. While no role to date has been described for SIRT1 in macrophage metabolism, various groups have described that SIRT1 promoted mitochondrial respiration in a range of non-myeloid cell lines (P. Bai et al., 2011; Rodgers et al., 2005; Zaini et al., 2018), therefore one could hypothesise that SIRT1 overexpression would promote an intact Krebs cycle and hence an anti-inflammatory phenotype in macrophages. Additionally, AMPK and mTOR, the activity of which was upregulated in SIRT^{oe} macrophages, have also been described to promote mitochondrial biogenesis (Morita et al., 2013; Zong et al., 2002). In this work we did not measure OXPHOS in SIRT^{oe} BMDM therefore we do not know if their mitochondrial respiration is intact or disrupted. Recently, several groups observed that pro-inflammatory macrophages that respire using both an intact OXPHOS and aerobic glycolysis are hyperinflammatory compared to pro-inflammatory macrophages that respire using aerobic glycolysis alone (Di Gioia et al., 2020; Lauterbach et al., 2019). Interestingly, in the study by the Hiller group, the authors observed that pro-inflammatory macrophages possessed the same Krebs cycle breaks, measured by accumulating citrate and succinate, as well as increased aerobic glycolysis and intact mitochondrial respiration (Lauterbach et al., 2019). If SIRT1 overexpression promotes mitochondrial respiration as is described in the literature, the use of aerobic glycolysis, OXPHOS and a disrupted Krebs cycle could all be contributing to the hyperinflammatory phenotype that we observe in our SIRT1 overexpressing cells. Additionally, increased mitochondrial biogenesis by SIRT1 overexpression could mean that SIRT^{oe} macrophages have more mitochondria available for ROS production, which could in turn be driving Nlrp3 inflammasome activation (Hughes & O'Neill, 2018).

Taken together, the results of this chapter suggest that macrophages from SIRT^{oe} mice are more pro-inflammatory than WT macrophages in response to LPS. Using a combination of *in vivo* and *in vitro* approaches to study SIRT1 overexpression in macrophage function in response to endotoxin, here we propose that SIRT^{oe} macrophages promote liver inflammation in response to LPS challenge using a mechanism involving the upregulation of mTOR signaling which results in

increased activation of Nlrp3 inflammasome, underpinned by defective autophagy and a broken Krebs cycle. This could contribute to aggravating liver injury, as observed in SIRT^{oe} mice in response to LPS *in vivo.*

4.5 Future work

In this chapter we showed that SIRT^{oe} animals had increased liver damage in response to two models of endotoxin challenge and we proposed that the detrimental phenotype observed was driven by macrophage SIRT1 overexpression. To definitively confirm the role of SIRT1 overexpression in macrophages, and not another cell type, in the pathological response to endotoxin treatment, macrophage transplantation could be performed of WT and SIRT^{oe} isolated macrophages into recipient animals. After successful engraftment, the animals could be treated with LPS/GalN to investigate the role of SIRT^{oe} macrophages in endotoxin induced liver damage. If the livers of animals transplanted with SIRT^{oe} macrophages have more damage and inflammation, this will definitively confirm that macrophage SIRT1 overexpression promotes liver injury in response to endotoxin.

In this chapter, we identified that SIRT^{oe} animals have increased inflammasome activation in the liver in response to LPS. An *in vivo* experiment to definitively confirm the involvement of the Nlrp3 inflammasome in the pathogenesis of septic liver injury in SIRT^{oe} animals could be carried out where mice are pre-treated with the Nlrp3 inflammasome inhibitor MCC950 prior to LPS/GalN challenge. If liver damage appears reduced, this would confirm the role of the Nlrp3 inflammasome. BMDM from SIRT^{oe} mice could be pre-treated with MCC950 *in vitro*, prior to LPS stimulation, and the expression of Nlrp3 and IL1β in the presence of this inhibitor could be measured to confirm that the Nlrp3 inflammasome drives inflammation in SIRT1 overexpressing macrophages.

Finally, to investigate whether the knockout of SIRT1 in cells of the myeloid lineage, including macrophages, will have a protective effect in the liver in response to endotoxin SIRT$^{mye-/-}$ mice can be treated with LPS/GalN. If the livers are protected from liver damage and inflammation then this will confirm the detrimental role of SIRT1 overexpression in cells of the myeloid lineage in response to endotoxin.

Above we proposed several potential mechanisms of how macrophage SIRT1 overexpression could be promoting Nlrp3 inflammasome activation. We proposed increased mTOR activation, inhibited autophagy and metabolic rewiring could result in increased Nlrp3 inflammasome activation. The Beraza laboratory has performed mechanistic studies using rapamycin treatment on BMDM and identified that IL1β production in SIRT^{oe} macrophages is dependent on mTOR. In this thesis, we identified that SIRT1 overexpression could be promoting

inflammasome activation through activation of JNK and mTOR. To exclude the involvement of JNK, *in vitro* experiments of culturing BMDM from WT and SIRT^{oe} mice with the specific inhibitor of JNK (SP600125) and then LPS stimulation and investigation of Nlrp3 inflammasome activation would elucidate the mechanism of how SIRT1 overexpression promotes Nlrp3 inflammasome activation in macrophages. *In vivo*, pre-treatment of WT and SIRT^{oe} mice with SP600125, as well as with rapamycin, followed by LPS/GalN injection would confirm the biological relevance of the mechanism.

We identified that SIRT1 overexpressing macrophages have increased S6 phosphorylation, which is an indicator of increased mTOR pathway activity. While inhibition of mTOR with rapamycin was performed by the Beraza laboratory, whether SIRT1 overexpression affects S6K1 remains undefined. S6 is phosphorylated by S6K1 and S6K1 is a target of SIRT1 (Hong et al., 2014). Pre-treatment of BMDM with the S6K1 inhibitor PF-4708671 prior to LPS treatment will help to elucidate whether the increased inflammation observed in SIRT^{oe} mice is driven by S6K1 or is an upstream event.

In this chapter we described that SIRT^{oe} macrophages undergo metabolic changes in response to LPS treatment. While aerobic glycolysis and a broken Krebs cycle are hallmarks of pro-inflammatory macrophages (Van den Bossche et al., 2017), the metabolic effects are exaggerated in SIRT^{oe} cells. Further characterisation of the metabolic state of the SIRT^{oe} macrophages using Seahorse technology would determine the metabolic capacity of these cells. In this work we did not investigate the mitochondria of the SIRT^{oe} macrophages. Literature suggests that SIRT1 promotes mitochondrial respiration and biogenesis (P. Bai et al., 2011; Rodgers et al., 2005; Zaini et al., 2018). Experiments to investigate mitochondrial dynamics using mitotracker and mitochondrial potential using TMRM would elucidate this in BMDM from SIRT^{oe} animals.

This chapter focused on the effect of macrophage SIRT1 overexpression in response to LPS therefore we investigated the pro-inflammatory phenotype of macrophages and did not characterise whether SIRT1 overexpression affects the ability of macrophages to undergo polarization into an anti-inflammatory state. *In vitro* experiments using IL4 to induce an anti-inflammatory state of SIRT^{oe} BMDM and investigation of cytokine production would determine whether SIRT1 overexpression affects the resolution of inflammation.

Chapter 5 – Defining the role of myeloid SIRT1 overexpression in the pathogenesis of cholestasis

5.1 Introduction and aims

We have previously demonstrated that SIRT1 expression is upregulated in human and murine cholestasis and that SIRT1 overexpression correlated with increased inflammation and liver damage in mouse models of bile duct destruction (Blokker et al., 2018). Several studies demonstrated that monocyte-derived macrophages, rather than liver resident Kupffer cells, drive inflammation during cholestatic liver diseases (Guicciardi et al., 2018; Karlmark et al., 2009). Increased intestinal permeability is a common pathological feature in cholestasis (Di Leo et al., 2003; Feld et al., 2006), and in chapter 3 of this thesis we established that bacterial endotoxin and bile acids synergise to promote hepatocyte cell death during cholestasis. DAMPs from dying hepatocytes have been described to activate macrophages in response to liver damage (Canbay et al., 2003) and bacterial endotoxin could also promote macrophage activation when it reaches the liver.

In the previous chapter of this thesis, we showed that SIRT^{oe} animals had increased liver damage in response to bacterial endotoxin (LPS) *in vivo* and that SIRT1 overexpressing macrophages had a pro-inflammatory phenotype in response to LPS stimulation *in vitro*. Taking all these together, we hypothesise that SIRT1 overexpression in macrophages may contribute to the inflammatory response in the context of cholestatic liver disease.

The aim of this chapter is to define the role of SIRT1 overexpression in myeloid cells in the progression of cholestatic liver disease. First, we performed an *in vitro* study to demonstrate that SIRT1 overexpression is upregulated in macrophages in response to cholestatic stimuli. Next, we carried out bone marrow transplantation from WT and SIRT^{oe} mice into WT recipient animals after which, we induced cholestasis by BDL and analysed the liver parenchyma.

5.2 Results

5.2.1 Bile acids and endotoxin synergize to upregulate SIRT1 expression in macrophages *in vitro*

During cholestasis, there are two main insults in the liver. The first is the accumulation of bile acids where bile acids can reach a concentration of 200nmol/g in the mouse liver after BDL (Y. Zhang et al., 2012). The second is the translocation of intestinal bacteria to the liver which occurs

due to increased intestinal permeability in the absence of the anti-microbial function of bile (Urdaneta & Casadesús, 2017). Previous work by Hao and colleagues described that bile acids and endotoxin can synergize to activate the Nlrp3 inflammasome in macrophages during cholestasis (Hao et al., 2017). In chapter 4 we showed that SIRT1 expression is upregulated in BMDM in response to endotoxin and that $SIRT^{oe}$ macrophages have an increased pro-inflammatory response to LPS treatment *in vitro*.

Based on work presented in chapter 4, we hypothesised that bile acids and LPS synergise to activate SIRT1 in macrophages during cholestasis. To mimic the environment of the cholestatic liver, the accumulation of bile acids and the presence of bacteria which translocates from a permeable intestine, *in vitro*, we treated BMDM from WT animals with 100µM of a primary bile acid (CDCA), a secondary bile acid (DCA) and LPS, individually and in tandem. We found that while treatment with LPS for three hours increased SIRT1 expression 1.5 times as we showed in chapter 4, treatment with both stimuli, LPS and bile acids upregulated SIRT1 gene expression up to 3 times (figure 5.1A). This finding translated to the protein level, as we found that protein expression of SIRT1 increased 4 times in response to 24 hours of treatment with DCA and LPS (figures 5.1B and C). Our results show that bile acids and LPS synergise to promote SIRT1 expression in macrophages.

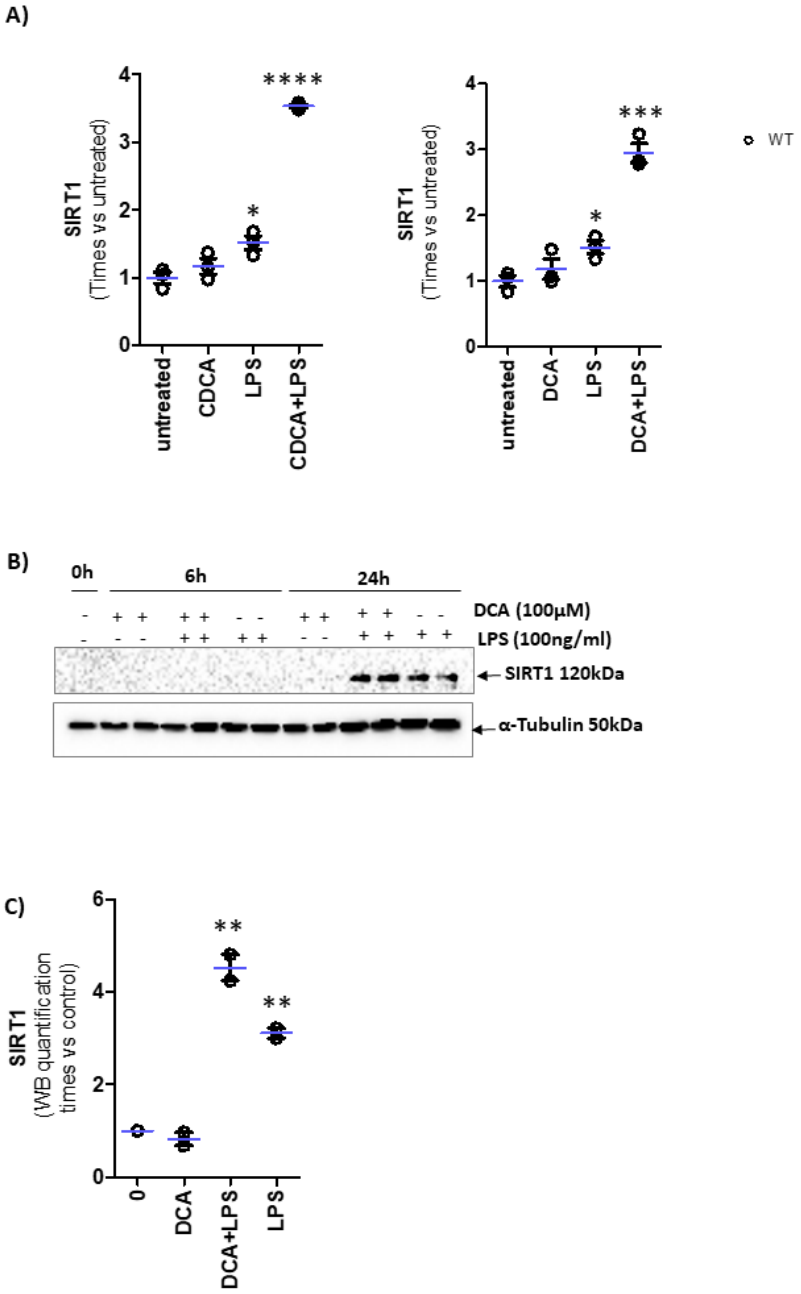


Figure 5.1 SIRT1 is upregulated in WT BMDM in response bile acids and endotoxin. *A) SIRT1 gene expression is upregulated in response to three hours of treatment with CDCA, DCA, LPS and CDCA and LPS, DCA and LPS. B) Western blotting showing that the protein expression of SIRT1 is upregulated in BMDM response to bile acid and LPS treatment. C) Quantification of band intensity of western blot B 24 hours after treatment. Upregulated SIRT1 expression correlates with increased gene expression of pro-inflammatory cytokines. BMDM from WT mice were treated with CDCA, DCA, LPS or both CDCA and LPS for 24 hours. Error bars represent standard error of the mean (n=3). Statistical significance was determined using Students' t.test * P ≤ 0.05 ** P ≤0.01 *** 0.001 P ≤ ****P ≤ 0.0001. Data is representative of three independent experiments.*

Next, we investigated whether this upregulation of SIRT1 expression correlated with increased gene expression of pro-inflammatory cytokines. At the gene expression level, the increase of SIRT1 in response to bile acids and LPS associated with increased expression of the pro-inflammatory cytokines TNFα and IL1β (figure 5.2). Treatment with bile acids alone resulted in a small increase in the upregulation of pro-inflammatory cytokine gene expression, treatment with LPS resulted in a large increase of expression and treatment with both resulted in the highest increase of pro-inflammatory cytokines (figure 5.2). The upregulation of SIRT1 expression in response to bile acids and endotoxin correlated with increased expression of the pro-inflammatory cytokines TNFα and IL1β.

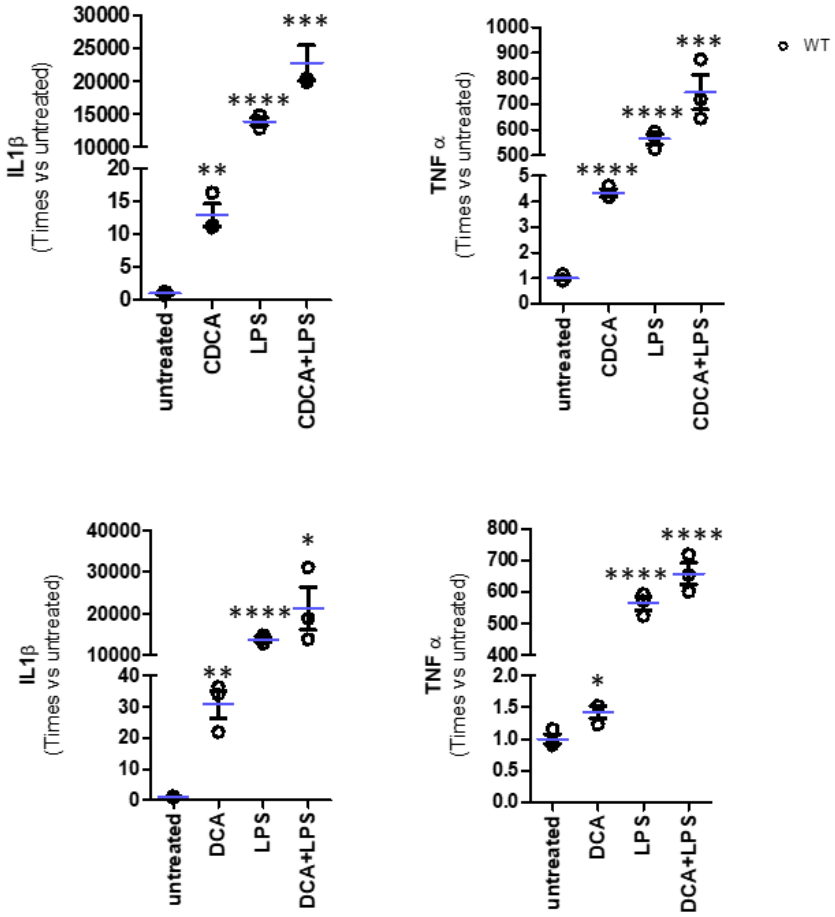


Figure 5.2 Upregulated SIRT1 expression correlates with increased gene expression of pro-inflammatory cytokines in response to bile acids and LPS. *BMDM from WT mice were treated with CDCA, DCA, LPS or both CDCA and LPS for three hours. Gene expression is significantly increased upon treatment, the highest increase is when cells are treated with LPS and bile acids. Significance was determined using Students' t test. Error bars represent standard error of the mean (n=3). * P ≤ 0.05 ** P ≤0.01 *** 0.001 P ≤ ****P ≤ 0.0001. Data is representative of three independent experiments.*

This *in vitro* study shows that bile acids and LPS synergise to promote SIRT1 expression in macrophages and this increase in SIRT1 expression associates with an increase in pro-inflammatory cytokine expression.

5.2.2 Myeloid SIRT1 overexpression promotes the pathogenesis of cholestasis

5.2.1 Generation of PEPC+WT and PEPC+SIRT^{oe} allograft model

To investigate the biological relevance of the SIRT1 overexpression, which we observed in BMDM in response to cholestatic stimuli *in vitro*, in myeloid cells in the pathogenesis of cholestatic liver disease *in vivo,* we performed bone marrow transplantation, in collaboration with the Rushworth laboratory (University of East Anglia, UK). Bone marrow transplantation is a technique that is widely used to study the effects of immune cells (Duran-struuck & Dysko, 2009) and we chose this technique to investigate the biological relevance of the SIRT1 increase we observed in BMDM in our *in vitro* study because the bone marrow is a source of myeloid cell progenitors which give rise to BMDM when differentiated with M-CSF *in vitro* (Weischenfeldt & Porse, 2008).

The peptidase C variant B (PepCboy) mice are a congenic C57BL/6J mouse strain which possess the differential pan leukocyte marker CD45.1 (Saga & Litman, 1985). Standard C57BL/6J mouse strains commonly utilised in laboratory research, including our SIRT^{oe} strain, carry the CD45.2 allele.

We transplanted the bone marrow of WT and SIRT^{oe} animals into recipient PepCboy mice (figure 5.3). In our experimental setting, we used WT or SIRT^{oe} animals as the donors and the PepCboy mice as the recipients. The donor (CD45.2) immune cells can be easily distinguished from the recipient's immune cells (CD45.1) by using specific monoclonal antibodies for CD45.1 and CD45.2 antigens for flow cytometry.

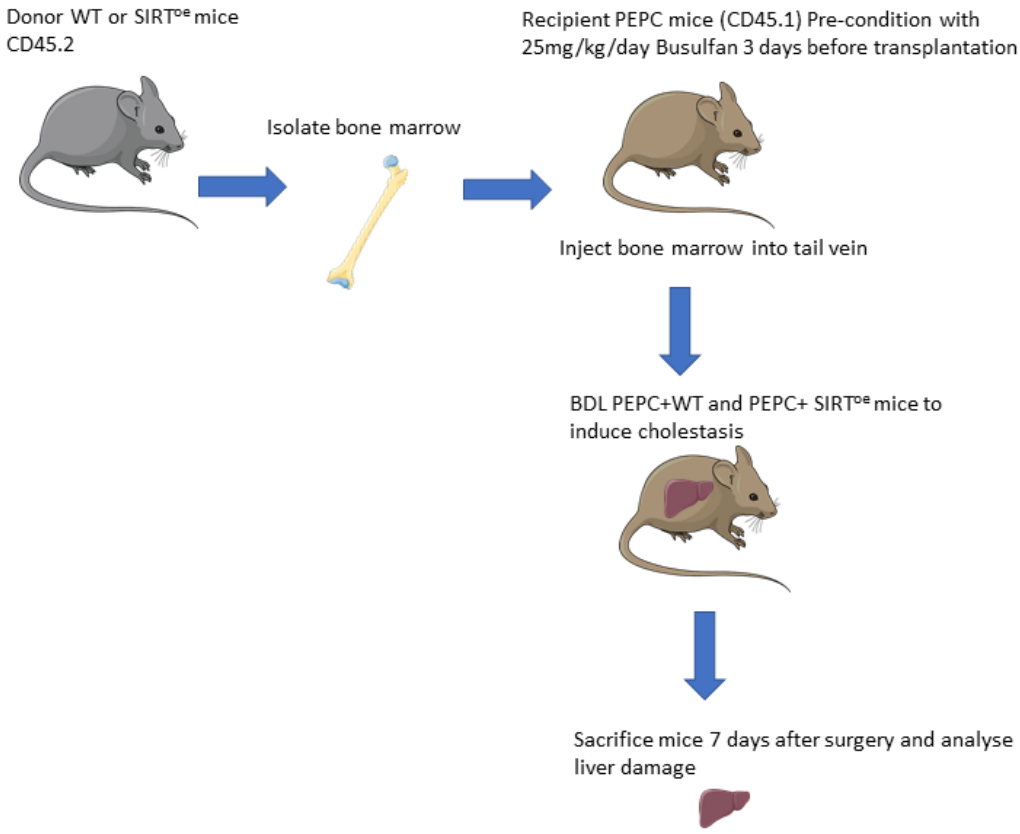


Figure 5.3 Schematic representation of the generation of PEPC+WT and PEPC+ SIRT^{oe} mice. *Bone marrow of WT or SIRT^{oe} mice (CD45.2) was injected into the tail vein of PEPC Boy mice (CD45.1). PEPC Boy mice were treated with busulfan to deplete endogenous haematopoietic stem cells prior bone marrow transplantation. Four weeks post engraftment, BDL surgery was performed on the animals to induce cholestasis and they were sacrificed 7 days after the surgery.*

PepCboy mice were treated with busulfan prior transplantation to deplete their endogenous haematopoietic stem cell population (Montecino-Rodriguez & Dorshkind, 2020). Bone marrow of WT or SIRT^{oe} mice was then injected into the tail vein of the PepCBoy mice to render (PEPC+WT) and (PEPC+SIRT^{oe}) mice respectively. Successful engraftment was confirmed by the Rushworth laboratory by flow cytometry of blood samples obtained by tail vein sampling (S. Rushworth, personal communication). Post engraftment, we performed BDL to induce obstructive cholestasis (figure 5.3). Mice were sacrificed 7 days after the surgery, where we confirmed successful engraftment (90% of cells) of WT and SIRT^{oe} bone marrow using flow cytometry of liver isolated immune cells for CD45.2 (transplanted) and CD45.1 (resident) immune cells (figure 5.4).

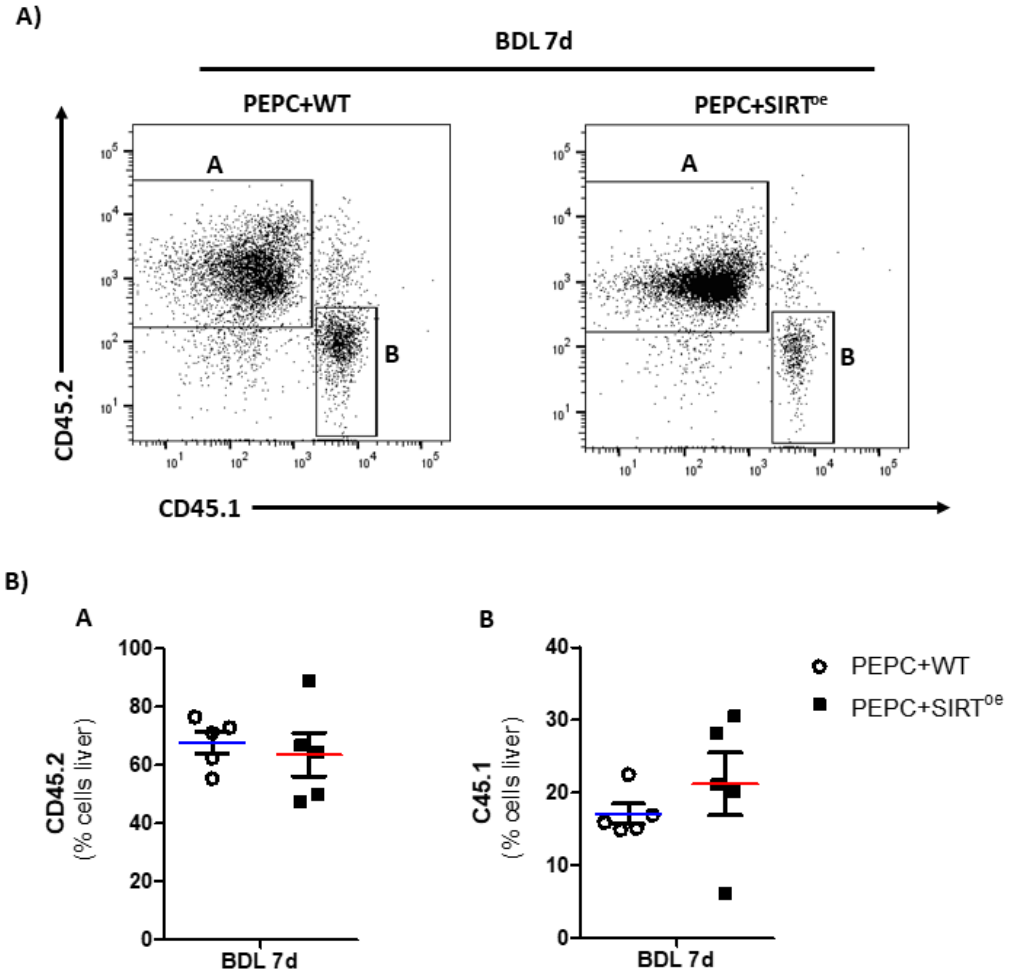


Figure 5.4 Engraftment of liver isolated immune cells of PEPC boy mice after 7 days of BDL. *Bone marrow transplantation was carried out. Bone marrow from WT and SIRT^{oe} mice was transplanted into recipient PEPC boy mice followed by BDL. Mice were sacrificed 7 days after BDL surgery. Immune cells from WT and SIRT^{oe} mice are of the CD45.2 lineage. Immune cells of the PEPC boy mice are of the CD45.1 lineage. Up to 90% engraftment was achieved. A) FACS plots showing the CD45.2 population (A) and the CD45.1 population (B). B) Plots showing numbers of CD45.2 (A) and CD45.1 (B) immune cells in the livers of PEPC+WT and PEPC+SIRT^{oe} mice*.

5.2.2 Transplantation of SIRT1 overexpressing myeloid cells promoted liver damage in response to obstructive cholestasis

To investigate the effect of SIRT1 overexpression in myeloid cells in response to cholestasis we analysed the serum of PEPC+WT and PEPC+SIRT^{oe} mice for markers of liver damage. PEPC+SIRT^{oe} mice showed increased levels of ALT and AST compared to PEPC+WT mice but comparable levels of ALP and bilirubin (figure 5.5) suggesting that SIRT1 overexpression in myeloid cells promotes liver damage in response to an equal severity of cholestasis (Fevery, 2008; Poupon, 2015).

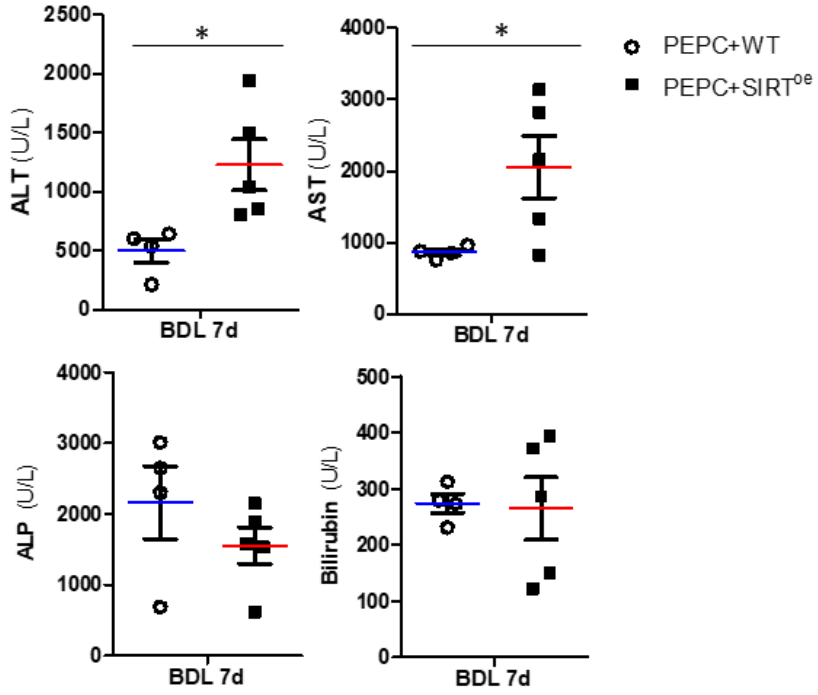


Figure 5.5 Transplantation of SIRT1 overexpressing macrophages results in increased liver injury in response to 7 days of BDL compared to WT macrophage transplantation. *Serum transaminases, ALT and AST, are significantly increased in PEPC+SIRT^{oe} mice compared to PEPC+WT animals, indicating a higher degree of liver injury in PEPC+SIRT^{oe} mice. ALP and bilirubin levels are comparable in both groups of animals suggesting an equal degree of cholestasis in response to BDL. * $p \leq 0.05$. Statistical significance was determined using Students' t.test. n= at least 4 mice. (PEPC+WT vs PEPC+SIRT^{oe}). Data shown is representative of three independent experiments.*

Analysis of liver histology by H and E staining revealed that PEPC+SIRT^{oe} mice had massive necrotic areas in the liver compared to PEPC+WT mice (figure 5.6) suggesting that myeloid SIRT1 overexpression promotes hepatocyte cell death during cholestasis.

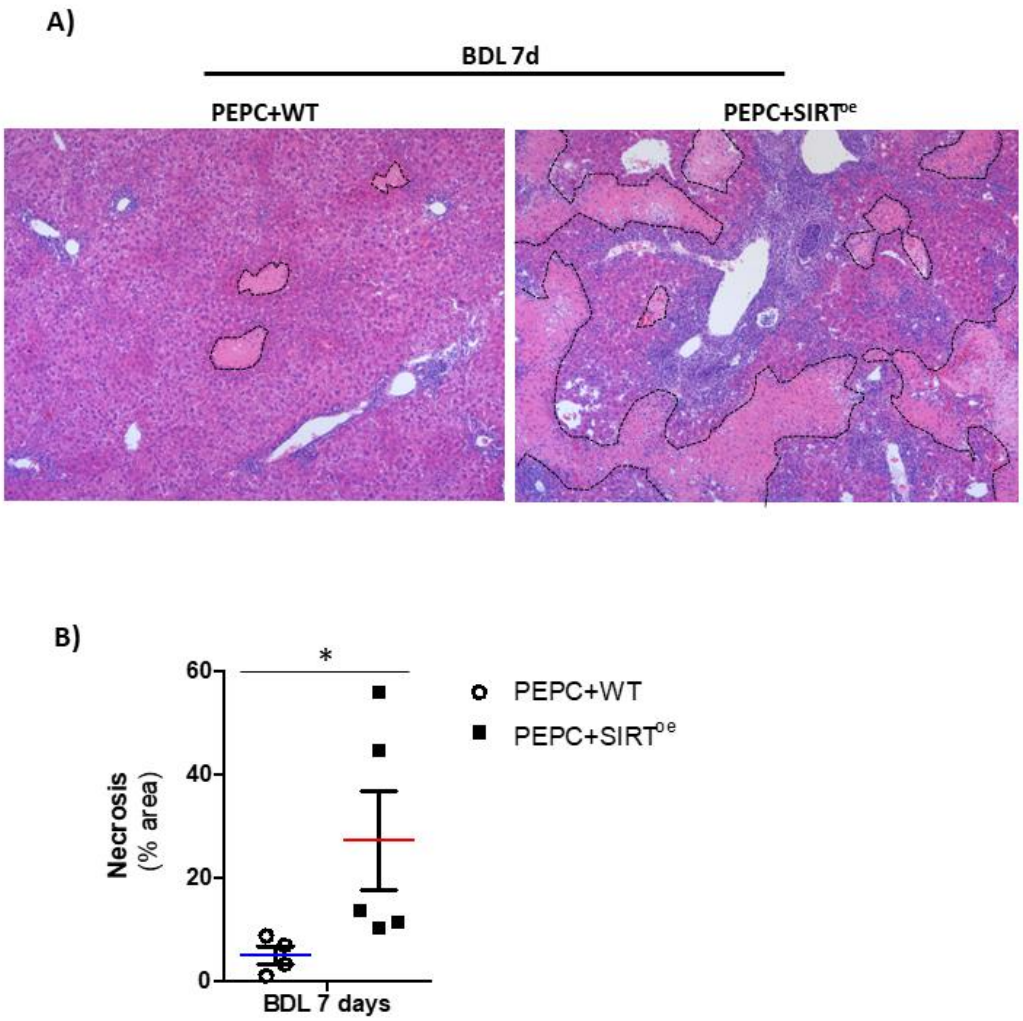


Figure 5.6 Transplantation of SIRT1 overexpressing macrophages results in increased liver damage in response to 7 days of BDL compared to WT macrophage transplantation. *A) H and E staining of liver tissue shows increased necrotic areas in PEPC+SIRT^{oe} mice compared to PEPC+WT animals. Images were taken using the 4x magnification. B) Image J quantification of necrotic areas shows a significantly higher degree of necrosis in PEPC+SIRT^{oe} mice compared to PEPC+WT mice. Necrosis is shown as % area of the image. * $p \leq 0.05$. Statistical significance was determined using Students' t.test. n= at least 4 mice. (PEPC+WT vs PEPC+SIRT^{oe}). Data shown is representative of three independent experiments.*

5.2.3 Transplantation of SIRT1 overexpressing myeloid cells correlated with increased Nlrp3 inflammasome activation

The worsening of the liver phenotype of PEPC+SIRT^{oe} mice correlated with increased activation of the Nlrp3 inflammasome in the liver as evidenced by increased cleavage of caspase 1 and IL1β in total liver extracts (figure 5.7).

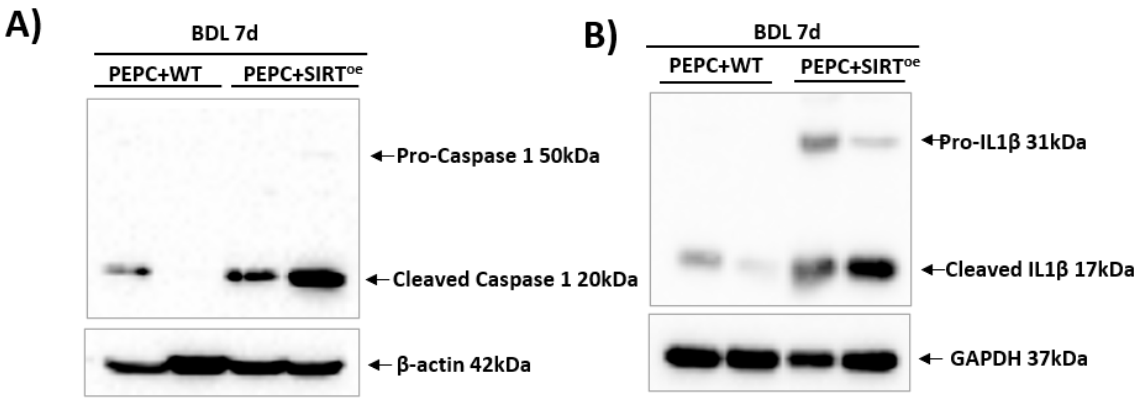


Figure 5.7 Transplantation of SIRT1 overexpressing macrophages correlates with Nlrp3 inflammasome activation in the liver in response to BDL*. Western blotting shows increased A) cleaved caspase 1 levels and B) cleaved IL1ß levels in the livers of PEPC+SIRT^{oe} mice compared to PEPC+WT animals 7 days after BDL surgery. Western blots shows representative results of n=4 mice.*

5.2.4 Transplantation of SIRT1 overexpressing myeloid cells correlated with increased ductular reaction in response to BDL

Ductular reaction is the proliferation of cholangiocytes around the bile ducts in response to the blockade of bile ducts which eventually leads to bile duct loss (Sato et al., 2019). It is a common pathological feature observed during cholestatic liver diseases and can be seen around the bile ducts by immunostaining for CK19.

Thus, we performed immunohistochemical analysis of CK19 which showed significantly increased ductular reaction in PEPC+SIRT^{oe} mice compared to PEPC+WT mice, indicating that SIRT1 overexpression in myeloid cells contributes to the pathogenesis of cholestatic liver disease (figure 5.8).

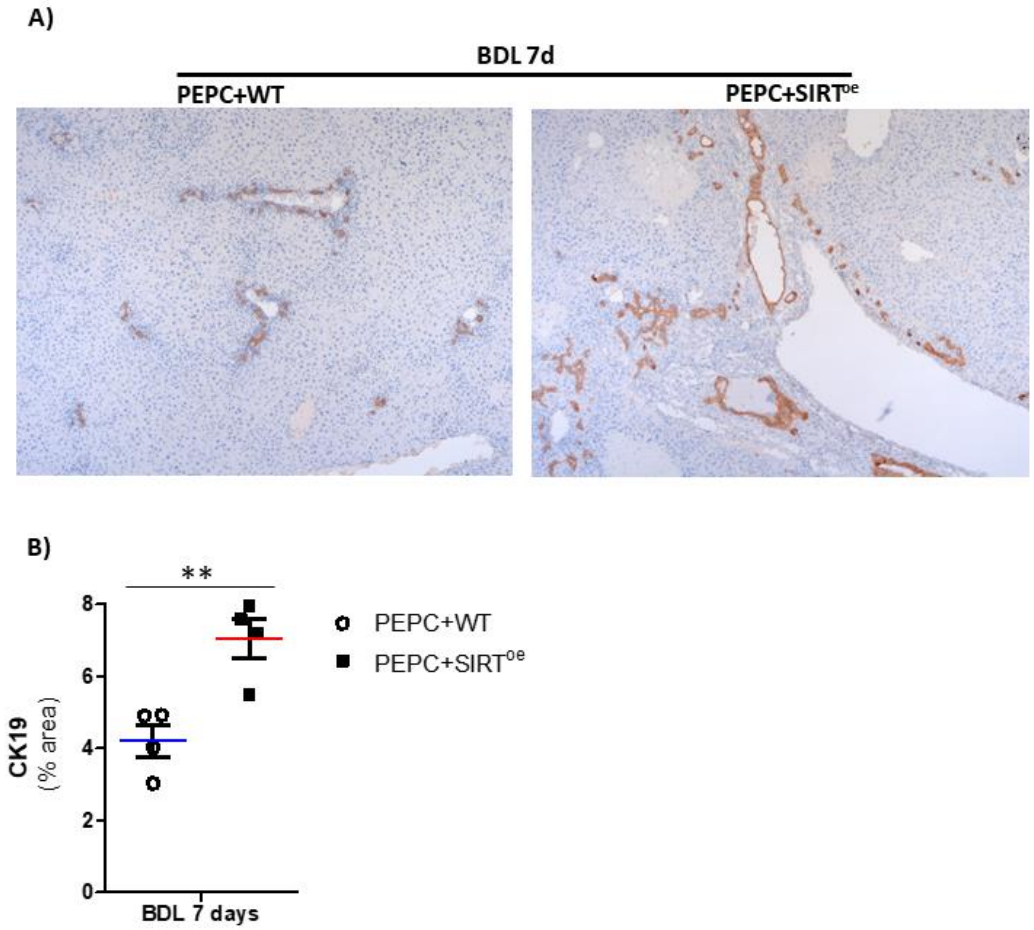


Figure 5.8 Transplantation of SIRT1 overexpressing macrophages results in increased ductular reaction in the animals in response to BDL. *A) CK19 immunohistochemistry shows increased cholangiocyte proliferation in the livers of PEPC+SIRT^{oe} mice compared to PEPC+WT animals 7 days after BDL surgery. Images were taken using the 4x magnification. B) Quantification of CK19 staining using image J software. CK19 staining is shown as % area of the image. ** p ≤ 0.01. Statistical significance was determined using Students' t.test. n= at least 4 mice. (PEPC+WT vs PEPC+SIRT^{oe}).*

5.2.5 Transplantation of SIRT1 overexpressing myeloid cells promoted fibrosis in response to obstructive cholestasis

Fibrosis is a hallmark of cholestatic liver disease and macrophages promote both collagen deposition and degradation during fibrogenesis (Henderson et al., 2020). Analysis of liver fibrogenesis by Sirius red staining and α-smooth muscle actin (α-SMA) immunofluorescence staining revealed a significant increase in fibrosis in PEPC+SIRT^{oe} mice compared to PEPC+WT mice (figures 5.9 and 5.10) suggesting that SIRT1 overexpression in myeloid cells promotes fibrogenesis during cholestasis.

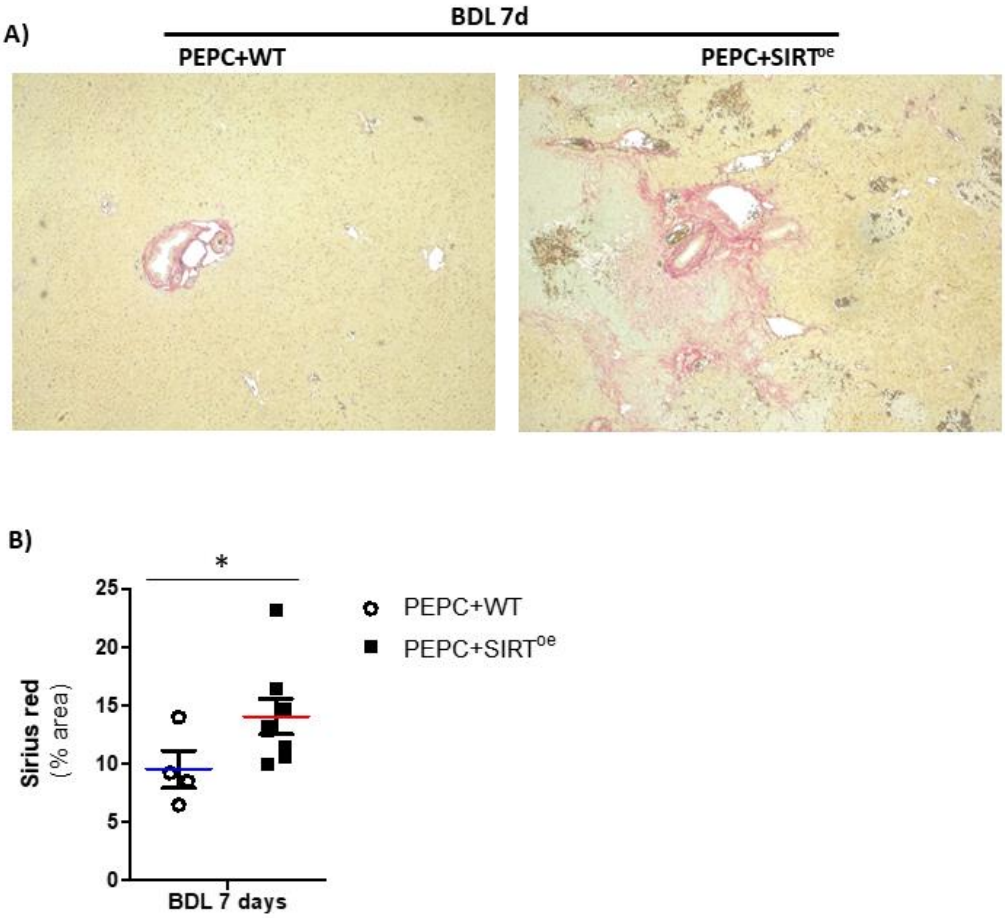


Figure 5.9 Transplantation of SIRT1 overexpressing macrophages results in increased fibrosis in the animals in response to BDL. *A) Sirius red staining of liver sections shows increased collagen deposition (red fibres) in the livers of PEPC+SIRT^{oe} mice compared to PEPC+WT animals 7 days after BDL surgery. Images were taken using the 4x magnification. B) Quantification of Sirius red staining using image J software. Sirius red staining is shown as % area of the image. * $p \leq 0.05$. Statistical significance was determined using Students' t.test. n= at least 4 mice. (PEPC+WT vs PEPC+SIRT^{oe}).*

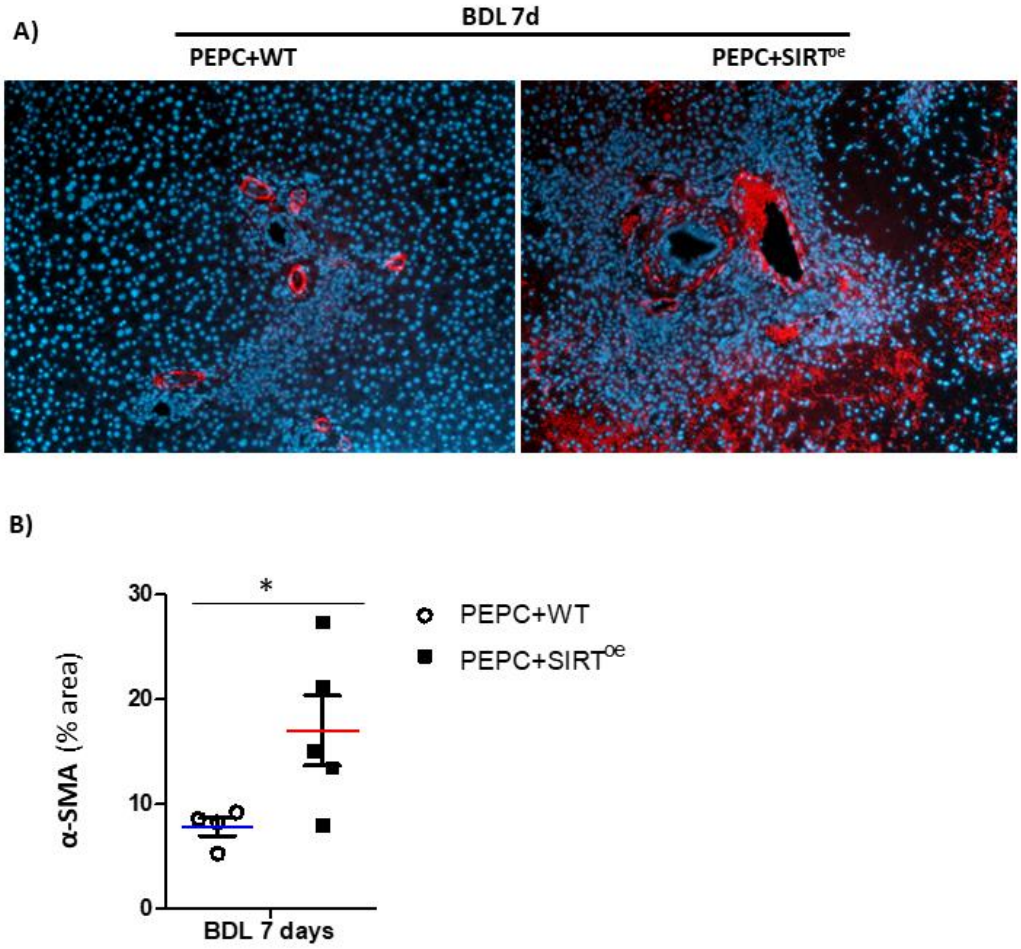


Figure 5.10 Transplantation of SIRT1 overexpressing macrophages results in increased fibrosis in the animals in response to BDL. *A) Immunofluorescence of α-SMA (red) and DAPI (blue) staining of liver sections shows increased collagen deposition (red fibres) in the livers of PEPC+SIRT^{oe} mice compared to PEPC+WT animals 7 days after BDL surgery. Images were taken using the 10x magnification. B) Quantification of α-smooth muscle actin staining using image J software. α-smooth muscle actin staining is shown as % area of the image. * p ≤ 0.05. Statistical significance was determined using Students' t.test. n= at least 4 mice. (PEPC+WT vs PEPC+SIRT^{oe}).*

In conclusion, our bone marrow transplantation experiments show that SIRT1 overexpression in myeloid cells promotes the pathogenesis of cholestatic liver disease by exacerbating liver damage and fibrosis.

5.3 Discussion

We have previously shown that SIRT1 is upregulated in hepatocytes of cholestatic patients and that SIRT1 overexpression correlates with increased inflammation and liver damage in murine

models of cholestasis (Blokker et al., 2018) but the role of myeloid SIRT1 overexpression in cholestasis remained undefined.

In this chapter, we demonstrate that macrophage SIRT1 overexpression promotes the pathogenesis of cholestasis. Here we show that bile acids and endotoxin synergise to upregulate SIRT1 expression in macrophages *in vitro*. Next, to investigate the biological relevance of this myeloid SIRT1 increase in response to cholestatic stimuli, we used bone marrow transplantation from SIRT1 overexpressing animals into recipient mice and we established that myeloid SIRT1 overexpression aggravates liver damage and fibrosis.

The role of bile acids in macrophage function is poorly defined in the literature as while some studies described that they promote an anti-inflammatory phenotype in macrophages (Guo et al., 2016; Haselow et al., 2013; Wammers et al., 2018) others described that bile acids synergise with endotoxin to promote Nlrp3 inflammasome activation in macrophages (Hao et al., 2017) and that pre-treatment with LPS sensitises macrophages to Nlrp3 activation in response to CDCA treatment (Gong et al., 2016). In this chapter we show that bile acids and LPS promote a greater upregulation in SIRT1 expression in BMDM than LPS alone, and that this upregulation correlated with increased production of pro-inflammatory cytokines in macrophages. Since we observed a significant upregulation in IL1β expression, and IL1β is produced by the Nlrp3 inflammasome, in response to bile acid and LPS treatment, our findings confirm previous work where bile acids and LPS have been described to promote Nlrp3 inflammasome activity (Gong et al., 2016; Hao et al., 2017), moreover, we are the first to show that an upregulation of SIRT1 activity associates with Nlrp3 inflammasome activation in macrophages. Our findings are not comparable to work from the Graf group as they used primary human peripheral blood mononuclear cells and a single bile acid, TLC, for their experiments (Haselow et al., 2013; Wammers et al., 2018) and we used mouse BMDM and two bile acids CDCA and DCA. Our results are comparable with Gong and colleagues who used CDCA to stimulate the immortalised mouse macrophage cell line J774A.1 (Gong et al., 2016) and Hao and co-workers who used a range of bile acids, including CDCA and DCA, in mouse peritoneal macrophages (Hao et al., 2017). It is interesting to note that studies by Haselow and Wammers used 50μM of TLC to stimulate macrophages (Haselow et al., 2013; Wammers et al., 2018) while we used 100μM of CDCA or DCA. Serum bile acids of patients have been described to reach up to 200μM (Trottier et al., 2011). 50μM is quite a low concentration of a bile acid to mimic cholestasis, perhaps the Graf group would have observed a pro-inflammatory phenotype in their macrophages if they used a higher concentration of TLC in their experiments.

Our *in vivo* study of bone marrow transplantation supported our previously published work where we described that SIRT1 overexpression is detrimental during cholestasis (Blokker et al.,

2018). We have previously shown that SIRT^{oe} animals had increased liver damage in response to BDL (Blokker et al., 2018) and in this thesis we have identified that myeloid SIRT1 overexpression promotes liver damage during cholestasis. Our previous study used constitutively overexpressing animals (Blokker et al., 2018), and, as we saw increased liver inflammation in cholestatic SIRT^{oe} mice, we hypothesised that SIRT1 overexpression may promote macrophage activation during cholestatic liver disease. Here, using bone marrow transfer, we show that myeloid SIRT1 overexpression promotes the pathogenesis of cholestasis. The results of this thesis support our previously published work in SIRT^{oe} animals (Blokker et al., 2018) as both experiments showed increased levels of ALT and AST and liver necrosis, as well as increased fibrosis, associated with SIRT1 overexpression.

While the results of the bone marrow transfer experiments shown here support most of our findings of Blokker et al., an interesting finding of this thesis is that myeloid SIRT1 overexpression promoted ductular reaction in response to BDL whereas in SIRT^{oe} animals, where SIRT1 was overexpressed in all cell types, including cholangiocytes, showed a decrease in ductular reaction compared to WT animals due to increased ductopenia (Blokker et al., 2018). In our study led by Blokker and colleagues, using transfection to overexpress SIRT1 in normal mouse cholangiocytes, we identified that SIRT1 overexpression attenuated cholangiocyte proliferation (Blokker et al., 2018). In accord with our previous findings, Pant and colleagues recently demonstrated that SIRT1 overexpression promoted cilia loss in cholangiocytes and that SIRT1 knockdown in a cholangiocarcinoma cell line resulted in upregulated CK19 expression (Pant et al., 2021). Additionally, the authors described that SIRT1 expression is upregulated in cholangiocytes of cholangiocarcinoma patients and identified that the treatment of rats with the SIRT1 inhibitor sirtinol reduced liver tumour size in the animals in a model of cholangiocarcinoma (Pant et al., 2021). Our previously published study and the work of Pant and colleagues suggests that cholangiocyte SIRT1 overexpression is detrimental in the context of liver disease (Blokker et al., 2018; Pant et al., 2021).

In this thesis, however, we show that myeloid SIRT1 overexpression promotes the proliferation of WT cholangiocytes in the PEPC+SIRT^{oe} animals during cholestasis. Pro-inflammatory macrophages contribute to ductular reaction (Best et al., 2016) through the activation of cholangiocyte proliferation via the secretion of pro-inflammatory cytokines such as IL1β and TNFα (Gadd et al., 2014). In the previous chapter we demonstrated that SIRT^{oe} macrophages were hyperactivated in response to LPS and in this chapter we found that, in WT BMDM stimulated with LPS and bile acids *in vitro*, SIRT1 expression is upregulated and this SIRT1 increase correlated with an increase in the transcription of TNFα and IL1β. *In vivo*, we showed that the transplantation of

SIRT^{oe} myeloid cells associated with increased Nlrp3 inflammasome activation in the livers of PEPC+SIRT^{oe} animals in response to BDL, further suggesting that myeloid SIRT1 overexpression promotes liver inflammation during cholestasis. Here we propose that the increased ductular reaction seen in the PEPC+SIRT^{oe} mice could be a result of the increased activation of the transplanted SIRT1 overexpressing myeloid cells as pro-inflammatory macrophages have been described to promote ductular reaction through the secretion of pro-inflammatory cytokines (Best et al., 2016; Gadd et al., 2014).

Fibrosis is a common feature of all liver disorders including cholestasis (Trautwein et al., 2015) and we found increased fibrogenesis in PEPC+SIRT^{oe} in response to BDL. Fibrosis occurs due to dysregulated wound healing in the liver and 7 days of BDL is an established time point to study the initiation phase of hepatic fibrogenesis (Tag et al., 2015).

Macrophages have dual effects as they contribute to the initiation and resolution of fibrosis in the liver (Pellicoro et al., 2014). Pro-inflammatory monocyte-derived macrophages are recruited to the liver during cholestasis where they differentiate into macrophages and promote inflammation and fibrogenesis (Guicciardi et al., 2018). In the previous chapter we demonstrated that SIRT^{oe} macrophages had increased production of pro-inflammatory cytokines and upregulated Nlrp3 inflammasome activity in response to LPS treatment. In accord with this, we found increased caspase 1 cleavage and IL1β production in the livers of PEPC+SIRT^{oe} mice in response to BDL. Increased activation of the Nlrp3 inflammasome has been described to promote fibrogenesis in the liver (Wree et al., 2014) therefore one mechanism how SIRT1 overexpression in macrophages could promote fibrogenesis during cholestasis could be via mechanisms involving the upregulation of Nlrp3 inflammasome activity.

In summary, in this chapter we show that bile acids and endotoxin upregulate macrophage SIRT1 expression *in vitro* and that *in vivo* macrophage SIRT1 overexpression contributes to the pathogenesis of cholestasis, possibly via mechanisms involving the Nlrp3 inflammasome.

5.4 Future work

A reciprocal bone marrow transplantation experiment would help to confirm the role of SIRT1 overexpressing myeloid cells in promoting the pathogenesis of cholestasis. WT bone marrow of PepCboy mice could be transplanted into SIRT^{oe} mice, followed by BDL after engraftment. If the liver damage appears attenuated in the SIRT^{oe} mice reconstituted with WT PepCboy bone marrow, the role of SIRT1 overexpression in the pathogenesis of cholestasis would be confirmed.

Chapter 6 – Defining the role of myeloid cell specific SIRT1 depletion during cholestasis

6.1 Introduction and aims

Several studies have shown that monocyte-derived macrophages contribute to the pathogenesis of cholestasis (Guicciardi et al., 2018; Karlmark et al., 2009). In this thesis, we demonstrate that SIRT1 overexpression promotes a pro-inflammatory phenotype in BMDM *in vitro* and liver damage in response to LPS *in vivo*. As well, we showed that SIRT1 overexpression in myeloid cells promoted the pathogenesis of cholestasis in the BDL model. Taking all these results together, we hypothesise that SIRT1 modulation in myeloid cells could be utilised as a potential therapeutic approach to treat cholestatic liver diseases.

The aim of this chapter is to investigate the effect of SIRT1 depletion in monocyte derived-macrophage function during cholestasis induced by BDL.

6.2 Results

6.2.1 Generation of $SIRT^{mye-/-}$ mice

To further characterise the role of SIRT1 in macrophage function during cholestasis, we used the Cre/Lox P system where Cre expression was placed under the control of lysozyme M (LysM) promoter (LysMCre) (Clausen et al., 1999). LysM is an antimicrobial enzyme which is expressed exclusively in cells of the myeloid lineage including monocytes, macrophages, neutrophils, dendritic cells and these mice have been widely used to study macrophage function (J. Shi et al., 2018). We generated a SIRT1 knockout in cells of the myeloid lineage LysM ($SIRT^{mye-/-}$) by crossing LysMCre mice with SIRT1 floxed animals (figure 6.1A). Compared to cre negative littermate controls ($SIRT^{mye+/+}$; herein WT), $SIRT^{mye-/-}$ mice had residual SIRT1 expression in cells of the myeloid lineage, BMDM (figure 6.1B).

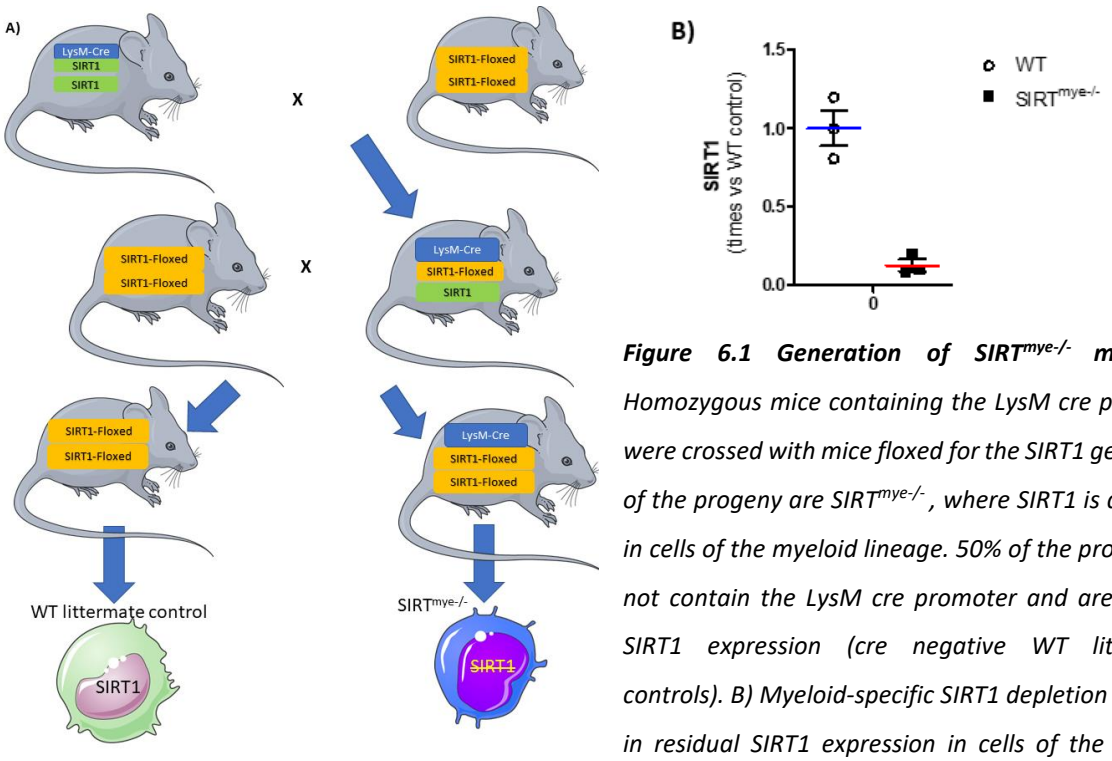


Figure 6.1 Generation of SIRT$^{mye-/-}$ mice. *A) Homozygous mice containing the LysM cre promoter were crossed with mice floxed for the SIRT1 gene. 50% of the progeny are SIRT$^{mye-/-}$, where SIRT1 is depleted in cells of the myeloid lineage. 50% of the progeny do not contain the LysM cre promoter and are WT for SIRT1 expression (cre negative WT littermate controls). B) Myeloid-specific SIRT1 depletion resulted in residual SIRT1 expression in cells of the myeloid lineage, bone marrow derived macrophages (untreated SIRT$^{mye-/-}$ vs WT littermate BMDM). Error bars represent standard error of the mean n= 3.*

6.2.2 Myeloid-specific SIRT1 depletion associated with a mild increase in liver damage in response to obstructive cholestasis

In response to cholestasis induced by BDL, SIRT$^{mye-/-}$ mice had increased liver damage, as indicated by mildly increased levels of serum transaminases ALT and AST, 7 days after BDL, compared to WT littermate controls, but the data did not reach statistical significance (figure 6.2). Both genotypes had comparable levels of ALP and bilirubin in the serum, suggesting an equal severity of cholestasis in both genotypes (Fevery, 2008; Poupon, 2015) 7 after BDL (figure 6.2).

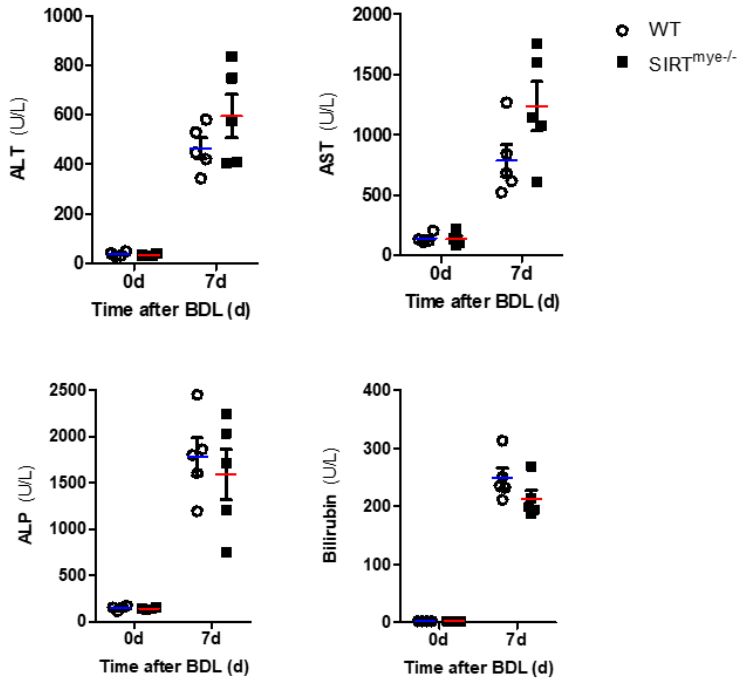


Figure 6.2. Myeloid SIRT1 depletion promotes liver injury in response to 7 days of BDL. *Mice with myeloid SIRT1 depletion had increased levels of ALT and AST serum transaminases compared to WT littermate controls. Both genotypes had comparable levels of the cholestasis serum markers ALP and bilirubin. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 (SIRT$^{mye-/-}$ vs WT littermate). Error bars represent standard error of the mean (n=6).*

As expected in response to BDL, bile acids were elevated in the liver and serum (Y. Zhang et al., 2012). Both genotypes had comparable levels of total bile acid pool size in the liver and the serum (figure 6.3) and comparable gene expression of bile acid transporters in the liver (figure 6.4). Histological examination of necrotic areas by H and E staining revealed an equal degree of necrosis in both genotypes 7 days after BDL (figure 6.5).

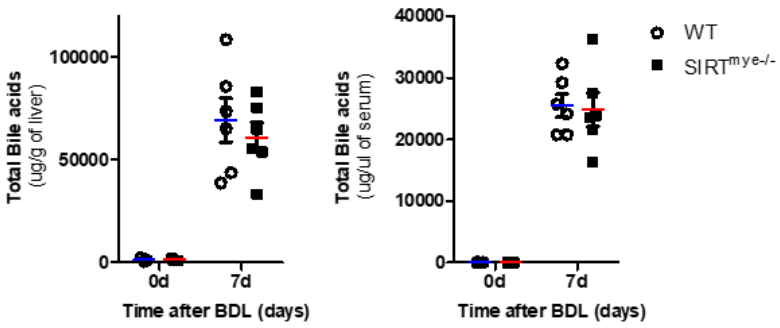


Figure 6.3 Quantification of bile acid pool in the liver and serum of SIRT$^{mye-/-}$ mice and WT littermate controls. *SIRT1 myeloid-depleted and WT mice had comparable levels of bile acid accumulation in the serum and liver in response to 7 days of BDL. Error bars represent standard error of the mean (n=6). (SIRT$^{mye-/-}$ vs WT littermate).*

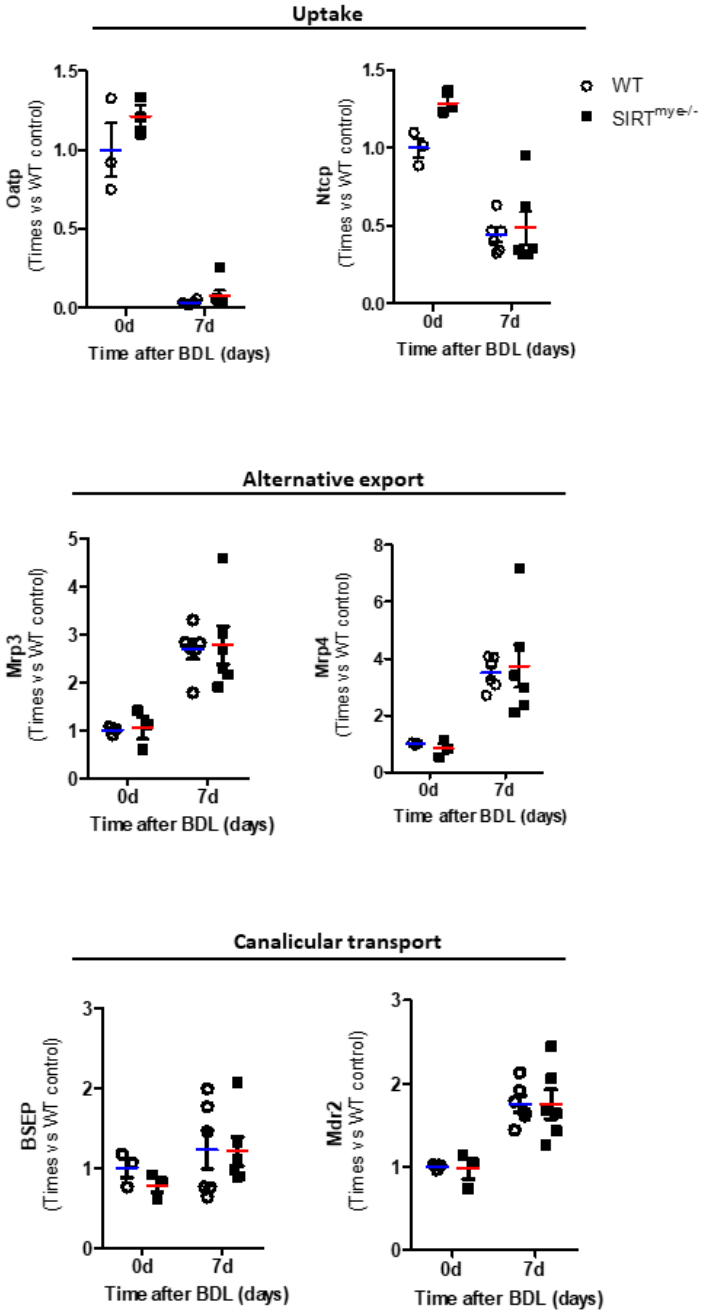


Figure 6.4 SIRT$^{mye-/-}$ mice and WT littermate controls had comparable bile acid metabolism in response to 7 days of BDL. *Gene expression analysis of bile acid transporters. WT and SIRT$^{mye-/-}$ mice had comparable levels of bile acid uptake, alternative export and canalicular transport. Error bars represent standard error of the mean (n=6). (SIRT$^{mye-/-}$ vs WT littermate).*

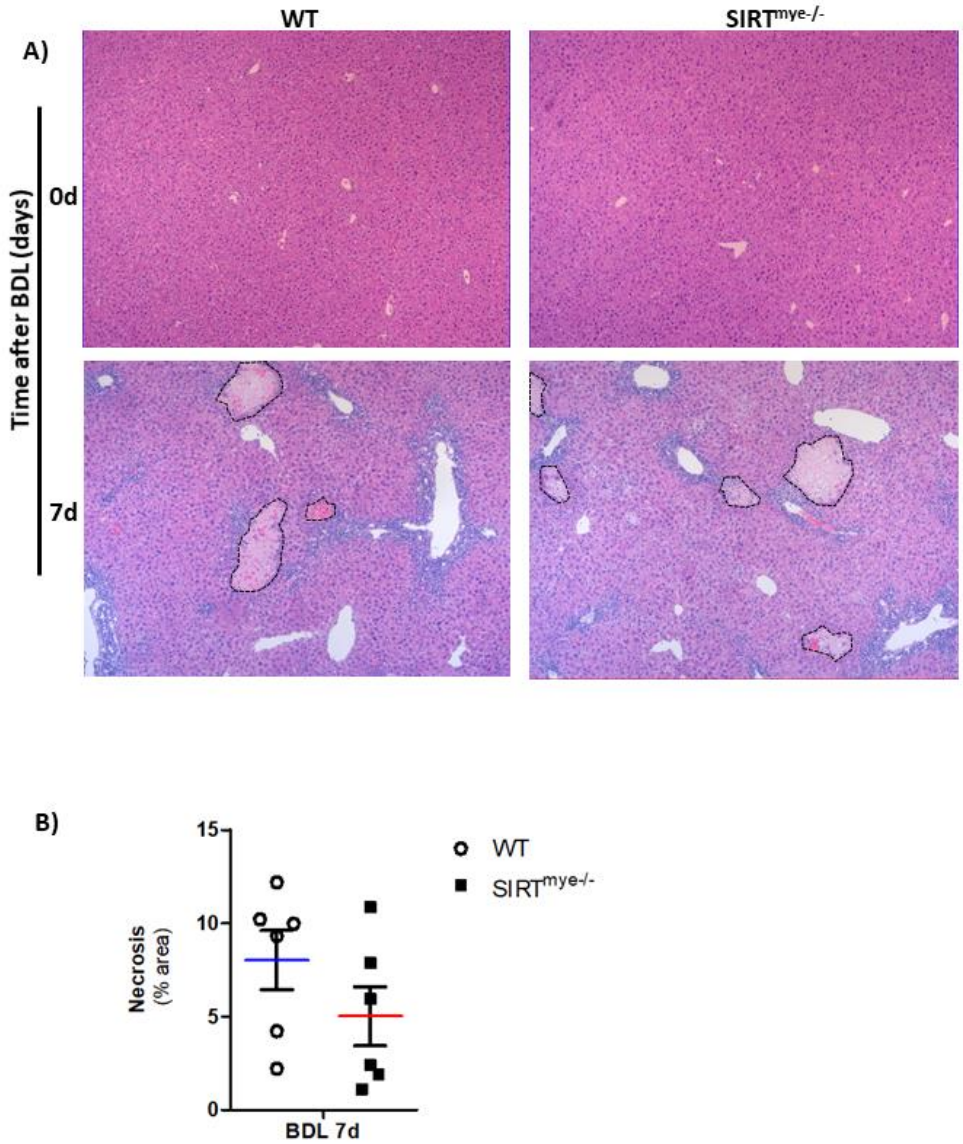


Figure 6.5 Myeloid SIRT1 depletion has no effect on liver injury in response to BDL. *Mice with myeloid SIRT1 depletion had equal necrotic areas compared to WT littermate controls. A) H and E staining of liver sections. B) Quantification of necrotic areas using Image J software. Error bars represent standard error of the mean (n=6). (SIRT$^{mye-/-}$ vs WT littermate).*

In summary, the results suggest that myeloid SIRT1 depletion associated with mildly increased liver damage 7 days after BDL.

6.2.3 Myeloid-specific SIRT1 depletion had little effect on inflammation in response to BDL

We found no significant differences in the numbers of liver infiltrating macrophages in response to BDL in WT vs SIRT$^{mye-/-}$ mice 7 days after BDL (figure 6.6), indicating that SIRT1 depletion did not affect macrophage recruitment during cholestasis.

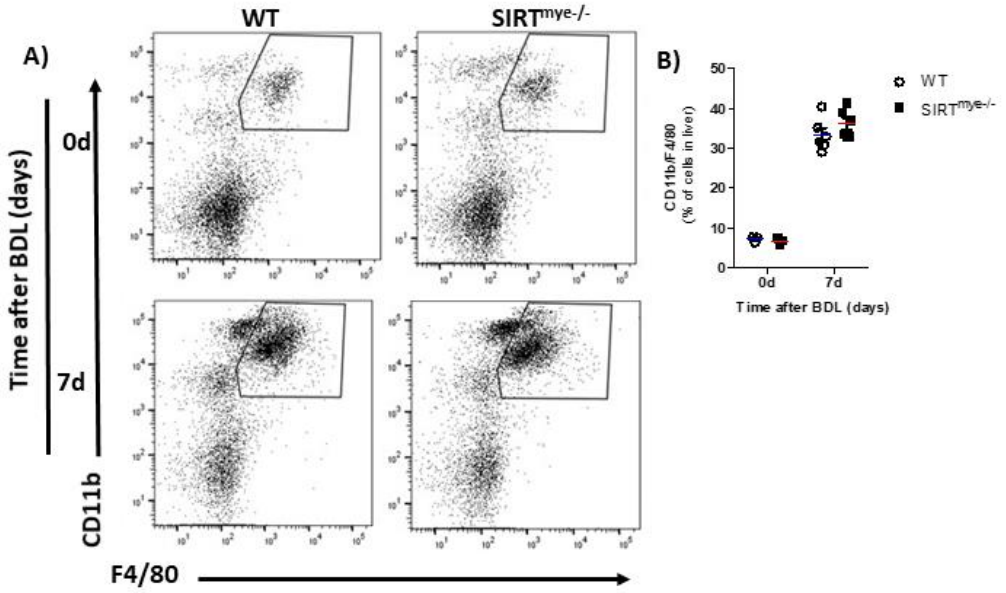


Figure 6.6 Livers of myeloid-specific SIRT1 depleted mice and littermate controls had equal numbers of macrophages in response to BDL*. A) FACS plots of liver isolated immune cells B) quantification of CD11b/F4/80 positive cells. Error bars represent standard error of the mean (n=6). (SIRT$^{mye-/-}$ vs WT littermate).*

In line with this, we found comparable levels of the pro-inflammatory cytokines TNFα and IL1β in the liver (figures 6.7A) as well as similar inflammasome activation as livers of both genotypes had comparable levels of IL1β at the mRNA and protein level (figure 6.7). The expression of the enzyme NOS2 and receptors for monocyte recruitment CCR1, CCR2, CCR5, CX3CR1 was comparable between both genotypes 7 days after BDL (figure 6.7). Interestingly, we found significantly decreased levels of CCL2 mRNA in the livers of SIRT$^{mye-/-}$ animals compared to WT littermate controls while this didn't associate with decreased macrophage recruitment to the livers of SIRT$^{mye-/-}$ mice.

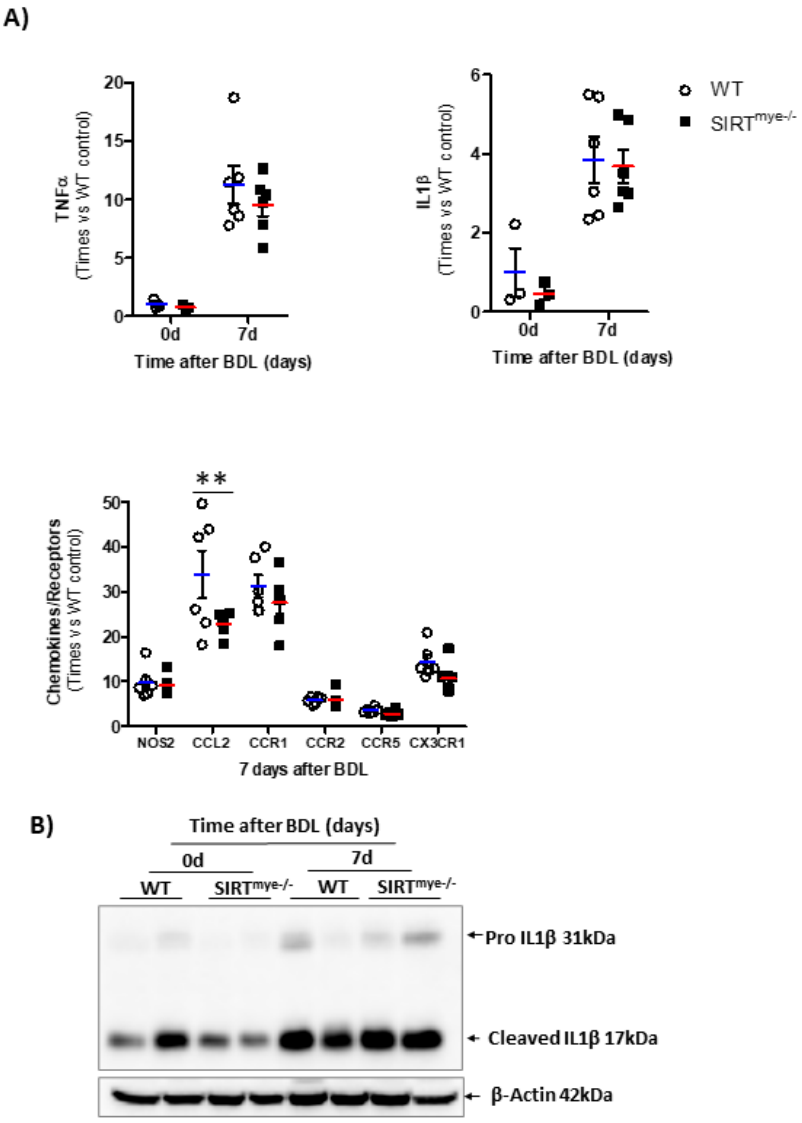


Figure 6.7 SIRT$^{mye-/-}$ mice and WT Cre negative littermate controls had comparable liver inflammation in response to BDL. *A) Gene expression of pro-inflammatory cytokines TNFα and IL1β is comparable in response to BDL. Expression levels of NOS2 and chemokine receptors CCR1, CCR2, CCR5 and CXC3R1 were comparable between genotypes. CCL2 expression was significantly decreased in SIRT$^{mye-/-}$ mice compared to WT. Error bars represent standard error of the mean (n=6). Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** 0.001 P ≤ ****P ≤ 0.0001 B) IL1β protein levels are comparable between the genotypes in response to BDL. Western blots shows representative results of n=6 mice.*

In summary, myeloid SIRT1 depletion had little effect on liver inflammation during cholestasis.

6.2.4 Myeloid-specific SIRT1 depletion correlated with increased ductular reaction in response to BDL

Proliferation of cholangiocytes around the bile ducts is a common pathological feature of cholestasis which leads to bile duct loss as the disease progresses (Sato et al., 2019). SIRT$^{mye-/-}$ mice had a statistically significant increase in ductular reaction in the liver 7 days after BDL surgery, compared to WT animals, as examined by CK19 immunohistochemistry (figure 6.8). This result suggests that myeloid SIRT1 depletion contributes to the progression of cholestasis.

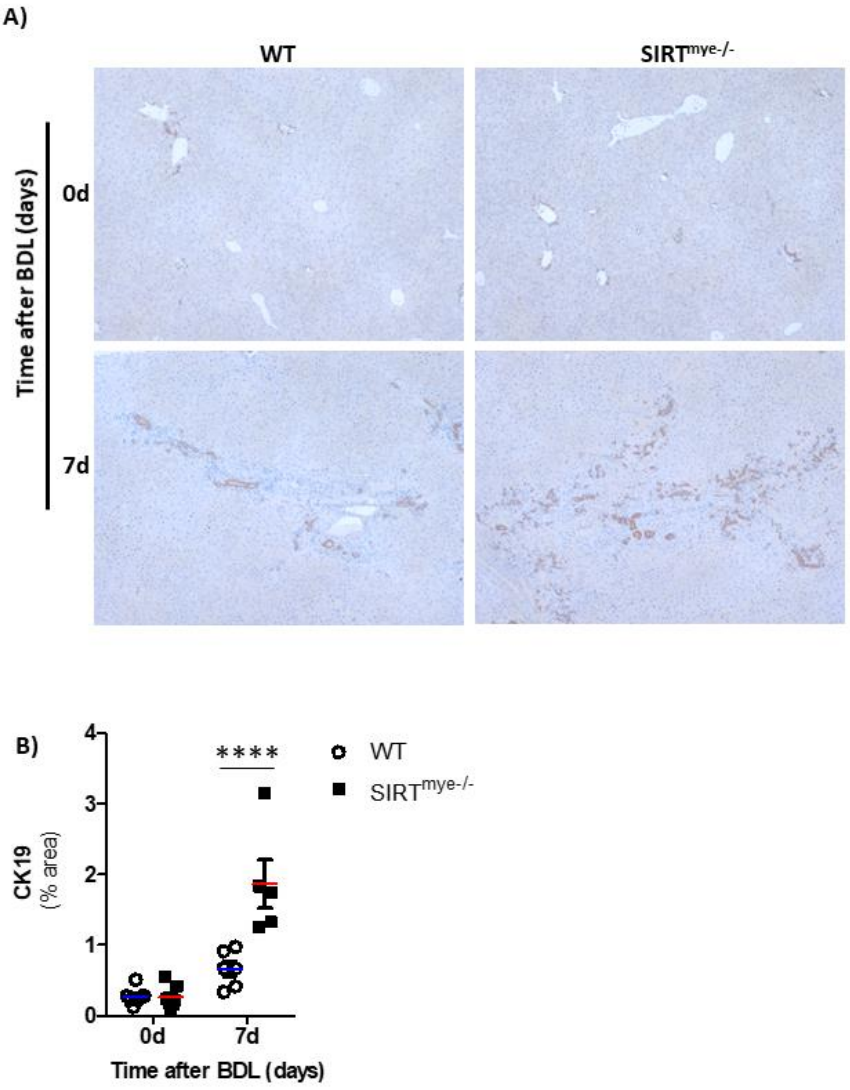


Figure 6.8 SIRT$^{mye-/-}$ mice had increased ductular reaction compared to littermate controls in response to BDL. *A) CK19 immunohistochemistry shows increased ductular reaction in SIRT$^{mye-/-}$ mice 7 days after BDL. Images were taken using the 4x magnification. B) Image J quantification of CK19 area shows a significant increase in ductular reaction in SIRT$^{mye-/-}$ animals. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 **** P ≤ 0.0001 (SIRT$^{mye-/-}$ vs WT littermate). Error bars represent standard error of the mean (n=6).*

6.2.5 Myeloid-specific SIRT1 depletion correlated with increased fibrogenesis in response to BDL

Fibrosis is a common pathological feature of cholestatic liver disease and macrophages promote both fibrogenesis and fibrolysis during disease (Trautwein et al., 2015). SIRT$^{mye-/-}$ mice exhibited significantly increased liver fibrogenesis, as evidenced by Sirius red staining (figure 6.9) and α-SMA immunofluorescence staining (figure 6.10) 7 days after BDL surgery.

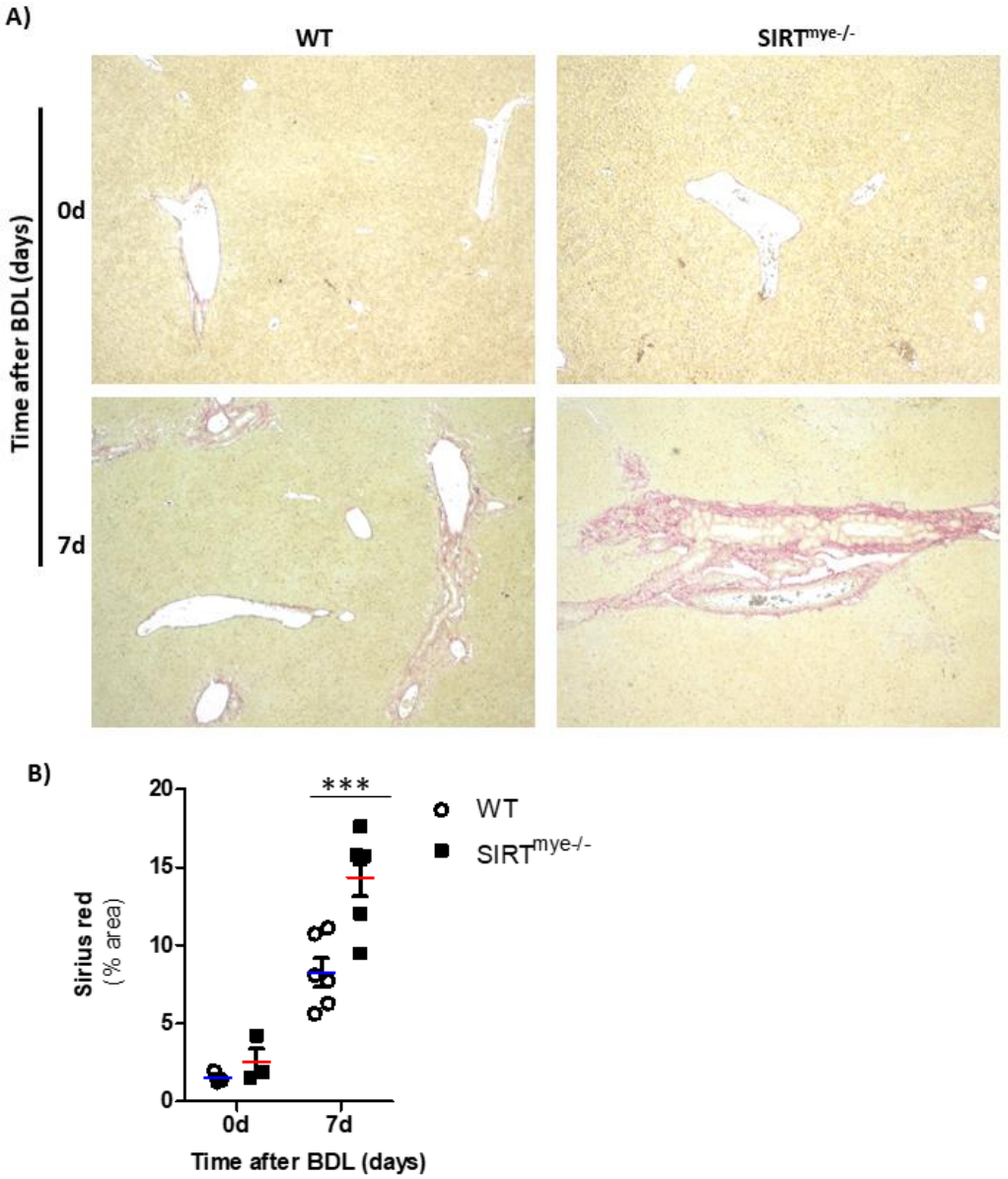


Figure 6.9 Livers of SIRT$^{mye-/-}$ mice had increased fibrogenesis compared to littermate controls in response to 7 days of BDL. *A) Sirius red staining of liver sections shows increased collagen deposition (red fibres) shows increased fibrogenesis in SIRT$^{mye-/-}$ mice 7 days after BDL. Images were taken using the 4x magnification. B) Image J quantification of the stained area shows a significant increase in fibrogenesis in SIRT$^{mye-/-}$ animals. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 ** P ≤0.01 *** P ≤ 0.001 (SIRT$^{mye-/-}$ vs WT littermate). Error bars represent standard error of the mean (n=6).*

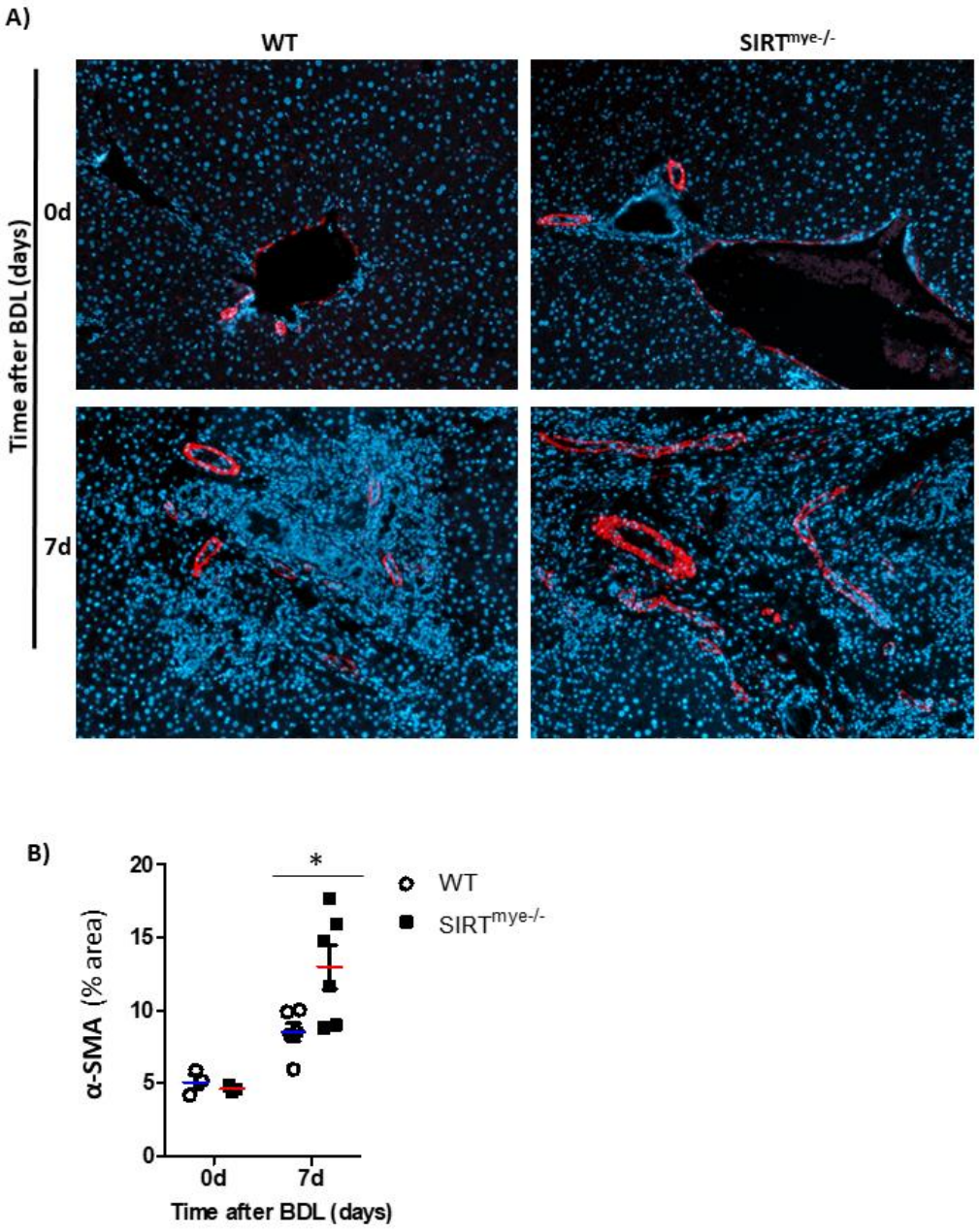


Figure 6.10 Livers of SIRT$^{mye-/-}$ mice had increased fibrogenesis compared to littermate controls in response to 7 days of BDL. *A) α-SMA (red) and DAPI (blue) immunofluorescence staining shows increased fibrogenesis in SIRT$^{mye-/-}$ mice 7 days after BDL. Images were taken using the 10x magnification. B) Image J quantification of α-SMA area shows a significant increase in fibrogenesis in SIRT$^{mye-/-}$ animals. Significance was determined by 2-way ANOVA with Bonferroni post test. * P ≤ 0.05 (SIRT$^{mye-/-}$ vs WT littermate). Error bars represent standard error of the mean (n=6).*

Overall, our results suggest that myeloid SIRT1 depletion is detrimental during cholestatic liver disease.

6.3 Discussion

In this chapter we have demonstrated that myeloid SIRT1 depletion contributes to the pathogenesis of cholestasis by promoting ductular reaction and fibrosis.

Our study is the first one to use $SIRT^{mye-/-}$ animals to study the role of SIRT1 in cells of the myeloid lineage during cholestasis. Several murine studies described that myeloid SIRT1 depletion promoted liver damage in response to LPS induced sepsis (X. Bai et al., 2018; Schug et al., 2010) and high fat diet feeding (Roh et al., 2015; Schug et al., 2010). In accord with those studies, we found mildly increased liver transaminase activity in response to BDL in $SIRT^{mye-/-}$ animals, while necrosis levels were comparable between both genotypes. Cholestasis induced by BDL is a different model of liver damage from high fat diet feeding or LPS induced sepsis, where liver damage is induced by triglyceride accumulation in hepatocytes (Kazankov et al., 2019) and LPS toxicity (Korneev, 2019), respectively. In our context of obstructive cholestasis, where liver damage is a result of bile acid accumulation in the liver and bacterial translocation from the intestine (Mariotti et al., 2018), myeloid SIRT1 may be behaving differently than in the previous studies due to a difference in the induction of liver injury.

Interestingly, we found comparable levels of pro-inflammatory cytokines and chemokine receptors in the livers of both genotypes, which was surprising as SIRT1 depletion has been described to promote inflammation (Yeung et al., 2004). We found a significant decrease in the pro-inflammatory cytokine CCL2 but this did not correlate with decreased macrophage numbers in the livers of $SIRT^{mye-/-}$ mice in response to BDL in our 7 day timepoint. We did not perform enzyme-linked immunosorbent assays for CCL2 protein expression in the livers of our animals therefore we do not know if the protein expression of CCL2 was reduced in $SIRT^{mye-/-}$ mice. From our gene expression results, we can speculate that the protein levels of CCL2 would be reduced in $SIRT^{mye-/-}$ animals compared to WT at a later BDL time point than 7 days, therefore there may be reduced liver macrophage recruitment 10 to 14 days after BDL, further BDL experiments would clarify this.

Ductular reaction is caused by cholangiocyte proliferation of bile ducts damaged by bile acid accumulation during cholestasis (Sato et al., 2019). Macrophages have been previously described to promote ductular reaction and fibrosis in a mouse model of cholestasis as the depletion of macrophages with liposomal chlodronate attenuated ductular reaction in response to DDC feeding (Best et al., 2016). We found a significant increase in bile duct proliferation in $SIRT^{mye-/-}$ mice in response to BDL than in the WT littermate controls. Macrophages can promote ductular reaction, independently of bile acid load, inflammation or liver injury, by inducing cholangiocyte apoptosis (Alabraba et al., 2008) or by the secretion of the cytokine TWEAK, which stimulates HPCs to produce CK19 (Bird et al., 2013). We did not investigate TWEAK expression in this work, $SIRT^{mye-}$

$^{/-}$ macrophages could be secreting more TWEAK that WT macrophages and thus promoting ductular reaction independently of inflammation.

TWEAK has also been described to promote fibrogenesis by stimulating ECM production by HSC (Dwyer et al., 2014). Fibrosis is the accumulation of ECM, which is produced by HSC in response to signals from macrophages, hepatocytes and cholangiocytes (Karlsen et al., 2017). While we are the first to describe that SIRT$^{mye-/-}$ animals had increased fibrogenesis in a model of cholestasis, Roh and colleagues have previously described that SIRT$^{mye-/-}$ mice had increased fibrogenesis in response to high fat diet feeding (Roh et al., 2015). Our findings, and those of Roh et al. suggest that SIRT1 depletion of cells of the myeloid lineage promotes liver fibrogenesis irrespective of disease aetiology (Roh et al., 2015). Roh and colleagues associated the increased fibrogenesis of the SIRT$^{mye-/-}$ mice with increased inflammation, which they defined as increased macrophage infiltration and increased nuclear translocation of NFκB (Roh et al., 2015). Roh et al. did not show any data on pro-inflammatory cytokine expression and, in our study, we did not observe any differences in inflammation between SIRT$^{mye-/-}$ animals and littermate controls in response to BDL. If SIRT$^{mye-/-}$ macrophages produce more TWEAK, this could explain the increased fibrogenesis we observed.

Studies in other cell types described that the depletion of SIRT1 promoted fibrosis. Knockout of SIRT1 in kidney epithelial cells promoted renal fibrosis (Simic et al., 2013) and depletion of SIRT1 in HSC promoted liver fibrosis in mice in response to BDL and CCL_4 treatment as SIRT1 deacetylated enhancer of zeste homolog 2 and PARPγ to prevent the activation of HSC (M. Li et al., 2017, 2018). Several studies proposed that the pro-fibrogenic cytokine TGFβ is hyperactivated in the absence of the deacetylase activity of SIRT1 (X. Z. Huang et al., 2014; Simic et al., 2013). In our model of SIRT1 depletion in cells of the myeloid lineage, monocyte-derived macrophages in the livers of the SIRT$^{mye-/-}$ mice could be producing more TGFβ than the WT littermate controls. This TGFβ increase could be promoting more HSC activation in the SIRT$^{mye-/-}$ and hence the increased fibrogenesis which we have observed. Additionally, the TGFβ increase could be promoting increased ductular reaction which we observed in SIRT$^{mye-/-}$ animals as macrophage TGFβ promotes cholangiocyte proliferation (Locatelli et al., 2016).

In conclusion, in this chapter we show that myeloid-SIRT1 depletion contributes to the progression of cholestasis.

6.4 Future work

We have identified that SIRT$^{mye-/-}$ mice have increased ductular reaction and fibrosis at 7 days of BDL compared to WT animals despite having an equal degree of liver inflammation. We proposed that SIRT$^{mye-/-}$ animals could be producing more TWEAK or more TGFβ than WT, therefore

qPCR analysis could reveal this. If SIRT$^{mye-/-}$ have more TGFβ, SIRT$^{mye-/-}$ mice could be pre-treated with the TGFβ inhibitor galunisertib prior to BDL. If the animals have less fibrosis than untreated SIRT$^{mye-/-}$ mice, this finding would confirm that SIRT1 depletion in monocytes promotes fibrosis by TGFβ activation.

Additionally, BMDM from WT and SIRT$^{mye-/-}$ mice could be treated with bile acids and LPS to investigate the effect of myeloid SIRT1 knockout on macrophages under cholestatic conditions. We found comparable levels of pro-inflammatory cytokines in the livers of WT and SIRT$^{mye-/-}$ animals in response to 7 days of BDL. If the levels of pro-inflammatory cytokines are comparable in BMDM from WT and SIRT$^{mye-/-}$ animals in response to bile acid and LPS treatment *in vitro*, this would confirm that myeloid SIRT1 depletion has no effect on inflammation in response to cholestatic stimuli.

Chapter 7- Discussion

7.1 Thesis summary

In this thesis, we demonstrate that the microbiome associates with increased liver damage and macrophage infiltration in response to cholestatic liver injury and that endotoxin sensitises hepatocytes to bile acid induced cell death during cholestatic liver disease. Next, we show that SIRT1 overexpression promotes macrophage activation *in vitro* and liver damage *in vivo* in murine models of endotoxin challenge and that myeloid SIRT1 overexpression promotes the pathogenesis of cholestasis. Finally, we show that myeloid SIRT1 depletion associates with disease pathogenesis in response to BDL. In conclusion, we propose that fine tuning of macrophage SIRT1 is essential to maintain liver health as overexpression or depletion of macrophage SIRT1 is detrimental during cholestasis.

7.2 The role of microbiome-derived endotoxin in the pathogenesis of cholestasis

Cholestatic liver diseases associate with changes in the composition of the microbiome (Iwasawa et al., 2017; Kummen et al., 2017; Quraishi et al., 2017) however the pathological role of these changes has been poorly characterised to date and generated conflicting results.

Tabibian and colleagues described that the lack of a microbiome and associated innate immune cell recruitment promoted cholestatic liver damage in Mdr2$^{-/-}$ animals rederived in a GF environment (Tabibian et al., 2016). Most recently, GF mice displayed increased inflammation and liver damage in response to BDL than mice colonised with altered Schaedler's flora (Juanola et al., 2021). However, the rederivation of another mouse model of bile duct injury NOD.c3c4 into a GF environment prevented liver damage (Schrumpf et al., 2017), suggesting that the presence of the microbiome actively contributes to cholestatic liver disease.

More recently, faecal microbiome transplantation of the microbiome of cholestatic Mdr2$^{-/-}$ animals into recipient mice promoted Nlrp3 inflammasome activation, intestinal permeability and liver injury (Liao et al., 2019). Importantly, this unique study showed that PSC patients have increased Nlrp3 activity and subsequent IL1β production in the liver (Liao et al., 2019).

In this thesis, we show that the transplantation of a WT microbiome into GF mice promoted macrophage recruitment to the liver in response to cholestatic liver injury. Using the MCC950 inhibitor of the Nlrp3 inflammasome and chlodronate loaded liposomes to deplete macrophages in cholestatic GF+WT animals, further work from the Beraza laboratory identified that when activated by the microbiome, the Nlrp3 inflammasome in macrophages promotes the pathogenesis of cholestasis (Isaacs-Ten et al., 2020). Our work, and work from the Trautwein group identified the

Nlrp3 inflammasome as a druggable target in cholestatic liver diseases (Isaacs-Ten et al., 2020; Liao et al., 2019).

7.3 SIRT1 overexpression in macrophages promotes inflammation and liver damage in response to endotoxin

This thesis was underpinned by our previous findings where we described that SIRT1 overexpression correlated with increased macrophage infiltration and inflammation in murine models of cholestasis (Blokker et al., 2018). The gut/liver axis is critical during cholestasis as translocating bacteria can reach the liver and promote inflammation and damage (Di Leo et al., 2003; Feld et al., 2006). Following on from the work of Blokker et al., the Beraza group identified that SIRT^{oe} animals had increased intestinal permeability in response to BDL (N. Beraza, personal communication). The increased inflammation we previously described in SIRT^{oe} animals could be a result of a higher bacterial load reaching the liver due to increased intestinal permeability that promotes an increased pro-inflammatory response of SIRT^{oe} macrophages to endotoxin or by the higher sensitivity of SIRT^{oe} hepatocytes to endotoxin and/or inflammatory mediators. In this thesis, we investigated the role of macrophage SIRT1 overexpression in response to an equal dose of endotoxin *in vivo* and *in vitro*. In this work we described that SIRT^{oe} animals had increased liver damage in response to liver injury with LPS/GalN and LPS alone and, *in vitro*, SIRT^{oe} BMDM exhibited a pro-inflammatory phenotype characterised by increased production of pro-inflammatory cytokines, Nlrp3 inflammasome activation and metabolic rewiring.

Our *in vivo* results suggested that SIRT1 overexpression promoted Nlrp3 inflammasome activation therefore we looked at mechanisms which could upregulate Nlrp3 activity. Nlrp3 inflammasome can become activated through the transcription factor NFκB which upregulates the transcription of its components Nlrp3 and pro-caspase 1 (Swanson et al., 2019). SIRT1 has been described to inhibit NFκB activity (Schug et al., 2010; Shen et al., 2009; Yeung et al., 2004), therefore we explored other pathways. There are several pathways independent of NFκB which can activate Nlrp3 activity. Nlrp3 inflammasome activity can be promoted by phosphorylation of its components by the kinase JNK (Hara et al., 2013; Song et al., 2017). Additionally, defects in autophagy can promote IL1β production through the Nlrp3 inflammasome (C. S. Shi et al., 2012). Finally, metabolism can regulate inflammasome activation (Hughes & O'Neill, 2018) and upregulation of glycolysis by mTOR is another mechanism of Nlrp3 inflammasome activation (Moon et al., 2015).

Firstly, we established that the nuclear translocation of the pro-inflammatory transcription factor NFκB was delayed in BMDM SIRT^{oe} mice, not completely inhibited as proposed by the mechanistic studies of Yeung and colleagues (Yeung et al., 2004), this result suggests that the pro-

inflammatory phenotype we observed occurred independently of NFκB mediated transcription. The delay of NFκB expression in SIRT^{oe} BMDM in response to LPS was further confirmed by other colleagues from the Beraza laboratory (N. Beraza, personal communication). After establishing that SIRT1 overexpression drives inflammation in macrophages independently of NFκB, we explored other mechanisms.

When the kinase JNK becomes activated by phosphorylation by TAK1 (O'Neill et al., 2013), it phosphorylates and activates a range of pro-inflammatory mediators including the Nlrp3 inflammasome (Hara et al., 2013; Song et al., 2017). Therefore, we investigated pJNK expression *in vivo* in the livers of WT and SIRT^{oe} animals in response to LPS/GalN treatment and in BMDM *in vitro* in response to LPS challenge and we found that SIRT1 overexpression promoted JNK activity. The SIRT1 homolog SIRT2 has been described to activate JNK by deacetylation of K153 (Sarikhani et al., 2018). SIRT1 overexpression could be driving JNK activity by deacetylation of K153 on JNK and hence promoting Nlrp3 inflammasome activity.

SIRT1 is a well described positive regulator of autophagy through various mechanisms including the upregulation of AMPK activity (F. Lan et al., 2008), as pAMPK is essential for the initiation of autophagy (J. Kim et al., 2011). We found that SIRT^{oe} BMDM had increased AMPK phosphorylation compared to WT BMDM, basally and in response to LPS therefore we expected to see increased autophagy flux in SIRT^{oe} cells. Thus, we investigated autophagosome formation in response to LPS treatment in WT and SIRT^{oe} BMDM and we, surprisingly, found that autophagosome formation appeared delayed in BMDM from SIRT^{oe} animals. This was an intriguing discovery as several studies described that SIRT1 promotes autophagy in mouse embryonic fibroblasts (Lee et al., 2008), HEK293 cells (Sun et al., 2015) and the immortalised cervical cancer cell line HeLa and HEK293 (R. Huang, Xu, Wan, et al., 2015) transiently transfected with SIRT1 overexpressing plasmids. Our study is more biologically relevant to the role of SIRT1 in macrophages as we used BMDM extracted from SIRT^{oe} animals where overexpression was under the BAC promoter (Pfluger et al., 2008). Indeed, the findings of this thesis that support impaired autophagy in SIRT^{oe} BMDM were confirmed by further work from the Beraza laboratory which identified accumulation of p62 in BMDM from SIRT^{oe} animals in response to LPS treatment (N. Beraza, personal communication). The accumulation of the adaptor protein p62 is a marker of failed cargo degradation due to impaired autophagy (Katsuragi et al., 2015). In accord with the findings of the Beraza group, a very recent study identified that SIRT1 deacetylates p62 at K295 and this deacetylation promotes p62 accumulation and mTOR activation in a mouse model of hepatocellular carcinoma (L. Feng et al., 2021).

Autophagy induction can be stopped by the phosphorylation of the autophagy initiator complex ULK1 at S757 by mTOR (J. Kim et al., 2011). Work from the Beraza laboratory has identified increased phosphorylation of ULK1 at S757 in SIRT^{oe} BMDM in response to endotoxin (N. Beraza, personal communication). These findings confirm increased mTOR activity which we observed in response to LPS treatment in SIRT^{oe} BMDM, where we found increased phosphorylation of S6, a target of S6K1 which is activated by mTOR (Weichhart et al., 2015). However, SIRT1 itself has been described to promote S6K1 activity by deacetylating the C terminal domain of S6K1 (Hong et al., 2014), independently of mTOR. Recent work from the Beraza group has identified that IL1β production by SIRT^{oe} BMDM is dependent on mTOR. Treatment of SIRT^{oe} BMDM with the mTOR inhibitor rapamycin, in conjunction with LPS stimulation for 24 hours, resulted in a significant decrease of IL1β protein production in SIRT^{oe} BMDM compared to WT (N. Beraza, personal communication). Our findings complement a previous report where pharmacological inhibition of SIRT1 suppressed mTOR mediated pro-inflammatory cytokine production in the mouse lung in response to LPS-induced lung injury (J. Huang et al., 2017).

Finally, we identified that macrophage SIRT1 overexpression promoted metabolic changes in response to LPS treatment. Metabolic rewiring is known to underpin the pro-inflammatory phenotype of macrophages (Van den Bossche et al., 2017), including the promotion of Nlrp3 activity (Hughes & O'Neill, 2018). SIRT^{oe} BMDM had increased glycolytic activity in response to LPS treatment, indicated by increased lactate production. Increased glycolysis could promote Nlrp3 activity via mTOR (Moon et al., 2015), which we know promotes inflammation in SIRT^{oe} macrophages. Pro-inflammatory macrophages are characterised by a disrupted Krebs cycle, where accumulation of the metabolites citrate and succinate is used to support anti-bacterial functions of macrophages (Ryan et al., 2019). Work presented in this thesis and data generated by external collaborators (K. Hiller, personal communication) has identified that SIRT^{oe} macrophages produce significantly more citrate and succinate in response to endotoxin challenge than WT macrophages. Succinate has been described to promote Nlrp3 inflammasome activation (Y. Li et al., 2016) thereby increased IL1β production could also be supported by metabolic changes in SIRT^{oe} macrophages.

The work shown in this thesis and further work from the Beraza group has identified that SIRT1 overexpression promotes macrophage activation in response to endotoxin by an mTOR mediated mechanism. Despite being activated by opposing stimuli, where SIRT1 is activated during starvation and mTOR is activated during nutrient abundance, SIRT1 has been described to activate mTOR to promote biomass generation during low energy states such as intestinal stem cell self-renewal during caloric restriction (Igarashi & Guarente, 2016), muscle hypertrophy (Gombos et al., 2021) and for pro-inflammatory cytokine production during LPS induced lung injury (J. Huang et al.,

2017). During cholestasis, there is a loss of bile flow from the liver to the intestine and since bile is essential for the digestion of lipids, there is a decrease in the digestion and absorption of dietary lipids and this affects the energy status of the liver. Recently, the activation of AMPK in hepatocytes was shown to drive lipid loss from the cholestatic liver (Irungbam et al., 2020). An earlier study by Moustafa and colleagues identified that the restoration of lipid metabolism by high fat diet feeding or treatment with the therapeutic bile acid NorUDCA protected Mdr2$^{-/-}$ mice from cholestatic liver injury and correlated with decreased macrophage activation and pro-inflammatory cytokine production (Moustafa et al., 2012). We have previously proposed that SIRT1 is upregulated in the cholestatic liver due to decreased energy in the liver, which occurs due to deficient lipid absorption (Blokker et al., 2018; Irungbam et al., 2020; Moustafa et al., 2012). Taken together, we propose that during cholestasis SIRT1 is upregulated due to the low energy status of the liver and, in response to cholestatic liver injury, SIRT1 promotes mTOR activation in macrophages to drive the rapid generation of biomass required for production of pro-inflammatory cytokines needed to initiate the inflammatory response in the liver. As inflammation persists, further liver damage then occurs, promoted by SIRT1 overexpression in macrophages via mTOR activation (figure 7.1).

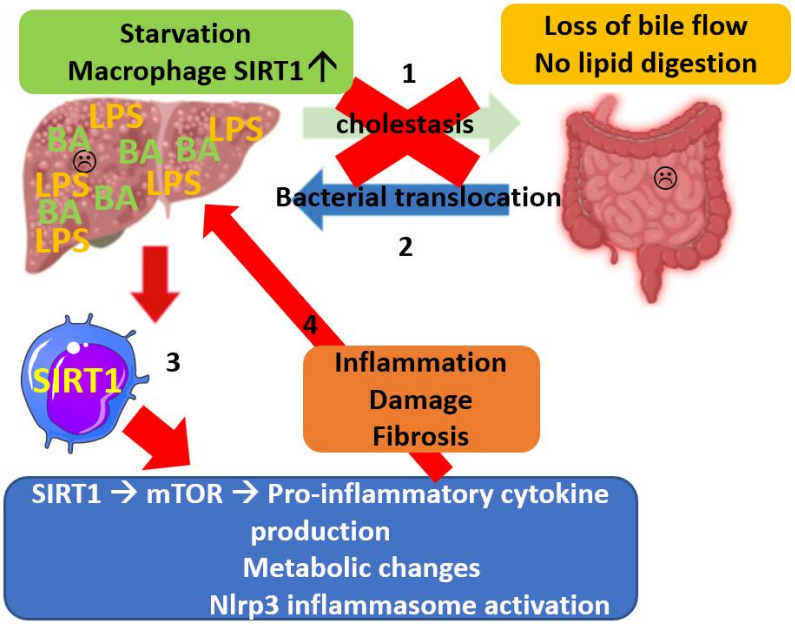


Figure 7.1 Macrophage SIRT1 overexpression in the liver contributes to liver injury during cholestasis. *1. During cholestasis, bile acids (BA) accumulate in the liver and do not flow to the intestine causing liver injury and intestinal dysbiosis. Additionally, the lack of bile in the intestine causes impaired lipid digestion which results in a low energy status in the liver. 2. Due to increased intestinal permeability, bacterial products (LPS) translocate to the intestine where they contribute to the inflammatory response. 3. The low energy status of the liver, as well as the dual action of BA and LPS causes the upregulation of SIRT1 expression in macrophages. In order to respond to the bioenergetic demands of the inflammatory response, SIRT1 activates mTOR and this contributes to pro-inflammatory cytokine production, metabolic changes and Nlrp3 activation in macrophages. 4. As the inflammatory response persists, further liver damage occurs which leads to fibrosis.*

7.4 SIRT1 overexpression in myeloid cells promotes the pathogenesis of cholestasis

After establishing a pro-inflammatory role of SIRT1 overexpression in macrophages, we used bone marrow transplantation followed by BDL to establish that SIRT1 overexpression in myeloid cells promotes the pathogenesis of cholestasis. We found increased liver damage and massive areas of necrosis in the livers of PEPC+SIRT^{oe} animals compared to PEPC+WT mice. The ability of macrophages to clear dying cells is essential to the resolution of liver injury (Canbay et al., 2003). We described that BMDM from SIRT^{oe} animals have a delayed phagocytic capacity of the model organism zymosan compared to WT macrophages. Previous investigations of SIRT1 in phagocytosis generated conflicting reports as some groups described that pharmacological activation of SIRT1 did not affect phagocytosis (Crotty Alexander et al., 2013) while others described that SIRT1 promoted phagocytosis in peritoneal macrophages (R. Zhang et al., 2010). The reduced phagocytosis phenotype which we describe is in agreement with our context of SIRT1 overexpression, and in accord with the overactive pro-inflammatory phenotype we described in SIRT^{oe} macrophages in this thesis. Pro-inflammatory macrophages have been described to have reduced phagocytic capacity compared to anti-inflammatory macrophages (Leidi et al., 2009; Schulz et al., 2019). Transfer of anti-inflammatory macrophages reduced necrotic areas in the liver as they cleared necrotic hepatocytes by phagocytosis in a murine model of acetaminophen overdose (Starkey Lewis et al., 2020). In our PEPC+SIRT^{oe} mice, reduced phagocytic clearance of dying hepatocytes by transplanted SIRT^{oe} macrophages could result in the large necrotic areas we observed in the livers of PEPC+SIRT^{oe} animals after BDL. The presence of DAMPs from dying hepatocytes stimulates collagen production by HSC and contributes to liver fibrosis (An et al., 2020). We showed that PEPC+SIRT^{oe} mice had increased fibrosis compared to PEPC+WT animals. The increased presence of necrotic hepatocytes in PEPC+SIRT^{oe} animals could contribute to increased fibrogenesis by activation of HSC.

Phagocytosis is essential to the resolution of fibrosis in the liver as it reprograms macrophages to secrete ECM-degrading MMPs (Ramachandran et al., 2012). Since we demonstrated that macrophage SIRT1 overexpression results in delayed phagocytosis, the production of MMPs could be delayed in PEPC+SIRT^{oe} animals compared to PEPC+WT mice and this could contribute to increased fibrosis in PEPC+SIRT^{oe} mice.

We have previously described that macrophages promote the pathogenesis of cholestasis via the activation of the Nlrp3 inflammasome (Isaacs-Ten et al., 2020). In this thesis we show that SIRT1 overexpression in macrophages promotes Nlrp3 inflammasome activation in response to LPS and BDL. This increased inflammasome activation associated with increased pathogenesis of cholestasis in our PEPC+SIRT^{oe} mice in response to BDL. During cholestasis, there are two stimuli of

macrophage activation, bile acid accumulation in the liver due to the loss of bile flow (Fickert & Wagner, 2017) and bacterial endotoxin which translocates to the liver from a permeable intestine (Wiest et al., 2017). Bile acids have been described to act as DAMPs which promote Nlrp3 inflammasome activation during cholestasis (Gong et al., 2016; Hao et al., 2017). Hao and colleagues reported that FXR is a key negative regulator of the Nlrp3 inflammasome in macrophages during cholestasis (Hao et al., 2017). We have previously described that SIRT1 overexpression promoted FXR degradation in hepatocytes during cholestasis (Blokker et al., 2018). In this thesis we did not explore FXR status in SIRT1 overexpressing macrophages, however we hypothesise that SIRT1 overexpression may promote FXR degradation in macrophages during cholestasis. The absence of FXR in $SIRT^{oe}$ macrophages could be promoting activation of the Nlrp3 inflammasome in response to bile acids and translocating bacterial endotoxin in PEPC+$SIRT^{oe}$ mice, and $SIRT^{oe}$ animals, following BDL surgery. Intestinal LPS was found to activate the Nlrp3 inflammasome in macrophages to produce IL1β and macrophage-derived IL1β was found to promote cholestasis through the downregulation of FXR expression in hepatocytes (El Kasmi et al., 2018). The increased IL1β produced by $SIRT^{oe}$ macrophages could be driving FXR downregulation in hepatocytes, and hence, increased cholestatic liver injury which we observed in $SIRT^{oe}$ and PEPC+$SIRT^{oe}$ mice following BDL.

Work presented in this thesis suggests that the modulation of macrophage SIRT1 could be a potential therapeutic approach for the treatment of cholestatic liver disease.

7.5 Myeloid SIRT1 depletion contributes to the pathogenesis of cholestasis

In this thesis, we have shown that myeloid SIRT1 overexpression promotes macrophage activation and the progression of cholestasis.

Several studies demonstrated the role of myeloid SIRT1 depletion in various inflammatory diseases, generating conflicting reports (Roh et al., 2015; Schug et al., 2010; Woo et al., 2016). To evaluate whether myeloid SIRT1 depletion could be a viable therapeutic option, we induced obstructive cholestasis in $SIRT^{mye-/-}$ mice and found that myeloid-specific SIRT1 depletion is detrimental during cholestasis. While we did not observe a difference in pro-inflammatory cytokine production between the genotypes, we found increased liver damage and disease pathogenesis in $SIRT^{mye-/-}$ animals, indicated by mildly elevated serum transaminases, significantly increased ductular reaction and fibrogenesis 7 days after the BDL surgery. TWEAK production by macrophages has been described to promote both ductular reaction (Bird et al., 2013) and fibrogenesis (Dwyer et al., 2014) in mouse models of liver disease and therefore, the worse phenotype during cholestasis in $SIRT^{mye-/-}$ animals could be driven by increased TWEAK production.

Recent work by Li and colleagues described that SIRT1 expression was downregulated in monocyte-derived macrophages isolated from PBC patients (Y. Li et al., 2020), however, as the peripheral blood mononuclear cells were differentiated *in vitro* for 7 days, the decrease of SIRT1 observed could be due to the cells being in culture for 7 days. The SIRT1 of monocytes circulating in the bloodstream may be differently regulated than the SIRT1 of monocyte-derived macrophages which reach the cholestatic liver, where the bile acids and intestinal-derived endotoxin may synergise to upregulate SIRT1 expression, as we demonstrated in chapter 5 of this thesis, using BMDM stimulated with bile acids and LPS *in vitro*. Perhaps in the late stages of cholestatic liver diseases, when cholestasis associates with sepsis (Chand & Sanyal, 2007) and bacteria reach the bloodstream, an increase in bacterial LPS along with a high concentration of bile acids in the serum could upregulate SIRT1 expression in blood monocytes.

In the work by Li and colleagues, using the poly I:C model to induce cholestasis, the authors described that SIRT1 is downregulated in the liver and liver isolated macrophages during cholestasis and that the restoration of SIRT1 signaling with pharmacological SIRT1 activators reduced liver damage (Y. Li et al., 2020). While their finding supports our reports that the fine tuning of SIRT1, not overexpression or ablation promotes liver health, their finding that SIRT1 is downregulated in the liver response to cholestasis is contradictory to our previous work (Blokker et al., 2018). While administration of poly I:C for 16 weeks has been described as a model of the initiating events of PBC by one group, where liver damage was driven by IFNα mediated inflammation (Okada et al., 2005), it is not a widely accepted model of cholestasis (Nevzorova et al., 2020) unlike the models we used to demonstrate SIRT1 upregulation, BDL and DDC (Blokker et al., 2018), which are widely accepted models of bile acid metabolism disorders where liver damage is driven by bile acid accumulation (Fickert et al., 2014). In the study of Li and colleagues, SIRT1 may have been differently regulated due to the differences in the induction of liver damage (Y. Li et al., 2020).

We have previously described that high doses of bile acids are necessary to induce SIRT1 upregulation (Blokker et al., 2018) and bile acids likely did not reach high concentrations in the poly I:C model used by Li et al. (Y. Li et al., 2020). Whilst the findings of Li et al. are interesting, they are not comparable to our findings due to the differences in the models of liver damage (Blokker et al., 2018; Y. Li et al., 2020). However, despite the differences in the models, the work of Li and colleagues supports the findings of chapter 6 of this thesis, as both studies demonstrate that loss of SIRT1 expression in macrophages is detrimental during cholestatic liver diseases (Y. Li et al., 2020).

Our fascinating results demonstrated that the transfer of the bone marrow from SIRT^{oe} animals and the depletion of SIRT1 from myeloid cells contributed to the pathogenesis of

cholestasis in response to BDL. Pre-clinical studies have shown that bone marrow transfer associated with reduced liver fibrosis in a CCL_4 model (Luo et al., 2019), and BMDM transfer improved liver fibrosis in response to CCL_4 (Thomas et al., 2011) and BDL (Ma et al., 2017). Protocols for autologous macrophage transplantation have been developed and well tolerated in fibrotic patients (Moroni et al., 2019) and new technologies have been invented for the repolarization of macrophages (Iv et al., 2020; Klichinsky et al., 2020). Our results suggest that the modulation, not overexpression or depletion, of SIRT1 in cells of the myeloid lineage could be an interesting therapeutic opportunity for the treatment of cholestatic liver diseases.

7.6 Thesis conclusion and impact

In this thesis, we show that microbiome-derived endotoxin is essential for macrophage recruitment during cholestasis. Additionally, we demonstrate that SIRT1 promotes macrophage activation and regulates liver inflammation in response to endotoxin and cholestatic liver injury. Finally, we propose that the loss of SIRT1 in myeloid cells is detrimental during cholestasis. The overall conclusion of this work is that SIRT1 expression must be finely regulated in macrophages to preserve liver health as overexpression or depletion of macrophage SIRT1 contributes to the pathogenesis of cholestasis. Our work makes an important contribution to the fields of hepatology and immunology as we are the first to propose the role of SIRT1 as a mechanism to promote inflammation in macrophages during cholestatic liver disease. The fundamental knowledge generated from this work can be used to develop therapeutics to target macrophage SIRT1 to treat cholestatic liver diseases. This thesis will be a valuable source of information for future research of SIRT1 in the context of inflammation.

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Appendix

List of publications

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PhD candidate's name is in bold and underlined