Genetic and Epigenetic Determinants of Alcoholic Liver Disease

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Alcoholic liver disease (ALD) is a significant and growing global health problem, responsible for over 10000 deaths per year in the UK alone. Clinical liver failure can result from gradual, chronic depletion of the hepatocyte pool and replacement with fibrous tissue in cirrhosis or from rapid, acute hepatocellular dysfunction secondary to inflammation in acute alcoholic hepatitis (AAH) which carries a mortality of up to 35% on first presentation. Corticosteroid therapy has shown some benefit in AAH but its utility is limited by uncertainty in patient selection and poor clinical response in a proportion of cases. Our current understanding of AAH pathogenesis attributes hepatocellular dysfunction to the action of supra-physiological concentrations of pro-inflammatory cytokines. Evidence from animal and human studies suggests that the major source of cytokine release is the hepatic macrophage or Kupffer cell responding to an increased concentration of bacterial endotoxin in portal blood following an ethanol-mediated increase in gut permeability. However, this enhanced and sustained inflammatory response is at odds with the normal response in the liver in which endotoxin tolerance allows bacterial components to be cleared from the blood without an inflammatory response. This study set out to investigate factors that determine the enhanced inflammatory response in AAH and its response to therapy.

Genetic analysis revealed a single nucleotide polymorphism in a component of the endotoxin response pathway (the Toll-like receptor adapter molecule MAL) associated weakly with advanced disease in both ALD and the related condition non-alcoholic steatohepatitis. Different alleles associated with advanced disease in the two conditions, suggesting divergent importance of signalling pathways in their pathogenesis. Assays in AAH patients demonstrated that their lymphocyte steroid sensitivity was impaired relative to normal controls, correlated with clinical markers of steroid responsiveness, improved in recovery and could be improved by *ex vivo* supplementation with theophylline, a known recruiter of histone deacetylases. The role of histone modifications in the enhancement of inflammatory responses in ethanol was investigated in a human macrophage cell-line model which revealed increased histone acetylation at pro-inflammatory cytokine gene promoter regions associated with potentiated cytokine responses to endotoxin after culture in ethanol or its metabolite acetate. This effect was abrogated by knockdown of acetyl-coA synthetases, suggesting that increased synthesis of acetyl-coA from acetate is crucial for histone acetylation and consequent increased cytokine production after ethanol exposure.

These findings suggest that while genetic predisposition may have some effect on innate immune responses in the pathogenesis of alcoholic liver disease, the more significant contribution is likely to come from gene-environment interactions such as modulation of histone acetylation by products of ethanol metabolism. This epigenetic relationship between metabolism and gene expression in inflammation, mediated by histone deacetylases such as the sirtuin proteins, may be a novel therapeutic target in ALD and potentially also in other inflammatory conditions.

γGT	γ-glutamyltransferase
4-MP	4-methylpyrazole
AAH	acute alcoholic hepatitis
ABC	antibody binding capacity
Acetyl-CoA	acetyl coenzyme A
ACSS	acyl-coenzyme A synthetase short chain
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
ALD	alcoholic liver disease
ALDH	aldehyde dehydrogenase
ALP	alkaline phosphatase
ALT	alanine transaminase
AP-1	activator protein 1 (heterodimer of c-Fos and c-Jun)
APS	ammonium persulphate
ARDS	adult respiratory distress syndrome
AST	aspartate transaminase
ATP	adenosine triphosphate
BAC	blood alcohol concentration
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CBP	CREB binding protein
CCD	charge-coupled device
CCK	cholecystokinin
CD	cluster of differentiation
CER	cytoplasmic extraction reagent
ChIP	chromatin immunoprecipitation
COPD	chronic obstructive pulmonary disease
COX-2	cyclo-oxygenase 2
CpG	cytosine-phosphate-guanine
CREB	cAMP response element binding
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CYP	cytochrome P450
$ddiH_2O$	double deionised (‘MilliQ’) water
DEPC	diethyl procarbonate
diH_2O	deionised water
DLPC	dilinoleoylphosphatidylcholine
DMEM	Dulbecco’s modified Eagle’s medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphates

DPI	diphenylene iodonium
ECBL	early change in bilirubin level
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
EU	endotoxin units
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FFA	free fatty acids
FRET	fluorescence (or Förster) resonance energy transfer
g	acceleration due to gravity
GAHS	Glasgow alcoholic hepatitis score
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GILZ	glucocorticoid-induced leucine zipper
GR	glucocorticoid receptor
GROα	GRO1 oncogene product (CXCL1)
GSH	reduced glutathione
GSSG	oxidised glutathione, glutathione disulphide
HAT	histone acetyl transferase
HBsAg	hepatitis B surface antigen
HCV	hepatitis C virus
HDAC	histone deacetylases
HDL	high density lipoprotein
HER	hydroxyethyl radical
HFE	haemochromatosis gene
HLA	human lymphocytic antigen
HRP	horseradish peroxidise
HRS	hepatorenal syndrome
HSC	hepatic stellate cell
hsp90	heat shock protein 90
IC_{50}	concentration achieving 50% inhibition of proliferation
IFN	interferon
IKK	IκB kinase
IL	interleukin
I_{max}	maximum inhibition of proliferation
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	inducible nitric oxide synthase

INR	international normalised ratio
IRAK	IL-1 receptor-associated kinase
IRF3	IFN-regulatory factor 3
ISRE	IFN-stimulated response element
IκB	inhibitor of NF-κB
JNK	c-Jun N-terminal kinase
KC	Kupffer cells
K_m	Michaelis constant
LAL	limulus amoebocyte lysate
LBP	LPS binding protein
LFT	liver function tests
LPS	lipopolysaccharide
LSS	lymphocyte steroid sensitivity
MAL	MyD88 adaptor-like
MAPK	mitogen-activated protein kinase
MAT	methionine adenosyltransferase
MCD	methionine-choline deficient diet
MCP-1	monocyte chemoattractant protein-1
mDF	modified hepatitis discriminant function
MDR	multi-drug resistance
MELD	model for end-stage liver disease
MEOS	microsomal ethanol oxidising system
MFI	median fluorescence intensity
MIP-1α	macrophage inflammatory protein-1 alpha
MIP2	macrophage inflammatory protein 2
MnTBAP	Manganese(III)tetrakis(4-Benzoic acid)porphyrin chloride
mRNA	messenger RNA
MSD	MesoScale Discovery platform
MTA	5'-methylthioadenosine
MyD88	myeloid differentiation factor 88
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEMO	NF-κB essential modulator
NER	nuclear extraction reagent
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOD	nucleotide oligomerisation domain
OPD	O-phenyldiamine

OR	odds ratio
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBC	primary biliary cirrhosis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDCE2-ILD	pyruvate dehydrogenase complex E2 inner lipoyl domain
PDE	phosphodiesterase
PHA	phytohaemagglutinin
PI3K	phosphoinositide-3-kinase
PKA	protein kinase A
PPAR	peroxisome proliferator activated receptor
PT	prothrombin time
qRT-PCR	quantitative reverse-transcriptase PCR
RANTES	regulated upon activation, normal T-cell expressed and secreted (CCL5)
RBC	red blood corpuscle
RCT	randomised controlled trial
RFLP	restriction fragment length polymorphism
RIG-I	retinoic acid inducible gene-like RNA helicase (also RLH)
RIP1	receptor-interacting protein 1
RISC	RNA induced silencing complex
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640 medium
RSV	respiratory syncitial virus
SAH	s-adenosylhomocysteine
SAMe	s-adenosylmethionine
SDS	sodium dodecyl sulphate
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SOD2	manganese superoxide dismutase
SREBP	sterol response element binding protein
SST	short synacthen test
SYBR	Synergy Brands Incorporated
TAB	TAK binding protein
TACE	TNFα converting enzyme
TAE	Tris-acetate-EDTA buffer
TAK1	transforming growth factor-activated kinase

TBK1	TRAF-family-member-associated NF-κB activator-binding kinase 1
TBS	tris-buffered saline
TEMED	N,N,N,N-tetramethylethylenediamine
TGF	transforming growth factor
TIPSS	transjugular intrahepatic portosystemic shunt stent
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNFα	tumour necrosis factor alpha
TRAF6	tumour necrosis factor receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	toll/IL-1 receptor domain-containing adaptor inducing IFN-β
Tris	tris(hydroxymethyl)aminomethane
TSA	trichostatin A (HDAC inhibitor)
TUNEL	terminal uridine deoxynucleotidyl transferase dUTP nick-end labelling
UC	ulcerative colitis
ULN	upper limit of normal
UPR	unfolded protein response
WCC	white cell count

LEGO™ tackling the stigma of jaundice

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This thesis is dedicated to Matthew and Robert whose early arrival lent a new perspective to the research, to Alice and Edward whose early arrival had a similar effect on the writing-up, and to their mum.

1 Introduction

One may drink wine and be nothing the worse for it; on another, wine may have effects so inflammatory as to injure him in both body and mind.

Samuel Johnson (1776), quoted in the first clinical description of acute alcoholic hepatitis (Beckett, Livingstone et al. 1961).

1.1 Introduction to Acute Alcoholic Hepatitis

1.1.1 Epidemiology of ethanol-related liver disease

Alcoholic liver disease (ALD) is a significant and growing health problem worldwide. Alcohol consumption is responsible for 4% of the global burden of disease, a proportion similar to that of tobacco and hypertension (Room, Babor et al. 2005; Rehm, Taylor et al. 2006). In the UK alone it was responsible for 8380 deaths in 2004, with the death rate having more than doubled since 1991 (ONS 2006). The rate of increase in mortality from ALD in the UK is now the highest in Europe (Leon and McCambridge 2006).

The relationship between alcohol dose and liver disease was studied in 13 285 subjects in Copenhagen (Becker, Deis et al. 1996). There was dose-dependent relationship between weekly alcohol consumption and incidence of alcohol-induced liver disease and alcohol-induced cirrhosis, with the relative risk of disease becoming significantly greater than 1 at 7-13 standard drinks per week for women and 14-27 standard drinks per week for men. These data support the current medical recommendations for 'safe' alcohol consumption in the UK of no more than 14 drinks per week for women and no more than 21 drinks per week for men. A standard drink ("unit") contains 10ml (7.9g) of absolute ethanol in the UK. There is no international standard drink and national standards vary, with a unit being 10g of ethanol in Ireland, 12g in France and Denmark and 14g in the USA (Tapson 2004). Thus the level of drinking from the Copenhagen study at which risk of liver disease is increased is above 20 UK units per week for women and 41 UK units for men.

The distinction between ALD and the alcohol dependence syndrome is not always well appreciated by the general public, and the adjective 'alcoholic' is used indiscriminately in both scientific literature and the mass media to refer to any or all of alcohol addiction, alcohol-related physical disease and heavy drinking without disease. 58% of ALD patients have no significant dependence on alcohol and only 9% have severe dependence. They are a different demographic group from patients whose primary problem is alcohol dependence. ALD patients tend to drink less and are more likely to have started drinking heavily for social reasons rather than as a response to social or psychological stress (Smith, White et al. 2006). Thus the use of the term 'alcoholic' in relation to this disease is misleading and could be prejudicial. 'Ethanol-

related liver disease' and 'ethanol-related steatohepatitis' are preferable terms but have not become common currency even within the field so the traditional names will be used here for clarity.

1.1.2 Individual susceptibility

The majority of individuals appear to be remarkably resistant to the damaging effects of alcohol on the liver. Above the apparent 'safe limits' the risk of ALD increases in proportion to intake, but of individuals consuming over 200g per day only 20% were found to have cirrhosis after 13 years consumption, and 50% at 20 years (Lelbach 1975). Thus in the drinking population susceptibility factors are just as important to the development of disease as alcohol intake. Susceptibility is influenced by genetic and environmental factors, some of which have been elucidated (Day 2006). Twin studies suggest that genetic factors account for at least 50% of individual susceptibility (Hrubec and Omenn 1981) to ALD, although this may be largely accounted for by a shared genetic predisposition to heavy drinking (Reed, Page et al. 1996).

The most obvious environmental influence on susceptibility is dose of alcohol, and the dose-dependent relationship described above was replicated in the Dionysos study which surveyed the dietary habits and health outcomes of the entire population of two Northern Italian towns (Bellentani, Saccoccio et al. 1997). This also revealed an effect of pattern of drinking independent of absolute levels of consumption, with an increased risk of ALD associated with drinking outside meal times, drinking more than one variety of alcoholic beverage and drinking every day rather than just at weekends. The influence of diet has received some attention, with a diet high in fat and low in carbohydrate associated with increased risk of cirrhosis in drinkers (Rotily, Durbec et al. 1990), and all stages of ALD shown to be increased in heavy drinkers who were also obese (Naveau, Giraud et al. 1997). There has been some data to suggest that wine drinkers are relatively protected from liver damage (Becker, Gronbaek et al. 2002) although an elegant further study has demonstrated that wine drinking is associated with a healthier diet, which may account for the apparent benefit (Johansen, Friis et al. 2006). Cigarette smoking independently increases the risk of ALD, with a one packet a day habit trebling the risk of cirrhosis (Klatsky and Armstrong 1992). The same studies have repeatedly demonstrated a protective effect of coffee drinking on alcohol-related cirrhosis (Klatsky, Morton et al. 2006), the significance of which will be discussed later (section 1.6.5).

In the determination of genetic susceptibility factors, the requirement for an environmental trigger (hazardous drinking) and the social impact of the disease mean that ALD does not lend itself to family linkage studies, and the majority of genetic information comes from case-control, candidate gene allele-association studies. These have suffered from a shortage of validation studies in independent cohorts and, where validation has occurred, lack of reproducibility due to ethnic variations and non-comparable control populations (type 1 error). Small cohort sizes

have resulted in underpowered studies and the risk of false negative results (type 2 error) (Day 2006; Stickel and Osterreicher 2006).

Female sex is the most obvious genetic risk factor for ALD, with women developing accelerated disease compared to men at the same level of alcohol consumption (Ashley, Olin et al. 1977). Alcohol is predominantly water-soluble and has a smaller volume of distribution in women who have a lower total body mass and a higher proportion of body fat, resulting in higher blood alcohol concentrations (Schenker 1997). Rodent studies suggest that there may be a female predisposition to endotoxaemia and lipid peroxidation, perhaps as a result of an oestrogen-dependent increase in gut permeability, which could result in increased inflammation (Nanji, Jokelainen et al. 2001). A testosterone-mediated reduction in pro-inflammatory TLR4 receptors on macrophages may have a protective effect in males (Rettew, Huet-Hudson et al. 2008). Diet does not appear to be responsible for the gender difference (Wagnerberger, Schafer et al. 2008).

The larger candidate gene studies with positive associations have identified susceptibility polymorphisms in genes encoding components of the innate immune response. Study of the C-259T single nucleotide polymorphism (SNP) in the gene for the bacterial lipopolysaccharide receptor CD14 in 381 Finnish men with moderate or high ethanol consumption showed that the T allele was associated with increased risk of alcoholic hepatitis and cirrhosis. The relative risk of cirrhosis was 3.08 for CT and 4.17 for homozygous TT (Järveläinen, Orpana et al. 2001). However, the association was not reproduced in a UK cohort (Leathart, Day et al. 2001). The only positive association to have been validated in an independent population is the G-238A SNP in the tumour necrosis factor alpha (TNFα) promoter region. The A allele was associated with alcoholic steatohepatitis in 150 UK patients with biopsy-proven ALD (Grove, Daly et al. 1997) and also with cirrhosis in 149 male Spanish alcoholics (Pastor, Laso et al. 2005).

1.1.3 Spectrum of ALD

The spectrum of ALD ranges from simple steatosis (fatty liver) through steatohepatitis to fibrosis and eventually cirrhosis (**Figure 1-1**). Progression through the stages can be asymptomatic and go unnoticed unless abnormal liver blood tests are detected in known or covert heavy drinkers. Patients may not present to health services until they experience decompensation of end-stage cirrhosis when the mass of remaining hepatocytes is insufficient to maintain hepatic function and the symptoms of liver failure, jaundice, ascites, encephalopathy and bleeding become apparent. Others present earlier in the spectrum with an episode of symptomatic acute alcoholic hepatitis (AAH) with fever, hepatomegaly, leucocytosis and clinical and laboratory features of liver failure (Stewart and Day 2003).

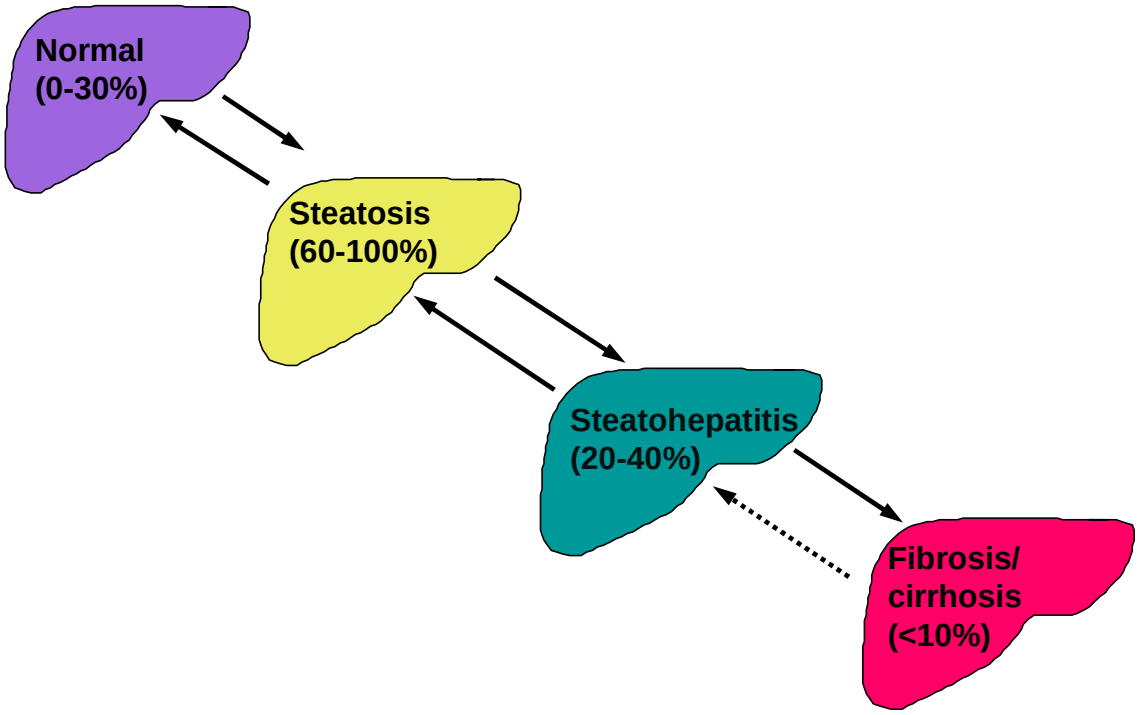


Figure 1-1 Histological spectrum of ALD

Figures in brackets indicate frequency of findings in biopsy series of unselected heavy drinkers consuming at least twice recommended limits (Stewart and Day 2003).

1.1.4 Clinical features of acute alcoholic hepatitis

The association between alcohol excess and chronic liver disease was described as early as 1793 (Baillie 1793) and the term 'cirrhosis' coined 23 years later (Laennec 1819). The ability of alcohol to cause '*acute* necrosis of the liver' was known at the outset of modern medical practice (Osler 1892) but it was in 1961 that doctors at the Royal Free Hospital, London, coined the term 'acute alcoholic hepatitis' to describe the clinical syndrome that they observed in seven patients in whom jaundice developed after sustained alcohol consumption (Beckett, Livingstone et al. 1961). They described a syndrome of jaundice, fever, anorexia, nausea and vomiting with tender hepatomegaly on examination and leucocytosis on blood testing. Histology showed fatty change with inflammatory infiltrate, bile stasis and fibrosis with established cirrhosis in four patients. Since then, AAH has become a well-recognised component of the spectrum of ALD. It is important to note that the description 'acute alcoholic hepatitis' is used differently by clinicians and pathologists, with the former using it to refer to the syndrome described above while the latter apply it to the constellation of histopathological features, without requirement for the presence of the clinical syndrome (Lucey 2002).

AAH patents commonly present with symptoms that are not specific to the liver - abdominal pain, gastrointestinal upset (anorexia, nausea and vomiting, diarrhoea), lethargy and pyrexia.

The majority have tender, smooth hepatomegaly and may have a hepatic bruit on auscultation. A hyperdynamic circulation can lead to palmar erythema, high-volume pulse character and moderate hypotension with tachycardia. Liver insufficiency may be present and demonstrated by jaundice, encephalopathy and bruising, and portal hypertension can manifest as ascites and peripheral oedema. A proportion of patients present with complications of their liver disease such as variceal bleeding or spontaneous bacterial peritonitis. AAH patients are frequently malnourished.

Up to 40% of patients undergo acute clinical deterioration after admission with worsening liver function tests (LFTs) and onset of encephalopathy, renal failure or variceal bleeding. It has been postulated that the withdrawal of ethanol-related calories on admission reduces liver blood flow leading to increased metabolic stress, or that optimum liver perfusion relied on the vasodilatory effect of acetaldehyde (*via* adenosine) which ceases when alcohol is withdrawn (Hardison and Lee 1966).

1.1.5 Laboratory findings

The most frequent positive laboratory finding in ALD is an elevated γ-glutamyltransferase (γGT) which is seen in over 90% of hazardous drinkers. However, this elevation is primarily due to microsomal induction and is largely independent of the severity of liver disease (Wu, Slavin et al. 1976). It has been used to monitor for recidivist drinking in abstinent patients (Pol, Poynard et al. 1990). Blood alcohol estimation can confirm the suspicion of hazardous drinking, with morning levels greater than 100mg/100ml or a level greater than 150mg/100ml without apparent intoxication being strongly suggestive of chronic heavy drinking (Stewart and Day 2006).

The serum transaminase enzymes alanine transaminase (ALT) and aspartate transaminase (AST) are the classical markers of hepatocellular damage. However, the elevations in serum levels in ALD differ from those in other liver diseases in that they are generally of lower magnitude (AST up to five times the upper limit of normal (ULN), ALT up to twice ULN) and the AST:ALT ratio is greater than one in 90% of patients, and frequently greater than two (Cohen and Kaplan 1979). This is often used to help differentiate between alcohol and other aetiologies of liver injury. ALT is predominantly found in the cytoplasm of hepatocytes while AST has cytosolic and mitochondrial forms and a wider tissue distribution. Synthesis of both ALT and AST requires vitamin B6 (pyridoxine) and B6 deficiency is frequently present in chronic heavy drinking. However, ALT synthesis is more sensitive to B6 depletion than AST, which contributes to the elevated AST:ALT ratio seen in ALD. This is compounded by the presence of mitochondrial injury in ALD, which further raises the serum AST (Diehl, Potter et al. 1984).

Blood parameters associated with hepatic insufficiency are low serum albumin, elevated serum bilirubin, low serum urea and prolonged prothrombin time. Portal hypertension and vasodilatory relative hypovolaemia lead to activation of the renin-angiotensin-aldosterone system and

consequent dilutional hyponatraemia and renal potassium loss leading to hypokalaemia. Macrocytosis is seen in 85% of those drinking more than 80g of alcohol daily, but folate or vitamin B12 deficiency is present in only a third, with the primary cause being the direct toxic effect of ethanol on the developing erythroblast (Wu, Chanarin et al. 1975). Falling mean corpuscular volume is often used as a marker of abstinence in patients undergoing treatment, although there are many contradictory studies of its usefulness, particularly after hospitalisation when it may fall whether there is abstinence or not, and in cirrhosis where it may remain elevated (Shaw, Worner et al. 1979) (Pol, Poynard et al. 1990). Neutrophil leucocytosis is a frequent feature of AAH and thrombocytopenia due to splenic and hepatic sequestration of platelets is common when cirrhosis and portal hypertension are present.

The histological features of alcoholic hepatitis (**Figure 1-2**) have been extensively described (Galambos 1972) and debated, and an international consensus (Baptista 1981) identified the following features as obligatory for the diagnosis:

Evidence of liver cell damage: usually ballooning degeneration of hepatocytes or necrosis. Mallory bodies are intracytoplasmic inclusions, staining purple-red on haematoxylin and eosin, which are frequently present but not essential for diagnosis. They consist of microtubule debris, principally keratin 8 and 18, ubiquitin and p62, which accumulate as a result of overload or dysfunction of the hepatocyte proteasome (Zatloukal, French et al. 2007).

Inflammatory cell infiltrate: neutrophils tend to predominate

Fibrosis: pericellular distribution produces a lattice-like or ‘chickenwire’ appearance

Perivenular distribution of lesions: centrilobular / zone 3, though in cirrhotic liver they tend to be isolated to the periphery of nodules.

The following are also seen but are not considered necessary for diagnosis: macrovesicular steatosis, apoptotic bodies, bile duct proliferation, cholestasis, giant mitochondria. Alcoholic hepatitis can occur in an already cirrhotic liver, when the features above will be accompanied and distorted by the architectural features of cirrhosis (Alexander, Lischner et al. 1971).

Although both the clinical syndrome and the histological findings are well described, there is no correlation between the severity of the histology and the severity of the clinical presentation (Hislop, Bouchier et al. 1983) and the presence of the clinical syndrome does not accurately predict the histological features (Austin, Kaye et al. 2006). A study of 61 patients demonstrated that after cessation of drinking histology reverted to normal in 27%, progressed to cirrhosis in 18%, and persisted as hepatitis in 55% for up to three years. Of those who continued to drink 38% progressed to cirrhosis and 62% had persistent hepatitis (Mendenhall 1981). In a study of 122 patients with alcohol-related cirrhosis, the presence of alcoholic hepatitis on biopsy actually

had a favourable prognostic significance, presumably because of the associated potential for further recovery (Pessione, Ramond et al. 2003).

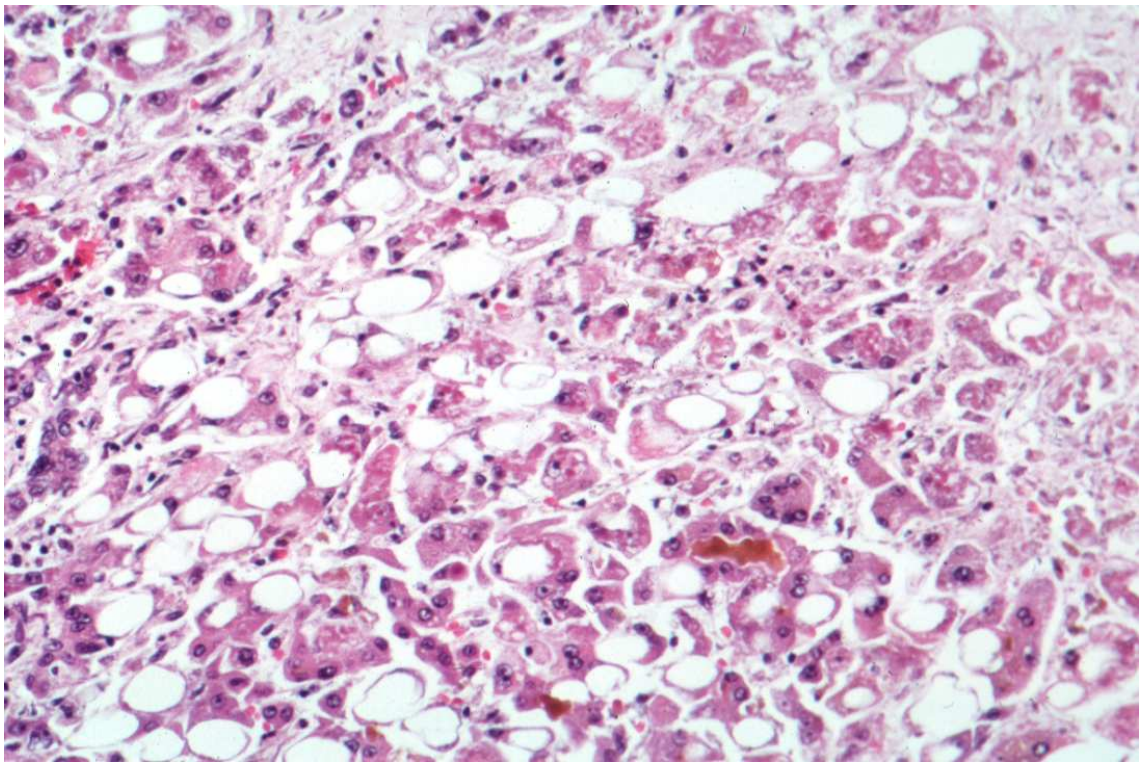


Figure 1-2 Histological features of alcoholic hepatitis

Photomicrograph showing ballooning degeneration of hepatocytes with Mallory hyaline, neutrophil infiltration, macrovesicular steatosis and cholestasis*. Image: PEIR, University of Alabama Birmingham.*

1.1.6 Natural history and prognosis

AAH carries a poor prognosis with early death occurring from infection, hepatic encephalopathy, hepatorenal failure or haemorrhage. The 28-day mortality is up to 35% (Maddrey, Boitnott et al. 1978) (Carithers, Herlong et al. 1989) (Mathurin, Mendenhall et al. 2002) (Akriviadis, Botla et al. 2000). Histological features at presentation to not predict prognosis (Elphick, Dube et al.) so a number of clinical scoring systems have been devised to identify patients at high risk of death in whom interventions are most likely to have a net benefit.

The first scoring system for predicting prognosis in chronic liver disease was published by Child and Turcotte in 1964 and estimated mortality after surgery for portal hypertensive haemorrhage from an aggregate score of five measures of hepatocellular dysfunction: encephalopathy grade, ascites, serum bilirubin, serum albumin and nutritional status (Child and Turcotte 1964). It was subsequently shown to predict mortality in medically-treated cirrhosis (Christensen, Schlichting et al. 1984). The score was modified in the 1970s, replacing assessment of nutritional status

with the more objective criterion of PT prolongation, as illustrated in **Table 1-1** (Pugh, Murray-Lyon et al. 1973), and it is this version which is commonly found in clinical use.

	1 point	**2 points**	**3 points**
Encephalopathy grade	0	1-2	3-4
Ascites	Absent	Slight	Moderate
Bilirubin (µmol/l)	17-34	35-50	>50
Albumin (g/l)	>35	28-35	<28
PT prolongation (s)	1-4	4-10	>10

		Percentage survival		
Total Score	**Grade**	**1 year**	**5 years**	**10 years**
5-6	A	84	44	27
7-9	B	62	20	10
10-15	C	42	21	0

Table 1-1 Child-Pugh score for mortality in chronic liver disease

Calculation of score and grade and associated percentage survival (Howdle 2006)

The hepatitis discriminant function of Maddrey was devised to predict 28-mortality in AAH and was derived from a cohort of 55 patients randomised to corticosteroid treatment or placebo. The laboratory parameters independently associated with death were the prolongation of the prothrombin time and the serum bilirubin (Maddrey, Boitnott et al. 1978). The same group refined the score to produce a modified discriminant function (mDF, **Equation 1-1**) which was used to identify patients with poor prognosis for a further corticosteroid trial (Carithers, Herlong et al. 1989). AAH patients with mDF>32 had a 35% mortality without treatment.

$$mDF = 4.6\left(PT - PT_{control}\right) + \frac{bilirubin}{17.1}$$

Equation 1-1 Modified hepatitis discriminant function

PT prothrombin time in seconds, bilirubin in μmol/litre (Carithers, Herlong et al. 1989)

The model for end-stage liver disease (MELD, **Equation 1-2**) score was developed to predict outcome after transjugular intrahepatic portosystemic shunt stent (TIPSS) procedures for portal hypertension and was subsequently validated for mortality in all chronic liver disease (Malinchoc, Kamath et al. 2000) (Kamath, Wiesner et al. 2001). When applied to AAH and compared to mDF>32, cut-off values of 11 and 21 achieved similar sensitivity and better specificity for predicting 30- and 90-day mortality respectively (Sheth, Riggs et al. 2002) (Dunn, Jamil et al. 2005). In a larger study, MELD increase in the first week of hospitalisation out-performed Child-Pugh score and mDF in predicting in-hospital mortality, and admission MELD>21 had the best sensitivity (91%) and specificity (85%) for death (Srikureja, Kyulo et al. 2005).

$$MELD = 3.8 \times \log_e(\text{bilirubin}) + 11.2 \times \log_e(\text{INR}) + 9.6 \times \log_e(\text{creatinine}) + 6.4 \times (\text{aetiology})$$

Equation 1-2 Model for end-stage liver disease

Bilirubin and creatinine in mg/dl, aetiology = 0 for cholestatic or alcoholic liver disease, 1 for all other diagnoses (Kamath, Wiesner et al. 2001).

The Glasgow alcoholic hepatitis score (GAHS) was an attempt to derive an easily-calculated score that would predict mortality based on readily-available clinical variables. It was derived in a cohort of 241 patients and validated in a further 195 patients, identifying the known prognostic factors of bilirubin, PT and renal function, and adding age and peripheral blood white blood cell count (**Table 1-2**). It was found to have a slightly lower sensitivity than mDF and MELD in predicting mortality, but a higher specificity, and its accuracy was not affected by whether the diagnosis of alcoholic hepatitis was biopsy-proven or made on clinical criteria (Forrest, Evans et al. 2005). The GAHS can be used to compensate for the relatively low specificity of the other scoring systems in selecting high-mortality patients who may benefit from corticosteroid treatment: in patients with mDF>32, only those who also had GAHS≥9 had improved survival when treated with corticosteroids (Forrest, Morris et al. 2007).

	1 point	2 points	3 points
Age	<50	≥50	-
WCC (x10^9/l)	<15	≥15	-
Urea (mmol/l)	<5	≥5	-
PT ratio or INR	<1.5	1.5-2.0	>2.0
Bilirubin (μmol/l)	<125	125-250	>250

Table 1-2 The Glasgow alcoholic hepatitis score

In patients with mDF>32, a total score of ≥9 is associated with a 28-day survival of 52% which improves to 78% with corticosteroid treatment. WCC white cell count, INR international normalised ratio (Forrest, Morris et al. 2007).

GAHS and mDF can predict short-term survival, but in those who survive the acute episode long-term survival depends on a number of different factors. Liver function continues to be a significant determinant of survival, as demonstrated by the prognostic usefulness of the Child-Pugh and MELD scores in chronic ALD (Christensen, Schlichting et al. 1984) (Kamath, Wiesner et al. 2001). Histological severity becomes more relevant for determining long-term prognosis, with the degree of fibrosis being particularly significant (Alexander, Lischner et al. 1971). A patient whose hepatitis resolves and leaves him without cirrhosis has a particularly good prognosis, whereas cirrhotics with even clinically mild hepatitis have a significantly poorer outcome (Goldberg, Mendenhall et al. 1986). Progression to cirrhosis occurs more frequently in women than men (Pares, Caballeria et al. 1986). Achievement and maintenance of abstinence from alcohol can reduce the risk of progression to cirrhosis, particularly in males with histologically mild disease, and even in those with established cirrhosis abstinence will reduce the risk of hepatic decompensation and death (Mendenhall 1981) (Alexander, Lischner et al. 1971) (Pares, Caballeria et al. 1986).

1.1.7 Treatment of AAH

1.1.7.1 Abstinence

Abstinence for alcohol is the only factor that consistently improves long-term survival (Powell and Klatskin 1968) (Alexander, Lischner et al. 1971) and histology (Pares, Caballeria et al. 1986). In some, achievement of abstinence requires the input of an addiction psychiatry service or pharmacological adjuncts such as disulfiram, acamprosate or naltrexone. In others the provision of information about their illness and a structured 'brief intervention' counselling episode from a trained healthcare professional is sufficient (Kaner, Beyer et al. 2007). AAH patients are a different (though overlapping) population from those with alcohol dependence syndrome and so a reasonable proportion should achieve abstinence with clinical advice and brief interventions (Smith, White et al. 2006). This is confirmed by the fact that 50% of ALD patients will abstain or reduce their alcohol intake to a non-harmful level when advised to do so by a physician at first presentation, and that this reduction in intake is associated with improved survival (Day 1996). However, an admission with AAH is often an individual's first presentation with ALD and the high mortality of AAH means that many do not survive to reap the benefits of abstinence. Thus there is an imperative for treatments that are effective in improving survival in the acute situation.

1.1.7.2 Corticosteroids

Corticosteroids suppress inflammation through a variety of molecular pathways but have the adverse effects of increased susceptibility to infection, gastrointestinal bleeding and slowed tissue healing, so it is vital to select the patients with the highest risk of mortality in whom the survival benefit of steroid therapy will outweigh the attendant risks. For this reason mDF>32 has generally been used to select patients for inclusion in steroid trials. Trials have generally included relatively small numbers of patients and produced contradictory results. An initial meta-analysis (Christensen and Gluud 1995) suggested that there was no benefit, but a later meta-analysis of the three largest and most recent placebo-controlled trials showed a significant benefit with 84% survival with corticosteroid treatment compared to 65% survival on placebo (Mathurin, Mendenhall et al. 2002).

Steroid responsiveness has been shown to be greater in the group of patients with a peripheral blood neutrophilia and a significant neutrophil infiltrate on biopsy (Mathurin, Duchatelle et al. 1996). In addition, it was noted that those patients who survived on steroids showed a fall in their serum bilirubin in the first week of treatment and the value of this 'early change in bilirubin level' (ECBL) in predicting which patients will go on to respond to therapy has been verified in further studies (Mathurin, Abdelnour et al. 2003) (Morris and Forrest 2005). More recently the ECBL has been included in a new validated prognostic score for patients on steroid treatment for severe AAH – the Lille model (Louvet, Naveau et al. 2007).

1.1.7.3 Pentoxifylline

Pentoxifylline (Oxypentaphylline) is a methylxanthine phosphodiesterase inhibitor that is used for symptom relief in peripheral vascular disease because of its ability to increase RBC deformability and increase flow in stenosed blood vessels. It has been found to have an anti-cytokine effect, reducing transcription of the TNFα gene. A randomised controlled trial (RCT) showed improved short-term survival on pentoxifylline (75%) compared to placebo (54%) in patients with mDF>32 (Akriviadis, Botla et al. 2000). In both this and a further study, the increased survival corresponded with a reduction in the incidence of hepatorenal syndrome (HRS) in the pentoxifylline-treated group (Karnam and Reddy 2001). A recent study found no benefit from switching patients to pentoxifylline if they failed to respond to corticosteroids with an ECBL at 7 days (Louvet, Diaz et al. 2007).

1.1.7.4 Biological anti-TNFα agents

TNFα is strongly implicated in the pathogenesis of AAH(Felver, Mezey et al. 1990)so there was hope that the novel biological anti-TNFα agents would be effective therapy. Unfortunately, in a RCT which randomised 36 corticosteroid-treated patients to infliximab or placebo the two-month mortality in the infliximab group was higher (39 v 18%, not significant), possibly due to the increased frequency of severe infections in that group (Naveau, Chollet-Martin et al. 2004). However, the dose of infliximab used was three administrations of 10mg/kg, which is at least twice that used in other inflammatory conditions. A pilot study of the TNFα-receptor:F_c fusion protein etanercept suggested tolerability and possible efficacy (Menon, Stadheim et al. 2004), but a further large-scale trial followed and showed increased mortality in the etanercept-treated group (Boetticher, Peine et al. 2008). There is evidence from animal studies that TNFα is particularly crucial for hepatocyte regeneration in the ethanol-exposed liver (Akerman, Cote et al. 1993), so the balance of anti-inflammatory and regenerative effects may not favour anti-TNFα therapy once liver injury is established and regeneration is vital for restoration of function.

1.1.7.5 Antibiotics

The role of gut-derived bacterial endotoxin in ALD and the beneficial effect of gut sterilisation in animal models lead to a RCT of the non-absorbable antibiotic paromomycin in 50 patients. There was no difference in outcome between placebo and verum groups (Bode, Schafer et al. 1997). Although antibiotics can prevent the onset of AAH in rodents, their use in established human disease may be too late in the pathogenic process.

1.1.7.6 Enteral nutrition

AAH patients are commonly nutritionally compromised at presentation. A Spanish RCT randomised 71 patients to 28 days of total enteral nutrition *via* nasogastric tube or prednisolone

40mg and followed them for one year. Mortality during treatment was similar in the two groups, though deaths in the enteral nutrition tended to occur earlier than on steroids (7 v 23 days). Amongst those who survive the first month, mortality during follow up was higher in the steroid group (37 v 8%, p=0.04) with the majority of deaths being from infection (Cabré, Rodríguez-Iglesias et al. 2000). This suggests that corticosteroid therapy may delay mortality in the acute episode but nutritional replacement is necessary to improve long-term survival.

1.1.7.7 Liver transplantation

The scarcity of donor organs creates an imperative that they should be used where the chance of survival without transplantation is low and the chance of healthy post-transplantation survival is highest. Unlike end-stage cirrhotic ALD in abstinent patients, liver transplantation is not usually offered for AAH because of concerns about equitable donor organ allocation and the effect on public opinion of using donor organs for actively- or recently-drinking patients (Everhart and Beresford 1997) (Neuberger 1998). Most transplantation policies require a period of abstinence of at least 3 months to allow time for spontaneous recovery that would make transplantation unnecessary (Veldt, Laine et al. 2002) and to address concerns about recidivism and the physical and psychiatric co-morbidities that would reduce peri- and post-operative survival (Bathgate 2006).

An initial study suggested that the histological finding of active alcoholic hepatitis in the explanted liver was associated with a higher rate of post-transplant recidivism, alcohol-related graft loss and death (Conjeevaram, Hart et al. 1999). Two subsequent larger studies have found no association, consistent with the low positive predictive value of histological findings for the presence of active drinking in apparently abstinent patients (Tomé, Martinez-Rey et al. 2002) (Wells, Said et al. 2007). However, is unclear whether these findings that support transplantation in histological alcoholic hepatitis can be extrapolated to a population of recent drinkers with clinically apparent and severe AAH (Lucey 2002). A French trial is under way to determine whether transplantation improves survival in patients with clinically severe AAH who fail to respond to corticosteroids with an ECBL (Mathurin 2005).

1.2 Introduction to innate immunity

The human innate immune system is the first line of defence against invading pathogens. Its remarkable ability to respond rapidly to a wide range of micro-organisms is essential for survival. It combats and contains infection at the point of entry, signals danger to other systems, and allows time for the T- and B-cells of the more finely-tuned, antigen-specific, adaptive immune system to become effective. In evolutionary terms, a vigorous innate immune response will have conferred a survival advantage to our hominid ancestors when the loss of the thick fur characteristic of other primates left the skin vulnerable to frequent injuries and contamination (Opal and Huber 2002). Although the molecular and cellular components of the innate immune

system differ little between mammalian species, the human response to microbial components is one of the most sensitive (Heumann, Glauser et al. 1998) (Smirnova, Poltorak et al. 2000). The disadvantage of such a vigorous response is an increased susceptibility to exaggerated inflammation when the innate immune system is activated systemically or locally.

The innate response is triggered by activation of cells equipped to respond to pathogens or specific pathogen components – cells of the macrophage/monocyte lineage, natural killer cells, dendritic cells and endothelial cells. The activated cells secrete inflammatory mediators including cytokines (most importantly tumour necrosis factor alpha (TNFα), interleukin 1 (IL-1), and IL-6), chemokines (such as IL-8), prostaglandins and histamine. These mediators act on vascular endothelial cells to cause nitric oxide-mediated vasodilatation, increased vascular permeability and neutrophil recruitment into tissues. The coagulation cascade is activated locally with up-regulation of endothelial tissue factor and decrease in thrombomodulin and its antithrombotic product, activated protein C. Reactive oxygen species are generated from activated neutrophils, tissue effects of nitric oxide and cytokine-induced alterations in cellular metabolism. The cumulative effect of these responses can result in tissue damage as well as pathogen neutralisation (Opal and Huber 2002).

1.2.1 Danger signals

Until recently our understanding of inflammation lacked a description of the mechanism by which cells of the innate immune system recognise and respond to microbial threats. By definition, these cells lack the elegant (but relatively slow and energetically costly) antigen-specific receptor systems that characterise the adaptive immune response. In recent years our understanding of immune reactivity has changed from the pure discrimination of ‘self’ from ‘non-self’ epitopes to an appreciation of the importance of specific ‘danger’ signals in initiating, directing and modulating the immune response (Matzinger 2002). Danger signals can come from internal sources that indicate tissue damage or invasion, such as products of cell lysis, coagulation or complement cascades, or from exogenous material such as microbial surface molecules or genetic material. It is to these danger signals that the innate immune system responds. The fact that both microbial and internal danger signals can trigger the response explains the similarity of the sepsis syndrome to systemic inflammation with a non-infective precipitant such as trauma, burns or pancreatitis (Pisetsky 2007).

Recognition of microbial danger signals requires a receptor system that responds to evolutionarily-conserved structural components of micro-organisms, so that an organism cannot use genetic variability to escape detection. The components that allow micro-organisms to trigger the immune response are termed pathogen-associated molecular patterns or PAMPs. Typical PAMPs include lipopolysaccharide and peptidoglycan from the cell walls of Gram negative and Gram positive bacteria respectively, bacterial flagellin and microbial DNA and RNA. Several classes of PAMP receptors have been discovered to date, including Toll-like

receptors, NOD and RIG-I receptors (Janeway and Medzhitov 2002). Different PAMP receptors can have pro- or anti-inflammatory effects depending on the cell type and the other stimuli present, and the balance of pro- and anti-inflammatory signalling determines the cellular response (Henson 2005).

1.2.2 Toll-like receptors

Toll-like receptors (TLRs) are the principal PAMP receptors in the innate immune system and obtained their unusual name because of their similarity to the receptor Toll (German for 'funky' or 'cool') in the fruit fly *Drosophila*. Toll was initially a cause for scientific excitement when it was found to be responsible for dorso-ventral body patterning in *Drosophila*, but in addition was later shown to form part of the fly's immune defence against fungal infections. This phylogenetically ancient system of pathogen detection is highly conserved in evolution, with similar receptors occurring not only in humans and invertebrates, but also in plants such as tobacco (Armant and Fenton 2002).

Eleven different TLRs have been identified in mammals. The first to have its involvement in pathogen recognition demonstrated, and the most studied, is TLR4 which responds to the most powerful stimulant of innate immune responses, Gram negative bacterial endotoxin (lipopolysaccharide, LPS). This was established through study of two strains of mice that fail to mount a septic response to large doses of endotoxin and which were shown to have a loss-of-function mutation in the gene for TLR4 (Hoshino, Takeuchi et al. 1999). Subsequently, other TLRs and their ligands have been identified and these are summarised in **Table 1-3**. Some TLRs are able to respond to microbial ligands on their own, but in many cases the response depends on the interaction of several different molecules at the cell surface. TLR dimers are required for signalling through TLR4 (homodimers of two TLR4 molecules) and TLR2 (heterodimers with either TLR1 or TLR6, with the combination determining the ligand specificity of the receptor complex). In addition, LPS signalling through TLR4 requires the interaction of several other molecules at the receptor complex; LPS is delivered to the receptor by soluble LPS binding protein (LBP) and effective receptor activation requires the presence of at least two additional molecules, CD14 and MD2 (Takeda and Akira 2005).

There is differential subcellular localisation of individual TLRs. TLR2 and TLR4 are expressed on the cell surface where they are most likely to encounter material from microbial cell walls. TLR3 and TLR9 are located within endosomes where they are most likely to encounter their ligands in the lytic products of phagocytosed micro-organisms (Takeda and Akira 2005). Human monocytes and Kupffer cells express a wide range of TLRs. The pattern of TLR expression in different peripheral blood leukocyte populations implies specific roles in each population. Although $CD14^+$ cells express less TLR3, TLR9, and TLR10 than other cells, they express the highest levels of TLR2, TLR4, TLR5, and TLR8, and levels of TLR1, TLR6, and TLR7 are comparable to those of $CD19^+$ cells (Zarember and Godowski 2002).

At first the triggering of immune responses by a relatively small range of receptors and ligands might seem crude. However, most micro-organisms present more than one TLR ligand, so it is likely that microbes with differing patterns of molecular motifs can cause differential activation of a number of TLRs, allowing differential responses to various classes of pathogen (Bekeredjian-Ding, Roth et al. 2006).

Toll-like receptor	**Ligands**
TLR1 (heterodimer with TLR2)	Triacylated lipopeptides, lipomannans from M. tuberculosis
TLR2 (often dimer with TLR1 or 6)	Lipoproteins, peptidoglycans, lipoteichoic acids, yeast zymosan
TLR3	Double-stranded RNA
TLR4 (homodimer plus CD14 & MD2)	Lipopolysaccharide, heat shock proteins, pneumolysin, RSV coat proteins, heparan sulphate fragments, fibrinogen peptides
TLR5	Flagellin
TLR6 (heterodimer with TLR2)	Diacylated lipopeptides
TLR7	Responds to synthetic nucleosides and imidazoquinoline anti-virals; native ligand is thought to be single-stranded RNA in endosomes
TLR8	As for TLR7
TLR9	Bacterial DNA – unmethylated CpG motifs
TLR10	Ligand unknown but TLR10 expressed in lung and B lymphocytes
TLR11	Uropathogenic bacteria in mice; absent in humans

Table 1-3 Toll-like receptors and their known ligands

(Takeda and Akira 2005)

1.2.3 TLR signalling pathways

Understanding of the signalling pathway through which TLR ligation leads to activation of a cell and secretion of inflammatory mediators has advanced considerably in the last few years. The end product of intracellular signal transduction is activation of transcription factors which translocate to the nucleus and modulate transcription of target genes. The principal transcription factor in inflammation is nuclear factor kappa B (NF-κB) which up-regulates transcription of genes for inflammatory mediators such as TNFα, interleukins and COX-2. Other transcription factors under TLR regulation induce pro-apoptotic, anti-apoptotic and even anti-inflammatory gene transcription, though how the differential effects of these pathways are modulated is not yet well understood.

Apart from TLR3, all TLRs signal down a common pathway accessed with slight inter-receptor variations *via* the adaptor molecule MyD88. The various signalling intermediates have been identified and are likely to be the targets of future immunomodulatory therapies in sepsis and inflammatory disease, and so are summarised in **Figure 1-3**. TLR2 and 4 rely on a further adapter molecule, MAL (also known as TIRAP), to facilitate their interaction with MyD88 (Fitzgerald, Palsson-McDermott et al. 2001; O'Neill and Bowie 2007).

MyD88 recruits a kinase, IRAK-4, and facilitates its phosphorylation of IRAK-1. IRAK-1 then associates with TRAF-6 to activate the TAK1/TAB complex which in turn enhances the activity of the IκB kinase (IKK) complex. NF-κB is held inactive in the cytoplasm by its inhibitor IκB. The IKK complex phosphorylates IκB, leading to its degradation and the release of free NF-κB which can translocate to the nucleus. There NF-κB undergoes phosphorylation and associates with other transcription regulators to activate inflammatory gene transcription.

TLR3 and TLR4 can also access a separate MyD88-independent pathway to inflammatory gene transcription using the adaptor molecules TRAM and TRIF. This pathway leads to a slower activation of NF-κB and also to transcription of genes for the type 1 interferons *via* a different transcription factor, IRF3 (Takeda and Akira 2005).

NF-κB is not the only intracellular signalling pathway activated by inflammatory stimuli. Signalling through the mitogen-activated protein kinase (MAPK) pathways is also increased by TLR ligation (Guha and Mackman 2001). The three main subtypes of MAP kinases are extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun N-terminal kinase (JNK) and p38. These activate other transcription factors such as activator protein 1 (AP-1, c-Fos/c-Jun) (Dong, Davis et al. 2002). It is the balance of the signals through each of the intracellular pathways that determines the combination and proportional expression of genes transcribed and hence the cellular response to inflammatory stimulation. This balance depends not only on the stimulus itself but also on the cross-talk between pathways, the presence of other molecular modulators of signalling and the prevailing intracellular conditions.

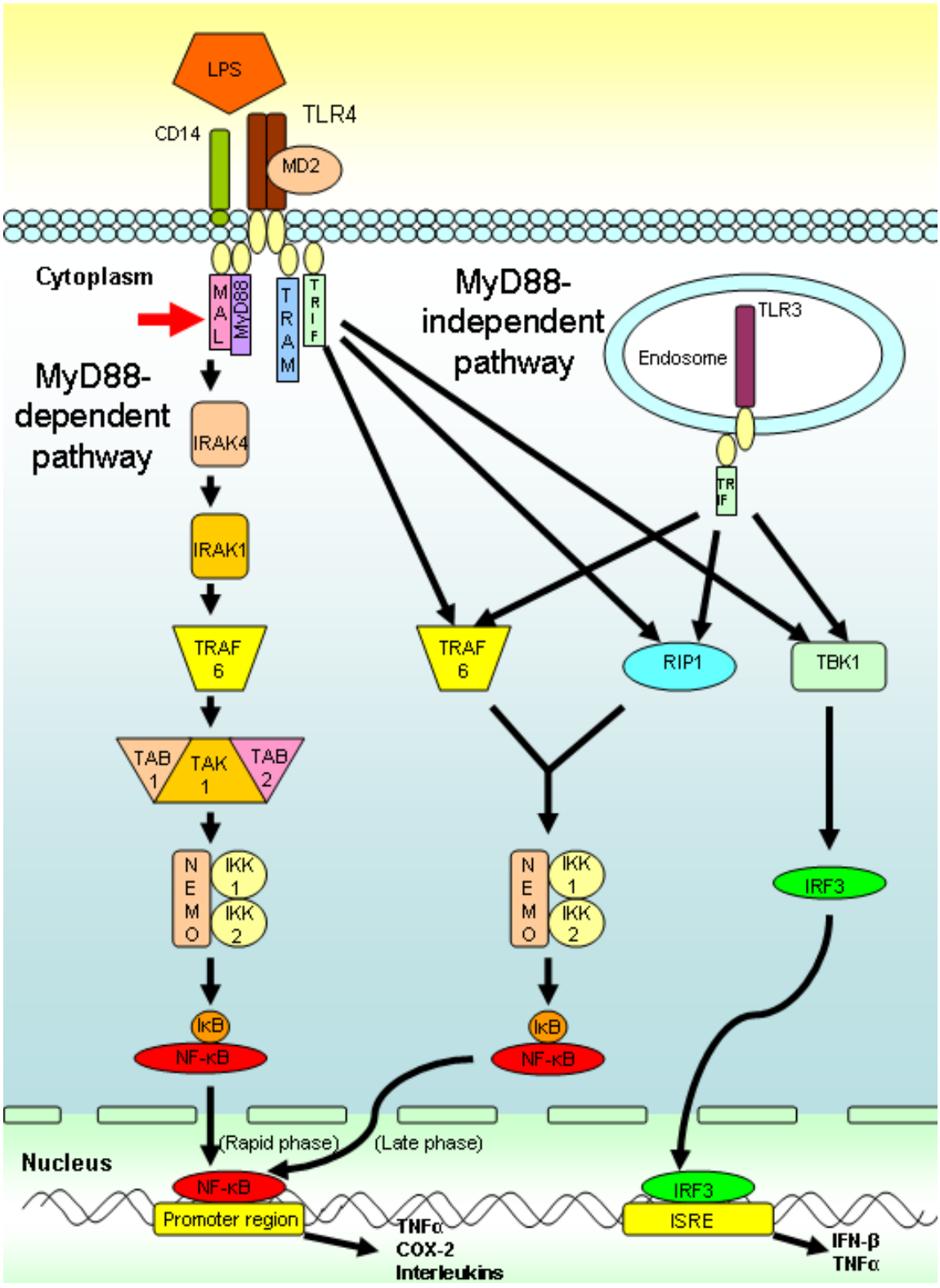


Figure 1-3 Signalling through toll-like receptor 4 and NF-κB

Intracellular signalling through other TLRs uses the MyD88-dependent pathway with small variations. LPS lipopolysaccharide, MyD88 myeloid differentiation factor 88, MAL MyD88 adaptor-like, TRAM TRIF-related adaptor molecule, TRIF toll/IL-1 receptor domain-containing adaptor inducing IFN-β, IRAK IL-1 receptor-associated kinase, TRAF6 tumour

necrosis factor receptor-associated factor 6, TAK1 transforming growth factor-activated kinase, TAB TAK binding protein, IKK IκB kinase, NEMO NF-κB essential modulator, IκB inhibitor of NF-κB, NF-κB nuclear factor-κB, RIP1 receptor-interacting protein 1, TBK1 TRAF-family-member-associated NF-κB activator-binding kinase 1, IRF3 IFN-regulatory factor 3, ISRE IFN-stimulated response element.

1.2.4 Regulation of TLR signalling

TLR activation can trigger a rapid and vigorous inflammatory response, so it is not surprising that TLR signalling is subject to regulation at multiple levels (Liew, Xu et al. 2005). Some regulatory molecules are constitutively expressed in tissues and plasma, while others are induced by activation of the TLR signalling pathway and so provide negative feedback regulation. There is negative feedback within the signalling pathway itself with the gene for the inhibitor IκB being under direct control of a NF-κB-binding promoter sequence, so NF-κB activation results in increased IκB concentration and subsequent down-modulation of the NF-κB effect (Han and Ulevitch 2005).

1.2.5 TLR response tolerance

Repeated observations have demonstrated that the natural history of an episode of sepsis consists of the initial inflammatory phase of vigorous innate immune responses, and then a period of relative immune suppression in which the individual is at increased susceptibility to further infections. These secondary infections tend not to elicit as vigorous an inflammatory response as the initial infection and can insidiously become widespread. This is paralleled by the responses of isolated monocytes which, after an initial stimulation with LPS, show diminished pro-inflammatory cytokine responses to repeat stimulation. This phenomenon of 'endotoxin tolerance' has also been demonstrated with other TLR responses, with prior exposure to a TLR ligand producing diminished responses to the same TLR ligand (termed 'homotolerance') and, in some cases, to other TLR ligands ('heterotolerance') (Sato, Nomura et al. 2000) (Dobrovolskaia, Medvedev et al. 2003).

The mechanism of TLR tolerance is still being investigated and some of the regulators of TLR signalling mentioned above have been implicated in its aetiology. Other mechanisms may involve down-regulation of surface TLRs (Nomura, Akashi et al. 2000), although this is not a consistent observation (Medvedev, Henneke et al. 2001), or nuclear events that suppress the transcription of pro-inflammatory genes (Yoza, Hu et al. 2006). Recent work has suggested that endotoxin tolerance is not simply an all-or-nothing 'off switch' for inflammation, but rather a state of immune 'reprogramming' - a switch to more anti-inflammatory cytokine profiles with modulation of LPS-sensitivity, so that markedly increased doses can still induce an inflammatory response (Broad, Jones et al. 2006). Tolerance is certainly a complex

phenomenon, with LPS from different species (differing principally in the proportion and accessability of the biologically active lipid A moiety) and different prior stimuli inducing differential tolerising effects (Martin, Katz et al. 2001) (Tsujimoto, Ono et al. 2006). It is becoming clear that the surrounding cytokine milieu can modulate the effect of tolerance with, for instance, interferon-γ restoring LPS sensitivity in some systems (Adib-Conquy and Cavaillon 2002). Additionally, the *in vitro* responses of individual cell types do not necessarily correlate with the *in vivo* effects; cooperative networks of cells determine the net effect, which depends on the pattern of TLR stimulation and the tissue environment (Morris, Parker et al. 2006).

Endotoxin tolerance may have developed as a protective mechanism to avoid death from the cytokine storm associated with severe sepsis. However, endotoxin tolerance may be significant in situations other than sepsis. Organ systems such as the gut (Abreu, Fukata et al. 2005) and liver (Knolle and Gerken 2000) which are exposed to tonic levels of TLR ligands from commensal microbes may rely on the tolerance mechanisms to physiologically elevate their threshold for activation and prevent unwanted inflammation. Both homo- and heterotolerance can be observed in the normal liver (Slotta, Scheuer et al. 2006), and both Kupffer cells (Hafenrichter, Roland et al. 1994) and sinusoidal epithelial cells (Uhrig, Banafsche et al. 2005) contribute to the effect. Kupffer cells appear to be the principal mediators of hepatic endotoxin tolerance, through their secretion of the anti-inflammatory cytokine IL-10 (Knolle, Schlaak et al. 1995).

1.2.6 Adapter molecule polymorphisms and disease severity

A case control study in 6106 individuals from the UK, Vietnam and Africa studied 33 SNPs in the gene encoding the adapter molecule MAL which interacts with TLR4 and TLR2 and identified an association with invasive pneumococcal disease, malaria, tuberculosis and bacteraemia for a SNP encoding a serine to leucine substitution at ser180 (S180L). Being heterozygous for S180L was protective against disease. Molecular analysis revealed that the S180L variant protein is unable to bind TLR2, reducing intracellular signalling and attenuating the inflammatory response. This suggests that wild-type homozygosity is associated with a more vigorous inflammatory response which can be damaging to the individual, and S180L homozygosity is associated with inadequate host defence and fatal invasive microbial disease, which would account for the absence of this combination in developing-world populations. The heterozygous state appears to have the host defence response ‘just right’ to eliminate pathogens without deleterious enhancement of inflammation (Khor, Chapman et al. 2007). About a quarter of the UK population are heterozygous for S180L. With strong evidence for involvement of TLR4 in ALD and suggestions that free fatty acids (FFA) may signal through TLR2 (Lee, Zhao et al. 2004) which could cause inflammation and insulin resistance in non-alcoholic steatohepatitis (NASH) (Senn 2006), it is tempting to speculate that the SS homozygotes might be more susceptible to these two liver diseases.

1.3 Pathogenesis of acute alcoholic hepatitis

Although the epidemiological link between ethanol consumption and liver disease is clear, the pathogenic mechanisms of ethanol liver injury and the variation in individual susceptibility are still areas of investigation. Current thinking is that in susceptible individuals AAH occurs as a result of the combination of cellular stress generated by the products of ethanol metabolism and a consequent dysregulated innate immune response to gut-derived endotoxin.

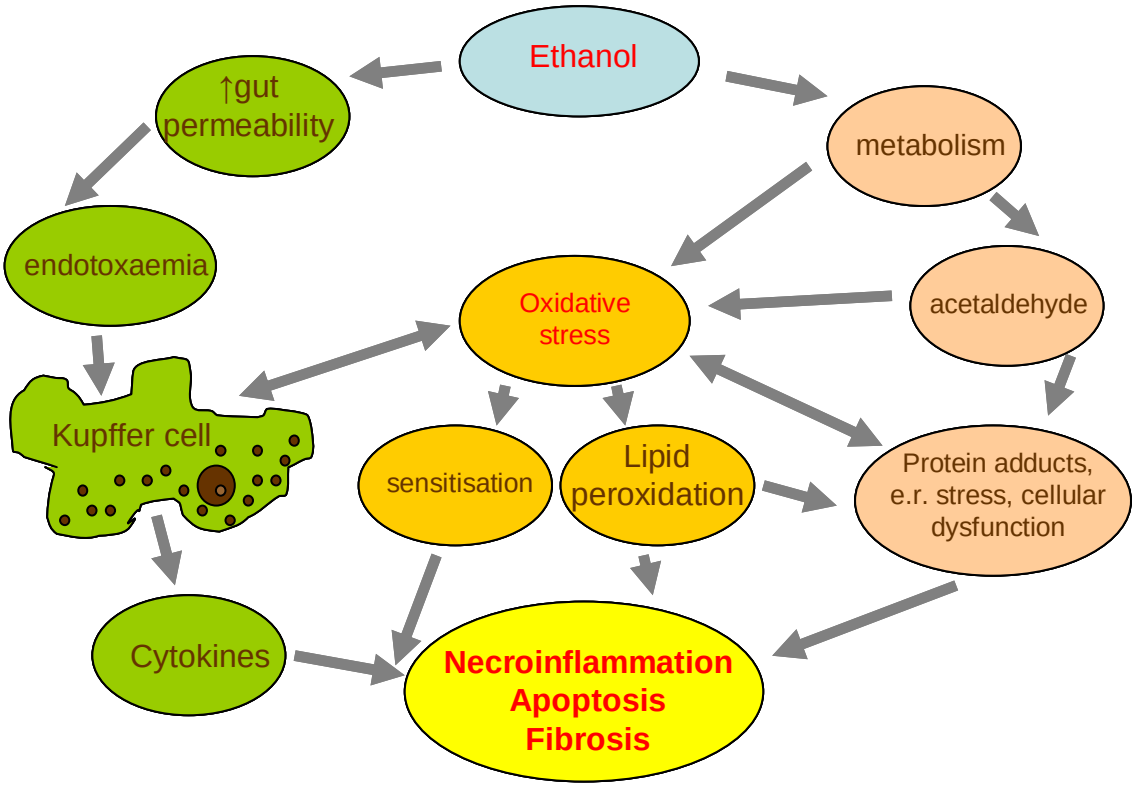
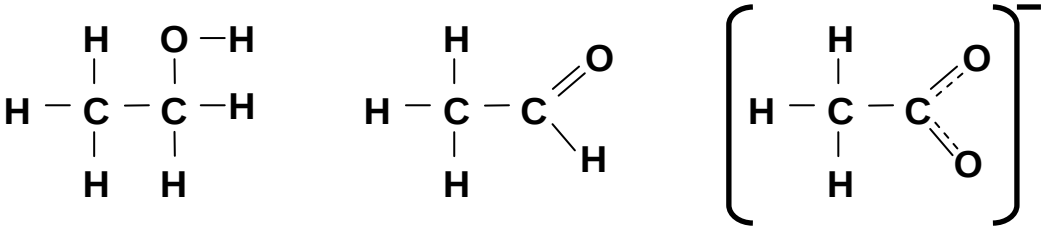


Figure 1-4 Overview of the pathogenesis of acute alcoholic hepatitis

1.3.1 Ethanol metabolism

One unit of alcohol (8g or 10ml absolute ethanol in the UK) produces a peak blood alcohol concentration (BAC) of 10-15mg/100ml at approximately 20 minutes after ingestion (Tapson 2004). Absorption is by simple diffusion, predominantly in the duodenum. Ethanol is a polar molecule and poorly lipid soluble, so its distribution in the body depends on organ blood flow and water content. 90% of absorbed ethanol undergoes oxidative metabolism to carbon dioxide and water, principally in the liver. Ethanol metabolism follows zero-order kinetics with a rate of 100mg/kg/hour, which is at most doubled by the enzyme-inducing effects of chronic heavy consumption.

Ethanol metabolism is a three-stage process: first, ethanol is oxidised to acetaldehyde in the cytoplasm, then acetaldehyde is oxidised to acetate in the mitochondria, and finally acetate is oxidised to CO_2 and water in the peripheral tissues, primarily lung and muscle.



Ethanol Acetaldehyde Acetate

Figure 1-5 Chemical structures of intermediates in ethanol metabolism

The oxidation of ethanol to acetaldehyde occurs through three separate mechanisms. In most cell types the greatest contribution is made by the enzyme alcohol dehydrogenase (ADH) which converts ethanol to acetaldehyde using the cofactor nicotinamide adenine dinucleotide (NAD).

$$CH_3CH_2OH + NAD^+ \rightarrow CH_3CHO + NADH + H^+$$

Equation 1-3 Action of alcohol dehydrogenase

The reduced cofactor NADH must be oxidised back to NAD^+ in the mitochondria, which is associated with the oxidative phosphorylation of ADP to yield a molecule of ATP. This recycling of NAD by the mitochondria is the rate-limiting step in ethanol metabolism and accounts for its zero-order kinetics. The reduced NAD:NADH ratio means less NAD^+ is available for glycolysis and the citric acid cycle, leading to an accumulation of acetyl co-enzyme A (acetyl-CoA) and its precursor, pyruvate. An alternative route for the conversion of NADH to NAD^+ is the reduction of pyruvate to lactic acid, which can result in lactic acidosis and a shortage of pyruvate for gluconeogenesis with consequent hypoglycaemia.

The second mechanism involves the microsomal ethanol oxidising system (MEOS), the principal component of which is cytochrome P450 2E1 (CYP2E1), a haem-containing enzyme localised in the smooth endoplasmic reticulum (ER), and concentrated in the Perivenular region of the liver. CYP2E1 has a K_m 10-20 times that of ADH, so its contribution for ethanol

metabolism in moderate drinking is small, but the cytochrome is inducible by ethanol so its contribution becomes more significant in heavy and chronic consumption.

$$CH_3CH_2OH + ½O_2 \rightarrow CH_3CHO + H_2O$$

Equation 1-4 Action of CYP2E1

The reaction requires donation of electrons by cytochrome P450 reductase which is itself reduced by the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to give $NADP^+$. NADPH is key to the maintenance of antioxidant defences by keeping cellular glutathione in its reduced form (GSH) rather than its oxidised form (GSSG). $NADP^+$ is recycled to NADPH by an ATP-requiring process.

The third mechanism of ethanol oxidation involves catalase which is located in peroxisomes.

Acetaldehyde is metabolised to acetate by aldehyde dehydrogenase (ALDH), primarily in mitochondria. This process also consumes NAD^+. Acetate is available for acetyl-CoA synthesis in the liver or can diffuse out of the liver for eventual metabolism to CO_2 and water in peripheral tissues (Stewart and Day 2006).

$$CH_3CHO + NAD^+ + H_2O \rightarrow CH_3COO^- + NADH + 2H^+$$

Equation 1-5 Action of aldehyde dehydrogenase

1.3.2 Steatosis

The macrovesicular steatosis of ALD has a number of sources. The reduced NAD:NADH ratio leads to accumulation of acetyl-CoA which is diverted to synthesis of free fatty acids (FFA) and glycerol-3-phosphate, leading to an increase in triglycerides. Acetaldehyde inhibits peroxisome proliferator activated receptor (PPAR) α, which reduces β-oxidation of FFA and allows their accumulation. The effect of ethanol on the cellular microtubule transport system inhibits export of triacylglycerol from the cell.

Steatosis is exacerbated by the proinflammatory cytokine TNFα, as demonstrated by the reduced steatosis in ethanol-fed mice deficient in the TNF receptor (Yin, Wheeler et al. 1999),

which suggests that inflammatory cytokines are active at the earliest stage of the disease, rather than being a later event in the pathogenic pathway. The severity of early steatosis correlates with later cirrhosis (Teli, Day et al. 1995). There is good evidence that steatosis is not just an associated observation but an active contributory factor to inflammatory liver injury by increasing lipid peroxidation under conditions of oxidative stress and sensitising hepatocytes to LPS-induced injury (Day and James 1998). FFA stimulation of TLR2 could potentially also contribute to the inflammatory response (Lee, Zhao et al. 2004).

1.3.3 Oxidative stress

Oxidative stress occurs when the generation of oxidising species overcomes intrinsic antioxidant defences, with the potential for cellular dysfunction and tissue damage. Reactive oxygen species (ROS) include the superoxide anion radical ($SO^{\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyethyl radical (HER) and the peroxynitrite radical ($ONOO^{\cdot-}$). The principal antioxidant defences are superoxide dismutase (SOD) enzymes and glutathione, which is oxidised to GSSG by antioxidant systems such as GSH peroxidise. Consequences of oxidative stress include peroxidation of polyunsaturated fatty acids in membrane lipoproteins, disrupting membrane integrity and leading to cell death. ROS can also form adducts to cellular proteins which impairs protein folding and function. Additionally ROS can initiate inflammatory signalling cascades with activation of NF-κB.

Ethanol induces oxidative stress by both generating ROS and depleting antioxidant defence.

1.3.3.1 Importance of oxidative stress in AAH

Products of lipid peroxidation can be found in the blood and livers of patients with AAH. In the liver they are concentrated in the perivenular region, correlating with the distribution of liver injury, and in the blood their concentration correlates with the severity of the histological inflammation. Diets that promote oxidative stress increase liver injury in animal models of AAH, and animals with gene knockouts for components of antioxidant defence such as SOD and methionine adenosyltransferase (MAT) suffer accelerated liver injury. Techniques to reduce oxidative stress in animal models of AAH all reduce liver injury, though antioxidant replenishment has not been shown to be effective in humans with severe AAH (Stewart, Prince et al. 2007), perhaps because the inflammatory process is well-established by the time of presentation and the window of opportunity for antioxidant replacement has passed.

1.3.3.2 Sources of oxidative stress in AAH

By virtue of its oxygen-splitting catalytic mechanism, CYP2E1 is a potent source of ROS and is upregulated in chronic heavy ethanol consumption. Incomplete catalysis can result in the release of the highly reactive HER instead of acetaldehyde. Inhibition of CYP2E1 with diallyl sulphide reduces lipid peroxidation (Albano, Clot et al. 1996).

The mitochondrial respiratory chain produces superoxide during the reoxidation of NADH to NAD^+. Mitochondrial manganese-SOD-2 converts SO to hydrogen peroxide which is then broken down to carbon dioxide and water by GSH peroxidise. In conditions of inflammation, TNFα increases the rate of ROS generation from the respiratory chain. In experimental animals, viral transfection of SOD has been shown to reduce liver injury, NF-κB activation and cytokine production (Wheeler, Kono et al. 2001).

In endotoxin-induced inflammation, inducible nitric oxide synthase (iNOS) is upregulated by LPS and TNFα. The nitric oxide (NO) produced binds to cytochrome c oxidase and increases SO production. The interaction between superoxide and NO yields the peroxynitrite radical ($ONOO^-$) which can nitrosylate proteins, altering their ultrastructure and function. iNOS-knockout animals get reduced lipid peroxidation, fewer reactive nitrogen species and less liver injury in response to ethanol feeding.

Kupffer cells (KC), the resident macrophages in the liver, are a significant source of oxidative stress during inflammation, producing free radicals from NADPH oxidase as part of their defence against microorganisms. The NADPH oxidase p47phox subunit knockout mouse produces fewer free radicals and had minimal liver damage in response to ethanol feeding (Wheeler, Kono et al. 2001). KC also secrete chemokines which recruit neutrophils to the liver, which in turn produce more ROS. KC cytokine output can also increase oxidative stress through directly hepatotoxic mediators such as TNFα which increases oxidative metabolism in hepatocytes but blocks electron transport, so increasing ROS generation from the respiratory chain. Blocking KC activity with gadolinium chloride significantly reduces the rate of lipid peroxidation (Niemela, Parkkila et al. 2002).

1.3.3.3 The methionine cycle and antioxidant defence

Glutathione (γ-glutamylcysteinylglycine, GSH) is the principal antioxidant in human cells, scavenging free radicals and ROS and becoming oxidised to GSSG as the thiol groups on two GSH form a disulphide bond. The cellular GSH:GSSG ratio is normally greater than 10. GSSG is restored to GSH by glutathione reductase, a process requiring NADPH. Glutathione reductase is abundant in cytoplasm but not found in mitochondria which must export GSSG and import GSH. GSH also complexes and detoxifies xenobiotics *via* glutathione-s-transferase and can be conjugated with NO to allow tissue-specific modulation of NO effects (Wu, Fang et al. 2004).

Ethanol diminishes the available GSH by production of ROS, by depletion of NADPH and also by reducing production of its precursor cysteine in the methionine cycle. The rate-limiting step in GSH synthesis is γ-glutamylcysteine synthetase activity but this is upregulated under conditions of oxidative stress *via* a NF-κB-dependent pathway and cysteine availability becomes the limiting factor. Cysteine is synthesised from s-adenosylmethionine (SAMe) in the methionine

cycle and transulphuration pathway. SAMe is not only the first substrate of the cycle but also a vital co-factor for the reactions recycling homocysteine to methionine or diverting it down the transulphuration pathway to make cysteine (Mato and Lu 2005). A high ratio of SAMe to s-adenosylhomocysteine (SAH) is also required for transmethylation reactions (methylation of DNA, RNA, phospholipids and proteins such as histones) which are vital for cellular function (Yi, Melnyk et al. 2000). Ethanol reduces the SAMe:SAH ratio by three processes. Firstly, acetaldehyde inhibits the activity of the methionine synthetase enzyme (Kenyon, Nicolaou et al. 1998). Secondly, ethanol reduces transcription of most of the other enzymes in the methionine cycle (Halsted, Villanueva et al. 2002). Thirdly, SAMe synthesis requires folate and chronic heavy drinking is associated with folate deficiency due to impaired absorption (Villanueva, Devlin et al. 2001), with a high-ethanol folate deficient diet producing steatohepatitis in micropigs (Villanueva and Halsted 2004).

Methionine supplementation has been shown to reduce ethanol-induced liver injury in a rodent model (Parlesak, Bode et al. 1998) but SAMe replacement was ineffective in established ALD in humans (Mato, Cámara et al. 1999). Subsequent work on the micropig model suggested that the contribution of SAMe depletion to GSH depletion was small compared to the effect of consumption by ROS scavenging (Villanueva, Esfandiari et al. 2006) but whether this holds true in the protein-deficient diet of some heavy drinkers is unknown. A reduced SAMe:SAH ratio may also contribute to liver injury by leading to hypomethylation and consequent expression of pro-apoptotic genes, predisposing the cell to programmed death in response to TNFα (Song, Zhou et al. 2004).

1.3.3.4 Endoplasmic reticulum stress

A more recently discovered mechanism for ethanol-induced cellular stress is the process of endoplasmic reticulum (ER) stress. The ER is the main site of mRNA translation and protein synthesis, post-translational modification and transport. Failure of normal protein folding leads to accumulation of unfolded protein in the ER. This ‘ER stress’ triggers an adaptive response known as the ‘unfolded protein response’ (UPR). The UPR upregulates ER proteins and increases lipid and cholesterol biosynthesis to supply more ER membrane *via* the transcription factor sterol response element binding protein 1c (SREBP1c) (Ji, Chan et al. 2006), while reducing the synthesis of other proteins and eventually activating pro-apoptotic mechanisms so that a cell overcome by unfolded protein will apoptose (Ji, Mehrian-Shai et al. 2005) (Ji and Kaplowitz 2006). In ALD the UPR may be triggered by elevated levels of homocysteine disrupting disulphide bonds in folded proteins, or potentially by acetaldehyde forming protein adducts which then fail to fold normally (Ji and Kaplowitz 2003).

Under these conditions a decrease in mitochondrial GSH has been observed, and this has been suggested to be a result of impaired transport of GSH across the mitochondrial inner membrane under conditions of increased membrane cholesterol and decreased membrane fluidity as a

consequence of the UPR. Mitochondria lack glutathione reductase so their capacity to scavenge ROS depends on the rate of GSH import from the cytosol (Fernandez-Checa and Kaplowitz 2005). The redox state of the cell can activate or modulate signalling pathways that lead to inflammatory or apoptotic responses (Han, Hanawa et al. 2006) and ER stress can activate the pro-apoptotic JNK MAP kinase through a mechanism involving the TNF receptor TNFR1 (Yang, Kim et al. 2006). Depletion of intracellular GSH inhibits activation and nuclear translocation of NF-κB and elevated GSSG inhibits its DNA binding and transactivation capacity (Mihm, Galter et al. 1995). Work from the Kaplowitz group suggests that the ultrastructural pattern of GSH depletion influences the hepatocyte response to inflammatory stimuli such as TNF: mitochondrial GSH depletion increases ROS in response to TNF and leads to necrotic cell death; depletion of cytoplasmic GSH leads to oxidation of NF-κB and reduces its DNA binding and pro-inflammatory, anti-apoptotic transcripts, so c-Jun transcripts predominate and the cell dies by apoptosis; in the absence of GSH depletion, NF-κB transcripts predominate and the hepatocyte is resistant to apoptosis (Matsumaru, Ji et al. 2003). Further work with ethanol-fed TNFR knockout mice analysed the contribution of inflammatory stimuli to the cellular response and established that TNFα made a significant contribution to cell death and a small contribution to steatosis, but elevated homocysteine and ER stress occurred independently of TNFα (Ji, Deng et al. 2004).

1.3.4 Adaptive immune responses

Some clinical features of ALD suggest that adaptive immune responses might play a role in its pathogenesis: abstinent patients who later return to drinking have a rapid recurrence of AAH in a manner suggestive of immunological memory; AAH responds to the immunosuppressive effects of steroids in a proportion of cases; most histological specimens show some degree of lymphocytic infiltration; ALD is often associated with elevated serum immunoglobulin and in some cases circulating autoantibodies (Stewart and Day 2003). However, the presence of autoantibodies does not correlate with disease severity and no HLA associations have been detected (McFarlane 2000).

Self proteins can be covalently modified by the reactive products of ethanol metabolism so that they are no longer recognised as self and so become immunogenic antigens. Both acetaldehyde-adducted proteins (Holstege, Bedossa et al. 1994) and anti-adduct antibodies (Niemela, Klajner et al. 1987) have been identified in ALD patients, with adducts predominating in zone 3 of the liver and circulating antibodies being highest in AAH patients.

The effect of ethanol-induced oxidative stress and consequent lipid peroxidation can also generate new immunoreactive antigens (Mottaran, Stewart et al. 2002). Reactions between ethanol-derived free radicals and the enzymes involved in their generation can result in adducts such as the hydroxyethyl radical (HER) adducted to CYP2E1. Serum from ALD patients has been shown to contain antibodies to both this adduct and to native CYP2E1, suggesting an

autoimmune response to a self protein through the phenomenon of epitope spreading. The autoantibodies were associated with a loss-of-function mutation in the gene for cytotoxic T-lymphocyte-associated protein 4 (CTLA4) which is an inhibitory regulator of immune responses associated with a range of autoimmune diseases (Vidali, Stewart et al. 2003). In addition to autoantibodies, T-cell responses to adducts have also been demonstrated in ALD patients (Stewart, Vidali et al. 2004).

It has been shown that Kupffer cells are necessary for both adduct formation and liver injury in response to alcohol (Niemela, Parkkila et al. 2002) suggesting that the innate immune response is still key to the pathogenesis of ethanol-induced liver injury and the adaptive immune reactivity is at most a secondary response, and possibly of little pathogenic significance. More recent work has clarified whether the T-cell and antibody reactivity is a direct cause of liver injury or an immune epiphenomenon arising in an already inflamed liver. The CTLA4 polymorphism has been shown to correlate closely with anti-CYP2E1 autoantibodies and with the histological degree of lymphocyte infiltration in ALD patients. However, the CTLA polymorphism is not associated with the development of ALD, having the same frequency in ALD patients, disease-free heavy drinkers and normal controls. This Mendelian randomisation approach suggests that the immune reactivity is due to the CTLA4 polymorphism but is not part of the pathogenesis of ALD (Stewart, Daly et al. 2006).

1.3.5 Apoptosis

There is increasing evidence that apoptotic mechanisms and DNA fragmentation play an early role in the pathogenesis of ALD, and are a participant in liver injury, rather than purely its end result.

Not all investigations support a key role for apoptosis in ALD, and the highly disorganised structural changes in hepatocytes typical of alcoholic hepatitis are cited as evidence that the orderly deletion of cells by apoptosis is not driving pathogenesis. Animal work suggests that the intracellular environment of the ethanol-exposed hepatocyte favours pro-survival, pro-inflammatory NF-κB activity over the pro-apoptotic effects of the JNK transcription factors (Koteish, Yang et al. 2002).

However, increased incidence of apoptosis as identified by terminal uridine deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) is seen in rats that have undergone continuous intragastric ethanol feeding (Yacoub, Fogt et al. 1995) and in biopsies from ALD patients where apoptosis is greatest in areas of greatest tissue damage and around ballooning hepatocytes (Zhao, Laissue et al. 1997). It correlates with histological grade and serum bilirubin, suggesting a key role in pathogenesis (Natori, Rust et al. 2001). In AAH the apoptotic index has been found to correlate with mDF (Ziol, Tepper et al. 2001).

In a rat model, apoptosis has been shown to be an early event in ALD, detectable before any other evidence of liver injury (Deaciuc, Fortunato et al. 1999). Ethanol feeding has been shown to sensitise cells to Fas-mediated apoptotic killing (Minagawa, Deng et al. 2004). The antiapoptotic effect of NF-κB relies on it being maintained in its reduced state by thioredoxin and the NADPH depletion of chronic ethanol consumption diminishes the available thioredoxin so favouring apoptosis (Day 2001). Further study has demonstrated that hepatocytes of ethanol-fed rats appear to be in a state of 'pre-apoptosis' with increased TUNEL staining suggesting DNA fragmentation but no upregulation of apoptosis proteins. The addition of LPS resulted in a huge increase in TUNEL and upregulation of caspases and other protein mediators of apoptosis. It was suggested that in response the combined insult of ethanol and endotoxin the primary mode of cell death is apoptosis, and the histologically-apparent necrosis only develops because the apoptosis rate outstrips the rate of phagocytosis of apoptotic cells (Deaciuc, D'souza et al. 2001). Cellular glutathione status may also determine the mode of cell death (section 1.3.3.4). In addition, proteasome dysfunction occurs in chronic ethanol exposure, thought to be a consequence of oxidative stress or protein hyperacetylation, and this leads to apoptosis, probably because the inhibitory protein IKB accumulates and sequesters NF-κB, pushing the balance of transcription in favour of the pro-apoptotic JNK pathway (Joshi-Barve, Barve et al. 2003).

Apoptosis releases IL-8 which recruits neutrophils to the liver and contributes to inflammatory injury (Day 2001). Apoptosis can induce further liver injury when Kupffer cell phagocytosis of apoptotic bodies stimulates additional pro-inflammatory and pro-fibrotic cytokine secretion (Canbay, Feldstein et al. 2003). Clearance of apoptotic bodies *via* the liver-specific asialoglycoprotein receptor is diminished by alcohol, leading to accumulation of apoptotic end-products (McVicker, Tuma et al. 2002). KC from ethanol-fed rats have an enhanced cytokine response to these apoptotic bodies with a six-fold increase in TNFα and a 60% increase in IL-6 (McVicker, Tuma et al. 2007). Thus apoptosis is a consequence, an enhancer and possibly a trigger of inflammation in the liver.

1.3.6 Innate immune responses

It is clear that the cellular consequences of ethanol metabolism described above are necessary but not sufficient to cause ALD. The other critical component is an exaggerated innate immune response to gut-derived endotoxin (Enomoto, Ikejima et al. 2000). The individual components of this response are discussed below.

1.3.6.1 Cytokines and chemokines

There is considerable evidence for the key role of cytokines and chemokines in ALD and, in particular, AAH. Serum concentrations of pro-inflammatory cytokines are elevated in ALD patients and are particularly high in AAH, correlating with clinical severity and prognosis. Patient

monocytes have an exaggerated cytokine response to ex-vivo administration of LPS. Genetic polymorphisms of components of the cytokine signalling mechanism are associated with disease or its severity. In animal models, gene knockouts for cytokines and their receptors, and treatment with anti-cytokine antibodies all reduce or abolish ethanol-related liver injury. The relevant studies are summarised in **Table 1-4** and **Table 1-5**.

Cytokine / chemokine	**Principal relevant findings**	**Reference**
TNF	Monocytes harvested from AAH patients produce twice as much TNF in response to LPS than controls.	(McClain and Cohen 1989)
TNF IL-1	Plasma TNF and IL-1α and β measured at admission and 30 days later in 23 severe AAH patients. 82% of patients with elevated serum TNF died within two years, but 100% of those with low TNF survived. TNF was not elevated in controls. IL-1 α was elevated in AAH but IL-1 β was not and neither correlated with survival.	(Felver, Mezey et al. 1990)
TNF IL-1	TNF was found to be elevated in 21 patients with severe AAH compared to controls. TNF correlated with serum bilirubin, creatinine and probability of death but not with measured endotoxaemia. IL-1 was not elevated.	(Bird, Sheron et al. 1990)
TNF IL-6	PBMC from ethanol cirrhosis patients showed enhanced TNF and IL-6 production in response to LPS compared to control PBMC. The difference resolved after 7 days in ethanol-free culture.	(Deviere, Content et al. 1990)
TNF IL-1 IL-6	All three cytokines are higher in abstinent cirrhotics and AAH patients than in disease-free heavy drinkers, with concentrations correlating with markers of liver injury, albumin and immunoglobulins. IL-6 time courses closely follow acute injury and fall during recovery, while TNF and IL-1 are slower to fall due to the metabolic consequences of injury.	(Khoruts, Stahnke et al. 1991)
TNF IL-10	Compared to controls, monocytes from ethanol cirrhotic patients produce more TNF but significantly less IL-10. Supplementation of IL-10 reduces TNF output and anti-IL-10 antibodies increase TNF, suggesting that TNF changes are secondary to opposite changes in IL-10.	(Le Moine, Marchant et al. 1995)
TNF IL-10	Adenosine release during ethanol liver injury should increase IL-10 and reduce TNF but increased adenosine deaminase in the liver of heavy drinkers reduced this anti-inflammatory effect.	(Le Moine, Quertinmont et al. 1999)
TNF	TNF was found to be elevated in alcoholic cirrhosis and correlates with plasma LPS, soluble TNF receptor and Child-Pugh grade.	(Hanck, Rossol et al. 1998)
TNF	TNF mRNA was increased by polymeric IgA, which also enhanced PBMC TNF output in response to LPS.	(Deviere, Vaerman et al. 1991)
TNF	Peripheral blood mononuclear cells (PBMC) from patients with ethanol cirrhosis were found to be a source of TNF and soluble TNF receptors with levels correlating with Child-Pugh grade. PBMC were in an activated state without addition of LPS.	(Hanck, Glatzel et al. 2000)

Cytokine / chemokine	**Principal relevant findings**	**Reference**
TNF IL-6 IL-8 IL-10	LPS, LPS binding protein, IL-6 and IL-8 are all elevated relative to controls in the acute phase of AAH, and are particularly high in eventual non-survivors, but fell during recover in survivors. IL-10 was also high in non-survivors. TNF was undetectable in all patients.	(Fujimoto, Uemura et al. 2000)
TNF	TNF mRNA was increased in monocytes from AAH patients, and increased further on stimulation with LPS. This was associated with increased nuclear NF-κB binding, primarily p65 and p50 subunits.	(Hill, Barve et al. 2000)
TNF	TNF receptor density was found to be increased by ethanol.	(McClain, Hill et al. 2002)
TNF	SNP at position -238 in TNF receptor gene is associated with cirrhosis in heavy drinkers	(Grove, Daly et al. 1997)
TNF	Findings above replicated in a separate cohort	(Pastor, Laso et al. 2005)
IL-6 IL-2 IFNγ	IL-6 was elevated in ethanol cirrhosis and has negative correlation with IL-2 and IFNγ which were both suppressed.	(Deviere, Content et al. 1989)
IL-6	IL-6 was measured in 58 patients with severe AAH and found to be elevated compared to controls and to correlate with markers of severity and probability of death.	(Sheron, Bird et al. 1991)
IL-6	Consecutive IL-6 measurements in 30 AAH patients demonstrated that serum levels correlated with markers of severity and fell during recovery.	(Hill, Marsano et al. 1992)
IL-8	Serum IL-8 was found to be increased in 40 AAH patients compared to ethanol-dependent controls. IL-8 fell with recovery.	(Hill, Marsano et al. 1993)
IL-8	IL-8 is elevated in AAH patients relative to ethanol cirrhosis, other liver diseases and healthy controls. IL-8 correlated with histological neutrophil infiltration and markers of severity,	(Sheron, Bird et al. 1993)
IL-8	IL-8 is released by hepatocytes and Kupffer cells in response to TNF and is under control of a NF-κB promoter	(Hill, McClain et al. 1998)
IL-8	Lipid peroxidation products stimulated IL-8 secretion from human PBMC	(Jayatilleke and Shaw 1998)

Cytokine / chemokine	Principal relevant findings	Reference
IL-8	The effect of ethanol on increasing IL-8 production was shown to be dose-dependent and to begin at ethanol concentrations associated with light-moderate drinking. In heavy drinking, ethanol increased IL-8 more in women than in men.	(Gonzalez-Quintela, Campos et al. 2007)
IL-8 MCP-1 MIP-1α	AAH and alcoholic cirrhosis were found to be associated with distinct patterns of chemokine expression on biopsy, with AAH showing more extensive parenchymal chemokine staining.	(Afford, Fisher et al. 1998)
IL-10	Il-10 gene polymorphism reduces IL-10 secretion and increases likelihood of liver disease in heavy drinkers.	(Grove, Daly et al. 2000)
IL-18	PBMC from patients with ethanol cirrhosis showed enhanced production of IL-18 mRNA and protein without additional LPS stimulation. It was suggested that this was part of a 'pre-sensitising' mechanism. IL-18 is secreted by a caspases-dependent, MyD88-independent mechanism (Seki, Tsutsui et al. 2001) so may be activated by cellular stress.	(Hanck, Manigold et al. 2001)
MCP-1	Monocytes from AAH patients constitutively produced MCP-1 and had enhanced production in response to LPS, which was reduced by n-acetylcysteine which replenishes GSH	(Devalaraja, McClain et al. 1999)
MCP-1 MIP-1α	Serum MCP-1 concentrations were raised in AAH patients and reflected severity of hepatic inflammation. Monocytes from AAH patients showed increased secretion of MCP-1 and MIP-1α, suggesting both systemic and hepatic sources were contributing to the raised serum MCP-1.	(Fisher, Neil et al. 1999)
GROα RANTES	In an examination of differential chemokine expression in liver disease, GROα (a CXC chemokine) was found to be increased in biopsies from AAH patients and correlated with neutrophil infiltration. RANTES (a CC chemokine) was found to be increased in biopsies from viral hepatitis.	(Maltby, Wright et al. 1996)

Table 1-4 Human studies of cytokines and chemokines in ALD

Cytokine / chemokine	Principal relevant findings	Reference
TNF	Chronically ethanol-fed rats had higher transaminases and higher serum TNF after iv administration of LPS than isocaloric non-ethanol fed controls. Acute ethanol gavage had the opposite effect to chronic ethanol feeding – inflammatory responses were suppressed.	(Honchel, Ray et al. 1992)
TNF	Rats subjected to continuous intragastric ethanol feeding show increased TNF transcription in parallel with histological liver injury.	(Nanji, Zhao et al. 1994)
TNF	Ethanol-fed rats treated with polyclonal anti-TNF antibodies showed no change in cytokine transcription or liver steatosis but had reduced ALT and reduced liver inflammation and necrosis.	(Iimuro, Gallucci et al. 1997)
TNF	TNF receptor 1 (TNFR1) knockout mice subjected to ethanol feeding did not display elevated ALT or histological liver injury, but wild-type and TNFR2 knockouts did.	(Yin, Wheeler et al. 1999)
TNF IL-6 TGF	KC transcription of TNF, IL-6 and TGF was increased in rats after 17 weeks of intragastric ethanol feeding, and was accompanied by inflammations and fibrosis on histology.	(Kamimura and Tsukamoto 1995)
IL-6	IL-6 knockout mice were found to be susceptible to ethanol-induced steatosis and liver injury, in contradiction to its assumed role in pathogenesis. Ethanol-fed IL-6-/- mice displayed elevated products of oxidative stress and increased liver injury, observations which were reversed by administration of exogenous IL-6. IL-6 supplementation also reduced liver injury in ethanol-fed rats. It is suggested that IL-6 exerts a protective effect by upregulating anti-oxidant metallothionein proteins.	(El-Assal, Hong et al. 2004)
MIP-2 CD18 ICAM-1	Ethanol-fed rats showed increased MIP-2, increased liver expression of ICAM-1 and enhanced neutrophil expression of its ligand CD18. Liver injury was reduced by treatment with anti-CD18 antibodies.	(Bautista 1997)
Chemokines	CXC chemokines predominate early in ethanol liver injury and induce neutrophil infiltration, while CC chemokines predominate later and attract monocytes which might contribute to fibrosis.	(Bautista 2000)

Table 1-5 Animal studies of cytokines and chemokines in ALD

Analysis of single cytokines in inflammatory conditions can be problematic because it is rare that a single cytokine is individually responsible for the inflammatory injury, but rather the physiological effect will depend on the net balance of the prevailing cytokine milieu. In addition, individual cytokines may have both harmful and beneficial effects, the balance of which will

depend on concentration, duration of exposure and the physiological context in which they act. TNFα is a powerful early inducer of inflammation, but also promotes liver regeneration. IL-6 has pleiotropic effects, being largely pro-inflammatory, but having anti-inflammatory effects in certain contexts. IL-10 is anti-inflammatory but can also promote fibrosis.

A dichotomy exists between the observed effects of acute and chronic alcohol on cytokine production. The studies above show enhanced proinflammatory responses after chronic persistent ethanol exposure in humans, animals and cell lines. However, the effect of acute ethanol administration is to suppress TNFα secretion in response to LPS and downregulate TNF receptors in rats (Nelson, Bagby et al. 1989) (D'Souza, Nelson et al. 1994). Acute alcohol administration was shown to have a tolerising effect on the innate immune response to LPS in rat livers (Bautista and Spitzer 1996). In human cells, this has been shown to be a result of inhibition of p65 phosphorylation by IKKβ (Mandrekar, Jeliazkova et al. 2007). Zhang and colleagues investigated the time course and mechanism of the transition between anti- and pro-inflammatory responses in the human macrophage-like cell line MonoMac6; emergence of the enhanced TNFα response to LPS took six days of culture in ethanol-containing media, was associated with increased ROS generation, and was abrogated by addition of ROS scavenging compounds (Zhang, Bagby et al. 2001).

1.3.6.2 Increased gut permeability

The stimulus for innate immune activation in ALD has been postulated to be endotoxaemia due to increased intestinal permeability, and this 'leaky gut' hypothesis is supported by circumstantial evidence in humans and more direct evidence in animals. In health, the tight junctions of the intestinal epithelium limit free movement of immunostimulatory molecules from the gut to the internal environment, and the liver is exposed to a low tonic level of gut-derived endotoxin *via* the portal vein, which it clears from the circulation without inflammatory response (Knolle and Gerken 2000). Ethanol is known to reduce gut motility and increase the intestinal bacterial load with small intestinal bacterial overgrowth (Bode and Bode 2003). Intestinal permeability has been shown to be increased in heavy drinkers with liver disease compared to heavy drinkers without disease and non-drinkers with other liver diseases; both gastroduodenal permeability to sucrose and intestinal permeability to lactulose and mannitol were elevated (Keshavarzian, Holmes et al. 1999). ALD patients have subsequently been shown to have increased intestinal permeability to large molecules such as high-molecular weight polyethylene glycol and bacterial endotoxin (Parlesak, Schäfer et al. 2000).

The aetiology of the increased permeability appears to be the effect of the ethanol metabolite acetaldehyde on epithelial tight junctions. Acetaldehyde increases the paracellular epithelial permeability of the caco-2 monolayer, an *in vitro* model of intestinal barrier function, by inhibition of protein tyrosine phosphatases. The unopposed action of kinases results in hyper-phosphorylation of the tight junction proteins zona occludens-1, E-cadherin and beta catenin

leading to tight junction disruption (Atkinson and Rao 2001). The acetaldehyde effect can be ameliorated *in vitro* by L-glutamine acting *via* an epidermal growth factor receptor (EGFR)-dependent mechanism (Seth, Basuroy et al. 2004). The effect of acetaldehyde has been demonstrated in human colonic mucosa, with similar protective effects of L-glutamine and EGF (Basuroy, Sheth et al. 2005). It is likely that the source of acetaldehyde in the human colon is ethanol which has been absorbed in the proximal small bowel and then equilibrated across the colonic epithelium where it is metabolised by enteric bacteria which possess ADH but relatively little ALDH, leading to acetaldehyde accumulation (Rao, Seth et al. 2004).

It has even suggested that the only relevant site of action of ethanol in the pathogenesis of ALD might be the enteric mucosa, with the tissue-damaging oxidative and metabolic stress in the liver being secondary solely to the increased load of bacterial components from the gut rather than the effect of hepatic ethanol metabolism (Bode and Bode 2005). Additional studies in germ-free animal models would be needed to investigate this. Strategies to improve the enteric barrier such as zinc supplementation have been shown to moderately reduce liver injury in ethanol-fed rodents (Lambert, Zhou et al. 2003), but in human experiments reduction of the enteric bacterial load with the non-absorbable antibiotic paromomycin, although producing a transient dip in measured plasma endotoxin, did not improve markers of liver damage (Bode, Schafer et al. 1997). It remains possible that the increased intestinal permeability may, at least in part, actually be secondary to the liver inflammation, with Kupffer-cell-derived cytokines worsening the intestinal mucosal injury. Rats whose Kupffer cells were inactivated with gadolinium chloride before intravenous LPS treatment demonstrated reduced small intestinal injury (Gong, Wu et al. 2002), suggesting that there may be a two-way relationship between liver inflammation and intestinal permeability, and perhaps the two processes worsen each other in a vicious circle of inflammation.

1.3.6.3 Endotoxaemia

Measurement of bacterial lipopolysaccharide in biological fluids is by no means straightforward. Although referred to as a single biochemical entity, LPS is a heterogeneous group of molecules that differ in molecular weight, degree of branching and content of lipid A, the major immunostimulatory component. The inflammatory potency of LPS is further modified by its interaction with plasma proteins which can sequester it (albumin, high density lipoprotein HDL) or facilitate its binding to TLR4 (LPS binding protein LBP). Hence there is no consistent relationship between quantity of LPS and its biological effect. Bioassays have been developed to quantify biologically-relevant LPS activity in terms of ‘endotoxin units’ (EU) and these have replaced the traditional method of determining whether a given sample is pyrogenic when injected into a rabbit. The most widely adopted test that attempts to achieve a degree of reproducibility is the limulus amoebocyte lysate assay (LAL) which in its various commercially-available forms relies on the coagulation response to endotoxin of blood cells from the horseshoe crab *Limulus polyphemus*, more recently coupled to a chromogenic substrate.

Although the LAL assay is a robust tool for LPS determination in simple fluid and injectable medications, its accuracy and reproducibility diminish in complex matrices such as plasma, and its utility is further hampered in samples from jaundiced patients as the absorption peak of bilirubin is close to that of the chromogenic substrate.

A study that attempted to overcome these limitations compared plasma LPS in 85 ALD patients (65 cirrhotic) and 15 patients with liver disease of another cause using individual standard curves for each sample and demonstrated significantly higher plasma LPS in the ALD group (Fukui, Brauner et al. 1991). A further study in cirrhotic patients alone showed that plasma LPS correlated with Child-Pugh score, serum TNFα and soluble TNF receptor concentration (Hanck, Rossol et al. 1998). A smaller study that looked specifically at AAH patients detected elevated LPS in 14 patients compared to healthy controls, with particularly high levels in one of two fatal cases and in the one patient with clinically severe disease (Fujimoto, Uemura et al. 2000). Another small study demonstrated elevated LPS and LBP in all stages of ALD and noted that HDL which can bind LPS and protect against inflammatory responses was elevated in early and minimal disease but reduced in advanced cirrhosis (Schafer, Parlesak et al. 2002). However, other work has demonstrated that plasma LPS actually correlated better with ethanol consumption than with disease, weakening the evidence for its pathogenic role in humans (Urbaschek, McCuskey et al. 2001).

In animal studies, rats on the Lieber-DeCarli ethanol-containing diet developed hepatic steatosis alone while littermates on the same diet challenged with *E. coli* LPS developed focal necrotising hepatitis. Littermates given LPS challenge in the absence of ethanol developed no liver lesion (Bhagwandeen, Apte et al. 1987). In alcohol-fed mice plasma LPS correlated with histological liver injury, serum transaminases and serum TNFα, and was reduced by oral zinc supplementation, with evidence of reduced small intestinal mucosal injury (Lambert, Zhou et al. 2003). A role for shifts in the balance of endotoxin-neutralising and endotoxin-potentiating factors was suggested by analysis of 105 ALD cases in which plasma LPS correlated with progression of liver injury and fell during recovery in survivors. Elevated LBP and reduced HDL characterised clinically severe cases. In an accompanying animal study an extra high dose of ethanol on top of chronic ethanol feeding mimicked this picture and was associated with transiently impaired LPS clearance which may be responsible for the acute liver injury (Fukui 2005).

1.3.6.4 Gut flora

The apparent importance of enteral endotoxin in pathogenesis has lead to investigation of how manipulation of gut flora might modulate disease. Weekly enteral LPS supplementation in ethanol-fed rats was shown to cause portal and systemic endotoxaemia with increased TNFα, serum transaminases and worsening histological inflammation and necrosis (Mathurin, Deng et al. 2000). The probiotic lactobacillus was shown to reduce plasma LPS and reduce severity of

experimental liver disease in rats (Nanji, Khettry et al. 1994). In the rat intragastric feeding model of ALD, gut sterilisation with neomycin and polymixin B reduced measured plasma LPS and also serum AST and histological inflammatory scores (Adachi, Moore et al. 1995). This result did not translate into human therapeutics, however, with the non-absorbable antibiotic paromomycin failing to improve liver function in ALD despite an initial reduction in measured plasma LPS (Bode, Schafer et al. 1997). It remains possible that gut flora manipulation could have benefit in humans but by the time human ALD becomes clinically apparent the window for modulating the process with antibiotics has closed.

1.3.6.5 Kupffer cells

Kupffer cell inflammatory responses are the link between portal endotoxaemia and the elevated cytokines characteristic of AAH. TNFα is exclusively produced by the monocyte-macrophage lineage and Kupffer cells form the largest population of this line (Decker, Lohmann-Matthes et al. 1989). KC are present in the hepatic sinusoid lumen and the perisinusoidal space of Disse, maximising both their exposure to circulating material and their ability to interact with hepatocytes, other non-parenchymal cells and other immune cells (Roberts, Ganey et al. 2007). As a component of the reticuloendothelial system, KC clear cellular and other debris (including bacterial components) from the blood and help modulate downstream immune responses to such material. Their key role in ethanol-mediated liver injury in animals is clear from experiments in which the KC toxin gadolinium chloride virtually abolishes liver injury in a rat model of ALD (Adachi, Bradford et al. 1994). This was confirmed by later work which also demonstrated that the KC response to LPS could be potentiated by oestrogens, suggesting a further explanation for the increased susceptibility to ALD in females (Thurman 1998).

The KC response to LPS can be modified by ethanol. Rat KC isolated 24 hours after ethanol dosing demonstrate a sensitised response to LPS in terms of calcium fluxes and TNFα secretion. This was associated with upregulation of the LPS co-receptor CD14 and the effect could be blocked by enteral antibiotics, suggesting that bacterial components actually played a part in ethanol sensitisation of the KC inflammatory response (Enomoto, Ikejima et al. 1998). Ethanol may also reduce the ability of KC to clear endotoxin from blood. Chronic ethanol exposure was shown to enhance secretion of proinflammatory chemokines from KC but to inhibit their chemotactic and phagocytic potential, resulting in reduced clearance of circulating innate immune stimuli (Bautista 2002).

A critical observation in the study of AAH is the enhanced KC proinflammatory response to LPS seen after chronic ethanol exposure. Both rat KC and AAH patient peripheral blood mononuclear cells (PBMC) produce more TNFα in response to LPS stimulation than control cells, and this enhancement of the inflammatory response can be reduced by treatment with antioxidants (Hill, Devalaraja et al. 1999). A role for KC oxidant stress in this process is confirmed by the fact that inhibition of KC oxidant species generation either pharmacologically

or by genetic knockout can reduce liver injury from ethanol (Wheeler, Kono et al. 2001; Wheeler, Kono et al. 2001). Ethanol may induce oxidative stress in KC by similar mechanisms to its action in hepatocytes: KC are capable of ethanol metabolism, although at a slower rate than hepatocytes, and, in rat at least, the oxidant-generating CYP2E1 can be induced in KC by chronic ethanol exposure (Cao, Mak et al. 2002).

1.3.6.6 TLR4 / LPS receptor complex

Increases in KC surface TLR4 and CD14 are observed after chronic ethanol exposure (Enomoto, Ikejima et al. 1998; Zuo, Gong et al. 2003), though it is not clear whether this increase is directly responsible for the enhanced cytokine response to stimulation. What is clear is that the LPS receptor complex (TLR4, CD14 and MD2 interacting with LBP) is critical for the development of ethanol-related liver injury. C3H/HeJ mice have a complete absence of TLR4 and when subjected to four weeks of intragastric ethanol feeding have lower ALT, significantly less histological liver injury and reduced TNFα expression compared to control animals (Uesugi, Froh et al. 2001). CD14 knockout mice have been shown to be similarly protected from ethanol liver injury (Yin, Bradford et al. 2001), as have LBP knockout animals (Uesugi, Froh et al. 2002).

In humans at C-T single nucleotide polymorphism at position -159 in the CD14 promoter was found to be significantly associated with advanced ALD in a Finnish cohort. The T allele had an odds ratio (OR) of 2.48 (p=0.018) for alcoholic hepatitis and OR of 3.45 (p=0.004) for cirrhosis (Järveläinen, Orpana et al. 2001). However, these findings were not reproduced in two further cohorts in the UK and Portugal (Leathart, Day et al. 2001; Martins, Cortez-Pinto et al. 2005).

1.3.6.7 NF-κB

Multiple transcription factors mediate the downstream effects of TLR4 signalling and NF-κB remains one of the best studied and is responsible for the widest range of cellular responses to stimulation. Among its many cellular roles, NF-κB modulates gene expression in response to TLR ligation in KC and in response to TNF receptor ligation in hepatocytes. NF-κB activity as measured by electrophoretic mobility shift assay (EMSA) was increased in livers of rats subjected to intragastric ethanol feeding relative to rats on an isocaloric control diet, and was associated with elevated plasma LPS, increased chemokine secretion and more severe histological liver injury (Nanji, Jokelainen et al. 1999). A human study then demonstrated that monocyte NF-κB activity by EMSA was increased in AAH patients relative to controls. LPS treatment resulted in a greater increase in NF-κB activity in the monocytes from patients than in those from controls and TNFα production at both the mRNA and protein level was greater in patient samples. Super shift assays showed that both p50 and p65 subunits of NF-κB were active and able to bind their consensus sequence in the samples (Hill, Barve et al. 2000). A commentary on this paper suggests that AAH can be considered a “gene expression disease” in which “aberrant activation of proinflammatory genes perpetuates a chronic inflammatory

process", and cites the efficacy of glucocorticoids in AAH in support of dysregulated NF-κB-driven pro-inflammatory gene expression as the key pathogenic mechanism in AAH (Szabo 2000). Glucocorticoids induce transcription of the NF-κB regulatory molecule IKBα (Scheinman, Cogswell et al. 1995) and the glucocorticoid receptor also interacts directly with p65, blocking its DNA-binding domain (Funder 1997; Wissink, van Heerde et al. 1997).

Further evidence of a causative role for NF-κB in AAH comes from experiments in which rats were subjected to adenoviral transfection with the transgene for IKB superrepressor and then underwent ethanol feeding. The treated animals had reduced NF-κB activation, TNFα production and ALT relative to controls exposed to an irrelevant transgene. The protective effect diminished once the transgene was no longer detectable. The NF-κB inhibition had no effect on oxidative stress as measured by 4-hydroxynonenal concentrations, suggesting that any contribution from oxidative stress occurs upstream or independently of NF-κB (Uesugi, Froh et al. 2001). NF-κB activation has been shown to precede liver injury in the rat intragastric feeding model of ALD, supporting a role for it in pathogenesis of liver injury (Jokelainen, Reinke et al. 2001).

1.3.6.8 Neutrophils

The infiltrate of polymorphonuclear cells is one of the most prominent histological features of AAH and results from the enhanced secretion of pro-neutrophilic CXC chemokines such as IL-8 (Bautista 2002). Transmigrating neutrophils can adhere to hepatocytes undergoing cellular stress *via* β_2-integrin on the neutrophil and ICAM-1 upregulated on the hepatocyte surface. Contact is followed by neutrophil respiratory bust and degranulation, which are both additional sources of oxidative stress, leading to hepatocyte death (Ramaiah and Jaeschke 2007). Recent evidence suggests that neutrophil responses in AAH are dysregulated, possibly as result of stimulation by increased circulating endotoxin, resulting in a state of persistent respiratory burst with impaired phagocytic capacity (Mookerjee, Stadlbauer et al. 2007). This could reduce the specificity of the response and result in more widespread tissue damage and impaired clearance of further proinflammatory products of cellular disintegration.

1.3.7 Working model of innate immune responses in AAH

The various findings about the role of the innate immune response in the pathogenesis of acute alcoholic hepatitis have been brought together, largely in the work of the late Ron Thurman and his group, into a working model of inflammatory responses in AAH. This states that ethanol encourages small intestinal bacterial overgrowth and increases intestinal permeability, resulting in an increased load of Gram negative bacterial endotoxin reaching the liver *via* the portal vein. There the endotoxin activates KC which have been rendered more susceptible to activation by the cellular effects of ethanol metabolism which include increased ROS and facilitated opening of calcium channels (Iimuro, Ikejima et al. 1996).The vigorous KC response releases cytokines

and chemokines which recruit neutrophils, alter hepatocyte metabolism and alter blood flow leading to centrilobular hypoxia, ROS release, hepatocyte injury and death (Thurman 1998). Later modifications de-emphasised the role of hypoxia, suggesting that the effect of ethanol metabolism and cytokines on hepatocytes was sufficient for ROS generation (Enomoto, Ikejima et al. 2000).

There is, however, a paradox inherent in this endotoxin-induced inflammation in a normally endotoxin-tolerant organ. The liver is exposed to tonic levels of LPS in portal blood, which it clears appropriately from the circulation without inflammatory response (Broad, Jones et al. 2006). Indeed, Kupffer cells are themselves key to the maintenance of hepatic endotoxin tolerance (Knolle, Schlaak et al. 1995; Knolle and Gerken 2000). Consequently, the liver inflammation of AAH is already being referred to by leaders in the field as “failure of hepatic endotoxin tolerance” (McClain, Hill et al. 2002; Mandrekar, Pruett et al. 2005).

There is a further paradox in the contrast between this ethanol-induced enhancement of inflammatory responses and the widely documented and clinically evident suppressive effect of ethanol on immune responses including TLR signalling down both the MyD88-dependent and independent pathways (Pruett, Zheng et al. 2004). The Thurman group was the first to demonstrate a biphasic effect of ethanol on KC responses with early suppression (referred to by them as ‘tolerance’) and later sensitisation to LPS (Enomoto, Ikejima et al. 1998; Enomoto, Ikejima et al. 2000). They suggested that both these processes are endotoxin-dependent and it is conceivable that *in vivo* a first dose of ethanol increases gut permeability and delivers an increased endotoxin concentration to the KC which then become tolerant to that dose and unable to respond to similar concentrations until the metabolic effects of prolonged ethanol metabolism supervene and result in sensitisation and enhanced inflammatory responses. However, it is unlikely that LPS tolerance is solely responsible for the initial anti-inflammatory or immunosuppressive effect of ethanol. The effects of acute ethanol on membrane dynamics may alter TLR component clustering in lipid rafts, diminishing TLR responsiveness (Pruett, Zheng et al. 2004). Additionally, the impaired neutrophil phagocytic capacity observed in AAH may also contribute to the increased susceptibility to invasive bacterial disease in heavy drinkers despite the associated enhancement of cytokine responses (Mookerjee, Stadlbauer et al. 2007).

The biphasic effect of ethanol on inflammatory responses was further investigated *in vitro* in the human macrophage cell line MonoMac6. This demonstrated that the cells did not require prior endotoxin exposure to undergo early ‘tolerance’ and later ‘potentiation’ of cytokine responses to LPS in ethanol. Acute ethanol inhibited TNFα production by a post-translational method likely to be inhibition of TNFα converting enzyme (TACE), but chronic ethanol exposure for six days or more potentiated TNFα release, a phenomenon that could be reversed by antioxidant supplementation (Zhang, Bagby et al. 2001).

It is worth noting that, in the strict sense of the term, 'failure of endotoxin tolerance' in ethanol has not been demonstrated experimentally. This would require sequential exposure to two equal doses of LPS and observation that the cytokine response to the second was not diminished ('tolerised') to the same degree in the ethanol-exposed setting as in controls. However, the observation of enhanced cytokine responses to low doses of LPS in AAH patients and experimental animals, that will inevitably have had prior endotoxin exposure, would certainly be consistent with failure of the normal mechanisms of endotoxin tolerance and with the concept of AAH as a 'gene expression disease' in which proinflammatory gene expression is uncoupled from its normal regulatory mechanisms resulting in disorganised and deleterious inflammatory responses.

1.4 Control of inflammatory responses in alcoholic hepatitis

1.4.1 Factors shown to enhance cytokine output in ethanol

Inflammatory responses are regulated at multiple levels and it is likely that the dysregulation seen with chronic ethanol exposure involves effects at more than one point in the inflammatory response mechanism. Various studies have implicated different factors in the development of enhanced KC responses to LPS in ethanol.

1.4.1.1 Oxidative stress

Oxidative stress secondary to ethanol metabolism has been clearly implicated in hepatocyte damage (section 1.3.3) but evidence has also accumulated that it may sensitise KC to LPS stimulation (Arteel 2003). It has been shown to have a role in the ethanol-dependent upregulation of CD14 (Wheeler and Thurman 2003), and antioxidants and glutathione supplementation reduced TNFα output in AAH patient PBMCs and rat KC (Hill, Devalaraja et al. 1999). N-acetylcysteine, already in therapeutic use for paracetamol toxicity, has been suggested as a suitable antioxidant and GSH replenisher, but its *in vitro* behaviour in serum-free conditions is different from that in serum when it auto-oxidises and develops pro-oxidant properties, increasing p38 MAP kinase and JNK phosphorylation (Chan, Riches et al. 2001).

The antioxidant dilinoleoylphosphatidylcholine (DLPC) was found to reduce the TNFα response from ethanol-fed rat KC and this effect was associated with reduced ERK1/2 and p38 phosphorylation, increased cytoplasmic IKBα and decreased nuclear p65. An ERK inhibitor had a similar effect to the DLPC but a p38 inhibitor did not, suggesting that oxidative stress may be increasing TNFα *via* ERK-dependent activation of NFKB p65 (Cao, Mak et al. 2002).

In a rat model of haemorrhagic shock, oxidative stress has been shown to recruit TLR4 to the cell membrane with an associated increased responsiveness to LPS. Inhibitors of intracellular calcium or cytoskeletal activity reduced the effect suggesting, perhaps unsurprisingly, that

exocytosis had a significant role in the mechanism. Fluorescence resonance energy transfer (FRET) showed the TLR4 to be concentrated in lipid rafts in the plasma membrane and lipid raft blockers could also reduce the effect of oxidative stress on LPS sensitivity (Powers, Szaszi et al. 2006). One might speculate that when ethanol is the source of oxidative stress, the associated ER stress might increase the cholesterol content of the plasma membrane and hence increase lipid rafts, amplifying the effect.

1.4.1.2 S-adenosylmethionine depletion

SAMe depletion is already thought to play a critical role in hepatocyte injury in ALD (section 1.3.3.3) and may also contribute to enhanced cytokine responses in KC. Rats depleted in SAMe by the methionine-choline deficient (MCD) diet display increased TNFα responses to LPS at both the mRNA and protein level with more severe histological liver injury, both of which could be attenuated by SAMe supplementation. SAMe supplementation lowered LPS-induced TNFα secretion in RAW264.7 murine macrophages and in monocytes from AAH patients in a dose-dependent manner while concurrently increasing the anti-inflammatory cytokine IL-10 (McClain, Hill et al. 2002). Reduction in the SAMe:SAH ratio in mice had a similar effect on TNFα (Song, Zhou et al. 2004).

Work using TNFα promoter reporter constructs suggested that SAMe down-regulates TNFα at a transcriptional level. This down-regulation was not affected by mutation of the NF-κB binding sites or by over expression of p65 or its co-activator p300 suggesting that SAMe is working downstream of NF-κB in the mechanism of transcriptional control. There were no associated changes in DNA methylation and the non-methylating SAMe metabolite 5'-methylthioadenosine (MTA) had the same effect on TNFα transcription as SAMe suggesting that the SAMe effect is not related to its role in DNA methylation (Veal, Hsieh et al. 2004). It is possible that SAMe's role in replenishing antioxidant defence mechanisms and maintaining proteasome function (McClain, Barve et al. 2005) has a favourable effect on control of TNFα transcription downstream of p65/p300 activation.

1.4.1.3 TLRs and their signalling intermediates

Ethanol has been shown to upregulate KC TLR4 in rats (Zuo, Gong et al. 2003) and TLR1,2,4,6,7,8 and 9 in mice (Gustot, Lemmers et al. 2006). In the latter study antibiotics were shown to attenuate liver injury without reducing TLR expression, suggesting that the TLR upregulation was a consequence of the ethanol rather than the endotoxin. The NADPH oxidase inhibitor diphenylene iodonium (DPI) reduced ROS and prevented TLR upregulation, suggesting that the TLR expression resulted from oxidative stress. In the physiological setting TLRs rarely act alone but respond in concert to the various PAMPs that result from microbial ingress. The character of the response will depend on the variety and intensity of stimulation of the different TLRs with some signals potentiating the response to ligation of other TLRs and others inhibiting

them ('cross-tolerance'). There is also evidence of variation in the pattern of intracellular signalling when TLRs are activated together (Broad, Jones et al. 2006). A study of the effect of acute ethanol administration on signalling suggested that it attenuated the TNFα response to ligation of TLR4 alone but augmented the response to simultaneous ligation of TLR2 and 4, with reduced ERK signalling after the single stimulus and enhanced JNK and AP-1 signalling after dual stimulation (Oak, Mandrekar et al. 2006).

The biphasic effect of ethanol on inflammatory responses has been shown to correlate with changes in the activity of the signalling intermediate IRAK which is low in mice after acute exposure to ethanol and LPS at one hour, but increases when the LPS is administered after 21 hours of ethanol feeding (Yamashina, Wheeler et al. 2000).

The MAP kinase pathway has been investigated several times in this context. LPS stimulation of RAW264.7 macrophages results in increased stability of TNFα mRNA and consequent increased protein expression, but this effect can be reversed by inhibitors of p38 MAPK (Brook, Sully et al. 2000). P38 and ERK1/2 signalling have both been found to be increased after chronic ethanol exposure (Kishore, McMullen et al. 2001; Nagy 2003). Four weeks of ethanol feeding enhanced the TNFα mRNA and protein response to LPS in rats and this was associated with ERK1/2 activation and recruitment of egr-1 to the TNFα promoter, an effect that could be fully blocked by ERK inhibition. In this study ethanol actually reduced NF-κB activity and had no effect on AP-1 (Kishore, Hill et al. 2002). Dominant negative transgenes for egr-1 and ERK prevented the LPS-stimulated TNFα mRNA accumulation in ethanol (Shi, Kishore et al. 2002). Egr-1 is an immediate early gene that coordinates cellular responses to stressors and activates genes for many other pro-inflammatory proteins in addition to TNFα and may be a future therapeutic target in ALD (Pritchard and Nagy 2005). ERK1/2 activity and TNF production can also be diminished in parallel by DPI, suggesting that ROS many be driving the increased ERK activation (Thakur, Pritchard et al. 2006).

The PPARγ agonist pioglitazone has been shown to reduce the enhanced TNFα production by 70% in ethanol-fed rats. There was no associated change in gut permeability, suggesting that the sensitising mechanism was antagonised directly (Enomoto, Takei et al. 2003).

1.4.1.4 Post-translational modification of inflammatory mediators

Changes in total TNFα mRNA may result from changes in transcription rate or changes in the stability of the transcript. Nuclear run-on experiments in ethanol-exposed and control mouse KC and RAW264.7 cells suggested that ethanol did not actually significantly increase TNFα transcription but rather increased the half-life of the transcript resulting in increased protein production. This stabilisation could be prevented by a p38 inhibitor or by transfection with a dominant negative p38 (Kishore, McMullen et al. 2001).

1.4.1.5 Adenosine signalling

Tissue inflammation has a local negative feedback mechanism through adenosine signalling *via* A2 receptors. There are four known adenosine receptors, all G-protein coupled: A1 and A3 inhibit adenylate cyclase, while A2A and A2B activate it by $G\alpha_s$ activity. A2A predominates on leucocytes and vascular endothelium. In conditions of inflammation and cellular stress, ATP consumption outstrips production and adenosine is liberated into the extracellular space. There it provides a feedback mechanism to limit tissue damage *via* A2A receptors by inducing local vasodilatation and reducing leucocyte reactivity through increases in intracellular cAMP (Hasko, Pacher et al. 2006). The biological significance of this mechanism was demonstrated in A2A knockout mice that had significantly increased susceptibility to liver damage by concavalin A and lipopolysaccharide (Ohta and Sitkovsky 2001). The cAMP drives protein kinase A (PKA) to phosphorylation of CREBP leading to transcriptional suppression at pro-inflammatory genes. KC from ethanol-fed rats show reduced $G\alpha_s$ protein, reduced cAMP in response to A2 agonists and reduced PKA activity. However, no difference in PKA nuclear translocation and CREBP phosphorylation could be detected between ethanol-fed animals and controls so the role of A2 receptors and cAMP in ethanol-related inflammation remains unclear (Aldred and Nagy 1999).

1.4.1.6 Phosphodiesterase 4B

An alternative route to cAMP depletion is increased breakdown and recent work has identified phosphodiesterase 4B (PDE4B) as having a role in models of ALD. Chronic ethanol exposure significantly decreased cAMP in KC and peripheral monocytes and was associated with increased NF-κB transcriptional activity and increased TNFα mRNA and protein. The ethanol exposure was shown to have increased expression of the LPS-inducible PDE4B which degrades cellular cAMP. Selective inhibition of PDE4B with rolipram abrogated the TNFα response to LPS in both control and ethanol-exposed cells. The potential of selective PDE4B inhibitors to reduce inflammation in human AAH is likely to be under investigation soon (Gobejishvili, Barve et al. 2008).

1.4.2 Similar phenomena in other organ systems

While the co-localisation of high concentrations of bacterial endotoxin in portal venous blood and high rates of ethanol metabolism account for the fact that the inflammation of AAH is most apparent in the liver, the tissue distribution of both ingested ethanol and macrophages means that, if the mechanisms discussed above are genuine, one would expect to see exaggerated inflammatory responses after chronic ethanol exposure in other physiological systems. This is indeed the case, and the effects of ethanol on inflammation are apparent in both the lung and the pancreas.

The effect of heavy drinking on pulmonary inflammation was identified in the 1990s with a study that showed that chronic excess alcohol increases the risk of adult respiratory distress syndrome (ARDS) in intensive care from 22% to 43% ($p<0.001$), increasing to 52% in patients with sepsis. Patients with ARDS and alcohol excess were more likely to die than those who did not drink to excess (65% V 36%, $p=0.003$) (Moss, Bucher et al. 1996). Glutathione depletion was implicated in the pathogenesis of this phenomenon, with the heavy drinkers showing significantly reduced pulmonary GSH concentrations and a greater proportion of it in the oxidised form (Moss, Guidot et al. 2000). The lung cannot synthesise GSH and relies on the liver for its supply (Foreman, Hoor et al. 2002). A subsequent rat model of ARDS after chronic ethanol displayed increased lung TNFα and mitochondrial GSH depletion with increased ROS and apoptosis in type II alveolar cells. Inflammation in this model could be ameliorated by procysteine but not by n-acetylcysteine, suggesting that mitochondrial GSH depletion was a key factor in pathogenesis (Brown, Harris et al. 2001). Interestingly, there is even some evidence of a biphasic effect of ethanol on inflammatory responses in the lung with acute ethanol down-regulating alveolar macrophage iNOS within 2.5 hours (Kato, Negoro et al. 2005).

The pancreas also typically shows prolonged and dysregulated inflammatory responses after chronic ethanol exposure, but so far the mechanisms suggested have been different. NF-κB activation in pancreatic acinar cells relies on both PKC δ and ε. Low concentrations of cholecystokinin (CCK) only activate PKC δ but ethanol will activate PKC ε and so can synergise with CCK to activate inflammatory gene transcription in acute pancreatitis (Satoh, Gukovskaya et al. 2006).

There are also other instances of enhanced cytokine responses to TLR ligands in diseases unrelated to alcohol. Monocytes from patients with primary biliary cirrhosis (PBC), a disease that features impaired mitochondrial energetics (Hollingsworth, Newton et al. 2008), have increased IL-1, IL-6, IL-8 and TNFα responses to a panel of TLR ligands (Mao, Lian et al. 2005). Acute renal failure, particularly when secondary to powerful oxidants such as in rhabdomyolysis or cisplatin nephrotoxicity, is also associated with macrophage hyper-responsiveness to LPS (Zager, Johnson et al. 2006).

1.4.3 Contrasting effects of acute and chronic ethanol

The biphasic effect of ethanol on inflammatory responses is not always well recognised in the literature, leading to unresolved controversies about whether heavy alcohol drinking is immunosuppressive or pro-inflammatory with limited recognition that it can be both, either sequentially (Enomoto, Ikejima et al. 1998) or concurrently (Mookerjee, Stadlbauer et al. 2007).

The initial work by Enomoto *et al* (1998) suggested that both the early hyporesponsiveness and the later hyperresponsiveness to TLR ligands were abrogated by antibiotics and so were dependent on endotoxin. Similar animal work corroborated these findings with acute ethanol

administration down-regulating TLR4 in mouse liver after 2-6 hours, a decrease which could be prevented by gut sterilisation with antibiotics and which was not seen in RAW264.7 murine macrophage cell line given ethanol in the absence of bacterial components (Nishiyama, Ikejima et al. 2002). These findings are consistent with the established concept of endotoxin tolerance with acute ethanol in the whole-animal model causing an initial rise in portal endotoxaemia to which the KC become tolerant and hence hyporesponsive to early LPS challenge until the metabolic effects of prolonged ethanol exposure take effect and hyperresponsiveness supervenes. This would suggest that chronic ethanol causes true 'failure of endotoxin tolerance'.

However, there is contradictory evidence, some from the same group as the initial description, that acute ethanol can diminish inflammatory responses to LPS in cell-based models without prior endotoxin exposure. Work in the human macrophage cell line MonoMac6 (Zhang, Bagby et al. 2001) and in isolated mouse KC (Yamashina, Wheeler et al. 2000) shows that pre-treatment with ethanol alone can blunt the cytokine response to LPS. This suggests that the acute effect of ethanol is not purely endotoxin tolerance, although it remains possible that the ethanol, a microbial product itself, contained some previously-undetected TLR ligands which induced tolerance. Support for a more direct, endotoxin-independent mechanism for the anti-inflammatory effect of acute ethanol comes from a study in which it reduced responses to stimulation of all mouse TLRs, apparently through inhibition of a type 1 interferon autocrine amplification loop which is particularly important for enhancement of signalling from TLR3 and down the MyD88-independent pathway from TLR4 (Pruett, Zheng et al. 2004). In addition, the *in vivo* situation is certainly more complex than the single-ligand techniques used experimentally, with stimulation of multiple TLRs resulting in potentiation and cross-tolerance of signalling and the potential for ethanol to be exerting an influence at any or all of the intersections (Oak, Mandrekar et al. 2006).

1.5 Steroids in the control of inflammation

Both endogenous and exogenous (pharmaceutical) glucocorticoids are likely to influence the course of AAH.

1.5.1 Mechanism of steroid action

Glucocorticoids are able to cross the plasma membrane and bind the cytoplasmic glucocorticoid receptor (GR) which has its principal actions in the nucleus. The actions of the GR vary with cell type (Truss and Beato 1993) and depend on the presence of other transcription factors and regulatory elements, the phosphorylation state of the GR and the tertiary organisation of DNA into nucleosomes (Beato, Truss et al. 1996) as well as whether the GR is in its monomeric or dimeric state (Dewint, Gossye et al. 2008). In its dimeric form GR can activate gene transcription, for instance interacting with ERK1/2 to increase transcription of IL-10 in response

to LPS (Xia, Peng et al. 2005). Monomeric GR is sufficient for its role in antagonism of activation of pro-inflammatory genes. It can reduce NF-κB and AP-1 activity by competitive binding of the coactivator cAMP response element binding protein (CBP) which blocks access to CBP's transcription activating histone acetyl transferase (HAT) activity (Ito, Barnes et al. 2000). This can achieve approximately 50% suppression of transcription, but maximal suppression is achieved by GR recruiting histone deacetylase (HDAC) 2 which deacetylates chromatin and suppresses transcription (section 1.6) (Ito, Jazrawi et al. 2001).

1.5.2 Steroid sensitivity

In AAH corticosteroid therapy has been demonstrated to have a moderate effect on outcome in the group at highest risk of death, reducing mortality from 35% to 16% (Mathurin, Mendenhall et al. 2002). However, a sixth of patients in this group perish despite treatment, and the applicability of corticosteroid therapy is limited by concerns about heightened risks of sepsis and gastrointestinal haemorrhage. Steroid responsiveness in AAH is indicated by the early change in the serum bilirubin level (ECBL) (section 1.1.7.2) with those patients whose bilirubin has not fallen by the seventh day of treatment having a particularly high mortality and gaining no benefit from continuation of therapy (Mathurin, Abdelnour et al. 2003). Treatment outcomes could be improved by early identification of the 27% of patients who are unlikely to benefit from steroids or by strategies to improve steroid sensitivity in this group.

Steroid insensitivity is not unique to AAH. It has been well characterised in inflammatory skin diseases and ulcerative colitis (UC) where it is evident as a reduced maximum inhibitory effect of dexamethasone on *ex vivo* phytohaemagglutinin (PHA)-stimulated lymphocyte proliferation. Impaired lymphocyte steroid sensitivity (LSS) in this assay reliably predicted clinical response to glucocorticoid therapy in acute UC with a maximum inhibition of proliferation (I_{max}) of 60% or less observed in all treatment failures and 3/5 incomplete responders but in none of those with a complete clinical response (Hearing, Norman et al. 1999). In that study there was no significant difference in LSS measured in patients three months after their acute admission, suggesting that reduced LSS was a function of the disease activity rather then an intrinsic property of the patients. However, steroid insensitivity is seen in roughly similar proportions of patients in many inflammatory diseases and a study that measured LSS by the same method in a healthy population showed a wide variation in lymphocyte steroid responsiveness, implying that at least some of the clinical steroid insensitivity in inflammatory disease is a result of individual physiology rather than a consequence of disease (Hearing, Norman et al. 1999). IL-2 has been implicated as a disease-specific determinant of steroid insensitivity in moderate and severe UC with high concentrations leading to impaired nuclear translocation of GR and increased glucocorticoid export *via* the multi-drug resistance (MDR) transporter (Creed, Norman et al. 2003). IL-2 blockade with the monoclonal antibody basiliximab is showing clinical promise in reversing steroid insensitivity and improving clinical outcome in UC (Creed, Probert et al. 2006).

Other factors can influence steroid sensitivity, including the acetylation status of heat shock protein 90 (hsp90) which acts a molecular chaperone in the nuclear translocation of the activated GR. If hsp90 becomes hyperacetylated due to inhibition of HDAC6 its ability to interact with GR is impaired and this is manifest as a 100-fold shift to the right in the steroid dose-response curve (Murphy, Morishima et al. 2005). This is of particular interest when one starts to consider the role of acetylation changes in AAH (section 1.6). Another molecule already studied in AAH is the glucocorticoid-induced leucine zipper (GILZ) which inhibits NF-κB responses to LPS in macrophages and which has been shown to be expressed at a lower level in the livers of patients with severe AAH than in heavy drinkers without AAH. 48 hours of prednisolone 40mg/day was shown to increase GILZ expression in circulating monocytes from patients and reduce their LPS-induced secretion of TNFα and regulated upon activation, normal T cell-expressed secretion (RANTES) chemokine. It was suggested that impaired GILZ expression could be responsible for the enhanced inflammatory responses that lead to AAH, in the form of reduced sensitivity to endogenous glucocorticoids (Hamdi, Bigorgne et al. 2007).

1.5.3 Endogenous steroids

Although it is exogenous pharmaceutical steroids that are first thought of in the context of AAH, there is emerging evidence that endogenous glucocorticoids are important and often relatively deficient in liver disease, and mechanisms that modulate steroid sensitivity will be as relevant to endogenous steroids as to exogenous ones.

In septic shock, adrenal insufficiency as assessed by the short synacthen test (SST), which measures the increase in cortisol in response to exogenous corticotrophin stimulation, is associated with haemodynamic instability and poor prognosis which can be improved by administration of exogenous glucocorticoids (Annane, Sebille et al. 2002). Similar relative adrenal insufficiency has been observed in 62% of patients with acute liver dysfunction and correlated with severity and outcome (Harry, Auzinger et al. 2002). Corticosteroid replacement was found to reduce vasopressor requirements in patients with hypotensive acute liver dysfunction without overall increase in survival (Harry, Auzinger et al. 2003). Cirrhotic patients suffering severe sepsis showed blunted response to corticotrophins in 50% with the low responders having significantly higher mortality (Tsai, Peng et al. 2006). Relative adrenal insufficiency can be present in cirrhosis in the absence of sepsis or cardiovascular collapse and has now been observed in up to 95% of patients requiring liver transplantation leading to the suggestion of a ‘hepatoadrenal syndrome’ separate from the adrenal effects of sepsis (O'Beirne, Holmes et al. 2007).

This evidence suggests that endogenous glucocorticoid responses to TLR-driven inflammation (sepsis, fulminant liver failure) are frequently inadequate in liver disease and it is possible that the same holds true in the TLR-driven inflammation of AAH with relative inadequacy of endogenous anti-inflammatory effects contributing to pathogenesis.

Chronic heavy drinking has been shown to affect the hypothalamo-pituitary-adrenal axis producing higher peak serum cortisol and diminished diurnal variation (Badrick, Bobak et al. 2007). This higher sustained basal exposure might render immune processes less responsive to additional exogenous glucocorticoid in times of physiological crisis.

1.6 Chromatin modification in the control of inflammation

The initiation and perpetuation of inflammation is the result of the net effect of the multiple modifiable and inter-dependent processes described above on the transcription of the key inflammatory mediators, cytokines and chemokines. As a result, gene transcription is the final common pathway for nearly all pro-inflammatory processes. This transcription is intimately linked to and regulated by covalent chromatin modifications. When this final pathway is tightly-regulated, the effect of perturbations in upstream signalling will be reduced, and if this final pathway became dysregulated the potential for chaotic, disproportionate and deleterious responses would be increased. This makes it a critical process in the study and treatment of disordered inflammatory responses.

In its unactivated state DNA is tightly coiled around histone protein octamers and this chromatin is compacted into a closed tertiary structure from which the histone tails protrude but in which the DNA is inaccessible to polymerases that would produce gene transcription. Gene activation by transcription factors involves co-activator proteins with histone acetyl transferase (HAT) activity and these acetylate key lysine residues in the histone tails. The negatively-charged acetyl groups favour a conformational change in chromatin that allows RNA polymerases access to the DNA, facilitating gene transcription. Termination of transcription is mediated through histone deacetylases (HDAC) which release free acetate and allow the chromatin to resume its closed, untranscribed conformation (Kimura, Matsubara et al. 2005) (**Figure 1-6**). There is a variety of HDACs able to modulate inflammatory gene transcription including class I and II HDACs which can be recruited by transcriptional repressors such as the activated glucocorticoid receptor (Barnes, Adcock et al. 2005), and class III HDACs, known as sirtuins (SIRT), which are active in the presence of NAD^+ and are thought to mediate the effects of nutrient supply on gene expression and cellular ageing (Lavu, Boss et al. 2008).

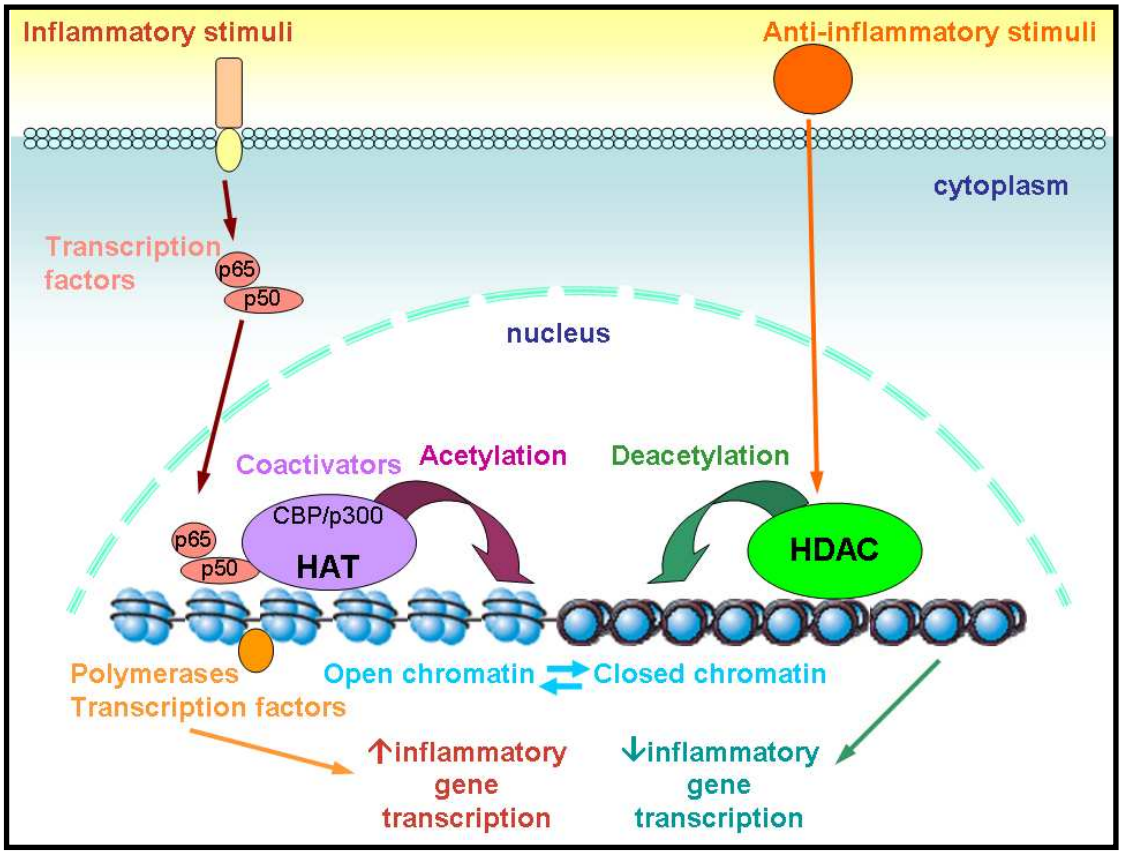


Figure 1-6 Histone acetylation control of inflammatory gene transcription

Transcriptional activation of a given gene will depend on the net balance of HAT and HDAC activity, and it is by influencing these activities that transcriptional activators and repressors have their effects. In the control of gene expression, acetylation changes occur downstream of most of the mechanisms that regulate cell signalling. Thus pathological processes that directly alter this 'final common pathway' can have a profound effect on gene expression, relatively independent of variations in signalling. The influence of such processes is well established in carcinogenesis and there is emerging evidence of their importance in inflammation.

The effect of histone acetylation on gene transcription will depend not just on the total quantity of acetyl groups but also on which histone monomers are acetylated, which residues within the monomer are acetylated, and the number of acetyl groups at a particular residue (Grunstein 1997). For instance, acetylation of histone H4 is a permissive event, generally necessary but not sufficient for transcription, so the promoter regions of genes that a given cell needs to activate frequently or quickly will already have a high content of acetyl-histone H4. By contrast, acetylation of histone H3 is an activation event and generally will only occur in high concentration at promoters during initiation of transcription (Eberharter and Becker 2002). Other covalent chromatin modifications including DNA and histone methylation and histone

phosphorylation or ubiquitination can also influence gene transcription by recruitment of further co-activator or co-repressor complexes.

These epigenetic changes represent a level of control of gene behaviour separate from the activity of transcription factors and the genetic code itself. The pattern of epigenetic modifications will influence cell and organism phenotype, often more so than variations in the DNA base-pair sequence. Thus epigenetic changes are important modulators of cellular differentiation and behaviour and at least some components of this 'epigenetic code' are transmissible in somatic and germ line division.

The evolutionary basis for these mechanisms is that they allow patterns of gene expression and responses to stimulation and signalling to be modified by environmental factors such as available nutrients. The phenotype changes are much faster than those brought about by mutation and natural selection, can alter physiology within a generation, and confer improved survival to the exposed individuals and to subsequent generations. Furthermore, there is evidence that they can respond to chemical changes in ingested plants, often the earliest indication of changing environmental conditions, and so adapt pre-emptively to environmental stress (Howitz and Sinclair 2008). However, the major selection pressure promoting these mechanisms will be nutrient shortage so they will have evolved to cope with famine rather than excess food, and may not be attuned to adapt to compensate for the effect of energy over-supply under circumstances such as obesity, parenteral feeding and ethanol consumption.

1.6.1 Histone acetylation in transcriptional control of inflammatory genes

An epigenetic basis for dysregulated inflammatory responses was first studied in intestinal epithelial cells in investigation of the role of commensal bacterial in modulating gut inflammation. Butyrate, a bacterial metabolite, is a natural inhibitor of HDACs and it was demonstrated that this inhibition lead to increased histone acetylation and an enhanced response to LPS stimulation in terms of secretion of the chemokine macrophage inflammatory protein 2 (MIP2) (Ohno, Lee et al. 1997). The ability of dietary and bacterial short chain fatty acids to modulate inflammation in this way could account for the effects of diet and intestinal flora on Crohn's disease and necrotising enterocolitis (Sanderson 2004).

1.6.2 Histone acetylation and Theophylline in COPD

A link between chromatin modifications, steroid responsiveness and oxidative stress in the control of inflammatory responses has been established in the smoking-related airway inflammation of chronic obstructive pulmonary disease (COPD). The glucocorticoid receptor acts in part by recruitment of HDAC2 to actively transcribed pro-inflammatory genes resulting in suppression of transcription (Ito, Barnes et al. 2000). This HDAC recruitment is necessary for maximal suppression of transcription; the GR partial agonist mifepristone, which activates the

GR without HDAC recruitment, achieves only 50% reduction in pro-inflammatory gene transcription despite effective inhibition of NF-κBp65-associated HAT activity (Ito, Jazrawi et al. 2001). This GR-recruitable HDAC activity is significantly reduced in the presence of smoking-associated oxidative stress, most likely because of nitrosylation of tyrosine residues in the HDAC molecule. However, the HDAC activity and suppression of inflammatory gene transcription can be restored by the addition of theophylline, a methyl xanthine drug commonly used as a bronchodilator because of its action in inhibiting phosphodiesterases (PDE) in bronchial smooth muscle leading to cAMP accumulation, protein kinase A activation and muscle relaxation. Interestingly, the beneficial effect of theophylline on HDAC activity occurred at concentrations lower than those necessary to achieve PDE inhibition or antagonism of adenosine receptors, the other major pharmacological action of theophylline, suggesting that it is acting by another mechanism (Ito, Lim et al. 2002).

Further investigation into the theophylline effect revealed that PBMC from COPD patients show reduced steroid sensitivity compared to normal controls when assayed by IL-8 response to TNFα stimulation after treatment with increasing doses of glucocorticoid. The COPD steroid insensitivity could be mimicked by inducing oxidative stress in control cells with hydrogen peroxide. Theophylline was shown to restore normal steroid responsiveness in these cells but not in cells in which HDAC2 had been knocked down by RNA interference, confirming that HDAC2 was involved in the theophylline effect. Peroxide-induced oxidative stress was associated with activation of the signalling kinase AKT and theophylline inhibited this phosphorylation event which is normally dependent on phosphoinositide-3-kinase (PI3K). In smoke-exposed mice a PI3K inhibitor and knockdown of PI3Kδ each restored steroid responsiveness, suggesting that theophylline is restoring HDAC2 activity through PI3Kδ inhibition (Marwick, Caramori et al. 2009). Earlier, separate *in vitro* work had established that both theophylline and caffeine, another methyl xanthine, could inhibit PI3K lipid kinase activity and that PI3Kδ was the isoform most sensitive to this effect (Foukas, Daniele et al. 2002).

However, it is worth noting that AKT signalling can increase p300 HAT activity and can be induced by HDAC inhibition (Liu, Denlinger et al. 2006) so there remains doubt as to whether PI3K activation or HDAC2 impairment is the primary event in steroid insensitivity, and as to where theophylline is exerting its effect. More confusingly, pharmacological HDAC inhibition can actually decrease inflammatory responses to TLR ligation, probably due to impaired recruitment of transcription factors to pro-inflammatory genes (Bode, Schroder et al. 2007), a finding which emphasises the need for caution in interpreting the effects of blunt interference with a global mechanism of transcriptional regulation on individual downstream events.

The majority of work in COPD has focussed on the role of HDAC2 but recent work has also suggested there may be a contribution from the NAD^+-dependent class 3 HDACS, the sirtuins (Yang, Wright et al. 2007).

1.6.3 Effect of ethanol on histone acetylation

The enhanced cytokine responses to TLR ligation seen in AAH could have a similar origin to those in COPD. In addition, clinical trial data suggests at least a proportion of AAH patients have relative steroid insensitivity and again this could have an epigenetic basis. Ethanol metabolism induces oxidative stress which could lead to HDAC inhibition, hyperacetylation of histones and consequent increased transcription of pro-inflammatory genes. In addition, the hepatic end product of ethanol metabolism is acetate which in high concentrations may be able to influence the balance of HAT and HDAC activity to favour inflammatory gene expression.

The specific role of histone acetylation in ethanol-enhanced inflammatory responses has yet to be studied. However, evidence is emerging that ethanol does increase acetylation of cellular proteins, including core histones. This was first demonstrated in isolated rat hepatocytes incubated with 5-200mM ethanol for 24h which showed a dose- and time-dependent increase in acetyl-histone H3 at lysine 9 (AcH3K9) as measured by immunoblotting (Park, Miller et al. 2003). This increase was mimicked by the HDAC inhibitor trichostatin A (TSA) and abrogated by inhibitors of ethanol metabolism 4-methylpyrazole (ADH inhibitor) and cyanamide (ALDH inhibitor), emphasising the importance of ethanol metabolism at least as far as acetate in the mechanism. Similar studies performed in ethanol-exposed rat hepatic stellate cells (HSC) showed a similar dose- and time-dependent increase in AcH3K9 with a peak of 86-fold at 200mM ethanol and 72h, perhaps slower than in hepatocytes due to the lower rate of ethanol metabolism in HSC (Kim and Shukla 2005). *In vivo* work with ethanol-fed rats confirmed the increased AcH3K9 in hepatocytes. This was associated with increased HAT activity which was shown to be particularly active for the H3K9 modification. Chromatin immunoprecipitation (ChIP) demonstrated that this modification was associated with the ADH1 gene, the expression of which was upregulated by ethanol. The increased HAT activity was not reduced by inhibitors of the ERK and JNK signalling pathways, suggesting that it may occur through a process distinct from normal upstream signalling events (Park, Lim et al. 2005). Further experiments with bolus ethanol injection showed that the increases in histone acetylation were greatest in liver (6-fold), lung (3-fold) and testes (3-fold) but there was no effect in kidney, brain, heart, pancreas, stomach, colon or blood vessels (Kim and Shukla 2006).

The effect of alcohols of differing carbon chain length has been recently studied. Alcohols with up to eight carbons were able to increase HAT activity and H3K9 acetylation in cultured primary rat hepatocytes, with the peak activity induced by butanol. Inhibitors of alcohol metabolism abrogated the effect. The carboxylic acid metabolites of these alcohols had a similar effect on HAT activity but propionate and butyrate were also observed to modestly reduce HDAC activity (Choudhury and Shukla 2008). It appears that both HAT activation and HDAC inhibition occur and either or both could be responsible for the observed changes in histone acetylation.

Histones are not the only cellular proteins to be acetylated in the presence of ethanol. Recent work has shown increased acetylation of hepatocyte microtubule proteins in ethanol which was associated with impaired recruitment of HDAC6 and abrogated by 4-MP (Shepard, Joseph et al. 2008). This cytoskeletal acetylation may contribute to proteasome dysfunction in AAH and to the formation of Mallory bodies. Another recent investigation demonstrated that total liver acetylated lysine in both whole liver and mitochondrial fractions was increased five-fold after six weeks of ethanol feeding in rodents and the mechanism was not reliant on CYP2E1 or SIRT3 (Picklo 2008).

1.6.4 Putative mechanisms of ethanol-induced histone acetylation

The existing data make feasible the idea that ethanol's enhancement of inflammatory cytokine responses to TLR stimulation, a key component of AAH pathogenesis, occurs as a result of increased histone acetylation at pro-inflammatory gene loci after prolonged ethanol exposure and metabolism. A mechanism which favours net increase in histone acetylation could be expected to produce insensitivity to endogenous and exogenous glucocorticoids and so facilitate prolongation and propagation of the inflammatory response. From what is already known, a number of mechanisms by which ethanol could increase histone acetylation might be postulated.

Increased upstream signalling. As discussed above, ethanol exposure can increase TLR receptor density and, through oxidative and ER stress, potentiate signalling in the NF-κB pathway. This will lead to increased recruitment of co-activators with associated HAT activity to inflammatory gene promoter sites and increased histone acetylation as part of the normal transcription process. However, this would only cause activation-dependent histone acetylation and there would not be acetylation of histones before the arrival of the activating stimulus as observed in ethanol, and it would not result in acetylation of other proteins. It could still be debated as to whether the significant contribution to enhanced transcription came from increased transcription factor pathway signalling, increased ground-state histone acetylation leaving a gene 'primed' for greater transcription, or impaired deacetylation leading to ineffective termination of transcription, or whether all three mechanisms contribute to the observed effect. The clinical pattern of inflammation in AAH is of an inflammatory response that has been uncoupled form its normal regulatory mechanisms with a prolonged, 'smouldering' inflammation atypical of a classical 'septic' response, perhaps hinting at a role for impaired deacetylation.

HDAC inhibition. The presence of ROS and the adducted products of oxidative reactions are thoroughly documented in ALD, so it is entirely conceivable that nitrosylation of HDACs and consequent inhibition of function occurs in AAH in the same way as in COPD. The cellular perturbations of ER stress, mitochondrial dysfunction and increased acetaldehyde might all have an additional detrimental effect on HDAC activity. In addition, ethanol metabolism results in significant elevations in the concentration of free acetate which may impair HDAC enzymatic

activity by end-product inhibition. Furthermore, ethanol metabolism results in depletion of NAD^+ which could lead to inhibition of the NAD^+-dependent class 3 HDACs, the sirtuins. Recent studies have confirmed that ethanol can reduce both the expression and the activity of sirtuins in liver (Lieber, Leo et al. 2008; You, Liang et al. 2008).

Acetyl-coenzyme A. The acetate produced by ethanol metabolism is an obvious candidate for causing histone acetylation events. However, free acetate is not available for use by HAT and must be converted to acetyl-coA to be deployed for histone acetylation. This is achieved by acetyl-coA synthetase enzymes which are present in macrophages (Fujino, Ikeda et al. 2003) and which can increase the pool of acetyl-coA available for protein acetylation. The acetate may also reduce the total HDAC activity through sirtuin inhibition; free acetate will not affect NAD^+ but once converted to acetyl-coA it can enter the Krebs cycle and convert NAD^+ to NADH in the same way as if it had come from glycolysis or fatty acid oxidation.

PI3K activation. PI3K activation of AKT was implicated in the HDAC2 inhibition and enhanced cytokine responses of COPD and there is evidence that ethanol can also increase PI3K activity (Liu, Tian et al. 2002). Furthermore, AKT signalling can inhibit nicotinamidase synthesis and so increase the cellular ratio of nicotinamide:NAD^+ leading to reduced sirtuin activity (Kassi and Papavassiliou 2008), and the potent sirtuin activator resveratrol is a PI3K inhibitor (Frojdo, Cozzone et al. 2007).

A positive feedback loop may contribute to perpetuation of inflammation through impaired deacetylation. The question of how concerted bursts of multiple inflammatory cytokine transcription could occur in sepsis despite the global moderating effect of HDAC activity was addressed experimentally by measuring changes in HDAC1 after stimulation with other inflammatory mediators. This revealed that TNFα signalling results in depletion of HDAC1 by an IKK2-dependent mechanism, leading to increased acetylation and transcription of other genes (Gopal, Arora et al. 2006). As elevated TNFα is a prominent feature of AAH and correlates strongly with clinical severity and outcome, its role in damping one of the critical epigenetic regulators of multiple gene expression might be of particular relevance. It could be postulated that in chronic ethanol the various impediments to HDAC activity described above will sensitise the cell to the effect of TNFα-induced HDAC1 depletion, amplifying its effect on cytokine gene transcription and contributing to the enhanced inflammatory response.

A further contributing mechanism might be one specific to HDAC6 and SIRT2 which have a role in the cellular trafficking of proteins destined for autophagy and marked for lysis by ubiquitination. By binding the ubiquitin moiety of an unneeded, often misfolded protein and then deacetylating α-tubulin in the microtubule network, HDAC6 can activate transport of proteins to aggresomes for degradation by autophagy. Deacetylated microtubules have enhanced motility so HDAC inhibition by ethanol would lead to impaired intracellular transport and protein degradation, presumably worsening the cellular stress associated with the unfolded protein

response to adducted proteins. HDAC6 also has a role in maintaining HSP90 in its deacetylated form in which it can stabilise the glucocorticoid receptor and preserve the cellular actions of steroids. Consequently, HDAC6 inhibition impairs GR signalling and reduces steroid sensitivity (Boyault, Sadoul et al. 2007). The ability of ethanol to inhibit HDAC6 in hepatocytes and cause microtubule dysfunction has been demonstrated (Shepard, Joseph et al. 2008), but the association with steroid sensitivity has not been investigated.

1.6.5 Probable benefits of methyl xanthines in ALD

To suggest that the enhanced inflammatory responses seen in ethanol have a basis in HDAC inhibition and impaired glucocorticoid responsiveness similar to that seen in COPD implies that ethanol-induced enhancement of inflammation and steroid insensitivity might also respond to the HDAC-activating effects of theophylline. Theophylline has yet to be studied in ALD, but the related methyl xanthine compounds caffeine and pentoxifylline each have evidence of benefit.

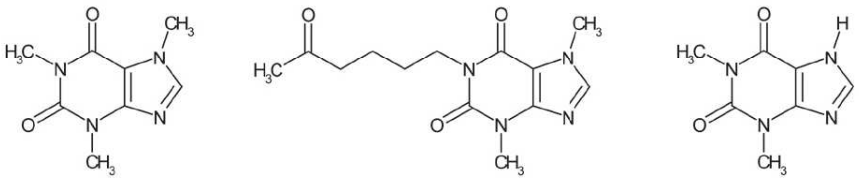


Figure 1-7 Chemical structures of methyl xanthines

The benefits of pentoxifylline in AAH are discussed in Section 1.1.7.3. Caffeine is likely to be responsible for the impressive protective effect of coffee drinking against ethanol cirrhosis. This was most recently described in an epidemiological study of 125580 individuals followed for over 15 years in which drinking four or more cups of coffee a day reduced the relative risk of alcoholic cirrhosis to 0.2 ($p<0.001$), a finding which was absent in cirrhosis of other causes (Klatsky, Morton et al. 2006). Coffee contains many biologically active compounds (including antioxidants and cafestol which boosts cellular glutathione) but the most plentiful and the most likely to be responsible for the hepatoprotective effect is caffeine. The fact that the protective effect was not observed with tea in this study goes against caffeine being responsible. However, tea consumption in the study cohort was low, and other studies have demonstrated lower transaminase levels in tea as well as coffee drinkers (Ruhl and Everhart 2005).

Caffeine's direct inhibition of phosphodiesterases (PDE) increases cAMP and consequent anti-inflammatory signalling through PKA, and this effect is either enhanced or diminished by its antagonism of cell surface adenosine receptors, depending on the class of receptor expressed (section 1.4.1.5). Chronic caffeine exposure causes persistent A2A receptor antagonism and

subsequent up-regulation. It could be postulated that this would increase leucocyte sensitivity to the anti-inflammatory effects of adenosine, protecting coffee-drinkers from tissue damage.

Caffeine is metabolised by hepatic CYP1A2 to its principal metabolites paraxanthine (80%), theobromine (15%) and theophylline (4%) which have similar but distinct effects on PDE and adenosine receptors. Theophylline has been shown to have an antifibrotic effect in lung, acting *via* the cAMP-PKA pathway to reduce TGF-β conversion of fibroblasts to myofibroblasts and suppressing collagen synthesis by reduction of COL1 mRNA in chronic obstructive pulmonary disease (Yano, Yoshida et al. 2006). The same group has demonstrated that this pathway is not only antifibrotic but also antiapoptotic in radiological contrast-induced nephropathy (Yano, Itoh et al. 2003). However, the circulating concentration of theophylline generated from metabolism of normal coffee intake is not high enough to cause detectable PDE inhibition, although the theophylline is generated in the liver, so local tissue concentrations may be significantly higher. The further anti-inflammatory mechanism of theophylline described in section 1.6.2 with recruitment of HDAC to silence transcriptionally-active pro-inflammatory genes was observed at lower concentrations than those necessary for PDE inhibition or A2A receptor antagonism (Ito, Lim et al. 2002).

More recently, caffeine itself has been shown to have an antifibrotic effect on mouse liver *in vivo*. Caffeine pre-treatment reduced fibrosis in mice challenged with carbon tetrachloride (Chan, Montesinos et al. 2006).

Thus evidence is accumulating to support the notion that methyl xanthines have a beneficial effect in inflammatory and fibrotic disease, and that they might modulate disease progression in ALD, but the therapeutic potential of this class in AAH and the mechanism, epigenetic or otherwise, through which it might act in that context remains to be discovered.

1.7 Plan of investigation

1.7.1 Thesis

This investigation developed from the thesis that the augmented acute inflammatory responses characteristic of acute alcoholic hepatitis have both a genetic and an epigenetic basis, or, more generally, that the machinery of energy metabolism and host defence (both critically present in the liver) are linked at an epigenetic level so that the character and intensity of inflammatory responses reflect not only the product of receptor signalling cascades shaped by individual genetic background but also the susceptibility of the mechanism to environmental influences. This allows the organism to modulate its inflammatory response according to the prevailing or anticipated energy supply, which might provide a survival advantage in the face of combined pressures of starvation and infection. This adaptability, however, brings with it vulnerability to the amplifying and perpetuating effects of metabolic excesses on inflammatory responses which

can be exemplified by acute alcoholic hepatitis. Modulation of these epigenetic interactions has potential for therapeutic benefit in inflammatory disease.

1.7.2 Hypothesis

Three avenues of investigation were explored with separate but related hypotheses:

- That the functional S180L polymorphism in the TLR adapter molecule MAL, a critical genetic determinant of TLR-triggered disease, will contribute to the pathogenesis of alcoholic liver disease and that other liver disease associated with excess energy metabolism, non-alcoholic steatohepatitis (Chapter 3).

- That acute alcoholic hepatitis is characterised by reduction in the sensitivity of immune responses to glucocorticoid inhibition and that this steroid insensitivity can be ameliorated by the epigenetic modulator theophylline (Chapter 4).

- That the enhanced macrophage cytokine response to endotoxin seen in acute alcoholic hepatitis occurs through uncoupling of cytokine gene transcription from its normal regulatory mechanisms by increased histone acetylation at proinflammatory gene promoter regions, as a consequence of the interaction of ethanol metabolism with the epigenetic mechanisms of transcriptional regulation (Chapter 5).

2 General Methods and Materials

2.1 Laboratory Procedure

All experimental procedures were conducted according to the Control of Substances Hazardous to Health (COSHH) regulations. All laboratory work was carried out in compliance with the 'University Safety Policy' and the 'Departmental Safety Policy'. Laboratory procedures were performed in accordance with the Newcastle University publications 'Safe Working with Biological Hazards', and 'Safe Working with Chemicals in the Laboratory'. Experiments involving radioactivity were carried out in accordance with the regulations outlined in 'Local Rules for the use of unsealed sources of radioactivity in the RVI, the Medical School, the Dental Hospital and the Dental School – University of Newcastle upon Tyne' and also 'A Basic Guide for Radiation Workers'. Tissue culture was carried out in compliance with the regulations for containment of class II pathogens.

2.2 Cell Culture

All cells were grown and maintained in a humidified atmosphere at 37ºC with 5% CO_2 (IncuSafe MCO-17AI, Sanyo, Japan). Cell lines were passaged in a containment level II microbiological safety cabinet (Envair, Haslingden, UK) every 5-7 days at a ratio of 1:5 depending on growth. Cells were grown in 25, 75 and150cm^2 culture flasks (Corning, Stone, UK and Greiner Bio-One, Frickenhausen, Germany). Cells in suspension were pelleted by centrifugation at 400g for 5 minutes at room temperature in a bench-top centrifuge (Sorvall legend RT, Thermo Scientific, Waltham MA) unless otherwise stated. Adherent cells were washed in phosphate buffered saline (PBS; Lonza, Wokingham, UK) and detached by incubation with pre-warmed 167g/l trypsin / 67g/l ethylenediaminetetraacetic acid (EDTA; Lonza) solution for 5 minutes and then washed twice by centrifugation in PBS.

2.2.1 Culture media

Media were supplemented with 10% fetal calf serum (FCS; Lonza) from a single batch that had been tested for its ability to maintain cell viability and confirmed endotoxin-free. FCS was heat-inactivated to denature complement proteins by heating to 56 ºC for 30 minutes and then sterile filtered through a 0.22µm membrane (Millipore, Billerica MA) before use. All media were supplemented with 2mM L-glutamine (Lonza). Additional supplements were made as required. Antimicrobials were not routinely added to media, it being thought preferable when studying inflammatory responses to avoid low-grade microbial contamination which might not be visually apparent due to the bacteriostatic effect of antibiotics but still capable of causing significant TLR stimulation, and to rely on scrupulous aseptic technique alone. All cultures showing signs of microbial contamination were destroyed and experiments repeated with fresh cultures. The

media used in this study were Roswell Park Memorial Institute 1640 (RPMI; Lonza) and Iscove's Modified Dulbecco's Medium with High Glucose (IMDM; ATCC, Rockville MD).

2.2.2 Cell counting and viability

Cells were counted using a Neubauer Improved haemocytometer (Hawksley, Lancing, UK). 10µl of cell suspension was allowed to diffuse beneath the coverslip and examined at x20 magnification. Cells in the central large square were counted and the total multiplied by 10^4 to give the number of cells per millilitre.

Viability was assessed by trypan blue exclusion. 10µl of cell suspension was mixed with 10µl 0.2% trypan blue (Sigma, Poole, UK) and examined immediately in a haemocytometer. At least 100 cells were counted in each determination. Cells that failed to exclude trypan blue were classified as non-viable.

2.2.3 Cryopreservation

Cell lines were expanded and cryopreserved at low passage number. Approximately $2x10^6$ cells were pelletted and resuspended in 2ml freezing medium (normal media supplemented with 10% demethylsulphoxide (DMSO; Sigma) to prevent cell damage from ice crystal formation) in a 2ml cryovial (Corning). Cryoinjury was further prevented by controlled cooling in an isopropanol-jacketed freezing container ('Mr Frosty'; Nalgene, Rochester NY) which cools no faster than 1ºC per minute in a -80ºC freezer. Frozen cells were transferred to liquid nitrogen (-196ºC) for long term storage.

Cells were retrieved from cold storage by rapid thawing to 37ºC in a water bath, washed and pelleted three times to remove DMSO. Pellets were resuspended in 10ml normal media, divided between two $25cm^2$ flasks and incubated for 48h to allow growth factor accumulation before decanting to $75cm^2$ flasks and adding media to a total of 20ml.

2.2.4 Cells

Cultured cell lines are an established tool for the study of cellular responses. They are generally derived from human or animal neoplasms and the malignant transformation confers an ability to divide repeatedly in culture and a robustness that allows them to survive in experimental conditions that are only approximately physiological. Howvever, this neoplastic transformation and tolerance of suboptimal culture conditions increases the chance of nonphysiological responses which limits the utility and generalisability of such model systems.

2.2.4.1 THP-1

This is a monocyte cell line obtained from the American Type Culture Collection of cell cultures (catalogue number TIB-202; ATCC) which was derived from peripheral blood monocytes from a one year old male with acute monocytic leukaemia in 1978. It was maintained in RPMI with 2mM L-glutamine and 10% FCS.

2.2.4.2 Monomac-6

Monomac-6 is a monocyte-macrophage cell line obtained from the German Collection of Microorganisms and Cell Cultures (catalogue number ACC124; DSMZ, Braunschweig, Germany) which was derived from peripheral blood monocytes from a 64 year old man with relapsed acute monoblastic leukaemia (FAB M5) in 1985. This line differs from THP-1 and most other monocytic lines in that it displays features of mature macrophages without requirement for pre-treatment with phorbol esters (Ziegler-Heitbrock, Thiel et al. 1988). It was maintained in RPMI with 2mM L-glutamine, 10% FCS, 9µg/ml human insulin (Sigma), 1mM sodium pyruvate (Gibco, Paisley, UK) and 100µM non-essential amino acids (Gibco).

2.2.4.3 HeLa

HeLa is an epithelial line gratefully received from Dr Graeme O'Boyle at Newcastle University. It was established from the human cervical carcinoma of a 31 year old woman in 1951. It was maintained in RPMI with 2mM L-glutamine and 10% FCS.

2.2.4.4 Peripheral blood mononuclear cells

Peripheral blood mononuclear cells were separated from fresh blood taken from study subjects and healthy volunteers. 1µl of 1000u/ml heparin (Sigma, confirmed endotoxin-free in house) per ml of blood was added and mixed thoroughly. The sample was then diluted in an equal volume of serum-free RPMI and layered on top of Lymphoprep density gradient medium (1.077 ± 0.001g/ml; Axis-Shield, Norway) in Leucosep cell-porous membrane tubes (Greiner Bio-One). Centrifugation at 800g for 20 minutes with no brake yielded a pellet of erythrocytes and neutrophils and partition of PBMCs at the plasma/Lymphoprep interface. These were aspirated and washed twice. Contaminating erythrocytes were eliminated by incubation in red cell lysis buffer (8.3g NH_4Cl, 1.0g $KHCO_3$, 1.8ml 5% EDTA, filter sterilised at 0.2µm and made up to 1000ml with ddiH_2O) for 15 minutes at room temperature before a final wash. PBMC were then maintained in RPMI with 2mM L-glutamine and 10% FCS.

2.2.5 Mycoplasma testing

Mycoplasma infection can modify cellular responses and is undetectable by light microscopy. Cell lines were screened for all known *Mycoplasma*, *Acholeplasma* and *Ureaplasma* species

every six months using the VenorGem Mycoplasma Detection Polymerase Chain Reaction (PCR) Assay (Minerva Biolabs, Berlin, Germany). This uses PCR primers specific for the highly-conserved 16S ribosomal RNA coding region of the *Mycoplasma* genome and can detect as little as 1fg of *Mycoplasma* DNA.

100µl of cell culture was heated to 95ºC for 5 minutes and briefly centrifuged to pellet debris. PCR reactions were prepared using 5µl primer/dNTP mix, 0.2µl Taq polymerase 5U/µl (Sigma), 5µl 10x reaction buffer (100mM Tris-HCl pH 8.5, 500mM KCL and 30mM $MgCl_2$), 35.8µl DNA-free water, 2µl internal control DNA (191bp) and 2µl of the boiled sample or 2µl DNA-free water (negative control) or 2µl DNA fragment from *Mycoplasma orale* (278bp, positive control). PCR was run in a thermal cycler (Thermo Hybaid, Ashford, UK): samples were heated to 94ºC for 2 minutes followed by 35 cycles of 94ºC for 30 sec (denaturation), 55ºC for 30 sec (primer annealing) and 72ºC for 30 sec (primer extension). 8µl of PCR products were mixed with 2µl of 5x loading buffer (70% Tris-acetate-EDTA (TAE) buffer, 30% glycerol, 0.001% bromophenol blue) and electrophoresed in a 1% agarose gel containing ethidium bromide (all Sigma). The internal control produces a band at 191bp in all completed reactions and a second band around 270bp indicates the presence of *Mycoplasma* (**Figure 2-1**).

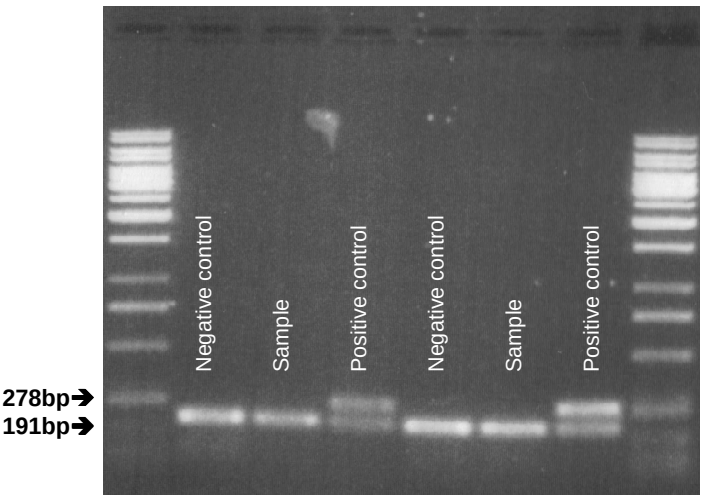


Figure 2-1 Mycoplasma testing by PCR

Each reaction contains internal control DNA producing an amplicon of 191bp.

2.2.6 Endotoxin testing of media and reagents

Endotoxin (particularly LPS) contamination of media or reagents could alter cellular responses to stimulation either by activating cells or by inducing endotoxin tolerance. The original method for establishing that fluids and medications for biological or clinical use were 'non-pyrogenic' was to inject a sample into a rabbit and observe it for fever. This has been superseded by the

limulus amoebocyte lysate (LAL) assay based on the coagulation response of derivatives of the innate immune cells of the horseshoe crab. Endotoxins and even lipopolysaccharides are a heterogenous group of molecules, so bioassays like this which measure a biological effect of endotoxin rather than an absolute quantity have particular utility. However, although the LAL assay is well validated for simple fluids, its limitations are more apparent in complex matrices such as those containing serum (Broad 2007). A novel bioassay that more closely mimics the conditions of the experiments uses cell lines that either naturally or by transfection express endotoxin receptors, and this natural cellular endotoxin responsiveness is coupled to a reporter system that produces a colour change in the presence of an endotoxin dose sufficient to engage downstream signalling from TLRs.

HEK-Blue4 cells (Invivogen, Toulouse, France) are a stably transfected clone of the human embryonic kidney (HEK) 293 cell line expressing the LPS receptor complex TLR4, CD14 and MD2 with a gene for secretable alkaline phosphatase (ALP) under the control of a NF-κB promoter. LPS binding results in ALP secretion which catalyses a colour change in the detection medium. THP-1Blue (Invivogen) are THP1 cells transfected with a similar reporter gene and so respond in the presence of any of a range of TLR ligands rather than LPS alone. Their sensitivity can be increased by treatment with phorbol myristate acetate which induces macrophage differentiation, or by over-expression of CD14 (THP1Blue-CD14).

Media were tested for LPS contamination at 6-monthly intervals or before large experiments using HEK-Blue4 cells. Further testing using THP1Blue cells was undertaken by Dr John Taylor in Newcastle University Dental School Host Response Group. HEK-Blue4 cells were grown in the recommended medium (Dulbecco's Modified Eagle's Medium (DMEM) with 4.5g/l glucose, 10% FCS, 200mM L-glutamine, 20,000U/l penicillin, 20mg/l streptomycin, 100mg/ml normocin and 1x HEK-Blue selection mix) to 80% confluency. Cells were detached by trypsin-EDTA, washed and resuspended in HEK-Blue detection medium at a concentration of 1-1.25x10^5 cells/ml. An LPS standard curve was constructed using *E. coli* K12 LPS from 0.1ng/ml to 100µg/ml. 20µl of each standard or sample was transferred to a 96-well plate (Sarstedt, Newton NC) in triplicate. 200µl of cell suspension in detection medium was added to each well with fresh pipette tips for each well to avoid cross-contamination and incubated at 37ºC in 5% CO_2 for 18 hours. Plates were inspected for blue colour change in the positive control wells and none in the negative controls. The presence of LPS in samples was detected by a blue colour change in the media, and quantified against the standard curve by measuring absorbance at 620nm (**Figure 2-2**).

This system was able to detect LPS concentrations above 0.1ng/ml. However, the response saturated at 10ng/ml giving a two-$\log_{10}$ dynamic range which was not useful for LPS quantification. An additional limitation of this system is its inapplicability to biological samples such as serum because the ALP activity in the sample induces a colour change in the substrate

even in the absence of LPS. Various combinations of heat-inactivation and dilution were investigated but none allowed useful measurement of serum LPS.

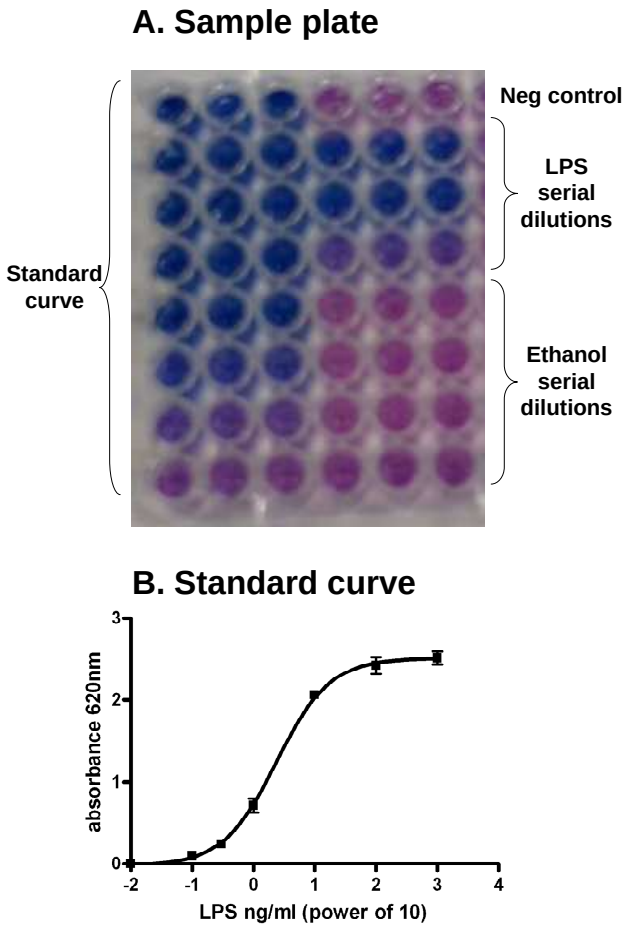


Figure 2-2 LPS detection by HEK-Blue4 cells

The standard curve illustrates the narrow dynamic range of this system which limits its use for LPS quantification.

2.3 Ethanol

2.3.1 Ethanol concentrations

The blood alcohol concentration at the legal limit for driving in the UK is 80mg/dl (17.3mM, 0.102%). Concentrations over 400mg/dl can cause loss of consciousness and death from respiratory suppression or cardiac arrhythmia in ethanol-naïve drinkers. However, chronic heavy drinkers can achieve concentrations of over 500mg/dl (108mM, 0.64%) with only moderate impairment of psychomotor function (Jones 1999). Concentrations in portal vein and liver tissue are likely to be higher but fluctuate with intake. 400mg/dl (86mM, 0.5%) was used as an estimate of median liver concentration in very heavy drinkers.

2.3.2 Ethanol measurement

The ethanol concentration in samples and media was measured by its ability to reduce potassium dichromate $K_2Cr_20_7$ (Jetter 1950). In acidified dichromate solution the ethanol is oxidised to acetate and water with reduction of the chromium(VI) to chromium(III) which produces a colour change from yellow to greenish blue.

Ethanol was measured using the Quantichrom Ethanol Assay Kit (BioAssay Systems, Hayward CA) according to manufacturer's instructions. Samples were deproteinated by adding 125µl of sample to 250µl 10% trichloroacetic acid and centrifuging at 15000g for 5 minutes at room temperature to pellet proteinaceous debris. 100µl of supernatant was transferred to a 96-well plate in triplicate and 100µl acidified potassium dichromate solution added and incubated for 8-30 minutes at room temperature until a colour change was observed. The reaction was stopped by addition of sodium hydroxide-containing stop solution and the blue colour measured spectrophotometrically at 595nm in a MRX II plate reader (Dynex Technologies, Chantilly VA). Concentrations were calculated from a standard curve in the range 0-2% ethanol (**Figure 2-3A**). The suitability of the assay for cell cultures was verified using samples spiked with a known concentration of ethanol.

2.3.3 Ethanol culture conditions

The evaporation of ethanol from solution at 37℃ is significant and could lead to loss of ethanol from the culture media during a prolonged incubation. The decay of ethanol concentration from 0.5% in media at 37℃ was determined by serial meas urements over 72h, but had fallen below the detection limit of the assay of 0.1% by 24h. Sealing culture vessels to minimise evaporation is not an option in this situation as CO2 exclusion over 7 days would significantly limit the buffering capacity of the media. One solution would be replacement of fresh 0.5% ethanol containing media every 24h, and this has the advantage of mimicking the diurnal variation in most human ethanol intake. However, for suspension cells this would involve daily manipulation and maybe even centrifugation of cultures with attendant risks of bacterial inoculation and cell activation. An alternative method is the use of an ethanol vapour incubator as pioneered by the Kolls group (Zhang, Bagby et al. 2001). In this system the required concentration of ethanol is added to the culture media and twice this concentration is added to the water in the humidification tray of the incubator. Ethanol evaporates from the tray and fills the incubator with ethanol vapour which equilibrates with the dissolved ethanol in solution so that the vapour pressure of ethanol in the incubator maintains the ethanol concentration in culture media at the required level. This system was optimised for use in our specific incubator. Sterile 1% ethanol with 160µl/l water bath treatment (Sigma) was added to the humidification tray with variation in volume and replacement interval. It was found that 500ml 1% ethanol replaced every 48h and any time the incubator door was opened maintained cell culture ethanol concentration close to 0.5% (**Figure 2-3B**).

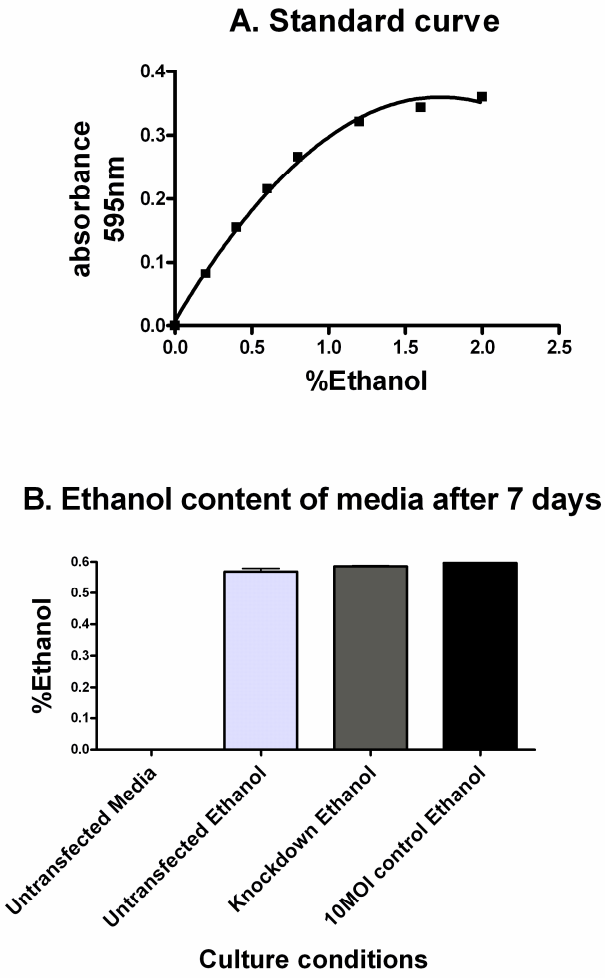


Figure 2-3 Potassium dichromate ethanol assay

An example standard curve is shown in (A). An example of regular monitoring of ethanol concentration in media (from ACSS knockdown experiments in section 5.2.10.2) is illustrated in (B). 0.5% ethanol = 86mM.

2.4 LPS stimulation

LPS stimulation was achieved with purified *E. coli O111:B4* LPS (Invivogen) to achieve a predominant TLR4 stimulation. LPS was stored at 4℃ at a concentration of 10µg/ml in endotoxin-tested glass or polystyrene vessels to minimise loss of LPS due to adhesion to the vessel wall. Stocks were vortexed at full speed for 5 minutes immediately prior to use to release adherent LPS into solution. Dose-response curves for THP-1 and MonoMac6 cells revealed a stimulating concentration of 10ng/ml produced an IL-6 response in the middle of the dynamic range of cytokine expression so this concentration was used for subsequent experiments (**Figure 2-4**). A single batch of LPS was used for all experiments.

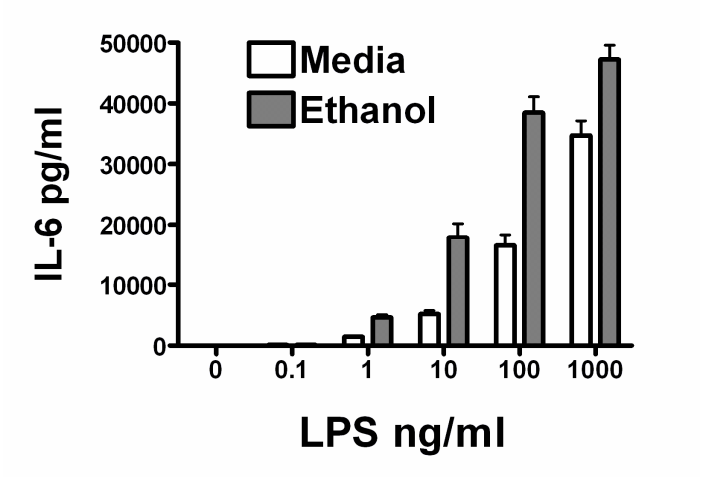


Figure 2-4 IL-6 dose response of MonoMac6 cells to LPS in normal media and 86mM ethanol

2.5 Protein detection by Western blotting

Western immunoblotting was used to identify the presence and estimate relative concentrations of intracellular proteins in samples.

2.5.1 Preparation of cell lysates

Lysis was achieved using proprietary extraction reagents M-PER for whole cell lysis and NE-PER for separate nuclear and cytoplasmic fractions (both Pierce Biotechnology, Rockford IL). To minimise protein loss from protease release and activation, all stages were performed at 4℃ or on ice using chilled reagents and vessels. Protease inhibitors (Roche Complete Mini tablets) were added to M-PER, cytoplasmic extraction reagent (CER) I and nuclear extraction reagent (NER). Cells were harvested and centrifuged at 400g for 10 minutes and washed twice in ice-cool PBS to remove FCS that would interfere with later protein determination. Supernatants were discarded and pellets resuspended in 1ml PBS and transferred to 1.5ml centrifuge tubes.

For whole cell lysates, cells were pelleted by centrifugation at 2500g for 10 minutes and the supernatant discarded. The pellet was resuspended in 1ml M-PER and mixed by pipetting. After gentle shaking for 10 minutes debris was pelleted by centrifugation at 15000g for 15 minutes and the supernatant transferred to a new cooled tube.

For separate nuclear and cytoplasmic fractions cells were pelleted at 400g for 10 minutes and the supernatant aspirated to near-dryness. The pellet was resuspended in 100µl CER I and vortexed at maximum speed for 15 seconds then incubated on ice for 10 minutes. 5.5µl CER II was added and vortexed for 5 seconds, incubated for 1 minute and then vortexed for 5 seconds again before pelleting nuclei and debris by centrifugation at 15000g for 5 minutes. The

supernatant (cytoplasmic extract) was transferred to a new cooled tube and the nuclear pellet resuspended in 50µl NER and vortexed for 15 seconds every 10 minutes for 40 minutes. The sample was centrifuged at 15000g for 10 minutes and the supernatant (nuclear extract) transferred to a new cooled tube. Reagent volume were varied depending on pellet size and desired protein concentration but the ratio of CER I:CER II:NER of 200:11:100 was maintained.

2.5.2 Determination of protein concentration

Total protein concentration in each sample was determined by the Bradford assay (Bradford 1976) which utilises a colour change in Coomassie reagent when its blue anionic form is stabilised by binding to amino groups in the protein. 1µl of each lysate was added to 250 µl of reagent in triplicate in a 96-well plate with gentle agitation for 10 minutes. Absorbance was measured at 595nm in a plate reader and converted to an equivalent protein concentration from a bovine albumin standard curve (**Figure 2-5**).

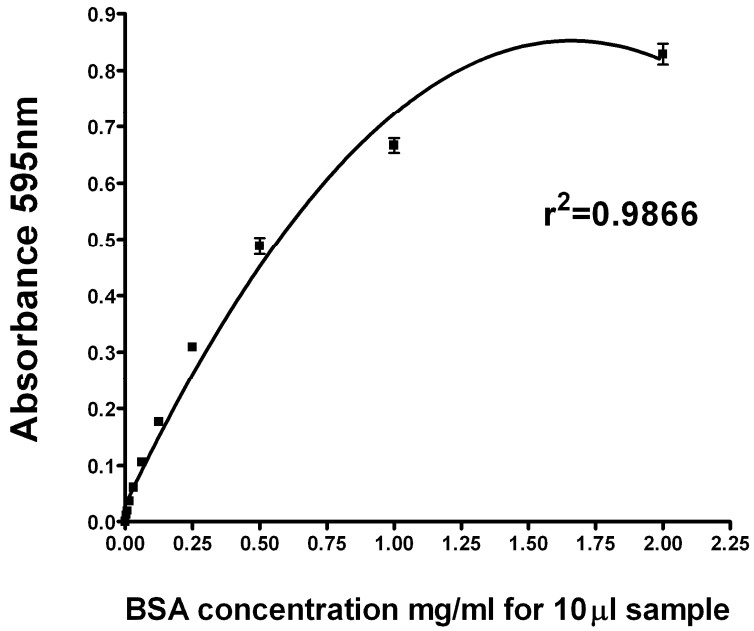


Figure 2-5 Sample standard curve for protein determination by Coomassie reagent (Bradford assay)

2.5.3 Sample preparation

Samples were equalised for total protein concentration and mixed with sample buffer (0.125M Tris pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.001% bromophenol blue, 10% β-mercaptoethanol) to give a final equalised total protein concentration of 2-5mg/ml. Samples were heated to 95℃ on a hot block (Techne, Stone UK) for 5 minutes to ensure protein

denaturation and then cooled and briefly centrifuged before use or storage at -20℃ for up to a month or -80℃ for longer.

2.5.4 Polyacrylamide gel electrophoresis (PAGE)

Proteins were separated by electrophoresis in denaturing (SDS-containing) polyacrylamide gels. The percentage of acrylamide was varied depending on the molecular weights of the proteins of interest but a typical method to make four 0.75mm mini gels (Hoefer, Holliston MA) would consist of 12.5% running gel (7.8ml ddiH_2O, 10.4ml acrylamide-bisacrylamide (Sigma), 6.3ml buffer 'B' (1.5M TrisHCl pH 8.8), 250µl SDS 10%, 250µl ammonium persulphate (APS) 10%, 12.5µl N,N,N,N-tetramethylethylenediamine (TEMED)) and 4% stacking gel (8.9ml ddiH_2O, 2.0ml acrylamide-bisacrylamide, 3.8ml buffer 'D' (0.5M TrisHCl pH 6.8), 150µl SDS 10%, 150µl APS 10%, 7.5µl TEMED). Electrophoresis buffer was made at 10x concentration (30.3g TrisHCl, 144.2g glycine, 10g SDS made up to 1000ml with ddiH_2O) and diluted for use. Samples were introduced to each lane of the gel with 5µl protein molecular weight standards (Fermentas, Burlington, Canada) in the first lane. Electrophoresis took place at 35mA per gel, 300V until sufficient separation of standards was achieved (20-40 minutes).

2.5.5 Immunoblotting

Proteins were electroblotted from the PAGE gels on to methanol-soaked nitrocellulose membranes (Immobilon, Millipore, Billerica MA) in N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (2.213g CAPS, 100ml methanol, pH 11, made up to 1000ml with diH_2O) at 250mA, 300V for 2 hours or 50mA, 300V overnight in a water-cooled transfer tank (Hoefer). Non-specific protein binding was blocked by incubating the membrane in 5% fat-free milk (Marvel, Premier Foods, St Albans UK) made up in PBS with 0.05% Tween-20 (Sigma) (PBS-Tween) for 1 hour on a rocker (Stuart, Stone UK) at room temperature. Membranes were probed for proteins of interest by hybridisation with appropriate primary antibodies in 5% milk overnight at 4℃ or for 2 hours at room temperature. After three washes in PBS-Tween membranes were incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibodies in 3% milk for 45 minutes at room temperature. Hybridised protein-antibody bands were visualised using a chemiluminescent HRP substrate (SuperSignal West Pico (Thermo Scientific) or Immobilon Western Chemiluminescent Substrate (Millipore) where increased sensitivity was required) and autoradiography. The use of a second antibody probe for a constitutively expressed protein such as β-actin can control for unequal loading of samples.

2.5.6 Analysis of blots

Semiquantitative analysis of relative protein concentration in autoradiographs was performed by band densitometry using Scion Image software (Scion Corp, Frederick MD).

2.5.7 Copper staining of membranes

Membranes were stained to confirm equal loading and even transfer with copper phthalocyanine 3',4',4'',4'''-tetrasulfonic acid (Sigma) 0.05% in 12mM HCl and destained in 10mM HCl until bands were clearly resolved (**Figure 2-6**).

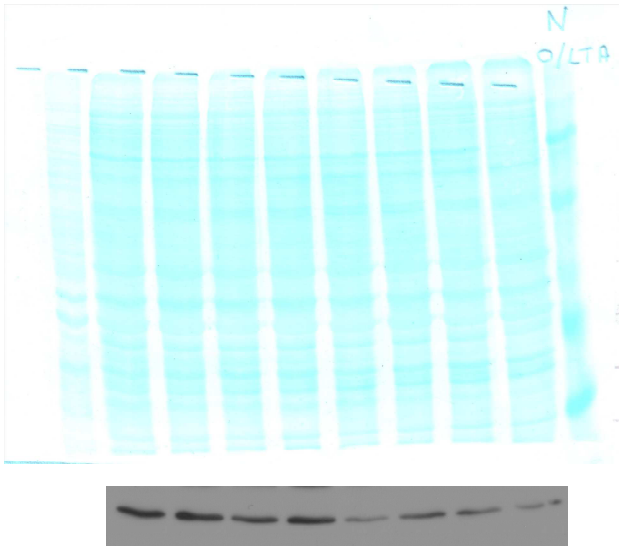


Figure 2-6 Copper stained Western immunoblot membrane

The copper staining confirms equal protein loading in this Western of nuclear lysates probed for NF-κBp65. Lanes 1-4 tolerised by poly-I:C, lanes 5-8 tolerised by LPS.

2.6 Protein determination by immunoassays

Quantitative measurements of secreted cytokines were initially made by enzyme-linked immunosorbent assay (ELISA) and later in multiplex by the MesoScale Discovery (MSD) platform.

2.6.1 Enzyme-linked immunosorbent assay

The sandwich ELISA allows specific measurement of protein concentrations over a 2-3 $\log_{10}$ dynamic range. A clear flat-bottomed well is coated with an excess of a 'capture' antibody and then non-specific protein binding to the well is blocked so that only epitopes specific to the capture antibody will bind. The sample is incubated in the well to allow binding of the protein of interest and then unbound sample is washed away. A 'detection' antibody, ideally one that binds a different epitope of the same protein, is incubated and binds the captured protein. Either the detection antibody or a further secondary antibody is coupled to an enzyme-based reporter system, such as a biotinylated antibody able to strongly bind streptavidin-conjugated HRP which

catalyses a colour change in a substrate at a rate proportional to the concentration of captured protein. When the reactions are simultaneously stopped, the wavelength-specific absorbance reflects the concentration of analyte in each sample. Serial dilution of protein standards subjected to the same incubations as the samples can be used to construct a standard curve and derive the concentration of the protein of interest in each sample.

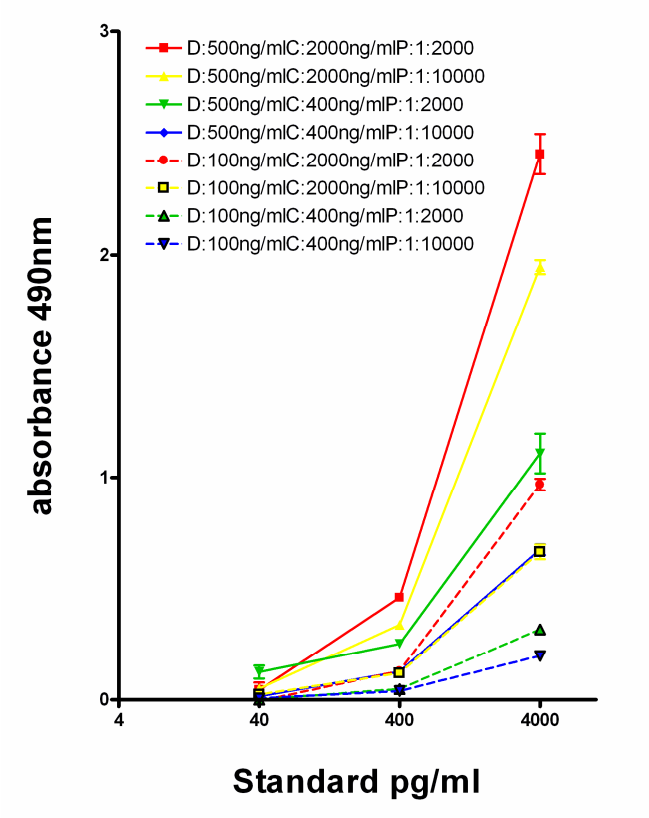


Figure 2-7 ELISA optimisation by checkerboard titration

Two dilutions of detection antibody (D), capture antibody (C) and peroxidise (P) were assayed in every possible combination against 4 10x dilutions of protein standard. The combination giving the best signal within the dynamic detection range of the plate reader while avoiding wasting reagents is selected for subsequent experiments.

Cytokines were assayed using antibody pairs optimised in a 'checkerboard' titration to find the combination of concentrations of enzyme, capture and detection antibodies that gives the best signal and widest dynamic range while avoiding wastage (**Figure 2-7**). For the assay, capture antibodies were incubated in wells of a 96-well plate in coating buffer (Na_2CO_3 1.59g, $NaHCO_3$ 2.53g, pH 9.4 made up to 1000ml with ddiH_2O) overnight at 4℃ before washing three times in PBS-Tween and blocking with 5% bovine serum albumin (BSA, Sigma) in PBS for 1-2 hours at room temperature. Cell culture supernatants and standards (seven doubling dilutions of a top standard of 1-2ng/ml plus a zero standard) were added in triplicate and incubated overnight at 4℃. Where possible, all samples from a single expe riment or timepoint were assayed on the

same plate against the same standard curve to minimise the effect of inter-plate variations. Plates were incubated with detection antibody, streptavidin-HRP (both for 2 hours at room temperature in 0.5% BSA in PBS-Tween) and substrate (0.012% hydrogen peroxide and 0.43mg/ml O-phenyldiamine (OPD) in 0.1M citrate buffer pH5), washing three times before each step. The substrate was observed for colour change and the reaction stopped with concentrated (2M) sulphuric acid before measuring absorbance at 490nm in a plate reader (Dynex). Absorbance was corrected for background and cytokine concentrations calculated from the standard curve using Prism 4 (GraphPad, LaJolla CA) and Excel (Microsoft Corporation, Redmond WA).

2.6.2 MesoScale Discovery Platform

MSD (MesoScale, Gaithersburg, MD) is proprietary system that adapts the ELISA principles to allow multiplex measurement of several cytokines from the same sample in the same well and to improve the sensitivity and dynamic range of the assay. In the MSD 96-well plate the capture antibody is bound to a carbon electrode in the floor of the well. The finely irregular surface and high absorptive capacity of carbon mean that a high concentration of antibody can be achieved, so increasing the range of detectable analyte concentrations. Wells can contain more than one antibody electrode, allowing multiplex cytokine measurement from each sample. Instead of a colour-changing enzymatic reaction, the detection antibody is coupled to an electrochemoluminescent label ('sulfo-tag') which, in the presence of the required buffer, emits light at a 620nm when an electric charge is applied to the carbon electrode. This light is detected by a charge-coupled device (CCD) camera within the MSD analyser and converted to an analyte concentration by use of a standard curve. The electrochemoluminescent system minimises background signals because the stimulus (electricity) is different from the output signal (light). Only the active component of the buffer is depleted by the electrochemoluminescent reaction; the sufo-tag is returned to its ground state allowing multiple cycles of electrical stimulation to increase the luminescence and hence the sensitivity of the assay (**Figure 2-8**).

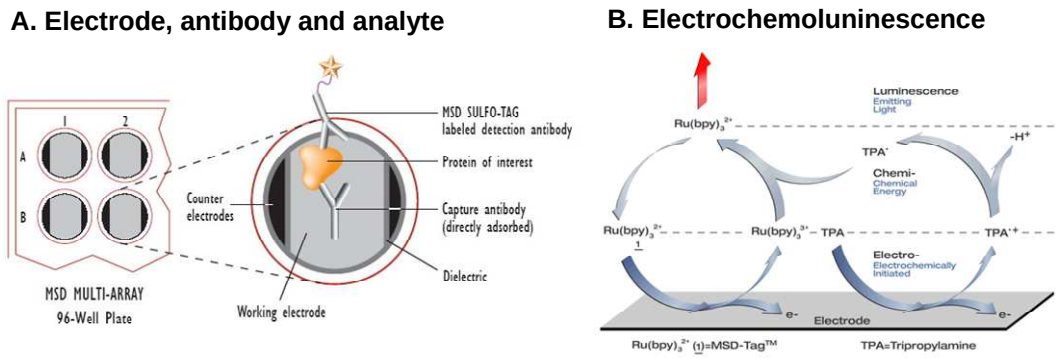


Figure 2-8 MesoScale Discovery Platform multiplex immunoassay

20µl of sample or standard (seven 10x dilutions of a top standard and a zero standard) was added to wells in duplicate and incubated at room temperature for 2 hours with vigorous shaking at 200rpm in a mechanical shaker (Stuart). Sulfo-tag-coupled detection antibody cocktail was added in excess without washing and incubated for a further 2 hours with shaking. The plate was then washed three times with PBS-Tween before the addition of read buffer and processing in the MSD Sector analyser. Cytokine concentrations in each well were calculated from the standard curve using MSD Workbench software (MesoScale).

2.7 Cell surface molecules by flow cytometry

2.7.1 General principles

Flow cytometry measures the physical characteristics of cells as they pass through the apparatus one at a time in a fluid stream. Fluorescence-activated cell sorting (FACS) is a refinement of the technique in which additional information is gathered from the cell's natural or chemically-induced fluorescence properties, allowing identification and quantification of different populations of cells.

Fluid is propelled through the analyser in two concentric streams. The core stream contains the cells moving in single file to intercept a stationary laser light while the faster-moving sheath stream maintains the cells in their precisely determined trajectory through the beam. The light is reflected and refracted by the cell and by its internal structures and this scattered light is detected and measured by the analyser. The degree of scatter in the plane of the beam is called forward scatter (FSC) and is proportional to the size of the cell while scatter at right angles to the plane of the beam is called side scatter (SSC) and is proportional to the cell's granularity.

Additional information can be gathered by labelling cells with dyes or fluorochrome-conjugated antibodies which allow detection of specific cell surface antigens. When a fluorochrome is stimulated by light of a particular wavelength it becomes excited and returns to its unexcited state by emission of light of lower energy and longer wavelength. The flow cytometer employs excitation lasers to activate the fluorochromes and the resulting fluorescence is reflected and filtered into photomultiplier tubes where it induces a current proportional to its intensity. The wavelength specificity of the photomultiplier is determined by its associated filters. The induced currents are amplified and digitised to give a readout of fluorescence intensity and cells can be grouped and counted accordingly and the median fluorescence intensity (MFI) of populations calculated.

Fluorochromes must be selected so that their emission and excitation wavelengths have sufficient spectral separation to allow the fluorescence to be detected. Furthermore, fluorochromes do not emit at a single wavelength but have a spectrum that will activate different

detectors to different extents. This means that when more than one fluorochrome is used on a cell there is a chance that their emission spectra will overlap and the signal at one fluorochrome's peak wavelength will be artificially increased by photons from the periphery of another fluorochrome's spectrum. To mitigate against these effects an 'electronic compensation' algorithm must be set up at the outset of an experiment. The signals from single-fluorochrome labelled cells are compared with those from identical double-labelled cells. The degree of spectral overlap is calculated at each fluorochrome's peak wavelength and the contribution from other fluorochrome's subtracted from the signal at that wavelength.

2.7.2 Staining and quantification of cell surface antigens

FACS was performed using an LSR II benchtop flow cytometer (Becton Dickinson, Rutherford NJ) with FACSDiva software. Cells were harvested and washed in PBS with 5% FCS and transferred to FACS tubes in which they were centrifuged at 400g for 5 minutes and the supernatant discarded. Optimum antibody concentrations had been determined in titration experiments which identified the minimum concentration of antibody necessary to saturate all binding sites. Cells were resuspended in 200µl of primary antibody prepared in PBS/FCS and incubated for 30 minutes at 4℃ in the dark. If the primary antibody was not fluorochrome-conjugated, the cells were washed twice and resuspended in 50µl of fluorochrome-conjugated secondary antibody, prepared and incubated as before. Cells were washed twice and resuspended in 250µl PBS/FCS for analysis. Non-specific binding of antibody was controlled for by incubating identical samples with isotype-matched non-specific primary antibodies. For each sample the population of normal-sized viable cells was identified and gated on a FSC/SSC plot and at least 10000 events recorded from this population. For double-stained cells electronic compensation was applied to minimise the effect of spectral overlap. MFI for each antigen of interest was recorded in duplicate for each experimental condition, corrected for non-specific binding by subtracting the MFI of the corresponding isotype control, and compared across samples.

To quantify the actual number of antigen binding sites per cell (eg number of TLR4 receptors per cell) the same concentration of antibody was incubated with Quantum Simply Cellular kits (Bangs Laboratories Inc, Fisher IN) which consist of five populations of beads 7-9µm in diameter, one blank and four labelled with increasing amounts of anti-immunoglobulin antibody specific for the primary antibody used to stain the cells. Each population has a known antibody binding capacity (ABC) and, once the beads had been incubated and analysed in an identical manner to the cells, a standard curve of MFI against ABC was plotted and used to calculate the ABC (ie number of antigen binding sites) of the cells (**Figure 2-9**).

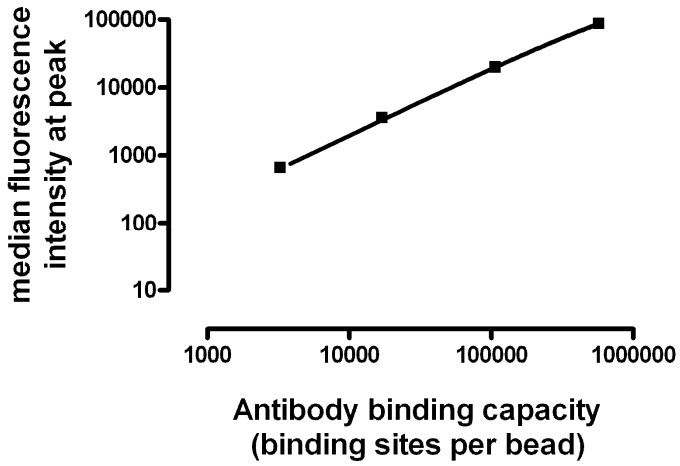


Figure 2-9 Standard curve for quantification of cell surface TLR4

Curve constructed using beads of known antibody binding capacity can be used to calculate the number of antibody binding sites and by inference number of surface TLR4 per cell.

2.8 Nucleic acid extraction

2.8.1 DNA extraction

DNA was isolated from cells by phenol-chloroform extraction. Cells are lysed by detergent or by a chaotropic agent such as guanidinium thiocyanate. The sample is mixed with a equal volume of water-saturated phenol at neutral pH (this will leave RNA and DNA in the aqueous phase; acid phenol will remove DNA from the aqueous phase or precipitate it on the interphase, and alkaline phenol will degrade RNA) and vortexed to mix thoroughly. The sample is centrifuged at 15000g to separate the aqueous and phenolic phases and the upper aqueous phase is aspirated to a new tube. The upper aqueous phase contains the nucleic acids and the proteins and lipids remain in the phenol. The phenol extraction can be repeated twice to improve purity. Traces of phenol in the sample will interfere with downstream applications such as PCR so these are removed with chloroform (or chloroform:isoamyl alcohol in a ratio 24:1 to prevent foaming). An equal volume of chloroform is vortexed and centrifuged with the sample and the upper aqueous layer aspirated and then the process repeated to ensure purity. DNA is then precipitated from the aqueous sample by the addition of 2-3 volumes of absolute ethanol. Cooling the sample can enhance precipitation. The sample is centrifuged at 15000g and the supernatant aspirated to dryness. The DNA pellet is air-dried and resuspended in ddiH_2O or TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) which maintains neutral pH and chelates divalent cations to inhibit any DNase activity for long term storage.

2.8.2 RNA extraction

All water used in RNA work was treated with diethyl procarbonate (DEPC) to inactivate RNases. A 0.01% solution of DEPC in ddiH_2O was incubated for 24 hours and then autoclaved to inactivate the DEPC. RNase contamination of reagents was minimised by careful technique, the use of RNase-free plasticware, the wearing of gloves and cleaning of the workspace and equipment with RNaseZAP (Applied Biosystems, Foster City CA).

Two methods for RNA extraction were compared. Trizol (Invitrogen) is a single-step extraction reagent for RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi 1987). Pelletted cells are resuspended in 1ml Trizol per 10^6 cells and incubated for 5 minutes to ensure complete dissociation of proteins from RNA. Phenolic and aqueous phases are then separated by addition of chloroform 0.2ml per millilitre of Trizol and centrifugation at 15000g. The upper aqueous phase is aspirated and can be subjected to an additional chloroform cleaning step if it remains turbid. RNA is precipitated from the aqueous phase by addition of isopropanol at half the original Trizol volume and centrifugation. The supernatant is aspirated and the RNA pellet washed in 70% ethanol in DEPC water before centrifugation, air-drying and resuspension in DEPC water. The second method involves RNA isolation by adsorption to silica membranes in centrifuge spin columns, the Absolutely RNA Microprep kit (Stratagene, LaJolla CA). Pelleted cells are resuspended in 100µl of a guanidium thiocyanate lysis buffer containing β-mercaptoethanol to ensure ribonuclease inactivation. An equal volume of 70% ethanol is added and vortexed for 5 seconds and the sample transferred to a spin column and forced through a silica-based membrane by centrifugation at maximum speed. The RNA binds to the silica and is retained. Contaminating proteins are eluted from the membrane by 3 centrifugation washes with salt-containing buffers and the RNA is then eluted into a clean tube by centrifugation with 30µl of elution buffer. The two methods were compared on identical samples and the RNA yield and purity were substantially higher from the spin column method which was also less time-consuming so this method was used for all future extractions.

RNA integrity was confirmed by electrophoresis in a 1% agarose gel in tris-acetate-EDTA (TAE) buffer (40mM tris-acetate, 1mM EDTA, pH8; 50x stock solution contains 242g tris-HCl, 57.1 ml glacial acetic acid, 100ml 0.5M EDTA, pH 8, made up to 1000ml with ddiH_2O) containing ethidium bromide to enable visualisation of RNA under ultraviolet light. 1µl samples were diluted to 8µl and mixed with 2µl loading buffer (70% TAE, 30% glycerol, 0.001% bromophenol blue) for electrophoresis at 110V, 400mA. Distinct bands of ribosomal RNA confirm integrity (**Figure 2-10**).

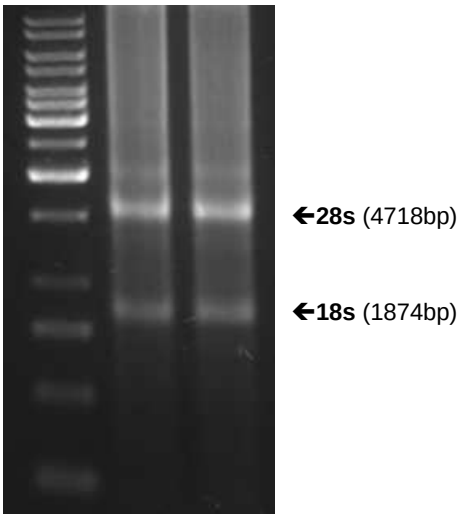


Figure 2-10 Representative RNA agarose gel

RNA integrity is assessed by electrophoresis in a 1% agarose gel. Sharp bands for 28s and 18s RNA confirm integrity.

2.8.3 Nucleic acid quantification

Nucleic acids in solution can be quantified spectrophotometrically by their absorbance at 260nm with an optical density (OD) of 1.0 at this wavelength equating to 50ng/µl of dsDNA or 40ng/µl of RNA. Proteins have a peak optical density at 280nm and an OD_{260}/OD_{280} ratio of 1.8-2.0 confirms that protein contamination is proportionately low and sample purity is high. Nucleic acid samples were quantified using a Nanodrop spectrophotometer (Thermo Scientific) with a 1µl sample, referenced to a blank sample of buffer without nucleic acid.

2.9 Polymerase chain reaction

The polymerase chain reaction (PCR) allows the amplification of a specific DNA sequence through repeated cycles of thermal denaturation, primer annealing and polymerisation using the unusually thermostable DNA polymerase derived from the marine bacterium *Thermus aquaticus* (*Taq*) whose ecological niche is on the border of hot undersea geological vents (Mullis and Faloona 1987).

For larger runs (8 samples or more) a master mix was made up with the following components (all Bioline, London UK) in the ratio 200µl $ddiH_2O$: 20µl 10x PCR buffer concentrate (100mM TRIS-HCl, pH8.3 at 25ºC, 500mM KCl, 15mM MgCl2, and 0.01% gelatin) : 2µl 100x mixed forward and reverse primers : 2µl 100x deoxynucleoside triphosphates (dNTP) mix : 1µl 200x Taq polymerase. 25µl of master mix was transferred to each reaction tube and 1µl of DNA sample (template) added to each with one tube left as a no-template control to ensure the system was free of additional DNA contamination. Samples were transferred to a thermal cycler

and underwent 35 cycles of denaturation at 94℃ for 60 seconds, annealing at a temperature appropriate for the primers for 30 seconds, and extension at 72℃ for 60 seconds per kilobase in the expected amplicon. For smaller runs a ready-made master mix (Platinum Super Mix HiFi, Invitrogen) was used with 1µl template and 1µl of each primer added to 45µl of mix and subjected to a 2 minute 94℃ activation step and then 35 cycles of denaturation at 94℃ for 30 seconds, annealing at temperature appropriate for the primers for 30 seconds, and extension at 72℃ for 60 seconds per kilobase. 1µl of each PCR product was run on a 2% agarose gel against a DNA ladder (Fermentas) to confirm successful amplification, amplicon size and the absence of amplification in the no-template control.

2.10 Quantitative reverse-transcriptase PCR

Expression of specific genes can be compared between samples by quantitative reverse-transcriptase PCR (qRT-PCR). This involves the conversion of mRNA to complementary cDNA by the action of an RNA-dependent DNA polymerase or reverse transcriptase enzyme. The cDNA then undergoes PCR with primers specific to the transcript of interest. Ideally, these primers will be exon-spanning so their complementary sequences appear only in the cDNA and not in any contaminating genomic DNA that may remain in the sample so only the cDNA is amplified. This PCR can be made semi-quantitative by stopping the reaction after a defined number of cycles and comparing the fluorescence intensity of the amplicons from each sample in an ethidium bromide agarose gel. Those samples with a higher number of transcripts (ie increased gene expression) will produce more PCR amplicons for a given number of cycles (before reagents are exhausted in the exponential phase of amplification) and this may be detected as increased fluorescence.

Improved quantification can be achieved if, instead of end-point analysis, kinetic monitoring of PCR product synthesis in real time is employed through the use of fluorescent markers of PCR amplicon formation which generate a signal proportional to the quantity of product at the end of each PCR cycle ('Real Time PCR', Applied Biosystems, Foster City CA). The fluorescent markers can be non-specific dsDNA dyes such as Synergy Brands (SYBR) Green which fluoresce when bound to double stranded DNA. However, these will give increased signal in the presence of non-specific PCR products and primer-dimers. A more amplicon-specific readout is given by the use of hydrolysis probes such as TaqMan probes (Applied Biosystems). These are designed to bind to specific consensus sequences between the PCR primers and feature a high energy fluorophore at their 5' end with a low energy quencher molecule at the 3' end. The fluorophore is stimulated by a laser of appropriate wavelength but while the probe is intact no photons are emitted and the excitation energy is instead transferred to the quencher by Förster (or fluorescence) resonance energy transfer (FRET). The *Taq* polymerase copies the DNA template from its primer in the 3'-5' direction and its 5' exonuclease activity hydrolyses the probe, separating the fluorophore from its quencher and allowing it to fluoresce in response to stimulation. Thus fluorescence increases in proportion to the accumulation of PCR products

(**Figure 2-11**). In the initial cycles of the reaction the increase in fluorescent signal between cycles is too small to detect. This signal is designated the baseline and is the same for all samples. A fixed threshold (T) can be set above this line and as PCR products increase exponentially the signal from each sample will double with each cycle, eventually crossing the T value. An important parameter for the quantification of template in a sample is the sample's threshold cycle (C_T) which is the cycle number at which the fluorescence signal crosses the fixed threshold T. When all signals double with each cycle, the samples with a greater initial amount of template will reach T sooner and have lower C_T values.

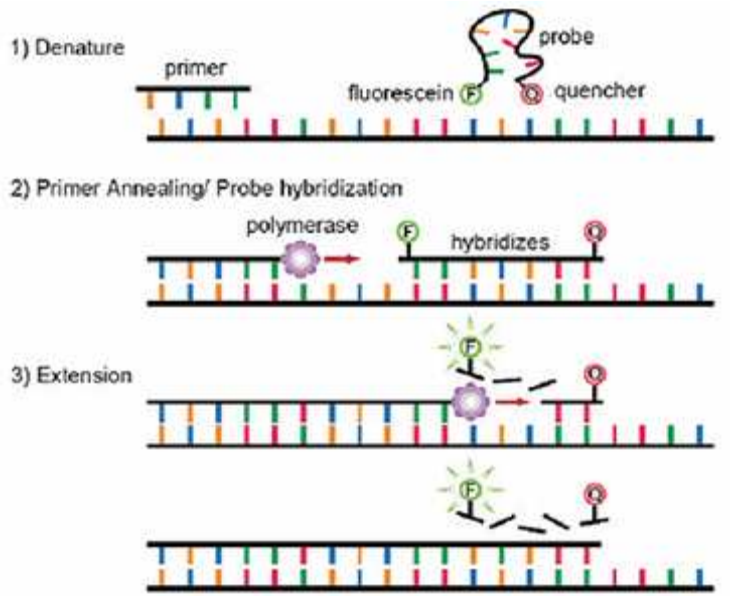


Figure 2-11 Fluorescence monitoring of PCR product synthesis by use of hydrolysis probes

The sequence-specific probe hybridises to the template DNA strand between the forward and reverse primers. When excited by laser the fluorophore (F) reporter transfers its energy to the quencher (Q) so no light is emitted. During chain extension the 5'-exonuclease activity of the *Taq* polymerase cleaves the probe and the reporter is separated from the quencher, allowing it to fluoresce in response to stimulation.

C_T values will be very sensitive to small differences in the total cDNA concentration in each sample, so the C_T values for the transcript of interest are normalised to the C_T values of a transcript that should not vary between samples, a 'housekeeping gene' that is constitutively expressed at the same level in cells and is not influenced by the experimental conditions. The difference between the C_T of the transcript of interest and the C_T of the reference transcript for each sample is its ΔC_T. ΔC_T can then be compared between samples to give $\Delta\Delta C_T$, the corrected difference (in cycles) between the samples' threshold crossing points. Since each cycle is equivalent to a doubling in the quantity of DNA, the relative quantity of DNA in one sample compared to another will be given by $2^{\Delta\Delta CT}$. If all samples are compared to a reference

(unstimulated) sample then this sample will have a $\Delta\Delta C_T$ of 0 and hence a relative DNA quantity of 1, and all other samples will produce values of transcript abundance relative to that sample (i.e. 3 times as many transcripts should give a value of 3).

2.10.1 cDNA preparation

Total RNA was extracted from samples as described above. cDNA was prepared using AffinityScript multiple temperature reverse transcriptase (Stratagene). Up to 5µg of total RNA was mixed with 3µl of random hexamer primers (0.1µg/µl) and made up to 15.7µl with DEPC water, incubated at 65℃ and cooled to room tempera ture to allow primers to anneal to RNA. This was followed by addition of 2µl 10x RT buffer, 0.8µl dNTP mix (25mM each dNTP), 0.5µl ribonuclease inhibitor (40U/µl) and 1µl AffinityScript RT. The reaction was incubated at 25℃ for 10 minutes to allow primer extension and then at 42℃ for 60 minutes for full cDNA synthesis before inactivating the RT at 70℃ for 15 minutes.

cDNA synthesis was verified by PCR for the constitutively expressed transcript encoding pyruvate dehydrogenase complex E2 inner lipoyl domain (PDCE2-ILD) and electrophoresis in agarose gel. cDNA was stored at -20℃ for up to 4 w eeks and -80℃ longer term until use.

2.10.2 Optimisation of Real Time PCR

The $\Delta\Delta C_T$ method for quantification of transcripts relies on the fact that each PCR product is being amplified with equal reaction efficiency, and that none is proceeding faster or slower than the others so that initial transcript abundance is the sole determinant of C_T. This was validated by generating dilution curves for each housekeeping gene and transcript of interest. 18s ribosomal RNA was selected as the endogenous control housekeeping gene in all experiments because expression of the more commonly used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been shown to be reduced by ethanol(de la Monte, Ganju et al. 1999). 18s has previously been validated as an endogenous control in ethanol-exposed cells(Li, Zhang et al. 2001). Serial 10x dilutions of cDNA were made and 2µl of each transferred to a Micro-amp optical 96-well reaction plate (Applied Biosystems) over ice in triplicate for each transcript of interest. Master mixes for each transcript of interest were made up so as to minimise the variation in primer, probe and polymerase concentrations between wells. Each 25µl reaction received 12.5µl 2x TaqMan universal PCR master mix (Roche/Applied Biosystems), 1.25µl primer/probe mix and 9.25µl DEPC water. Plates were sealed, briefly centrifuged to collect droplets, and transferred to an ABI Prism 7000 thermal cycler and sequence detection system (Applied Biosystems) for Real Time analysis. Polymerase was activated by incubation at 95℃ for 10 minutes and this was followed by 50 cycles of denaturation at 95℃ for 15 seconds and annealing/extension at 60℃ for 60 seconds.

C_T was plotted against relative cDNA concentration (log_{10} scale) for each transcript of interest and r^2 and the slope of each graph was calculated (**Figure 2-12**). A slope of -3.33 indicates optimum PCR efficiency. It was confirmed that the lines were parallel, meaning that reactions were of equal efficiency and the $\Delta\Delta C_T$ method was valid.

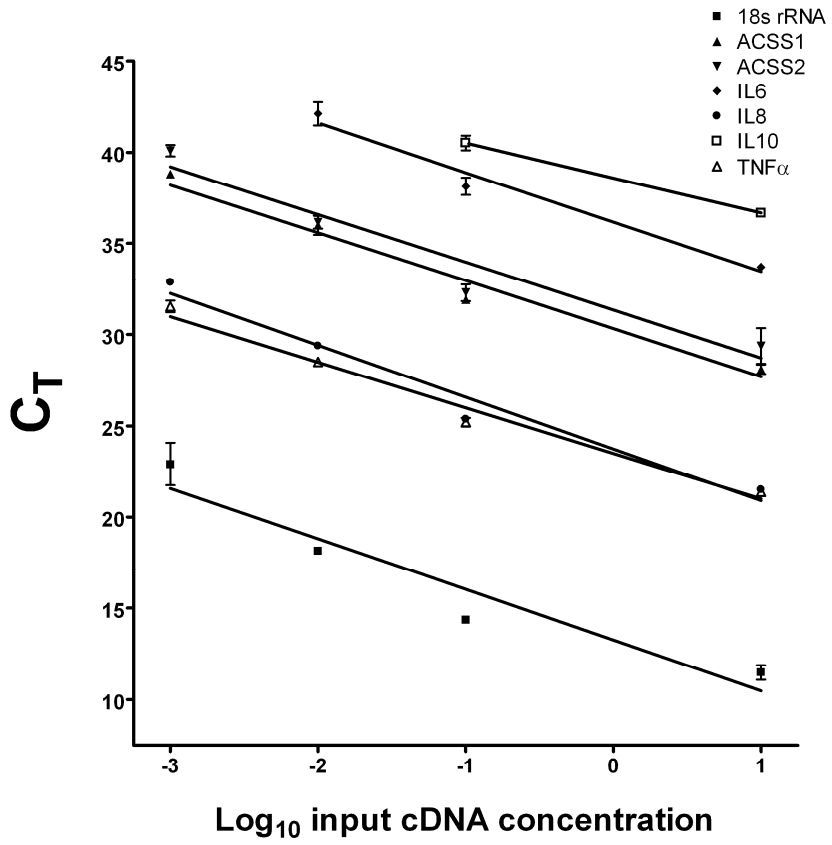


Figure 2-12 Validation plot for qRT-PCR primers used in this study

Although slopes are significantly greater than -3.33 (suggesting some inhibition of PCR at higher template concentrations), all slopes except that of IL-10 do not show significant difference, confirming that the $\Delta\Delta C_T$ method for calculation of relative transcript abundance is valid for those targets.

2.10.3 Analysis of Real Time PCR

All primers and hydrolysis probes for cytokine and enzyme work were purchased from Applied Biosystems and are summarised in Appendix 2. cDNA was transferred to an optical 96-well plate in triplicate for each transcript of interest and master mixes were added as above to a total reaction volume of 25µl. Plates were sealed, centrifuged and incubated as above. Real Time data was captured and analysed using Prism 7000 software (Applied Biosystems). The baseline signal was defined and threshold set above baseline so as to intersect with the exponential phase of each reaction plot. C_T values were calculated and imported to Excel (Microsoft

Corporation) for calculation of relative transcript abundance which was displayed using Prism 4 (Graph Pad). PCR products were resolved by electrophoresis in 4% agarose gel and visualised by ethidium bromide fluorescence to confirm amplification.

2.10.4 Statistical considerations

Statistical analysis must be performed on the $\Delta\Delta C_T$ values rather than the values of relative transcript abundance because the exponential element will distort the variance. $\Delta\Delta C_T$ is ΔC_T minus an arbitrary number so its standard deviation (SD) will not change. The standard deviation of ΔC_T will be the square root of the sum of the variances of the C_T of the transcript of interest and the C_T of the housekeeping gene transcript. The means and standard deviations of the ΔC_T values can then be used to test for statistical significance.

Error bars for graphs of relative transcript abundance will be asymmetrical because of the exponential component and the upper and lower bars must be calculated separately. The upper bar will go to $2^{mean\Delta\Delta CT+SD}$ and the lower bar will go to $2^{mean\Delta\Delta CT-SD}$.

3 Adapter molecule polymorphisms in ALD and NASH

3.1 Introduction

Both alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are responsible for a significant global health impact and evidence to date suggests that they have similar pathogenic mechanisms. In each there is a probable contribution to hepatocyte injury from reactive oxygen species, endoplasmic reticulum stress and pro-inflammatory cytokine release and in each probability of severe liver injury increases in the presence of obesity and insulin resistance (Day 2006).

3.1.1 Role of Toll-like receptors in ALD and NASH

An aetiological role for gut-derived bacterial endotoxin signalling through Toll-like receptors (TLR) is well established in ALD and evidence for a similar pathogenic component in NAFLD is now accumulating. In ALD gut permeability is known to be increased (Keshavarzian, Holmes et al. 1999; Parlesak, Schäfer et al. 2000) and plasma lipopolysaccharide (LPS) is elevated in all stages of the disease, correlates with severity and outcome, and falls in recovery (Fukui, Brauner et al. 1991; Hanck, Rossol et al. 1998; Fujimoto, Uemura et al. 2000; Schafer, Parlesak et al. 2002; Fukui 2005). The LPS receptor TLR4 is upregulated by chronic ethanol treatment (Zuo, Gong et al. 2003), and mice deficient in TLR4 or its co-receptor CD14 have less liver injury after ethanol feeding than wild types (Uesugi, Froh et al. 2001; Yin, Bradford et al. 2001). In NAFLD increased gut permeability has recently been demonstrated and increased circulating LPS has been detected (Miele, Valenza et al. 2009). Patients with jejunoileal bypass surgery have a high incidence of advanced liver disease due to non-alcoholic steatohepatitis (NASH) and this can be prevented by treatment of blind-loop bacterial overgrowth with antibiotics (Drenick, Fisler et al. 1982). The enteric bacterial load is increased in both NASH and type II diabetes patients (Wigg, Roberts-Thomson et al. 2001). In rodent models, TLR4 deficiency protects against liver injury in the methionine-choline deficient diet model of NASH (Rivera, Adegboyega et al. 2007) while the ob/ob mouse model displays hepatic steatosis but does not develop steatohepatitis until treated with LPS (Yang, Lin et al. 1997).

3.1.2 Genetic susceptibility to ALD and NASH

The risk factors for ALD and NAFLD (heavy drinking, metabolic syndrome) are well-established and extremely common yet both diseases display a relatively low prevalence of advanced disease relative to the prevalence of risk factors. Less than 10% of heavy drinkers will develop cirrhosis (Stewart and Day 2003), and even in those drinking over 200g of ethanol per day the incidence of cirrhosis is only 20% at 13 years and 50% at 20 years (Lelbach 1975). A similar pattern is seen in NAFLD where even in type II diabetic patients with two additional risk factors

the prevalence of NASH does not rise above 20% (Wanless and Lentz 1990; Erbey, Silberman et al. 2000). The inter-individual differences in disease susceptibility are the focus of ongoing research with identification of susceptibility factors allowing counselling of at-risk individuals and helping to direct attention to components of the pathogenic mechanism that have potential as novel targets for therapy. Evidence that a proportion of the individual excess risk of ALD has a genetic basis comes from the clear ethnic variation in susceptibility (Caetano and Clark 1998; Stinson, Grant et al. 2001; Klatsky, Morton et al. 2006) and from twin-twin concordance studies in 15924 male twin pairs aged 51-61 which showed that cirrhosis had a genetic risk over and above the genetic risk for alcoholism and suggested that approximately 50% of the variance in susceptibility was due to additive genetic effects (Hrubec and Omenn 1981). Support for a genetic susceptibility to advanced NASH comes from evidence of family clustering (Struben, Hespenheide et al. 2000) and from ethnic variation in susceptibility (Caldwell, Harris et al. 2002; Browning, Kumar et al. 2004).

A number of candidate gene association studies have focussed on polymorphisms affecting LPS signalling through TLR4 (**Figure 1-3**). The ligated TLR4 activates two distinct intracellular signalling pathways. The principal and most rapid signalling is down the 'MyD88-dependent pathway' which is coupled to TLR4 *via* the adapter molecule MyD88 and a second adapter MAL (Yamamoto, Sato et al. 2002). The adapters recruit a cascade of intracellular kinases which results in translocation of the proinflammatory transcription factor NF-κBp65 to the nucleus where it activates transcription of genes for tumour necrosis factor α, interleukins, cyclo-oxygenase 2 and other pro-inflammatory mediators. This pathway is shared with TLR2. The 'MyD88-independent pathway' is a second, slower signalling cascade linked to TLR4 activation by distinct adapter molecules TRAM and TRIF. This cascade is shared with TLR3 which responds to double-stranded RNA within endosomes. Signalling down this pathway results in transcription of type 1 interferons and a slower, sustained activation of NF-κBp65 and its target genes (Takeda and Akira 2005).

Polymorphisms of TLR4 and its co-receptor CD14 have been shown to be associated with altered inflammatory responses in humans. When studied in NAFLD the loss of function asp299gly mutation in TLR4 was found to protect against fibrosis and necroinflammation with odds ratios of 1.8 and 2.8 respectively (Bloomgarden 2005). A gain of function C-T single nucleotide polymorphism (SNP) at position -159 in the CD14 gene increases both soluble and membrane-bound CD14 and was found to be associated with increased histological severity of NAFLD with odds ratios of 2.4 for fibrosis and 2.1 for necroinflammation (Day 2002; Brun, Castagliuolo et al. 2006). Loss of function SNPs in the cytoplasmic endotoxin receptor NOD2 which are associated with Crohn's disease have also been found to be more common in advanced NAFLD with an odds ratio of 2.3 and it has been suggested that the mechanism is *via* increased small intestinal bacterial overgrowth (Bloomgarden 2005). Studies have not revealed any significant association between TLR4 and NOD2 SNPs and advanced ALD and of three studies into the CD14 SNP in ALD only one found a positive association and this was limited by

containing only 48 cases making a type 1 error possible (Leathart, Day et al. 2001; de Alwis and Day 2007).

3.1.3 A functional polymorphism of the TLR adapter MAL

A functional SNP has been identified in *TIRAP* (MIM 606252), the gene encoding MAL, the adapter molecule necessary for signalling down the MyD88-dependent pathway from TLR4. This C-T SNP (rs8177374) encodes a serine to leucine change at position 180 (S180L) which impairs the ability of MAL to interact with the upstream adapter MyD88. A case-control study in 6106 individuals from the UK, Vietnam and Africa with invasive pneumococcal disease, malaria, tuberculosis or bacteraemia showed that heterozygous carriage of S180L associated independently with each of the diagnoses. Heterozygosity was protective against each diagnosis with homozygous mutants being of extremely low prevalence, particularly in developing world populations. The authors concluded that heterozygosity for the loss of function SNP was protective against excessively vigorous inflammatory responses which could be more harmful to the wild-type host than the invading microorganisms against which they were directed. By contrast homozygous mutants' responses were inadequate to clear infections and many succumbed in early life accounting for their low frequency (Khor, Chapman et al. 2007).

In light of the evidence for a key role for TLR4 signalling in the pathogenesis of NASH and ALD, a mutation that attenuates this signalling might be expected to reduce the severity of liver injury. It was hypothesised that the 'low activity' T allele would be:

1. less common in advanced NASH compared to simple steatosis

2. less common in advanced ALD compared to heavy drinking controls

3.2 Specific methods

3.2.1 Patients and Characteristics

Patients and controls were recruited in North East England, UK, over a 10-year period and DNA samples were banked. To maximise statistical power all banked samples were used in this study. NAFLD patients were recruited from a regional specialist clinic and numbered 264. All NAFLD diagnoses were confirmed by clinical assessment and liver biopsy and histological features were scored using a modified Brunt scoring system by a single experienced pathologist (Brunt, Janney et al. 1999). 105 of the patients had steatosis without any fibrosis and were compared to the 159 with fibrosis present on biopsy. ALD patients were recruited from Hepatology clinics and from in-patients in a regional liver centre and numbered 382. All had advanced disease with cirrhosis or acute alcoholic hepatitis. Diagnoses were made by clinical assessment and confirmed by liver biopsy in over 80% of cases; the patients who were not biopsied all had clinical evidence of advanced disease (ascites, jaundice, prolonged

prothrombin time, hypoalbuminaemia or encephalopathy) with clear history of alcohol excess and exclusion of other causes of cirrhosis on blood tests (HBsAg, HCVAb, autoantibodies, immunoglobulins, α_1-antitrypsin, caeruloplasmin, ferritin +/- HFE genotype). Biopsy features were scored using a semi-quantitative system by a single experienced pathologist (Section 8.4). Controls were recruited from patients attending a regional alcohol addiction service and had a history of prolonged heavy drinking (at least 80g ethanol/day for at least 10 years) without evidence of liver disease. Controls numbered 188 and were not subjected to liver biopsy but had persistently normal liver function tests (save for elevated γ-glutamyltransferase) on at least two separate occasions with no clinical evidence of liver disease. Heavy-drinking controls were used to avoid the confounding factors associated with the use of matched community controls, specifically identification of genes that predispose to alcoholism rather than liver disease and increased probability of type 2 error due to controls that possess the predisposing mutation but have not developed liver disease due to inadequate ethanol exposure (Stickel and Osterreicher 2006). Patients consented to storage and testing of their genetic material.

3.2.2 Automated genotyping

Samples were genotyped for presence of the S180L MAL SNP by competitive allele-specific PCR in a commercial facility (Kbiosciences, Hertfordshire, UK). The PCR technique involves two pairs of primers with one of each pair overlapping the SNP at its 3' end, one binding the C allele and one binding the T allele. The extreme sensitivity to 3' end mismatches of the *Taq* polymerase used for the PCR means that primers that have hybridised to their specific complementary allele will be preferentially extended while those that have hybridised with a mismatch will not. The allele-specific primers are constructed with 5' tail sequences specific to each primer. The reaction mix contains fluorescence resonance energy transfer (FRET) cassette oligonucleotides complementary to each primer tail. When these are intact in solution or bound to the tails their fluorophore is held in close proximity to a quencher which absorbs energy from an excited fluorophore and prevents emission of photons. However, once primer extension begins the endonuclease activity of the *Taq* cleaves the FRET cassette and the fluorophore is liberated from the quencher and can emit fluorescence in response to stimulation. The total fluorescence at each wavelength will depend on successful extension of the associated allele-specific primer and therefore on the presence of that allele in the DNA sample (Mokry and Cuppen 2008). Blind duplicates, plate-identifying blank wells and Hardy-Weinberg equilibrium tests were used as quality control tests.

3.2.3 Restriction fragment length polymorphism analysis

A validation sample of 27 was genotyped in-house by restriction fragment length polymorphism (RFLP) analysis. This relies on amplification of the SNP-containing sequence by PCR followed by digestion of the PCR products with a restriction enzyme that will cleave one allele but not the other. The digested products are separated by electrophoresis and examined for the presence

of smaller (cleaved) and/or larger (uncleaved) product, corresponding to the presence of each allele. In this case the SNP did not occur within a recognised restriction site so one was induced by site-directed mutagenesis. One primer contained a mid-sequence single base mismatch close to the SNP which did not significantly reduce primer hybridization but which, when propagated by PCR, produced a product with a sequence containing a restriction site in the presence of one allele but not in the presence of the other allele. Primers are described in **Table 3-1**. These primers induced a BstXI restriction site in the mutant allele. 1µl of each DNA sample was subjected to PCR using the above primers with *Taq* polymerase and buffer (Bioline, London UK)) and dNTP (Bioline) in a thermal cycler (Techne, Burlington NJ) for 35 cycles with an annealing temperature of 60ºC. Products were digested with BstXI (New England BioLabs, Ipswich MA) in the manufacturer's supplied buffer supplemented with 1% bovine serum albumin at 55ºC overnight. Digest products were separated by electrophoresis on 4% agarose (Sigma) gel and visualised by ethidium bromide (Sigma) fluorescence in a Gel Logic gel documentation system and images recorded with Kodak molecular imaging software (Carestream Health, New Haven CT).

Forward	CTCCAGGGGCCGAGGGCTGCACCATCCCC[C→A]TGCTG
Reverse	TACTGTAGCTGAATCCCGTTCC

Table 3-1 Primers for RFLP analysis of S180L SNP in *TIRAP* (MAL) gene

The C-to-A substitution in the forward primer induces a BstXI restriction site in the mutant T allele by site-directed mutagenesis.

3.2.4 Statistical analysis

Genotype frequencies were compared between groups and significance tested using a two-tailed Chi-squared test (Prism4, GraphPad Software Inc, LaJolla CA) with $p<0.05$ considered significant.

3.3 Results

3.3.1 Patient characteristics

Patient characteristics are summarised in **Table 3-2** and **Table 3-3**. The expected associations between advanced disease and age and features of the metabolic syndrome in NAFLD were observed.

	ALD	Heavy drinking controls
n	382	188
Male	273 (71%)	132 (70%)
Mean age	49	43
C:C genotype	264 (69%)	145 (77%)

Table 3-2 ALD patient and control characteristics

	No fibrosis	Any fibrosis
n	105	159
Male	75 (71%)	92 (60%)
Mean age	47	52
Diabetes mellitus	23 (22%)	77 (48%)
Mean BMI	33	35
ACE inhibitor / A2RB	11 (10%)	24 (15%)
Metformin	9 (9%)	19 (12%)
C:C genotype	65 (62%)	115 (72%)

Table 3-3 NAFLD patient and control characteristics

3.3.2 Validation of genotype data

The outsourced genotyping correctly identified blank wells and was fully consistent between blinded duplicates. Genotype frequencies did not differ significantly from Hardy-Weinberg equilibrium (Hardy 1908). In the alcohol group allele frequencies were C 0.854 T 0.146 and genotype frequencies were C:C 0.716 (Hardy-Weinberg prediction 0.729) C:T 0.272 (0.249) and T:T 0.011 (0.021). In the NAFLD group allele frequencies were C 0.841 T 0.159 and genotype frequencies were C:C 0.703 (Hardy-Weinberg prediction 0.707) C:T 0.276 (0.268) and T:T 0.021 (0.025). Sample BstXI digests of C:C, C:T and T:T genotypes are illustrated in **Figure 3-1**. The in-house validation is shown in **Figure 3-2** and with a single exception was consistent with the outsourced sequencing.

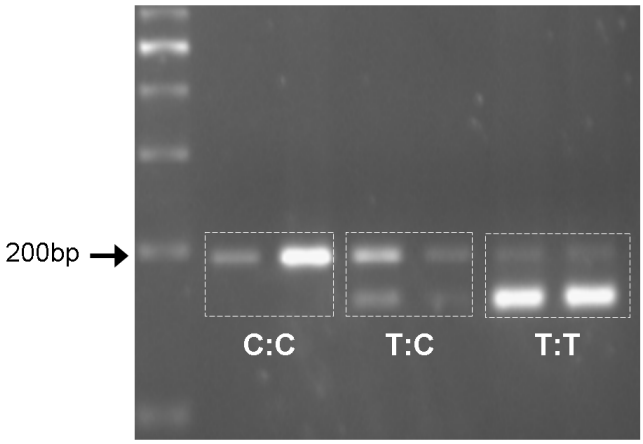


Figure 3-1 Sample BstXI digests of MAL genotypes

Two different samples of each genotype are shown. The restriction site in the mutant T allele PCR product results in a smaller fragment.

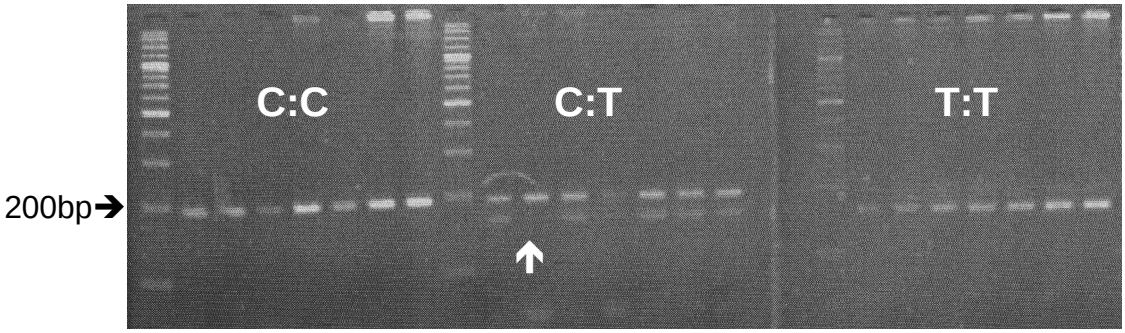


Figure 3-2 Validation of genotypes

With a single exception (arrowed) the in-house validation was consistent with the out-sourced genotyping.

3.3.3 Genotype analysis

The 'high signalling activity' C:C genotype was more common in NAFLD patients whose biopsies showed any degree of fibrosis than in those with no fibrosis (72% v 62%, p=0.038). The relative risk of fibrosis for those possessing the C:C genotype in this cohort was 1.22 (0.97-1.54) with an odds ratio of 1.61 (0.95-2.72) (**Figure 3-3**). There was no significant difference in genotype frequency with any other recorded histological or clinical marker.

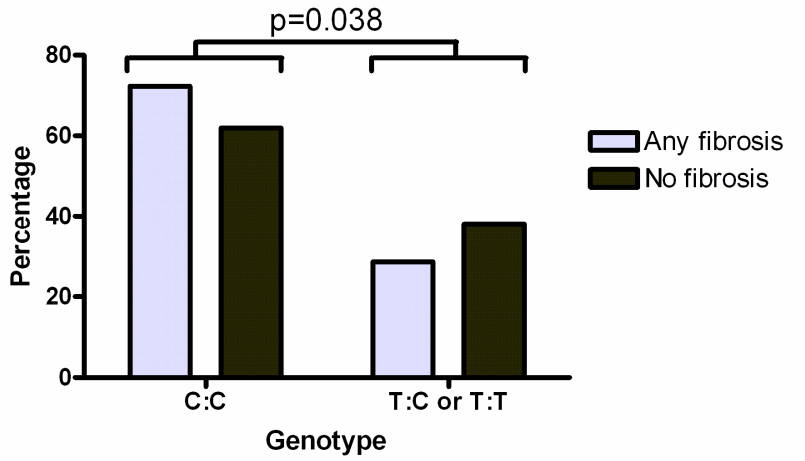


Figure 3-3 Distribution of fibrotic NASH by MAL genotype in NAFLD

In the ALD group there was evidence that the SNP was associated with reduced inflammatory responses with the C:C genotype more frequent in patients whose biopsies showed moderate to severe macrophage infiltration than in those in whom macrophage infiltration was mild or absent (77% v 62%, p=0.045) (**Figure 3-4**). The relative risk of moderate-severe macrophage infiltration for those possessing the C:C genotype was 1.74 (0.89-3.42) with an odds ratio of 2.03 (0.89-4.63). However, when genotype frequencies were compared between advanced ALD patients and heavy-drinking controls, there was no evidence of the 'low signalling activity' T allele providing protection against ALD. In fact, the attenuated-signalling C:T and T:T genotypes were slightly more common in ALD patients than in controls (31% v 23%, p=0.046) (**Figure 3-5**). The odds ratio for possession of the C:C genotype in advanced ALD compared to heavy drinking controls was 0.66 (0.44-0.99). There was no correlation between genotype and the histological diagnosis of alcoholic hepatitis (p=0.43).

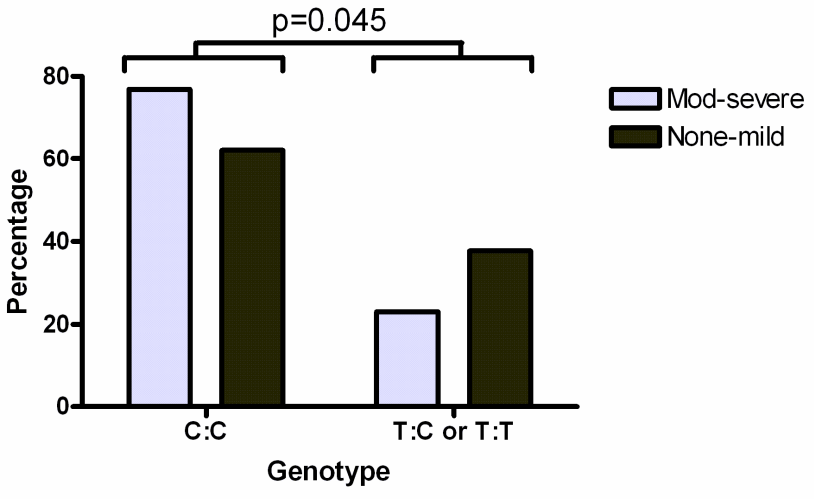


Figure 3-4 Distribution of macrophage infiltration by MAL genotype in ALD

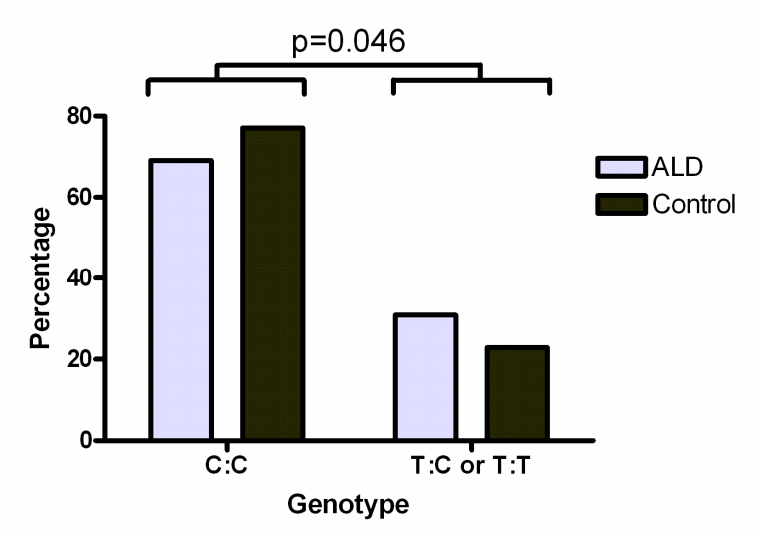


Figure 3-5 Distribution of ALD patients and controls by MAL genotype

3.4 Discussion

3.4.1 Divergent findings in ALD and NASH

Prior evidence strongly suggests a key role for endotoxin signalling through TLR4 in the pathogenesis of advanced NASH and ALD (Uesugi, Froh et al. 2001; Rivera, Adegboyega et al. 2007). The S180L SNP in the adapter molecule MAL has been shown to be functional at both the molecular and the population genetics level with the low signalling activity resulting from carriage of the T allele reducing damaging inflammatory responses to microbial components in a range of infectious diseases (Khor, Chapman et al. 2007). In the present study a small protective effect of the T allele in NAFLD was detected but there was also a paradoxical association between the low signalling activity T allele and the presence of advanced ALD in heavy drinkers. This occurred despite evidence that the high signalling activity C allele was associated with macrophage infiltration on biopsy in the ALD population.

3.4.2 Limitations of this study

There are potential explanations for these observations. Firstly, the relatively high p values raise the possibility of type 1 error and the associations between the SNP and advanced liver disease in our populations may have arisen by chance. Small sample size remains a problem in all candidate gene studies in ALD and NAFLD and large banks of patient DNA do not exist for more powerful studies. If these findings are replicated in a different cohort then the evidence for the importance of this SNP in ALD or NAFLD will be considerably strengthened. A further limitation of this approach is that it is unable to control for duration of exposure or total cumulative dose of alcohol or calories, or for body mass index. Similarly it cannot control for behavioural factors so any effects of the SNP on alcohol or total calorie intake cannot be

differentiated. It is also impossible to differentiate any effects of obesity in the ALD group and effects of drinking in the NAFLD group. The effect of patrient age on susceptibility to advanced disease cannot be separated out in this study, and may mask a stronger effect of a functional SNP in younger ALD or NAFLD patients. A larger study could stratify patients and controls by age, BMI and alcohol intake and examine for differential influence of the SNP across strata, but this is not possible with the present data set.

3.4.3 Implications of the findings

If S180L status does not significantly influence susceptibility to advanced NASH or ALD, does this cast doubt on the role of TLR signalling in these diseases? It may do, but it should be borne in mind that these diseases have complex pathogenic mechanisms in which TLR signalling is at most a component. It is possible that the presence of some TLR signalling, no matter how weak, is permissive for disease progression and the effect of variations in signalling strength are insignificant in the face of the severe cellular stress associated with alcohol metabolism or NASH. It is of note that the diseases surveyed in the initial establishment of the functionality of the SNP were ones that may rely more on TLR2 than TLR4 signalling (Khor, Chapman et al. 2007). TLR2 is entirely reliant on the MyD88 pathway for signalling whereas TLR4 also has the MyD88-independent pathway which will be unaffected by the SNP (Takeda and Akira 2005). Hence the impact of the SNP may be reduced in diseases in which TLR4 is the more important receptor.

Alternatively, if these observations describe a true differential effect of the MAL SNP in NAFLD and ALD, it may point to a divergence of pathogenic mechanisms with the MyD88-dependent pathway important in NASH and the MyD88-independent pathway important in ALD. If MyD88-independent signalling were enhanced by ethanol then individuals with the SNP who have reduced signalling down the rapid MyD88-dependent pathway will be relatively protected from NASH but will rely on the ethanol-modifiable MyD88-independent pathway for signalling from TLR4 and so be more susceptible to the inflammation-enhancing effects of ethanol and hence to advanced ALD.

3.4.4 The findings in context

Recent laboratory evidence has emerged to support this differential importance of the two TLR4 signalling pathways in NASH and ALD. The role of MyD88-dependent pathway signalling in the methionine-choline deficient diet rodent model of NASH has been confirmed by a study in which both TLR4 and MyD88 knockouts reduced liver injury, particularly when the knockout was restricted to bone-marrow derived cells (Szabo, Velayudham et al. 2008). However, in a similar study in which TLR4 and MyD88 knockout animals were fed the Lieber-DeCarli ethanol-containing diet, TLR4 deficiency protected against liver injury but MyD88 deficiency did not, suggesting that MyD88-dependent signalling was not necessary for the development of ethanol

liver injury (Hritz, Mandrekar et al. 2008). Other work has shown that the augmented proinflammatory cytokine release from macrophages in ethanol-fed rodents is associated with increased activity of interferon regulatory factor 3 and can be abolished by knockout of the MyD88-independent pathway adapter molecule TRIF (Zhao, Dong et al. 2008). An independent study confirmed that either TLR4 or IRF3 deficiency ameliorated ethanol liver injury in mice but MyD88 deficiency did not (Szabo, Hritz et al. 2008).

The effect of genetic variation in TLR signalling pathways observed here is consistent with this growing body of laboratory data about TLR signalling pathways in NASH and ALD. If confirmed in independent cohorts, these data support the role for gut-derived endotoxin in the pathogenesis of advanced NASH and ALD in humans, and add human genetic evidence to existing laboratory evidence for the differential importance of the MyD88-dependent and independent signalling pathways in these two diseases with implications for development of future therapies.

3.4.5 Genetic susceptibility reconsidered

Despite numerous candidate gene case-control studies in ALD, very few have demonstrated a significant specific genetic basis for the variation in susceptibility to advanced disease. This may be a consequence of unsuccessful identification of candidate genes or a result of relatively small sample sizes increasing the probability of type 2 error (Stickel and Osterreicher 2006). A hypothesis-free genome-wide association study is under way in Australia and may yet identify previously unexpected genetic determinants. However, it is equally possible that the genetic contribution to ALD susceptibility is not as great as previously thought. When the cohort of 15924 twin pairs aged of 51-61in which a genetic component to advanced ALD was originally identified was re-investigated 16 years later at the ages of 67-77 a significant independent genetic risk for ALD could no longer be detected (Reed, Page et al. 1996). Similarly, a contribution from a loss-of-function SNP in the manganese superoxide dismutase (SOD2) gene to susceptibility to advanced ALD was significant in a younger sample but the effect disappeared as the cohort aged and individuals carrying the SNP died while individuals without the SNP also developed advanced disease (Nahon, Sutton et al. 2005). One might contend that ethanol is a powerful environmental toxin and that, while genetic susceptibility factors might influence the development of cirrhosis at an earlier time or a lower cumulative dose in a minority of drinkers, for the majority cumulative intake, pattern of drinking and environmental factors will be the major determinants of susceptibility to advanced disease. To understand these determinants better requires a study of gene-environment interactions at the molecular level, for which attention must shift from genetic to epigenetic mechanisms.

4 Steroid sensitivity in acute alcoholic hepatitis

4.1 Introduction

4.1.1 Corticosteroids, inflammation and immunity in AAH

It is well established that AAH is characterised by a significant innate immune inflammatory response with high levels of pro-inflammatory cytokines, including IL-6 and TNF-α, observed in patients (Bird, Sheron et al. 1990; Sheron, Bird et al. 1991). There is also well documented antigen-specific, lymphocyte-mediated adaptive immune system activity in ALD livers (Stewart, Vidali et al. 2004), although the pathogenic significance of the adaptive immune response in ALD remains uncertain (section 1.3.4). The presence of this inflammatory element to AAH provides an obvious potential approach to therapy through anti-inflammatory drugs such as corticosteroids. Indeed, in meta-analysis corticosteroid therapy has been demonstrated to have a moderate effect on outcome in the group at highest risk of death, reducing one month mortality from 35% to 16% (Mathurin, Mendenhall et al. 2002). However, a sixth of patients in this group die despite treatment, and the applicability of corticosteroid therapy is limited by concerns about heightened risks of sepsis and gastrointestinal haemorrhage. Steroid responsiveness in AAH is indicated by an early change in the serum bilirubin level (ECBL) with those patients whose bilirubin has not fallen by the seventh day of treatment having a particularly high mortality and gaining no benefit from continuation of therapy (Mathurin, Abdelnour et al. 2003). ECBL has been incorporated into the Lille model to identify patients unlikely to benefit from continued corticosteroid therapy (Louvet, Naveau et al. 2007). Treatment outcomes could be improved by early identification of the 27% of patients who are unlikely to benefit from steroids or by strategies to improve steroid sensitivity in this group.

4.1.2 Steroid sensitivity in inflammatory disease

Steroid un-responsiveness is not unique to AAH. It has been well characterised in inflammatory skin diseases and ulcerative colitis where it is evident as a reduced maximum inhibitory effect of dexamethasone on *ex vivo* phytohaemagglutinin-stimulated lymphocyte proliferation (Hearing, Norman et al. 1999). Its prevalence is higher in chronic obstructive pulmonary disease (COPD) in which it has been shown to result from smoking-induced abnormalities in transcriptional regulation of pro-inflammatory genes. This occurred through a reduced capacity of the glucocorticoid receptor to recruit histone deacetylases (HDACs) to actively transcribed pro-inflammatory genes due to the inhibitory effect of smoking-induced oxidative stress (Barnes, Ito et al. 2004). The traditional bronchodilator theophylline was shown to counteract this effect, exerting an anti-inflammatory effect by improving HDAC recruitment to silence pro-inflammatory genes (Barnes 2005), even at 10^{-5}M, a concentration at which clinically useful bronchodilator effects do not occur.

4.1.3 Markers, causes and treatments for steroid insensitivity in AAH

A biological marker for corticosteroid insensitivity in AAH could have clinical value in identifying earlier those patients unlikely to respond to steroid therapy who may benefit from avoidance or earlier cessation of steroids or rapid consideration for trials of experimental therapies. Analysis of steroid responsiveness at the cellular level will help identify the cause of steroid insensitivity and whether it occurs as a consequence of ethanol exposure and/or inflammation or is an inherent characteristic of individuals who develop AAH, possibly contributing to its aetiology. Studying the effect of known modulators of steroid responsiveness will not only help identify potential future therapies but will also provide hints as to the molecular basis for enhanced inflammatory responses and steroid insensitivity in AAH. A beneficial effect from an HDAC recruiting molecule such as theophylline would add to the nascent body of evidence that histone acetylation and impaired deacetylase activity are together a key component of the pathogenesis of ALD.

This study set out to explore corticosteroid insensitivity in AAH using an *ex vivo* approach and to test the hypothesis that *in vitro* corticosteroid sensitivity can be improved by the actions of theophylline.

4.2 Specific Methods

4.2.1 Patients and controls

Patients with a primary diagnosis of acute alcoholic hepatitis admitted to Newcastle upon Tyne Hospitals NHS Foundation Trust between October 2006 and March 2008 were invited to participate in the study if they fulfilled the accepted criteria for corticosteroid therapy (Maddrey discriminant function >32, equating to a 35% 28-day mortality without treatment (Carithers, Herlong et al. 1989)). Patients were excluded if they had gastrointestinal bleeding, sepsis, recent surgery or inflammatory disease or were already on immuno-modulatory therapy. Alternative causes of liver dysfunction were excluded by duplex ultrasonography and blood testing for hepatitis A, B, C and E, ferritin and HFE genotype, caeruloplasmin, alpha-1-antitrypsin, immunoglobulins, thyroid stimulating hormone, antinuclear antibody, anti-smooth muscle antibody, antimitochondrial antibody, anti-liver/kidney microsomal antibody, anti-soluble liver antigen antibody and anti-neutrophil cytoplasmic antibody. Liver biopsy was performed only when there was thought to be a significant probability of it altering clinical management or outcome. In patients who were not biopsied the presence of coexisting cirrhosis was determined either from diagnostic imaging or from the presence of varices at endoscopy. Age- and sex-matched controls were recruited locally. Control exclusion criteria were alcohol intake greater than 40 units per week for men and 30 units per week for women, active infection or inflammatory disease, surgery, trauma or immunomodulatory therapy. Patient and control characteristics are summarised in **Table 4-1**. None of the study subjects were receiving

corticosteroid therapy at the time of their participation in the study. Patients were treated with oral Prednisolone 40mg daily for 28 days. Treatment was discontinued early in eight patients, four due to evidence of poor clinical response and four due to concern about sepsis or bleeding. Lymphocyte steroid sensitivity was assessed at recruitment and, for surviving patients who attended follow-up, at 4-weekly intervals for 6 months. The study protocol was prospectively approved by the Local Research Ethics Committee and informed consent was obtained from or on behalf of each participant.

	AAH Patients (n=12)	**Normal Controls (n=12)**
Male	10	10
Age (years)	47 (34-60)	51 (34-65)
Weekly ethanol (units)	117 (20-560)	19 (7-40)
Maddrey DF	61 (32-141)	
Biopsied	2	0
Biopsy evidence of cirrhosis	2	
Circumstantial evidence only	4	

Table 4-1 AAH patient and control characteristics.

Figures are mean (range). DF discriminant function

4.2.2 Lymphocyte steroid sensitivity assay

The lymphocyte steroid sensitivity (LSS) assay was adapted from that of Hearing *et al* (1999(i)). Peripheral blood mononuclear cells (PBMCs) are isolated and T lymphocytes are induced to proliferate by the action of phytohaemagglutinin (PHA), a plant lectin found in red kidney and cannellini beans which binds and cross-links the T cell receptor (Chilson and Kelly-Chilson 1989). PHA-induced proliferation is inhibited by serial increasing concentrations of dexamethasone and proliferation at each dexamethasone concentration measured by incorporation of radioactive tritiated (H^3) thymidine. Proliferation is plotted against dexamethasone concentration and a curve fitted from which I_{max}, the maximum percentage inhibition of proliferation (a measure of steroid efficacy), and $IC_{50,}$ the concentration of dexamethasone required to achieve 50% of maximal inhibition (a measure of steroid potency), can be calculated. I_{max} and IC_{50} can be compared between groups and correlated with clinical parameters.

PBMCs were isolated from 25ml of blood by density centrifugation (Lymphoprep, Axis-Shield, Norway, Leucosep tubes, Greiner Bio-One, Germany) and washed three times in phosphate-

buffered saline. $4x10^5$ cells were added to each of 24 wells of a 96-well tissue culture plate in a final volume of 200µl RPMI medium with 2mM L-glutamine and 10% fetal bovine serum (Lonza, UK). Seven triplicates were stimulated with PHA (Sigma) at a final concentration of 5µg/ml. Six of these triplicates were treated with dexamethasone (Sigma) in serial 1 in 10 dilutions to give final concentrations from 10^{-6} to 10^{-11}M. For the initial patient samples the reaction was duplicated in the presence of 10^{-5}M theophylline (Sigma) (**Figure 4-1**). Plates were incubated at 37ºC in 5% CO_2 for 72 hours. Proliferation was measured by incorporation of tritiated thymidine (Amersham Biosciences, UK). 5.29nmol (37kBq of activity) was added to each well and incubated for a further 16 hours. Well contents were aspirated on to filter mats (Wallac, Finland) by a cell harvester (Harvester 96 Mach II, Tomtec, Hamden CT) and radioactivity of the aspirate determined by β-counter (MicroBeta TriLux, Perkin Elmer, Waltham MA).

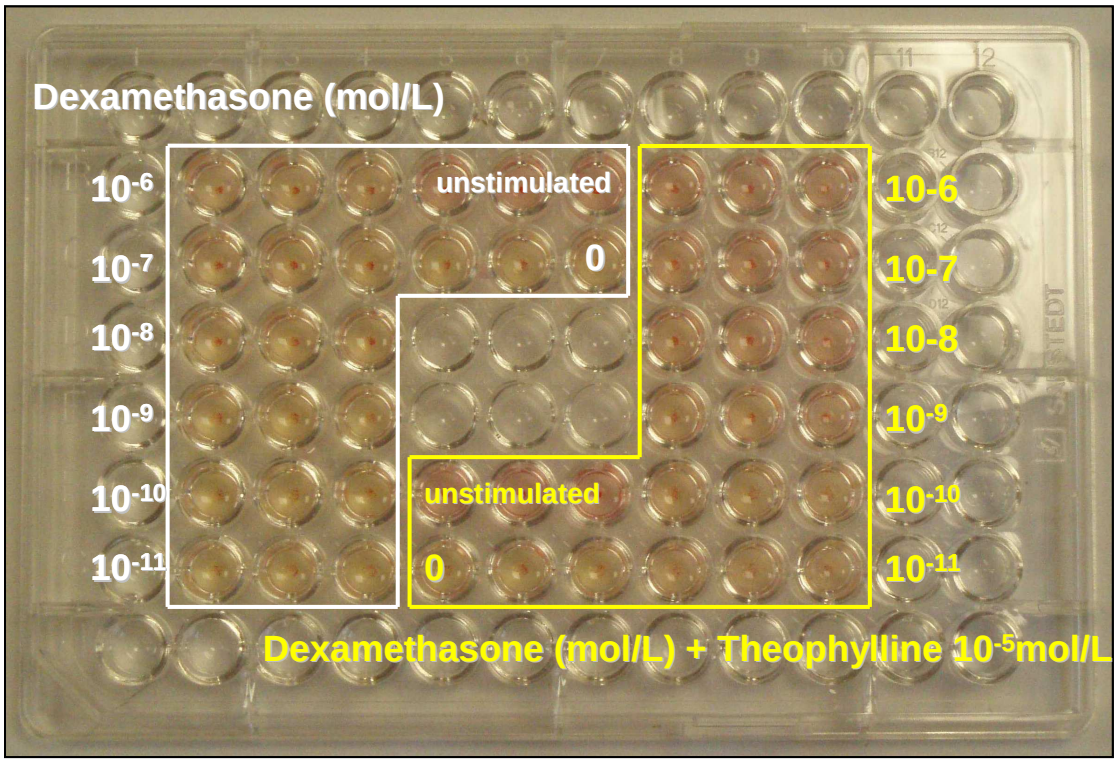


Figure 4-1 Culture plate for lymphocyte steroid sensitivity assay

4.2.3 Data interpretation

Incorporated ^{3}H-thymidine activity counts were corrected for background and normalised to uninhibited proliferation without dexamethasone. Proliferative response was plotted against dexamethasone concentration, a curve was fitted and I_{max} and IC_{50} were calculated (Prism 4, Graph Pad, LaJolla CA)(Hearing, Norman et al. 1999). Mean(±standard error) I_{max} was compared between groups using the Mann-Witney U test (unpaired observations) and Wilcoxon signed-rank test (paired observations) with $p<0.05$ considered to be significant.

4.3 Results

4.3.1 Steroid sensitivity is reduced in AAH

Representative control and patient lymphocyte steroid sensitivity curves are shown in **Figure 4-2.** Mean *ex-vivo* steroid sensitivity as determined by I_{max} was significantly lower in AAH patients than in controls (67(±4.5)% v 95(±2.3)%, p<0.0005; **Figure 4-3**) suggesting a significantly greater level of corticosteroid insensitivity in AAH patients than normal controls. Groups did not differ significantly in mean IC_{50} (18.0(±10.1) versus 9.1(±1.7) nmol/l, p=0.98, (**Figure 4-4**).

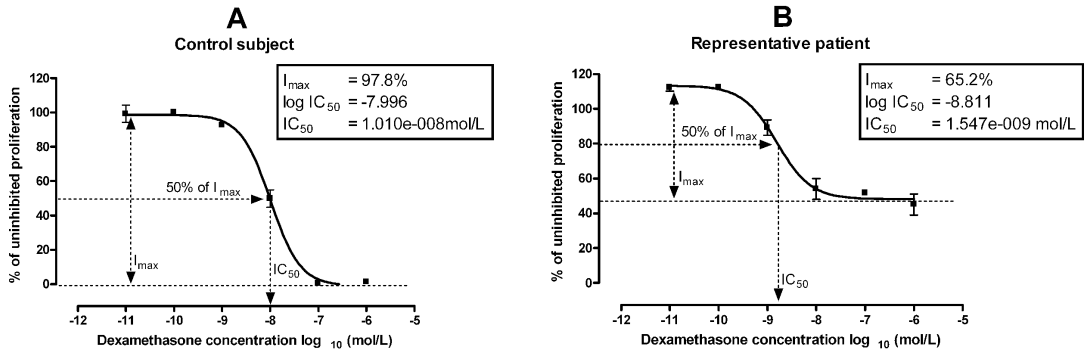


Figure 4-2 Representative patient and control LSS curves

In peripheral blood mononuclear cells from control subjects (A) and patients with acute alcoholic hepatitis (B) T lymphocyte proliferation was stimulated with phytohaemagglutinin and suppressed with increasing concentrations of dexamethasone. At each concentration proliferation was measured by incorporation of a radio-labelled nucleotide into new cells and expressed as a percentage of uninhibited proliferation in the absence of dexamethasone. For each subject the maximum inhibition of proliferation (I_{max}) and the dexamethasone concentration required to achieve 50% of maximal inhibition (IC_{50}) were calculated. Values are mean +/- SEM.

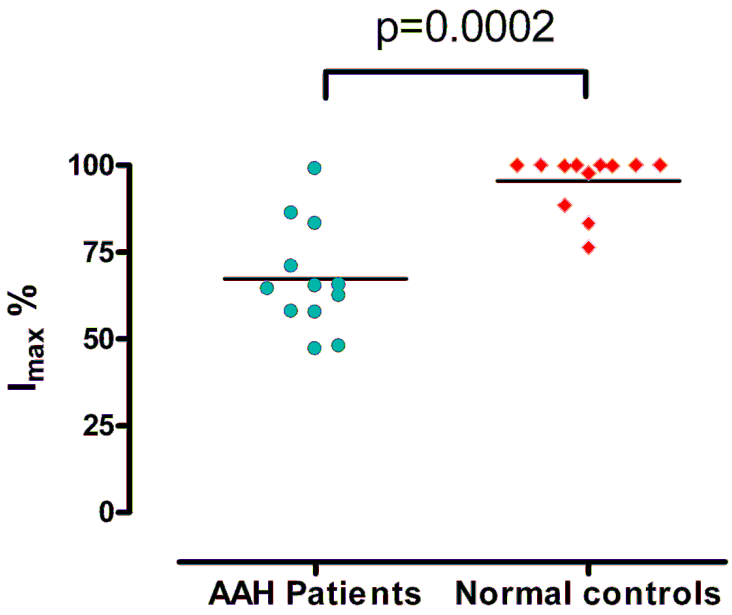


Figure 4-3 Lymphocyte steroid sensitivity in acute alcoholic hepatitis patients and normal controls (efficacy)

Steroid sensitivity is measured in terms of I_{max}, the maximal percentage inhibition of proliferation by dexamethasone. Bars represent mean I_{max} in each group.

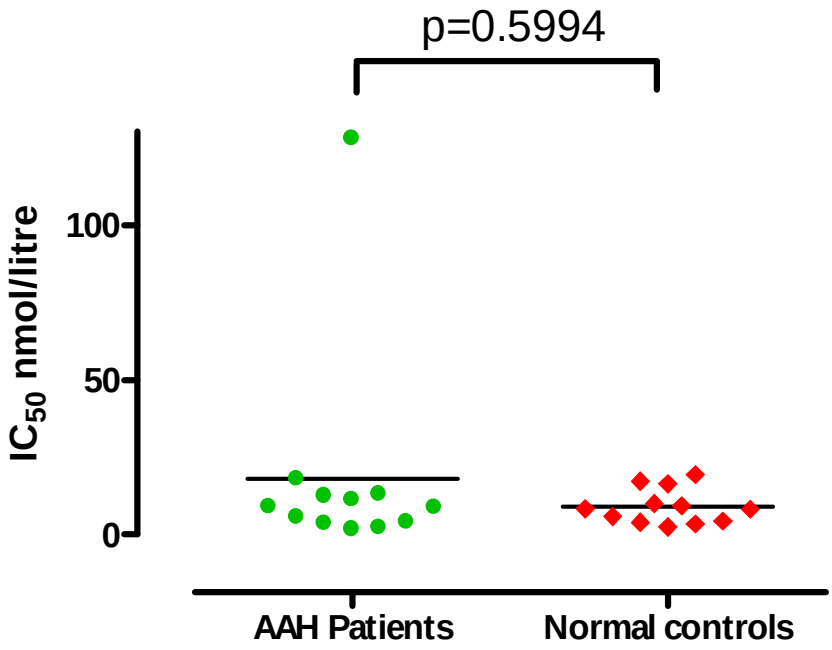


Figure 4-4 Lymphocyte steroid sensitivity in acute alcoholic hepatitis patients and normal controls (potency)

Steroid sensitivity is measured in terms of IC_{50}, the concentration of dexamethasone required for 50% of maximal inhibition of PHA-induced proliferation. Bars represent mean IC_{50} in each group.

4.3.2 LSS correlates with clinical markers of steroid responsiveness

Within the AAH group mean I_{max} was greater in those patients who showed an early change in bilirubin level, an established clinical marker of glucocorticoid responsiveness in AAH, than in those whose bilirubin had not fallen by seven days (74(±5.8)% versus 58(±4.7)%, $p<0.05$, **Figure 4-5**) suggesting that corticosteroid insensitivity in the *in vitro* proliferation is a predictor of subsequent clinical response to corticosteroid therapy. However, when the full Lille model was calculated there was no significant difference in I_{max} between patients with Lille<0.45 (good prognosis) and those with Lille≥0.45 (poor prognosis) (67(±6.4)% versus 69(±4.8)%, $p=0.81$).

In this study no relationships were seen between I_{max} and smoking status ($p=0.32$), reported weekly alcohol intake or disease severity as measured by serum bilirubin, prothrombin time, creatinine, albumin or discriminant function, Child-Pugh (Christensen, Schlichting et al. 1984), MELD (Dunn, Jamil et al. 2005) or Glasgow Alcoholic Hepatitis (Forrest, Evans et al. 2005) scores ($r^2<0.15$ for each).

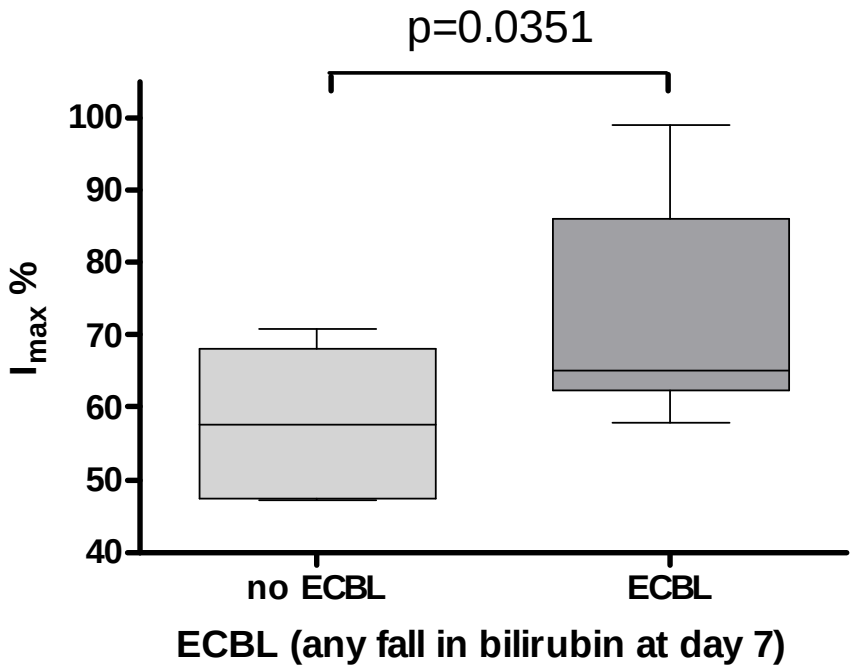


Figure 4-5 Clinical correlation of *ex vivo* steroid sensitivity

Relationship between *ex vivo* lymphocyte steroid sensitivity expressed as I_{max} and early change in bilirubin level (ECBL), a clinical marker of steroid responsiveness in acute alcoholic hepatitis defined as any fall in bilirubin at day 7. Boxes represent the interquartile range divided at the median with whiskers covering the whole range of the data. n=5 for no ECBL, n=7 for ECBL.

4.3.3 Measured steroid sensitivity improved during recovery from AAH

Within the AAH group eight patients survived and seven attended for at least one follow-up appointment. Of these, six reported abstinence from alcohol since admission. Mean I_{max} in this group was significantly higher during recovery than at presentation (92(±4.9)% v 70(±7.4)%, p=0.01, **Figure 4-6**).

4.3.4 Steroid sensitivity increased in the presence of 10^{-5}M theophylline

The addition of theophylline 10^{-5}M (a concentration previously demonstrated to modulate inflammation in COPD (Barnes 2005)) to the lymphocyte steroid sensitivity reaction resulted in an increase in I_{max} measured at presentation (86(±6.6)% v 67(±5.0)%, p<0.05, **Figure 4-7**). In one caseI_{max} fell in the presence of theophylline; this was a 54-year ols male with prior evidence of portal hypertension and a serum bilirubin of 530 who did not survive, but was not an outlier for any baseline characteristics.

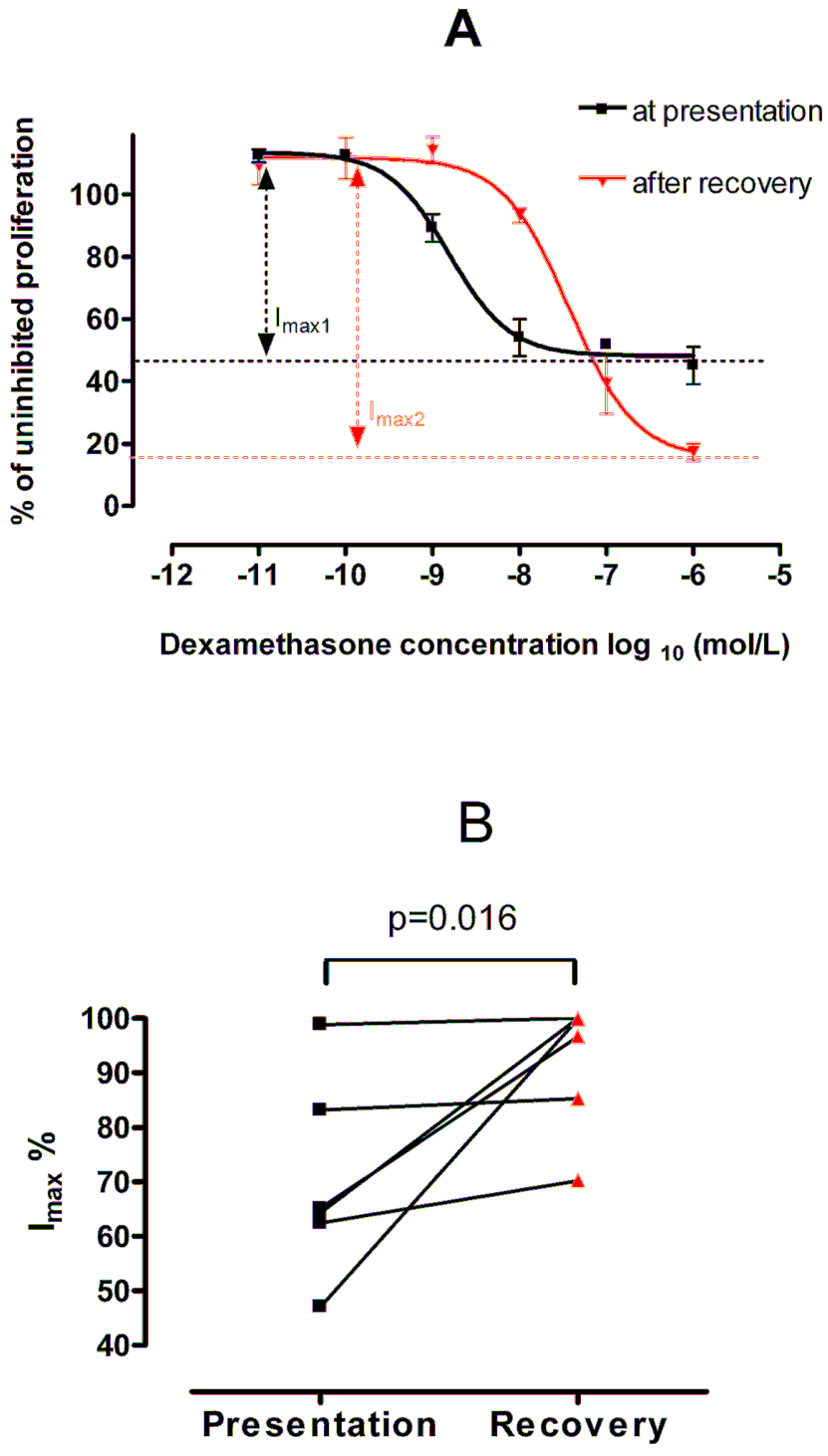


Figure 4-6 Change in lymphocyte steroid sensitivity after recovery from acute alcoholic hepatitis

Representative steroid sensitivity curves for the same patient at presentation and after recovery are illustrated in A. Steroid sensitivity in terms of I_{max} is plotted at presentation and after recovery for surviving patients attending follow-up in B.

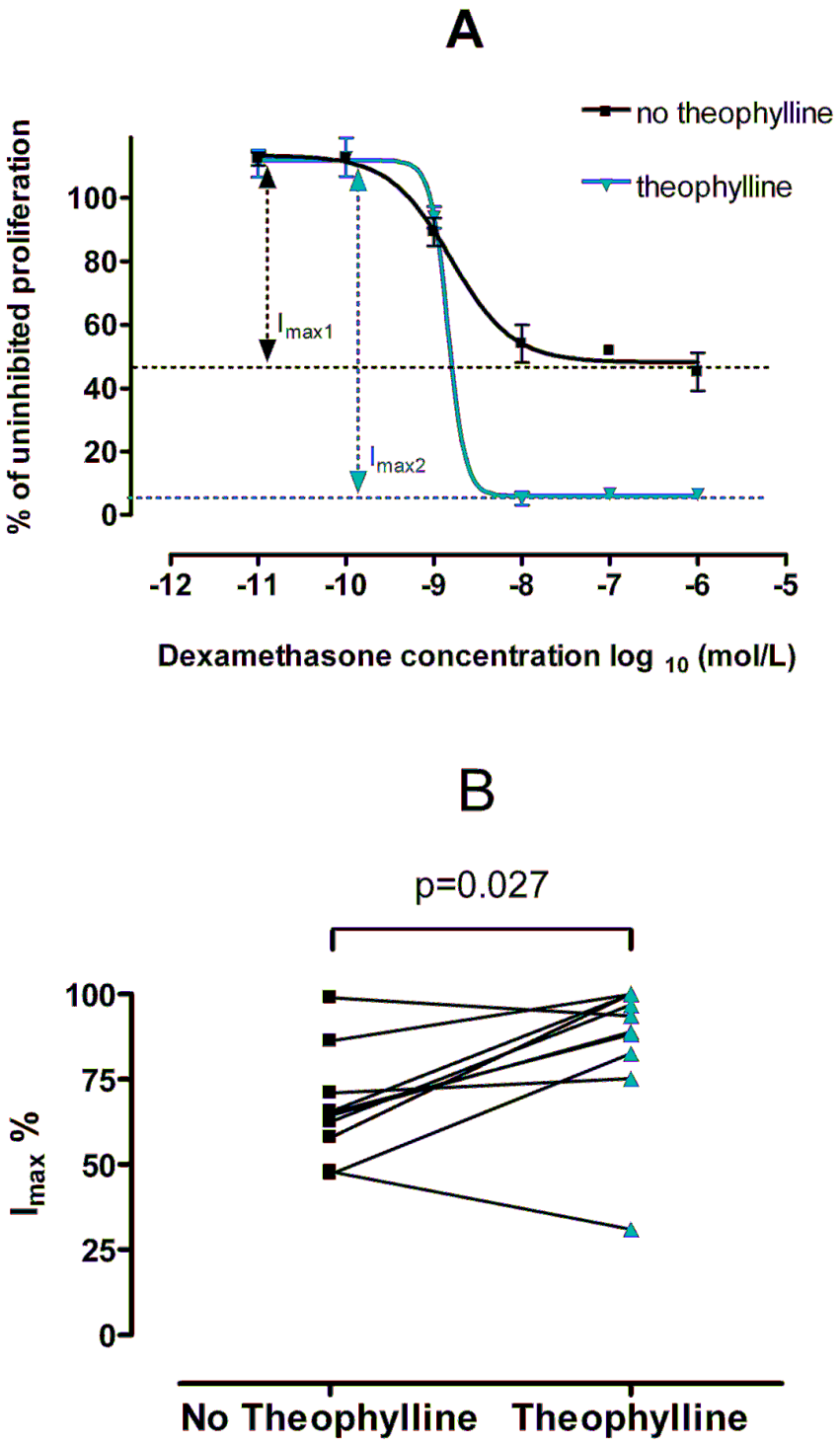


Figure 4-7 Change in lymphocyte steroid sensitivity with addition of theophylline

Representative steroid sensitivity curves for a patient's lymphocytes with and without theophylline 10^{-5}M are illustrated in A. Steroid sensitivity in terms of I_{max} is plotted in the absence and presence of 10^{-5}M theophylline in B.

4.4 Discussion

4.4.1 Improving and expanding the use of existing therapies

Certain diseases, frequently those with substantial social or economic components to their aetiology, suffer comparatively low research investment despite their relatively high contribution to total human suffering. The prospects for development of entirely novel therapeutic compounds for such conditions are poor and so alternative strategies must be sought to help improve clinical outcome. One strategy is to rationalise the use of existing treatments to maximise therapeutic benefit either by improving response or minimising exposure to potentially hazardous unwanted effects. Another strategy is to uncover new therapeutic effects of existing pharmaceutical compounds which might be applicable to different disease mechanisms. Many of the compounds in pharmaceutical use will have more than one biological action which can be exploited in a different therapeutic arena. The advantages of this approach over *de novo* drug development are that compounds with an established safety profile can proceed to trials and licensing faster, and the lower cost, particularly of off-patent compounds, can improve patient access to treatment. This study has uncovered new observations about glucocorticoid responsiveness in patients with AAH and identified a potential new role for the off-patent compound theophylline its treatment.

4.4.2 Ex vivo steroid responsiveness and its clinical correlates

The sensitivity of mitogen-stimulated lymphocyte proliferation to glucocorticoids is a well-established correlate of clinical steroid responsiveness in asthma, rheumatoid arthritis, renal transplantation and ulcerative colitis (Langhoff, Ladefoged et al. 1986; Corrigan, Brown et al. 1991; Kirkham, Corkill et al. 1991; Hearing, Norman et al. 1999). Lymphocyte proliferative responses to liver derived antigens are a feature of human alcoholic liver disease (Stewart, Vidali et al. 2004) and steroid therapy is effective in a proportion of patients with AAH (Mathurin, Mendenhall et al. 2002). This study investigated *ex vivo* lymphocyte steroid sensitivity in patients with clinically severe AAH and found it to be significantly lower than in age- and sex-matched controls.

As is the case in steroid-resistant ulcerative colitis, the reduced steroid sensitivity in AAH was apparent in differences in the maximum inhibition of proliferation I_{max}, a measure of steroid efficacy, rather than in the IC_{50}, which measures steroid potency (Hearing, Norman et al. 1999). This implies that steroid resistance is unlikely to be overcome simply by increasing the steroid dose. A limitation of measuring steroid responses in this way is that the ^{3}H-thymidine assay measures reduction in DNA synthesis after steroid exposure and ignores any contribution that an increased rate of apoptosis might make to glucocorticoid suppression of the total lymphocyte pool.

The clinical marker of any fall in bilirubin by day 7 on treatment (ECBL) has been shown to identify patients who are clinically steroid responsive with a 95% 6-month survival after treatment, as opposed to those with no fall in bilirubin whose 6-month survival is 25% and who do not benefit from continuation of glucocorticoid therapy (Mathurin, Abdelnour et al. 2003). In our study mean steroid sensitivity as measured by I_{max} was significantly lower in those patients who showed no ECBL, suggesting that, as in UC, I_{max} is an *ex vivo* correlate of clinical steroid responsiveness in AAH. The Lille model was found to be a more sensitive predictor of poor prognosis on corticosteroid therapy than ECBL in two French cohorts (Louvet, Naveau et al. 2007) but there was no significant difference in I_{max} between prognositic groups determined by the Lille model in this study. A larger follow-up study could compare this assay with the Lille model for utility in deciding about continuation of steroid therapy. Further refinement of this technique might allow ineffective steroid therapy to be stopped at day 4 or earlier, helping to minimise steroid-related morbidity. This strategy could be further refined by developing a steroid sensitivity assay based on inhibition of PBMC cytokine responses to LPS rather than T cell proliferation. This would have the advantages of measuring a response that has been more convincingly demonstrated to be key to the pathogenesis of AAH (Bird, Sheron et al. 1990), and of providing a result within, for example, 24 hours for TNFα expression as opposed to 4 days for lymphocyte proliferation.

4.4.3 Origins and consequences of steroid resistance in AAH

The significant prevalence of reduced I_{max} in patients relative to controls strongly indicates that steroid resistance is a feature of AAH. However, there is known to be wide inter-individual variation in steroid sensitivity with studies suggesting that up to 30% of the healthy population would fail to respond to steroid therapy for severe inflammatory conditions (Hearing, Norman et al. 1999). This raises the question of whether the patients with AAH are those who are intrinsically ‘steroid resistant’ (suggesting that this intrinsic steroid insensitivity might predispose to severe AAH in heavy drinkers) or whether steroid resistance occurs as a consequence of earlier events in the pathogenesis of AAH. The observation that in survivors of AAH I_{max} tends to increase (though not always normalise) during recovery suggests that steroid resistance is at least in part a consequence rather than a cause of the development of AAH. An interesting focus for further study would be whether steroid insensitivity in other inflammatory conditions correlates with alcohol intake, and whether heavy drinkers without inflammatory disease demonstrate relative steroid insensitivity by the same assay.

Several of the pathogenic features of AAH have the potential to reduce steroid sensitivity. Glucocorticoid modulation of inflammatory responses results from the ability to control transcription of pro-inflammatory genes. Inflammatory and immune cells respond to stimuli by increased transcription factor activity at promoter regions of genes controlling cell activation, proliferation and secretion of pro-inflammatory mediators such as cytokines and chemokines. Transcription factors recruit co-activators including those with histone acetyltransferase (HAT)

activity. These acetylate the histone proteins around which the DNA is coiled. The increase in negatively charged acetyl groups favours a more open chromatin conformation which allows polymerases access to the DNA and gene transcription is initiated. One of the actions of the activated glucocorticoid receptor is to oppose this process by inhibiting HAT activity and recruiting HDACs to deacetylate the histones, causing the chromatin to revert to a closed conformation and transcription to cease (summarised in **Figure 1-6**). Failure of HDAC recruitment leads to steroid insensitivity and ongoing inflammation. The oxidative stress associated with cigarette smoking has been shown to have this effect in COPD (Barnes, Ito et al. 2004). No association between I_{max} and smoking history was observed in our patient sample. However, ethanol metabolism is itself a potent source of reactive oxygen species which may impair the ability of the glucocorticoid receptor to recruit HDACs to 'switch off' transcription of pro-inflammatory genes by the same mechanism described in COPD. Alternatively, the increased concentrations of free acetate generated by ethanol metabolism may favour increased histone acetylation and enhanced pro-inflammatory gene expression. This is investigated in more detail in Chapter 5.

The discovery of impaired corticosteroid sensitivity has implications not only for steroid-treated AAH but also for our understanding of the propagation of inflammation during AAH pathogenesis. If the development of AAH is associated with steroid resistance then the inflammatory response in AAH will be similarly resistant to the moderating effects of the body's endogenous glucocorticoids. Furthermore, there is evidence that patients with both acute and chronic liver diseases experience a high prevalence of hypoadrenalism (Harry, Auzinger et al. 2002; O'Beirne, Holmes et al. 2007) and the effect of this relative endogenous glucocorticoid deficiency on inflammation will be compounded by any associated glucocorticoid resistance. The prevalence of relative hypoadrenalism in AAH could be investigated with a short synacthen test in a cohort of patients and compared with normal and heavy-drinking controls.

4.4.4 The theophylline effect

In this *ex vivo* assay, the addition of theophylline improved (but not always normalised) steroid sensitivity as measured by I_{max}. Theophylline has a similar effect on steroid sensitivity in COPD where it has been shown to improve HDAC recruitment at the same concentration (10^{-5}M) (Barnes 2005) which is below the plasma concentration associated with a clinically significant bronchodilator action. The same mechanism could be responsible for the increased I_{max} observed in the AAH patient samples. Alternatively, theophylline may be exerting its effect through phosphodiesterase inhibition as a role has recently been demonstrated for PDE4B in the enhanced inflammatory responses observed after chronic ethanol exposure (Gobejishvili, Barve et al. 2008). A third possibility is that theophylline acts through its upregulation of anti-inflammatory adenosine A2a receptors (Ohta and Sitkovsky 2001). However, significant phosphodiesterase and adenosine receptor effects are unlikely at the relatively low concentration of theophylline used in this study (Barnes 2005). Of interest is the fact that

theophylline is a hepatic metabolite of caffeine and coffee drinking has been shown to protect against advanced alcoholic liver disease in large epidemiological studies with an average intake of four cups a day reducing the relative risk of alcoholic cirrhosis by 80% compared to those who drank no coffee (Klatsky, Morton et al. 2006). Furthermore, pentoxifylline, another methyl xanthine compound similar in structure to both caffeine and theophylline with documented anti-TNFα properties, has been shown in trials to improve survival in AAH (Akriviadis, Botla et al. 2000) but has not been tested in combination with corticosteroids. In light of the findings above, it remains possible that pentoxiphylline's efficacy is due, at least in part, to increased sensitivity to endogenous corticosteroids.

4.4.5 Implications of these findings

This study highlights the possibility for existing, inexpensive treatments to be used more effectively in a common and frequently fatal condition. Early and more accurate determination of steroid sensitivity has the potential to rationalise corticosteroid treatment to minimise unwanted effects and maximise clinical benefit. The potential of low dose theophylline to increase steroid sensitivity suggests that a clinical trial of its use in acute alcoholic hepatitis could inform and improve future clinical practice. The low theophylline concentration required to show an effect *ex vivo* suggests that the dose required in a clinical trial could be low (eg 200mg per day), reducing the risk of theophylline toxicity and reducing the need for monitoring plasma theophylline levels in eventual clinical practice. A further potential clinical strategy would be to investigate a role for IL-2 receptor blockade in improving steroid sensitivity as this has shown some early promise *in vitro* and *in vivo* in ulcerative colitis (Creed, Norman et al. 2003; Creed, Probert et al. 2006). However, the addition of a second immunosuppressant in a disease in which the majority of deaths, with or without steroid treatment, are ultimately due to sepsis is likely to be considered an unacceptable clinical hazard.

The potential of theophylline, a known HDAC recruiter, to contribute to the control of inflammation in AAH lends credence to the concept that histone acetylation and HDAC inhibition have a role in AAH pathogenesis. This concept will be explored further in the next chapter.

5 Ethanol, acetate and acetylation in inflammation

5.1 Introduction

5.1.1 Enhanced innate immune responses in AAH

Our current understanding of the pathogenesis of AAH attributes hepatocellular dysfunction to the action of supra-physiological concentrations of pro-inflammatory cytokines on hepatocytes that are already suffering oxidative and endoplasmic reticulum stress due to the reactive products of ethanol metabolism (McClain, Song et al. 2004). The major source of cytokine release is thought to be the hepatic macrophage or Kupffer cell responding, *via* Toll-like receptors (TLR), to the increased concentration of bacterial endotoxin in portal blood that results from an ethanol-mediated increase in gut permeability (Thurman 1998).

Evidence for the role of endotoxin, TLRs and cytokines in this mechanism is well established (Mandrekar and Szabo 2009). Increased gut permeability is a feature of ALD and plasma lipopolysaccharide (LPS) is elevated in all stages of ALD, levels correlating with clinical severity and outcome. The principal LPS receptor, TLR4, is upregulated by chronic ethanol treatment in humans and both C3H/HeJ mice lacking TLR4 and animals deficient in the CD14 co-receptor show relative protection from ethanol-induced liver injury in comparison with wild-type animals (Uesugi, Froh et al. 2001; Yin, Bradford et al. 2001). AAH patients have significantly elevated serum cytokines, particularly IL-6, IL-8 and TNFα, with levels correlating with prognosis (Bird, Sheron et al. 1990; Sheron, Bird et al. 1991; Hill, Marsano et al. 1993). Their *ex vivo* monocyte responses to LPS are significantly enhanced relative to controls and this LPS hyper-responsiveness can be reproduced *in vitro* by exposure of the human macrophage cell line MonoMac6 to ethanol for six days (Zhang, Bagby et al. 2001).

The enhanced and sustained inflammatory response seen in AAH is, however, in complete contradistinction to the normal processing of portal endotoxin by the liver (McClain, Hill et al. 2002). The liver is normally subject to tonic endotoxin exposure *via* the portal vein and it is effective at clearing this endotoxin from the blood without an inflammatory response, The phenomenon of 'endotoxin tolerance' thereby renders endotoxin-exposed Kupffer cells refractory to further LPS stimulation, maintaining an anti- rather than pro-inflammatory cytokine output (Knolle, Schlaak et al. 1995). It is therefore somewhat unexpected that the pro-inflammatory response to endotoxin in AAH should be so disproportionately high, particularly considering that it is the Kupffer cells themselves which are key to maintaining hepatic endotoxin tolerance (Knolle and Gerken 2000). It has become increasingly clear, therefore, that the enhancement of cytokine gene expression and perpetuation of the inflammatory response is the key event in the pathogenesis of AAH (Szabo 2000) and the inflammation in AAH has been considered a 'failure of endotoxin tolerance' (McClain, Hill et al. 2002).

Despite its clear importance for the pathogenesis of AAH, the mechanism for enhanced inflammatory cytokine release in this disease remains unclear. This study addresses the novel hypothesis that the enhanced inflammatory cytokine response results from the direct effect of ethanol metabolism on the final common pathway of cytokine gene transcriptional regulation by histone acetylation.

5.1.2 Histone acetylation and deacetylation

In its un-transcribed state DNA is tightly coiled around histone protein octamers and the resulting chromatin is compacted into a closed tertiary structure from which the histone tails protrude, but in which the DNA is inaccessible to polymerases involved in gene transcription. Gene activation by transcription factors involves co-activator proteins with histone acetyl transferase (HAT) activity that acetylate key lysine residues in the histone tails. The negatively-charged acetyl groups cause a conformational change in chromatin that allows RNA polymerases access to the DNA, facilitating gene transcription. Termination of transcription is mediated through histone deacetylases (HDAC) which release free acetate and allow the chromatin to resume its closed, un-transcribed conformation (Kimura, Matsubara et al. 2005) (**Figure 1-6**). Various HDACs are able to modulate inflammatory gene transcription, including class I and II HDACs which can be recruited by transcriptional repressors such as the activated glucocorticoid receptor, and class III HDACs, known as sirtuins (SIRT), which are active in the presence of NAD^+ (Lavu, Boss et al. 2008). Ethanol has been demonstrated to increase total histone acetylation in rat liver (Kim and Shukla 2006) with increased HAT and reduced HDAC activity (Choudhury and Shukla 2008) and separate investigations have established that both SIRT expression and activity can be inhibited by ethanol in the liver (Lieber, Leo et al. 2008; You, Liang et al. 2008).

5.1.3 Acetate, acetyl-coA and acetyl-coA synthetases

Hepatocyte ethanol metabolism produces free acetate as its end-product which, largely in other tissues, can be incorporated into acetyl-coenzyme A (acetyl-coA) for use in Krebs cycle oxidation, fatty acid synthesis or as a substrate for protein acetylation (Yamashita, Kaneyuki et al. 2001). The synthesis of acetyl-coA from acetate is catalysed by the acetyl-coA synthetases (Fujino, Ikeda et al. 2003), recently re-designated acyl-coenzyme A synthetase short-chain family members 1 and 2 (ACSS1 (UniProt Q9NUB1) and ACSS2 (Q9NR19)) (Watkins, Maiguel et al. 2007). ACSS1 is found in mitochondria and expressed in, amongst others, skeletal and cardiac muscle and inflammatory cells but not hepatocytes. It supplies acetyl-coA predominantly for Krebs cycle oxidation. ACSS2 is a cytoplasmic enzyme of ubiquitous expression which is abundant in hepatocytes and pancreas and which largely supplies acetyl-coA for fatty acid synthesis and protein acetylation (Fujino, Ikeda et al. 2003).

The role of acetyl-coA synthesis in control of inflammation has not previously been investigated and could open a novel field of study into the relationship between cellular energy supply and inflammatory disease. This study tests the hypothesis that ethanol enhances macrophage cytokine production by uncoupling gene transcription from its normal regulatory mechanisms through increased histone acetylation, and that the conversion of the ethanol metabolite acetate to acetyl-coA is crucial to this process.

5.2 Specific Methods

5.2.1 Cell culture

The human monoblastic cell line MonoMac6 (DSMZ, Braunschweig, Germany, ACC124), an established human cell-line which displays features of mature macrophages and has been previously used to model Kupffer cell responses in ethanol (Zhang, Bagby et al. 2001), was grown in RPMI-1640 medium with appropriate supplements (section 2.2). The use of a cell line to model human macrophage responses has technical advantages over the use of primary human monocytes or Kupffer cells. It allows use of a pure population without the need for separation processes which can produse unwanted activation of cell signalling pathways. It provides a genetically and epigenetically consistent cell population without the influence of individual human variation in nutrient supply and exposure to ethanol, glucocorticoids and TLR ligands. The robustness of a cell line means that it will continue to proliferate under the sub-physiological conditions of culture media and the stress of ethanol exposure in which primary cells did not tolerate a 9-day protocol. However, a cell line model has limitations in this context and these arise from the neoplastic character of the cells. The development of malignant potential is associated with an ability of cells to proliferate in the face of restricted energy and oxygen supply and this can occur through altered flux through metabolic pathways and increased tolerance to the effects of oxidative stress. Furthermore, the aberrant gene expression characteristic of a neoplastic cell line is likely to result from alterations in epigenetic control of transcription. For these reasons there is no guarantee that results form this robust and convenient model will be generalisable, and promising data should be followed up with corroborative investigation in primary human cells or whole animal models.

Ethanol exposure was achieved in fresh media with 86mM (0.5%, 400mg/dl) ethanol (VWR, Poole, UK). This is five times the legal blood alcohol limit for driving in the UK and equivalent to heavy drinking in humans (Jones 1999). Ethanol concentration was maintained by using ethanol vapour in the incubator to prevent evaporation of ethanol from culture media (section 2.3.3) and monitored by potassium dichromate reduction assay (BioAssay Systems, Hayward CA). For acetate culture experiments, media were supplemented with 1mM sodium acetate (Sigma) and replaced every 48h to minimise fluctuations in acetate concentration. 1mM is an achievable acetate concentration in an individual metabolising ethanol at the concentration used (Mascord, Smith et al. 1992).

5.2.2 Characterisation of ethanol metabolic pathways

RNA extraction and qRT-PCR (section 2.10) were performed to confirm that the ethanol-exposed cells expressed the necessary enzymes for ethanol metabolism. Primers and probes are detailed in Appendix 2. PCR products were resolved by electrophoresis in a 4% agarose gel and visualised by ethidium bromide fluorescence in ultraviolet light.

5.2.3 LPS stimulation and cytokine determination

After seven days incubation cells were resuspended in fresh medium at $2x10^6$/ml and stimulated with *E. coli O111:B4* LPS (InvivoGen) at a final concentration of 10ng/ml. For mRNA determination, nucleic acids were isolated in binding columns (Stratagene, LaJolla CA) and cytokine transcripts measured by quantitative reverse-transcriptase PCR using AffinityScript reverse transcriptase with random primers (Stratagene) and TaqMan polymerase, primers and probes in a AB7000 cycler using 18s RNA as the endogenous control (all Applied Biosystems, Foster City CA) (section 2.10). Primers and probes are detailed in Appendix 2. Cytokine protein determination was achieved by multiplex electrochemoluminescent immunosorbent assay using the MesoScale Discovery System (MesoScale, Gaithersburg, MD) (section 2.6.2).

The reversibility of the effect of ethanol on cytokine production was assessed by repeating the LPS stimulation and cytokine measurements on cells that had received seven days ethanol exposure and then been returned to normal media without ethanol for 0-7 days.

The effects of the antioxidants Manganese(III) tetrakis(4-Benzoic acid) porphyrin chloride (MnTBAP, Alexis, Lausanne, Switzerland) 300µM (Szabo, Day et al. 1996) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma) 100µM (Wu and Cederbaum 1999) and of the SIRT1 activator resveratrol (Biomol, Plymouth Meeting PA) 200µM (Yang, Wright et al. 2007) were assessed by supplementing media with the appropriate concentration during the 7-day incubation with or without ethanol, and then performing LPS stimulation and cytokine measurement as above.

5.2.4 TLR4 surface receptor density

Surface TLR4 was measured by FACS (section 2.7) after staining with AlexaFluor647-conjugated anti-human TLR4 antibody (eBioscience, San Diego CA, diluted 1 in 2.5) and quantified by calibration with Quantum Simply Cellular beads (Bangs Laboratories Inc).

5.2.5 TLR4 response tolerance

The ability of ethanol incubation to overcome tolerance to LPS stimulation was investigated by incubation of MonoMac6 cells for seven days with and without 86mM ethanol. One flask of each condition was given a first stimulation with LPS 10ng/ml and another flask left unstimulated.

After 12 hours cells were washed, resuspended in the appropriate medium and returned to the incubators for 72 hours. Cells were then washed, counted and transferred to 24-well plates at a density of 10^6/ml for a second LPS stimulation: one triplicate of each condition was left unstimulated, one triplicate was given LPS 10ng/ml and one triplicate LPS 100ng/ml. Plates were incubated for 12 hours and then supernatants harvested and assayed for TNFα by ELISA (antibodies and standards from PeproTech, Rocky Hill NJ) (section 2.6).

5.2.6 Immunofluorescence microscopy

After 0-6 days in 86mM ethanol, cells were adhered to slides by centrifugation at 1000rpm for 3 minutes (Cytospin3, Shandon, Runcorn, UK). Adherent cells were fixed and permeablised in ice-cold methanol for 10 minutes, acetone (both VWR) for 30 seconds, washed three times in PBS and transferred to 0.5% triton X-100 (Sigma) for 10 minutes before washing and briefly air-drying. Slides were blocked overnight in 5% BSA at 4℃, then washed and incubated with primary antibodies to total acetyl lysine (Cell Signalling Technology, Danvers MA, 1 in 200), acetyl-histone H3 (Upstate, Lake Placid NY, 5µg/ml) and acetyl-histone H4 (Upstate, 10µg/ml) in 0.5% BSA at 4ºC for 18 hours. Slides were washed and incubated with FITC-conjugated secondary antibody (goat anti-rabbit IgG, Sigma, 1 in 200) for 2 hours at room temperature in the dark and counterstained with DAPI (Vectashield Hardset, Vector Laboratories, Burlingame CA). The effect of ethanol metabolism on the staining pattern was assessed by incubation in 86mM ethanol supplemented with the alcohol dehydrogenase inhibitor 4-methylpyrazole 1mM (Sigma).

5.2.7 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was used to detect ethanol-induced changes in histone acetylation at specific pro-inflammatory cytokine gene promoter regions. The principle of the assay is that chromatin is digested into individual mononucleosomes (single histone octamers with their associated DNA). Antibodies specific for a given histone modification are used to precipitate mononucleosomes from the chromatin solution, producing a precipitate that is enriched with DNA segments that were associated with histones displaying the modification (eg acetylated histone H3). DNA is extracted from the precipitate and analysed for the gene promoter region of interest by quantitative (Real Time) PCR (**Figure 5-1**). The degree of enrichment for the promoter region of interest can then be compared between treatment groups.

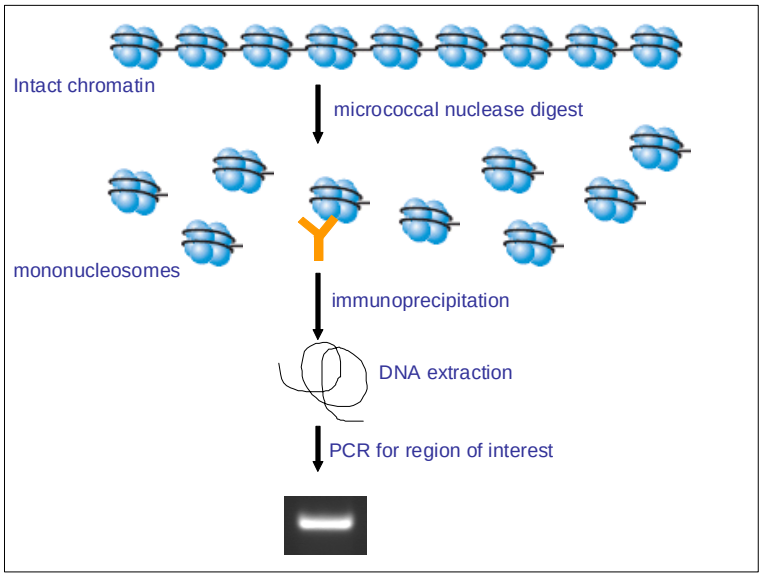


Figure 5-1 Chromatin immunoprecipitation (ChIP)

Mononucleosomes are separated by nuclease digestion and then precipitated using an antibody specific for the chromatin modification of interest. The resulting precipitate will be enriched with DNA from promoters associated with the modification, which can be quantified by qPCR. Figure adapted from one by Dr Jelena Mann (Newcastle University).

ChIP experiments were performed with Dr Jelena Mann, Cell Signalling Group, Newcastle University Institute of Cellular Medicine, who optimised the nuclease digestion, designed the primers and analysed the Real Time data. All buffers were supplemented with 5mM sodium butyrate to prevent deacetylation during the preparation and extractions were performed at 4℃ in the presence of protease inhibitors to minimise protein loss.

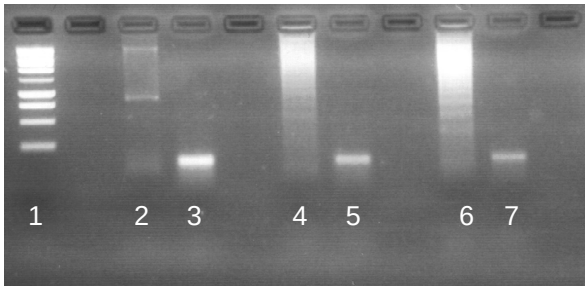


Figure 5-2 Chromatin digestion for ChIP

For each condition the left lane contains DNA from undigested chromatin and the right lane DNA from nuclease-digested chromatin. A single sharp band confirms digestion to mononucleosomes. Lane 1 markers; lane 2,3 butyrated HeLa; lane 3,4 Monomac6 normal medium; lane 6,7 MonoMac6 86mM ethanol.

Cells were subjected to gentle lysis by stirring in 0.5% Tween-40 in TBS buffer (10mM Tris-HCl pH7.5, 3mM $CaCl_2$, 2mM $MgCl_2$, 5mM Na butyrate) for 45 minutes followed by release of nuclei in a Dounce homogeniser. Intact nuclei were isolated by sucrose density centrifugation and equalised to a DNA concentration of 0.5mg/ml in digestion buffer (0.32M sucrose, 50mM Tris-HCl pH7.5, 4mM $MgCl_2$, 1mM $CaCl_2$, 5mM Na butyrate, protease inhibitors). Chromatin was digested by micrococcal nuclease (Amersham, Little Chalfont, UK) 50U/ml at 37℃ to yield a mononucleosome suspension. After 5 minutes digestion was halted by the addition of EDTA to a final concentration of 5mM. A 50µl aliquot of each sample underwent phenol extraction and electrophoresis to confirm adequate digestion (**Figure 5-2**). DNA concentration was measured spectrophotometrically and 300µg aliquots were diluted with twice the volume of incubation buffer (50mM NaCl, 20mM Tris-HCl pH 7.5, 5mM EDTA, 20mM Na butyrate, protease inhibitors) and were pre-cleared with 100µl Zysorbin staphylococcal protein A membranes (blocked with salmon sperm DNA and acetylated BSA) (Invitrogen) by incubation at 4℃ for 20 minutes on a turntable (Stuart). The membranes were pelleted by centrifugation and three aliquots of each supernatant, each containing 100µg DNA, were incubated with 10µl of anti-acetyl H3 antibody, anti-acetyl H4 antibody or irrelevant isotype control antibody (all Upstate) overnight on a turntable at 4ºC. Antibody-bound mononucleosomes were precipitated out using Zysorbin as above and the precipitates were washed by being allowed to settle through three buffers of increasing salt concentrations to remove any unbound protein or DNA (50mM Tris-HCl pH7.5, 10mM EDTA, 5mM Na butyrate, 50mM/100mM/150mM NaCl). Antibody-mononucleosome complexes were eluted from Zysorbin in incubation buffer containing 1% SDS to give mononucleosome-containing supernatant from which DNA was extracted using the phenol-chloroform method (section 2.8.1) and precipitated in ethanol at -20℃ overnight with 0.3M sodium acetate and 1µl glycogen to improve the yield. The washed and air-dried DNA pellet was redissolved in TE buffer.

The DNA extracts underwent conventional PCR with an annealing temperature of 55℃ and primers for the promoter regions of the IL-6 and TNFα genes. The PCR products were resolved in a 2% agarose gel and visualised by ethidium bromide fluorescence in ultraviolet light. The relative concentrations of IL-6 and TNFα promoter DNA in the extracts were quantified by SYBR Green Real Time PCR and compared between ethanol-exposed and control cells for each histone modification. Primers are detailed in **Table 5-1**.

	IL6	**TNFα**
Forward	GAGCAGTGGCTTCGTTTCAT	TGTCCAGGGCTATGGAAGTC
Reverse	TTGGGGAAAGTGAGGTCATC	TTTCATTCTGACCCGGAGAC

Table 5-1 Primers for IL-6 and TNFα ChIP

5.2.8 HAT and HDAC activity

HAT and HDAC activity were determined in nuclear lysates (section 2.5.1) from cells after seven days culture in 86mM ethanol, 1mM sodium acetate or normal medium.

5.2.8.1 HAT activity

HAT activity was measured by an ELISA-based method (Millipore, Temecula CA) in which wells of a 96-well plate were coated with unacetylated histone H3, except for a standard curve which was coated with known quantities of acetylated histone H3. Wells were blocked with 3% BSA and then incubated for 25 minutes at 30℃ with nuclear extract containing 40µg total protein in the presence of HAT buffer, an excess of acetyl-coA and sodium butyrate to inhibit any HDAC activity in the sample. One triplicate was given this cocktail without the nuclear extract as a control. In addition, two tripilicates containing lysate from cells that had not been exposed to ethanol or acetate were treated with 86mM ethanol and 1mM acetate during the incubation to investigate the effect of acute exposure on HAT activity. After incubation wells were washed and acetylation was measured as captured protein would be in an ELISA with an anti-acetyl lysine detection antibody coupled to HRP producing absorbance at 450nm in a tetramethylbenzidine (TMB) substrate. The quantity of protein acetylated by the lysate was quantified by use of the standard curve in Prism 4 (Graph Pad).

5.2.8.2 HDAC activity

HDAC activity was measured by colour change on deacetylation of an acetylated substrate (Biomol) according to the manufacturers' instructions. A standard curve was constructed by serial dilutions of a deacetylated standard substrate and transferred to a 96-well plate in triplicate. Other triplicates received fully acetylated substrate in the proprietary buffer and 5µl (7.5µg total protein) nuclear lysate. Again, one triplicate received control lysate with 86mM ethanol and another control lysate with 1mM acetate. The plate was incubated at 37℃ for 30 minutes before reactions were stopped by the addition of the HDAC inhibitor trichostatin A (TSA) 2µM and 50µl of a developer solution sensitive to the deacetylated substrate added to each well. The developer was incubated for a further 15 minutes before the plate was read at 405nm and the results imported to Prism 4 for quantification of deacetylated substrate from the standard curve.

5.2.9 ACSS1 and 2 determinations

ACSS1 and 2 transcripts were measured by qRT-PCR as above. Protein determination was by Western blotting (section 2.5) using anti-ACSS1 (Abnova, Taipei, Taiwan) and anti-ACSS2 (Atlas, Stockholm, Sweden) primary antibodies with anti-beta-actin (Abcam, Cambridge, UK) used to confirm equal loading. Secondary antibodies were HRP-conjugated goat anti-mouse

IgG and goat anti-rabbit IgG (Sigma) and bands were identified using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford IL) for ACSS2 and Immobilon Western chemiluminescent substrate (Millipore, Billerica MA) for ACSS1. Band densitometry was performed using Scion Image (Scion Corp, Frederick MD).

5.2.10 ACSS 1 and 2 knockdown

To confirm that conversion of acetate to acetyl-CoA is crucial to the acetylation-mediated potentiation of inflammatory responses in ethanol, short hairpin RNA (shRNA, Sigma) knockdown of ACSS 1 and 2 was performed. shRNA is introduced into target cells as a DNA vector which becomes integrated and produces the shRNA *via* RNA polymerase III under the influence of a U6 promoter. The hairpin constructs are cleaved to small interfering RNAs (siRNA) which activate the RNA induced silencing complex (RISC) to degrade the corresponding mRNA and prevent translation of the target molecule. Stable knockdowns can be constructed by the co-transduction of a gene for resistance to the toxic effects of the antibiotic puromycin.

5.2.10.1 Optimisation of shRNA knockdown

Amenability to knockdown was assessed in MonoMac6 cells by termination of protein synthesis with cycloheximide 10µg/ml for 0-5 days. Cell lysates were assayed for persistence of ACSS1 and 2 by Western blotting as above and this demonstrated no detectable protein three days after termination of protein synthesis, suggesting that knockdown should be effective in reducing ACSS1 and 2 protein (**Figure 5-3**).

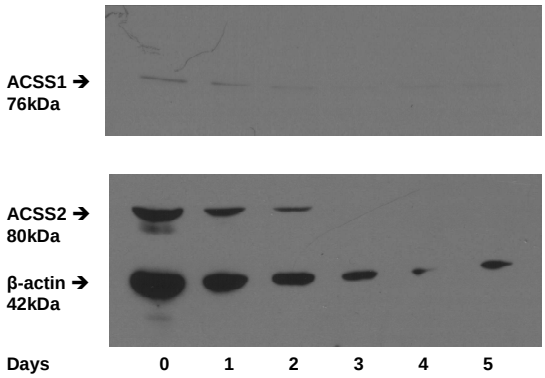


Figure 5-3 Persistence of ACSS1 and 2 in cycloheximide

Cycloheximide inhibits protein synthesis. Absence of ACSS 1 and 2 after 3 days suggests that cellular levels are dependent on new synthesis and so should be amenable to knockdown.

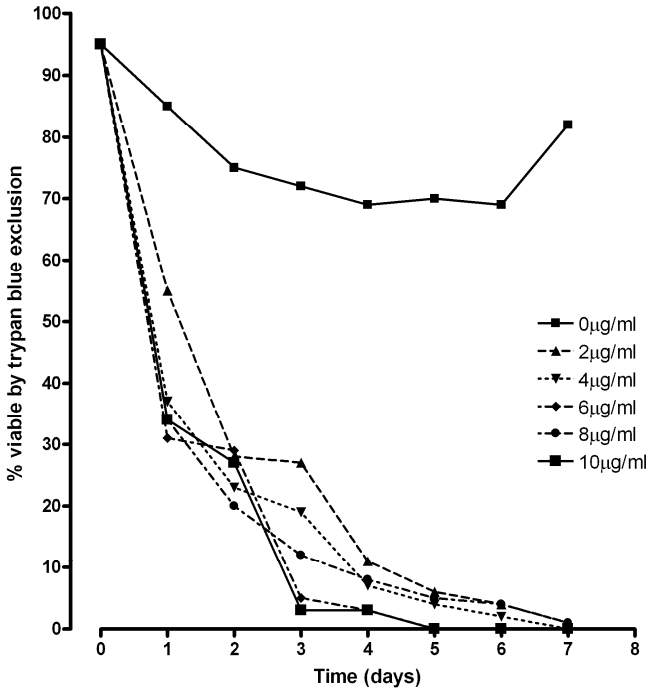


Figure 5-4 Killing curves for MonoMac6 cells in puromycin

MonoMac6 cells were cultured in the indicated range of puromycin concentrations and percentage viability was assessed at 24 hour intervals by trypan blue exclusion. 5μg/ml was selected as the minimum concentration likely to achieve 100% kill in 5 days.

A puromycin kill curve was constructed to identify the minimum concentration of puromycin necessary to kill all untransduced cells. Viability at each concentration was assessed on a daily basis by trypan blue exclusion (**Figure 5-4**). 5μg/ml was selected as the appropriate concentration for selection of stably transduced lines.

The shRNA vector for ACSS 1 or 2 was delivered by lentiviral particles. Hexadimethrine bromide (Sigma) can increase transduction efficiency by neutralising the charge repulsion between virions and sialic acid moieties on the cell surface but is toxic to some cell lines. MonoMac6 cells were tested for hexadimethrine bromide toxicity by trypan blue exclusion at the recommended concentration of 8μg/ml and showed no loss of viability.

A multiplicity of infectivity (MOI) of 5 (i.e. 5 lentiviral particles per cell) was initially selected and achieved successful transduction. Five candidate vectors were provided for each target and were assayed in triplicate on $1.6x10^4$ cells in a 96-well culture plate. Vectors were added to cell suspensions in the presence of hexadimethrine bromide 8μg/ml and incubated at 37℃ in 5% CO_2 for 18 hours. Suspensions were then aspirated, washed in fresh media and pelleted by centrifugation to remove remaining viral particles that could stimulate inflammatory responses. Cells were resuspended in fresh media and viability measured by trypan blue exclusion before

returning to the incubator for a further 24 hours. Suspensions were then aspirated and assayed for ACSS1 and 2 mRNA knockdown by qRT-PCR. Vectors producing the most effective knockdown were selected for larger scale transduction (**Figure 5-5**).

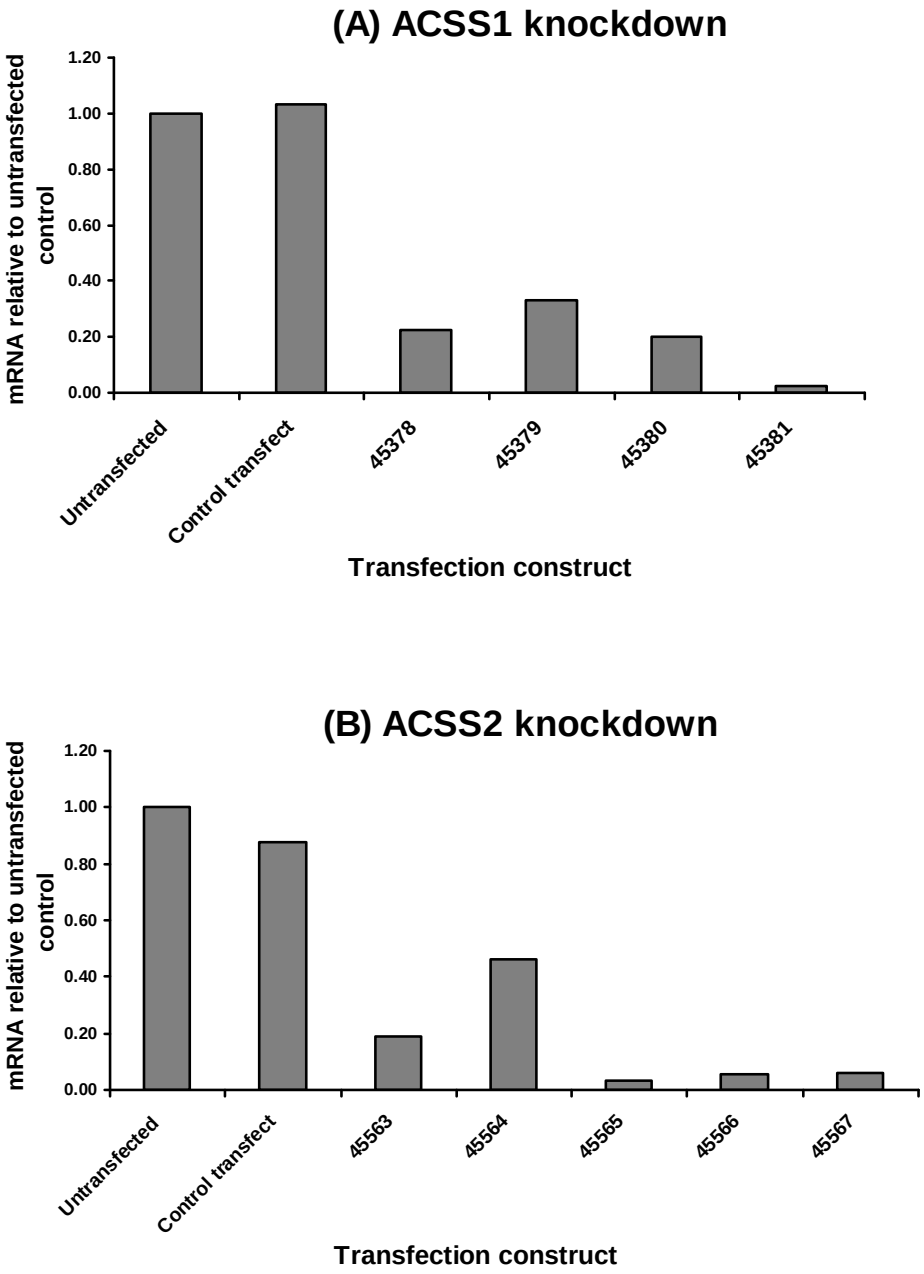


Figure 5-5 Testing of ACSS 1 and 2 knockdown constructs

ACSS1 and 2 transcripts were assayed by qRT-PCR. Constructs 45380 and 45565 were selected for creation of stable knockdowns.

5.2.10.2 Creation and assay of stable knockdowns and controls

1.6x105 cells were transduced in triplicate as above at a MOI of 5 with the selected vectors to achieve knockdown of ACSS1, ACSS2 and a double knockdown ACSS1+2. To control for the effect of shRNA generation on cellular function in accordance with the principles laid down by the Horizon symposium (2003), control cells were transduced with a vector for an irrelevant shRNA transcript at 5MOI to control for the single knockdowns and at 10MOI to control for the double knockdown. 48 hours after transduction, viability was confirmed by trypan blue exclusion and cell suspensions were transferred to 25cm^2 culture flasks in selection medium containing puromycin 5µg/ml. Cell were propagated into 75cm^2 flasks and after 5 passages in selection medium they were assayed for stable knockdown by qRT-PCR and Western blotting (**Figure 5-6**).

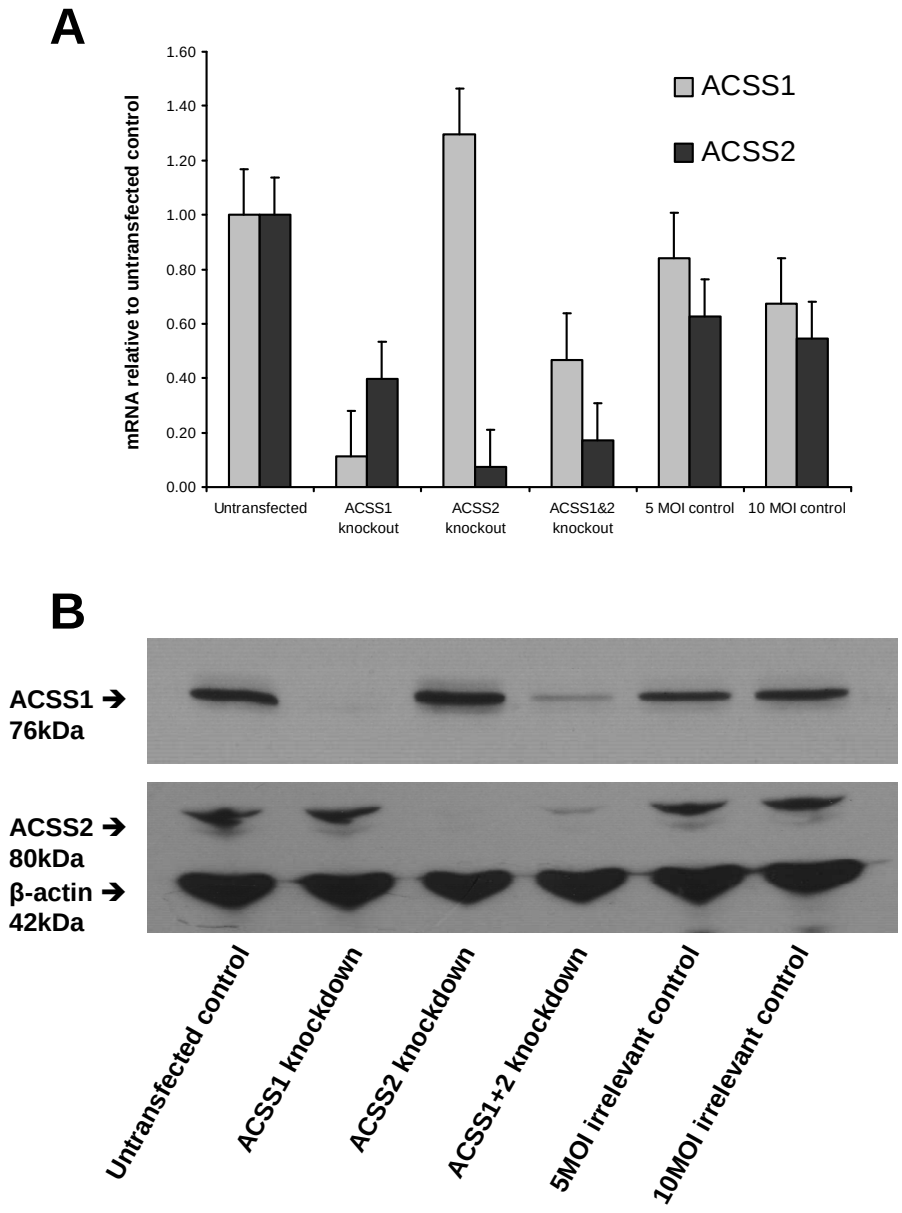


Figure 5-6 Stable knockdowns after 5 passages in selection medium

Knockdowns were assayed after 5 passages in puromycin 5µg/ml by qRT-PCR (A) and Western blotting (B) which confirm knockdown at both the mRNA and the protein level.

Stable knockdowns and controls were subjected to ethanol incubation and LPS stimulation as above and cytokine output was assayed by MSD. Stability of the ethanol culture system was confirmed in transduced cells by dichromate assay. Cytokine responses to LPS from transduced cells were compared to un-transduced and to the appropriate irrelevant transcript control.

5.2.11 Statistical analysis

Numerical results were expressed as means of at least three samples and statistical significance assessed by the Mann-Whitney U-test. Statistical testing of Real Time results is discussed in section 2.10.4.

5.3 Results

5.3.1 Ability of MonoMac6 to metabolise ethanol

qRT-PCR confirmed that ethanol-exposed MonoMac6 cells expressed transcripts for forms of ADH, ALDH and CYP2E1 sufficient for metabolism of ethanol in a similar way to human macrophages (Wickramasinghe 1986) (**Figure 5-7**).

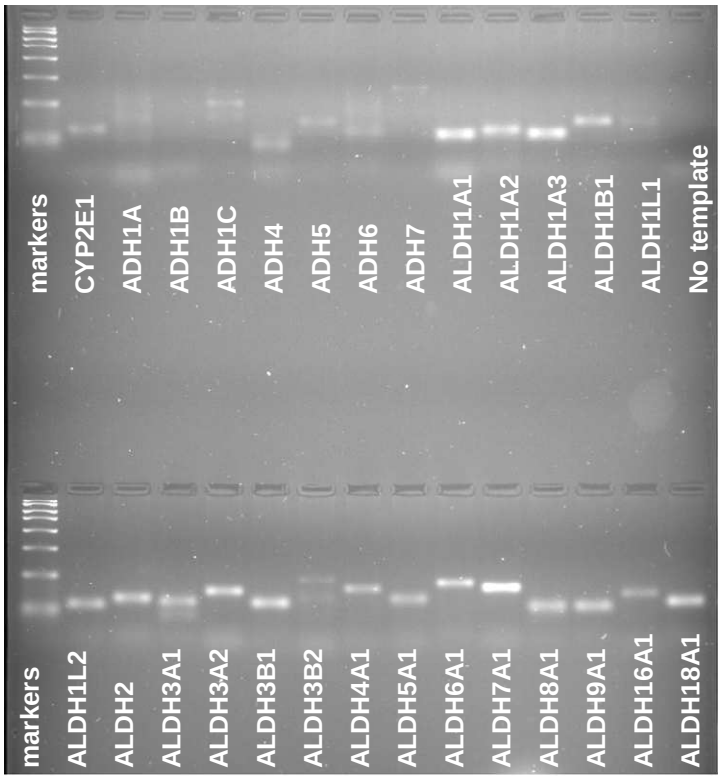


Figure 5-7 Ethanol metabolising enzymes expressed by MonoMac6 cells

qRT-PCR products resolved in 4% agarose gel after 45 cycles amplification. CYP2E1 cytochrome P450 2E1, ADH alcohol dehydrogenase, ALDH aldehyde dehydrogenase.

5.3.2 Enhancement of inflammatory cytokine responses by ethanol

Monomac6, an established human macrophage cell-line modelling Kupffer cell responses in ethanol (Zhang, Bagby et al. 2001), was maintained in a validated constant-exposure ethanol

culture system at an ethanol concentration of 86mM, equivalent to human blood concentrations after heavy drinking. This system demonstrated enhancement of the cytokine response to *E coli* LPS 10ng/ml compared to cells grown in normal medium. This was not seen with acute ethanol exposure but after seven days culture in ethanol significant augmentation of IL-6, IL-8 and TNFα release following LPS exposure (**Figure 5-8A**) was observed. Cytokine mRNA expression was also increased (**Figure 5-8B**). The effect of ethanol on cytokine output was reversible with transfer of hyper-responsive cells from ethanol to normal medium causing the cytokine response to LPS to normalise within four days (**Figure 5-9**). The culture system adopted therefore replicates previous reports of the augmentation of inflammatory cytokine release in the context of chronic ethanol exposure in animal models and human patients with AAH, and demonstrates that this results, at least in part, from an increase in the level of inflammatory cytokine gene transcription.

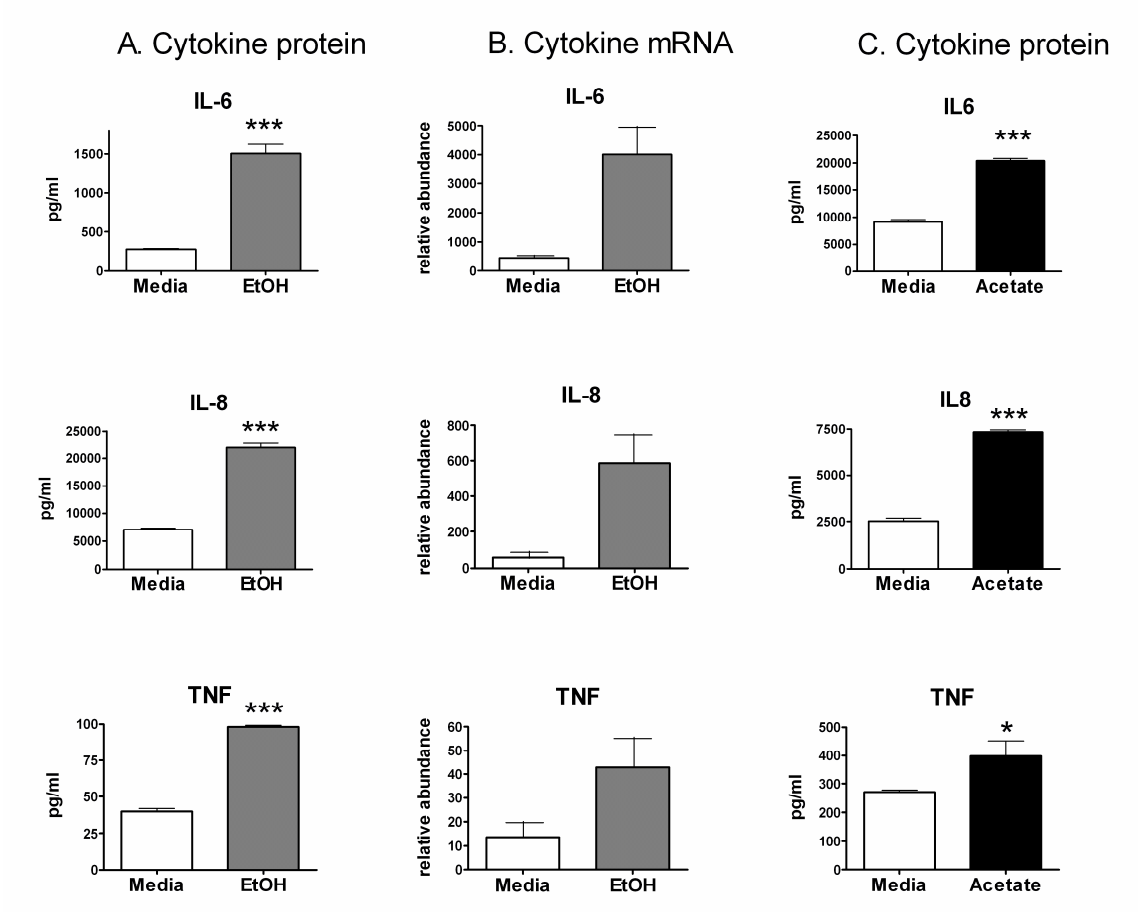


Figure 5-8 Enhanced cytokine responses to LPS in ethanol and acetate

Monomac6 cells were cultured in normal media or in the presence of 86mM ethanol (A, B) or 1mM acetate (C) for 7 days and then stimulated with *E. coli* LPS 10ng/ml. Cells were harvested for cytokine mRNA determination by qRT-PCR at 60minutes (B) and supernatants for cytokine protein determination by multiplex immunoassay at 48 hours (A, C). Values shown are mean + SEM for 3 independent determinations.

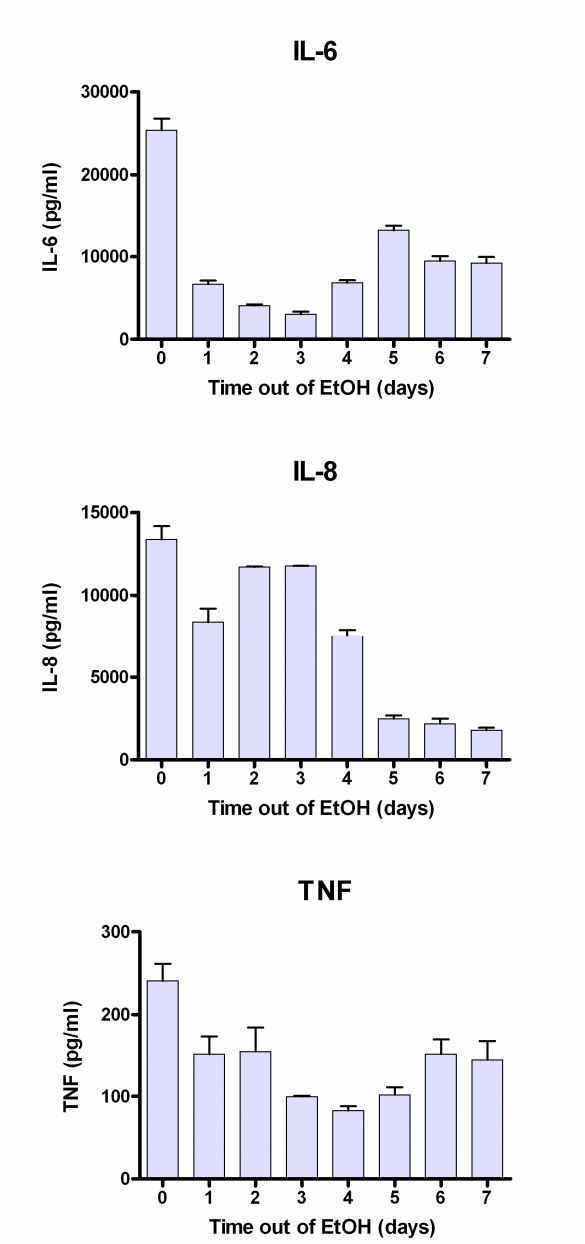


Figure 5-9 Recovery of normal cytokine response after ethanol removal

Monomac6 cells were cultured with 86mM ethanol for 7 days, washed and transferred to normal media for 0-7 days before stimulation with LPS 10ng/ml. Supernatants were harvested for cytokine protein determination by multiplex immunoassay after 48 hours.

5.3.3 Increased TLR4 but maintained endotoxin tolerance in ethanol

FACS analysis demonstrated increased surface TLR4 after seven days culture in 86mM ethanol and this was increased further by exposure to LPS (**Figure 5-10**). ELISA demonstrated an enhanced TNFα response to a single LPS 10ng/ml stimulation in the ethanol-exposed cells, consistent with the findings of the MSD analysis above. However, in cells that had received

earlier LPS exposure 72 hours previously, the TNFα response to re-challenge with LPS was strongly suppressed in both the presence (p=0.004) and absence (p=0.009) of ethanol, despite high levels of TLR4 expression in both groups (**Figure 5-10**). Stimulation with a higher LPS concentration of 100ng/ml did not induce a TNFα response in tolerised cells. This suggests that cells became tolerant to LPS in both the presence and absence of ethanol. Although this preservation of tolerance at a cellular level is not consistent with the prolonged inflammation seen at a whole organism level, it does confirm that increased surface expression of TLR4 is not the key determinant of the enhanced cytokine responses seen in ethanol.

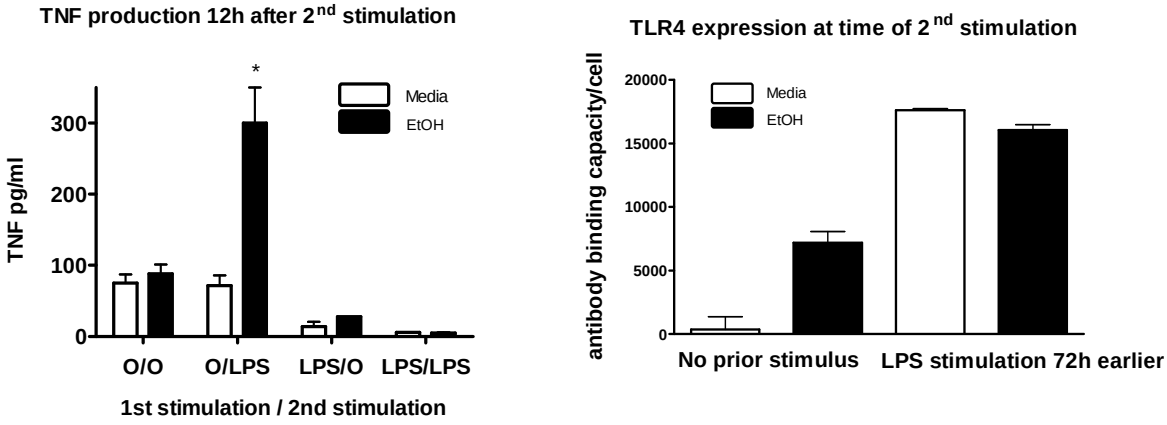


Figure 5-10 LPS tolerance and changes in surface TLR4 in ethanol

LPS-naïve cells show an enhanced TNFα response to LPS in 86mM ethanol compared to normal media, but in LPS-exposed cells the response to a second LPS stimulus is tolerised in both ethanol and normal media (left panel). Ethanol culture is associated with increased surface TLR4 but the response to a second LPS stimulus shows tolerance despite even greater TLR4 expression in both ethanol and normal media (right panel).

5.3.4 Global acetylation increases in ethanol

The next investigation examined whether the enhanced inflammatory responses associated with chronic ethanol exposure were associated with increased histone acetylation. Immunofluorescence microscopy for total acetylated lysine residues, acetyl-histone H3 and acetyl-histone H4 revealed a time-dependent increase in acetylation over six days culture in 86mM ethanol (**Figure 5-11**). Co-culture with the inhibitor of ethanol metabolism 4-MP in the ethanol-containing medium reduced the acetylation staining to baseline, suggesting that ethanol metabolism rather than simply ethanol exposure was responsible for the acetylation changes. This observation demonstrates that ethanol metabolism by mononuclear cells is associated with increased histone acetylation, with a time course similar to the cytokine enhancement, and which is dependent on the metabolism of ethanol.

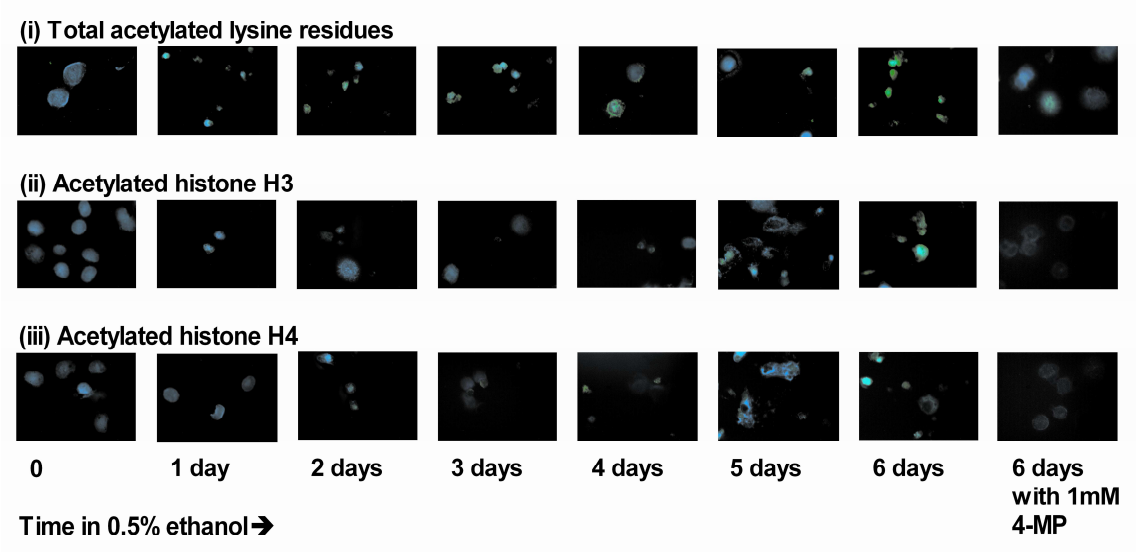


Figure 5-11 Global histone acetylation changes in ethanol

Monomac6 cells were cultured in 86mM ethanol and examined for acetylation modifications (green) after 0-6 days by immunofluorescence microscopy. Modifications stained were total acetylated lysine residues (top row), acetylated histone H3 (middle row) and acetylated histone H4 (bottom row). Nuclei were stained blue with DAPI. The effect of ethanol metabolism on the modifications was demonstrated by co-culture with the alcohol dehydrogenase inhibitor 4-methylpyrazole (4-MP) for 6 days (right column).

5.3.5 Increased histone acetylation at specific cytokine gene promoters

The immunofluorescence microscopy had revealed global increases in histone acetylation. To determine whether this specifically included increased acetylation of the crucial promoter regions of pro-inflammatory cytokine genes chromatin immunoprecipitation was performed on cells cultured in ethanol and control cells cultured in normal medium. The immunoprecipitates produced by anti-acetyl-histone H3 and anti-acetyl-histone H4 antibodies from the monococcal nuclease-digested chromatin of ethanol-exposed cells were enriched for DNA from the promoter regions of the IL-6 and TNFα genes relative to immunoprecipitates from unexposed cells (**Figure 5-12**). This confirmed that increased histone H3 and H4 acetylation was present at these pro-inflammatory cytokine gene promoters after 7 days culture in 86mM ethanol, providing a mechanism for increased cytokine transcription in response to LPS stimulation.

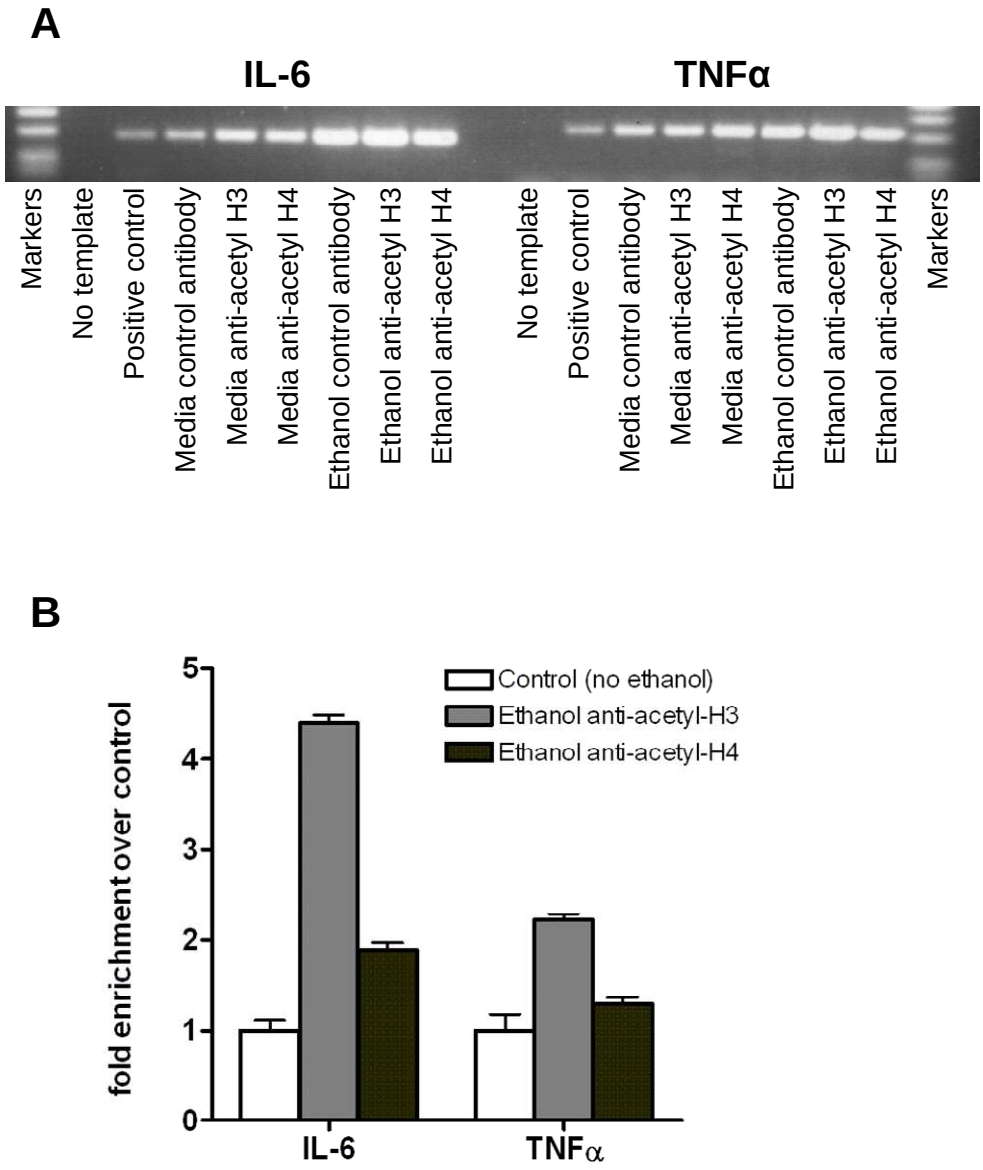


Figure 5-12 Promoter-specific histone acetylation changes in ethanol

Histone H3 and H4 acetylation at IL-6 and TNFα promoters after 6 days in 86mM ethanol was studied by chromatin immunoprecipitation. Immunoprecipitates generated using anti-acetyl-histone H3 and H4 antibodies were assayed by qPCR for enrichment of promoter regions of IL-6 and TNFα genes relative to precipitates from cells cultured in normal media. Enrichment of the precipitate from a given sample with DNA from the IL-6 and TNFα promoters reflects the degree to which these were associated with histones bearing the modification of interest. (A) PCR products from amplification of immunoprecipitated DNA with primers for the IL-6 and TNFα promoter regions resolved in 2% agarose gel. (B) Relative concentrations of IL-6 and TNFα promoter DNA in the extracts quantified by SYBR Green Real Time PCR and compared between ethanol-exposed and control cells for each histone modification.

5.3.6 Reproduction of the ethanol effect by acetate

A potential mechanism for the effect of ethanol exposure on histone acetylation status would be through increased exposure to acetate (the principal hepatic metabolite of ethanol). In order to address this mechanism the extent to which co-culture with acetate could replicate the ethanol effect on histone acetylation was explored. When cells were cultured in 1mM acetate for seven days and then stimulated with LPS an augmentation of cytokine release was observed similar in magnitude to that seen in the ethanol-exposed cells for IL-6, IL-8 and TNFα (**Figure 5-8C**). That exposure to acetate can replicate the enhanced cytokine responses seen following prolonged ethanol metabolism suggests that exposure to acetate (or one of its metabolites) is likely to be critical for increased histone acetylation in the context of ethanol exposure/AAH.

5.3.7 HAT and HDAC activity

It was then tested whether ethanol or acetate were acting by influencing the balance of HAT and HDAC activity in the cells. Addition of 86mM ethanol or 1mM acetate to fresh lysate of MonoMac6 cells significantly reduced HDAC activity within 30 minutes and produced a non-significant increase in HAT activity, a situation favouring net increase in histone acetylation (**Figure 5-13**).

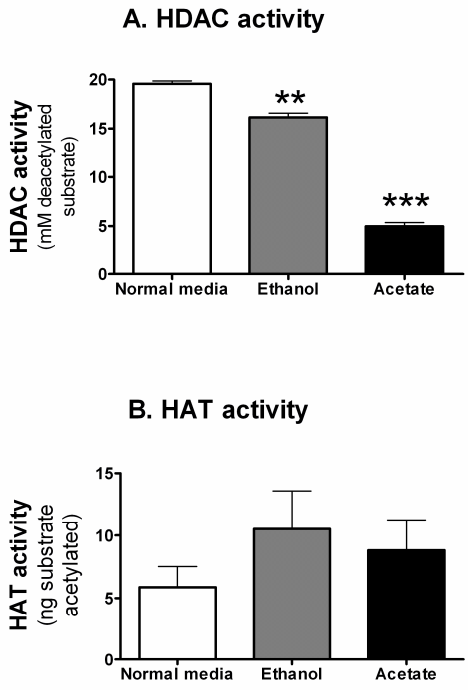


Figure 5-13 HDAC and HAT activity in ethanol and acetate

Monomac6 nuclear lysates were incubated with control media, 86mM ethanol or 1mM acetate for 30min at 37ºC. Substrate deacetylation or acetylation was quantified as described in the text. Values shown are mean + SEM for 3 independent determinations.

Nuclear lysates from cells cultured in 86mM ethanol or 1mM acetate for seven days suggested a trend towards reduced HDAC and increased HAT activity but this did not achieve statistical significance (**Figure 5-14**).

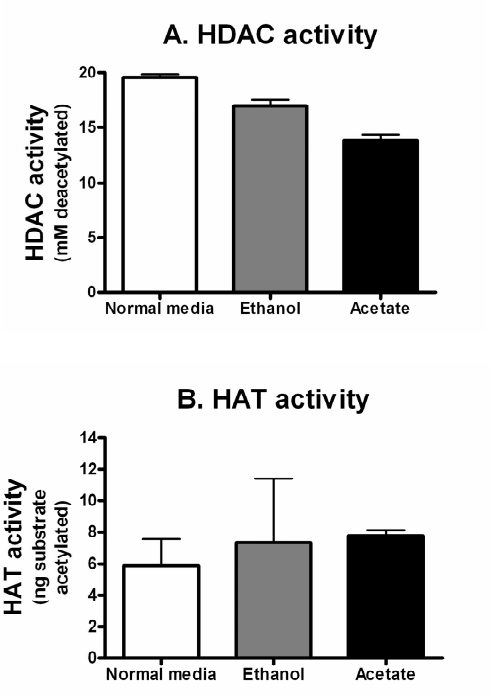


Figure 5-14 HDAC and HAT activity after 7 days in ethanol and acetate

Monomac6 cells were incubated in control media alone or with 86mM ethanol or 1mM acetate for 7 days. HDAC and HAT activity of nuclear lysates was quantified as described in the text. Values shown are mean + SEM for 3 independent determinations.

5.3.8 Induction of ACSS1 and 2 by ethanol and acetate

Free acetate has little metabolic activity and is more likely to influence cellular responses as the metabolically active acetyl-coA, synthesised from acetate by ACSS1 and 2. ACSS 1 and, to a lesser extent, ACSS 2 transcripts were more abundant in cells incubated in 86mM ethanol for seven days than in control cells (**Figure 5-15A**). At the protein level, Western immunoblotting identified induction of ACSS1 from six days culture in ethanol. A similar induction was observed in 1mM acetate but was apparent at 24 hours (**Figure 5-15B**). Although an increase in ACSS 2 was identified by band densitometry this was not sufficient to be apparent on visual examination of the blots and is therefore likely to be artefactual. These findings demonstrate, for the first time, that macrophages have the potential to increase synthesis of metabolically active acetyl-coA during ethanol exposure, making additional acetyl-coA available for use by HAT enzymes and the Krebs cycle.

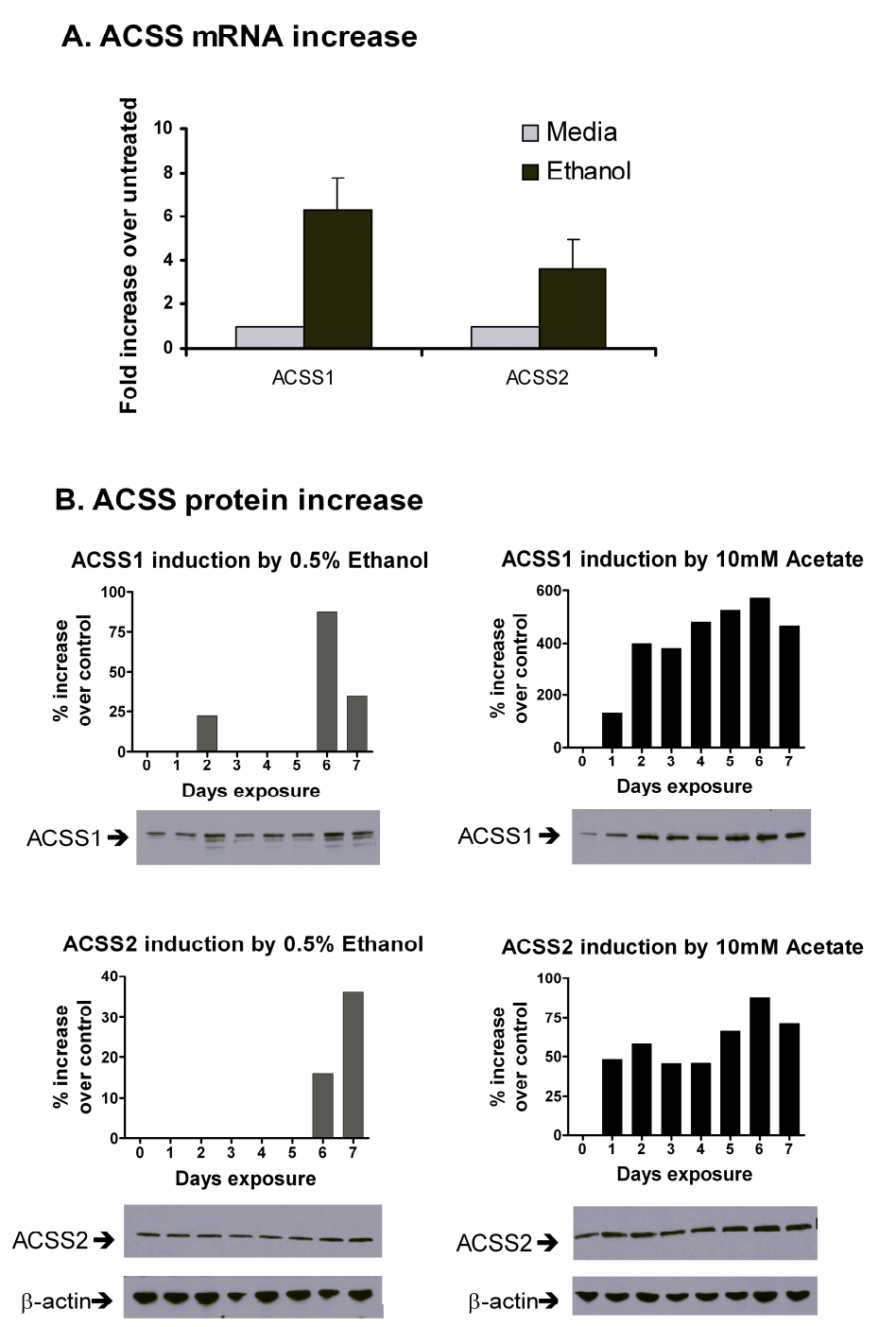


Figure 5-15 Induction of ACSS 1 and 2 by ethanol and acetate

(A) Monomac6 cells were cultured in normal media or media with 86mM ethanol for 7 days and assayed for ACSS1 and 2 mRNA by qRT-PCR. Values shown are mean + SEM for 3 independent determinations. (B) Monomac6 cells were cultured in 86mM ethanol or 1mM acetate for 0-7 days before lysis and proteins were separated by SDS-PAGE and identified by immunoblotting for ACSS1 and 2 with β-actin to control for differences in loading. Increases in band density over untreated cells were quantified relative to β-actin by band densitometry.

5.3.9 Effect of antioxidants and sirtuin activators

The antioxidants MnTBAP and trolox did not show any consistent effect upon cytokine output in this model system. Co-culture with the SIRT1 activator resveratrol reduced the LPS cytokine response virtually to zero in both ethanol-exposed and control cells. This lead to suspicion that the resveratrol was contaminated with LPS and producing a tolerance effect.

5.3.10 ACSS 1 and 2 knockdown abrogates the effect of ethanol

Western immunoblotting confirmed stable knockdown of ACSS1, ACSS2 and the double ACSS1+2 knockdown at the protein level (**Figure 5-16A**). The enhancement of cytokine output after incubation in 86mM ethanol was markedly diminished by ACSS knockdown, most significantly in the double ACSS1+2 knockdown cells. Cytokine output from the double knockdown cells was significantly lower than from the cells transduced with irrelevant transcript shRNA constructs at an equal multiplicity of infectivity (**Figure 5-16B**). These findings corroborate the idea that the augmented inflammatory cytokine response seen in alcoholic hepatitis occurs as a direct consequence of the metabolic effects of ethanol exposure modifying the transcriptional regulation of these cytokines *via* acetate and its conversion to acetyl-coA.

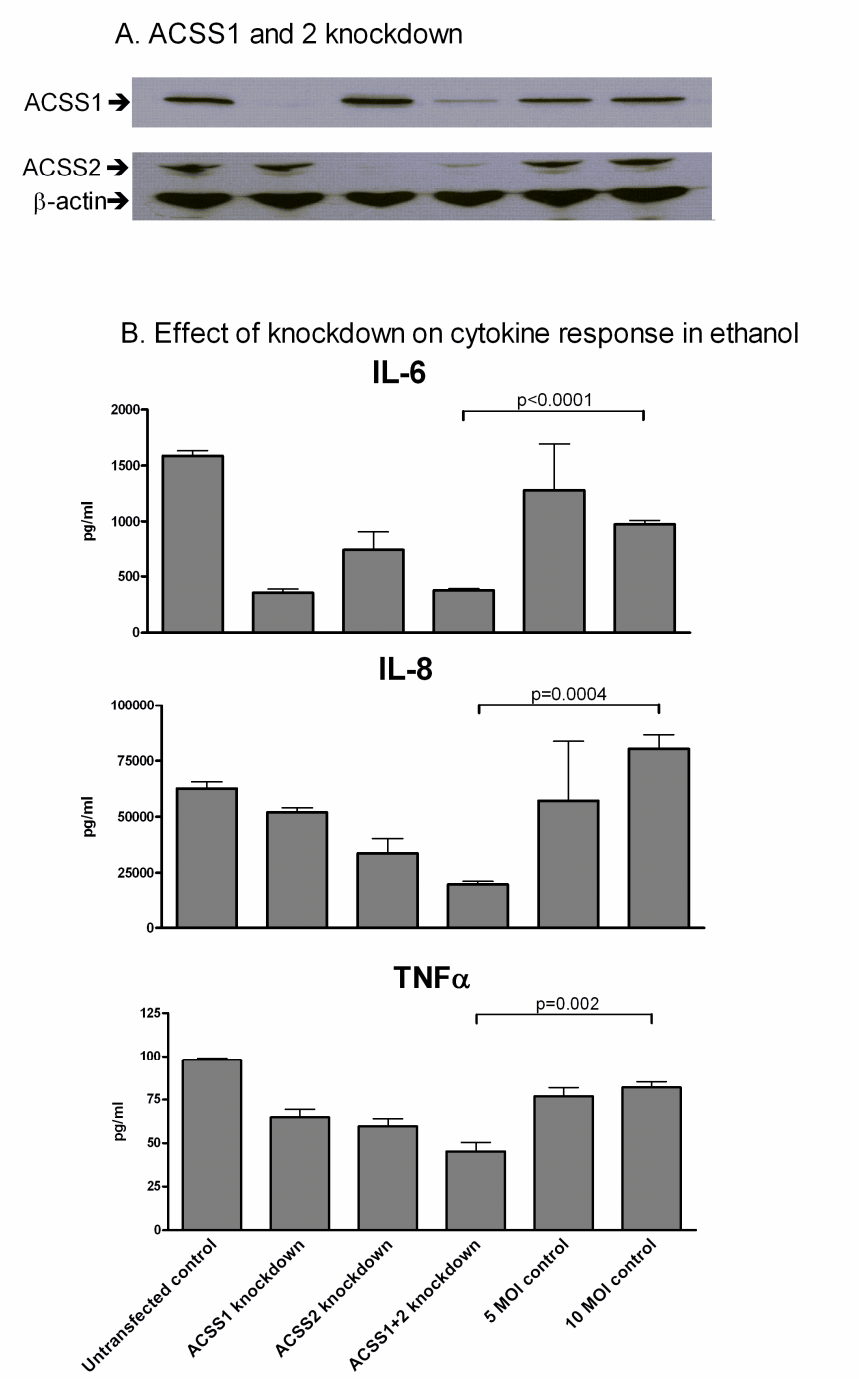


Figure 5-16 Effect of ACSS1 and 2 knockdowns on inflammatory cytokine responses in ethanol

Effective knockdown of ACSS1 and 2 protein expression was confirmed by Western blotting with β-actin as a loading control (A). Stable knockdowns, controls and untransfected cells were cultured in 86mM ethanol for 7 days and then stimulated with *E. coli* LPS 10ng/ml. Supernatant cytokines were determined by MSD immunoassay at 48 hours. Cytokine output was compared between knockdowns and their relevant controls. Values shown are mean + SEM for 3 independent determinations.

5.4 Discussion

5.4.1 Cytokines, tolerance and potential tissue effects

The enhanced cytokine response to bacterial endotoxin in AAH, ethanol-fed animals and ethanol-exposed cells is already established, and the MonoMac6 cells studied here displayed the same enhanced TNFα response reported elsewhere (Zhang, Bagby et al. 2001). The multiplex assay technology available allowed demonstration of a similar pattern of enhancement in IL-6 and IL-8, both shown to be of pathological significance in human AAH (Sheron, Bird et al. 1991; Sheron, Bird et al. 1993).

The disease-related cytokine pattern has been described as a failure of normal hepatic endotoxin tolerance (McClain, Hill et al. 2002), and this is an appropriate term for what is observed at the whole-organ level. However, although this cell line based model showed enhancement of cytokine transcription and release in ethanol, it also displayed normal tolerance to a second LPS stimulus. This may have been unexpected, but it is in keeping with the hypothesis that ethanol's key effect is at the level of gene expression rather than TLR receptor signalling. The majority of the endogenous regulatory molecules currently thought to play a role in the establishment of TLR response tolerance act on signalling intermediates in the TLR/NF-κB pathway and as such will silence inflammatory pathways upstream of any chromatin changes (Liew, Xu et al. 2005). It is conceivable that changes in the balance of acetylation and deacetylation mechanisms cannot alter gene expression when tolerance diminishes the initiation effect of activating transcription factors.

This persistence of a strong tolerising effect of prior LPS exposure in this model requires discussion as this could negate the effect of ethanol's enhancement of cytokine release in the physiological context of fluctuating continual LPS exposure from portal venous blood. Indeed, repeated LPS exposure might be considered to be a more physiological stimulus than a single dose. The term 'endotoxin tolerance' is used to describe phenomena in isolated cells, organ systems and whole organisms that are associated but not necessarily equivalent. In the isolated cell internal regulators of receptor signalling pathways and autocrine and paracrine effects of anti-inflammatory mediators can be enough to silence the cytokine response to a second LPS stimulus (Knolle, Schlaak et al. 1995; Liew, Xu et al. 2005). However, in a more physiological microenvironment stimulation of multiple TLRs by the mixture of microbial products in portal blood might overcome intracellular tolerance mechanisms (Broad 2007). Furthermore, the anatomical positioning of inflammatory cells and the flow of their surrounding fluid might reduce the autocrine effect of anti-inflammatory mediators. There is a substantial literature supporting the concept that macrophages from ethanol-exposed humans and rodents do not behave in a tolerised manner despite increased circulating LPS (Section 1.3.6). Further work to dissect out determinants of tolerised responses in these cells would include detailed timecourse

experiments, exposure to multiple TLR ligands, measurement of IL-10 and modulation of the cells' microenvironment by frequent media changes or use of a flow culture system.

Even with multiplex cytokine measurements, *in vitro* models like this can only inform us about the likely dysregulation of cytokine production in the inflamed, ethanol-exposed liver. They cannot tell us how this disordered cytokine milieu will affect the organ (or the organism) as a whole. It would be entirely reasonable to suggest that global hyperacetylation of the sort suggested by the immunofluorescence staining could influence most cellular mechanisms and enhance expression of multiple genes, including anti-inflammatory mediators, implying that the net effect of the prevailing cytokine milieu may not necessarily be pro-inflammatory. Findings with respect to the anti-inflammatory cytokine IL-10 are not presented above for simplicity but it showed similar enhancement at the mRNA and protein level with increased promoter histone acetylation on ChIP after ethanol incubation. It cannot be assumed, however, that increased IL-10 would 'cancel out' the effect of proinflammatory cytokines. Indeed, it is equally possible that the combined effects of augmented and unmodulated pro- and anti-inflammatory influences would be particularly deleterious for hepatocytes, hepatic stellate cells and endothelial cells. The profibrotic effects of some anti-inflammatory mediators are well established (Purohit and Brenner 2006). Furthermore, the same mechanisms that lead to enhanced expression of cytokine genes in macrophages may also act in other liver and immune cells to influence the expression of genes downstream of the cytokine receptors and hence the cellular response (activation, migration, apoptosis etc) to those cytokines. Only *in vivo* studies will help to answer these questions.

5.4.2 Increased histone acetylation

The observation of increased histone acetylation in a macrophage cell line after ethanol treatment is consistent with the findings of other groups that have recently demonstrated that ethanol increases histone acetylation in hepatocytes (Park, Miller et al. 2003), hepatic stellate cells (Kim and Shukla 2005) and whole rat tissues (Kim and Shukla 2006). There is also recent evidence that ethanol can reduce total HDAC activity (Choudhury and Shukla 2008). However, the present study is the first demonstration of ethanol modulation of gene expression in inflammatory cells by a mechanism dependent on histone acetylation. This increased acetylation could, in principle, arise through a number of routes. Ethanol metabolism, particularly at higher concentrations, produces a significant burden of reactive oxygen species (ROS) (Wheeler, Kono et al. 2001) and endoplasmic reticulum (ER) stress (Ji and Kaplowitz 2006). ROS can directly activate transcription factors such as NF-κBp65 (Cao, Mak et al. 2002) and oxidative and ER stress can favour a pro-inflammatory transcription factor milieu (Han, Hanawa et al. 2006). NF-κBp65 will recruit HAT co-activators to pro-inflammatory gene promoters and increase histone acetylation. Additionally, oxidative stress is known to inhibit HDAC recruitment to actively transcribed chromatin (Barnes, Ito et al. 2004). However, critically, these data have shown that exposure to acetate, the principle hepatic end-product of ethanol

metabolism, can fully mimic the effects on cytokine production seen with ethanol. As cytokine potentiation can occur without the ROS-generating metabolism of ethanol to acetate then oxidative stress cannot be solely responsible for the enhanced inflammatory response to ethanol. Antioxidants have been demonstrated to reduce the effect of ethanol on TNFα secretion in this model by another group (Zhang, Bagby et al. 2001) but the present study failed to corroborate these findings.

These data demonstrated that both ethanol and its metabolite acetate could reduce HDAC activity in a cell-free system. Free acetate is the end product of histone deacetylation, so acetate may increase histone acetylation through end-product inhibition of HDACs. Acetate may also increase histone acetylation through increased HAT activity. This could be through increased substrate supply (though for this acetate must be in the form of acetyl-coA) or indirectly through reduced HDAC activity. HDACs have a role in deacetylation of NF-κBp65 leading to a reduction in its ability to recruit HAT co-activators, so reduced HDAC activity can lead to increased HAT recruitment (Quivy and Van Lint 2004).

5.4.3 ACSS, acetyl-coA and sirtuins

Ethanol and acetate might also influence total HDAC activity by modulating the activity of sirtuins. These are class III HDACs whose activity is dependent on the presence of NAD^+ and which are increasingly recognised as a vital link between energy supply, gene expression, cellular activity and cellular ageing (Lavu, Boss et al. 2008). In this study the SIRT1 activator resveratrol did appear to inhibit cytokine responses to LPS in both ethanol and normal medium, although its mechanism of action in this case has not been fully elucidated. Metabolism of ethanol to acetate results in NAD^+ depletion which will reduce sirtuin and hence total HDAC activity. Free acetate will not affect NAD^+, but once converted to acetyl-coA it can enter the Krebs cycle and convert NAD^+ to NADH in the same way as if it had come from glycolysis or fatty acid oxidation. Acetate is converted to acetyl-coA through the action of the ACSS 1 and 2 enzymes whose activity is also dependent on sirtuin activity (North and Sinclair 2007). Acetyl-coA synthetases can be induced by acetate in prokaryotes (Bräsen and Schönheit 2004), although free acetate can also downregulate ACSS 1 and 2 through reduced SREBP transcription (Sakakibara, Yamauchi et al. 2006). These experiments demonstrated upregulation of ACSS 1 in this human cell line by acetate and, at a slower rate, by ethanol. Knockdown of ACSS 1 and 2 by shRNA significantly diminished ethanol's enhancement of cytokine responses to LPS and this implies that the supply of acetyl-coA from free acetate by ACSS enzymes makes a significant contribution to the increased inflammatory cytokine responses seen after chronic ethanol exposure.

Consideration should be given to the relative rates of metabolism of ethanol and acetate in different cell types. Ethanol metabolism in hepatocytes is rapid and inducible with plentiful alcohol and aldehyde dehydrogenases and cytochrome p450 2E1, but the majority of the

resulting acetate diffuses out of the hepatocyte (Yamashita, Kaneyuki et al. 2001). Acetate is metabolised in other tissues where it is converted to acetyl-coA by ACSS1 and 2 and either enters the Krebs cycle to yield carbon dioxide and water or is deployed for fatty acid synthesis or protein acetylation. Although the macrophage does metabolise ethanol (Wickramasinghe 1998), it cannot achieve the high rates of the hepatocyte, but it can produce acetyl-coA from acetate due to plentiful ACSS1 (Fujino, Ikeda et al. 2003). One could therefore speculate that in an ethanol-exposed whole liver the major determinant of enhanced cytokine production from Kupffer cells might be exogenous acetate released in high concentration by nearby ethanol-metabolising hepatocytes, rather than the lower concentrations generated within the Kupffer cell itself. This would mean that the effect observed *in vitro* in this isolated macrophage cell line would be magnified in the physiological setting.

In summary, these findings are evidence for a mechanism of enhanced inflammation in acute alcoholic hepatitis in which acetyl-coA synthetases are upregulated and convert the ethanol metabolite acetate to an excess of acetyl-coA which increases pro-inflammatory cytokine gene histone acetylation by increased substrate concentration and, potentially *via* NAD+ and sirtuins, HDAC inhibition, leading to enhanced gene expression and perpetuation of the inflammatory response. The clinical implication of these findings is that modulation of ACSS or specific HDAC or sirtuin activity might affect the clinical course of alcoholic liver injury in humans. If HDAC activators or inhibitors of ACSS 1 and 2 can modulate ethanol-associated histone changes without affecting the flow of acetyl-coA through the normal metabolic pathways then they would have potential as therapeutic options in acute alcoholic hepatitis.

6 Final discussion

6.1 Aims and outcomes of the project

This study was designed to explore the thesis that the augmented acute inflammatory responses characteristic of acute alcoholic hepatitis have both a genetic and an epigenetic basis and occur as a result of the interaction of metabolic and inflammatory processes at the level of gene expression. Investigation was developed around three principle hypotheses.

- That the functional S180L polymorphism in the TLR adapter molecule MAL, a critical genetic determinant of TLR-triggered disease, will contribute to the pathogenesis of both alcoholic liver disease and that other liver disease associated with excess energy metabolism, non-alcoholic steatohepatitis (Chapter 3).

Investigation confirmed an association between the MAL polymorphism and both cirrhotic ALD and advanced fibrotic NASH. Unexpectedly, different alleles associated with advanced disease in the two aetiologies. The C allele, associated with strong pro-inflammatory signalling from TLR2 and TLR4, was more common in advanced NASH while the T allele, associated with attenuated signalling, was more common in advanced ALD. It was postulated that this divergence could be due to individuals carrying the T allele relying more on an alternative (MyD88-independent) signalling pathway which is more susceptible to modulation by ethanol. Unexpected as they were, these findings corresponded with recently-published molecular biology work that suggested differential importance of the two signalling pathways in models of NASH and ALD.

The relatively high p values associated with these findings highlighted the possibility of a type 1 error, and it remains possible that future validation experiments in a separate cohort will not confirm the association between the polymorphism and advanced disease. This would not be unusual in ALD in which multiple studies have succeeded in identifying very few convincing genetic determinants. It is suggested that the genetic component to ALD susceptibility is likely to be small and significant only in those who develop advanced disease at an early age or relatively low cumulative ethanol dose. It is likely that environmental and epigenetic effects are the more significant determinants of ALD susceptibility in the population. It is particularly intriguing that several of the principal environmental factors influencing ethanol cirrhosis – smoking, diet/obesity/insulin resistance and caffeine intake – now have identifiable potential to influence histone acetylation, by HDAC inhibition, sirtuin inactivation and HDAC recruitment respectively.

- That acute alcoholic hepatitis is characterised by reduction in the sensitivity of immune responses to glucocorticoid inhibition and that this steroid insensitivity can be ameliorated by the epigenetic modulator theophylline (Chapter 4).

Measured *ex vivo* lymphocyte steroid sensitivity was observed to be significantly suppressed in AAH patients relative to normal controls and relative to the surviving patients after recovery. The *in vitro* addition of theophylline to the assay significantly improved steroid sensitivity. Confirmation of these findings and refinement of the technique in a larger cohort could influence therapy in AAH by minimising ineffective corticosteroid exposure and exploring the therapeutic potential of theophylline.

It was inferred that impaired steroid sensitivity was acquired in the pathogenesis of AAH. The effect of 10^{-5}M theophylline on this pathogenesis-related phenomenon, the fact that one of the few chemical effects of theophylline likely to be evident at this concentration is HDAC recruitment, and the fact that similar theophylline-responsive steroid insensitivity in COPD has an association with histone acetylation and HDAC inhibition all lent credibility to the theory that ethanol-driven HDAC inhibition and increased histone acetylation were aetiological factors in AAH.

- That the enhanced macrophage cytokine response to endotoxin seen in acute alcoholic hepatitis occurs through uncoupling of cytokine gene transcription from its normal regulatory mechanisms This occurs through increased histone acetylation at proinflammatory gene promoter regions, as a consequence of the interaction of ethanol metabolism with the epigenetic mechanisms of transcriptional regulation (Chapter 5).

In a cell-line model ethanol was confirmed to enhance cytokine responses to endotoxin at both the protein and the transcriptional level. Ethanol was demonstrated to have increased global protein and particularly histone acetylation, including in chromatin at the promoter regions of the specific cytokine genes. The effect of ethanol on cytokine responses was reproduced by acetate, the end product of ethanol metabolism, suggesting that acetate is involved in AAH pathogenesis. Acetyl-coA synthetases which produce metabolically active acetyl-coA from free acetate were demonstrated to be upregulated by ethanol and acetate and knockdown of these enzymes abrogated the effect of ethanol on cytokine production. This suggested that acetyl-coA, the key molecule of cellular energy release and storage, was also key to the modulation of inflammatory responses by ethanol.

The data indicated that this process was associated with reduced HDAC activity and there was circumstantial evidence for the involvement of the NAD+ dependent HDACs, the sirtuins. Acetyl-coA can provide the substrate for histone acetylation but also deplete NAD+ by its Krebs cycle metabolism. This could result in sirtuin inhibition, reduction in the rate of histone deacetylation and enhanced gene expression (**Figure 6-1**). The identification of a possible pathway from ethanol through acetate, ACSS, acetyl-coA, sirtuins and histone acetylation to enhanced gene expression and inflammatory responses is a novel concept, and one whose implications widen when it is considered that other sources of acetyl-coA including carbohydrates and triglycerides might also be able to modulate gene expression *via* the same pathway.

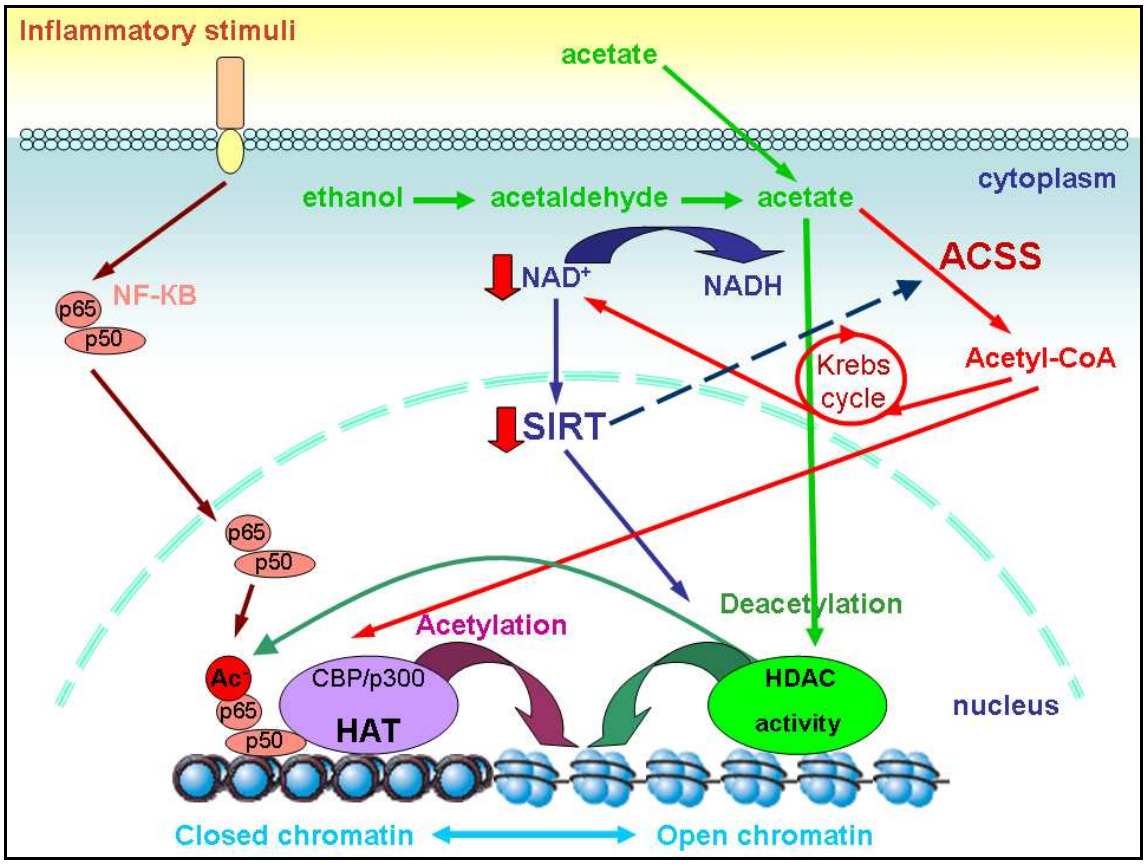


Figure 6-1 Potential roles of acetate, ACSS1 and 2 and sirtuins in ethanol-induced enhancement of inflammatory gene expression

Acetate is generated from ethanol metabolism in the inflammatory cell and in adjacent hepatocytes and may reduce HDAC activity by end-product inhibition (light green arrows). Acetyl-coA synthetases (ACSS) convert the acetate to acetyl-coA, the form in which it is a substrate for Krebs cycle metabolism and for histone acetylation by HATs (red arrows). Both ethanol and acetyl-coA metabolism deplete NAD+, leading to inactivation of sirtuin (SIRT) HDAC activity and hence reduced total HDAC activity (navy arrows). Reduced HDAC activity prolongs the acetylation of NF-κBp65 and hence its ability to recruit HATs to inflammatory genes in response to inflammatory stimuli (dark green arrow). The net effect of changes in HDAC and HAT activity is to increase and prolong histone acetylation and hence inflammatory gene transcription. Active sirtuins also increase ACSS activity (North and Sinclair 2007), which may provide a negative feedback limb in this pathway (dashed navy arrow).

6.2 Implications of the findings

The role of histone acetylation and epigenetics in the effects of alcohol has developed into a new and burgeoning field of study during the course of this project (Shukla, Velazquez et al.

2008; Shepard and Tuma 2009).The project's findings could add to this field, providing additional evidence for the role of epigenetics in ALD, reporting a role for histone acetylation in ethanol's enhancement of inflammatory responses for the first time, and postulating new therapeutic mechanisms in theophylline, Acetyl-coA synthetase inhibition and sirtuin activation. While the others require much more detailed laboratory investigation, theophylline, an established therapy with a known safety profile, is well placed for use in initial clinical studies in steroid-treated AAH.

The measurement of *ex vivo* steroid sensitivity has the potential to improve use of existing treatments by avoiding ineffective corticosteroid exposure, and hopefully identify early those patients who should be offered clinical trials of new therapeutic agents. However, the technique will require considerable development, refinement and validation before it can find use as a clinical decision-making tool.

In the last 20 years much of the research in ALD has focussed on the effect of ethanol on receptors, pathways and signalling intermediates and a clear role in modulation of signalling, largely through induction of oxidative and ER stress, was emerging. New discoveries regarding the role of ethanol in transcriptional regulation do not make its effects on signalling pathways irrelevant. The presence of TLR4 and NF-κB have been shown to be critical for the development of ethanol-induced liver injury and it would seem unlikely than even a hyperacetylated inflammatory gene promoter would initiate significant tissue damage in their absence. However, it may become clear that the signalling effects of ethanol are necessary but not sufficient for the development of liver injury and its downstream effects on gene expression may have equal importance. In therapeutic terms, it is possible that specific modulators directed at this downstream component of the pathogenic mechanism might be better tolerated with fewer off-target effects that might limit their clinical usefulness. However, it is unlikely that all the clinical effects of chemical interference with mechanisms of gene expression will be benign and predictable.

The close coupling of energy metabolism and modulation of inflammation and gene expression hinted at by these findings and by the very existence of sirtuins is likely to be a fascinating and fertile area of research in the years to come. It has been shown that through their NAD+ dependence sirtuins mediate the positive effects of calorie restriction on longevity, metabolism, mitochondrial function and insulin sensitivity, promote physical activity and moderate illness behaviour in animals (Baur, Pearson et al. 2006; Lagouge, Argmann et al. 2006). Sirtuin activation can mimic the cellular effects of calorie restriction and therefore has potential as a therapeutic strategy in diseases associated with energy over-supply, particularly type II diabetes mellitus and the metabolic syndrome (and by inference NASH), but could potentially also mitigate the effects of ethanol-induced increases in acetyl-coA and decreases in NAD+.

6.3 The findings in context

The findings and discussion above beg the question of what the evolutionary basis could be for such a deleterious response to plentiful energy supply.

Nutritional excess may be common now but has not been a frequent event during evolutionary time. More often starvation has been the driver for natural selection, and phenotypes that can survive and function despite starvation have been more likely to pass their genetic traits on to subsequent generations. During times of calorie deficiency, individuals would benefit from mechanisms that limit unnecessary gene expression and the energy-consuming effects of vigorous inflammatory responses. Increased aerobic capacity, physical activity and reduced illness behaviours would allow them to persist longer in the search for food. Longevity in the face of starvation increases the animal's chance of surviving long enough to encounter a mate and pass on its genetic material. Where nutrition is plentiful, individuals will live closer together and often compete to mate. The priorities change to strength development, protein synthesis and effective inflammation, healing responses and illness behaviour to limit activity and promote recovery. After mating the genetic imperative for longevity becomes less important.

With this in mind it is understandable that mechanisms responding to nutritional excess might lead to enhanced inflammation, reduced lifespan and lassitude, all features of the metabolic syndrome as well as NASH and ALD. Interestingly, another liver condition, primary biliary cirrhosis (PBC) is associated with autoimmunity and lassitude and recently has been demonstrated to be associated with impairment of mitochondrial biogenesis, which could be postulated to lead to failure of NAD+ regeneration and accumulation of acetyl-coA (Hollingsworth, Newton et al. 2008).

6.4 Future directions

Clinical directions for this work include evaluation of theophylline as an adjunctive treatment in corticosteroid-treated AAH and refinement of biomarkers of steroid responsiveness in this condition. Small molecule sirtuin activators are already being studied in type II diabetes and have shown some early promise in reduction of steatosis in a rodent model of NASH (Yamazaki, Usui et al. 2009). Clinical trials in human NASH would be a possible next step.

Scientific directions include studying histone acetylation, gene expression and activities of sirtuins and ACSS in human patients and animal models of ALD and NASH. Evaluation of small molecule sirtuin activators in models of ALD might inform future clinical studies. Detailed study of the relationship between energy metabolism and inflammation could inform future work beyond the field of liver and ethanol-related disease.

6.5 Synthesis

Even the most basic multicellular organism, the social amoeba *Dictyostelium discoideum*, shows specialisation of cells that mediate both of the crucial aspects of interaction with the environment – nutrition and host defence (Chen, Zhuchenko et al. 2007). This co-localisation of metabolism and immunity is phylogenetically preserved and anatomically localised in the liver. However, it is only in recent years that the importance of the liver and hepatic metabolism in immunity and inflammation has emerged as a field of study (O'Farrelly 2004). The findings of this project emphasise the importance of this metabolism-inflammation interaction in the pathogenesis of a common human disease. The potential role of acetyl-coA, the key molecule of cellular energetic and biosynthetic pathways, in the modulation of the inflammatory changes associated with acute alcoholic hepatitis further reinforces the intimate relationship between metabolism and immunity and the role of the liver as the intersection of these vital processes.

7 References

(2003). "Whither RNAi?" Nat Cell Biol **5**(6): 489-490.

Abreu, M. T., M. Fukata, et al. (2005). "TLR Signaling in the Gut in Health and Disease." J Immunol **174**(8): 4453-4460.

Adachi, Y., B. U. Bradford, et al. (1994). "Inactivation of Kupffer cells prevents early alcohol-induced liver injury." Hepatology **20**(2): 453-460.

Adachi, Y., L. E. Moore, et al. (1995). "Antibiotics prevent liver injury in rats following long-term exposure to ethanol." Gastroenterology **108**(1): 218-224.

Adib-Conquy, M. and J. M. Cavaillon (2002). "Gamma interferon and granulocyte/monocyte colony-stimulating factor prevent endotoxin tolerance in human monocytes by promoting interleukin-1 receptor-associated kinase expression and its association to MyD88 and not by modulating TLR4 expression." J Biol Chem **277**(31): 27927-27934.

Afford, S. C., N. C. Fisher, et al. (1998). "Distinct patterns of chemokine expression are associated with leukocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis." J Pathol **186**(1): 82-89.

Akerman, P. A., P. M. Cote, et al. (1993). "Long-term ethanol consumption alters the hepatic response to the regenerative effects of tumor necrosis factor-alpha." Hepatology **17**(6): 1066-1073.

Akriviadis, E., R. Botla, et al. (2000). "Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: A double-blind, placebo-controlled trial." Gastroenterology **119**(6): 1637-1648.

Albano, E., P. Clot, et al. (1996). "Role of cytochrome P4502E1-dependent formation of hydroxyethyl free radical in the development of liver damage in rats intragastrically fed with ethanol." Hepatology **23**(1): 155-163.

Aldred, A. and L. E. Nagy (1999). "Ethanol dissociates hormone-stimulated cAMP production from inhibition of TNF-alpha production in rat Kupffer cells." Am J Physiol Gastrointest Liver Physiol **276**(1): G98-106.

Alexander, J., M. Lischner, et al. (1971). "Natural history of alcoholic hepatitis. II. The long-term prognosis." American Journal of Gastroenterology **56**(6): 515-525.

Annane, D., V. Sebille, et al. (2002). "Effect of Treatment With Low Doses of Hydrocortisone and Fludrocortisone on Mortality in Patients With Septic Shock." JAMA **288**(7): 862-871.

Armant, M. and M. Fenton (2002). "Toll-like receptors: a family of pattern-recognition receptors in mammals." Genome Biology **3**(8): reviews3011.3011 - 3011.3016.

Arteel, G. E. (2003). "Oxidants and antioxidants in alcohol-induced liver disease." Gastroenterology **124**(3): 778-790.

Ashley, M. J., J. S. Olin, et al. (1977). "Morbidity in alcoholics. Evidence for accelerated development of physical disease in women." Arch Intern Med **137**(7): 883-887.

Atkinson, K. J. and R. K. Rao (2001). "Role of protein tyrosine phosphorylation in acetaldehyde-induced disruption of epithelial tight junctions." Am J Physiol Gastrointest Liver Physiol **280**(6): G1280-1288.

Austin, A. S., P. Kaye, et al. (2006). "Heterogeneity of liver histology in severe alcoholic hepatitis diagnosed on clinical criteria." Gut **55**(suppl_2): a1-119 abstract 180.

Badrick, E., M. Bobak, et al. (2007). "The Relationship between Alcohol Consumption and Cortisol Secretion in an Aging Cohort." J Clin Endocrinol Metab: jc.2007-0737.

Baillie, M. (1793). The Morbid Anatomy of Some of the Most Important Parts of the Human Body. London, Johnson.

Baptista, A. (1981). "Alcoholic liver disease: morphological manifestations. Review by an international group." Lancet **1**(8222): 707-711.

Barnes, P. J. (2005). "Theophylline in Chronic Obstructive Pulmonary Disease: New Horizons." Proc Am Thorac Soc **2**(4): 334-339.

Barnes, P. J., I. M. Adcock, et al. (2005). "Histone acetylation and deacetylation: importance in inflammatory lung diseases." Eur Respir J **25**(3): 552-563.

Barnes, P. J., K. Ito, et al. (2004). "Corticosteroid resistance in chronic obstructive pulmonary disease: inactivation of histone deacetylase." Lancet **363**: 731-733.

Basuroy, S., P. Sheth, et al. (2005). "Acetaldehyde disrupts tight junctions and adherens junctions in human colonic mucosa: protection by EGF and L-glutamine." Am J Physiol Gastrointest Liver Physiol **289**(2): G367-375.

Bathgate, A. J. (2006). "Recommendations for alcohol-related liver disease." The Lancet **367**(9528): 2045-2046.

Baur, J. A., K. J. Pearson, et al. (2006). "Resveratrol improves health and survival of mice on a high-calorie diet." Nature **444**(7117): 337-342.

Bautista, A. P. (1997). "Chronic alcohol intoxication induces hepatic injury through enhanced macrophage inflammatory protein-2 production and intercellular adhesion molecule-1 expression in the liver." Hepatology **25**(2): 335-342.

Bautista, A. P. (2000). "Impact of alcohol on the ability of Kupffer cells to produce chemokines and its role in alcoholic liver disease." Journal of Gastroenterology and Hepatology **15**(4): 349-356.

Bautista, A. P. (2002). "Chronic alcohol intoxication primes Kupffer cells and endothelial cells for enhanced CC-chemokine production and concomitantly suppresses phagocytosis and chemotaxis." Front Biosci **7**: a117-125.

Bautista, A. P. (2002). "Neutrophilic infiltration in alcoholic hepatitis." Alcohol **27**(1): 17-21.

Bautista, A. P. and J. J. Spitzer (1996). "Cross-tolerance between acute alcohol intoxication and endotoxemia." Alcohol Clin Exp Res **20**(8): 1395-1400.

Beato, M., M. Truss, et al. (1996). "Control of transcription by steroid hormones." Ann N Y Acad Sci **784**: 93-123.

Becker, U., A. Deis, et al. (1996). "Prediction of risk of liver disease by alcohol intake, sex, and age: A prospective population study." Hepatology **23**(5): 1025-1029.

Becker, U., M. Gronbaek, et al. (2002). "Lower risk for alcohol-induced cirrhosis in wine drinkers." Hepatology **35**(4): 868-875.

Beckett, A. G., A. V. Livingstone, et al. (1961). "Acute alcoholic hepatitis." British Medical Journal **5260**: 1113-1119.

Bekeredjian-Ding, I., S. I. Roth, et al. (2006). "T Cell-Independent, TLR-Induced IL-12p70 Production in Primary Human Monocytes." J Immunol **176**(12): 7438-7446.

Bellentani, S., G. Saccoccio, et al. (1997). "Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group." Gut **41**(6): 845-850.

Bhagwandeen, B., M. Apte, et al. (1987). "Endotoxin induced hepatic necrosis in rats on an alcohol diet." Journal of Pathology **152**(1): 47-53.

Bird, G., N. Sheron, et al. (1990). "Increased plasma tumor necrosis factor in severe alcoholic hepatitis." Ann Intern Med **112**(12): 917-920.

Bloomgarden, Z. T. (2005). "Second World Congress on the Insulin Resistance Syndrome." Diabetes Care **28**(6): 1518-1523.

Bode, C. and J. C. Bode (2003). "Effect of alcohol consumption on the gut." Best Practice and Research Clinical Gastroenterology **17**(4): 575-592.

Bode, C. and J. C. Bode (2005). "Activation of the Innate Immune System and Alcoholic Liver Disease: Effects of Ethanol per se or Enhanced Intestinal Translocation of Bacterial Toxins Induced by Ethanol?" Alcoholism: Clinical and Experimental Research **29**(s2): 166S-171S.

Bode, C., C. Schafer, et al. (1997). "Effect of Treatment with Paromomycin on Endotoxemia in Patients with Alcoholic Liver Disease;A Double-Blind, Placebo-Controlled Trial." Alcoholism: Clinical and Experimental Research **21**(8): 1367-1373.

Bode, K. A., K. Schroder, et al. (2007). "Histone deacetylase inhibitors decrease Toll-like receptor-mediated activation of proinflammatory gene expression by impairing transcription factor recruitment." Immunology **122**(4): 596-606.

Boetticher, N. C., C. J. Peine, et al. (2008). "A randomized, double-blinded, placebo-controlled multicenter trial of etanercept in the treatment of alcoholic hepatitis." Gastroenterology **135**(6): 1953-1960.

Boyault, C., K. Sadoul, et al. (2007). "HDAC6, at the crossroads between cytoskeleton and cell signaling by acetylation and ubiquitination." Oncogene **26**(37): 5468-5476.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-254.

Bräsen, C. and P. Schönheit (2004). "Regulation of acetate and acetyl-CoA converting enzymes during growth on acetate and/or glucose in the halophilic archaeon Haloarcula marismortui." FEMS Microbiology Letters **241**(1): 21-26.

Broad, A. (2007). Endotoxin tolerance in context: microenvironments, measurements and toll-like receptor interactions. Institute of Cellular Medicine. Newcastle upon Tyne, Newcastle University. **PhD:** 328.

Broad, A., D. Jones, et al. (2006). "Toll-like receptor (TLR) response tolerance: a key physiological 'damage limitation' effect and an important potential opportunity for therapy." Current Medicinal Chemistry **13**(21): 2487-2502.

Brook, M., G. Sully, et al. (2000). "Regulation of tumour necrosis factor [alpha] mRNA stability by the mitogen-activated protein kinase p38 signalling cascade." FEBS Letters **483**(1): 57-61.

Brown, L. A., F. L. Harris, et al. (2001). "Chronic ethanol ingestion potentiates TNF-alpha-mediated oxidative stress and apoptosis in rat type II cells." Am J Physiol Lung Cell Mol Physiol **281**(2): L377-386.

Browning, J. D., K. S. Kumar, et al. (2004). "Ethnic differences in the prevalence of cryptogenic cirrhosis." Am J Gastroenterol **99**(2): 292-298.

Brun, P., I. Castagliuolo, et al. (2006). "Increased risk of NASH in patients carrying the C(-159)T polymorphism in the CD14 gene promoter region." Gut **55**(8): 1212.

Brunt, E. M., C. G. Janney, et al. (1999). "Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions." Am J Gastroenterol **94**(9): 2467-2474.

Cabré, E., P. Rodríguez-Iglesias, et al. (2000). "Short- and long-term outcome of severe alcohol-induced hepatitis treated with steroids or enteral nutrition: A multicenter randomized trial." Hepatology **32**(1): 36-42.

Caetano, R. and C. L. Clark (1998). "Trends in alcohol-related problems among whites, blacks, and Hispanics: 1984-1995." Alcohol Clin Exp Res **22**(2): 534-538.

Caldwell, S. H., D. M. Harris, et al. (2002). "Is NASH underdiagnosed among African Americans?" Am J Gastroenterol **97**(6): 1496-1500.

Canbay, A., A. E. Feldstein, et al. (2003). "Kupffer cell engulfment of apoptotic bodies stimulates death ligand and cytokine expression." Hepatology **38**(5): 1188-1198.

Cao, Q., K. M. Mak, et al. (2002). "Dilinoleoylphosphatidylcholine decreases LPS-induced TNF-alpha generation in Kupffer cells of ethanol-fed rats: respective roles of MAPKs and NF-kappaB." Biochemical and Biophysical Research Communications **294**(4): 849-853.

Carithers, R. J., H. Herlong, et al. (1989). "Methylprednisolone therapy in patients with severe alcoholic hepatitis. A randomized multicenter trial." Ann Intern Med **110**(9): 685-690.

Chan, E. D., D. W. H. Riches, et al. (2001). "Redox Paradox: Effect of N-Acetylcysteine and Serum on Oxidation Reduction-Sensitive Mitogen-Activated Protein Kinase Signaling Pathways." Am. J. Respir. Cell Mol. Biol. **24**(5): 627-632.

Chan, E. S., M. C. Montesinos, et al. (2006). "Adenosine A(2A) receptors play a role in the pathogenesis of hepatic cirrhosis." Br J Pharmacol **148**(8): 1144-1155.

Chen, G., O. Zhuchenko, et al. (2007). "Immune-like Phagocyte Activity in the Social Amoeba." Science **317**(5838): 678-681.

Child, C. G. and J. G. Turcotte (1964). Surgery and portal hypertension. The liver and portal hypertension. C. G. Child. Philadelphia, W.B. Saunders Co.**:** 50.

Chilson, O. P. and A. E. Kelly-Chilson (1989). "Mitogenic lectins bind to the antigen receptor on human lymphocytes." European Journal of Immunology **19**(2): 389-396.

Chomczynski, P. and N. Sacchi (1987). "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction." Anal Biochem **162**(1): 156-159.

Choudhury, M. and S. D. Shukla (2008). "Surrogate alcohols and their metabolites modify histone H3 acetylation: involvement of histone acetyl transferase and histone deacetylase." Alcohol Clin Exp Res **32**(5): 829-839.

Christensen, E. and C. Gluud (1995). "Glucocorticoids are ineffective in alcoholic hepatitis: A meta-analysis adjusting for confounding variables." Gut **37**(1): 113-118.

Christensen, E., P. Schlichting, et al. (1984). "Prognostic value of Child-Turcotte criteria in medically treated cirrhosis." Hepatology **4**(3): 430-435.

Cohen, J. A. and M. M. Kaplan (1979). "The SGOT/SGPT ratio--an indicator of alcoholic liver disease." Dig Dis Sci **24**(11): 835-838.

Conjeevaram, H. S., J. Hart, et al. (1999). "Rapidly progressive liver injury and fatal alcoholic hepatitis occurring after liver transplantation in alcoholic patients." Transplantation **67**(12): 1562-1568.

Corrigan, C. J., P. H. Brown, et al. (1991). "Glucocorticoid resistance in chronic asthma. Glucocorticoid pharmacokinetics, glucocorticoid receptor characteristics, and inhibition of peripheral blood T cell proliferation by glucocorticoids in vitro." Am Rev Respir Dis **144**(5): 1016-1025.

Creed, T. J., M. R. Norman, et al. (2003). "Basiliximab (anti-CD25) in combination with steroids may be an effective new treatment for steroid-resistant ulcerative colitis." Aliment Pharmacol Ther **18**(1): 65-75.

Creed, T. J., C. S. Probert, et al. (2006). "Basiliximab for the treatment of steroid-resistant ulcerative colitis: further experience in moderate and severe disease." Aliment Pharmacol Ther **23**(10): 1435-1442.

D'Souza, N. B., S. Nelson, et al. (1994). "Expression of tumor necrosis factor-alpha and interleukin-6 cell-surface receptors of the alveolar macrophage in alcohol-treated rats." Alcohol Clin Exp Res **18**(6): 1430-1435.

Day, C. (2002). "CD14 promoter polymorphism associated with risk of NASH." Journal of Hepatology **36**(Supplement 1): 21-21.

Day, C. P. (1996). "Moderate alcohol intake is not deleterious in patients with alcoholic liver disease." Hepatology **24**(Supplement): 443A.

Day, C. P. (2001). "Apoptosis in alcoholic hepatitis: a novel therapeutic target?" Journal of Hepatology **34**(2): 330-333.

Day, C. P. (2006). "Genes or environment to determine alcoholic liver disease and non-alcoholic fatty liver disease." Liver International **26**(9): 1021-1028.

Day, C. P. and O. F. W. James (1998). "Hepatic steatosis: Innocent bystander or guilty party?" Hepatology **27**(6): 1463-1466.

de Alwis, N. M. W. and C. P. Day (2007). "Genetics of alcoholic liver disease and nonalcoholic fatty liver disease." Semin Liver Dis **27**(1): 44-54.

de la Monte, S. M., N. Ganju, et al. (1999). "Differential effects of ethanol on insulin-signaling through the insulin receptor substrate-1." Alcohol Clin Exp Res **23**(5): 770-777.

Deaciuc, I. V., N. B. D'souza, et al. (2001). "Inhibition of Caspases In Vivo Protects the Rat Liver Against Alcohol-Induced Sensitization to Bacterial Lipopolysaccharide." Alcoholism: Clinical and Experimental Research **25**(6): 935-943.

Deaciuc, I. V., F. Fortunato, et al. (1999). "Modulation of caspase-3 activity and Fas ligand mRNA expression in rat liver cells in vivo by alcohol and lipopolysaccharide." Alcohol Clin Exp Res **23**(2): 349-356.

Decker, T., M. L. Lohmann-Matthes, et al. (1989). "Comparative study of cytotoxicity, tumor necrosis factor, and prostaglandin release after stimulation of rat Kupffer cells, murine Kupffer cells, and murine inflammatory liver macrophages." J Leukoc Biol **45**(2): 139-146.

Devalaraja, M. N., C. J. McClain, et al. (1999). "Increased monocyte MCP-1 production in acute alcoholic hepatitis." Cytokine **11**(11): 875-881.

Deviere, J., J. Content, et al. (1989). "High interleukin-6 serum levels and increased production by leucocytes in alcoholic liver cirrhosis. Correlation with IgA serum levels and lymphokines production." Clin Exp Immunol **77**(2): 221-225.

Deviere, J., J. Content, et al. (1990). "Excessive in vitro bacterial lipopolysaccharide-induced production of monokines in cirrhosis." Hepatology **11**(4): 628-634.

Deviere, J., J. P. Vaerman, et al. (1991). "IgA triggers tumor necrosis factor alpha secretion by monocytes: a study in normal subjects and patients with alcoholic cirrhosis." Hepatology **13**(4): 670-675.

Dewint, P., V. Gossye, et al. (2008). "A Plant-Derived Ligand Favoring Monomeric Glucocorticoid Receptor Conformation with Impaired Transactivation Potential Attenuates Collagen-Induced Arthritis." J Immunol **180**(4): 2608-2615.

Diehl, A. M., J. Potter, et al. (1984). "Relationship between pyridoxal 5'-phosphate deficiency and aminotransferase levels in alcoholic hepatitis." Gastroenterology **86**(4): 632-636.

Dobrovolskaia, M. A., A. E. Medvedev, et al. (2003). "Induction of In Vitro Reprogramming by Toll-Like Receptor (TLR)2 and TLR4 Agonists in Murine Macrophages: Effects of TLR "Homotolerance" Versus "Heterotolerance" on NF-kappaB Signaling Pathway Components." J Immunol **170**(1): 508-519.

Dong, C., R. J. Davis, et al. (2002). "MAP Kinases in the Immune Response." Annual Review of Immunology **20**(1): 55-72.

Drenick, E. J., J. Fisler, et al. (1982). "Hepatic steatosis after intestinal bypass--prevention and reversal by metronidazole, irrespective of protein-calorie malnutrition." Gastroenterology **82**(3): 535-548.

Dunn, W., L. H. Jamil, et al. (2005). "MELD accurately predicts mortality in patients with alcoholic hepatitis." Hepatology **41**(2): 353-358.

Eberharter, A. and P. B. Becker (2002). "Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics." EMBO Rep **3**(3): 224-229.

El-Assal, O., F. Hong, et al. (2004). "IL-6-deficient mice are susceptible to ethanol-induced hepatic steatosis: IL-6 protects against ethanol-induced oxidative stress and mitochondrial permeability transition in the liver." Cell Mol Immunol **1**(3): 205-211.

Elphick, D. A., A. K. Dube, et al. (2007). "Spectrum of Liver Histology in Presumed Decompensated Alcoholic Liver Disease." The American Journal of Gastroenterology **102**(4): 780-788.

Enomoto, N., K. Ikejima, et al. (1998). "Alcohol causes both tolerance and sensitization of rat Kupffer cells via mechanisms dependent on endotoxin." Gastroenterology **115**(2): 443-451.

Enomoto, N., K. Ikejima, et al. (2000). "Role of Kupffer cells and gut-derived endotoxins in alcoholic liver injury1." Journal of Gastroenterology and Hepatology **15**(s1): 20-25.

Enomoto, N., Y. Takei, et al. (2003). "Prevention of Ethanol-Induced Liver Injury in Rats by an Agonist of Peroxisome Proliferator-Activated Receptor-{gamma}, Pioglitazone." J Pharmacol Exp Ther **306**(3): 846-854.

Erbey, J. R., C. Silberman, et al. (2000). "Prevalence of abnormal serum alanine aminotransferase levels in obese patients and patients with type 2 diabetes." Am J Med **109**(7): 588-590.

Everhart, J. E. and T. P. Beresford (1997). "Liver transplantation for alcoholic liver disease: a survey of transplantation programs in the United States." Liver Transpl Surg **3**(3): 220-226.

Felver, M. E., E. Mezey, et al. (1990). "Plasma Tumor Necrosis Factor Alpha Predicts Decreased Long-Term Survival in Severe Alcoholic Hepatitis." Alcoholism: Clinical and Experimental Research **14**(2): 255-259.

Fernandez-Checa, J. C. and N. Kaplowitz (2005). "Hepatic mitochondrial glutathione: transport and role in disease and toxicity." Toxicology and Applied Pharmacology **204**(3): 263-273.

Fisher, N. C., D. A. Neil, et al. (1999). "Serum concentrations and peripheral secretion of the beta chemokines monocyte chemoattractant protein 1 and macrophage inflammatory protein 1alpha in alcoholic liver disease." Gut **45**(3): 416-420.

Fitzgerald, K. A., E. M. Palsson-McDermott, et al. (2001). "Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction." Nature **413**(6851): 78-83.

Foreman, M. G., T. T. Hoor, et al. (2002). "Effects of chronic hepatic dysfunction on pulmonary glutathione homeostasis." Alcohol Clin Exp Res **26**(12): 1840-1845.

Forrest, E. H., C. D. J. Evans, et al. (2005). "Analysis of factors predictive of mortality in alcoholic hepatitis and derivation and validation of the Glasgow alcoholic hepatitis score." Gut **54**(8): 1174-1179.

Forrest, E. H., A. J. Morris, et al. (2007). "The Glasgow alcoholic hepatitis score identifies patients who may benefit from corticosteroids." Gut **56**(12): 1743-1746.

Foukas, L. C., N. Daniele, et al. (2002). "Direct effects of caffeine and theophylline on p110 delta and other phosphoinositide 3-kinases. Differential effects on lipid kinase and protein kinase activities." J Biol Chem **277**(40): 37124-37130.

Frojdo, S., D. Cozzone, et al. (2007). "Resveratrol is a class IA phosphoinositide 3-kinase inhibitor." Biochem J **406**(3): 511-518.

Fujimoto, M., M. Uemura, et al. (2000). "Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: relation to severity of liver disturbance." Alcoholism: Clinical and Experimental Research **24**(4): 48S-54S.

Fujino, T., Y. Ikeda, et al. (2003). "Sources of Acetyl-CoA: Acetyl-CoA Synthetase 1 and 2." Current Medicinal Chemistry - Immunology, Endocrine & Metabolic Agents **3**: 207-210.

Fukui, H. (2005). "Relation of Endotoxin, Endotoxin Binding Proteins and Macrophages to Severe Alcoholic Liver Injury and Multiple Organ Failure." Alcoholism: Clinical and Experimental Research **29**(s2): 172S-179S.

Fukui, H., B. Brauner, et al. (1991). "Plasma endotoxin concentrations in patients with alcoholic and non-alcoholic liver disease: reevaluation with an improved chromogenic assay." Journal of Hepatology **12**(2): 162-192.

Funder, J. W. (1997). "Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance." Annual Review Of Medicine **48**: 231-240.

Galambos, J. (1972). "Natural history of alcoholic hepatitis. 3. Histological changes." Gastroenterology **63**(6): 1026-1035.

Gobejishvili, L., S. Barve, et al. (2008). "Enhanced PDE4B expression augments LPS-inducible TNF expression in ethanol-primed monocytes: relevance to alcoholic liver disease." Am J Physiol Gastrointest Liver Physiol **295**(4): G718-724.

Goldberg, S., C. Mendenhall, et al. (1986). "VA Cooperative Study on Alcoholic Hepatitis. IV. The significance of clinically mild alcoholic hepatitis--describing the population with minimal hyperbilirubinemia." American Journal of Gastroenterology **81**(11): 1029-1034.

Gong, J. P., C. X. Wu, et al. (2002). "Intestinal damage mediated by Kupffer cells in rats with endotoxemia." World J Gastroenterol **8**(5): 923-927.

Gonzalez-Quintela, A., J. Campos, et al. (2007). "Serum concentrations of interleukin-8 in relation to different levels of alcohol consumption." Cytokine **38**(1): 54-60.

Gopal, Y. N. V., T. S. Arora, et al. (2006). "Tumour necrosis factor-alpha depletes histone deacetylase 1 protein through IKK2." EMBO Reports **7**(3): 291-296.

Grove, J., A. K. Daly, et al. (1997). "Association of a tumor necrosis factor promoter polymorphism with susceptibility to alcoholic steatohepatitis." Hepatology **26**(1): 143-146.

Grove, J., A. K. Daly, et al. (2000). "Interleukin 10 promoter region polymorphisms and susceptibility to advanced alcoholic liver disease." Gut **46**(4): 540-545.

Grunstein, M. (1997). "Histone acetylation in chromatin structure and transcription." Nature **389**(6649): 349-352.

Guha, M. and N. Mackman (2001). "LPS induction of gene expression in human monocytes." Cellular Signalling **13**(2): 85-94.

Gustot, T., A. Lemmers, et al. (2006). "Differential liver sensitization to Toll-like receptor pathways in mice with alcoholic fatty liver." Hepatology **43**(5): 989-1000.

Hafenrichter, D., C. Roland, et al. (1994). "The Kupffer cell in endotoxin tolerance: mechanisms of protection against lethal endotoxemia." Shock **2**(4): 251-256.

Halsted, C. H., J. A. Villanueva, et al. (2002). "Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig." Proc Natl Acad Sci U S A **99**(15): 10072-10077.

Hamdi, H., A. Bigorgne, et al. (2007). "Glucocorticoid-induced leucine zipper: A key protein in the sensitization of monocytes to lipopolysaccharide in alcoholic hepatitis." Hepatology **46**(6): 1986-1992.

Han, D., N. Hanawa, et al. (2006). "Mechanisms of Liver Injury. III. Role of glutathione redox status in liver injury." Am J Physiol Gastrointest Liver Physiol **291**(1): G1-7.

Han, J. and R. J. Ulevitch (2005). "Limiting inflammatory responses during activation of innate immunity." Nature Immunology **6**: 1198-1205.

Hanck, C., M. Glatzel, et al. (2000). "Gene expression of TNF-receptors in peripheral blood mononuclear cells of patients with alcoholic cirrhosis." J Hepatol **32**(1): 51-57.

Hanck, C., T. Manigold, et al. (2001). "Gene expression of interleukin 18 in unstimulated peripheral blood mononuclear cells of patients with alcoholic cirrhosis." Gut **49**(1): 106-111.

Hanck, C., S. Rossol, et al. (1998). "Presence of plasma endotoxin is correlated with tumour necrosis factor receptor levels and disease activity in alcoholic cirrhosis." Alcohol Alcohol. **33**(6): 606-608.

Hardison, W. and F. Lee (1966). "Prognosis in acute liver disease of the alcoholic patient." N Engl J Med **275**: 61-66.

Hardy, G. H. (1908). "Mendelian Proportions in a Mixed Population." Science **28**(706): 49-50.

Harry, R., G. Auzinger, et al. (2002). "The clinical importance of adrenal insufficiency in acute hepatic dysfunction." Hepatology **36**(2): 395-402.

Harry, R., G. Auzinger, et al. (2003). "The effects of supraphysiological doses of corticosteroids in hypotensive liver failure." Liver International **23**(2): 71-77.

Hasko, G., P. Pacher, et al. (2006). "Shaping of monocyte and macrophage function by adenosine receptors." Pharmacol Ther.

Hearing, S. D., M. Norman, et al. (1999). "Predicting therapeutic outcome in severe ulcerative colitis by measuring in vitro steroid sensitivity of proliferating peripheral blood lymphocytes." Gut **45**(3): 382-388.

Hearing, S. D., M. Norman, et al. (1999). "Wide Variation in Lymphocyte Steroid Sensitivity Among Healthy Human Volunteers." J Clin Endocrinol Metab **84**(11): 4149-4154.

Henson, P. M. (2005). "Dampening inflammation." Nature Immunology **6**: 1179-1181.

Heumann, D., M. P. Glauser, et al. (1998). "Molecular basis of host-pathogen interaction in septic shock." Curr Opin Microbiol **1**(1): 49-55.

Hill, D., L. Marsano, et al. (1992). "Increased plasma interleukin-6 concentrations in alcoholic hepatitis." Journal of Laboratory and Clinical Medicine **119**(5): 547-552.

Hill, D. B., S. Barve, et al. (2000). "Increased monocyte nuclear factor-kappaB activation and tumor necrosis factor production in alcoholic hepatitis." Journal of Laboratory and Clinical Medicine **135**(5): 387-395.

Hill, D. B., R. Devalaraja, et al. (1999). "Antioxidants attenuate nuclear factor-kappa B activation and tumor necrosis factor-alpha production in alcoholic hepatitis patient monocytes and rat Kupffer cells, in vitro." Clinical Biochemistry **32**(7): 563-570.

Hill, D. B., C. J. McClain, et al. (1998). "Use of Transfected Liver Cells to Evaluate Potential Mechanisms of Alcohol-Induced Liver Injury." Alcoholism: Clinical and Experimental Research **22**(4): 785-788.

Hill, D. L. B., L. S. Marsano, et al. (1993). "Increased plasma interleukin-8 concentrations in alcoholic hepatitis." Hepatology **18**(3): 576-580.

Hislop, W. S., I. A. Bouchier, et al. (1983). "Alcoholic liver disease in Scotland and northeastern England: presenting features in 510 patients." Q J Med **52**(206): 232-243.

Hollingsworth, K. G., J. L. Newton, et al. (2008). "Pilot study of peripheral muscle function in primary biliary cirrhosis: potential implications for fatigue pathogenesis." Clin Gastroenterol Hepatol **6**(9): 1041-1048.

Holstege, A., P. Bedossa, et al. (1994). "Acetaldehyde-modified epitopes in liver biopsy specimens of alcoholic and nonalcoholic patients: localization and association with progression of liver fibrosis." Hepatology **19**(2): 367-374.

Honchel, R., M. B. Ray, et al. (1992). "Tumor Necrosis Factor in Alcohol Enhanced Endotoxin Liver Injury." Alcoholism: Clinical and Experimental Research **16**(4): 665-669.

Hoshino, K., O. Takeuchi, et al. (1999). "Cutting Edge: Toll-Like Receptor 4 (TLR4)-Deficient Mice Are Hyporesponsive to Lipopolysaccharide: Evidence for TLR4 as the Lps Gene Product." J Immunol **162**(7): 3749-3752.

Howdle, P. D. (2006). History and physical examination. Comprehensive clinical hepatology. B. R. Bacon, J. G. O'Grady, A. M. Di Bisceglie and J. R. Lake. Philadelphia, Elsevier Mosby**:** 61-71.

Howitz, K. T. and D. A. Sinclair (2008). "Xenohormesis: sensing the chemical cues of other species." Cell **133**(3): 387-391.

Hritz, I., P. Mandrekar, et al. (2008). "The critical role of toll-like receptor (TLR) 4 in alcoholic liver disease is independent of the common TLR adapter MyD88." Hepatology **48**(4): 1224-1231.

Hrubec, Z. and G. S. Omenn (1981). "Evidence of genetic predisposition to alcoholic cirrhosis and psychosis: twin concordances for alcoholism and its biological end points by zygosity among male veterans." Alcoholism: Clinical and Experimental Research **5**: 207–215.

Iimuro, Y., R. M. Gallucci, et al. (1997). "Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat." Hepatology **26**(6): 1530-1537.

Iimuro, Y., K. Ikejima, et al. (1996). "Nimodipine, a dihydropyridine-type calcium channel blocker, prevents alcoholic hepatitis caused by chronic intragastric ethanol exposure in the rat." Hepatology **24**(2): 391-397.

Ito, K., P. J. Barnes, et al. (2000). "Glucocorticoid Receptor Recruitment of Histone Deacetylase 2 Inhibits Interleukin-1beta -Induced Histone H4 Acetylation on Lysines 8 and 12." Mol. Cell. Biol. **20**(18): 6891-6903.

Ito, K., E. Jazrawi, et al. (2001). "p65-activated histone acetyltransferase activity is repressed by glucocorticoids: mifepristone fails to recruit HDAC2 to the p65-HAT complex." J Biol Chem **276**(32): 30208-30215.

Ito, K., S. Lim, et al. (2002). "A molecular mechanism of action of theophylline: Induction of histone deacetylase activity to decrease inflammatory gene expression." PNAS **99**(13): 8921-8926.

Janeway, C. A. and R. Medzhitov (2002). "Innate Immune Recognition." Annual Review of Immunology **20**(1): 197-216.

Järveläinen, H. A., A. Orpana, et al. (2001). "Promoter polymorphism of the CD14 endotoxin receptor gene as a risk factor for alcoholic liver disease." Hepatology **33**(5): 1148-1153.

Jayatilleke, A. and S. Shaw (1998). "Stimulation of monocyte interleukin-8 by lipid peroxidation products: a mechanism for alcohol-induced liver injury." Alcohol **16**(2): 119-123.

Jetter, W. W. (1950). "Modified dichromate method for determination of ethyl alcohol in biologic tissue." Am J Clin Pathol **20**(5): 473-475.

Ji, C., C. Chan, et al. (2006). "Predominant role of sterol response element binding proteins (SREBP) lipogenic pathways in hepatic steatosis in the murine intragastric ethanol feeding model." J Hepatol **45**(5): 717-724.

Ji, C., Q. Deng, et al. (2004). "Role of TNF-alpha in ethanol-induced hyperhomocysteinemia and murine alcoholic liver injury." Hepatology **40**(2): 442-451.

Ji, C. and N. Kaplowitz (2003). "Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice." Gastroenterology **124**(5): 1488-1499.

Ji, C. and N. Kaplowitz (2006). "ER stress: can the liver cope?" J Hepatol **45**(2): 321-333.

Ji, C., R. Mehrian-Shai, et al. (2005). "Role of CHOP in Hepatic Apoptosis in the Murine Model of Intragastric Ethanol Feeding." Alcoholism: Clinical and Experimental Research **29**(8): 1496-1503.

Johansen, D., K. Friis, et al. (2006). "Food buying habits of people who buy wine or beer: cross sectional study." BMJ **332**(7540): 519-522.

Jokelainen, K., L. A. Reinke, et al. (2001). "NF-kappaB activation is associated with free radical generation and endotoxaemia and precedes pathological liver injury in experimental alcoholic liver disease." Cytokine **16**(1): 36-39.

Jones, A. W. (1999). "The drunkest drinking driver in Sweden: blood alcohol concentration 0.545% w/v." J Stud Alcohol **60**(3): 400-406.

Joshi-Barve, S., S. S. Barve, et al. (2003). "Inhibition of proteasome function leads to NF-kappaB-independent IL-8 expression in human hepatocytes." Hepatology **38**(5): 1178-1187.

Kamath, P. S., R. H. Wiesner, et al. (2001). "A model to predict survival in patients with end-stage liver disease." Hepatology **33**(2): 464-470.

Kamimura, S. and H. Tsukamoto (1995). "Cytokine gene expression by Kupffer cells in experimental alcoholic liver disease." Hepatology **22**(4): 1304-1309.

Kaner, E. F., F. Beyer, et al. (2007). "Effectiveness of brief alcohol interventions in primary care populations." Cochrane Database Syst Rev(2): CD004148.

Karnam, U. S. and K. R. Reddy (2001). "A toast to pentoxifylline." The American Journal of Gastroenterology **96**(5): 1635-1637.

Kassi, E. and A. G. Papavassiliou (2008). "Could glucose be a proaging factor?" J Cell Mol Med **12**(4): 1194-1198.

Kato, H., M. Negoro, et al. (2005). "Effects of acute ethanol administration on LPS-induced expression of cyclooxygenase-2 and inducible nitric oxide synthase in rat alveolar macrophages." Alcohol Clin Exp Res **29**(12 Suppl): 285S-293S.

Kenyon, S. H., A. Nicolaou, et al. (1998). "The effect of ethanol and its metabolites upon methionine synthase activity in vitro." Alcohol **15**(4): 305-309.

Keshavarzian, A., E. W. Holmes, et al. (1999). "Leaky gut in alcoholic cirrhosis: a possible mechanism for alcohol-induced liver damage." The American Journal of Gastroenterology **94**(1): 200-207.

Khor, C. C., S. J. Chapman, et al. (2007). "A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis." Nat Genet **39**(4): 523-528.

Khoruts, A., L. Stahnke, et al. (1991). "Circulating tumor necrosis factor, interleukin-1 and interleukin-6 concentrations in chronic alcoholic patients." Hepatology **13**(2): 267-276.

Kim, J.-S. and S. D. Shukla (2006). "Acute in vivo effect of ethanol (binge drinking) on histone H3 modifications in rat tissues." Alcohol Alcohol. **41**(2): 126-132.

Kim, J. S. and S. D. Shukla (2005). "Histone h3 modifications in rat hepatic stellate cells by ethanol." Alcohol Alcohol **40**(5): 367-372.

Kimura, A., K. Matsubara, et al. (2005). "A Decade of Histone Acetylation: Marking Eukaryotic Chromosomes with Specific Codes." J Biochem **138**(6): 647-662.

Kirkham, B. W., M. M. Corkill, et al. (1991). "Response to glucocorticoid treatment in rheumatoid arthritis: in vitro cell mediated immune assay predicts in vivo responses." J Rheumatol **18**(6): 821-825.

Kishore, R., J. R. Hill, et al. (2002). "ERK1/2 and Egr-1 contribute to increased TNF-alpha production in rat Kupffer cells after chronic ethanol feeding." Am J Physiol Gastrointest Liver Physiol **282**(1): G6-15.

Kishore, R., M. R. McMullen, et al. (2001). "Stabilization of Tumor Necrosis Factor alpha mRNA by Chronic Ethanol. Role of A+U-rich elements and p38 mitogen-activated protein kinase signaling pathway." J. Biol. Chem. **276**(45): 41930-41937.

Klatsky, A. L. and M. A. Armstrong (1992). "Alcohol, smoking, coffee, and cirrhosis." Am J Epidemiol **136**(10): 1248-1257.

Klatsky, A. L., C. Morton, et al. (2006). "Coffee, Cirrhosis, and Transaminase Enzymes." Arch Intern Med **166**(11): 1190-1195.

Knolle, P., J. Schlaak, et al. (1995). "Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge." Journal of Hepatology **22**(2): 226-229.

Knolle, P. A. and G. Gerken (2000). "Local control of the immune response in the liver." Immunol Rev **174**: 21-34.

Koteish, A., S. Yang, et al. (2002). "Chronic Ethanol Exposure Potentiates Lipopolysaccharide Liver Injury Despite Inhibiting Jun N-terminal Kinase and Caspase 3 Activation." J. Biol. Chem. **277**(15): 13037-13044.

Laennec, R. (1819). De l'auscultation mediate. Paris, Brosson et Chaude.

Lagouge, M., C. Argmann, et al. (2006). "Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha." Cell **127**(6): 1109-1122.

Lambert, J. C., Z. Zhou, et al. (2003). "Prevention of Alterations in Intestinal Permeability Is Involved in Zinc Inhibition of Acute Ethanol-Induced Liver Damage in Mice." J Pharmacol Exp Ther **305**(3): 880-886.

Langhoff, E., J. Ladefoged, et al. (1986). "Recipient lymphocyte sensitivity to methylprednisolone affects cadaver kidney graft survival." Lancet **1**(8493): 1296-1297.

Lavu, S., O. Boss, et al. (2008). "Sirtuins - novel therapeutic targets to treat age-associated diseases." Nat Rev Drug Discov **7**(10): 841-853.

Le Moine, O., A. Marchant, et al. (1995). "Role of defective monocyte interleukin-10 release in tumor necrosis factor-alpha overproduction in alcoholic cirrhosis." Hepatology **22**(5): 1436-1439.

Le Moine, O., E. Quertinmont, et al. (1999). "Blunted anti-inflammatory response to adenosine in alcoholic cirrhosis." J Hepatol **31**(3): 457-463.

Leathart, J. B., C. P. Day, et al. (2001). "No association between functional SNPs in the endotoxin receptors CD14 and TLR4 and alcoholic liver disease (ALD): is endotoxin important in the pathogenesis of ALD in humans?" Hepatology **34**: 459A.

Lee, J. Y., L. Zhao, et al. (2004). "Saturated fatty acid activates but polyunsaturated fatty acid inhibits Toll-like receptor 2 dimerized with Toll-like receptor 6 or 1." J Biol Chem **279**(17): 16971-16979.

Lelbach, W. K. (1975). "Cirrhosis in the alcoholic and its relation to the volume of alcohol abuse." Ann N Y Acad Sci **252**: 85-105.

Leon, D. A. and J. McCambridge (2006). "Liver cirrhosis mortality rates in Britain from 1950 to 2002: an analysis of routine data." Lancet **367**: 52–56.

Li, H.-S., J.-Y. Zhang, et al. (2001). "Rat mitochondrial ATP synthase ATP5G3: cloning and upregulation in pancreas after chronic ethanol feeding." Physiol. Genomics **6**(2): 91-98.

Lieber, C. S., M. A. Leo, et al. (2008). "Effect of chronic alcohol consumption on Hepatic SIRT1 and PGC-1alpha in rats." Biochem Biophys Res Commun **370**(1): 44-48.

Liew, F. Y., D. Xu, et al. (2005). "Negative regulation of toll-like receptor-mediated immune responses." Nature Reviews Immunology **5**(6): 446-458.

Liu, J., Z. Tian, et al. (2002). "Dose-dependent activation of antiapoptotic and proapoptotic pathways by ethanol treatment in human vascular endothelial cells: differential involvement of adenosine." J Biol Chem **277**(23): 20927-20933.

Liu, Y., C. E. Denlinger, et al. (2006). "Suberoylanilide hydroxamic acid induces Akt-mediated phosphorylation of p300, which promotes acetylation and transcriptional activation of RelA/p65." J Biol Chem **281**(42): 31359-31368.

Louvet, A., E. Diaz, et al. (2007). "Early switch to pentoxifylline in patients with severe alcoholic hepatitis is inefficient in non-responders to corticosteroids." J Hepatol.

Louvet, A., S. Naveau, et al. (2007). "The Lille model: a new tool for therapeutic strategy in patients with severe alcoholic hepatitis treated with steroids." Hepatology **45**(6): 1348-1354.

Lucey, M. R. (2002). "Is liver transplantation an appropriate treatment for acute alcoholic hepatitis?" J Hepatol **36**(6): 829-831.

Maddrey, W. C., J. K. Boitnott, et al. (1978). "Corticosteroid therapy of alcoholic hepatitis." Gastroenterology **75**(2): 193-199.

Malinchoc, M., P. S. Kamath, et al. (2000). "A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts." Hepatology **31**(4): 864-871.

Maltby, J., S. Wright, et al. (1996). "Chemokine levels in human liver homogenates: Associations between GRO alpha and histopathological evidence of alcoholic hepatitis." Hepatology **24**(5): 1156-1160.

Mandrekar, P., V. Jeliazkova, et al. (2007). "Acute Alcohol Exposure Exerts Anti-Inflammatory Effects by Inhibiting IkappaB Kinase Activity and p65 Phosphorylation in Human Monocytes." J Immunol **178**(12): 7686-7693.

Mandrekar, P., S. Pruett, et al. (2005). "RSA 2004: combined basic research satellite symposium - session two: toll-like receptors and organ damage." Alcohol Clin Exp Res **29**(9): 1744-1748.

Mandrekar, P. and G. Szabo (2009). "Signalling pathways in alcohol-induced liver inflammation." J Hepatol **50**(6): 1258-1266.

Mao, T. K., Z.-X. Lian, et al. (2005). "Altered monocyte responses to defined TLR ligands in patients with primary biliary cirrhosis." Hepatology **42**(4): 802-808.

Martin, M., J. Katz, et al. (2001). "Differential Induction of Endotoxin Tolerance by Lipopolysaccharides Derived from Porphyromonas gingivalis and Escherichia coli." J Immunol **167**(9): 5278-5285.

Martins, A., H. Cortez-Pinto, et al. (2005). "Are genetic polymorphisms of tumour necrosis factor alpha, interleukin-10, CD14 endotoxin receptor or manganese superoxide dismutase associated with alcoholic liver disease?" Eur J Gastroenterol Hepatol **17**(10): 1099-1104.

Marwick, J. A., G. Caramori, et al. (2009). "Inhibition of PI3Kdelta restores glucocorticoid function in smoking-induced airway inflammation in mice." Am J Respir Crit Care Med **179**(7): 542-548.

Mascord, D., J. Smith, et al. (1992). "Effects of increasing the rate of alcohol metabolism on plasma acetate concentration." Alcohol Alcohol. **27**(1): 25-28.

Mathurin, P. (2005). "Is alcoholic hepatitis an indication for transplantation? Current management and outcomes." Liver Transpl **11**(Suppl 2): S21-24.

Mathurin, P., M. Abdelnour, et al. (2003). "Early change in bilirubin levels is an important prognostic factor in severe alcoholic hepatitis treated with prednisolone." Hepatology **38**(6): 1363-1369.

Mathurin, P., Q.-G. Deng, et al. (2000). "Exacerbation of alcoholic liver injury by enteral endotoxin in rats." Hepatology **32**(5): 1008-1017.

Mathurin, P., V. Duchatelle, et al. (1996). "Survival and prognostic factors in patients with severe alcoholic hepatitis treated with prednisolone." Gastroenterology **110**(6): 1847-1853.

Mathurin, P., C. L. Mendenhall, et al. (2002). "Corticosteroids improve short-term survival in patients with severe alcoholic hepatitis (AH): individual data analysis of the last three randomized placebo controlled double blind trials of corticosteroids in severe AH." Journal of Hepatology **36**(4): 480-487.

Mato, J. M., J. Cámara, et al. (1999). "S-Adenosylmethionine in alcoholic liver cirrhosis: a randomized, placebo-controlled, double-blind, multicenter clinical trial." Journal of Hepatology **30**(6): 1081-1089.

Mato, J. M. and S. C. Lu (2005). "Homocysteine, the bad thiol." Hepatology **41**(5): 976-979.

Matsumaru, K., C. Ji, et al. (2003). "Mechanisms for sensitization to TNF-induced apoptosis by acute glutathione depletion in murine hepatocytes." Hepatology **37**(6): 1425-1434.

Matzinger, P. (2002). "The danger model: a renewed sense of self." Science **296**(5566): 301-305.

McClain, C., S. Barve, et al. (2005). "Dysregulated Cytokine Metabolism, Altered Hepatic Methionine Metabolism and Proteasome Dysfunction in Alcoholic Liver Disease." Alcoholism: Clinical and Experimental Research **29**(s2): 180S-188S.

McClain, C. J. and D. A. Cohen (1989). "Increased tumor necrosis factor production by monocytes in alcoholic hepatitis." Hepatology **9**(3): 349-351.

McClain, C. J., D. B. Hill, et al. (2002). "S-Adenosylmethionine, cytokines, and alcoholic liver disease." Alcohol **27**(3): 185-192.

McClain, C. J., D. B. Hill, et al. (2002). "Monocyte activation in alcoholic liver disease." Alcohol **27**(1): 53-61.

McClain, C. J., Z. Song, et al. (2004). "Recent Advances in Alcoholic Liver Disease IV. Dysregulated cytokine metabolism in alcoholic liver disease." Am J Physiol Gastrointest Liver Physiol **287**(3): G497-502.

McFarlane, I. G. (2000). "Autoantibodies in alcoholic liver disease." Addiction Biology **5**(2): 141-151.

McVicker, B. L., D. J. Tuma, et al. (2007). "Effect of Chronic Ethanol Administration on the in Vitro Production of Proinflammatory Cytokines by Rat Kupffer Cells in the Presence of Apoptotic Cells." Alcoholism: Clinical and Experimental Research **31**(1): 122-129.

McVicker, B. L., D. J. Tuma, et al. (2002). "The effect of ethanol on asialoglycoprotein receptor-mediated phagocytosis of apoptotic cells by rat hepatocytes." Hepatology **36**(6): 1478-1487.

Medvedev, A. E., P. Henneke, et al. (2001). "Induction of Tolerance to Lipopolysaccharide and Mycobacterial Components in Chinese Hamster Ovary/CD14 Cells Is Not Affected by Overexpression of Toll-Like Receptors 2 or 4." J Immunol **167**(4): 2257-2267.

Mendenhall, C. L. (1981). "Alcoholic hepatitis." Clin Gastroenterol **10**(2): 417-441.

Menon, K. V. N., L. Stadheim, et al. (2004). "A Pilot Study of the Safety and Tolerability of Etanercept in Patients with Alcoholic Hepatitis." The American Journal of Gastroenterology **99**(2): 255-260.

Miele, L., V. Valenza, et al. (2009). "Increased intestinal permeability and tight junction alterations in nonalcoholic fatty liver disease." Hepatology **49**(6): 1877-1887.

Mihm, S., D. Galter, et al. (1995). "Modulation of transcription factor NF kappa B activity by intracellular glutathione levels and by variations of the extracellular cysteine supply." Faseb J **9**(2): 246-252.

Minagawa, M., Q. Deng, et al. (2004). "Activated natural killer T cells induce liver injury by Fas and tumor necrosis factor-alpha during alcohol consumption." Gastroenterology **126**(5): 1387-1399.

Mokry, M. and E. Cuppen (2008). "The Atp1a1 Gene From Inbred Dahl Salt Sensitive Rats Does Not Contain the A1079T Missense Transversion." Hypertension **51**(4): 922-927.

Mookerjee, R. P., V. Stadlbauer, et al. (2007). "Neutrophil dysfunction in alcoholic hepatitis superimposed on cirrhosis is reversible and predicts the outcome." Hepatology **46**(3): 831-840.

Morris, G. E., L. C. Parker, et al. (2006). "Cooperative molecular and cellular networks regulate Toll-like receptor-dependent inflammatory responses." FASEB J. **20**(12): 2153-2155.

Morris, J. M. and E. H. Forrest (2005). "Bilirubin response to corticosteroids in severe alcoholic hepatitis." European Journal of Gastroenterology & Hepatology. **17**(7): 759-762.

Moss, M., B. Bucher, et al. (1996). "The role of chronic alcohol abuse in the development of acute respiratory distress syndrome in adults." JAMA **275**(1): 50-54.

Moss, M., D. M. Guidot, et al. (2000). "The effects of chronic alcohol abuse on pulmonary glutathione homeostasis." Am J Respir Crit Care Med **161**(2 Pt 1): 414-419.

Mottaran, E., S. F. Stewart, et al. (2002). "Lipid peroxidation contributes to immune reactions associated with alcoholic liver disease." Free Radic Biol Med **32**(1): 38-45.

Mullis, K. B. and F. A. Faloona (1987). "Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction." Methods Enzymol **155**: 335-350.

Murphy, P. J. M., Y. Morishima, et al. (2005). "Regulation of the Dynamics of hsp90 Action on the Glucocorticoid Receptor by Acetylation/Deacetylation of the Chaperone." J. Biol. Chem. **280**(40): 33792-33799.

Nagy, L. E. (2003). "Recent Insights into the Role of the Innate Immune System in the Development of Alcoholic Liver Disease." Experimental Biology and Medicine **228**(8): 882-890.

Nahon, P., A. Sutton, et al. (2005). "Genetic dimorphism in superoxide dismutase and susceptibility to alcoholic cirrhosis, hepatocellular carcinoma, and death." Clin Gastroenterol Hepatol **3**(3): 292-298.

Nanji, A., U. Khettry, et al. (1994). "Lactobacillus feeding reduces endotoxemia and severity of experimental alcoholic liver disease." Proc Soc Exp Biol Med **205**(3): 243-247.

Nanji, A. A., K. Jokelainen, et al. (2001). "Increased severity of alcoholic liver injury in female rats: role of oxidative stress, endotoxin, and chemokines." Am J Physiol Gastrointest Liver Physiol **281**(6): G1348-1356.

Nanji, A. A., K. Jokelainen, et al. (1999). "Activation of nuclear factor kappa B and cytokine imbalance in experimental alcoholic liver disease in the rat." Hepatology **30**(4): 934-943.

Nanji, A. A., S. Zhao, et al. (1994). "Use of reverse transcription-polymerase chain reaction to evaluate in vivo cytokine gene expression in rats fed ethanol for long periods." Hepatology **19**(6): 1483-1487.

Natori, S., C. Rust, et al. (2001). "Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis." Journal of Hepatology **34**(2): 248-253.

Naveau, S., S. Chollet-Martin, et al. (2004). "A double-blind randomized controlled trial of infliximab associated with prednisolone in acute alcoholic hepatitis." Hepatology **39**(5): 1390-1397.

Naveau, S., V. Giraud, et al. (1997). "Excess weight risk factor for alcoholic liver disease." Hepatology **25**(1): 108-111.

Nelson, S., G. J. Bagby, et al. (1989). "The effects of acute and chronic alcoholism on tumor necrosis factor and the inflammatory response." J Infect Dis **160**(3): 422-429.

Neuberger, J. (1998). "Transplantation for alcoholic liver disease: a perspective from Europe." Liver Transpl Surg **4**(5 Suppl 1): S51-57.

Niemela, O., F. Klajner, et al. (1987). "Antibodies against acetaldehyde-modified protein epitopes in human alcoholics." Hepatology **7**(6): 1210-1214.

Niemela, O., S. Parkkila, et al. (2002). "Effect of Kupffer cell inactivation on ethanol-induced protein adducts in the liver." Free Radic Biol Med **33**(3): 350-355.

Nishiyama, D., K. Ikejima, et al. (2002). "Acute ethanol administration down-regulates toll-like receptor-4 in the murine liver." Hepatology Research **23**(2): 130-137.

Nomura, F., S. Akashi, et al. (2000). "Cutting Edge: Endotoxin Tolerance in Mouse Peritoneal Macrophages Correlates with Down-Regulation of Surface Toll-Like Receptor 4 Expression." J Immunol **164**(7): 3476-3479.

North, B. J. and D. A. Sinclair (2007). "Sirtuins: a conserved key unlocking AceCS activity." Trends in Biochemical Sciences **32**(1): 1-4.

O'Beirne, J., M. Holmes, et al. (2007). "Adrenal insufficiency in liver disease - what is the evidence?" J Hepatol **47**(3): 418-423.

O'Farrelly, C. (2004). "Immunoregulation in the liver and its extrahepatic relevance." J Pediatr Gastroenterol Nutr **39 Suppl 3**: S727-728.

O'Neill, L. A. and A. G. Bowie (2007). "The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling." Nat Rev Immunol **7**(5): 353-364.

Oak, S., P. Mandrekar, et al. (2006). "TLR2- and TLR4-Mediated Signals Determine Attenuation or Augmentation of Inflammation by Acute Alcohol in Monocytes." J Immunol **176**(12): 7628-7635.

Ohno, Y., J. Lee, et al. (1997). "Macrophage inflammatory protein-2: chromosomal regulation in rat small intestinal epithelial cells." Proc Natl Acad Sci U S A **94**(19): 10279-10284.

Ohta, A. and M. Sitkovsky (2001). "Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage." Nature **414**(6866): 916-920.

ONS. (2006). "Alcohol-related deaths - rates rise since early 1990s." Retrieved 27.07.2006, 2006, from http://www.statistics.gov.uk/CCI/nugget.asp?ID=1091&Pos=1&ColRank=1&Rank=192.

Opal, S. and C. Huber (2002). "Bench-to-bedside review: Toll-like receptors and their role in septic shock." Critical Care **6**(2): 125 - 136.

Osler, W. (1892). The Principles and Practice of Medicine. Edinburgh, Young J Pentland**:** 444.

Pares, A., J. Caballeria, et al. (1986). "Histological course of alcoholic hepatitis. Influence of abstinence, sex and extent of hepatic damage." Journal of Hepatology **2**(1): 33-42.

Park, P.-H., R. Miller, et al. (2003). "Acetylation of histone H3 at lysine 9 by ethanol in rat hepatocytes." Biochemical and Biophysical Research Communications **306**(2): 501-504.

Park, P. H., R. W. Lim, et al. (2005). "Involvement of histone acetyltransferase (HAT) in ethanol-induced acetylation of histone H3 in hepatocytes: potential mechanism for gene expression." Am J Physiol Gastrointest Liver Physiol **289**(6): G1124-1136.

Parlesak, A., C. Bode, et al. (1998). "Free Methionine Supplementation Limits Alcohol-Induced Liver Damage in Rats." Alcoholism: Clinical and Experimental Research **22**(2): 352-358.

Parlesak, A., C. Schäfer, et al. (2000). "Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease." Journal of Hepatology **32**(5): 742-747.

Pastor, I. J., F. J. Laso, et al. (2005). "-238 G to A polymorphism of Tumor Necrosis Factor Alpha Gene (TNFA) is Associated with Alcoholic Liver Cirrhosis in Alcoholic Spanish Men." Alcoholism: Clinical and Experimental Research **29**(11): 1928-1931.

Pessione, F., M. J. Ramond, et al. (2003). "Five-year survival predictive factors in patients with excessive alcohol intake and cirrhosis. Effect of alcoholic hepatitis, smoking and abstinence." Liver Int **23**(1): 45-53.

Picklo, M. J., Sr. (2008). "Ethanol intoxication increases hepatic N-lysyl protein acetylation." Biochem Biophys Res Commun **376**(3): 615-619.

Pisetsky, D. S. (2007). "The Role of Nuclear Macromolecules in Innate Immunity." Proc Am Thorac Soc **4**(3): 258-262.

Pol, S., T. Poynard, et al. (1990). "Diagnostic value of serum gamma-glutamyl-transferase activity and mean corpuscular volume in alcoholic patients with or without cirrhosis." Alcohol Clin Exp Res **14**(2): 250-254.

Powell, W. J., Jr. and G. Klatskin (1968). "Duration of survival in patients with Laennec's cirrhosis. Influence of alcohol withdrawal, and possible effects of recent changes in general management of the disease." Am J Med **44**(3): 406-420.

Powers, K. A., K. Szaszi, et al. (2006). "Oxidative stress generated by hemorrhagic shock recruits Toll-like receptor 4 to the plasma membrane in macrophages." J. Exp. Med. **203**(8): 1951-1961.

Pritchard, M. T. and L. E. Nagy (2005). "Ethanol-Induced Liver Injury: Potential Roles for Egr-1." Alcoholism: Clinical and Experimental Research **29**(s2): 146S-150S.

Pruett, S. B., Q. Zheng, et al. (2004). "Acute exposure to ethanol affects Toll-like receptor signaling and subsequent responses: an overview of recent studies." Alcohol **33**(3): 235-239.

Pugh, R. N., I. M. Murray-Lyon, et al. (1973). "Transection of the oesophagus for bleeding oesophageal varices." Br J Surg **60**(8): 646-649.

Purohit, V. and D. A. Brenner (2006). "Mechanisms of alcohol-induced hepatic fibrosis: A summary of the Ron Thurman Symposium." Hepatology **43**(4): 872-878.

Quivy, V. and C. Van Lint (2004). "Regulation at multiple levels of NF-kappaB-mediated transactivation by protein acetylation." Biochemical Pharmacology - Proceedings from

the 6th and 7th international conferences, Signal Transduction 2004 and Chromatin 2004 **68**(6): 1221-1229.

Ramaiah, S. K. and H. Jaeschke (2007). "Role of neutrophils in the pathogenesis of acute inflammatory liver injury." Toxicol Pathol **35**(6): 757-766.

Rao, R. K., A. Seth, et al. (2004). "Recent Advances in Alcoholic Liver Disease I. Role of intestinal permeability and endotoxemia in alcoholic liver disease." Am J Physiol Gastrointest Liver Physiol **286**(6): G881-884.

Reed, T., W. F. Page, et al. (1996). "Genetic Predisposition to Organ-Specific Endpoints of Alcoholism." Alcoholism: Clinical and Experimental Research **20**(9): 1528-1533.

Rehm, J., B. Taylor, et al. (2006). "Global burden of disease from alcohol, illicit drugs and tobacco." Drug Alcohol Rev **25**(6): 503-513.

Rettew, J. A., Y. M. Huet-Hudson, et al. (2008). "Testosterone Reduces Macrophage Expression in the Mouse of Toll-Like Receptor 4, a Trigger for Inflammation and Innate Immunity." Biol Reprod **78**(3): 432-437.

Rivera, C. A., P. Adegboyega, et al. (2007). "Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis." J Hepatol **47**(4): 571-579.

Roberts, R. A., P. E. Ganey, et al. (2007). "Role of the Kupffer Cell in Mediating Hepatic Toxicity and Carcinogenesis." Toxicol. Sci. **96**(1): 2-15.

Room, R., T. Babor, et al. (2005). "Alcohol and public health." Lancet **365**(9458): 519-530.

Rotily, M., J. P. Durbec, et al. (1990). "Diet and alcohol in liver cirrhosis: a case-control study." Eur J Clin Nutr **44**(8): 595-603.

Ruhl, C. and J. Everhart (2005). "Coffee and Tea Consumption Are Associated With a Lower Incidence of Chronic Liver Disease in the United States." Gastroenterology **129**(6): 1928-1936.

Sakakibara, S., T. Yamauchi, et al. (2006). "Acetic acid activates hepatic AMPK and reduces hyperglycemia in diabetic KK-A(y) mice." Biochemical and Biophysical Research Communications **344**(2): 597-604.

Sanderson, I. R. (2004). "Short Chain Fatty Acid Regulation of Signaling Genes Expressed by the Intestinal Epithelium." J. Nutr. **134**(9): 2450S-2454.

Sato, S., F. Nomura, et al. (2000). "Synergy and Cross-Tolerance Between Toll-Like Receptor (TLR) 2- and TLR4-Mediated Signaling Pathways." J Immunol **165**(12): 7096-7101.

Satoh, A., A. S. Gukovskaya, et al. (2006). "Ethanol sensitizes NF-kappaB activation in pancreatic acinar cells through effects on protein kinase C-epsilon." Am J Physiol Gastrointest Liver Physiol **291**(3): G432-438.

Schafer, C., A. Parlesak, et al. (2002). "Concentrations of lipopolysaccharide-binding protein, bactericidal/permeability-increasing protein, soluble CD14 and plasma lipids in relation to endotoxaemia in patients with alcoholic liver disease." Alcohol Alcohol. **37**(1): 81-86.

Scheinman, R. I., P. C. Cogswell, et al. (1995). "Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids." Science **270**(5234): 283-286.

Schenker, S. (1997). "Medical consequences of alcohol abuse: is gender a factor?" Alcoholism: Clinical and Experimental Research **21**(1): 179-181.

Seki, E., H. Tsutsui, et al. (2001). "Lipopolysaccharide-induced IL-18 secretion from murine Kupffer cells independently of myeloid differentiation factor 88 that is critically involved in induction of production of IL-12 and IL-1beta." J Immunol **166**(4): 2651-2657.

Senn, J. J. (2006). "Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes." J Biol Chem **281**(37): 26865-26875.

Seth, A., S. Basuroy, et al. (2004). "L-Glutamine ameliorates acetaldehyde-induced increase in paracellular permeability in Caco-2 cell monolayer." Am J Physiol Gastrointest Liver Physiol **287**(3): G510-517.

Shaw, S., T. M. Worner, et al. (1979). "Detection of alcoholism relapse: comparative diagnostic value of MCV, GGTP, and AANB." Alcohol Clin Exp Res **3**(4): 297-301.

Shepard, B. D., R. A. Joseph, et al. (2008). "Alcohol-induced alterations in hepatic microtubule dynamics can be explained by impaired histone deacetylase 6 function." Hepatology **48**(5): 1671-1679.

Shepard, B. D. and P. L. Tuma (2009). "Alcohol-induced protein hyperacetylation: mechanisms and consequences." World J Gastroenterol **15**(10): 1219-1230.

Sheron, N., G. Bird, et al. (1991). "Elevated plasma interleukin-6 and increased severity and mortality in alcoholic hepatitis." Clinical and Experimental Immunology **84**: 449-453.

Sheron, N., G. Bird, et al. (1993). "Circulating and tissue levels of the neutrophil chemotaxin interleukin-8 are elevated in severe acute alcoholic hepatitis, and tissue levels correlate with neutrophil infiltration." Hepatology **18**(1): 41-46.

Sheth, M., M. Riggs, et al. (2002). "Utility of the Mayo End-Stage Liver Disease (MELD) score in assessing prognosis of patients with alcoholic hepatitis." BMC Gastroenterology **2**(1): 2.

Shi, L., R. Kishore, et al. (2002). "Chronic Ethanol Increases Lipopolysaccharide-stimulated Egr-1 Expression in RAW 264.7 Macrophages. Contribution to enhanced tumor necrosis factor alpha production." J. Biol. Chem. **277**(17): 14777-14785.

Shukla, S. D., J. Velazquez, et al. (2008). "Emerging role of epigenetics in the actions of alcohol." Alcohol Clin Exp Res **32**(9): 1525-1534.

Slotta, Scheuer, et al. (2006). "Immunostimulatory CpG-oligodeoxynucleotides (CpG-ODN) induce early hepatic injury, but provide a late window for protection against endotoxin-mediated liver damage." Journal of Hepatology **44**(3): 576-585.

Smirnova, I., A. Poltorak, et al. (2000). "Phylogenetic variation and polymorphism at the Toll-like receptor 4 locus (TLR4)." Genome Biology **1**(1): research002.001 - 002.010.

Smith, S., J. White, et al. (2006). "Severe alcohol-induced liver disease and the alcohol dependence syndrome." Alcohol Alcohol **41**(3): 274-277.

Song, Z., Z. Zhou, et al. (2004). "S-adenosylhomocysteine sensitizes to TNF-alpha hepatotoxicity in mice and liver cells: a possible etiological factor in alcoholic liver disease." Hepatology **40**(4): 989-997.

Srikureja, W., N. Kyulo, et al. (2005). "MELD score is a better prognostic model than Child-Turcotte-Pugh score or Discriminant Function score in patients with alcoholic hepatitis." Journal of Hepatology **42**(5): 700-706.

Stewart, S., A. Daly, et al. (2006). Genetic evidence that immune mechanisms do not play a primary role in the pathogenesis of alcoholic liver disease (ALD): a mendelian randomisation study.

Stewart, S., M. Prince, et al. (2007). "A randomized trial of antioxidant therapy alone or with corticosteroids in acute alcoholic hepatitis." J Hepatol **47**(2): 277-283.

Stewart, S. F. and C. P. Day (2003). "The management of alcoholic liver disease." Journal of Hepatology **38**: s2-s13.

Stewart, S. F. and C. P. Day (2006). Alcoholic Liver Disease. Hepatology: A Textbook of Liver Disease. T. D. Boyer, WB Saunders Co Ltd.

Stewart, S. F., M. Vidali, et al. (2004). "Oxidative stress as a trigger for cellular immune responses in patients with alcoholic liver disease." Hepatology **39**(1): 197-203.

Stickel, F. and C. H. Osterreicher (2006). "The role of genetic polymorphisms in alcoholic liver disease." Alcohol Alcohol. **41**(3): 209-224.

Stinson, F. S., B. F. Grant, et al. (2001). "The critical dimension of ethnicity in liver cirrhosis mortality statistics." Alcohol Clin Exp Res **25**(8): 1181-1187.

Struben, V. M., E. E. Hespenheide, et al. (2000). "Nonalcoholic steatohepatitis and cryptogenic cirrhosis within kindreds." Am J Med **108**(1): 9-13.

Szabo, C., B. J. Day, et al. (1996). "Evaluation of the relative contribution of nitric oxide and peroxynitrite to the suppression of mitochondrial respiration in immunostimulated macrophages using a manganese mesoporphyrin superoxide dismutase mimetic and peroxynitrite scavenger." FEBS Letters **381**(1-2): 82-86.

Szabo, G. (2000). "New insights into the molecular mechanisms of alcoholic hepatitis: A potential role for NF-kappaB activation?" Journal of Laboratory and Clinical Medicine **135**(5): 367-369.

Szabo, G., I. Hritz, et al. (2008). "A novel, critical role of interferon regulatory factor 3 in alcoholic liver disease." Hepatology **48**(S1): 373A.

Szabo, G., A. Velayudham, et al. (2008). "Two faces of MyD88 expression in steatohepatitis: protection in liver parenchymal cells and mediation of damage in bone marrow-derived cells " Hepatology **48**(S1): 366A.

Takeda, K. and S. Akira (2005). "Toll-like receptors in innate immunity." Int Immunol **17**(1): 1-14.

Tapson, F. (2004). "Notes on Measures 6 - The Alcohol Content of Drinks." 1.4. Retrieved 7th January 2008, 2008, from http://www.cleavebooks.co.uk/dictunit/notes6.htm.

Teli, M. R., C. P. Day, et al. (1995). "Determinants of progression to cirrhosis or fibrosis in pure alcoholic fatty liver." Lancet **346**(8981): 987-990.

Thakur, V., M. T. Pritchard, et al. (2006). "Chronic ethanol feeding increases activation of NADPH oxidase by lipopolysaccharide in rat Kupffer cells: role of increased reactive oxygen in LPS-stimulated ERK1/2 activation and TNF-alpha production." J Leukoc Biol **79**(6): 1348-1356.

Thurman, R. G. (1998). "II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin." Am J Physiol Gastrointest Liver Physiol **275**(4): G605-611.

Tomé, S., C. Martinez-Rey, et al. (2002). "Influence of superimposed alcoholic hepatitis on the outcome of liver transplantation for end-stage alcoholic liver disease." Journal of Hepatology **36**(6): 793-798.

Truss, M. and M. Beato (1993). "Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors." Endocr Rev **14**(4): 459-479.

Tsai, M. H., Y. S. Peng, et al. (2006). "Adrenal insufficiency in patients with cirrhosis, severe sepsis and septic shock." Hepatology **43**(4): 673-681.

Tsujimoto, H., S. Ono, et al. (2006). "Differential toll-like receptor expression after ex vivo lipopolysaccharide exposure in patients with sepsis and following surgical stress." Clinical Immunology **119**(2): 180-187.

Uesugi, T., M. Froh, et al. (2001). "Delivery of I?B superrepressor gene with adenovirus reduces early alcohol-induced liver injury in rats." Hepatology **34**(6): 1149-1157.

Uesugi, T., M. Froh, et al. (2001). "Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice." Hepatology **34**(1): 101-108.

Uesugi, T., M. Froh, et al. (2002). "Role of Lipopolysaccharide-Binding Protein in Early Alcohol-Induced Liver Injury in Mice." J Immunol **168**(6): 2963-2969.

Uhrig, A., R. Banafsche, et al. (2005). "Development and functional consequences of LPS tolerance in sinusoidal endothelial cells of the liver." J Leukoc Biol **77**(5): 626-633.

Urbaschek, R., R. S. McCuskey, et al. (2001). "Endotoxin, Endotoxin-Neutralizing-Capacity, sCD14, sICAM-1, and Cytokines in Patients With Various Degrees of Alcoholic Liver Disease." Alcoholism: Clinical and Experimental Research **25**(2): 261-268.

Veal, N., C.-L. Hsieh, et al. (2004). "Inhibition of lipopolysaccharide-stimulated TNF-alpha promoter activity by S-adenosylmethionine and 5'-methylthioadenosine." Am J Physiol Gastrointest Liver Physiol **287**(2): G352-362.

Veldt, B. J., F. Laine, et al. (2002). "Indication of liver transplantation in severe alcoholic liver cirrhosis: quantitative evaluation and optimal timing." J Hepatol **36**(1): 93-98.

Vidali, M., S. F. Stewart, et al. (2003). "Genetic and epigenetic factors in autoimmune reactions toward cytochrome P4502E1 in alcoholic liver disease." Hepatology **37**(2): 410-419.

Villanueva, J. A., A. M. Devlin, et al. (2001). "Reduced folate carrier: tissue distribution and effects of chronic ethanol intake in the micropig." Alcohol Clin Exp Res **25**(3): 415-420.

Villanueva, J. A., F. Esfandiari, et al. (2006). "Abnormal Transsulfuration and Glutathione Metabolism in the Micropig Model of Alcoholic Liver Disease." Alcoholism: Clinical and Experimental Research **30**(7): 1262-1270.

Villanueva, J. A. and C. H. Halsted (2004). "Hepatic transmethylation reactions in micropigs with alcoholic liver disease." Hepatology **39**(5): 1303-1310.

Wagnerberger, S., C. Schafer, et al. (2008). "Is nutrient intake a gender-specific cause for enhanced susceptibility to alcohol-induced liver disease in women?" Alcohol Alcohol. **43**(1): 9-14.

Wanless, I. R. and J. S. Lentz (1990). "Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors." Hepatology **12**(5): 1106-1110.

Watkins, P. A., D. Maiguel, et al. (2007). "Evidence for 26 distinct acyl-coenzyme A synthetase genes in the human genome." J. Lipid Res. **48**(12): 2736-2750.

Wells, J. T., A. Said, et al. (2007). "The impact of acute alcoholic hepatitis in the explanted recipient liver on outcome after liver transplantation." Liver Transpl **13**(12): 1728-1735.

Wheeler, M. D., H. Kono, et al. (2001). "The role of kupffer cell oxidant production in early ethanol-induced liver disease,." Free Radical Biology and Medicine **31**(12): 1544-1549.

Wheeler, M. D., H. Kono, et al. (2001). "Delivery of the Cu/Zn-Superoxide dismutase gene with adenovirus reduces early alcohol-induced liver injury in rats." Gastroenterology **120**(5): 1241-1250.

Wheeler, M. D. and R. G. Thurman (2003). "Up-regulation of CD14 in Liver Caused by Acute Ethanol Involves Oxidant-dependent AP-1 Pathway." J. Biol. Chem. **278**(10): 8435-8441.

Wickramasinghe, S. N. (1986). "Observations on the biochemical basis of ethanol metabolism by human macrophages." Alcohol Alcohol **21**(1): 57-63.

Wickramasinghe, S. N. (1998). "Ethanol metabolism by macrophages: possible role in organ damage." Addiction Biology **3**(4): 405-412.

Wigg, A. J., I. C. Roberts-Thomson, et al. (2001). "The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis." Gut **48**(2): 206-211.

Wissink, S., E. C. van Heerde, et al. (1997). "Distinct domains of the RelA NF-kappaB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor." J Biol Chem **272**(35): 22278-22284.

Wu, A., I. Chanarin, et al. (1975). "Folate deficiency in the alcoholic--its relationship to clinical and haematological abnormalities, liver disease and folate stores." Br J Haematol **29**(3): 469-478.

Wu, A., G. Slavin, et al. (1976). "Elevated serum gamma-glutamyl-transferase (transpeptidase) and histological liver damage in alcoholism." Am J Gastroenterol **65**(4): 318-323.

Wu, D. and A. I. Cederbaum (1999). "Ethanol-Induced Apoptosis to Stable HepG2 Cell Lines Expressing Human Cytochrome P-4502E1." Alcoholism: Clinical and Experimental Research **23**(1): 67-76.

Wu, G., Y.-Z. Fang, et al. (2004). "Glutathione Metabolism and Its Implications for Health." J. Nutr. **134**(3): 489-492.

Xia, C. Q., R. Peng, et al. (2005). "Dexamethasone induces IL-10-producing monocyte-derived dendritic cells with durable immaturity." Scand J Immunol **62**(1): 45-54.

Yacoub, L. K., F. Fogt, et al. (1995). "Apoptosis and Bcl-2 Protein Expression in Experimental Alcoholic Liver Disease in the Rat." Alcoholism: Clinical and Experimental Research **19**(4): 854-859.

Yamamoto, M., S. Sato, et al. (2002). "Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4." Nature **420**(6913): 324-329.

Yamashina, S., M. D. Wheeler, et al. (2000). "Tolerance and Sensitization to Endotoxin in Kupffer Cells Caused by Acute Ethanol Involve Interleukin-1 Receptor-Associated Kinase." Biochemical and Biophysical Research Communications **277**(3): 686-690.

Yamashita, H., T. Kaneyuki, et al. (2001). "Production of acetate in the liver and its utilization in peripheral tissues." Biochim Biophys Acta **1532**(1-2): 79-87.

Yamazaki, Y., I. Usui, et al. (2009). "Treatment with SRT1720, a SIRT1 Activator, Ameliorates Fatty Liver with Reduced Expression of Lipogenic Enzymes in MSG Mice." Am J Physiol Endocrinol Metab(online publication ahead of print September 2009).

Yang, Q., Y.-S. Kim, et al. (2006). "Tumour necrosis factor receptor 1 mediates endoplasmic reticulum stress-induced activation of the MAP kinase JNK." EMBO Reports **7**(6): 622-627.

Yang, S.-R., J. Wright, et al. (2007). "Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF-kappaB in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging." Am J Physiol Lung Cell Mol Physiol **292**(2): L567-576.

Yang, S. Q., H. Z. Lin, et al. (1997). "Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis." Proc Natl Acad Sci U S A **94**(6): 2557-2562.

Yano, T., Y. Itoh, et al. (2003). "Cyclic AMP reverses radiocontrast media-induced apoptosis in LLC-PK1 cells by activating A kinase/PI3 kinase." Kidney Int **64**(6): 2052-2063.

Yano, Y., M. Yoshida, et al. (2006). "Anti-fibrotic effects of theophylline on lung fibroblasts." Biochem Biophys Res Commun **341**(3): 684-690.

Yi, P., S. Melnyk, et al. (2000). "Increase in Plasma Homocysteine Associated with Parallel Increases in Plasma S-Adenosylhomocysteine and Lymphocyte DNA Hypomethylation." J. Biol. Chem. **275**(38): 29318-29323.

Yin, M., B. U. Bradford, et al. (2001). "Reduced Early Alcohol-Induced Liver Injury in CD14-Deficient Mice." J Immunol **166**(7): 4737-4742.

Yin, M., M. D. Wheeler, et al. (1999). "Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice." Gastroenterology **117**(4): 942-952.

You, M., X. Liang, et al. (2008). "Involvement of mammalian sirtuin 1 in the action of ethanol in the liver." Am J Physiol Gastrointest Liver Physiol **294**(4): G892-898.

Yoza, B. K., J. Y.-Q. Hu, et al. (2006). "Induction of RelB Participates in Endotoxin Tolerance." J Immunol **177**(6): 4080-4085.

Zager, R. A., A. C. M. Johnson, et al. (2006). "Acute renal failure: determinants and characteristics of the injury-induced hyperinflammatory response." Am J Physiol Renal Physiol **291**(3): F546-556.

Zarember, K. A. and P. J. Godowski (2002). "Tissue Expression of Human Toll-Like Receptors and Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their Products, and Cytokines." J Immunol **168**(2): 554-561.

Zatloukal, K., S. W. French, et al. (2007). "From Mallory to Mallory-Denk bodies: what, how and why?" Exp Cell Res **313**(10): 2033-2049.

Zhang, Z., G. J. Bagby, et al. (2001). "Prolonged Ethanol Treatment Enhances Lipopolysaccharide/Phorbol Myristate Acetate-Induced Tumor Necrosis Factor-Alpha Production in Human Monocytic Cells." Alcoholism: Clinical and Experimental Research **25**(3): 444-449.

Zhao, M., J. A. Laissue, et al. (1997). "TUNEL-positive hepatocytes in alcoholic liver disease. A retrospective biopsy study using DNA nick end-labelling." Virchows Arch **431**(5): 337-344.

Zhao, X.-J., Q. Dong, et al. (2008). "TRIF and IRF-3 Binding to the TNF Promoter Results in Macrophage TNF Dysregulation and Steatosis Induced by Chronic Ethanol." J Immunol **181**(5): 3049-3056.

Ziegler-Heitbrock, H. W., E. Thiel, et al. (1988). "Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes." Int J Cancer **41**(3): 456-461.

Ziol, M., M. Tepper, et al. (2001). "Clinical and biological relevance of hepatocyte apoptosis in alcoholic hepatitis." Journal of Hepatology **34**(2): 254-260.

Zuo, G., J. Gong, et al. (2003). "Synthesis of Toll-like receptor 4 in Kupffer cells and its role in alcohol-induced liver disease." Chin Med J (Engl) **116**(2): 297-300.

8 Appendix

8.1 Antibodies used in this study

Antigen	Host / class	Conjugate	Manufacturer	Catalogue#	Lot#	Working dilution	Application
B-actin	rabbit	-	Abcam	ab8227-50	426698	1 in 5000	WB
ACSS1	mouse	-	Abnova	H00084532-B01	08263	1 in 1000	WB
ACSS2	rabbit	-	Atlas	HPA004141	R04111	1 in 2500	WB
TLR4	mouse IgG2aκ	AlexaFluor647	eBioscience	51-9917-73	E019647	1 in 2.5	FC
acetyl-lysine	rabbit	-	Cell Signalling Technology	9441S	7	1 in 200	IF
acetyl-histone H3	rabbit	-	Upstate	06-599	31994	5µg/ml	IF
acetyl-histone H4	rabbit	-	Upstate	06-598	31991	10µg/ml	IF

Table 8-1 Primary antibodies

WB Western Blotting; FC flow cytometry; IF immunofluorescence

Antigen	Host / class	Conjugate	Manufacturer	Catalogue#	Lot#	Working dilution	Application
Mouse IgG	goat IgG	HRP	Sigma	A3673	087K6014	1 in 5000	WB
Rabbit IgG	goat IgG	HRP	Sigma	A6154	086K60142	1 in 5000	WB
Rabbit IgG	goat IgG	FITC	Sigma	F1262	105K6070	1 in 200	IF

Table 8-2 Secondary antibodies

HRP horseradish peroxidase

Antigen	Host / class	Conjugate	Manufacturer	Catalogue#	Lot#	Working dilution	Application
Isotype	mouse IgG2aκ	AlexaFluor647	eBioscience	51-4724-80	E020012	1 in 10	FC

Table 8-3 Isotype control antibodies

8.2 Real Time primers and probes

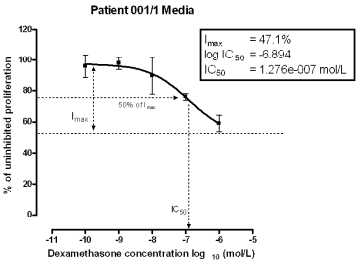
Target	Exon-spanning	Assay ID	Lot#	PCR efficiency
18s	-	Hs99999901_s1	467721	-2.775
TNFα	Yes	Hs00174128_m1	465479	-2.493
IL-6	Yes	Hs00174131_m1	466829	-2.723
IL-8	Yes	Hs99999034_m1	609148	-2.834
ACSS1	Yes	Hs00287264_m1	531983	-2.629
ACSS2	Yes	Hs00218766_m1	545764	-2.626
CYP2E1	Yes	Hs00559367_m1	607568	-
ADH1A	No	Hs00605167_g1	551822	-
ADH1B	Yes	Hs00605175_m1	545306	-
ADH1C	Yes	Hs00817827_m1	580597	-
ADH4	Yes	Hs00167415_m1	550113	-
ADH5	Yes	Hs00605185_m1	548804	-
ADH6	Yes	Hs00167423_m1	522585	-
ADH7	Yes	Hs00609447_m1	568774	-
ALDH1A1	Yes	Hs00946916_m1	622474	-
ALDH1A2	Yes	Hs00180254_m1	551423	-
ALDH1A3	Yes	Hs00167476_m1	542499	-
ALDH1B1	Yes	Hs00377718_m1	523325	-
ALDH1L1	Yes	Hs00201836_m1	575091	-
ALDH1L2	Yes	Hs00402876_m1	516746	-
ALDH2	Yes	Hs00355914_m1	573637	-
ALDH3A1	Yes	Hs00964880_m1	549124	-
ALDH3A2	Yes	Hs00166066_m1	316445	-
ALDH3B1	Yes	Hs00997594_m1	549124	-
ALDH3B2	Yes	Hs00167496_m1	520692	-
ALDH4A1	Yes	Hs00186689_m1	442990	-
ALDH5A1	Yes	Hs00542449_m1	549124	-
ALDH6A1	Yes	Hs00194421_m1	291558	-
ALDH7A1	Yes	Hs00609622_m1	546589	-
ALDH8A1	Yes	Hs00224021_m1	506316	-
ALDH9A1	Yes	Hs00997881_m1	593337	-
ALDH16A1	Yes	Hs00292269_m1	520333	-
ALDH18A1	Yes	Hs00913261_m1	549531	-

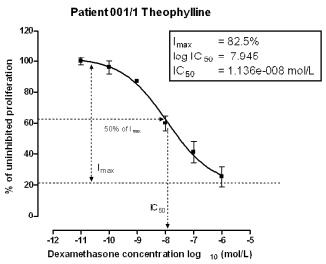
Table 8-4 Primers and probes

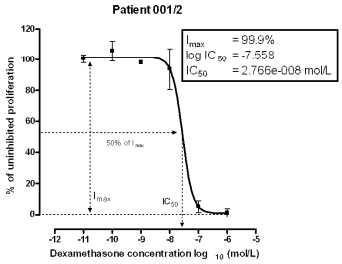
All primer/probe sets supplied by Applied Biosystems with FAM reporter dye and a non-fluorescent quencher.

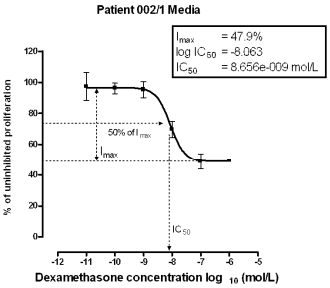
8.3 Lymphocyte steroid sensitivity graphs

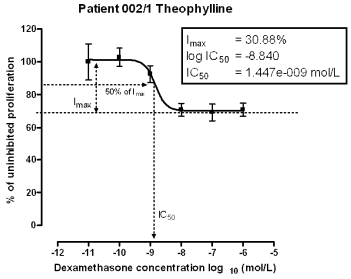
Presentation **Theophylline** **Recovery**

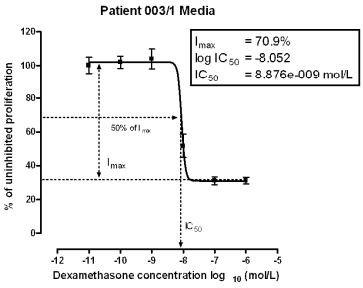


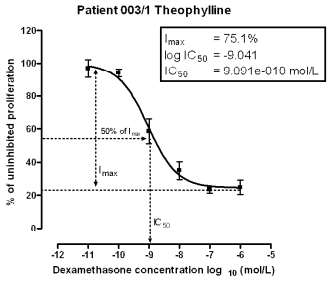


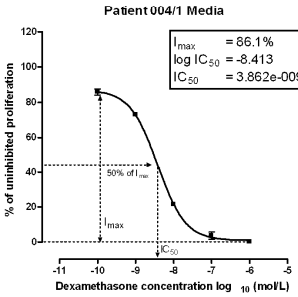


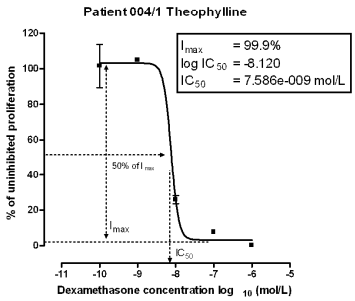


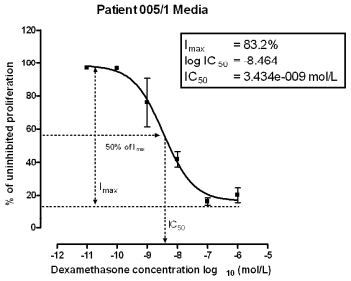
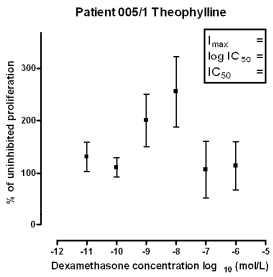
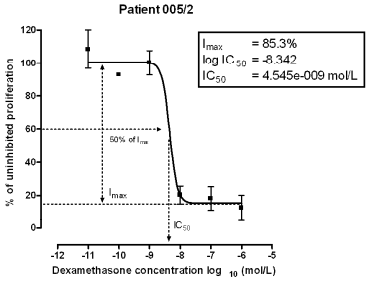
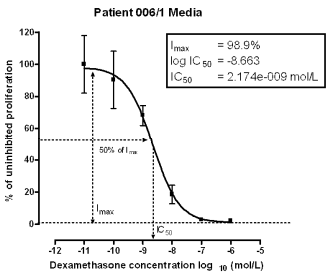
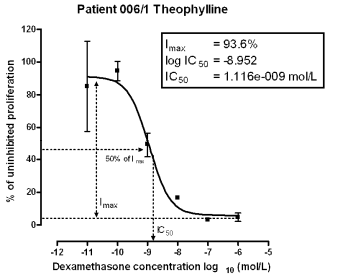
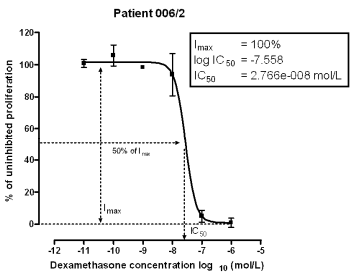
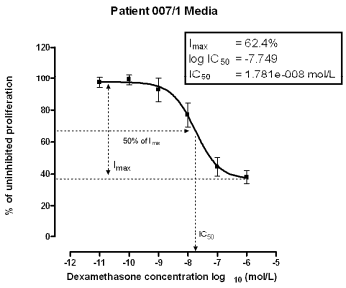
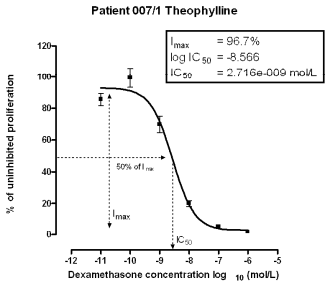
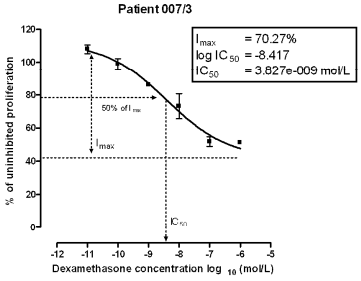
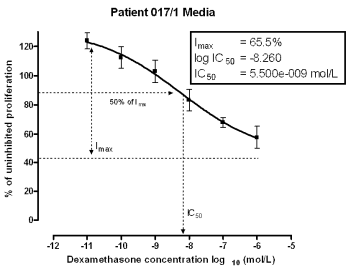
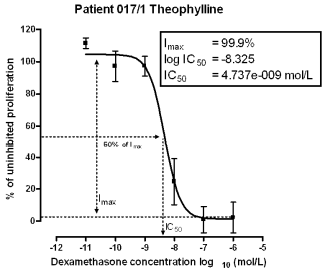
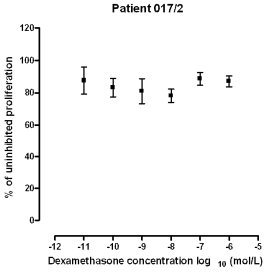




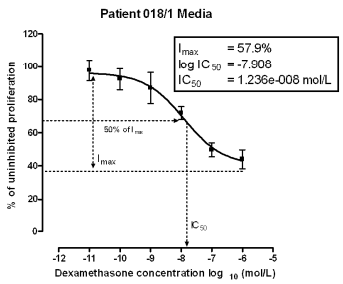


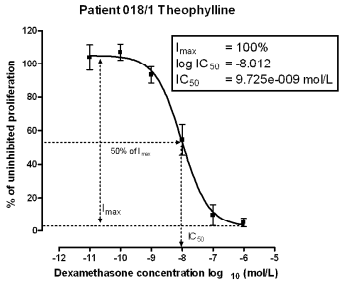


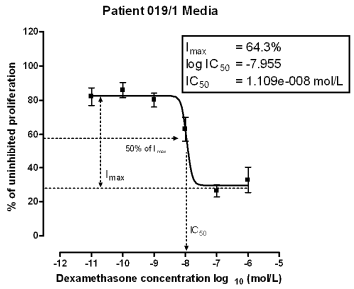


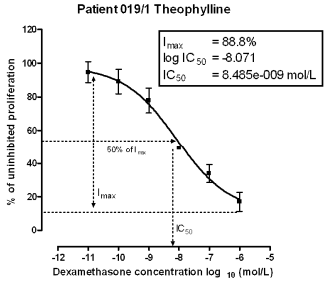
Presentation	Theophylline	Recovery
		
		
		
		

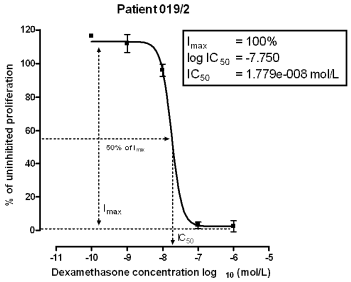
Presentation **Theophylline** **Recovery**

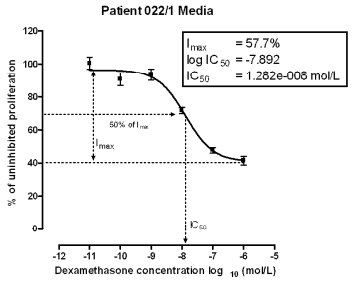


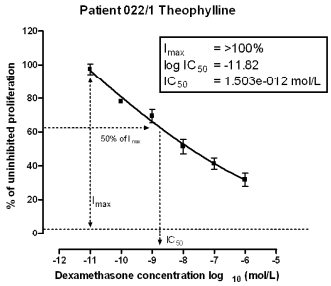


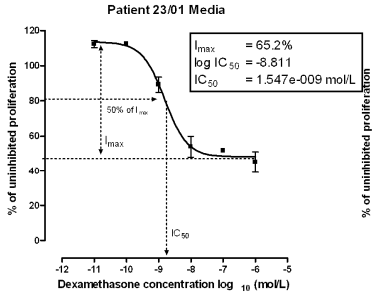


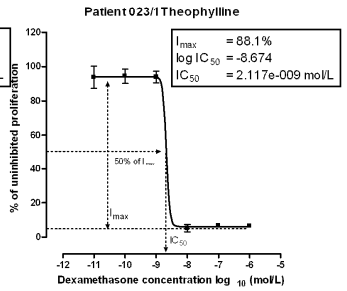


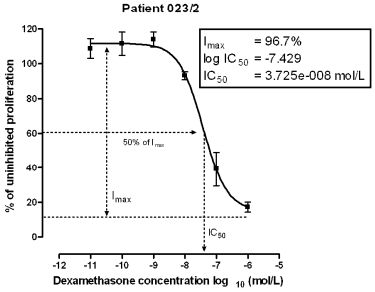












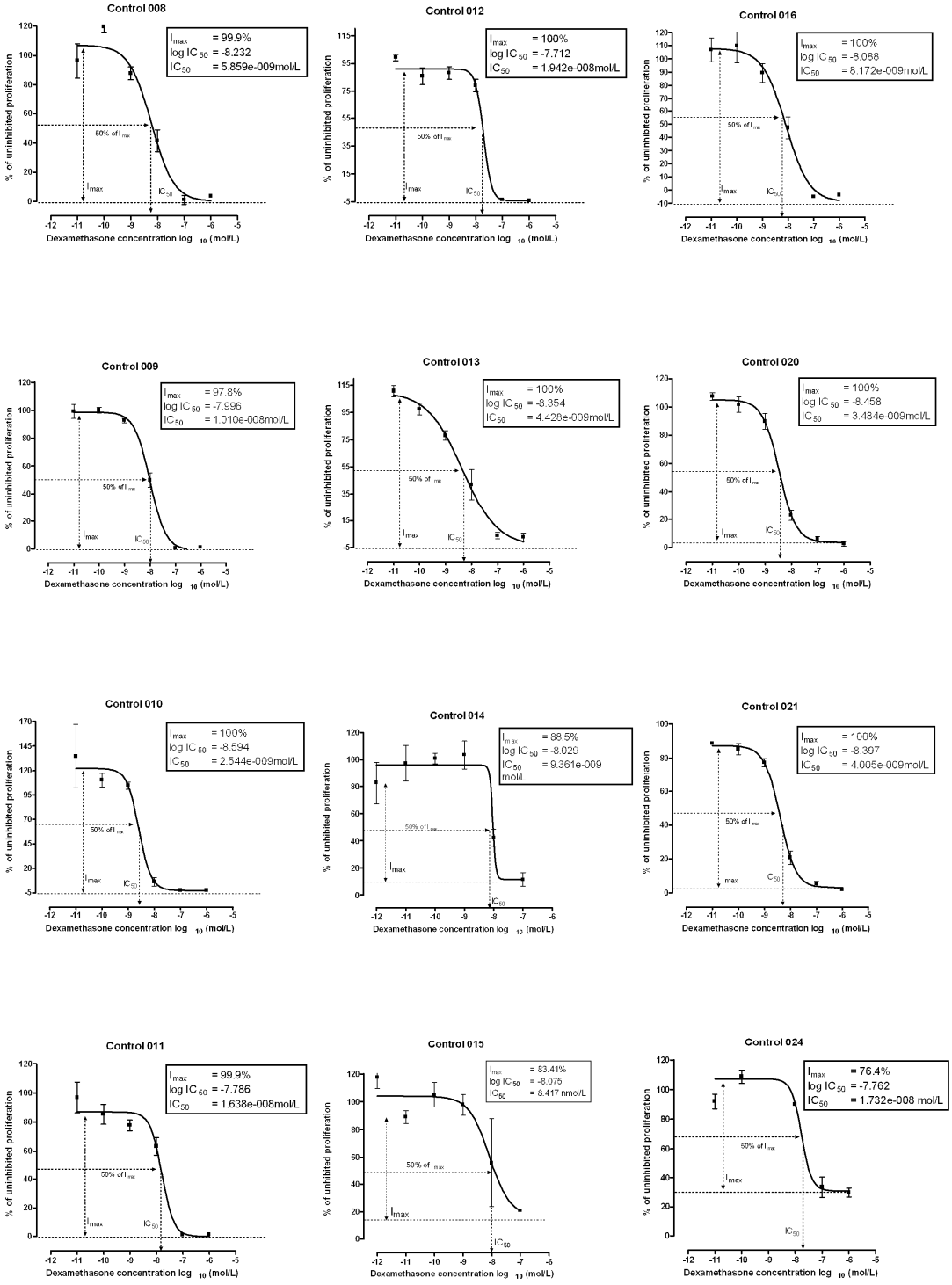


Figure 8-1 Individual lymphocyte steroid sensitivity plots for patients and controls

8.4 Histological scoring systems used in this study

Characteristic	0	1	2	3
Fat type		macrovesicular	microvesicular	mixed
Fat score	<5%hepatocytes	5-33%	33-66%	>66%
Ballooning	none	focal	abundant	
Mallory bodies	none	few	abundant	
Apoptotic bodies	none	few	abundant	
Polymorphs	absent	focal increase	generalised increase	aggregates
Lymphocytes	absent	focal increase	generalised increase	aggregates
Macrophages	normal numbers	Mild - Kupffer cell prominence	Moderate - generalised increase	Servere - aggregates
Plasma cells	absent	present		
Eosinophils	absent	present		
Cirrhosis	absent	present		

Table 8-5 Scoring system for alcoholic liver disease biopsies

8.5 Presentations of data from this study

American Association for the Study of Liver Disease Annual Meeting; San Francisco CA, 31 October – 4 November 2008

Enhanced inflammatory responses in alcoholic liver disease: is acetate the key? (Oral presentation)

A 'loss-of-function' mutation in TIRAP, the gene encoding the toll-like receptor adapter molecule Mal protects against fibrosis in NAFLD but not ALD. (Oral presentation)

British Association for the Study of the Liver Annual Meeting; Edinburgh, 10-12 September 2008

Enhanced inflammatory responses in alcoholic liver disease: is acetate the key? (Poster)

A 'loss-of-function' mutation in TIRAP, the gene encoding the toll-like receptor adapter molecule Mal protects against fibrosis in NAFLD but not ALD. (Poster)

American Association for the Study of Liver Disease Annual Meeting; Boston MA, 2-6 November 2007

Ethanol-induced histone acetylation: a novel mechanism for enhancement of inflammation in alcoholic hepatitis? (Presidential Poster of Distinction)

British Association for the Study of the Liver Annual Meeting; London, 12-14 September 2007

Ethanol-induced histone acetylation: a novel mechanism for enhancement of inflammation in alcoholic hepatitis? (Poster)

American Gastroenterological Association / British Society of Gastroenterology Joint Research Workshop: Inflammation, Repair and Fibrosis; London, 4-5 September 2007

Ethanol-induced histone acetylation: a novel mechanism for enhancement of inflammation in alcoholic hepatitis? (Oral presentation)

European Association for the Study of the Liver Annual Meeting; Barcelona, 11-15 April 2007

Effects of ethanol on endotoxin-mediated cytokine release: sensitisation via TLR4 but normal tolerance (Poster)

British Society of Gastroenterology Annual Meeting; Glasgow, 26-29 March, 2007

Lymphocyte steroid sensitivity in severe alcoholic hepatitis (Plenary poster)