MONOCYTE DYSFUNCTION IN ALCOHOLIC HEPATITIS

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Declaration of originality

I declare that this work is my own in this thesis and all else appropriately referenced.

Copyright declaration

Dedication

This thesis is dedicated my mother, father and sister Elizabeth, George and Anjana Varughese and to my new wife, Samantha.

Acknowledgements

Patience is an important virtue and I have learnt the importance of it over the course of these doctoral studies. It is a quality that my mother and father have shown me from childhood. I express my love and gratitude to them for it. It is also a quality embodied by my beautiful wife Sam over these last years, who is forever my ray of sunshine.

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Abstract

Severe alcoholic hepatitis (SAH) is the most florid form of alcohol-related liver disease (ALD). It is caused by hepatic inflammation after a prolonged period of heavy alcohol drinking. Causes of death include liver failure and infection. Inflammatory hepatic injury and systemic immunoparesis are therefore key features in disease pathogenesis.

This thesis seeks to evaluate this immune dysfunction in detail, focussing on a key component of the innate immune system, the circulating monocyte. A variety of techniques such as flow cytometry, Western blotting and polymerase-chain reaction (PCR) were used to characterise the phenotype and function of monocytes from peripheral blood. Dysfunction was then related to patient outcome and treatments administered by the randomised placebo controlled *Steroids and Pentoxyfilline for Alcoholic Hepatitis* (STOPAH) clinical trial.

Novel findings from this work include the identification of preserved uptake of bacteria by phagocytosis but defective monocyte oxidative burst and bacterial killing of *Escherichia coli*. Further, I show that the presence of this defect predicts the subsequent development of infection. In addition, this defect is associated with reduced expression of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme and may be treatable with *N*-acetylcysteine (NAC). Secondly, I identify an expanded population of an inflammatory intermediate monocyte subset in SAH that bears high expression of chemokine CC chemokine receptor type 5 (CCR-5), and may be amenable to targeted antibody therapy to reduce hepatic inflammation in SAH patients.

Publications arising from this work

Papers

- *Defective monocyte oxidative burst predicts infection in alcoholic hepatitis and is associated with reduced expression of NADPH oxidase*. **Vergis N**, Khamri W, Beale K, Sadiq F, Aletrari MO, Moore C, Atkinson SR, Bernsmeier C, Possamai LA, Petts G, Ryan JM, Abeles RD, James S, Foxton M, Hogan B, Foster GR, O'Brien AJ, Ma Y, Shawcross DL, Wendon JA, Antoniades CG, Thursz MR. Gut. 2016 Feb 9. pii: gutjnl-2015-310378. doi: 10.1136/gutjnl-2015-310378

- *Right of Left for COLonoscopy? Results of the ROLCOL study*. **Vergis N**, McGrath AK, Stoddart CH, Hoare JM. Am J Gastroenterol. 2015 Nov;110(11):1576-81. doi: 10.1038/ajg.2015.298

- *Prednisolone or Pentoxifylline for alcoholic hepatitis*. Thursz MR, Richardson P, Allison M, Austin A, Bowers M, Day CP, Downs N, Gleeson D, MacGilchrist A, Grant A, Hood S, Masson S, McCune A, Mellor J, O'Grady J, Patch D, Ratcliffe I, Roderick P, Stanton L, **Vergis N**, Wright M, Ryder S, Forrest EH; STOPAH Trial. N Engl J Med. 2015 Apr 23; 372(17):1619-28. doi: 10.1056/NEJMoa1412278.

- *Patients with acute-on-chronic liver failure have increased numbers of regulatory immune cells expressing the receptor tyrosine kinase MERTK*. Bernsmeier C, Pop OT, Singanayagam A, Triantafyllou E, Patel VC, Weston CJ, Curbishley S, Sadiq F, **Vergis N**, Khamri W, Bernal W, Auzinger G, Heneghan M, Ma Y, Jassem W, Heaton ND, Adams DH, Quaglia A, Thursz MR, Wendon J, Antoniades CG. Gastroenterology. 2015 Mar; 148(3): 603-615.e14. doi: 10.1053/j.gastro.2014.11.045

- *Blockade of PD1 and TIM3 restores innate and adaptive immunity in patients with acute alcoholic hepatitis*. Markwick LJ, Riva A, Ryan JM, Cooksley H, Palma E, Tranah TH, Manakkat Vijay GK, **Vergis N**, Thursz M, Evans A, Wright G, Tarff S, O'Grady J,

Williams R, Shawcross DL, Chokshi S.Gastroenterology. 2015 Mar;148(3):590-602.e10. doi: 10.1053/j.gastro.2014.11.041.

- *CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14(hi) /CD16(neg) monocytes: Expansion of CD14(hi) /CD16(pos) and contraction of CD14(lo) /CD16(pos) monocytes in acute liver failure.* Abeles RD, McPhail MJ, Sowter D, Antoniades CG, **Vergis N**, Vijay GK, Xystrakis E, Khamri W, Shawcross DL, Ma Y, Wendon JA, Vergani D. Cytometry A. 2012 Oct; 81(10):823-34. doi: 10.1002/cyto.a.22104

Presented abstracts

- High frequency of inflammatory CD16^{+} monocytes in alcoholic hepatitis can be reduced by treatment with prednisolone. Vergis N, Khamri W, Gill US, Blackmore LJ, Shawcross DL, Ma Y, Antoniades CG, Thursz MR. 2016 Lancet poster abstract

- Monocyte oxidative burst defect in alcoholic hepatitis is resistant to interferon gamma. Beale K, Vergis N, Sadiq F, Petts G, Khamri W, Antoniades CG, Thursz MR. *Gut* 2015; 64:A450 doi: 10.1136/gutjnl-2015-309861.984

- Acute alcoholic hepatitis and cellular Th1 immune responses to alcohol dehydrogenase. Blackmore LJ, Ryan JM, Huang Xiaohong, Hussain Munther, Traintafyllou E, Vergis N, Vijay GM, Antoniades CG, Thursz MR, Jassem W, Vergani D, Shawcross DL, Ma Y. 2015 Lancet

- Monocyte oxidative burst defect predicts risk of infection in alcoholic hepatitis and can be reversed by *N*-acetylcysteine. Vergis N, Khamri W, Antoniades CG, Thursz MR. 2014 Hepatology, Supplement

- Elevated levels of circulating bacterial DNA predict non-response to corticosteroid therapy in alcoholic hepatitis. Vergis N, Atkinson SR, S. Knapp, V. Patel, C.

Antoniades, E. Forrest, MR Thursz, Journal of Hepatology April 2015 Volume 62, Supplement 2, Page S770

- Functional defects in circulating monocytes may contribute to increased susceptibility to infection in alcoholic hepatitis. N. Vergis, C.G. Antoniades, V. Zingarelli, R.D. Abeles, Y. Ma, D. Shawcross, D. Patch, W. Rosenberg, P. Kennedy, G. Foster, A. Barnabas, S. Khan, K. Agarwal, M. Heneghan, J. O'Grady, D. Vergani, J. Wendon, M.R. Thursz. Journal of Hepatology April 2012 Volume 56, Supplement 2, Page S43

Organisation

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Abbreviations

βME	β-mercaptoethanol
1,2,3DHR	1,2,3-dihydrorhodamine
7-AAD	amino actinomycin D
ACLF	Acute on Chronic Liver Failure
ALD	alcohol-related liver disease
ALF	acute liver failure
ALT	Alanine Transferase
APC	allophycocyanin
ASH	Alcoholic steatohepatitis
BCA	91-bicinchoninic acid
bDNA	Bacterial DNA
C5a	complement factor 5a
CCL2	chemokine ligand 2
CCR	CC chemokine receptor
CD	Cluster of differentiation
CXCR	CXC chemokine receptor
DALY	disability adjusted life years
DAMP	damage-associated molecular pattern
DMSO	dimethyl sulfoxide
E. coli	Escherichia coli
ECBL	early change in bilirubin level
EDTA	Ethylenediaminetetraacetic acid
FasL	Fas ligand
FCS	fetal calf serum
FMO	fluorescence minus one FMO
G6PDH	Glucose-6 Phosphate Dehydrogenase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GILZ	glucocorticoid-induced leucine zipper
GM-CSF	Granulocyte macrophage colony stimulating factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GSH	glutathione
GSSG	glutathione disulphide
HMGB-1	high mobility group box-1
HRP	horseradish peroxidase
HSC	hepatic stellate cells
HSC	hepatic stellate cells
HSPs	heat shock proteins
ICAM	intracellular adhesion molecule
IFN	Interferon

IL	Interleukin
IQR	interquartile range
IRAK	interleukin-1 receptor associated kinase
IRF	Interleukin regulatory factor
KC	Kupffer cells
KCs	Kupffer cells
LDS	lithium dodecyl sulphate
Linneg	lineage negative gating strategy
LPS	Lipopolysaccharide
LPS-LBP	Lipopolysaccharide binding protein
LSECs	liver sinusoidal endothelial cells
MCP-1	monocyte chemotactic protein 1
MDF	Maddrey's discriminant function
MDF	Maddrey's discriminant function
MELD	model for end-stage liver disease
MFI	median fluorescence intensity MFI
MHC	Major histocompatibilty antigen
MiR	micro-RNA
MOB	monocyte oxidative burst defect
MSD	Mesoscale Discovery
NAC	*N*-acetylcysteine
NAC	N-acetylcysteine
NADLD	non-alcoholic decompensated liver disease
NADPH	nicotinamide adenine dinucleotide phosphate
NETs	neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
NP-40	nonyl phenoxypolyethoxylethanol-40
OR	Odds ratios
PAGE	Polyacrilamide gel electrophoresis
PBMCs	Peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCT	Procalcitonin
PD-L	Programmed death ligand
PE	phycoerythrin
PE-Cy7	phycoerythrin-Cy7
PerCP	Peridinin chlorophyll protein
PGE2	prostaglandin E2
pSTAT-1	phospho-Signal Transducer and Activator of Transcription-1
PTX	pentoxifylline
PVDF	polyvinylidene difluoride
qPCR	quantitateive polymerase-chain reaction
ROC	receiver operator characteristic curve
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute media
RT	room temperature

SAH	Severe alcoholic hepatitis
SAH-MOB	severe alcoholic hepatitis without monocyte oxidative burst defect
SAH+MOB	severe alcoholic hepatitis with monocyte oxidative burst defect
slan	6-sulfo LacNAc
SOCS-1	Suppressor of Cytokine Signalling-1
SR	scavenger receptors
STOPAH	*Steroids and Pentoxyfilline for Alcoholic Hepatitis*
TBST	Tween20
TGF	transforming growth factor
TLR	Toll like receptor
TNF-α	Tumour necrosis factor α
TRAIL	TNF related apoptosis inducing ligand
TRAIL	TNF related apoptosis inducing ligand
UK	United Kingdom
US	United States

Glossary

- **Lille score:** a prognostic marker of the change in bilirubin levels in the first week after prednisolone therapy for SAH (Louvet et al. 2007). It is given by the formula below. Lille is calculated as:

Exp(-R)/[1+exp(-R)] where

R = [3.19 - (0.101*age in years)] + (1.47*albumin at day 0 in g/dL) + [0.28215* (bilirubin at day 0 - bilirubin at day 7 in mg/dL)] - [0.206 * (if creatinine>=1.3 mg/dL at day 0)] - [0.11115*bilirubin day 0 in mg/dL] - (0.0096*Prothrombin Time in seconds at day 0)

- **Model for End-stage Liver Disease (MELD)**

9.57xln (creatinine(mg/dL)) + 3.78xln (bilirubin(mg/dL)) + 11.2xln(INR) + 6.43

- **Maddrey's discriminant function (MDF)**

(4.6 x (PT test - control))+(Bilirubin/17)

- **Statistical notation:**

*   p<0.05

**   p<0.01

***   p<0.001

****   p<0.0001

Introduction

Background

Alcoholic hepatitis is a condition of liver failure and jaundice associated with heavy and prolonged alcohol abuse (Lucey et al. 2009). When liver impairment reaches a defined threshold, the condition is known as severe alcoholic hepatitis (SAH). Up to 30% of patients suffering from SAH may die within 30 days of diagnosis (Maddrey et al. 1978).

Infection is responsible for a large proportion of deaths in SAH, suggesting immune dysfunction. Monocytes are key players in innate immunity. The phenotypic and functional characteristics of human monocytes in SAH patients have not so far been described. This thesis tests these characteristics with the aim of identifying defects in human monocyte function that might contribute to the hepatic inflammation and increased susceptibility to infection that is associated with SAH.

(a) Burden of Alcohol-related Liver Disease (ALD)

Mortality rates in liver disease have increased 400% since 1970. Liver disease is now the fourth commonest cause of death in the UK. 600,000 people in England and Wales have some form of liver disease of which 60,000 have cirrhosis. In patients less than 65 years of age, mortality rates have increased by over 500%. Liver disease is the now the third biggest cause of premature mortality – 62,000 years of working life are lost every year (Williams et al. 2014).

Alcohol is in the top 5 causes of death and disability globally; responsible for 5.9% of deaths and 5.1% of disability adjusted life years (DALYs) lost each year (World Health Organisation 2014). Europe is the heaviest drinking region in the world, with over 20% of the European population aged≥15 years reporting heavy episodic drinking. As a result alcohol is the main

cause of liver disease in Europe and the strongest risk factor for liver cirrhosis (Blachier et al. 2013). Alcohol related health problems cost the UK National Health Service £3.5 billion per year (Williams et al. 2014). Alcohol related harm costs the UK £21 billion per year (Home Office 2012).

Health problems related to alcohol drinking range from poisoning, mental and behavioural disorders and alcoholic cardiomyopathy but liver disease is responsible for the greatest morbidity and mortality. Liver disease attributable to alcohol accounts for 800,000 hospital admissions per year and is responsible for 6,500 deaths per year (House of Commons 2010).

The burden of liver disease attributable to the harmful use of alcohol is higher than other aetiologies (Blachier et al. 2013). In the UK approximately 14,700 hospital admissions each year are attributable to alcohol related liver disease (Potts & Verma 2012). The UK standardised mortality ratio from alcohol-related liver diseases is 12.5 and 6.1 per 100,000 in men and women respectively and accounts for a tenth of all deaths of those aged 40-50 years (Blachier et al. 2013; Potts & Verma 2012).

(b) **Spectrum of Alcohol-related Liver Disease**

ALD encompasses a range of hepatic manifestations of alcohol excess. These include hepatic fatty change, alcoholic hepatitis and hepatic fibrosis or cirrhosis.

The vast majority of patients who chronically abuse alcohol will accumulate fat in the liver, often referred to as fatty liver and histologically termed hepatic steatosis. Initially, hepatocytes demonstrate liposomes around the nucleus, termed microvesicular fatty change. As the condition progresses, the size of the vacuoles increase which pushes the

nucleus to the periphery and gives the cell a signet ring appearance known as macrovesicular fatty change. If alcohol drinking ceases, fatty liver disease is reversible.

(c) **Histologic steatohepatitis**

A proportion of those patients with hepatic steatosis who continue to abuse alcohol will develop steatohepatitis. Steatohepatitis is caused by biochemical and immunological mechanisms. Biochemical mechanisms centre on the metabolism of ethanol by the liver to generate acetaldehyde. Reactive oxygen species are by-products of this metabolism, figures 1 and 2. Immunological mechanisms are described in figure 3 and 4.
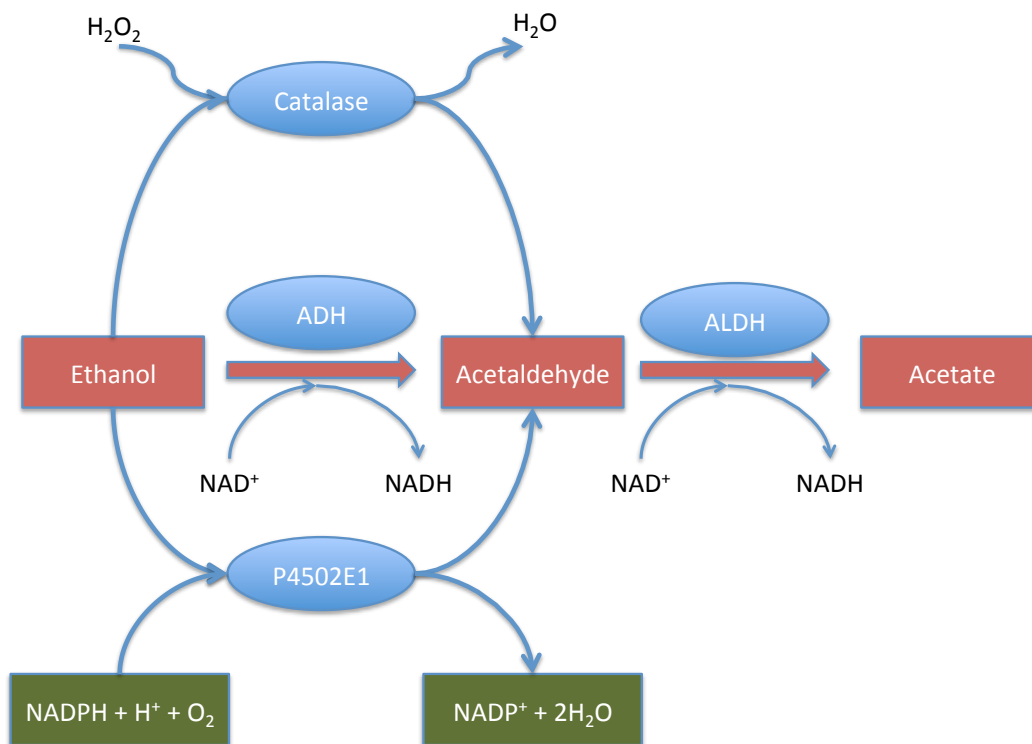
Figure 1: metabolism of ethanol leads to the generation of reactive oxygen species (ROS) through the activity of catalase and the enzyme cytochrome P4502E1. Acetate can be utilised within the Kreb's cycle to generate adenosine triphosphate (ATP)
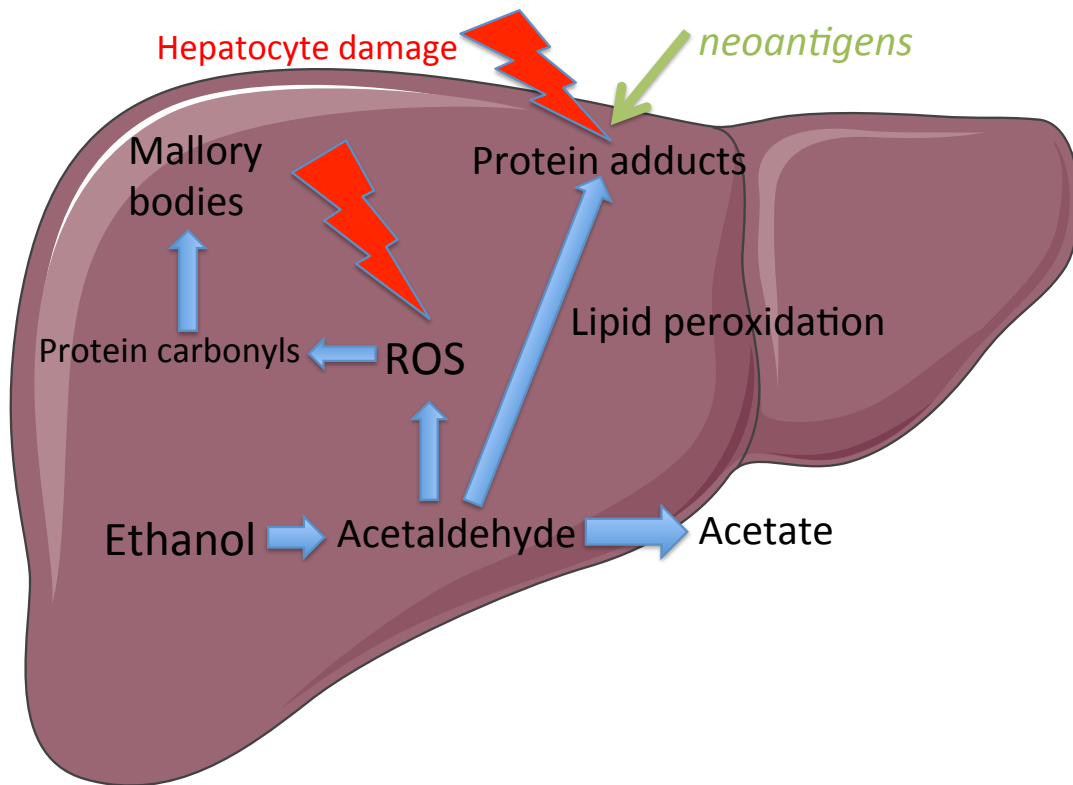
Figure 2: *key pathways in the pathogenesis of SAH*. **The metabolism of ethanol by the liver generates reactive oxygen species that can damage hepatocytes. In addition, acetalydehyde can bind to hepatocyte proteins via lipid peroxidation. Proteins adducts act as neoantigens. B cells can generate specific antibodies against these neoantigens. Adapted from** (Lucey et al. 2009)**.** *Figure created using Servier™ medical artwork*

Alcoholic steatohepatitis (ASH) is characterised by ballooning degeneration often associated with Mallory-Denk bodies, and a variable degree of, usually, neutrophilic inflammation. As the disease progresses fibrosis develops in a pericellular/sinusoidal pattern centred on central veins that can progress to bridging fibrosis and ultimately cirrhosis. The precise definition of steatohepatitis is inconsistent in published literature however: whilst the presence of hepatocyte ballooning is a consistent feature, the presence of Mallory Denk bodies and the type of inflammation may vary (Sakhuja 2014). In a recent study from Belgium, features of steatohepatitis were seen in 96% of patients with acute on chronic liver failure undergoing liver biopsy (Katoonizadeh et al. 2010).

Megamitochondria, if identified, carry prognostic information (Altamirano et al. 2014). The presence of canalicular cholestasis has been identified as an adverse prognostic factor. The degree of parenchymal neutrophil infiltration has been positively associated with response to corticosteroid (Spahr et al. 2011).

(d) **Alcoholic Hepatitis**

Alcoholic hepatitis is the clinical syndrome of liver failure and jaundice superimposed on histological steatohepatitis. The disease severity, manifest as cholestasis and liver failure, can be quantified using serum bilirubin and prothrombin time measurements to calculate Maddrey's discriminant function (MDF) score (see *Glossary* for formula). MDF≥32 is termed severe alcoholic hepatitis (SAH). SAH portends a high mortality rate within 30 days: studies conducted in the early 1980s suggest a mortality rate of 30-40% (Maddrey et al. 1978) while more recent studies document mortality of less than 20% within the first month (Thursz et al. 2015; Mathurin et al. 2013).

The exact prevalence of SAH in the UK is unknown. In the US, there were 45 hospitalisations per 106 citizens for SAH from 1998 to 2004 (Yang et al. 2008). A population-based cohort of 1951 patients with severe alcoholic hepatitis in Denmark revealed an incidence of 46 per 106 men and 34 per 106 women (Sandahl et al. 2011), figures that have increased since 1999. This increase in SAH in recent times is on a backdrop of falling annual per-capita sales of pure alcohol, by around 13%. However, the proportion of Danes with potentially harmful drinking rose from 8% to 11% and 14 to 18% for women and men, respectively. The biggest rise in incidence in SAH was in middle-aged women (Sandahl et al. 2011).

(e) **Causes of mortality in SAH**

SAH is a precipitant of acute on chronic liver failure (ACLF), a recently coined syndrome of acute decompensation of chronic liver disease combined with other organ failure that portends a high rate of death within two weeks (Moreau et al. 2013). It follows that the causes of death in SAH are common to other forms of ACLF and include variceal bleeding, hepatorenal syndrome, encephalopathy, liver failure and infection. In the recent STOPAH trial in which 268 patients died within 90 days, of which hepatic failure and infection accounted for the majority (Thursz et al. 2015).

A quarter of patients are already infected on admission to hospital with SAH and around half have developed infection by the end of their hospital stay (Louvet et al. 2009). Patients with SAH were almost fourfold more likely to develop culture positive infection than cirrhotic control groups without alcoholic hepatitis who were in turn more likely to develop organ failure and die (Mookerjee et al. 2007). Those patients that show biochemical evidence of response to seven days of corticosteroid therapy can expect two-month survival of 94%; this, however, falls to 51% if there is concurrent infection (Louvet et al. 2009). While prevention of mortality has been the goal of a number of therapeutic clinical trials aimed at improving hepatic inflammation (Akriviadis et al. 2000; Naveau et al. 2004; Mathurin et al. 2011; Thursz et al. 2015), prevention of infection has received comparatively little attention although it is the subject of on-going antibiotic-based clinical trials (Louvet *et al.* NCT02281929).

(f) **Hepatic inflammation and systemic immunoparesis co-exist in alcoholic hepatitis**

SAH is primarily a disease of an activated immune system causing inflammatory tissue damage. Concomitantly, there are immune defects that render the patient susceptible to infection. The remainder of this thesis is concerned with characterising this immune

dysfunction in SAH. The overarching aim is to identify both the exaggerated and deficient immune responses and focus on reversible immune targets for therapy.

Immune Dysfunction in SAH

Traditionally, alcoholic hepatitis was viewed as a biochemical disease on inception. Acetaldehyde, a toxic by-product of ethanol metabolism, is implicated in disease pathogenesis. The fact that SAH, once established, can progress for several days even in the absence of alcohol consumption suggests that immunological mechanisms may also play a role in pathogenesis.

(g) **Immune-Mediated Hepatic Injury**
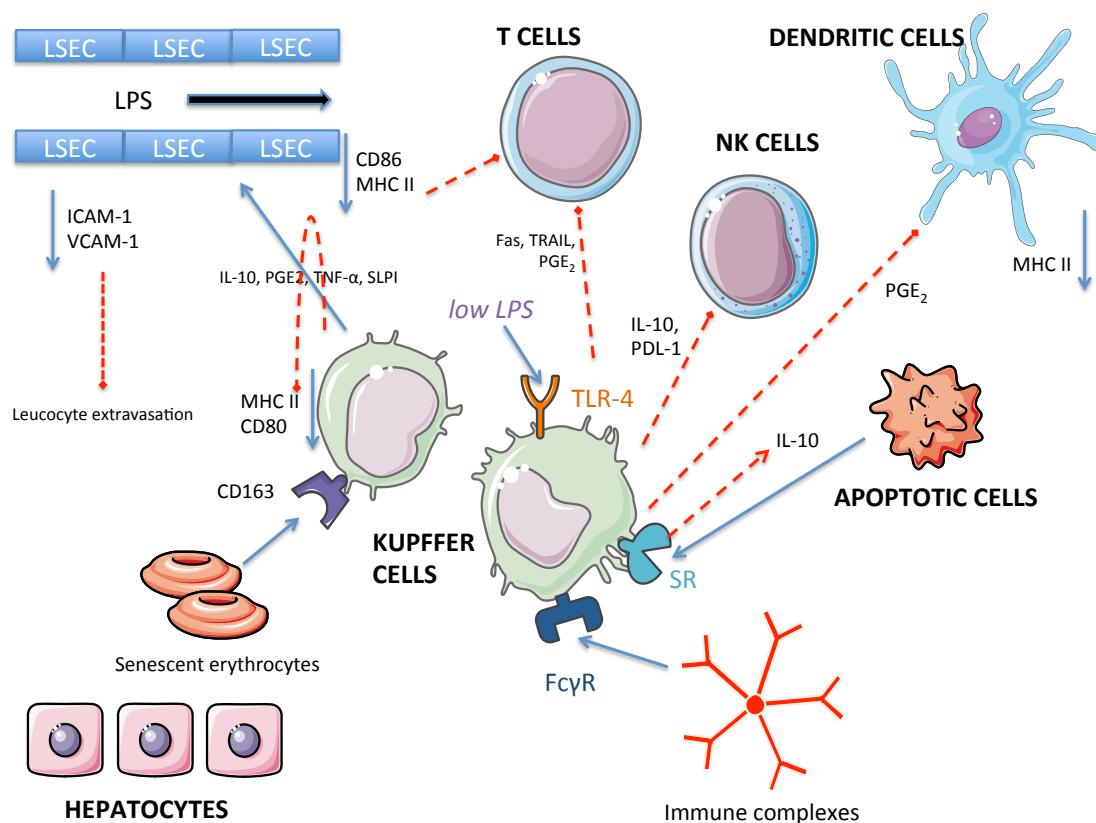
Figure 3: *Liver immune homeostasis. Phagocytosis and induction of tolerance are the two main functions of KCs during homeostatic conditions*. KCs are in close proximity to liver sinusoidal endothelial cells (LSECs). KCs express a broad range of scavenger receptors that makes them highly efficient at filtering endogenous and exogenous antigens. Complement receptors mediate removal of opsonised material. Immune complexes are

cleared by FcγRs. Engulfment of apoptotic cell constituents can induce IL-10, an immunosuppressant. Constant exposure to gut-derived LPS via TLR-4 also results in expression of IL-10 and prostaglandin E2 (PGE2) that can directly inhibit T and NK cells, mediating down-regulation of co-stimulatory proteins. Programmed death ligand (PD-L)-1 derived from KCs and release of mediators such as TNF related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL) induces apoptosis and can contribute to immune suppression by elimination of T cells and NK cells. Apoptotic cells are detected by scavenger receptors (SR) and may produce further IL-10 in response to phagocytosis of apoptotic cells. IL-10, PGE2 and TNF-α lead to reduced expression of adhesion molecules on LSECs, thereby limiting leucocyte recruitment. Adapted from (Zimmermann et al. 2012). *Figure created using Servier™ medical artwork*

The key cell populations to liver homeostasis are given in figure 3 (Zimmermann et al. 2012).

Despite a low throughput of endotoxin entering via the liver sinusoidal endothelial cells,

inflammatory homeostasis is achieved within the liver via the carefully controlled immune

response. During acute liver failure, the cell populations most important in liver injury are

Kupffer cells, neutrophils, T cells and NK cells, as shown in figure 4.
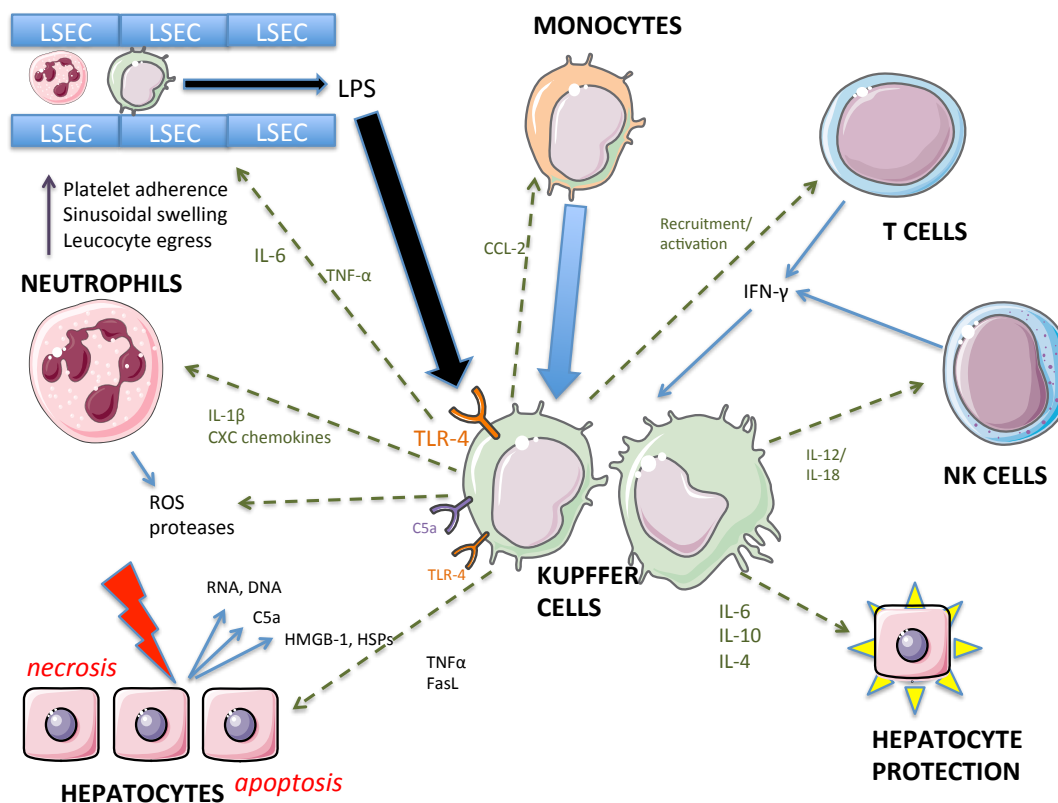
Figure 4: *Immune mediated injury in acute liver injury*. Hepatocyte releases DAMPs including heat shock proteins (HSPs) and high mobility group box-1 (HMGB)-1. These

DAMPs can bind to a variety of TLRs including TLR4. NF-κB is activated and pro-inflammatory cytokines, chemokines, reactive oxygen, and nitrogen species are generated. TNFα from KCs exacerbates liver injury by inducing hepatocyte apoptosis and can also disrupt the hepatic microcirculation through swelling of endothelial cells, sinusoidal platelet aggregation and recruitment of circulating leucocytes. KCs can secrete IL-1β and CXC chemokines (CXCL2 and CXCL8 (IL-8)). This attracts neutrophils that release ROS and proteases causing hepatocyte necrosis. CCL2, together with other CC chemokines can mediate liver influx of monocytes derived from the bone marrow, increasing KC numbers. IL-4, IL-6, and IL-10 secreted by KCs attempt to dampen hepatic injury. Adapted from (Zimmermann et al. 2012). *Figure created using Servier™ medical artwork*

Various aetiologies of liver injury such as drug toxicity, acute viral hepatitis B, or paracetamol poisoning can cause acute liver injury, and the excessive death of hepatocytes can causes severe hepatic inflammation. In murine models, Kupffer cells initiate and drive the inflammatory response by releasing factors such as TNFα, IL-6, IL1β, and MCP-1/CCL2 in addition to activating hepatic stellate cells and endothelial cells. These macrophage activities depend largely on the recruitment of monocytes into the liver, namely Ly6C$^+$ monocytes as a precursor of tissue macrophages. Some of this recruitment may be mediated by CCR2 and its ligand MCP-1, which can promote macrophage subset differentiation upon liver injury. On the contrary, the chemokine receptor CX3CR1 and ligand fractalkine can negatively regulate monocyte infiltration by controlling survival and differentiation into diverse subsets after acute liver injury (Zimmerman 2012).

For SAH, a condition of acute on chronic liver disease, such detailed characterisation of the immunological interplay occurring during liver injury are not available, in part due to a lack of an appropriate animal model of disease, see *Discussion*. In addition, liver biopsy specimens for human SAH are taken via the transjugular route: by nature only small samples of liver are obtained by this route and the potential for functional studies is therefore limited. Whole explant liver tissue is also difficult to acquire because many countries do not offer liver transplantation to patients who are actively drinking, which SAH patients are by definition. Where explant tissue is available because of pilot transplantation programmes, patients

have commonly received prednisolone therapy for prolonged periods that will critically modulate immune cell phenotype and function and therefore restrict inferences (Dubuquoy et al. 2015).

Despite their deficiencies, some mouse models are available with which to formulate hypotheses. The most popular of these are the Lieber deCarli method of alcohol feeding (Lieber & DeCarli 1989), intragastric infusion of alcohol pioneered by Tsukomoto and French (Tsukamoto et al. 2008), and the chronic binge model recently developed (Bertola et al. 2013), see also *Discussion.* Similar to models of acute liver injury, these models too suggest that innate immune cells are pivotal in liver injury.

For example, when Kupffer cells (KCs) are depleted by gadolinium chloride from animal models of ethanol-mediated liver injury, there is a reduction in early alcohol induced liver inflammation and necrosis (Adachi et al. 1994). Similarly, monoclonal antibody to CD18, a neutrophil cell-surface receptor that, in conjunction with β2 integrin, binds to intracellular adhesion molecule (ICAM)-1 on target hepatocytes, prevents neutrophil infiltration into hepatic tissue and abrogates alcohol induced liver injury (Bautista 1997). While KCs and neutrophils may produce pro-inflammatory cytokines and reactive oxygen species (ROS) respectively in SAH, T cells, B cells, Natural Killer cells and Natural Killer T cells may also contribute to initiation and propagation of hepatocyte damage in SAH. Each of these cells will be considered in turn.

(i) Innate Immune Dysfunction within the Liver

- **Early liver injury results from complement activation**

Animal models have implicated activation of the classical complement pathway by ethanol as a critical step in early ethanol-induced liver injury. Ethanol metabolism itself induces KC apoptosis: C1q, the recognition subunit of the first complement component, binds to

apoptotic KCs in ethanol fed mice and this activates the classical pathway by cleavage of the third component of the complement pathway (C3) (Cohen et al. 2010). Elevated C3a in plasma and C3b in liver has been demonstrated in ethanol-induced liver injury in rodent models (Pritchard et al. 2007; Bykov et al. 2006).

The importance of complement activation to alcoholic liver disease was highlighted by the observation that C3 knock-out mice do not develop hepatic steatosis in response to prolonged ethanol consumption (Bykov et al. 2007). In contrast, C5 knock-out mice are completely protected from ethanol-induced changes in ALT and inflammatory cytokines but still develop hepatic steatosis (Pritchard et al. 2007). C5a is a potent neutrophil chemo attractant that up regulates the neutrophil adhesion molecules β2integrin/CD18, thereby facilitating neutrophil recruitment to the inflamed liver (Abraham P. Bautista 2002).

In KCs, complement activation is a stimulus for KC derived TNFα production. C3-/- knockout mice were protected from the early, ethanol-induced increases in hepatic TNFα expression whilst mice deficient in C3a or C5a receptors showed had no increase in hepatic TNFα expression in response to ethanol. This increased production of TNFα was associated with a rise in hepatic triglycerides, plasma ALT, and 4-hydroxnonenal adducts (Roychowdhury et al. 2009). Taken together, these experimental data implicate both KC and neutrophils in complement-driven ethanol induced liver damage.

- **The Gut-Liver Axis, Endotoxin and Kupffer Cells**

The gut is an important component of human innate defence since it provides an anatomical barrier against enteric pathogens. In healthy individuals, gut integrity is maintained and only small amounts of bacterial endotoxin enter the liver via the portal vein. KCs are tolerant to low concentrations of endotoxin, and have been demonstrated to produce predominantly

anti-inflammatory mediators in such resting states (Knolle et al. 1995; Purohit et al. 2008), figures 3 and 5.
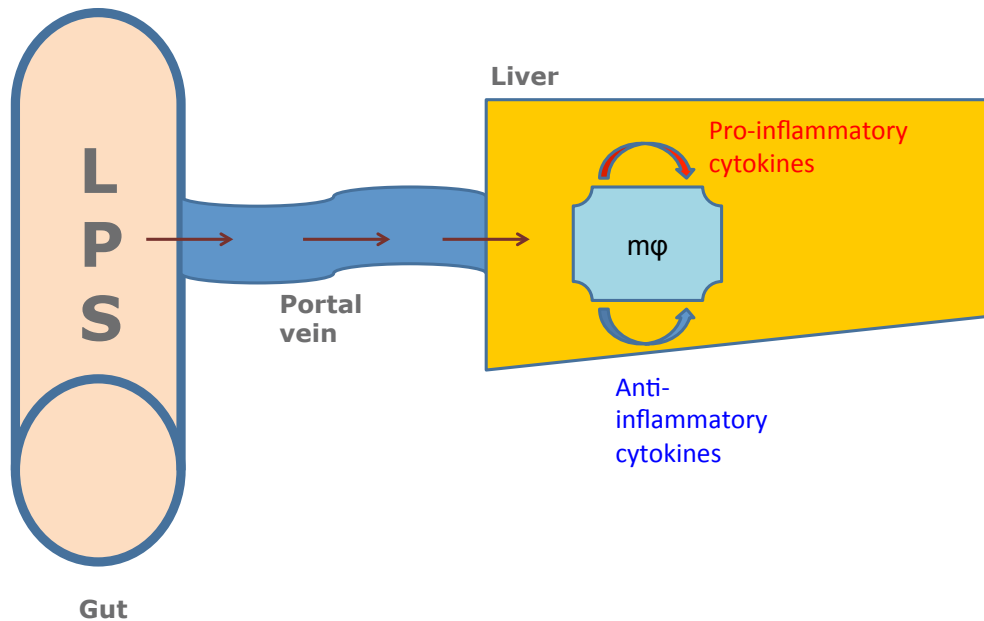
Figure 5: in health, gut membranes are intact and bacterial translocation of lipopolysaccharide (LPS) occurs at low levels. Hepatic macrophages (mφ) are tolerant to these low levels of bacterial translocation and there is minimal inflammatory activity within the liver.

Consumption of large doses of ethanol damages intestinal barriers of alcoholic patients, causing mucosal ulceration; haemorrhage into the lamina propria and disruption of gut epithelial tight junctions: such injury has been shown to contribute to increased gut permeability (Bode & Christian Bode 2003). In addition, small bowel bacterial overgrowth has been demonstrated in up to 50% of alcoholic patients (Bode et al. 1984). Finally, chronic ethanol feeding impairs the ability of Kupffer cells to phagocytose *E. coli* in animal models (A P Bautista 2002), implying diminished clearance of endotoxin by the liver during alcoholism.

Increased gut translocation of endotoxin and reduced hepatic clearance is thought to account for the increased systemic concentrations of endotoxin in patients with ALD (Lumsden et al. 1988; Bigatello et al. 1987), figure 4, although it should be noted that there are also reports that do not detect elevated serum endotoxin levels in patients with ALD (Triger et al. 1978; Mathurin et al. 2000). Animal studies indicate that elevations in circulating concentrations of LPS correlate with the extent of liver injury (Nanji et al. 1993; Mathurin et al. 2000), figure 6. In line with this, selective gut decontamination with antibiotics or repopulation with lactobacillus has been shown to reduce endotoxaemia and ameliorate alcohol induced liver injury (Adachi et al. 1995; Nanji et al. 1994).
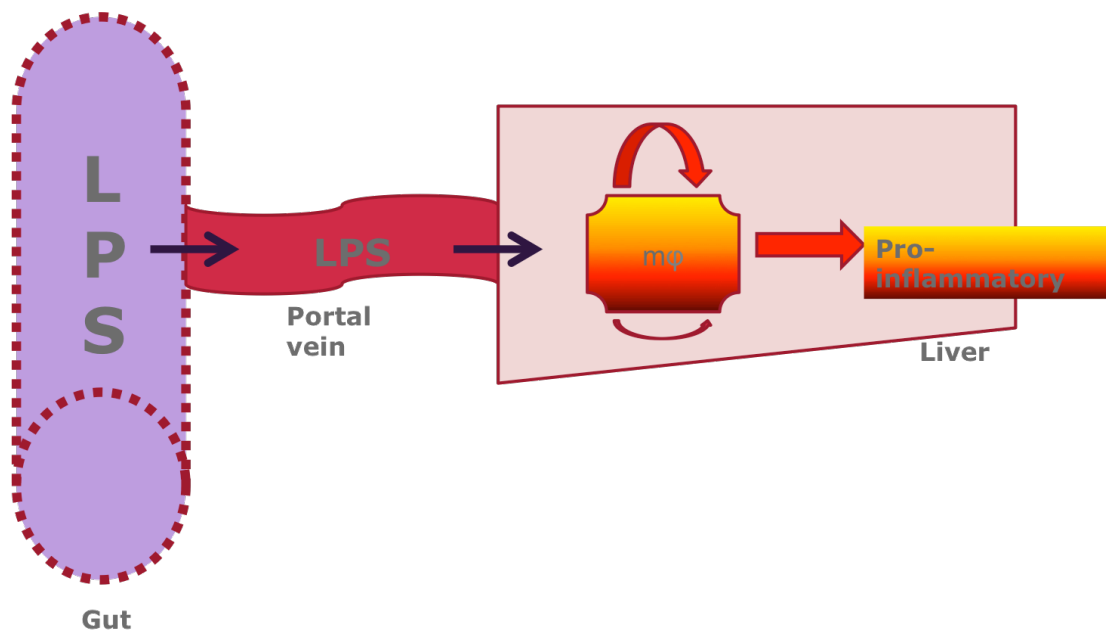
Figure 6: alcohol increases gut permeability, leading to leakage of endotoxin from the gut into the portal circulation. An increased load of endotoxin arriving at the liver may activate KCs to produce increased pro-inflammatory cytokines, which may cause inflammatory damage to hepatocytes

LPS binds to LPS-binding protein (LBP) in the circulation (Uesugi et al. 2002). LPS-LPB in turn binds to the Toll-Like Receptor 4 (TLR4) complex on KCs, activating both Myd88-dependent and Myd88-independent intracellular signalling pathways (Shimazu et al. 1999), figure 7. Activation of these cascades lead to the nuclear translocation of transcription

factors such as NF-κB and interleukin regulatory factor 3 (IRF3), which in turn lead to expression of pro-inflammatory cytokines and type 1 interferons (Pålsson-McDermott & O'Neill 2004). Release of such pro-inflammatory cytokines is believed to propagate hepatocellular injury during SAH.
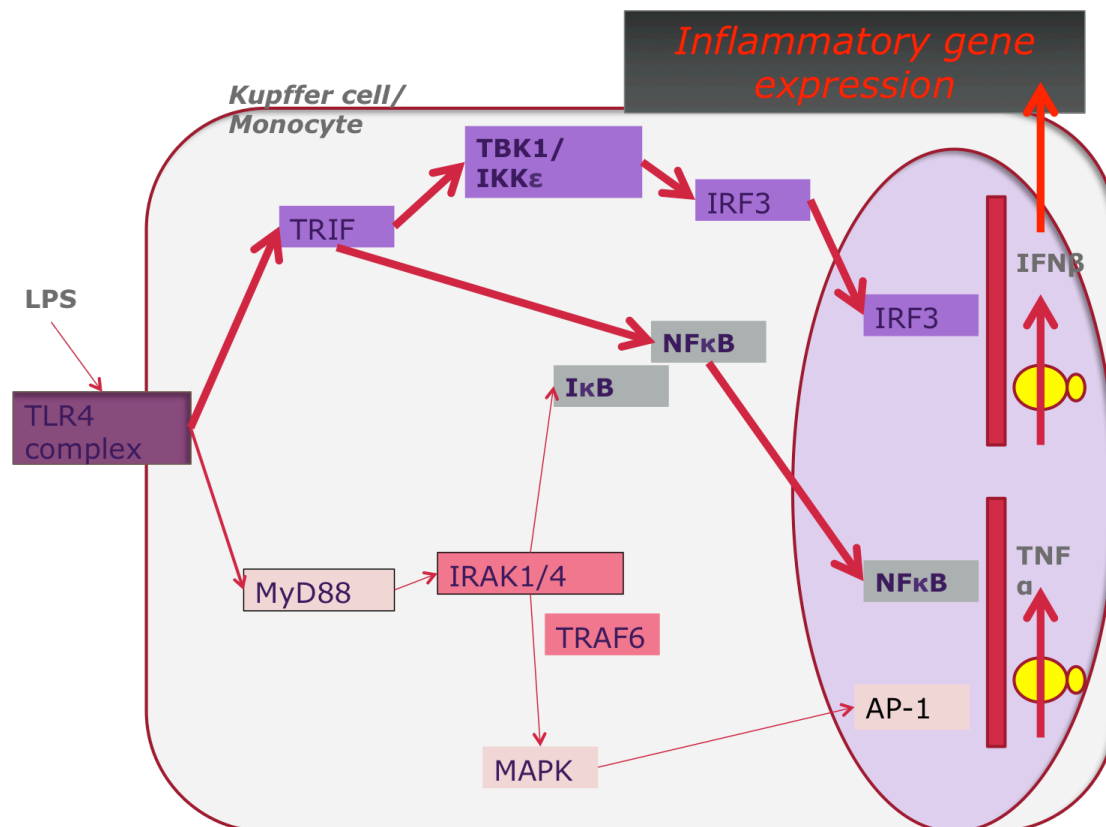
Figure 7: LPS binding to TLR-4 leads to an intracellular cascade involving TRIF (TIR domain containing adapter inducing interferon-β), Myd88 (myeloid differentiation primary response gene 88 (MYD88), IRAK1/4, mitogen-activated protein kinase (MAPK), TNF receptor associated factors (TRAF), TANK-binding kinase 1 (TBK1), IRF3, NK-κB and activator protein-1 (AP-1). This signalling cascade ultimately leads to transcription of various inflammatory genes such as TNF-α and IFN-β

It should be noted that, in animal models at least, acute and chronic ethanol exposure have opposing effects on Kupffer cells. Acute ethanol exposure is able to induce tolerance of macrophages, whereas chronic ethanol induces sensitisation to further LPS stimulation. Changes in the expression of an intracellular regulator interleukin-1 receptor associated

kinase (IRAK) and monocyte specific IRAK-M may underlie these observations (Mandrekar et al. 2009; Yamashina et al. 2000)).

There are sparse human data specifically regarding the role of KCs in the development of SAH. Data from human circulating monocytes in SAH are instructive, however. These monocytes demonstrate augmented production of pro-inflammatory mediators, both at baseline and upon endotoxin stimulation (McClain & Cohen 1989; Hill et al. 2000; Bala et al. 2011) and decreased production of IL-10 (Taïeb et al. 2000).

Emerging data suggesting that ethanol itself may be the driver of this by modulating both positive and negative regulators of the monocyte/macrophage inflammatory response to endotoxin (Messingham et al. 2002; Bala et al. 2011). In animal studies for example, monocyte micro-RNA (MiR)-155 production is increased by ethanol feeding and is associated with higher levels of TNFα, serum ALT levels and steatohepatitis (Bala et al. 2011).

In addition, in vitro studies using human circulating monocytes reveal that expression of IL-1R-associated kinase-monocyte (IRAK-M), a negative regulator of the pro-inflammatory response, is decreased by chronic alcohol, leading to increased expression of TNFα in response to LPS (Mandrekar et al. 2009).

Similarly, glucocorticoid-induced leucine zipper (GILZ), another negative regulator of the monocyte/macrophage pro-inflammatory response, is poorly expressed in the liver of patients with SAH. Moreover, circulating monocyte levels of this protein can be increased by treatment with glucocorticoids illuminating a mechanism of action of corticosteroid therapy in SAH (Hamdi et al. 2007).

Finally, the anti-inflammatory cytokine IL-10 has been implicated in the pathogenesis of SAH. Impaired monocyte IL-10 synthesis has been reported in alcoholic patients with cirrhosis (Le Moine et al. 1995). Circulating monocyte-derived IL-10 levels appear depressed in SAH when compared to patients with cirrhosis and healthy controls and return to normal after 28 days of corticosteroid therapy (Taïeb et al. 2000).

In parallel with the intracellular regulators of inflammatory gene expression hitherto described, emerging evidence credits epigenetic modulation of inflammatory gene expression with a role in the pathogenesis of SAH. Intriguingly, when human macrophages are cultured with ethanol, they display global histone acetylation, increased acetylation in the promoter regions of TNFα and IL-6 genes (Kendrick et al. 2010) and an enhanced pro-inflammatory response to LPS (Zhang et al. 2001).

Moreover, there is indirect evidence that circulating pro-inflammatory monocytes may be recruited into the liver during SAH via monocyte chemotactic protein 1 (MCP-1)/chemokine ligand 2 (CCL2), which showed 3-fold elevated hepatic gene expression when liver biopsies from patients with SAH were compared to that from control subjects (Dominguez et al. 2009).

- **Neutrophils, Oxidative Stress and Hepatic Injury**

Neutrophils are often seen in biopsy specimens and are implicated in disease pathogenesis. In some studies, the degree of neutrophil infiltration into the inflamed liver of SAH patients has positively correlated with disease severity in some studies (Taïeb et al. 2000; Abraham P. Bautista 2002) and negatively in other (Altamirano et al. 2014). Neutrophil recruitment in SAH is mediated by chemokines such as IL-8, Gro-α and osteopontin (OPN) (Maltby et al. 1996; Abraham P. Bautista 2002). Elevations in serum IL-8 reflect disease severity and

confer a poor outcome in patients suffering with SAH (Dominguez et al. 2009; Hill et al. 1993; Huang et al. 1996). Intrahepatic expression of IL-8 correlates with both the degree of hepatic neutrophil infiltration and severity of portal hypertension (Dominguez et al. 2009). More recently OPN, a potent chemokine secreted by activated lymphocytes and macrophages, has been implicated in neutrophil, monocyte and NKT cell recruitment to the liver (Ramaiah & Rittling 2008; Apte et al. 2005; Diao et al. 2004): in animal models, the level of neutrophilic inflammation and necrosis correlated well with levels of both intrahepatic and circulating levels of OPN (Apte et al. 2005; Banerjee et al. 2006). Moreover, OPN expression is up regulated in human disease and this has led to it being proposed as a therapeutic target in SAH (Morales-Ibanez et al. 2013).

Neutrophil transmigration into the inflamed liver requires the expression of the adhesion molecule β2integrin/CD18 that binds to ICAM-1 on the surface of hepatocytes (Bautista 1997). After extravasation, neutrophils attack the hepatic parenchyma in two ways: via the generation of ROS and via neutrophil-derived serine proteases. In support of a predominantly ROS-mediated mechanism, there is evidence of oxidative stress in hepatocytes during neutrophil attack (Gujral et al. 2004); a reduction of neutrophil-mediated injury after inhibition of NADPH oxidase (Gujral et al. 2004); and an enhancement of neutrophil-mediated injury in glutathione-1-peroxidase deficient mice (Jaeschke et al. 1999). NADPH oxidase is also present in activated macrophages (Robinson et al. 2004) and gene knockout studies indicate that KCs may also contribute to the generation of ROS (Kono et al. 2000).

ROS cause mitochondrial damage and activation of endoplasmic reticulum-dependent apoptosis (Ji & Kaplowitz 2003). The nutritional deficiencies often seen in alcoholic patients, and specifically the deficiencies in antioxidants such as hepatic glutathione, are likely to

worsen oxidative stress and augment hepatic inflammation, possibly via a reduction in nuclear factor-erythroid 2-related factor (nrf2) activity. Nrf2 is a leucine zipper transcription factor that is able to regulate intracellular redox balance by increasing expression of enzymes able to restore glutathione and control pro-inflammatory responses (Thimmulappa et al. 2006).

There are few data on the role of neutrophil-derived proteases in SAH. Neutrophil elastase is implicated in tissue damage in chronic obstructive airways disease (Demkow & van Overveld 2010), pancreatitis and cystic fibrosis (Janoff 1985) and can also impair neutrophil phagocytosis (Tosi et al. 1990). High levels of neutrophil elastase have been detected at the site of sterile injury in cirrhotic patients (Tritto et al. 2011) but such *in vivo* models of tissue injury have not yet been used in studies of SAH.

Products of hepatocyte necrosis such as high mobility group box 1 protein (HMGB1), a damage-associated molecular pattern (DAMP), add to the inflammatory milieu by enhancing TLR4-complex mediated pro-inflammatory cytokine production from KC as well as NADPH oxidase-mediated ROS generation from neutrophils (Scaffidi et al. 2002; Fan et al. 2007).

- **Natural Killer cells**

The liver is home to an abundance of natural killer (NK) cells (Nemeth et al. 2009). In contrast to their extensively studied role in viral hepatitis (Cheent & Khakoo 2011) with some limited clinical evidence of an anti-fibrotic role in hepatitis C infection (Morishima et al. 2006), there are few data on the contribution of NK cells to SAH. NK cells are rare or absent in histology from advanced alcoholic liver disease (Chedid et al. 1993). In addition, there is a well-documented inhibition of NK cells by ethanol via several mechanisms (Cook et al. 1997). Firstly, ethanol down regulates several of NK cell killing tools such as Fas ligand (FasL), TNFα related apoptosis inducing ligand (TRAIL), perforin and interferon (IFN)-γ

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(Arjona et al. 2004). Secondly, ethanol stimulates hepatic stellate cells (HSC) to produce transforming growth factor (TGF)-β which in itself can inhibit NK cell cytotoxicity (Cook et al. 1997). Thirdly, chronic ethanol stimulates suppressor of cytokine signalling 1 (SOCS-1) expression which further inhibits IFN-γ production (Norkina et al. 2008).

Notably, NK cells are a source of the anti-microbial, anti-inflammatory and pro-regenerative cytokine, interleukin IL-22 (Wolk et al. 2010; Aujla & Kolls 2009). Inhibition of NK cells by ethanol, may therefore impact adversely on outcome in SAH via suppression of IL-22.

- **Natural Killer T cells**

Few studies have examined NKT cell function in SAH. In contrast to NK cell populations, chronic alcohol consumption in mouse models leads to increased NKT cell numbers and activity concomitant with an induction of liver injury (Jaruga et al. 2004). NKT cells can be demonstrated to have both regulatory and effector functions and ethanol-induced injury can be delayed by depleting the NKT cell population in mouse liver (Minagawa et al. 2004). NKT are the major lymphocyte population in the liver and were essential for secondary concanavalin A (Con A) mediated T-cell damage in an animal model of chronic ethanol feeding, which also induced a significant increase in the number of NKT cells in the liver compared to pair-fed controls (Minagawa et al. 2004).

(ii) Adaptive Immune Cells within the Liver

In contrast to the vast array of animal and human data available with which to unpick the involvement of the innate immune system in SAH, few studies have examined adaptive responses. One of the key questions is how the innate immune system in SAH might stimulate adaptive immune cells. Ethanol consumption inhibits antigen presenting cell (APC) capabilities of monocytes and dendritic cells (Fan et al. 2011) classically responsible for linking innate and adaptive immunity by activating and regulating T cells and B cells.

- **T Lymphocytes and direct cytotoxicity within the liver**

Lymphocytes are commonly seen on liver histology in SAH patients (Colombat et al. 2002). The ratio of CD8^{+} to CD4^{+} T cells within the hepatic parenchyma is raised compared to peripheral blood in experimental alcoholic liver injury (Cao et al. 1999); a finding consistent with human studies (Chedid et al. 1993) and suggesting a role for cytotoxic T cells in alcohol-induced hepatocyte damage. This hypothesis is supported by the presence of T cells at sites of lobular inflammation (Chedid et al. 1993). The target of these cytotoxic T cells, and whether they express perforin, granzyme or FasL is unknown, but T cell responses specific to alcohol dehydrogenase have been implicated in alcohol-related cirrhosis (Lin et al. 2013).

Th17 cells have recently been detected in the liver parenchyma in SAH and the extent of Th17 infiltration to the liver correlates with prognostic scores in SAH (Lemmers et al. 2009). Th17 cells are effector CD4^{+} T cells that secrete IL-17, IL-21, IL-22, IL-6 and TNFα and their role in autoimmune hepatitis is well-characterised (Ouyang et al. 2008). There are IL-17 secreting cells present within the liver during alcoholic hepatitis, including T cells and neutrophils. The presence of these IL-17 secreting cells correlated with markers of liver function such as Maddrey's discriminant function (MDF), suggesting a role for these cells in inflammatory liver damage during SAH. The IL-17 receptor is also present on hepatic stellate cells, where they appear to be involved in the recruitment of neutrophils to the area. SAH patients had threefold greater IL-17^{+} cell number within the liver and correspondingly low plasma IL-17 levels compared with stable cirrhotic patients. The same authors went on to show that human hepatic stellate cells express the IL-17 receptor and, when stimulated, will produce CXC chemokines such as IL-8 and GROα that cause neutrophil chemotaxis into the liver in a dose-dependent fashion. Th17 cells may also have cytotoxic properties and they are found in areas of hepatocellular damage in SAH (Lemmers et al. 2009).

- **B cells and antibody-dependent cell-mediated cytotoxicity**

There are no reports of B cell infiltrates to the hepatic parenchyma in SAH (Chedid et al.

1993) but B cells may still play a role in immune-mediated liver injury in SAH. Acetaldehyde

is a by-product of ethanol metabolism and has the propensity to form adducts with hepatic

proteins; thereby generating neo-antigens, figure 2. Specific anti-acetaldehyde antibodies

have been discovered in both rats chronically exposed to alcohol and alcoholic patients

(Koskinas et al. 1992).

Similarly, lipid peroxidation that results from ethanol oxidation can generate

malondialdehyde (MDA), 4-hydroxynonenal and oxidised arachidonic acid (Albano 2006).

These compounds can also form adducts with proteins and are implicated in the

pathogenesis of SAH. The adducts are found in the livers of patients with ALD and are

localised to areas of hepatocyte damage (Niemela 2001). Titres of IgG antibodies to these

neo-antigens are raised in advanced human ALD (Rolla et al. 2000; Mottaran et al. 2002)

and a proportion of patients with advanced ALD have peripheral blood CD4^{+} T cells

responsive to MDA adducts (Stewart et al. 2004). Importantly, sera from patients with ALD

can induce antibody dependent cell-mediated cytotoxicity in ethanol-treated rat hepatocytes

when co-cultured with normal blood mononuclear cells (Clot et al. 1997). In addition, heavy

drinkers with raised levels of lipid peroxidation-derived antibodies are much more likely to

have elevated plasma TNFα levels (Vidali et al. 2008) and the combination of raised TNFα

and lipid peroxidation-derived antibodies is associated with an 11-fold increased risk of

developing advanced ALD (Vidali et al. 2008).

Systemic Immune Dysfunction in SAH and predisposition to Infection

Clinically, alcoholic hepatitis is striking for the paradox of how an inflammatory process that benefits from immunosuppressive therapy can co-exist with immunodeficiency and susceptibility to infection.

As already detailed, there are enhancements of many facets of the innate and adaptive immune response within the hepatic parenchyma in SAH, which collectively are more than capable of explaining the systemic inflammatory response syndrome (SIRS) that many patients present with. What is less clear however is whether inflammatory processes within the liver may adversely impact on the functionality of systemic immune cells without the liver. In this section, the dysfunction of circulating immune cells in SAH will be reviewed.

(h) **Circulating Monocyte Dysfunction**

A wealth of literature covering a broad range of systemic inflammatory conditions has described the phenomenon of endotoxin tolerance (ET). This is the observation, originally reported in rabbits (Beeson 1947) and more recently confirmed in human septic shock (Draisma et al. 2009; Heagy et al. 2003), stable liver cirrhosis (Lin et al. 2007), acute-on-chronic liver failure (ACLF) (Wasmuth et al. 2005) and acute liver failure (Antoniades et al. 2006) wherein peripheral blood monocytes demonstrate attenuated production of pro-inflammatory cytokines in response to repeated exposure to endotoxin. The phenomenon is thought to play an important role in the immune paralysis observed in critically ill patients and in vivo human studies have correlated ET with longer stays in intensive care units, increased ventilator requirements and a higher incidence of infection and mortality (Heagy et al. 2003).

Recent data have focussed on molecular mechanisms for the development of ET and, strikingly, these appear to bear similarities to mechanisms implicated in ethanol induced monocyte hypersensitivity detailed above (Kobayashi et al. 2002; van 't Veer et al. 2007; Nahid et al. 2011; Taganov et al. 2006; Tili et al. 2007). Bernsmeier *et al.* showed that Mer receptor tyrosine kinase (MERTK) signalling could be a mediator for the innate immune suppression seen in ACLF. The authors observed high numbers of MERTK expressing regulatory cells in patients with ACLF. These cells showed attenuated levels of pro-inflammatory cytokine production in response to lipopolysaccharide stimulation. When cells were treated with an inhibitor of MERTK signalling pro-inflammatory responses to endotoxin were restored (Bernsmeier et al. 2015).

Furthermore, recent data confirms that, during human paracetamol-induced acute liver failure (ALF), hepatic macrophages generate a predominantly anti-inflammatory hepatic microenvironment (Antoniades et al. 2012). It is tempting to speculate that anti-inflammatory, pro-regenerative mediators, produced within the liver to effect tissue healing, then spill into the systemic circulation and induce circulating immune cells to adopt anti-inflammatory characteristics that could leave the host susceptible to opportunistic infections. In the case of alcoholic liver injury, the gut leakage associated with SAH that is proven to lead to high portal and systemic endotoxaemia, provides an ideal platform for endotoxin tolerance to develop. However, immune mediators demonstrated to derive from previously healthy liver in the case of ALF, may have different expression patterns in the case of SAH, which is a disease affecting patients with chronic liver disease.

Despite these attractive hypotheses, there are currently no data suggesting that these phenomena are relevant in SAH. In fact, most studies evaluating peripheral blood monocyte function in patients with SAH show quite the opposite: that of an augmented pro-

inflammatory response to endotoxin stimulation (McClain et al. 1998; Hill et al. 2000). In line with these studies monocyte production of IL-10, an anti-inflammatory cytokine known to inhibit free radical production, is barely detectable in SAH but rises after 21 days of corticosteroid therapy (Taïeb et al. 2000).

Why studies involving peripheral monocytes from patients with SAH have so far failed to demonstrate ET is intriguing, but may simply be explained by the fact that most available data evaluates monocyte function soon after alcohol cessation, at the start of inpatient care: it is certainly possible that the ethanol-mediated sensitising effects on monocytes continue into a patient's hospital stay and time of research sampling. Alternatively, there may be other as yet unidentified agents able to abrogate endotoxin tolerance in SAH. Data evaluating monocyte function at different time-points in the natural history of the disease are needed to gain insight into the impact of cessation of alcohol on the restoration of liver function, susceptibility to infection and consequently outcome in SAH.

(i) **Circulating Neutrophil Dysfunction**

Ex vivo studies conducted over the last twenty years have suggested that circulating neutrophils in SAH have a lower threshold for oxidative burst activity (Mookerjee et al. 2007; Taïeb et al. 2000) suggesting a primed or pre-activated state when compared to cirrhotic or healthy controls. In the most recent study, this pre-activation of neutrophils correlated with subsequent development of culture-positive infection and predicted organ failure and 90-day mortality. Notably however, the oxidative burst generated by neutrophils in response to *E. coli* was the same in SAH patients as for cirrhotic and healthy control groups (Mookerjee et al. 2007).

In the same study, the phagocytic capacity of neutrophils was found to be depressed and this was similarly correlated with the development of infection and was a better predictor of organ failure and death. Incubation of normal neutrophils with patient's plasma or endotoxin led to pre-activation of neutrophil oxidative burst and decreased phagocytosis, pointing to plasma endotoxin as the culprit for neutrophil dysfunction (Mookerjee et al. 2007; Bohmer et al. 1992). Systemic neutrophil dysfunction may be the result of conditioning received in the liver in SAH, where endotoxin load is believed to be particularly high. The mechanism of action may involve modulation of neutrophil NAPDH oxidase assembly by LPS (DeLeo et al. 1998). Interestingly, there is also evidence that ammonia may induce neutrophil swelling, leading to depressed phagocytic function, and that this dysfunction is mediated by p38^{MAPK} (Shawcross et al. 2008).

TLR complexes have also been linked to neutrophil dysfunction in SAH. Neutrophil pre-activation and impairment of phagocytosis were abrogated by antibodies to CD14 (Mookerjee et al. 2007), a component of the TLR-4 complex, suggesting that the effects of endotoxin on neutrophil function are mediated by TLR-4 signalling. Subsequent work associated neutrophil pre-activation with increased neutrophil cell surface expression of TLR-2 and -4 and increased intracellular expression of TLR-9. Inhibiting these TLRs abrogated neutrophil pre-activation and increased chemokine receptor expression but did not prevent phagocytic dysfunction (Stadlbauer et al. 2008).

On the other hand albumin, a postulated endotoxin scavenger, was able to abrogate neutrophil pre-activation, normalise chemokine receptor expression, improved phagocytic capacity, and prevented the increases in neutrophil cell surface expression of TLR-2, TLR-4 and intracellular expression of TLR-9. At the time of sampling in the study, there was also indirect evidence that circulating neutrophils had reduced capacity to migrate into tissues in

SAH. Neutrophil pre-activation was associated with down-regulation of chemokine receptors, CXCR-1 and CXCR-2, which respond to IL-8, a chemo-attractant that has been heavily implicated in the pathogenesis of SAH (Dominguez et al. 2009) Similarly, impaired up regulation of CD11b expression, a β2-integrin involved in neutrophil chemotaxis, has been demonstrated in circulating neutrophils from SAH patients (Taïeb et al. 2000; Naveau et al. 2004). While control of neutrophil recruitment into an already inflamed liver may reduce liver inflammation, systemically it may also reduce recruitment to infected tissue (Cummings et al. 1999; Khandaker et al. 1998).

TNFα may also play a role in the ROS response of neutrophils to stimulation. Neutrophils from patients treated with infliximab and prednisolone had reduced ROS response to stimulation when compared to the stimulated response of neutrophils from patients that had been treated by prednisolone alone (Naveau et al. 2004). In addition, there was altered neutrophil adhesion molecule expression, depressed neutrophil IL-8 production, and it is likely that such neutrophil dysfunction contributed to the high rate of infection in patients treated with infliximab and corticosteroid combination therapy in this trial.

(j)   **Dendritic Cells and Impaired Antigen Presentation in SAH**

There is a paucity of data on dendritic cell (DC) function in SAH and antigen presenting faculties of the immune system in SAH generally. We are therefore reliant on animal models of chronic alcohol feeding, rather than SAH *per se*, which broadly suggest an impaired ability of DCs to stimulate Th1 T cell responses, via a reversible suppression of IL-12 production and an elevation in IL-10 (Mandrekar et al. 2004).

(k) **Circulating T cells have a Th1 and Th17 phenotype**

SAH patients are lymphopaenic (Støy et al. 2015). More specifically, the frequency of cytotoxic T and NK cells are reduced in SAH but the frequency of NKT cells was not different amongst patient groups. NK cell frequency correlated inversely with MELD and these cells had a reduced capacity to degranulate (Støy et al. 2015). These data conflict with animal models, wherein chronic ethanol feeding is associated with an expansion of activated circulating T-cells. In these models of disease, there is increased expression of HLA-DR and CD11c and *in vitro* stimulation through the T-cell receptor, notably without a second signal, produces a rapid burst of IFN-γ and TFNα resembling a Th1 response in both mice and humans (Laso et al. 1997; Cook et al. 2004). There is also increased expression of CD80 and CD86 on antigen presenting cells (APC) which, when bound to CD28 on T cells, provide second signals for T-cell activation (Cook et al. 2004). Similarly, when rats are chronically fed ethanol, intrahepatic T cells harvested and transferred to the peripheral circulation of an ethanol-naive animal, a pro-inflammatory T cell phenotype is induced in the ethanol-naive animal. Further injection of LPS leads to augmented IL-6 and TNFα production and liver injury (Cao et al. 1999).

There are few data on other populations of circulating lymphocytes in SAH. Expanded numbers of NK and NKT cells with enhanced cytotoxicity have been demonstrated, but B cell populations appear to be diminished (Laso et al. 1997).

(l) **Focus on Monocytes: Ontogeny**

The monocyte is a type of circulating white blood cell that is involved in innate immune responses such as phagocytosis, antigen presentation and cytokine production. Neutrophils and NK cells are also involved in these innate immune responses. Monocytes can differentiate further into a range of tissue macrophages and dendritic cells. Bloodstream

monocytes derive from precursors in the bone marrow and are subdivided into subsets that differ in size, trafficking and innate immune receptor expression and in their ability to differentiate following stimulation with cytokines or microbial molecules, figure 8. Human monocytes can be defined as $CD14^+CD16^-$ classical; $CD14^+CD16^+$ intermediate; $CD14^{lo}CD16^+$ non-classical monocytes, figure 9. When the gene transcriptome is analysed, $CD14^{lo}CD16^+$ monocytes are found to be counter-parts of $Ly6C^{lo}$ non-classical monocytes in the mouse, Conversely, $CD14^+CD16^-$ cells are the counter-parts of the $Ly6C^{hi}$ classical mouse monocytes (Cros et al. 2010) and express high levels of chemokine receptor CCR2 but low levels of CX_3CR_1. Recent work suggests that these non-classical monocytes exclusively express the surface marker 6-sulfo LAcNac (slan) (Thomas P. Hofer et al. 2015). These monocytes, often referred to as inflammatory monocytes, represent approximately 2-5% of circulating white blood cells in the mouse. Although the monocyte subsets identified in human and mice are not precisely overlapping, their differentiation and contribution to immune defence appear to be similar. There is a body of literature suggesting that monocytes mature through progression from subsets $Ly6C^{hi}$ to $Ly6C^{lo}$ in the mouse and from classical to intermediate to non-classical monocytes in the human, before differentiation into tissue macrophages (Strauss-Ayali et al. 2007).

Subset	Markers	Chemokine receptors	Functions
Mouse			
Ly6C^{hi}	CD11b$^+$CD115$^+$Ly6C^{hi}	CCR2^{hi}CX$_3$CR$_1$^{lo}	Pro-inflammatory and antimicrobial roles
Ly6C^{lo}	CD11b$^+$CD115$^+$Ly6C^{lo}	CX$_3$CR$_1$^{hi}CCR2^{lo}	Patrolling; early responses; tissue repair
Human			
Classical	CD14$^+$CD16$^-$	CCR2^{hi}CX$_3$CR$_1$^{lo}	Resemble Ly6C^{hi} murine monocytes based on gene-expression arrays
Intermediate	CD14$^+$CD16$^+$	CX$_3$CR$_1$^{hi}CCR2^{lo}	Pro-inflammatory roles
Non-classical	CD14$^+$CD16$^+$	CX$_3$CR$_1$^{hi}CCR2^{lo}	Patrolling; antiviral roles; resemble Ly6C^{lo} murine monocytes on transcriptome analysis

Table 1: monocyte heterogeneity in mice and humans
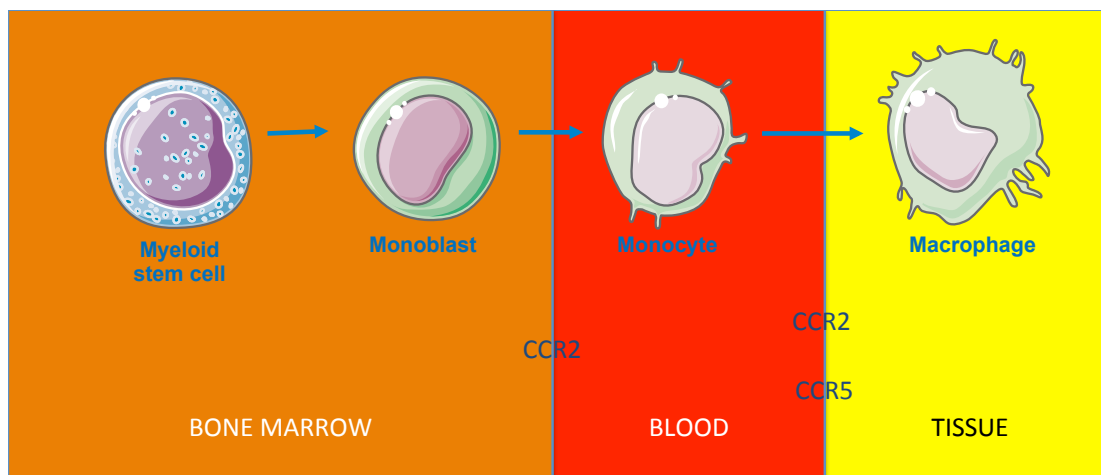
Figure 8: myeloid stem cells in the bone marrow develop into monoblasts. Using CCR-2, these monoblasts differentiate and emigrate into the blood where they exist in one of three monocyte subsets. Intermediate and non-classical monocytes are believed to be more mature, and more closely resemble tissue macrophages. Monocyte recruitment into tissues occurs using chemokine receptors CCR-2 and CCR-5. In liver tissues, macrophages are known as Kupffer cells. *Figure created using Servier™ medical artwork*
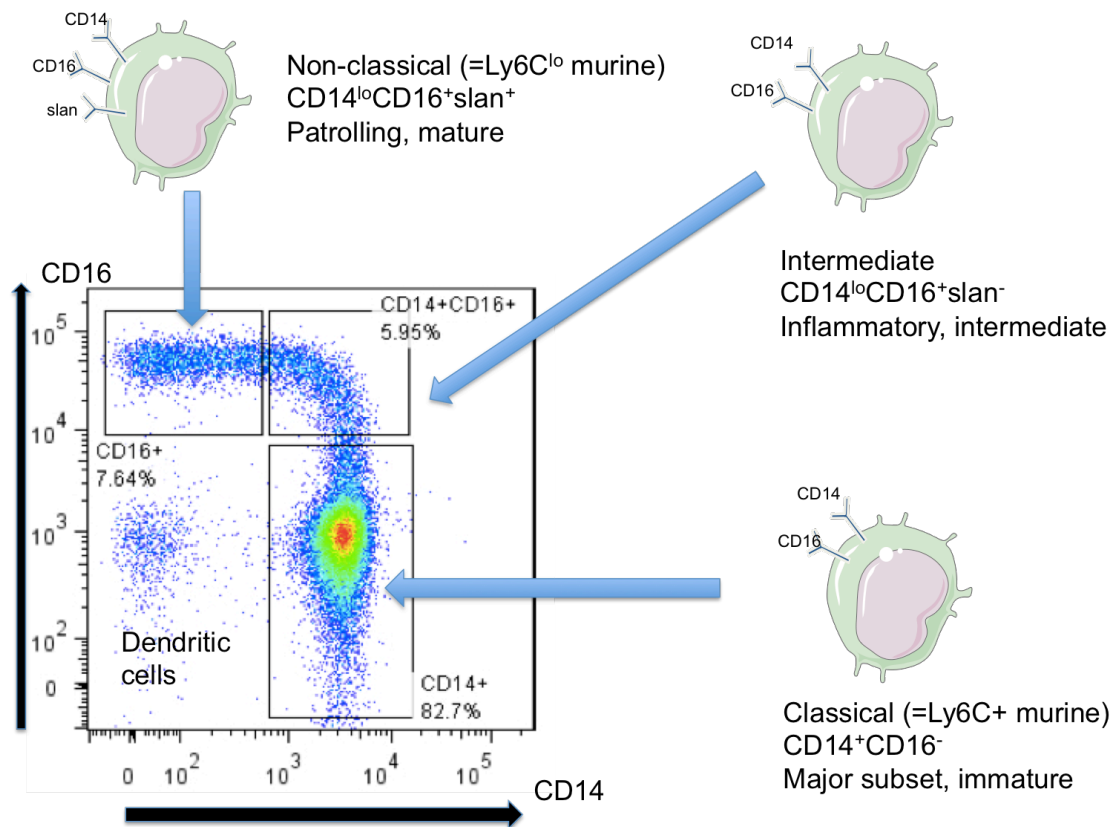
Figure 9: human circulating monocytes are divided into 3 subsets based on the expression pattern of CD14, CD16 and more recently, slan. This allows differentiation of classical, intermediate and non-classical monocytes by flow cytometry. *Figure created using Servier™ medical artwork*

Chemokine Receptor Expression

CCR2 is highly expressed on classical monocytes (Strauss-Ayali et al. 2007). Most nucleated cells express CCL2 in response to activation by pro-inflammatory cytokines or stimulation of innate immune receptors by a range of microbial molecules. Monocytes exit the bone marrow in a CCR2 dependent manner and are recruited to inflamed tissues (Strauss-Ayali et al. 2007). Many infections induce the expression of CCL2 and result in high levels of CCL2 in serum and within inflamed tissues. Monocytes also express CCR1 and CCR5 and these receptors bind to a variety of chemokines, including the shared ligands CCL3 and CCL5. CCR1 mediates the arrest of monocytes in the presence of shear flow whilst CCR5 contributes to monocyte spreading. Both CCR1 and CCR5 support transendothelial chemotaxis towards CCL5 (Shi & Pamer 2011).

Fungal Infections in SAH

A recent paper by Gustot *et al.* highlighted a high frequency of fungal infections in SAH patients (Gustot et al. 2014). In particular, the incidence of invasive aspergillosis in the cohort studied was 15/96 (16%). These patients were diagnosed after a median of 13 days from diagnosis of SAH. The site of infection most commonly isolated from lung. In those developing invasive aspergillosis, mortality at 90 days was 53% compared to 0% in those without invasive aspergillosis. The rate of candidiasis was 2%. However, this high rate of fungal infections was also associated with higher MELD and Intensive Care Unit stays, and so may be a marker of patients' sickness, rather than a factor that drives deterioration and death in SAH *per se*. The diagnosis of fungal infection crucially depends on the diagnostic criteria used to determine whether something is of causal, commensal or contaminant significance. Indeed, a previous study of 131 patients with SAH that reported a far lower incidence of 4.5% used different diagnostic criteria for aspergillosis (Magavero G. et al. 2012).

Rationale for focus of thesis

As described above, there are several possible avenues for investigation within immunology in alcoholic hepatitis in order to explain the apparent paradox of increased susceptibility to infection in the context of a pro-inflammatory hepatic state. The focus of this thesis however will be with the contribution of circulating monocyte dysfunction to susceptibility to infection in SAH.

SAH patients typically display features of both hepatic and systemic inflammation on admission to hospital, with correspondingly elevated serum levels of acute phase reactants such as C-reactive protein and inflammatory cytokines such as TNFα and IL1β, and

deranged serum liver transaminase enzymes. The high levels of inflammatory cytokines reported in SAH and other acute inflammatory conditions is likely to be produced by a variety of different immune cell types including monocytes, macrophages, neutrophils, NK cells, NKT cells and T cells (Støy et al. 2015; Albillos et al. 2014). However, despite the high levels of inflammatory cytokines reported in the serum of SAH patients, they are nonetheless highly susceptible to infection, and prompts further study as to whether there are specific immune defects within particular cell types. The largest contribution of inflammatory cytokine production is likely to come from cells of the innate immune system, such as monocytes and neutrophils.

Why monocytes and not neutrophils?

Neutrophils are key agents in the phagocytosis of pathogenic microorganisms. As well as phagocytosis and oxidative burst, neutrophils are also able to generate neutrophil extracellular traps (NETs), networks of extracellular fibres composed of neutrophilic DNA that is able to bind pathogens (Brinkmann et al. 2004). In addition, they are also important producers of inflammatory cytokines and reactive oxygen species that can cause hepatic immunopathology. Indeed as discussed in *Introduction*, patients with SAH often have histological features of neutrophilic infiltration. While mononuclear cells may also be present, neutrophils predominate. Neutrophilic infiltration corresponds to high levels of the inflammatory cytokine IL-8 seen in alcoholic hepatitis biopsy specimens and also on liver gene expression arrays (Abraham P. Bautista 2002). However, while there are a number of studies that address the phagocytic capabilities of neutrophils in SAH (Rajkovic & Williams 1986; Mookerjee et al. 2007; Tritto et al. 2011; Stadlbauer et al. 2008; Shawcross et al. 2008; Markwick et al. 2015) there have been comparatively few studies of monocyte phagocytosis. In addition, neutrophils are short-lived in culture. This presents a challenge to the assessment of functions involving prolonged co-culture, such as the generation of the

anti-inflammatory cytokine IL-10 in response to LPS. Indeed, stimulation periods of longer than 12 hours may be confounded by progressive cell death of neutrophils. Previous work in similar conditions of liver failure have shown defects in monocyte function (Antoniades et al. 2006; Wasmuth et al. 2005; Bernsmeier et al. 2015) but it is not clear whether these allied diseases are comparable to the unique situation of SAH, given the prevalence of significant chronic disease and the existence of alcohol as an important cofactor in SAH.

Why circulating monocytes and not hepatic or tissue macrophages?

Circulating monocytes, rather than tissue macrophages, were selected for study. Circulating monocyte function may be most relevant in situations of septicaemia, where there is haematogenous spread of bacteria from infected sites. In addition, circulating monocyte phenotype and function may be indicative of tissue macrophage phenotype and function. Access to tissue macrophages is challenging due to small amounts of liver tissue being available from trans-jugular liver biopsies that are performed in SAH patients, which are taken transjugularly in order to minimise bleeding risk. This is because patients with liver failure often have abnormalities of blood clotting, making percutaneous liver biopsy risky. Trans-jugular liver biopsy specimens are small, resulting in few tissue macrophages being available for co-culture work after collagenase degradation. As a result, the primary focus of this thesis is not to ascertain the mechanisms of hepatic injury in SAH, which would necessitate extraction of hepatic tissue macrophages. Rather, the primary focus will be to identify features of circulating monocyte phenotype and function that might explain the increased susceptibility to clinical infection observed in SAH patients. In addition, I expect to gain interesting clues as to the function of hepatic macrophages in SAH by studies of the phenotype and function of circulating monocytes.

Rationale for control groups

The control groups chosen for this study are healthy controls (HC), chronic liver disease (CLD) and severe alcoholic hepatitis (SAH). SAH patients are the group of interest, CLD patients are the pathological control group and HC subjects constitute "normal" controls. HCs will be age and sex-matched and are presumed to have normal liver function, although this will not be formally tested in this study. CLD patients share the features of chronic liver damage with SAH patients. These patients will all have alcohol as the aetiology of their liver disease, although they will be subdivided into patients who are drinking and patients who are abstinent, defined as not having consumed alcohol within 6 months of sampling. Patients who have been abstinent for at least 6 months are less likely to have features of steatohepatitis within the liver, although some reports indicate that steatohepatitis can be present in explants of patients undergoing liver transplantation 6 months after abstinence (Tomé et al. 2002). Within the CLD group, patients who are drinking may have steatohepatitis, but do not have the crucial features of liver failure and jaundice that are associated with SAH. Thus using CLD as a pathological control group allows us to separately estimate the effect of both alcohol drinking (comparing drinking CLD versus abstinent CLD patients) and steatohepatitis (comparing patients with SAH to abstinent CLD patients) on circulating monocyte function.

Other possible control groups include MELD-matched acute on chronic liver failure (ACLF) or ALF. Both ACLF and ALF might share similarities with SAH in terms of the inflammatory "spill-over" hypothesis. This is the theory that inflammation within large organs such as the liver is able to condition immune cells passing through it, as well as generate anti-inflammatory mediators that attempt to heal the injured organ. In the case of hepatic inflammation, these mediators may spill over into the external circulation where they are able

to induce anti-inflammatory phenotype and function to circulating immune cells, which may increase susceptibility to infection. This phenomenon is postulated to contribute to endotoxin tolerance seen in ALF, pancreatitis and severe sepsis, where high levels of anti-inflammatory mediators such as IL10, TGFβ and Secretary Leucocyte Protease Inhibitor (SLPI) in the serum are believed to act via intracellular signalling to reduce TLR-4 mediated responsiveness to lipopolysaccharide and reduce pro-inflammatory cytokine production via decreased activation of NF-κB (Draisma et al. 2009; Heagy et al. 2003; Antoniades et al. 2014). Monocyte function of ACLF patients without alcoholic hepatitis has recently been reported (Bernsmeier et al. 2015) Similarly, circulating monocyte function of ALF has also been studied (Antoniades et al. 2006). In these studies, impaired production of pro-inflammatory cytokines from monocytes was reported in ACLF and ALF patients, consistent with endotoxin tolerance.

When considering the suitability of ACLF and ALF cohorts as control groups, it is instructive to consider the two important cofactors of chronic liver dysfunction and alcohol drinking, both of which are known to impact on susceptibility to infection. Firstly, according to a recent study of SAH patients in Denmark around 40% of SAH patients had cirrhosis of the liver (Sandahl et al. 2011). Although this would imply 60% of patients with SAH do not have cirrhosis, one might reasonably assume that many of this remaining 60% of patients have advanced fibrosis of the liver at the very least. ALF patients would therefore not be an appropriate control group for SAH patients, since ALF patients have normal liver function prior to liver injury by definition.

Secondly, in the UK much ACLF is related to ALD, even if SAH is not a clinically obvious precipitant of organ failure in a large number. Many of these ACLF patients will have drunk alcohol to a degree similar to that consumed by SAH patients immediately prior to

admission. Thus it is likely that there will be alcoholic hepatitis in a large number of patients presenting with ACLF from other precipitants, like infection. Indeed in one study, 96% of patients presenting with alcohol related ACLF with recent onset jaundice (bilirubin >85umol/L) had some forms of alcoholic steatohepatitis on liver biopsy (Katoonizadeh et al. 2010). Thus, care must be taken when including patients with ACLF as a control group to ensure that the patients do not also have SAH. One option is to exclude ACLF patients who have ALD. However, in most UK centres the proportion of ACLF that does not involve ALD is small, which would result in small sample sizes for this control arm.

Aims

Monocyte phenotype and cytokine production

The phenomenon of endotoxin tolerance has been described in both acute and acute on chronic liver failure syndromes allied to SAH such as ALF and ACLF. This phenomenon is associated with impaired functionality of circulating monocytes, specifically in terms of inflammatory cytokine production. However, despite evidence of increased gut mucosal permeability in alcoholic hepatitis with high serum levels of lipopolysaccharide it is unclear whether endotoxin tolerance contributes to immune paresis in SAH. Endotoxin tolerance refers to a reprogramming of monocyte function, likely via epigenetic changes that lead to the down regulation of certain pro-inflammatory genes and the concomitant up regulation of particular anti-microbial genes(Medzhitov & Janeway Jr. 2000; Netea et al. 2016). A deactivated phenotype and function could be explained by endotoxin tolerance, a form of monocyte reprogramming, or monocyte exhaustion resulting from an immune system that is chronically activated. If a deactivated phenotype is discovered, its mechanism, and whether or not the response can be restored using immunotherapy will be explored. Reversal of defects by immunotherapy might help to determine whether the deactivated phenotype is due to monocyte exhaustion or due to active immunological reprogramming.

(m) **Hypothesis I**

Circulating monocytes exhibit a deactivated phenotype and function that may contribute to susceptibility to infection

(i) Objectives I

To examine phenotype and function of circulating CD14$^+$CD16$^-$, CD14$^+$CD16$^+$ and CD14^{lo}CD16$^+$ monocytes from patients with SAH and control groups:

i. before treatment

ii. if defects are identified, to stratify defects by the development of infection in order to find clinically relevant biomarkers

iii. and to evaluate the effect of prednisolone therapy on monocyte phenotype and function

Monocyte phagocytosis and oxidative burst

Previous studies have examined neutrophil phagocytosis in SAH and found defects that are associated with mortality (Rajkovic & Williams 1986; Mookerjee et al. 2007). One study has suggested that neutrophil phagocytic defects might be secondary to defective production of interferon-γ from T cells (Markwick et al. 2015)

(n) **Hypothesis II**

Defects in circulating monocytes associate with the development of infection in patients with SAH and can be treated with interferon-γ.

(i) Objectives II

To characterise, in circulating monocytes from patients with SAH:

i. bacterial uptake (phagocytosis)

ii. oxidative burst (bacterial killing)

iii. correlate defects to the subsequent development of infection

iv. and test the reversibility of defects with interferon-γ (IFN-γ)

METHODS

Patients and Sampling

(o) Regulatory approval

All patient recruitment and sample handling was conducted according to Good Clinical Practice and Human Tissue Act regulations. The National Research Ethics Committee (Reference Number 09/MRE09/59) and Imperial College Hospital Ethics Committee (12/LO/0167) approved the study. The patient's next of kin gave assent if they were unable to give informed consent themselves.

(p) Patient recruitment

132 subjects were recruited to the study from St Mary's, Royal London, Hammersmith, Charing Cross, Chelsea and Westminster, Royal Free and King's College Hospitals in London, United Kingdom, between January 2011 and September 2014.

(q) Patient Group Categorisation

Patients were categorised as follows: SAH (n=73); compensated alcohol-related cirrhotic patients (n=25), further subdivided into those who had been abstinent for at least 6 months (CLD, n=11), compensated actively drinking cirrhotic patients (dCLD, n=14) and healthy controls (HC, n=34).

Precise sample sizes for individual assays are given in figure legends. Phenotyping assays were performed in approximately 15 patients with SAH, 10 HCs and 10 CLD patients. Cytokine production assays were performed in 5 patients with SAH and 5 HCs. Phagocytosis uptake assays were performed in 20 patients with SAH and 30 HCs. Monocyte

oxidative burst (MOB) assays were performed in 42 patients with SAH, 26 CLD including 14 actively drinking and 11 abstinent CLD patients, and 34 HCs. MOB patients were further subdivided into patients with MOB defect (SAH+MOB, n=21) and patients without MOB defect (SAH-MOB, n=21). Luminometric assays of superoxide production were performed in 5 SAH patients with MOB defect and 5 HCs. Western blotting and PCR assays are specified with the figures, but in general had 5 patients with MOB defect compared to 5 patients without MOB defect and 5 HCs.

A power calculation for these exploratory laboratory assays was not performed *a priori.* HC subjects confirmed that they drank <10 units per week prospectively. Neither smoking status nor physical exercise was recorded prospectively.

(i) SAH patient criteria

All SAH patients had an alcohol consumption of >80g/day (men) or 60g/day (women) immediately prior to hospital admission, had bilirubin >80umol/L and Maddrey's Discriminant Function (MDF) ≥32. SAH patients satisfied clinical diagnostic criteria described in the *Steroids or Pentoxifylline for Alcoholic Hepatitis* (STOPAH) clinical trial protocol (Forrest *et al.* 2013). These criteria are described in Table 2. Of note, the attending physician controlled any infections before study entry with at least 48 hours intravenous antibiotics.

INCLUSION CRITERIA	• >age 18 years
	• serum bilirubin >80mmol/L
	• alcohol consumption >80g/day (men) >60g/day (women)
	• <4 weeks since admission to hospital
	• Maddrey's discriminant function ≥32
EXCLUSION CRITERIA	• >2 months abstinence
	• >3 months prior jaundice
	• no evidence hepatitis B or C infection, hepatocellular carcinoma nor biliary obstruction
	• pregnant or lactating women
	• serum creatinine >500μmol/L
	• current malignancy
	• AST>500 or ALT >300iu/L
	• Untreated sepsis

Table 2: Eligibility criteria for study entry

(ii) Chronic liver disease (CLD) patients

Chronic liver disease patients were stable ambulatory patients recruited from outpatient clinic. Baseline liver characteristics of these patients are listed in *Results*. All had a history of alcohol-related liver cirrhosis. Cirrhosis was diagnosed by previous liver biopsy or clinical presentation with typical ultrasound or computed tomography imaging.

CLD patients were subdivided into those who had been actively drinking for the 6 months prior to sampling (dCLD) and those who had been abstinent for 6 months prior to sampling (CLD).

(iii) *Healthy control subjects*

HC were age-matched members of clinical or university staff at St Mary's Hospital with no evidence of liver dysfunction.

(r) **Patient therapy for SAH**

58/73 (79%) of patients recruited to this study were also recruited to the STOPAH study. This was a multicentre, double blind, factorial (2 x 2) interventional trial, table 3. At the time of conception of the study, two therapies were thought to have therapeutic benefit: prednisolone and pentoxifylline. The role of both drugs, however, is controversial. Prednisolone is a glucocorticoid drug with anti-inflammatory properties administered as 40mg once a day for 28 days in SAH. Its benefit in SAH is unclear because multiple small and heterogeneous trials have yielded inconsistent results. One recent meta-analysis suggested a benefit for prednisolone in SAH (Mathurin et al. 2013). Pentoxifylline is a competitive non-selective phosphodiesterase inhibitor that can raise cyclic AMP and inhibits TNFα production (Marques et al. 1999), and can be administered at 400mg three times a day for 28 days in SAH. Only one previous placebo controlled trial has been published that shows benefit (Akriviadis et al. 2000). 64 sites in the UK participated in STOPAH and 1103 patients underwent randomisation. After withdrawals, data from 1092 patients was available for analysis.

Patients were randomised to one of four groups

Group A	Placebo / Placebo
Group B	Placebo / Prednisolone
Group C	Pentoxifylline / Placebo
Group D	Pentoxifylline / Prednisolone

Table 3: treatment group allocations in STOPAH

The primary endpoint was mortality at 28 days. Secondary endpoints included mortality at 90 days and 1 year.

15/73 (21%) patients were not recruited to the STOPAH study, but were nonetheless recruited for these doctoral studies. These patients were treated according to clinician preference: prednisolone, pentoxifylline, both or neither.

(s) **Paired Longitudinal Samples**

Ex vivo MOB was measured before (Day 0 MOB) and seven days after (Day 7 MOB) the start of therapy. In order to determine the effect of prednisolone on the MOB, after completion of the trial, treatment allocation data was released and used to compare results from patients given oral prednisolone to patients not given oral prednisolone.

(t) **Clinical, haematological and biochemical parameters**

Full blood count, international normalised ratio (INR) and prothrombin time, liver and renal function tests, C-reactive protein (CRP) and clinical variables including the prescription of intravenous antibiotics, were prospectively entered into an Excel™ database in coded form. Data was backed up onto an external hard drive. All devices were password protected and held within locked premises when unattended.

MDF, model for end-stage liver disease (MELD), Lille score and early change in bilirubin level (ECBL) were calculated according to calculations described in the *Glossary* section.

(u) **Definition of Infection**

The diagnosis of infection is clinically challenging. In order to categorise patients as accurately as possible for the purposes of this research, three approaches were evaluated as follows.

(i) Prescription of new intravenous antibiotics or a change in the type of antibiotic prescribed

This definition of infection will align with that proposed in the Albumin To PrevenT Infection in chronic liveR failure E (ATTIRE) study (ISRCTN 14174793). Specifically, patients who are prescribed new antibiotics or who are prescribed a change in antibiotics will be deemed to have developed new infection.

(ii) Development of culture positive infection

Patients were deemed to have developed infection if any of the following criteria were met in line with recently published studies: *i)* positive blood cultures, *ii)* ascitic neutrophil count >250cm^{-3} *iii)* consolidation on chest radiograph in conjunction with respiratory signs or laboratory markers of infection *iv)* diarrhoea with positive stool cultures for pathogenic bacteria *v)* cellulitis with fever or laboratory signs of infection *vi)* positive urine culture *vii)* intra-abdominal infections: diverticulitis, appendicitis, cholangitis with radiological evidence *viii)* secondary bacterial peritonitis: ascitic neutrophils >250cmm^{-3} in the presence of intra-abdominal source of peritonitis and multiple organisms cultured from ascitic fluid (Bajaj et al. 2012).

(iii) Measurement of circulating bacterial DNA levels

DNA was extracted from whole blood using the QIAmp DNA Blood spin columns according to the manufacturer's instructions (Qiagen, Germany). In brief, Proteinase K was mixed with 400μl of thawed whole blood and the mixture was incubated at 56°C for 10 minutes to lyse

the cells. DNA was extracted by centrifugation through spin columns. DNA purity was tested by A_{260}/A_{280} ratio. This is a ratio of the absorbance of maxima of nucleic acid (260nm) compared to the absorbance maxima of protein (280nm) (Wilfinger et al. 1997). A ratio of 1.8 is generally accepted for pure DNA; a ratio of 2.0 is generally accepted for pure RNA. It may be used as a guide to the level of protein contamination in extracted DNA samples. A 400μL sample of whole blood (approximately 5×10^6 leucocytes/mL) typically yields 12μg DNA in 400μL water (30ng/μL) with an A_{260}/A_{280} ratio of 1.7-1.9. Samples were then stored at -20°C.

To perform, PCR, 4μL of DNA extract was then mixed 3.5μL water, 1μL forward primer, 1μL reverse primer both at 10μM concentration, 0.5μL of Taqman probe, and 10μL Gene Expression Master Mix (Qiagen, Germany). The sequence for the forward primer was AACTGGAGGAAGGTGGGGAT (5'-3') and the sequence for the reverse primer was AGGAGGTGATCCAACCGCA (3'-5'). The probe was TACAAGGCCCGGGAACGTATTCACCG TAMRA probe, 6-FAM dye, at a concentration of 100pmol/μL (Life Technologies, USA).

Results were compared to standard curves of *E. coli* DNA at known concentrations (0.000008-0.008ng/μL). A total of 18 plates were run on a StepOne PCR machine (Applied Biosciences, UK) and standard curves are given in figure 10.
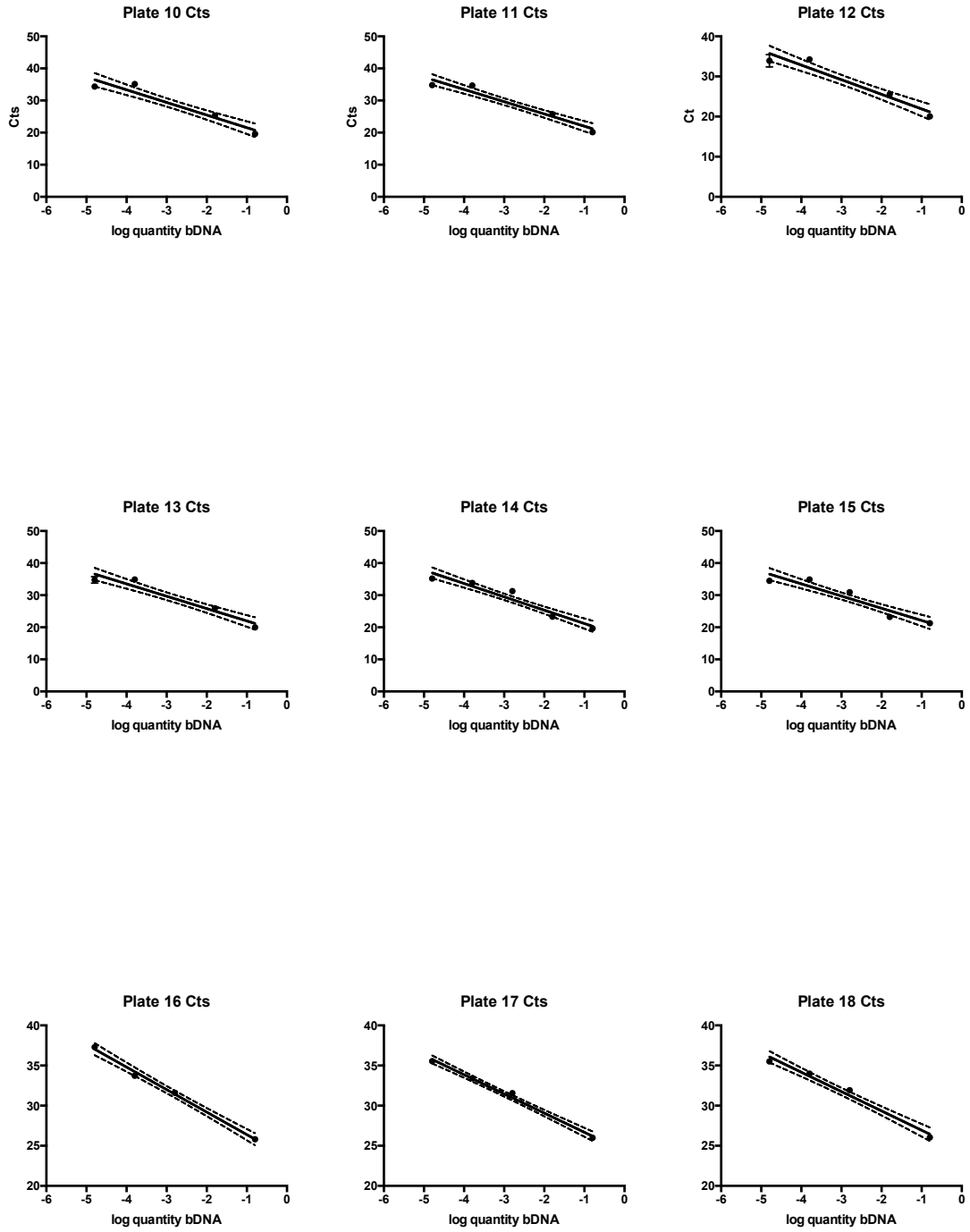
Figure 10: standard curves for 16S ribosomal bacterial DNA across 18 96-well plates

Flow Cytometric Assays

(v) Phenotyping of monocytes

Monocyte phenotyping was done on whole blood. 100μL of whole blood was lysed using 1mL of 1x eBioscience lysing solution in polypropylene tubes and incubated at room temperature (RT) for 10 minutes. Samples were spun for 5 minutes at 400g. Next, titrated volumes (1-10μL of fluorochrome conjugated monoclonal antibodies against CD14, CD16, CD163, CD64, CD206, DCIR, TLR2, TLR4, CCR2, CCR5, CCR9, CX3CR1, HLADR, CD86 and IFNγR1 (eBioscience, UK) were added to the lysed cell suspensions and incubated for 20 minutes at 4°C, table 4. Unbound antibody was washed with PBS and samples were analysed on a LSR II™ flow cytometer (BD, USA).

Significance of chosen monocyte markers

Monocyte surface marker and fluorochrome	Significance
CD14 (allophycocyanin, APC, eFluor780)	Expressed on all monocytes, but at low levels on non-classical monocytes. Not expressed on dendritic cells. Part of the TLR-4 receptor, which is also found on neutrophils
CD16 (phycoerythrin-Cy7, PE-Cy7)	Expressed on non-classical and intermediate monocytes Expressed on neutrophils and natural killer cells. Not expressed on dendritic cells Functions as an FcγR1, which binds the Fc portion of immunoglobulins that have attached to antigen specifically
6-Sulfo LAcNac (slan) (Fluorescein isothiocyanate, FITC)	Specifically identifies non-classical $CD14^{lo}CD16^{+}$ from $CD14^{+}CD16^{+}$ intermediate cells
HLA-DR (PE-Texas Red™)	Expressed exclusively on monocytes and dendritic cells Major Histocompatibility Class (MHC) II molecule involved in antigen presentation to adaptive immune system Associated with production of pro-inflammatory cytokines such as TNFα, IL-6 and IL1β and as a result regarded as an activation marker
CD86 (Peridinin chlorophyll protein,	Co-stimulatory molecule for activation

PerCP)	marker HLA-DR
CD163 (phycoerythrin, PE)	Scavenger receptor for degraded haemoglobin Up regulated in anti-inflammatory, reparative environments including after corticosteroid therapy
CD64 (allophycocyanin, APC	Fcγ receptor 1 that binds IgG. Constitutively expressed on macrophages and monocytes, but treatment of neutrophils with IFN-γ or GCSF can induce CD64 expression
DCIR (PE)	C-type lectin with roles in cell adhesion, signalling and inflammation.
CD206 (APC)	Also known as the mannose receptor that is expressed on macrophages and immature dendritic cells. Recognised glycans attached to proteins on the surface of some microorganisms (*candida albicans*, *pneumocystis jerivici*, *leishmania donovani*) and so acts as a pathogen recognition receptor. The receptor will internalise the bound pathogen and transports it to the lysosomes for degradation via the phagocytic pathway Has a further role as a scavenger receptor
TLR-2 (PE)	Pathogen recognition receptor that is expressed most abundantly on peripheral blood leucocytes (including monocytes, macrophages, neutrophils, T cells and B cells. Mediates host response to gram positive bacteria and fungi via stimulation of NFκB via production of TNFα and various interleukins
TLR-4 (APC)	Pathogen recognition receptors that can activate innate immunity by recognising gram negative and some gram-positive components (e.g. *Neiserria*). Other ligands include endogenous proteins such as heat shock protein, low density lipoprotein and β-defensins. The receptor is most abundantly expressed in the placenta and the myeloid cells such as monocytes and neutrophils.
CCR-2 (APC)	Chemokine receptor 2 is a receptor for monocyte chemoattractant protein 1 (MCP-1) which specifically mediates monocyte chemotaxis and infiltration into inflamed tissues
CCR-5 (PE)	Chemokine receptor 5 is a receptor for CCL3 and CCL4 (also known as

	Macrophage Inflammatory Protein (MIP) 1α and MIP1β) and CCL5 (also known as Regulated on Activation, Normal T cell Expressed and Secreted or RANTES). CCR5 is mainly expressed on T cells, macrophages, dendritic cells, eosinophils and microglia.
CCR-9 (PE)	Chemokine receptor for CCL25. It may play a role in the T cells of small intestine and colon, play a role in thymocyte recruitment.
CXR3CR1 (PerCP)	Chemokine receptor for fractalkine, involved in the adhesion and migration of leucocytes. It is known to play a role in the survival of monocytes and may negatively regulate monocyte infiltration into the liver
IFNγR1 (PE)	Binds to IFN-γ, the only Type 2 interferon. The human complex is a heterodimer of two chains IFN-γR1 and IFN-γR2. Upon activation, they are intimately linked with the JAK-STAT intracellular signalling pathway

Table 4: monocyte surface markers tested, fluorochrome conjugations and their significance

Gating Strategy

2 published gating strategies are available to identify monocytes, as distinct from other

circulating leucocytes and lymphocytes, in circulating human blood. A key objective is to

gate out neutrophils and NK cells that, like non-classical and intermediate monocytes,

express CD16. Gating strategies were kept consistent between samples and between

groups for individual assays.

1. ***lineage negative gating (lin^neg)***: utilised monoclonal antibody against T cells (CD3),

   B cells (CD20), neutrophils (CD66b) and NK cells (CD56) as an exclusion panel.

   Cells negative for this exclusion panel included monocytes and dendritic cells.

   Dendritic cells were further distinguished using CD14 and CD16, wherein dendritic

   cells were negative for CD14 and CD16, figure 11. This gating strategy was used for

   monocyte phenotyping assays.

2. ***HLA-DR gating***: HLA-DR positive cells were also shown to be negative for T cells, B cells, neutrophils and NK cells in a recent study (Abeles et al. 2012). HLA-DR positive cells can be further distinguished into monocytes and dendritic cells by the expression of CD14 and CD16, wherein dendritic cells are negative for CD14 and CD16 (figure 12A and 12B). This gating strategy was used for pHrodo™ phagocytosis, intracellular cytokine staining and phosphoflow assays in order to reduce the burden of fluorochromes and potential for fluorochrome spectral overlap.

Similarly, 2 gating strategies were employed to identify monocyte subsets were delineated by two methods.

A. The "fluorescence minus one" (FMO) technique can be used to delineate the border between populations that do or do not express a particular marker(Tung et al. 2007). This technique was used to delineate $CD16^+$ from $CD16^-$ populations. This technique was used in all flow cytometry experiments (figure 11C).

B. While the FMO technique is useful to separate expression from no expression in binary fashion, it cannot be used however to distinguish grades of expression, such as the $CD14^+$ vs $CD14^{lo}$ expression that delineates non-classical from intermediate monocytes. In 2015 a study was published that validated use of monoclonal antibody to cell surface 6-sulfo LAcNac (slan) to delineate the population of $CD14^{lo}CD16^+$ non-classical from the population of $CD14^+CD16^+$ intermediate monocytes(T. P. Hofer et al. 2015). This technique was not use in the experiments in this thesis, and is discussed here since it will be the preferred gating strategy for future work, figure 12D.

Dendritic cells were not analysed since these cells are not believed to play prominent roles in the innate immune response to bacterial infection.

5000 monocyte events were recorded in the flow cytometry assays. Results are expressed as positive proportion (%) when the aim was to identify cells bearing a specific marker. In all other instances, results are recorded as median fluorescence intensity (MFI), since flow cytometric data distributions were non-parametric in this study. Data was analysed using Flowlogic software (Inivai Technologies Pty Ltd, Australia).
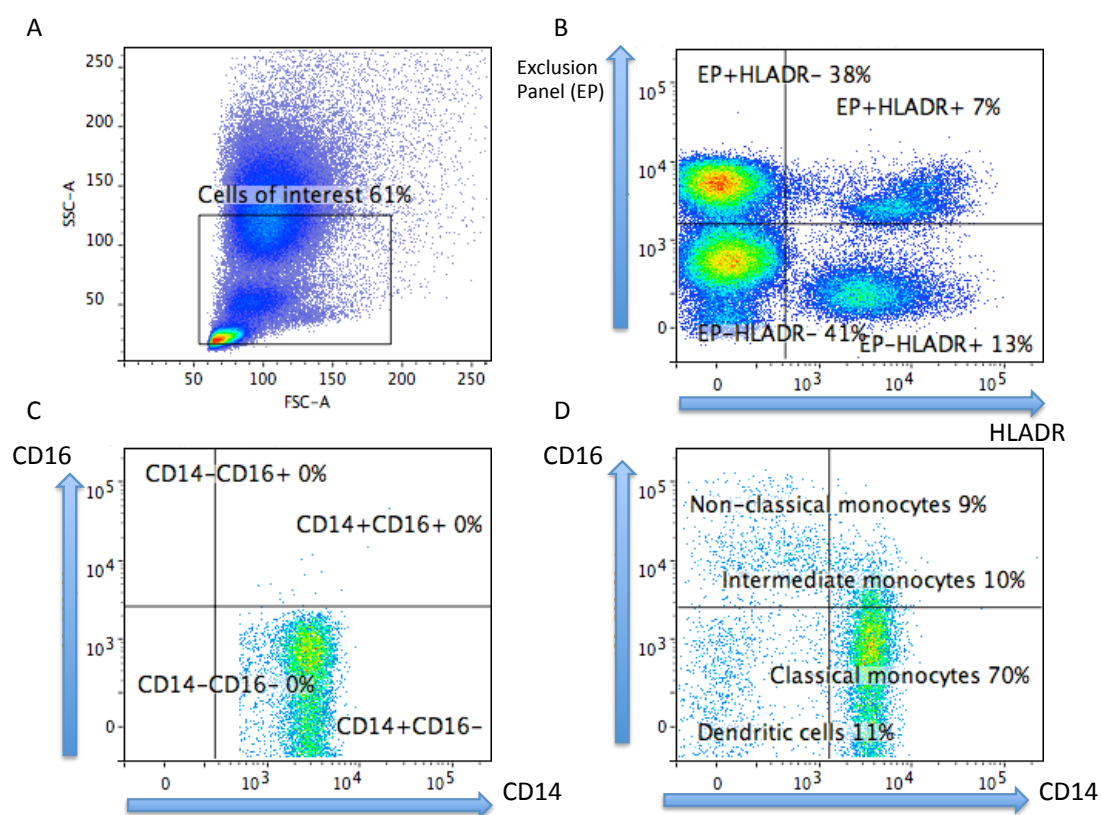
Figure 11: A. monocytes are identified on physical FSC vs SSC properties. B. HLA-DR positive cells may be distinguished from an exclusion panel that includes CD3, CD20, CD56 and CD66b. C. the Fluorescence Minus One (FMO) technique allows accurate delineation of CD16$^+$ from CD16$^-$ monocytes. D. Monocytes subsets can be identified by their relative expression of CD14 and CD16
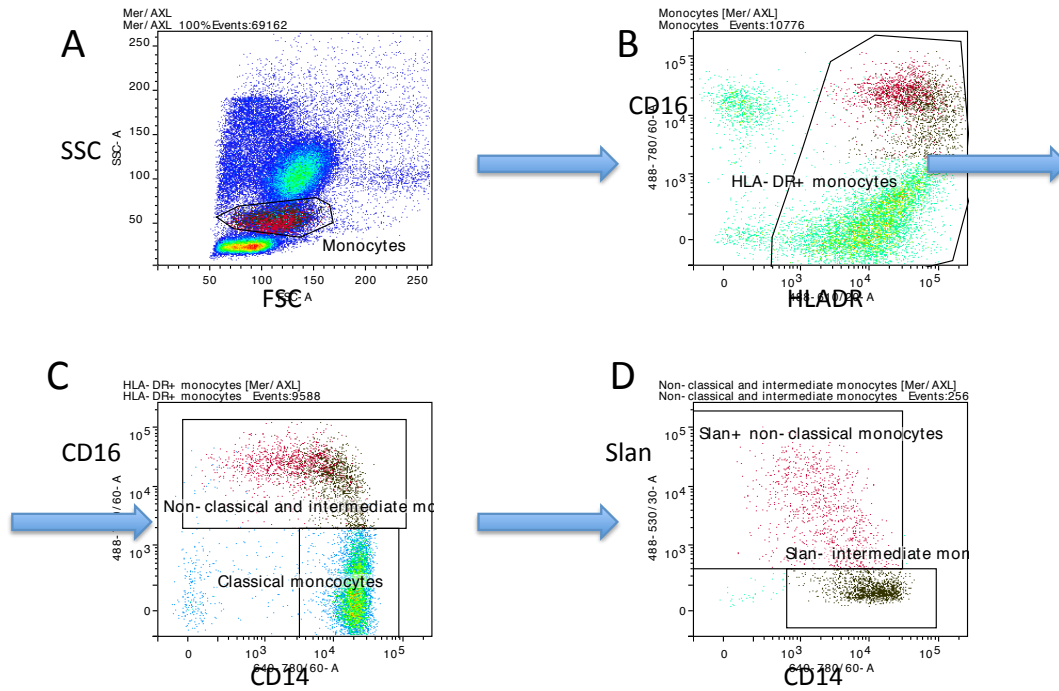
Figure 12: alternative technique of selecting non-classical from intermediate monocytes, using slan antibody staining. Non-classical, but not intermediate monocytes, stain for slan, allowing for their precise identification (T. P. Hofer et al. 2015). **This technique was not used in for experiments included in this thesis but is described as the preferred technique to identify non-classical monocytes in *Future Directions***

（w）**Phagocytosis Assay**

Phagocytosis by CD14⁺ monocytes in whole blood was assessed *ex vivo*. The ability of monocyte subsets to phagocytise *E. coli* was measured using a modified flow cytometry-based pHrodo™ assay according to the manufacturer's instructions (Life Technologies, UK). pHrodo™ is a proprietary conjugate that fluoresces brightly when the phagosome has been acidified, but is not fluorescent at neutral pH. It is thus sensitive for both the uptake of bacteria and successful acidification of the phagolysosome during phagocytosis.

pHRodo was performed according to manufacturer's instructions. Briefly, 100µL of whole blood was dispensed into test and control tubes. 20µL of *E. coli particles* were vortexed and added to the test condition. The tubes were incubated for 15 minutes at 37°C. After

incubation, cells were placed on ice and red cells were lysed. After centrifugation, cells were stained for monocyte markers CD14, CD16 and exclusion panel as described in *Monocyte Phenotyping* and figure 11. Cells were then acquired on a flow cytometer.

(x) **Oxidative Burst (MOB) Assay**

Oxidative burst by monocytes in whole blood was also assessed *ex vivo.* The ability of CD14$^+$ monocytes to oxidise 1,2,3-dihydrorhodamine (1,2,3DHR) to rhodamine in response to incubation with *E. coli* was measured using a modified flow cytometry-based Phagoburst™ assay according to manufacturer's instructions (Glycotrope, Germany) as follows.

100μL of whole blood was dispensed into test and control conditions. 20μL of fixed *E. coli* suspension ($2x10^7$ bacteria) was added to the test condition and 20μL of phosphate buffered saline (PBS) added to the control condition and both tubes were incubated at 37°C for 20 minutes. Subsequently, 1,2,3DHR was added to both tubes. The whole blood was lysed and fixed, centrifuged at 400g for 5 minutes and then washed in PBS. Cells were then stained for CD14 antibody and incubated at 4°C in the dark for 20 minutes. Unbound antibody was washed off and cells acquired on a flow cytometer. CD14$^+$ monocytes were identified first by physical properties and then by expression of CD14. CD14+ cells were gated as in the representative figure below and the oxidation of DHR 1,2,3 was quantitatively measured using the blue-green excitation light (488nm argon ion-laser), figure 13.

A. Control
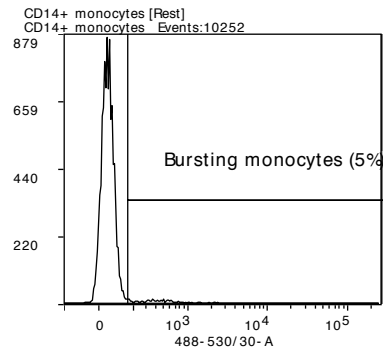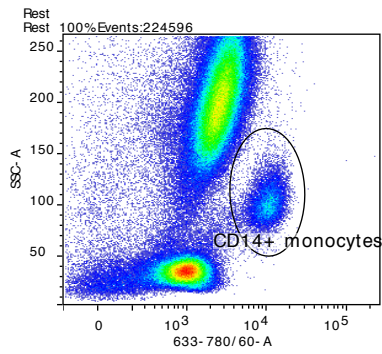
B. *E. coli*
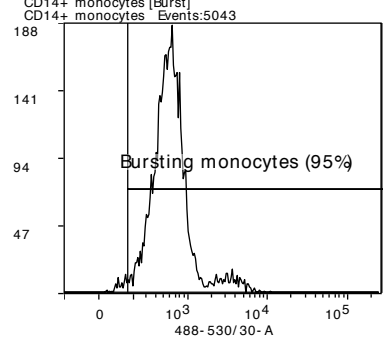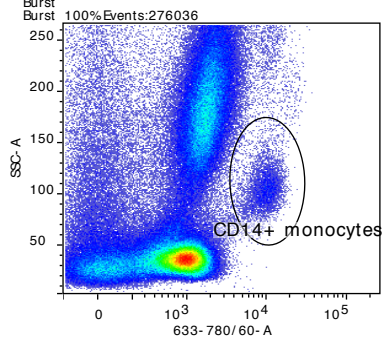
Figure 13: identification of CD14+ monocytes allows subseuqent assessment of oxidative burst in A. control and B. test conditions

(y) **Isolation of PBMCs**

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised fresh whole blood after Ficoll density gradient centrifugation according to established protocol. Briefly, whole blood was mixed 1:1 with PBS. Using an electrically powered 20mL pipette, the blood mixture was then layered above 15mL Ficoll-Paque (GE Healthcare, UK) in 50mL containers. The 50mL containers were then centrifuged at 2000g for 20 minutes at 20°C without break in order to separate red cell and plasma from a layer of PBMCs. The layer of PBMCs could then be aspirated from the Ficoll, washed in PBS, centrifuged initially at 600g and then at 400g. Pellets were suspended and then frozen in 10% dimethyl sulfoxide (DMSO), 20% fetal calf serum (FCS) and 70% Roswell Park Memorial Institute media (RPMI) using the Mr Frosty™ container, or used for fresh analyses. If frozen, PBMCs were rested for 4 hours at 37°C after thawing before functional analyses.

(z) **Intracellular Cytokine Staining**

1mL of PBMCs were suspended in 1mL RPMI and added to 24 well plates. 100ng/mL LPS (Sigma, UK, <1% protein impurity) was then added to test samples and an equivalent volume of PBS added to control samples. Protein inhibitor cocktail was also added to all wells, at a concentration of 2µL/mL (eBioscience, UK). All samples were then incubated for 6-12 hours. Intracellular cytokine staining and flow cytometry was then used to ascertain monocyte and monocyte subset specific responses to LPS stimulation, according to the manufacturer's instructions (eBioscience, UK). In brief, cells were first stained for viability using a fixable viability dye (eFluor 450, eBioscience, UK) [see *Viability Staining*]. Next, surface cell markers were stained with CD14, CD16 and HLA-DR monoclonal antibodies in order to identify monocyte subsets as described in *Monocyte Phenotyping*. Then, cells were fixed using 100µL of intracellular fixation buffer and incubated in the dark for 20 minutes at RT. Without washing, 2mL of 1x permeabilisation buffer was added to all wells. Samples

were centrifuged at 400g for 5 minutes. After discarding the supernatant, cells were stained in the recommended amount of fluorochrome labelled antibody for detection of intracellular antigen (TNFα, IL-6, IL-12, IL-1β, IFN-γ and IL-10) and incubated in the dark at RT for 20 minutes, table 5. After this incubation, 2mL of permeabilisation buffer was added and the samples were centrifuged for 400g at RT for 5 minutes. Cells were washed with 2mL of staining buffer and spun again at 400g for 5 minutes. Next cells were suspended in staining buffer and acquired on the flow cytometer. Flow cytometry data was analysed using Flowlogic software (Inivai Technologies Pty Ltd, Australia).

(aa) **Viability Staining**

Cells were tested for viability in one of two ways. For intracellular cytokine staining, cells were tested using the fixable viability dye eFluor450® (eBioscience, UK) according to manufacturer's instructions. Briefly, cells were washed 3 times in PBS to remove free amine groups. 1µL fixable viability dye then added per 1mL of cells suspension. The suspension was then incubated for 30 minutes and washed with PBS before further assays.

For all other experiments involving FACS, cells were stained with the dye 7-amino actinomycin D (7-AAD), according to manufacturer's instructions. In brief, cells were stained for 5 minutes with 5µL 7-AAD and fluorescence was detected by flow cytometry.

Cytokine and conjugated fluorochrome	Significance
TNF-α (allophycocyanin, APC)	Classical pro-inflammatory cytokine that may also play a role in tissue regeneration
Interferon-γ (allophycocyanin, APC)	Produced by T_H1 cells and known to promote bacterial killing in monocytes and macrophages via the JAK/STAT intracellular signalling pathway
IL-12 (Peridinin chlorophyll protein, PerCP)	IL-12 is produced by monocytes and macrophages and can stimulate T_H1 cells to produce Interferon-γ. It can enhance the cytotoxicity of NK cells and may have anti-angiogenic effects
IL-1β (phycoerythrin, PE)	IL-1β precursor is cleaved by cytosolic caspase 1 to form mature IL-1β. IL-1β mediates inflammatory responses and also has roles in cell proliferation, differentiation and apoptosis
IL-6 (Peridinin chlorophyll protein, PerCP)	Secreted by T cells and macrophages to stimulate immune response, especially during infection and trauma. IL-6 also has anti-inflammatory roles in inhibiting TNF-α and IL-1β, and by activation of IL-10
IL-10 (phycoerythrin, PE)	Anti-inflammatory cytokine that can down regulate T_H1 cytokine expression, MHC Class II antigens, can block NF-κB activity, and is involved in the regulation of the JAK-STAT pathway.

Table 5: Cytokines that were tested using the intracellular cytokine staining technique and their significance and fluorochrome conjugations

(bb) **Isolation of monocytes**

Monocytes were isolated using the Pan Monocyte Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer's instructions. In brief, thawed rested cells were suspended in 30µL MACS® buffer per 10^7 cells. MACS® buffer is a solution of bovine serum albumin suspended in a salt solution that contains ethylenediaminetetraacetic acid (EDTA). Next 10µL blocking antibody was added. Then, 10µL biotin-antibody cocktail was added per 10^7 cells. This antibody cocktail includes antibodies to T cells (CD3), B cells (CD19), NK Cells (CD56) and neutrophils (CD66a,c,e). The suspension was mixed by vortex and then incubated at 4°C for 5 minutes. Next, a further 30µL MACS buffer was added per 10^7 cells and 20µL anti-biotin microbeads were added per 10^7 cells. Samples were mixed and then

incubated at 4°C for a further 10 minutes. Magnetic separation takes place by passing the microbead-labelled cell suspension through a magnetic field, allowing labelled cells to adhere to the sides of an exclusion column, while unlabelled cells pass through, using the principle of negative selection. The flow-through is an untouched population of classical, intermediate and non-classical monocytes. The cells were pelleted by centrifugation at 400*g* and washed with PBS, before checking for purity and cell death. A representative figure of these parameters is given below in figure 14.
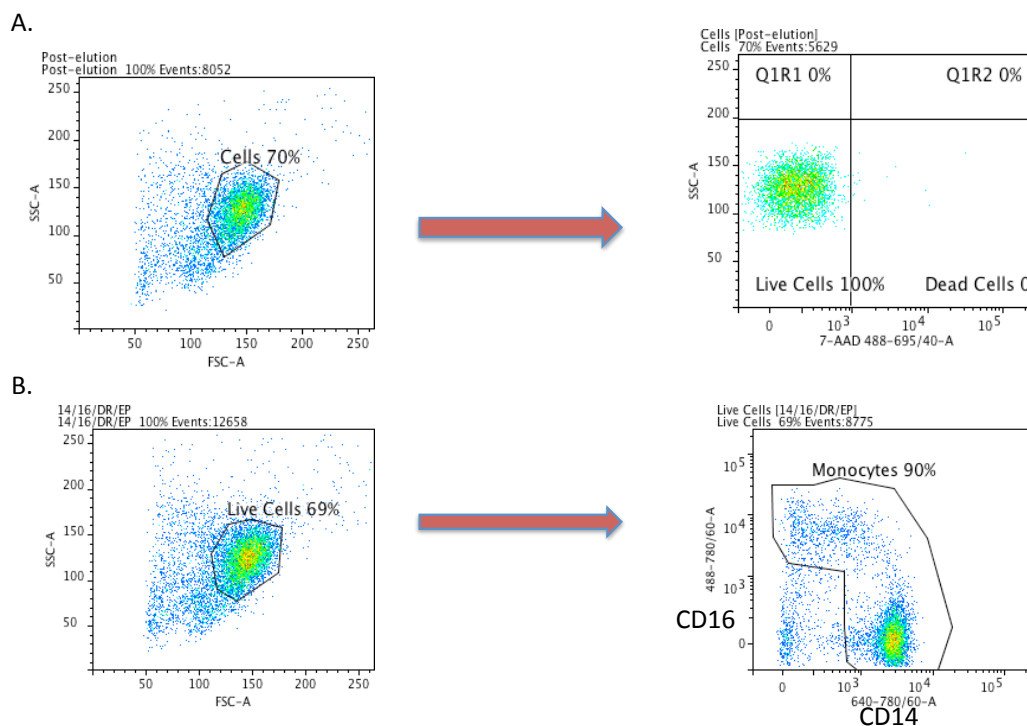
Figure 14: after negative selection using magnetic beads, cell populations were A. stained viability using 7-AAD, and then B. stained for purity using monoclonal antibody against CD14 and CD16

Phosphoflow of monocyte NF-κB transcription factors

Phosphoflow experiments were unable to detect differences between groups at concentrations of 100ng/mL. The concentration of LPS was therefore increased to 1µg/mL for phosphoflow experiments only. This was added to 200µL whole blood to stimulate

monocytes *ex vivo*, and compared to control samples treated with the equivalent volume of PBS. Cells were incubated for 20 minutes at 37°C. The reaction was quenched and the sample fixed. Phosphoflow was then performed according to manufacturers' instructions (BD Biosciences, US). This is described in brief as follows. After the stimulation period, 10 volumes of pre-warmed Lyse/Fix buffer was added to fix the cells immediately. Cells were then incubated for a further 10 minutes at 37°C. Next, cells were centrifuged at 600g for 6 minutes and supernatant removed. Cells were vortexed and then permeabilised by adding 1mL of chilled permeabilisation buffer (BD Biosciences, US). Cells were vortexed again and placed on ice for 30 minutes. Cells were then washed in 3mL staining buffer and centrifuged at 600g for 6 minutes a further 3 times. Next, cells were stained with 5µL anti-NF-κB antibody (eBiosciences, USA) and then incubated at RT for 60 minutes protected from light. These cells were then washed in staining buffer and centrifuged at 600g before aspiration of supernatant. Finally cell surface marker staining of CD14, CD16, HLA-DR and the exclusion panel was performed as described in *Monocyte Phenotyping* in order to identify monocyte subsets.

Interferon-γ (IFN-γ) and N-acetylcysteine (NAC) co-culture

1 million PBMCs were suspended in 1mL RPMI and incubated in wells of 24 well plates for 24 hours at 37°C in RPMI (Sigma, UK). The wells were supplemented with 10% autologous patient serum and 50ng/mL IFN-γ or 250µg/mL NAC, resulting in a total volume per well of 1.2mL. The ability of CD14$^+$ monocytes to oxidise 1,2,3-DHR to rhodamine in response to incubation with *E. coli* was measured using the flow cytometry-based Phagoburst assay described above (Glycotrope, Germany), but modified as follows. 20µL *E. coli* suspension (2×10^7 bacteria) was added to test wells and incubated at 37°C for 40 minutes. Next, 1mL of media was removed by aspiration. 20µL rhodamine substrate was then added to the remaining 200µL cell suspension. 1µL CD14 antibody was added and the suspension

incubated at 37°C for 20 minutes. After this incubation, 1mL of ice-cold PBS was added to the wells to detach them from the plastic wells and cells were scraped using a 1000µL pipette tip. The cell suspension was then centrifuged at 400g for 5 minutes and cells were acquired by flow cytometry.

Colorimetric, Fluorometric and Luminometric Assays

(cc) **Separating serum and plasma from whole blood**

Whole clotted blood was spun at 600g for 10 minutes in order to separate serum fractions. Aspiration was done using a Pasteur pipette. Plasma was obtained similarly from heparinised whole blood spun at 600g for 10 minutes. Plasma and serum samples were stored at -80°C until required for further analysis.

(dd) **Serum Cytokines**

Serum was used to measure the relative concentrations of a range of inflammatory cytokines using electrochemilluminescence within the Mesoscale Discovery (MSD) detection system multiplex (Gaithersburg, US). Pre-prepared 96-well multiplex plates allowed for simultaneous measurement of TNFα, IL-6, IL-1β, IL-12, Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), IL-2, IL-10 and osteopontin. In brief, serum from 20 SAH, 10 CLD and 20 HC subjects was thawed. MSD provides a plate pre-coated with capture antibodies on independent and well-defined spots. Detection antibodies to TNFα, IL-6, IL-1β, IL-12, GM-CSF, IL-2, IL-10 and osteopontin conjugated with electrochemilluminescent labels (MSD SULFO-TAG™) are added to wells. 25µL serum was then added to the wells. During incubation periods, analytes in the sample bind to capture antibodies immobilised on the working electrode surface. When the bound analytes further bind the detection antibodies a sandwich is completed. When loaded into an MSD™ instrument, a voltage applied across

electrodes causes the captured labels to emit light that can be measured, quantitatively, and correlated with the concentration of analyte in the sample.

(ee) Measurement of cytokines from stimulated PBMCs

PBMCs from SAH and HC patients were re-suspended in RPMI supplemented with 10% healthy AB serum. They were then incubated with 100ng/mL LPS (Sigma, UK) for 12 hours at 37°C. Cells were centrifuged at 400g for 10 minutes and supernatant was aspirated. Inflammatory cytokine levels from the supernatants were measure by electrochemilluminescence as described above.

(ff) Procalcitonin assay

Procalcitonin (PCT) was measured according to manufacturer's instructions (Abcam, UK). A standard procalcitonin curve was prepared by serial dilution. Serum samples were then diluted twofold. Each sample was assayed in duplicate. 100µL of sample or standard were added to each well that contained the primary capture antibody. Sample or standard was then incubated for 2.5 hours at RT. The solution was discarded and then washed 4 times with 1x wash solution. Next, 100µL biotinylated antibody was added to each well and incubated again at RT for 1 hour. 100µL of streptavidine solution conjugated to horseradish peroxidase (HRP) was next added and incubated for 45 minutes at RT. The wells were washed as previously. Wells were read at 450nm immediately. Mean absorbance was calculated for duplicate standards and samples. Values of PCT in samples were calculated by interpolation of the line of best fit described by the equation log(absorbance)=0.8*log(PCT)-3.4 to return a [PCT] >30 pg/mL, figure 14.
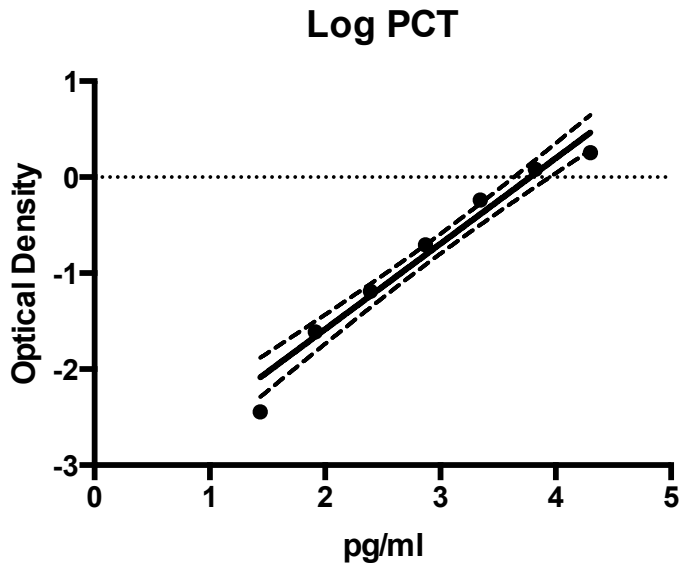
Figure 15: standard curve used for the procalcitonin assay

Monocyte superoxide production

Purified monocytes in RPMI, supplemented with 10% human AB serum, were co-cultured for 40 minutes at 37°C with *E. coli.* The monocyte: *E. coli* ratio was 1:100. 50,000 of these monocytes were then mixed with 100μL Diogenes Complete Enhancer (National Diagnostics, US) and superoxide was quantified by recording luminescence (relative light units) using a luminometer (FLUOstar OPTIMA, BMG Labtech). The intensity of light produced by Diogenes in the presence of superoxide is directly proportional to the O_2^- concentration.

(gg) **Measurement of GSH and GSSG, NADPH and NADH in serum**

(i) GSH and GSSG
A fluorometric kit (Abcam, UK) was used to quantify levels of oxidised and reduced glutathione in serum. The kit uses a proprietary non-fluorescent dye that becomes strongly fluorescent on reacting to the thiol groups of reduced glutathione (GSH). A thiol green

indicator solution is used to compare the fluorescence generated from standards of known concentrations of glutathione with the glutathione in test samples, figure 16.

(ii) NADPH and NADH

A colorimetric kit (Abcam, UK) was used to quantify the level of intracellular nucleotides NADP$^+$ and NADPH in serum. A standard curve of NADPH is used to calculate the concentration of NADPH in test samples, figure 17. NADPH is isolated from total NADP$^+$ and NADPH by decomposing NADP$^+$ by heating for 30 minutes at 60°C: under these conditions all NADP$^+$ will decompose while NADPH remains intact. A specific NADPH cycling mix and developer amplifies and detects NADPH in the test samples. NADP$^+$ can be calculated by subtracting total NADPH+NADP$^+$ from NADPH. The plate is read at 450nm after 4 hours incubation.
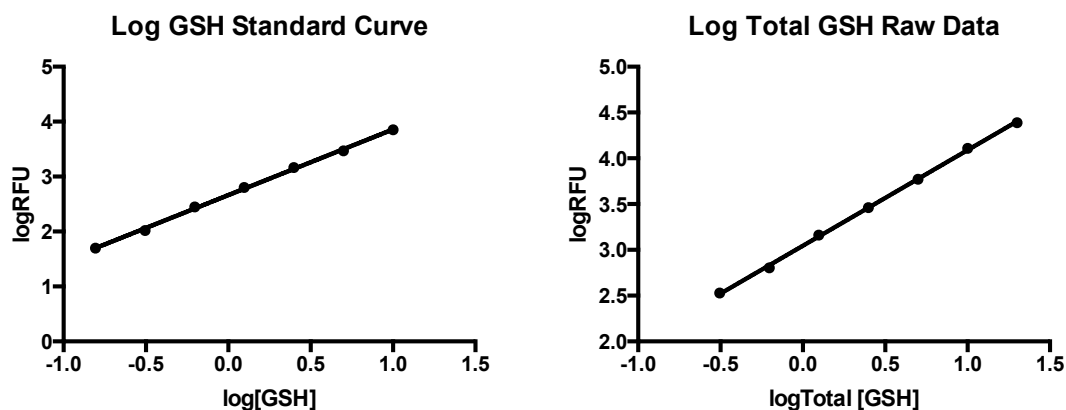
Figure 16: standard curves used in glutathione (GSH) and oxidised glutathione (GSSG) assays
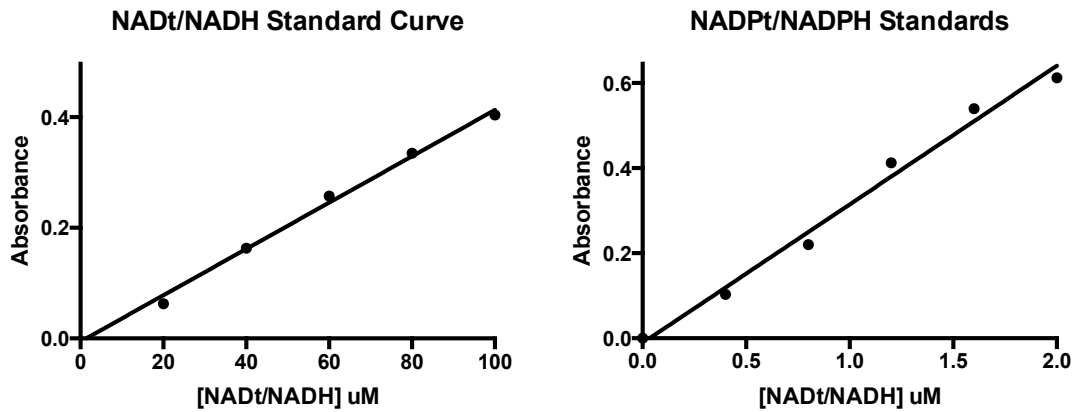
Figure 17: standard curves used for NADPH and NADH assays

(hh) **Measurement of intracellular G6PDH function**

Monocytes were isolated using magnetic separation as described above. Cells were then lysed using nonyl phenoxypolyethoxylethanol-40 (NP-40) buffer and intracellular enzyme function was immediately measured using the Glucose-6 Phosphate Dehydrogenase (G6PDH) Assay Kit (Abcam, UK). This kit measures the conversion of colourless glucose-6 phosphate, by G6PDG activity in the sample, to an intensely coloured product that absorbs at 450nm. The standard curve is drawn using a range of NADH concentrations. In brief, 50µl of sample was assayed in duplicate wells in a 96 well plate. A reaction mix containing substrate and developer is added to each well containing standards and test samples. Optical density at 450nm was measured immediately and then measured again after incubating the reaction at 37°C for 60 minutes protecting from light at all times. The difference between optical densities before and after the 60-minute incubation period is compared to the NADH standard curve to elucidate the amount of NADH liberated. Values were normalised to protein concentrations, which were estimated using the Bradford reagent. The Bradford reagent is coomassie brilliant blue that changes colour under acidic conditions, from red to blue when it binds to the protein being assayed. The bound form of the dye has a maximum absorption spectrum of 595nm. The absorption at 595nm of a

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standard curve of known concentrations of albumin is measured and used to interpolate

unknown concentrations of protein from their absorptions at 595nm(Bradford 1976).

Microbiological Assays
Bacterial killing assay

Bacterial killing in supernatant and lysate fractions was measured as previously described

(O'Brien et al. 2014). 100,000 monocytes were suspended in 200μL RPMI supplemented

with 10% human AB serum (not heat inactivated) in wells of a 96 well flat bottom plate

(NUNC, Thermoscientific US). 75μL of a stock of *E. coli* suspension (K12, Stratagene), at a

known concentration of $160x10^8$/mL, was then mixed with 25μL of human AB serum and

incubated for 30 minutes at 37°C. Bacteria at a concentration of $120x10^8$ bacteria/mL was

kept on ice to prevent further bacterial replication. 1μL of this suspension, containing 12

million bacteria, was added to the 100,000 monocytes per well, giving a monocyte: *E. coli*

ratio of 1:100. Depending on test condition, wells were then incubated for 1 hour at 37°C to

allow bacterial killing to occur, then placed on ice to stop further bacterial killing or

replication. The plates were then centrifuged at 4°C at 1000*g* in order to separate non-

phagocytosed bacteria (in the supernatant) from phagocytosed bacteria (in the monocyte

cell pellet). 180μL of supernatant was then aspirated from each well using a multi-channel

pipette and placed into a clean new 96 well plate. This plate was kept on ice.

After centrifugation and the aspiration of 180μL supernatant described above, 20μL of RPMI

containing the monocyte cell pellet remained at the bottom of the wells. This was suspended

in 200μL distilled water (1:10 dilution) at pH 11 to lyse the cells. This was to ensure

adequate phagocyte lysis according to published literature (Decleva et al. 2006). Lysate was

collected by cell scraping and lysates were placed on ice. Lysates and supernatants were

then plated in triplicate onto agar plates at dilutions of 1:10, 1:100 and 1:1000. A 10μL

inoculation loop was used to gently and evenly spread the bacteria on the surface of the

agar. Agar plates were incubated inverted, with agar on top, for 18 hours at 37°C. Colony-

forming units (CFU) were counted using cell counter, marker pen and coloured background.

Adjustments were made for dilution factors.
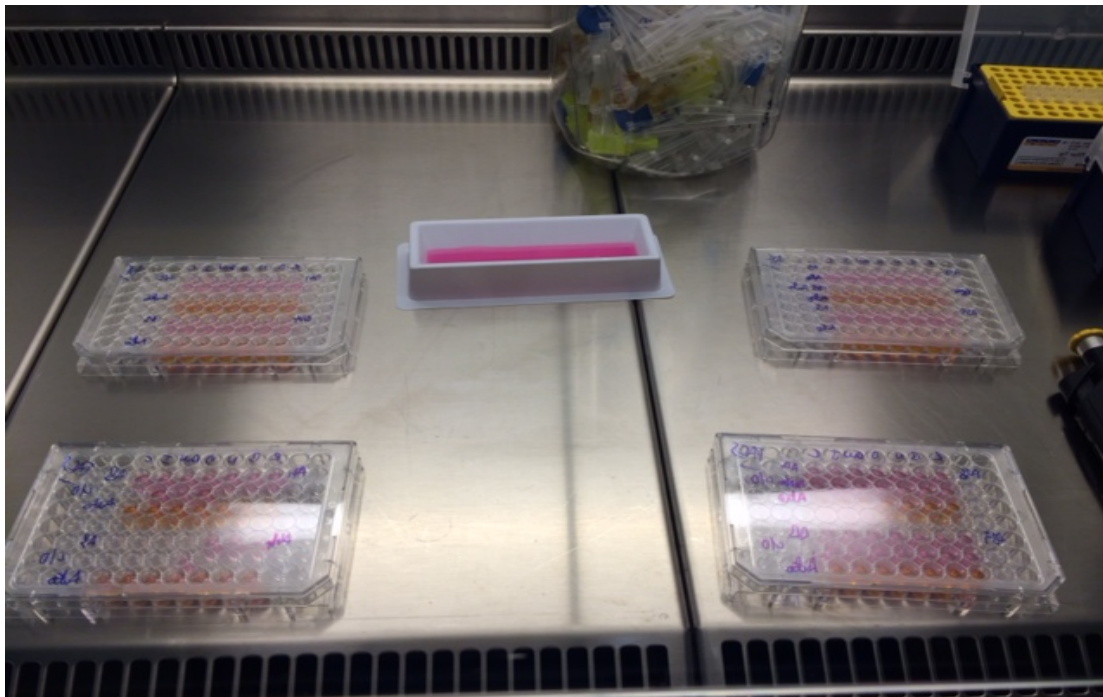
Figure 18: photograph of experimental set up for bacterial killing assays. *E coli* was incubated with CD14^{+} isolated monocytes 100:1 for 1 hour before centrifugation and separation of supernatant and cells. Cells were then lysed and both supernatant and lysate fractions were plated onto agar. Colony forming units were counted on agar after 18 hours incubation at 37°C
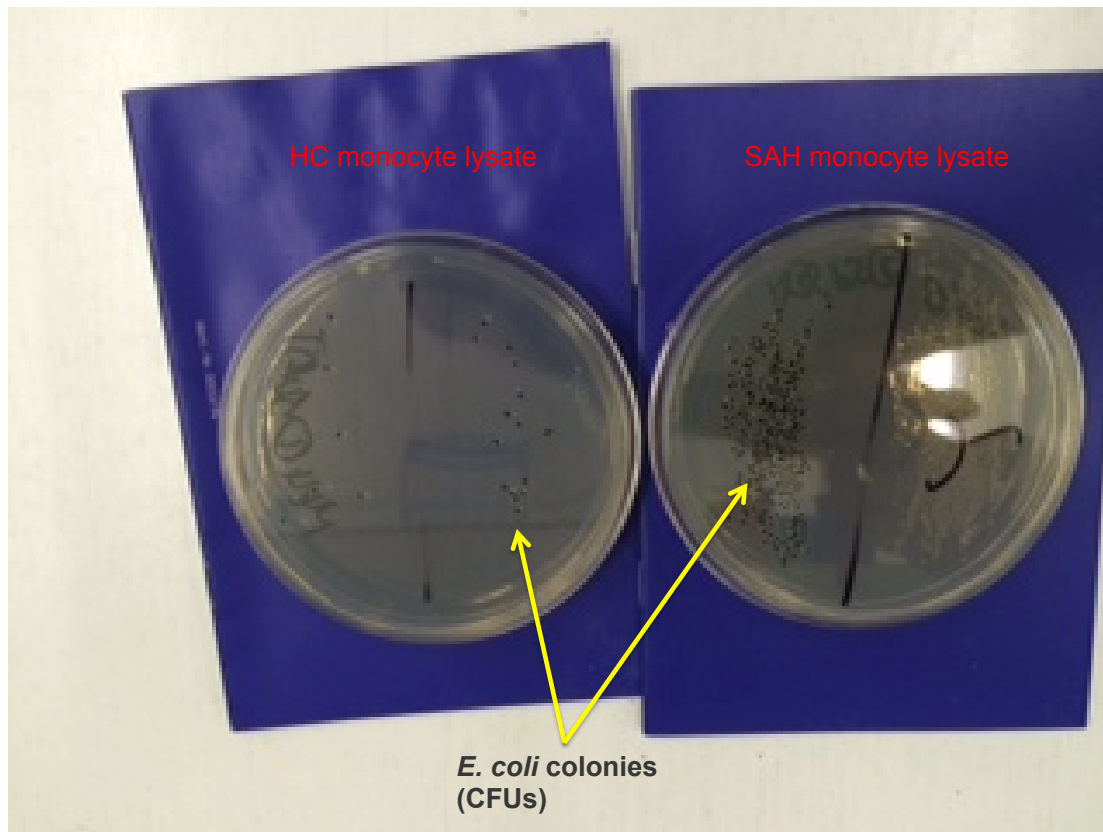
Figure 19:Photograph showing representative agar plates comparing *E. coli* CFUs from HC and SAH monocyte lysates

Molecular Assays

(ii) **Western blotting of monocyte G6PDH, pSTAT-1 and SOCS-1, Gp91phox and p47phox proteins**

After phagocytosis, monocytes secrete IL-12 that activates Th1 cells to generate IFN-γ. IFN-γ activates the IFN-γ receptor complex on the surface of monocytes. This initiates an intracellular signalling cascade resulting in phosphorylation of STAT-1. Phosphorylated STAT-1 is able to translocate to the nucleus where it activates expression, nuclear translocation, and transcriptional activity of IRF1 and IRF8, which are essential to activate the full microbicidal potential of monocytes and macrophages (Langlais et al. 2016) including transcription of the major NADPH oxidase subunit gp91^{phox}, figure 20. NADPH oxidase is a key component of monocyte oxidative burst, since it generates the superoxide radicals

required for bacterial killing within the monocyte phagolysosome. If there were defects in bacterial killing, we sought to identify whether these were due to deficiencies in STAT-1 or gp91^{phox} gene expression or translation.
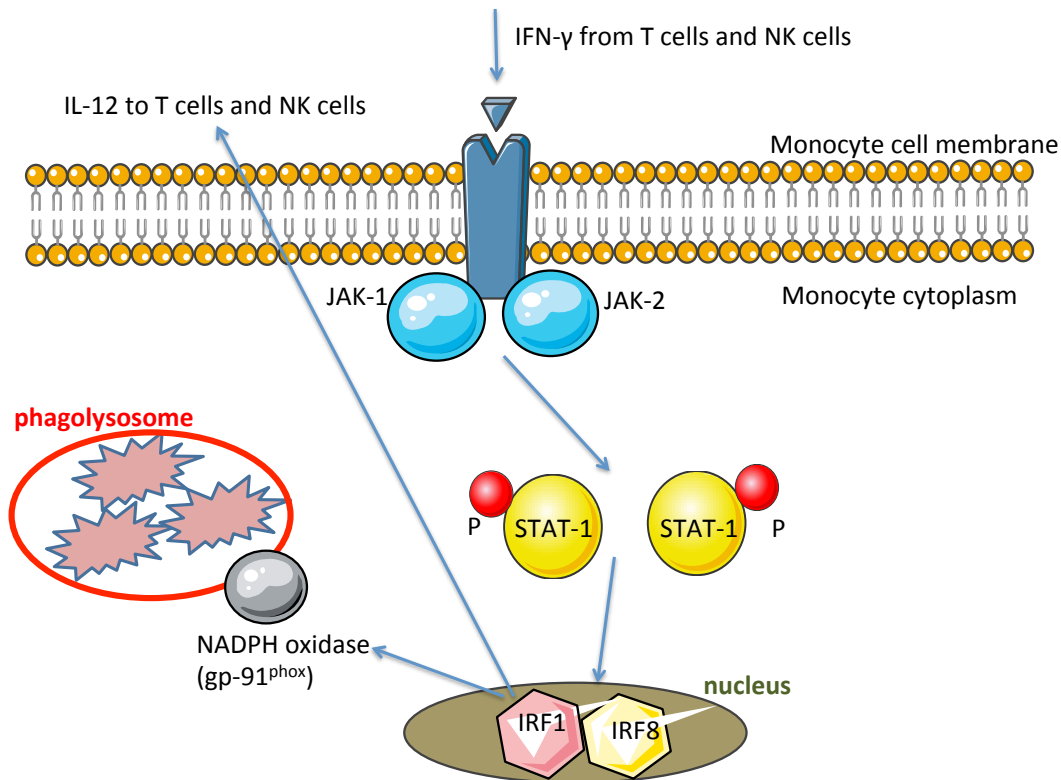
Figure 20: monocytes stimulated by bacterial products release IL-12. This stimulates T cells and NK cells to release IFN-γ. IFN-γ stimulates the IFN-γ receptor, which triggers intracellular signalling via JAK and the STAT-1 transcription factor. STAT-1 translocates to the nucleus where it activates regulatory factors IRF1 and IRF8, controlling gene transcription of NADPH oxidase subunits such as gp-91^{phox}. *Figure created using Servier™ medical artwork*

(i) Cell stimulation with IFN-γ

PBMCs were thawed and monocytes were isolated as described in *Isolation of Monocytes*. Cells were then rested for 4 hours at 37°C in X-VIVO medium (Lonza, Switzerland) in 15mL tubes. Next, cell suspension was transferred to 2mL sterile plastic *Eppendorf™* containers. Then, 50ng/mL IFN-γ was added to the containers for 20 minutes at 37°C. Cells were then

lysed with ice-cold nonyl phenoxypolyethoxylethanol-40 (NP-40) buffer (Invitrogen, UK) containing proteinase inhibitors (Sigma, UK). Lysate were stored at -80°C.

(i) Determining protein concentrations

The protein concentration of the cell extracts was determined prior to loading samples for polyacrylamide gel electrophoresis (PAGE). The Bradford reagent was first tested for its ability to estimate protein concentrations in the lysate. However, at higher concentrations of protein, the Bradford reagent appeared to saturate (figure 21), potentially leading to inaccurate protein estimation and consequent inaccurate estimate of the concentration of target protein per weight cellular protein by Western blotting.
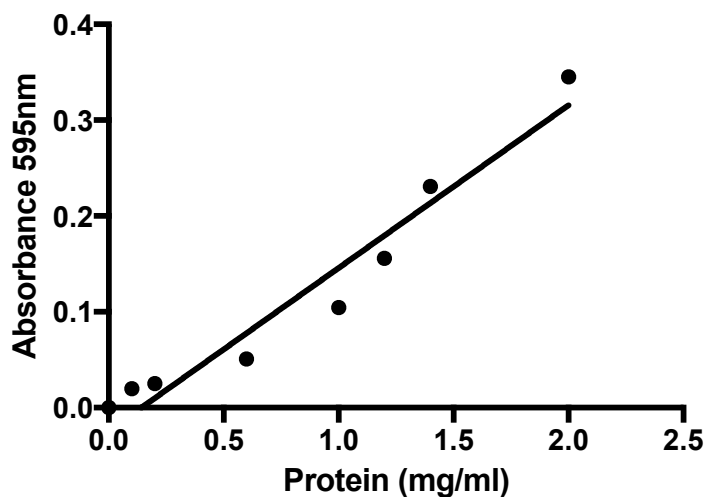
Figure 21: figure showing standard curve for Bradford reagent, demonstrating the non-linear relationship, particularly at higher concentrations of protein

The working range of the 91-bicinchoninic acid (BCA) assay is broader and able to estimate concentration of protein up to 2mg/mL on a linear scale (Pierce, Thermoscientific, UK). This colorimetric test was therefore used to measure the protein concentration of cell lysate samples before Western blotting. The procedure uses copper (II) sulphate and BCA in a 2-step process. Firstly, in the alkaline environment provided by the lysis buffer, the protein

reduces the copper of the blue $CuSO_4$ from Cu^{2+} to Cu^+. Secondly, the ligand BCA forms

chelates Cu^+ to form a purple solution, known as a Biuret reaction. The absorbance of light

at a wavelength of 570nm by this purple solution is read using colorimetrically, where the

absorbance value is proportional to the protein concentration in the sample (Smith et al.

1985). A representative standard curve for albumin is given below, figure 22.

BCA ASSAY
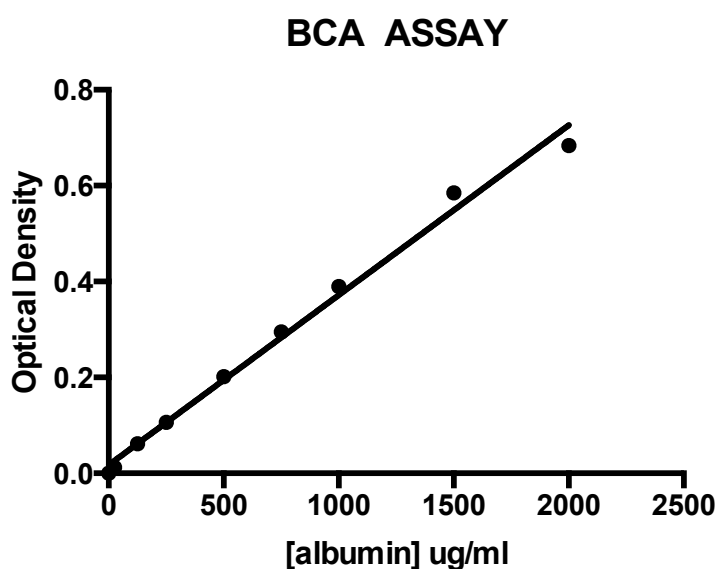
Figure 22: standard curve for albumin using the BCA technique

(ii) Sample conditioning

Before PAGE was performed, samples were conditioned to ensure separation by molecular

weight by reducing protein disulphide bonds and denaturing. Samples were therefore treated

with a mixture containing 500µL lithium dodecyl sulphate (LDS) (Nupage, Thermoscientific)

with 20µL β-mercaptoethanol (ME)(40%) as the reducing agent. 35µL of the LDS reducing

agent was added to 100µL sample, to give a final concentration of reducing agent in the

sample 10%. The LDS βME-sample mixture is then heated to 90°C for 5 minutes to

denature the protein. This mixture can be frozen at -80°C for long-term storage.

(iii) Polyacrilamide gel electrophoresis (PAGE)

Gels were loaded such that each lane had the same amount of protein (20µg) determined by the BCA assay. Gels are first rinsed with running buffer. Gel tanks were filled with running buffer (Tris/Glycine/SDS). A ladder was used in the first well, and each sample was loaded into a separate well. Protein was separated on 4-15% polyacrylamide pre-cast gels (Bio-Rad, UK) using electrophoresis at 100V. Once adequate separation was achieved, current was switched off and gels were transferred to transfer buffer. The wet transfer method was used. For this, a transfer sandwich is assembled using 4 sheets of filter paper sandwiching gel and polyvinylidene difluoride (PVDF) membrane (gel on anode). The cassette was placed in the transfer tank, with ice packs using a 100V for 30 minutes. After transfer, the PVDF membrane is rinsed with tris-buffered saline with Tween20 (TBST). Membranes are blocked with 3% BSA in TBST for 1 hour at RT.

Next, membranes are incubated overnight at 4°C on a roller with primary antibody including G6PDH (Abcam, UK), gp91^{phox} (Abcam, UK), phospho-Signal Transducer and Activator of Transcription-1 (pSTAT-1) (BD Bioscience, UK), and Suppressor of Cytokine Signalling-1 (SOCS-1) (Santa Cruz, US). Non-specifically bound primary antibody is washed off with TBST and horseradish peroxidase conjugated secondary antibody is applied for 1 hour at RT, targeted against the species-specific portion of the primary antibody. After further washing of the membrane with TBST, a chemiluminescent substrate (Supersignal West Pico, Thermoscientific) was added to react with the hydrogen peroxide. Images of the light emitted are captured in dedicated chamber. Next, the membrane was washed before probing for the loading controls of either glyceraldehyde 3-phosphate dehydrogenase GAPDH (Sigma, UK) or β-tubulin (Abcam, UK).

(jj) **Real-time polymerase chain reaction measurement of gp-91phox and STAT-1 gene expression**

(i) RNA extraction

Total RNA from isolated monocytes was extracted using the RNeasy™ Mini Kit (Qiagen, Germany) according to manufacturer's instructions. Briefly, cells were pelleted from suspension by centrifuging for 5 minutes at 400g. Supernatant was aspirated and cells were then lysed using a buffer containing 1% βME and guanidine isothiocyanate. 70% ethanol was then added to the homogenised lysate. Sample was then transferred to a spin column placed in a 2mL collection tube and after several washes and centrifugation steps at 8000g, RNA was extracted.

(ii) cDNA synthesis.

Complementary DNA was synthesised from RNA using the RETROscript™ Kit (Life Technologies, US) according to manufacturer's instructions. The resulting cDNA was stored at -20°C.

(iii) Quantitative real time polymerase chain reaction

qRT-PCR was performed using TaqMan Gene Expression assays specific for STAT-1 (Hs01013996_m1), SOCS-1 (Hs00864158_g1) and gp-91^{phox} (Hs00166163_m1) (Life Technologies, US). Beta actin was used as the endogenous control (Hs99999903_m1) (Life Technologies, USA). PCR reaction mixtures were: 1uL (20x) TaqMan gene expression master mix; 1ng cDNA template (pre-quantified using nanodrop), 5µL RNAse free water.

Assays were performed in 96 well plates on a StepOne Real-Time PCR System (Applied Biosciences, UK), using GAPDH and β-actin as housekeeping genes.

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(iv) Statistical Analysis

Continuous data are expressed as median, interquartile range (IQR) if data is non-parametric or mean (+/- standard deviation) if parametric. The significance of differences between medians or means was tested using Mann-Whitney test or Wilcoxon-pairs signed rank test for non-parametric data and paired or unpaired student t-tests for parametric data. Spearman's rank tests explored correlation. Proportions were compared using the χ^2 test. Odds ratios (OR) are expressed with 95% confidence intervals in parentheses. *P* values are two-tailed except where specified and *P*<0.05 was adopted as the threshold for statistical significance. P values were not corrected for multiple testing. In instances where the direction of association could be predicted *a priori* data was specified to be tested one-tailed for statistical significance. Data was analysed and graphs were drawn using Prism 6.0 (GraphPad, US).

Where data was non-parametrically distributed, box plots were used since they are able to demonstrate the direction of skew, where the box represents the interquartile range, the median is represented by a horizontal line and the whiskers are calculated by Tukey method in order to indicate outlying values.

Upper whiskers on a Tukey boxplot

The upper whisker represents the 75th percentile plus 1.5 times the IQR. If this value was greater than the largest value, then the upper whisker is drawn to the largest value. Otherwise, the upper whisker stops at the largest value less than the sum of the 75th percentile plus 1.5 times the IQR. Any values greater than this are plotted as individual points, and are suggested to be outliers that would otherwise misrepresent the distribution of the data.

If the 25th percentile minus 1.5 times the IQR is less than the smallest value in the dataset,

the lower whisker is drawn to the smallest value. Otherwise the lower whisker stops at the

lowest value that is greater than the 25th percentile minus 1.5 times the IQR. Any values less

than this are plotted as individual points and are suggested to be outliers.

Delta delta CT method to calculate the relative expression of gene of interest

The delta delta CT method was used to calculate the relative expression of the genes of

interest, namely STAT-1 and gp-91^{phox}. The calculation is given below in table 6, and relies

on taking mean C_T values from replicate PCR results, where C_T is the threshold cycle, which

is the cycle number at which the fluorescence generated within a reaction crosses the

threshold line. C_T values are logarithmic and are used either directly ($\Delta\Delta C_T$ method) or

indirectly (interpolation to standard curves).

ΔCt Value (Experimental)	ΔCt Value (Control)	Delta Delta Ct Value	Expression Fold Change
ΔCTE	**ΔCTC**	**ΔΔCt**	**2^-ΔΔCt**
Difference between gene of interest and housekeeping gene cycle number in test conditions	Difference between gene of interest and housekeeping gene cycle number in control conditions	Relative difference between cycle number in the gene of interest and housekeeping gene cycle number in test vs control conditions **ΔCTE- ΔCTC**	Exponential of the relative difference in cycle number between test and control conditions

Table 6: Calculating the fold change for gene expression using the delta delta CT method.

Standard curve method to detect circulating 16S ribosomal bacterial DNA

The mean C_T value was calculated from replicates and the circulating concentration of

bacterial DNA was interpolated from a standard curve of known concentrations of *E.coli.*

Results

Results I: Baseline characteristics and outcome of subjects

(kk) **Patient Baseline Characteristics**

Baseline patient characteristics are summarised in table 7. 65% SAH patients were male with mean age 48 years. The profile for CLD subjects was similar. The profile for HC subjects showed similar age but there was a greater proportion of women in the HC group compared to SAH and CLD.

In SAH patients, median MDF was 57 (41-76), MELD score 24 (22-27) and Lille Score 0.40 (0.12-0.65). In CLD patients, median MELD score was significantly lower at 12 (9-15) [$p<0.0001$]. Results highlight the marked liver impairment in SAH compared to CLD patents

	SAH	CLD	HC
Age /years	47 (41-56)	49 (47-56)	42 (36-53)
Male /%	65	70	30
MELD	24 (22-27)	12 (9-15)	n/a
CTP score (class)	10 (C)	7 (B)	n/a
INR	1.8 (1.5-2.0)	1.2 (1.2-1.4)	n/a
Bilirubin (µmol/L)	318 (200-460)	26 (8-44)	n/a
Albumin (g/L)	24 (19-33)	31 (28-36)	n/a
White cell count (x10^9/L)	8.9 (5.6-13.3)	4.9 (3.7-6.1)	n/a
Monocyte count (x10^9/L)	1.0 (0.6-1.5)	0.5 (0.4-0.7)	n/a
Neutrophil count (x10^9/L)	6.9 (3.8-10.3)	2.5 (2.4-3.7)	n/a
Alanine Transferase (iu/L)	42 (30-79)	21 (17-36)	n/a
Aspartate Transaminase (iu/L)	127 (100-164)	59 (34-79)	n/a
Serum creatinine (µmol/L)	69 (63-104)	63 (57-71)	n/a

Table 7: baseline characteristics of study participants

(i) Clinical Outcomes

SAH patients were recruited at a median 5 (3-8) days following admission. In 22 cases, a further follow-up sample of blood was obtained seven days after the institution of therapy. Therapy was determined by randomisation within the *STeroids Or Pentoxifylline for Alcoholic Hepatitis* (STOPAH) clinical trial in 74% (31/42) of patients and by clinical judgement in

patients not participating in the clinical trial. Overall, 52% (22/42) received prednisolone for 28 days and 48% (20/42) were treated without prednisolone.

As discussed in *Methods*, defining infection may be clinically challenging. Using the definition of infection according to a published recent study (Bajaj et al. 2012), within two weeks of initial sampling 40% (17/42) of SAH patients had developed *infection,* table 8. At 28 days, mortality rate of infected patients was 35% versus 0% of uninfected patients (odds ratio [OR] 48 [3-918]; *P*<0.0001). At 90 days, mortality rate of infected patients was 63% versus 15% of uninfected patients (OR 9 [2-46]; *P*<0.01).

Within two weeks of initial sampling, a higher proportion, 52% (22/42) of SAH patients, had been prescribed either new antibiotics or had been prescribed a change of antibiotics. All of these patients had developed *infection* as defined above. At 28 days, the mortality rate in patients who had been prescribed either new or a change of antibiotics was 29% versus 0% in those who had no change in antibiotic prescription. These data are summarised in table 8.

Maddrey's discriminant function (IQR)	57 (41-76)
Prednisolone therapy	52% (22/42)
No prednisolone therapy	48% (20/42)
Lille Score,(Louvet et al. 2007) (IQR)	0.4 (0.12-0.65)
Patients receiving antibiotics before initial sampling	50%
Patients with infection* before initial sampling	21%
Patients receiving new or a change of antibiotics within 2 weeks of initial sampling	50%
Patients developing nosocomial infection within 2 weeks of initial sampling (*infected patients**)	40%
28 day all-cause mortality	14%
28 day mortality of infected patients*	35%
28 day mortality of patients who were not infected*	0%
90 day all-cause mortality	31%
90 day mortality of infected patients*	63%
90 day mortality of patients who were not infected*	15%

Table 8: baseline SAH clinical characteristics and outcomes. Values are median average (interquartile range) unless otherwise stated. *Infected patients are defined according to consensus criteria (Bajaj et al. 2012).

(ii) Hepatic Function after 7 days prednisolone therapy

Data from the STOPAH study was used to evaluate the effect of prednisolone on liver function. A composite score of recovery of liver function used is termed the *Lille score* (for calculation, please see Glossary).

In those patients for whom a Lille calculation was available (*n*=702 patients), prednisolone was shown to reduce the chance of patients developing an adverse Lille score, suggesting that this anti-inflammatory corticosteroid may be able to improve liver function in the first week of therapy (odds ratio with vs without prednisolone of Lille score improvement is 0.58, CI 0.43-0.78; *p*<0.001), figure 23.

(iii) Causes of death in SAH

There were two prominent causes of death within the first 90 days of SAH.. The greatest proportions of deaths were caused by hepatic failure, followed by infection. However, multi-organ and renal failures were commonly quoted causes of death and it is probable infection contributed to a number of these deaths without record (table 9).

Cause of death within 90 days	Cases	%
Hepatic Failure	50	41
Infection	36	29
Renal	10	8
Multi-organ failure	9	7
Bleeding	7	6
Encephalopathy	4	3
Respiratory Failure	2	2
Cardiac arrest	2	2
Not available	2	2
Traumatic brain injury	1	1
Stroke	1	1

Table 9: causes of death in the STOPAH study

(II) **Types of Infection**

From the 42 patients included in the monocyte analysis, pneumonia was the commonest nosocomial infection, occurring in 59% (10/17 patients included in the monocyte analyses), followed by urinary tract infection at 18%. Pneumonia was also the commonest nosocomial infection recorded by Serious Adverse Event (SAE) reporting in the STOPAH study, occurring as 54/109 of all infectious SAEs (50%).

Organisms were grown from culture in 26 instances and these were predominantly catalase positive organisms of which *E. coli* was the most frequently isolated in 27% (7/26), figure 24.

Results II: Inflammation and circulating monocyte subsets in SAH

(mm)   **Serum cytokine levels in SAH**

Serum was collected from 20 SAH patients who underwent analysis in the monocyte study, and compared to serum from 10 CLD patients and 20 HC. The unequal sample sizes are noted but statistically valid for both non-parametric Mann Whitney U and Kruskal Wallis testing (Mann & Whitney 1947). In the case of unequal sample size, statistical power is slightly reduced, but as demonstrated below, effect sizes are large and variances relatively small between groups, and so the reduction in power is unlikely to alter conclusions.

High levels of both pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-12, osteopontin) and the anti-inflammatory cytokine IL-10 were recorded from the serum of patients with SAH before treatment compared to CLD and HC, figure 25.

(nn) **Circulating monocyte subset phenotype in SAH**

(i) Monocytosis in SAH

A monocytosis was observed in alcoholic hepatitis. Although the median number of circulating monocytes in CLD patients was elevated to the upper limit of the normal range, median total numbers of monocytes in SAH patients, however, were higher than both CLD and HC (1.0 vs 0.7 vs 0.5×10^9 cells; $P<0.003$), figure 26.

(ii) Expansion of Intermediate monocyte subset in SAH

$CD14^+CD16^+$ monocytes are termed intermediate monocytes and studies have shown their potential as inflammatory innate immune cells, believed to play roles in both inflammatory and infectious disorders (Ziegler-Heitbrock 2007a) such as chronic liver disease (Zimmermann et al. 2010), rheumatoid arthritis (Baeten et al. 2000), pancreatitis (Rahman et

al. 2004), tuberculosis (Antonelli et al. 2014) and sepsis (Fingerle et al. 1993a). We therefore sought to determine the number, phenotype and function of these cells in SAH.

The proportion of intermediate monocytes was increased in both SAH and CLD (11 vs 16 vs 6%, p=0.001) compared to monocytes from HC (figures 26 and 27). The proportion of intermediate monocytes did not correlate with composite scores of baseline liver function (MDF r=-0.22, p=0.3 and MELD r=-0.05, p=0.8) although it is possible that the sample sizes do not have sufficient power to definitively rule out correlation (figure 28).

The proportion of non-classical CD14^{lo}CD16$^+$monocytes was conversely diminished in SAH and CLD compared to HC, figure 29.

(iii) Pattern-recognition and IFN-γ receptors

Monocyte expression of pattern recognition receptors TLR-2 (classical: 7906 vs 8614 and intermediate: 9911 vs 9860MFI; P=ns) and TLR-4 (classical: 401 vs 365 and intermediate: 506 vs 568; p=ns) were equivalent in SAH as seen in HC, figures 30, 31, 32. The expression of these receptors was therefore not investigated in CLD patients.

(iv) Scavenger receptors

In order to dissect phagocytic functions in SAH, expression of monocyte Fcγ-receptors (Fcγ-receptor1/CD64) and scavenger receptors (CD36, CD206, CD163 and DCIR) were measured. Fcγ-receptor 1 expression was increased in SAH compared to controls CLD and HC (classical: 12599 vs 7987 vs 6002; intermediate: 10856 vs 6134 vs 5459MFI, p<0.001). Scavenger receptor expression was broadly equivalent in SAH compared to HC (CD163 classical: 457 vs 673; intermediate: 905 vs 773MFI; CD206 classical: 580 vs 547MFI and

intermediate: 1195 vs 985; CD36 classical: 1859 vs 2018; p=ns throughout) but elevated for DCIR (classical: 1939 vs 1045 vs 831; p<0.02) in SAH compared to CLD and HC, figure 33.

(v) Activation Markers

Monocyte expression of the activation marker HLA-DR has been shown, in studies of patients with sepsis, trauma and pancreatitis, to be a surrogate of the immunoparesis of endotoxin tolerance and also to correlate with nosocomial infections and short-term mortality (Draisma et al. 2009; Heagy et al. 2003).

In this thesis, there was a marked dichotomy of HLA-DR expression on circulating classical and intermediate monocytes, figure 34. Expression of activation markers HLA-DR and the co-stimulatory receptor CD86 were lower in classical monocytes from both SAH and CLD patients compared to HC (HLA-DR: MFI 2259 and 2666 vs 4151; p<0.01 and CD86: 1566 and 2042 vs 2155; p<0.01). Expression of HLA-DR was similarly depressed in non-classical monocytes compared to HC (p=0.01). In contrast, HLA-DR expression was selectively increased in the expanded population of intermediate monocytes of SAH patients when compared to expression on intermediate monocytes from CLD patients (intermediate monocytes: 11811 vs 5848; p<0.01), figures 34 and 35.

(vi) Inhibitory/Resolution markers

MerTK expression has been shown to negatively regulate monocyte inflammatory cytokine responses to LPS and postulated to be a mediator of endotoxin tolerance (Bernsmeier et al. 2015). MerTK expression was therefore measured in a further 6 patients with SAH. Expression was reduced on both classical (2109 vs 6417MFI; p=0.07) and intermediate monocytes (2290 vs 6082MFI; p=0.07) vs HC, figure 36.

(vii) Monocyte chemokine receptor expression

The expression of chemokine receptor CCR2 on monocytes was equivalent in patients with SAH and in HC (CCR2 classical: 296 vs 152; CCR2 intermediate: 222 vs 150MFI; p=ns), figure 37. The expression of CX_3CR1 was also preserved (SAH vs HC classical: 519 vs 785 and intermediate: 943 vs 1519; p=ns), figure 10. The expression of the tissue homing chemokine receptor CCR5, however, was raised in intermediate monocytes from SAH patients compared to HC (516 vs 177; p=0.01), figures 37 and 38.

(viii) Summary table of monocyte phenotypic markers in SAH patients

Surface marker	Classical	Intermediate	Non-classical	Function
CD163	←→	←→	←→	Scavenger receptor
CD64	↑	←→	←→	Scavenger receptor
CD206	←→	←→	←→	Scavenger receptor
DCIR	↑	←→	←→	Scavenger receptor
HLADR	↓	↑	↓	Activation marker
CD86	↓	←→	←→	Activation marker
TLR2	←→	←→	←→	Activation marker
TLR4	←→	←→	←→	Activation marker
IFN-γR	←→	↑	←→	Activation marker
MerTK	↓	↓	←→	Inhibitory marker
CCR2	←→	←→	←→	Chemokine receptor
CCR5	←→	↑	←→	Chemokine receptor
CX_3CR_1	←→	←→	↓	Chemokine receptor

Table 10: summary table of phenotypic markers in SAH patients compared to control groups. Overall, there was increased scavenger receptor expression on classical monocytes, but decreased expression of the activation markers HLADR and CD86. However, on the expanded population of intermediate monocytes there was increased expression of HLA-DR, IFN-γR and CCR5. TLR2 and TLR4 expression showed no change from controls

(ix) Effect of 7 days in vivo prednisolone therapy

Interestingly, when patients were treated with prednisolone *in vivo*, the proportion of intermediate monocytes decreased (15% before, 6% after: p=0.02) while there was a trend for classical monocytes to increase (85% to 89%; p=0.07). This phenomenon was not seen when patients were not treated with prednisolone (intermediate: 11% before, 10% after: p=0.8 and classical: 95% vs 90%; p=0.8), figure 39.

7 days treatment with prednisolone also selectively reduced the expression of the activation marker HLA-DR on intermediate monocytes, a phenomenon that was not seen in patients treated without prednisolone (loss of 8376MFI HLA-DR vs. gain of 1836MFI HLA-DR after 7 days therapy; p=0.06). Similarly, prednisolone did not reduce HLA-DR expression on the classical monocyte subset (p=0.5), figure 40.

(oo) Monocyte function

(i) Cytokine production

Monocyte production of pro-inflammatory cytokines in response to LPS [TLR-4 ligand] or *listeria monocytogenes* [TLR-2 ligand] was compared.

In line with previous studies of monocyte function in SAH, PBMCs overall responded to LPS stimulation with increased production of pro-inflammatory cytokines TNFα although this did not achieve statistical significance (HC vs SAH: 1155 vs 4957pgmL^{-1}; p=0.1), IL-6 (1304 vs 4894pgmL^{-1}; p=0.5) and diminished production of the anti-inflammatory cytokine IL-10 (465 vs 112; p<0.05), figure 41 and 42.

Interestingly, although IL-10 secretion in response to LPS was diminished at admission, during the course of inpatient stay levels of IL-10 production increased weekly, so that at the

end 4 weeks, IL-10 levels were approaching that seen in HC cells although these changes did not achieve statistical significance, figure 45.

Next, the monocyte-specific component of cytokine production in response to LPS was tested *ex vivo* using intracellular cytokine stains. This confirmed equivalent monocyte responses to LPS stimulation between SAH and HC in the total CD14$^+$ monocyte population (TNFα 300 vs 281MFI, IL-6 1571 vs 1319MFI, IL-12 703 vs 889MFI, IL1β 1359 vs 832MFI, IFN-γ 357 vs 1079MFI, IL-10 759 vs 608MFI; *p*=ns throughout). However, TNFα output was higher in intermediate when compared to classical monocytes (SAH classical vs SAH intermediate monocytes: 263 vs 419MFI, *p*=0.03), figure 43.

Phosphoflow confirmed that there were higher levels of phosphoNF-κB p65 subunit after 1μg LPS stimulation in intermediate compared to classical SAH monocytes, figure 44.

Results III: Phagocytic function in SAH

(ii) Bacterial uptake by phagocytosis is preserved in SAH

In line with preserved scavenger and Fc-γ receptor expression on monocytes in SAH described in the previous section, assays using the pH-Rodo™ technique confirmed preservation of phagocytic function in SAH. SAH monocytes were able to phagocytose *E. coli* equivalent to HC (classical: 95% vs 96%, intermediate 94% vs 93% and non-classical: 73% vs 73%; *p*=ns), figure 46.

(iii) Monocyte oxidative burst (MOB) is markedly impaired in SAH

Oxidative burst is the process by which microbes that have been phagocytosed are killed by the innate immune cell. More specifically, the NADPH oxidase enzyme complex generates superoxide and ROS that attack and destroy the invading microorganisms. While the pH

Rodo assay is able to assess uptake and internalisation of the microbe during the process of phagocytosis, it is not able to detect oxidative burst activity.

Intracellular killing of internalised bacteria using ROS was quantified by measuring 1,2,3dihydrorhodamine oxidation to fluorogenic rhodamine, figure 47. However, in response to *E. coli,* monocyte oxidative burst (MOB) was markedly impaired in SAH patients compared to CLD and HC in terms of both average ROS production and the proportion of monocytes able to generate oxidative burst to *E. coli* (SAH median (IQR): 879 (580-1832) MFI vs CLD 3688 (1776-4042) MFI vs HC 1521 (2939-1230) MFI; *P*<0.001 and SAH 69% vs HC 76%; *P*=0.02, although the proportion of monocytes generating oxidative burst was no different between SAH and CLD. Interestingly, reduced MOB was also noted in actively drinking cirrhotic patients when compared to abstinent cirrhotic patients (drinking CLD [dCLD] 676 (530-741) MFI vs CLD 3192 (1087-3828)MFI and dCLD 43% vs CLD 66%, *p*=0.003), figure 47.

The impaired ROS generation in SAH patients corresponded to a specific reduction in the production of superoxide (O_2^-) in response to incubation with *E. coli* (SAH vs HC: 202 vs 250 relative light units; *P*=0.02), figure 48. Moreover, impaired MOB resulted in defective intracellular killing of bacteria. Increased numbers of viable *E. coli* were enumerated from lysates of monocytes from SAH patients compared to HC monocytes (9 vs 446 CFU/mL; *p*=0.01). In particular, there were more colonies of *E. coli* enumerated from the lysates of SAH patients with MOB defect compared to the number of colonies enumerated from the supernatant, a phenomenon not seen in HC, supporting the hypothesis of defective intracellular bacterial killing rather than phagocytosis in SAH monocytes (3000 vs 315 CFU/mL; *P*=0.05), figure 48.

Reports of neutrophil oxidative burst have focussed on elevated levels of ROS production in unstimulated SAH neutrophils (Mookerjee et al. 2007). However in monocytes we are able to demonstrate that the unstimulated level of ROS production in circulating monocytes in SAH is similar to HC (175 vs 201 MFI; p=ns), figure 49.

(iv) MOB defect is associated with an increased risk of developing new infection

In view of previous data suggesting that the risk of contracting infection in SAH is dependent on liver function(Louvet et al. 2009), we explored the relationship between liver function, systemic inflammation and MOB in patients with SAH.

Day 0 MOB correlated inversely with white cell count (WCC) (r=-0.5, p=0.001) and CRP (r=-0.4, p=0.01) but there was no correlation with either static or dynamic markers of liver function such as serum bilirubin, MDF, model for end-stage liver disease (MELD), early change in bilirubin level (ECBL) or Lille Model(Louvet et al. 2007), figure 51. In contrast, a strong association between *ex vivo* MOB and the subsequent development of infection within 14 days was detected, figure 50. This association remained statistically significant at 90 days, figure 50.

SAH MOB had a broad interquartile range that overlapped that of HC (580-1832MFI vs 1230-2939MFI), figure 47. Those SAH patients with MOB below the 50th centile were deemed to possess a *MOB defect*, table 11 and figures 52 and 53. Using this 50th centile cut-off to define the presence of a MOB defect, the flow of patients from recruitment to final diagnosis is given in figure 54.

	Sensitivity /%	Specificity /%
<25th centile of HC	48	88
<50th centile of SAH	72	82
<25th centile of SAH	100	59

Table 11: sensitivity and specificity for MOB to predict the subsequent development of infection at 2 weeks at a range of cut-points

Patients with MOB defect were found to have an increased incidence of new infection within the following two weeks (OR 12 (3-55); p=0.0005, sensitivity 72%, specificity 82%, positive predictive value 0.86 (0.64-0.97); likelihood ratio 4; area under receiver operating characteristic curve of 0.86 (0.74-0.98; p<0.0001), figure 55. For comparison, the ROC curves for procalcitonin (PCT), CRP, WCC in predicting the subsequent development of infection are included (AUC 0.72, 0.73 and 0.75, p=0.02, 0.02 and 0.007, respectively), figure 55.

All patients below the 25th MOB centile developed infection within the subsequent two weeks, figure 52. In these SAH patients, deemed to possess a *severe* MOB defect, this higher sensitivity came at a cost of specificity (OR 71 (3.7-1367, p<0.0001); sensitivity 100%, specificity 59%, positive predictive value 78%, negative predictive value 100%, likelihood ratio 2.4), figure 52.

The development of new infection within the first two weeks was associated with an increased risk of death at 28 and 90 days (OR 48 (3-918); p<0.0001 and OR 9 (2-46); p=0.003, respectively). Accordingly, Day 0 MOB defect was also associated with mortality at 28 and 90 days (odds ratio 6.0 and 3.5, one-tailed p=0.044 and 0.041, respectively).

(v) *Prediction of infection by pre-treatment bacterial DNA levels*

We sought to determine whether pre-treatment bacterial DNA levels (bDNA) could predict the subsequent development of infection. We evaluated the level of bDNA in patients recruited to the STOPAH trial compared to the occurrence of a clinician recording a diagnosis of *sepsis* in the Clinical Records File within the first 7 days of admission.

263 cases were available for analysis. Of these, 63/263 (31%) had been newly diagnosed with sepsis within the first week of admission by the treating clinicians. Elevated pre-treatment bDNA levels were associated with an increased risk of developing *sepsis* within the subsequent 7 days (*p*=0.0042). The ability of a pre-treatment whole blood sample containing bDNA to predict the subsequent development of *sepsis* within the first 7 days showed an AUROC 0.70, *p*=0.003, figure 67. It should be noted however, that the definition of sepsis used within the Clinical Records File (CRF) in STOPAH differed from the term *infection* used in this thesis, based on published literature (Bajaj et al. 2012).

(vi) *Combining high bDNA levels with defective MOB at Day 0 to predict the risk of infection*

There was a negative correlation between bDNA levels and MOB (r=-0.13) but this relationship did not reach statistical significance. However, only 16 cases were available for analysis in this cohort.

Nonetheless, contingency analysis of patients who had elevated bDNA levels (above median) or defective MOB (below median) returned an odds ratio for the subsequent development of infection within the first two weeks of 32 (CI 1.4-726), *p*=0.004, sensitivity 67%, specificity 100%, figure 68. It is emphasised however, that the number of cases are small, raising the probability of a type 1 error.

(vii) In vivo prednisolone therapy does not depress MOB

We sought to determine whether the increased rate of infection seen in patients treated with prednisolone in the STOPAH trial could be attributed to an effect of prednisolone on MOB. We therefore measured MOB in sequential samples in SAH patients treated with or without prednisolone.

In vitro treatment with prednisolone did not alter MOB (*E. coli* vs *E. coli*+prednisolone: 564 vs 458MFI; p=0.75). This was confirmed by *in vivo* data showing that seven days prednisolone therapy had no effect on *ex vivo* phagocytosis or MOB (pre- vs post-prednisolone: 86% vs 92% for phagocytosis, p=0.69 and 74% vs 82% for MOB; p=0.16), figure 56. Similarly, patients not treated with prednisolone also demonstrated stable MOB (day 0 vs day 7: 59% vs 58%; p=ns), figure 56.

(viii) Prior use of antibiotics does not affect MOB

Non-absorbable antibiotics have been shown to reduce endotoxaemia in cirrhotic patients with hepatic encephalopathy (Bajaj et al. 2013). In addition, neutrophil phagocytic dysfunction was shown to be mediated by endotoxin (Mookerjee et al. 2007) and treatable with probiotics (Stadlbauer et al. 2008), indicating that bacterial translocation may interfere with the phagocytic function of innate immune cells and may be affected by antibiotic use. However in this study, prior treatment with systemic antibiotics had no impact on MOB in SAH patients (MOB in patients treated with antibiotics vs MOB in patients not treated with antibiotics: 994MFI vs 837MFI, p=0.36), figure 50.

(ix) Infections by catalase positive organisms were more common in SAH patients with MOB defect

Bacteria that are able to use the enzyme catalase to defend against H_2O_2 mediated attack during phagocyte oxidative burst are known as catalase positive organisms. We next sought to understand whether patients with MOB defect are more susceptible to catalase positive organisms compared to patients without MOB defect.

As previously discussed, *E. coli* was the organism grown most frequently by culture, followed by *candida albicans*, and together these two organisms comprised 50% of all positive cultures, figure 24. The majority of organisms grown by culture in SAH patients were catalase positive, figure 24. All of the catalase positive organisms were grown in samples from patients with defective MOB; in contrast, just 4 of the 8 (50%) samples that grew catalase negative organisms came from patients with defective MOB, figure 57. Conversely, none of the SAH patients with MOB >50th centile were infected by catalase positive organisms, figure 57. The odds ratio of MOB defect predicting subsequent infection with a catalase positive organism was 33 (*P*=0.007).

(pp) Mechanism of MOB defect

(i) Nicotinamide adenine dinucleotide phosphate (NADPH) substrate provision

We sought to determine the mechanism of impaired MOB in SAH patients. First, we focussed on the substrate of the key enzyme NADPH oxidase. NADPH is required by NADPH oxidase to generate superoxide and effect bacterial killing. However, serum NADPH levels were higher in SAH patients than HC, and levels were higher still in patients with a MOB defect compared to patients without, figure 59. Despite these serum levels suggesting plentiful supply, it has been reported that serum and intracellular levels of NADPH are unable, in themselves, to support phagocyte oxidative burst during phagocytosis of bacteria.

During MOB, the major source of intracellular NADPH is from the pentose phosphate pathway, and more specifically the G6PDH enzyme.

G6PDH maintains the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is important in maintaining the level of glutathione in cells, and is known for protecting red blood cells against oxidative damage. We next tested whether G6PDH dysfunction, resulting in inadequate generation of NADPH substrate, could be the cause of defective MOB. However, the capacity of intracellular G6PDH in generating NADPH was equivalent between HC and SAH patients, indicating that substrate availability for the NADPH oxidase enzyme is adequate in monocytes with defective MOB (17 vs 16nmol/min/mL; P=0.6), figure 59.

(ii) NADPH oxidase enzyme complex expression

gp-91^{phox} is the major subunit of the NADPH oxidase complex. Intracellular monocyte expression of gp-91^{phox} in SAH patients was therefore evaluated. The level of gp91^{phox} in the monocytes of SAH patients with MOB defect (SAH+MOB) was significantly reduced compared to SAH patients without MOB defect (SAH-MOB) by Western blotting (gp91^{phox}:GAPDH of SAH+MOB 0.35 arbitrary units vs SAH-MOB 0.53 arbitrary units; P=.04), figures 60A and 61. RT-PCR confirmed impaired gene expression of gp91^{phox} after IFN-γ stimulation in the monocytes of patients with MOB defect (gp91^{phox}:β-actin gene expression of SAH+MOB 0.58 arbitrary units vs SAH-MOB 2.00 arbitrary units; P=.03), figure 60B.

(iii) In vitro IFN-γ does not restore MOB in SAH

IFN-γ is able to potentiate oxidative burst on healthy monocytes. IFN-γ binding to IFN-γ receptor (IFN-γR) triggers a Janus Activated Kinase (JAK) signalling cascade that results in phosphorylation of Signal Transducer and Activator of Transcription (STAT)-1 (Schroder et

al. 2004) and activation of the NADPH oxidase complex. This cascade is negatively regulated by Suppressor of Cytokine Signalling (SOCS)-1 (Norkina et al. 2008). Accordingly, IFN-γ gene knockout renders mice susceptible to intracellular infections (Schroder et al. 2004). In addition, patients suffering from chronic granulomatous disease, in which loss-of-function-mutations in gp91^{phox} may be inherited, gain effective prophylaxis from opportunistic infection by treatment with subcutaneous IFN-γ (Mandel JS, Bond 1993). We therefore evaluated whether exogenous IFN-γ administration could restore defective *in vitro* MOB in patients with SAH.

Serum levels of IFN-γ were similar between SAH and HC (0.8 vs 2.4pg/mL, *p*=0.06), figure 25. Expression of IFN-γR1 was raised in SAH compared to HC (1049 vs 928MFI; *p*=0.05), figure 31. However, gene expression of STAT-1 in response to IFN-γ was diminished in all SAH patients with or without MOB (STAT-1:GAPDH gene expression of SAH 0.55 vs HC 0.97; *p*=0.05) and activation of STAT-1 with or without IFN-γ stimulation was similarly reduced in all SAH patients (unstimulated phosphoSTAT-1:GAPDH of SAH 0.23 vs HC 0.97; *p*<0.01, and IFN-γ-stimulated phosphoSTAT-1:GAPDH of SAH 0.96 vs HC 2.47; *p*=0.04), figure 60C, 61 and 60D.

Previously, *in vitro* studies from healthy volunteers had shown that high levels of Suppressor of Cytokine Signalling (SOCS)-1 protein can be induced within monocytes in response to alcohol drinking, potentially rendering monocytes refractory to IFN-γ stimulation(Norkina et al. 2008). Indeed in the current study, Western blotting demonstrated increased expression of this negative intracellular regulator of STAT-1 signalling(Schroder et al. 2004) in SAH monocytes (SOCS-1:GAPDH of SAH 0.38 vs 0.25; *p*=0.02), figures 60E and 61. It should be noted that increased SOCS-1 and impaired activation of STAT-1 was demonstrated in monocytes from all SAH patients, regardless of whether there was a MOB defect or not,

figure 60E. Consistent with these findings, SAH monocytes were resistant to 24 hours stimulation with IFN-γ *in vitro* (*E. coli* vs *E. coli* + IFN-γ: MOB 438 vs 512MFI; *p*=0.3), figure 60F.

(qq) N-acetylcysteine restores MOB

In vitro data demonstrates that NAC can enhance superoxide production in response to microbial cues within the phagocytes of patients with chronic renal failure(Witko-Sarsat et al. 2003). In line with this finding, clinical *in vivo* data demonstrates the utility of NAC in reducing the incidence of infections in SAH(Nguyen-Khac et al. 2011).

We therefore proceeded to test the utility of *in vitro* NAC in restoring defective MOB. 24 hours incubation with 250μg/mL NAC *in vitro* was able to restore MOB (*E. coli* vs *E coli* + NAC: 437 vs 672MFI; *p*=0.01), figure 62. NAC was able to improve mean MOB response by 54%. Accordingly, superoxide generation was increased by NAC treatment (193 to 231 relative light units), figure 62, and NAC-treated monocytes enumerated fewer viable *E coli* colonies when compared to lysates from SAH monocytes not treated by NAC (80 vs 520 *E coli* CFU), figure 63.

(i) Mechanism of the effect of NAC on MOB

Next we sought to investigate the mechanism of action of NAC in improving MOB. Numerous reports have attributed anti-inflammatory properties to NAC. Treatment during acute liver failure reduces death and the need for transplantation (Harrison et al. 1990). Certainly, levels of glutathione, an anti-oxidant that is restored by NAC, were depleted in the serum of SAH patients with MOB defect compared to controls, figure 64.

Indeed, when isolated monocytes were incubated with *E. coli* for 40 minutes and hydrogen peroxide levels were measured with and without NAC, a significant reduction in hydrogen peroxide levels was observed in NAC treated monocytes, figure 65. Paradoxically however, the generation of superoxide from monocytes incubated with *E. coli* had improved after NAC pre-treatment, figure 65.

We next tested the ability of monocytes to generate superoxide in different concentrations of hydrogen peroxide. Healthy monocytes showed a dose dependent decrease in superoxide production to E. coli according to the concentration of hydrogen peroxide that they were incubated in, figure 66. However, this relationship was not seen in SAH monocytes, with or without a MOB defect, figures 66A. 7-AAD staining quantified cell death caused by hydrogen peroxide and was shown not to be greater than 10% in this experiment, figure 66B. Finally, we compared hydrogen peroxide production between HC and SAH monocytes with MOB defect. SAH monocytes were shown to produce higher levels of hydrogen peroxide than HC monocytes, and NAC pre-treatment was show to reduce these levels of hydrogen peroxide, figure 66.

Data figures

A

Death within 28 days

B

Day 7 Lille score

C

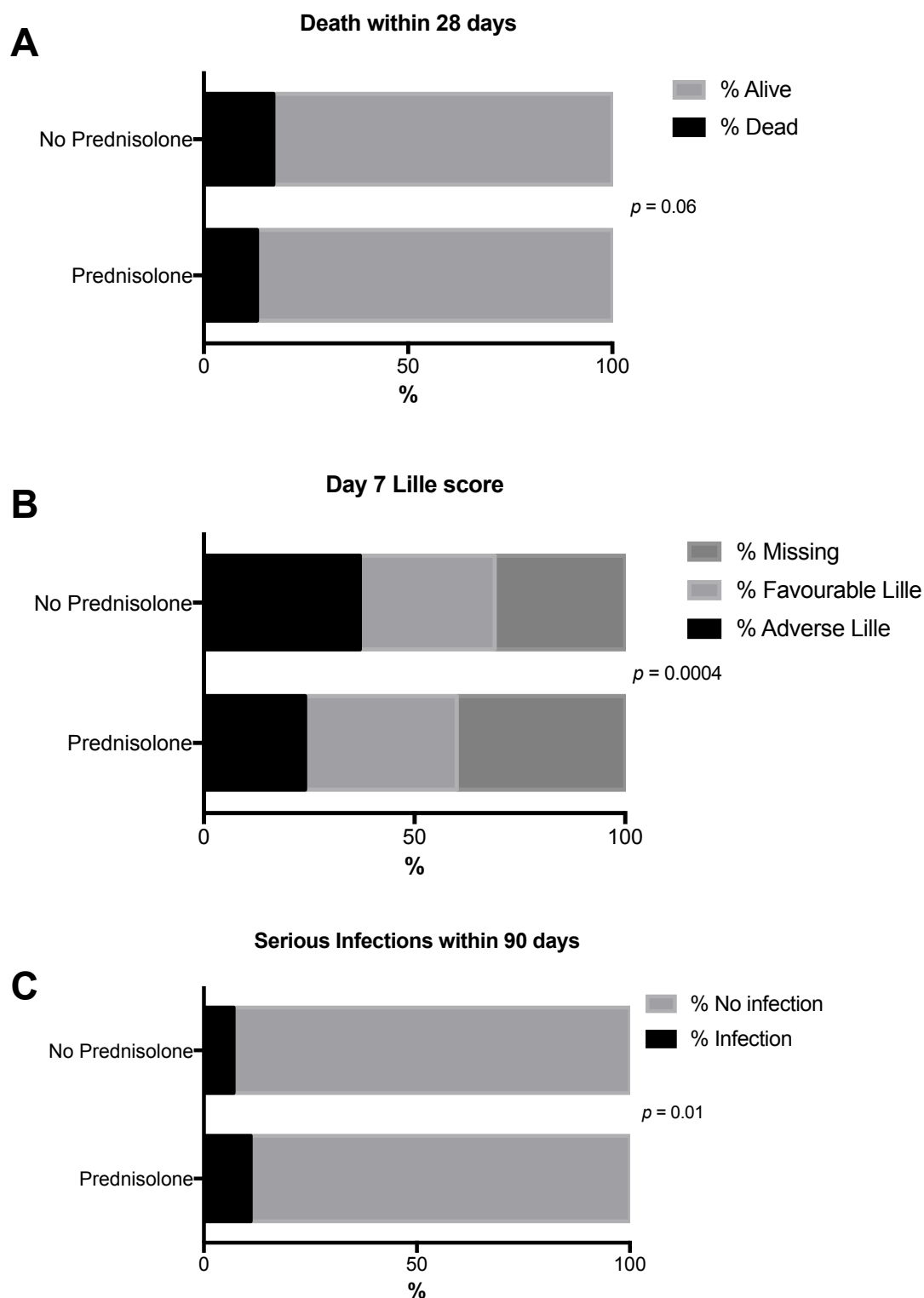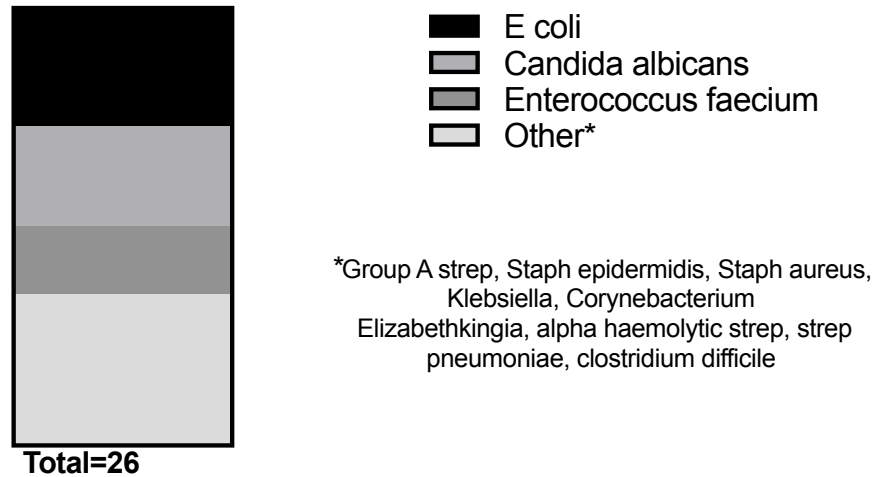### Serious Infections within 90 days

Figure 23: proportions of patients with adverse Lille score who received prednisolone therapy; 133/328 (40%) patients who received prednisolone had an adverse (>0.45) Lille score vs 202/374 (54%) patients who did not receive prednisolone. There was insufficient data to calculate Lille score in 390/1092 (36%) cases: 40% in prednisolone treated patients *vs.* 31% in patients not treated with prednisolone. Data drawn from the STOPAH study (n=1092). Comparison of A. death in prednisolone treated and prednisolone not treated patients in the STOPAH study

73/474 (13%) vs 95/450 (17%); B. serious infections in patients treated with and without prednisolone 59/488 (11%) vs 36/509 (7%); C. causes of death due to hepatic failure in patients treated with and without prednisolone 40/507 (7%) vs 47/498 (9%)
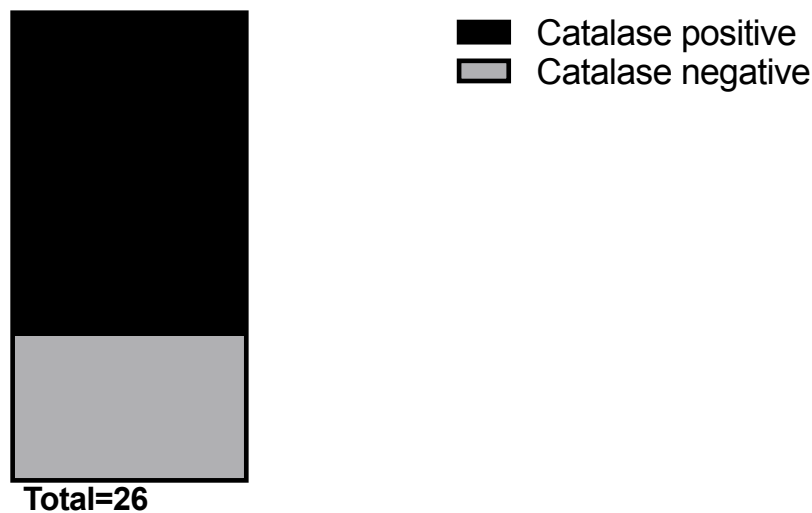
A

■ E coli
▥ Candida albicans
▨ Enterococcus faecium
▢ Other*

*Group A strep, Staph epidermidis, Staph aureus, Klebsiella, Corynebacterium Elizabethkingia, alpha haemolytic strep, strep pneumoniae, clostridium difficile

Total=26

B

■ Catalase positive
▨ Catalase negative

Total=26

Figure 24: A. diversity of bacteria grown from samples from patients participating in the monocyte substudy (*E. coli n*=7; *Candida albicans n*=6; *Enterococcus faecium n*=4; other *n*=9); B. proportion of catalase positive organisms grown from patients participating in the monocyte study (18 catalase positive vs 8 catalase negative)
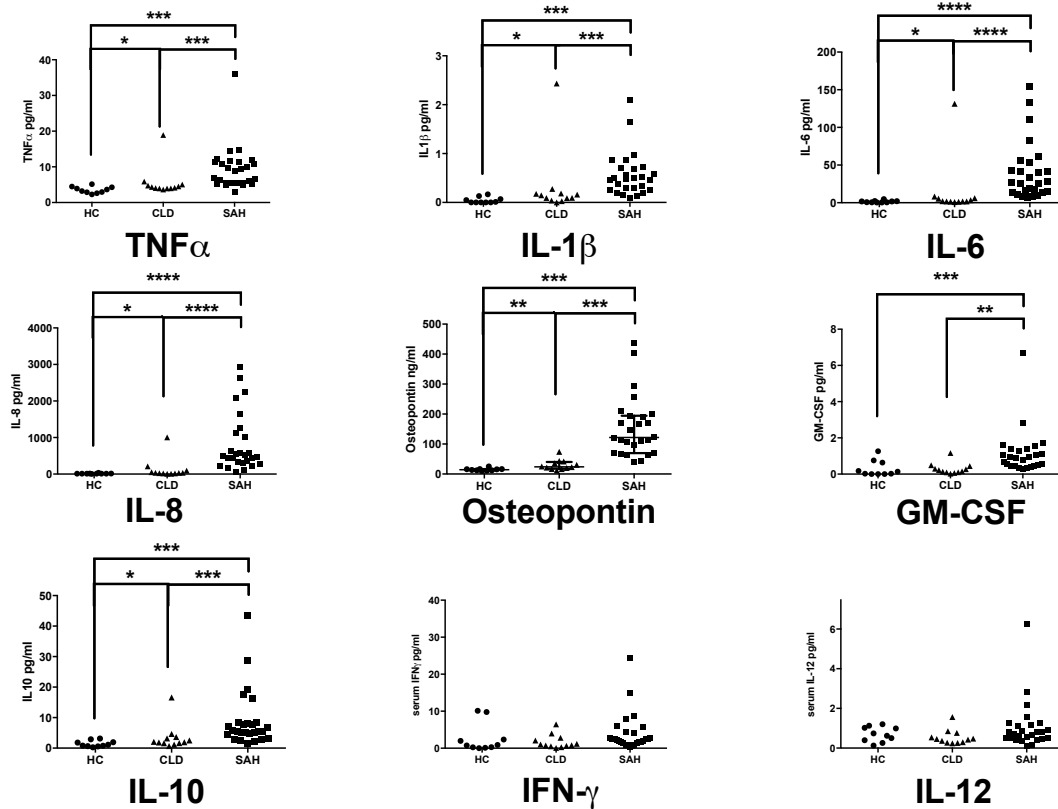
Figure 25: comparison of serum cytokine levels in SAH patients and control groups. Pro-inflammatory cytokines are TNF-α, IL-1β, IL-6 and IL-8. Osteopontin is a chemokine and cell survival factor and Granulocyte macrophage colony stimulating factor (GM-CSF) is a white blood cell growth factor that is part of the inflammatory cascade. IFN-γ and IL-12 are two key cytokines involved in signalling between monocytes and NK cells/T cells to drive monocyte oxidative burst, amongst other pro-inflammatory functions. SAH *n*=20, CLD *n*=10; HC *n*=10. * denotes *p*<0.05; ** denotes *p*<0.01; *** denotes *p*<0.001; **** denotes *p*<0.0001
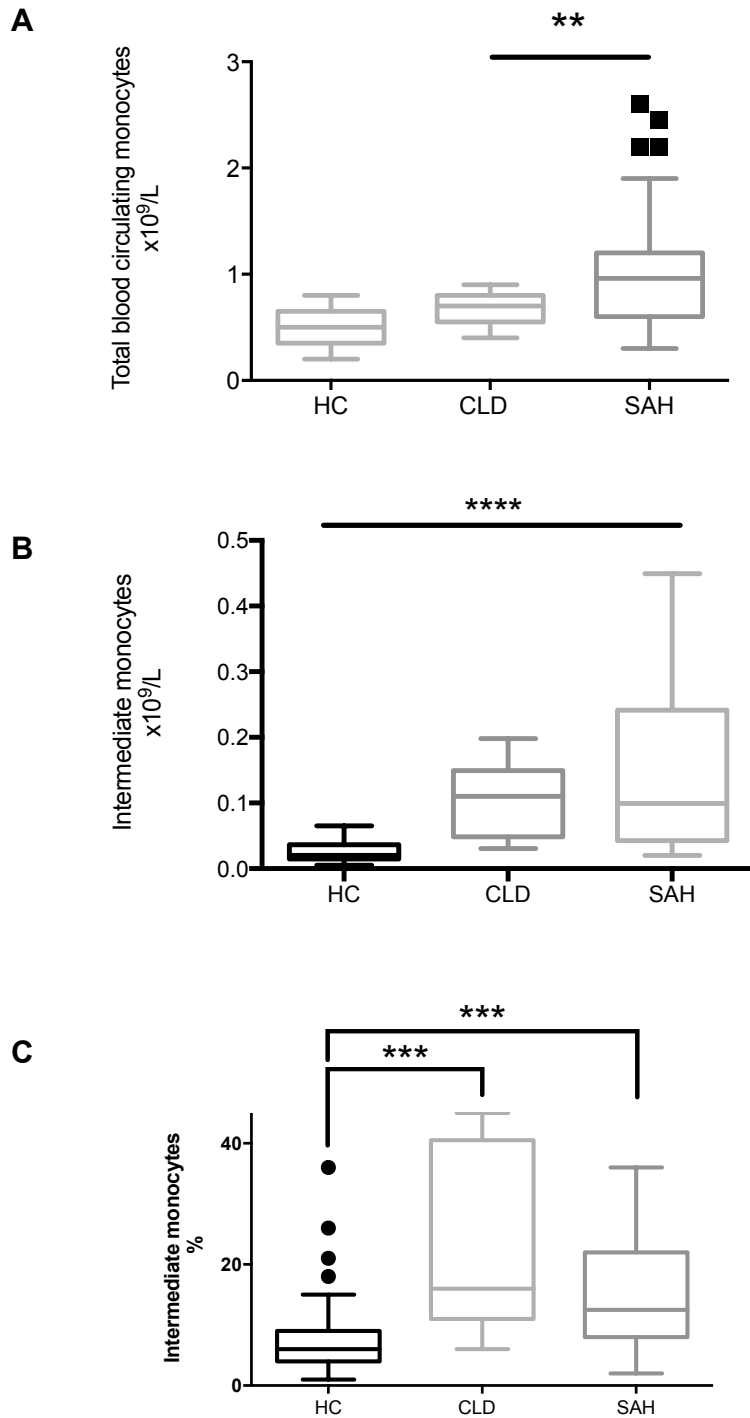
Figure 26: A. comparison of circulating monocyte frequency in whole peripheral blood from SAH (0.96x10^9/L, n=47), CLD (0.7x10^9/L, n=17) and HC (0.5x10^9/L, n=4) subjects; SAH vs CLD p=0.0031. B. comparison of intermediate monocyte subset frequency from SAH patients and control groups. The intermediate monocyte population is expanded in SAH monocytes (SAH n=28, HC n=18, CLD n=9, SAH vs HC p<0.0001). C. SAH and CLD patients have a higher proportion of intermediate monocytes (SAH n=28, HC n=18, CLD n=9, SAH vs HC p=0.001). Statistical tests by Kruskal Wallis and Mann Whitney U.
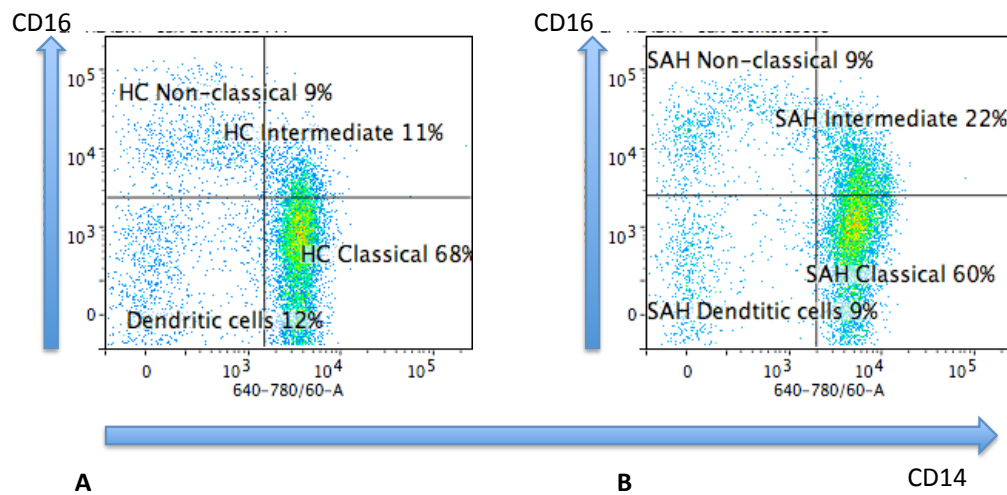
Figure 27: Representative FACS plots of the frequency distribution of classical, intermediate, non-classical monocytes and dendritic cells from whole blood. A. subset distribution in HC (68% classical, 11% intermediate, 9% non-classical) compared to B. showing an increased proportion of intermediate monocytes (11% vs 22%) and a decreased proportion of classical monocytes (68% vs 60%) in SAH patients compared to HC. Lineage negative (Lin[neg]) gating strategy
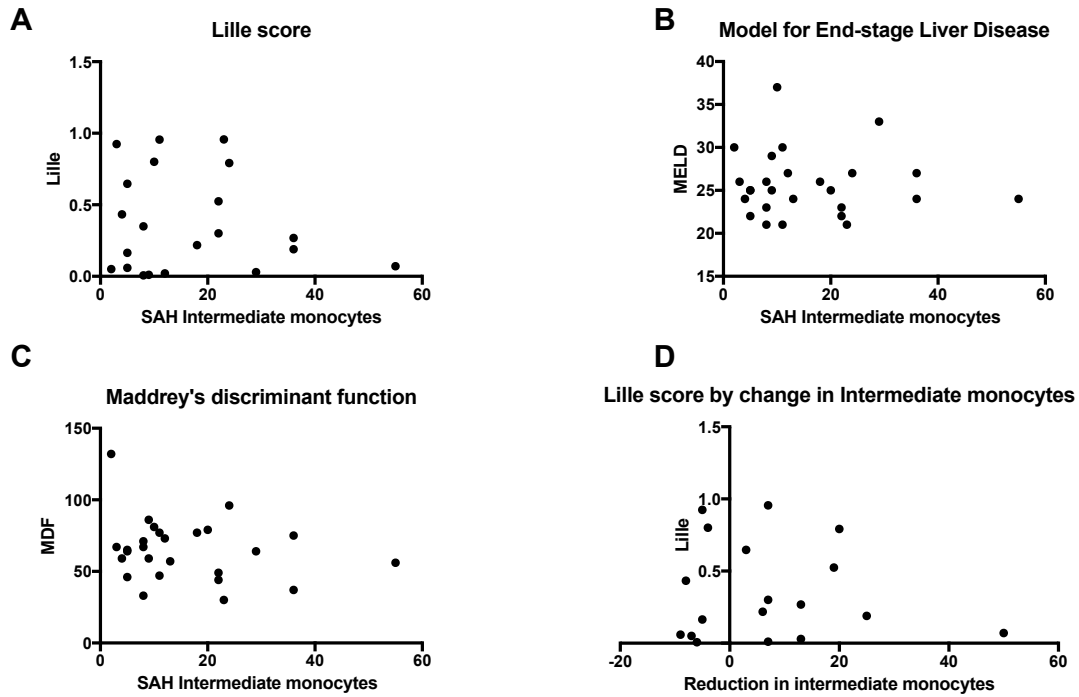
Figure 28: Scatter plots of the relationships between A. Lille score, B. MELD, C. Maddrey's discriminant function, and D. the change in frequency of intermediate monocytes from whole blood between day 0 and day 7 and the Lille score. *n*=21 and *p*>0.05 throughout by Spearman's rank correlation coefficient test
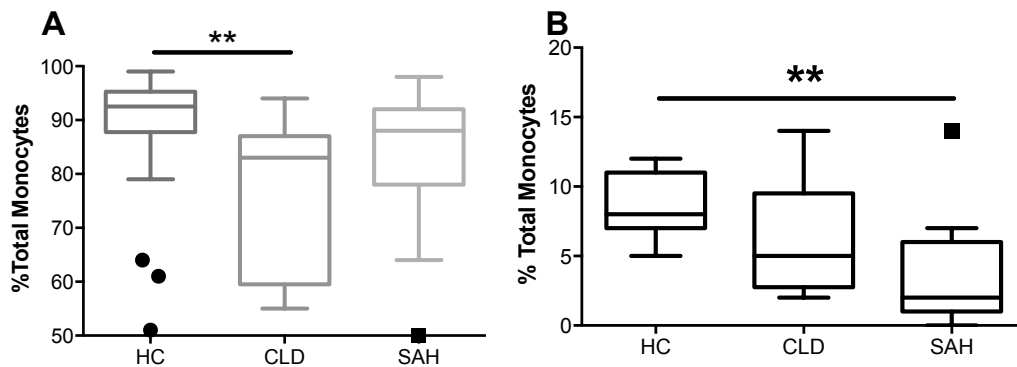

Figure 29: Comparison of the proportions of A. classical and B. non-classical circulating monocytes in SAH patients vs controls from whole blood (*n* SAH=19, CLD=16, HC=17; classical CLD vs HC *p*= 0.001; non-classical SAH vs HC *p*=0.002, CLD vs HC *p*=0.15; SAH vs CLD *p*=0.08, by Kruskal Wallis and Mann Whitney U tests. Lin[neg] gating strategy
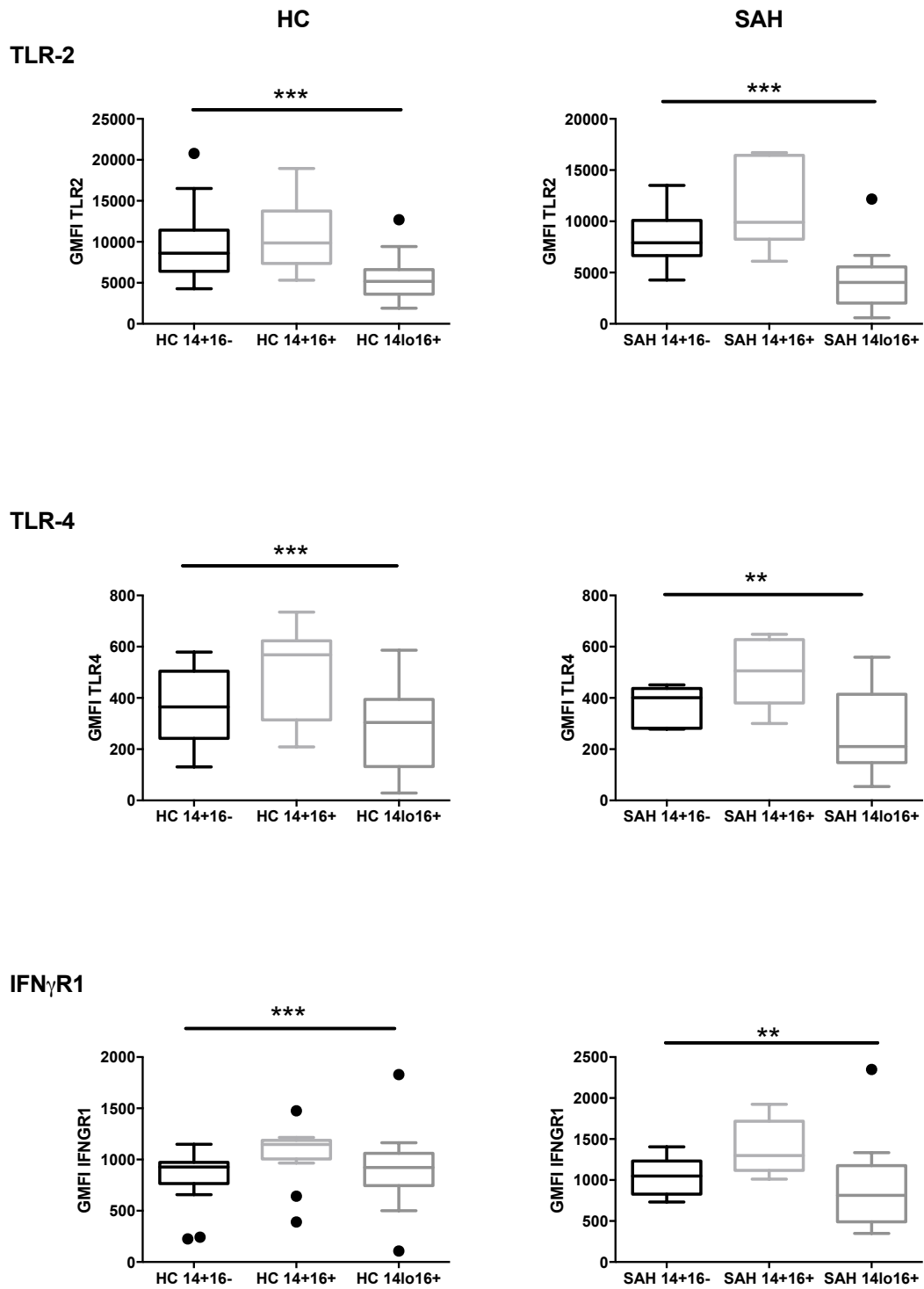
Figure 30: Profile of pattern recognition receptor expression between monocyte subsets within SAH and HC groups from whole blood; *n* **HC=14, SAH=10.** *p*<0.01 **throughout by Kruskal-Wallis test. Lin[neg] gating strategy**
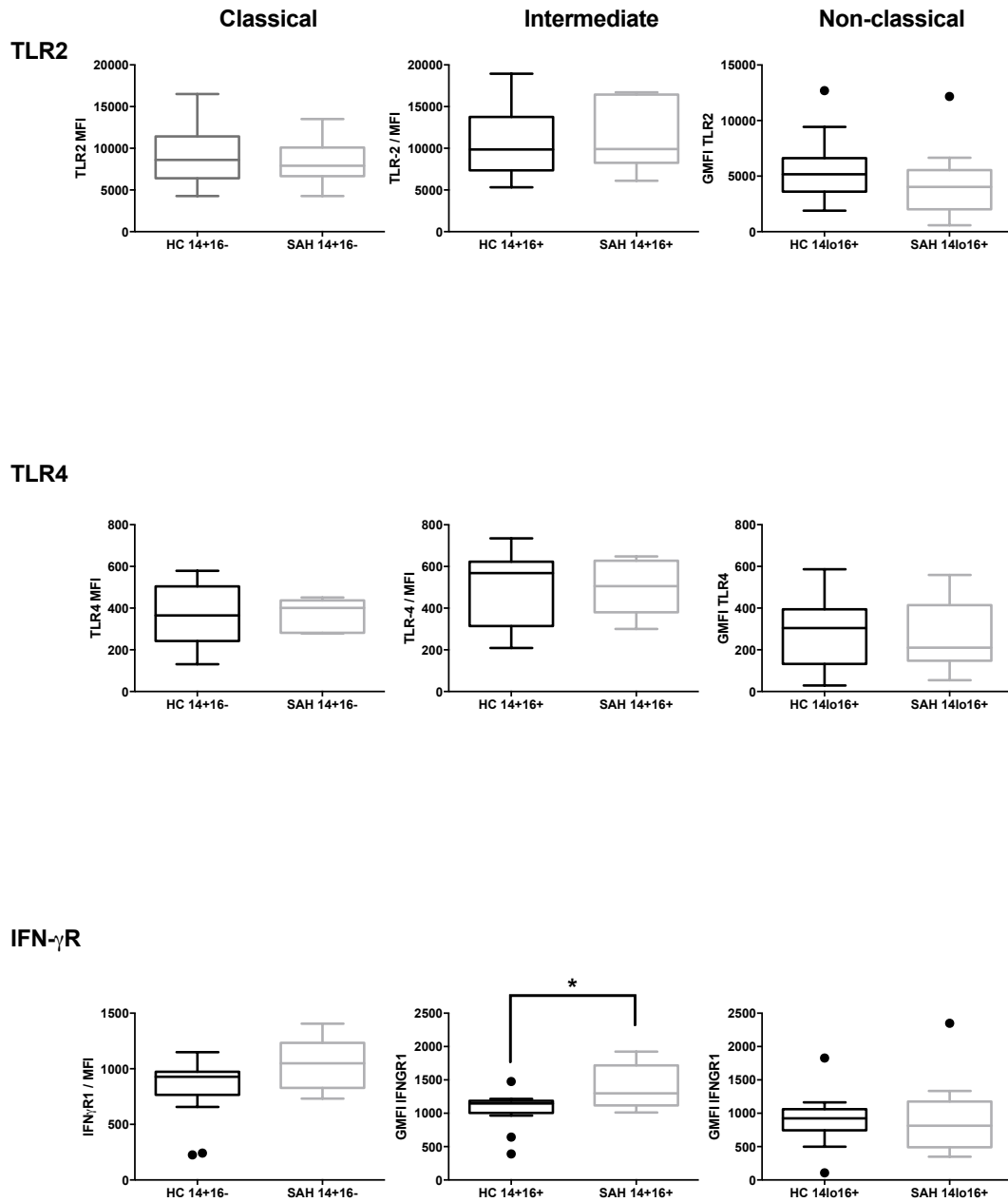
Figure 31: Comparison of expression of pattern recognition receptors between SAH and HC subjects (*n*=10 vs 14) from whole blood. Only IFN-γR1 showed significantly increased expression in SAH intermediate monocytes compared to HC (*p*=0.05). Lin[neg] gating strategy
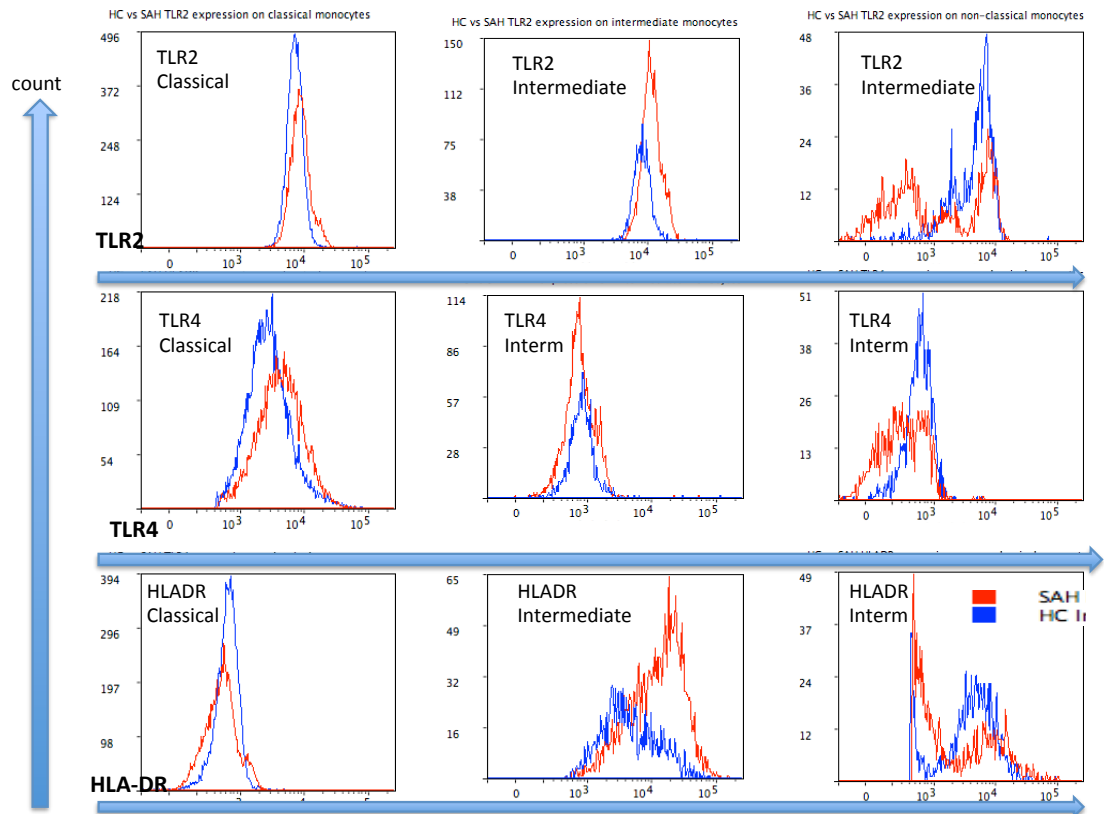
Figure 32: original representative FACS plots showing the relative expression of activation markers TLR-2, TLR-4 and HLA-DR on classical, intermediate and non-classical monocytes in SAH patients [red lines] vs HC controls [blue lines] from whole blood. As shown, there was no difference in expression of activation markers TLR-2 and TLR-4. HLA-DR showed increased expression on intermediate monocytes. Lin[neg] gating strategy
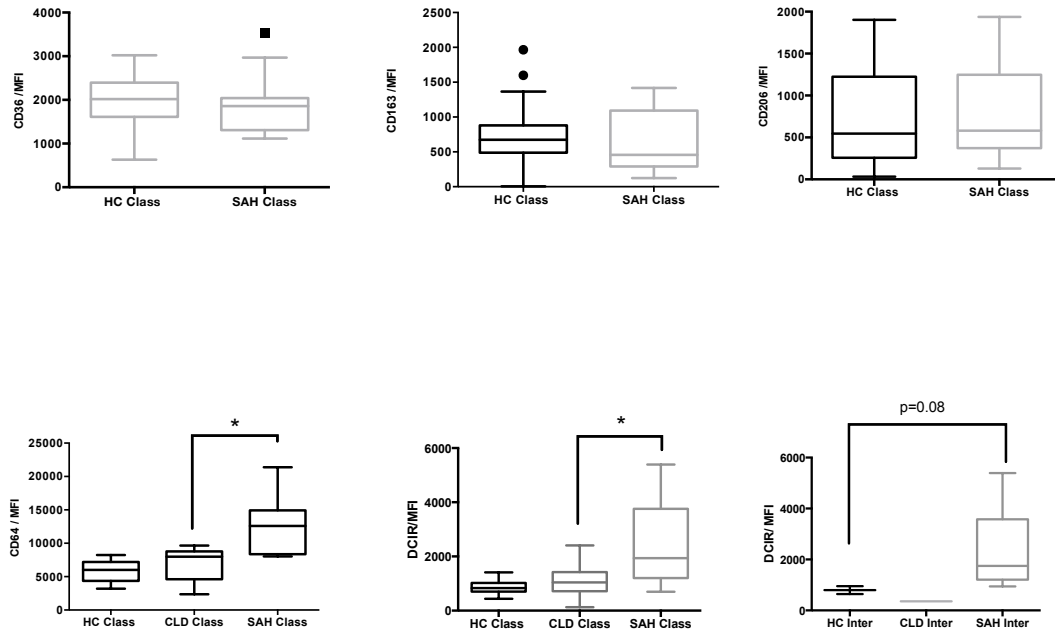
Figure 33: comparison of scavenger receptor expression in SAH vs CLD vs HC subjects (n=22 vs 7 vs 31) from whole blood. CD64 expression SAH vs CLD *p*=0.01, DCIR expression *p*<0.02 by Kruskal Wallis test. Lin^{neg} gating strategy
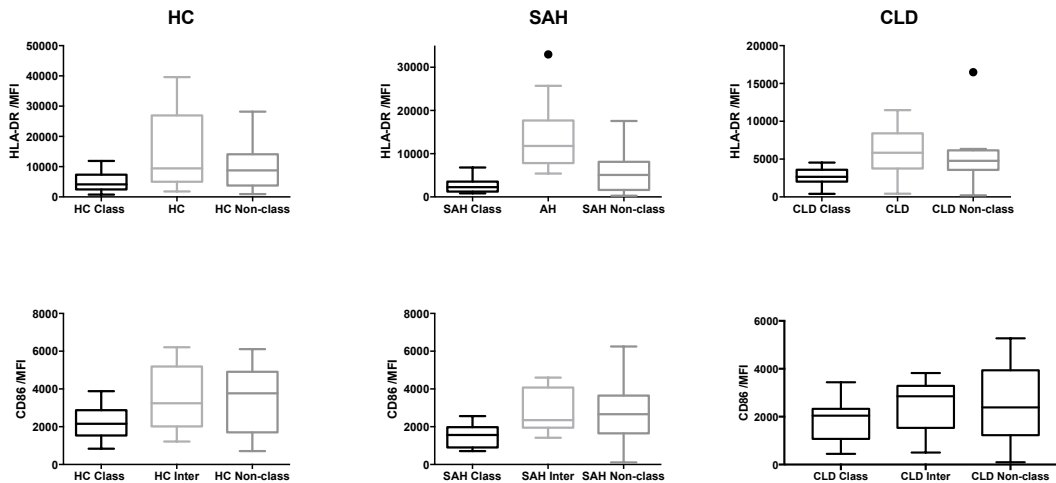
Figure 34: Comparison of expression of activation markers HLA-DR and CD86 in HC vs SAH vs CLD patients (*n*=33, 19, 7) showing the profile of expression between subsets from whole blood. Lin^{neg} gating strategy
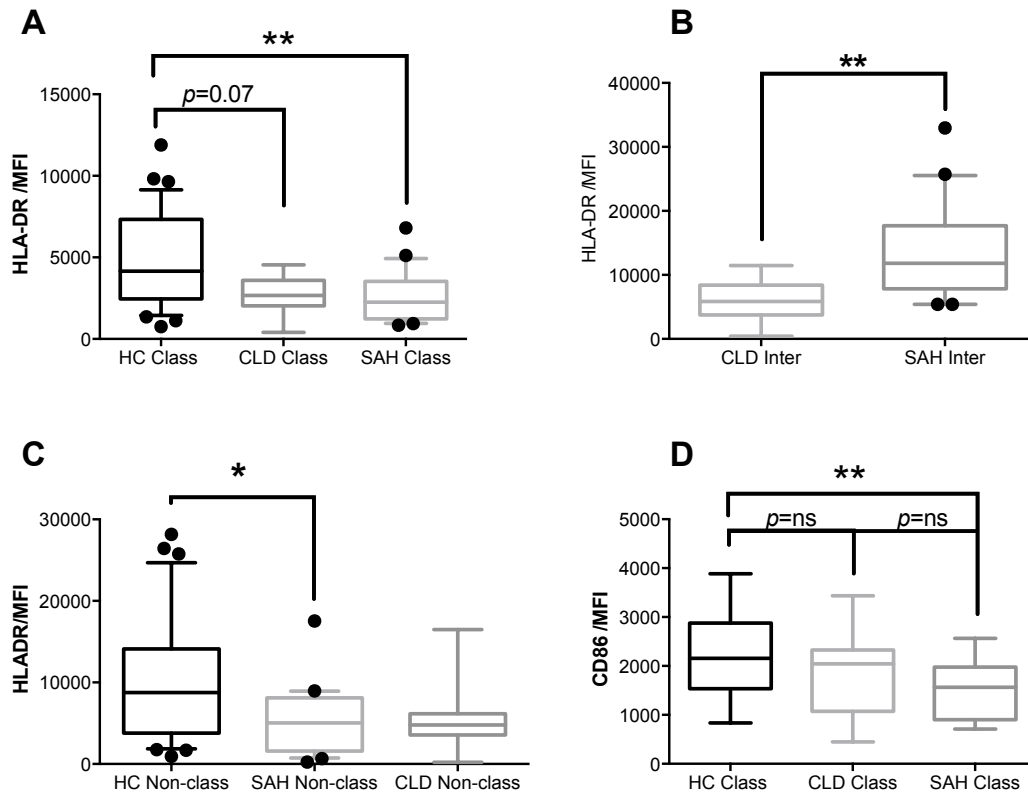
Figure 35: Expression of activation markers HLA-DR and CD86 in SAH vs CLD vs HC subjects (*n*=23, 34, 8) in whole blood. A. HLA-DR expression is reduced in SAH and CLD patients compared to HC, *p*<0.01 by Kruskal Wallis test. B. There was markedly increased expression of HLA-DR on intermediate monocytes compared to CLD and HC, *p*<0.01 by Mann Whitney U test. C. On non-classical monocytes, HLADR expression was reduced in SAH and CLD subjects (p=0.01) while D. CD86 expression was also reduced on SAH classical monocytes compared to HC, p<0.01 by Kruskal Wallis. Lin[neg] gating strategy
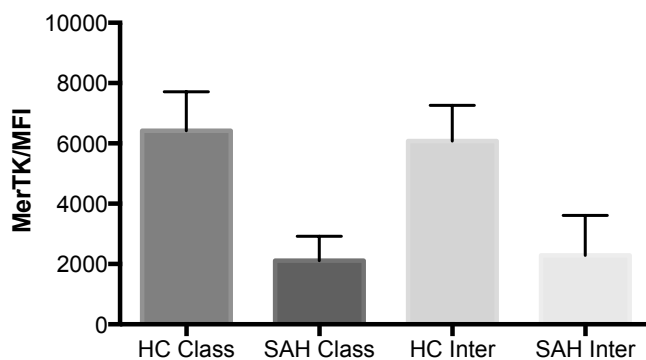
Figure 36: Comparison of MERTK expression in classical and intermediate monocytes from SAH (*n*=6) and HC (*n*=2) subjects, *p*=0.07) on PBMCs. Lin[neg] gating strategy

HC

SAH
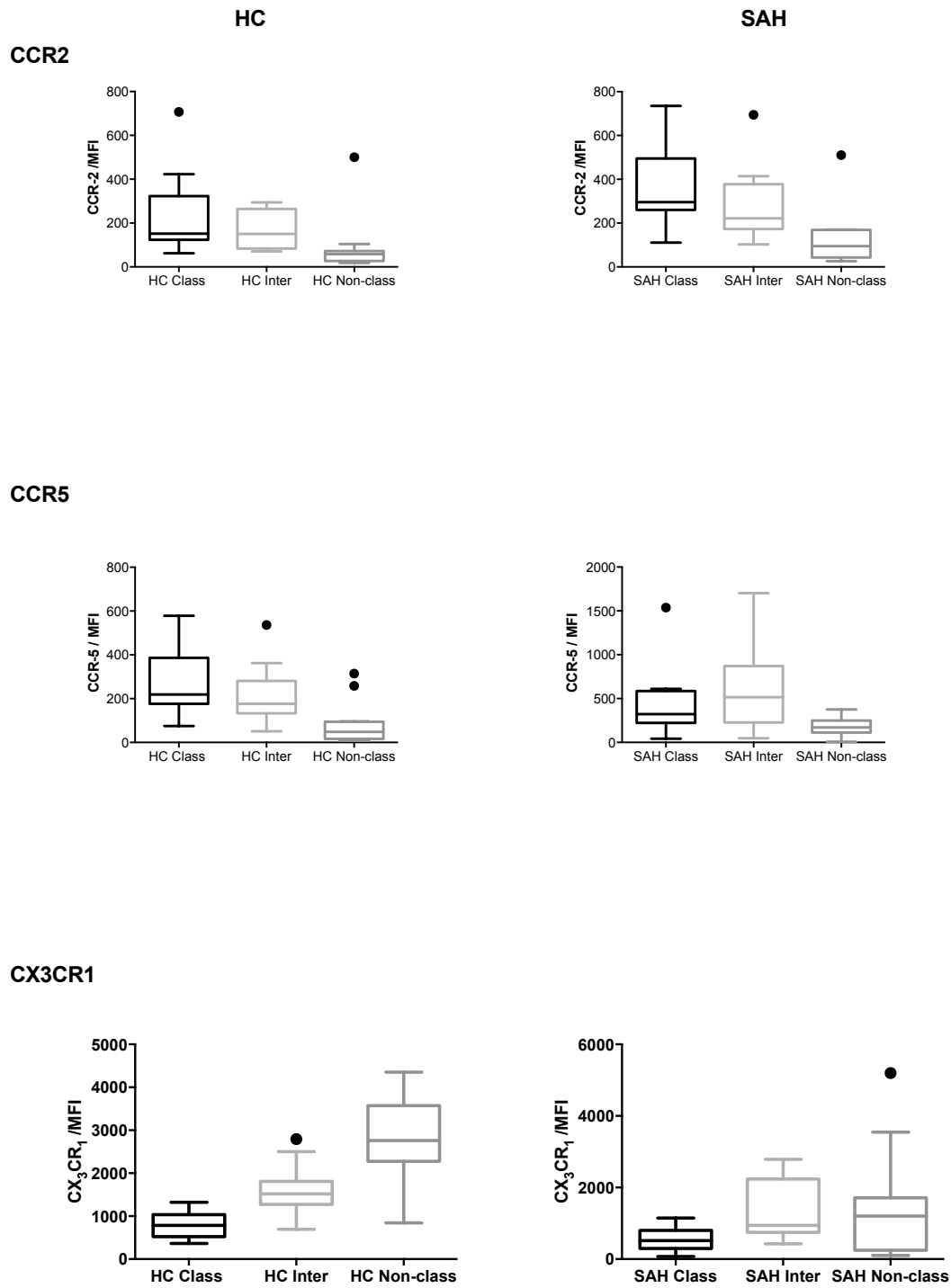
CCR2

CCR5

CX3CR1

Figure 37: Comparison of chemokine receptor profile for HC and SAH subjects (n=14, 10) for CCR-2, the receptor for monocyte chemotactic protein-1 (MCP-1) and CCR5 and CX_3CR_1, the fractalkine receptor and negative regulator of monocyte infiltration on whole blood. Lin^{neg} gating strategy
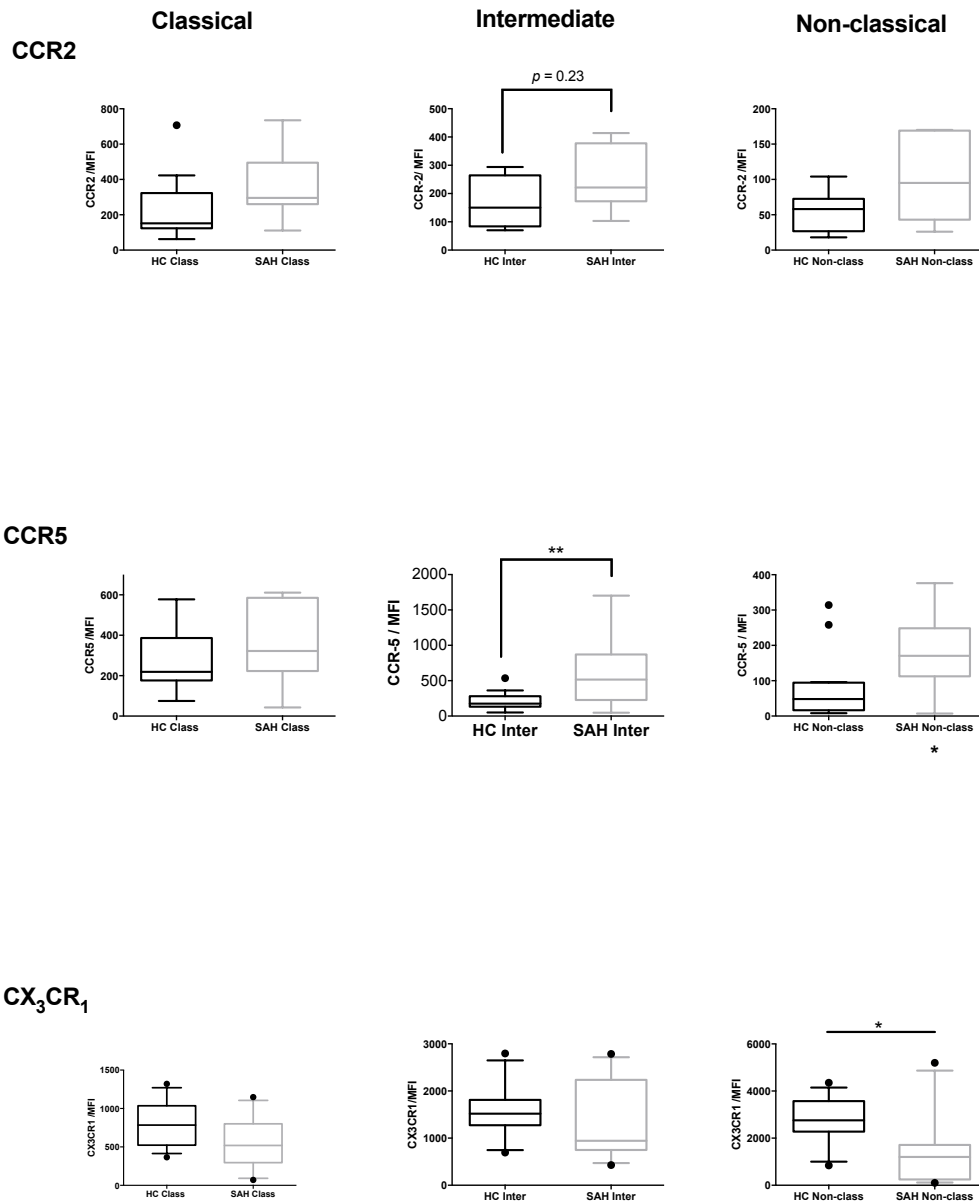
Figure 38: Comparison of the expression of chemokine receptors on classical, intermediate and non-classical monocytes in HC and SAH subjects (*n*=14, 10) on whole blood. An increased expression of CCR-5 was seen in intermediate SAH monocytes (*p*=0.01) by Mann Whitney U test. Lin^{neg} gating strategy
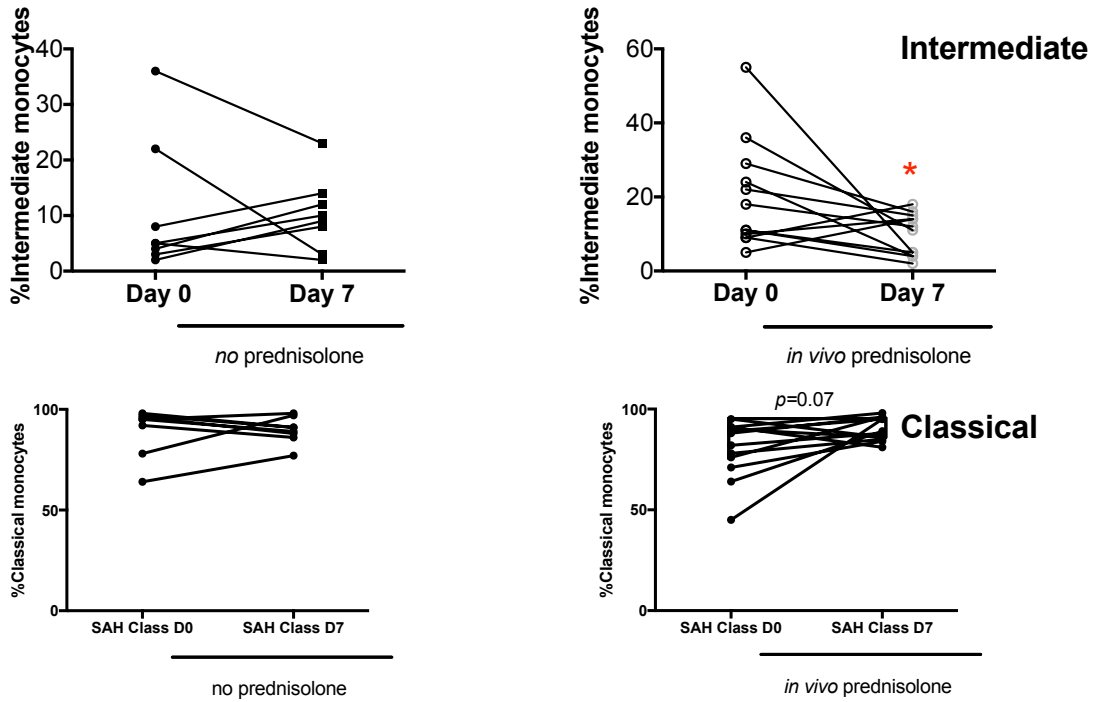
Figure 39: Comparison of the frequency of intermediate and classical monocytes before and 7 days after prednisolone therapy. Patients treated with prednisolone by random allocation had a reduction in the proportion of intermediate circulating monocytes (*p*=0.01) that was not seen in patients not treated with prednisolone (*p*=0.44 by Wilcoxon matched pairs signed rank test). Lin^neg gating strategy
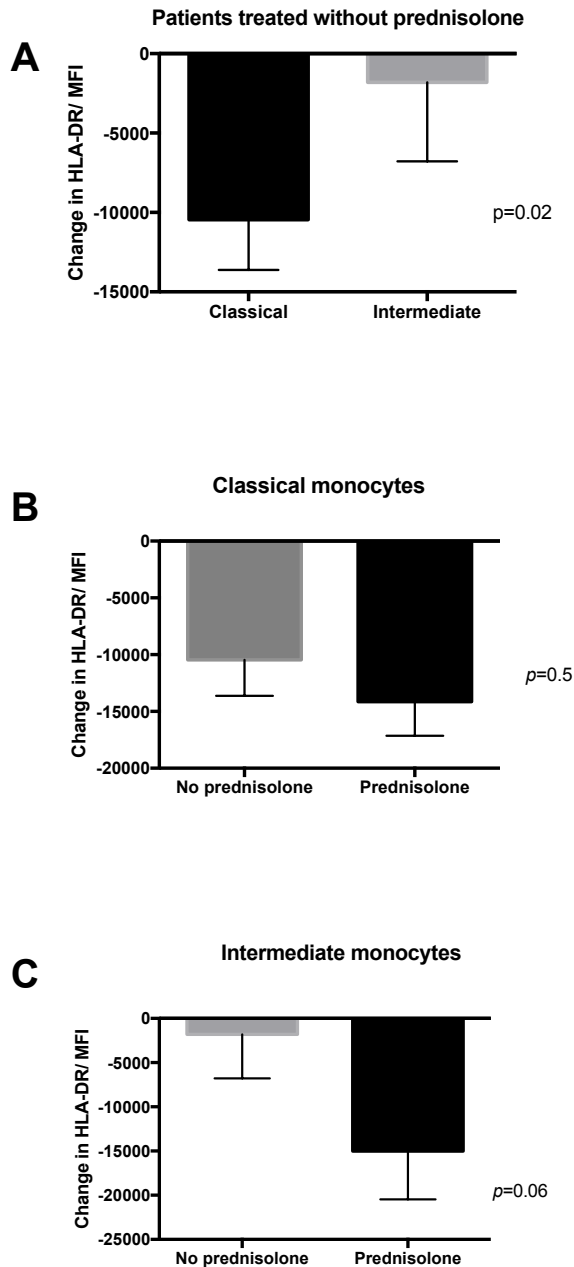
Figure 40: profile of the changes in expression of HLA-DR in the first 7 days, with and without prednisolone therapy. A. There is a fall in HLA-DR expression on classical monocytes in patients who were not treated with prednisolone during the natural history of SAH (vs intermediate monocyte HLA-DR expression, p=0.02). **B.** This fall in HLADR expression on classical monocytes was not significantly affected by prednisolone therapy, *p*=0.5. **C.** In contrast, expression of HLA-DR on intermediate monocytes did not decrease unless the patients were treated with prednisolone, *p*=0.06 by Mann Whitney U test. Lin[neg] gating strategy

TLR2 stimulation   **TLR4 stimulation**

TNFα

IL-6
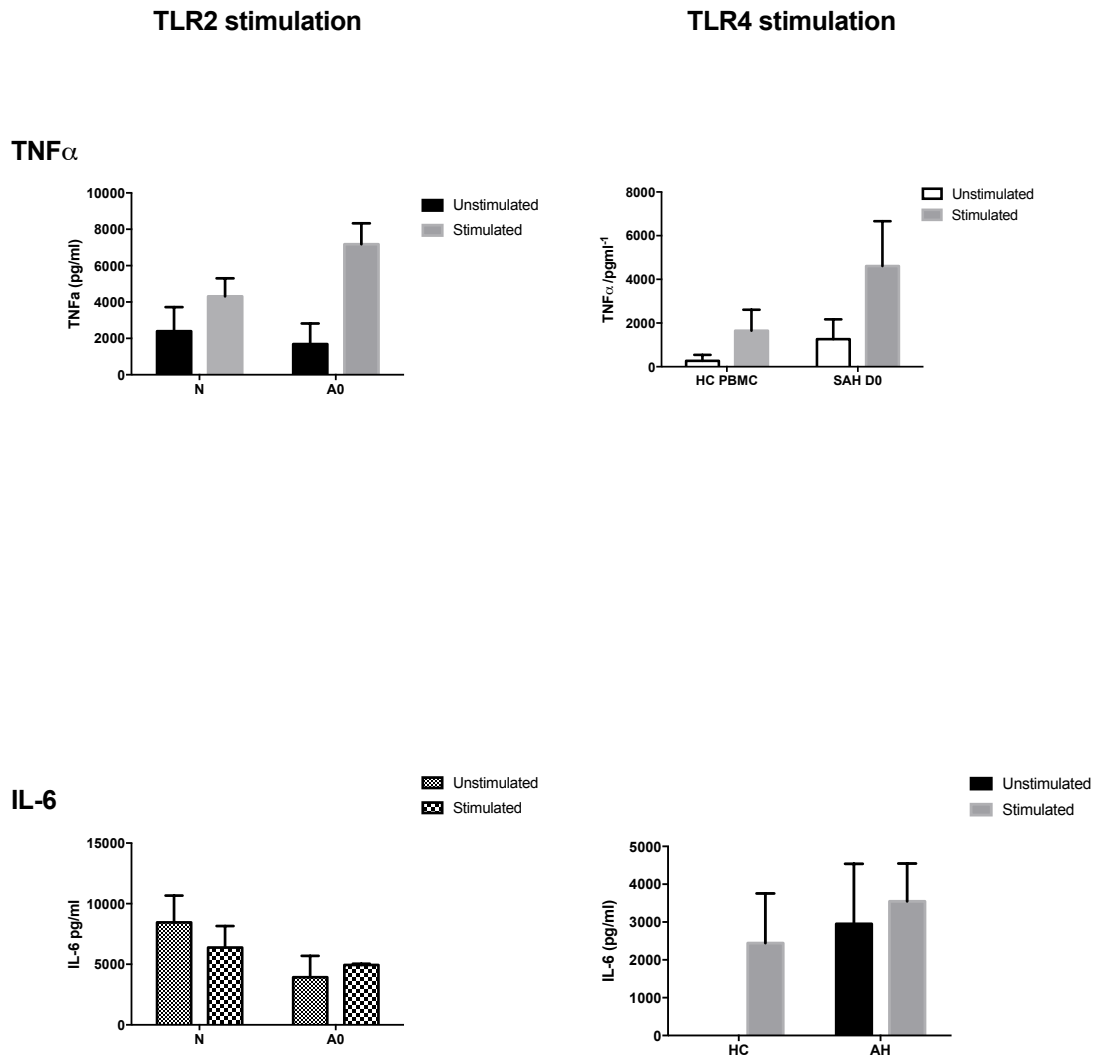
Figure 41: PBMCs stimulated with either *listeria monocytogenes* (TLR-2 stimulation) or 100ng/mL LPS (TLR-4 stimulation). Panels on left represent TLR2 stimulation while panels on the right represent TLR4 stimulation. Panels represent TNFα and IL-6 secretion respectively. *n*=4 in groups HC and SAH. Statistical comparisons made by Wilcoxon signed rank test. HLA-DR positive gating strategy
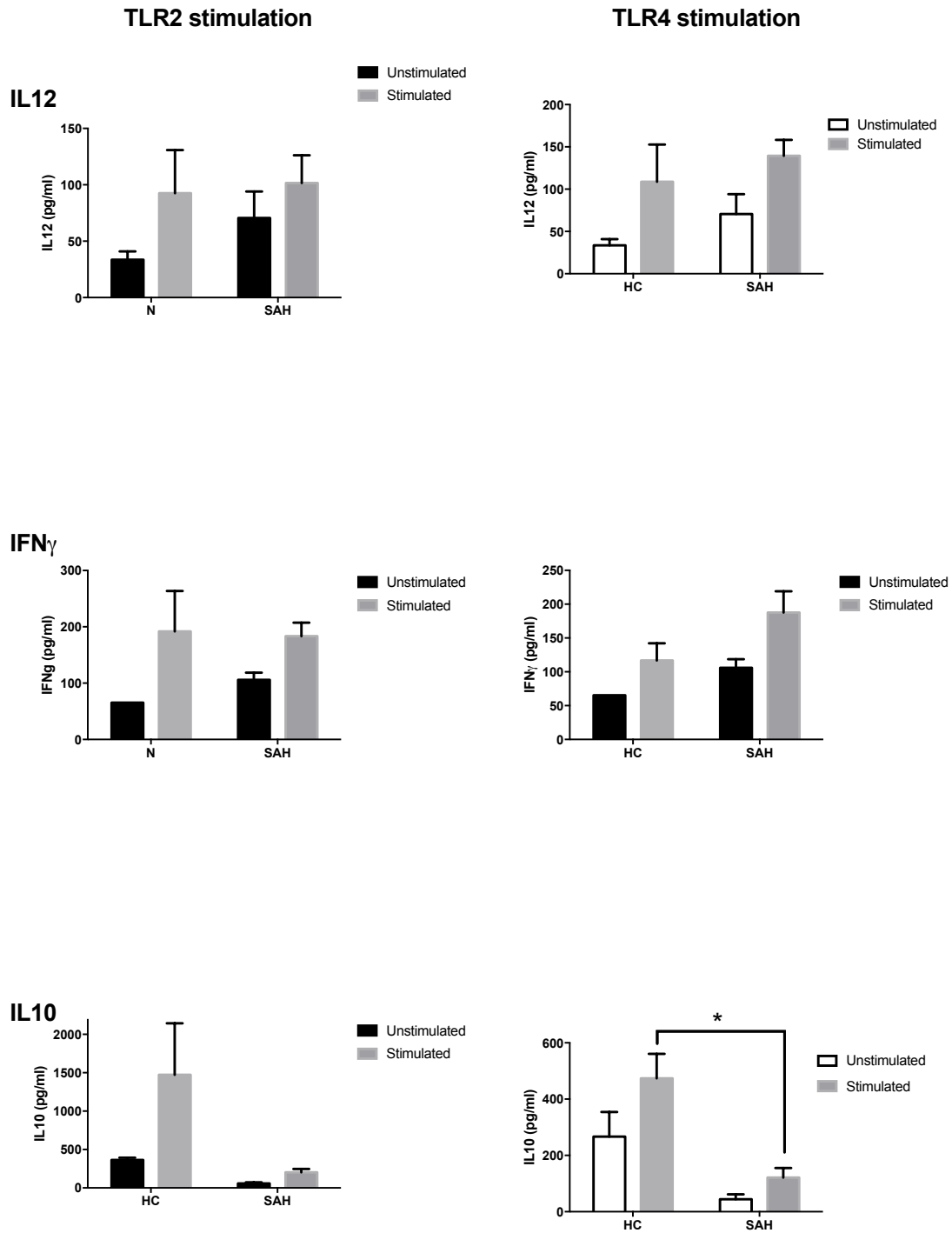
Figure 42: PBMCs stimulated with either *listeria monocytogenes* (TLR-2 stimulation) or 100ng/mL LPS (TLR-4 stimulation). Panels on left represent TLR2 stimulation while panels on the right represent TLR4 stimulation. Panels represent IL12, IFN-γ and IL10 secretion respectively. *n*=4 in groups HC and SAH. Statistical comparisons made by Wilcoxon signed rank test. HLA-DR positive gating strategy
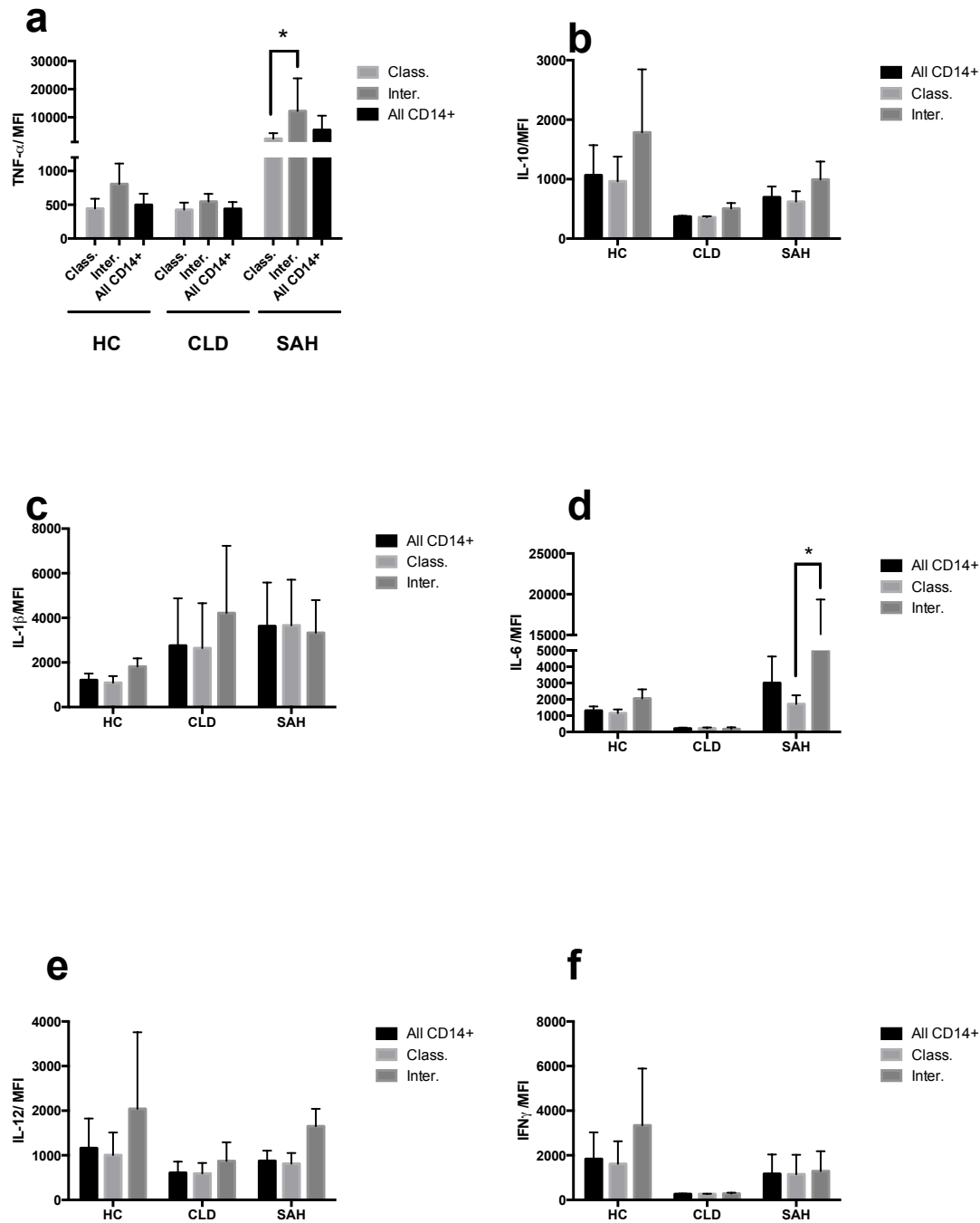
Figure 43: intracellular cytokine staining responses to 100ng/mL LPS in classical, intermediate and all CD14$^+$ cells from PBMCs. a. TNF-α, b. IL-10, c. IL-1β, d. IL-6, e. IL-12, f. IFN-γ. *n*=6 for SAH and *n*=7 for HC. Statistical comparisons made by Wilcoxon signed rank test. HLA-DR positive gating strategy

A

B

Unstimulated pNFkB
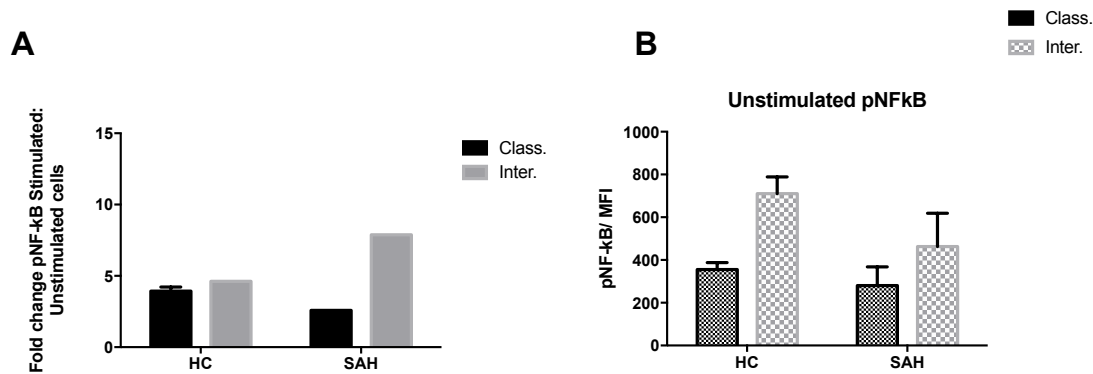
Figure 44: comparison of phosphorylation of NF-κB in monocytes from whole blood after activation with 1µg/mL LPS using the phosphoflow technique. A. In classical monocytes from SAH patients, there was impaired activation of NF-κB after LPS stimulation compared to HC. However, in intermediate monocytes from SAH patients the converse was true: there was greater activation of NF-κB in response to LPS stimulation compared to HC. B. Intermediate monocytes showed greater levels of pNF-κB compared to classical monocytes without stimulation. *n*=2 per group and these differences did not achieve statistical significance. HLADR positive gating strategy
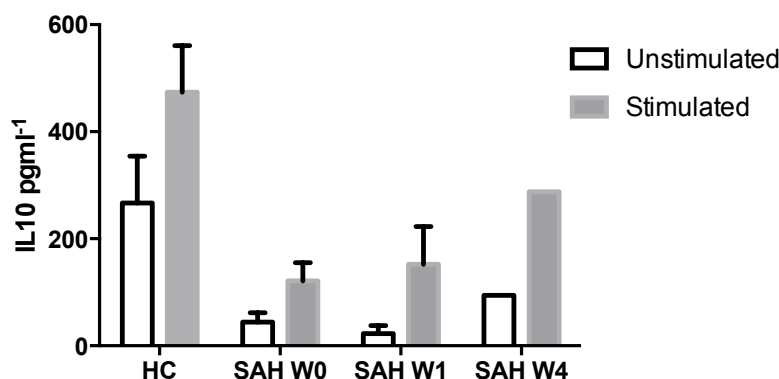
Figure 45: Secretion of IL-10 from PBMCs after 100ng/mL LPS in longitudinal samples from SAH patients pre-treatment (W0), after 1 week of therapy with prednisolone (W1) and after 4 weeks of therapy with prednisolone. *n*=4 HC and n=4 SAH at W0; n=3 at W1 and n=1 at W4. These differences did not achieve statistical significance. HLA-DR positive gating strategy

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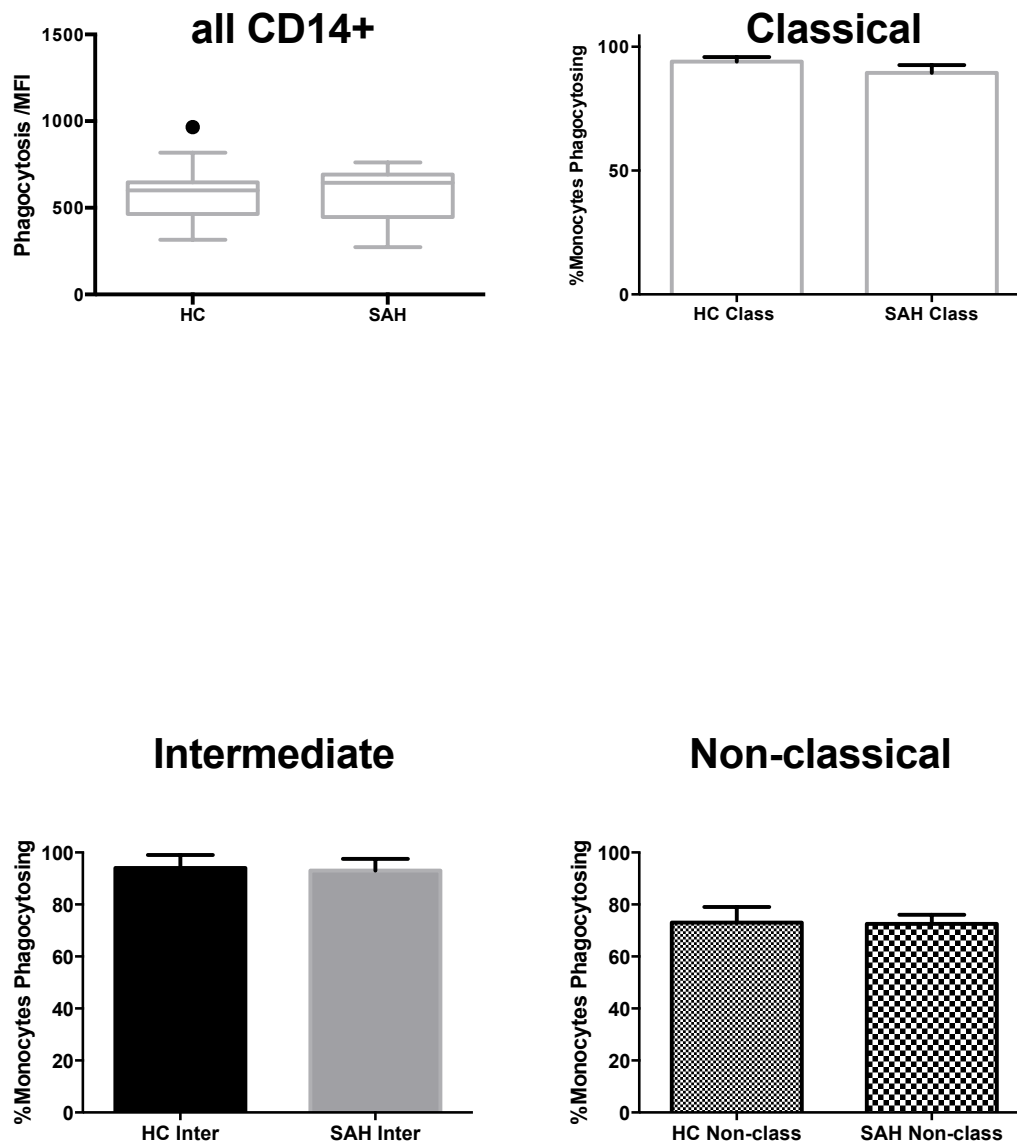
Figure 46: Comparison of ability of classical, intermediate, non-classical and all CD14⁺ monocytes to phagocytose *E. coli* using the pHRodo™ technique *ex vivo* using whole blood. *n*=30 HC and *n*=22 SAH. Statistical significance was compared using the Mann Whitney U test. HLA-DR positive gating strategy
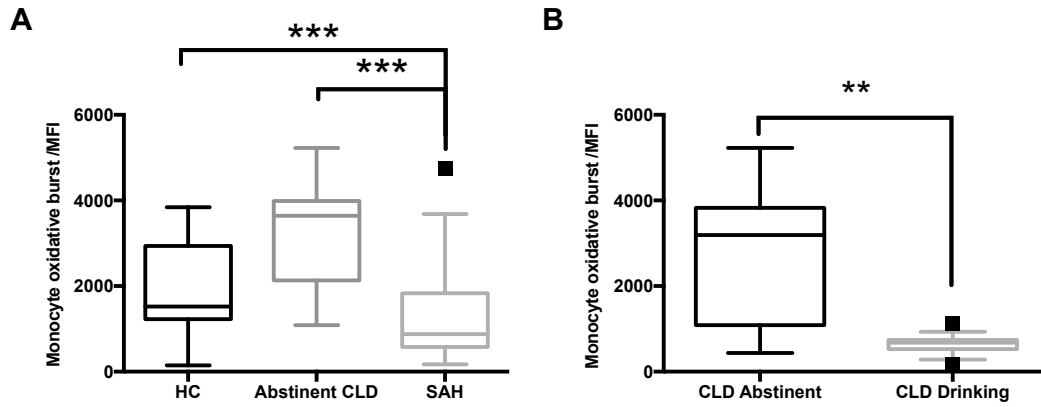
Figure 47: Comparison of oxidative burst (MOB) capabilities in CD14⁺ monocytes *ex vivo* from whole blood using the oxidation of rhodamine. *n*=42 SAH, *n*=11 abstinent CLD, *n*=14 drinking CLD and *n*=34 HC. A. MOB was depressed in SAH patients compared to CLD and HC and B. was also depressed in actively drinking CLD patients compared to abstinent patients. Comparisons were tested by Mann Whitney U test.
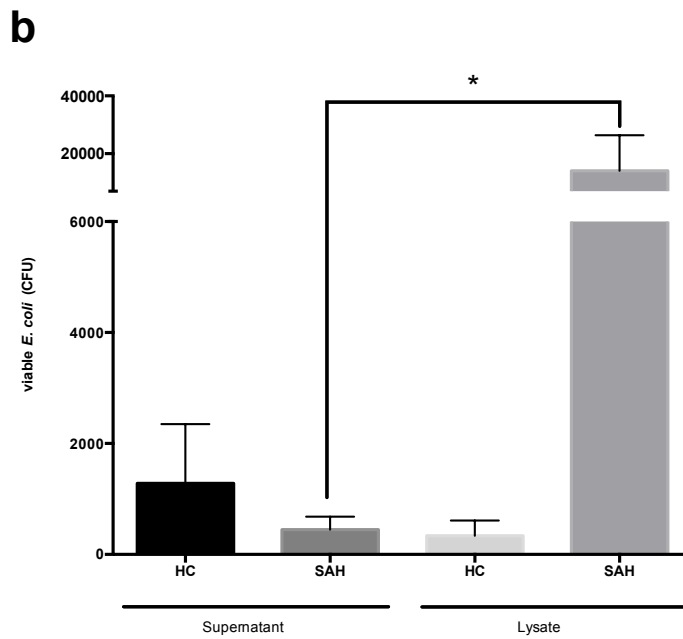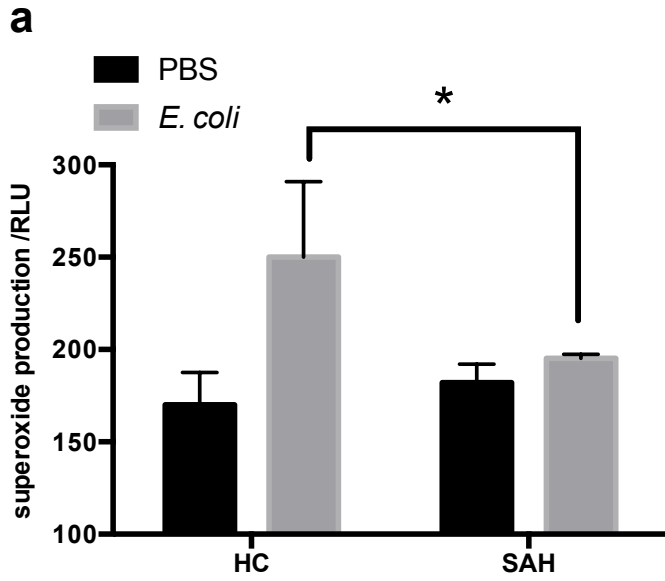
Figure 48: a. superoxide, measured by luminometry, was reduced in isolated CD14+ SAH monocytes incubated with _E. coli_ compared to HC. b. More _E. coli_ colony forming units were enumerated from the lysates of SAH monocytes compared to supernatants of SAH monocytes after _E. coli_ incubation, _n_=5 per group and comparisons were by Mann Whitney U for a. and Wilcoxon matched-pairs signed rank test for b.
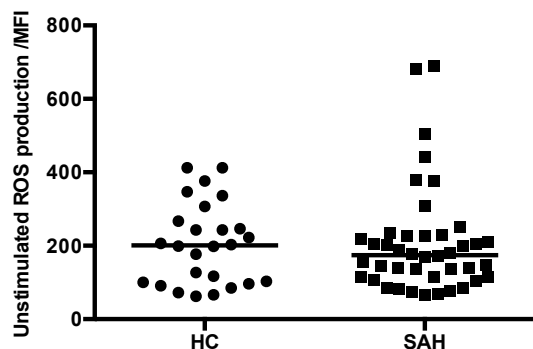
Figure 49: The generation of reactive oxygen species from monocytes from whole blood without stimulation from *E. coli* was also measured *ex vivo*. *n*=42 SAH and *n*=34 HC, comparisons by Mann Whitney U test.
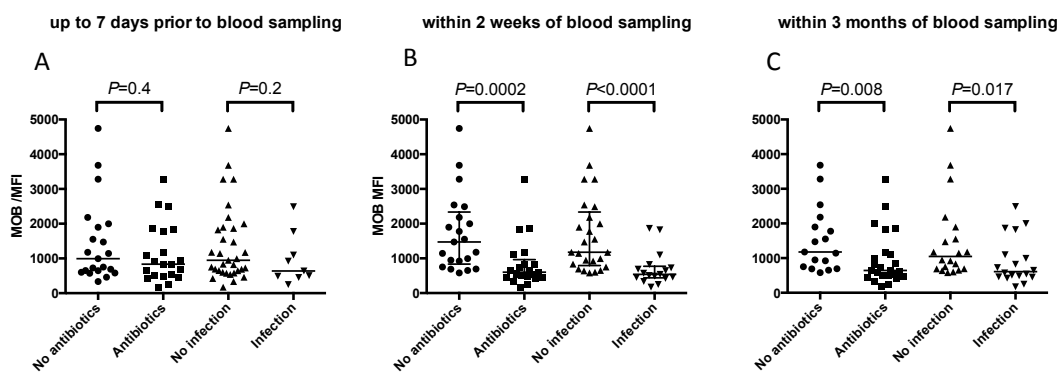
Figure 50: A. prescription of intravenous antibiotics and diagnosis of infection in the 7 days prior to MOB measurement; B. prescription of intravenous antibiotics and diagnosis of infection within two weeks of MOB measurement; C. prescription of intravenous antibiotics and diagnosis of infection within three months of MOB measurement.
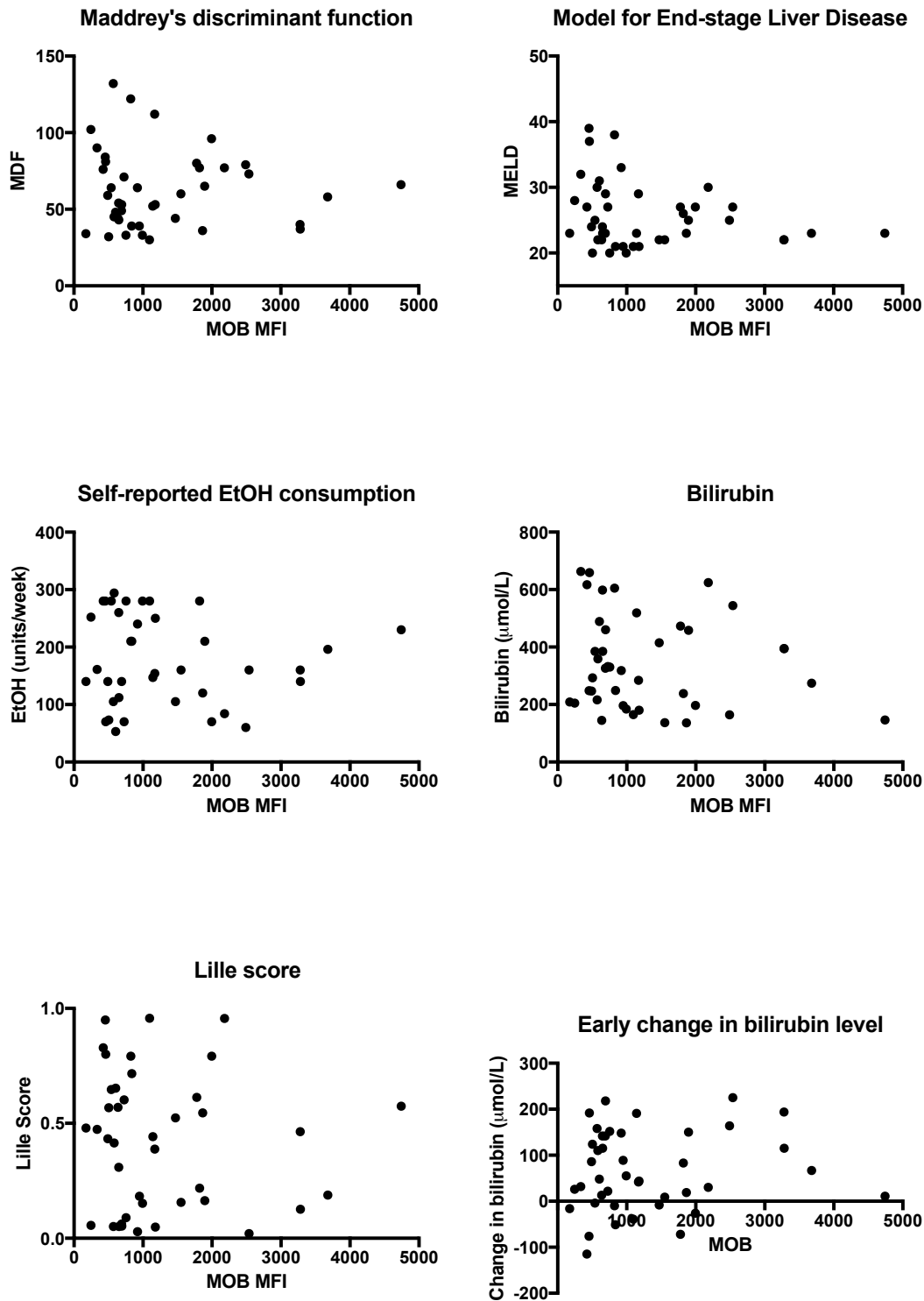
Figure 51: MOB was compared to a range of outcome measures as shown above, including self-reported alcohol consumption, bilirubin level, Lille score, early change in bilirubin level (ECBL). However no correlations were detected by Spearman's rank correlation.
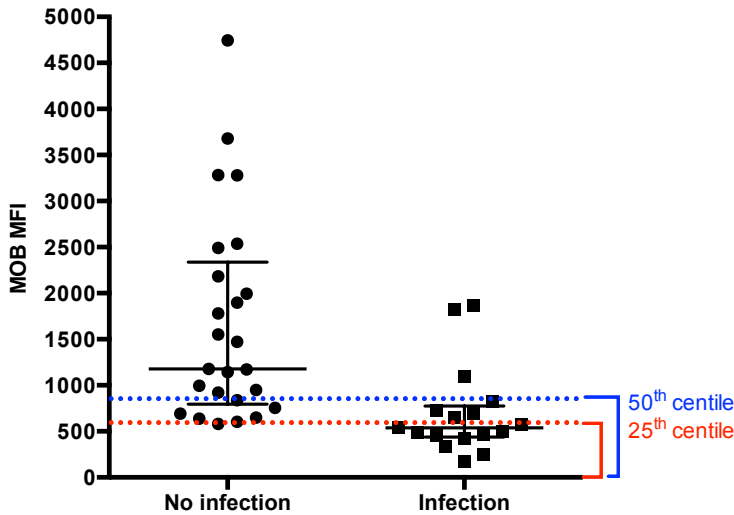
Figure 52: various cut-points were tested to identify the best sensitivity and specificity for MOB to predict infection. A cut-off at the 25th centile of SAH MOB resulted in 100% sensitivity but only 59% specificity. A cut-point at the 50th centile of the MOB distribution had 72% sensitivity and 82% specificity to predict the development of infection
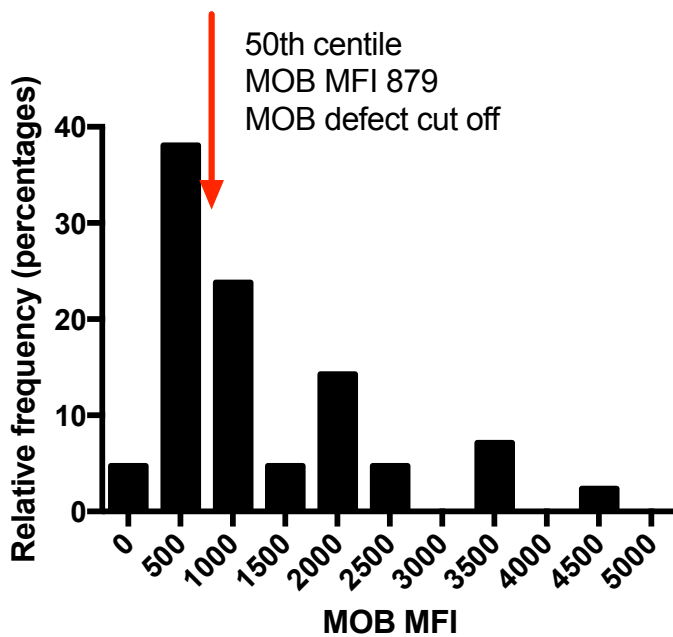
Figure 53: histogram demonstrating the skewed distribution of MOB in SAH patients. The red arrow shows the 50th centile cut-off for MOB defect.

Eligible patients
n=43

Excluded patients
n=1
(not histological SAH)

Patients in whom MOB was measured and whose infection status was prospectively recorded
n=42

MOB defect at Day 0
(< 50th centile)
n=21

No MOB defect at Day 0
(>50th centile)
n=21

Neither infection diagnosed nor new antibiotics prescribed
n=4

Neither infection diagnosed nor new antibiotics prescribed
n=16

Clinically treated for nosocomial infection
n=17

Clinically treated for nosocomial infection
n=5

New antibiotics prescribed only
(*culture-negative* infection)
n=3

New antibiotics prescribed only
(*culture-negative* infection)
n=2

Proven nosocomial infection
n=14

Proven nosocomial infection
n=3

Nosocomial infection did not develop
n=4

Nosocomial infection developed
n=14

Nosocomial infection developed
n=3

Nosocomial infection did not develop
n=16

Figure 54: flow of SAH patients through the study of MOB to predict subsequent *infection*: patients with MOB above and below the 50th centile on study entry are divided into two arms. The reference standard outcome is the development of proven nosocomial *infection* as defined by consensus criteria (Bajaj et al. 2012). Patients who are treated for nosocomial *infection* but for whom there was no radiological, microbiological or histological evidence of infection within the subsequent two weeks were deemed inconclusive (antibiotic treated *culture-negative infection*) and are removed from final analysis.

A

Infected
Not Infected

Odds ratio 12 (3-55)
P =0.0005
Positive predictive value 86%
Negative predictive value 67%
Likelihood ratio 4

B

C-reactive protein
WBC count
Procalcitonin
MOB
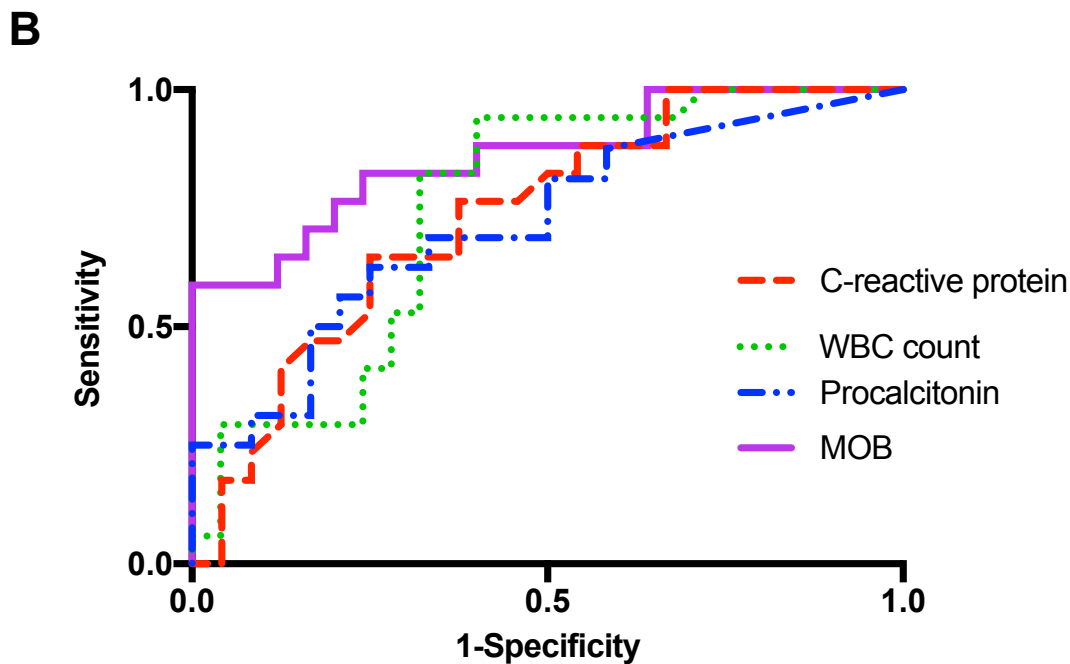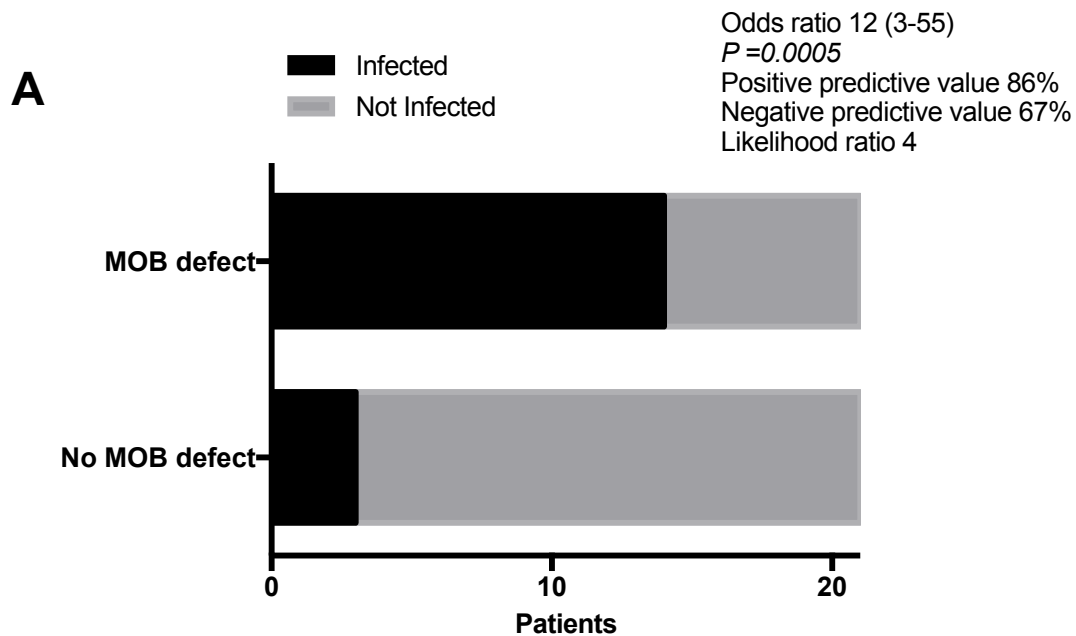
Figure 55: MOB defect predicts the subsequent development of infection, A. MOB defect predicted subsequent infection with high positive predictive value, c. Area Under Receiver Operating Characteristic (AUROC) curve for MOB was superior to white cell count, C-reactive protein and procalcitonin. (AUROC for MOB was 0.86 compared to 0.73, 0.75 and 0.72 for CRP, WCC and PCT, respectively).

A

B

no prednisolone

in vivo prednisolone
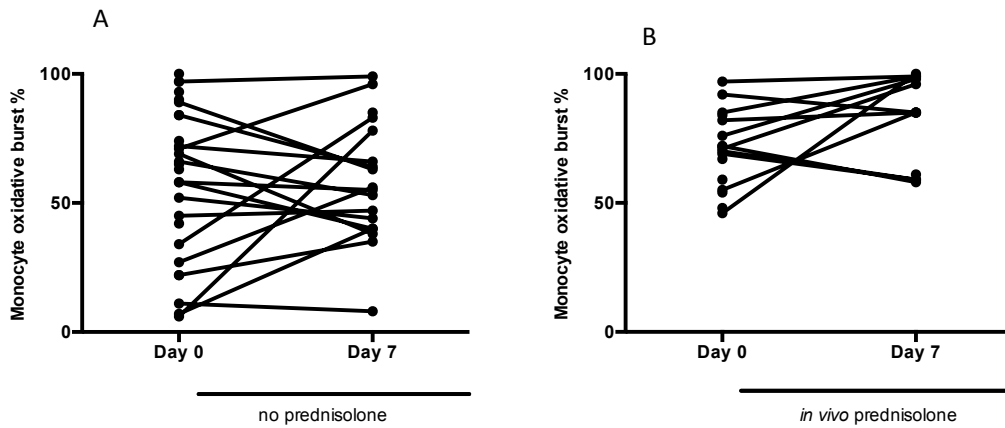
Figure 56: effect of oral prednisolone on whole blood *ex vivo* MOB over the first 7 days. A. Compares the change in MOB after 7 days without prednisolone, and B. compared the change in MOB after 7 days of *in vivo* prednisolone therapy. Statistical tests performed by Wilcoxon signed rank matched pairs test.
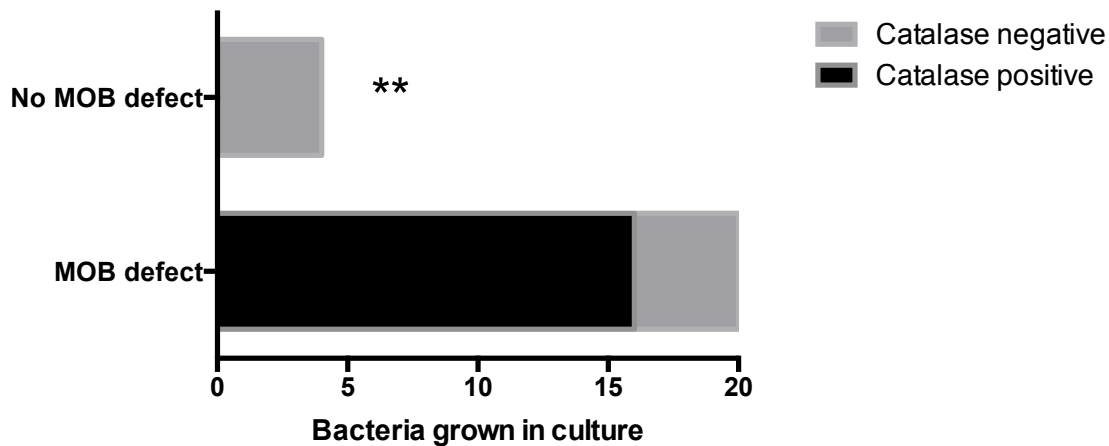
Figure 57: if patients with SAH had MOB defect the risk of contracting catalase positive organisms was substantially greater than contracting catalase negative organisms. This figure shows the association of MOB defect with the catalase status of the organism that infected the patient: 16/20 patients with MOB defect developed catalase positive infections vs 0/4 patients developed catalase positive infections that did not have defective MOB, *p*=0.0066
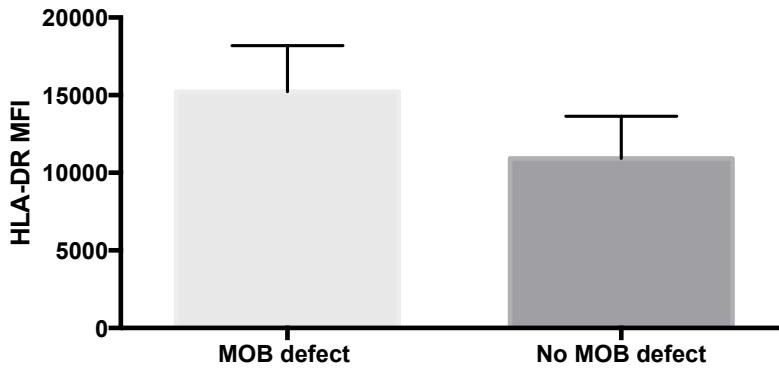
Figure 58: Comparison of HLA-DR expression on monocytes with and without MOB defect. *n*=6 in each group
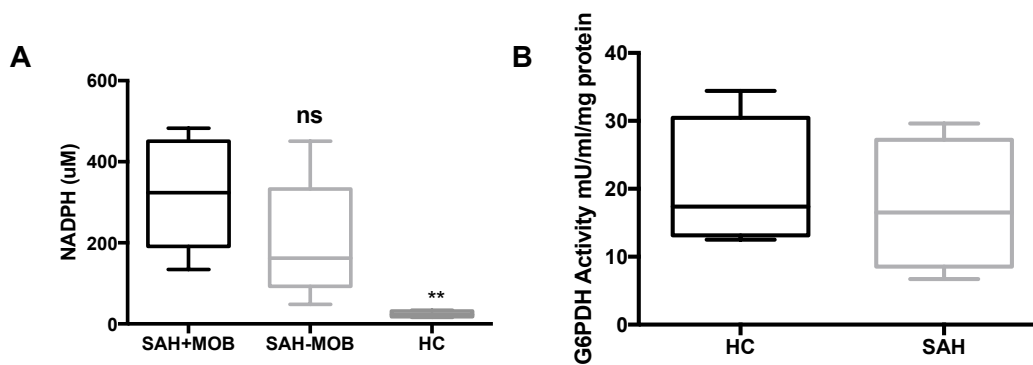
Figure 59: A. serum levels of NADPH in patients with (SAH+MOB) and without MOB defect (SAH-MOB) and HC, B. G6PDH activity in SAH patients compared to HC. *n*=8 in each group
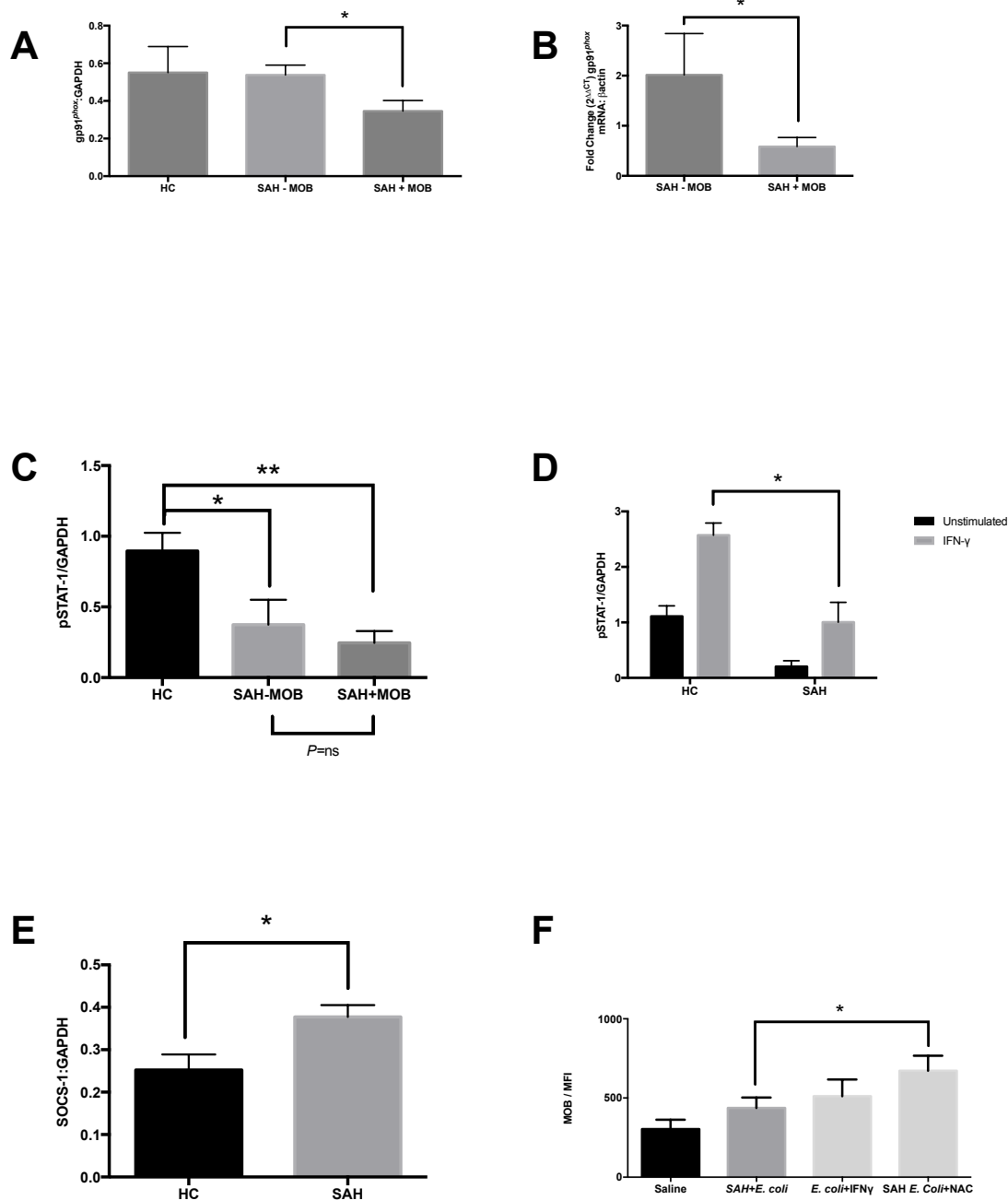
Figure 60: aberrant JAK-STAT signaling and reduced monocyte NADPH oxidase expression is associated with MOB defect and resistance to exogenous IFN-γ: A. SAH patients with MOB defect (SAH+MOB, n=6) have diminished levels of the gp91^{phox} subunit of the NADPH oxidase complex compared to SAH patients without MOB defect (SAH-MOB, n=6); B. RT-PCR demonstrated reduced gene expression of gp91^{phox} after IFN-γ stimulation in SAH+MOB, but not SAH-MOB monocytes; C. resting levels of phosphorylated STAT-1 are reduced in SAH monocytes with or without MOB defect; D. impaired activation of STAT-1 in response to IFN-γ in all SAH monocytes; E. suppressor of cytokine signaling SOCS-1 protein is present at high levels in all SAH monocytes compared to HC (n=5); F. exogenous IFN-γ stimulation does not improve MOB in vitro (n=6).
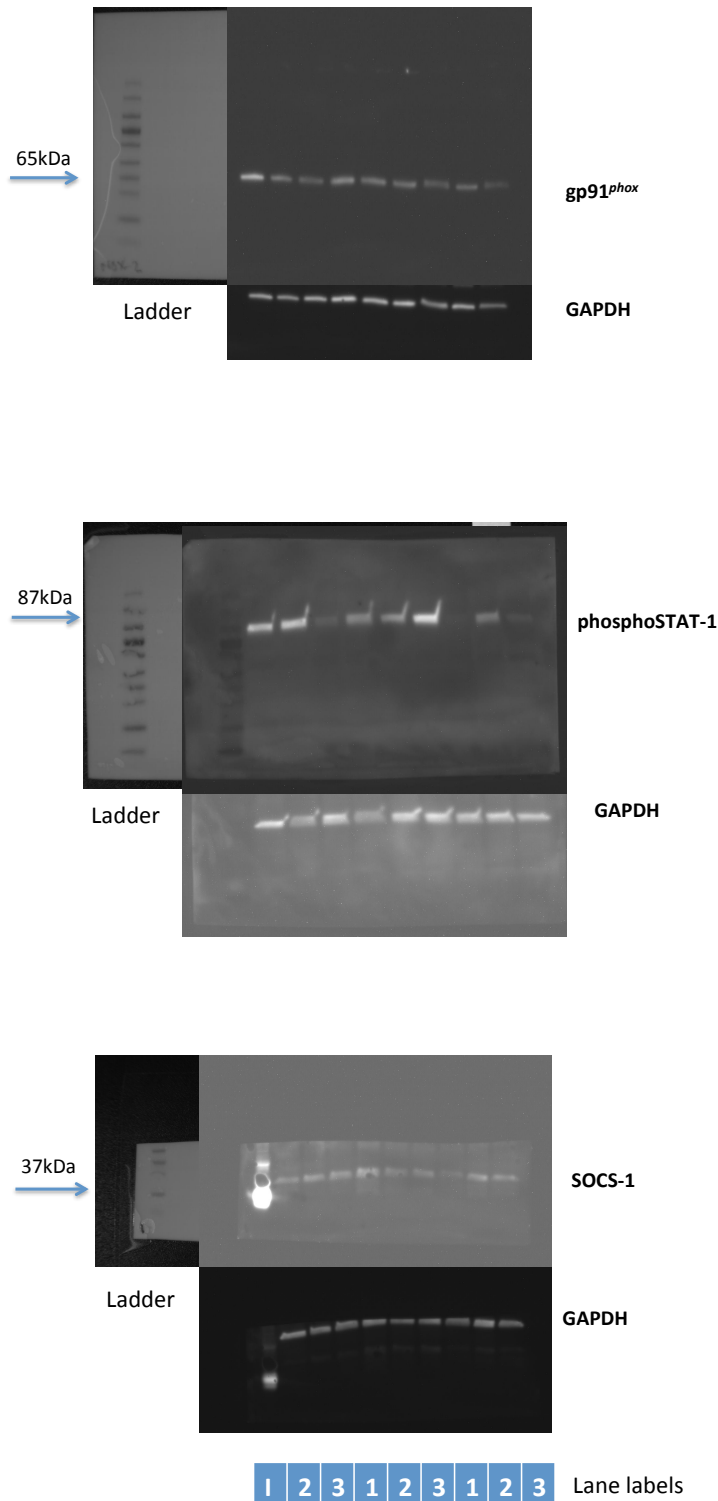
Figure 61: representative gels from Western blot analysis of CD14⁺ monocyte lysates from HC and SAH patients with an without MOB defect, *n*=6 in each group. Ladders shown on the left of the gel, indicating target protein at 65, 87 and 37kDa for gp91^{phox}, phosphoSTAT-1 and SOCS-1 respectively. GAPDH gel shown as the loading control, against which protein concentrations were normalised. Lane labels were 1=HC, 2=SAH monocytes without MOB defect (SAH-MOB) and 3=SAH monocytes with MOB

defect (SAH+MOB). Histograms for cumulative data are given in figure 51 with statistical analysis
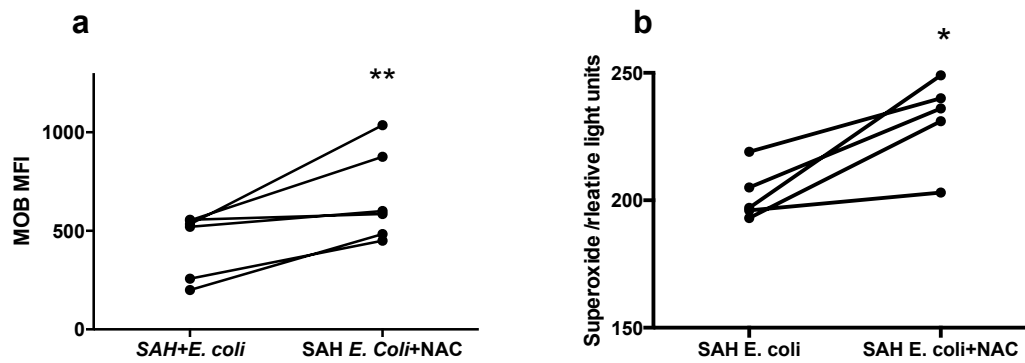
Figure 62: When monocytes are pre-treated with NAC for 24 hours before incubation with *E. coli,* they produce: a. greater MOB and b: greater amounts of superoxide. *n*=5, comparisons by Wilcoxon signed rank matched pairs test
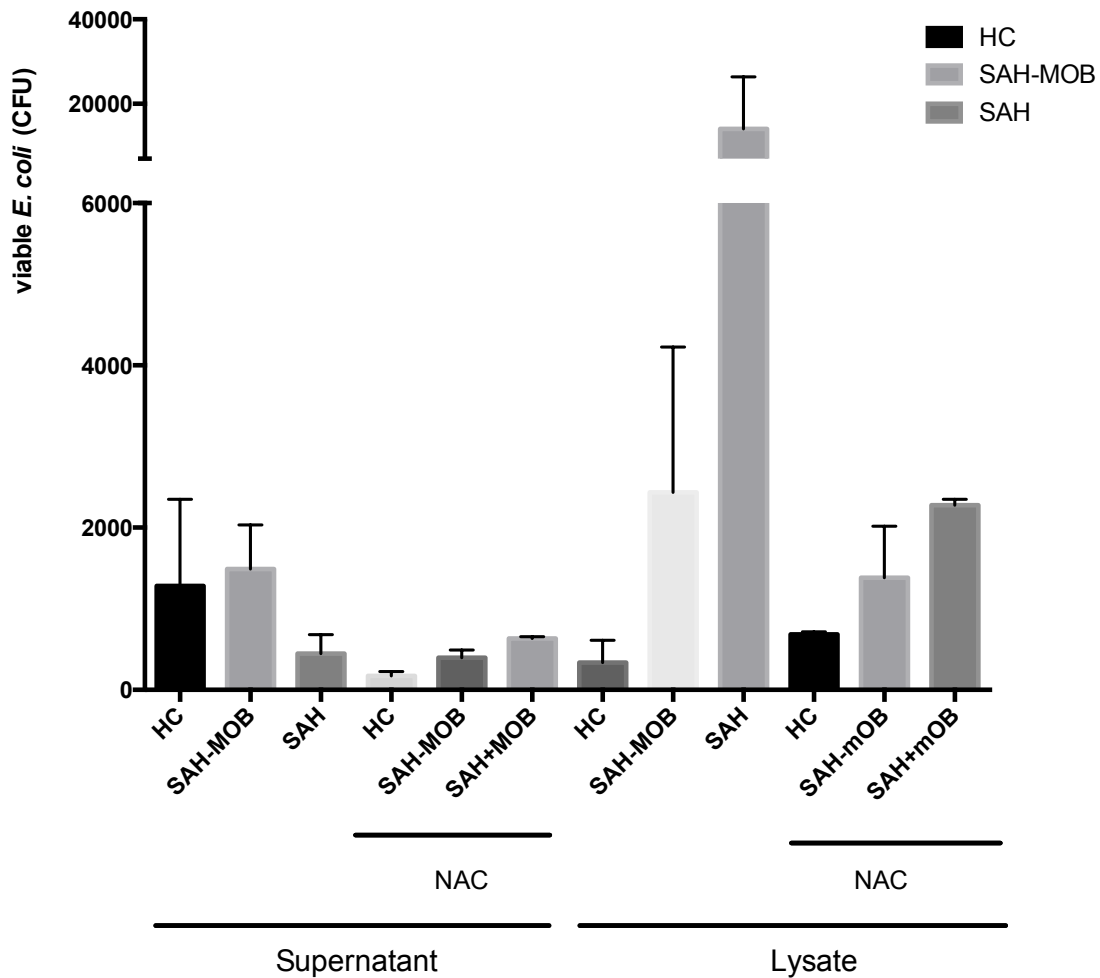
Figure 63: pre-treatment with NAC reduced the number of *E. coli* colony forming units enumerated from the lysates of SAH monocytes after incubation with *E. coli*. *n*=5 per group
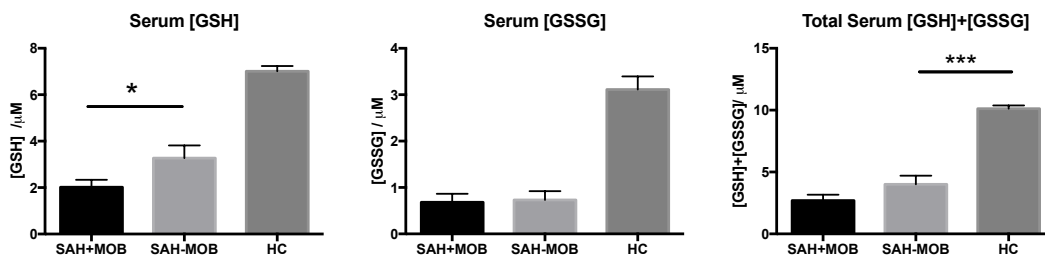
Figure 64: oxidised and reduced forms of glutathione, which NAC is known to replenish, were compared in the serum of patients with (SAH+MOB) and without) SAH-MOB) MOB defect. *n*=8 per group
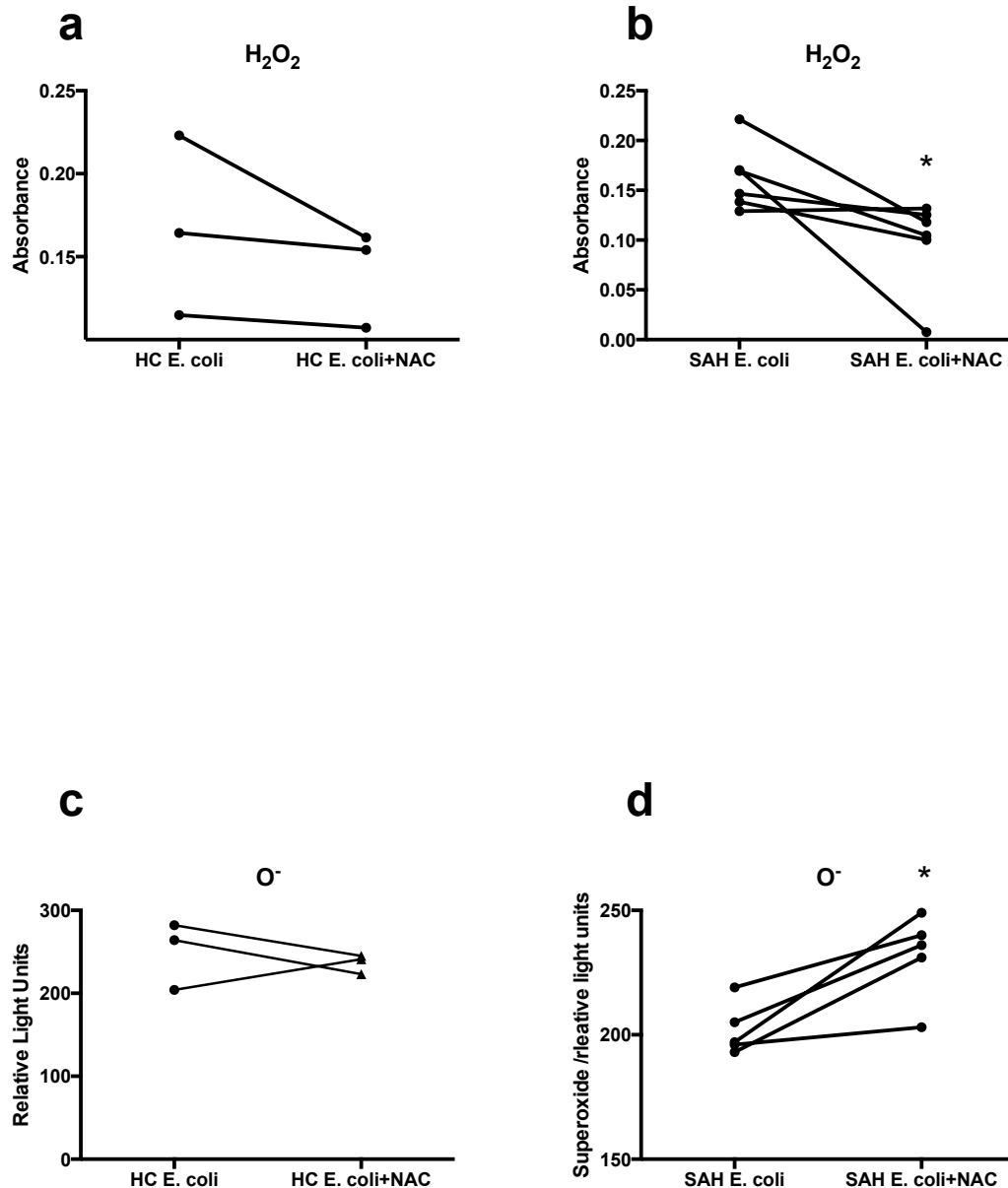
Figure 65: NAC pre-treatment of monocytes from patients with SAH and defective MOB induced changes to hydrogen peroxide and superoxide levels after monocyte oxidative burst. A. effect of 24hr pre-treatment with NAC on hydrogen peroxide levels in HC monocytes. B. effect of pre-treatment on hydrogen peroxide for monocytes from SAH patients with MOB defect, C. effect of 24hr pre-treatment with NAC on superoxide levels in HC monocytes. B. effect of pre-treatment on superoxide levels for monocytes from SAH patients with MOB defect. *n* as shown. Comparisons by Wilcoxon signed rank matched pairs test
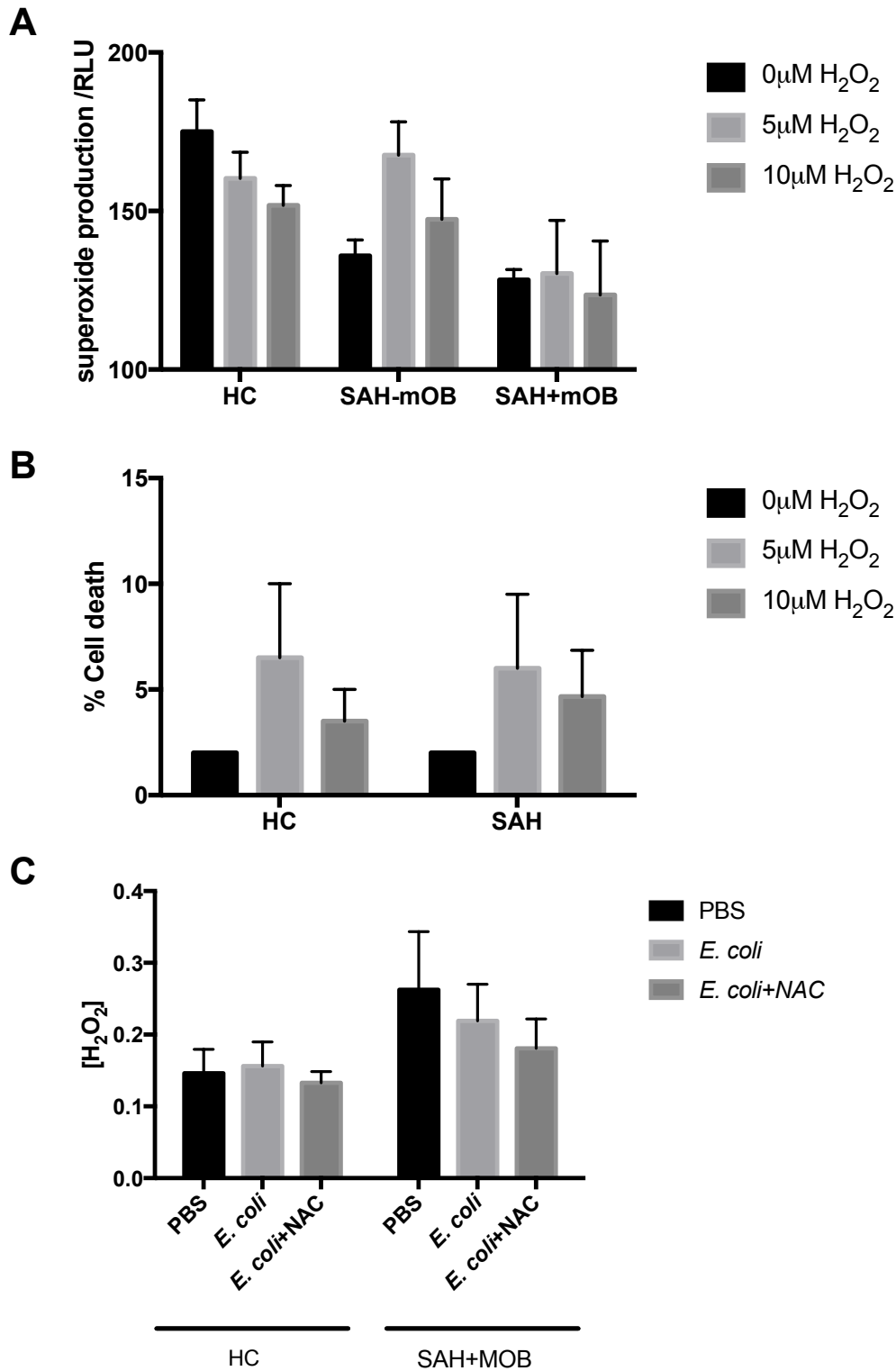
Figure 66: NAC is known to replenish stores of glutathione and reduce oxidative stress. In order to explain the improvement in MOB after NAC pre-treatment of monocytes, the effect of H_2O_2 on MOB was examined. A. Increasing concentrations of hydrogen peroxide decreased the MOB measured by superoxide production from monocytes, but only in HC monocytes. B. These finds were not readily explained by bystander damage from H_2O_2, since there was not an increase in cell death when similar concentrations of hydrogen peroxide were used. C. NAC pre-treatment of

monocytes reduced H_2O_2 levels in both HC and SAH after incubation with *E. coli,* confirming NAC's anti-oxidant effects in this setting

A   **Patients treated without prednisolone**

B   **Patients treated with prednisolone**

C   **Prednisolone treated patients**
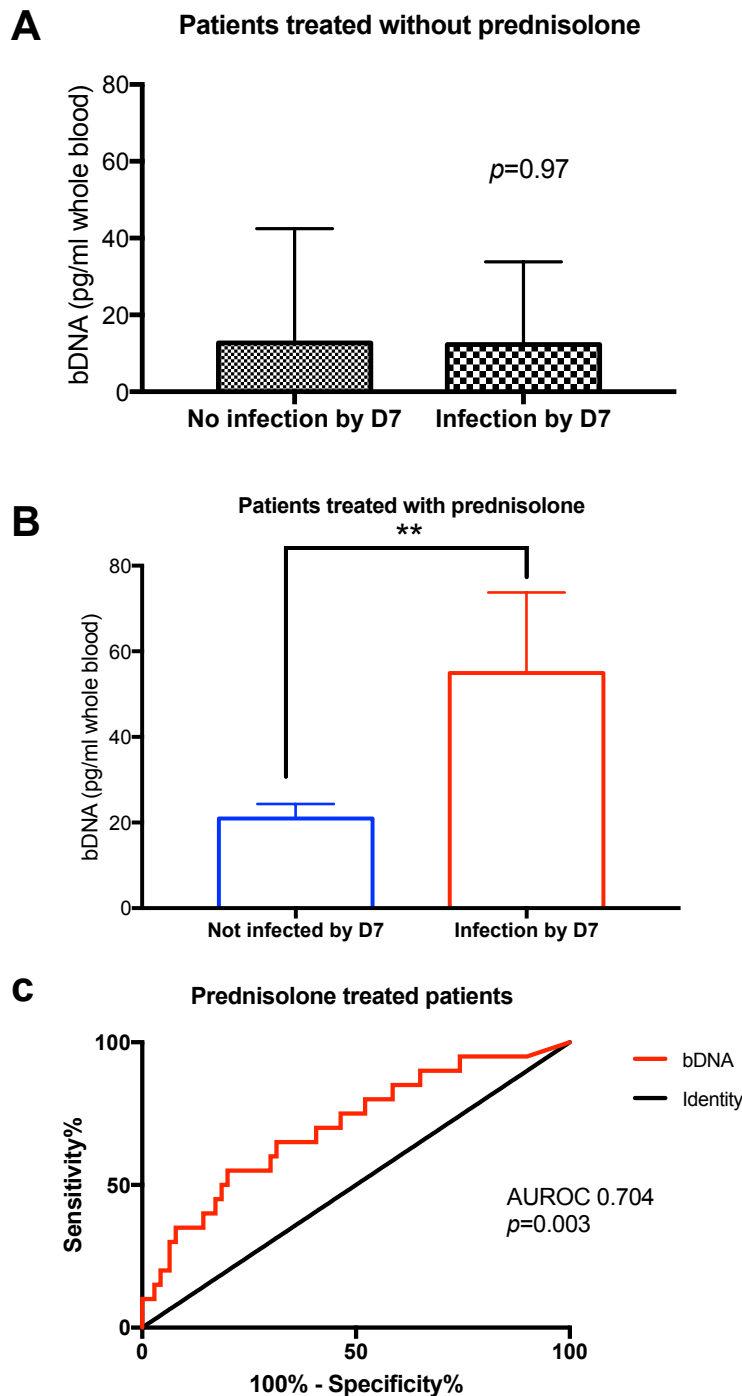
Figure 67: Bacterial DNA (bDNA) was measured by PCR in the whole blood of 731 patients recruited to the STOPAH study before treatment. A. Compares the level of bDNA in patients who did and did not subsequently develop infection in patients who did not subsequently receive prednisolone therapy, while B. compares bDNA levels in patients who received prednisolone (*p*=0.0042). C. Shows the receiver operator characteristic curve (ROC) for bDNA in the classification of patients who did and who did not develop infection. Area under ROC curve was 0.074, *p*=0.003.
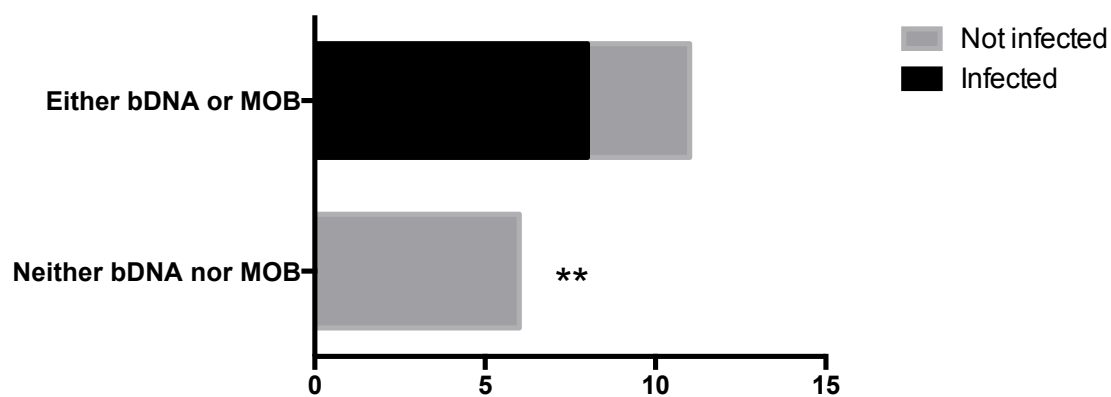
Figure 68: This figure demonstrated that if patients had low levels of bDNA and good MOB function they were highly unlikely to develop infection, compared to patients who had either high bDNA, defined as greater than 50th centile, or MOB defect

Discussion

SAH is a condition of inflammatory liver failure that contributes to high short-term mortality

(rr) **Inflammation and hepatic failure**

SAH is associated with severe functional liver impairment with median MELD score of 24. In the context of chronic liver disease, MELD scores of this magnitude portend a 3-month mortality of around 20% and consideration of referral for liver transplantation in order to avert death. The liver injury has a strong cholestatic component, with a median bilirubin 318 µmol/L compared to a median bilirubin in compensated cirrhotic control groups of 26 µmol/L. This hepatic failure is of serious consequence for patients, since clinicians stated hepatic failure as primary cause of death in 41% of death certificates by 90 days in the STOPAH study were caused by hepatic failure.

The 28-day mortality rate in the STOPAH study is lower than that reported by very early studies of SAH. For example, Theodossi *et al.* reported a mortality rate at 28 days of 40% in 1982 (Theodossi et al. 1982), followed by a series of papers in the 1990s reporting 28 day mortality rates above 30% (Carithers et al. 1989; Ramond et al. 1992; Cabre et al. 2000). More recently, large studies in 2011, 2013 and 2016 report 28 day mortality rates of 16%, 13%, and 18% respectively (Nguyen-Khac et al. 2011; Mathurin et al. 2013; Moreno et al. 2016), which is more in keeping with the mortality rate in STOPAH of approximately 16% (Thursz et al. 2015). There may be a number of reasons why the 28-day mortality rate is lower in more recent studies. Patient nutrition has improved over the last 30 years; indeed obesity is now a bigger challenge in the Western world than undernutrition(WHO 2000). In addition, dramatic improvement has been seen in the intensive care of liver failure patients, observations that have been most marked in the setting of acute liver failure (Bernal et al. 2015).

An important area of controversy is the use of biopsy in the diagnosis of SAH. The STOPAH trial used a series of clinical criteria to ensure a high likelihood of diagnosis of SAH, table 2. Some, but by no means all, of the past clinical trials of SAH have required liver biopsy confirming steatohepatitis for inclusion. In studies without defined clinical criteria for SAH, clinical diagnostic accuracy has been reported to be low. For example, in a study that recruited patients with ALD with either a worsening of jaundice, worsening of ascites, or worsening peripheral oedema, steatohepatitis was found in 31/68 cases (46%) (Mookerjee et al. 2007). Biopsies were performed in a subset of patients in the STOPAH study. For those patients who met STOPAH clinical criteria and who were biopsied as clinical routine, 89% (32/36) had histology consistent with steatohepatitis. If biopsy was performed for diagnostic uncertainty, clinical diagnostic accuracy fell to 79% (15/19 cases) (Petts et al. 2015). The utility of biopsy in SAH remains controversial, with only 10% reporting routine use of biopsy in a recent clinician survey(Singal et al. 2013).

The top two causes of mortality in the STOPAH study were hepatic failure and infection. However, classification of causes of death was limited by the terms used by clinicians on death certificates. For example, there may have been patients who died of multi-organ failure but who were categorised on death certificates as dying from "hepatic failure". Equally, patients who were categorised by clinicians on death certificates to die from multi-organ failure may have also died from infection.

This thesis has also demonstrated several features of systemic inflammation in SAH, such as leucocytosis and the monocytosis. There are raised serum markers of inflammation including C-reactive protein, procalcitonin and serum levels of inflammatory cytokines such as TNF-α, IL-1β, IL-8, IL-2, GM-CSF and osteopontin when compared to cirrhotic patients

and healthy controls. It is important to note that the anti-inflammatory, immunosuppressive corticosteroid prednisolone was shown to not only improve cholestasis in this condition but also reduce 28-day mortality, supporting the hypothesis that immunological inflammatory mediators are implicated in the cause of SAH.

An important limitation however is that the impact of pentoxifylline (PTX) was not studied in the analyses of phagocytosis and intermediate monocyte activity that were performed before and after 7 days of therapy. PTX is a phosphodiesterase inhibitor that can reduce TNFα levels (Deree et al. 2008). However, stratification of patients by both prednisolone and PTX therapy in these doctoral studies would have led to under powering of statistical analyses between groups. Although the numbers of PTX patients were randomly distributed between groups analysed in this thesis, analysis of the impact of PTX therapy on a larger cohort of SAH patients is undoubtedly of interest, and it is possible that the lower activity of intermediate monocytes after 7 days therapy could be attributed to PTX, rather than prednisolone therapy.

(ss) **Circulating intermediate monocytes are pro-inflammatory in SAH**

There are multiple previous reports of intermediate monocytes having inflammatory potential in a variety of infectious and hepatic and non-hepatic inflammatory human disorders (reviewed in (Ziegler-Heitbrock 2007b). Data presented in this thesis have revealed that, in SAH too, there is also an expansion of intermediate $CD14^+CD16^+$ monocytes. Whilst this was also seen in alcoholic compensated patients, the monocytosis prevalent in SAH compared to CLD inherently results in a higher frequency of these inflammatory cells in SAH compared to CLD. These monocytes are activated, with increased expression of the activation marker HLA-DR compared to CLD monocytes. Functionally, intermediate monocytes in SAH were demonstrated to produce greater amounts of pro-inflammatory

cytokines such as TNF-α, IL-1β and IL-6, but less IL-10 in response to TLR ligands. Through the course of the subsequent month, the capacity of monocytes to generate pro-inflammatory cytokines in response to LPS falls while capacity to produce IL-10 in response to LPS is seen to rise.

The exaggerated response to endotoxin did not support the original hypothesis that circulating monocytes in patients with SAH display features consistent with endotoxin tolerance. Given the large body of literature that supports the paradigm of increased bacterial translocation into the portal vein, secondary to alcohol drinking, and that this increased bacterial translocation might drive hepatic injury, this is an important negative finding. Its implications are twofold. Firstly, the fact that some features of monocyte function (pro-inflammatory cytokine production) are augmented whilst other facets are reduced (MOB) points away from a global down regulation of monocyte function that would be consistent with the hypothesis of innate immune exhaustion. Secondly, the results from this thesis bear similarities to the concept of monocyte training invoked in a recent article by Netea *et al.* The authors propose that after stimulation from bacteria or vaccines, which in this case might be products of bacterial translocation, innate immune cells display long-term changes in their epigenetic wiring. In the case of monocyte training, this leads to increased responsiveness upon secondary stimulation by microbial pathogens, resulting in an enhanced ability to eliminate infection(Saeed et al. 2014). Trained immunity differs from immunological memory in that it persists for weeks to months rather than years after the elimination of the initial stimulus.

The mechanism of this phenomenon is also different from classical immunological memory. Rather than gene rearrangement and proliferation of antigen-specific lymphocyte clones, the increased responsiveness to secondary stimuli is not specific to a particular pathogen. The

mechanism is epigenetic involving histone modifications and chromatic reconfigurations in particular(Foster et al. 2007). It is tempting to speculate that there is an equivalent parallel here, wherein the initial stimulus of either ethanol or bacterial products is able to increase responsiveness to other stimuli that act via TLR-4(Kendrick et al. 2010). Although monocyte oxidative burst function was depressed as will be discussed in the following chapter, there was a clear up regulation of Fcγ receptors I and III as well as excellent phagocytic capacities within SAH monocytes, in contrast to phagocytic uptake abilities reported from studies of neutrophil function(Mookerjee et al. 2007; Rajkovic & Williams 1986). Again, that specific facets of immune function were up regulated points away from the immune cell exhaustion hypothesis. In the case of endotoxin tolerance, gene-specific chromatin modifications are associated with the silencing of genes coding for inflammatory molecules while priming other genes coding for anti-microbial molecules (Foster et al. 2007). Indeed in the present study, monocyte expression of the activation marker HLA-DR was no different in patients with MOB defect compared to patients that had normal MOB, figure 58.

Murine work suggests that circulating inflammatory Ly6C^{hi} monocytes are able to infiltrate hepatic parenchyma, where they are involved in hepatic inflammation and fibrosis (Zimmermann et al. 2010). Human studies exploring resident hepatic macrophages in alcoholic liver disease have also identified an expanded population of CD16^{+} macrophages within the liver (Liaskou et al. 2013). In humans, alcoholic hepatitis is characterized by the presence of large amounts of chemokines including RANTES, a known ligand for CCR-5, as well as IL-8, Gro-α and CCL2 and CCL20 in the liver (Dominguez et al. 2009; Affò et al. 2014). It is noted that these chemokines are also known to play prominent roles in neutrophil recruitment into the liver.

(tt) **Circulating intermediate monocytes are reduced within 7 days by oral corticosteroid therapy**

Data in this thesis demonstrates that prednisolone therapy was able to reduce the number of circulating intermediate monocytes within seven days when compared with patients who were not treated with prednisolone. In addition, prednisolone induced phenotypic changes consistent with deactivation of intermediate monocytes during the course of prednisolone therapy.

The expression of the monocyte activation marker HLA-DR, which has previously been associated with LPS sensitivity (Wasmuth et al. 2005), was monitored in response to *in vivo* prednisolone therapy. While classical monocytes demonstrated a natural fall in HLA-DR levels over the course of the first week, this fall was not significantly influenced by prednisolone therapy. In contrast, HLA-DR expression on intermediate monocytes did not fall over the course of the first week in patients who were not treated with prednisolone. However, prednisolone therapy resulted in a significant fall in HLA-DR expression on intermediate monocytes. Thus, not only were intermediate monocytes reduced in number, but they may also have been deactivated by 7 days of prednisolone therapy.

Taken together with the improvement in prognostic markers seen in SAH patients treated with 7 days prednisolone, it is tempting to speculate that a mechanism by which prednisolone exerts its anti-inflammatory effects within the liver is by reducing the inflammatory activities of the intermediate monocyte subset, in a manner analogous to that previously reported by Fingerle *et al.* in sepsis (Fingerle-Rowson et al. 1998; Fingerle et al. 1993b).

Definitive conclusions regarding the possible role that intermediate monocytes play within the liver are difficult to make since circulating blood monocytes may display different phenotype and function to tissue hepatic macrophages, adapted according to microenvironment. As previously discussed, human hepatic macrophages are difficult to acquire from biopsy tissue in sufficient number. Immunohistochemistry on biopsy specimens that look for common phenotypic markers between blood monocytes and tissue macrophages may provide some clues (Liaskou et al. 2013), and will be the subject of future work.

(uu) **Circulating inflammatory intermediate monocytes express high levels of CCR-5**

Expression of the chemokine receptor CCR-5 was significantly raised in intermediate SAH monocytes, although elevations were also seen in classical monocytes. CCR2 was also raised albeit not reaching statistical significance. Elevations in the expression of CCR-5 have previously been associated with alcohol drinking. In a macaque monkey model of alcoholism and HIV, expression of CCR-5 was seen to elevate on CD16^{+} monocytes within 7 days of alcohol feeding (Marcondes et al. 2008). Specific antagonism of these receptors, in order to potentially reduce inflammatory monocyte recruitment to the liver, will also be the subject of future work.

(vv) **Evolution of circulating monocyte function after systemic inflammatory response syndromes**

Most patients in this study were sampled early in the course of the disease, a median of 5 days from admission, at which point monocytes displayed pro-inflammatory phenotype and function, exhibiting an expansion of intermediate monocytes that expressed greater amounts of HLA-DR and increased PBMC and monocyte derived inflammatory cytokines.

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Accordingly, levels of monocyte derived IL-10 were diminished at Day 0, in line with previous studies and increased during the course of treatment (Taïeb et al. 2000).

The fall in HLA-DR expression during the first week of SAH and the corresponding rise in IL-10 levels are in line with the view that patients admitted suffering from features of the systemic inflammatory response syndrome eventually develop a compensatory anti-inflammatory response syndrome (CARS), characterised by endotoxin tolerance, low HLA-DR expression, high IL-10 production, and increased expression of tyrosine kinase receptor MERTK(Bernsmeier et al. 2015). CARS have been associated with increased risk of nosocomial infection, longer stay in hospital and increased mortality (Heagy et al. 2003).

In longitudinal sampling, the rising levels of monocyte derived IL-10 produced in response to LPS stimulation and falling expression of HLA-DR described in this thesis suggest an evolving systemic inflammatory milieu during the course of SAH. However, 50% of patients were treated with the anti-inflammatory corticosteroid prednisolone. The mechanism of action of prednisolone has recently been reported to involve IRAK-M(Miyata et al. 2015; Julian et al. 2015), similar to mechanisms of endotoxin tolerance. Prednisolone contributed to a doubling of the rate of serious infections seen by 90 days in the STOPAH trial. It is tempting to speculate that while ameliorating intermediate monocyte mediated hepatic inflammation, prednisolone therapy concurrently exacerbated classical monocyte endotoxin tolerance, and hence contributed to the doubling of infections and the catch-up mortality associated with this drug in the STOPAH trial.

This dichotomy in monocyte inflammatory potential is further suggested by the expression of HLA-DR itself, which was significantly lower for classical monocytes in SAH than HC and CLD controls. The higher expression of HLA-DR discussed earlier was restricted to the

expanded population of intermediate monocytes. In line with this dichotomy, phosphoflow experiments demonstrated increased NF-kB activation in intermediate monocytes in response to LPS, but decreased activation in classical monocytes, although sample size for phosphoflow experiments was small. Further exploration of a possible dichotomy of monocyte phenotype and function evolving independently over the natural history of disease and in response to treatment is warranted. If proven, the effect of this evolution in monocyte function on patient outcomes, and whether these changes can be modified therapeutically, is a fascinating prospect.

Limitations I: monocyte phenotype and function experiments

(ww)   Circulating monocytes vs tissue macrophages

From the point of view of trying to explain hepatic injury in SAH, the main limitation to these studies has been that we are limited to the circulating compartment. Study of the intrahepatic compartment is required in order to prove that the intermediate monocytes identified are able to home to the liver via CCR-5 and cause inflammatory damage. This can be done using *in vitro* models of monocyte migration (Bernsmeier et al. 2015) to test whether antagonism of CCR-5 is able to successfully prevent monocyte migration and is the subject of future work.

There may also be important differences between circulating monocytes and tissue hepatic macrophages. The micro-environment within the liver may be markedly different to the systemic circulation, and this environment is known to influence macrophage phenotype and function(Shi & Pamer 2011). In a recent study of explanted liver tissue from patients with SAH not responding to prednisolone therapy, for example, low levels of TNFα and IL-6 were detected (Dubuquoy et al. 2015). This is in marked contrast to the high levels of serum TNFα and IL-6 that were found in the serum of patients included in these doctoral studies. Although these differences might be explained by prednisolone therapy in the former

compared to samples taken before treatment in the latter, they nonetheless open the question of whether the phenotype and function of circulating immune cells may be different to that found within the tissue because of different micro environments. The use of only circulating serum monocytes is an important limitation of this thesis in understanding mechanisms of pathology within the liver.

(xx) **Contributions from other immune cells**

In addition, the restriction in this thesis to one cell type means that the contribution from cells such as neutrophils, NK cells, T cells and B cells was not evaluated. For example, while there was no difference in TLR2 and TLR4 receptor expression on circulating monocytes in SAH patients compared to controls, different patterns of expression could be found on neutrophils or tissue macrophages. Previous studies of circulating neutrophils in SAH found down regulation of surface chemokine receptors CXCR-1 and CXCR-2 in SAH patients(Dominguez et al. 2009), in contrast to the increased expression of CCR5 seen in this thesis.

(yy) **Advantages and disadvantages of ex vivo vs in vitro experiments**

Ex vivo assays were used for monocyte phenotyping, phagocytosis and oxidative burst experiments. *Ex vivo* conditions may closely simulate the conditions of cells that function *in vivo.* However, there are several key differences to highlight before extrapolation to the human state is possible. Firstly, there is a marked temperature change when the blood leaves the body that may invoke phenotypic and functional changes within the cells. Although the *E. coli* are given to the monocytes at 37°C body temperature, it is important to note that the blood is static in the tube in which the incubation occurs, in contrast to flowing blood *in vivo.* Importantly, there is no interaction with endothelial cells. In addition, the

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activity of monocytes within whole blood may differ from the activity of monocytes in the body's tissues where the monocytes encounter pathogens, such as the lung.

Cytokine secretion assays were performed *in vitro* due to the need for long co-culture incubations. In *in vitro* experiments, the whole blood environment of the monocytes is removed and replaced with culture media. In addition, the media is supplemented by autologous serum, but how closely this environment represents either the tissue fluid environment of individual organs were macrophages are found and fight infection, or how closely it resembles conditions in the blood where monocytes also encounter pathogens, is unknown. There is likely to be a highly complex interplay of humoral factors, as well as cross-talk between various immune and non-immune cells, that is likely to shape the microenvironment of human macrophages when they are fighting infections in tissues. The precise environments in different organs and in different human disease states are unknown and therefore difficult to recapitulate, especially *in vitro*.

(zz) **Advantages and disadvantages of isolated monocytes vs PBMCS vs whole blood experiments**

Cross-talk between immune cells and the effect that this has on monocyte function is an important consideration for this thesis. The most faithful replication of blood cross-talk is given in the *ex vivo* whole blood oxidative burst and phagocytosis assays used. However, where multiple cell types are activated by the same stimulus, it is difficult to be sure whether the response seen in monocytes is a direct effect of the stimulus on the monocyte, rather than an indirect effect of the stimulus on say, neutrophils or T cells which could then trigger function in the CD14$^+$ monocytes identified. This was a potential problem for *ex vivo* whole blood experiments of MOB function and phagocytosis, as well as a problem in PBMC experiments of cytokine production, where pro-inflammatory cytokines could be generated from other sources such as T cells. However, monocytes are known to respond most

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strongly to LPS stimulation and it is likely that the greatest contribution of inflammatory cytokines in these stimulation experiments derived from monocytes. At the other end of the spectrum, effects on other cell types do not confound function that is demonstrated from monocytes that were isolated via negative magnetic bead separation. Where the individual monocyte subset contributions were required, isolated monocyte populations were used, whereas whole PBMC populations were used to measure overall cytokine production from SAH patients and controls.

There is considerable heterogeneity amongst healthy human volunteers, SAH patients and their pathological controls for the intracellular cytokine staining technique, which may, in part, explain why few significantly different changes were seen. As a result and in view of the relatively small sample sizes used to determine median average monocyte subset cytokine production, these results should be viewed with a degree of caution, and require expansion with larger sample sizes in order to make more definitive conclusions.

Although entry criteria to the study stipulated that patients were not on either prednisolone or pentoxifylline before recruitment, consideration was not given to other possible drugs that had recently been prescribed that may have influence monocyte function. One such example is the prior prescription of antibiotics, which through gut decontamination, may be able to influence the responsiveness of monocytes to stimulation.

(aaa)   **Association, not causation and the need for animal models**

A central limitation of much of the data discussed above is that it is inherently associative and correlative in nature, rather than capable of demonstrating causal relationships. Animal models of SAH would be instructive in this regard, but these are limited at present. The main issues with current animal models of SAH relate firstly to the fact that rodents have a natural aversion to ethanol drinking, and secondly that key enzymes involved in the hepatic

metabolism of ethanol differ significantly between mice and humans. So far, the ad libitum liquid diet feeding regime of the Lieber deCarli model (Lieber & DeCarli 1989) is able to generate liver steatosis, whilst the intragastric ethanol feeding model (Tsukamoto et al. 2008) generates steatosis, focal necrosis, and inflammatory foci but is technically challenging and expensive to conduct. The route of administration, by catheter placed percutaneously into the stomach, is also markedly different from the pattern of human alcohol consumption. Most recently, the chronic-binge model of ethanol feeding has been proposed. On this regimen, rodents receive an ethanol liquid diet for a "chronic" period of 10 days followed by a single binge of ethanol (5g/kg body weight). The model achieves a high blood alcohol level, transaminitis and a hepatic neutrophilic infiltration. The chronic period can be extended to 12 weeks and multiple binges can be used to create more severe liver injury. However, the hepatocellular damage and inflammation caused by this model is moderate and mimics early steatohepatitis rather than the florid necroinflammatory histology that is associated with SAH (Bertola et al. 2013).

Discussion II:

Monocyte oxidative burst defect predicts increased risk of infection in SAH

(bbb)   Susceptibility to infection and mortality in SAH

This thesis has confirmed a high rate of infection in SAH patients: 21% of SAH patients had developed infection before recruitment and 40% of patients developed nosocomial infection within two weeks of recruitment. Infection proved to be an important factor in patient outcome: remarkably, all patients who were not suspected of infection survived at least to 28 days, whilst the development of nosocomial infection rendered patients 48 times more likely to die within the first month.

Although fungal infections were prevalent in this study (of the 26 organisms cultured, 6, 23%, were *candida albicans*), there were no cases of invasive aspergillosis, in marked contrast to the 18% incidence of invasive aspergillosis that was seen in a recent French study (Gustot et al. 2014). Local prevalence and different diagnostic techniques may explain these discrepancies.

(ccc)   **Monocyte oxidative burst defect predicts infection in SAH**

Although there are many causes of immune paresis in SAH as detailed in the Introduction, novel data from this thesis demonstrate a marked impairment in the ability of circulating monocytes to kill bacteria that shares similarities with innate immune dysfunction in allied liver failure syndromes (O'Brien et al. 2014; Markwick et al. 2015). However, in contrast to the phagocytic profile seen in acute-on-chronic liver failure (Bernsmeier et al. 2015) and in neutrophil studies (Mookerjee et al. 2007; Rajkovic & Williams 1986), the defective bacterial killing that was identified in SAH monocytes is characterised by normal phagocytosis with profoundly impaired oxidative burst in a subset of patients who are demonstrably more likely to contract infection (Vergis et al. 2016).

The results indicate a wide spectrum of oxidative burst capacity in SAH patients that overlaps the range of oxidative burst capacity observed in healthy volunteers. This is not surprising, given that there are a substantial proportion of SAH patients who do not develop infection. In this regard, the close correlation of defective ex vivo MOB and susceptibility to infection within the subsequent two weeks is particularly encouraging. Various authors have highlighted the adverse impact of infection on prognosis in SAH (Louvet et al. 2009; Rudler et al. 2015). Antibiotics can reduce the incidence of infection in these patients (Rudler et al. 2015). However, concerns over antibiotic resistance have prevented routine prescribing of antibiotics in all SAH patients whilst awaiting the outcome of randomised trials

(clinicaltrial.gov NCT02281929). The strong positive predictive value of MOB for predicting the development of infection found in the current study offers a potential biomarker to rationalise use of prophylactic antibiotics and reduce the incidence of infection in SAH.

Although the data linking defective MOB to susceptibility to infection are compelling, there is no direct evidence of causation. The absence of adequate animal models is an obstacle to testing such hypotheses as described above. *In vivo* human studies to confirm causation require an agent that will reliably reverse defective oxidative burst. While IFN-γ is an attractive therapeutic candidate to restore MOB, data presented in the current study suggest that the efficacy that this drug has shown in the treatment of chronic granulomatous disease (CGD) (Mandel JS, Bond 1993) and tuberculosis (Condos et al. 1997; Dawson et al. 2009) is unlikely to extend to SAH patients.

Liver function was found not to correlate with MOB in patients with SAH. Indeed, the relationship between liver function and susceptibility to infection in SAH remains controversial(Louvet et al. 2009). Recent clinical studies suggest that susceptibility to infection in SAH is independent of liver function(Rudler et al. 2015), and our data of MOB is consistent with this conclusion.

What is known about monocyte oxidative burst in other liver failure states?

Recently, Bernsmeier *et al.* showed that in a group of patients with decompensated liver disease with similar liver function to SAH patients but without alcoholic hepatitis, monocyte oxidative burst responses to *E. coli* were preserved,(Bernsmeier et al. 2015). After removing patients with alcohol related aetiology to exclude all SAH patients from the control dataset, the MOB data comparing patients with non-alcoholic decompensated liver disease [NADLD]

(median MELD 25.5) with SAH patients (median MELD 24) is given in figure 69. Aetiologies

of these 24 NADLD patients are also listed in accompanying table.

Aetiology	Frequency
NAFLD	5
Cryptogenic	4
Autoimmune hepatitis	3
HCV	2
Alpha1 anti-trypsin deficiency	2
Wilson's	2
Other	6
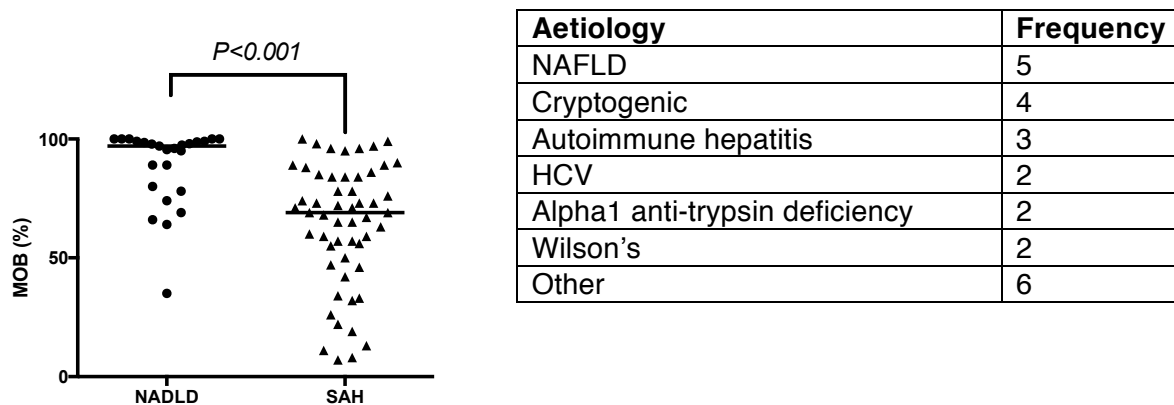
Figure 69: non-alcoholic decompensated liver disease NADLD group (n=24) patients that were matched by MELD score to SAH. Table showing the range of non-alcoholic aetiologies of liver disease in the MELD-matched NADLD group, adapted from (Bernsmeier et al. 2015)

Thus from this comparison, it appears that MOB defect is unique to patients with alcohol-related liver disease and appears to be independent of liver function and MELD score.

In contrast, the link between alcoholism *per se* and defective immunity to facultative intracellular organisms is more established (Jerrells & Sibley 1995). Specifically, infections by bacteria able to use catalase as a defence against phagocyte oxidative burst, known as catalase-positive organisms, are known to be prevalent in alcoholic patients (Storch et al. 1979; Forsblom et al. 2011; Guevara et al. 2009) and this was verified in the current study. The commonest nosocomial infection in this and other recent studies of SAH patients is pneumonia, which is often culture negative. Nosocomial pneumonia is most often caused by gram-negative bacilli and *staphylococcus aureus*, (Bennett et al. n.d.), both of which are catalase positive organisms. In addition, there appears to be a preponderance of pneumonia caused by *haemophilus influenza* and *klebsiella pneumoniae* bacteria in alcoholic patients,

both of which are also catalase positive (Jong et al. 1995). In addition, the association between alcoholism and the risk of contracting tuberculosis infection is frequently cited, and appears to be independent of socioeconomic status (Lonnroth K, Williams BG, Stadlin S, Jaramilo E 2008).

(ddd)   **N-acetylcysteine may restore MOB defect**

The *in vitro* results of NAC pre-treatment on MOB suggest a possible therapy for treatment of MOB defect if *in vivo* evidence of benefit could be obtained. How NAC might restore MOB defect is intriguing and will be the subject of future work. This hypothesis is of potential clinical interest, since a large randomised multi-centre trial recently found benefit in combining NAC with prednisolone therapy (Nguyen-Khac et al. 2011). The authors randomised 176 patients to receive either prednisolone or prednisolone combined with a 5-day regime of NAC similar to the regime proven to be of benefit in acute liver failure. Results showed a mortality benefit at 28 days (p=0.01) with a trend to benefit at 90 days (p=0.06) and 6 months (p=0.07).

How NAC is able to reverse MOB defect remains undetermined. Experiments that tested whether hydrogen peroxide treatment could reduce MOB presented in this thesis have small sample size and so firm conclusions cannot be made since there is a risk of a type 1 statistical error. Importantly however, the viability staining with 7-AAD showed that monocytes had not been killed as a result of the hydrogen peroxide therapy, suggesting a direct effect of hydrogen peroxide to boost MOB. The anti-oxidant properties of NAC are well documented as a scavenger of hypochlorous acid, hydroxyl radicals and hydrogen peroxide (Aruoma et al. 1989). Crucially however, NAC does not scavenge superoxide radicals (Aruoma et al. 1989). NAC has also been postulated to suppress activation of the transcription factor NF-kB (Oka et al. 2000), thereby reducing pro-inflammatory cytokine

production. Both of these features of NAC would likely result in improvement in hepatic injury and function which may reduce susceptibility to infection (Louvet et al. 2009). However, improvements in liver function are unlikely to explain our in vitro findings of restored MOB and bacterial killing in our isolated cell system. Rather, they point to a direct effect of NAC on monocytes. It is notable that in clinical studies, the later benefits of NAC in ALF could not be explained by improvements in liver function (Harrison et al. 1990). Similar to these data in ALF, we demonstrate that MOB in SAH is independent of classical markers of liver function encapsulated by MDF and MELD scores.

Although of small sample size, pre-treatment of monocytes with NAC appeared able to reduce hydrogen peroxide levels as expected, but, somewhat counter-intuitively, NAC boosted the superoxide response of monocytes to *E. coli* incubation. It is possible that high levels of oxidative stress in SAH monocytes, as a consequence of the disease process and seen as elevated levels of hydrogen peroxide, negatively inhibits the activity of NADPH oxidase in the generation of superoxide radicals required for bacterial killing. In line with this hypothesis, there was a dose-dependent decrease in superoxide production by monocytes incubated in increasing concentrations of hydrogen peroxide. NAC may be able to scavenge cytoplasmic hydrogen peroxide species, reduce negative regulation of monocyte oxidative burst and thereby boost bacterial killing.

(eee)   **Mechanism of MOB defect and resistance to IFN-γ reversal**

Various authors have documented defective neutrophil oxidative burst in SAH. Postulated mechanisms include reduced production of IFN-γ from T cells (Markwick et al. 2015) and impaired activation of the p47[phox] component of the neutrophil NADPH oxidase enzyme complex (Rolas et al. 2013). However, these data are the first to report defective monocyte oxidative burst with preserved monocyte phagocytic capabilities.

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Norkina *et al.*, found elevations in intracellular monocyte SOCS-1 and a corresponding decrease in monocyte STAT-1 activation after healthy volunteers had consumed ethanol (Norkina et al. 2008). In line with those data, in the present study we found that alcohol-drinking compensated cirrhotic patients, but not abstinent compensated cirrhotic patients, demonstrated defective MOB. This suggests that heavy drinking before SAH patients are admitted to hospital may contribute to impaired MOB. In this regard, we note that a recent retrospective population-based study, involving 633 cirrhotic patients followed for 10 years, identified active alcohol misuse as an independent predictor of infection-related acute on chronic liver failure (Sargenti et al. 2015).

However, alcohol alone appears insufficient to disrupt MOB. All SAH patients will have drunk large amounts of alcohol immediately prior to admission, and yet only a proportion present with defective MOB. Indeed in the current study, impaired activation of STAT-1 in SAH monocytes was observed whether or not the patient displayed a MOB defect ex vivo. Diminished expression of gp91^{phox}, however, was only demonstrated in patients with defective MOB. It is likely therefore that impaired IFN-γ signal transduction only partially explains the observed reduction in gp91^{phox} expression and other mechanisms, which remain to be identified, contribute to the clinical phenotype of impaired oxidative burst and increased susceptibility to infection.

Resistance to interferon-α therapy that is mediated by SOCS-1 is also seen in hepatocytes infected with hepatitis C virus (Read et al. 2015). Elsewhere, elevations in SOCS-1 have been implicated in a broad range of other persistent intracellular infections including *mycobacterium tuberculosis* (Carow et al. 2011), *leishmaniasis* (Srivastav et al. 2014), *toxoplasmosis* (Zimmermann et al. 2006), group A *streptococcus* (Wu et al. 2014), dengue

(Aslam et al. 2014) and Japanese encephalitis virus (Kundu et al. 2013). In these infections, the pathogen has evolved a mechanism to up regulate this important negative regulator of JAK-STAT signalling (Greenhalgh et al. 2002) in order to subvert pathogen killing within the innate immune cell, resulting in impaired clearance of the organism.

(fff) **High prevalence of pulmonary involvement in SAH**

It should be noted that lung infection accounted for the majority of nosocomial infection. An association between excessive alcohol drinking and pulmonary infection or inflammation (namely, pneumonia and adult respiratory distress syndrome) has previously been documented, and suggest a specific syndrome that may be induced by either alcoholism or alcoholic hepatitis. The immunological basis for this syndrome warrants further investigation, since both ARDS and pneumonia in the context of SAH result in substantial mortality.

(ggg)   **Prednisolone therapy did not affect MOB**

Seven days of oral prednisolone had no effect on MOB. Kaufmann et al. similarly found that in patients suffering from septic shock, neutrophil phagocytosis was unaffected by 24 hours intravenous hydrocortisone therapy (Kaufmann et al. 2008).

There is therefore insufficient data in this thesis to explain the increased incidence of infection seen in the STOPAH trial in patients treated with prednisolone. However, suppression of pro-inflammatory intermediate monocytes by prednisolone as a cause for the increased risk of infection is a possibility, given the data presented in this thesis, and will be the subject of future work.

Limitations II: Oxidative burst experiments

(hhh)   **Confounders of oxidative burst function**

There are multiple limitations to consider for the phagocytosis experiments. Firstly, there is a wide range of oxidative burst function for both healthy controls and SAH patients. This thesis has identified that alcohol drinking may be an important factor in this. Healthy control subjects were not precisely controlled for alcohol drinking, other than that they had confirmed that they had drunk less than 10 units in the previous week. In addition, smoking and exercise are factors known to impact on oxidative burst function in phagocytes and was not controlled for (Sorensen et al. 2004). Nutritional status is also likely to impact on oxidative burst function by changing the intracellular balance of anti-oxidants such as glutathione (Nelson et al. 2013). These factors were not recorded in either SAH or control groups, however. Such factors may have contributed to the broad variability seen in oxidative burst functions for all subjects.

(iii) **Difficulties in diagnosing clinical infection**

A further limitation stems from the difficulty in diagnosing clinical infection. While the criteria used in this study have a high specificity for infection (Bajaj et al. 2012), there is likely to be a large proportion of so-called culture negative infection that is undetectable with these criteria. Bacterial DNA offered some promise in being able to quantify culture negative infection, but area under receiver operator curves presented in this thesis represented only modest predictive value. Aseptic techniques for venepuncture before bDNA analysis has scope for improvement. In addition, specific statistical protocols can be developed for the identification of contaminant outliers.

(jjj) **Low sample sizes for mechanistic studies**

Sample sizes for the explorative, mechanistic assays used in MOB studies, such as Western blotting, qPCR and bacterial killing assays, were small, raising the possibility of type 1 statistical errors. Definitive conclusions would require larger sample sizes.

(kkk)   **Specificity of assays**

The Diogenes reagent used for the superoxide assay is highly sensitive for the free radical, but its take-up within cells is variable. Whether the output from these assays reflects intracellular phagolysosome superoxide levels or merely reflects secreted superoxide is contentious.

qPCR is known for highly sensitive and reproducible results. However, the delta delta C_T method used in this study has two potential errors associated with it. The first is that it relies on the assumption of 100% efficiency of amplification across all samples, which may not be true. Second, the PCR software automatically removes background fluorescence, and since background fluorescence is in fact unknown, this can lead to potential errors.

(lll) **Circulating monocytes vs tissue macrophages for infection**

An additional problem arises from the testing of circulating monocytes from blood. While it is true that the *E. coli* that was added to whole blood in the assays of this thesis invoked a robust oxidative burst response, it is also true that the phenotype and function of macrophages in tissues where pathogens are commonly encountered, such as the lung in pneumonia, may be manifestly different. For example, tissue macrophages may express increased levels of anti-inflammatory phenotypic markers such as CD163 and MERTK as part of a more mature or differentiating phenotype(Strauss-Ayali et al. 2007). These markers are associated with anti-inflammatory properties that may result in differing abilities to

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generate pro-inflammatory cytokines, phagocytose and perform oxidative burst than those demonstrated in this thesis. While some phagocytosis is likely to occur in the bloodstream in cases of septicaemia, the majority is likely to occur in the infected organ. In addition, the environment of a freely flowing bloodstream, where endothelial cell interactions are frequent, is likely to be very different to that encountered on static plastic surfaces.

Summary & Key Findings

1) *Severe alcoholic hepatitis is an inflammatory hepatic disorder* that portends high short-term mortality in young people due to:

a) hepatic failure

b) nosocomial infection

2) *The intermediate subset of circulating monocytes is expanded in severe alcoholic hepatitis.*

a) They bear a pro-inflammatory phenotype and function

b) They also express high levels of the chemokine receptor CCR-5 compared to controls

c) The frequency of this monocyte subset can be reduced by treatment with prednisolone

3) *A proportion of patients with SAH have defective monocyte oxidative burst that portends an increased risk of developing nosocomial infection.*

a) Defective monocyte oxidative burst is associated with increased expression of SOCS-1 and impaired expression of the NADPH oxidase enzyme

b) Defective monocyte oxidative burst can be reversed *in vitro* by treatment with N-acetylcysteine

Future Directions

Regulation of inflammatory cytokine output in alcoholic hepatitis

The finding that early in the course of SAH disease there is a pro-inflammatory phenotype and function of SAH monocytes, and later in the disease course, and in particular after prescription of corticosteroids, there is a deactivation of SAH monocytes, raises the question of how such monocyte training is regulated. Possible regulators of this switching from pro- to anti-inflammatory states, perhaps mirroring immunological changes from the systemic inflammatory response syndrome to the compensatory anti-inflammatory response syndrome, include monocyte surface receptors such as MerTK, intracellular receptors such as IRAK-M and epigenetic factors such as micro-RNA 122 or 155 leading to genetic modifications involving histone acetylation or chromatin remodelling(Foster et al. 2007). Indeed, the interplay of these factors in generating either trained or tolerant monocytes is likely to be complex and would necessitate both reductionist molecular approaches with knockout animal models and perhaps systems biological approaches to capture them.

CCR-5 mediated infiltration of inflammatory intermediate monocytes into the liver in SAH

Results suggesting that intermediate monocytes may be pivotal in the generation of inflammatory hepatic injury in SAH, via infiltration from the circulation using CCR-5, could be further studied using specific chemokine receptor antagonists and examining the effects in *in vitro* models of monocyte migration through endothelium. It also offers further avenues of investigation for potential therapy. Commercially available antagonists of CCR-5 and CCR-2, such as cenicriviroc (CVC), are currently undergoing clinical trials in the treatment of NASH (clinicaltrials.gov: NCT02217475), a condition histologically indistinguishable from alcoholic steatohepatitis. The possibility of selectively targeting the CCR-5 receptor in SAH is attractive. In the recent STOPAH study, prednisolone therapy resulted in a doubling in the number of serious infections reported within 90 days (Thursz et al. 2015). This may have resulted from impairments to immune defences to infection as "off-target" effects of

prednisolone therapy. Whilst CCR-5 antagonism may also impair host immune defence, pre-clinical trials and trials of HIV infected patients administered the drug CVC have not highlighted susceptibility to infection as a safety risk. Most studies of the role of chemokine receptors in the setting of infectious disorders highlight the importance of CCR-2 rather than CCR-5 (Shi & Pamer 2011). Targeting the CCR-5 receptor specifically with small molecular inhibitors such as aplaviroc, vicrivoc or maraviroc may therefore offer a more selective therapeutic strategy.

Mechanism of action of N-acetylcysteine in reducing susceptibility to infection in SAH

The finding that NAC reduced infection in SAH patients given prednisolone, and the utility of NAC in improving MOB *in vitro* presented in this study, raises the question of whether the mechanism of action of NAC in reducing infections is by improving MOB. This can be tested in a randomised controlled trial of prednisolone and NAC versus prednisolone therapy alone, to look for differences in the recovery of MOB function between the groups. If NAC is able to restore MOB *in vivo*, I aim to look at the mechanisms of this phenomenon, specifically testing the hypothesis of whether H_2O_2, as a surrogate of oxidative stress, acts as an immunological brake on MOB function as a means of protecting the host cell from further oxidative stress. NAC might therefore be effective by increasing glutathione reserves to relieve oxidative stress, and thereby allow restoration of MOB.

Animal models of acute and chronic liver injury

Limitations with animal models have already been described in *Discussion*. However, the molecular mechanisms underpinning SAH may nonetheless be suggested by cautious use of animal models. There are several options available. Neutrophilic infiltration into the murine liver can be seen as indicative of a good model of human SAH. Two models used in this context are the 3,5-diethoxycarbonyl-1,4-dihydrocollidine and griseofulvin models. These

produce ballooning of hepatocytes and accumulation of Mallory bodies but do not involve the administration of alcohol to the mice. Nrf2 knockout mice that receive the Lieber DeCarli alcohol diet are characterised by Kupffer cell and neutrophil infiltration into the liver. However, the most promising animal model of SAH is the chronic-binge model of ethanol feeding. This model increases serum ALT, TNFα, and has hepatic neutrophil infiltration without describing the other histological findings seen in SAH such as haptocyte ballooning. In addition, no murine models of alcohol liver damage have the cirrhotic changes that we commonly associate with SAH in humans, nor is jaundice a feature of any of the mouse models. These models combined with gene knock-outs of MerTK and IRAK-M, small molecule inhibitors of mir-155, CCR-5 and NADPH oxidase may provide vital clues as to whether these elements might be important for human monocyte training, monocyte infiltration into the liver and the oxidative burst defect discovered in this thesis.

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