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Modulation of the Immune Response by Current and Novel Treatments for Hepatitis C Virus

By

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ABSTRACT

FACULTY OF INFECTION, INFLAMMATION AND REPAIR
SCHOOL OF MEDICINE

MD

MODULATION OF THE IMMUNE RESPONSE BY CURRENT AND NOVEL TREATMENTS FOR HEPATITIS C VIRUS

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Hepatitis C virus (HCV) infection is a major global healthcare problem infecting 180 million people worldwide. Infection persists in 80% of patients and is thought to result from a failure to sustain an immune response to HCV. Current treatment with ribavirn and PEG-IFNα leads to a sustained viral clearance in 40-80% of patients. Despite its use for many years, the exact mechanisms of actions of ribavirin are poorly understood. These current treatments are poorly tolerated by many and therefore newer and more effective treatments are being investigated.

This thesis investigated how current and novel treatments were able to modulate the immune response looking at effects on the innate immune response including dendritic cells and NK cells and their ability to stimulate T cells and the adaptive immune response.

The aim of the work described in chapter 3 was to investigate the immunomodulatory effects of ribavirin on dendritic cell (DC) function. The results obtained suggest that ribavirin is able to modulate cytokine production from dendritic cells in response to maturation stimuli, in particular suppressing the production of IFNα from pDCS from both CHC and NHD patients but also TNFα, IL8 and IL10. In MoDCs from NHDs, ribavirin decreased CD40L-induced TNFα production but had no effect on other cytokines tested or on DC phenotype. These findings explain possible reasons behind the failure of monotherapy and dependence on co-administration of IFNα in treatment regimens.

The aim of the work in chapters 4 and 5 was to investigate the immunomodulatory effects of two novel treatments, a novel helminth protein, r*Ov*-ASP-1 and a TLR7 agonist, SM-360320.

rOv-ASP-1 showed evidence of DC phenotypic maturation, based on up-regulation of phenotypic markers (CD40, HLA-DR, CD83 and CD86) and enhanced pro-inflammatory cytokine production (IL-6, IL-8 and TNFα). It was also shown to stimulate proliferation of allogeneic $CD4^+$ T cells suggesting that the protein is able to enhance the accessory/antigen presentation by DCs. These findings suggest that this novel protein may be used as an effective immuno-stimulant to boost antigen specific responses or as a vaccine adjuvant.

SM-360320 was shown to enhance viral clearance and immune modulation with induction of 2'5'OAS gene expression, inhibition of replication of HCV replicons in Huh 7 cells, enhanced secretion of anti-viral and pro-inflammatory cytokines, up-regulation of NK cell activation. These findings suggest that SM-360320 might provide complementary and additional mechanisms of action to current HCV therapies making it a promising novel treatment.

Further investigation of these compounds is therefore warranted due to their potential widespread application in treating infectious diseases and immune mediated conditions

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Authors Declaration

The work done in chapters 3 and 4 were completed wholly by myself while in candidature for a research degree at Southampton University.

The work done in Chapter 5 was in part done jointly with others at the antiviral department of Pfizer, Sandwich, UK. All CHC patient blood samples were collected by myself at Southampton along with NHDs for the NK cell and pDC depletion studies, while the NHDs samples for the rest of the study had previously been collected at Pfizer. The samples were processed in Southampton and frozen for further analysis at Pfizer for the gene expression assays, HCV replicon and cytokine analysis. The NK cell work and the pDC depletion experiments were carried out by myself at Southampton. The statistics in this chapter were done by the statistician at Pfizer.

I can confirm that I have attributed published sources that I have consulted or quoted and acknowledged all main sources of help.

Part of this work has been published prior to submission as:

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Recombinant Ov-ASP-1, a Th1-biased protein adjuvant derived from the Helminth Onchocerca volvulus, can directly bind and activate antigen presenting cells. Journal Immunology 2009; 182(7) 4005-4016

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I would also like to thank my partner, Keith Miller and my parents for their ongoing encouragement throughout this research. Without this support this would not have happened. I now look forward to focusing on our next new arrival!

Chapter 1
Introduction

1.1 The Liver - Structure

The liver is the largest gland in the body. It weighs between 1200-1600g depending on size and sex. It is situated in the right upper quadrant of the abdominal cavity against the diaphragm.

1.1.1 Macroscopic Structure:

The liver is covered by the visceral peritoneum of Glisson's capsule, which thickens into ligaments connecting it to the diaphragm and abdominal wall - the falciform ligament superiorly, (which also separates the right and left lobes), the coronary, the right and left triangular and the round ligament (derived from the umbilical vein)(1).

It is divided by fissures into four lobes- the right (largest), left, quadrate and caudate. But it is divided functionally into 8 segments following the distribution of the portal pedicles and divisions of the right, middle and left hepatic veins.

Blood Flow:

The liver has a dual blood supply:

Hepatic Portal Vein –

Formed form the confluence of the splenic vein and superior mesenteric vein. It drains venous blood into the liver from the entire GI tract and supplies 75% of the liver's blood. The normal portal pressure is 5-8mmHg.

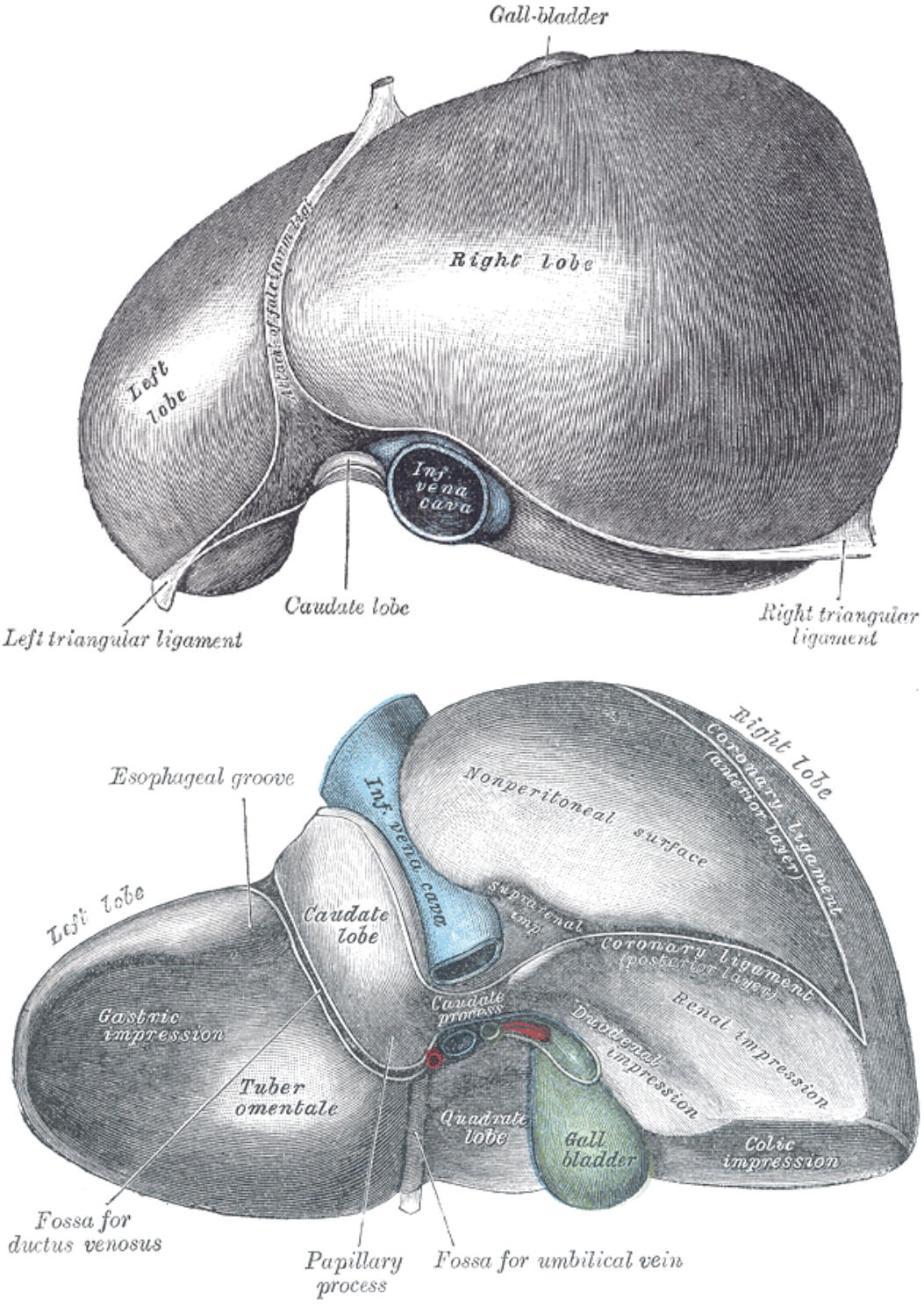
Hepatic Artery –

Originates from the celiac trunk as the common hepatic artery and supplies oxygenated blood to the liver. It supplies 25% of the total blood flow to the liver. Both vessels enter the liver via the hilum (porta hepatis). The blood from these vessels is distributed to the segments and passes into the sinusoids via the portal tracts.

Hepatic Vein –

The venous drainage from the liver is from the three hepatic veins. Short veins within lobes of the liver unite to form hepatic veins which join to the inferior vena cava.

Figure 1 Surface anatomy of the liver as viewed from superior aspect (a) and posterior/inferior aspect (b). *(Reproduced from Gray's Anatomy Online)*



1.1.2 Microscopic Structure

The basic architecture of the liver has classically been described as a lobular structure. The lobules are hexagonally shaped functional units of the liver consisting of a central tributary of the hepatic vein and at the periphery a portal tract containing the bile duct, portal vein radicle and hepatic artery branch. Columns of hepatocytes and sinusoids extend between these two systems.

More recently the acinar structure has been described (Figure 2). The acinus has been portrayed as the functional unit of the liver (1;2). Each acinus is centered on the portal triad with its terminal brach of portal vein, hepatic artery and bile duct (zone1). These inderdiginate with terminal hepatic veins of adjacent acini. The circulatory peripheries of acini (adjacent to terminal hepatic veins) (zone 3) suffer most injury and bridging fibrosis is located in this area.

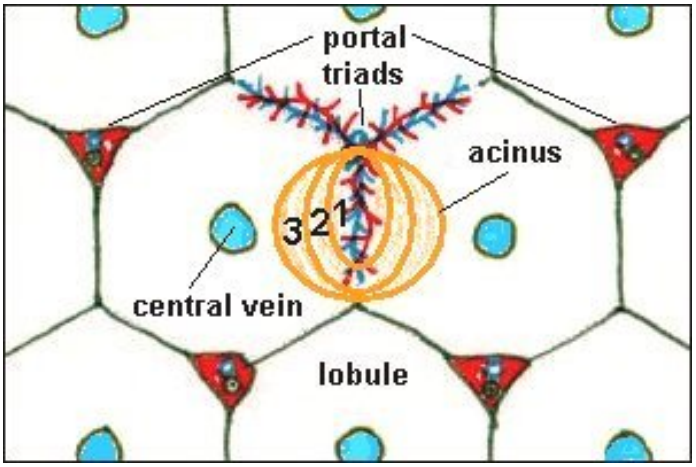
Sinusoids –
These are small blood vessels separated by rows of hepatocytes. The sinusoids are lined by endothelial cells. Kupffer cells are attached to the endothelium. The sub-endothelial space that lies between the sinusoids and hepatocytes is the space of Disse, which contains is filled with matrix rich laminin, type IV collagen and heparin sulphate proteoglycans as well as stellate cells (2). Sinusoids receive oxygen rich blood from hepatic artery and nutrients from the portal vein. Oxygen and nutrients diffuse through capillary walls to the liver cells.

Portal Tract –
At the corner of each lobule is a complex composed of branches of the portal vein, hepatic artery, bile duct and nerve.

Bile Duct – Bile is drained from liver cells by canaliculi. The intra lobular canalicular network drains into small ducts that unite to form the hepatic duct (main bile duct from the liver) which then joins the cystic duct and then the common bile duct.

Central Vein – Found in the middle of each lobule, receives blood from the portal vein and hepatic artery via the sinusoids and drains into the hepatic vein.

Figure 2. Diagram of a functional acinus: Zones 1, 2 and 3 represent areas supplied by blood with zone 1 being best oxygenated. Zone 3 is supplied from blood remote from afferent vessels and is in the microcirculatory periphery of the acinus(3).



1.1.3 Component cells of the liver

Hepatocyte

This is the most numerous cell in the liver making up 80% of the parenchyma. It is epithelial in origin and responsible for the specialised metabolic functions of the liver.

Sinusoidal Cells:

Endothelial cells

These are fenestrated cells which lie in the extracellular matrix of the space of Disse.

Kupffer Cell

These are highly mobile macrophages attached to the endothelial lining of the sinusoid, particularly in the peri-portal area. They are responsible for removing old and damaged blood cells or cellular debris as well as bacteria, viruses and tumour cells by phagocytosis. Kupffer cells are activated by a wide range of agents, including endotoxins, sepsis, shock, TNFα and IFNγ. The result of activation is the production of a range of products including cytokines - TNFα, IL-1, IL-6, IL-10

Hepatic stellate cell

These cells sit within the sub-endothelial space of Disse and have long cytoplasmic extensions. In normal liver they are the major storage site of retinoids which are retained in the cytoplasm as lipid droplets. Following liver injury, they loose their lipid droplets, proliferate, migrate to zone 3 of the acinus, change to a myelofibroblast-like phenotype and produce collagen type I, III and IV and laminin. Stellate cells also release matrix proteinases and inhibitory molecules of matrix proteinases (tissue inhibitor of metalloproteinases (TIMP). Collagenization of the space of Disse results in decreased access of protein-bound substrates to the hepatocyte.

1.2 The Liver – Function:

1.2.1 Protein Metabolism:

The liver is the principal site of synthesis of most circulating proteins with the exception of γ-globulins. The liver receives amino acids from the intestines and muscles and, by controlling the rate of gluconeogenesis and transamination, regulates levels in the plasma.

Around half of the circulating protein is albumin which is important for maintaining plasma oncotic pressure and transporting water-insoluble substances such as bilirubin, hormones and drugs. Other proteins produced in the liver include the carrier proteins, transferring and caeruloplasmin, clotting factors and components of the complement system.

Amino acids are degraded by transamination and oxidative deamination to produce ammonia, which is then converted to urea and excreted by the kidneys. This is the major pathway for the elimination of nitrogenous waste (2;4).

1.2.2 Carbohydrate Metabolism

Glucose homeostasis and the maintenance of the blood sugar is a major function of the liver. It stores approximately 80g of glycogen. In the immediate fasting state, blood glucose is maintained either by glucose released from the breakdown of glycogen (glycogenolysis) or by newly synthesized glucose (gluconeogenesis). Sources for gluconeogenesis are lactate, pyruvate, amino acids from muscle and glycerol from lipolysis of fat stores. In prolonged starvation, ketone bodies and fatty acids are used as alternative sources of fuel and the body tissues adapt to lower glucose requirement (2;4).

1.2.3 Lipid Metabolism

The liver has a major role in the metabolism of lipoproteins. It synthesizes very-low-density lipoproteins (VLDL) and high-density lipoproteins (HDL). Triglycerides are mainly of dietary origin but are also formed in the liver from circulating free fatty acids (FFAs) and glycerol and incorporated into VLDLs. Oxidation or *de novo* synthesis of FFA occurs in the liver, depending on the availability of dietary fat.

Cholesterol may be of dietary origin but most is synthesized from acetyl co-A in the liver, intestine and adrenal cortex (2;4).

1.2.4 Formation of bile

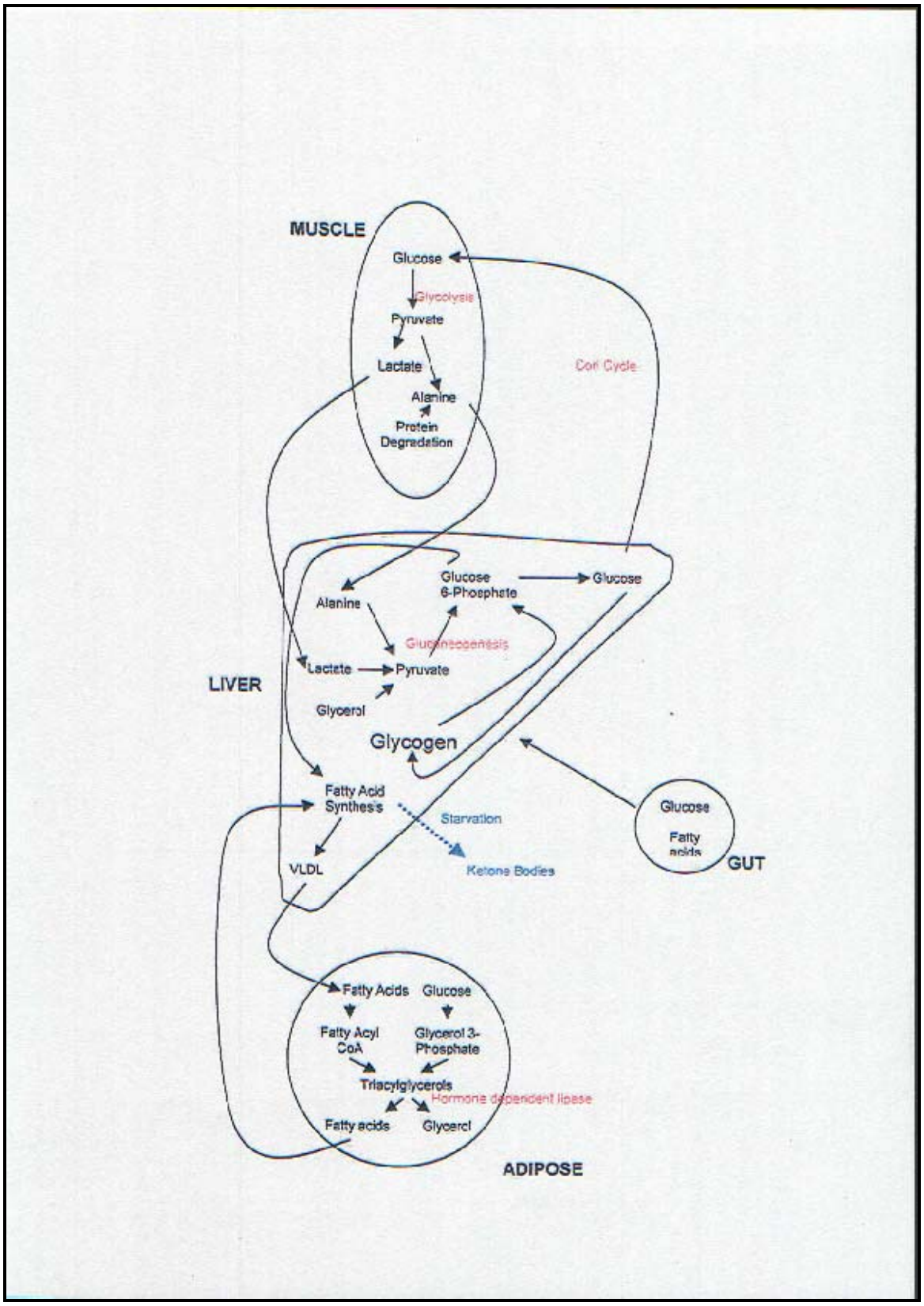
Bile consists of water, electrolytes, bile acids, cholesterol, phospholipids and bilirubin. Bile acids are synthesized from cholesterol and act as detergents; their main function is lipid solubilization. Bilirubin is produced mainly from the breakdown of haem containing proteins, especially haemoglobin. It is conjugated with glucuronic acid in the liver making it water soluble and excreted into the intestine with the bile (2;4).

1.2.5 Hormone and drug inactivation

The liver catabolises hormones such as insulin, glucagon, oestrogens, growth hormone, glucocorticoids and parathyroid hormone. It is also the prime target organ for many hormones e.g. insulin.

It is the most important site for the metabolism of drugs and alcohol. Fat soluble drugs are converted to water-soluble substances that facilitate their excretion in the bile or urine (2;4).

Figure 3 Metabolic pathways of the liver(5)



1.3 Hepatitis C - The Virus:

1.3.1 Structure:

Hepatitis C virus was identified in 1989 by Houghton *et al* (6) after recognition of the phenomenon of post-transfusional hepatitis following the administration of blood and blood products causing ”non-A, non-B hepatitis”. After extensive testing of serum from experimentally infected animals, the virus was cloned using molecular biology techniques.

The hepatitis C virus is an enveloped RNA virus that belongs to the Flaviviridae family and is the only member of the Hepacivirus genus (7). Its genome is a positive sense single stranded RNA 9.6kb length that comprises a long open reading frame (ORF) encoding for a large polyprotein, flanked on either side by 5’ and 3’ non coding regions,.

The viral genome is translated as a single polyprotein, which is subsequently cleaved by viral and host proteases to produce both structural and non-structural proteins of the virus (8;9) (figure 4).

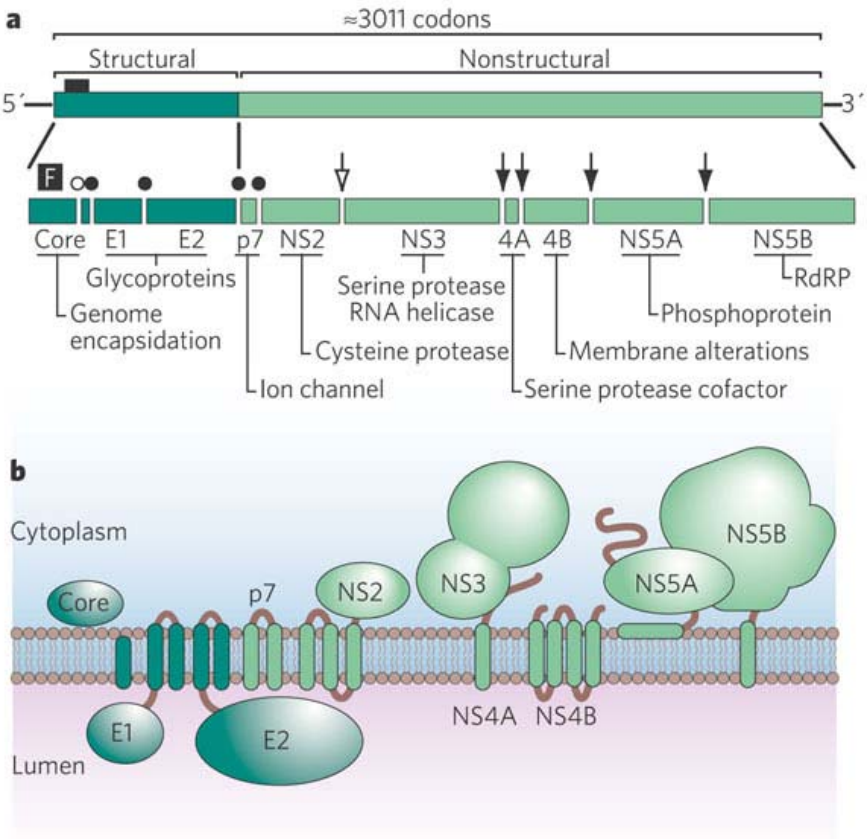
Structural proteins include core protein, which forms most of the nucleocapsid and glycosylated membrane protiens E1 and E2 that form the viral particle. The structural region is followed by the small integral membrane protein, p7, currently thought to be a viroporin (an ion channel that mediates membrane permeability and secretion) The remainder of the genome includes non-structural proteins NS2, NS3, NS4, NS4B, NS5A and NS5B which co-ordinate intracellular processes of the virus life cycle including proteolytic activity, formation of replication complexes, RNA binding and RNA-dependant RNA polymerase (10)

Two viral peptidases play a part in post-translational processing of the polyprotein—the HCV proteins NS2 and NS3/4A. NS2 and the N-terminal domain of NS3 form NS2-3 proteinase, a zinc-dependent metalloproteinase that cleaves the site between NS2 and NS3 in the polyprotein(11). NS3 contains a serine protease, RNA helicase and Nucleotide triphosphatase (NTPase) activities. The coupled NTPase and helicase unwinds duplex RNA structures and has a crucial role in viral replication, whereas the serine protease (which requires the cofactor NS4A) is implicated in polyprotein processing(12)

The non-structural protein NS5B is the RNA-dependent RNA polymerase that catalyses replication of the viral genome. Negative-sense RNA intermediates are formed, which serve as templates for synthesis of new positive-sense RNA(13). These RNA strands are either encapsulated to form new viruses or used as mRNA for viral protein synthesis. The mechanism by which new virions are assembled and released is poorly understood although it probably entails interaction of the core protein with genomic RNA(14)

At each end of the genome are short untranslated regions (UTR) – 5'and 3', required for replication of the genome. The long UTR at the 5' end of the genome is the most conserved of the genome, allowing it to be used as a diagnostic locus (genotype) and marker (HCV RNA) by PCR. Within this region is a translation initiation locus termed an internal ribosomal entry site (IRES) is found. Translation of viral proteins is dependant on IRES which comprises a complex RNA structure element that interacts directly with the 40S ribosomal subunit during translation initiation.

Figure 4. Structure of HCV Virus



1.3.2 Life cycle of HCV

The HCV lifecycle is not completely understood. The virus replicates in the cytoplasm of host cells using its own RNA dependant RNA polymerase in combination with the hosts own protein manufacturing apparatus (figure 5). The functions of some of its proteins have been characterized.

Cell Entry:

HCV entry into hepatocytes is a highly coordinated and multistep process requiring viral and host cell factors. The viral envelope glycoproteins E1 and E2 are essential for HCV entry. Lipoproteins have also been shown to associate with the viral particle and interfere with viral entry. The role of E1 in HCV entry is not completely understood, while several E2 domains play pivotal roles in HCV entry i.e. putative domain binding of two HCV entry factors, CD81 and scavenger receptor class B type 1 (SCAR-B1).

Host factors mediating viral attachment and binding to hepatocytes involve several cell surface molecules that interact with HCV and have been identified as putative receptors for HCV, including CD81, SCAR-B1 binding lectins, low-density lipoprotein receptor (LDLR), highly sulfated heparan sulfate, DC-SIGN and L-SIGN, claudin 1 and occludin (15-17).

In vivo, HCV enters the liver through the sinusoidal blood. Capture of circulating HCV particles by liver sinusoidal cells may thus facilitate the viral infection of neighboring hepatocytes which are not in direct contact with circulating blood. This process may be mediated by DC-SIGN, which is expressed in Kupffer cells that localize close to liver sinusoidal endothelial cells (LSEC) and hepatocytes, and L-SIGN that is highly expressed in LSEC. DC-SIGN and L-SIGN have been shown to bind envelope glycoprotein E2 with high affinity. On hepatocytes, HS glycosaminoglycans represent first attachment sites that may help to concentrate the virus on the target cell surface and allow further interactions with other host factors triggering viral entry (18).

CD81 is a ubiquitously expressed tetraspanin in hepatocytes as well as many other cell types. CD81 has been the first molecule described to interact with a soluble truncated form of HCV E2 and to be a critical host cell factor for viral entry (18).

Human SR-BI is an 82 kDa glycoprotein with a large extracellular loop highly expressed in the liver. SR-BI binds a variety of lipoproteins (HDL, LDL, oxLDL) and is involved in bi-directional cholesterol transport at the cell membrane. SR-BI may play a dual role during the HCV entry process, during both binding and post-binding steps.

CLDN1 and OCLN, are four transmembrane proteins, both identified as host cell factors critical for HCV hepatocyte entry. They are critical components of tight junctions (TJ) regulating paracellular permeability and polarity.

All these host entry factors have been shown to be required for productive HCV infection and suggests that HCV entry may be mediated through the formation of a tightly orchestrated HCV-entry factor complex at the plasma membrane (18). CD81 requires a co-receptor or modulator for HCV entry (15;19), since other cell types expressing CD81 alone are not vulnerable to HCV infection.

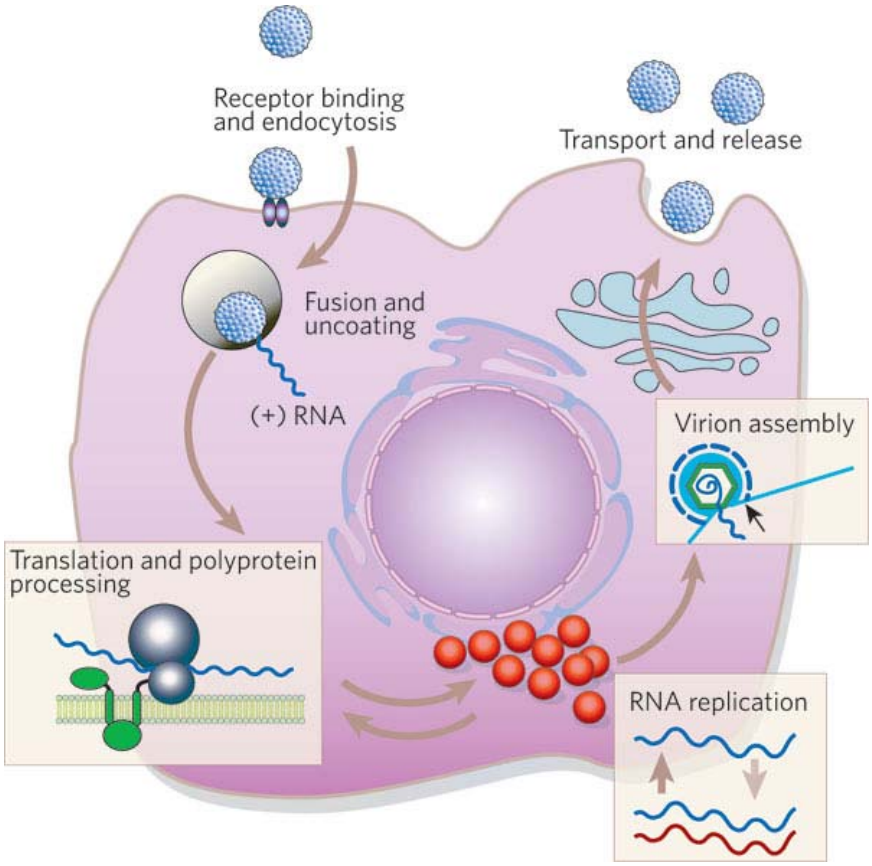
In addition to the presence of specific entry factors in hepatocytes, absence of a particular inhibitor might also contribute to the hepatotropism of HCV. EWI-2wint, is expressed in several cell lines but not in hepatocytes. Ectopic expression of EWI-2wint in a hepatoma cell line susceptible to HCV infection blocked viral entry by inhibition of the interaction between HCV envelope glycoproteins and CD81(20).

Once attached, the virus-receptor complex is internalized. This is thought to be dependant on claritin-mediated endocytosis (18). This transports the virus-receptor complex into early and late endosomes. The acidic pH in endosomes provides an essential cue that triggers penetration and uncoating. Fusion between viral and endosomal membranes is followed by release of the viral nucleic acid into the host cell cytoplasm where translation and replication take place. HCV particles are then assembled and released from the host cell. An alternative route of viral entry is direct cell–cell transmission to infect neighboring cells which is resistant to neutralizing antibodies (18).

Translation of the viral RNA is facilitated by an internal ribosome entry site (IRES) in the highly conserved 5’ untranslated region of the genome, to which 40S ribosome binds. Viral proteins are produced and HCV forms a membrane-associated replication complex, composed of viral proteins, replicating RNA, altered cellular membranes and additional host cell factors. A specific membrane alteration, referred to as the membranous web,

was recently identified as the site of RNA replication in Huh-7 cells harboring subgenomic HCV replicons (21). Intracellular membranes therefore play a crucial role in HCV replication. Recent data underline the importance of a specific lipid environment for HCV RNA replication with the use of lipid rafts, which are microdomains that are enriched in cholesterol and sphingolipid. They are known to play a critical role in many biological processes such as regulators and organizing centers of signal transduction and membrane traffic pathways(22).

Figure 5. HCV life cycle of infection (9)



1.3.3 Viral Heterogeneity:

HCV has a high rate of genetic mutation due to RNA replication being highly error prone, due to lack of a proofreading function capacity of the RNA dependant RNA polymerase, resulting in nucleotide mis-incorporation. Many of these errors are lethal to the virus resulting in a nonfunctional genome or a replication incompetent virus (lethal mutants) but others persist and account for the tremendous viral diversity that is characteristic of HCV.

1.3.4 Quasi-species:

Certain nucleotide substitutions may lead to changes in the amino acid sequence that confer survival advantage to the virus, either by improving replication efficiency or allowing immune evasion through epitope changes. Thus a single host will be infected with multiple quasi-species of the virus, usually with one dominant strain. Quasi-species are families of different, but highly similar, strains that develop within an infected host over time. Quasispecies are genetically related but sequence distinct, with minor differences in <5% of the viral genome (9). There is evidence that quasi-species may be important in the persistence of virus, natural history of infection and the response to treatment. The genetic diversity of HCV has obvious implications for vaccine development.

1.3.5 Genotypes:

Over decades and centuries, the degree of HCV diversity has evolved into several distinct genotypes of the virus. HCV is currently divided into 6 major genotypes each divided further into subtypes within these genotypes varying up to 35% in their nucleotide sequence (23). Within any infected individual there are further minor variants or quasispecies, which are viral variants that differ slightly and emerge under selective pressures of host immunity and drug treatment (24).

The evolution of genotypes has probably been influenced by several factors, including immune selection, infection patterns, and replication efficiency and population migration. For this reason there is a distinct geographical distribution of the six HCV genotypes as shown in figure 6. Some strains are distributed worldwide, whereas others are found only in specific geographic regions. HCV genotypes 1a, 1b, and 3a are highly prevalent "epidemic" strains that are found globally. These strains spread swiftly around the world during the 20th century, most likely through infected blood and blood products, injecting

drug use and invasive medical procedures, and have relatively low levels of genetic variation. In contrast, other HCV strains exhibit high local genetic variation, long-term local persistence but are found in restricted geographic areas. These "endemic" strains reflect long-term transmission at low levels in particular populations and represent the source populations for the epidemic strains. Genotypes 1, 2, and 4 appear to be endemic to regions of West and Central Africa and the Middle East, whereas divergent endemic strains of genotypes 3 and 6 are found in Southeast Asia (25).
In the US, genotype 1a and 1b together account for two thirds of HCV (57% 1a and 17% 1b). Another 15% are affected by genotype 2 (3.5% 2a and 11.5% 2b) and 7% with genotype 3a. Genotype 6 accounts for 3% while Genotype 4 only 1%.

In England, 50% are infected with genotype 1, with 3a being the next most prominent and then genotype 2, together making up another 40-50% of cases. In Europe, genotype 1 is prominent and found in 15-30% of infected Europeans. Genotype 3 is also prominent in Europe, especially in Scandinavia but is most common in India and the Far East. Genotype 4 is found most exclusively in the Middle East and Africa, Genotype 5 in South Africa and Genotype 6 in South East Asia.

However, the picture described above is not static and has changed during the last 15–20 years, due to a combination of several factors including eradication of transfusion-associated infections, improvement in health-care-related standards and a sharp increase in immigration from endemic areas(26).

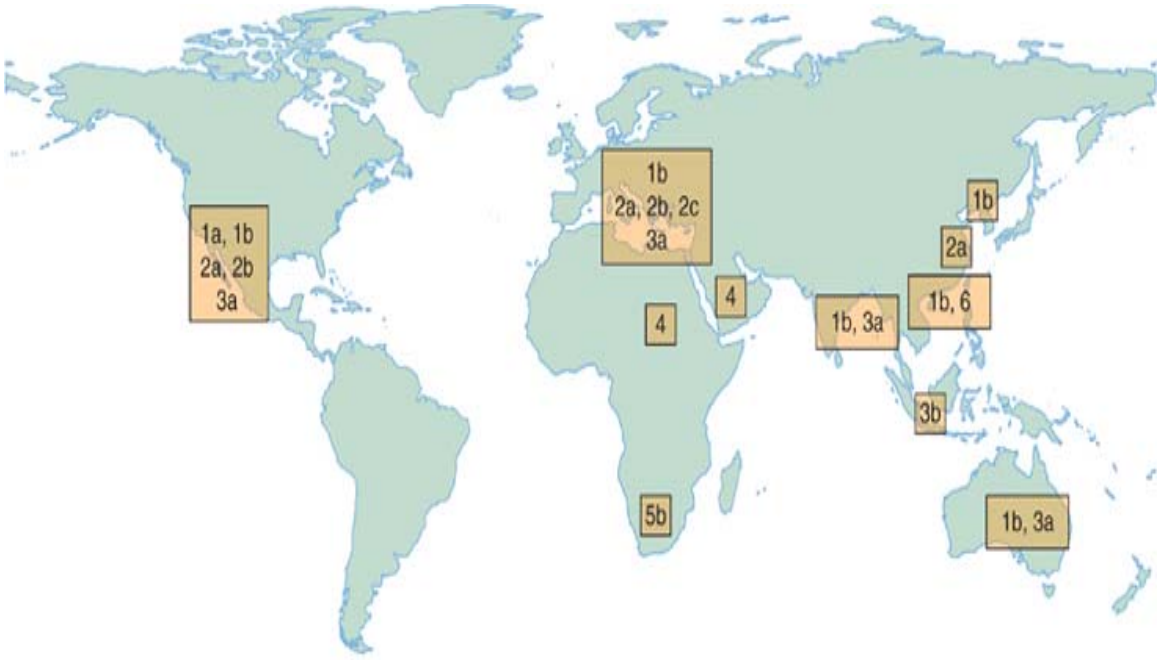
With respect to Europe, since blood products screening was introduced, IVDU has become the main risk factor for HCV transmission and has switched the HCV genotype distribution found in Europe from 1b and 2 among blood donors and young patients to IVDU-related genotypes 1a, 3a and 4. Even among IVDUs, relative genotype distribution is rapidly changing with genotype 4 spreading into Central and Northern Europe and increasing in Southern Europe (26).

Several European countries have sustained high net migration rates for decades, but uncontrolled migratory flows from developing countries are more recent and a substantial proportion in the EU have arrived during the last 15 years. In some countries up to 12% of the population is composed of immigrants and in some countries such as Spain, this is a

new phenomenon. This immigration from endemic areas diversifies HCV genotype distribution with unusual subtypes being identified (1g, 2e 3k, 4a and 4k) (27).

HCV genotypes are clinically significant and relevant to clinical management of chronic HCV infection. They are epidemiologically relevant as genotypes previously in different locations are indicative of the spread of infection. Furthermore, the HCV genotype is the strongest predictive parameter for sustained virological response to interferon based therapy.

Figure 6. Geographical distribution of hepatitis C virus genotype (Adapted from Fang JW, Chow V, Lau JY: Virology of hepatitis C virus. Clin Liver Dis 1997 (1) 493-514)



1.4 Hepatitis C - The Disease:

1.4.1 Routes of infection

Hepatitis C is a parenterally transmitted virus. The commonest route of infection varies with the geographical region studied.

In the UK, the commonest risk factor for transmission of hepatitis C is intravenous drug use (IVDU). In addition needle sharing, sharing of other drug paraphernalia – such as foils and spoons, has been implicated in HCV transmission. It is estimated that 80-90% of injection drug users become positive for anti-hepatitis C antibodies after repeated injections suggesting almost universal exposure in this population.

Exposure to blood or blood products (clotting factors, platelets, immunoglobulins etc) prior to the introduction of screening in 1991, is the next most common mode of transmission in the UK. The risk of HCV infection from blood transfusion prior to 1991 was low but immunoglobulins and clotting factors produced by pooling serum from multiple donors carried a much higher risk. Almost 100% of UK haemophiliacs have evidence of HCV exposure from infected factor VIII. With current screening methods, HCV transmission through transfusion of contaminated blood products has been virtually eliminated.

Other well recognised modes of transmission with less well defined transmission rates include vertical transmission which carries about a 5% transmission rate at the time of delivery and is two to four-fold higher in mothers co-infected with HIV (28). Currently there are no randomised controlled trials to suggest that caesarean section offers an advantage over vaginal delivery (29). The rate of sexual transmission is controversial and in a stable heterosexual relationship is thought to be low (1-3%)(30). Co-infection with HIV, duration of the relationship or chronic liver disease may be factors that increase the risk. Rates of transmission are higher with certain high-risk behaviours amongst men who have sex with men (30). Other modes of transmission include tattooing, body piercing and occupational exposure (needlestick) and sharing toothbrushes and razors.

A potential risk factor can be identified in approximately 90% of persons with HCV infection. In the remaining 10%, no recognized source of infection can be identified, although most persons in this category are associated with low socioeconomic level(31).

1.4.2 Diagnosis:

A combination of serological and molecular assays is needed to diagnose HCV infection. In clinical practice, the usual approach is to test initially for antibodies to HCV (anti-HCV). These antibodies can be detected by enzyme-linked immunoassays (ELISA), with false positives excluded using supplementary reverse immunoblot assay (RIBA)(32). Both of these tests use recombinant viral proteins to detect HCV specific antibodies in the serum. The sensitivity and specificity is high, however, there are greater false positive rates in low risk groups and the tests are less sensitive and specific in immuno-compromised population(33). Antibodies are usually detectable an average of 8 weeks after infection.

Hepatitis C viraemia is documented using HCV ribonucleic acid (RNA). HCV RNA can be measured qualitatively or quantitatively by either reverse transcription PCR or branched DNA (bDNA) assays.

Qualitative assays:
The qualitative PCR assays report results as the presence or absence of HCV RNA. These assays are considered the most sensitive tests for the diagnosis of HCV infection.

Quantitative assays:
Quantitation of HCV RNA titers, i.e., measuring the amount of HCV RNA per millilitre of serum, can be estimated with the use of quantitative reverse transcriptase PCR (RT-PCR) assays or branched-chain DNA (bDNA) assays. Several quantitative HCV RNA assays are commercially available, with the most sensitive assays currently being able to measure down to about 10IU/ml. These assays help to predict the likelihood of response to treatment and the change in the level of HCV RNA during treatment can be used to monitor response.

There is a window from infection to positive PCR of 1-4 weeks during which all PCR tests may be negative despite infection.

1.4.3 Acute infection

Acute hepatitis C infection is usually asymptomatic. About 20% of patients with acute HCV may develop clinical symptoms which may include fever, malaise, weakness, anorexia and jaundice. Acute HCV infection can be severe but fulminant hepatic failure is extremely rare, but may be more common in patients with underlying chronic hepatitis B virus infection(34).

HCV RNA is usually detectable in the serum within 1-4 weeks after exposure. Serum aminotransferases (ALT) begin rising 2-8 weeks after exposure and often reach levels of greater than 10 times the upper limit of normal(35). In self-limited acute hepatitis C, symptoms can last several weeks and subside as ALT and HCV RNA levels decline.

Detectable HCV specific antibodies develop 1 to 3 months after exposure. Almost all patients eventually develop the antibody to HCV, however, titres can be low or undetectable in immunodeficient patients(34).

Spontaneous and sustained virological clearance occurs in between 15-30% of infected individuals. Clinical symptoms are more frequent in those who successfully clear the virus but may be mistaken for a ‘flu like’ illness.

1.4.4 Chronic Infection

85% of HCV infected patients develop chronic infection defined as the persistence of HCV RNA in the blood 6 months after the onset of acute infection. Once chronic infection is established the annual rate of spontaneous clearance is negligible. The majority of these patients are unaware of their acute infection.

Chronic infection may give non-specific symptoms of fatigue, malaise and vague abdominal pains. Frequently the diagnosis of chronic HCV is made when routine blood tests reveal a raised alanine transferase (ALT). A persistently raised and fluctuating ALT is often the only indication of chronic infection. However, in many cases the ALT will be normal, even in cases with significant liver damage.

Chronic infection is associated with positive anti-HCV antibodies in the serum together with persistently detectable HCV RNA by PCR.

1.4.5 Natural history of chronic hepatitis C

The natural history of chronic hepatitis C has been difficult to clearly define because of the long course of the disease and the difficulty in identifying the onset of disease in many patients. Several studies have provided estimates of the proportion of patients with chronic infection who develop cirrhosis within 20 years (35-42) and suggest approximately 20 to 30 percent of chronically infected individuals develop cirrhosis over a 20- to 30-year period of time but the outcome is variable (figure 7). However, a more recent meta-analysis conducted on patients with a variety of etiologies for HCV infection estimated the 20-year cirrhosis prevalence to range from 7% to 18% (43). Chronic HCV is the most common cause of chronic liver disease and the most frequent indication for liver transplantation.

Several factors have been shown to be important determinants of disease progression in individual patients including excessive alcohol intake, concomitant diseases associated with liver injury (hepatitis B, steatohepatitis), concomitant HIV infection, advanced histological grade, persistently elevated aminotransferase activity, male gender, older age, ethnicity, obesity, immunosupression and certain HLA halotypes. Patients who acquire the disease from a blood transfusion may be at increased risk for disease progression compared to those infected via other modes (44). However, this relationship has not been confirmed in all studies with others showing no correlation of prognosis with the source of HCV (39).

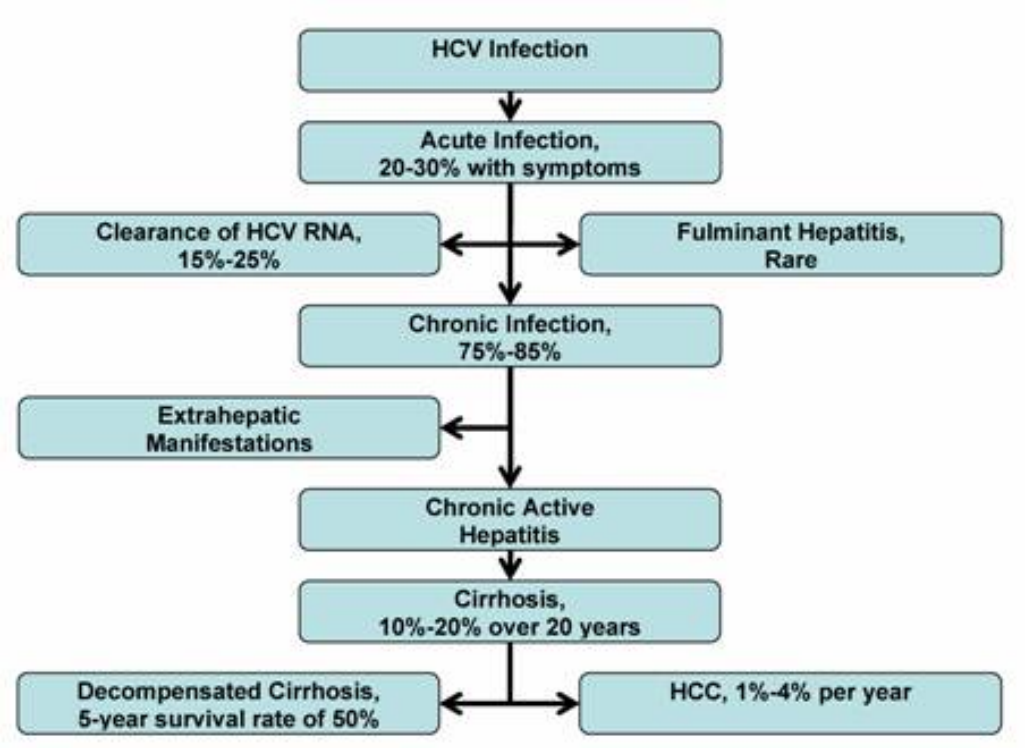
Alcohol - Alcohol consumption appears to be one of the most influential factors driving fibrosis progression in patients with chronic hepatitis C. There is convincing evidence that higher levels of alcohol consumption contributes to the development of progressive liver disease and a higher prevalence of cirrhosis (45).

Age at onset of infection - Several studies have shown a significant association between the rate of fibrosis and the age at time of infection with a significantly higher stage of fibrosis in those infected at an older age (>40 years) (46).

Sex – The mechanism by which sex affects disease progression is not understood; however, an antifibrogenic role for oestrogens has been proposed. The menopause has been associated with a high stage of fibrosis, and hormone replacement therapy has been shown to have a protective role(47).

Co-infection with HIV and HBV – Co-infection with HCV and HIV is very common among hemophiliacs and injection drug users. HIV seropositivity and low CD4 count appears to accelerate HCV liver fibrosis(47). Conversely, HCV has been associated with a faster progression of HIV to acquired immunodeficiency syndrome (AIDS)(48). Similarly, HCV co-infection with HBV also exhibits higher rates of progression to cirrhosis.

Figure 7 The natural history of HCV (42)



1.4.6 Extra hepatic manifestations of chronic hepatitis C

A number of extrahepatic diseases have been associated with chronic HCV infection(49). Approximately 1%-2% of HCV-infected individuals will develop extrahepatic manifestations. HCV shows particular lymphotropism in addition to hepatotropism, and this effect could account for many extrahepatic manifestations(50). Most cases appear to be directly related to the HCV infection but may be from the underlying immune stimulation caused by chronic infection. They can involve multiple organ systems including renal, dermatological, haematological and rhematological:

Haematologic diseases – essential mixed cryoglobulinemia is the commonest extrahepatic condition and cryoglobilins are found in 50% of patients with chronic HCV. However, only 25-30% develop clinical symptoms (51) ranging from fatigue, skin rashes, purpura, arthralgias, Raynaud's phenomenon, vasculitis, proliferative glomerularnephritis, and peripheral neuropathies. The clinical manifestations are thought to be caused by immune complex deposition in various organs. There is also an association between chronic HCV and non-Hodgkin's and Hodgkin's lymphoma.

Renal disease - particularly membranoproliferative glomerulonephritis

Autoimmune disorders – thyroiditis, Sjogren's syndrome and seronegative arthritis

Dermatologic conditions - porphyria cutanea tarda, lichen planus and vitiligo

1.4.7 Assessment of severity of liver damage

Acute HCV infection is not commonly clinically apparent and serological liver function tests are used to assess severity. Biochemical and haematological markers are used to monitor the severity of the hepatitis, whilst PCR is used to identify virological responses. Liver biopsy is seldom required. Any hepatocellular damage associated with the acute hepatitis will resolve completely within 3-6 months if there is sustained viral clearance.

Chronic HCV infection is usually silent and conventional biochemical and haematological markers are of little value. Clinical history and examination may reveal the duration of infection and clinical evidence of chronic liver disease but liver biopsy at present still remains the gold standard for staging liver disease and facilitates detection of secondary pathologies. Liver tissue is obtained by percutaneous, transjugular or intra operative

biopsy. There are several validated scoring systems used to assess the severity of liver damage, allowing intra and inter-patient comparisons to be drawn(52;53).

However, a liver biopsy has many limitations. Because it is invasive and causes significant pain in at least 30% of cases, it is usually not welcomed by patients. There is a small but significant morbidity and mortality rate. In addition, it can only sample a small portion of the liver and is thereby susceptible to sampling error and inter- and intra-observer variation in pathology reporting. It also provides only a static picture of liver architecture in a dynamic disease process.

These issues provided the rationale for the development and use of non-invasive methods for the assessment of liver fibrosis to compliment or replace biopsy and may provide a more rapid and cost-effective means of identifying patients with more advanced fibrosis and cirrhosis, in determining prognosis and informing treatment options in patients with CHC.

A number of tools are currently available or in development for the non-invasive assessment of hepatic fibrosis. Cross-sectional imaging studies such as CT and MRI scans can reliably demonstrate features of advanced liver disease such nodularity and signs of portal hypertension. However, the resolution of hepatic parenchyma with any of the available modalities is insufficient to determine the earlier stages of fibrosis. Transient hepatic elastography is a novel technology demonstrating promise as a non-invasive means of fibrosis determination(54).

Serum markers of liver fibrosis may allow dynamic calibration of fibrosis, and may be more cost effective. The diagnostic accuracy appears greatest and most promising in the studies using a panel of markers(55). Currently there are three serum markers that have been most extensively validated: Fibrospect(56), Fibrotest(57), marketed in the United States by Labcorp and the European Liver Fibrosis Study Group panel(58) using a diagnostic algorithm developed by Bayer HealthCare Diagnostics. All three assays demonstrate significant accuracy in differentiating none or early fibrosis (METAVIR F0-F1) from advanced fibrosis (F2-F4) for HCV but are particularly accurate for detecting advanced fibrosis/cirrhosis (F3,4)(59). Only the ELF test has regulatory approval.

1.5 The Immune Response

1.5.1 Innate Immunity:

The innate immune response forms the first line of defence by a host against pathogens, providing a non specific immediate response. It is directly activated in the early stages by infectious agents, inflammation or tumour and its defensive properties can limit proliferation and spread of pathogen within the body. The onset of the innate immune response is immediate and does not require conditioning through prior exposure to pathogens. The innate immune response delivers two main functions - direct killing of pathogens and initiation and modulation of adaptive immune responses.

It was previously thought that innate immunity was involved only with phagocytosis of pathogens in a non-specific manner, digestion of these and presentation of pathogen-derived antigen to T cells involved in acquired immunity. However, more recently it has been shown that the innate immune system has a greater degree of specificity than previously thought having evolved to recognise conserved pathogen-associated molecular patterns (PAMPS) by proteins termed pathogen-recognition receptors (PRRs). PPRs include toll like receptors and RIG1 like helicases. Toll like receptors (TLRs) expressed on cell surfaces of innate cells are a particularly important group of PPRs (see chapter 1.5.1.3) allowing discrimination between self and foreign pathogens. Recognition of invading pathogens triggers cytokine production and up-regulation of co-stimulatory molecules and phagocytosis, leading to activation of T cells (60). A wide range of innate mechanisms operate which are not programmed by repeated exposure to infection.

The major constituents of innate immunity are cellular and non cellular components. Cellular components include dendritic cells (see chapter 1.5.1.1), NK cells and NKT cells (see chapter 1.5.1.2), neutrophils, granulocytes, monocytes, macrophages and other inflammatory cells including eosinophils and basophils as well as epithelial cells such as the skin, lung and gut. Non cellular aspects include the complement system and acute phase reactants including CRP and fibronectin.

The Interferon system:

The interferon family is subdivided into two subfamilies: Type I and Type II. Type I interferons (IFNs) are a family of monomeric cytokines with an amino acid similarity of 30-

80%, very similar three-dimensional structure (5-alpha helix-bundle), that use the same receptor (IFNAR) to initiate a signaling response. The type I IFNs present in humans are IFN-α, IFN-β and IFN-ω.

Type I IFNs have many roles including their ability to confer an antiviral state on cells, anti-proliferative effects and immuno-regulatory activities. Secreted IFNs are recognised by neighbouring cells and cause them to express potent antiviral proteins. This slows virus multiplication and the organism buys time for establishment of adaptive immune responses. Induced IFNs lead to a number of secondary effects that link the innate immune response with adaptive immune responses. These include the activation of NK cells and cytotoxic lymphocyte responses as well as maturation of plasmacytoid DCs (pDCs).

All type I IFNs bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. Binding to IFNAR leads to dimerization and the activation of the receptor associated Janus-activated kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2). The activated kinases phosphorylate the signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2). The phosphorylated STATs dimerize, associate with IFN-regulatory factor 9 (IRF9) and Interferon-stimulated gene factor 3 (ISGF3G) to form a complex termed ISGF3 transcription factor which enters the nucleus. ISGF3 binds to the IFN stimulated response element (IRES) on cellular DNA to activate the transcription of multiple interferon stimulated genes (ISGs), including the genes for RNA-activated serine/threonine protein kinase, 2’5’-oliogoandenylate synthetase (2’5’OAS), Mx protein GTPases (Mx), RNA-specific adenosine deaminase 1 (ADAR1) and RNA-dependant protein kinase (PKR)(61;62) each of which induce an antiviral state through different pathways.

PKR is a well characterized ISG. PKR activation inhibits mRNA translation by phosphorylating the translation initiation factor 2α. Mx1 is another well characterized human ISG which inhibits RNA synthesis and alters cellular vesicle trafficking. 2’5’ OAS is an important ISG which is stimulated by dsRNA and catalyses the synthesis of 2,5-oligoadenylates (2,5-A). 2,5,-A activates ribonuclease L (RNAse L) which leads to suppression of translation through cleavage and subsequent inactivation of viral RNA(61;62). Levels of induction of 2’5-OAS have been used in studies as a surrogate marker of IFNα activity(63).

1.5.1.1 Dendritic Cells:

Dendritic cells were first discovered by Ralph Steinman and Zanvil Cohn over three decades ago(64). Dendritic cells (DCs) are potent antigen presenting cells, capable of priming naïve T cells, leading to the activation of adaptive immunity(65). DCs, therefore, play a pivotal role linking innate and adaptive immunity.

They are produced by haemopoetic stem cells or CD34 progenitors in the bone marrow. Dendritic cells reside at sites of interaction between the individual and the environment, such as skin and mucosal surfaces and also circulate in blood. In the periphery, resident dendritic cells are found in an immature form acting as sentinels, being on alert for microbial pathogens that they can bind and internalize. In the immature state they are very effective at antigen capture and processing with low expression of MHC and co-stimulatory molecules on their surface.

DCs recognize pathogens through pathogen recognition receptors (PRRs) e.g. toll like receptors (TLRs, see 1.5.1.3), mannose receptors and C type lectins. Upon encountering pathogen, they undergo maturation process that includes acquisition of migration capacity to T cell areas in secondary lymphoid tissues (spleen and lymph nodes) and potent T cell stimulatory ability via up-regulation of cell surface co-stimulatory molecules (CD83, CD86 and CD80) and MHC class I and II(66). This is accompanied by production of cytokines from DCs (IL12, TNFa, IFNa and IL10) which are essential for the polarization of T cells towards a Th1 or Th2 immune response. They also produce chemokines which favour lymphocyte recruitment and activation. Ultimately, these events result in enhanced antigen presentation and a greater capacity to promote T cell proliferation and maintains the adaptive immune response(66;67).

DCs provide naïve T cells with two signals required for their activation(68). The first signal is the antigen-specific signal received as a result of binding of the T cell receptor to peptide presented by the MHC molecule. The second is provided by co-stimulatory molecules such as CD83 and CD86, which are expressed by DCs and which trigger CD28 expressed on naïve T cells. Naïve CD4+ T cells differentiate in Th1 or Th2 cells dependant on the density of peptide presented, co-stimulatory cells expressed and cytokines secreted by DCs. DCs, in turn, then activate NK cells (see chapter 1.5.1.2) to drive the non-specific innate immune response. This maturation process is coupled with downregulation of dendritic cell capacity for antigen uptake.

Subtypes of dendritic cells:

Two distinct groups of dendritic cells have been identified, myeloid (mDC) and plasmacytoid (pDC). Although the common functions are antigen presentation and T lymphocyte activation, they differ in surface markers, migratory patterns and cytokine production(69). The ability of DCs to respond to certain pathogens relates to the TLR expression. The 2 subsets of DCs are distinct in their TLR expression as well as cytokine production profile as discussed below.

Myeloid dendritic cells (mDCs):

Myeloid DCs are $CD11_c^+$, HLA DR^+, CD14-, BDCA1+, CD83+ and express myeloid markers CD13, CD33 derived from a myeloid bone marrow precursor. The receptors that have been identified on mDCs include TLR 2, 3, 4, 5, 7 and 8, C type lectin family of transmembrane glycoproteins: Blood Dendritic Cell Antigen 1 and 3 (BDCA 1 and 3), DC-SIGN, DC-LAMP and the Mannose receptor.

They are found at a frequency of 0.5-1% in peripheral blood(70). They play an important role in adaptive anti viral immune responses and are required for priming T cells in vivo.

In peripheral tissues they act as sentinels, where they alert the immune system to pathogens and then initiate immune response in secondary lymphoid organs by processing viral antigens into peptides for MHC presentation to T cells(71). They are more potent at antigen presentation and stimulation of T cell response than pDCs.

mDCs recognise virus-derived double stranded RNA (dsRNA). Activated mDCs produce high levels of pro-inflammatory cytokines, especially IL12 which is essential for effective innate immune responses(72) and drives T helper type 1 (T_H1) polarization(73) and the production INFγ from the Th1 cells. There is flexibility for them to give rise to Th2 responses but they produce relatively little INFα in response to viral stimulus.

Plasmacytoid dendritic cells (pDC):

Plasmacytoid DCs are HLA-DR bright, CD123bright and CD11c-. They are derived from lymphocytes (as shown by the presence of preTCR α chain) and have a plasma cell like

morphology with rich endoplasmic reticulum. They are a rare subset of blood cells making up only 0.2 – 0.5% of PBMC's(74).

These cells were originally termed 'natural IFN producing cells' (NIPCs) as they were found to be the cell that produced the majority of the type 1 IFN (IFNα, IFN-ß, IFN, IFN-λ) and a broad spectrum of IFNα subtypes in response to enveloped viruses. Although many cell types in the body are capable of producing type I IFN, these lineage-negative cells were found to be particularly potent, with a single cell able to produce 10-100 times more than most other cells in response to a viral stimulus with higher amounts seen with a strong stimulus such as Herpes Simplex virus.

In addition to IFNs, TLR-activated pDCs produce high levels of TNFα and IL-6 in response to enveloped viruses, bacterial components (75) i.e. CpG oligonucleotides and synthetic TLR7 agonists, as well as waves of chemokines including CCL2, CCL3, CCL5, CXCL10 and IL-8. However, they do not produce significant amounts of IL-12p70 (76). pDC also express the recently described IL-28a, IL-28b and IL-29 in response to viral stimulation or to TLR7 and −9 agonists.

Their main functions are directed against viral infections and they represent the key cells in the innate immune responses. In the early stages of viral infection, pDCs promptly secrete massive amounts of type I IFN (77), activating NK cells, NKT cells, B cells, T cells and mDCs. pDCs, once activated, differentiate into mature DCs and play a major role in adaptive immunity, acting as professional antigen presenting cells with the ability to induce the proliferation of naïve T lymphocytes (65) but their efficiency at stimulating T cells compared to mDCs is controversial (67;78).

In health they are found in primary lymphoid organs and not in peripheral tissues, unlike mDCs. They migrate to and accumulate in inflammatory sites and are driven by inflammatory stimuli(69).

Human pDCs uniquely express only TLR7 and 9 which are expressed in their endosomal compartment. This exclusive TLR repertoire allows them to be specialized in microbial nucleic acid sensing, only responding to some DNA and RNA viruses (See TLRs 1.5.1.3). They have also been found to express unique markers, DC Ag-2 (BDCA-2) and DC-Ag-4 (BDCA-4) belonging to the C type lectin family (79).

1.5.1.2 NK Cells:

NK cells represent an important lymphocyte population of the innate immune system and comprise 10-15% of PBMCs. They play an important part in the first line of defence against viral infections, rapidly recognising and killing virus-infected cells via perforin release and induction of apoptosis and also secreting inflammatory cytokines, such as IFNγ, which have direct antiviral effects, inhibiting viral replication as well as activating and polarizing Th1 and cytotoxic lymphocyte responses(80;81). Therefore they provide a pivitol link between innate and adaptive immunity.

NK cells express CD56, the 140kDa isoform of the neural cell adhesion molecule (NCAM). NK cells are not a homogeneous cell population and can be divided into several subsets according to functional and phenotypic differences.

The major subset of NK cells (90%) in peripheral blood dimly expresses CD56 and shows co-expression of CD16 (82) with a low affinity receptor for the IgG ($CD56^{dim}$ / $CD16^{+}$). This subset represent about 7% (2-14%) of all PBMCs and 90% are found in the peripheral blood (75). This cell population also expresses killer cell immunoglobulin-like receptor (KIRs) and CD94-associated lectin-like NKG2 receptors and have homing markers for inflamed peripheral sites. Consequently, they exhibit strong antibody-dependant cell-mediated cytotoxicity (ADCC) and natural cell-mediated cytotoxicity(81;83) but have low cytokine production capacity.

Another subset of NK cells is characterised by high expression of CD56 and is negative for CD16 ($CD56^{bright}$ / $CD16^{-}$). These are a minor subset of NK cells in peripheral blood (10%) and represent about 1-2% of all PBMCs. They express homing markers for secondary lymphoid tissues(81) where they accumulate and are found predominantly in the liver. They lack the expression of killer cell immunoglobulin-like receptors (KIRs), contain low levels of perforin, and for this reason they neither show antibody-dependant cell-mediated cytotoxicity (ADCC), nor strong natural cell-mediated cytotoxicity. However, they have high cytokine production capacity, unlike $CD56^{dim}$CD16+ cells and thus it is suggested that they have an immunoregulatory role. They predominantly secrete INF-γ(81;84;85) (as well as TNFα, GM CSF, IL10 and IL13) which aids reciprocal interactions with other cells of the innate and adaptive immunity, mainly dendritic cells and T cells(86;87).

NK cell receptors:

The functional activities of NK cells are precisely controlled by a variety of stimulatory, co-stimulatory and inhibitory receptors(88). NK receptors regulate NK cell mediated cytotoxicity and cytokine production with a balance between stimulatory and inhibitory receptors belonging to the immunoglobulin-like receptor and C-type-lectin receptor families. The receptors bind to various ligands on target cells to regulate NK response. The dominant default feature of NK class 1 receptors is inhibition and there needs to be downregulation of these inhibitory signals or multiple activating signals to override these.

Receptor classes:

Immunoglobulin class: (Ligands HLA-A,B,C)
Killer Cell Immunoglobulin Like Receptor (KIR)
KIRnDSx – activating
KIRnDLX – inhibitory

Lectin like receptors: (Ligands MIC-A and MIC-B)
Natural Killer group 2, member A (NKG2A) / CD94 – inhibitory
Natural Killer group 2, member C NKG2C / CD94 – activating
Natural Killer group 2, member D NKG2D / CD94 – activating

Natural Cytotoxicity Receptors:
NKp30, NKp44 and NKp46

Others:
ILT2 /LIR1 (leukocyte inhibitory Receptor)

NK cell function:

The main functions of NK cells are cytotoxicity of tumour and virus infected cells and cytokine release. NK cells are the primary anti-viral effector population of the innate immune system (89). They are activated early after viral infection by type I IFN and IL-12 and in turn rapidly secrete large amounts of cytokines such as IFN-γ and chemokines, IP-10 and MIP-1, which may have direct effects on the hosts' immune response as well as serving to recruit other cell types involved in host defence. IFN-γ is considered the prototypic NK-cell cytokine and its production by NK cells is known to shape the T_H1

immune response, activate APCs with subsequent activation and maintenance of adaptive immune responses as well as activating macrophage killing. IFN-γ also has anti-proliferative effects on viral and malignant transformed cells.

Production of IFN-γ from NK cells requires 2 signals(89) and one of these almost always includes IL-12. The second can be IL-1, IL-2, IL-15 or IL-18, or engagement of an NK-activating receptor such as CD16 (FcγRIIIa) or NKG2D. These cytokines are released from DCs, monocytes and macrophages making interaction between these cells important.

$CD56^{dim}$ NK cells have the ability to lyse target cells without prior stimulation and without the need for antibody recognition(89). NK cells do not have Ag specific receptors, but mediate MHC-unrestricted cytolysis of susceptible cells. NK cells recognise infected cells in an antigen independent manner and destroy them due to their cytotoxic activity. They use two main pathways to induce apoptosis of target cells(90): granule exocytosis and death-receptor engagement. NK cells can also be directly activated to induce antibody-dependant cellular cytotoxicity (ADCC)(81). NK cell mediated cytotoxicity is regulated through a balance between inhibitory and activating receptors.

NK cells and DC cross talk:

Interactions between DCs and NK cells have been documented in a variety of settings with emerging evidence of complex bidirectional crosstalk between the two cell types which help each to acquire their complete functions both in the periphery and secondary lymphoid organs(90).

Mature DCs are able to stimulate NK cells via the production of cytokines, IL-12, IL18, IL-15 and IFNα and through the expression of NK activating ligands with subsequent activation of adaptive immune responses. IL-12 and IL-18 promote optimal cytokine production by NK cells while IL15 has been shown to promote NK cell survival and differentiation in vivo. Mature mDCs are a major source of IL-12 which enhances NK cell-mediated cytotoxicity and IFN-γ production. Type I IFN secreted by pDCs are crucial for DC-induced NK-cell activation and have an important role in the induction of NK cell cytotoxicity by modulating the expression of ligands for the NK cell receptor NKG2D(91). DCs may also activate NK cells indirectly by promoting the expansion of antigen-specific T cells, which secrete IL-2, which in turn activates NK cells.

NK cells can reciprocally regulate the function of DCs. NK cells produce TNF-α which is involved in maturation of DCs leading to the subsequent activation of the adaptive immune response. It has been reported that immature DCs are uniquely susceptible to NK cell mediated cytolysis while mature DCs are protected (91). NK cells may also play a role in both differentiation of adaptive T cells as well as their subsequent proliferation.

1.5.1.3 Toll Like Receptors:

Toll like receptors (TLRs) were first discovered in Drosophilia (fruit fly), and were found to be involved in their immunity to fungal infections. They were discovered in mammals in 1997 and at least 11 TLRs have been identified in mammals(92).

TLRs are a type of pathogen recognition receptor (PRR). They are type 1 intergral transmembrane proteins critical to innate immune responses and are the first line of defence against pathogens.

Conserved pathogen-associated molecular patterns (PAMPs) are recognised by TLRs expressed on the cell surface of immune cells which include macrophages, monocytes, dendritic cells and B cells. PAMPS come from a range of sources including bacterium, viruses, fungal and protozoan components.

TLR Structure:

The structure of all the TLRs is similar. They all have an extracellular ligand recognition and binding domain that contains leucine-rich repeats. All have a single transmembrane domain. The cytoplasmic domain is homologous to the interlukin-1 receptor and is called a Toll/IL-1 receptor or TIR domain.

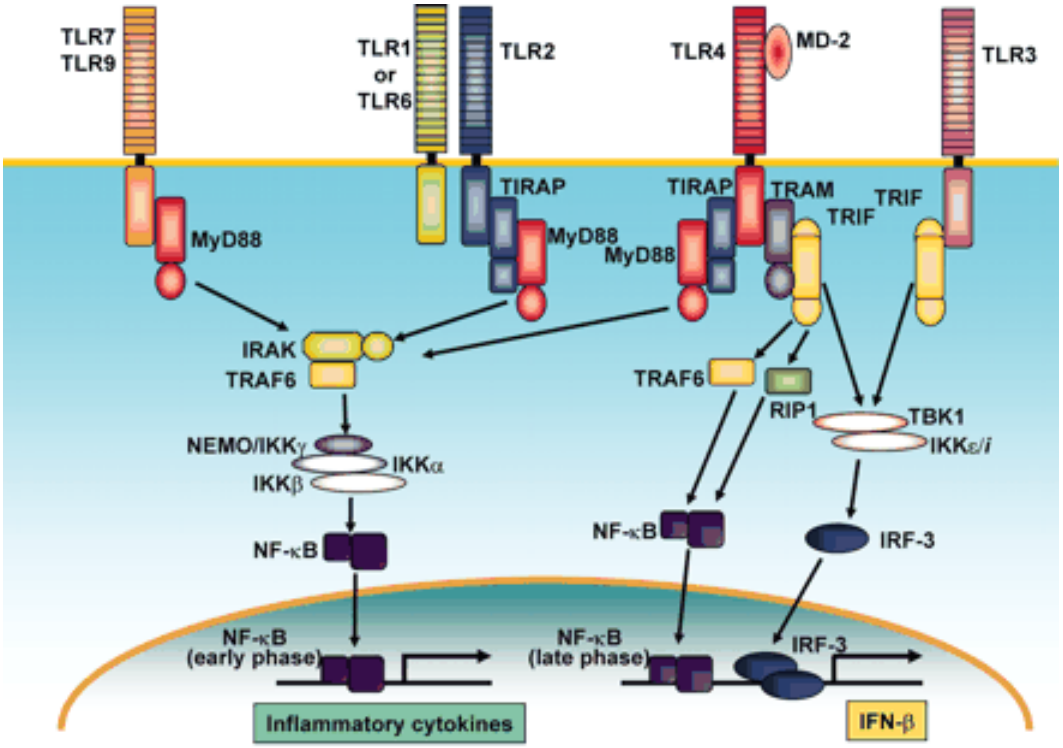
TLR Signalling:

After PAMP recognition by TLRs, expressed on the surface of effector cells of the innate immune response, signalling pathways are activated which originate from the cytoplasmic portion of TLRs, the Toll/IL-1 receptor (TIR) domain. Downstream signals are transmitted via the recruitment of TIR containing adaptor proteins including myeloid differentiation

factor-88 (MyD88), the TIR-associated protein (TIRAP), the TIR domain-containing adaptor-inducing IFN (Trif) and the Toll-receptor-associated molecule (TRAM)(93) with the downstream activation of transcription factors including the NFkB protein complex and AP-1 (Figure 8)(93-97). These transcription factors regulate a multitude of genes, including those encoding pro-inflammatory cytokines, chemokines, antimicrobial genes and type 1 IFNs through induction of transcription factors IRF3 and IRF7.

In addition, upregulation of MHC molecules and co-stimulatory molecules (CD40, CD54, CD80, CD86) results in activation and maturation of antigen presenting cells (APCs). In turn, these APCs, dendritic cells being the most potent, can present antigen, initiate T cell priming and contribute to the establishment of an adaptive immune response. Thus these stimuli serve to link innate and adaptive immunity.

Figure 8. TLR signalling pathway (93)



Which signalling pathway is activated following TLR engagement varies depending on the recruitment or not of adaptor protein (MyD88).

MyD88 dependant pathway:

The MyD88-dependant pathway is the best studied pathway (Figure 8). Activation of all TLRs, except TLR 3, by pathogen triggers recruitment of MyD88 adaptor molecule (contained within the TIR domain), either by itself (TLR5, 7, 8 and 9) or together with adaptor molecule TIR domain-containing adaptor molecule (TIRAP; TLR 1/2, 2/6 and 4)(92;96). MyD88 contains a C terminal TIR domain and structural region, the N terminal death domain, which allows it to associate with molecules that further transmit signals.

Upon stimulation of TLRs, the MyD88 adaptor connects through interlukin-1 receptor associated kinases 1 and 4 (IRAK1 and IRAK4), tumour necrosis factor receptor associated factor 6 (TRAF6) and transforming growth factor beta activated protein kinase 1 (TAK1). These molecules are linked to at least three major downstream pahways (84): the NF-kB pathway, the pathway involving mitogen-activated protein kinases (MAPKs) and the Interferon regulatory factor (IRF) pathways.

The NF-kB pathway:

NF-κB is a protein complex which plays a key role in regulating the immune response to infection. All proteins of the NF-κB family share a Rel homology domain in their N-terminus. There are five proteins in the mammalian NF-κB family (NFKB1, NFKB2, RELA, RELB and REL).

In unstimulated cells, the NF-κB dimers are sequestered in the cytoplasm by a family of inhibitors, called IκBs (Inhibitor of κB). Activation of the TAK1 adaptor may also lead to activation of the inhibitor of NF-kappa B kinase (IKK). Once activated, the IKK complex induces phosphorylation and subsequent degradation of IkB (98). This phosphorylation reaction dissociates IkB from nuclear factor-kB (NF-kB) allowing the NF-kB complex to translocate into the nucleus where it induces genes encoding proinflammatory proteins (IL6, IL-1B, TNFa, IL12p40) and co-stimulatory molecules.

The MAPK pathway:

MyD88 connects through IRAK 1 and 4, TRAF6 and TAK1. TAK1 phosphylates members of the MAPK kinase family which subsequently activate JNK and p38MAPK.
This complex cascade culminates in activation of the hetrodimeric transcription factors known as AP-1 and the production of pro-inflammatory cytokines and costimulatory molecules.

IRF pathway:

This pathway leads to type I IFN production and subsequently to induction of IFN-responsive genes. The pathway is activated through TLR7, 8 and 9 by inducing the transcription factors, interferon regulatory factors, IRF3 and 7 (figure 9).

IRF3 is a constitutively expressed protein that resides in the cytoplasm in unstimulated cells, but virus infection triggers IRF3 phosphorylation which allows IRF3 to form a homodimer, translocate into the nucleus, and bind to DNA to regulate expression of IFNβ and other IFN-stimulated genes (ISGs)(99).

IRF7 is structurally the most similar to IRF3, whose activation is also controlled by phosphorylation. IRF7 is constitutively expressed by pDCs(100) but weakly in other unstimulated cells but dramatically induced by stimulation with type I IFN or other stimuli. Activation of pDCs through TLR 7 or 9 activates adaptor molecule, MyD88, which directly interacts with IRF7 through its death domain(67). This interaction seems to induce IRF7 phosphylation and activation by forming a complex containing adaptor molecule TRAF6, and protein kinases IRAK1 and IRAK4. IRAK4 facilitates phosphorylation of IRAK1. Activated IRAK1 then causes the phosphorylation of IRF7 and TRAF6 mediates ubiquitination required for IRF7 activation. IRF7, once activated, is translocated to the nucleus where it regulates expression of target genes. ISRES (IFN-stimulated response element) found in the promoters of genes that are regulated by type 1 IFNs, is bound by IRF7.

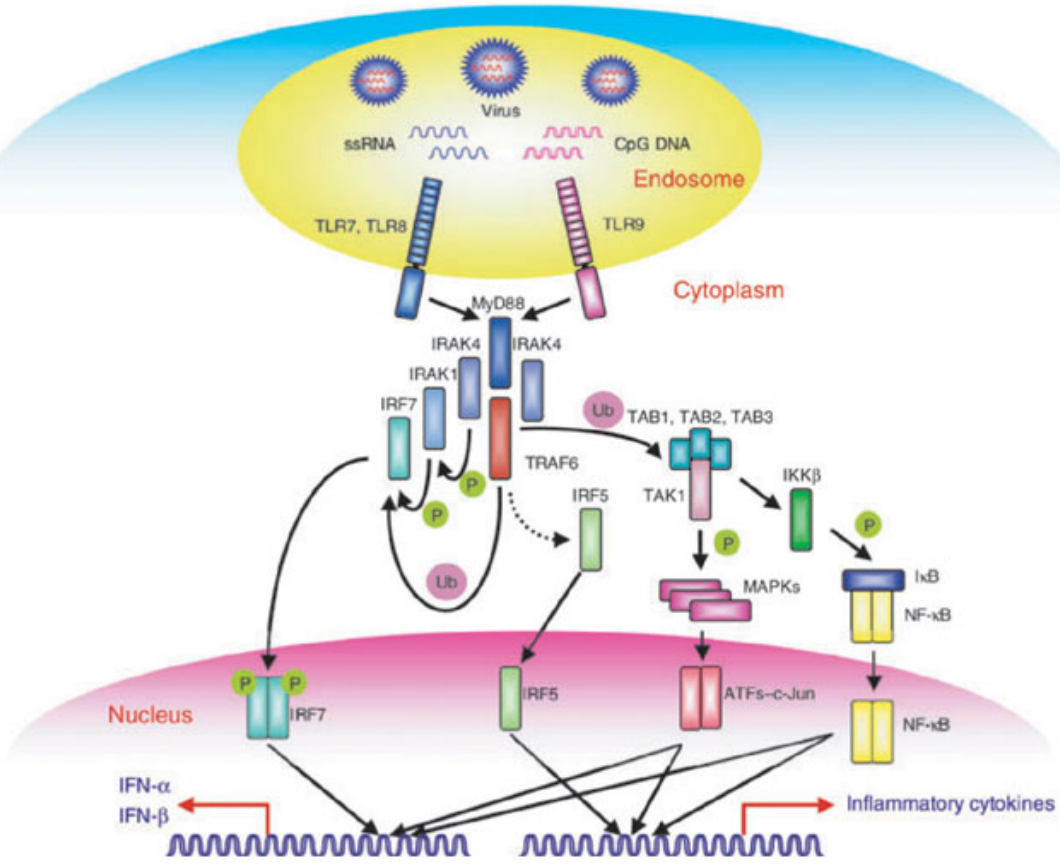
TRAF6 may also interact with TRAF-family-member-asoociated NF-kappaB activator-binding kinase 1 (TBK1), which activates interferon regulatory factors, IRF3 and IRF7, leading to production of type 1 Interferons.

IRF5 is also required for proinflammatory cytokine induction through TLR 7, 8 and 9. IRF5 associates with MyD88 and translocates to the nucleus in response to TLR activation. In the nucleus it binds to typical interferon-stimulated response element motifs in promoter regions of cytokine genes causing them to be expressed. This culminates in nuclear translocation of NFκB with activation of mitogen activated protein (MAP) and transcription of immunnologically relevant genes.

MyD88 Independent Pathway(93):

There is a MyD88 *independent* pathway, used by TLR3 and TLR4, which culminates in activation of NF-kB and induction of IFN-inducible genes and co-stimulatory molecules. The pathway utilizes adaptors, TRIF (TIR domain containing adaptor inducing interferon b), TRAM**,** TIRAP (TIR domain containing adaptor molecule/Mal). TIRAP is also involved in My88-independent activation of IRF 3 to induce INF type 1. In response to stimulation, IRF3 is phosphorylated by IKKs and TBK1 and then translocates to the nucleus to induce mainly INF β.

Figure 9 Recognition of viral nucleic acids by TLR7, TLR8 and TLR9 in pDCs with production of IFNα and proinflammatory cytokines (67)



Specific TLRs:

Each TLR family recognises molecular patterns associated with a specific class of microbial agents(101) .

TLR1, TLR6 and TLR10 form heterodimers with TLR2. The hetrodimer of TLR2 with TLR1 is expressed on mDCs and the TLR2 and TLR6 hetrodimer is expressed on mast cells and they signal via the MyD88 pathway with the TIRAP adaptor. The hetrodimers recognize fungi (Sarcomyces cereviciea, Candida albicans, and Aspergillus fumigatus), parasites (Trypanosoma cruzi), Gram positive bacteria-derived peptidoglycan (PGN) and bacterial lipoproteins, lipoarabinomannan from *Mycobacterium tuberculosis*, Treponema pallidum-derived glycolipid and lipoteichoic acid. TLR2 can also recognize certain viruses such as cytomegalovirus, measles virus and core and NS3 proteins of HCV.

TLRs 3, 7, 8 and 9 are intracellular receptors specialized in the recognition of viral nucleic acids and recognize ligands in endosomes and lysosomes.

TLR3 detects double stranded RNA (dsRNA)(102). It may also bind to synthetic double stranded RNA such as polycytidylic acid (Poly I:C). TLR3 is expressed on B and T lymphocytes and NK cells. It signals via a MyD88 *independent* pathway (Trif) and induces production of type 1 IFN in addition to inflammatory cytokines

TLR-7, 8 and 9 are located on the endosomal membrane and are homologous.
TLR-7 and TLR8 are very similar in their ligand binding and signaling. TLR7 is expressed in B cells and plasmacytoid dendritic cells. There is some controversy as to whether myeloid DCs express TLR-7. TLR8 is expressed in mDCs and mast cells. They recognize synthetic imidazoquinolone-like molecules, guanosine analogues such as loxoribine and single-stranded RNA derived from viruses(103). Both signal directly through MyD88. Hepatitis C is a single-stranded ss-RNA virus and therefore susceptible to detection by TLR7 and 8. It induces a rapid IFN response with a rapid induction of IFN-regulated genes, such as 2’5’oligoadenylate synthetase.

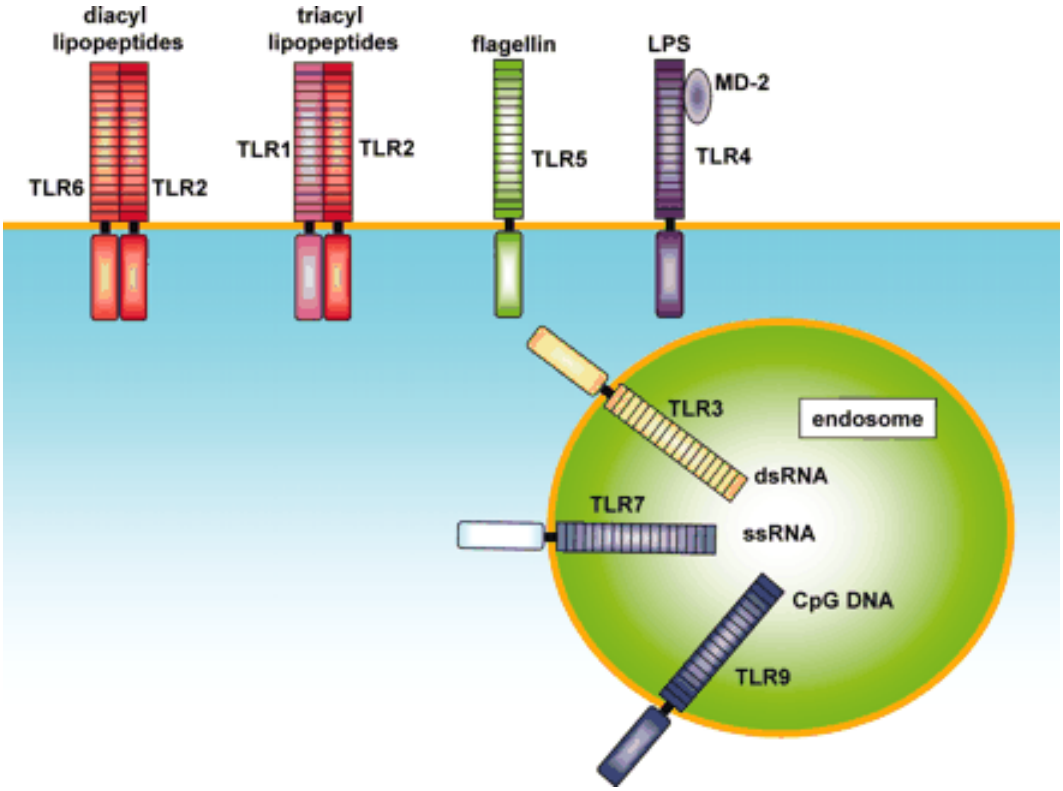
TLR 9 recognises unmethylated 2’-deoxyribo (cytidine-phosphate-guanosine (CpG) DNA(104). Bacterial DNA contains unmethylated CpG motifs, which confer immunostimulatory activity. In vertebrates, the frequency of CpG motifs are severely reduced and the cysteine residues of CpG motifs are highly methylated, leading to

abrogation of the immunostimulatory activity. Bacterial DNA and synthetic ODN containing unmethylated CpG-dinucleotide (CpG-DNA) lead to activation of TLR9 which is expressed on pDCs, B cells and T cells. TLR9 subfamily transmit signals by solely utilizing MyD88.

The principal ligand for TLR4 is the lipopolysaccharide (LPS) of gram-negative bacteria(105). Additional ligands include the fusion protein of respiratory syncytial virus, Cryptococcus neoformans and the plant product Taxol. TLR4 is expressed on mDCs and mast cells and its signaling utilizes 2 pathways, namely, MyD88 with TIRAP but it may also induce type 1 IFN through the MyD88 independent pathway via TRAM and Trif.

Bacterial flagellin has been identified as a TLR5 ligand(106). It is expressed on mDCs and transduces signals via MyD88.

Figure 10. Human TLRs and their PAMPs (94)



1.5.2 Adaptive Immunity:

The adaptive immune response is composed of humoral immune responses (antibodies produced by B cells) and cellular immune response (CD4 and CD8 T cells), most important in viral infections. It is characterised by specificity, flexibility and memory and is required for resolution of infection.

Flexibility is provided by the unique way in which T and B cell recognise antigens. Unlike innate responses, which use a fixed repertoire of inherited receptors, T and B cells have the capacity to undergo a recombination of antigen receptor genes to create novel and unique antigen receptors capable of recognising virtually any antigen. Memory is provided through the long term persistence of B and T cells that have encountered antigen that retain the capacity to deliver rapid and specific responses to re-exposure to antigens.

1.5.2.1 The Humoral Immune Response:

B lymphocytes (B cells) are the effector cells of the humoral immune response. The principal function of B cells is to make antibodies against soluble antigens which they express on their surface. Antibody expressed on the B cell membrane acts as the B cell receptor. B cell activation requires not only recognition of antigen but also clonal proliferation and differentiation into plasma cells (Naive B cells can be activated in a T-cell dependent or independent manner, but two signals are always required to initiate activation). Secreted antibodies bind to antigens on the surfaces of invading microbes (such as viruses or bacteria), which flags them for destruction.

B cell activation depends on one of three mechanisms:
T cell-independent (polyclonal) activation where the B cell is stimulated directly by antigen to produce antibodies.
T cell- independent activation, in which macrophages present several of the same antigen in a way that causes cross-linking of antibodies on the surface of B cells.
T cell-dependant activation in which an antigen presenting cell (APC) presents a processed antigen to a helper T (T_H) cell, priming it. When a B cell processes and presents the *same* antigen to the *primed T_H cell*, the T cell releases cytokines that activate the B cell. Signal 1 is between the B lymphocyte expressed antigen and the TCR. Signal2

is a co-stimulatory signal, in this case between the CD40 surface molecule and its ligand, CD40L on the T_H2 lymphocyte.

Antibodies:

Also, known as immunoglobulins, these are glycoproteins synthesized and secreted by plasma cells that are derived from B cells.

Structure: Immunoglobulins are composed of four polypeptide chains, 2 heavy and 2 light, bound together by di-sulphide bonds (figure 11).

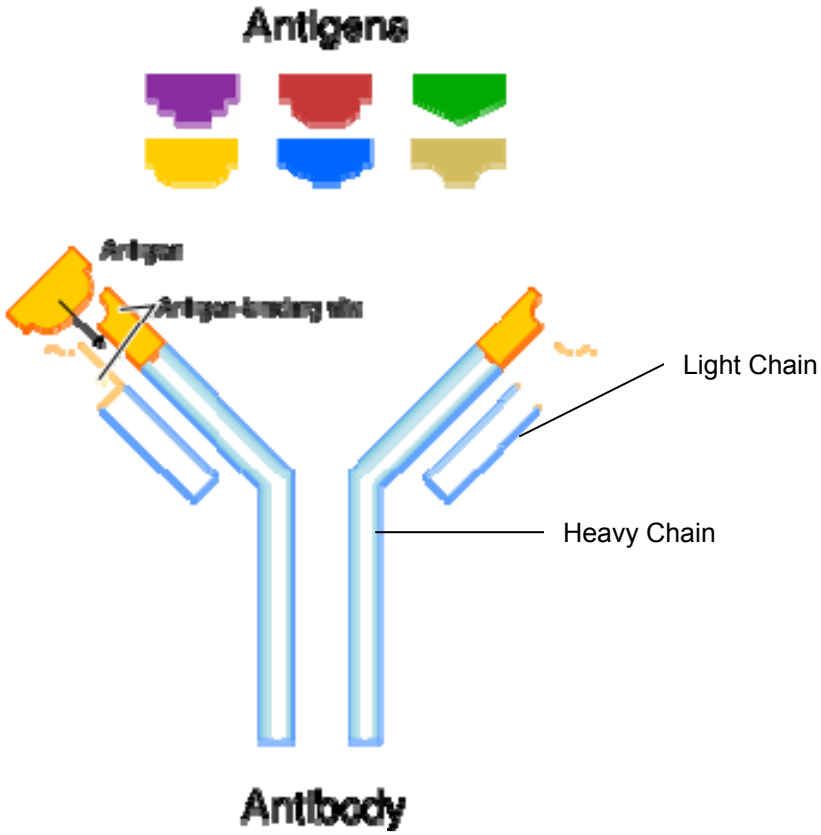
Heavy chains: There are five types of mammalian Immunoglobulin heavy chain denoted by the Greek letters: α, δ, ε, γ, and μ. The type of heavy chain present defines the *class* of antibody; these chains are found in IgA, IgD, IgE, IgG, and IgM antibodies, respectively.

Heavy chains contain one variable domain and three or four constant domains, depending on the class of the immunoglobulin. The constant region is identical in all antibodies of the same isotype, but differs in antibodies of different isotypes. The variable region of the heavy chain, at the amino-terminal domain, differs in antibodies produced by different B cells, but is the same for all antibodies produced by a single B cell or B cell clone.

Light Chains: In mammals there are two types of light chain, which are called lambda (λ) and kappa (κ). A light chain has one constant domain and one variable domain. Each antibody contains two light chains that are always identical.

*Fab and Fc Regions***:** The antigen binding (Fab – Fragment, antigen binding) region is composed of the combined amino-terminal ends of the heavy and the light chain representing the hypervariable domains of one heavy and one light chain of the antibody. The carboxy-terminal portions of the heavy chains trigger various effector functions following combination of the immunoglobulin molecule with its specific antigen and is termed the Fc (fragment crystallizable) region.

Figure 11: Structure of an immunoglubulin molecule



Function:

Antibodies are used by the immune system to identify and neutralize foreign objects like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target. By binding their specific antigens, antibodies can cause agglutination and precipitation of antibody-antigen products, prime for phagocytosis by macrophages and other cells, block viral receptors, and stimulate other immune responses, such as the complement pathway.

1.5.2.2 Cell Mediated Immunity:

T cells are lymphocytes that play a central role in cell mediated immunity. T lymphocytes mature in the thymus and respond to antigen encountered on the surface of infected host cells. T cells can only recognise and be activated by antigen bound to Major Histocompatibility Complex (MHC) or Human Leukocyte Antigen (HLA) Molecules.

T Cell Subsets:

There are several different subsets of T lymphocytes(107):

1. *T Helper (T_H) cells*

T helper cells (T_H cells) recognise antigens presented by MHC class II molecules on the surface of antigen presenting cells and subsequently CD4 T cells perform multiple effector functions, including direct activation of macrophages and antigen-specific B cells as well as activation of CD8 T cells in a cytokine dependant manner. The cytokines they secrete during the initial T_H-APC interaction provoke proliferation of further T_H lymphocytes and depending on the cytokine signal received, these cells differentiate into either T_H1 or T_H2 cells which in turn secrete characteristic groups of cytokines:

T_H1 cells secrete proinflammatory cytokines: INFγ, TNFβ and IL2 and promote cell mediated immunity and cytotoxic T cells as well as delayed hypersensitivity responses while T_H2 cells secrete IL4, IL5, IL6, and IL13 and promote humoral immune responses.

The cytokine profiles of these two T_H subsets are mutually inhibitory making a switch from cell mediated to humoral immunity or *vice versa* a rare occurrence once dominance is established. Uncontrolled T_H1 and T_H2 responses can cause chronic inflammatory, autoimmune disease and allergy respectively.

In T_H cells the defining accessory molecule is CD4 which is invariable and therefore conserved between individuals of a given species but unable to specifically recognise variable ligands such as antigens.

2. *Cytotoxic T (T_C) cells*

Cytotoxic T cells recognise antigens presented by MHC class I molecules. Subsequently, T_C cells perform different effector functions, such as the killing of infected target cells

through perforin mediated direct cytolysis and the secretion of cytokines such as IFNγ and TNFα that can inhibit viral replication without killing the infected cell.
T_C cells express the invariable accessory molecule CD8. This is either a hetrodimer of di-sulphide linked α and β invariant chains or homodimer with α chains. The CD8 molecule binds to the class I MHC molecule and acts to stabilize the TCR bond to the peptide MHC complex and transduce an activating signal to the T cell (108;109).

3. *Memory T cells*
These cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They are capable of rapid clonal expansion to generate large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise two subtypes: central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells). Memory cells may be either CD4+ or CD8+.

4. *Regulatory T cells*

Formerly known as suppressor T cells, these cells are crucial for the establishment of immunological self-tolerance and maintenance of immune hameostasis(110;111). Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus. Two major classes of CD4+ regulatory T cells have been described, including the naturally occurring T_{reg} cells and the adaptive T_{reg} cells.

Naturally occurring T_{reg} cells (also known as $CD4^+CD25^+FoxP3^+$ T_{reg} cells) constitutively express the interlukin (IL)-2 receptor α chain (CD25) and arise in the thymus. Their development and function depend on the expression of the transcription factor FOXP3. Naturally occurring T_{reg} cells can be distinguished from other T cells by the presence of an intracellular molecule called FoxP3 which is a master regulator of T_{reg} function. Mutations of FoxP3 result in T_{reg} deficiency(112).

Adaptive T_{reg} cells may originate during a normal immune response in response to newly encountered T cell epiptopes in the absence of co-stimulatory signals(113). They include IL-10-secreting T regulatory 1 (Tr1) cells, transforming growth factor (TGF)-β-secreting T helper (Th)3 cells, certain γ/δ T cell receptor (TCR)-expressing $CD4^-CD8^-$ T cells, and $CD8^+CD28^-$ T cells.

5. *Natural Killer T cells (NKT cells)*

These cells are a special kind of lymphocyte that link the adaptive immune system and the innate immune system. Unlike conventional T cells that recognize peptide antigen presented by MHC molecules, NKT cells express both CD56 and T cell receptor. They recognize glycolipid antigen presented by a molecule called CD1d. Once activated, these cells can perform functions ascribed to both T_h and T_c cells (i.e., cytokine production and expression of cytolytic/cell killing molecules). They interact with antigen presenting cells and impact on the adaptive immune response.

6. Gamma delta T cells (γδ T cells)

These cells represent a small subset of T cells that possess a distinct T cell receptor (TCR) on their surface which is made up of one γ-chain and one δ-chain. This group of T cell are found at their highest abundance in the gut mucosa, within a population of lymphocytes known as intraepithelial lymphocytes (IELs) (114).

The antigenic molecules that activate γδ T cells are still largely unknown. However, γδ T cells are unusual in that they do not seem to require antigen processing and MHC presentation of peptide epitopes although some recognize MHC class IB molecules. Furthermore, γδ T cells are believed to have a prominent role in recognition of lipid antigens. Human Vγ9/Vδ2 T cells, which constitute the major γδ T cell population in peripheral blood, are unique in that they specifically and rapidly respond to a set of non-peptidic phosphorylated metabolites precursors of cholesterol, collectively named phosphoantigens. Phosphoantigens are produced by virtually all living cells.

Mature γδ T cells are divided into functionally distinct subsets. Like other T cell subsets bearing invariant TCRs, such as CD1d-restricted Natural Killer T cells, γδ T cells exhibit several characteristics that place them at the border of the innate and adaptive immune system. On one hand, γδ T cells may be considered a component of adaptive immunity in that they rearrange TCR genes to produce junctional diversity and will develop a memory phenotype. However, the various subsets may also be considered part of the innate immunity where a restricted TCR may be used as a pattern recognition receptor (115;116). Recent work has shown that human Vγ9/Vδ2 T cells are also capable of

phagocytosis, a function previously exclusive to innate cells such as neutrophils, monocytes and dendritic cells.

The T cell Receptor (TCR):

The TCR is a hetrodimer consisting of alpha and beta chains in 95% of T cells, covalently joined by di-sulphide bonds (figure 12). Activation by engagement with antigen and MHC molecule, results in a series of biochemical events mediated by associated enzymes, co-receptors and specialized accessory molecules.

Each chain of the TCR is a member of the immunoglobulin superfamily and possesses one N-terminal immunoglobulin (Ig)-variable (V) domain, one Ig-constant (C) domain, a transmembrane/cell membrane-spanning region, and a short cytoplasmic tail at the C-terminal end.

Analogous with antibody, the TCR variable domain is responsible for antigen recognition and comprises V, D and J segments.

There are three hypervariable or complementary determining regions (CDRs) on each chain of the TCR (α and β), but there is an extra region in the V domain on the β chain responsible for binding super-antigens (e.g. enterotoxins). CDR3 on both chains of the TCR at the V-(D)-J junctions contain random nucleotide additions and therefore have the most sequence variability. It is the main CDR responsible for recognizing processed antigen while CDR1 and CDR2 (and sometimes CDR3) are thought to recognise the MHC complex.

Variation of TCR antigen binding regions are also generated both genetically through gene diversity of alleles encoding V and J regions and through somatic mutation through recombination of V and J regions giving rise to greater than 10^{25} different TCRs(117)

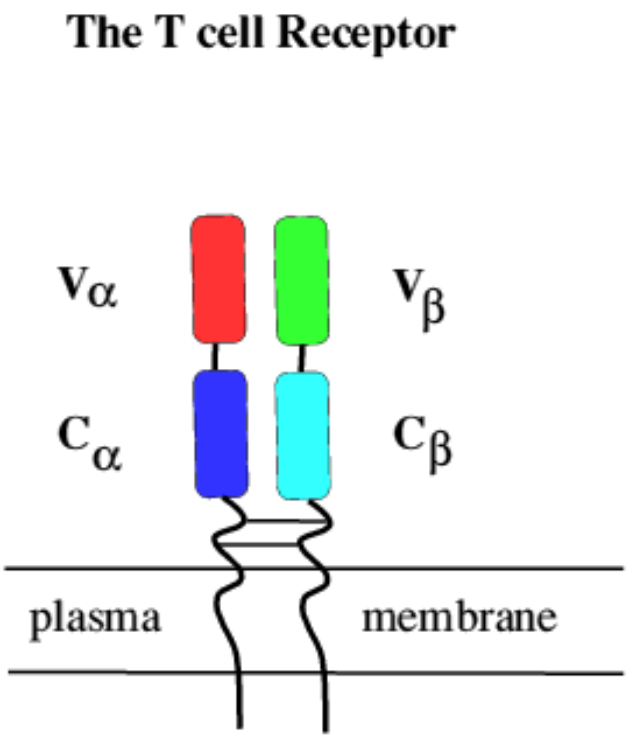
The constant domain of the TCR domain consists of short connecting sequences in which a cysteine residue forms disulfide bonds, which forms a link between the two chains. It is responsible for membrane binding to the cell.

The transmembrane region of the TCR is composed of positively-charged amino acids. It is thought that this allows the TCR to associate with other molecules like CD3, that possess three distinct chains (γ, δ, and ε) in mammals and the ζ-chain. These accessory

molecules have negatively-charged transmembrane regions and are vital for triggering intracellular signalling from the TCR leading to gene activation; the cytoplasmic tail of the TCR is extremely short, making it unlikely to participate in signaling. The CD3- and ζ-chains, together with the TCR, form what is known as the *T cell receptor complex*.

The signal from the T cell receptor complex is enhanced by simultaneous binding of the MHC molecules by a specific co-receptor. On T_H cells, this co-receptor is CD4 that exclusively binds the class II MHC while on T_C cells, the co-receptor is CD8 that is specific for class I MHC. The co-receptor not only ensures the specificity of the TCR for the correctly-presented antigen but also allows prolonged engagement between the antigen presenting cell and the T cell and recruits essential molecules (e.g., LCK) inside the cell that are involved in the signaling of that activated T lymphocyte.

Figure 12. Structure of the T cell receptor



T Cell Activation:

Although the specific mechanisms of activation vary slightly between different types of T cells, the "two-signal model" in CD4+ T cells holds true for most.

The first signal is provided by binding of the T cell receptor to a short peptide presented by the major histocompatibility complex (MHC) on another cell, usually antigen presenting cells. This ensures that only a T cell with a TCR specific to that peptide is activated.

The second signal comes from co-stimulation, in which surface receptors on the APC are induced by a relatively small number of stimuli, usually products of pathogens. The only co-stimulatory receptor expressed constitutively by naïve T cells is CD28, so co-stimulation for these cells comes from the CD80 and CD86 proteins on the APC. The second signal allows the T cell to respond to an antigen. Without it, the T cell becomes anergic, and it becomes more difficult for it to activate in future. This mechanism prevents inappropriate responses to self, as self-peptides will not usually be presented with suitable co-stimulation.

1.6 The Immune Respone in Hepatitis C:

Acute hepatitis C infection is spontaneously cleared by 20% of patients. The remainder develop chronic infection. The host’s immune response is important in determining its ability to clear the virus either spontaneously or in response to treatment.

Multiple arms of the host response, including the innate, humoral and cellular immune responses, have been implicated in determining the outcome of infection with HCV. Interaction between the innate and adaptive immune response plays a pivotal role in perpetuation or clearance of HCV infection.

To replicate and spread successfully, viruses direct various strategies to evade host defences. These measures seem effective since 85% progress to persistent infection and up to 60% do not respond to IFN therapy or relapse once stopped.

1.6.1 Innate Immune Response in HCV:

The innate immune response forms the first line of defense against pathogens providing a non specific immediate response. There is accumulating evidence that HCV affects the early innate immune responses preventing timely and appropriate T cell priming.

Innate immune cells recognise patterns of infectious agents (PAMPs) through receptors expressed on the hosts cells e.g. TLR receptors, thereby directing the selection of antigen by T and B lymphocytes and secretion of cytokines by T_H lymphocytes that promote appropriate host response to infection.

1.6.1.1 Dendritic Cell dysfunction:

Dendritic cells are key components of the innate immune system (Chapter 1.5.1.1). They activate and shape the immune response and link innate and adaptive immunity. As professional antigen presenting cells they are the most efficient activators of naïve T cells. Consequently their functions may be targeted by viruses.

Evidence points towards DC dysfunction in patients with chronic hepatitis C that may compromise their capacity to mount and sustain an effective antiviral immune response, thus leading to viral persistence.
Blood DCs are scarce and cannot be expanded readily *in vitro*. Blood monocytes have been widely used as a DC source in studies, developing into DCs in the presence of GM-CSF and IL-4. Cultured monocyte-derived DCs (MoDCs) are thought to most closely resemble myeloid DCs.

The first studies of DC function in chronic HCV found that MoDCs had an immature phenotype (reflected by the pattern of cell surface markers) and impaired maturation and T cell allostimulatory function(118-121). The direct mechanism underlying the defective function of these dendritic cells has not been clearly identified and varies between studies. However, more recent studies have found no defect in MoDCs from patients with CHC(122-124). MacDonald *et a*l(125) found only a subtle defect that was overcome by increasing the magnitude of the maturational stimulus or DC numbers. The differences among these studies may be explained in part by differences in culturing conditions.

It has been pointed out that data from studies using MoDCs may not be reflect the situation *in vivo(126)* where liver DCs are derived, not from monocytes, but from circulating precursors, the myeloid and plasmacytoid DCs (mDC and pDC respectively).

More recently, studies have looked at circulating dendritic cells directly *ex vivo*, and have shown, both pDCs and mDCs to be numerically and functionally impaired in CHC patients(127-130) with a reduced allostimulatory function, reduced production of IFNα and IL12 and increased priming of IL10-producing T cells. It has also been reported that while numbers of DCs are reduced in the peripheral compartment, increased numbers of DCs have been shown in the livers of HCV patients, suggesting hepatic DC sequestration(121;127;131;132).

Other studies(131;133-135) including data from Southampton(136) have shown that the DC subpopulations are differentially affected in chronic HCV with selective impairment of pDCs production of IFNα. These findings would impact on both innate and adaptive immunity and favor viral persistence. However, these findings are controversial with others finding fully functional circulating plasmacytoid DCs(137;138) and myeloid DCs(138) although reduced in numbers.
In patients with CHC it has been shown that the allostimulatory function of DCs return to normal after treatment induced HCV clearance(119;122;127) However, DC function in these patients was not monitored regularly throughout treatment. Itose *et al* (139) followed patients through treatment and found impaired DC function and lower pDC ratio in non-responders and transient responders compared to sustained responders.

In subjects who had spontaneously resolved HCV infection in the past, frequencies and function of circulating DCs have been found to be comparable to normal subjects(127). These findings together reveal evidence of HCV-induced DC disability and that dendritic cell function may be restored by antiviral therapy.

While these studies suggest a relationship between chronic HCV infection and impaired DC function, with impaired T cell polarization ability, it is not clear if DC dysfunction does result from a direct suppression by the virus or is a secondary consequence of the chronic inflammation. Indeed, a reverse correlation between frequencies of pDCs and mDCs and serum ALT levels has been demonstrated in CHC, suggesting that the liver disease *per se* may lead to reduction in peripheral blood DCs(127).

To further address the causal relationship between CHC and DC dysfunction, several studies(119;127;129;131;137) have looked at DC function in patients with other forms of liver disease including hepatitis B, primary sclerosing cholangitis, autoimmune hepatitis and primary biliary cirrhosis, drug induced hepatitis and steatohepatits and one looked at patients with generalized bacterial infection. Although inconclusive, these studies suggest that different types of hepatic inflammation may cause similar impairment in DC function to those seen in chronic HCV with a paucity of peripheral DCs possibly due to impaired DC mobilization from the bone marrow, or by increasing DC migration to the liver. It is not totally clear therefore if the defect seen in DCs in HCV is directly induced by the virus as an immune evasion strategy.

As discussed, reduction in frequency and impaired function of DCs in CHC patients has been reported by several groups, with some conflicting results on whether either mDCs, pDCs, both subsets or neither are affected. Plausible explanations which may explain the mechanisms underlying any dysfunction that has been reported include: 1) direct HCV infection of DC. The HCV genome has been reported to have been isolated from Mo-DC or blood DC from chronically infected patients (119;138) or 2) the presence of circulating HCV proteins that affect DC function and number.

1.6.1.2 NK Cell dysfunction:

NK cells are the first line of defense against viral infection. Once activated, they secrete inflammatory cytokines, such as IFNγ, which inhibits replication of HCV through a non-cytolytic mechanism (Chapter 1.5.1.2). They play an important role to prevent persistent HCV infection.

The phenotypes and/or functional activities of various populations of these cells have been reported to be impaired in patients with chronic HCV infection(140-146). While many studies found reduced frequency of NK cells associated with significant reductions in NK cytolytic activity in patients with chronic HCV infection(140;143;146) others found no decrease in frequency or cytolytic activity(147;148) and one found a normal frequency but a lower level of spontaneous cytotoxicity than normal controls(141) which may be due to the reported depletion in the cytotoxic $CD56^{dim}$ NK subset with expansion of the IFNγ producing $CD56^{bright}$ NK subset in CHC patients(149).

The functional activities of NK cells are controlled by a variety of stimulatory, co-stimulatory and inhibitory receptors. HCV may have also developed strategies to impair NK cell function by expression of the inhibitory receptor CD94/NKG2A which has been shown to be up-regulated on NK cells in patients with CHC(150). This correlates with an impaired capacity to activate dendritic cells, a reduced ability to kill hepatoma cells and reduced interferon-gamma production(151).

Others have suggested that HCV can modulate NK cell activity, having evolved mechanisms by which it can inhibit the responses of NK cells either directly by binding of the HCV envelope-2 protein to CD81(152;153) or indirectly by inducing the expression of inhibitory ligands for NK cells(154;155).
HCV may also inhibit NK cell activation by altering the functional activities of mDCs and/or pDCs (119;156;157). T cells that exhibit NK phenotypes and functions, including $CD56^+$ T cells(142;146) and natural killer T (NKT) cells expressing invariant Vα24Vβ11 T cell receptors(142;158), have also been reported to be depleted in blood and/or livers of patients with chronic HCV infection.

Finally, the NK cell mediated innate immune response may be genetically programmed to determine the chance of spontaneously resolving acute HCV infection. Subjects homozygous for a polymorphism in the inhibitory NK receptor KIR2DL3 and its ligand HLA-c1, predicted to lessen inhibition, were 2-3 times more likely to spontaneously resolve infection(159). However, this was a retrospective study and functional differences have not been described in acute infection.

1.6.2 Adaptive Immune Response in HCV:

The adaptive immune response is required for resolution of the infection and involves a mechanism for enhancing specificity of recognition and generating immunological memory. HCV persistence and disease progression may be related to inhibition in the adaptive immune response as well as to a failure of CD4+ T cells to support an effective CD8+ T cell response.

1.6.2.1 Cell Mediated Response:

A large amount of evidence points to a crucial role for cellular immune responses in determining the outcome of HCV(160).

Clearance of the HCV is attributed to strong early HCV specific CD4 T_H1 and CD8 T_C cell response against a variety of HCV-peptide epitopes. Conversely, in chronic infection the HCV-specific T cell response is less vigorous(161) and only transitory with a gradual loss of the CD4+ and CD8+ responses to HCV and contraction of the range of peptide epitopes recognized. In patients followed longitudinally from the time of infection it is failure to sustain these responses that correlates with progression to CHC.

Viral persistence and progression to chronic infection is also associated with an altered balance of T_H1 and T_H2 CD4+ T cell responses. Whereas a strong T_H1 response is considered important for the clearance of infection, in patients with CHC, the HCV-specific CD4+ T cell response is biased towards the production of T_H2 cytokines and regulatory cytokines, notably IL10(160).

Both T cell subsets secrete T_H1 cytokines including IL2, INFγ and TNFα in response to viral pathogens. A T_H1 cytokine response is important in the early phase of the viral infection with the aim of controlling the infection. Later in the infection, T cells differentiate into T_H 2 T cells, producing IL4, IL5, IL6, IL13 and IL10, providing help for humoral immune responses and limiting/ controlling the host T_H1 response thereby protecting the host from continual inflammatory response.

Viral Evasion Strategies from adaptive immune responses:

Various mechanisms of virus specific T cell failure leading to HCV persistence have been suggested including impaired antigen induction of T cells, T cell exhaustion, HCV escape mutation, functional anergy or T cell stunning and induction of regulatory T cell function.

1. Inefficient induction of T cells

Dendritic cell dysfunction reported in HCV (chapter 1.6.1.1) may lead to ineffective T cell priming leading to insufficient responses to clear the virus(161).

2. Mutability of RNA genome:

The HCV virus, like most RNA viruses, exist as quasispecies – a collection of related but generally distinct viral variants – due to their highly error prone replication mechanisms due to lack of proof reading activity of the HCV RNA polymerase. The existence of quasispecies may provide the necessary diversity to the virus and facilitate escape from specific host-immune responses with mutations of antigenic epitopes leading to reduced recognition by $CD8^+$ T cells. This is thought to be one of the major viral evasion strategies and an explanation for the persistence of HCV infection(162;163). It has been suggested that T cell escape occurs early during infection since chronically infected patients have been shown not to diversify their escape mutations after several years of follow up. Resolution of acute HCV is associated with overall decrease in viral quasispecies.

3. T cell exhaustion

HCV has a high rate of replication with the production of overwhelming quantities of HCV antigens. Exhaustion of an initially vigorous response is thought to result from chronic stimulation of immune response.

4. Functional Anergy:

Recent studies have shown that HCV-specific CD8+ T cells may be functionally impaired, or anergic, in chronic disease(164) and consistent with this loss of function, may exhibit an immature phenotype resulting in impaired proliferative state, cytotoxic capacity, and ability to secrete TNFα and IFNγ upon stimulation, referred to as stunned phenotype. CD8+ T cell dysfunction has been seen early in the course of acute infection irrespective of outcome. However those who progressed to chronic, CD8+ T cell function remained suppressed compared to those with self limited disease there was recovery of CD8+ T cell function(164).

5. Increased Regulatory T cell (Tregs) function:

Tregs may be natural or induced after prolonged antigenic stimulation. They actively control induction and effector functions of other immune cells by suppressing their functional activity via contact dependant mechanisms as well as by secreting immunosuppressive cytokines such as IL-10 and TGF-B. It is likely that they play a role in preventing immune mediated pathology during chronic HCV. The frequency of naturally occurring CD4+CD25+ Tregs in the periphery is reported to be higher in HCV infected patients when compared to recovered or uninfected patients(165). In addition, there is

growing evidence of Treg activity resulting in active suppression of CD4+/CD8+ T cell response by secreting IL-10(165), thus favouring HCV persistence and limited liver injury.

1.6.2.2 Humoral Immune response:

HCV infection results in antibody production to various HCV proteins in nearly all immunocompetent patients. HCV-specific antibodies are usually detectable 7-8 weeks after infection. Whether antibodies neutralize HCV infectivity is still unclear and the role of antibodies in protection against HCV have been questioned since in chimpanzees and humans, antibodies do not prevent re-infection and do not correlate with a favourable outcome(166).

Chronic infection is characterised by high titres of HCV-specific antibodies. No antibodies conferring immunity to HCV has been detected. Spontaneous viral clearance after acute infection has been described in patients with hypogammaglobulinemia, arguing that antibodies are not essential for HCV clearance.

In the natural course of HCV infection humoral immunity seems to fail as it coexists with high HCV titres and is therefore insufficient to block HCV entry into cells and clear infection. The precise contributions of natural antibodies to viral elimination still remain to be determined.

1.7 Treatment in Chronic Hepatitis C Infection:

1.7.1 Current Treatment for CHC:

The current standard of care for patients with chronic hepatitis C (CHC) is combination therapy with pegylated (PEG) interferon alpha and ribavirin. For many years, the mainstay of treatment was monotherapy with conventional interferon alpha, which led to a low sustained virological response rate (SVR) of 8% for a treatment period of 6 months, rising to 27% when treated for 12-24 months(167).

The introduction of ribavirin in combination with Interferon α in the late 1990s was a major breakthrough in the treatment of HCV infection. Although ribavirin monotherapy had been shown to be ineffective(168;169), combination therapy was consistently shown to improve response rates with a 40% chance of eradicating the HCV virus(170;171) and its most striking effect to decrease the relapse rate after stopping treatment(172).

The effectiveness of treatment is related to genotype of the virus with sustained viral response being achieved by approximately 80% of patients with genotype 2 or 3 infection(173) and approximately 50% of those with genotype 1 with an overall sustained virological response (SVR) rates of 50-60%(174). Introduction of the pegylated form of interferon α increased the SVR compared to conventional interferon in combination with ribavirin(174-176)

The current treatment strategy for individuals infected with genotypes 2 and 3 is 24 weeks of combined pegylated interferon α and ribavirin. For patients infected with other genotypes, virological response is ascertained by quantification of HCV-RNA viral load after 12 weeks of treatment (169). Individuals with an early virological response (defined as >2 $\log_{10}$ decrease in HCV-RNA level) continue for a total of 48 weeks, while those who do not attain this level stop.

Prior to August 2006, NICE guidance recommended treatment only for those patients with histological evidence of moderate or severe disease, or those with significant symptoms of hepatitis C virus (HCV) infection sufficient to impair quality of life. Following revision of the NICE guidance in August 2008 (177), patients with mild disease are now considered

eligible for treatment also without the need for prior liver biopsy and this should increase the numbers of patients eligible for treatment.

Anti-viral therapy for HCV with ribavirin and PEG interferon is cost effective with improved response rates resulting in a decreased rate of disease progression and liver related complications.

The mechanisms by which IFNα and ribavirin act against HCV are not well defined. The importance of understanding the biological pathways, cellular effects and antiviral activities of these agents is important in development of new effective treatments against HCV infection.

1.7.1.1 Mode of Action of IFNα:

Interferons are a family of pleiotropic cytokines with antiviral, anti-proliferative and immuno-modulatory properties. The interferon family is subdivided into two subfamilies: Type I and Type II. Type I interferons are a family of monomeric cytokines with an amino acid similarity of 30-80%, very similar three-dimensional structure (5-alpha helix-bundle), that use the same receptor (IFNAR) to initiate a signalling response (see IFN signaling). The type I interferons present in humans are IFN-α, IFN-β and IFN-ω.

IFNα is a type 1 interferon which is spontaneously expressed at low levels under normal physiological conditions and whose expression is highly enhanced after exposure to viruses. It is produced by a wide variety of cells, including fibroblasts, epithelial cells and hepatocytes but it is particularly abundantly expressed by pDCs, also known as interferon-producing cells (IPCs) and these are probably the major source in most viral infections. IFNα has been used in the treatment for hepatitis C for many years, initially as monotherapy and is known to have direct anti-viral effects as well as a number of immunomodulatory activities that can enhance antiviral immune responses.

Anti-viral effects

The ability of interferon to establish an antiviral state is their fundamental property and essential for survival against viral infection. Mice deficient in IFNalpha/beta are extremely susceptible to viral infections. Interferons interfere with a number of steps in the viral replication cycle including cell entry, transcription, RNA stability, initiation of translation, maturation and assembly.

IFNα has potent antiviral activity but does not act directly on the virus or replication complex. Instead it acts on interferon-stimulated genes (ISGs) which establish a non-virus-specific anti viral state within the cell(178). Circulating IFNα binds to IFN cell surface receptor subunits (IFNAR), leading to their dimerization and the activation of the receptor associated Janus-activated kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2). The activated kinases phosphorylate the signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2). The phosphorylated STATs dimerize, associate with IFN-regulatory factor 9 (IRF9) and Interferon-stimulated gene factor 3 (ISGF3G) to form a complex termed ISGF3 transcription factor which enters the nucleus. ISGF3 binds to the IRES on cellular DNA to activate the transcription of multiple ISGs. Hundreds of genes are induced by type 1 IFN, many related to antiviral activity and inflammatory cell responses.

Immunomodulation

It has been suggested that IFNα enhances humoral immunity by increasing proliferation of B cells, which secrete antibodies against invading antigens.

With regards to the cellular immune response, IFNα supports the proliferation, functional activity and survival of certain T cell subsets which might promote a T-helper 1 (T_H1) over T_H2 phenotype. IFNα enhances the CD8+ cytotoxic T cell (T_C cell) response by upregulating the expression of MHC class I molecules on the surface of the cells. MHC I molecules are responsible for presenting viral antigens to T_C cell, thereby triggering the destruction of the infected cell. Additionally, Th1 cells are responsible for amplifying CD8+ T_C cells in response to IL-12 and induce proliferation. Type I interferons appear to upregulate IL-12Rβ, a component of IL-12 receptor on T_H cells that allows T cells to respond to IL12 and thereby develop into T_H1 cells, enhancing the T_H1 response further. IFNα also promotes memory T cell proliferation, prevents T cell apoptosis and may prevent immune exhaustion.

IFNα has also been reported to induce the innate immune response as well as development of adaptive immunity. NK cells are capable of lysing target cells without prior sensitization (see chapter 1.5.1.2). They act very rapidly upon viral infection and together with interferons are considered the first line of defence against viral infections and tumours. Interferon alpha has been shown to augment NK activity in vitro thus boosting the innate immune responses.

Evidence regarding the effects of IFNα on DC function is contradictory. It is thought that IFNa may increase the expression of MHC class II molecules increasing presentation of antigen to helper T cells. It may promote DC differentiation/activation(179;180) and survival and stimulate DC maturation with enhanced co stimulatory marker expression(181;182) but some reports suggest it has inhibitory effects on these processes(183-185). Enhanced DC maturation leads to enhanced T cell activation and has a major role in the development of adaptive immune responses.

Similarly, while some studies show that DCs generated in the presence of IFNα stimulated IFNγ production by T_H1 cells(180;186;187), while others show that IFNα is a poor inducer of IL12p70 production by DCs, instead favouring IL10 release(183;187) and the induction of regulatory T cells(187). The ultimate fate of DCs with IFNα seems determined by the presence or absence of other influencing factors(182) and the differentiated state of the DC.

Antiproliferative effects:

Interferons have the ability to arrest cell growth. It seems that both direct and indirect (via the immune system) inhibition of the tumor cells might be involved, however, the exact mechanism of action is unclear. No specific genes have been linked to the antiproliferative activity of interferons, however, STAT1 is believed to be involved since it is often deficient in tumours. The mechanism of cellular arrest in not known, but it is likely to target components of the cell-cycle control apparatus, including induction of CDK inhibitors such as p15/16 and p21WAF1/Cip or the decrease in levels of cyclin D.

1.5.1.2 Mode of Action of Ribavirin:

Ribavirin, first discovered in 1970, is a purine nucleoside analogue that is activated or metabolized through intracellular phosphylation to form the monophosphate (RMP), diphosphate (RDP) and triphosphate (RTP), the active form of the drug.

The mechanisms of action of ribavirin are poorly understood but several have been proposed from in vitro studies(178;182;188)

1. Direct inhibition of viral RNA polymerases:

RTP, the active metabolite of ribavirin has been implicated in the inhibition of the activity of many viral polymerases at high doses causing a direct effect on RNA replication. The misincorporation of RTP by RNA polymerases could lead to premature primer chain termination and inhibition of replication. RTP has been shown to be incorporated by HCV RdRp into viral RNA opposite cytosine or uridine, resulting in significant block of RNA elongation(189).

Against the role of ribavirin in direct inhibition of viral replication is the lack of significant first phase decline of plasma HCV RNA with the onset of ribavirin therapy that is seen with IFN therapy and any decline that is seen seems to be transient. It is unlikely therefore that direct inhibition of HCV-RNA replication is the major mechanism of action against hepatitis C.

2. Inhibition of IMPDH

RMP can cause a depletion of intracellular GTP pools, essential for viral RNA synthesis, by competitively inhibiting the hosts enzyme inosine 5'-monophospahate dehydrogenase (IMPDH), essential for *de novo* synthesis of GTP(190). GTP depletion may influence viral replication in 2 ways:

First, it may inhibit viral RNA replication because of lack of an adequate supply of GTP, a critical building block required for RNA synthesis and second, it may promote the incorporation of RTP in place of GTP and thus increase the error rate during replication, contributing to the mutagenic activity of ribavirin.

3. Mutagenesis

RNA viruses typically exist as quasispecies – a collection of related but not identical genomes – due to their highly error prone replication mechanisms due to lack of proof reading activity of the HCV RNA polymerase. The existence of quasispecies may provide the necessary diversity to the viruses and facilitate escape from specific host-immune responses and drug therapy. However, if the error rate during replication crosses a threshold, the quasispecies may be driven into error catastrophe, resulting in meltdown of genetic information.

Ribavirin is known to increase the error rate during viral replication by the metabolite RTP being incorporated in place of guanosine or adenosine in viral RNA strands by viral RNA polymerases. This erroneous incorporation may inhibit chain elongation and, in the extreme situation, cause chain termination. Thus ribavirin may potentially drive viruses to extinction by inducing an error catastrophe(191). Several recent studies demonstrate that ribavirin has an early effect in elevating mutation rates, lending support to mutagenesis as a key mechanism of the antiviral(192-197). However these findings are conflicting with others finding no increase in mutational frequency(198-200).

3. Immunomodulation:

With the clinical observation that ribavirin monotherapy had minimal effect on HCV viraemia despite significant reduction in ALT levels(201), it was proposed that ribavirin may exert its effect on the host immune response.

Previous studies using mitogen stimulated PBMCs(202-205) or animal models(206-208) have shown that ribavirin promotes T_H1 type responses either by suppressing Th2 cytokine profile(206) and/or by increasing T_H1 cytokine response with production of tumour necrosis factor and IFNγ and decrease in IL10 (202-205;207;208). A T_H1 bias implies a stronger cellular rather than antibody response against infection and accumulating evidence has shown that an early T_H1 immune response leads to viral clearance, whereas T_H2 response favours chronic evolution.

In patients receiving treatment with ribavirin and IFNα, studies have shown that a favourable response was associated with induction of a T_H1 cytokine production with elevated IFNγ and IL-12 responses, in contrast to non responsders who showed a decrease in INFγ and IL12 and an increase in IL-10 expression(209)

Thus ribavirin can modulate the immune system but how this is achieved and whether it is relevant to responses to treatment remain unclear.

4. TLR agonist:

As ribavirin is a purine analogue, it may excert immunomodulatory activities via toll-like receptor (TLR). However, ribavirin was not found to stimulate TLR7 or TLR8-mediated signalling in cells transfected with these TLRs at the biologically active concentrations (1-5uM) (210)

Ribavirin monotherapy has been shown to have minimal effect on HCV viraemia but is associated with improvements in serum aminotransferases (211) suggesting that it may have an effect on inflammation and the host immune response, downregulating cell-mediated immune responses. This, however, goes against the notion that ribavirin induces T_H2 to T_H1 shift, since this would increase cell mediated immune response.

When used in combination with IFNα, ribavrin doubles the SVR, primarily through the prevention of virological relapse after the discontinuation of therapy (212) This is consistent with findings from viral kinetic studies of ribavirin mainly targeting the characteristic second and/or third phases of viral decay (190). The improved SVR with combination therapy suggests they act synergistically to achieve the initial clearance of virus. It may act by shortening the half life of infected cells in the presence of IFNα.

Ribavirin appears to be pleotropic agent with many intrinsic mechanisms that can influence its overall antiviral properties. Understanding of the key mechanisms of action of ribavirin will help design better therapy for patients with HCV.

1.7.2 Novel Treatment for CHC:

Although combination therapy with PEG-IFN α and ribavirin has significantly improved SVR rates with sustained response rates of 54-56%, still a large number of patients do not have any lasting improvement with treatment. Furthermore, treatment is expensive, associated with a wide spectrum of side effects and complications and contraindicated in many patients due to co-morbidities, leading to a relatively small number of patients being offered treatment and a large number discontinuing treatment due to adverse events.

For this reason there is continuing need for novel anti-HCV therapies to improve response rates and reduce adverse effects. A number of new treatments for HCV are undergoing clinical trials (Table 1). HCV is highly mutable and direct antiviral strategies are associated with drug resistance suggesting that future regimens will continue to include immunomodulatory drugs. Better understanding of the immunological factors determining successful versus failed anti-HCV treatment would aid identifying candidates for new HCV treatments directed at the immune response.

Table 1. Novel treatments and their development phase

	Development Phase
Ribavirin analogues	
viramidine	III
Merimepodib	II
Protease Inhibitors	
BILN 2061 or ciluprevir	II
VX-950 or telaprevir	III
SCH 503034 or bocepravir	II
Nucleoside polymerase inhibitors	
NM283 or valopicitabine	IIb
R1626	II
R7128	I
Non-nucleoside polymerase inhibitors	
JTK-109 and JTK-003	II
HCV-796	II
HCV-759	I
GS-9190	I
Cyclophilin B inhibitors	
DEBIO-025	II
α glucosidase inhibitors	
Celgosivir or MX-3253	II
Oligonucleotides	
ISIS 14803	II
AM-4065	II
RNA interference	
BLT-HCV	I
Immune Modulators	
ANA245 or isatorabine	I
Resiquimod	II
CPG 10101 or Actilon	II
ANA773	I

1.7.2.1 New Interferon Molecules:

New forms of IFNα are being developed with more potent antiviral effects and possibly more potent immunomodulatory effects as well as enhanced bioavailability and better pharmokinetics with less peaks and troughs.

Albuferon (albumin-linked IFNa) is a recombinant single polypeptide molecule, coded by the fusion of the human serum albumin (HAS) and the IFN-α genes. It has been shown to induce a significant biphasic decline in HCV replication with benefits of less frequent dosing(213). It is well tolerated with comparible efficacy to current treatment standard of care and is now in phase 3 trials.

Consensus interferon (CIFN) is a recombinant type 1 IFN containing 166 amino acids. CIFN was derived by scanning the sequences of several natural alpha IFNs and assigning the most frequently observed amino acid at each corresponding position. Therapy with CIFN together with ribavirin seems to have promising results especially in difficult patients with cirrhosis(214) and non-responders to previous therapies(215).

Omega IFN is a new type-1 interferon that has been designed for continuous delivery by an implantable device.

IFN lambda (IL-28A, B, IL-29) is a type III IFN that binds to a unique receptor. In phase Ib trials it has been shown to be well tolerated with significant antiviral activity(216)

1.5.2.2 Modified or improved forms of ribavirin:

The main dose limiting side effect of ribavirin is haemolytic anaemia and alternatives to ribavirin with fewer side effects are under development.

IMPDH inhibitors are being evaluated as antiviral drugs, since this enzyme catalyzes an essential step in the biosynthesis of guanine nucleotides. However, they have so far proven disappointing with none or minimal direct antiviral efficacy.

A prodrug of ribavirin, viramidine (taribavarin), becomes activated in the liver (limiting exposure to erythrocytes) and has demonstrated significant antiviral activities and erythrocyte-sparing properties. However, SVR rates have been lower when compared to ribavirin(217).

1.5.2.3 Specifically Targeted Antiviral Therapy (STAT-C):

With an improved understanding of the molecular structure of the HCV, its component proteins, the various phases of the replication cycle of the virus and the new culture methods and animal models for HCV, specific small molecules, inhibitors of the viral enzymes have been developed, so called STAT-C (specifically targeted antiviral therapy; table 2).

The HCV virus exists as quasi-species in nature, with high replication rate in vivo and long duration of disease. Therefore, drug resistant mutants may already pre-exist in HCV-infected patients. These new inhibitors that target directly the enzymes entailed in HCV replication are likely to prompt emergence of drug-resistant strains and this is a major hurdle to overcome with these new agents. Combination therapy has been shown to be more effective and less prone to resistance development than monotherapy, as seen in HIV.

Table 2 STAT-C agents in development for HCV

STAT-C Agents	Mechanism	Phase of Study
GS9132	Protease inhibitor	Failed (renal toxicity)
BILN 2061	Protease inhibitor	Failed (cardiac toxicity)
MK7009	Protease inhibitor	Phase 1
BI12202	Protease inhibitor	Phase 1
ITMN-191 (R7227)	Protease inhibitor	Phase 1
TMC435350	Protease inhibitor	Phase 2
Telaprevir (VX-950)	Protease inhibitor	Phase 3
Boceprevir (SCH-503034)	Protease inhibitor	Phase 3
Valopicitabine (NM283)	NucPolymerase	Failed (gastrointestinal effects)
MK-0608	NucPolymerase	Preclinical
R7128	NucPolymerase	Phase 1
R-1626	NucPolymerase	Phase 2
HCV-796	NNPoylmerase	Failed (hepatotoxicity)
GS-9190	NNPolymerase	Phase 1
VCH-759	NNPolymerase	Phase 1
NIM811	Cyclophilin inhibitor	Phase 1
DEBIO-025	Cyclophilin inhibitor	Phase 2

Inhibition of translation:

- Protease Inhibitors – (***NS3-4A Serine)***

NS3/4A protease is an attractive target because it plays a part in viral replication(218) and provokes abrogation of induction of the interferon and cytokine pathways by cleavage of the adaptor molecule TRIF and CARDIF/MAVS. This process favours viral propagation and presumably, chronicity in HCV infection.

Two classes of inhibitor have been developed: (1) peptidomimetic and (2) non-peptidic molecules and several have been evaluated in clinical trials: BILN 2061 (Ciluprevir) was one of the first but trials were halted due to cardiac toxicity in animal models(219), however, these results provided 'proof of concept' for the efficacy of targeting the NS3/NS4 potease. Further trials have shown that monotherapy with these agents is not possible due to development of resisiatance and INFα and ribavirin are required to enhance antiviral activity and reduce the frequency of resistance.

The leading candidate at present is telaprevir (VX-950), a reversible, selective, and specific peptidomimetic inhibitor of the NS3/NS4a protease, which is orally bioavailable(220). Combination of telaprevir with IFNα2a has shown enhanced efficacy with a significant reduction in HCV RNA(221) and triple therapy with ribavirin augments the response further with less resistance developing (222). Two phase II studies – PROVE 1 and 2 – have shown safety and efficacy of the triple regimen in patients with untreated chronic HCV (genotype 1)(223;224) and the PROVE 3 study is now looking at triple therapy in non-responders to previous treatment .

A second protease inhibitor, boceprevir (SGH 503034), which is peptidomimetic, has shown efficacy in combination with pegylated IFNα in patients with genotype 1 infection that were previous non-responders to conventional treatment (RESPOND-1 study)(225) and in genotype 1 treatment naïve patients the SVR rate was almost doubled when used in combination with IFNα and ribavirin (SPRINT-1 study)(226). Phase III trials are now underway in non-responders and treatment naïve patients (RESPOND II and SPRINTII respectively).

Others in phase III trials at present include ITMN-191(227), MK-7009, BI2202 and TMC435350(228).

Inhibition of viral replication:

1. Direct inhibition of RNA-dependant RNA polymerase

The three dimensional crystal structure of HCV RNA dependant RNA polymerase has been solved. This enzyme is essential for viral replication and no host equivalent exists, making it an attractive target. Two structurally distinct classes of inhibitors with different modes of action have been reported: (1) nucleoside analogues which generally target the polymerase active site in a competitive manner and typically show broad-spectrum activity; and (2) non-nucleoside analogue molecules which have much greater specificity and act either by direct interference with the active site or by binding to an allosteric site and preventing the initiation process. Many of these compounds have neen discontinued due bone marrow and GI toxicity, lack of efficacy or the development of resistance.

- Nucleoside polymerase Inhibitors

NM-283 (valopicitabine) is the oral prodrug of the nucleoside analogue 2'-C methylcytidine, which undergoes intracellular phosphorylation to form the active inhibitor. It was found to be a promising polymerase inhibitor in clinical trials with reasonable antiviral activity but high side effect profile halted drug development (229).

Others include R1626(230), a pro-drug of the nucleoside analogue R1479, which is a potent inhibitor of HCV replication *in vitro* and has shown encouraging results in a phase II study. An oral nucleoside analogue, R7128(231;232), a pro drug of PSI-6130 has shown encouraging results in efficacy with the absence of additional toxic effects.

Viral resistance is a major issue with these molecules and enforces the need to combine these new treatments with 'old therapies' in order to control the emergence of resistant strains.

- Non-nucleoside Inhibitors (NNIs)

The non-nucleoside polymerase inhibitors are drugs which bind allosterically on the enzyme surface near to its active site to disturb its structure and function. HCV-796, the first NNI of the HCV polymerase, showed significant HCV antiviral activity against multiple genotypes(233). However, the development of this drug was discontinued due to findings in a phase II study showing hepatotoxicity. GS-9190 is a novel non-nucleoside HCV NS5b polymerase inhibitor with potent antiviral activity (234).

2. Inhibition of host co-factors

- cyclophilin inhibitor:

Cyclophilin B promotes RNA binding activity of the HCV RNA-dependent RNA polymerase (NS5B) in vitro. Early observations with cyclosporine, an immunosuppressive drug with CYP-inhibiting activity, showed anti-HCV activity in vitro. Non-immunosuppressive cyclophilin inhibitors have subsequently been developed, including DEBIO-025(235;236) whose clinical profile was encouraging in phase I trials. NIM811, another non-immunosuppressive cyclophilin antagonist, has also entered early-phase clinical studies with encouraging preclinical studies.

Inhibition of protein synthesis*:*

- IRES Inhibitors –

Translation of HCV RNA is initiated by internal entry of ribosomes into the IRES. IRES inhibitors include small interfering RNAs (siRNA), anti-sense oligonucleotides and ribozymes.

Small interfering RNAs (siRNA) – RNA interference is a natural process used by all eukaryotic cells to recognize and destroy abnormal or exogenous RNA. In mammalian cells, introduction of double stranded RNA (dsRNA) activates the interferon pathway and induces non-specific degradation of RNA, inhibition of mRNA translation and cell death. Induction of RNA interference process in mammalian cells is possible by small dsRNA (called siRNA) and this strategy is being exploited as antiviral therapy. The siRNA drug BLT-HCV, which targets three different HCV sequences has entered clinical trials(237;238)

Anti-sense oligonucleotides are short synthetic nucleotide sequences that bind an RNA target forming RNA–RNA (antisense RNA) or RNA–DNA (antisense DNA) hybrids which result in inhibition of RNA translation and/or replication of viral proteins. ISIS 14803 is a 20-base antisense oligonucleotide that is complementary to the HCV translation initiation region within the IRES. Promising results were seen in early phase II trials but subsequent aminotransferase flares and poor viral efficacy led to discontinuation of further studies(239). A phase II study of another compound, AV-4065, is underway.

Ribozymes are synthetic nuclease-resistant catalytic RNA molecules taken up by the liver where they cleave specific HCV sequences. Unfortunately studies with the ribozyme RPI-13919 (heptazyme), designed to cleave the HCV IRES from the 5'-UTR region of the HCV genome were halted because of toxicity in primates(240).

One of the major issues that will determine the success of these drugs is the efficient delivery of the synthetic polymers to the appropriate cells *in vivo*.

1.7.2.4 Immune Modulators - *Toll Like Receptor Agonists*

The experience with IFN-based therapies has demonstrated that HCV infection can be eradicated by agents that stimulate the hosts innate and adaptive immunity. In hepatocytes, there are two major pathways of host defence: one is the retinoic acid-inducible gene I (RIG-I) and the other is the Toll-like receptor (TLR) (chapter 1.5.1.3).

TLR agonists are known to selectively induce the interferon system through activation of TLRs, a group of pattern–recognition receptors that play a crucial role in antigen recognition. The induction a range of different type 1 interferon subtypes triggers the activation of intracellular antiviral responses and a T_H1-adaptive immune response. TLR 3, 7, 8 and 9 are known to be responsible for detection of viral molecules.

Stimulation of the appropriate TLRs by artificial ligands may therefore help restore the innate and adaptive immune functions that are dysfunctional in hepatitis C infected hosts and help to eradicate HCV infection.

Recently TLR7 and TLR9 agonists have been shown to have clinical efficacy against HCV and both are highly expressed on plasmacytoid dendritic cells which are known secrete high amounts of endogenous IFNα.

TLR-7 Agonists:

The natural ligands for TLR7 are patterns within viral single stranded RNAs but several synthetic compounds that are structurally related to nucleic acids are also recognised. These include imidazoquinolines, loxoribine and bropirimine. TLR-7 stimulation leads to activation of the transcription factor NF-kB through a MyD88 dependant signaling pathway

resulting in activation of innate immune cells and the secretion of pro-inflammatory cytokines, especially IFNα.

Isatoribine (ANA245; Anadys Pharmaceuticals) is a guanine nucleoside analogue whose immunostimulatory activity depends on its agonistic activity on TLR-7. It has been shown to induce a modest reduction in viral replication in some HCV infected patients with evidence of immune modulation as shown by upregulation of the interferon-response gene, 2'5'OAS and increased production of IFNα, IP10 and neopterin (a marker of immune activation produced by activated macrophages) (241). Following these encouraging results an oral prodrug ANA975, was being investigated(242). The prodrug enables convenient oral dosing while avoiding the GI symptoms associated with the oral route. However, ongoing clinical trials with ANA245 and ANA975 were suspended in 2007 for safety reasons because of information obtained from preclinical toxicology studies consistent with intense immune stimulation in animals even at dose levels where desired immunostimulatory effects were not measurable(243).

Clinical investigation of the TLR7 mechanism for the treatment of chronic hepatitis C has now resumed with investigation of ANA773, an orally administered prodrug of a novel TLR7-specific agonist. Results from pre-clinical pharmacology studies have shown that ANA773 can elicit desired immune responses and that the profile of response can be modulated by both dose and schedule of administration. Results of recently completed 13-week GLP toxicology studies have shown that with every-other-day dosing of ANA773, immune stimulation of a magnitude believed to confer therapeutic potential can be achieved without adverse toxicology findings. The immune stimulation observed with every-other-day dosing of ANA773 in monkeys included induction of IFNα and interferon dependent responses at levels that are sustained over 13 weeks of dosing. Phase 1 trials have shown a dose related IFN-dependant response and was typically well tolerated in healthy volunteers and trials in HCV patients are now underway (244).

Resiquimod, a TLR 7 and 8 agonist has been assessed in CHC patients and shown some reduction in HCV RNA levels(245) but was also associated with intense adverse events because of IFNα induction.

SM-360320 (9-benzyl-8-hydroxy-2-(2-methoxyethoxy)adenine) is a specific TLR-7 agonist and a potent inducer of INFα *in vivo* and *in vitro*. Further studies have shown it also to be

effective in activating anti-HCV responses and reducing HCV-RNA levels *in vitro (246)*. It was used in this study to further explore its effects in HCV.

Results from clinical trials have indicated that TLR7 agonists have poor tolerability when administered orally compared with either topical or intravenous administration. Adverse events with the oral administration often include significant GI symptoms by virtue of the large amount of immune tissue associated with the gut wall. Doses sufficient to achieve effective systemic concentrations lead to side effects including nausea, sickness, gastroenteritis and localized systemic effects.

TLR-9 Agonists:

TLR9 is expressed in human B cells and pDCs and signals through a MyD88-dependant signaling pathway to activate NF-kB leading to the activation of DCs and secretion of INFα and pro-inflammatory cytokines, IL-12 and TNFα. TLR-9 recognizes unmethylated cytidine-phosphate-guanosine (CpG) rich sequences which are present frequently in bacterial and viral DNA which can be mimicked by synthetic CpG oligonucleotides (CpG-ODNs) which contain unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs). These CpG motifs are present at a 20-fold greater frequency in bacterial DNA compared to mammalian DNA. They have been used for therapeutic purposes to stimulate TLR-9.

Different classes or subtypes of CpG-ODN have been described and the immunostimulatory activity of the CpG-ODN is dependant on the sequences surrounding the CpG dinucleotide as well as the secondary structure formed by the ODN(247).
Type A ODNs (2216) have been shown to activate pDCs to mature, upregulate costimulatory molecules and to secrete type-1 interferons, but they are not potent activators of B cells. These CpG-ODNs contain a central palindromic sequences allowing for the potential of secondary structure formation.
Type B CpG-ODNs (2006) contain 3 human CpG motifs and are potent activators of B cells mediating cytokine and immunoglobulin secretion. These ODNs do not strongly activate pDCs and are therefore not associated with high levels of IFNα secretion but they still promote survival and maturation of pDCs. They lack the secondary-structure forming palindromic sequences and rather it is the dinucleotide flanking purines and pyrimidines that influence the immunostimulatory activity.

Type C CpG-ODNs (M362) combine the features of type A and B, enhancing the proliferation of B cells and high induction of pDCs inducing IFNα secretion as well as TNFα and IL12.

CPG10101, (Actilon^{TM}, COLEY Pharmaceutical Group, USA) is an investigational, class C synthetic ODN that contains immunostimulatory CpG motifs optimized for activation of human TLR9 and is being developed as an antiviral and enhancer drug for treatment of chronic HCV infection. It been shown to activate and mature B cells and pDCs in both healthy volunteers and HCV patients, and to stimulate production of cytokines, including those with known antiviral activity such as IFNα (248;249).

In a phase 1B trial it was found to induce moderate HCV RNA load reductions in infected patients with elevation of IFNα and β levels(250). In relapsed and non-responder patients to previous ribavirin and Interferon, reductions in HCV-RNA were significantly greater in the CPG combined treatment group(251).

Clinical studies with Actilon have however been ceased at present, suggesting possible exhaustion of limitation in endogenous IFNα producing capacity in CHC (251).

The therapeutic application of TLR agonists should be evaluated with some caution however because they may have multiple detrimental effects. Excessive production or imbalanced amounts of TLR agonist induced cytokines may favour the development of non-infectious or autoimmune diseases.

Other Immunomodulatory Drugs

Several interleukins have been investigated, with encouraging results. Interleukin 10, an anti-inflammatory drug, given to patients with no response to IFN can reduce aminotransferase levels and improve liver histological findings(252) and interleukin 29 has shown promise in animal work. Other immunomodulators include Histamine dihydrochloride which inhibits phagocyte-derived oxidative stress and inflammation and Thymosin which promotes T cell maturation and NK cells.

1.7.2.45 Specific Vaccine Therapy

Attempts are being made to develop both prophylactic and therapeutic vaccines capable of stimulating functional CD4 and CD8 T-cell responses in chronic hepatitis C patients. Development of such vaccines has so far been unsuccessful predominately because of the rapid evolution of the virus which continuously generates mutants that escape immune surveillance. All new agents will be heavily influenced by their ability to inhibit all viral variants and prevent emergence of escape mutants

Preclinical and early human studies indicate that therapeutic vaccines using various forms of HCV recombinant polypeptide together with various adjuvants have been investigated (253). Some genes have been found to be more mutable than others. The HCV envelope proteins, E1 and E2 have been shown to have the best potential for serving as efficacious vaccines. When administered with an adjuvant, these recombinant antigens have been found to evoke robust anti-envelope antibody and T-cell responses, giving chimpanzees partial protection from becoming chronically infected.

Vaccines designed to augment the immune response to HCV are in development. INO-101, a proprietary E1 protein is being tested with alum adjuvant as a therapeutic vaccine and shown to enhance both humoral and cellular immune responses. It did not affect viral load but was able to slow disease progression (254). IC-41 is a proprietary combination vaccine that has been shown to increase T-cell responses and reduce viral load by an average of 60% in hepatitis C in those unresponsive to previous treatments (255).

Another vaccination strategy makes use of attenuated or defective viral vectors expressing one or more of the HCV genes for priming humoral or cellular immune responses. Adenovirus(256), vaccinia virus (257) and canarypox derived vectors are all used for this purpose.

T-cell vaccine based on the HLA and HCV genotype cross-reactivity (258) and dendritic cell-based vaccines(259) are novel and very promising approaches for therapeutic immunization in patients with CHC. In particular, dendritic cell vaccination is able to elicit potent activation of antigen-specific cellular immunity against HCV proteins. In recent studies, immunization of mice with beads coated with NS5 protein and anti-DEC205-

endocytosis receptor of dendritic cell was able to induce a significant cellular immune response(260;261).

Preventative vaccination

A phase 1 trial of a candidate vaccine demonstrated safety and significant antibody and lymphocyte proliferation response in healthy volunteers(262). A T cell HCV vaccine was protective against acute infection in chimpanzees(263). A third vaccine, based on recombinant HCV-like particles produced in insect cells, also controlled HCV challenge in a chimpanzee model(264).

Hepatitis C immunoglobulin

Unlike hepatitis B, no effective immunoglobulin prophylaxis exists for hepatitis C. Passive immunization with hyper-immune hepatitis C immunoglubulins (HCIg) has been shown to decrease HCV replication and reduce aminotransferase levels in chimpanzees with CHC(265;266). Several experimental immunoglobulin preparations are in various stages of testing with current development focused in liver transplantation. Civacir is an antibody preparation specifically targeted to HCV. It is currently in clinical trials for the prevention of HCV infection in transplanted livers(267;268). HepeX-c is a HCV specific monoclonal antibody preparation in early-phase clinical trial for the prevention of re-infection in HCV-positive patients undergoing transplantation (269).

Chapter 2
Materials and Methods

2.1 Materials:

2.1.1 General Consumables

Standard tissue culture plasticware, including flat-bottomed 6, 12, 24, 48 and 96 well tissue plates and 15 and 50ml Falcon tubes were from Corning (Dorset, UK) and were chosen for low endotoxin levels. U botton and V-bottom plates 96 well plates were from Millipore (Watford, UK).

2.1.2 Buffers and Solutions

Stock solutions:

Sterile pyrogen-free water	Pharmacy, Southampton General Hospital, Southampton, UK
Rothwell Park Memorial Institute (RPMI) [1640] medium,without L-glutamine and phenol red	Invitrogen, Paisley, Scotland,
10x Phosphate Buffered Saline (PBS), sterile.	Gibco, Invitrogen
Bovine serum albumin (BSA)	Miltenyi Biotec, Bisley, UK
0.5M ethylenediaminetetraacetic acid (EDTA)	Invitrogen, Paisley, Scotland
Foetal calf serum (FCS), low endotoxin	Hyclone, Perbio Science UK Ltd, Tattenhall, UK. Heat inactivated at 56^0C, 30 mins.
Human AB serum (HAB)	Sigma Chemical Company Ltd, Poole, UK. Heat inactivated at 56^0C, 30 mins

***Stock solutions were used to prepare*:**

Sterile phosphate buffered saline (PBS):	Prepared from 10x PBS stock diluted 1:10 with sterile pyrogen free water
Running Buffer	1x PBS / 2mM EDTA / 0.5% BSA
Rinsing Buffer	1x PBS / 2mM EDTA
Culture Medium	RPMI 1640 supplemented with 10% FCS, in 2mM L-Glutamine, 80IU/ml Penicillin, 80IU/ml Streptomycin (Sigma, Poole, UK)
FACS wash	1x PBS, 1%BSA, 0.1% sodium azide
FACS block	FACS wash containing 100ug/ml Chromepure human IgG (Jackson Immunoreserch, Stratech Scientific Ltd, Suffolk, UK)

2.1.3 Cytokines and cell culture additives

Recombinant human (rh)GM-CSF	R & D Systems, Abingdon, UK (100 000IU/ml)
Recombinant human (rh) IL-4	R & D Systems, Abingdon, UK (1ug/ml)

Stock solutions of the cytokines were prepared by dissolving the lyophilized powder in sterile PBS containing 0.1% Human AB serum and filter-sterilised through 0.2µm syringe filters. Solutions were aliquoted and frozen.

2.1.4 Antibodies:

Monoclonal antibodies used for flow cytometric analysis of cell surface markers are listed below. The antibodies were directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or allophycocyanin (APC) and all from BD Biosciences, Oxford, UK unless stated.

Isotype controls	Mouse IgG2a FITC
	Mouse IgG1 APC
	Mouse IgG1 FITC
	Mouse IgG1 PE
Cell surface markers	Mouse anti-human HLA-DR FITC
	Mouse anti-human HLA Class I APC
	Mouse anti-human CD40 APC
	Mouse anti-human CD86 APC
	Mouse anti-human CD83 APC
	Mouse anti-human CD4 FITC
	Mouse anti-human CD69 FITC
	Mouse anti-human CD56 APC (Miltenyi Biotech, UK)
	Mouse anti-human CD3 PE
Intra-cellular Cytokines	Mouse anti-human IFN-gamma FITC

2.1.5 Stimuli

Tetanus Toxoid	Calbiochem, USA
Lipopolysaccharride	Sigma, Poole, UK
Loxoribine	InvivoGen, San Diego, USA
r*Ov*-ASP-1	New York blood centre, NY, USA
ODN M362	InvivoGen, San Diego, USA
Ribavirin	Provided by iQur Ltd, Southampton, UK
CD40L- transfected CHO cells	Gift from Professor M. Glennie (Tenovus, Southampton, UK)
TLR7 agonist, SM360320	Pfizer, Sandwich, UK
Interferon alpha 2a	PBL Biomedical Laboratories, NJ, US

2.1.6 Cell Purification / Isolation

CD14 Microbeads	Miltenyi Biotech, Surrey, UK
BDCA-4 dendritic cell isolation kit	Miltenyi Biotech, Surrey, UK
Naïve CD4 T cell isolation kit	Miltenyi Biotech, Surrey, UK
Pan T cell isolation kit	Miltenyi Biotech, Surrey, UK

2.1.7 Identification and Classification of Subjects:

2.1.7.1 Recruitment of Subjects

Ethical approval for this study was obtained from Southampton and S.W Hants Joint Research Committee (ref: 05/Q1703/45).

Subjects falling into the following groups were investigated:

1. *Normal, uninfected volunteers*
   Healthy controls were identified from volunteers within the research unit and medical school. Volunteers were deemed to be normal and uninfected with HCV if they did not present with any current or previous risk factors for HCV infection. They were not tested for evidence of HCV or any other blood-borne virus. Written, informed consent was taken. Included in this group were patients with hereditary haemochromatosis for whom regular phlebotomy is part of their treatment, all of whom were screened for blood-borne viruses.

2. *Subjects with CHC infection*
   Chronic hepatitis C was diagnosed on the basis of detection of HCV viraemia by PCR testing (Cobas Amplicor HCV Monitor test, Roche Molecular Systems, NJ, USA). Subjects with CHC were sourced from the pool of past and present patients attending the Hepatology Clinics at the Southampton University Hospitals NHS Trust and Royal Bournemouth and Christchurch NHS Trust. As part of the routine screening of patients with suspected CHC, most patients had already undergone a liver biopsy and PCR-based tests to determine the presence of HCV RNA in the serum and the HCV genotype. The subjects with ongoing HCV-infection had *either*
   - Not yet received anti-HCV treatment, *or*
   - Had failed to respond to prior treatment which had ceased *at least* 6 months before recruitment in the study.

   All subjects gave written, informed consent.

2.1.7.2 Phlebotomy

6mls and 90ml of blood was collected into vacutainers attached to a butterfly cannula containing sodium citrate and EDTA respectively in an appropriate clinical area using asceptic technique. Blood tubes were labeled for identification and transported to either the containment level 3 suite (if HCV infected) or to the containment level 2 laboratory (for NHDs) in a sealed polythene bag.

2.1.8 Gathering Clinical Information

Clinical information on each patient or volunteer was gathered by direct questioning. Additional details on the CHC patients were obtained from medical records and computerized pathology record system. Data collected is shown in table 3. The patients all gave prior consent to examination of their records for information relevant to the study. The information gathered on the CHC patients was entered into a database for analysis.

Table 3: Clinical information collected from patients

Healthy Volunteers	***Hepatitis C patients***
Age	Age
Sex	Sex
Ethnicity	Ethnicity
	Route of infection
	Alcohol consumption
	ALT
	Genotype and Viral Load
	Liver Biopsy IPA score
	Concurrent medical conditions
	Previous treatment for HCV

2.2 Methods

All centrifugations were performed in a Mistral 3000i refrigerated centrifuge (Jepson Bolton Laboratory Equipment, Watford, UK) unless otherwise stated. All incubations at 37^0 C, 5% CO_2 were performed in a Heraeus 6000 series incubator (Kendro Laboratory, Stevenage, UK).

2.2.1 Cell Purification:

2.2.1.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC):

Freshly drawn EDTA-treated blood was diluted 1:1 with RPMI 1640 (no additives), pre-equilibrated to room temperature. In sterile 50ml falcon tubes, 40mls of diluted blood was carefully overlayed onto 10mls Lymphoprep (Robbins Scientific, Solihull, UK) and centrifuged at 1550rpm for 20-25 minutes at room temperature, with the brake off. PBMCs were recovered from the interface between the lymphoprep and serum using a plastic Pasteur pipette, transferred into a 50ml falcon tube and diluted with RPMI. Cells were spun at 1400rpm for 10 minutes at room temperature. The cell pellet was resuspended in sterile running buffer (see buffers and solutions) and spun at 1100rpm for 10 minutes at 4^0C, to remove platelets. Cells were resuspended in 10mls of sterile running buffer and a live cell count performed using trypan blue in PBS and a cell counter. The required number of cells were removed, spun at 4^0C and the pellet resuspended into culture medium at the required density. If they were to be stored for future use, they were cryopreserved in 90% FCS and 10% sterile culture grade dimethyl sulfoxide (DMSO, HybriMAX, Sigma).

2.2.1.2 Isolation of Monocytes from PBMCs:

Freshly isolated PBMCs (section 2.2.1.1) were resuspended in cold running buffer (80µl / 1.0 x 10^7 PBMCs). CD14 Microbeads (20 µl / 1 x 10^7 PBMCs), were added and incubated for 15 minutes at 4-8 ^{0}C. The cells were then washed with running buffer and centrifuged at 1250rpm for 10 minutes at 4 ^{0}C. Up to 10^8 cells were resuspended in 500ul of running buffer and loaded onto the Auto MACS (Miltenyi Biotech, Surrey, Uk). The separation programme, POSSEL, was selected and the positive fraction collected from port 'pos1'. The positive fraction was resuspended in running buffer to 10mls and a live cell count performed using trypan blue exclusion.

2.2.1.3 Isolation of Plasmacytoid Dendritic cells from PBMCs:

Freshly isolated PBMCs (2.2.1.1) were washed and pelleted. The pellet was resuspended in cold running buffer (300µl / 10^8 PBMCs). 100µl of FcR Blocking reagent was added per 10^8 PBMCs, followed by 100µl of anti-BDCA-4 Microbeads per 10^8 PBMCs. This mixture was incubated for 15 minutes at 6-12 ^{0}C. The cells were then washed in running buffer, spun at 1250rpm and resuspended in 500µl cold running buffer per 10^8 cells. The cells were loaded onto the AutoMACS (Miltenyi Biotech) and the separation programme, POSSEL was selected. The positive fraction was collected at port 'pos2'. A live cell count was performed using tryphan blue exclusion and cells spun and resuspended in culture medium at the required density.

2.2.1.4 Isolation of T Cells from PBMCs

Naïve CD4 T cell Isolation:

Freshly isolated PBMCs (2.2.1.1) were washed and pelleted. Cells were resuspended in running buffer. Biotin-antibody was added (10µl /10^7 cells) and left in fridge for 10 minutes. Running buffer was then added (30µl / 10^7 cells) followed anti-biotin beads (20µl / 10^7 cells) and left in the fridge for a further 15 minutes. Cells were washed with running buffer, spun at 1200rpm and resuspended in 500µl of running buffer per 10^7 cells. The cells were loaded onto the AutoMACs (Miltenyi Biotech) and the separation programme, DEPLETE was selected. The negative fraction was collected from port 'neg1' for naïve CD4 cells. A live cell count was taken using trypan blue exclusion and cells spun and resuspended in culture medium at 2 x 10^6 cells/ml.

Pan T cell Isolation:

Freshly isolated PBMCs (2.2.1.1) were washed and pelleted. Cells were resuspended in running buffer (40µl / 10^7 cells). Biotin-antibody was added (10µl / 10^7 cells) and left in fridge for 10 minutes. Running buffer was then added (30µl / 10^7 cells) followed anti-biotin beads (20µl / 10^7 cells) and left in the fridge for a further 15 minutes. Cells were washed with running buffer, spun at 1200rpm and resuspended in 500ul of running buffer per 10^7 cells. The cells were loaded onto the AutoMACs (Miltenyi Biotech) and the separation programme, DEPLETE was selected. The negative fraction was collected from port 'neg1' for enriched T cells. A cell count was taken and cells spun and resuspended in culture medium at 2 x 10^6 cells/ml.

2.2.2 Monoctye-Derived Dendritic Cell Cultures:

Purified monocytes (2.2.1.2) were resuspended in culture medium at 1 x 10^6 cells/ml in sterile flat bottomed, 6-well tissue culture plates at 3mls per well. The culture medium was supplemented with 50ng/ml rhGM-CSF and 1000IU/ml rhIL4. Cells were incubated at 37^0C and 5% CO_2. After 2 days the cultures were supplied with 0.5mls of fresh medium containing 50ng/ml GM-CSF and 1000IU/ml IL4 per well and this was repeated after another 3 days, when MoDCs were harvested to be used as immature MoDCs.

2.2.3 Stimulation of Mo-DCs and pDCs

2.2.3.1 *Stimulation of MoDCs:*

Day 5 immature MoDCs (section 2.2.2) were harvested, washed and resuspended in culture medium supplemented with IL4 and GM-CSF (1 x 10^5 cells/ml). Cells were plated into a 24-well, flat bottomed tissue culture plate (2mls / well). MoDCs were cultured with medium alone or in a 1:1 ratio with CD40 ligand-transfected Chinese hamster ovary cells (hCD40L-CHO) with or without different concentrations of ribavirin (4, 20, 100 and 500μM). As known stimuli of maturation, comparison was made with the TLR4 ligand lipopolysaccharide (100 μg/ml) and the TLR7/8 agonist, loxoribine (250μM). For the Helminth protein study (chapter 4), Mo-DCs were exposed to rOv-ASP at concentration of 5ug/ml alone or in a 1:1 ratio with a CD40L-transfected cell line. Cells were incubated at 37^0C in 5% CO_2.

2.2.3.2 Stimulation of pDCs

Freshly isolated pDCs were plated into 96 well (200μl /well) cell culture plates at a concentration of 2 x 10^4 / ml. pDCs were cultured under the following conditions; (1) medium alone (2) in a 1:1 ratio with hCD40L-CHO cells, (3) with 1μM M362, a TLR9 agonist or (4) 250μM loxoribine, a TLR7 agonist each with or without different concentrations of ribavirin (4, 20, 100 and 500μM). Cells were incubated at 37^0C in 5% CO_2.

2.2.4 Immunostaining Techniques for Flow Cytometry

2.2.4.1 Staining MoDCs and pDCs for Cell Surface Markers:

MoDCs and pDCs cultured for 48 and 24 hours respectively in the presence or absence of additional stimuli (2.2.3.1 and 2.2.3.2), were harvested to investigate their phenotype. Each treatment was harvested into a 15ml falcon tube and spun in the centrifuge at 1200rpm at 4^0C for 10 minutes. The cells were resusupended in FACS block (2.1.2), allowing 50µl for each staining condition to be carried out. 50µl of the blocked cells were transferred into the required number of wells on a v-bottom 96 well plate and incubated on ice for 30 minutes. Antibodies were diluted with FACS block to optimal concentrations for flow cytometry and 50ul of appropriate antibody added to 50ul of block already in the well. Cells were incubated on ice for 30 minutes. The cells were washed 2 times in 200µl FACS wash and spun at 1200rpm at 4 c for 7 minutes. The supernatant was removed and cells resuspended in 150µl FACS wash (2.1.2) with 50ul 4% paraformaldehyde (PFA) to fix the cells in order to retain the fluorescence. The cells were transferred to micronic tubes and placed on ice in the dark pending analysis by FACS.

2.2.4.2 2 Colour Flow cytometric analysis of surface molecules:

Fluorescently labeled cell suspensions were passed through a Fluorescence-Activated Cell Scanner (FACScalibur© FACS machine, B D Biosciences, Oxford, UK) and cell populations were analysed using Cell Quest© Software (BD Biosciences) and WinMDI. Unlabelled cells were used to set the sensitivity of the fluorescence detectors to appropriate levels, such that on a histogram the peak of cells lay in the first decade of a four log scale. Singly-stained cells labeled with FITC were used to compensate the fluorescence signal in FL1. Cells stained with PE were used to compensate the fluorescence signal in FL2. This was done using the standard compensation circuits on the instrument. MoDCs and pDCs were typically detected using forward and side scatter settings and gated to exclude contaminating lymphocytes.

2.2.5 Identification of apoptotic cells and cell death by flow cytometry

2.2.5.1 Immunostaining of apoptotic cells for flow cytometry:

Apoptotic cells were determined by their ability to bind annexin V. Freshly isolated pDCs were plated into 96 well cell culture plates at a concentration of 2 x 10^4/ml in complete medium in the presence of M362 with and without ribavirin at different concentration (500uM, 100uM, 20uM and 4uM) for 24 hours.

Cultured cells were harvested into 15ml Falcon tubes and centrifuged at 1200rpm for 10 minutes. The supernatant was aspirated and each culture condition resuspended in 300µl cold PBS and divided into 3 wells of a v-bottomed 96 well plate. The cells were washed twice in cold PBS at 1200rpm, 4 c for 10 minutes and resuspended into 100µl of binding buffer (BD Biosciences, Oxford, UK). Annexin V-FITC (BD Biosciences) alone (5µl) was added to one well, PI (BD Biosciences) alone (5µl) to another well, and PI in combination with annexin V-FITC in the final well. Following incubation for 15minutes at room temperature in the dark, 100µl of binding buffer was added to the wells. Cells were transferred to micronic tubes and a further 50µl binding buffer added to them. Apoptotic cells were analysed within two hours using a FACScan cytometer.

2.2.5.2 Flow cytometric identification of apoptotic cells:

pDCS were detected on forward and side scatter and gated to eliminate cell debris. PI-labeled cells were used to compensate the fluorescence signal in FL2 and cells stained with annexin V-FITC were used to compensate the fluorescence in FL1. Early apoptotic cells were identified as cells showing labeling for annexin-V but impermeable to PI. Late apoptotic cells were identified as cells showing labeling for annexin-V and permeable to PI. Live cells were identified as showing no labeling for annexin –V and impermeable to PI. Five thousand events for each preparation were collected.

2.2.6 Techniques for Cytokine Analysis

2.2.6.1 *Enzyme Linked Immuno-Sorbent Assays*

Commercially available Human IFN-α multi-species Enzyme-Linked Immunosorbent Assay (ELISA) (PBL Biomedical Laboratories, NJ, US) was used to detect IFNα.

ELISAs were performed at room temperature according to the supplier's instructions using the solutions supplied with the kit. In brief, supernatant were harvested after 24 hours of culture. The supernatants were harvested and stored at -20 c until ELISAs were undertaken.

A standard curve from 0 – 5000pg/ml was constructed by serial dilutions of the Human Interferon Alpha Solution in the dilution buffer. Assay samples were diluted to optimal concentrations with dilution buffer. 100µl of the assay samples or standards prepared above were placed in individual wells of the microtitre plate in duplicate (figure 13). The plate was sealed and incubated for one hour in a closed chamber at 24^0C. After the first incubation, the contents of the plate was emptied and the wells washed once only with the Final Wash Solution, filling each well when washing. After washing, the plate was inverted and blotted on lint-free absorbent paper and the plate tapped dry.

The antibody solution was diluted with 2.9µl of antibody concentrate added to 1ml of dilution buffer for each strip of microtitre plate. 100µl of this was added to each well and the plate sealed and incubated for 1 hour at 24^0C and then emptied and washed 3 times as before. HRP Conjugate concentrate was diluted 1:80 and 100µl added to each well. The plate was covered and incubated for a further 1 hour at 24^0C and then emptied and washed four times as before. During the incubation, the TMB solution was warmed to 24^0C and 100µl added to each well. The plate was covered and incubated for 15minutes at 24^0C in the dark to allow the colour reaction to develop. Before the negative on the standard curve began to change colour, the reaction was stopped by the addition of 100µl of 'stop' solution to each well. The optical density of each well was then determined using a microtiter plate reader set to 450nm with a reference filter at 570nm.

No differences were identified from samples on the edge of the plates.

Figure 13: Layout for IFNα ELISA:

	1	2	3	4	5	6	7	8	9	10	11	12
A	BK	BK										
B	BK	BK										
C	S1	S1										
D	S2	S2										
E	S3	S3										
F	S4	S4										
G	S5	S5										
H	S6	S6										

BK- Blank, S1-6: Standards

2.2.6.2 *Multiplex Fluorescent Bead Immunoassay (FBI):*

The Bender MedSystems human Th1/Th2 10plex kit II (Bender MedSystems, Burlingame, CA) allows simultaneous measurement of up to 10 cytokines (TNFα, IFNγ, IL-1B, IL2, IL4, IL5, IL6, IL8, IL10, IL12p70). The kit was used at room temperature, according to the manufacturers' instructions using the solutions supplied with the kit.

The contents (50mls) of the 10x assay buffer concentrate was added to 450mls of distilled water and stored at 2-8^{0}C. The standard was prepared by taking each vial of standard for the 10 cytokines and centrifuging briefly. Each standard was reconstituted with distilled water, according to the labels on the vial to give 400ng/ml stock. The vial was vortexed to ensure complete solubilisation and left to stand for 10 minutes.

Tubes were labeled from 1 to 7. 100ul of assay buffer was added to each. To tube 1, 10µl of each of the 10 reconstituted standards was added and mixed well. 50µl from standard 1 was transferred to tube 2 and mixed thoroughly. 50µl was transferred from standard 2 to tube 3 and this was continued until 50µl has been transferred from tube 6 to 7. The vials containing the fluorescent beads for all 10 cytokines were vortexed for 5 seconds each. To make sufficient bead mixture for a full 96-weel plate, 150µl of each of the 10 bead sets were transferred into one tube. The vials containing the biotin conjugates specific for all 10 cytokines were vortexed briefly. 300µl of each of the 10 biotin conjugates was added to the same tube. The filter plate was prepared by wetting each well wit 50µl of assay buffer and then aspirating using a vacuum manifold, blotting the bottom of the plate after filtration.

12.5µl of the appropriate standard or blank was added as follows:

A1 and A2: standard 1, B1: standard 2, C1: standard 3, D1: standard 4, E1: standard 5, F1: standard 6, G1: standard 7, H1: assay buffer as blank.

12.5µl of each undiluted test supernatant was added from B2 onwards. To all wells, 12.5µl of 10-plex bead mixture and 25µl of biotin-conjugate mixture was added. The plate was covered with adhesive film, wrapped in foil to exclude light and incubated for 2 hours at room temperature on a microplate shaker at 500rpm.

After incubation, the wells were emptied using a filtration manifold and 100µl of assay buffer was added to each well and then emptied using the vacuum manifold. This was repeated once more.

The streptavidin-PE (SA-PE) was prepared by diluting with assay buffer at 1:31.35

50µl of assay buffer was added to each well followed by 25µl SA-PE. The plate was covered, wrapped in foil and incubated for 1 hour at room temperature on the microplate shaker at 500rpm. The plate was emptied using the vacuum manifold and 100µl of assay buffer added to each well twice as before. 150µl of assay buffer was added to each well and using the multichannel pipette the beads in each well were resuspended and the contents of each of the wells were transferred into micronic tubes and a further 100µl assay buffer added to each micronic tube. Cytokines were identified using flow cytometry (FACSCalibur) and analysed using CellQuest software.

2.2.7 Lymphocyte Proliferation Assays:

2.2.7.1 Allogeneic Mixed Lymphocyte Reaction (MLR):

Proliferation of T cells in response to stimulated MoDCs was compared using MLR in the helminth study (chapter 4).

Day 5 immature MoDCs (2.2.2) were stimulated with r*Ov*-ASP-1 at 5ug/ml for 48 hours. Allogeneic naïve CD4 T cells were isolated as previously described (2.2.1.4) and resuspended in complete medium (containing HAB serum) at a concentration of 2 x 10^6/ml and plated into 96-well flat bottomed plates. MoDCs were serially diluted in complete medium (from 8 x 10(4)/ml – 625/ml) and 100µl added to 100µl of T cells resulting in ratio of DC to T cell of 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280 (in triplicate). Controls were also prepared containing T cells or DCs alone.

The cells were incubated at 37^0c for 5 days before 1µCi tritiated [methyl-H^3} thymidine (GE Healthcare) was added to each well. Following 18 hours incubation at 37^0c, 5% CO_2, thymidine incorporation was stopped by washing the plate. Using a 96-well plate cell harvester the cells from each plate were harvested onto separate glass fibre filters. The filters were air dried and 2ml Liquid Scintillant (Microscint 40, Perkin Elmer LAS, UK) added. T cell proliferation was quantified by measuring H3-thymidine incorporation as counts per minute (cpm) by a β-Plate reader (Packard Top Count NXT (Model 9902V), Groningen, Netherlands).

2.2.7.2 Proliferation assay for Antigen specific T cell responses :

To determine if r*OV*-ASP1 treatment enhances the ability of MoDCs to support TT-induced T cell proliferation by autologous T cells Chapter 4) we measured antigen specific T cell proliferation by tritiated [methyl-H^3} thymidine incorporation.

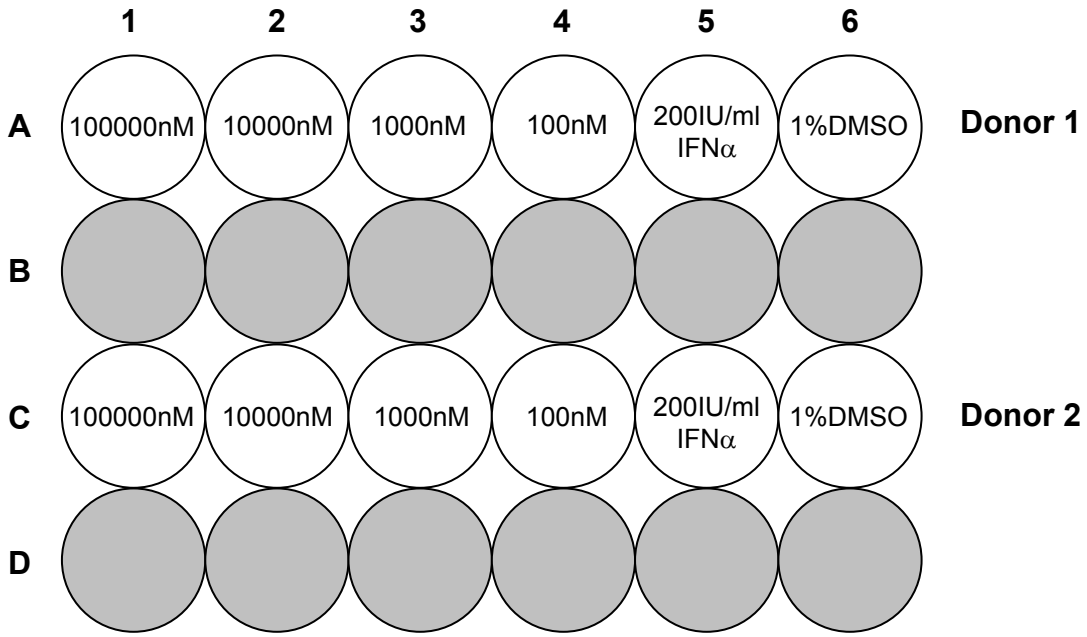
Day 5 MoDCs (2.2.2) were cultured (0.5 x 10^5) in complete media alone, or with tetanus toxoid (1µg/ml). After 6 hours, r*Ov*-ASP-1 (5µg/ml) was added to one of the wells of the two groups and cells cultured for a further 24 hours. Autologous Pan T cells were isolated as previously described (2.2.1.4) and resuspended in complete medium (containing HAB serum) at a concentration of 2 x 10^6/ml. Purified T cells were co-cultured in triplicates with the different MoDCs groups at various DC : T cell ratios (1:2, 1:10, 1:50 and 1:250) in U bottom 96-well plates for 5 days at 37^0c, 5% CO_2. During the last 18 hours of incubation, the cultures were pulsed with1µCi/ml ^{3}H-thymidine. Thymidine incorporation was stopped after 18 hours by washing the plate and using a 96-well plate cell harvester the cells from each plate were harvested onto separate glass fibre filters and the incorporated tritium thymidine was measured on a β plate reader to determine induction of antigen specific T cell proliferation.

2.2.8 Evaluating the antiviral and immunostimulatory actions of TLR Agonist in chronic hepatitis C.

2.2.8.1 *Preparation of assay plates*

TLR7 agonist (SM360320) stock (2.1.5) was diluted 10 times in a serial dilution in phosphate buffered saline (PBS) + 0.1% dimethyl sulphoxide (DMSO). IFNα stock (2.1.5)was also diluted 10 times in PBS, 10% foetal calf serum and 1% DMSO. The background controls for these experiments were PBS with 0.1% DMSO.
Compounds were aliquoted into 24 well assay plates as shown in figure 14 at 50ul/well for anti-viral action assays and 100ul/well for NK assays. Assay plates were prepared in advance of blood collections and stored at -20 C until used.

Figure 14 24-well plate layout for stimulating whole blood with TLR7 agonist



NB: Molarities of the stimuli solutions are shown. In the final assay, these will be diluted 1/10 by addition of whole blood or PBMCs.

2.2.8.2 Assay set up:

Pre-prepared assay plates (2.2.8.1) containing the compound were thawed at room temperature in a class II hood for at least 30 minutes prior to addition of blood or PBMCs to allow equilibration of the plates to room temperature.

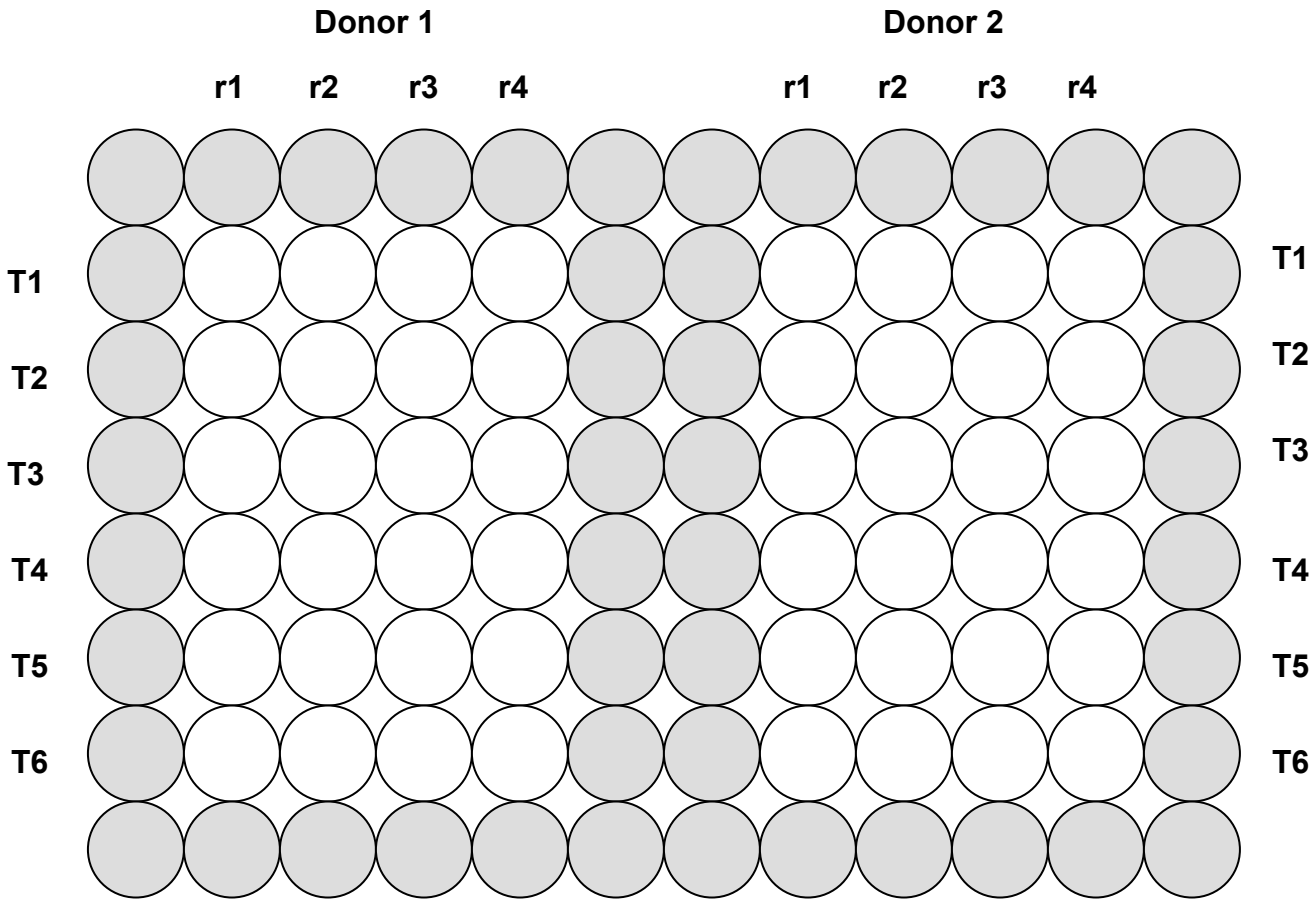
Aliquots of fresh blood (450ul) collected into sodium citrate vacutainers (2.1.7.2) were transferred into 24-well assay plates containing 50µl pre-prepared agonist, SM-360320, IFNα or no stimulus according to the plate plan (figure 14). Final concentrations of SM-360320 were 0.1µM – 10µM and IFNα was 20IU/ml. The plate was sealed using a breathable membrane, placed on an orbital shaker and incubated for 5 hours at 37^0C / 5% CO_2.

2.2.8.3.1 ***Harvest of samples:***

After the incubation period, aliquots (4 x 25µl) of the stimulated blood from each well were transferred to a U bottomed 96 well plate as shown in layout below (figure 15) and mixed thoroughly with multichannel pipette with MagMAX lysis/binding solution (130µl/well of MagMax 96 blood lysis buffer (Ambion) mixed with isopropanol in a 1:1 ratio). Fresh blood from NHDs (180µl) was transferred to 96-well plates containing 20µl of SM-360320 (1µM), IFNα 20IU/ml) or no stimulus. One aliquot of stimulated blood was removed into MagMax buffer.

The blood remaining in the 24 or 96 well assay plates was transferred into 2ml screw capped eppendorf tubes and spun (5 minutes at 500rpm) and the plasma was removed and frozen at -80^0C. The remaining cell pellet was resuspended in the residual plasma (~300µl) and 1.3mls RNALater (Ambion) was added to stabilize the samples. These samples were also frozen at -80 C pending further analysis for gene expression, cytokine analysis, and HCV replicon assays.

Figure 15: 96-well plate layout for gene expression analysis



T1: treatment 1, 10000nM TLR7
T2: treatment 2, 1000nM TLR7
T3: treatment 3, 100nM TLR7
T4: treatment 4, 10nM TLR7
T5: treatment 5, 20IU/ml IFNa 2
T6: treatment 6, 1% DMSO

2.2.8.4 Evaluating the anti-viral effects of TLR7 agonist, SM-360320

2.2.8.4.1 Gene Expression Analysis (performed at Pfizer):RNA extraction and analysis by PCR was done to determine the induction of IFN-inducible genes e.g. 2'-5' OAS:

After thawing, RNA was extracted from blood mixed with MagMAX lysis/binding solution using the MagMAX 96 well blood RNA isolation kit (Ambion; #1837) according to the manufacturer's instructions.

Complementary deoxyribonucleic acid (cDNA) was prepared using high capacity cDNA archive kits (Applied Biosystems, USA) using 10mL RNA in 20mL reactions. Each reaction contained 2mL of 10x reverse transcriptase (RT) buffer, 0.8mL of 25 x deoxyribonucleotide triphosphate (dNTP) mixture, 2 mL of 10 x random primers, 1 mL of Multiscribe RT (50U / mL) and 4.2 mL of RNase free water.

2'5'OAS gene expression was quantified using real time taqman Quantitative Polymerase Chain Reactions (QPCR) using 'assay on demand reagents' (Applied Biosystems, USA) in multiplex reactions as described by the manufacturer. Each reaction contained 12.5 µL of Taqman mix, 1.25 µL of 20 x actin reagents and 20 x 2'5'OAS reagents, 5 µL of cDNA and 5 µL of RNase free water. β-actin and 18S were used as housekeeping genes. QPCR amplification reactions were performed on a 7900 real time PCR machine (Applied Biosystems) over 40 cycles; annealing at 60ºC for 45 seconds, primer extension at 72ºC for 1 minute and denaturation at 94ºC for 20 seconds.

2.2.8.4.2 HCV replicon assay (Performed at Pfizer)

The presence of soluble factors with anti-viral activities was measured by evaluating their ability to inhibit HCV replication in an HCV-replicon system.

Huh7 cell culture and cell harvest: Huh7 1b replicon cells were cultured in flasks ($T225cm^2$) to confluency of around 80% in Huh7 media (RPMI, 10% FCS, 5mls non-essential amino acids, 5 mLs sodium pyruvate, 5 mLs Penicillin/streptomycin and 5 mLs geneticin). Spent media was poured off the flasks and cells were rinsed with 20mls sterile PBS. After rinsing, 5mls of accutase was applied to the cells and the excess was disposed of. After 5 minutes, flasks were tapped to remove cells from flask wall and 40mls of assay medium (Huh7 medium without geneticin) was added to the cells.

Cells were centrifuged (5 minutes at 1000rpm) and the supernatant was removed. Cells were resuspended in assay medium (25mL), counted and seeded at 111,111 cells/ml. 90 uL was seeded into each well of both a 96 well white and clear bottom plate. Plates were incubated at 37°C/5% CO_2 in a humidified incubator.

Supernatants (10 uL) from whole blood ex vivo experiments were added to the replicon cells – one white and one clear plate. Plates were incubated in a humidified incubator as above for 24 hours.
Cellular Proliferation assay: To each white plate, 100 uL of Bright-Glo mix – lyophilized substrate reconstituted in bright-glo assay buffer (Promega, #E2650) was added. Luminescence was read using an Analyst plate reader (using default luminescence programme).

WST-1 cellular viability assay: To each clear plate, 100 uL of WST-1 diluted 1:3 with PBS at RT was added. Plates were incubated in a humidified incubator for 1-4 hours and absorbance was read at 450nm on the Wallac victor using the photometry>abs 450 at 0.1second.

HCV Replicon levels were then quantified using a luciferase reporter assay (270).

2.2.8.4.3 Cytokine analysis (Performed at Pfizer):

Levels of pro-inflammatory cytokines IFNα, IP-10, TNFα, IL-1 IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70 and IFNγ were determined using a custom multiplex electrochemiluminescence Enzyme-Linked ImmunoSorbent Assay (ELISA) as described by the manufacturer (Meso Scale Discovery). Harvested plasma from whole blood ex vivo assays was used to quantify production of cytokines. Aliquots of plasma (25µL) from whole blood ex vivo assays were added to custom 10-plex multispot ultrasensitive cytokine detection plates (MS600, MSD, 9238 Gaither Road, Gaithersburg, Maryland 20877, USA). Only values that fell between the highest and lowest concentrations on the standard curve were used. Below the limit of quantification (BLQ) is defined as less than or equal to 20 pg/ml, and any results below that were reported as BLQ.

2.2.8.5 Evaluating the immunomodulatory actions of SM-360320

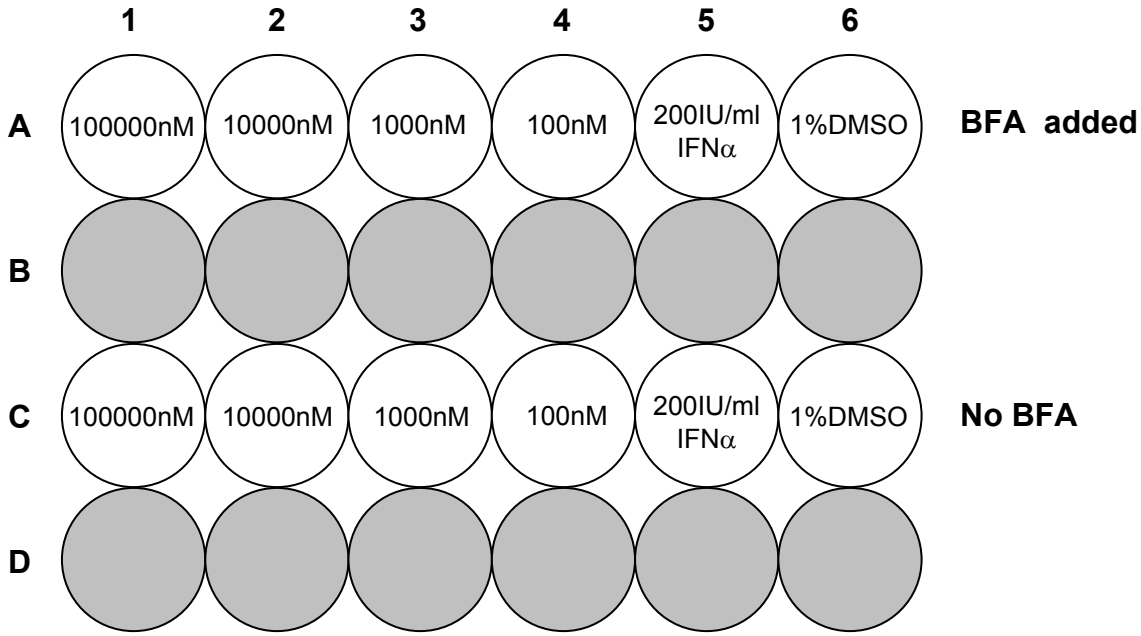
2.2.8.5.1 *NK cell Experiments*

NK cell Activation:

Previously cryopreserved PBMCs (2.2.1.1) were thawed rapidly in a pre-warmed waterbath at 37°C. Thawed cells were added drop-wise to 10mls RMPI 1640 with 20% FCS and 2mM L-glutamine and 1% penicillin-streptomycin and left to rest for 5 hrs at 37°C in a 5% CO2 humidfied incubator prior to the assay.

Cells were resuspended to 2 x 10^6 / ml in RPMI 1640 (no phenol) containing 10% FCS, 2mM L-glutamine & 1% PS. 900ul PBMCs were plated into duplicate wells of a 24 well plate containing 100µl pre-prepared agonist per well, 100µl IFNα2b.or 100µl of dilutent only (2.2.8.1). After 5 hours incubation at 37°C, 1ul of Brefeldin-A (Sigma, Poole, Uk) was added to one well of each duplicate (figure 16). Cells were then incubated for a further 18 hours.

Figure 16 24-well plate layout for NK Cell Activation



NK Cell Surface Staining **:**

After a total culture period of 24 hours, PBMCs were harvested and labelled for four-colour flow cytometry to identify activated $CD56^+$ $CD16^+$ NK cells on the basis of cell surface CD69 expression and cytoplasmic interferon gamma (IFNγ) expression by intracellular staining.

Cells were resuspended in FACS block, allowing 50µl for each staining condition to be carried out. 50µl of the blocked cells were transferred into the required number of wells on a v-bottom 96 well plate and incubated on ice for 30 minutes.

Antibodies (CD56-APC, CD4-FITC, CD69-FITC, CD3-PE) and their isotypes (IgG1-APC, -FITC, -PE) were diluted with FACS block to optimal concentrations for flow cytometry and 50ul of appropriate antibody added to 50ul of block already in the well. Cells were incubated on ice for 30 minutes. The cells were washed 2 times in 200µl FACS wash and spun at 1200rpm at 4 c for 7 minutes. The supernatant was removed and cells resuspended in 200µl FACS wash (2.1.2). The cells were transferred to micronic tubes (table 4) and placed on ice in the dark pending analysis using FACS Calibur flow cytometer with cell quest software.

Intracellular IFNγ Staining

For intracellular staining, the cells to which BFA was added were harvested, blocked on ice and surface stained as above. Once washed twice with FACS wash, they were resuspended in 100ul fixation/permeabilisation buffer (BD Biosciences, Oxford, UK) and incubated on ice for a further 20 minutes. After washing with permeabilisation buffer, cells were stained with FITC-labelled anti-human IFNγ or IgG1 (50ul added to each well), diluted to the optimal concentration and left on ice for a further 40 minutes in the dark. After staining cell were washed twice with permeabilisation buffer, resuspended in FACS wash and stored on ice in the dark pending FACS analysis.

Table 4 Sample plate set up for NK cell staining

Cells without BFA:

Tube	Surface Stain
1	Buffer only
2	IgG1FITC + IgG1PE + IgG1APC (1/30)
3	CD4 FITC (1/30)
4	CD3 PE (1/30)
5	CD56 APC (1/30)
6	CD56APC + CD3PE+ CD69FITC (1/30) (1/30) (1/50)
7	CD56APC + CD3PE+ IgG1FITC (1/30) (1/30) (1/500)

Cells with BFA:

Tube	Surface Stain	Intracellular Stain
1	Buffer only	Buffer only
2	IgG1FITC + IgG1PE + IgG1APC (1/30)	Buffer only
3	CD56APC + CD3PE (1/30)	IgG1FITC ICCS (1/500)
4	CD56APC + CD3PE (1/30)	IFNgFITC (1/500)

Fluorescence Activated Cell Scanning of stained PBMCs

Unlabelled PBMCs (tube 1) were used to set the sensitivity of the fluorescence detectors to appropriate levels, so that on a histogram the peak of the cells lay in the first decade of a four-log scale. Singly-stained cells with CD4-FITC, CD3-PE were used to compensate the fluorescence signal in FL1 and FL2 and cells stained with CD3-PE and CD56-APC were used to compensate the fluorescence signal in FL4. This was done using the standard compensation circuits on the instrument. PBMCs were gated using forward / side scatter characteristics. NK cells were identified as cells showing strong labeling for CD56 but negative for CD3 and a second gate was formed around these cells. The amount of IFN gamma production produced from these cells was determined by setting a histogram marker at <1% of the appropriate isotype control, typically on the first decade of the 4-log scale. Five thousand events were acquired per sample and analysed using WinMDI software.

2.2.8.5.2 *Plasmacytoid Dendritic cell Depletion Assays:*

To determine the contribution of pDCs to any anti-viral or immunostimulatory effects of SM360320 on cHCV-PBMCs, whole PBMCs and PBMCs from which BDCA4^{+} pDCs had been depleted by immunomagnetic separation were stimulated with SM360320 and the efficacy compared in the HCV-replicon assay, gene expression analysis, cytokine secretion and NK cell activation.

PBMCs were prepared from freshly drawn EDTA-treated venous blood (2.2.1.1). pDC were isolated from the PBMCs (2.2.1.3) and the negative and positive fraction retained fro use. The % purity of the pDC depletion was assessed by FACS analysis. Whole PBMCs and PBMCs from which BDCA^{+} pDC were depleted were resuspended at 5.55 x 10^5 in culture medium and added to pre-prepared 96 well plate (180uL / well) containing the TLR7 agonist and IFNα. pDCs alone were resuspended at 2.76 x 10^5 and added to the same plate (180uL / well). The plate was sealed using a breathable membrane, placed on an orbital shaker and incubated for 5 hours at 37^0C / 5% CO_2.

After the incubation, the plate was spun (5 minutes at 1200rpm) and the supernatant removed and stored in a fresh U bottom 96 well plate. The cell pellets were resuspended in 150µl Quagen RLT Lysis buffer (Ambion). All samples were frozen at -80 C pending further analysis at Pfizer for gene expression, cytokine analysis, and HCV replicon

assays. NK cell activation experiments comparing whole PBMCs with pDC deplete PBMCs were done using previously cryopreserved cells with staining as in 2.2.8.5

Chapter 3

The effects of Ribavirin on dendritic cell maturation and cytokine production

3.1 Introduction:

Chronic infection with hepatitis C virus (HCV) is a major cause of liver related morbidity and mortality with infection persisting in 80% of patients and is thought to result from a failure to sustain an immune response against HCV(35).

Dendritic cells (DCs) are professional antigen presenting cells characterized by their exceptional capacity to activate naïve T cells and prime adaptive immunity. DCs play a pivotal role linking innate and adaptive immunity(65;67). They recognize pathogens through pathogen recognition receptors (PRRs) including toll like receptors (TLRs), leading to DC activation and maturation(67). Activated DCs then activate NK cells, thus driving the non-specific innate immune response, whilst simultaneously presenting antigen to initiate T cell mediated adaptive immunity.

There are two functionally distinct subsets of DCs; mDC and pDC (chapter 1.5.1.1). pDCs are the most abundant producer of type-1 interferons in the body which they produce in large quantities during viral infections(271). mDCs prime T_H1 responses via IL12 production but also produce TNFα, IL10 and IFN-ß but not IFNα. Monocyte derived dendritic cells (MoDCs) are commonly used *in vitro* as a model of myeloid DCs due to the larger numbers that can be obtained and their close resemblance.

Evidence points towards dendritic cell dysfunction in patients with chronic hepatitis C, with impaired maturation and impaired ability to stimulate T cells which may compromise their capacity to mount, and sustain, an effective antiviral immune response, thus leading to viral persistence(118-121;125;127-131;133-136).

The current mainstay of treatment for CHC is ribavirin in combination with Pegylated IFNα, leading to sustained viral eradication in 40-80% of infected patients depending on the HCV genotype. Ribavirin is a purine nucleoside analogue that is activated or metabolised through 5' phosphylation to the monophosphate (RMP), diphosphate (RDP) and biologically active triphosphate (RTP) form. Despite its effective use for the last 40 years, the mechanisms of action of ribavirin are poorly understood but several hypotheses have been proposed(188). These include direct inhibition of viral RNA replication, inhibition of the enzyme inosine monophosphate dehydrogenase (IMPDH) and mutagenesis causing viral replication error catastrophe. Ribavirin has also been suggested to have immunomodulatory effects, shifting the T-helper 1 cytokine balance (T_H1)/ T_H2) toward T_H1 from T cells resulting in improved viral clearance.

Ribavirin monotherapy has minimal effect on HCV viraemia but is associated with improvements in serum aminotransferases(272) suggesting that it may have an effect on inflammation and the host immune response. In combination with IFNα however, ribavirin increases the proportion of patients that clear the virus whilst on treatment from 27% with IFNα monotherapy(167) to 41% with combination therapy(171). However its most striking effect is that it prevents relapse after cessation of treatment. This latter effect is unexplained but also plays a significant role in newer treatment regimes that include HCV protease inhibitors with pegylated interferon and ribavirin(221;223;273).

3.2 Aims

We set out to investigate the immunomodulatory effects of ribavirin on DC function in order to elucidate the mechanisms behind the prevention of relapse following treatment. Freshly isolated pDCs and cultured MoDCs from both NHDs and CHC patients were exposed *in-vitro* to ribavirin alone or to a combination of ribavirin and a maturation stimulus (CD40L, TLR9 agonist TLR7 agonist). The effects on cytokine secretion from DCs were studied as well as the expression of DC maturation markers (HLA-DR, HLA-A,B,C, CD83, CD86, CD40).

3.3 Materials and Methods:

Blood samples were collected from 21 uninfected normal healthy donors (NHDs) (18 male and 3 female, median age 53 years, range 25-67) who had no risk factors for blood borne viruses. Ten patients with chronic hepatitis C virus infection (4 female, 6 male, median age 54 years, range 37-61 years) were recruited from the hepatology clinics run by Southampton University Hospitals National Health Service Trust. All patients had detectable HCV RNA (4 genotype 1, and genotype 6 “non-1”). Patients were excluded if they had received treatment for HCV within 6 months prior to the study or tested positive for other blood borne viruses, including HBV and HIV.

Monocytes and pDCs were purified by positive selection from PBMCs using anti-CD14 conjugated and BDCA-4 magnetic microbeads respectively, in conjunction with an AutoMACS. Monocyte-derived dendritic cells (MoDCs) were generated by culturing monocytes in medium supplemented with rhGM-CSF (50ng/mL) and rhIL-4 (1000IU/mL) for 5 days.

Immature MoDCs and freshly isolated pDCs were cultured under the following conditions; (1) medium alone (2) in a 1:1 ratio with Chinese hamster ovary cells (hCD40L-CHO cells), (3) with 1μM M362, a TLR9 agonist or (4) 250μM loxoribine, a TLR7 agonist each with or without different concentrations of ribavirin (4, 20, 100 and 500μM). Cells were diluted in complete medium and incubated at 37^0C in 5% CO_2.

The cytokines produced by stimulated MoDCs and pDCs were assessed in cell culture supernatants after 24 hours of culture using human IFN-α multi-species ELISA and the Multiplex Fluorescent Bead Immunoassay (FBI) (IL12p70, IL10, IL8, IL6, IL1β and TNFα, IFNg, IL2, IL4, IL5). Three thousand events were acquired using FACSCalibur dual laser flow cytometer and analysed using CellQuest software.

After 48 hours and 24 hours of culture, MoDCs and pDCs respectively, were harvested and labelled for two-colour flow cytometry to identify their cell surface phenotype

Cell death was determined by FACS analysis with annexin V FITC in combination with propidium iodide after pDCs had been cultured in the presence of M362 (1μM) with and without ribavirin at different concentration (500uM, 100uM, 20uM and 4uM) for 24 hours.

For a full description of the methods, see *Materials and Methods* chapter.

Statistical Analysis:

Data was analyzed using GraphPad Prism version 4.0 for windows (GraphPad Software, San Diego, CA, USA) with unpaired *t*-tests and one way ANNOVA test applied with Dunnett's post test analysis.

3.4 Results:

3.4.1 *Ribavirin has no effect on IFNα production from immature NHD-pDCs:*

Freshly isolated pDCs from NHDs (*n* = 5) were cultured for 24 hours with; (1) medium alone (2) in a 1:1 ratio with CD40L transfectants (3) with TLR9 agonist, M362 or (4) with the TLR7 agonist, loxoribine in the presence or absence of ribavirin at different concentrations. Unstimulated pDCs produced negligible amounts of IFNα which were unaltered by the addition of ribavirin (Fig. 17A).

3.4.2 Ribavirin suppresses IFNα production from stimulated NHD-pDCs:

When pDCs (*n*=5) were stimulated with CD40L, IFNα production was significantly increased. However, the addition of ribavirin, at all concentrations, significantly reduced the production of IFNα by NHD-pDCs in a dose-dependent manner in response to CD40L (fig. 17B). The same effect was observed when either a M362 or loxoribine was used in combination with ribavirin (Figs. 17C and D respectively).

3.4.3 *Ribavirin suppresses TNFα, IL8 and IL10 from CD40L-stimulated NHD-pDCs*

Using the Multiplex Fluorescence Bead Immunoassay (FBI), we tested the effects of ribavirin on other cytokines (IL12p70, IL10, IL8, IL6, IL1β and TNFα, IFNγ, IL2, IL4, IL5) produced by CD40L-stimulated pDCs from NHDs (*n*=7). Ribavirin was found to reduce the production of TNFα, IL8 and IL10 from stimulated NHD-pDCs (Fig. 18). The effects of ribavirin on production of these cytokines by loxoribine or M362 has not been studied.

Fig. 17 Effect of ribavirin on IFNα production from pDCs:

(A) Unstimulated pDCs from NHDs produced negligible amounts of IFNα which were unaltered by ribavirin. (B) Ribavirin, at all concentrations tested, significantly reduced the amount of IFNα produced by NHD-pDCs in combination with CD40 ligation. When (C) a TLR9 agonist (M362) and (D) a TLR7 agonist (loxoribine) were used as alternative stimuli of IFNα production, ribavirin again reduced IFNα production. (analyzed using one way ANNOVA and Dunnett's post test anaysis) *p=<0.05; **p=<0.01

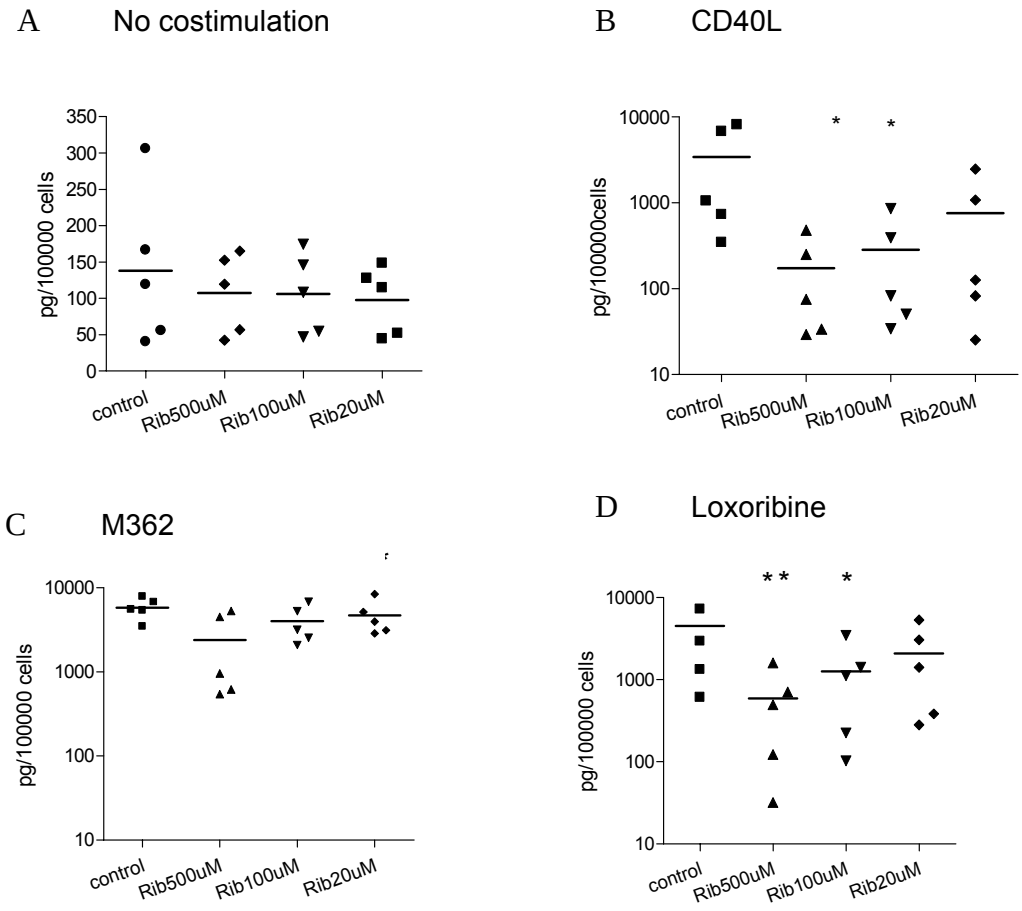
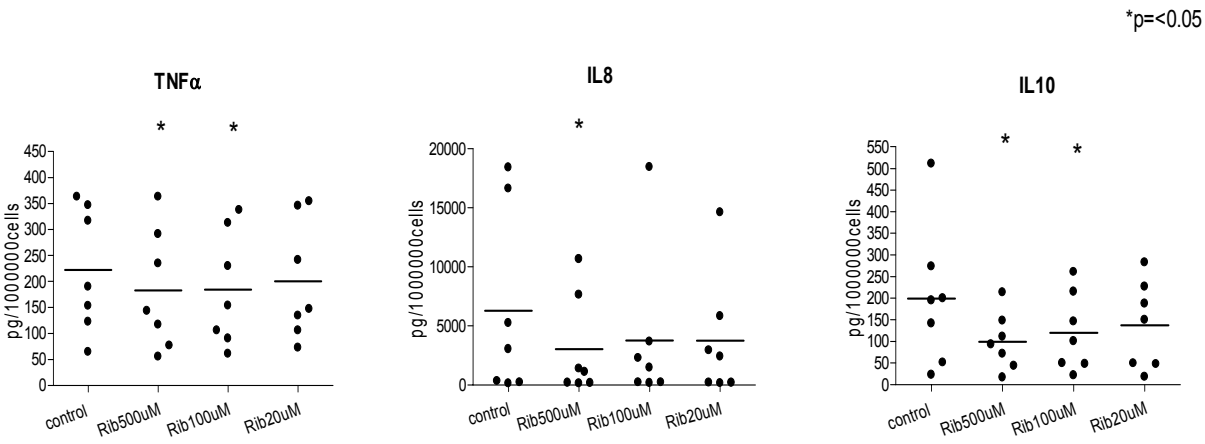


Fig 18. NHD-pDCs stimulated with CD40L and ribavirin show suppressed production of TNFα, IL8 and IL10. (analyzed using one way ANNOVA and Dunnett post test anaysis)



3.4.4 Ribavirin does not cause cell death or apoptosis in ex-vivo pDCs

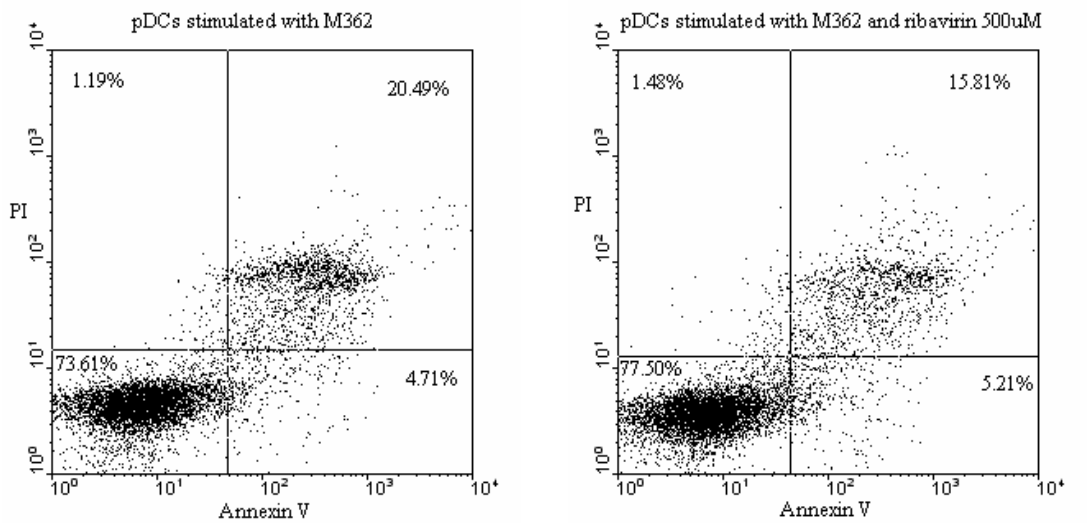
To ensure that the suppressive effects of ribavirin on pDC cytokine production were not due to induction of apoptosis or cell death, freshly isolated pDCs from NHDs were stimulated for 24 hours with M362 in the presence or absence of ribavirin, and then stained with annexin V and propidium iodide.

Figure 19A shows that the inclusion of ribavirin, did not result in increased apoptosis or cell death. After 24 hours of culture without ribavirin, 20% of pDCs were dead compared to 15%, when the highest dose of ribavirin (500μM) was added. The experiment was repeated 5 times and ribavirin did not significantly alter the proportions of live, apoptotic or dead cells at any of the test doses (Fig. 19B).

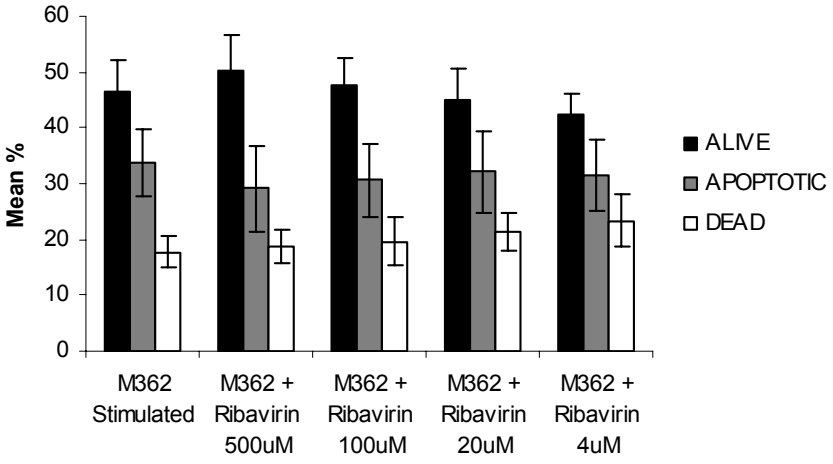
Fig 19. Cytokine changes in NHD-pDCs stimulated with M362 and ribavirin are not attributable to increased apotosis or cell death:

Apoptosis and cell death were assessed by flow cytometric staining for annexin V and PI (A). The left lower quadrant shows the number of viable cells (annexin-V and PI negative). The right lower quadrant shows the number of cells undergoing apoptosis (annexin-V positive and PI negative). The right upper quadrant shows the number of dead cells or those in late stages of apoptosis (annexin-V and PI positive). The experiment was repeated 5 times and ribavirin did not significantly alter the proportions of live, apoptotic or dead cells (B).

A



B



3.4.5 Ribavirin upregulates stimulus induced CD86 expression from NHD-pDCS

To ensure that the suppression of cytokine production from pDCs was not a ubiquitous reduction in all proteins, we looked at the effects of ribavirin on cell surface expression of CD86 and HLA class 1. Both molecules are normally highly expressed by stimulated pDCs. pDCs from NHDs (*n* = 5) were cultured for 24 hours with M362 (1μM) in the presence or absence of ribavirin and cells subsequently stained for two colour flow cytometry. We found that in pDCs stimulated with M362, ribavirin further upregulated the expression of CD86 (Figure 20A) (*p=<0.05) but had no effect on HLA class I expression (Figure 20B).

3.4.6 Ribavirin suppresses IFNα, TNFα, IL8 and IL10 from stimulated CHC-pDCs:

To investigate if these effects of ribavirin were also demonstrated in CHC-pDCs, freshly isolated pDCs from CHC patients (*n* = 5) were cultured for 24 hours in a 1:1 ratio with CD40L and the effects of ribavirin on IFNα and cytokine production were assessed. As seen in NHDs, IFNα production by pDCs from CHC patients was significantly reduced at 500μM,100μM and 20μM of ribavirin (Figure 21) and TNFa, IL8 and IL10 production were also significantly suppressed (Fig. 22).

Fig 20. Ribavirin upregulates stimulus induced CD86 expression from NHD-pDCS:
NHD pDCs (*n* =5) stimulated with 1μM M362 and ribavirin further upregulated the expression of CD86 in a dose dependent manner (Figure 20A) (*p=<0.05) but had no significant effect on HLA class I expression (Figure 20B).
MFI; mean fluorescence intensity; bars show mean.

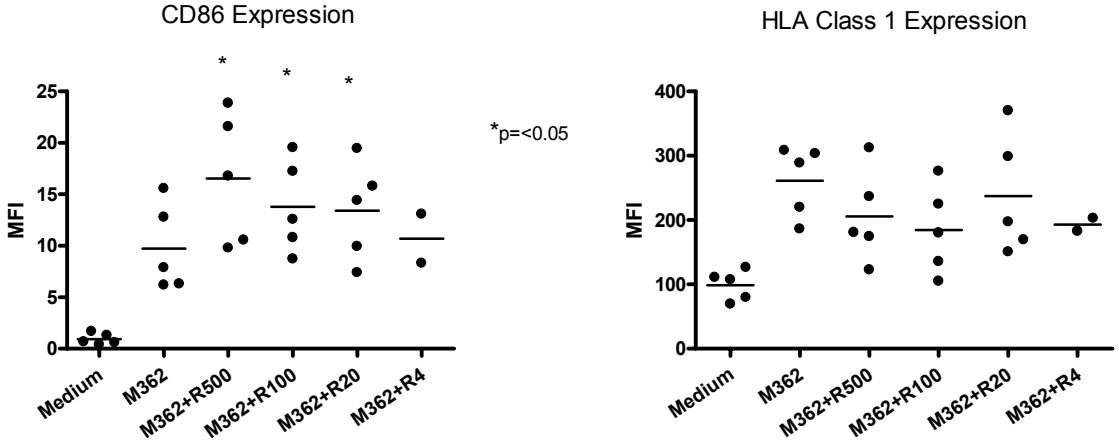


Fig 21. Ribavirin suppresses IFNα from stimulated CHC-pDCs:
pDCs from CHC patients show reduced IFNα production following CD40 ligation in the presence of ribavirin as seen in NHD-pDCs (*p=<0.05).

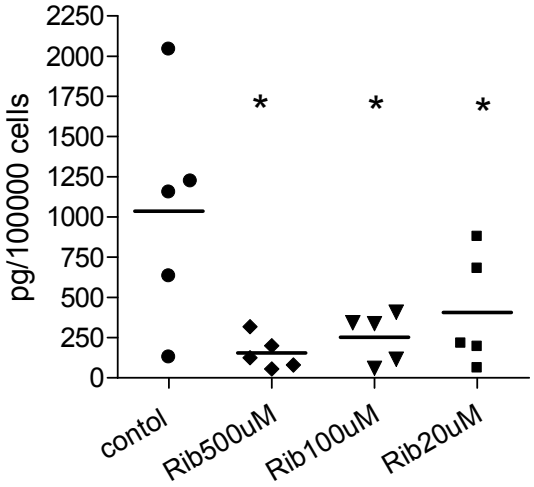
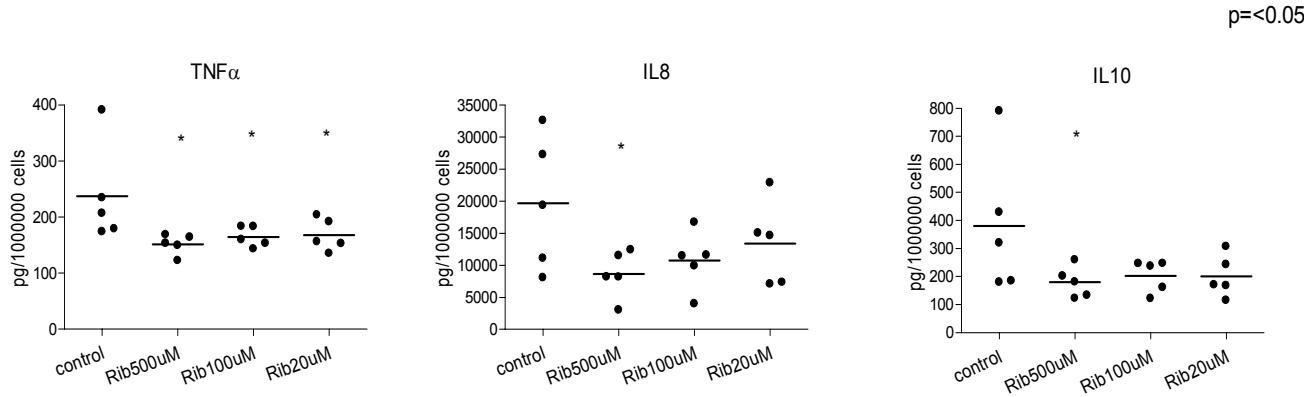


Fig 22. Ribavirin suppresses TNFα, IL8 and IL10 from stimulated CHC-pDCs:
In CHC-pDCs stimulated with CD40L (*n*=5), ribavirin was found to significantly suppress the production of TNFα, IL8 and IL10 by pDCs, mirroring the effects seen in NHDs.



3.4.7 *Ribavirin had no effect on unstimulated MoDC maturation:*

MoDCs from NHDs (*n*= 8) were cultured with ribavirin at different concentrations for 48 hours without any additional stimulus. Irrespective of the concentration used, ribavirin alone had no effect on levels of expression of phenotypic markers CD40, CD86, CD83 and HLA-DR on MoDCs (data not shown).

3.4.8 Effects of ribavirin on cytokine production from MoDCs:

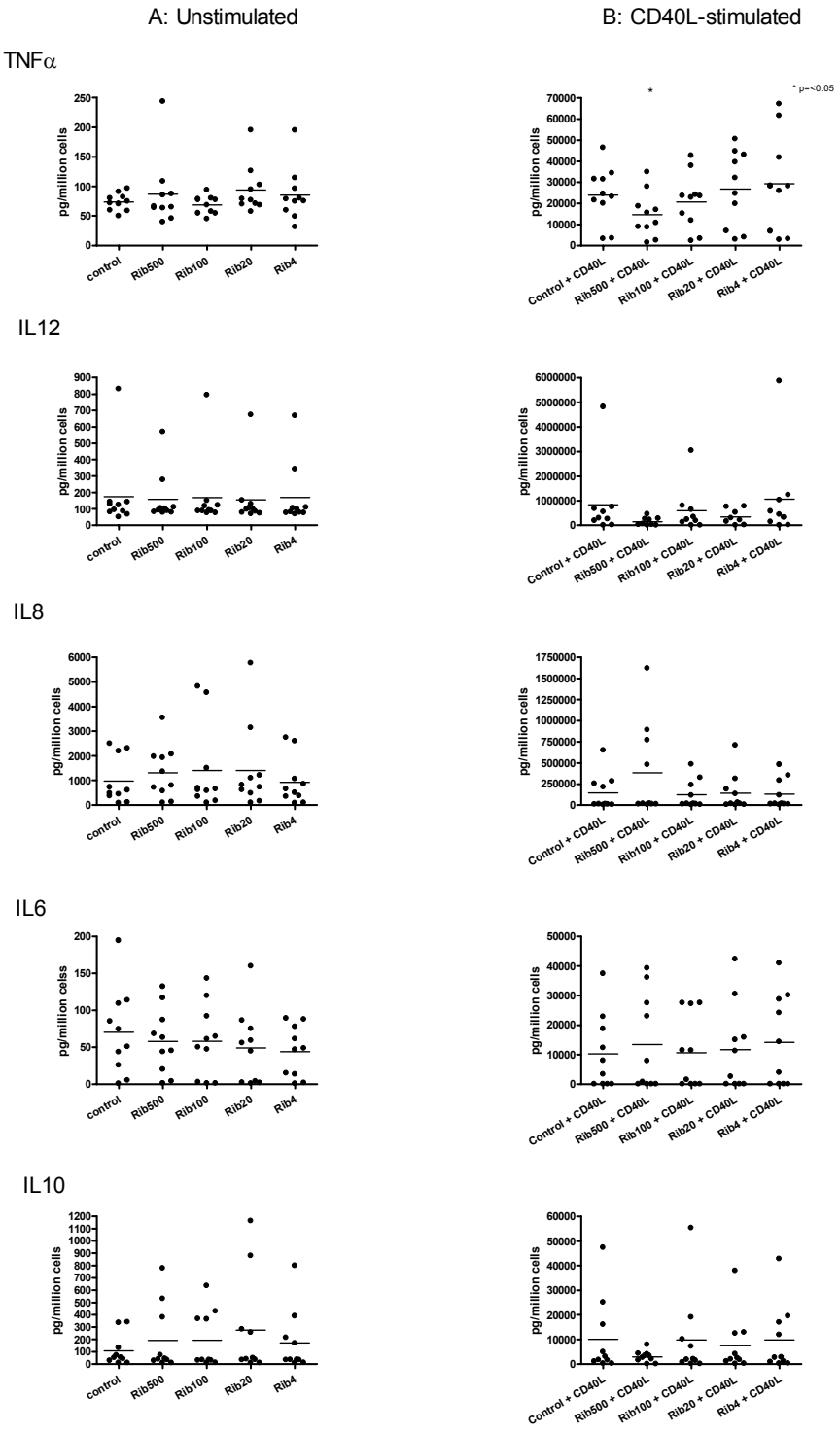
MoDCs from NHDs (*n* =10) were cultured with or without CD40L for 24 hours in the presence or absence of ribavirin at different concentrations.

In immature NHD-MoDCs, cultured with medium alone, ribavirin did not significantly alter the secretion of any tested cytokine (Fig 23a). When immature MoDC were cultured for 24hours with CD40L, CD40 transfectants ligation stimulated the production of TNFα, IL8, IL6, IL12 and IL10 (Fig 23b) but not that of IL1β, IL2, IL5 or IFNγ. The addition of ribavirin at a concentration of 500μM significantly suppressed the production of this CD40L-induced TNFα production (Fig 23b). The effect of ribavirin was less pronounced at lower doses. Ribavirin did not however, significantly alter the CD40L-induced production of the 9 other cytokines tested.

Fig 23. Effects of ribavirin on cytokine production from MoDCs:

A) In immature NHD-MoDCs, ribavirin had no effect on secretion of any tested cytokine.

B) MoDCs cultured with CD40L enhanced the secretion of TNFα, IL8, IL6, IL12 and IL10.



3.5 Discussion:

With the clinical effects of ribavirin still largely unexplained, we set out to investigate the immunomodulatory effects of ribavirin on dendritic cell function by looking principally at the effects on cytokine production.

In this study we have shown that ribavirin modulates dendritic cell cytokine production in response to a maturation stimulus. In pDCs from NHDs, ribavirin significantly suppressed stimulus-induced IFNα, TNFα, IL8 and IL10 and these findings were mirrored in pDCs from CHC patients. In MoDCs from NHDs ribavirin decreased CD40L-induced TNFα production but had no effect on other cytokines tested or phenotype.

The ribavirin induced suppression of TNFα and IL8 from MoDC and pDC shown in this study may indicate an anti-inflammatory effect of ribavirin and may, in part, explain the reported reduction in hepatic inflammation with ribavirin monotherapy. It has been shown that ribavirin alone transiently improves biochemical and histological response in the absence of significant reduction in HCV load(274).

The reduction of IFNα from ribavirin-treated pDCs may be of particular physiological importance since pDCs, unlike MoDCs, are the major source of INFα in the human body producing large quantities of type 1 IFN in response to viral infection. Several studies(131;133-136) have found pDCs from patients with CHC have impaired production of IFNα. This defect will have an impact on both innate and adaptive immunity and favour viral persistence. Our finding that ribavirin is unable to stimulate IFNα production from pDCs together with the further impairment in IFNα production from pDCs treated with ribavirin correlates with the requirement for exogenous IFNα in the effective treatment of CHC and may, in part, explain why ribavirin alone has no beneficial effects on HCV viral loads. Whether ribavirin is actually exacerbating the IFNα defect in CHC-pDCs needs further investigation in larger cohorts to explore the exact mechanisms. It would be interesting to investigate whether the addition of exogenous IFNα can overcome the suppressive effects of ribavirin on cytokine production from dendritic cells.

The opposing actions of ribavirin and TLR agonists in IFNα production demonstrated in this study may have implications if they are to be used in combination in the future

treatment of CHC. The study undertaken by Coley with Actilon the TLR9 agonist, CpG10101(248) (currently withdrawn from further drug development at present) looked at the effects of this compound alone, in combination with ribavirin or IFNα or all combined and found ribavirin combined with CPG10101 was less effective than when combined with exogenous IFNα or all 3 together, strengthening the importance of continued use of exogenous IFNα in future treatments.

As a purine nucleoside analogue, it has been hypothesized that ribavirin may exert immunomodulatory activities via TLR stimulation on dendritic cells(247). Our findings do not support this hypothesis, with ribavirin having no effect on either phenotype or cytokine production when administered alone to either pDCs or MoDCs. In stimulated DCs, ribavirin was still unable to further enhance cytokine production, but in fact reduced the production of IFNα, TNFα, IL8 and IL10 from pDCs and TNFα from MoDCs.

To ensure the suppression of cytokine production from ribavirin-treated DCs was not a ubiquitous downregulation of all proteins, we looked at the effects of ribavirin on CD86 and HLA class I expression, both of which are highly expressed on stimulated pDCs. Our results show that ribavirin had no significant effect on Class I expression and actually enhanced CD86 surface expression. The enhancment of CD86 surface expression was interesting and could reflect a subtle maturation effect of ribavirin. This should be explored further, looking at the other markers of pDC maturation such as increases in surface expression of MHC and co-stimulatory molecules, CD83 and CD80 as well as CD86. The expression of these co-stimulatory molecules is essential for effective activation of T lymphocytes. In previous studies it has been shown that DCs from CHC patients have impaired maturation with an immature phenotype(118-121;125;127-131;133-136). It is possible that ribavirin can overcome this effect and thereby restore antigen presentation and priming of T cells. In the absence of evidence of any other mechanism whereby ribavirin may enhance viral clearance and sustained response to interferon it may be a subtle effect on DC maturation that accounts for the profound clinical effects of ribavirin when used in conjunction with IFNα in the treatment of HCV infection and further studies will need to be carried out. The effect of dendritic cells treated with ribavirin on T cell allo-stimulation and the consequence for NK cell activation to see if ribavirin has any effect on DC antigen presentation function also needs further investigation.

Only one other study has examined the effects of ribavirin on DC maturation and function(275) and focused only on MoDCs. Using ribavirin at a physiological concentration of 120μM, they found that ribavirin suppressed IL-12 and IL-10 in addition to TNFα when co-administered with poly I:C as a stimulus. They assessed cell culture supernatants at multiple time points up to 48 hours and the suppression of IL-12 was seen at 32 hours and maximal suppression of IL-10 at 36 hours. In our study, supernatants were assessed at only one time point, at 24 hours which may be a reason for seeing no effect on IL12 in this study. However, they too found that ribavirin had no effect on phenotypic maturation in either stimulated or immature MoDCs.

A number of studies in PBMCs have shown that ribavirin has an effect on mitogen-induced cytokine production, with some studies showing enhancement and others inhibition(202-205). Furthermore some studies have shown ribavirin to induce a T_H1 profile(202;205;206), while others showed no impact on cytokine production(276) or induction of a T_H2 profile. We have shown reduction in both T_H1 (TNFα, IL8 and IL6) and also T_H2 (IL10) and the significance of this remains unexplained. Some of the inhibitory effects of cytokines seen in PBMCs are thought to be secondary to the known antiproliferative effects of ribavirin on T cells(203). DCs, however, do not proliferate during stimulation so this is unlikely to be the case in this study.

This study is limited by small numbers but has shown that ribavirin is able to modulate cytokine production from dendritic cells, in particular suppressing the production of IFNα. It is clear from recent trials with protease inhibitors Telaprivir (221;223;277) and Bocepravir(226) that ribavirin will continue to be required in future anti-viral regimes to improve SVR and prevent relapse. If this is the case, the findings in this study highlight the importance of the need for the continued use of IFNα therapy in this combination to overcome the suppression we have identified.

The mechanisms underlying the suppression of IFNα by ribavirin and the possible effects of ribavirin on pDC maturation should be explored further. By studying a larger cohort of CHC patients it may also be possible to assess differences in the effects on cytokine production from sustained viral responders and non-responders to treatment.

Summary of results:

Results from pDCs in NHDs and CHC:

1. Effects on IFNα production:
    - Ribavirin had no effect on IFNa production from immature pDCs
    - Ribavirin suppressed IFNa production from stimulated pDCs in NHDs (stimulated with CD40L, TLR9 or TLR7 agonist)
    - Ribavirin suppressed IFNa production from stimulated pDCs in CHC (stimulated with CD40L – not tested with TLR7 or TLR9 agonist)

2. Effects on other cytokines from pDCs:
    - Ribavirin suppressed TNFa, IL8 and IL10 from stimulated pDCs in NHDs and CHC (Stimulated with CD40L – not tested with TLR7 or TLR9 agonist)

3. Suppressive effects were not due to apoptosis or cell death:
    - Addition of ribavirin to NHD-pDCs stimulated with TLR9 agonist did cause cell death or apoptosis.

4. Suppressive effects were not ubiquitous to all proteins:
    - CD86 expression was found to be enhanced by ribavirin

Results from MoDCs in NHDs:

1. Effect on MoDC maturation:
    - Ribavirin had no effect on maturation of unstimulated MoDCs (effect on stimulated MoDCs has not yet been assessed)

2. Effect on cytokine production:
    - Ribavirin had no effect on cytokine secretion from unstimulated MoDCs
    - Stimulation of MoDCs with CD40L enhanced secretion of IL12, TNFa, IL6 and IL10 and ribavrin was found to suppress the CD40L-induced TNFa production but did not effect the other cytokines

Chapter 4

Activation of Human Dendritic Cells and Enhancement of Antigen Specific T Cell Immunity by a Novel Helminth-Derived Immunostimulant.

4.1 Introduction:

With the increasing prevalence of chronic hepatitis C there is a pressing need for safe and effective immunotherapies that will drive the hosts immune effector mechanisms towards viral clearance. Persistence of the virus is thought to result from failure to mount a vigorous and sustained $CD4^+$ and $CD8^+$ T cell response with contraction of the range of peptide epitopes recognized culminating in the inability to eliminate infection and subsequent disease chronicity(163).

Immunostimulants represent an emerging class of drugs that are designed to effectively and appropriately amplify naturally occurring immune responses against infectious pathogens and tumor cells. Dendritic cells are professional antigen presenting cells and with their exceptional capacity to activate naïve T cells upon maturation, they are the prime initiators of acquired immune responses with the production of antigen specific effector T cells in response to viral and other pathogens(65). Dendritic cells therefore appear to serve as a relevant target of immunostimulants.

Currently available treatment for HCV, with interferon alpha and ribavirin, leads to a sustained viral eradication in only 40-80% of patients depending on the HCV genotype(167). Treatment is associated with multiple side effects and complications that often result in discontinuation of treatment and a large number not being offered treatment. The use of immune stimulators might prove to be an effective treatment either alone or in combination with existing or new anti-virals.

O*nchocerca* ***v****olvulus* is a filarial parasite that causes human onchocerciasis, more commonly known as river blindness, mainly in West Africa.

Activation-associated secreted proteins (ASPs) of parasitic nematodes are produced by the infective larval stage and are highly immunogenic. ASPs have been studied as potential anti-helminth vaccine components, especially hookworm ASP(278-281). Recombinant secreted protein of the helminth *Onchocerca volvulus*, r*Ov*-ASP-1, is typical of nematode ASPs in that it is intrinsically immunogenic(282), highly charged and contains highly conserved cysteine rich domain with structural homology to pathogenesis-related proteins in plants and also vespid venom antigen 5 (283).

During the course of experiments evaluating **R**ecombinant ***O**nchocerca **v**olvulus* **a**ctivation-associated **s**ecreted **p**rotein-**1** *(*r*Ov*-ASP-1) as a possible vaccine candidate against onchocerciasis in humans, the 25kD protein was also shown to be a potent adjuvant for inducing Th2 and Th1-associated antibodies to protein (ovalbumin), polypeptide (HIV-1 gp120-CD4 chimera) and small peptide (from SARS-CoV spike protein) antigens in vaccinated mice(284). Unusually for a helminth product, rOv-ASP-1 as an adjuvant with ovalbumin strongly biased antibody and cellular responses to Th1-type (IFN γ) in vaccinated mice(284).

4.2 Aims:

Having shown that rOv-ASP-1 is a potent vaccine adjuvant in mice, the aim of this study was to explore the mechanisms of action of r*Ov*-ASP-1 and assess its potential as a human therapeutic by testing its ability to stimulate human dendritic cells (DCs), looking at the effect of r*Ov*-ASP-1 on expression of DC maturation markers (HLA-DR, CD83, CD86, CD40 and BDCA-2) and cytokine secretion using *ex vivo*- isolated plasmacytoid DCs (pDCs) and cultured, monocyte-derived DCs (MoDCs) from normal healthy donors (NHDs) and to determine if Ov-ASP-1-treated DCs are capable of enhancing MoDC antigen-presenting capacity. If Ov-ASP-1 was able to enhance *in vitro* responses, it may be potentially used to boost antigen-specific responses in chronic infections such as HCV.

4.3 Materials and Methods:

Blood samples were collected from 20 uninfected normal healthy donors with no known risk factors for blood-borne virus infection. 14 were male and 6 were female. The median age was 52 (range 26-68).

Monocytes (n=14) and pDCs (n=6) were purified by positive selection from PBMCs using anti-CD14 conjugated and BDCA-4 magnetic microbeads respectively, in conjunction with an AutoMACS. Monocyte-derived dendritic cells (MoDCs) were generated by culturing monocytes in medium supplemented with rhGM-CSF (50ng/mL) and rhIL-4 (1000IU/mL) for 5 days.

Immature MoDCs and freshly isolated pDCs were cultured in media alone or in a 1:1 ratio with a CD40 ligand-transfected Chinese hamster ovary cells (hCD40L-CHO) with or without with 5 µg/ml r*Ov*-ASP-1 or 100 ng/ml LPS, used as a positive control.

After 24 hours of culture, MoDCs and pDCs were harvested and labelled for two-colour flow cytometry to identify their cell surface phenotype.

The cytokines produced by stimulated MoDCs and pDCs were assessed in cell culture supernatants after 24 hours of culture using ELISA (IFNα) and a cytometric bead assay (IL12p70, IL10, IL8, IL6, IL1β and TNFα, IFNg, IL2, IL4, IL5).

To establish if r*Ov*-ASP-1 was able to enhance MoDC antigen-presenting capacity a mixed lymphocyte reaction assay was performed and proliferation was measured by H-thymidine incorporation.

For a full description of the methods see *Materials and Methods* chapter.

Statistical analysis

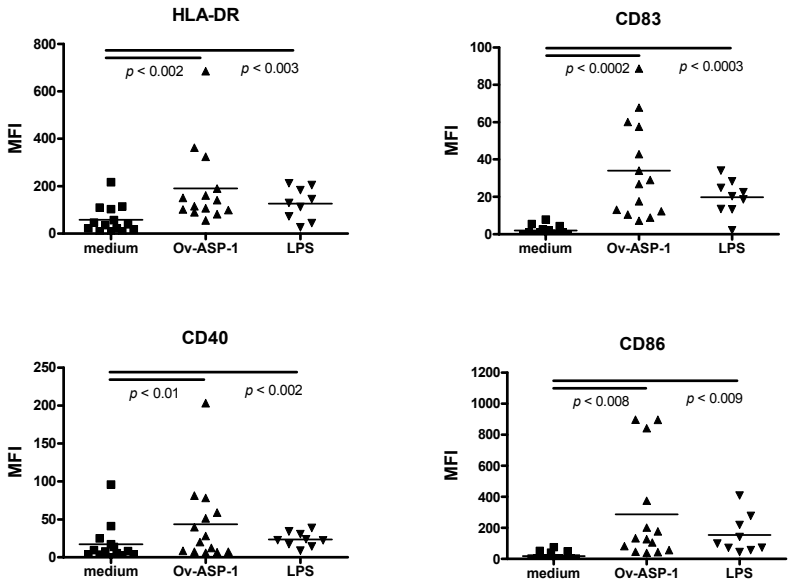
Paired *t*-tests were used to compare means. Analyses were performed using Prism version 4·0 for Windows (GraphPad Software, San Diego, CA, USA).

4.4 Results:

4.4.1 r*Ov-ASP-1 induces phenotypic maturation of monocyte-derived dendritic cells from normal healthy donors*

It is well known that TLRs activate signal transduction cascades leading maturation of DCs that includes expression of co-stimulatory molecules on their surface. To determine if rOv-ASP-1 induced DC maturation, MoDCs (n=14) were cultured with or without recombinant rOv-ASP-1 at an optimal concentration of 5µg/ml for 48 hours. Using FACS analysis we measured expression of surface molecules that are characteristically upregulated in DCs after stimulation. As shown in figure 24, all four phenotypic markers that were tested increased after treatment with r*Ov*-ASP-1.

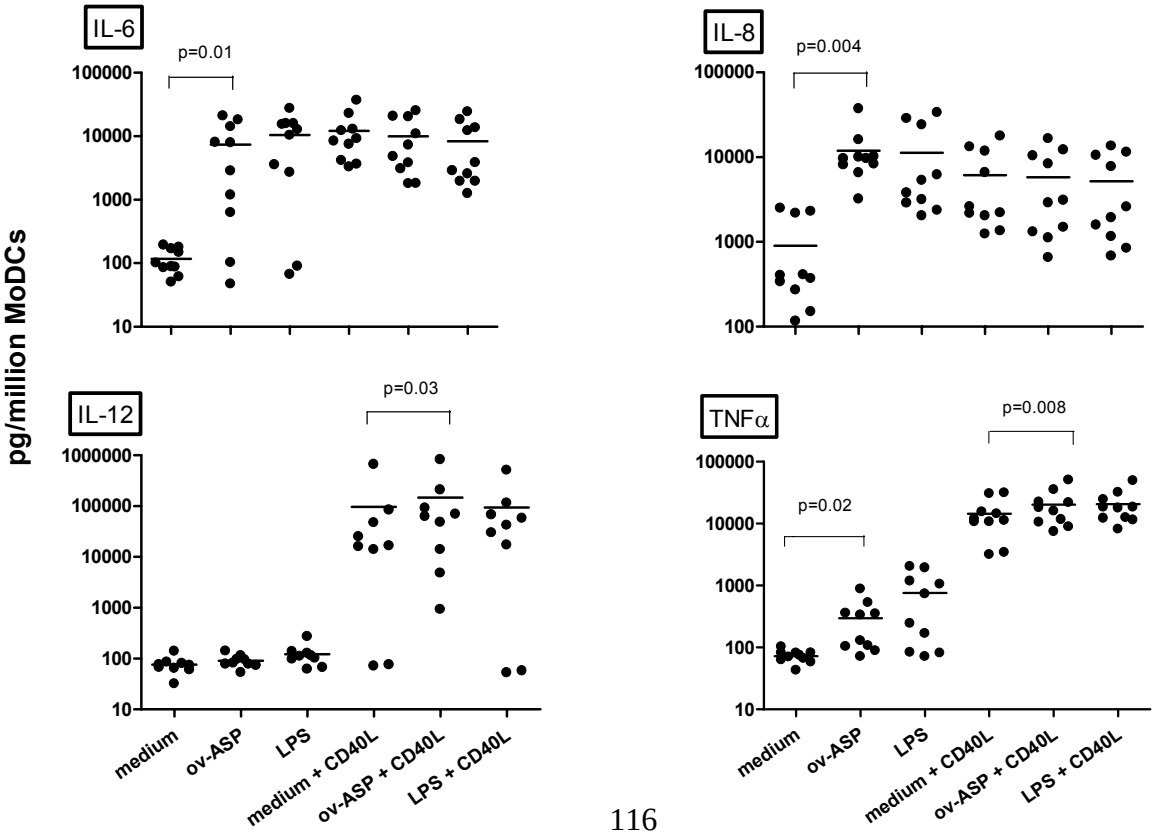
Fig 24. Phenotypic maturation by rOv-ASP-1 treated MODCs: Phenotypic maturation by r*Ov*-ASP-1 treated MODCs. MoDCs (N = 14) were cultured with or without 5 µg/ml rOv-ASP for 24 hours. Using FACS analysis the expression of maturation markers CD40, CD83, CD86 and HLA-DR were measured. All four of the maturation markers were significantly upregulated after treatment with r*Ov*-ASP-1. LPS was used as a positive control (n=9)



4.4.2 rOv-ASP-1 stimulates proinflammatory cytokines from monocyte-derived dendritic cells

MoDCs from NHDs (n=10) were cultured with or without recombinant r*Ov*-ASP-1 at an optimal concentration of 5µg/ml for 24 hours and cytokine production was determined. r*Ov*-ASP-1 alone was found to stimulate significant amounts of pro-inflammatory cytokines, IL6, IL8 and TNFα (fig 25) but not IL-12. However, when MoDCs were co-stimulated with CD40L, significant enhancement of IL-12 was seen. Co-stimulation with CD40L also enhanced the r*Ov*-ASP-1 induced secretion of IL-10 and TNFα. A wide variation in response was seen between donors with some secreting much higher levels of cytokines than others. There was no significant induction of other cytokines tested IL2, IL4, IL5 or IL1ß.

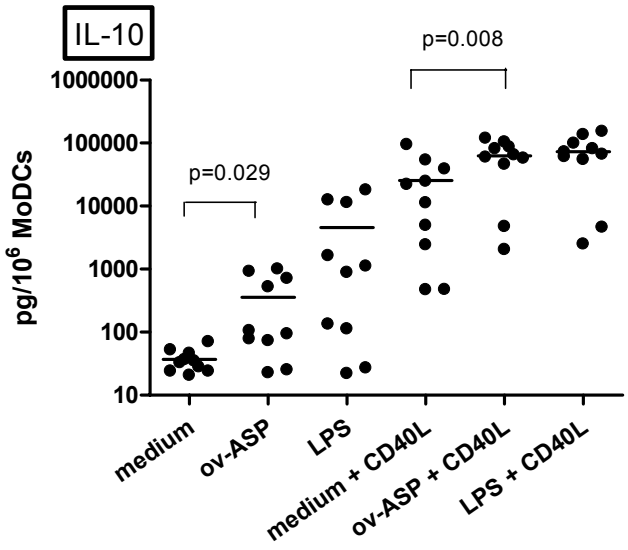
Fig 25. Production of proinflammatory cytokines from r*Ov*-ASP-1 treated MoDCs with and without CD40L: MoDCs were cultured with media alone or in a 1:1 ratio with a CD40 ligand-transfected Chinese hamster ovary cells (hCD40L-CHO) with or without 5µg/ml rOv-ASP. LPS was used as a positive control. rOv-ASP alone stimulated significant amounts of pro-inflammatory cytokines, IL-6, IL-8, and TNFα but not IL-12. However, when MoDCs were co-stimulated with CD40L, significant enhancement of IL-12 secretion was seen.



4.4.3 r*Ov-ASP-1 stimulates an anti-inflammatory cytokine from monocyte-derived dendritic cells.*

MoDCs cultured with r*Ov*-ASP-1 were also found to significantly stimulate the production of the anti-inflammatory cytokine, IL-10 (Figure 26).

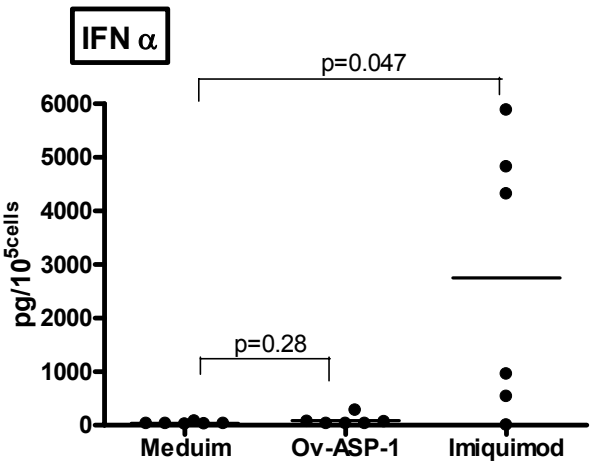
Fig 26. Production of IL-10 from r*Ov*-ASP-1 treated MoDCs with and without CD40L:
MoDCs stimulated with r*Ov*-ASP significantly stimulated the production of the anti-inflammatory cytokine, IL-10 and this was further enhanced when MoDCs were co-stimulated with CD40L



4.4.4 r*Ov-ASP-1 has no effect on pDC maturation or Interferon alpha production.*

pDCs are the major producers of type 1 interferons. We therefore tested the effects of r*Ov*-ASP-1 on the production of IFNα from pDC after 24 hours of culture. As shown in figure 27, r*Ov*-ASP-1 did not stimulate the production IFNα. r*Ov*-ASP-1 also had no effect on levels of expression of phenotypic markers on pDCs (either HLA-DR or co-stimulatory molecules BDCA 2, CD83 or CD86)

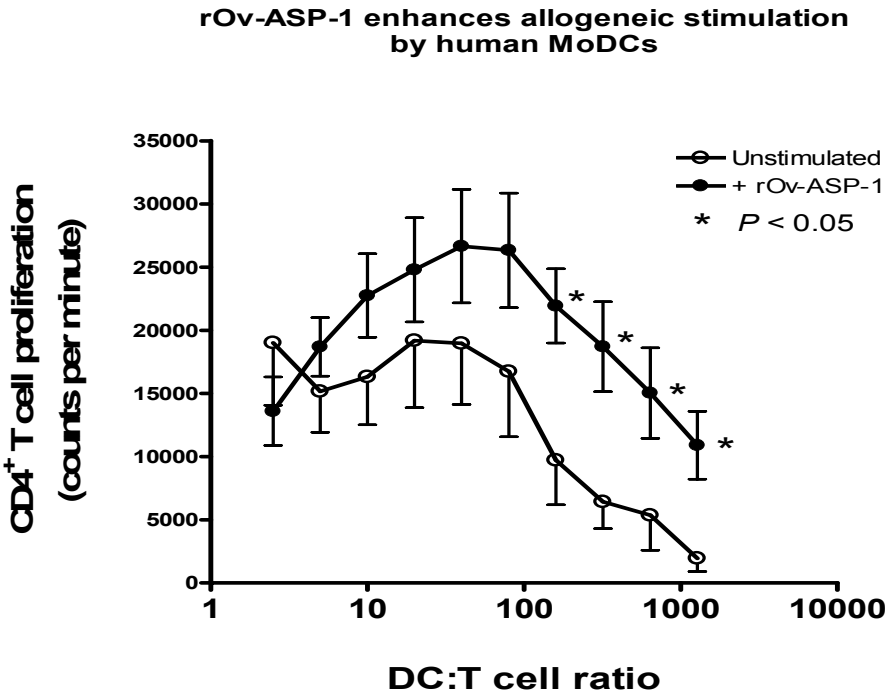
Fig 27. **Production of IFNα from r*Ov*-ASP-1 treated pDCs:** pDC (n=5) stimulated with 5µg/ml rOv-ASP did not enhance IFNα secretion



*4.4.5 r**Ov-ASP enhances the allo-stimulatory function of MoDCs:***

To assess if r*Ov*-ASP treatment of MoDCs was also able to enhance their accessory / antigen presentation function, we used the mixed lymphocyte reaction (MLR) assay with allogeneic antigens. Figure 28 shows the results of the MLR experiments indicating that MoDCs treated with 5ug/ml of r*Ov*-ASP enhanced their ability to stimulate proliferation of allogeneic $CD4^+$ T cells.

Fig 28. **r*Ov*-ASP-1 treated MoDCs have enhanced allo-stimulatory function:** MoDCs treated with *5* µg/ml r*Ov*-ASP-1 showed enhanced ability to stimulate the proliferation of allogenic $CD4^+$ T cells. The $CD4^+$ T cell proliferation (counts per minute) in response to r*Ov*-ASP-1 is shown. Each point represents the mean (s.d) of results from 5 individuals **P<0.05*



4.5 Discussion:

r-*Ov*-ASP is a novel immunostimmulant derived from *Onchocera volvulus*, the helminth parasite that causes onchocerciasis (river blindness). r-*Ov*-ASP has previously been shown to be a potent vaccine adjuvant in mice(284). In this study we investigated the immunostimulatory effects of this protein on human DCs from NHDs and show evidence of phenotypic maturation, enhanced cytokine production and enhanced allo-stimulatory function from MoDCs.

It has previously been shown that among human PBMCs, r*Ov*-ASP primarily binds to monocytes (93-98%) and B cells (41-94%), the major APCs in peripheral blood[11], suggesting r*Ov*-ASP may interact with specific receptors presented on the surface of the APCs to activate the immune cells. In human PBMCs, r*Ov*-ASP has been shown to be a potent inducer of Th1-type pro-inflammatory cytokines (IFNγ and TNF-α) and the immunoregulatory (IL-10) cytokine secretion(285).

The effects of the rOv-ASP-1 on DCs, the major antigen presenting cell involved in priming T-cell responses, has not been previously described. Dendritic cells recognize PAMPS through TLRs. TLRs are important pattern recognition receptors (PPRs) of the innate immune system, recognizing various invading pathogens (PAMPS) expressed on the cell surface of immune cells. After PAMP recognition by TLRs, signalling pathways are activated resulting in recruitment of adaptor proteins such as myeloid differentiation factor 88 (MyD88) and down stream activation of transcription factors, NFkB and mitogen activated protein kinases (MAPK) with production of pro-inflammatory cytokines(93). TLR signalling stimulates the activation and maturation of DCs with expression of co-stimulatory molecules and secretion of cytokines which drive polarization of CD4+ T_H1 or T_H2 cells towards the T_H1 or T_H2 phenotype. DCs provide a central link between innate and adaptive immunity(286).

It is thought from previous studies that r*OV*-ASP-1 activity is mediated through TLR signaling(285) and other human parasite-derived products have also been shown to stimulate immune responses via TLR pathways(287;288). In human PBMCs, rOV-ASP-1 has been shown to activate TLR2 and TLR4 to induce production of pro-inflammatory cytokines (IFNγ) with inhibition of IFNγ secretion from human PBMC reported by anti-TLR2 and anti-TLR4 antibodies(285). r*Ov*-ASP-1 was also found to trigger the activation

of HEK293 cells transfected with TLR2 and TLR4 but not with TLR9(285). Although TLR2 and 4 mediate a response to a different set of PAMPs, they share a common activation pathway through the TIR signaling domain resulting in activation of NFkB and MAPK.

In this study we have shown that r*OV*-ASP activities are mediated by its ability to activate APCs. We showed phenotypic maturation of rOv-ASP-1 treated MoDCs based on up-regulation of phenotypic markers (CD40, HLA-DR, CD83 and CD86). rOV-ASP stimulated significant amounts of pro-inflammatory cytokine, IL6, IL-8 and TNFα but not IL-12. The r*OV*-ASP induced secretion of IL-12 was found to be CD40-dependant, with significant enhancement of IL-12 only being observed after providing MoDCs with co-stimulation through the CD40-CD40L pathway. Co-stimulation through the CD40-CD40L pathway also enhanced the rOV-ASP-1 induced secretion of IL-10 and TNFα. These results provide evidence that rOV-ASP-1 may support DC-induced adaptive immune responses by multiple distinct mechanisms. The CD40-CD40L pathway dependant IL-12 production by DCs may be important for the development of T_H1 responses and cytotoxic T lymphocyte generation. IL-12 is primarily produced by myeloid DCs and macrophages and T cell and NK cell secretion of IFN γ is mostly induced by IL-12.

r*Ov*-ASP not only enhanced pro-inflammatory cytokine production from MoDCs but also significantly stimulated the production of the anti-inflammatory cytokine IL-10. The enhanced secretion of IL-10 has previously been seen in rOV-ASP-1 stimulated PBMCs(285) and also in vaccinated mice where, despite theoretical presence of Ov-ASP-1 induced IL-10 *in vivo* (based on stimulation of normal mouse splenocyte *in vitro*), the protein has potent Th1-inducing immunostimulatory activity(284). IL-10 of DC origin plays a central role in the regulation of T-cell responses and is capable of inhibiting synthesis of pro-inflammatory cytokines like IFNγ, TNFα and IL2. It is possible that in natural infection with *O. Volvulus* larvae, co-induction of IL-10 helps to limit damaging effects of too potent a Th1 response.

Based on the profile of cytokines stimulated by r*Ov*-ASP-1 it has been hypothesized that the r*Ov*-ASP-1 protein may contain several Th1 PAMPS that bind to different receptors on APCs and activate differently the secretion of Th1 cytokines and IL-10 through distinct pathways. Structural homology analyses of r*Ov*-ASP-1 with the crystallized closely-related hookworm secreted protein Na-ASP-2 suggested that r*Ov*-ASP-1 has three distinct domains(289) each of which could contain distinct bioactive sites. If this is the case and

different domains induce IL-10 and pro-inflammatory cytokines, then it may be possible to express a subunit that stimulates the pro-inflammatory cytokines without concurrent IL-10 secretion or subunits that differentially activate TLR2 or TLR4 pathways.

We found that r*Ov*-ASP enhanced the ability of MoDCs to stimulate proliferation of allogeneic $CD4^+$ T cells and r*Ov*-ASP-1 primed DCs have been shown to have the ability to induce IFNγ secretion from naïve autologous PBMCs. This suggests the protein is able to enhance DCs accessory/antigen presentation function and suggests its ability to boost the activation of an adaptive immune response. Further studies need to be done looking at antigen specific responses.

The observed effects of r*Ov*-ASP on DCs described in this study suggest several potential applications for the use of the protein which are discussed below, but in the first instance, the subunits of the protein would need to be expressed or synthesized and the bioactivities determined.

There has been a recent resurgence of interest in new and improved vaccine adjuvants. The interest has been stimulated by the need for new vaccines to combat problematic pathogens causing AIDS, SARS, TB, HCV and 'flu as well as those that could be employed as biological weapons in terrorist attacks. Recombinant protein-based or subunit based vaccines with adjuvants as stimulants are becoming more popular as they are less broadly immunogenic than traditional live or killed whole organism based vaccines. However, finding high potency immunostimulatory adjuvants capable of safely boosting both humoral and cellular immune responses is a challenge. At present, alum is the only adjuvant approved for human use world-wide but it is mainly associated with Th2 responses and has little capacity to stimulate cellular (Th1) immune responses(290).

Ov-ASP-1 has previously been shown to stimulate high tires of IgG1 (Th2) and IgG2a (Th1) in vaccinated mice which exceeded the efficacy of alum or MPL and TDM adjuvants(282;284;291). Unlike alum, it has the ability to stimulate a mixed Th1/Th2 with a strong Th1-biased immune response. It has been shown to have potential adjuvanticity in inducing immune responses of recombinant subunit-based vaccines, synthetic peptides, as well as in improving the immune efficacy of commercial inactivated vaccines (haemorragic fever with renal syndrome (HFRS), Influenza and Rabies)(291).

The key property of adjuvants is their ability to induce DC maturation and activation. In this study we have demonstrated that r*Ov*-ASP activates DCs to the same degree as an established TLR agonist, LPS. These findings further substantiate the potential of the use of r*Ov*-ASP as a novel human innate vaccine adjuvant in humans that can boost the activation of an adaptive immune response. It has been shown to stimulate both antibody and cellular responses which is an important challenge for vaccine development and which few experimental adjuvants are capable. It may be possible to express a subunit with optimal Th1 inducing ability without the concurrent IL-10 secretion or one which specially activates TLR2 or TLR4 pathways.

The ability of r*Ov*-ASP-1 to stimulate human DCs may enable it to be used as a human therapeutic and as a DC activator where DC function is impaired by a pathogen such as HCV. In the case of chronic HCV infection, where DC function has been reported to be impaired by the virus, r*Ov*-ASP-1, or more likely a subunit, could enhance DC-driven T cell anti-HCV responses that would shift the virus-host equilibrium in favor of viral clearance. In support of this, recent *in vitro* evidence showed that r*Ov*-ASP-1 was able to enhance and/or induce anti-HCV Core IFNγ responses in patients with chronic HCV infection (292) Our data have shown that in pDCs treated with r*Ov*-ASP, there was no effect seen in either maturation or cytokine production. Human pDCs express TLR7, 8 and 9 and so these findings support previous findings that r*Ov*-ASP-1 signals through TLR2 and TLR4. Our findings of an effect of r*Ov*-ASP-1 on MoDCs but not pDCs indicate that r*Ov*-ASP-1 could directly activate the closely-related myeloid DC subset *in vivo*, which share at least two of the Toll-like receptors (TLRs) through which r*Ov*-ASP-1 signals (TLR2 and TLR4) and not the plasmacytoid compartment. Although the latter is the most abundant and rapid source of IFN-α, other DCs and antigen presenting cells can also make this cytokine, albeit in lower amounts. The liver-resident DC population is mainly myeloid in type and could, therefore, be susceptible to activation by r*Ov*-ASP-1 resulting in restored or enhanced ability to present antigen.

In a previous study (292) it was shown that r*Ov*-ASP-1 induced IFNγ secretion from purified $CD56^+$ cells but not CD4+ or CD8+ cells. The CD56+ cells were critical to the immunostimulatory effect of the protein and dependant on contact between CD56+ and CD56- fractions of PBMCs, most likely between APCs, including DCs, in the CD56- fraction. It is possible, therefore, that as well as being an adjuvant for acquired immune responses, r*Ov*-ASP-1 has possible applications for the direct stimulation of NK cells and

could be used as a therapeutic immunostimulant either systemically or, where appropriate, topically. With respect to the liver, we have not tested the effects of r*OV*-ASP on NKT cells which are abundant in this organ and it is possible that the protein may have activating properties on these cells *in situ.*

The potential therapeutic uses of rOv-ASP-1 are not restricted to pro-inflammatory immune stimulation. We found that r*Ov*-ASP-1 also enhances secretion of large amounts of IL-10, a potent immunoregulatory cytokine which may also be valuable in limiting inflammatory and autoimmune diseases. This would require expressing a subunit that is responsible for the induction of IL-10 without pro-inflammatory cytokines and could be could particularly useful if administered topically, locally or targeted to an affected organ using a tissue-specific antibody.

In conclusion, this study shown the ability of r*Ov*-ASP-1 to stimulate human DCs and these findings suggest that the immunostimulatory properties of this protein may be mediated by its ability to activate antigen presenting cells, in particular MoDCs. The findings extends the immunostimulatory activity of Ov-ASP-1 beyond the previous reported findings of the adjuvanticity of the parasite protein for primary immune responses in vaccinated mice(282;284) to enhancing human responses.

Summary of Findings:

1. Phenotypic maturation of MoDCs
    - Shown by up-regulation of maturation markers, HLA-DR, CD83, CD86 and CD40

2. Stimulation of Pro-inflammatory cytokines from MoDCs
    - rOV-ASP1 alone stimulated IL8, IL6 and TNFα but not IL12
    - IL12 secretion required co-stimulation with CD40L
    - CD40L co-stimulation also further enhanced TNFα secretion

3. Stimulation of anti-inflammatory cytokine from MoDCs
    - rOV-ASP alone stimulated IL10 secretion
    - CD40L co-stimulation further enhanced this IL10 secretion

4. No effect seen on pDCs

- No effect on pDC phenotypic maturation as shown by maturation markers, HLA-DR, BDCA 2, CD83 or CD86
- No effect on IFNα secretion from pDCs

5. Enhanced allo-stimulatory function of MoDCS

- Enhanced ability to stimulate proliferation of allogeneic CD4+ T cells

Chapter 5

The TLR7 agonist SM-360320 induces a robust antiviral and immunostimulatory response in chronic hepatitis C infection

5.1 Introduction:

The current mainstay of treatment for CHC is ribavarin in combination with pegylated (PEG)-IFNα, leading to sustained viral eradication in 40-80% of infected patients, primarily depending on the HCV genotype(167). The current treatment is associated with significant side effects including flu-like symptoms, depression and injection site reactions and is also not suitable for a number of patients(178). Alternative novel strategies for the treatment of CHC are therefore required.

The host's innate and adaptive cellular immune responses are believed to be critical in determining the outcome of HCV infection(161;161;163). The NK cell-mediated innate immune response may be both genetically programmed to determine the chance of spontaneously resolving acute HCV infection (159) and may be directly modulated by HCV itself, leading to impaired NK cell function (293). Dendritic cells (DCs) are professional antigen presenting cells characterized by their exceptional capacity to activate naïve T cells and prime adaptive immunity. DCs play a pivotal role linking innate and adaptive immunity(67). Two functionally distinct subsets of DCs are recognised; myeloid (mDC) and plasmacytoid (pDC). pDCs are the most potent producer of type-1 interferons in the body and release large quantities during viral infections(294).

In terms of the adaptive immune response, successful viral clearance during acute infection has been attributed to a robust HCV-specific $CD8^+$ T cell effector response and a predominantly Th1-polarised $CD4^+$ anti-HCV response(161). A failure to sustain this early anti-HCV T cell response correlates with viral persistence and the development of CHC.

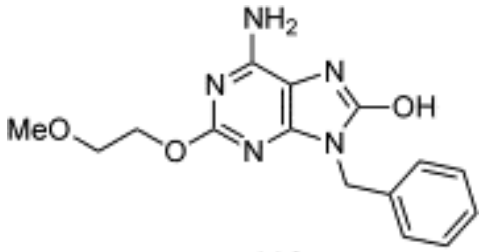
Toll-like receptors (TLRs) are a family of pattern recognition receptors that play a key role in innate immunity(93). TLRs are activated by a range of pathogen-associated molecular patterns (PAMPs) which are conserved among pathogens. TLRs 3, 7 and 9 are intracellular receptors specialized in the recognition of viral nucleic acids and recognize ligands in endosomes and lysosomes. TLR3 recognizes double stranded RNA (dsRNA)

and is expressed on B and T lymphocytes and NK cells. TLR7 recognizes synthetic imidazoquinolone-like molecules, guanosine analogues and single-stranded RNA derived from viruses while TLR9 recognizes unmethylated 2'-deoxyribo (cytidine-phosphate-guanosine (CpG) DNA. TLR 7 and 9 are expressed by pDCs and B cells. On binding, they induce a signaling cascade resulting in the induction of type I interferons and proinflammatory cytokines, which drive an inflammatory response and activate the adaptive immune system(94).

TLR agonists have been investigated for the treatment of chronic hepatitis C as a means of inducing endogenous IFNα production by stimulating immune cells with efficient activation of innate and acquired immunity even in the presence of the immune dysfunction induced by CHC infectioṇ. Activation of pDCs is important for HCV treatment because these activated cells are not only the primary producer of the cytokine IFNα, but also present antigen to initiate T cell mediated adaptive immunity.

In short term monotherapy trials, the TLR7 agonist Isatoribine(241) and TLR9 agonist, CpG 10101 (Actilon)(250) have been shown to have clinical efficacy against HCV, reducing HCV viral load with evidence of immune modulation as shown by upregulation of the interferon-response gene, 2'5'OAS and increased production of IFNα and IP10 in both sudies but also neopterin (a marker of immune activation produced by activated macrophages) in the Isatoribine trial.

Figure 29: **Structure of SM-360320**: Selectivity – TLR7 specific
Plasma protein binding (PPB) – 75%



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5.2 Aims:

The aim of this study was to investigate both the anti-viral and the immunostimulatory actions that might be responsible for HCV clearance *in vitro.* The effects of the TLR7 agonist, SM-360320 (9-benzyl-8-hydroxy-2-(2-methoxyethoxy)adenine) (295) on innate immunity was studied and the responses were compared in blood from NHDs and patients with CHC, both treatment naïve and previous non-responders to treatment.

5.3 Materials and Methods:

Blood samples were collected from chronic hepatitis C patients recruited from the hepatology clinics run by Southampton University Hospitals NHS Trust and Royal Bournemouth and Christchurch NHS Trust. All patients had detectable HCV RNA determined by quantitative PCR (Cobas Amplicor HCV Monitor test, Roche Molecular Systems, NJ, USA). Patients were excluded if they had received treatment within 6 months or less prior to the study or tested positive for other blood bourn viruses, including HBV and HIV. 60 patients were Interferon treatment naïve and 20 were previous non-responders (NR) to treatment. A further 80 uninfected NHDs, who had no risk factors for blood borne viruses were also recruited.

Clinical data from all three groups can be seen in table 5.

Stimulating whole blood:

Aliquots of fresh blood (450µl) collected into sodium citrate were transferred into 24-well assay plates containing 50µl pre-prepared agonist, SM-360320, IFNα or no stimulus. Final concentrations of SM-360320 were 0.1µM – 10µM and IFNα was 20IU/ml IFNα. The plates were sealed, placed on an orbital shaker and incubated for 5 hours at 37^0C. Aliquots (4 x 25µl) of the stimulated blood from each well were transferred to 96 well plates and mixed with MagMAX lysis/binding solution.

The blood remaining in the 24 well plate was spun (5 min at 500rpm) and plasma removed and frozen at -80^0C. The remaining cell pellet was resuspended in the residual plasma (~300µl) and 1.3mls RNALater (Ambion) was added to stabilize the samples. These samples were also frozen at -80 C pending further analysis for gene expression, cytokine analysis, and HCV replicon assays which were undertaken at Pfizer, Sandwich.

PBMC isolation and storage:

Blood collected into EDTA containing vacutainers was separated immediately and PBMC isolated (see chapter 2.2.1.1) Cells were frozen and stored at -80^{0}C while awaiting further use.

Gene Expression Analysis:

After thawing, RNA was extracted from blood mixed with MagMAX lysis/binding solution using the MagMAX 96 well blood RNA isolation kit according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was prepared using high capacity cDNA archive kits. 2'5'OAS gene expression was quantified using real time taqman QPCR using 'assay on demand reagents' (Applied Biosystems, USA) as described by the manufacturer. 2'5' OAS gene expression was maeasured relative to the background house keeper gene, β-actin, giving the 'relative quantitiy'. This was analysed using the delta-delta-ct method of analyzing Taqman. (This work was done at Pfizer)

Cytokine analysis

Levels of pro-inflammatory cytokines, TNFα, IL-1β, IL-2, IL-6, IL-8, IL-12p70, anti-viral cytokines, IFNγ and IFNα and regulatory cytokines, IL-10 and IL-4 were determined in plasma using a custom multiplex electrochemiluminescence Enzyme-Linked ImmunoSorbent Assay (ELISA) as described by the manufacturer (Meso Scale Discovery). (This work was done at Pfizer).

HCV replicon assay

The presence of soluble factors with anti-viral activities in treated plasma was measured by evaluating the ability to inhibit HCV replication in an HCV-replicon system.
Huh7 1b replicon cells were cultured in flasks. Supernatants (10 uL) from whole blood ex vivo experiments were added to the replicon cells and incubated for 48 hours at 37^{0}C. HCV Replicon levels were then quantified using a luciferase reporter assay (270). (This work was done at Pfizer).

NK cell activation & intracellular IFNγ staining

Previously cryopreserved PBMCs were thawed and rested in incubators for five hours at 37^{0}C prior to the assay. Cells were resuspended to 2 x 10^6/ml in RMPI1640 (no phenol). 900µl PBMCs were plated into duplicate wells of a 24 well plate containing 100µl pre-

prepared agonist per well, 100µl IFNα2b.or 100µl of dilutent only. After 5 hours incubation at 37°C, 1µl of Brefeldin-A was added to one well of each duplicate. Cells were then incubated for a further 18 hours. After a total culture period of 24 hours, PBMCs were harvested and labelled for four-colour flow cytometry to identify activated $CD56^+$ $CD16^+$ NK cells on the basis of cell surface CD69 expression and cytoplasmic interferon gamma (IFNγ) expression by intracellular staining.

pDC Depletion

For 5 NHDs and 5CHC patients, PBMCs were prepared from freshly drawn blood (se Chapter 2.2.1.1). pDCs were isolated from the PBMCs by positive selection (see chapter 2.2.1.3). Whole PBMCs, PBMCs from which $BDCA4^+$ pDCs were depleted, and pDCs alone were stimulated with SM360320 (10uM-0.01uM) in sealed plates for 5hours at 37^0C and gene expression analysis, cytokine production and NK cell activation was assessed as above. The % purity of the pDC depletion was assessed by FACS analysis.

For a full description of the methods, see *Materials and Methods* chapter 2.

Statistical Analysis:

All statistical analysis was undertaken by a statistician at Pfizer. GraphPad PRISM (V5) and a Pfizer Inc proprietary add-in to Microsoft Excel 2003 were used to perform initial data analysis and visualisations. Statistical analyses were performed using SAS (V8.02), GenStat (V10) and Spotfire DecisionSite 9.0.

In order to satisfy the assumptions of the analysis methods used, the data from the gene expression, cytokine and pDC depletion analyses were analysed on the $\log_{10}$ scale and data from NK-cell Activation were analysed on the logit scale. Plots of the data were used to check the statistical assumptions and any unusual features such as outliers were investigated.

For gene expression, cytokine analyses, NK-cell activation and pDC depletion experiments all transformed responses were analysed using a linear mixed model that allowed for donor-to-donor variation and investigated differences between the three donor groups, the stimuli and the effects of pDC depletion. Specific pairwise comparisons between the stimuli and groups were quantified on the transformed scale and back-transformed to give geometric means, ratios in means, 95% confidence intervals

(excluding NK-cell activation data) and p-values. All tests were performed at the 5% significance level.

For the HCV replicon assay the distribution of the collected Replicon data did not lend itself to the modelling techniques used for the other responses. Therefore the multivariate technique K-means clustering was applied to identify similar donor profiles, where a profile contains the responses exhibited by a donor across the 6 stimuli. A number of different clusters and similarity measures were investigated.

Table 5: Subjects clinical characteristics

		CHC		*NHDs*
		Naive (n=60)	**NR (n=20)**	**n=80**
Age	**Median**	46	45	42
	Range	27-87	36-70	22-61
Gender	**Male**	46(77%)	16(80%)	54(68%)
	Female	14(23%)	4(20%)	26(32%)
Race	**Caucasian**	57	17	Unknown
	Asian	1	3	
Genotype[a]	**G1**	28(47%)	14(70%)	N/A
	G2/3	28(47%)	5(25%)	
	G4	1(1%)	1(5%)	
	Unknown	3(5%)	0(0%)	
Viral load[b]	**Median**	7.91E+05	5.52E+06	N/A
	Range	(7.02E+02-2.32E+07)	(7.25E+05-2.06E+07)	
Disease Severity[c]	**Mild**	19(32%)	5(25%)	N/A
	Moderate	14(23%)	1(5%)	
	Severe	14(23%)	7(35%)	
	No Biopsy	13(22%)	7(35%)	
ALT (IU/L) (10-40)	**Median**	62	58.5	N/A
	Range	10-169	22-252	
Risk for HCV	**IVDU**	33	7	N/A
	Blood Product	14	9	
	Other	11	4	
Alcohol intake[d]	**Low**	33(55%)	16(80%)	N/A
	Moderate	11(18%)	3(15%)	
	Heavy	16(27%)	1(5%)	

[a] HCV genotype determined using CPA accredited PCR-based 'Genotyping for Treatment Assay' (iQur Ltd, UK).

[b] Viral load determined by quantitative PCR (Cobas Amplicor HCV Monitor test, Roche Molecular Systems, USA) quoted as copies/ml. [c] Based on histological analysis of biopsies for inflammation and fibrosis. In the absence of histological evidence, disease status was determined by clinical criteria including physical examination, diagnostic imaging and laboratory indices.

[d] Low, <7 units/week; Moderate, 7-14 units/week; Heavy, >14 units/week

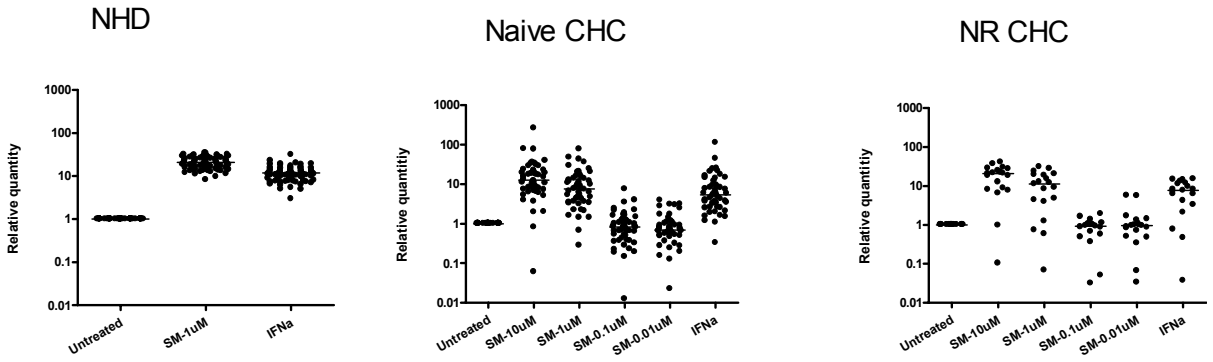
5.4 Results:

5.4.1 Induction of 2’5 OAS Gene Expression:

2’5’OAS gene expression is shown as ‘relative quantity’ The ‘relative quantity’ of 2’5’OAS is relative to the background house keeper gene, β-actin, analyzed using the delta-delta-ct method of analyzing Taqman rather than analyzing arbitrary copy number. The β-actin levels were not found to be reduced by either treatments.

Culture of whole blood, from all three groups, with 20IU/ml IFNα or SM-360320 at dose of 1μM significantly induced 2’5’OAS gene expression (p=<0.01; Fig 30). The magnitude of the response was higher in blood from NHD compared to CHC (p=<0.01). However, in the CHC patients, a larger variation in the induction of 2’5’ OAS was observed across the samples compared to NHDs with some patients giving a 100-fold induction. There was no significant difference observed between treatment naïve and NR patient groups.

Fig. 30: Expression of 2’5 OAS: SM-360320 (10μM and 1μM) and IFNα (20IU/ml) enhanced expression of 2’5’OAS in all 3 patient groups. Expression was significantly less in the CHC groups compared to NHD (p<0.01 for all).



5.4.2 Induction of Cytokine Production:

1μM SM-360320 stimulated secretion of all the antiviral, anti-inflammatory and regulatory cytokines tested (p=<0.001) in whole blood from NHDs and CHC patients with a dose response clearly shown. A wider range of secretion of individual cytokines was noted from CHC patients as compared to NHDs (Figure 31). It enhanced the secretion of IP-10, IFNγ and IL6 to the highest levels.

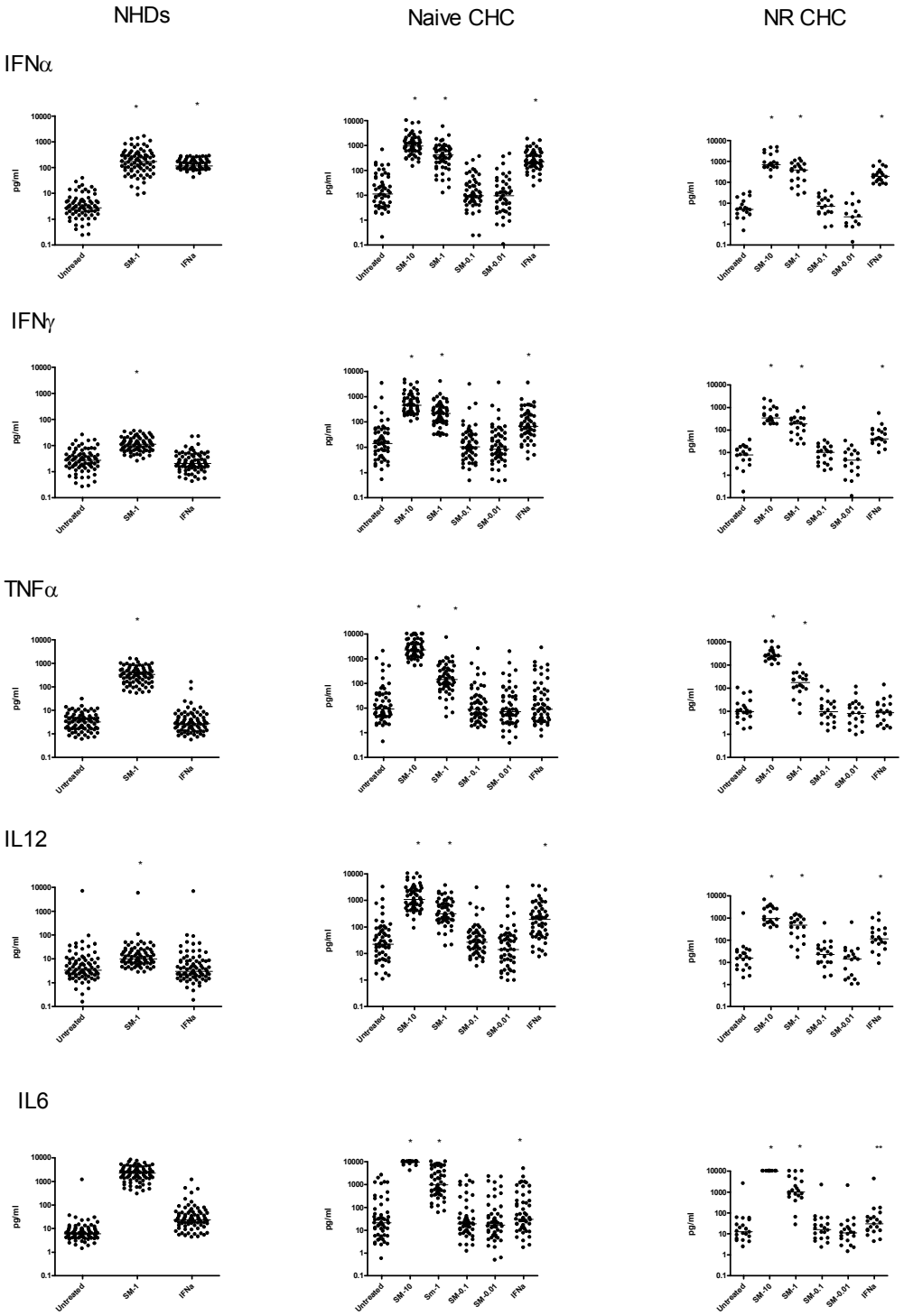
20IU/ml IFNα and was found to stimulate significant amounts of IFNα, IFNγ, IL-6, IP-10 and IL-12 (p=<0.001) in blood from CHC patients and IFNα, IL-6, IP-10 and IL-1β in blood from NHDs but had no effect on the other cytokines tested (IL-1β, IL-8, TNFα, IL-4 and IL-10 in CHC patients and IFNγ, TNFα, IL-12, IL-8, IL-4 and IL-10 in NHDs)

SM-360320 enhanced secretion of TNFα, IL-6 and IL-1β to higher levels than were observed from NHD- blood and CHC-blood on incubation with IFNα. In contrast, similar levels of endogenous IFNα and IP-10 were secreted on incubation with both compounds, although the MSD assay may be detecting the exogenous IFNα making this data difficult to interpret.

On average, naïve CHC-blood induced higher levels of the cytokines when stimulated with either compound as compared to non-responder CHC-blood, however this did not reach statistical significance (p=>0.10)

The untreated levels of all the cytokines secreted was higher from CHC-blood than NHD-blood (p=<0.001).

Fig 31. Cytokine secretion: Secretion of anti-viral, pro-inflammatory and regulatory cytokines by PBMCs treated with TLR7 agonist SM360320 and IFNα. * indicate significant enhancement of cytokine production compared to unstimulated levels.



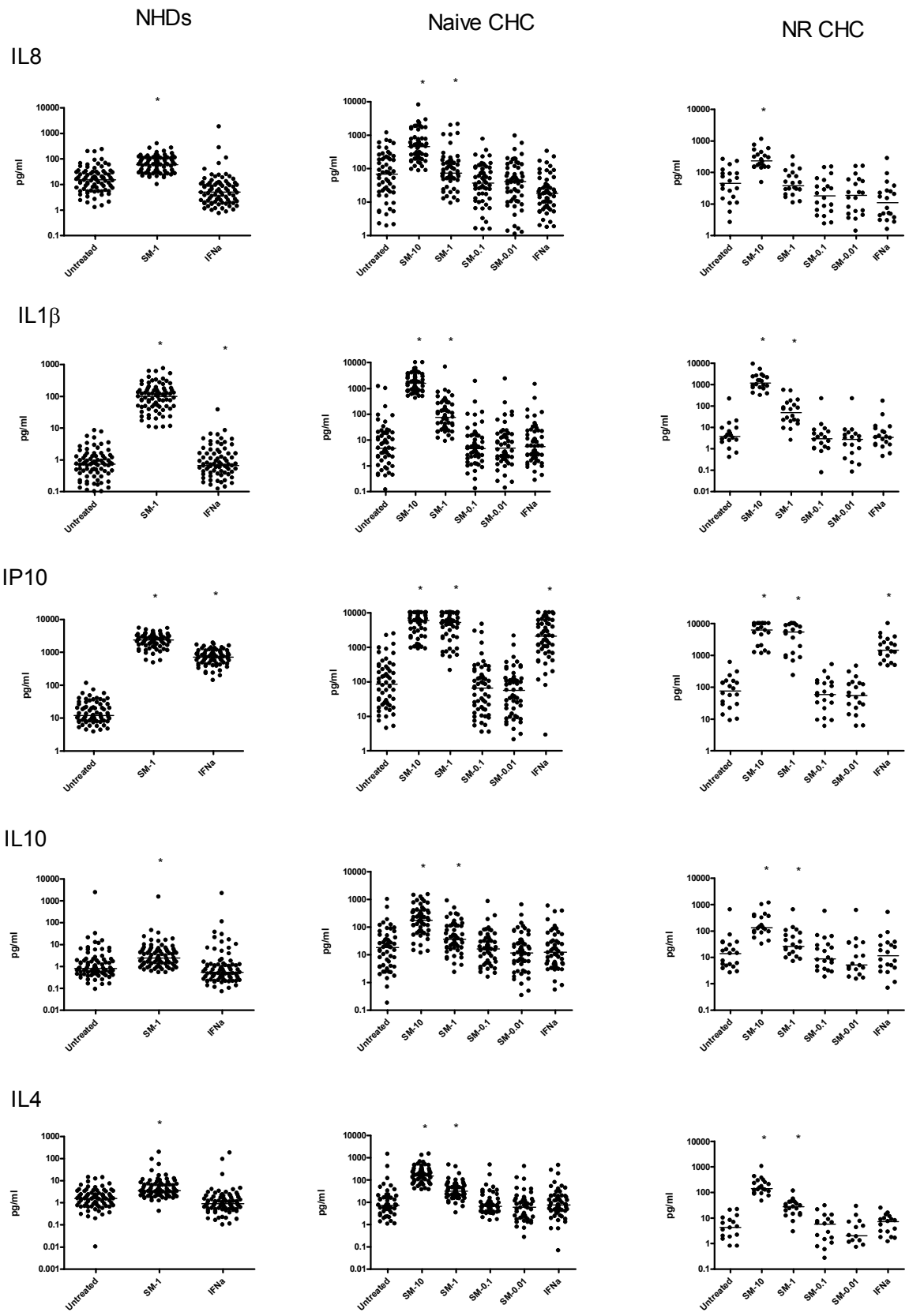

NHDs
Naive CHC
NR CHC
IL8
IL1β
IP10
IL10
IL4
pg/ml
Untreated
SM-10
SM-1
SM-0.1
SM-0.01
IFNa

Table 5: **Induction of cytokine production:** Cytokines were induced in all 3 subject groups. Data presented here showing mean responses and significance over untreated for 10 cytokines tested in NHD, Naive and NR subject groups. The ratio of geometric means and 95 % confidence intervals are also presented.

Cytokine	Subject Group	Treatment SM-1μM	Geometric Means	P-value (vs. untreated)	Ratios of Geo Means	95% Confidence Intervals
IP-10	NHD	Untreated	10.4	-	-	-
		SM	1803.0	<0.001	174.2	(148.3, 204.6)
		IFNα	529.7	<0.001	51.2	(43.6, 60.1)
	Naïve	Untreated	83.6	-	-	-
		SM	4036.5	<0.001	48.3	(37.4, 62.4)
		IFNα	1749.8	<0.001	20.9	(16.2, 27.0)
	NR	Untreated	66.5	-	-	-
		SM	3372.9	<0.001	50.7	(33.2, 77.4)
		IFNα	1566.8	<0.001	23.6	(15.4, 36.0)
IFNα (2a)	NHD	Untreated	1.7	-	-	-
		SM	94.4	<0.001	54.5	(43.6, 68.0)
		IFNα	81.1	<0.001	46.8	(37.5, 58.4)
	Naïve	Untreated	12.0	-	-	-
		SM	338.8	<0.001	28.2	(19.8, 40.3)
		IFNα	241.0	<0.001	20.1	(14.1, 28.7)
	NR	Untreated	6.0	-	-	-
		SM	269.2	<0.001	45.2	(25.0, 81.5)
		IFNα	205.1	<0.001	34.4	(19.1, 62.1)
TNFα	NHD	Untreated	1.9	-	-	-
		SM	185.8	<0.001	99.8	(86.9, 114.6)
		IFNα	1.5	<0.01	0.8	(0.7, 0.9)
	Naïve	Untreated	13.4	-	-	-
		SM	179.5	<0.001	13.4	(10.5, 17.2)
		IFNα	12.1	NS	0.9	(0.7, 1.2)
	NR	Untreated	10.4	-	-	-
		SM	141.3	<0.001	13.5	(9.0, 20.4)
		IFNα	8.8	NS	0.8	(0.6, 1.3)
IL-12p70	NHD	Untreated	2.8	-	-	-
		SM	7.2	<0.001	2.6	(2.2, 3.0)

		IFNα	2.3	<0.05	0.8	(0.7, 0.9)
	Naïve	Untreated	27.2	-	-	-
		SM	357.3	<0.001	13.2	(10.1, 17.2)
		IFNα	161.4	<0.001	5.9	(4.5, 7.8)
	NR	Untreated	15.7	-	-	-
		SM	311.9	<0.001	19.8	(12.7, 30.8)
		IFNα	125.3	<0.001	8.0	(5.1, 12.4)
IL-1β	NHD	Untreated	0.5	-	-	-
		SM	43.8	<0.001	96.7	(81.6, 114.6)
		IFNα	0.3	<0.001	0.7	(0.6, 0.8)
	Naïve	Untreated	5.6	-	-	-
		SM	86.9	<0.001	15.5	(11.2, 21.3)
		IFNα	6.2	NS	1.1	(0.8, 1.5)
	NR	Untreated	4.8	-	-	-
		SM	45.7	<0.001	9.5	(5.5, 16.5)
		IFNα	4.0	NS	0.8	(0.5, 1.4)
IL-4	NHD	Untreated	1.2	-	-	-
		SM	2.4	<0.001	2.1	(1.8, 2.4)
		IFNα	0.6	<0.001	0.5	(0.5, 0.6)
	Naïve	Untreated	8.2	-	-	-
		SM	31.8	<0.001	3.9	(2.9, 5.2)
		IFNα	8.3	NS	1.0	(0.8, 1.3)
	NR	Untreated	4.7	-	-	-
		SM	23.7	<0.001	5.0	(3.1, 8.2)
		IFNα	5.8	NS	1.2	(0.8, 2.0)
IL-6	NHD	Untreated	4.5	-	-	-
		SM	1419.1	<0.001	317.0	(257.5, 390.1)
		IFNα	13.6	<0.001	3.0	(2.5, 3.8)
	Naïve	Untreated	32.5	-	-	-
		SM	1306.2	<0.001	40.2	(28.5, 56.7)
		IFNα	61.2	<0.001	1.9	(1.3, 2.7)
	NR	Untreated	17.3	-	-	-
		SM	1137.6	<0.001	65.8	(37.3, 116.1)
		IFNα	35.5	<0.001	2.1	(1.2, 3.6)
IL-8	NHD	Untreated	9.8	-	-	-
		SM	40.7	<0.001	4.2	(3.6, 4.8)
		IFNα	3.2	<0.001	0.3	(0.3, 0.4)

	Naïve	Untreated	55.8	-	-	-
		SM	87.5	<0.001	1.6	(1.2, 2.0)
		IFNα	17.2	<0.001	0.3	(0.2, 0.4)
	NR	Untreated	39.0	-	-	-
		SM	43.9	NS	1.1	(0.7, 1.7)
		IFNα	11.4	<0.001	0.3	(0.2, 0.4)
IL-10	NHD	Untreated	0.9	-	-	-
		SM	1.6	<0.001	1.7	(1.5, 2.0)
		IFNα	0.6	<0.001	0.6	(0.6, 0.7)
	Naïve	Untreated	17.1	-	-	-
		SM	46.3	<0.001	2.7	(2.2, 3.3)
		IFNα	16.5	NS	1.0	(0.8, 1.2)
	NR	Untreated	13.6	-	-	-
		SM	33.7	<0.001	2.5	(1.8, 3.5)
		IFNα	11.7	NS	0.9	(0.6, 1.2)

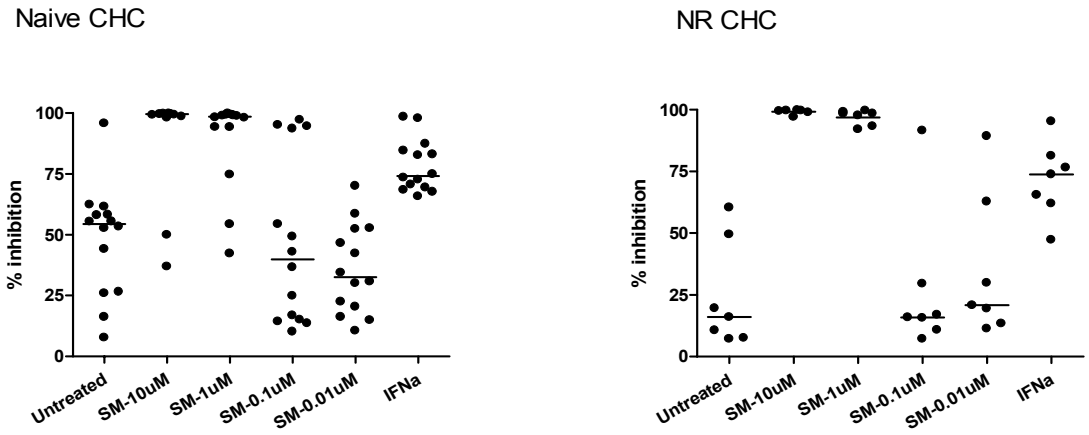
5.4.3 Inhibition of HCV Replication:

Plasma derived from blood derived from treatment naïve and NR patients that had been incubated with 10μM and 1μM SM-360320 significantly inhibited replication of HCV replicons in Huh-7 cells (p=<0.05) shown by reduction of reduce luciferase reporter expression to baseline levels in an HCV replicon assay (Fig 32). This is consistent with the high levels of IFNα found in the plasma (338.8pg/ml-naive and 269pg/ml-NR) (Fig 31).

The antiviral activity of plasma from IFNα treated blood was lower than that of SM-360320 treated blood (median = 73% inhibition *vs* 99% inhibition) possibly due to degradation of exogenous IFNα and/or lower induction of endogenous IFNα. Some antiviral activity was observed in plasma derived from untreated blood. In the naïve CHC patients, two distinct groups were identified by K-means clustering, with some patients reaching maximal inhibition at a lower concentration of agonist than others. When comparing naïve and N.Rs, some naïve patients gave replicon responses that were more consistent with the N.R group than others.

Blood from NHDs has not been analysed in the replicon assay.

Fig. 32 Inhibition of HCV replication – Plasma from SM-360320 (10μM and 1μM) treated PBMCs significantly inhibited replication of HCV replicons in Huh-7 cells (p=<0.05).The antiviral potential of plasma from IFNα treated blood was less effective.

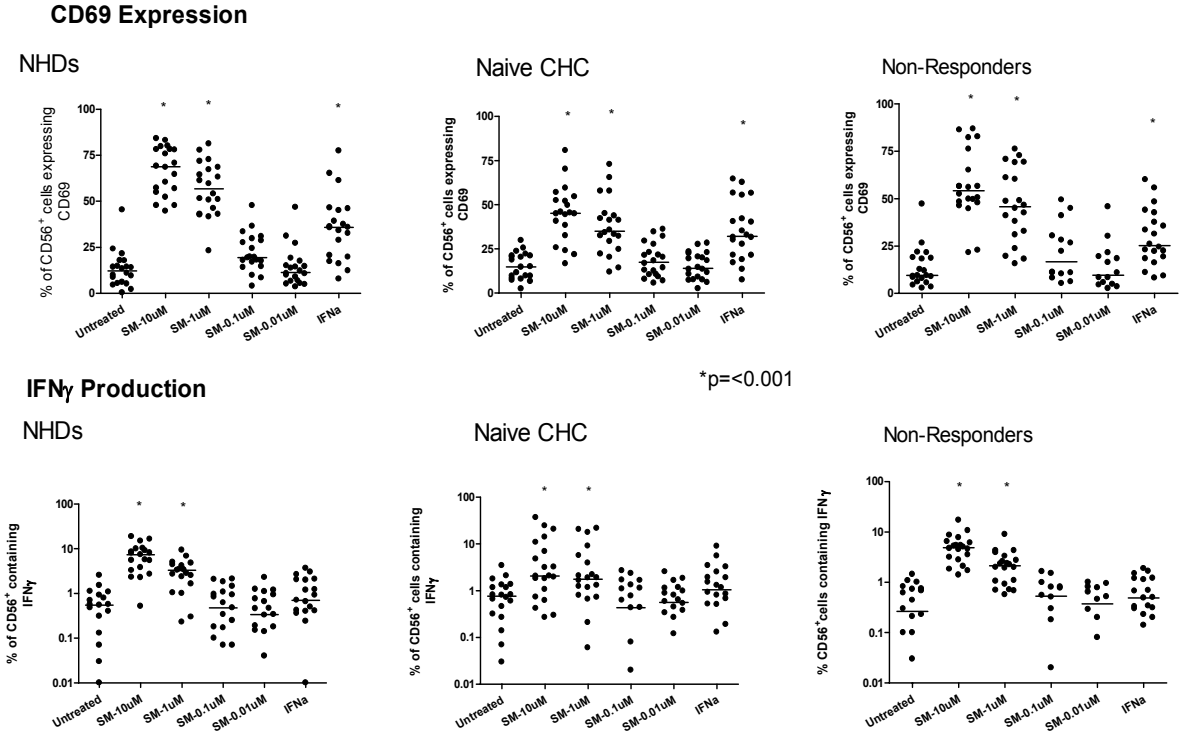


5.4.4 Induction of NK Cell Activation:

NK cells were identified by FACS as CD3-CD56+ and the percentage of these cells expressing CD69 (as a marker of activation) or containing intracellular IFNγ was determined. Surface expression of CD69 was studied on cells that had not been exposed to Brefaldin-A. In all 3 subject groups, the TLR7 agonist resulted in a dose-dependent increase in NK cell activation (Fig. 33). CD69 expression and IFNγ production were significantly enhanced by both 10μM and 1μM SM-360320 (p=<0.001) in all subject groups and also 0.01μM in NHDs. IFNα also enhanced CD69 expression in all subject groups (p=<0.001) but was less effective than the highest concentrations of SM-360320 (p=<0.05) and did not significantly enhance IFNγ production (p=>0.1).

Comparing the 3 groups treated with 10μM SM-360320, CD69 expression by treatment naïve CHC-NK cells was significantly lower than that seen in NHD-NK cells (p=<0.05). A similar trend was also seen between NHD-NK cells and NR CHC-NK cells but this was not statistically significant (p=>0.1)

Fig. 33 Induction of NK cell activation: TLR7 agonist enhanced NK cell activation in all 3 patient groups in a dose dependant manner with greater effects seen in NHD than CHC patients.



5.4.5 The Role of pDCs:

To investigate the role of pDCs in IFNα production and 2'5 OAS gene expression the effect of depleting pDCs from PBMCs prior to exposure to the agonist, was studied in a subset of patients (NHD *n*=5, CHC *n*=5). Secretion of IFNα and 2'5' OAS expression from whole NHD-PBMCs was induced on incubation with SM-360320 (10μM and 1μM) or 20IU/ml IFNα (p=<0.05). Levels of IFNα induction were reduced on depletion of pDCs from the NHD-PBMCs stimulated with SM360320 (p=<0.05), confirming the importance of this cell type for TLR7 agonist responses. However, no significant difference in 2'5' OAS expression was seen with pDC depletion (Fig. 34A). In the CHC group there was a wide variation of responses in the untreated samples of the whole PBMCs making the results from pDC depletion difficult to interpret for IFNα secretion. However, on depletion of pDCs from CHC-PBMCs, there was a significant reduction in 2'5' OAS expression with SM360320 (10μM and 1μM) and 20IU/ml IFNα (p=<0.001) (Fig 34B). The efficiency of depletion of pDCs from PBMCs was 68% on average.

To investigate whether NK cell activation was occurring indirectly in response to agonist-induced IFNα production by plasmacytoid DCs (pDCs), or directly in response to the agonist, the effect of depleting pDCs from PBMCs prior to exposure to the agonist, was studied and cells stained for CD69 expression and intracellular IFNγ production. In preliminary experiments in the uninfected subject group, NK cell activation (assessed by CD69 expression and IFNγ production) was induced by the top doses of TLR7 agonist and was partially impaired by pDC depletion with reduced IFNγ secretion.

Fig 34. Effect of pDC depletion on IFNα production and 2'5' OAS expression:

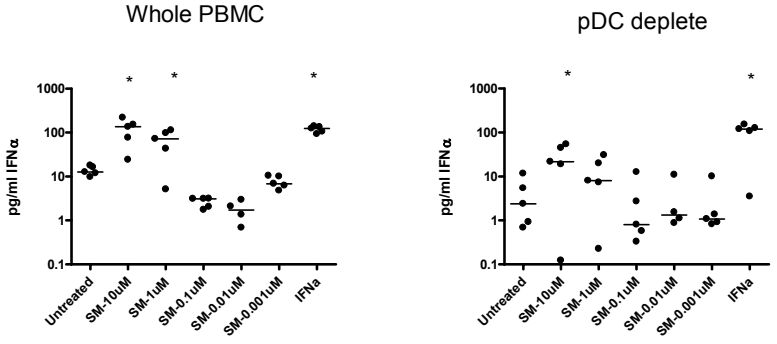
A. In NHDs a reduced secretion of IFNα was seen from PBMC depleted of pDCs stimulated with SM360320. No significant change was seen in 2'5' OAS expression when pDCs were depleted

B. In CHC patients untreated levels of IFNα secretion in whole PBMCs were highly variable making interpretation on pDC depletion difficult. However, reduced expression of 2'5' OAS was seen on depletion of pDCs.

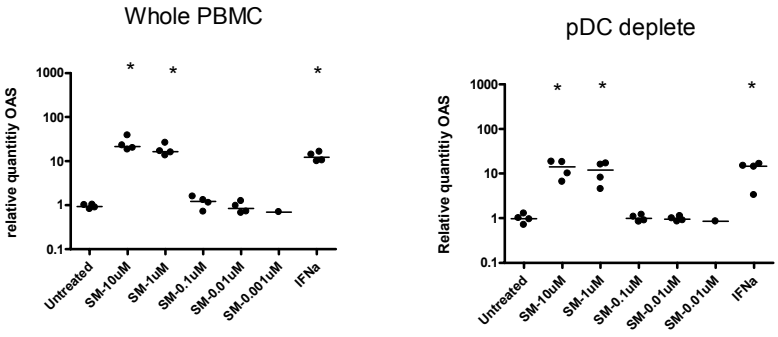
* indicate statistically significant enhancement or inhibition of IFNα production from untreated levels in NHD and CHC patients.

34A: NHDs

IFNα secretion:

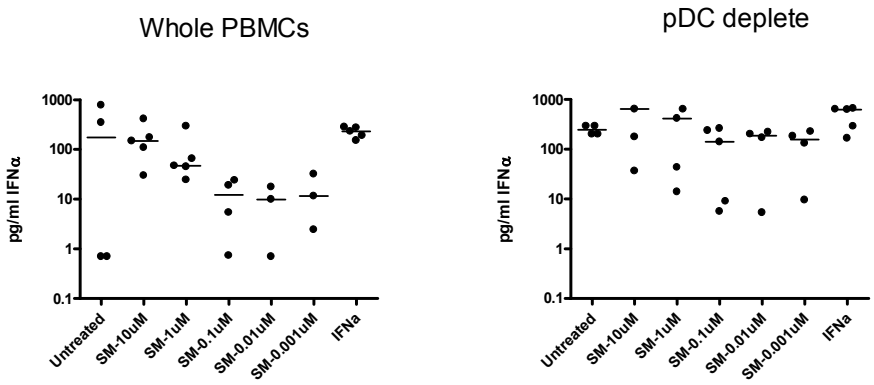


2'5' OAS Expression:

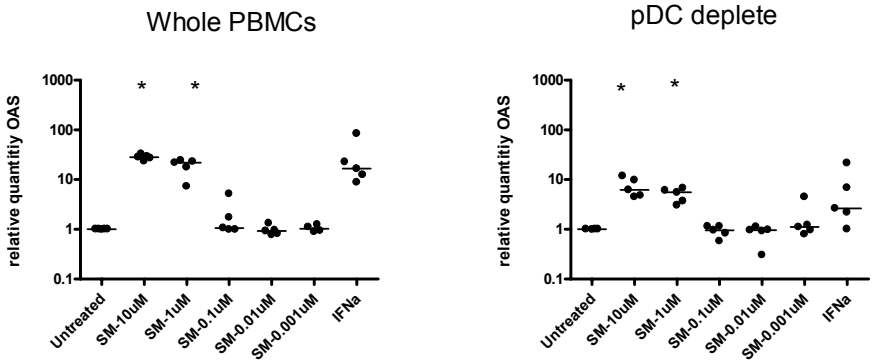


34B: CHC patients

IFNα secretion:



2'5' OAS Expression:



5.5 Discussion:

The need to overcome immune dysfunction seen in HCV and induce strong HCV-specific T-cell responses to clear the virus has prompted evaluation of immunomodulators in CHC treatment. The aim of this study was to investigate both the antiviral and immunostimulatory actions of the TLR7 agonist, SM-360320 on cells derived from the blood of CHC -patients. We have shown that SM-360320 was able to induce a robust *ex vivo* innate immune response in blood from treatment naïve and NR-CHC-patients.

On average blood from naïve-CHC patients produced higher levels of all cytokines compared to NRs but no differences were observed in the cytokines secreted. In the replicon assay, blood from some of the naïve patients showed responses more like the NR-patients which may indicate they will be non-responders to treatment. CD69 expression on NK-cells was higher in NHDs than CHC-patients with no significant difference between naïve and NRs. There were no differences observed in 2'5'OAS expression between the patient groups.

TLR7 expression is limited to certain tissues (296) and a restricted set of human immune cells, notably pDCs and B cells. TLR7 agonists are not thought to have direct antiviral effects (297) but following TLR engagement induce pDCs to produce large quantities of type 1 IFNs and more modest amounts of various other cytokines e.g. IL-6 and TNFα. Induction of type 1 IFNs through activation of TLRs on pDCs is a crucial part of the host's innate immune response to viral infection which results in the production of a wide range of downstream antiviral effector molecules such as interferon stimulated genes: 2'5'OAS, RNase-L, MxA, and signaling chemokines such as IP-10. As a consequence of the secretion of type 1 IFNs, a number of secondary effects are also induced such as stimulation of NK-cells as well as maturation of pDCs to potent antigen-presenting cells, augmenting their ability to stimulate T-cells and thus activating both the innate and adaptive immune responses.

In this study we investigated the antiviral effects of SM-360320 by looking at the induction of 2'5OAS, induction of cytokines and the antiviral activity of plasma from treated blood in HCV replicon cells.

Plasma from blood treated with SM-360320 inhibited replication of HCV-replicons in Huh-7 cells reducing luciferase reporter expression to baseline levels. This may indicate the potential of TLR7 agonists to reduce viral load mediated by the antiviral activities of agonist-induced IFNα. The expression 2'5'OAS was found to be increased with TLR7 treatment and secretion of antiviral cytokines, IFNα and IFNγ and pro-inflammatory cytokines from whole blood were significantly enhanced. *In vivo* this would be predicted to promote a Th1 immune response and favour viral clearance.

NK-cells play an important part in the first line of defence against viral infections, rapidly recognising and killing virus-infected cells and also secreting inflammatory cytokines, such as IFNγ which have direct antiviral effects as well as activating and polarising Th1 and cytotoxic lymphocyte responses (298;299). Therefore they provide a pivotal link between innate and adaptive immunity. Several groups have shown that TLR7 agonists induce NK-cell activation (CD69 expression and IFNγ production) and enhance their cytotoxic effector function (300-303). In this study we found that SM-360320 was able to significantly upregulate NK-cell activation and was more effective than IFNα treatment. The effect of SM-360320 on NK-cells was lower in CHC-patients than NHDs.

NK-cells express TLR 2, 3, 5 and 6 but little TLR 4, 7, 8 and 9 (300). It is not clear therefore if NK-cell activation by TLR7 agonists is caused by direct activation of TLR7 receptor in NK-cells or if they act indirectly and secondary to activation of other cells, in particular pDCs, and cytokines produced by these cells. It has previously been observed that TLR7-induced NK-cell activation is crucially dependant on contact with cytokines (e.g. IFNα or IL-12) produced by accessory cells in the blood (301-303).

To explore this further, we depleted pDCs, the main IFNα producing cells, from PBMCs and assessed the effects of the TLR7 agonist on NK-cell activation. In preliminary experiments it has been shown that NK-cell activation was partially impaired supporting evidence of an indirect effect and dependence on cytokines from accessory cells in the peripheral blood.

To determine the contribution of pDCs to any antiviral or immunostimulatory effects of SM-360320, the effects of pDC depletion on cytokine secretion and 2'5'OAS induction were assessed. As would be expected, depletion of pDCs from NHD-PBMCs resulted in a reduction of IFNα secretion however a corresponding reduction in 2'5'OAS induction was

not found to be significant. In CHC-PBMCs, 2'5'OAS induction was found to be decreased but the IFNα data is difficult to interpret due to large variability in the whole PBMC group.

Defects in CHC-pDC IFNα production have been described in a number of studies (136;304-309) and it would be interesting to explore the effects of the TLR7 agonist directly on pDCs to see if it has the ability to induce pDC maturation and reverse the defects described.

Current treatment for CHC is based on the combination of PEG-IFNα and ribavirin. Both appear to possess antiviral and some immunomodulatory activities (310;311). When comparing IFNα, at a concentration representative of plasma levels typically found during IFN therapy, to the TLR7 agonist in this study we show that, SM-360320 at 1μM induced pro-inflammatory cytokines and 2'5'OAS to higher levels than IFNα, was more effective in the replicon assay and induced NK-cell activation to significantly higher levels based on percentage of circulating NK cells expressing CD69, which has also been shown with a TLR9 agonists (312). Therefore, comparison of immunostimulatory and antiviral activities show that SM-360320 may provide similar or better antiviral effects and modulation of the innate immune response. Irrespective of the efficacy of SM-360320 in vitro it must be recognized that preclinical and clinical trials may reveal adverse effects that may make the compound impractical or less attractive than IFNα.

Clinical studies with TLR7/8 agonists (313-316) have shown a modest reduction in viral replication in HCV infected patients as well as induction of the immune response with enhanced secretion of IFNα, IP-10, neopterin and enhanced expression of ISGs, 2'5' OAS and ISG15. They were also shown to functionally activate NK cells *in vitro* and conditioned media from the TLR treated PBMCs inhibited HCV replication in a replicon system (317).The virological response correlated with the induction of the ISGs indicating that they induce their anti-HCV effect through induction of an immune response. These findings are therefore similar to what I have shown in this study.

While stimulating TLRs could re-establish an appropriate immune response to infection, caution must be taken as inappropriate stimulation can lead to inflammation and autoimmunity with unwanted stimulation of cytokines causing side effects. In general side effects of TLR agonists have been mild, with far fewer problems than less subtle immune stimulators such as IFNs (318), however, development of several TLR7 agonists have

been halted due to intense immune stimulation found in 13 week toxicology studies . In this study the effective in vitro dose of SM-360320 was found to be 1μM.

Although direct-acting HCV-specific drugs (e.g. protease inhibitors) may provide superior antiviral effects, a probable advantage of TLR agonists is the relatively low emergence of drug resistance that is a particular challenge in HCV with its high genetic diversity and mutation rate. Drug resistance is less likely because the molecular target of these agents is the host receptor rather than a virally encoded target. Furthermore, because TLR7 activation initiates a broad immune response, including both innate and adaptive responses, it is likely that multiple aspects of viral replication are simultaneously attacked.

In summary, the TLR7 agonist, SM-360320 has been shown to possess effective antiviral and immunostimulatory effects on whole blood from CHC donors and might provide complementary and additional HCV therapies making it a promising novel treatment. It is likely that HCV therapy will continue to require the presence of an effective immunomodulatory agent and TLR7 provides a candidate target mechanism for this role.

Summary of findings:

Induction of 2'5'OAS:

- SM360320 (10μM and 1μM) and IFNα induced 2'5'OAS expression in NHD and CHC patients
- A higher response was seen in NHDs compared with CHC patients
- No difference was seen between NR and naïve CHC patients

Induction of cytokine production:

- SM360320 stimulated secretion of all cytokines tested (10μM and 1μM) in NHD and CHC patients
- IFNα stimulated secretion of IFNα, IFNγ, IL-6, IP-10 and IL-12 in CHC patients and IFNα, IL-6, IP-10 and IL-1β in NHDs
- Higher levels of IL-1β and IL-6 were produced from SM360320 treated blood compared to IFNα treated blood
- Naïve CHC blood induced higher levels of cytokines than NR CHC blood
- Untreated levels of all cytokines were higher from CHC patient blood

Inhibition of HCV replication:

- SM360320 treated blood inhibited replication of HCV replicons in Huh-7 cells
- SM360320 treated blood had a higher antiviral activity than IFNα treated blood

Induction of NK cell activation:

- SM360320 enhanced CD69 expression and IFNγ production in a dose dependant manner
- IFNα was less effective at enhancing CD69 expression than SM360320
- INFα did not enhance INFγ production
- NHDs enhanced CD69 expression greater than naïve CHC patients

The role of oDCs:

- SM360320 and IFNα enhanced secretion of IFNα and expression of 2’5’OAS from whole PBMCs from NHDs
- In NHDs, secretion of IFNα was reduced when pDCs were depleted while 2’5’OAS expression remained unchanged
- IN CHC patients, 2’5OAS expression was reduced when pDCS were depleted while the results of IFNα secretion ae difficult to interpret

Chapter 6
Discussion

6.1 Introduction:

Chronic infection with hepatitis C virus (HCV) is a major cause of liver related morbidity and mortality. It has infected 180 million people worldwide. Infection is spread parenterally and persists in 80% of patients. Substantial evidence has emerged to support the role of the host immune response in the outcome and pathogenesis of acute and chronic HCV infection. It has been accepted that successful viral clearance by the host is largely attributable to induction of innate immune responses with robust induction of type I IFN, antiviral cellular effectors and NK cells(159;319-321) and also the development of broad and sustained adaptive T cell responses directed to HCV antigenic determinants.

Dendritic cells (DCs) are professional antigen presenting cells characterized by their exceptional capacity to activate naïve T cells and prime adaptive immunity. DCs play a pivotal role linking innate and adaptive immunity(65;67). They recognize pathogens through pathogen recognition receptors (PRRs) including toll like receptors (TLRs), leading to DC activation and maturation. Activated DCs then activate NK cells, thus driving the non-specific innate immune response, whilst simultaneously presenting antigen to initiate T cell mediated adaptive immunity. DC dysfunction has been implicated as a mechanism that enables persistent viral infection via impaired priming of naïve T cells and subsequent adaptive immune response.

NK cells play an important part in the first line of defence against viral infections, rapidly recognising and killing virus-infected cells via perforin release, by induction of apoptosis and also by secreting inflammatory cytokines, such as IFNγ, which have direct antiviral effects, inhibiting viral replication as well as activating and polarizing Th1 and cytotoxic lymphocyte responses(80;81). Therefore they provide a pivotal link between innate and adaptive immunity. The phenotypes and/or functional activities of various populations of these cells have been reported to be impaired in patients with chronic HCV infection (140-146).

I set out to explore the how current and novel treatments for hepatitis C are able to modulate the immune response looking specifically at their effects on dendritic cells, NK cells and their ability to stimulate T cells and an adaptive immune response in both NHDs and CHC patients.

In the following sections, the findings made during the course of the project are summarized and discussed in relation to the future direction of treatment for CHC.

6.2 Summary of Findings:

6.2.1 Effect of ribavirin on DC maturation and cytokine production

The current mainstay of treatment for CHC is ribavirin in combination with PEG- IFNα. Ribavirin is a purine nucleoside analogue which continues to remain critical in the treatment of CHC. Despite its effective use for the last 40 years, its mechanisms of action are still poorly understood but several hypotheses have been proposed (188). These include immunomodulatory effects, shifting the T-helper 1 cytokine balance (T_H1)/ T_H2) toward T_H1 resulting in improved viral clearance.

Some studies have previously shown suppressive effects of ribavirin on PBMCs but only one study has examined the effects of ribavirin on dendritic cells and focused on moncyte derived DCs that represent a model of myeloid DCs(322). They found that ribavirin suppressed IL-12, TNFα and IL-10 secretion when co-administered with poly I:C as a stimulus but had no effect on phenotypic maturation.

In this thesis, I set out to investigate further the immunomodulatory effects of ribavirin and looked at its effects on pDCs freshly extracted from blood, which have not previously been evaluated. pDCs are the main producers of IFNα in the body. I explored the effects of ribavirin on pDC function and cytokine production.

I have shown that ribavirin is able to modulate cytokine production from dendritic cells in response to a maturation stimulus, in particular suppressing the production of IFNα from pDCS from both CHC and NHD patients but also TNFα, IL8 and IL10. In MoDCs from

NHDs, I have shown that ribavirin decreased CD40L-induced TNFα production but had no effect on other cytokines tested following this stimulus, or on DC phenotype.

The finding that ribavirin is unable to stimulate IFNα production from pDCs together with the further impairment in IFNα production from pDCs treated with ribavirin correlates with the requirement for exogenous IFNα in the effective treatment of CHC and may, in part, explain why ribavirin alone has little beneficial effect on HCV viral loads.

At present it seems likely that ribavirin will continue to be needed in the future treatment of CHC alongside newer anti-virals, to achieve SVR and prevent relapse, as suggested in recent HCV protease inhibitor trials (206; 208; 256). The findings in this thesis suggest that exogenous IFNα therapy will be required if ribavirin is continued to be used as part of a combination therapy. Furthermore, the opposing actions of ribavirin and TLR agonists in IFNα production demonstrated in this study may have implications if they are to be used in combination in the future treatment of CHC. Finally, the ribavirin induced suppression of TNFα and IL8 from MoDC and pDC shown in this study may indicate an anti-inflammatory effect of ribavirin and may, in part, explain the previously reported reduction in hepatic inflammation with ribavirin monotherapy.

6.2.1.1 Future Work:

- Further investigation into the suppression of IFNα by Ribavirin using a larger cohort of patients and with exploration into the mechanisms underlying this defect.

- Explore any possible maturation defect of pDCs caused by ribavirin by looking at the effects on CD86, CD80 and HLA-DR expression.

- Explore if ribavirin has any effect on DC antigen presentation by looking at the effect of ribavirin treated DCs on T cell allo-stimulation and the effect on NK cell activation.

- Comparison of effects of ribavirin on sustained responders and previous non-responders to treatment.

6.2.2 Activation of Human Dendritic Cells and Enhancement of Antigen Specific T Cell Immunity by a Novel Helminth-Derived Immunostimulant.

Current treatment for CHC leads to sustained viral eradication in 40-80% of infected patients depending on the HCV genotype and is associated with multiple side effects and complications. These often result in discontinuation of treatment and account for a large proportion of patients not being offered treatment. With the increasing prevalence of chronic hepatitis C there is a pressing need for safe and effective immunotherapies that will drive the host's immune effector mechanisms towards viral clearance. Immunostimulants represent an emerging class of drugs that are designed to effectively and appropriately amplify naturally occurring immune responses against infectious pathogens.

O*nchocerca* ***v****olvulus* is a filarial parasite that causes human onchocerciasis (River Blindness). Recombinant secreted protein of the helminth *Onchocerca volvulus*, r*Ov*-ASP-1, is typical of nematode ASPs in that it is intrinsically immunogenic(282). r*Ov*-ASP-1 has been shown to stimulate murine immunocytes in earlier work (274).

In this study, I assessed the potential of r*Ov*-ASP-1 as a human therapeutic by testing its ability to stimulate human dendritic cells from NHDs looking at its effect on DC phenotype and cytokine production and I investigated if it is able to enhance DC antigen presentation capacity by using proliferation assays.

I have shown that r*Ov*-ASP-1 activities are mediated by its ability to activate APCs. I have shown evidence of phenotypic maturation, based on up-regulation of phenotypic markers (CD40, HLA-DR, CD83 and CD86) and enhanced proinflammatory cytokine production (IL-6, IL-8 and TNFα). r*Ov*-ASP-1 not only enhanced pro-inflammatory cytokine production from MoDCs but also significantly stimulated the production of the anti-inflammatory cytokine IL-10. The r*Ov*-ASP-1 induced secretion of IL-12 was found to be CD40-dependant and co-stimulation through the CD40-CD40L pathway also enhanced the rOV-ASP-1 induced secretion of IL-10 and TNFα. These results provide evidence that rOV-ASP-1 may initiate DC-induced adaptive immune responses by multiple distinct mechanisms.

I also found that r*Ov*-ASP-1 enhanced the ability of MoDCs to stimulate proliferation of allogeneic $CD4^+$ T cells suggesting the protein is able to enhance DCs accessory/antigen presentation function.

With the findings of the effects of r*Ov*-ASP-1 on DCs shown in this study, there are several potential applications for the use of the protein including use as a novel innate vaccine adjuvant that can boost the activation of an adaptive immune response. The key property of adjuvants is their ability to induce DC maturation and activation which I have shown in this study. r*Ov*-ASP-1 has also previously been shown to stimulate a mixed Th1/ Th2 with a strong Th1-biased immune response which is an important challenge for vaccine development and which few experimental adjuvants are capable.

The ability of r*Ov*-ASP-1 to stimulate human DCs may enable it to be used as a human therapeutic and as a DC activator where DC function is impaired by a pathogen. In the case of chronic HCV infection, where DC function has been reported to be impaired by the virus, r*Ov*-ASP-1, or more likely a subunit, may potentially be used to boost antigen-specific responses and favor viral clearance.

Finally, the findings that r*Ov*-ASP-1, enhances secretion of large amounts of IL-10, a potent immunoregulatory cytokine may also be valuable in inflammatory and autoimmune diseases. This would require expressing a subunit that is responsible for the induction of IL-10 without pro-inflammatory cytokines and be could particularly useful if administered topically, locally or targeted to an affected organ using a tissue-specific antibody.

6.2.2.1 Future Work:

- Investigate the antigen specific responses of r*Ov*-ASP to recall antigens (influenza haemagglutinin, tetanus toxoid) and recombinant HCV antigens (core and NS3, from a genotype 1b isolate) measured by ^{3}H-thymidine incorporation.

- Explore the effects of *Ov*-ASP-1 in CHC patients.

6.2.3 Evaluating the antiviral and immunostimulatory actions of a TLR7 agonist in chronic hepatitis C infection

TLR agonists have been investigated in the treatment of chronic hepatitis C as a means of inducing endogenous IFNα production and efficient activation of innate and acquired immunity. The potential ability of TLR agonists to stimulate immune cells even in the presence of the immune dysfunction induced by CHC infection makes them important therapeutic targets with the aim that they could be used to activate pDCs to enhance endogenous IFNα production to clear HCV infection.

In this study, I investigated both the anti-viral and the immunostimulatory actions of TLR7 agonist, SM-360320 on the innate immunity, looking at its effects on 2'5' OAS gene expression, HCV replicon inhibition, cytokine production and NK cell activation and comparing the effect of the agonist on blood from treatment naïve CHC patients, previous NRs to treatment and NHDs.

I have shown that SM-360320 induces 2'5'OAS gene expression, inhibits replication of HCV replicons in Huh 7 cells and stimulates secretion of anti-viral and pro-inflammatory cytokines, all suggesting a potential mechanism for enhanced viral clearance through increased IFNα production and promotion of a Th1 immune response.

I have also shown that SM-360320 is able to up-regulate NK cell activation as shown by enhanced CD69 expression and IFNγ secretion. NK cells play an important part in the first line of defence against viral infections, with direct cytotoxicity of virus infected cells and secretion of cytokines, such as IFNγ which have direct antiviral effects as well as activating and polarizing a Th1 immune response and again favouring viral clearance.

I investigated the effects of pDC depletion on cytokine secretion and 2'5 OAS induction to explore the contribution of pDCs to any anti-viral or immunostimulatory effects of SM360320. Depletion of pDCs from NHD-PBMCs resulted in a reduction of IFNα secretion suggesting activation of pDCs through TLR7. However, in CHC patients the results were difficult to interpret and needs to be explored further with a larger number of patients.

The effects of SM-360320 on whole blood and NK cell activation were found to be greater than IFNα suggesting that SM-360320 may provide similar or better antiviral effects and modulation of the innate immune response than IFNa.

The findings in this study suggest that SM-360320 might access complementary and additional mechanisms of action to current HCV therapies making it a promising novel treatment. However, irrespective of the efficacy of SM-360320 i*n vitro* it must be recognized that preclinical and clinical trials may reveal adverse or differing effects that may make the compound impractical or less attractive than IFNα.

6.2.3.1 Future work:

- Explore the effects on the TLR7 agonist on purified pDCs from CHC patients to determine if it has sufficient potency to reverse the defect in CHC-pDC IFNα production reported in a number of studies. Determine if it has the ability to induce pDC maturation with upregulation of HLA-DR, CD80, CD86 and CD40.

- To assess the immunostimulatory effects of SM360320 on acquired immunity by looking at T cell proliferation in response recall antigens (influenza haemagglutinin, tetanus toxoid) and recombinant HCV antigens (core and NS3, from a genotype 1b isolate) measured by ^{3}H-thymidine incorporation to demonstrate if the agonist increases the capacity of antigen presenting cells to induce reactive T cells and promote antigen specific T helper type 1 cell-mediated immune response.

- Further explore the effect of pDC depletion in IFNα production and 2’5’ OAS expression in CHC patients with a larger cohort of patients.

- Take TLR7 agonist, SM-360320 through preclinical testing, pharmo-toxicology studies and into phase I clinical trials.

6.3 Conclusions:

HCV remains a significant global healthcare challenge. The host's immune response is pivotal in the natural clearance of infection, in disease pathogenesis of CHC and in response to treatment.

The current mainstay of treatment for CHC is PEG-IFNα in combination with ribavirin, relying on immune stimulation from the IFNα while the mechanism of action of ribavirin remains poorly understood. Work in this thesis has done little to advance the understanding of these mechanisms beyond showing a reduced production of IFNα from pDCs and explaining possible reasons behind the failure of monotherapy and dependence on co-administration of IFNα in treatment regimens.

IFNα alone is only modestly effective and toxic, promoting the search for other therapeutic agents. Here we have shown that TLR7 agonists have the potential to reproduce many of the effects of exogenous IFNα with evidence of inhibition of viral replication and immune modulation. TLR7 agonists may have a role in replacing IFN and may be used in conjunction with directly acting antivirals. Used alone, they may have an advantage over direct antiviral agents such as protease inhibitors with the risk of drug resistance that is a particular challenge in HCV with its high genetic diversity and mutation rate. The molecular target of these agents is the host receptor rather than a virally encoded target making drug resistance less likely.

The novel helminth-derived protein, r*Ov*-ASP-1, investigated in this thesis has also shown promise as an effective immunostimulant to boost antigen specific responses or as a vaccine adjuvant.

Further investigation of these compounds is therefore warranted due to their potential widespread application in treating infectious diseases and immune mediated conditions.

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Appendix 1

The effects of Ribavirin on dendritic cell maturation and cytokine production

Running head: Effect of ribavirin on dendrtitic cells

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Abstract:

Background: Ribavirin, in combination with interferon alpha (IFNα), is the current treatment for chronic hepatitis C (CHC). However, the mechanisms of ribavirin's action are still poorly understood.
Aim: We investigated the immunomodulatory effects of ribavirin on dendritic cells which are potent antigen presenting cells playing a pivotal role linking innate and adaptive immunity.
Methods: Using freshly isolated plasmacytoid DCs (pDCs) and cultured, monocyte-derived DCs (MoDCs) the effect of ribavirin alone or in combination with the maturation stimuli CD40L, TLR9 agonist or TLR7 agonist was tested. The expression of DC

maturation markers (HLA-DR, CD83, CD86 & CD40) and cytokine secretion by normal healthy donors (NHDs) and CHC patients was examined.

Results: In pDCs, ribavirin upregulated the expression of CD86 (p=<0.05) but had no effect on HLA class I expression. Production of IFNα by NHD and CHC was significantly reduced by ribavirin at all concentrations (p=<0.05) following pDC stimulation with CD40L, TLR9 or TLR7 agonists. Ribavirin also caused a significant reduction in IL8 (p=<0.05) and TNFα (p=<0.05). The suppressive effects of ribavirin on cytokine production were not due to induction of pDC apoptosis or cell death. In MoDCs, ribavirin had no effect on maturation but was found to significantly suppress the production of TNFα (p=0.05).

Conclusions: The suppression of TNFα production from MoDCs and pDCs treated with ribavirin may contribute to the reduction in hepatic inflammation seen with ribavirin monotherapy. Ribavirin's suppression of endogenous IFNα production from activated pDC explains the requirement for exogenous IFNα in therapeutic regimes for CHC.

Introduction:

Chronic infection with hepatitis C virus (HCV) is a major cause of liver related morbidity and mortality affecting 170 million people worldwide. Infection persists in 80% of patients and is thought to result from a failure to sustain an immune response against HCV[1].

Dendritic cells (DCs) are professional antigen presenting cells characterized by their exceptional capacity to activate naïve T cells and prime adaptive immunity. DCs play a pivotal role linking innate and adaptive immunity[2,3]. They recognize pathogens through pathogen recognition receptors (PRRs) including toll like receptors (TLRs), leading to DC activation and maturation[3]. Activated DCs then activate NK cells, thus driving the non-specific innate immune response, whilst simultaneously presenting antigen to initiate T cell mediated adaptive immunity.

There are two functionally distinct subsets of DCs; myeloid (mDC) and plasmacytoid (pDC). pDCs are the most abundant producer of type-1 interferons in the body which they produce in large quantities during viral infections[4]. Myeloid DCs produce IL12 to prime T_H1 responses. Monocyte derived dendritic cells (MoDCs) are commonly used *in vitro* as a model of myeloid DCs due to the larger numbers that can be obtained and their close resemblance.

Evidence points towards dendritic cell dysfunction in patients with chronic hepatitis C, with impaired maturation and impaired ability to stimulate T cells which may compromise their capacity to mount, and sustain, an effective antiviral immune response, thus leading to viral persistence[5-18].

Ribavirin is a purine nucleoside analogue that is activated or metabolised through 5' phosphylation to the monophosphate (RMP), diphosphate (RDP) and biologically active triphosphate (RTP) form. Despite its effective use for the last 40 years, the mechanisms of action of ribavirin are poorly understood but several hypotheses have been proposed[19,20]. These include direct inhibition of viral RNA replication, inhibition of the enzyme inosine monophosphate dehydrogenase (IMPDH) and mutagenesis causing viral replication error catastrophe as well as immunomodulatory effects, shifting the T-helper 1 cytokine balance (T_H1)/ T_H2) toward T_H1 from T cells resulting in improved viral clearance.

Ribavirin monotherapy has minimal effect on HCV viraemia but is associated with improvements in serum aminotransferases[21] suggesting that it may have an effect on inflammation and the host immune response. In combination with IFNα however, ribavirin increases the proportion of patients that clear the virus whilst on treatment from 27% with IFNα monotherapy[22] to 41% with combination therapy[23]. However its most striking effect is that it prevents relapse after cessation of treatment. This latter effect is unexplained but also plays a significant role in newer treatment regimes that include HCV protease inhibitors with pegylated interferon and ribavirin[24-26].

We set out to investigate the immunomodulatory effects of ribavirin on DC function in order to elucidate the mechanisms behind the prevention of relapse following treatment. Freshly isolated pDCs and cultured MoDCs from both NHDs and CHC patients were exposed *in-vitro* to ribavirin alone or to a combination of ribavirin and a maturation stimulus (CD40L, TLR9 agonist TLR7 agonist). These data show that ribavirin does induce profound changes to DC derived cytokine profiles.

Materials and Methods:

Human subjects:

Ethical approval was obtained from Southampton and South-West Hampshire Joint Research Ethics Committee and all patients gave informed consent in writing prior to participating in the study.

Blood samples were collected from 21 uninfected NHD (18 male and 3 female, median age 53 years, range 25-67) who had no risk factors for blood borne viruses. Ten patients with CHC virus infection (4 female, 6 male, median age 54 years, range 37-61 years) were recruited from the hepatology clinics run by Southampton University Hospitals National Health Service Trust. All patients had detectable HCV RNA (4 genotype 1, and genotype 6 "non-1"). Patients were excluded if they had received treatment for HCV within 6 months prior to the study or tested positive for other blood borne viruses, including HBV and HIV.

Purification of plasmacytoid DCs from peripheral blood:

Freshly drawn blood was obtained from NHD and CHC subjects in 300ml and 100ml volumes respectively. Blood was collected into K_3 ethylenediamine tetraacetic acid (EDTA). PBMCs were prepared immediately using Lymphoprep density gradient centrifugation (Robbins Scientific, Solihull, UK). Plasmacytoid DCs (pDCs) were isolated from the PBMCs by positive selection with MACS BDCA-4 Blood Dendritic Cell Isolation Kit (Miltenyi Biotec, Bisley, UK), according to the manufacturer's recommended protocol, in conjunction with an AutoMACS machine (Miltenyi Biotec).

Generation of immature MoDCs:

50mls of freshly drawn blood was obtained from NHD subjects and collected into EDTA and PBMCs prepared immediately using Lymphoprep density gradient centrifugation. Monocytes were isolated by positive selection with anti-CD14 conjugated magnetic MACS beads (Miltenyi Biotec), using an AutoMACS.

Monocytes (1 x 10^6cells/ml) were cultured in 6 well plates (Greiner Bio-One, Stonehouse, UK) in 3ml per well of complete medium [RMPI-1640 without phenol red (Invitrogen, Paisley, UK), with 2 mmol/l L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin (Sigma, Poole, UK) and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Perbio Science UK Ltd, Tattenhall, UK)], containing 50ng/ml recombinant

human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and 1000 IU/ml rhIL4 (both from R&D Systems, Abingdon, UK). After 2 and 5 days 0.5ml of culture medium was replaced with fresh medium containing cytokines.

Stimulation of MoDCs and pDC with ribavirin

Day 5 immature MoDCs were plated into 24 well (500µL / well) cell culture plates (Greiner Bio-One) at a concentration of 2 x 10^5/ml. MoDCs were cultured with medium alone or in a 1:1 ratio with CD40 ligand-transfected Chinese hamster ovary cells (hCD40L-CHO)), (gift from Professor M. Glennie, Tenovus Laboratories, Southampton, UK) with or without different concentrations of ribavirin (4, 20, 100 and 500µM) (provided by iQur Ltd, Southampton, UK).

Freshly isolated pDCs were plated into 96 well (200µl /well) cell culture plates at a concentration of 2 x 10^4 / ml. pDCs were cultured under the following conditions; (1) medium alone (2) in a 1:1 ratio with hCD40L-CHO cells, (3) with 1µM M362, a TLR9 agonist (InvivoGen, San Diego, CA) or (4) 250µM loxoribine, a TLR7 agonist (InvivoGen) each with or without different concentrations of ribavirin (4, 20, 100 and 500µM). Cells were diluted in complete medium and incubated at 37^0C in 5% CO_2

Determination of DC maturation:

After 24 hours and 48 hours of culture, pDCs and MoDCs respectively were harvested and labelled for two-colour flow cytometry to identify their cell surface phenotype.

Briefly, cells were resuspended in PBS containing 0.1% Sodium Azide (Sigma), 1% BSA (Sigma) and 100µg / ml human IgG (Jackson Immunoresearch, Suffolk, UK) and blocked on ice for 30 minutes. MoDCs were then stained using allophycocyanin (APC) - conjugated monoclonal antibodies against CD40, CD86 and CD83 (all from BD Biosciences, Oxford, UK) or fluorescein isothiocyanate (FITC)-labeled anti-human HLA-DR (BD Biosciences). pDCs were stained using APC- conjugated monoclonal antibodies against HLA class 1 or FITC-labeled CD86 all diluted to their optimal concentrations or with equivalent concentrations of APC- or FITC- labelled isotype controls (murine IgG1 and IgG2b; BD Biosciences). After staining, cells were washed, fixed in 1% paraformaldehyde and stored on ice in the dark pending data analysis using a FACSCalibur flow cytometer with Cell Quest software (BD Biosciences).

Measurement of cytokine production:

The cytokines produced by stimulated MoDCs and pDCs were isolated by centrifugation (300xg) as cell culture supernatants after 24 hours of culture.

Production of IFNα from pDCs was determined using a human IFN-α multi-species Enzyme-Linked Immunosorbent Assay (ELISA), following the 'extended range' protocol of the manufacturer (PBL Biomedical Laboratories, NJ, US). This recognizes 13 of 14 IFN-α isoforms, the exception being IFN-αF.

Concentrations of IL12p70, IL10, IL8, IL6, IL1β and TNFα, IFNγ, IL2, IL4, IL5 in MoDC and pDC culture supernatants were simultaneously quantified using the Multiplex Fluorescent Bead Immunoassay (FBI), according to the manufacturer's instructions (Bender MedSystems). 3000 events were acquired using a FACSCalibur dual laser flow cytometer and analysed using CellQuest software.

Determination of cell death and apoptosis:

Freshly isolated pDCs were plated into 96 well cell culture plates at a concentration of 2 x 10^4/ml in complete medium in the presence of 1μM M362 with or without ribavirin at different concentrations (500μM, 100μM, 20μM and 4μM) for 24 hours. Cell death was determined by FACS analysis with annexin V FITC in combination with propidium iodide, used according to the instructions of the manufacturer (BD Pharmingen).

Statistical Analysis:

Data was analyzed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) with unpaired *t*-tests and one way ANNOVA test applied with Dunnett's post test analysis.

Results:

Results from pDCs:

Ribavirin has no effect on IFNα production from immature NHD-pDCs:

Freshly isolated pDCs from NHDs (*n* = 5) were cultured for 24 hours with; (1) medium alone (2) in a 1:1 ratio with CD40L transfectants (3) with TLR9 agonist, M362 or (4) with the TLR7 agonist, loxoribine in the presence or absence of ribavirin at different concentrations. Unstimulated pDCs produced negligible amounts of IFNα which were unaltered by the addition of ribavirin (Fig. 1A).

Ribavirin suppresses IFNα production from stimulated NHD-pDCs:

When pDCs (*n*=5) were stimulated with CD40L, IFNα production was significantly increased. However, the addition of ribavirin, at all concentrations, significantly reduced the production of IFNα by NHD-pDCs in a dose-dependent manner in response to CD40L (fig. 1B). The same effect was observed when either a M362 or loxoribine was used in combination with ribavirin (Figs. 1C and D respectively).

Ribavirin suppresses TNFα, IL8, IL10 and IFNα from CD40L-stimulated NHD-pDCs

Using the Multiplex Fluorescence Bead Immunoassay (FBI), we tested the effects of ribavirin on other cytokines (IL12p70, IL10, IL8, IL6, IL1β and TNFα, IFNγ, IL2, IL4, IL5) produced by CD40L-stimulated pDCs from NHDs (*n*=7). Ribavirin was found to reduce the production of TNFα, IL8 and IL10 from stimulated NHD-pDCs (Fig. 2). The effects of ribavirin on production of these cytokines by loxoribine or M362 has not been studied.

Ribavirin does not cause cell death or apoptosis in ex-vivo pDCs

To ensure that the suppressive effects of ribavirin on pDC cytokine production were not due to induction of apoptosis or cell death, freshly isolated pDCs from NHDs were stimulated for 24 hours with M362 in the presence or absence of ribavirin, and then stained with annexin V and propidium iodide.

Figure 3A shows that the inclusion of ribavirin, did not result in increased apoptosis or cell death. After 24 hours of culture without ribavirin, 20% of pDCs were dead compared to 15%, when the highest dose of ribavirin (500μM) was added. The experiment was repeated 5 times and ribavirin did not significantly alter the proportions of live, apoptotic or dead cells at any of the test doses (Fig. 3B).

Ribavirin upregulates stimulus induced CD86 expression from NHD-pDCS

To ensure that the suppression of cytokine production from pDCs was not a ubiquitous reduction in all proteins, we looked at the effects of ribavirin on cell surface expression of CD86 and HLA class 1. Both molecules are normally highly expressed by stimulated pDCs. pDCs from NHDs (*n* = 5) were cultured for 24 hours with M362 (1μM) in the presence or absence of ribavirin and cells subsequently stained for two colour flow cytometry. We found that in pDCs stimulated with M362, ribavirin further upregulated the expression of CD86 (Figure 4A) (*p=<0.05) but had no effect on HLA class I expression (Figure 4B).

Ribavirin suppresses IFNα, TNFα, IL8, IL10 and IFNα from stimulated CHC-pDCs*:*

To investigate if these effects of ribavirin were also demonstrated in CHC-pDCs, freshly isolated pDCs from CHC patients (*n* = 5) were cultured for 24 hours in a 1:1 ratio with CD40L and the effects of ribavirin on IFNα and cytokine production were assessed. As seen in NHDs, IFNα production by pDCs from CHC patients was significantly reduced at 500μM,100μM and 20μM of ribavirin (Figure 5) and TNFa, IL8 and IL10 production were also significantly suppressed (Fig. 6).

<u>Effects on MoDCs</u>

Ribavirin had no effect on unstimulated MoDC maturation:

MoDCs from NHDs (*n*= 8) were cultured with ribavirin at different concentrations for 48 hours without any additional stimulus. Irrespective of the concentration used, ribavirin alone had no effect on levels of expression of phenotypic markers CD40, CD86, CD83 and HLA-DR on MoDCs (data not shown).

Effects of ribavirin on cytokine production from MoDCs:

MoDCs from NHDs (*n* =10) were cultured with or without CD40L for 24 hours in the presence or absence of ribavirin at different concentrations.

In immature NHD-MoDCs, cultured with medium alone, ribavirin did not significantly alter the secretion of any tested cytokine (Fig 7a). When immature MoDC were cultured for 24hours with CD40L, CD40 transfectants ligation stimulated the production of TNFα, IL8, IL6, IL12 and IL10 (Fig 7b) but not that of IL1β, IL2, IL5 or IFNγ. The addition of ribavirin at a concentration of 500μM significantly suppressed the production of this CD40L-induced TNFα production (Fig 7b). The effect of ribavirin was less pronounced at lower

doses. Ribavirin did not however, significantly alter the CD40L-induced production of the 9 other cytokines tested.

Discussion:

With the clinical effects of ribavirin still largely unexplained, we set out to investigate the immunomodulatory effects of ribavirin on dendritic cell function by looking principally at the effects on cytokine production.

In this study we have shown that ribavirin modulates dendritic cell cytokine production in response to a maturation stimulus. In pDCs from NHDs, ribavirin significantly suppressed stimulus-induced IFNα, TNFα, IL8 and IL10 and these findings were mirrored in pDCs from CHC patients. In MoDCs from NHDs ribavirin decreased CD40L-induced TNFα production but had no effect on other cytokines tested or phenotype.

The ribavirin induced suppression of TNFα and IL8 from MoDC and pDC shown in this study may indicate an anti-inflammatory effect of ribavirin and may, in part, explain the reported reduction in hepatic inflammation with ribavirin monotherapy. It has been shown that ribavirin alone transiently improves biochemical and histological response in the absence of significant reduction in HCV load[21].

The reduction of IFNα from ribavirin-treated pDCs may be of particular physiological importance since pDCs, unlike MoDCs, are the major source of INFα in the human body producing large quantities of type 1 IFN in response to viral infection. Several studies[14-18] have found pDCs from patients with CHC have impaired production of IFNα. This defect will have an impact on both innate and adaptive immunity and favour viral persistence. Our finding that ribavirin is unable to stimulate IFNα production from pDCs together with the further impairment in IFNα production from pDCs treated with ribavirin correlates with the requirement for exogenous IFNα in the effective treatment of CHC and may, in part, explain why ribavirin alone has no beneficial effects on HCV viral loads. Whether ribavirin is actually exacerbating the IFNα defect in CHC-pDCs needs further investigation in larger cohorts to explore the exact mechanisms. It would be interesting to investigate whether the addition of exogenous IFNα can overcome the suppressive effects of ribavirin on cytokine production from dendritic cells.

The opposing actions of ribavirin and TLR agonists in IFNα production demonstrated in this study may have implications if they are to be used in combination in the future treatment of CHC. The study undertaken by Coley with Actilon the TLR9 agonist, CpG10101[28] (currently withdrawn from further drug development at present) looked at the effects of this compound alone, in combination with ribavirin or IFNα or all combined and found ribavirin combined with CPG10101 was less effective than when combined with exogenous IFNα or all 3 together, strengthening the importance of continued use of exogenous IFNα in future treatments.

As a purine nucleoside analogue, it has been hypothesized that ribavirin may exert immunomodulatory activities via TLR stimulation on dendritic cells[29]. Our findings do not support this hypothesis, with ribavirin having no effect on either phenotype or cytokine production when administered alone to either pDCs or MoDCs. In stimulated DCs, ribavirin was still unable to further enhance cytokine production, but in fact reduced the production of IFNα, TNFα, IL8 and IL10 from pDCs and TNFα from MoDCs.

To ensure the suppression of cytokine production from ribavirin-treated DCs was not a ubiquitous downregulation of all proteins, we looked at the effects of ribavirin on CD86 and HLA class I expression, both of which are highly expressed on stimulated pDCs. Our results show that ribavirin had no significant effect on Class I expression and actually enhanced CD86 surface expression. The enhancement of CD86 surface expression was interesting and could reflect a subtle maturation effect of ribavirin. This should be explored further, looking at the other markers of pDC maturation such as increases in surface expression of MHC and co-stimulatory molecules, CD83 and CD80 as well as CD86. The expression of these co-stimulatory molecules is essential for effective activation of T lymphocytes. In previous studies it has been shown that DCs from CHC patients have impaired maturation with an immature phenotype[5-18]. It is possible that ribavirin can overcome this effect and thereby restore antigen presentation and priming of T cells. In the absence of evidence of any other mechanism whereby ribavirin may enhance viral clearance and sustained response to interferon it may be a subtle effect on DC maturation that accounts for the profound clinical effects of ribavirin when used in conjunction with IFNα in the treatment of HCV infection and further studies will need to be carried out. The effect of dendritic cells treated with ribavirin on T cell allo-stimulation and the consequence for NK cell activation to see if ribavirin has any effect on DC antigen presentation function also needs further investigation.

Only one other study has examined the effects of ribavirin on DC maturation and function[30] and focused only on MoDCs. Using ribavirin at a physiological concentration of 120μM, they found that ribavirin suppressed IL-12 and IL-10 in addition to TNFα when co-administered with poly I:C as a stimulus. They assessed cell culture supernatants at multiple time points up to 48 hours and the suppression of IL-12 was seen at 32 hours and maximal suppression of IL-10 at 36 hours. In our study, supernatants were assessed at only one time point, at 24 hours which may be a reason for seeing no effect on IL12 in this study. However, they too found that ribavirin had no effect on phenotypic maturation in either stimulated or immature MoDCs.

A number of studies in PBMCs have shown that ribavirin has an effect on mitogen-induced cytokine production, with some studies showing enhancement and others inhibition[31-34]. Furthermore some studies have shown ribavirin to induce a T_H1 profile[31,34,35], while others showed no impact on cytokine production[36] or induction of a T_H2 profile. We have shown reduction in both T_H1 (TNFα, IL8 and IL6) and also T_H2 (IL10) and the significance of this remains unexplained. Some of the inhibitory effects of cytokines seen in PBMCs are thought to be secondary to the known antiproliferative effects of ribavirin on T cells[32]. DCs, however, do not proliferate during stimulation so this is unlikely to be the case in this study.

This study is limited by small numbers but has shown that ribavirin is able to modulate cytokine production from dentritic cells, in particular suppressing the production of IFNα. The findings in this study therefore highlight the importance of the continued use of exogenous IFNα therapy if ribavirin is used and the recent trials of HCV protease inhibitor Telaprivir[24-26] and Bocepravir[27] have indicated that ribavirin will continue to be required in future anti-viral regimes to achieve SVR and prevent relapse. The mechanisms underlying the suppression of IFNα by ribavirin and the possible effects of ribavirin on pDC maturation should be explored further. By studying a larger cohort of CHC patients it may also be possible to assess differences in the effects on cytokine production from sustained viral responders and non-responders to treatment.

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Declarations of interest

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FIGURE LEGENDS

Fig. 1 (A) Unstimulated pDCs from NHDs produced negligible amounts of IFNα which were unaltered by ribavirin. (B) Ribavirin, at all concentrations tested, significantly reduced the amount of IFNα produced by NHD-pDCs in combination with CD40 ligation. When (C) a TLR9 agonist (M362) and (D) a TLR7 agonist (loxoribine) were used as alternative stimuli of IFNα production, ribavirin again reduced IFNα production
*p=<0.05; **p=<0.01

Fig 2. NHD-pDCs stimulated with CD40L and ribavirin show suppressed production of TNFα, IL8 and IL10.

Fig 3. Cytokine changes in NHD-pDCs stimulated with M362 and ribavirin are not attributable to increased apotosis or cell death. Apoptosis and cell death were assessed by flow cytometric staining for annexin V and PI (A). The left lower quadrant shows the number of viable cells (annexin-V and PI negative). The right lower quadrant shows the number of cells undergoing apoptosis (annexin-V positive and PI negative). The right upper quadrant shows the number of dead cells or those in late stages of apoptosis (annexin-V and PI positive).
The experiment was repeated 5 times and ribavirin did not significantly alter the proportions of live, apoptotic or dead cells (B).

Fig 4. NHD pDCs (*n* =5) stimulated with 1μM M362 and ribavirin further upregulated the expression of CD86 in a dose dependent manner (Figure 4A) (*p=<0.05) but had no significant effect on HLA class I expression (Figure 4B).
MFI; mean fluorescence intensity; bars show mean.

Fig 5. pDCs isolated from CHC patients also show reduced IFNα production following CD40 ligation in the presence of ribavirin as seen in NHD-pDCs (*p=<0.05).

Fig 6. In CHC-pDCs stimulated with CD40L (*n*=5), ribavirin was found to significantly suppress the production of TNFα, IL8 and IL10 by pDCs, mirroring the effects seen in NHDs.

Fig 7. In immature NHD-MoDCs, ribavirin had no effect on secretion of any tested cytokine (7a). When MoDCs were cultured with CD40L, CD40 transfectants ligation stimulated the production of TNFa, IL8, IL6, IL12 and IL10 (fig 7b). Inclusion of ribavirin at a concentration of 500uM was found to significantly suppress CD40L-induced TNFa production but had no effect on the CD40L-induced production of the other cytokines.

Fig. 1

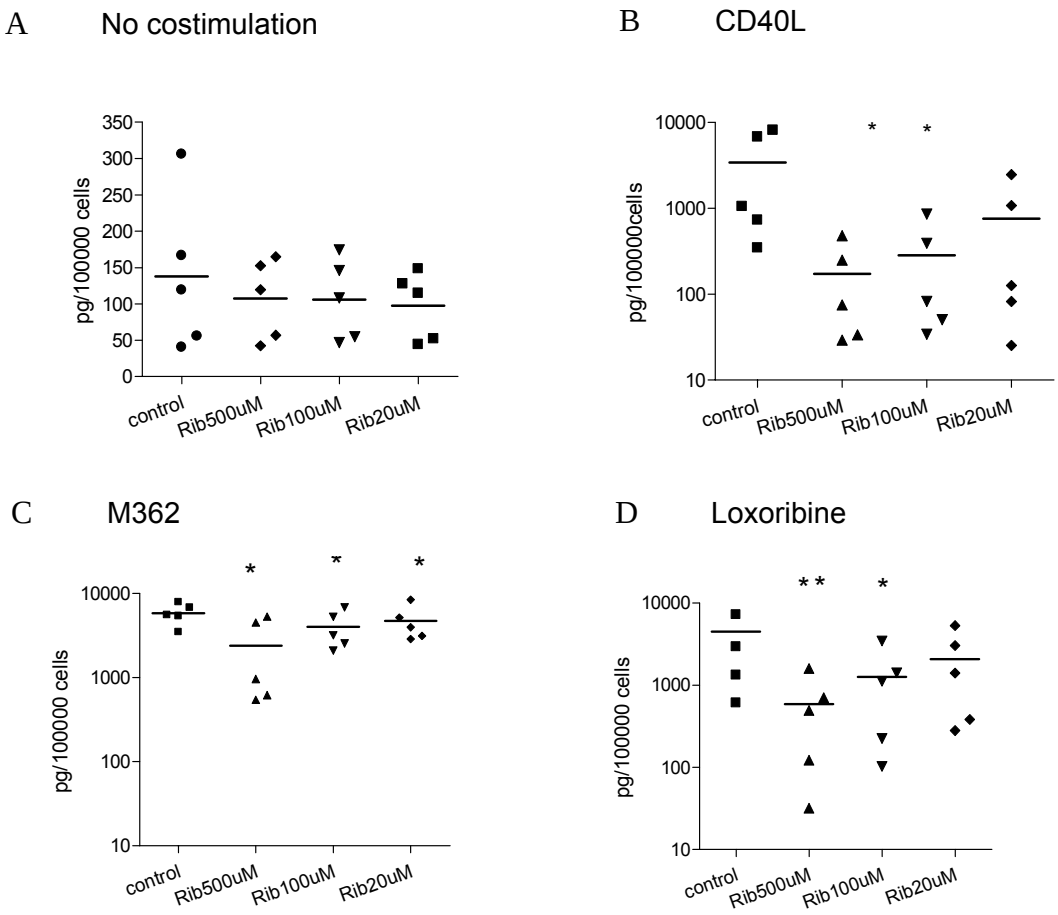


Fig. 2

*p=<0.05

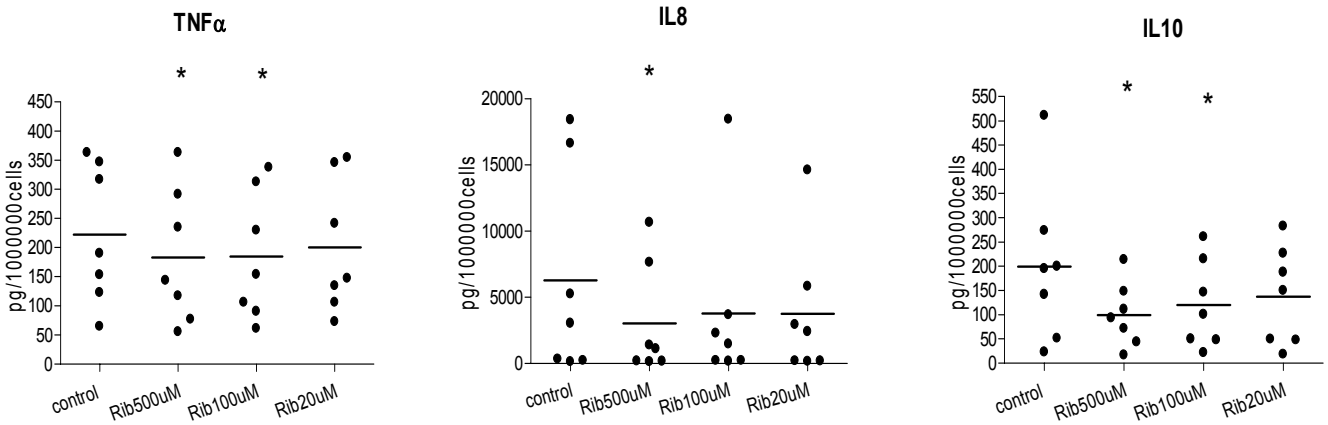
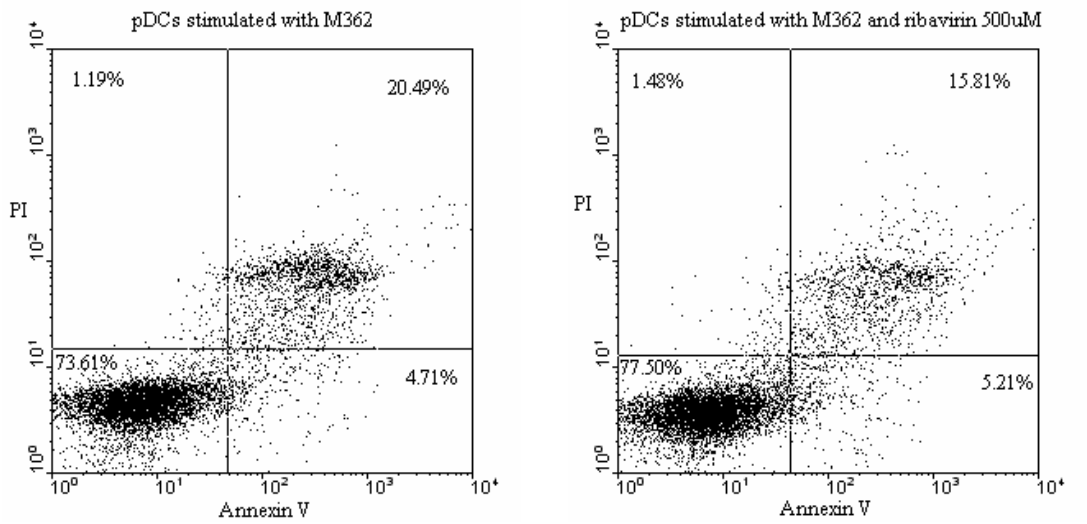


Fig. 3

A



B

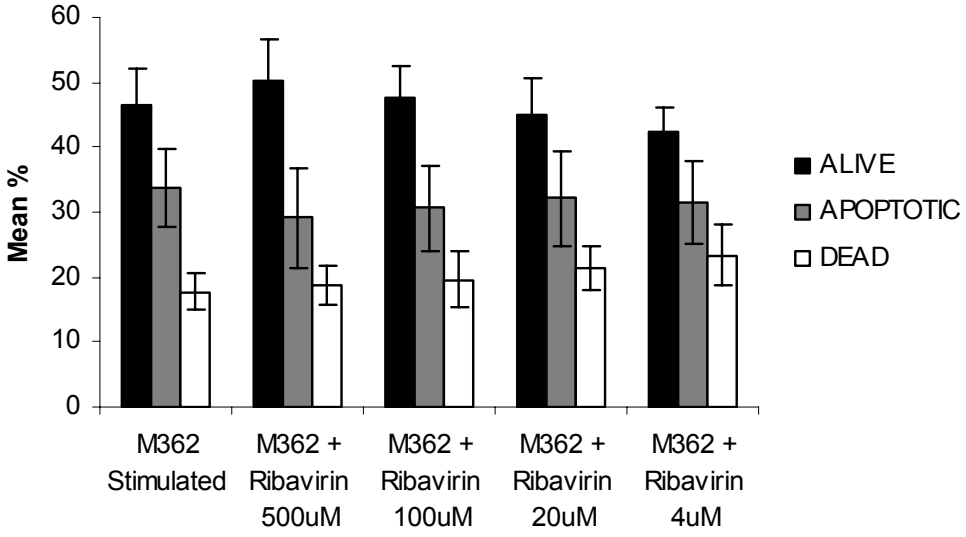

60
50
40
30
20
10
0
Mean %
ALIVE
APOPTOTIC
DEAD
M362 Stimulated
M362 + Ribavirin 500uM
M362 + Ribavirin 100uM
M362 + Ribavirin 20uM
M362 + Ribavirin 4uM

Fig. 4

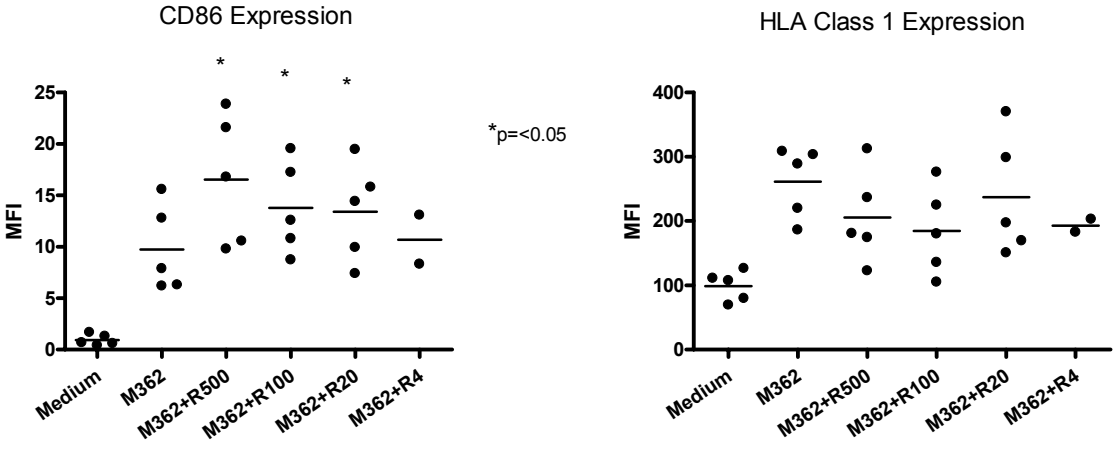

CD86 Expression
MFI
25
20
15
10
5
0
*
*
*
*p=<0.05
Medium
M362
M362+R500
M362+R100
M362+R20
M362+R4
HLA Class 1 Expression
MFI
400
300
200
100
0
Medium
M362
M362+R500
M362+R100
M362+R20
M362+R4

Fig 5.

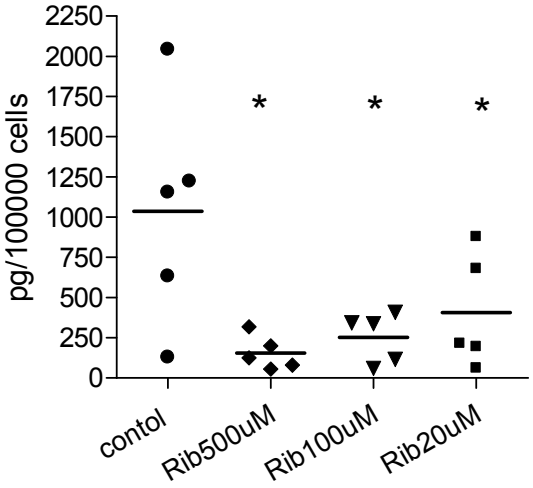


Fig. 6

p=<0.05

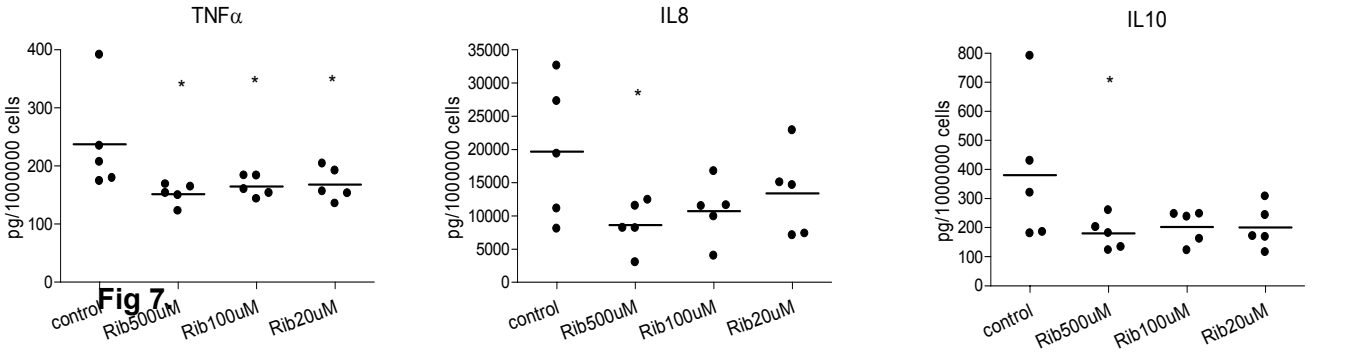
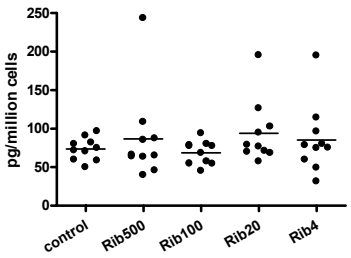


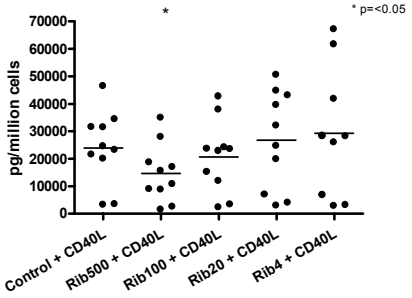
Fig 7.

A: Unstimulated

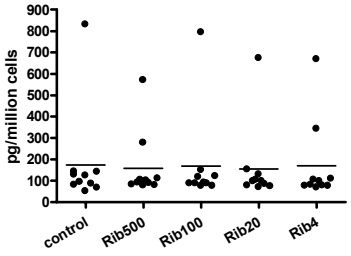
B: CD40L-stimulated

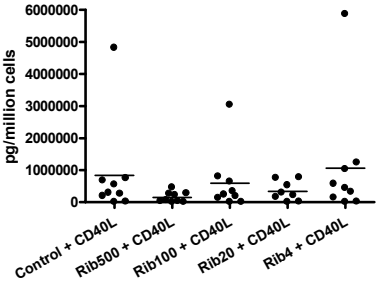
TNFα



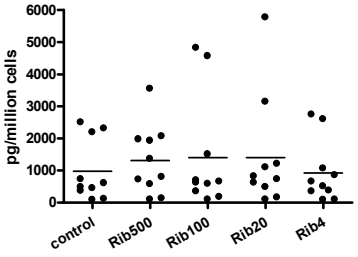


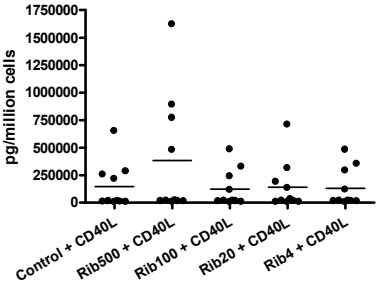
IL12



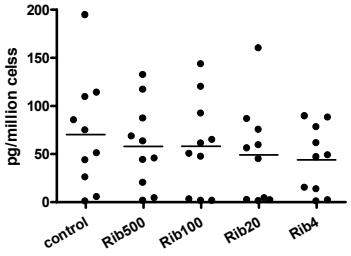


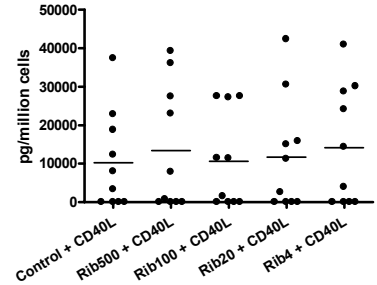
IL8



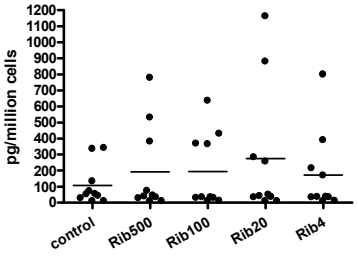


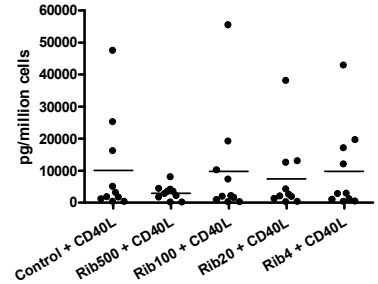
IL6





IL10





Appendix 2

Recombinant *Ov*-ASP-1, a Th1-biased Protein Adjuvant Derived from the Helminth *Onchocerca volvulus*, Can Directly Bind and Activate Antigen-presenting Cells

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Running title: Ov-ASP-1, a Th1-biased innate protein adjuvant

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Abstract

We previously reported that r*Ov*-ASP-1, a recombinant *Onchocerca volvulus* activation associated protein-1, was a potent adjuvant for recombinant protein or synthetic peptide-based antigens. In this study, we further evaluated the adjuvanticity of r*Ov*-ASP-1 and explored its mechanism of action. Consistently, recombinant full-length spike protein (rS) of SARS-CoV or its receptor-binding domain (rRBD) in the presence of r*Ov*-ASP-1 could effectively induce a mixed but Th1-skewed immune response in immunized mice. It appears that r*Ov*-ASP-1 primarily bound to the antigen-presenting cells (APCs) among human peripheral blood mononuclear cells (PBMCs) and triggered Th1-biased pro-inflammatory cytokine production probably via the activation of monocyte-derived dendritic cells (DCs) and the toll-like receptor TLR2 and TLR4, thus suggesting that r*Ov*-ASP-1 is a novel potent innate adjuvant.

Keywords: *Onchocerca volvulus*, innate adjuvant, vaccine, antigen-presenting cells, TLR-2; TLR-4, SARS-CoV

Introduction

Recently, recombinant proteins and synthetic peptides have been considered as alternative approaches for vaccine development, but their poor immunogenicity emphasizes a major unmet need for immunostimulatory adjuvants capable of safely boosting both humoral and cellular immune responses. Currently, alum remains the only adjuvant approved for human use in the majority of countries worldwide. Although alum is able to induce a good antibody (Th2) response, it has little capacity to stimulate strong cellular (Th1) immune responses which are so important for protection against many pathogens (1). In addition, alum has the potential to cause severe local and systemic side-effects that limit its wide application for various vaccines in humans.

Protozoan and helminth parasites have proven to be a source of molecules which have potent regulatory and, sometimes, stimulatory effects on the immune systems of their mammalian hosts (1-4). Some of these molecules were shown to contain pathogen associated molecular patterns (PAMP) that bind to endocytic-pattern recognition receptors (PRRs) on antigen-presenting cells (APCs). Several helminth products were also reported to act as adjuvants in experimental vaccine models, but they preferentially induced Th2-type immune responses (5-8). For example, proteins secreted by adult *Nippostrongylus brasiliensis* (NES) induced strong Th2 responses in mice immunized with hen egg

lysozyme (5). Similarly, lacto-*N*-fucopentaose III (LNFPIII), a carbohydrate found on the surface of the eggs of a human parasite, *Schistosoma mansoni*, acted as a Th2 adjuvant for human serum albumin when injected intranasally, subcutaneously or intraperitonealy into mice (6, 7). In a recent report, a 19 aa synthetic peptide (GK-1) from *Taenia crassiceps* cysticerci was shown to also have a capacity of an adjuvant (8). When it was co-administered with the inactivated anti-influenza vaccine in both young and aged mice, it induced increased levels of anti-influenza antibodies in aged mice before and after infection, reduced the local inflammation that accompanied influenza vaccination itself and favored virus clearance after infection in both young and aged mice.

Activation-associated secreted proteins (ASP) of parasitic nematodes are highly immunogenic and have been extensively studied as potential vaccine components against their corresponding parasites, with the ASPs from hookworms being the major vaccine candidates tested in human trials (9-15). Like other filarial nematodes, *Onchocerca volvulus* worms secrete a large number of immunoregulatory molecules to subvert the immune responses and minimize severe pathology and thus enabling their ability to establish a chronic infection in humans that can last more than 15 years (16). The native ASP-1 protein of *O. volvulus* (*Ov*-ASP-1) is located in the secretory granules of the glandular oesophagus of the infective third-stage larvae (L3) and is predicted to have multiple immunoregulatory functions (2, 17). Our previous studies demonstrated that recombinant *Ov*-ASP-1 (r*Ov*-ASP-1) is not only a protective antigen in vaccinated mice against *O. volvulus* L3s, but also a potent adjuvant for bystander proteins (2, 17-19). In ovalbumin (OVA)-immunized mice, r*Ov*-ASP-1 exceeded the efficacy of alum or MPL+TDM adjuvants in terms of end-point total IgG or IgG1 and IgG2a antibody titers (18). Interestingly, IgG isotype responses to OVA were of a mixed Th1/Th2-associated antibody profile but with a Th1 dominance and the spleen cell cytokines were exclusively of the Th1-type (18). We have also shown that although rOv-ASP-1 induced similar level of IgG1 responses as alum when formulated with HBsAg as the bystander vaccine protein, it clearly induced significantly higher levels of IgG2a and IFN-g-producing T cell responses against HBsAg than alum. Furthermore, Ov-ASP-1 improved both IgG1 and IgG2a responses to three commercial inactivated vaccines ((haemorrhagic fever with renal syndrome (HFRS), Influenza and Rabies) when used separately or in combination (20). In comparison with the other helminth-derived adjuvants shown to be strong inducers of Th2 responses to bystander proteins in vaccines (5-7, 21), the property of r*Ov*-ASP-1 to induce both Th1 and Th2-associated antibody responses and depending on the antigen also a Th1-biased antibody and cellular responses highlights its potential utility as an

effective adjuvant against many existing and emerging pathogens, for whom the protective responses are of the Th1 type.

In this study, we further evaluated the adjuvanticity of r*Ov*-ASP-1 and explored its mechanism of action. Consistently, r*Ov*-ASP-1 effectively boosted recombinant SARS-CoV spike protein (rS) or its receptor-binding domain (rRBD) to induce a mixed but Th1-skewed immune response in the immunized mice. It appears that r*Ov*-ASP-1 bound primarily to the APCs among human peripheral blood mononuclear cells (PBMCs) and triggered Th1 biased pro-inflammatory cytokines probably via the activation of monocyte-derived dendritic cells (MoDCs) and the toll-like receptor TLR2 and TLR4, thus suggesting that rOv-ASP-1 is a novel potent innate adjuvant.

Materials and Methods

Preparation of recombinant Ov-ASP-1

The recombinant *Ov*-ASP-1 protein (r*Ov*-ASP-1) was expressed as a histidine-tagged protein in *E-coli* and purified as previously described (18). The purified r*Ov*-ASP-1 was tested negative in a *Limulus* amoebocyte lysate assay. A quantitative LPS testing by Cambrex Bio Science (Baltimore, MD, USA) showed that purified r*Ov*-ASP-1 contained less than 0.25 endotoxin units per milligram of the protein (25 pg endotoxin/mg). We considered it as an LPS-free stock and used it in all described experiments.

Immunization of mice

Mouse animal protocols were approved by the Institutional Animal Care and Use Committee at the New York Blood Center. The recombinant full-length S protein (rS) and its receptor-binding domain (rRBD) of SARS-CoV Urbani (accession number AY278741) were expressed by recombinant baculovirus system as previously described (22). Four BALB/c mice or five C57BL/6 (6 wks old) per group were subcutaneously immunized with 20 μg of rS or rRBD resuspended in PBS plus 25 μg r*Ov*-ASP-1 or MLP+TDM adjuvant as recommended (Sigma, Saint Louis, MO) and boosted with 10 μg of the same antigen with the corresponding adjuvant or PBS at 3-wk intervals. In a previous study (18) we found that 2.5 □g/mouse of r*Ov*-ASP-1 adjuvant was not significantly active for antibody responses, 10 □g/mouse gave adjuvanticity intermediate to the final dose that we have chosen to use (25 □g/mouse) and 50 □g/mice was not better than 25 □g/mouse. The source of r*Ov*-ASP-1 in this study is the same as that used in our previous studies and therefore we also used 25 □g/mice in this study. Pre-immune sera were collected prior to

immunization and antisera were collected 7 days after each boost. Sera were kept at 4□ before use.

Enzyme-linked immunosorbent assay (ELISA)

The reactivity of mouse antisera with rS protein was determined by ELISA. Briefly, 1 μg/ml rS protein was used to coat 96-well microtiter plates (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4 □ overnight. After blocking with 2% non-fat milk, serially diluted mouse sera were added and incubated at 37 □ for 1 h, followed by four washes with PBS containing 0.1% Tween 20. Bound antibodies were detected with goat anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 antibodies (Sigma) as previously described (18). Then, biotinylated rabbit anti-goat IgG and extravidin peroxidase conjugate (both from Sigma, 1:2000 dilution for 1 h at 37□) were added sequentially. After washes, the reaction was visualized by addition of the substrate 3,3',5,5'-tetramethylbenzidine (TMB) and the absorbance at 450 nm was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC).

SARS pseudovirus and neutralization assay

A SARS-CoV pseudovirus system was developed in our laboratory as previously described (23, 24). In brief, HEK293T cells were co-transfected with a plasmid encoding the S protein corresponding to SARS-CoV Tor2 isolate and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE) by using FuGENE 6 reagents (Boehringer Mannheim). Supernatants containing SARS pseudovirus were harvested 48 h post-transfection and used for single-cycle infection of human or civet ACE2-transfected 293T (293T/ACE2) cells. Briefly, 293T/ACE2 cells were plated in 96-well tissue-culture plates at 10^4 cells/well and grown overnight. The supernatants containing SARS pseudovirus were preincubated with serially diluted antisera from vaccinated mice at 37 □ for 1 h before adding to the 293T/ACE2 cells. The cultures were fed with fresh medium 24 h later and then incubated for an additional 48 h. The cells were washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega). Relative light units (RLU) were determined immediately in the Ultra 384 luminometer (Tecan US). Values are presented as the antibody titers that provide 50% neutralization of rS binding to ACE2.

Stimulation of mouse splenocytes and measurement of cytokines

Spleens were harvested from the immunized mice and single-cell suspensions of splenocytes were obtained from the pooled spleens by mincing through cell strainers. Erythrocytes were lysed with ammonium chloride solution. Splenocytes were resuspended in complete RPMI 1640 medium to 1 X 10^6 cell/ml and 0.2 X10^5 were plated to 96-well U bottom plates for culture. The cells were stimulated with 1 μg/ml rRBD protein for 24 h. Cell culture supernatants were collected after stimulation and mouse cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and TNF-α) were quantified by Cytometric Bead Array (CBA) kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol.

IFN-γ ELISPOT assay

Specific IFN-γ -producing cells from the splenocytes of vaccinated mice were detected by BD™ ELISPOT Mouse IFN-γ ELISPOT Set according to the manufacturer's protocol (BD Biosciences). In brief, 96-well ELISPOT plates were coated with anti-IFN-γ monoclonal antibody (mAb) overnight at 4 □, and blocked by the Blocking Solution (RPMI-1640 containing 10% FBS). Single-cell suspensions prepared from the spleens of vaccinated mice were added to the wells at the concentration of 2 x 10^5 cells/well. Cells were incubated for 24 h in the presence or absence of the rRBD (1μg/ml), followed by washes with PBS. The cells were sequentially incubated with biotinylated anti-mouse IFN-γ mAb for 2 h at room temperature (RT), streptavidin-conjugated horseradish peroxidase (HRP) for 1 h at RT and 3,3',5,5'-tetramethylbenzidine (TMB) substrate solutions for around 15 min, with extensive washes between incubations. The spots of IFN-γ producing cells were counted by ELISPOT reader system.

Flow cytometry-based binding assay

r*Ov*-ASP-1 and a similarly expressed control protein, chloramphenicol acetyl transferase (CAT), were labeled with biotin using the FluoReporter® Mini-biotin-XX Protein Labeling Kit (Molecular Probes). Fresh healthy donor PBMCs (N = 4) were isolated by density gradient centrifugation over Ficoll (Sigma). Five μg of biotinylated r*Ov*-ASP-1 or CAT were then incubated with 1x10^6 human PBMCs at 37 □ for 30 min. After washing the cells at 4□ the cells were incubated with streptavidin FITC (Amersham Pharmacia Biotech, Uppsala, Sweden) for 20 min followed by 30 min incubation with PE or APC-labeled antibodies (BD Pharmingen) directed against surface markers on PBMCs (T-cells, monocytes, natural killer (NK) cells and B cells). As controls for non-specific binding, r*Ov*-ASP-1-biotin labeled cells were incubated with appropriate APC or PE labeled isotype and

species-matched control antibodies. After 3 washes the cells were resuspended in FACS buffer and analyzed using 2-3 color FACS analysis using a Becton Dickinson FACS CANTO flow cytometer and the DiVa software (NYBC FACS facility services).

Stimulation of human naïve PBMCs by rOv-ASP-1

Fresh human naïve PBMCs were isolated by density gradient centrifugation over Ficoll (Sigma). For cytokine production, PBMCs (N =14) were cultured in quadruplicate in round-bottomed 96-well culture plates at 4 x 10^5/well in RPMI-1640 medium containing 10% FBS, 25 mM HEPES, 2 mM L-glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin (all from Sigma). The cells were cultured for 5 days in the presence of r*Ov*-ASP-1 (5 μg/ml) with or without polymyxin B at 37 □ in a humidified 5% CO_2 incubator. In preliminary experiments, 5 μg/ml of LPS-free r*Ov*-ASP-1 was established as an optimal dose for cytokine stimulation. For control purposes, we also treated PBMCs with recombinant CAT (5 μg/ml). In previous studies we have established that CAT and other recombinant *O. volvulus* proteins expressed similarly in *E. coli* did not have any immunostimulatory activity. Cytokines (IFN-γ, TNF-α, IL-4, IL-5, IL-10) in the cell culture supernatants were measured using a human Th1/Th2 CBA kit (BD Biosciences) according to the manufacturer's protocol. To examine the potential involvement of the TLRs on the immunostimulatory activity of r*Ov*-ASP-1 on PBMCs, we tested the inhibitory activity of anti-TLR antibodies (TLR2 and TLR4) on the IFN-γ secretion from PBMCs stimulated by r*Ov*-ASP-1. PBMCs were pre-incubated with 10 μg/ml of Goat anti-TLR2, -TLR4 or -IL-4 antibodies (eBiosciences) at 37□ for 1 hr before adding r*Ov*-ASP-1 (5 μg/ml) to each treated well. Anti-IL-4 antibodies served as unrelated control antibody and untreated wells served as the positive control for maximum IFN-γ secretion in the presence of r*Ov*-ASP-1. The concentration of IFN-γ in the culture supernatants was tested by ELISA (25). Amounts of secreted cytokine are expressed in pg/ml per cultured wells.

Stimulation of MoDCs with rOv-ASP-1

Blood samples were collected from uninfected normal healthy donors (NHD) with no known risk factors for blood-borne virus infection (11 were male and 3 were female and the median age was 52). Ethical approval was obtained from Southampton and South-west Hampshire Joint Research Ethics Committee and all volunteers gave informed consent in writing prior to participating in the study. Fifty ml of freshly drawn blood was obtained from 14 NHD subjects. Blood was collected into K_3-ethylenediamine tetraacetic acid (EDTA) and separated immediately by centrifugation over Lymphoprep (Robbins

Scientific, Solihull, UK). PBMCs were recovered and monocytes then isolated using the MACS $CD14^+$ isolation kit (Miltenyi Biotec, Bisley, UK), according to the manufacturer's recommended protocol. $CD14^+$ cells were positively selected from PBMC using an AutoMACS machine (Miltenyi Biotec). Monocytes (10^6 cells/ml) were cultured in six-well plates (Greiner Bio-One, Stonehouse, UK) in 3 ml per well of complete RPMI medium [RPMI-1640 without phenol red (Invitrogen, Paisley, UK), with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, Poole, UK) and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Perbio Science UK Ltd, Tattenhall, UK)], containing 50 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (rhGM-CSF) and 1000 IU/ml rhIL-4 (both from R&D Systems, Abingdon, UK). After 2 days and 5 days 0.5 ml of culture medium was replaced with fresh cytokines. The day 5 immature DCs (2×10^5) were then cultured in complete media [RPMI-1640 without phenol red (Invitrogen, Paisley, UK), with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, Poole, UK) and 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Perbio Science UK Ltd, Tattenhall, UK)] alone or with 5 µg/ml r*Ov*-ASP or 100 ng/ml LPS in 48-well cell culture plates. After 24hrs of stimulation, DCs were stained for two-color flow cytometry (FACSCalibur dual laser flow cytometer, BD Biosciences) and analyzed using CellQuest software (BD Biosciences) and the supernatants were measure for cytokine secretion. For each condition, cells were resuspended and incubated on ice for 30 min in 50 µl wash buffer [phosphate-buffered saline (PBS)/0·05% NaN_3/0·5% bovine serum albumin (BSA)] containing 100 µg/ml human Fcγ fragments (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) ('blocking buffer'). Cells were then stained with 50 µl of antigen-presenting cell (APC)-labeled anti-human CD83, CD86, CD40 (BD Biosciences) or CD303-APC (BDCA2; Milltenyi Biotec Bisley, UK) or fluorescein isothiocyanate (FITC)-labeled anti-human HLA-DR (BD Biosciences), all diluted to their optimal concentrations in blocking buffer or APC- or FITC-labelled isotype controls (murine IgG1 and IgG2b; BD Biosciences) at equivalent dilutions. After staining cells were washed, then fixed in 1% formaldehyde and stored on ice in the dark pending analysis. Using two-color analysis, the presence of the surface markers, HLA-DR, CD83, CD86 and CD40 on immature and mature MoDCs was determined by collecting ≥ 40 000 events in the total cell population. All positive staining was compared with appropriate isotype controls. Multiple cytokines in the 24 h culture supernatants from the stimulated MoDCs were also measured using a human Th1/Th2 Cytometric Bead Array (BD Biosciences, San Jose, CA).

To measure cytokine in response to CD40 ligation, 2 × 10^5 washed immature or TNF-α-matured MoDCs were added to 2 × 10^5 human CD40 ligand-transfected Chinese hamster ovary cells (hCD40L-CHO) or wild-type cells (wt-CHO) (both gifts from Professor M. Glennie, Tenovus Laboratories, Southampton, UK) adhering to the wells of 48-well cell culture plates in a final culture volume of 0.5 ml complete RPMI-1640 medium with or without 5 µg/ml r*Ov*-ASP or 100 ng/ml LPS. Further wells contained MoDCs, hCD40L-CHO or wt-CHO cells alone. After 24 h, supernatants were harvested and centrifuged for 10 min at 2500 *g*, then frozen pending further analysis using a human Th1/Th2 Cytometric Bead Array as described above.

Mixed lymphocyte reaction assay (MLR)

To establish if r*Ov*-ASP was able to enhance MoDC antigen-presenting capacity a mixed lymphocyte reaction assay (MLR) was performed and proliferation was measured by H-thymidine incorporation. Day 5 MoDCs isolated from 5 NHDs were cultured (1 x 10^5) in complete medium supplemented with IL-4 and GM-CSF with or without r*Ov*-ASP (5µg/ml) for 24-48 h. DCs were then thoroughly washed before adding to naive $CD4^+$ T cells to ensure all r*Ov*-ASP was washed off. As a result, the T cell response will be detected if presented by the stimulated DCs. As responder cells in the MLR, naive $CD4^+$ T cells were isolated by negative selection from 5 allogeneic normal healthy donors using a $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer's recommended protocol. Purified $CD4^+$ T cells (2 x 10^5) were co-cultured in triplicates with MoDCs at various DC:T cell ratios (1:20—1:80) in U bottom 96-well plates in complete RMPI-1640 for 5 days at 37 □, 5% CO_2. During the last 18 h of incubation, the cultures were pulsed with 1 µCi/ml ^{3}H-thymidine. Cells were then harvested and the incorporated tritium thymidine was measured on a β plate reader (Topcount, Packard, Groningen, Netherlands) to determine induction of the allogeneic naive CD4 T cell proliferation.

TLR2, TLR4 and TLR9 activation

Stable lines of human embryonic kidney cells (HEK) 293 expressing surface toll-like receptor (TLR) constructs (TLR2, TLR4 or TLR9) were obtained under Material Transfer Agreement from Dr. D. Golenbock (University of Massachusetts Medical School, Worchester, MA). These HEK cells naturally lack TLR2, TLR4, TLR9 expression and genetic complementation with TLR constructs renders the cells responsive to the respective TLR ligands (323;324). The cells were maintained in Dulbecco's modified Eagle medium (GIBCO Invitrogen, Carlsbad, USA) supplemented with 10 % fetal calf serum, 100 U/ml penicillin (PAA, Pasching, Austria), 0.1 µg/ml streptomycin (PAA) and

200 mM L-glutamine (Gibco Invitrogen) in a 5% saturated CO_2 atmosphere at 37□. Cells were plated at 5 x 10^4 cells per well in flat bottom 96-well tissue-culture plates and incubated for 3 h to allow adhesion. Cells were then stimulated with r*Ov*-ASP-1 or control TLR ligands (Pam3CysK4; a bacterial lipoprotein analog for TLR2; LPS for TLR4; or the synthetic DNA analog CpG-ODN2216 for TLR9) at 37□ for 18 h. The culture supernatants were collected for quantitation of IL-8 production using commercial ELISA kit (R&D systems). Values are expressed in pg/ml per well.

NF-κB-dependent reporter gene assay

HEK293 cells, either with or without stable transfection with hCD14, were maintained in media including sodium pyruvate and supplemented with 10 % fetal calf serum (FCS; Biochrom, Berlin, Germany), 100 U/ml penicillin (PAA, Pasching, Austria), 0.1 µg/ml streptomycin (PAA) and 200 mM L-glutamine (Gibco Invitrogen). Transient transfection of HEK293 cells was performed using FuGENE 6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instruction. Cells were plated in 12-well plates with 1 x 10^5 cells per well. When cultures reached 50% to 80% confluence, they were incubated overnight with plasmids containing NF-κB reporter luciferase (120 ng) and, to control for differences in transfection efficiency, with RSV-β-galactosidase (40 ng). Cells were co-transfected either with hTLR4 (2 ng) and hMD2 (40 ng), or with hTLR2 (40 ng). After 24 h, cells were stimulated with various amounts of r*Ov*-ASP-1 or control ligands in DMEM with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 200 mM L-glutamine. The control ligands for TLR2 were: Triacylated lipopeptides (Pam3CSK4; LP3) and diacylated lipopeptides (Pam2CSK4; LP2) (EMC Microcollections, Tübingen, Germany) as previously described (26). LPS (Sigma) was used as the ligand for TLR4. After 20 h of stimulation, luciferase activity was measured in cell extracts with the Luciferase Reporter Gene Assay kit (Roche Diagnostics) according to the manufacturer's instruction. Luciferase and β-galactosidase activity were estimated with chemiluminescence assays (Luciferase and β-Gal Reporter Gene Assay (Roche Diagnostics) and results were expressed as the ratio of luciferase to β-galactosidase and graphed as the mean +/- SD of triplicate experiments.

Statistical analysis

Paired *t*-tests and, where appropriate, Mann–Whitney tests were used to compare means. Analyses were performed using Prism version 4·0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

rOv-ASP-1 potentiates both Th1 and Th2-associated IgG2a and IgG1 responses

The adjuvanticity of r*Ov*-ASP-1 was firstly evaluated by using recombinant SARS-CoV S protein (rS) as a model antigen. Four Balb/C mice were immunized four times with the rS in the absence or presence of r*Ov*-ASP-1 or with a control adjuvant, MLP+TDM. As shown in Fig. 1A, both adjuvants, r*Ov*-ASP-1 and MLP+TDM, augmented the IgG antibody responses to rS protein versus (vs.) rS alone in terms of total IgG titers. Further comparison between the r*Ov*-ASP-1 and MLP+TDM-induced responses by IgG isotypes (Table 1) shows: 1) similar IgG1 (1:656,100); and 2) three-fold higher IgG2a (656,100 vs. 218,700) and three-fold higher IgG2b (218,700 vs. 72,900) end point titers in the presence of r*Ov*-ASP-1. Each adjuvant/antigen model performed differently depending on the adjuvant – a skewed Th2 response with MLP+TDM (IgG1/IgG2a=3), but a mixed Th1/Th2 (IgG1/IgG2a=1) response with r*Ov*-ASP-1 with a dominance of Th1 antibodies (IgG2a and IgG2b). These results indicate that r*Ov*-ASP-1 induced a more Th1 skewing antibody response than MLP+TDM. Moreover, when the neutralizing activities of the antibodies were analyzed (Fig. 1B), it appeared that the antisera from the mice immunized with rS in the presence of r*Ov*-ASP-1 contained similar levels of the neutralizing antibodies against infection by SARS pseudovirus (1/29,096) as those from the mice administered with rS plus MLP+TDM adjuvant (1/33,960). Therefore, r*Ov*-ASP-1 is a potent adjuvant for SARS-CoV rS protein and can induce functional antiviral antibodies.

rOv-ASP-1 effectively induced both humoral and Th1- dominant cellular immune responses against the rRBD of SARS-CoV S protein

We have previously shown that the RBD of SARS-CoV is a major target of neutralizing antibodies (22, 27-29) and that recombinant RBD-based vaccines can induce protective immunity in the immunized animals (23, 30-33). Here, the recombinant RBD was further used to test the adjuvanticity of r*Ov*-ASP-1 in comparison with the MLP+TDM adjuvant in C57BL/6 mice. As shown in Fig. 1C, both immunization protocols elicited similar high titers of antibodies against the rS protein after three vaccinations. The pooled splenocytes from each group of immunized mice were stimulated with the rRBD (1 μg/ml) as a recall antigen and the production of cytokines were measured by CBA kit (Table 2). In previous vaccines studies using r*Ov*-ASP-1 as the adjuvant and rS or rRBD as the bystander antigen (32, 34), we found that the variation between individual mice was very low and therefore we are confident that that the results we obtained using the pooled spleens are a good representation of what would have been the outcome if we have had used

individual mice. Notably, r*Ov*-ASP-1 exceeded the efficacy of MPL+TDM adjuvant in boosting the production of Type I proinflammatory cytokines (IL-2, IFN-γ, TNF-α, and IL-6). Secretion of the Th1/regulatory cytokine IL-10 was elevated but less than that induced by MPL+TDM. There was no significant recall induction of the IL-4 or IL-5 cytokines by rRBD. The highly dominant IFN-γ recall response was further supported by enumerating the number of the IFN-γ producing cells in splenocytes from the vaccinated mice cultured for 24 h post-in the presence of rRBD. As shown in Fig. 2, RBD/ASP-1 vaccination induced significantly a higher frequency of the IFN-γ-producing cells than those of RBD/MPL+TDM and PBS groups ($P<0.01$). These results suggested that r*Ov*-ASP-1 is a potent adjuvant inducing not only strong humoral response but also an RBD specific Th1-biased cellular response.

rOv-ASP-1 binds primarily to antigen-presenting cells among human PBMCs

Our data have clearly demonstrated that r*Ov*-ASP-1 is a potent vaccine adjuvant that induces a Th1-dominant cellular response and depending on the bystander antigen we also found that it can elicit a dominant Th1-associated antibody response (18, 20). To explore the possible mechanism of r*Ov*-ASP-1 adjuvanticity, we analyzed first the binding profile of biotinylated r*Ov*-ASP-1 with human PBMCs (N = 4) by flow cytometry (Fig. 3). We found that biotin-labeled r*Ov*-ASP-1 bound mostly to monocytes (93-98% of CD14+ or 87-90% of CD3-CD4+ ^{low}) and to B cells (41-94% of CD19+). A small fraction of other cells also bound r*Ov*-ASP-1: 1-3% CD4+ (CD3+CD4+^{high}), 3.9-8% of $CD8^+$ T cells and 0.5-9% of NK cells. As a control, biotinylated-CAT did not bind to more than 2% of any leukocyte population (data not shown). These results demonstrated that r*Ov*-ASP-1 primarily binds to the APCs, monocytes and B cells, among human PBMCs, suggesting that r*Ov*-ASP-1 may interact with the specific receptors presented on the surface of the APCs to activate the immune cells.

rOv-ASP-1 stimulated human PBMCs to produce proinflammatory cytokines

We then determined whether r*Ov*-ASP-1 can trigger cytokine production of human PBMCs from normal healthy donors. As shown in Fig. 4, r*Ov*-ASP-1 protein stimulated significant production of Th1-type cytokines, IFN-γ and TNF-α, and the immunoregulatory T cell cytokine, IL-10. No significant Th2 cytokines (IL-4 or IL-5) were detected in the PBMC culture supernatants, although the IL-5 response was somewhat increased. Inhibition of LPS activity using polymyxin B (20 μg/ml) had no effect on the bioactivity of r*Ov*-ASP-1 on human PBMCs (Fig. 4F). In contrast, it appeared that the activity of the

recombinant control protein, CAT, was due to contamination by LPS, as its cytokine-inducing activity could be abolished by polymyxin B. Therefore, the r*Ov*-ASP-1 protein appears to be a potent inducer of pro-inflammatory (IFN-γ and TNF-α) and immunoregulatory (IL-10) cytokine secretion from the normal human PBMCs.

rOv-ASP-1 induces phenotypic maturation of MoDCs and stimulates the secretion of proinflammatory and anti-inflammatory cytokines

It is well known that TLRs activate signal transduction cascades leading to expression of co-stimulatory molecules on DCs and the secretion of IL-12 following recognition of their respective ligands (35). To determine if r*Ov*-ASP-1 induced DC maturation, MoDCs from NHD (N = 14) were cultured with or without r*Ov*-ASP-1 at 5 µg/ml for 24 h. Using FACS analysis we measured expression of surface molecules that are characteristically upregulated in DCs after stimulation. As shown in Figure 5, all four phenotypic markers that were tested increased after treatment with r*Ov*-ASP-1. MoDCs from NHDs (N = 9-10) were then cultured with or without r*Ov*-ASP-1 (5 µg/ml) for 24 h and cytokine production was determined. Notably, r*Ov*-ASP-1 stimulated significant amounts of the proinflammatory cytokines, IL-6, IL-8, IL-2 and TNF-α but not IL-12 (Fig. 6). There was no significant induction of other cytokines tested IL-2, IL-4, IL-5 or IL-1ß but the MoDCs cultured with r*Ov*-ASP-1 significantly stimulated also the production of the anti-inflammatory cytokine, IL-10. Notably, the r*Ov*-ASP-1 induced secretion of IL-12 was CD40-dependent; significant enhancement of IL-12 secretion was observed only after providing the MoDCs with co-stimulation through the CD40-CD40L pathway (i.e. by including CD40L-expressing CHO cells). Co-stimulation through the CD40-CD40L pathway also enhanced the r*Ov*-ASP-1 induced secretion of IL-10 and TNF-□(Fig. 6; immature + CD40L vs. rOv-ASP-1 + CD40L). These results provide evidence that r*Ov*-ASP-1 may initiate DC-induced adaptive immune responses by multiple distinct mechanisms.

To assess if r*Ov*-ASP-1 treatment of MoDCs was also able to enhance their accessory/antigen presentation function, we used the mixed lymphocyte reaction (MLR) assay with allogeneic antigens. Figure 7A shows the results of the MLR experiments indicating that MoDCs treated with 5ug/ml of r*Ov*-ASP-1 enhanced their ability to stimulate proliferation of allogeneic $CD4^+$ T cells. Moreover, r*Ov*-ASP-1 primed DCs were able to induce IFN-γ secretion from naïve PBMCs (Fig. 7B). The ability of r*Ov*-ASP to stimulate human DCs further substantiates the prospect of its use as an innate vaccine adjuvant in humans that can boost the activation of an adaptive immune response.

The rOv-ASP-1-induced IFN-γ secretion is TLR2 and TLR4 dependent

Previous studies have indicated that signaling from TLR on APCs after ligand binding can induce subsequently the production of pro-inflammatory cytokines from T cells and NK cells and provide help to B cells, and thus this mechanism is thereby thought to contribute considerably to the enhanced antibody and cellular-enhancing effects of innate vaccine adjuvants (36, 37). The secretion of IFN-□ by T cells and NK cells is mostly induced by IL-12, which is primarily produced by DCs and macrophages (38-40). Therefore, we investigated whether r*Ov*-ASP-1 can activate TLR2 and/or TLR4 presented on the surface of APCs and thereby result in pro-inflammatory cytokine production. To test this hypothesis, PBMCs from three healthy donors were treated with r*Ov*-ASP-1 in the absence or presence of anti-TLR2, anti-TLR4 or anti-IL-4 antibodies, and the secretion of IFN-γ was then measured by ELISA. As shown in Fig. 8, the IFN-γ secretion was significantly inhibited with both anti-TLR2 and anti-TLR4 antibodies and not with anti-IL-4 antibodies, suggesting that the r*Ov*-ASP-1-induced IFN-γ secretion from human PBMCs is TLR2 and TLR4 dependent.

rOv-ASP-1 triggered cellular activation via TLR2 and TLR4

The above results suggest that the r*Ov*-ASP-1-induced pro-inflammatory cytokine production from human PBMCs might be dependent on TLR2 and TLR4 activation. We then used the stable lines of HEK293 cells expressing TLR2, TLR4 or TLR9 to test its potential to activate TLR directly. Each of the cell lines was respectively incubated with r*Ov*-ASP-1 at the indicated amounts and the TLR activation was expressed by human IL-8 production. As shown in Fig. 9, the specific control ligands for TLR2, TLR4 or TLR9 stimulated the cells expressing the appropriate receptors, while r*Ov*-ASP-1 activated both TLR2 and TLR4-expressing cells at dose-dependent manners but not TLR9. The wild-type HEK293 cells, which do not expression TLR2 and TLR4, were unresponsive to the r*Ov*-ASP-1 (data not shown) in the IL-8 release assay.

The r*Ov*-ASP-1 cellular activation via TLR2 and TLR4 was further determined by a more sensitive NF-κB-dependent reporter gene assay. Consistently, r*Ov*-ASP-1 strongly induced luciferase activity in the TLR2 or TLR4-transfected HEK293 cells in a dose-dependent manner (Fig. 10). These data strongly support the view that both TLR2 and TLR4 are involved in transducing r*Ov*-ASP-1 stimulation.

Discussion

The identification of new adjuvants that stimulate both antibody and cellular responses is an important challenge for vaccine development. In particular, there is a demand for safe and non-toxic adjuvants able to stimulate cellular (Th1) immunity for fighting against the existing and emerging pathogens. We have previously shown that r*Ov*-ASP-1 has potent adjuvant effects for several vaccine antigens including recombinant proteins, synthetic peptides and commercial inactivated vaccines (18, 20). Importantly, r*Ov*-ASP-1 could induce a mixed Th1/Th2 response with a Th1-biased antibody profile against some of the bystander antigens in the immunized mice. In the present work, the adjuvanticity of r*Ov*-ASP-1 was further evaluated in comparison with MPL+TDM adjuvant using the recombinant SARS-CoV S and RBD proteins, which have been shown to induce in the presence of the MLP+TDM adjuvant potent neutralizing antibodies and have thereby been considered as an ideal SARS vaccine candidates (22, 30). Our present study has shown that r*Ov*-ASP-1 also was highly effective in eliciting functional antibody responses in immunized mice. Its adjuvanticity to the SARS-CoV S protein exceeded the MPL+TDM adjuvant, especially in the induction of Th1 associated IgG2a and IgG2b antibody isotype responses. Furthermore, we have demonstrated that *Ov*-ASP-1 is a potent adjuvant also for the induction of Th1-biased cellular response. Impressively, immunization with the rRBD protein and r*Ov*-ASP-1 significantly promoted the IFN-γ and TNF- production in the immunized mice. These results further confirmed that r*Ov*-ASP-1 can be used as a potent vaccine adjuvant for the recombinant protein-based vaccine antigens which are generally far less immunogenic than old fashion vaccines consisting of live or killed whole organisms.

The molecular mechanism underlying adjuvant activity has been poorly understood for a long time, but this field has been rapidly evolving since the discovery of the TLR family of proteins and their corresponding innate ligands. TLRs are prominent pattern recognition receptors (PRR) of the innate immune system recognizing various invading pathogens through conserved motif named PAMPs (36). Interaction of PAMPs with TLR on the surface of APCs initiates a signaling cascade resulting in the recruitment of adaptor proteins such as the myeloid differentiation factor 88 (MyD88) and the downstream activation of NF-κB and mitogen-activated protein kinases (MAPK) (35). Consequently, the TLR signaling stimulates the activation and maturation of APCs including the regulated presentation of antigens, up-regulation of costimulatory molecules and secretion of proinflammatory chemokines and cytokines. These events mediate not only innate but ultimately also adaptive immunity (37, 41). Vaccinations with adjuvants

that mimic TLR ligands are advantageous as they are capable of eliciting positive effects across the entire spectrum of innate and adaptive immunity. Therefore, innate immune signals mediated by TLRs have been thought to contribute significantly to the antibody-enhancing effects of vaccine adjuvants as well as their ability to elicit cellular protective immunity. Previous studies have demonstrated that human parasite-derived products can also stimulate the immune responses via the TLR pathways. For example, a schistosome-specific phosphatidylserine was shown to activate TLR2 and affect DCs such that mature DCs gained the ability to induce the development of IL-10-producing regulatory T cells (42). The *T. gondii* profilin activate DCs through TLR11, which was required for parasite-induced IL-12 production and optimal resistance to infection *in vivo* (1). Furthermore, the adjuvant candidate lacto-*N*-fucopentaose III (LNFPIII) from *S. mansoni* acted as an innate Th2 adjuvant via TLR4 signaling (43, 44).

To explore the mechanism of r*Ov*-ASP-1 adjuvanticity, we initially analyzed the binding profile of r*Ov*-ASP-1 with normal human PBMCs by a flow cytometry-based assay. It was found that r*Ov*-ASP-1 binds primarily to the human monocytes and B cells, the major APCs in the peripheral blood. Significantly, r*Ov*-ASP-1 stimulated human PBMCs to produce Th1-type pro-inflammatory cytokines (IFN-γ and TNF-α) and the immunoregulatory IL-10 cytokine. It has been known that IFN-γ is mainly released by activated Th1 cells and NK cells while TNF-α is mainly produced by macrophages but also by a broad variety of other cell types including Th1 cells. Both cytokines are involved in the regulation of the immune and inflammatory responses. Recently, we have shown that r*Ov*-ASP-1 induced IFN-γ secretion from purified CD56+ cells but not from CD4+ or CD8+ cells (25). Moreover, this immunostimulatory effect was dependent on contact between CD56+ and CD56- fractions of PBMC, most likely between APCs, including DCs, in the CD56- fraction.

Interestingly, r*Ov*-ASP-1 also induced significant IL-10 secretion from naïve human PBMCs. This cytokine is mainly expressed by monocytes and Th2 cells and is capable of inhibiting synthesis of proinflammatory cytokines like IFN-γ, IL-2, TNF-α and GM-CSF made by cells such as macrophages and the Th1 cells. As r*Ov*-ASP-1 could not stimulate human PBMCs to produce Th2-type IL-4 and IL-5 cytokines, it is possible that a monocytes subpopulation is responsible for the secretion of IL-10. Initial studies of PBMCs vs. monocytes have indicated that monocytes secrete IL-10 in the presence of r*Ov*-ASP-1 through an unknown as yet mechanism. Based on the profile of cytokines stimulated by r*Ov*-ASP-1 we hypothesize that the *Ov*-ASP-1 protein (~23 kDa) may contain few putative Th1 PAMPs that bind to different receptors on APCs that activates

differently the secretion of Th1 cytokines and IL-10 through distinct pathways. Innate recognition of PAMP signals APCs, typically human monocyte-derived dendritic cells (DCs), to express costimulatory molecules and secrete cytokines, which drive the polarization of naïve CD4+ Th type 1 and type 2 cells toward the Th1 or Th2 phenotype (45). Th1 cells express IFN-γ and TNF-α that instruct B cells to produce antigen-specific IgG2a, whereas Th2 cells produce IL-4, IL-5 and IL-13 to promote IgG1 and IgE class switching.

Human monocyte-derived DCs treated with the parasite protein r*Ov*-ASP-1 have shown evidence of phenotypic maturation, enhanced cytokine production and enhanced allo-stimulatory function. These findings confirm the immunostimulatory properties of this protein are mediated by its ability to activate APC population. The ability of r*Ov*-ASP to stimulate human DCs further substantiates the prospect of it use as an innate vaccine adjuvant in humans that can boost the activation of an adaptive immune response. The ability of r*Ov*-ASP-1 to stimulate human DCs may enable it to be used as a human therapeutic. In the case of chronic HCV infection, where DC function has been reported to be impaired by the virus, r*Ov*-ASP-1 could enhance DC-driven T cell anti-HCV responses that would shift the virus-host equilibrium in favor of viral clearance. In support of this, recent *in vitro* evidence showed that r*Ov*-ASP-1 was able to enhance and/or induce anti-HCV Core IFN-γ responses in patients with chronic HCV infection (25).

The adjuvant activity of r*Ov*-ASP-1 appears to be mediated by TLR signaling presented on the APCs. In agreement with this hypothesis, the IFN-γ secretion from human PBMCs could be significantly inhibited by both anti-TLR2 and anti-TLR4 antibodies. Consistently, r*Ov*-ASP-1 also triggered the activation of HEK293 cells transfected with TLR2 and TLR4 but not with TLR9 in dose-dependent manners. Previous studies indicate that each TLR mediates the response to specific PAMPs shared by a set of molecular structures (41). Among the TLRs, TLR2 mediates the response to the most diverse set of molecular structures including lipoproteins, peptidoglycan and lipoteichoic acids, while it is generally accepted that TLR4 mainly responds to LPS from Gram-negative bacteria. But, they share a common activation pathway mediated through their Toll-IL-1R (TIR) signaling domain resulting in activation of NF-κB and MAPK. Previous results have also demonstrated that a number of pathogen-derived PAMPs-containing ligands could simultaneously activate TLR2 and TLR4 (46-48), such as the major surface protein of *Wolbachia* endosymbionts in filarial nematodes (325) and the glycosylphosphatidylinositols derived from *T. gondii* (46). Structural homology analyses of *Ov*-ASP-1 with the crystallized closely-related hookworm secreted protein *Na*-ASP-2

suggest that *Ov*-ASP-1 has three distinct functional domains (49). It is possible that such distinct subdomains of *Ov*-ASP-1 may contain distinct bioactive sites. If this is in case, we may be able to express a subunit that stimulates IFN-γ without concurrent IL-10 secretion or specially activates TLR2 or TLR4 pathways. To further corroborate the involvement of TLR2 and TLR4 in rOv-ASP-1 bioactivities and thus back our indirect studies of anti-TLR2 and anti-TLR4 antibodies, additional studies using TLR2 and TLR4 knockout mice are needed. These would include testing if the adjuvanticity of r*Ov*-ASP-1 is lost in these mice and if innate cytokines production and DC maturation is reduced.

In conclusion, r*Ov*-ASP-1 has potential to be developed as a novel innate vaccine adjuvant since it can bind to APCs, matures and activates DCs and subsequently induces proinflammatory cytokines leading to a Th1-dominant immune response, potentially via TLR2 and TLR4 activation.

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Figure legends

Fig. 1. Antibody responses of mice immunized with recombinant SARS-CoV S protein or its RBD in the absence or presence of the r*Ov*-ASP-1 or the MLP+TDM adjuvant. (A) Reactivity of mouse antisera with rS protein. The antisera were collected from mice before immunization and 7 days after each boost and tested by ELISA at 1/1000 dilution. (B) Neutralization of SARS pseudovirus infection by mouse antisera from each rS immunization group. Infection of 293T/ACE2 cells by SARS pseudovirus was determined in the presence of antisera at a series of 2-fold dilutions, and 50% neutralization was calculated for each sample. * indicate $P<0.01$ compared to the group of rS alone. (C) Titers of anti-rS antibodies from the rRBD-immunized mice. The antisera were tested by ELISA at a series of dilution.

Fig. 2. Detection of IFN-γ-producing cells by ELISPOT. Splenocytes from vaccinated mice were stimulated with SARS-CoV rRBD. Frequencies of IFN-γ-producing cells are expressed as mean ± SE of cytokine spot-forming cells (SFC)/10^6 cells of 5 independent experiments. * indicates $P<0.05$ compared to RBD/MPL+TDM or PBS group.

Fig. 3. Binding of biotinylated r*Ov*-ASP-1 to human PBMC as determined by flow cytometry. Cells were gated on lymphocytes (R1) (a). The isotype specific antibodies to IgG-PE or IgG-APC were used as control (b and c). Cells were stained with rOv-ASP-1 biotin-Streptavidin FITC and for T cells with CD3/CD4/CD8(d-f), for monocytes with CD14 (g), for B-cells with CD19 (h) and for NK cells with CD56 (i). Figure 4 is a representative of the binding profile of PBMCs from one normal individual.

Fig. 4. Cytokine secretion of human PBMCs stimulated by r*Ov*-ASP-1. Human PBMCs were collected from normal healthy donors (n=14), and treated with or without r*Ov*-ASP-1 (5 μg/ml) for 5 days. The culture supernatants were tested by human CBA kits for cytokines (a) IFN-γ; (b) TNF-α; (c) IL-4; (d) IL-5; (e) IL-10. * indicate $P<0.01$ compared to untreated control wells. (f) IFN-γ and IL-10 secretion of human PBMCs triggered by r*Ov*-ASP-1 or recombinant CAT in the presence or absence of polymycin B (20 μg/ml). The r*Ov*-ASP-1 induced cytokine secretion was not inhibited by polymycin B.

Fig. 5. Phenotypic maturation of MoDCs treated by r*Ov*-ASP-1. MoDCs (N = 14) were cultured with or without 5 μg/ml r*Ov*-ASP for 24 hours. Using FACS analysis the expression of maturation markers CD40, CD83, CD86 and HLA-DR were measured. All four of the maturation markers were upregulated after treatment with r*Ov*-ASP-1. LPS was used as a positive control.

Fig. 6. Cytokine production from r*Ov*-ASP-1 treated MoDCs with and without CD40L. MoDCs (N = 9-10) were cultured for 24 hours with media alone or in a 1:1 ratio with a CD40 ligand-transfected Chinese hamster ovary cells (hCD40L-CHO) with or without 5 μg/ml r*Ov*-ASP. LPS was used as a positive control. r*Ov*-ASP alone stimulated significant amounts of pro-inflammatory cytokines, IL-6, IL-8, IL-2 and TNF-α but not IL-12. However, when MoDCs were co-stimulated with CD40L, significant enhancement of IL-12 secretion was seen. MoDCs stimulated with r*Ov*-ASP significantly stimulated the production of the anti-inflammatory cytokine, IL-10 and this was further enhanced when MoDCs were co-stimulated with CD40L.

Fig 7. r*Ov*-ASP-1 treated MoDCs have enhanced allo-stimulatory function. (A) MoDCs treated with 5 μg/ml r*Ov*-ASP-1 showed enhanced ability to stimulate the proliferation of allogenic $CD4^+$ T cells. The $CD4^+$ T cell proliferation (counts per minute) in response to r*Ov*-ASP-1 is shown. Each point represents the mean (+ s.d.) of results from 5 individuals. **P<0.05.* (B) r*Ov*-ASP-1-primed human DCs induced IFN-γ secretion from naïve PBMCs. MoDCs were treated with 2 μg/ml r*Ov*-ASP-1 and then washed and co-cultured (1×10^4/well) with autologous normal PBMCs (2×10^5/well) in round-bottomed 96-well plate for 5 days. The level of IFN-γ secretion was determined by ELISA. Streptolysin O (SLO) served as a positive immunopotentiator.

Fig. 8. Inhibition of r*Ov*-ASP-1-induced IFN-γ secretion from human PBMC (n=4) by anti-TLR antibodies. The PBMCs were pre-incubated with anti-TLR2, anti-TLR4 or IL-4 antibodies (10 μg/ml) at 37°C for 1 hr before adding 5 μg/ml r*Ov*-ASP-1 for stimulation over a 5 day period. The concentration of IFN-γ in the culture supernatants was tested by ELISA. *indicate P<0.05 when compared to the r*Ov*-ASP-1 untreated cells.

Fig. 9. Activation of TLR2 and TLR4 by r*Ov*-ASP-1 in a dose-dependent manner. Human HEK293 cells expressing TLR2 (A), TLR4 (B) or TLR9 (C) were stimulated with 1 μg or 5 μg/ml of rOv-ASP-1 for 18h and the activation was measured by human IL-8 secretion.

TLR control ligands (Pam3CSK4 for TLR2, LPS for TLR4, and ODN2216 for TLR9) activated the secretion of IL-8 from the appropriate cell lines as expected.

Fig. 10. The r*Ov*-ASP-1-induced cellular activation as determined by NF-κB-dependent reporter gene assay. HEK293 cells were co-transfected with an NF-κB-dependent luciferase reporter construct and the expression plasmids for TLR2 (A) or TLR4/MD2 (B). The cells were challenged with increasing amounts of r*Ov*-ASP-1. The control ligands for TLR2 were Pam3CSK4 (LP3) and Pam2CSK4 (LP2); LPS was used as a ligand for TLR4. The luciferase activity was quantified after 20 h of stimulation.

Figure 1

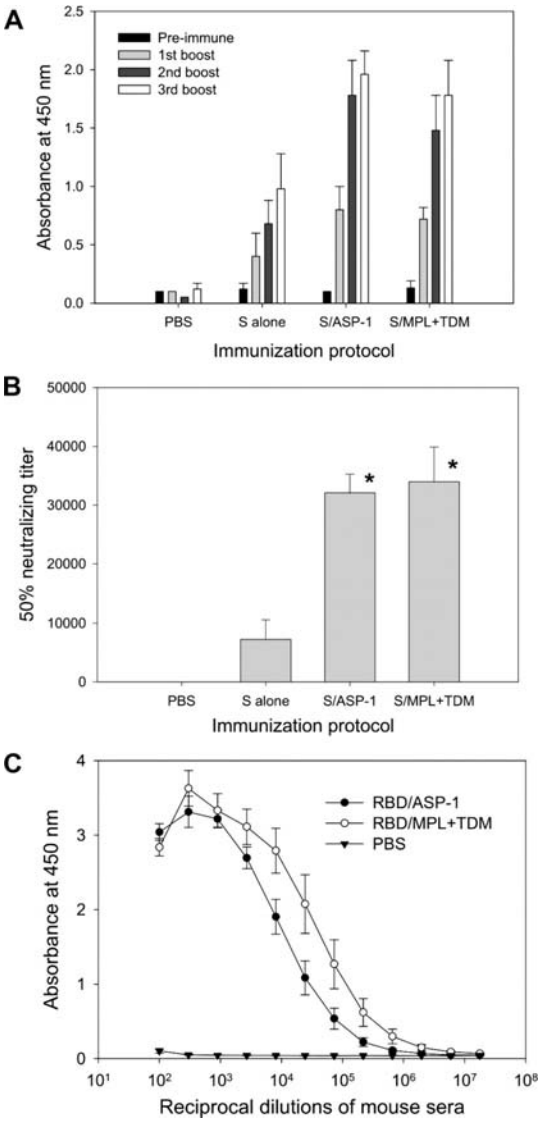

Figure 2

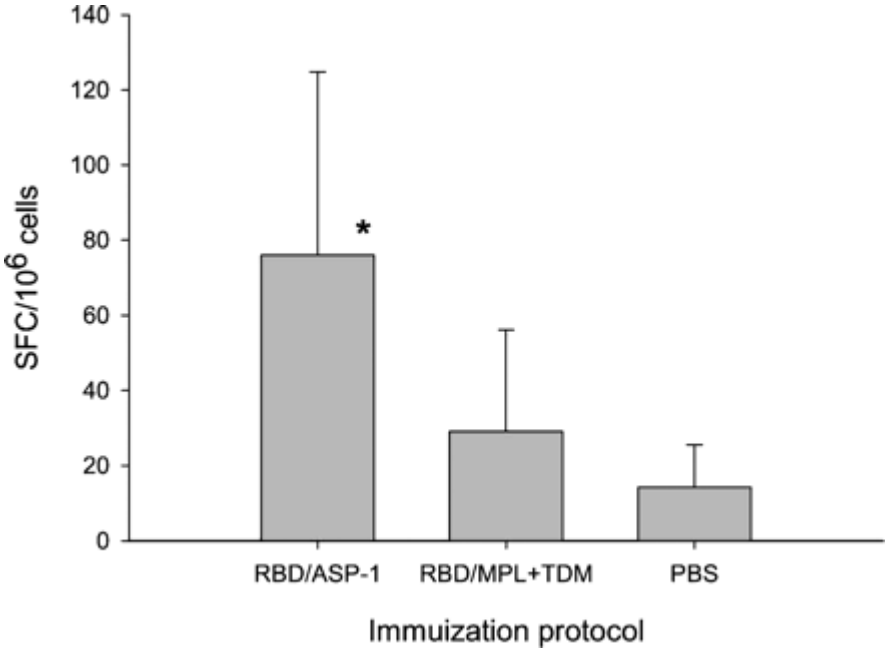

Figure 3

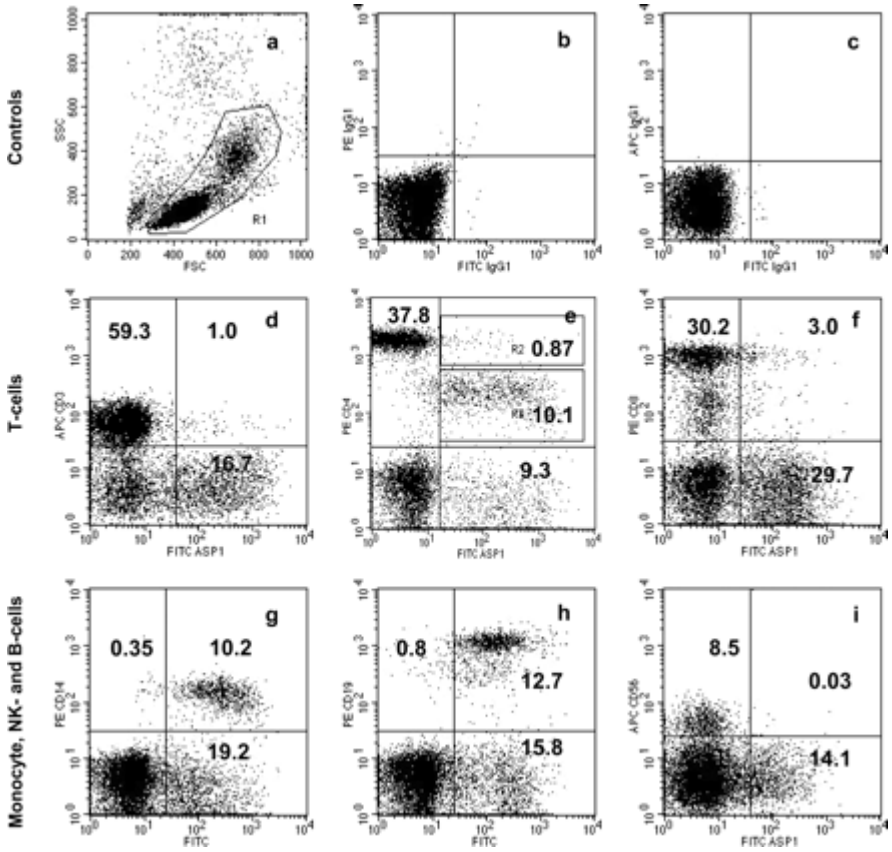

Figure 4

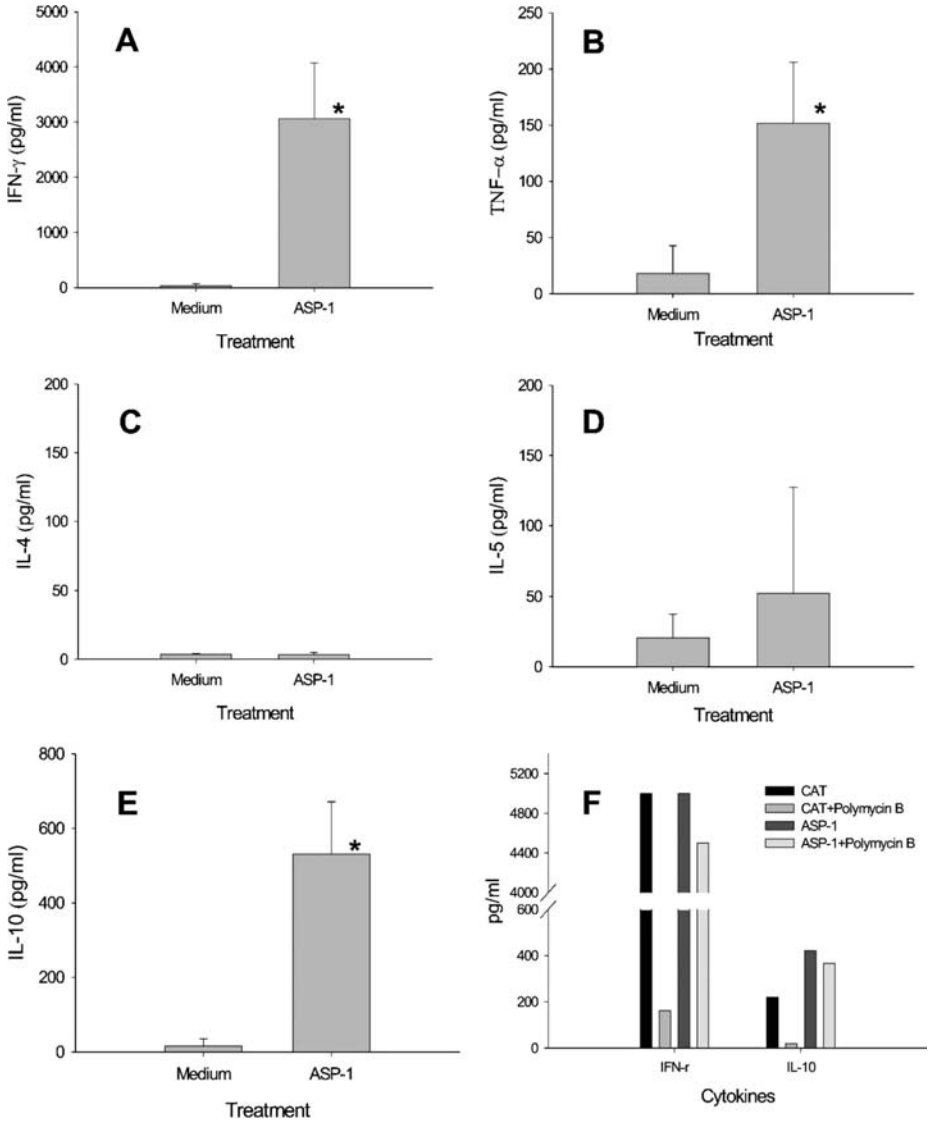
A
IFN-γ (pg/ml)
5000
4000
3000
2000
1000
0
Medium
ASP-1
Treatment
*
B
TNF-α (pg/ml)
250
200
150
100
50
0
Medium
ASP-1
Treatment
*
C
IL-4 (pg/ml)
200
150
100
50
0
Medium
ASP-1
Treatment
D
IL-5 (pg/ml)
200
150
100
50
0
Medium
ASP-1
Treatment
E
IL-10 (pg/ml)
800
600
400
200
0
Medium
ASP-1
Treatment
*
F
pg/ml
5200
4800
4400
4000
600
400
200
0
CAT
CAT+Polymycin B
ASP-1
ASP-1+Polymycin B
IFN-r
IL-10
Cytokines

Figure 5

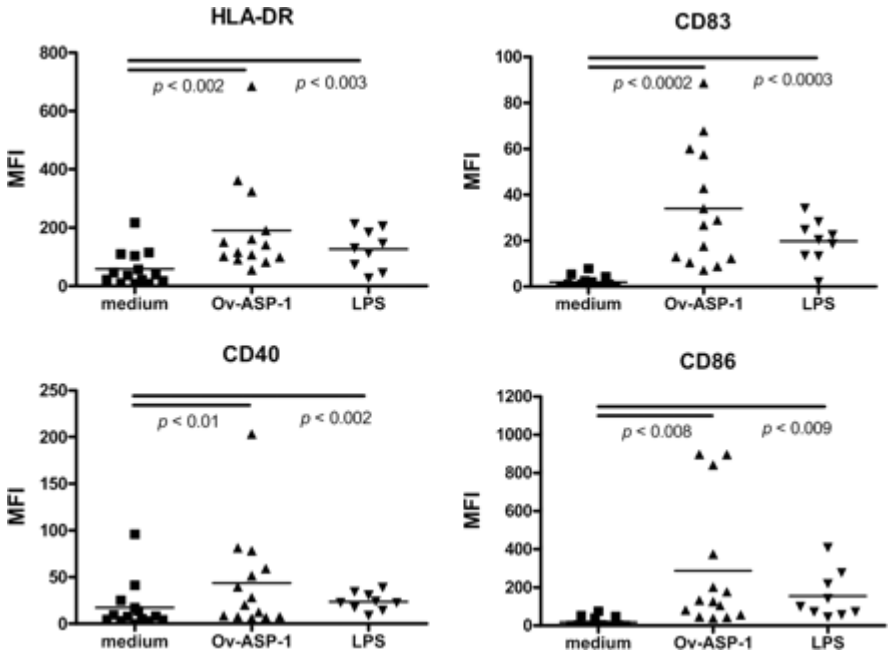

Figure 6

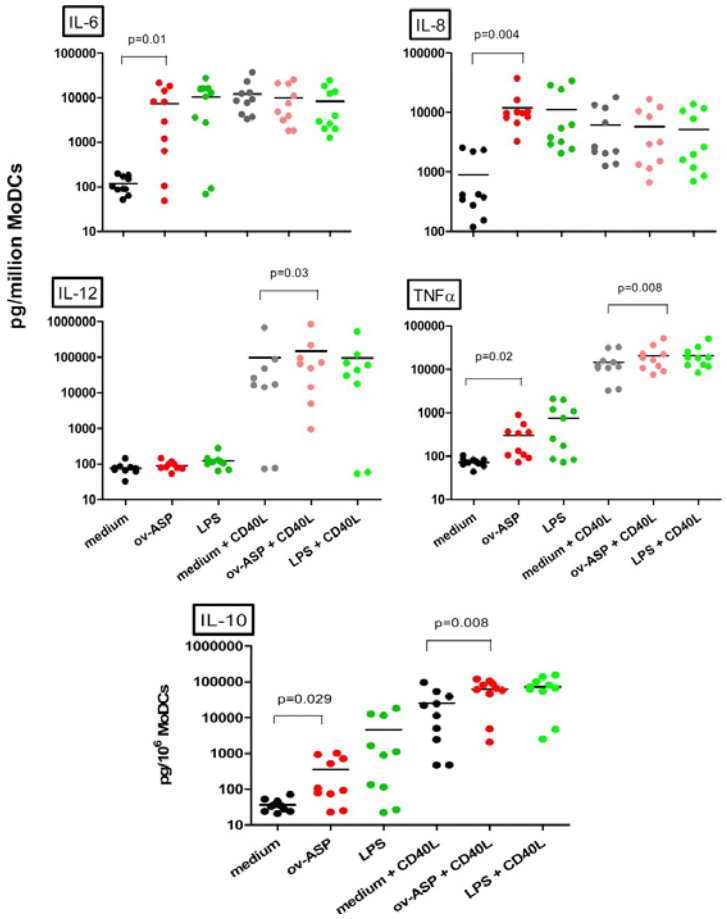

Figure 7

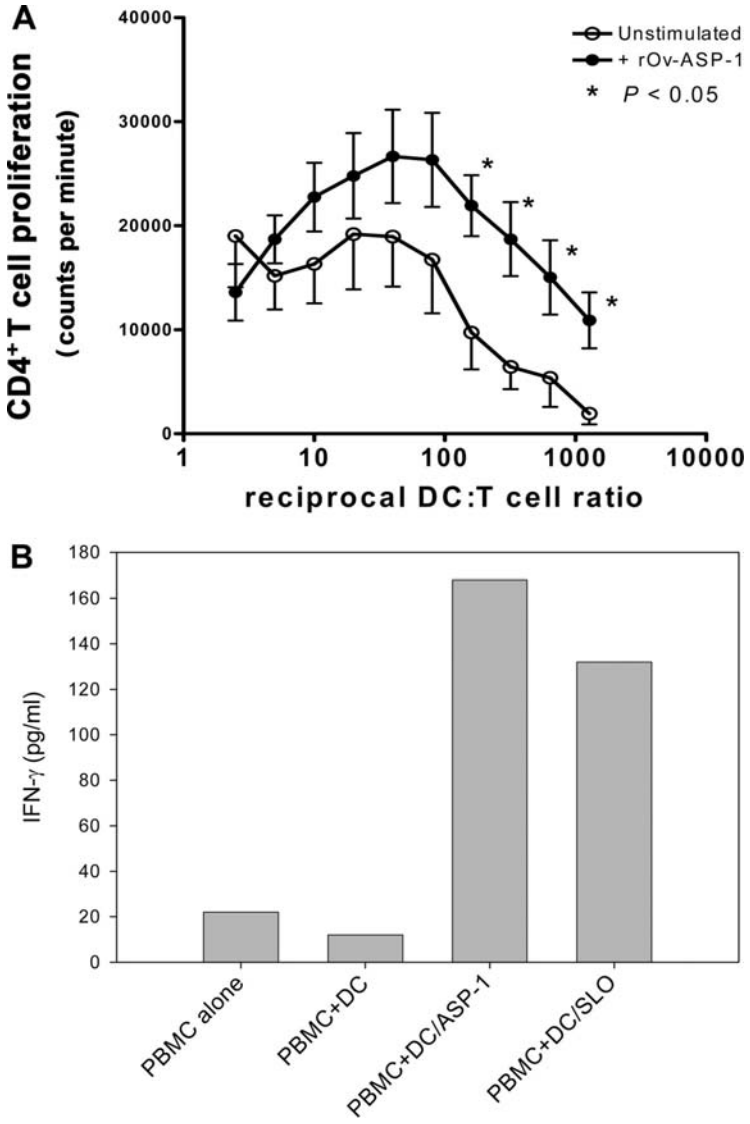
A
CD4+ T cell proliferation
(counts per minute)
40000
30000
20000
10000
0
Unstimulated
+ rOv-ASP-1
* P < 0.05
1
10
100
1000
10000
reciprocal DC:T cell ratio
B
IFN-γ (pg/ml)
180
160
140
120
100
80
60
40
20
0
PBMC alone
PBMC+DC
PBMC+DC/ASP-1
PBMC+DC/SLO

Figure 8

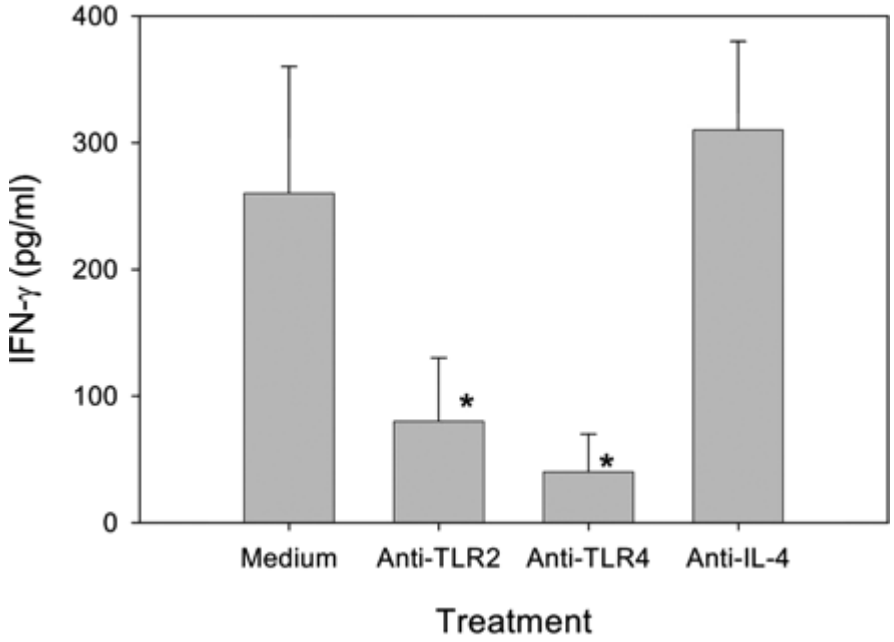

Figure 9

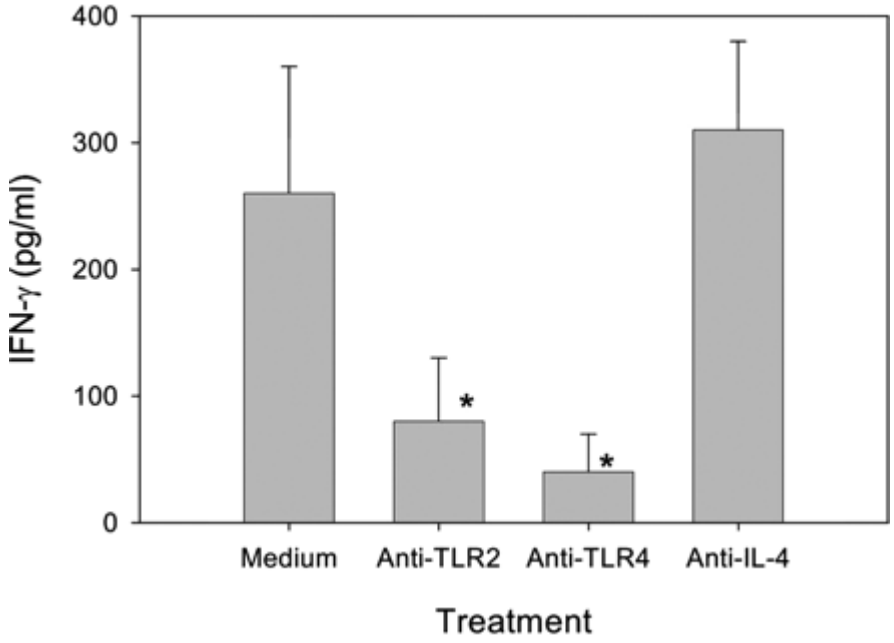

Figure 10

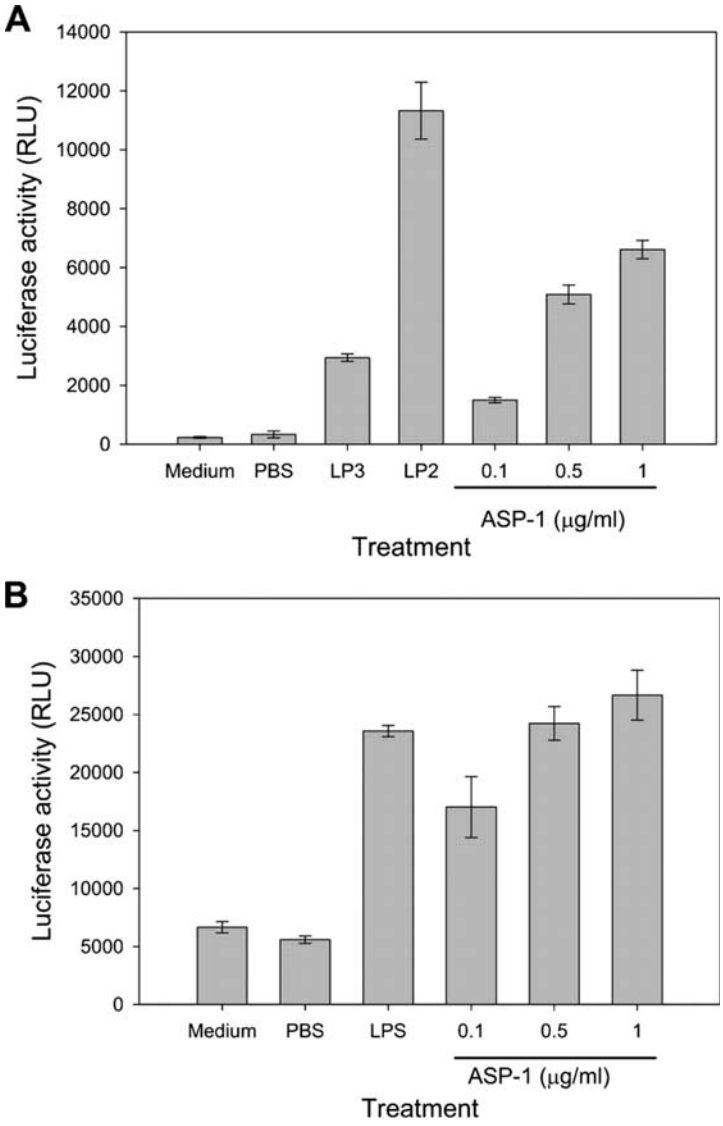
A
Luciferase activity (RLU)
14000
12000
10000
8000
6000
4000
2000
0
Medium
PBS
LP3
LP2
0.1
0.5
1
ASP-1 (μg/ml)
Treatment
B
Luciferase activity (RLU)
35000
30000
25000
20000
15000
10000
5000
0
Medium
PBS
LPS
0.1
0.5
1
ASP-1 (μg/ml)
Treatment

Table 1. Isotyping of antibodies against SARS rS protein after the second boost*

	Immunization protocol		
Isotype	rS alone	rS/r*Ov*-ASP-1	rS/MPL+TDM
IgG1	72,900	656,100	656,100
IgG2a	2,700	656,100	218,700
IgG2b	2,700	218,700	72,900
IgG3	300	8,100	2,700

* The end-point titers are the mean of 4 mice per group. As induction of high titer of IgG1 is considered indicative of a Th2-type immune response, high IgG2a, IgG2b and IgG3 titers are typical of a Th1-type response. Apparently, rS alone induced a Th2 dominant response while rS/r*Ov*-ASP-1 is a mixed but Th1 dominant.

Table 2. Cytokine secretion from mouse splenocytes stimulated *ex vivo* by rRBD

	Immunization protocol		
Cytokine (pg/20,000 cells)	rRBD/PBS	rRBD/r*Ov*-ASP-1	rRBD/MPL+TDM
IL-2	40.6	108.0	24.9
IL-4	0.0	5.3	6.3
IL-5	1.6	6.8	20.8
IFN-γ	75.5	950.9	267.6
TNF-α	16.8	66.9	24.3
IL-6	9.2	90.4	18.6
IL-10	7.6	41.5	51.3

Appendix 3

The TLR7 agonist SM-360320 induces a robust antiviral and immunostimulatory responses in chronic hepatitis C infection

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Abstract

Background and Aims: Toll-like receptor 7 (TLR7) agonists offer therapeutic potential in chronic hepatitis C (CHC). Their antiviral activity is mediated through induction of innate and stimulation of adaptive immune responses. We compared responses *in-vitro* using blood from normal healthy donors (NHDs) and patients with CHC who were treatment naïve (TN) or non-responders (NR) to interferon.

Methods: Peripheral blood from 60 TN, 20 NR CHC patients and 80 NHDs was stimulated *in-vitro* with the TLR7 agonist, SM-360320 or Interferon-α (IFNα). Cytokine secretion, antiviral gene expression, Hepatitis C virus (HCV) replicon inhibition, and NK cell activation were quantified.

Results: Basal cytokine levels were higher in CHC than NHD blood with TN-CHC higher than NR-CHC. SM-360320 and IFNa induced expression of all tested cytokines in all groups. Both treatments increased expression of 2'5'OAS in all groups; NHDs were highest. SM-360320 treated CHC blood plasma reduced HCV replication to baseline and was more effective than IFNα. SM-360320 enhanced NK-cell activation in all groups ($p=<0.001$) and was more effective than IFNα ($p=< 0.05$). CD69 expression was lower on CHC NK cells than NHDs. Depletion of pDCs from NHD- PBMCs reduced IFNα production on incubation with SM-360320 and IFNα.

Conclusions: SM-360320 induced innate immune responses in blood from CHC patients suggesting the potential use of TLR7 agonists in treatment of CHC. The lower response in NR-CHC blood may indicate an underlying defect in the TLR7 mediated IFNα pathway in these patients.

Introduction

Chronic infection with hepatitis C virus (CHC) is a major cause of liver related morbidity and mortality. Infection persists in 80% of patients and is thought to result from a failure to sustain an immune response against HCV (35). The current standard of care for Hepatitis C (HCV) pegylated (PEG)-Interferon-α (IFNα) with Ribavirin, leading to viral clearance in 40-80% of patients, primarily depending on genotype (167). Current treatment is associated with significant side effects including flu-like symptoms, depression and injection site reactions (178). Novel strategies for the treatment of CHC are therefore required.

The host's innate and adaptive cellular immune responses are believed to be critical in determining the outcome of HCV (161;161;163). The natural killer (NK) cell-mediated innate immune response may be genetically programmed to determine the chance of spontaneously resolving acute HCV infection (159) and directly modulated by HCV itself, leading to impaired NK-cell function (326). Dendritic cells (DCs) are professional antigen presenting cells characterised by their exceptional capacity to activate naïve T-cells and prime adaptive immunity. DCs play a pivotal role linking innate and adaptive immunity (67). Two functionally distinct subsets of DCs have been described; myeloid (mDC) and plasmacytoid (pDC). pDCs are the most potent producer of type-1 interferon's in the body and release large quantities during viral infections (327).

In terms of the adaptive immune response, successful viral clearance during acute infection has been attributed to a robust HCV-specific $CD8^+$ T-cell effector response and a predominantly Th1-polarised $CD4^+$ anti-HCV response (161). A failure to sustain this early anti-HCV T-cell response correlates with viral persistence and development of CHC. Toll-like receptors (TLRs) are a family of pattern recognition receptors that play a key role in innate immunity (93). TLRs are activated by a range of pathogen-associated molecular patterns conserved among pathogens. TLR3, 7 and 9 are intracellular receptors specialized for recognition of viral nucleic acids, recognizing double stranded-RNA, synthetic imidazoquinolone-like molecules, guanosine analogues and single-stranded-RNA derived from viruses and unmethylated 2'-deoxyribo cytidine-phosphate-guanosine (CpG) DNA respectively. TLR7 and 9 are expressed by pDCs and B cells. On binding, they induce a signalling cascade which induces type I interferon's and pro-inflammatory cytokines, which drive inflammatory responses and activate the adaptive immune system (94).

TLR agonists have been investigated for the treatment of CHC as a means of inducing endogenous IFNα even in the presence of the immune dysfunction induced by CHC infection. Activation of pDCs is important for HCV treatment because these activated cells not only primarily produce IFNα, but also present antigen to initiate T-cell mediated adaptive immunity.

In short term monotherapy trials, the TLR7 agonist Isatoribine (241), TLR7 agonist Resiquimod (1) (328)and the TLR9 agonist, CpG 10101 (Actilon) (250) have been shown to have clinical efficacy against HCV with reduced viral load and evidence of immune modulation.

The aim of this study was to investigate the antiviral and the immunostimulatory actions that might be responsible for HCV clearance *in vitro.* The effects of the TLR7 agonist, SM-360320 (9-benzyl-8-hydroxy-2-(2-methoxyethoxy)adenine) (TLR7 specific and 75% bound in human plasma) (295) and IFNα on innate immunity was studied. Responses were compared in blood from normal healthy donors (NHDs) and patients with CHC, both treatment naïve (TN) and previous non-responders (NR) to treatment.

Materials and Methods

Study subjects:

Ethical approval was obtained from Southampton and South-West Hampshire Joint Research Ethics Committee and all patients gave informed consent in writing prior to participating in the study

Whole blood was collected from CHC-patients recruited from hepatology clinics run by Southampton University Hospitals and Royal Bournemouth and Christchurch NHS Trusts. All patients had detectable HCV-RNA determined by quantitative PCR (Cobas Amplicor HCV Monitor test, Roche Molecular Systems, USA). Patients were excluded if they had treatment within 6 months prior to the study or tested positive for other blood borne viruses, including HBV and HIV. Sixty patients were treatment naïve and 20 were previous NR to treatment. A further 80 uninfected NHDs, without risk factors for blood borne viruses were recruited internally within Pfizer. Clinical data from the groups is in Table 1.

Whole Blood Stimulation

Fresh blood collected in sodium citrate vacutainers was aliquoted (450µl for CHC and 180µl for NHDs) into 24 or 96 well assay plates respectively containing 50µl or 20µ pre-prepared, SM-360320, IFNα or no stimulus. Final concentrations of SM-360320 were 0.1µM – 10µM (1µM for NHDs) and IFNα was 20IU/ml. Plates were sealed, placed on an orbital shaker and incubated for 5 hours at 37^0C. Aliquots of 25µl (n=4 CHC and n=1 NHDs) of the stimulated blood from each well were transferred into 96 well plates and mixed with 130µl of MagMAX lysis/binding solution (Ambion) in a 1:1 ratio with isopropanol.

The remaining blood was spun (5 min at 500rpm), plasma removed and frozen at -80^0C. The remaining cell pellet for the CHC-blood was re-suspended in the residual plasma (~300µl) and 1.3ml RNALater (Ambion) was added to stabilize the samples. These samples were also frozen at -80 C pending further analysis.

PBMC isolation and storage:

Blood collected into EDTA containing vacutainers was separated immediately by centrifugation over Lymphoprep (Robbins Scientific, Solihull, UK). Peripheral blood mononuclear cells (PBMCs) were recovered from the interface, cells counted using trypan

blue and then cryopreserved in 90% FCS and 10% sterile culture grade dimethyl sulfoxide (DMSO, HybriMAX, Sigma) and stored at -80^{0}C while awaiting further use.

Gene Expression Analysis

After thawing, RNA was extracted from blood/MagMAX lysis/binding mixture using the MagMAX 96-well blood RNA isolation kit (Ambion) according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was prepared using high capacity cDNA archive kits (Applied Biosystems, USA) using 10µl RNA in 20µl reactions (2µl reverse transcriptase (RT) buffer, 0.8µl deoxyribonucleotide-triphosphate mix, 2µl random primers, 1µl Multiscribe RT and 4.2µl RNase free water). 2'5'OAS gene expression was quantified using real time taqman QPCR using 'assay on demand reagents' (Applied Biosystems, USA) as described by manufacturer. QPCR amplification reactions were performed on a 7900 real time PCR machine (Applied Biosystems) over 40 cycles (annealing 60ºC for 45secs, primer extension 72ºC for 1min and denaturation 94ºC for 20sec). 2'5'OAS gene expression was measured relative to the house keeper gene, β-actin. Data were analysed using the delta-delta-ct method for relative quantity (329).

Cytokine analysis

Levels of pro-inflammatory cytokines, TNFα, IL-1β, IL-2, IL-6, IL-8, IL-12p70, antiviral cytokines, IFNγ and IFNα and regulatory cytokines, IL-10 and IL-4 were determined in aliquots of 25 µl plasma from the whole blood assays using a custom ultrasensitive 10-plex electrochemiluminescence ELISA as described by the manufacturer (Meso Scale Discovery, USA). Values were calculated within the standard curve.

HCV replicon assay

The presence of soluble factors with antiviral activities in treated plasma was measured in an HCV-replicon system. Huh7 1b replicon cells were cultured in flasks. Supernatants (10µl) from whole blood ex vivo experiments were added to the replicon cells and incubated for 48 hours at 37°C. Replicon levels were quantified using a luciferase reporter assay (270).

NK-cell activation & intracellular IFNγ staining

Cryopreserved PBMCs were thawed and rested for 5 hours at 37°C prior to assay. Cells were resuspended to 2 x 10^6/ml in RMPI[1640] (no phenol). PBMCs (900µl) were plated

into duplicate wells of a 24 well plate containing 100µl SM-360320, IFNα or diluent only per well. After 5 hours, 1µl of Brefeldin-A (Sigma, UK) was added to one well of each duplicate. Cells were incubated for a further 18 hours. After 24 hours, PBMCs were harvested and labelled to identify activated $CD56^+$ $CD16^+$ NK-cells for cell surface CD69 expression and cytoplasmic IFNγ expression by intracellular staining. Antibodies (CD56-APC, CD4-FITC, CD69-FITC, CD3-PE from BD Biosciences) and their isotypes (IgG1-APC, -FITC, -PE) were diluted with FACS block to optimal concentrations for flow cytometry and 50µl of appropriate antibody added 1:1 with FACs block in the well. Cells were incubated on ice for 30 minutes, washed, then fixed in 1% formaldehyde and stored on ice in the dark pending analysis.

pDC Depletion

From 5 NHDs (from Southampton) and 5 CHC-patients, PBMCs were prepared from blood as above. pDCs were isolated from PBMCs by positive selection using a MACS BDCA-4 blood dendritic cell isolation kit (Miltenyi Biotec, UK), according to the manufacturer's instructions, in conjunction with an AutoMACS (Miltenyi Biotec). Whole PBMCs, PBMCs depleted of pDCs, and pDCs alone were stimulated with SM-360320 (10µM-0.01µM; Pfizer, Sandwich) in sealed plates for 5 hours at 37°C and gene expression analysis, cytokine production and NK-cell activation was assessed as above. The % purity of the pDC depletion was assessed by FACS analysis.

Statistical Analysis

GraphPad PRISM (V5) and a Pfizer Inc proprietary add-in to Microsoft Excel 2003 were used to perform initial data analysis and visualisations. Statistical analyses were performed using SAS (V8.02), GenStat (V10) and Spotfire DecisionSite 9.0.
In order to satisfy the assumptions of the analysis methods used, the data from the gene expression, cytokine and pDC depletion analyses were analysed on the $\log_{10}$ scale and data from NK-cell Activation were analysed on the logit scale. Plots of the data were used to check the statistical assumptions and any unusual features such as outliers were investigated.

For gene expression, cytokine analyses, NK-cell activation and pDC depletion experiments all transformed responses were analysed using a linear mixed model that allowed for donor-to-donor variation and investigated differences between the three donor groups, the stimuli and the effects of pDC depletion. Specific pairwise comparisons

between the stimuli and groups were quantified on the transformed scale and back-transformed to give geometric means, ratios in means, 95% confidence intervals (excluding NK-cell activation data) and p-values. All tests were performed at the 5% significance level.

For the HCV replicon assay the distribution of the collected Replicon data did not lend itself to the modelling techniques used for the other responses. Therefore the multivariate technique K-means clustering was applied to identify similar donor profiles, where a profile contains the responses exhibited by a donor across the 6 stimuli. A number of different clusters and similarity measures were investigated.

Results

Induction of 2'5'OAS

IFNα (20IU/ml) and SM-360320 (1μM) significantly induced 2'5'OAS in whole blood from all 3 groups (p=<0.01 for all; Figure 1). The level of the response with both stimuli was higher in blood from NHD compared to CHC (p=<0.001 for all). However, in CHC-patients, a larger variation in 2'5'OAS induction was observed across the samples compared to NHDs with some patients giving a 100-fold induction. No significant difference was observed between treatment naïve and NR-patient groups (p=>0.10).

Induction of Cytokines

SM-360320 (1μM) stimulated secretion of all cytokines tested in whole blood from NHDs and CHC-patients (p=<0.001 for all at 1μM; Figure 2 and Table 2) in a dose response manner. A wider range of secretion of individual cytokines was noted from CHC-patients compared to NHDs.

IFNα (20IU/ml) stimulated significant amounts of IFNα, IFNγ, IL-6, IP-10 and IL-12p70 (p=<0.001 for all) in blood from CHC-patients and IFNα, IL-6 and IP-10 in blood from NHDs (p=<0.001 for all). No effect was observed with IFNα on IL-1β, IL-8, TNFα, IL-4 and IL-10 in CHC-patients and IFNγ, TNFα, IL-12, IL-8, IL-4 and IL-10 in NHDs.

Higher levels of IL-1β and IL-6 were observed from NHD-blood and CHC-blood with 1μM SM-360320 compared to IFNα. In contrast, similar levels of endogenous IFNα and IP-10

were secreted on incubation with both compounds, although exogenous IFNα may have been detected making interpretation difficult.

On average, naïve-CHC blood induced higher levels of the cytokines compared to NR-CHC blood with either compound; however this did not reach statistical significance (p=>0.10). The untreated levels of all cytokines was higher in CHC-blood than NHD-blood (p=<0.001 for all).

Inhibition of HCV Replication

Plasma derived from blood treated with SM-360320 (10μM and 1μM) from CHC-naïve and CHC-NR patients inhibited replication of HCV replicons in Huh-7 cells shown by reduction of luciferase reporter expression to baseline levels (Figure 3). This is consistent with the high levels of IFNα found in the plasma (338.8pg/ml-naive and 269pg/ml-NR) (Table 2).

The antiviral activity of plasma from IFNα treated blood was lower than that with SM-360320 (medians 73% *vs.* 99% inhibition) possibly due to degradation of exogenous IFNα and/or lower induction of endogenous IFNα. Some antiviral activity was observed in plasma derived from untreated blood. In naïve CHC-patients, two distinct groups were identified by K-means clustering, with some patients reaching maximal inhibition at a lower concentration of agonist than others. Some naïve patients gave replicon responses that were more consistent with the NR group than others (Figure 3). Blood from NHDs has not been analysed in the replicon assay.

NK-cell Activation

CD3-CD56+ NK-cells were identified by FACS and % expression of CD69 (as a marker of activation) or intracellular IFNγ was determined. Surface expression of CD69 was studied on cells without Brefeldin-A exposure. In all 3 subject groups, the TLR7 agonist resulted in a dose-dependent increase in NK-cell activation (Figure 4). CD69 expression and IFNγ production were significantly enhanced by both 10μM and 1μM SM-360320 in all subject groups (p=<0.001) and CD69 in NHDs by 0.01μM (p=<0.001). IFNα also enhanced CD69 expression in all subject groups (p=<0.001) but was less effective than the highest concentrations of SM-360320 (p=<0.05) and did not significantly enhance IFNγ production (p=>0.1).

Comparing treatment with 10μM SM-360320, CD69 expression in naïve NK-cells was significantly lower than that in NHD NK-cells (p=<0.05 for all). A similar trend was also

seen between NHD NK-cells and NR-CHC NK-cells but this was not statistically significant (p=>0.1)

The Role of pDCs

Secretion of IFNα and 2'5'OAS expression was observed with 1μM SM-360320 or 20IU/ml IFNα in whole NHD-PBMCs (p=<0.01). IFNα induction was reduced on depletion of pDCs from NHD-PBMCs stimulated with SM-360320 (p=<0.05 for all), confirming the importance of this cell type for TLR7 agonist responses. However, no significant difference in 2'5'OAS expression was seen with pDC depletion (Figure 5.). In the CHC group there was a wide variation of responses in the untreated samples of the whole PBMCs making the results from pDC depletion difficult to interpret for IFNα secretion. However, on depletion of pDCs from CHC-PBMCs, there was a significant reduction in 2'5'OAS expression with 1μM SM-360320 and 20IU/ml IFNα (p=<0.001 for all). The efficiency of depletion of pDCs from PBMCs was 68% on average.

To investigate whether NK-cell activation was occurring indirectly in response to agonist-induced IFNα production by plasmacytoid DCs (pDCs), or directly in response to the agonist, the effect of depleting pDCs from PBMCs prior to exposure to the agonist, was studied and NK assessed as above. In preliminary experiments in NHDs, NK-cell activation was induced by the top doses of SM-360320 and was partially impaired by pDC depletion with reduced IFNγ secretion (data not shown).

Discussion

The need to overcome immune dysfunction seen in HCV and induce strong HCV-specific T-cell responses to clear the virus has prompted evaluation of immunomodulators in CHC treatment. The aim of this study was to investigate both the antiviral and immunostimulatory actions of the TLR7 agonist, SM-360320 on cells derived from the blood of CHC -patients. We have shown that SM-360320 was able to induce a robust *ex vivo* innate immune response in blood from treatment TN and NR-CHC-patients.

Blood from TN-CHC patients produced higher levels of all cytokines compared to NRs but no differences were observed in the cytokines secreted. In the replicon assay, blood from some of the TN patients showed responses more like the NR-patients which may indicate they will be non-responders to treatment. CD69 expression on NK-cells was higher in

NHDs than CHC-patients with no significant difference between TN and NRs. There were no differences observed in 2'5'OAS expression between the patient groups.

TLR7 expression is limited to certain tissues (18) and a restricted set of human immune cells, notably pDCs and B cells. TLR7 agonists are not thought to have direct antiviral effects (19) but following TLR engagement induce pDCs to produce large quantities of type 1 IFNs and more modest amounts of various other cytokines e.g. IL-6 and TNFα. Induction of type 1 IFNs through activation of TLRs on pDCs is a crucial part of the host's innate immune response to viral infection which results in the production of a wide range of downstream antiviral effector molecules such as interferon stimulated genes: 2'5'OAS, RNase-L, MxA, and signaling chemokines such as IP-10. As a consequence of the secretion of type 1 IFNs, a number of secondary effects are also induced such as stimulation of NK-cells as well as maturation of pDCs to potent antigen-presenting cells, augmenting their ability to stimulate T-cells and thus activating both the innate and adaptive immune responses.

In this study we investigated the antiviral effects of SM-360320 by looking at the induction of 2'5OAS, induction of cytokines and the antiviral activity of plasma from treated blood in HCV replicon cells.

Plasma from blood treated with SM-360320 inhibited replication of HCV-replicons in Huh-7 cells reducing luciferase reporter expression to baseline levels. This may indicate the potential of TLR7 agonists to reduce viral load mediated by the antiviral activities of agonist-induced IFNα. The expression 2'5'OAS was found to be increased with TLR7 treatment and secretion of antiviral cytokines, IFNα and IFNγ and pro-inflammatory cytokines from whole blood were significantly enhanced. *In vivo* this would be predicted to promote a Th1 immune response and favour viral clearance.

NK-cells play an important part in the first line of defence against viral infections, rapidly recognising and killing virus-infected cells and also secreting inflammatory cytokines, such as IFNγ which have direct antiviral effects as well as activating and polarising Th1 and cytotoxic lymphocyte responses (20;21). Therefore they provide a pivotal link between innate and adaptive immunity. Several groups have shown that TLR7 agonists induce NK-cell activation (CD69 expression and IFNγ production) and enhance their cytotoxic effector function (22-25). In this study we found that SM-360320 was able to significantly

upregulate NK-cell activation and was more effective than IFNα treatment. The effect of SM-360320 on NK-cells was lower in CHC-patients than NHDs.

NK-cells express TLR 2, 3, 5 and 6 but little TLR 4, 7, 8 and 9 (22). It is not clear therefore if NK-cell activation by TLR7 agonists is caused by direct activation of TLR7 receptor in NK-cells or if they act indirectly and secondary to activation of other cells, in particular pDCs, and cytokines produced by these cells. It has previously been observed that TLR7-induced NK-cell activation is crucially dependant on contact with cytokines (e.g. IFNα or IL-12) produced by accessory cells in the blood (23-25).

To explore this further, we depleted pDCs, the main IFNα producing cells, from PBMCs and assessed the effects of the TLR7 agonist on NK-cell activation. In preliminary experiments it has been shown that NK-cell activation was partially impaired supporting evidence of an indirect effect and dependence on cytokines from accessory cells in the peripheral blood.

To determine the contribution of pDCs to any antiviral or immunostimulatory effects of SM-360320, the effects of pDC depletion on cytokine secretion and 2’5’OAS induction were assessed. As would be expected, depletion of pDCs from NHD-PBMCs resulted in a reduction of IFNα secretion however a corresponding reduction in 2’5’OAS induction was not found to be significant. In CHC-PBMCs, 2’5’OAS induction was found to be decreased but the IFNα data is difficult to interpret due to large variability in the whole PBMC group.

Defects in CHC-pDC IFNα production have been described in a number of studies (26-32) and it would be interesting to explore the effects of the TLR7 agonist directly on pDCs to see if it has the ability to induce pDC maturation and reverse the defects described.

Current treatment for CHC is based on the combination of PEG-IFNα and Ribavirin. Both appear to possess antiviral and some immunomodulatory activities (33;34). When comparing IFNα, at a concentration representative of plasma levels typically found during IFN therapy, to the TLR7 agonist in this study we show that, SM-360320 at 1μM induced pro-inflammatory cytokines and 2’5’OAS to higher levels than IFNα, was more effective in the replicon assay and induced NK-cell activation to significantly higher levels (also been shown with a TLR9 agonist) (35). Therefore, comparison of immunostimulatory and antiviral activities show that SM-360320 may provide similar or better antiviral effects and

modulation of the innate immune response. Irrespective of the efficacy of SM-360320 in vitro it must be recognized that preclinical and clinical trials may reveal adverse effects that may make the compound impractical or less attractive than IFNα.

Clinical studies with TLR7/8 agonists (13;36-38) have shown a modest reduction in viral replication in HCV infected patients. While stimulating TLRs could re-establish an appropriate immune response to infection, caution must be taken as inappropriate stimulation can lead to inflammation and autoimmunity. In this study the effective in vitro dose of SM-360320 was found to be 1μM.

Although direct-acting HCV-specific drugs (e.g. protease inhibitors) may provide superior antiviral effects, a probable advantage of TLR agonists is the relatively low emergence of drug resistance that is a particular challenge in HCV with its high genetic diversity and mutation rate. Drug resistance is less likely because the molecular target of these agents is the host receptor rather than a virally encoded target. Furthermore, because TLR7 activation initiates a broad immune response, including both innate and adaptive responses, it is likely that multiple aspects of viral replication are simultaneously attacked.

In summary, the TLR7 agonist, SM-360320 has been shown to possess effective antiviral and immunostimulatory effects on whole blood from CHC donors and might provide complementary and additional HCV therapies making it a promising novel treatment. It is likely that HCV therapy will continue to require the presence of an effective immunomodulatory agent and TLR7 provides a candidate target mechanism for this role.

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Figure / table Legends:

Table 1: Clinical characteristics of CHC-Naïve, and CHC-NR and information on NHDs.

Table 2: Cytokines were induced in all 3 subject groups. Mean responses and significance over untreated for 10 cytokines tested in NHD, Naive and NR groups. The ratio of geometric means and 95% confidence intervals are also presented.

Figure 1: SM-360320 (10μM and 1μM) and IFNα (20IU/ml) enhanced expression of 2'5'OAS in all groups. Expression was significantly less in the CHC-patients compared to NHD ($p<0.01$).

Figure 2: Cytokines secreted by PBMCs treated with TLR7 agonist SM-360320 and IFNα. * indicate significant increase of cytokine production over untreated.

Figure 3: **Plasma from SM-360320 (10μM and 1μM) treated PBMCs significantly inhibited replication of HCV replicons in Huh-7 cells (p=<0.05).**The antiviral potential of plasma from IFNα treated blood was less effective.

Figure 4: SM360320 enhanced NK-cell activation in all 3 groups in a dose dependant manner with greater effects seen in NHD than CHC-patients.

Figure 5: Effect of pDC depletion from PBMCs.

A) Reduced IFNα was observed in pDC depleted PBMCs in NHDs following SM-360320 treatment but no significant change in 2'5'OAS expression. B) In CHC-patients, untreated levels of IFNα in whole PBMCs were highly variable making interpretation on pDC-depletion difficult. However, reduced expression of 2'5'OAS was seen on depletion of pDCs. * indicate statistically significant enhancement or inhibition of IFNα production from untreated levels in NHD and CHC-patients.

Table 1.

		CHC		*NHDs*
		Naive (n=60)	**NR (n=20)**	**n=80**
Age	**Median**	46	45	42
	Range	27-87	36-70	22-61
Gender	**Male**	46(77%)	16(80%)	54(68%)
	Female	14(23%)	4(20%)	26(32%)
Race	**Caucasian**	57	17	Unknown
	Asian	1	3	
Genotype[a]	**G1**	28(47%)	14(70%)	N/A
	G2/3	28(47%)	5(25%)	
	G4	1(1%)	1(5%)	
	Unknown	3(5%)	0(0%)	
Viral load[b]	**Median**	7.91E+05	5.52E+06	N/A
	Range	(7.02E+02-2.32E+07)	(7.25E+05-2.06E+07)	
Disease Severity[c]	**Mild**	19(32%)	5(25%)	N/A
	Moderate	14(23%)	1(5%)	
	Severe	14(23%)	7(35%)	
	No Biopsy	13(22%)	7(35%)	
ALT (IU/L) (10-40)	**Median**	62	58.5	N/A
	Range	10-169	22-252	
Risk for HCV	**IVDU**	33	7	N/A
	Blood Product	14	9	
	Other	11	4	
Alcohol intake[d]	**Low**	33(55%)	16(80%)	N/A
	Moderate	11(18%)	3(15%)	
	Heavy	16(27%)	1(5%)	

[a] HCV genotype determined using CPA accredited PCR-based 'Genotyping for Treatment Assay' (iQur Ltd, UK).

[b] Viral load determined by quantitative PCR (Cobas Amplicor HCV Monitor test, Roche Molecular Systems, USA) quoted as copies/ml.

[c] Based on histological analysis of biopsies for inflammation and fibrosis. In the absence of histological evidence, disease status was determined by clinical criteria including physical examination, diagnostic imaging and laboratory indices. [d] Low, <7 units/week; Moderate, 7-14 units/week; Heavy, >14 units/week

Table 2.

Cytokine	Subject Group	Treatment SM-1μM	Geometric Means	P-value (vs. untreated)	Ratios of Geo Means	95% Confidence Intervals
IP-10	NHD	Untreated	10.4	-	-	-
		SM	1803.0	<0.001	174.2	(148.3-204.6)
		IFNα	529.7	<0.001	51.2	(43.6, 60.1)
	Naïve	Untreated	83.6	-	-	-
		SM	4036.5	<0.001	48.3	(37.4, 62.4)
		IFNα	1749.8	<0.001	20.9	(16.2, 27.0)
	NR	Untreated	66.5	-	-	-
		SM	3372.9	<0.001	50.7	(33.2, 77.4)
		IFNα	1566.8	<0.001	23.6	(15.4, 36.0)
IFNα (2a)	NHD	Untreated	1.7	-	-	-
		SM	94.4	<0.001	54.5	(43.6, 68.0)
		IFNα	81.1	<0.001	46.8	(37.5, 58.4)
	Naïve	Untreated	12.0	-	-	-
		SM	338.8	<0.001	28.2	(19.8, 40.3)
		IFNα	241.0	<0.001	20.1	(14.1, 28.7)
	NR	Untreated	6.0	-	-	-
		SM	269.2	<0.001	45.2	(25.0, 81.5)
		IFNα	205.1	<0.001	34.4	(19.1, 62.1)
TNFα	NHD	Untreated	1.9	-	-	-
		SM	185.8	<0.001	99.8	(86.9, 114.6)
		IFNα	1.5	<0.01	0.8	(0.7, 0.9)
	Naïve	Untreated	13.4	-	-	-
		SM	179.5	<0.001	13.4	(10.5, 17.2)
		IFNα	12.1	NS	0.9	(0.7, 1.2)
	NR	Untreated	10.4	-	-	-
		SM	141.3	<0.001	13.5	(9.0, 20.4)
		IFNα	8.8	NS	0.8	(0.6, 1.3)
IL-12p70	NHD	Untreated	2.8	-	-	-
		SM	7.2	<0.001	2.6	(2.2, 3.0)
		IFNα	2.3	<0.05	0.8	(0.7, 0.9)
	Naïve	Untreated	27.2	-	-	-
		SM	357.3	<0.001	13.2	(10.1, 17.2)
		IFNα	161.4	<0.001	5.9	(4.5, 7.8)

	NR	Untreated	15.7	-	-	-
		SM	311.9	<0.001	19.8	(12.7, 30.8)
		IFNα	125.3	<0.001	8.0	(5.1, 12.4)
IL-1β	NHD	Untreated	0.5	-	-	-
		SM	43.8	<0.001	96.7	(81.6, 114.6)
		IFNα	0.3	<0.001	0.7	(0.6, 0.8)
	Naïve	Untreated	5.6	-	-	-
		SM	86.9	<0.001	15.5	(11.2, 21.3)
		IFNα	6.2	NS	1.1	(0.8, 1.5)
	NR	Untreated	4.8	-	-	-
		SM	45.7	<0.001	9.5	(5.5, 16.5)
		IFNα	4.0	NS	0.8	(0.5, 1.4)
IL-4	NHD	Untreated	1.2	-	-	-
		SM	2.4	<0.001	2.1	(1.8, 2.4)
		IFNα	0.6	<0.001	0.5	(0.5, 0.6)
	Naïve	Untreated	8.2	-	-	-
		SM	31.8	<0.001	3.9	(2.9, 5.2)
		IFNα	8.3	NS	1.0	(0.8, 1.3)
	NR	Untreated	4.7	-	-	-
		SM	23.7	<0.001	5.0	(3.1, 8.2)
		IFNα	5.8	NS	1.2	(0.8, 2.0)
IL-6	NHD	Untreated	4.5	-	-	-
		SM	1419.1	<0.001	317.0	(257.5, 390.1)
		IFNα	13.6	<0.001	3.0	(2.5, 3.8)
	Naïve	Untreated	32.5	-	-	-
		SM	1306.2	<0.001	40.2	(28.5, 56.7)
		IFNα	61.2	<0.001	1.9	(1.3, 2.7)
	NR	Untreated	17.3	-	-	-
		SM	1137.6	<0.001	65.8	(37.3, 116.1)
		IFNα	35.5	<0.001	2.1	(1.2, 3.6)
IL-8	NHD	Untreated	9.8	-	-	-
		SM	40.7	<0.001	4.2	(3.6, 4.8)
		IFNα	3.2	<0.001	0.3	(0.3, 0.4)
	Naïve	Untreated	55.8	-	-	-
		SM	87.5	<0.001	1.6	(1.2, 2.0)
		IFNα	17.2	<0.001	0.3	(0.2, 0.4)
	NR	Untreated	39.0	-	-	-

		SM	43.9	NS	1.1	(0.7, 1.7)
		IFNα	11.4	<0.001	0.3	(0.2, 0.4)
IL-10	NHD	Untreated	0.9	-	-	-
		SM	1.6	<0.001	1.7	(1.5, 2.0)
		IFNα	0.6	<0.001	0.6	(0.6, 0.7)
	Naïve	Untreated	17.1	-	-	-
		SM	46.3	<0.001	2.7	(2.2, 3.3)
		IFNα	16.5	NS	1.0	(0.8, 1.2)
	NR	Untreated	13.6	-	-	-
		SM	33.7	<0.001	2.5	(1.8, 3.5)
		IFNα	11.7	NS	0.9	(0.6, 1.2)

Figure 1:

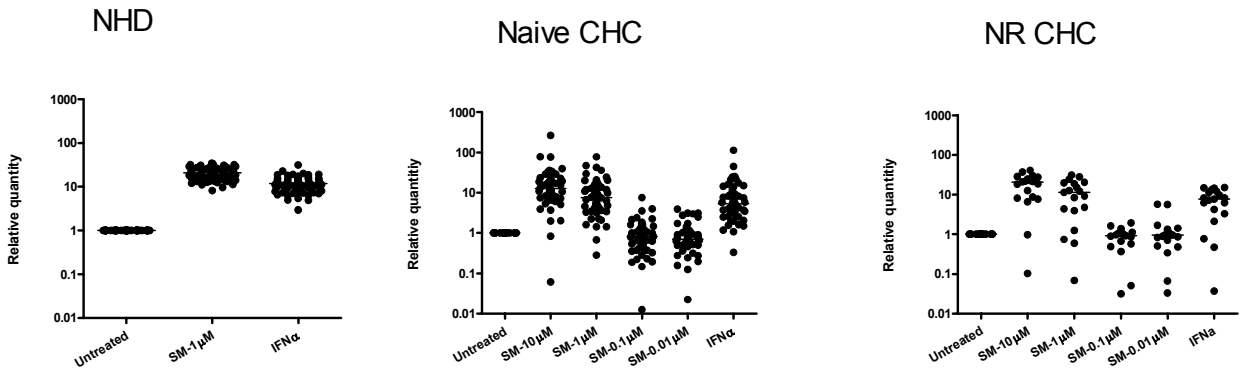

NHD
Naive CHC
NR CHC
Relative quantity
Relative quantity
Relative quantity
1000
100
10
1
0.1
0.01
Untreated
SM-1µM
IFNα
Untreated
SM-10µM
SM-1µM
SM-0.1µM
SM-0.01µM
IFNα
Untreated
SM-10µM
SM-1µM
SM-0.1µM
SM-0.01µM
IFNa

Figure 2:

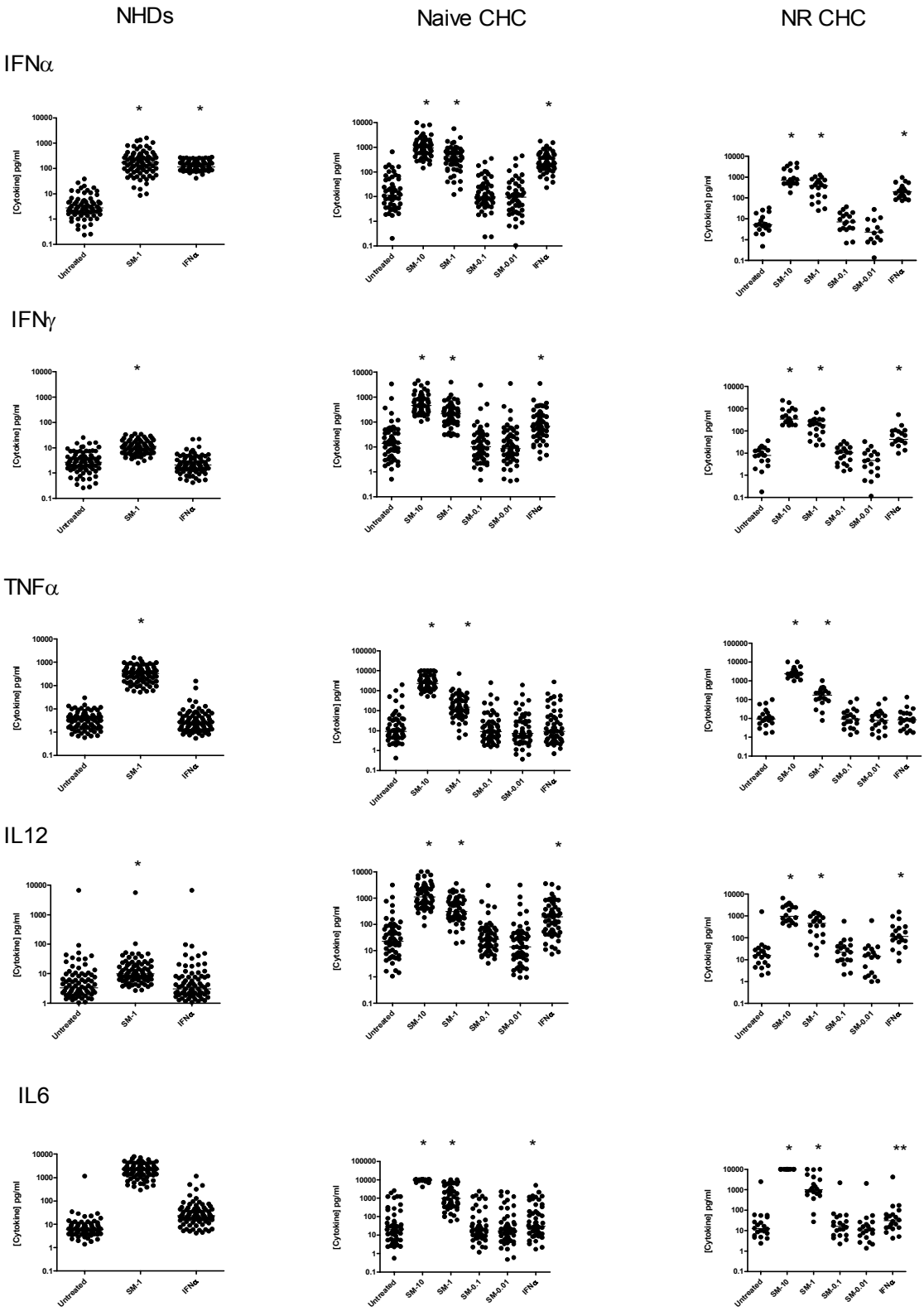

NHDs
Naive CHC
NR CHC
IFNα
IFNγ
TNFα
IL12
IL6
[Cytokine] pg/ml
Untreated
SM-10
SM-1
SM-0.1
SM-0.01
IFNα

Figure 3:

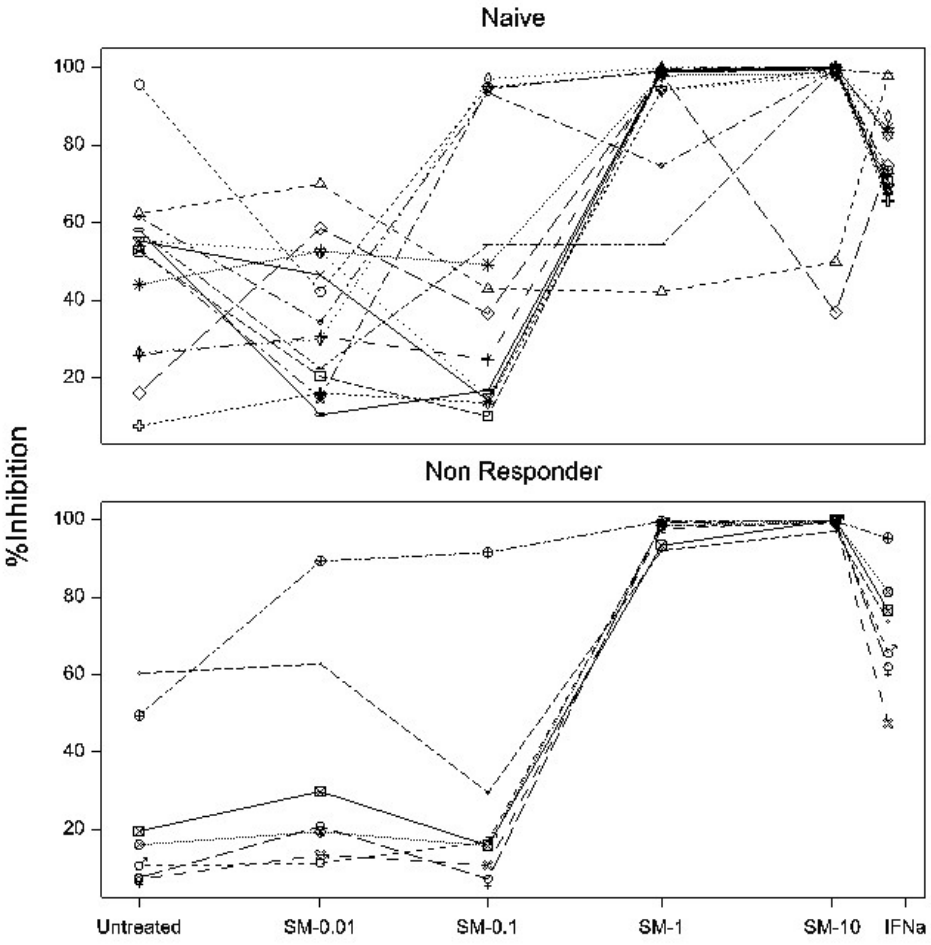

Naive
Non Responder
%Inhibition
100
80
60
40
20
Untreated
SM-0.01
SM-0.1
SM-1
SM-10
IFNa

Figure 4:

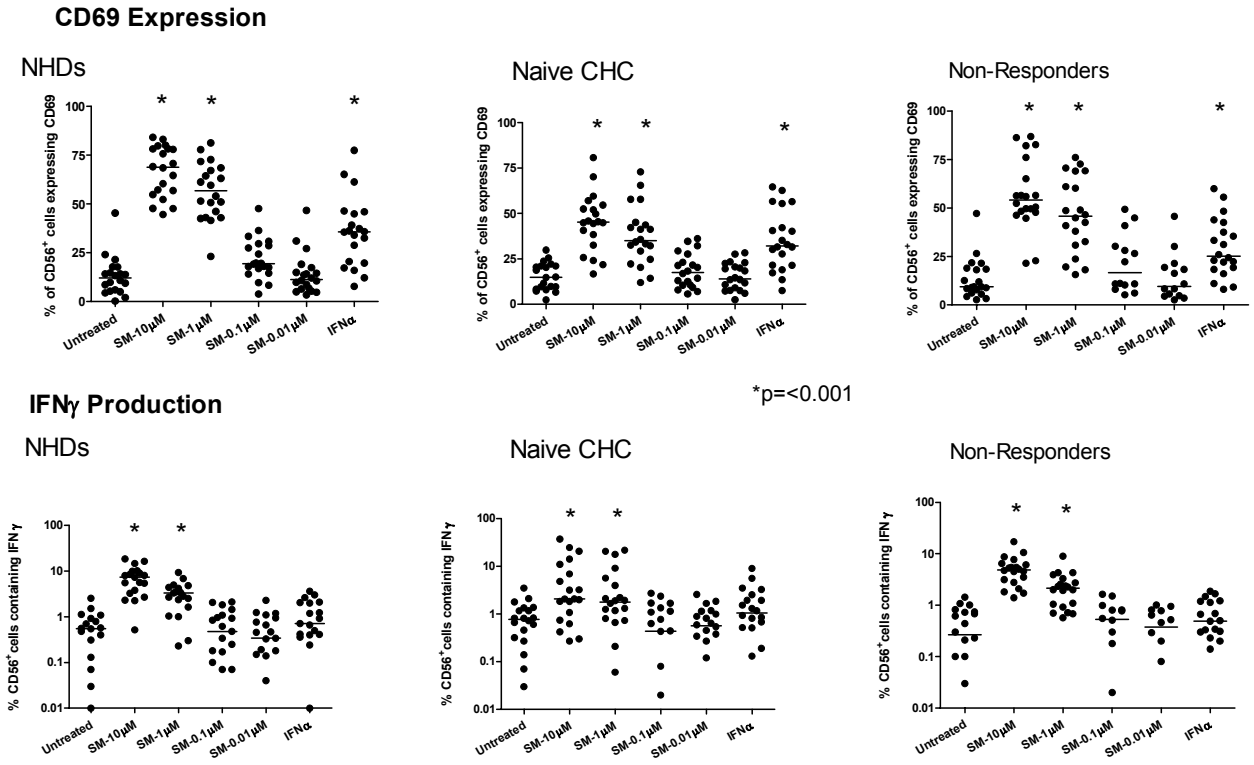
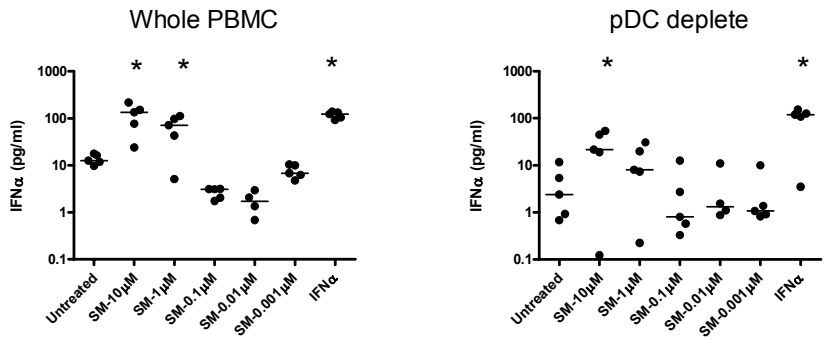

CD69 Expression
NHDs
Naive CHC
Non-Responders
% of CD56+ cells expressing CD69
Untreated
SM-10μM
SM-1μM
SM-0.1μM
SM-0.01μM
IFNα
*p=<0.001
IFNγ Production
NHDs
Naive CHC
Non-Responders
% CD56+cells containing IFNγ

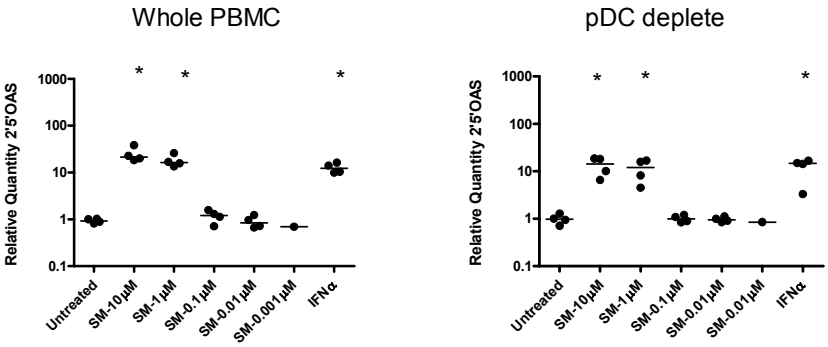
Figure 5:

A: NHDs

IFNα secretion:

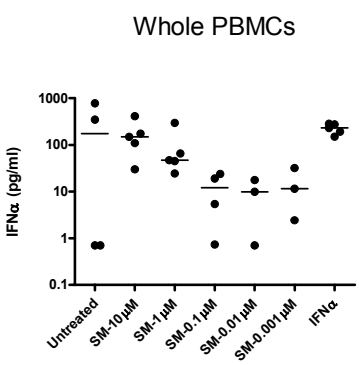


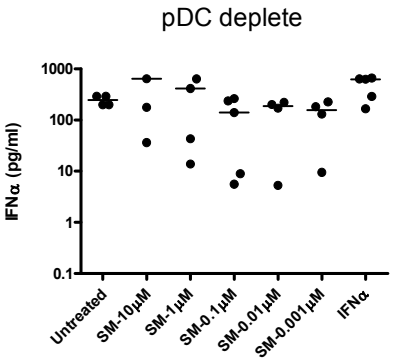
2'5' OAS Expression:



B: CHC

IFNα secretion:





2'5' OAS Expression: