Investigation into the role of novel interferon lambdas in the treatment of viral disease, primarily hepatitis C

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Rhiannon May Lowe

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Academic supervisor: Professor Mark R. Thursz

Industrial supervisor: Dr Peter Morley

Hepatology Section
School of Medicine
Imperial College London
St Mary's Campus
Norfolk Place
Paddington
London, W2 1PG

Abstract

Interferon lambdas (IFNλs), termed IFN-λ1, IFN-λ2 and IFN-λ3, or IL-29, IL-28A and IL-28B are a recently identified family of cytokines with antiviral activity. IL-28A/B and IL-29 bind to a novel heterodimeric receptor complex formed between IL-28 receptor (IL-28R) and IL-10 receptor (IL-10R2). Type I IFNs are used therapeutically in the treatment of chronic hepatitis B and C; however only ~30% of patients with hepatitis B virus will be successfully treated and only ~60% of patients with chronic HCV. New interventions are therefore required to address this unmet medical need and this thesis aimed to evaluate the potential use of IFNλs in treating viral infection.

A range of *in vitro* antiviral assays were developed to determine which viruses were inhibited by IFNλs. Results showed IL-28A and IL-29 have antiviral effects with HCV 1a and 1b replicons and HBV. No antiviral effect was demonstrated against dengue, RSV or HIV. Gene expression stimulated by IFNλ was compared with IFNα; and the effects of IFNλ against HCV were investigated. The types of genes induced, and the kinetics of gene induction were similar between the type I and type III IFNs in the HCV replicon cell line. With the parental cell line, the interferon signalling pathway was the most greatly affected by IFNα, IL-28A and IL-29, but IL-29 strongly regulated the antigen presenting pathway compared with IFNα. IL-28R distribution was determined to investigate the tissue and cellular distribution of IFNλ responsive cells. IL-28R was expressed in epithelial tissues, lymphoid tissue, spleen, liver, kidney and thymus, with majority of IL-28R expression on macrophages and dendritic cells.

The differences between type I and type III IFNs need to be further investigated but these differences identified provide a rationale for exploring the use of type III IFNs as an alternative to IFNα in the treatment of viral diseases.

Statement of Originality

All work is my own unless acknowledged and all else is appropriately referenced.

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Abbreviations used

%	-	Percent
<	-	Less than
>	-	Greater than
±	-	Plus or minus
?	-	Less than or equal to
?	-	Greater than or equal to
~	-	Approximately
± s.d.	-	Plus or minus standard deviation
°C	-	Degrees Celsius
µg	-	Microgram
µL	-	Microlitre(s)
µM	-	Micromolar
A	-	Adenine
ANOVA	-	Analysis of variance
C	-	Cytosine
CALR	-	Calreticulin
CD (34)	-	Cluster of differentiation
cDNA	-	Complementary DNA
CH3	-	Methyl group
CLIP	-	Class II-associated invariant chain peptide
CNX	-	Calnexin
cRNA	-	Complementary RNA
Ct	-	Cycle threshold
Da	-	Dalton
DDX58	-	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (also RIG-I)
DMSO	-	Dimethylsulphoxide
DNA	-	Deoxyribonucleotide acid
DTT	-	Dithiothreitol
dUTP	-	Deoxyuridine Triphosphate
e.g.	-	For example
EC50	-	Effective concentration 50 percent
EDTA	-	Ethylenediaminetetraacetic acid
EGF	-	Epidermal growth factor

ELF2	-	E74-like factor 2
EPSTI1	-	Epithelial stromal interaction 1
g	-	Gram(s)
G	-	Guanine
G1P3	-	Interferon, alpha-inducible protein 6
GM-CSF	-	Granulocyte macrophage colony-stimulating factor
GPCR	-	G protein coupled receptors
GSK	-	GlaxoSmithKline
HCl	-	Hydrochloric Acid
HETE	-	5-Hydroxyeicosatetraenoic acid
HLA (A)	-	Human leukocyte antigen
HPETE	-	Arachidonic acid 5-hydroperoxide
IFI (6)	-	Interferon, alpha-inducible protein
IFIH1	-	Interferon induced with helicase C domain 1
IFIT1	-	Interferon-induced protein with tetratricopeptide repeats 1
IFITM(1)	-	Interferon induced transmembrane protein 1
Ig (G)	-	Immunoglobulin
IGF-1	-	Insulin-like growth factor 1
IMS	-	Industrial methylated spirit
IPA	-	Ingenuity pathways analysis
ISGF3G	-	Interferon regulatory factor 9
IU	-	International Unit
LPS	-	Lipopolysaccharide
M	-	Molar
MAPK	-	Mitogen-activated protein kinase
mg	-	Milligram(s)
mL	-	Millilitre(s)
mM	-	Millimolar
mRNA	-	Messenger RNA
nM	-	Nanomolar
Oligo(s)	-	Oligonucleotide(s)
OPD	-	O-phenylenediamine
PARP9	-	Poly (ADP-ribose) polymerase family, member 9
PBMC	-	Peripheral blood mononuclear cell
PCR	-	Polymerase chain reaction

PEG	-	Polyethylene glycol
pg	-	Picogram
pH	-	Potential Hydrogen
PLSCR1	-	Phospholipid scramblase 1
RNA	-	Ribonucleotide acid
rpm	-	Revolutions per minute
RT-QPCR	-	Real-time quantitative PCR
SUMO-1	-	Small Ubiquitin-like Modifier
SUPT16H	-	Suppressor of Ty 16 homolog
T	-	Thymine
TGF	-	Tumor growth factor
Th1	-	T helper cell
TNF	-	Tumor necrosis factor
TRAF	-	TNF receptor-associated factor
T-Reg	-	Regulatory T cell
VEGF	-	Vascular endothelial growth factor

Chapter 1

1. General Introduction

1.1 Introduction to interferons and the discovery of type III interferons

Type I interferons (IFNs) have been considered the gold standard for antiviral protection since their discovery over 50 years ago[1]. Interferons (IFNs) are defined by their ability to induce resistance to viral infection. However the recent discovery of type III interferons may add a new dimension to conventional treatment regimes.

Interferon lambdas (IFNλs) are newly identified cytokines jointly discovered in 2002/3[2,3] termed IFN-λ1, IFN-λ2 and IFN-λ3[2] or interleukin (IL) IL-29, IL-28A, IL-28B respectively[3]. These cytokines are the first novel IFN family (IFN type III) defined in more than 20 years[4]. Initial studies showed that interferon lambdas had antiviral properties *in vitro* with encephalomyocarditis virus (EMCV)[3], stimulated interferon sensitive genes (ISGs)[2,3] and both groups described that the activity of the interferon lambdas is achieved through a novel receptor interferon-lambda receptor 1 (IFN-λR1)[2] also known as interleukin-28 receptor (IL-28R)[3].

Interferon lambdas share similar expression patterns with type I IFNs and trigger common signal transduction cascades and sets of stimulated genes. Both type I and type III IFNs share many biological activities, including the ability to induce an antiviral state in cells. IFNs protect cells from virus infection, directly by inducing ISGs such as 2', 5'-oligoadenylate synthetase 1 (2', 5'-OAS) and myxovirus resistance-A (MxA) and intracellular proteins[5] and, indirectly, by inducing major histocompatibility complex (MHC) class I antigen expression on and activation of antigen-presenting cells, stimulating dendritic cell maturation and activating macrophages and natural killer cells[6].

There are three families of interferons: type I interferons, like interferon alpha family (IFNαs) and interferon beta (IFNβ), which show potent antiviral activity; type II interferon like interferon gamma (IFNγ), which shows weak antiviral activity but is a strong activator of cellular immune responses; and now type III

interferons with novel IFNλs. In humans, the type I IFN family comprises at least 13 nonallelic IFNα genes, IFNβ, IFNω, IFNκ and the limitin gene[7]; type II is IFNγ and the novel type III interferons IL-28A, IL-28B and IL-29. IL-28A, IL-28B and IL-29 are weakly related to type I IFNs at the amino acid level but have a genetic structure more similar to IL-10. IL-28A and IL-28B share 96% amino acid identity and IL-29 shows 81% homology to IL-28[3]. Genes for all three members of the IFNλ family are found on chromosome 19 (q13.13 region)[3], whereas the genes for all type I IFNs are clustered on human chromosome 9[8,9]. The gene for IFNγ is located on chromosome 12[10]. Type I IFN genes lack introns, but the coding regions of the IFNλ genes are interrupted by 4 introns, and the positions of the introns with respect to the protein reading frames are conserved for the IFNλ genes and for genes encoding IL-10-related cytokines[11]. Even though the amino acid identity of type III IFNs is lower than even the most distant members of the type I IFN family, and the fact that they are clustered on different chromosomes, the conserved cysteine pattern and amphipathic profile[3] of the IFNλs suggest they belong to the helical cytokine family and appear to be an evolutionary link between IL-10 and type I IFNs.

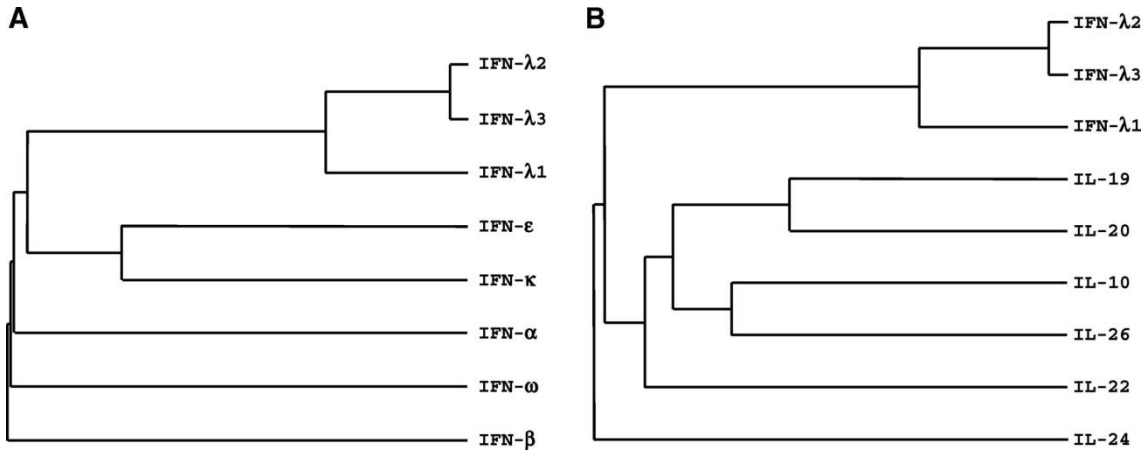


Figure 1.1: A phylogenetic alignment of the class II cytokine family genes

Figure taken from Donnelly *et al*[12] Alignment of the human interferon-λ (IFN-λ) genes with either (A) the human type I IFN genes or (B) the human interleukin-10 (IL-10)-related cytokines was used to generate a phylogenetic tree for the class II cytokine genes.

IFN-λ genes are clustered together on human chromosome 19 (19q13.13 region) or murine chromosome 7 (7A3 region). There are three functional IFN-λ genes in the human genome IFN-λ1 (IL-29), IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B) and 1 pseudogene IFN-λ4ψ, whereas there are only 2 functional IFN-λ

coding genes in the murine genome: IFN-λ2 (IL-28A) and IFN-λ3 (IL-29), mIFN-λ1Ψ and mIFN-λ4Ψ genes are pseudogenes. Studies have shown that the mutated mIFN-λ1 gene does not encode a functional IFN-λ1 protein[13]. The high degree of homology between the IFN-λ genes suggesting that the genes evolved from a common predecessor, IFN-λ3 (IL-28B) gene is almost identical to the IFN-λ2 (IL28A) gene not only in the coding region but also in the upstream and downstream flanking sequences. The promoters of the IFN-λ2 and IFN-λ3 genes are very similar and share several common elements with the IFN-λ1 promoter, suggesting that all 3 genes are likely to be regulated in a similar manner[14,15].

Type I and type III IFN genes have similar expression patterns, this is likely to be due to common regulatory elements in the promoters of the type I and type III IFN genes. Promoters of IFN-λ genes have predicted binding sites for transcription factors: AP1 (dimeric factor containing members of the JUN, FOS, ATF and MAF protein families), nuclear factor κB (NF-κB) and interferon regulatory factors (IRFs), crucial for the induction of type I and type III IFN expression[14,15]. IFN-λ1 is controlled by either IRF3 or IRF7, but importantly IFN-λ2/3 like IFNα genes are more dependent on IRF7[15]. IRF3 is ubiquitously expressed in cells and upon direct viral activation upregulates IFN-λ1 and IFNβ representing early response genes. IFN-λ2/3 gene expression is mainly controlled by IRF7, thus resembling those of IFNα genes with delayed kinetics conforming to a positive feed-back mechanism. The regulation of type III IFNs may differ in mice, however, since there is no functional IFN-λ1 gene in the murine genome[13]. Co-expression of type I and type III IFNs in response to diverse viruses and various TLR agonists has been shown by multiple cell types but plasmacytoid dendritic cells (pDCs) constitutively express IRF7, enabling these cells to rapidly produce high levels of type I and type III IFNs upon stimulation[16]. Differences in type I and type III IFN expression have been reported, including IFN-λs were shown to be the main IFN type produced by both murine and human airway epithelial cells in response to various respiratory viruses[17,18], a model is proposed for IFN-λ1 gene regulation, in which IRF and NF-κB activate gene expression independently via spatially separated promoter elements[19] showing type III IFNs can be induced through IRFs or NF-κB unlike IFNα which needs multiple transcription factors. Though IFN-λ is typically

activated by viral infections, showing that it is an important part of the innate immune response, activation of TLR-4 by bacterial LPS has been shown to induce IFN-λ in DCs[16,19] highlighting a role of IFN-λ in the modulation of the immune response. Interferon lambdas also have a role in antiviral immunity through modulation of both the maturation and differentiation of immune cells. Differentiation of monocytes into dendritic cells leads to upregulation of IL-28R and an increased ability to express IFN-λ[20,21,22,23]. When DCs are then exposed to IFN-λ, increased maturation and migration capacity are induced[22]. Interferon lambda influences the effects of DCs on interactions with T cells. DCs treated with IFN-λ preferentially expand regulatory T cells, which negatively regulate the immune response, and promote self-tolerance[20,22]. IFN-λ appears to be primarily focused on biasing T cell differentiation against Th2 development and Th2 cytokine secretion but also modulating Th1 cells [24,25,26,27,28]although this role is not fully clear. Interferon lambdas are an important part of the innate immune response and have a role in the modulation of the adaptive immune response.

1.2 Antiviral properties of interferon lambdas

Like IFNα, IL-28A and IL-29 have antiviral properties and potentially could be used as or incorporated into another therapeutic approach to treating viral disease. Initial studies by Sheppard *et al* in 2003 showed that IL-28A and IL-29 induced antiviral effects in response to encephalomyocarditis virus (EMCV) and stimulated interferon sensitive genes *in vitro* (Figure 1.2)[3].

As IFNλs had been shown to have antiviral properties it was an aim of this thesis to investigate the antiviral properties of IFNλs *in vitro* against a panel of viruses including hepatitis C (HCV), hepatitis B (HBV), human immunodeficiency virus (HIV), dengue fever and respiratory syncytial virus (RSV), with a primary focus on HCV.

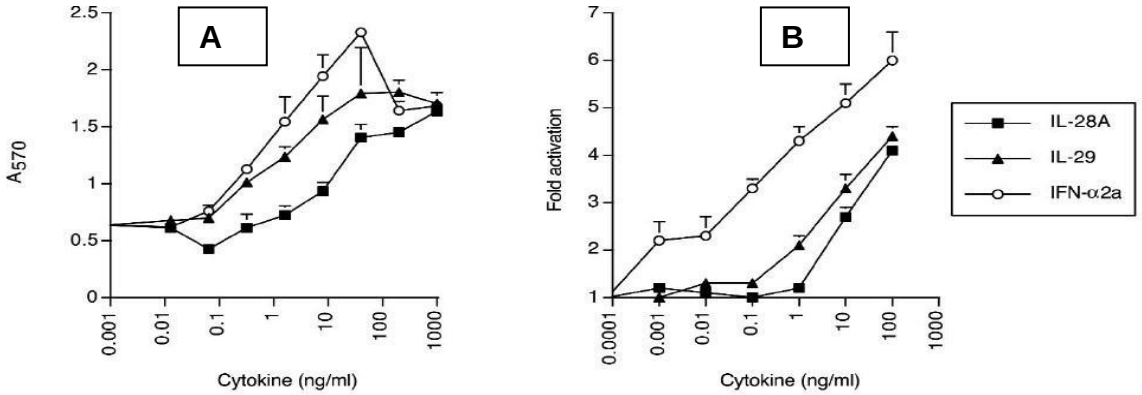


Figure 1.2: Graphical representations of IFNα, IL-28A and IL-29

EMCV assay **A** and interferon sensitive gene induction with ISG **B**

A Antiviral activity of IL-28A and IL-29. IL-28A, IL-29 and IFNα2a (IFNα) were added at varying concentrations to HepG2 cells before EMCV infection and dye-uptake was measured. Mean ±s.d. A570 values, which were directly proportional to antiviral activity, are shown (n = 3).

B IL-28A and IL-29 signal through the IFN-stimulated response element (ISRE). HepG2 cells were transfected with an ISRE reporter plasmid and luciferase activity was analysed after treatment with varying concentrations of IL-28A, IL-29 or IFNα2a. Fold activation was determined by dividing the relative light units (RLU) of each experimental sample by the RLU of media alone. Mean ±s.d. data are shown (n = 2). Figure taken from Sheppard[3].

Hepatitis C (HCV) has a positive-sense single stranded RNA genome and is classified as a *flavivirus*. The hepatitis C virus particle consists of a core of genetic material (RNA), surrounded by an icosahedral protective shell of protein, and further encased in a lipid (fatty) envelope of cellular origin. Two viral envelope glycoproteins, E1 and E2, are embedded in the lipid envelope[29]. The HCV genome is 9600 nucleotides and comprises of two non-coding regions in 5' and 3' flanking a large reading frame which codes for a polyprotein of 3000 amino acids; this polyprotein is further cleaved into structural (C, El, E2) and non- structural (NS1, NS2, NS3, NS4, NS5) proteins. The positive RNA acts as a cap-independent messenger; the transcription is mediated by the NS5 RNA polymerase. After the maturation step, the virion is liberated by budding through the cytoplasmic membrane.

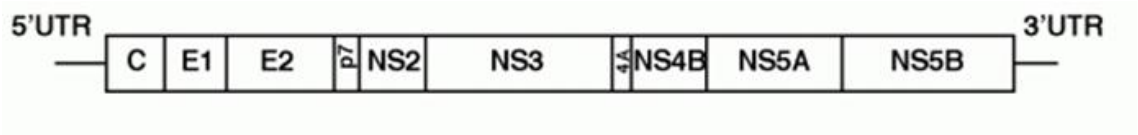


Figure 1.3: Organization of Flaviviridae genome HCV

Figure adapted[30]to show organization of Flaviviridae genome, HCV showing structural proteins made by the hepatitis C virus include Core protein, E1 and E2; nonstructural proteins include NS2, NS3, NS4, NS4A, NS4B, NS5, NS5A, and NS5B.

The structurural proteins made by HCV are further described below. Core protein is a highly basic, RNA-binding protein, proteins of various sizes (17 to 23 kDa) are described but the 21-kDa core protein (P21) appeared to be the predominant form[31]. The core protein contains three distinct predicted domains: an N-terminal hydrophilic domain of 120 aa (domain D1), principally involved in RNA binding and nuclear localization, a C-terminal hydrophobic domain of about 50 aa (domain D2), responsible for core protein association with endoplasmic reticulum (ER) membranes, and the last 20 or so aa that serve as a signal peptide for the downstream envelope protein E1[32]. Envelope proteins E1 and E2 are highly glycosylated and have a key role in cell entry, the p7 protein, a 63 amino acid membrane spanning protein which locates itself in the endoplasmic reticulum, is dispensable for viral genome replication but plays a critical role in virus morphogenesis[33]. NS2 protein is a 21-23 kDa

transmembrane protein with protease activity, NS2 is a short-lived protein that loses its protease activity after self-cleavage from NS3 and is degraded by the proteasome[34]. NS3 is 67 kDa protein whose N-terminal has serine protease activity and whose C-terminal has NTPase/helicase activity. NS4A is a cofactor of NS3 protease activity. NS3-4A also bears additional properties through its interaction with host cell pathways and proteins that may be important in the lifecycle and pathogenesis of infection, NS3-NS4A protease is a popular viral targets for anti-HCV therapeutics[35]. NS4B is a small (27 kDa) hydrophobic integral membrane protein with 4 transmembrane domains. It is located within the endoplasmic reticulum and plays an important role for recruitment of other viral proteins[36]. NS5A is a hydrophilic phosphoprotein which plays an important role in viral replication, modulation of cell signaling pathways and the interferon response. The NS5B protein (65 kDa) is the viral RNA dependent RNA polymerase. Until recently when the complete replication of hepatitis C virus in cell culture was demonstrated[37], the lack of a robust tissue culture system for HCV meant that HCV was studied using a replicon system[38], where the effects of IFNs on HCV replication *in vitro* can be studied[39]. Replication of HCV involves several steps. The virus replicates mainly in the hepatocytes of the liver, where each infected cell produces approximately 10 virions per day[40] and NS5B produces mutations at rate 10^{-4} per nucleotide[41], this key in using current direct acting antivirals (DAA) in the treatment of HCV, the risk of rapid development of resistance, without the use of cotreatment of IFN. Entry into host cells occur through interactions between virions and cell-surface molecules CD81, LDL receptor, SR-BI, DC-SIGN, Claudin-1, and Occludin[42]. HCV uses portions of the intracellular machinery to replicate[43]. The HCV genome is translated to produce a 3000aa protein, which is processed by viral and cellular proteases to produce three structural and seven nonstructural (NS) proteins. The NS proteins then recruit the viral genome into an RNA replication complex. RNA replication takes places via the viral RNA-dependent RNA polymerase NS5B, which produces a negative strand RNA intermediate. The negative strand RNA then serves as a template for the production of new positive strand viral genomes. Nascent genomes can then be translated, further replicated or packaged within new virus particles. New virus particles are thought to bud into the secretory pathway and are released at the cell surface.

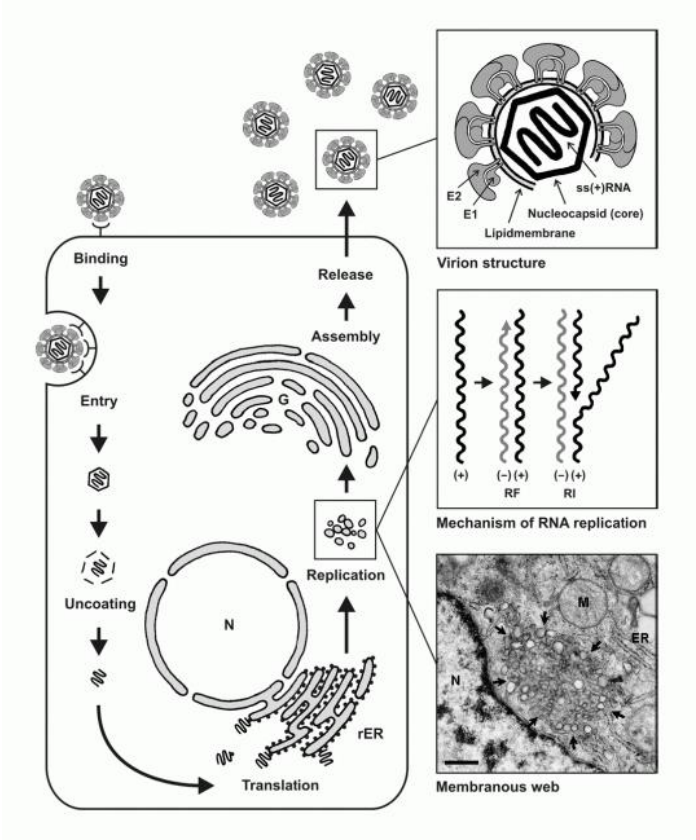


Figure 1.4: Hypothetical HCV replication cycle

This figure is taken from Bartenschlager et al., 2004[30]. HCV particles bind to the host cells via a specific interaction between the HCV envelope glycoproteins and a yet unknown cellular receptor. After the viral genome is liberated from the nucleocapsid and translated at the rough ER, NS4B induces the formation of membranous vesicles (referred to as the membranous web; EM in the lower right). These membranes are supposed to serve as scaffolds for the viral replication complex. After genome amplification and HCV protein expression, progeny virions are assembled. Newly produced virus particles may leave the host cell by the constitutive secretory pathway. The upper right panel of the figure shows a schematic representation of an HCV particle. The middle panel shows a model for the synthesis of negative-stranded (−) and positive stranded (+) progeny RNA via a double-stranded replicative form (RF) and a replicative intermediate (RI).

HCV is sensitive to IFNα, IFNβ and IFNγ[44] but at the time this project was initiated (August 2004) there was not any published data on the role of type III interferons in HCV. Pegylated IFNα in combination with ribavirin is the current standard of care for the treatment of patients chronically infected with HCV; it results in a sustained virological response (defined as clearance of circulating

HCV RNA at 6 months post-treatment) in ~50% of HCV patients[45]. However, combination therapy remains less effective against infections caused by HCV genotype 1 which constitute ~75% of all HCV infections in the developed world. IFNα therapy is administered intravenously and has side effects such as flu-like symptoms, anorexia, depression, haemolytic anaemia and myelosuppression that can be severe and sometimes dose limiting. Approximately 500 million people worldwide are living with either hepatitis B or hepatitis C[46]. This represents 1 in 12 people, and was the basis for the 2008 'World Hepatitis Day Am I Number 12?' campaign. HCV affects ~ 170 million people worldwide, with a seroprevalence in the United States and Japan of ~2%, and in Western Europe of 1-1.9%[47] (Figure 1.5). HCV infection can lead to liver fibrosis, cirrhosis and liver cancer[14].

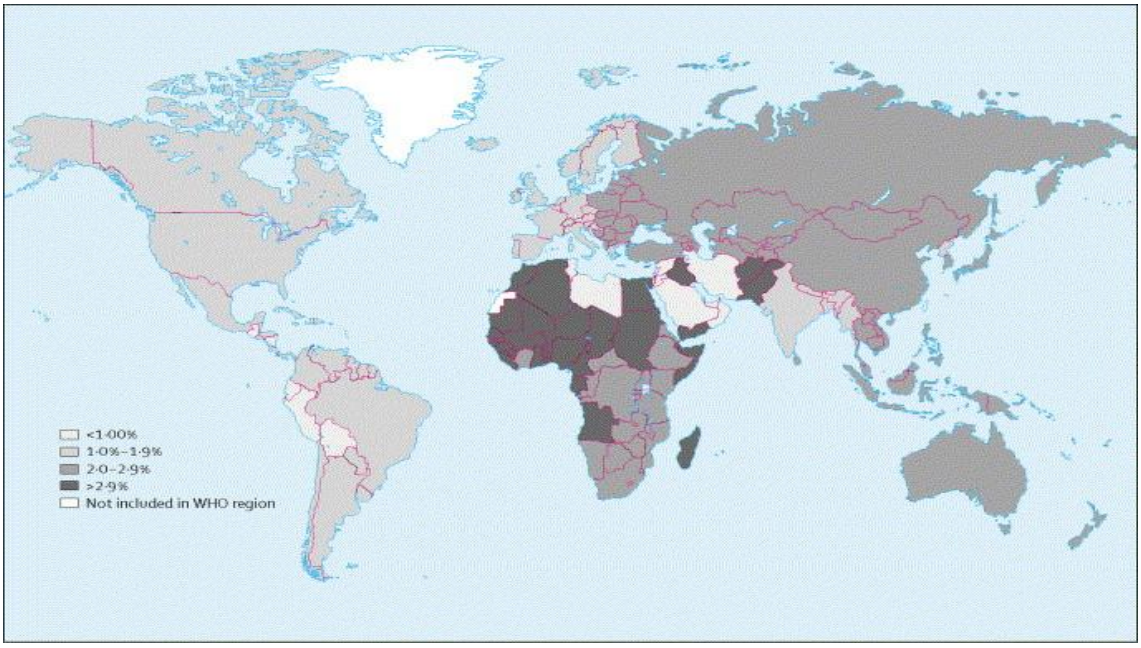


Figure 1.5: Geographical representation of HCV prevalence

Percentage of the population infected with HCV globally. Figure from Shepard 2005[48]

The HCV genome exhibits a high degree of variability, especially in the E2/NS1, El, NS3 and NS5b regions, resulting in at least 6 genotypes of HCV numbered from 1 to 6 in Simmonds' classification[49] and over 100 subtypes. Genotype 1, 1a and 1b accounts for 60% of all HCV cases in USA and Europe and in Japan 73% is 1b. Geneotype 2 is 8% in Europe; Genotype 3 is 60% in India and 58%

in Iran. Genotype 4 has been associated with 20% of chronic infections worldwide and is the genotype in 7% of Northern Europe HCV cases and 24% of Southern Europe, Genotype 4a makes up 90% of HCV cases in Egypt, Egypt has 13% of its population diagnosed with HCV. Genotype 5 is in 40% of the HCV cases in South Africa and genotype 6 is a diverse genotype now including genotypes originally classes as 7,8, and 11, it is the genotype in 30% of HCV cases in Asia and 20% in China. Genotype is clinically important in determining potential response to therapy, for example genotypes 1 and 4 are less responsive to interferon-based treatment than are the other genotypes (2, 3, 5 and 6)[49].

Hepatitis B, which is caused by the hepatitis B virus (HBV), affects 350 million people worldwide and is responsible for one million deaths each year. HBV infection, like HCV, can lead to liver fibrosis, cirrhosis and liver cancer. Hepatitis B is the primary cause of liver cancer (60-80%), (WHO 2006). HBV is part of the *hepadnaviridae* (hepatitis DNA viruses) family. Hepatitis B is typically treated with interferon alpha (IFNα) which results in ~30% seroconversion, or nucleoside reverse transcriptase inhibitors (NRTIs) such as adefovir, entecavir, lamivudine, telbivudine, and tenofovir. Lamivudine, a nucleoside analogue, results in ~40% seroconversion, compared with 5% seroconversion with no treatment. As with HCV, there is a need for new treatments with increased efficacy. The effect of HBV and IFNα has been studied *in vivo*[50]. IFNλs have been shown to reduce levels of HCV mRNA in the HCV replicon system and reduce expression of HBV DNA in murine cells[51].

Human immunodeficiency virus (HIV) is an RNA reverse transcribing virus, part of the *retroviridae* family of viruses. Approximately 40 million people worldwide are living with HIV (WHO statistics 2005). HIV infection leads to the loss of CD4 cells, which can result in immunosuppression (Acquired immune deficiency syndrome (AIDS)). There are currently a variety of treatments for HIV: IFNα, cell fusion inhibitors, reverse transcriptase inhibitors and protease inhibitors. Treatment options are often limited due to cross resistance. Overall prevalence of HIV drug resistance is on the increase[52], so alternative therapies are still being developed for the treatment of HIV.

Dengue fever is a major threat to public health in many tropical and subtropical countries. There are an estimated 50 million infections per year (WHO 2006). Dengue is a *flavivirus* which circulates as four major serotypes and is passed on from an infected mosquito bite. Dengue haemorrhagic fever and dengue shock syndrome are the more severe manifestations of dengue infection and primarily occur on re-infection with a virus of differing serotype to the initial infection. IFNα in the dengue replicon system has been shown to have no antiviral effect and that dengue virus specifically inhibits IFNα signalling by the down-regulation of signal transducers and activators of transcription protein (STAT) STAT2 expression[53], however the effects on dengue with IFNλs remain to be determined.

Respiratory syncytial virus (RSV) is a single stranded RNA *paramyxovirus*, which is the same family as para-influenza, measles and rubella. RSV infection is recognised as the leading cause, of bronchiolitis and pneumonia in infants and young children[54]. RSV is highly contagious and in immunocompromised patients can lead to persistent infection. Treatment options are currently limited to monoclonal antibody based therapy and supportive care; ribavirin has been used historically.

Type III interferons have also been implicated as having antiviral properties in a range of viruses: DNA viruses; poxvirus[55], murine cytomegalovirus (CMV)[56,56], hepatitis B virus[57,51,58] and herpes simplex virus 1 and 2[59], the single stranded (ss) (+) RNA viruses EMCV[60,3], West Nile virus[57] and hepatitis C virus[57,61,62,51], as well as the ss (−) RNA viruses influenza-A virus[63] and vesicular stomatitis virus,[62]. IL-28A and IL-29 has also been shown to modulate anti-viral proteins like 2′5′-oligoadenylate synthetase[2,3], MxA protein, and IFN-inducible double stranded (ds) RNA-activated protein kinase, and multiple interferon sensitive genes (ISGs)[56,57,2,64,62,65].

1.3 IL-28 receptor and signalling

IFNα and IFNβ (type I IFNs) bind to a specific and distinct heterodimeric receptor composed of IFNARα and IFNARβ. Binding of IFNα or IFNβ to their receptor leads to the activation of two receptor-associated tyrosine kinases, Janus kinase (Jak)1 and tyrosine kinase (Tyk)2; this is followed by tyrosine

phosphorylation of the Signal Transducers and Activators of Transcription (STAT)1 and STAT2 proteins. Phosphorylated STAT1 and STAT2 combine with IFN-regulatory factor 9 (IRF-9) to form the trimeric IFN-stimulated gene factor-3 (ISGF-3) complex, which, upon translocation to the nucleus, binds to the cis element IFN-stimulated response element (ISRE), upstream of IFN-inducible genes, and modulates their transcription[66,67].

Type I, II and III IFNs are class II cytokine receptor ligands (CRF2) which also include interleukin 10 (IL-10) and IL-10 related proteins[11].The activity of the type III IFNs is achieved through a heterodimeric receptor complex which, is formed between IL-28A receptor (IL-28R) and IL-10 receptor (IL-10R2). The promiscuous IL-10R2 is also part of the IL-10 and IL-22 receptor complex and is involved in signal transduction for IL-10 and IL-22 (Figure 1.6). Other key ligands have now been shown to include IL-28A, IL-28B and IL-29[2,3].

IFNλs were initially shown to activate both STAT1 and STAT2 and the downstream signalling pathways including interferon sensitive gene complex ISGF3 and ISRE, similar to those activated by IFNα and IFNβ[2]. IFNλs like IFNα and IFNβ have been shown to activate STAT3, 4 and 5[68]. Some of IFNα and IFNβ immunomodulatory functions are mediated via STAT4[69].

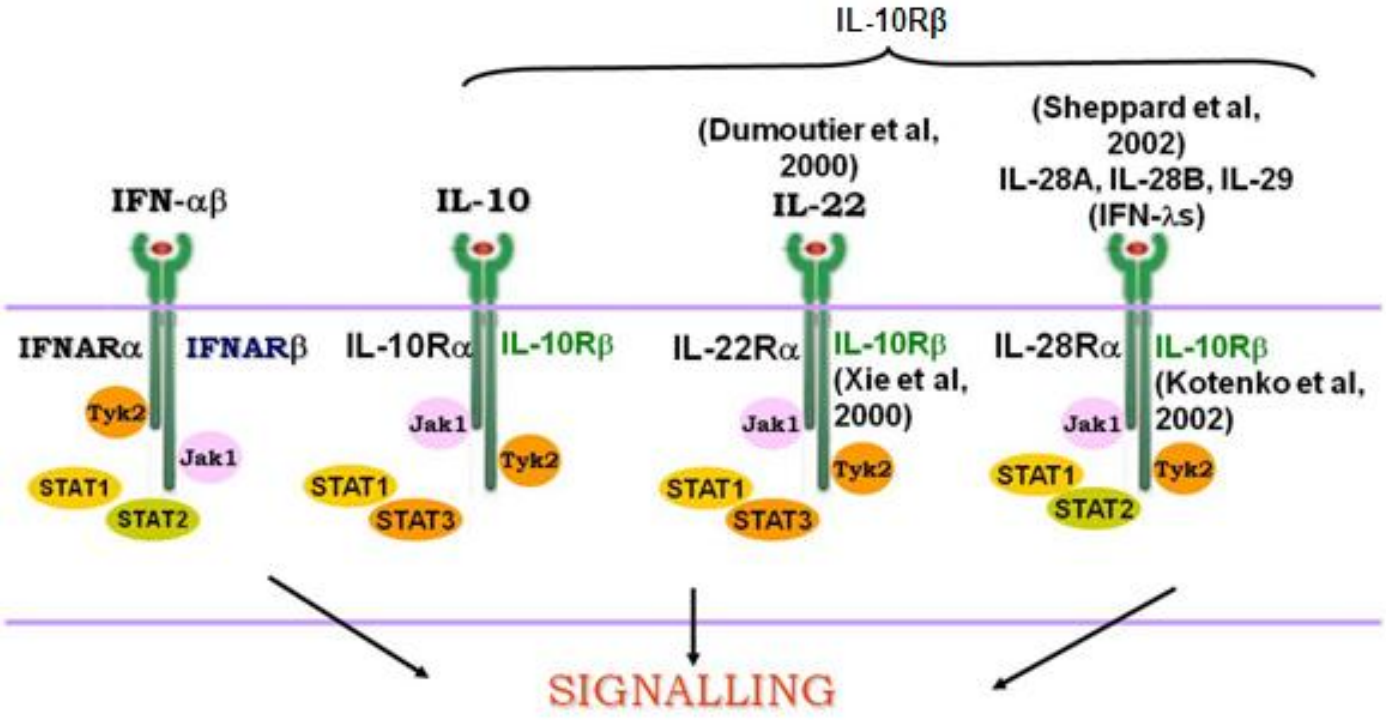


Figure 1.6: IL-10Rβ is a promiscuous cytokine receptor

Figure 1.6 adapted from Dumoutier, Sheppard, Kotenko and Xie[2,3,70,71] shows that part of this heterodimeric receptor is the promiscuous interleukin 10 receptor beta (IL-10Rβ) which has a role in cytokine binding with not only the IFNλs but IL-10 and IL-22. However the downstream signalling cascade shows that IFNλs cause STAT1 and 2 activation. This signalling pathway shares similarities with the IFNAR receptor.

Despite signalling through distinct receptor complexes, type I and type III IFNs trigger similar signalling pathways (Figure 1.7), leading to the activation of a transcriptional complex designated ISGF3 which is a unique and crucial mediator of type I and type III IFN-induced biological activities. ISGF3 binds to the ISRE in the promoters of ISGs leading to gene transcription.

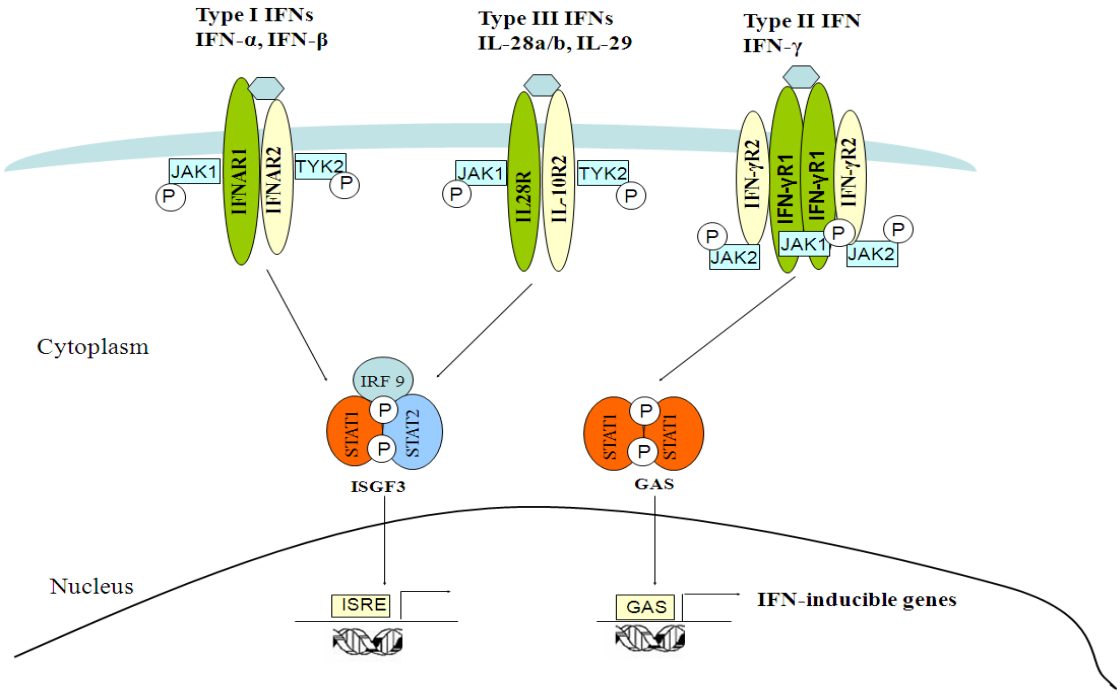


Figure 1.7: Type III IFN receptor signalling compared to type I and II

Type I, II and III IFN receptor binding leads to activation of STAT, the translocation of STAT into the nucleus activates either interferon-stimulated genes (ISG) with a promoter interferon-stimulated response element (ISRE) or γ-activated sequence (GAS). Adapted Vilcek, J. Nat Immunol[4].

As well as the Jak-STAT pathway type I and type III IFNs can also induce signalling through mitogen-activated protein kinase (MAPK) cascades: stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK); extracellular signal-regulated kinase (ERK)-1/2; and mitogen-activated protein kinase p38 [56,65].

The heterodimeric receptor IFNAR1/2 for IFN-α and IFN-β is present on all nucleated cells[72]. IL-10R2 is ubiquitously expressed[73], whereas initial RNA expression data showed IL-28R is limited to specific tissues and cell types[2]. IL-

28R has been shown to be present in intestinal epithelial cells, MCA205, a fibrosarcoma cell line[56] and type III IFN expression has been shown in monocyte–derived dendritic cells (MDDC)[74,75,76], plasmacytoid dendritic cells (pDC)[74,76] and human primary macrophages where induction of type III IFNs required pre-treatment with IFNα[74,75,76]. Other studies showed *in vivo* experiments highlighting primary targets of type III IFNs are epithelial cells of the respiratory, gastro-intestinal and reproductive tracts[59,77,78]. IL-28R is predominantly expressed in epithelial cells and specific subsets of immune cells[77,79,78,80].

1.4 Transcriptomic analysis gene activation profiles and signalling pathways of IL-28A and IL-29

Transcriptional (mRNA) expression patterns have been shown for IFNα, β and γ[81], and for IL-29 in a HCV replicon cell line[61] and Raji cells[65]. Zhou[65] investigated gene expression at an early time point, 4 hours, with 200 IU of IFNα and 10ng/mL of IL-29 in triplicate on Affymetrix gene chips. 27 unique genes with greater than a two fold change were seen with IL-29 compared with 313 IFNα induced genes. Significance Analysis of Microarrays analysis (SAM) (Stanford University) showed that only 10 genes were unique to IFNα, the majority of genes were also present in the IFNλ dataset but due to IFNλ signal being weaker they did not reach the 2 fold cut off. IFNλ was shown to induce ISGs, none of which were determined to be unique to IFNλ. Marcello[61] also used Affymetrix microarray analysis, but used 5ng/mL IFNα and 10ng/mL of IL-29 in an HCV replicon system at looked at 3, 12 and 24 hour time points. Marcello showed in a dataset of 66 genes (being induced at least 2 fold in either IFNα or IL-29 at one or more time points); that IFNα and IL-29 induce most of the same genes (with no unique genes being expressed with IFNλ) but that over time the majority of IFNλ genes continue to increase by 24 hours whereas expression of IFNα genes are mainly decreased by 24 hours. Doyle[57] also reported that IL-29 stimulates nearly identical patterns of gene expression as IFNα as analysed by microarray. Microarray analysis as documented in this thesis was initiated prior to the publication of microarray studies described above[57,61,65]. There is still a need to fully identify the antiviral effects and modes

of action of type III interferons by pathway expression analysis. Microarray analysis described in this thesis additionally investigated IL-28A versus IFNα and work was carried out in a HCV HuH7 parent cell line.

Induction of similar sets of ISGs enables Interferon lambdas and IFNα to induce similar biological activities and functions primarily inducing an antiviral state in cells.

1.5 Alternative therapies for HCV and IFN lambda as a therapy

Chronic infection with hepatitis C virus affects 1:12 or approximately 3% of the world's population and causes approximately 350,000 deaths annually[46]. Pegylated interferonα (IFNα) in combination with the nucleoside analogue ribavirin is the current standard of care for the treatment of patients chronically infected with hepatitis C (HCV). Combination therapy results in a sustained virological response (defined as clearance of circulating HCV RNA at six months post-treatment) of only ~50% of patients with HCV genotype 1 infection[82]. IFNα therapy is administered parenterally and has side effects such as flu-like symptoms, anorexia, depression[83] and myelosuppression[84] that can be severe and often dose limiting[85]. In recent years, a number of new drugs against HCV have emerged. Direct-acting antivirals (DAAs) are specifically designed to inhibit viral targets, and host-targeted antivirals block host factors that are important for the viral lifecyle. Alternative interferons could be effective antiviral agents, without the side effects of IFNα.

Direct-acting antivirals, NS3/4A protease inhibitors inhibit viral protease NS3/4A is required for the cleavage of downstream, nonstructural proteins, including the NS5A protein and the NS5B RNA-dependent RNA polymerase (RdRp). In May 2011, NS3/4A protease inhibitors, telaprevir and boceprevir, were approved by the US Food and Drug Administration for the treatment of patients with chronic genotype 1 HCV infection. With the current DAAs there is rapid resistance due to rapid mutations of HCV[40,41,86,87]. The current DAAs are administered with peginterferon-alfa-2a and ribavirin to prevent resistance to the DAAs. Both telaprevir and boceprevir highly improve rates of SVR of HCV infected patients but there are limitations, designed to target genotype 1 HCV they have differential efficiency across the genotypes[88,89,90], they are also limited by side

effects, rash (Telaprevir) and anaemia and pharmcokinetic properties meaning that they have to be taken with fatty meals every 8 hours.

RdRp inhibitors inhibit the active site of polymerase activity, Preliminary results from Phase II trials reported rates of EVR >80% among patients with HCV genotype 1 or 4 infection who received mericitabine in combination with SOC PEG-IFN and RBV47; no viral rebound or resistance mutations were observed by week 12[91]. These inhibitors could be used without IFN. miR-122 inhibitors inhibit miR-122 which is a host liver-specific microRNA required for HCV replication. Miravirsen is in Phase IIa trials; and significantly reduces viral load after 8 weeks of therapy without significant adverse effects[92] but there are concerns with microRNAs with off target effects. Host factor cyclophilins are important for viral replication, Alisporivir is in advanced clinical trials, it inhibits viral replication by disrupting the interaction between cyclophilin A and NS5A[93,94]. Alisporivir combined with PEG-IFN and RBV, led to an SVR in 76% of HCV genotype 1 infection patients compared to 55% with SOC[94].

Zymogenetics licensed lambda interferons to Novo-Nordisk and now Bristol-Myers Squibb for viral infections including HCV. A pegylated form of IFN-λ1 (IL-29) is currently in clinical trials for the treatment of chronic HCV infection and initial reports shows less severe side effects than pegylated IFNα, phase 1b dose-escalation study, PEG−IFN-λ1 inhibited HCV without significant systemic toxicity and phase 2b data showed that the highest doses of PEG−IFN-λ1, when combined with RBV, produced a complete EVR in 30% of patients with HCV genotype 1 infection, compared with 28% of the patients who received the SOC[95,96]. Clinical development: A recent press release from Bristol-Myers Squibb (19 April 2012) showed that peginterferon lambda-1a (Lambda) plus ribavirin achieved sustained virologic response rates 24 weeks post-treatment (SVR24) that were comparable to peginterferon alpha-2a plus ribavirin in phase IIb EMERGE clinical trial in 118 treatment-naïve patients chronically infected with genotype 2 or (HCV). There may be a role for IL-28B treatment in the future, four Genome Wide Association studies have highlighted the significance of IL-28B within the innate immune response to HCV[97,98,99,100]. Two protective SNPs were shown to be associated with a SVR to IFNα/Ribavirin in patients with HCV genotype 1. These were the T allele of rs8099917 and the C allele

of rs12979860 located 8 kilo bases (kb) and 3 kb respectively upstream of the IL-28B gene. In a study of rapid viral response (RVR) (undetectable HCV RNA at 4 weeks) rates in patients with HCV Genotype 1 and 4, of the RVRs 100% of carriers of the protective rs12979860 C allele, and 64% of non-RVR individuals expressed the non-protective genotype T allele of rs8099917[101].

The potential broad roles of IFN-λs in immune function may also mean they play a future role in autoimmunity and cancer therapy. Type III IFNs like type I IFNs, may potentially be used for the treatment of other inflammatory or autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE)[102], or multiple sclerosis (MS)[103]. IFN-λs have been shown to have potent antitumor activities in murine models of cancer[104,105,106], highlighting potential as anti-cancer therapy.

1.6 Introduction summary

The biological significance of IFNλs remains to be fully determined. This thesis shows the investigation of antiviral activity of IFNλs, the similarities and differences between the type I and type III IFNs determined by microarray analysis, and the distribution of IL-28R and the significance of limited distribution in bone marrow assays.

1.7 Hypothesis

“Current treatment of antiviral diseases with type I IFNs can be inadequate. As IFNλs have antiviral activity and use a different receptor and signalling transduction pathways to IFNα, IFNλ therapy could be useful in the management of viral infections.”

1.8 Thesis Aims

1. Investigation of the antiviral activity of IL-28A and IL-29 on a panel of viruses *in vitro* and their effects on IFN sensitive gene models.
2. Transcriptomic analysis to determine gene activation profiles and signalling pathways of IL-28A and IL-29.
3. Investigation of IL-28R tissue expression in a panel of mouse and human tissues.
4. Further investigations to support hypothesis that IFNλ therapy could be an alternative to IFNα.

Chapter 2

2. Materials and methods

2.1 Investigation of the antiviral activity of IL-28A and IL-29 using antiviral and interferon sensitive gene model assays

2.1.1 Hepatitis C comparison of antiviral activity of IFNα, IFNβ, IFNγ, IL-28A and IL-29

The antiviral effects of interferon lambdas were investigated in a HCV replicon model which comprises of a HuH7 hepatoma derived cell line stably transfected with an autonomously replicating subgenomic HCV RNA[38]. Established stable replicon assays for HCV genotypes 1a and 1b were used; constructs are shown (Figure 2.1). Later work used clones of HCV genotype 1b with an integral luciferase; the structure is shown (Figure 2.2) and method is described (Section 2.1.2).

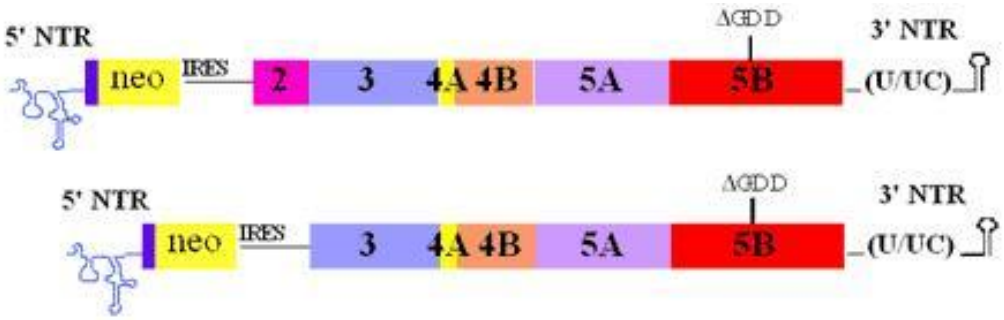


Figure 2.1: HCV replicon structure

HCV replicon RNA in HuH7 hepatoma cells, replicons of HCV genotypes 1a (top) and 1b (bottom). Non structural viral (NS) proteins are expressed 5 prime (') and 3' nontranslated regions (NTR), neo is the neomycin phosphotransferase gene used with G418 to select cells containing the replicon, NS2- envelope protein not present in the 1a replicon, NS3 serine protease, NS4A and 4B cofactors of NS3, NS5A interaction with cellular proteins and NS5B RNA dependant RNA polymerase.

HuH7 cells containing either genotype 1a[107] or 1b[38] replicons were cultured in: Dulbecco's Modified Eagle Medium (DMEM) without sodium pyruvate,

4500mg/L Glucose and Pyridoxine hydrochloride (HCl), supplemented with 10% foetal bovine serum, 1% penicillin, 100µg/mL streptomycin, 2mM L-glutamine (left), 1% non-essential amino acids and 500µg/mL G418 (Geneticin) (addition of G418 selects cells with replicon) (all reagents from Invitrogen). 96-well flat bottomed microtitre plate (Nunc) containing 3 x 10^4 cells per well were incubated with compounds as detailed below in a humidified incubator at 37°C and 5% CO_2 for 72 hours.

Serial dilutions of IFNα, IFNβ, IFNγ (PBL biomedical laboratories) IL-28A and IL-29 (R&Dsystems) were made up in culture medium as described above without the addition of G418. IFNα2a was used in all experiments and called IFNα. IFNα, IFNβ dilutions were optimised to 1000, 500, 250, 125, 63, 32, 16, 8, 4, 2 international units (IU)/mL, IFNγ 2000 to 4 IU/mL, IL-28A 200ng/mL to 4ng/mL and IL-29 1000ng/mL to 2ng/mL. Controls were included on each plate, these were: no primary antibody controls, cell control wells without the addition of compound and in house proprietary assay controls were included as a negative control and positive controls respectively. IFNα, IFNβ, IFNγ, IL-28A and IL-29 were run in duplicate at each dilution on the plate and each plate was run in triplicate over 3 separate occasions to allow for variability of cell passage.

An Enzyme-linked immunosorbent assay (ELISA) was performed on the plates to detect NS5A protein using murine anti-HCV NS5A primary antibody. Medium was aspirated followed by a PBS wash and aspirate; the cells were then fixed for 5 minutes in methanol (MeOH): acetone (1:1). The fixative was removed and further washed in PBS. This was followed by a blocking step with 2% skimmed milk in PBS plus 0.05% tween 20 at 37^{o}C for 1 hour. Blocking buffer was removed and primary antibody, 50µL of murine anti-HCV NS5A (Virostat) was diluted to 0.5µg/mL in blocking buffer and added to the plate, with the exception of no primary antibody wells (blocking buffer only) then incubated at 37^{o}C for 2 hours. Primary antibody was removed and plates were washed three times with wash buffer, PBS plus 0.05% tween 20. 50µL of horseradish peroxidase-conjugated secondary antibody, rabbit anti-mouse immunoglobulin G (IgG) (Dako) was diluted 1:1000 in blocking buffer and incubated for 1 hour at 37^{o}C. Secondary antibody was then removed and plates were washed five times with wash buffer. The next step was to allow a colour to develop by incubating the

plates for 30 minutes with 50µL OPD/peroxidase substrate in urea buffer. The colour reaction was stopped with the addition of 25µL of 2 molar (M) sulphuric acid. Absorbance was then measured at 490 nanometers (nm). Cells were also stained with 5% carbol fuchsin to visually assess cytotoxicity.

2.1.2 Determination of IC_{50} values for four luciferase HCV replicon clones

Values for 10 x IC_{50} concentrations in the replicon cell lines were determined to normalise for the antiviral activities of proteins IFNα, IL-28A and IL-29. Four luciferase replicon cell lines were compared for sensitivity with IFNα, IL-28A and IL-29 these were: 1a1_19, 1a3II, 1b 2.2, 1b2.5, and the most sensitive selected to carry through into transcriptomics analysis (Section 2.2.1).

HuH7 HCV luciferase replicon cells were routinely maintained in DMEM, 4500mg/L Glucose and Pyridoxine HCl supplemented with 10% foetal bovine serum, 100IU/mL penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 1% non-essential amino acids and 500µg/mL Geneticin. (All reagents from Invitrogen). The assay media conditions were as above minus Geneticin. 1 x 10^6 cells per well of each of the HuH7 HCV luciferase replicon cells were cultured in 96-well plates at 37°C. Dilutions of IFNα, IL-28A and IL-29 were made up in triplicate. For each luciferase cell line, 5 x 10^4 confluent cells in a 50µL volume per well were added to compound made up to 50µL with medium. The plates were incubated for 18 hours at 37^{o}C. Supernatent was removed and 25µL of Steady-Glo (Promega) was added per well and the plate was left in the dark for 15 minutes before reading chemiluminescence at 450nm. The half maximal inhibitory concentration (IC_{50}) was defined as the concentration of each treatment which reduced luciferase signal by 50% compared with untreated cells.

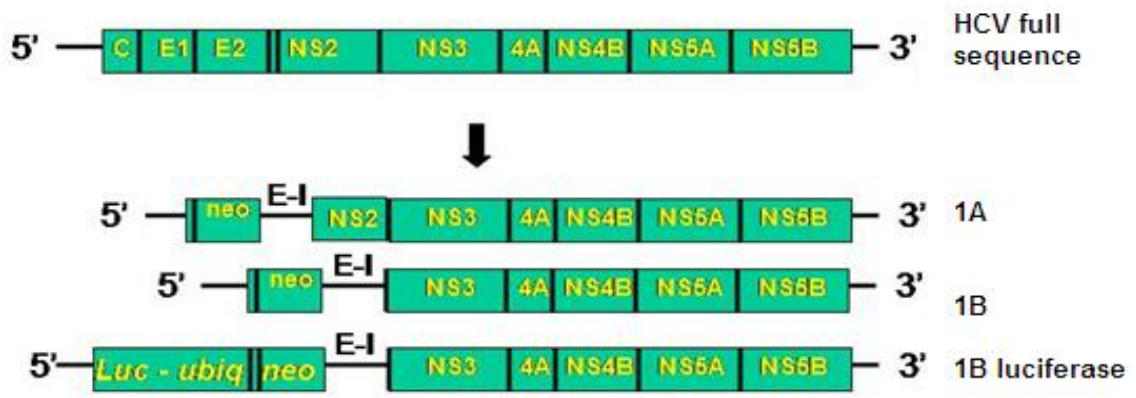


Figure 2.2: HCV luciferase replicon clones structure

Clones 1a1_19, 1a3II and 1b 2.2, 1b2.5 were provided by Thomas Zurcher GSK, all clones had a 5' lucifease unit (luc-ubiq). Neo is the neomycin phosphotransferase gene used with G418 to select cells containing the replicon, NS2- envelope protein not present in the 1a replicon, NS3 serine protease, NS4A and 4B cofactors of NS3, NS5A interaction with cellular proteins and NS5B RNA dependant RNA polymerase.

2.1.3 IL-28A, IL-29 and IFNα synergy

1b replicon cell line assay as described (Section 2.1.1) was used as results showed (Section 3.1.1) that the 1b cell line was most sensitive to interferons tested. Serial dilutions of IL-28A 200ng/mL to 4ng/mL and IL-29 1000ng/mL to 2 ng/mL were made up in culture medium alone and then in addition with 0.09ng/mL of IFNα (IC_{50} concentration as determined in experiments performed (Section 2.1.1)). To determine antiviral activity in the HCV 1b replicon cell line of IFNα at 0.09ng/mL this was also run separately on its own as a control. Controls were included on each plate, these were: no primary antibody controls, cell control wells without the addition of compound and in house proprietary assay controls were included as a negative control and positive controls respectively. IL-28A and IL-29 were run in duplicate at each dilution on the plate and each plate was run in triplicate over at 2, 4, 6, 8, 16 and 24 hours.

2.1.4 IFN sensitive gene models 6-16 and ISG56

To investigate type III interferon modulation of interferon sensitive genes (ISGs), two ISG assays were used: interferon sensitive gene 56 (ISG56) and interferon inducible gene 6-16 (G1P3).

ISG56 alternatively named interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), a 293 cell line (a continuous line of transformed human embryonic kidney cells) with ISG56 promoter construct was used. ISG56 is the most highly induced IFN sensitive gene (30-100 fold induction), the assay is robust over several logs of IFN concentration and response is dependent on intact receptor and signal transduction pathways. 293 cells were cultured in DMEM + 4500mg/mL glucose, 20mM L-Glutamine, Pyruvate (Sigma-Aldrich), 10% foetal calf serum, 100 IU/mL penicillin, 100µg/mL streptomycin and 500µg/mL G418. 5×10^4 cells were cultured for 18 hours with serial dilutions of IFNα, IFNβ and IFNγ, 1000, 500, 250, 125, 63, 32, 16, 8, 4, 2 IU/mL and serial dilutions for IL-28A ranging from 200ng/mL to 4ng/mL and IL-29 from 1000ng/mL to 2ng/mL. Untreated cell and virus controls were also included. IFNs were run in duplicate and the experiment was performed in duplicate. Supernatants were removed and 25µL of Steady-Glo, (Promega) was added per well and the plate was left in the dark for 15 minutes at room temperature before optical density (OD) measurement at 405nm.

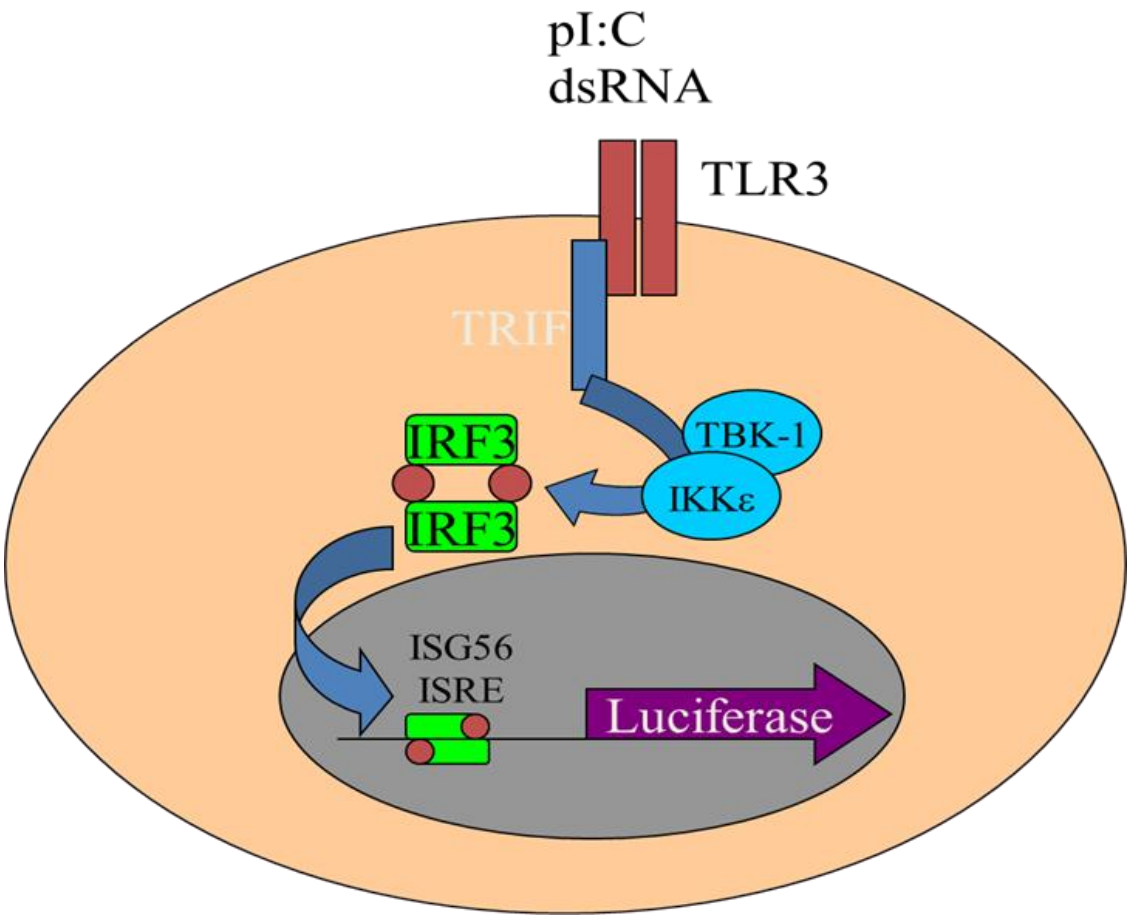


Figure 2.3: ISG56-luciferase reporter

The ISG56-luciferase reporter assay is used to show binding to Toll-like receptor 3 (TLR3), inducing antiviral response resulting in ISG56 induction and involving TANK-binding kinase 1(TBK-1), interferon regulatory factor 3 (IRF3) leading to modulation of ISG56.

Interferon, alpha-inducible protein 6 (6-16) alias IFI6; 6-16; FAM14C; G1P3; IFI-6-16; IFI616 an interferon sensitive gene model was also used to investigate type III interferon activity, this cell line was maintained at GSK. Human glioblastoma T98G cells were transfected with a plasmid containing a gene coding for soluble alkaline phosphatase under control of an interferon-inducible promoter. The assay was set up as described above for ISG56.

2.1.5 Hepatitis B (HBV)

Antiviral effects of IL-28A and IL-29 compared with IFNα were investigated in human hepatocellular carcinoma HepG2 (2.2.15) cell line stably transfected with

HBV subtype ayr maintained at the Department of Medicine, Imperial College. HepG2 cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in Minimum Essential Medium Eagle media (MEME) supplemented with 10% foetal calf serum, and 50µg/mL kanamycin. Cells were subcultured once a week by detaching the cells with pancreatin (0.5mg/mL) followed by change of medium on the following day. 8 well plates containing 5 x 10^4 cells per well were incubated with IFNs in a humidified incubator at 37°C and 5% CO_2 for 1 week. Concentrations of IFNs used were: 100, 1000 IU/mL for IFNα, 200, 2000ng/mL for IL-28A, 100, 1000ng/mL for IL-29 and untreated. Total DNA was extracted from each well using a Wizard® Genomic DNA Purification kit according to manufacturer's instructions.

(http://www.promega.com/~/media/Files/Resources/Protocols/Technical%20Manuals/0/Wizard%20Genomic%20DNA%20Purification%20Kit%20Protocol.pdf.)
Antiviral activity was detected using quantitative real-time polymerase chain reaction (PCR) (TaqMan®) primers (Proligo) and probes (Applied Biosystems). This was performed in the presence of the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and all amplifications were performed in quadruplicate.

Table 2.1: Hepatitis B virus probe and primer sequences

HBV1	Sequence
Forward primer	5'GGACGGAAACTGCACTTGTATTC-3'
Reverse primer	5'GACTGAGGCCCACTCCCATAG-3'
Probe FAM/ TAMRA	5' CCATCATCCTGGGCTTTCGCAAGATT-3'

TaqMan® probes consist of a fluorophore (e.g. 6-carboxyfluorescein, (FAM)) covalently attached to the 5'-end of the oligonucleotide probe and a quencher (e.g. tetramethylrhodamine, (TAMRA)) at the 3'-end.

2.1.6 Real-Time PCR assay overview

TaqMan® (Applied Biosystems) is the trademark name of the PCR-based, fluorogenic 5'-nuclease assay, which allows for the real-time quantitation of DNA/RNA, allelic discrimination and pathogen detection. This can include

SybrGreen and fluorogenic probes (TaqMan®). Most PCR included in the thesis is covered by these two assays and run on Applied Biosystems 7900 PCR machines, the other PCR assay described in this thesis also uses SybrGreen dye but run on a different proprietary PCR machine Corbett Life Science Rotor-Gene real-time PCR machine (Corbett Life Science).

2.1.6.1 Sybr Green 1 dye

Sybr Green 1 dye: binds to the minor groove of DNA and is fluorescent when bound. As the PCR progresses through the cycles and more PCR product is accumulated so more dye binds resulting in an increase in fluorescence. By plotting fluorescence against cycle number an amplification plot is produced which provides a more accurate picture of the PCR rather than measuring end product. To ensure the specificity of the primers, dissociation curve analysis is carried out on the PCR'd samples. This collects the fluorescence levels associated with the melting point of the PCR product already produced and it allows detection of non-specific priming and primers dimers in the template free sample.

2.1.6.2 Fluorogenic 5' nuclease assay (TaqMan®)

The fluorogenic 5' nuclease assay uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR. The reporter dye is incorporated on the 5' end and the quencher on the 3' end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The 5´–3´ nucleolytic activity of the AmpliTaq Gold DNA Polymerase cleaves the probe between the reporter and the quencher only if the probe hybridises to the target. The probe fragments are then displaced from the target, and polymerisation of the strand continues. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any non-specific amplification is not detected.

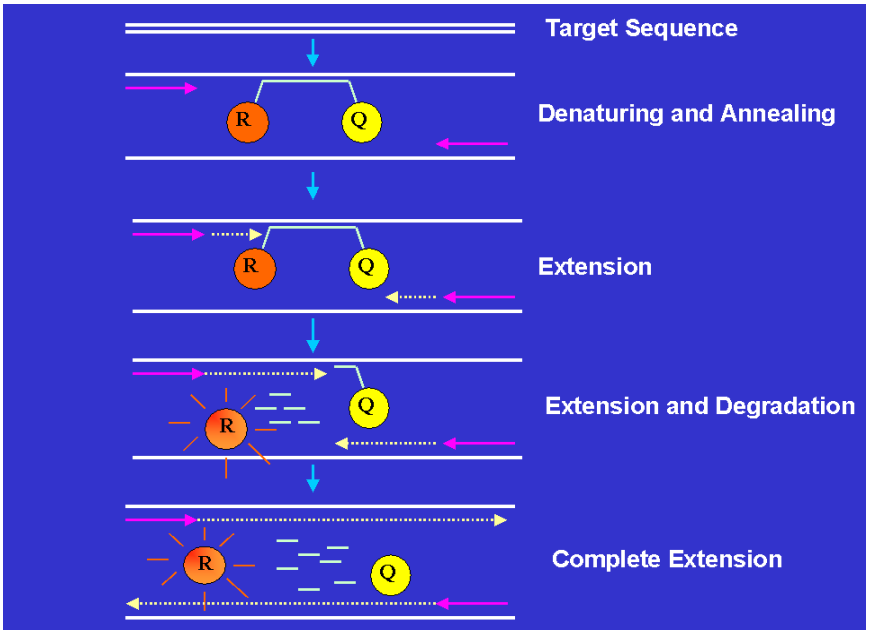


Figure 2.4: TaqMan® Real-time PCR process

Real time PCR process probe containing R-reporter and Q- quencher, anneals to target sequence, during the extension phase the reporter is detached and can be measure at every cycle of the PCR. Taken from http://ukgwrdweb1.ggr.co.uk/TaqMan

2.1.6.3 Primer and probe design

Primer sequence and where applicable probe sequences were designed using NCBI entrez nucleotide website:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide

Primers and probe are designed to the gene of interest using Applied Biosystems Primer Express software. This software has some of the necessary requirements for acceptable probes and primers built in. The exception to this was IL-28R assay was on demand assay predesigned (Applied Biosystems).

2.1.6.4 PCR reagents

DNA or Total RNA was prepared from the tissue or cells being investigated and normally added at 50ng concentration. Copy number relative changes or a genomic standard curve was prepared for the species being investigated. This allowed quantification of gene copy number. Primers were diluted to a working concentration of 10μM, and probe to 5μM. Universal PCR Master Mix (Applied Biosystems) was added at 2 x concentration, it contains the AmpliTaq Gold DNA polymerase, AmpErase® Uracil N-glycosylase (UNG), deoxynucleotide

triphosphates (dNTPs), Passive reference ROX, and optimised buffer components. AmpErase UNG is present in the mix as it can prevent the reamplification of carryover PCR products by removing any uracil incorporated into single or double stranded DNA. ROX is a passive reference dye present in the master mix and allows for the normalisation of amplification across wells due to master mix variability.

Multiplexing allowed normalisation of the gene of interest to an endogenous control whose levels are assumed not to change with treatment, reducing the well to well template variability within the data, e.g. GAPDH, b-actin, 18S ribosomal RNA.

2.1.6.5 PCR cycling parameters

PCR cycling parameters unless stated were 40 cycles of:
50^{o}C for 2 minutes
95^{o}C for 10 minutes
95^{o}C for 15 seconds
60^{o}C for 1 minute

For Sybr Green Dissociation Curve analysis one cycle is added at the end of run.
95^{o}C for 15 seconds
60^{o}C for 20 seconds
95^{o}C for 15 seconds

2.1.6.6 Data Analysis

Key parameter for data analysis is the Threshold cycle (Ct): this is the cycle at which the amplification of the gene is in the exponential phase. The baseline was set to ensure that signal is not discarded as noise. The standard curve was checked to ensure reproducibility and satisfactory linear regression. No Template Control (NTC) wells were viewed to assess cross contamination. The starting copy number of the gene in each sample was calculated from the standard curve by the software, and then exported into Excel (Microsoft), for further data analysis including normalisation to endogenous control if applicable.

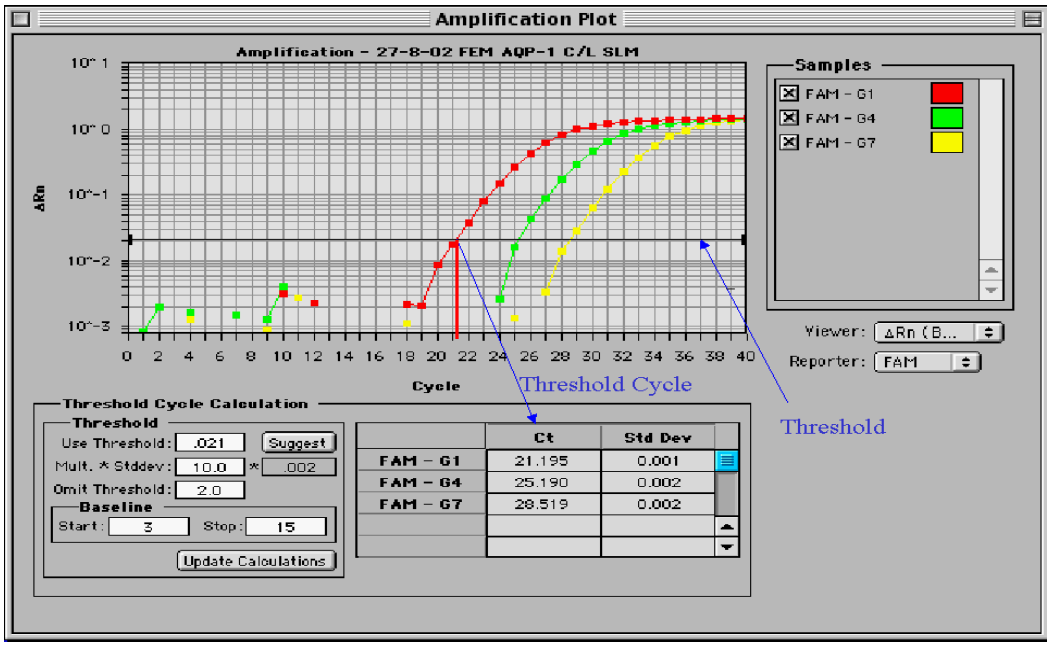


Figure 2.5: TaqMan® Real-time PCR amplification plot

Adapted from TaqMan® data plot 7900 Applied Biosystems.

2.1.7 Human immunodeficiency virus (HIV)

An established method was adapted at GSK to look at antiviral effects of IFNα, IFNβ, IFNγ, IL-28A and IL-29. The assay used MT4 T lymphocyte derived cells infected with HIV virus HXB2. MT4 cells were split 1:4 two days before required using phenol red indicator media (RPMI) plus 10% Hyclone foetal bovine serum and Penicillin/streptomycin/L-Glutamine (100units/mL,100µg/mL and 2mM respectively). Cells were used at a concentration of 8 x 10^5 per mL and 2.4 x 10^6 cells were used per infection. Cells were harvested by spinning at 400g for 5 minutes at room temperature and re-suspended in RPMI medium without phenol red indicator containing the supplements as above.

MT4 cells were infected with HIV virus HXB2 at a concentration of 100 x 50% Tissue Culture Infectious Dose ($TCID_{50}$) per million cells for one hour. IFNs were then added to the cells. Serial dilutions of IFNα were optimised to 400, 200,100, 50, 25, 12, 6, 3, 1.5 IU/mL, IFNβ were optimised to 3200, 1600, 800, 400, 200, 100, 50, 25, 12 IU/mL and serial dilutions were made for IFNγ ranging from 25600 to 2 IU/mL, for IL-28A from 7.68mg/mL to 4ng/mL and for IL-29 from

1mg/mL to 2ng/mL. IFNs were run in quadruplicate on the plate and each plate was run in triplicate over different days. Non infected cells were used as a cell control and virally infected cells with media in place of drug were run as a virus control. The cells were incubated for 120 hours and then treated with Dimethyl thiazolyl diphenyl tetrazolium salt (MTT). Plates were read between 540-590nm and the IC_{50} for IFNs calculated.

Category 3 lab training was required in order to work with HIV virus in the GSK Stevenage labs; and was completed before work using HIV was initiated.

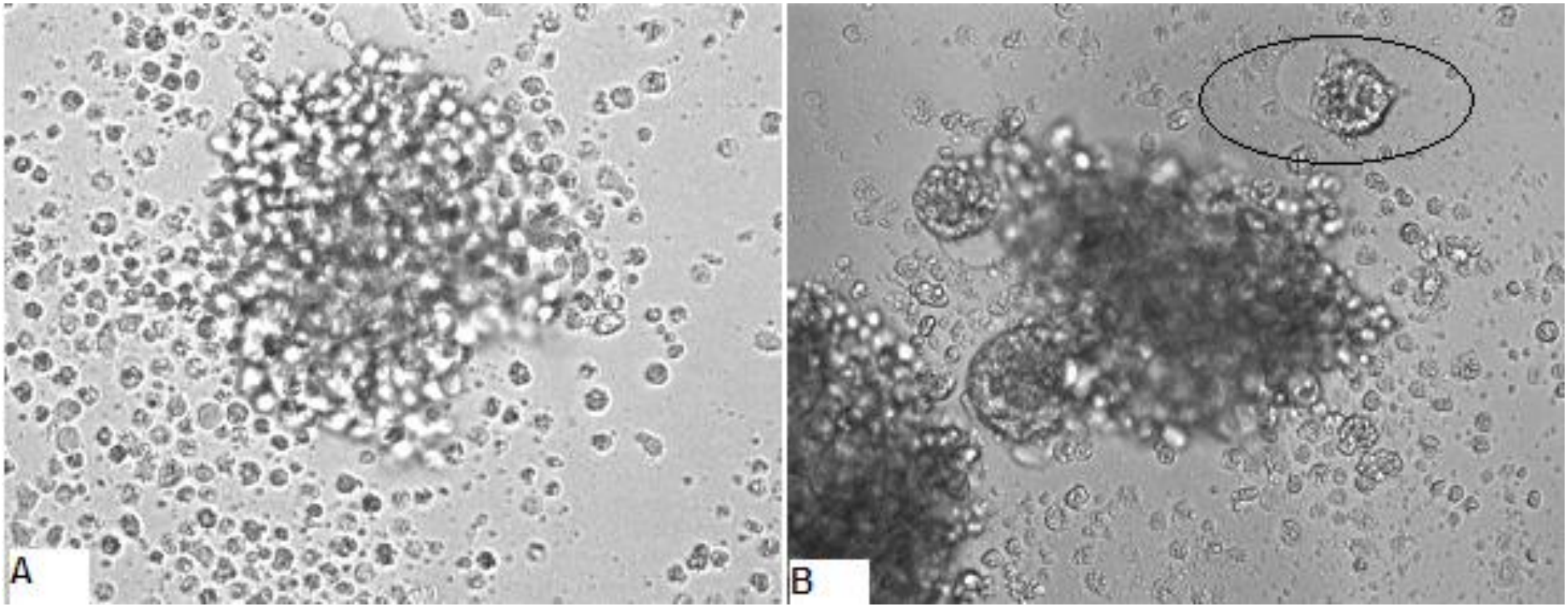


Figure 2.6: MT-4 cells uninfected and infected with HXB2

An example of MT-4 cells uninfected and infected with HXB2;

A shows normal MT-4 cells in suspension, single cells and common clumping,

B shows HXB2 infected cells, the cells circled show one cell full of HXB2 virus and the outline of a burst cell.

2.1.8 Dengue fever virus

A cell line stably expressing dengue virus replicon established in Professor Graham Foster's laboratory at Queen Mary's School of Medicine and Dentistry was used to test the antiviral properties of type III interferons. K562 (human chronic myeloid leukaemia) cell line stably expressing the dengue virus replicon ΔCprME-PAC2A was used.

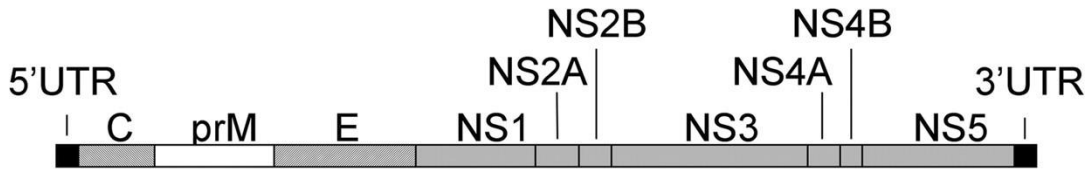


Figure 2.7: Schematic showing plasmid pDENΔCprME-PAC2A

Dengue virus type 2 infectious clone cDNA (in plasmid pDVWS601)[108] containing a single open reading frame carrying three structural genes (C, core; prM, premembrane; E, envelope), seven nonstructural (NS) genes, and flanking 5' and 3' untranslated regions (UTR).

2.1.8.1 Cell Culture

Cells stably expressing dengue virus replicon RNA were generated by transfection with ΔCprME-PAC2A RNA and then propagation in RPMI containing 10% foetal bovine serum (FBS) and 3μg of puromycin (Sigma-Aldrich) per mL. Cells were removed from puromycin selection and checked for replicon expression before use by indirect immunofluorescence of the dengue virus NS1 protein with a specific monoclonal antibody (5H5.4)[109]. K562 cells without replicon were continuously maintained in the same medium without puromycin[53]. K562.ΔCprME-PAC2A cells were grown in the presence of 0, 100, 1,000 IU/mL for IFNα, IFNβ and IFNγ, 0, 200, 2000ng/mL, 0, 100, 1000ng/mL for IL-28A and IL-29 respectively for 24 hours.

2.1.8.2 Total RNA extraction

Total RNA extraction from suspension cell cultures was carried out using the SV Total RNA Isolation System (Promega). Samples were centrifuged for 5 minutes and culture media was removed. Cell pellets were washed with 1mL of sterile PBS and centrifuged for a further 5 minutes. The PBS was removed and 150μL

SV Lysis Buffer added to each tube. The lysates were transferred into RNase/DNase free 1.5mL Eppendorf tubes. Genomic DNA was sheared by passing the lysates through a 19-gauge blunt-ended needle four times and placed on ice. Lysates were stored at -80ºC until required for total RNA extraction.

2.1.8.3 Total RNA extraction using the SV Columns

Lysates were thawed and 350µL transferred to a fresh RNase/DNase free 1.5mL Eppendorf. 700 µL of SV Dilution Buffer was added, mixed by inversion, and the mixture incubated at 70^{o}C for 3 minutes. Following centrifugation at 12,000 x *g* for 10 minutes, the supernatant was transferred to a sterile 1.5mL Eppendorf containing 400µL of 95% v/v ethanol (Sigma-Aldrich). This mixture was loaded onto two SV RNA Isolation Tube Assemblies which were centrifuged at 14,000 x *g* for 1 minute. Liquid in the collection tubes was discarded, 600µL of SV RNA Wash Solution applied to each column and the SV RNA Isolation Tube Assemblies centrifuged at 14,000 x *g* for 1 minute. The collection tubes were emptied and 50µL of SV DNase Incubation Mix (prepared from 50µL of SV Yellow Core Buffer, 5µL of SV 0.09M $MgCl_2$ and 5µL of SV DNase I enzyme) applied to the surface of each column membrane. SV RNA Isolation Tube Assemblies were incubated at room temperature for 15 minutes to allow DNA digestion to proceed, after which 200µL of SV DNase Stop Solution was added to each column. SV RNA Isolation Tube Assemblies were centrifuged at 14,000 x *g* for 1 minute, 600µL of SV RNA Wash Solution applied to each column and then centrifuged again at 14,000 x *g* for 1 minute. Liquid in the collection tubes was discarded and a final wash carried out by applying 300µL of SV RNA Wash Solution to each column and centrifuging the SV RNA Isolation Tube Assemblies at 14,000 x *g* for 2 minutes (to eliminate ethanol carry-over).

The column within each SV RNA Isolation Tube Assembly was transferred to a fresh 1.5mL RNase/DNase free Eppendorf tube and 100µL of SV Nuclease Free Water applied to the membrane. Columns were incubated at room temperature for 1 minute and the total RNA eluted by centrifuging the column/tube assemblies at 14,000 x *g* for 1 minute. The columns were

discarded and eluted total RNA from replicate columns pooled prior to storage at -80^{o}C until required.

2.1.8.4 Total RNA Quantification and Quality Control

The concentration, yield and purity of each total RNA sample was determined using ultraviolet (UV) spectrophotometry. The integrity of each total RNA sample was determined by agarose gel electrophoresis.

2.1.8.5 Quantification Using UV Spectrophotometry

Spectramax 190 UV Microtitre Plate Reader (Molecular Devices) was used for measuring UV absorbance of total RNA samples. The absorbance of each total RNA sample (at an appropriate dilution in Molecular Grade Water) at 260nm (peak nucleic acid absorbance) and 280nm (peak protein absorbance) was measured and the A_{260}/A_{280} ratio determined to indicate the level of protein contamination. Total RNA samples with an A_{260}/A_{280} ratio of 1.8 to 2.1 were considered of acceptable quality. The total RNA concentration was derived using the following formula:

RNA concentration in µg/µL = ((A_{260} x 40*) x dilution factor)/1000

*Where 40 = the RNA extinction coefficient

2.1.8.6 Quality determination using agarose gel electrophoresis

A 1% weight/volume agarose gel was prepared by melting 1g of Molecular Biology Grade Agarose (Sigma-Aldrich) in 100mL of 1 x Tris-borate Ethylenediaminetetraacetic acid (EDTA) (TBE) buffer (45mM tris-borate, 2mM EDTA) (Sigma-Aldrich). The melted gel was allowed to cool to approximately 60ºC before adding 5µL of 10mg/mL ethidium bromide solution (Sigma-Aldrich) (final concentration of 0.5µg/mL). The gel was mixed gently by swirling, poured onto a gel tray (containing two gel combs) and allowed to set for 30 minutes at room temperature. The set gel was transferred to an electrophoresis tank (Life Technologies) and submerged in 1 x TBE buffer.

Total RNA samples were prepared by mixing 5µL of each total RNA sample with 1µL of 6 x Gel Loading Buffer (Sigma-Aldrich) and placed on wet ice until required. Samples were loaded into wells within the gel and electrophoresis

carried out for 30-45 minutes at 100 volts. To visualise total RNA staining, the gel was visualised using the GeneGenius Bioimager gel documentation system (Syngene) and the presence of intact discrete 18S and 28S ribosomal RNA (rRNA) bands indicated that the total RNA was of acceptable quality. If the rRNA bands were not present, or appeared degraded, the total RNA was discarded and a fresh isolation carried out.

2.1.8.7 QPCR

Extracted RNAs were treated with RQ1 RNase-free DNase (Promega) to degrade both double stranded and single-stranded DNA, and reverse transcribed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (Promega) using random decamer primers. PCR reactions were performed and analysed on a Rotorgene instrument (Corbett Life Science) by the use of custom primers and a fluorescent probe specific for dengue virus NS1 and housekeeping gene GAPDH was analysed in the same samples by the use of specific primers and QuantiTect SYBR green (QIAGEN).

Table 2.2: Dengue virus probe and primer sequences

	Sequences		
Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Probe /equivalent (5'-3')
Dengue virus NS1	CTGAAGTGTGGCAGT GGGATT	CTTCAAAGCTAGCTTCA GCTATCCA	CACAGACAACGTGCAC ACATGGACAGA
GAPDH	ACAGTCCATGCCATCA CTGCC	GCCTGCTTCACCACCTT CTTG	QuantiTect SYBR green

Table 2.2 shows probe and primer sequences used for RT-PCR analysis to detect dengue virus NS1 and housekeeping gene GAPDH.

2.1.9 Respiratory Syncytial Virus (RSV)

A tissue culture model of chronic viral infection using an RSV virus infected transformed human B cell line established in Professor Graham Foster's laboratory at Queen Mary's School of Medicine and Dentistry, was used to test the antiviral properties of type III interferons.

2.1.9.1 RSV cell culture

Transformed B cells (10^6 in 10 mL RPMI medium) were incubated with 2 x 10^8 viral particles for a total of 14 days leaving the cells undisturbed. Chronic infection was assessed by direct cell staining and by quantitative PCR. After incubation for 14 days, greater than 90% of the cells expressed RSV antigens. Viral RNA was detected in the supernatant. The cells were maintained in culture for 16 weeks and passaged every two weeks (cells split 1:3 and the media exchanged). Cells in six well plates at 0.5 x 10^6 cells per well were treated with IFNα, IFNβ and IFNγ at 1000 IU/mL, IL-28A at 2µg/mL, IL-29 at 1µg/mL and untreated to assess the effects of antiviral treatment. Plates were incubated at two time points: 48 hours and 96 hours.

Total RNA was extracted and quantified as described (Section 2.1.8.2 – 6) and reversed transcribed using M-MLV RT (Promega). A quantitative PCR reaction was performed and analysed by Rotorgene (Corbett Life Science) using primers designed to the WSX1 RSV virus. The values obtained for RSV concentration were normalised against GAPDH.

Table 2.3 Probe and primer sequences for RSV

	Sequences	
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
RSV	GATATGCCTATAACAAATGATCAG	GATACTGATCCTGCATTGTCAC
GAPDH	ACAGTCCATGCCATCACTGCC	GCCTGCTTCACCACCTTCTTG

Table 2.3 shows probe and primer sequences used for RT-PCR analysis to detect RSV and housekeeping gene GAPDH.

2.2 Transcriptome analysis in HCV replicon cells treated with type I and type III IFNs

2.2.1 Determination of IC_{50} values for 4 replicon clones

Values for 10 x IC_{50} concentrations in the replicon cell lines were determined to normalise for the antiviral activities of proteins IFNα, IL-28A and IL-29. Four luciferase replicon cell lines were compared for sensitivity with IFNα, IL-28A and IL-29 these were: 1a1_19, 1a3II, 1b 2.2, 1b2.5, and the most sensitive selected to carry through into transcriptomics analysis. Dilutions of IFNα, IL-28A and IL-29 were made up in triplicate. For each luciferase cell line, 5 x 10^4 confluent cells in a 50µL volume per well were added to compound made up to 50µL with medium. The plates were incubated for 18 hours at 37^{o}C. Supernatent was removed and 25µL of Steady-Glo (Promega) was added per well and the plate was left in the dark for 15 minutes before reading chemiluminescence at 450nm. The IC_{50} was defined as the concentration of each treatment which reduced luciferase signal by 50% compared with untreated cells.

2.2.2 Cell culture and treatment

Parental HuH7 and HuH7 HCV 1a3II luciferase replicon cells were routinely maintained in DMEM, 4500mg/L Glucose and Pyridoxine HCl supplemented with 10% foetal bovine serum, 100IU/mL penicillin, 100µg/mL streptomycin, 2mM L-glutamine, 1% non-essential amino acids and 500µg/mL Geneticin. (All reagents from Invitrogen). The assay media conditions were as above minus Geneticin. 1 x 10^6 cells per well of parental HuH7 and HuH7 HCV 1a3II luciferase replicon cells were cultured in 6-well plates at 37°C. Four treatments were used: untreated, 10 x IC_{50} for IFNα, IL-28A and IL-29 at time points of 4, 8, 16 and 24 hours. Each condition was performed in triplicate. Values for 10 x IC_{50} concentrations in the replicon cell lines were determined to normalise for the antiviral activities of proteins IFNα, IL-28A and IL-29.

2.2.3 Isolation of RNA

Culture media was removed and monolayers were washed with 1mL of sterile PBS. The PBS was removed and 1mL TRIzol® Reagent (Invitrogen) 4°C added to each well. Cells were scraped with a sterile tissue culture scraper, the lysates

were collected in RNAse/DNAse free 1.5mL Eppendorf tubes. Genomic DNA was sheared by passing the lysates through a 19-gauge blunt-ended needle four times and placed on ice. Lysates were stored at -80ºC until required for total RNA extraction. Total RNA was isolated the SV Total RNA Isolation System (Promega) and stored at -80°C until required (description of method section 2.1.8.2).

All RNA samples were analysed by the Agilent Bioanalyzer 2100 Lab-On-A-Chip Nano 6000 chip (Section 2.2.6.6) to determine the integrity and by UV spectrometer; OD A_{260} and A_{280} for accurate RNA concentration determination.

2.2.4 Quantitative QPCR pre-screen

Real-time PCR was performed on the RNAs isolated above and data analysed to assess the suitability of the time points and concentrations of the IFNs in the assay before running the samples of full genome Affymetrix chips. Applied Biosystems assay on demand were used for interferon sensitive genes: Interferon-induced GTP-binding protein (IFIT1), myxovirus (influenza virus) resistance 1 (MX1), 2'-5'-oligoadenylate synthetase 1 (OAS1) and an interferon inducible gene 6-16 (G1P3). Five micrograms of each RNA sample was reverse transcribed to cDNA using SuperScript II first strand cDNA synthesis (Life Technologies). The relative levels of target transcripts were measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Serial dilutions of human genomic DNA (Novagen) were used to generate a standard curve for quantitation. GAPDH and b-actin were used as housekeeping genes to normalise the data. Real-time data was collected and analysed using Sequence Detection System (SDS) software Version 2.0 (Applied Biosystems).

2.2.5 Affymetrix GeneChips® Expression analysis

Affymetrix analysis was carried out using RNA from PBS, IL-28A, IL-29 and IFNα treated samples in both replicon and parental HuH7 cells at time points of 8,16 and 24 hours with each in triplicate (72 chips in total). Analysis of 12 additional chips was performed at the 4 hour time point in parental cells only due to lack of RNA in the 4 hour time point in the replicon cells, making a total of 84 chips in the experiment.

The samples were hybridised to the Human Genome HG-U133plus2 GeneChip (Affymetrix). This array enables the analysis of the expression levels of over 47,000 transcripts and variants including 38,500 well characterised genes. Further information on the Human Genome HG-U133plus2 GeneChip is available at: (http://www.affymetrix.com/products/arrays/specific/hgu133.affx).

Briefly the standard expression GeneChips® possess 22 different 25mer oligo probes (termed a 'probe set') for each gene represented, which are distributed at locations remote to each other. The probe sets are 3'-biased and designed to give the optimal balance between sensitivity and specificity for the target gene. To evaluate any potential for non-specific hybridisation, each 'perfect match' (PM) probe feature has an adjacent paired 'mismatch' (MM) probe feature. The MM probe differs in sequence from the PM probe by a single nucleotide, located in the centre of the 25mer. The MM features serve as controls that allow the subtraction of non-specific hybridisation signals (non-specific hybridisation for the PM and MM features should be roughly equivalent) from the PM signal to derive the true signal for a particular gene.

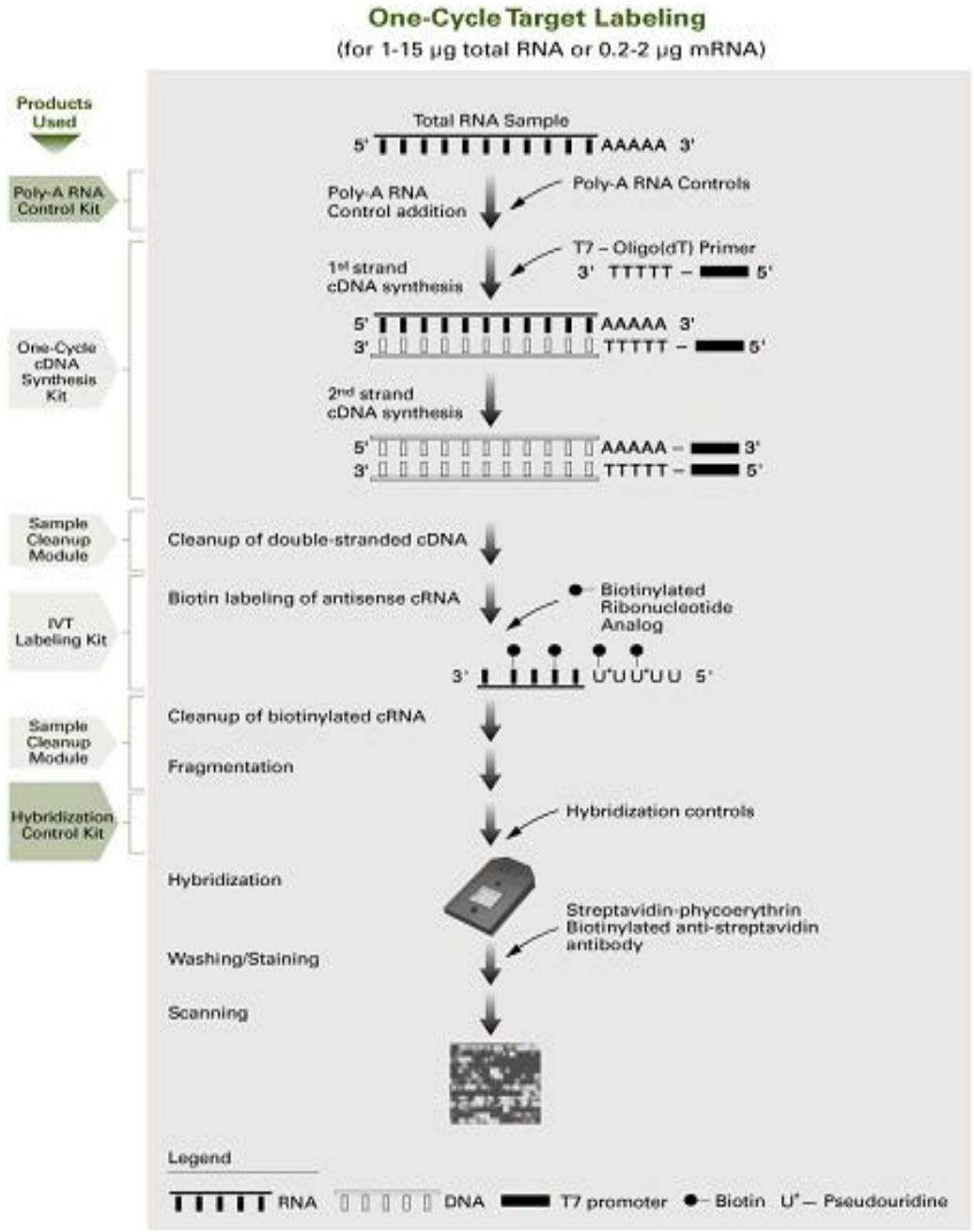


Figure 2.8: Standard eukaryotic gene expression assay

Labelled cRNA targets derived from mRNA of an experimental sample are hybridised to nucleic acid probes attached to the quartz wafer chip. By monitoring the amount of label associated with each DNA location, it is possible to infer the relative abundance of each RNA species represented (Affymetrix image library - http://affymetrix.com).

Reverse transcription, second-strand synthesis, and probe generation were accomplished by standard Affymetrix protocols full details can be found at: (http://www.affymetrix.com/support/technical/manual/expression_manual.affx) and are described briefly below.

2.2.6 Preparation and Quality Control of cRNA Targets for Hybridisation to GeneChips®

2.2.6.1 Preparation Double-Stranded cDNA

Double-stranded cDNA (ds cDNA) was prepared from total RNA using the One-Cycle cDNA Synthesis Kit (900431, Affymetrix).

The following was added to a 0.75mL RNase/DNase free Eppendorf tube:

- 2µL of T7-(dT)24 primer (100pmol/µL), 5µg of total RNA in 9µL of Nuclease Free Water.

The tubes were then centrifuged briefly and incubated at 70°C for 10 minutes to allow total RNA denaturation and primer hybridisation. The tubes were cooled to 4°C and the following added to each reaction:

- 4µL of 5 x First Strand cDNA Buffer, 2µL 0.1M DTT, 1µL 10mM dNTP mix and 2µL Superscript II RT (200U/µL)

The tubes were centrifuged briefly and incubated at 42°C for 1 hour (to allow first strand synthesis to proceed). The tubes were cooled to 4°C and the following added to each reaction:

- 91µL of nuclease free water, 30µL of 5 x Second Strand cDNA Buffer, 3µL 10mM dNTP mix, 1µL of *E. coli* DNA ligase (10U/µL), 4µL of *E. coli* DNA polymerase (10U/µL) and 1µL of *E. coli* RNase H (2U/µL)

The tubes were centrifuged briefly and incubated at 16°C for 2 hours (to allow second strand synthesis to proceed). Two microlitres of T4 DNA polymerase was added to each reaction, the tubes centrifuged briefly and incubated at 16°C for 15 minutes. To stop the reaction, 10µL 0.5M EDTA (Fluka) was added to each tube and the contents mixed well.

2.2.6.2 ds cDNA cleanup

ds cDNA was cleaned-up using the ds cDNA Cleanup Kit (Affymetrix). 600µl of Binding Buffer was added to each ds cDNA sample, mixed thoroughly and applied to a ds cDNA Spin Column Assembly. The Assemblies were centrifuged at 14,000 x *g* for 1 minute and the flow through discarded. The columns were

transferred to a fresh 2mL collection tube, 750µL of cDNA Wash Buffer added and the tube assemblies centrifuged at 14,000 x *g* for 1 minute. The flow through was discarded and the tube assemblies centrifuged at 14,000 x *g* for 5 minutes with the caps open (to completely dry the columns). The columns were transferred to a 1.5mL Collection Tube and 25µL of cDNA Elution Buffer applied to the membrane. The columns were centrifuged at 14,000 x *g* for 1 minute and the purified ds cDNA stored at -20°C until required for the *in vitro* transcription (IVT) reaction.

2.2.6.3 IVT reaction

Biotinylated complementary RNA (cRNA) targets were generated from ds cDNA using the IVT Labelling Kit (Affymetrix). The following were added to a 0.75mL RNase/DNase free Eppendorf tube:

- 20µL of ds cDNA sample, 4µL of 10 x IVT Labelling Buffer, 12µL of IVT Labelling NTP Mix and 4µL of IVT Labelling Enzyme Mix.

The tubes were centrifuged briefly and incubated at 37°C for 16 hours to allow IVT to proceed. The cRNA was either stored at -20ºC until required, or subjected to cRNA Cleanup.

2.2.6.4 cRNA Cleanup

Biotinylated-cRNA was cleaned-up using the cRNA Cleanup Kit (Affymetrix). To each cRNA sample, 55µL of Nuclease Free Water and 5µL of 3M Sodium Acetate (Fluka) were added. 350µL of cRNA Binding Buffer and 250µL 100% ethanol were added to each cRNA sample, mixed thoroughly and applied to a cRNA Spin Column Assembly. The assemblies were centrifuged at 14,000 x *g* for 15 seconds. The flow through was reapplied to the columns and assemblies centrifuged again at 14,000 x *g* for 15 seconds. The columns were transferred to a fresh 2mL collection tube, 500µL of cRNA Wash Buffer added and the tube assemblies centrifuged at 14,000 x *g* for 15 seconds and the flow through discarded. This step was repeated, followed by a final centrifugation at 14,000 x *g* for 1 minute. The columns were transferred to a 1.5mL Collection Tube and 50µL of Nuclease Free Water applied to the membrane. The columns were incubated at room temperature for 1 minute and centrifuged at 14,000 x *g* for 1

minute to elute the cRNA. The purified cRNA was stored at -20°C until required for the quantification and fragmentation.

2.2.6.5 Quantification of IVT Products and Fragmentation

UV spectrophotometry was used to determine the cRNA concentration and yield. For cRNA samples where sufficient yield and concentration had been achieved, fragmentation was carried out using the Fragmentation Buffer as supplied with the cRNA Cleanup Kit. An appropriate dilution of each cRNA sample in Nuclease Free Water was prepared. The absorbance at A_{260} and A_{280} was measured and the adjusted cRNA yield calculated using the following formula:

Adjusted cRNA yield (µg) = A – (B x C)

Where:

A = Total yield of cRNA according to A_{260} result (µg).

B = Starting amount of total RNA used in cDNA reaction (µg).

C = Fraction of cDNA used in IVT reaction.

For example: Start with 5µg of total RNA, use 80% (or 4µg) in the IVT, and the cRNA yield is 45µg. The calculation would therefore be:

45 - (5 x 0.8) = 41µg

To determine the adjusted concentration, the adjusted yield (in µg) is divided by the total volume (µL). A volume of each cRNA sample containing 15µg (based on the adjusted concentration) was transferred to a 0.75mL RNase/DNase free Eppendorf tube. 0.25µL of Fragmentation Buffer was added for each 1µL of cRNA and the tube incubated at 94°C for 35 minutes and then cooled to 4°C for 5 minutes using a DNA Engine Tetrad PCR machine (Bio-Rad). Fragmented cRNA samples were stored at -20°C until required. To determine whether cRNA fragmentation was successful, the size of cRNA and fragmented cRNA samples were assessed using the RNA 6000 Nano Labchip Kit (Agilent).

2.2.6.6 Gel preparation and RNA 6000 Nano Labchip Priming

RNA 6000 Nano Gel Matrix was prepared by placing 550µL into a Spin Filter and centrifuging at 1,500 x *g* for 10 minutes at room temperature. On the day of use, a 65µL aliquot of filtered RNA 6000 Nano Gel Matrix was transferred to a RNase free Eppendorf tube and 1µL of RNA Nano 6000 Dye Concentrate

added (Gel-Dye Matrix). The Gel-Dye Matrix was mixed by vortexing and centrifuged at 13,000 x *g* for 10 minutes at room temperature. A RNA 6000 Nano Labchip was placed on the Priming Station (Agilent) and 9µL of Gel-Dye Matrix carefully pipetted into the 'priming well'. The plunger of the Priming Station was positioned at the 1mL graduation and the manifold closed firmly over the Labchip. The plunger was firmly depressed until it was held by the clip and priming allowed to proceed for 30 seconds, after which the clip was released. The plunger allowed to recoil for 5 seconds and then pulled back to the 1mL graduation. The Priming Station was opened, the Labchip removed and 9µL of Gel-Dye Matrix carefully pipetted into each of the three 'gel' wells. Labchip priming was completed by adding 5µL of RNA 6000 Nano Marker Buffer to wells 1-12 and the 'ladder' well.

2.2.6.7 Sample Preparation, Loading and Running the Labchip

Frozen cRNA and fragmented cRNA samples were thawed and diluted 1/10 with Molecular Biology Grade Water. Two microlitres of RNA Nano 6000 Ladder and each diluted cRNA/fragmented cRNA sample was transferred to a 0.75mL RNase free Eppendorf tube and heat denatured at 70ºC for 2 minutes. The tubes were immediately chilled on ice and the contents centrifuged to the bottom at 14,000 x *g*. One microlitre of heat denatured sample was added to each sample well; the same volume of heat denatured RNA Nano 6000 Ladder was added to the 'ladder' well. The RNA Nano 6000 Labchip was placed on the Labchip Vortexer (IKA) and vortexed for 1 minute at 2,400 rpm. The loaded Labchip was placed into the 2100 Bioanalyzer (Agilent). The 'Nano 6000 Assay' protocol was selected within 2100 Expert Software version B.02.02 (Agilent) and electrophoresis commenced. Once the electrophoresis was complete the results file was saved and the size profiles of each cRNA and respective fragmented cRNA sample inspected visually.

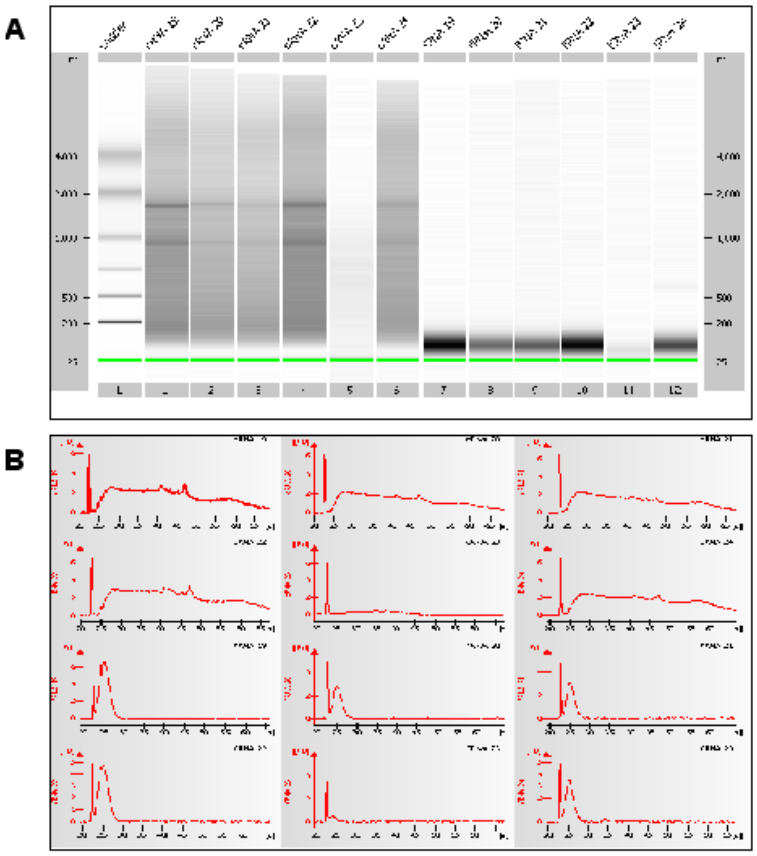


Figure 2.9: Representative RNA 6000 Nano Labchip cRNA Quality Assessment Images

A Gel View and **B** Electropherogram View L = RNA 6000 Nano Marker Ladder. 1-6 = cRNA samples. 7-12 = fragmented cRNA samples.

2.2.6.8 Hybridisation of cRNA Targets to Affymetrix GeneChips® and Scanning

Fragmented cRNA targets were hybridised to GeneChips® using the GeneChip® Hybridisation, Wash and Stain Kit (Affymetrix). All reagents were used as supplied. The required number of GeneChips® were allowed to equilibrate to room temperature for a minimum of 1 hour. The GeneChip® was filled with 250µl Pre-Hybridisation Buffer. GeneChips® were placed in a rotisserie rack and incubated at 45°C for a minimum of 10 minutes with rotation (~60rpm) in a GeneChip® Incubator (Affymetrix).

While pre-hybridisation proceeded, hybridisation cocktails were prepared. Reagents (Table 2.4) were added to a 1.5mL RNase/DNase free Eppendorf tube. The hybridisation cocktails were incubated at 99ºC for 5 minutes followed

by 45ºC for 5 minutes using a DNA Engine Tetrad PCR machine (Bio-Rad). Tubes were centrifuged at 14,000 x *g* for 10 minutes to remove particulates and stored at room temperature just prior to hybridisation.

Table 2.4: Reagents and Volumes for Hybridisation Cocktail Preparation

Reagent	Human Genome HG-U133plus2 GeneChip®	Final Concentration
Fragmented cRNA	15µg	0.05µg/µL
Control Oligo B2	5µL	50pM
20 x Hyb Controls	15µL	1.5, 5, 25 and 100pM spikes
DMSO	30µL	
2 x Hyb Mix	150µL	1 x
Nuclease Free Water	Up to 300µL	

GeneChips® were removed from the incubator and the Pre-Hybridisation Buffer removed. 200µL hybridisation cocktail was applied to the GeneChip®. GeneChips® were placed in a rotisserie rack and incubated at 45°C for 16 hours with rotation (~60rpm) in a GeneChip® Incubator (Affymetrix).

2.2.6.9 Washing and Staining of Affymetrix GeneChips®

GeneChips® were removed from the incubator and the hybridisation cocktail removed. The GeneChip® was filled with 250µL Wash Buffer A. For each GeneChip® to be stained, 600µL of Staining Cocktail 1, 600µL of Staining Cocktail 2 and 800µL of Array Holder Buffer were dispensed into 1.5mL Eppendorf tubes on the fluidic station. The Experimental details and barcode for each GeneChip® were entered in Experiment Manager within Affymetrix Microarray Suite (MAS). To commence staining and washing, GeneChips® were loaded into cassette holders on the appropriate Fluidics Station 450. When the wash protocol was completed, the GeneChip® was removed for scanning.

2.2.6.10 Scanning of Affymetrix GeneChips®, Primary Analysis and Quality Assessment

To scan the GeneChips®, Scanner Control within MAS was opened and the 'autoloader' function selected. Stained GeneChips were loaded onto the autoloader of a GeneChip® Scanner 3000 (Affymetrix) and scanning commenced by clicking 'start scan' within Scanner Control. The GeneChips® were automatically scanned, producing a .dat file. GeneChip® .dat files that were of acceptable visual quality were analysed using the 'batch analysis function' within GeneChipA® operating software (GCOS) to generate .chp, .cel and .rpt files. Hybridisation quality was assessed by reviewing the metrics generated within the .rpt file and the quality assessed according to the thresholds (Table 2.5).

Table 2.5: GeneChip® hybridisation quality control metrics

Quality Indicator	Metric	Description
Background values	Average Background	Values range 20-100 intensity units
Background values	Raw Q value	Overall indication electrical noise
Hybridisation controls	*Bio*B (1.5pM final) *Bio*C (5pM final) *Bio*D (25pM final) *Cre* (100pM final)	Prokaryotic gene spikes. *Bio*B equates to 1:100,000 copies and is at the limit of assay sensitivity. *Bio*B should be called 'present' in 50% of arrays. *Bio*C, D and C*re* should always be called present.
Internal control genes	Beta-actin GAPDH	Used to assess input RNA quality. Ratio 3':5' ratio should be <3 for at least one gene.
Percent present	%P	The number of probe sets called 'present' expressed as a percentage of the total probe sets on the GeneChip®.
Scaling and normalisation factors	SF	Indicates the factor used to normalise the array to the Target Intensity Signal.

2.2.6.11 Downstream Analysis of Transcriptomic Data

To undertake comparison analyses of different treatment conditions, the normalised .chp file for GeneChips® of acceptable quality (as defined in Table 2.5) were imported into Rosetta Resolver® Version 6.0 (Merck Sharpe Dohme). Rosetta Resolver® is an integrated microarray data repository system that enables the storage, annotation and statistical analysis of GeneChip® experiments[110]. Upon import of .chp files Rosetta Resolver® associates individual probe set intensities with gene annotations. To analyse the data, Rosetta Resolver® uses a proprietary error model that is specific to the GeneChip® type being analysed[111]. The concept of the error model is to account for experimental error without the need to perform large numbers of replicate experiments. The error model is an empirical intensity-based method for obtaining a conservative estimate of the signal variability within a small replicate data set. To determine the average signal, and whether a particular probe set is significantly expressed above background (absolute analysis), Rosetta Resolver® computes the average PM minus MM value for the each probe pair. As part of this analysis the error model is used to define the variability of each probe set intensity. Significance of expression is determined by comparing the average signal of each probe set to the average signal of negative controls within the GeneChip®. Probe sets where the error-normalised difference in signal is significantly different are called as present (or significantly expressed).

To undertake comparative analysis between control ('baseline') and treatment ('test'), replicate arrays are defined for any number of groups and the 'RatioBuild' function used. The RatioBuild function automatically performs comparisons between all pairs of baseline and test groups and produces a consensus result for ratio of change (relative to baseline) and significance of change. During the course of this analysis, a global normalisation is applied to all replicate GeneChips® and ratio of change computed from the group mean intensity for each probe set. To determine the significance of change, the error of the ratio of change is computed by combining the modelled error of individual probe sets and the variability of the actual replicates signals. With increasing numbers of experimental replicates, the relative contribution that the modelled error makes to the analysis decreases. The analysed data can then be exported by using the 'TrendSet' function within Rosetta Resolver®. The user defines all

comparisons within the data set and selects thresholds for ratio of change and significance (P-value). An arbitrary threshold can also be set for the number of comparisons in which the ratio of change and significance thresholds must be met. This data can then be exported to Excel or as tab-delimited files for downstream data analysis.

The complexity of the data generated by microarray experiments necessitates the use of bioinformatics tools to aid interpretation and help confer biological meaning. To this end, transcriptional data exported from Rosetta Resolver® was analysed using biological theme analysis tools. This provides a high level overview of the functional classes of genes and pathways significantly overrepresented within a data set.

Two alternative methods, GeneGo MetaCore™ pathways analysis software (http://www.genego.com/metacore.php) and Ingenuity Pathway Analysis (IPA) (Ingenuity Inc.) (Version 2.1), were used.

IPA segregates groups of genes within the query set into networks defined by known interactions amassed from the literature[112]. In addition, biological themes are conferred to the networks by again utilising a modified version of the Fisher exact. IPA uses proprietary 'function terms' to assign overrepresented gene categories within the gene list. IPA (and GeneGo) are able to identify canonical pathways that are significantly regulated within the data[113]. Ingenuity Pathway Analysis Base (IPKB) has a knowledge base of ~1.4 million findings.

Expression data sets containing gene identifiers (Entrez Gene ID) and their corresponding expression values with a cut off p-value of 95% or higher and fold changes were uploaded into IPKB or GeneGo. The genes identified as differentially expressed were included in the analysis and utilised to search for biological networks.

2.3 Distribution of type III interferon receptor in a panel of mouse and human tissues –genomic analysis

2.3.1 TaqMan® quantative PCR for IL-28R RNA detection in mouse

Total cellular RNA was extracted using the SV RNA Isolation kit (Promega), (Section 2.1.8.2-6). Using the Bioanalyser RNA 6000 Nano Chip (Agilent), the integrity of the RNA samples was assessed. 1µg of total RNA was reverse transcribed (RT) using the Superscript™ First-Strand Synthesis System for Reverse Transcription RT-PCR (Invitrogen). Three tissue replicates of 10ng/µL cDNA per tissue were pooled and then used in triplicate for TaqMan® analysis. IL-28R specific RNA levels were quantified using an ABI Prism 7900 sequence detector (Applied Biosystems). RT-PCR was performed using mastermix (1x) and IL-28R assay on demand, Mm00558035_m1 (Applied Biosystems): 18S ribosomal RNA used as the endogenous control in a multiplex reaction, RT reactions were incubated for 30 minutes at 50^{o}C followed by the activation of Taq polymerase at 95^{o}C for five minutes. After cooling for two minutes, 40 cycles of PCR were performed with cycling conditions of 15 seconds at 95^{o}C, 40 seconds at 50^{o}C and 30 seconds at 72^{o}C. A relative standard curve using known standards of genomic human DNA was run in parallel and real-time amplification signals were analysed using SDS software (version 1.71 Applied Biosystems).

2.3.2 Gene Logic human tissue microarray screen

The BioExpress Human Atlas Suite (Gene Logic Inc), comprising of 2695 normal human samples belonging to 101 tissue types profiled[114] using human U133A & B chips (Affymetrix)[115,114,116] was used to look at gene expression of IL-28R, IL-10R and IFNAR1.

2.3.3 Human inflammatory cell microarray screen

A GSK proprietary panel of mRNA from human primary cells was used to investigate IL-28R expression using microarray technology, developed by GSK. The panel included peripheral blood mononuclear cells (PBMCs), plasmacytoid dendritic cells (pDC), myeloid dendritic cells (mDC), alveolar macrophages, macrophages, mast cells, B cells, CD4+ T cells and CD8+ T cells, (unstimulated

and stimulated). The Affymetrix human HG-U133-Plus 2 chips were used to profile expression data on mRNA extracted from these cells, IL-28R (TIG45563) data is shown from this screen.

2.3.4 Isolation & treatment of cells for human inflammatory cell microarray screen

pDC and mDCs (>95%), CD4+ T cells, CD8+ T cells and CD4+ CD25+ regulatory T cells were isolated from PBMCs (three donors); separated using Accuspin Histopaque-1077 columns (Sigma-Aldrich) and purified using magnetically labelled beads (Miltenyi Biotec). pDCs and mDCs were magnetically labelled with BDCA-4 and BDCA-1 microbeads respectively. Untouched CD4+ T cells were prepared by magnetic depletion of other cells within PBMC using antibodies to CD8, CD11b, CD16, CD19, CD36, CD56. In a second step naïve CD4+ T cells were isolated from CD4+ T cells by depletion of CD45RO+ T cells (>90% CD4+ CD45RA+ T cells). Isolated CD8+ T cells are depleted of CD8+CD56+cells. Non-CD8+ T cells are indirectly magnetically labelled by using a cocktail of biotin-conjugated antibodies (CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, TCR γ/δ, and CD235a), and the CD8+ T Cell MicroBead cocktail. Isolation of highly pure CD8+ T cells was achieved by depletion of magnetically labelled cells. CD4+CD25+ regulatory T cells were isolated by indirectly magnetically labelling non-CD4+ cells (by using a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ, and CD235a (glycophorin A)). In a second step CD4+CD25+ regulatory T cells are directly labelled with CD25 MicroBeads and isolated by positive selection from CD4+ T cells. Primary tonsillar B cells from three donors (>98%) were isolated by negative selection, non-B cells were labelled (CD2, CD14, CD16, CD36, CD43, CD235a). Macrophages derived from monocytes were prepared from PBMCs (three donors), separated using Accuspin Histopaque-1077 columns and purified using positive selection CD14 beads. Cells were resuspended in RPMI, 10% foetal calf serum, penicillin/streptomycin +L-glutamine and 100ng/mL Macrophage colony-stimulating factor (M-CSF) and incubated for four days to differentiate into macrophages. Mast cells were derived from cord blood mononuclear cells (AllCells), cells were cultured in Iscove's modified Dulbecco's medium (Invitrogen), supplemented with 5% foetal

bovine serum, 1% penicillin/streptomycin, human stem cell factor (100ng/mL), human IL-6 (50ng/mL) and IL-10 (10ng/mL) for 14 weeks.

Cells were treated with a wide range of inflammatory stimuli. pDCs and mDCs were untreated. B cells were unstimulated or stimulated with CD40 ligand or anti B cell receptor (anti-IgM) co-stimulation assay at four time points; 0, 24, 48 and 72 hours. Macrophages were cultured with vehicle alone, TNF (10ng/mL), LPS (10ng/mL), immune complex (IgG coated beads) and cytomix (TNF (10ng/mL), LPS (10ng/mL) and IFNγ (10ng/mL)) for 2, 6 and 24 hours. T cells treatments were CD4+ and CD8+ cells resting; 1, 6 and 24 hours post anti-CD3/CD28. Mast cells were primed for 6 days with human IL-4 (10ng/mL) and human myeloma IgE, lambda (1µg/mL) / human myeloma IgE, kappa (1µg/mL), cells were triggered for 1 hour with anti-IgE (1.5µg/mL), control with no triggering.

2.3.5 Human tissues and whole blood

Human tissue and whole blood samples were collected from anonymised donors. Ethics committee approval was provided and all patients gave informed consent.

2.4 Distribution of type III interferon receptor in a panel of mouse and human tissues, protein expression analysis

2.4.1 Immunohistochemistry overview

Immunohistochemistry (IHC) is a powerful investigative tool used to detect presence and location of antigens (e.g. proteins) in cells of a tissue (histo) section using antibodies specific for the antigen of choice. Antigen : antibody interaction is visualised using chromogenic detection; where enzyme conjugated antibody cleaves substrate resulting in coloured product at location of target protein, or fluorescent detection using fluorophore conjugation to specific antibody. In optimising IHC protocols, several stages need consideration and optimisation; these include antigen retrieval, blocking, primary antibody choice and incubation temperature and time, direct labelling or indirect labelling, choice of secondary antibody and detection method enzyme, substrate. Dual staining where multiple primary antibodies are used needs

further consideration, care needs to be taken to ensure compatibility of methods so both primary antibodies can be visualised.

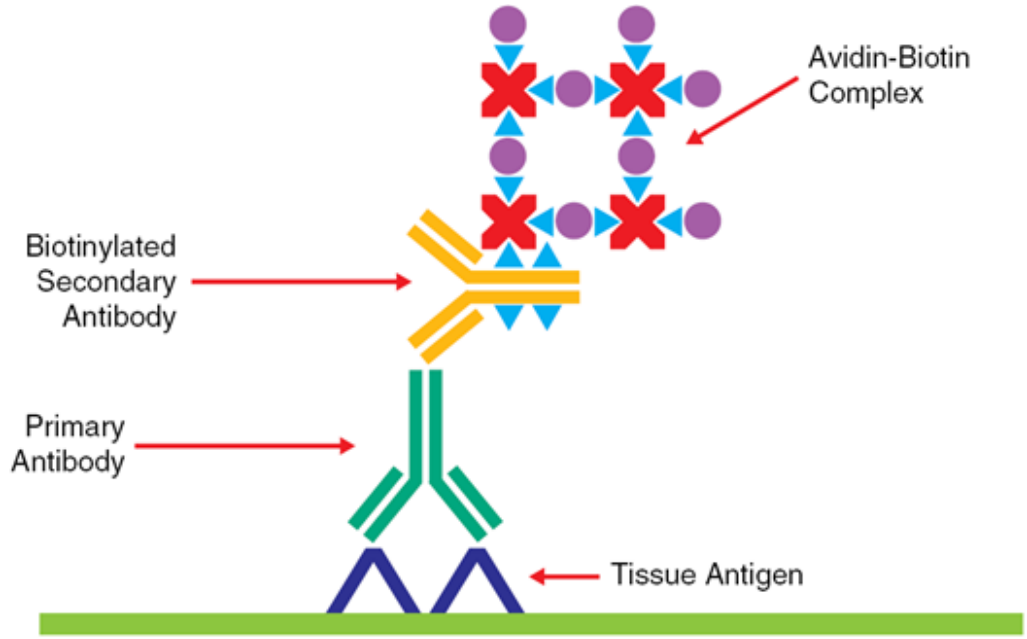


Figure 2.10: Avidin-Biotin Complex (ABC) method

Diagram representation of immunohistochemistry using ABC method - adapted from Dako IHC Staining methods Fifth Edition.

2.4.2 Immunohistochemical staining - IL-28R single stain in mouse and human

IL-28R protein expression in a selected panel of mouse and human tissues was determined by immunohistochemistry. Optimal staining parameters for IL-28R were initially worked up in mouse spleen using two IL-28R antibodies (Capralogics and Abcam). Frozen tissues were evaluated first and then adapted for use in paraffin fixed tissues (formalin fixed for human). Experiments were designed to determine the optimal dilutions that yielded specific positive immunohistochemical staining with minimal non-specific background staining. The following conditions were optimised: dilution range of 4µg/mL to 0.4µg/mL, +/- antigen retrieval, +/- additional avidin/biotin blocking steps, +/- copper sulphate to enhance staining. Preliminary experiments to optimise rat IL-28R antibody (Capralogics) in mouse spleen showed non-specific staining so only IL-28R antibody (Abcam) was evaluated further. Immunohistochemistry method

was developed using IL-28R goat anti-mouse polyclonal antibody (Abcam) (amino acid residues corresponding to 44-72 of N-terminus of mouse CRF-2-12 (IL-28R) protein: LPGLGSPPNVT YFVTYQSYIK TGWRPVEH. Tissue sections were de-waxed in xylene (Sigma-Aldrich) for 5 minutes and rehydrated in industrial methylated sprit (IMS) (Sigma-Aldrich) to distilled water, 2 x 5 minutes. Antigen retrieval was carried out using a low pH buffer; slides were microwaved (Antigen retrieval programme, Milestone Mega TT microwave (Analytix)) in 1500mL of diluted antigen unmasking solution (Vector Laboratories). Slides were washed in water and mounted into sequenza cassettes and slide racks (Thermo Shandon), then rinsed in TBS* buffer for 5 minutes. Peroxidases were blocked with peroxidase blocking solution (Dako) to block endogenous peroxidises for 10 minutes. Non-specific binding of protein was blocked using normal rabbit serum (Dako) (diluted 1:5 TBS) for 10 minutes, followed by a rinse in TBS for 5 minutes and then avidin and biotin blocks (Vector) (4 drops per mL of TBS) for 15 minutes with a TBS wash in between. Slides were incubated with IL-28R antibody at 0.8µg/mL diluted in TBS for 1 hour at room temperature followed by a 5 minute TBS wash. Slides were incubated for 30 minutes with biotinylated rabbit anti-goat secondary antibody (Dako) at 13µg/mL TBS and then washed with TBS for 5 minutes. Slides were developed using streptavidin horseradish peroxidise (HRP) and 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Dako). Staining was enhanced using 4% Copper Sulphate (Sigma-Aldrich) (diluted in distilled water) for 5 minutes, washed in TBS for 5 minutes and then counterstained in Mayers haematoxylin (Sigma-Aldrich) for 5 minutes. Slides were ‘blued’ in running tap water and taken through IMS and xylene washed before being cover slipped using Entellen® mounting medium (Merck Sharpe and Dohme). A negative control goat IgG and a negative control (no antibody) for assessing the biological system were performed for every tissue.

* TBS Tris Buffered Solution 50mM Tris, 0.15M NaCl, pH 7.4. (Dako).

2.4.3 Immunohistochemical staining- IL-28R dual stain in mouse

IL-28R dual staining in mouse tissues was additionally performed with mouse and rat monoclonal antibodies for macrophage identification; 1) Mac387 (ab22506 Abcam) which recognises the L1 or Calprotectin molecule expressed

by granulocytes, monocytes and by tissue macrophages and 2) F4/80 (ab6640 Abcam) which is expressed on a wide range of mature tissue macrophages including Kupffer cells, Langerhans and macrophages located in cords and marginal zone of the spleen. Macrophages are white blood cells derived from monocytes. Macrophages have a phagocytic role, secrete cytokines and are involved in antigen processing and presentation to other cells. OX-62a dendritic cell antibody (ab36444 Abcam) and CD79a b cell marker antibody (Dako 7050) were also used during the optimisation of dual staining protocol but not carried through as the methods for antigen retrieval for OX-62 and IL-28R antibodies were not compatible. All antibodies above were optimised as a single assay and then evaluated for compatibility as a dual assay with IL-28R.

IL-28R dual staining in mouse tissues was performed with 5µg/mL Mac387 (Abcam) and 10µg/mL F4/80 (Abcam), mouse and rat monoclonal antibodies for macrophage identification.

Tissue sections were de-waxed in xylene for 5 minutes and rehydrated in industrial methylated spirit (IMS) to distilled water, 2 x 5 minutes. Antigen retrieval was carried out using a low pH buffer; slides were microwaved (Antigen retrieval programme, Milestone Mega TT microwave (Analytix,)) in 1500mL of diluted (1:10 TBS) antigen unmasking solution (Vector). Slides were washed in water and mounted into sequenza cassettes and slide racks (Thermo Shandon), then rinsed in TBS buffer for 5 minutes. Peroxidases were blocked with peroxidase blocking solution (Dako) to block endogenous peroxidises for 10 minutes. Non-specific binding of protein was blocked using normal rabbit serum (Dako) (diluted 1:5 TBS) for 10 minutes, followed by a rinse in TBS for 5 minutes and then avidin and biotin blocks (Vector) (4 drops per mL of TBS) for 15 minutes with a TBS wash in between. Slides were incubated with IL-28R antibody at 0.8µg/mL diluted in TBS for 1 hour at room temperature followed by a 5 minute TBS wash. Slides were incubated for 30 minutes with biotinylated rabbit anti-goat secondary antibody (Dako) at 13µg/mL TBS and then washed with TBS for 5 minutes. Slides were developed using streptavidin horseradish peroxidase (HRP) and 3, 3-diaminobenzidine tetrahydrochloride (DAB) (Dako). Staining was enhanced using 4% Copper Sulphate (Sigma-Aldrich) (diluted in distilled water) for 5 minutes.

Sections were incubated for 1 hour with the primary antibody MAC387 (5µg/µL Mouse tissue 1µg/µL Human tissue) or F4/80 10µg/mL at room temperature. Slides were incubated for 30 minutes with biotinylated rabbit anti-rat secondary antibody (Dako) at 13µg/mL. The sections were then developed using either VECTASTAIN® ABC-Alkaline Phosphatase (AP) reagent and Vector Red (Vector Laboratories) for MAC387 or HRP followed by VECTASTAIN® ABC-Very Intense Purple® (VIP) (Vector Laboratories) F4/80, and counter-stained with either haematoxylin (Sigma-Aldrich) or methyl green (Vector Laboratories). Slides were taken through IMS and xylene washed before being cover slipped using mount with glycergel mounting medium (Dako). A matched isotype control goat IgG for IL-28R comparison and rat IgG2b for MAC387/F4/80 comparison and a negative control (no antibody) for assessing the biological system were performed for every tissue.

2.4.4 Toluidine blue histochemical stain in mouse

To identify mast cells toluidine blue was used on a section of mouse uterus and compared to serial sections stained with IL-28R antibody and a goat IgG isotype matched control. Mast cells are connective tissue cells with cytoplasm filled with granules comprising predominantly of histamine and heparin. Tissues containing glycosaminoglycans (GAGs) e.g. heparin GAGs, can be stained with basic (cationic) dye, toludine blue. This results in mast cells displaying a purple/violet ‘metachromatic’ colour and blue background staining. Toluidine blue dye structure is shown below.

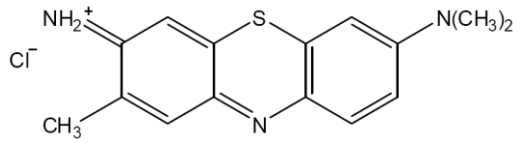


Figure 2.11: Structure of toluidine blue

5µm paraffin sections were dewaxed using xylene and rehydrated in IMS and then distilled water. A toluidine blue working solution was made fresh (1g toluidine blue and 100ml 70% alcohol, mixed 1:10 with a 1% sodium chloride solution (0.5g sodium chloride and 50mL distilled water pH adjusted to 2-2.5 using glacial acetic acid) (all reagents Sigma-Aldrich). Sections were stained in toluidine blue working solution for 3 minutes. Washed 3 times in distilled water

and then dehydrated quickly in 95% ethanol and then 100% ethanol. The sections were then cleared in xylene and cover slipped using Entellen® mounting medium (Merck, Sharp and Dohme).

2.4.5 Flow Cytometric Analysis for tissue IL-28R determination

Mouse tissues: spleen, liver, heart, brain, kidney were homogenised using Covaris focused acoustic system (Kbioscience). IL-28R antibody (Abcam) was biotinylated with an ImmunoProbe Biotinylation kit (Sigma-Aldrich) according to manufacturer's instructions:

http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/bk101bul.Par.0001.File.tmp/bk101bul.pdf

Biotinylated IL-28R was then added to streptavadin phycoerythrin conjugate (R&D systems) and then incubated with tissue homogenate (~5 x 10^6/mL) for 15 minutes at room temperature. Goat IgG antibody was used as a negative control. Cell pellets were suspended in CellWash (Becton Dickinson), centrifuged at 2100rpm for 3 minutes, supernatant decanted and this process was repeated until visibly clear of red blood cells. Labelled cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson). A homogeneous population of cells was gated and analysed using FlowJo Version 4.5.4 software (Tree Star).

2.5 Bone marrow assays

2.5.1 Murine bone marrow in vitro assay

This work was carried out in collaboration with David Brott, AstraZeneca, US all cell work was completed at AstraZeneca, US. Methods were adapted from those described by Brott & Pognan (2009)[117], briefly:

Cell lines & Cultures: M1 cell line (ATCC TIB-192) derived from Mus muscululs spontaneous myeloid leukemia with the cell type being myeloblast was used to evaluate myelotoxicity potential. M1 cells were maintained by splitting cultures twice a week to 1-2 x 10^5 cells/mL in RPMI-1640 (Sigma Aldrich) and 10% foetal bovine serum (Sigma Aldrich).

The HCD57 epo-dependent erythroleukemia cell line was kindly provided by Dr. Spiveck at Johns Hopkins University. HCD57 cells were maintained by splitting

cultures twice a week to 2-5 x 10^5 cells/mL in Iscove's Modified Dulbecco's Medium containing 30% foetal bovine serum, L-glutamine (Sigma Aldrich), 10pg/mL mouse recombinant erythropoietin (R&D Systems) and 20µM beta-mercaptoethanol (Qiagen).

Assay: M1 and HCD57 cells were seeded at 5,000 cell per well in 50µL media. Compounds were dissolved and diluted in appropriate media with 50µL added to the appropriate wells. Mouse IFNα (R&D systems (PBL)) and mouse IL-28A and IL-28B (R&D systems). IFNα, IL-28A and IL-28B were run in triplicate and over duplicate plates, serial dilutions were prepared 0.002, 0.004, 0.008, 0.16, 0.031, 0.063, 0.125, 0.25, 0.5, 1 and 2µM. Assays were cultured at 37^{o}C and 5% CO_2 for approximately 72 hours. After adding 100µL of CellTiter Glo (Promega) to each well, relative luminescence was determined and IC_{50} calculated.

2.5.2 Human bone marrow

2.5.2.1 Gene expression

CD34+ hematopoietic cell samples were obtained from five human umbilical cords (purity >60%) and three bone marrow donors (Lonza, purity >95%) - CD3+ T-cells, CD19+ B-cells, CD11c+ DCs and CD14+ monocytes/macrophage cells were purified from peripheral blood from four normal donors using magnetic beads protocols for separation (Miltenyi Biotec, purity >80%). Cells were analysed for IFNAR1 (Hs01066118_m1 FAM labelled assay on demand (Applied biosystems)) and IL-28R (Hs00417120_m1 FAM labelled assay on demand (Applied biosystems)), IL-10RB (Hs00175123_m1 FAM labelled assay on demand (Applied biosystems)) gene expression using TaqMan® using b-actin house keeper gene (Hs00417120_m1 VIC labelled assay on demand (Applied biosystems)).

2.5.2.2 Human bone marrow CD34+ cell assays

A colony forming cell (CFC) assay was used to determine the ability of hematopoietic progenitor bone marrow CD34+ cells to proliferate and differentiate into erythroid or myeloid colonies after two weeks in a methylcellulose-based semi-solid media (StemCell Technologies) in response

to cytokine stimulation. 0, 0.03125, 0.0625, 0.125μM of IFNα (Sigma-Aldrich), IL-28A (R&D), IL-28B (R&D) and IL-29 (R&D) were added in duplicate. Colony forming unit burst forming unit-erythroid (CFU-BFU), CFU-granulocyte, macrophage (CFU-GM) and CFU-granulocyte, erythrocyte, macrophage, megakaryocyte (GEMM) colonies formed were enumerated and characterised according to their unique morphology.

For proliferation assays, 50,000 CD34+ cells (Lonza) were plated in 200 μL/well of culture medium: DMEM, 10% Hyclone foetal bovine serum and Penicillin/streptomycin, L-Glutamine (100units/mL, 100μg/mL and 2mM respectively). Cells were stimulated with TPO (100ng/mL, Preprotech), IL-3 (60ng/mL, Preprotech), SCF (100ng/mL, Preprotech), Flt3 ligand (300ng/mL, Preprotech) and incubated with serial dilutions of 0.03125, 0.0625, 0.125μM of IFNα (Sigma-Aldrich), IL-28A (R&D), IL-28B (R&D) and IL-29 (R&D) in triplicate for 72 hours. After three days of culture 40μL of Cell Titer 96 aqueous solution (Promega) was added to each well to measure the number of viable cells after proliferation. After 2 hours of incubation at 37oC- the absorbance was measured at 490nm on a 680 microplate reader (BioRad). Stimulated samples exposed to IFN-α or IFN-□□were normalised with respect to the OD of untreated controls.

2.5.2.3 Human tissues and whole blood

Human tissue and whole blood samples were collected from anonymised donors. Ethics committee approval was provided and all patients gave informed consent. San Raffaele Telethon Institute for Gene Therapy Biologic, specimens from umbilical cord blood and peripheral blood of healthy donors were collected for research purpose and used on informed consent in agreement with the rules defined by approved protocols TIGET01 and TIGET PERIBLOOD.

Chapter 3

3. Investigation of the antiviral activity of IL-28A and IL-29 using antiviral and interferon sensitive gene model assays

3.1 Introduction

At the time of this work being undertaken IL-29 and IL-28A had been shown to have antiviral properties in an EMCV model[3] and sensitivity was shown with interferon sensitive gene assays[2,3], but other antiviral activity against a virus or using a model/replicon had not been published.

The aim of this chapter was to evaluate the *in vitro* antiviral activity of IL-28A and IL-29 across a range of viruses spanning a cross section of viruses from all seven classes classified using Baltimore classification[118] compararing their antiviral properties with each other and with IFNα primarily. IL-28B was not tested in these assays as at the time it was not commercially available and was deemed to be similar to IL-28A (96% amino acid identity).

This Chapter looked aty the antiviral activity of IL-28A and IL-29 in the viruses below, class II and III were not covered:

I: dsDNA viruses: Papillomaviridae – Human Papillomarvirus HPV-16 and HPV31

II: ssDNA viruses (+)sense DNA (e.g. Parvoviruses)

III: dsRNA viruses (e.g. Reoviruses)

IV: (+)ssRNA viruses (+)sense RNA: Flaviviridae - Dengue and Hepatitis C, Togaviridae –Semliki Forest virus)

V: (−)ssRNA viruses (−)sense RNA: Paramyxoviridae - Respiratory Syncytical virus.

VI: ssRNA-RT viruses (+)sense RNA: Retroviridae - Human Immunodeficiency virus

VII: dsDNA-RT viruses: Hepadnaviridae - Hepatitis B

Effects of IL-28A and IL-29 were also investigated in IFN sensitive gene models IFIT6-16 and ISG-56.

IFNλs were tested in a wide range of viral cell assays and models in order to elicit interferon lambdas antiviral properties. IFNα was used as a comparison in all assays with IFNα and IFNγ being additional comparisons in the; 1a and 1b HCV replicon assays, HIV, dengue fever replicon assay, RSV assay and ISG assays. Positive sense RNA virus HCV (*flavivirus*) using replicon models were tested as a priority: as type I interferons have antiviral properties it was likely that interferon lambdas may also have been shown to reduce levels of HCV mRNA in the HCV replicon system. Human liver cells HuH7 containing either genotype 1a or 1b HCV replicons were tested in an ELISA assay, and later experiments were performed using two genotype 1a and two genotype 1b replicon luciferase clones in a common parent HuH7 cell (developed by Thomas Zurcher, GSK). HCV is responsive to IFNα, IFNβ and IFNγ[119] but at the time this project was initiated (August 2004) there was no published work to show the role of type III interferons in HCV.

Pegylated IFNα, in combination with ribavirin, is the current standard of care for the treatment of patients chronically infected with HCV; it results in a sustained virological response (defined as clearance of circulating HCV RNA at 6 months post-treatment) in ~50% of patients[10]. However, combination therapy remains less effective against infections caused by HCV genotype 1 which constitute ~75% of all HCV infections in the developed world[10]. New interventions are therefore required to address this unmet medical need and the discovery of interferon lambdas presents the opportunity to explore their roles in antiviral therapy.

Dengue was tested as a virus in the same family as HCV, *flavivirus,* this assay, like the hepatitis C assay, used a replicon model. A further range of viruses were investigated: DNA virus HBV (*Hepadnaviridae)*, negative sense RNA virus Respiratory Synctial Virus (RSV) and the RNA transcribing virus Human Immunodeficiency Virus (HIV) (*Retroviridae*) to give a broad spectrum of different families of viruses. Other viruses: Influenza and Human papillomavirus HPV-16, HPV-31, Semliki Forest virus were considered and even work instigated but are not reported here.

Additional work in this chapter: IFNα and IL-28A, IL-29 were evaluated together in a HCV 1a replicon assay over a time course of 2, 4 ,6, 8, 16 and 24 hours, in

order to determine any synergistic effects of the type I interferons with type III interferons.

Effects of IL-28A and IL-29 were also investigated in IFN sensitive gene models IFIT6-16 and ISG-56 to see if interferon lambdas had a role in regulation of interferon sensitive genes. ISG56 is the most highly induced IFN sensitive gene (30-100 fold induction), and is stimulated by both IFNα and IFNβ, but not IFNγ, IFIT6-16 is also induced by type I IFNs but the role of IFNλ modulation was unknown.

3.1.1 Hepatitis C comparison of antiviral activity of IFNα, IFNβ, IFNγ and interferon lambdas IL-28A and IL-29 in two replicon assays

HuH7 cells containing either genotype 1a or 1b HCV replicons were stained with 5% carbol fuchsin to visually assess cytotoxicity. Cytotoxicity based on cell shape was not seen with any of the interferons tested at any concentration.

The half maximal inhibitory concentration (IC_{50}) values for all interferons were determined as the concentration of each treatment which showed 50% inhibition of the NS5A ELISA signal in HCV 1a and 1b replicon cell lines compared with untreated cells. IC_{50} values were determined for positive control, IFNα, IFNβ, IFNγ, IL-28A and IL-29. % inhibition values were determined according to the following formula:

% Inhibition = 100 - [100x (mean concentration – mean cell control) / (mean cell control – mean no primary antibody control)]

IC_{50} values for replicon assays of genotype 1b were determined; IFNα 18 IU/mL, IFNβ 50 IU/mL, IFNγ 54 IU/mL, IL-28A 160ng/mL and IL-29 11ng/mL. IC_{50} values for genotype 1a replicon assays were as follows; IFNα 86 IU/mL, IFNβ 89 IU/mL, IFNγ 148 IU/mL, IL-28A 201ng/mL and IL-29 19ng/mL.

For comparison purposes IU/mL values for IFNα, IFNβ and IFNγ were converted to pg/mL using the following formula:

[1 x 10^9 ÷ (Lot specific activity)] x (Lot Concentration (IU/mL) = pg/mL)

IC_{50} values for IFNα, IFNβ, IFNγ, IL-28A and IL-29 all in ng/mL units are shown below (Table 3.1) and determined from IC_{50} plots (Figure 3.1).

Table 3.1 IC_{50} values for IL-29, IL-28A, IFNα, IFNβ and IFNγ

	IL-29 Mean (SD)	IL-28A Mean (SD)	IFNα Mean (SD)	IFNβ Mean (SD)	IFNγ Mean (SD)
1a	20 (1.6)	202 (20.1)	0.4 (0.03)	7.3 (0.8)	7.2 (0.6)
1b	11 (2.0)	161 (9.4)	0.09 (0.01)	5 (0.4)	6 (0.7)

Table 3.1 shows IC_{50} values in ng/mL determined in two HCV replicon cell lines 1a and 1b for: IL-29, IL-28A, IFNα, IFNβ and IFNγ.

The results show that all IFNs had some potency against the HCV 1a and 1b replicons *in vitro*. IL-29 was shown to be ~10 x more potent than IL-28A against both replicons, IL-29 was shown to be in the same level of magnitude with potency in both cell lines comparable to IFNβ and IFNγ. IFNα was shown to approximately 100 times more potent than IL-29 and ~1000 times more potent than IL-28A.

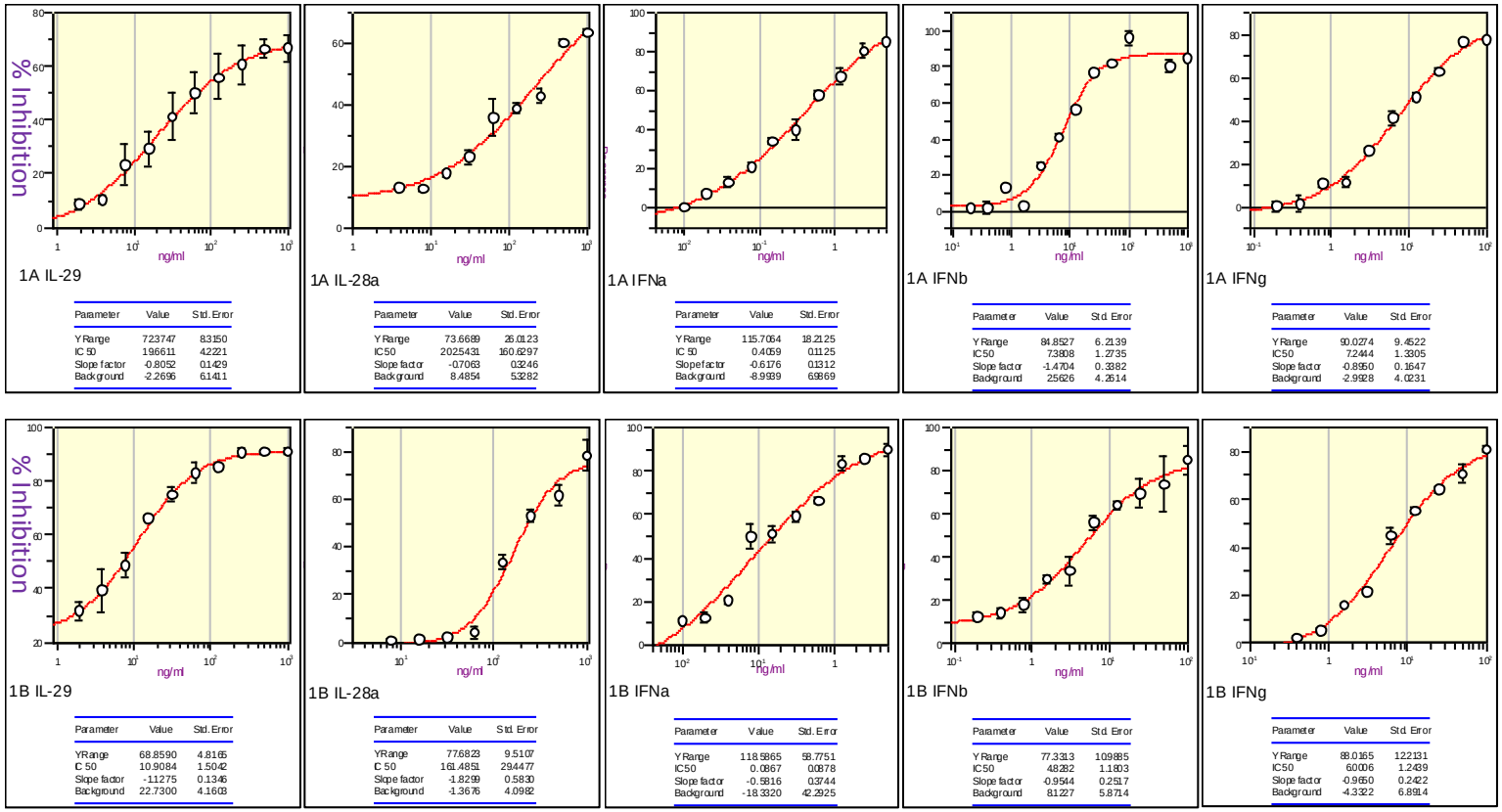


Figure 3.1: IC_{50} plots for IFNα, IFNβ, IFNγ, IL-28A and IL-29

Figure 3.1 shows IC_{50} plots for IFNα, IFNβ, IFNγ, IL-28A and IL-29 in HCV replicon cell assays 1a and 1b, values in ng/mL (Graffit V5).

3.1.2 Determination of IC_{50} values for luciferase HCV replicon clones

Four luciferase HCV replicon clones 1a1_19, 1a3II and 1b 2.2, 1b2.5 were tested for their antiviral sensitivity to IL-28A and IL-29 compared to IFNα. These cells were all developed in the same parental HuH7 cell line. Log dose plots are shown (Figure 3.2) for clones 1a3II and 1b2.5 showing good curve fits for IL-29 and IFNα and due to solubility issues the best fit for IL-28A is shown (Figure 3.2).

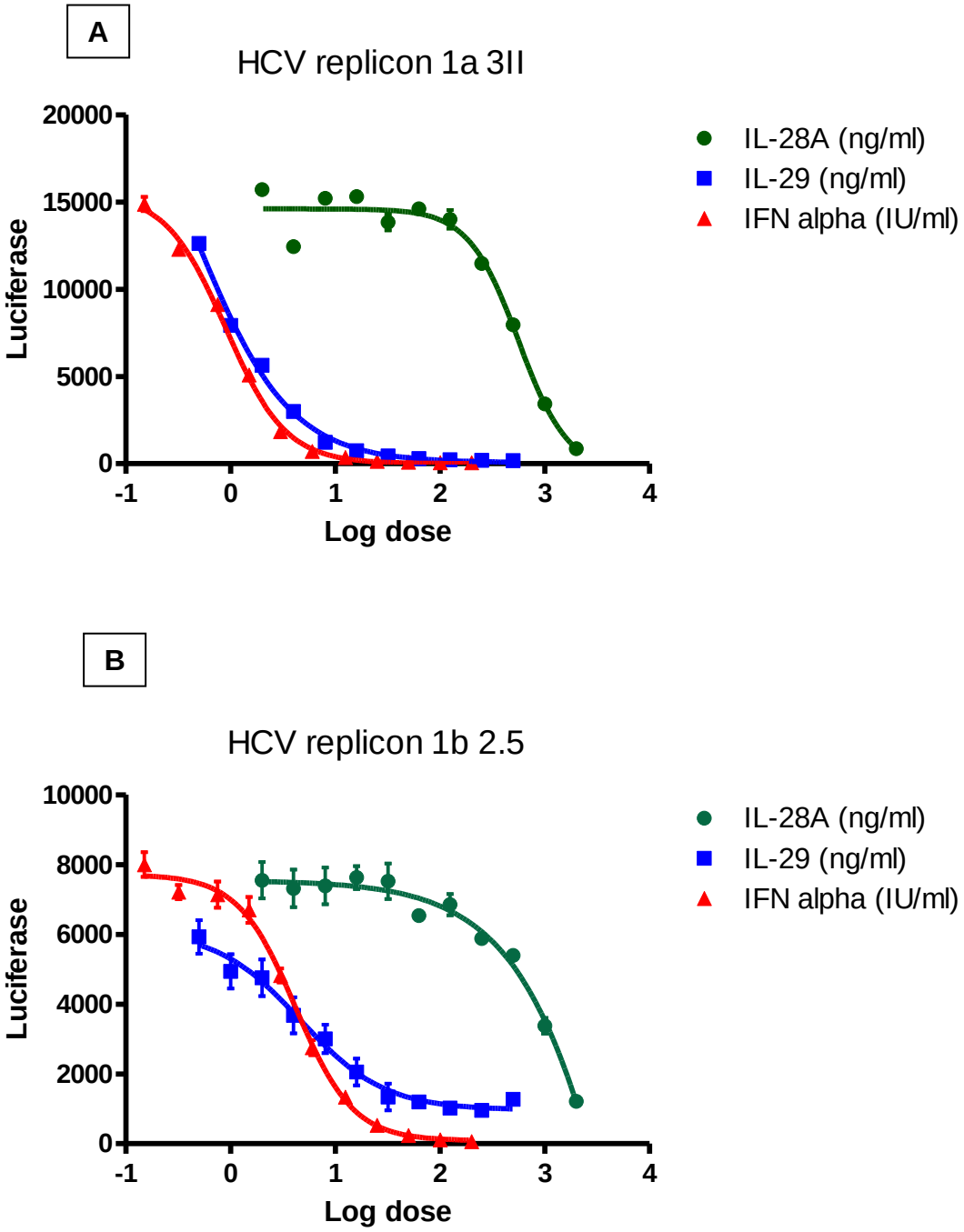


Figure 3.2: IC_{50} plots for HCV replicon assays 1a3II and 1b2.5

Figure 3.2: IC_{50} plots are shown for luciferase assays: **A** 1a3II and **B** 1b2.5 for IL-28A, IL-29 ng/mL and IFNα IU/mL (PRISM).

All four HCV replicon clone assays 1a1_19, 1a3II and 1b 2.2, 1b2.5 were assessed and the IC_{50} values in ng/mL with IL-28A, IL-29 and IFNα shown (Table 3.2).

Table 3.2: IC_{50} values for IL-28A, IL-29 and IFNα in HCV replicon clones

Clone	IL-28A IC_{50} (ng/mL) Mean (SD)	IL-29 IC_{50} (ng/mL) Mean (SD)	IFNα IC_{50} (ng/mL) Mean (SD)
1a 1_19	46.6 (2.9)	0.7 (0.08)	0.005 (0.0002)
1a3II	21.8 (5.6)	0.55 (0.03)	0.003 (0.0001)
1b 2.2	118 (16.5)	2.7 (0.17)	0.004 (0.0002)
1b2.5	227 (19.9)	4.6 (0.31)	0.01 (0.003)

Mean IC_{50} values for IL-28A, IL-29 and IFNα are shown for HCV replicon clones 1a1_19, 1a3II and 1b 2.2, 1b2.5 in ng/mL.

Replicon clone 1a3II was most sensitive to all tested interferons having the lowest IC_{50} value for IL-28A, IL-29 and IFNα. IC_{50} values for replicon clone 1a3II were used for later gene expression work (Section 4.2).

3.1.3 IL-29 and IFNα synergy

IFNα and IL-28A, IL-29 were evaluated together in a HCV 1b replicon assay over a time course of 2, 4 ,6, 8 16 and 24 hours, in order to determine any synergistic effects of the type I interferons with type III interferons. Serial dilutions of IL-28A 200ng/mL to 4ng/mL and IL-29 1000ng/mL to 2 ng/mL were tested with and without 0.09ng/mL of IFNα (IC_{50} concentration as determined in experiments performed (Section 2.1.1). To confirm baseline expression of IFNα at 0.09ng/ml this was also run separately. Results showed an additive effect only, not a synergistic effect. This was seen across all doses with IL-28A and IL-29 at all tested time points.

3.1.4 IFN sensitive gene models 6-16 and ISG56

To investigate type III interferon modulation of interferon sensitive genes (ISGs), two ISG assays were used; interferon sensitive gene 56 (ISG56) and interferon inducible gene 6-16 (G1P3).

Half maximal effective concentration (EC_{50}), representing the concentration of a compound where 50% of its maximal effect is observed, were determined for both ISG models.

Table 3.3: EC_{50} values for IFNs in ISG models

IFN	ISG56 EC_{50} Mean (SD)	6-16 EC_{50} Mean (SD)
IL-28A	439ng/mL (34.7)	>2.0µg/mL
IL-29	115ng/mL (18.4)	>1.0µg/mL
IFNα	50 IU/mL (3.2) (0.16ng/mL)	48 IU/mL (4.6) (0.15ng/mL)
IFNβ	121 IU/mL (14.3)	120 IU/mL (0.89)
IFNγ	350 IU/mL (22.0)	500 IU/mL (35.7)

In the Interferon sensitive assays, sensitivity was shown in the ISG56 assay to IL-28A and IL-29 but not in the 6-16 interferon sensitive gene model. These experiments suggested different modes of action for IL-28A and IL-29 compared with IFNα, however Affymetrix gene expression data suggested that there was expression of the IFIT6-16, alias G1P3 with IFNα, IL-28A and IL-29 treatment at 8, 16 and 24 hour time points.

Data below is taken from replicon data (Section 4.5.2, Table 4.8).

Table 3.4: Subsection of Table 4.7 to highlight G1P3 expression

Symbol	IFN**α**			IL-29			IL-28A		
	8h	16h	24h	8h	16h	24h	8h	16h	24h
G1P3	3.2	82.1	42.1	6.7	100	96.5	4.5	100	43

This suggests that whilst the 6-16 cell model is not sensitive to type III IFNs, there is gene expression of 6-16 shown with Affymetrix and TaqMan® technology.

3.1.5 Hepatitis B (HBV)

Antiviral effects of IL-28A and IL-29 compared with IFNα were investigated in human hepatocellular carcinoma HepG2.2.15 cell line stably transfected with HBV subtype ayr. Concentrations of IFNs tested were: IFNα: 100, 1,000 ng/mL, IL-28A: 200, 2000 ng/mL and IL-29: 100, 1000 ng/mL, compared to untreated. Preliminary results shown (Figure 3.3) indicated that both IL-29 and IL-28A have antiviral properties in the HepG2 (2.2.15) cells stably transfected with HBV subtype ayr. These results show that IFNα has a greater antiviral effect of IL-29 on HBV compared to IL-28A. Initial data shows IL-29 having a greater antiviral effect on HBV than IL-28A. A publication by Robek[51] superseded further work on HBV.

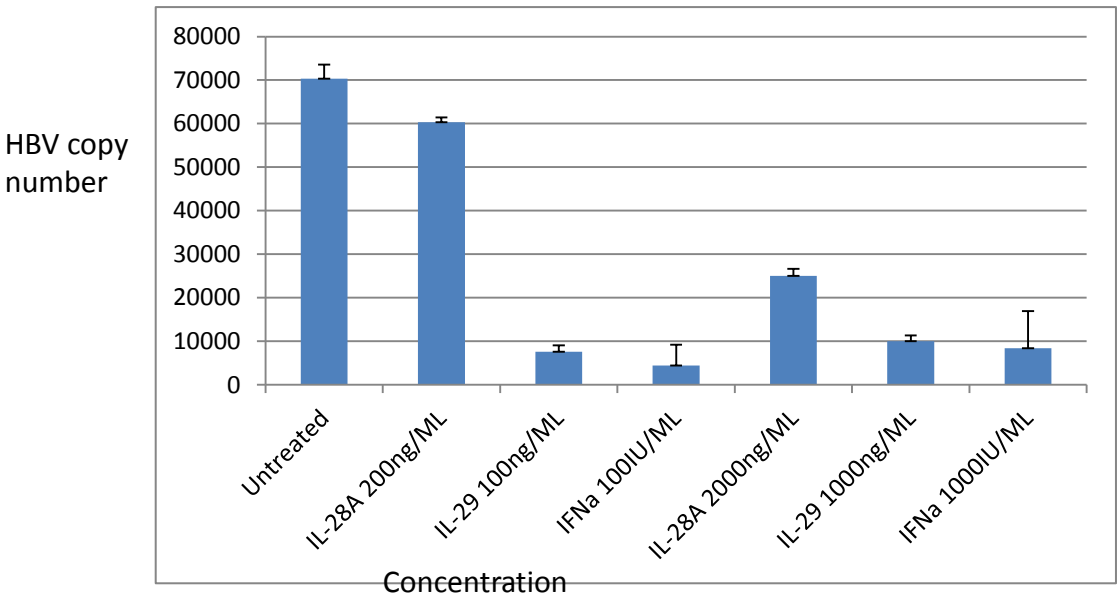


Figure 3.3: Antiviral effects of IL-28A and IL-29 with HBV

Antiviral effects of IL-28A and IL-29 compared with IFNα were investigated in human hepatocellular carcinoma HepG2.2.15 cell line stably transfected with HBV subtype ayr. IL-28A and IL-29 tested at low and high doses (200, 2000 and 100, 1000 ng/ml respectively) and compared with IFNα low and high dose dose (100, 1000 IU/ml). Copy number was determined from the mean of the 4 replicate samples using the following equation: 10-1(Ct-40)/3.5 where 10-1 is the inverse log, Ct is cycle threshold, 40 is number of total cycles and 3.5 is PCR efficiency.

3.1.6 Human immunodeficiency virus (HIV)

IFNα, IFNβ, IFNγ have been shown to have antiviral properties against HIV[120], and are used as positive controls and as comparisons to investigate the antiviral effects of IL-28A and IL-29 with HIV.

Data (Figure 3.4) shows: a strong antiviral effect of IFNα and IFNβ on HIV, IFNγ has a weak antiviral effect and IL-28A and IL-29 have no antiviral effects even at very high concentrations. IC_{50} values were determined as: IFNα 2.8 IU/mL, IFNβ 43.2 IU/mL and IFNγ 140 IU/mL. There were no effects even at a high dose of 7.68mg/mL for IL-28A and 1mg/mL for IL-29; interestingly no toxicity was observed at these doses. In this cell system, type III lambdas do not seem to have an antiviral effect even at very high doses.

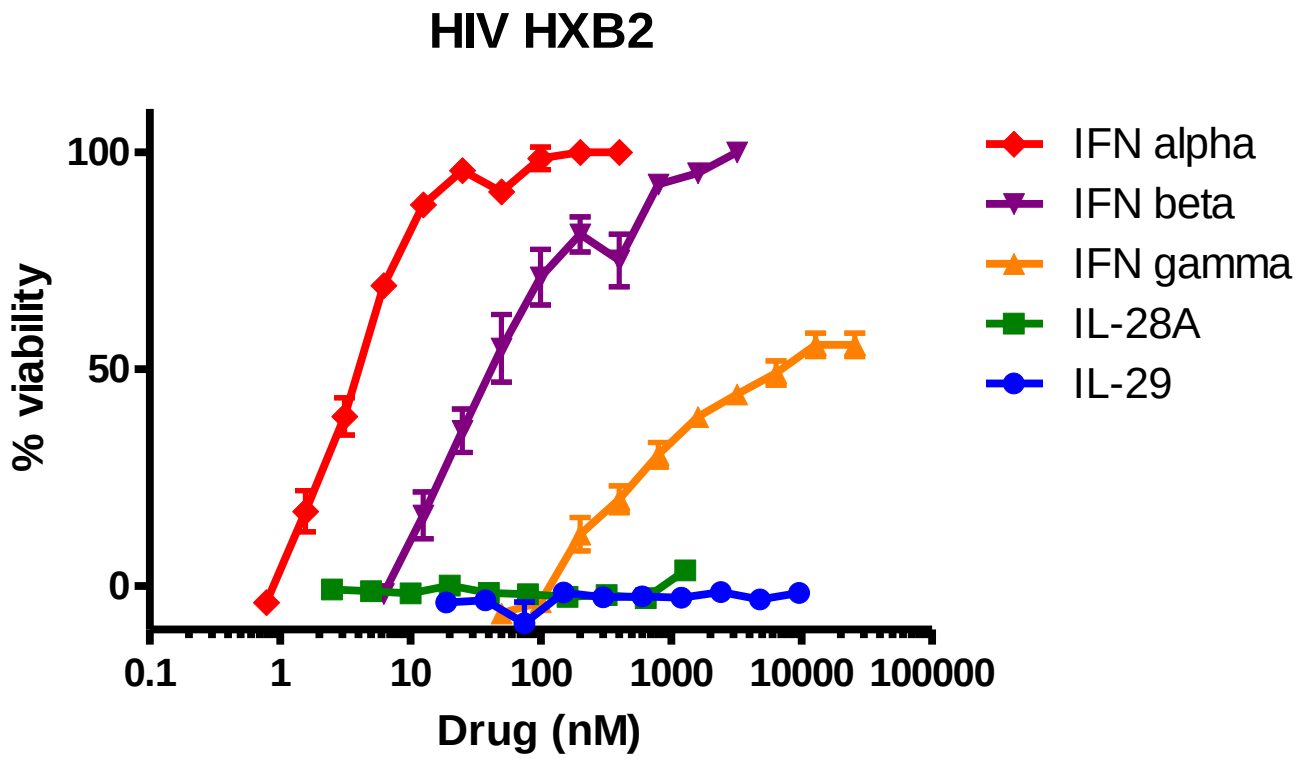


Figure 3.4: Antiviral effects of IL-28A and IL-29 against HIV virus HXB2

The antiviral effects of IFNα, IFNβ, IFNγ, IL-28A and IL-29 in and MT4 cell line infected with HIV virus HXB2. The graph shows % viability of MT4 cells against log dose concentration of IFNα, IFNβ, IFNγ, IL-28A and IL-29. (PRISM).

3.1.7 Dengue Fever Virus assay

K562 myeloid/erythroid (human chronic myeloid leukaemia) cell line stably expressing the dengue virus replicon ΔCprME-PAC2A was used to test the antiviral properties of type III interferons, compared to IFNα, IFNβ and IFNγ. 100, 1,000 IU/mL of IFNα, IFNβ and IFNγ, 200, 2000ng/mL of IL-28A and 100, 1000ng/mL of IL-29 were tested against dengue virus replicon with an incubation of 24 hours. Results show for all IFNs at all concentrations that dengue virus RNA levels do not significantly vary from that of untreated cells, results shown (Figure 3.5).

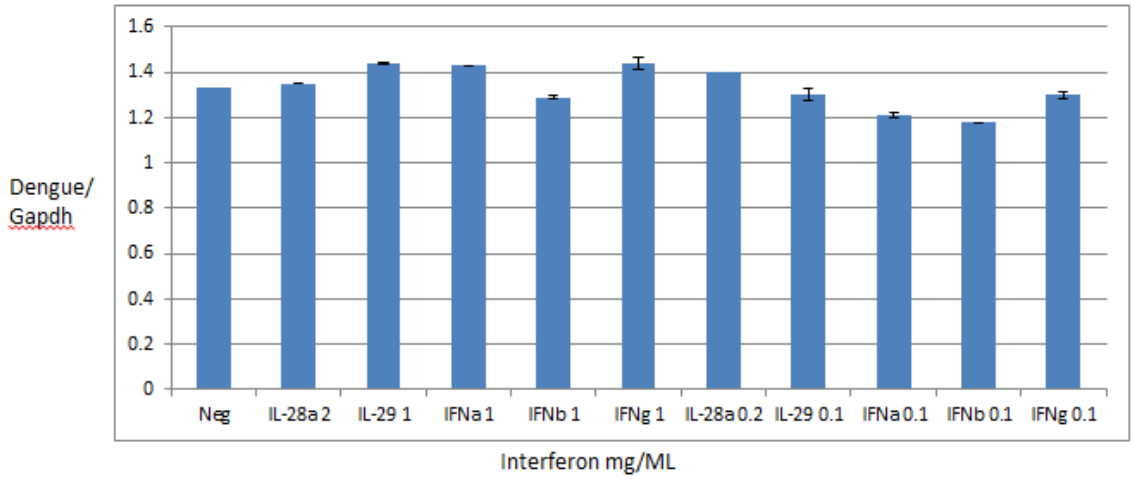


Figure 3.5: Dengue virus replicon

Dengue RNA levels were measured by quantitative PCR and normalised to GAPDH mRNA levels; the data demonstrated no significant difference between untreated sample (negative control) and IFNα, IFNβ, IFNγ, IL-28A and IL-29 treatment, indicating that Interferon lambdas IL-29 and IL-28A do not have antiviral effects in a dengue replicon system.

3.1.8 Respiratory Syncytial Virus (RSV)

Transformed B cells infected with RSV WSX1 viral particles were treated with IFNα, IFNβ and IFNγ at 1000 IU/mL, IL-28A at 2µg/mL, IL-29 at 1µg/mL and untreated to assess the effects of antiviral treatment. Cells were incubated at two time points: 48 hours and 96 hours.

No test samples differed significantly from control, suggesting that IFNs have no effect on the RSV WSX1 model. The negative results above may also be

explained by RSV RNA being very sensitive to manipulation and the cell model being quite difficult to maintain stable RSV levels.

3.1.9 Discussion

It has been shown that type I IFNs (IFNα, IFNβ, IFNЄ, IFNκ and IFNω in humans) are modulators of antiviral defence by many groups[121,122,123], Type II IFN, IFNγ is also an important mediator of antiviral defence but not as its primary function which is stimulation of the immune responses[124,125]. The role of type III interferons, IL-28A, IL-28B and IL-29 in antiviral disease is still not fully known.

HCV- *in vitro* assays show that IL-28A and IL-29 have a direct antiviral effect against HCV in the HCV genotype 1a and 1b replicon systems (ELISA). Despite having similar molecular weights (IFNα 19.5kDa, IL-29 21.9kDa and IL-28A 20.8kDa) IFNα was shown to be ~100 fold more potent against HCV replicons 1a and 1b than IL-29 and 1000 fold more potent than IL-28A, suggesting that IFNα is more potent against HCV than type III IFNs. IFNβ and IFNγ showed similar potency to IL-29 in both 1a and 1b replicon assays and IL-29 was shown to be ~10 times more potent in both assays than IL-28A. Replicon 1b assay was more sensitive to all IFNs tested; IFNα, IFNβ, IFNγ, IL-29 and IL-28A.

With an alternative luciferase replicon assay model for HCV 1a replicon clones, 1a 1_19, 1a3II and 1b replicon clones 1b 2.2 and 1b2.5, similar results were shown with comparison of IFNα to IL-28A and IL-29 as with the HCV genotype 1a and 1b replicon systems (ELISA). IFNα was shown to be ~100 fold more potent than IL-29 (1000 fold in 1b 2.2) and over 1000 fold more potent than IL-28A. IFNα showed similar IC_{50} values in all the four replicon assays but IL-28A and IL-29 were ~10 times more potent against the 1a replicons than the 1b replicons which may show a difference in mode of antiviral action against the HCV sequence. Replicon clone 1a3II was shown to be most sensitive to IFNα, IL-28A and IL-29 and chosen for further investigation of pathway analysis (gene chip experiments). Robek[51] demonstrated that IL-29 and IL-28A inhibits replication of subgenomic and full-length HCV replicons in HuH7 cells and this was also shown later by Doyle[57] and Marcello[61].

Due to IL-28A and IL-29 using a different receptor (IL-28R rather than IFNR1), IL-28A and IL-29 were tested in the HCV 1b replicon assay to determine any synergistic effects of the type I and type III interferons together. Results described only showed an additive effect not a synergistic effect, this was observed at all time points 2, 4, 6, 8, 16 and 24 hours. An additive effect though could show that IL-29 when used with IFNα could be good as a dual therapy as opposed to IFNα alone as the results demonstrate greater potency against HCV with both IFNa and IL-29 together. These results were confirmed by Marcello *et al* in 2006, his group also saw an additive effect not a synergistic effect of IL-29[61].

Two interferon sensitive gene assays ISG56 and 6-16 were tested with IL-29 and IL-28A and compared to IFNα, IFNβ and IFNγ. In the ISG56 assay (Human Embryonic Kidney 293 cells (HEK cells)) IL-28A and IL-29 were shown to be ~1000 times less potent for activation of the ISG promoter in this model than IFNα and interestingly significantly less sensitive at modulation of ISG56 than IFNβ and IFNγ. In the HCV replicon assays IL-29 showed similar antiviral protective effects as IFNβ and IFNγ. IL-28A has been shown to have ~10 times less antiviral effect in the replicon assays than IL-29 but in the ISG56 assay, IL-29 and IL-28A showed EC_{50} values in the same magnitude. In the interferon sensitive assays, sensitivity was shown in the ISG56 assay with IL-28A and IL-29 but not in the 6-16 assay. It was initially thought the ISG experiments described above suggest different modes of action for IL-28A and IL-29 compared with IFNα, but results later (Chapters 5 and 6) showed IL-28R had limited receptor distribution, and difference seen in reporter activity might be due to restricted receptor expression. Interferon lambdas have also been shown to induce expression of 2'5-OAS[2] and MxA[126] by other groups Kotenko *et al* and Brand *et al*.

All cells described in Chapter 3 were tested by TaqMan® for presence of IL-28R gene, Human T98G cells (glioblastoma multiforma tumor cells) (6-16 assay cells) did not show signal for IL-28R.

Initial data in a human HepG2 cell line stably transfected with HBV subtype ayr showed that both IL-29 and IL-28A had antiviral effects against HBV but significantly lower in antiviral activity than IFNα. The antiviral effect of IL-28A

compared to IL-29 was significantly lower. HBV data was consistent with data from Robek[51], where IL-29 was shown to inhibit HBV replication in a murine model (HBV-Met cells). However, a later study suggested that the antiviral activity of type III IFNs against HBV may be limited in human cells; Kotenko *et al* found limited antiviral properties with IL-29 in human hepatoma cells stably transfected with HBV subtype adwR9[127].

No antiviral effects were observed with IL-28A or IL-29 in HIV, RSV assays or against the dengue replicon system. The presence of IL-28 receptor gene expression was confirmed by TaqMan® real-time PCR in these systems, implying that the specific type III receptor could be present, so an obvious lack of receptor is unlikely to be the reason for the lack antiviral response in these systems. There was no cytotoxicity observed with the type III IFNs in the HIV assays at concentrations of IL-28A and IL-29 up to 7.68mg/mL and 1mg/mL respectively. This initially was of great interest as IL-28A and IL-29 not having any antiviral activity against HIV *in vitro* could have indicated that the lambdas were functionally different to IFNα. To date limited studies have described the effects of IFN-λ on HIV-1 replication and disparate results were reported, the effect of interferon lambdas remains controversial. Hou *et al* demonstrated inhibition of HIV-1 replication in macrophages by IL-29 and IL-28A[128], with Liu *et al* showing that IL-29 inhibition of HIV in macrophages is via the JAK-STAT pathway[129]. Another study showed pretreatment of uninfected PBMCs or CD4+ T-cell lines with IFN-λ improved the expression of HIV-1 receptor and co-receptors that increase viral binding and replication[130] and in July 2012 Tian *et al* demonstrated IL-28A and IL-29 treatment induced an antiviral state in cultured primary T-cells, suppressing HIV-1 integration whereas *in vivo* IL-29 showed limited in vivo repression of viral production in CD4+ T cells[131].

It is possible to speculate that the IFN signal transduction pathway with type I, II and III IFNs is blocked in the dengue virus replicon system. Jones *et al* showed that IFNα in the same dengue replicon system had no antiviral effect and that dengue virus specifically inhibits IFNα signalling by the down-regulation of STAT2 expression[53]. Whilst HCV is very sensitive to the antiviral cascade induced by IFNα, in contrast, the related viruses West Nile virus (WNV) and

Japanese encephalitis virus are resistant to IFNα therapy. WNV has been shown to block the IFN signal transduction pathway[132].

Results from the RSV assay showed no differences between IFNα, IL-28A, IL-29 compared with negative control, suggesting that IFNs have no effect on the RSV WSX1 model. The negative results above may also be explained by RSV RNA being very sensitive to manipulation and the cell model being quite difficult to maintain stable RSV levels. There are also reports to show that RSV inhibits IFNα signalling, IFNγ production and type III interferon expression[133].

The antiviral effects in other viral systems have been demonstrated for the IFNλs both *in vitro* and *in vivo*: IL-28A and IL-29 in an *in vivo* poxvirus infection model[55], IL-29 reduced influenza A titre *in vitro*[134], IL-28A and IL-29 in an *in vitro* murine CMV system[56], IL-29 had antiviral effects against herpes simplex virus (HSV-1) *in vitro* comparable to that of IFNα[135], IFNλs have antiviral effects *in vivo* and not *in vitro* with HSV-2[136] highlighting the possibility that the majority of IFNλs antiviral effects against HSV may involve immune modulation. Taken together, these studies supported the value of studying IL-28A and IL-29 as a possible therapeutic approach to viral disease. Whilst there have been several studies looking at the antiviral properties of IFNλs in RNA viruses there is a gap in research on the effects of IFNλs with DNA viruses.

Chapter 4

4. Transcriptome analysis in parent HuH7 cells and HCV replicon cells treated with type I and type III IFNs

4.1 Introduction

Type III interferons (IFNs) are a newly identified class of cytokines with antiviral activity[1,2]. It is not yet clear how the function of these cytokines differs from the type I IFNs. This chapter therefore sets out to investigate how the pattern of gene induction by type III IFNs (IL-28A and IL-29) differs from that of type I and whether type III IFNs might have a superior therapeutic profile. IFNs protect cells from viral infection, directly by inducing interferon sensitive genes (ISGs) such as 2', 5'-oligoadenylate synthetase 1 (2', 5'-OAS) and myxovirus resistance-A (MxA) and intracellular genes and, indirectly, by inducing major histocompatibility complex (MHC) class I antigen expression on and activation of antigen-presenting cells, by stimulating dendritic cell maturation and activating macrophages and natural killer cells.

IFNλs were initially shown to activate both STAT1 and STAT2 and the downstream signalling pathways including interferon sensitive gene complex ISGF3 and ISRE1[3] , similar to those activated by IFNα and IFNβ. However the mechanisms of IFNλs still need to be fully elucidated.

The objectives of this chapter were to:

1) identify which genes are regulated by type III IFNs

2) to see how this profile differs from type I IFNs and

3) to see the effect of IFNs on human hepatoma cell line HuH7 HCV replicon cells compared with parental HuH7 cells.

By comparing the transcriptional levels of genes in tissues or cells from 'control' and 'test' states, it is possible to gain insight into the mechanisms and pathways underlying the comparative phenotypes observed. There are a multitude of techniques available to researchers to undertake these types of transcriptional

investigation, although they can be broadly defined as ‘open systems’ and ‘closed systems’. Open transcriptional systems have the advantage of not requiring any prior sequence knowledge of the genes under investigation, examples are: ‘fragment display’ (FD)[137], ‘tag sequencing’ and ‘subtractive/competitive hybridisation’ techniques. In general, the main disadvantage associated with open transcriptional systems is their relatively low throughput, both in terms of numbers of gene modulations that can be positively identified and the number of transcriptional comparisons that can be made.

Closed transcriptional systems include an equally diverse array of techniques, although the shared attribute is the requirement for prior sequence knowledge of the gene(s) under investigation. Closed systems include techniques that are only amenable to investigating the transcriptional levels of single (e.g. Northern blots and the ribonuclease protection assay) or small subsets of genes (e.g. real-time quantitative PCR (RT-QPCR)). At the other end of the spectrum, they include those capable of simultaneously measuring the expression levels of thousands of genes, i.e. Affymetrix GeneChips® microarrays, leading to the development of ‘transcriptomics’, broadly defined as the rapid and quantitative comparison of large-scale mRNA expression profiles in biological systems. Combined with the huge advances in genome sequencing and gene annotation made in recent years, they have led to the advantages of ‘open’ transcriptional systems becoming more or less obsolete. As genome sequencing of an increasing number of model species is completed, it is becoming practicable to use microarrays to measure transcription at a virtually genome-wide scale. This has enabled the generation of huge volumes of data, faster than ever before.

Data (Section 3.1.2) showed that the HCV replicon system would be a suitable model for transcriptomic analysis of the IL-28A and IL-29 as the replicon cell line showed sensitivity to IL-28A and IL-29. To determine gene activation profiles and the signalling pathways of IL-28A and IL-29 transciptomic and pathway analysis were performed using Affymetrix GeneChips® analysis and pathway analysis.

4.2 Determination of 10 x IC_{50} for most IFN sensitive replicon cell line

Results of the four replicon cell lines were compared and IC_{50} values determined (Section 3.1.2, Table 3.2),1a3II results highlighted below (Table 4.1)

Table 4.1: IC_{50} values in ng/mL of IL-28A, IL-29 and IFNα in HCV replicons

Clone	IL-28A IC_{50} (ng/mL)	IL-29 IC_{50} (ng/mL)	IFNα IC_{50} (ng/mL)
1a1_19	46.6	0.7	0.005
1a3II	21.8	0.55	0.003
1b2.2	118	2.7	0.004
1b2.5	227	4.6	0.01

HCV replicon clone 1a3II was most sensitive to interferons having the lowest IC_{50} value with IL-28A, IL-29 and IFNα. 10 x the IC_{50} level was calculated as: 218ng/mL, 6ng/mL and 0.03ng/mL for IL-28A, IL-29 and IFNα respectively. These 10 x IC_{50} values were used to treat parent HuH7 cells and HuH7 HCV 1a3II luciferase replicon cells for use in transcriptome analysis.

4.3 TaqMan® analysis

Real-time PCR was performed on the RNAs isolated from HuH7 cells and HuH7 HCV 1a3II luciferase replicon cells treated with 10 x IC_{50} values of IL-29, IL-28A and IFNα at 4, 8, 16 and 24 hour time points The data was analysed to assess the suitability of the time points and concentrations of the IFNs in the assay before running the samples of full genome Affymetrix chips.

TaqMan® analysis showed IFIT1 expression in all IL-29, IL-28A and IFNα samples showing that all RNAs were suitable for further Affymetrix genechip® analysis.

TaqMan® analysis showed greatest gene expression change with IFIT1, MX1, OAS1 and G1P3 at the 8, 16 and 24 hour time points (Figure 4.1).

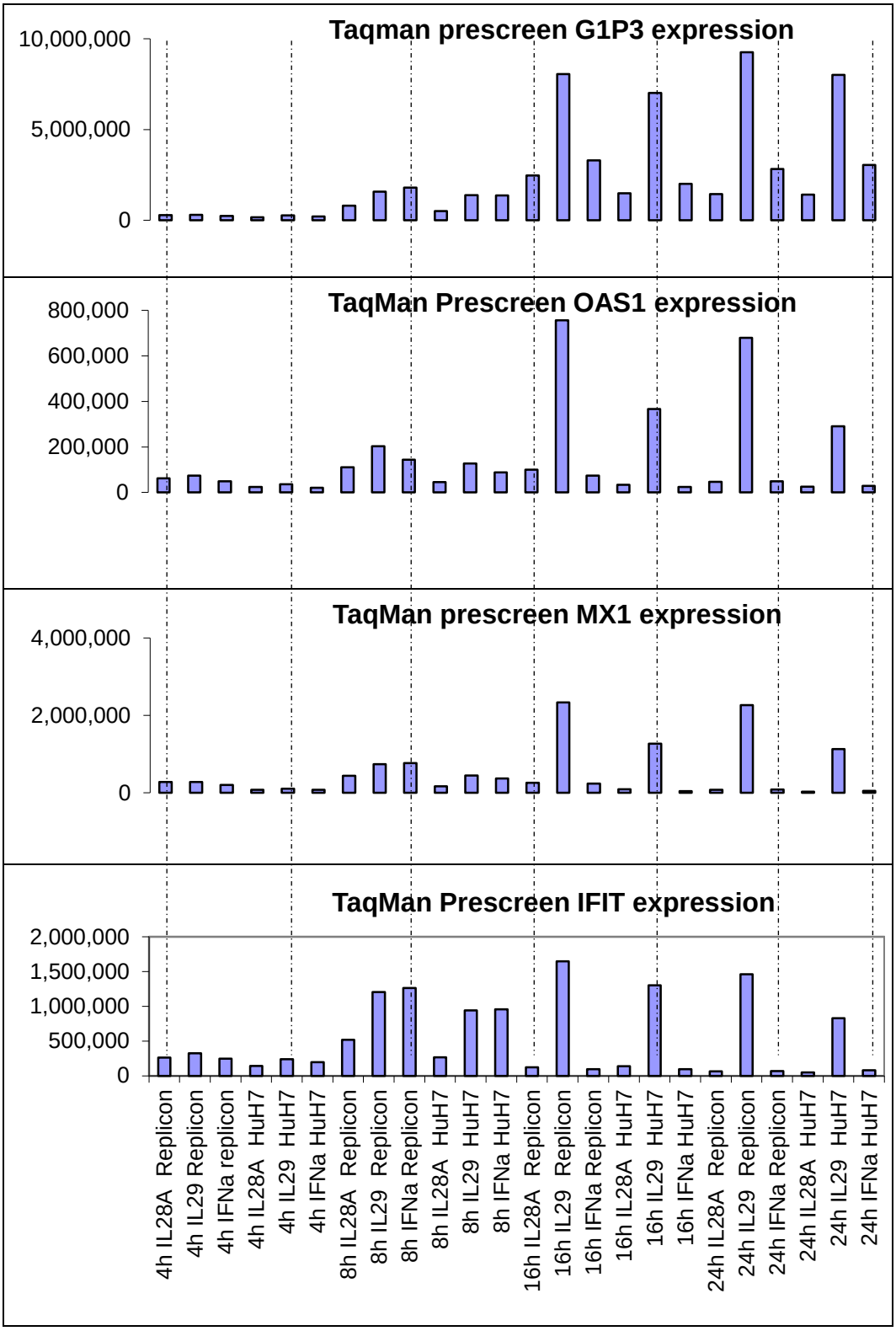


Figure 4.1: TaqMan® expression of IFITI, MX1, OAS1 and G1P3

TaqMan® expression of IFITI, MX1, OAS1 and G1P3 compared with house keeping genes b-actin and GAPDH in parent HuH7 and HuH7 HCV replicon cells treated with 10 x IC_{50} values of IFNα, IL-28A and IL-29 at 4, 8, 16 and 24 hour time points.

4.4 Parent HuH7

Individual data and pathway analysis was undertaken for all treatments and time points. Data was analysed to a set criteria: in Rosetta Resolver (2.2.6.11), an ANOVA was generated for each compound versus PBS control, the genes were filtered to include genes with fold change >1.5 and >minus 1.5, and genes were included if their p-value was <0.05 (>95%). Lists were generated based on the above criteria of upregulated and downregulated genes for IL-28A, IL-29 and IFNα at each time point. Lists were also generated of genes that were common and unique between IL-28A, IL-29 and IFNα in both HuH7 parent cells and HuH7 HCV replicon cells. Gene lists were then put into Ingenuity and GeneGo for pathway analysis.

4.4.1 Parent HuH7 versus HCV replicon

The Affymetrix dataset (P-value >95% Fold change -1.5< and >1.5) is represented numerically in a series of Venn diagrams (Figure 4.2), showing gene numbers common and unique to IFNα, IL-28A and IL-29 at all time points in both the parent HuH7 and HCV replicon datasets.

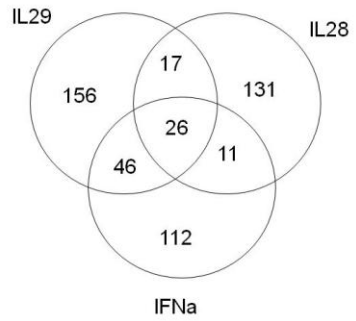
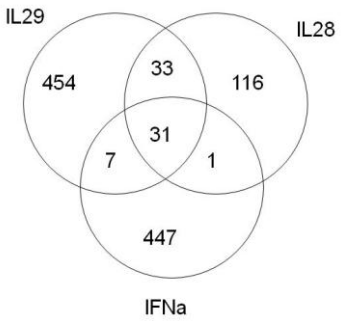
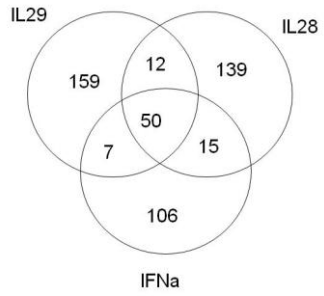
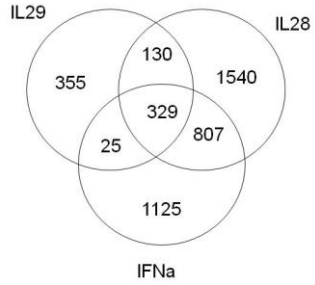
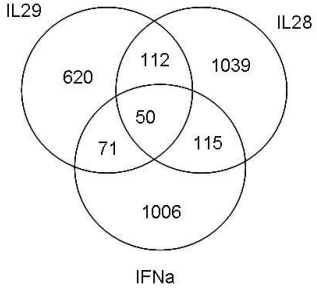
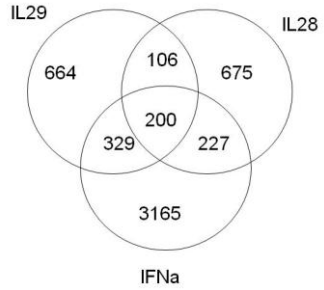
8 hours	16 Hours	24 Hours
Replicon		
		
Parent		
		

Figure 4.2: Venn diagrams

The number of genes expressed by IL-28A, IL-29 and IFNα across the 8, 16 and 24 hour time points from both replicon and parental datasets are represented in Venn diagrams. (P-value >95%, fold change -1.5< and >1.5)

Results from the data presented as Venn diagrams (Figure 4.2) show at the 8 hour time point show a 90, 92 and 56% reduction in gene expression for genes unique to IFNα IL-28A and IL-29 respectively in replicon compared to parent line. Similar reductions in gene expression are also seen at the 16 hour and 24 hour time points. The Venn diagrams showed a trend between parent and replicon datasets of more genes being unique to an individual interferon than there were genes in common between the interferons.

Principal component analysis (PCA) is a mathematical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components. The first principal component (PCA1) shows the largest possible variance e.g. accounts for as much of the variability in the data as possible, this will be followed by PCA2 and so on. PCA1 is shown (Figure 4.3) and illustrates that time accounts for the biggest variable in the data, followed by cell type replicon versus parent in PCA2 plot (Figure 4.4). PCA1 plot shows 4 hour and 16 hour clustering together and 8 hour data being separate for replicon and parent cell lines. PCA2 shows replicon cells separating from normal parent HuH7cells.

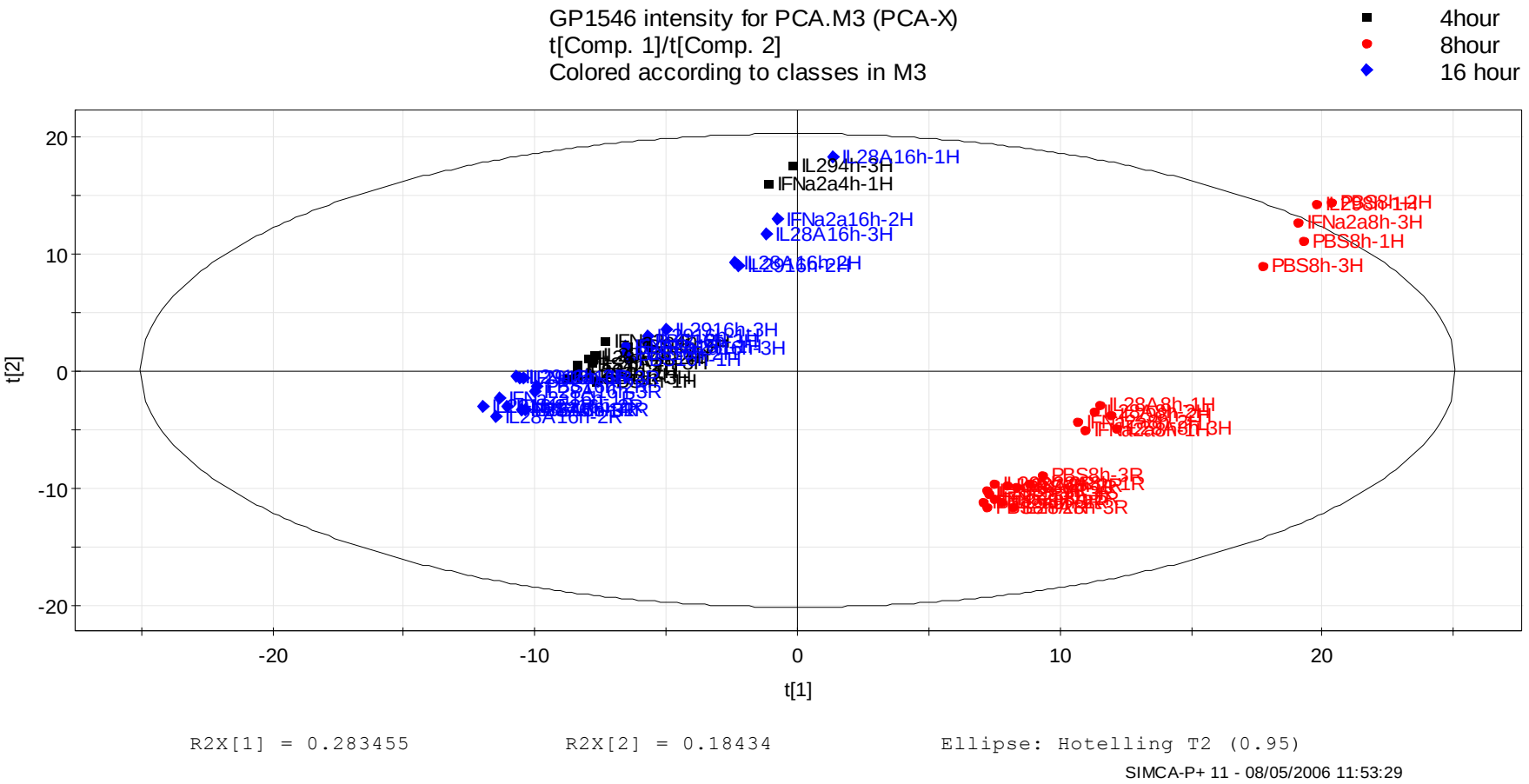


Figure 4.3: PCA plot –PC1 Vs 2 by time point

The first principal component (PCA1) shows the largest possible variance time accounts for the biggest variable in the data, PCA1 plot shows 4 hour and 16 hour clustering together and 8 hour data being separate for replicon and parent cell lines.

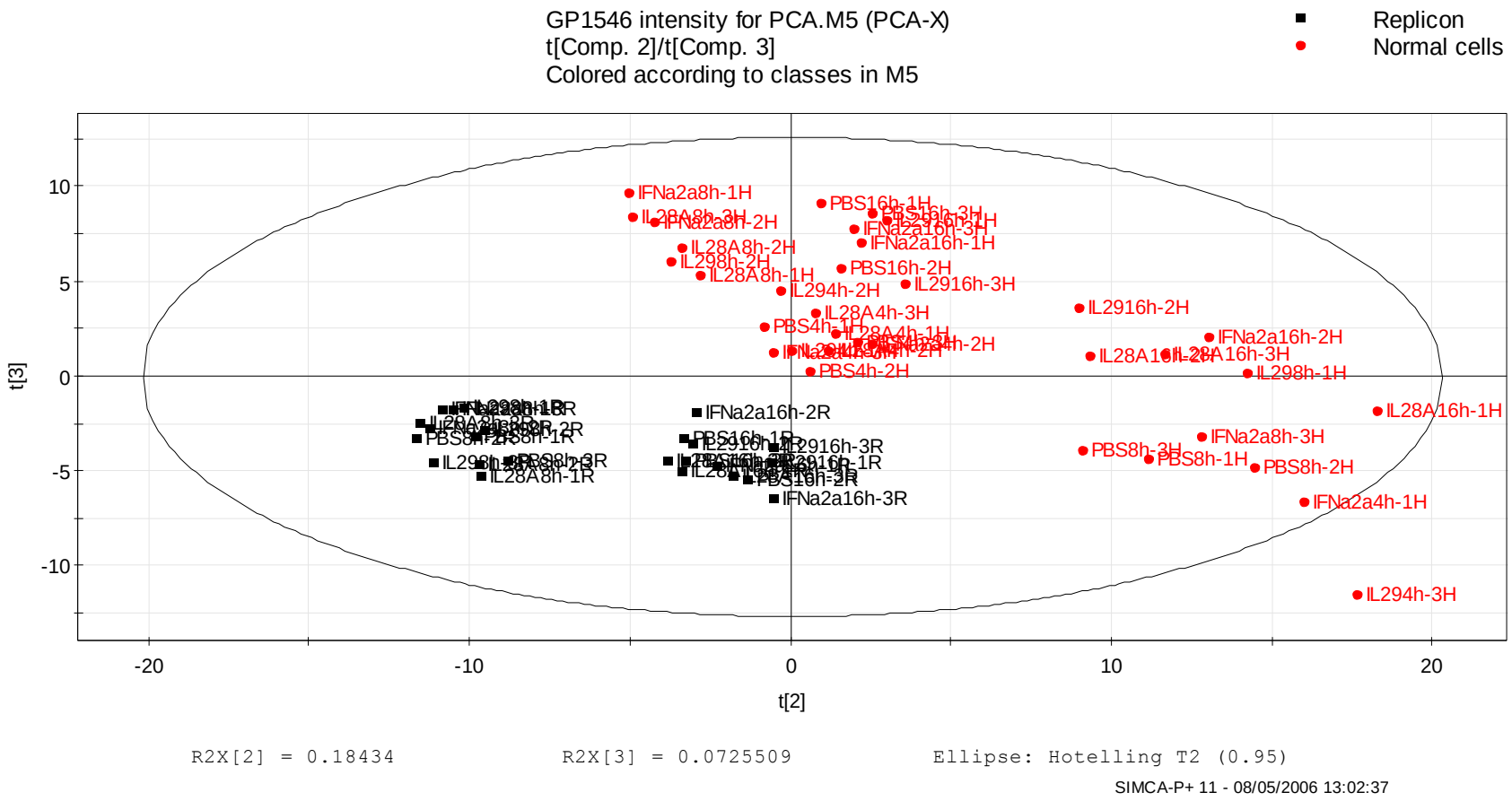


Figure 4.4: PCA plot –PC2 Vs 3 by cell type

PCA2 shows replicon cells separating from normal parent HuH7cells.

4.4.2 Parent HuH7 signature genes

The top 20 expressed genes in the parent cell line with IFNα, IL-28A and IL-29 treatments at 4, 8, 16 and 24 hour time points in relation to IL-29 expression 8 and 24 hour time points are shown below (Table 4.2).

Table 4.2: Table to show top 20 expressed genes in parent HuH7 cells

Symbol	IFNα				IL-29				IL-28A			
	4h	8h	16h	24h	4h	8h	16h	24h	4h	8h	16h	24h
G1P3	100	79	74	74	100	100	100	100	84	52	69	48
IFI6	-	79	-	74	-	100	-	100	-	52	-	48
IFIT1	16	6.6	3.9	7.1	100	100	100	100	14	7.2	4.6	3.4
MX1	8.8	2.9	2.9	2	66	51	53	46	7.7	5.2	2.7	1.9
IFITM3	11	12	6.3	10	26	38	18	40	8.5	7.9	6	5.6
IFIH1	-	-	-	5.2	34	-	30	30	3.9	5	-	3.4
IFITM1	15	16	3.6	6.9	67	82	22	25	15	9.2	3.8	4.5
ISG15	-	5	-	5.4	-	18	-	20	-	3.3	-	2.4
OAS1	4.7	-	2.6	-	49	14	38	19	6.1	-	3.1	-
ISGF3G	6.6	7.7	3.6	6.1	14	17	12	16	6.2	7.4	3.9	3.1
SUPT16H	-	49	-	-7.6	-	29	12	-	-	70	3.1	-
STAT1	3.4	14	2	-2.2	11	28	12	-	2.7	11	2.1	-
IFI27	-	-	-	-	12	-	14	9.7	-	-	-	-
IFITM2	4.5	2.8	2.6	3.9	9.3	8.1	7.2	9.4	3.5	2.3	2.9	2.6
OAS3	2	1.7	-	-	14	8.4	14	8.6	2.1	-	-	-
PARP9	2.3	-	1.9	2.1	7.3	2.3	8.7	5.8	2.3	-	2	-
PLSCR1	2.5	2.8	1.6	-	8.7	8.6	8.4	5.5	2.4	2.6	1.6	-
DDX58	3.4	-	-	-	20	5.1	14	5	-	-	-	-
IFI35	2.6	-	2.8	2.1	5.7	2.7	9.3	5	2.4	-	2.8	1.8
EPSTI1	2.4	-	-	-	11	5.6	7.2	4.7	2.5	-	-	-

Table 4.2 shows the top 20 expressed genes in the parent cell line with IFNα, IL-28A and IL-29 treatments at 4, 8, 16 and 24 hour time points in relation to IL-29 expression at 8 and 24 hour time points. Genes are >1.5 fold up or down with one or more

treatments. Values represent fold activation compared with PBS treatment. Genes highlighted in yellow are also present in the replicon table (Table 4.8).
(-) = <1.5 fold induction.

There are highly expressed interferon stimulated genes (ISG) common to IFNα and IFNλs both in the replicon and the parent HuH7 cell lines, these include G1P3, IFI6, IFIT, IFITM1, MX1, IFITM3, STAT1, ISG15, ISGF3G and OAS1 as shown in Table 4.8. The only top 20 gene determined by IL-29 at the 24 hour time point to not be represented in the entire replicon dataset (genes expressed > or < 1.5 fold change) was SUPT16H which plays a role in general transcription, it has positive and negative effects on gene expression. Required for the appropriate synthesis during heat shock and continued expression of cyclin genes that determine the passage through start during cell cycle control it may act as an acidic activator.

4.4.3 Parent HuH7 pathway analysis IPA

To demonstrate the mechanisms of IFNλs, pathway analysis was performed using Ingenuity and GeneGo pathway analysis tools. The top 7 pathways determined by Ingenuity pathway analyses scoring for IFNα, IL-28A and IL-29 at the 4 hours and IFNα and IL-29 at 8 hours are shown in Figures 4.5 and 4.6 respectively. For type I and type III IFNs at 4 hours, interferon signalling is the most strongly induced pathway determined by Ingenuity Pathway Analysis, complement coagulation pathway is the second most strongly induced pathway for IL-28A, and protein ubiquitination pathway is the second most strongly induced pathway for both IFNα and IL-29. After this, IL-29 induces immune modulation pathways such as antigen presentation pathway and complement and coagulation cascades predominantly, in contrast to IFNα that predominantly regulated cell signalling pathways. At 8 hours interferon signalling is the top pathway for IL-29 followed by antigen presentation and complement coagulation pathways, for IFNα there are gene expression changes in multiple signalling pathways: integrin, IGF-1, Jak/Stat, SAPK/JNK signalling pathways.

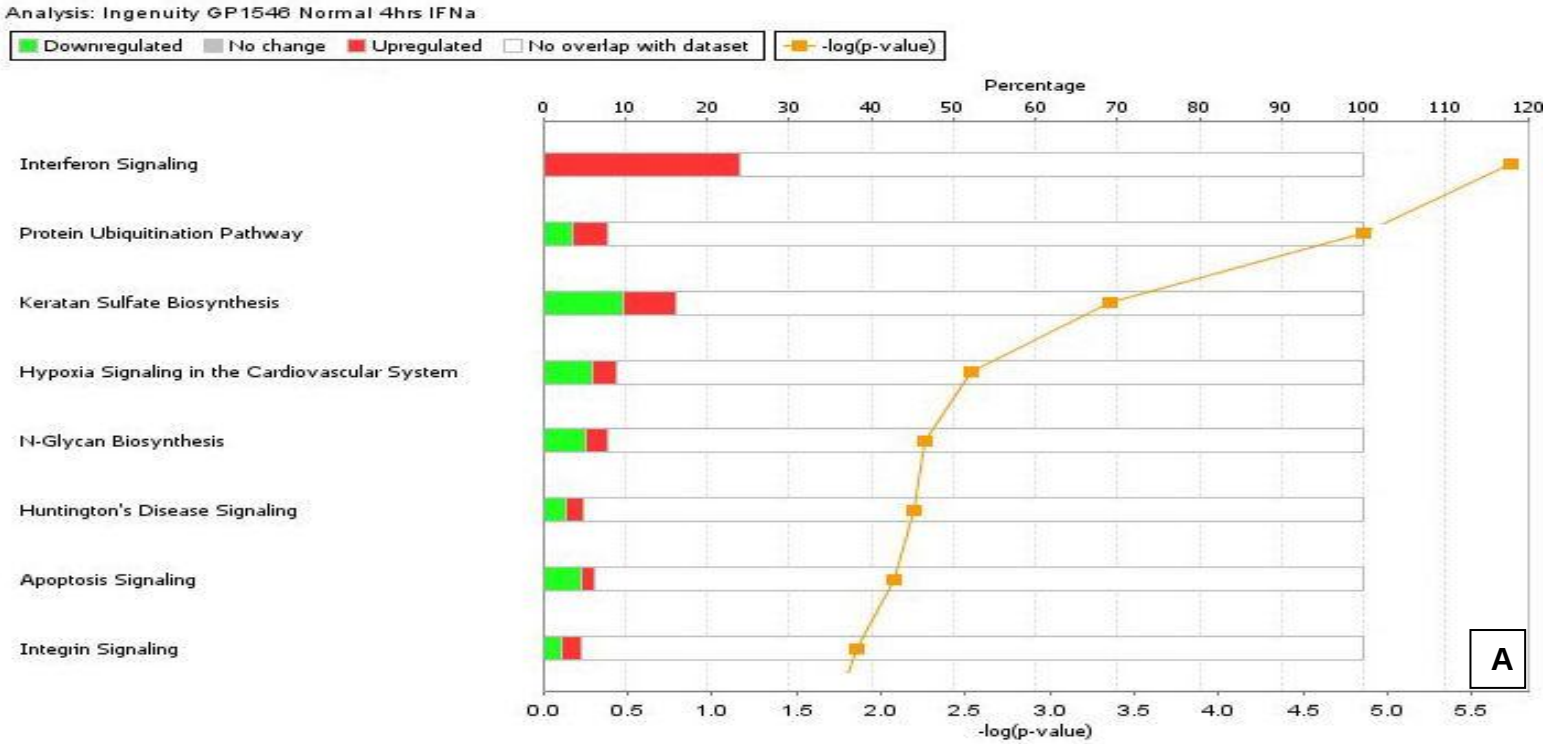


Figure 4.5 A: Parent HuH7 cell line pathway analysis at 4 hours for IFNα

Using IPA for the Parent HuH7 cell line at the 4 hour time point, showing the top seven pathways for: **A** IFNα. Pathway analysis using IPA for the parent cell line at the 4 hour time point graphical representation of the top seven pathways for IFNα showing up and down regulated genes associated to the pathways IPA gives a score shown by the trend line and –log scale determined by expression level and number of genes expressed in pathway/total number of genes within pathway. Percentage is number of genes modulated in pathway; red represents up regulated genes and green represents down regulated genes.

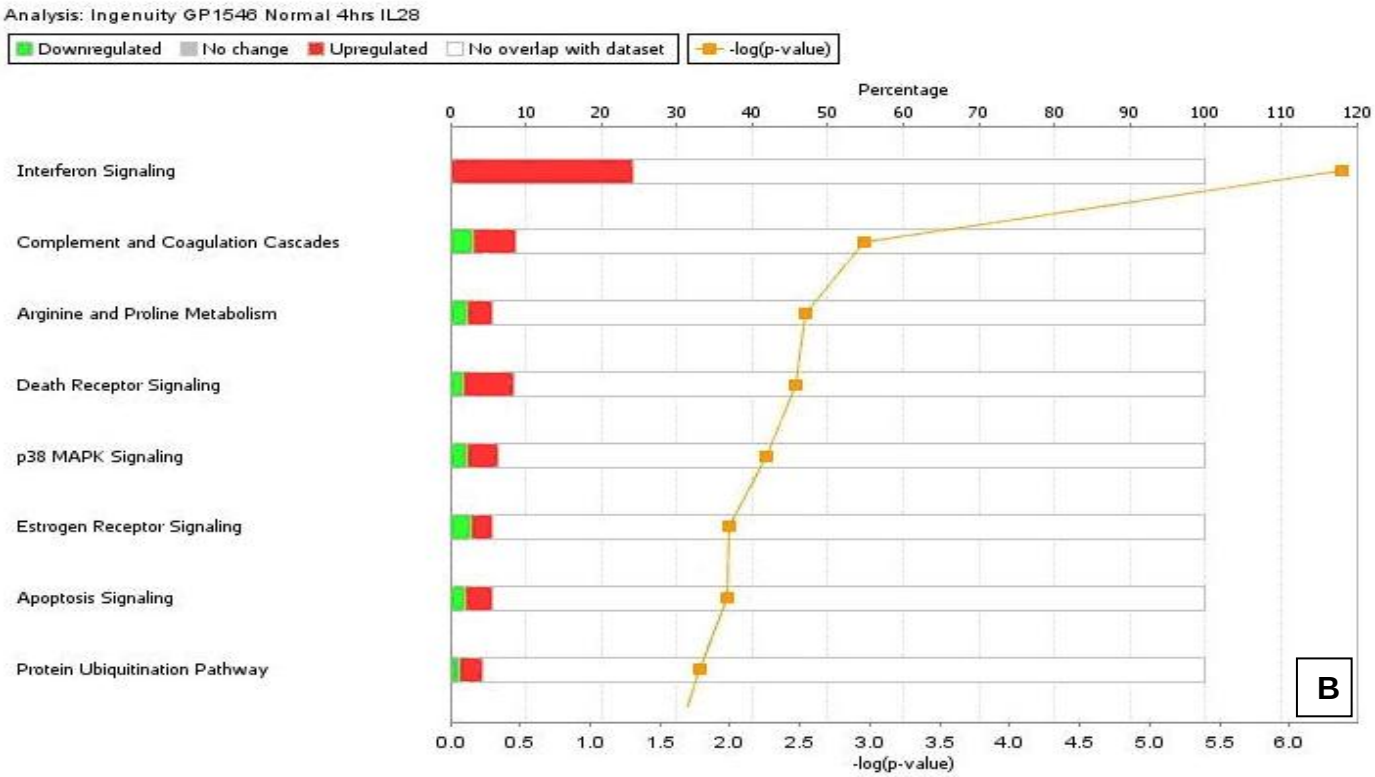


Figure 4.5 B: Parent HuH7 cell line pathway analysis at 4 hours for IL-28A

Using IPA for the Parent HuH7 cell line at the 4 hour time point, showing the top seven pathways for: **B** IL-28A. Pathway analysis using IPA for the parent cell line at the 4 hour time point graphical representation of the top seven pathways for IL-28A showing up and down regulated genes associated to the pathways IPA gives a score shown by the trend line and –log scale determined by expression level and number of genes expressed in pathway/total number of genes within pathway. Percentage is number of genes modulated in pathway; red represents up regulated genes and green represents down regulated genes.

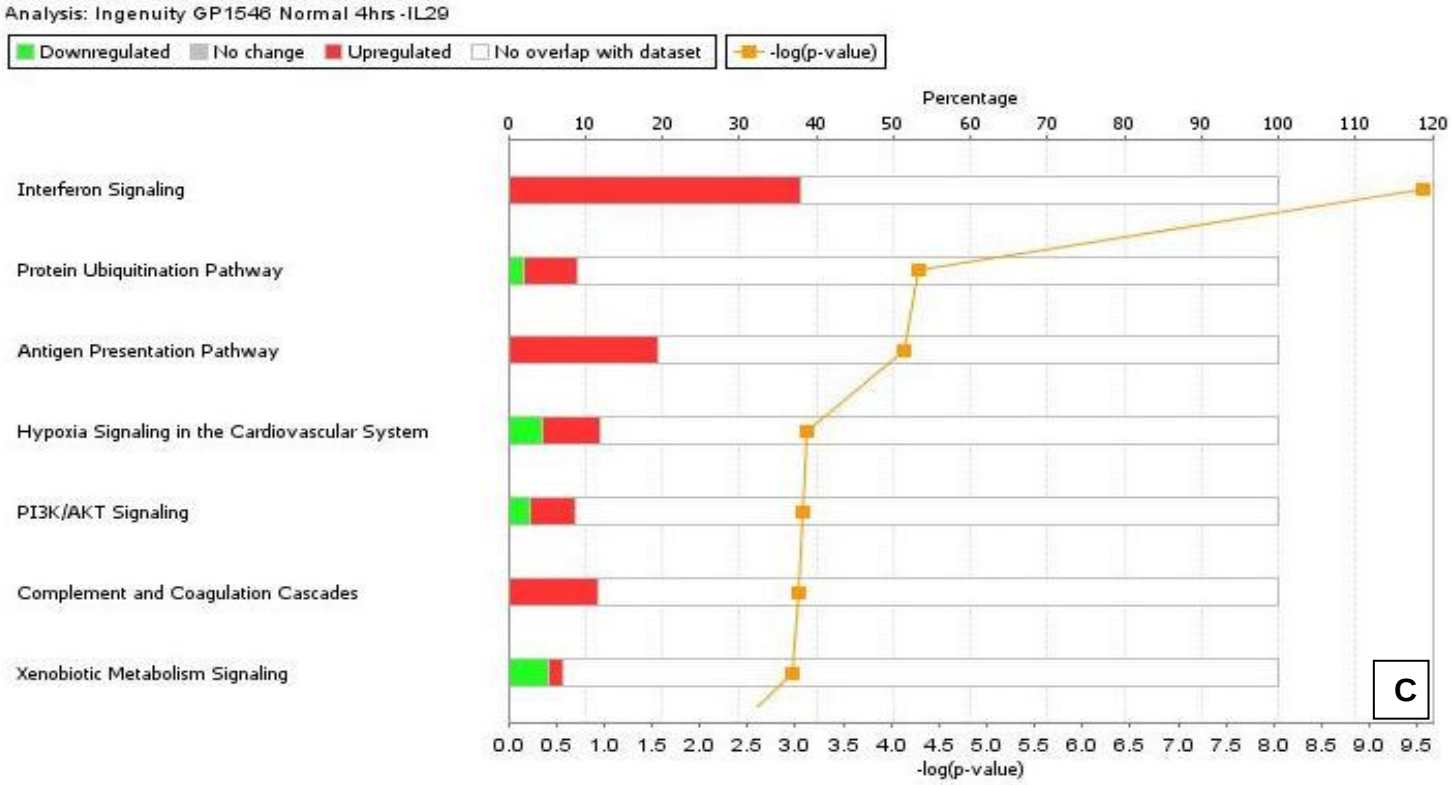


Figure 4.5 C: Parent HuH7 cell line pathway analysis at 4 hours for IL-29

Using IPA for the Parent HuH7 cell line at the 4 hour time point, showing the top seven pathways for: **C** IL-29. Pathway analysis using IPA for the parent cell line at the 4 hour time point graphical representation of the top seven pathways for IL-29 showing up and down regulated genes associated to the pathways IPA gives a score shown by the trend line and –log scale determined by expression level and number of genes expressed in pathway/total number of genes within pathway. Percentage is number of genes modulated in pathway; red represents up regulated genes and green represents down regulated genes.

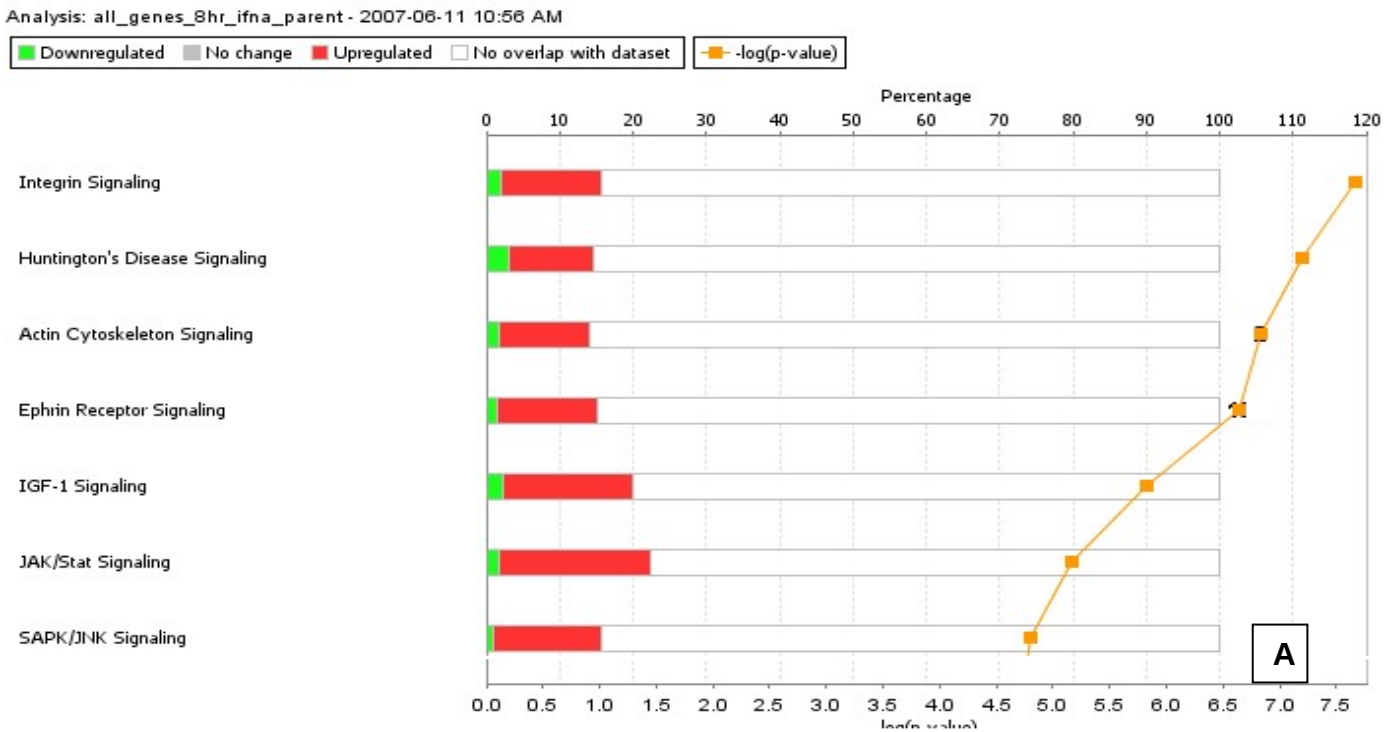


Figure 4.6 A: Parent HuH7 cell line pathway analysis at 8 hours for IFNα

Using IPA for the Parent HuH7 cell line at the 8 hour time point, showing the top seven pathways for: **A** IFNα. Pathway analysis using IPA for the parent cell line at the 8 hour time point graphical representation of the top seven pathways for IFNα showing up and down regulated genes associated to the pathways IPA gives a score shown by the trend line and –log scale determined by expression level and number of genes expressed in pathway/total number of genes within pathway. Percentage is number of genes modulated in pathway; red represents up regulated genes and green represents down regulated genes.

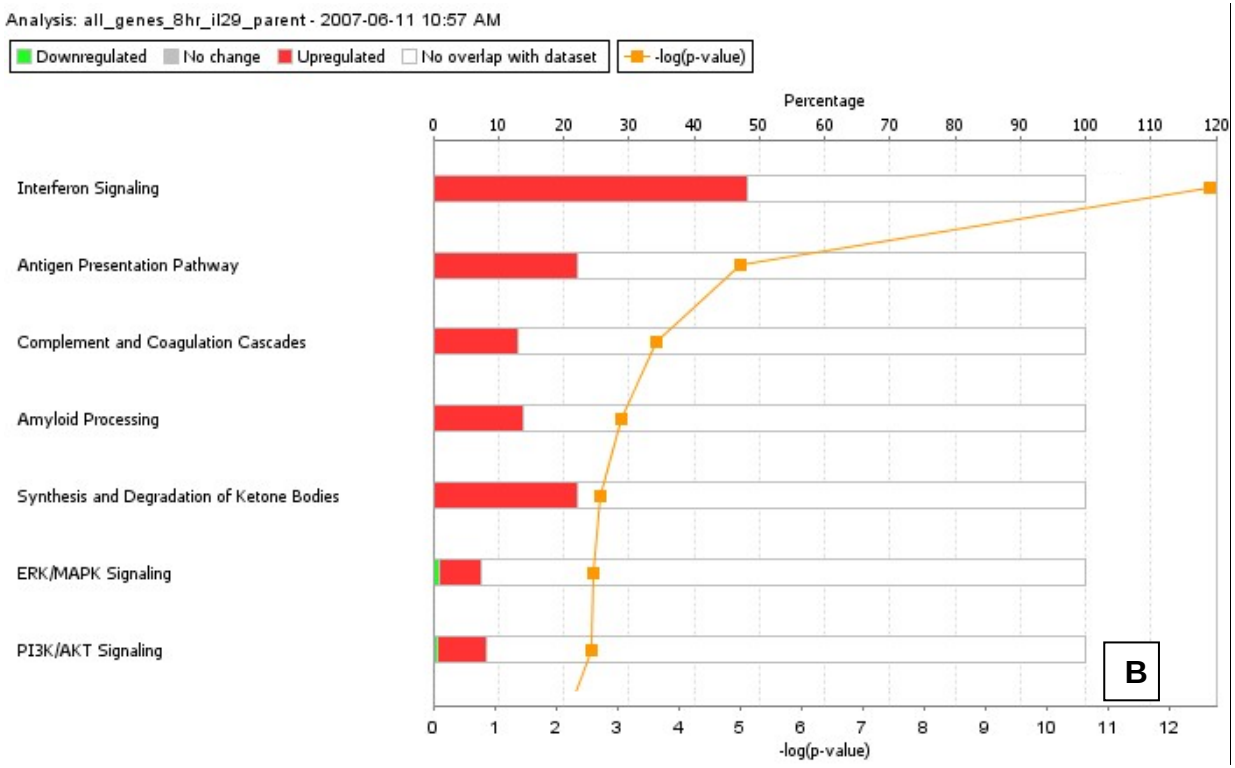


Figure 4.6 B: Parent HuH7 cell line pathway analysis at 8 hours for IL-29

Using IPA for the Parent HuH7 cell line at the 8 hour time point, showing the top seven pathways for: **B** IL-29. Pathway analysis using IPA for the parent cell line at the 8 hour time point graphical representation of the top seven pathways for IL-29 showing up and down regulated genes associated to the pathways IPA gives a score shown by the trend line and –log scale determined by expression level and number of genes expressed in pathway/total number of genes within pathway. Percentage is number of genes modulated in pathway; red represents up regulated genes and green represents down regulated genes.

4.4.4 Parent HuH7 unique IL-29 gene analysis

Genes unique for IFNα belong to predominately signalling pathways; Jak/Stat, EGF, IGF-1, IL-2, GM-CSF, FGFF, IL-4 and IL-6. These unique genes for IFNα include: PIK3CB, PIK3R1, SOS2, SOCS2, MAP3K1. Genes unique to IL-28A belong to IGF-1, EGF, TGF, VEGF, IL-2, IL-6 and IL-4 signalling pathways. IL-29 unique genes belong to interferon signalling as the top pathway but are followed by antigen presentation and complement and coagulation cascades; unique genes at the 8 hour time point include HLA-A, HLA-B, HLA-C, HLA-F, HLA-G, PLG, CFB and SERPINE1. Antigen presenting gene expression with IFNα and IL-29 treatments are shown (Table 4.3). Antigen presenting genes are shown to be expressed at earlier time points in cells treated with IL-29 compared to cells treated with IFNα.(Table 4.3)

Table 4.3: Antigen presenting genes IL-29 versus IFNα.

Symbol	IFNα				IL29			
	4h	8h	16h	24h	4h	8h	16h	24h
HLA-A	-	-	-	-	1.5	1.5	1.6	-
HLA-B	-	-	-	1.9	2.6	2.9	2.9	2.9
HLA-C	-	-	-	1.6	2.5	2.4	3.2	2.6
HLA-DRA	-	-	-	-	-	3.6	-	-
HLA-DRB4	-	-	-	-	-	-	-	3.2
HLA-E	-	-	-	-	2.6	1.6	3.9	2.3
HLA-F	-	-	-	-	-	1.6	-	-
HLA-G	-	-	-	-	1.5	2.7	1.6	-
PSMB8	-	-	-	-	-	3.2	-	-
TAP1	-	-	-	2.8	-	2.7	3.3	4.5

(-) = <1.5 fold induction

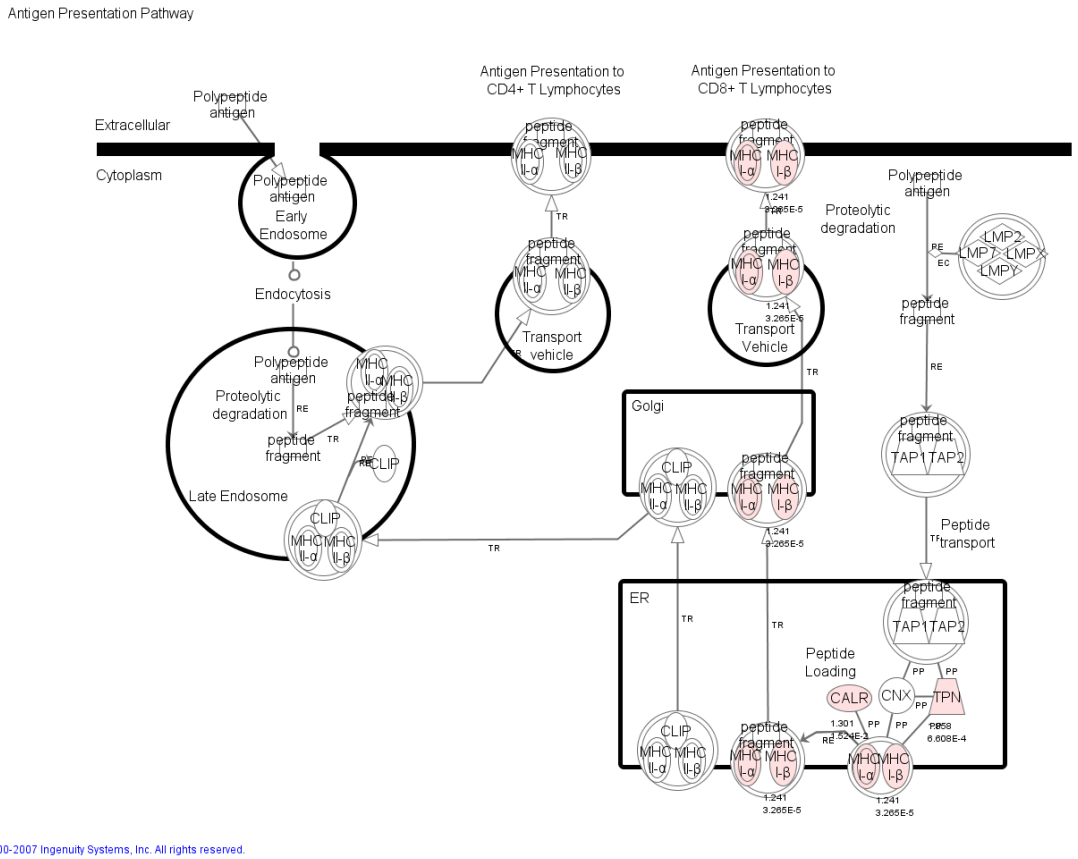


Figure 4.7: A top pathway - antigen presentation pathway determined by Ingenuity for genes unique to IL-29 at 8 hours

Antigen processing is a biological process that prepares antigens for presentation to T lymphocytes. This process involves two distinct pathways for processing of antigens from an organism's own (self) proteins or intracellular pathogens (e.g. viruses), or from phagocytosed pathogens (e.g. bacteria); subsequent presentation of these antigens on class I or class II MHC molecules is dependent on which pathway is used. Both MHC class I and II are required to bind antigen before they are stably expressed on a cell surface. Red correspondes to up regulation of gene.

An Ingenuity pathway map for a top pathway antigen presentation pathway is shown (Figure 4.7) determined by genes unique to IL-29 at 8 hours. Other pathway analysis tools were used including GeneGo, data shown below (Section 4.4.5).

4.4.5 Parent HuH7 pathway analysis GeneGo

The top 10 pathways with genes uniquely regulated by IL-29 at 24 hours are shown in Table 4.4. Genes unique to IL-29 treatment at 24 hours induce IFNα, IFNβ and IFNγ pathways, so any gene changes with IL-29 at 24 hours may be due to secondary mechanisms, e.g. feedback from genes expressed at early time points. These pathways are also shown in a GeneGo pathway map (Figure 4.8).

Table 4.4: Pathway analysis for HuH7 cell line GeneGo

Genego Map - Unique genes to IL-29 at 24 hours	p-Value	Genes ↓	Genes ↑
RhoA regulation pathway (extension, GEFs/GAPs)	0.000267	6	34
IL9 signaling pathway	0.001799	5	33
Propionate metabolism p.2	0.01824	3	21
Mitochondrial ketone bodies biosynthesis and metabolism	0.02321	2	9
HETE and HPETE diosynthesis and metabolism	0.02636	4	42
IFN gamma signaling pathway	0.02765	5	63
Gs-a Specific GPCRs (in brain)	0.04279	3	29
Putative SUMO-1 pathway	0.04279	3	29
Ascorbate metabolism	0.04535	5	72
IFN alpha/beta signaling pathway	0.04662	3	30

Pathway analysis for genes unique to IL-29 at the 24 hours shows the top 10 pathways for IL-29 determined by GeneGo.

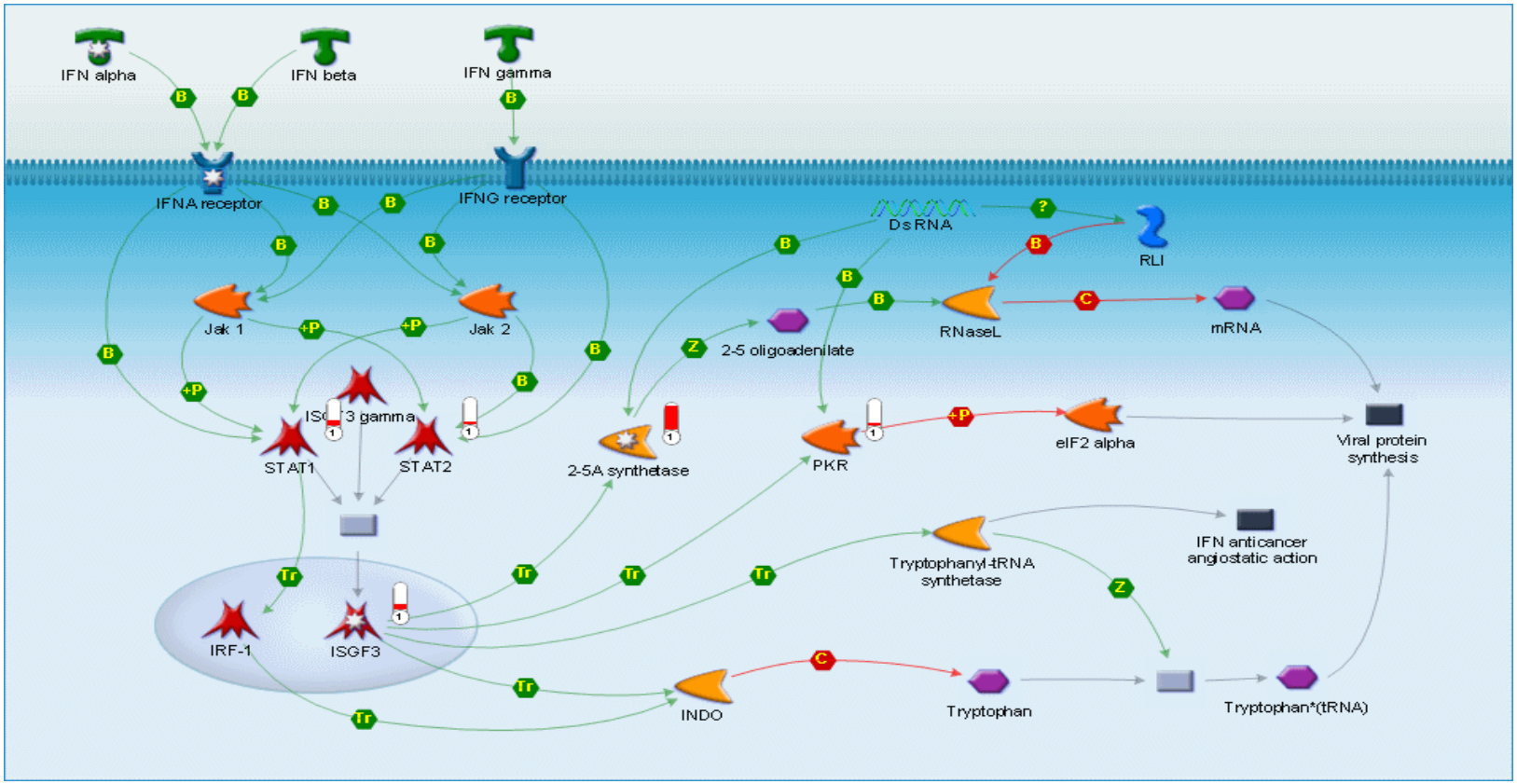


Figure 4.8: Top pathway interferon signalling determined by GeneGo for genes unique to IL-29 at 24 hours.

This figure is a schematic produced in GeneGo, bars with one represent IL-29 upregulation, the higher the red in the bar –the higher the gene expression e.g. 2-5A synthetase is highly upregulated by IL-29 and not up-regulated significantly (≥ 1.5 fold) at this time point by IFNα.

4.4.6 Additional unique gene changes –cytokine induction, TLR and MAPK signalling

There are differences in cytokine induction between the types of interferons: IL-24 is down regulated by type III IFNs, IL-27 is up regulated by type I IFN. IL-27R is up regulated by type I IFN and down regulated by IL-29.

TLR7 signalling genes unique to IL-28A in the parent cell line at the 8 hour time point are: TLR7, TRAF4, TRAF5 and IRAK1BP1 (-2.9, 1.9, -1.9 and 1.7 fold change respectively) and at the 24 hour time point IRF7 and TRAF31P3 were expressed (-3.2 and -2.9).

There are notable differences between the IFNs with MAPK signalling regulation in the parent cell line IFNα induces a far greater number of the MAPK genes than IL-29. For IFNα, 9 genes are expressed at the 8 hour time point and 16 genes are expressed at the 24 hour time point, for IL-29 this is 3 and 2 genes respectively. IL-28A has more similarities with IFNα with the regard to expression of MAPK genes with expression of 15 MAPK genes at the 8 hour time point but only 4 genes at the 24 hour time point.

4.4.7 Parent HuH7 kinetics

Table 4.5 shows the kinetics of expression in a selection of genes in the parent cell line 8, 16 and 24 hours after treatment with IFNα, IL-28A and IL-29 treatments. The IFNs shown similar trends to each other over the time points e.g. IFI6 was shown to be expressed with IFNα, IL-29 and IL-28A at 8 hours and 24 hours but not at 4 and 16 hour time points.

Table 4.5 Data subset: to illustrate kinetics in parent HuH7 cells

Symbol	IFNα				IL-29				IL-28A			
	4h	8h	16h	24h	4h	8h	16h	24h	4h	8h	16h	24h
G1P3	100	79.3	74.2	74	100	100	100	100	84.3	51.7	68.7	48.2
IFI6	-	79.3	-	74	-	100	-	100	-	51.7	-	48.2
IFITM3	11.4	11.8	6.3	10.1	26.4	37.9	17.6	40.4	8.5	7.9	6	5.6
ISG15	-	5	-	5.4	-	18.4	-	19.7	-	3.3	-	2.4
OAS1	4.7	-	2.6	-	48.8	13.6	38.2	18.8	6.1	-	3.1	-
STAT1	3.4	13.5	2	-2.2	11	28.2	11.8	-	2.7	10.9	2.1	-

(-) = <1.5 fold induction.

4.5 HCV replicon

4.5.1 Comparison of parental data with replicon data to investigate the effects of HCV on IL-28A, IL-29 and IFNα gene modulation

RNA from time point 4 hours with the replicon cells was not of sufficient quality to use in Affymetrix genechip® analysis.

HCV replicon inhibited gene induction by both type I and type III IFNs; this is seen at 8, 16 and 24 hour time points in the Venn diagrams (Figure 4.2), where there is a substantial decrease in the numbers of genes expressed in the replicon system from those expressed in the parent system. Parent HuH7 cells are more sensitive to interferons IFNα, IL-28A and IL-29 compared to the replicon cells. Gene expression is generally higher in the parent cells than the replicon cells, as shown for IF16 in Table 4.7. This is also highlighted in the Venn diagrams (Figure 4.2). Gene expression detail is shown below (Tables 4.6 and 4.7).

Table 4.6: Comparison of replicon versus parent HuH7 cells

	Replicon	Parent	Replicon	Parent
Symbol	IFNα	IFNα	IL-29	IL-29
G1P3	3.2	79.3	6.7	100
IFIT1	20	6.6	29.8	100
IFI6	-	79.3	9.8	100
MX1	2.3	2.9	3.4	53.1
ISGF3G	15	7.7	18.3	12.1
IFIH1	6	-	6.6	30
IFITM3	-	11.8	4	17.6
ISG15	2.6	5	3.1	-
IFITM1	-	16.1	-	22.2

Comparison of replicon cells versus parent HuH7 cells at 8 hours with IFNα and IL-29

Table 4.7: Sensitivity of parent HuH7 cell line versus replicon

IF16 fold change	8hr IFNα	8hr IL-29	24hr IFNα	24hr IL-29
Parent	79	>100	73	>100
Replicon	0	9.7	52	41.9

4.5.2 HCV replicon signature genes

The top 20 expressed genes in the replicon cell line with IFNα, IL-28A and IL-29 treatments at 8, 16 and 24 hour time points in relation to IL-29 expression at 24 hour time point are shown below (Table 4.8).

Table 4.8: A selection of top 20 expressed genes in the replicon cells

Symbol	IFN**α**			IL-29			IL-28A		
	8h	16h	24h	8h	16h	24h	8h	16h	24h
IFIT1	21	21	100	30	100	100	13	78	64
G1P3	3.2	82	42	6.7	100	97	4.5	100	43
IFI6	-	-	52	9.8	100	42	-	54	20
MX1	2.3	2.3	30	3.4	18	35	-	12	17
ISGF3G	15	15	22	18	14	21	15	12	16
IFIH1	6	6	23	6.6	23	15	4.4	13	9.5
IFITM3	-	-	7.6	4	4.9	12	3	3.5	4
ISG15	2.6	2.6	11	3.1	14	9.9	-	7.4	5.4
IFITM1	-	-	7.1	-	9.9	8.7	-	5.6	4.3
ADAMTS13	-	-	-	-	-	7.7	-	-	6.7
OAS1	3.2	3.2	5.3	6.2	10	7.4	3.8	5.4	4.1
DDX58	3.4	3.4	7.9	3.7	11	6.5	-	4.8	3.6
PRIC285	2.2	-	6.8	3.1	5.1	6.4	3.1	3.4	3
OAS3	3.4	3.4	8	5.1	6.4	5.3	-	-	2.8
PARP9	-	-	4.9	2.9	-	4.9	-	-	3
STAT1	3.2	3.2	5.4	3.5	5.2	4.8	-	3.2	3.3
PLSCR1	-	-	4.2	-	4.6	4.2	-	-	2.9
PML	-	-	-	-	-	4	-	-	-
IFI27	-	-	-	-	-	3.5	-	-	-
IFITM2	-	-	-	-	-	3.3	-	-	-

Table 4.8 shows a selection of top 20 expressed genes in the replicon cell line with IFNα, IL-28A and IL-29 treatments at 8, 16 and 24 hour time points in relation to IL-29 expression at the 24 hour time point. Genes are >1.5 fold activation with one or more treatments compared with PBS treatment. 100 represents 100 fold change or greater, Genes highlighted in yellow are also present in the parent table. (-) = <1.5 fold induction.

The only top 20 gene determined by IL-29 at the 24 hour time point to not be represented in the entire replicon dataset (genes expressed > or < 1.5 fold change) was ADAMTS13 which prevents thrombosis through abnormal clotting.

There are highly expressed interferon stimulated genes (ISG) common to IFNα and IFNλs both in the replicon and the parent HuH7 cell lines, these include G1P3, IFI6, IFIT, IFITM1, MX1, IFITM3, STAT1, ISG15, ISGF3G and OAS1.

4.5.3 HCV replicon kinetics

To compare gene expression trends in the replicon cells treated with IFNα, IL-28A and IL-29 at 8, 16 and 24 hours, several types of data analysis was performed on the replicon data set. Cluster analysis was generated using Rosetta Resolver of all replicon fold change data greater than 2 fold up or down regulated and > 99% confidence; a total of 881 genes. The data clustered by groups of genes which share similar properties is shown in Figure 4.9.

The up regulated data from the entire replicon data set where a gene is above 2 fold change in relation to PBS control in a minimum of one time point, based on their fold regulation in response to IFNα at 8 hours. Data is represented visually in more detail as a Heat Map (Figure 4.10) and in further detail for MX1, IFIT1 and ISGF3G genes in trend plots (Figure 4.11).

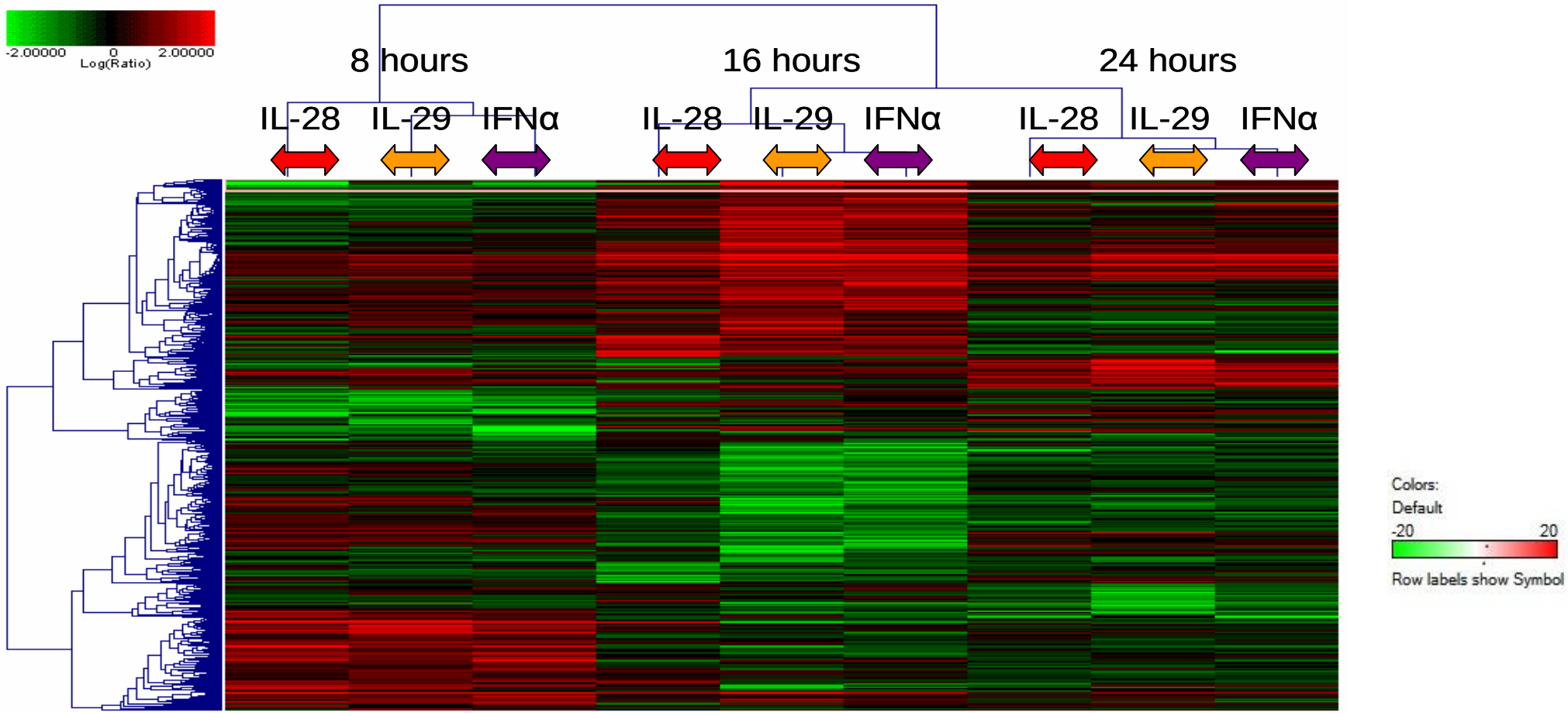


Figure 4.9: Cluster analysis plot

Cluster analysis of 640 genes regulated significantly ($p<0.01$) regulated ±1.5-fold with either IFNα, IL-29 or IL-28A at 8, 16 and 24 hours.

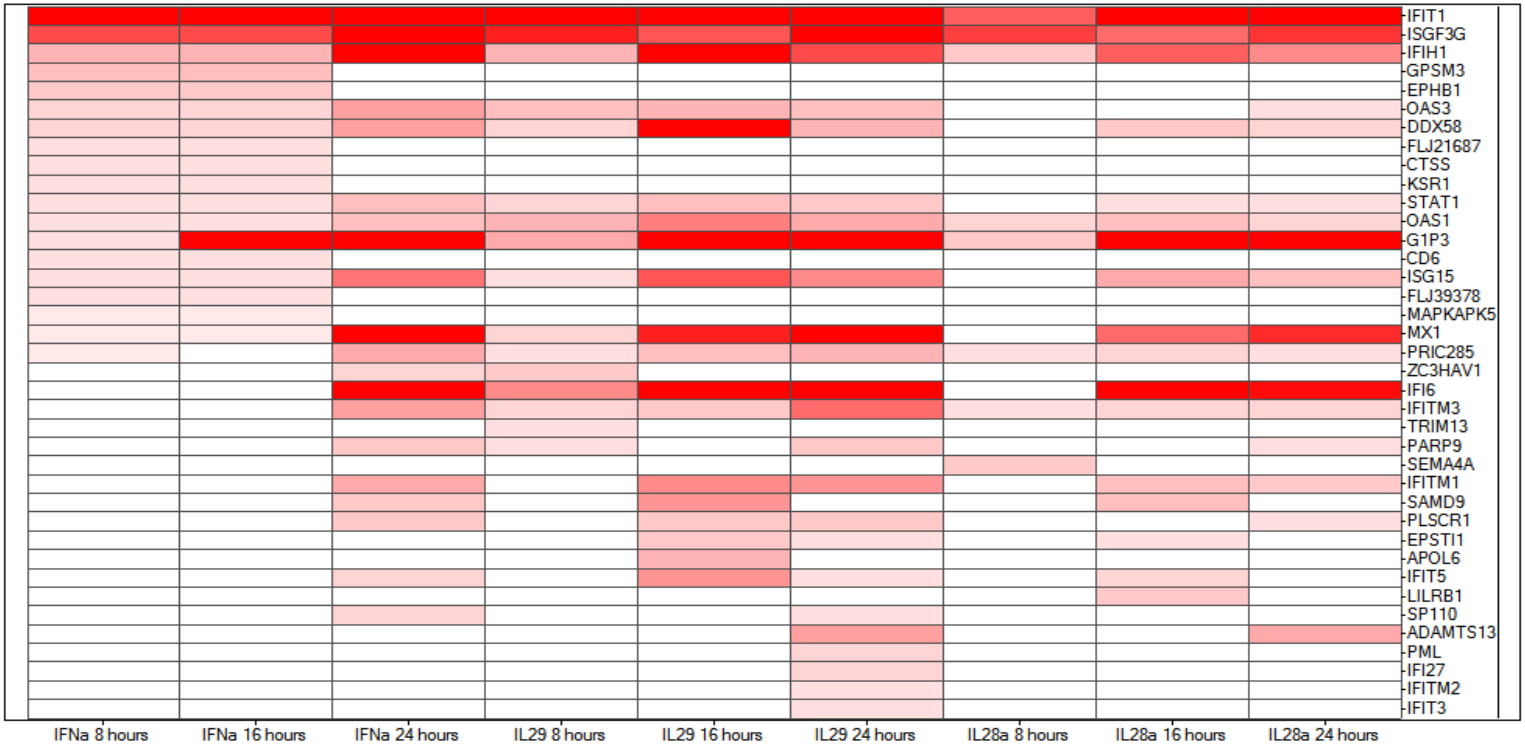


Figure 4.10: Heat map

A visual summary of the replicon gene data. In the heat map, genes are sorted in decreasing order based on their fold regulation in response to IFNα at 8 hours. The heat map represents the entire up regulated data set for any one gene whose expression was above 2 fold compared with PBS treatment with one or more IFNs at one or more time points, p-value <0.5. To show low induction values, the maximum heat intensity (red) was set to a 20 fold induction value.

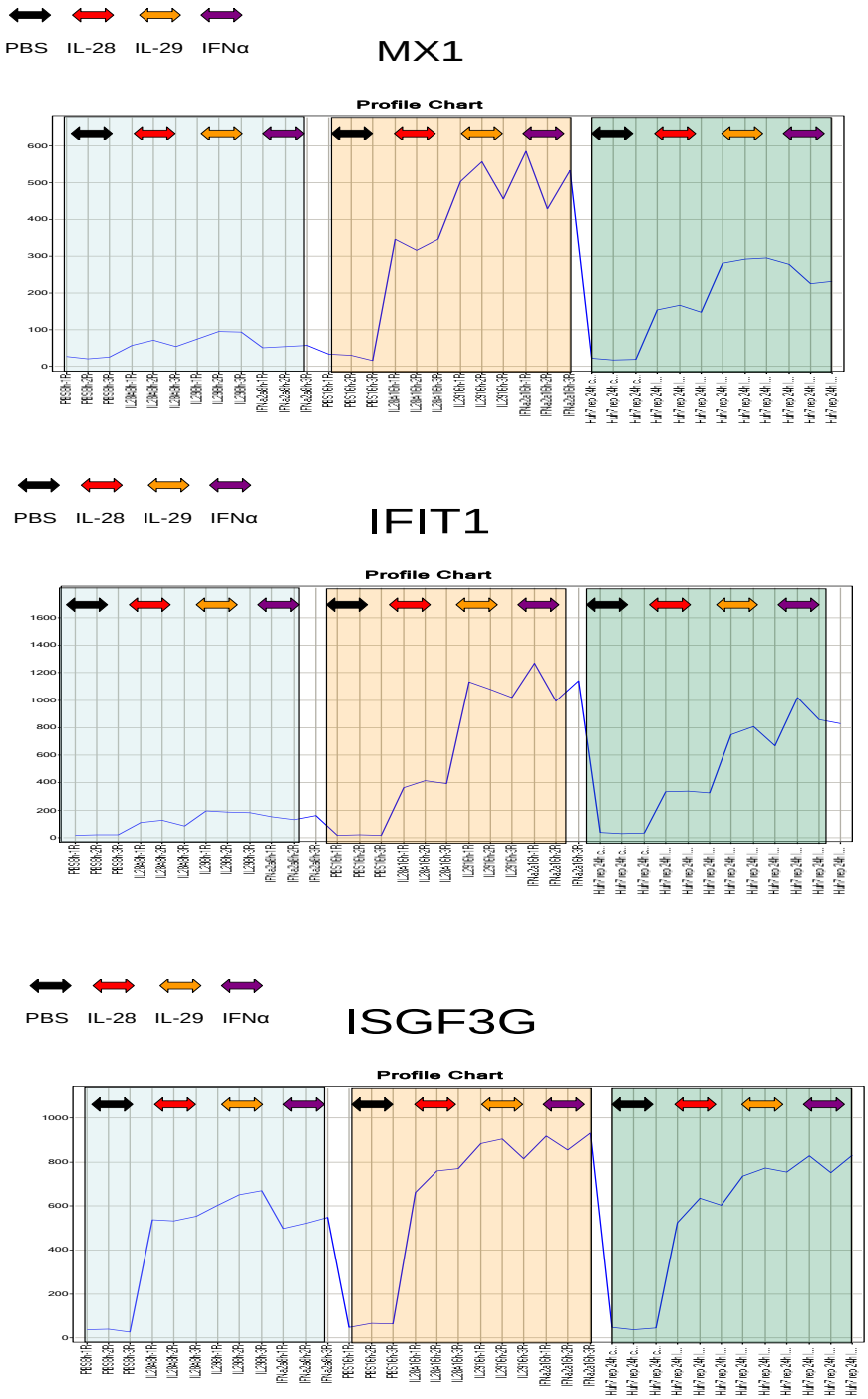


Figure 4.11: Trend plots

Trend plots were used as an effective, efficient oversight screening tool to visualise control (PBS) versus treatments IL-28A, IL-29 and IFNα over 3 timepoints 8 hour (blue box), 16 hour (orange box) and 24 hour (green box). Trend plots for three genes Mx1, IFIT1 and ISGF3G are shown.

Trend plots and trend analysis show that IFNα and IL-28A and IL-29 show the same trends, e.g. for Mx1 (MxA) there is low expression with IL-28A, IL-29 and

IFNα at 8 hours, this peaks at 16 hours and levels off at 24 hours for all IFNs tested (IL-28A, IL-29 and IFNα).

4.6 Discussion

There are many similarities in both gene modulation between type I and type III IFNs. When looking at the genes that are expressed most highly in both the parent HuH7 and the replicon systems IFNλs and IFNα show largely overlapping sets of interferon stimulated genes (ISGs). Although antiviral mechanisms of type I and III differ, they may actually involve similar ISG responses. The individual gene expression results from the replicon data set shown here showed good concordance with data published by Marcello *et al* [61], with 45/66 differentially regulated genes as a response to IFNα and 49/66 as a response to IL-29. Zhou *et al,* Doyle *et al* and Marcello *et al* have all shown similar sets of ISGs being expressed by type I and type III IFNs[65,57,61].

Genes common to both the replicon and parent gene expression were highlighted (in Tables 4.2 and 4.8). The majority of interferon stimulated genes (ISG) stimulated in the replicon cell line by IFNα, IL-28A and IL-29 are also stimulated in the parental cell line; these ISGs include: G1P3, IFIT1-5, ISG15 and ISGF3G.

Despite this there are more differences in the pattern of expressed genes between the IFNλs and IFNα than genes expressed in common as shown in the Venn diagrams (Figure 4.2). The Venn diagrams showed a trend between parent and replicon datasets of there being more genes being unique to an individual interferon than genes in common between the interferons. This is different to the findings of other groups [61,65]. The unique genes shown in the Venn diagrams are generally expressed at low levels.

For the majority of genes in both the replicon and the parent datasets, the kinetics of gene expression was the same for type I and type III IFNs, which is highlighted in subset of data (Table 4.5) GIP3, IF16, IFITM3, ISG15, OAS1, STAT1. The table shows that gene modulation with IFNα, IL-28A and IL-29 shows similar patterns over the time courses even being expressed at some time points and not at all in other time points as shown with IFI6, and ISG15.

The Marcello study[61] showed in the replicon system that the majority of type III stimulated genes continue to increase at 24 hours and with IFNα the same genes tend to decrease by the 24 hour time point. In contrast the data shown here demonstrates that the kinetics of the type I and III IFNs show similar patterns of gene expression. HCV replicon inhibited gene induction by both type I and type III IFNs; this is seen at 8, 16 and 24 hour time points, where there is a substantial decrease in the numbers of genes expressed in the replicon system from those expressed in the parent system. There are also multiple genes for which the expression levels are lower in the replicon data than that of the parent. The NS3/4A protease encoded by HCV has been shown to cleave the adaptor in the RIG-I-like receptor (RIG-I) pathway IPS1 thus disrupting the signalling to type I IFN[138]; this data indicates that signalling to type III IFN is also disrupted. Downstream genes from RIG-I are affected in the replicon system including MAPK genes which show lower abundance in number in the replicon system than in the parent system.

Pathway analysis was carried out for the replicon dataset but due to limited gene numbers in the replicon dataset more focus was placed on the larger parental cell line dataset. At the earliest time point 4 hours, for type I and type III IFNs interferon signalling is the most strongly induced pathway determined by Ingenuity Pathway Analysis, complement coagulation pathway is the second most strongly induced pathway for IL-28A, and protein ubiquitination pathway is the second most strongly induced pathway for both IFNα and IL-29. At 8 hours after interferon signalling pathway, IL-29 induces immune modulation pathways such as antigen presentation pathway and complement and coagulation cascades predominantly, compared with IFNα mostly regulating cell signalling pathways. Interferon signalling was not represented in the top 7 pathways by IFNα at 8 hours potentially showing that IFNα is less specific in its signalling pathways than IL-29.

The antigen presenting pathway regulated in the parent cell line showed gene expression of MHC class I antigens to be up regulated with IFNa and IL-29, but IL-29 up regulated MHC class expression at much earlier time points (4,8,16 hours and 24 hours) than IFNα (24 hours). MHC class I antigen expression was shown to be up-regulated following type I or type III IFN addition[2]. Up regulation

of MHC class I expression may lead to increased recognition of virus-infected cells by the immune system.

Genes unique for IFNα belong to predominately signalling pathways; Jak/Stat, EGF, IGF-1, IL-2, GM-CSF, FGFF, IL-4 and IL-6. These unique genes for IFNα include: PIK3CB, PIK3R1, SOS2, SOCS2, MAP3K1. Genes unique to IL-28A also belong to IGF-1, EGF, TGF, VEGF, IL-2, IL-6 and IL-4 signalling pathways. IL-29 unique genes belong to interferon signalling as the top pathway but interestingly are followed by antigen presentation and complement and coagulation cascades; unique genes at the 8 hour time point include HLA-A, HLA-B, HLA-C, HLA-F, HLA-G, PLG, CFB and SERPINE1.

Genes unique to IL-29 treatment at 24 hours induce IFNα, IFNβ and IFNγ pathways, so any gene changes with IL-29 at 24 hours may be due to secondary mechanisms, e.g. feedback from genes expressed at early time points. 2-5A synthetase is of note being significantly (≥ 1.5 fold) expressed by IL-29 but not IFNα at 24 hours, this gene encodes a member of the 2-5A synthetase family, essential proteins involved in the innate immune response to viral infection.

Gene expression analysis here suggests that IFNλs and IFNα have primarily complementary functions, signalling through common pathways enabling IFNλs and IFNα to induce similar biological activities in particular antiviral resistance mediated by very similar sets of interferon sensitive genes, this has also been shown by other groups[65,57,61]. The kinetics of IL-29 response has been shown to differ from IFNα by Marcello with gene expression data[139] also demonstrating in Huh-7 hepatocellular carcinoma cells, that IFN-λ induces STAT-1 and STAT-2 more rapidly than IFNα[64,139] this is also seen in HaCaT keratinocytes[63] these studies have also shown although the subsequent transcriptional response is slightly delayed, the increase in ISG expression induced by IFNλ is stronger and more prolonged than the response activated by IFNα[64,139]. There are several studies although Jak/STAT signaling mediates the primary functions of IFNλ, other pathways are also activated by the receptor including ERK-1/2, mitogen activated protein kinase (MAPK) and Akt in intestinal epithelial and colorectal cancer-derived cell lines[126], activation of MAPKs was also observed in Raji cells following treatment with IFNλ[65]. These results indicate that IFNλ can induce

multiple signaling pathways that may contribute to its activity as an antiviral and immunomodulatory cytokine and complement the findings here.

Where this research differs from that published is that IL-29 is more specific in its modulation and by modulating alternative pathways such as the antigen presentation and processing pathways at early time points may prove to be a useful therapeutic option for the management of chronic viral infection such as HCV. IL-28R is not as widely expressed as the type I IFN receptor which could mean a more specific target. IL-28R was shown to be found primarily on antigen presenting cells (Chapters 5 & 6). Coupled with IL-29 modulating the antigen presenting pathway at earlier time points than IFNα could prove to have a superior therapeutic profile to IFNα.

Chapters 5 & 6

5. IL-28R distribution using genomic analysis

5.1 Introduction

The aim of this chapter was to study the expression of IL-28 receptor (IL-28R) in a panel of tissues and cells in order to identify type III IFN targeted tissues and cells. Tissues and cells containing IL-28R were identified using real-time PCR and gene expression data for IL-28R was analysed.

IFNα and IFNβ (type I IFNs) bind to a heterodimeric receptor comprising an alpha component (IFNAR1) and a beta component (IFNAR2). Although the activities of type III IFNs are similar to type I IFNs, IFNλ signalling is achieved through a specific and distinct heterodimeric receptor complex which is formed between IL-28A receptor (IL-28R / IFN-λR1) and IL-10 receptor (IL-10R2)[2,3]. Similarly to type I IFNs, binding of type III IFNs to their receptor leads to the activation of the Jak:STAT pathway. This is upstream of IFN-inducible genes and modulates their transcription.

The heterodimeric receptor IFNAR1/2 for IFNα and IFNβ is present on all nucleated cells[72]. IL-10R2 is ubiquitously expressed[73], whereas initial RNA expression data showed that IL-28R is limited to specific tissues and cell types[3]. It is the aim of this chapter to conduct a comprehensive exploration of IL-28R distribution using gene expression techniques in both human and murine tissues and immunohistochemical techniques in both human and murine tissues are described in chapter 6.

5.2 IL-28R gene expression in mouse tissue

IL-28R gene expression was studied in a panel of mouse tissues using real-time PCR (Figure 5.1). IL-28R was shown to be expressed predominantly in epithelial tissue, lymph nodes and spleen, as was seen with the protein expression of IL-28R. High expression; greater than 1,000,000 copies per 50ng of cDNA was seen in mandibular and messenteric lymph nodes and gut tissues

(stomach, duodenum, jejunum, ileum and colon). Expression was also detected in epididymis, spleen, thyroids, kidney, lung, pancreas, prostate, oesophagus, ovaries, peripheral nerve and pituitary gland. Tissues with minimal expression were: liver, bone marrow, adrenals, heart, seminal vesicles and cerebellum. Tissues (not recorded in Figure 5.1) with no detectable expression were: aorta, bladder, eyes, harderian gland, skeletal muscle, skin and spinal cord.

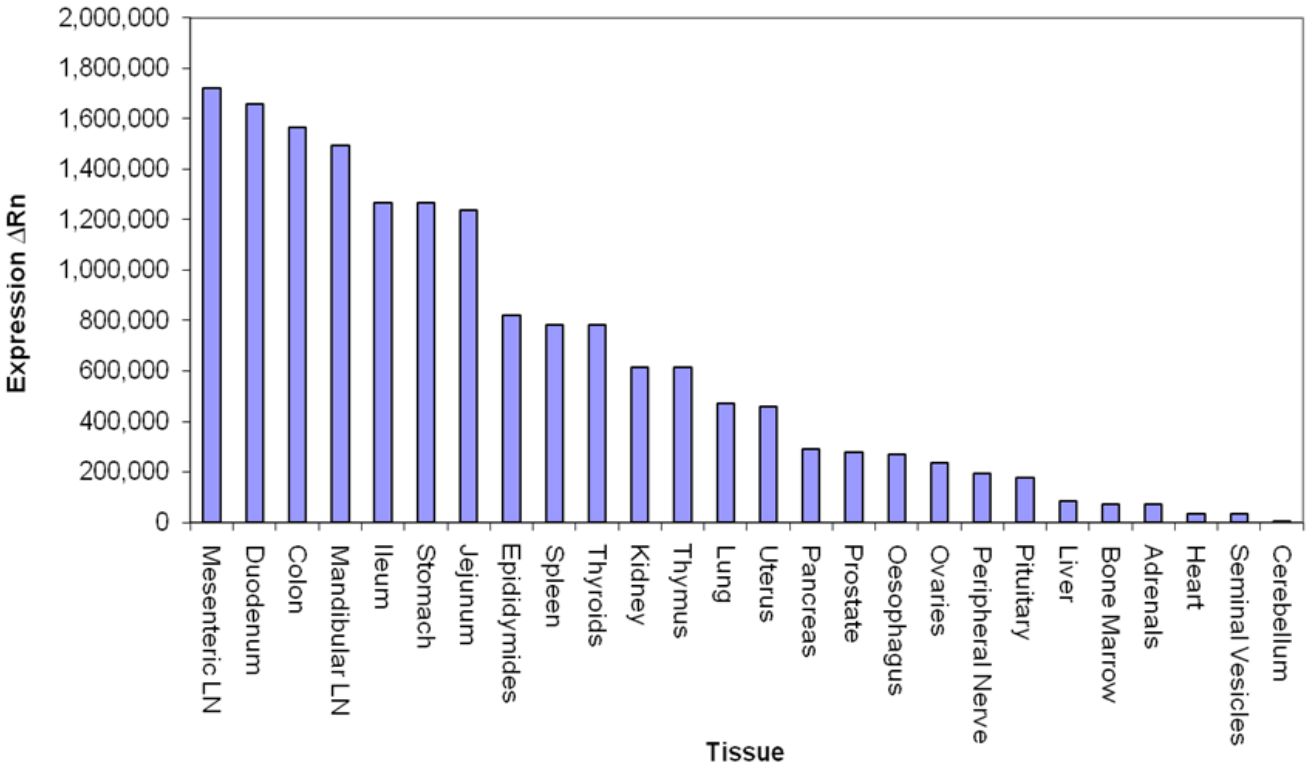


Figure 5.1: TaqMan® IL-28R

Quantative RT-PCR analysis of IL-28R expression in a range of mouse tissues, shown as magnitude of fluorescence signal ΔRn and measured in copy numbers, 3 samples for each tissue were pooled prior to PCR analysis.

5.3 Gene Logic human tissue microarray screen

Using data from the Gene Logic human tissue microarray database, gene expression of IL-28R (TIG45563), IL-10RB (TIG1871) and IFNAR1 (TIG1823) were compared across a panel of human tissues (Figure 5.2 A-C). This data showed that while IL-10R and IFNAR mRNAs were ubiquitously expressed across tissues (with the exception of brain IL-10RB and skeletal muscle IFNAR1 shown in red), several tissues were negative for IL-28R (left and right ventricles, blood, cervix, exo/endo cervix, brain, thymus, bone, ovary, testis, placenta, uterus, bladder, adipose, aorta and vein shown in red). Gene Logic data for IL-28R in human tissues showed gene expression primarily in epithelial tissue, lymph nodes and spleen,). Tissues expressing IL-28R (shown in blue): small intestine, ileum, jejunum, pancreas, thyroid, lymph node, gallbladder, colon, rectum, liver, prostate, seminal vesicle, cecum, lung, skin, kidney, spleen, stomach, skeletal muscle, bronchus, breast, heart, oesophagus, adrenal gland, fallopian tube.

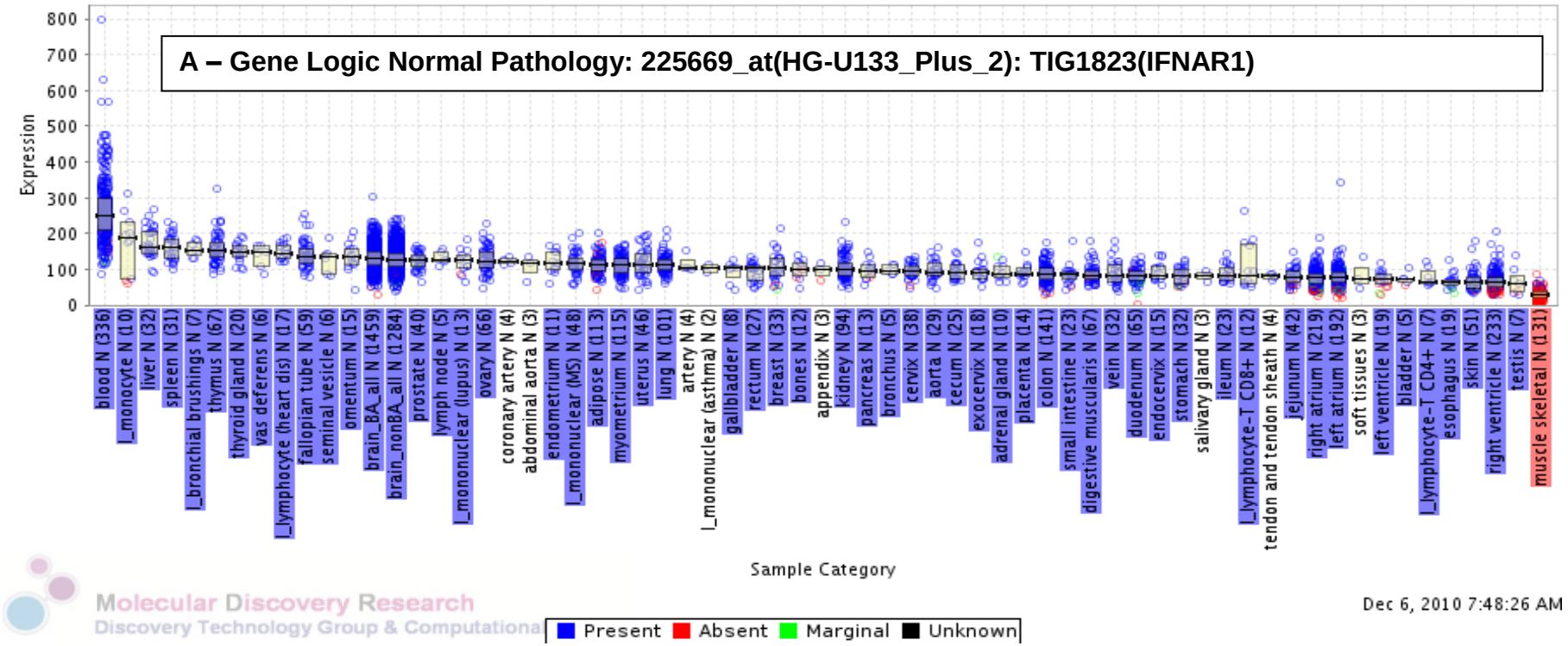


Figure 5.2 A: Gene Logic graph showing IFNAR1 expression in a range of human cell types and tissues

Affymetrix data retrieved from a database of human gene expression data across a range of human cell types and tissues using the bioinformatics tool Gene Logic. Tissues highlighted in blue show significant expression and tissue highlighted in red insignificant expression.

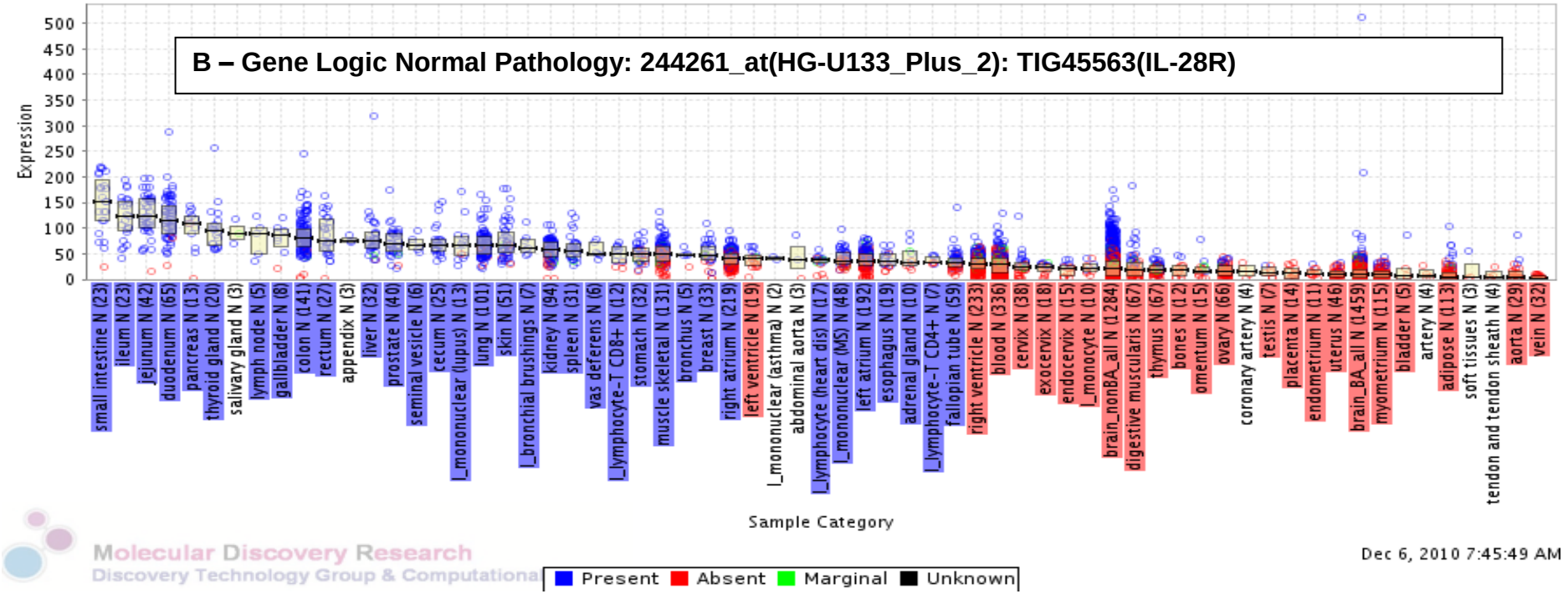


Figure 5.2 B: Gene Logic graph showing IL-28R expression in a range of human cell types and tissues

Affymetrix data retrieved from a database of human gene expression data across a range of human cell types and tissues using the bioinformatics tool Gene Logic. Tissues highlighted in blue show significant expression and tissue highlighted in red insignificant expression.

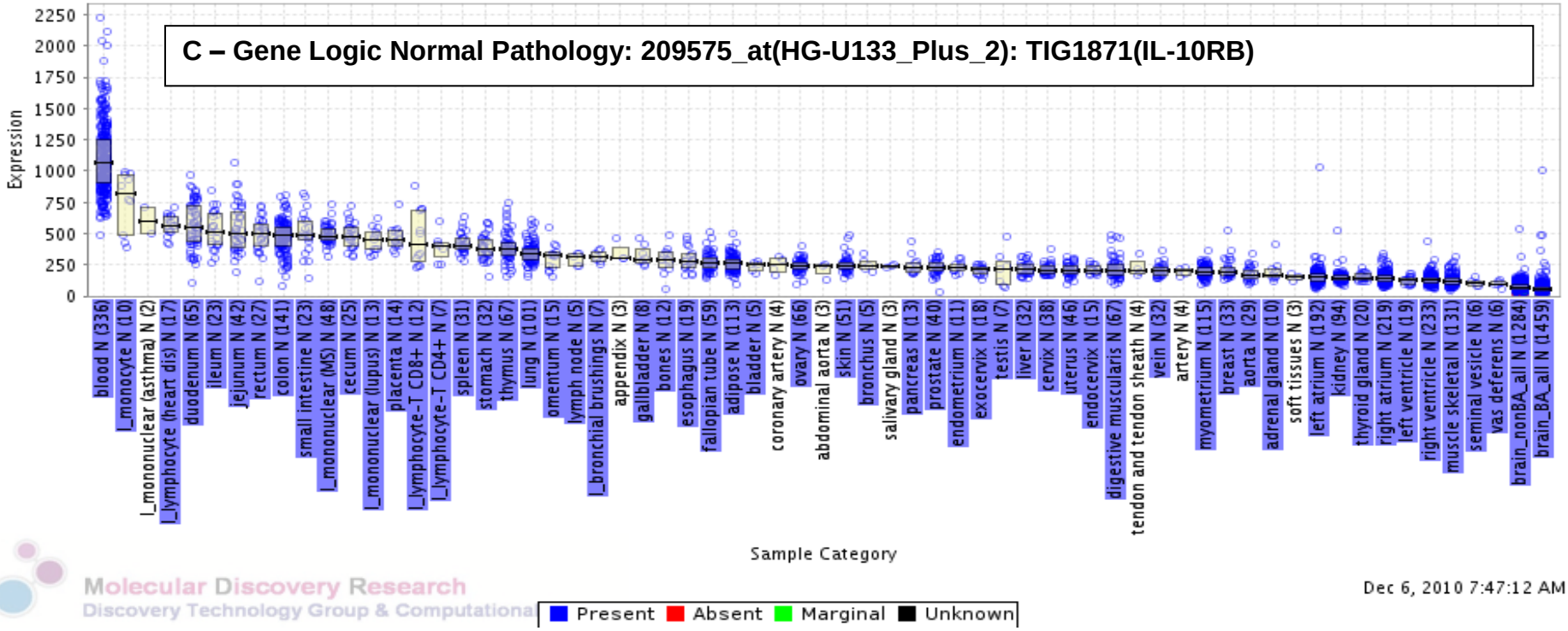


Figure 5.2 C: Gene Logic graph showing IL-10RB expression in a range of human cell types and tissues

Affymetrix data retrieved from a database of human gene expression data across a range of human cell types and tissues using the bioinformatics tool Gene Logic. Tissues highlighted in blue show significant expression and tissue highlighted in red insignificant expression.

5.4 Inflammatory cell microarray screen

In order to investigate the expression of IL-28R in immune and inflammatory cells in more detail we looked at mRNA extracted from a panel of human cells (Figure 5.3). The graph demonstrates the distribution of IL-28R on a panel of cells both with and without a variety of inflammatory stimuli. The data reveal that IL-28R is highly expressed on stimulated and untreated B cells and alveolar macrophages. In non-alveolar macrophages only those stimulated for 24 hours with immune complex (IgG coated beads) showed significant IL-28R expression. Unstimulated pDCs have a higher level of IL-28R expression than unstimulated mDCs. Stimulated and resting CD8+ T cells show low levels of IL-28R expression. Similarly resting PBMCs express IL-28R at low levels while CD4+ T cells and mast cells do not significantly express IL-28R.

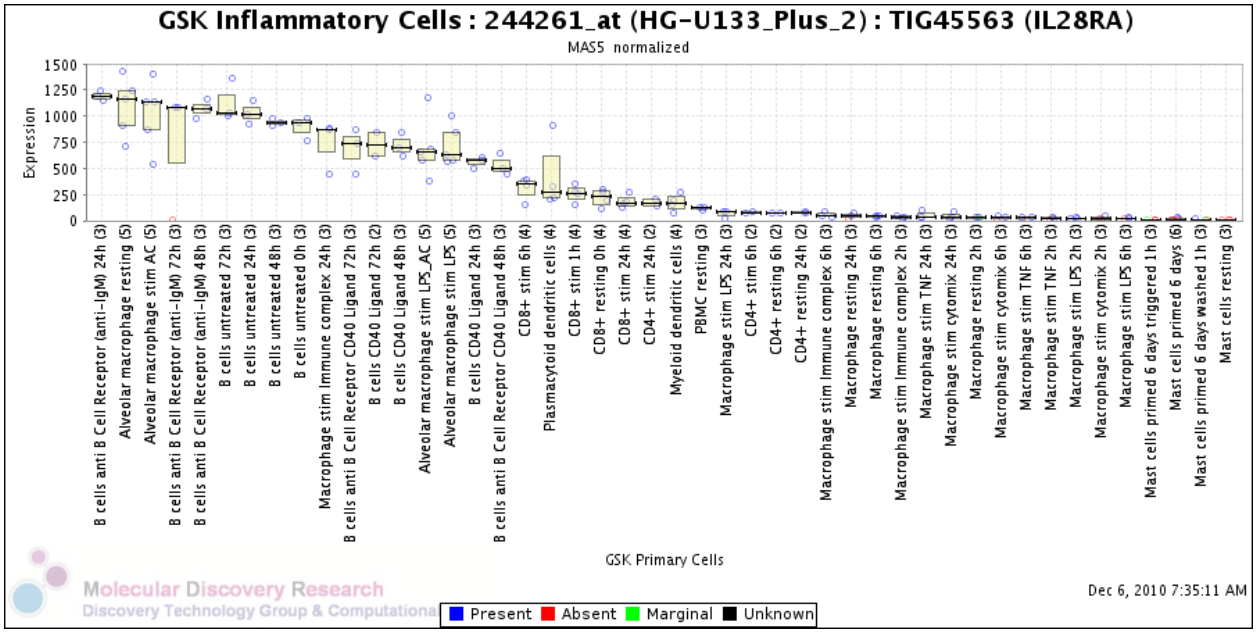


Figure 5.3: GSK Inflammatory cell screen showing IL-28R gene expression

Inflammatory cell screen: B cells were unstimulated or stimulated with CD40 ligand or anti B cell receptor (anti-IgM) co-stimulation assay at four time points; 0, 24, 48 and 72 hours. Macrophages were cultured with vehicle alone, TNF (10ng/mL), LPS (10ng/mL), immune complex (IgG coated beads) and cytomix (TNF (10ng/mL), LPS (10ng/mL) and IFNγ (10ng/mL) for 2, 6 and 24 hours. T cells treatments were CD4+ and CD8+ cells resting; 1, 6 and 24 hours post anti-CD3/CD28. Mast cells were cultured with human stem cell factor (100ng/mL), human IL-6 (50ng/mL) and IL-10 (10ng/mL), then primed for 6 days with human IL-4 (10ng/mL), human myeloma IgE, lambda (1µg/mL) and human myeloma IgE, kappa (1µg/mL). Cells were triggered for 1 hour with anti-IgE (1.5µg/mL), control with no triggering. pDCs and mDCs; no treatment. Numbers in brackets indicate number of replicates.

5.5 Discussion

IL-28R gene expression was shown in a panel of mouse tissues using real-time PCR (Figure 5.1). High levels of IL-28R gene expression were seen in mandibular and messenteric lymph nodes and gut tissues (stomach, duodenum, jejunum, ileum and colon). Expression was also detected in epididymis, spleen, thyroid, kidney, lung, pancreas, prostate, oesophagus, ovaries, peripheral nerve and pituitary gland. Tissues with minimal expression were: liver, bone marrow, adrenals, heart, seminal vesicles and cerebellum. Tissues with no detectable expression were: aorta, bladder, eyes, harderian gland, skeletal muscle, skin and spinal cord.

The distribution of IL-28R, IFNAR1 and IL-10RB expression in human tissues was shown by mRNA expression profiles in the Affymetrix datasets.

Whilst type I IFN receptor and IL-10RB are expressed on virtually all cell types, type III IFN receptor expression exhibits more restricted cellular distribution. Type III IFN receptor is expressed primarily in epithelial tissue, lymph nodes and spleen, tissues expressing IL-28R in detail were: small intestine, ileum, jejunum, pancreas, thyroid, lymph node, gallbladder, colon, rectum, liver, prostate, seminal vesicle, cecum, lung, skin, kidney, spleen, stomach, skeletal muscle, bronchus, breast, heart, oesophagus, adrenal gland, fallopian tube.

Patterns of IL-28R gene expression in mouse tissues (summarised in Figure 5.1) were compared with those found in human tissues (illustrated in Figure 5.2 B). In both human and mouse high levels of IL-28R was expressed in lymph nodes, spleen and gut epithelial tissues, low levels of IL-28R were seen in brain and bone marrow, a notable difference in IL-28R expression between human and mouse was in liver where the levels in human were a lot higher than in mouse (mouse expression levels of IL-28R were low). This was also shown in Chapter 6 where compared with human liver, mouse liver was found to have very restricted IL-28R staining (mainly in Kupffer-like cells). The tissues above are complex in nature and contain a wide variety of cell types –some of these were investigated further in an inflammatory cell microarray screen (Figure 5.3) and immunohistochemistry was carried out on various tissues (Chapter 6) to try and identify specific cell types which express the IL-28R.

An inflammatory cell microarray screen was used to investigate the expression of IL-28R in human immune and inflammatory cells. Data from this screen showed that IL-28R is highly expressed on stimulated and untreated B cells and alveolar macrophages. In non-alveolar macrophages only those stimulated for 24 hours with immune complex (IgG coated beads) showed significant IL-28R expression. Unstimulated pDCs have a higher level of IL-28R expression than unstimulated mDCs. Stimulated and resting CD8+ T cells show low levels of IL-28R expression. Similarly resting PBMCs express IL-28R at low levels while CD4+ T cells and mast cells do not significantly express IL-28R[140]. While type I IFN receptors are expressed on virtually all cell types, results described above show that type III IFN receptor expression exhibits a more restricted cellular distribution.

6. Distribution of IL-28R, protein expression

6.1 Introduction

The aim of this chapter was to study the expression of IL-28 receptor (IL-28R) in a panel of mouse/human tissues and cells in order to identify type III IFN targeted tissues and cells. Tissues and cells containing IL-28R were identified using immunohistochemistry (IHC), dual staining IHC methods and FLOW.

The heterodimeric receptor IFNAR1/2 for IFNα and IFNβ is present on all nucleated cells[72]. IL-10R2 is ubiquitously expressed[73], whereas initial RNA expression data showed IL-28R is limited to specific tissues and cell types[3]. IL-28R has been shown to be present in intestinal epithelial cells[56,6,141], MCA205, a fibrosarcoma cell line[142] and type III IFN expression has been shown in monocyte–derived dendritic cells (MDDC)[74,75,76], plasmacytoid dendritic cells (pDC)[74,76] and human primary macrophages where induction of type III IFNs required pre-treatment with IFNα[74,75,76].

6.2 Immunohistochemistry for IL-28R protein expression in murine lymphoid and epithelial tissues

Positive IL-28R expression in mouse tissues (summarised in Table 6.1 and illustrated in Figure 6.2 A-H). Tissue type was confirmed /scored by peer review from two pathologists Dr Rob Goldin and Dr Chris Clarke. IL-28R was found to be widely expressed in epithelial tissues such as stomach, small and large intestine where baso-lateral staining of the epithelial cells and strongly staining individual cells was found in the lamina propria. In lymphoid tissue and spleen approximately 30% of cells visibly expressed IL-28R. This was confirmed by flow cytometry analysis on mouse spleen showing that ~60% of spleen cells stained positively for the IL-28R (Figure 6.1).

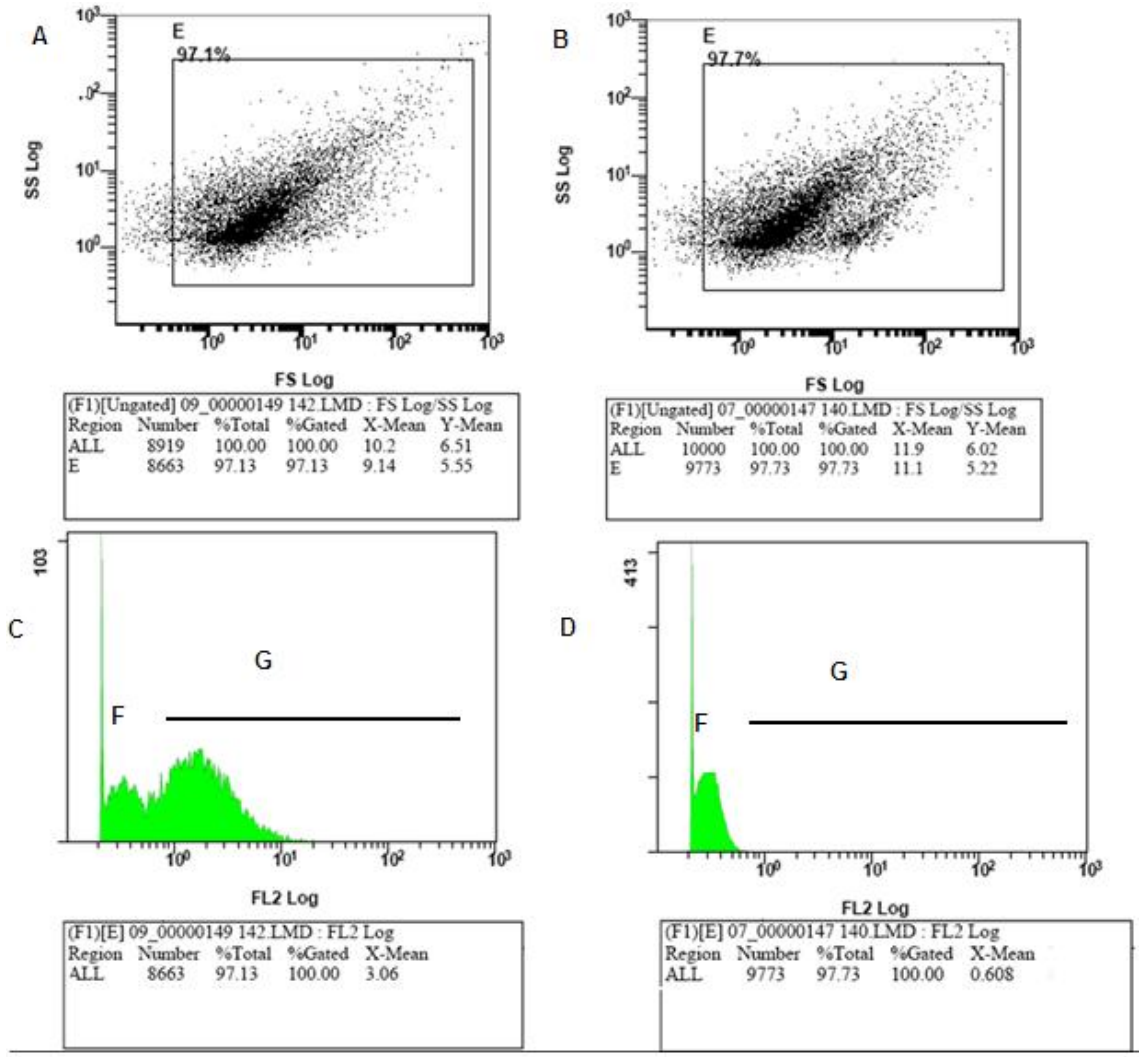


Figure 6.1: Flow cytometry showing IL-28R expression in mouse spleen

Analysis of a murine spleen sample using IL-28R antibody by flow cytometry. **A** shows normal scatter plot for treated cells, **B** shows normal scatter plot for control. SS = side scatter and FS = forward scatter. **C** shows positive cells beyond negative point as shown in **D**. **D** = negative. (**F** = negative cells, **G** = positive cells) FL2 log shows PE-IL-28R.

In the spleen IL-28R positive cells were distributed throughout the red and white pulp but predominately in the red pulp, where antigen-presenting and phagocytic cells such as dendritic cells and macrophages are concentrated. In lymph nodes IL-28R expression was shown throughout the cortex and medulla with notable presence in the sub-capsular sinus. The sub-capsular sinus area is the receiving area for afferent (incoming) lymph and is populated by 'activated' dendritic cells from stimulated regional tissues. In the mouse liver, scattered

non-parenchymal cells lining the hepatic sinusoids express IL-28R; these are likely to be Kupffer cells or stellate cells.

The following mouse tissues showed no observable IL-28R protein expression: adrenal gland, bladder, brain, eyes, harderian gland, heart, lungs, peripheral nerve, pituitary, prostate, seminal vesicles, skeletal muscle, skin, salivary glands, pancreas, spinal cord, testis, tongue and trachea.

Table 6.1: Location of IL-28R protein expression in mouse tissues

Tissue	Positive staining*	Likely Cell type	Likely background stain**
Stomach	Occasional interstitial cell 'endocrine' granular cells in basal area	macrophage	Glandular epithelial cells
Duodenum	Occasional cell in lamina propria	macrophage	-
Thymus	Scattered cells throughout cortex and medulla	dendritic cell/ macrophage	
Gall bladder	Occasional cell in subepithelial lamina propria		-
Liver	Occasional small cells with oval nuclei, sinusoidal spaces	Kupffer cell	-
Epididymis	Occasional small interstitial cell	macrophage	epithelium
Kidney	Rare interstitial cell in cortex	macrophage	-
Uterus	Occasional cell with positive cytoplasmic granules and small non-granlular cells – subepithelial and myometrium	mast cells	-
Spleen	Scattered cells throughout red pulp and white pulp/PALS	dendritic cell/ macrophage	-
Large & small intestine	Occasional cell in lamina propria		-
Peyer's patches	Occasional cell	dendritic cell/ macrophage	
Blood	Occasional cell	dendritic cell/ macrophage	-

* Typically represented by sharply-defined, intense cytoplasmic/membrane staining. ** Typically represented by pale 'wash' effect

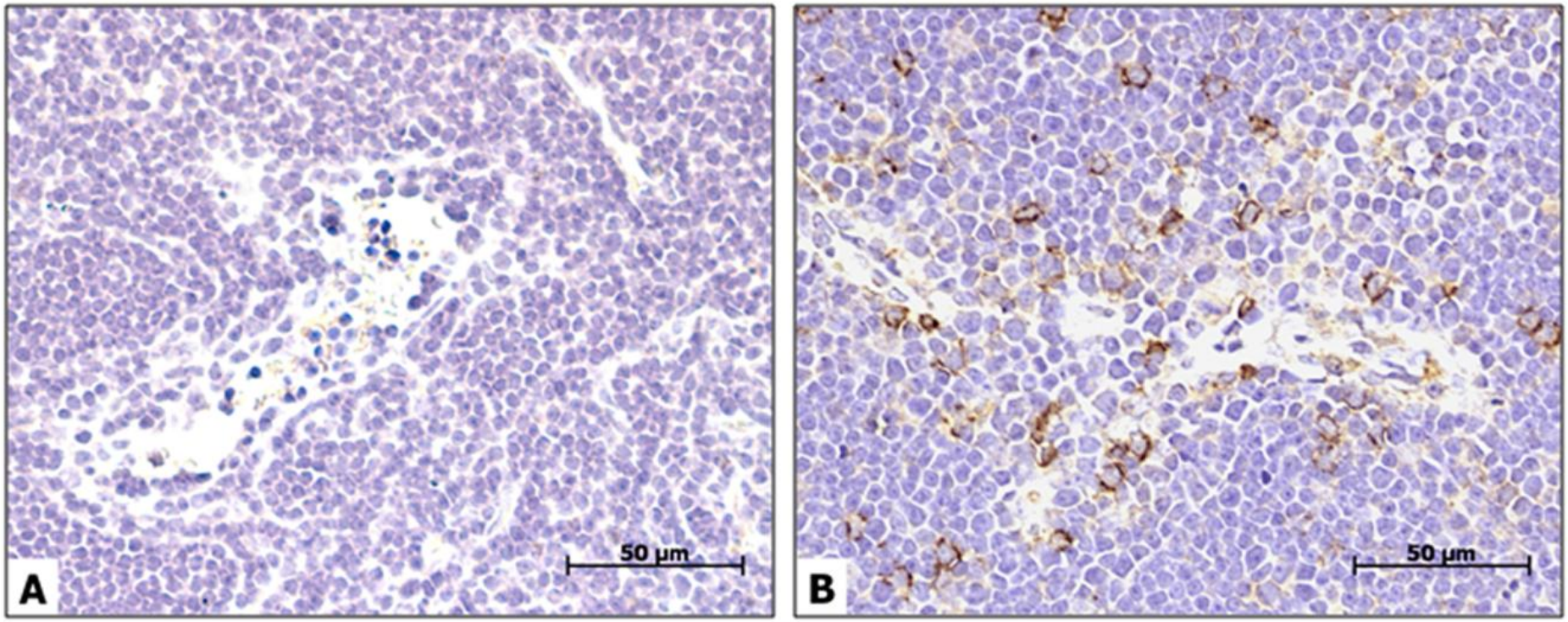


Figure 6.2 A and B: IL-28R distribution in mouse spleen using Immunohistochemistry

IHC specific staining of IL-28R is represented by sharply-defined, intense cytoplasmic/membrane staining in a selection of mouse tissues. **A** Spleen goat IgG negative control. **B** Spleen showing scattered positive cells throughout the marginal zone.

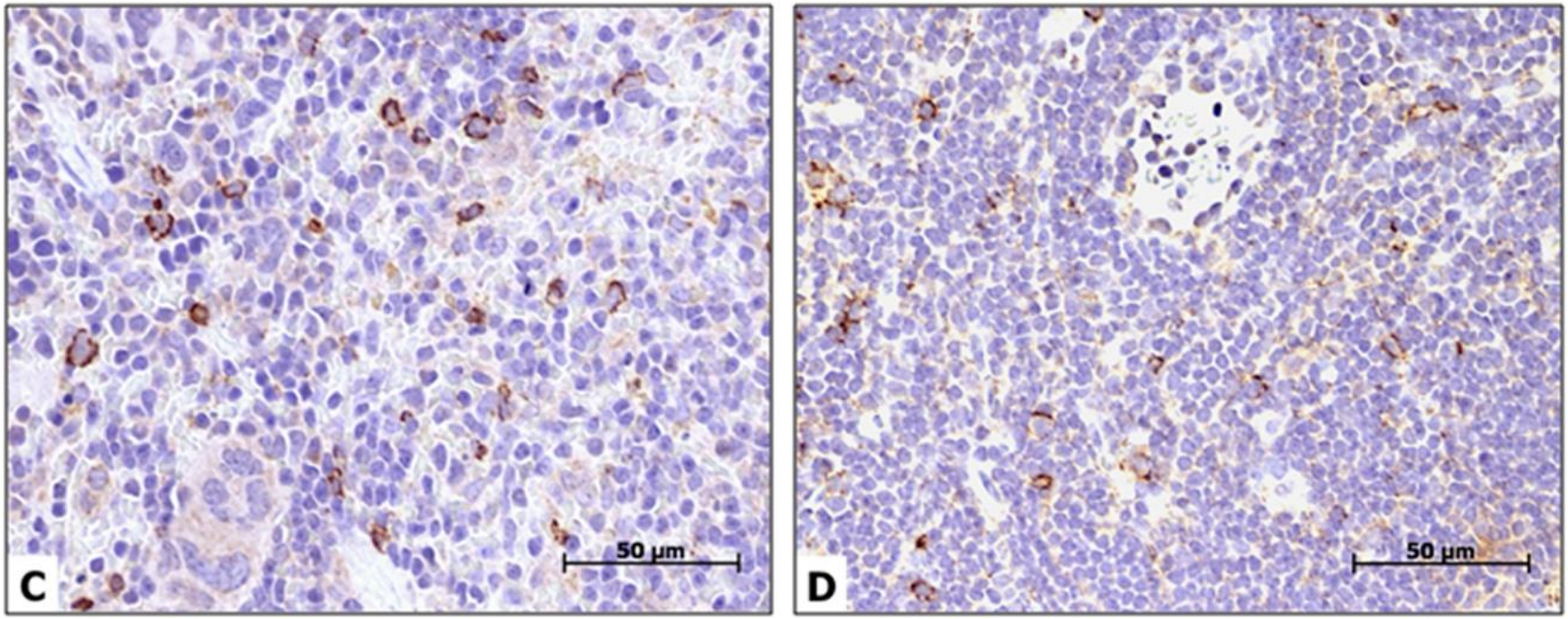


Figure 6.2 C and D: IL-28R distribution in mouse thymus and mesenteric lymph node using Immunohistochemistry

IHC specific staining of IL-28R is represented by sharply-defined, intense cytoplasmic/membrane staining in a selection of mouse tissues. **C** Thymus: scattered cells throughout cortex **D** Mesenteric lymph node.

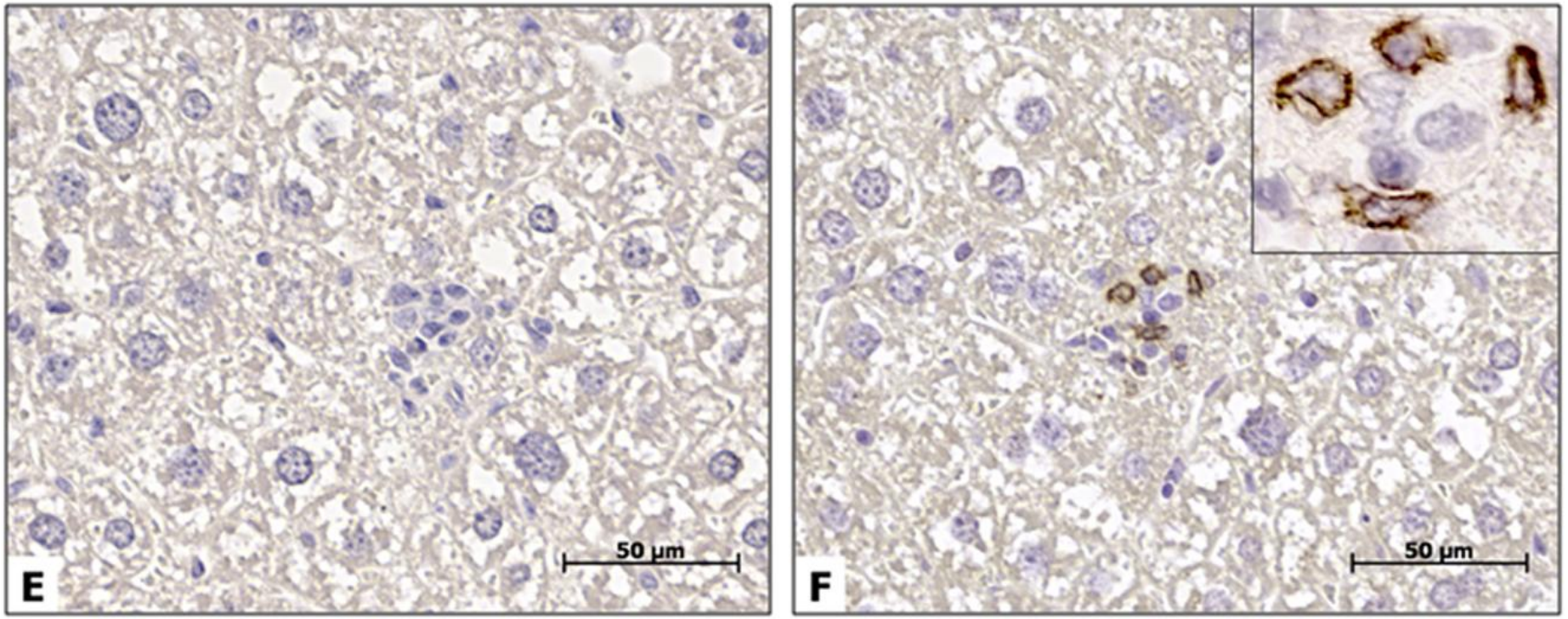


Figure 6.2 E and F: IL-28R distribution in mouse liver using Immunohistochemistry

IHC specific staining of IL-28R is represented by sharply-defined, intense cytoplasmic/membrane staining in a selection of mouse tissues. **E** Liver goat IgG negative control. **F** Liver: occasional small cells stained.

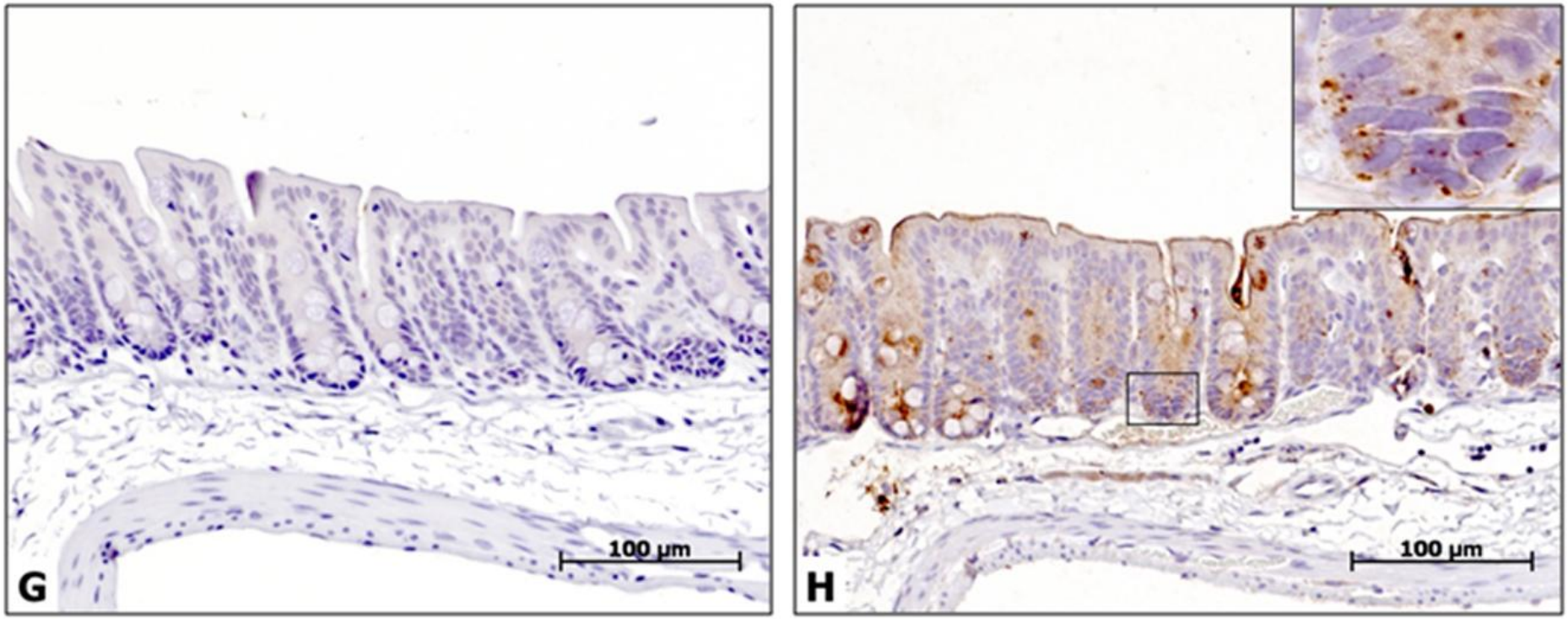


Figure 6.2 G and H: IL-28R distribution in mouse colon using Immunohistochemistry

IHC specific staining of IL-28R is represented by sharply-defined, intense cytoplasmic/membrane staining in a selection of mouse tissues. **G** Colon: goat IgG negative control. **H** Colon: cells stained at base of crypt and non-specific staining of the brush border.

6.3 Toluidine blue histochemical stain in mouse showed mast cells express IL-28R

In the uterus, IL-28R staining was found in cells with granular cytoplasm which stained positively for the mast cell stain toluidine blue on serial sections.

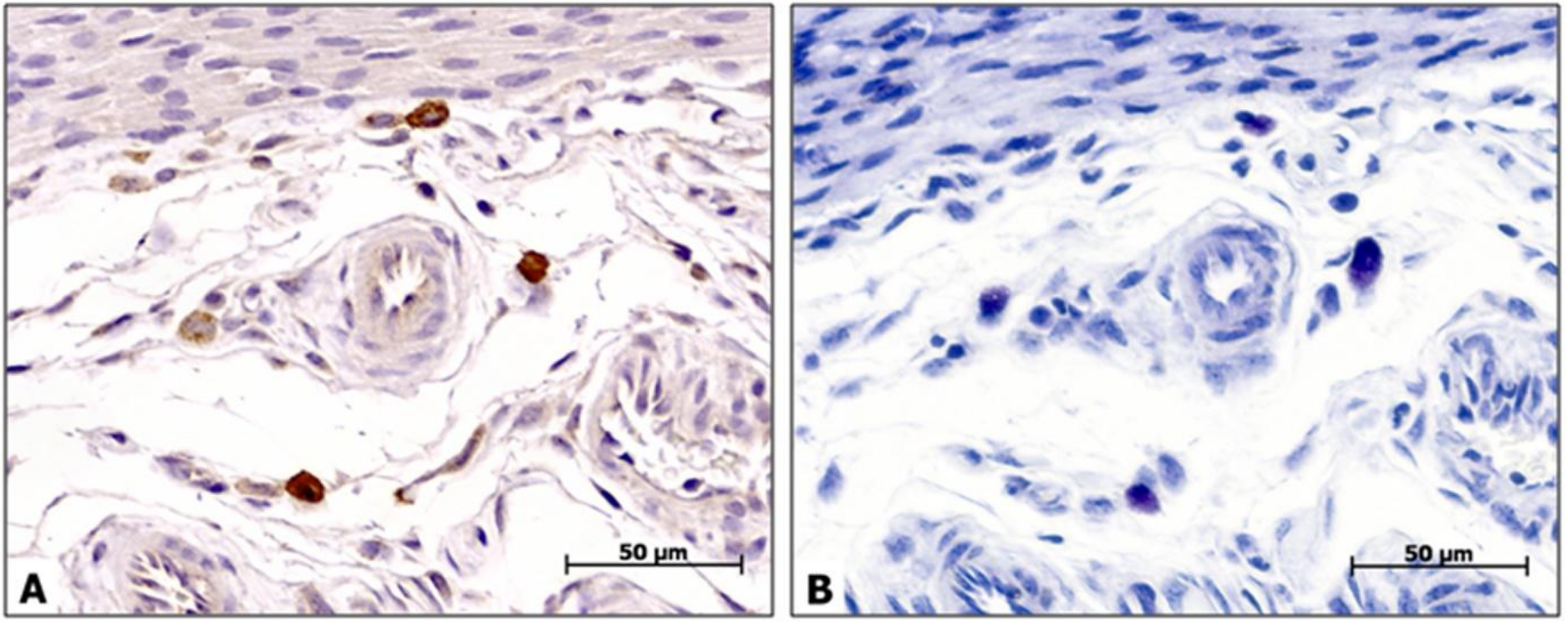


Figure 6.3: IL-28R distribution in mast cells

Mouse uterus **A** IL-28R staining in granular cells these were confirmed to be mast cells with a serial section stained with toludine blue **B.**

6.4 Dual staining- IL-28R and macrophage markers

The distribution of IL-28R staining in mouse tissues suggested expression of the IL-28R in macrophages and dendritic cells. Mouse monoclonal antibodies Mac 384 and F4/80 were used for macrophage identification. Dual staining (illustrated in Fig. 6.4) revealed that a sub-population of macrophages/DCs express IL-28R in normal mouse spleen but with a different pattern of staining in lymph node.

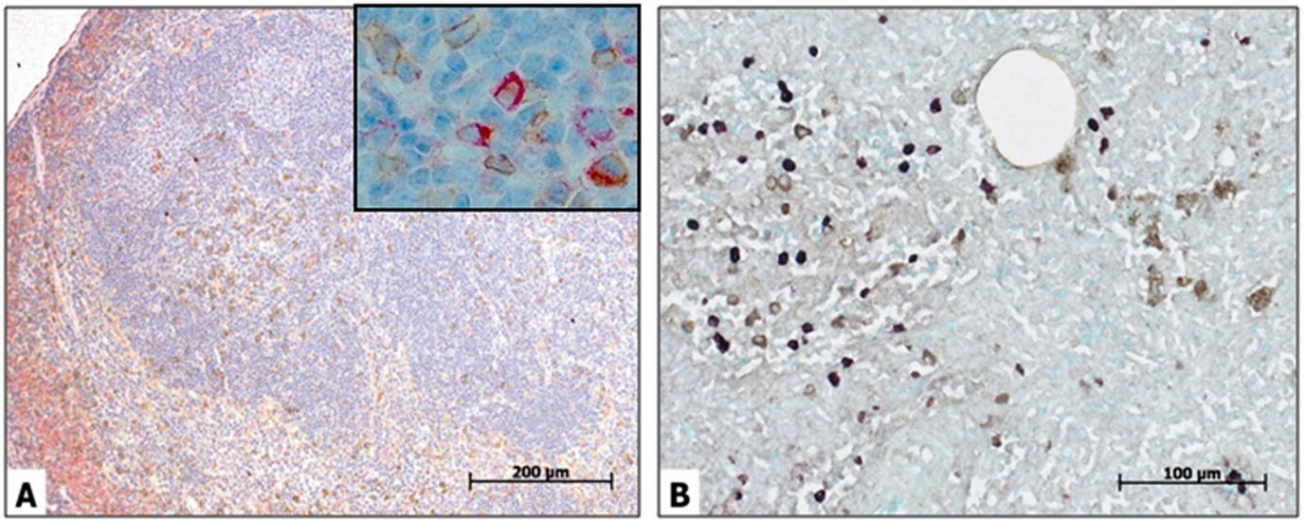


Figure 6.4: Dual staining

IL-28R and macrophage markers **A** mouse spleen stained with Mac 387 and IL-28R **B** mouse lymph node stained with F4/80 and IL-28R. Red stained, Mac 387+ macrophages are predominantly in the marginal zone and cords. Cells demonstrating IL-28R staining (brown cytoplasmic) were distributed throughout the red pulp. A subset of IL-28R positive cells stain with Mac 387 indicating IL-28R distribution on phagoctic cells; dendritic cells and macrophages. However, in lymph node **B** F4/80-positive macrophages, stained with Very Intense Purple appear to be a separate population of cells from those which stained (brown) for IL-28R.

6.5 IL-28R protein expression in normal and diseased human tissues

Human IL-28R protein expression was determined in a selection of relevant normal tissues and diseased tissues (Figure 6.5 A-F). In normal liver parenchyma, cells expressing IL-28R are relatively sparse and presumed to be Kupffer cells and some macrophages. In contrast, the number of IL-28R expressing cells in hepatitis C infected liver are increased, particularly around inflammatory infiltrates and portal areas. In addition to the inflammatory cells there appears to be low level expression of IL-28R in some hepatocytes. In fatty liver IL-28R staining was seen mainly at the edge of the cell membranes and in sinusoidal cells presumed to be Kupffer cells and macrophages. In a section of well differentiated hepatocellular carcinoma, IL-28R expression was observed in large pleomorphic cells of uncertain origin which may be mega macrophages. In ileal sections most of the positive IL-28R staining was within the lamina propria, expressed by lymphocytes. There was some IL-28R expression in cells close to the bottom of the crypt near the paneth cells. Some staining also occurred in the enterocytes but goblet cells were not involved. In sections of synovial tissue taken from patients with rheumatoid arthritis there was increased cellularity due to an influx of inflammatory cells. IL-28R staining is taken up by some of the spindle shaped/fibroblast like synovial cells and some macrophages.

Patterns of IL-28R expression in mouse tissues (summarised in Table 6.1 and illustrated in Figure 6.2 A-H) were compared with those found in human tissues (illustrated in Figure 6.5 A-F). Human tissues showed a similar pattern of staining for IL-28R in uterus, ileum and lymph nodes with predominantly macrophages/DCs staining. Compared with human liver, mouse liver was found to have very restricted IL-28R staining (mainly in Kupffer-like cells), while in human cells staining of the hepatocytes as well as macrophages/DCs was seen. This was further enhanced in diseased tissue where there was an increased expression of IL-28R in hepatitis, fatty liver and liver tumor tissues in comparison with normal liver.

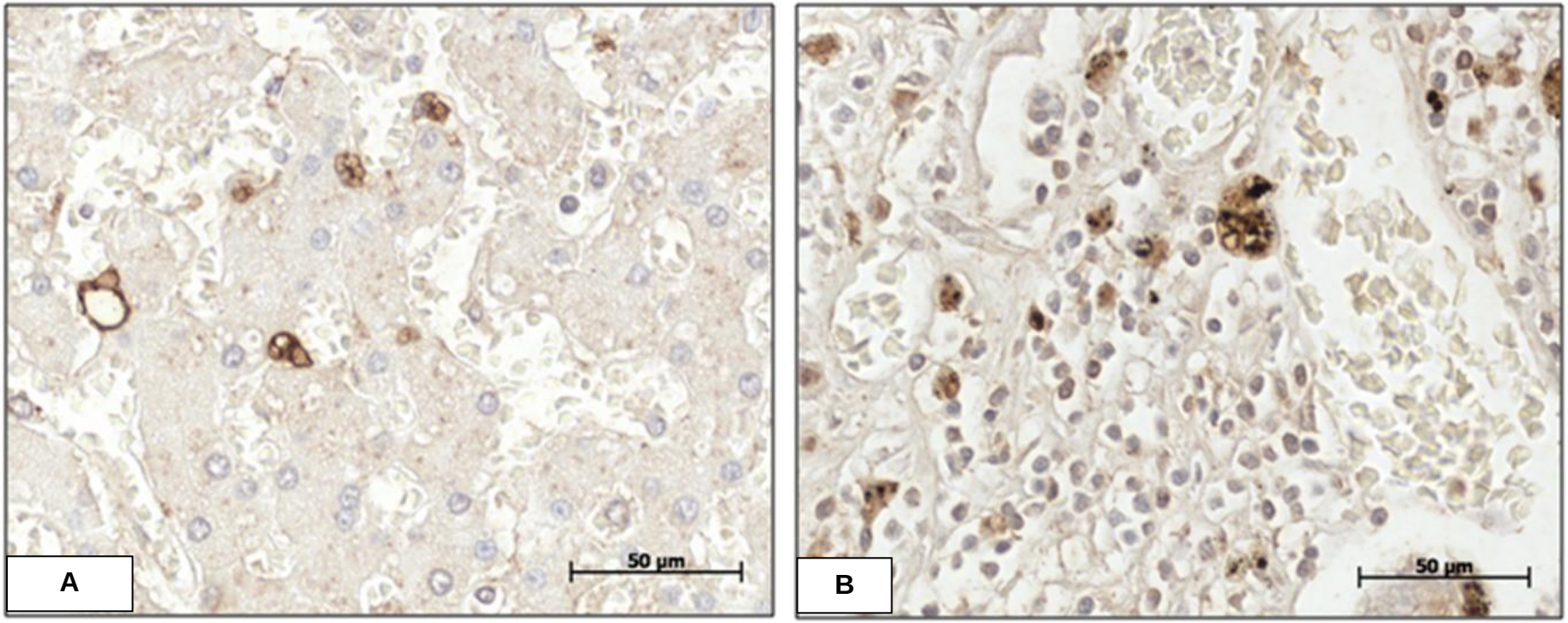


Figure 6.5 A and B: Human diseased liver and IL-28R expression using Immunohistochemistry

IHC specific staining of IL-28R in a selection of human tissues, **A** Normal liver showing IL-28R expression on mixed population of cells including hepatocytes. **B** Hepatitis C infected liver.

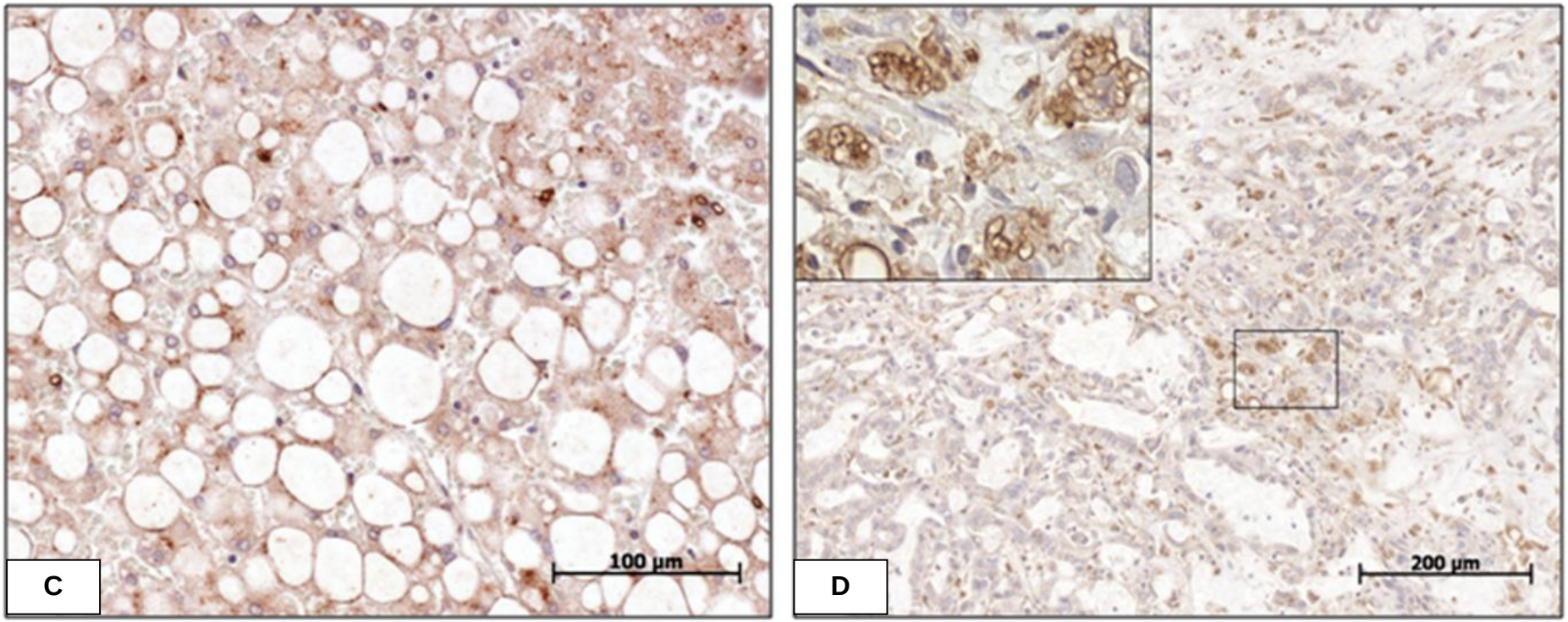


Figure 6.5 C and D: Human diseased liver and IL-28R expression using Immunohistochemistry

IHC specific staining of IL-28R in a selection of human tissues. **C** Fatty liver (macrovesicular steatosis). **D** Hepatocellular carcinoma.

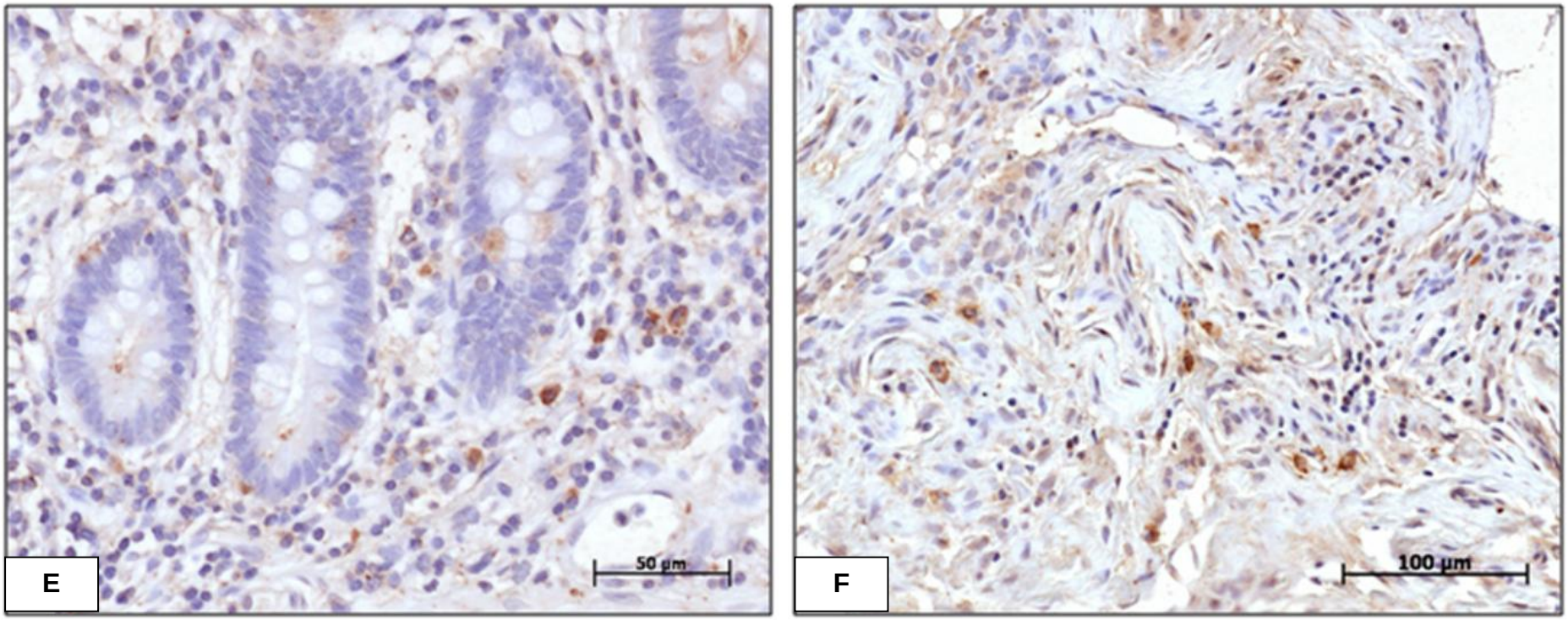


Figure 6.5 E and F: Human ileum and synovium and IL-28R expression using Immunohistochemistry

IHC specific staining of IL-28R in a selection of human tissues, **E** Normal ileum. **F** Synovium - rheumatoid arthritis.

6.6 Discussion

While type I IFN receptors are expressed on virtually all cell types, results (Chapter 5 and 6) show that type III IFN receptor expression exhibits a more restricted cellular distribution. Gene array data confirms that the IL-10RB component of the receptor is ubiquitously expressed but the IL-28R component is not. As both components are required for IFNλ signalling the distribution of the IL-28R is likely to determine which cells or tissues respond to this class of interferon.

In the panel of murine tissues, immunohistochemical analysis shows that IL-28R is expressed at highest levels by cells in lymph nodes and spleen. There is moderate cellular staining in the epithelial tissue of the gastrointestinal tract and low numbers of cells expressing IL-28R in the epididymis, uterus, thymus and kidney. Flow cytometric analysis revealed that ~60% of splenic and lymph node cells expressed IL-28R. The pattern of staining in these tissues, particularly the staining of cells in the sub-capsular regions of lymph nodes suggested that antigen presenting cells express IL-28R at high density. Dual staining with Mac 387 and F4/80 illustrate that some but not all tissue macrophages express IL-28R at high density. Interestingly, mast cells also appear to express IL-28R at high density. The immunohistochemical findings are effectively replicated in the IL-28R gene expression data (Figure 5.1). The distribution of IL-28R expression in mouse tissues was confirmed by TaqMan® PCR.

In human tissues we first analysed the distribution of IL-28R using microarray screens which showed a broadly similar tissue expression pattern to the mouse. One major exception was a much greater expression in the human liver than in the mouse. Hepatocytes were seen to express IL-28R in human tissue but not in murine tissue. A low level of IL-28R expression in mouse liver has also been reported by other investigators[78].

Although IL-28R is highly expressed by macrophages, DC and lymphocytes, neither human nor murine bone marrow was found to express the IFNλ receptor. This may have important consequences if IFNλ is used therapeutically. Up to 20% of patients treated for chronic HCV infection with IFNα have to reduce the dose due to the consequences of bone marrow

suppression. From our data it appears unlikely that IFNλ would have any significant impact on bone marrow cells and therefore neutropenia and thrombocytopaenia are less likely to occur.

Analysis of IL-28R mRNA in resting and activated inflammatory cells suggests that the IL-28R is responsive to inflammatory stimuli in some cell types. To some extent this is also seen in the comparison of IL-28R expression in normal and diseased liver tissue where there is a significant increase in the number of IL-28R expressing cells in non-alcoholic hepatitis and chronic HCV infection. However, in these tissues it is difficult to determine whether increased IL-28R expression is due to IL-28R induction or recruitment of IL-28R-expressing inflammatory cells into the diseased tissue.

In summary we have demonstrated that the distribution of the IL-28R is restricted in comparison to the IFNα receptor. Differences in receptor expression may result in a different profile of therapeutic effects and a reduction of adverse events if patients are treated with IFNλ.

IL-28R was found to be expressed in many mouse tissues including epithelial tissues such as stomach, and small and large intestine where there is baso-lateral staining of the epithelial cells and in addition, strongly staining individual cells were found in the lamina propria. In the liver, scattered non-hepatocyte cells lining the hepatic sinusoids appear to express IL-28R. These may be Kupffer cells or stellate cells. In lymphoid tissue and spleen approximately 5% of cells expressed the IL-28R strongly. In the spleen these were distributed throughout the red and white pulp. In lymph nodes these were scattered throughout the cortex and medulla. There was a notable presence of cells in subcapsular sinus, this is an area of a lymph node that is the receiving area for afferent (incoming) lymph and hence tends to be populated by 'activated' dendritic cells from stimulated regional tissues. The distribution of staining suggested expression of the IL-28R in macrophages and dendritic cells could be confirmed by staining for these cells on serial sections and/or by flow cytometry analysis of these cell types. In the uterus, staining was found in cells with granular cytoplasm which stained positively for the mast cell stain toluidine blue on serial sections. The distribution of IL-28R expression in human tissues was shown by TaqMan® PCR and correlated directly with mRNA expression

profiles in the Affymetrix datasets. Gene expression analysis showed additional tissues likely to be expressing IL-28R including lung, heart and thyroid.

Whilst type I IFN receptors are expressed on virtually all cell types (IFNαR1 Gene Logic data), type III IFN receptor expression exhibits more restricted cellular distribution. Type III IFN receptor is expressed in epithelial tissue, lymph nodes and spleen. Macrophages and/or dendritic cells appear to express the receptor at high levels. Preliminary evidence shows IL-28R is predominately expressed on antigen presenting cells. If confirmed this suggests that therapeutic manipulation of these cells may be achieved by use of IFNλ without affecting a wide range of cell types as seen with IFNα. However none of the tissues in these experiments were stimulated or infected: IL-28R might be inducible in other cell types and tissues that are stimulated or infected.

Chapter 7

7. Bone marrow

7.1 Introduction

Although IFN-α is a potent antiviral agent, the broad expression of the type I IFN receptor results in responses in many organs apart from the target tissue, leading to serious complications include the development of neutropenia and lymphopenia resulting from IFNAR1 expression in hematopoietic cells[84], depression resulting from CNS effects[83], and constitutional symptoms (fevers, chills, myalgias). Lymphopenia, is the condition of having an abnormally low level of lymphocytes in the blood and neutropenia is characterised by an abnormally low number of neutrophils, neutrophils account for 50-70% of circulating white blood cells and act as the primary defence against infection, destroying bacteria. Patients with neutropenia are more susceptible to bacterial infections which can lead to sepsis without treatment and often results in discontinuation of IFNα treatment.

Although IL-28R is highly expressed by macrophages, DC and lymphocytes, neither human nor murine bone marrow was found to express the IFNλ receptor (Data from Chapter 5 and 6). The limitation of type III IFN receptor expression suggests that some side effects may be avoided by type III IFN administration. This may have important consequences if IFNλ is used therapeutically. Up to 20% of patients treated for chronic HCV infection with IFNα have to reduce the dose due to the consequences of bone marrow suppression. From data in Chapters 5 & 6 it appears unlikely that IFNλ would have any significant impact on bone marrow cells, this chapter set out to investigate this further.

Initially Professor Myrtle Y Gordon's group and Dr Hayley Cordingley's groups at Imperial College, London were contacted for a collaboration to look at interferon lambdas in human bone marrow assays, the groups at Imperial were no longer doing these assays and could not reinstate. Commercial options were also considered but could not be funded by GSK. Through various contacts within GSK it was found that Astra Zeneca were doing bone marrow assays

using mouse cell lines, this was a good starting point to investigate the properties of IL-28A and IL-28B.

Work in collaboration with David Brott, Astra Zeneca was done to look at IFNs in murine myeloid and erythroleukemia cell lines. IL-28A and IL-28B were tested in myeloid M1 and HCD57 erythroleukemia murine cell lines and compared to IFNα. M1 mouse cells were used to look at differentiation into M1 macrophage-like cells (promoting a Th1 response –innate immune system) and HCD57, an erythroleukemia cell line derived from a mouse infected at birth with Friend murine leukemia virus, the cells proliferate in response to Epo to erythrocytes (red blood cells).

IL-29 is not expressed in mice so only IL-28A and IL-28B were tested in the mouse cell lines.

The mouse bone marrow data was very limited but data showed that it was worth pursuing collaboration with a group that did bone marrow research in humans. Human bone marrow work was performed in collaboration with Alessandro Aiutis lab, University of Rome using a gene therapy connection.

Initially IL-28R gene expression in comparison with IFNAR was evaluated in hematopoietic stem cells and lymphoid lineages (Bone marrow derived CD34+ and CD3+ T-cells, CD19+ B-cells, CD11c+ DCs and CD14+ monocytes/macrophage cells were purified from peripheral blood) to determine IL-28R distribution. To determine if *in vitro* there was a myelosuppressive effect of IFNα or interferon lambdas IL-29, IL-28A and IL-28B, human CD34+ cells were tested in colony forming cell (CFC) assays and using a CD34+ proliferation assay showed the viability of CD34+ cells in the presence of interferon lambdas and IFNα.

IL-28B was evaluated in the experiments above as it is commercially available (not the case when work was undertaken in Chapter 3 and 4) and of key interest as 3 independent genome-wide association studies have identified single nucleotide polymorphisms in the IL-28B gene region to be associated with response of patients with chronic hepatitis C to pegylated IFN-α treatment[143,100,144], showing a role for IL-28B unique to that of IL-29 or IL-28A.

7.2 Mouse cells

The myeloid M1 cell line did not respond appropriately so only data from the HCD57 epo-dependent erythroleukemia cell line assay is shown below.
The concentration that decreased the cell number by 50% (IC_{50}) was calculated for IFNα, IL-28A and IL-28B from the data shown in Figure 7.1 and used to determine whether a compound was a bone marrow toxicant. A compound was deemed positive if it had an IC_{50} <1µM.

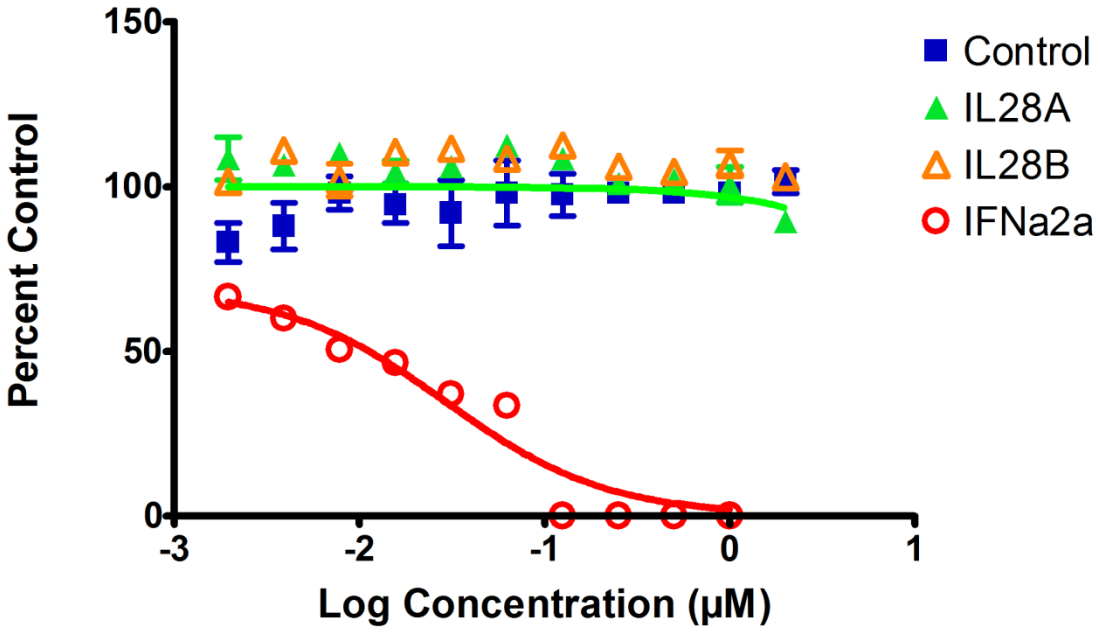


Figure 7.1: Mouse bone marrow data
The values in the graph are percent of control (100 means same relative luminescence [cell number] as in the control well; 50 means 50% less relative luminescence [cell number] compared to the control well). From these values (triplicate wells for each concentration) the IC_{50} was calculated, IFNα is 0.04µM and IL-28A and IL-28B >2µM.

In the HCD57 epo-dependent erythroleukemia cell line assay the IC_{50} for IFNα is 0.04µM or 40nM. IL-28A and IL-28B have IC_{50} values of >2µM (highest concentration tested due to solubility). This data shows that IFNα has an IC_{50} value of <1µM which shows that it is classed as a bone marrow toxicant in the

HCD57 erythroleukemia cell line assay. IL-28A and IL-28B was not associated with any toxicity at 2µM. IL-29 was not tested as it is not expressed in mouse.

7.3 Human cells - IL-28R gene expression in comparison with IFNAR in hematopoietic stem cells and lymphoid lineages

The expression of IFNAR1 and IL-28R were tested on purified CD34+, CD3+, CD19+, CD14+ and CD11c+ purified from healthy donors. The expression levels for the IFNAR1 were higher than the expression of IL-28R, with CD34+, CD3+, CD14+ and CD11c+ cells but not with CD19+ cells where receptor expression for IL-28R and IFNAR1 were shown to be similar. IL-28R expression was detected in very low levels in peripheral myeloid cells CD11c+ but not detected in CD14+ cells or bone marrow derived CD34+ cells, Figure 7.2. High levels of IL-10R2 expression were detected in all cell types (data not shown).

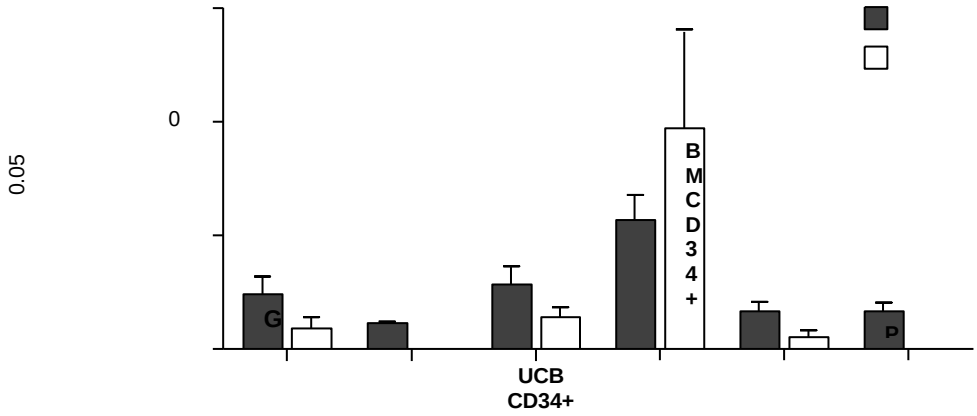


Figure 7.2: Human IFNαR1 and IL-28R expression

Quantitative RT-PCR analysis of IL-28R and IFNAR1 expression in human hematopoietic stem cells and peripheral lymphoid lineages. Umbilical cord (UCB) and bone marrow (BM) CD34+ cells, peripheral blood (PB) CD3+, CD19+, CD11c+ and CD14+ cells. Gene expression data calculated as ΔCt in comparison with b-actin housekeeping control, concentration in µM.

7.4 Colony forming capacity and proliferation of human CD34+ cells in vitro

To assess a possible myelosuppressive effect of IFNα or interferon lambdas, human CD34+ cells were tested in colony forming cell (CFC) assays. As shown in Figure 5B, IL-29, IL-28A and IL-28B did not inhibit the capacity of BM CD34+

cells to form erythroid or myeloid colonies (BFU, GM and GEMM) with the concentrations tested. In comparison the capacity of the plated cells to proliferate and form colonies is significantly reduced by the presence of all three IFNα concentrations tested, Figure 7.3.

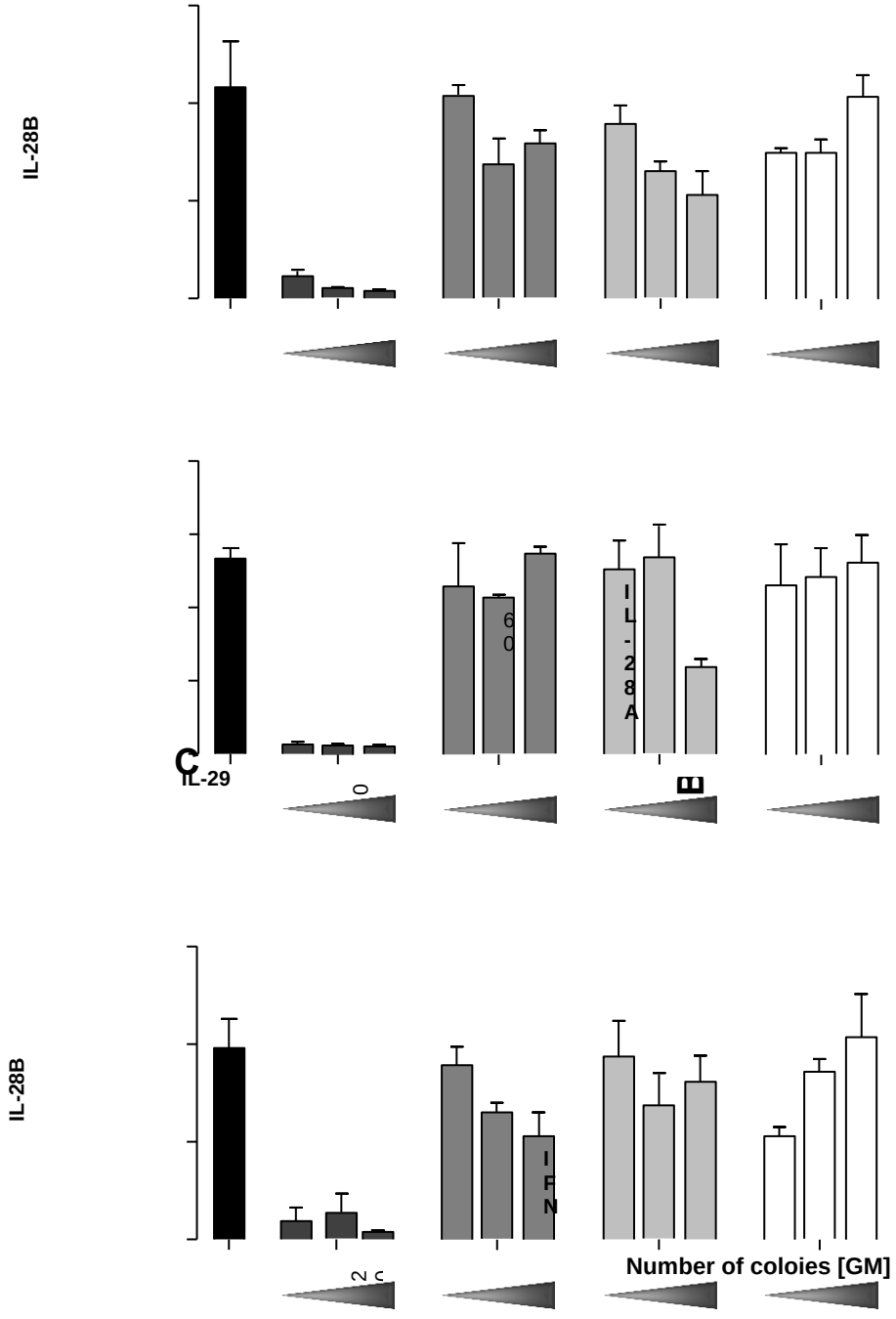


Figure 7.3: Colony forming cell (CFC) assay

1000 BM CD34+ cells/ well after 14 days in methylcellulose with 0.03125, 0.0625 and 0.125µM IL-29, IL-28A, IL-28B and IFNα stimulation. (A) BFU colonies= burst-forming unit erythroid= erythroid progenitor cells. (B) GM colonies= granulocyte, monocyte =

precursor for monoblasts and myeloblasts. (C) GEMM colonies= granulocyte, erythrocyte, monocyte, megakaryocyte= multipotential progenitor cells.

In addition the CD34+ proliferation assay showed the viability of CD34+ cells after cytokine stimulation is lower for IFNα compared to the interferon lambdas (Figure 7.4), this comparible to the results seen for the mouse bone marrow data, Figure 7.1.

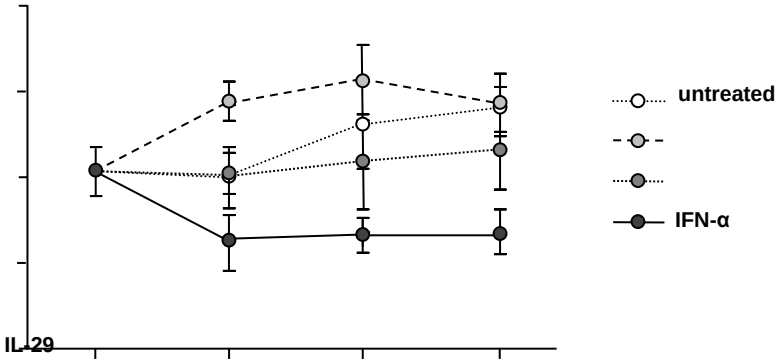


Figure 7.4: CD34+ cells' viability after proliferation

50,000 bone marrow CD34+ cells/ well after cytokine stimulation (with TPO, SCF, Flt3, IL-3) and proliferation for 3 days in the presence of 0.03125, 0.0625 and 0.125μM IFNα, IL-29, IL-28A and IL-28B.

7.5 Bone marrow cell discussion

In the murine HCD57 erythroleukemia cell line assay data showed that IFNα has an IC_{50} value of <1μM which shows that it is classed as a bone marrow toxicant in the HCD57 erythroleukemia cell line assay and IL-28A and IL-28B show no toxicity at 2μM. IL-29 is not expressed in the mouse so work to look at IL-29 was done in a human CD34+ assay.

Results for the IFNαR1 and IL-28R gene expression on purified CD34+, CD3+, CD19+, CD14+ and CD11c+ cells show the expression levels for the IFNR1 is higher in most cell types than IL-28R, including CD34+. Most importantly, IL-28R expression was not detected in peripheral myeloid cells.

In vivo exposure to IFNα acting through IFNαR1 is therefore likely to have a greater effect on immune cell development and function as compared to the three interferon lambdas IL-29, IL-28A and IL-28B. The IL-28R is not only expressed at lower levels as compared to IFNαR1, but its expression is completely absent in peripheral blood myeloid cells (CD14+, CD11c+). This differential expression could contribute to a greater myeloablative action of IFNα as compared to lambdas *in vivo*.

Consistently, the capacity of BM CD34+ cells to proliferate and form colonies was severely not compromised by the presence of IFN lambdas IL-29, IL-28A and IL-28B. But in vitro exposure to IFNα lowered the viability of CD34+ cells after cytokine stimulation and completely abolished their colony forming capacity. These findings are consistent with the findings that both human and murine bone marrow only expressed IFNAR but not the IL-28 receptor, thereby indicating an increased sensitivity of bone marrow haematopoietic stem cells to IFNα. It can be hypothesised that IFN-α exerts an inflammatory effect also on hematopoietic stem cells, thereby altering their quiescence state. Changes in their cellular turnover and increased proliferation are likely to lead to an exhaustion of their differentiation capacity. This effect might well underlie their abolished colony formation capacity in vitro and cause a myeloablative effect after treatment with IFNα. The conserved viability and colony forming capacity in the presence of interferon lambdas on the other hand indicates that this unwanted side effect could be prevented by administration of the interferon lambdas. Although the IL-28R was found expressed by macrophages, DC and lymphocytes in the peripheral blood, its expression was lower than the IFNAR in all cell types and comparable in B cells.

Chapter 8

General Discussion

8.1 Antiviral properties of interferon lambdas

Interferon lambdas show a clear role in antiviral response, with EMCV, vesicular stomatitis virus, herpes simplex virus 1, influenza A virus, cytomegalovirus, HIV, HBV, and HCV are all sensitive to the antiviral effects of IFNλ[136,145,57,127,2,58,51,3,146,65]. Data here shows *in vitro* assays show that IL-28A and IL-29 have an antiviral effect against HCV in the HCV genotype 1a and 1b replicon systems. IFNα was shown to have ~100 times greater antiviral activity than IL-29, and IL-29 had ~10 times greater antiviral effect in the HCV 1a and 1b replicon system than IL-28A. Robek demonstrated that IL-29 and IL-28A inhibit replication of subgenomic and full-length HCV replicons in HuH7 cells. IFNα and type III interferons were tested in the HCV 1b replicon assay to determine any synergistic effects of the type I and type III interferons together: results showed an additive effect not a synergistic effect, also seen with IL-29 and IFNα by Marcello *et al* [61]. An additive effect though could show that IL-29 when used with IFNα could be good as a dual therapy as opposed to IFNα alone. In the interferon sensitive assay, sensitivity was shown in the ISG56 assay with IL-28A and IL-29: interferon lambdas have also been shown to induce expression of 2'5-OAS[2] and MxA[126]. Initial data in a human HepG2 cell line stably transfected with HBV subtype ayr showed that both IL-29 and IL-28A had antiviral effects against HBV but significantly lower in antiviral activity than IFNα. IL-29 was shown to inhibit HBV replication in a murine model Robek[51]. No antiviral effects were observed with IL-28A or IL-29 in the HIV, RSV assays or in the dengue replicon system, Therefore, the inability of IFNλ to inhibit these viruses may be due to the cell line having limited IL-28R distribution or their inherent insensitivity to the antiviral effects of IFNs in general, rather than a specific shortcoming of the IFNλ response.

8.2 Gene Expression

There are many similarities in gene modulation by type I and type III IFNs. When looking at the genes that are expressed most highly in both the parent HuH7 and the replicon systems IFNλs and IFNα show largely overlapping sets of interferon stimulated genes (ISGs). Although antiviral mechanisms of type I and III differ, they may actually involve similar ISG responses. Other groups have shown similar sets of ISGs being expressed by type I and type III IFNs[65,57,61]. Our data was different to these groups in that IL-28A was also tested in HuH7 and the replicon systems, and that parent HuH7 cells were used in gene expression analysis. Marcello and Zhou used HCV replicon cells and Doyle used $HepG_2$ cells for microarray analysis and all groups compared IL-29 with IFNα.

The majority of interferon stimulated genes (ISG) stimulated in the replicon cell line by IFNα, IL-28A and IL-29 are also stimulated in the parental cell line. For the majority of genes in both the replicon and the parent datasets, the kinetics of gene expression was the same for type I and type III IFNs. Marcello[61] showed in the replicon system that the majority of type III stimulated genes continue to increase at 24 hours and with IFNα the same genes tend to decrease by the 24 hour time point. In contrast the data shown here demonstrates that the kinetics of the type I and III IFNs show similar patterns of gene expression. HCV appears to influence IFNλ induced gene expression similar to IFNα induced gene expression. HCV replicon inhibited gene induction by both type I and type III IFNs; this may be due to the NS3/4A protease encoded by HCV cleaving the adaptor in the RIG-I-like receptor (RIG-I) pathway IPS1 thus disrupting the signalling to type I IFN[138]; this data indicates that signalling to type III IFN is also disrupted. Downstream genes from RIG-I are affected in the replicon system including MAPK genes which show lower abundance in number in the replicon system than in the parent system.

Pathway analysis showed in the parent HuH7 cells at the earliest time point tested, 4 hours, for type I and type III IFNs interferon signalling is the most strongly induced pathway. At 8 hours after interferon signalling pathway, IL-29 induces immune modulation pathways such as antigen presentation pathway

and complement and coagulation cascades predominantly, compared with IFNα mostly regulating cell signalling pathways. Interferon signalling was not represented in the top 7 pathways by IFNα at 8 hours potentially showing that IFNα is less specific in its signalling pathways than IL-29. This gene expression analysis suggests that IFNλs and IFNα have primarily complementary functions but where IL-29, by modulating alternative pathways such as the antigen presentation and processing pathways at an early time point, may prove to be a useful therapeutic option for the management of chronic viral infection such as HCV.

8.3 IL-28R distribution

While type I IFN receptors are expressed on virtually all cell types, results show that type III IFN receptor expression exhibits a more restricted cellular distribution. This is also reported by other groups showing not all cell types respond to interferon lambdas including fibroblasts and endothelial cells[13].

In the panel of murine tissues, immunohistochemical analysis shows that IL-28R is expressed at highest levels by cells in lymph nodes and spleen and epithelial tissue of the gastrointestinal tract. Type III interferons have been shown to be the main interferon giving antiviral protection of intestinal epithelial cells against GI viruses identifying a critical role of IFN-λ in the epithelial antiviral host defense[147] and possibly maintenance of GI tract homeostasis, linking this role to high level of IL-28R expression in gut tissue. A high level of IL-28R on intestinal epithelial cells has been shown to be of importance. Antiviral protection of intestinal epithelial cells against gastrointestinal viruses mainly relies on the type III antiviral system[147]. This study showed mice lacking functional IL-28R had impaired control of oral rotavirus infection, which infects intestinal epithelial cells. Additionally it was demonstrated that systemic administration of IL-29 not IFNα, induced an antiviral state in these cells resulting in suppression of rotavirus replication. The immunohistochemical findings were confirmed in the IL-28R gene expression data. The unique functional tissue-specificity of IFN-λs is due to the cell type-restricted pattern of IFN-λR1 expression it was shown here the unique of staining in these tissues, particularly the staining of cells in the sub-capsular regions of lymph nodes

suggested that antigen presenting cells express IL-28R at high density. Dual staining with Mac 387 and F4/80 illustrated that some but not all tissue macrophages express IL-28R at high density. Literature also shows that unlike IFNα IFN-λR1 is primarily expressed in epithelial cells and specific subsets of immune cells[59,57,13,148,77,79,78,80,23]. In human tissues the distribution of IL-28R using microarray screens showed a broadly similar tissue expression pattern to the mouse. One major exception was a much greater expression in the human liver than in the mouse. Hepatocytes were seen to express IL-28R in human tissue but not in murine tissue, this may account for the IFN-λ antiviral system appearing to play minimal if any role in the protection of mice against hepatotropic viruses[148].

IHC data didn't not show IL-28R in lung but stimulated alveolar macrophages showed high gene expression of IL-28R, type III IFN system is shown to have an important role in asthma and respiratory viral infections[149,140].

Neither human nor murine bone marrow was found to express the IFNλ receptor in significant levels. This may have important consequences if IFNλ is used therapeutically. Up to 20% of patients treated for chronic HCV infection with IFNα have to reduce the dose due to the consequences of bone marrow suppression. It appears unlikely that IFNλ would have any significant impact on bone marrow cells and therefore bone marrow suppression is less likely.

In summary the distribution of the IL-28R is restricted in comparison to the IFNα receptor. IFN-λs have a specialized role in diseases of epithelial tissue, and in the treatment of viral infections of these and other responsive tissues. Limited IL-28R may explain the findings that IFN-λ independently of IFNα cannot provide full protection against systemic virus infections; these viruses infect cells that are not responsive to type III IFNs. Restriction of IL-28R receptor expression may result in a different profile of therapeutic effects and fewer or milder adverse events if patients are treated with IFNλ rather than IFNα.

8.4 Bone marrow experiments

In a murine HCD57 erythroleukemia cell line assay, IFNα was classed as a bone marrow toxicant while IL-28A and IL-28B were shown to not cause toxicity. In a murine HCD57 erythroleukemia cell line assay, IFNα was classed as a bone marrow toxicant while IL-28A and IL-28B were shown to not cause toxicity. Consistently, the capacity of BM CD34+ cells to proliferate and form colonies was not compromised by the presence of IFN lambdas IL-29, IL-28A and IL-28B. But in vitro exposure to IFNα lowered the viability of CD34+ cells after cytokine stimulation and completely abolished their colony forming capacity. These findings are consistent with the finding that both human and murine bone marrow only expressed IFNAR but not the IFN-λ receptor, thereby indicating an increased sensitivity of bone marrow haematopoietic stem cells to IFNα. The conserved viability and colony forming capacity in the presence of interferon lambdas, indicates that this unwanted side effect could be prevented by administration of the latter. Although the IL-28R was found expressed by macrophages, DC and lymphocytes in the peripheral blood, its expression was lower than the IFNAR in all cell types and comparable in B cells. These findings might therefore have important consequences if IFNλ is used therapeutically. Up to 20% of patients treated with IFN-α for chronic HCV infection have to reduce the dose due to the consequences of bone marrow suppression. From data in Chapter 7 it appears unlikely that IFNλ would have any significant impact on bone marrow cells and therefore neutropaenia and thrombocytopaenia are less likely to occur. In support of this hypothesis phase 1b data from Zymogenetics showed that repeated dosing with PEG-IFN-λ was well tolerated with minimal constitutional symptoms and in contrast to the effects of type I interferons, there were no significant decreases from baseline values in neutrophil or platelet counts[96,150,116].

8.5 IFNλs as a therapy

Type III interferons have been implicated as having antiviral properties in a range of viruses: DNA viruses; poxvirus[55], murine cytomegalovirus (CMV)[56,56],

hepatitis B virus[57,51,58] and herpes simplex virus 1 and 2[59], the single stranded (ss) (+) RNA viruses EMCV[60,3], west nile virus[57] and hepatitis C virus[57,61,62,51], as well as the ss (−) RNA viruses influenza-A virus[63] and vesicular stomatitis virus[62]. Chronic infection with hepatitis C virus causes approximately 350,000 deaths annually[46]. Pegylated interferonα (IFNα) in combination with the nucleoside analogue ribavirin is the current standard of care for the treatment of patients chronically infected with hepatitis C (HCV). There is a role for Interferon lambdas in the treatment of HCV, data described here showed IL-29 and IL-28A have an antiviral effect against HCV in the HCV genotype 1a and 1b replicon systems. An additive effect of IFNα and IL-29 or IL-28A treatment could show that IFN lambdas when used with IFNα could be good as a dual therapy as opposed to IFNα alone.

The data presented shows that by modulating alternative pathways such as the antigen presentation and processing pathways at an early time point and having a limited receptor distribution it may prove to be a superior therapeutic option for the management of HCV chronic viral infection, causing fewer and/or milder adverse events. Up to 20% of patients treated for chronic HCV infection with IFNα have to reduce the dose due to the consequences of bone marrow suppression, this data shows IFNλ does not have a significant impact on bone marrow cells and therefore neutropaenia and thrombocytopaenia are less likely to occur. In support of this hypothesis, phase 1b data from Zymogenetics showed repeated dosing with PEG-IFNλ was well tolerated with minimal constitutional symptoms and in contrast to effects of type I interferons, there were no significant decreases from baseline values in neutrophil or platelet counts[96,116]. Latest data from Bristol Myers Squibb Co. showed peginterferon lambda achieved SVR24 rates comparable to peginterferon alpha with fewer flu-like and musculoskeletal symptoms in phase IIb study in treatment-naïve genotype 2 or 3 hepatitis C patients.

There is a potential role for IL-29 instead of IFNα in the treatment regime with direct-acting antivirals (DAAs), NS3/4A protease inhibitors, telaprevir and boceprevir. With the current DAAs there is rapid resistance due to rapid mutations of HCV[40,41,86,87]. The current DAAs are administered with peginterferon-alfa-2a and ribavirin to prevent resistance to the DAAs. Both

telaprevir and boceprevir highly improve rates of SVR of HCV infected patients but there are limitations, designed to target genotype 1 HCV they have differential efficancy across the genotypes[88,89,90], they are also limited by side effects, rash (Telaprevir) and anaemia, replacement of IFNα with IL-29 could reduce side effects caused by IFNα and may reduce side effects attributed to the DAA as this may be exasperated by IFNα. Other HCV therapies are currently being evaluated using co treatment of IFNα , these include RdRp inhibitors which inhibit the active site of polymerase activity, preliminary results from Phase II trials reported rates of EVR >80% among patients with HCV genotype 1 or 4 infection who received mericitabine in combination with SOC PEG-IFN and RBV47; may not need the co-administration of IFNs and host factor cyclophilins, Alisporivir is in advanced clinical trials, it inhibits viral replication by disrupting the interaction between cyclophilin A and NS5A[93,94]. Alisporivir combined with PEG-IFN and RBV, led to an SVR in 76% of HCV genotype 1 infection patients compared to 55% with SOC[94]. There may be a role for Interferon lambdas to replace IFNα as a co treatment but the aim with these therapies is to have an interferon free regime.

A pegylated form of IFN-λ1 (IL-29) is currently in clinical trials for the treatment of chronic HCV infection and initial reports shows less severe side effects than pegylated IFNα and similar antiviral activity[95,96]. Caution would be needed if administrating interferon lambda with HCV/HIV co infection as the role of IFNλs is still unclear, whilst IL-29 and IL-28A are reported to inhibit HIV-1 replication in macrophages[151] pretreatment of uninfected PBMCs or CD4+ T-cell lines with IFN-λ improved the expression of HIV-1 receptor and co-receptors that increase viral binding and replication[130].

There may be a role for IL-28B treatment in the future, four Genome Wide Association studies have highlighted the significance of IL-28B within the innate immune response to HCV[97,98,99,100]. Two protective SNPs were shown to be associated with a SVR to IFNα/Ribavirin in patients with HCV genotype 1. In a study of rapid viral response (RVR) rates in patients with HCV Genotype 1 and 4, of the RVRs 100% of carriers of the protective rs12979860 C allele, and 64% of non-RVR individuals expressed the non-protective genotype T allele of rs8099917[101].

The potential broad roles of IFN-λs in immune function may also mean they play a future role in autoimmunity and cancer therapy. Type III IFNs like type I IFNs, may potentially be used for the treatment of other inflammatory or autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE)[102], or multiple sclerosis (MS)[103], IFN-λ therapy may represent a novel approach to prevention and/or treatment of respiratory virus-triggered asthma exacerbations[149,140]. IFN-λs have been shown to have potent antitumor activities in murine models of cancer[104,105,106], highlighting potential as anti-cancer therapy.

8.6 Future work

Further experiments could be designed looking at the effects of interferon lambdas on antigen presenting cells, as experiments above have suggested that the interferon lambdas not only modulate alternative pathways such as the antigen presentation and processing pathways at an early time point compared with IFNα but their receptor IL-28R is also found primarily on antigen presenting cells. Maturation of dendritic cells (DCs) (generated from buffy coats) can be investigated by applying a fixed dose of antigen to DCs, treating the DCs with IFNλ, IFNα and no interferon and looking at T-cell responses. The current, immunomodulatory activities of IFNλs are still not defined and studies to date show opposing functions e.g. DC-mediated stimulation of either T-reg proliferation[22] or towards Th1 differentiation[140]. There is value in continuing to look at the properties of IL-28A and IL-28B as well as IL-29 and look at their broader roles in immune function including autoimmunity and cancer therapy.

8.7 Conclusions

Interferon lambdas (IFNλs), termed IFN-λ1, IFN-λ2 and IFN-λ3, or IL-29, IL-28A and IL-28B are a recently identified family of cytokines with antiviral activity. Type I IFNs are used therapeutically in the treatment of chronic hepatitis B and C; however only ~30% of patients with hepatitis B virus will be successfully treated and only ~60% of patients with chronic HCV. New interventions are

therefore required to address this unmet medical need and this thesis aimed to evaluate the potential use of IFNλs in treating viral infection.

A range of *in vitro* antiviral assays were developed to determine which viruses were inhibited by IFNλs. Results showed IL-28A and IL-29 have antiviral effects with HCV 1a and 1b replicons and HBV. No antiviral effect was demonstrated against dengue, RSV or HIV. Gene expression stimulated by IFNλ was compared with IFNα; and the effects of IFNλ against HCV were investigated. The types of genes induced, and the kinetics of gene induction were similar between the type I and type III IFNs in the HCV replicon cell line. With the parental cell line, the interferon signalling pathway was the most greatly affected by IFNα, IL-28A and IL-29, but IL-29 strongly regulated the antigen presenting pathway compared with IFNα. IL-28R distribution was determined to investigate the tissue and cellular distribution of IFNλ responsive cells. IL-28R was expressed in epithelial tissues, lymphoid tissue, spleen, liver, kidney and thymus, with majority of IL-28R expression on macrophages and dendritic cells.

In mouse HCD57 erythroleukemia cell line assay, IFNα was classed as a bone marrow toxicant and IL-28A and IL-28B were shown to not cause toxicity. Human BM CD34+ cells ability to proliferate and form colonies was not compromised by the presence of IFN lambdas IL-29, IL-28A and IL-28B whilst IFNα completely abolished their colony forming capacity. Human and murine bone marrow only expressed IFNAR but not the IL-28 receptor, thereby indicating an increased sensitivity of bone marrow haematopoietic stem cells to IFNα. The conserved viability and colony forming capacity in the presence of interferon lambdas, indicates that this unwanted side effect could be prevented by administration of the latter.

These findings might therefore have important consequences if IFNλ is used therapeutically. Up to 20% of patients treated with IFNα for chronic HCV infection have to reduce the dose due to the consequences of bone marrow suppression. From our data it appears unlikely that IFNλ would have any significant impact on bone marrow cells and therefore neutropenia and thrombocytopenia are less likely to occur. In support of this hypothesis phase 1b data from Zymogenetics showed that repeated dosing with PEG-IFN-λ was well tolerated with minimal constitutional symptoms and in contrast to the

effects of type I interferons, there were no significant decreases from baseline values in neutrophil or platelet counts[53, 63].

In summary differences in receptor expression and gene expression compared to IFNα may result in a different profile of therapeutic effects and adverse events if patients are treated with IFN-λ.

Appendix I

A data CD containing Affymetrix gene chip raw data is enclosed.

Appendix II Suppliers

Below are listed the names and contact details of most suppliers or materials and reagents used in this study.

Abcam	www.abcam.com
Affymetrix	www.affymetrix.com
Agilent	www.agilent.com
AllCells	www.allcells.com
Analytix	www.analytix.co.uk
Applied Biosystems	www.appliedbiosystems.com
Becton Dickinson	www.bdbiosciences.com
Bio-Rad	www.bio-rad.com
Capralogics	www.capralogics.net
Corbett Life Science	www.corbettlifescience.com
Dako	www.dako.com
Eppendorf	www.eppendorf.co.uk
Fluka	www.sigmaaldrich.com/Fluka
Gene Logic Inc.	www.genelogic.com
Gibco	www.invitrogen.com
IKA	www.ika.com
Ingenuity Inc.	www.ingenuity-inc.org
Invitrogen	www.invitrogen.com
Kbioscience	www.kbioscience.co.uk
Life Technologies	www.lifetechnologies.com
Lonza	www.lonza.com/research
Merck, Sharpe and Dohme	www.msd-uk.com
Miltenyi Biotec	www.miltenyibiotec.com
Molecular devices	www.moleculardevices.com
Novagen	www.merckmillipore.com/novagen
Nunc	www.nuncbrand.com
PBL	www.interferonsource.com
Proligo	www.sigmaaldrich.com

Promega	www.promega.com
Qiagen	www.qiagen.com
R&D systems	www.rndsystems.com
Sigma-Aldrich	www.Sigma-Aldrich.com
Syngene	www.syngene.com
Thermo Shandon	www.thermoscientific.com
Tree Star	www.treestar.com
Vector Labratories	www.vectorlabs.com
Virostat	www.virostat-inc.com

Appendix III Publications

TALKS:

Rhiannon Lowe Talk entitled 'Use of IHC to show distribution of IL-28R'. 9th European Histopathology Forum (19-21st April 2010) - Stratford on Avon. Awarded best new speaker award.

PUBLICATIONS:

Jaimini Mistry, **Rhiannon Lowe**, Christian Weise, Mikala Skydsgaard and Zuhal Dincer.Morphometric assessment of minipig skin thickness in relation to age, sex and anatomical location using a computerised system. 8th European Histopathology Forum (20th - 22nd April 2009) - Stratford on Avon

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