Innate resistance to hepatitis C virus infection in the Irish anti-D cohort

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By

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Declaration

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General Abstract

Even within homogenous populations, outcome following exposure to a virus can vary substantially. This continuum of infection outcome spans viral resistance, spontaneous resolution and chronic infection, with further immunological and pathological heterogeneity observed within each of these subgroups. This heterogeneity is particularly evident in hepatitis C virus (HCV) outbreaks. HCV candidate viral resistors, also known as exposed seronegative (ESN), remain antibody (Ab) and PCR negative following viral exposure, and are identifiable based on risk or documented exposure. Spontaneous resolvers (SR) are a distinct group that clear infection via an adaptive immune response, and are identifiable as Ab-positive but PCR-negative. The final group, those with chronic infection (CI), are identifiable as Ab-positive and PCR-positive. Study of the full spectrum of outcome in a single group has been stymied by the lack of suitable cohorts exposed to a virus in a controlled manner.

Between 1977-79 in Ireland, over 2000 Rhesus (RhD)-negative females were exposed to HCV following receipt of HCV-contaminated anti-D immunoglobulin. Analysis of records from the Irish Blood Transfusion Service (IBTS)indicated that there were three infection groups: ESNs, SRs and CI. Work over the past 40 years has focused on SRs and CIs as they were easily identifiable based on clinical assays. However, up to 611 (47%) women tested Ab and PCR-negative following exposure to HCV out of 1293 women who received highly infectious batches of anti-D. Through a nationwide media campaign we sought recruit women from each of these groups to study correlates of protective immunity. We successfully recruited and record-matched 34 ESN women, 98 seropositive women (SP; 48 SR and 50 CI [All the women with CI were treated successfully and achieved a sustained virological response; SVR]). We hypothesised that ESN women from the anti-D cohort have an enhanced innate immune response that protected them from infection with HCV.

To test for anti-HCV antibodies in our ESNs, and to test the hypothesis that ESN donors may also be protected against other common viral pathogens, we used VirScan, a novel technology to explore the history of viral infection. We compared antibody levels against 206 human pathogens in our subgroups. We observed no significant differences in

antibody levels to any virus other than HCV. HCV Abs were detectable in 84% (16/17) of the SVR donors. Raised antibodies to some HCV epitopes were detected in 16% (3/19) of SR women. No HCV Ab positivity was detected in the ESN donor group. To further confirm the absence of a HCV adaptive immune response in our ESNs, we used IFNγ ELISpots to assess the HCV-specific T memory response. We did not find any T cell activity to HCV peptides in our ESNs or controls but observed some positivity in the SRs and SVRs.

Assessing the immune response in humans is often confounded by labour intensive technologies with handling steps that can compromise reproducibility. We therefore opted to use standardised whole blood stimulations using the TruCulture system previously used by the *Milieu Interieur Cohort (MIC).* We invited n=18 ESN women, n=19 SR and n=17 SVR women from our cohort to donate a blood sample for stimulation with a panel of viral ligands including polyIC (TLR3), R848 (TLR7), ODN (TLR9) and IFNα2 (IFNAR1/2). Analysis of stimulated whole blood was carried out using Luminex and Simoa proteomics and NanoString transcriptomics. We found that ESNs had an increased polyIC induced type I interferon (IFN-I) signature compared with SP donors as well as increased production of several pro-inflammatory cytokines including CCL8, CXCL11 and IL-6. ESN, SRs and CI all had similar responses to stimulation with R848 or IFNα2.

To assess the potential genetic differences in innate immune response genes associated with resistance to HCV infection we genotyped our full cohort, alongside additional age and sex matched donors from previous studies, for tagSNPs in TLR3 (rs3775291), IRF3 (rs7251) and IFNAR1 (rs2257167; n ESN = 38, n SR = 63, n SVR = 77 and n unexposed control [UC] = 119). Using this approach, we identified an association between rs2257167 and resistance to HCV in our cohort. Subsequent analysis of transcriptomic data from UC donors in our cohort and the MIC showed that rs2257167 is a baseline expression quantitative trait locus (eQTL) for IFNAR1. Analysis of NanoString data from the MIC showed that rs2257167 is associated with increased interferon stimulated gene (IRG) responses following stimulation influenza virus (IAV), polyIC and LPS compared with the wild-type IFNAR1 receptor. rs2257167 is associated with decreased non-IRG pro-inflammatory responses to these stimuli.

Differences in the innate immune response may also impact the ability to spontaneously resolve infection. Seropositive women include those who cleared the virus themselves (SRs) and those who required therapeutic intervention to clear infection (SVR). SP women responded similarly to stimulation with R848, polyIC or IFNα2. However, in response to ODN, we observed differential expression of over 50 genes between SR and SVRs. Further interrogation of the differences between groups showed enrichment for IFN-I signalling in ESNs and SVRs compared to SRs. Using gene signature scores, the SRs had a reduced IFN-I signature. This was also reflected at the protein level, with SRs having decreased IFNα production compared to SVRs.

Association studies during the COVID-19 pandemic uncovered interesting associations between blood groups and risk of hospitalisation and severe disease. As our cohort is comprised of RhD-negative females, and appeared to have increased viral resistance compared with other cohorts, we sought to determine whether RhD status impacts on the whole blood immune response. We analysed genetic, proteomic and transcriptomic data previously generated on the 1000 person MIC in response to stimulation with LPS, polyIC and IAV. No study had yet explored the impact of RhD on the immune response. Donors were classified as RhD-negative or -positive using the rs590787 SNP in RHD and split into males and females. We found that RhD-negative males have increased responsiveness to IAV stimulation compared with RhD-positive males. Analysis of this response, using gene set enrichment analysis, showed increased IFNγ signalling in the RhD-negative males but not in RhD-negative females. This increased IFNγ response may explain, at least in part, the protective association of RhD-negativity with viral infection in males.

Collectively, the work here identifies for the first time, a unique cohort of HCV resistant individuals from the Irish anti-D cohort. Based on data from the IBTS, we show that viral resistance may be more common than originally thought- with up to 50% of individuals displaying natural resistance to HCV infection. We identify a TLR3-induced IFN-I signature as being associated with viral resistance, and uncover novel genetic associations with viral protection. We also show that resolution of acute HCV is associated with an reduced IFN-

I response to ODN. Our work here underscores the power of whole blood analysis when coupled with robust multiplex assays to define the signatures of induced immune responses in small well-defined cohorts of volunteers.

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Outputs

Publications from this thesis

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2. Jamie A. Sugrue, Cliona O'Farrelly. **Uncovering resistance to hepatitis C virus infection: scientific contributions and unanswered questions in the Irish anti-D cohort.** MDPI Pathogens 2021 (*Submitted)*.

Papers

1. Jamie A. Sugrue, Megan Smith, Celine Posseme, Bruno Charbit, The Milieu Interieur Consortium, Nollaig Bourke, Darragh Duffy, Cliona O'Farrelly. **Rhesus negative males have an enhanced IFNγ-mediated immune response to influenza A virus.** Genes and Immunity 2021 (*Submitted)*.

2. Jamie A. Sugrue, Celine Posseme, Bruno Charbit, Vincent Bondet, Nollaig Bourke, Darragh Duffy, Cliona O'Farrelly. **Enhanced TLR3-induced type I interferon signature and cytokine production in hepatitis C virus resistant women from the Irish anti-D cohort.** Journal of Hepatology 2021 (*Submitted)*.

3. Jamie A. Sugrue, Celine Posseme, Bruno Charbit, Vincent Bondet, Nollaig Bourke, Darragh Duffy, Cliona O'Farrelly. **Reduced TLR9 type I interferon responses in females who spontaneously cleared hepatitis C virus infection.** Journal of Infectious Diseases (*in preparation*).

4. Jamie A. Sugrue, Celine Posseme, Darragh Duffy, Nicole deWeerd, Paul Bastard, Jean-Laurent Casanova, Paul Hertzog, Nollaig Bourke, Cliona O'Farrelly**. The V141L variant of the type I interferon receptor IFNAR1 alters the response to stimulation and is associated with resistance hepatitis C virus infection.** *(In preparation).*

Presentations

Oral presentations

- Invited talk - Federation of Clinical Immunological Societies, California, United States.

Poster Presentations

- Irish Society for Immunology Annual Meeting 2018
- The International Symposium on Hepatitis C Virus and Related Viruses 2019
- Sex Differences in the Immune System 2021
- The International Symposium on Hepatitis C Virus and Related Viruses 2021
- Host Pathogen Conference 2021

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Other publications

1. Orla M. Finucane, Jamie A. Sugrue, Ana Rubio-Araiz, Maie Victoire Guillot-Sestier, Marina A. Lynch. **The NLRP3 inflammasome modulates glycolysis by increasing PFKFB3 in an IL1β dependent manner in macrophages.**

2. Liam Townsend, Adam H. Dyer, Karen Jones, Jean Dunne, Aoife Mooney, Fiona Gaffney, Laura O'Connor, Deirdre Leavy, Kate O'Brien, Joanne Dowds, Jamie A. Sugrue, David Hopkins, Ignacio Martin-Loeches, Cliona Ni Cheallaigh, Parthiban Nadarajan, Anne Marie McLaughlin, Nollaig M. Bourke, Colm Bergin, Cliona O'Farrelly, Ciaran Bannan, Niall Conlon. **Persistent fatigue following SARS-CoV-2 infection is common and independent of severity of initial infection.** Plos one. 2020;15(11):e0240784.

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4. Celine Posseme, Alba Llibre, Bruno Charbit, Vincent Bondet, Vincent Rouilly, Violaine Saint André, Jeremy Boussier, Jacob Bergstedt, Nikaïa Smith, Liam Townsend, Jamie A. Sugrue, Clíona Ní Cheallaigh, Niall Conlon, Maxime Rotival, Michael S Kobor, Estelle Mottez, Stanislas Pol, Etienne Patin, Matthew L. Albert, Lluis Quintana-Murci, Darragh Duffy, Milieu Intérieur Consortium. **Early IFNβ secretion determines variable downstream IL-12p70 responses upon TLR4 activation in health and disease.** *In review*, Cell Reports. https://papers.ssrn.com/sol3/papers.cfm?abstract_id=3965084

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Abbreviations

Ab	Antibody
BCR	B cell receptor
cDC	Conventional dendritic cell
CI	Chronic infection
CTL	Cytotoxic T lymphocyte
DAA	Direct acting antivirals
DAMP	Damage associated molecular patterns
ESLD	End stage liver disease
ESN	Exposed seronegative
HC	Healthy control
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IAV	Influenza A virus
IBTS	Irish Blood Transfusion Service
IFN	Interferon
IgG	Immunoglobulin
IL-6	Interleukin 6
IL8	Interleukin 8
IRES	Internal ribosome entry site
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
IRG	Interferon response gene
IVDU	Intravenous drug user
JAK	Janus kinase
KIR	Killer immunoglobulin receptor
LDLR	Low density lipoprotein receptor
LD	Linkage disequilibrium
MAF	Minor allele frequency
MAPK	Mitogen activated protein kinase

MDA5	Melanoma differentiation associated protein
mDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MIC	Milieu interieur cohort
NCR	Non-coding region
NK	Natural killer cell
NS	Non-structural
OAS1	2'-5'-Oligoadenylate Synthetase 1
ODN	Oligodeoxynucleotides
PAMP	Pattern associated molecular pattern
pDC	Plasmacytoid dendritic cell
PI3K	Phosphoinositide 3-kinases
PKR	Protein kinase R
polyIC	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
PWID	People who inject drugs
R848	Resiquimod
RhD	Rhesus
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
SES	Socioeconomic status
SNP	Single nucleotide polymorphism
SP	Seropositive
SR	Spontaneous resolver
SRB1	Scavenger receptor B 1
STAT	Signal transducer and activator of transcription
SVR	Sustained virological responder
TCR	T cell receptor
TLR	Toll like receptor
TNFα	Tumour necrosis factor α
TRIF	TIR-domain-containing adapter-inducing interferon
UC	Unexposed control

Chapter 1: General introduction

The primary function of the immune system is maintenance of a homeostatic balance within our bodies by protecting against pathogenic viruses and bacteria when challenged, and maintaining the host milieu in equilibrium when at rest [1]. Natural variation is a feature of the human immune response that has been largely overlooked [2,3]. Genome wide analysis over the past ten years has revealed that individual heterogeneity in infection outcome is due to both heritable and non-heritable factors that affect the immune system [4,5]. This variation has likely evolved from selective pressure arising from differential viral exposure by human populations and subsequent admixture breeding over generations [3,4]. For example, carriers for sickle cell anaemia, have some protective advantage against malaria and as a result, frequencies are higher in regions where malaria is endemic [6]. Understanding variation in the immune system is important on multiple fronts- it likely underpins, at least in part, differential responses to vaccines and medications.

As illustrated by the COVID-19 pandemic, the response to viral infection is also heterogenous [7]. Some individuals, despite lacking typical risk factors, are susceptible to severe disease, while others, despite exposure to a high viral load, appear to be naturally resistant to SARS-CoV-2 infection [8]. Virus resistant individuals such as these have been described in other viral infections, including HIV and HCV [9,10]. While the focus has historically been on immunological susceptibility to viral disease, there is growing interest in viral protection and resistance. Studying resistant individuals may shed light on new pan or virus specific antiviral mechanisms, inform novel vaccine strategies and provide tools for identifying individuals who might be protected during future epidemics [11].

Innate antiviral immunity

The human immune system is comprised of several effector systems, cells and molecules that protect against infection, while also discriminating between self and non-self-antigens. Based on differences in specificity, kinetics and memory capacity, the human immune system is divided into two branches – the innate and the adaptive arms [12].

The innate immune response is the first line of defence against viral infection. Key effector cells involved in the acute viral response are cells of myeloid origin including monocyte subsets as well as granulocytes such as neutrophils, eosinophils and basophils. Lymphoid cells such as plasmacytoid dendritic cells (pDCs), Natural Killer (NK) cells and γδ T Cells are also important players [12].

Pattern recognition receptors

Activation of the immune system is contingent upon its ability to detect antigens via a number of germline encoded pattern recognition receptors (PRRs). These receptors bind to conserved molecular structures on pathogens known as pathogen associated molecular patterns (PAMPs), or to immune activation products derived from damaged or dying tissues or cells known as damage associated molecular patterns (DAMPs) [13].

The four main families of PRRs include the toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) like receptors (RLRs), nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin-like receptors (CTLLRs). The key antiviral receptors include the endosomal TLRs, TLR3, 7, 8 and 9, which bind double stranded (ds) RNA, ssRNA and DNA with CpG motifs respectively (**Table 1.1**) [14].

Members of the RLR family include RIG-I and MDA5 and are essential for innate recognition of viruses and production of type I interferons (IFN-I). RIG-I is located in the cytoplasm and detects ssRNA and dsRNA [15]. MDA5 also detects dsRNA from viruses. Ligation of PRRs induces a signalling cascade that ultimately causes activation of the transcription factors NF-κB and IFN-regulatory factors (IRFs) that bind to response elements in the promoter regions of IFN-I and -III genes thus causing upregulation of interferon production to elicit potent antiviral activity [15].

Toll-like receptor	Ligand
TLR1	Peptidoglycan, lipopeptides
TLR2	Lipoproteins, peptidoglycans
TLR3	dsRNA
TLR4	Lipopolysaccharide
TLR5	Flagellin
TLR6	Lipopeptides (with TLR2)
TLR7	ssRNA
TLR8	Viral, bacterial RNA
TLR9	CpG sequences (DNA)
TLR10	Listeria, IAV

Table 1.1. TLRs and their ligands.

Type I interferons

IFN-Is are highly conserved proteins involved in innate and adaptive antiviral immune responses. In humans, IFN-I is a multigene family of pleiotropic cytokines comprised of 13 IFNα subtypes, 1 IFNβ, and several other less well defined IFN-Is including IFNε, IFNκ, IFNω [16]. IFN-Is are activated by the innate immune system immediately on detection of a threat, particularly when a virus is sensed. This rapid and robust IFN-I immune response which activates and regulates a wide range of biological mechanisms, is required for successful early control of viral infection and is crucial for the activation of long lasting and more specific adaptive immune responses [17]. However, this ability to activate such pleiotropic biological mechanisms means that IFN-I responses, from their initial activation to their ability to induce downstream signalling, requires tight regulatory control mechanisms. Subtle variations to these responses can have marked physiological effects [18,19].

IFN-I is produced in response to ligation of PRRs including the toll like receptors (TLRs) 3, 7/8 and 9, and the DNA/RNA sensors RIG-I, MDA5 and cGAS-STING [20]. These pathways converge to activate the IRF transcription factors, chiefly IRF3 and IRF7 [21]. Binding of dsRNA to TLR3 results in the activation of a downstream signalling pathway involving the adaptor proteins TRIF and TRAF, which activate TANK binding kinase 1 (TBK1) and IκB

Kinase-e (IKKε) activity to phosphorylate and activate the transcription factor IRF3 [22]. Activation of IRF7 can also occur and is required for robust IFN-I production [23]. While IRF3 is expressed at high levels in homeostatic conditions, IRF7 is more lowly expressed, and is induced following ligation of the TLRs 7,8 and 9 through MyD88 signalling [24,25]. IFN-I also activates IRF7, particularly in pDCs; IRF3 is essential for upregulation of IFN-I genes during the early stages of infection and for potentiating the overall IFN-I response via positive-feedback with IRF7. Initial events result in upregulation of IFNβ and IFNα4, which act in a positive feedback loop to upregulate additional IFNα subtypes and interferon regulated genes (IRGs) via IRF7 (**Fig. 1.1**) [24].

pDCs which are found in most tissues of the human body, are the most potent producers of IFN-I [26,27]. Other cell types in the human body are also capable of producing IFN-I, including lymphoid populations and non-immune cells such as epithelial cells, fibroblasts and neurons [28,29]. Canonical IFN-I signalling occurs via a heterodimeric complex composed of IFNAR1 and IFNAR2 and expressed on most nucleated cells in the human body. Ligation of the receptor complex activates the JAK-STAT pathway, which in turn acts to upregulate 1000s of IRGs [30–32]. Other pathways activated via IFNAR1/2 ligation include the MAPK and PI3K pathways, which leads to a broader range of effects yet to be fully elucidated and discussed elsewhere [17,33]. IFN-Is are also critical in shaping the metabolic shift required to mount a successful immune response [34]. IFN-I and IRGs are tightly regulated by negative regulators including IRG15, USP18 and SOCS proteins which act to switch off IFN-I signalling [35]. While IRG induction is important for protection against viral infection as well as certain bacterial and protozoan infections, uncontrolled, or inappropriate IRG activation can lead to the development of several autoimmune disease states [17,36,37].

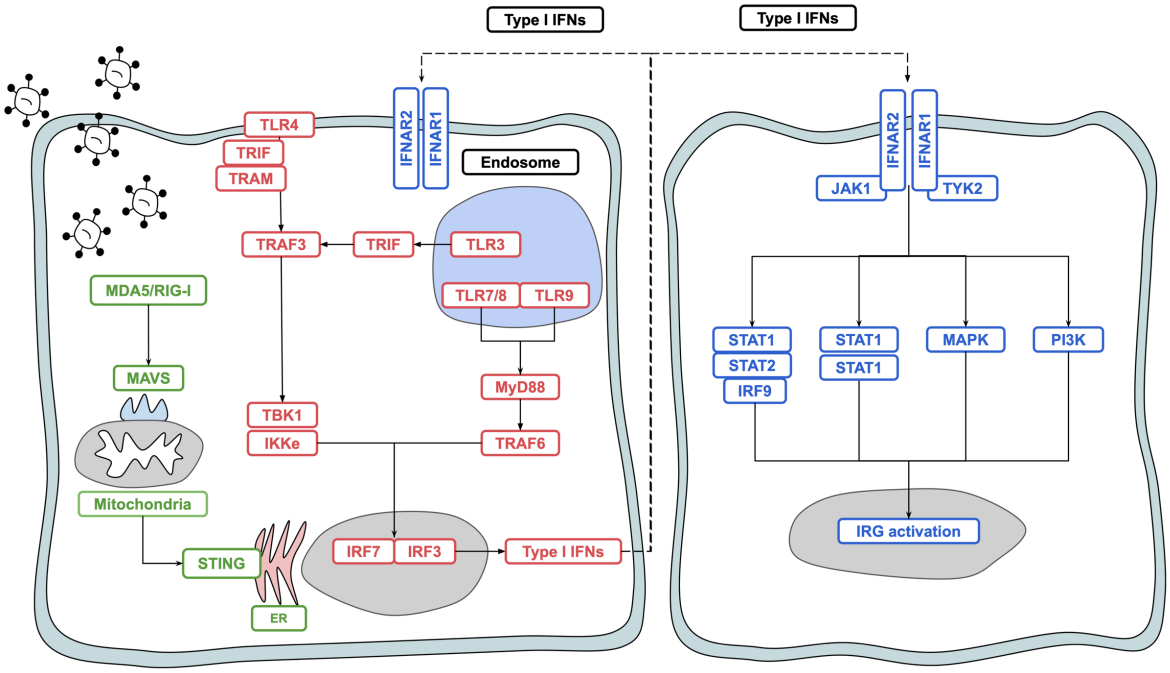


Figure 1.1. Overview of type I interferon induction and signalling. *Activation of PRRs such as TLR4, TLR3, TLR7, TLR8 and RIG-I results in signal transduction and activation of the transcription factors IRF3 and IRF7, leading to production of IFN-I. IFN-I binds to the IFNAR1/IFNAR2 heterodimer and signals in both a paracrine and autocrine manner via the JAK/STAT pathway to upregulate interferon regulated genes which act to protect the host against noxious agents such as viruses and bacteria.*

IFN-I in viral infection

During viral infection, IFN-I exerts both antiproliferative and cytotoxic effects on cells in order to limit viral replication. While it appears that different viruses can upregulate various modules of IRGs, activation of IFN-Is and subsequent signalling appears to be largely similar [20]. Following viral exposure, pattern recognition receptors are activated by double stranded RNA, single stranded RNA, and DNA. Ligation of these receptors triggers a signalling cascade which culminates in the upregulation of IFN-Is followed by IRGs [20]. IRGs can act directly or indirectly and at multiple levels to disrupt the viral life cycle and inhibit viral entry. Several viruses have evolved mechanisms to directly subvert the induction and activity of IRGs, a clear indicator of their importance in impeding viral replication [38].

IFN-I also induces survival and maturation of dendritic cells to enhance antigen presentation and upregulates costimulatory molecules including CD40, CD80, CD86. These cells and molecules act in concert to control viral infection [17]. IFN-I derived from pDCs forms part of an important T and B lymphocyte axis that is key to an adaptive immune response and antibody production [39]. Excessive IFN-I can be detrimental and inhibit or blunt an appropriate antibody response; mechanisms underpinning these observations have yet to be elucidated [17,40–42].

Type II interferons

Although production of IFNγ is restricted to specialised immune cells, its receptor, IFNGR1, is widely expressed [43]. Direct antiviral functions of IFNγ are limited; instead, it promotes an immune response through activation of cellular components of the innate and adaptive arms of the immune system.

Type III interferons

The type III IFNs, consisting of IFNλ1-4 in the human, are expressed in tissues such as the liver and at barrier sites including the mucosal surface of the respiratory tract [44]. They are induced early following pathogen exposure and act to rapidly limit infection. Rapid pathogen elimination also aids in limiting immunopathology associated with prolonged engagement of the immune response. Ligation of the IFNλ receptor, IFNλR1/IL10R2, which is largely expressed on immune cells, leads to activation of the JAK-STAT pathway and upregulation of several hundred IRGs. IFNλ activity suppresses IL17 and IL1 responses [45].

Cellular components of the innate immune response

Production of these antiviral cytokines is facilitated largely by cells of the innate immune system found in both the circulation and in tissues throughout the human body.

Granulocytes

Neutrophils are the most abundant of the granulocyte family. They play an important role in phagocytosis during infection [46]. They also possess the unique ability to extravasate

their DNA matrix studded with proteins such as α-defensin, which can bind to viruses such as HIV-1 and promote viral clearance [46]. It is increasingly apparent that there are several neutrophil subpopulations with differential functions. Discrimination of neutrophils on the basis of high CD16 expression ($CD16^{bright}$) and low CD16 expression ($CD16^{dim}$) may reflect differential maturation states of the neutrophil [47]. In sepsis it has been reported that $CD16^{dim}$ neutrophils have reduced ability to generate ROS and decreased ability to interact with opsonized bacteria, while their $CD16^{bright}$ counterparts show increased antimicrobial activity and increased ROS production [48].

Monocytes

Monocyte subsets also have important functions in the early innate immune response to viral infection. They account for between 5-10% of circulating leukocytes. Like neutrophils, monocytes are professional phagocytes that are important for antigen presentation. Monocyte subsets are defined on the basis of CD14 and CD16 expression [49,50]. The classical monocytes (CD14++CD16-) are the most abundant monocyte subset. In response to inflammatory stimuli have the potential to differentiate into another specialised cell type known as monocyte derived dendritic cells (mDCs) [51]. Intermediate monocytes are defined as CD14+CD16+. Their function is less well understood, but they are known to release high levels of ROS upon activation and are highly inflammatory. The third and final subset are the non-classical monocytes, which are CD16++CD14. This non-classical subset are important for eliciting a proinflammatory response by secreting cytokines in response to insult or injury. They are also important for antigen presentation and T cell stimulation [50]. They mostly differentiate into macrophages in response to inflammatory stimuli. The phagocytic and differentiation potential of monocytes make them important in viral infection for robust activation of adaptive responses by cross presentation [52,53].

Dendritic Cells

There are two main DC subsets in circulation, the conventional DCs (cDC1 and cDC3) which are derived from monocytes or a macrophage/DC precursor (MDP) lineage and the pDCs from the lymphoid lineage. In response to stimulation, DCs upregulate the costimulatory molecule CD86 and the MHC class II molecule HLA-DR, which are often used as surrogate

activation markers [49]. DCs are professional antigen presenting cells (APCs) and are key mediators of antiviral immunity [54]. The major function of cDC subsets is to sense and capture microbes and migrate to the lymphoid organs to prime naive T cells and regulate B cell and NK cell response. cDC priming of T cells is usually by IL-12 which drives T cells to produce IFNγ. cDC1s are potent producers of chemotactic agents and cDC3s are thought to be the most efficient at cross presentation [54]. pDCs have especially important antiviral roles and are the most potent producers of IFN-I in the body, producing 1000 times more IFN-I than any other cell type. This ability to produce such a large of amount of IFN-I is largely dependent on their high expression of TLR7 and TLR9 [54,55].

Natural Killer Cells

NK cells are innate immune cells derived from a common lymphoid progenitor that do not require antigen processing and presentation for activation but instead have the ability to directly kill virally infected or transformed cells [56]. This ability is based on the integration of positive and negative signals from the interaction of NK cell receptors with their cognate ligands on target cells. Activation of NK cells occurs if the balance of signal tips towards activation. Unlike conventional T cells, NK cells express a number of germline encoded receptors such as the killer immunoglobulin like receptors (KIRs) that recognise conserved self and non-self-antigens. Recognition of self induces tolerance, whereas failure to identify self-antigens like MHC class I causes activation - 'missing self' [57].

γδ T Cells

These are unconventional innate T cells with a semi-invariant T cell receptor (TCR) composed of γ and δ glycoprotein chains. In total, γδs account for just 0.5 to 5% of total human leukocytes in circulation. There are two main subsets of human γδs differentiated by the γδ chain - Vδ1 and Vδ2. The Vγ9Vδ2 subset responds to phospho antigens such as HMB-PP and isopentenyl pyrophosphate (IPP) and are important for tumour surveillance and antiviral responses [58]. The non-Vγ9Vδ2s are less well characterised but are thought to exert antiviral activity by responding to stress ligands upregulated on the surface of virally infected cells [59,60].

Adaptive immunity to viral infection

In contrast to early activation of the innate immune response which occurs hours to days after infection, there is a typically a long delay before the adaptive response becomes detectable. This delay is highly variable depending on both the host and virus in question [61]. The adaptive immune response is mediated by B and T lymphocytes and is composed of both cellular and humoral components. In contrast to the innate immune response which relies upon a limited number of germline encoded receptors, the adaptive response has a much larger repertoire of receptors [62]. This is because the antigen receptors of the adaptive immune response are encoded by genes that are assembled by somatic gene rearrangement by germline gene elements to form intact TCR and B cell receptor (BCR) genes. This means that a few hundred genes can result in millions of combinations [62].

B Cells

B lymphocytes are CD19+ and recognise antigen presented by APCs via immunoglobulins expressed on their cell surface as BCRs [49]. The BCR interaction with an antigen induces differentiation of the B cell into a plasma cell, which then sheds the BCR as an antigen specific antibody and undergoes clonal expansion [62]. Only a small number of antibodies produced are able to inhibit virus binding, entry or uncoating and are known as neutralising antibodies.

T Cells

T cell activation requires presentation of antigen by APCs via MHCI/II, co-stimulation by molecules like CD80/86 and cytokine production (**Figure 1.2**) [63]. T cells recognise specific antigen epitopes via the T cell receptor (TCR) co-expressed with CD4 or CD8 (**Figure 1.2**).

CD8+ cytotoxic T lymphocytes (CTLs) are activated via MHC class I and have potent antiviral activity. CD4+ T cells can be skewed to a Th1 or Th2 phenotype. Both phenotypes are important in promoting antibody secretion by B cells, though Th1 states are associated with control of intracellular bacteria and Th2 for controlling parasitic infections [63]. It is possible to have a robust T cell response capable of clearing infection without an accompanying antibody response [64,65].

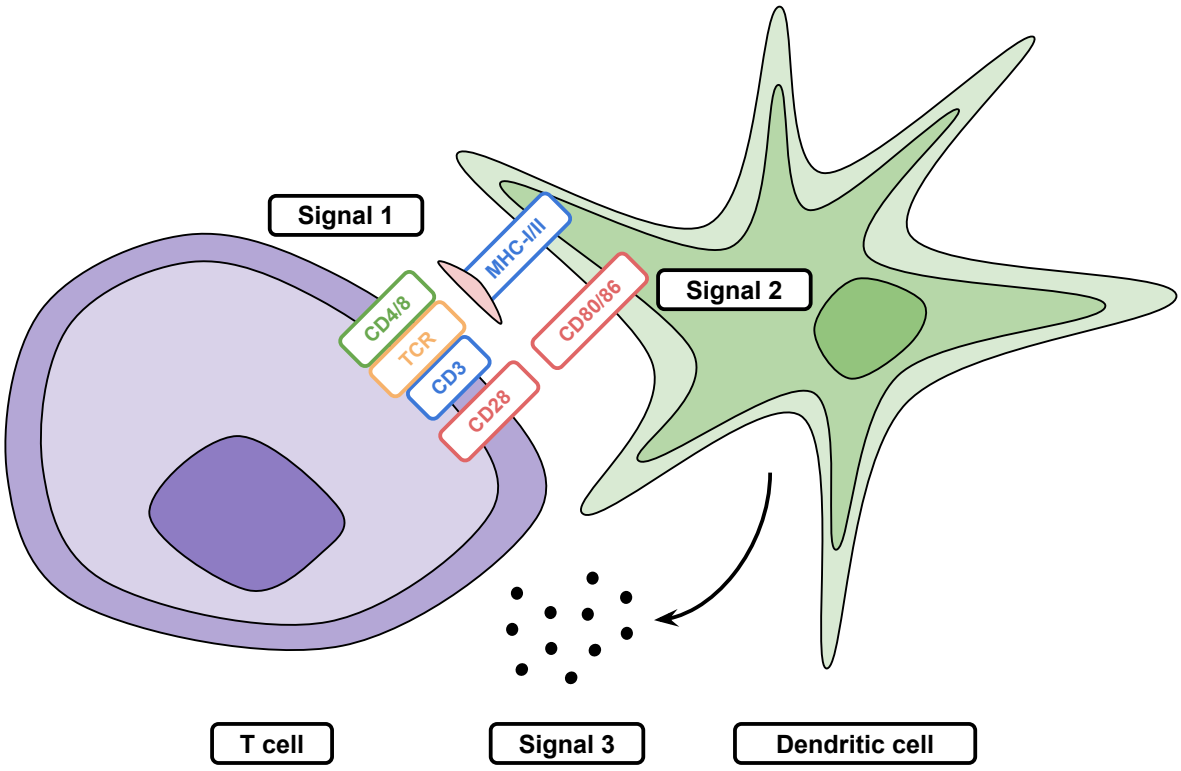


Figure 1.2. T cell activation by dendritic cells. *Signal 1: The CD3:TCR complex and CD8 or CD4 interact with MHC-I or MHC-II – peptide complex. Signal 2: Co-stimulation by molecules such as CD80/86. Signal 3: Cytokines binding to their cognate receptor on the T cell.*

Immune system variation

The human immune response described here exhibits remarkable heterogeneity that is driven by both heritable and non-heritable factors [66]. However, the impact of this heterogeneity on outcome following exposure to pathogens such as viruses is largely unknown. This lack of understanding of immune system heterogeneity and its relationship with clinical outcome during viral outbreak stems, in part, from a dearth of suitable cohorts for which the full spectrum of clinical outcomes are identifiable. During the 1970s however, several documented outbreaks of HCV infection occurred for which records of exposure exist. Outcome following exposure to HCV and the clinical course of those who develop infection vary dramatically.

Hepatitis C virus

30 years since its discovery, HCV still represents a significant public health burden. There are a predicted 70 million people infected worldwide, though only 20% of cases have been diagnosed [67,68]. The current diagnostic approach for HCV is arduous and unsuited to areas like Asia and Africa where HCV is endemic. It is based on serological screening for HCV-specific antibodies, followed by nucleic acid testing to detect presence of HCV RNA (20). HCV is responsible for 260,000 new liver cancers each year and approximately 365,000 deaths per annum. Although curative direct acting antiviral (DAA) drugs have been on the market since 2013, only a small percentage of infected individuals have been treated [69]. The poor treatment rate for HCV is largely due to the huge cost associated with the required medication. As it is a blood borne virus, typical at risk populations include intravenous drug users (IVDUs), men who have sex with men and those exposed through contaminated blood products (nosocomial infections) [70]. There is currently no vaccine for HCV, and the available drugs do not prevent reinfection [71]. Therefore, repeat infections especially in groups such as IVDUs are likely to increase in the absence of a prophylactic vaccine [72]. The World Health Organisation aims to fully eradicate HCV by 2030, a goal that is unlikely to be met if the current climate of HCV diagnosis and treatment doesn't significantly improve [72,73]. Chronic HCV infection is associated with progressive liver inflammation, fibrosis and cirrhosis leading to eventual hepatocellular carcinoma and death in a number of infected patients (**Figure 1.3**). HCV vaccine development is widely held to be a key factor in eradicating HCV globally [74].

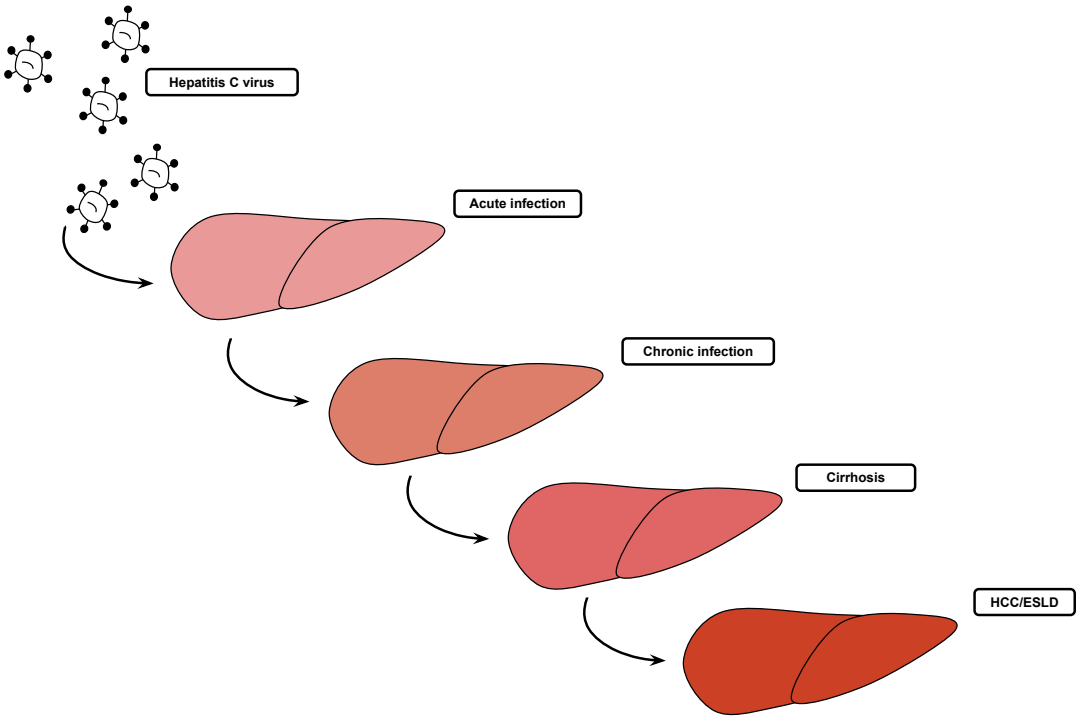


Figure 1.3. Overview of HCV Progression. *Infection with HCV is associated with a variety of outcomes, highly dependent on the genotype. Of those who are exposed to HCV a reported minority innately clear infection. A further 20-50% of infected individuals use their adaptive immune system to clear infection. Up to 80% of individuals exposed develop chronic infection with 10-20% developing cirrhosis and of those, 1-5% developing hepatocellular carcinoma (HCC) or end stage liver disease (ESLD).*

HCV genome and virology

HCV is a positive sense single stranded hepatotropic RNA virus of the Flaviviridae family, to which dengue, West Nile and zika virus also belong. There are seven major HCV genotypes with further subdivisions into 67 subtypes based on the nucleotide sequence of the core and NS5B regions [75]. The most infectious HCV genotype is that which women from the Irish anti-D cohort were exposed to in the 1970s, genotype 1b, and is typically associated with progression to chronic infection in about 90% of cases [75,76]. Other genotypes appear to be less infective with only 30-50% progressing to chronic infection [75]. However, the absence of well documented known infected populations internationally means that these figures may not be accurate.

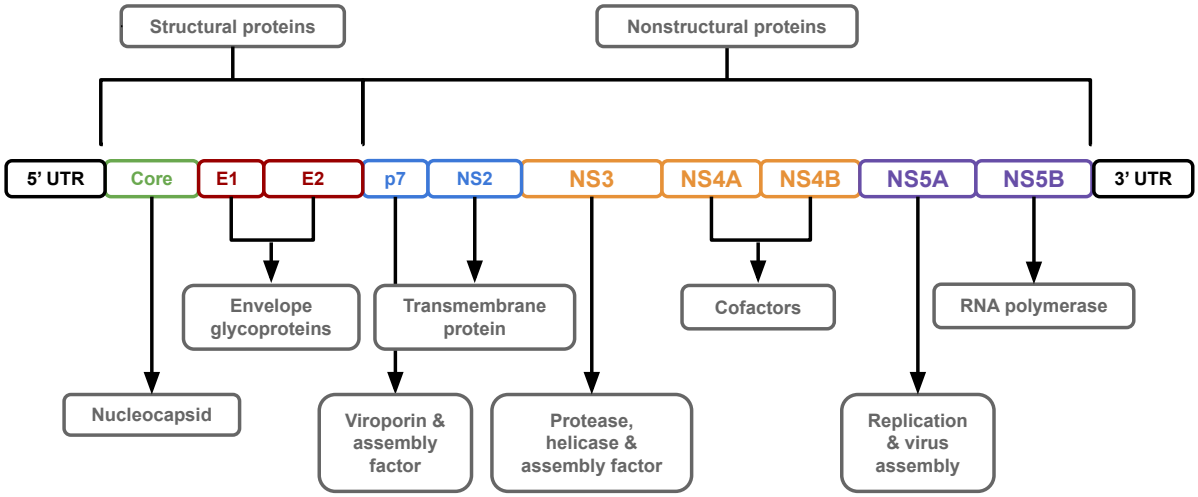


Figure 1.4. The HCV polyprotein. *The HCV genome is flanked by a 5' and 3' untranslated region (UTR) and produces 10 structural (core, E1, E2, p7) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B).*

HCV has a small single stranded genome of about 9600 nucleotide base pairs in length, encoding a large polyprotein of ten viral proteins (**Fig. 1.4**) [77]. The HCV viral genome is composed of a 5' non coding region (NCR), and an open reading frame and a 3' NCR. The 5' NCR contains the internal ribosome entry site (IRES) involved in cap-independent translation of RNA. The structural proteins encoded in the HCV genome are the core and envelope proteins E1 and E2. The non-structural proteins are p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B RNA dependent RNA polymerase (**Fig. 1.4**) [78]. Proteins encoded by the virus have multifunctional and synergistic functions important for replication and survival including ion channel formation, and immune suppression [79]. The HCV NS5A RNA dependent RNA polymerase is highly error prone and contributes to the large degree of genetic diversity seen in HCV. This makes vaccine development for HCV an extremely challenging prospect. It also increases the likelihood that HCV could eventually evolve resistance to therapy, including DAAs [80]. Indeed, reports of this occurring are already emerging [81,82].

The HCV E1-E2 heterodimer is important for HCV entry into the target cell, and is known to bind several receptors including CD81, the high density lipoprotein receptor: scavenger receptor class B type 1 (SRB1), claudin-1, low-density lipoprotein receptor (LDL-R) and

many more. Upon binding to the receptor, HCV enters the cell via clathrin mediated endocytosis and fusion of viral and endosomal membranes, allowing for viral uncoating and introduction of the viral genome into the cellular cytoplasm. HCV RNA then undergoes protein translation and replication [79]. As HCV is a positive sense RNA virus the viral RNA can act directly as an mRNA. Replication occurs on the endoplasmic reticulum and requires formation of a lipid raft and the induction of profound metabolic changes in the host cell [79].

The immune response to hepatitis C virus

The immune response to HCV is multifaceted and complex. Despite HCV RNA being detectable in the blood at just one week post infection, HCV specific immune responses are not detectable for several weeks [61].

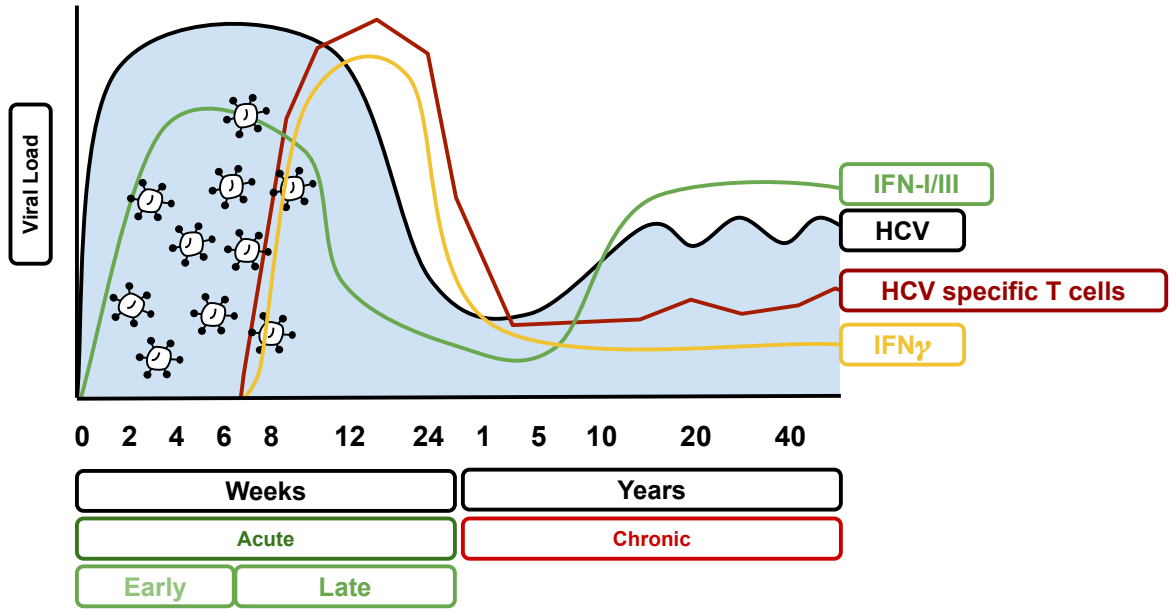


Figure 1.5. Timeline of the immune response to HCV. *Early in infection, the HCV viral load rapidly increases. While IRG upregulation occurs early following exposure, it appears to have limited effect on viral load. HCV specific T cell responses are detectable between 6 to 8 weeks post infection. This is accompanied by an increase in IFNγ production and a rapid reduction in viral load.*

Hepatocytes detect HCV infection primarily though the receptors TLR3, MDA5 and RIG-I and upregulate IFN-I and IFN-III (**Fig. 1.6**) [83]. The importance of PRRs in the detection of HCV is reflected by the role of SNPs in TLR9 in spontaneous resolution of HCV [84]. The IFNs can act in both an autocrine and paracrine manner to upregulate IRGs early in infection. IRGs induced by IFN-I and -III such as OAS1 and PKR attempt to limit HCV infection. PKR, a serine/threonine kinase plays an important role in inhibition of HCV replication through phosphorylation of the elF1α complex, which inhibits host protein synthesis [85].

Production of the IFN cytokines also results in activation of NK cells, which are highly enriched in the human liver and likely play an important role in control of HCV and other hepatotropic viruses. Activated NK cells produce IFNγ and TNFα which can inhibit HCV by limiting spread of infection to neighbouring cells [86,87].

Myeloid populations in the liver such as Kupffer cells are also activated and produce cytokines such as CXCL10 and IFNγ to limit HCV infection. Vγ9Vδ2 unconventional T cells have been shown to inhibit HCV replication *in vitro,* and low frequencies of Vγ9Vδ2 in circulation have been associated with HCV persistence and lower IFNγ production [88]. Other pro-inflammatory cytokines have been shown to be important in protection against HCV, including interleukin-6 (IL-6) and interleukin-8 (IL-8), which have both been associated with resistance to HCV infection [89]. Chemokines such as IL-8 are important in recruiting peripheral immune cells such as neutrophils from the blood to the site of infection [90].

Bridging the innate and adaptive immune responses to HCV are the DC populations. pDCs detect HCV in a TLR7 specific manner through transfer of exosomes containing HCV viral RNA from an infected cell to the pDC to produce IFN-I [91]. Frequencies of pDCs in the blood correlate well with infection outcome [92].

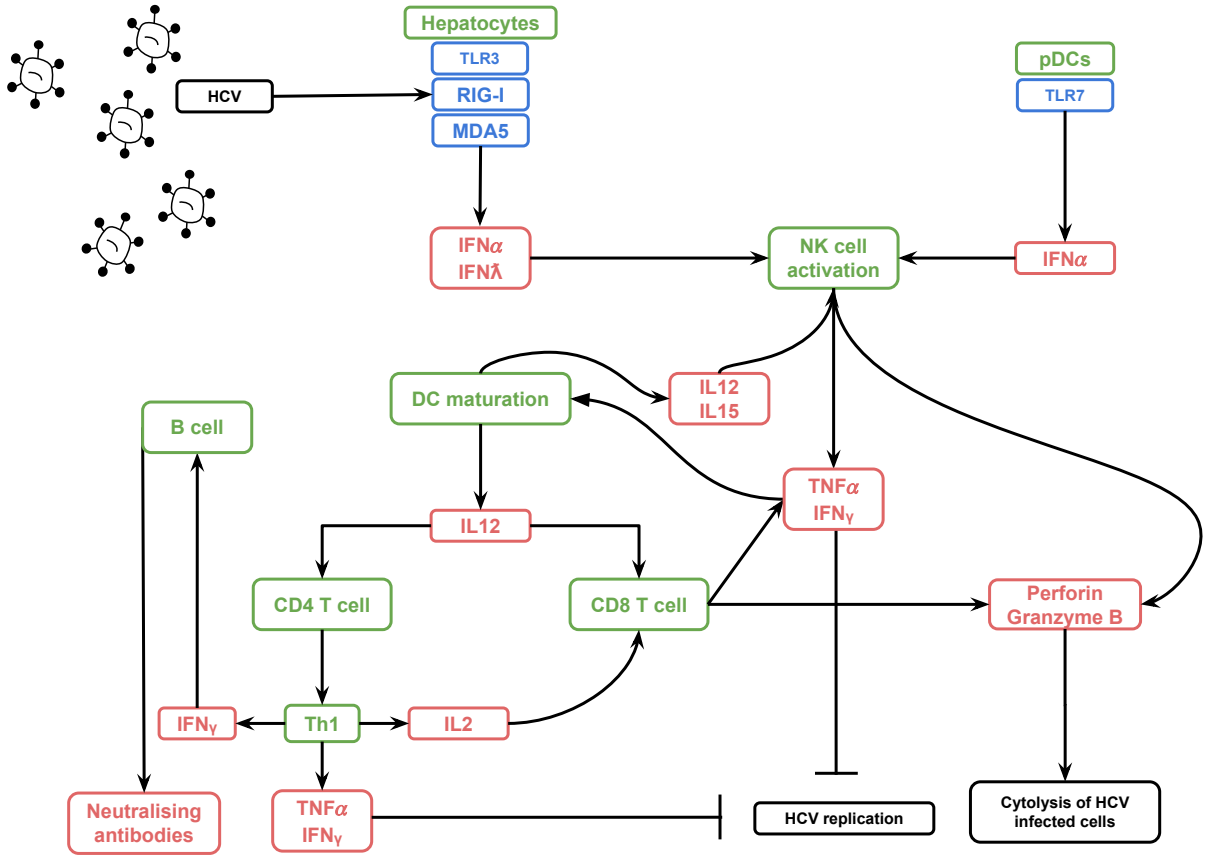


Figure 1.6. Overview of the innate and adaptive immune response to HCV. *HCV infection is detected by hepatocytes expressing RIG-I, TLR3 and MDA5, which activate downstream signalling pathways to produce IFN-I and -III. IFN-I is also produced by pDCs expressing TLR7. IFNs can inhibit HCV replication and activate NK cells to produced TNFα and IFNγ, which can also limit HCV replication and induce maturation of other DC subsets to produce IL-12 and IL-15, which act in a positive feedback loop to increase NK cell activity. Production of granzyme B and perforin by NK cells kills virally infected cells. CD4+ T cells support Ab production by B cells through IFNγ production. IFNγ produced from T cells can also inhibit HCV replication. IL2 produced by CD4+ T cells supports CD8+ T cell activities, which include production of TNFα and IFNγ to inhibit HCV replication and perforin and granzyme B to lyse virally infected cells. Cellular components are in green and cytokine components are in red.*

Adaptive immunity in HCV

B cells in HCV

In acute infection most infected individuals produce antibodies against epitopes in the structural (core, E1 and E2) and non-structural proteins (**Fig. 1.4**), however the majority of these antibodies are non-neutralising and fail to inhibit viral binding, entry or uncoating [93]. The importance of antibodies in HCV infection is not clear, reports suggest production of neutralising antibodies early in infection is associated with spontaneous resolution of HCV. However, virus clearance has also been described in the absence of antibodies and in patients with hyperglobulinaemia [61,94]. Other studies indicate that broadly neutralising antibodies (bnAbs), those with the ability to neutralise multiple HCV genotypes, correlate well with infection outcome [95,96]. However, HCV patients often fail to produce bnAbs in a substantial quantity. The role of B cells themselves, not just their effector molecules, remains obscure and is the subject of ongoing research, in particular work is focused on why B cells fail to produce substantial levels of bnAbs during HCV infection in the hope that greater insight will further HCV vaccine efforts [74,97].

T cells

While the importance of B cells and antibodies in HCV is somewhat nebulous, there is a clear link between a potent T cell response to HCV infection and successful viral clearance. DCs and other APCs present peptides via MHCI or MHCII to CD8+ or CD4+ T cells. HCV specific T cell responses are usually detected no earlier than 6-8 weeks following initial infection. This delay appears to be a feature of hepatotropic viruses in general, not just HCV [98,99]. Temporally, the emergence of HCV-specific T cells is linked to a dramatic reduction in viral load and onset of liver disease. CD4+ T cells support the antiviral activity of CD8+ T cells (**Fig. 1.5**) [83,98]. Both cell types adopt an activated state and increase PD-1, CD38 and CTLA4 expression. Following successful viral clearance and consequent cessation of antigenic stimulation, CD4 and CD8+ T cells upregulate memory markers including CD127 and downregulate marks of activation [100]. HCV specific T cells can persist for decades following viral clearance [101]. Despite the presence of HCV specific T memory cells, re-infection is possible due to the emergence of escape mutations in the virus [102].

Hepatitis C virus immune evasion

Though humans are capable of eliciting a potent immune response, only a minority of individuals successfully clear HCV infection, either innately or adaptively. In part, this is due to HCV's ability to evade and suppress the intended immune response [38]. HCV proteins can interfere with many aspects of the immune system. NS3 and NS4A can inhibit TLR3 signalling by cleaving the molecule TRIF, thus preventing NFκB and IRF3 activation and subsequent IFN-I production. As shown by our group, HCV functions as an E3 ligase and induces degradation of STAT proteins, therefore limiting interferon signalling. This is particularly pronounced in HCV genotype 1b [38]. HCV proteins such as NS5A and E2 can target proteins derived from IRG upregulation such as PKR and OAS [103]. HCV proteins also stimulate IL-10 and TGFβ production by monocytes, thereby driving a T regulatory response [104].

HCV treatment and vaccine development

While some individuals are able to clear HCV infection without medical intervention, a substantial number of individuals progress to chronic infection and require treatment. Historically HCV infection was treated with a combination of pegylated IFNα (peg-alfa) and the antiviral drug ribavirin, a synthetic nucleoside analogue of ribofuranose. Exogenous peg-alfa attempted to boost the host antiviral immune response to clear HCV infection, while ribavirin was incorporated into HCV viral RNA and inhibited RNA synthesis and inhibiting normal virus replication [105]. This early treatment regime for HCV was poorly tolerated and elicited favourable results in a minority of individuals following prolonged treatment (~30%) [106]. Treatment side effects included retinopathy, depression, increased risk of developing autoimmune diseases to name but a few [107]. Stratifying patients using biomarkers of likely responders and non-responders was a priority for researcher in the early 2000s. Since 2010 however, novel DAA drugs have been widely available. These drugs function by directly inhibiting the action of non-structural proteins, and have revolutionised HCV treatment- a typical dosing regime is just 12 weeks with a cure rate of 95-100% with few reported adverse events [108]. Successfully treated individuals are said to be sustained virological responders (SVR).

Efforts to develop HCV vaccines have been hampered by the vast diversity seen in HCV, owing to the error prone RNA polymerase, coupled with differential pressures exerted by the host immune responses driving further quasi species and diversity [109,110].

Immune scarring following HCV clearance

The advance in HCV therapy using DAAs has created new opportunities to study resolution of chronic infection following treatment with a well-tolerated and standardised therapy. Though successful at clearing infection, the restoration of immune homeostasis following therapy is heterogeneous and reports are conflicting [111]. Chronic HCV infection results in widespread immune dysfunction characterised by increased expression of exhaustion markers on immune cell subsets, increased proinflammatory cytokines at baseline and a compromised ability to respond to infection .

Reports suggest that innate immune restoration occurs following treatment with DAAs [112]. Following therapy, T cell subsets from some patients appear to return to a homeostatic norm, characterised by a reduction in exhaustion markers [113]. Metabolically however, some reports indicate that mitochondria in T cells regain normal functionality, while others suggest persistent defects [114]. In chronic HCV infection, expansion of Treg cells occurs, and these cells do not appear to return to normal levels following clearance of infection, though patients were only followed for one year post therapy so it is possible that complete restoration occurs at a later timepoint [115].Normalisation of IFN-I signalling also appears to occur in T cells, as indicated by a reduction in the IRGs IRG15 and OAS1 from single cell RNA-sequencing experiments [116]. $\gamma\delta$ T cells have an activated phenotype in chronic HCV infection and a reduced ability to respond to stimulus- the persistently activated phenotype appears to be alleviated following loss of HCV antigen stimulation, but the stunted cytokine response appears to persist [117].

A significant loss of MAIT cells occurs during chronic HCV infection. Interestingly, and as described for many other viral infections, MAIT cells do not seem to recover fully following viral clearance, although some functional restoration has been described [118–120]. The consequences, if any, of this failure to recover MAIT cells are not yet known.

The Irish anti-D cohort

Viral transmission that occurs during the course of medical examination or treatment, referred to as iatrogenic infection, was a significant contributor to the historical spread of HCV infection worldwide during the 1970s, 80s and 90s, prior to the identification of HCV in 1989 [121,122]. The lack of specific molecular or immune-based detection assays for HCV resulted in several instances of transmission due to the medical use of contaminated blood or blood-derived products, most frequently in haemophiliac populations, and iatrogenic transmission remains a risk in both developing and developed countries[123,124]. The introduction of effective screening for HCV in the 1990s resulted in disappearance of iatrogenic HCV infections across the globe. Those at high risk of infection with HCV nowadays are intravenous drug users who exchange needles [125].

With the development of diagnostic assays specific for HCV, several countries initiated large scale studies to identify and test individuals exposed to infectious viruses like HCV via contaminated blood donations or blood-derived products [73,126,127]. These studies attempted to trace the recipients of HCV-positive blood donations and blood-derived products, screening all individuals who were potentially exposed to these products. In doing so, these studies identified a spectrum of outcomes following HCV exposure that ranged from resistance (exposed seronegative; ESN), spontaneous resolution (SR) and chronic infection (CI; **Fig. 1.7**)[126].

In Ireland, a major iatrogenic transmission event occurred between 1977-1979. The outbreak centred on the distribution of HCV contaminated anti-D immunoglobulin (Ig) preparations to pregnant Irish Rhesus-negative women [128–130]. Prophylactic anti-D Ig is administered to mitigate the risk of haemolytic disease of the foetus and new-born, which can lead to foetal anaemia, jaundice, and stillbirth [131]. The Irish anti-D Ig HCV outbreak occurred following the use of HCV-infected plasma from a single donor who had symptoms or risk factors indicative of potential HCV infection [130].

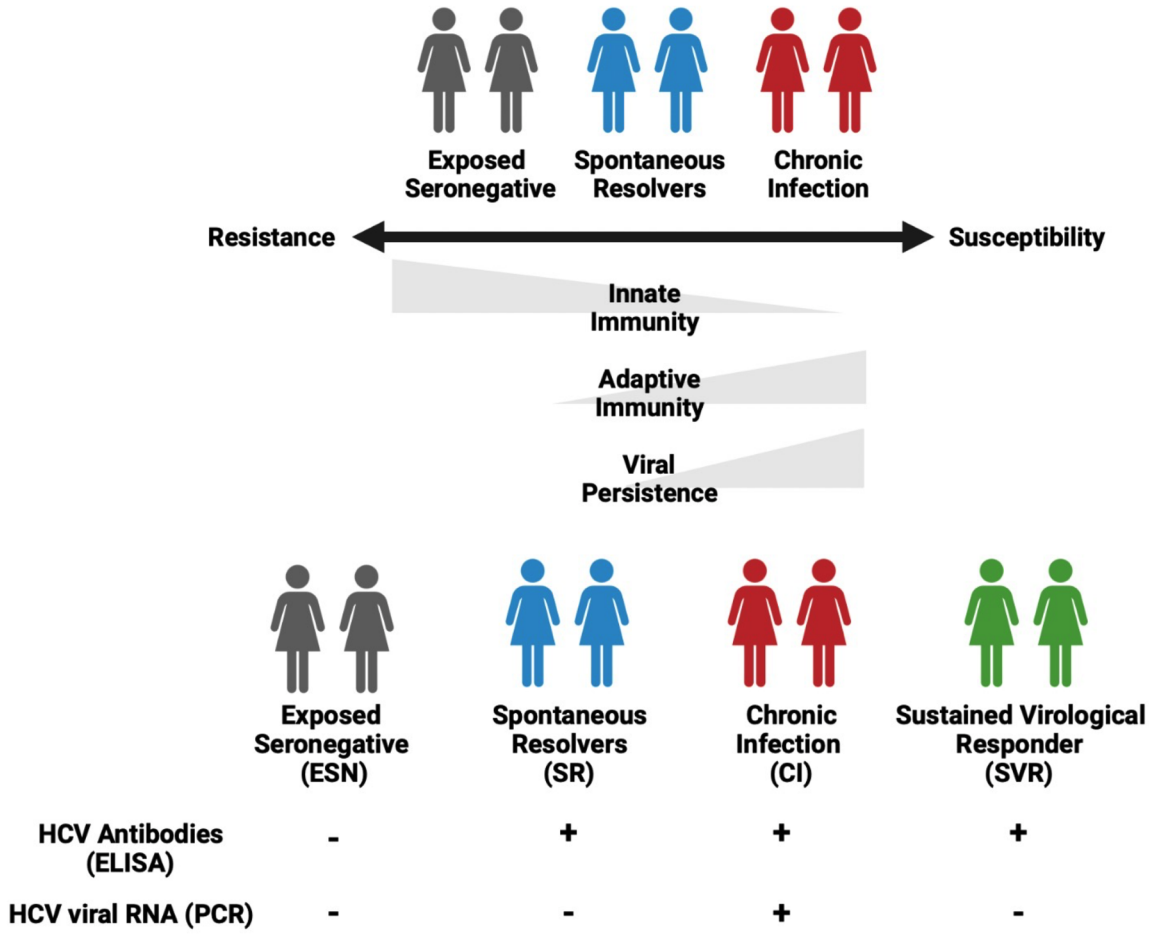


Figure 1.7. Spectrum of outcome following exposure to HCV. *50-80% of individuals exposed to HCV progress to chronic infection, many clear infection and some do not become infected. Those who successfully clear infection via an innate immune response are known as exposed seronegative individuals (ESNs), while those who clear infection through engagement of their adaptive immune systems are known as spontaneous resolvers (SR). Those who were chronically infected but successfully clear infection following treatment are known as sustained virological responders (SVRs). ESN = HCV Ab-negative, HCV RNA-negative; SR = HCV Ab-positive, HCV RNA-negative; Chronic infection = HCV Ab-positive, HCV RNA-positive; SVRs = HCV Ab-positive, HCV RNA-negative (following treatment).*

The anti-D cohort as a model to understand HCV infection

Since the widespread recognition of the Irish HCV outbreak in the early 1990s a significant body of research has been undertaken. This includes almost 70 primary peer-reviewed research articles published in leading international academic and clinical journals

including Gut, NEJM, The Lancet, Hepatology, PNAS, and Gastroenterology. These studies have contributed to an epidemiological evidence-base that has fundamentally changed how HCV infection is managed in clinical settings worldwide as well as providing a detailed understanding of the on-going evolution of the HCV genome and the role that host factors play in influencing the outcome of infection (**Table 1.2**). As HCV replicates poorly in culture, studies of high risk cohorts such as these have been paramount in shaping our knowledge of HCV and HCV infection.

Study Type	Major Research Findings
Clinical & Molecular Epidemiology	The HCV sequence in Irish anti-D Ig-recipients arose from a single-strain, distinct from circulating HCV strains in Ireland, confirming single-source outbreaks due to the use of contaminated blood in the preparation of anti-D Ig [132,129]
	The development of liver pathology and HCV disease progression is slow in the absence of additional life-style risk factors [126,133]
	There is a low risk of transmission to children born around the time of their mother's infection but transmission to other family-contacts is less likely [134]
Viral Genetics	Immune pressure is the dominant driver of viral evolution early in infection while evolution towards a consensus ancestor sequence (with higher fitness) is dominant in the late stages of chronic infection[135]
	The major HCV genotypes were estimated to have diverged between 500–2000 years ago, requiring on-going transmission of HCV in historical human populations [136]
	Evasion of host immune pressure comes at a significant cost to viral fitness and following transmission events the virus reverts to a consensus sequence with higher fitness[137]
Infection & Immunity	The HLA-DRB1*01, -DQB1*0501, -DRB1*0401, HLA-DRB1*15, -A*03, -B*27, and -Cw*01 alleles are associated with spontaneous resolution of HCV infection[138,139]
	Immune pressure requires both a protective host HLA allele and a specific viral immunodominant epitope – the HLA-B*27 allele is only effective in genotype 1 HCV infection and is neutral when the infectious strain harbours escape variants[140–142]
	Polymorphisms in innate immune genes (*IFNL3*, *KIR2DS3* and *IFIH1*) are associated with spontaneous resolution of HCV infection[143,144]

Table 1.2. Major findings from the Irish Anti-D Cohort.

Uniqueness of the Irish anti-D cohort

Animal studies of viral infection require giving a defined amount of a defined infectious agent at the same time to all study participants. Studies that emulate this set up in humans (challenge trials) such as these are contentious and rare [145]. However, the Irish anti-D-related HCV outbreak replicates key criteria of the ideal infection study. These key aspects have provided a silver-lining to this national tragedy, enabling research that has fundamentally changed how we view and investigate HCV infection world-wide. The 1977-

79 outbreak was linked to a single HCV-infected individual, whose blood donation was used in the preparation of anti-D Ig [130]. Viruses of the single, contaminating HCV strain were distributed amongst hundreds of batches of anti-D Ig, and administered to thousands of Irish women (**Table 1.3**). All the exposed individuals were women of child-bearing age and were predominantly of an Irish ethic origin. Collectively this group of individuals were in relatively good health, with low rates of alcohol consumption, and few lifestyle risk factors associated with HCV transmission [146]. These factors resulted in a highly homogeneous cohort of individuals with similar exposure histories and lengths of infection.

	1977-79 Outbreak
No. of contaminated batches	12 (4062 vials)
No. of high risk batches (>30% Ab+)	6
Genotype of HCV involved	1b
No. of chronically infected women from high risk batches	356
No. of spontaneous resolvers from high risk batches	326
No. of potentially resistant (ESN) women from high risk batches	611

Table 1.3. Details of the HCV 1977-79 outbreak from Irish Blood Transfusion Service (IBTS). (Anon. 2012. *National Hepatitis C Database for Infection Acquired through Blood and Blood Products: 2012 Report*. Retrieved (http://www.hpsc.ie/AZ/Hepatitis/HepatitisC/HepatitisCDatabase/BaselineandFollow-upReports/).

A total of 4,062 batches of potentially-contaminated anti-D Ig were produced during the 1977-1979 outbreaks (**Table 1.3**) [129,147]. There was also significant variability in infectivity amongst different anti-D Ig batches associated with the 1977-79 outbreak; six batches in particular were highly infectious [147]. Amongst those individuals that became chronically infected it has been estimated that 13.9% have signs of liver disease, 11.3% have liver cirrhosis, 1.2% have developed liver cancer or HCC, and 2.6% have died from liver disease [148].

Previous infection and immunity studies of HCV from the anti-D cohort

The impact of host genetics on viral infection and the host immune response is difficult to assess. Clinical heterogeneity (such as ethnic backgrounds, age at infection, viral genotype, and mode of infection) hampers direct comparisons between human cohorts [149]. In this sense the Irish anti-D Ig HCV outbreak provides a unique cohort of homogeneous individuals, and allows the investigation of host immune factors without a number of confounding factors that influence immune responses.

The Irish anti-D Ig HCV outbreak was ideal to perform studies investigating genetic associations between immune-related genes and spontaneous resolution of HCV infection. The first study of this type focussed on the human leukocyte antigen (HLA) complex, which encodes a highly variable array of important antigen-presenting molecules that are known to influence host response to infection. The HLA-DRB1*01 allele was associated with individuals who spontaneously cleared the virus compared to those who developed chronic infection (27.4% vs. 7.1%, *p*=0.001, odds ratio OR=4.9) [138]. Subsequent studies also identified associations between the HLA-DRB1*0401, HLA-DRB1*15, HLA-DQB1*0501, HLA-A*03, HLA-B*27, and HLA-Cw*01 alleles and viral clearance [139,150]. Of all the alleles HLA-B*27 had the strongest association (odds ratio [OR] 7.99; **Table 1.2**) [150].

This HLA-B*27 allele allows the presentation of a single immunodominant viral peptide epitope (spanning residues 421–429 of the NS5B protein) to CD8+ T cells, yet despite the strong immune pressure, escape variants are rarely seen [140]. Artificial variants that disrupted HLA-B*27 binding could completely abolish T cell cross-recognition however these viral variants showed a dramatic reduction in replication efficiency [140]. The protective HLA-B*27 allele is dependent on viral genotype; non-genotype 1 HCV strains lack the consensus immunodominant HLA-B*27 epitope and the protective influence of the HLA-B*27 allele is lost [141]. Certain genotype 1b HCV strains also harbour immune escape variants in this epitope and the HLA-B*27 allele has no protective influence during infection with these viral strains [151]. By comparing multiple individuals with the same HLA alleles it is possible to identify a 'molecular footprint' of immune pressure and identify novel epitopes [152,153]. This information is particularly important for the development of

HCV vaccination strategies to generate immune responses specific for immunodominant epitopes that will be recognised by the majority of the vaccinated population.

While the HLA complex is a major determinant of susceptibility, other polymorphisms in immune-related genes, which correlate with the outcome of HCV infection, have been identified from the Irish anti-D Ig HCV outbreak. Dring and colleagues investigated the impact of NK cell-associated killer cell Ig-like receptors (KIR) genes as well as a SNP near the type III IFN gene, IFNλ3 (previously IL28B; rs12979860) [143]. They found the NK cell gene KIR2DS3 and the IL28B 'T' allele were associated with chronic infection (odds ratio (OR) 1.90 and 7.38, respectively) [143]. Hoffmann and colleagues investigated polymorphisms in IFIH1 (interferon induced with helicase C domain 1; also known as MDA-5), an intracellular receptor which recognises viral entry into a host cell [144]. The IFIH1 signalling pathway is critical for the recognition of HCV infection and is suppressed by the virus. Two polymorphisms in the IFIH1 gene correlated with spontaneous resolution of infection, due to an increased antiviral response [144]. While substantial information has been garnered from studying these women on the SRs and CIs, the ESNs in this cohort have never been studied.

Thesis rationale, hypothesis and objectives

Outcome following exposure to hepatitis C virus is highly variable, however the reasons for this variability are poorly understood. A major outbreak of hepatitis C virus infection occurred in Ireland in the 1970s, in which 1000s of otherwise healthy females were exposed to HCV. Three groups were identifiable from the population – those who were exposed and remained seronegative (ESN), resisting infection, those who spontaneously resolved infection (SR) and those who developed chronic infection (CI). Here we sought to recruit these women to our study to further understand the genetic and biological mechanisms that might have contributed to the variable clinical outcomes described. We hypothesised that a particularly potent innate immune response protected ESN women against infection. We hypothesised that common SNPs in the innate immune system contributed to this particularly potent innate immune response. As the rates of apparent resistance were high in women from Irish anti-D cohort, all of whom were Rhesus negative, we hypothesised that RhD-negativity is protective against viral infection.

Overall objective:

To test the innate immune response of HCV ESNs and virus susceptible (SR and SVR) women to understand correlates of virus resistance in the Irish anti-D cohort.

The specific aims of this thesis were:

1. To assess the whole blood innate immune response in ESN women compared with SR and SVR donors. To test the absence of a detectable adaptive immune response in ESN women in our cohort.
2. To compare the peripheral whole blood immune response in SR and SVR donors.
3. To investigate potential genetic associations with resistance using a gene candidate approach.
4. To determine whether Rhesus antigen status is associated with an altered innate immune response.

Reagent	Supplier
Bovine serum albumin (BSA)	VWR Chemicals
Dimethyl Sulfoxide (DMSO)	Sigma
Ethanol	Sigma
Ficoll-Paque	GE Healthcare
HBSS	Sigma-Aldrich
Penicillin-streptomycin	Gibco Invitrogen
RPMI 1640 + L-glutamine	Gibco Invitrogen
Sodium azide	Sigma-Aldrich
Trypan blue	Sigma
TriZol LS	Thermo Fisher
Quantiflour RNA system kit	Promega
2D barcoded tubes	Thermo Fisher
Tween 20	Sigma
Phosphate buffered solution	Gibco Invitrogen

Table 2.1. List of general reagents used.

Reagent	Supplier
10 ml plastic pipettes	Cruinn Diagnostics (Grenier)
24-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)
25 ml plastic pipettes	Cruinn Diagnostics (Grenier)
5 ml plastic pipettes	Cruinn Diagnostics (Grenier)
Cryovials	Thermo Scientific Nunc
Nalgene™ cryo 1°C freezing container	Thermo Scientific Nunc
Polystyrene FACS tubes	BD Biosciences
Red blood cell lysis buffer	Sigma
RPMI 1640 + L-glutamine	Gibco (Biosciences)
Sterile phosphate buffered saline (PBS)	Gibco (Biosciences)

Table 2.2. List of cell culture reagents used.

Equipment	Company
BD Fortessa	BD Biosciences
Graphpad Prism	Graphpad Software Inc
FlowJo	Treestar Inc
NanoDrop 2000 spectrophotometer	Thermo Fisher
StepOnePlus RT-PCR Machine	Applied Biosystems StepOnePlus
Ncounter digital analyser	NanoString
NanoString prep station	NanoString
Luminex MAGPIX	Luminex
Sioma Analyser	Quanterix
Agilent 2100 bioanalyzer	Agilent
AID ELISpot Reader	Autoimmun Diagnostika

Table 2.3. List of equipment and software programs used.

Reagent	Supplier
Millipore MAIP plates	Merck, Germany
Human IFNγ ELISpot Kit	Mabtch, Sweden
HCV peptide pools	ProImmune, Oxford
CEF peptide pools	ProImmune, Oxford
Concanavalin A	Sigma
BCIP/NBT	Sigma

Table 2.4. List of ELISpot reagents.

TruCulture	Stimulant	Concentration	Supplier
Null/Negative Control	NC	---	MyriadRBM
R848	R848	1μM	Invivogen
Poly I:C	PIC	20μg/ml	Invivogen
Interferon α_{2b} (Intron A)	IFNα	1,000 IU/ml	Merck
ODN2216/CpG-A	ODN	25μg/ml	Invivogen

Table 2.5. List of stimuli used for TruCulture.

Antibody	Conjugate	Catalogue Number	Supplier
CD45	BV510	563204	BD Biosciences
CD19	APC-Cy7	557791	BD Biosciences
CD3	AF700	344822	MSC
CD4	AF488	317420	MSC
CD8α	BV785	301046	BioLegend
CD56	BV650	364057	BD Biosciences
CD14	BV421	301830	MSC
CD16	PeCy7	557744	BD Biosciences
Live/Dead	Live/Dead Red	423109	MSC
BDCA1/CD1c	FITC	331518	MSC
BDCA2/CD303	PerCP-Cy5.5	354210	MSC
CD86	PE	305438	MSC
HLA-DR	V500	561224	BD Biosciences
BDCA3/CD141	BV711	563155	BD Biosciences
BDCA4/CD304	APC	354505	MSC
Compensation beads	BV711	563155	BD Biosciences
CountBright count beads	---	C36950	Invitrogen

Table 2.6. List of reagents, antibodies and fluorochromes used for flow cytometry.

Ethics

Individuals enrolled in this study provided written informed consent in line with the Declaration of Helsinki. Ethical approval for the study was granted by the Trinity College Dublin Faculty of Health Sciences Ethics Committee.

Classifying batches as low and high risk

Twelve batches of anti-D were made in 1977-1979 containing serum from a single HCV infected donor. Records are only available on 64% of vials. The batches had varying viral loads [147]. Six of the batches: 246, 238, 245, 237, 252 and 250 had significant levels of virus, ranging from 12,400 genome equivalents per vial to 200,000. Vials from these batches accounted for 98% of the chronic infections, and so were deemed high risk (**Table 2.7**).

Batch	Genomes/ Vial	Vials Issued	Vials Traced	People Tested	People Tested -ve	Ab +ve	PCR +ve
239	<400	372	131	117 (31)	116 (99)	1 (1)	0
244	<400	259	156	134 (52)	129 (96)	5 (4)	0
247	<400	305	197	165 (54)	153 (93)	12 (7)	3 (2)
243	<400	332	217	191 (57)	187 (98)	4 (2)	0
251	<400	273	139	115 (42)	110 (96)	5 (4)	1 (1)
240	9,600	401	274	224 (56)	219 (98)	5 (2)	1 (1)
246	12,400	251	152	133 (53)	92 (69)	41 (31)	12 (9)
238	16,000	371	281	258 (70)	70 (27)	187 (73)	106 (41)
245	56,000	464	347	302 (65)	201 (67)	101 (33)	60 (2)
237	92,000	430	247	225 (52)	64 (28)	160 (71)	75 (33)
252	100,000	308	216	191 (62)	73 (38)	115 (60)	60 (31)
250	200,000	296	228	191 (65)	111 (58)	78 (42)	43 (23)
Total		**4062**	**2585 (64)**	**2246 (55)**	**1525 (68)**	**714 (32)**	**361 (16)**

Table 2.7. Details of infected batches from the Irish Blood Transfusion Service. *Table showing the number of HCV contaminated vials of anti-D issued and traced. The viral load associated with each batch is also indicated. The number of women who were traced and tested are shown, along with the numbers of women who tested antibody (Ab) or PCR positive. Figures in brackets are percentages.*

Recruitment of women from the Irish anti-D cohort

Ethics did not permit direct contact with donors via the IBTS, therefore a national recruitment campaign for this study was launched in 2017, followed by an additional social media campaign in 2018. Researchers appeared on national and local television and radio as well as in print newspapers, asking for women who received contaminated anti-D between 1977-79, regardless of their infection status, to contact us. Together with the more traditional media campaign, an online campaign was also launched, which included a website (viralresistanceproject.com) as well as Twitter and Facebook pages. An overview of the recruitment campaign is shown in **Fig. 2.1**.

Matching recruited donors to their IBTS records

Once individuals had contacted us about participation in the study, they were screened by phone. Those who met the study criteria by being Rhesus -negative, having received anti-D immunoglobulin between 1977-79, and agreeing to participate, were sent a study pack via the postal service. The pack contained a consent form, patient information leaflet and saliva collection kit (Isohelix, Cell Projects Ltd., United Kingdom). When the pack was returned with a completed consent form, the Irish Blood Transfusion Service (IBTS) was contacted on behalf of the recruited study member to access their anti-D records and batch information. Exposure data and batch information for each volunteer were sought. Where available, details of each volunteer's anti-D vial were identified and each individual was stratified as either "high risk" if they received a vial from one of the highly infectious batches: 246, 238, 245, 237,252 and 250, or as 'unexposed' if they received a vial of anti-D from an uncontaminated batch. Volunteers for whom no data were available were excluded from the study.

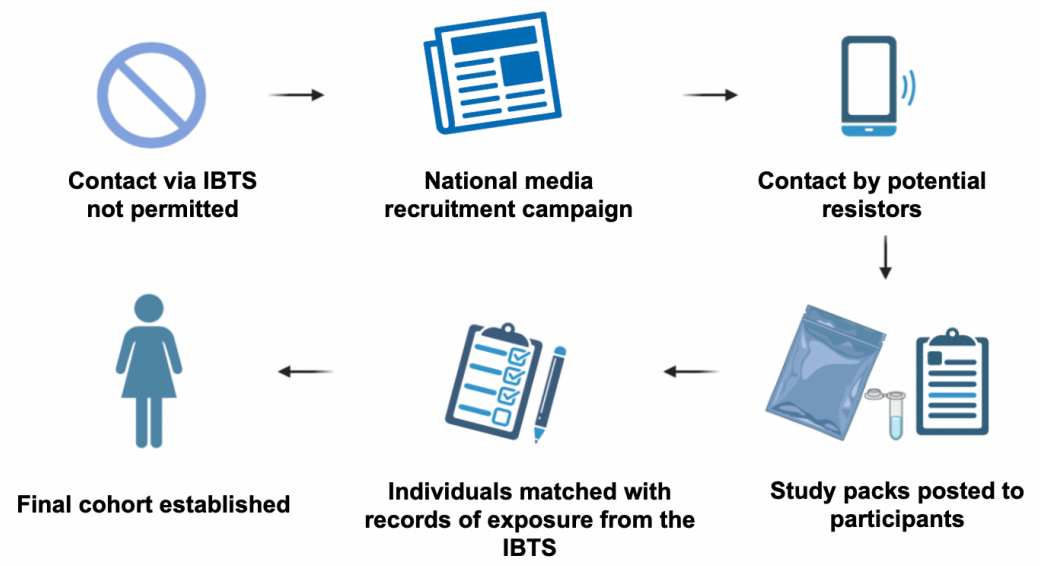


Figure 2.1. Overview of recruitment campaign methodology.

Questionnaires

Included in the study pack posted to each potential participant was a questionnaire. This was used to gather basic clinical and demographic information on donors before being enrolled in the cohort.

Biological Analysis

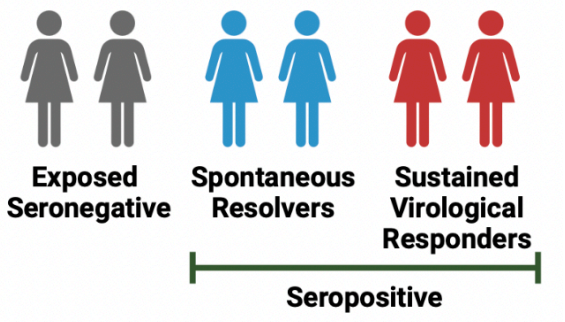


Figure 2.2. HCV infection groups and potential groupings.

Grouping of recruited donors for biological study

We invited 18 ESN, 17 SVR and 19 SR women to donate a blood sample for the project. For interrogation of the ESN immune response we compared ESNs with donors who were susceptible to HCV infection by grouping the SRs with SVRs as seropositive (SP; **Fig. 2.2**).

To assess potential differences in the SR and SVR response in our cohort we analysed the three infection groups separately.

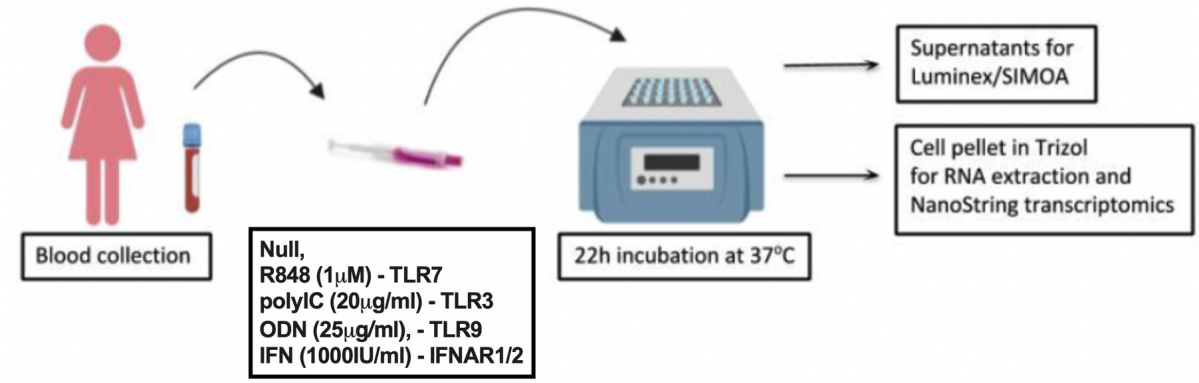


Figure 2.3 Overview of TruCulture workflow.

TruCulture whole blood stimulation

TruCulture tubes (Myriad RBM, Austin, TX, USA) were thawed and brought to room temperature. Peripheral blood was drawn into 9ml lithium heparin collection tubes and transferred to a biological safety cabinet. 1ml of blood was aliquoted into each tube within 15 minutes of blood draw as previously described [154]. The tubes were preloaded with our chosen stimuli and included: IFNα2 (1,000IU/ml), polyIC (20μg/ml), R848 (20μM) and a null unstimulated tube. Following incubation for 22hrs at 37°C, supernatants were collected as per manufacturers protocol and stored at -80°C for later cytokine analysis. The cell pellet was stored in 2ml of Trizol LS (Qiagen) for later RNA extraction and transcriptomic analysis (**Fig. 2.3**).

Immunophenotyping using flow cytometry

Blood was collected in 9ml sodium heparin tubes and transferred to a biological safety cabinet. 200μl of whole blood was aliquoted into polystyrene FACs tubes and washed with FACs buffer (1X PBS; 5% FBS; 0.1% Sodium Azide) by centrifugation (400 x g; 5 minutes). Supernatants were suctioned off and the pellet resuspended in 100μl of FACs buffer containing the appropriate antibody cocktail. Cells were incubated for 30minutes at 4°C while protected from light. Unbound antibodies were removed by washing with 1ml FACs

buffer and centrifugation (400 x g; 5 minutes). Cells were resuspended in 2ml of red blood cell lysis buffer for 15 minutes at room temperature. Samples were centrifuged to remove red cell lysis buffer and washed in FACs buffer to remove any red cell lysis buffer residue. Samples were stained with relevant live dead antibody for 20 minutes at 4^{o}C. Cells were washed with 500μl FACs buffer. Samples were resuspended in 200μl FACs buffer and 200μl of the sample was transferred to fresh FACs tubes containing 20μl of count beads before acquisition on the cytometer. For each experiment essential controls were used. Unstained cells were used to adjust forward and side scatter settings on the cytometer. Single stained controls (BD Compensation Beads stained with each individual fluorochromes used) were required to adjust compensation settings, while FMO controls (samples stained with all of the fluorochrome labelled antibodies in the experiment bar one) were used to set negative gates on cytometry plots.

Absolute cell counts by flow cytometry

To determine absolute cell counts in our whole blood samples we included count beads in our stained samples for flow cytometry. 25μl of count bead suspension (CountBright, Invitrogen) was added to the 200μl final volume of FACS buffer and the samples were acquired on the LSR Fortessa. The equation below was used to calculate the absolute number of cell subsets in each tube.

$$[(\text{population count} / \text{bead count})] \times (**\#\ \text{of beads per } \mu l)/\text{total test volume}] = \text{cells per } \mu l$$

**CountBright beads were provided a concentration of 20,800 beads per μl

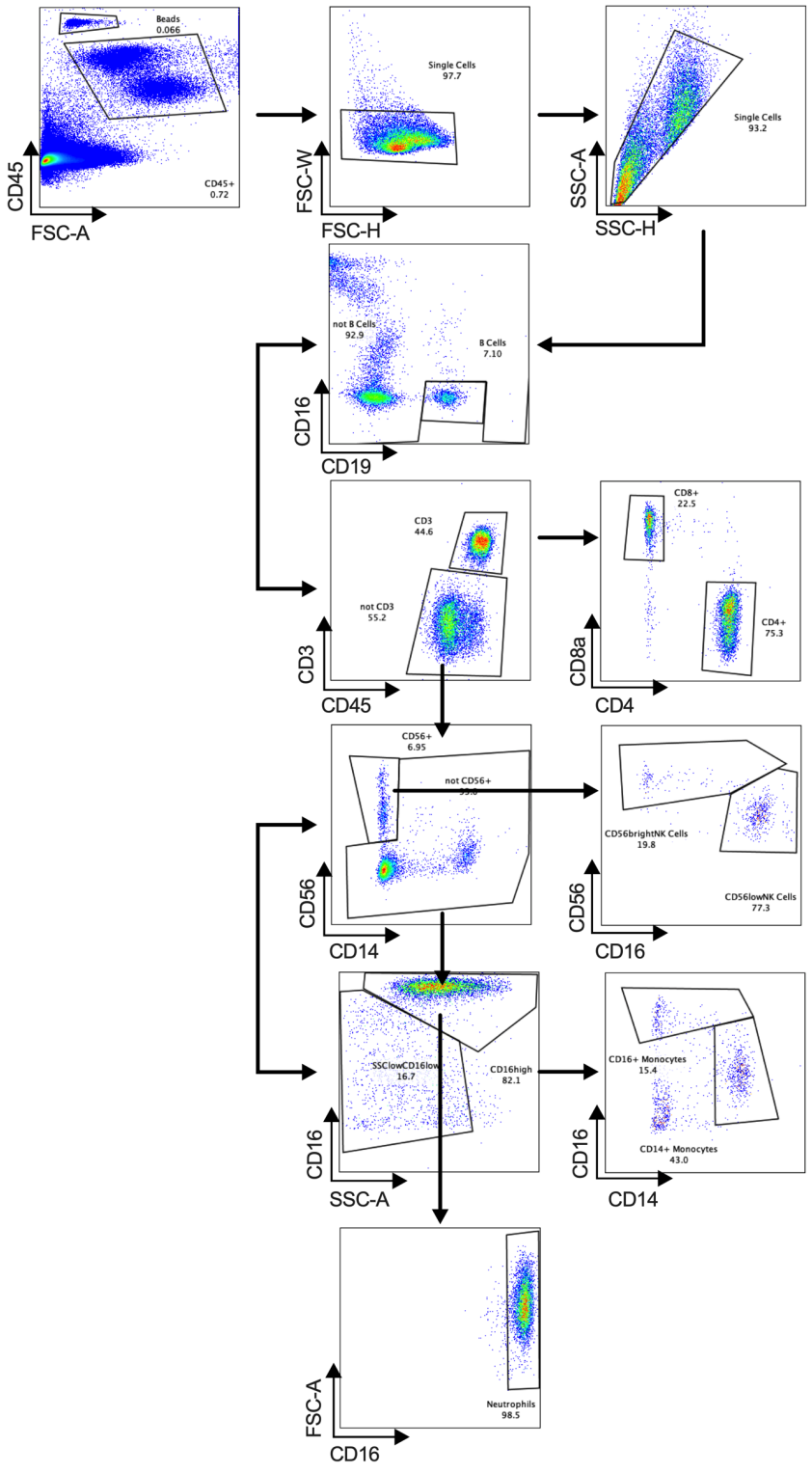

Beads
0.066
CD45+
0.72
CD45
FSC-A
Single Cells
97.7
FSC-W
FSC-H
Single Cells
93.2
SSC-A
SSC-H
Single cells
not B Cells
92.9
B Cells
7.10
CD16
CD19
CD19+ B cells
CD3
44.6
not CD3
55.2
CD3
CD45
CD8+
22.5
CD4+
75.3
CD8a
CD4
CD8+ T cells
CD4+ T cells
CD56+
6.95
not CD56+
CD56
CD14
CD56brightNK Cells
19.8
CD56lowNK Cells
77.3
CD56
CD16
CD56+CD16++ NK cells
CD56++CD16+ NK cells
SSClowCD16low
16.7
CD16high
82.1
CD16
SSC-A
CD16+ Monocytes
15.4
CD14+ Monocytes
43.0
CD16
CD14
CD16+ monocytes
(non classical)
CD14+ monocytes
(classical)
Neutrophils
98.5
FSC-A
CD16
Neutrophils

Figure 2.4. Gating strategy for lineage panel. *Immune cells were gated as CD45+. Doublets were excluded based on FSC and SSC properties. B cells were identified as CD19+. CD19- cells were divided into lymphocytes and non-lymphocytes based on CD3 expression . CD3+ lymphocytes were further divided into CD8+ and CD4+ T cells. CD3-CD45+ were divided as CD56+ into the NK cell subsets based on CD16 expression. Monocytes subsets were gated as SSC-low and divided based on CD14+ and CD16+. The CD16+ SSC-A high population was selected and neutrophils selected based on CD16 expression.*

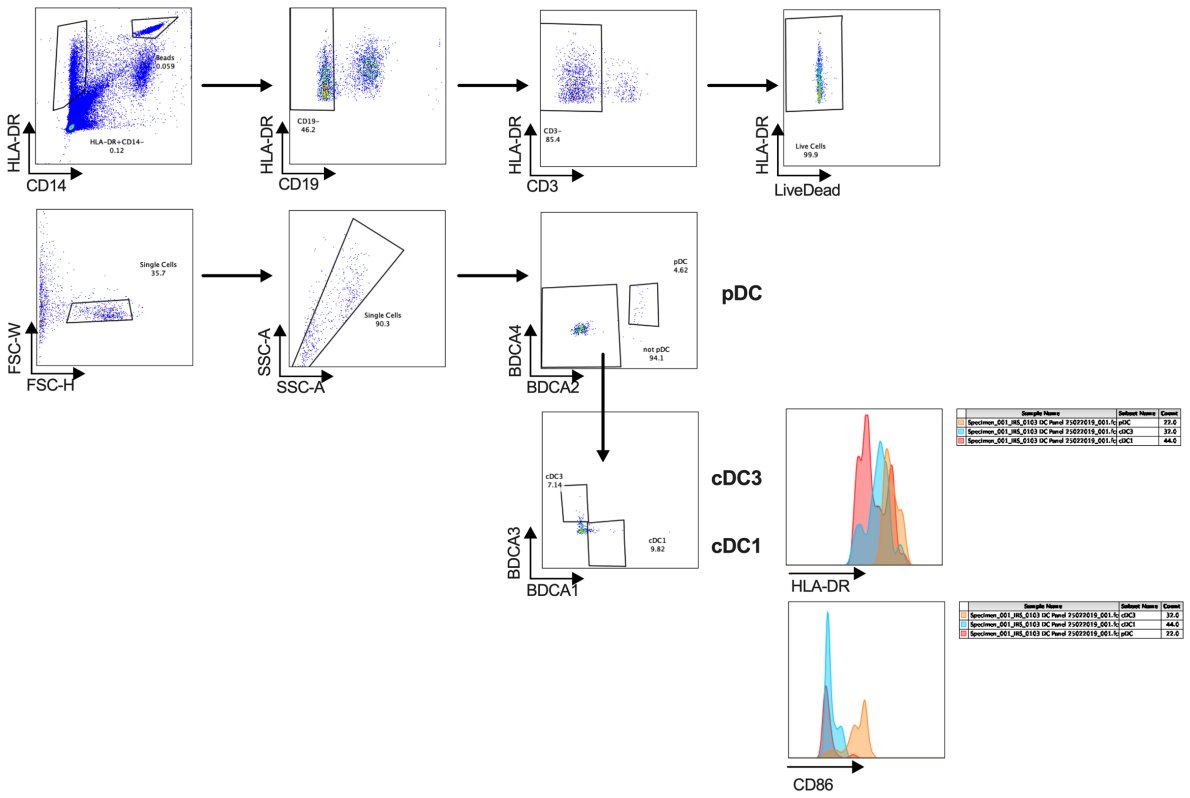


Figure 2.5. Gating strategy for DC panel. *DC subsets were gated as follows: HLA-DR+CD14-. Dead cells were excluded along with CD19+ and CD3+ subsets. Doublets were excluded using FSC and SSC parameters. pDCs were gated as BDCA2+BDCA4+. Conventional DC1s (cDC1) were subsequently gated as BDCA1+ and cDC3s were gated as BDCA3+. Representative plots of HLA-DR and CD86 on the three subsets are shown.*

Protein quantification using Luminex proteomics

Cytokines levels in the supernatants of the TruCulture whole blood stimulations were assessed using Luminex xMAP technology. We designed a custom panel targeting cytokines previously shown to be important in the control of HCV infection using the ThermoFisher panel design tool (**Table 2.8**).

Protein	Role in HCV	Reference
IL1β, TNFα, TNFβ, IFNγ	Inhibit HCV replication; may play role in viral clearance.	Laidlaw et al.[86] Liu et al.[155] Frese et al.[87] Bradley et al.[156]
CCL2, CCL3, CCL8	Initial effector cell recruitment	Fahey et al.[157]
IL8	Neutrophil recruitment	Fahey et al.[157]
CXCL10, CXCL11	Th1 recruitment and retention; may play a role in viral clearance	Zeremski et al.[158]
IL12p70, IL18, IL10	Cell-mediated antiviral response; may play a role in viral clearance	Flynn et al.[159] Sharma et al.[160] Sobue et al.[161]
IL-6, IL17	Play roles in viral clearance as well as liver fibrosis	Paquissi et al.[162]

Table 2.8. Cytokine targets chosen for the custom Luminex panel.

Samples were centrifuged (10,000rpm x 15 minutes at 4°C) and diluted 1 in 5 in a final volume of 110µl. 100µl of supernatants or assay standards were added to wells in a 96 well plate. 25µl of the microparticle cocktail containing coded magnetic beads conjugated to capture antibodies that bind the analyte of interest were then added, the plate was covered and the samples-beads mix were incubated overnight on a plate shaker (800rpm). Excess sample was washed off following incubation using 100µl of wash buffer. Plates were washed using a magnetic holder to pull down the beads and limit their loss. 50µl of

a biotin-antibody cocktail was then added to each well and the plate incubated on a shaker (1 hour; RT; 800rpm). The wash step was repeated as before, and 50µl of streptavidin-PE was added to each well, at which point the plate was again incubated on the shaker (30 minutes; RT; 800rpm). The plate was then washed as before and the microparticles resuspended in 100µl of wash buffer for acquisition on a Luminex xMAP.

Analysis of Luminex data

Induced cytokines with a q value (FDR corrected p value) of <0.01 relative to the null condition were included in downstream analysis for the relevant stimulus. Variables were rescaled to range between 0 and 1 and spider plots were generated in R using the package fmsb.

Protein quantification using Simoa ultrasensitive ELISAs

Simoa ultra-sensitive digital ELISAs were used to analyse IFNα concentrations in the supernatants of the stimulated whole blood experiments and in plasma from women in our cohort. IFNβ was also quantified using Simoa in the supernatants of polyIC stimulated and ODN stimulated blood. Briefly, samples were centrifuged (10,000rpm x 15 minutes at 4°C), diluted 1:3 and distributed in a 96 well plate alongside relevant standard. Samples and standards were incubated with magnetic beads conjugated to antibodies targeting either IFNα or IFNβ. The plate was then loaded into the Simoa HD-X analyser and run. The limit of detection for IFNα was 0.246 fg/ml and 0.011 pg/ml for IFNβ. Plasma cytokines were assessed using the Quanterix Corplex SP-X panel according to manufacturer's instructions.

Gene expression analysis

Gene expression in the TruCulture whole blood stimulations was assessed using the NanoString human immunology panel version 2. This robust quantification method allows for quantification of 560 genes through direct RNA hybridisation, without reverse transcription or laborious handling steps.

RNA extraction and quality control

Total RNA from stimulated whole blood was extracted using a semi-automated extraction system as previously described [163]. Samples were extracted in random sets of 96. Cell pellets preserved in Trizol were thawed on ice and centrifuged (2000rpm x 5 minutes at 4°C). Pellets were mechanically detached from the base of the tube by banging them on a hard surface. Samples were vortexed at 2000rpm for 5 minutes and centrifuged again (3500rpm x 5 minutes at 4°C). The samples were loaded into the racks of the vacuumed Freedom EVO workstation (Tecan). The sample name and relevant position was noted. The extractions were performed using the NucleoSpin 96 miRNA kit (Macherey-Nagel). 600µl of the clear phase from each tube loaded into the Tecan was automatically taken up and mixed with 900µl of ethanol in a deep well plate.

This mixture was transferred to the 96 well silica membrane plate and vacuum aspiration applied to facilitate RNA binding to the membrane. Membranes were subsequently washed with 600µl of the buffer MW1, aspirated, and washed with 700µl of MW2. The silica membranes were dried by centrifugation (10,000xg for 10 minutes; RT). RNA was eluted in 60µl of RNase-free water into plates of 2D barcoded tubes. Aliquots were prepared for RNA quantification, quality assessment and NanoString. The remaining samples were stored at -80°C.

RNA quality and quantity assessment

RNA was quantified using the Quantiflour RNA system kit (Promega). Briefly, 1X TE buffer was prepared by diluting the 20X stock 1:20 with nuclease free water. A working solution was prepared by diluting the RNA dye 1:400 in the 1X TE buffer. Standards were prepared by serially diluting the 100ng/ml standard 7 times with 1X TE buffer (50ng/ml to 0.78125ng/ml). 200µl of the RNA dye was distributed to each well in a 96 well plate and 10µl of sample, standard or 1X TE (blank) added. The plate was vortexed briefly, and incubated for 5 minutes protected from light. Fluorescence was measured at an excitation wavelength of 485nm using the Tecan Infinite F200 pro plate reader. RNA concentrations in the unknown samples were determined using the standard curve.

The RNA integrity number (RIN) for each sample was determined using the Agilent RNA 6000 Nano kit (Agilent) and Bioanalyser. In short, the RNA dye was brought to room temperature. The RNA dye was vortexed for 10 minutes and centrifuged briefly. 1μl of dye was added to 65μl of pre-filtered gel. The solution was vortexed (13000xg for 10 minutes; RT). A new RNA chip was taken and 9μl of the gel-dye mix was added to the relevant chamber and pressurised for 30 seconds. The chip was opened and 9μl of the remaining gel-dye mix was added to the appropriate wells. 5ml of the green RNA marker was added to the 12 sample wells and the well marked ladder. To the sample wells 1ml of sample was added. 1ml of the ladder was added to the well marked with the ladder symbol. The chip was vortexed for 1 minute at 2000rpm and read in the Agilent 2100 Bioanalyser within 5 minutes of its preparation. Samples with a RIN > 5 were used for NanoString.

mRNA quantification using NanoString transcriptomics

Extracted RNA was subsequently used for NanoString transcriptomics using the human immunology panel v2. NanoString uses a biotinylated capture probe combined with a reporter probe containing a hybridisation region of about 50 nucleotides specific to the target of interest. The reporter probe contains a six spot fluorescent barcode and each spot can be one of four different colours. Each gene is assigned a unique colour combination. The assay counts individual barcodes which represent individual RNA molecules.

NanoString involves 4 key steps outlined below:

1. Hybridisation – binding of RNA to capture and reporter probes.
2. Purification – removal of excess probes from the system.
3. Immobilisation – binding of biotinylated capture probe RNA complex to the imaging surface.
4. Counting – bound and aligned complexes are scanned using an automated fluorescence microscope and labelled barcodes are individually counted.

Samples were processed 12 at a time. RNA was diluted to 20ng/ml and a thermocycler pre-heated to 65^{o}C. Reporter and capture probes were thawed to room temperature. A master mix containing 5μl of hybridisation buffer and 3ml of the reporter probes was added to each well of a 12 well stip. 5μl of sample was added to the each well, followed by 2μl of the capture probes. The strips were capped and mixed by inverting and then placed in a thermocycler at 65^{o}C overnight for 16 hours.

Following hybridisation, samples were transferred in the 12 well strips to the nCounter automatic prep station, where excess probes were removed through a two-step magnetic bead based purification. The purified complexes were eluted and immobilised on a cartridge for counting using the nCounter Digital Analyser. Count data was exported as reporter code count (RCC) files.

Data normalisation

RCC files were imported into nSolver, the NanoString analysis software and checked for quality control flags (binding density, linearity of positive controls, limit of detection for positive controls, total counts and field of view counted). Each NanoString codeset contains positive and negative probes as well as housekeeping genes. The positive and negative probes are used to control for potential experimental differences across runs, while the housekeeping genes can be used to correct for differences in RNA input between samples. We selected housekeeping genes stably expressed across all samples using the geNorm method (*ALAS1, EEF1G, G6PD, HPRT1, POL2RA, PPIA, RPL19, SDHA, TBP*). Principal component analysis of the positive and negative probes indicated batch effects between sample runs. Prior to downstream analysis, these batch effects were corrected using the removeBatchEffect function in the Limma package in R.

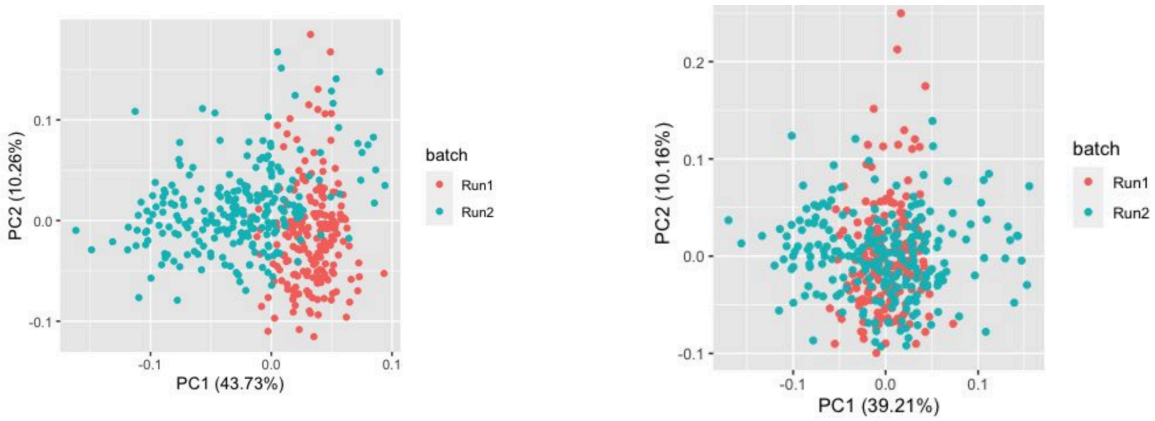


Figure 2.6. PCAs showing positive and negative probes before and after batch correction.

Differential gene expression analysis

Differentially expressed genes between groups were assessed in R studio. Lowly expressed genes with counts <4 in 75% of samples were filtered out. This reduced the gene list for downstream analysis to 519. Given the small size of our cohort and our interest in comparing the induced immune response to stimuli between donors groups we focused on induced genes for downstream analysis. Induced gene lists were generated per stimulus using paired t tests between null – stimulated conditions followed by FDR correction to generate a q value (FDR adjusted p value). Genes with a q value of 0.01 or less were included as "induced" genes. The number of genes retained for each stimulation are shown in **Table 2.9**.

Stimulus	Number of induced genes (q<0.01)
R848	440
polyIC	387
ODN (2216/CpG-A)	306
IFNα2	383

Table 2.9. Number of genes induced per stimulus. *Table showing the number of induced genes per stimulus. Genes with a q value of <0.01 following a paired t test between the unstimulated and stimulated condition were retained.*

Normality and variance of the dataset were assessed in R studio using Shapiro-Wilk tests and Flinger-Killeen tests respectively. For two group comparisons we used non-parametric

unpaired t tests followed by FDR adjustment. A q value of 0.1 was taken as significantly differentially expressed between groups. For multigroup comparisons we used non-parametric ANOVAs (Kruskal-Wallis test) followed by FDR adjustment.

Plotting the data – volcano, heatmap, PCA, kinetic plots

Data visualisation was carried out using R studio. Principal component analysis was carried out using the package "prcomp". Volcano plots were generated using the package "EnhancedVolcano" and heatmaps were generated using the package "pheatmap".

Gene set enrichment analysis (GSEA)

Pathways enriched between groups were assessed using the gene set enrichment algorithm (GSEA version 4.0.3, Broad Institute). The dataset was read into the GSEA application along with a pathway dataset built from the NanoString Immunology panel version 2 annotation file. Number of permutations was set to 2000, the 'classic' enrichment statistic was used the minimum set of genes per pathway was set to 5 and the max set to 200. The remaining parameters were run using the default settings. Significant pathways were visualised by plotting the -log10 p values as bar charts in Prism (version 8).

Gene signature score analysis

Gene signature scores, previously described using the same workflow in an independent cohort from the *Milieu Interieur* Cohort (MIC), were used to assess differences in specific cytokine induced gene modules (IFNγ, IFN-I, TNFα, IL1β) [164]. Gene scores for each stimulus are calculated as the average gene level Z scores per sample using $\log_2$ fold change (polyIC, R848, IFNα2) or expression data (null condition). A secondary IFN-I gene score from the Molecular Signature Database was used to validate findings with the MIC derived score [165]. The secondary signature was downloaded from the MSigDB website and the gene IDs converted to gene names. As the NanoString panel includes just 560 genes, it was not possible to include all genes from the secondary IFN-I signature as they were not on the panel – therefore a modified version was used. Genes included in the calculation of each gene score are shown in **Table 2.10**.

Gene	Signature	Source
STAT2	IFN-I	Urrutia et al., 2016
IFI35	IFN-I	Urrutia et al., 2016
TNFSF13B	IFN-I	Urrutia et al., 2016
TNFSF10	IFN-I	Urrutia et al., 2016
IRF7	IFN-I	Urrutia et al., 2016
IFITM1	IFN-I	Urrutia et al., 2016
CCR1	IFN-I	Urrutia et al., 2016
CXCL10	IFN-I	Urrutia et al., 2016
MX1	IFN-I	Urrutia et al., 2016
BST2	IFN-I	Urrutia et al., 2016
IFIH1	IFN-I	Urrutia et al., 2016
CCL8	IFN-I	Urrutia et al., 2016
B2M	MSigBD_IFN-I_response	Mostafavi et al., 2016
BST2	MSigBD_IFN-I_response	Mostafavi et al., 2016
CASP1	MSigBD_IFN-I_response	Mostafavi et al., 2016
CASP8	MSigBD_IFN-I_response	Mostafavi et al., 2016
CCRL2	MSigBD_IFN-I_response	Mostafavi et al., 2016
CD74	MSigBD_IFN-I_response	Mostafavi et al., 2016
CSF1	MSigBD_IFN-I_response	Mostafavi et al., 2016
CXCL10	MSigBD_IFN-I_response	Mostafavi et al., 2016
CXCL11	MSigBD_IFN-I_response	Mostafavi et al., 2016
HLA_C	MSigBD_IFN-I_response	Mostafavi et al., 2016
IFI35	MSigBD_IFN-I_response	Mostafavi et al., 2016
IFIH1	MSigBD_IFN-I_response	Mostafavi et al., 2016
IFIT2	MSigBD_IFN-I_response	Mostafavi et al., 2016
IFITM1	MSigBD_IFN-I_response	Mostafavi et al., 2016
IL15	MSigBD_IFN-I_response	Mostafavi et al., 2016
IL4R	MSigBD_IFN-I_response	Mostafavi et al., 2016
IL7	MSigBD_IFN-I_response	Mostafavi et al., 2016
IRF1	MSigBD_IFN-I_response	Mostafavi et al., 2016
IRF7	MSigBD_IFN-I_response	Mostafavi et al., 2016
LAMP3	MSigBD_IFN-I_response	Mostafavi et al., 2016
MX1	MSigBD_IFN-I_response	Mostafavi et al., 2016
PSMB8	MSigBD_IFN-I_response	Mostafavi et al., 2016
PSMB9	MSigBD_IFN-I_response	Mostafavi et al., 2016
SELL	MSigBD_IFN-I_response	Mostafavi et al., 2016
STAT2	MSigBD_IFN-I_response	Mostafavi et al., 2016
TAP1	MSigBD_IFN-I_response	Mostafavi et al., 2016

Gene	Signature	Source
C3	TNFα	Urrutia et al., 2016
CCL4	TNFα	Urrutia et al., 2016
CD44	TNFα	Urrutia et al., 2016
CD83	TNFα	Urrutia et al., 2016
IRAK2	TNFα	Urrutia et al., 2016
NFKB2	TNFα	Urrutia et al., 2016
NFKBIA	TNFα	Urrutia et al., 2016
RELB	TNFα	Urrutia et al., 2016
SOCS3	TNFα	Urrutia et al., 2016
SRC	TNFα	Urrutia et al., 2016
TNFAIP3	TNFα	Urrutia et al., 2016
CDKN1A	IFNγ	Urrutia et al., 2016
CXCL9	IFNγ	Urrutia et al., 2016
HLA-DMB	IFNγ	Urrutia et al., 2016
HLA-DPA1	IFNγ	Urrutia et al., 2016
HLA-DPB1	IFNγ	Urrutia et al., 2016
HLA-DRA	IFNγ	Urrutia et al., 2016
IDO1	IFNγ	Urrutia et al., 2016
JAK2	IFNγ	Urrutia et al., 2016
RARRES3	IFNγ	Urrutia et al., 2016
SLAMF7	IFNγ	Urrutia et al., 2016
SOCS1	IFNγ	Urrutia et al., 2016
CCL2	IL1β	Urrutia et al., 2016
LILRB1	IL1β	Urrutia et al., 2016
NFKB1	IL1β	Urrutia et al., 2016
NFKBIZ	IL1β	Urrutia et al., 2016
POU2F2	IL1β	Urrutia et al., 2016
IL6	IL1β	Urrutia et al., 2016
IL1B	IL1β	Urrutia et al., 2016
CCL20	IL1β	Urrutia et al., 2016
IL1A	IL1β	Urrutia et al., 2016
CXCL2	IL1β	Urrutia et al., 2016

Table 2.10. List of genes used to generate the gene signatures for IFN-I, IFNγ, IL1β, TNFα and the secondary IFN-I signature. *Gene signature scores for each stimulus are calculated as the average gene level Z scores per sample using log_2 fold change (polyIC, R848, IFNα2) or expression data (null condition).*

PBMC isolation and cryopreservation

Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood collected in 9ml sodium heparin tubes. In a biological safety cabinet (BSC), blood was diluted 1:1 with sterile HBSS in a 50ml Falcon tube, carefully layered on top of 15ml of sterile Ficoll-Paque (GE Healthcare) and centrifuged (900 x g; 15 minutes; -20^{o}C) with break set to one. This density dependent separation results in the formation of a 'buffy coat' between the erythrocytes and plasma. The buffy layer was removed using a sterile Pasteur pipette, added to a fresh falcon tube and made up to 50ml with wash buffer (HBSS; 5% FBS) and centrifuged at 300 x g for 20 minutes at -20^{o}C with break set to 9. During this spin a 4ml aliquot of the remaining HBSS/plasma supernatant was removed and stored at -80^{o}C. Following centrifugation the supernatant was discarded and 30ml of fresh wash buffer was added and the sample was centrifuged at 300 x g for 15 minutes at 20^{o}C. The supernatants were once again discarded and the pellet resuspended in 5ml complete RPMI culture medium + 2.05mM L-Glutamine + 10% FBS + 5% PenStrep (GIBCO, Life Technologies).

Cells were counted using trypan blue staining on a haemocytometer and a light microscope. An aliquot of cells was diluted in Trypan blue. The haemocytometer was covered with a glass cover slip and 10µl of the cell/Trypan blue solution was pipetted under the cover slip. Sample was visualised under a light microscope. The number of live cells in the four grid corners was counted. The number of cells present was calculated using the following calculation:

(number of cells counted / number of corners counted)
(10^4)(dilution factor) = number of cells per ml.

Cells were again pelleted at 400 x g for 5 minutes at 20^{o}C. Freezing medium (50 % RPMI, 40% FBS, 10% DMSO) was prepared. The cell pellet was then resuspended in the freezing medium at a concentration of 10-25×10^6 cells per ml. Cells were transferred to cryovials, and 10% DMSO was added. The cryovials were placed in a Mr Frosty 1°C/minute freezing container containing isopropanol and stored at -80°C overnight. Cryovials were subsequently transferred to liquid nitrogen tanks for long-term storage.

Serum collection and preservation

During sample collection, one red topped 5ml serum tube was also collected. These tubes were centrifuged at 2000xg for ten minutes. Serum was stored at -80^{o}C until use.

ELISpots

For detection of T memory cell responses IFNγ ELISpots were performed on PBMCs isolated from the resistance cohort. In a BSC, Millipore MAIP plates were pre-wetted by adding 15µl of 35% ethanol to each well. The plates were flicked to remove excess liquid and were washed with 200µl sterile water three times. Waste water was decanted into sterile reservoirs in the BSC. Coating solution was prepared with anti-human IFNγ monoclonal antibody clone 1-D1K, diluted 1 in 200 in PBS at 75µl per well. Plates were coated with diluted coating antibody using a multichannel pipette and incubated in the fridge overnight at 4^{o}C. Plates were removed from the fridge, transferred to a biological safety cabinet and washed five times with 200µl of sterile PBS. Plates were blocked using 200µl of cRPMI per well and stored at 37^{o}C for two hours. Peptide pools of HCV, CMV, EBV and Influenza virus (CEF peptides; ProImmune), concanavalin A, were prepared in sterile cRPMI. A DMSO control diluted to contain the same amount of DMSO as the peptide solutions was also prepared. Peptides were mixed by gently swirling the tube. The blocked plates were removed from the incubator and inverted over a trough to decant the blocking solution. 50µl of the peptide solution was added to each well in triplicate. 50µl of the DMSO control was also added in duplicate, along with duplicate blank wells containing 100µl of cRMPI alone. Freshly isolated donor PBMCs were made up to a concentration of 4x10^{6} cells/ml. 50µl of the resuspended PBMC solution was added to each well. The plate was mixed gently by rotating the plate in a figure 8 pattern. The plate was covered and plated in the incubator overnight (37^{o}C).

ELISpot development

The plate was washed 7 times with 200µl of PBS Tween20 wash buffer. A biotinylated mouse anti-human IFNγ antibody was diluted to 0.5µg/ml in assay diluent (0.5% BSA in PBS) and 50µl of the prepared antibody was added to each well and incubated at RT for 2 hours. Immediately before the end of the incubation a solution of alkaline phosphatase

conjugated anti-biotin antibody diluted 1:1000 in assay diluent (0.5% BSA in PBS) was prepared. The plate was washed 4 times with 200µl of PBS Tween20 wash buffer and blotted dry on a paper towel. 50µl of the ALP solution was transferred to each well and incubated at room temperature for 2 hours. At the beginning of the incubation period the appropriate aliquot of BCIP/NBT stock solution as removed from the fridge and placed at room temperature while protected from light. Following the 2 hour incubation, the plate was washed 4 times with PBS Tween20 and dried on a paper towel. The BCIP/NBT was sterile filtered using a 22µm filter at room temperature and 50µl was added to each well for 20 minutes, or until distinct dark spots appeared. To stop colour development, the plates were washed 3 times each using dH20. The plates were dried and placed faced down overnight at room temperature to dry.

Reading ELISpots

Dark spots in each well were counted using an ELISpot reader. The mean number of spot forming cells per 1 x 10^6 input PBMCs was calculated by multiplying the mean number of spots for each set of replicates by 1x10^6/cell per well input.

Antibody profiling

Antibody profiling in serum from women in our cohort was carried out using VirScan, a novel technology designed to test for antibodies against all viruses known to have a tropism for human cells. VirScan technology is based on a T7 bacteriophage display system expressing epitopes from human viruses. Sera are incubated with these epitope expressing bacteriophages and antibodies present in the serum can bind to the relevant epitopes. Antibody-phage complexes are immunoprecipitated, lysed and sequenced to uncover which epitopes have been targeted. For VirScan n=18 ESN, n=19 SR, n=17 SVR, n=29 UC.

VirScan was carried out in the lab of Professor Petter Brodin at the Karolinska Institute. Normalised data was analysed for differences between groups by me.

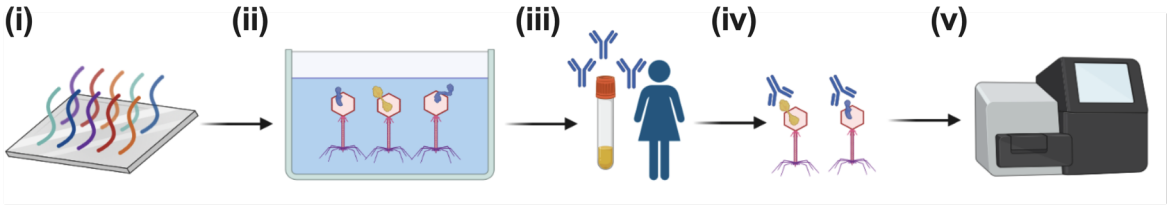


Figure 2.7. Overview of VirScan methodology. *(i) Customised DNA synthesis: DNA sequences of viral protein fragments are synthesised as an array on a solid surface. (ii) T7 bacteriophage display: protein fragments encoded by the DNA sequences are cloned into bacteriophages and expressed on the surface of the phage capsids. (iii-iv) Donor serum containing antibodies is incubated with the protein fragment expressing bacteriophages. Antibodies bind to protein targets they recognise. Bound and unbound antibodies are separated using magnetic beads. (v) Next generation sequencing: the DNA sequences in the antibody-phage complexes are barcoded and amplified using PCR. The PCR products are sequenced using high throughput DNA sequencing and analysed using a bioinformatics pipeline.*

Total IgG quantification

To normalise levels of serum immunoglobulin inputted for each sample, total IgG levels were quantified using the IGG-2 Roche system according to the manufacturer's instructions.

VirScan

Serum normalised for total IgG content was incubated in duplicate alongside bead controls with bacteriophages presenting 56-mer amino acid linear peptides that overlap by 28 amino acids to encompass the whole genomes of 206 viral species and 1,276 viral strains and 115,753 epitopes. Following incubation, the IgG-phage immunocomplexes were pulled down using magnetic beads. The collected bacteriophages were subsequently lysed, PCR amplified, barcoded and pooled for sequencing using the Illumina NextSeq Kit v2.

Analysis of VirScan data

Reads were matched to the original library sequences using Bowtie and reads counted using SAMtools. Next, a binning strategy as described by Mina, et al was applied to determine the positivity of each epitope in serum samples compared to control beads [205]. Briefly, epitopes were grouped into hundreds of bins so that the reads from bead samples forms a uniform distribution. These bins were then used for epitopes from serum samples. A Z score was calculated for each epitope from each serum sample with the mean and standard deviation from beads samples within each bin. An epitope was considered a positive hit only when the Z scores from both replicates were larger than 3.5. Z scores were used at an epitope level to provide a relative quantification. A higher Z score is related to a higher antibody titre. The number of significant epitopes of a viral species compared to an experimental negative control were summed to calculate a virus score for the specific virus (virusHit). The epitopes are a combination of the UniProt entry and the position (start and end). To determine sample level positivity for viruses of interest epitopes for each virus were plotted as heatmaps. Positive samples were identified based on the breadth of positivity or based on positive epitope signals that were absent in samples known to be negative (spurious hits).

Genetic analysis of HCV cohort

The cohort was genotyped for SNPs of interest in TLR3, IRF3 and IFNAR1. SNPs representing haplotypes (tagSNPs) were selected based on previous literature associations and minor allele frequencies (MAFs).

TagSNP selection and function prediction software

TagSNPs were chosen using the SNPinfo tool from the National Institute of Health (NIH; https://snpinfo.niehs.nih.gov/snpinfo/snptag.html). The gene name was inputted and the genotype data from dbSNP used, selecting the European population. The minimum number of valid genotype pairs required to calculate linkage disequilibrium (LD) was set to 5 and the LD threshold was set to 0.8. MAFs were set between 5-50%.

Selected SNPs were assessed using the function prediction software SIFT, Provean and Polyphen-2.

Saliva collection and DNA isolation

DNA isolation was carried out using the protocol and materials from Isohelix GeneFix™ Saliva DNA Mini Kit: GSS-50 (Cell Projects). Saliva collection kits were sent to study participants. 2ml of saliva was deposited into the 10ml collection tube which contained 2ml of stabilising solution provided. Participants were asked to ensure sample was not collected with 30 minutes of eating, smoking or brushing teeth. The tubes were then packaged and returned to the lab by post. The returned tubes were vortexed and 1ml of the solution aliquoted into a 2ml Eppendorf. 15µl of proteinase K was added to the tube and vortexed. The tube was incubated at 60°C for 60 minutes. 1ml of elution buffer was preheated to 70°C for later use. 1ml of ethanol was added to the Eppendorf and vortexed. A GeneFix™ Mini DNA column was placed on top of a new collection tube (1.5ml microcentrifuge column). 700µl of the initial mix was added to the column. The column on the 2ml Eppendorf was centrifuged at 12000 x g for 1 minute. The flow through was discarded and this was repeated until all the initial solution was run through the column. 750µl of wash buffer was added to the column and centrifuged again at the same speed and duration as previously. This was step was repeated discarding the flow through each time. The column was centrifuged for 3 minutes at the same speed to remove all traces of ethanol. The column was placed on a new collection tube and 50µl of preheated elution buffer was added to the centre of the filter. This was incubated at room temperature and then centrifuged at the previous speed for 1 minute. The resulting filtrate was transferred to a 1.5ml Eppendorf and labelled with the sample ID and date and stored at -20^{o}C.

DNA quantification and analysis

A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, UK) was used to determine the concentration and purity of eluted DNA in each sample. Sample purity was assess by taking the ratio of absorbance measured at 260 and 280 nm.

SNP genotyping

DNA from the recruited cohort plus samples previously collected for a pilot study and a previous HCV study in the lab were selected for SNP genotyping. In total 38 ESN, 63 SR, 77

CI/SVR and 119 HC were genotyped. Genotyping for rs3775291, rs7251 and rs2257167 was performed by quantitative polymerase chain reaction (qPCR) using the Applied Biosystems StepOnePlus RT-PCR machine. Taqman mastermix (Applied Biosystems) and SNP primer, and DNA samples were thawed on ice. A mastermix was made up using 2.5µl Taqman mastermix, 0.25µl SNP assay mix and 1.75µl DNase/RNase free molecular grade water per sample to be plated. 4.5µl of the mastermix was added to each well of a 96 well plate. 0.5µl DNA sample was added to the relevant well. In the negative control wells 0.5µl of water was added. The plate was sealed with a sterile plate cover and centrifuged briefly to remove bubbles and ensure the solution was at the bottom of the well. The plate was then placed in the RT-PCR machine. The PCR machine was set up by naming the experiment, changing the settings to genotyping and running a fast time lasting 40 mins. Negative control wells were also labelled. The volume was changed to 5µl and the pre and post-read times changed to 2 minutes. The details of each phase of the qPCR run are shown below in **Table 2.11**. SNPs were called automatically as homozygous wild-type, heterozygous or homozygous variant. Differences in the frequencies of genotypes or alleles between groups were assessed using Chi-square tests for dominant and recessive models.

Stage	Temperature (°C)	Duration	Phase
Pre-PCR Read	25	120	Holding
Initial Denaturation	95	20	
Denature	95	3	Cycling (X40)
Annealing	60	20	
Post-PCR Read	25	120	Holding

Table 2.11. Thermocycle conditions for fast genotyping on Applied Biosystems StepOnePlus RT-PCR machine.

Multiple sequence alignments

IFNAR1 alignments were carried out using Clustalω. The amino acid sequences of human (NP_000620.2), mouse (AAH43935.1), chimpanzee, chicken and zebrafish were retrieved

from GenBank with the signal peptide sequence removed and inputted into the Clustalω online tool.

SNP visualisation using PyMOL

The IFNAR1 protein structure "3S98 human IFNAR1" was downloaded from www.rcsb.org/pdb/home/home.do in PDB format (.gz). The file was read into the PyMOL application, and the commands "cartoon", "hide lines" and "hide waters" were selected. The amino acid valine at position 141 was selected in the protein sequence and the colour changed to indicate the position at which rs2257167 changes the amino acid (to leucine).

The Milieu Interieur Cohort overview

The Milieu Interieur Cohort (MIC) consists of a large cohort of 1000 individuals, 500 males and 500 females, stratified by ages 20 to 69 with 100 in each group. Through a collaboration with Dr Darragh Duffy (co-coordinator of the MIC) at the Institut Pasteur, Paris, we were fortunate to have access to some of the datasets previously generated on the cohort. We were able to analyse several of these datasets in novel ways to ask questions of relevance to our viral resistance cohort.

The datasets we had access to and the means by which they were generated are outlined below.

The samples and data used in the MIC were formally established as the *Milieu Interieur* biocollection (NCT03905993), with approvals by the Comité de Protection des Personnes – Sud Méditerranée and the Commission nationale de l'informatique et des libertés (CNIL) on April 11, 2018. The study was designed and conducted in accordance with the Declaration of Helsinki and good clinical practice, with all subjects giving informed consent.

SNP genotyping

All participants were genotyped through a genome-wide SNP array, using HumanOmniExpress and HumanExomeBeadChips [66]. After imputation using the 1000

genomes project imputation reference panel, a final dataset of 5,265,361 SNPs were obtained.

TruCulture stimulation, NanoString and Luminex proteomics

In the same manner as our cohort, blood from each person in the MIC was stimulated with a panel of agonists for 22 hours using the TruCulture whole blood stimulation system. Gene expression was also assessed using the NanoString human immunology panel v2. Luminex proteomics was used to quantify secreted cytokines in the supernatants of the TruCulture stimuli. We had access to NanoString datasets from stimulations with polyIC (25μg/ml), LPS (10ng/ml), and the live influenza A virus (H1N1 PR8; 100 HAU).

Immunophenotyping of Milieu Interieur donor whole blood

Whole blood from the 1000 donors was collected and transported to Paris within 6 hours of blood draw. Here, the blood was stained using premixed antibody cocktails for flow cytometry to enumerate major circulating immune cell populations, including B cells, T cells and monocytes. Gating strategies and antibody details have previously been published [49]. Samples were acquired on using a MACSQuant analyser.

Serology of Milieu Interieur donors

The serological response to 15 antigens from common human pathogens were assessed using clinical grade serological assays. Qualitative and quantitative IgG response to cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus 1 and 2 (HSV1; HSV2), varicella zoster virus (VZV), Helicobacter pylori (H. pylori), Toxoplasma gondii (T. gondii), IAV, measles, mumps, rubella, and hepatitis B virus (HBV) were assessed.

RhD analysis

For the RhD study, Rhesus status (RhD-positive or -negative) was determined based upon the presence or absence of the rs590787 polymorphism in the gene sequence of each individual of the MIC [166]. Flow cytometry data from MIC was analysed by RhD status using parametric unpaired t tests with FDR correction. NanoString datasets from null, LPS,

polyIC and IAV were analysed using linear regression, including CMV as a covariate in the model. Serological data was analysed by RhD status using non-parametric t tests.

Statistical analysis

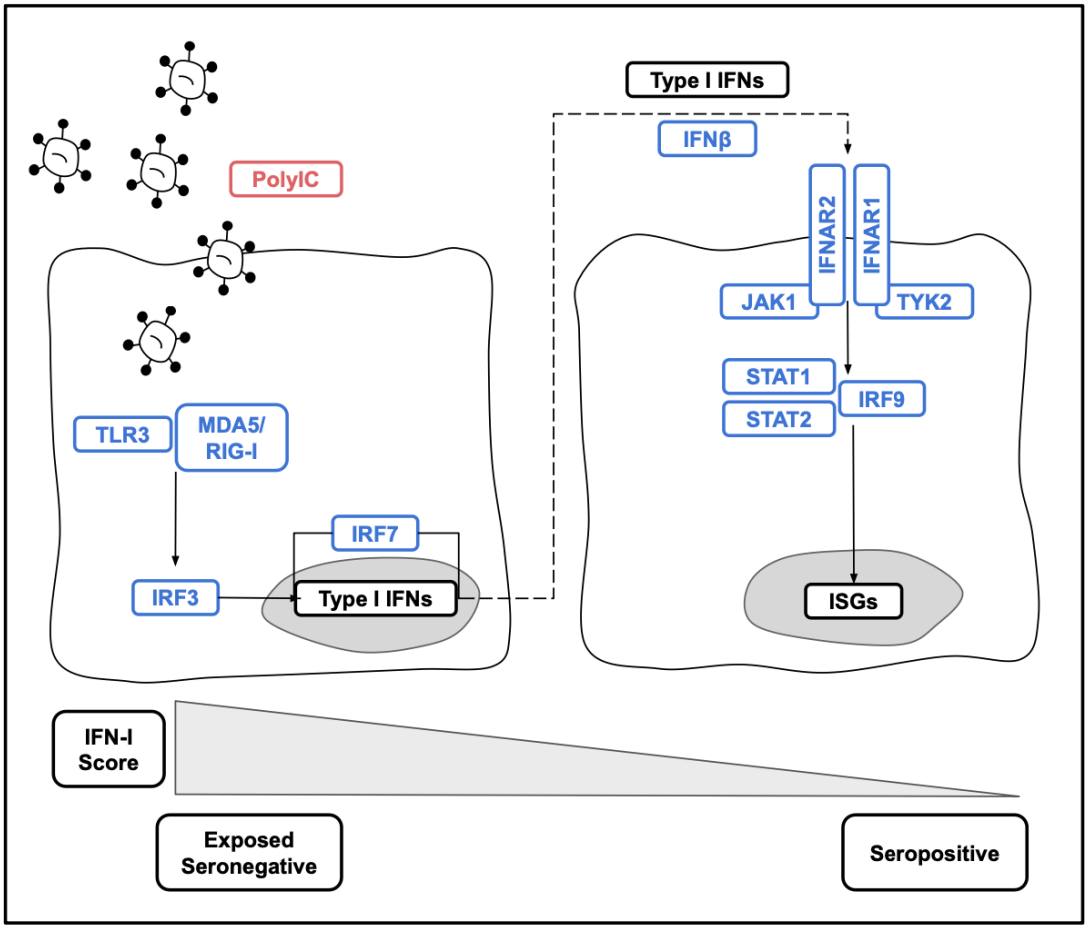
Normality of data was tested using QQ plots and Shapiro-Wilk tests. For two group comparisons of normally distributed data, paired or unpaired t tests were used. For two group comparisons of non-normally distributed data Mann-Whitney U tests were used. For comparisons greater than two groups one way ANOVAs or Kruskal-Wallis tests were used for normally distributed and non-normally distributed datasets respectively.

Chapter 3: Recruitment and assessment of innate resistance to hepatitis C in women in the Irish anti-D cohort

Abstract

Natural resistance to viral infection is an overlooked outcome following hepatitis C virus (HCV) exposure. Between 1977-79, over 1,200 Rhesus negative Irish women were exposed to highly infectious batches of HCV contaminated anti-D immunoglobulin. Data from this period indicate that just over 50% of recipients who received vials of anti-D from highly infectious batches became infected and developed HCV specific antibodies. No previous study has explored mechanisms of viral resistance in the women who did not become infected. A national campaign was launched to recruit Rhesus negative women who had received HCV contaminated anti-D in 1977-179. To test for antibodies against HCV and other viruses we used VirScan. We performed standardized *ex vivo* whole blood stimulation (TruCulture) assays with viral ligands and used NanoString transcriptomics and Luminex and Simoa proteomics to assess antiviral responses. We used cytokine gene signature scores after stimulation to examine cytokine specific responses in our cohort. 34 exposed seronegative (ESN) women who tested PCR and antibody negative following high viral exposure were successfully recruited to the study, as well as 98 seropositive SP women. We found anti-HCV antibody positivity in the SVR and SR groups only. ESN women had a higher polyIC (TLR3) induced type I interferon (IFN-I) gene signature when compared with SP donors. Production of several inflammatory cytokines, including CCL8, CCL2 and IL-6 in response to polyIC stimulation was increased in ESN compared to SP women. In contrast, transcriptomic and cytokine responses to other ligands (R848, IFNα2) were similar in both groups. This study identifies a specific enhanced IFN-I gene signature in response to the viral mimic polyIC in ESN women compared to SP donors. This enhanced antiviral responsiveness may have contributed to innate immune protection against HCV infection.

Graphical abstract



Introduction

Susceptibility to viral infection in the human population is highly variable, yet the reasons for this discordance are unknown [167]. For example, following exposure to HCV, between 50-80% of infected individuals progress to chronic infection, and test both PCR positive for HCV RNA and anti-HCV antibodies [168]. Additionally, a significant percentage of individuals are able to clear HCV infection within 6 months of becoming infected through engagement of their adaptive immune system, and test PCR negative for HCV RNA, but remain HCV antibody positive (spontaneous resolution) [168]. As both of these groups have evidence of seroconversion, they are known collectively as seropositive (SP), and, as they are readily identifiable using routine clinical assays, they have been well studied over the past 25 years [147,169,170].

There also exists a third group, ESNs, who lack detectable evidence of past infection as measured by conventional HCV detection assays, testing both HCV PCR negative and anti-HCV antibody negative despite known exposure to the virus [10,171]. Currently, ESNs are only identifiable through analysis of risk, or documented outbreaks of infection and are consequently less well studied than seropositive individuals. While iatrogenic outbreak events are rare in the modern era, since the introduction of effective viral screening technologies for blood products and donor organs, several historical outbreaks of HCV have occurred due to failures in decontamination procedures, or prior to the discovery of certain viruses eg. HCV and HIV. These natural experiments present useful opportunities to identify ESN individuals and to study the host factors that contribute to differential susceptibility and potential resistance to viral diseases in a relatively controlled manner [10]. Understanding the mechanisms of resistance to viral infection could have major implications for design of vaccines and novel antiviral therapeutics.

An enhanced innate immune response is thought to be sufficient to clear infection without engagement of the adaptive immune system in ESNs [10]. The innate immune response to HCV is multifaceted and complex, any part of which could contribute to HCV resistance. HCV viral RNA is detected by several pattern recognition receptors, including TLR3, RIG-I, MDA5 and TLR7 [61]. Ligation of these receptors induces synthesis and secretion of type I (IFN-I) and type III (IFNL) interferons, which act early following viral exposure to inhibit HCV replication through induction of interferon stimulation genes (IRGs) and activation of immune cells [61].

A major outbreak of HCV occurred in Ireland between 1977-79 in Ireland, when over 2,000 Rhesus negative women were exposed to highly infectious batches of HCV contaminated anti-D immunoglobulin [147]. The anti-D was retrospectively discovered to have been contaminated by blood donations from a single individual infected with genotype 1b HCV [147]. Seropositive individuals from this outbreak were readily identifiable and have been studied extensively over the last three decades [169]. The cohort has proven to be a rich source of HCV related data, helping to identify several factors associated with progression to chronic disease or spontaneous resolution of infection [84,172]. In this transmission episode, the recipients were all healthy, non-immunocompromised females, of a similar

age and ethnic origin and the amount of immunoglobulin administered was consistent in all recipients [147]. All individuals were infected from a single variant of HCV present in a single plasma donor. The only variables in this transmission episode was the amount of virus associated with each batch and host genetic factors. To date, no study has investigated resistance to HCV during this outbreak.

Previous studies on exposed seronegative individuals

Most work on ESN cohorts to date has been carried out on people who inject intravenous drugs (PWIDs) who are known to share needles and other injection paraphernalia with those who are HCV positive [10]. Typically, this high risk behaviour results in a low dose exposure to HCV infection that results in chronic infection [10]. Despite this, these ESN individuals fail to contract the infection themselves. Studies to date of ESNs in PWID cohorts have found interesting differences in key cellular and secreted processes (**Table 3.1**) [10,89,173].

Most work in PWID ESNs has centred around NK cell functionality. Lloyd and co. have reported increased counts of CD69+CD56dim and CD69+CD56bright NK cells – indicating a more 'active' phenotype [173]. They also showed greater IFNγ production and less CD107a (a degranulation marker) upregulation in NK cells derived from ESN donors *in vitro* [174]. Other NK cell work carried out on Canadian PWIDs found no difference in NK cells at baseline [175]. Nor did they find differences in the expression of IFNγ production from PBMCs as measured by ELISpot in response to HCV peptides[175]. This finding has been contradicted in other studies on ESN cohorts where robust antigen specific T cell responses as measured by ELISpots have been found, implicating enhanced T cell responsiveness as the mechanism by which individuals are protected from HCV infection [65,174,176,177]. Also reported in PWID ESN cohorts is an increased frequency of KIR2DL3+NKG2A- NK cell populations compared with healthy controls, SVR and SR populations [178]. This cell population is uninhibited by HLA-E ligation and so produces more IFNγ. KIR2DL3 HLA-C1 homozygosity has also been associated with exposed seronegativity [179].

Fewer studies on the cytokine profiles of these individuals have been carried out, although there are reports of increased IL-6, IL-8 and TNFα in the serum [10]. SNPs in the IL-12B gene that increase IL-12 production have also been associated with resistance [180]. NK cells from ESN donors produce more IFNγ and TNFα on stimulation compared with either SR or SVR groups [181].

Like the CCR5 mutation seen in HIV-1 resistance it would be plausible to think a similar observation might be made in a human population who resist HCV infection and perhaps a polymorphism in CD81 the receptor for HCV might explain resistance [182]. However, this does not appear to be the case with HCV. Studies by two groups found CD81 to be very highly conserved with no genetic alterations [183]. Other work found SNPs in both claudin-6 and occludin in two ESN individuals, though later functional studies showed that these SNPs did not confer resistance *in vitro* [184,185]. Further work refuting the idea that HCV resistance is mediated by entry receptor mutations comes from Matthew Cramp's group at Plymouth. Through a look back study, 1340 individuals who received a HCV contaminated blood transfusion Cramp's group were identified and 8 recruited individuals with an ESN phenotype. Whole exome sequencing of this group showed no enrichment for SNPs in any HCV entry receptors [186].

Further work on this cohort corroborates studies on PWIDs and shows enhanced NK cell functionality with increased NKp30, NKp80 and KIR2DL3 expression and increased cytotoxic responses with IL-2 [10]. Occupational exposure to HCV via needle stick injury demonstrated early NK cell activation and increased NKp44, NKp46 and CD122 expression as important mediators of resistance to infection [94]. Pilot work by our group on a smaller cohort exposed to both genotype 3a and genotype 1b of HCV via contaminated anti-D found ESNs had decreased monocyte counts as well as increases in IL-8 and IL-18 compared with SR and SVR [187]. We did not observe an increase NKp30 expression [187].

Route of Exposure	Mechanisms of Resistance
Blood Transfusion	NK cell counts have been reported to be increased, alongside an increase in NK cell functionality and increased NKp30, NKp80 and KIR2DL3 expression on both NK cell subsets. In response to IL2, NK cells from ESNs have increased levels of cytotoxicity. Some reports of detectable IFNγ ELISpots in response to HCV peptides [10].
	No enrichment for SNPs in HCV entry receptors in ESNs [186].
Occupational Exposure	ESNs following needle stick injury had early NKT activation and increased serum cytokine responses. ESNs also had increased CD122, NKp44, NKp46 and NKG2A expression, cytotoxicity and IFNγ production. This robust response correlated with a strong HCV specific T cell response [94].
	ESNs following needle stick injury in Germany had robust CD4+ T cell response to HCV [60].
	Robust anti-HCV T cell response also noted in needle stick injury ESN individuals as measured by ELISpot for IFNγ [94].
People Who Inject Drugs	ESNs had distinct lipidomic profiles compared to HCV susceptible individuals [188].
	ESNs had increased counts of CD69+CD56dim NK cells and an increased number of NKp30+ CD56bright CD16+ NKs with greater IFNγ production but less CD107a expression. ESNs had no difference in their ELISpot responses compared to CIs. There was no association between IL28B, HLA-C or KIR2DL3 and resistance to HCV infection [173,189].
	PWID ESNs have robust HCV specific T cell responses as measured by ELISpot [174,176,177].
	ESNs had increased KIR2DL3+NKG2A- NK cells compared to controls, CIs and SRs. These NKs are not inhibited by HLA-E ligation and therefore produce greater IFNγ in response to stimulation [178].
	The 1188A/C polymorphism of IL-12B, C allele and CC genotype are associated with HCV resistance [179].
	Claudin-6 and occludin variants were found in an ESN individual but were not sufficient for resistance *in vitro*. CD81 appears to be very highly conserved with no genetic alterations found in a study of ESNs cases [183–185].
	IL28B genotype rs12979860 CC is not associated with resistance but ESNs have higher homozygosity for KIR2DL3 HLA-C-1 [175,179].
	Increased IL-6, IL-8 and TNFα in the ESNs compared with HC, CI and SR. Increased IFNα in the untreated CI [10].
	Enhanced IFNγ, TNFα production and degranulation by CD56dim NK cells in ESNs [181]. No baseline differences in NK cells between ESNs and SPs [175].
Anti-D Cohorts	Decreased monocyte counts in ESNs. Increased IL-8 and IL-18 in ESNs. Enhanced NK cell function - greater IFNγ production [187].
	No significant increase in NKp30, or changes in CD56bright or CD56dim NK cell counts. No difference in degranulation but CD56dim NK cells produced more IFNγ when stimulated [187].

Table 3.1. Summary of results from previous ESN studies

Chapter hypothesis

An enhanced innate immune response protected ESN women from the Irish anti-D cohort from infection with HCV.

Chapter aims

1. To identify and recruit HCV susceptible and resistant women from the Irish anti-D cohort.
2. To assess the adaptive response to HCV in our recruited donors.
3. To compare the whole blood response to a panel of viral ligands in HCV susceptible and resistant women.

Results

Identifying ESN women in the Irish anti-D cohort

Six of the twelve contaminated batches contained significant viral loads (batch numbers 246, 238, 245, 237,252 and 250; **Fig. 3.1a**). Between 31% and 71% of the recipients of vials from these batches were antibody positive. As indicated in Figure 1, 682 (53%) women tested positive for HCV antibodies indicating that they had been infected. However, 611 (47%) women who received a vial of anti-D from a high risk, highly infectious batch, tested negative for both HCV RNA and anti-HCV antibodies suggesting that they had resisted infection (**Fig. 3.1b**). These women had all been told at the time in 1993 that they had not been infected and would not be contacted again by the IBTS.

a

Anti-D Batch	Genomes/Vial	Vials Issued	Vials Traced	No. Tested	No. Tested -ve	No. Antibody +	PCR +ve
246	12400	251	152	133 (53)	92 (69)	41 (31)	12 (9)
238	16000	371	281	258 (70)	70 (27)	187 (73)	106 (41)
245	56000	464	347	302 (65)	201 (67)	101 (33)	60 (2)
237	92000	430	247	225 (52)	64 (28)	160 (71)	75 (33)
252	100000	308	216	191 (62)	73 (38)	115 (60)	60 (31)
250	200000	296	228	191 (65)	111 (58)	78 (42)	43 (23)
Total	----	2120	1471 (69)	1300 (61)	611 (47)	682 (52)	356 (27)

b

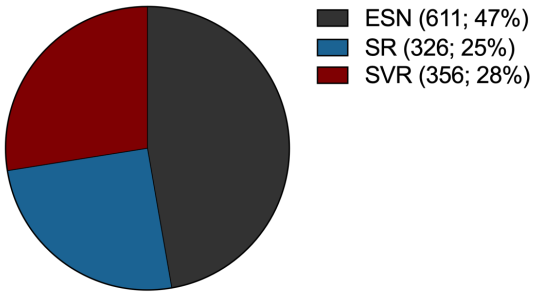


Figure 3.1. Infectivity rates of HCV contaminated anti-D from the Irish Blood Transfusion Service. *(a) Table obtained from the IBTS showing the six high risk batches of contaminated anti-D (see materials and methods). Figures in brackets are percentages. (b) Pie chart showing total numbers and percentages of exposed seronegative women (ESNs), spontaneous resolvers (SRs) and sustained virological responders (SVRs) from the six high risk batches.*

Recruitment of women who were exposed to contaminated HCV between 1977-79 the campaign & matching recruits to IBTS records

Ethics consultants advised that it would be unethical to approach any of the women who had been informed that they would not be contacted again by the IBTS. It was decided therefore to use a national communications campaign to invite these women to take part in our study Following the national media recruitment campaign, approximately 700 women volunteered to participate (**Fig. 3.2**). On screening these 700 volunteers by phone, 450 were deemed eligible to participate and were sent a study pack containing further information about the study, a saliva collection kit for later DNA extraction, a consent form and a pre-addressed and stamped envelope to return the completed pack. Of the 450 sent out, 395 (88%) were returned. Once participants had returned their completed consent form, matched batch records were obtained from the IBTS. Of the 395 study packs returned, batch records were available for 234 members of the cohort. These included 34 ESN donors who received anti-D from one of the highly infectious batches, 48 donors who spontaneously resolved infection and 50 recipients who had a previous chronic infection (now SVR having cleared HCV with therapy)[190]. We grouped the SR and SVR individuals together as seropositive (SP) as they had serological evidence of past infection. We also recruited 102 women who received an uncontaminated batch of anti-D in the same period (unexposed controls; UC).

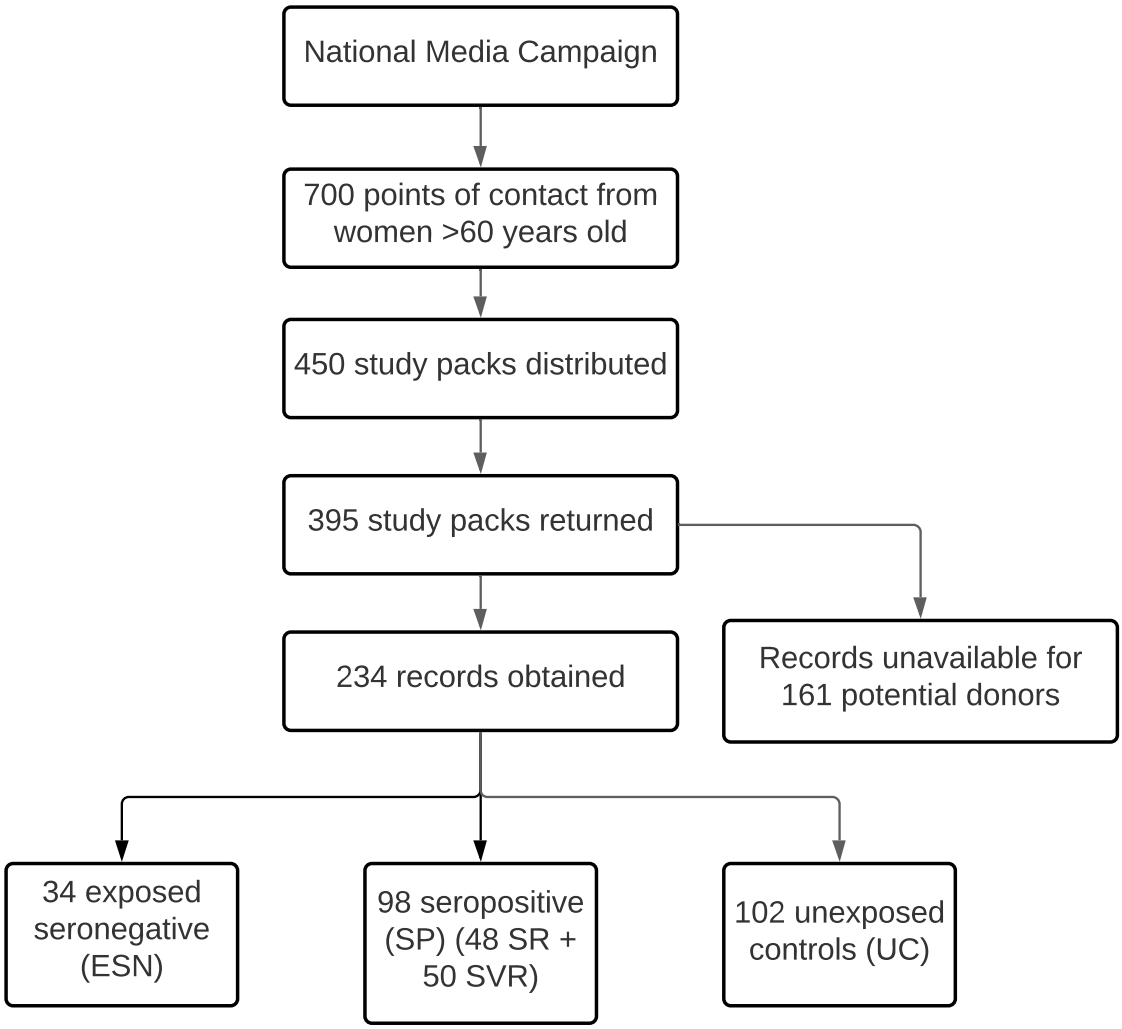


Figure 3.2. Summary of recruitment process and overview of final cohort. *A national campaign was run to recruit women who received anti-D between 1977-79 to our study. 700 individuals contacted the group about the study. 450 study packs were sent to eligible participants. 395 study packs were returned and batch details were retrieved and matched for 234 women. 102 women who received an uncontaminated batch, 34 ESN and 98 SP with details of batch records were recruited.*

Cohort characteristics

Basic demographic and clinical data were collected on the full cohort at the time of consent (**Table 3.2**). Both infection outcome groups, ESN and SPs, were all female, all Rhesus negative, were similarly aged and had all been pregnant. ESN women appeared to have a higher incidence of third level education compared with the SP donors (61% Vs 28%). SP donors reported increased incidence of liver disease compared to ESNs, despite lack of active HCV infection. SP donors also reported higher incidence of chronic fatigue and fibromyalgia. Clinically the two groups were otherwise similar.

Characteristic	Exposed Seronegative	Seropositive	Statistic
Total Recruited	34	98	-
Age (years; mean +/- SD)	71.6 +/- 5.1	71.6 +/- 4.95	ns
Education (no.>3rd level/no.<3rd level)	11/19	10/36	*
Clinical Questionnaire			
Acute Symptoms After Anti-D	4 (12%)	9 (9%)	ns
Liver Disease	1 (3%)	36 (37%)	****
Diabetes	3 (9%)	11 (11%)	ns
Osteoarthritis	11 (32%)	47 (48%)	ns
Rheumatoid Arthritis	2 (6%)	11 (11%)	ns
Chronic Fatigue	1 (3%)	55 (56%)	****
Fibromyalgia	2 (6%)	30 (31%)	**
Lichen Planus	1 (3%)	9 (9%)	ns
Inflammatory Bowel Disease	3 (9%)	20 (20%)	ns
Lupus	0 (0%)	8 (8%)	ns
Sjogren's Syndrome	2 (6%)	14 (14%)	ns

Table 3.2. Clinical characteristics of the full cohort. *Self-reported clinical data from ESN and SP donors in our recruited cohort. Differences were assessed using Chi-square tests.*

Sustained virological responders have persistent hepatitis C virus antibodies

To asess the cohort for anti-HCV antibodies we emloyed a virome wide antibody scan with the ability to detect IgG antibodies directed against 206 human pathogens and 115,753 epitopes (VirScan). Human serum containing antibodies was incubated with a bacteriophage library expressing peptides. Following incubation, antibodies complexed to bacteriophage were isolated using magentic beads, lysed and sequenced to identify the IgG target epitope (**Fig. 3.3a)**. Sequence data was aligned and processed to generate a "virusHit" for each viral species. A "virusHit" is the number of significant epitopes of a virus species compared to an experimental negative control. To test for differences in antibodies between ESN, SR, SVRs and UCs we performed a one way non-parametric ANOVA adjusting for multiple testing using an FDR correction ($q<0.01$). All viral antibodies with the exception of HCV were similar between groups. SVR donors had the highest level of HCV antibody positivity (**Fig. 3.3b**). In line with published data from conventional serological assays the most frequently targeted viruses in our cohort included Epstein-Barr virus (EBV; herpesvirus 4), cytomegalovirus (CMV; human herpesvirus 5), herpresvirus 1 and Rhinovirus A (**Fig. 3.3b**). 48.81% of donors were anti-CMV antibody positive (**Fig. 3.4a**). 86.9% of donors were seropositive for antibodies against HSV-1 (**Fig. 3.4b**). 96.43% of donors were seropositive for antibodies against EBV (**Fig. 3.4c**).

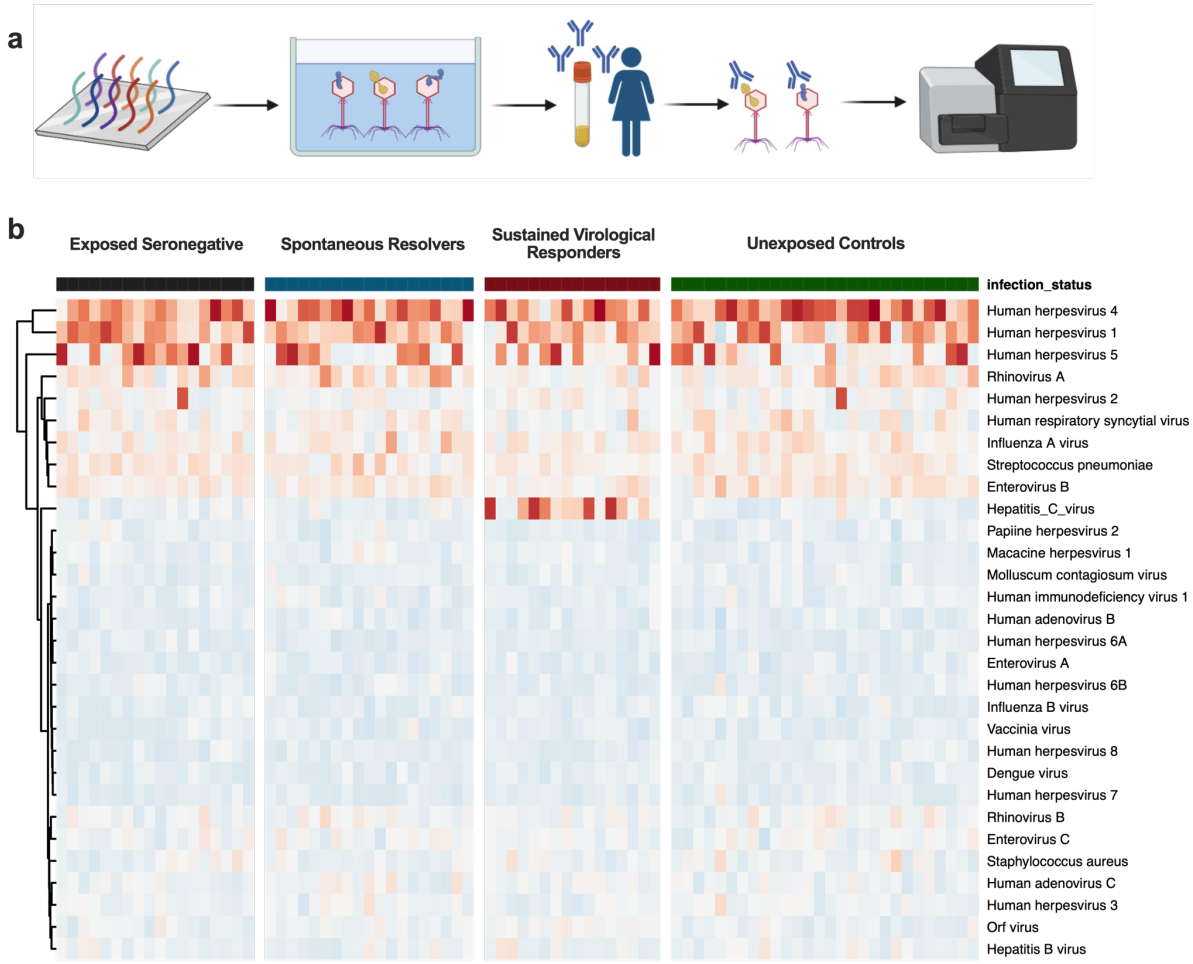
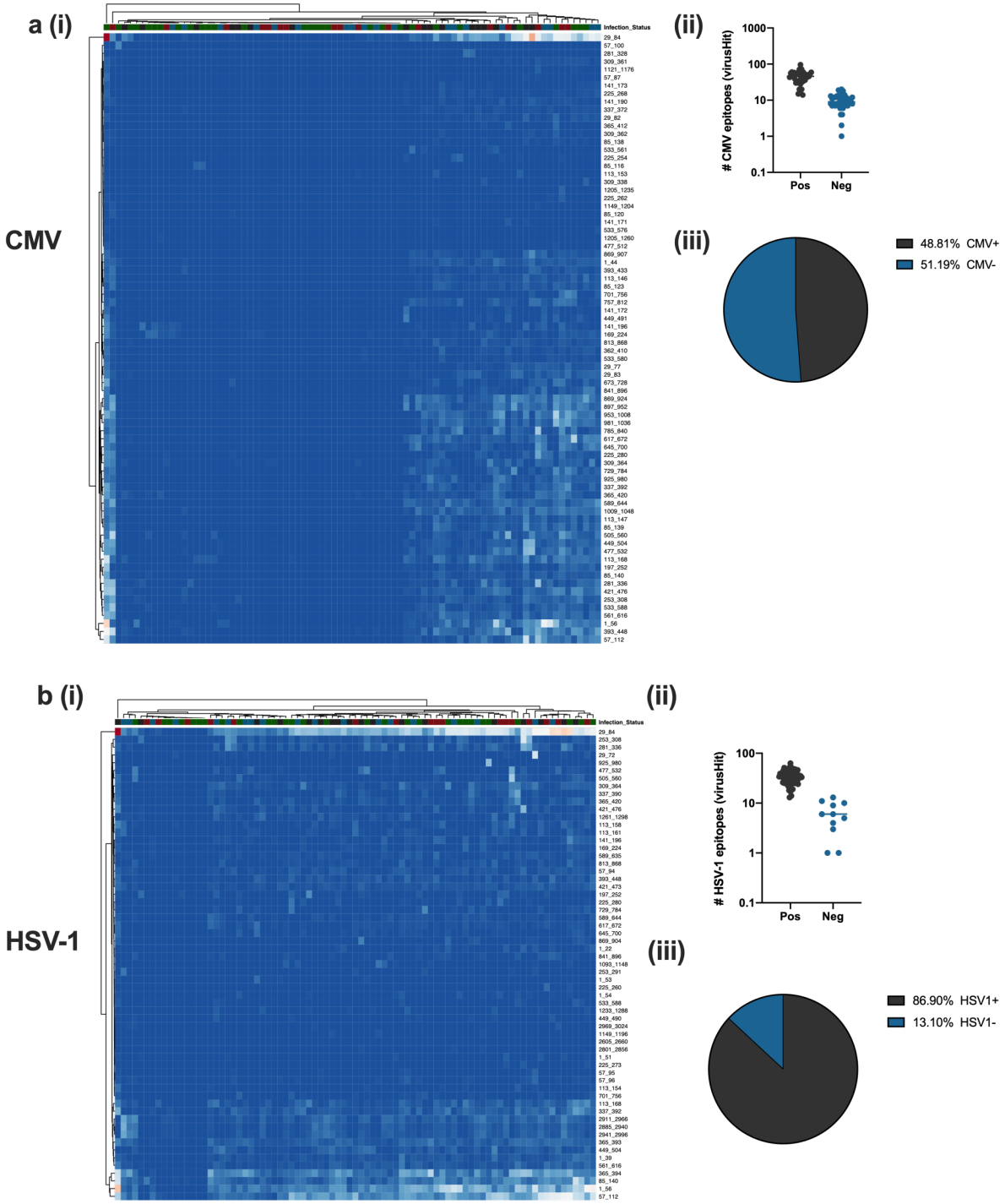


Figure 3.3. Global profiling of antibodies from the viral resistance cohort. *(a) Overview of the workflow for VirScan. (b) Heatmap showing the top 30 virus hits in samples from our cohort. Red indicates high expression, while blue represents low expression. Comparisions between ESNs, SRs, SVRs and UCs were made using Kruskal-Wallis tests followed by FDR correction (q>0.01).*


a (i)
(ii)
CMV
(iii)
Infection_Status
CMV epitopes (virusHit)
1000
100
10
1
0.1
Pos
Neg
48.81% CMV+
51.19% CMV-
b (i)
(ii)
HSV-1
(iii)
Infection_Status
HSV-1 epitopes (virusHit)
100
10
1
0.1
Pos
Neg
86.90% HSV1+
13.10% HSV1-

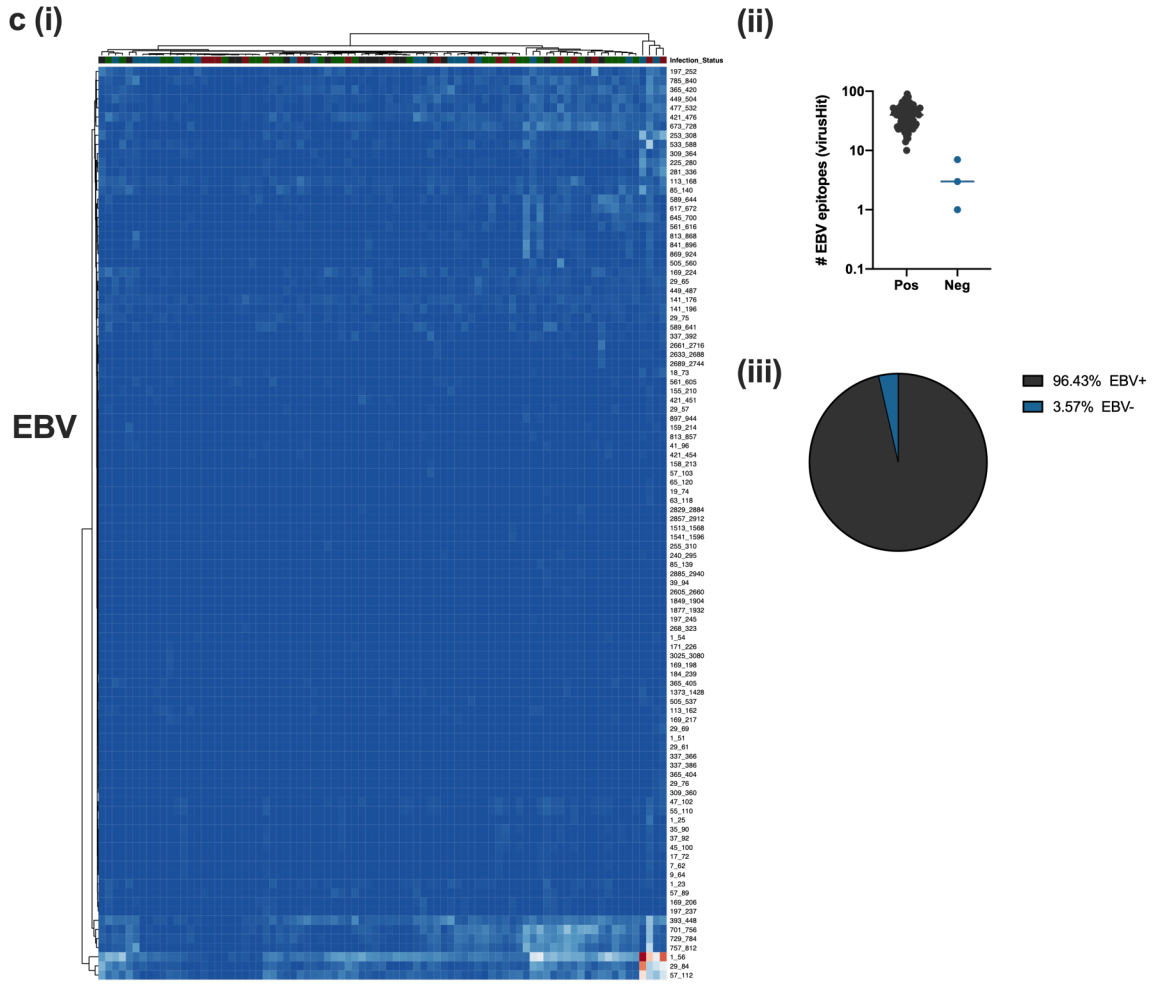


Figure 3.4. Heatmaps of common viruses in the cohort. *VirScan was used to assess the history of viral infection in our cohort. (a(i)) Heatmap of epitope Z scores for CMV. (a(ii, iii)) dot plot and (iii) pie chart showing the number and percentage of CMV antibody positive and negative donors. (b(i)) Heatmap of epitope Z scores for HSV-1. (b(ii, iii)) dot plot and (iii) pie chart showing the number and percentage of HSV-1 antibody positive and negative donors. (c(i)) Heatmap of epitope Z scores for EBV. (c(ii, iii)) dot plot and (iii) pie chart showing the number and percentage of EBV antibody positive and negative donors. The name of the epitopes is a combination of the UniProt entry and the start and end position. The color intensity is representative of the number of epitopes targeted, with red being high and dark blue being low.*

SRs and SVRs target different HCV epitopes

VirScan enables more nuanced analysis of which epitopes are tageted by antibodies. We sought to investigate what individual epitopes were targeted in our infection groups. At an epitope level, Z scores were used to show relative quantification, wherein a higher Z score is related to a higher antibody titre. In total there are 3,382 HCV epitopes in the VirScan library. As some epitopes targeted are shared across several HCV genotypes, we merged those with the same start and end site by adding the Z score for each genotype together. Individual HCV epitopes are shown as a heatmap (**Fig. 3.5a)**. Epitopes, including 2325-2380 and 2745-2800, found in the age matched RhD-negative UCs were thought to be spurious hits due to cross reactivity. Based on the heatmap, 3/19 (16%) SRs appeared to have anti-HCV antibodies, while 16/17 (84%) of SVRs appeared to be anti-HCV antibody positive. Interestingly, there are some epitopes, including 29-84 that are shared between both SVRs and SRs, while antibodies against other epitopes including 57-112, 309-364 and 2157-2212 are only found in the SVRs (**Fig. 3.5a**).

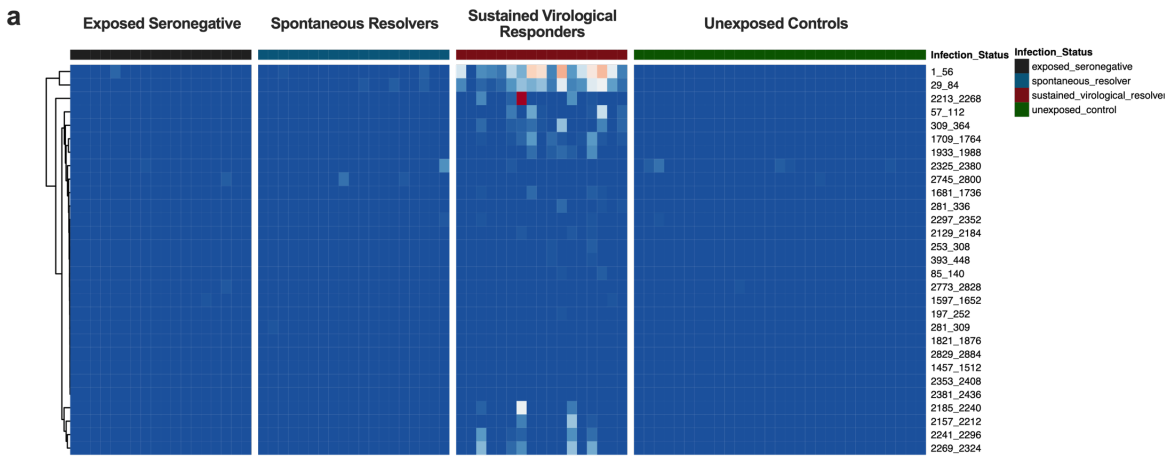


Figure 3.5. Differences in HCV epitopes targetd by spontaneous resolvers and sustained virological responders*. (a) Heatmap of summed Z scores from all HCV genotypes with overlapping epitopes. The name of the epitopes is a combination of the UniProt entry and the start and end position. The color intensity is representative of the number of epitopes targeted, with red being high and dark blue being low.*

HCV specific T cells are detectable in SR and SVR donors

Some studies have shown that clearance of HCV in the absence of antibodies is achieved by a potent T cell resposne. HCV specific T memory cell responses can persist for years following viral exposure, while antibody levels wane after about 20 years. Here we wanted to check for HCV specific T memory cells in our cohort using ELISpot IFNγ assays. We used an HCV peptide mix and a peptide pool of CMV, EBV and Flu (CEF) virus peptides as a positive control. Using a standardised cut-off derived from the UC group of the mean + 3 x the standard deviation we found detectable T cell responses in both the SR and SVR groups. We did not see any HCV specific IFNγ T cell response in the ESN cohort (**Fig. 3.6**). All four infection groups responded similiarly to the CEF peptide pool.

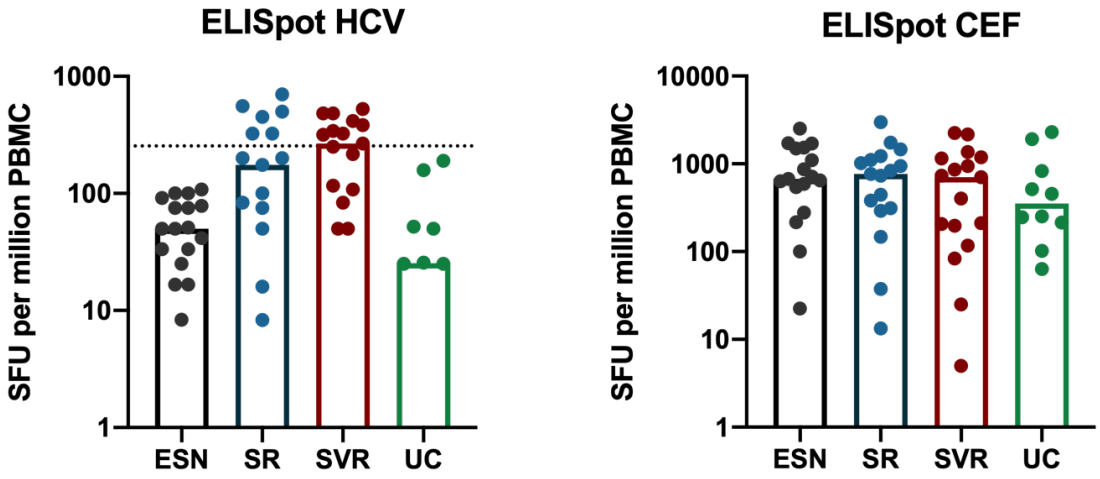


Figure 3.6. ELISpot analysis of HCV specific T cells. *IFNγ producing T cell counts per 10^6 PBMCs in ESNs, SRs, SVRs and UCs following stimulation with ProMix™ HCV and CEF peptide pools shown on a log_{10} axis. The dashed line represents the mean + 3xSD of the UC group. The median per group is shown. Details of peptide pools used are shown in Appendix table 1.*

Innate immune stimulation of whole blood induces gene expression.

Fresh whole blood from ESNs (n=18) and SPs (n=36) was stimulated using the TruCulture system to assess potential differences in the induced immune responses between ESN and SP volunteers (**Fig. 3.7a**). A panel of viral agonists with a focus on the induction and regulation of type I interferon (IFN-I) related antiviral immunity was chosen: IFNα2, which acts via the IFNAR1/2 complex to induce a direct IFN-I response; R848 via TLR7/8, as well as polyIC which acts via TLR3 and RIG-I to induce an indirect IFN-I response. Cell pellets and supernatants were collected from stimulated whole blood for later gene expression and cytokine analysis. As expected, the selected stimuli induced changes in immune gene expression. The unstimulated control separated from the stimulated conditions along PC1, which accounted for 33% of the variance in the data (**Fig. 3.7b**). Induced gene expression changes showed stimulus-specific patterns. R848 was the most potent stimulus and clustered separately from the other two stimuli along PC2, accounting for 10% of the variance (PCA; **Fig. 3.7b**). IFNα2 and polyIC clustered separately along PC4, accounting for 5% of the variance. A heatmap showing the differential gene induction between the 4 stimuli is shown in **Fig. 3.7c**.

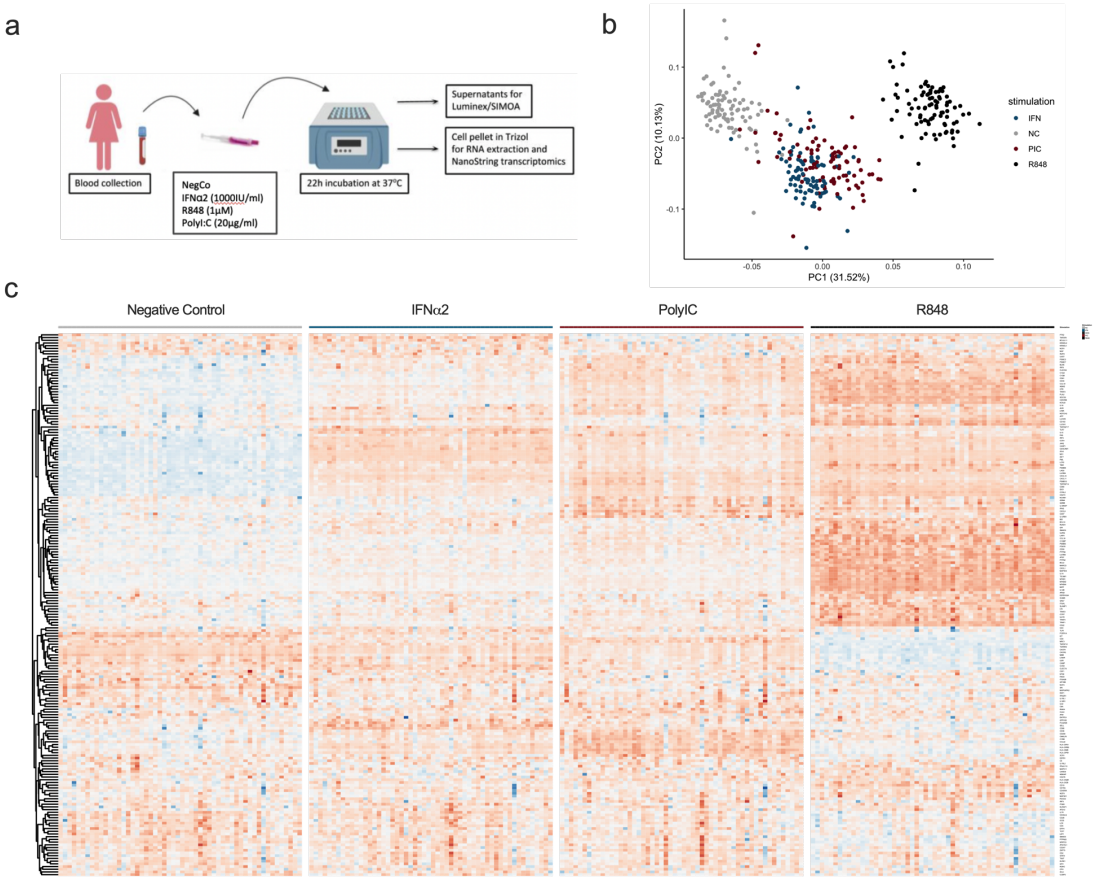


Figure 3.7. Innate immune stimuli induce changes in gene expression in whole blood from all donors. *Whole blood from ESN (n=18) and SP (n=36) women was stimulated with a panel of viral ligands (R848, polyIC, IFNα2). (a) Overview of experimental workflow. (b) Principal component analysis (PCA) plot of log_2 normalised NanoString transcriptomic data. The percentage variance captured in PC1 and PC2 are indicated in brackets. Each point on the plot represents a single donor for the colour denoted stimulation. (c) Heatmap of NanoString transcriptomic data (n=200 genes) showing negative control (NC) and stimuli induced genes for IFNα2, polyIC and R848.*

Gene expression in unstimulated whole blood from ESN and SP women.

A key question in this study was whether there were any differences in immune gene expression at baseline between ESN vs SP participants. We found that in the absence of stimulation, gene expression in the ESN and SP groups was similar, with no significant differences observed between groups, following FDR correction ($q>0.1$; **Fig. 3.8a**). To further analyse more specific components of the innate immune response, and given the relatively small size of our cohort, we employed previously defined gene signatures. These have proven to be useful in defining stimulus specific differences in small cohorts. Gene signatures associated with specific responses were previously defined in a separate healthy control cohort by the MIC using the same TruCulture and NanoString workflow [164]. By applying these gene signatures to our unstimulated samples, we saw similar baseline IL1β, IFN-I, IFNγ and TNFα – specific gene signatures in our ESN and SP groups, suggesting that baseline differences in the parameters examined are not responsible for the differences observed in infection outcome (**Fig. 3.8b-e**). To probe the basal immune response further we also assessed plasma cytokine levels from ESN and SP donors. Following FDR correction, we saw no significant differences in plasma cytokines between ESN and SP donors (**Fig. 3.9**).

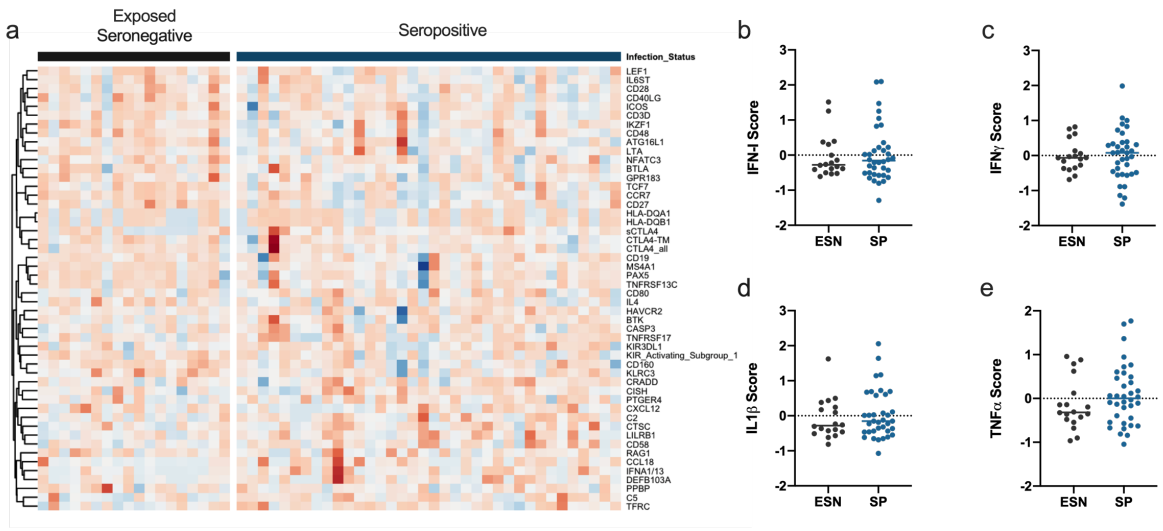


Figure 3.8. Gene expression was similar in unstimulated whole blood from ESN and SP women. *Unstimulated whole blood was incubated at 37°C for 22 hours. (a) Heatmap showing the top 50 genes with the lowest q values when comparing the ESN and SP donors in the null condition (q<0.99). (b-d) Gene signature scores for (b) IFN-I, (c) IFNγ, (d) IL1β and (e) TNFα in the unstimulated null condition for the two infection groups. Data are presented with median shown as a solid line. Comparisons between ESNs and SPs were made using Mann-Whitney U tests.*

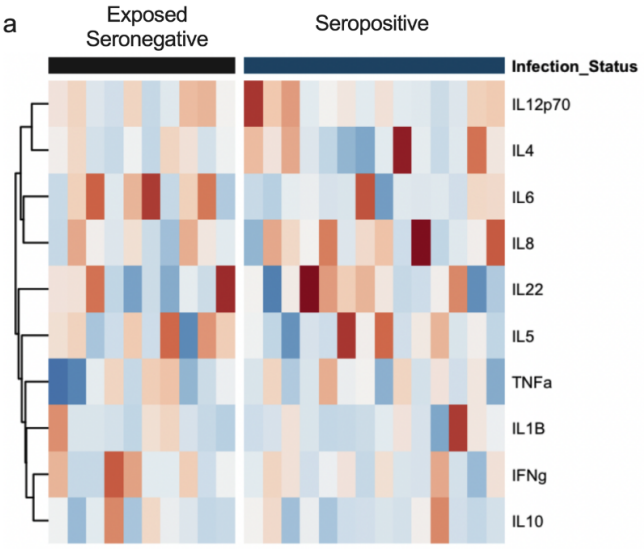


Figure 3.9. No difference in plasma cytokine levels between ESN and SP donors. *(a) Heatmap of plasma cytokines quantified using the Quanterix Corplex panel. Groups were compared using Mann-Whitney U tests with FDR correction.*

HCV resistant women have an increased IFN-I gene signature in response to poly IC stimulation but not to R848 or IFNα2.

TLR3, TLR7 and IFNAR1/2 induced responses, particularly those of type I interferon associated antiviral immunity have been shown to be important in the control of HCV infection. We therefore focused on activation of these key antiviral pathways in our cohort. We compared induced gene expression following stimulation with either polyIC, R848 and IFNα2 between ESN and SP donors. Overall gene expression of ESN and SP donors was similar, following FDR correction (**Fig. 3.10a, b, c**). We then focused our analysis on the IFN-I gene signature for all stimuli. ESN donors and SP donors were similar after R848 and IFNα2 stimulation (**Fig. 3.10d, e**). Interestingly however, we saw a significantly increased IFN-I signature score following polyIC stimulation in the ESN group when compared to the SP donors ($p<0.05$; **Fig. 5f**). A heatmap showing the genes included in the IFN-I signature score for the polyIC condition is shown in **Fig 3.11a**. To further examine the polyIC response we compared gene signature scores for IL1β, TNFα and IFNγ, however we saw no significant differences between ESNs and SPs (**Fig. 3.11b, c, d**). The increased polyIC IFN-I signature in ESNs was confirmed using a secondary gene signature from the MolSigDB Hallmark Gene Set (**Fig. 3.12**).

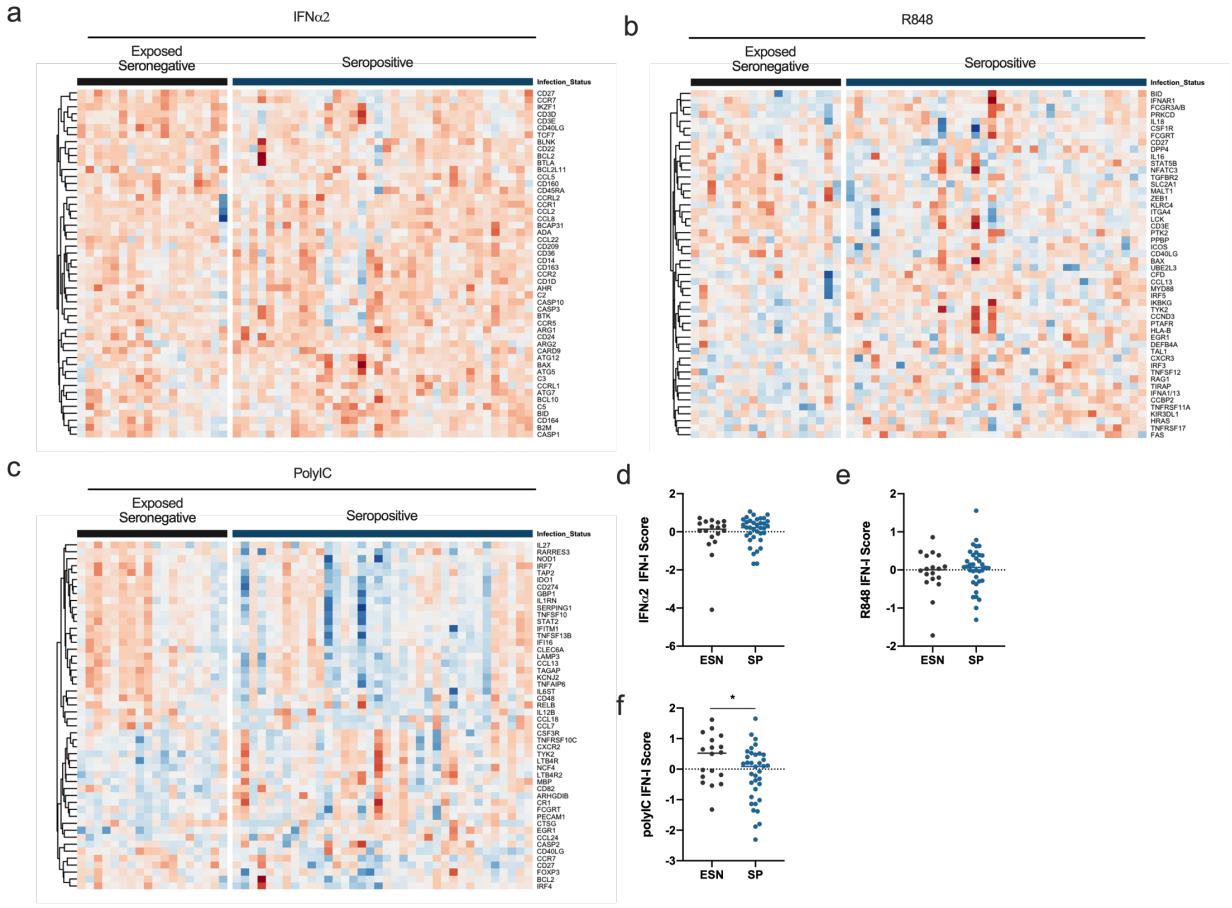


Figure 3.10. PolyIC induced higher IFN-I responses in exposed seronegative women. *Fresh whole blood was stimulated with IFNα2, R848 or polyIC for 22 hours at 37^{o}C and changes in gene expression assayed using NanoString transcriptomics. (a-c) Heatmaps showing the top 50 genes with the lowest q values when comparing the response to stimulation between ESN and SP donors. (a) IFNα2, p value < 0.16, q value (FDR adjusted p value) < 0.99 (b) R848, p value < 0.15, q value < 0.99, (c) polyIC, p value < 0.06, q value < 0.65. (d-f) MIC derived IFN-I score in ESN and SP donors following stimulation with (d) IFNα2 (e) R848 and (f) polyIC (*, p<0.05). Data are presented with median line reported as a solid line. Comparisons between ESNs and SPs were made using Mann-Whitney U tests (*, p<0.05).*

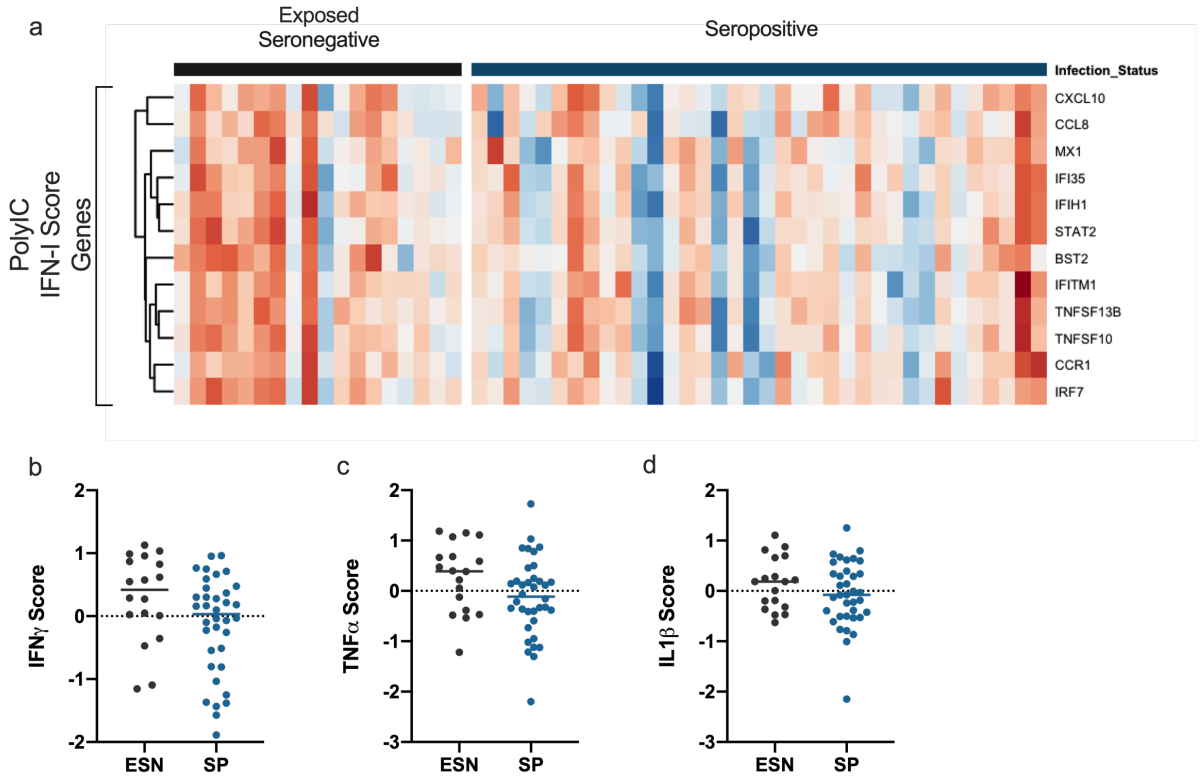


Figure 3.11. Expression of IFN-I genes after polyIC stimulation. *Whole blood was stimulated with polyIC and gene expression was quantified using NanoString transcriptomics. (a) Heatmap of genes used to calculate the MIC polyIC induced IFN-I score. (b-d) MIC derived gene signature scores for the polyIC stimulated condition: (b) IFNγ (c) TNFα and (d) IL1β. Data are presented with median line reported as a solid line. Comparisons between ESNs and SPs were made using Mann-Whitney U tests (p>0.05).*

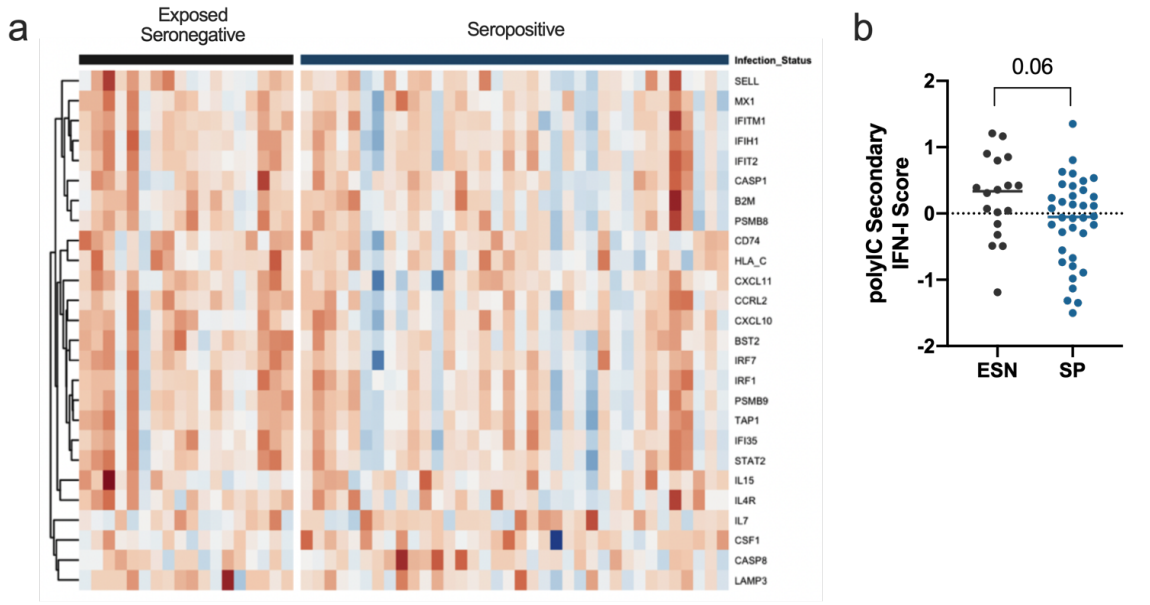


Figure 3.12. Increased secondary IFN-I signature in ESNs compared to SPs. *A secondary IFN-I signature score was calculated based on gene expression from polyIC stimulated whole blood. (a) Heatmap showing genes in the MolSigDB Hallmark IFN-I response signature in the polyIC condition. (b) Plot of MolSigDB IFN-I signature in the ESN and SP donors. Median line is shown as a solid line. Comparisons between ESNs and SPs were made using Mann-Whitney U tests.*

ESN women have increased cytokine production following polyIC stimulation

We also measured polyIC induced cytokines known to be important in the control of HCV infection using a Luminex multiplex assay. Several cytokines including CCL8, CXCL11, CCL2 and IL-6 were found to be significantly increased in whole blood from ESN women stimulated with polyIC compared to stimulated blood from SP women (**Fig. 3.13a**). To assess whether the altered IFN-I score was due to changes in IFN-I protein levels we quantified IFNα2 and IFNβ using Simoa digital ELISA. No significant differences in IFNα or IFNβ protein were observed between infection groups (**Fig. 3.13b, c**). IFNα and IFNβ positively and significantly correlated with the polyIC induced IFN-I score (**Fig. 3.13d, e**).

Differences in immune cell numbers in whole blood might account for the increased IFN-I signature observed. Using flow cytometry we quantified the major circulating immune cell populations in whole blood. However, we saw no significant differences in the numbers of immune cells measured between ESN and SP donors, including in key immune cells involved in the antiviral innate immune response such as natural killer cells and monocytes (**Fig. 3.14**). We also hypothesised that the increased polyIC IFN-I signature in the ESN women may have been due to altered baseline components of the polyIC signalling pathway or negative regulator expression in the null condition. We saw no significant differences in the expression of any of these components (**Fig. 3.15**; **Fig. 3.16**). Taken together, these data reveal an increased polyIC induced IFN-I gene signature, with increased polyIC induced IFN related protein cytokines independently of IFN-I protein secretion or differences in cell numbers.

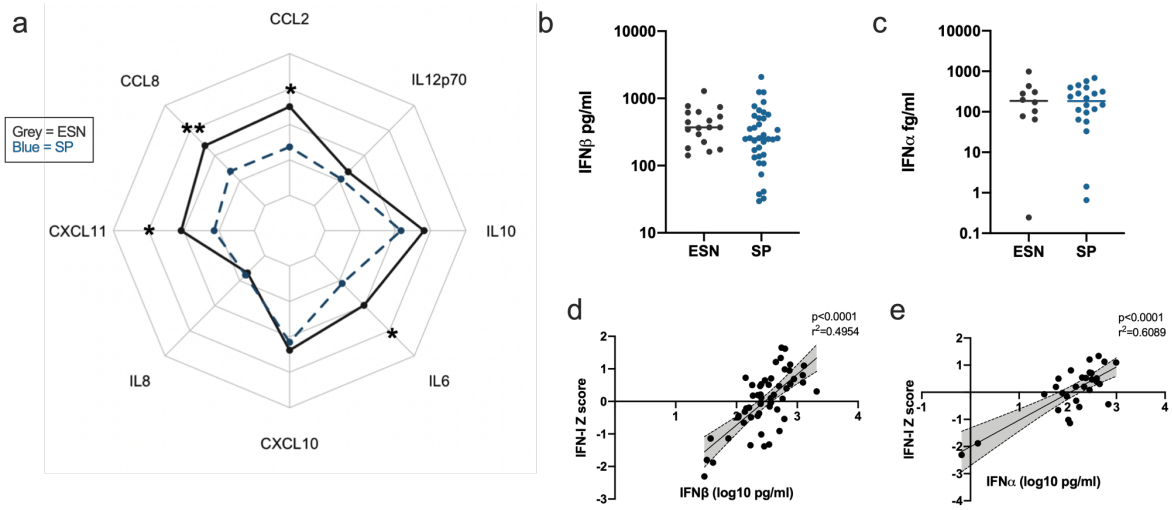


Figure 3.13. Inflammatory cytokine production is increased in polyIC stimulated blood from ESN women. *Secreted cytokines from polyIC stimulated whole blood were quantified using Luminex and Simoa. (a) Spider plot of polyIC induced cytokines differentially expressed between ESN and SP donors (*, p<0.05, **, p<0.01). (b, c) IFNβ (b) and IFNα (c) protein levels measured using Simoa digital ELISA in supernatants from ESN and SP women after polyIC stimulation. Data are presented on a log_{10} scale with median line reported as a solid line. (d, e) Correlation between IFNβ (d) and IFNα (e) protein levels and the polyIC induced IFN-I score. For Luminex and IFNα Simoa n = 10 ESN, 20 SP, for everything else n = 18 ESN, 36 SP. Comparisons between ESNs and SPs were made using Mann-Whitney U tests (* p<0.05, ** p<0.01).*

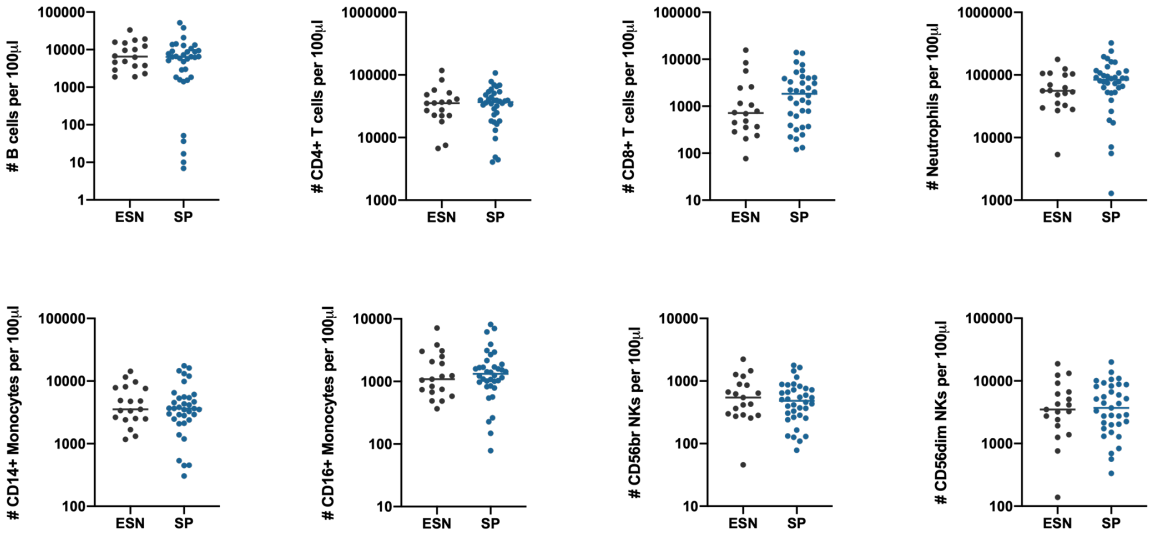


Figure 3.14. Immunophenotyping of whole blood. *Major circulating immune cell populations in whole blood were quantified using flow cytometry. Data is presented as counts per 100µl on a log_{10} scale with median shown as a solid line. Comparisons between ESNs and SPs were made using Mann-Whitney U tests ($p>0.05$).*

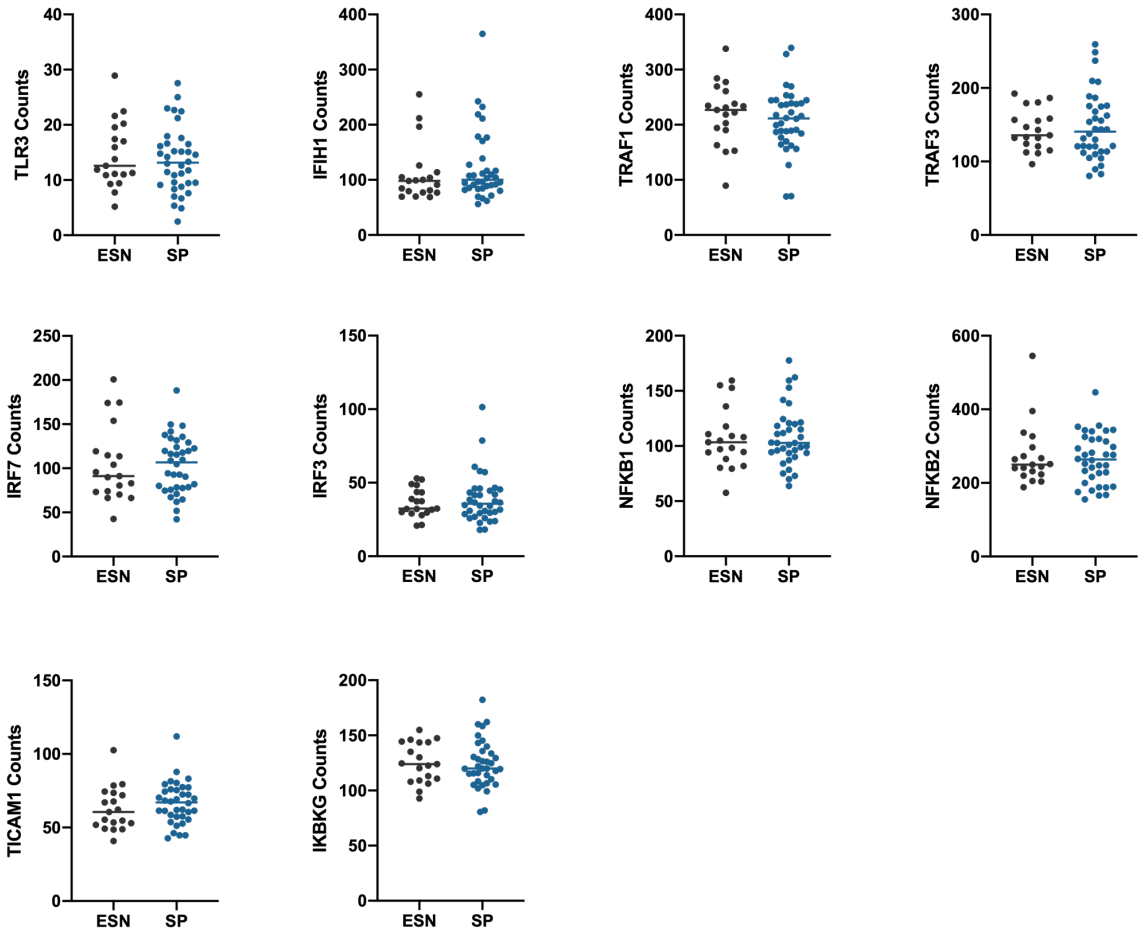


Figure 3.15. Expression of TLR3 components in the unstimulated condition. *NanoString transcriptomics was performed on unstimulated whole blood. Represented here are the available components of the TLR3 signalling pathway. Data is presented as counts with median shown as a solid line. Mann-Whitney U tests were used to compare counts between ESN and SP donors (p>0.05).*

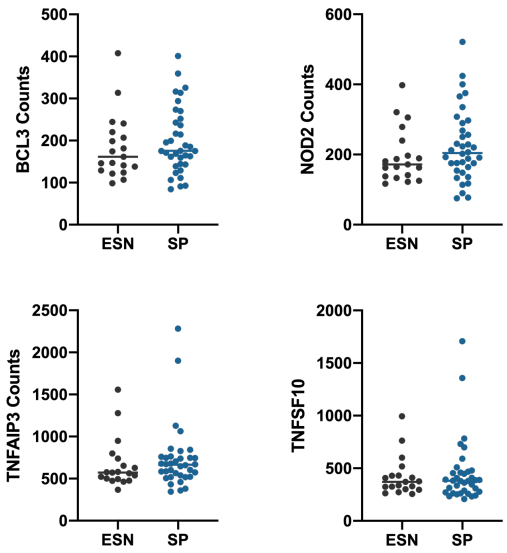


Figure 3.16. Expression of negative regulators of TLR3 in unstimulated whole blood. *NanoString transcriptomics was performed on unstimulated whole blood. Represented here are the available negative regulators of the TLR3 signalling pathway. Data is presented as counts with median line shown. Mann-Whitney U tests were used to compare counts between ESN and SP donors (p>0.05).*

Discussion

In the present study we identify ESN women in the Irish anti-D cohort for the first time. We reveal an enhanced polyIC induced IFN-I gene signature in these women compared to SP donors. We also uncover increased polyIC induced cytokine production in ESN women compared to SP women. This enhanced polyIC responsiveness may have been protective against HCV infection.

Individuals who have been exposed to a virus but remain uninfected are critical to understanding the mechanism of protection against viral infection. However, systematic identification and recruitment of ESN individuals are major obstacles to studies of viral resistance. This is possibly why levels of resistance to any viral infection have never been defined. Indeed dogma in the HCV field holds that up to 80% of people who are infected with HCV become chronically infected [167]. In this study, analysis of records from the women who received vials of highly infectious batches of HCV contaminated anti-D, but remained PCR negative and Ab negative, suggest that up to 50% of women in our Irish cohort might be resistant to HCV infection. This has major public health considerations as it is likely that HCV infectivity, as well as infectivity of other viral infections, is overestimated if individuals without a detectable antibody response are overlooked.

Other studies of viral resistance to HCV have focused primarily on individuals who engage in high risk behaviour rather than exploring potential viral resistance in healthy populations . These studies estimate exposed seronegativity to be more in the region of 10% [10]. However, these cohorts are typically more heterogenous than ours often including mixed sexes, age groups and ethnicities and viral origin. A key point to emphasise is that our cohort is entirely female, and it is well documented that females have greater resistance to infection by several viruses, including HCV [191]. Indeed, previous work by our group has shown sex specific differences in spontaneous resolution of HCV [84]. Furthermore, follow up work involving our group on the Irish anti-D cohort showed reduced HCV related disease in women who were still chronically infected, suggesting that females also experience milder HCV related illness than males [169].

Using VirScan, we found that SVR women had the highest level of anti-HCV Ab positivity. This is to be expected as SVRs cleared infection more recently than SRs. We saw no differences in Ab positivity for other viruses between our groups. Interpretation of our ELISpot results is difficult due to the relatively high background signal in unexposed control donors. However, is appears that anti-HCV T cell responses were detectable in both SRs and SVRs, and that SRs had a higher level of HCV-specific T cell positivity than Ab positivity. This is in line with previous reports showing that detectable HCV specific T cell responses persist longer than Abs [101].

Here, we found an increased polyIC IFN-I signature in whole blood from our viral resistant ESN women compared to SP individuals. As SP women have been stratified as 'virus susceptible' they are a natural comparison group to our virus resistant ESN women. All SP donors in our cohort successfully cleared HCV infection. It appears as though viral clearance following acute infection or therapy restores 'normal' innate immune homeostasis [112]. Whether this is also the case for the adaptive immune response is unclear [113,192]. Work in this area is ongoing and is the subject of additional studies. Given the heterogeneity in the wider population, inclusion of an additional UC group in this study would detract from the power of our cohort, as it would not be possible to determine how control women would be stratified as a result of response to viral infection.

The enhanced polyIC responsiveness was also seen in a second IFN-I gene signature from MolSigDB and at the protein level, with greater upregulation of several key cytokines known to be important in the control of HCV observed, including IL-6, CCL8, CXCL11 and CCL2. We saw no difference in plasma concentrations of inflammatory cytokines. This is in contrast to previous ESN studies, however, given the unique homogeneity of our cohort and the confounding factors typically associated with other ESN studies it is not surprising that our results differ.

The increased polyIC induced IFN-I score could not be explained by differences in circulating immune cell populations or by differences in the gene expression of baseline components of the TLR3-RIG-I pathways or negative regulators. Nor could they be explained by differences in induction of IFN-I proteins. It is possible that the increased IFN-

I signature in the ESN group could be attributed to differences in the methylation status or phosphorylation of key downstream signalling proteins [193].

The TLR3 pathway has been shown to be key in controlling HCV infection, and single nucleotide polymorphisms (SNPs) in proteins associated with this pathway have previously been associated with increased susceptibility to HCV infection [194,195]. In particular, the induction of IFN-I by TLR3 is key to rapid clearance of viral infection. Impaired TLR3 mediated induction of IFN-I through loss of function mutations has rendered individuals susceptible to otherwise innocuous infections. It is therefore plausible that host genetic factors associated with variability in the TLR3 mediated response could explain the ESN phenotype observed in our study. Indeed, a polymorphism in TLR3, rs3775291, has previously been associated with resistance to HIV infection in a Spanish cohort [196]. Investigations into the genetic factors associated with the TLR3 response will be the subject of further studies.

Further work in an independent cohort will strengthen the observations made in the present study, and ongoing studies are focused on understanding the observed difference in the polyIC IFN-I response.

Here, for the first time we report ESN individuals, previously overlooked, in the Irish anti-D cohort. We suggest that resistance to HCV infection in females may be up to 5 times greater than previous studies indicate. We uncover enhanced polyIC responsiveness at protein and gene levels in stimulated whole blood from women enrolled in our study. Our findings of increased resistance amongst females have major public health implications. Our biological findings reaffirm the importance of IFN-I in the control of viral infection and begin to provide evidence that heterogeneity in the human immune response has real world effects that may translate to differential susceptibility to infection.

Chapter 4: The whole blood immune response and spontaneous resolution of hepatitis C virus infection

Abstract

In many people, Hepatitis C virus (HCV) infection results in rapid immune activation followed by a profound period of immunosuppression. Spontaneous resolution occurs in approximately 30% of HCV infections, mediated by an effective polyclonal adaptive immune response. Features of adaptive immunity underlying spontaneous resolution (SR) of infection have been well characterised; however the role of innate immune activity remain to be fully elucidated. We sought to examine the role of the whole blood innate immune response in spontaneous clearance of HCV in women recruited from the anti-D cohort recruited to our study. We used the TruCulture whole blood *ex vivo* stimulation assay, coupled with gene expression analysis and quantification of cytokine secretion by Luminex and SIMOA. At baseline, and following stimulation with polyIC, R848 and type I interferon, transcriptomic and protein levels were similar in SRs and women who were chronically infected (now cured following therapy; sustained virological response; SVR) and women who appeared to clear infection without involvement of the adaptive immune response (exposed seronegative; ESN). However, following stimulation with ODN, a TLR9 ligand, we found that SRs had reduced upregulation of several immune genes when compared with either ESNs or SVRs. To further interrogate this differential response, we used cytokine signature scores, previously defined in a separate cohort of healthy individuals using the same assay system, and found a reduced type I interferon (IFN-I) response score in SRs compared with the SVR and ESN donors. The signature scores for IFNγ, TNFα and IL1β responses were similar in all groups. The difference in IFN-I was recapitulated using gene set enrichment analysis. We also saw reduced IFNα protein production in response to ODN in SRs compared to SVRs. pDC counts and activation states were similar between infection groups. These findings point towards a decreased TLR 9 mediated IFN-I response in women who spontaneously resolved HCV infection. This phenotype could be the result of prior-HCV infection or reflect a phenotype which pre-dates HCV exposure and contributed to spontaneous resolution.

Introduction

While 47% of donors in the anti-D cohort exposed to high risk batches of HCV contaminated anti-D immunoglobulin appeared to resist infection, the remaining 53% either spontaneously resolved infection (28%) or developed chronic infection (25%). In the previous chapter we grouped both SRs and previously CI donors together as seropositive, since both had serological evidence of infection and were susceptible to HCV. While useful in interrogating differences between virus resistant and susceptible individuals, outstanding questions remain about whether or not differences exist between the TLR responses of SRs and SVRs separately. Chronically infected women and HCV SRs have been studied extensively since the first outbreaks were described in the early 1990s. This is because they were readily identifiable from cohorts known to have been exposed to HCV by conventional antibody assays. While much has been learned about the ability to spontaneously resolve HCV infection, the role of innate immunity has been largely overlooked. In part, this is due to a dearth of suitably sensitive technologies to overcome and limit technical noise [149]. In this particular chapter, we were interested in assessing differences between the IFN-I and TLR responses in SR donors.

TLRs and HCV infection

HCV infection is sensed by the endosomal TLRs 3,7/8 and 9, which detect dsRNA, ssRNA and CpG DNA respectively [61]. Ligation of these TLRs results in production of IFN-Is and subsequent IFNAR1/2 signalling via the JAK-STAT pathway [17]. Detection of HCV by these receptors results in a rapid innate immune response that is also important in shaping the adaptive antiviral immune response [17]. Numerous SNP association studies have highlighted the importance of TLR responses in SR and CI risk in HCV.

Several SNPs in TLR3, including rs18709026, rs3775291 and rs3775296 have been associated with HCV infection outcome. In particular, rs3775291 has been relatively well studied given its heterologous associations with multiple disease states. This SNP results in an amino acid substitution from leucine to phenylalanine at position 412 and increases TLR3 expression. The wild type (CC) genotype has been associated with reduced TLR3 expression and increased risk of progression to chronic HCV infection [195].

Rs5743836 in TLR9 has previously been associated with spontaneous resolution of HCV in females only [84]. This sexually dimorphic association is due to the SNP location in an estrogen response element in the TLR9 promoter region. Females are also known to have higher rates of spontaneous resolution of HCV than males, and this female specific TLR9 association may contribute [84,197].

In TLR7, the A allele of rs179008 has been associated with spontaneous resolution of HCV in both males and females, but with differing magnitudes [198]. This difference in magnitude of effect could be contributed to by the increased TLR7 expression described in females [199]. The TLR7 gene is located on the X chromosome and does not undergo X chromosome inactivation, consequently certain immune cell populations from females have higher expression of TLR7 compared to immune cells from males. This increased sex difference in TLR7 expression may also contribute to the increased SR of HCV described for females [199].

While these genetic studies have indicated a role for the TLR responses in SR and CI of HCV, differences in the biological TLR responses remain to be fully described.

Chapter hypothesis

SRs and SVRs exhibit differential responses to whole blood stimulation with viral ligands.

Chapter aims

1. To determine whether or not there are differences in the biological response to viral ligands between SR and SVR donors using NanoString.
2. To further assess potential differences using GSEA and gene signature scores
3. To compare the cytokine responses of SR and SVR donors

Results

The response to R848, polyIC and IFNα2 is similar in SRs and SVRs

Whole blood from SRs (n=19) and SVRs (n=17) was stimulated with a panel of viral ligands targeting TLR3 (polyIC), TLR7 (R848) and IFNAR1/2 (IFNα2). The response to stimulation was assessed using the NanoString human immunology panel v2. Following FDR correction, both baseline expression and the response to all three stimuli was similar between SRs and SVRs (**Fig. 4.1a, b, c, d**).

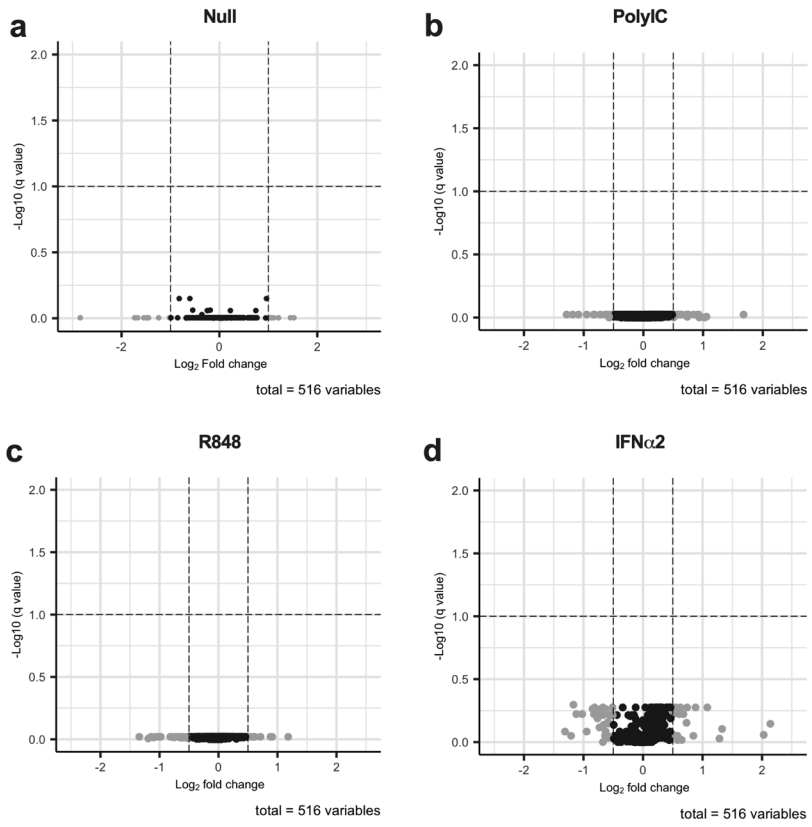


Figure 4.1. Responses to R848, polyIC or IFN responses are similar in SR and SVR donors. *Whole blood was stimulated with either R848, polyIC or IFNα2 for 22 hours using the TruCulture system. Gene expression was quantified using NanoString. (a-d) Volcano plots showing no significant differences in RNA expression between SRs or SVRs for (a) IFNα2, (b) polyIC or (c) R848 (q>0.1, Mann-Whitney U test with FDR correction).*

Spontaneous resolvers have a reduced TLR9 response

Additionally, whole blood was stimulated with ODN (CpG-A), a TLR9 agonist. NanoString transcriptomics was used to assess differences in the response to ODN between ESNs, SRs and SVRs. By non-parametric ANOVA, we observed differential expression of 69 genes between the three groups ($q<0.2$, **Fig. 4.2a**). Gene expression comparisons between ESNs and SRs showed no significant differences following FDR correction ($q>0.1$, **Fig. 4.2b**). However, comparisons between SRs and SVRs showed differential expression of 55 immune genes (**Fig. 4.2c**). This decreased response to ODN stimulation is further illustrated in a kinetic plot showing a reduction in the level of transcription in the SRs (**Fig. 4.2d**). PCA of ODN stimulated data (**Fig. 4.2e**) Gene set enrichment analysis of the transcriptional data showed significant enrichment for pathways involved in the adaptive immune response and IFN-I signalling in the non-SR groups (**Fig. 4.2f**).

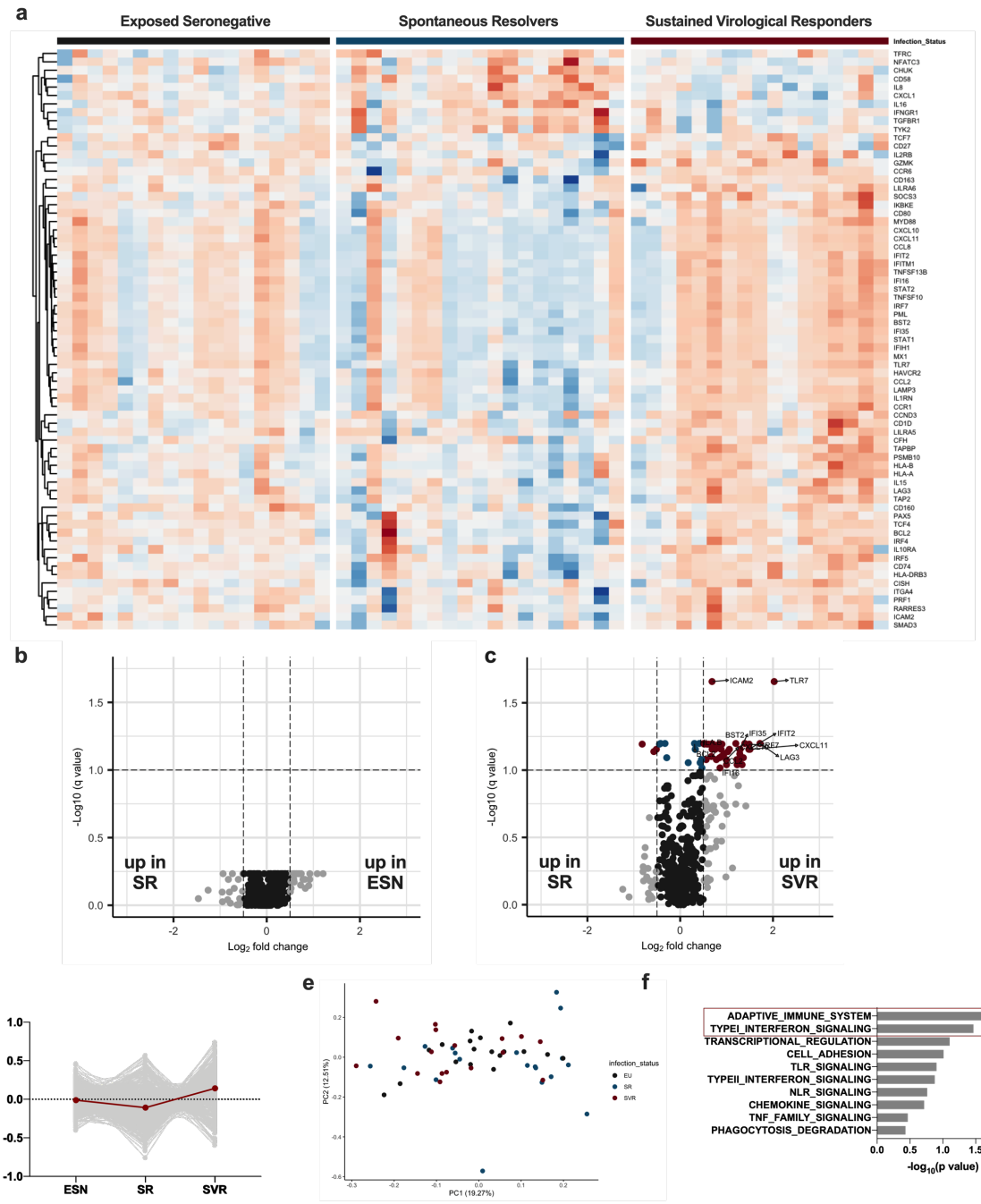

a
Exposed Seronegative
Spontaneous Resolvers
Sustained Virological Responders
Infection_Status
TFRC
NFATC3
CHUK
CD58
IL8
CXCL1
IL16
IFNGR1
TGFBR1
TYK2
TCF7
CD27
IL2RB
GZMK
CCR6
CD163
LILRA6
SOCS3
IKBKE
CD80
MYD88
CXCL10
CXCL11
CCL8
IFIT2
IFITM1
TNFSF13B
IFI16
STAT2
TNFSF10
IRF7
PML
BST2
IFI35
STAT1
IFIH1
MX1
TLR7
HAVCR2
CCL2
LAMP3
IL1RN
CCR1
CCND3
CD1D
LILRA5
CFH
TAPBP
PSMB10
HLA-B
HLA-A
IL15
LAG3
TAP2
CD160
PAX5
TCF4
BCL2
IRF4
IL10RA
IRF5
CD74
HLA-DRB3
CISH
ITGA4
PRF1
RARRES3
ICAM2
SMAD3
b
-Log10 (q value)
up in SR
up in ESN
Log2 fold change
c
ICAM2
TLR7
BST2
IFI35
IFIT2
CXCL11
LAG3
IFI6
up in SR
up in SVR
Log2 fold change
d
Normalised mean expression
ESN
SR
SVR
e
PC2 (12.51%)
PC1 (19.27%)
infection_status
EU
SR
SVR
f
ADAPTIVE_IMMUNE_SYSTEM
TYPEI_INTERFERON_SIGNALING
TRANSCRIPTIONAL_REGULATION
CELL_ADHESION
TLR_SIGNALING
TYPEII_INTERFERON_SIGNALING
NLR_SIGNALING
CHEMOKINE_SIGNALING
TNF_FAMILY_SIGNALING
PHAGOCYTOSIS_DEGRADATION
-log10(p value)

Figure 4.2. Whole blood responsiveness to ODN stimulation is decreased in donors who spontaneously resolved infection when compared to SVRs. *Whole blood was stimulated with ODN (25μg/ml for 22 hours at 37°C). (a) Heatmap showing genes ordered by hierarchical clustering with q values (FDR adjust p value) <0.2 following non-parametric ANOVA between ESNs (n=18), SRs (n=19) and SVRs (n=17). Upregulated genes are in red, while downregulated genes are shown in blue. (b,c) Volcano plots of differentially expressed genes between (b) SRs and ESNs and (c) SRs and SVRs (Mann Whitney U test, q<0.1). (d) Kinetic plot of ODN inducted genes between infection groups. Each grey line represents a single gene. The median line is shown in red. (e) PCA of genes from ODN stimulated whole blood. Each dot represents a single donor. (f) GSEA of transcriptomic data (p<0.05).*

Specific disruption to the type I interferon pathway in spontaneous resolvers

To further assess whether specific modules of gene expression are disrupted in response to ODN in SR women, we employed gene signature scores, previously developed in an independent healthy cohort. Applying these gene signatures to the ODN NanoString transcript data we found decreased expression of ODN induced IFN-I mediated gene expression in the SRs compared with both ESNs and SVRs (**Fig. 4.3a(i)**). No differences in the gene signature scores for IFNγ, IL1β and TNFα in the ODN data were observed (**Fig. 4.3a(ii-iv)**). Individual genes involved in the type I interferon gene signature score are shown as a heatmap (**Fig. 4.3b**).

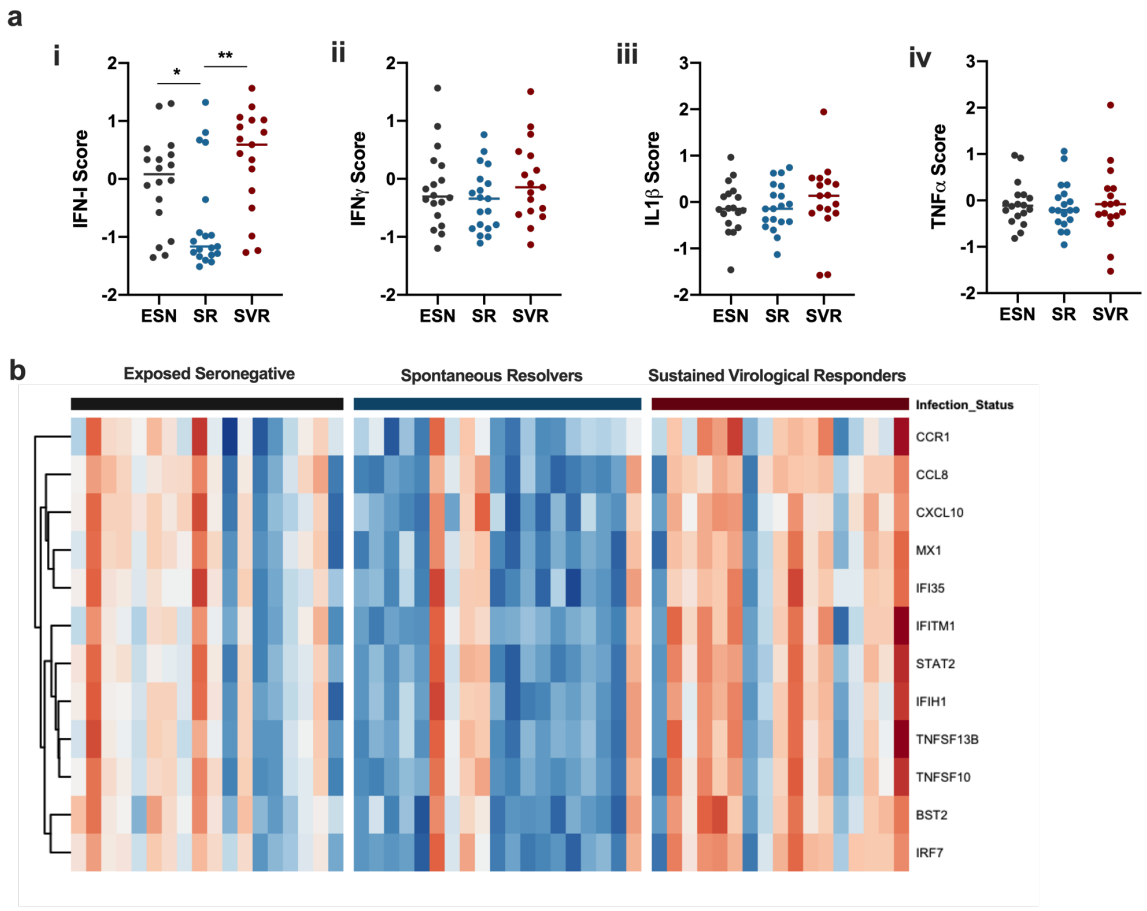


Figure 4.3. Reduced Type I interferon specific scores in the spontaneous resolvers. *Whole blood was stimulated with ODN (25μg/ml for 22 hours at 37°C). (a(i-iv) Previously defined gene signatures were applied to the ODN transcriptomic data. (a(i-iv)) Gene signatures for (i) IFN-I, (ii) IFNγ, (iii) IL1β and (iv) TNFα are shown. (e) Heatmap showing genes used to generate the IFN-I gene score. ESN, SR and SVR gene signature scores were compared using Kruskal-Wallis tests (*p<0.05, **p<0.01).*

Activation status and frequency of circulating dendritic cells are similar in all groups

The most potent producers of IFN-I in circulation in the human are pDCs. Given the specific IFN-I signature reduction observed in SR donors in our cohort we sought to ascertain the activation status and frequency of pDCs in circulation in our cohort. Comparing pDC counts between ESNs, SRs and SVRs we saw no significant difference in the numbers of pDCs (**Fig. 4.4a(i)**). We used the MFI of HLA-DR and CD86 as surrogate markers of activation on pDCs and found similar expression of both molecules on pDCs from the three infection groups (**Fig. 4.4a(ii, iii)**). We also assessed the frequencies and activation states of the two other major DC subsets in circulation, cDC1s and cDC3s. Similar to pDCs, we saw no significant difference in the counts or activation states of cDC1s and cDCs between the three infection groups (**Fig. 4.4a(i-iii), b(i-iii)**).

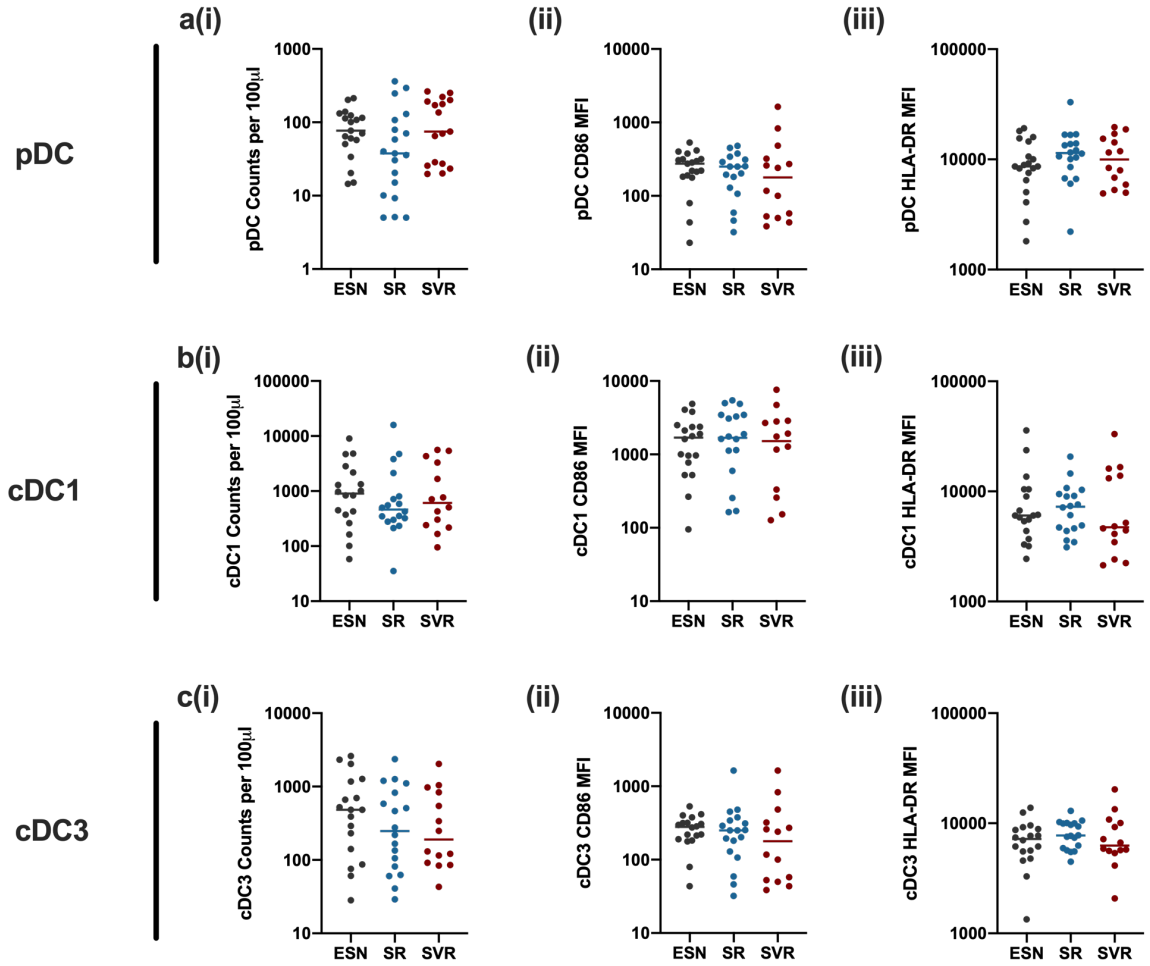


Figure 4.4. DC counts and their activation status are similar across the three infection groups. *Circulating DC subsets were quantified using flow cytometry and their activation assessed using CD86 and HLA-DR MFI as surrogates. (a(i)) pDC counts per 100µl in ESNs, SRs and SVRs. CD86 (a(ii)) and HLA-DR (a(iii)) expression on pDCs. (b(i)) cDC1 counts per 100µl in ESNs, SRs and SVRs. CD86 (b(ii)) and HLA-DR (b(iii)) expression on cDC1s. (c(i)) cDC3 counts per 100µl in ESNs, SRs and SVRs. CD86 (c(ii)) and HLA-DR (c(iii)) expression on cDC3s. Data is presented on a log_{10} axis with the median line shown as a solid line. ESN, SR and SVR DC counts and activation states were compared using Kruskal-Wallis tests ($p>0.05$).*

Spontaneous resolvers have reduced IFNα protein production following ODN stimulation

Differences in the expression of TLR9 signalling components at baseline might have affected the response to ODN. We therefore assessed the NanoString data from the null stimulation for differences in baseline expression of genes involved the TLR9 signalling pathway, however, we saw no significant differences (**Fig. 4.5**). Given there were no apparent differences in pDC counts or activation states we sought to investigate whether differences in ODN induced IFN-I protein production contributed to the reduced IFN-I score in SRs. IFNα and IFNβ protein levels were quantified in the supernatants from ODN treated whole blood using the Simoa ultrasensitive digital ELISA (**Fig. 4.6a, b**). SRs produced less IFNα in response to ODN stimulation compared to SVRs (**Fig. 4.6a**). There was no difference in the production of IFNβ in response to ODN between ESNs, SRs and SVRs (**Fig. 4.6b**). The ODN induced milieu of non-IFN-I inflammatory cytokines measured was similar between ESNs, SRs and SVRs (**Fig. 4.6c**). The levels of IFNα protein correlated positively and significantly with the IFN-I signature score, suggesting that the reduced IFN-I score may be attributable to reduced IFNα protein production in response to ODN by SRs (**Fig. 4.6d**).

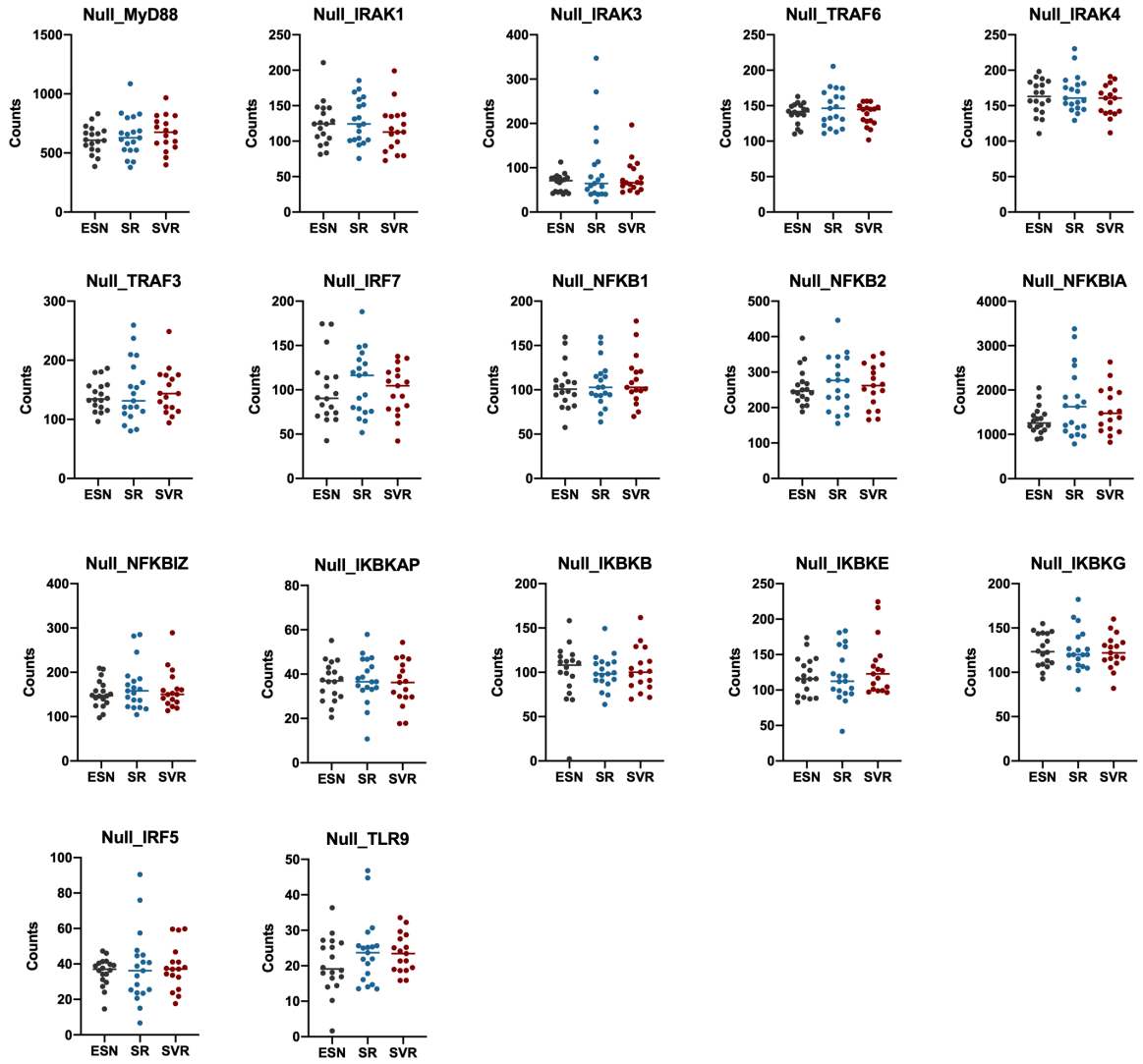


Figure 4.5. No difference in signalling components of the TLR9 signalling pathway. *1ml of whole blood was aliquoted into the negative control TruCulture tube and incubated overnight (22 hours at 37°C). NanoString transcriptomics was used to quantify RNA expression. Baseline signalling components of the TLR9 pathway are shown as counts with the median line shown as a solid line. Counts for each group were compared using Kruskal-Wallis tests (p>0.05).*

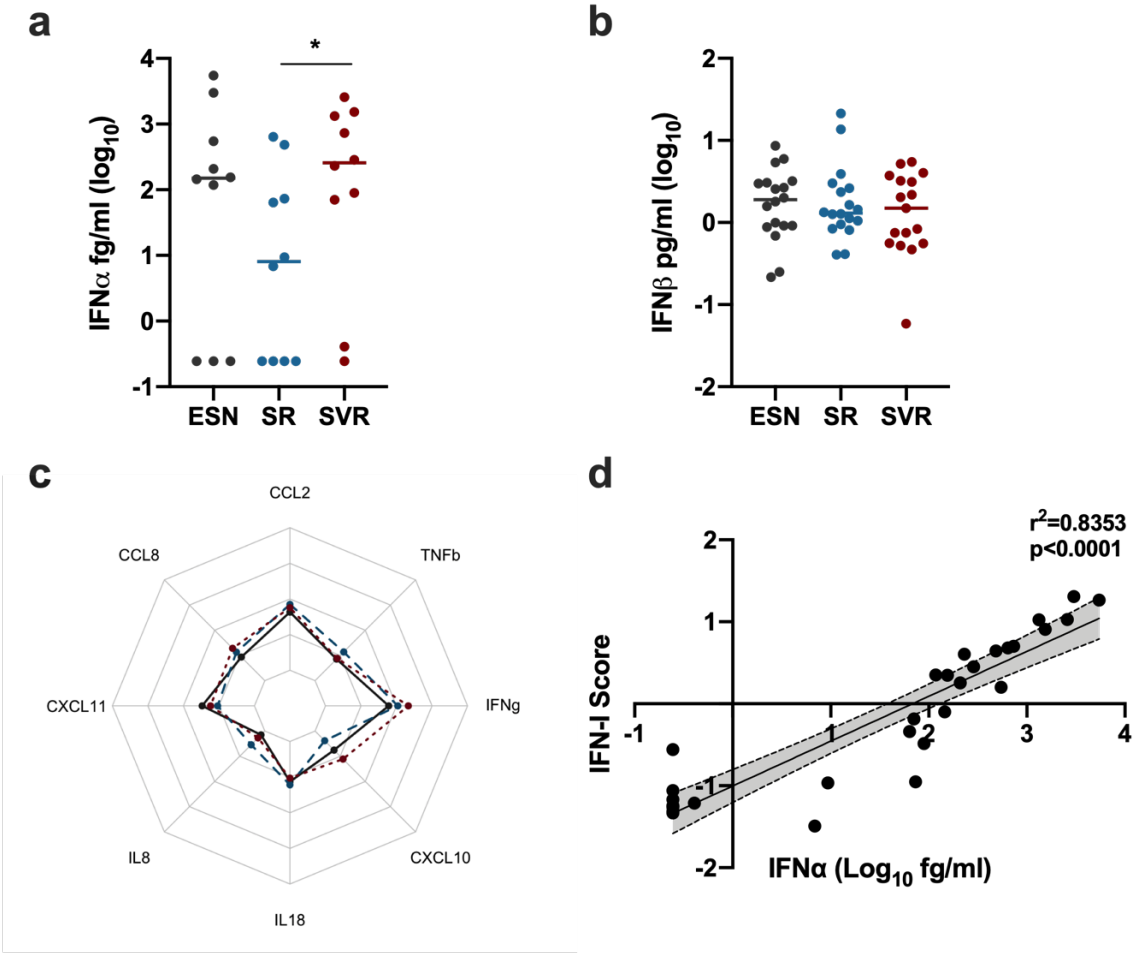


Figure 4.6. SRs have reduced IFNα production following ODN stimulation. *Whole blood was stimulated with ODN (25μg/ml for 22 hours at 37^{o}C). Secreted cytokines were quantified using*

*(a, b) Simoa ultrasensitive digital ELISA and (c) Luminex. (a) IFNα and (b) IFNβ protein concentration in ODN stimulated whole blood. Data is shown as log_{10} of the protein concentration. (c) Spider plot of ODN induced cytokines in whole blood. (d) IFNα levels correlate positively and significantly with the ODN induced IFN-I gene signature score (*p<0.05, linear regression).*

Discussion

In a unique cohort of HCV-exposed women, we sought to assess the role of the innate immune response in adaptive clearance of HCV infection. To this end, we investigated the whole blood innate immune response to conventional viral ligands including polyIC, R848, IFNα2 and ODN. The transcriptomic response to polyIC, R848 and IFNα2 was similar in women who spontaneously resolved infection and in women who were chronically infected. Surprisingly however, following ODN stimulation we found several differentially expressed genes between SRs and the other two groups (ESNs and SVRs). Further analysis of the ODN transcriptomic data showed differential expression of 55 genes in the SRs and SVRs. Using gene set enrichment analysis we found enrichment for IFN-I signalling in the ESNs and SVRs compared to the SRs. Applying gene signature scores we found a specific reduction in the IFN-I gene signature score in the SRs compared to the SVRs and ESNs. This reduction in the IFN-I gene signature was reflected by a reduction in IFNα, but not IFNβ, protein production by SRs.

Stimulation of whole blood ex vivo allows for identification of altered innate immune responses in SRs not observed in the absence of stimulation. The use of sensitive technologies that limited handling steps in our study enabled detection of signatures in a relatively small cohort, however, validation in an independent larger group would strengthen observations made in this study.

Reduced IFN-I in response to ODN in the SRs is a surprise, as previous work has demonstrated the importance of IFN-I in shaping the adaptive immune response. However, more recent work has shown that a potent innate IFN-I response can be detrimental to the adaptive response, possibly through inhibition of CD5 expression on B cells and suppression of antibody production [17,41,42]. It is therefore possible that the reduced IFN-I observed in this study was sufficient to support an adaptive immune response without secondary inhibition/disruption. Though the induction of IFNα by ODN is low, small differences in IFNα have been shown to have important clinical consequences, as seen in SLE, where femtomolar differences in IFNα correlate with disease severity [19].

We saw no difference in the frequencies or activation states of pDCs in the SRs compared with ESNs or SVRs. The reduced IFN-I production and response in the SRs could be underpinned by genetic differences in the in the TLR9 pathway that reduce IFNα production and subsequent IRG upregulation, or by differences in phosphorylation of key signalling proteins or transcription factors such as IRF7.

The metabolic ability of cells to support cytokine production has emerged as a key regulator of the immune response over the past decade [200]. In a human pDC cell line stimulated with CpG-A for 12 hours it was shown that pDCs increase lactate production and the extracellular acidification rate (ECAR), as well as key glycolytic genes lactate dehydrogenase, HIF1α and hexokinase 2 [201]. Increases in these features are indicative of an increase in glycolysis. Inhibition of glycolysis using the glucose analogue, 2-deoxy-glucose, impaired the TLR9 induced IFNα production. It is therefore possible a reduced glycolytic capacity in the SRs contributed to the reduced IFNα production and reduced IRG upregulation described here.

While it is possible that the differences described here stem from a pre-HCV phenotype that increased the propensity to spontaneously resolve infection, it is also plausible that infection with HCV resulted in an altered immune state in the SRs that persisted following successful viral clearance. HCV immune evasion strategies result in widespread disruption to the immune system.

Work from several groups suggests that HCV induced changes can persist following curative therapy, particularly affecting the adaptive arm of the immune system [111]. Most of these studies have focused on treatment following chronic infection. From these studies it appears that some sequelae of HCV infection persist despite loss of antigenic stimulation. MAIT cells do not seem to recover fully following viral clearance, although some functional restoration appears to occur [120]. NK cell receptor diversity appears to be persistently reduced, and mitochondria of T cells do not regain normal functionality in all individuals treated with DAAs [114,202].

While these studies have focused on the restoration of immunity following chronic infection, fewer works have explored the impact of acute infection on the immune response post resolution of infection. One study has shown that the soluble immune mediator profile of people treated acutely for HCV infection does not return to homeostatic levels [203]. Recent *in vitro* work using a model of acute HCV infection suggests that differences in the epigenome of individuals treated with DAAs persist and may explain some of the persistent immune defects seen post-acute HCV infection. No study has yet explored whether natural resolution of HCV infection results in a phenotype similar to that reported using acute infection models. However, the notion that virally induced changes persist in the absence of infection is not new and has been described for other infections including measles and CMV [204,205].

To our knowledge, the study presented in this chapter is one of the first to point towards people able to clear acute HCV infection as having a unique induced immune signature in whole blood. This could be reflective of a phenotype that makes someone susceptible to infection, but also helps to clear the infection with an adaptive immune response. Alternatively, acute HCV infection may have lasting impact on the induced immune response.

Chapter 5: Investigating the genetics of resistance to hepatitis C virus infection

Abstract

Resistance to viral infection is an oft overlooked and understudied outcome following exposure to viral infection. Previously, our group has recruited a cohort of women exposed the hepatitis C virus following receipt of contaminated anti-D immunoglobulin. Studies of the cohort, involving three infection outcomes – resistant (exposed seronegative; ESN), spontaneous resolvers (SRs) and those who were previously chronically infected but cleared infection following therapy (sustained virological responders; SVRs) uncovered a signature of viral resistance in the ESNs that implicated an enhanced polyIC induced type I interferon gene signature in whole blood. Here, using a gene candidate approach we sought to assess the genetics of viral resistance. The cohort was genotyped for tagSNPs in the TLR3-IFN-I pathway. Using this approach we found that a SNP in IFNAR1, rs2257167 (V141L), was associated with increased resistance to HCV infection in the dominant model (p=0.02; Odds ratio = 3.54; 95% confidence interval = 1.21 to 9.82). Analysis of transcriptomic data from two independent healthy female control cohorts revealed increased IFNAR1 mRNA expression in whole blood in those carrying at least one copy of the variant C allele compared to those homozygous for the wild-type G allele. Analysis of whole blood stimulation data in 500 females from the *Milieu Interieur* cohort showed increased IRG upregulation in response to stimulation with LPS, polyIC and the live influenza A virus (IAV) in those carrying the variant allele. Conversely, GG individuals had an increased pro-inflammatory response to stimulation with LPS, polyIC and IAV. The increased pro-inflammatory response observed in GG donors may have contributed to resistance to HCV infection by ESNs in the anti-D cohort.

Introduction

Earlier work presented in this thesis uncovered a polyIC induced IFN-I gene signature that appeared to be associated with resistance in our cohort. Resistors from the cohort also appeared to produce greater levels of several cytokines including CCL2, CCL8, IL-6 and CXCL11 in response to polyIC compared to women who were susceptible to infection (those who were chronically infected or those who spontaneously resolved infection; collectively seropositive). Interestingly, the whole blood response to other antiviral stimuli (ODN, IFN and R848) was similar across the three infection groups. The enhanced polyIC response we observed in our cohort could be due to differences in the key immune genes in this pathway.

Heritable factors and their influence on immune system variation

Both autoimmune and infectious disease have strong immunogenetic associations. Historical focus of genetic influences on enhanced resistance to infection has been on MHC variability and monogenic variants, including CCR5delta in HIV, FUT2 in norovirus and DARC in malaria. Monogenic variants resulting in primary immunodeficiencies that increase susceptibility to viral infections include IRF loss of function mutations seen in influenza, herpes simplex encephalitis and COVID-19 [206–214]. In the context of autoimmunity, monogenic variants have also been described and include IFIH1 and DNASE1 in SLE [215]. While causative variants have been explored widely in these settings, they are unlikely to have more ambiguous roles as absence or gain of function in genes are deleterious and often incompatible with a normal health span.

Monogenic disease associated variants are typically uncommon. In contrast, sequence differences that arise due to SNPs are commonly found in the genome [216]. SNPs are defined as either synonymous, wherein a change in a nucleotide base does not result in an alteration in the amino acid composition of a protein (codon degeneracy), or non-synonymous (missense), where the amino acid sequence of a protein is altered. Both types of SNPs can contribute to human health and disease [217,218]. Different selective pressures driven by variable disease burdens across populations has led to substantial variation in the minor allele frequencies (MAFs) of SNPs. Studies of SNPs have provided

valuable insight into the roles of specific residues in the function of IFN-I related genes and proteins [219].

SNPs in the TLR3-IFN-I pathway

Several SNPs in the TLR3-IFN-I pathway have previously been associated with increased susceptibility to infectious diseases. The rs3775291 SNP in TLR3 has been of particular interest globally given its wide and heterologous associations, including HIV, SLE, type I diabetes and idiopathic pulmonary fibrosis [196,220–222]. This is a non-synonymous variant in which a cytosine is replaced with a thymine, leading to a change in the amino acid in the ectodomain at position 412 from a leucine to a phenylalanine (L412F). C is the major allele, whereas T is the minor. The SNP exhibits substantial population variation – the minor T allele is present in just 3% of Africa donors from the 1000 genomes project, while it is present in 33% of east Asian populations [223].

The TLR3 SNP has also previously been associated with increased resistance to HIV infection in highly exposed seronegative (HESN) intravenous drug users exposed to HIV [196]. Sironi et al. genotyped two independent cohorts and found the frequency of individuals carrying at least one phenylalanine allele is significantly higher in HESN individuals compared to a matched controls [196]. The SNP has also been associated with SLE risk and more strongly with development of type I diabetes mellitus [220,221].

Analysis of GTEX data shows rs3775291 to be an expression quantitative trait loci (eQTL) for TLR3 expression, meaning that it effects TLR3 mRNA levels. Functional analysis of peripheral blood mononuclear cells (PBMCs) in the Sironi study showed variant CT and TT donors to have reduced replication of HIV compared to WT CC donors [196]. This reduced HIV replication was accompanied by increased immune activation denoted by marked increases in IL6, CCL3 and the activation marker CD69. In response to a TLR3 agonist, donors with the minor allele CT and TT also had increased upregulation of these markers [196].

Additionally, it appears this polymorphism is associated with enhanced general TLR responsiveness, suggesting that tonic signalling through TLR3 may be important for TLR

expression levels and subsequent antiviral activity and IFN-I production [29]. The dual autoimmune and viral resistance association is likely underpinned by the increased TLR3 expression and consequent enhanced immune activation.

Further downstream and of potential interest is rs7251, a non-synonymous SNP in IRF3 involving a base change from cytosine to guanine leading to an amino acid substitution at the final position of 427 from a threonine (ACC) to a serine (AGC; T427S) [223]. G encodes a serine and is the major allele in most populations with an allele frequency ranging from 50% to 67%, except in Africans where it is the minor allele with a frequency of 29%. G is considered to be the derived risk allele, whereas C appears to be the ancestral allele. The SNP is a blood *cis* eQTL for IRF3 and leads to increased IRF3 expression (GTEX data;[224]). The resultant increased basal expression of IRF3 is likely to lead to a "heightened" immune state that, in one context could contribute to resistance to viral infection, while in another could increase the risk of immune dysregulation and development of autoimmune disease. This notion is reflected in previous association studies in which one study linked the CG and CC genotypes with increased persistence of HPV infection – conversely suggesting that the GG genotype may be associated with increased clearance of HPV infection [225]. A recent meta-analysis involving 7,212 cases and 13,556 controls found that the G allele is significantly associated with SLE risk, in particular increased risk of developing an SLE associated inflammatory condition called lupus nephritis [224]. This link between the G allele and resistance to HPV infection as well as SLE risk supports our hypothesis of a shared genetic signature underlying viral resistance and development of autoimmunity.

SNPs in IFNAR1 and IFNAR2 have been associated with several disease states; associations appear to be highly context and disease specific with the same SNP reported to have both positive and negative effects on disease outcome depending on whether the disease is due to viral infection or autoimmunity. For example, rs2257167 (V141L) in IFNAR1 has been associated with both spontaneous resolution of hepatitis B virus infection, resistance to respiratory virus infection and increased pain in lung cancer and risk of developing multiple sclerosis and vitiligo in females [226–230]. This dual association with both autoimmune susceptibility and protection from viral infections is interesting as it shows

that a SNP which is thought to be pathogenic in one instance can be protective in another. Rs2257167 results in a valine to leucine substitution at position 141 in the SD2 domain of AR1 [226]. As with several other SNPs, the MAF varies dramatically between populations, suggesting a functional consequence driven by different evolutionary pressures [223]. While investigations into its functional impact are lacking, limited reports suggest that rs2257167 increases IFNAR1 expression.

Sex and SNPs in the type I interferon pathway

Of note, rates of viral resistance and spontaneous clearance of infection are higher in females than in males. Incidence of autoimmune diseases such as multiple sclerosis, SLE and rheumatoid arthritis are also higher in females [231]. Indeed, the transcriptomic signature described by Tsang et al that is predictive of vaccine responses and autoimmune disease flares is also higher in females [39]. Despite well-described physiological sexually dimorphic disease associations, studies often fail to appropriately address sex differences. Genome wide association studies analysed by sex have uncovered sex specific SNP associations [232].

Differences in MAFs are unlikely to account for sex specific SNP associations as no large sex differences in SNP MAFs have been described, rather it has been proposed that dimorphism in genotype effects exists between sexes [233]. This is evinced in a study by Fischer et al. wherein they describe a female specific association between a SNP in the TLR9 promoter region (rs5743836) and spontaneous clearance of HCV infection [84]. TLR9 is important in the detection of viral DNA and upregulation of IFN-I. This SNP maps close to an area in the promoter region coregulated by the transcription factors NFκB and the estrogen receptor alpha (ERα), suggesting that the association differences observed could be due to differential regulation by the female sex hormone and hormone response elements within the TLR9 gene [84].

Functional work on whole blood stimulated with ERα activators on WT and variant female donors showed homozygous WT donors downregulated TLR9 within 3 hours following treatment, whereas in the heterozygotes and homozygous variant donors the

downregulation was significantly lower. Negative regulation of TLR9 by oestrogen could explain the attenuation of autoimmune disease often observed during pregnancy when high oestrogen levels are maintained. High oestrogen and a reduction in TLR9 expression during pregnancy could also help explain the increased susceptibility to viral infection during pregnancy [234]. Indeed, PBMCs from pregnant women stimulated with HRV43, a human rhinovirus showed significantly reduced IFNα production compared with non-pregnant women [235]. IFNα also appears to be positively regulated by female sex hormones [236].

As females have two X chromosomes, and males have only one X and one Y, the second X chromosome in females is transcriptionally silenced so as to achieve dosage compensation between the sexes [237]. Several immune genes are found on the X chromosome and are not silenced, therefore a further likely contributor to sexually dimorphic effects of SNPs is escape of X chromosome inactivation in females. TLR7, a PRR involved in the IFN-I response and detection of ssRNA, is one such example; immune cells from females express higher levels of TLR7 and produce more IFNα as a consequence [199]. Non-synonymous SNPs in the TLR7 gene therefore likely exert different effects between sexes. Indeed, this phenomenon has been described for the rs179008 SNP which appears not to impact on IFNα production in response to R848 stimulation in males, yet reduces IFNα levels in females [238]. A key point to note is that sex differences described in humans may not be present in non-human mammals and caution is required when attempting to dissect sex differences in animal models and extrapolate findings to humans [239]. Further explorations of SNPs in the IFN-I pathway and their associations with autoimmune and infectious disease ought to consider males and females separately in order to appropriately discern potential sex effects.

Here, we searched the NIEHS tagSNP database for tagSNPs in TLR3, IRF3 and IFNAR1 that provided adequate gene coverage. We genotyped our cohort for three SNPs in TLR3 (rs3775291), IRF3 (rs7251) and IFNAR1 (rs2257167) and tested for associations with resistance to viral infection. On identifying an association with rs2257167 and resistance to HCV infection we analysed transcriptomic data from whole blood stimulations with LPS,

influenza A virus and polyiC from the 1000 person MIC as well as stimulation data with ODN, IFN, R848 and polyIC from healthy age matched controls recruited alongside women from the anti-D cohort to determine the functional consequences of rs2257167.

Chapter hypothesis

SNPs in the TLR3-IFN-I pathway are associated with infection outcome following exposure to HCV in the Irish anti-D cohort.

Chapter aims

1. Genotype the cohort for tagSNPs in the TLR3-IFN-I pathway and test for associations with outcome following exposure to HCV.
2. Assess the functional consequences of selected SNPs *in silico.*
3. Assess the functional consequences of selected SNPs using healthy unexposed donors from the viral resistance cohort and the MIC.

Results

tagSNP selection in the TLR3-IFN-I pathway

To evaluate whether genetic variation in the TLR3-IFN-I pathway contributed to resistance to hepatitis C virus infection we searched the NIEHS tagSNP database for nonsynonymous SNPs in TLR3, IRF3 and IFNAR1 (**Fig. 5.1**). We identified three SNPs of interest at a high enough frequency to warrant further exploration in our cohort- TLR3 rs3775291 (L412F), IRF3 rs7251 (T427S) and IFNAR1 rs2257167 (**Fig. 5.1a, b, c**). Previous genetic association studies involving these SNPs are summarised in **Table 5.1**.

Gene Name	SNP ID	Alleles (Major/ Minor)	Amino Acid Change	Associations	Refs.
TLR3	rs3775291	C>T	Leu412Phe	Resistance to HIV-I infection	Sironi, M.[196]
				Increased risk of SLE development	Laska, M. [220]
				Increased risk of type I diabetes mellitus	Assman, T.S. [221]
				Increased risk of idiopathic pulmonary fibrosis	O'Dwyer, D.N. [222]
IRF3	rs7251	C>G	Thr427Ser	Increased risk of SLE development	Zhang, F. [224]
				Increased clearance of HPV infection	Wang, S.S. [225]
IFNAR1	rs2257167	G>C	Val141Leu	Spontaneous resolution of HBV infection	Zhou, J. [226]
				Increase in lung cancer pain	Reyes-Gibby, C.C. [228]
				Increased risk multiple sclerosis	Leyva, L. [229]
				Increased risk of female vitiligo	Traks, T.[230]

Table 5.1. List of selected SNPs and their previous disease associations.

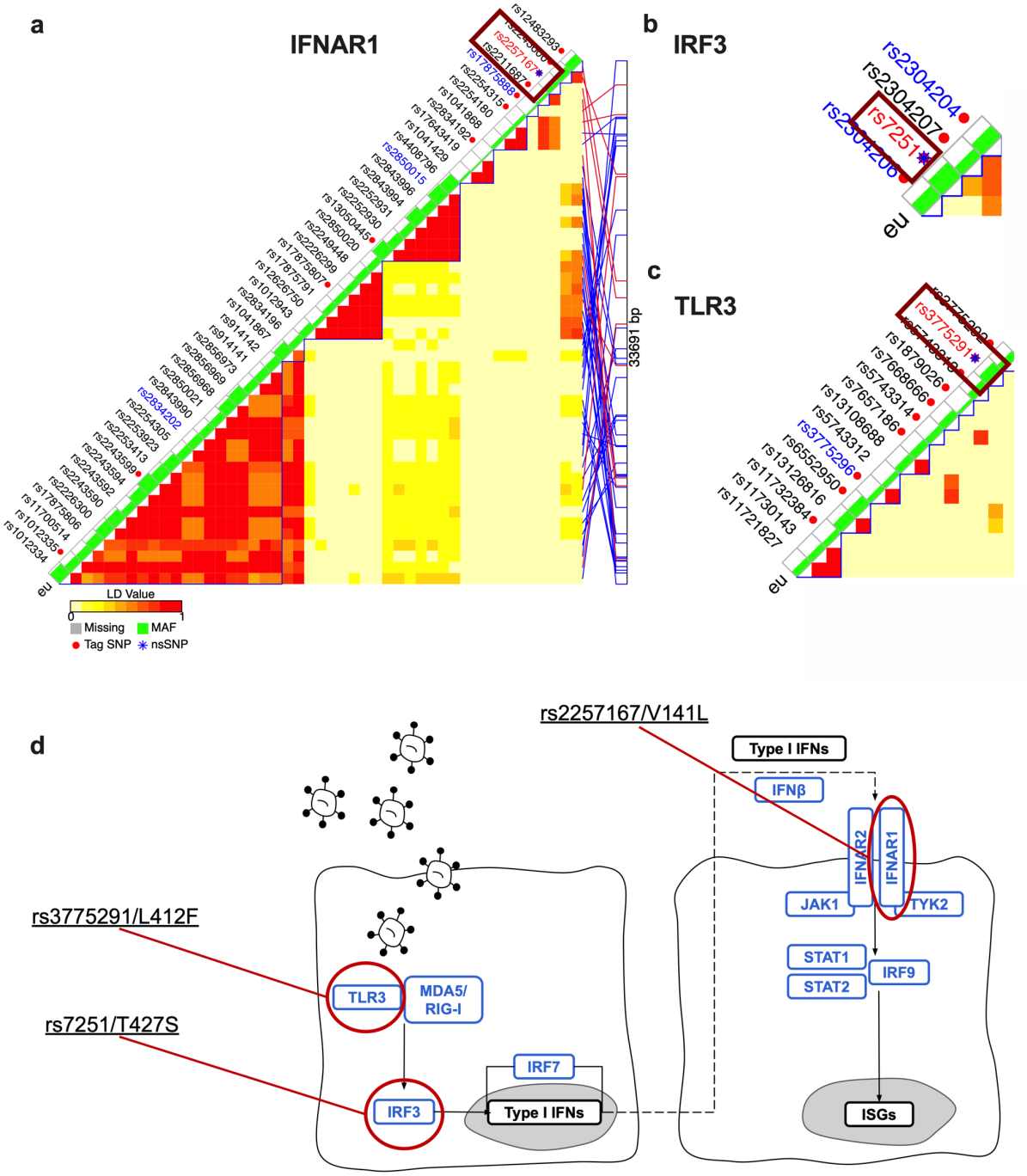


Figure 5.1. tagSNPs selection for IFNAR1, IRF3 and TLR3. *The SNPinfo tagSNP selection database was used to search for tagSNPs in linkage disequilibrium that provided adequate gene coverage. The selected tagSNPs for (a) IFNAR1, (b) IRF3 and (c) TLR3 are denoted by the red and blue asterisk and are highlighted in the red box. (d) Diagrammatic representation of the TLR3-IFN-I pathway with selected tagSNPs indicated.*

The IFNAR1 rs2257167 GG genotype is associated with protection against HCV infection.

Women in our cohort were genotyped for TLR3 rs3775291, IRF3 rs7251 and IFNAR1 rs2257167. The estimated MAF for rs3775291 and rs7251 is 32% and 33% respectively. The MAF for rs2257167 is 13% and the overall MAF in our cohort is 13%. Association testing for additive and dominant models showed no significant differences between groups for either the TLR3 or IRF3 SNPs (**Table 5.2**). However, association testing for IFNAR1 rs2257167 showed a significant association in the dominant model between the GG genotype and resistance to HCV infection when comparing to the healthy control (HC) group in our cohort with an odds ratio of 3.54 ($p<0.05$, 95% confidence interval of 1.21 to 9.82; **Table 5.3**). The wild-type GG genotype (Val141) appears to be associated with protection against HCV infection. Allele counts for the TLR3, IRF3 and IFNAR1 SNPs were compared between groups. Similar to the genotype frequencies, an association between ESNs and the IFNAR1 SNP was observed when comparing to HC donors (**Table 5.4).**

			Additive		Dominant	
TLR3_rs3775291	ESN	SR	p value	Odds ratio (95% CI)	p value	Odds ratio (95% CI)
CC	22 (58)	30 (48)	0.6	-	0.32	1.51 (0.65 to 3.36)
CT	13 (34)	26 (41)				
TT	3 (8)	7 (11)				
TLR3_rs3775291	ESN	CI				
CC	22 (58)	44 (57)	0.95	-	0.94	1.03 (0.46 to 2.18)
CT	13 (34)	28 (36)				
TT	3 (8)	5 (7)				
TLR3_rs3775291	ESN	HC				
CC	22 (58)	56 (47)	0.5	-	0.24	1.55 (0.72 to 3.27)
CT	13 (34)	50 (42)				
TT	3 (8)	13 (11)				
IRF3_rs7251	ESN	SR				
CC	20 (53)	27 (43)	0.48	-	0.34	1.48 (0.67 to 3.24)
CG	16 (42)	29 (46)				
GG	2 (5)	7 (11)				
IRF3_rs7251	ESN	CI				
CC	20 (53)	41 (53)	0.38	-	0.95	0.98 (0.44 to 2.19)
CG	16 (42)	26 (34)				
GG	2 (5)	10 (13)				
IRF3_rs7251	ESN	HC				
CC	20 (53)	58 (49)	0.45	-	0.68	1.17 (0.59 to 2.39)
CG	16 (42)	46 (39)				
GG	2 (5)	15 (12)				

Table 5.2. Association of TLR3 SNP rs3775291 and IRF3 SNP rs7251 and resistance to hepatitis C virus infection. *Women in the cohort were genotyped for the rs7251 SNP in IRF3 and the TLR3 SNP rs3775291. Chi-square tests were used to assess differences between ESN donors and SRs, CIs and HCs in our cohort (p>0.05).*

			Additive		Dominant	
IFNAR1_rs2257167	ESN	SR	p value	Odds ratio (95% CI)	p value	Odds ratio (95% CI)
GG	34 (89)	48 (76)	-	-	0.09	2.66 (0.79 to 7.81)
GC	4 (11)	14 (22)				
CC	0 (0)	1 (2)				
IFNAR1_rs2257167	ESN	CI				
GG	34 (89)	58 (75)	-	-	0.07	2.78 (0.91 to 8.01)
GC	4 (11)	18 (23)				
CC	0 (0)	1 (1)				
IFNAR1_rs2257167	ESN	HC				
GG	34 (89)	84 (71)	-	-	0.02 *	3.54 (1.21 to 9.82)
GC	4 (11)	32 (27)				
CC	0 (0)	3 (2)				

Table 5.3. Association of the IFNAR1 SNP rs2257167 and resistance to hepatitis C virus infection. *DNA samples were genotyped for the IFNAR1 rs2257167 SNP. Chi-square tests were used to assess differences in the frequencies of this SNP between infection groups (*, p<0.05).*

a

IFNAR1_rs2257167	#G	#C
ESN	72	4
SR	110	16
CI	134	20
HC	200	38

Comparision	p value	Odds ratio	95% CI
ESN Vs SR	0.09	2.62	0.9 to 7.43
ESN Vs CI	0.07	2.69	0.9 to 7.48
ESN Vs HC	0.02	3.42	1.21 to 9.19

b

TLR3_rs3775291	#C	#T
ESN	57	19
SR	86	40
CI	116	38
HC	162	76

Comparision	p value	Odds ratio	95% CI
ESN Vs SR	0.31	1.39	0.76 to 2.62
ESN Vs CI	0.96	0.98	0.51 to 1.83
ESN Vs HC	0.25	1.41	0.79 to 2.56

c

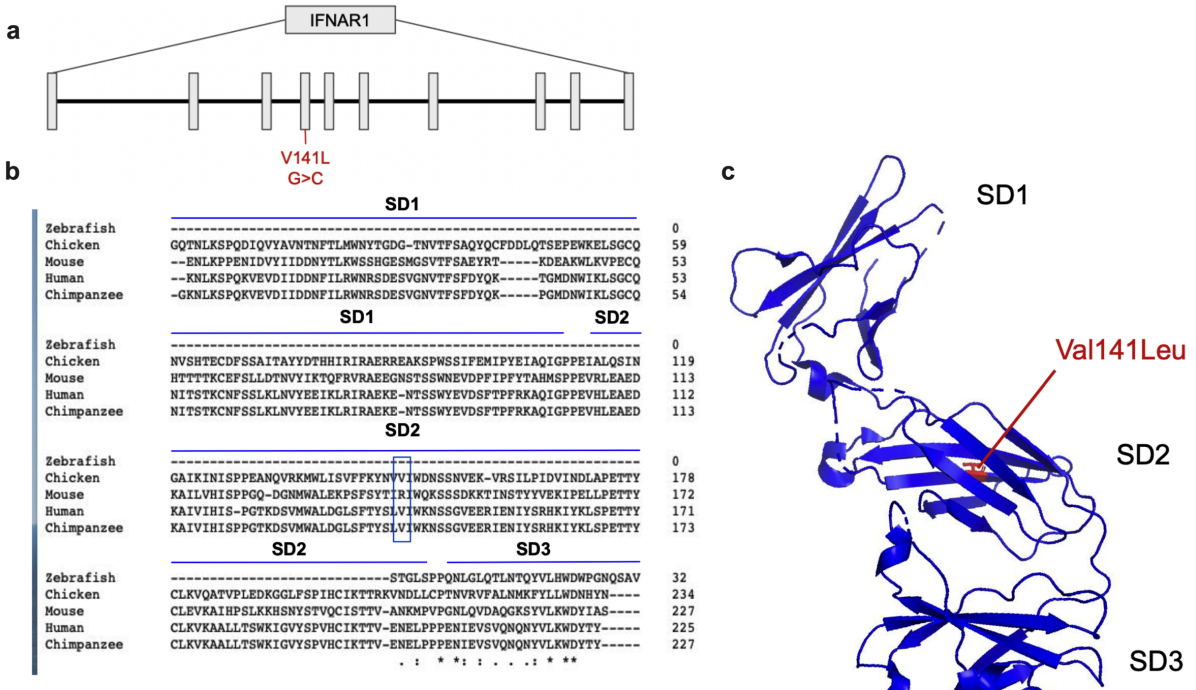
IRF3_rs7251	#C	#G
ESN	56	20
SR	83	43
CI	108	46
HC	162	76

Comparision	p value	Odds ratio	95% CI
ESN Vs SR	0.25	1.45	0.76 to 2.65
ESN Vs CI	0.58	1.19	0.65 to 2.27
ESN Vs HC	0.35	1.31	0.74 to 2.34

Table 5.4. Allele counts for IFNAR1 rs2257167, TLR3 rs3775291 and IRF3 rs7251. *The number of wild-type and variant alleles were counted for (a) IFNAR1 rs2257167, (b) TLR3 rs3775291 and (c) IRF3 rs7251. Comparisons of allele counts were made between infection groups using Chi-square tests (*, p<0.05).*

V141L is located in the SD2 domain of IFNAR1.

Given the association between rs2257167 and HCV resistance we sought to assess the functional consequences of the SNP using available bioinformatic tools. The SNP is located in exon 4 of IFNAR1 (**Fig. 5.2a**). Using Clustalω we found the wild-type Val141 to be conserved across multiple species, including chickens and chimpanzees, but not in the mouse (**Fig. 5.2b**). PyMOL was used to localise the SNP to the SD2 domain of IFNAR1 (**Fig. 5.2c**). Function prediction software such as SIFT, Provean and Polyphen-2 were used to estimate the functional consequences of the amino acid change. All three methods predicted the SNP to have benign or neutral consequences (**Fig. 5.2d**).



d

Prediction tool	Score	Prediction
SIFT	0.235	Tolerated
Provean	-1.509	Neutral
Polyphen-2	0.364	Benign

Figure 5.2. Bioinformatic analysis of V141L. *(a) Diagram localising rs2257167 to exon 4 of IFNAR1. (b) Protein alignment of V141L using Clustalω. (c) Localisation of rs2257167 using PyMOL. (d) Table of results from function prediction analysis using SIFT, Provean and Polyphen-2. The Genbank accession numbers are as follows: mouse = AAH43935.1, human = NP_000620.2, zebrafish = A0FJH7, chicken = Q9YHW0.*

Rs2257167 is an expression quantitative trait loci for IFNAR1.

To assess the impact of the SNP on the expression of IFNAR1 at baseline and in response to stimulation we analysed data from healthy donors from the viral resistance cohort and the MIC. Prior to any downstream analysis we sought to determine whether there was a difference in IFNAR1 at baseline and in response to stimulation between males and females, as sex is known to impact on the immune system. In donors from the MIC we found increased expression of IFNAR1 in whole blood from females in the null condition (**Fig. 5.3a**). In response to stimulation, we saw an increased expression of IFNAR1 in the MIC males compared to the females in response to LPS, but saw no significant difference between sexes in response to polyIC or IAV. For subsequent analysis we focused solely on female donors in the MIC. In the healthy control donors from the viral resistance cohort we saw increased expression of IFNAR1 RNA in unstimulated whole blood in females with the GC.CC genotype compared to the GG donors (**Fig. 5.4a**). We next looked at data from the MIC. The increased IFNAR1 expression in whole blood from GC.CC donors observed in our cohort was recapitulated in the MIC, indicating rs2257167 is a cis-acting eQTL for IFNAR1 (**Fig. 5.4b**). In response to stimulation with polyIC, R848, ODN or IFN in healthy females from the viral resistance cohort we saw no significant difference in the expression of IFNAR1 between genotypes (**Fig. 5.4c**). This finding was extended in response to polyIC and IAV in the MIC (**Fig. 5.4d(i, ii)**). However, in response to LPS, GG donors appeared to have increased IFNAR1 expression compared to the GC.CC donors in the MIC (**Fig. 5.4d(iii)**).

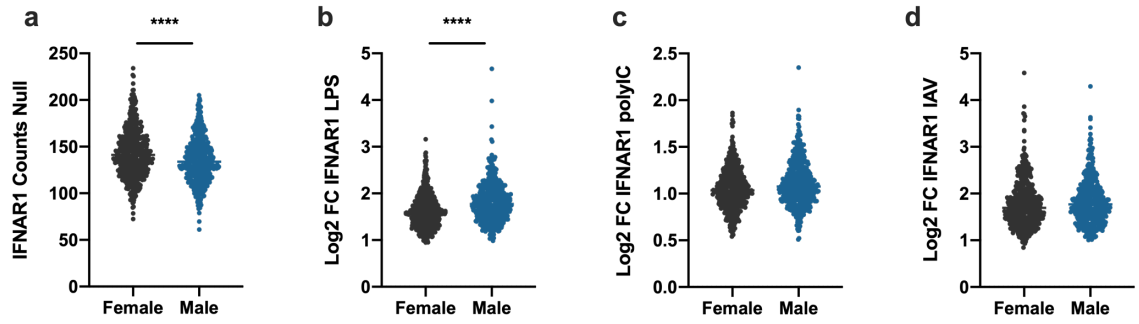


Figure 5.3. IFNAR1 expression is higher in females in the null condition. *Whole blood from the milieu interieur 1000 person cohort was aliquoted into (a) negative control TruCulture tubes or tubes containing (b) LPS, (c) polyIC or (d) IAV and incubated (37°C, 22 hours). RNA from each condition was analysed using NanoString transcriptomics (human immunology panel v2). The log_2 fold change expression of IFNAR1 above the unstimulated condition was calculated in response to LPS, polyIC and IAV stimulation. The median line is shown as a solid line. Unpaired t tests were used to compare expression between males and females (***p<0.001).*

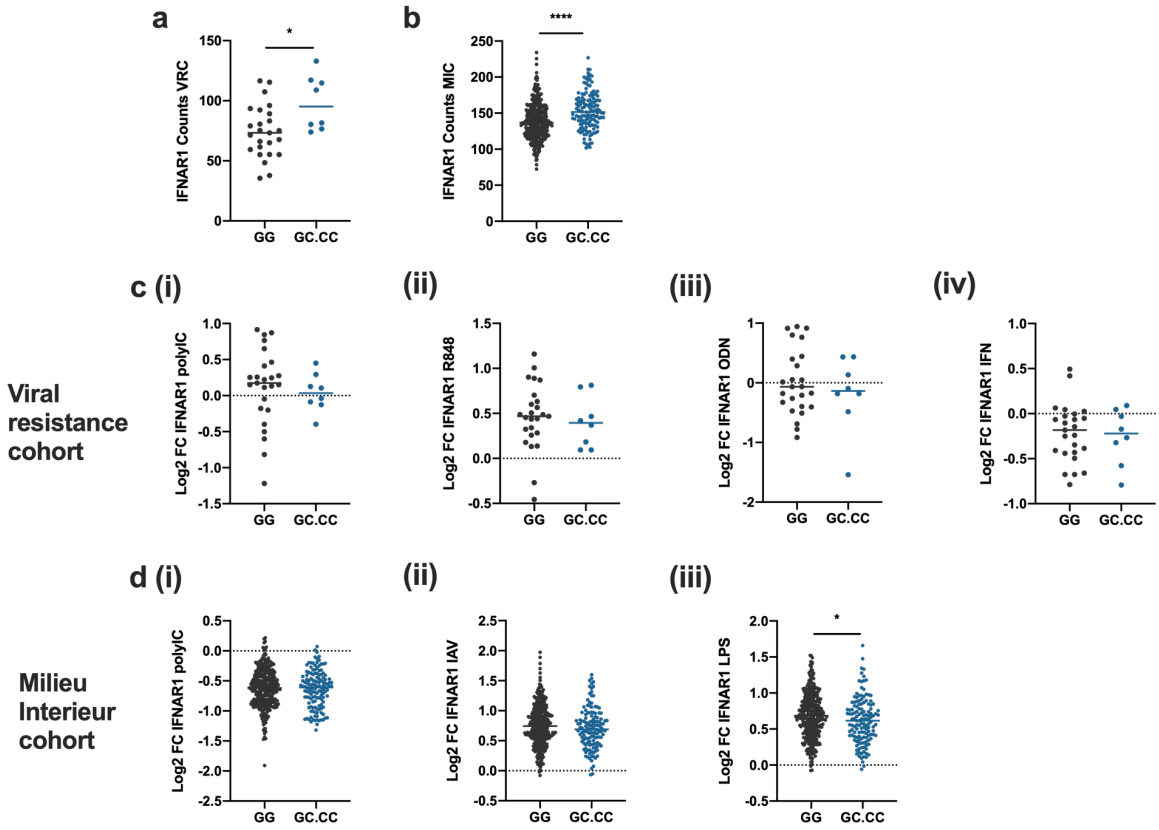


Figure 5.4. rs2257167 is cis-acting baseline expression QTL (eQTL) for IFNAR1. *Whole blood NanoString data from healthy donors in the viral resistance cohort and females from the MIC was analysed for IFNAR1 expression at baseline and in response to stimulation. IFNAR1 RNA expression in the null condition from donors in (a) the viral resistance cohort and (b) the MIC. (c(i-iv)) IFNAR1 expression in healthy donors from the viral resistance cohort in response to (i) polyIC, (ii) R848, (iii) ODN and (iv) IFN as a log_2 fold change above the unstimulated expression. (d(i-iii))) IFNAR1 expression in female donors from the MIC in response to (i) polyIC, (ii) IAV and (iii) LPS. The median line is shown as a solid line. Unpaired t tests were used to compare expression between GG and GC.CC donors (*p<0.05, ***p<0.001).*

Rs2257167 alters the IAV induced immune response in whole blood.

To assess the potential downstream consequences of rs2257167 we analysed NanoString transcriptomic data from whole blood stimulated with LPS, polyIC and the live IAV from females in the MIC. We opted to focus our analysis on this cohort to maximise power and the likelihood of detecting potentially subtle differences between genotypes. We first analysed data from these donors in an unbiased manner using a FDR adjusted unpaired t tests, comparing GG donors with those carrying at least one C allele grouped together (GC and CC). A q value of <0.1 was taken as significant. Using this approach we found differential expression of 16 genes between GG and GC.CC donors in the IAV stimulation (**Fig. 5.5a**). There were no significant differences between GG and GC.CC donors in response to polyIC or LPS (**Fig. 5.5b, c**).

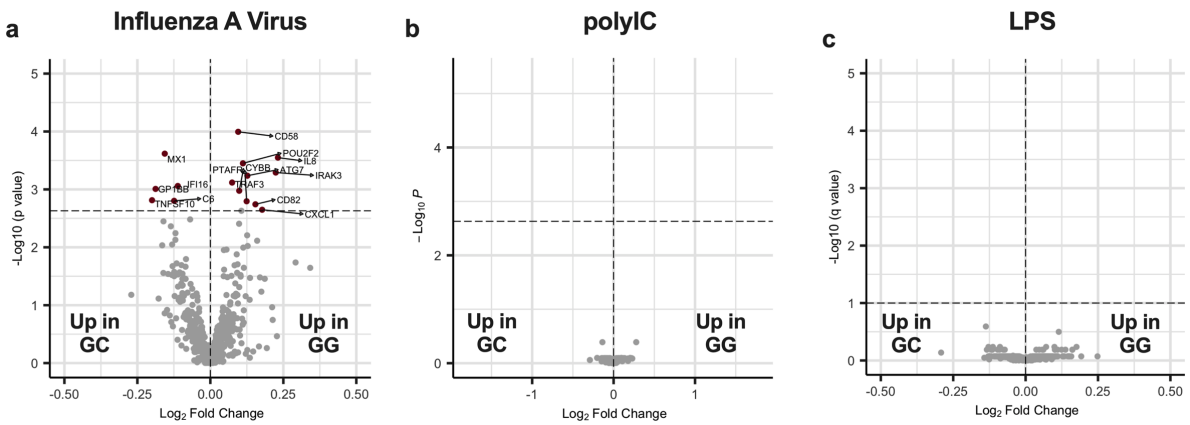


Figure 5.5. rs2257167 is associated with an altered response to IAV stimulation. *NanoString data from whole blood stimulated with live IAV, polyIC and LPS from females in the MIC was analysed for differences in the response to stimulation between GG and GC.CC donors. (a) Volcano plot showing differential expression of 16 genes between GG and GC.CC donors in response to IAV stimulation (q<0.1, unpaired t test with FDR correction). (b, c) Volcano plot showing no significant differences between GG and GC.CC donors in response to stimulation with (b) polyIC and (c) LPS (q>0.1, unpaired t test with FDR correction).*

Rs2257167 alters the IFN-I gene signature score for LPS and IAV.

To determine whether or not rs2257167 impacts on specific modules of gene expression we applied gene signature scores to the MIC whole blood NanoString stimulation data. These scores are generated using the average Z scores of specific genes from gene-sets known to be induced in a distinct manner by the cytokine of interest and were previously devised in an independent healthy cohort. Here we examined the gene signature scores for IL1β, IFN-I, TNFα and IFNγ. In response to stimulation with IAV and LPS, GC.CC donors have increased IFN-I gene signature scores compared to GG donors (**Fig. 5.6a**). There was no difference in the IFN-I score between genotypes in response to polyIC stimulation (**Fig. 5.6a**). There were no significant differences in the gene signature scores of IFNγ, TNFα or IL1β between genotypes in response to any of LPS, IAV or polyIC (**Fig. 5.6b-d**).

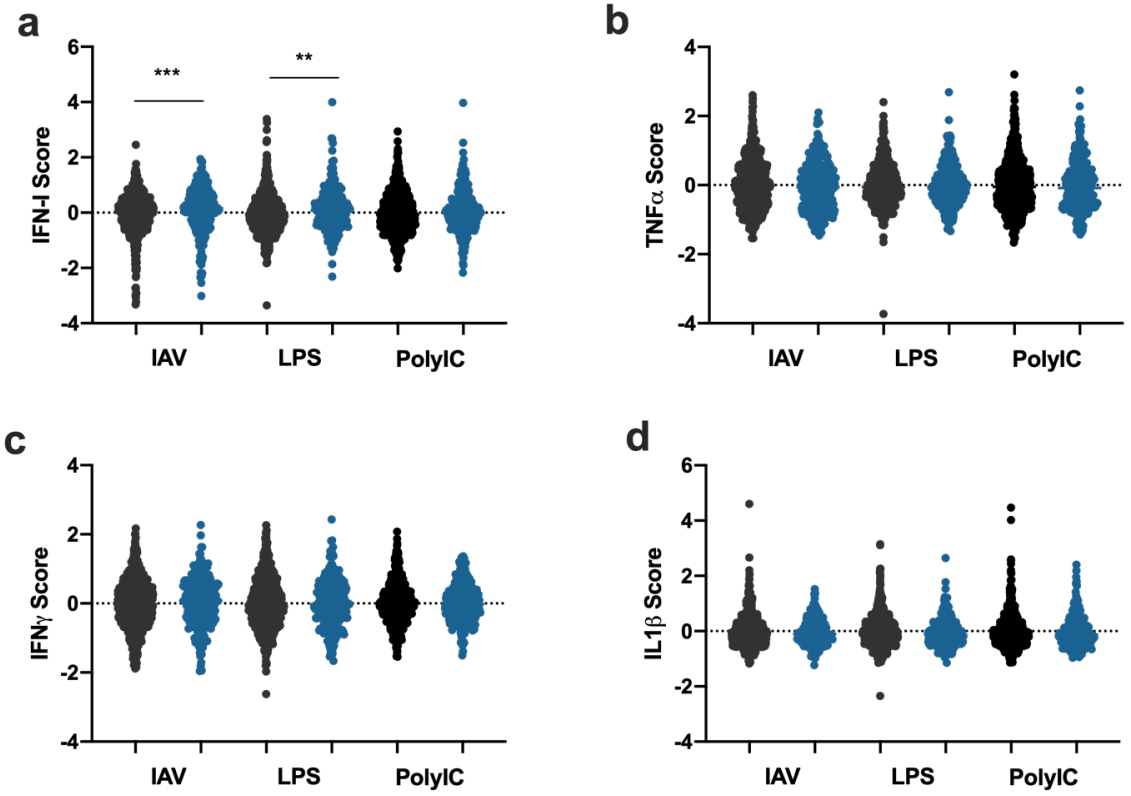


Figure 5.6. Increased IFN-I gene signature in response to IAV and LPS stimulation. *Whole blood from females in the MIC was stimulated with IAV, LPS or polyIC for 22 hours and gene expression analysed using the NanoString transcriptomics human immunology panel v2. Gene signature scores were applied to NanoString whole blood stimulation data. (a-d) Gene signature score in response to IAV, LPS and polyIC stimulation in GC.CC donors compared with GG donors for (a) IFN-I, (b) TNFα, (c) IFNγ and (d) IL1β (**, p<0.01, *** p<0.001; unpaired t test). Black = GG and Blue = GC.CC.*

Rs2257167 increases IRG upregulation in response to stimulation with LPS, IAV and polyIC.

To assess the downstream consequences of rs2257167 at an individual gene level we selected a subset of IRGs from the NanoString panel and examined their upregulation in the MIC in response to whole blood stimulation with LPS, influenza A virus (IAV) and polyIC. These stimuli are known to increase IFN-I protein expression which acts via the IFNAR1/IFNAR2 complex to upregulate IRGs. In response to these stimuli we observed an increase in the expression of several IRGs in the GC.CC donors compared with those of the GG genotype (**Fig. 5.7**).

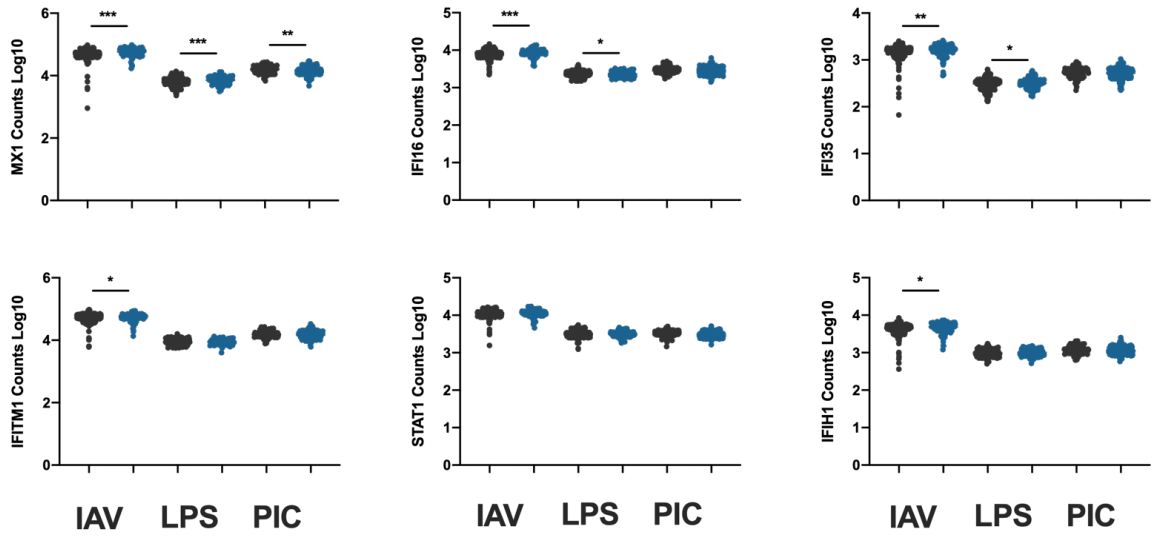


Figure 5.7. rs2257167 in IFNAR1 increases the IRG response to several PRR agonists. *Whole blood was stimulated with IAV, LPS and polyIC for 22 hours and gene expression analysed using the NanoString transcriptomics human immunology panel v2. GG donors are shown in black, while GC.CC donors are shown in blue. GC.CC donors have increased gene expression of several IRGs compared with GG donors (*p<0.05, **p<0.01, ***p<0.001; unpaired t test). Data is presented as log_{10} counts. Black = GG and Blue = GC.CC.*

Rs2257167 decreases the non-IRG proinflammatory response

To further dissect the impact of rs2257167 on the inflammatory response we examined a subset of non-IRG genes in the NanoString panel in females from the MIC. These included NFKBIA, IL10, IL1β, IL6, IL8 and TRAF1. In response to stimulation with IAV, LPS and polyIC donors with the GG genotype had increased expression of several non-IRG inflammatory genes including NFKB, IL8 and IL6 compared to those with at least one copy of the variant C allele (GC.CC; **Fig. 5.8**).

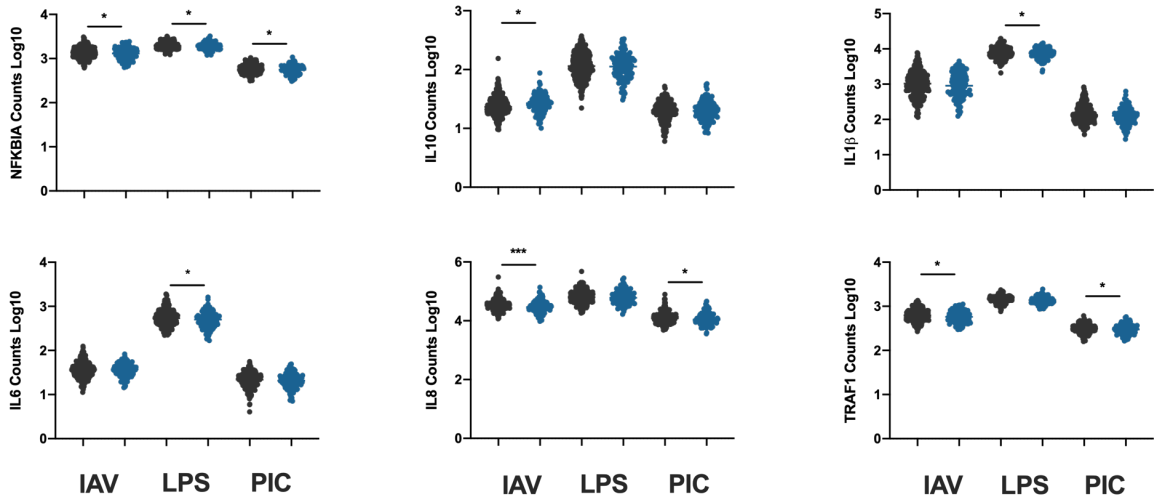


Figure 5.8. rs2257167 decreases the inflammatory response to several viral ligands. *Whole blood was stimulated with IAV, LPS and polyIC for 22 hours and gene expression analysed using the NanoString transcriptomics human immunology panel v2. GG donors are shown in black, while GC.CC donors are shown in blue. GC.CC donors have increased gene expression of several IRGs compared with GG donors (*p<0.05, **p<0.01, ***p<0.001; unpaired t test). Data is presented as log_{10} counts. Black = GG and Blue = GC.CC.*

Discussion

Despite exposure to highly infectious batches of HCV contaminated anti-D immunoglobulin in the 1970s, up to 50% of the women who were exposed remained negative for both HCV viral RNA and anti-HCV antibodies. The genetic reasons contributing this apparent natural resistance to HCV infection remained to be explored. Here, we identify a SNP in IFNAR1, rs2257167 as being associated with resistance to viral infection in this cohort.

Given the relatively small size of our cohort we opted to use a gene candidate approach to limit correction for false discovery. Based on our biological results showing an enhanced TLR3-IFN-I gene signature and the literature we chose tagSNPs in TLR3 (rs3775291), IRF3 (rs7251) and IFNAR1 (rs2257167). Each of these SNPs had previously been associated with enhanced susceptibility or resistance to viral infection [195,196,229]. We opted not genotype the cohort for the IFNL4 locus previously associated with SR of HCV, as previous studies of ESNs did not find an association between resistance and this locus [179]. Using this gene candidate approach we identified an association with the G allele of rs2257167 and resistance to HCV infection in the our cohort.

Using function prediction software we found the predicted consequences of rs2257167 to be non-deleterious and benign. Recent work showed that function prediction software is poorer at detecting positive impacts of SNPs compared to those that are deleterious [240]. Given that GWAS studies have shown the SNP to be associated with outcome of several clinical conditions and infections, including cancer and HBV, it seems likely that the SNP has significant biological consequences [219,226].

Having identified an association with IFNAR1 and resistance we examined NanoString transcript data from the MIC. Previous work has shown that several components of the IFN-I pathway, including IFNAR2 are increased on pDCs from females, however studies of IFNAR1 have been less clear [241]. We found that IFNAR1 expression is increased in whole blood from females compared to males. This increased IFNAR1 expression may contribute, along with several other genes impacted by sex, to the enhanced protection against infection described in this cohort and elsewhere [242,243].

Examining the biology of rs2257167 in healthy donors from both our viral resistance project cohort and donors from the MIC we found that the SNP was associated with increased IFNAR1 mRNA expression in whole blood. Surprisingly, our findings in whole blood are in contrast to those seen in the GTEX database – a browsable dataset of integrated transcriptomic and genomic data (https://gtexportal.org/home/snp/rs2257167). In the GTEX dataset, GC and CC donors have reduced IFNAR1 expression in several tissues including oesophagus, artery and brain. Tissues from GG donors had higher IFNAR1 expression in the GTEX dataset. Unfortunately for this SNP, data from whole blood in the GTEX dataset was unavailable. Previous studies of rs2257167 showed either no difference in IFNAR1 expression or increased expression on monocytes from GC.CC donors [219,244]. Both valine and leucine are branch chain amino acids of similar structure, however it has been suggested that the larger leucine structure may act to stabilise IFNAR1 expression on the cell surface and increase IFNAR1 expression [245].

The reasons behind the discordance between our data and the GTEX data are unknown but may be linked to haplotypic differences in IFNAR1. Rs2257167 is in LD with a large number proximal intronic and distal promoter SNPs in IFNAR1, many of which could impact on the expression of the receptor (as well as downstream signalling) [227]. The contrasting results between our viral resistance cohort and MIC data compared with GTEX data could also be due to tissue specific SNP effects [246]. Reports suggest differences in the cytokine milieu, architecture and epigenetic landscape of tissues can influence the impact of genetic variants locally. From an HCV perspective, this could be an important consideration when extrapolating results obtained in the blood to other tissues such as liver.

Using PyMOL we localised the SNP to the SD2 domain of IFNAR1. Although distal to the IFN-I binding sites, the SNP appears to impact on downstream IRG upregulation following IFNAR1 binding and signalling. It is hypothesised that the V141L may interact with adjacent amino acid residues such as Trp143 or Tyr188 [219]. Furthermore, the SD2-SD3 regions of IFNAR1 contains a hinge region that facilitates a conformational change following IFN-I binding [247]. The larger leucine could displace proximal amino acids

important for IFN-I binding and alter interdomain motility. The increased IFNAR1 expression in the null condition may explain the altered IRG response to stimulation with TLR and IFNAR1/2 ligands. It is also possible that the SNP affects binding of an accessory protein such as TYK2, thereby altering downstream signalling.

Upon analysing the downstream effects of the SNP we found that the SNP appeared to have contrasting effects on the proinflammatory and IRG responses to whole blood stimulation with LPS, IAV and polyIC in the MIC. It is plausible that the increased IRG response observed in those carrying a C allele could inhibit the pro-inflammatory response. Indeed, this phenomena has previously been described, where IFN-I can suppress specific proinflammatory modules through a number of mechanisms. Upregulation of IL10 by IFN-I, can inhibit IL1β, TNFα and IL12 signalling, IFN-I can downregulate the IFNγ receptor, IFNGR1, thereby limiting IFNγ signalling. IFN-I can further limit IL1β signalling through induction of IL1RA, the IL1 receptor antagonist [17]. We did not observe the same downstream SNP effects in healthy donors from the viral resistance cohort. This is may be due to the size of the viral resistance cohort and the resultant reduction in power, as the differences between GG and GC.CC donors are subtle. The upregulated pro-inflammatory cytokines in the GG genotype, associated with HCV resistance, include IL-6 and IL-8 [187]. These cytokines have previously been shown to be important in control of HCV infection.

Not all stimuli impacted IRGs in the same manner, and significant heterogeneity was observed in the expression patterns of IRGs within each stimulus. These inconsistencies are likely due to the 22 hour time point that was chosen. Following pathogen exposure in vivo, IFN-I is rapidly produced to upregulate IRGs and limit infection within the early hours. While the 22 hour time point chosen for our whole blood stimulations was optimal for capturing upregulation of several genes, an earlier time point would be more appropriate in understanding the impact of rs2257167 on IRG expression [164].

The differences between GG and GC.CC females were most apparent following IAV stimulation in the MIC. IAV is a live virus and engages several TLRs, including TLR7/8, TLR10 and TLR3 [248]. Detection of IAV by multiple TLRs may explain why differences in genotypes

of the rs2257167 SNP are most pronounced with this stimulation [249]. The stimulus specific differences in the effects of rs2257167 could be explained by the types of IFN-I induced by ligation of different TLRs. Although all IFN-I signal via the same IFNAR1/2 heterodimer, they do so with different affinities and engage different amino acid residues on the receptor [250].

The presence of a single C allele was sufficient to alter IFNAR1 expression and downstream IRG expression. As there were just 14 CC donors in the MIC and no CC donors for which we had biological data in our viral resistance cohort we opted to group GC and CC donors together. However, additional analysis of GG versus GC versus CC donors showed a stepwise increase in IFNAR1 expression in an allele dosage manner. *In vitro* analysis of rs2257167 in cell culture models will be important in understanding the effects of the SNP on phosphorylation of downstream signalling components of the JAK-STAT pathway.

Importantly the association described here is one of many potential contributors to the resistant phenotype in our cohort. Given the high level of resistance reported (47%) in the anti-D cohort the mechanisms contributing to the phenotype are likely to be many and polygenic, wherein small effects across multiple genes result in a protective phenotype. A similar synergistic effect has been described for individuals at risk of chronic HCV infection [143]. As the SNP does not appear to be associated with an increase in the IFN-I gene signature it is unlikely to underlie, at least in full, the enhanced polyIC-IFN-I gene signature described for the ESNs in the previous chapter.

Here we have identified an association between the GG genotype of the rs2257167 SNP in IFNAR1 and resistance to HCV infection in the Irish anti-D cohort. We show that the SNP is associated with increased IFNAR1 expression at baseline, and appears to exert different effects on the IRG and pro-inflammatory responses. The increased pro-inflammatory response to stimulation with LPS and IAV in the GG donors may have played a role in protection against HCV infection in the ESN women in our cohort.

Chapter 6: Investigating the impact of Rhesus status on the immune response

Abstract

The Rhesus D antigen (RhD) has been associated with susceptibility to several viral infections. Reports suggest that RhD-negative individuals are better protected against infectious diseases and have overall better health. However, potential mechanisms contributing to these associations have not yet been defined. Here, we used transcriptomic and genomic data from the *Milieu Interieur* cohort of 1000 healthy individuals to explore the effect of RhD on immune responses. We used the rs590787 SNP in the RHD gene to classify the 1000 donors as either RhD-positive or -negative. Whole blood was stimulated with LPS, polyIC, and the live influenza A virus and the NanoString human immunology panel of 560 genes used to assess donor immune response and to investigate sex specific effects. Using regression analysis, we observed no significant differences in responses to polyIC or LPS between RhD-positive and -negative individuals. However, upon sex-specific analysis, we observed over 30 differentially expressed genes (DEGs) between RhD-positive (n=401) and RhD-negative males (n=78). Interestingly these Rhesus-associated differences were not seen in females. Further investigation, using gene set enrichment analysis, revealed enhanced IFNγ signalling in RhD-negative males. This amplified IFNγ signalling axis may explain the increased viral resistance previously described in RhD-negative individuals.

Introduction

The Rhesus blood group antigen

The Rhesus D blood group antigen (RhD) system is an important clinical factor in transfusion and obstetric medicine. RhD status is determined based on the presence or absence of the rhesus antigen, a transmembrane protein found on the surface of red blood cells [251]. The function of the RhD antigen is largely unknown, although it may play a role in maintaining erythrocyte membrane integrity or transport of ammonium and carbon dioxide [252–254]. The rhesus protein is highly immunogenic and resulting antibodies can induce severe adverse reactions in RhD-negative individuals should they encounter the D antigen following an unmatched blood transfusion. RhD-negative women can also be sensitised during pregnancy with an RhD-positive foetus or during delivery, often leading to haemolytic disease of the new born in subsequent pregnancies with RhD-positive foetuses [255].

RhD and the immune system

Although less well studied, RhD status is also known to influence several other health outcomes [256]. An agnostic analysis of 1217 disease states found an association between RhD status and hypertension during pregnancy [256]. Several studies have demonstrated that RhD-positive and -negative subjects differ in resistance to the pathological effects of aging, fatigue, and smoking [257]. RhD-negative individuals also appear to be protected against infection, including latent toxoplasmosis [257]. RhD status also appears to affect susceptibility to SARS-CoV-2; in a study of 14,112 donors, RhD-negative individuals had a lower risk of initial infection, intubation and death- suggesting a protective role for RhD negativity [258]. RhD negativity varies substantially across different populations and may potentially confer an as yet unknown fitness advantage [259]. A major challenge in determining potential effects of RhD status on antiviral immunity is the lack of large, relevant human cohort studies with the power to detect potentially subtle immune differences.

The overall aim of the *Milieu Interieur* study is greater understanding of the determinants of variation in the immune responsiveness of healthy adult humans [260]. Previous work

from MI and others has shown that sex and CMV serostatus are key drivers of variation in the human immune response [66,261]. Substantial genomic and transcriptomic data has been generated on the cohort to date and analysed using agnostic approaches [66,262]. Access to data from the MIC presents a unique opportunity to study the impact of RhD status on the immune response.

Classifying RhD status

Here, we used genotype data on the rs590787 single nucleotide polymorphism (SNP) in RhD to classify individuals from the MI study as either RhD-positive or -negative, an approach that has previously been used to determine RhD status in several studies [166,263,264]. Individuals who are RhD-negative are homozygous for the recessive alleles (CC), while RhD-positive individuals are heterozygous or homonymous dominant (CT, TT). Genomic data was integrated with transcriptomic data from whole blood stimulated with bacterial and viral ligands, LPS and polyIC, and the live influenza A virus, to investigate whether Rhesus status was associated with induced immune responses. We were particularly interested to compare RhD-positive and -negative males and females, as sex is emerging as a key factor in determining outcomes to viral infection, especially SARS-CoV-2 and influenza [265].

Chapter hypothesis

RhD-negativity is associated with an enhanced innate immune response that is protective against infection.

Chapter aims

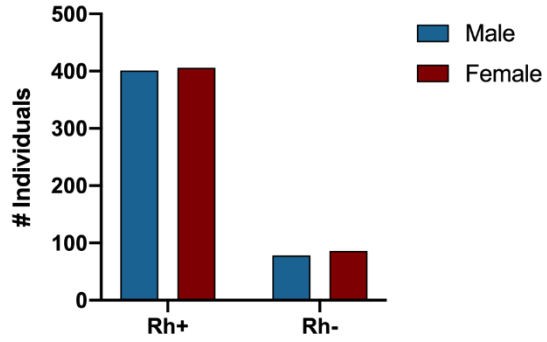
1. To compare the frequency of RhD-positivity and -negativity between males and females from the MIC using the rs590787 SNP as a marker.
2. To compare the whole blood immune response to the PRR ligands LPS, polyIC and IAV between RhD-positive and -negative individuals using NanoString transcriptomics and Luminex proteomics.
3. To compare serological data for common viral infections between RhD-positive and -negative individuals from the MIC.

Results

Rhesus status distribution is similar between males and females.

The rs590787 SNP in the RhD gene, was used to determine the RhD status of donors in our cohort **(Fig.6.1)** [166]. There was no difference in the distribution of RhD-positive or -negative individuals between males and females in our cohort **(Fig. 6.1a)**. 83% of donors in the MI cohort were RhD-positive **(Fig. 6.1b)**. The genotype frequencies of the Rhesus SNP rs590787 were compared to those from the 1000 genomes cohort [266]. The genotype frequencies in the MI cohort were similar to those observed for Europeans in the 1000 Genome Study **(Fig. 6.1c)**.

a



b

Rhesus Status	Male	Female	Total
Rh+	401	406	807 (83%)
Rh-	78	86	164 (17%)
Total	479	492	971

c

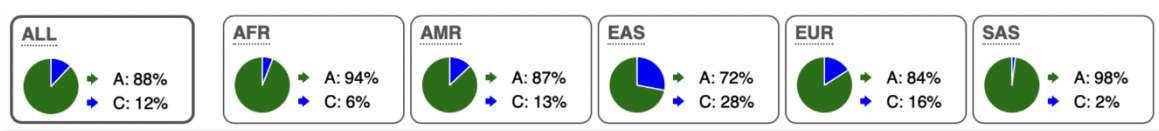


Figure 6.1. Rhesus phenotype distribution of all individuals in the Milieu Interieur cohort. *RhD status was determined based on rs590787, a SNP in the RHD gene, using the Human Exome Bead Chip.* ***(a)*** *Frequencies of RhD-positive and -negative individuals in males and females from the MIC.* ***(c)*** *Genotype frequencies for the European cohort from the 1000 Genome Project. The numbers in brackets are percentages.*

Rhesus factor does not affect immune gene expression at baseline or in response to stimulation with PRR ligands in whole blood.

Whole blood was incubated for 22 hours and expression of 560 immune genes quantified using NanoString transcriptomics. Gene expression in the unstimulated condition was similar between all RhD-positive and -negative individuals. Given the important sex-specific differences emerging regarding pathogen susceptibility, we also investigated sex-specific effects. The cohort was also stratified by sex and assessed for differential gene expression between RhD-positive and -negative males and females. Baseline immune gene expression was similar between RhD-positive and -negative males and females **(Fig. 6.2(i))**.

To assess specific TLR induced immunity, namely TLR3 and TLR4, we stimulated whole blood with the widely used ligands, polyIC and LPS. PolyIC is a dsRNA mimic that acts via TLR3 to upregulate an IFN-I response [267]. LPS is a bacterial ligand that can activate both the IFN-I response, as well as other proinflammatory pathways leading to upregulation of key mediators including IL-6, TNFα and COX2 [268]. Using regression analysis, we found the response to both polyIC and LPS to be similar in RhD-negative and -positive individuals whether male or female (**Fig. 6.2(ii), (iii)**). Data is presented as a heatmap with 100 randomly selected genes shown. Differences in cell counts can have a major impact on the whole blood transcriptional response [262]. Therefore, we compared major circulating immune populations between RhD-positive and -negative individuals. Following FDR correction, we observed no significant differences in any cell population examined (**Table. 6.1**).

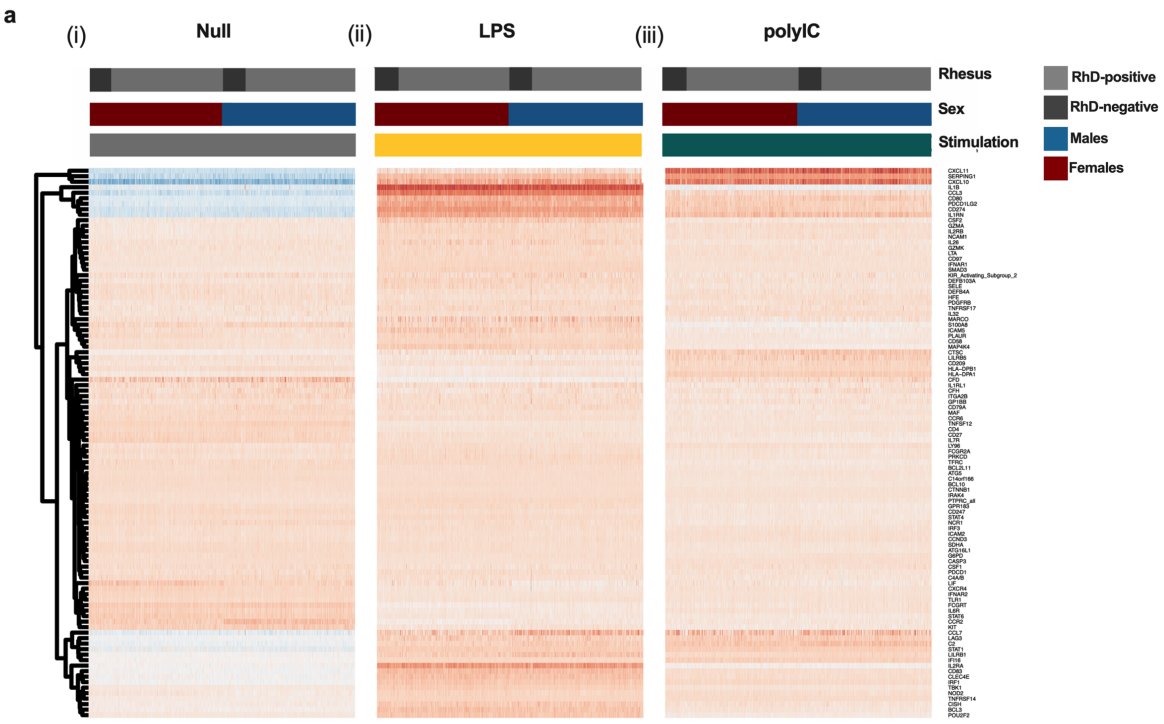


Figure 6.2. Rhesus antigenicity does not affect immune gene expression at baseline or in response to stimulation with PRR ligands in whole blood. *Immune gene expression in unstimulated, LPS and polyIC stimulated whole blood was assessed using NanoString transcriptomics. Comparisons between RhD-positive and -negative individuals in all donors, females only and males only showed no significant differences (q>0.1 , regression analysis with FDR correction) for* ***(i)*** *Null,* ***(ii)*** *LPS or* ***(iii)*** *polyIC. Shown in the heatmap are 100 representative genes selected at random.*

Immune cell type	p value	FDR adjusted p value (q value)
CD45+	0.29	0.71
CD19+ B cells	0.66	0.71
CD3+ cells	0.62	0.71
CD4+ T cells	0.58	0.71
CD8b+ T cells	0.84	0.84
CD4-CD8- T cells	0.45	0.71
NK cells	0.12	0.36
CD56+ NK cells	0.56	0.71
CD16++ NK cells	0.1	0.36
CD8+CD4+ T cells	0.09	0.36
Monocytes	0.04	0.31
CD14+ monocytes	0.03	0.31
CD16+ monocytes	0.62	0.71
PMN	0.37	0.71
Neutrophils	0.48	0.71

Table 6.1. No difference in numbers of major circulating immune cell populations between RhD-positive and -negative donors. *Immune cell were quantified using flow cytometry. Potential differences in the numbers of these circulating immune cells were assessed using regression analysis including CMV serostatus as a covariate, p values were adjusted using the FDR correction (q>0.1).*

Increased IFNγ mediated responses in RhD-negative males only.

Infection with a live virus results in the activation of several PRRs and downstream pathways that upregulate pro-inflammatory cytokines and antiviral mechanisms, and help clear infection [269]. To determine whether the Rhesus factor has an impact on the antiviral response to a live virus, we stimulated whole blood with the live influenza A virus. No significant difference was observed between RhD-positive and -negative individuals when comparing the entire cohort, or when looking at females only **(Fig. 6.3a)**. Interestingly, however, when examining the whole blood response to influenza A virus between RhD-positive and -negative males only, we observed differential expression of 35 immune related genes (**Fig. 6.3b, Fig. 6.3c**; q <0.1, regression analysis with FDR correction). To further interrogate differences in the immune response to influenza A between RhD-positive and -negative individuals we used gene set enrichment analysis. Using this approach we found the IFNγ pathway to be significantly enriched in RhD-negative males (**Fig. 6.3d)**. We did not observe a difference in IFNγ protein levels between RhD-positive and -negative individuals in response to IAV stimulation in all donors, males only and females only (**Fig. 6.4**)

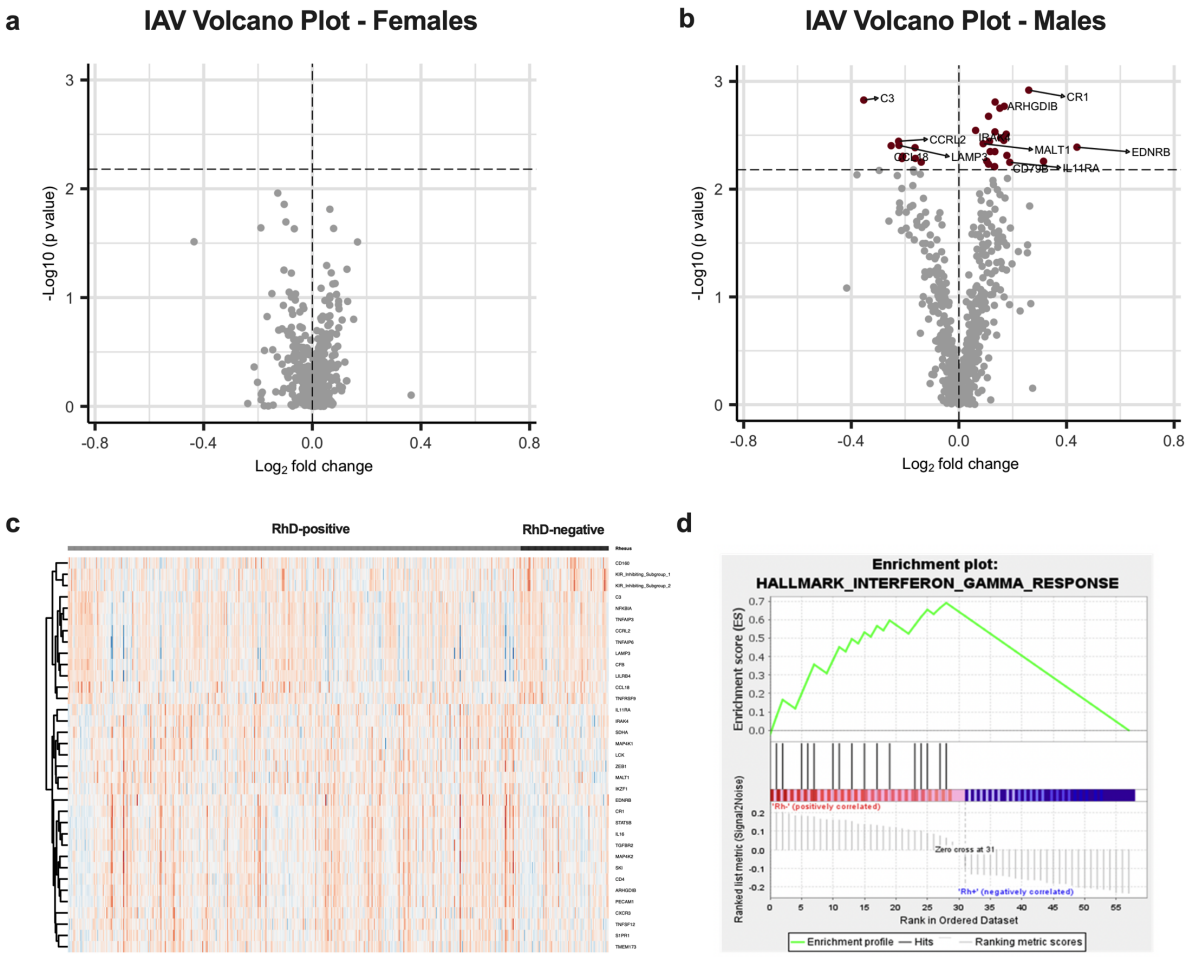


Figure 6.3. Following influenza A virus stimulation, 35 genes were differentially expressed between RhD-positive and -negative males. *Whole blood was stimulated with influenza A virus and gene expression assessed using NanoString.* ***(a)*** *Volcano plot showing no differentially expressed genes between RhD-positive and -negative females (q>0.1, regression analysis with FDR adjustment).* ***(b)*** *Volcano plot of the differentially expressed genes between RhD-positive and -negative donors in the male group (q<0.1, regression analysis with FDR adjustment).* ***(c)*** *Heatmap of DEGs from RhD-negative males.* ***(d)*** *Enrichment plot indicating upregulation of the IFNγ pathway in the RhD-negative males (p<0.0001).*

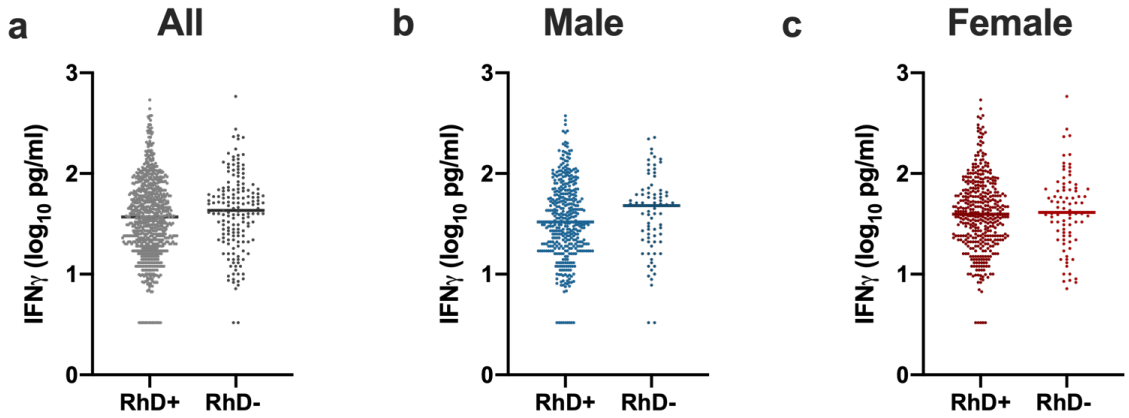


Figure 6.4. No difference in IFNγ protein expression between RhD-positive and -negative donors in response to influenza A virus stimulation. *Following stimulation with influenza A virus, supernatants were collected and IFNγ protein levels quantified using Luminex proteomics. (a-c) Influenza A virus induced IFNγ protein levels from RhD-positive and -negative donors for (a) all, (b) males only and (c) females only. The median line is shown as a solid line (p>0.05, unpaired t test).*

RhD status is not associated with differences in antibody positivity against common viral infections.

As RhD-negative males had an increased IFNγ response and given previous studies have shown protective associations between RhD-negativity and infection, we sought to assess potential differences in seropositivity between RhD-positive and -negative individuals using previously generated serology data from the MIC [270]. No differences in the seropositivity between RhD-positive and -negative males were observed (**Fig. 6.5a**). However, in RhD-negative females, we found an increase in the EBV antibody titre of RhD-negative females (**Fig. 6.5b)**.

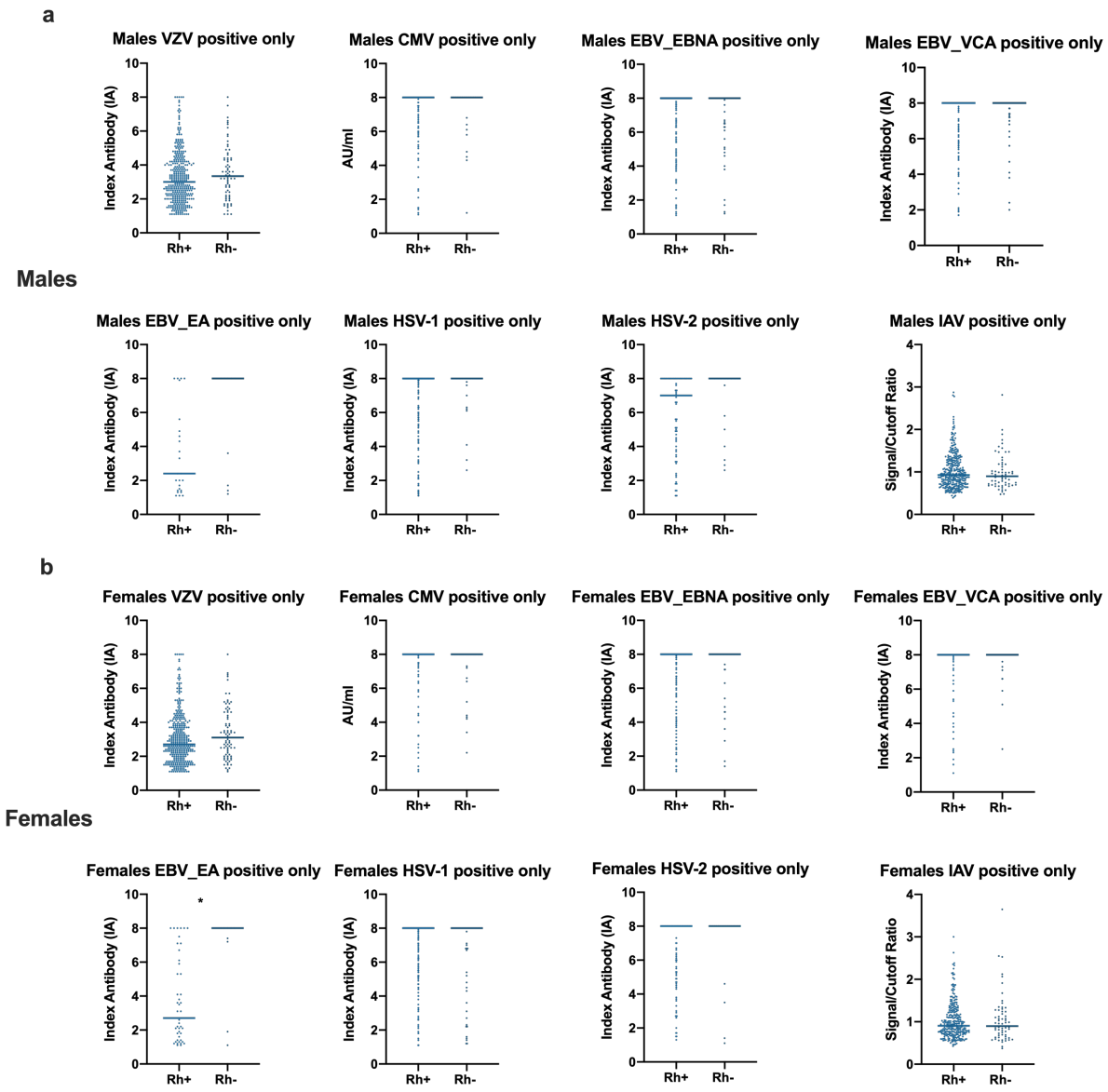


Figure 6.5. Serological analysis of MI donors by sex and RhD antigen status. *Seropositivity for antibodies against common viruses was assessed in donors from the MIC by RhD status. (a) serology for male donors from the MIC. (b) serology for female donors from the MIC. Comparisons between RhD-positive and -negative donors were made using Mann Whitney U tests (*p<0.05). The median line is shown as a solid line.*

Discussion

In a French cohort of 1000 well characterised healthy individuals, we investigated the impact of RhD on the innate immune response. Following stimulation with live Influenza A virus, we found differential expression of 35 immune genes between RhD-positive and -negative males. In contrast RhD-negative and positive women had similar responses to influenza A virus. Further analysis of the differentially expressed genes revealed enrichment for IFNγ signalling in RhD-negative males. The increased IFNγ signalling found here in RhD-negative males may begin to explain their reduced susceptibility to infection.

Several association studies have found a relationship between RhD-negativity and enhanced resistance to viral infection [256–258,271]. However, systemic analysis of immune activity by RhD status had never been carried out. RhD-negative individuals have reduced risk of initial infection with SARS-CoV-2, as well as decreased risk of both intubation and death [258]. The increased IFNγ signalling that we find in RhD-negative donors could contribute to reduced susceptibility to SARS-CoV-2 infection and pathology.

This study analysed transcriptomic data from studies using well described PRR ligands, LPS and polyIC, which are mimics relevant to bacterial and viral infection. We also chose to include a more complex live stimulus, the influenza A virus. The responses to the bacterial and viral mimics in RhD-positive and -negative individuals were similar across all groups. Studies of cells from other blood types including the Lewis (Le) group found no differences in expression in responses to LPS between Le+ and Le- individuals in either males or females [272]. Another study found associations between the inheritance of polymorphisms in genes that encode and regulate the expression of the Lewis blood antigens and protection against infections with *Helicobacter pylori* [273]. This suggests that analysis focused exclusively on specific TLR ligands may not be sufficient to identify differential effects of blood groups on the innate immune response to infectious agents. Studies using whole organisms may be more informative.

Here, using stimulation with the influenza A we observed significant differential expression of 35 immune genes between RhD-positive and -negative males. Influenza A

viral pathogenesis has already been described as different between sexes, with males at risk of developing more severe disease compared with females of a similar age [243]. Recent studies have shown that females mount a more robust immune response and have increased production of several key inflammatory proteins known to be important in control of viral infection [242]. This more potent innate immune response in females could mask potential differences in RhD-positive and -negative females in our system.

The distribution of blood groups, including RhD, varies globally- this may explain, at least in part, the regional differences in disease occurrence. RhD-negativity is particularly enriched in East Asian populations (28% RhD-negative) compared with European or African populations (16% and 6%, respectively) [256]. This may reflect the different historic disease burdens on these populations. As the cohort used in our study includes only donors of Western European descent, more cohorts should be analysed to include individuals of difference ethnicities to further probe the differences in the immune response between RhD-positive and -negative individuals.

While non-communicable disease states have been studied in the context of blood antigens, viral illness is often overlooked as it is typically transient and often underreported [274]. Further association studies exploring the relationships between blood types and viral infection are warranted to fully understand the contribution of blood type to risk of severe viral disease. Findings from these studies may inform clinical management of patients based on RhD status. Recently, Alsten et al. looked at differences in 96 circulating inflammatory proteins between several blood groups. While RhD was not among those examined, differences were found in the circulating inflammatory profiles of ABO and Duffy blood types [275].

In this study we found evidence of an enhanced IFNγ mediated immune response in RhD-negative males. IFNγ has been shown to be important in the control of several viral infections, including Ebola and SARS-CoV-2 [276,277]. The enhanced signature observed in RhD-negative individuals in our cohort may contribute to the increased resistance to infection described in these individuals. The enhanced gene signature was not reflected at the protein level. However, this discrepancy could be explained by the relatively low

induction of IFNγ by influenza in our system, or by differences in the kinetics of IFNγ protein and its downstream transcripts. Increased responsiveness to IFNγ by RhD-negative individuals could also explain the enhanced IFNγ response in the absence of an increase in IFNγ protein.

Although modest, the differences between RhD-positive and -negative males were widespread and may contribute to the increased viral resistance reported elsewhere. Several genes related to natural killer cells, including CD160 and KIRs, were among those that are upregulated in RhD-negative individuals. NK cells, potent IFNγ producers and cytotoxic lymphocytes, are key players in control of viral infection. While there was no difference in NK cell counts in our cohort, the differences observed in marker expression could be indicative of an increased activation state and IFNγ production [278]. These findings are the first to shed light on the potential immune differences between RhD-positive and -negative individuals and support the use of systemic investigations to understand the impact of blood types on the whole blood immune response.

Chapter 7: Final Discussion

In this thesis, we identified and recruited women from the Irish anti-D cohort who appeared to have resisted hepatitis C virus infection. Examination of records published by the IBTS suggests that 611 (47%) of the 1293 women exposed to highly infectious vials of HCV contaminated anti-D appeared to resist infection, testing negative for both HCV viral RNA and anti-HCV antibodies. The remaining 53% is comprised of those who cleared infection adaptively (25%) and those who progressed to chronic infection and required therapeutic intervention (28%). The dogma has held that that 50-80% of HCV infected individuals progress to chronic infection but there is little consensus around how many become infected when exposed to HCV [10]. This is because it is difficult to establish exposure to this blood borne virus. Few cohorts such as the anti-D cohort exist where it has been established that a specific number of individuals were exposed to the virus by injection of HCV infected blood product. Direct comparison of expected numbers is difficult, however here our data suggest that approximately half of the people exposed did not become infected.

Several reasons may contribute to this high level of resistance. Female sex is a likely contributor to the high degree of viral resistance observed in the cohort - females appear to be more protected against viral infection [197].The HCV disease course described in those who progressed to chronic infection was milder than expected based on data from other cohorts [169].Female sex may also have contributed to this phenomenon. It is well established that females are at a greater risk of developing autoimmune diseases and similar genes may be involved in both processes [279]. Efforts to unpick mechanisms underpinning this enhanced antiviral response in females have uncovered TLR7 as a likely contributor; TLR7 is expressed on the X chromosome and escapes X chromosome inactivation, therefore TLR7 expression is increased on immune cells in females [199].

Additionally, women in the cohort were all pregnant at the time of infection. The immune system plays an important role in pregnancy, and immune system dysregulation can compromise fertility [280]. For instance, reports suggest that women with unexplained fertility issues have a dysregulated IL-17 cytokine axis both locally and systemically, and

that those with autoimmune conditions such as SLE and RA often suffer from additional fertility and pregnancy problems [280–282]. Therefore, selecting for women who are fertile with the "healthy" immune response required for pregnancy could contribute to the high degree of viral resistance observed in our cohort.

The half-life of anti-HCV antibodies is a further important consideration in interpreting the high degree of resistance observed in this cohort. Work from Barbara Rehermann's group in the 2000's showed that attrition of HCV antibodies occurs stochastically, and that 18 out of 43 previously Ab-positive individuals were Ab-negative 18-20 years following viral clearance [101]. It is therefore plausible that a percentage of the resistors in the anti-D cohort were once antibody positive with antibodies that were particularly effective at clearing HCV infection. While these factors may have contributed to resistance, we hypothesised that a particularly potent innate immune response protected these women against HCV infection without generating a detectable adaptive immune response.

To study the immune system of these resistant women, we first had to recruit them to our study. Initially we had hoped the IBTS would contact all 611 potential resistors on our behalf and invite them to participate in the study. However, following consultation with ethicists, it was determined that recontacting these women in such a manner would be unethical as it could cause distress (the IBTS had previously stated they would not contact the women directly again following receipt of a second negative antibody test.) This presented as a major challenge in the initial stages of our study.

To overcome the ethical issue, we conducted a national media recruitment campaign. Newspaper articles were published on the project and members of the lab appeared on national television and radio to speak about the study and invite individuals who thought that they were exposed to HCV contaminated anti-D during the 1977-79 period to contact the lab about participating in the study. Ultimately we recruited 38 ESN women for whom we had records of documented exposure from the IBTS. This represents just 6% of the 611 potential ESN women from the full anti-D cohort. Though we had study packs from 395 eligible individuals returned to us, we were able to match just 234 women to their anti-D records held by the IBTS. This drop off was largely due to poor record keeping in the 1970s.

Given the age demographic of the women we were attempting to contact (60+ years) we felt the campaign was successful.

Once matched to their records, we invited women from our cohort to donate blood samples which were used to isolate DNA for genetic studies and to perform analysis of innate and adaptive immune responsiveness. We used a recently developed technology from Stephen Elledge's group with a high sensitivity and specificity for HCV antibodies to look for the presence of anti-HCV antibodies in the serum of our donors [283]. This technology uses over 115,000 viral epitopes and allowed us to look at antibodies against 203 viruses simultaneously. We detected no HCV antibody positivity in our ESN donors. Interestingly, some SR donors retained some HCV positivity 40 years after clearance of infection. The majority of SVR donors, most of whom cleared HCV following therapy in the last 10 years, were HCV Ab positive. Further analysis of HCV antibodies retained by those SRs is of great interest as previous work has shown antibody attrition occurs within 20 years following resolution of HCV infection [283]. In accordance with our hypothesis that ESN donors cleared or prevented infection independently of antibodies, we also found no HCV specific T cell responses in our ESN donors. HCV specific T cells persisted within the SRs. The relatively increased persistence of HCV specific T cells compared to Abs is interesting and is in line with previous reports suggesting HCV specific T cells persist longer than anti-HCV Abs [284].

Also noted in our antibody study, CMV seropositivity was relatively low. This phenomenon has been previously described, with Ireland having the lowest CMV seropositivity in the developed world [285,286]. It is estimated that just 30-40% of Irish females are CMV seropositive, while in non-Irish populations of a similar age this reaches up to 90% [287]. Here we found that 48% of females in our cohort are CMV seropositive. Age is an independent risk factor for CMV seropositivity, and the mean age of our cohort is 72 years- which may explain the slight increase in CMV seropositivity we see in our recruited women [288].

Having confirmed the absence of an HCV specific adaptive response in our cohort, we next sought to examine the induced whole blood innate immune response to a panel of viral

agonists, including polyIC, R848, IFN and ODN, using NanoString transcriptomics and Luminex proteomics. Of these stimuli, we observed a specific-polyIC induced IFN-I gene signature in the ESN donors compared to SP donors. We decided to group SR and SVR donors together as seropositive, as both were susceptible to infection with HCV, and we did we not observe any significant differences in the response to stimulation with R848, polyIC or IFN between them. We decided not to include an UC comparison group in this setting as we felt they would detract from the value of the cohort, in that the rest of the cohort had been stratified by infection outcome following HCV exposure, while in an unexposed group they would be more heterogenous and include ESN, SR and SVR individuals.

The increase in polyIC responsiveness in the ESNs was also observed at the protein level, where ESNs upregulated a number of inflammatory proteins including CCL8, CXCL11, IL-6 and CCL2 more than the SP donors. The importance of TLR3 in RNA virus immunity is underscored by recent work by Casanova et al. who showed TLR3 LOF leads to severe COVID19 disease, and also by genetic association studies between SNPs in TLR3 and susceptibility to infection [195,211]. Despite HCV being hepatotropic, the peripheral immune response is important in controlling infection - immune cells in the blood are the first to encounter HCV following transmission via blood to blood contact [289]. Peripheral immune cells are also recruited to the liver during infection, and several studies have demonstrated that interindividual differences in the peripheral immune response correlate well with infection outcome [92,290,291].

Although we saw no difference in responses to polyIC, R848, IFN and null stimulation between SRs and SVRs, when stimulating whole blood with ODN, we observed several interesting differences in the induced response. Notably, SR donors had reduced upregulation of several genes compared to SVRs. Using GSEA, we compared differences in pathways between SRs, ESNs and SVRs. In both the ESNs and SVRs, we observed enrichment for IFN-I signalling compared to the SRs. This was recapitulated in gene signature scores, where we observed a decreased IFN-I gene signature in the SRs compared to the ESNs and SVRs. We saw no difference in the gene signature score for IL1β, TNFα or IFNγ. pDCs are the main producers of IFNα in the human, however we saw

no difference in the counts or activation states (as measured by HLA-DR and CD86 MFI) of pDCs in our cohort. Lastly, we found that the SRs produced less IFNα protein in response to stimulation with ODN compared with the SVRs. This correlated positively and significantly with the ODN IFN-I gene signature score, suggesting that the reduced protein may account for the reduced IFN-I signalling observed.

The reduction in IFN-I signalling and IFNα protein was a surprise. Based on previous reports suggesting that IFNα blockade resulted in increased Ab titres following vaccination, it is possible that reduced TLR9 responsiveness would have led to less IFN-I and may have led to a better Ab response in the SRs [40]. Recent reports from *in vitro* work suggest that HCV induced immune perturbations can persist even after resolution of acute HCV infection [111,192]. It is possible that the reduction in TLR9 responsiveness observed in SRs in our cohort is due to their past acute HCV infection and is the subject of ongoing investigations. In this study, we used ODN2216 (CpG-A), one of three classes of ODN. ODN2216 leads to both IFN-I production and NFκB activation [292]. Whether SRs also have a muted response to other ODNs will be interesting to explore. We did not observe any difference in TLR9 expression between SRs and other groups in our NanoString data. This is in line with previous reports indicating that TLR9 expression is important for spontaneous resolution of HCV [84].

Using DNA isolated from saliva collected from the full cohort, we next examined genetic factors that may have contributed to viral resistance. Based on the finding of increased polyIC IFN-I responses in the ESNs, we chose to genotype our cohort for three tagSNPs in the TLR3-IFN-I pathway, namely rs3775291 in TLR3, rs7251 in IRF3 and rs2257167 in IFNAR1. These SNPs had previously been identified as risk loci for autoimmune or infectious disease [195,224,293]. Using this gene candidate approach, we identified an association between rs2257167 and resistance to HCV infection in our cohort. Analysis of NanoString transcriptomic data from unstimulated whole blood in healthy donors from the viral resistance cohort and the 1000 person Milieu Interieur cohort showed rs2257167 to be an eQTL for IFNAR1. Analysis of transcript data from whole blood stimulated with polyIC, LPS and IAV showed that donors of the GC.CC variant genotype had increased

expression of several ISGs including IFIH1 and IFITM1. Interestingly, wild type GG donors appeared to have increased expression of non-IRG genes including IL6 and IL8 in response to these stimuli. This increased pro-inflammatory phenotype may have aided in resistance to viral infection in the cohort. Indeed, cytokines such as IL-6 have been shown to be important in the control of HCV infection [294].

This finding may seem contrary to the increased polyIC-IFN-I signature also described in ESN donors, however, it is important to note that rs2257167 is in lineage disequilibrium with several other SNPs in IFNAR1 that may also impact on the response to stimulation, and that our approach is limited here by choosing just three SNPs to genotype in the cohort. The association with rs2257167 and resistance may not impact on the increased polyIC-IFN-I signature described. Indeed, there is no difference in the polyIC-IFN-I gene signature between GG and GC.CC genotypes in the viral resistance cohort healthy controls or in the MIC.

Access to a such a large secondary healthy control cohort of well characterised individuals for whom a raft of demographic and biological data has been collected is a hugely valuable resource. The 1000 healthy individuals in the MIC have been genotyped for over 5 million SNPs, have whole blood stimulated with 38 ligands and NanoString and Luminex carried out on a selection of the same [66,262]. They have also extensive demographic and clinical information [260]. We leveraged data from the MIC once again to study the impact of Rhesus status on the immune response.

Given the high degree of viral resistance observed in the anti-D cohort, which consists entirely of RhD-negative females, we hypothesised that RhD-negativity is associated with an 'enhanced' immune response. We again used data from the MIC to test our hypothesis. The transcriptomic response to stimulation with LPS and polyIC was similar between RhD-negative and -positive males and females. Of note, we found that RhD-negative and -positive males had differential expression of about 30 genes. When analysed using GSEA, RhD-negative males had an enrichment for IFNγ signalling compared with RhD-positive males. Though not the focus of the study, we observed several differences in the responses to LPS and polyIC between males and females. RhD-negativity has been

associated with protection from other viral infections including SARS-CoV-2 in both males and females [258]. The fact that we only see a difference in RhD-negative males in this study is surprising, however the NanoString human immunology panel applied to the stimulated whole blood is a somewhat biased technology, with just 569 genes included in the panel. Broadening the analysis using RNA-seq may reveal other differences in immune activity between RhD-negative and -positive females also. Despite its critical role in transfusion medicine, the function of RhD is as yet unknown. Some reports suggest it is important in maintaining membrane integrity or in transport of ammonia [295]. Recent work has shown that RBCs, which express the RhD protein in RhD-positive individuals, have an immunological function through expression or TLR9 [296]. It is possible that RhD's impact on membrane integrity or in waste transport could impact on the RBC immune response.

The value of whole blood analysis coupled with multiplexing is illustrated in this study of RhD, and indeed throughout the rest of this thesis. Whole blood analysis is more physiological as it allows for the inclusion of all circulating cells, not just those isolated during a PBMC preparation. This is of particular importance in the RhD study, as RhD is expressed on RBCs, which would typically be excluded in traditional immunological studies.

Future directions

The work presented in this thesis has generated several new lines of research for our group, and has developed an important basis for future studies in the area of viral resistance.

Despite the small size, observations made about the whole blood immune response in our cohort are interesting. Though identification of such a group would be arduous, validation work in a secondary cohort would strengthen our results. There are likely women from the German anti-D cohort who were exposed to HCV and resisted infection. Application of our recruitment strategy and scientific methodology to such a group may yield interesting results.

The addition of even a small number of donors from a secondary cohort would also aid in the identification of more robust genetic signatures of resistance using whole exome or genome sequencing. Utilising a large healthy control comparison cohort (eg. TILDA or Irish individuals in the UK Biobank) would help overcome issues of power.

Genotyping an additional cohort for the rs2257167 SNP in IFNAR1 would be a useful validation step. *In vitro* assessment of the IFNAR1 variant by transfecting it into IFNAR1null cells would also help disentangle the functional effects of the specific SNP from potential LD SNP effects.

Our finding that the SRs and SVRs retain HCV specific T cells and antibodies following clearance of HCV could be important in informing novel HCV vaccine strategies. As VirScan does not provide information on neutralisation capacity, work should first focus on this area.
This could be carried out *in vitro* using conventional neutralisation assays. From a T cell perspective, the persistence of these HCV specific T cells should be further validated using conventional flow cytometry techniques, or using novel technologies like TScan. Using TCR sequencing, one could determine what epitopes are targeted by the HCV specific T cells, and carry out *in vitro* work exploring the ability of these cells to control or protect against HCV infection.

The finding of reduced ODN responsiveness in the SRs in our cohort is surprising. We have several hypotheses that warrant further exploration. The metabolic change induced in response to PRR ligation is important for the production of cytokines such as IFNα. We propose that the reduced TLR9 response observed in our SRs is potentially due to a reduced ability to support TLR9 induced IFNα production. This metabolic disruption could be interrogated by assessing pDC metabolism using single cell RNA-seq and novel technologies such as SCENITH, which allows for assessment of metabolism at a single cell level.

Our finding of differential susceptibility to HCV infection based on education status is interesting and could be explored further in the milieu interieur donors, for whom

extensive demographic information has been collected. Analysis of NanoString and Luminex data generated on the whole blood stimuli from the MIC by low and high socioeconomic status including important covariates such as CMV and age could shed light on the impact of SES on the induced immune response. Exploration of the impact of different blood groups, not just RhD is also possible using the MIC.

Additionally, through leveraging cohorts like the MIC, one could look at the genetic determinants of ODN responsiveness by dividing donors into low and high responders and matching those responders with the genomic data on the cohort to find SNPs associated with the ODN response. Following identification of such SNPs, one could genotype our cohort for the relevant SNPs. This would help determine whether the reduced ODN response was genetically encoded and thus a state that proceeded HCV infection. Infection of iPSC derived hepatocytes cocultured with donors PBMCs harbouring these SNPs could help determine whether these SNPs impact on the ability to SR HCV *in vitro.*

Final conclusions

Here we have described for the first time, innate resistance to HCV in the Irish anti-D cohort. We identified a polyIC-IFN-I signature as being associated with resistance. These findings may prove important in epidemiological studies that estimate population level immunity and viral exposure [64,297]. We also uncover an association between a SNP in IFNAR1 and protection against HCV infection in our cohort. We describe for the first time, reduction in TLR9 IFN-I mediated immunity in SR individuals. Interestingly, we also find differences in HCV-specific antibody positivity between SRs and SVRs. HCV therapy has evolved dramatically over the last decade, however, no vaccines are yet available. The evolution of drug resistance is also a major concern [298]. Harnessing the findings in this study could help in designing novel antibody therapies or vaccines. This thesis demonstrates the value of systematically studying the human whole blood induced immune response in understanding variability in infection outcome.

Chapter 8: References

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Appendix

Peptide	Sequence	Epitope source	HLA allele restriction
1	ATDALMTGF	HCV NS3 1436-1444	A*01:01
2	ATDALMTGY	HCV NS3 1435-1443	A*01:01
3	ATDALMTGY	HCV NS5B 2594-2602	A*02:01
4	CINGVCWTV	HCV NS3 1073-1081	A*02:01
5	CINGVCWTV	HCV NS3 1073-1081	A*02:01
6	DLMGYIPAV	HCV core 132-140	A*02:01
7	DLMGYIPLV	HCV core 132-140	A*02:01
8	GLQDCTMLV	HCV NS5B 2727-2735	A*02:01
9	KLSGLGINAV	HCV NS3 1406-1415	A*02:01
10	KLVALGINAV	HCV NS3 1406-1415	A*02:01
11	LLFNILGGWV	HCV NS4B 1807-1816	A*02:01
12	VLSDFKTWL	HCV NS5A 1987-1995	A*02:01
13	YLLPRRGPRL	HCV core 35-44	A*02:01
14	RVCEKMALY	HCV NS5B 2588-2596	A*03:01
15	AYSQQTRGL	HCV NS3 1031-1039	A*24:02
16	DPRRRSRNL	HCV core 111-119	B*07:02
17	GPRLGVRAT	HCV core 41-49	B*07:02
18	HSKKKCDEL	HCV NS3 1395-1403	B*08:01
19	CPNSSIVY	HCV E1 207-214	B*35:01
20	HPNIEEVAL	HCV NS3 1359-1367	B*35:01
21	REISVPAEIL	HCV NS5A 2266-2275	B*40:01

Appendix table 1. Details of the HCV peptide pool used for IFNγ ELISpots.