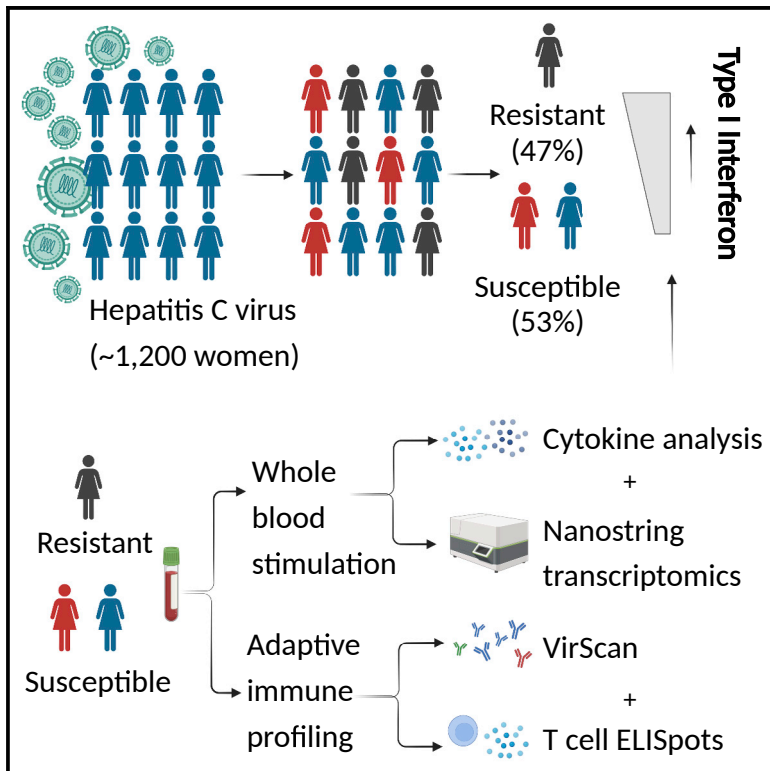**Article**

Enhanced TLR3 responsiveness in hepatitis C virus resistant women from the Irish anti-D cohort

Graphical abstract

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In brief

Natural resistance to infection is an often-overlooked outcome after exposure to HCV. Here, Sugrue et al. describe HCV resistance in a cohort of women accidentally exposed to HCV in the 1970s and find increased TLR3-induced type I activity in women who resisted infection.

Highlights

- Resistance to HCV is common in the Irish anti-D cohort

- VirScan provides a comprehensive assessment of all previous viral infections

- Resistance to HCV is associated with increased TLR3-induced IFN-I activity in blood

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Article

Enhanced TLR3 responsiveness in hepatitis C virus resistant women from the Irish anti-D cohort

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SUMMARY

Natural resistance to infection is an overlooked outcome after hepatitis C virus (HCV) exposure. Between 1977 and 1979, 1,200 Rhesus D-negative Irish women were exposed to HCV-contaminated anti-D immunoglobulin. Here, we investigate why some individuals appear to resist infection despite exposure (exposed seronegative [ESN]). We screen HCV-resistant and -susceptible donors for anti-HCV adaptive immune responses using ELISpots and VirScan to profile antibodies against all know human viruses. We perform standardized *ex vivo* whole blood stimulation (TruCulture) assays with antiviral ligands and assess antiviral responses using NanoString transcriptomics and Luminex proteomics. We describe an enhanced TLR3-type I interferon response in ESNs compared with seropositive women. We also identify increased inflammatory cytokine production in response to polyIC in ESNs compared with seropositive women. These enhanced responses may have contributed to innate immune protection against HCV infection in our cohort.

INTRODUCTION

Susceptibility to viral infection in the human population is highly variable, yet the reasons for this discordance are unknown.[1] For example, after exposure to hepatitis C virus (HCV), reports suggest that between 50% and 80% of infected individuals progress to chronic infection, and test both polymerase chain reaction (PCR) positive for HCV RNA and anti-HCV antibodies.[2] Additionally, a significant percentage of individuals are able to clear HCV infection through the engagement of their adaptive immune system, and test PCR negative for HCV RNA, but remain HCV antibody positive (spontaneous resolvers [SR]).[2] 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.[3–5]

There also exists a third group, those who appear to naturally resist infection, known as "exposed seronegative" (ESN) individuals, 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.[6,7] Currently, ESNs are only identifiable through analysis of risk, or documented outbreaks of infection, and are consequently less well studied than SP 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 because of failures in decontamination procedures or before the discovery of certain viruses, including HCV and HIV[8–10] These natural experiments present unique 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.[6] Understanding the mechanisms of resistance to viral infection could have major implications for public health strategies as well as design of vaccines and antiviral therapeutics.

An enhanced innate immune response is thought to be sufficient to clear infection without engagement of the adaptive immune system in ESNs.[6] 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 Toll-like receptor 3 (TLR3), RIG-I, MDA5, and TLR7.[11] Ligation of these receptors induces synthesis and secretion of type I (interferon I [IFN-I]) and type III (IFNL) IFNs, which act early following viral exposure to inhibit HCV replication through induction of IFN-stimulated genes and activation of immune cells.[11]

Previous studies of HCV-resistant individuals have identified factors related to innate immunity that may have contributed to protection against infection. HCV ESN individuals, identified in a risk-based cohort of people who inject drugs (PWIDs), have

elevated serum levels of the proinflammatory cytokines interleukin 6 (IL-6) and IL-8 compared with SP individuals.[12] Also described among PWIDs, are HCV ESN individuals with increased natural killer (NK) cell activation markers, and increased IFN-γ production.[13] This enhanced NK cell phenotype has also been described in HIV ESN women who remained HIV negative status despite repeated exposure to HIV through sex work in Benin.[14]

A limitation of ESN studies to date has been the reliance on peripheral blood mononuclear cells (PBMCs). While this has enabled the discovery of the phenotypes outlined above, PBMC preparations are poorly reproducible and often induce technical noise from handling steps, which may preclude or mask subtleties in the immune response.[15] Standardized whole blood stimulation technologies have been developed to overcome these limitations and have enabled the identification of stimulus induced differences in other cohorts and disease states that would otherwise have been undetectable.[16,17] We proposed that these cutting edge whole blood stimulation technologies would uncover additional mechanisms of innate resistance against HCV.

A major outbreak of HCV occurred in Ireland between 1977 and 1979 in Ireland, when more than 1,200 RhD-negative women were exposed to highly infectious batches of HCV-contaminated anti-D immunoglobulin.[4] The anti-D was retrospectively discovered to have been contaminated by blood donations from a single individual infected with genotype 1b HCV.[4] SP individuals from this outbreak were readily identifiable and have been studied extensively over the last three decades.[3] 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.[18,19] 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.[4] 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.[20]

In this study, we have identified ESN women from the Irish ant-D cohort and recruited them to our project. We used ELISpots to profile IFN-γ T cell responses to HCV peptides and used VirScan to assess the history of viral exposure in our cohort. We stimulated whole blood from 18 ESN and 36 SP women *ex vivo* with a panel of antiviral ligands and quantified gene expression changes and cytokine responses. A greater understanding of the factors that protected these women from infection by HCV could provide greater insight into mechanisms of innate resistance to viral infection.

RESULTS

Identifying ESN women in the Irish anti-D cohort
Six of the 12 contaminated batches contained significant viral loads (batch numbers 246, 238, 245, 237, 252, and 250; Figure 1). Between 31% and 71% of the recipients of vials from these batches were antibody positive. As indicated in Figure 1, 682 women (52%) tested positive for HCV antibodies, indicating that they had been infected. However, women 611 (48%) 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. 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 Irish Blood Transfusion Service (IBTS).

Recruitment of women who were exposed to contaminated HCV between 1977 and 1979 the campaign and 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. After the national media recruitment campaign, approximately 700 women volunteered to participate (Figure 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 packs 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 sustained virological responders [SVRs] having cleared HCV with therapy).[21] We also recruited 102 women who received an uncontaminated batch of anti-D in the same period (unexposed controls [UC]).

Cohort characteristics
Basic demographic and clinical data were collected on the full cohort at the time of consent (Table 1). All three infection outcome groups—ESNs, SRs, and SVRS—were all female, all RhD negative, similarly aged, and had all been pregnant. SVR donors reported an increased incidence of liver disease compared with ESNs and SRs, despite lack of active HCV infection. SR and SVR donors also reported a higher incidence of chronic fatigue and fibromyalgia (Table 1). Clinically, the three groups were otherwise similar.

Assessment of adaptive immunity in our recruited cohort
We invited a subgroup of our larger cohort to donate a blood sample for additional analyses. Participants were selected for additional sampling on the basis of their ability to travel and proximity to the sampling center. To assess the cohort for anti-HCV antibodies, we used a virome-wide antibody scan with the ability to detect IgG antibodies directed against 206 human pathogens and 115,753 epitopes (VirScan;[22]). Serum from 18 ESNs, 19 SRs, 17 SVRs, and 29 UCs containing antibodies was incubated with a bacteriophage library expressing peptides from all known human viruses and some bacterial species, including *Streptococcus pneumoniae* and *Staphylococcus aureus*. After incubation, antibodies complexed to bacteriophages were isolated

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

ESN (611; 47%)
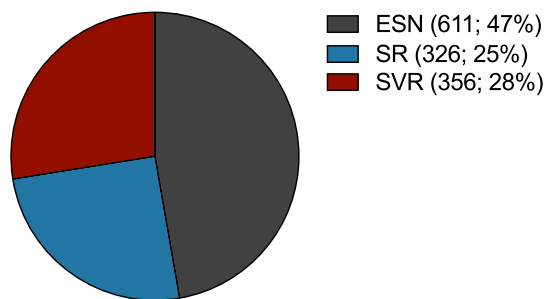SR (326; 25%)
SVR (356; 28%)

Figure 1. Infectivity rates of HCV contaminated anti-D from the IBTS
(A) Table obtained from the IBTS showing the six high risk batches of contaminated anti-D. Figures in brackets are percentages.
(B) Pie chart showing total numbers and percentages of ESNs, SRs, and SVRs who received a vial of HCV anti-D from a highly infectious batch.

using magnetic beads, lysed and sequenced to identify the IgG target epitope (Figure 3A). Sequence data were aligned and processed to generate a "virusHit" for each viral species. A virusHit is the number of significant epitopes of a virus species compared with an experimental negative control. To test for differences in antibodies against all pathogens in the VirScan library between ESN, SR, SVRs, and UCs, we performed a one-way non-parametric ANOVA adjusting for multiple testing using a false discovery rate (FDR) correction (q < 0.01). Based on this analysis, all antibodies with the exception of HCV were similar between groups. SVR donors had the highest level of HCV antibody positivity (Figure 3B).

VirScan facilitates a more nuanced analysis of which epitopes are targeted by antibodies. 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 sites by adding the Z score for each genotype together. Individual HCV epitopes are shown as a heatmap (Figure S1). 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 of 19 SRs (16%) seemed to have anti-HCV antibodies, while 16 of 17 SVRs (94%) seemed appeared to be anti-HCV antibody positive. Interestingly, there are some epitopes, including 29–84 that persist in both SVRs and SRs, while antibodies against other epitopes including 57–112, 309–364, and 2157–2212 are only detected in the SVRs (Figure S1). 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), herpesvirus 1, and rhinovirus A (Figure S2). Of the donors, 48.81% were anti-CMV antibody positive (Figure S2), 86.9% of the donors were SP for antibodies against HSV-1 (Figure S2), and 96.43% of the donors were SP for antibodies against EBV (Figure S2).

Some studies have shown that the clearance of HCV in the absence of antibodies is achieved by a potent T cell resposne.[23] HCV-specific T memory cell responses can persist for years after viral exposure, while antibody levels wane after approximately 20 years.[24] Here we sought to assess HCV-specific T memory cells in our cohort using ELISpot IFN-γ 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 standardized cut-off derived from the age- and gender-matched UC group (n = 10) of the mean +3× the SD we found detectable T cell responses in both the SR and SVR groups. We did not see any HCV-specific IFN-γ T cell response in the ESN cohort (Figure 3C). All four infection groups responded similarly to the CEF peptide pool (Figure 3D).

Innate immune stimulation of whole blood induces changes in gene expression
Having confirmed the absence of a detectable adaptive response in the ESNs, we sought to assess the innate antiviral response of ESNs and virus susceptible women (SRs and SVRs) in our cohort. Fresh whole blood from ESNs (n = 18), SRs (n = 19), and SVRs (n = 17) was stimulated using the TruCulture system to assess potential differences in the induced immune responses between ESN
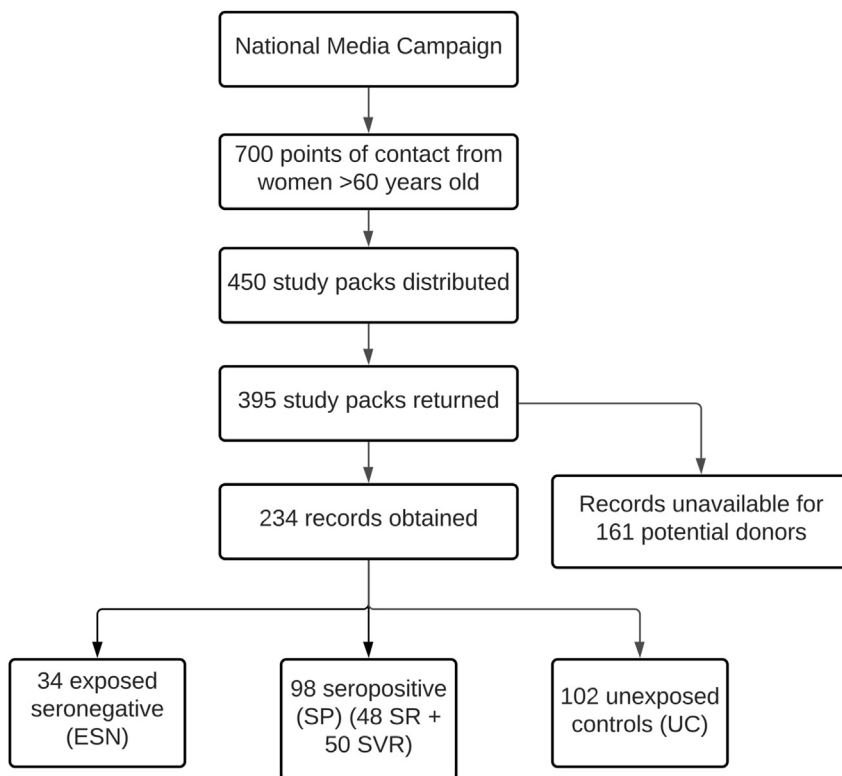
in the ESN and SP groups was similar, with no significant differences observed between groups, after FDR correction (q > 0.1; Figure 5A). To further analyze more specific components of the innate immune response, and given the relatively small size of our cohort, we used previously defined gene signatures. These gene signatures 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 Milieu Interieur Cohort (MIC) using the same TruCulture and NanoString workflow.[25] By applying these gene signatures to our unstimulated samples, we saw similar baseline IL1β, IFN-I, IFN-γ, and tumor necrosis factor α (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 (Figures 5B–5E). To probe the basal immune response further we also assessed plasma cytokine levels from ESN and SP donors. After FDR correction, we saw no significant differences between ESN and SP donors (Figure S3B).

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

TLR3-, TLR7-, and IFNAR1/2-induced responses, particularly those of IFN-I-associated antiviral immunity have been shown to be important in the control of HCV infection.[26,27] We, therefore, focused on the activation of these key antiviral pathways in our cohort. We compared induced gene expression after stimulation with either polyIC, R848, and IFNα2 between ESN and SP donors. The overall gene expression of ESN and SP donors was similar, after FDR correction (Figures 6A–6C). 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 (Figures 6D and 6E). Interestingly, however, we saw a significantly increased IFN-I signature score after polyIC stimulation in the ESN group when compared with the SP donors (p < 0.05; Figure 6F). A heatmap showing the genes included in the IFN-I signature score for the polyIC condition is shown in Figure S4A. To further examine

and HCV-susceptible volunteers (Figure 4A). A panel of antiviral agonists with a focus on the induction and regulation of IFN-I-related antiviral immunity was chosen: IFNα2, which acts via the IFNAR1/2 complex to induce a direct IFN-I response and 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 (Figure 4B). Induced gene expression changes showed stimulus-specific patterns (Figure 4C). R848 was the most potent stimulus and clustered separately from the other two stimuli along PC2, accounting for 10% of the variance (principal component analysis; Figures 4B and 4C). IFNα2 and polyIC clustered separately along PC4, accounting for 5% of the variance.

Gene expression in unstimulated whole blood from ESN and SP women

Both SRs and SVRs responded similarly to stimulation with our panel of viral agonists (Figure S3A). We grouped SRs and SVRs together as SP for subsequent analysis, as we were interested in investigating differences in those who resisted infection and those who were susceptible. A key question in this study was whether there were any differences in immune gene expression at baseline between ESN and SP participants that might have contributed to differential susceptibility to HCV infection. We found that, in the absence of stimulation, gene expression

Table 1. Clinical characteristics of the full cohort

Characteristic	ESN	SRs	SVRs	Statistic
Total Recruited	34	48	50	
Age (years; mean ± SD)	71.6 ± 5.1	71.8 ± 4.5	71.4 ± 5.4	ns
Clinical questionnaire				
Acute symptoms after anti-D receipt	4 (12%)	7 (15%)	2 (4%)	–
Liver disease	1 (3%)	3 (6%)	33 (66%)	****
Diabetes	3 (9%)	5 (10%)	6 (12%)	ns
Osteoarthritis	11 (32%)	24 (50%)	23 (46%)	ns
Rheumatoid arthritis	2 (6%)	1 (2%)	10 (20%)	**
Chronic fatigue	1 (3%)	24 (50%)	31 (62%)	****
Fibromyalgia	2 (6%)	13 (27%)	17 (34%)	*
Lichen planus	1 (3%)	1 (2%)	8 (16%)	*
Inflammatory bowel disease	3 (9%)	9 (19%)	11 (22%)	ns
Lupus	0 (0%)	3 (6%)	5 (10%)	–
Sjogren's syndrome	2 (6%)	4 (8%)	10 (20%)	ns

Self-reported clinical data from ESN, SR, and SVR donors in our recruited cohort. Differences were assessed using χ^2 tests.

the polyIC response, we compared gene signature scores for IL1β, TNFα, and IFN-γ; however, we saw no significant differences between ESNs and SPs (Figures S4B–S4D). This suggests that the differences between ESN and SP donors in TLR3 responsiveness are specific to IFN-I. This increased polyIC IFN-I signature in ESNs was confirmed using a secondary gene signature from the MolSigDB Hallmark Gene Set (Figures S4E and S4F).

ESN women have increased cytokine production after polyIC stimulation

We also measured polyIC-induced cytokines that are known to be important in the control of viral infection using a Luminex multiplex assay.[28–30] Consistent with the increased IFN-I score, several key cytokines including CCL8, CXCL11, CCL2, and IL-6 were found to be significantly increased in whole blood stimulated with polyIC from ESNs compared with stimulated blood from SP women (Figure 7A). 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 the infection groups (Figures 7B and 7C). IFNα and IFNβ positively and significantly correlated with the MIC polyIC induced IFN-I score (Figures 7D and 7E).

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 NK cells and monocytes (Figure S5). We also hypothesized that the increased polyIC IFN-I signature in the ESN women may have been due to altered baseline components of the polyIC signaling pathway or negative regulator expression in the null condition. We saw no significant differences in the expression of any of these components (Figure S6).

Previous work has shown that the IFNL3 genotype is strongly associated with HCV clearance and the response to polyIC in hepatocytes.[31,32] To determine whether the IFNL3 genotype is also associated with resistance to HCV, we genotyped our full cohort for rs12979860. The T allele of rs12979860 is in strong linkage disequilibrium with the ΔG allele of the dinucleotide variant, rs368234815 (r^2 = 0.92 in the European population), a frameshift mutation that forms an open reading frame and leads to the creation of a novel type III IFN known as IFNL4. Rs12979860_CC and rs368234815_TT donors do not express a functional IFNL4 protein and are associated with HCV clearance.[32] We compared the allele frequencies of rs12979860 between the infection groups in our cohort. Compared with SVRs and UCs, we found that SRs had a reduced frequency of the CT and TT genotype (p < 0.0001, odds ratio [OR] = 0.14 [95% confidence interval (CI), 0.05–0.36]; p = 0.0014, OR = 0.29 [95% CI, 0.13–0.63]). After an adjustment for multiple testing, we did not see a significant association between HCV viral resistance and IFNL3 genotype (Table S1).

Taken together, these data reveal an increased polyIC induced IFN-I gene signature, with increased polyIC-induced IFN-related protein cytokines independent of IFN-I protein secretion, IFNL3 genotype, or differences in cell numbers.

DISCUSSION

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

Using VirScan, we found that SVR women had the highest level of anti-HCV Ab positivity. This is to be expected; SVRs cleared infection more recently than SRs. While there seemed to be differences in the epitopes targeted by antibodies between SRs and SVRs, this is likely due to attrition of antibodies in the
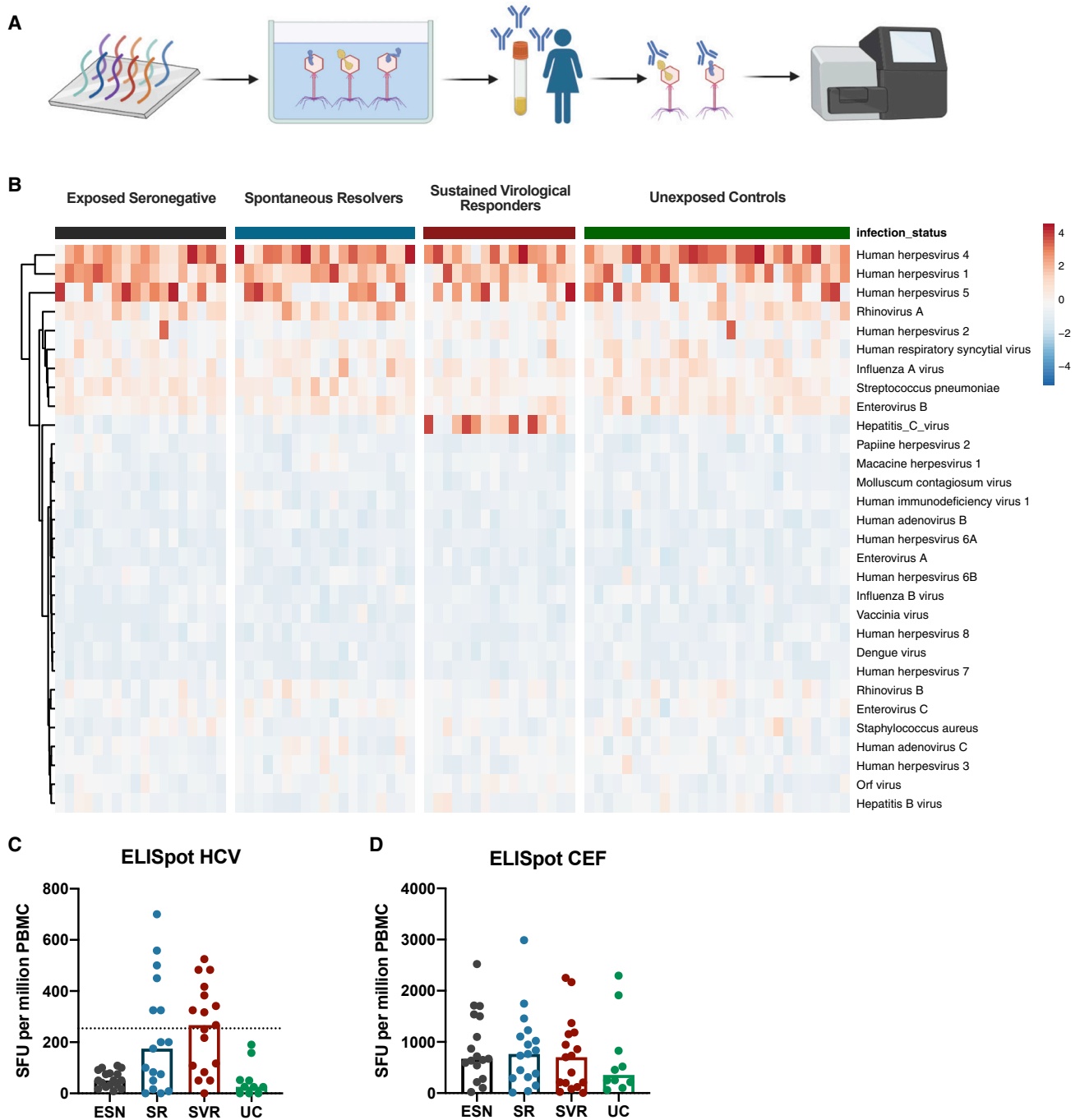
Figure 3. Adaptive immunity in the recruited cohort

(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. Comparisons between ESNs, SRs, SVRs, and UCs were made using Kruskal-Wallis tests followed by FDR correction (q > 0.01; n = 18 ESN, n = 19 SR, n = 17 SVR, and n = 29 UC).

(C and D) IFN-γ-producing T cell counts per 10^6 PBMCs in ESNs, SRs, SVRs, and UCs after stimulation with ProMix (C) HCV and (D) CEF peptide pools shown on a linear axis. For ELISpots, comparisons between ESNs, SRs, SVRs, and UCs were made using the Kruskal-Wallis tests (p > 0.05). The dashed line represents the mean + 3 × SD of the UC group. The median per group is shown.
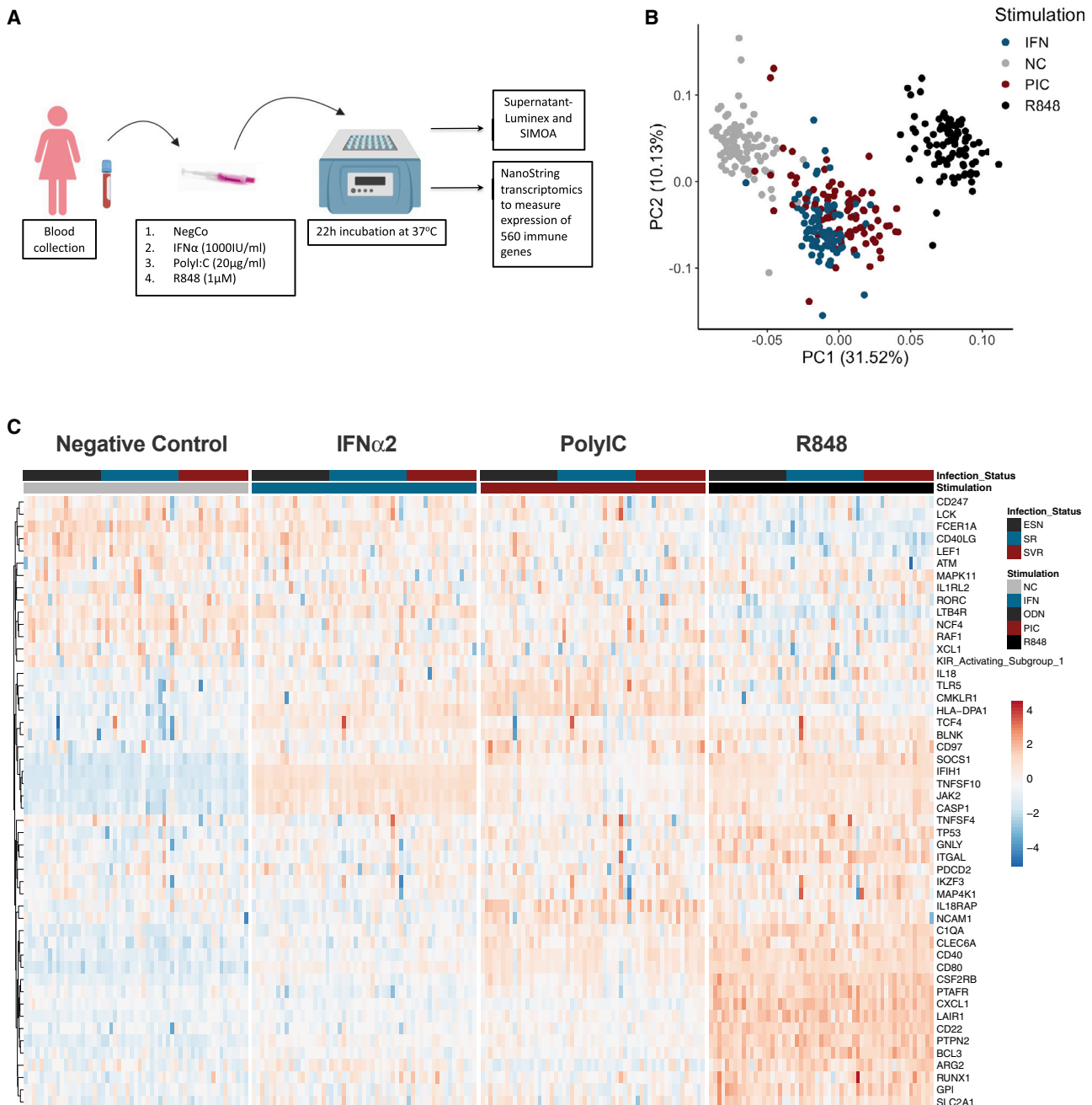
Figure 4. Innate immune stimuli induce changes in gene expression in whole blood from all donors

Whole blood from ESN (n = 18), SR (n = 19) and SVR (n = 17) women was stimulated with a panel of antiviral agonists (R848, polyIC, IFNα2).

(A) Overview of experimental workflow.

(B) Principal component analysis plot of log₂ normalized 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 color denoted stimulation.

(C) Heatmap of NanoString transcriptomic data (n = 50 genes) showing negative control (NC) and stimuli induced genes for IFNα2, polyIC, and R848.

SRs overtime.[33] Indeed, those epitopes that are targeted by antibodies in the SRs are among those most strongly targeted in the SVRs. We saw no differences in Ab positivity for other viruses between our groups. Anti-HCV T cell responses were detectable in both SRs and SVRs. 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.[24]

Individuals who have been exposed to a virus but remain uninfected are critical to understanding the mechanism of
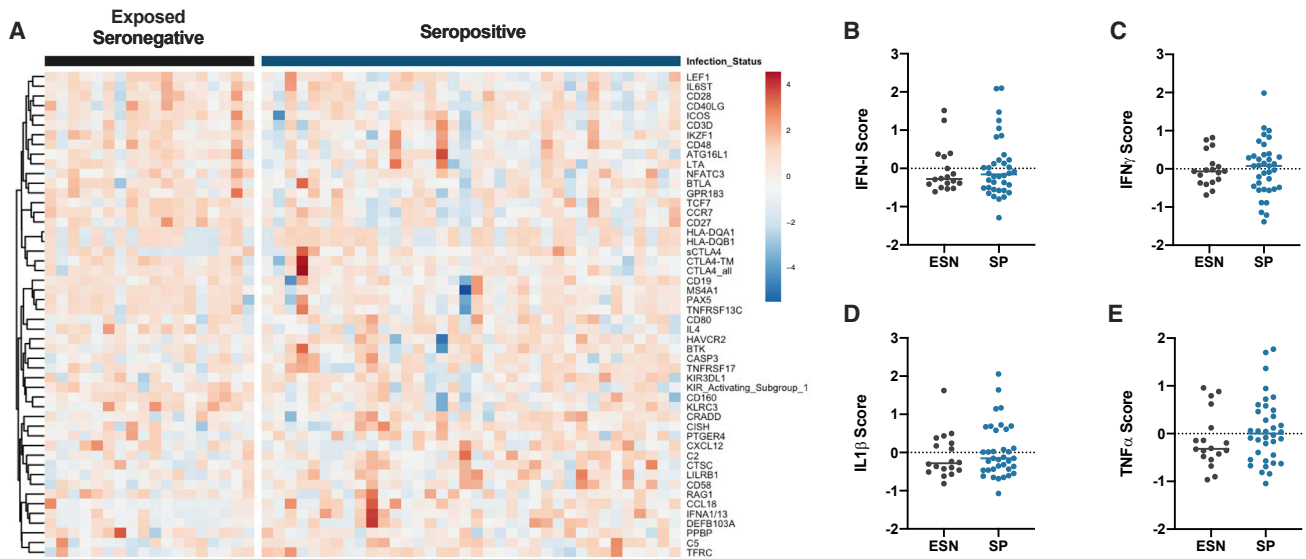
Figure 5. Gene expression was similar in unstimulated whole blood from ESN and SP women
Unstimulated whole blood was incubated at 37°C for 22 h.
(A) Heatmap showing the top 50 genes with the lowest q values when comparing the ESN (n = 18) and SP (n = 36) donors in the null condition (q < 0.99).
(B–D) Gene signature scores for (B) IFN-I, (C) IFN-γ, (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.

protection against viral infection. However, systematic identification and recruitment of ESN individuals are major obstacles to studies of viral resistance. Indeed dogma in the HCV field holds that up to 80% of people who are infected with HCV become chronically infected.[1] 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 antibody negative, suggest that up to 50% of women in the Irish cohort might be resistant to HCV infection. This has major public health considerations; 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 behaviors, rather than exploring potential viral resistance in healthy populations. These studies estimate exposed seronegativity to be more in the region of 10%.[6] However, these cohorts are typically more heterogeneous than ours, often including mixed sexes, age groups and ethnicities, and viral origin. A key point to emphasize is that our cohort is entirely female, and it is well documented that females have greater resistance to infection by several viruses, including HCV.[34] Indeed, previous work by our group and several others has shown gender-specific differences in spontaneous resolution of HCV.[18,35,36] Furthermore, follow-up work involving our group on the Irish anti-D cohort showed decreased HCV-related disease in women who were still chronically infected, suggesting that females also experience milder HCV-related illness than males.[3]

While relative homogeneity among our donors is a strength of our cohort, it also limits the direct extrapolation of findings to the general population. It is also important to note that our donors were in the peripartum period after HCV exposure, and it is

well known that pregnancy results in significant changes to the immune system.[37] Postpartum, women can experience flares in autoimmune disease.[38,39] Reports indicate that the restoration of immune homeostasis after pregnancy is associated with a decrease in viral load in the context of HCV infection.[40] It is, therefore, possible that alterations in the immune response in the peripartum period contributed to the high level of resistance we have described in the anti-D cohort. This further limits direct translation of our findings of high resistance to the wider population.

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 for our virus-resistant ESN women. All SP donors in our cohort successfully cleared HCV infection. It seems as though viral clearance following acute infection or therapy restores normal innate immune homeostasis.[41] Some reports suggest that an adaptive immunity disequilibrium, particularly affecting T cells, persists after SVR.[42–45] While the SP cohort are a natural comparison group, it is possible that past HCV infection in these donors contributes to the reduced IFN-I signature we see in these individuals compared with our ESNs. Indeed, the increased reports of liver disease in the SVRs may also contribute to the difference in polyIC responsiveness that we have observed. However, given the response to stimulation for all ligands was similar between SRs and SVRs, it seems unlikely that persistent liver disease reported by SVRs (66% liver disease), but not by the SRs (6% liver disease), is responsible for the reduced IFN-I response in the SPs compared with the ESNs. A potential solution to this caveat is the inclusion of a healthy UC group; however, given the heterogeneity in the wider population, the inclusion of an additional UC group in
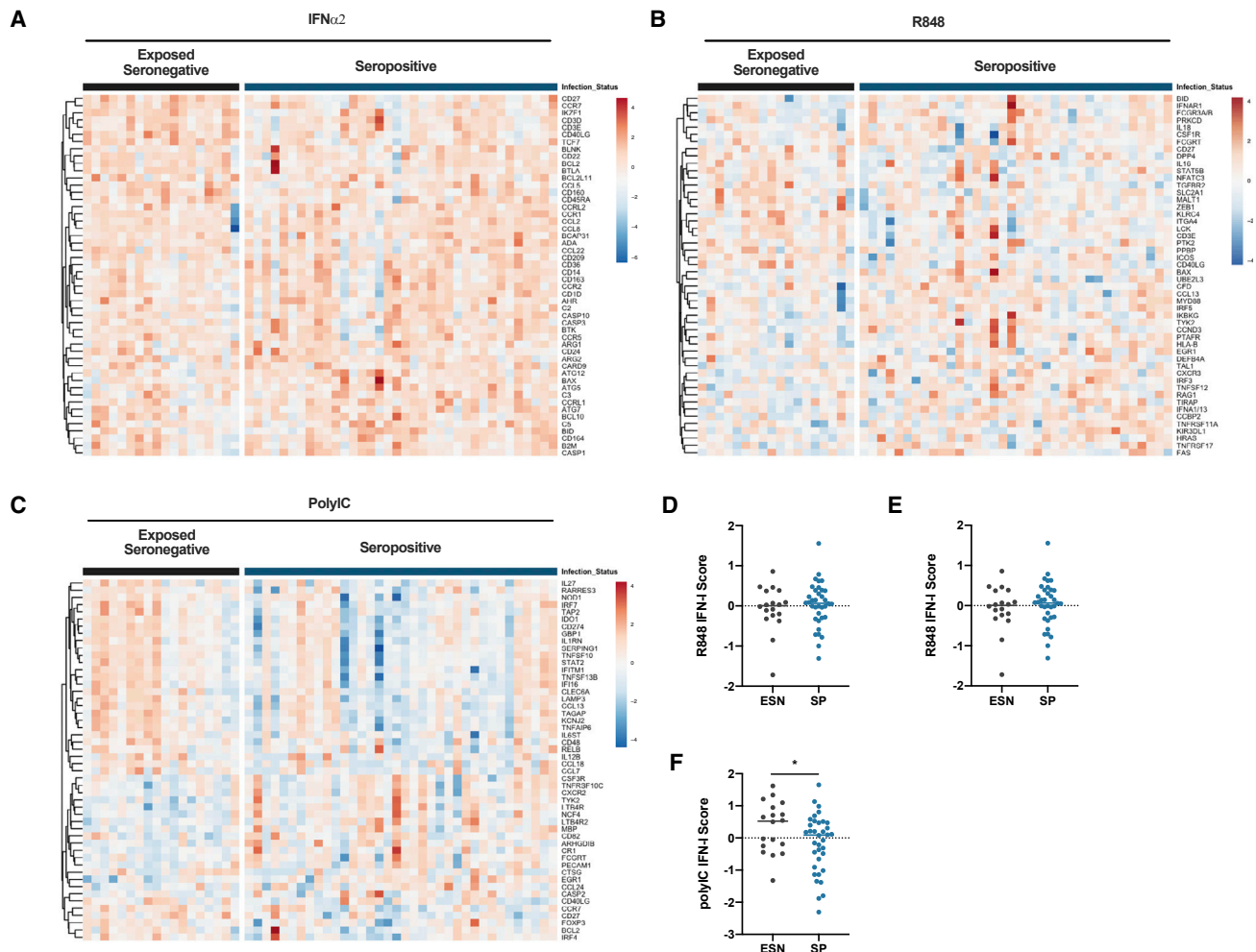
Figure 6. PolyIC-induced higher IFN-I responses in ESN women

Fresh whole blood was stimulated with IFNα2, R848, or polyIC for 22 h at 37°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 with stimulation between ESN and SP donors. (A) IFNα2, p < 0.16, q value (FDR-adjusted p value) < 0.99 (B) R848, p < 0.15, q < 0.99, and (C) polyIC, p < 0.06, q < 0.65.

(D–F) MIC-derived IFN-I score in ESN and SP donors after 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).

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 HCV infection.

The enhanced polyIC responsiveness was also seen in a second IFN-I gene signature from MolSigDB and at the protein level, with increased upregulation in the ESNs of several key cytokines known to be important in the control of viral infection observed, including IL6, CCL8, CXCL11, and CCL2.[29,30] We saw no difference in the 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 IFNL3 genotype, 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 signaling proteins.[46] More than 40 years have elapsed since the women in our cohort were originally exposed to HCV. Whether this increased IFN-I response was present at the time of infection is not known, and research into the stability of immunotypes for more than 5 years is lacking.[47]

In line with previous work, we did not find an association between IFNL3 genotype and resistance to HCV.[48] We recapitulated the findings from several other groups that SRs had the lowest frequency of the CT and TT genotypes, which reinforces
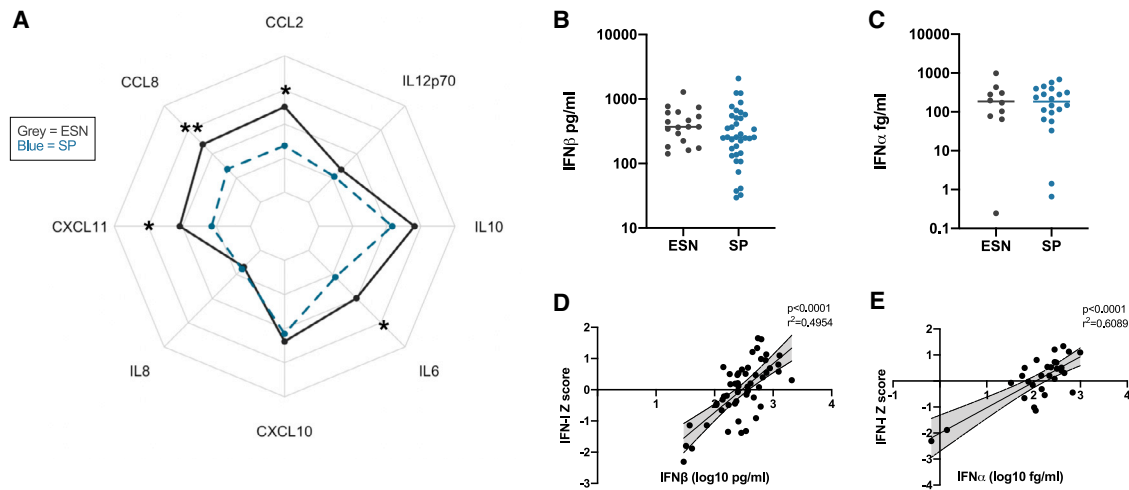
Figure 7. Inflammatory cytokine production is increased in polyIC stimulated blood from ESN women

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 and 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 and 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).

our belief that despite the relatively small size, our cohort is a good model of HCV infection.[32,49,50] How the absence of IFNL4 contributes to the spontaneous resolution of HCV is not yet fully understood. However, recent work suggests that IFNL4, when present, is not well secreted, and induces ER stress, which impairs antigen presentation.[51] Given this link with adaptive immunity it is not surprising that we did not find an association between IFNL4 and innate resistance.

The TLR3 pathway has been shown to be key in controlling HCV infection, and single nucleotide polymorphisms in proteins associated with this pathway have previously been associated with increased susceptibility to HCV infection.[52,53] In particular, the induction of IFN-I by TLR3 is key to rapid clearance of viral infection.[54–56] Impaired TLR3-mediated induction of IFN-I through loss-of-function mutations has rendered individuals susceptible to otherwise innocuous infections.[57] 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.[58,59] Investigations into the genetic factors associated with the TLR3 response will be the subject of further studies.

Ongoing latent and previous viral infections can impact on the immune response and susceptibility to other viral infections.[60,61] Individuals who are CMV SP exhibit substantial alterations in immune cell populations, exemplified by a dramatic increase in the number of CD4+ and CD8+ effector memory T cells, and are reported to have an increased risk of developing severe viral disease, including coronavirus disease 2019.[62,63] Using VirScan, we saw no differences in seropositivity for any virus except HCV, however it is possible that the women from our infection groups had different viral exposures at the time of infection

that contributed to altered susceptibility to infection during the 1977–1979 period.

Here, we identified ESN individuals in the Irish anti-D cohort. We suggest that resistance to HCV infection in females may be up to five times greater than previous studies indicate. We uncover enhanced polyIC responsiveness at the protein and gene levels in stimulated whole blood from women enrolled in our study. Our findings of increased resistance among 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.

Limitations of the study

This study has a number of limitations. Our overall sample size for women exposed to HCV was limited (n = 18 ESN, n = 19 SR, and n = SVR). We were, therefore, not sufficiently powered to compare ESNs, SRs, and SVRs separately in our analysis of the whole blood immune response. As the cohort consists of older females we were also limited by the blood volume that we were approved to draw. This limited the number of analyses that could be performed in the present study.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2022.100804.

AUTHOR CONTRIBUTIONS

J.A.S. designed and performed experiments, analyzed and interpreted data and wrote the manuscript. C.P., Z.T., C.P., B.C., and V.B. performed specific experiments. P.B., N.M.B., and D.D. designed experiments and interpreted data. N.M.B. and D.D. provided guidance and edited the manuscript. C.O.F. conceived the study, obtained funding, and wrote the manuscript. All authors contributed to the manuscript revision, and read and approved the submitted version.

DECLARATIONS OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

One or more of the authors of this paper self-identifies as a member of the LGBTQIA+ community. One or more of the authors of this paper self-identifies as living with a disability. We support inclusive, diverse, and equitable conduct of research.

REFERENCES

1. Yee, L.J. (2004). Host genetic determinants in hepatitis C virus infection. Genes Immun. 5, 237–245. https://doi.org/10.1038/sj.gene.6364090.

2. Westbrook, R.H., and Dusheiko, G. (2014). Natural history of hepatitis C. J. Hepatol. 61, S58–S68. https://doi.org/10.1016/j.jhep.2014.07.012.

3. Garvey, P., Murphy, N., Flanagan, P., Brennan, A., Courtney, G., Crosbie, O., Crowe, J., Hegarty, J., Lee, J., McIver, M., et al. (2017). Disease outcomes in a cohort of women in Ireland infected by hepatitis C-contaminated anti-D immunoglobulin during 1970s. J. Hepatol. 67, 1140–1147. https://doi.org/10.1016/j.jhep.2017.07.034.

4. Lawlor, E., Power, J., Garson, J., Yap, P., Davidson, F., Columb, G., Smith, D., Pomeroy, L., O'Riordan, J., Simmonds, P., et al. (1999). Transmission rates of hepatitis C virus by different batches of a contaminated anti-D immunoglobulin preparation. Vox Sang. 76, 138–143.

5. Wiese, M., Fischer, J., Löbermann, M., Göbel, U., Grüngreiff, K., Güthoff, W., Kullig, U., Richter, F., Schiefke, I., Tenckhoff, H., et al. (2014). Evaluation of liver disease progression in the German hepatitis C virus (1b)-contaminated anti-D cohort at 35 years after infection. Hepatology 59, 49–57. https://doi.org/10.1002/hep.26644.

6. Shawa, I.T., Felmlee, D.J., Hegazy, D., Sheridan, D.A., and Cramp, M.E. (2017). Exploration of potential mechanisms of hepatitis C virus resistance in exposed uninfected intravenous drug users. J. Viral Hepat. 24, 1082–1088. https://doi.org/10.1111/jvh.12720.

7. Swann, R.E., Mandalou, P., Robinson, M.W., Ow, M.M., Foung, S.K.H., McLauchlan, J., Patel, A.H., and Cramp, M.E. (2016). Anti-envelope antibody responses in individuals at high risk of hepatitis C virus who resist infection. J. Viral Hepat. 23, 873–880. https://doi.org/10.1111/jvh.12568.

8. Yee, T.T., Griffioen, A., Sabin, C.A., Dusheiko, G., and Lee, C.A. (2000). The natural history of HCV in a cohort of haemophilic patients infected between 1961 and 1985. Gut 47, 845–851. https://doi.org/10.1136/gut.47.6.845.

9. Rouet, F., Nouhin, J., Zheng, D.-P., Roche, B., Black, A., Prak, S., Leoz, M., Gaudy-Graffin, C., Ferradini, L., Mom, C., et al. (2018). Massive iatrogenic outbreak of human immunodeficiency virus type 1 in rural Cambodia, 2014–2015. Clin. Infect. Dis. 66, 1733–1741. https://doi.org/10.1093/cid/cix1071.

10. Kenny-Walsh, E. (1999). Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. N. Engl. J. Med. 340, 1228–1233. https://doi.org/10.1056/NEJM199904223401602.

11. Heim, M.H., and Thimme, R. (2014). Innate and adaptive immune responses in HCV infections. J. Hepatol. 61, S14–S25. https://doi.org/10.1016/j.jhep.2014.06.035.

12. Warshow, U.M., Riva, A., Hegazy, D., Thurairajah, P.H., Kaminski, E.R., Chokshi, S., and Cramp, M.E. (2012). Cytokine profiles in high risk injection drug users suggests innate as opposed to adaptive immunity in apparent resistance to hepatitis C virus infection. J. Viral Hepat. 19, 501–508. https://doi.org/10.1111/j.1365-2893.2011.01574.x.

13. Sugden, P.B., Cameron, B., Mina, M., and Lloyd, A.R.; HITS investigators (2014). Protection against hepatitis C infection via NK cells in highly-exposed uninfected injecting drug users. J. Hepatol. 61, 738–745. https://doi.org/10.1016/j.jhep.2014.05.013.

14. Zhao, N.Q., Vendrame, E., Ferreira, A.-M., Seiler, C., Ranganath, T., Alary, M., Labbé, A.C., Guédou, F., Poudrier, J., Holmes, S., et al. (2020). Natural killer cell phenotype is altered in HIV-exposed seronegative women. PLoS One 15, e0238347.

15. Duffy, D., Rouilly, V., Braudeau, C., Corbière, V., Djebali, R., Ungeheuer, M.-N., Josien, R., LaBrie, S.T., Lantz, O., Louis, D., et al. (2017). Standardized whole blood stimulation improves immunomonitoring of induced

immune responses in multi-center study. Clin. Immunol. *183*, 325–335. https://doi.org/10.1016/j.clim.2017.09.019.

16. Rodrigues, K.B., Dufort, M.J., Llibre, A., Speake, C., Rahman, M.J., Bondet, V., Quiel, J., Linsley, P.S., Greenbaum, C.J., Duffy, D., et al. (2020). Innate immune stimulation of whole blood reveals IFN-1 hyper-responsiveness in type 1 diabetes. Diabetologia *63*, 1576–1587. https://doi.org/10.1007/s00125-020-05179-4.

17. Llibre, A., Smith, N., Rouilly, V., Musvosvi, M., Nemes, E., Posseme, C., Mabwe, S., Charbit, B., Mbandi, S.K., Filander, E., et al. (2020). Tuberculosis alters immune-metabolic pathways resulting in perturbed IL-1 responses. Preprint at bioRxiv, 2020.12.17.423082. https://doi.org/10.1101/2020.12.17.423082.

18. Fischer, J., Weber, A.N.R., Böhm, S., Dickhöfer, S., El Maadidi, S., Deichsel, D., Knop, V., Klinker, H., Möller, B., Rasenack, J., et al. (2017). Sex-specific effects of TLR9 promoter variants on spontaneous clearance of HCV infection. Gut *66*, 1829–1837, LP – 1837. https://doi.org/10.1136/gutjnl-2015-310239.

19. Barrett, S., Goh, J., Coughlan, B., Ryan, E., Stewart, S., Cockram, A., O'Keane, J.C., and Crowe, J. (2001). The natural course of hepatitis C virus infection after 22 years in a unique homogenous cohort: spontaneous viral clearance and chronic HCV infection. Gut *49*, 423–430. https://doi.org/10.1136/gut.49.3.423.

20. Sugrue, J.A., and O'Farrelly, C. (2022). Uncovering resistance to hepatitis C virus infection: scientific contributions and unanswered questions in the Irish anti-D cohort. Pathogens *11*, 306. https://doi.org/10.3390/pathogens11030306.

21. Ji, F., Yeo, Y.H., Wei, M.T., Ogawa, E., Enomoto, M., Lee, D.H., Iio, E., Lubel, J., Wang, W., Wei, B., et al. (2019). Sustained virologic response to direct-acting antiviral therapy in patients with chronic hepatitis C and hepatocellular carcinoma: a systematic review and meta-analysis. J. Hepatol. *71*, 473–485. https://doi.org/10.1016/j.jhep.2019.04.017.

22. Xu, G.J., Kula, T., Xu, Q., Li, M.Z., Vernon, S.D., Ndung'u, T., Ruxrungtham, K., Sanchez, J., Brander, C., Chung, R.T., et al. (2015). Viral immunology. Comprehensive serological profiling of human populations using a synthetic human virome. Science *348*, aaa0698. https://doi.org/10.1126/science.aaa0698.

23. Heller, T., Werner, J.M., Rahman, F., Mizukoshi, E., Sobao, Y., Gordon, A.M., Sheets, A., Sherker, A.H., Kessler, E., Bean, K.S., et al. (2013). Occupational exposure to hepatitis C virus: early T-cell responses in the absence of seroconversion in a longitudinal cohort study. J. Infect. Dis. *208*, 1020–1025. https://doi.org/10.1093/infdis/jit270.

24. Takaki, A., Wiese, M., Maertens, G., Depla, E., Seifert, U., Liebetrau, A., Miller, J.L., Manns, M.P., and Rehermann, B. (2000). Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. Nat. Med. *6*, 578–582. https://doi.org/10.1038/75063.

25. Urrutia, A., Duffy, D., Rouilly, V., Posseme, C., Djebali, R., Illanes, G., Libri, V., Albaud, B., Gentien, D., Piasecka, B., et al. (2016). Standardized whole-blood transcriptional profiling enables the deconvolution of complex induced immune responses. Cell Rep. *16*, 2777–2791. https://doi.org/10.1016/j.celrep.2016.08.011.

26. Marcello, T., Grakoui, A., Barba–Spaeth, G., Machlin, E.S., Kotenko, S.V., Macdonald, M.R., and Rice, C.M. (2006). Interferons α and λ inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterology *131*, 1887–1898. https://doi.org/10.1053/j.gastro.2006.09.052.

27. Zhou, Z., Hamming, O.J., Ank, N., Paludan, S.R., Nielsen, A.L., and Hartmann, R. (2007). Type III interferon (IFN) induces a type I IFN-like response in a restricted subset of cells through signaling pathways involving both the Jak-STAT pathway and the mitogen-activated protein kinases. J. Virol. *81*, 7749–7758. https://doi.org/10.1128/JVI.02438-06.

28. Laidlaw, S.M., Marukian, S., Gilmore, R.H., Cashman, S.B., Nechyporuk-Zloy, V., Rice, C.M., and Dustin, L.B. (2017). Tumor necrosis factor inhibits spread of hepatitis C virus among liver cells, independent from interferons.

Gastroenterology *153*, 566–578.e5. https://doi.org/10.1053/j.gastro.2017.04.021.

29. Melchjorsen, J., Sørensen, L.N., and Paludan, S.R. (2003). Expression and function of chemokines during viral infections: from molecular mechanisms to in vivo function. J. Leukoc. Biol. *74*, 331–343. https://doi.org/10.1189/jlb.1102577.

30. Velazquez-Salinas, L., Verdugo-Rodriguez, A., Rodriguez, L.L., and Borca, M.V. (2019). The role of interleukin 6 during viral infections. Front. Microbiol. *10*, 1057.

31. Lin, C.-Y., Chen, J.-Y., Lin, T.-N., Jeng, W.-J., Huang, C.-H., Huang, C.-W., Chang, S.-W., and Sheen, I.-S. (2011). IL28B SNP rs12979860 is a critical predictor for on-treatment and sustained virologic response in patients with hepatitis C virus genotype-1 infection. PLoS One *6*, e18322. https://doi.org/10.1371/journal.pone.0018322.

32. Prokunina-Olsson, L., Muchmore, B., Tang, W., Pfeiffer, R.M., Park, H., Dickensheets, H., Hergott, D., Porter-Gill, P., Mumy, A., Kohaar, I., et al. (2013). A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. Nat. Genet. *45*, 164–171. https://doi.org/10.1038/ng.2521.

33. Toyoda, H., Kumada, T., Kiriyama, S., Sone, Y., Tanikawa, M., Hisanaga, Y., Kuzuya, T., Honda, T., Hayashi, K., Nakano, I., et al. (2005). Changes in hepatitis C virus (HCV) antibody status in patients with chronic hepatitis C after eradication of HCV infection by interferon therapy. Clin. Infect. Dis. *40*, e49–e54. https://doi.org/10.1086/428128.

34. Jacobsen, H., and Klein, S.L. (2021). Sex differences in immunity to viral infections. Front. Immunol. *12*, 720952. https://doi.org/10.3389/fimmu.2021.720952.

35. Grebely, J., Page, K., Sacks-Davis, R., van der Loeff, M.S., Rice, T.M., Bruneau, J., Morris, M.D., Hajarizadeh, B., Amin, J., Cox, A.L., et al. (2014). The effects of female sex, viral genotype, and IL28B genotype on spontaneous clearance of acute hepatitis C virus infection. Hepatology *59*, 109–120. https://doi.org/10.1002/hep.26639.

36. Bakr, I., Rekacewicz, C., El Hosseiny, M., Ismail, S., El Daly, M., El-Kafrawy, S., Esmat, G., Hamid, M.A., Mohamed, M.K., and Fontanet, A. (2006). Higher clearance of hepatitis C virus infection in females compared with males. Gut *55*, 1183–1187. https://doi.org/10.1136/gut.2005.078147.

37. Pazos, M., Sperling, R.S., Moran, T.M., and Kraus, T.A. (2012). The influence of pregnancy on systemic immunity. Immunol. Res. *54*, 254–261. https://doi.org/10.1007/s12026-012-8303-9.

38. Abu-Raya, B., Michalski, C., Sadarangani, M., and Lavoie, P.M. (2020). Maternal immunological adaptation during normal pregnancy. Front. Immunol. *11*, 575197. https://doi.org/10.3389/fimmu.2020.575197.

39. Jara, L.J., Medina, G., Cruz-Dominguez, P., Navarro, C., Vera-Lastra, O., and Saavedra, M.A. (2014). Risk factors of systemic lupus erythematosus flares during pregnancy. Immunol. Res. *60*, 184–192. https://doi.org/10.1007/s12026-014-8577-1.

40. Coss, S.L., Torres-Cornejo, A., Prasad, M.R., Moore-Clingenpeel, M., Grakoui, A., Lauer, G.M., Walker, C.M., and Honegger, J.R. (2020). CD4+ T cell restoration and control of hepatitis C virus replication after childbirth. J. Clin. Invest. *130*, 748–753. https://doi.org/10.1172/JCI123623.

41. Burchill, M.A., Roby, J.A., Crochet, N., Wind-Rotolo, M., Stone, A.E., Edwards, M.G., Dran, R.J., Kriss, M.S., Gale, M., Jr., and Rosen, H.R. (2017). Rapid reversal of innate immune dysregulation in blood of patients and livers of humanized mice with HCV following DAA therapy. PLoS One *12*, e0186213. https://doi.org/10.1371/journal.pone.0186213.

42. Alanio, C., Nicoli, F., Sultanik, P., Flecken, T., Perot, B., Duffy, D., Bianchi, E., Lim, A., Clave, E., van Buuren, M.M., et al. (2015). Bystander hyperactivation of preimmune CD8+ T cells in chronic HCV patients. Elife *4*, e07916. https://doi.org/10.7554/eLife.07916.

43. Hensel, N., Gu, Z., Sagar, Wieland, D., Jechow, K., Kemming, J., Llewellyn-Lacey, S., Gostick, E., Sogukpinar, O., Emmerich, F., et al. (2021). Memory-like HCV-specific CD8(+) T cells retain a molecular scar after

cure of chronic HCV infection. Nat. Immunol. 22, 229–239. https://doi.org/10.1038/s41590-020-00817-w.

44. Khera, T., Du, Y., Todt, D., Deterding, K., Strunz, B., Hardtke, S., Aregay, A., Port, K., Hardtke-Wolenski, M., Steinmann, E., et al. (2022). Long-lasting imprint in the soluble inflammatory Milieu despite early treatment of acute symptomatic hepatitis C. J. Infect. Dis. 226, 441–452. https://doi.org/10.1093/infdis/jiab048.

45. Du, Y., Khera, T., Strunz, B., Deterding, K., Todt, D., Woller, N., Engelskircher, S.A., Hardtke, S., Port, K., Ponzetta, A., et al. (2022). Imprint of unconventional T-cell response in acute hepatitis C persists despite successful early antiviral treatment. Eur. J. Immunol. 52, 472–483. https://doi.org/10.1002/eji.202149457.

46. Zhang, G., deWeerd, N.A., Stifter, S.A., Liu, L., Zhou, B., Wang, W., Zhou, Y., Ying, B., Hu, X., Matthews, A.Y., et al. (2018). A proline deletion in IFNAR1 impairs IFN-signaling and underlies increased resistance to tuberculosis in humans. Nat. Commun. 9, 85. https://doi.org/10.1038/s41467-017-02611-z.

47. Brodin, P., and Davis, M.M. (2017). Human immune system variation. Nat. Rev. Immunol. 17, 21–29. https://doi.org/10.1038/nri.2016.125.

48. Knapp, S., Warshow, U., Ho, K.M.A., Hegazy, D., Little, A.M., Fowell, A., Alexander, G., Thursz, M., Cramp, M., and Khakoo, S.I. (2011). A polymorphism in IL28B distinguishes exposed, uninfected individuals from spontaneous resolvers of HCV infection. Gastroenterology 141, 320–325.e2. https://doi.org/10.1053/j.gastro.2011.04.005.

49. Terczyńska-Dyla, E., Bibert, S., Duong, F.H.T., Krol, I., Jørgensen, S., Collinet, E., Kutalik, Z., Aubert, V., Cerny, A., Kaiser, L., et al. (2014). Reduced IFNλ4 activity is associated with improved HCV clearance and reduced expression of interferon-stimulated genes. Nat. Commun. 5, 5699. https://doi.org/10.1038/ncomms6699.

50. Shebl, F.M., Pfeiffer, R.M., Buckett, D., Muchmore, B., Chen, S., Dotrang, M., Prokunina-Olsson, L., Edlin, B.R., and O'Brien, T.R. (2011). IL28B rs12979860 genotype and spontaneous clearance of hepatitis C virus in a multi-ethnic cohort of injection drug users: evidence for a supra-additive association. J. Infect. Dis. 204, 1843–1847. https://doi.org/10.1093/infdis/jir647.

51. Chen, Q., Coto-Llerena, M., Suslov, A., Teixeira, R.D., Fofana, I., Nuciforo, S., Hofmann, M., Thimme, R., Hensel, N., Lohmann, V., et al. (2021). Interferon lambda 4 impairs hepatitis C viral antigen presentation and attenuates T cell responses. Nat. Commun. 12, 4882. https://doi.org/10.1038/s41467-021-25218-x.

52. Ferreira, A.R., Ramos, B., Nunes, A., and Ribeiro, D. (2020). Hepatitis C virus: evading the intracellular innate immunity. J. Clin. Med. 9, E790. https://doi.org/10.3390/jcm9030790.

53. Al-Anazi, M.R., Matou-Nasri, S., Abdo, A.A., Sanai, F.M., Alkahtani, S., Alarifi, S., Alkahtane, A.A., Al-Yahya, H., Ali, D., Alessia, M.S., et al. (2017). Association of toll-like receptor 3 single-nucleotide polymorphisms and hepatitis C virus infection. J. Immunol. Res. 2017, 1590653. https://doi.org/10.1155/2017/1590653.

54. Gao, D., Ciancanelli, M.J., Zhang, P., Harschnitz, O., Bondet, V., Hasek, M., Chen, J., Mu, X., Itan, Y., Cobat, A., et al. (2021). TLR3 controls constitutive IFN-β antiviral immunity in human fibroblasts and cortical neurons. J. Clin. Invest. 131, 134529. https://doi.org/10.1172/JCI134529.

55. Wang, N., Liang, Y., Devaraj, S., Wang, J., Lemon, S.M., and Li, K. (2009). Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. J. Virol. 83, 9824–9834. https://doi.org/10.1128/JVI.01125-09.

56. Menezes, M.C.S., Veiga, A.D.M., Martins de Lima, T., Kunimi Kubo Ariga, S., Vieira Barbeiro, H., de Lucena Moreira, C., Pinto, A.A.S., Brandao, R.A., Marchini, J.F., Alencar, J.C., et al. (2021). Lower peripheral blood Toll-like receptor 3 expression is associated with an unfavorable outcome in severe COVID-19 patients. Sci. Rep. 11, 15223. https://doi.org/10.1038/s41598-021-94624-4.

57. Lim, H.K., Seppänen, M., Hautala, T., Ciancanelli, M.J., Itan, Y., Lafaille, F.G., Dell, W., Lorenzo, L., Byun, M., Pauwels, E., et al. (2014). TLR3 deficiency in herpes simplex encephalitis: high allelic heterogeneity and recurrence risk. Neurology 83, 1888–1897. https://doi.org/10.1212/WNL.0000000000000999.

58. Sironi, M., Biasin, M., Cagliani, R., Forni, D., De Luca, M., Saulle, I., Lo Caputo, S., Mazzotta, F., Macías, J., Pineda, J.A., et al. (2012). A common polymorphism in TLR3 confers natural resistance to HIV-1 infection. J. Immunol. 188, 818–823. https://doi.org/10.4049/jimmunol.1102179.

59. Sugrue, J.A., Bourke, N.M., and O'Farrelly, C. (2021). Type I interferon and the spectrum of susceptibility to viral infection and autoimmune disease: a shared genomic signature. Front. Immunol. 12, 757249.

60. Kempkes, B., and Robertson, E.S. (2015). Epstein-Barr virus latency: current and future perspectives. Curr. Opin. Virol. 14, 138–144. https://doi.org/10.1016/j.coviro.2015.09.007.

61. van den Berg, S.P.H., Lanfermeijer, J., Jacobi, R.H.J., Hendriks, M., Vos, M., van Schuijlenburg, R., Nanlohy, N.M., Borghans, J.A.M., van Beek, J., van Baarle, D., et al. (2021). Latent CMV infection is associated with lower influenza virus-specific memory T-cell frequencies, but not with an impaired T-cell response to acute influenza virus infection. Front. Immunol. 12, 663664.

62. Shrock, E., Fujimura, E., Kula, T., Timms, R.T., Lee, I.-H., Leng, Y., Robinson, M.L., Sie, B.M., Li, M.Z., Chen, Y., et al. (2020). Viral epitope profiling of COVID-19 patients reveals cross-reactivity and correlates of severity. Science 370, eabd4250. https://doi.org/10.1126/science.abd4250.

63. Patin, E., Hasan, M., Bergstedt, J., Rouilly, V., Libri, V., Urrutia, A., Alanio, C., Scepanovic, P., Hammer, C., Jönsson, F., et al. (2018). Natural variation in the parameters of innate immune cells is preferentially driven by genetic factors. Nat. Immunol. 19, 645. https://doi.org/10.1038/s41590-018-0105-3.

64. Mina, M.J., Kula, T., Leng, Y., Li, M., de Vries, R.D., Knip, M., Siljander, H., Rewers, M., Choy, D.F., Wilson, M.S., et al. (2019). Measles virus infection diminishes preexisting antibodies that offer protection from other pathogens. Science 366, 599–606. https://doi.org/10.1126/science.aay6485.

65. Duffy, D., Rouilly, V., Libri, V., Hasan, M., Beitz, B., David, M., Urrutia, A., Bisiaux, A., LaBrie, S.T., Dubois, A., et al. (2014). Functional analysis via standardized whole-blood stimulation systems defines the boundaries of a healthy immune response to complex stimuli. Immunity 40, 436–450. https://doi.org/10.1016/j.immuni.2014.03.002.

66. Bondet, V., Rodero, M.P., Posseme, C., Bost, P., Decalf, J., Haljasmägi, L., Bekaddour, N., Rice, G.I., Upasani, V., Herbeuval, J.-P., et al. (2021). Differential levels of IFNα subtypes in autoimmunity and viral infection. Cytokine 144, 155533. https://doi.org/10.1016/j.cyto.2021.155533.

67. Liberzon, A., Birger, C., Thorvaldsdóttir, H., Ghandi, M., Mesirov, J.P., and Tamayo, P. (2015). The molecular signatures Database Hallmark gene Set collection. Cell Syst. 1, 417–425. https://doi.org/10.1016/j.cels.2015.12.004.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-pan-IFNα (capture)	ImmunoQure	8H1 clone
Anti-pan-IFNα (detector)	ImmunoQure	12H5 clone
Anti-pan-IFNβ (capture)	PBL	ref. 710322-9
Anti-pan-IFNβ (detector)	PBL	ref. 710323-9
Anti-CD45	BD	ref. 563204; RRID:AB_2738067
Anti-CD19	BD	ref. 557791; RRID:AB_396873
Anti-CD3	BioLegend	ref. 344822; RRID:AB_2563420
Anti-CD56	BD	ref. 364057
Anti-CD16	BD	ref. 557744; RRID:AB_396850
Anti-CD14	BioLegend	ref. 301830; RRID:AB_10959324
Anti-CD4	BioLegend	ref. 317420; RRID:AB_571939
Anti-CD8α	BioLegend	ref. 301046; RRID:AB_2563264
LiveDead Red	BioLegend	ref. 423109
Anti-BDCA1	BioLegend	ref. 331518; RRID:AB_2073403
Anti-BDCA2	BioLegend	ref. 34210
Anti-CD86	BioLegend	ref. 305438; RRID:AB_2564164
Anti-HLA-DR	BD	ref. 561224; RRID:AB_10563765
Anti-BDCA3	BD	ref. 563155; RRID:AB_2738033
Anti-BDCA4	BioLegend	ref. 354505; RRID:AB_11219393
Biological samples		
Viral resistance cohort	Trinity College Dublin	REC 171111
Chemicals, peptides, and recombinant proteins		
Trizol LS	BioSciences	ref. 10296028
Critical commercial assays		
NegControl TruCulture tube	Myriad RBM	ref. 782-001291
PolyIC TruCulture tube	Myriad RBM	ref. 782-001282
R848 TruCulture tube	Myriad RBM	ref. 782-001264
IFNα TruCulture tube	Myriad RBM	ref. 782-001277
NanoString human immunology panel v2	NanoString	N/A
Custom Luminex panel	R&D Systems	N/A
IFNL3/4 genotyping assay (rs12979860)	ThermoFisher	ref. 4351379 (C___7820464_10)
Deposited data		
Cytokine data	This manuscript	https://doi.org/10.17632/m65wh4vc9z.1
NanoString data	This manuscript	https://doi.org/10.17632/z4nr7p7ry7.1
Software and algorithms		
RStudio version 4.04	RStudio	N/A

RESOURCE AVAILABILITY

Lead contact
Further information and request for resources and reagents should be directed to and will be fulfilled by the lead contact Cliona O'Farrelly (ofarrecl@tcd.ie).

Materials availability

No materials were developed in the context of this study.

Data and code availability

- Data availability

Cytokine data presented in this manuscript is available at https://dx.doi.org/10.17632/m65wh4vc9z.1. NanoString data is available at https://data.mendeley.com/datasets/z4nr7p7ry7. Additional data reported in this paper will be shared by the lead contact upon request.

- Code availability

This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

Donors in this study were recruited through a national media campaign. Ethical approval for this study was given by the Faculty of Health Sciences Research Ethics Committee (Reference number 171111). Demographic information relevant to this study is detailed in Table 1.

Recruitment of resistant volunteers

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 our research group. 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.

Informed consent and ethical approval

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 HCV contaminated anti-D batches as high and low risk

Twelve batches of anti-D were made in 1977–1979 containing serum from a single HCV infected donor. Between 251 and 464 vials were made for each batch. The batches had varying viral loads.[4] 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.

Matching donors to 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 RhD 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 batch 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 of anti-D. Those who received a low risk batch were not included in the study. Volunteers for whom no data were available were excluded from the study.

Questionnaire

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.

METHOD DETAILS

Antibody profiling using 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. 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.[64] Briefly, epitopes were grouped into hundreds of bins so that the reads from bead samples formed 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). Heatmaps of "virusHits" and epitopes were generated in R using the package pheatmap. Comparisons between infection groups for antibodies against all pathogens in the VirScan library using one-way non-parametric ANOVAs (Kruskal-Wallis tests) followed by Dunn's post hoc test with an FDR correction.

PBMC extraction and IFNγ ELISpot
Peripheral blood mononuclear cells (PBMCs) were isolated from venous blood collected in 9mL sodium heparin tubes. For detection of T memory cell responses, IFNγ ELISpots were performed on isolated PBMCs stimulated with the ProMix HCV peptide pool and the ProMix CEF peptide pool (CMV, EBV and influenza virus; ProImmune Limited, Oxford, UK). All peptides were dissolved in DMSO and used at a final concentration of 3 μg/mL. DMSO and concanavalin A (5 μg/mL) were used a negative and positive controls respectively. Plates were read and analysed using an AID ELISpot reader (AID GmbH, Germany). The cut off for positivity was determined based off the mean + 3xSD of the UC group.

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.[65] The tubes were preloaded with our chosen stimuli: IFNα2 (1,000IU/mL), polyIC (20 μg/mL), R848 (20μM) and a null unstimulated tube. Following incubation for 22 hrs 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.

Cytokine analysis
Secreted cytokines were measured in the supernatants of TruCulture tubes using a custom Luminex panel (R&D Systems). 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. IFNα2 and IFNβ were quantified using the highly sensitive Simoa digital ELISA (Quanterix) with HomeBrew assays developed as previously described.[66] The limit of detection for IFNα2 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
Total RNA was extracted as previously described.[25] The RNA integrity number (RIN) for each sample was determined using the Agilent RNA 6000 Nano kit (Agilent) and Bioanalyser. Gene expression was quantified using the NanoString human immunology panel v2 (NanoString). Samples were processed 12 at a time. RNA was diluted to 100ng and a thermocycler pre-heated to 65°C. Reporter and capture probes were thawed to room temperature. A master mix containing 5μL of hybridisation buffer and 3μL 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 placed in a thermocycler at 65°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 (NanoString). Count data was exported as reporter code count (RCC) files. RCC files were imported into the NanoString nSolver software for normalisation using positive and negative probes. Differences in RNA input were corrected using housekeeping genes selected using the geNorm method (*ALAS1, EEF1G, G6PD, HPRT1, POL2RA, PPIA, RPL19, SDHA, TBP*). Lowly expressed genes with counts <4 in 75% of samples were filtered out. 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.

Differential gene expression analysis
Differential gene expression between ESN and SP groups was determined using Mann-Whitney U tests in R, followed by an FDR correction using the Benjamini-Hochberg method. Heatmaps were generated in R using the package pheatmap.

Gene signature scores

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).[25] 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 (MolSigDB) was used to validate findings with the MIC derived score.[67]

Flow cytometry

Immune cell populations were quantified using flow cytometry. Briefly, 200μL of fresh whole blood was washed in PBS and incubated with the relevant antibodies for 20 minutes at room temperature. Cell suspensions were washed with PBS and the cell pellet resuspended in red blood cell lysis buffer at 37°C for 15 minutes. Lysis buffer was removed by centrifugation and the cells washed again in FACS buffer (PBS + 0.5% FBS). Cells were resuspended in a final volume of 200μL of FACS buffer and 25μL count beads (CountBrightt^{TM}, ThermoFisher) were added. Stained cells were acquired on the LSR Fortessa (BD Biosciences). Plots were analysed using FlowJo^{TM} (version 10.7.1).

DNA isolation and rs12979860 genotyping

DNA was isolated from saliva returned by study participants via the postal service in Ireland. DNA isolation was carried out using the protocol and materials from Isohelix GeneFix^{TM} Saliva DNA Mini Kit: GSS-50 (Cell Projects, United Kingdom). Donors were genotyped by quantitative polymerase chain reaction (qPCR) using the Applied Biosystems StepOnePlus RT-PCR machine for the rs12979860 SNP upstream of IFNL3 (TaqMan, Applied Biosystems, USA, C__7820464_10) in a total volume of 5μL. 20 additional unexposed control donors and 9 additional SVR donors for whom we had DNA were included in the genotype analysis. The total number of donors included for the genetic analysis is indicated in Table S1. Genotype frequencies between groups were assessed using Chi-square tests. The significance level was adjusted with a Bonferroni correction (α/n, where a is the original significance level and n is the number of tests performed; $0.05/12 = 0.004$). A $p < 0.004$ was considered significant.

QUANTIFICATION AND STATISTICAL DATA ANALYSIS

Data analysis

Data analysis was carried out using Prism (version 8.2.1) and RStudio (R4.0.4 version). F tests and Shapiro-Wilk tests were used to determine variance and normality respectively. Chi-square tests were used to compare differences in demographic data. Unpaired t tests were used to compare gene signature scores between groups. Mann-Whitney U tests were used to compare differences between individual genes and proteins between infection groups. Except where otherwise specified, a p value < 0.05 was considered significant.