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IFNL3 mediates interaction between innate immune cells: Implications for hepatitis C virus pathogenesis

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Abstract

Common *IFN lambda 3* (*IFNL3*) variants have been demonstrated to affect spontaneous and treatment-induced clearance of hepatitis C virus (HCV) infection. The functional basis of these genetic variants has yet to be determined. Data examining the effect of IFNL3, specifically, in innate immune cells is lacking. Here, we determined the expression of *IFNL3* and its receptor *IFNLR1* in blood immune cell subsets and in HCV-infected livers. Next we assessed their sensitivity to IFNL3. All participants were genotyped for the IFNL3 SNPs *rs8099917* and *rs12979860*. Importantly, unstimulated blood immune cells express significantly higher levels of *IFNL3* than HCV liver biopsies. Plasmacytoid dendritic cells (pDCs) are the predominant producers of *IFNLR1*, especially in response to IFN-α. PBMCs, monocytes and pDCs all respond to IFNL3 based on *MxA* up-regulation. No differences in *IFNL3* expression levels between *rs8099917* or *rs12979860* genotypes were detected. This is the first study to show peripheral blood pDCs to be the main producers of IFNL3, especially compared with HCV-infected livers. This makes innate immune cells the key players in determining the functional significance of *INFL3* polymorphisms in patients with HCV.

Keywords

IFNL3, hepatitis C, IFN, innate immunity

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Introduction

Several genome-wide association studies (GWAS) have described single nucleotide polymorphisms (SNPs) upstream of IFN lambda 3 [*IFNL3* (previously known as *IL28B*); *IFNL3* will be used here to comply with this recent nomenclature change by the HUGO Gene Nomenclature Committee] that are highly predictive of response to pegylated IFN-α and ribavirin therapy in patients with chronic hepatitis C virus (HCV) genotype 1 infection.[1–3] Both *rs12979860* and *rs8099917* were subsequently shown to be associated with natural clearance of HCV.[4–6] An association of the SNPs with rapid virological response to IFNA-based therapy[7] and early viral kinetics[8] suggests that *IFNL3* genetic differences lead to altered innate immune cell responses.

IFNL3 encodes IFNL3, a member of the type III IFN family (IFNL1, IFNL2, IFNL3), which is functionally closely related to the type-I IFNs, and induces antiviral activity both *in vitro* and *in vivo*.[9,10] Both families signal through the same JAK/STAT pathway leading to phosphorylation of STAT1 and STAT2, and subsequent assembly of the IFN stimulated gene factor-3

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transcription factor. This, in turn, induces IFN-stimulated genes (ISGs). All type III IFNs signal through the same heterodimeric receptor complex. This is composed of an IFN-lambda specific chain [IFN-lambda-receptor-1 (IFNLR1)] and IL-10R2.[9,10] IL-10R2 is ubiquitously expressed, while IFNLR1 has been suggested to have restricted tissue expression, predominately on epithelium-like-tissues including, fibroblasts, endothelial cells and adipocytes.[11] *IFNLR1* gene generates several splice variants including a full-length, membrane-bound IFNLR1 and secreted soluble IFNLR1 protein.[12] Previous work has demonstrated expression of *IFNLR1* mRNA in human immune cells, especially B, T and NK cells.[11] However, these immune cells were shown to express a relatively more soluble receptor, which can act as an inhibitor to IFNL activity.[11] Studies have shown that the IFNLs exhibit both *in vitro*[13] and *in vivo*[14] antiviral activity against HCV. There is also *in vitro* evidence that IFNA induces expression of IFNL genes, and that both cytokines appear to enhance the activity of the other.[15] This synergism has recently been confirmed *in vitro* against HCV.[16]

The available functional data on the IFNLs make a strong case that the SNPs identified in the GWAS tag haplotypes with functional significance. Since their publication in 2009, many studies have therefore sought to determine how the risk haplotype may affect immune function and, ultimately, HCV clearance. To date, the exact biological mechanism remains elusive. It has been proposed that the haplotype SNPs may affect response to transcription factors and thus alter endogenous levels of IFNL3 affecting spontaneous HCV clearance and treatment response. However, increased levels of IFNL3 in PBMCs has been detected in some,[2,3] but not all, studies.[1]

The SNPs predicting treatment response in HCV patients tag *IFNL3* and not *IFNL2* or *IFNL1*. However, studies examining IFNL3 specifically are lacking and so we examined for the expression of *IFNL3* and *IFNLR1* in immune cell subsets and in HCV-infected liver. Immune cell subsets, including NK cells, monocytes, monocyte-derived dendritric cells (moDCs) and plasmacytoid dendritic cells (pDCs) were analysed under control settings and after stimulation with IFNA. In addition we assessed if these immune cells were sensitive targets for IFNL3. Finally, we examined for a link between *IFNL3* expression levels and *IFNL3* genotype in different immune cell subsets.

Materials and methods

Study cohort

Twenty healthy control participants were recruited for large-volume blood collection for the generation of immune cell subsets, as outlined below. Liver biopsies from 38 chronic HCV genotype 1 patients were obtained for RNA extraction.

Ethical approval was obtained from the Human Research Ethics Committees of the Sydney West Area Health Service and the University of Sydney. All participants gave written informed consent to take part.

Separation of immune cell subsets and in vitro *generation of moDCs*

EDTA tubes of blood were collected from healthy participants and separation of PBMCs on Ficoll-Paque was performed. A number of cell subsets were isolated from the PBMCs, including monocytes (Negative selection-STEMCELL Technologies, Melbourne, Australia, and Miltenyi Biotec Magnetic Cell Sorting, Miltenyi Biotec, Sydney, Australia), pDCs (EasySep Human plasmacytoid DC Enrichment Kit; Negative selection-STEMCELL Technologies) and NK-cells (Negative selection-STEMCELL Technologies). moDCs were generated from CD14-positive separated cells by a 6-d culture in granulocyte macrophage-CSF (67 ng/ml) (eBioscience, San Diego, CA, USA) and recombinant human IL-4 (17 ng/ml).[17] Purity was checked by flow cytometry using recommended Abs after cell separations and was $> 90\%$ in each case.

Stimulation experiments

Purified cell populations were cultured in 96-well plates at a concentration of $1–1.5 \times 10^5$ cells per ml with media alone, IFNA 1000 IU/ml (Roferon-A, IFN-α-2 a; Roche, Sydney, Australia), recombinant human IFNL3 100 ng/ml (R&D Systems, Minneapolis, MN, USA), or IFNA and IFNL3. After 24 h, the cell pellets were captured in Cell-to-signal lysis buffer (Ambion, Austin, TX, USA) and stored at -80°C until RNA extraction.

IFNL3 genotyping

All healthy controls were genotyped for the *rs8099917* SNP by methods previously reported[2] or by Taqman genotyping (Applied Biosystems, Carlsbad, CA, USA). The *IFNL3* rs12979860 SNP was genotyped using a custom-made Taqman genotyping kit.

Gene expression by qPCR

RNA was extracted from the immune cell pellets and liver biopsies using the RNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer instructions. RNA quality and concentration was assessed using and Agilent 2100 Bioanalyser (Agilent, Waldbronn, Germany). cDNA was prepared using Superscript III, RnaseOut, Oligo (dT) and random primers (Invitrogen; Melbourne, Australia) in a

Mastercycler gradient 5331 (Eppendorf AG, Hamburg, Germany). Reverse transcription was performed at 50°C for 45 min. Gene expression was measured by qPCR using newly, previously unpublished, custom-designed primers (Sigma Aldrich; Sydney, Australia) specific for *IFNL3* (forward: 5'CCCAAAAAAGGAGTCCCCTG-3'; reverse: 5'-GGTTGCATGACTGGCGGA-3'). Specificity for *IFNL3* was confirmed on sequencing of the PCR product (Supplementary Figure S1). In addition, primers for *IFNLR1* were designed (forward: 5'-CTAAGCCCACCTGCTTCTTG-3'; reverse: 5'-GTCAGTTCCTTTTGGGGACA-3'). These primers detected both splice forms of *IFNLR1*; the membrane-associated and soluble form (Supplementary Figure S2). The ratio of membrane-bound receptor to soluble isoforms was determined from amplified gene products using an Agilent Bioanalyzer (Agilent). *MxA* (forward: 5'-GCCGGCTGTGGATATGCTA-3'; reverse: 5'-TTTATCGAAACATCTGTGAAAGCAA-3') was selected as the candidate IFN-stimulated gene (ISG) given published associations with HCV treatment outcomes.[18] *GAPDH* primers (forward: 5'-TCCACCACCCTGTTGCTGTA-3'; reverse: 5'-ACCACAGTCCAGCCATCAC-3') were utilised as the housekeeping gene. Amplification was measured using Power Syber (Life Technologies, Carlsbad, CA, USA). Gel electrophoresis was used to confirm the absence of gDNA products from the PCR reactions. Expression was measured using C_T values, normalised to that of *GAPDH* ($\Delta C_T = C_T$ (*GAPDH*)- C_T (target) and then expressed as $2^{-\Delta CT}$. C_T values were less than 30, and all amplifications were done in duplicate.

Statistics

GraphPad Prism v. 5.0 (GraphPad Software, La Jolla, CA, USA) was used to perform the analyses, which included (1) data being transformed for normality ($\log(Y \times 10^6)$—D'Agostino-Pearson omnibus test was performed on all data sets to confirm normality; (2) ANOVA with Bonferroni's post-hoc testing for multiple comparison to compare *IFNL3* and *IFNLR1* mRNA expression between different cell subsets and for rs12979860; and (3) paired *t*-test for comparison of *IFNLR1* and *MxA* mRNA expression in immune cells with and without stimulation, and *MxA* mRNA expression based on the rs12979860 SNP. A two-sided *P*-value < 0.05 was considered significant.

Results

IFNL3 *and* IFNLR1 *expression in immune cell subsets and HCV-infected liver biopsies*

IFNL3 and *IFNLR1* mRNA expression was determined in each of the immune cell subset samples and in HCV-infected liver biopsies (Figure 1). All unstimulated immune cells examined (PBMCs, NK cells, monocytes, moDCs and pDCs) exhibited significantly higher *IFNL3* mRNA expression than HCV-infected liver biopsies (Figure 1A).

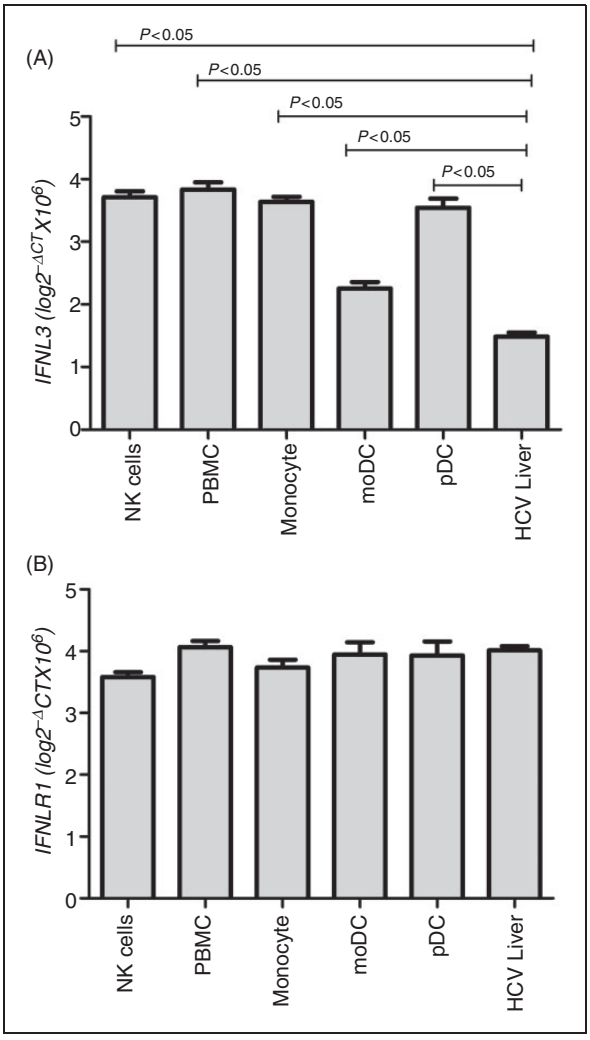

Figure 1. (A) *IFNL3* mRNA expression as determined by qPCR in unstimulated immune cell subsets [NK cells ($n = 18$), PBMCs ($n = 18$), monocytes ($n = 16$), moDCs ($n = 17$) and pDCs ($n = 15$)] and HCV-infected liver biopsies ($n = 38$). NK cells, PBMCs and pDCs expressed significantly higher ($P < 0.05$) levels of *IFNL3* mRNA compared with liver biopsy samples from HCV patients using ANOVA and Bonferroni's post-hoc correction for multiple testing. (B) *IFNLR1* mRNA expression as determined by qPCR in unstimulated immune cell subsets [NK cells ($n = 15$), PBMCs ($n = 15$), monocytes ($n = 13$), moDCs ($n = 14$) and pDCs ($n = 14$)] and HCV-infected liver biopsies ($n = 38$). There is no significant difference in expression levels between immune cell subsets and HCV-infected liver biopsies using ANOVA and Bonferroni's post-hoc correction for multiple testing. Error bars represent SEM.

To test if these differences may have been due to differences in expression of the housekeeping gene *GAPDH* rather than *IFNL3*, we also compared expression using RNA-seq (Supplementary Figure S3), which

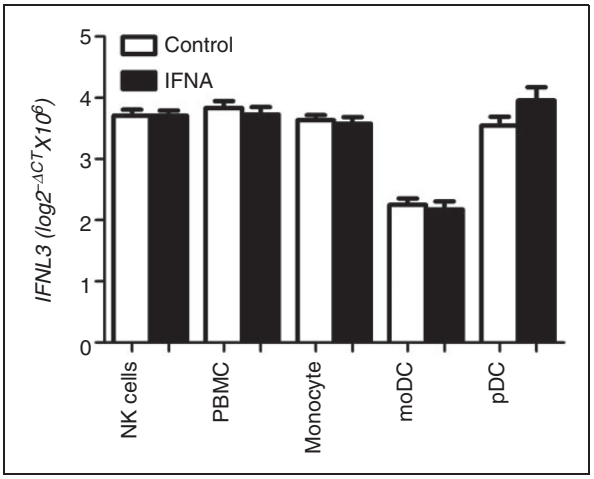

Figure 2. *IFNL3* mRNA expression as determined by qPCR in immune cell subsets under control conditions and after stimulation with IFNA. NK cells ($n = 18$), PBMCs ($n = 18$), monocytes ($n = 16$), moDCs ($n = 17$) and pDCs ($n = 15$). pDCs were the predominant producers of *IFNL3* based on mRNA expression in the context of IFNA. Error bars represent SEM.

confirmed there was much lower IFNL3 expression in HCV-infected hepatocytes than pDCs. There was no significant difference in *IFNLR1* mRNA expression between any of the immune cell subsets and HCV infected liver biopsies (Figure 1B).

pDCs were the dominant cell type to express *IFNL3* mRNA; however, this was not significantly higher than the other immune cell subsets. There was no significant up-regulation in *IFNL3* after IFNA stimulation in any of the immune cell subsets (Figure 2).

pDCs exhibited the highest expression levels of *IFNLR1* in the context of IFNA treatment. *IFNLR1* levels were significantly higher in IFNA-treated pDCs compared with IFNA-treated NK cells ($P < 0.05$), monoctyes ($P < 0.05$) and moDCs ($P < 0.05$) (Figure 3). A significant up-regulation of *IFNLR1* expression after IFNA stimulation was observed in NK cells ($P < 0.05$), PBMCs ($P < 0.0001$) and pDCs ($P < 0.001$). pDCs exhibited the largest up-regulation of *IFNLR1* in response to IFNA (Figure 3A). In order to ascertain if this resulted in functional receptor on the cell surface, we determined the ratio of membrane-bound receptor to soluble isoforms (Figure 3B). For the monocytes, pDCs and moDCs the majority of the *IFNLR1* detected was the isoform encoding the membrane-associated *IFNLR1*.

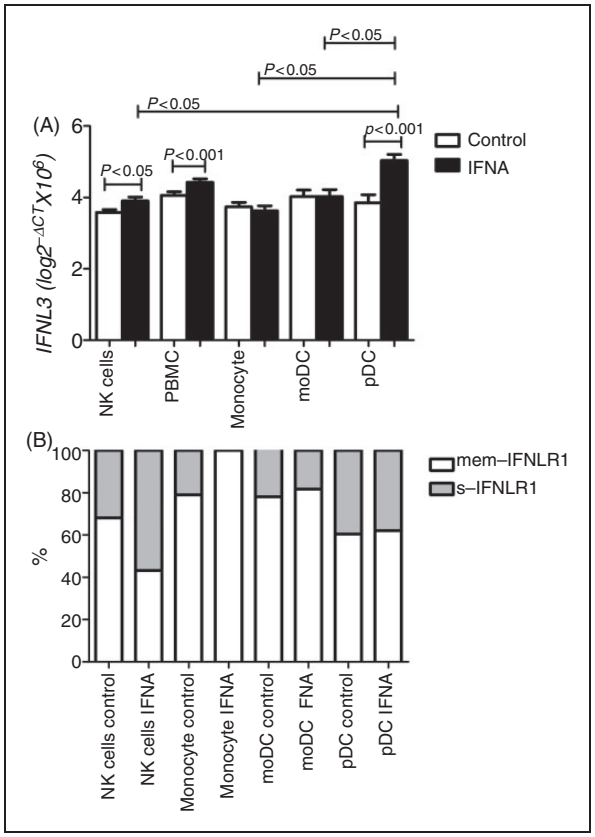

Figure 3. (A) *IFNLR1* mRNA expression as determined by qPCR in immune cell subsets under control conditions and after stimulation with IFNA. NK cells ($n = 15$), PBMCs ($n = 15$), monocytes ($n = 13$), moDCs ($n = 14$) and pDCs ($n = 14$). pDCs ($P < 0.0001$), NK-cells ($P < 0.05$) and PBMCs ($P < 0.005$) all demonstrate a significant up-regulation of *IFNLR1* mRNA after IFNA stimulation using paired *t*-test. IFNA-stimulated pDCs were the predominate producers of *IFNLR1* mRNA expressing significantly higher levels than stimulated all the other stimulated cell subsets ($P < 0.05$) using ANOVA and Bonferroni's post-hoc correction for multiple testing. Error bars represent SEM. (B) Bioanalyser analysis showing relative expression of membrane-bound (*memIFNLR1*) and soluble-form (*sIFNLR1*) of *IFNLR1* in immune cell subsets.

Immune cell subset response to IFNL3

To examine the responsiveness of immune cell subsets to IFNL3, cells were stimulated with IFNA and IFNL3, either individually or combined for 24 h. To determine if cells were functionally responsive, expression of *MxA* was used as a marker ISG (Figure 4). Again, PBMCs ($P < 0.05$), pDCs ($P < 0.05$) and monocytes ($P < 0.05$) all responded to IFNL3, as measured by *MxA* up-regulation. Importantly, at these doses, IFNA was more effective than IFNL3 in up-regulating the expression of *MxA* in NK cells ($P < 0.0001$), PBMCs ($P < 0.0001$), pDCs ($P < 0.05$) and monocytes ($p < 0.0001$).

Association of IFNL3 genotype with immune cell subset IFNL3 expression

In the cell subsets examined, there was no statistically significant correlation between *IFNL3* expression and the *rs8099917* SNP (data not shown) or the *rs12979860* SNP (Figure 5) in untreated cells or after stimulation with IFNA. However, there was a high degree of inter-

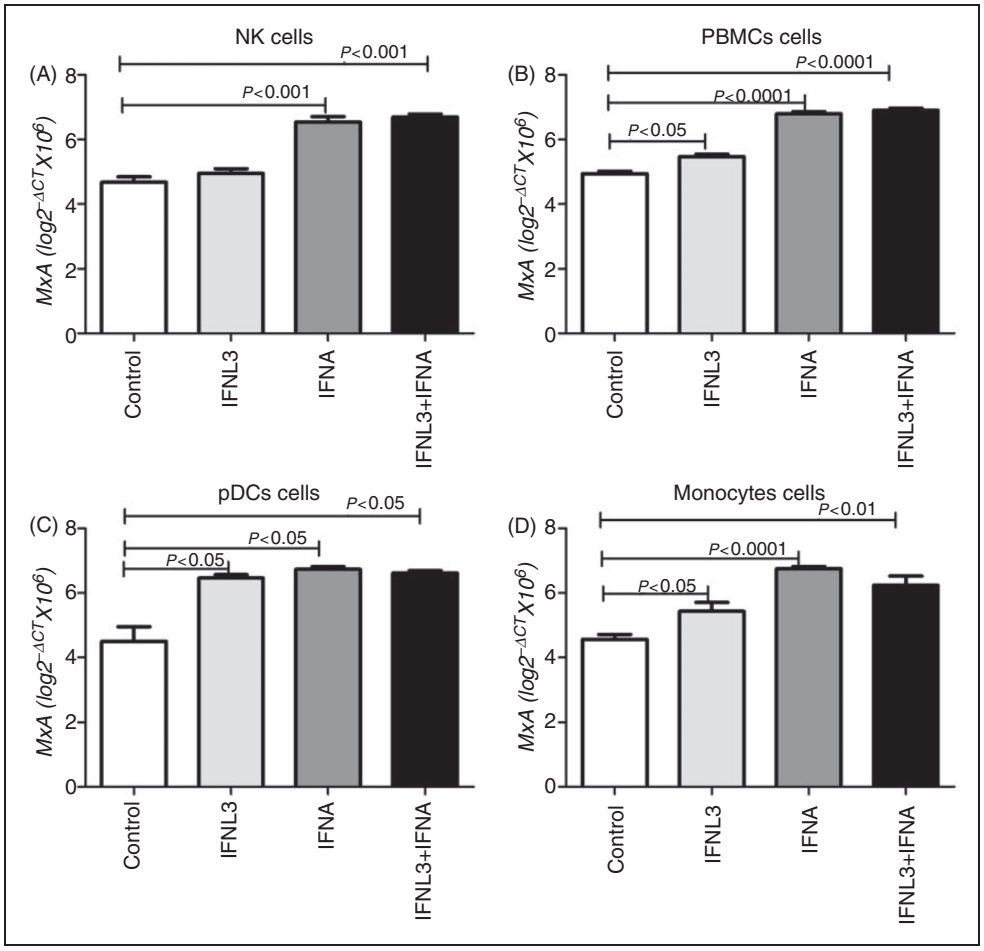

Figure 4. *MxA* expression in immune cell subsets in response to treatment with IFNL3, IFNA and IFNL3 plus IFNA. (A) NK cells, (B), PBMCs (C), pDCs and (D) monocytes. A statistically significant up-regulation in *MxA* in response to IFNL3 is demonstrated in PBMCs ($P < 0.05$), pDCs ($P < 0.05$) and monocytes ($P < 0.05$), but not in NK cells using paired *t*-test. Error bars represent SEM.

individual variation in the levels of *IFNL3* expression—up to 500-fold in some stimulated cell subsets.

Discussion

In this study we focused exclusively on *IFNL3* and showed significantly higher *IFNL3* mRNA expression levels in immune cell subsets compared with HCV-infected liver biopsies. pDCs, in particular, but also other immune cells (PBMCs, NK cells) up-regulate *IFNLR1* in response to IFNA, and is found in this context predominately expressed as the functional membrane-bound form in these cells. This is in contrast to previous studies that demonstrate relatively more soluble *IFNLR1* in immune cells (including T, B and NK cells). This study suggested that the soluble *IFNLR1* may act as an antagonist to limit signalling through the membrane-bound *IFNLR1*.[11] Thus, our findings of expression of the signalling competent form of *IFNLR1* on immune cells is of functional significance and is in keeping with our results that immune cells respond to IFNLR1. We show that PBMCs, monocytes and pDCs are sensitive to IFNL3 as evidenced by up-regulation of the ISG *MxA*. In contrast with traditional thinking we show that *IFNLR1* expression is not significantly higher in HCV-infected liver biopsies compared with immune cell subsets.

Previous work has produced conflicting results on whether or not immune cells are a target for type III IFNs. Several studies have failed to show a response to IFNLs (IFNL1 and/or IFNL2) by a variety of immune cells, including B-cells, T-cells, NK cells and monocytes.[11,19] Indeed, this putative lack of response and reported restricted tissue expression of the receptor, IFNLR1, was heralded as a potential advantage in the development of a pegylated form of IFNL1 (PEG-rIL-29), which was thought to exhibit fewer systemic side effects than IFNA. *In vivo* studies performed in cynomolgus monkeys treated with PEG-rIL-29 demonstrated a lack of induction of antiviral genes by peripheral blood mononuclear cells, such as *MxA*.[14] In contrast, several other human studies have revealed a direct effect of type III IFNs on monocytes,[20,21] DCs[19] and T-cells.[20,22,23] These studies all used IFNL1 and or IFNL2 to assess and define response to the 'IFNLs'. Human studies focusing on immune cell responses to

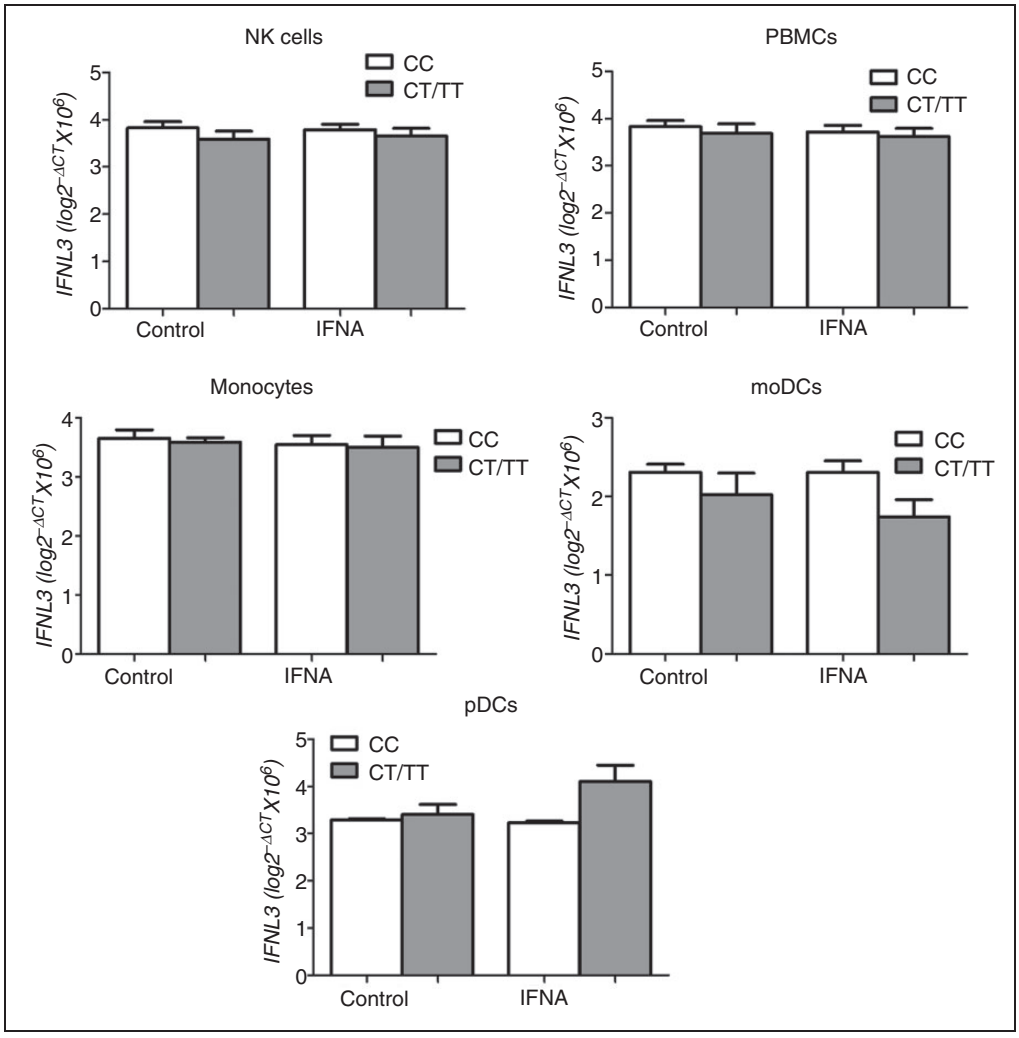

Figure 5. *IFNL3* mRNA expression as determined by qPCR in unstimulated and IFNA-stimulated immune cell subsets for rs12979860 responder (CC) and non-responder (TT/CT) genotypes. No significant difference based on rs12979860 genotype is detected for *IFNL3* expression using ANOVA and Bonferroni's post-hoc correction for multiple testing. Error bars represent SEM.

IFNL3, specifically, are lacking. Here we have demonstrated that PBMCs, monocytes and pDCs are all responsive to IFNL3 as assessed by *MxA* up-regulation. This has important implications, including the use of IFNL3 as a therapy and the likelihood of a positive feedback loop existing for the production of IFNL3, particularly by pDCs within HCV-infected livers and the potential for an amplified response with IFNA therapy.

We further examined the hypothesis that the *IFNL3* genotype alters expression levels of *IFNL3* as several studies have suggested that the protective alleles may be associated with higher IFNL3 expression.[2,3,24] The limitations of the evidence to date is that there is little work examining IFNL3 owing to the difficulties in developing assays that do not also detect IFNL2 (the two cytokines share 98.2% sequence idendity).[25] Furthermore, most of the work has concentrated on IFNL levels in whole blood, serum or liver, which are known to be relatively low. We sought to overcome these limitations by designing primers specific for IFNL3 and by examining the immune cells known to produce high levels of IFNL. We did not identify a difference in expression levels between *IFNL3* genotypes in the immune cell subsets isolated.

Some of the limitations of our study include the use of healthy control participants owing to the volumes of blood required for many of the cell separations. This would not be feasible in HCV-infected patients. It is therefore still possible that expression level differences may exist only in specific settings, for example pDCs within the liver of HCV-infected patients that have been primed and activated by chronic infection, or in the context of IFNA therapy. Another limitation of this study is that we focus primarily on mRNA expression of *IFNL3*. Ideally, results would be validated with protein expression. Owing to the sequence homology and low level expression, however, assays for IFNL3 are of

only limited value. Finally, a recent publication has detected a variant upstream of *IFNL3* associated with a novel gene, *IFNL4*, which is associated with impaired clearance of HCV.[26] However, *IFNL4* has only been detected (transiently), in polyI:C-stimulated primary human hepatocytes, and its role of innate immune cells has not yet been determined.

In conclusion, our results demonstrate that *IFNL3* is more highly expressed in unstimulated immune cell subsets than in HCV-infected liver biopsies, and PBMCs, monocytes and pDCs all respond to IFNL3. *IFNLR1* expression in immune cells is comparable to HCV-infected liver biopsies. Considering that pDCs and blood effector cells up-regulate ISGs in response to IFNL3, we propose that the IFNL3 effect on HCV viral clearance is mediated via blood immune cells migrating to the liver after they have been activated in an IFNL3-dependent manner. Thus, innate immune cells should be the focus of future work to determine the functional significance of the *IFNL3* haplotypes.

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Conflict of interest

The authors do not have any potential conflicts of interest to declare.

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