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REVIEW

Gene–disease association with human *IFNL* locus polymorphisms extends beyond hepatitis C virus infections

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Interferon (IFN) lambda (IFN-λ or type III IFN) gene polymorphisms were discovered in the year 2009 to have a strong association with spontaneous and treatment-induced clearance of hepatitis C virus (HCV) infection in human hosts. This landmark discovery also brought renewed interest in type III IFN biology. After more than half a decade since this discovery, we now have reports that show that genetic association of *IFNL* gene polymorphisms in humans is not limited only to HCV infections but extends beyond, to include varied diseases such as non-alcoholic fatty liver disease, allergy and several other viral diseases including that caused by the human immunodeficiency virus. Notably, all these conditions have strong involvement of host innate immune responses. After the discovery of a deletion polymorphism that leads to the expression of a functional IFN-λ4 as the prime 'functional' variant, the relevance of other polymorphisms regulating the expression of IFN-λ3 is in doubt. Herein, I seek to critically address these issues and review the current literature to provide a framework to help further understanding of IFN-λ biology.

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INTRODUCTION

In the year 2003, two different groups reported on the presence of three novel genes closely placed to each other, on human chromosome 19 that coded for interferons (IFNs) with potent antiviral properties.[1,2] These genes due to their relatedness to the interleukin 10 (IL-10) family were initially christened *IL-28A*, *IL-28B* and *IL-29*, and subsequently changed to *IFNL2*, *IFNL3* and *IFNL1*.[3] The genes encode IFNλ-2, IFNL-λ3 and IFNL-λ1, respectively; together with the newly discovered IFNL-λ4 (or IFNL4) they constitute the type III IFNs or the lambda IFNs (IFNL-λs). IFNL-λ1, 2 and 3 activate antiviral responses through the JAK–STAT (janus kinase–signal transducer and activator of transcription) pathway by utilizing a distinct receptor complex made of a heterodimer formed between IFN-λR1 and IL-10R2.[2,3] Subsequent studies showed that unlike the receptors that bind to type I IFNs, the IFN-λ receptors were expressed on selective cell types mainly of epithelial origin, hepatocytes and some immune cells.[3–6] The discovery of IFN-λs was seminal in the sense that it showed the presence of an alternate system to the well-known type I IFNs (IFN-α and IFN-β) that the different nucleated cells in the body, especially the ones on epithelial surfaces, can utilize to combat viral infections. Later studies in mice have shown that type III IFNs form a strong barrier at the host–environment interface, which encompasses large regions of epithelial lining to the respiratory, gastrointestinal and urogenital tracts of mammals.[7–10]

A major boost in the area of IFN-λ research came after another discovery in the year 2009. Three independent groups conducted genome-wide association studies (GWASs) involving treatment response to chronic hepatitis C virus (HCV) infections, in three different geographical regions of the world, and reported that single-nucleotide polymorphisms (SNPs) in the *IFNL* locus (Figure 1), had strong association with treatment-induced HCV clearance irrespective of ethnicity and geographical location of the hosts.[11–13] The search for a 'causative' or a 'functional' SNP at the *IFNL* locus that could give a biological explanation for the HCV-GWAS results was taken up rigorously by several groups, but none seem to have given a better explanation to the HCV-IFN-λ 'puzzle' than the group from the National Institutes of Health, USA, that discovered the presence of another *IFNL* upstream to *IFNL3*, named as *IFNL4* (refs 14,15; Figure 1; in Figure 1b, the alleles for the respective SNPs are shown as beneficial (B) or non-beneficial (NB) with respect to the studies on HCV (reviewed in ref. 15; the major allele for each of the SNPs depicted in Figure 1b has been shown to be the beneficial one in HCV infections). Functional IFN-λ4 is expressed only in a subset of individuals, due to a frameshift-causing deletion polymorphism (*IFNL4-ΔG;* rs368234815) in the first exon of *IFNL4* (Figure 1).[14] The presence of the alternate allele (*IFNL4-TT)* renders *IFNL4* a pseudogene and this allele is seen in ~50% of the European population and in most of the east Asian population, but *IFNL4* is a functional gene (*IFNL4-ΔG* allele) in majority (~95%) of the African population,[16] suggesting that human evolution has played an active role in elimination of a functional IFN-λ4 in the human species.[17] In fact, the pseudogene shows strong positive selection in human evolution, whereas the functional gene is conserved in other mammalian species except in mice and rats where the gene is completely absent.[17]

In this article, I review the literature on genetic association studies that have shown the involvement of the HCV-GWAS SNPs in non-HCV disorders that involve both viral diseases and some non-infectious conditions. I also update the progress on transcriptional studies of the *IFNL4* gene and examine whether the functional IFN-λ4-generating SNP is sufficient to explain the molecular mechanism of causality in the diseases it is associated with, and whether the other *IFNL* locus SNPs (mainly the ones

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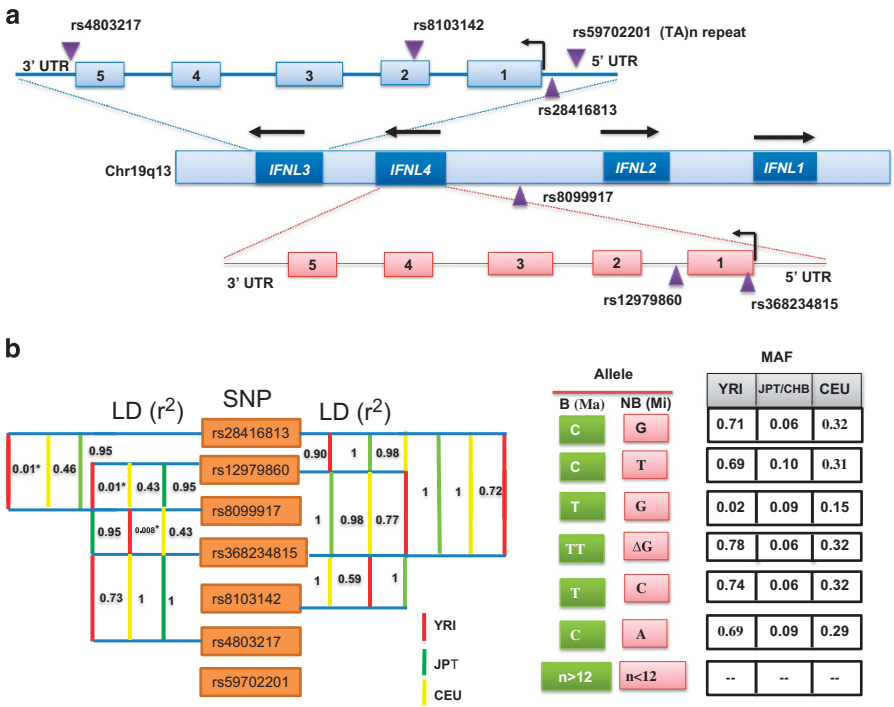

Figure 1. Gene structure at the *IFNL* locus. (**a**) The SNPs that will be discussed in the text are shown. Arrows indicate the direction of the open reading frames of genes. UTR, untranslated region. (**b**) The linkage disequilibrium (LD; r^2) values and minor allele frequency (MAF) are shown for the SNPs depicted in **a**. The alleles for the respective SNPs are shown as beneficial (B) or non-beneficial (NB) with respect to the studies on HCV (reviewed in ref. 15). The minor allele (Mi) is the non-beneficial allele (that leads to expression of a functional IFN-λ4 and also lowers expression of IFN-λ3) and the major allele (Ma) is the beneficial allele (that does not produce a functional IFN-λ4 and is associated with higher levels of expression of IFN-λ3) for all SNPs, again with respect to HCV studies.[15] The information on LD values has been obtained from 1000 Genomes Project reference panel (http://www.1000genomes.org), October 2014 release; some LD (r^2) values for YRI (marked with *) were obtained from ref. 14. The MAF values were obtained from dbSNP (National Center for Biotechnology Information). In Asian population, MAFs of rs12979860, rs8099917 and rs4803217 are from JPT population; and MAFs of rs368234815, rs8103142 and rs28416813 are from CHB-JPT populations. YRI, Yoruba in Ibadan, Nigeria; CEU, residents with ancestry from northern and western Europe; CHB/JPT, Han Chinese in Beijing, China/Japanese in Tokyo, Japan; —, MAF information not available for the TA repeat polymorphism rs59702201.

regulating IFN-λ3 expression) may have any functional roles to play in the observed phenotypes.

GENETIC ASSOCIATION OF *IFNL* LOCUS POLYMORPHISMS IS NOT LIMITED TO HCV INFECTIONS: INNATE IMMUNITY IS THE KEY

IFNL-λs and innate immunity against viruses

Innate and adaptive immunity are the two indispensable arms of the mammalian immune system. Although we had a clearer understanding of the principles of functioning of the adaptive immunity arm, a lack of advanced molecular techniques and incomplete understanding of molecular mechanisms made us remain unaware of the intricacies of functioning of the innate immunity arm, for a long time.[18] With the advent of superior molecular biology techniques and the discovery of the pathogen-associated molecular pattern (PAMP) or pattern recognition receptors,[19] we now have better understanding of how nucleated cells can differentially recognize different classes of pathogens and propagate signals to their surroundings, in the process raising the immediate alarm in the host.[19] Large strides were made in the area of molecular recognition of viral PAMPs and signal transduction that leads to raising of antiviral states within virus-infected cells.[10,20] The epithelial cells, being at the interface between the host and the environment in the respiratory, gastrointestinal and the urogenital tract, are not only prone to a variety of viral infections but are strategically located to respond and propagate alarm signals to the underlying immune cells (Figure 2). However, the primary function of the epithelium is to provide a physical barrier between the underlying lamina propria and the lumen of the cavity or the exterior. Even though they can sense and respond to PAMPs and damage-associated molecular patterns,[21] the epithelial cells are not professional immune cells and due to their high level of differentiation, may lack the plasticity required to send out amplified and prolonged signals to the lamina propria. Therefore, a crucial link still remained missing about how an adaptive immune response is shaped within distantly located lymph nodes that have obligatory 'immune-rich environments', by taking cues from signals generated by viral infections at the epithelium (Figure 2).

The discovery of the new class of effector immune cells called the innate lymphoid cells (ILCs) may seem to have provided the answer to this puzzle (Figure 2). ILCs are derivatives of common lymphoid precursors along with T and B cells.[22] These cells are stationed near the epithelial surfaces in larger numbers and respond to signals from the surrounding cells by secreting cytokines and chemokines in large quantities, thereby acting as signal amplifiers in both health and disease.[23] The ILCs are considered the innate immunity counterparts to the various Th (T helper) cell (CD4+ (cluster of differentiation)) subsets of the adaptive immune system (Figure 2; ref. 24). For example, ILC2s respond to IL-33 generated from influenza virus-infected epithelial cells by secreting IL-5 and IL-13, both known inducers of Th2 immunity.[25] The natural killer cells, now classified as ILC1 cells, are considered the Tc (T cytotoxic) cell (CD8+) counterparts.[24] Even though most studies so far on ILCs have been on mice, ILCs have also been characterized in a variety of human tissues[26] and are deregulated in many human diseases.[27] With emerging roles of type III IFNs at the epithelium,[8,10,28] ILCs and IFN-λs now seem to be the major players in innate immunity at barrier surfaces.[10,29]

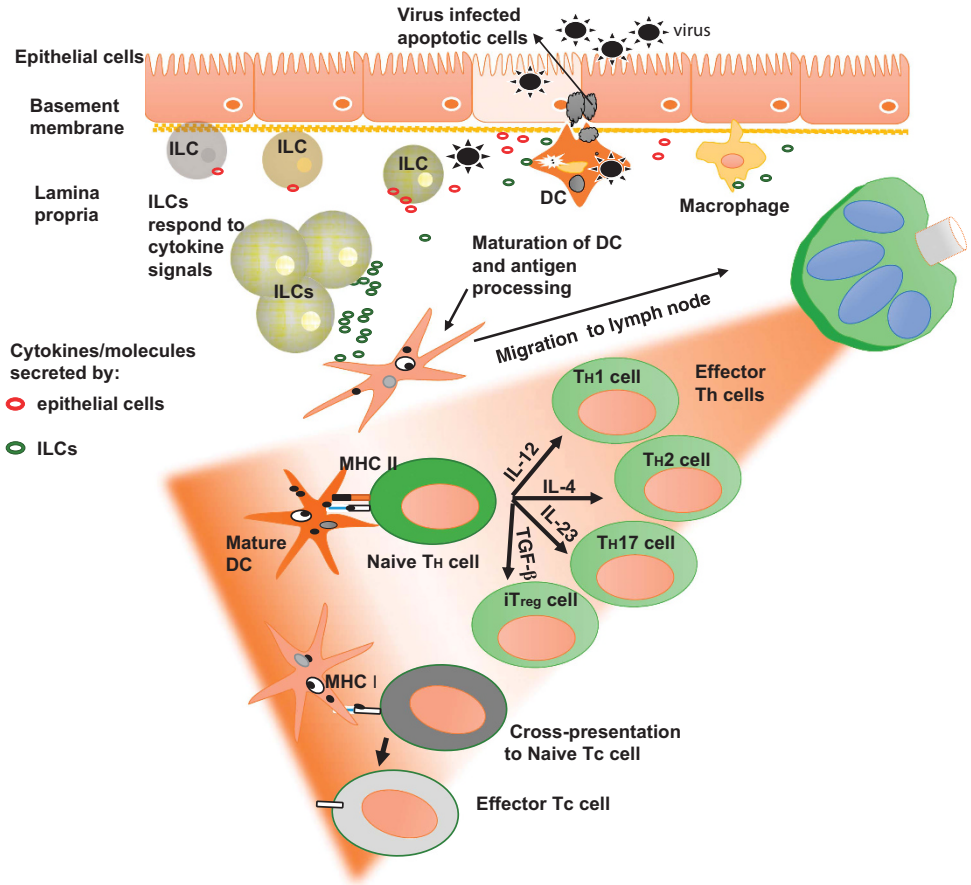

Figure 2. Immune responses to viral infections of the epithelia. Resident macrophages and dendritic cells (DCs) form the first line of cellular resistance to invading viruses. The newly discovered innate lymphoid cells (ILCs)[22] may have important roles due to their ability to respond to signaling molecules/alarmins secreted by the epithelial cells. ILCs diversify and respond by secreting large amounts of effector cytokines and chemokines locally. These effector molecules can potentially lead to the polarization of DCs and macrophages, and therefore may be critical in shaping the adaptive immunity mediated by T-helper (Th) and T-cytotoxic (Tc) cells that develop in the nearby lymphatic tissue (green lobed structure). MHC, major histocompatability complex.

How ILCs respond and interact with the epithelial cells during viral infections and how they cross-talk with other immune cells at the barrier surfaces in shaping and maintaining the optimal Th responses, will form the next exciting wave in innate immunity research.

Similarly, research on the production, regulation and functions of IFN-λs in viral infections has also been exciting in the last decade. Human IFN-λs are secreted (except IFN-λ4 that is poorly secreted[30]) in response to the detection of viral RNA intermediates from the cytoplasm of epithelial cells via the toll-like receptor and retinoic acid inducible gene I-like receptor) pathways.[4,7,31] IFN-λs are also known to be secreted by macrophages, plasmacytoid dendritic cells, monocyte-derived dendritic cells and hepatocytes.[4,6,32–34] The IFN-λR1 receptor is expressed on limited cell types including epithelial cells, hepatocytes, B cells and monocytes.[5,35] There is currently no information on whether the newly discovered ILCs secrete any of the IFN-λs and whether they express IFN-λR1. IFN-λs act in a paracrine and/or autocrine manner to raise an antiviral state in the infected and to-be-infected cells by reprogramming the target cell gene expression patterns.[28,36] The *IFNL* SNPs would have functional roles if they can affect the diversification of innate and adaptive immune cell subsets. Diversification of ILC subsets will lead to the polarization of dendritic cells and macrophages and will eventually influence the Th1/Th2 balance by favoring either a Th1 or a Th2 response (Figure 3).[37] Although there is no evidence for this belief, it is known that the *IFNL* SNPs do affect the expression of IFN-λ3 (ref. 15) and that they give rise to a new IFN (IFN-λ4).[14] IFN-λ1, 2 and 3 are known to deflect the Th1/Th2 balance to a Th1 predominant mode *in vitro* and also *in vivo* in humans and mice[38,39] (reviewed in ref. 37). No such studies are reported for IFN-λ4. One hypothesis is that different levels of IFN-λ3 expression dictated by the underlying genetic polymorphisms are responsible for eliciting a Th1 or a Th2 response.[37] The role played by IFN-λ4 in this process is only speculative at this stage (Figure 3). How IFN-λs interact with the newly discovered ILCs is also unknown. A recent report showed that rotavirus infection in mice is controlled by IL-22 produced by ILC3s for which the presence of an intact IFN-λ signaling pathway is required.[40] An even more important question in humans will be on what role does the *IFNL* SNPs, and therefore IFN-λ4, play in the diversification and functioning of ILCs.

IFN-λ4 apart from being antiviral to HCV[14] is also known to inhibit other flaviviruses such as dengue virus, yellow fever virus[41] and human corona viruses.[30] Some of the other IFN-λs are known to be induced by several human pathogens including *M. tuberculosis*,[42] human papilloma virus,[43] influenza virus[44] and human metapneumovirus (also induces IFN-λ4),[45] and have clear antiviral activities against some but not other viruses in mice (reviewed in ref. 46). In a recent finding, murine IFN-λ3 but not IFN-α/IFN-β was responsible for protecting mice against norovirus persistence in mice colon, strikingly, even in the absence of T and B lymphocytes.[9] Similar results were seen in reovirus infections of mice colon.[8] In conclusion, IFN-λs are potent antiviral molecules and their cross-talk with innate immune cells can potentially

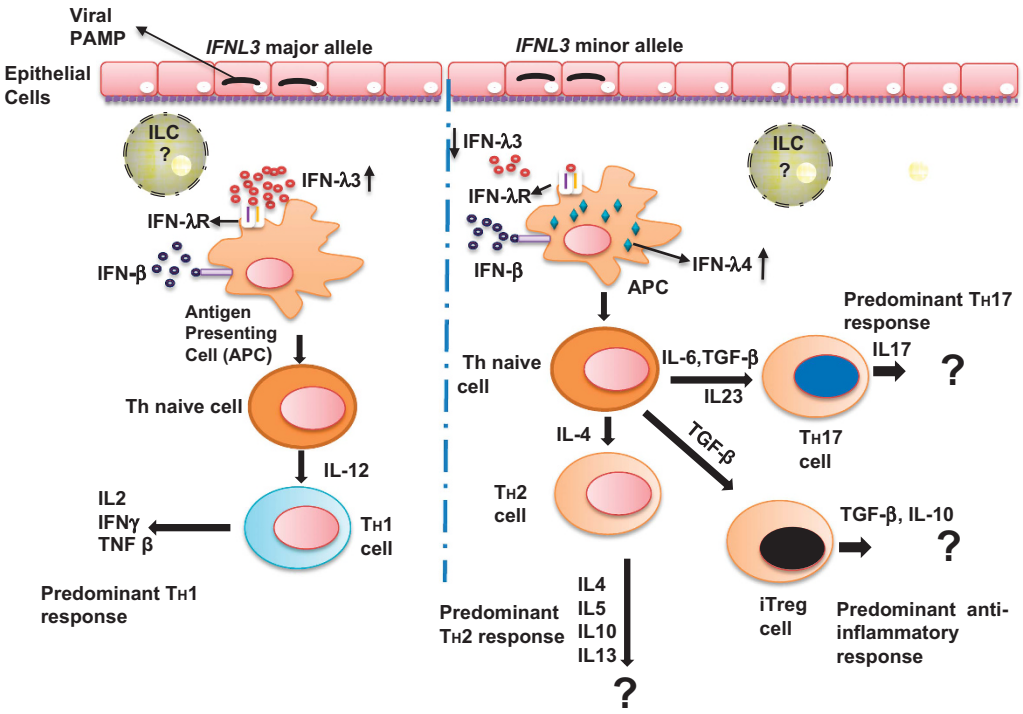

Figure 3. *IFNL* SNPs influence Th1/Th2 responses by affecting expression of IFN-λ3 and IFN-λ4. The model is based on the hypothesis proposed by Egli *et al.*[37] Although IFN-λ3 is known to upregulate Th1 responses and simultaneously downregulate Th2 responses,[86] the role played by IFN-λ4 is unknown. As the SNPs that can potentially affect IFN-λ3 expression (rs59702201, rs28416813 and rs4803217; reviewed in ref. 15) are in strong LD with rs368234815 that generates a functional IFN-λ4, lower levels of IFN-λ3 expression (caused by minor/non-beneficial alleles) will be accompanied by expression of a biologically active IFN-λ4, and therefore should not favor a predominant Th1 response. However, experimental proof is lacking for the role of IFN-λ4 in affecting the Th1/Th2 balance (denoted as ?). The role of the newly discovered innate lymphoid cells (ILCs)[22–24] may be critical in the pathway. IL, interleukin; PAMP, pathogen-associated molecular patterns; TGF, transforming growth factor. The APCs in a viral infection are predominantly dendritic cells (DCs).

orchestrate innate immunity against several viruses and they may be particularly important at the barrier surfaces. IFN-λs also modulate adaptive immunity by affecting Th1/Th2 balance, tilting it to a Th1-favoring response that is required for clearing viral infections. The function of the newly discovered IFN-λ4 in these immune processes remains to be determined.

Association of IFNL locus polymorphisms with human diseases

In cognizance of the potential that IFN-λs may hold in innate immunity, researchers across the world have got intrigued with the *IFNL* SNPs and have started to test them in candidate gene case–control studies in both infectious and non-infectious diseases. So far, association has been reported with non-alcoholic fatty liver disease (NAFLD), allergy and infections with several viruses.

Cytomegalovirus. Cytomegalovirus (CMV) is a herpes virus that infects plasmacytoid dendritic cells and epithelial cells in humans causing chronic infections and is especially a problem in immunosuppresed individuals. Egli *et al.*[47] tested the association of the *IFNL* SNP rs8099917 with CMV replication in a small number of solid organ transplant patients ($n = 38$) who were seronegative for CMV (but received an organ transplant from a CMV-seropositive donor) and who had stopped receiving antiviral prophylaxis. They found that the minor allele at rs8099917 (G) was associated with decreased CMV replication ($P = 0.036$) in a dominant model of inheritance. Further, their *in vitro* studies provided evidence for a beneficial effect of the minor allele against CMV replication.[47] A study in CMV-seropositive kidney transplant patients[48] also found that the minor allele (T) at rs12979860 had a dominant beneficial effect against CMV replication. Although in another study in allogenic stem cell transplant recipients,[49] the minor allele T at rs12979860 protected recipients against CMV infection in a recessive model of inheritance.

Two more studies have also reported on the association of *IFNL* SNPs with CMV.[50,51] The first report by Bibert *et al.*[50] tested for the occurrence of CMV retinitis among those human immunodeficiency virus (HIV)-infected patients who were at risk of developing CMV retinitis due to low CD4 counts and CMV seropositivity. They found that carriers of two copies of the minor allele (ΔG/ΔG), increased the risk of getting CMV retinitis ($P = 0.007$) in multivariate regression models. In the second report by Manuel *et al.*,[51] solid organ transplant patients were tested for the association of rs368234815 with cumulative incidence of CMV replication. The results showed that the minor allele homozygotes (ΔG/ΔG), only in the pre-emptive antiviral therapy group but not among those receiving antiviral prophylaxis, had higher incidence of CMV replication. These results are indeed very interesting and sound, but the paradoxical findings of the five groups in terms of the model of inheritance of *IFNL* SNPs raise some questions. Although two groups showed that their results fit best with a recessive model of inheritance of *IFNL* SNPs[50,51] in affecting CMV replication/retinitis wherein minor homozygosity was non-beneficial, the other three studies show an opposite trend where the minor allele had a beneficial effect against CMV replication in both patients and cell culture experiments, involving either dominant[47,48] or recessive models of inheritance.[49] In stark contrast, a dominant model of inheritance (of the non-beneficial *IFNL* SNP minor allele) has consistently given the best explanation on the observed phenotypes in association studies with both spontaneous clearance and IFN-based treatment response in chronic HCV

infections.[11–13,15] This discrepancy within the CMV studies may be partly due to statistical fluctuations owing to low sample sizes,[47,50] and high heterogeneity among patient groups, end points and antiviral regimens that are followed in the different studies. The discrepancy needs be resolved by well-designed replication studies to gain a proper understanding of the role of *IFNL* SNPs in CMV replication and disease.

Human T-lymphotrophic virus. Human T-lymphotrophic virus (HTLV)-1 is an ancient retrovirus causing chronic infections in humans and is associated with adult T-cell leukemia/lymphoma. It is also associated with inflammatory disorders such as HTLV-1-associated myelopathy/tropical spastic papaparesis (HAM/TSP), HTLV-associated arthropathy and several other related disorders including rheumatoid arthritis. A Spanish study reported for the first time that an *IFNL* SNP (rs12979860) was associated with HAM/TSP in a small number of patients ($n = 41$) who had HTLV-1 proviral DNA in their blood cells.[52] They showed that the presence of the minor allele made 12/41 patients to have a sixfold higher risk ($P = 0.03$) of having symptoms of HAM/TSP compared with asymptomatic carriers ($n = 29$) in a dominant model of inheritance. However, proviral DNA load was a confounder in this association; covariate analysis suggested that both the SNP and proviral DNA load were linked in their association with HAM/TSP. The minor allele-carrying genotypes (CT and TT) were indeed having higher proviral DNA in their blood cells compared with the major homozygotes ($P = 0.01$). The major drawback of this study seems to be the low sample number; although the strength of the study was that there was significant effect of the minor allele on proviral DNA copy number, a functional association that could be directly linked with the antiviral role of IFN-λ3. It is known that the *IFNL* SNP minor alleles are associated with decreased expression of IFN-λ3 (reviewed in ref. 15, although an opposite effect is evident in chronic HCV infections, where the minor allele carriers have low baseline virus levels[11]). In a later study from Brazil,[53] both rs8099917 and rs12979860 were tested in a cohort consisting of 229 HTLV-positive subjects (93 HAM/TSP patient and 136 asymptomatic carriers). The minor allele G of rs8099917 was significantly associated with HAM/TSP in both univariate and multivariate analysis with a recessive model of inheritance ($P < 0.001$). In this study, the proviral DNA load as a covariate did not seem to interfere with the association of rs8099917 with HAM/TSP unlike the previous Spanish study.[52] With respect to rs12979860, the minor allele T was associated with HAM/TSP only as a heterozygote in univariate analysis ($P = 0.01$) and weakly in multivariate analysis ($P = 0.06$).

However, a series of studies have also reported conflicting results to the above two reports on the association of *IFNL* SNPs with HTLV-1-associated diseases. First, Sanabani *et al.*[54] show clearly a lack of association of *IFNL* SNP rs12979860 with HAM/TSP and/or adult T-cell leukemia/lymphoma. This Brazilian study had more number of samples ($n = 112$) than the Spanish study of Trevino *et al.* ($n = 41$).[52] They also did not find any correlation of proviral DNA load with rs12979860 genotypes. Another report from Brazil with a sample size of 79 also reflected similar findings, where they found no association of rs12979860 with HAM/TSP and proviral DNA load.[55] This study also compared 300 healthy controls with 79 HTLV-1-positive subjects and found no association with rs12979860 and HTLV-1 infection. Yet another recent study from Brazil analyzed the genotypes at rs8099917, rs12979860 and rs8103142 in 300 healthy controls and 96 HTLV-1-infected individuals, and found no association with HTLV-associated arthropathy and any of the three SNPs when tested individually.[56] When they carried out haplotype analysis, they found some association ($P = 0.01$) with HTLV-1 infection and one of the seven haplotypes (CCT) involving the three SNPs (rs8099917, rs12979860 and rs8103142); with HTLV-associated arthropathy and another haplotype (TTG; $P = 0.05$). They also found an association of the three SNPs individually with levels of some cytokines (such as IFN-γ) and proviral DNA load ($P < 0.05$).[56] However, no multiple testing corrections seem to have been carried out in their analysis, thus severely undermining the results.[56] Further, a Japanese study also failed to see any association with adult T-cell leukemia/lymphoma and rs8099917 and also with HTLV-1 and HCV mono- or co-infections.[57] Last, a study from France on 95 HTLV-1-positive subjects of Afro-Carribean lineage compared the distribution of genotypes of rs12979860 and the IFN-λ4-generating SNP rs368234815, and found no association with HAM/TSP.[58] In summary, the results so far are not entirely convincing on a true association of the *IFNL* SNPs with HTLV infection-related diseases. Further, in the two reports[52,53] that did see an association, the models of inheritance used to fit the phenotype data do not agree with each other, raising doubts on the underlying functionality of the observed genetic associations. Therefore, more functional characterization of the observed association may be needed to rule out false positivity.

Hepatitis B virus. If at all there is another human disease where *IFNL* SNPs were expected to have associations as strong as that of HCV infections, it was the case of hepatitis B virus (HBV). This expectation is because both viruses are hepatotropic and cause chronic infections; both diseases can be effectively treated using IFN-α even though they differ in their PAMP ligands recognized by innate immune receptors.[59,60] Mixed results have been obtained about the role of *IFNL* SNPs in both spontaneous clearance and IFN-α-induced clearance of HBV and the literature until the year 2013–2014 has been reviewed elsewhere.[61–63] More studies have also been conducted since then (results from 10 of them are summarized in Supplementary Table 1), but have largely failed to resolve the conflict.

Two studies have also reported on a lack of association of *IFNL* SNPs on spontaneous as well as IFN-induced clearance of hepatitis D virus, a co-infecting satellite virus that requires HBV for replication and assembly.[64,65] Although consistent results were obtained across numerous studies with HCV–*IFNL* SNP association mainly because they had only virological end point phenotypes[15] that correlated well with serological and biochemical parameters of the infection, several drawbacks in case of HBV studies may be responsible for the inconsistent results. Some of them are: presence of different HBV genotypes as mixed infections, with some genotypes (genotype D[62]) showing better association than others; variation in treatment regimens (IFN alone or with nucleotide analogs); end point phenotypes varying from serology to viral load, to biochemical parameters without a common quantitative parameter to assess the phenotype; prolonged clinical course of the disease with fluctuating virological, serological and biochemical markers; and prevalence rates of disease differing in different ethnicities/populations, to name a few. The reports that have shown an association of *IFNL* SNPs with HBV spontaneous or treatment-induced clearance are largely in agreement with the results from HCV studies in terms of the model of inheritance (Supplementary Table 1). Although the conflicting data so far on HBV-*IFNL* SNP association do lead to doubts about its true positive nature, the data are also not fully supportive of the notion that the association may be false positive. In fact it has been argued that if properly assessed, the association between HBV disease progression and *IFNL* SNPs could have clinical value.[62] But it appears that the effect of the *IFNL* SNPs on HBV persistence and/or progression within the human host is highly variable; it may involve more complex interactions with other variables and genes than was observed in chronic HCV infections.

Human immunodeficiency virus. Another important viral disease that has been tested for the influence of *IFNL* SNPs is acquired

immunodeficiency syndrome (AIDS) caused by HIV. Particularly, it was of interest to see how the *IFNL* SNP beneficial alleles are distributed in a unique group of HIV-infected individuals that can suppress the virus from replicating to high levels (defined as elite controllers/suppressors or natural viral suppressors) and defer the progression to AIDS (called as long-term non-progressors) without any antiretroviral therapy; and another unique group that remains HIV-seronegative despite being at high risk for infection due to intravenous drug use (highly exposed seronegative or exposed seronegative)). More interestingly, the natural viral suppressor patients have also been known to efficiently clear HCV in HCV-HIV co-infections, compared with controls in both African Americans[66] and Caucasians.[67]

A few reports have come up in this area, some showing no correlation of HIV infection/disease progression to the *IFNL* SNPs, whereas others see clear association.[68–74] One of the earlier reports tested the association of rs12979860 in 291 high-risk seronegative and 1221 HIV-positive subjects comprising both white and black subjects in the USA.[68] No association was evident for either HIV positivity or for disease progression within the infected subjects. In another study reported by Rallon *et al*.,[69] the association of rs12979860 was tested in two different cohorts. The first comprised of 30 long-term non-progressors and 38 typical progressors to AIDS; the second included 29 exposed seronegative and 29 HIV-positive partners. Thus, the study tested both HIV progression and protection, although in a small number of patients. No significant difference in distribution of genotypes between cases and controls was evident in both cohorts, although the beneficial CC genotype was more frequent in the exposed seronegative patients compared with HIV-positive patients (62% vs 45%), suggesting that there may be a protective role for the CC genotype against HIV that was not detected in the study, likely due to inadequate power. Another study from the USA tested whether the beneficial CC genotype at rs12979860 was over-represented in 25 African-American elite controllers/suppressors compared with HIV-infected patients with high viral loads, and found no statistically significant difference.[70] Confirming this report, Sajadi *et al.*[71] also found that CC genotype (for rs12979860) was not significantly over-represented in 48 natural viral suppressors of African-American origin compared with HIV-positive ($n = 124$) and -negative controls ($n = 173$).

Three reports published subsequently have seen association with rs12979860 and rs368234815, and spontaneous control of HIV and/or AIDS progression.[72–74] Interestingly, all three studies were carried out on whites/Caucasians, whereas the reports that failed to see an association described above were mostly carried out on African Americans (except that by Rallon *et al*[69] that used white subjects) or mixed group of blacks and whites.[68] First, Machmach *et al.*[72] found an association of rs12979860 with spontaneous HIV control when tested on 53 white natural viral suppressors and 389 matched non-controllers at $P = 0.02$ after correcting for occurrence of HLA-B57 (human leukocyte antigen) protective alleles (which also have independent association with HIV and HCV spontaneous clearance) and gender, in multivariate analysis. Second, Machmach *et al.*[73] confirmed and extended their results in another report, where they found the association of rs368234815 with AIDS progression. Last, a recent study carried out on a well-characterized Spanish white cohort of HCV-seropositive men exposed to HIV infection through shared needles shows a clear association of IFN-λ4-generating rs368234815 with HIV positivity.[74] The study had 213 men who were HIV seropositive and 188 were highly exposed seronegative. They found that the protective TT/TT genotype was over-represented in the highly exposed seronegative group (0.49 vs 0.41; $P = 0.006$). Further, this association had no interaction with the HIV-protective CCR5 (C–C chemokine receptor type 5) deletion ($P = 0.7$). Interestingly, all three studies that found an association show data that the non-beneficial minor alleles are following a recessive model of inheritance,[72–74] suggesting a common underlying functionality in the observed genetic association between the three studies. This is however different from the dominant model of inheritance seen in HCV studies,[11–13,15] suggesting that the two viruses may have different interactions with IFN-λ-driven immune responses. However, unlike in the case of CMV studies discussed above,[47–51] all three HIV studies[72–74] show that the minor alleles are non-beneficial, similar to the observations in chronic HCV infections.[15] In summary, it is evident that *IFNL* SNPs do associate with HIV replication and disease progression, even though in an ethnicity-specific manner. It appears that the beneficial alleles of *IFNL* SNPs have protective roles in whites/Caucasians. In African Americans, more studies with the functional IFN-λ4-generating SNP rs368234815 rather than rs12979860 will reveal any true association. This is especially true as the two SNPs are not in strong linkage disequilibrium (LD) in this population (Figure 1b).

Herpes simplex virus. Herpes simplex virus resides inside the human host latently in sensory neurons, but gets reactivated due to the altered host immunity and replicates efficiently in epithelial cells causing genital or oral lesions (the latter is referred to as 'cold sores'). An association of rs12979860 with the recurrence and severity of herpes simplex virus-1-induced 'cold sores' was reported from a study on a small number of individuals ($n = 57$).[75] A recent report carried out on a large number of individuals ($n = 2192$ for genital herpes; $n = 1511$ for oral herpes) clearly found no association of rs368234815 with recurrence of genital or oral herpes episodes.[76] Among other phenotypes, the latter report ruled out an effect of the SNP on 'frequency of recurrence' of oral herpes, it however did not rule out an effect of the SNP on severity of infection.

Non-infectious diseases and miscellaneous conditions. *IFNL* SNPs have also been tried as candidate gene SNPs in some non-infectious inflammatory diseases. The association of *IFNL* SNPs with NAFLD was earlier reported by Petta *et al*.[77] This study with 160 subjects identified an independent association with the CC genotype at rs12979860 and the severity of lobular inflammation (CC genotype positively correlated with severity of inflammation) in a cohort of patients with NAFLD ($P = < 0.001$). The study was conducted taking lead from previous reports of increased hepatic necroinflammation in HCV patients with the CC genotype at rs12979860.[78] Another report showed no such association in 195 Caucasian biopsy-confirmed NAFLD patients.[79] However, all the patients in this cohort were obese (with body mass index > 30), whereas in the previous study only 40% were obese. After the latter report, Petta *et al.*[80] revisited their data and indeed found an association with the *IFNL* SNP only with their non-obese patients ($n = 94$; $P = 0 < 0.001$) but not with the obese patients ($n = 66$; $P = 0.13$). These initial doubts seem to have been resolved with a recent report by Eslam *et al.*[81] who worked on a relatively large number of NAFLD patients ($n = 488$) and confirmed the association of the CC genotype at rs12979860 with increased severity of liver inflammation and fibrosis ($P = < 0.0001$). They also found a similar strong association with hepatic inflammation and fibrosis in separate cohorts of viral hepatitis C ($n = 3129$) and B ($n = 555$), strongly suggesting that even though the major allele genotype CC is beneficial against HCV and HBV, it is also responsible for excess inflammation of the liver in viral and non-viral hepatitis. All four studies[77–81] report on dominant models of inheritance for the minor alleles; but unlike in the context of chronic HCV infections,[11–13] the minor alleles in NAFLD are beneficial rather than being non-beneficial, as they are responsible for less severe hepatic inflammation.

Extending the potential role for *IFNL* SNPs in inflammatory diseases, there is also a recent report that showed association of rs12979860 and allergy in children aged < 5 years.[82] Although the

study involved a small sample size (non-allergic, $n = 35$; allergic cohort 1, $n = 35$; food allergy cohort 2, $n = 30$), a large effect size (odds ratio = 4.56, $P = 0.004$, for cohort 1) was seen wherein the non-beneficial T allele at rs12979860 was over-represented in the allergy group in a dominant model of inheritance. Another interesting finding in this study was that the effect of the *IFNL* SNP was more pronounced in females than males. This report suggests that the non-beneficial *IFNL* SNP minor allele-carrying genotypes may predispose children to an allergic phenotype by skewing the Th1/Th2 balance to a Th2 predominant one. This extrapolation is also based on the findings from a recent study on mice model of allergic asthma, which showed that IFN-λ2 was able to rescue mice from allergy by suppressing Th2 cytokines.[39] These conclusions would also suggest a role for *IFNL* SNPs in immune response to respiratory viral infections, which account for disease exacerbations in conditions such as asthma.[83] A study carried out on infants with respiratory syncytial virus-induced bronchiolitis shows no association of r12979860 and rs8099917 with viral load or other clinical features.[84] Interestingly, the non-beneficial TT genotype of rs12979860 was associated with early age of hospitalization in the infants ($P = 0.005$). More studies are awaited on the association of *IFNL* SNPs with allergy and allergy-related diseases. In the autoimmune disorder multiple sclerosis, rs8099917 and rs12979860 did not show any association with IFN-β treatment.[85]

As evidence for a potential role of IFN-λs and *IFNL* SNPs in adaptive immune responses, a study has linked *IFNL* SNPs with development of effective vaccine response against human influenza virus in immunosuppressed individuals.[86] The study shows that minor allele (G)-carrying individuals at rs8099917 show better vaccine responses (odds ratio = 1.99; $P = 0.038$) in a dominant model of inheritance, and that T cells from minor allele-carrying individuals produce more IL-4 (a Th2 cytokine). This study also showed that recombinant IFN-λ3 increased Th1 responses from human peripheral blood mononuclear cells while inhibiting Th2 responses (Th2 cytokines are needed for effective seroconversion).[86]

TRANSCRIPTION STUDIES ON THE *IFNL4* GENE

Studies have accumulated evidence on the different transcription factors (TFs) that bind and drive transcription from all four *IFNL* genes. Roles for several virus-inducible TFs such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), IFN regulatory factor-3 and IFN regulatory factor-7 have been defined for *IFNL1*, *2* and *3* genes, and have been reviewed elsewhere.[28,87,88] We recently showed that apart from these three TFs, specificity protein 1 (a GC-rich DNA-binding TF) also has a role in driving transcription from the *IFNL4* promoter in A549 cells[89] (Figure 4a). A CpG island of ~ 1.5 kb is located upstream of the *IFNL3* gene and overlapping the *IFNL4* gene, and also includes two of the most important *IFNL* SNPs rs12979860 and rs368234815. Epigenetic regulation of the transcriptional activity at the *IFNL* locus is likely to involve this CpG island and needs further exploration.[90,91]

Even though *IFNL4* messenger RNA (mRNA) was shown to be highly expressed from stimulated primary hepatocytes in the original report that described the discovery of *IFNL4*,[14] subsequent studies have not seen robust expression levels of *IFNL4* gene in human samples. For example, Amanzada and others found *IFNL4* transcripts expressed at 4.3–5.6-fold lower levels than *IFNL2/3* mRNA in liver biopsies of HCV-infected patients.[92] In another report, *IFNL4* mRNA was detectable in only in 7/23 and 8/23 patient-derived peripheral blood mononuclear cells that were stimulated or not with IFN-poly(I:C), respectively.[93] All 23 patients carried at least one copy of the functional IFN-λ4-generating allele, ΔG. In the report by Liang and colleagues also, *IFNL4* transcripts were seen in only 33/70 liver biopsies from HCV-infected patients.[94] Furthermore, Lu *et al*.[41] found that only 2% of the transcripts generated from poly(I:C)-stimulated primary hepatocytes derived from two heterozygous (TT/ΔG at rs368234815) individuals, represented functional IFN-λ4 transcripts that originated from the ΔG allele. Similarly, they also found no expression of the full-length functional IFN-λ4 mRNA transcripts from poly(I:C)-stimulated A549 cells. It is possible that the qPCR assays utilized in these studies may be suffering from technical difficulties in dealing with several different mRNA isoforms of *IFNL4* gene.[14] All the four studies described above have used primer sets where at least one of the primers binds to the exon/intron/exon–intron junctions (Figure 4b). Using a primer set that uniquely binds to the 3′-untranslated region of *IFNL4* mRNA, IFN-λ4 induction was seen at levels similar to that of IFN-λ2/3, upon human metapneumovirus infection of A549 cells by Banos-Lara *et al*.[45] We used the same primer set and found similar high expression levels upon poly(I:C)/HCV RNA transfection in A549 cells.[89] Two other studies have reported no such problems in amplifying *IFNL4* transcripts

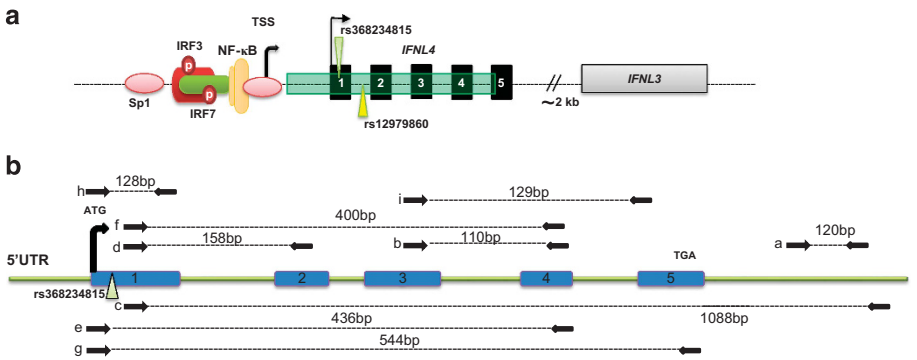

Figure 4. Transcription studies on *IFNL4* gene expression. (**a**) A schematic of the *IFNL4* promoter showing the different transcription factors (TFs) that were recently discovered[89] that are important in driving the expression of *IFNL4* gene. The gene structure of *IFNL4* is shown as having five exons with the positions of the two SNPs also indicated. Tall arrow indicates translation start site. The transparent triangle (green) shows the CpG island (position: chr19:39737690-39739288; band: 19q13.2; CpG count-137, UCSC genome browser, https://genome.ucsc.edu) that covers most of the exonic region and a part of the 5′-UTR (untranslated region) over a length of ~ 1.5 kb. IRF, IFN regulatory factor; P, phosphorylated TFs; TSS, transcription start site. (**b**) Schematic representation of primer-binding sites used in several recent studies to measure the expression of *IFNL4*. A schematic of the *IFNL4* gene locus in chromosome 19q13 is depicted. Position of SNP rs368234815, which creates functional IFN-λ4, is shown. Black standing arrow represents the translational start site (ATG). Primer-binding sites of six different studies (eight primer pairs in total) are shown (a, refs 45,89; b, ref. 94; c, ref. 41; d–g, ref. 93; h, refs 14,96; i, ref. 92). Expected amplicon size of each primer pair is shown as dotted line between two small black arrows. The primer sequences are listed in Supplementary Table 2. TGA, translational stop site.

from liver biopsies.[95,96] Although Honda *et al.*[96] used the allele-specific Taqman assay described earlier,[14] Konishi *et al.* do not provide the primer sequence information that they used in their Taqman assays.[95] The inconsistency in the above reports in detection of *IFNL4* transcripts (either the full-length functional IFN-λ4 isoform or the other isoforms) suggests that RNA-sequencing may be the optimal approach to measure *IFNL4* transcripts. Furthermore, the pre-mRNA splicing mechanism that leads to the expression of different IFN-λ4 isoforms[14] under different stimulation and cell-type conditions needs to be examined.

THE RELEVANCE OF OTHER 'IFN-λ3 FUNCTIONAL SNPS' AFTER THE DISCOVERY OF FUNCTIONAL IFN-λ4-GENERATING SNP

Till date, three functional SNPs have been identified that regulate *IFNL3* gene transcription/translation (referred to as 'IFN-λ3 functional SNPs' in this section). Although rs28416813 is a SNP present at ~37 nucleotides upstream of the start codon of *IFNL3* and is known to affect downstream gene expression by differentially binding to NF-κB,[97] rs4803217 was identified in the 3′-untranslated region region of *IFNL3* (Figure 1a) that affects stability of the mRNA by interfering with AU-rich element decay (AMD).[98] Both these SNPs are in high LD with each other and with rs12979860 and were predicted to be two of the four potential causal SNPs from among the HCV-GWAS hits.[15,99] A recent study defines another mechanism by which rs4803217 affects the stability of RNA by remodeling its secondary structure.[100] Furthermore, a third functional SNP is the TA repeat polymorphism rs59702201, originally reported by the Mizokami group.[101] This SNP located within the proximal promoter affects transcription of *IFNL3*, depending on the number of repeats present.[101] Recent reports have confirmed the significance of this SNP in association studies involving chronic HCV infections.[102–104] Apart from several reports that showed genotype-dependent differences in expression levels of IFN-λ3 (reviewed in ref. 15) recent reports also have confirmed this finding in *ex vivo* and *in vivo* conditions.[94,105] These results suggest that IFN-λ3 expression levels dictated by the alleles present at the three functional SNPs may have a role in the observed phenotype in health and disease.

However, the discovery of the IFN-λ4-generating SNP (rs368234815, referred to as 'IFN-λ4 functional SNP' in this section) has overshadowed the importance of the 'IFN-λ3 functional SNPs', as most of the new reports have chosen to test for the 'IFN-λ4 functional SNP' rather than rs12979860, which is the tag-SNP for the 'IFN-λ3 functional SNPs'.[15] Another SNP within the coding region of *IFNL4* that leads to a non-synonymous mutation in the functional IFN-λ4 protein is thought to be a better marker of association along with rs368234815, in chronic HCV infections.[106] So the question arises: is the IFN-λ4-generating SNP the sole functional SNP or the other SNPs regulating the expression of IFN-λ3 also have independent roles? The answer to this question will be difficult to obtain under *in vivo* conditions due to high LD between these SNPs in most populations that precludes any attempts to assess their independent effects (Figure 1b). A recent report compared the strength of association of rs4803217 and rs368234815 in IFN treatment response in HCV-infected African Americans, who have the lowest reported LD values among all ethnicities between rs368234815 and rs12979860 SNPs (Figure 1b).[107] They found that rs368234815 was more strongly associated with the outcome than rs4803217.[107] In another independent report, Lu *et al.* applied multivariate regression to test the independence of rs368234815 from rs4803217 in predicting response to IFN treatment in HCV patients of African-American decent.[100] They found that correcting for the effect of s368234815 on rs4803217 abolished the latter SNP's association with treatment response, whereas correcting for the effect of the latter SNP reduced the strength of association of the former SNP (from $P = 0.004$ to 0.065).[100] This could have resulted due to the low sample size ($n = 169$); nevertheless, more results are awaited to conclude that IFN-λ4-generating SNP is the sole functional SNP and that the observations giving credence to the functionality of the other 'IFN-λ3 functional SNPs may only be artifacts.

Studies so far point to the functional IFN-λ4-generating SNP rs368234815 as the prime causal variant at the human *IFNL* locus. Therefore, a detailed investigation is needed on the function of IFN-λ4 as both an antiviral cytokine in different viral diseases and as a potential player in diversification and maintenance of innate and adaptive immune cell subsets. In case of IFN treatment response in chronic HCV infections, even though the IFN-λ4 -generating SNP can explain a large amount of variance in the observed phenotypes,[107] questions still remain on its role in spontaneous clearance of HCV. It is known that expression of functional IFN-λ4 is associated with high levels of IFN-stimulated gene (ISG) expression in non-responders who further fail to upregulate their ISG expression upon IFN-α treatment.[15] However, such an elegant explanation is lacking in the case of spontaneous clearance of HCV (Figure 5). Although the majority of studies on spontaneous HCV clearance are with rs12979860 and not rs368234815, the fact that these two SNPs are in high LD in most populations[14] allows us to extrapolate the results. So, how does the expression of a functional IFN-λ4 in patients who get an acute HCV infection will lead them to become chronically infected? One explanation could be that similar to the IFN treatment non-responders group, the subjects expressing functional IFN-λ4 also have higher baseline levels of ISGs, which do not get further upregulated upon acute HCV infection. Having a higher basal innate immune response may have helped those populations with higher frequency of the functional IFN-λ4-generating allele in dealing with prevalent viral infections. Higher baseline levels of ISGs in people who can express a functional IFN-λ4 may be offering protection against excess inflammation of the liver in hepatitis of non-viral origin such as NAFLD.[78–81] However, as is evident from studies on spontaneous and treatment-induced clearance of HCV, such a pre-emptive state may become non-beneficial in dealing with hepatitis of viral origin. Further, there may be other conditions that we do not yet know in which expression of a functional IFN-λ4 may be beneficial. Therefore, an epidemiological investigation to assess basal ISG expression levels, in humans both in health and disease, and their correlation with expression of IFN-λ4, is needed. Further, studies are also needed to examine why hepatic IFN-λ3 levels are lower in the beneficial allele/genotype-carrying individuals during chronic HCV infection and how this phenomenon is reversed once treatment is initiated (Figure 5c; ref. 94).

Although IFN-λ1, 2 and 3 are known to associate with a Th1 response,[38,39,86] no information is available in this regard for IFN-λ4. If the hypothesis that higher levels of IFN-λ3 expression (due to presence of major alleles) will lead to a Th1 predominant response is to be believed, then by extrapolation, IFN-λ4 should promote Th2 responses (Figure 3; as 'IFN-λ3 functional SNPs' and 'IFN-λ4 functional SNP' are in strong LD and the minor allele will give rise to IFN-λ4). This explanation is difficult to reconcile with the observations that IFN-λ4, except for being a poorly secreted IFN, requires the same dimeric receptor for signaling and can stimulate not only similar set of ISGs but also at similar levels when compared with IFN-λ3.[14,30,36] Besides, both IFN-λ4 and IFN-λ3 have similar antiviral potencies *in vitro*.[14,41] Therefore, expression of IFN-λ4 and its associated ISGs in a subset of individuals carrying minor alleles is less likely to favor a completely opposite phenotype to that seen in those carrying major alleles that induce higher levels of IFN-λ3 expression.

CONCLUSIONS

Human *IFNL* SNPs came to the limelight after genetic studies showed their relevance in chronic HCV infections in the year 2009.

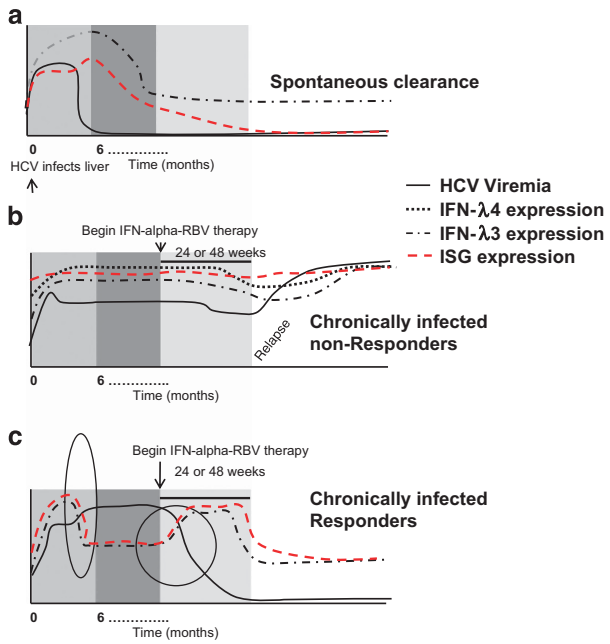

Figure 5. IFN-λ3 and IFN-λ4 expression, and spontaneous and IFN-based therapy-induced clearance of HCV infections in humans. The schematic is a summary and interpretation of data available so far from different studies (reviewed in ref. 15) dealing with HCV infections. All patients who get infected by HCV can be divided in to the three groups shown in the schematic. (**a**) Patients who spontaneously clear HCV are known to have higher levels of IFN-λ3 protein in their serum compared with patients who carry on with the infection[108] and it is likely that their IFN-stimulated gene (ISG) levels are also influenced by the IFN-λ3 levels, thus helping to clear the virus infection. Once patients get chronically infected with HCV, they undergo IFN-α-ribavirin treatment for 24–48 weeks. (**b**, **c**) Those who do not respond to this therapy have high frequency of the functional IFN-λ4-generating allele ΔG at rs368234815 (ref. 14) and therefore are expected to express IFN-λ4.[94] A recent study has documented that type III IFNs including IFN-λ2/3 and IFN-λ4 expression levels correlate with ISG expression in the liver of patients undergoing anti-HCV therapy.[94] The IFN-λ2/3 and IFN-λ4 levels and their associated ISG levels are higher in the liver of patients who will eventually not respond to the therapy[94] compared with those who will respond, suggesting that an unknown effect inhibits the expression of IFN-λ3 in the latter group of patients (shown as an ellipse in **c**). It seems that IFN-α-ribavirin treatment induces the expression of IFN-λ3 once treatment begins (depicted as a circle in **c**) preferably in those patients who will eventually respond to the treatment[94] (These patients have lower frequency of the functional IFN-λ4-generating alleles[14] and hence are shown without IFN-λ4 expression (**c**)). This induction is further dependent on whether the patients carry beneficial alleles at rs12979860. The patients who have the beneficial alleles show higher increase in their hepatic IFN-λ3 levels than those who do not, once treatment is initiated.[94] A previous report also had seen similar changes in serum IFN-λ3 levels.[109] The ISG levels are shown in correlation with IFN-λ3 expression.

Since then, case–control studies in humans have been reported involving *IFNL* SNPs and several other viral diseases, NAFLD, allergy and even in vaccine responses. Although it appears that the associations are well replicated (such as in NAFLD and AIDS) and strong in some diseases (such as in pediatric allergy), conflicting reports weaken the association in some (such as those involving HTLV and HBV), whereas further studies are needed in others (such as those involving CMV and herpes simplex virus) to clear discrepancies. The IFNL-λs may have critical roles in shaping innate and adaptive immunity in general and in viral infections in particular. However, unlike their effect in HCV infections, the *IFNL* SNPs may involve interactions with variables other than just standard end point phenotypes in many of the reported diseases/conditions. Therefore, future studies should aim at dissecting these interactions to arrive at meaningful results. Well-designed replication studies and functional studies to strengthen the genetic association results are required to arrive at firm conclusions. Importantly, IFN-λ4 has emerged as the key causal link to the genetic associations, but many questions remain on its functional role in the diversification and shaping of innate and adaptive immune responses in viral infections and other inflammatory conditions.

CONFLICT OF INTEREST

The author declare no conflict of interest.

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