The A946T variant of the RNA sensor IFIH1 mediates an interferon program that limits viral infection but increases the risk for autoimmunity

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The single-nucleotide polymorphism rs1990760 in the gene encoding the cytosolic viral sensor IFIH1 results in an amino-acid change (A946T; IFIH1T946) that is associated with multiple autoimmune diseases. The effect of this polymorphism on both viral sensing and autoimmune pathogenesis remains poorly understood. Here we found that human peripheral blood mononuclear cells (PBMCs) and cell lines expressing the risk variant IFIH1T946 exhibited heightened basal and ligand-triggered production of type I interferons. Consistent with those findings, mice with a knock-in mutation encoding IFIH1T946 displayed enhanced basal expression of type I interferons, survived a lethal viral challenge and exhibited increased penetrance in autoimmune models, including a combinatorial effect with other risk variants. Furthermore, IFIH1T946 mice manifested an embryonic survival defect consistent with enhanced responsiveness to RNA self ligands. Together our data support a model wherein the production of type I interferons driven by an autoimmune risk variant and triggered by ligand functions to protect against viral challenge, which probably accounts for its selection within human populations but provides this advantage at the cost of modestly promoting the risk of autoimmunity.

Autoimmune diseases arise from a complex interplay of genetic and environmental factors1. The anti-viral immune system includes several pathways in which the nexus of genetics, the environment and autoimmunity has become evident. Type I interferons and viral infection have each been associated with autoimmune diseases, including systemic lupus erythematosus (SLE) and type-1 diabetes (T1D)2–4, and genome-wide association studies have linked genes encoding products important for anti-viral immunity to those disorders. That association network emphasizes the delicate balance in selective pressure to mediate a more effective anti-viral response while limiting the risk for autoimmunity sequelae. A striking example of that is IFIH1 (‘interferon-induced helicase C-domain-containing protein 1’; also known as MDA5), a critical upstream component in the innate response of nearly all cell types to RNA viruses, including those in the Picornaviridae and Flaviviridae families5–7. IFIH1 contains two N-terminal caspase-activation-recruitment domains and a DExD/H-box helicase domain followed by a C-terminal domain8. While the full repertoire of ligands recognized by IFIH1 remains to be defined, published work has shown that those viral families generate both long, double-stranded RNA (dsRNA) and branched, high-molecular-weight RNA that includes both dsRNA and single-stranded RNA, which stimulate signaling9,10. Upon coordinated recognition of dsRNA by the C-terminal and helicase domains, IFIH1 undergoes conformational changes that lead to its oligomerization and assembly into filaments2 and association of its caspase-activation-recruitment domain with the signaling effector MAVS (‘mitochondrial anti-viral signaling protein’). That initiates signaling events that ultimately promote the transcription of genes encoding type I interferons and hundreds of interferon-stimulated genes (ISGs).

Gain-of-function missense mutations in IFIH1 that alter the helicase domain of IFIH1 have been identified in subjects with the rare, early-onset auto-inflammatory disorder Aicardi–Goutières syndrome, a type I interferonopathy11. The various mono-allelic mutations identified consistently alter the dsRNA- or ATP-binding sites within the helicase domain. Mutations that result in an activated IFIH1 helicase domain have also been identified in Singleton-Merten Syndrome, a rare disorder characterized by variable penetrance of dental and skeletal abnormalities and aortic and cardiac calcification12. In addition, an activating mutation in IFIH1 (resulting in the amino-acid substitution R779H), previously described in Aicardi–Goutières

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syndrome, has been identified in a single subject with early-onset SLE and immunoglobulin A (IgA) deficiency\(^\text{15}\). Similarly, N-ethyl-N-nitrosourea–mediated mutagenesis in mice has led to the identification of an interferon-dependent inflammatory disease that results from a gain-of-function Ifih1 mutation that alters the IFIH1 helicase domain\(^\text{16}\), although this specific mutation has not been associated with human disease.

In contrast to those penetrant, rare autosomal-dominant disorders, genome-wide association studies have identified both protective variants and risk variants of IFIH1 in various common human autoimmune diseases\(^\text{15-18}\). One study has reported a significant allelic association with risk for T1D at rs1990760, a non-synonymous coding variant of IFIH1 (ref. 15). Subsequent studies have reported similarly significant associations of this single-nucleotide polymorphism (SNP) with other autoimmune diseases, including SLE, as well as suggestive findings or regional associations with many additional disorders, including psoriasis, rheumatoid arthritis, multiple sclerosis and vitiligo, indicative of a basic role for IFIH1 in the genesis of autoimmunity\(^\text{17-21}\). Subsequent re-sequencing of IFIH1 in patients with T1D and healthy control subjects has revealed that rare loss-of-function variants (including rs35667974, which leads to the amino-acid substitution I923V) are associated with protection from T1D\(^\text{16}\). In contrast to either rare loss-of-function variants or dominant mutations that promote auto-inflammatory disease, the rs1990760 risk allele is common, with an allele frequency of ~57% in European populations\(^\text{22}\), and it is predicted to result in an amino-acid change from alanine to threonine at codon 946 (A946T) within the C-terminal domain of IFIH1 (called ‘IFIH1\(^\text{R746}\)’ here) of unknown functional relevance.

While various studies have replicated and expanded the range of disorders associated with the IFIH1\(^\text{R746}\) autoimmune risk variant, there is little information about its effect on protein function and/or signaling activity. Most notably, its mechanistic effect(s) on viral control and immunotolerance in vivo remain(s) unknown. In this study, we defined the role of IFIH1\(^\text{R746}\) as a partial gain-of-function variant that established the capacity for enhanced protection against specific viral challenges while concurrently promoting the risk of autoimmunome disease via heightened basal and ligand-dependent signaling. We demonstrated that IFIH1\(^\text{R746}\) promoted increased production of type I interferons in vitro and in vivo. Consistent with those findings, IFIH1\(^\text{R746}\) enhanced anti-viral responses in vitro and viral control in vivo. In parallel, through two models of autoimmune disease, we found that the variant led to an inflammatory state that promoted autoimmunity and functioned in concert with an additional common human autoimmunome risk allele.

**RESULTS**

Enhanced interferon signaling of PBMCs expressing IFIH1\(^\text{R746}\)

To evaluate both the role of genetic variation at IFIH1 on the risk of T1D and the relationships of the various polymorphic sites reported to be associated with autoimmunity at this locus, we re-analyzed genotyping data from the Type 1 Diabetes Genetics Consortium\(^\text{23-24}\). Four rare variants (rs35744605, rs72650663, rs35667974 and rs35732034)\(^\text{18}\), as well as the two common non-synonymous coding variants (rs1990760 and rs3747517, which encode the amino-acid substitutions A946T and H843R, respectively), were tested separately and as haplotypes for association with T1D. Of note, the common coding variant rs3747517 has also been reported to be associated with T1D, multiple sclerosis and SLE\(^\text{17,25-26}\). Family-based single-marker analysis indicated that presence of guanine at rs3747517 (‘G allele’) or adenine at rs1990760 (‘A allele’) was associated with increased risk for T1D (Table 1). Haplotype analysis identified three haplotypes (H1, H2 and H4) that were significantly associated with T1D (Table 2). The H1 haplotype carrying the rs3747517 ‘G allele’ and the rs1990760 ‘A allele’ conferred risk for T1D, whereas the H2 and H4 haplotypes carrying the non-risk nucleotides of rs3747517 (adenine) and rs1990760 (guanine) protected against T1D (Table 2). In summary, the H1 risk haplotype encoded arginine at position 843 and threonine at position 946 of IFIH1 (called ‘IFIH1\(^\text{R843}-\text{T946}\)’ or simply ‘IFIH1\(^\text{R}\)’) here. Additionally, this risk haplotype has been found to be in a moderate linkage disequilibrium and to be associated with psoriasis\(^\text{16,27-28}\).

To directly assess the effect of expression of the rs1990760 risk variant on primary human hematopoietic cells, we obtained PBMCs from healthy donors homozygous for the risk ‘A allele’ at rs1990760 encoding IFIH1\(^\text{R746}\) (IFIH1\(^\text{R}\)/IFIH1\(^\text{R}\)) or a non-risk ‘A allele’ at rs1990760 (IFIH1\(^\text{N}\)/IFIH1\(^\text{R}\)). We isolated the PBMCs and cultured them with medium alone or stimulated them with the artificial dsRNA ligand poly(I:C). PBMCs from all subjects had similar basal expression of IFIH1 mRNA (Supplementary Fig. 1a) that increased after treatment with poly(I:C) (Fig. 1a and Supplementary Fig. 1b). We found no difference between cells of the two IFIH1 genotypes in their ability to upregulate IFIH1 transcription in response to poly(I:C) (Fig. 1a and Supplementary Fig. 1a,b). Following stimulation with poly(I:C), PBMCs from IFIH1\(^\text{R}/\text{R}\) donors had higher expression of IFNβ1 mRNA, which encodes interferon-β (IFN-β), than that of PBMCs from IFIH1\(^\text{N}/\text{N}\) donors (Fig. 1b). PBMCs from donors expressing the variant protein IFIH1\(^\text{R843}-\text{T946}\), which represents the product of the identified risk haplotype H1, also had higher expression of IFNβ1 mRNA than that of donors expressing IFIH1\(^\text{R842}\) alone (Fig. 1c). That finding was thus probably attributable to the co-occurrence of substitutions of Arg843 and Thr946. On the basis of these observations, we call the H1 haplotype (encoding IFIH1\(^\text{R843}-\text{T946}\)) simply ‘IFIH1\(^\text{R}\)’ below (for further detailed explanation of genotypes, Supplementary Table 1).

We next sought to determine if expression of IFIH1\(^\text{R}\) was associated with evidence of enhanced type I interferon signaling in vivo. Using a custom high-throughput quantitative PCR assay to measure the expression of mRNA from candidate ISGs, we found that PBMCs from healthy donors homozygous for the risk haplotype (IFIH1\(^\text{R}/\text{R}\)) showed significantly higher basal expression of 9 of 38 candidate

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**Table 1** Single-marker family-based association analysis of IFIH1 markers

| dbSNP ID (137) | Location | cDNA change | Amino acid change | MAF | Family (n) | z score | P value |
|---------------|----------|-------------|-------------------|-----|-----------|---------|---------|
| rs35744605    | Exon 10  | c.1879G>T   | p.Glu627T         | 0.0034 | 36 | -0.792 | 0.43 |
| rs72650663    | Exon 11  | c.2105C>T   | p.Thr702I         | 0.0029 | 26 | 0.854 | 0.39 |
| rs3747517     | Exon 13  | c.2528A>G   | p.H843R           | 0.27(A) | 1592 | -3.925(A) | 0.000087 |
| rs35667974    | Exon 14  | c.2767A>G   | p.I923V           | 0.0087 | 84 | -2.902 | 0.0037 |
| rs35732034    | Intron 14| c.2807+1G>A | –                 | 0.0061 | 66 | -1.300 | 0.19 |
| rs1990760     | Exon 15  | c.2836G>A   | p.A946T           | 0.41(G) | 1755 | -4.369(G) | 0.000012 |

Analysis of IFIH1 markers in T1D-affected sibling pairs and trio families of European ancestry (quantification, column 6) (data collected by the Type 1 Diabetes Genetics Consortium), showing accession codes from the Single Nucleotide Polymorphism Database (dbSNP), and the type and location of each variant (based on RefSeq accession codes NM_022168.2 and NP_071451.2), with z scores and P values calculated for the minor alleles (MAF, minor allele frequency).

A is the minor allele (opposite to the dbSNP allele); G is the minor allele (opposite to the dbSNP allele).
ISGs than that of PBMCs from healthy donors homozygous for the non-risk haplotype (IFIH1NR/NR); none of the ISGs were significantly downregulated in IFIH1R/R PBMCs relative to their expression in IFIH1NR/NR PBMCs (Fig. 1d.e and Supplementary Fig. 1c.d).

Furthermore, we quantified upregulated genes per donor to assess the presence of a type I interferon signature marked by ISGs. IFIH1R/R donors exhibited greater number of ‘interferon signature’ genes that were upregulated than did IFIH1NR/NR donors (Fig. 1f). Hence, IFIH1R/R led to an ‘interferon signature’ in healthy human subjects. Together these results showed that cells from subjects carrying the IFIH1 risk haplotype exhibited both basal and ligand-triggered enhanced IFIH1 activity and basal type I interferon signaling.

**IFIH1R promotes basal and ligand-triggered activity**

In anticipation of establishing a knock-in mouse model (described below), we generated a series of cDNA expression constructs encoding mouse IFIH1 (mIFIH1) (Fig. 2a) to assess the function of candidate variants in vitro following overexpression in HEK293T human embryonic kidney cells. Ifih1 in C57BL/6 mice encodes IFIH1 with arginine at position 843; thus, cDNA encoding IFIH1 with arginine at position 843 and threonine at position 946 (mIFIH1R) precisely modeled the human H1 risk haplotype encoding IFIH1R843T946 (Fig. 2a). Control constructs included mIFIH1NR (the naturally occurring ‘non-risk’ (NR) Arg843 variant) and mIFIH1P (with the amino-acid substitution I923V16,29 encoded by the rare rs5667974 variant that is protective (P) against T1D) (Table 1). While transfection of cells with each construct yielded a similar amount of mIFIH1 (Fig. 2b,c and Supplementary Fig. 2a,b), transfection of cells with the mIFIH1R construct led to an increase of two-fold in basal IFNB1 mRNA relative to that of cells transfected with the mIFIH1NR (control) construct (Fig. 2d). That result was consistent with a published report showing the human IFIH1T946 variant is a gain-of-function mutant14. Next we explored the ability of mIFIH1R to respond to RNA ligands. Cells expressing each mIFIH1 construct were transfected with poly(I:C), and type I interferons were measured. Cells expressing the mIFIH1P construct exhibited modestly but consistently higher expression of poly(I:C)-inducible IFNB1 mRNA than that of cells transfected with mIFIH1NR (Fig. 2e,f). In contrast, transfection of cells with the ‘protective’ mIFIH1P construct ablated both basal expression of IFNB1 mRNA (Fig. 2d) and poly(I:C)-triggered expression of IFNB1 mRNA10,29 (Supplementary Fig. 2c,d).

We next assessed IFIH1 activity in response to viral challenge. Following transfection of the various mIFIH1 constructs described above, HEK293T cells were infected with encephalomyocarditis virus (EMCV), a picornavirus specifically recognized by IFIH1 (ref. 30). Cells expressing mIFIH1R had two- to threefold higher virus-triggered expression of IFNB1 mRNA than that of cells expressing mIFIH1NR, at 9 h after infection (Fig. 2g); in contrast, cells expressing mIFIH1P exhibited no such response to viral challenge (Fig. 2g). In a second test, cells expressing mIFIH1R had higher expression of type I interferons than that of cells expressing mIFIH1NR, concomitant with lower viral load, following infection with West Nile virus (Supplementary Fig. 2d–g), which also triggers the induction of type I interferons, in part through IFIH1 (ref. 6). These combined results demonstrated that mIFIH1R promoted heightened basal and ligand-triggered signaling and enhanced responsiveness to RNA viruses that express IFIH1 ligands during their replication cycle.

**Enhanced type I interferons and viral protection of mice**

To gain better understanding of the function of IFIH1R in both autoimmune disease and viral control, we generated a mouse strain with an Ifih1 allele that precisely mimicked the human IFIH1R (IFIH1R843T946) haplotype. We used homologous recombination to generate founder mice on the non-autoimmune-prone C57BL/6 genetic background (Supplementary Fig. 3a,b). Gene targeting achieved the variant-specific coding change (encoding the substitution A946T) in exon 15 of Ifih1 without affecting Ifih1 mRNA (Supplementary Fig. 3c). When we intercrossed heterozygous Ifih1NR/NR mice, we observed partial embryonic death, with substantially fewer homozygous Ifih1R/R offspring than expected (Fig. 3a). While newborn mice heterozygous (Ifih1NR/R) or homozygous (Ifih1R/R) for the risk allele (the mouse equivalent of the H1 haplotype allele) seemed to be healthy and exhibited normal weight gain and a normal frequency of lymphoid and myeloid subsets (data not shown), they exhibited a trend for a greater number of splenocytes than that of their Ifih1RNR/NR (control) littermates at 2–12 months of age (Supplementary Fig. 3d). Similar to our findings for human PBMCs ex vivo, splenocytes from Ifih1R/R mice had higher basal expression of Ifnb1 mRNA and mRNA from multiple ISGs, including Mx1, Ifit1 and Ifit5, than that of splenocytes from Ifih1NR/NR mice (Fig. 3b,c).

To investigate whether the H1 haplotype conferred an in vivo advantage against viral pathogens, we assessed the ability of Ifih1R/R mice to control an EMCV challenge. After intraperitoneal injection of EMCV, ~75% of the Ifih1NR/NR mice succumbed to EMCV infection within 8 d (Fig. 4). However, both Ifih1NR/R mice and Ifih1R/R mice were significantly more protected against EMCV than were their Ifih1NR/NR littermates. Together our findings showed that IFIH1R mice, whether Ifih1NR/R or Ifih1R/R, manifested elevated basal IFIH1 activity, which led to an ‘ISG signature’ and a modest auto-inflammatory state. In parallel, they had enhanced resistance to challenge with an IFIH1-specific, RNA viral pathogen.

**IFIH1R promotes disease in mouse autoimmune models**

We were interested in determining whether IFIH1R has a causal role in the promotion of autoimmune disease. Streptozocin (STZ), a DNA-alkylating agent that is selectively toxic to pancreatic β-cells, can induce T1D in C57BL/6 mice. We used an STZ dosing regimen that did not affect the concentration of glucose in the blood of the Ifih1NR/NR

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**Table 2** Family-based haplotype association analysis of IFIH1

| Haplotype | rs35744605 | rs72650663 | rs3747517 | rs35667974 | rs35732034 | rs1990760 | Freq | Family (n) | z score | P value |
|-----------|------------|------------|-----------|------------|------------|------------|------|------------|---------|---------|
| H1        | 2 G        | 2 C        | 2 G Arg   | 2 A        | 2 G        | 2 A Thr    | 0.571| 1,678      | 4.797   | 1.61 x 10^-6 |
| H2        | 2 G        | 2 C        | 1 A His   | 2 A        | 2 G        | 1 G Ala    | 0.269| 1,500      | 3.309   | 9.35 x 10^-4 |
| H3        | 2 G        | 2 C        | 2 G Arg   | 2 A        | 2 G        | 1 G Ala    | 0.141| 1,009      | -1.119  | 0.26    |
| H4        | 2 G        | 2 C        | 1 A His   | 1 G        | 2 G        | 1 G Ala    | 0.009| 86         | -3.022  | 0.0025  |
| H5        | 1 T        | 2 C        | 2 G Arg   | 2 A        | 2 G        | 2 A Thr    | 0.003| 36         | -0.815  | 0.42    |
| H6        | 2 G        | 2 C        | 2 G Arg   | 2 A        | 1 A        | 2 A Thr    | 0.003| 40         | -0.980  | 0.33    |
| H7        | 2 G        | 1 T        | 2 G Arg   | 2 A        | 1 A        | 2 A Thr    | 0.003| 26         | -0.854  | 0.39    |
| H8        | 2 G        | 2 C        | 1 A His   | 2 A        | 2 G        | 2 A Thr    <0.001| 2  | -0.999     | 0.32    |

Analysis of IFIH1 in T1D-affected sibling pairs and trio families of European ancestry (quantification, column 6) (data collected by the Type 1 Diabetes Genetics Consortium), showing nucleotides in the minor allele (1) or major allele (2), with the amino acid encoded below, and the frequency of the haplotype (Freq).
**Figure 1** IFIH1R mediates a modest increase in poly(I:C)-triggered production of IFN-β and leads to a ‘basal type I interferon signature’. (a,b) Quantitative RT-PCR analysis of IFIH1 mRNA (a) and IFNβ1 mRNA (b) in PBMCs obtained from healthy IFIH1NR/NR donors (NR/NR) or IFIH1R/R donors (R/R) (risk R or non-risk NR) alleles for rs1990760, which encodes A946T and stimulated for 24 h in vitro with poly(I:C) (1 µg/ml); results were normalized to those of POLR2A mRNA (control) and are presented relative to baseline expression (a) or were normalized to the baseline expression of IFIH1 mRNA (b). (c) Quantitative RT-PCR analysis of IFNβ1 mRNA in PBMCs obtained from healthy IFIH1NR/NR or IFIH1R/R donors (alleles as in a), all homozygous for risk alleles encoding H843R (below plot), and then stimulated for 24 h in vitro with poly(I:C) (1 µg/ml); results presented as in b. (d-f) Custom high-throughput quantitative PCR assay of mRNAs from ISGs in PBMCs from IFIH1NR/NR or IFIH1R/R donors (alleles as in a), all homozygous for risk alleles encoding H843R, showing the quantification of ISGs, among 38 candidate ISGs, with significantly (P < 0.05) or ‘borderline’ (P < 0.1) higher expression (key) in cells from IFIH1R/R donors than in those from IFIH1NR/NR donors (d), the expression of various ISGs (vertical axes) with significantly differential expression in cells from IFIH1R/R donors relative to that in cells from IFIH1NR/NR donors (presented as log-transformed values, standardized to the expression of endogenous control genes (e), and the frequency (numbers in plot) of donors with various numbers of ISGs (key) with a difference in expression of over twofold relative to the median expression in cells from IFIH1NR/NR donors (f). Each symbol (a–c,e) represents an individual donor; small horizontal lines indicate the mean (a–c) or median (e), NS, not significant (P > 0.05); *P < 0.05 (Mann-Whitney U-test (one-tailed) (a–c) or Welch’s t-test (e)). Data are from two experiments with a combined total of n = 20 IFIH1NR/NR donors and n = 20 IFIH1R/R donors (a,b), n = 4 IFIH1NR/NR donors and n = 20 IFIH1R/R donors (c) or n = 17 IFIH1NR/NR donors and n = 25 IFIH1R/R donors (d-f).

(control) littermates of Ifih1R/R and Ifih1NR/NR mice (Fig. 5a,b). However, Ifih1R/R mice treated with that same regimen had a greater incidence of diabetes than that of Ifih1NR/NR mice, while Ifih1NR/NR mice showed an intermediate incidence (Fig. 5a,b). As human variants do not occur in isolation, we next intercrossed the IFIH1R mouse with mice of another risk model consisting of a single-nucleotide change in the gene encoding the phosphatase PTPN22 (Pttn22)31. This strain (called ‘PTPN22R’ here) mimics the extensively studied human autoimmune risk variant rs2476601 that leads to an amino-acid substitution in a key interacting domain of the phosphatase. PTPN22R mice on a mixed 129/BL6 background exhibit more STZ-induced diabetes than that of their littermates expressing the ‘non-risk’ (NR) Pttn22 gene (PTPN22R31). For these experiments we used PTPN22R mice that had been fully backcrossed onto the C57BL/6 background and challenged mice of single or combined genotype with STZ to assess potential synergy in the development of T1D. Ifih1NR/NR/Pttn22NR/RR mice exhibited a low rate of diabetes development, while Ifih1NR/R/Pttn22NR/NR mice and Ifih1NR/NR/Pttn222NR/RR (control) mice failed to develop disease (Fig. 5c,d). In contrast, Ifih1NR/R/Pttn22NR/RR mice displayed a greater incidence and rate of diabetes than that of mice of the other genotypes assessed (Fig. 5c,d). The Ifih1NR/R/Pttn22NR/RR (compound heterozygous) mice displayed early-stage and late-stage focal or focally extensive pancreatitis and pancreatic atrophy (Supplementary Fig. 4b) not seen in the diabetic Ifih1NR/RR/Pttn22NR/RR (heterozygous) mice (Supplementary Fig. 4a). Ifih1NR/RR/Pttn22NR/RR mice also exhibited atrophic and sclerotic pancreatic tissue that contained remnants of pancreatic ducts with damaged islets embedded within fibrous tissues or scattered among clusters of fibroblasts and inflammatory cells (Supplementary Fig. 4b). Together these data demonstrated an additive effect in vivo of two human risk alleles in promoting autoimmune disease.

As IFIH1R is also associated with risk for SLE in humans, we next investigated whether the mouse allele directly promoted lupus; for this we used the BM12 model of lupus. The BM12 strain (derived from the C57BL/6 strain) has a three-amino-acid change in the major histocompatibility complex class II molecule H2-Ab1β (ref. 32). Adoptive
transfer of BM12 CD4+ T cells into C57Bl/6 recipients initiates autoimmune germinal-center responses that generate autoantibodies directed against dsDNA and the Smith ribonucleoprotein (smRNP) within ∼3 weeks of cell transfer. We transferred CD4+ T cells from BM12 mice into Ifih1R/R or Ifih1NR/R mice or their Ifih1NR/R (control) littermates. All strains displayed similar titers of autoantibodies at 3 weeks after cell transfer (Fig. 5ef and Supplementary Fig. 4c). In contrast, IgG and IgG2c autoantibodies directed against dsDNA and smRNP remained elevated in Ifih1R/R mice at 12 weeks after cell transfer, whereas those titers returned to baseline in Ifih1NR/R mice (Fig. 5ef and Supplementary Fig. 4c). Similar trends were observed for Ifih1R/R mice (Fig. 5ef and Supplementary Fig. 4c). Additionally, we observed a trend toward greater spleen size in Ifih1R/R mice than in Ifih1NR/R mice (Supplementary Fig. 4d). The sustained, BM12-cell-triggered autoantibody response observed in Ifih1R/R mice might mimic the autoantibody responses of patients with SLE who have a ‘type I interferon signature’ and is consistent with clinical features of patients with SLE in whom anti-dsDNA titers have been shown to correlate with the Ifih1 rs1990760 risk allele35. Together our findings demonstrated that Ifih1R was able to facilitate autoimmune disease in response to a triggering event and was able to function coordinately with additional risk variants in altering tolerance.

Enhanced activity of IFIH1R toward self RNA ligands

Published work has demonstrated that in addition to identifying ligands of viral dsRNA and single-stranded RNA, IFIH1 might be triggered by dsRNA structures within endogenous RNA26–39. We hypothesized that the observed embryonic survival defect (Fig. 3a) and increased production of basal type I interferons (Fig. 3c,d) associated with mice with the allele encoding IFIH1R, which were housed in specific-pathogen-free conditions, might have been due...
to enhanced responsiveness of IFIH1R to self RNA ligands. We tested our hypothesis in a cell line engineered to limit the processing of self RNA: HEK293T cells lacking the dsRNA-specific adenosine deaminase ADAR1 (ADAR-null cells). ADAR1 performs adenosine-to-inosine RNA editing that is proposed to destabilize duplexes formed from inverted repetitive elements within self RNAs and thereby prevents IFIH1 from sensing those cytoplasmic RNAs.\textsuperscript{36,37} Consistent with that role, bi-allelic missense mutations in human ADAR, like dominant IFIH1 mutations, result in a severe congenital interferonopathy\textsuperscript{40}, and disruption of ADAR in HEK293T cells leads to a substantial increase in signaling after overexpression of IFIH1 (ref. 37). We therefore assessed the effect of the candidate mIFIH1 constructs described above (Fig. 2a) in control (ADAR-sufficient) and ADAR-null 293T cells. As noted above (Fig. 2), the mIFIH1\textsuperscript{NR} and mIFIH1\textsuperscript{R} constructs yielded similar amounts of mIFIH1 protein, as ‘read out’ by both the geometric mean fluorescent intensity of green fluorescent protein (GFP) and frequency of cells expressing a cis-linked gene encoding a GFP reporter (Fig. 6a,b). Recapitulating our results reported above, ADAR-sufficient (control) HEK293T cells expressing mIFIH1\textsuperscript{R} had higher basal expression of IFNB1 mRNA than that of ADAR-sufficient HEK293T cells expressing mIFIH1\textsuperscript{NR} (control 293T cells) (Fig. 6c). However, expression of IFNB1 mRNA was significantly (~50-fold) higher in ADAR-null cells expressing mIFIH1\textsuperscript{R} than in control 293T cells, and that enhancement was further amplified in ADAR-null cells expressing mIFIH1\textsuperscript{NR} (~100-fold higher than that of control 293T cells) (Fig. 6d,e), consistent with the recognition of accumulated self dsRNA ligands. Together these results indicated that IFIH1R was hypersensitive to the RNA self ligands present in the ADAR-null cells and thereby generated a stronger type I interferon signal.

\textbf{DISCUSSION}

While IFIH1 has been linked to multiple autoimmune disorders, the functional effect of this isoform on immune systems has remained largely unexplored. Notably, despite strong evidence of risk for autoimmunity, the frequency of this risk haplotype is relatively high in...
some human populations, which suggests that it might also provide a selective advantage in the setting of infectious challenge. In this study, we comprehensively assessed the function of IFIH1\textsuperscript{R} both in vitro and in vivo in mouse and human PBMCs. Our findings demonstrated that IFIH1\textsuperscript{R} facilitated enhanced ligand-dependent viral sensing while also increasing the risk for autoimmunity. First, we found that PBMCs from healthy donors expressing IFIH1\textsuperscript{R} exhibited increased ligand-triggered production of type I interferons. Strikingly, PBMCs from people homozygous for risk alleles also showed significantly higher baseline expression of ISGs than that of PBMCs expressing IFIH1\textsuperscript{A946}, consistent with a ‘basal type I interferon signature’. Our data showed that IFIH1\textsuperscript{R} mediated increased sensitivity to self RNA ligands. In contrast to earlier work, our combined data demonstrated that the IFIH1\textsuperscript{T946} variant exhibited enhanced ligand–dependent signaling in vivo and in vitro relative to that of the IFIH1\textsuperscript{A946} variant. Consistent with our findings, a published report has shown that overexpression of human IFIH1\textsuperscript{T946} promotes greater basal levels of type I interferons than does overexpression of IFIH1\textsuperscript{A946} (ref. 14). However, in contrast, that study reported that the IFIH1\textsuperscript{T946} variant is unable to recognize RNA ligands or trigger signaling, due to a lack of ATPase activity.

Notably, in addition to indicating an enhanced response to viral RNAs, our data also indicated an enhanced response to RNA self-ligands in the context of the biological effect of IFIH1\textsuperscript{R}. Through the use of ADAR-null cells, which generate more endogenous self RNAs, we provided evidence that IFIH1\textsuperscript{R} recognized both synthetic dsRNA and viral RNA ligands, which led to an increase mRNA encoding in type I interferons (relative to that induced by IFIH1\textsuperscript{A946}), as assessed in three different systems in vivo and/or in vitro. The differences between our work here and that of earlier studies\textsuperscript{11} might reflect different time courses and/or reagents. Of note, the requirement for the ligand–triggering of IFIH1 activity might reflect the location of missense mutation within IFIH1 with constitutive activation from mutations that alter the helicase domain\textsuperscript{44} versus a mutation that alters the C-terminal domain (IFIH1\textsuperscript{T946}) that functions to modulate responsiveness to RNA ligands. Additional structural modeling will be needed to address these possibilities.

Notably, in addition to indicating an enhanced response to viral RNAs, our data also indicated an enhanced response to RNA self-ligands in the context of the biological effect of IFIH1\textsuperscript{R}. Through the use of ADAR-null cells, which generate more endogenous self RNAs, we provided evidence that IFIH1\textsuperscript{R} recognized and displayed heightened activity associated with the risk allele was modest, a low increase in interferons correlated with a disease phenotype. Similarly, type I interferons are frequently too low to be measured consistently, which has led to the use of an ‘interferon signature’ as biomarker for Aicardi–Goutières syndrome\textsuperscript{45}. Whether IFIH1\textsuperscript{R} recognizes different ligands or a greater breadth of ligands relative to the ligands recognized by IFIH1\textsuperscript{NR} remains to be determined. Overall, our data support the hypothesis that IFIH1\textsuperscript{R} leads to an enhanced ‘interferon state’ through the recognition of self RNAs and that this might increase the risk for autoimmune disease with or without concomitant viral challenge.
Our viral-challenge data support the possibility that the IFIH1 R843-T946 haplotype might have been subject to positive selection in humans, given its ability to promote an improved response to viral infection. Published population-genetics studies have noted a high degree of nucleotide diversity and strong population differences at IFIH1. Action of positive selection on IFIH1 has been proposed for the SNP rs10930046 (which encodes the substitution R460H). A study of intracellular pattern-recognition RIG-I-like receptors has reported a second signature of positive selection at rs3747517 (which encodes the substitution R843H) in African and Asian populations. Furthermore, haplotypes of IFIH1 encoding histidine at position 843 and threonine at position 946 (IFIH1 R843-T946) have been reported to correlate with the resolution of infection with hepatitis C virus. Notably, we found that IFIH1 R843-T946 was very rare in our data, consistent with other reports. Thus, at the population level, the effect of the common risk haplotype (IFIH1 R843-T946) on the response to viral challenge and risk of autoimmune disease is probably important, whereas the effect of the rare IFIH1 R843-T946 haplotype would be negligible. Overall, our functional and population data suggest that non-synonymous substitutions at IFIH1 might act as a double-edged sword, protecting from illness and promoting illness at the same time, and that this might affect their frequency in the population.

Notably, we identified a partial embryonic survival defect in the homozygous Ifih1 T777/R843 mice, which raised the possibility of a similar effect on human fertility. However, the frequency of the IFIH1 T777 allele would suggest that this is unlikely. Furthermore, increased fetal loss has not been reported for more-severe gain-of-function variants that lead to early-onset interferonopathies. In contrast, embryonic death has been described in several mouse strains with alterations in the IFIH1 signaling cascade. Thus, we anticipate that the threshold for type I interferons to mediate embryonic death differs in mice versus humans.

In summary, we have identified the function for the IFIH1 T777/R843 risk variant in regulating a key antiviral sensing pathway in which it both defended against viral infection and bolstered responses to self RNA. Our data have provided a demonstration of the power of a combinatorial approach that made use of studies of healthy human subjects in association with mouse modeling to assess the effect of candidate genome-wide-association-study variants and to functionally model combinatorial effect of additional variants; this is an important consideration, as disease-associated genetic variants probably do not function in isolation. Our combined work has provided new insight into functional mechanism(s) in the setting of endogenous gene expression and candidate disease initiators. Application of this approach to other risk or protective variants might promote a more rapid advance in the understanding of human disease mechanism.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.A.G., C.H., A.E.S. and Y.G. designed and performed experiments, analyzed data and wrote and/or edited the manuscript; J.S.E. designed and performed experiments and analyzed data; E.A., T.A., C.C., X.D., S.K., K.P., K.C. and M.O. developed required models, strains or reagents and/or performed experiments; D.L., D.B.S., R.G.J., P.C. and M.G. analyzed data and edited the manuscript; H.B. designed and interpreted human-subject studies; and D.J.R. conceived of and supervised the study, interpreted data and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Plasmids and cultured cells. Mouse cDNA corresponding to Ifih1 nucleotides 326–3403 of the National Center for Biotechnology Information Genbank accession code NM_027835.3. This was cloned into pRRL.MND.T2A.GFP, a plasmid backbone for lentivirus that also effectively expresses proteins when transiently transfected. Point mutations for mIFIH1 and mIFIH1 were made to correspond to rs35667974 and rs1990760 using Quikchange II XL Site-Directed Mutagenesis Kit (Agilent Technologies).

ADAR-null and untargeted HEK293T cells were generated as described.

Cre/penetrated PBMCs were obtained from 85 participants in the Benaroya Research Institute (BRI) Healthy Control Registry and Biorepository. Subjects were selected based on IFIH1 genotype and the absence of autoimmune disease or any family history of autoimmunity. 38 subjects were homozygous for the IFIH1 rs1990760 non-risk (NR) variant, and 47 were homozygous for the IFIH1 rs1990760 risk (R) variant. Subjects were age matched (mean age: NR group, 35.3 ± 18.8 years; R group, 35.2 ± 19.2 years) and sex matched as far as possible (NR group, 22 males and 16 females; R group, 25 males and 22 females). Genotyping for the IFIH1 SNPs rs1990760/C/T (Ala946Thr) and rs3747517/G/A (His843Arg) was performed using major-groove-binding Eclipse genotyping probes (ELITech Group, Inc., Logan UT). All genotyping assays were validated using HapMap DNAs of known genotype. Samples were ‘blinded’ for analysis but were provided in a manner that guaranteed that samples from both groups would be tested on each assay day. DNA from control subjects was genotyped for the IFIH1 SNPs rs1990760/C/T (Ala946Thr) and rs3747517/G/A (His843Arg) using major-groove-binding Eclipse genotyping probes (ELITech Group, Inc., Logan UT). All genotyping assays were validated using HapMap DNAs of known genotype. Informed consent was obtained from all participants and the research protocols were approved by the Institutional Review Board at BRI.

All cell lines and primary cell were cultured at 37 °C in a humidified environment with 5% CO2. HEK293T cells were cultured in DMEM, 10% FCS (FCS), and 1% Glutamax (Gibco). PBMCs were cultured in RPMI with 10% FCS, 1% Glutamax. HEK293T cells were a gift from St. Jude's Research Institute and are annually tested for mycoplasma.

Cell transfection and stimulation with IFIH1 ligands. 1.5 × 10^6 HEK 293T cells in 1 ml medium underwent transient transfection with 1 μg plasmid DNA using Fugene 6 (Promega) (1 μg DNA to 3 μl of Fugene). After 15 h, cells were either transfected with (1.25 μg) high-molecular-weight poly(I:C) (InvivoGen) using a TransIT-mRNA Transfection kit (Mirus Bio) in serum-free DMEM following manufacturer’s instructions or were infected with virus as previously described.

In brief, EMCV K strain at 10 MOI or West Nile virus strain TX02 at 5 MOI was added to serum-free RPMI for 1 h, then virus-containing medium was removed and cells were cultured for times indicated in the figures, in standard conditions. Cells were collected and RNA or protein was extracted (described below).

For primary human PBMCs, stimulations were performed in 96-well round-bottomed tissue culture plates (2 × 10^6 cells in media/well). HEK293T cells were transfected as described above. For poly(I:C) stimulation, cells were left untreated (medium alone) or were transfected with 1 μg poly(I:C) using the TransIT-mRNA transfection kit (Mirus Bio) according to the manufacturer’s instruction, followed by standard culturing. 24 h after stimulation, cells were collected and RNA was extracted (described below).

Quantitative RT-PCR. RNA was extracted from cell pellets or mouse spleenocytes (described below) using the RNeasy Mini Kit (Qiagen) and the manufacturer’s instructions. For mouse spleenocytes and HEK293T cells, cDNA was made from RNA using a Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Real-Time PCR was performed on the cDNA using iTaq Universal Syber Green Supermix (Bio-Rad) and the BioRad C1000 Thermal Cycler (Supplementary Table 2). For primary PBMCs, cDNA was generated using Superscript III Reverse transcriptase and Oligo(dT)20 Primer (Invitrogen). TaqMan multiplex real-time PCR was performed for analysis of transcripts encoding human IFN-β and IFIH1 using FAM-labeled probes (Thermo-Fisher) Hs01077958_s1 (IFNB1) and Hs0170332_m1 (IFIH1).

Transcript Levels of RNA polymerase II were determined as endogenous control using VIC-labeled probe Hs001722187_m1. PCR was run on a 7500 Fast Real-Time PCR system (Applied Biosystems).

Fluidigm assay. Total RNA from human PBMCs were converted to cDNA (High Capacity RT kit, Life Technologies) and preamplified by limited PCR (PreAmp Master Mix, Life Technologies) with a pool of 96 primers (DeltaGene, Fluidigm). We assessed 38 ISGS in this pool of 96 primers. Preamplified cDNAs were treated with Exonuclease I (New England BioLabs) and were diluted to remove unused primers and dNTPs, then were loaded onto a 96.96 Dynamic Array IFC for real-time PCR analysis on a BioMarkHD (Fluidigm). Analysis was performed, using Fluidigm's Real Time PCR Analysis Software to determine Ct values, using linear (derivative) baseline correction and auto-detected, assay-specific threshold determination. Ct values were standardized to those of three housekeeping genes (DOCK2, EEF1A1 and FAM105B) that showed high correlation across samples to correct for sample input differences. Four samples from same donor and same draw were run on both Fluidigm assays. The mean of the Ct values were used for analysis. Human sample size was based on availability of IFIH1rs1990760 and IFIH1rs3747517 subjects.

Immunoblot analysis and antibodies. HEK293T cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Igepal, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDF)) with 1 μM okadaic acid and 1 μM phosphatase inhibitor mixture II (both EMD-Millipore) and 10 μM protease inhibitor cocktail (P-8340, Sigma-Aldrich). Whole-cell lysates were separated by 10% SDS-PAGE, transferred to Immobilon x (EMD-Millipore) and analyzed immunoblot using the following primary antibodies: rabbit anti-MA5 (Cell Signaling: #5231; 1:1,000), mouse anti-β-actin (Cell signaling: #3700; 1:1,000), and goat antibody to the West Nile virus protein NS3 (R&D Systems: AF2907; 1:1,000). Anti-goat (926-32214), anti-mouse (925-32210) and anti-rabbit (926-32211,925-68021) IRDye (LI-COR Biociences; 1:15,000) were used as secondary antibodies. Membranes were imaged by the Odyssey Infrared Imager (LI-COR Biociences) and quantified by ImageJ software.

Mice. A construct designed to generate a T946A mutation in exon 15 of Ifih1 by homologous recombination in C57BL/6j mice was generated and injected by Biocytogen as follows. The construct contained a neomycin-resistance selection cassette between two FRT sequences that would be inserted into intron 16 after successful recombination.loxP sites also added between intron 15 and 16 to allow for lineage-specific deletion were not used. The construct was introduced into C57BL/6j embryonic stem (ES) cells and clones were obtained by limited dilution and G418 selection. Clones with successful integration of the knock-in template into the locus were confirmed by Southern blot and PCR analysis of genomic DNA. Successfully targeted clones were injected into BALB/c blastocysts and were subsequently transferred into pseudopregnant females. One clone gave rise to a line with germine transmission of the allele. The mutation was confirmed by sequencing of exon 15 (Supplementary Fig. 2), and PCR was used to genotype all litters (using the following primers: 5’-AGAATCTCATTTCTTGGTGCGGC-3’ and 5’-GTTCTTCTTGTATGCTGCTATG-3’). BM12 mice (B6-C-H2-Ab1m12/KH(Egfj)) were purchased from Jackson Laboratory. All mice used in the BM12 cell-transfer experiments were females at 11–20 weeks of age. Ptpn22+/- mice have been previously described. No statistical methods were used to determine sample size. No randomization or blinding was done during experimental setup. All strains were maintained in a specific-pathogen-free facility, and studies were performed in accordance with procedures approved by the Institutional Animal Care and Use Committees of Seattle Children's Research Institute or the University of Washington.

Analysis of mouse tissues. Mouse serum was tested by ELISA for reactivity to calf thymus dsDNA (Sigma-Aldrich) and sm-RNP (ATR1-10; Arotech Diagnostics Limited), which has previously been described.
To obtain RNA samples for quantitative PCR, mouse spleens were digested with collagenase Type 4 (Worthington Biochemical), followed by calcium chelation and RBC lysis, and were made into single-cell suspensions.

Insulin was detected by immunohistochemistry in paraffin-embedded pancreas-tissue sections using a Leica Bond MAX Automated Immunostainer (Leica Microsystems) and the primary antibody guinea pig anti-insulin (Dako-A0564; 1:4,000), with the secondary antibodies rabbit anti-guinea pig (Abcam-102356; 1:1,000) and goat anti-rabbit poly-HRP (Bond Polymer Refine detection-DS9800; Leica Microsystems). Visualization was with DAB-Bond Polymer Refine (Leica Microsystems-DS9800).

**In vivo infection.** Mice were infected intraperitoneally with 100 plaque-forming units of EMCV K strain diluted in phosphate buffered saline (PBS) in 200 µl total volume. Recipients were monitored twice daily for 14 d and were assigned scores for clinical signs as follows: 1 = lethargic; 2 = hunched and/or scruffy coat; 3 = weakness in 1 limb; 4 = weakness in >1 limb; 5 = moribund. Mice with a clinical score of 4 or more were euthanized. Recipients were all males and were 6–22 weeks of age.

**STZ-induced diabetes.** Mice received daily intraperitoneal injections of STZ (Sigma-Aldrich) at a dose of 40 mg/kg for 5 consecutive days, or 55 mg/kg for 4 consecutive days. Following the final injection, peripheral blood glucose concentrations were measured two to three times per week throughout the experiment using a Contour Glucose Meter (Bayer). Mice with blood glucose concentrations above 250 mg/dl for two consecutive reads or three of five consecutive reads were considered diabetic. Any mouse that exhibited blood glucose concentrations >500 mg/dl were euthanized. All mice were 7–12 weeks old.

**Statistical analysis.** All statistical analysis were performed using GraphPad Prism version 6.01 unless specified above. All specific statistical tests and P-values are indicated in the relevant figures. To assess statistical significance between two groups with not normally distributed data sets, the Mann Whitney U-test was used. For normally distributed data sets, the Student’s t-test and the Welch’s t-test (for unequal s.d. were used). When three groups were analyzed, we used either a one-way ANOVA or Kruskal-Wallis test.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon request. Uncropped gels are presented in Supplementary Figure 5.

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