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TYPE I INTERFERON IN HUMAN AUTOIMMUNITY

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Timothy B. Niewold

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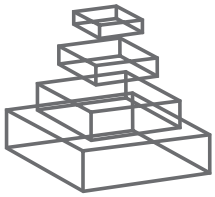

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TYPE I INTERFERON IN HUMAN AUTOIMMUNITY

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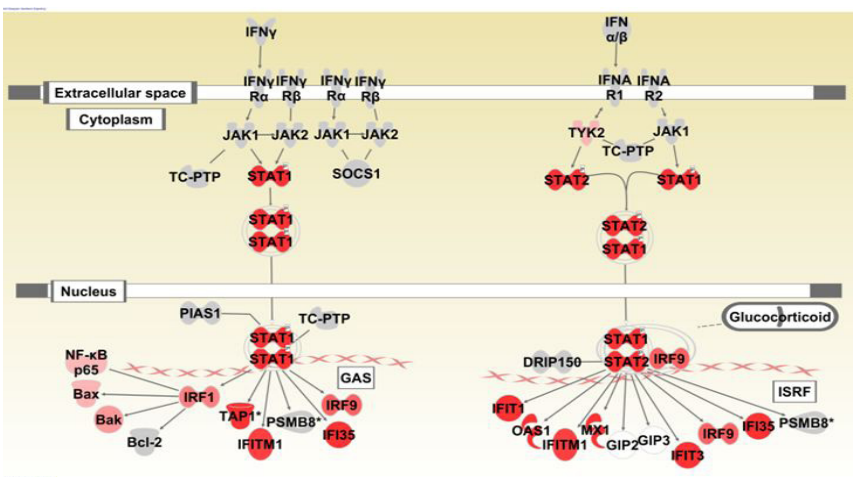

Activation of the type I interferon system in systemic lupus erythematosus (Copyright: Ko K, Koldobskaya Y, Rosenzweig E and Niewold TB)

The type I interferon system plays a critical role in host defense in health, and a growing body of literature suggests that type I interferon is a critical mediator of human autoimmune disease. Type I interferons function as a bridge between the innate and adaptive immune systems, and as such play an important role in setting thresholds for response against self antigens. Many investigators have focused on the role type I interferons play in autoimmune disease. This fascinating and rapidly growing body of literature encompasses many different autoimmune diseases, including systemic lupus erythematosus, type I diabetes, multiple sclerosis, and others. In this Research Topic, we provide a comprehensive overview of the various roles type I interferons play in autoimmune diseases, with a focus on human immunology.

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Type I interferon in human autoimmunity

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Keywords: interferons, systemic lupus erythematosus, Sjogren's syndrome, multiple sclerosis, scleroderma, systemic, type I diabetes, autoimmune thyroid disease

The type I interferon system plays a critical role in host defense in health, and a growing body of literature suggests that type I interferon is a critical mediator of human autoimmune disease (1). Type I interferons function as a bridge between the innate and adaptive immune systems, and as such play an important role in setting thresholds for response against self antigens. Many investigators have focused on the role type I interferons play in autoimmune disease. This fascinating and rapidly growing body of literature encompasses many different autoimmune diseases, including systemic lupus erythematosus, type I diabetes, multiple sclerosis, and others. Type I interferons play differing roles in human autoimmune conditions. For example, in the autoimmune diseases, systemic lupus erythematosus and Sjogren's syndrome, increased interferon alpha signaling plays a pathogenic role (2, 3). Interestingly, interferon beta is used as a therapeutic in multiple sclerosis, an autoimmune disease of the central nervous system (4). Both interferon alpha and beta signal through the same type I interferon receptor and share many similarities in downstream signaling, suggesting that the disparate activities of type I interferons in lupus and multiple sclerosis relate to differences in the underlying disease processes and immunoregulation in these two diseases. In this Research Topic, a series of articles provides a comprehensive overview of the various roles type I interferons play in autoimmune diseases, with a focus on human immunology.

This Research Topic features a number of Original Research Articles, including a study by Mavragani et al. examining type I interferon levels in the organ-specific autoimmune disorders type I diabetes and autoimmune thyroid disease (5). They demonstrate high type I interferon levels in both of these autoimmune conditions, supporting the idea that high levels of type I interferon are detectable in organ-specific autoimmune conditions in addition to systemic autoimmune disorders. Clark et al. investigate genetic polymorphisms in the interferon regulatory factor 5 (IRF5) gene (6). This gene has been associated with susceptibility to systemic lupus erythematosus (7), and they demonstrate four distinct promoter regions have differential activity. Ko et al. study type I interferon-induced gene expression in patients with systemic lupus erythematosus (8). They demonstrate that the expression of type I interferon-induced genes in lupus immune cells differs significantly between ancestral backgrounds, which corresponds to clinical differences in the disease between ancestral backgrounds. A Methods article by Feng et al. examines public domain gene expression data to document patterns of type I interferon-induced gene expression and infer both positive and negative regulation by transcription factors (9).

The Research Topic also features a number of Review Articles focusing on various disease states. Liu et al. review murine models of systemic lupus erythematosus that are interferon-inducible, providing model systems of autoimmunity related to type I interferon (10). Wu et al. review the role of type I interferon in systemic sclerosis, a distinct autoimmune disease characterized by thickening and fibrosis of the skin, which shares a type I interferon signature with other autoimmune conditions (11). Li et al. review the evidence supporting a role for type I interferon in the pathogenesis of Sjogren's syndrome, spanning genetic associations, gene expression studies, and clinical features of the disease (12). Reder et al. review the contrasting role of type I interferon in multiple sclerosis and systemic lupus erythematosus and other autoimmune conditions (13). In multiple sclerosis, type I interferon levels are low (14), and administration of recombinant type I interferon is an effective treatment. They review the evidence supporting multiple sclerosis as a low interferon autoimmune disease, and speculate on immunological features that might underlie this striking difference. Shrivastav et al. review the role of nucleic acid receptors in type I interferon generation in systemic lupus erythematosus (15), a disease characterized by pathological activation of the type I interferon pathway. These articles taken together provide an overview of many of the ways type I interferons have been implicated in human autoimmune disease, providing a fascinating window into the biology of the human immune system gone wrong.

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Increased serum type I interferon activity in organ-specific autoimmune disorders: clinical, imaging, and serological associations

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Background: Activation of the type I interferon (IFN) pathway has been implicated in the pathogenesis of systemic autoimmune disorders but its role in the pathogenesis of organ-specific autoimmunity is limited. We tested the hypothesis that endogenous expression of type I IFN functional activity contributes to the pathogenesis of autoimmune thyroid disease (ATD) and type I diabetes (T1DM).

Methods: We studied 39 patients with ATD and 39 age and sex matched controls along with 88 T1DM patients and 46 healthy matched controls respectively. Available clinical and serological parameters were recorded by chart review, and thyroid ultrasound was performed in 17 ATD patients. Type I IFN serum activity was determined in all subjects using a reporter cell assay. The rs1990760 SNP of the interferon-induced helicase 1 gene was genotyped in ATD patients.

Results: Serum type I IFN activity was increased in patients with ATD and T1DM compared to controls (*p*-values: 0.002 and 0.04, respectively). ATD patients with high type I IFN serum activity had increased prevalence of antibodies against thyroglobulin (anti-Tg) and cardiopulmonary manifestations compared to those with low IFN activity. Additionally, the presence of micronodules on thyroid ultrasound was associated with higher type I IFN levels. In patients with T1DM, high IFN levels were associated with increased apolipoprotein-B levels.

Conclusion: Serum type I IFN activity is increased in ATD and T1DM and is associated with specific clinical, serological, and imaging features. These findings may implicate type I IFN pathway in the pathogenesis of specific features of organ-specific autoimmunity.

Keywords: type I interferon, autoimmune thyroid disease, organ-specific autoimmunity, type I diabetes

INTRODUCTION

Autoimmune thyroid diseases (ATD), including Hashimoto's thyroiditis (HT) and Graves' disease (GD), as well as type I Diabetes Mellitus (T1DM) are prototype organ-specific autoimmune disorders characterized by loss of immunological tolerance against thyroid and β-cell pancreatic antigens, lymphocytic infiltration of the thyroid gland and the insulin producing pancreatic islands, and various degrees of organ dysfunction (1, 2).

Autoimmune thyroid disease and T1DM share common features with systemic autoimmune disorders, such as multifactorial etiology involving both genetic and environmental factors, female predominance (ATD), and familial aggregation associated with other organ-specific or systemic autoimmune disorders (3–5). Despite the fact that ATD is classically considered as a disease that predominantly affects the thyroid gland, non-specific systemic features such as musculoskeletal complaints, sicca symptomatology, pregnancy loss, and various neurological manifestations may also occur (6). Taken together, these observations suggest that clinically different autoimmune phenotypes might share common pathogenetic pathways.

While increasing evidence over the last few years suggests a dominant role for the type I interferon (IFN) pathway in the pathogenesis of many systemic autoimmune disorders such as systemic lupus erythematosus (SLE) and Sjogren's syndrome (7, 8), limited data are available regarding the role of the IFN-α pathway in the pathogenesis of organ-specific autoimmune disorders (5). Recent studies have suggested that the Ala946Thr polymorphism of the interferon-induced helicase 1 gene (IFIH1) (SNP ID rs1990760) is associated with type I diabetes (T1DM), GD, and Addison's disease (9, 10). Recent data also support the protective role of rarer IFIH1 alleles against T1DM (10). The IFIH1 gene, also known as the melanoma differentiation-associated 5 (MDA-5), encodes

a putative RNA helicase implicated in sensing of viral RNA and generation of antiviral responses (11). In SLE, risk alleles of the IRF5, IRF7, and IFIH1 genes have been associated with high type I IFN levels and distinct autoantibody profiles (12, 13).

Given that development of thyroid autoimmunity and to a lesser extent T1DM, either separately or in combination, has been previously described after IFN-α treatment (14–18), we hypothesized that activation of the type I IFN pathway may contribute to the pathogenesis of these organ-specific autoimmune disorders. To test this hypothesis, type I IFN activity was measured in sera of patients with ATD, T1DM, and healthy controls (HC), using a sensitive functional assay, and its presence was related to various clinical, biochemical, morphological, and genetic indices.

PATIENTS AND METHODS

STUDY PARTICIPANTS

Thirty-nine patients with ATD (13 with GD and 26 with HT) and 39 age and sex matched HC without evidence of underlying autoimmune disease along with 88 patients with T1DM and 46 HC matched for sex and age were studied. Study participants were followed in the Department of Pathophysiology, University of Athens (ATD patients), and the General Pediatric Hospital Ag.Sophia (TIDM patients) (19). Study subjects signed an informed consent form prior to enrollment in the study. All patients underwent a complete medical history and physical examination. Baseline hematological and biochemical profiles were performed and detailed medical therapy was recorded in all patients.

All ATD participants completed a specific questionnaire addressing symptoms/signs related to systemic autoimmune diseases. Symptoms/signs and parameters recorded included skin manifestations, musculoskeletal features, Raynaud's phenomenon, sicca symptoms, renal involvement, hematological manifestations (autoimmune hemolytic anemia, leucopenia, thrombocytopenia) cardiovascular and/or pulmonary features (pulmonary hypertension, pulmonary fibrosis, pleuritis, pericarditis, coronary artery disease), and neurological complications (headaches, stroke, white matter microangiopathy, transverse myelitis, cranial/peripheral neuropathy). The presence or absence of autoantibodies to thyroid antigens, including antibodies to thyroglobulin (anti-Tg) and thyroid peroxidase (anti-TPO), thyroid stimulating hormone receptor (TSHR), as well as thyroid stimulating hormone (TSH) levels at the time of diagnosis were also recorded. The normal range of TSH values was 0.5–5 (mU/L). On this basis, TSH levels were defined as high and low (>5 and <0.5 mU/L, respectively).

Patients with T1DM were suffering from no other disease and/or DM related complications and were not taking any medications other than insulin. Biochemical parameters that were particularly recorded in patients with T1DM included cholesterol, triglycerides, HbA1c, apolipoprotein-A and -B as well C-reactive protein (CRP) levels.

Serum from ATD and T1DM patients and controls was collected and stored at −80°C until assayed. Informed consent was obtained from ATD patients and controls as well as from the parents of both diabetic and healthy subjects, according to the Declaration of Helsinki. The study has been approved by the Ethics Committee of Athens University Medical School.

SERUM TYPE I IFN ACTIVITY

Type I IFN activity was measured in sera derived from ATD, T1DM, and HC using a reporter cell assay, which measures the ability of serum to upregulate IFN-inducible genes in an IFN sensitive cell line as previously described (20). In brief, cells of the WISH epithelial cell line (ATCC) express the type I IFN receptor and are highly responsive to type I IFN. WISH cells were plated at a density of cells/mL in 96 well plates in Minimal Essential Media (Cellgro, Herndon, VA, USA) with 10% fetal calf serum (FCS). The cells were then cultured with 50% patient serum for 6 h. Recombinant human IFN-α (IFNaA; BioSource International, Camarillo, CA, USA) and media were used as positive and negative controls respectively. Subsequently, total cellular mRNA was purified from stimulated cells at the end of the culture period using the Qiagen TurboCapture oligo-dT coated 96 well plate system as per manufacturer protocol (Qiagen, Valencia, CA, USA) and was reverse-transcribed to cDNA immediately following purification using the Superscript III reverse transcriptase system from Invitrogen (Carlsbad, CA, USA). Quantitative real-time polymerase chain reaction (PCR) was then used to quantify specific cDNAs using the Bio-Rad SYBR Green intercalating fluorophore system with a Bio-Rad I-cycler thermocycler and fluorescence detector (Bio-Rad, Hercules, CA, USA). Primers for genes highly induced by type I IFN signaling-interferon induced with tetratricopeptide repeats 1 (IFIT-1, Forward CTCCTTGGGTTCGTCTATAAATTG; Reverse AGTCAGCAGCCAGTCTCAG), Protein kinase R (PKR) (Forward CTTCCATCTGACTCAGGTTT; Reverse TGCTTCTGACGGTATGTATTA), interferon-induced protein with tetratricopeptide repeats 3 (IFIT-3, Forward GGCAGACAGGAAGACTTCTGAAGAACA; Reverse TGACTGCCCTCT-GTGTCTCTGCT), myxovirus (influenza virus) resistance 1 (MX-1, Forward TACCAGG ACTACGAGATTG-Reverse TGCCAGGAAGGTCTATTAG) were used in the PCR reaction on the WISH cell derived cDNAs. The housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Forward CAACGGATTTGGTCGTATT; Reverse GATGGCAACAA-TATCCACTT) was also quantified in the cDNA samples to control for background gene expression. The type I IFN-induced genes are compared with housekeeping gene expression to determine relative expression. The relative expression is then normalized to the relative expression of the respective genes in unstimulated cells from the same population. Type I IFN activity was calculated as the average relative expression of IFN-inducible genes (IFIT-1 and PKR in the thyroid cohort and IFIT-3, PKR, MX-1 in the diabetes cohort). The cut-off for high serum type I IFN activity among patient samples was defined as the mean plus 1 SD of the IFN activity score of sera from healthy donors (HD) (cut-off for high activity = 1.29, for ATD patients and 0.87 for T1DM patients).

IFIH1 GENOTYPING

The rs1990760 SNP in IFIH1 was genotyped in ATD patients using real-time PCR with Applied Biosystems Assays-by-Design Taqman primer and allele specific fluorescent labeled probes. Reactions were run using 10 ng of genomic DNA along with primers and probes on an ABI 7900HT PCR machine per manufacturer protocol. Genotype calls were made from clustering diagrams at >99% certainty, and the call rate was >90%.

AUTOANTIBODY ASSAYS

Anti-Ro/SSA and anti-RNP/Sm antibodies in the ATD patients were determined by commercial ELISA (Diamedix, FL, USA). Anti-Tg and anti-TPO autoantibodies were measured in the same laboratory using a two-site immunoluminometric assay (Dia-Sorin, LIASON analyzer, normal range: <100 and <25 IU/mL respectively). TRAbs were measured using commercial kit (Dia-Sorin Inc., Stillwater, MN, USA, cut-off value: 10%).

THYROID ULTRASONOGRAPHY

Thyroid ultrasonography was carried out in 17 ATD patients using a high-resolution apparatus (Logic-Book XP, General Electric Co., USA) equipped with a 6–11 MHz broadband linear array probe by a single operator who was unaware of the diagnosis.

High-sensitivity color flow Doppler sonography was used to estimate the intraparenchymal blood flow pattern. The vascularity index and the hypoechogenicity index were calculated for all images as previously described (21). Thyroid volume was measured and the presence of nodules was also recorded [micronodules (diameter <10 mm) and macronodules (diameter >10 mm)]. In addition, thyroid blood flow was also measured at the inferior thyroid artery (16).

STATISTICAL ANALYSIS

Two-group comparisons of continuous data were assessed using unpaired *t*-test on non-parametric Mann–Whitney test for normally or not normally distributed data respectively. Fisher's exact two-tailed test was used for categorical variables. Correlations between quantitative variables were performed by Spearman's rho test. Our multivariate analysis consisted of a stepwise logistic regression that was used to identify independent variables that could be associated with high or low type I IFN serum activity in ATD patients. The variables entered in the multivariate model were those shown to be statistically significantly different between high and low IFN groups based on the bivariate analysis ($p < 0.05$).

RESULTS

ATD COHORT

Type I IFN activity in ATD patients and controls

In order to explore whether activation of the type I IFN pathway occurs in the context of ATD, type I IFN serum activity was determined by a reporter cell assay (please see Patients and Methods for details) in 39 ATD patients (26 with HT, 12 with GD) and 39 HC of similar age, sex, and race distribution (**Table 1**). Patients with ATD had increased type I IFN activity compared to HC (mean ± SD: 1.2 ± 0.4 vs. 0.9 ± 0.4, $p = 0.002$) (**Figure 1A**). Following these findings patients with ATD were subdivided further according to type I IFN activity into those with "high" (13 patients) and "low" (26 patients) IFN score.

Clinical and serological correlates of type I IFN activity among ATD patients

In order to determine whether high serum type I IFN activity is associated with the presence of any clinical and serological parameters, we compared the "high" and "low"-IFN score ATD patients using bivariate analysis. Comparisons between the two groups were performed for demographic variables, systemic manifestations, thyroid function, and antibodies. As shown in **Table 2**, anti-Tg antibodies were present in almost all patients of the "high" IFN group, compared to less than 50% of the "low" IFN patients ($p = 0.013$).

Table 1 | Demographic characteristics of the study subjects.

	ATD patients ($n = 39$)	Healthy controls ($n = 39$)	*p*-Value
No. of subjects	39	39	
Mean age ± SD (years)	48.9 ± 14.4	47.6 ± 11.2	ns
Female to male ratio	4.6:1	4.6:1	ns
Mean disease duration ± SD (years)	5 ± 5.2	NA	NA
% Caucasians	100	100	1
No of patients with GD	12	NA	NA
No of patients with Hashitoxicosis	1	NA	NA
No of patients with HT	26	NA	NA

GD, Grave's disease; HT, Hashimoto's disease; ATD, autoimmune thyroid disease; SD, Standard Deviation; NA, not applicable; ns, no significant.

Cardiopulmonary manifestations were significantly more frequent in the "high"-IFN ATD group (46 vs. 8%, $p = 0.011$) and included shortness of breath on exertion in two individuals (possibly related to heart failure), pericarditis in one individual, asthma in two cases, and interstitial lung disease in another one. In the low IFN group two cases of asthma were reported. Autoantibodies against Ro/SSA and RNP/Sm nucleoproteins, previously shown to be associated with high type I IFN activity in SLE patients (22), were negative in all ATD subjects (data not shown). No correlations were found between type I IFN levels and autoantibodies to TSHR ($r = -0.163$, $p = 0.396$) by Spearman's correlation test. Logistic regression analysis revealed an independent association of high IFN-α activity with the presence of anti-Tg antibodies (OR = 17.69, 95% CI: 2.05–560.9) and cardiopulmonary manifestations (OR = 15.34, 95% CI: 1.95–335.5) respectively.

Type I IFN activity and thyroid ultrasonographic pattern

We next sought to explore whether type I IFN activity serum levels were associated with ultrasonographic parameters of the thyroid gland. While no significant associations were detected between type I IFN levels and the various ultrasonographic indices examined including thyroid volume, vascularity and hypogenicity indexes, macronodules and inferior thyroid artery blood flow (data not shown), higher type I IFN activity was found in patients with ATD and thyroid micronodules ($p = 0.04$) (**Figure 1B**).

IFIH1 genotypes and IFN levels in ATD patients

To determine whether the IFIH1 risk variant is associated with higher type I IFN levels, the rs1990760 SNP in IFIH1 was genotyped. No association was found between genotypes and type I IFN levels (data not shown). However, a trend toward higher prevalence of the T risk allele among patients with low TSH levels compared to those with high TSH levels (77.7 vs. 50%, OR = 3.5, CI: 0.9–13.3, $p = 0.07$) was found, consistent with the previously reported association of the T allele of the IFIH1 with GD (10).

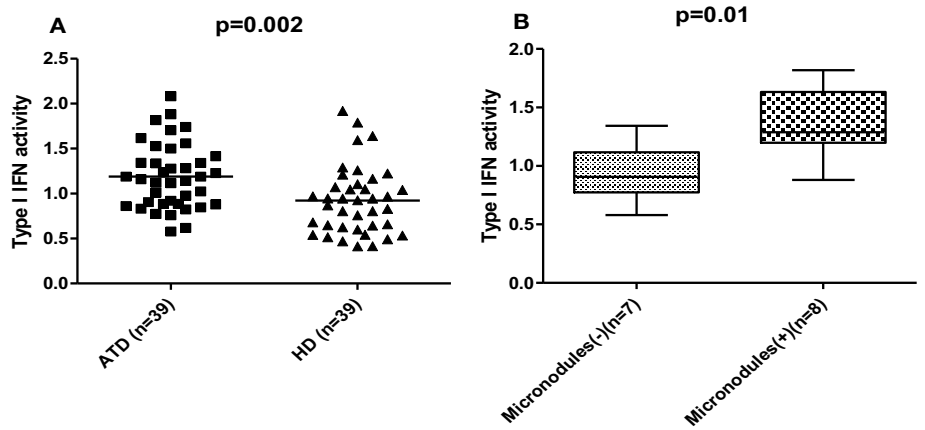

FIGURE 1 | (A) Increased type I interferon (IFN) activity in patients with autoimmune thyroid disease (ATD) compared to healthy donors (HD). Serum type I IFN activity was assessed using a sensitive reporter cell assay in 39 patients with autoimmune thyroid disease (ATD) and 39 age-sex matched healthy controls (HC). Results are expressed as an IFN score, as described in materials and methods. Symbols represent individual subjects; horizontal lines represent the mean; *p*-values were calculated by unpaired *t*-test. **(B)** Significantly higher serum type I IFN activity levels in ATD patients with ultrasonographic presence of thyroid micronodules. Serum type I IFN activity assessed using a sensitive reporter cell assay was found to be higher in ATD patients characterized by the presence of micronodules on ultrasound ($n = 8$) compared to those without such nodules ($n = 7$). Data are shown as box plots. Each box represents the 25–75th percentiles. Lines inside the box represent the median. Lines outside the box represent the 10th and the 90th percentiles; *p*-values were calculated by unpaired *t*-test. Micronodules (+): presence of micronodules, micronodules (−): absence of micronodules.

T1DM COHORT

Type I IFN activity in T1DM patients and controls-clinical and laboratory correlates

In order to investigate whether type I IFN pathway is activated in the setting of T1DM, serum type I IFN activity was determined in 88 pediatric T1DM patients and 46 controls of similar age, sex, and body mass index (BMI) distribution (**Table 3**) by the previously described reporter cell assay. As shown in **Figure 2**, type I IFN activity was found to be significantly increased in pediatric patients with T1DM compared to age-sex matched controls (mean ± SD:1.1 ± 2.2 vs. 0.6 ± 0.3, $p = 0.04$). Of interest, apolipoprotein-B levels were higher in T1DM patients with high type I IFN activity. No other associations with clinical and/or serological data were observed (**Table 4**).

DISCUSSION

While several lines of evidence suggest a central role for the type I IFN pathway in the pathogenesis of a number of systemic autoimmune disorders, more limited data are available regarding its contribution to the pathogenesis of organ-specific autoimmune disease and its potential association with distinct clinical or serological phenotypes. The current study provides evidence of elevated serum type I IFN activity in approximately one third of patients with ATD and one fifth of those with T1DM, using a functional IFN assay.

Designation of ATD and T1DM patients as patients with "high" or "low" type I IFN activity allowed us to test the hypothesis that type I IFN pathway activation identifies ATD and T1DM patients with distinct clinical and serological characteristics. Following both bivariate and multivariate analysis, the presence of anti-TG, but not anti-TPO was associated with a high IFN status among ATD patients. Of interest, a recent study identified SNP (_1623A/G) of the TG gene – previously identified as a major ATD susceptible variant – to modify a binding site for the IFN-induced transcription factor interferon regulatory factor-1 (IRF-1), leading to increased promoter activity and increased TG levels, a major antigenic target for ATD (23). However, the presence of such genetic variant was not assessed in our patient population. Additionally, stimulation of rat thyroidal cells with IFN-α has been shown to lead to persistent (up to 48 h) increase of TG levels through TG promoter activation suggesting that IFN-induced upregulation of the TG autoantigen could lead to generation of antigen specific serum reactivities. Though upregulation of TPO was also observed, this was limited to 24 h and seemed to be independent from the activation of the TPO promoter (24). As no antibodies against Ro/SSA and RNP/Sm antigens were found, those antibodies cannot be implicated in the induction of type I IFN, as has been proposed for SLE (22).

Interestingly, high IFN status was also associated with increased prevalence of cardiopulmonary features. Although IFN-α has been previously linked to atherosclerotic risk and a negative effect of IFN-α on vascular endothelial cells has been demonstrated (25, 26), the pathophysiological implication of this association in ATD will require further investigation. ATD patients with increased type I IFN levels exhibited mostly micronodular sonographic appearance which is related to the presence of lymphocytic aggregates with germinal centers and/or transformed follicular oxyphilic cells (27). Given that type I IFN has been previously associated with B-cell activation and immunoglobulin class switching (28), the relation of micronodulation to type I IFN activity could reflect the contribution of the latter in the pathophysiology of ATD disease. Whilst no significant associations were detected between levels of type I IFN activity and genotypes of the IFIH1 gene, a trend toward increased prevalence of the T risk allele among patients with low

Table 2 | Comparison of ATD patients with low or high type I IFN activity in bivariate analysis.

Variable	Type I IFN activity		
	Low ($n=26$)	High ($n=13$)	p
Mean type I IFN activity score	0.98 ± 0.19	1.6 ± 0.23	<0.0001
DEMOGRAPHICS			
Age, years	47.08 ± 15.16	52.91 ± 12.33	0.278
Disease duration, years	4.32 ± 4.78	6.54 ± 6.03	0.318
No of females	22/26 (84.6%)	10/13 (76.9%)	0.666
SYSTEMIC MANIFESTATIONS			
Skin manifestations	8/25 (32%)	7/13 (53.8%)	0.295
Musculoskeletal manifestations	7/25 (28%)	6/13 (46.1%)	0.30
Oral ulcers	6/25 (24%)	0/11 (0%)	0.147
Raynaud's phenomenon	4/25 (16%)	1/13 (7%)	0.642
Sicca symptoms	5/25 (20%)	3/13 (23.1%)	1
Cardiopulmonary	2/25 (8%)	6/13 (46.1%)	0.011
Renal	0/25 (0%)	0/12 (0%)	1
Hematological	0/24 (0%)	0/11 (0%)	1
Neurologic			
Headaches	13/25 (52%)	2/13 (15.3%)	0.039
Other*	2/25 (8%)	1/13 (7.7%)	1
ABS AGAINST THYROID ANTIGENS			
Positive anti-Tg autoAbs	12/25 (48%)	11/12 (91.6%)	0.013
Positive anti-TPO autoAbs	21/25 (84%)	11/12 (91.6%)	1
TYPE OF THYROID DISORDER			
Graves' disease	8/26 (30.7%)	4/13 (30.7%)	1
Hashimoto's/Hashitoxicosis	18/26 (69.3%)	9/13 (69.3%)	1
THYROID FUNCTION TESTS			
High TSH	12/26 (46.1%)	8/13 (61.5%)	0.5
Low TSH	9/26 (34.6.9%)	4/13 (30.7%)	1

Other (includes stroke, white matter microangiopathy, transverse myelitis, cranial/peripheral neuropathy).*

Table 3 | Characteristics of the T1DM patients and healthy subjects.

	Healthy subjects ($n=46$)	T1DM patients ($n=88$)	p
Age (years)	10.53 ± 0.64	12.12 ± 0.57	ns
Female to male ratio	0.8:1	1.1:1	ns
Body mass index (kg/m^2)	21.16 ± 0.20	20.79 ± 0.58	ns
Diabetes duration (months)	–	57.82 ± 7.83 (0–184)	NA

ns, no significant; NA, not applicable.

TSH levels (defined as <0.5 IU/mL) compared to those with high TSH levels was noted. This finding is consistent with the previously reported association of the T risk allele of the IFIH1 with GD (10).

The association between type I IFN and thyroid disease was first appreciated in 1985 in patients treated with IFN-α for breast cancer (29). Since then, a large number of studies have revealed a high incidence of thyroid abnormalities in IFN-α treated patients,

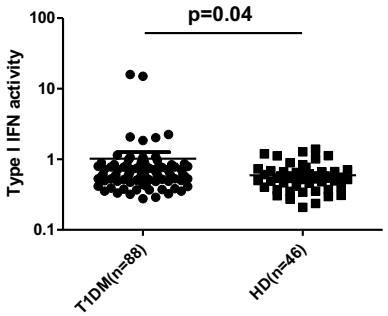

FIGURE 2 | Increased type I IFN activity in patients with type 1 diabetes mellitus (T1DM) ($n=88$) compared to healthy donors (HD) ($n=46$). Serum type I IFN activity was assessed using a sensitive reporter cell assay as previously described. Symbols represent individual subjects; horizontal lines represent the mean; *p*-values were calculated by non-parametric Mann–Whitney test.

Table 4 | Comparison of TIDM patients with low or high type I IFN activity in bivariate analysis.

Variable	Type I IFN activity		
	Low ($n=72$)	High ($n=16$)	p
Mean type I IFN activity score	0.57 ± 0.15	3.06 ± 4.86	<0.0001
Age (years)	11.96 ± 0.74	13.00 ± 1.03	0.500
Female to male ratio	1.2	1.3	1
Body mass index (kg/m^2)	20.90 ± 0.67	20.21 ± 1.43	0.640
Diabetes duration (months)	59.19 ± 9.25	48.45 ± 17.26	0.583
Total cholesterol (mg/dL)	165.51 ± 5.31	167.27 ± 7.61	0.869
HDL (mg/dL)	59.16 ± 1.74	59.55 ± 2.94	0.915
LDL (mg/dL)	91.95 ± 4.06	90.00 ± 5.48	0.810
Total triglycerides (mg/dL)	70.51 ± 6.31	73.00 ± 10.03	0.847
Lipoprotein (a) (mg/dL)	12.86 ± 1.84	13.50 ± 3.62	0.873
Apolipoprotein-A (mg/dL)	126.46 ± 4.85	137.57 ± 10.70	0.305
Apolipoprotein-B (mg/dL)	81.63 ± 4.16	91.43 ± 1.62	0.037
Urine microalbumin (mg/L)	7.33 ± 0.99	6.83 ± 1.73	0.813
Serum creatinine (mg/dL)	0.64 ± 0.021	0.65 ± 0.06	0.977
Serum urea (mg/dL)	29.59 ± 1.08	31.73 ± 4.94	0.681
CRP (mg/dL)	5.00 ± 1.21	5.91 ± 3.03	0.744
HbA1c (%)	8.08 ± 0.27	7.77 ± 0.42	0.599

ranging from development of thyroid autoantibodies to overt ATD such as GD or HD (30). IFN-α treatment has been shown to exacerbate preexisting thyroid autoimmunity by increasing the titers of antithyroid antibodies (31). Although unclear, the potential mechanisms through which IFN-α might promote the development of thyroid autoimmunity are multiple, including facilitation of antigen presentation through increased expression of the adhesion and costimulatory molecules ICAM-1, B7.1, and MHC class I antigens on thyrocytes, activation of cytotoxic T-cells, promotion of autoantibody production through direct and indirect effects on B-cell and immunoglobulin class switching, upregulation of thyroid specific antigens, and direct toxicity on thyroidal cells

(18, 24). Recently, functional sensors detecting both exogenous and endogenous signals were detected in thyroid cells promoting induction of innate immune responses including activation of type I IFN pathway. In particular, stimulation of thyroid cells with Toll-like receptor ligands led to activation of the interferon-beta (IFN-β) promoter (32). Though therapeutic administration of IFN-α in patients with HCV infection has been mainly associated with induction of ATD, several reports derived from the multiple sclerosis (MS) literature suggest IFN-β as an inducer of ATD among MS patients possibly through stimulation of CXCL10 secretion by thyrocytes (33, 34). Unfortunately, in the present study, exploring whether type I IFN activity was mainly related to IFN-α or IFN-β components was not included in the initial design of the study.

Recent findings have revealed an upregulation of IFN-α inducible genes in peripheral blood mononuclear cells in patients with GD, which correlated with TSHR messenger RNA and protein levels of HLA-DR and IFN-α (35). Although stimulation of primary cultured thyrocytes with recombinant human IFN-α resulted in increased expression of MHC-II antigens and TSHR in these patients, no serum IFN type I activity was detected in the samples tested. IFN-α levels – measured by a commercially available enzyme immunoassay – were also found to be increased in a small cohort of patients with several thyroid disorders including 12 Grave's disease and four patients with HT. No associations with distinct clinical, serological, or imaging findings were reported (36). Finally, data from a recent report revealed heightened levels of the type I IFN-inducible myxovirus resistance protein A in thyroid tissue derived from early HT patients further reinforcing the implication of the type I IFN pathway in ATD pathogenesis (37).

Activation of the type I IFN pathway was also confirmed in our pediatric diabetic cohort – the largest so far tested, with approximately 20% of patients demonstrating raised serum type I IFN levels, by a sensitive bioassay. The contribution of type I IFN in the pathogenesis of autoimmune T1DM has been previously postulated in both human and animal studies (38–40). Earlier data from a relatively small cohort of mixed adult and pediatric populations demonstrated raised IFN-α in peripheral blood at the mRNA and protein level which correlated well with blood enteroviral RNA, implying a role of enteroviral infection in the pathogenesis of T1DM (39). No clinical or serological associations of type I IFN pathway with disease related biomarkers were reported in that study. On the other hand, endogenous nucleic acids derived from apoptotic pancreatic β cells were also proposed as potential triggers of IFN-α production by plasmacytoid dendritic cells, leading to activation of autoreactive CD4+ T-cells which ultimately lead to destruction of insulin producing pancreatic islets (41). Of interest, in a recently reported animal model of virus induced T1DM, defective function of viral sensors with impaired type I responses was associated with development of the disease and was related to defective clearance of a virus directed against the beta cells of the pancreas (42).

In the current study, apolipoprotein-B levels – previously shown to be associated with pronounced atherosclerotic risk (43, 44) – were increased in the high IFN group in our T1DM cohort. Of interest, apolipoprotein-B is a member of the APOBEC family of proteins, many of which are regulated by type I interferons and particularly IFN-α (45, 46). Whilst it is not known whether increased apolipoprotein-B levels are directly induced by type I IFN, such a probability remains, providing an additional mechanism by which type I IFN might contribute to the pathogenesis of atherosclerosis (47).

The demonstration of type I IFN activity in serum of patients with ATD and T1DM, supports its role in the pathogenesis of both organ-specific and systemic autoimmune disorders. While the reasons for the tissue specificity in the autoimmune process remain elusive, the identification of the type I IFN pathway as a common pathogenetic denominator among distinct and diverse autoimmune phenotypes may explain the similarities found in patients with IFN-related disorders, such as strong familial aggregation and female predominance, and contribute to the identification of unifying underlying determinants of autoimmunity. Familial aggregation of both high IFN and ATD in SLE families could support a case for IFN being causal in both (48).

In conclusion, the findings of the present study support a role for type I IFN in the pathogenesis of organ-specific autoimmune disorders, particularly in ATD and T1DM patients. These data extend our current list of type I IFN-related autoimmune disorders and may provide insight into shared pathogenic factors and suggest new targets for therapeutic intervention.

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Activation of the interferon pathway is dependent upon autoantibodies in African-American SLE patients, but not in European-American SLE patients

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Background: In systemic lupus erythematosus (SLE), antibodies directed at RNA-binding proteins (anti-RBP) are associated with high serum type I interferon (IFN), which plays an important role in SLE pathogenesis. African-Americans (AA) are more likely to develop SLE, and SLE is also more severe in this population. We hypothesized that peripheral blood gene expression patterns would differ between AA and European-American (EA) SLE patients, and between those with anti-RBP antibodies and those who lack these antibodies.

Methods: Whole blood RNA from 33 female SLE patients and 16 matched female controls from AA and EA ancestral backgrounds was analyzed on Affymetrix Gene 1.0 ST gene expression arrays. Ingenuity Pathway Analysis was used to compare the top differentially expressed canonical pathways amongst the sample groups. An independent cohort of 116 SLE patients was used to replicate findings using quantitative real-time PCR (qPCR).

Results: Both AA and EA patients with positive anti-RBP antibodies showed overexpression of similar IFN-related canonical pathways, such as IFN Signaling ($P = 1.3 \times 10^{-7}$ and 6.3×10^{-11} in AA vs. EA respectively), Antigen Presenting Pathway ($P = 1.8 \times 10^{-5}$ and 2.5×10^{-6}), and a number of pattern recognition receptor pathways. In anti-RBP negative (RBP−) patients, EA subjects demonstrated similar IFN-related pathway activation, whereas no IFN-related pathways were detected in RBP−AA patients. qPCR validation confirmed similar results.

Conclusion: Our data show that IFN-induced gene expression is completely dependent on the presence of autoantibodies in AA SLE patients but not in EA patients. This molecular heterogeneity suggests differences in IFN-pathway activation between ancestral backgrounds in SLE. This heterogeneity may be clinically important, as therapeutics targeting this pathway are being developed.

Keywords: systemic lupus erythematosus, interferon alpha, autoantibodies, ancestral background, interferon gamma

INTRODUCTION

Systemic lupus erythematosus (SLE) is a heterogeneous disease characterized by complex genetic contributions and activation of a number of immune system pathways (1–3). Recent advances in human genetic studies have helped us better understand the immunopathogenesis of the disorder (4, 5). Multiple candidate gene association studies and genome wide association studies have led to discovery of more than 30 susceptibility loci throughout the whole genome, most of which are involved in three main pathways in lupus pathogenesis: abnormal clearance of nuclear debris and immune complexes, over-activation of innate immune system through Toll-like receptor (TLR) and type I interferon (IFN) signaling, and aberrant adaptive immune response through B and T cell signaling (6, 7). Moreover, gene expression microarray studies have been instrumental in defining important aspects of the complex immunological pathogenesis in human subjects (8, 9). Several gene expression analyses in SLE have found up-regulation of IFN-inducible genes (IFIGs) in more than 50% of patients (10–13), and others have shown differential expression of genes involved in several pathways including inflammation, apoptosis, DNA repair, and T cell activation (12, 14–17).

Interferon-α is a pleiotropic type I IFN which plays a key pathogenic role in lupus development (18). It is an anti-viral cytokine which is regulated by endosomal pattern recognition receptors (PRRs) such as TLRs or cytosolic PRRs like RIG-I like receptors (RLRs) (18, 19). It has the potential to break self-tolerance by

Abbreviations: AA, African-American; ANA, antinuclear antibodies; Anti-dsDNA, anti-double-stranded DNA; Anti-RBP, anti-RNA-binding protein; Anti-RNP, anti-ribonucleoprotein; Anti-Sm, anti-smith; EA, European-American; HA, Hispanic-American; IFIGs, IFN-inducible genes; IFN, interferon; IPA, ingenuity pathway analysis; PRRs, pattern recognition receptors; qPCR, quantitative real-time PCR; RBP+, anti-RBP antibody positive; RBP−, anti-RBP antibody negative; RLRs, RIG-I like receptors; SLE, systemic lupus erythematosus; TLR, toll-like receptor; UCMC, University of Chicago Medical Center.

inducing dendritic cell differentiation which in turn leads to activation of autoreactive T and B cells, thus linking innate and adaptive immune systems (20, 21). IFN-α is a heritable risk factor in SLE (22, 23) and some people who have received recombinant human IFN-α as a treatment for viral hepatitis C or malignancy have developed *de novo* SLE which resolves upon discontinuation of the IFN-α treatment (24). These data strongly support a causal role for IFN-α in SLE pathogenesis. Increased activity of IFN-α has been associated with presence of various SLE-associated autoantibodies, both anti-double-stranded DNA (anti-dsDNA) and anti-RNA-binding protein (anti-RBP) antibodies along with different organ involvement such as hematologic, renal, and central nervous systems (10, 25, 26). However, longitudinal studies have not confirmed the association between increases in IFIG expression and disease flare (27, 28). It seems that patients with high IFN-α have more severe disease and a higher rate of flare on average, but the changes in IFN-α levels in circulation do not correlate closely or quantitatively with changes in measures of disease activity over time.

Systemic lupus erythematosus is both more prevalent and more severe in African-American (AA) populations than in European-American (EA) populations, and disease manifestations are variable amongst different ancestral backgrounds (29–32). AA and Hispanic-American (HA) patients are likely to have more active SLE, with an earlier age at onset, than EA patients (31, 32). Anti-ribonucleoprotein (anti-RNP) and anti-Smith (anti-Sm) antibodies are more prevalent in AA patients than in EA and HA (30, 32), and a number of genetic variants are associated with autoantibody profiles in different ancestral groups (33, 34). Moreover, compared to EA patients, HA, and AA patients have a higher incidence of SLE-related renal disease, associated with anti-dsDNA and anti-RNP antibodies (31, 35). Additionally, some of the genetic factors associated with SLE are not shared between AA and EA patients (36–39). These data all support the idea that molecular and biological differences should exist in SLE patients of different ancestral backgrounds. We have shown that overall serum IFN-α activity is higher in SLE patients of non-European ancestry as compared to European ancestry, either directly or indirectly through an increased prevalence of anti-RBP antibodies (40, 41). In this study, we compare peripheral blood gene expression between AA and EA SLE patients taking into account the differences in autoantibody profile, and we find a striking difference in the activation of the IFN pathway between the two groups.

MATERIALS AND METHODS

PATIENTS, SAMPLES, AND DATA COLLECTION

Serum samples were obtained from 149 female SLE patients from the University of Chicago Medical Center (UCMC) ($n = 119$) and NorthShore University Health System ($n = 30$). All cases fulfilled the American College of Rheumatology criteria for the diagnosis of SLE (1, 42), and the data regarding the presence or absence of these criteria as well as of SLE-associated autoantibodies [anti-nuclear antibodies (ANA), and anti-Ro, anti-La, anti-Sm, anti-RNP, and anti-dsDNA antibodies] were available for all patients. Forty-nine unrelated females who were screened by medical record review for the absence of autoimmune disease were used as controls. They were of similar age ($P = 0.21$) as the SLE cases. All subjects provided informed consent, and the study was approved by the institutional review boards at the Mayo Clinic and University of Chicago.

DETECTION OF AUTOANTIBODIES

Antibodies to Ro, La, Sm, and RNP for all samples were measured by ELISA methods (INOVA Diagnostics, San Diego, CA, USA) at UCMC at the time of serum and RNA sampling, and standard clinical laboratory cutoff points were used to categorize them as positive or negative. Anti-dsDNA antibodies were measured using Crithidia luciliae immunofluorescence at UCMC, and detectable fluorescence was considered positive.

GENE EXPRESSION ANALYSIS

Thirty-three SLE cases and 16 age-matched controls were selected for microarray gene expression analysis. The cases were subdivided into AA and EA patients, and those with positive anti-RBP antibodies and those without as described in **Table 1**. Whole blood from the subjects was collected in PAX gene tubes (Qiagen), and RNA was purified in spin columns per manufacture recommendations. The RNA was analyzed on Affymetrix Gene 1.0 ST gene expression arrays, which were run in the University of Chicago Microarray Core facility. These intensity data were normalized through Affymetrix Expression Console software. Data from the microarray experiment have been deposited in the GEO database, accession number GSE50635.

Quantitative real-time PCR (qPCR) was used to validate the hypotheses generated from the microarray data with an independent replication cohort. The RNA of whole blood from 60 AA SLE patients, 47 anti-RBP antibody positive (RBP+), and 13 anti-RBP antibody negative (RBP−), and 56 EA SLE patients, 21 RBP+ and 24 RBP− along with 25 AA and 8 EA controls was purified using Qiagen RNeasy kit. cDNA was synthesized from total mRNA, and qPCR was used to measure relative transcript expression using SYBR Green dye on an ABI 7900HT thermal cycler.

STATISTICAL ANALYSIS

For each ancestry, the anti-RBP antibody status was used as a dichotomous variable, and each subgroup was compared to respective controls from the same ancestral background. Following normalization, the mean microarray gene expression values along

Table 1 | Samples and data collection for microarray analysis*.

		SLE cases**		Non-autoimmune controls
		RBP+	RBP−	
European-American	Female	8	8	8
African-American	Female	9	8	8

**There was no difference in age amongst the groups.*

***All SLE cases fulfilled the ACR criteria for SLE. Anti-RBP (anti-Ro, anti-La, anti-Sm, and anti-RNP) antibodies were measured by ELISA, and anti-dsDNA antibody levels were measured using Crithidia luciliae immunofluorescence.*

RBP+, anti-RNA-binding-protein (RBP) antibody positive; RBP−, RBP antibody negative.

with standard deviations were calculated for each subgroup and used to calculate the fold changes between subjects and controls. Values were compared between the groups using the two-tailed Student's unpaired *t*-test. Similar comparisons were made with the qPCR data, but this time, all group results were expressed in medians and compared using Mann–Whitney tests as they did not follow Gaussian distributions. For both microarray and qPCR analyses, *P* values less than 0.05 were considered significant.

CANONICAL PATHWAY ANALYSIS

From the microarray data, the differentially expressed genes with a cutoff *P* value of 0.05 along with their respective fold changes were analyzed further through Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com) to compare the top canonical pathways amongst the sample groups (**Table 1**). The IPA canonical pathway analysis identified the pathways from the IPA Knowledge Base that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in two ways: (1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway was displayed. (2) Fisher's exact test was used to calculate a *P* value determining the probability that the association between the genes in the dataset and the canonical pathway was explained by chance alone.

RESULTS

DEMOGRAPHICS AND PRESENCE OF ANA AND ANTI-dsDNA ANTIBODIES

The average age of all subjects included in the microarray portion of the study was 43.5 ± 10.6 years. When the subjects and controls were divided into subgroups according to ancestry and the presence of anti-RBP antibodies (**Table 1**), there was no statistical difference in age amongst the subgroups including controls. There was lower prevalence in anti-dsDNA antibodies in RBP− AA patients (77% of RBP+ vs. 31% of RBP− had anti-dsDNA antibodies, $P = 0.005$). In EA subjects, this difference was much less pronounced, and was not statistically significant (66% of RBP+ vs. 54% of RBP− had anti-dsDNA antibodies, $P = 0.4$). This is in concordance with our previous large-scale analyses in SLE, in which we have also found that anti-RBP and anti-dsDNA antibodies are more correlated in AA as compared to EA SLE patients (40). All subjects were female and all tested positive for ANA.

IFN-RELATED CANONICAL PATHWAY ACTIVATION IN AA vs. EA SLE PATIENTS

Top 10 canonical pathways from each subgroup are shown in **Table 2**. Many immune system associated pathways were associated with the cases, and similar to previous studies, type I IFN-related pathways were the most differentially expressed when comparing cases vs. controls except in RBP− AA group. We examined in greater detail the six canonical pathways which were type I IFN-related across patients from the two different ancestral backgrounds studied. As shown in **Table 3**, the microarray analysis of all SLE cases vs. controls demonstrated all six IFN-related canonical pathways significantly involved. The same pattern was also observed for all EA cases vs. controls. However, to our surprise, the associations between the IFN-related canonical pathways and AA SLE cases were not as strong as only three out of six pathways were found to be significant. This is despite that fact that high circulating levels of type I IFN are more common in AA SLE patients (40).

IFN-RELATED CANONICAL PATHWAY ACTIVATION IS NOT SEEN IN RBP− AA PATIENTS

To explore this further, we looked at the associations between IFN-related canonical pathways within SLE patient subgroups stratified by both ancestry and the presence or absence of anti-RBP antibodies. As shown in **Table 4**, all six type I IFN-related pathways were activated in both AA and EA RBP+ patients. The key pathway difference was found between AA and EA patients who were RBP−. RBP− EA patients demonstrated activation of all six IFN-related canonical pathways, whereas not a single type I IFN pathway was significantly involved in the RBP− AA patients.

IFN-INDUCED GENE EXPRESSION PATHWAY DIAGRAMS IN AA vs. EA PATIENTS

In **Figure 1**, we show pathway diagrams generated in IPA software of the type I and type II IFN pathways, with genes that were up-regulated in cases vs. controls shaded red. It is striking that none of the genes illustrated downstream of the type I and type II IFN receptors are up-regulated in the RBP− AA patients, while in the RBP− EA patients, many IFN-induced genes are over-expressed. It is also interesting that STAT1 over-expression is observed in the RBP+ subjects regardless of ancestral background, and this is not observed in the RBP− patients from either ancestral background.

REPLICATION STUDY CONFIRMS THE DEPENDENCE OF IFN-INDUCED GENE EXPRESSION UPON PRESENCE OF ANTI-RBP ANTIBODIES IN AA PATIENTS, BUT NOT EA PATIENTS

Three IFIGs (IFIT1, MX1, and PKR) were selected for qPCR analysis to replicate the microarray observation with regards to the association between anti-RBP antibodies and IFN-related gene expression across different ancestral backgrounds. These genes were quantified in whole blood mRNA from an independent cohort of 116 SLE patients and 33 controls. As shown in **Figure 2**, the pattern observed mirrors the microarray data. All three genes were up-regulated in both EA and AA RBP+ patients. In the RBP− patients, there is essentially no increase in IFN-induced gene expression in AA patients, while the expression of these genes, in particular PKR, is increased in the RBP− EA SLE patients. Because anti-dsDNA antibodies have been associated with high IFN-α (25, 26), it is important to determine whether anti-dsDNA antibodies are contributing to the induction of IFN-induced gene expression we observe in our RBP− EA patients. As noted above, the RBP− EA subjects were more likely to have anti-dsDNA antibodies than the RBP− AA subjects. When we looked at the RBP negative patients in the qPCR replication cohorts with regard to presence or absence of anti-dsDNA antibodies, there was no significant difference in IFIT1, MX1, or PKR over-expression in the AA subjects. In RBP− EA patients, however, over-expression of IFN-induced genes (IFIT1 and PKR) was observed in the anti-dsDNA antibody positive patients but not in the anti-dsDNA antibody negative group (**Figure 3**). Thus, in RBP− EA subjects, the anti-dsDNA

Table 2 | Top 10 canonical pathways from each subgroup vs. matching controls through IPA from microarray data.

All cases	All EA	All AA	EA RBP+	EA RBP−	AA RBP+	AA RBP−
EIF2 signaling	Interferon signaling	EIF2 signaling	Interferon signaling	Antigen presentation pathway	EIF2 signaling	Regulation of IL-2 expression in activated and anergic T lymphocytes
Interferon signaling	Antigen presentation pathway	Activation of IRF by cytosolic pattern recognition receptors	Activation of IRF by cytosolic pattern recognition receptors	OX40 signaling pathway	Regulation of eIF4 and p70S6K signaling	Glucocorticoid receptor signaling
Antigen presentation pathway	Role of pattern recognition receptors in recognition of bacteria and viruses	Angiopoietin signaling	Antigen presentation pathway	Autoimmune thyroid disease signaling	mTOR signaling	CD28 signaling in T helper cells
Activation of IRF by cytosolic pattern recognition receptors	Retinoic acid mediated apoptosis signaling	Regulation of eIF4 and p70S6K signaling	Role of pattern recognition receptors in recognition of bacteria and viruses	Allograft rejection signaling	Activation of IRF by cytosolic pattern recognition receptors	T cell receptor signaling
IL-12 signaling and production in macrophages	Activation of IRF by cytosolic pattern recognition receptors	Hypoxia signaling in the cardiovascular system	IL-15 production	Interferon signaling	Interferon signaling	Glycosphingolipid biosynthesis - globoseries
mTOR signaling	Graft-vs.-host disease signaling	Role of RIG1-like receptors in antiviral innate immunity	Retinoic acid mediated apoptosis signaling	Graft-vs.-host disease signaling	Apoptosis signaling	Biosynthesis of steroids
Role of pattern recognition receptors in recognition of bacteria and viruses	Dendritic cell maturation	mTOR signaling	Role of RIG1-like receptors in antiviral innate immunity	Cytotoxic T lymphocyte-mediated apoptosis of target cells	Colorectal cancer metastasis signaling	April mediated signaling
TNFR2 signaling	IL-15 production	Hereditary breast cancer signaling	Communication between innate and adaptive immune cells	Crosstalk between dendritic cells and natural killer cells	TNFR2 signaling	Reelin signaling in neurons
Production of nitric oxide and reactive oxygen species in macrophages	Autoimmune thyroid disease signaling	Role of PI3K/AKT signaling in the pathogenesis of influenza	Dendritic Cell maturation	Type I diabetes mellitus signaling	IL-8 signaling	Glycosphingolipid biosynthesis – neolactoseries
IL-15 production	Communication between innate and adaptive immune cells	TNFR2 signaling	Starch and sucrose metabolism	Dendritic cell maturation	P2Y purigenic receptor signaling pathway	Mitotic roles of polo-like kinase

IPA, ingenuity pathway analysis; EA, European-American; AA, African-American; RBP+, anti-RNA-binding-protein (RBP) antibody positive; RBP−, RBP antibody negative; eIF2, eukaryotic initiation factor 2; IRF, interferon-regulatory factor; mTOR, mammalian target of rapamycin; TNFR2, tumor necrosis factor receptor 2; eIF4, eukaryotic initiation factor 4; RIG1, retinoic acid-inducible gene 1; PI3K, phosphatidylinositol 3-kinase; OX40 = CD134.

Table 3 | *P* values for IPA IFN-related canonical pathways from microarray data.

	All SLE cases ($n = 33$)	EA SLE cases ($n = 16$)	AA SLE cases ($n = 17$)
Interferon signaling	1.53×10^{-10}	6.55×10^{-12}	0.29
Activation of IRF by cytosolic pattern recognition receptors	6.14×10^{-5}	2.74×10^{-5}	8.46×10^{-5}
Role of RIG1-like receptors in antiviral innate immunity	1.63×10^{-4}	5.85×10^{-7}	0.044
Role of PKR in interferon induction and antiviral response	0.020	0.038	0.091
Role of pattern recognition receptors in recognition of bacteria and viruses	0.0097	0.0064	0.0011
Communication between innate and adaptive immune cells	0.0098	0.0013	Not listed

IPA, ingenuity pathway analysis; IFN, interferon; SLE, systemic lupus erythematosus; EA, European-American; AA, African-American; IRF, interferon-regulatory factor; RIG1, retinoic acid-inducible gene 1; and PKR, protein kinase R.

Table 4 | *P* values from IPA IFN-related canonical pathways from microarray data.

	EA RBP+	EA RBP−	AA RBP+	AA RBP−
Interferon signaling	5.8×10^{-11}	10.6×10^{-5}	1.3×10^{-7}	0.063
Activation of IRF by cytosolic pattern recognition receptors	1.1×10^{-6}	0.016	2.86×10^{-8}	0.25
Role of RIG1-like receptors in antiviral innate immunity	2.0×10^{-5}	0.030	0.0014	Not listed
Role of PKR in interferon induction and antiviral response	0.0073	0.042	9.0×10^{-4}	Not listed
Role of pattern recognition receptors in recognition of bacteria and viruses	1.8×10^{-4}	0.028	5.8×10^{-4}	0.31
Communication between innate and adaptive immune cells	0.0043	0.0064	0.023	Not listed

IPA, ingenuity pathway analysis; IFN, interferon; EA, European–American; AA, African-American; RBP+, anti-RNA-binding-protein (RBP) antibody positive; RBP−, RBP antibody negative; IRF, interferon-regulatory factor; RIG1, retinoic acid-inducible gene 1; and PKR, protein kinase R.

antibody status did have an effect on expression of IFIGs, and thus anti-dsDNA antibodies are contributing to the IFN-induced gene expression in this group. Strikingly, anti-dsDNA antibodies had no impact upon IFN-induced gene expression in the RBP− AA group. This was somewhat unexpected and reinforces the idea of RBP antibody dependence in the AA ancestral background.

DISCUSSION

To our knowledge, this was the first study to show differential gene expression patterns in various subgroups of SLE patients stratified by ancestral background and presence or absence of anti-RBP antibodies. Through microarray whole genome expression with pathway analysis followed by independent qPCR validation, we demonstrated that activation of IFN-related pathways depended on presence of anti-RBP antibodies in AA patients, but not in EA patients. The results also support the model suggested by our previous study in which African ancestry increases the likelihood of SLE-associated autoantibody formation, leading to higher IFN-α activity (41). In the present study, we observe a similar dependence of IFN-induced gene expression upon anti-RBP antibodies in AA patients, and this is not shared with the EA patients, and this novel observation should be confirmed in larger cohorts.

Autoantibody immune complexes present in SLE patients have been implicated as major endogenous IFN-inducers, likely via the endosomal TLR and IFN regulatory factor pathways (43–45). Our data would suggest that the classical activation of IFN-related pathways observed in SLE patients is highly dependent upon anti-RBP antibodies in AA SLE patients, and this dependence is not shared by EA SLE patients. The additional IFN-pathway activation observed in EA subjects is partly due to the presence of anti-dsDNA antibodies. As shown in **Figure 3** there are a number of anti-dsDNA and anti-RBP negative EA patients that show over-expression of IFN-induced genes, while in AA SLE patients lacking RBP antibodies, IFN-induced gene expression resembles the AA control population. This heterogeneity in the dependence of IFN-related pathways on autoantibody profile may reflect differential activation of the TLR pathway is SLE patients of different ancestral backgrounds. Anti-RBP antibodies would be expected to activate the RNA-sensing TLRs, while anti-dsDNA antibody immune complexes would be expected to activate TLR 9. Genetic variations in the TLR pathway genes such as IRF5 and IRF7 have been associated with risk of SLE, and with gain of function within the type I IFN pathway (46, 47). In our previous study looking at genetic variation at the IRF7/PHRF1 locus, we observed two different high IFN genetic effects in AA subjects, while we saw only one in EA subjects (46). This example demonstrates genetic diversity between world populations in the TLR/IRF system, and could support the idea that this pathway may more prominent in AA subjects and help to explain the findings we report here. Additionally, many of the genetic polymorphisms we have discovered that are associated with increased type I IFN in SLE patients differ between ancestral backgrounds (34, 48), and this would also support the idea that the pathway will be more or less prominent in different ancestral backgrounds.

There were EA SLE patients that had increased expression of IFN-induced genes who did not have either anti-RBP or anti-dsDNA autoantibodies. These data suggest that IFN-related pathways may be activated through different mechanisms in this

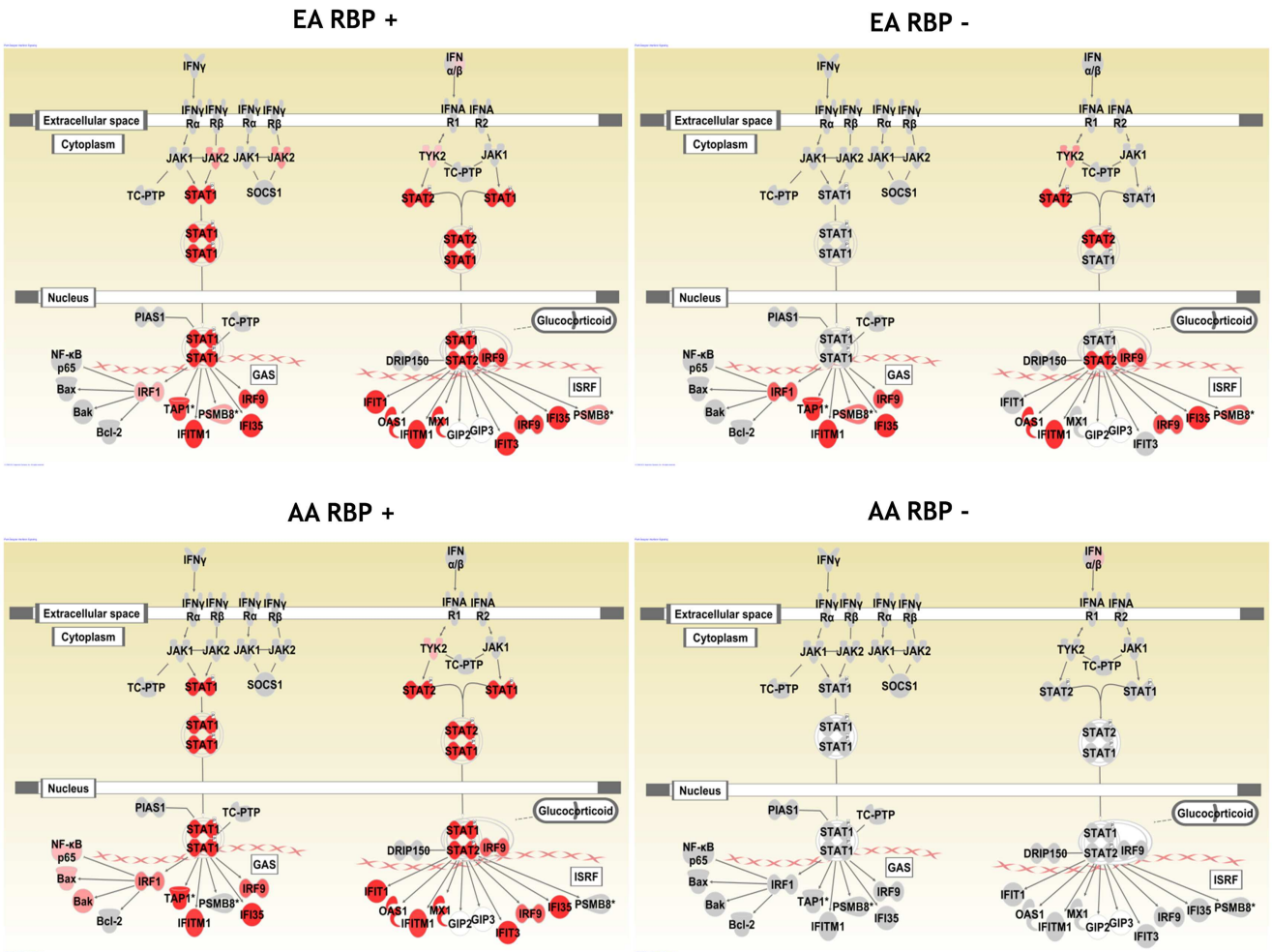


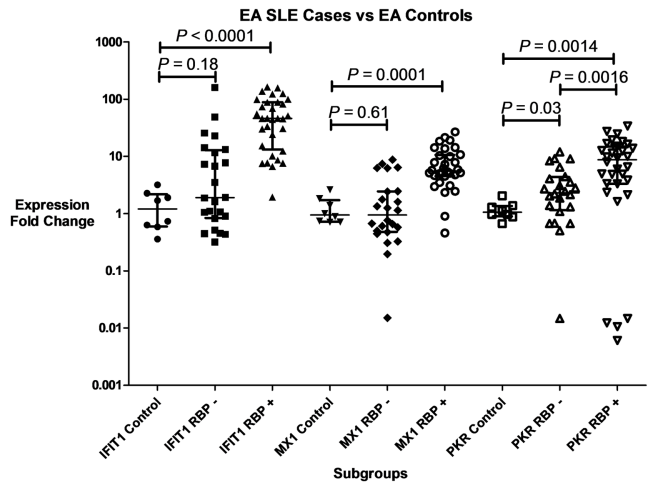
FIGURE 1 | Pathway diagram illustrating the type I and type II IFN pathways in SLE patient subsets. Genes which are up-regulated are shaded red, with increasingly dark red shading indicating a greater degree of over-expression in cases as compared to controls of the same ancestral background. AA, African-American; EA, European-American; RBP+, anti-RNA-binding-protein (RBP) antibody positive; and RBP−, RBP antibody negative. Pictures generated using Ingenuity Pathway Analysis software.

ancestral background. All patients in our study had ANA, and it may be that other nuclear antigen/autoantibody complexes could have triggered the TLR/RLR system leading to IFN-pathway activation in these subjects. It is also possible that other molecules such as HMGB1, which can bind with immune complexes, may be activating an inflammatory cascade in plasmacytoid dendritic cells resulting in activation of IFN pathways (49). Another possibility is that there is an increased sensitivity to IFN signaling or a downstream activator of IFN-induced gene expression in these patients. We have observed some SLE patients in previous studies that have high IFN-induced gene activity in their PBMC with essentially normal circulating type I IFN activity from the same sample (50, 51).

This surprising diversity in IFN-pathway activation between different SLE patient subgroups is relevant to clinical care, as therapeutics directed at IFN or IFN-related pathways are being actively developed (52). It seems likely that these IFN-pathway targeting therapeutics will be characterized by heterogeneity in treatment response, and our results may suggest some groups that are likely to be better responders that could be predicted without having to run a gene expression chip prior to therapy. The RBP− AA group is very interesting in this regard, as it seems that this patient group may represent a distinct subset of SLE patients which is not as IFN-dependent as other groups of SLE patients. This may represent a significant difference in disease pathogenesis, which could be important in planning targeted therapies.

CONCLUSION

Systemic lupus erythematosus is a heterogeneous disease with differences in disease incidence, clinical manifestations, serological findings, and genetic risk factors between ancestral backgrounds (7, 29–32, 36–39). Perhaps it is not very surprising to find heterogeneity in the activation of molecular pathways between ancestral groups, and it seems likely that different



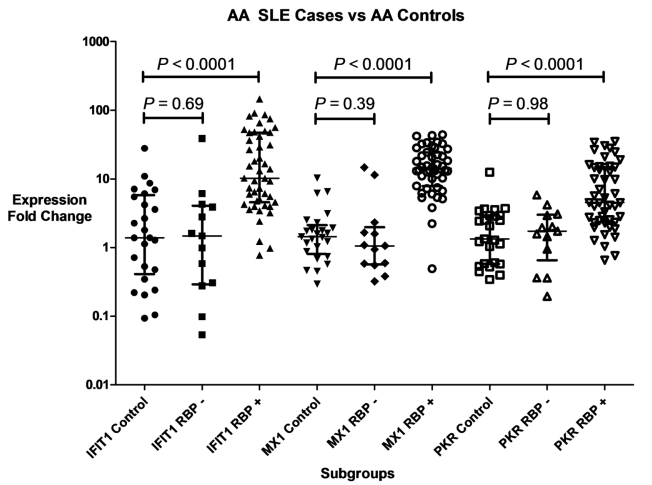
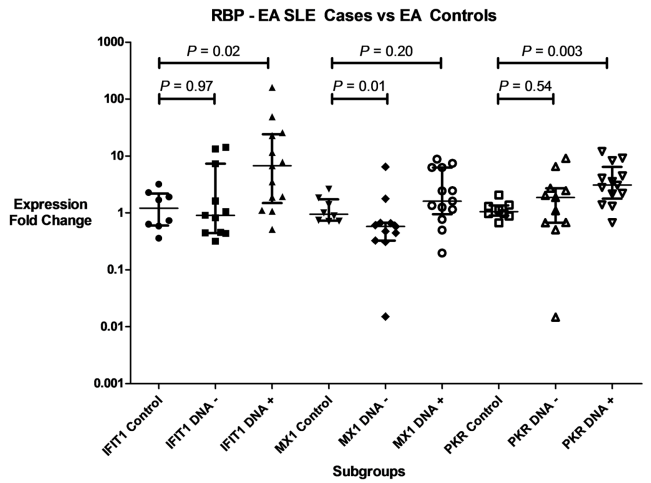


FIGURE 2 | Type I IFN-induced gene expression in SLE patient subgroups and controls. Expression of three genes (IFIT1, MX1, and PKR) are shown in both patients with anti-RNA-binding protein antibodies (RBP+), and those who lack those antibodies (RBP−). Central tendency shown is a median, with error bars representing the interquartile range. *P* values generated by Mann–Whitney *U* test.



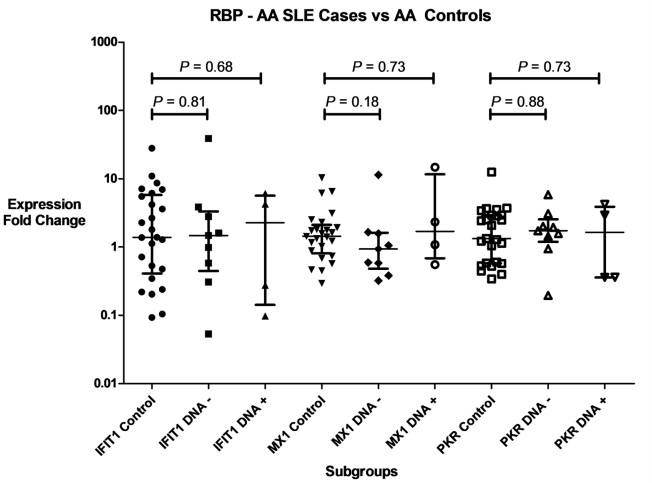


FIGURE 3 | Type I IFN-induced gene expression in RBP−SLE patient subgroups and controls in regards to of anti-dsDNA antibodies. Expression of three genes (IFIT1, MX1, and PKR) are shown in both patients with anti-dsDNA antibodies (DNA+), and those who lack those antibodies (DNA−). Central tendency shown is a median, with error bars representing the interquartile range. *P* values generated by Mann–Whitney *U* test.

pathogenic factors will be relevant in RBP− AA patients as compared to the RBP+ SLE patients. SLE is a complex autoimmune disease, and understanding heterogeneity in the molecular pathogenesis in lupus will be crucial in informing therapeutic and diagnostic strategies. This study demonstrates the relevance of careful patient characterization and including patients from more than one ancestral background in biological studies of SLE.

AUTHOR'S CONTRIBUTIONS

Kichul Ko participated in the design of the study and its coordination, screened for cases and controls, lead the statistical analysis, and drafted the manuscript. Yelena Koldobskaya helped screen for cases and controls, and collected microarray data. Elizabeth Rosenzweig helped generate qPCR data. Timothy B. Niewold was the senior scientist overseeing the study and helped with study design, statistical analysis, and manuscript drafting.

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Four promoters of IRF5 respond distinctly to stimuli and are affected by autoimmune-risk polymorphisms

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Introduction: Autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis affect millions of people worldwide. Interferon regulatory factor 5 (IRF5) contains polymorphisms associated with these autoimmune diseases. Two of these functional polymorphisms are found upstream of the IRF5 gene. rs2004640, which is a single nucleotide polymorphism and the CGGGG insertion/deletion (indel) were studied. IRF5 uses four different promoters for its four first exons: 1A, 1B, 1C, and 1D. Each promoter was analyzed, including functional differences due to the autoimmune-risk polymorphisms.

Results: IRF5 promoters were analyzed using ChIP-Seq data (ENCODE database) and the FactorBook database to define transcription factor binding sites. To verify promoter activity, the promoters were cloned into luciferase plasmids. Each construct exhibited luciferase activity. Exons 1A and 1D contain putative PU.1 and NFkB binding sites. Imiquimod, a Toll-like receptor 7 (TLR7) ligand, was used to activate these transcription factors. IRF5 levels were doubled after imiquimod treatment ($p < 0.001$), with specific increases in the 1A promoter (2.2-fold, $p = 0.03$) and 1D promoter (2.8-fold, $p = 0.03$). A putative binding site for p53, which affects apoptosis, was found in the promoter for exon 1B. However, site-directed mutagenesis of the p53 site showed no effect in a reporter assay.

Conclusion: The IRF5 exon 1B promoter has been characterized, and the responses of each IRF5 promoter to TLR7 stimulation have been determined. Changes in promoter activity and gene expression are likely due to specific and distinct transcription factors that bind to each promoter. Since high expression of IRF5 contributes to the development of autoimmune disease, understanding the source of increased IRF5 levels is key to understanding autoimmune etiology.

Keywords: IRF5, alternative promoters, autoimmune disease risk, interferon, systemic lupus erythematosus

INTRODUCTION

Alternative splicing is a method of making different transcripts from one genomic region. One type of alternative splicing involves the use of multiple first exons. This is termed alternative promoter splicing, since each first exon must have its own promoter. Alternative promoter splicing occurs in around half of human genes (1).

The gene interferon regulatory factor 5 (IRF5) is a transcription factor which controls immune signaling, cytokine expression, the cell cycle, and apoptosis (2–5). It exhibits alternative promoter splicing and has four different first exons that are currently known. The start codon for IRF5 is in exon 2, therefore the use of different first exons does not directly alter the protein sequence. Instead the four alternative promoters are four pathways to make the same protein. The first exons are 1A, 1B, 1C, and 1D.

The IRF5 gene contains several GWAS-identified polymorphisms associated with autoimmune diseases. Among them, most do not have an assumed effect. Although IRF5 contains several polymorphisms associated with autoimmunity, only four have been identified as functional polymorphisms (6). Two of these are in the promoter or untranslated regions of IRF5 where the polymorphisms may have a direct effect on IRF5 expression: a single nucleotide polymorphism (SNP) near exon 1B called rs2004640, and a copy-number variant near exon 1A called rs77571059 (**Figure 1**). The rs77571059 polymorphism is an insertion/deletion (indel) of 5 bp, and is commonly referred to as a CGGGG indel. This study examines the promoters of IRF5, with information on how these two functional polymorphisms play a role in IRF5 expression. A general trend of these polymorphisms is to increase levels of IRF5.

The rs2004640 SNP is a G or T polymorphism near the 3′ end of exon 1B. The SNP is within the splice junction, such that when the G allele is present, the splice junction is not recognized and exon 1B cannot be spliced onto exon 2 (8). Unspliced transcripts are usually targeted by non-sense mediated decay (9). The risk T allele at this locus is associated with systemic lupus erythematosus (SLE) in multiple ethnic groups (8, 10–13) rheumatoid arthritis (14, 15), systemic sclerosis (16), multiple sclerosis (17), ulcerative

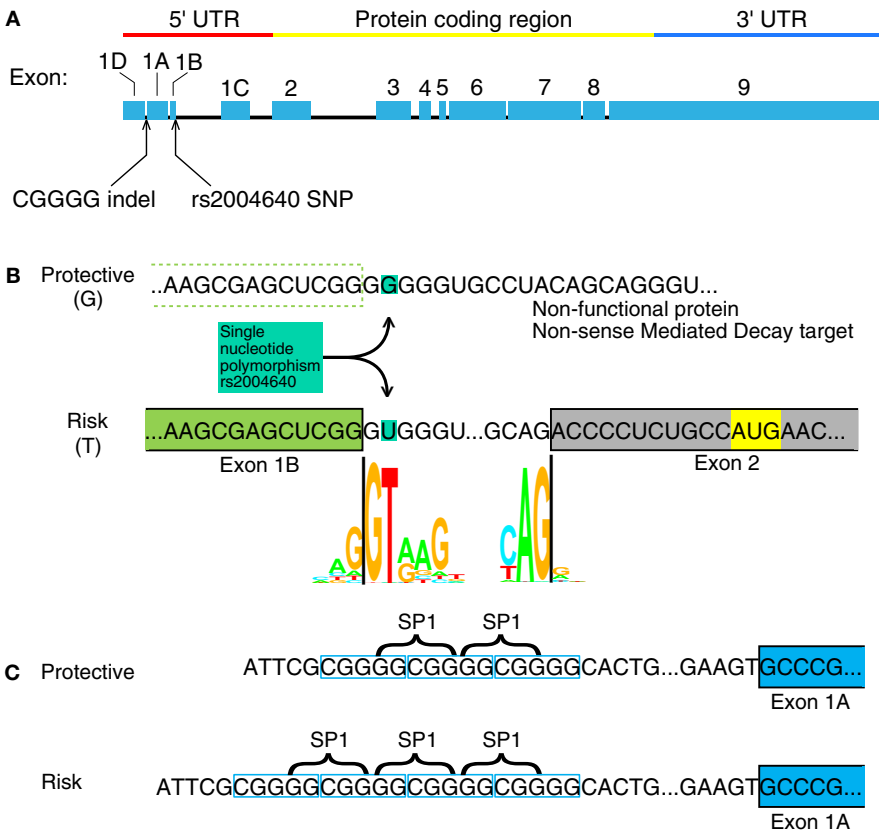

FIGURE 1 | Interferon regulatory factor 5 mRNA and the position of the rs2004640 SNP and CGGGG indel. **(A)** rs2004640 is at the splice acceptor site for exon 1B, and the CGGGG indel is 64 bp upstream from the transcription start site for exon 1A. The genomic region of IRF5 is drawn to scale, but with introns reduced in size 10:1. The protein coding and untranslated regions are shown above. Only one first exon is used per RNA transcript; therefore each first exon corresponds to the untranslated region for that transcript. **(B)** The position of the rs2004640 SNP on pre-mRNA. Before splicing, the messenger RNA has either a U (encoded by the risk T allele) or G. The colored letters shown in the WebLogo (the nucleotide stacks of varying heights represent the consensus recognition sites for the spliceosome. The height of the stack represents how often those nucleotides are found at that position, and thus the high GT represents a strong preference for recognizing GT at the intron boundary. This matches in the risk T allele (GT at the intron boundary), but not the protective allele (GG at the intron boundary). A person homozygous for the protective allele cannot splice IRF5 mRNA that begins with exon 1B. Instead of a functional protein, the resultant mRNA would encode a non-functional protein and be targeted for non-sense mediated decay. Splice junction WebLogos are from Stephens and Schneider (7). **(C)** The CGGGG indel is an insertion/deletion of a CGGGG repeat upstream of exon 1A, and it is part of exon 1A's promoter. When there are four copies, additional SP1 transcription factors (which bind to GGCGG) can bind to the promoter, altering transcription levels. UTR, untranslated region; SNP, single nucleotide polymorphism.

colitis (18), and Sjögren's syndrome (19). Autoimmune-risk haplotypes that include rs2004640 exhibit high IRF5 levels (6, 20), as well as high levels of IFNα and TNFα (21, 22).

The CGGGG indel (rs77571059) is found 64 bp upstream of the transcription start site for exon 1A. Each allele has either three (3×) or four copies (4×) of the CGGGG repeat sequence. The 4× copy-number variant allows binding of additional SP1 transcription factors (23). This 4× variant is associated with SLE (10), Sjögren's syndrome (24), multiple sclerosis (17), Crohn's disease and ulcerative colitis (18), and acute coronary syndrome (25). The CGGGG 4× variant is associated with increased expression of IRF5 itself (23), as well as TNFα, IL-12p40, IL-8, IL-1b, and IL-10 (22).

Interferon regulatory factor 5 exons 1A, 1B, 1C, and 1D each have a distinct transcriptional start site, and as is the case with every first exon, each exon 1 of IRF5 has its own promoter. IRF5's four promoters have not been thoroughly characterized, although previous studies on the 1A and 1C exons' promoters revealed that they are controlled in part by an IRF element (IRFE) and an interferon stimulatory response element (ISRE), respectively (26). Herein, we identify and characterize a putative promoter for exon 1B, and hypothesize that the 1B promoter would be active and regulated by stimuli that activate IRF5. We further hypothesize that the 1B promoter would be regulated by p53.

Autoimmune diseases are caused by environmental triggers in those with a genetic propensity. Increases in IRF5 expression due to these promoter polymorphisms could lead to an autoimmune-risk state. A hallmark of lupus and those at genetic risk for lupus is the presence of heightened levels of interferon and interferon-response genes; the interferon signature (27). IRF5 is a key gene in the interferon response to viral infection. IRF5 is a transcription factor whose activation leads to the interferon signature and the control of multiple genes involved in inflammation and immunity (28). It is primarily expressed in B cells, monocyte-derived cells, and plasmacytoid dendritic cells (pDCs) (2).

For SLE, an environmental trigger is likely to be Epstein–Barr virus (EBV) infection (29, 30). EBV infection affects IRF5 and IRF7 signaling, and has been associated with lupus through several different mechanisms (30–32). Interestingly, EBV infection alters IRF5 splicing to produce a dominant negative variant, suppressing the interferon response (33). For these studies, EBV-infected B cells are used, because cells with the appropriate genotypes can be immortalized and used in multiple experiments. As B cells, these cells are relevant to autoimmune disease and express IRF5. The incorporation of EBV into the model cells means that our results must be interpreted with caution, as it is possible that the major effects of these risk polymorphisms regulate or alter EBV infection, not IRF5 directly. These results must therefore be interpreted with caution. However, if it is found to be the case that these IRF5 polymorphisms affect EBV infection, that would likely provide even more exciting directions to pursue given the potential relationship between EBV infection and lupus.

RESULTS

IRF5'S FOUR PROMOTERS

Interferon regulatory factor 5 uses one of four first exons for each molecule of mRNA – 1A, 1B, 1C, or 1D. Whether or not one of the four first exons of IRF5 would be actively transcribed depends on the cellular transcription factors that are able to bind it. A putative IRF5 exon 1B promoter sequence was identified by using the encyclopedia of DNA elements chromatin immunoprecipitation sequencing (ENCODE ChIP-Seq) data set (34). This analysis includes a list of transcription factors known to bind to the putative promoter sequence. An analysis of the promoters for each of the other three first exons of IRF5 was performed using the same database. This list represents results from many experiments which show transcription factors that bind to this genomic region of DNA (**Figure 2A**).

The transcription factors listed have also been associated with specific binding sites. WebLogos, which visualize consensus

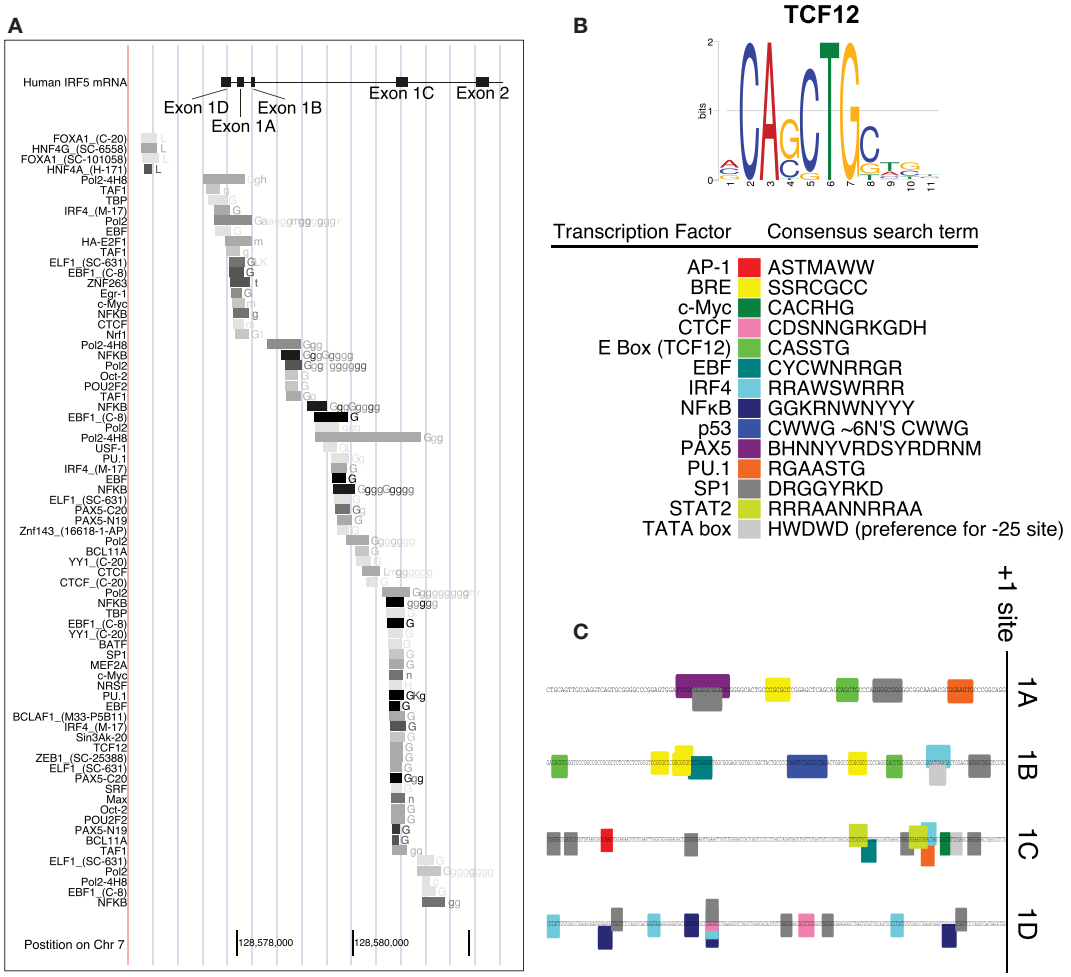

FIGURE 2 | Promoter analysis of each first exon of IRF5. **(A)** ENCODE data shows results of ChIP-Seq analyses in the promoter region of IRF5. **(B)** The consensus search terms generated from FactorBook, with the TCF12 consensus binding site as an example (35). This data was used to manually define the nucleotide search terms shown. **(C)** The final analysis of potential binding sites is shown along the genomic DNA promoter sequences, with color-coded boxes representing the binding sites or transcription factors shown in the key. AP, activator protein; BRE, B-response element; CTCF, CCCTC binding factor; TCF, transcription factor; EBF, early B cell factor; IRF, interferon regulatory factor; NFκB, nuclear factor kappa light chain enhancer of activated B cells; PAX, paired box; PU, purine rich; SP, specificity protein; STAT, signal transducer and activator of proteins; TATA, thymidine adenine.

binding sites (36), were generated *de novo* for each ENCODE transcription factor tested, and compiled in the online database FactorBook (35). The consensus sites were converted manually into an ambiguous DNA code search term, where for example W (weak) represents an A or a T nucleotide (**Figure 2B**). The consensus search term was then used to search the proximal promoters (∼200 bp upstream from the +1 sites) to encounter a proposed binding site. Consensus search term screening was performed using MEGA (37).

Several transcription factors' binding sites were found in the regions upstream of transcription start sites (**Figure 1C**). The start sites were taken from reference sequences for exons 1A, 1B, and 1C, and the sequence for variant 12 of IRF5 for exon 1D (no reference sequence exists at present for exon 1D). The source sequences are GenBank IDs NM_002200.3, NM_032643.3, NM_001098627.2, and EU258897.1 for exons 1A, 1B, 1C, and 1D, respectively. The workflow and results are shown in **Figure 2**, with transcription factor 12 (TCF12) as an example.

EACH IRF5 PROMOTER EXHIBITS TRANSCRIPTIONAL ACTIVITY

The promoters for the four first exons of *IRF5* contain different potential transcription factor binding sites. The 1A promoter contains putative binding sites for paired box 5 (PAX5), PU.1, SP1, and TCF12 which binds to enhancer boxes (E boxes). An extra SP1 binding site appears in those with the CGGGG 4× indel. Exon 1B's promoter was the only IRF5 promoter with a p53 binding site. This is discussed in more detail below. 1B also has SP1, TCF12, IRF4, and early B cell factor (EBF) sites. The 1C promoter was the only promoter with STAT2, activator protein 1 (AP1), and Myc binding sites; it also has SP1 and IRF4 sites. The 1D promoter evaluation showed potential binding sites for only four transcription factors: SP1, CCCTC binding factor (CTCF), IRF4, and NFκB.

To determine activity levels of each promoter, they were cloned using PCR and inserted into luciferase reporter plasmids. In addition to the 1B, 1C, and 1D promoters, there are two distinct versions of the 1A promoter, representing the two rs77571059 polymorphisms. One has the 4× variant of the CGGGG indel ($1A_{risk}$), and the other has the 3× variant ($1A_{protective}$). The 1B promoter was cloned using nested PCR to avoid an inverted repeat sequence located ∼2 kbp upstream. The inverted repeat is 1.8 kbp in length, and the two copies have 82.8% identity (34).

A luciferase assay was performed using the pGL4 plasmid. The promoters of IRF5 were inserted upstream of the luciferase gene and promoter activity was evaluated by measuring luminescence. The activity levels of the promoters were analyzed in several cell types since distinct transcription factors would be active in different cell types. Three types of immune cells were used: lymphoblastoid cell lines (LCLs), EBV-transformed human B cells that were generated from three healthy volunteers; U937 cells, a commercially available human monocyte cell line; and Jurkat cells, a commercially available human T cell line. Jurkat cells were used as the negative control, since T cells do not express high levels of *IRF5*. Cells were electroporated with each of the IRF5 promoter luciferase plasmids. A second plasmid, which expresses enhanced green fluorescent protein (eGFP), was cotransfected as a transfection control for each construct (38). Values for luciferase expression were then normalized to the fluorescence level to account for transfection efficiency.

Luciferase assay results showed that the 1A promoters ($1A_{risk}$ and $1A_{protective}$) demonstrated significantly higher transcriptional activity than the other three promoters in LCL and Jurkat cells ($p = 0.0009$ and $p = 0.016$, respectively) (**Figure 3**). As expected,

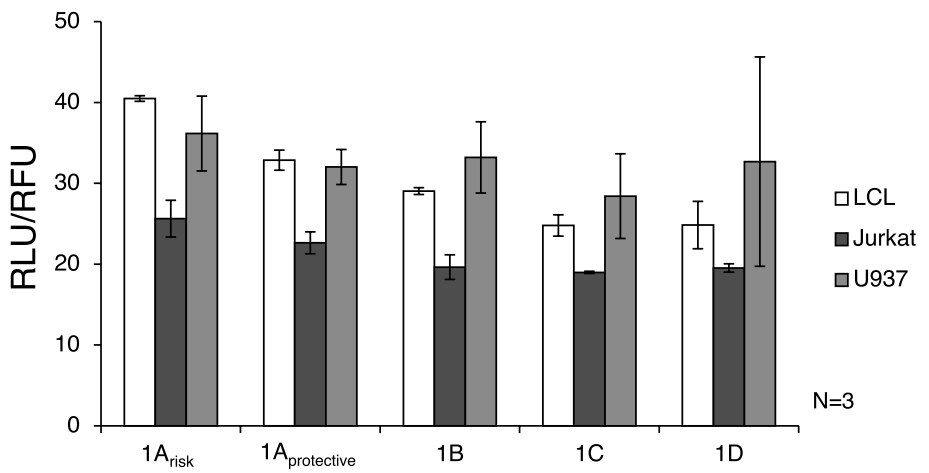

FIGURE 3 | Interferon regulatory factor 5 promoter activity in immune cells. The luciferase plasmids were transfected by electroporation of three cell types: LCL, U937 cells, and Jurkat cells. A control GFP-encoding plasmid was also used in each sample to normalize transfection efficiency. ANOVA analysis revealed statistically significant variation between groups ($p = 0.014$); therefore *t*-tests were used to determine where the variation was found. The levels of transcription were significantly lower in Jurkat cells compared to LCL and U937 cells ($p < 0.01$). IRF5 is not highly expressed in T cells such as Jurkat cells, but is normally expressed in B cells and monocytes (39). The 1A promoters ($1A_{risk}$ and $1A_{protective}$) displayed higher activity than the 1B, 1C, or 1D promoters in LCL and Jurkat cells ($p = 0.0009$ and $p = 0.016$, respectively). In LCLs the $1A_{risk}$ promoter activity was higher than $1A_{protective}$ promoter activity ($p = 0.019$). The putative 1B promoter acted like the 1A promoter in that expression was significantly higher in LCLs than in Jurkat cells ($p = 0.027$). LCL, lymphoblastoid cell line; RLU, relative luminescence units; RFU, relative fluorescence units. In some samples, the first exon was not detectable, which is why there is some variation in sample number.

expression from all IRF5 promoters was significantly lower ($p < 0.01$) in Jurkat cells when compared to U937 or LCL cells. When comparing LCL to U937 cells, there was no significant difference in IRF5 promoter activity ($p = 0.38$).

The autoimmune-risk polymorphisms affected the activity of the promoters. In LCLs the $1A_{risk}$ promoter activity was significantly higher than $1A_{protective}$ promoter activity ($p = 0.019$). The 1B promoter, which is only relevant when the risk allele rs2004640 is present, showed activity in LCLs and U937 cells, indicating that it is an active promoter in the same cell types as the other promoters.

THE 1A AND 1D PROMOTERS ARE AFFECTED BY TLR7 LIGATION

The levels of IRF5 expression increase due to several signaling pathways, one of which is the Toll-like receptor 7 (TLR7) pathway. Endosomal TLRs such as TLR7 require the ligand to first be endocytosed into the cells, and then merged with the endosome that contains TLR7. Most endosomal TLRs bind to nucleic acids.

Toll-like receptor 7 ligation is an important method of activation for pDCs (40, 41). pDCs can produce large amounts of interferon alpha in response to immunostimulatory molecules such as nucleic acids. This is an important activation pathway in autoimmune disease (42). Single stranded RNA is the natural agonist for TLR7. TLR7 can also be activated by small synthetic compounds such as the imidazoquinolines, namely imiquimod and resiquimod. Imiquimod is a TLR7 ligand and resiquimod is a ligand for TLR7 and TLR8 (43). Imiquimod is used clinically as a topical cream as a form of treatment for genital warts and certain cancers. It activates the immune system, recruiting inflammatory mediators to kill the virus-infected or cancerous cells (44).

To verify that imiquimod treatment was stimulating the cells through TLR7, gene expression of interferon-response genes and cytokine gene expression were monitored using real-time PCR. Imiquimod stimulation led to significantly increased expression of the interferon-induced genes CCR7 and NOXA, while expression of the calreticulin was not significantly affected (**Figure 4**). Expression of the genes for the cytokine IL-6 was substantially upregulated (71-fold increase, $p = 0.028$). IL-6 expression is a common readout for stimulation through TLR7 (45, 46). Expression of the cytokine IL-10 is also significantly, though slightly, increased (1.5-fold, $p = 0.038$) after treatment with imiquimod (**Figure 4**). These results indicate that imiquimod treatment did in fact stimulate the cells.

Imiquimod treatments were performed to determine the effects of stimulation on the activity of each IRF5 promoter. First exon-specific quantitative PCR was used to determine changes in the levels of each first exon after imiquimod stimulation. Cells were treated with imiquimod at 25 μg/ml for 24 h, and then cDNA was prepared from an RNA extract of treated cells. This was done for LCLs generated from 20 healthy individuals. As expected, IRF5 levels increased when cells were treated with imiquimod – a 1.9-fold increase when normalized to the housekeeping gene, β-glucuronidase (β-GUS) (**Figure 5A**).

The amounts of each first exon were also measured and compared to β-GUS by quantitative PCR. Several samples yielded undetectable levels of first exon transcripts, and were thus not included in the analysis, resulting in the variation in sample number noted in **Figure 5**. The levels of exons 1A and 1D increased by at least twofold after treatment with imiquimod when compared to β-GUS (**Figure 5B**).

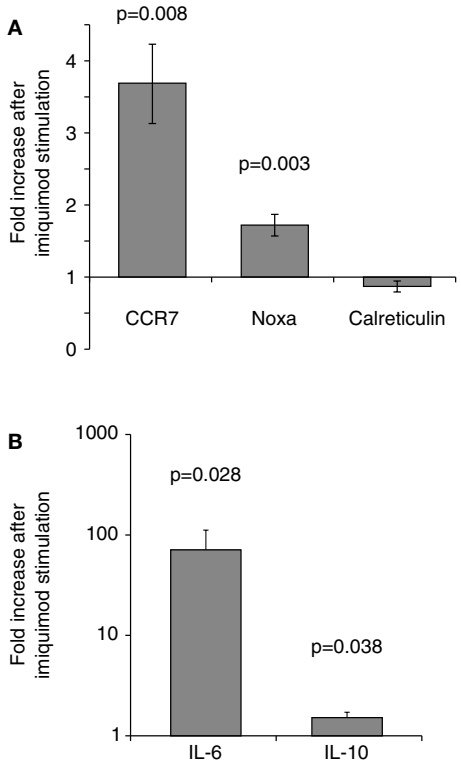

FIGURE 4 | Imiquimod stimulates LCLs to express cytokines and interferon-response genes. LCLs were generated by EBV infection of cells from healthy volunteers. Cells were treated with imiquimod. **(A)** Expression of the interferon-response genes CCR7, NOXA, and Calreticulin were measured using SYBR Green real-time PCR. The figure shows the fold increase in gene expression after imiquimod stimulation for each gene. CCR7 and NOXA were significantly upregulated after imiquimod stimulation ($p = 0.008$ and $p = 0.003$, respectively). **(B)** Expression of cytokine RNA was measured using SYBR Green real-time PCR. IL-6 expression was upregulated by 71-fold after imiquimod stimulation ($p = 0.028$). IL-10 expression was also significantly upregulated ($p = 0.038$), although to a much lesser extent, at 1.5-fold. $N = 12$ for each experiment.

The effect of the rs2004640 polymorphism on imiquimod stimulation on was examined using real-time PCR. LCLs with risk or protective genotypes were stimulated with imiquimod, and the change in expression of interferon-stimulated genes was compared between risk and protective cells. IRF5 expression was higher in risk cells by nearly 1.7-fold (p-0.021). CCR7 did not increase as much after imiquimod stimulation in the risk cells compared to the protective (0.77-fold, $p = 0.05$). However, the risk cells demonstrated a small increase in NOXA expression after imiquimod stimulation, and the Calreticulin levels decreased less (by 1.35-fold, $p = 0.05$) in the risk cells than in the protective (**Figure 6**). These results show a small, but consistent increase in responsiveness to imiquimod stimulation in the cells with the risk allele.

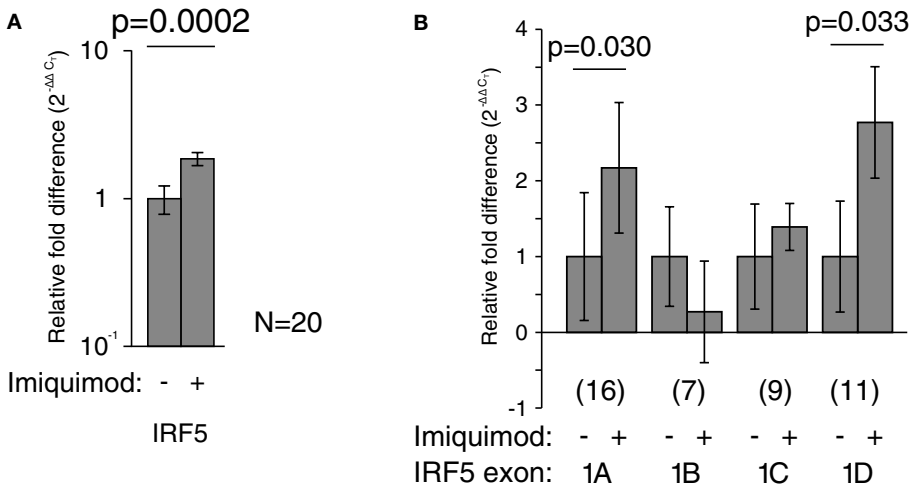

FIGURE 5 | Imiquimod caused increased IRF5 transcription through exons 1A and 1D. All mRNA levels were measured in LCLs generated from healthy individuals. Levels were determined by TaqMan-based quantitative PCR using the $2^{-\Delta\Delta CT}$ method. **(A)** The levels of IRF5 were 1.9-fold higher in treated cells ($p = 0.0002$). **(B)** The levels of exon 1A increased 2.2-fold ($p = 0.030$) and exon 1D increased by 2.8-fold ($p = 0.033$). All fold-increase values were normalized to the β-GUS housekeeping gene. The numbers in parentheses indicate the sample size. Analysis of variance was performed including each first exon and stimulation state as groups. This analysis revealed statistically significant variation ($p < 0.0001$). Statistical significance between individual groups was determined by paired *t*-test. IRF, interferon regulatory factor.

The effect of EBV infection on IRF5 expression and imiquimod stimulation were analyzed. Ramos cells, a B cell line that is similar to LCL but is EBV negative, were stimulated with imiquimod and expression of IRF5 was compared to IRF5 expression in LCLs. After two experiments, there was a <1.2-fold difference in IRF5 expression between the EBV positive and EBV-negative cell lines. After imiquimod stimulation, there was similarly only a very small difference between the EBV positive LCL and EBV-negative Ramos cells (**Figure 6B**).

THE rs2004640 SNP'S ROLE IN p53 BINDING AND ACTIVATION

Mutagenesis of the 1B promoter p53 binding site did not alter transcriptional activity.

The promoter analysis described above revealed a potential p53 binding site. p53 binds as a tetramer to two copies of the sequence rrrCwwGyyy, with a spacer of 0–13 nt between the copies (189). A close match to this sequence was found in the 1B promoter (**Figure 7B**). This is suggestive because of the potential role IRF5 may play in apoptosis dysregulation in SLE. IRF5 is also proapoptotic in a p53-independent manner (47), and thus if p53 activates IRF5, apoptosis levels would be additively altered. Should p53 can control the 1B promoter, apoptosis would be altered in rs2004640 risk cells because the 1B promoter is only used in cells with the rs2004640 risk allele.

To test whether activated p53 protein can indeed bind to the p53 binding site, the plasmid which contains the 1B promoter was mutated using site-directed mutagenesis. Mutations were made to the wild-type sequence such that p53 should not be able to bind. The consensus binding site contains four conserved C or G bases which were mutated to A or T on the luciferase plasmid (**Figure 7B**). The wild type and p53-mutant luciferase plasmids were transfected by electroporation into three different LCLs generated from healthy volunteers. After 24 h to allow for plasmid expression, cells were either treated with etoposide or left untreated for 48 h. The levels of luciferase activity stayed fairly constant in the wild-type plasmid when treated with the etoposide. However, when the p53 binding site was mutated, thus inhibiting binding of p53, there is an slight, but non-significant increase in activity when treated with the etoposide versus being left untreated (**Figure 7A**). This finding suggests that if p53 does in fact bind to the IRF5 exon 1B promoter, it is likely inhibitory rather than stimulatory.

DISCUSSION

The CGGGG indel polymorphism within the 1A promoter has previously been shown to alter transcription factor binding. When cells have the 4× variant, an additional SP1 binding site is created. This has been shown to increase IRF5 in PBMCs (48), but decrease 1A-specific IRF5 transcripts in thymic cells (49). Both versions of the 1A promoter showed activity in HEK293T cells and Raji cells. As expected, SP1 sites were found in our analysis of the 1A promoter, including an extra SP1 binding site in those with the CGGGG 4× indel. SP1 is active during development, cell growth, apoptosis, differentiation, and immune and DNA damage responses (50).

The 1A promoter has a PAX5 binding site, a gene that activates B cells at early, but not late stages of development (51). There is an E box, and TCF12 is a member of the basic helix-loop-helix group of transcription factors which binds to E boxes (52). TCF12 was shown to bind somewhere in the promoter region of IRF5 in the ENCODE dataset (34), and the putative E box in 1A's promoter is a likely site. TCF12 is known to be expressed in B cells and T cells (39). A PU.1 site is in the 1A promoter as well; PU.1 activates gene expression during B cell development and in myeloid cells (53).

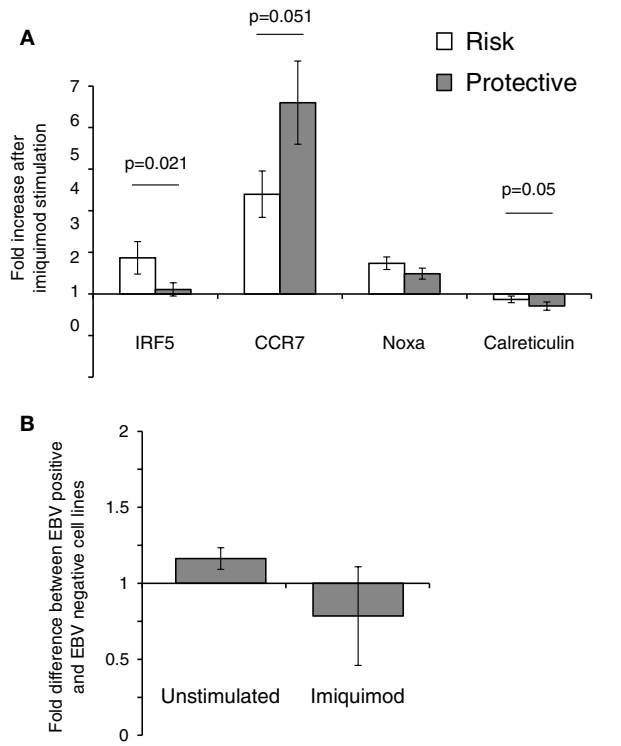

FIGURE 6 | The 2004640 risk allele affects responsiveness to TLR7 stimulation. **(A)** LCLs with either risk or protective genotypes were treated with imiquimod to stimulate TLR7. Expression of interferon-response genes before and after stimulation was compared using SYBR Green real-time PCR. RNA input was normalized for each sample using the housekeeping gene GAPDH. Increase after imiquimod stimulation for risk and protective cells is shown. IRF5 expression is higher after imiquimod stimulation in the risk cells, by 1.67-fold ($p = 0.021$). Although Calreticulin expression decreases in both the risk and protective cells, it does so less in the risk cells (p-0.05). CCR7 increases less in the risk cells after imiquimod treatment ($p = 0.05$) $N = 12$ **(B)**. EBV status does not dramatically effect IRF5 levels. IRF5 expression was compared between LCLs and Ramos cells, a Burkitt's lymphoma-derived B cell line that is EBV negative. IRF5 levels were not dramatically different between cell lines $N = 2$.

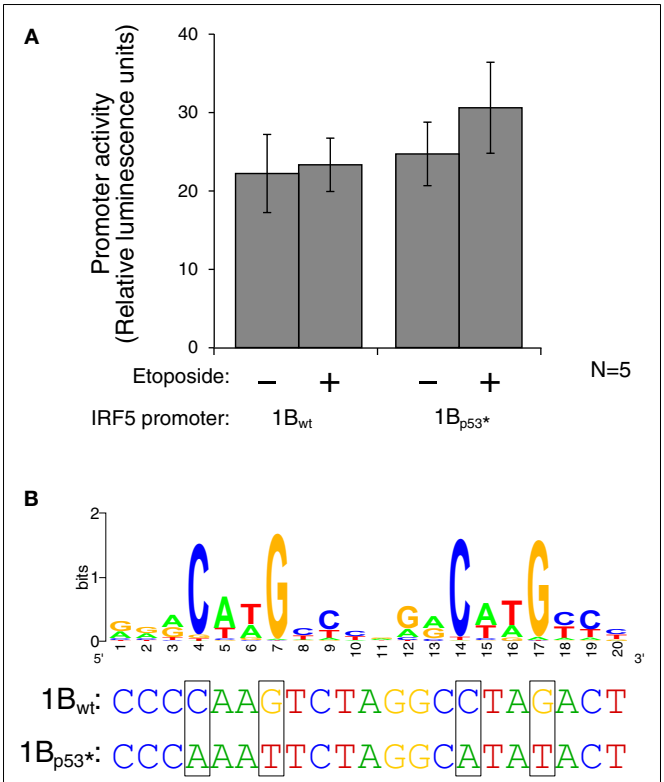

FIGURE 7 | Interferon regulatory factor 5 1B promoter activity is not regulated by direct p53 binding. **(A)** Different LCLs generated from healthy volunteers were electroporated with the $1B_{wt}$ or the $1B_{p53*}$ promoter luciferase plasmid. Cells were treated with 0.5 mM etoposide or left untreated. Despite the presence of a putative p53 binding site, DNA damaging treatment did not affect promoter activity; neither did mutation of the p53 site. **(B)** The putative p53 binding site in IRF5's exon 1B promoter, with a WebLogo of the p53 consensus binding site (29) to indicate important bases and matches. The height of the base represents the frequency of that nucleotide. Site-directed mutagenesis was performed to mutate the binding site at the critical C and G bases as shown. UV, ultraviolet; wt, wild type; *, mutant.

The 1A promoter showed increased activity when cells were stimulated with the TLR7 agonist imiquimod. This may be through the PU.1 site through IRF7. IRF7 is known to be activated by TLR7 (54), and PU.1 binds to a similar $GAAN_{(N)}GAA$ motif to IRFs. Further work is necessary to determine in which cell types or with which stimuli the 1A promoter is most active, and in what instances the CGGGG 4× variant alters this activity.

A previous report by Mancl et al. evaluated the 1A and 1C promoters (26). The 1A promoter was activated by herpes simplex, Newcastle disease and vesicular stomatitis viruses in PBMCs, Daudi, and THP-1 cells; respectively; as evidenced by increased transcription of IRF5. A luciferase reporter gene assay also showed that IRF5's 1A promoter is constitutively active and contains an IRFE consensus binding site. However, the promoter region used was a 596-bp region determined by a 5′ rapid amplification of cDNA ends (5′RACE) experiment and is 939 bp upstream of the GenBank reference sequences for exon 1A, and even extends past the 1D exon by 714 bp. The results of their luciferase assay cannot therefore be compared with the promoter analysis performed in this work. This work narrows the DNA regions studied and separates them into each of the four unique promoter elements, demonstrating that each are active promoters. This work also confirms that the 1A promoter is the strongest and is activated by imiquimod, and that the 1D promoter is also strongly activated by this stimulus.

The ability of a cell to use and splice the 1B exon is independent of its promoter usage. The 1B promoter is active in persons with both the risk and protective polymorphisms at rs2004640, yet the protective sequence would result in a non-sense transcript, as splicing would not be possible. The risk T allele allows for the exon 1B transcript to be spliced onto exon 2 and this is evidenced by the correlation between the risk T allele and increased levels of both IRF5 and exon 1B usage. The effects of the ability to use the 1B promoter can also be seen in the increased responsiveness of the cells containing the risk allele to imiquimod.

Interferon regulatory factor 5's 1B promoter was predicted to contain a p53 binding site. The only promoter tested which increased in activity after inducing DNA damage was the 1B promoter. The others showed a reduction in luciferase activity (data not shown). The mutated version of the 1B promoter, which contained an altered p53 binding site, showed a slight increase in luciferase activity instead of a decrease, likely suggesting that any p53 binding to this promoter region is inhibitory. The 1B promoter contains SP1, IRF4, TCF12, and EBF binding sites. EBF is a B cell-specific transcription factor (55). Further work is necessary to reveal the stimuli or cell types that use the 1B promoter, as well as the combinations of transcription factors that drive transcription.

During cloning experiments dealing with exon 1B and its promoter, several sequencing reactions showed <100% sequence identity to the target. It was soon discovered that the primers were annealing to an upstream inverted repeat sequence. This repeat necessitated nested PCR for cloning the 1B promoter. The repeat length is 1.8 kbp, and the two copies have 82.8% identity (56). The function of this repeat is unknown, but repeated sequences can act as decoys for transcription factors, lowering transcription of the intended target (57).

Usage of exon 1C is lower in cells with the rs2004640 autoimmune-risk factor. The 1C promoter contains putative SP1, IRF4, and EBF sites. It was the only promoter with AP1, Myc, and STAT2 binding sites. AP1 is a heterodimer of Fos and Jun proteins, among others, which are common in immune signal transduction (58). Myc is a proto-oncogene, and is essential for B cell proliferation (59). STAT2, when complexed with STAT1 and IRF9, is known to be activated by type I interferon (60). The STAT2 binding sites agree with a previous report on the 1C promoter of IRF5 by Mancl et al. which said the promoter is interferon responsive (61). The current analysis identified the same STAT2 binding site in the 1C promoter. The difference in the two analyses is the assumed placement of the initiation site. The analysis by Mancl et al. uses 5′RACE to determine the initiation site and they calculate the STAT2 binding site is 96 bp downstream of that transcription initiation site. According to our initiation site – taken from the GenBank reference sequences which use exon 1C – the site was 47 bp upstream of the initiation site. Also of note, cells treated with imiquimod had lower 1C levels in proportion to the total IRF5.

Usage of exon 1D is lower in cells with the rs2004640 T allele and in cells with the CGGGG 4× allele. The 1D promoter evaluation showed only four transcription factors' binding sites: CTCF, IRF4, NFκB, and SP1. NFκB is a target of TLR7 (62), and thus the promoter should be activated by imiquimod treatment. This was the case, and the 1D promoter nearly tripled in usage after imiquimod treatment. The IRF5 promoter analysis also showed a CTCF binding site. It is interesting that the 1D promoter is the furthest exon in the 5′ direction, and has putative CTCF sites, since CTCF is known to block the spread of CpG methylation by acting as an insulator (63). This may keep the other first exons – which are downstream and have high GC content – free from heterochromatin.

Interferon regulatory factor 5 is proapoptotic in a p53-independent manner (64, 65). This does not preclude modulation by p53, and a p53 enhancer site in exon 2 of IRF5 has been shown to activate IRF5 (66). p53 is a main regulator of apoptosis. Exon 1B's promoter was the only one with a putative p53 binding site, and cells with the rs2004640 risk T allele are the only cells that can use exon 1B. Also, p53 can act as both a repressor and activator of transcription depending on local factors (67). However, in our assay, p53 did not significantly regulate the 1B promoter.

Epstein–Barr virus infection is a necessary complicating factor when using LCLs as B cell lines. This is especially important since EBV has been shown to affect IRF5 function (33, 68). The effect of EBV in this study was limited by using EBV-infected LCLs as both our risk and protective cell lines. Since cell lines of both genotypes are transformed with EBV, the differences observed should be comparable and the effect of EBV excluded. However, given the importance of EBV infection in IRF5 activity and the development of lupus, viral effects cannot be simply discounted. There is a chance that differential effects of the risk polymorphisms on EBV infection processes are affecting IRF5 activity. These effects would be difficult to differentiate from direct effects on IRF5 activity. Either way, however, the results would be interesting and merit further investigation.

Autoimmune diseases are complex, multifactorial disorders with both genetic and environmental influences. The promoter variations examined in these experiments are strongly linked to risk for autoimmune diseases, including lupus, multiple sclerosis, and rheumatoid arthritis. Despite much effort, there has not been a dramatic effect associated with these polymorphisms, or really, most of the polymorphisms associated with autoimmune disease. Rather than diminishing their importance, however, the somewhat small effects observed here speak to the fine balance of the immune system. It is likely that even relatively small changes in gene regulation can lead to an imbalance in tolerance or activation of immune cells. Also, these genes are intertwined with other pathways and systems to provide a complex fabric controlling the level of immune responsiveness.

MATERIALS AND METHODS

PLASMID CONSTRUCTION AND LUCIFERASE ASSAY

All vectors were sequenced to confirm the proper sequence. The plasmid pMax-GFP (Clontech) expresses the eGFP fluorescent protein, and it was used to measure transfection efficiency. Electroporations were performed using a Nucleofector device (Lonza). The electroporation buffer was 5 mM KCl, 15 mM $MgCl_2$, 15 mM HEPES, 140 mM Na_2HPO_4, pH 7.2. Transfected cells were lysed and assayed for fluorescence levels before assaying luciferase activity using the Luciferase Assay System (Promega) on a Fusion αHT plate reader (Packard). Luciferase activity was evaluated in proportion to the transfection efficiency.

CELL LINES

Peripheral blood samples were obtained from healthy volunteers after informed consent following a protocol approved by the IRB at Brigham Young University. Peripheral blood mononuclear cells were isolated using lymphocyte separation medium (Mediatech). These cells were induced to form LCLs by incubation with EBV (B95-8 strain) and 2 ng/ml cyclosporin A (Tocris Biosciences). U937 and Jurkat cells were a kind gift from Dr. Kim O'Neill. Cell lines were maintained in RPMI (Sigma) with 10% fetal bovine serum (PerBio) with penicillin/streptomycin/amphotericin (Calbiochem) at 5% CO_2 and passaged at least weekly.

GENOTYPING OF VOLUNTEERS AND FORMATION OF PAIRED SAMPLES

Genomic DNA was extracted (Qiagen) from peripheral blood mononuclear cells and genotyped using TaqMan reagents Applied Biosystems (ABI) on a StepOnePlus real-time PCR machine (ABI) at the rs2004640 SNP (ABI SNP Assay C9491614). Homozygous risk or protective individuals were matched by gender and ethnicity. Heterozygotes were not included in the study. The primers and PCR conditions are in **Table A1** in Appendix.

CELL TREATMENTS

The TLR7 ligand imiquimod (R-837) was used to stimulate cells for some experiments. Cells were treated for 24 h with 25 μg/ml imiquimod (InvivoGen). cDNA preparation, quantitative PCR, primers, probes, and conditions are described elsewhere in the Section "Materials and Methods." Etoposide was used at 0.1 and 1 mM concentrations and applied for 48 h. 5FU was used at 1.5 mg/ml, and the activating antibodies to TRAIL and Fas were used at 1 and 5 μg/ml, respectively. All treatments used 10^6 cells per milliliter.

cDNA LIBRARIES AND PCR

About 8×10^6 cells were used for each condition in each experiment. cDNA preparations were made by extracting RNA using the RNaqueous system (Ambion), followed by DNase treatment (Promega). One thousand nanograms RNA was per condition was then reverse transcribed using SuperScript III reverse transcriptase (Invitrogen Life Technologies). One hundred nanograms cDNA preparations were used as template for quantitative PCR using TaqMan reagents (ABI), or SYBR green reagents For gene expression studies, input RNA levels were normalized using primers to the housekeeping gene GAPDH for SYBR green experiments and β-GUS for TaqMan experiments. For cloning of 5′UTRs and promoters the template genomic DNA from Section "Genotyping of Volunteers and Formation of Paired Samples" was used, with the NEB High GC PCR kit. Primers were purchased from Integrated DNA Technologies. Sequences and PCR conditions are available in **Table A1** in Appendix.

SEQUENCING

Plasmid sequencing used purified plasmid DNA and a primer upstream of the insertion site. Sequencing reactions used Big Dye terminator reagents and the 3730xl DNA analyzer (ABI). See **Table A1** in Appendix for primers.

STATISTICAL ANALYSIS

A paired *t*-test was used to compare means for mRNA expression. Paired *t*-test was used for luciferase levels. An alpha value of 0.05 and two-tailed *p* values were used in all cases. For experiments using more than two comparisons, ANOVA was used to determine if statistically significant differences were present. Statistical analysis was performed using Data Analysis Plus software (Keller Statistics). ANOVA was performed using the CSBJU online calculator (http://www.physics.csbsju.edu/stats/).

PROMOTER ANALYSIS

An analysis of the promoters for each of the four first exons of IRF5 was performed using the ENCODE ChIP-Seq data set (34) for determining actual binding factors on the genomic region, followed by determining a consensus site using the WebLogo data in FactorBook (35). The consensus site was then used to search the proximal promoters (∼200 bp upstream from the +1 sites) to encounter a proposed binding site. Consensus site screening was performed using a custom searches of ambiguous nucleotides with MEGA (37). This involved searching using the find function, which allows for searching using the ambiguous nucleotide code. For example, a search for GAW would highlight both GAA and GAT.

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APPENDIX

Table A1 | List of primers and PCR conditions.

TAQMAN-BASED QUANTITATIVE PCR PRIMERS
IRF5 exon 2 RT fwd: CCACCTCAGCCCTACAAGAT
IRF5 probe: FAM-TCCAATGGCCCTGCTCCCAC-TAMRA
IRF5 exon 3 RT rev: CTCCTCTCCTGCACCAAAAG
IRF5 1A TaqMan RT fwd: ACGCAGGCGCACCGCAGACA
IRF5 1B RT fwd: AGCTGCGCCTGGAAAGCGAGC
IRF5 1C TaqMan RT fwd: AGGCGGCACTAGGCAGGTGCAAC
IRF5 1D RT fwd: GAGGCTCAGCCCGGATCTGC
IRF5 exon 1 probe: FAM-CCATGAACCAGTCCATCCCAGTGGCTCCCACC-TAMRA
IRF5 exon 2 common RT rev: TCGTAGATCTTGTAGGGCTGAGGTGGCA
β-Glucuronidase fwd: CTCATTTGGAATTTTGCCGATT
β-Glucuronidase probe: FAM-TGAACAGTCACCGACGAGAG-TAMRA
β-Clucuronidase rev: CCGAGTGAAGATCCCCTTTTTA
Conditions: 52°C, 95°C for 10 min, 52 cycles of (95°C for 15 s, 65°C* for 1 min) with 500 nM primers, 250 nM probe
SYBR green quantitative PCR primers and conditions
GAPDH fwd: TGCACCACCAACTGCTTAGC
GAPDH rev: GGCATGGACTGTGGTCATGAG
CCR7 fwd: GCTCCAGGCACGCAACTT
CCR7 rev: GACCACAGCGATGATCACCTT
Calreticulin fwd: GCAGCAGAAGGGGGTGGTGT
Calreticulin rev: GTCCTGGGGGCAGGGGAGAA
NOXA fwd: GCTGTCCGAGGTGCTCCAGTT
NOXA rev: AGCGTTCTTGCGCGCCTTCT
IRF5 fwd: CCACCTCAGCCCTACAAGAT
IRF5 rev: CTCCTCTCCTGCACCAAAAG
Conditions: 95°C for 10 min, 40 cycles of (95°C for 15 s and 60°C for 1 min)
PROMOTER CLONING PRIMERS[†]
IRF5 1A prom fwd: CTGCgctagcCAGGTCAGTGCGGGGC
IRF5 1A prom rev: CCTGagatctACTTCCGCGTCTTGCCGC
Conditions: 94°C for 30 s, 40 cycles of (94°C for 15 s, 62.0°C for 1 min, 68°C for 30 s), 68°C for 5 min
IRF5 1B prom fwd: GCGCgctagcGACAGGTGGGTCCCGGCCGC
IRF5 1B prom rev: GCAGagatctGCGGACCCCGCCCTACTCCA
Nested PCR first round: IRF5 1A prom fwd + IRF5 1B prom rev
Conditions: 94°C for 30 s, 40 cycles of (94°C for 15 s, 59.3°C for 1 min, 68°C for 30 s), 68°C for 5 min
Nested PCR second round: IRF5 1B prom fwd + IRF5 1B prom rev
Conditions: 94°C for 30 s, 40 cycles of (94°C for 15 s, 66.0°C for 1 min, 68°C for 30 s), 68°C for 5 min
IRF5 1C prom fwd: TAGTgctagcGCTGGTTTCCTCAGGTCCT
IRF5 1C prom rev: CAGAagatctCAGCCCTGCCCTGGCCT
Conditions: 94°C for 30 s, 40 cycles of (94°C for 15 s, 60.8°C for 1 min, 68°C for 2 min), 68°C for 5 min
IRF5 1D prom fwd: ACATgctagCACCTGCTGCCTGTTGACC
IRF5 1D prom rev: TGGCagatctGTCATTTGACAACCCC
Conditions: 94°C for 30 s, 40 cycles of (94°C for 15 s, 59.4°C for 1 min, 68°C for 1 min), 68°C for 5 min
pGL4 sequencing fwd: CTAGCAAAATAGGCTGTCCC

**Primer annealing temperatures were 60°C for β-glucuronidase; 65°C for IRF5, 1A, and 1B; 66°C for 1C and 69°C for 1D.*

[†] PCR for these GC-rich promoters was performed using a high-GC kit (NEB) according to package instructions, with 10% enhancer solution included for all reactions except exon 1D.

All are listed in 5′ to 3′ orientation. Restriction enzyme cut sites or overhangs are indicated in lowercase. FAM, fluorescein amidite; IRF5, interferon regulatory factor 5; RT, real time; TAMRA, carboxytetramethylrhodamine.

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Bioinformatics analysis of the factors controlling type I IFN gene expression in autoimmune disease and virus-induced immunity

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Patients with systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) display increased levels of type I interferon (IFN)-induced genes. Plasmacytoid dendritic cells (PDCs) are natural interferon producing cells and considered to be a primary source of IFN-α in these two diseases. Differential expression patterns of type I IFN-inducible transcripts can be found in different immune cell subsets and in patients with both active and inactive autoimmune disease. A type I IFN gene signature generally consists of three groups of IFN-induced genes – those regulated in response to virus-induced type I IFN, those regulated by the IFN-induced mitogen-activated protein kinase/extracellular-regulated kinase (MAPK/ERK) pathway, and those by the IFN-induced phosphoinositide-3 kinase (PI-3K) pathway. These three groups of type I IFN-regulated genes control important cellular processes such as apoptosis, survival, adhesion, and chemotaxis, that when dysregulated, contribute to autoimmunity. With the recent generation of large datasets in the public domain from next-generation sequencing and DNA microarray experiments, one can perform detailed analyses of cell-type specific gene signatures as well as identify distinct transcription factors (TFs) that differentially regulate these gene signatures. We have performed bioinformatics analysis of data in the public domain and experimental data from our lab to gain insight into the regulation of type I IFN gene expression. We have found that the genetic landscape of the *IFNA* and *IFNB* genes are occupied by TFs, such as insulators CTCF and cohesin, that negatively regulate transcription, as well as interferon regulatory factor (IRF)5 and IRF7, that positively and distinctly regulate *IFNA* subtypes. A detailed understanding of the factors controlling type I IFN gene transcription will significantly aid in the identification and development of new therapeutic strategies targeting the IFN pathway in autoimmune disease.

Keywords: type I interferons, bioinformatics, autoimmunity, transcriptional regulation, transcription, genetic

INTRODUCTION

Patients with autoimmune diseases, such as systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS), display increased expression of type I interferon (IFN)-induced genes. Plasmacytoid dendritic cells (PDC), as natural IFN-producing cells, are considered to be a primary source of IFN-α in such diseases (1, 2). The type I IFN family consists of multiple members, including 14 IFN-α subtypes, -β, -ε, -κ, -ω, -δ, and -τ. These members may have autocrine effects on the IFN-producing cells themselves, such as PDCs, and paracrine effects on neighboring cells, as well as systemic effects on distant immune cells (3). IFNs can be added directly to cell cultures and molecular profiling performed to understand their biologic effect. For instance, the direct treatment of peripheral blood mononuclear cells (PBMCs) with 0.6 pM of IFN-α, -β, or IFN-ω led to the increased expression of about 200 genes (4). Broadly speaking, an IFN gene signature should include all of these genes. These genes can be functionally classified into antiviral pathways, apoptosis control, cell surface receptor expression, chemokine/cytokine expression, and components of IFN signaling pathways.

Although methods of bioinformatics analysis are not yet intensively used in immunology research, the field is changing fast and significant information can now be obtained from the public domain for the analysis of mechanisms controlling type I *IFN* gene expression. This report explores several elements of translational bioinformatics analysis, specifically addressing the biological questions relevant to how type I IFN expression is regulated in autoimmune disease. We collected publically available microarray gene expression datasets in Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) and performed data mining and pathway analysis. With the growing datasets in public repository that are shared in the research community, the integrative analysis of experimental data and disease profiling data sets has become an important approach to our understanding of autoimmune disease pathology at the molecular level. In this study, we have also used human datasets from

the Encyclopedia of DNA Elements (ENCODE) to understand the epigenetic codes that control the type I IFN gene cluster. This information can be used as a reference to guide future experiments that focus on epigenetic changes in more relevant human immune cell populations such as monocytes and dendritic cells. Understanding the regulation and epigenetic control of type I IFN expression will be useful for the development of new therapeutic interventions targeting the IFN pathway in autoimmune disease.

MATERIALS AND METHODS

MATERIALS

Gene expression microarray data were retrieved from NCBI's GEO through series accession numbers GSE17762 and GSE10325. Data were loaded with GEO query and limma R packages from the Bioconductor project. Alternatively, GEO2R, an interactive web tool, was used. Next-generation sequencing datasets from multiple cell lines and cell types were retrieved from the ENCODE Project[1].

METHODS

In brief, for the analysis of microarray data, gene symbols and value of log fold changes for individual genes were extracted from NCBI's GEO and Ingenuity IPA software was used to perform pathway analysis. For next-generation sequencing datasets, ENCODE offers a few software tools for analyzing the data. One relevant tool is factor book, which organizes all the information associated with individual transcription factors (TFs) (5). Although useful, it should be noted that the current lack of information on human primary immunocytes limits one's ability to analyze individual genes/gene clusters and therefore limits the value and/or relevance of some of these datasets.

The following information provides a brief summary of methods used for the analysis of next-generation sequencing data. For example, the epigenome analysis of the *IFNA* gene cluster was performed using a variety of resources for data visualization. In brief, the genetic region was located and retrieved in UCSC genome browser using URL http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr9:21000000-21550000. Methylated/unmethylated CpGs data was retrieved using Methylation-sensitive restriction enzyme sequencing (MRE-seq) and MeDIP-seq loaded from http://genome.ucsc.edu/cgi-bin/hgTrackUi?g=ucsfBrainMethyl. Methyl Reduced Representation Bisulfite Sequencing (RRBS) tracks were loaded from http://genome.ucsc.edu/cgi-bin/hgTrackUi?g=wgEncodeHaibMethylRrbs, samples used include all cells in the following list: http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=342586899&c=chr9&g=wgEncodeRegTfbsClusteredV2. Histone modification data, including H3K4me3 was loaded from http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=342586899&c=chr9&g=wgEncodeReg. For the analysis of CTCF and other relevant TFs, we selected TFs and cell types by adding tracks from http://genome.ucsc.edu/cgi-bin/hgTrackUi?db=hg19&g=wgEncodeAwgTfbsUniform. TF binding peaks were either calculated using ENCODE pre-processed data with a False Discovery Rate of 1% or mapped to human genome hg37 using CLC Genomics Workbench software 5.5, followed by peak calling using Model-based Analysis for ChIP-Seq (MACS).

[1]http://genome.ucsc.edu/ENCODE/

RESULTS AND DISCUSSION

RELATIONSHIP BETWEEN THE TYPE I IFN GENE SIGNATURE AND CLINICAL AUTOIMMUNE BIOMARKERS

We have performed an in depth bioinformatics analysis of genes regulated by type I IFNs, as well as the mechanisms controlling type I IFN expression, in autoimmune diseases using publically available datasets. In many cases, we found that IFN-induced genes directly explain the presence of clinical biomarkers that appear in patients with autoimmune diseases. For example, we found that IFN-α increases the expression of interleukin (IL)-15 and its receptor IL-15Rα in PBMCs. IL-15, that is primarily expressed by activated monocytes and dendritic cells, binds to IL-15Rα (CD359) on accessory cells and is trans-presented to T cells that express functional IL-15Rα, composed of IL-2/15Rβ (CD122) and γc chains. Several groups have reported elevated IL-15 levels in the sera of SLE patients, however, the functional consequence of IL-15Rα activation in SLE remains to be studied (6). In addition to IL-15 and IL-15Rα, IFN-β moderately upregulates *IL-7* and *CD59* transcripts in PBMCs. IL-7 is a survival factor for naïve, early effector, and memory $CD4^+$ and $CD8^+$ T cells. It is primarily produced by fibroblastic reticular cells (FRCs), a mesenchymal cell population found in the stromal environment of lymphoid organs. In SLE patients, soluble (s)IL-7R concentrations were found to be elevated in the serum and raised levels of sIL-7 were detected in patients with lupus nephritis (LN) that reflected activation of kidney tissue cells (7). Receptor blockade by anti-IL-7Rα in MRL-Fas^{lpr} lupus mice resulted in alleviation of dermatitis, lymphadenopathy, splenomegaly, and total serum IgG2a; yet, only a marginal reduction in IgG2a autoantibodies was found (8). CD59 are glycosylphosphatidylinositol-anchored proteins with complement inhibitory properties that prevent the terminal polymerization of the membrane attack complex. Increased numbers of CD55- and CD59-lymphocytes and CD59-granulocytes were found in SLE patients as compared with controls (9).

PATHWAY ACTIVATION BY TYPE I IFNs

Type I IFNs may play a pathological role in autoimmune disease through their ability to regulate key signaling pathways important in the innate immune response. For instance, we found that IFN-α upregulates the expression of Toll-like receptors (*TLR*)*-3* and *TLR-7*, as well as the critical cofactor myeloid differentiation primary response protein 88 (*MyD88*). IFN-α also enhances the expression of interferon regulatory factor (*IRF*)*2*, which competitively inhibits IRF1-mediated transcriptional activation of *IFNA* and *B* genes. As compared to IFN-α, the effect of IFN-β on gene expression extends to *TLR-1*, *TRAF/TANK*, *IRF4*, and *IRF1*. We also found in our analysis that the human dual specificity mitogen-activated protein kinase kinase 5 (*MAP2K5*) can be up-regulated by IFN-α/IFN-β and mitogen-activated protein kinase kinase 8 (*MAP3K8*) can be induced by IFN-β. Since p38 MAPK acts up-stream of type I IFN-induced STAT (signal transducers and activators of transcription) 1 signaling (10, 11), the up-regulation of *MAP3K8* or *MAP2K5* may provide further hints toward the biologic effects of type I IFN on cells. For example, MAP3K8 has

been shown to promote the production of tumor necrosis factor (TNF)-α and IL-2 during T lymphocyte activation. It is also known that addition of IFN-α with anti-CD3 antibodies results in enhanced T helper (Th)1 responses that associate with enhanced phosphorylation of STAT1 (12).

It is well-known that IFN-α has pro-apoptotic effects in many cancer cell types including myeloma (13), renal cell carcinoma (14), and glioma (15). It is also known that monocytes stimulated with IFN-α express functional TNF-related apoptosis-inducing ligand (TRAIL), which is capable of killing myeloma cells (16). IFN-α also increases the expression of functional FasL exclusively on natural killer (NK) cells (17). The functional clustering of genes regulated by IFN-β, using DAVID tools, revealed a number of genes that control apoptosis, including *caspase 1*, *8*, and *10*, *TRAIL* (*TNFSF10*), and *FADD [Fas (TNFRSF6)-associated via death domain]*.

THE TYPE I IFN GENE SIGNATURE IN SLE B CELLS AND T CELLS

Disease biomarkers or disease gene signatures provide important clues for our understanding of disease pathogenesis and aid in the identification and development of new therapeutic strategies for treatment. High-throughput screening technologies, such as DNA microarrays, have been used to profile disease signatures in PBMCs from SLE patients (18), and subsequently, in specific subsets such as monocytes, neutrophils, T cells, and B cells. The presence of a type I IFN gene signature in PBMC of SLE patients has been recognized for nearly 35 years now (19). However, not all IFN-inducible genes that have been identified by *in vitro* assays can be detected *in vivo* in PBMCs isolated from SLE patients. About 20 IFN-inducible genes were consistently found to be highly expressed in PBMC from SLE patients (18). In our analysis of SLE B and T cells, we found that approximately 10 IFN-inducible genes were consistently and highly expressed. The gene transcriptional signatures that appear to overlap between cell types include *Mx1*, *ISGF-3*, *PRKR*, *IFIT1*, and *IFI44* in cells that have been either exposed to type I IFNs *in vivo* or *in vitro*. This gene signature has been used as a readout for the type I IFN bioassay and is considered a measure of the "IFN-α activity score" in patients with SLE and other inflammatory or autoimmune diseases (19, 20).

Intensive pathway analyses with KEGG[2], BioCarta[3], and GenMAPP[4] have shown up-regulated activation markers on SLE T cells and genes that correlate with STAT1 expression (21). Using IPA[5] analysis of independent datasets, we also found groups of genes in the network that strongly correlate with STAT1, suggesting a persistent and strong effect downstream of type I IFNs in SLE T cells. Furthermore, IFN response factor consensus sequences (ISREs) can be found up-stream of the start sites of each of the genes in the type I IFN gene signature. Our independent analysis also indicated groups of up-regulated genes in SLE T cells that can be modulated by STAT4. Genome-wide mapping of STAT4 and IRF 5 occupancy in immune cells from SLE patients by chromatin immunoprecipitation combined with next-generation sequencing (ChIP-seq) revealed the possible cooperation of high mobility group-I/Y, specificity protein 1, and paired box 4 with IRF5 and STAT4 in transcriptional regulation (22). As noted above, IFN-regulated pathways derived from *in vitro* data do not always align with microarray datasets obtained from primary cells of SLE patients. In this regard, short-term IFN treatment has been shown to promote apoptosis signaling via TRAIL pathways. However, anti-apoptotic signatures, including elevation of caspase 8 and FADD-like apoptosis regulator (*CFLAR*), were identified in lupus T cells (21). Our bioinformatics pathway analysis identified additional genes, such as *BIRC5*, that participate in the B cell anti-apoptotic pathway in cells isolated from SLE patients. Given that apoptosis and the clearance of apoptotic material have been implicated in SLE pathogenesis, further research detailing the in depth analysis and mapping of these anti-apoptotic pathways in PBMC subsets will be of significant importance to our understanding of SLE pathogenesis.

GENETIC LANDSCAPE OF THE TYPE I IFN CLUSTER

The human type I *IFN* gene cluster spans approximately 450 kb on chromosome 9p22. *IFNB* and *IFN*ε define the boundaries of the cluster, with all other type I *IFN* genes, except *IFNk*, distributed between these borders. This gene cluster also contains *KLHL9*, which is a substrate-specific adaptor of the BCR (BTB-CUL3-RBX1) E3 ubiquitin ligase complex that functions in cell division. Studies of virus-induced type I IFN production in murine fibroblasts indicates the presence of an immediate-early response gene, *IFNA4*, which is induced rapidly and without the need for ongoing protein synthesis, and *IFNA2*, *5*, *6*, and *8*, that display delayed induction, are induced more slowly, and require cellular protein synthesis. In CpG-stimulated human PDCs, *IFNA5*, *IFNA10*, *IFNA4*, *1/13*, *21*, *14*, *16*, and *6* transcription can be detected within 2 h. *IFN21* and *IFNA16* levels are dramatically up-regulated further after 8 h suggesting an efficient positive feedback loop regulating expression of these two genes. Recent analysis of data from the ENCODE Consortium suggests that this important gene cluster may be controlled by epigenetic regulation supporting new mechanistic insight and a basis for the design of experiments focused on this aspect of type I IFN gene regulation.

Methylation

Indeed, there has already been significant data in the literature to support the mechanism(s) of epigenetic regulation in autoimmune diseases. In particular, DNA from SLE T cells was found to be less methylated than control DNA from normal T cells by measuring the cellular deoxymethylcytosine content (23). Interestingly, non-T cells from lupus patients displayed normal DNA methylation levels (24). Decreased DNA methyltransferase (DNMT) activity in lupus T lymphocyte nuclear proteins was considered to be responsible for the observed DNA hypomethylation in lupus T cells. Patients with lupus had significantly lower levels of *DNMT1* mRNA, but not *DNMT3A* or *DNMT3B*, as compared with healthy controls (25). A preliminary analysis of microarray data from immature monocyte-derived dendritic cells (MDDCs) revealed that they express abundant amounts of *DNMT1*, which is downregulated after LPS stimulation. The methylation status of DNA from SLE PDCs and the levels of *DNMT1* expression in this important IFN-α producing cell type are not currently known.

[2]www.genome.ad.jp
[3]www.biocarta.com
[4]www.genmapp.org
[5]www.ingenuity.com

In general, hypermethylation in the promoter of a gene is associated with gene suppression, while hypomethylation is linked to gene expression; methylation within the gene body is also associated with gene expression. Two next-generation sequencing technologies have recently been developed for the analysis of gene methylation – methylated DNA immunoprecipitation sequencing (MeDIP)-seq, to detect methylated CpGs (26, 27), and MRE-seq, to detect unmethylated CpGs (28). Integrative methodologies that combine both MeDIP-seq and MRE-seq can differentiate hypermethylation, intermediate, and hypomethylation regions of DNA. An integrative analysis of *KLHL9* indicates that the CpG islands of the *KLHL9* promoter are highly hypomethylated (**Figure 1**). These islands are highly conserved since they were found to be present in virtually all cell types queried. Combining these data with ChIP-seq histone modification data in the same tissues, we found that hypomethylated CpGs of *KLHL9* are occupied by significant levels of trimethylated lysine 4 on histone H3 (H3K4me3) (**Figure 1**). Two other hypomethylated regions in the type I IFN cluster, located in the genomic region between *IFNA2* and *IFNA8*, have relative low levels of enrichment for H3K4me3 peaks (**Figure 1**). H3K4me3 is a histone modification that accumulates at the transcription-start site (TSS) of active genes and is believed to be important for transcription activation. Loss of H3K4me3 occurs at TSSs and leads to gene transcriptional inactivation as a result of promoter hypermethylation. The occupancy of H3K4me3 in the promoter of *KLHL9* may ensure the protection of CpG islands from methylation. In contrast, the other two hypomethylated sites that are located quite far from the TSSs of *IFNA2* and *IFNA8*, may not be functional for transcription. DNA methylation by RRBS from various cell types, including B cells, failed to reveal strong methylation signals in the *IFNA* gene cluster. One exception to this is that MeDIP-seq defined methylation peaks were found to be distributed between *IFNA* genes from brain tissue.

Thus far, data do not support that methylation is the likely major mechanism by which *IFNA* gene expression is suppressed in most non-IFN-producing cells. Further experimental studies will be necessary to determine whether constructive hypomethylation, as well as H3K4me3 occupancy, is important for regulating *IFNA* gene transcription in IFN-producing cells such as monocytes, and PDCs.

Chromatin structure

There are multiple *IFNA* and *IFNB* genomic regions that have open chromatin structure in an evolutionally conserved pattern across species and most human cell types. Since DNase I hypersensitive sites (DHSs) reflect the local openness and accessibility of chromatin, chromatin structure or accessibility of *IFNA* clustering may be similar among different cells. In general, hypersensitive sites are found only in the chromatin of cells in which the associated gene is being expressed, and do not occur when the gene is inactive. Therefore, mapping DHSs within nuclear chromatin is a powerful method of identifying genetic regulatory elements (29). However, the distribution of DHSs in promoters and other gene regions of similarly expressed genes differs among different chromosomes. Furthermore, silenced genes have a more open chromatin structure than previously thought and DHSs in 3′-untranslated regions (3′-UTRs) have been shown to negatively correlate with gene expression levels (30), thus going against the standard dogma. Bioinformatics analysis of DHSs in the *IFN* gene cluster between different cell types revealed a highly conserved pattern (**Figure 2**);

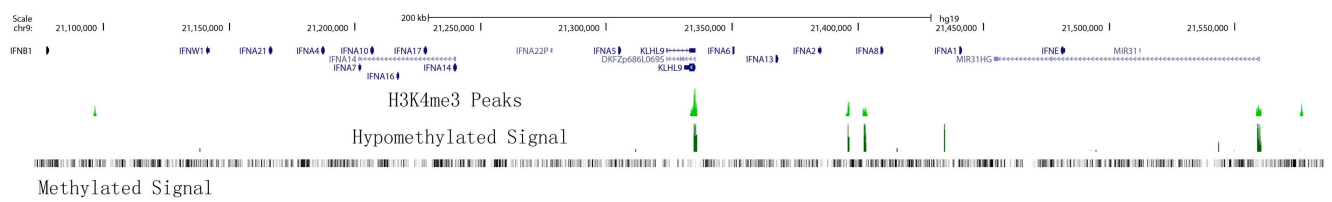

FIGURE 1 | H3K4me3 peaks and methylation tracks on the type I *IFN* gene cluster. Members of the type I *IFN* gene cluster are shown and illustrated proportionally according to Human (*Homo sapiens*) Genome hg19. H3K4me3 peaks and UCSC DNA methylation tracks are shown for a human B cell line.

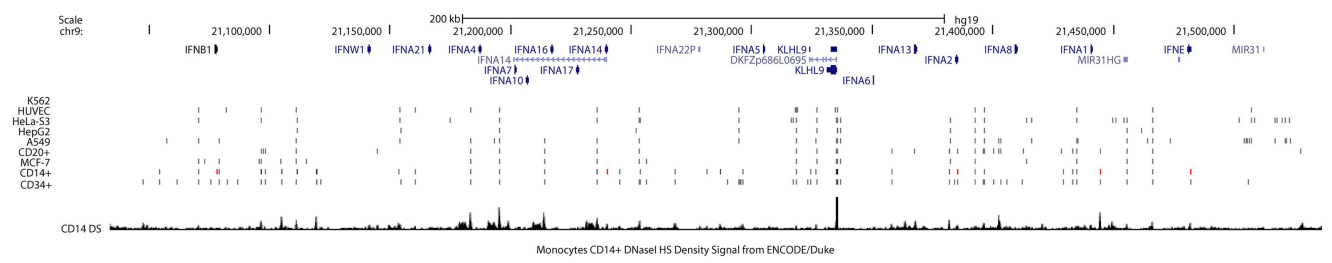

FIGURE 2 | DNase I hypersensitivity sites in the type I *IFN* gene cluster are highly conserved between cell types. Members of the type I *IFN* gene cluster are shown and illustrated proportionally according to Human (*Homo sapiens*) genome hg19. Cell lines and cell types analyzed are listed on the left side. Short vertical lines below the gene track indicate the open chromatin position marked by DNaseI hypersensitivity sites from ENCODE/OpenChromatin (Duke University) for each cell type. Red lines indicate the novel sites identified between cell types. The DNase I hypersensitivity signal peaks for $CD14^{+}$ monocytes are shown at the bottom for reference to chromatin marks.

however, we found additional DHSs in $CD14^+$ monocytes that can produce type I IFNs. We also found that $CD34^+$ stem cells have more DHSs close to promoters within the *IFN* gene cluster (**Figure 2**). These data support the presence of unique cell-specific chromatin structures which may play important regulatory roles in the control of type I IFN expression.

Histone modification

Modifying the chromatin template at a particular gene locus can also serve as an important mechanism of gene transcriptional activation that exhibits cell-type specific expression patterns. The functional importance of histone acetylation in type I IFN production has been supported by studies that show increased IFN-β expression in cells treated with histone deacetylase inhibitors, such as Trichostatin A (TSA) (31), and decreased IFN-β expression in murine macrophages where the binding of bromodomain-containing BET (bromodomain and extraterminal) transcriptional regulators to acetylated histones was inhibited (32). Di- or tri-methylation of H3K9 is capable of suppressing gene expression not only passively, by inhibiting acetylation, but also actively, by recruiting transcriptional repressors of the heterochromatin protein 1 (HP1) family. We found that H3K9me2 occupancy at *IFN* and *ISG* promoters is inversely correlated with gene expression. Furthermore, human MDDCs that are capable of producing type I IFNs, as compared with human lung fibroblasts that do not, show decreased H3K9me2 occupancy at the *IFNB* promoter. In the absence of G9A, a methyltransferase for H3K9me2, non-professional IFN-producing cells were shown to be converted into potent IFN-β producers (33). Together, these data support the importance of histone modifications in the regulation of type I IFN expression.

H3K27me3, on the other hand, are found to be associated with the repression of gene transcription in a cell-type specific manner. Polycomb Repressive Complex 2 (PRC2) is a histone methyltransferase that catalyzes tri-methylation of Histone 3 at Lysine 27 (H3K27me3) (34). A detailed profile of H3K27me3 peaks reveal that broad peaks at TSS are associated with transcriptional suppression while skewed peaks up-stream of the TSS may not be suppressive (35). Indeed, we found that *IFNA* regions in B cells, which are incapable of producing IFN-α, are widely occupied with H3K27me3, as shown by the substantive peaks found along the gene cluster (**Figure 3**). In contrast, ChIP-seq data from monocytes demonstrate that H3K27me3 peaks occupy some *IFNA* genes, such as *IFNA2*, *IFNA14*, and intergenic regions between *IFNA2* and *IFNA8*, while the remaining *IFNA* genes were not suppressed by H3K27me3.

As mentioned above, current dogma holds that H3K4me3 represents a chromatin landmark that is present at the TSS for genes that are either actively transcribed or permissive for gene transcription. However, H3K4me3 are not sufficient to license cells to produce IFN-α. For example, multiple H3K4me3 occupancy peaks can be identified in the IFN regulatory regions in B cells that do not express IFN-α. A good comparison would be with PDCs, yet the histone codes are not yet available for this cell type. In PDCs, TLR-7 signaling quickly turns on transcription of *IFNB*, *IFNA2*, *IFNA8*, and *IFNA14* genes at 30 min post-stimulation with peak levels being achieved at this time point. In comparison, peak levels of *IFNA5*, *IFNA6*, *IFNA10*, *IFNA13*, and *IFNA21* were observed around 4 h post-stimulation (36). Based on our bioinformatics analysis, we reason that transcriptional suppression by H3K27me3, if it exists in PDCs in a pattern similar to that found in $CD14^+$ monocytes, may not be functional in PDCs or can quickly be replaced by H3K4me3 after TLR-7 activation. Alternatively, the *IFN* gene cluster in PDCs may not have H3K27me3 markers. It is not known whether chromatin change is necessary for *IFNA* transcriptional activation or whether chromatin status is responsible for differentially transcribed type I *IFN* genes. Further studies in human PDCs will be required to address this.

Transcription factors regulating basal repression of IFNA gene expression

The transcriptional repressor CTCF (11-zinc finger protein) or CCCTC-binding factor is thought to regulate the 3-dimensional (3D) structure of chromatin by binding strands of DNA together and forming DNA loops (37). CTCF represses gene expression by blocking the interaction between enhancers and promoters (38). This phenomenon may serve as a chromatin barrier to block the spread of heterochromatin structures and set boundaries between active chromatin regions marked by histone H2A acetyl Lys5 (H2AK5ac) and repressive regions marked by H3K27me3 (39). The cohesin complex, consisting of cohesion proteins SMC1, SMC3, SCC3, and the α-kleisin SCC1, may contribute to CTCF-mediated repression. Many CTCF/cohesin binding sites are located at promoter regions suggesting a joint regulatory role for these factors (40). Although most cohesin sites overlap with CTCF, a significant proportion of each factor's sites are independent of the other, implying CTCF-independent functions of cohesin as well as cohesin-independent CTCF functions. Bioinformatics analysis of CTCF ChIP-seq data from ENCODE cell lines identified several CTCF insulators that are basally located in the promoters and intergenic regions of *IFNA5*, *A1*, *A2*, and *A8* (**Figure 4A**). In gum fibroblast cells (AG09319), we found five CTCF binding sites in the region covering *IFNA14*, *A17*, *A16*, *A10*, *A7*, and *A4*. There is only one CTCF/SMC3 binding site in the *IFNB* gene. The regulatory region of the *IFNA2* gene contains two CTCF binding sites. The second CTCF site yields co-binding with SMC3, suggesting

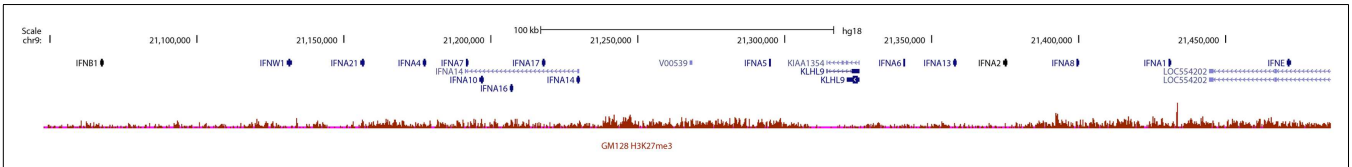

FIGURE 3 | H3K27me3 peaks on the type I *IFN* gene cluster. Members of the type I *IFN* gene cluster are shown and illustrated proportionally according to Human (*Homo sapiens*) Genome hg19. H3K27me3 peaks were found along the entire region of the *IFN* gene cluster in the human B cell line GM128.

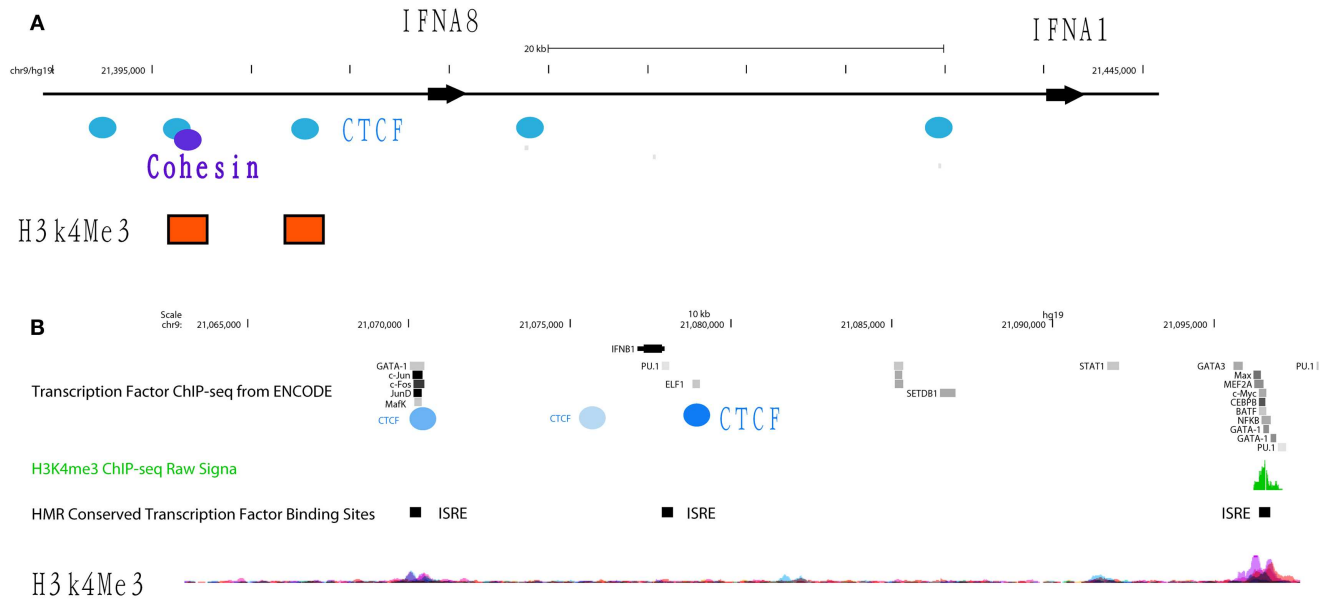

FIGURE 4 | Differential occupancy for insulators CTCF and cohesin on the *IFNA8* and *IFNB* genes. **(A)** The genomic regions of *IFNA8* and *IFNA1* are shown proportionally according to Human (*Homo sapiens*) Genome hg19. CTCF (blue circles) and cohesin (purple circles) occupancy positions are marked according to transcription factor ChIP-seq datasets from ENCODE. Orange squares indicate positions enriched with H3K4Me3 signals. **(B)** The genomic region of *IFNB* is shown with transcription factor binding sites from the ENCODE database. Experimentally verified CTCF binding sites are shown by the blue circles, with the darker shades of blue denoting signal intensity; light blue – low intensity, dark blue – high intensity. IFN-stimulated response elements (ISRE) are labeled with black squares. Peaks showing H3K4me3 signals are shown.

the cohesin complex may function in this *IFN* genomic region. Based on these data, we speculate that CTCF may indeed function as an *IFNA* suppressor and block promoter activation.

Within the *IFNA* gene cluster, we have yet to identify any other TF in the ENCODE datasets that basally occupies the promoter regions between TSSs and the proximal CTCF sites. In contrast, multiple TFs, such as NF-κB and PU.1 (in B cells), do constitutively occupy regions up-stream of CTCF sites that control individual *IFNA* genes. CTCF binding sites are not conserved but cell-type specific. While the majority of cells show CTCF occupancy up-stream of the *IFNA2* gene, binding is absent in fibroblast cells. Similarly, at the *IFNB* promoter, CTCF binding was identified in some B cells lines, HeLa cells, MCF-7, and osteoblast cells, but not in any fibroblast cell lines or A549 lung carcinoma cells. Lack of binding of this insulator may render fibroblast cells to produce type I IFNs upon the appropriate stimulation, such as viral infection, thus supporting that CTCF binding to the *IFNA* gene may be regulated. In this regard, dexamethasone treatment in A549 cell lines induces CTCF to bind to the *IFNA8* promoter. Finally, the discrepancy of CTCF binding patterns in Epstein–Barr virus (EBV)-transformed B cell lines suggests that viral infection may interfere with CTCF function. It is known that CTCF/cohesin occupancy is essential for IFN-gamma (*IFNg*) gene transcription (41). Thus, this complex may have a similar function and be important for regulating *IFNB* gene transcription via maintaining the 3D chromatin structure of the *IFNB* locus in fibroblast cells (**Figure 4B**). Based on these data, we propose that the DNA regions in the *IFN* gene cluster that contain CTCF occupancy may be subject to control by this factor to ensue *IFNA* transcription during viral or viral-like challenges in IFN-producing cells. This region may also be used as a landmark to demarcate the promoter region that spans from a TSS to the CTCF binding sites and enhancer regions located up-stream of CTCF binding site.

Transcription factors that regulate induction of IFNA gene expression

Interferon regulatory factors, as their name suggests, have been long known to regulate type I *IFN* gene expression (42). Of the nine mammalian IRF family members currently identified to date, IRF7 has garnered the most attention for its role in regulating *IFNA* gene expression (3). IRF7 is highly expressed in human PDCs and allows bypass of the classic autocrine feedback loop that is regulated by IFN-β (43). IRF7 was also shown to be required for murine PDCs to produce an antiviral IFN immune response (44). Similarly, IRF5 has been implicated in the regulation of type I *IFN* gene expression (45). Early data in human cell lines revealed the regulation of type I *IFN* genes and IFN-stimulated genes (ISGs) by IRF5 in response to virus (46). Later data in mice supported these findings. For example, splenic PDCs from mice lacking *Irf5* were shown to produce less type I IFNs in response to virus infection (47). IRF5 has also been recently reported to regulate IFN-β production in myeloid dendritic cells downstream of the mitochondrial antiviral-signaling protein (MAVS) (48). Furthermore, recent studies demonstrate that IRF5 and NF-κB p50 are key co-regulators of IFN-β and IL-6 expression in TLR9-mediated activation of human PDCs (49). Although both of these IRF family members have been implicated as key regulators of IFN-α production, no ChIP-seq data is available to support these findings. Interestingly, data from the aforementioned STAT4/IRF5 ChIP-seq datasets in PBMCs did not support the direct regulation of type I

IFN expression by IRF5 since no peaks were detected in the IFN gene cluster after immunoprecipitation with anti-IRF5 antibodies (22). In this case, PBMCs were stimulated with either IFNa2 or SLE immune complexes before immunoprecipitation with anti-IRF5 or anti-STAT4 antibodies. In the case of IRF7, a cursory review of the literature and publicly available datasets indicate that no ChIP-seq data is currently available for this TF. We have recently performed IRF5 and IRF7 ChIP-seq in human PDCs stimulated with virus. Our unpublished data indicate that these two TFs bind to different regions in the *IFNA* gene cluster (**Figure 5**). These data support the distinct and differential roles for IRF5 and IRF7 in type I *IFN* gene regulation (45, 49). With regard to autoimmune diseases such as SLE and SS that display a pathogenic type I *IFN* gene signature, determination of the mechanisms by which these two IRF family members cooperatively and distinctly regulate *IFNA* subtype expression in the critical IFN-α producing cell types will be important for the design of new therapeutic strategies targeting these two factors.

CONCLUDING REMARKS

With the recent generation of large datasets in the public domain from next-generation sequencing and DNA microarray experiments, others and we have begun to perform detailed analyses of cell-type specific gene signatures as well as identify distinct TFs that differentially regulate these gene signatures in a cell type- and disease-specific manner. This report describes a sample workflow and method of integrative analysis to inspect, clean, and model data from GEO and ENCODE with the goal of highlighting information and knowledge discovery at the gene cluster level. We demonstrate that this method can extract valuable information including downstream pathway analysis, DNA methylation, chromatin structure, histone modification, and TF binding to a gene of interest (in our case, type I *IFNA*s). This report summarizes data from our bioinformatics analysis of the type I *IFN* gene cluster using data in the public domain and experimental unpublished data from our lab (**Tables 1** and **2**). We have found that the genetic landscape of the *IFNA* and *IFNB* genes are occupied by TF, such as insulator CTCF and cohesin, that negatively regulate transcription, as well as IRF5 and IRF7, that positively and distinctly regulate the *IFNA* subtypes. This information can be used as a reference to guide future experiments that focus on proving and/or disapproving these novel regulatory mechanisms that control type I *IFN* expression. A detailed understanding of the factors controlling type I *IFN* gene transcription will significantly aid in the identification and development of new therapeutic strategies targeting the IFN pathway in autoimmune disease.

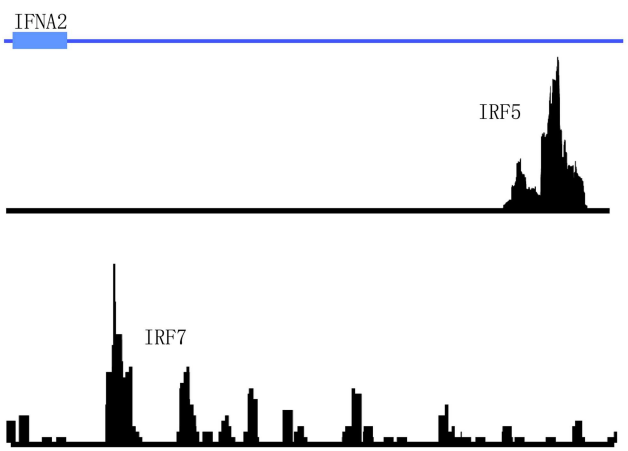

FIGURE 5 | Differential binding of IRF5 and IRF7 to the *IFNA2* gene in human primary PDCs stimulated with virus. The genomic region of *IFNA2* is shown with IRF5 and IRF7 ChIP-seq peaks plotted according to their enrichment positions. Briefly, human primary PDCs were stimulated with Herpes simplex virus (HSV) for 4 h and cells cross-linked and harvested for immunoprecipitations with anti-IRF5 or anti-IRF7 antibodies.

Table 1 | Results from computational pathway analysis of microarray data sets.

Genes and pathways	*Ex vivo* type I IFN treatment	In SLE patients
IL-15 and its receptor IL-15Rα	Up-regulated	Up-regulated
IL-7	Up-regulated	Up-regulated
CD59	Up-regulated	Up-regulated
MAP kinase	MAP kinase (ERK2) activity at up-stream of STAT1, MAP2K5, MAP2K5 are up-regulated	Unknown
TLR pathway (*TLR-3, 7, 1, TRAF/TANK, IRF4,* and *IRF1)*	Up-regulated	TLR-7 up-regulated[a]
STAT	STAT1	STAT1, STAT4
Apoptotic pathways	Up-regulated *caspase 1, 8,* and *10, TRAIL, FADD*	Up-regulated anti-apoptotic genes including *BIRC5*

[a] *Indicates data from Ref. (50).*

The following list of genes and pathways were predicted to be active in PBMCs treated with type I IFN ex vivo and in SLE patients.

Table 2 | Results from the computational analysis of ENCODE next-generation sequencing data on the type I *IFN* gene cluster.

Epigenetic markers	Factors that affect type I IFN gene cluster
Chromatin structure	Monocytes display more DNase I hypersensitivity sites within gene cluster
Methylation	Methylation not found in non-IFN-producing cells; hypomethylated CpG island identified in cluster
Histone modification	H3K4Me3, H3K27me3, H3K9me2
Conserved transcription factor binding site	HMR conserved transcription factor binding sites computed with the Transfac Matrix Database (v7.0) identified ISRE sites
Transcription factors	IRF5, IRF7
Insulator	CTCF, SMC3, cohesin complex

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The role of type 1 interferon in systemic sclerosis

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Systemic Sclerosis (Scleroderma, SSc) is an autoimmune disease characterized by vasculopathy, inflammation, and fibrosis that can lead to loss of organ function. Type I interferons (IFNs) are family of cytokines that mitigate the deleterious effects of viral and bacterial infections in the innate immunity system. Past several years, research efforts have been focused on the role of type I IFN and IFN-inducible genes in the pathogenesis of SSc. Polymorphisms in the Interferon regulatory factor (*IRF*)*-5*, *IRF7*, and *IRF8* are associated with SSc, Similarly, polymorphism of Signal Transducer and Activator of Transcription (STAT)-4, has been established as a genetic risk factor of SSc. IRFs and STAT4 proteins are key activators of type I IFN signaling pathways. An IFN signature (increased expression and activation of IFN-regulated genes) has been observed in the peripheral blood and skin biopsy samples of patients with SSc. Furthermore, a plasma IFN-inducible chemokine score correlated with markers of disease severity and autoantibody subtypes in SSc. In this review, we summarize our current knowledge of the role of type I IFNs and IFN-inducible genes in the pathogenesis of SSc and their potential role as biomarkers and therapeutic targets.

Keywords: systemic sclerosis, innate immunity, type 1 IFN, interferon regulatory factor, IFN-inducible cytokines and chemokines

INTRODUCTION

Systemic sclerosis (Scleroderma, SSc) is characterized by immune dysregulation, fibrosis, and vasculopathy although its pathogenesis is not completely understood (1). Disease morbidity and mortality remain high (2, 3). There is no definite cure for SSc and the available treatments have limited efficacy. The major hurdle in developing effective therapies for SSc is an incomplete understanding of disease pathogenesis. A better understanding of SSc pathogenesis is important for identifying more targeted and effective therapeutic approaches.

Recently, there has been an increasing interest in the role of type I interferons (IFNs) in pathogenesis and severity of SSc. IFNs are a heterogeneous family of multifunctional cytokines. They were originally identified as proteins responsible for induction of cellular resistance to viral infections. Type I IFNs include IFN-α, -β, and -ω, and alleviate the effects of viral and bacterial infections in the innate immunity system (4, 5). Type I IFN subtypes-α and -β share common multicomponent, cell surface receptors, and elicit a similar range of biological responses, including antiviral, anti-proliferative (6), and immune modulatory activities.

In this review, we summarize the current knowledge about the role of type I IFN and its inducible genes in the SSc pathogenesis and biomarker development.

INNATE IMMUNITY AND SSc

The innate immune system is the first line of host defense against pathogens. It plays an important part in triggering inflammation and promoting development of fibrosis in many organ systems. The dominant cellular components of innate immunity are mainly neutrophils, macrophages, and dendritic cells. These cells sense pathogens and destroy them, followed by secretion of pro-inflammatory chemokines and cytokines to activate T cells and other components of adaptive immune system. There is an increasing evidence for activation of the innate immune system in SSc. Cells involved in the innate immune system are detected at the end organ damage site of SSc (7, 8). Being the first cells in line in the defense against pathogens of any sort, the antigen presenting cells (APCs) are often considered the most influential cell of the innate immune system. The specific nature of these APC and how they contribute to the development of fibrosis is still unclear. The perivascular infiltrates in the non-lesional skin of SSc patients mainly consists of macrophages/monocytes and CD4+ T cells suggesting that the aberrant or dysregulated immune system precedes fibrosis (9–11). Alternatively activated macrophages are present in SSc skin biopsies (8, 12, 13), this type of macrophages are potentially important source of profibrotic cytokines including transforming growth factor β (TGF-β) which contribute to resolving inflammation and promoting wound healing (14). The sub-classification of macrophages into classically activated M1 macrophages and alternatively activated M2 macrophages is also of special interest in SSc because the M1 type is clearly more inflammatory and the M2 type is thought to be more involved in tissue remodeling and profibrotic phenotypes. M2 macrophages highly express several receptors such as hemoglobin scavenger receptor (CD163), class A scavenger receptor (CD204), and mannose receptor (CD206) (15, 16). SSc patients show significantly higher serum soluble CD163 levels, and the number of CD163+ and CD204+ activated M2 macrophages is significantly greater in SSc skin (17, 18). The role of M2 macrophages for the development of fibrosis in SSc is still speculative, further studies are needed to clarify the potential mechanism of M2 macrophages in this disorder.

In this non-specific immune system, mast cells, basophils, and natural killer (NK) T cells play more specialized immune functions. For instance, dermal mast cell number density was

significantly higher in diffuse SSc patients than in unaffected controls (19, 20). Electron microscopy (EM) with immunogold labeling in skin biopsy samples revealed that patients with progressive SSc (worsening skin thickening and/or organ function in the year preceding biopsy) had higher number of mast cells. Furthermore, mast cell vesicles containing active TGF-β in patients with SSc showed higher level of degranulation than those from unaffected controls (21). The number of basophils, a circulating counterpart of mast cells was increased in SSc patients. Spontaneous histamine releasability, its reactivity to IgE and response to IL-3 were increased in basophiles from patients with SSc (22). On the other hand, the absolute number and proportion of NK T cells were decreased in patients with SSc which possibly can lead to down-regulation of the normal immune response (23). Altogether, these observations implicate a dysregulated immune system in the pathogenesis of SSc.

In line with those observations, large efforts have been made to find the genetic risk factors for abnormal immune system in SSc. These studies independently replicated genetic risk factors such as *STAT4* (24–26), *BLK* (27–30), *BANK1* (31, 32), Interferon regulatory factor (*IRF*)*-5* (33, 34), *IRF-7*, and *-8* (35–38), and the T cell receptor zeta-chain (*CD247*) (26) which are involved in innate and adaptive immune system.

TYPE I IFNs AND SSc

Type I IFNs are important key regulators of the innate immune system. They modulate immune cell differentiation and proliferation, as well as inflammatory cytokine production. Recent studies have provided considerable evidence that implicates a dysregulation in type I IFN and IFN-inducible genes in the pathophysiology of autoimmune diseases including SSc (39–43). SSc shares this common characteristic with systemic lupus erythematosus (SLE) (44). Anti-IFNα mAb, sifalimumab, was evaluated in a phase Ia study with an open-label extension of 67 SLE patients with moderately active disease. Sifalimumab caused dose-dependent inhibition of type I IFN-induced mRNAs in whole blood and corresponding changes in related proteins in affected skin. Exploratory analyses showed consistent trends toward improvement in disease activity (44). In a follow-up phase Ib randomized, controlled trial with 161 SLE patients, no statistically significant differences in clinical activity between sifalimumab and placebo were observed. However, when adjusted for excess burst steroids, change in disease activity, and complement levels from baseline showed a positive trend over time (45).

Approximately, half of SSc patients have an increased expression of IFN-regulated genes (termed the "IFN signature") in their peripheral whole blood cells (46). Recent studies demonstrated activation of type I IFN system was present in SSc sera and plasmacytoid dendritic cells (pDCs) were the main source of IFN-α production (47, 48). Tan et al. first reported a distinct transcript pattern of dysregulated type I IFN-inducible genes in peripheral blood cells (whole blood) from patients with SSc (40). This finding was subsequently confirmed in peripheral blood mononuclear cells (41). The development of SSc has been reported in patients undergoing IFN treatment (49). Furthermore, a randomized, placebo-controlled trial of subcutaneous IFN-α injection in patients with early SSc showed that treatment with IFN-α resulted in worsening lung function and a smaller degree of improvement in skin thickening scores compared to placebo (50). Although the presence of an activated IFN system could be demonstrated, the exact mechanism by which the dysregulated type I IFN signaling contributes to the pathophysiology of fibrosis in SSc is still unknown.

The innate immune system responds rapidly to the presence of certain motifs or patterns that microbes possess commonly such as unmethylated DNA rich in CpG dinucleotides, dsRNA, and bacterial cell wall components via pattern recognition receptors (PRRs) (51). These PRRs are widely expressed on cells of the immune system, as well as endothelial, epithelial, and mesenchymal cells including fibroblasts. Some of the most prominent PRRs are the Toll-like receptors (TLRs). TLRs located on various cellular membranes to sense exogenous and endogenous danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), and play a critical role in innate immune responses. TLR activation triggers production and secretion of several inflammatory cytokines including type I IFNs (52, 53). The TLR family includes both extracellular and endosomal receptors. The first is based on the cell surface like TLR-2, -4, -5, and -6 and recognize patterns found primarily on bacteria, mycobacteria, fungal, and parasitic organisms (54), the latter is located in endosome like TLR-7 and -8. TLR-9 is localized in the endoplasmic reticulum and translocated to the endosome upon response to bacteria DNA. They recognize a wide variety of pathogen components, and all the TLRs except TLR3 signal through the adaptor molecule MyD88, activate and stimulate type I IFN production. Bhattacharyya et al. reported that TLR4 was overexpressed in the skin and lung tissues, as well as explanted skin fibroblasts from patients with SSc (55). Our recent findings revealed that TLR3 expression was upregulated in patients with SSc and IFN-α2 induced an up-regulation of TLR3 in human dermal fibroblasts which is more prominent in SSc patients than in unaffected control subjects (56). These findings are suggesting an important role for TLR activation via type I IFNs in fibroblast biology.

INTERFERON REGULATORY FACTORS AND SSc

Interferon regulatory factors are best characterized as transcriptional regulators of type I IFNs and IFN-inducible genes and play a pivotal role in regulation of many facets of innate and adaptive immune response (57). This family is composed of nine members: IRF1, IRF2, IRF3, IRF4 (also known as LSIRF, PIP, or ICSAT), IRF5, IRF6, IRF7, IRF8 (also known as ICSBP), and IRF9 (also known as ISGF3γ) (58, 59). As transcriptional factors, each IRF contains a well-conserved DNA-binding domain which is located at the amino terminus and forms a helix-turn-helix-motif. This region recognizes a consensus DNA sequence known as the IFN-stimulated response element (ISRE) in the promoters of targeted genes (59–61). These IRFs coordinate the expression of type I IFNs and type I IFN-inducible genes (57). Several genetic polymorphisms have been associated with SSc in multiple case–control studies and a few family studies (**Table 1**). Some of these genetic variants are associated with susceptibility for development of SSc, while others act as disease modifiers. Recent genome-wide association studies (GWASs) also confirmed IRFs as genetic susceptibility loci in autoimmune diseases.

Table 1 | Polymorphisms in the interferon regulatory factors associated with systemic sclerosis.

IRF	Chromosome (human)	Expression cells	SSc associated SNPs	Reference
IRF5	7q32	B cells; DCs; monocytes	rs2004640; rs2280714; rs10954213; rs3757385	Radstake et al. (26), Dieude et al. (31), Sharif et al. (33)
IRF7	11p15.5	B cells; fibroblasts; pDCs; monocytes	rs1131665; rs4963128; rs702966; rs2246614	Carmona et al. (35)
IRF8	16q24.1	B cells; macrophages; CD8α + DCs; pDCs; T cells	rs11642873; rs2280381	Gorlova et al. (36), Terao et al. (37), Martin et al. (38)

IRF5 is a transcription factor which induces the transcription of IFN-α and other early pro-inflammatory cytokines (62, 63). *In vitro* experiments have shown that in virus-infected cells, IRF5 is activated by phosphorylation, resulting in nuclear translocation and stimulation of IFN-α (64). Initial analysis of the role of IRF5 in the innate antiviral response utilizing IRF5 mutant mice showed impairment of interleukin-6 (IL-6) and TNF-α production in splenic dendritic cells. IRF5 mutant mice are highly sensitive to viral infection and show lower levels of type I IFN in the serum. IFN production was also impaired in the infected macrophages from IRF5 mutant mice (65, 66). Genetic variants of IRF5 are associated with SSc susceptibility (67–69).

The minor allele of the *IRF5* single-nucleotide polymorphism (SNP) rs4728142 was shown to be predictive of longer survival in the two independent SSc cohorts. The association of this SNP with survival was independent of age at disease onset, disease type, and autoantibody profiles (33). This minor allele was also associated with lower IRF5 transcript expression in monocytes of patients and controls suggesting functional relevance of rs4728142 or it associated SNPs for IRF5 expression.

IRF7 is one of transcription factors involved in IFN signaling pathways which is activated by TLRs TLR3/7/9 or retinoic acid-inducible gene 1 (RIG-1) in response to nucleic acid (both DNA and RNA) immune complexes. Activated IRF7 leads to secretion of a large amount of type I IFN (70). Its expression can potentially be enhanced via a positive feedback loop through IFN receptor and ISGF3 activation, leading to increased IRF7 over-expression and subsequently additional IFNα, transcription (71). IRF7 is essential for the induction of IFN-α/β genes via the virus-activated, MyD88-independent pathway and the TLR-activated, MyD88-dependent pathway (72). Inactive IRF7 resides in the cytoplasm. With pathogenic stimulation, IRF7 is phosphorylated, activated, and translocated into the nucleus, where it forms a transcriptional complex with other co-activators and binds to promoter regions of target genes including IFN-α/-β (73, 74). IRF7 also regulates the pro-inflammatory cytokine IL-6 in pDCs and monocytes (75, 76). The viral induction of MyD88-independent IFN-α/β genes is severely impaired in IRF7 null fibroblasts. Consistently, markedly decreased serum IFN-α level were also observed in IRF7 null mice (72). These studies demonstrated the importance of IRF7 dependent systemic IFN response for the innate immunity. Furthermore, recent genetic studies have established IRF7 as a susceptibility locus in SLE (77–80). Similarly, our group recently reported that a functional variant in the IRF7 exonic region, rs1131665 was associated with SSc (35). These findings support that IRF7 may represent a common risk factor for systemic autoimmune disease processes, including SSc. Microarray studies revealed up-regulation of IRF7 mRNA level in whole peripheral blood cells from SSc patients with early diseases (40). Another independent study showed no statistically significant difference in IRF7 transcript levels in PBMCs of SSc patients compared to controls by quantitative PCR analysis (81). However, patients with late stage disease and a smaller sample size were investigated in this study. Further investigations are needed to determine the contributory role of IRF7 in pathogenesis of SSc.

IRF8 is another immune cell specific IRF family member. It participates in the MyD88-dependent signaling pathway through interaction with TRAF6 (82). IRF8 is required for the induction of Type I IFN genes by viruses and TLR ligands in DCs (83). IRF8 is known to be involved in the development of dendritic cells (84). IRF8 also promotes B cell differentiation (85). Recently, the *IRF8* SNP, rs11642873 was identified as a risk factor for limited and anti-centromere positive SSc patients in a large GWAS follow-up study conducted in European and North-American cohorts (36). Another independent study identified rs2280381 polymorphism in IRF8 as a susceptibility locus of SSc in the Japanese population (37). The association of *IRF8* genetic variants with SSc supports possible involvement of B cells and dendritic cells in the development of SSc. However, the role and importance of B cells or dendritic cells in the fibrotic component of SSc has not been well established (86–88).

Further fine-mapping and functional studies are crucial for elucidating the role of genetic variants in the IRFs in the pathogenesis of SSc.

INTERFERON INDUCIBLE CYTOKINES AND CHEMOKINES IN SCLERODERMA

Interleukin-6 is one of the most prominent cytokines activated by IFN pathway. It is involved in the pathogenesis of many immune-mediated diseases including SSc (89–91). IL-6 is a classic inflammatory cytokine produced by various cells and involved in B cell differentiation, induction of acute phase proteins in liver cells, proliferation, and differentiation of T cells (92, 93). By binding to the IL-6 receptor (IL-6R)-α chain and the signal transducing component gp130 (CD130), pleiotropic IL-6 activates downstream signaling mediated by STAT1 or STAT3 through tyrosine phosphorylation. Previous studies have shown that IL-6 plays an important role in the initiation and promotion of fibrosis (94, 95). Production of IL-6 and soluble IL-6R by cultured peripheral blood mononuclear cells were significantly higher in patients with SSc

and soluble IL-6R levels significantly correlated with the severity of pulmonary fibrosis in patients with SSc (96). Serum IL-6 levels might be predictive of disease progression in Interstitial lung disease associated with SSc (97). IL-6 shifts T cells from regulatory response to pathogenic Th17 response (98), and promotes the differentiation of CD4+ cells to a profibrotic Th2 type while suppressing Th1 differentiation (99). IL-6 stimulation induces increased collagen production in dermal fibroblasts (100, 101). These studies demonstrate that IL-6 is involved in the pathogenesis of SSc and may contribute to progression of fibrosis and disease severity in SSc.

A combined score of the plasma IFN-inducible chemokines, IFNγ-inducible protein 10 (IP-10/CXCL10), and IFN-inducible T cell α chemoattractant (I-TAC/CXCL11) highly correlated with the IFN gene expression signature in SSc patients in the Genetics versus Environment in Scleroderma Outcome Study (GENISOS) cohort study (102). As expected, SSc patients had higher IFN-inducible chemokine scores than age-, gender-, and ethnicity-matched controls. Among 266 SSc patients, the IFN-inducible chemokine score was associated with presence of anti-U1 RNP antibodies while patients with anti-RNA polymerase III antibodies had lower levels of this chemokine score. The lower IFN chemokine levels in patients with anti-RNA polymerase III antibodies might be of important biological significance because these antibodies are associated with presence of diffuse cutaneous involvement and absence of severe interstitial lung disease. The IFN-inducible chemokine score was not associated with disease duration, disease type, or other auto-antibodies. The chemokine score correlated positively with the concomitantly obtained scores on the Medsger Severity Index for muscle, skin, and lung involvement, as well as creatine kinase levels in SSc. There was also a negative correlation with forced vital capacity and diffusing capacity for carbon monoxide. These results support the aforementioned findings that the IFN activation is associated with the more severe form of SSc. There was no significant change observed in the IFN-inducible chemokine score over time in SSc patients. The fact that the IFN chemokine score did not show a consistent trend of change and that it was not associated with disease duration at the baseline visit indicates that the IFN signature is a stable marker for the more severe subtype of disease rather than a time-dependent immune dysregulation that improves after the initial phase of SSc (102).

CONCLUSION

There are many distinct immunological and molecular mechanisms that can contribute to pathogenesis and progression of SSc. Dysregulated innate and adaptive immune responses are major contributors to fibrosis and disease severity of SSc. This review summarized a possible role of type I IFN and IFN-inducible genes in pathogenesis of SSc, and provides support for a link between type I IFN and fibrosis in SSc. Potential role of type I IFN or IFN-inducible genes as treatment targets or biomarkers in SSc need to be further explored. A better understanding of the relationship between type I IFN and fibrosis could bring us closer to the ultimate goal of reversing or slowing the fibrotic process and regenerating the normal end organ tissue in SSc.

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Aberrant type I interferon regulation in autoimmunity: opposite directions in MS and SLE, shaped by evolution and body ecology

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Studying the action of mechanisms of type I interferon (IFN) provides the insight to elucidate the cause and therapy for autoimmune diseases. There are high IFN responses in some diseases such as connective tissue diseases, but low responses in multiple sclerosis. Distinct IFN features lead us to understand pathology of a spectrum of autoimmune diseases and help us to search genetic changes, gene expression, and biomarkers for diagnosis, disease progression, and treatment response.

Keywords: interferon-beta, Devic's disease, multiple sclerosis, neuromyelitis optica, phospho-serine-STAT1, SLE, statins, Trex1

PROLOGUE

EVOLUTION OF TYPE I INTERFERONS: IMPLICATIONS FOR AUTOIMMUNITY AND CELL PROTECTION

Interferons (IFNs) were present in early bony fish during the Devonian Period, 400,000,000 years ago. Fish have branched into more species than all other vertebrates combined, and changes in their proteins evolved in parallel with taxonomic diversity. Sequence and structural similarities in fish IFNs suggest that there was an earlier ancestor in common with mammals. Present-day fish IFN has introns and in that way resembles human IFN-lambda, a type III IFN. Mammalian type I IFN has no introns, and may have arisen from retrotransposition of spliced RNA to intronless DNA with the same sequence as the parent gene (1). DNA without introns is more easily duplicated, leading to many subtypes. Transcription from DNA without introns is rapid and can bypass mechanisms that can be derailed by virus infections. Antiviral IFN genes would therefore have a selection advantage during fevers.

Interferons vary widely between species of present-day fish. Salmon have 11 subtypes of type I and III IFN; zebrafish have two group 1 and two group 2 subtypes. Subtypes of IFN likely evolved to effectively target different viruses in vertebrates. Viruses bind toll-like receptors (TLRs) 3, 7, 8, and 9 and retinoic acid-inducible gene-I (RIG-I) receptors. These fish receptors then induce IFNs which activate or induce type I IFN receptors (IFNARs), and in turn, signaling proteins (JAK1, TYK2), transcription factors (STAT1), and many IFN-stimulated gene (ISG) proteins (such as IRF, MxA, PKR, and viperin) (2). All are present in humans.

The human type I IFN family comprises 13 IFN-α, 1 IFN-β, 1 IFN-κ (keratinocytes), 1 IFN-ω (**Table 1**). IFN-τ is a related variant found only in the ruminant trophectoderm, important early in pregnancy. IFN-δ is produced by pig trophoblasts. IFN-ζ/limitin is present in mice.

Humans have three type III IFN-λs (*aka* IL-29, IL-28A, and IL-28B) which bind a different receptor (IL-10Rβ and IL-28Rα chains), often on epithelial cells and liver cells. IFN-lambda has introns. Only restricted cell types express the unique type III IFNAR, and thus it may have fewer side effects. It is produced by plasmacytoid dendritic cells (pDCs) and induces IL-6, IL-8, and IL-10, and activates type II monocytes. It is anti-proliferative, but only for several tumor cell subtypes.

Viruses were likely to have been early targets of IFNs in evolutionary history. There is intense selective pressure to rapidly destroy viruses and virus-infected cells. Human interferons induce proinflammatory cytokines and chemokines such as CXCL10 (IP10), and turn on hundreds of anti-viral genes such as MxA, viperin, 2′,5′-OAS, and PKR. IFNs also activate cytolytic T cells, NK cells, Th1 cells, macrophages, and cells from other tissues, and induce apoptosis of infected cells. (Control of inflammation is discussed below.)

Interferons regulate a 1000 genes (3, 4). Many of these genes are not anti-viral. Some IFN-regulated genes shape the innate and adaptive immune systems, some modify transcription, some are anti-proliferative and pro-apoptotic to combat cancer, other genes control fertility, fatty acid oxidation, free radical neutralization, energy metabolism, and cell protection and tissue repair. These functions are a leap beyond the anti-viral role of IFNs. Virus-independent benefits suggest that IFN responses evolved to "clean up the mess" after a virus infection (Ed Croze, personal communication, 2007). Subnormal IFN levels, as in multiple sclerosis (MS), could disturb immune regulation and also diminish cell protection. Therapy of MS with type I IFNs reverses these disturbances.

Table 1 | Interferons in mice vs. humans.

	Mouse	Man
Type I IFNs	14 IFN-α genes	13 IFN-α genes (12 proteins)
19 kDa, 165–166 aa	Four have alleles that differ between strains	
	10 Glycosylated	2 Glycosylated
	IFN-α4 promoter binds IRF3	IFN-α4 promoter binds IRF3
20 kDa, 22 kDa glycosylated, 166 aa	1 IFN-β	1 IFN-β
	IFN-β promoter binds IRF3	IFN-β promoter binds IRF3
22 kDa and glycosylated, 187 aa	1 IFN-ε	1 IFN-ε
25 kDa, 180 aa	1 IFN-κ	1 IFN-κ
20 kDa, 172 aa	1 IFN-ω	None
19 kDa, 182 aa	1 IFN-ζ (limitin)	1 IFN-ζ (limitin)
Type II IFN	1 IFN-γ	1 IFN-γ
17 kDa		
Type III IFN		
21 kDa, 26–35 glycosylated	None	IFN-l1 (IL-29)
22 kDa, 24 glycosylated	IFN-l2 (IL-28A)	IFN-l2 (IL-28A)
21 kDa, 24 glycosylated	IFN-l3 (IL-28B)	IFN-l3 (IL-28B)
Signaling	**P-STAT proteins**	**P-STAT proteins**
STAT1a 91 kDa	P-Y701-STAT1	P-Y701-STAT1
	P-S727-STAT1	P-S727-STAT1
STAT1b 84 kDa	P-Y701-STAT1	P-Y701-STAT1
STAT2 113 kDa	P-Y689-STAT2	P-Y690-STAT2
	No Y833 – truncated (76)*	P-Y833-STAT2
	No Y841 – truncated	P-Y841-STAT2
	No STAT2 induction of P-STAT4 (76)	P-STAT2 induces P-STAT4
STAT4 86 kDa	Type I IFNs induce Th1	P-STAT4 induces IFN-g, Th1
		Type I IFNs induce Th1, but reports in MS are mixed
EAE vs. MS	**EAE**	**MS**
The target	Antigen-induced	No known antigen (ADEM is Ag-induced)
Therapy	Improvement with pure IFN-β	Most improve with IFN-β therapy (∼85% have a low IFN signature) (∼15% have higher IFN signature; less response to IFN-β therapy)

**"Humans do not provide a good model for mouse immunology" from Ref. (76).*

IFN SIGNALING, KINETICS, AND SPECIES DIFFERENCES

The primary source of IFN is from pDCs (IFN-α > IFN-β production), fibroblasts – the major producers of IFN-β (IFN-β > IFN-α), macrophages, and endothelial cells. pDC are only 1% of DC. Myeloid dendritic cells (mDCs) secrete IL-12 and other cytokines, but only small amounts of IFN.

Interferons signal through a rapid cascade. Type I IFN is secreted within an hour of stimulation with virus or poly(IC), a synthetic analog of viral double-stranded RNA (dsRNA). Viral nucleic acids are recognized by pattern receptors (described below) that activate IRF3, which then turns on IFN-β and IFN-α1 synthesis. These first subtypes of IFN bind IFNARs and activate IRF7 in surrounding cells to induce multiple other type I IFN subtypes/species.

The cell surface type I IFNAR has two-chains, IFNAR1 and IFNAR2 which activate TYK2 and JAK1. Phospho-JAK1 and TYK2 then phosphorylate STAT1 (P-Tyr701-STAT1) and STAT2 (P-Tyr-STAT2) proteins which complex with IRF9 (p48) (ICSBP) to form IFN-stimulated gene factor 3 (ISGF3). Like type I IFNs, IFN-lambda induces ISGF3. Within 10 min after IFN activates its receptor, the ISGF3 binds the IFN-stimulated response element (ISRE) of a large group of gene promoters. DNA-bound P-Y-STAT1 is sometimes then phosphorylated on serine 727 within ∼10 min (forming P-S-STAT1) (5) which boosts the signal for a subset of IFN-regulated genes. STAT2 has no serine phosphorylation site.

TYPE I IFN REGULATION AND LOCALIZATION OF IFN PRODUCTION

Once the receptors above are activated, a sequence of intracellular signals amplifies IFN secretion. IRF3 is phosphorylated, binds to DNA promoters, and then IFN-β and IFN-α1 (IFN-α4 in mice) are rapidly secreted. These two IFNs then bind the type I IFNAR on the same and other cells and activate IRF7 which binds to promoters for other IFN-α subtypes.

Other intracellular signal transduction molecules modify the JAK-STAT pathway, including, PI3K, CRKL, RAP1, PKC-δ and ε, and p38 in the MAPK cascade (6). These converging proteins are cell-specific. These pathways enhance effects of type I IFNs, but perhaps the most important interaction is with IFN-γ.

Type II IFN-γ is the prototypic immunoregulatory Th1 cytokine, but it is only distantly related to type I IFNs and has only weak anti-viral effects. It activates STAT1 on tyrosine. P-Y-STAT1 homodimers bind to the gamma-activated site (GAS), present in a set of promoters that are different from the type I IFN ISRE that is activated by the STAT1/STAT2/IRF9 complex. IFN-γ-activated P-Y-STAT1 can interact with IFN-β-induced P-Y-STAT1. Preincubation with IFN-γ "primes" cells for a more vigorous response to IFN-β.

Interferon signaling differs between mice and men. In humans, activation of STAT2 then phosphorylates STAT4, which can turn on more cytokine genes. In some mouse strains, however, STAT2 is truncated, and does not activate STAT4 (**Table 1**). Thus, the murine experimental allergic encephalomyelitis (EAE) model of MS suffers from being an antigen-specific model of a disease with no known antigen, and because inflammation in EAE is regulated by interferon signaling that is missing part of the human signaling cascade.

Some downstream genes are rapidly induced (e.g., anti-viral and immunoregulatory genes); others take longer to plateau (e.g., genes with secondary induction, e.g., dual oxidase 2) (4). Kinetics also vary between cells. For instance, IL-10 mRNA production by activated monocytes is maximal at 4–8 h and is inhibited by IFN-β. In activated T cells, the peak is at 24 h and IL-10 mRNA is amplified by IFN-β (7).

Interferon-β, compared to the individual subtypes of IFN-α, induces a larger number of genes in human fibrosarcoma cells, and does so more quickly (3). IFN-β binds to the IFNAR for a longer time than IFN-α (8), explaining the differential gene induction. Perhaps because of the greater number, but more balanced portfolio, of induced genes, IFN-β has fewer side effects than equivalent anti-viral doses of IFN-α. IFN-β is also more effective than IFN-α in some therapies. Despite the traditional use of IFN-α subtype to treat some forms of cancer, IFN-β is actually more potent against several types of cancer at equivalent anti-viral titers of both IFNs (9). It is also more effective in MS therapy. (Cytoprotective effects are discussed with MS, below.)

Does IFN-β cross the blood-brain barrier (BBB)? Although CSF IFN levels are only 1/1000 of serum levels in a normal monkey (10), the damaged BBB in MS and EAE could allow IFN to cross. In humans, circumstantial evidence suggests that IFN-β has direct effect on the CNS. IL-10 levels in CSF rise after IFN-β-1a therapy (11) and black hole formation is reduced by IFN-β-1b (12). In mice with EAE, IFN-induced mRNA is clearly present in the CNS, after controlling for the effects of EAE and IFN-induced mRNA outside the CNS (13). Thus, IFN-β may have direct effects on brain cells in MS.

TRIGGERS FOR TYPE I IFN PRODUCTION

Virus components that induce type I IFNs include exogenous virus RNA or DNA and associated proteins, vaccines which are attenuated viruses or contain virus components, and endogenous retroviruses that make up ∼8% of human DNA. Exuberant responses to viruses or to abnormally processed DNA from dying cells and their nucleic acids can activate the immune system and lead to autoimmune diseases such as systemic lupus erythematosus (SLE).

Receptor families recognize conserved pathogen-associated molecular patterns (PAMPs; i.e., "danger" from viral nucleic acids and other motifs) and viruses. These receptors include TLR, RIG-I, melanoma differentiation-associated protein 5 (MDA5), CD11b/CD18 (Mac1), STING (DNase II), as well as the Trex system. Each virus can be detected by multiple PAMP receptors.

Toll-like receptors were originally characterized in fruit flies, where they recognize a developmental growth factor and control antimicrobial responses in adult flies. In humans, 10 types of TLR recognize bacterial components, RNA, and DNA. Fibroblasts express TLR3 on their surface. Other, intracellular, sensors detect viruses after they are internalized or generated inside cells. They also sense abnormally processed nucleic acids in connective tissue diseases after Fc receptors internalize DNA-Ab complexes. TLR3 is endosomal in monocytes and mDCs (TLR3 is not in pDC). Virus RNA is recognized after phagocytosis and internalization, or after enveloped viruses penetrate the cell by endocytosis. After binding dsRNA or pIC, human TLR3 is activated within acidified cytoplasmic endosomes (14). TLR3 activates TRIF, and then kinases (IKKε, TBK1) that phosphorylate IRF3 and IRF7 (below). TLR7 and TLR8 bind single-stranded RNA (ssRNA), poly-IC, and imiquimod. TLR9 binds intracytoplasmic viral or bacterial CpG-rich DNA. TLR7, 8, and 9 are present in pDC.

The RNA helicases, RIG-I and MDA5, bind short and long viral dsRNA, or pIC. Homologs of all of these receptors, with the exception of the OAS system, are present in fish where they sense pathogens and induce IFNs and many ISG.

IRF3 is constitutively expressed at high levels in most cells. IRF7 is present at only low levels, mainly in immune cells especially pDC, but is necessary for the initial induction of IFNs. Activated IRF3 induces IFN-β and IFN-α1 (IFN-α4 in mice) which prime the type I IFN system for a much stronger response. After virus exposure, rapidly secreted IFN-β binds the IFNAR and induces and activates intracellular IRF7 (at 4 h), while IRF3 is degraded. IRF7 broadens the response by inducing multiple IFN-α genes in pDC which and activate pDC, T cytolytic, Th1, and NK cells. In parallel, virus-exposed conventional DC and monocytes secrete cytokines such as IL-12 plus low amounts of IFN-α1 and IFN-β.

TLR7, 8, and 9 are expressed at high levels in pDC, and activate MyD88. These TLRs activate IRF7 which induces transcription of some IFN-β plus high levels of multiple types of IFN-α in pDC (15). Ligation of RIG-I and MDA5 activate transcription factors IRF3 and NF-κB, which travel to the nucleus and initiate transcription of type I IFNs.

CONSEQUENCES OF EXCESS STIMULATION BY DNA, RNA, BACTERIA, AND CYTOKINES

Influenza infections elevate type I IFNs in serum and pulmonary secretions. In Sjögren's syndrome, foci of inflammation in salivary glands are positive for IFN-α, and serum IFN levels are elevated. In SLE and Sjögren's disease, high interferon levels and lack of immune regulation cause damage to target organs. Surprisingly,

because it is an immunologically privileged site, the brain also exhibits IFN expression or binding. An early paper showed IFN-α-positive macrophages, IFN-γ-positive astrocytes and microglia, and occasional IFN-β-positive astrocytes and macrophages, within active chronic MS brain lesions (16). During chronic hepatitis C therapy, IFN-α (5 MU TIW) reduces PET activity by 10% in the pre-frontal cortex (17), and 3 MU TIW causes gradual slowing of VEP over 1 year (18). Hepatitis C in these patients may have had additive effects with IFN-α. In contrast, VEP slowing is likely not seen with IFN-β therapy, as P300 potentials are stable or improved (19). Excessive CNS IFN levels cause encephalopathy in Aicardi–Goutières syndrome, Cree encephalitis, and cerebral malaria, *v.i.* (20), and possibly in SLE and Sjögren's disease.

The Trex system is an intracellular monitor for products of endogenous retroviruses. Trex1 (DNAse III) is the major 3′,5′-exonuclease in humans. The single-exon gene codes for a cytosolic protein that is induced by the IRF3-dependent IFN-stimulated response to foreign DNA (21). It edits DNA by stripping off one stand of ssDNA and metabolizes intranuclear DNA; residual ssDNA that escapes from the nucleus can trigger an immune response. Remnants of ancient retroviruses human endogenous retrovirus (hERV) that have incorporated into the DNA comprise ∼8% of the human genome, and retroelements may outnumber our genes by 100-fold (21). These hERV are not complete virions, but portions can be transcribed, and do generate ssDNA fragments and proteins. hERV DNA is degraded by Trex1. In Trex1 knockout mice, 22% of the DNA in inflamed myocardium is coded by endogenous retroviruses, vs. 7% in wild type mice (21), indicating that Trex1 is needed to destroy these retroviral genes. Reverse-transcribed DNA that induces IFN production is the principle cause of autoimmunity in these mice. Defects in Trex1 lead to high circulating levels of foreign DNA, which triggers type I IFN production and autoantibody production.

Trex1 deficiency causes constitutive activation of the systems that control ATM-dependent double-strand breaks and cell cycle checkpoints. Induction of ATM for DNA-damage monitoring also activates p53 and BRCA1, leading to fewer tumors, self-renewal of hematopoietic stem cells, thymocyte survival, but less apoptosis of autoreactive immune cells. This could amplify autoimmune disease.

A defective Trex1 gene is common in Canadian Cree Indians, and causes excessive interferon production. This leads to Cree Indian Encephalopathy, or Aicardi–Goutières syndrome, an encephalopathy associated with lupus-like symptoms (22). Excessive CSF IFN-α (42 IU/ml) in affected children (Pierre Lebon, Paris) mimicked congenital viral infection, but associated chilblains (pernio) pointed to SLE and autoimmunity, and IFN-α. These children have: (1) progressive microcephaly and failure to attain neurodevelopmental milestones, beginning in early infancy; (2) recurrent viral, bacterial, and fungal infections; (3) cerebral atrophy, white matter attenuation, and calcifications of basal ganglia, white matter, and/or cerebellum on CT scan; (4) perivascular chronic inflammatory infiltrates in cerebral hemisphere white matter and hyperplasia of vascular endothelial linings; and (5) polyclonal hypergammaglobulinemia. They sometimes have corroborative features such as: (1) dystonic posture; (2) systemic autoimmune abnormalities – high ALT and Abs to cardiolipin, ssDNA, dsDNA, and RNA-protein complexes; (3) splenomegaly and lymphadenopathy; (4) CSF pleocytosis with high CSF IFN-α and Ig; high blood CD8 "suppressor" and B cells; (5) intermittent hyperpyrexia; (6) chronic active Epstein–Barr virus (EBV) or CMV infection or persistent viral excretion; (7) similarly affected siblings; and (8) acrocyanosis with autoamputation (23, 24). With this "chilblain lupus" from Trex1 deficiency, cold exposure causes cyanosis of toes and fingers because of damage to capillaries. Eighty percent of families with Aicardi–Goutières syndrome have mutations in one of four nuclease genes – the exonuclease Trex1 [chromosome 3p21 (*AGS1*)] or the genes for all three components of the ribonuclease H2 enzyme complex (*AGS2, 3*, and *4*).

In SLE, apoptotic products of PMN, T cells, and macrophages are not cleared correctly by macrophages. Apoptotic blebs contain modified chromatin, and neutrophil extracellular traps are released by dying neutrophils (NETosis). Abnormally processed nucleic acid-containing debris circulates as phospho-DNA that is recognized as a virus, or Ab-DNA neoantigens, that activate FcR and TLR of DC and the BCR of B cells. Chromatin, double-stranded DNA, and RNA-binding ribonucleoproteins activate an autoimmune circuit and production of IFN-α/β by pDCs and anti-dsDNA Abs by autoreactive B cells.

Excess local IFN-α damages the CNS. Encephalopathy develops when IFN-α is overexpressed in astrocytes (25). Transgenic mice develop early mineralization around blood vessels in the thalamus at 2 months, calcium crystals in cerebellum at 12 months, and perivascular CD4+ T cell infiltrates in the CNS. Some pediatric infections lead to high CSF IFN-α ("TORCH," from Toxoplasmosis, Other, Rubella, CMV, HSV), with sequelae of CNS calcifications and brain atrophy. Chronic exposure to IFN-α in cultured astrocytes increases GFAP expression, reduces proliferation, and causes hypertrophy and activation (25, 26), reflecting the changes from high CNS IFN-α in Aicardi–Goutières syndrome.

In MS, HERV DNA and antibodies to HERV proteins appear in serum, CSF, and brain (27). Activated immune cells release HERV nucleic acids into the cytosol. This could induce type I IFNs in immune cells or the CNS in some MS patients.

"Interferon inducers" also generate non-IFN cytokines and proteins. For instance, pIC is a potent stimulus for lymphocyte production of ACTH and other proteins processed from the pro-opiomelanocortin (POMC) precursor molecule (28). IFN-α2 triples serum ACTH and cortisol 5 h after injections in patients with hepatitis B; flu-like symptoms do not correlate with induction (29). In contrast, IFN-β-1b therapy of MS does not elevate cortisol (30).

Environmental agents and drugs can modify IFN effects. Vitamin A activates STAT1 and synergizes with IFN-β (31). Oral vitamin D3 regulates 63 genes, 62 of these were also regulated by IFN-β-1b therapy in early MS (Munger, uncorrected data analysis of BENEFIT study; personal communication, CSMC, 2013). Statins, which lower cholesterol and are anti-inflammatory, surprisingly block IFN signaling. They reduce formation of P-Y-STAT1 and IFN-β-induced MxA production *in vitro* and *in vivo* (32), and allow attacks of MS when added to ongoing IFN-β-1a therapy (33) (below).

The response to exogenous or endogenous triggers has to be tightly controlled, or unchecked immune responses will destroy

the host (34). Excessive responses to influenza, as in the 1918 pandemic, lead to death from severe pneumonitis. Weak immune responses, as in never-exposed youths or in octogenarians, do not control the virus. Prior immune education usually allows rapid clearance, manageable immune reaction, and tolerance/regulation that allows the inflammation to subside. Tolerance should be under stronger selective pressure than the actual anti-pathogen response (34, 35). As a virus is cleared, the immune system tempers inflammation with regulatory T cells, inhibitory receptors for immunoglobulin Fc on immune effector cells, apoptosis and autophagy of target cells, anti-inflammatory cytokines, and induction of intracellular suppressors of cytokine signaling (SOCS) proteins. IFNs regulate each of these immune functions, and regulation of each, had to evolve over eons.

Prevention of anti-self responses in an individual are honed over a lifetime by environmental events which generate a complex immune repertoire to combat danger. If the environmental guidance is missing, holes in the repertoire can trigger autoimmunity to self antigens. If certain antigens or levels of interferon are in excess, immune responses could be excessive and can cross react with self, triggering autoimmunity.

DISEASE WITH HIGH SERUM TYPE I IFN LEVELS AND HIGH RESPONSES TO IFN: SLE, CNS SJÖGREN'S SYNDROME, NMO, AND A MINORITY OF MS PATIENTS

Early reports identified a unique "acid labile" type I IFN in serum of patients with SLE and HIV infection (36). Type I IFN is resistant to pH 2, but IFN-γ is destroyed on exposure to acid. Acid sensitivity may be from have aberrant glycosylation of some subtypes of IFN-α in connective tissue disease and HIV. More recent studies of patients with SLE demonstrate significant increases in serum type I IFN activity (37, 38) and excessive signatures for IFN-induced RNA in white blood cells [in Ref. (37)]. Responses to self DNA or viruses are inappropriately regulated in hereditable complex traits linked to single nucleotide polymorphisms (SNPs) in IFN-regulatory genes (TYK2, IRF5, STAT4, TNFAIP3, and TREX1) and diverse mutations in Trex1 in 0.5–3%. In support, lupus-prone mice that are transgenic for TLR7.1 and have excessive levels of TLR7 have more autoantibodies and early severe lupus. High IFN-α levels correlate with SLE disease activity and severity in some studies (24). Clinically relevant, therapy with type I IFN causes *de novo* SLE or worsening of preexisting disease.

Nearly all parts of the body can be affected in SLE from the antinuclear antibodies and symptoms derived from high serum IFN levels. A constellation of damage affects skin, mucocutaneous tissue, joints, kidneys, lungs, heart, and blood vessels – with immune-complex vasculitis and thrombotic occlusions. CNS lesions are rare, but occasionally lupus myelopathy develops with devastating vasculitis, inflammation, swelling, and demyelination over many cord segments. In addition, serum type I IFN levels are elevated. SLE problems that are possibly related to high serum IFN levels include lymphopenia, myalgia and muscle weakness, joint pain with modest infusions, significant constitutional symptoms – headache, body ache, malaise, and fatigue that can herald neuropsychiatric problems (poor memory, mood swings, seizures, and psychosis – without strokes or vasculitis, although there is underlying small vessel vasculopathy in SLE brains). Cognitive problems are often unrelated to SLE exacerbations, suggesting a second mechanism of damage. Perhaps high levels of type I IFN, and abnormally processed nucleic acids induce autoantibodies that are specific to certain regions of the brain and interfere with neuronal function (39) (**Table 2**). With disruption of the BBB by stress or epinephrine, neurons in the amygdala are damaged by Abs (anti-NR2) to orphan NMDA receptors. With BBB disruption from infections, auto-Abs damage hippocampal neurons and disrupt spatial memory. In both cases the damage is excitotoxic, without inflammation, due to Ab-mediated stimulation of the NMDA receptor.

Interferon levels are high in serum and salivary glands in Sjögren's disease, a connective tissue disorder with dry eyes and mouth (sicca) from inflammation of the lacrimal and salivary glands, along with synovitis, vasculitis, and neuropathy. Classic primary Sjögren's syndrome typically appears in middle-aged women. CNS involvement is usually not part of primary Sjögren's, but a CNS variant was recently described (Javed, below). Secondary Sjögren's syndrome coexists with SLE, polyarteritis nodosa, rheumatoid arthritis, scleroderma, polymyositis, and neuromyelitis optica (NMO) (below). Glands are infiltrated by foci of memory CD4 T cells (secreting IL-10 and IFN-γ), macrophages, and mast cells near activated epithelial cells (IL-1β, IL-6, TNF-α). A small number of infiltrating activated B cells produce high levels of immunoglobulin, some directed against rheumatoid factor, SSA/Ro, SSB/La, anti-nuclear antigens, aquaporin 5, and the M3 muscarinic receptor. The surprising overlap between CNS Sjögren's syndrome and NMO (often considered an "MS variant") is discussed below.

MULTIPLE SCLEROSIS: EPIDEMIOLOGY, IMMUNOLOGY, AND ROLE OF IFN

Multiple sclerosis is an inflammatory CNS disease with no clear etiology, pleomorphic clinical and MRI appearance, and huge variation in clinical course, with a transition from bi-yearly relapses and remissions to a progressive form after ∼8–15 years (40). Clinical attacks plus the 10× more frequent subclinical events seen on MRI cause cumulative damage. Attacks last 2–6 weeks, affect any part of the CNS, and often resolve to near-baseline function. MRI lesions enhance with Gd, from leakage through the BBB and perhaps pinocytosis by activated endothelial cells of post-capillary venules. Lesions enhance for a month, then the residual high-water T2 signal fades slowly or becomes a permanent black hole reflecting damage to axons, neurons, and oligodendroglia. Therapy with IFN-β and some other drugs prevent Gd+ lesions but also prevent black hole formation after Gd+ lesions appear. This suggests that MS therapies may have neuroprotective effects.

Multiple sclerosis is more likely to arise when a first degree relative has MS (10- to 20-fold increase), and in smokers, the obese (2×), in those with little exposure to sunlight or with low vitamin D levels (2×), and when first EBV infection is delayed to adolescence. Once MS develops, exacerbations are more frequent in smokers (1.6×), and after infections by many different viruses or after vaccination with certain attenuated live viruses such as yellow fever (41). IFN-β therapy does not prevent virus infections, but it does diminish the residual clinical deficit after a virus-induced exacerbation of MS (42).

Table 2 | Characteristics of demyelinating disease.

	MS (40)	SLE	CNS Sjögren's (58)	NMO (77, 78)
MRI brain lesions	Periventricular, Dawson's fingers	Gray and white matter lesions	Centrum seniovale	Hypothalamic and periventricular in <10%
	"Random" WM + GM, but predilection for certain areas			
	Small to large lesions	Small WM, rare large vasculitic, and CVA lesions	Small lesions	Medium to large lesions, later in course
MRI cord lesions	<1 Segment	Rare extensive myelopathy	>3 Segments, also smaller lesions (58)	>3 Segments; longitudinally extensive
	Often subpial or acentric		Central cord	Central cord
Relapse rate	q 2 year	∼Once per 5 year, on therapy	Similar to NMO	Frequent (∼2×/year) early in the course for NMO + patients
Progression	PPMS at onset in 10%	Stepwise and gradual multi-organ failure	Progressive sicca symptoms	None
	RRMS becomes SPMS @ 8–15 year	∼Once per 5 year, on therapy		
Pathology CNS	Demyelination > axonal loss	"Vasculopathy" > cognitive changes	Demyelination < axonal damage	Demyelination < axonal damage
	Many lesions will repair	Rare arteriopathy	Vasculopathy	Vasculopathy
	Destruction by CD8 T cells and monocytes	Cells include PMN and Eos	Severe	Severe and destructive
				No repair
Serum marker	No marker	Anti-dsDNA	SSA/SSB 40% Anti-AQP-4 50%	Anti-AQP-4 60–75%
Target Ag	Unknown	Abnormally processed DNA and RNA	Nucleic acids, AQP-5 Minor salivary gland inflammation in 100%	AQP-4
Serum type I IFN	Low IFN-α/β	High IFN-α/β	High IFN-α/β	High IFN-α/β
IFN-β Response by MNC	Low	High	High	High
CSF	High IgG	∼Normal	∼Normal	∼Normal
OGCB	90%	10%	∼10%	20%
Triggers for exacerbation	Virus, vaccination for yellow fever	Virus Type I IFNs	Virus, possibly Possibly type I IFNs	Virus; UTI (AQP-Z) (79) Possibly type I IFNs
	Low vitamin D	Sunlight		
	Smoking: MS onset and exacerbations	Smoking: SLE onset and exacerbations	Possibly smoking	Possibly smoking
Therapy	IFN-β, glatiramer, natalizumab, fingolimod, fumarate, teriflunomide, alemtuzumab, rituximab, laquinimod Sunlight; vitamin D potentiates IFN-β-1b GI parasites Gout is rare	Hydroxychloro-quine, steroids, chemotherapy	Rituximab, steroids, chemotherapy	Rituximab, steroids, chemotherapy
Pregnancy	Benefit, perhaps from estriol	Worse	Unknown	Unknown
Linked diseases	?Thyroid	Connective tissue diseases	Connective tissue diseases	Connective tissue diseases
	?Ulcerative colitis	Aicardi–Goutieres		

AQP, aquaporin; GI, gastrointestinal; GM, gray matter; NMO, neuromyelitis optica; UTI, urinary tract infection; WM, white matter.

Multiple sclerosis usually begins in the reproductive years and is thee times more common in women than men. A subset of patients, perhaps 25%, has a high IFN signature as well as more clinical and MRI attacks before therapy, and these patients often do not respond to IFN-β therapy (43, 44). They have excessive activation of monocytes and mDCs and a high type I IFN-induced gene signature that predicts 70% of non-responders compared to patients with normal IFN signatures. Non/poor-response was defined as one or more attacks of 2 years on therapy (43) or three or more new or worse MRI lesions (44). However, since high disease and MRI activity predicts more future activity, these patients should be considered partial-responders, in the absence of an untreated control group.

Immune studies and SNPs on GWAS implicate many genes involved in immunoregulation or IFN signaling. HLA-DR2 has the strongest GWAS odds ratio at 2.0; others are only 1.1–1.4, but do include IFN-regulated genes such as Tyk2, 2′,5′-oligoadenylate synthase (OAS1), IRF5, MxA, and many IFN-affected immune response genes (40, 45). This suggests that environmental influences and the education of the immune system are critical in the development of MS and its course. A Th1 bias is characteristic of MS. As a consequence, cancers, virus infections, and allergies is less frequent than expected in MS patients (40). A shift to Th2 immunity, seen with glatiramer therapy or after parasite infestation, reduces exacerbations of MS. IFN-β, however, does not simply cause a shift to Th2 immunity – type I IFNs typically enhance Th1 immunity (46). In the CSF of IFN-β treated patients, however, IL-10 is elevated (11), perhaps from IFN-β stimulation of activated T cells (7).

It would seem that MS and connective tissue disease could have a common etiology because they are "autoimmune." However, MS only rarely coincides with SLE (47), NMO is a separate entity, and the majority of MS patients benefit from IFN-β therapy instead of worsening (48). Importantly, the vigorous signature in SLE, Sjögren's, and NMO, contrasts with the subnormal serum type I IFN activity and WBC responses to IFN in most MS patients (**Table 2**).

MULTIPLE SCLEROSIS: LOW SERUM TYPE I IFN ACTIVITY AND WEAK RESPONSES TO IFN

The first patients that were placed on commercially available IFN-β showed induction of IFN-g (Th1), IL-10 (Th2) (46), and the IFN-stimulated proteins IRF-1, IRF-2, and 2′,5′-oligoadenylate synthetase (2′,5′-OAS) in mononuclear cells (MNCs) (49) (**Figure 1**). However, before any therapy, IFN regulation is abnormal in MS. Levels of these ISG products were actually low, and after IFN-β injections, levels rose only to levels seen in unstimulated control MNC. It was apparent that IFN responses were subnormal in MS, and that IFN-β therapy corrected IFN-induced protein levels back to the normal range. Extensive experiments using flow cytometry of IFNAR expression, Western blots of P-Y-STAT1, gel shift assays (EMSA), transfection of MNC with an IFN-responsive human ISRE reporter gene, and SHP levels to measure potential dephosphorylation of *P*-tyrosine, all showed that P-Y-STAT1 levels and P-STAT1 binding to DNA were normal and or above normal [Reder et al., 2000, unpublished and (32)] (**Figure 2**). Despite normal levels of P-Y-STAT1, however, induction of the above-mentioned ISG and MxA, an IFN-induced anti-influenza protein, was subnormal. It was later discovered there was a second STAT1 phosphorylation site at serine 727 (50). In unstimulated MNC from therapy-naive patients, P-S-STAT1 levels were low in MS (32). P-S-STAT1 was also poorly induced by IFN-β in cultured MNC, and this low P-S-STAT1 correlated with low levels of ISG. This indicated that there is a fundamental defect in IFN regulation that underlies MS, and that is likely to have consequences for immune regulation, therapy, and CNS repair.

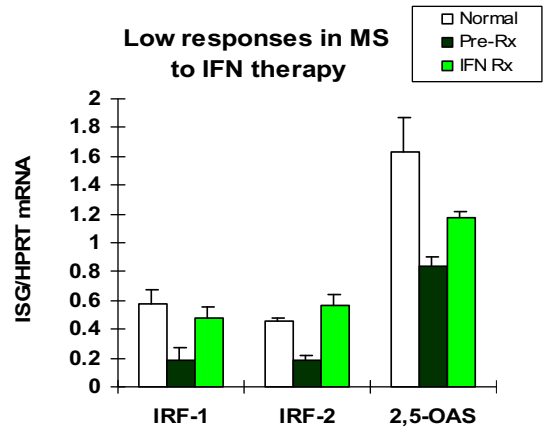

FIGURE 1 | Basal IFN-regulated mRNA levels in therapy-naïve stable RRMS (black bars) are lower than in healthy controls (white). IFN-β therapy induces mRNA production in MS MNC (Pre-Rx vs. IFN Rx), but levels are still at or below levels in *unstimulated* normal MNC (white). MNC assayed before and 3 months after *in vivo* IFN-β-1b (8 MU sq qod). Early genes are IFN-regulatory factor-1 (IRF-1; enhances signaling) and IRF-2 (negative regulator), and later gene is 2′,5′-oligo-adenylate synthetase (2′,5′-OAS, an IFN-α/β-induced anti-viral enzyme). (Pre vs. Rx: IRF-1 and IRF-2, $p < 0.02$; 2′,5′-OAS, $p < 0.03$; paired *t*-test). (Avg ± SEM for IFN-stimulated gene/HPRT; RT-PCR) $N = 5$ RRMS patients for IRFs, and 10 for 2′,5′-OAS, vs. 9 normal (46).

P-S-STAT1 levels are low in MNC during exacerbations and progression (32). *In vitro*, induction of therapy-naïve MNC with IFN-β induces phosphorylation of STAT1 on serine in healthy controls and in patients with stable RRMS. In contrast, P-S-STAT1 is not induced during exacerbations and progression. A subset of downstream genes are not induced during the IFN-resistant state seen in active MS. MxA and viperin induction is diminished, but other ISG such as PKR have normal expression (32, 38).

The subdued IFN response that underlies clinical disease activity in therapy-naïve MS patients has consequences for immune regulation, and may also predict future disease activity. The IFN-resistant state appears to be corrected by IFN-β therapy. IFN-β injections increase IRF-1, IRF-2, 2′,5′-OAS, MxA, and viperin to near normal levels (**Figures 1** and **2**) (32, 46). Type I IFNs, themselves, are important IFN-stimulated proteins. IFN-β injections cause a rise in serum IFN-β that peaks at 30 min and then declines. This elevation is soon followed by a second, prolonged rise in serum type I IFN activity, presumably from newly induced IFN-α plus more IFN-β (38). Finally, IFN-β therapy of MS restores defective CD8 regulatory cell function (51), increases expression of the inhibitory ILT3 protein on monocytes (52), and reduces expression of costimulatory molecules on B cells (53).

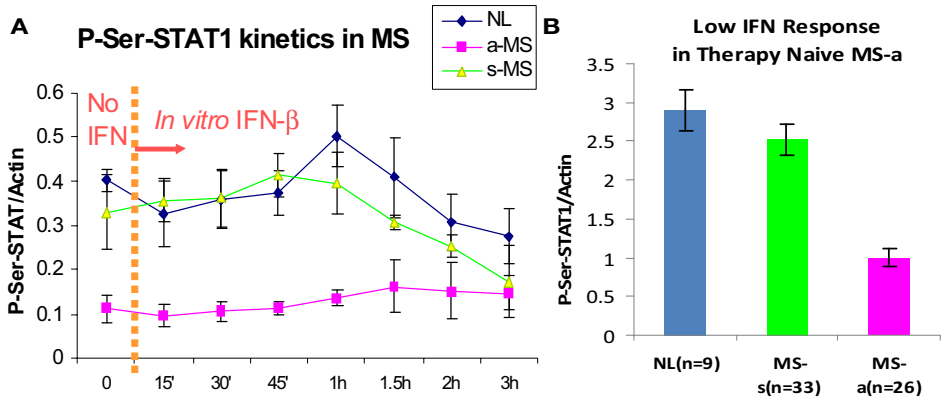

FIGURE 2 | (A) P-S-STAT1 is markedly reduced in MNC from therapy-naïve, clinically active MS at baseline (left of dashed line), and after stimulation with 160 U/ml IFN-β (right of dashed line). **(B)** Area under curve of IFN-β-induced P-S-STAT1 is high in stable RRMS and healthy controls (NL) at 60′ vs. active MS (8 NL, 7 RRMS-s, 7 RR + SPMS-a) ($p < 0.001$ active/progressive MS vs. NL; ANOVA with repeated measures) (32).

Interferon-β-treated patients with ostensibly stable MS, yet weak responses to their IFN injections, are more likely to have attacks in the future (54). These weak responses parallel the effect of neutralizing antibodies (NAbs) to IFN-β, where high-titer serum NAb correlate with more MRI lesions during IFN therapy, presumably from lower circulating IFN-β levels. However, effects on clinical activity are difficult to demonstrate, likely due to complex pharmacokinetic effects of Abs to IFN (55).

Would more frequent or higher doses of IFN-β reverse the IFN signaling defect in those patients refractory to conventional doses of IFN-β? In a very large study, patients with stable RRMS did not have fewer relapses or MRI lesions from double doses of every-other-day IFN-β-1b. Thus, the approved (single) dose of IFN-β is optimal in early, stable RRMS. However, the higher dose was more effective in preventing black hole development (12). The IFN signaling defect is less common in stable RRMS than during active and progressing MS (12). With thrice-weekly IFN-β-1a, there is no difference in outcome between 22 and 44 μg doses in RRMS with low EDSS (early MS), but when the EDSS is >3.5 (later or more severe MS), the 44-μg dose is superior in preventing relapses and progression (56). This suggests complex interactions between IFN-β dose and injection frequency, NAb, and form or severity of MS.

Unexplored issues remain with effects of excessive or defective IFN signature and responses to therapy. Is the incidence of infections and cancer reduced by IFN therapy? In a large, but only 2-year, study, there were fewer bladder infections and cancers in the intramuscular IFN-β-1a subgroup compared to placebo and fingolimod (57). Does correction of low IFN levels restore immunity and promote neuroprotection? Is the aging process slowed with normal or high levels of IFN-β, a protein that is cell-protective?

IFN REGULATION IN SLE, SJÖGREN'S SYNDROME, AND NMO IS OPPOSITE OF REGULATION IN MS

Neuromyelitis optica is a demyelinating disease that until recently was simply considered a severe variant of MS. We have argued that NMO is actually much closer to SLE and Sjögren's syndrome, and that it differs fundamentally from MS (**Table 2**) (47, 58).

CNS Sjögren's syndrome affects younger women, 18–40 years-old, predominantly non-white, whereas the primary form affects 45–55-year-old women. The clinical appearance is similar to NMO (Devic's disease), with devastating spinal cord or optic nerve lesions. Onset of devastating CNS symptoms is over hours to days, may follow infections, and recovery is often incomplete. Spinal cord lesions in CNS Sjögren's syndrome and NMO span 3 or more vertebral segments, are central or holocord, are demyelinating but also necrotic, are sometimes so severe that there is spinal cord swelling. Optic nerve lesions are also highly destructive, and can have bilateral onset. In contrast, MS lesions are smaller, are <1 cord segment long, are often acentric or subpial, are not necrotic, and typically recover partially or completely, and optic neuritis is unilateral.

The type I IFN signature is supra-normal in NMO, but diminished in MS (38). NMO patients who were partially treated with low-dose steroids or plaquenil have normal levels of serum type I IFN activity (6× greater than in MS), and *in vitro* induction of P-Y-STAT-1, MxA, and viperin is excessive compared to healthy controls (**Figure 3**). In contrast, therapy-naïve MS patients have low levels of type I IFN activity and subnormal responses to IFN-β *in vitro*.

Neuromyelitis optica is now defined by MRI criteria – longitudinally extensive cord MRI lesions, and by serology – NMO-IgG positive. However, some cord lesions are too short on MRI and less devastating than expected, and only 60–70% of suspected NMO patients are NMO-IgG positive. Moreover, there is significant clinical overlap with CNS Sjögren's disease, but here only 50% are NMO-IgG positive. Using serum type I IFN activity as a discriminator, ROC analysis shows a 35-fold ability to discriminate between NMO/CNS Sjögren's and MS. This and other tests that could help discriminate between NMO and MS would aid in therapeutic decisions. This is important because disease mechanisms and responses to therapy differ between the two. For instance, IFN-β does not benefit or worsens NMO, but IFN-β is therapeutic in MS.

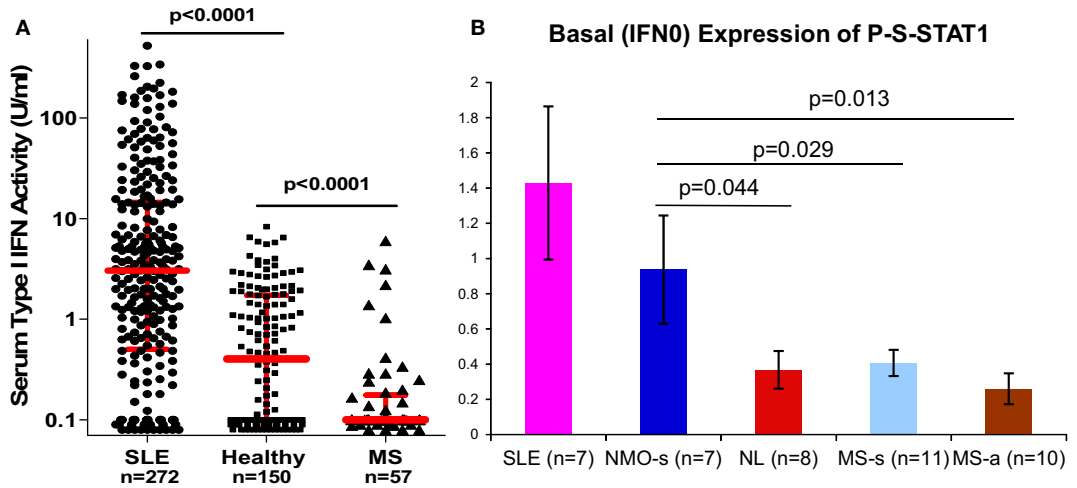

FIGURE 3 | (A) Serum IFN-α/β activity is low in MS vs. NL, and high in SLE. IFN-α/β activity was obtained from real-time PCR of three expressed genes in WISH epithelial cells. $N = 272$ SLE on minimal Rx, 150 healthy controls, 57 stable RRMS naïve to Rx. Red bars = log median ± interquartile range (Kruskal–Wallis test). Medians = 3.1 for SLE, 0.4 for NL, and 0.1 for MS. Ninety percent of MS levels are <0.5 U/ml. In NMO, IFN-α/β is in the normal or SLE range. IFN-α/β detection is 0.1–0.5 U/ml, i.e., 20× more sensitive than many available assays (38). **(B)** P-S-STAT1 levels in MNC from SLE, stable NMO on minimal therapy, normal controls, stable RRMS, and active RRMS. Unpaired *t*-test with equal variance.

IFN-β THERAPY OF MS: COGNITIVE BENEFITS, PROLONGED SURVIVAL, INDUCTION OF ANTIOXIDANTS AND POSSIBLE NEUROPROTECTION

One third of patients with pediatric-onset MS and clinically isolated demyelinating syndromes (CISs) are abnormal on neuropsychological testing. Untreated MS patients have significant cognitive loss over time, to the point of falling several *z*-scores below expected levels over 20 years (59). IFN-β improves cognition and slows cognitive loss in MS. The benefit was significant in the 2-year pivotal trials and later studies for all three forms of IFN-β-1. In the original IFN-β-1b trial, 32 patients tested over 2 years showed a dose-dependent improvement in visual-spatial performance compared to placebo (19). Benefit correlated with diminished MRI activity. The periventricular predilection of MS lesions could selectively disrupt the input and output of the calcarine cortex. We hypothesized that IFN-β quelled inflammation in these sites to enhance visuospatial performance (60).

Sixteen of these patients were studied in a long-term follow-up, 16 years after starting IFN-β (61). They did not remain in the original placebo, 50 μg, and 250 μg qod subgroups for more than 3 years, because at study end in 1993, all of these patients remained on IFN-β or started IFN-β *de novo* when the drug was approved. Fifteen of 16 patients were taking IFN-β at the 16-year point. The natural history of progressive decline of cognition in MS would lead one to expect loss of cognitive function after this long period, but there was no or only minimal cognitive decline at 16 years. This indicates that IFN-β-1b had a pronounced long-term benefit on cognition in active RRMS.

Interferon-β-1b therapy prevents death. In the pivotal IFN-β-1b study, there was a 5-year randomized disparity in placebo vs. IFN-β treatment, and then all patients started standard-of-care treatments. Five years of IFN-β therapy increased later survival by 7 years over a 21-year observation, a 47% reduction in mortality (62). What is the mechanism of the profound effect of IFN-β on survival in MS? Baseline pre-therapy male sex, high T2 MRI burden of disease, and high EDSS all were increased in those who died, but IFN-β-1b had an independent effect (nearly 50% reduction in mortality) for each variable. On-study responses such as new relapses, T2 lesions, or neutralizing Ab titers, did not significantly change mortality. This suggests that IFN-β has effects on MS that are beyond the usual trial readouts of relapses and MRI lesions.

Over the first 16 years of this study, those who were on IFN-β-1b therapy for >80% of the time (restricted to a subset of the original 250 μg IFN-β group), compared to those treated only <10% of the time (a subset of the original placebo) were ∼60% less likely to require a cane or wheelchair or to develop SPMS (63). The delay of onset of SPMS in the low medication usage group was 12 year, vs. 18 year in the high medication usage group. Note that these data are based on correlations, and other factors could influence medication use.

Interferon-β's benefit on mortality in MS cannot be explained by a simple Th1–Th2 shift. Could IFN-β induce genes that would protect or repair brain cells? The likelihood that type I IFNs evolved to have cytoprotective and neuroprotective effects was introduced in the Prolog. Can we measure gene induction after IFN-β therapy?

Multiple sclerosis patients with carefully defined clinical disease activity who had been on prolonged IFN-β-1b therapy were studied more than 60 h after their last injections, and then exactly 4, 18, and 42 h after an injection of IFN-β. RNA from MNC was run on Affymetrix Hu133A and also all-exon arrays. IFN caused a rapid (4 h) and intermediate (18 h) induction of ISGs at 4 and 18 h, and levels then fell back to baseline at 42 h (33). Surprisingly, after the 60-h washout, there remained an RNA signature for upregulation of scores of genes that control fatty acid oxidation, apoptosis, energy metabolism, and cytoprotection, such as Nrf2 (64). These

patients had been on IFN-β-1b therapy for 2–18 years. It is likely that long-term IFN-β therapy alters the set-point for control of cell metabolism and neuroprotection in blood and CNS cells. Glucocorticosteroids, stress, smoking, and obesity increase oxidative stress. Some of these environmental factors increase MS severity. Perhaps induction of cytoprotective genes explains some of the benefits of IFN-β-1b therapy on MRI black hole formation, cognition, and survival.

Multiple sclerosis brain lesions are an ominous predictor of shorter survival (62). MRI lesions predict mortality in untreated and treated MS patents. IFN-β therapy reduces new MRI T2 and new Gd+ lesions by 85% (48, 65) and reduces formation of permanent T1 MRI lesions ("black holes") (12). Black holes are a marker for inflammatory damage as well as lack of repair. IFN-β therapy prevents inflammation and may enhance repair. IFN-β also increases NAA concentration in brain neurons on MRS imaging (66), presumably by rescuing unhealthy neurons from death. IFN-β-1b therapy in MS has delayed benefits on cognition (19). Though speculative, fewer MRI lesions during therapy may increase survival. CNS lesions could disrupt trophic outputs to the rest of the body or disrupt immune regulation. For instance, sympathetic nerves bathe the spleen and secondary lymphoid organs with inhibitory catecholamines and neuropeptides. Spinal cord lesions that disrupt these SNS pathways will disinhibit the immune system, provoke and amplify autoimmune disease, and correlate with presence of progressive forms of MS (67, 68). Thus, new MRI lesions, permanent T1 black holes, cold purple feet from SNS damage in SPMS, cognitive loss, and even death may be interrelated phenomena and appear to be thwarted by IFN-β therapy.

COMBINATION THERAPY WITH OTHER AGENTS CAN PERTURB THE IFN SIGNALING PATHWAY

Intrinsic or disease-specific responses to IFN can modify the course of an autoimmune disease, but can be altered by exogenous triggers or by IFN-β therapy. Viruses for instance, cause exacerbations of MS, likely by activating not just IFNs, but many other facets of immunity. Could other agents modify intrinsic IFN levels or the effects of therapy? Two examples include statins and vitamin D.

Statins lower cholesterol, but are also anti-inflammatory. It would seem reasonable to combine statins with IFN-β to treat the inflammation of MS. Statins appeared to reduce MRI lesions (69). However, only active patients entered this uncontrolled study, and the 41% reduction in MRI activity is what would be expected due to regression to the mean without any therapy (70). Several small studies seemed to show benefit of combining statins and IFN-β. However, a controlled trial of RRMS patients on subcutaneous IFN-β-1a therapy who were then randomized to IFN-β alone or IFN-β plus high-dose atorvastatin (40–80 mg/day) showed that the combination provoked MRI and clinical exacerbations (33). A larger study of IFN-β-1a ± atorvastatin showed similar trends, especially in a composite score of MRI and clinical MS activity (71). The presumed synergy between two anti-inflammatory agents did not materialize because statins actually block IFN signaling. *In vitro*, statins cause a dose- and time-dependent block of IFN-β-induced phosphorylation of STAT1 (38) (**Figure 4**). *In vivo*, patients on various IFN-β therapies plus various forms of statin were tested after washing out both drugs and then performing IFN-β induction kinetics after: (1) adding IFN-β alone, and later repeating with (2) IFN-β plus high-dose statins. A dose of statins blocked IFN-β formation of P-Y-STAT, but not P-S-STAT1, and inhibited production of downstream proteins such as MxA and viperin. This cautionary tale suggests that statins could block the benefit of IFN-β, and that if both are needed, then the posology of therapy should be adjusted so statins are given at a time when they will have the least effect on the IFN injection.

Vitamin D has pleiotropic effects on immunity. It enhances macrophage function, but also induces IL-10. Serum vitamin D levels vary, due to lifestyle, skin color, and seasonal sunlight. Low levels are linked to onset of MS, and exacerbations once MS has developed. Could this inexpensive, sometimes free, agent be added to IFN-β? Several small studies show no additive effects, including one that was designated as class I evidence by the journal, Neurology (72). Unfortunately, a significant mismatch in the baseline demographics favored IFN-β monotherapy and the results are not at all conclusive. Another study showed no additive effect with subcutaneous IFN-β-1a (73). In a larger controlled

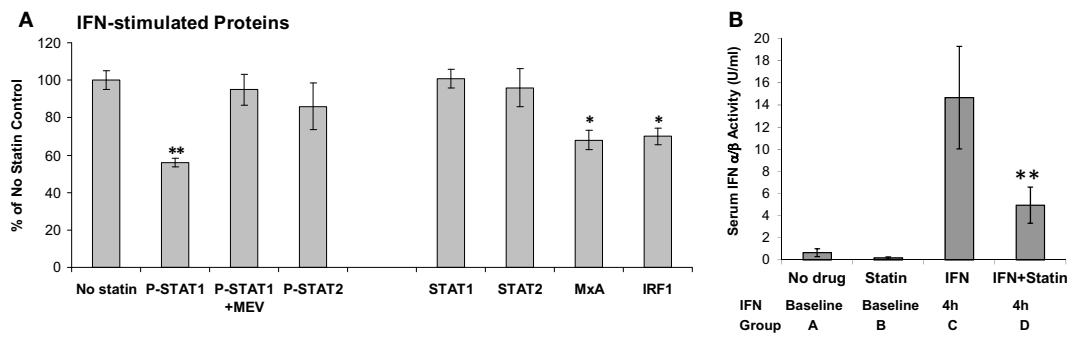

FIGURE 4 | (A) *In vitro* atorvastatin reduces IFN-β effects. MNC from 21 therapy-naïve RRMS were pretreated 24 h with 10 μM atorvastatin, then induced with 160 U/ml IFN-β-1b for 45 min (phosphorylated/activated P-STAT transcription factors) and for 24 h (induced unphosphorylated STAT1, STAT2, also MxA and IRF-1). Proteins quantified with Western blots, normalized with actin. Percentage change = statin-treated vs. no-statin (100%). $*p < 0.05$, $**p < 0.001$ vs. no-statin control. MEV = 100 μM mevalonate to reverse statin effect. Mean ± SEM; 21 replications (38). **(B)** *In vivo* statins reduce IFN-β therapy induction of serum type I IFN activity. Sera were obtained at 8 a.m. after statin washout or long-term statin alone, and then exactly 4 h after IFN-β injections or high-dose statins plus 4 h IFN-β. Fourteen stable RRMS. $**p < 0.001$ vs. IFN alone (paired *t*-test). Mean ± SEM (38).

study, however, vitamin D3 added to IFN-β-1b reduced new MRI lesions compared to IFN-β alone (74). IFN-β also appears to increase serum vitamin D levels (75). Many other studies of vitamin D plus IFN-β in MS, and vitamin D alone in SLE, are in progress. It is hoped that current, partially effective and expensive, therapies will be enhanced by a second inexpensive agent.

SUMMARY

- Type I Interferons (IFN-α/β) control viruses, cancer, cell proliferation, and immunity
- Type I IFNs were present in early fish, 400 million years ago
- Interferons evolved complex responses to viruses over eons; vertebrate survival likely was enhanced by cytoprotective effects of IFNs
- Interferons regulate ∼1000 genes
- Plasmacytoid dendritic cells produce IFN-α and some IFN-β; fibroblasts produce IFN-β
- The IFNAR activates the STAT1 transcription factor on tyrosine, allowing DNA binding
- Some genes are further induced by a second phosphorylation on serine (P-S-STAT1)
- Viruses bind TLR, RIG-I, and MDA5. These activate IRF3, then IFN-β and IFN-α1, then IRF7, then many subtypes of IFN-α
- Viruses, exogenous, and perhaps endogenous, trigger IFN production
- High serum and organ IFN levels are linked to SLE, Sjögren's syndrome, Aicardi–Goutières, and Cree encephalitis, as well as CNS Sjögren's and NMO
- Interferon-α treatment triggers and amplifies SLE
- Serum IFN levels are low in MS
- Responses to *in vitro* IFN-β are low in MNCs from untreated MS patients during clinical activity and progression
- Responses to *in vivo* IFN-β therapy are low in MS during clinical activity and progression, measured by gene induction and activation of transcription factors
- Low responses to IFN during clinically active MS are linked to low levels of P-S-STAT1
- Interferon-β is therapeutic in MS, and may be most effective in patients with low IFN responses
- Some drugs such as statins, block type I IFN signaling, and worsen MS
- The high IFN responses in NMO and CNS Sjögren's disease can be used to differentiate them from the low responses in MS

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Nucleic acid sensors and type I interferon production in systemic lupus erythematosus

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The characteristic serologic feature of systemic lupus erythematosus (SLE) is autoantibodies against one's own nucleic acid or nucleic acid-binding proteins – DNA and RNA-binding nuclear proteins. Circulating autoantibodies can deposit in the tissue, causing inflammation and production of cytokines such as type 1 interferon (IFN). Investigations in human patients and animal models have implicated environmental as well as genetic factors in the biology of the SLE autoimmune response. Viral/Bacterial nucleic acid is a potent stimulant of innate immunity by both toll-like receptor (TLR) and non-TLR signaling cascades. Additionally, foreign DNA may act as an immunogen to drive an antigen-specific antibody response. Self nucleic acid is normally restricted to the nucleus or the mitochondria, away from the DNA/RNA sensors, and mechanisms exist to differentiate between foreign and self nucleic acid. In normal immunity, a diverse range of DNA and RNA sensors in different cell types form a dynamic and integrated molecular network to prevent viral infection. In SLE, pathologic activation of these sensors occurs via immune complexes consisting of autoantibodies bound to DNA or to nucleic acid-protein complexes. In this review, we will discuss recent studies outlining how mismanaged nucleic acid sensing networks promote autoimmunity and result in the over-production of type I IFN. This information is critical for improving therapeutic strategies for SLE disease.

Keywords: systemic lupus erythematosus, nucleic acid sensor, type 1 interferon, TLR, DNA, RNA

INTRODUCTION

The normal immune system strikes a delicate balance between defense against foreign invasion and the prevention of misdirected responses against self-antigens. Sometimes, this intricate balance becomes faulty due to genetic, environmental, or other factors leading to breakdown of self-tolerance and the onset of an autoimmune disorder. Systemic Lupus Erythematosus (SLE) is a prototype autoimmune disease that affects the skin, kidney, musculoskeletal, and hematologic systems and is characterized by presence of various autoantibodies against self-components, especially double-stranded DNA (dsDNA) and RNA-binding nuclear proteins. Amongst SLE patients, the female to male ratio is 9:1, suggesting that sex-related factors are important in the development of the disease (1, 2). Many genetic factors have been strongly associated with disease susceptibility (3, 4). Exposure to several viruses and bacterial infections, and also UV light are known to trigger SLE (5). Thus, it is considered that SLE occurs when an environmental trigger acts on a genetically predisposed individual, leading to a loss of tolerance toward native proteins (6). Multiple immune system abnormalities contribute to the pathogenesis of SLE, including abnormal clearance of apoptotic cells and immune complexes, over-production of type I interferon (IFN), reduced thresholds for B and T lymphocyte activation, and production of autoantibodies against self-antigens (7). These autoantibodies are directed against nucleic acids and RNA-binding proteins such as Ro, La, and Sm (8). Tissue damage is mediated in part by deposition of immune complexes in the affected organs, followed by activation of downstream inflammatory pathways mediated by complement and FcR engagement of innate immune cells (9). Viruses such as Cytomegalovirus (CMV), Epstein–Barr (EBV), and Parvovirus B19 are frequently involved as environmental triggers in lupus. Hypomethylated bacterial and viral DNA are potent inducers of immune responses through TLR signaling cascade finally leading to type 1 IFN over-expression, B cell activation, production of autoantibodies, and interleukin (IL)-6 (10).

Many patients with SLE have high circulating levels of type I IFN (11). Some individuals treated with IFN-α for chronic viral infections developed *de novo* SLE that was resolved when IFN-α was withdrawn (12, 13). Additionally, within SLE families abnormally high IFN-α levels have been found clustered (14). A recent genome-wide association study has identified additional novel genetic loci associated with high serum IFN-α in SLE patients (15, 16). Taken together, these data support the idea that genetically determined endogenous elevations in IFN-α predispose to human SLE.

HOW DOES LUPUS START?

The etiology of lupus is considered to be multifactorial involving multiple genes and environmental factors such as infections, hormones, and drugs (**Figure 1**) (17). It is considered that unrestrained immune response to apoptotic cells and decreased disposal of apoptotic material are important initiators of the autoimmune response in SLE. Genomic DNA is not accessible to the immune system under standard conditions as it is safely sequestered in the nucleus or in mitochondria under the tight control of DNA damage and repair response systems. However,

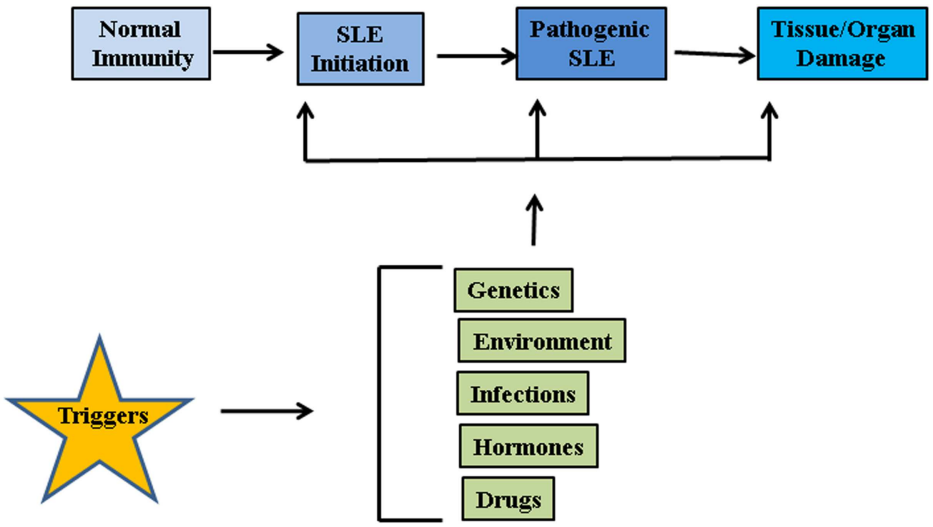

FIGURE 1 | Factors associated with SLE pathogenesis. Genetic factors, environmental influences such as radiations, repeated infections, hormonal imbalances, and certain drugs may act on innate immune system and disrupt the intricate balance between protection against foreign invasion and self-defense.

when cells die through apoptosis, apoptotic bodies containing fragmented cellular material and abnormal surface antigens, circulate in the body enabling the immune system to access new epitopes (18). Under normal conditions cellular mechanisms exist to ensure that apoptotic debris is not immunogenic to self, but these mechanisms can fail. It seems likely that defective clearance of apoptotic material and modifications to DNA such as hypomethylation can promote SLE (19). Recent reports suggest that neutrophil extracellular traps (NETs) are a potent stimulus for type 1 IFN release by plasmacytoid dendritic cells (DCs), and play an important role in propagation of the lupus phenotype (20–23). Neutrophils are specialized immune cells that are rapidly recruited to sites of inflammation in response to microbial infections. One of the mechanisms of neutrophil action is the formation of "NETs" (24). NETs are made of processed chromatin bound to granular and selected cytoplasmic proteins. NETs are released by neutrophils to control microbial infections (24). This release of chromatin is the result of a unique form of cell death, called "NETosis." Material derived from NETosis can contribute to SLE by serving as source of autoantigen, propagating inflammation, and tissue damage (21, 23, 25, 26). In an interesting recent study, Sangaletti et al. suggested that NETs may provide antigens to DCs and in this way promote immune responses against neutrophil antigens in the autoimmune disease small vessel vasculitis, which is characterized by antibodies against cytoplasmic proteins in neutrophils (23). It is possible that NETs may provide nuclear antigens to immune cells in a similar way in SLE.

Pathways through which our own nuclear material is able to induce pro-inflammatory responses are a topic of active research. At least three distinct types of nucleic acid recognition receptors are recognized: (1) the toll-like receptors (TLRs), which recognize nucleic acids on the plasma membranes and endosomes; (2) the nucleotide binding and oligomerization domain (NOD) receptors (NLRs), which monitor the cytosolic compartment and also interact with TLR pathways; and (3) the retinoid acid inducible gene (RIG)-I-like receptors that recognize RNA or DNA in the cytoplasm (RLRs). Many of these receptors may directly or indirectly participate in the pathogenesis of SLE (27).

TOLL-LIKE RECEPTOR MEDIATED SIGNALING IN LUPUS

Toll-like receptors are major components of the innate immune system that activate multiple inflammatory pathways and coordinate systemic defense against microbial pathogens. Data from animal models and human patients suggest that improper engagement of TLR pathways by endogenous or exogenous ligands may lead to the initiation of autoimmune responses and tissue injury (28). Endosomal TLRs (TLR-3, -7, -8, and -9) are potent activators of DCs and B cells. TLR-3 is specific for double-stranded RNA (dsRNA), TLRs-7 and -8 for single-stranded RNA (ssRNA), and TLR-9 is specific for dsDNA (29, 30). TLRs are expressed predominantly in DCs, B cells, macrophages, monocytes, and neutrophils. Cell surface receptors, such as the B cell receptor (BCR) and FcγRIIa, facilitate the endocytosis of nucleic acid containing material or immune complexes (31, 32). Chromatin-containing immune complexes can stimulate B cells up to 100-fold more effectively than complexes without nucleic acids apparently due to collective engagement of BCR and TLR (31–34). Thus, dual engagement of the BCR and the TLR can induce abnormal activation of B cells and break immune tolerance. In human lupus, an increased proportion of B cells and monocytes expressed TLR-9 among patients with active SLE compared to patients with inactive disease (35). TLR activation in combination with T cell derived IL-21 markedly increased B cell differentiation into plasma cells (36).

All TLR family members, including TLRs-7, -8, -9 are type I membrane proteins composed of a ligand-binding ectodomain containing 18–25 tandem copies of leucine-rich repeats (LRRs), a transmembrane domain, and a conserved

cytoplasmic toll/interleukin-1 receptor (TIR) domain. Ligand-induced dimerization and conformational rearrangement of the TIR domains leads to the creation of two symmetry-related sites which allow binding of the cognate signaling adaptor molecules (37, 38). Two main adaptors are utilized by TLRs, namely Myeloid Differentiation Factor-88 (MyD88) (TLR-7, -8, and -9) and TIR domain-containing adaptor inducing IFN-β (TRIF) (TLR-3). These adaptors mediate the recruitment of a series of kinases that lead to the formation of specific macromolecular signaling platforms for inflammatory reactions. IL-1 receptor-associated kinase 4 (IRAK-4) is recruited to MyD88 and is activated after recruitment (38). IRAK-4, in turn, activates IL-1 receptor-associated kinase 1 (IRAK 1) via phosphorylation (39, 40). These activated kinases recruit tumor necrosis factor receptor-associated factor 6 (TRAF-6), which is an E3 ubiquitin ligase required for activation of NFκB by freeing it from its inhibitor, I kappa B (IκB) (41). In addition to this, interferon regulatory factors (IRFs) IRF5 and IRF7 are recruited to the MyD88/IRAK/TRAF6 complex, where they become phosphorylated and activated (42, 43). Ultimately, the transcription factors NFκB and IRF5 and IRF7 are activated and translocate into the nucleus where they initiate gene transcription and production of pro-inflammatory cytokines and type I IFN (**Figure 2**) (43–45). Unlike TLR-7, -8, and -9, TLR-3 signaling is MyD88-independent and utilizes adaptor protein TRIF (46). TRIF also recruits additional proteins necessary for downstream signaling, including TRAF-family member-associated NFκB-activator-binding kinase 1 (TBK1), TRAF3, and receptor-interacting protein 1 (RIP1) (40). TRIF interaction with TBK1 is necessary for the activation of IRF-3, which is a transcription factor involved in the production of interferon beta (IFN_β). (47). TLR-3 can also activate NFκB by the interaction of TRIF with TRAF-6 or RIP1 (40, 48) leading to up-regulated IFNα production and secretion of other pro-inflammatory cytokines.

GENETIC FACTORS ASSOCIATED WITH TLR-DEPENDENT IFNα PATHWAY IN LUPUS

One of the most striking immune system abnormalities in SLE patients is the frequent up-regulation of the type I IFN pathway (49, 50). IFNα is critical player in SLE progression and severity, and has been shown to induce the production of autoantibodies when administered to non-SLE patients (12, 51). An interesting report describes remission of SLE in a patient which was attributed to unresponsiveness to both TLR-7 and -9 stimulation after development of common variable immunodeficiency – (CVID-) like disease (52). Genetic variations in many of the components of the TLR signaling pathway have been associated with SLE, such as TLR-7, IRF5, IRF7, IRF8, IRAK1, and TNFAIP3 (53–59). Three of the nine genes in the IRF family have been genetically

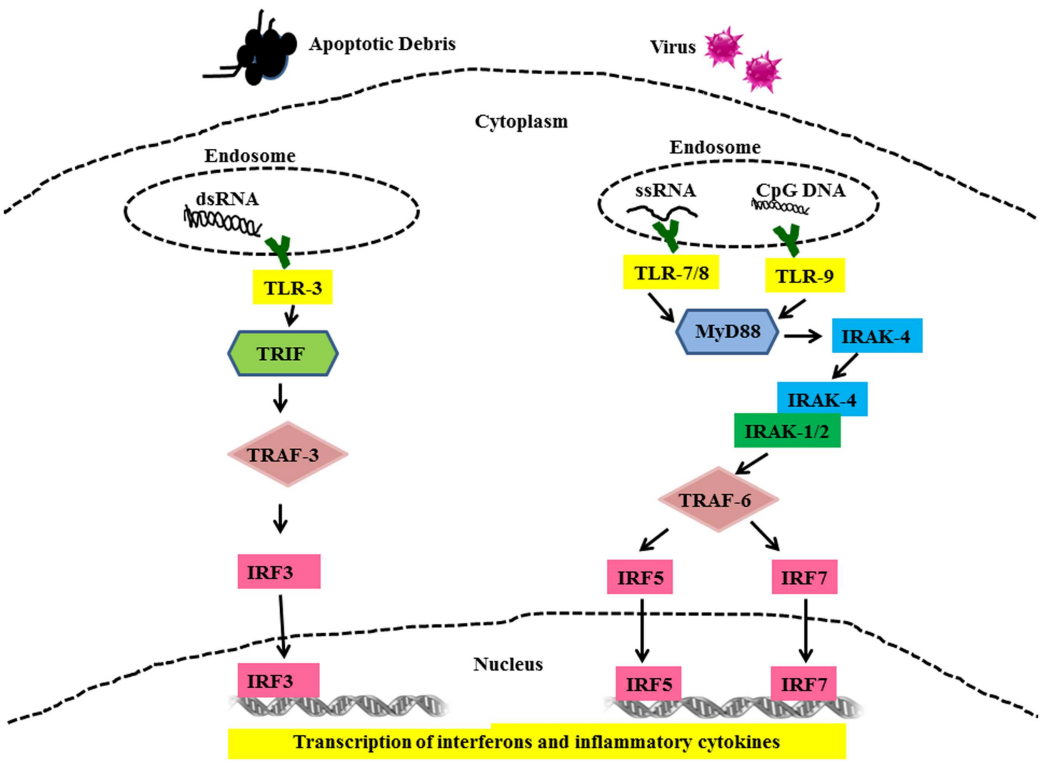

FIGURE 2 | Toll-like receptor mediated signaling in SLE. Cells use TLRs as sensors to detect the presence of viruses and apoptotic debris via TLR-3, -7, -8, and -9. Nuclear material is trafficked to the endosome triggering TLRs signaling. Binding of cognate ligands to these TLRs recruits MyD88, a main signaling intermediate involved in TLR-7, -8, and -9 signaling. MyD88 recruits interleukin-1 receptor-associated kinase (IRAK)-4. IRAK-4 binds and phosphorylates IRAK-1, which in turn recruits Tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6. IRF5 and IRF7 are then shuttled to the nucleus and these events set the stage for the transcription of IFN-α and other pro-inflammatory cytokines. TLR-3 signaling is MyD88-independent and utilizes TRIF and TRAF3 as signaling intermediates finally leading to activation of IRF3 and production of IFN-α and other pro-inflammatory cytokines.

associated with SLE (60). Additionally, some of these genetic polymorphisms have been associated with increased type I IFN in SLE patients, supporting the idea that these genetic variations modulate the output of the TLR pathway (42, 60–64). The implication of these genes in SLE strongly supports the primary relevance of the TLR and IFNα pathway in the disease phenotype (63, 65). Additionally, many of these genetic polymorphisms in the TLR pathway are associated with the formation of autoantibodies (62–64, 66), supporting the concept of a feed-forward loop in which genetic variations in the TLR pathway enhance autoantibody production, and then the autoantibodies form immune complexes which stimulate the TLR pathway and result in increased type I IFN production in the setting of the same genetic variations. The TLR pathways are important in B cell maturation, and it is possible that genetically programed TLR pathway over-activity could promote autoantibody formation in B cells. Then after immune complexes are formed, these stimulate the TLR pathway in DCs and macrophages, and the same polymorphisms promote increased cytokine output from these cells.

TOLL-INDEPENDENT SIGNALING IN LUPUS

SIGNALING THROUGH RIG-1 LIKE RECEPTORS IN LUPUS

After viruses enter the cytoplasm and start replicating, infected host cells can sense and activate anti-viral responses in response to viral nucleic acids. This sensing occurs in the cytoplasm, and is independent of the cell surface and endosomal TLRs. Thus far, three cytosolic RNA helicases have been identified, RIG-I (retinoic acid – inducible gene I), MDA5 (melanoma differentiation – associated gene 5), and LGP2 (laboratory of genetics and physiology 2) that act as RNA sensors to mediate TLR-independent IFN-α/β induction in the presence of replicating RNA viruses (37, 67). Unlike membrane-bound TLRs, RLRs reside in the cytoplasm and sense cytoplasmic RNA. RIG-I contains tandem caspase recruitment domain (CARD)-like regions at its N-terminus and the central DExD/H helicase domain which has an ATP-binding motif and a C-terminal repressor domain which binds to RNA (68, 69). MDA5 contains tandem CARD-like regions and a DExD/H helicase domain, but it is unknown whether the C-terminal region of MDA5 really functions as repressor domain. LGP2 contains a DExD/H helicase domain and a repressor domain, but lacks the CARD-like region. LGP2 was suggested to be a negative regulator of RNA virus-induced responses, because the LGP2 repressor domain binds to that of RIG-I and suppresses signaling by interfering with the self-association of RIG-I (70, 71). Findings suggest that RIG-I and MDA5 have specificities in their detection of RNA viruses, through recognition of distinct viral RNA structures. RIG-I can recognize ssRNA bearing a 5′-triphosphate moiety (72, 73). In the case of self-RNA, 5′-triphosphate structures are removed or masked by a cap structure, which suggests a discrimination mechanism between self- and non-self RNA. RIG-I and MDA5 can distinguish dsRNA by size; RIG-I can bind short dsRNA whereas MDA5 can bind long dsRNA (74). Although LGP2 was considered a negative regulator, LGP2-deficient mice exhibited complicated phenotypes (75) and higher levels of type I IFN in response to polyinosinic: polycytidylic acid (Poly I:C) and vesicular stomatitis virus (VSV), but decreased type I IFN following encephalomyocarditis virus (EMCV) infection, suggesting that LGP2 can negatively or positively regulate RIG-I and MDA5 responses depending on the type of RNA virus (75).

Ligand binding to RLRs induces conformational changes leading to association with mitochondrial-associated IFN-β promoter stimulator 1 (IPS-1) through card-card domain interactions (76–79). IPS-1 then recruits TRAF3, which activates TANK-binding kinase 1 (TBK1) and IκB kinase (IKK) – related kinases IKKε (80). This leads to the phosphorylation and nuclear translocation of IRF-3 and -7 resulting in the transcription of IFN type 1 genes (81, 82) (**Figure 3**). IPS-1 also interacts with FAS-associated death domain protein (FADD) and receptor-interacting protein 1 (RIP-1) (76), which activate caspase-8 and caspase-10, resulting in NF-κB activation and production of inflammatory cytokines (83, 84). Genetic studies in SLE have strongly implicated the RLR pathways in SLE susceptibility. Variants in both MDA5 and IPS-1 have been associated with SLE susceptibility and with altered activation of the type I IFN pathway in SLE patients *in vivo* (85, 86). This again supports the idea that multiple nucleic acid recognition pathways are involved in SLE pathogenesis.

SIGNALING THROUGH NUCLEOTIDE BINDING AND OLIGOMERIZATION DOMAIN (NLR) RECEPTORS IN LUPUS

The NOD (NLR) family of receptors are key molecules that drive inflammatory responses by forming a multi-protein complex called "inflammasome." The inflammasome drives the processing and release of cytokines such as the pro-inflammatory cytokines IL-1β and IL-18. Several inflammasome complexes have been identified in recent years. Of the known inflammasomes, NLRP3, absent in melanoma 2 (AIM2), and IFN inducible protein 16 (IFI16) inflammasomes have been linked to immune responses to intracellular DNA, as well as bacterial and viral infections (87). IL-1β is important in activating neutrophils, macrophages, DCs, and T cells, whereas IL-18 is crucial for IFN-γ production by NK cells and T cells (88). IL-1β and IL-18 are regulated at both transcriptional and post-translational levels. Upon transcriptional induction by TLRs and other sensor systems, IL-1β and IL-18 are synthesized as inactive precursor proteins, which are subsequently processed by the cysteine protease caspase-1 (IL-1β converting enzyme) (89). Conversion of procaspase-1 into an enzymatically active form, caspase-1, occurs upon formation of a multi-protein inflammasome complex (89). Previous reports have suggested that the NLRP3 inflammasome is involved in mediating the inflammatory responses to both DNA and RNA viruses (90, 91). In human SLE macrophages, NETs induce robust activation of the NLRP3 inflammasome (92).

Several groups independently identified AIM2 as a receptor for cytosolic DNA that leads to caspase-1 activation and IL-1β secretion (93, 94). AIM2 binds cytosolic DNA of self and non-self origin, including bacterial, viral, and mammalian DNA, in a sequence-independent manner (95). Recent evidence indicates that the AIM2-related protein IFI16 also forms an inflammasome complex following Kaposi sarcoma – associated herpes virus infection of endothelial cells (96). Several groups independently identified STING as a key component of the DNA-sensing pathway (97, 98). STING/MITA translocates to perinuclear regions where it interacts with TBK1 to relay downstream signals to IRF3 (**Figure 4**). STING deficiency in macrophages or DCs leads to a

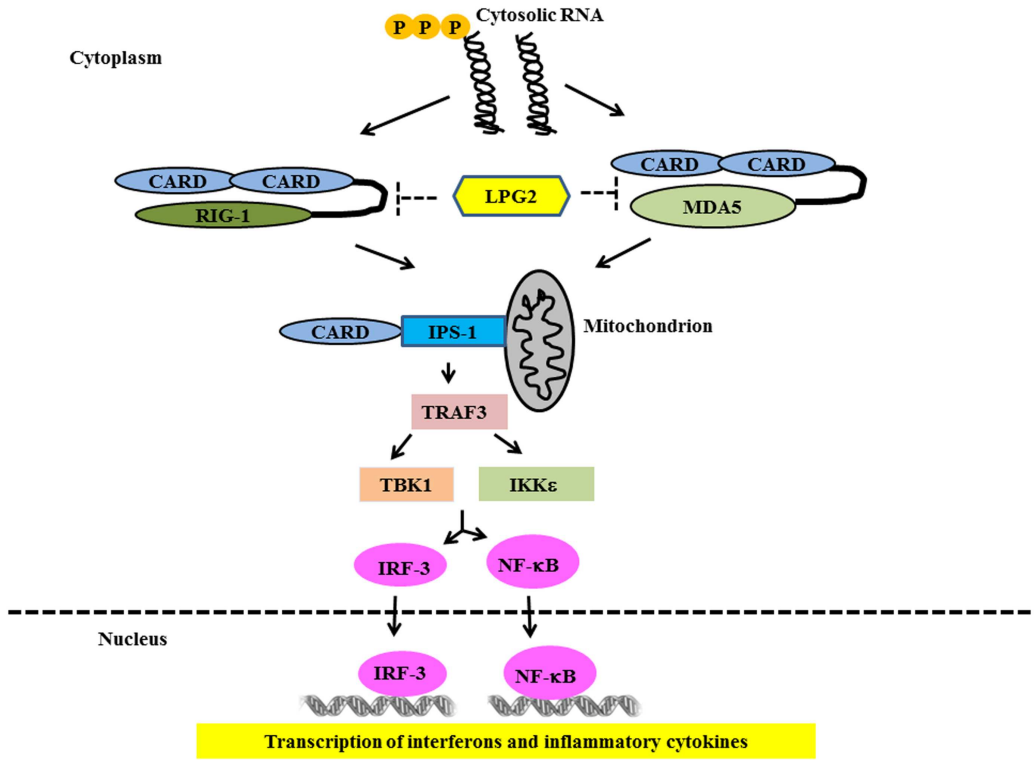

FIGURE 3 | Signaling through RIG-1 like Receptors in lupus. Following recognition of the cytosolic RNA, RIG-I, and MDA5 associate with the adapter IPS-1 via CARD-like domains. IPS-1 is localized to the mitochondrion and initiates signaling leading to activation of IRF3 and NFκB that finally lead of over-production of type 1 IFN and other inflammatory cytokines.

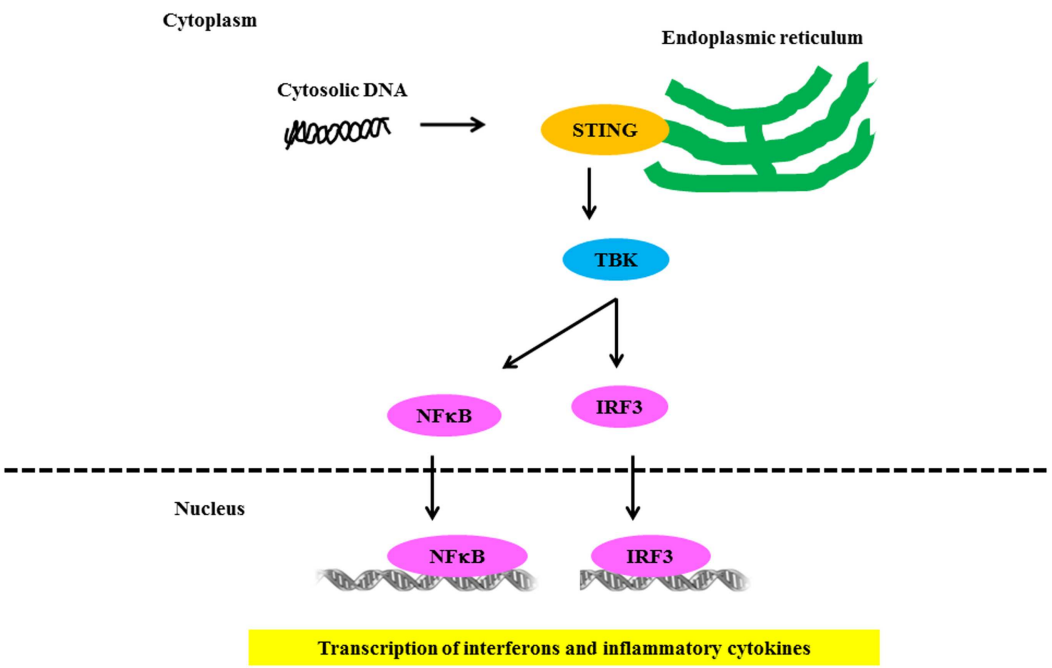

FIGURE 4 | Signaling through NLR receptors in lupus. Intracellular DNA following microbial infection or phagocytosis of immune complexes can potentially trigger the assembly of NLRs. The nucleic acid-induced signaling pathway converges on the adaptor STING and the kinase TBK1, which phosphorylates IRF3 to mediate downstream signaling events leading to transcriptional induction of type 1 IFN and other inflammatory cytokines.

markedly impaired type I IFN response to B-DNA and immunostimulatory DNA or to infection with DNA viruses, including HSV-1, human CMV, and vaccinia virus (97, 98). Initial studies showed that STING also interacted with components of the RNA-recognition machinery, such as RIG-I, where it was linked to type I IFN induction in response to VSV, a negative-strand RNA virus (97, 99). Murine models support the relevance of AIM2 in susceptibility to lupus-like disease in the NZB × NZW mouse (100).

OTHER CYTOSOLIC NUCLEIC ACID SENSORS

DNASE-I, II, AND III

Production of type I IFN and inflammatory cytokines are important for protecting the host against infections; however overstimulation of innate immune pathways can induce autoimmune disease (101). Normally, host nucleic acid is limited to the nucleus and mitochondria whereas; host cellular DNA/RNA sensors are localized in the cytoplasmic compartment. Thus, accidental activation of inflammatory cytokine pathways by host defense sensors is largely averted. However, faulty clearance of self-nuclear material from apoptotic/necrotic bodies can cause improper activation of cytokines including type I IFN production.

One level of self-defense is provided by cellular endonucleases, such as Dnase-I, Dnase-II, and Dnase-III/Trex-1, which are involved in the clearance of extracellular, lysosomal, and cytosolic DNA, respectively. Genetic deficiencies of Dnase-I have been identified in SLE patients (102), and *Dnase I* – deficient mice develop a lupus-like syndrome (103). Dnase-I defects lead to the accumulation of extracellular DNA produced by apoptotic and necrotic cells, which is immunogenic and can lead to type I IFN production (101, 104). Dnase-II is expressed in lysosomes, where it degrades DNA from engulfed apoptotic/necrotic cells (105). Dnase-II knockout mice are embryonically lethal. However, they are viable on the IFNR1 knockout background, indicating that type I IFN mediates the lethality of Dnase-II genetic deficiency (101, 106). This finding supports the concept that inefficient nucleic acid degradation promotes type I IFN excess and subsequent SLE disease. Dnase-III is another nuclease that is normally involved in the clearance of cell-intrinsic ssDNA (107, 108). DNAse-III is 3′-5′ exonuclease and is localized to the endoplasmic reticulum. In the absence of DNAse-III, there is an accumulation ∼60-bp ssDNA, believed to be produced during replication, which leads to the activation of ATM-dependent DNA-damage associated checkpoint pathways (109). Stetson et al. (110) revealed a role for DNAse-III in preventing cell-intrinsic initiation of autoimmunity. Trex-1 substrates are ssDNA, which are either the by-products of replication and/or reverse transcribed from endogenous retroelements. Loss of function mutations in the human DNAse-III gene cause Aicardi–Goutieres Syndrome (AGS) (111, 112). Different rare DNAse-III mutations also cause monogenic chilblain lupus, and common genetic variations in DNAse-III have also been associated with risk of SLE, suggesting that a common mechanism may underlie these disorders (113–115).

OTHER DNA AND RNA SENSORS

DNA-dependent activator of IRFs (DAI) is another cytoplasmic DNA sensor capable of activating IRF-3 and NF-κB, resulting in type I IFN production. DAI interacts directly with dsDNA *in vitro* and this interaction in turn enhances DAI association with IRF-3. DAI-induced IRF-3 phosphorylation is dependent on TBK1 (47, 116). Recently, Zhang et al. (117) reported that DAI expression is predominantly increased in SLE patients as well as in activated lymphocyte-derived self-apoptotic DNA (ALD-DNA)-induced lupus mice. ALD-DNA could induce the dimerization/oligomerization of DAI and activate DAI signaling pathways via regulating calcium signaling, thus resulting in aberrant macrophage activation and lupus nephritis, implying the possible mechanisms for the recognition and regulation of ALD-DNA-induced pathological macrophage activation in the context of SLE disease (117).

Recently, Kondo et al. (118) identified MRE11 as a sensor for exogenous dsDNA, which is required for STING trafficking and type I IFN induction. The report reveals that MRE11 contributes to recognition of a broad spectrum of dsDNA and MRE11-mediated intracellular DNA recognition is to respond to damaged host cells, rather than defense against foreign pathogens (118). DDX41 is another DExD/H-box helicase that can interact with synthetic dsDNA through the DEAD domain *in vitro* and DDX41 is required for DNA-dependent induction of type I IFN in myeloid DCs through a pathway dependent on STING and TBK1 (119).

Found in the cytoplasm, RNA polymerase III is known to transcribe AT-rich DNA into dsRNA transcripts characterized by uncapped 5′-triphosphate moieties. This can act as a ligand for RIG-I. Subsequently, RIG-I signals via IPS-1 to induce the expression of type I IFN and other cytokines (72, 120). Ku80 is an abundant nuclear protein that is known to bind dsDNA with high affinity.

A recent study (121) identified Ku70, as the newest member of the cytosolic DNA-sensing machinery with in IFN production. Ku70 was identified as a DNA-binding protein in HEK-293 cells by DNA-affinity purification followed by mass spectrometry. Notably, Ku70 is involved in the production of type III IFN (λ_1), but not type I IFN (α or β) in response to a variety of transfected DNA (>500 bp) in HEK-293 (121). It seems likely that we will continue to identify additional DNA and RNA sensors, and that some of these novel mediators will also play a role in SLE pathogenesis.

CONCLUSION

In recent years, there has been tremendous progress in understanding how cells recognize and respond to microbial threats. Many DNA and RNA sensors have been identified that are dedicated to detection and elimination of microbial infection and clearing cellular damage. Sometimes these beneficial immune responses lose their fidelity and thus contribute to pathogenesis of autoimmune diseases. It is striking that many of the classical components of these pathways have been genetically associated with risk of SLE. This emphasizes the primary importance of nucleic acid handling and innate immune sensors in the pathogenesis of SLE. In SLE, it seems likely that stimulation of these pathways occurs via the combined contribution of microbial nucleic acids as well as self-tissue-derived stimuli. Work from our group and others supports a model in which immune complexes containing nucleic acid and free nucleic acid are a micro-environmental factor that cooperates with genetic

variation in the nucleic acid sensing pathways to produce immune system dysregulation and risk of SLE (62). Understanding the molecular mechanisms of how the innate nucleic acid recognition system is dsyregulated in SLE will suggest new therapeutic avenues directed toward the inhibition of nucleic acid recognition by their sensors, downstream signaling events, and inhibition of end-stage mediators. This will lead to the new era of molecular medicine for the treatment of intractable autoimmune diseases like SLE.

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Interferons in Sjögren's syndrome: genes, mechanisms, and effects

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Sjögren's syndrome (SS) is a common, progressive autoimmune exocrinopathy distinguished by dry eyes and mouth and affects ∼0.7% of the European population. Overexpression of transcripts induced by interferons (IFN), termed as an "IFN signature," has been found in SS patients. Four microarray studies have been published in SS that identified dysregulated genes within type I IFN signaling in either salivary glands or peripheral blood of SS patients. The mechanism of this type I IFN activation is still obscure, but several possible explanations have been proposed, including virus infection-initiated and immune complex-initiated type I IFN production by plasmacytoid dendritic cells. Genetic predisposition to increased type I IFN signaling is supported by candidate gene studies showing evidence for association of variants within IFN-related genes. Once activated, IFN signaling may contribute to numerous aspects of SS pathophysiology, including lymphocyte infiltration into exocrine glands, autoantibody production, and glandular cell apoptosis. Thus, dysregulation of IFN pathways is an important feature that can be potentially used as a serum biomarker for diagnosis and targeting of new treatments in this complex autoimmune disease.

Keywords: interferon signature, Sjögren's syndrome, gene expression profiling, microarrays, type I interferon, genetic association, mechanisms, biomarker

INTRODUCTION

Sjögren's syndrome (SS) is a chronic autoimmune disease that primarily affects middle-aged women with an estimated prevalence of ∼0.7% in European populations (1, 2). SS is characterized by infiltration of lymphocytes into glandular tissues, typically the salivary and lacrimal glands, leading to xerostomia (dry mouth) and keratoconjunctivitis sicca (dry eye). The resulting pathology can be debilitating, and target organ damage may be so severe that moisture production is virtually non-existent. However, manifestations of SS are not limited to exocrinopathy; other common extraglandular features include fatigue, arthritis, Raynaud's phenomenon, and an increased incidence of non-Hodgkin B cell lymphoma (3). Two autoantibodies targeting ribonucleoproteins, anti-Ro/SSA, and anti-La/SSB, are detected in 60–70% of SS patients and are important to disease diagnosis (4, 5).

The etiology and pathogenesis of SS are still unclear, partially due to the complexity and heterogeneity of disease mechanisms. Recently, the dysregulation of interferon (IFN) signaling pathways, especially upregulation of type I IFN-inducible genes, has been observed in salivary glands and peripheral blood in a subset of SS patients (6–10). Type I IFNs, including IFNα and IFNβ, are key immune mediators involved in viral defense and activation of immune responses (11). Viral infection has long been suspected to trigger SS (12), and abnormal elevations in type I IFN signaling may reflect an important role for viral infection in disease pathogenesis. Additionally, genetic association studies indicate the importance of multiple genetic loci within IFN pathways. Here, we review the identification of the type I IFN "signature" through high-throughput techniques, and discuss potential mechanisms and functions of dysregulated IFN signaling in SS.

IDENTIFICATION OF THE "IFN SIGNATURE" IN SS

Many powerful, high-throughput techniques have emerged in the last few decades and have revealed important insights into mechanisms of complex human diseases. Gene expression profiling (GEP) studies using microarrays represent one of the most widely used approaches to determine global transcriptome differences between patients and healthy controls. "Signatures" of disease have been defined that represent clusters of co-expressed genes, often within a biological network, that may serve as biomarkers for disease diagnosis, classification, and drug response prediction. The "IFN signature," first described in a GEP study of systemic lupus erythematosus (SLE), has been defined by the overexpression of type I IFN-inducible genes (13). Subsequent studies have demonstrated similar signatures in other autoimmune diseases, such as rheumatoid arthritis (RA), systemic sclerosis (SSc), myositis, and SS (14).

Four microarray studies have been published in SS to date that describe the overexpression of type I IFN-inducible transcripts in minor salivary glands or peripheral blood from SS patients (7–10). Hjelmervik et al. (7) published the first GEP in SS using a relatively low-density 16K microarray to identify a whole transcriptome signature in biopsy samples of minor salivary glands from 10 SS patients and 10 controls who experienced subjective oral dryness. They successfully clustered 19 out of the 20 subjects into the correct group by using the top 200 differentially expressed transcripts, in which numerous type I IFN-regulated genes were represented,

including *IFI27*, *ISG12*, *GBP2*, *IFITM1*, and *IRF8*. Subsequently, Gottenberg et al. (8) also identified distinct gene expression patterns involving IFN pathways (both type I and type II) in salivary glands of SS patients by comparing seven cases and seven controls. Specifically, 23 genes were IFN-inducible, including genes in the antiviral IFN-induced transmembrane protein (IFITM) family (*IFITM1*, *IFITM2*, and *IFITM3*) and genes in the Toll-like receptor (TLR) family (*TLR8* and *TLR9*) that play a fundamental role in pathogen recognition and activation of innate immunity (15). All showed significantly increased expression in SS patients. Interestingly, the only two known IFN-inducible genes that showed decreased expression in SS salivary glands, *CCL18* and *SOCS3*, are involved in the inhibition of inflammatory processes. The overexpression of IFN-inducible genes was supported by the detection of plasmacytoid dendritic cells (pDC), the most potent producer of type I IFNs (16), in salivary glands of patients with SS, but none in the glands of controls. These results suggest that pDC activation may play a role in SS pathogenesis, which is discussed later in this review.

Upregulation of 11 IFN-inducible genes has been identified by Pérez et al. (10) through a microarray study of epithelial cells from salivary glands of nine SS patients and six controls. Notably, three of these genes belong to the IFN regulatory factor (IRF) family (*IRF7*, *IRF8* or *ICSBP1*, and *IRF9*). IRFs are pivotal transcriptional regulators of type I IFN and IFN-inducible genes, and are important in cellular differentiation of hematopoietic cells (17). This GEP study also identified dysregulation of apoptotic pathways in SS epithelial cells, which are now thought to be involved in local auto-antigen production and tissue damage in the salivary glands of SS patients (18, 19). Additionally, they identified six genetic loci associated with SS using microsatellite markers, with five of the association signals falling within regions where differentially expressed genes were found, such as *IL6*, *CD44*, and *IRF9*. These results support a genetic contribution to the dysregulated IFN pathways observed in SS.

The IFN signature has also been observed in peripheral blood of SS patients. Emamian et al. (9) detected upregulation of IFN-inducible genes in peripheral blood of a subset of SS patients by comparing 21 cases and 23 controls followed by replication in an independent dataset of 17 SS cases and 22 controls. *IFI35*, *MX1*, *OAS1*, *IRF7*, and *OAS2* were among the top differentially expressed genes and are known to be induced by IFNs. The authors also showed that the expression levels of most IFN-inducible genes were positively correlated with anti-Ro/SSA and anti-La/SSB titers. Although the relationship between IFN pathway activation and autoantibody production is unclear, these results provide a link for both innate and adaptive immune responses to the pathogenesis of disease. These results also suggest that the IFN signature can be potentially used as a disease biomarker for a subgroup of SS patients with certain clinical features that includes the production of anti-Ro/SSA and anti-La/SSB.

All of these microarray studies have consistently identified differentially expressed genes in IFN-mediated signaling pathways. As shown in **Table 1**, there are several differentially expressed genes found to be common across multiple studies and multiple tissue types, such as *IFITM1*, *IFI44*, *MX1*, *IRF7*, and *IRF8*, suggesting both local and systemic dysregulation of IFN signaling pathways in SS patients (**Table 1**). Each study also revealed unique dysregulated genes, partially due to the different types of arrays, relatively limited sample sizes, different quality control processes, and sample heterogeneity between studies.

Table 1 | Differentially expressed IFN-inducible genes found in common from the gene expression profiling studies in SS.

	Hjelmervik et al. (T score)	Gottenberg et al. (Fold change)	Pe´rez et al. (Mean difference with $\log_2$ scale)	Emamian et al. (Average fold change)	Gene function
IFITM1	−4.98	2.52	1.13	1.83	Block early stages of viral replication
IFITM3		1.88		1.96	
IFIT2			1.11	2.39	Inhibits expression of viral mRNA lacking 2′-O-methylation
IRF7			0.93	2.18	Important transcription regulators of type I IFN and IFN-inducible genes
IRF8	−4.40		1.47		
IRF9	−5.72		0.74		Important to cell differentiation
IFI16			0.99	1.56	Modulates p53 function and inhibits cell growth via Ras/Raf pathway
IFI27	−6.29			15.83	Mediates IFN-induced apoptosis
IFI44	−4.74			3.49	Antiproliferative, associated with HCV infection
MX1			1.15	3.85	A GTPase with antiviral activity against a wide range of RNA viruses and some DNA viruses
SP110	−5.05			1.85	Regulates gene transcription

Additional studies have identified increased activation of type I IFN-mediated genes in SS patients by candidate gene approaches (20–24). Increased IFN-inducible gene expression profiles have been detected in saliva and tears as well as particular cell types from SS patients (25, 26). However, results for the expression levels of IFNα itself in SS patients are controversial (6, 27). This may be due to the different techniques used in separate studies, a mixture of cell types in each experiment, and the heterogeneity of SS patients involved.

MECHANISMS OF ELEVATED IFN-MEDIATED SIGNALING IN SS

Although virtually all cells can produce type I IFNs in response to viral and bacterial infection, pDCs are the most potent IFN-producing cells, making up to 1000-fold more type I IFNs than other cell types (28). The detection of activated pDCs in salivary glands of SS patients but not in controls makes pDCs prominent candidates for the local production of type I IFNs that may promote the formation of inflammatory foci (8). Activated pDCs have also been found in the target organs of other autoimmune diseases (29, 30). Interestingly, Wildenberg et al. (26) found that, although the number of pDCs is decreased in the blood of SS patients, supposedly due to the migration of pDCs to peripheral sites, the cell surface activation marker CD40 is significantly overexpressed on pDCs from SS patients. Possible explanations of sustained activation of pDCs in SS include chronic exogenous stimulation and constitutive expression of pro-inflammatory transcription factors, such as IRF5 and IRF7, in SS patients (31).

Type I IFNs are induced transiently by viral infection and elicit antiviral effects. The pDCs can be rapidly induced to produce IFNα upon stimulation by RNA and DNA through TLR7 and TLR9, respectively (32). Thus, an initial viral infection is suspected to trigger the production of type I IFN by pDCs. The contribution of viral infection to elevated IFN signaling in SS is unknown; however, a number of viruses have been thought to contribute to SS pathogenesis, including Epstein–Barr virus (EBV), cytomegalovirus (CMV), hepatitis B virus (HBV), and hepatitis C virus (HCV) (12). Several mechanisms have been hypothesized regarding possible infectious triggers of SS, such as antigenic molecular mimicry. For example, infection with EBV results in the production of EBV nuclear antigen-1 (EBNA-1). Immune response against EBNA-1 can generate antibodies that cross-react with SS-associated autoantigens, such as anti-Ro/SSA (33). These antibodies may undergo epitope spreading and may ultimately become pathogenic in SS.

Autoantibodies and autoantigen-specific B cells have been detected in the salivary glands of SS patients (34, 35) and may involve in the production the type I IFNs through the formation of immune complexes. Båve et al. (6) have found that the combination of autoantibodies to RNA-binding proteins and material released by apoptotic cells can induce IFNα production by pDCs. This event is probably triggered by the interaction of RNA-containing immune complexes with Fcγ receptor IIa (FCGRIIA) on the surface of pDCs. Lövgren et al. (36) have described the production of IFNα by pDCs stimulated using U1 snRNA combined with IgG from patients with SLE. This response can be inhibited by FCGRIIA antagonists or RNase, suggesting a role for the RNA component as well as FCGRIIA in the immune complex-induced IFNα production by pDCs.

Another role of autoantibodies, especially anti-Ro52, in promoting IFN signaling is based on the function of their target autoantigens. Ro52, also known as tripartite motif-containing protein 21 (TRIM21), is an IFN-inducible E3 ubiquitin-protein ligase that promotes ubiquitination and proteasomal degradation of IRF3 and IRF7 (37, 38). After induction by IFNs following TLR signaling, Ro52 exerts a negative role on IFN signaling and prevents further inflammatory damage. Therefore, autoantibodies against Ro52 may interrupt the negative feedback of type I IFN signaling. Indeed, anti-Ro52 from SS patients is able to inhibit the E3 ligase activity of Ro52 by blocking the E3/E2 interface (39). Additionally, tissue inflammation and systemic autoimmunity in Ro52 knockout mice is thought to be induced by overproduction of pro-inflammatory cytokines (40). These results may well explain the correlation between the IFN signature and autoantibody positivity in SS patients (9). However, anti-Ro alone does not seem sufficient to induce high IFNα activity, given the fact that patients with disease are more likely to have high serum IFN activity than asymptomatic individuals with autoantibodies (41). Therefore, the contribution of autoantibodies to the elevated IFN signaling warrants further study.

GENETICS RISK FACTORS IN IFN PATHWAYS

A possible model for SS development is that an initial viral infection induces the production of type I IFNs and genetic susceptibility factors in certain individuals promote prolonged activation of the IFN system. Genetic predisposition to SS is supported by family aggregation of disease as well as a few twin studies (42–45). Genetic risk variants within or near IFN-regulated genes could possibly predispose patients to increased IFN signaling by (1) the constitutive expression of IFN-inducible genes or (2) the induction of loss-of-function inhibitors within IFN pathways. Genetic studies in SS have relied primarily on candidate gene approaches, focusing on those genes with biological plausibility for a role in SS etiology or evidence of association in other autoimmune diseases (46). The most convincing associations outside the HLA in SS found by candidate gene studies are within the regions of *IRF5* and *STAT4* (47–49), both of which are involved in IFN signaling. IRF5 is a transcription factor mediating type I IFN responses in various immune-related cells (17). Upon viral infection, IRF5 induces the transcription of IFNα and other pro-inflammatory cytokines, including IL12 p40 subunit, IL6, and TNFα (50). Genetic association within the *IRF5* region has been established in other autoimmune diseases, including SLE, RA, ulcerative colitis, primary biliary cirrhosis (PBC), and SSc (51–55). STAT4 is also a critical transcription factor involved in signaling initiated by type I and type II IFNs. It is required for the development of Th1 cells from naive CD4+ T cells and IFNγ production in response to IL12 (56, 57). The association of variants in *STAT4* has also been well established in other inflammatory diseases (58–60). Ongoing genome-wide association studies in SS have firmly established these loci as well as other genes that promote susceptibility to disease and may contribute to the dysregulation of IFN-inducible genes (61).

EFFECTS OF THE OVEREXPRESSION OF TYPE I IFN-INDUCIBLE GENES IN SS

Type I IFNs are key regulators of human immune systems and exert a broad effect on immune responses and autoimmunity (11). Overexpression of type I IFN-inducible genes in the salivary glands and peripheral blood of SS patients may influence many aspects of SS pathophysiology. Epithelial cells from the salivary glands of SS patients play an active role in promoting immune responses, including increased expression of MHC molecules and co-stimulatory molecules, such as B7 and CD40 (62–65). Many T cell-attracting and germinal center-forming chemokines, such as CXCL10, IL-8, and CXCL13, have also been found to be expressed in epithelial cells from the salivary glands of SS patients (22, 66, 67). Thus, these cells acquire antigen-presenting characteristics, mediating the recruitment, activation, and differentiation of the infiltrating inflammatory cells (68). Many of these molecules are induced by IFNα and IFNβ.

Another cytokine induced by type I IFNs in both salivary gland epithelial cells and peripheral blood monocytes is B cell activating factor (BAFF) (69, 70). BAFF is important in B cell activation, proliferation, and differentiation and has been found to be overexpressed in SS patients (71). Increased expression of BAFF has been observed in salivary gland epithelial cells from SS patients compared with those from healthy controls upon stimulation by IFNα, but not IFNγ or TNFα, suggesting a specific role of type I IFNs in B cell dysfunction in SS (72).

As mentioned above, autoantigen Ro52, or TRIM21 can be induced by IFNα in cultured human B cells and peripheral blood mononuclear cells (73). After upregulation, Ro52 translocates from the cytoplasm to the nucleus and initiates IFNα-induced apoptosis through intrinsic caspase-3. IFNα can also induce the expression of pro-apoptotic molecules, including Fas and FasL (74), and the increased expression of Fas and FasL has been identified in salivary glands from SS patients (18, 75). But this effect is not limited to type I IFNs: IFNγ and TNFα are also potent inducers of these pro-apoptotic molecules in salivary glands (18, 76). Concordantly, elevated levels of epithelial apoptotic cell death have been detected in the minor salivary gland tissues of SS patients (77). In addition, Ro52, Ro60, and La48 redistribute to apoptotic blebs and are exposed to the surface of cells undergoing apoptosis (78). Thus, apoptotic cells are also a main source of the autoantigens that form immune complexes in the salivary gland tissue of SS patients that further boost local IFNα production.

CONCLUSION

It is widely acknowledged that SS patients have elevated type I IFN signaling that may contribute to the pathogenesis of this complex disease. Hundreds of genes can be induced by IFNs and are mediated by IFN-related pathways in different cell types, which makes it difficult to attribute disease pathology to the malfunction of a single gene or certain gene family. An efficient approach for unraveling mechanisms of complex diseases is to use multiple tools integrated into systems approaches to answer a specific biological question. High-throughput techniques, such as microarray and genome-wide genotyping arrays, provide us with exceptional opportunities to comprehensively analyze dysregulated IFN networks.

It is worth noting that the IFN signature is only observed in a subset of SS patients who tend to be autoantibody positive (9). This heterogeneity is obviously important to consider and may explain the controversial results of recent IFN-targeting therapies and drug trials for SS (79–83). Eventually the IFN signature in SS may be used as a tool to identify high-risk individuals for preventative strategies, to help diagnose SS through its use as a serum biomarker in lieu of cumbersome and costly routine diagnostic processes currently used in research and clinical practice, and to predict the efficacy of treatment by IFN-related therapies.

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IFNα inducible models of murine SLE

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The role of type I interferons (IFNs) in SLE pathogenesis has been a subject of intense investigation in the last decade. The strong link between type I IFNs and SLE was initially provided by *ex vivo* studies showing that exposure of peripheral blood mononuclear cells to immune complexes from SLE patients elicits a signature of IFN inducible genes and was then further highlighted by human genetic studies. The mechanisms by which type I IFNs, especially IFN alpha (IFNα), modulate the immune system and exacerbate SLE have been largely elucidated through studies in mouse lupus models. In this review, we discuss the characteristics of several such models in which disease is accelerated by ectopically expressed IFNα. We also summarize several studies which tested therapeutic interventions in these models and discuss the advantages and disadvantages of using IFNα accelerated models to study experimental treatments for lupus.

Keywords: interferon α, lupus, nephritis, mouse model, inflammation

INTRODUCTION

In the last decade, type I interferons (IFNs) have received particular attention for their role in the pathogenesis of systemic lupus erythematosus (SLE). The induction of anti-dsDNA antibodies and development of lupus-like symptoms in a small number of IFN treated patients with cancer or infectious diseases suggested a causal link between this cytokine and SLE (1). The discovery of the "IFNα signature," which refers to the augmented expression of a group of IFNα induced genes, in peripheral blood mononuclear cells (PBMCs) from active lupus patients further highlighted the essential role of type I IFNs in the disease (2, 3). The IFN signature is induced in healthy PBMCs by SLE plasma containing nucleic acid associated immune complexes and this induction is inhibited by anti-IFNα antibody (4). Furthermore, polymorphisms in several genetic loci that are involved in the toll-like receptor (TLR)/IFN signaling pathway are associated with SLE risk (5, 6). These reports establish the important role of IFNα in the pathogenesis of SLE and are the basis for the development of drugs that target Type I IFNs or their receptor.

Type I IFNs are produced by several different cell types and are of major importance in anti-viral defense. In conventional dendritic cells, Type I IFN production is triggered by a several mechanisms including activation of endosomal TLR3 and binding to cytosolic nucleic acid receptors [reviewed (7) – **Table 1**]. By contrast, plasmacytoid dendritic cells (pDCs) are a major source of Type I IFN in SLE. Opsonized apoptotic material or circulating immune complexes of self-nucleic acids and autoantibodies are taken up by pDCS through the Fcγ receptor (8) and their nucleic acid components can then traffic to the endosome where they interact with TLR7 or TLR9. The adaptor molecule MyD88 is then recruited and this results in phosphorylation of IRAK1 and activation of the transcription factor IRF7 that induces IFN production. pDCs rapidly produce large amounts of IFNs owing to their constitutive expression of IRF7 (1, 9–11). Recent studies have shown that cytosolic DExD/H-Box helicases can sense cytoplasmic DNA (12) and initiate type I IFN production in human pDCs through the IRF7 pathway (13). The interaction between DNA and TLR9 is facilitated by a nuclear DNA binding protein HMGB1 (14) which is a component of neutrophil derived neutrophil extracellular traps (NETs) released from dying neutrophils in SLE (15). Other components of NETs protect nucleic acids from degradation and enhance their ability to form stable immune complexes with SLE related autoantibodies (16).

Type I IFNs can be induced in conventional DCS and macrophages following activation of TNFR1 and LTβR receptors. In addition, intracytoplasmic nucleic acids may trigger cytoplasmic receptors and activate a mitochondrial membrane pathway culminating in phosphorylation of IRF7 and Type I IFN production [reviewed (7)]. A complete description of the molecular pathways involved in type I IFN production in SLE is beyond the scope of this article but is the subject of several recent reviews (7, 17).

Type I IFNs have profound effects on the innate and adaptive immune systems [reviewed (7, 17, 18) – **Table 1**]. Serum from SLE patients induces monocytes from healthy donors to acquire a DC-like phenotype and become potent activators of T cells (19) in an IFNα dependent manner. Furthermore, IFNα acts on conventional DCs to enhance their production of an important B cell survival factor, B cell activating factor (BAFF) (20, 21). IFNα upregulates TLR7 expression on B cells, which in turn mediates increased expression of TACI, a receptor for BAFF (22). Its dual role in promoting BAFF production of DCs and enhancing the responsiveness of B cells to BAFF makes IFNα an important modulator of the fate of autoreactive B cells. Furthermore, IFNα drives B cell differentiation into CD138+ plasmablasts; terminal differentiation into Ig-secreting plasma cells is mediated by IL-6, another cytokine produced by activated pDCs (23). Finally, type I IFNs stimulate CD4 T cells to enhance antigen-specific B cell responses and prevent activated T cell death in mice (24, 25). These immunological features of type IFNs may all contribute to the pathogenesis of SLE.

Direct evidence for the essential role of IFNs in SLE was achieved through studies using lupus-prone mice that are

Table 1 | Induction and pro-inflammatory effects of Type I interferons.

Cell type	Effect	Mechanisms	Reference
B cells	Induction of autoantibodies	Enhancement of response to TLR activation Upregulation of MHCII, CD86, CD69	(18, 35)
	Induction of germinal centers	Increased class switch to pathogenic isotypes IgG2a and IgG3 Dysregulation of CD62L expression with increased shuttling of antigen from the MZ to the follicles	
	Induction of plasma cells	Enhanced crosstalk with IL-6 signaling Induction of miR-15a and repression of PAX5 Increased expression of BLIMP and XBP	
	Induction of short-lived plasma cells	Decreased bone marrow expression of CXCL12 and VCAM-1	(34)
Conventional dendritic cells	Release of Type I IFN in response to TLR3 activation	TRAF3 mediated recruitment of TBK1, IKKε, and IRF3, leading to IRF3 phosphorylation	(7, 17)
	Release of Type I IFNS through receptors for cytosolic nucleic acids	RIG-I mediated recruitment of the MAVS adapter and mitochondrial membrane assembly of a TRAF3, TBK1, and IKKε signalosome leading to IRF3 phosphorylation IFI16 mediated induction of a STING, TBK, IRF3-dependent pathway	
	Priming for antigen presentation	Upregulation of MHC and costimulatory molecules Increased expression of CCR7	
	Release of cytokines including BAFF	TRAF6 mediated NFκB activation	
Plasmacytoid dendritic cells	Rapid release of high concentrations of Type IFN in response to immune complexes	High levels of endosomal TLR7, 8, and 9 and activation of the MyD88 adaptor Constitutive expression of IRF7 Formation of late endosomes	
T cells	CD4 T cell stimulation	Increased IFNγ production Enhanced survival	(7, 24)
	Priming for induction of CD8 killer cells	Increased cross-presentation Increased gene transcription Enhanced responsiveness to IL-2 and IL-15	
	Decreased Treg function	Downregulation of intracellular cAMP	(62)

genetically deprived of type I IFN signaling or treated with exogenous type I IFNs. *Ifnar1* gene deficiency largely protects lupus-prone mice from disease onset or attenuates disease severity (26–29). Conversely, transient overexpression of exogenous IFNα accelerates disease progression in all lupus-prone mice tested to date. This makes these models not only useful tools to understand the role of IFNs in SLE, but also useful platforms to test potential therapies for SLE.

IFNα ACCELERATED LUPUS MOUSE MODELS

NZB/W F1 MICE

New Zealand black/New Zealand white (NZB/W) F1 mice are a widely used animal model for lupus; they mimic human lupus in several aspects including gender specificity, the appearance of circulating anti-dsDNA antibodies, renal deposition of immune complexes and the development of fatal glomerulonephritis. They do not develop skin disease or hematologic manifestations and thus have been used primarily to study SLE nephritis. NZB/W F1 mice develop proteinuria at a median age of 37 weeks and die by the age of 1 year (30, 31). Although NZB/W F1 mice do not develop detectable levels of circulating IFNα (20), the IFN signature can be detected in splenic cells of pre-autoimmune NZB/W F1 mice (32). The disease-initiating activities of IFNα in NZB/W F1 mice were suggested by a report that treatment with poly IC, a TLR3 agonist, accelerates the disease in these mice (33). More recently, a single injection of an adenovirus expressing IFNα (Ad-IFNα) has been shown to accelerate the production of circulating anti-dsDNA antibodies, renal deposition of immune complexes, onset of proteinuria, and death in NZB/W mice in a dose dependent manner (20, 34). The accelerated clinical manifestations are associated with a vastly enhanced germinal center reaction, increased serum levels of pro-inflammatory cytokines, and the induction of T cell expression of IL-21 (34). This pro-inflammatory environment is associated with expanded B cells, CD4 T cells, and DCs (34) and loss of B10 cells (35). Furthermore, IFNα virus injection induces elevated serum levels of BAFF and increased TLR7 expression on splenic B cells (20–22, 34). Interestingly, although NZB/W F1 mice normally possess a proportion of long-lived autoreactive plasma cells in the spleen and BM, treatment with Ad-IFNα skews the differentiation of autoreactive B cells almost completely toward short-lived plasma cells [(34, 36) reviewed in (37)]. This appears to be due to a decrease in bone marrow expression of CXCL12 and VCAM-1, both of which are components of the bone marrow plasma cell niche (34). Finally, in contrast to conventional

mice, Ad-IFNα treated NZB/W F1 mice have far less renal interstitial leukocyte infiltration. This is due to reduced renal expression of pro-inflammatory chemokines such as CXCL13 and intrinsic defects of leukocyte migration toward these chemokines (38).

Most of these features have also been reported in Ad-IFNα treated New Zealand Mixed 2328 mice (39). However, despite a large increase in T cell numbers, these mice do not develop a preferential expansion of memory T cells following IFN treatment or substantial glomerular macrophage infiltration as they age, suggesting these two features may not be driven by type I IFNs. In addition to the immune effects of Type I IFNs in this model, administration of Ad-IFNα has a detrimental effect on the vasculature, causing impairment of endothelium-dependent vasorelaxation, a decrease in maturation of endothelial progenitor cells into mature endothelial cells, increased platelet activation, and accelerated thrombus formation, suggesting a potential role for IFN in the accelerated atherosclerosis associated with SLE (40).

Studies using cell depletion or mice with genetic deficiencies have shown that disease acceleration by IFN is dependent on T cells (NZB/W mice) (34), B cells (NZM2328 mice) and BAFF (NZM2328 mice) (39).

NZW/BXSB MICE

Male NZW/BXSB mice carry two active copies of the TLR7 gene. They develop anti-RNA and anti-phospholipid autoantibodies, severe inflammatory nephritis and anti-phospholipid syndrome with thrombocytopenia, myocardial infarcts, and cardiomyopathy (41, 42). The survival of these mice is prolonged by prophylactic treatment with anti-IFNAR antibody, suggesting the disease process is driven by IFNα (43). In contrast, female mice with a single active copy of TLR7 develop late onset nephritis, but not anti-phospholipid syndrome (42, 44). Administration of Ad-IFNα induced high titers of circulating anti-phospholipid, anti-Sm/RNP, and anti-DNA autoantibodies and markedly accelerated nephritis and death, but not anti-phospholipid syndrome in female NZW/BXSB mice (44). These IFNα induced effects were accompanied by a striking increase in activated B and T cells in the spleen. Using female NZW/BXSB mice bearing the site-directed anti-cardiolipin/DNA autoantibody V_H transgene 3H9, IFNα has been shown to relax the stringency for selection against autoreactivity of the antigen selected B cell repertoire (45).

B6.SLe123

B6.Sle123 mice, that possess three SLE susceptibility loci, spontaneously develop highly penetrant severe systemic autoimmunity and fatal glomerulonephritis beginning at 6 months of age. Young pre-autoimmune mice that were treated with IFNα quickly developed renal immune complex deposition and nephritis, accompanied by increased serum levels of pro-inflammatory cytokines such as TNFα and IL-6, activation of DCs, B cells, and T cells, as well as an enhanced germinal center response (46). As in the strains discussed above, renal leukocyte infiltration was not affected by IFNα treatment.

Collectively, these studies show that excess IFNα accelerates progression of glomerulonephritis in most lupus models. The acceleration and severity of the disease is dose dependent, allowing researchers to control of the duration of their study. IFNα induces a T dependent and enhanced germinal center response and exhibits characteristics of the disease in the conventional strain. For instance, IFNα induces anti-dsDNA antibodies in NZB/W F1 mice and anti-RNA antibodies in BXSB mice, these being the predominant specificities in the respective strains (20, 34, 44). Of interest is the skewing of the antibody response from long-lived to short-lived plasma cells in the NZB/W model, a feature associated with alterations of the bone marrow environment. However, IFN acceleration is associated with less renal inflammatory cell infiltration compared to its spontaneous counterpart. This is probably due to the short disease course which does not allow these features to develop to the same extent as in the conventional mice. It is also important to note that other major manifestations of human SLE including skin, hematologic, and neurologic disease cannot be addressed using these models.

IFNλ ACCELERATION OF SLE

IFNλ is a family of Type III IFNs that mediate their biologic activities through a receptor that is expressed predominantly on epithelial cells and induce a similar pattern of gene expression as Type I IFNs (47). Treatment of NZB/W mice with a continuous infusion of IFNλ did not exacerbate disease, however the addition of IFNλ to a low dose of IFNα modestly accelerated proteinuria onset (35).

ASSOCIATION OF TYPE I IFNs WITH ALTERATION IN miRNAs

Type I IFN production can be regulated by miRNAs. For example, underexpression of miR-146a was found to correlate with disease activity and with an IFN signature. miR-146a targets multiple components of the IFN signaling pathway such that its deficiency results in overexpression of IFN inducible genes. Importantly, administration of TLR agonists or of Type I IFN induced expression of miR-146a indicating a physiologic feedback loop that may be dysregulated in SLE (48). Delivery of miR-146a to lupus PBMCs *in vitro* reduced the expression of IFN inducible genes (48) and delivery to lupus-prone BXSB mice *in vivo* reduced the production of pro-inflammatory cytokines and autoantibodies (49). Thus miR-146a reduction is a biomarker for disease activity and a potential therapeutic target. Another interesting observation in the NZB/W model is the IFN induced expression of miR-15a in the spleens of treated mice; this is associated with downregulation of PAX5 and the emergence of autoantibodies and plasma cells. Since PAX5 is a negative regulator of miR-15a, the upregulation of miR-15a may be an early biomarker for IFN induction of plasma cells (35).

USING THE IFNα ACCELERATED LUPUS MODEL TO TEST THERAPEUTICS FOR SLE NEPHRITIS

IFNα KINOID

IFNα kinoid is an IFNα derived immunogen that triggers a strong but transient production of neutralizing antibody against IFNα (50). In a proof of principle experiment, prophylactic administration of kinoid delayed IFNα induced immune complex formation, proteinuria, and death in NZB/W F1 mice (50). It is worth noting that not all kinoid immunized mice mounted a substantial

humoral response to IFNα and only the ones with antibody levels above a certain threshold showed delayed clinical manifestations. Moreover, a sustainable protective effect required prolonged production of anti-IFNα antibody, suggesting that periodic booster injections might be required to achieve a long-term antibody dependent clinical benefit. The success of this study led to the development of a human IFNα kinoid that induces antibodies neutralizing all 13 subtypes of human IFNαs (51). This kinoid has been shown to reduce the IFNα signature in lupus patients (52).

BIOLOGIC THERAPIES

TACI-Ig is a fusion protein that inhibits the BAFF/APRIL signaling pathway. The treatment of pre-autoimmune NZB/W F1 mice with TACI-Ig significantly delayed proteinuria onset and substantially prolonged the survival of the mice (53). TACI-Ig treatment achieved a similar clinical outcome in IFNα induced NZB/W F1 mice, although the survival benefit was only apparent when the treatment was started concomitant with IFN administration and was no longer effective if it was delayed until autoantibodies emerged (54). TACI-Ig treatment did not affect germinal center formation, autoantibody production, renal deposition of immune complexes, or pro-inflammatory cytokine expression in lymphoid organs however it was associated with a decrease in renal inflammation, prevention of activation of resident renal macrophages, and a decrease in renal and serum levels of TNF (54).

CTLA4-Ig, a drug that interrupts CD28-B7 interactions, prevents disease onset in NZB/W F1 mice (55, 56). In contrast, CTLA4-Ig at standard-dose failed to prevent or delay the onset of nephritis in Ad-IFNα treated mice despite preventing T and B cell activation, GC formation, and the production of pathogenic IgG2a anti-dsDNA antibodies (54). Resistance to standard-dose CTLA4-Ig was associated with the persistence of pathogenic IgG3 autoantibodies that were attenuated only after administration of high-dose CTLA4-Ig. Although the mice treated with high-dose CTLA4-Ig eventually died of nephritis, this treatment markedly delayed proteinuria onset and protected the mice from interstitial inflammation.

Ad-IFNα treatment in NZB/W F1 mice results in elevated renal expression and increased serum levels of TNFα concomitant with the onset of nephritis, making these mice an ideal model to study the efficacy of TNF receptor 2 (TNFR2)-Ig (57). TNFR2-Ig treatment delayed the onset of nephritis and prolonged survival of IFN accelerated mice without affecting autoantibody production or systemic immune activation. Similar to the observations with TACI-Ig, the therapeutic effect of TNFR2-Ig was achieved through inhibiting the renal response to immune complex deposition. The upregulation of a panel of chemokines in response to renal immune complex deposition was blocked by TNFR2-Ig treatment, resulting in diminished recruitment of periglomerular and interstitial F4/80^{hi} macrophages. In addition, renal endothelial cell activation and oxidative stress were decreased in the mice treated with TNFR2-Ig.

CD137 (4-1BB) is an inducible T cell costimulatory receptor belonging to the TNF receptor superfamily. It is expressed on activated CD4 and CD8 T cells, and promotes the proliferation of these cells. Treatment of NZB/W F1 mice with an agonistic anti-CD137 antibody significantly delays the onset of nephritis and prolongs survival (58). The therapeutic benefit is associated with inhibition of IgG but not IgM anti-dsDNA antibodies (58), consistent with an effect of anti-CD137 on T cell dependent but not T cell independent humoral responses (59). In preliminary experiments, we have shown that anti-CD137 antibody treatment of IFN induced NZB/W mice markedly delays the formation of germinal centers and the development of IgG2a and IgG3, but not IgM anti-dsDNA antibodies and greatly protects the kidneys from glomerular and interstitial injury (unpublished data), similar to the observations in NZB/W F1 mice that develop disease spontaneously. As with TACI-Ig, maximal benefit was achieved if the anti-CD137 treatment was started within 1 week of administration of Ad-IFNα.

REMISSION INDUCTION THERAPIES

Triple therapy with cyclophosphamide (CTX), anti-CD40L, and CTLA4-Ig induces remission in a high percentage of NZB/W F1 mice with established nephritis (60). A similar percentage of Ad-IFNα treated mice entered remission after this therapy but they relapsed rapidly (54). Mice treated with high-dose Ad-IFNα relapsed faster than mice treated with low dose Ad-IFNα and the latter relapsed faster than conventional NZB/W F1 mice (54, 60). Production of anti-dsDNA antibodies and glomerular deposition of IgG immune complex in Ad-IFNα treated NZB/W F1 mice were markedly reduced (54) by triple therapy, consistent with the observation that IFNα induces predominantly short-lived plasma cells which are susceptible to cytotoxic reagents (34). Nevertheless, in the high-dose IFN group, new autoreactive plasma cells formed as soon as the triple therapy drugs dissipated from the serum and this was associated with reaccumulation of renal immune complexes and rapid disease relapse. This is in contrast to conventional NZB/W F1 mice whose renal deposition of immune complexes was not reversed by the triple therapy (60) but whose renal response to immune complex deposition was markedly attenuated.

THE UTILITY OF THE IFNα ACCELERATED LUPUS MODEL

Studies of therapeutic interventions in many strains of lupus-prone mice, such as NZB/W mice, are hampered by the stochastic disease onset and the length of time needed for the mice to develop spontaneous disease. In contrast, IFNα induced disease has a relatively synchronized onset and highly reproducible disease progression, allowing the study of therapies on defined stages of disease including remission induction studies. In addition, the IFNα induced model requires less time to develop clinical manifestations and has a shorter window from disease onset to death, allowing the therapies to be tested in a compact time frame.

At the same time, one needs to be aware that Ad-IFNα treated NZB/W F1 mice are not merely a hastened version of the spontaneous lupus model but they possess some distinct features. For instance, Ad-IFNα treated NZB/W F1 mice almost completely lack the long-lived plasma cells that exist in abundance in the spleen and bone marrow of the conventional NZB/W F1 mice (34) and

therefore may not be a suitable model to test therapies targeting these pathogenic cells. Similarly, the modest renal infiltration with inflammatory cells renders the IFNα model less attractive for studies focused on inhibitors of leukocyte trafficking. Finally Ad-IFNα treatment also alters the response of disease to therapies that are highly effective in the spontaneous disease. Such therapies are for the most part only effective in the IFN model if they are administered prophylactically. This may reflect a more dynamic inflammatory environment in IFNα induced disease, rendering the disease more resistant to therapeutic intervention. Whether IFN signature^{hi} patients are similarly more resistant to drug interventions than IFN signature^{lo} patients needs to be determined in the context of clinical trials. It is of interest in this regard that preliminary studies of one anti-IFN agent showed no effect on autoantibodies and demonstrated efficacy only in the IFN signature^{lo} patient group (61, 63). By contrast, preliminary data from a second trial of a different anti-IFN agent showed a trend towards a better outcome in the patients with a high IFN signature (64). Further clinical trials are in progress. Overall Ad-IFNα treated mice are a reliable but stringent model to test new therapies for lupus nephritis. The difference in their response to therapies may help to predict proper intervention for patients with an IFN signature.

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