Research Article

Effects of IRF5 Lupus Risk Haplotype on Pathways Predicted to Influence B Cell Functions

Joel M. Guthridge,[1] Daniel N. Clark,[2] Amanda Templeton,[1] Nicolas Dominguez,[1] Rufei Lu,[1] Gabriel S. Vidal,[1] Jennifer A. Kelly,[1] Kenneth M. Kauffman,[3] John B. Harley,[3] Patrick M. Gaffney,[1] Judith A. James,[1,4] and Brian D. Poole[1,2]

[1] *Arthritis and Clinical Immunology Program, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, OK 73104, USA*

[2] *Department of Microbiology and Molecular Biology, Brigham Young University, 857 WIDB, Provo, UT 84604, USA*

[3] *Division of Rheumatology, Cincinnati Children's Hospital, 3333 Burnet Avenue, Cincinnati, OH 45229, USA*

[4] *Departments of Medicine and Pathology, The University of Oklahoma Health Sciences Center, 1100 N. Lindsay, Oklahoma City, OK 73104, USA*

Correspondence should be addressed to Brian D. Poole, bpoole@gmail.com

Both genetic and environmental interactions affect systemic lupus erythematosus (SLE) development and pathogenesis. One known genetic factor associated with lupus is a haplotype of the interferon regulatory factor 5 (*IRF5*) gene. Analysis of global gene expression microarray data using gene set enrichment analysis identified multiple interferon- and inflammation-related gene sets significantly overrepresented in cells with the risk haplotype. Pathway analysis using expressed genes from the significant gene sets impacted by the *IRF5* risk haplotype confirmed significant correlation with the interferon pathway, Toll-like receptor pathway, and the B-cell receptor pathway. SLE patients with the *IRF5* risk haplotype have a heightened interferon signature, even in an unstimulated state ($P = 0.011$), while patients with the *IRF5* protective haplotype have a B cell interferon signature similar to that of controls. These results identify multiple genes in functionally significant pathways which are affected by IRF5 genotype. They also establish the IRF5 risk haplotype as a key determinant of not only the interferon response, but also other B-cell pathways involved in SLE.

1. Introduction

Systemic lupus erythematosus is a complex disease with multifactorial etiology and pathogenesis. Studies in identical twins indicate that concordance for lupus is approximately 40%, indicating a strong but not exclusive genetic component [1, 2]. Recent genetic analyses have identified more than thirty candidate genes that are associated with lupus risk [3–18]. *IRF5* was found to be associated with lupus by multiple independent groups in a variety of populations [10, 13–15, 19, 20]. *IRF5* risk haplotypes may function at the crossroads of environmental risk, such as virus infection, and cellular immune responses. At least three polymorphisms of *IRF5*

have been identified that contribute independently to the risk for lupus, which together constitute the lupus risk haplotype [10, 21]. Although the majority of the polymorphisms that have been associated with lupus are in nontranslated regions, they may affect several facets of *IRF5* activity, including splicing, RNA stability, transcription factor binding, and apoptosis [9, 10, 15, 21, 22].

IRF5 is important in the production of and response to interferon alpha (IFNα), which is heightened in lupus. IFNα is produced by dendritic cells, macrophages, B cells, and other cell types, primarily in response to virus infection [23, 24]. Dendritic cells have been shown to produce IFNα in response to incubation with immune complex-containing

sera from SLE patients [25], especially patients that have the risk haplotype for *IRF5* [19]. Additionally, serum interferon levels, as well as the interferon response signature, are increased in patients with the risk haplotype [19, 26]. *IRF5* is an especially interesting candidate for a genetic risk factor in lupus because it acts in pathways that control many of the cellular and immune responses to environmental factors, such as infection, which may contribute to lupus.

One putative environmental agent that is strongly associated with risk for lupus is Epstein-Barr virus (EBV) infection. Lupus has been associated with prior EBV infection in both pediatric and adult populations [27–33]. EBV expresses antigens that are immunologically cross-reactive with significant lupus autoantigens such as Sm and nRNP [34–39]. However, since over 95% of adults are infected with EBV, determining why EBV could contribute to lupus in certain individuals but not others has proven challenging. The identification of *IRF5* and other genetic risk factors for lupus open the possibility that the lupus-associated genetic polymorphisms in one or more of these genes works in concert with environmental factors culminating in the increased observed risk for developing lupus. Previous work has shown that pediatric lupus patients have broadened, more cross-reactive humoral immune responses to EBV than controls [40]. EBV is also not as well controlled in lupus patients as it is in controls, with increased viral load and altered T-cell responses [41, 42]. Differences in viral infection or the response to viral infection conferred by genetic factors such as *IRF5* polymorphisms may in part explain these observations.

Since B cells are the primary host cell for EBV infection, we used B cells and EBV interactions as a model to study the impact of *IRF5* genotype on downstream B-cell responses. For this study, we examined differences in B-cell gene expression between naïve B cells from individuals with the *IRF5* risk haplotype and those with the protective or neutral haplotypes at both basal levels and after exposure to EBV. Naïve B cells were chosen because they are the cell type in which EBV establishes latent infection [43]. We found multiple networks of genes that were enriched for differential expression, as well as individual gene expression differences. Most importantly, we identified different expression patterns of interferon response genes in lupus patients based on the *IRF5* risk haplotype. Understanding these differences will aid in determining mechanisms through which the genetic risk conferred by the *IRF5* risk haplotype is manifested.

2. Materials and Methods

2.1. Study Participants. Genotypes were previously collected on samples obtained from the Oklahoma Rheumatic Disease Resource Cores Center (ORDRCC) at the Oklahoma Medical Research Foundation. Previously enrolled subjects were contacted for study participation based upon their *IRF5* risk and protective haplotypes using genotypes at single nucleotide polymorphisms rs2004640 and rs10954213. Five *IRF5* high-risk (3 controls, 2 patients) and five *IRF5* nonrisk (2 controls, 3 patients) sex- and race- matched individuals

were recruited. The study was approved by the institutional review board at OMRF and OUHSC, and informed consent was obtained from all subjects in the study.

2.2. B-Cell Stimulation. Peripheral blood mononuclear cells were separated by density gradient centrifugation from the peripheral blood of volunteers. Naïve B cells were isolated using the MACS Naïve B Cell Isolation Kit II (Miltenyi Biotec Inc). Untouched naïve B cells were incubated at a 1 : 1 (v/v) ratio with either virus-free media or infectious EBV for 16 hours. Virus preparations were in the form of B95-8 cell culture supernatant. The same preparation of supernatant was used for all assays.

2.3. Gene Expression Profiling. Total cellular mRNA was purified from lysates of infected and mock-infected cells using the Ambion RNaqueous-Micro Kit (Applied Biosystems, Austin, TX, USA) according to the manufacturer's protocol and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc.). cRNA amplification and labeling with biotin were performed using the Illumina TotalPrep RNA amplification kit protocol (Ambion, Austin, TX, USA) on an aliquot of 200 ng of total RNA. Whole genome expression analysis was performed using the Illumina HumanRef-8 v.3 gene expression chip (24,526 transcripts) following the Illumina Whole-Genome Expression Protocol.

2.4. Statistical and Pathway Analysis. The microarray data were analyzed using gene set enrichment analysis, and pathway analysis to investigate changes in gene networks. These analyses were followed by comparison of individual gene expression differences inside these networks. Raw expression data was first normalized using the MDAT toolbox [44]. Gene Set Enrichment Analysis software (Molecular Signatures Database) was used to determine whether an *a priori* functionally defined set of genes showed statistically significant, concordant differences between two phenotypes (*IRF5* risk and nonrisk haplotypes) [45, 46]. Significant gene sets were identified by an enrichment score, which reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes, and a false discovery rate (FDR) of <25%. We focused our subsequent pathway analysis on the subset of enriched genes ($n = 368$) from the statistically significant gene sets.

Pathway analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems, http://www.ingenuity.com/). A data set containing gene identifiers and corresponding expression values was uploaded into in the application. Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. The expression values entered were the normalized log (intensity) values of *IRF5* nonrisk and risk haplotype individuals, respectively.

Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways 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 genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway; (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 is explained by chance alone. All associations are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Human and mouse orthologs of a gene are stored as separate objects in the Ingenuity Pathways Knowledge Base, but are represented as a single node in the network. Heat maps were created with previously mentioned expression values in Spotfire software.

3. Results

3.1. Enrichment of Lupus-Related Gene Sets by IRF5 Risk Haplotype.
Using existing genotyping data for single-nucleotide polymorphism rs2004640 (T: risk, G: protective) and rs10954213 (A: risk, G: protective) [10, 11, 20, 21, 47] the frequency of the risk, protective, and neutral *IRF5* haplotypes were examined in 1,390 SLE patients and 2,039 controls enrolled in a large cohort of European American female (EA) SLE patients from the Lupus Family Registry and Repository (LFRR: http://lupus.omrf.org/). A significant enrichment for the risk and risk-neutral haplotypes was found in lupus patients ($P < 0.0001$), as expected (Table 1). Using these results, five individuals with the risk or risk-neutral haplotypes and five with the protective or protective-neutral haplotypes were recruited from the local SLE collections through the ORDRCC for further study. These included two SLE patients and three controls in the risk groups, and three SLE patients and two controls with protective and protective-neutral haplotypes. The risk group was enriched for controls so that the effects of the *IRF5* haplotypes could be better studied in the absence of potential confounding genetic influences or factors related to lupus.

In all, 368 genes were found to be significantly ($P < 0.05$) differentially expressed between the risk and the nonrisk individuals. Gene set enrichment analysis was used to look for gene pathways overrepresented when comparing expression data from the risk haplotype and the protective haplotype phenotypic groups in the case or control groups separately. This analysis examines 6,769 *a priori* defined functional gene sets [46, 48]. In the unaffected controls with the risk haplotype, nine gene sets were enriched with a false discovery rate (FDR) <25% (Table 2), and 19 were enriched with a nominal P value <0.01, but an FDR >25% (Supplemental Table 2 which available online at doi:10.1155/2011/594056). In the lupus patients with the risk haplotype five gene sets were enriched with an FDR <25%. However, four of these were different versions of interferon-alpha gene sets. The fifth was the lupus-related interferon response signature (Table 2). Seven gene sets were enriched with a nominal P value <0.01 but a FDR >25% in the SLE risk haplotype cells, including the Toll-like receptor (TLR) gene set (Supplemental Table 1).

Fewer gene sets were as highly enriched in the protective haplotype cells. No gene sets were significantly enriched at an FDR level <25% in either the unaffected controls or the lupus patients with the protective haplotype. However, 39 gene sets were enriched at $P < 0.01$ in the SLE patients with the protective haplotype, and 35 gene sets were enriched at $P < 0.01$ level in the controls with the protective haplotype (Supplemental Tables 3 and 4).

3.2. Identification of Lupus-Related Pathways Differentially Affected by IRF5 Haplotype.
Genes from the enriched gene sets described above which also demonstrated differences in expression in the previous analysis were included in a global pathway analysis using the Ingenuity Pathway Analysis system. This analysis uses the curated Ingenuity Knowledge Base to associate sets of genes and expression data with established gene pathways. Fisher's exact test was used to quantify the degree of association with these pathways. When the cells with the risk haplotype (both stimulated and unstimulated) were compared to those with the non-risk haplotypes, three canonical pathways were found with statistically significant ($P < 0.01$) association: the interferon (Figure 1), Toll-like receptor (Figure 2), and B-cell receptor (Figure 3) pathways. Interestingly, all three of these pathways have significant implications for lupus. These three canonical pathways and relative changes in expression following EBV exposure are represented in Figures 1–3 and Table 3.

3.3. Identification of Individual Genes Differentially Expressed by IRF5 Haplotype.
Several genes in the interferon pathway exhibited differential expression between either the risk and protective haplotypes or the EBV exposed and unexposed conditions. Genes with differential expression were selected based on inclusion in a significantly associated pathway, average expression values of at least thirty for one condition, and differential expression of at least 1.5-fold. Differential expression comparisons were done both with the unstimulated and the EBV-infected states (Table 3). Several genes were differentially expressed in the interferon pathway, including interferon-induced transmembrane protein 1 (*IFITIM1*), signal transducer and activator of transcription 1 (*STAT1*), IFNα receptor 2 (*IFNAR2*), 2'–5'-oligoadenylate synthetase 1 (*OAS1*), and *MX1*. The expression patterns of these genes varied based on *IRF5* haplotype and EBV infection status (Figure 1).

IFITM1 was more strongly expressed in the risk cells than in the nonrisk in the unstimulated condition (2.1-fold difference). When the cells were exposed to EBV, this difference disappeared, a result of a greater increase in expression (4.8-fold) in the nonrisk cells than the risk (2.8-fold). *STAT1* was slightly underexpressed in the risk cells than in the nonrisk prior to EBV exposure (1.3-fold), but after EBV exposure it was more highly expressed in the risk cells (1.3-fold). *IFNAR2* acted in the opposite manner; its expression was higher in the risk cells in the unstimulated condition (1.7-fold), but higher in the nonrisk cells after EBV infection (1.3-fold). IFNAR2 is an interferon receptor that contributes directly to the response to interferon, making this

TABLE 1: Frequency of *IRF5* haplotypes in lupus patients and healthy controls. Haplotype frequencies observed in controls and systemic lupus erythematosus (SLE) patient cohort at single-nucleotide polymorphisms rs2004640 (*T* : risk, *G:* protective) and rs10954213 (*A:* risk, *G:* protective).

Haplotype	Allele 1	Allele 2	Frequency (patients) $n = 1390$	Frequency (controls) $n = 2039$	SLE risk
1	TA	TA	0.2576	0.1947	Risk/risk
2	TA	TG	0.4460	<0.0001	Risk/neutral
3	GA	GG	0.2108	0.2737	Protective/protective
4	GG	TG	0.0165	0.1810	Protective/neutral

TABLE 2: Effect of the IRF5 risk haplotype on the expression of gene sets. Gene set enrichment analysis showed gene sets enriched in the risk haplotypes of either SLE-unaffected controls or SLE patients without EBV infection. Genes shown have a false discovery rate (FDR) of <25%.

Gene set name	Affected pathways or cellular conditions	No. of genes	Normalized enrichment score	*P* value	FDR *q*-value
Unaffected controls					
CROONQUIST_IL6_STROMA_UP	IL-6 exposure	37	−2.026	0.0018	0.038
PASSERINI_INFLAMMATION	Inflammation	23	−1.751	0.0112	0.212
PASSERINI_PROLIFERATION	Proliferation	62	−1.782	<0.0001	0.224
ADIP_DIFF_CLUSTER2	Differentiation	41	−1.752	0.004	0.225
CROONQUIST_RAS_STROMA_DN	Ras activation	21	−1.757	0.0038	0.229
UVB_NHEK3_C6	UV light exposure	27	−1.763	0.0039	0.234
HOHENKIRK_MONOCYTE_DEND_DN	Dendritic cell maturation	121	−1.724	0.0348	0.239
LEE_DENA_UP	Murine liver cancer	59	−1.769	<0.0001	0.241
ZUCCHI_EPITHELIAL_DN	Breast cancer metastasis	44	−1.785	0.0117	0.245
SLE patients					
IFNALPHA_HCC_UP	IFNα	29	−1.968	0.0038	0.042
IFNALPHA_NL_HCC_UP	IFNα	18	−1.878	0.0096	0.063
RADAEVA_IFNA_UP	IFNα	49	−1.897	0.0059	0.07
IFNALPHA_NL_UP	IFNα	27	−1.847	0.0099	0.075
BENNETT_SLE_UP	SLE	28	−1.785	0.0082	0.138

gene very interesting in the context of interferon regulation and responsiveness. *OAS1* was overexpressed in the risk cells compared to the nonrisk cells in both the unstimulated and EBV-exposed conditions (1.5-fold and 2.2-fold, resp.), as was *MX1* (1.6- and 1.8-fold).

The TLR pathway also contained several genes that were differentially expressed between the risk and protective haplotype-containing cells (Figure 2). Fos and myeloid differentiation primary response gene 88 (*MyD88*) are both under expressed in the unstimulated risk cells compared to the nonrisk (2.2- and 1.8-fold, resp.). Both of these genes switch from being downregulated in the risk cells before EBV exposure to upregulated in the risk cells after EBV exposure (1.3- and 1.2-fold, resp.). Another very interesting gene that was differentially expressed in the TLR pathway is tumor necrosis factor α-induced protein 3 (*TNFAIP3*). It is under expressed by 1.3-fold in the risk cells in the resting condition. After EBV exposure, expression is even more unbalanced, with 1.9-fold under expression in the risk cells.

Genes of interest that are overexpressed in the risk cells in the TLR pathway without EBV exposure include *CD14* (3.2-fold), lymphocyte antigen 96 (*LY96,* or *MD-2*) (2.3-fold), and *TLR1* (1.7-fold).

The B-cell receptor (BCR) pathway exhibited differences in gene expression due to the *IRF5* risk haplotype (Figure 3).

CD79A and *CD79B*, which together form part of the BCR, were both downregulated 2.2-fold in the uninfected risk cells, but this difference disappeared after EBV infection. Ras-related C3 botulinum toxin substrate 1 (*RAC1*), a gene involved in lymphocyte differentiation and survival [49], was overexpressed in risk cells under all conditions (1.5-fold in mock infected cells, 1.7-fold in EBV infected cells). Expression of the signaling protein *AKT1* and the transcription factor *NFκB2* were downregulated in EBV infected cells with the *IRF5* risk haplotype (1.9- and 3.4-fold, resp.). In three other genes, phosphatidylinositol 3 kinase catalytic subunit α (*PIK3CA*), nuclear factor of activated T cells 5 (*NFAT5*), and glycogen synthase kinase 3β (*GSK3B*), the risk haplotype had
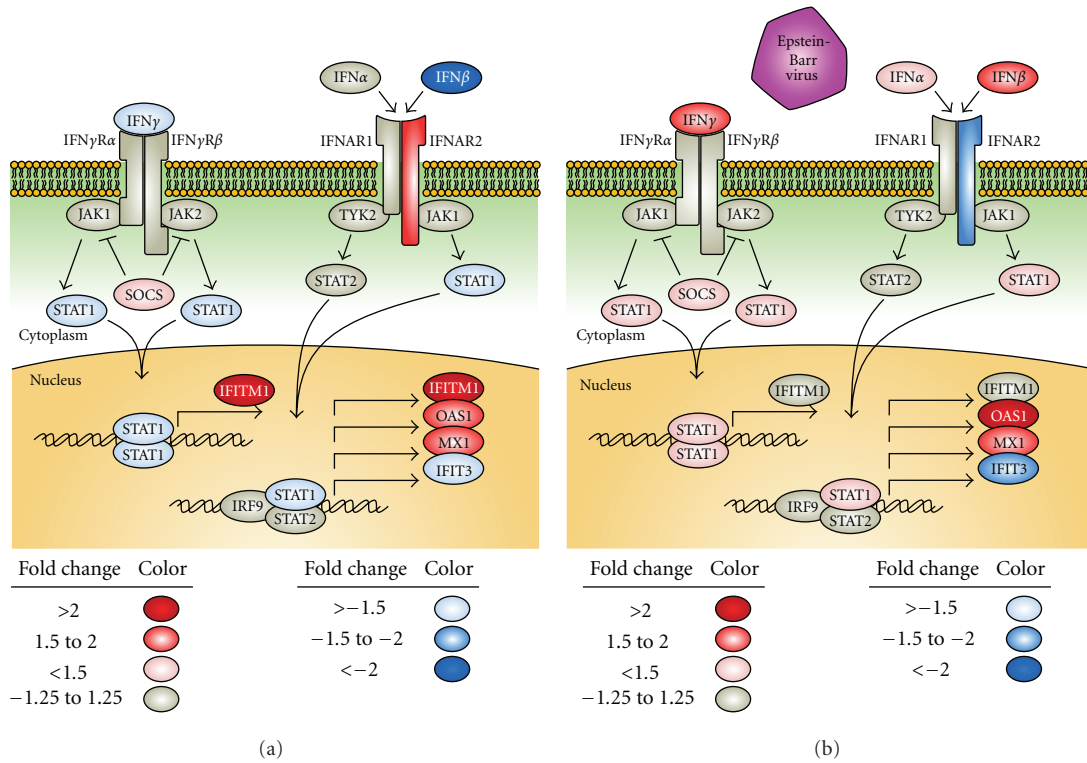
FIGURE 1: Association of the canonical interferon pathway with the *IRF5* haplotype. Naïve B cells were either exposed to EBV for 16 hours or left unstimulated. Whole-genome expression levels were compared between cells with the risk and protective haplotypes using the Illumina platform. Significant association of the data set with the canonical IFN pathway was discovered using Ingenuity Pathway Analysis ($P < 0.01$). Interactions between genes in the interferon pathway based on Ingenuity Pathway Analysis are shown for both the unstimulated (a) and the EBV-infected conditions (b). Blue gene symbols represent genes with relatively lower expression in the cells with the risk haplotype, while genes with red shading are upregulated in the risk cells. IFIT3: IFN-induced protein with tetratricopeptide repeats 3, SOCS: suppressor of cytokine signaling.

a 1.5- to 1.8-fold increase in expression. However, when EBV was present, the risk haplotype showed a decrease of 1.6- to 1.9-fold of the same genes.

3.4. The Interferon Response Signature in Patients Depends on Haplotype.
Lupus patients have a heightened interferon response signature in the peripheral blood [50–52]. This signature is heritable and is associated with the *IRF5* risk haplotype [19, 26]. When we examined genes included in the interferon response signature, we found an interesting association with the *IRF5* risk haplotype. Cells from the SLE patients with the risk haplotype had an interferon response signature under all conditions, whether exposed to EBV or not. However, the cells from SLE patients with the protective haplotype did not exhibit an interferon signature without EBV infection. The difference in expression of the interferon response genes between the unstimulated patient risk and the unstimulated patient protective cells was statistically significant ($P = 0.011$) (Figure 4). The risk haplotype cells derived from control individuals did not have heightened baseline expression of interferon response genes. After exposure to EBV, these cells developed an interferon response signature that was similar to that seen in the baseline and EBV-infected risk-haplotype lupus patients.

Interestingly, the patients with the protective haplotype did not develop a strong interferon response signature even after exposure to EBV, indicating that the *IRF5* protective haplotype is dampening the response to interferon compared to the risk haplotype (Figure 4).

4. Discussion

The *IRF5* gene has been associated with risk for lupus. These findings demonstrate that the lupus-associated polymorphisms in the *IRF5* gene have wide-reaching effects on B-cell responses to infection. The gene sets that were enriched in the risk haplotypes included interferon-related sets, which is encouraging considering that the genotype being examined is *IRF5*. Multiple gene sets that are related to lupus were enriched in the cells with the risk haplotypes, including IFNα sets, interleukin- (IL-) 6, inflammation, proliferation, and monocyte and dendritic cell genes, in addition to the SLE-related interferon gene set. The finding that these gene sets are the most strongly enriched in the risk haplotype indicates that the *IRF5* risk haplotype has a strong influence on interferon signaling and inflammation, processes that are at the core of SLE. The finding that the most enriched gene sets were associated with interferon and lupus also indicates

TABLE 3: Genes exhibiting differential expression between risk and nonrisk cells in the canonical pathways identified through ingenuity pathway analysis. Fold up/down column is positive in the case that the gene expression is higher in the risk haplotype cells, and negative in the case that gene expression is higher in the nonrisk cells. PIK3CA: phosphoinositide-3-kinase, catalytic, alpha; RAC1: Ras-related C3 botulinum toxin substrate 1. *The *IFNB1* gene is found in both the interferon and Toll-like receptor pathways.

Gene pathway	Gene symbol	Mock infected 16 hours				EBV live virus infected 16 hours			
		Avg. nonrisk	Avg. risk	Ratio	Fold up/down	Avg. nonrisk	Avg. risk	Ratio	Fold up/down
Interferon	IFNB1*	104.95	3.89	0.04	−26.99	5.39	9.15	1.70	1.70
	STAT1	71.08	52.84	0.74	−1.35	184.29	244.12	1.32	1.32
	OAS1	56.04	84.55	1.51	1.51	49.87	107.78	2.16	2.16
	MX1	3179.14	5161.02	1.62	1.62	3409.35	6199.20	1.82	1.82
	IFNAR2	959.14	1637.11	1.71	1.71	1437.47	1094.97	0.76	−1.31
	IFITM1	439.88	944.41	2.15	2.15	2128.61	2602.68	1.22	1.22
Toll-like receptor	IFNB1*	104.95	3.89	0.04	−26.99	5.39	9.15	1.70	1.70
	FOS	116.48	53.84	0.46	−2.16	90.05	120.60	1.34	1.34
	MYD88	281.53	152.57	0.54	−1.85	181.06	226.62	1.25	1.25
	TNFAIP3	439.96	321.29	0.73	−1.37	728.35	377.23	0.52	−1.93
	TLR1	26.50	46.76	1.76	1.76	67.59	116.37	1.72	1.72
	LY96 (MD-2)	560.79	1274.32	2.27	2.27	1128.47	1208.54	1.07	1.07
	CD14	401.17	1272.53	3.17	3.17	139.53	450.09	3.23	3.23
B-Cell receptor	CD79B	2222.90	991.48	0.45	−2.24	797.14	827.44	1.04	1.04
	CD79A	41.32	19.08	0.46	−2.17	6526.62	5973.72	0.92	−1.09
	RAC1	1407.29	930.84	0.66	−1.51	4.36	2.64	0.61	−1.65
	MAPK9	105.87	71.02	0.67	−1.49	62.50	80.35	1.29	1.29
	AKT1	512.79	353.65	0.69	−1.45	13.02	6.74	0.52	−1.93
	NFKB2	6.44	4.85	0.75	−1.33	495.75	147.18	0.30	−3.37
	PIK3CA	25.86	40.05	1.55	1.55	79.96	49.71	0.62	−1.61
	NFAT5	123.74	222.63	1.80	1.80	5.14	2.70	0.52	−1.91
	GSK3B	68.58	123.65	1.80	1.80	177.23	91.07	0.51	−1.95

that these results are unlikely to be false positives obtained by chance, since the variable being studied is an interferon-affecting gene.

The gene set enrichment analysis techniques that were used are valuable because they identify not only individual genes, but also how strongly pathways that include those genes and the interactions between them are affected by the experimental conditions. This allows a much broader look into gene networks than looking only at individual genes. These studies point to the *IRF5* risk haplotype having a wide influence on interferon and inflammation. The results identify targets for future investigation into the function of the *IRF5* polymorphisms as well as other genetic influences on lupus.

As was the case with the gene set enrichment analysis, the identification of the interferon and Toll-like receptor pathways through Illumina pathway analysis suggests that the results are robust, as these are pathways that would be expected to be modulated by the underlying *IRF5* haplotype of the donor. Interferon alpha is an extremely important cytokine in lupus [53]. These studies suggest that the interferon alpha pathway is strongly affected by genetic

variation in the *IRF5* gene, and show multiple genes that could potentially be targets for understanding interferon in lupus or potential therapeutic targets. Toll-like receptors are involved in response to infection through the recognition of pathogen-associated molecular patterns. Additionally, Toll-like receptors are important in the pathogenesis of lupus. They are capable of recognizing endogenous nucleic acids in the context of immune complexes found in lupus patient sera, thereby stimulating dendritic cell maturation and interferon alpha production [25, 54–60], a process which also involves *IRF5* itself [25]. *IRF5* is a very interesting transcriptional regulator in that it acts as both an activator when homodimerized and blocks activation when heterodimerized with *IRF7* [61, 62]. This mechanism of action may help to explain how some of these pathways can exhibit relative upregulation or downregulation depending on the other conditions in the cell.

One of the more unexpected findings of this study was the modulation of the B-cell receptor pathway by the *IRF5* haplotype. The B-cell receptor is important in the recognition of antigen and the survival, maturation, and proliferation of B cells. B cells produce the autoantibodies
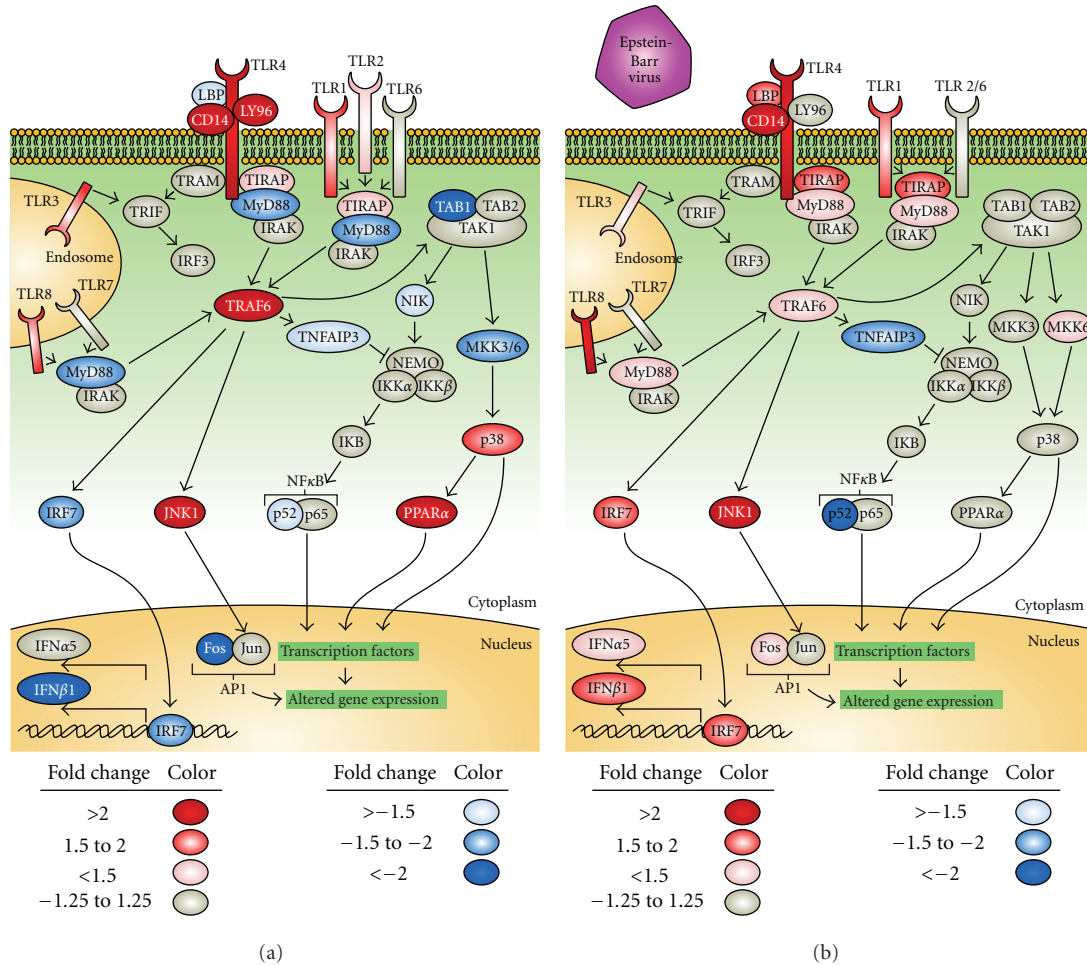
FIGURE 2: Association of the canonical Toll-like receptor pathway with *IRF5* haplotype. Differential regulation of the TLR pathway was seen using Ingenuity analysis ($P < 0.01$). Unstimulated conditions are shown on the left, and EBV-exposed conditions on the right. Genes with altered expression based on haplotype are shown as either red or blue. IKB: inhibitor of NFκB, IKK: IKB kinase, JNK: c-Jun N-terminal kinase, LBP: lipopolysaccharide-binding protein, MKK: mitogen activated protein kinase kinase, NIK: NFκB inducing kinase, PPARα: peroxisome proliferator-activated receptor α, TAB1: transforming growth factor β-activated kinase 1, TIRAP: Toll/IL-1 receptor domain containing adaptor protein, and TRAF6: tumor necrosis factor receptor-associated factor 6.

involved in lupus, as well as being important for antigen processing and presentation and T-cell activation. Differences in the activation threshold or other effects that may be seen with altered B-cell receptor gene expression may be very important to breaking self-tolerance or other aspects of B cell biology involved in SLE. Of particular interest in this respect is the recent finding that *IRF5* controls antibody class switching to IgG2A, allowing lupus-like autoimmunity in mice [63]. The B-cell receptor and Toll-like receptor pathways are involved in antibody class switching, and the genes that were modulated by *IRF5* variation in this study could represent mechanisms through which the *IRF5* risk haplotype may contribute to class switching or other similar variations in humans.

The interferon response signature has been identified as a common feature in lupus. These studies examined how polymorphisms in the *IRF5* gene affected the interferon response signature in both patients and controls. Interestingly, SLE patients with the risk haplotype demonstrated an interferon signature in both the infected and uninfected cells, while a strong interferon response was not found in the patients without the risk haplotype even when stimulated by EBV exposure. The controls with the risk haplotype lacked the interferon response signature in the basal state, but developed it after exposure to EBV, as would be expected. These findings suggest that the *IRF5* risk haplotype is integral for the interferon response signature in both patients and controls. They also indicate that other factors contribute to a basal interferon response in lupus patients, since the *IRF5* risk haplotype was not sufficient for the response signature to be present in the unstimulated control cells, as it was in the patients with the risk haplotype.

Cells were infected with EBV for two reasons. The first was to identify differences in gene expression patterns when

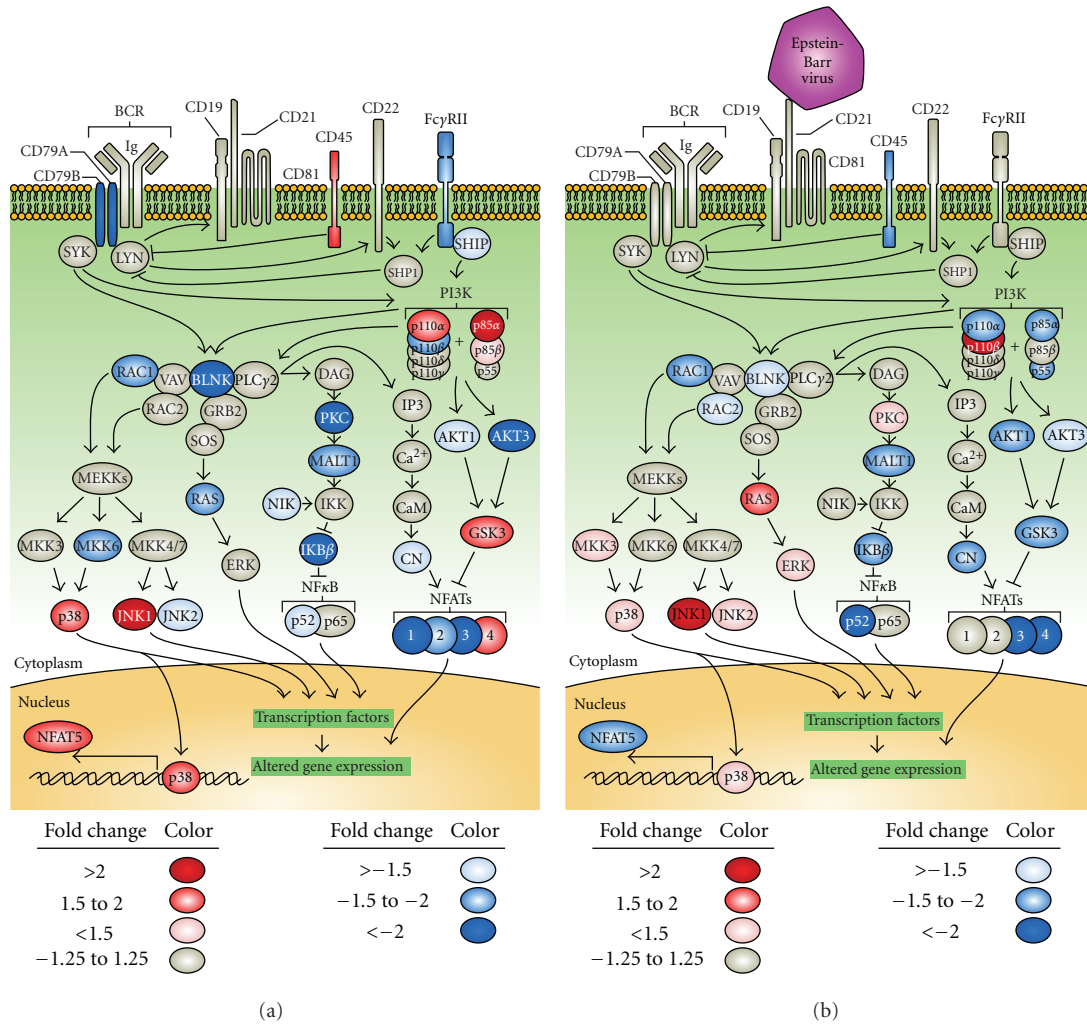(a)   (b)

FIGURE 3: Association of the canonical B-cell receptor pathway with *IRF5* haplotype. BCR pathway genes demonstrate a significant enrichment of changes in expression levels based on *IRF5* haplytype, as determined by Ingenuity analysis ($P < 0.01$). The unstimulated condition is shown on the left, and the EBV-exposed condition is shown on the right. Genes with altered expression based on haplotype are shown as either blue or red. BLNK: B cell linker, CAM: Calmodulin, CN: Calcineurin, ERK: extracellular signal-regulated kinase, FCGR2B: fragment crystallizable *γ* receptor 2B, INPP5D: inositol polyphosphate-5-phosphatase D (SHIP), MALT1: mucosa associated lymphoid tissue lymphoma translocation gene 1, and PKC: protein kinase C.

cells were stimulated with a biologically relevant trigger for interferon production. The second reason was to identify areas that may start to explain the differences in EBV infection and response in lupus. Gene expression was examined for genes in the three pathways found to be significant by ingenuity analysis. In several cases, (*IFITM1, IFNAR2, LY96, PIK3CA, NFAT5,* and *GSK3B*) the baseline level of gene expression was higher in the risk cells, but after EBV infection, the gene expression was comparatively increased in the protective cells. In other genes, including *CD79A, CD79B, STAT1, MyD88,* and *Fos,* expression was lower in the risk cells but the difference diminished or reversed after EBV infection. Expression of one gene, *TNFAIP3*, was lower in the risk than in the protective haplotype subject unstimulated cells and was comparatively diminished further after EBV infection. These differences suggest several areas of investigation to

understand differences in B cell biology in lupus and show that the *IRF5* haplotype affects multiple genes related to EBV infection and response.

Although a detailed analysis of each gene involved in these pathways is beyond the scope of this paper, the genes with expression differences between the risk and protective haplotypes are suggestive in several instances. One of the genes identified with promise to affect lupus is *TNFAIP3*. This gene is a transcription factor that is produced in response to inflammation. It has been shown to be critical to limiting inflammation by terminating NFκB responses [64].Variants have recently been associated with risk for lupus and other autoimmune and inflammatory diseases [65–71], and it is often suppressed in tumors, especially lymphomas [66, 72, 73]. Other promising genes identified by these experiments include *STAT4, IFITM1,* and *IFNAR2,*
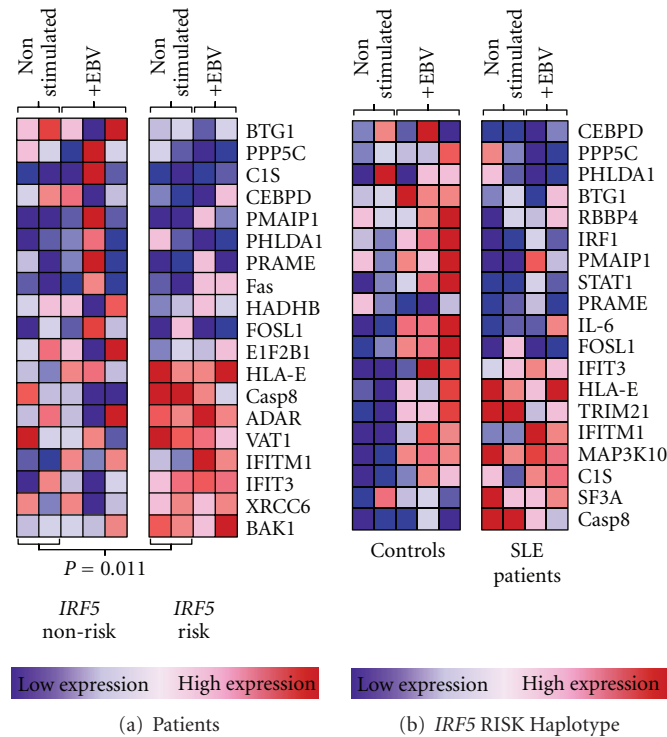
FIGURE 4: Dependence of the interferon response signature on *IRF5* haplotype in SLE patients. Interferon signature genes identified in gene set enrichment analysis were compared between patients and controls, and between lupus patients with the *IRF5* risk and nonrisk haplotypes. Each column represents expression of genes of interest in individual subjects. (a) Interferon signature genes of 3 *IRF5* nonrisk and 2 *IRF5* risk SLE patients. Gene expression comparisons are between *IRF5* risk and nonrisk SLE patients, either nonstimulated or EBV infected. (b) Gene set enrichment of 3 control *IRF5* risk haplotype individuals and 3 SLE patient *IRF5* risk haplotype individuals. Gene expression comparisons between *IRF5* risk patients and controls from nonstimulated and EBV-infected B cells. ADAR: adenosine deaminase, BAK: Bcl2-antagonist/killer, BTG: B cell translocation gene, C1S: complement component 1S, CASP: caspase, CEBPD: CCAAT/enhancer-binding protein δ, eIF2B: eukaryotic translation initiation factor 2B, FOSL: Fos-related antigen, HADHB: hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase β subunit, HLA: human leukocyte antigen, PHLDA: pleckstrin homology-like domain family A, PMAIP1: phorbol-12-myristate-13-acetate-induced protein 1 (Noxa), PRAME: melanoma antigen preferentially expressed in tumors, RBBP: retinoblastoma binding protein, SF3A: splicing factor 3A, TRIM: tripartite motif, VAT: vesicle amine transport, and XRCC: X-ray repair cross-complementing (Ku70).

which are all involved in the response to interferon, and several B cell signaling genes, including *NFAT5*, *GSK3B*, and *NFκB2*.

Although EBV was used in part to simulate an infected state in B cells, EBV itself could be involved in the etiology of lupus by affecting several pathways. The three pathways identified here are all involved in EBV infection. EBV may stimulate these pathways through several mechanisms, including both infection and binding of virions to the receptors involved in these pathways. Although the effect of EBV infection on differential gene expression was somewhat variable, for many of the genes examined in this study there was overexpression in the risk cells, which subsequently diminished after EBV infection. This pattern, as well as that seen with the interferon response signature, suggests that the *IRF5* risk haplotype makes these cells appear more activated in the resting state. Because of this heightened activation state, there is less difference in the response to EBV infection in the risk cells, with the nonrisk cells often catching up

to or passing the risk cells in expression of several genes following viral infection. An activated basal state would be likely to promote inappropriate cellular responses and possibly heightened sensitivity to self-antigens, including those recognized by TLRs.

These findings identify several key pathways that are affected by the *IRF5* risk haplotype and are involved in the B cell response to antigen stimulation and viral infection. Many of the genes involved in these pathways have definite potential to alter the response to EBV infection and affect the development of lupus. These merit further investigation. Since all of these pathways are likely to be involved in the development of lupus, further comparison of these pathways in other cell types such as plasmacytoid dendritic cells will be beneficial to understanding the origins and pathogenesis of lupus. It will also be beneficial to examine more closely the role of EBV in regulating expression of these genes, through the use of EBV mutants, and to dissect the role of *IRF5* in each pathway and gene set identified.

Acknowledgments

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