Cutting Edge

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Cutting Edge: Autoimmune Disease Risk Variant of STAT4 Confers Increased Sensitivity to IFN-α in Lupus Patients In Vivo[1]

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Increased IFN-α signaling is a primary pathogenic factor in systemic lupus erythematosus (SLE). STAT4 is a transcription factor that is activated by IFN-α signaling, and genetic variation of STAT4 has been associated with risk of SLE and rheumatoid arthritis. We measured serum IFN-α activity and simultaneous IFN-α-induced gene expression in PBMC in a large SLE cohort. The risk variant of *STAT4* (T allele; rs7574865) was simultaneously associated with both lower serum IFN-α activity and greater IFN-α-induced gene expression in PBMC in SLE patients in vivo. Regression analyses confirmed that the risk allele of *STAT4* was associated with increased sensitivity to IFN-α signaling. The IFN regulatory factor 5 SLE risk genotype was associated with higher serum IFN-α activity; however, *STAT4* showed dominant influence on the sensitivity of PBMC to serum IFN-α. These data provide biologic relevance for the risk variant of STAT4 in the IFN-α pathway in vivo. ***The Journal of Immunology,*** **2009, 182: 34–38.**

The pathogenesis of systemic lupus erythematosus (SLE)[3] is driven by a combination of genetic risk factors and environmental events that lead to an irreversible break in immunologic self-tolerance. IFN-α is a pleiotropic type I IFN with the potential to break self-tolerance by activating APCs after uptake of self-material (1). Serum IFN-α levels are frequently elevated in SLE patients (2). Additionally, a number of patients treated with recombinant human IFN-α for malignancy and chronic viral hepatitis have developed de novo SLE, which typically resolves after the IFN-α is discontinued (3). These data suggest a potential role for IFN-α in SLE susceptibility.

In previous work, we have demonstrated that abnormally high serum IFN-α activity is a common heritable trait within SLE families in both healthy and SLE-affected members (4). The serum IFN-α trait showed wide variance in SLE patients, and 40–50% of patients did not have significantly high serum IFN-α activity as compared with healthy donors (4). Microarray gene expression studies of SLE patient PBMC suggest that up to 90% of SLE patients demonstrate overexpression of IFN-α-induced transcripts (5). The contrast between these two data sets suggests that some mediators downstream of IFN-α binding to the receptor could play a role in the frequent up-regulation of IFN-α-induced transcripts observed in SLE patient PBMC. It is possible that increased sensitivity to IFN-α in some SLE patients may result in increased IFN-α-induced gene expression in PBMC in the absence of abnormally high serum IFN-α activity.

The rs7574865 single nucleotide polymorphism (SNP) in the *STAT4* gene has been associated with risk of SLE in individuals of European ancestry (6), and the same SNP has been associated with risk of rheumatoid arthritis in individuals of European and Asian ancestry (6, 7). STAT4 is a transcription factor that binds to the type I IFN receptor and is activated by phosphorylation following receptor ligation (8). We hypothesized that risk variants of STAT4 may influence SLE susceptibility by augmenting downstream IFN-α-induced signaling. In this study, we examine simultaneous measurements of both serum IFN-α activity and downstream IFN-α-induced gene expression in SLE patient PBMC to determine the influence of *STAT4* genotype in vivo.

Genetic variants of IFN regulatory factor (IRF) 5 have been associated with the risk of SLE in large scale genetic association studies in both European and African-American ancestral backgrounds (9, 10). IRF5 is a transcription factor that can induce transcription of both IFN-α and IFN-α-induced genes (11). In recent work, we have shown that the *IRF5* SLE risk haplotype

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; IFIT, IFN-induced protein with tetratricopeptide; IRF, IFN regulatory factor; MX, myxovirus resistance; PKR, dsRNA-activated protein kinase; RBP, RNA-binding protein; SNP, single nucleotide polymorphism.

was associated with increased serum IFN-α activity in SLE patients (12), suggesting that IRF5 may exert its influence on disease susceptibility via increased IFN-α production. IRF5 can also induce transcription of IFN-α-induced genes however, and in this study we examine the influence of *IRF5* genotype in combination with *STAT4* genotype on both serum IFN-α activity and downstream IFN-α-induced gene transcription.

Materials and Methods

Patients and samples

Serum and genomic DNA samples from 270 SLE patients (143 of African American ancestry, 101 of European ancestry, and 26 of Asian ancestry) were obtained from the Translational Research Initiative at the Department of Medicine at University of Chicago, the Hospital for Special Surgery (HSS) Lupus Family Registry, and the HSS Lupus Registry. Seventy-four of these patients had PBMC samples available from the time of blood draw. One hundred forty-one healthy donor serum samples were used to standardize the IFN-α assay, as previously described (4). The study was approved by the institutional review boards at both institutions, and informed consent was obtained from all subjects in the study.

Reporter cell assay for IFN-α

The reporter cell assay for IFN-α has been described in detail elsewhere (4, 13). In this assay, reporter cells were used to measure the ability of patient sera to cause IFN-induced gene expression. The reporter cells (WISH cells, no. CCL-25; American Type Culture Collection) were cultured with 50% patient sera for 6 h and then lysed. cDNA was made from total cellular mRNA and cDNA was then quantified using real-time PCR. Forward and reverse primers for the genes MX-1 (myxovirus resistance 1), PKR (dsRNA-activated protein kinase), and IFIT-1 (IFN-induced protein with tetratricopeptide 1), which are known to be highly and specifically induced by IFN-α, were used in the reaction (4).

PBMC processing and transcript analysis

PBMC were obtained from SLE patients and processed immediately following phlebotomy. Cells were lysed, and cDNA was made from total cellular RNA. cDNA transcripts were then quantified using real-time PCR with the same primers for IFN-α-induced genes used above (4). See Ref 2 for further details regarding PBMC transcript analysis.

Real-time PCR data analysis

PCR data from both WISH cells and PBMC were analyzed in the same way. The relative expression of each of the three tested IFN-induced genes was calculated as a fold increase compared with its expression in either WISH cells cultured with medium alone or compared with healthy donor PBMC expression levels, respectively. The PCR data were log-normally distributed, and we performed $\log_{10}$ transformation to normalize the relative expression data. We also used an algorithm to generate a single IFN-α score from the three genes tested in the WISH cells and PBMC. Healthy unrelated donor sera ($n = 141$) were tested in the WISH assay, and PBMC from 28 healthy donors were assayed for IFN-α-induced gene expression. The mean and SD of the log-transformed IFN-α-induced gene relative expression in healthy individuals was calculated for both WISH assay and PBMC gene expression. The number of SD above the mean of healthy donors was calculated for each gene tested in patient samples. The number of SD above healthy donors was then summed for the three genes, and this number was reported as the score used to measure IFN-α-induced gene expression (4).

Genotyping

Subjects were genotyped at the rs7574865 SNP in *STAT4* and the rs2004640, rs3807306, rs10488631, and rs2280714 SNPs in *IRF5* using TaqMan Assays-by-Design primers and probes (Applied Biosystems). SNP genotyping was performed with >99% success among registry samples. Observed SNP genotypes did not deviate from Hardy-Weinberg proportions ($p > 0.5$ for all SNPs; supplemental Table I).[4] Haplotype structure of IRF5 in European ancestry SLE subjects is shown in supplemental Fig. 1.

Statistical analysis

χ^2 distribution was used to analyze categorical data, and unpaired *t* test or nonparametric Mann-Whitney U was used to compare quantitative IFN-α data between two genotype subgroups as appropriate given the distribution of the data. Analysis of quantitative data across multiple genotype subgroups that demonstrated a consistent dose-response trend was done using a linear model, with equal weighting of genotype categories on the *x*-axis and log-transformed PCR data on the *y*-axis. Genotype categories were arrayed on the *x*-axis by increasing SLE risk designation, and *p* values were calculated as the probability that a horizontal line (null hypothesis) was a better fit of the data than a straight line with a nonzero slope between genotype categories using the extra sum-of-squares F test. Linear regression was used to analyze simultaneous serum IFN-α activity and PBMC IFN-α-induced gene activity data, and *p* values were calculated using the extra sum-of-squares F test for a difference in slope or *y*-intercept of the best fit line.

[4] The online version of this article contains supplemental material.

Results and Discussion

STAT4 SLE risk variant is associated with lower serum IFN-α in SLE patients in vivo

All 270 SLE patients were genotyped at *STAT4* (rs7574865), and allele frequencies are shown in supplemental Table I. *STAT4* allele frequencies differed significantly by ancestry (supplemental Table I), consistent with available HapMap allele frequency data (www.ncbi.nlm.nih.gov/SNP/). When all subjects were analyzed together, those with the homozygous nonrisk GG genotype had higher serum IFN-α than those with the GT genotype ($p = 0.016$), and the small group of patients with the TT genotype were not significantly different (supplemental Fig. 2).

The T allele of *STAT4* is associated with anti-dsDNA Abs in European ancestry individuals (14), and in our multiethnic cohort we saw this association as well ($\chi^2 = 5.8$, $p = 0.016$ in European ancestry; $\chi^2 = 9.8$, $p = 0.0022$ in all ancestries pooled). In previous work, we have shown that SLE patients with either anti-dsDNA Abs or anti-RNA-binding protein (RBP) Abs had higher IFN-α than those lacking these Abs (4). Patients with both anti-dsDNA and anti-RBP Abs had higher serum IFN-α than those with only one of these two types of autoantibodies (4). When SLE patients were stratified by anti-dsDNA and anti-RBP Abs, the relationship between *STAT4* genotype and serum IFN-α was refined (Fig. 1). A dose-response relationship between the T allele and lower serum IFN-α was observed in patients with either anti-RBP Abs or anti-dsDNA Abs as well as patients who were positive for both types of autoantibodies. SLE patients lacking both anti-RBP and anti-dsDNA Abs had low serum IFN-α activity in general and showed a nonsignificant trend toward lower serum IFN-α in risk allele carriers. Each IFN-α-induced gene tested in the reporter cell assay showed the same pattern when analyzed by *STAT4* genotype and autoantibody status (separate plots for IFIT-1, MX-1, and PKR are shown in supplemental Fig. 3). When patients are separated by ancestral groups, the same trends were observed (supplemental Fig. 4).

STAT4 SLE-risk allele is associated with increased sensitivity to IFN-α signaling

When IFN-α-induced transcripts were quantified in PBMC from 74 of the SLE patients, subjects with the SLE-risk TT genotype of *STAT4* had increased IFN-α-induced gene expression compared with the GG genotype (supplemental Fig. 5, *A–C*). This was striking, as the PBMC were isolated from the same blood sample which would be expected to have lower serum IFN-α. A similar trend was seen in each ancestral background analyzed separately (Supplemental Fig. 5*D*).

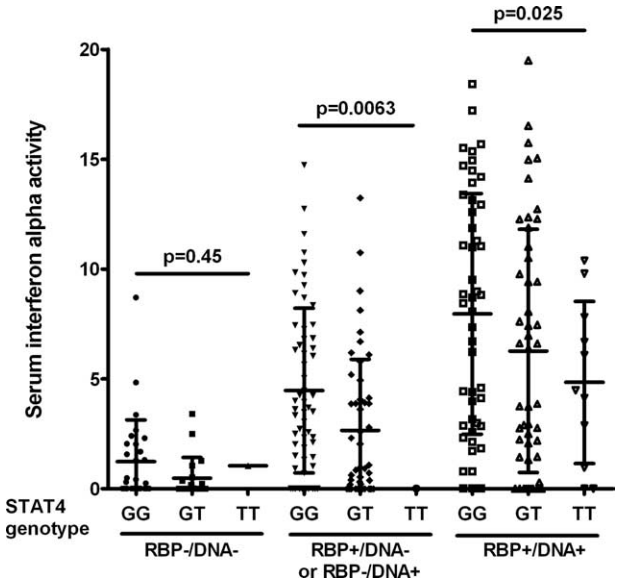

FIGURE 1. Serum IFN-α activity in SLE patients in relation to STAT4 genotype. SLE patients are stratified by *STAT4* rs7574865 genotype and presence or absence of anti-RBP or dsDNA autoantibodies, with *p* values calculated using the extra sum-of-squares F test. Serum IFN-α activity is measured as a summed score of IFN-α-induced gene expression in the reporter cells (see *Materials and Methods*). RBP−/DNA− indicates subjects lacking both anti-RBP and anti-dsDNA Abs; RBP+ or DNA+ indicates subjects with either anti-RBP or anti-dsDNA Abs; and RBP+/DNA+ indicates subjects with both anti-RBP and anti-dsDNA Abs. Lines show the mean with SD, *p* values were corrected for multiple comparisons using a Bonferroni correction.

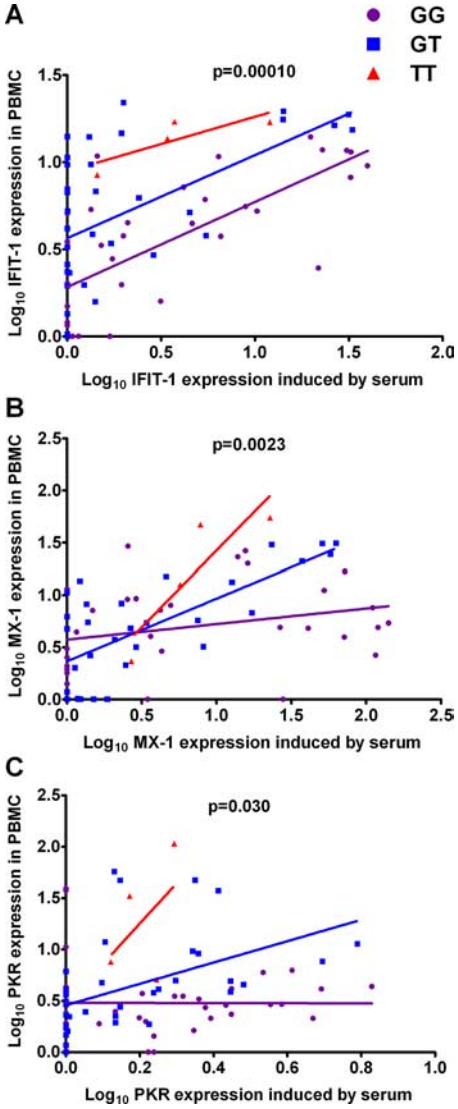

FIGURE 2. IFN-α-induced gene expression in PBMC vs serum IFN-α activity in SLE patients stratified by *STAT4* genotype. Multiple linear regression plots showing PBMC IFN-α-induced gene expression vs induction of the same gene by serum in reporter cells in simultaneous samples. All patients are stratified by *STAT4* genotype and the best-fit lines and slopes for each genotype as determined by linear regression, with a *p* value by extra sum-of-squares F test for a difference in either *y*-intercept (IFIT-1) or slope (MX-1 and PKR).

We next analyzed IFN-α-induced gene transcription in PBMC as compared with the ability of sera from the same sample to induce transcription of the gene in the reporter cells. This analysis allows simultaneous comparison of functional IFN-α activity in the sera and downstream IFN-α gene expression in PBMC in vivo. For each of the three tested IFN-α-induced genes, the relationship between functional IFN-α activity in serum vs the actual amount of IFN-α-induced gene transcription observed in PBMC was significantly different by *STAT4* genotype (Fig. 2). STAT4 risk allele carriers showed greater IFIT-1 expression at all levels of serum IFN-α activity. *STAT4* risk allele carriers also had greater induction of MX-1 and PKR transcription with increasing serum IFN-α activity. A number of patients with the SLE risk genotypes of *STAT4* had high IFN-α-induced gene expression in PBMC despite having very little serum IFN-α activity in simultaneous samples (Fig. 2).

STAT4 exerts a dominant influence over IRF5 upon sensitivity to IFN-α

We have previously shown that the SLE risk variant of *IRF5* was associated with higher serum IFN-α activity in SLE patients in vivo and that this association was limited to those patients with either anti-RBP or anti-dsDNA autoantibodies (12). When serum IFN-α activity was analyzed in SLE patients stratified by both *STAT4* and *IRF5* genotypes, no influence of IRF5 on serum IFN-α was observed in patients with the protective GG genotype of *STAT4*. In contrast, patients carrying the SLE risk T allele of *STAT4* showed a differential pattern of serum IFN-α by *IRF5* genotype, with the *IRF5* SLE risk allele carriers having increased serum IFN-α as compared with nonrisk allele carriers ($p = 0.0002$ and $p = 0.013$ in African American and European ancestry, respectively; supplemental Fig. 6). The rs3807306 SNP in *IRF5* is a shared SLE susceptibility locus in both African-American and European ancestral groups (9, 10), and the SLE-risk variant of this SNP is associated with differential serum IFN-α activity in both ancestral backgrounds (Ref. 12 and T. B. Niewold, J. B. Harley, M. K. Crow, unpublished data). When simultaneous serum IFN-α activity and downstream IFN-α-induced gene expression data were analyzed with multiple linear regression, *STAT4* genotype showed a dominant effect over the *IRF5* risk variant rs3807306 (Fig. 3).

In this study, we show that the SLE risk variant of *STAT4* is associated with increased sensitivity to IFN-α in SLE patients in vivo, providing a biologic relevance for *STAT4* variants within the IFN-α pathway. Higher serum IFN-α in subjects with the nonrisk GG genotype of *STAT4* may indicate that *STAT4* genotype influences the threshold required for IFN-α to result in

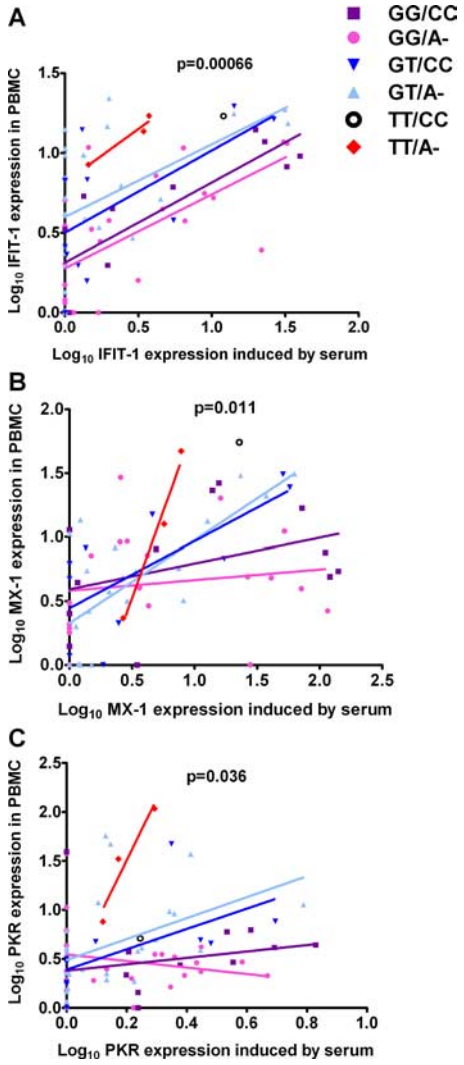

FIGURE 3. Serum IFN-α activity and serum vs PBMC data in SLE patients stratified by both *STAT4* and *IRF5* genotype and autoantibody status. Multiple linear regressions showing PBMC IFN-α-induced gene expression vs induction of the same gene by serum in reporter cells in simultaneous samples. *p* value by extra sum-of-squares F test for a difference in either *y*-intercept (IFIT-1) or slope (MX-1 and PKR).

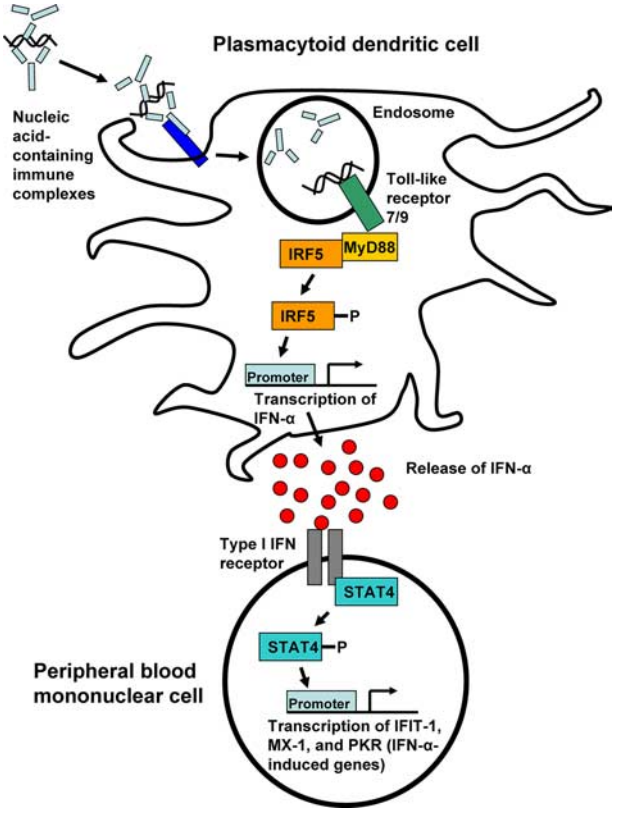

FIGURE 4. Proposed model of IFN-α signaling in SLE including autoantibodies, *IRF5*, and *STAT4*. Anti-RBP and anti-dsDNA Abs form immune complexes containing nucleic acid, which can be taken into plasmacytoid dendritic cells via Fc receptors and result in ligation of endosomal TLRs. Downstream TLR signaling is augmented by *IRF5* SLE risk variants, resulting in increased IFN-α production. IFN-α is released, and *STAT4* SLE risk variants result in increased sensitivity to IFN-α downstream of the type I IFN receptor.

SLE susceptibility. If the risk of SLE due to IFN-α is dose dependent, then subjects with the nonrisk genotype may require higher levels of serum IFN-α to develop clinical SLE, and those SLE patients with nonrisk *STAT4* genotypes would have higher serum IFN-α activity when compared with the general SLE cohort. Alternatively, the nonrisk genotype of *STAT4* may result in impaired feedback regulation of IFN-α, and decreased sensitivity to IFN-α could result in overproduction.

We saw the associations with serum IFN-α activity and *STAT4* genotype only in those patients who had one or more of the anti-RBP or anti-dsDNA Abs. A number of investigators have suggested that the nucleic acid component of anti-RBP and anti-dsDNA immune complexes trigger IFN-α production via ligation of endosomal TLRs 7 and 9, respectively reviewed in Ref. 15). Thus, autoantibodies are expected to increase IFN-α production, and in SLE cohorts these autoantibodies are strongly associated with high serum IFN-α activity (4). Similar to previous studies (14), we see an association between STAT4 genotype and anti-dsDNA Abs. This association could be due to synergy between increased IFN-α production due to anti-dsDNA Abs in the microenvironment coupled with increased sensitivity to IFN-α due to *STAT4* risk genotype. We saw a trend toward association of the *STAT4* SLE risk allele with anti-RBP Abs in our study ($p = 0.16$). Previous studies do not report data regarding anti-RBP Abs and *STAT4*; however, a potential association between the two could represent a similar synergy between IFN-α production and sensitivity.

A recent study reported an additive interaction between the risk alleles of *IRF5* and *STAT4* in SLE susceptibility (14). In our study, we see that *IRF5* risk genotypes are associated with higher serum IFN-α in subjects with *STAT4* SLE-risk genotypes, and no effect of IRF5 genotype is noted in the *STAT4* nonrisk genotype subjects. These effects on serum IFN-α are observed in subjects with either anti-RBP or anti-dsDNA Abs, suggesting that autoantibodies as well as genotypes at these two loci cooperate to result in dysregulation of IFN-α in vivo and subsequent risk of SLE. Our data suggest that *IRF5* and *STAT4* impact the IFN α pathway at different points in human disease. The *IRF5* genotype plays a role in modulating IFN-α production in lupus patients, while *STAT4* genotype appears to be more important in regulating sensitivity to IFN-α. A potential model of *IRF5*, *STAT4*, and autoantibodies in SLE pathogenesis is shown in Fig. 4. Our data support a central role for dysregulation of the IFN-α pathway in SLE susceptibility and may inform both diagnostics and therapeutics targeting the IFN-α pathway in SLE.

Disclosures

K. A. Kirou and M. K. Crow have a patent pending for the serum IFN-α assay used in this work. The other authors have no financial conflicts of interest.

References

1. Blanco, P., A. K. Palucka, M. Gill, V. Pascual, and J. Banchereau. 2001. Induction of dendritic cell differentiation by IFN-α in systemic lupus erythematosus. *Science* 294: 1540–1543.
2. Kirou, K. A., C. Lee, S. George, K. Louca, I. G. Papagiannis, M. G. Peterson, N. Ly, R. N. Woodward, K. E. Fry, A. Y. Lau, et al. 2004. Coordinate overexpression of interferon-α-induced genes in systemic lupus erythematosus. *Arthritis Rheum.* 50: 3958–3967.
3. Niewold, T. B., and W. I. Swedler. 2005. Systemic lupus erythematosus arising during interferon-α therapy for cryoglobulinemic vasculitis associated with hepatitis C. *Clin. Rheumatol.* 24: 178–181.
4. Niewold, T. B., J. Hua, T. J. Lehman, J. B. Harley, and M. K. Crow. 2007. High serum IFN-α activity is a heritable risk factor for systemic lupus erythematosus. *Genes Immun.* 8: 492–502.
5. Bennett, L., A. K. Palucka, E. Arce, V. Cantrell, J. Borvak, J. Banchereau, and V. Pascual. 2003. Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. *J. Exp. Med.* 197: 711–723.
6. Remmers, E. F., R. M. Plenge, A. T. Lee, R. R. Graham, G. Hom, T. W. Behrens, P. I. de Bakker, J. M. Le, H. S. Lee, F. Batliwalla, et al. 2007. STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. *N. Engl. J. Med.* 357: 977–986.
7. Lee, H. S., E. F. Remmers, J. M. Le, D. L. Kastner, S. C. Bae, and P. K. Gregersen. 2007. Association of STAT4 with rheumatoid arthritis in the Korean population. *Mol. Med.* 13: 455–460.
8. Tyler, D. R., M. E. Persky, L. A. Matthews, S. Chan, and J. D. Farrar. 2007. Preassembly of STAT4 with the human IFN-α/β receptor-2 subunit is mediated by the STAT4 N-domain. *Mol. Immunol.* 44: 1864–1872.
9. Graham, R. R., C. Kyogoku, S. Sigurdsson, I. A. Vlasova, L. R. Davies, E. C. Baechler, R. M. Plenge, T. Koeuth, W. A. Ortmann, G. Hom, et al. 2007. Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. *Proc. Natl. Acad. Sci. USA* 104: 6758–6763.
10. Kelly, J. A., J. M. Kelley, K. M. Kaufman, J. Kilpatrick, G. R. Bruner, J. T. Merrill, J. A. James, S. G. Frank, E. Reams, E. E. Brown, et al. 2008. Interferon regulatory factor-5 is genetically associated with systemic lupus erythematosus in African Americans. *Genes Immun.* 9: 187–194.
11. Barnes, B. J., P. A. Moore, and P. M. Pitha. 2001. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon α genes. *J. Biol. Chem.* 276: 23382–23390.
12. Niewold, T. B., J. A. Kelly, M. H. Flesch, L. R. Espinoza, J. B. Harley, and M. K. Crow. 2008. Association of the IRF5 risk haplotype with high serum interferon-α activity in systemic lupus erythematosus patients. *Arthritis Rheum.* 58: 2481–2487.
13. Niewold, T. B., T. L. Rivera, J. P. Buyon, and M. K. Crow. 2008. Serum type I interferon activity is dependent on maternal diagnosis in anti-SSA/Ro positive mothers of neonatal lupus patients. *Arthritis Rheum.* 58: 541–546.
14. Sigurdsson, S., G. Nordmark, S. Garnier, E. Grundberg, T. Kwan, O. Nilsson, M. L. Eloranta, I. Gunnarsson, E. Svenungsson, G. Sturfelt, et al. 2008. A common STAT4 risk haplotype for systemic lupus erythematosus is over-expressed, correlates with anti-dsDNA production and shows additive effects with two IRF5 risk alleles. *Hum. Mol. Genet.* 17: 2868–2876.
15. Ronnblom, L., M. L. Eloranta, and G. V. Alm. 2006. The type I interferon system in systemic lupus erythematosus. *Arthritis Rheum.* 54: 408–420.