

# A loss-of-function variant of the antiviral molecule MAVS is associated with a subset of systemic lupus patients

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Dysregulation of the antiviral immune response may contribute to autoimmune diseases. Here, we hypothesized that altered expression or function of MAVS, a key molecule downstream of the viral sensors RIG-I and MDA-5, may impair antiviral cell signalling and thereby influence the risk for systemic lupus erythematosus (SLE), the prototype autoimmune disease. We used molecular techniques to screen non-synonymous single nucleotide polymorphisms (SNPs) in the MAVS gene for functional significance in human cell lines and identified one critical loss-of-function variant (C79F, rs11905552). This SNP substantially reduced expression of type I interferon (IFN) and other proinflammatory mediators and was found almost exclusively in the African-American population. Importantly, in African-American SLE patients, the C79F allele was associated with low type I IFN production and absence of anti-RNA-binding protein autoantibodies. These serologic associations were not related to a distinct, functionally neutral, MAVS SNP Q198K. Hence, this is the first demonstration that an uncommon genetic variant in the MAVS gene has a functional impact upon the anti-viral IFN pathway *in vivo* in humans and is associated with a novel sub-phenotype in SLE. This study demonstrates the utility of functional data in selecting rare variants for genetic association studies, allowing for fewer comparisons requiring statistical correction and for alternate lines of evidence implicating the particular variant in disease.

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex disease with a multi-factorial etiology. An important role for a genetic

contribution to lupus susceptibility in humans is supported by the observation that SLE demonstrates strong familial clustering and occurs more frequently in monozygotic twins than in dizygotic twins (Crow, 2009; Sebastiani & Galeazzi, 2009). Large-scale genetic association studies have been successful, identifying a number of single nucleotide polymorphisms (SNPs) associated with a diagnosis of SLE (Graham et al, 2009; Harley et al, 2008). These case-control associations are typical of complex human disease, with odds ratios <1.7 for the majority of the loci discovered to date. In fact, much of the heritability of SLE remains to be explained, and it is likely that rare and uncommon variants will contribute to some of this unexplained heritability as these variants may not be captured well in large-scale case-control studies. Alternate approaches for gene discovery will be important to facilitate future genetic discoveries in SLE.

A significant pathogenic role for type I interferon (IFN) in lupus pathogenesis was initially suggested in the late 1970's

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**Abbreviations:** EBV, Epstein-Barr virus; IAV, influenza A virus; RBP, RNA-binding protein; SeV, Sendai virus; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism; TLR, toll-like receptor; ACR, American College of Rheumatology.

based on demonstrations of increased IFN activity in the blood of lupus patients with active disease (Hooks et al, 1979). Those observations have been supported in recent years by microarray studies showing elevated expression of a broad spectrum of IFN-inducible genes in peripheral blood cells of many lupus patients (the 'IFN signature'). Immunologic studies to determine the cause of increased production of IFN in SLE have focused on the capacity of nucleic acid-containing immune complexes to access intracellular toll-like receptors (TLRs) and initiate a signalling cascade resulting in transcription of IFN. The association of autoantibodies targeting RNA-associated proteins, such as anti-Ro and anti-Sm, with high expression of IFN-inducible genes has directed attention to TLR7 and its downstream signalling components, including the transcription factor IFN regulatory factor 5 (IRF5). Polymorphisms in IRF5 and its partner IRF7 are among those recently shown to be associated with a diagnosis of SLE, particularly in those patients with serum autoantibodies specific for RNA-binding proteins or DNA.

In spite of the well-documented studies concerning high levels of IFN- $\alpha$  in lupus patients with nucleic acid-targeted autoantibodies, there are SLE patients who have low or normal levels of IFN activity or IFN-inducible gene expression. Those IFN low patients account for as many as 50% of patients in some cross-sectional studies, although the number might be lower if patients are followed longitudinally (Baechler et al, 2003; Crow et al, 2003; Kirou et al, 2004). It is not known if those individuals reflect a distinct pathogenic mechanism or clinical phenotype. Thus, it would be of great interest to understand whether alternative genetic susceptibility factors, signalling pathways and immunologic mediators are associated with those patients who have *bona fide* SLE, yet are IFN low.

Viruses may stimulate some of the early events in SLE pathogenesis (Ramos-Casals et al, 2008), and Epstein-Barr virus (EBV) is the most extensively studied trigger (Poole et al, 2006; Posnett, 2008). In that regard, it is of note that EBV-encoded small RNAs induce type I IFN *via* the viral sensor RIG-I, a component of the TLR-independent innate immune pathway (Samanta et al, 2006). Another recent study showed that MAVS, an adaptor transducing RIG-I signalling, interacts with 'Eyes absent 4' (EYA4) protein to induce type I IFN expression in response to self-DNA (Okabe et al, 2009), a candidate inducer of SLE (Deane & Bolland, 2006). Moreover, in a mouse model of lupus, transient exposure to a RIG-I ligand aggravates lupus nephritis *via* IFN signalling (Allam et al, 2008). Taken together, these data draw attention to a potential role for a TLR-independent, RIG-I/MAVS-dependent pathway in type I IFN production and altered immune system regulation in lupus pathogenesis.

Consequently, this prompted us to search for functional SNPs of human MAVS to further investigate the role of this antiviral molecule in SLE. We used molecular techniques to screen non-synonymous SNPs in the MAVS gene for functional significance in human cell lines. We identified one critical loss-of-function MAVS variant (C79F) among eight described in the NCBI database, which was found mostly in African-derived chromosomes. Most significantly, we revealed that the C79F allele is associated with SLE in African-American lupus patients defined by an absence of autoantibodies specific for RNA-binding

proteins (anti-RBP). Moreover, a functional role for the C79F allele in humans *in vivo* is suggested by an association with lower serum type I IFN activity in SLE patients, which mirrors our *in vitro* data. This study supports the utility of a 'function first, genetic epidemiology second' approach for studying candidate genes in which some knowledge of the candidate's pathway function is already available. This is particularly important for rare variants, as standard unbiased techniques result in statistical correction for hundreds or thousands of comparisons, frequently abolishing the potential for rare variants to demonstrate independently significant associations.

## RESULTS

### Genetic variation in human MAVS.

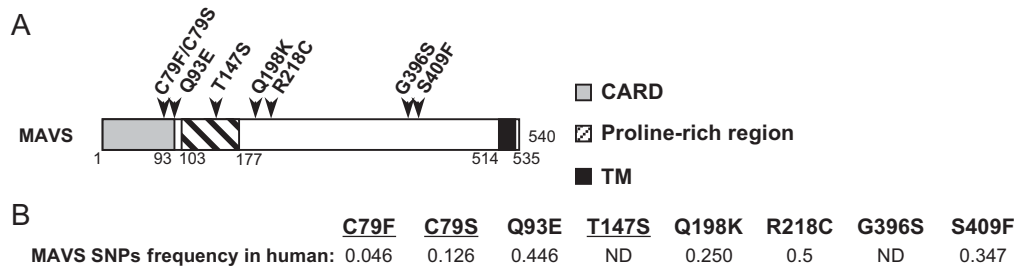
Information collected in January 2009 from the NCBI SNP database indicated that at least 301 SNPs are present in the human MAVS gene (mRNA NM\_020746). Among them, 12 are situated within coding sequences but only 8 result in amino acid substitutions, *i.e.* C79F, C79S, Q93E, T147S, Q198K, R218C, G396S and S409F (Fig 1A and Table S1).

Sequence alignments showed that only three SNPs affect amino acids that are conserved in all analysed species (underlined on Fig 1B and alignment provided in Supplementary Fig S1), *i.e.* C79F, C79S and T147S, whereas R218 and G396 residues were conserved from horse to human only. Notably Q93E, Q198K, R218C and S409F are the four most frequent SNPs in human MAVS, being present in 25.0–50% of the population according to the NCBI SNP database (Fig 1B and Supplementary Fig S1).

Although C79F and C79S alter a conserved residue, both are relatively frequent in humans. The C79F SNP was present in 4.6% of the HapMap samples genotyped, with the major frequency of the C79F allele in sub-Saharan Africans (27.6%), in comparison with European (1.7%) and Asian (0%) ethnic groups. Information collected from the NCBI SNP database revealed that the C79S allele was detected in African-Americans (9.4%) but not in European-Americans. All genotyped individuals available in this database were heterozygous for C79F and either homozygous or heterozygous for C79S.

### C79F SNP uniquely impairs MAVS-mediated innate immune signalling.

Plasmids encoding individually the eight variants were generated by site-directed polymerase chain reaction (PCR) mutagenesis using a WT MAVS (accession number NM\_020746) vector as a template. We first studied the impact of C79F, C79S, Q93E, T147S, Q198K, R218C, G396S and S409F mutations on the expression pattern of MAVS protein (SNP nucleotide positions are reported on Table S1). To avoid a non-specific cell-type effect, WT and SNPs MAVS were expressed in two different human cell lines, *i.e.* the bronchial epithelial cell line BEAS-2B and the embryonic kidney cell line HEK 293T. The advantage of such an approach is that MAVS expression is studied in stable and homogeneous cellular environments, thus excluding any additional genetic or epigenetic effect that can be observed by using human primary cells.



**Figure 1. SNPs reported in the human MAVS gene.**

- A.** Schematic representation of major domains of MAVS (signalling CARD in grey, proline-rich region with stripes and the transmembrane domain in black; adapted from references (Hiscott et al, 2006; Potter et al, 2008) with the position of non-synonymous SNPs, *i.e.* C79F (rs11905552), C79S (rs11908032), Q93E (rs17857295), T147S (rs45563035), Q198K (rs7262903), R218C (rs45437096), G396S (rs4552913), S409F (rs7269320).
- B.** Frequency according to NCBI SNP database of SNPs in human populations [which includes the four populations initially studied in the HapMap project: European (Northern and Western European, HapMap-CEU), Asian (Han Chinese in Beijing, HapMap-HCB and Japanese in Tokyo, HapMap-JPT), and Sub-Saharan African (Yoruba in Ibadan, Nigeria, HapMap-YRI); see also <http://www.hapmap.org> for additional information]. SNP affecting amino acid residues conserved in all mammals studied from mouse to human are underlined (see Supplementary Fig S1 for ClustalW alignment; N.D.: not determined).

Western-blotting and flow cytometry analyses revealed that none of the SNPs altered MAVS expression profile, with the exception of C79F SNP which seemed to result in a slightly increased protein level (Fig 2A and B). Next, we determined whether non-synonymous SNPs could alter MAVS-induced IFN-mediated antiviral and/or NF- $\kappa$ B-mediated pro-inflammatory signalling in the two foregoing cell types (Fig 2C and D). In agreement with previous studies (Kawai et al, 2005; Moore et al, 2008; Seth et al, 2005; Zhao et al, 2007), WT MAVS constitutively induced IFN as well as NF- $\kappa$ B responses relative to control cells (*i.e.* transfected with an empty vector). Interestingly, among all SNPs assessed, only those affecting the cysteine 79 residue similarly impacted MAVS-dependent signalling in both cell types. Indeed, C79S and C79F SNPs resulted in a limited and major reduction of MAVS reporter activity, respectively. The loss-of-function effect of C79F MAVS variant was further confirmed by measuring expression of endogenous inflammatory and antiviral mediators such as IL-8, RANTES and IFN- $\beta$  in HEK 293T cells (Fig 2E–F), and RANTES in BEAS-2B cells (Fig 2G); ( $n = 3$ ,  $p < 0.0001$ ). Based on Fig 2A's data mentioned above and in Fig 2H showing that C79F MAVS and WT MAVS are both located in the mitochondria, we can argue that C79F loss-of-function is neither due to protein degradation nor to a cellular mislocalization. Altogether, our results identified for the first time a natural variant affecting MAVS function and revealed the critical role of the cysteine 79 residue in MAVS activity.

#### C79F SNP alters both MAVS-dependent antiviral and pro-inflammatory responses to virus infection.

To confirm the relevance of the loss-of-function effect of C79F MAVS in virus-infected cells, we examined the responsiveness of WT or C79F MAVS to replicative influenza A (IAV) and Sendai (SeV) viruses (Fig 3A and B). While WT MAVS highly enhanced antiviral and pro-inflammatory responses triggered by both viral infections (relative to control infected cells), C79F SNP dramatically impaired these responses ( $n = 3$ ,  $p < 0.0001$ ). Remarkably, C79F SNP did not result in a complete loss-of-

function effect after viral infection, as indicated by the slightly but significantly enhanced response relative to control cells after IAV infection ( $p < 0.0001$  considering IFN- $\beta$  and NF- $\kappa$ B activities,  $n = 3$ ). Nevertheless, our findings show that the presence of the C79F SNP clearly decreases the magnitude of MAVS-dependent immune responses in virus-infected cells.

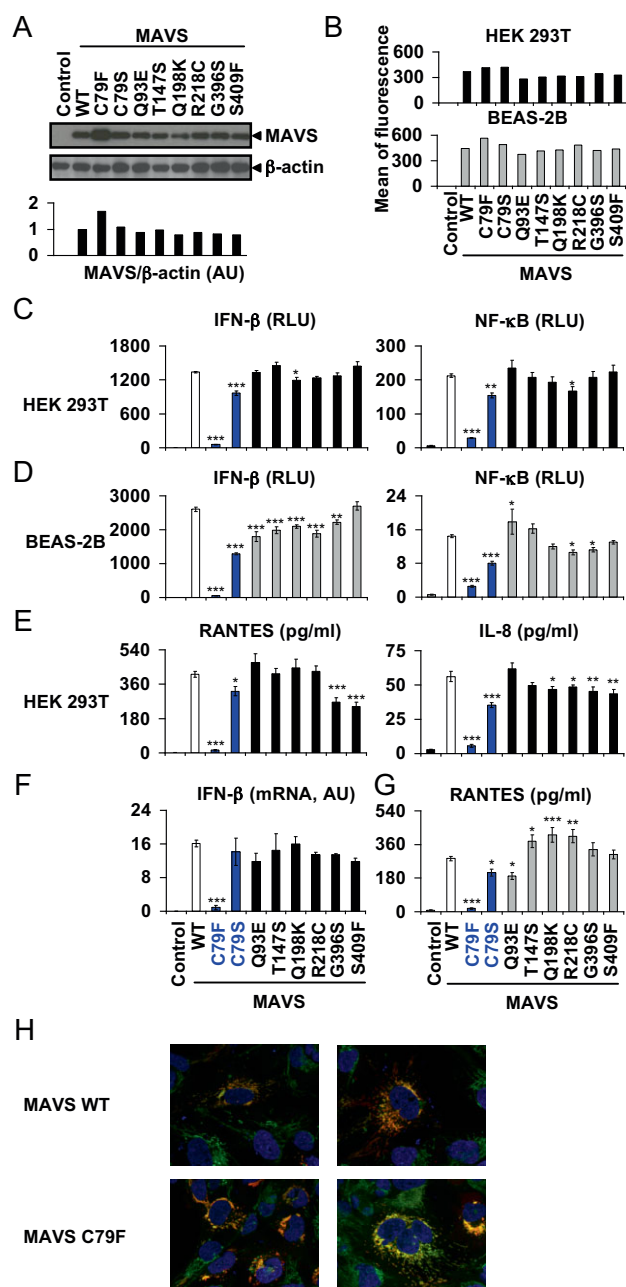
#### C79F SNP exerts a down-regulatory effect and affects TRAF3 binding to MAVS.

Next, we investigated the mechanism by which C79F SNP resulted in alteration of MAVS-mediated immune responses. We hypothesized that such a mutation might alter MAVS conformation, thus modifying its interaction with downstream signalling molecules. Among them, tumour necrosis factor receptor-associated factor 3 (TRAF3) is a critical MAVS signalling effector (Nakhaei et al, 2009; Saha et al, 2006). Interestingly, co-immunoprecipitation assays of WT or mutant MAVS with TRAF3 demonstrated that the C79F variant (but not C79S) resulted in a significantly reduced interaction of MAVS with TRAF3 ( $p = 0.012$ ; Fig 4A and B).

We further assessed whether the C79F loss-of-function variant might have a down-regulatory effect in *trans*. Figure 4 C–E show that C79F MAVS resulted in a potent alteration of antiviral and inflammatory signalling induced either by a constitutively active module of RIG-I [*i.e.* RIG-I 2CARD (Pothlichet et al, 2009; Yoneyama et al, 2004); Fig 4C], by WT MAVS (Fig 4D) or by TRAF3 molecules (Fig 4E). Thus, these co-expression experiments indicated that the C79F MAVS allele might exert a major negative effect on WT MAVS-dependent signalling and therefore suggested that heterozygous individuals for WT and C79F MAVS alleles might have an impaired RIG-I/MAVS signalling.

#### C79F MAVS SNP is associated with a distinct SLE serologic phenotype in African-American patients.

Next, we examined the impact of the C79F MAVS SNP in SLE, the prototype systemic autoimmune disease that is characterized by immune alterations typical of responses to virus-derived stimuli



**Figure 2. C79F SNP severely alters MAVS signaling.**

**A, B.** Expression of wild-type (WT) and mutant MAVS proteins as assessed by western-blotting (using HEK 293T cells; panel A) and flow cytometry (using BEAS-2B and HEK 293T cells; panel B). MAVS/ $\beta$ -actin proteins ratio are also represented in panel A.

**C-G.** BEAS-2B or HEK 293T cells were co-transfected with either a NF- $\kappa$ B- or IFN- $\beta$ -luciferase-reporter plasmid (100 ng/well) and a vector encoding WT or mutant MAVS (100 ng/well) or an empty vector (control) and a  $\beta$ -galactosidase (100 ng/well for BEAS-2B cells and 20 ng/well in HEK 293T cells) expression vector (this latter served for verifying transfection efficiency). Data were collected 18 h post-transfection and are expressed as the mean of three independent experiments ( $\times 10^{-3}$ )  $\pm$  SEM of promoter-driven RLU normalized to  $\beta$ -galactosidase activity of triplicate samples (C and D). Using the supernatants collected from samples shown in panels C and D, IL-8 and/or RANTES release was measured by ELISA (E and G). Data are mean of three independent experiments  $\pm$  SEM done in triplicate. (F) HEK 293T cells were transfected with a vector encoding WT or SNPs MAVS or an empty vector (control) and endogenous IFN- $\beta$  mRNA expression was analysed by qRT-PCR. Results are expressed as fold-increase of IFN- $\beta$  mRNA level normalized with  $\beta$ -actin level and relatively to C79F MAVS-transfected cells. Data are mean  $\pm$  SEM of samples obtained in three independent experiments. AU, arbitrary units.

**H.** Both C79F and WT MAVS proteins have a similar mitochondrial localization. BEAS-2B cells were transfected with WT or C79F MAVS constructs. Twenty-four hours later, mitochondria were labelled with Mitotracker (Green) and then cells were fixed and permeabilized. Cells were visualized with a fluorescence microscope (with Apotome). MAVS protein is detected (Red) and nucleus are labelled with DAPI (Blue). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

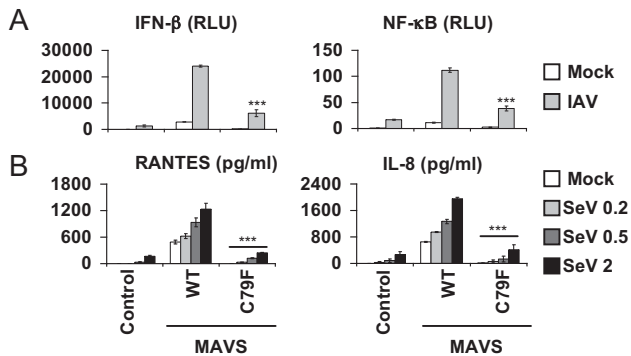
frequencies for the C79F SNP are consistent with the NCBI SNP database. The C79F allele was found at increased frequency in African-American and European-American SLE patients compared to the controls in these backgrounds, *i.e.* 12.6 and 1.3%, respectively (Table 1). This SNP was rare in Hispanic-American SLE patients (1.7% frequency) and was not found in the small number of Asian-American SLE patients studied (Supplementary Table S2).

When we examined the frequency of the C79F MAVS allele in SLE patients *versus* controls from either African or European origins, we found no strong evidence for an overall case-control genetic association (European ancestry  $p = 0.043$ , OR = incalculable due to zero individuals in the control population; African-American ancestry  $p = 0.26$ , OR = 1.3; calculations based upon data in Supplementary Table S2). Our previous studies have revealed strong associations of SLE-risk loci within sub-groups of SLE patients defined by their production of defined autoantibody specificities (Niewold et al, 2008a; Salloum et al, 2010). Thus, we used logistic regression models to detect relationships between the MAVS SNPs and autoantibody traits in the SLE patient cohort.

We found that the C79F allele was especially enriched in African-American SLE patients who lacked anti-RBP antibodies (OR = 2.6,  $p = 0.00084$  when compared to RBP+ patients; Table 1), and a similar trend was observed in the European-American SLE patients (OR = 3.2) although this was not statistically significant probably due to the low frequency of the C79F allele in European ancestry. RBP+ SLE patients of both African- and European-American ancestry had C79F allele

(Crow, 2009; Kariuki et al, 2009; Niewold et al, 2008b; Sebastiani & Galeazzi, 2009). We postulated that the C79F MAVS variant might regulate type I IFN production in SLE patients and consequently influence disease pathogenesis and clinical phenotype. To test this hypothesis, we studied the C79F and Q198K SNPs (the latter being neutral in our functional assay) in genomic DNA from 520 American SLE patients and 510 control individuals from several ethnic groups. Full genotype and allele frequencies are shown in Supplementary Table S2, and allele frequency summary data and analyses are shown in Table 1.

The allele encoding C79F MAVS was present in African-American control individuals at a frequency of 10.2%, and was not found in our European-American controls. These allele



**Figure 3. C79F SNP impairs MAVS-mediated immune signalling in response to influenza A and Sendai viruses.**

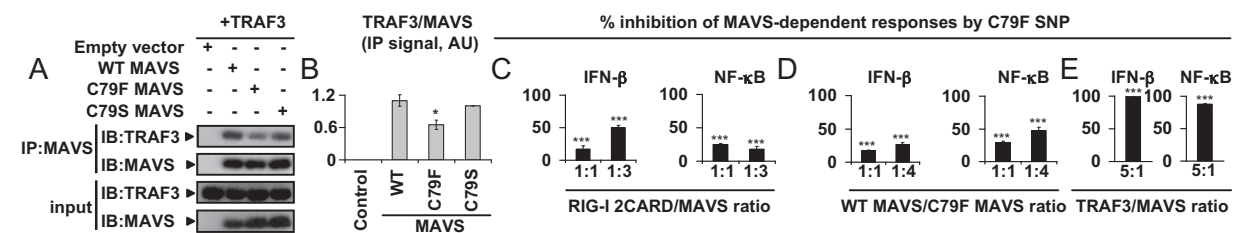
**A.** WT or C79F MAVS were expressed in BEAS-2B cells co-transfected with IFN-β or NF-κB-dependent luciferase and β-galactosidase reporter plasmids. Six hours later, cells were infected with influenza A virus (IAV; MOI = 1) or not (white bars, Control). Luciferase and β-galactosidase activities were analysed 16 h post-infection. Data are expressed as in Fig 2C and D.

**B.** WT or C79F MAVS were expressed in HEK 293T cells before infection with Sendai virus (SeV; 0.2–2 HAU/well) or not (white bars, Control), and RANTES and IL-8 concentrations were assessed in the supernatants by ELISA. Data are mean ± SEM of three independent experiments done in triplicate. \*\*\**p* < 0.001.

frequencies which were very similar to controls (Table 1). To check for any potential confounding due to admixture in our African-American population, we performed a principal component analysis using genotype data from 12 SNPs which confer information about ancestry including all cases and controls (Table S3). The first component derived from this analysis provides a quantitative variable indicating the degree of European- versus African-derived genetic ancestry. The intersection of the *x*- and *y*-axis provides a strong separation between those of African-American and European-American ancestry (Kariuki et al, 2010). Inclusion of this variable in our regression models did not significantly change the results for C79F in African-Americans (RBP- versus RBP+ SLE—OR = 2.95, *p* = 0.00080; RBP- SLE versus controls—OR = 2.30, *p* = 0.0034). Similarly, the case-control analyses did not change significantly with inclusion of the ancestry covariate. Thus, the C79F result is not a spurious association arising from differences in proportional admixture in the subjects analysed. It can be concluded that the C79F allele is very significantly associated with the absence of autoantibodies specific for RBP in African-American SLE patients.

We also examined our data for possible associations between the American College of Rheumatology (ACR) clinical criteria for the diagnosis of SLE and presence or absence of the C79F allele using a multivariate logistic regression model in 109 African-American SLE patients with complete data for these characteristics (see methods). There was a trend towards association of the following clinical variables with the C79F allele: presence of malar rash, arthritis and neurologic disease, and absence of oral ulcers (Supplemental Table S3). These variables were not highly correlated with each other, and an additive model including all four clinical features was the best fit for the association (*p* = 0.019). This result is preliminary due to the low number of patients with complete clinical data, which could explain the absence of significant association of C79F MAVS with each of these four variables separately. The prevalence of clinical features in the 109 subjects African-American subjects analysed is shown in Supplemental Table S3.

In contrast, the Q198K MAVS SNP was not associated with autoantibody profile or clinical disease features in our SLE patients in logistic regression models (Table 1 and Supplemental



**Figure 4. C79F SNP exerts a down-regulatory effect and alters TRAF3 binding to MAVS.**

**A.** C79F SNP alters the interaction of MAVS with TRAF3 protein. This interaction was assessed 24 h post-transfection by immunoblotting of HEK 293T cells samples co-transfected with vectors expressing WT, C79F or C79S Flag-MAVS and Myc-TRAF3 or an empty vector as control. MAVS was immunoprecipitated (‘IP’) with an anti-Flag antibody and TRAF3 was immunoblotted (‘IB’) with an anti-Myc antibody. A representative western-blot out of three different experiments with similar results is shown.

**B.** Shown is the quantification of western-blot signals. Data are mean ± SEM of TRAF3 over WT or mutant MAVS ratio obtained in three independent experiments. AU, Arbitrary Unit; \**p* = 0.012).

**C.** C79F MAVS inhibits RIG-I 2CARD-induced IFN-β and NF-κB-dependent luciferase reporter activity. HEK 293T cells were co-transfected with 100 ng of RIG-I 2CARD and 100 ng (ratio 1:1) or 300 ng (ratio 3:1) of an empty vector or WT or C79F MAVS vectors.

**D.** C79F MAVS exerts a down-modulatory effect on WT MAVS-dependent signalling. HEK 293T cells were co-transfected with the above-mentioned reporter plasmids and different ratio of WT and C79F MAVS. An empty vector was used to maintain the total plasmid quantity constant at 100 ng.

**E.** C79F MAVS co-expression with TRAF3 inhibits MAVS-dependent IFN-β antiviral response and NF-κB-dependent signalling as revealed by specific luciferase reporter assays with HEK 293T cells co-transfected with 100 ng of TRAF3 and 20 ng of C79F or WT MAVS expression vector. Data are mean ± SEM of percentage of inhibition of MAVS-dependent responses by C79F SNP of three (Fig 4B–D) or four (Fig 4E) independent experiments done in triplicate. \*\*\**p* < 0.001.

**Table 1. C79F MAVS, but not Q198K, is associated with absence of autoantibodies against RNA binding proteins (RBP) in African-American SLE**

Ancestry	C79F minor allele freq. (MAF)				C79F MAF comparison		Q198K MAF		Q198K MAF comparison
	All SLE cases	RBP+ SLE	RBP- SLE	Control	RBP- SLE vs. RBP+ SLE	RBP- SLE vs. controls	All SLE cases	Control	SLE vs. controls
African-American	0.126	0.101	0.224	0.102	OR = 2.6 (1.5–4.6) $p = 0.00084$	OR = 2.5 (1.5–4.4) $p = 0.00051$	0.187	0.200	OR = 0.9 (0.7–1.2) $p = 0.61$
European-American	0.013	0.007	0.020	0	OR = 3.1 (0.3–30.4) $p = 0.37$	OR = N/A* $p = 0.021$	0.128	0.155	OR = 0.8 (0.5–1.2) $p = 0.32$

Shows minor allele frequencies (MAF) of the C79F and Q198K polymorphisms in SLE patients, controls, and SLE patient subgroups defined by anti-RBP antibodies. Odds ratios (OR) and p-values are calculated for the difference in allele frequency between anti-RBP negative and anti-RBP positive SLE patients, as well as anti-RBP negative patients compared to controls. p-values less than 0.0084 withstand Bonferroni correction for multiple comparisons in this analysis. Numbers of subjects are as follows: African-American SLE: 253, African-American Controls: 313, European-American SLE: 152, European-American Controls: 197.

Table S2), and there were no significant differences in allele frequency at this SNP in SLE patients or controls (Table 1). These data did not change following inclusion of the co-variate designating proportional admixture (data not shown). Thus, the C79F SNP which demonstrates a strong influence on type I IFN responses *in vitro* is associated with disease phenotype in SLE, while the Q198K allele which does not impact type I IFN responses *in vitro* is not significantly associated with either SLE or subphenotypes within the disease.

We have previously shown that the RBP+ phenotype is positively correlated with type I IFN activity in SLE serum (Niewold et al, 2007). To investigate the functional implications of the association of the C79F allele with RBP- African-American patients, we analysed serum type I IFN activity in lupus patients defined based on presence or absence of the C79F allele. In agreement with the *in vitro* data shown in Figs 2–4, we found that samples from African-American subjects with the C79F SNP had significantly lower type I IFN activity ( $p = 0.0032$ , Fig 5A), and a similar non-significant trend was observed in the small number of European ancestry subjects carrying this allele (Fig 5B). Remarkably, while serum type I IFN activity was similar between RBP+ African-American patients regardless of expression of the C79F allele ( $p = 0.43$ , Fig 5C), in patients who were RBP-, IFN activity was significantly lower in those carrying the C79F allele ( $p = 0.0013$ , Fig 5C). Also, we saw no difference in serum IFN activity in SLE patients related to the Q198K allele ( $p \geq 0.37$  in both African- and European-American patients and RBP- and RBP+ patients, Fig 5D–F), which mirrors our *in vitro* data in which this allele showed no impact on IFN production and is in agreement with the absence of association of Q198K SNP with RBP phenotype.

## DISCUSSION

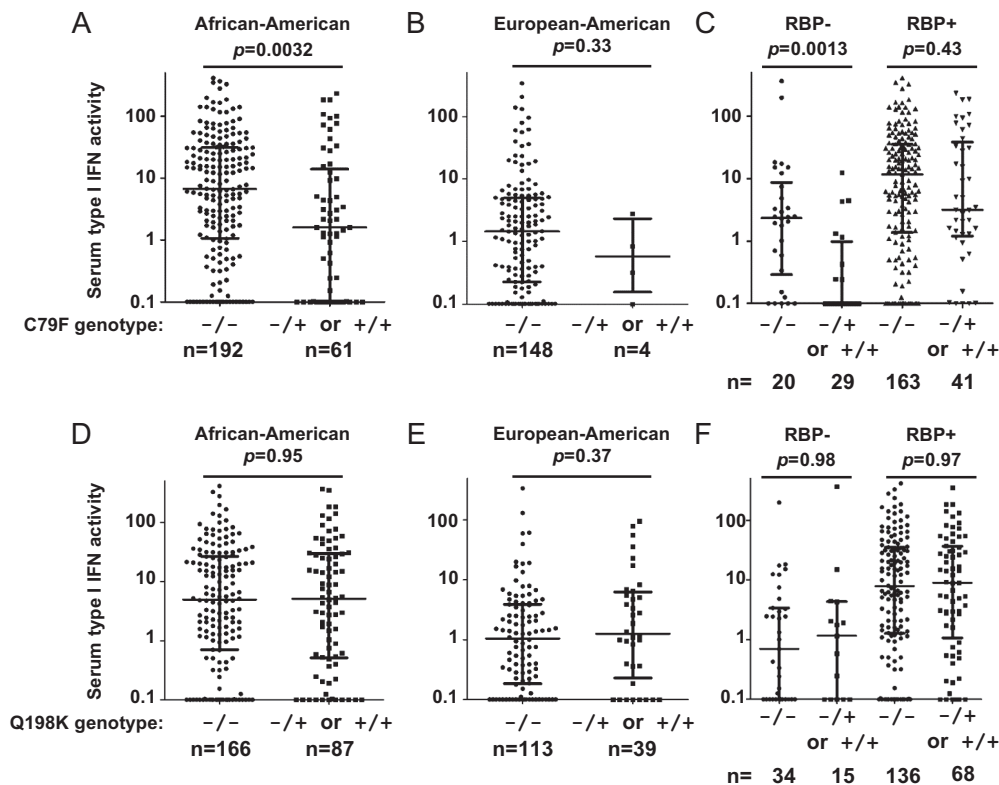
SLE is often considered the prototype systemic autoimmune disease. The pathogenesis of SLE is driven by a combination of environmental events and genetic risk factors that lead to an irreversible break in immunologic self-tolerance (Crow, 2009; Sebastiani & Galeazzi, 2009). Many common genetic variations

have been linked to SLE susceptibility in recent studies (Botto et al, 1998; Hinds et al, 2005). These include variations located in genes involved in innate immunity, which could also account for some of the observed human variability in response to pathogens and/or autoimmune triggers. In regard to genes encoding CARD-containing proteins, mutations in the peptidoglycan receptors NOD1 and NOD2 have been associated with several inflammatory and autoimmune disorders, including Crohn's disease (Werts et al, 2006). Remarkably, no human disease has yet been linked to the antiviral molecule MAVS.

Using a molecular screening strategy, we identified one critical coding-change variant in the MAVS gene which significantly altered function. In functional and biochemical assays, the C79F MAVS SNP substantially impaired MAVS-dependent antiviral and pro-inflammatory responses to two distinct viruses. Also, we demonstrated that this loss-of-function effect of the C79F SNP was not due to a modified MAVS protein expression but likely to a compromised MAVS-TRAF3 interaction.

By screening known coding-change polymorphisms for function, we were able to perform a directed genetic association study largely focused on this functional variant and including another nearby coding-change variant which lacked functional significance in our assays for comparison. In this portion of the study, we found that C79F MAVS contributes to pathogenesis in a distinct subset of African-American SLE patients defined by absence of autoantibodies specific for RBP. A functional role for the C79F MAVS allele in altered immune system regulation was indicated by the lower production of type I IFN in those RBP- African-American patients expressing that MAVS variant. The association between C79F MAVS allele and this SLE subgroup was further emphasized by demonstrating an absence of such link when considering another MAVS SNP, *i.e.* Q198K, this variant being neutral in our functional assays. Hence, our results are the first to reveal a contribution of impaired signalling through the MAVS pathway to SLE pathogenesis.

A recent study showed that a putative inducer of SLE, namely self-DNA (Deane & Bolland, 2006), could induce type I IFN signalling through EYA4 and MAVS interaction. However, in contrast to what might be predicted in view of the large literature supporting increased IFN- $\alpha$  in SLE, the lupus association



**Figure 5. C79F MAVS, but not Q198K, is associated with low type IFN activity in African-American SLE patients.**

- A, B.** Serum type I IFN in African- and European-American SLE patients, respectively, stratified by C79F genotype.
- C.** Serum type I IFN activity in African-American SLE patients stratified by both anti-RBP antibodies and C79F genotype.
- D, E.** Serum type I IFN in African- and European-American SLE patients, respectively, stratified by Q198K genotype.
- F.** Serum type I IFN activity in African-American SLE patients stratified by both anti-RBP antibodies and Q198K genotype.

Dot-plots are represented with the median and error bars show the interquartile range.  $p$ -values by Mann-Whitney U test, with values  $<0.01$  being significant after Bonferroni correction for multiple comparisons. (+) means that SNP is present and (-) means that is absent.

observed in our study was in patients with low plasma type I IFN activity.

Incidentally, our results constitute the first demonstration that MAVS is associated with type I IFN signalling *in vivo* in humans *per se*. This finding is critical in regard to previously reported cases of murine and human deficiencies in the TLR pathway. *In vitro* studies and experiments using MyD88-deficient mice showed that MyD88 is a crucial adaptor downstream of most of the TLRs, resulting in severe immunodeficiency. By contrast, MyD88-deficient humans are only sensitive to a small set of infectious diseases (von Bernuth et al, 2008) suggesting a greater redundancy in this pathway and potentially a greater importance of non-TLR mediated immunity such as that provided by the MAVS/RIG-I/MDA-5 pathway. Hence, our study and future work on the functional impact of MAVS polymorphisms in humans may help to demonstrate important human variability in the immune response to viral infection or virus-triggered autoimmune diseases.

Our preliminary analysis of the clinical features of SLE that were associated with the C79F SNP in African-American patients suggested a potential distinct clinical picture when compared to that previously described in lupus patients with high expression

of IFN-inducible genes. While increased frequency of renal disease and more severe disease as assessed by disease activity score are seen in lupus patients with high level of IFN pathway activation (Baechler et al, 2003; Kirou et al, 2005), the analysis performed on our African-American patients with the C79F SNP indicated a possible association with a different clinical pattern which included malar rash, arthritis, neurologic disease and the absence of oral ulcers. Consistent with the interpretation that genetic variants associated with distinct molecular pathways might characterize lupus patients with distinct clinical phenotypes, an analysis of the lupus features associated with a polymorphism in the TNFAIP3 gene, an inhibitor of the TLR pathway, showed associations with renal disease and hematologic manifestations but not with skin disease or arthritis (Bates et al, 2009). Thus, future large-scale studies of the frequency of the C79F SNP, along with immunologic and clinical characterization of lupus in a replication cohort of RBP- and RBP+ African-American patients defined based on the C79F allele, will likely provide clues to the underlying mechanisms that account for clinical subphenotypes that are based on altered function of either the TLR-dependent or TLR-independent pathways.

## MATERIALS AND METHODS

### Viruses

Influenza/A/Scotland/20/74 (H3N2) virus was previously described (Pothlichet et al, 2008). Sendai virus (Cantell strain, ATCC VR-907 Parainfluenza 1) was a kind gift of E. Meurs.

### Plasmids construction and site-directed mutagenesis

pcDNA3-Flag-MAVS and pcDNA3-Myc-MAVS plasmids were a kind gift of Dr. Z. Chen and Dr. J. Hiscott, respectively. pCI-V5-WT 2CARD (encodes the RIG-I aminoacids 2-229 fragment) and pcDNA3-Myc-TRAF3 plasmids were a kind gift of Dr. E. Meurs. SNPs-containing MAVS plasmids were made using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene) and pcDNA3-Myc-MAVS as a template. All constructs were entirely sequenced to confirm that no unintended mutations were generated during PCR reaction.

### Expression analysis

To evaluate WT or mutant MAVS expression levels, the human bronchial epithelial cell line BEAS-2B and the human embryonic kidney cell line HEK 293T were transfected with 100 ng of an expression vector for 18 h or an empty vector as a control and were further processed for Western-blotting as previously described (Pothlichet et al, 2008) or for flow cytometry using the following antibodies: anti-Flag antibody (2  $\mu$ g/ml) and Alexa488-conjugated secondary antibody (4  $\mu$ g/ml, A11001, Molecular probes). Protein ratios were determined using ImageJ software. To analyse protein subcellular localization, BEAS-2B cells were transfected with WT or C79F MAVS constructs. Twenty-four hours later, mitochondria were labelled with Mitotracker (100 nM) and then cells were fixed with PFA 4%. Fixed cells were washed twice in PBS and permeabilized with 1% Triton X-100. Cells were incubated with anti-FLAG M2 antibody at a dilution of 1:200 for 1 h and then washed three times. Cells were incubated with Cy3 goat anti-mouse IgG at a dilution of 1:200 for 1 h. Cells were washed three times and then were incubated with DAPI for 5 min and then mounted on glass slides. Cells were visualized with a fluorescence Zeiss Apotome microscope.

### Cell culture, transfection, ELISA, luciferase assays and quantitative RT-PCR analyses.

Detailed protocols were described before (Le Goffic et al, 2007; Pothlichet et al, 2008). Data obtained in luciferase assays are expressed as the mean ( $\times 10^{-3}$ ) of relative luciferase units (RLU) normalized with  $\beta$ -galactosidase activity of at least three independent experiments with triplicate samples.

### Analysis of MAVS interaction with TRAF3 by a co-immunoprecipitation assay

HEK 293T cells seeded in 6 well-plates ( $6.6 \times 10^5$  cells/well) were transiently co-transfected with 1  $\mu$ g of Flag-tagged MAVS and Myc-tagged TRAF3 expression vectors. After cell disruption, 600  $\mu$ g of cell lysates were pre-cleared with 15  $\mu$ l of protein G sepharose 4 beads (GE Healthcare). Next, co-immunoprecipitation of Flag-tagged MAVS was performed by incubating cleared cell lysates with 3  $\mu$ g of monoclonal anti-Flag M2 antibody, followed by the addition of 15  $\mu$ l of protein G sepharose 4 beads. Beads were then collected by centrifugation before resuspension in 30  $\mu$ l of denaturing buffer.

Samples were then analysed by immunoblot as described in reference (Pothlichet et al, 2008). Anti-Flag M2 (1/10000) and anti-Myc (Santa Cruz, 1/2000) were used for immunoblotting.

### SLE patient and control samples

Serum and genomic DNA samples were obtained from the Translational Research Initiative in the Department of Medicine (TRIDOM) at the University of Chicago and the Hospital for Special Surgery Lupus Registries. Of the 520 SLE patients, 253 were African-American, 152 were European-American, 86 were Hispanic-American, and 29 were of Asian-American ancestry. In the two groups of patients that were analysed in subgroups based upon autoantibody profiles, the number of RBP+ versus RBP- patients was as follows: European-American 75 RBP- and 77 RBP+, African-American 49 RBP- and 204 RBP+. Genomic DNA samples from 313 African-American and 197 European-American controls from the TRIDOM registry were also studied, and these subjects were screened by medical record review for the absence of autoimmune or inflammatory disease. The study was approved by the institutional review board at each institution, and informed consent was obtained from all subjects in the study.

### Genotyping of SLE patients and Controls

SLE patients were genotyped at the rs6116065, rs11905552, rs7262903 and rs3746660 SNPs in the MAVS gene (see Supplementary Fig S2 for location of these SNPs within the gene and correlations between SNPs represented as  $D'$  values). Rs11905552 is the C79F coding change SNP, while rs7262903 is the Q198K coding change SNP. Controls were genotyped at the rs11905552 and rs7262903 coding change SNPs. Genotyping was performed using Applied Biosystems Taqman Assays-by-Design primers and probes on an Applied Biosystems 7900HT PCR machine per manufacturer protocol. Genotyping was successful in >98% of subjects at each SNP, and only subjects with complete data at both C79F and Q198K were included in the analyses. Each genotyping scatter plot was reviewed individually for quality control, and experiments were done in duplicate with allelic calls made only when the duplicates were consistent. Genotype frequencies at the SNPs studied did not deviate significantly from the expected Hardy-Weinberg proportions ( $p$  value for deviation from Hardy-Weinberg equilibrium was  $\geq 0.22$  for all markers in each ancestral background).

### Reporter cell assay for type I IFN activity

The reporter cell assay for type I IFN has been described in detail elsewhere (Niewold et al, 2007; Niewold et al, 2008b). Reporter cells were used to measure the ability of patient sera to cause IFN-induced gene expression. The reporter cells (WISH cells, ATCC #CCL-25) were cultured with 50% patient sera for 6 h, and then lysed. Messenger RNA (mRNA) was purified from cell lysates, and complementary DNA (cDNA) was made from total cellular mRNA. cDNA was then quantified using real-time PCR using an Applied Biosystems 7900HT PCR machine with the SYBR Green fluorophore system. Forward and reverse primers for the genes MX1, PKR and IFIT1, which are known to be highly and specifically induced by type I IFN, were used in the reaction (Niewold et al, 2007). GAPDH was amplified in the same samples to control for background gene expression. The amount of PCR product of the IFN-induced gene was normalized to the amount of product for the housekeeping gene GAPDH in the same sample. The



## The paper explained

### PROBLEM:

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disorder. It is characterized by a limited number of therapeutic options and is caused by a combination of genetic risk factors and environmental events that lead to an irreversible break in immunologic self-tolerance. Moreover, a growing body of evidence supports the pivotal role of immune system mechanisms similar to those activated by viral infections in the induction or exacerbation of SLE. Here, we hypothesized that people may be predisposed to SLE via inheritance of a genetic alteration that influences a key antiviral molecule, namely MAVS, which is downstream of the viral sensors RIG-I and MDA-5.

### RESULTS:

We screened variants from public domain databases to identify genetic polymorphisms (coined “SNP” for “single nucleotide polymorphism”) that may result in functional effects on the MAVS protein. Through a series of *in vitro* studies, we characterized a single SNP that strongly alters MAVS protein

function. Then, we tested this SNP for association with SLE using patients from several ethnic cohorts who were carefully characterized for clinical features and immunologic manifestations of systemic autoimmunity. We identified an association between this MAVS SNP and African-American SLE patients with specific immune characteristics: absence of certain autoantibodies and low levels of type I interferon.

### IMPACT:

Our study identifies a potential role for the immune system pathway involved in viral responses in a subgroup of African American people who develop SLE. These results demonstrate the value of careful clinical and immunologic characterization of patients in identifying genetic risk factors for disease. They also justify further study of potential viral stimuli and the relevant immune response pathways, including MAVS-dependent signalling, as important mechanisms and potential therapeutic targets in SLE.

relative expression of each of the three tested IFN-induced genes was calculated as a fold increase compared to its expression in WISH cells cultured with media alone. Results from the IFN assay were standardized to a healthy multi-ancestral reference population as previously described, and a serum IFN activity score was calculated based upon the mean and SD of the reference population (Niewold et al, 2007). All IFN and autoantibody data were generated by persons who were blinded to the genotype results.

### Measurement of autoantibodies

Lupus antibodies to common human targets, including anti-Ro, anti-La, anti-Sm and anti-RNP were measured in SLE serum samples by enzyme-linked immunosorbent assay (ELISA) methods in the University of Chicago and Hospital for Special Surgery clinical laboratories, respectively. Standard clinical lab cut-off points were used to categorize samples as positive or negative. Anti-dsDNA antibodies were measured using *Crithidia luciliae* immunofluorescence at both registry sites, and detectable fluorescence was considered positive.

### Statistical analysis

Statistical differences were tested using a one-way ANOVA followed by a Fisher test, with a threshold of  $p < 0.05$  in Figs 2–4. In these figures, data are mean  $\pm$  SEM of at least three independent experiments performed in triplicate and asterisk symbols mean  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ . Chi-square distribution and Fisher's exact test were used to analyse categorical genotype data, and non-parametric Mann–Whitney U was used to compare quantitative type I IFN data between genotype subgroups in Fig 5. Backward logistic regression models were used to detect associations between autoantibodies and MAVS SNPs in the SLE cohort. Screening models incorporated each of the four SNPs with a single autoantibody trait in each ancestral background separately. Follow up models discarded

non-significant variables and assessed potential interactions between significantly associated variables. Six autoantibody traits were assessed in total (Ro, La, Sm, RNP, dsDNA and the 'RBP' category which represents subjects positive for at least one of the RNA-binding protein autoantibodies Ro, La, Sm or RNP). To account for potential differences related to proportional ancestry in admixed populations, we performed a principal component analysis on data from 12 SNPs which confer information about genetic ancestry which were genotyped in all cases and controls as described in Kariuki et al (2010), similar to the approach outlined in Parra et al (1998). The first principal component in this analysis provided strong separation of self-reported European- versus African-American ancestry, and this component was included in logistic regression analyses as a covariate to control for any differences in the degree of admixture between cases and controls which could potentially confound genetic association analyses.

Clinical variables were also analysed for association with functional MAVS SNP genotypes in the African-American SLE patients using logistic regression, and in this case carriage of the C79F and Q198K minor alleles was the outcome variable in a backwards logistic regression model which included each of the 11 ACR clinical criteria for the diagnosis of SLE as predictor variables (Tan et al, 1982). These variables were coded as positive if the criterion for that clinical feature had ever been met, in accordance with the diagnostic criteria (Tan et al, 1982). For the clinical variables analysis, complete data was available for approximately 50% of the African-American cohort, and subjects with incomplete clinical data were excluded from analysis. Clinical variables which demonstrated at least nominal evidence for association ( $p < 0.3$ ) were kept in the model. In the case of C79F, four clinical variables demonstrated some nominal and independent evidence for association. Both additive and multiplicative models were tested using these variables to predict carriage of C79F, and the

additive model provided a better fit ( $p = 0.019$ ). As three features were positively correlated and one was inversely correlated, the final additive model using equal weighting was as follows: malar + arthritis + neurologic disease – oral ulcers.  $p$ -Values shown in the paper are uncorrected for multiple comparisons, and we considered results significant when the  $p$ -values remained significant following a Bonferroni correction for multiple comparisons correcting for the number of regressions or number of analyses performed ( $p < 0.0083$  in the six autoantibody logistic regressions,  $p < 0.01$  in the six serum type I IFN analyses, and  $p < 0.025$  in the two functional SNP-ACR clinical variable logistic regressions).

## Author contributions

Conceived and designed the experiments: JP, TBN, MKC, MS. Performed the experiments: JP, TBN, DV, BS. Analysed the data: JP, TBN, DV, MKC, MS. Wrote the paper: JP, TBN, MKC, MS.

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Supporting information is available at EMBO Molecular Medicine online.

The author declares that he has no conflict of interest.

## For more information

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<http://www.ncbi.nlm.nih.gov/About/primer/snps.html>

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