Published in final edited form as:

Arthritis Rheumatol. 2016 November ; 68(11): 2697–2707. doi:10.1002/art.39733.

Prion-like MAVS aggregation in lupus patients associates with increased interferon-I

Wen-Hai Shao, PhD[1], **Daniel H. Shu, B.A.**[1], **Yuxuan Zhen, M.A. Ed**[1], **Brendan Hilliard, PhD**[1], **Stephen O. Priest, PhD**[1], **Matteo Cesaroni, PhD**[2], **Jenny P-Y. Ting, PhD**[3], and **Philip L. Cohen, MD**[1]

[1]Rheumatology Division, Department of Medicine, Temple University, Philadelphia, PA, USA

[2]Fels Institute for Cancer Research and Molecular Biology, Temple University School of Medicine, Philadelphia, Pennsylvania, USA

[3]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

Abstract

Objective—Increased levels of Type I interferon (IFN-I) and IFN-I-regulated genes are found in patients with systemic lupus erythematosus (SLE) and may be central to its pathogenesis. The mitochondrial adaptor protein MAVS is a key regulator of IFN-I that undergoes a dramatic prion-like aggregation and self-propagates the activation signal from viral RNA to amplify downstream IFN production. We wondered if such MAVS aggregates might play a role in the sustained increased production of IFN-I in SLE.

Methods—Peripheral blood mononuclear cells (PBMCs) were isolated and mitochondrial extracts were prepared. MAVS aggregation was detected with semi-denatured agarose gel electrophoresis (SDD-AGE) and confirmed by immunofluorescence staining. MAVS-associated signaling proteins were analyzed by Western blot. MAVS aggregation-associated gene expression signature was analyzed by microarray.

Results—Blood cells from 22 of 67 SLE patients were found to have essentially all of their MAVS in a high molecular weight aggregated form. None of six rheumatoid arthritis patients and only three of 33 healthy controls had abnormal MAVS. The MAVS-aggregate positive SLE patients had significantly higher serum levels of IFN-β and significantly increased auto-antibodies against Sm and U1RNP, compared to MAVS-aggregate negative patients. Gene array data revealed

Address correspondence to: Philip L. Cohen, Temple University, Section of Rheumatology, Department of Medicine, MERB, Room 454, 3500 N. Broad St, Philadelphia, Pennsylvania 19140, USA. philco@temple.edu. Wen-Hai Shao, Temple University, Section of Rheumatology, Department of Medicine, MERB, Room 456, 3500 N. Broad Street, Philadelphia, Pennsylvania 19140, USA. wshao@temple.edu.

AUTHOR CONTRIBUTIONS

All authors approved the final version to be published. Drs. Philip L. Cohen and Wen-Hai Shao had full access to all of the data and the accuracy of the data analysis.

Study conception and design. Philip L. Cohen, Wen-Hai Shao.

Acquisition of data. Daniel H. Shu, Yuxuan Zhen, Brendan Hilliard, Stephen O. Priest

Analysis and interpretation of data. Philip L. Cohen, Wen-Hai Shao, Matteo Cesaroni, Jenny P-Y Ting.

a characteristic gene expression pattern in these patients, with altered expression of genes involved in IFN signaling and membrane trafficking.

Conclusion—Persistent MAVS aggregates may lead to increased IFN-I production and result in unmitigated signals leading to autoimmunity.

Patients with systemic lupus erythematosus (SLE) have elevated type I interferon (IFN-I) and IFN-inducible gene expression, the “IFN signature”, implicated in disease etiology and activity. Type I interferon production is regulated to a considerable extent by Toll-like receptor signaling, and abnormalities in this pathway have been described in lupus patients (1, 2). The more recently described RIG-I signaling pathway also plays an important role in IFN-I production. The mitochondrial antiviral signaling protein MAVS is required for this pathway of innate anti-viral defense (3–6). RIG-I/MDA5 recognizes viral dsRNA and undergoes a conformational change to induce the activation of MAVS, ultimately engaging nuclear factor κ-B (NFκ-B) and IRF3/7 activation through TRAF6/3, respectively (7). Coordinated activation of these transcription factors triggers inflammatory cytokine and IFN-I production. Supporting the notion that RIG-I signaling is important in respond to viral infection *in vivo*. MAVS deficient mice are severely compromised in anti-viral defense (8).

The RIG-I pathway may contribute to increased IFN inducible gene activation in SLE, resulting in increased disease activity. Transient exposure to a RIG-I ligand aggravates murine lupus nephritis via IFN signaling (9). Polymorphisms in *Ifih1* (IFN-induced helicase 1 gene, encoding MDA5) are associated with susceptibility to autoimmune diseases. Constitutively activated MDA5 (Gly821Ser) leads to a murine SLE-like phenotype, with increased IFN-I and IL-6, lymphocyte infiltration, complement deposition, and nephritis. The SLE-like disease requires functional MAVS (10). Overexpression of MAVS in fish cells causes constitutive induction of IFN and IFN-stimulated genes (ISGs) (11). Polymorphisms of human MAVS are associated with SLE susceptibility and manifestations (12). A loss-of-function variant (C79F) of MAVS is associated with low levels of IFN-I in SLE patients, together with absence of RNA-protein binding autoantibodies (13).

Recently, Hou et al discovered that MAVS forms remarkable prion-like aggregates that propagate RIG-I signaling (14). Aggregated MAVS is detergent- and protease-resistant, and mediates signal transduction by autocatalytic conformational conversion of the adapter. We wondered if inappropriate or persistent MAVS aggregation might lead to increased IFN-I production, immune stimulation, and systemic autoimmunity in SLE. Our findings indicate that in a significant fraction of SLE patients, there is MAVS aggregation in peripheral blood cells, raising the possibility that this abnormality reflects persistent MAVS signaling and underlies type I interferon production, contributing to the development of SLE.

PATIENTS AND METHODS

Study populations

Patients were from the Lupus Clinic at Temple University Hospital. The studies were approved by the Temple University Institutional Review Board. After informed consent was given, we obtained blood from patients who satisfied diagnostic criteria of the ACR (American College of Rheumatology) for SLE and RA. Disease activity was assessed by the

SLEDAI activity index and determined on the day of blood draw. 67 SLE patients (64 females and 3 males), 6 RA patients and 33 normal age-, sex-, and race-matched controls were enrolled in the study 2012–2014. Of these, 22 were Hispanic, 38 African-American, 1 Asian, and 5 Caucasian.

Serology

Anti-nuclear antibodies, anti-dsDNA, anti-Sm, anti-RNP, anti-cardiolipin antibodies, serum C3 and C4, and antibodies to SS-A and SS-B were measured in our clinical laboratories and also at RDL Laboratories (Santa Monica, CA) using standard clinical protocols.

PBMC preparation and MAVS aggregation detection

Peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll/Hypaque sedimentation. Crude mitochondria and cytosolic extracts were obtained through differential centrifugation as described (14). Briefly, we added buffer A (10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 0.25 M D-mannitol, and Pierrs EDTA-free protease inhibitor cocktail) into 3×10^6 PBMCs and then lysed by repeated douncing. Cell debris was removed by centrifugation (1000 × g, 5 min) and the supernatants were then centrifuged again at 10,000 × g for 10 min at 4°C to obtain the supernatant (cytosolic extracts, S5) from the pellet (mitochondria enriched samples, P5). MAVS aggregation was detected with SDS-AGE according to previous publications (14). In brief, P5 and S5 were suspended in 1× sample buffer (0.5 × TBE, 10% glycerol, 2% SDS, and 0.0025% bromophenol blue) and loaded onto a vertical 1.5% agarose gel. We then ran electrophoresis in 1 × TBE buffer supplied with 0.1% SDS and transferred to Immobilon membranes for immunoblotting.

Microscopy

PBMCs from lupus patients and normal controls were incubated with 200 nM of MitoTracker Red (Life Technologies) in pre-warmed RPMI-1640 for 45 minutes in the dark. Cells were then washed, fixed, and permeabilized with BD Cytoperm buffer. Rabbit anti-MAVS antibody was diluted into the Per/Wash buffer and incubated for 20 minutes with cells. FITC-conjugated anti-rabbit secondary antibody was added after washing. Images were captured with a Zeiss confocal microscope (LSM 510META, Germany).

IFN-β analysis

IFN-β was detected using the human IFN-β ELISA kit (Fujirebio Inc. Tokyo, Japan) following the manufacture's procedure. In brief, the antibody-coated microplate was washed and incubated with serum and enzyme-linked secondary antibody for 2 hours at room temperature. Color developer was then added into each well after 3 washes. The reaction was stopped after 30 minutes. Absorbance was read at 450nm with a reference at 620nm.

Gene expression analysis

Total RNA was extracted from all SLE and control PBMCs and the cRNA was run on HT-HG-U133-plus (Affymetrix) oligonucleotide microarrays containing ~47,000 human transcripts (15). RMA function was applied to correct for background and to normalize the raw expression values. A linear model to the data was fitted in order to identify

Differentially Expressed (DE) genes (16). A Support Vector Machine (SVM) with Recursive Feature Selection was applied to determine which genes divided MAVS aggregates positive from negative samples. The top 30 genes were selected from the SVM analysis and clustering analysis was performed using Pearson correlation.

Western blotting

Western blot analysis was performed using standard procedures. Antibodies against MAVS (1:1000, Santa Cruz), IRF3, pho-IRF3 (1:1000, Abcam), NLRC3, NLRX1 (1:2000, Ting Lab (17)), TRAF3 (1:200, Santa Cruz), TRAF6, Stat1 (1:500, Santa Cruz), PCBP2 and ARCH5 (1:500, Abcam), gC1qR (1:1000, Santa Cruz), β-actin (1:200,000, Abcam) were used.

Statistics

Western blot data were analyzed using the ImageStudio software (Li-Cor, Lincoln, NE). Intensity differences between groups were tested using the Mann-Whitney U test. A p value of less than 0.05 was considered to be significant. Data are shown as median with interquartile range. Chi square with Yates correction was used to compare prevalence of autoantibodies between groups.

RESULTS

MAVS aggregates in the PBMCs of SLE patients

SLE patients fulfilled American College of Rheumatology (ACR) criteria. We analyzed MAVS aggregation status in PBMCs of 67 SLE patients and 33 controls. Mitochondria-enriched P5 samples were lysed and separated by vertical semi-denaturing agarose gel electrophoresis (SDD-AGE) to detect prion particles (Figure 1) (14). To our initial surprise, over a third of patients with SLE showed aggregation of the MAVS protein when analyzed using the SDD-AGE method. The aggregation was usually marked, with most of the MAVS protein present in aggregate form. To judge whether individual samples were aggregate-positive or negative, we arbitrarily considered a ratio of ≥10 for relative intensity (compared to β-actin protein levels) as positive aggregation (Figure 1). To confirm our initial observation, we measured the protein levels of MAVS with conventional, fully denatured SDS-PAGE and compared MAVS levels to those of the housekeeping gene β-actin. We found no correlation between the total levels of MAVS protein and the presence of MAVS aggregation (Figure 1B and C). We also detected MAVS aggregation using multiple anti-MAVS antibodies from different sources and obtained consistent results (Figure 1B and C). Cells from 32.8% (22 out of 67) SLE patients had a prion-like high molecular weight form of MAVS, compared to 9% (3 out of 33) of normal controls (Figure 1A, B, C, and data not shown). None of PBMC from six RA patients meeting ACR/EULAR criteria showed the high-molecular weight smear indicating MAVS aggregation (Figure 1D).

We had the opportunity to examine additional samples from nine SLE patients over the course of about a year to address the question of whether MAVS aggregation was a stable characteristic or if it changed over time. For three MAVS aggregation positive patients, only one remained positive when assayed a second time. Four MAVS aggregation negative

patients remained negative when sampled a second time, and two negative patients showed MAVS aggregation when examined at a later time. Thus, MAVS aggregation is not a stable phenotype for lupus patients, but changes over time.

To visualize MAVS aggregation in cells, we stained PBMCs with MitoTracker-PE, rabbit anti-MAVS, and then Alexa-488 anti-rabbit IgG. Confocal fluorescence microscopy revealed that Alexa-MAVS formed clusters that partially overlapped with MitoTracker from MAVS-aggregates positive PBMCs detected by SDD-AGE, but not from MAVS-aggregates negative samples. Mitochondria from MAVS aggregates positive PBMCs tended to cluster together, suggesting a role of mitochondrial membrane association (Figure 1E). In Sendai virus-infected fibroblasts, disulfide bonds were required for the aggregated form of MAVS (14). We found that incubation with β-mercaptoethanol reduced the high-molecular weight MAVS aggregates to the low-molecular weight range (Figure 1F), confirming this requirement for SLE patients.

MAVS aggregates are associated with down-regulation of the MAVS-regulatory protein C1qr and MARCH5, yet levels of NLRC3 and NLRX1 are the same in aggregates-positive and aggregates-negative individuals

We asked whether MAVS aggregates might reflect lack of a down-regulatory effect of known inhibitors of this protein. MAVS regulating proteins in the cytoplasmic preparation (S5) and mitochondria portions (P5) were analyzed by Western blot and the levels of several proteins known to affect MAVS were examined. We found that cytoplasmic C1qr, a negative regulator of MAVS signaling (18), was significantly reduced in the PBMCs of SLE patients with aggregated MAVS, compared to MAVS aggregation-negative SLE patients and normal controls (Figure 2A). Down-regulation of C1qr may be one of the mechanisms leading to impaired inactivation of MAVS in some SLE patients. MARCH5 and PCBP2 (poly(rC) binding protein 2) are recently identified MAVS-linked proteins (19, 20). Association of MAVS with either MARCH5 or PCBP2 promotes proteasome-mediated degradation of MAVS. Western results revealed a significant decrease of MARCH5 in MAVS aggregation positive SLE patients compared to the MAVS aggregation negative SLE patients (Figure 3). It is of note that MARCH5 only binds MAVS when it forms aggregates (20). Cytoplasmic Stat1 levels were also significantly low in MAVS aggregation positive SLE patients, compared to both normal controls and SLE patients without MAVS aggregation (Figure 2A). Stat1 activation was associated with translocation into the nucleus (21). Decreased cytoplasmic levels of Stat1 in MAVS aggregation positive SLE patients may reflect its activation and translocation into nucleus. There was no difference in Stat1 levels in the P5 samples (Figure 2B).

MAVS activation recruits adaptor proteins (TRAF3 and TRAF6) that activate transcription factors. Induction of the NF-κB pathway occurs via the recruitment of TRAF6 (22). Western blot results revealed no difference in protein levels of TRAF6 between normal controls and SLE patients, but TRAF6 levels were low in the PBMCs of SLE patients with aggregated MAVS (Fig. 2A), a trend only observed for the cytoplasmic preparations (S5) but not the mitochondria samples (P5). In contrast, TRAF3 levels were significantly higher in the mitochondria preparations (P5) from MAVS aggregation positive SLE patients compared to

normal controls (Figure 2B). MAVS interacts with TRAF3, which leads to the subsequent phosphorylation of IRF3. IRF3 dimers translocate to the nucleus and bind the interferon stimulated response elements (ISRE) (22). We noted an increased level of IRF3 in PBMCs from SLE patients compared to normal controls, indicating active IFN signaling in SLE patients (Figure S1A). However, the activated form of IRF3 (Ser396 phosphorylated IRF3, p-IRF3) was no different in SLE patients with or without MAVS aggregation (Figure S1A).

NLRC3 plays inhibitory roles during inflammation. It may interact with the RIG-I-MAVS pathway via stimulator of interferon genes (STING) (23). Enhanced NF-κB activation was observed in *nlrc3*$^{-/-}$ macrophages. NLRC3 can also regulate TRAF6 activation by modulating its K63-linked ubiquitination (23). K63 ubiquitin chains bind and activate RIG-I (3). Furthermore, MAVS aggregation leads to recruitment of TRAF6, promoting an inflammatory response. We analyzed protein levels of NLRC3 by Western blot. A slightly increased level of NLRC3 seemed to associate with aggregated MAVS, but the difference did not achieve statistical significance (Figure S1B). Further experiments will be needed to define whether NLRC3 regulates MAVS aggregation/activation. NLRX1 is another negative regulator of IFN-I (17) and inhibits the interaction between MAVS and RIG-I. NLRX1 did not seem to be involved in MAVS aggregation, as there was no difference in protein levels of NLRX1 between MAVS aggregate positive and negative SLE patients (Figure S1B). Surprisingly, we found expression of NLRX1 in cytoplasmic preparations from SLE patients (Figure S1B), although the majority of the protein does reside in the mitochondria. However, previous work was performed in mouse cells and not PBMC, thus this finding will need to be followed up with a more complete survey of expression in patients and in normals.

MAVS aggregates are associated with increased circulating IFN-I

Next, we examined the clinical significance of MAVS aggregates in SLE. We postulated that persistent MAVS aggregates might upregulate type I IFN production in SLE patients and consequently influence disease pathogenesis and clinical phenotype. To evaluate levels of IFN-I in the lupus cohort with positive MAVS aggregates, we first aligned our RNA-seq data to the human genome hg19 (24). Patients were clustered based on the expression of 27 interferon signature genes (25). Three major clusters were detected: high, moderate/medium, and low interferon signature. Frequency of MAVS aggregation positive patients was higher in the high interferon signature cluster (60% (6/10) of Agg+ in the high signature cluster compared to 30% (6/20) of Agg- in the same signature cluster) (Figure 4A). We then measured the IFN-β levels in plasma samples collected along with the PBMCs. We found significantly elevated IFN-β levels in MAVS Agg+ patients compared to both the Agg- SLE patients and the normal controls (Figure 4B). Taken together, MAVS aggregates showed a correlation with type I IFN responses in SLE.

A characteristic gene expression pattern in MAVS aggregates positive SLE patients

To identify a possible gene expression signature unique to MAVS aggregates positive SLE patients, we analyzed PBMCs from 27 MAVS SLE patients and 21 age, ethnicity, and sex-matched controls. We performed clustering analysis using the RNA-seq data. Patients with PBMC MAVS aggregation clustered together, suggesting that MAVS aggregation led to a specific differential gene expression pattern. 20 genes were up-regulated and 10 genes were

down-regulated in MAVS Agg+ SLE patients, compared with MAVS Agg- SLE patients (Figure 5). The most commonly up-regulated transcripts corresponded to DNA/protein binding and organelle trafficking (*Sdcbp2, Klc4, Kncn, and Cnnm3*). *Pias2*, a member of the protein inhibitor of activated STATs (PIAS) family, was down-regulated. Pias2 encodes a transcriptional co-regulator in the STAT pathway and p53 pathway, both playing roles in SLE pathogenesis. Notably, three genes (*Fkbp1a, Gimap1,* and *Sox4*) classified by gene ontology analysis are involved in T-cell activation, lymphocyte activation and leukocyte activation.

Disease Phenotype in MAVS-aggregate positive vs. negative SLE patients

We looked at disease activity and at the spectrum of organ involvement in patients with and without MAVS-aggregation. We compared demographics, drug therapy, and clinical features of disease between the two groups and found no significant differences, nor were there differences in SLEDAI (not shown) between groups. We analyzed serological data from MAVS aggregates-positive SLE patients and compared them to the MAVS aggregates negative SLE patients. Data are shown in Table I. All SLE patients were positive for anti-nuclear antibodies (ANA). Autoantibodies are recorded as positive or negative as recorded in the medical record (Table I). Using this measure, patients with MAVS aggregation were significantly more likely to have had antibodies to U1RNP and Sm. They were significantly less likely to have antibodies to cardiolipin. All of the stored plasma samples (from blood samples taken at the time MAVS aggregation was assessed) were subsequently re-analyzed by a commercial laboratory (RDL, Santa Monica, CA). As seen in Table I bottom, when this cohort of samples was analyzed, there were no significant differences in the prevalence of the panel of autoantibodies when aggregate positive patients were compared to aggregate-negative patients.

DISCUSSION

The connection between infections and autoimmunity has been known for decades. Microbial invasion is detected by cellular sensors and initiates immune responses through cytokine production. Cytosolic RIG-I-like helicases (RIG-I and MDA5) specifically bind viral RNAs and trigger a robust antiviral response through MAVS (26). Active MAVS undergoes significant biochemical changes, leading to its aggregation into large prion-like fibers, resistant to detergents and proteinases (22). Prion-like MAVS catalyzes further polymerization of native MAVS proteins, mediating downstream signaling and IFN production (27). Structural and biophysical analyses of MAVS indicate its respective prion conversion involves an all-or-none transition from monomers to polymers (10, 28, 29). Indeed, all MAVS aggregates in our study are essentially in the high-molecular form. The initial activation of the RIG-I pathway in SLE may be provoked by viral infection. The persistence of MAVS aggregates may reflect a decreased ability to degrade poorly soluble prion-like aggregates resulting from RIG-I signaling. Degradation of MAVS aggregates occurs by several mechanisms and may be required to shut down the IFN antiviral response. Delayed or impaired clearance of aggregated MAVS may represent a disease mechanism leading to increased steady-state IFN-I pathway activation.

MAVS aggregation was found in over a third of patients with SLE. Data regarding disease phenotype in MAVS-aggregate positive versus MAVS-aggregate negative patients were inconclusive. There was no apparent difference between aggregate positive and negative patients in disease manifestations, nor in disease activity as measured by SLEDAI. Clinical laboratory testing suggested an increased prevalence of anti-Sm and U1RNP antibodies; yet this was not seen when patient samples obtained at a different time were analyzed as a cohort. There was a trend toward statistical significance of the anti-Sm and U1RNP findings. Methodological differences between the two sets of autoantibody assays may explain results, as well as the fact that they were from samples taken at different times. Further study of the clinical significance of MAVS aggregation is needed.

It is of note that a few apparently normal individuals had evidence of MAVS aggregation. These people might be responding abnormally to common minor viral illnesses or might have constitutive MAVS aggregation that might predispose them to autoimmune disease. Further study of MAVS aggregation in these normal individuals might reveal an increased tendency to develop autoimmune disease.

Mutations in RIG-I like receptors may also provoke IFN-I secretion. The RLRs are usually expressed at very low levels. The sensitive and robust switch of MAVS to its prion state perpetuates downstream MDA5-mediated antiviral signaling (27). The constitutively activated form of MDA5 exerts a strong phenotype of autoimmunity in mice without viral infection. The autoimmune phenotype is MAVS and IFN-I dependent, as deficiencies of IFN-I receptor and MAVS abrogated the lupus-like clinical manifestations, respectively (10). Remarkably, human alleles of the same MDA5 mutation exhibited similar downstream effects, including constitutive IFN production. In MRL/lpr mice, exposure to 5′-triphosphate RNA aggravates autoimmunity and lupus nephritis (9). MAVS aggregates observed in our lupus patients may be triggered by the upstream RLRs.

Our patients showed a characteristic pattern of gene activation by microarray analysis of 47,000 genes. Principal Component Analysis revealed altered expression of genes involved in protein-protein interaction, vesicular trafficking, E3 ligase binding, and Stats inhibition. KLHL23 is an E3 ligase binding protein. E3 ligase mediated MAVS degradation is an important mechanism in regulating MAVS aggregates. Increased KLHL23 in MAVS aggregates patients may suggest a compromised mechanism toward the impaired degradation of MAVS polymer. The ability of mitochondria to fuse properly and maintain an adequate membrane potential is crucial for MAVS signaling. Of 20 genes showing enhanced expression, five (*Cyth4*, *Cyp4b1, Hoxc9, Kncn,* and *Klc4*) mediate the regulation of protein sorting and membrane trafficking. Interestingly, expression of *Pias2* (encoding a member of the protein inhibitor of activated Stat) was down regulated in MAVS aggregates positive patients. PIAS2 has a critical role as a transcriptional regulator of Stat-signaling (30). C1qr was significantly low in the MAVS aggregation positive SLE patients, compared to normal controls and MAVS aggregation negative SLE patients. Since C1qr plays an inhibitory role in MAVS activation/aggregation, lower C1qr may indicate a defect in controlling MAVS aggregation. We are particularly interested in the significantly decreased protein levels of MARCH5 in MAVS aggregation positive SLE patients as MARCH5 only binds to the aggregated form of MAVS (20). This decreased MARCH5 protein may account for the

persistent MAVS aggregation in our SLE patients. However, this abnormality deserves further study. Finally, it would also be important to analyze the signaling proteins with total protein extracts instead of cytosol and mitochondria fraction. Western blot with nuclear extractions may reveal the importance of IRF3 activation in the SLE Agg+ PBMCs.

Our finding of the aggregated form of MAVS in the PBMCs of SLE patients provides a potential mechanism linking innate immunity in the development of autoimmune disease, and may explain the elevated type I interferon levels in SLE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by the US National Institutes of Health (grant R21AR065692 to P.L.C; grant DK095067 to W.H.S.), and the Alliance for Lupus Research (P.L.C.).

We are grateful to Dr. Timothy B. Niewold for assistance with the analysis of IFN-I.

References

1. Pascual V, Farkas L, Banchereau J. Systemic lupus erythematosus: all roads lead to type I interferons. Curr Opin Immunol. 2006; 18(6):676–82. [PubMed: 17011763]
2. Theofilopoulos AN. TLRs and IFNs: critical pieces of the autoimmunity puzzle. J Clin Invest. 2012; 122(10):3464–6. [PubMed: 23154274]
3. Seth RB, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell. 2005; 122(5):669–82. [PubMed: 16125763]
4. Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, et al. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nature immunology. 2005; 6(10):981–8. [PubMed: 16127453]
5. Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, et al. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature. 2005; 437(7062):1167–72. [PubMed: 16177806]
6. Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB. VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol Cell. 2005; 19(6):727–40. [PubMed: 16153868]
7. West AP, Shadel GS, Ghosh S. Mitochondria in innate immune responses. Nature reviews Immunology. 2011; 11(6):389–402.
8. Sun Q, Sun L, Liu HH, Chen X, Seth RB, Forman J, et al. The specific and essential role of MAVS in antiviral innate immune responses. Immunity. 2006; 24(5):633–42. [PubMed: 16713980]
9. Allam R, Pawar RD, Kulkarni OP, Hornung V, Hartmann G, Segerer S, et al. Viral 5′-triphosphate RNA and non-CpG DNA aggravate autoimmunity and lupus nephritis via distinct TLR-independent immune responses. European journal of immunology. 2008; 38(12):3487–98. [PubMed: 19009528]
10. Funabiki M, Kato H, Miyachi Y, Toki H, Motegi H, Inoue M, et al. Autoimmune disorders associated with gain of function of the intracellular sensor MDA5. Immunity. 2014; 40(2):199–212. [PubMed: 24530055]
11. Biacchesi S, LeBerre M, Lamoureux A, Louise Y, Lauret E, Boudinot P, et al. Mitochondrial antiviral signaling protein plays a major role in induction of the fish innate immune response against RNA and DNA viruses. Journal of virology. 2009; 83(16):7815–27. [PubMed: 19474100]
12. Liu X, Jiao Y, Wen X, Wang L, Ma C, Gao X, et al. Possible association of VISA gene polymorphisms with susceptibility to systemic lupus erythematosus in Chinese population. Molecular biology reports. 2011; 38(7):4583–8. [PubMed: 21127988]

13. Pothlichet J, Niewold TB, Vitour D, Solhonne B, Crow MK, Si-Tahar M. A loss-of-function variant of the antiviral molecule MAVS is associated with a subset of systemic lupus patients. EMBO Mol Med. 2011; 3(3):142–52. [PubMed: 21268286]
14. Hou F, Sun L, Zheng H, Skaug B, Jiang QX, Chen ZJ. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell. 2011; 146(3):448–61. [PubMed: 21782231]
15. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics. 2004; 20(3):307–15. [PubMed: 14960456]
16. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic acids research. 2015; 43(7):e47. [PubMed: 25605792]
17. Allen IC, Moore CB, Schneider M, Lei Y, Davis BK, Scull MA, et al. NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRAF6-NF-kappaB signaling pathways. Immunity. 2011; 34(6):854–65. [PubMed: 21703540]
18. Xu L, Xiao N, Liu F, Ren H, Gu J. Inhibition of RIG-I and MDA5-dependent antiviral response by gC1qR at mitochondria. Proc Natl Acad Sci U S A. 2009; 106(5):1530–5. [PubMed: 19164550]
19. Xia P, Wang S, Xiong Z, Ye B, Huang LY, Han ZG, et al. IRTKS negatively regulates antiviral immunity through PCBP2 sumoylation-mediated MAVS degradation. Nat Commun. 2015; 6:8132. [PubMed: 26348439]
20. Yoo YS, Park YY, Kim JH, Cho H, Kim SH, Lee HS, et al. The mitochondrial ubiquitin ligase MARCH5 resolves MAVS aggregates during antiviral signalling. Nat Commun. 2015; 6:7910. [PubMed: 26246171]
21. Gambin A, Charzynska A, Ellert-Miklaszewska A, Rybinski M. Computational models of the JAK1/2-STAT1 signaling. JAKSTAT. 2013; 2(3):e24672. [PubMed: 24069559]
22. Moresco EM, Vine DL, Beutler B. Prion-like behavior of MAVS in RIG-I signaling. Cell Res. 2011; 21(12):1643–5. [PubMed: 21931357]
23. Schneider M, Zimmermann AG, Roberts RA, Zhang L, Swanson KV, Wen H, et al. The innate immune sensor NLRC3 attenuates Toll-like receptor signaling via modification of the signaling adaptor TRAF6 and transcription factor NF-kappaB. Nature immunology. 2012; 13(9):823–31. [PubMed: 22863753]
24. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 2009; 25(9):1105–11. [PubMed: 19289445]
25. Tcherepanova I, Curtis M, Sale M, Miesowicz F, Nicolette C. Results of a randomized placebo controlled phase IA study of AGS-009, A humanized anti-interferon-alpha monoclonal antibody in subjects with systemic lupus erythematosus. Ann Rheum Dis. 2012; 71:536–7.
26. Berke IC, Li Y, Modis Y. Structural basis of innate immune recognition of viral RNA. Cell Microbiol. 2013; 15(3):386–94. [PubMed: 23110455]
27. Cai X, Chen ZJ. Prion-like polymerization as a signaling mechanism. Trends Immunol. 2014; 35(12):622–30. [PubMed: 25457352]
28. Reikine S, Nguyen JB, Modis Y. Pattern Recognition and Signaling Mechanisms of RIG-I and MDA5. Front Immunol. 2014; 5:342. [PubMed: 25101084]
29. Potter JA, Randall RE, Taylor GL. Crystal structure of human IPS-1/MAVS/VISA/Cardif caspase activation recruitment domain. BMC Struct Biol. 2008; 8:11. [PubMed: 18307765]
30. Shuai K. Modulation of STAT signaling by STAT-interacting proteins. Oncogene. 2000; 19(21): 2638–44. [PubMed: 10851063]
31. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010; 26(1):139–40. [PubMed: 19910308]

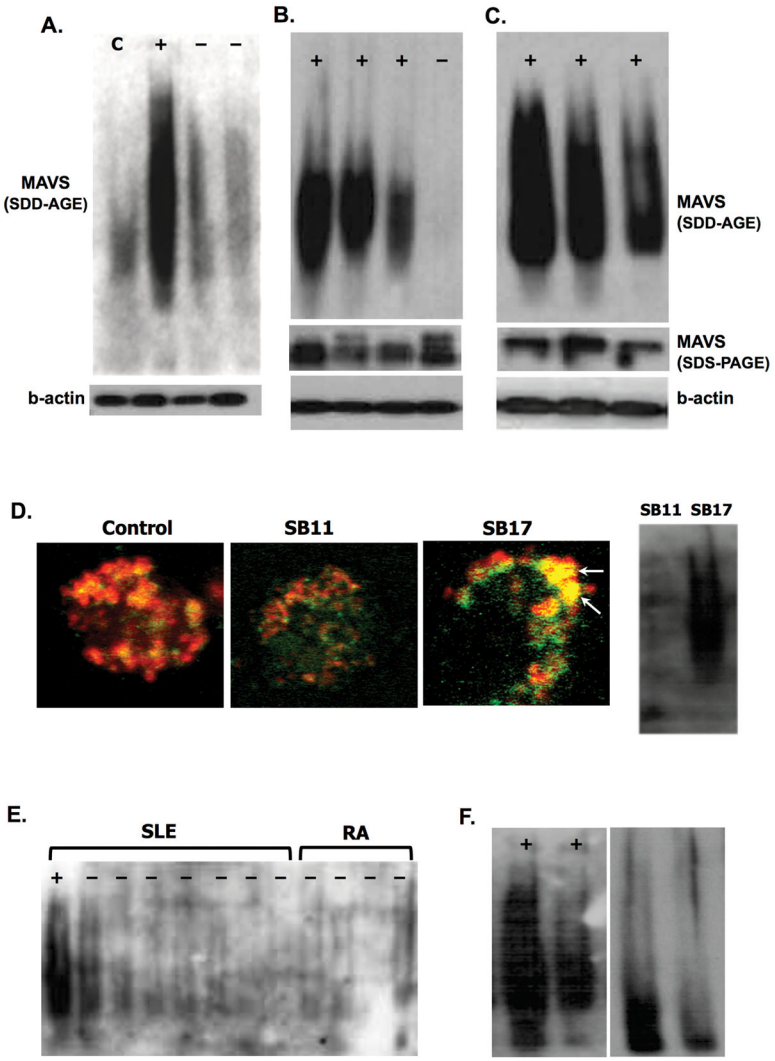

Figure 1. MAVS aggregation in SLE patients

A. MAVS aggregation was assessed by SDD-AGE ("**c**". Jurkat T cell control; "+". Aggregate positive; "−", Aggregate negative). **B** and **C,** MAVS aggregation was detected and confirmed both in SDD-AGE and SDS-PAGE gel with antibodies from Abcam **(B)** and Santa Cruz **(C).** β-actin was used to indicate the total protein loading. **D.** Visualization of MAVS aggregates from lupus patients. Normal control (left), aggregate-negative patient (SB11 shown in SDD-AGE, right panel), and aggregate-positive lupus patient (arrows, SB17 shown in SDD-AGE, right panel). MAVS aggregation were shown in yellow clusters (merge of MAVS-FITC and MitoTracker-Red) under confocal microscope. **E.** PBMC samples from lupus patients (let) and RA patients (right) were analyzed by SDD-AGE. **F.** 2 MAVS aggregate positive samples were treated with β-mercaptoethanol and analyzed by SDS-AGE in parallel. Data are representative of 67 SLE patients and 33 controls.

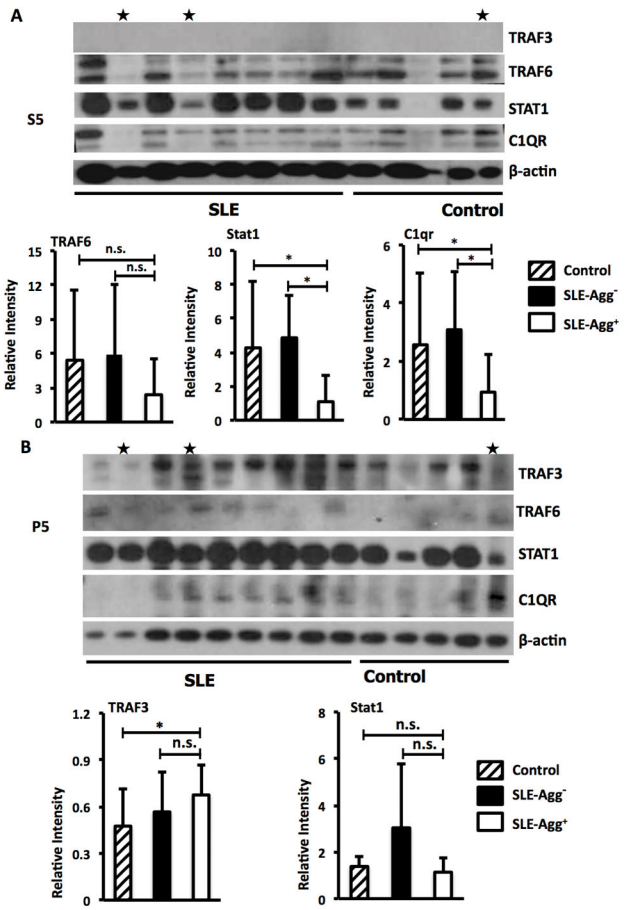

Figure 2. Levels of protein involved in MAVS signaling

MAVS associated proteins in the cytoplasm samples (S5, **A**) and mitochondria samples (P5, **B**) were analyzed by Western blot. Representative Western images are shown (MAVS aggregates positive samples were indicated with '★'). Densitometry analysis to quantify ratio of indicated protein to b-actin is shown at the bottom. Values are expressed as median with interquartile range. Statistical test is the Mann-Whitney test, * $p<0.05$.

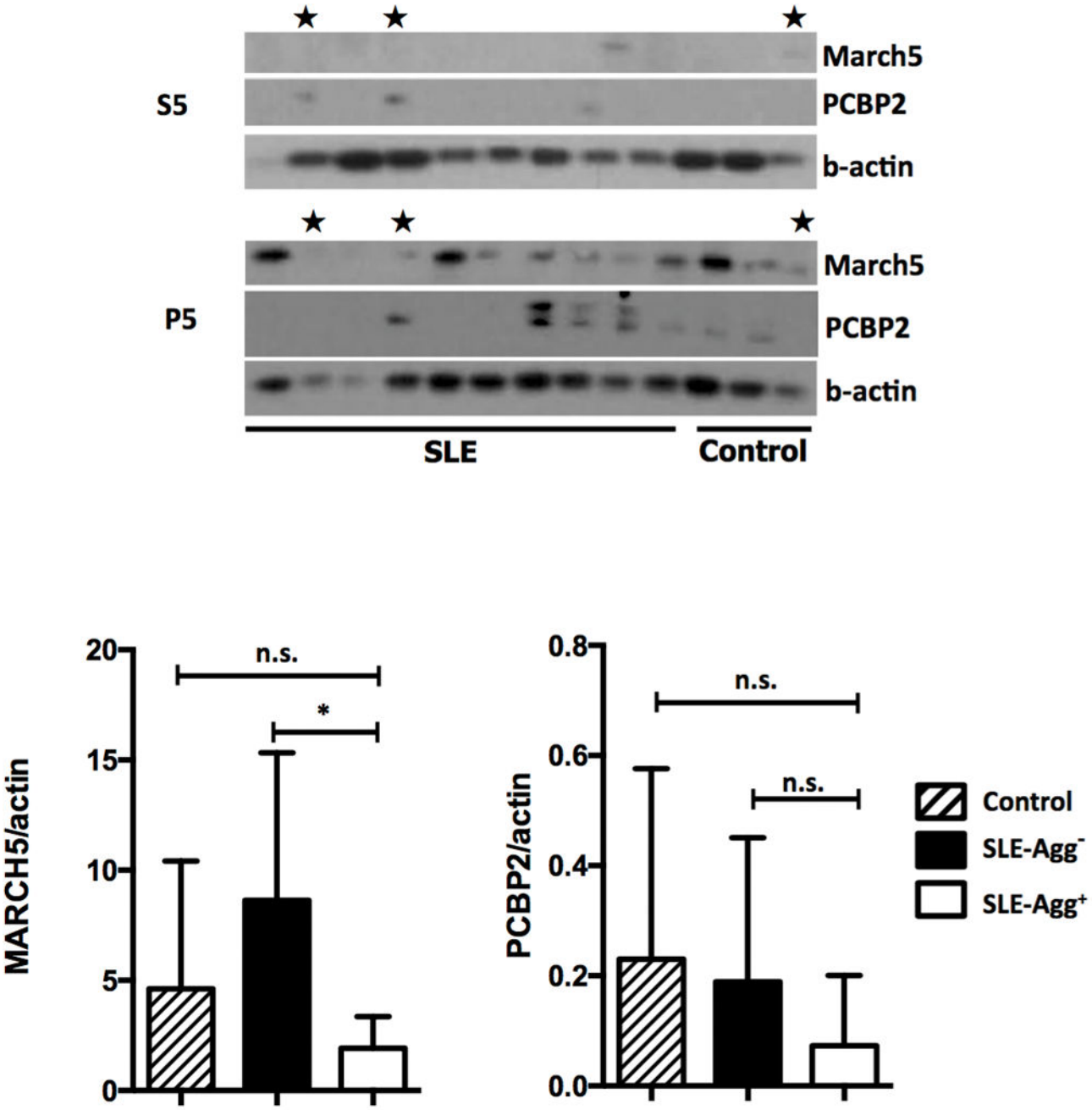

Figure 3. Decreased MARCH5 is associated with MAVS aggregation

MAVS negative regulators PCBP2 and MARCH5 were analyzed by Western blot in the cytoplasm samples (S5) and mitochondria samples (P5). Representative Western images are shown (MAVS aggregates positive samples were indicated with '★'). Densitometry analysis to quantify ratio of indicated protein to β-actin is shown at the bottom. Values are expressed as median with interquartile range. Statistical test is the Mann-Whitney test, * $p<0.05$.

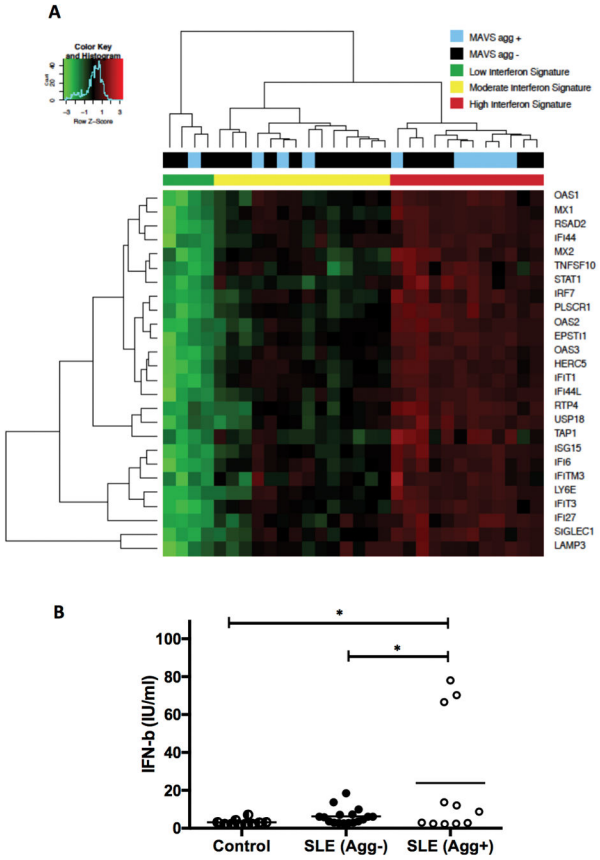

Figure 4. Increased IFN-I signature in MAVS Agg+ SLE patients

A. RNA-seq data (100-bp paired-end reads) were aligned to the human genome hg19 using TopHat (24). Libraries normalization was performed and FPKM for each RefSeq gene was calculated using Edger, a package in R (31). 27 interferon related genes, used by ARGOS in their clinical trial were used to cluster patients based on expression of this interferon signature (25). **B.** IFN-β levels in the plasma samples of SLE patients and normal controls were measured using a human IFN-β ELISA kit (Fujirebio Inc. Tokyo, Japan). Results were expressed as individual data points with means ±SEM. Statistical test is the unpaired *t* test, * $p<0.05$.

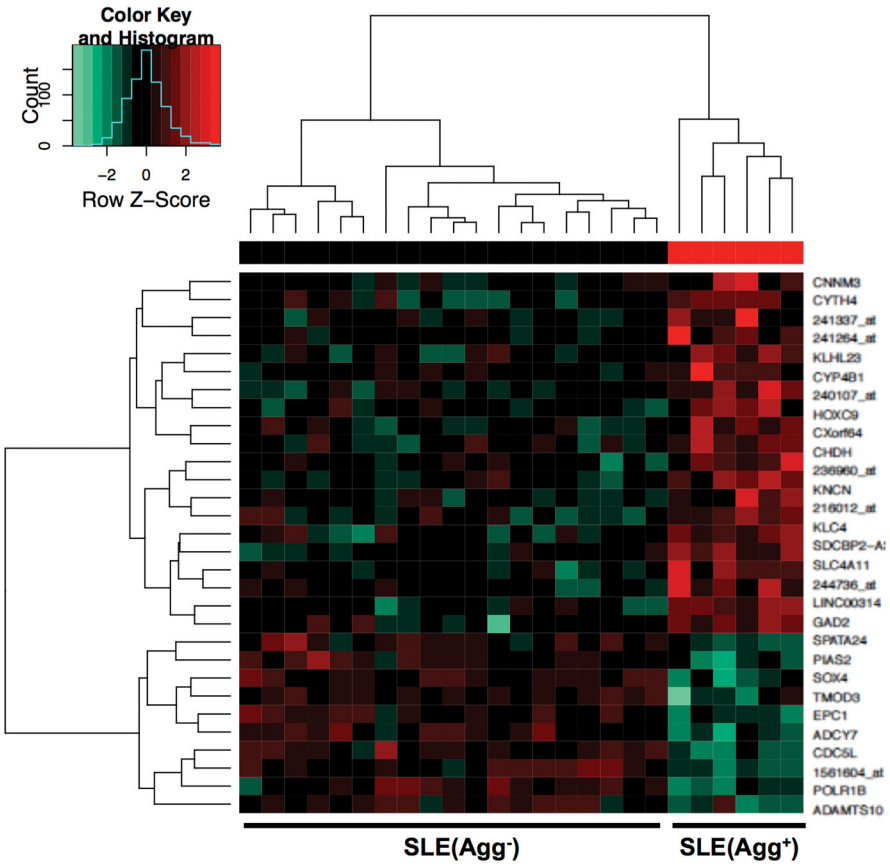

Figure 5. Signature gene expression pattern in MAVS aggregation positive patients

Microarray was run on Affymetrix HT-HG-U133-plus (15). A Support Vector Machine (SVM) with Recursive Feature Selection was applied to determine which genes divided MAVS Agg+ from Agg- samples. The top 30 genes were selected from the SVM analysis and clustering analysis was performed using Pearson correlation.

Table I

Demographic and serological characteristic of MAVS aggregation positive & negative SLE patients.

Parameter	MAVS aggregate negative (n=45)	MAVS aggregate positive (n=22)
Age	40.58 (± 13.78)	52.24 (± 12.73)
Race		
White: 8.9%	8.9%	4.5%
African American	53.33%	63.6%
Latino	35.6%	27.3%
Asian	2.2%	0.0%
Other	0	4.5%
Treatment		
Hydroxychloroquine	84.4%	90.95
Mycophenolate	35.6%	27.7%
Prednisone and dose	48.9% (12.3±14.2mg)	63.4% (15.08±9.0mg)
Aspirin	22.2%	42.9%

Serology from medical records							
	Aggregate Negative			**Aggregate Positive**			**Significance**
	Total number	**Positive (number)**	**Positive (%)**	**Total number**	**Positive (number)**	**Positive (%)**	
ANA	41	39	95.1	22	22	100.0	
ds-DNA Ab	42	27	64.3	18	13	72.2	
U1RNP	25	9	36.0	15	13	86.7	p<0.01
Sm	25	8	32.0	15	10	66.7	p<0.05
Ro/SSA	31	19	61.3	20	15	75.0	
La/SSB	28	9	32.1	18	9	50.0	
Cardiolipin	27	12	44.4	16	2	12.5	p<0.05
Lupus anticoagulant	24	4	16.7	13	3	23.1	
Anti-b2 microglobulin	24	11	45.8	14	6	42.9	
Anti-phospholipid Ab	9	6	66.7	3	1	33.3	
Lupus Nephritis	45	20	44.4	23	5	21.7	

Serology from medical records						
	Aggregate Negative			Aggregate Positive		Significance
	Total number	Positive (number)	Positive (%)	Total number	Positive (number)	Positive (%)	
Anti-Phospholipid Syndrome	12	6	50.0	5	3	60.0	
RF	5	3	60.0	2	0	0.0	
Serology performed by RDL laboratories							
ANA	41	41	100.0	22	22	100.0	
ds-DNA Ab	41	20	48.8	22	16	72.7	P=0.12
U1RNP	41	22	53.7	22	16	72.7	P=0.42
Sm	41	15	36.7	22	11	50.0	P=0.42
Ro/SSA	41	19	46.3	22	15	68.2	P=0.12
La/SSB	41	7	17.1	22	6	27.3	
Cardiolipin IgG	41	1	2.4	22	1	4.5	
Cardiolipin IgA	41	1	2.4	22	1	4.5	
Cardiolipin IgM	41	2	4.9	22	2	9.1	