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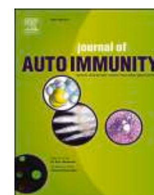

Splicing machinery is profoundly altered in systemic lupus erythematosus and antiphospholipid syndrome and directly linked to key clinical features

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A R T I C L E I N F O

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A B S T R A C T

Objectives: To characterize the splicing machinery (SM) of leukocytes from primary antiphospholipid syndrome (APS), systemic lupus erythematosus (SLE) and antiphospholipid syndrome with lupus (APS + SLE) patients, and to assess its clinical involvement.
Methods: Monocytes, lymphocytes and neutrophils from 80 patients (22 APS, 35 SLE and 23 APS + SLE) and 50 HD were purified, and 45 selected SM components were evaluated by qPCR-microfluidic array. Relationship with clinical features and underlying regulatory mechanisms were assessed.
Results: APS, SLE and APS + SLE leukocytes displayed significant and specific alterations in SM-components (SMC), associated with clinical features [autoimmune profiles, disease activity, lupus nephritis (LN), and CV-risk markers]. A remarkable relationship among dysregulated SMC in monocytes and the presence of LN in SLE was highlighted, revealing a novel pathological mechanism, which was further explored. Immunohistology analysis of renal biopsies highlighted the pathological role of the myeloid compartment in LN. Transcriptomic analysis of monocytes from SLE-LN(+) *vs* SLE-LN(−) identified 271 genes differentially expressed, mainly involved in inflammation and IFN-signaling. Levels of IFN-related genes correlated with those of SMC in SLE-LN (+). These results were validated in two external SLE-LN(+) datasets of whole-blood and kidney biopsies. In vitro, SLE-LN(+)-serum promoted a concomitant dysregulation of both, the IFN signature and several SMC, further reversed by JAKinibs treatment. Interestingly, IFNs, key inflammatory cytokines in SLE pathology, also altered SMC. Lastly, the over/down-expression of selected SMC in SLE-monocytes reduced the release of inflammatory cytokines and their adhesion capacity.
Conclusion: Overall, we have identified, for the first time, a specific alteration of SMC in leukocytes from APS, SLE and APS + SLE patients that would be responsible for the development of distinctive clinical profiles.

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1. Introduction

Primary Antiphospholipid Syndrome (APS), systemic lupus erythematosus (SLE) and antiphospholipid syndrome with lupus (APS + SLE) are three narrowly related autoimmune disorders that share common and distinctive clinical and serological features, which have major implications for the therapy.

APS is a rare and complex autoimmune disorder characterized by recurrent venous and/or arterial thrombosis and pregnancy complications in the presence of antiphospholipid antibodies (aPL), including anticardiolipin antibodies (aCL), anti-beta2glycoprotein I (anti-β2GPI) antibodies, and lupus anticoagulant (LA) [1]. APS pathogenesis was centered on aPL-mediated coagulation and fibrinolysis disruption but an interplay between inflammatory and thrombotic mechanisms has been increasingly recognized involving aPL-mediated platelet, monocyte and endothelial cell activation, adhesion molecule alterations and pro-inflammatory cytokines activation, complement and neutrophils activation, and tissue factor upregulation [2].

SLE is a multisystemic autoimmune disease that affects numerous organs such as the skin, joints, lungs, heart, kidneys, and nervous system. Its etiology is multifactorial and includes genetic and environmental factors, as well as several epigenetic, hormonal and immunoregulatory mechanisms. The involved pathophysiological mechanisms include decreased immune tolerance, production of antibodies, deposition of immune complexes on target tissues, and activation of the complement system [3].

Approximately one-third of SLE patients with persistently positive aPL develop arterial or venous thrombotic events, the so-called SLE-associated APS [4]. In addition to classic thrombotic events, severe manifestations of SLE, such as alveolar hemorrhage, renal microangiopathy, myelitis, adrenal insufficiency and cognitive impairment, are also more common among SLE patients with positive aPL and/or APS.

Long-term follow-up studies of SLE patients have shown more severe damage in patients with coexisting APS vs those without APS and a significantly lower cumulative survival at 15 years in SLE + APS [5].

Therefore, it is of great relevance to clearly define each clinical entity and to evaluate the patients based upon a complete knowledge of the underlying disease processes.

To date, although extensive findings have been accomplished regarding the genetic and epigenetic factors that predispose to the development and/or progression of these pathologies [6], no study has so far fully characterized the potential role of posttranscriptional mechanisms such as the alternative splicing.

Splicing is a sequential and coordinated process of RNA editing by which the introns of an immature RNA are excised and the exons are combined to generate mature RNAs. Splicing recognizes exon–intron boundaries and is catalyzed and controlled by a complex ribonucleoprotein complex known as spliceosome [7]. The spliceosome consists of an intricate network of at least 300 proteins associated to a discrete core of five uracil-rich small nuclear ribonucleoproteins (U1, U2, U4, U5, and U6 snRNPs). Proper spliceosome assembly at the splice sites (ss) is the core reaction mechanism. Although splicing may be constitutive or alternative, around 95% of human genes undergo physiological processes of alternative splicing, which consist in the differential reorganization of the exons to generate various mature RNAs, thus allowing the generation of alternative protein species. Alternative splicing is a pivotal mechanism widely acknowledged to regulate gene expression and promote protein diversity. The maintenance of functional immune responses requires protein diversity and flexibility, which are essential in alternative splicing regulation. Many genes involved in innate or adaptive immune signaling undergo varying degrees of alternative splicing. Ergun et al. determined by RNA sequencing and microarray that alternatively spliced isoforms occur in 60% of all T- or B-lymphocyte genes [8]. Based on spliced gene functions, alternative splicing may affect the physiological function of the immune system in different ways.

The correct function of the splicing machinery is an essential mechanism to maintain normal cell physiology and whole-body homeostasis, so that minimal alterations in this tightly regulated process can lead to the generation of deficient proteins, contributing to many human disorders, including autoimmune diseases. Several studies have investigated the roles of alternative splicing in autoimmunity mechanisms. Among them, immune-related genes encoding IFN regulatory factor 5 (IRF5), T-cell receptor ζ (TCRζ) chain, leukocyte immunoglobulin-like receptors (LILRs), B-cell scaffold protein with ankryn repeats 1 (BANK1), Ras guanyl releasing protein 1 (RasGRP1), among others, are best-studied examples of alternative splicing that affect both the adaptive- and innate immune signaling-related genes associated with SLE [9]. However, the altered expression of spliceosome components in immune cells as well as the pathological roles of dysregulated splicing machinery components (SMC) in APS, SLE and APS + SLE is still unknown.

With these premises, the aims of this study were, firstly, to identify shared and differential changes in the splicing machinery of immune cells from APS, SLE and APS + SLE patients, and their involvement in the activity and clinical profile of these autoimmune disorders. We also aimed at characterizing the underlying molecular mechanisms of these alterations and their involvement in immune cells activity.

2. Materials and methods

2.1. Patients and controls

One hundred and eighty patients and fifty healthy donors (HD) were included in the study (during a 48-month period) after obtaining approval from the ethics committee of the Reina Sofia University Hospital (Córdoba, Spain), and involving two patients' cohorts. The first cohort comprised 22 primary APS patients, 35 SLE patients and 23 APS + SLE patients, as well as 50 HD, who's clinical and laboratory details are displayed in Table 1. All patients fulfilled the American College of Rheumatology criteria for the classification of APS, SLE and APS + SLE respectively [10,11]. Patients and HD provided written informed consent. None of the HD had a history of other autoimmune diseases, atherosclerosis, or thrombosis.

The second cohort consisted of 50 SLE patients diagnosed with lupus nephritis on adequate renal biopsy specimens, including 42 women and 8 men, with a mean age of 31 ± 11 years, median disease activity (SLEDAI-R) of 7,5 ± 4,9, and a chronicity index of 2,2 ± 2,1. Following the international society of Nephrology/Renal pathology society (ISN/RPS) classification (based on light microscopy, immunofluorescence and electron microscopy findings from renal biopsy specimens) [12], more than 75% of patients were categorized as class III or IV lupus nephropathy.

The study was conducted in accordance with the Declaration of Helsinki principles.

Blood sample collection, assessment of clinical and biological parameters, B-mode ultrasound IMT measurements and immunohistochemical study in renal biopsies are detailed in online Supplementary materials.

2.2. Analysis of splicing machinery components by qPCR dynamic array based on microfluidic technology

Total RNA from monocytes, neutrophils and lymphocytes was isolated using TRIsure (Bioline, Memphis, TX, USA) followed by a DNAse treatment (RQ1DNAse, Promega; Wisconsin, USA) ensuing manufacturer's instructions. RNA purity and concentration were evaluated using Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). 1000 ng of total RNA were retrotranscribed using NZY Reverse Transcriptase kit (NZYTech, Lisboa, Portugal) using random hexamer primers [13]. A 48.48 Dynamic Array (Fluidigm, San Francisco, CA, USA) was used to assess the expression of 45 selected transcripts of the

Table 1
Clinical and analytical profiles of APS, SLE and APS + SLE patients and HD recruited to the study.

Clinical parameters	HD (n = 50)	APS (n = 22)	APS + SLE (n = 23)<	SLE (n = 35)
Woman/man	42/8	14/8	21/2	29/6
Age, years (mean ± SD)	47 ± 15	53 ± 10	52 ± 9	42 ± 12
Time of evolution (mean ± SD)	–	10 ± 7	15 ± 9	14 ± 9
aGAPSS (mean ± SD)	–	7 ± 4	7 ± 4	–
SLEDAI (mean ± SD)	–	–	0,4 ± 0,8	2 ± 2
Renal involvement, n (%)	–		4/23 (17%)	19/35 (54%)
Obstetric morbidities, n (%)	–	7/14 (50%)	12/21 (57%)	6/29 (20%)
Thrombosis, n (%)	–	13/22 (60%)	23/23 (100%)	4/35 (11%)
Recurrences, n (%)	–	9/22 (40%)	8/23 (34%)	2/35 (5%)
Arterial Hypertension, n (%)	–	16/22 (72%)	17/23 (73%)	6/35 (17%)
Hyperlipidaemia, n (%)	–	13/22 (60%)	18/23 (78%)	10/35 (28%)
Laboratory parameters				
LA positivity, n (%)	–	19/22 (86%)	18/23 (78%)	–
aCL-IgG/IgM positivity, n (%)	–	7/22 (32%)	7/23 (30%)	2/35 (5%)
Anti-B2GPI-IgG/IgM positivity, n (%)	–	11/22 (50%)	4/23 (17%)	4/35 (11%)
Anti-dsDNA positivity, n (%)	–	0/22 (0%)	7/23 (30%)	17/35 (48%)
CRP, mg/dL (mean ± SD)	1 ± 3	3 ± 5	6 ± 10	8 ± 23
ESR, mm/h (mean ± SD)	8 ± 5	13 ± 15	20 ± 15	23 ± 21
Treatments				
Corticoids, n (%)	–	3/22 (12%)	16/23 (50%)	20/35 (57%)
Antimalarials, n (%)	–	2/22 (7%)	15/23 (47%)	20/35 (57%)
Anticoagulants/ Antiplatelets, n (%)	–	10/22 (45%)	14/23 (44%)	7/35 (22%)

major and minor spliceosome and associated splicing factors as previously reported [14–18]. *(See online* Supplementary materials*).*

2.3. Bioplex assay of the inflammatory profile in plasma of APS, SLE and APS plus SLE patients

(See online Supplementary materials).

2.4. Transfection studies to evaluate the effects of altered SMC on the immune cell's activity

Peripheral blood leukocytes subsets from SLE patients (as the most prevalent of the diseases evaluated) were used for transfection studies. Briefly, 500,000 cells/well were transiently overexpressed with *MAGOH, TRA2B or KHDRBS1* (OHu20035, Genscript, Leiden, The Netherlands) vectors for 24 h using Lipofectamine 2000 (Life Technologies). Empty pCDNA3.1^{+} (mock, Life Technologies, Grand Island, NY, USA) was used as negative control. Effectiveness of transfections was assessed by RT-PCR. Chemotaxis, proliferation and NETosis were further assessed (see online Supplementary materials).

2.5. In vitro studies

Two sets of *in vitro* experiments were developed to interrogate mechanistically the role of the altered SM in SLE.

1. Treatment of HD monocytes with both IFNalpha and IFNgamma, key cytokines linked to the pathophysiology of SLE, to assess their involvement in the aberrant expression of the altered SMC observed *in vivo* in SLE patients.

Five hundred thousand cells/well from HD peripheral blood monocytes were treated with either, IFNα (10 ng/mL) or IFNγ (100 ng/mL) for 24 h, and effects on SMC were assessed by RT-PCR.

2. Treatment of HD monocytes with serum from SLE patients with lupus nephritis, either, in the presence or in the absence of two JAK inhibitors to evaluate its effects on the expression of SMC.

Five hundred thousand cells/well from HD peripheral blood monocytes were treated with 10% serum from SLE patients with nephropathy for 24 h. In addition, these treatments were performed in the absence or in the presence of either, Tofacitinib (10 μg/mL) or Baricitinib (10 μg/mL), which were added 2 h before the incubation with SLE serum, and effects on SMC were assessed by RT-PCR.

2.6. Transfection studies to evaluate the effects of overexpression or silencing of SMC on SLE-LN(+) patients' monocyte activity

Monocytes from SLE-LN(+) patients (500,000 cells/well) were processed to transiently overexpressed PTB (ThermoFisher Scientific, Dreieich, Germany) or silenced RBM17 (ThermoFisher Scientific) for 24 h.

Then, effectiveness of transfections was assessed by RT-PCR. Cell-adhesion was evaluated using Vybrant cell adhesion kit (Molecular Probes Inc, Leiden, The Netherlands).

In this set of experiments, a 27-plex panel of secreted inflammatory molecules was further assessed in the supernatant of cultured SLE-monocytes (Bioplex).

2.7. NanoString array and RT-PCR

(See online Supplementary materials for details).

2.8. Characterization of biological functions and pathways of altered genes

Genes differentially expressed in monocytes were functionally classified and used for identifying enriched pathways and functional interconnected networks using the genetic ontology enrichment analysis (http://geneontology.org/).

2.9. Public datasets analyses

Public data of various external cohorts of SLE patients (E-MTAB-2713; GSE99967; GSE32591) were analysed as independent cohorts to gain insight in the splicing alteration and its association with relevant genes involved in SLE pathology. (See online Spplementary materials).

2.10. Statistical analysis

Data were expressed as mean ± SEM or median ± IQR according to data distribution, evaluated using Kolmogorov-Smirnov test. Student's *t*-test or Mann-Whitney rank sum test were used to assess statistical differences in unpaired data, and paired t tests and Wilcoxon matched-pairs signed rank tests for paired data. The chi-square test was used to associate qualitative variables. Correlations were evaluated by Spearman's correlation test. For adjusting the p-values towards multiple hypothesis testing, a Benjamini Hochberg-based false discovery rate (FDR) was applied. Statistically significant differences were considered at p-value<0.05.

Data analyses were performed using SPSS 24.0 (IBM, Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA).

3. Results

3.1. The splicing machinery is profoundly altered in APS, SLE and APS plus SLE patients

Patients with primary APS, SLE or APS + SLE displayed a significant and specific alteration in the SMC compared with HD, including major and minor spliceosome components and splicing factors (Fig. 1A–F).

All leukocytes' subtypes mainly showed a reduced expression of several SMC, that was further specific for each cell-type and pathology. However, a few numbers of SMC were commonly altered on each leukocyte subset, including MAGOH, TRA2B or KHDRBS1, among others. Monocytes seemed to be the leukocyte subset more deeply altered (Fig. 1D–F). These results were validated in an external dataset of monocytes from an independent lupus cohort (E-MTAB-2713) [19] (Supplementary Fig. 1).

3.2. Altered expression of SMC was related to key clinical features on each disease

Correlation studies allowed to identify significant and disease-specific correlations among levels of altered SMC and clinical features such as autoimmune profiles (i.e. titres of aCL, anti-Beta2-GPI and anti-dsDNA antibodies), the disease activity scores (SLEDAI and aGAPSS), presence of lupus nephritis, and CV-risk markers including CIMT, hyperlipidaemia (atherogenic index and ApoB/A ratio), and arterial hypertension (Fig. 2).

Interestingly, a remarkable relationship among dysregulated SMC in monocytes and the presence of lupus nephritis was highlighted, pointing to the potential key involvement of this novel altered mechanism in this particular cell type in the development of renal damage in SLE patients, which was further explored in this study.

3.3. Restoration of altered levels of SMC modulated key functional endpoints in leukocytes

Next, we aimed to prove whether restoration of altered levels of splicing machinery factors might have a positive impact in the altered activity of leukocytes. Specifically, we evaluated the effects of *MAGOH, TRA2B* and *KHDRBS1* overexpression (Fig. 3). These genes were selected based on their altered and common low expression in the three evaluated pathologies and their associations with relevant clinical features previously observed (Figs. 1–2, and Supplementary Fig. 1).

The overexpression of these genes (Supplementary Fig. 2A) reversed the pathological leukocytes activity to normal-like levels. Specifically, overexpression of MAGOH reduced chemotaxis in monocytes (Supplementary Fig. 2B), the overexpression of TRA2B inhibited cell proliferation in lymphocytes (Supplementary Fig. 2C), and finally, the overexpression of KHDRBS1 promoted a downregulation of NETosis features, involving reduced cell-free nucleosomes generated by neutrophils (Supplementary Fig. 2D).

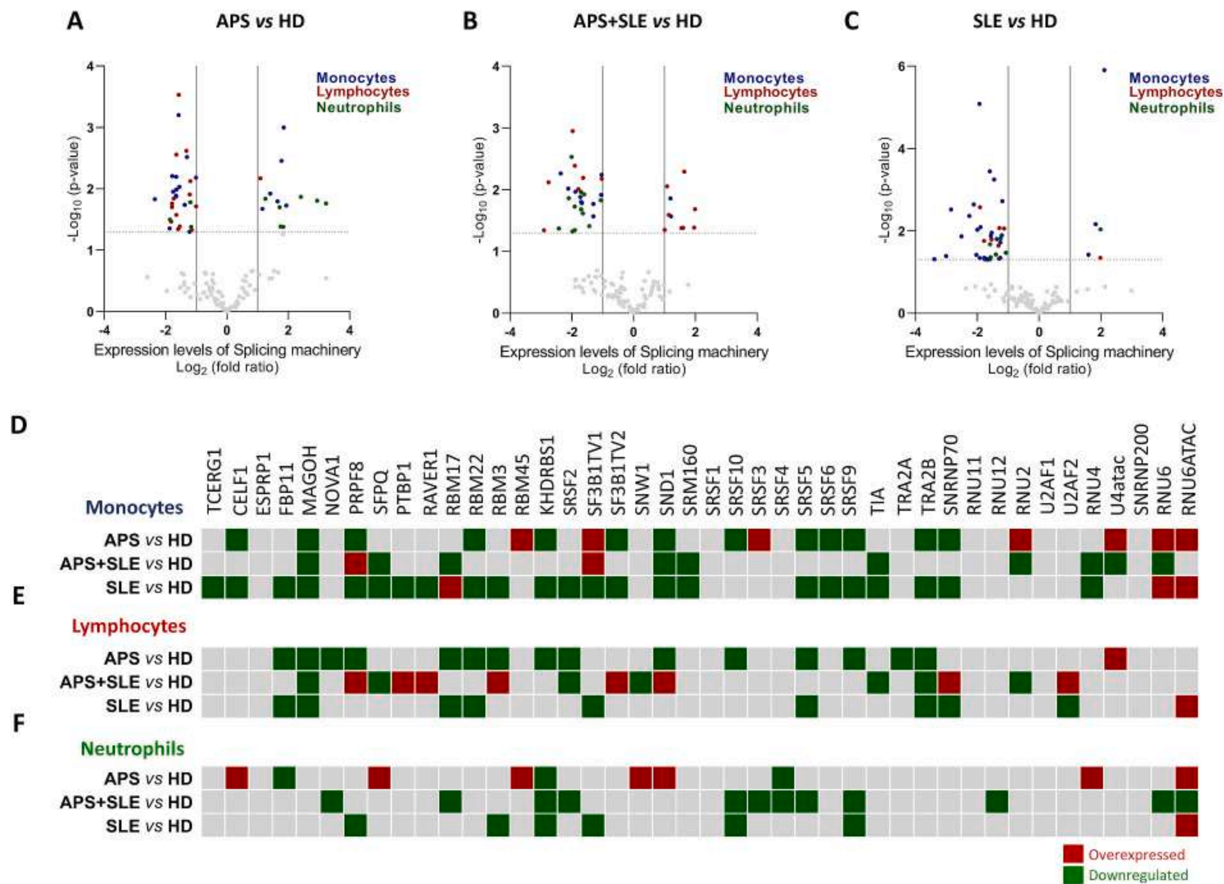

Fig. 1. The splicing machinery is profoundly altered in APS, SLE and APS plus SLE patients.
Expression levels of major and minor spliceosome and associated splicing factors were quantified through a 48.48 Dynamic Array (Fluidigm) in monocytes, lymphocytes and neutrophils from 22 APS patients, 23 APS plus SLE patients, 35 SLE patients, and 50 HD. Volcano plots showing the expression levels of SMC in monocytes, lymphocytes and neutrophils from (A) APS vs HD, (B), APS plus SLE vs HD and (C), SLE vs HD. (D, E and F) Heat maps showing differential expression in the splicing machinery components from different leukocyte subsets between APS, APS plus SLE and SLE patients and HD (log10 fold change). Green and red colours represent downregulated and upregulated splicing machinery elements, respectively, showing significant differences ($p < 0.05$).

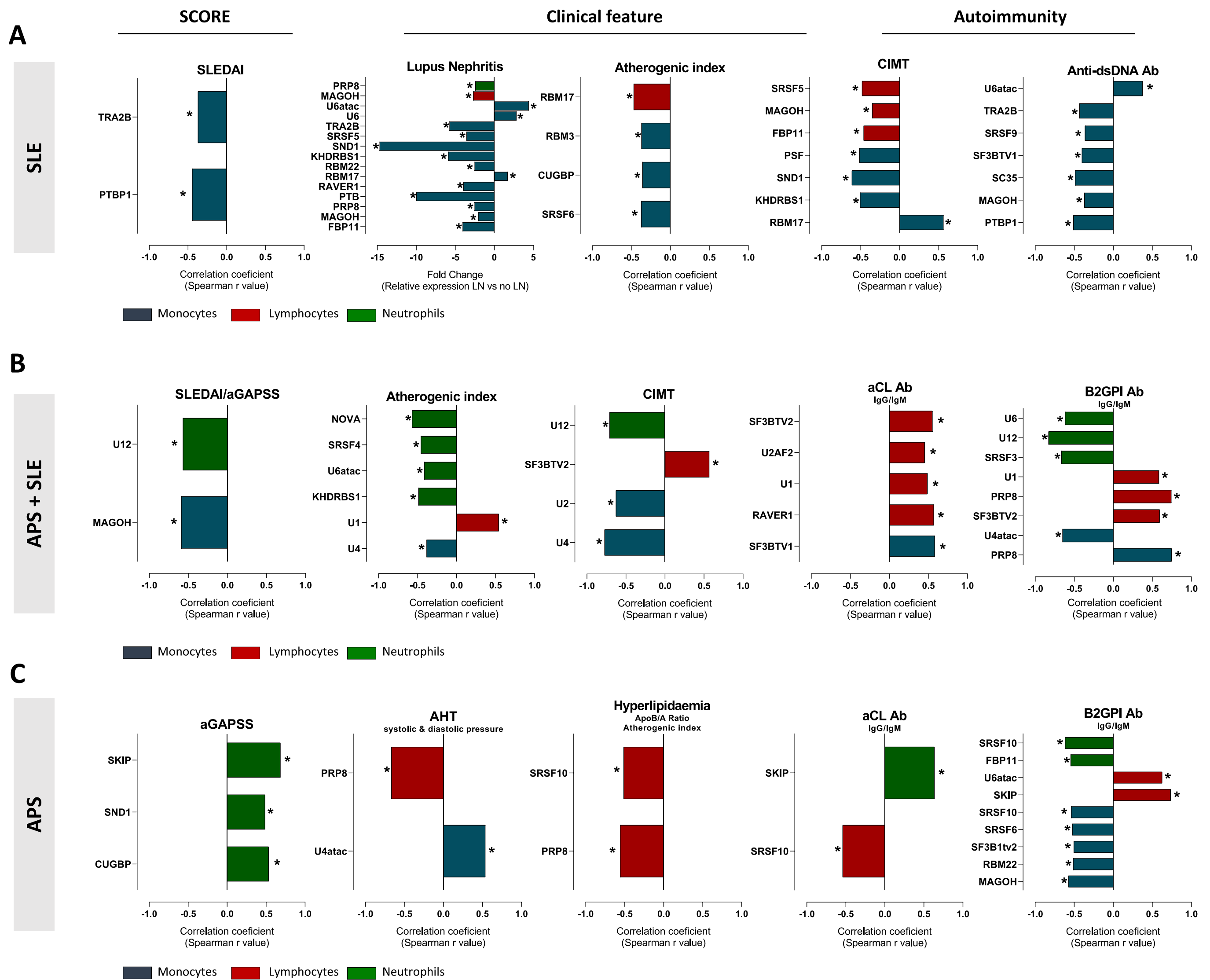

Fig. 2. Altered expression of SMC was related to key clinical features on each disease.
Correlation (Spearman) and association analyses between spliceosome components in each leukocyte subtype and clinical/analytical profiles of APS, APS + SLE and SLE patients. Those showing a p < 0,05 are shown.

3.4. Simultaneous alterations of splicing machinery and key transcriptional signatures were identified in monocytes from SLE patients with lupus nephropathy

To unravel the role of monocytes in lupus nephropathy, immunohistochemical analysis of renal biopsies from an independent cohort of SLE patients with LN was carried out. The analysis showed the presence of macrophage infiltration in all samples, most of them with a relevant CD68 labelling percentage between 6 and 50% (Supplementary Fig. 3A and B).

Furthermore, the percentage of macrophage infiltration was associated with the biopsy chronicity index (fibrosis), the renal activity index and the levels of C1q and C3 deposited in the kidney (Supplementary Fig. 3C).

These results evidenced the presence of macrophage infiltration associated with renal damage in SLE patients, highlighting the potential pathological role of the myeloid compartment in the development of lupus nephritis.

To gain further insight on this association, the transcriptomic profiles of monocytes from SLE patients with LN [LN(+)] *vs* patients without LN [LN(−)] were evaluated in an exploratory array analysis. Thus, 271 genes were differentially expressed between both groups, including 152 overexpressed and 119 downregulated (Fig. 3A, Supplementary Table 1). Functional analysis of the differentially expressed genes showed that the main enriched pathways were associated with inflammatory processes such as cytokine-mediated signalling, cellular response to cytokine stimuli, cellular response to type I and type II interferons, and interferon (IFN) signalling (Fig. 3B).

Among the deranged genes found in SLE-LN(+) patients from the exploratory analysis, a specific gene signature was selected for validation based on its higher differential expression vs LN(−) and its association with the main pathways altered in this disease, such as the IFN pathway. qPCR analysis in the whole cohort of patients validated the alteration of IFN-pathway-related genes in monocytes from SLE-LN(+) (Fig. 3C). Interestingly, the expression of IFN-related genes was inversely correlated with the levels of a number of SMC, which was particularly more prominent in SLE-LN(+) patients (Fig. 3D). These particular results were further validated in an external dataset of whole blood from lupus patients, where a strong relationship among SMC and IFN-related genes was again identified in SLE-LN(+) patients (GSE99967) (Fig. 3E).

We hypothesized that if these associations observed in peripheral

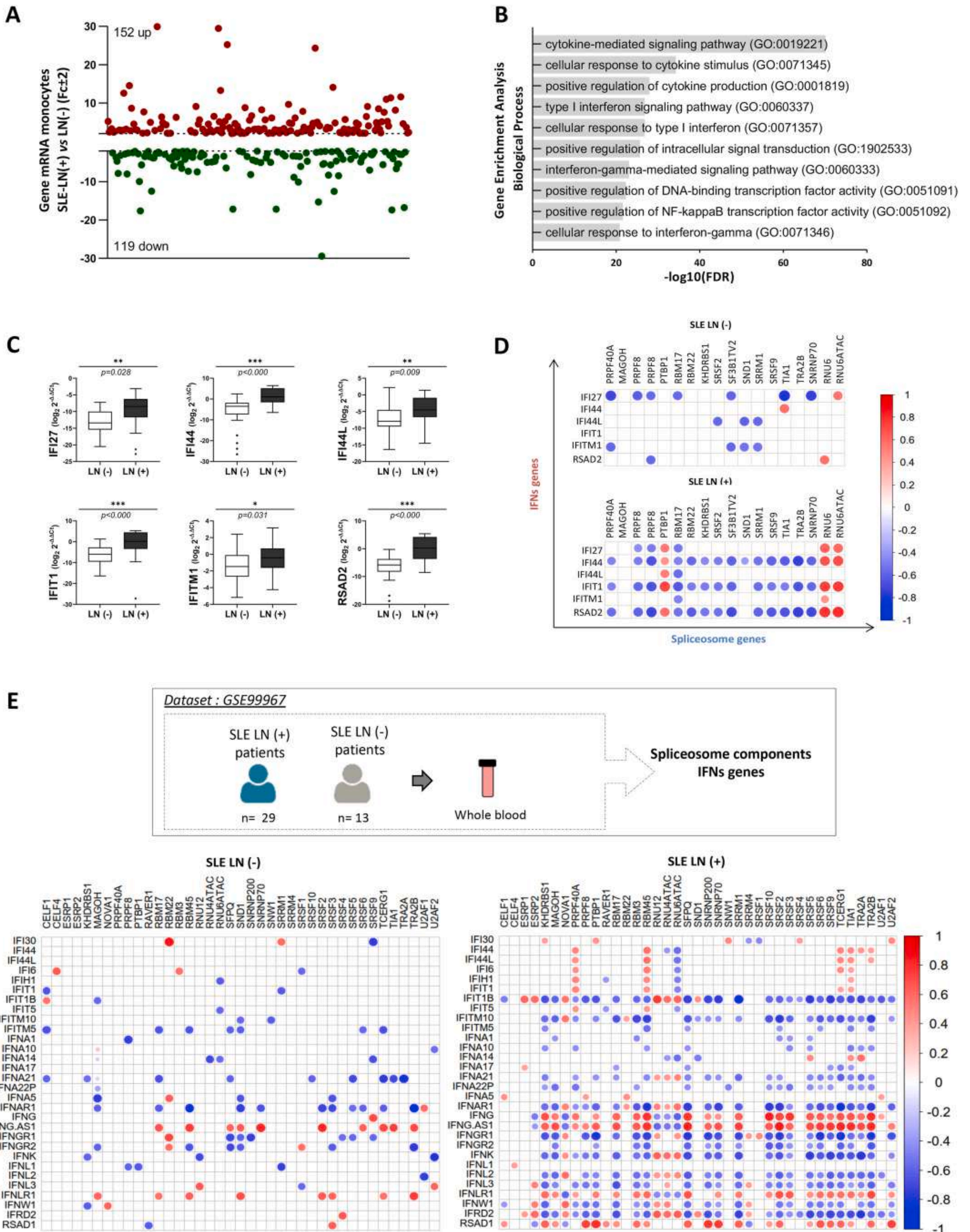

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Fig. 3. Simultaneous alterations of splicing machinery and transcriptome in monocytes from SLE patients with lupus nephropathy.
(A) Scatter plot of the gene expression profile in monocytes from SLE-LN(+) versus SLE-LN(-) patients. The expression levels of 152 genes were upregulated, while 119 were downregulated (Fold change (FC),>2). (B) Main signalling pathways enriched by gene ontology (GO) enrichment analysis of the altered gene expression signature in SLE-LN(+) monocytes. (C) Validation in the whole SLE cohort of altered genes belonging to the IFN signature between SLE-LN(+) and SLE-LN(-) patients. *p < 0.05, **p < 0.01, ***p < 0.001. (D) Correlation analysis between levels of altered SMC and levels of genes belonging to the IFN signature in monocytes from SLE-LN(+) and SLE-LN(-) patients. (E) Results from correlation studies were validated in a public dataset (GSE99967) of SLE patients involving gene expression microarrays to contrast, in whole peripheral blood, the transcriptomic profiles of active SLE-LN(+) vs SLE-LN(-) patients.

blood are important in the pathogenesis of SLE, they might also be detectable in diseased tissue. Thus, we analysed an external published transcriptomic dataset from kidney biopsies of lupus nephritis patients (GSE32591), both extending our findings to the tissue level and providing further validation in independent cohorts. We identified a simultaneous alteration of genes belonging to both, the splicing machinery and the IFN signature in the kidney of SLE-LN(+) patients, which were further significantly correlated (Supplementary Fig. 4).

3.5. Serum from SLE-LN(+) patients altered the transcriptomic and spliceosome profiles of healthy monocytes, which were reversed by JAKinibs

Increased levels of serum cytokines and chemokines related to the macrophage response (IL-6, IL-8 and GM-CSF), leukocyte chemotaxis (IP-10, MIP-1b, RANTES), and lymphocyte activity (IFNγ, IL-2, IL-9, and IL-12), were identified in SLE-LN(+) patients in relation to those without LN (Fig. 4A).

Besides, altered levels of various of these inflammatory mediators in SLE-LN(+) patients were found closely linked to those of either, some members of the IFN pathway, and several altered SMC (Fig. 4B), highlighting the involvement of an additional player like inflammation in these pathological processes. These results prompted us to perform mechanistic studies to test this hypothesis.

Specifically, monocytes from HD were cultured in the presence of serum from SLE-LN(+) patients and HD. Serum from SLE-LN(+) patients altered the expression of several genes belonging to the IFN signature, as well as a large number of SMC (Fig. 4C), suggesting that several

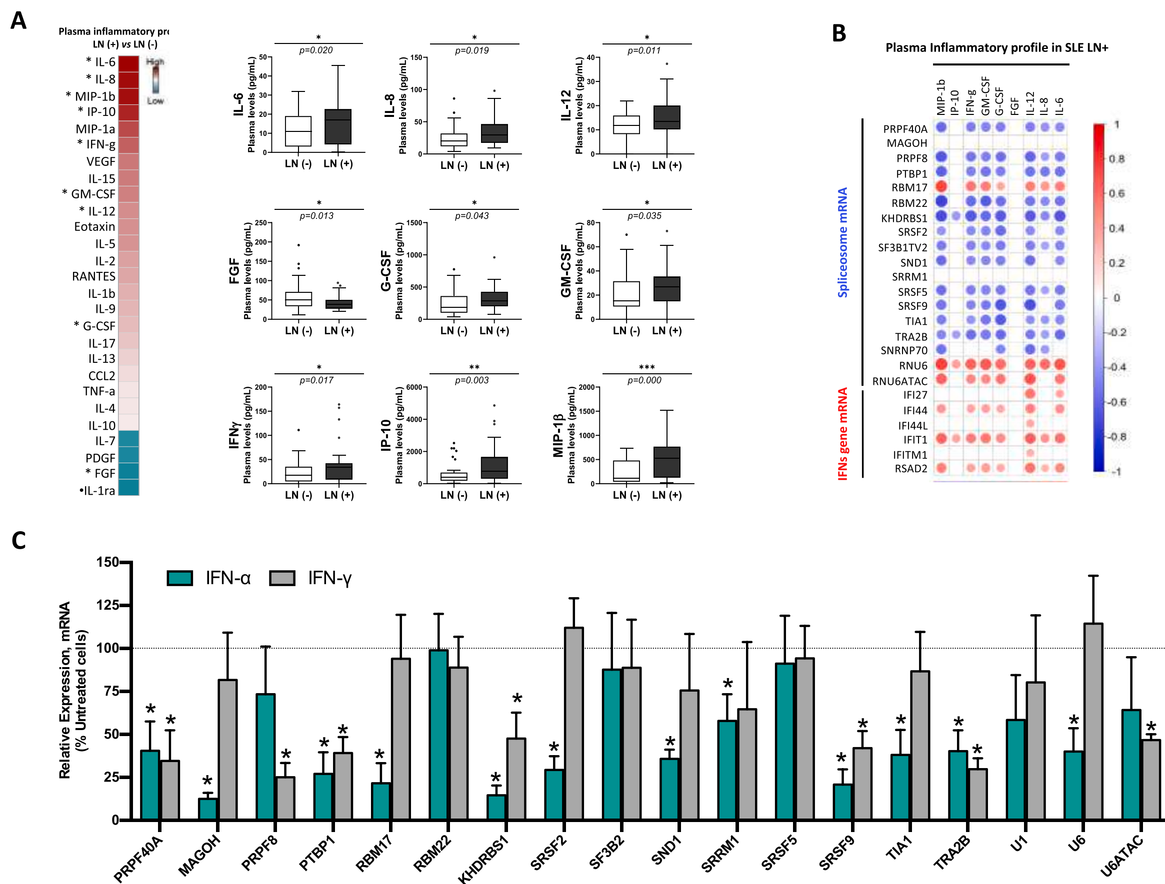

Fig. 4. Serum from SLE patients with Lupus nephropathy induces altered transcriptomic and spliceosome profiles in HD monocytes.
(A) Heatmap showing differential expression levels of 27 cytokines/chemokines/adhesion molecules in the plasma of SLE-LN(+) vs SLELN(-) patients. Bar graphs show levels of the inflammatory mediators displaying differential expression. Boxes indicate the interval between the 25th and 75th percentiles and horizontal bars inside boxes indicate median. Whiskers indicate the interval of data within 1.5 x interquartile ranges(IQR). Black circles indicate data points outside 1.5 x IQR)(*p < 0.05, **p < 0.01, ***p < 0.00). (B) Diagram showing the correlation analysis between the relative expression of SMC and IFNs' genes signature with serum levels of inflammatory mediators differentially expressed between SLE-LN(+) and SLE-LN(-) patients. Red or blue circles in each square represent positive or negative Spearman correlation coefficients respectively. Color and size scales are defined for the ranges delimited by the correlation coefficients, where darker colour and greater size represent higher correlation coefficients, while lighter color and lower size indicate lower correlation coefficients. (C) 500,000 cells/well from HD peripheral blood monocytes were treated with IFN-alfa or IFN-gamma for 24 h. The expression levels of SMCs were analysed by RT-PCR. Data obtained was compared with untreated cells, which was used as control and set at 100% in each panel. (*p < 0.05)

inflammatory and autoimmune mediators present in the serum of SLE patients with renal involvement might be responsible for these molecular alterations.

The preincubation of HD monocytes with two JAK inhibitors (either Tofacitinib or Baricitinib) promoted the reversion of the serum-induced altered expression of several SMC to the control levels, although in a specific way depending on the inhibitor administered (Supplemental Fig 5).

3.6. In vitro treatment with either, IFNα or IFNγ dysregulated the SM in monocytes

To further evaluate the potential influence of the IFN-pathway on the SME dysregulation observed in SLE patients, monocytes from HD were treated with either, IFNα or IFNγ. Both interferons promoted a specific and significant dysregulation in most SM components observed altered *in vivo* in SLE, pointing at a relevant role of these cytokines in the control of SM (Fig. 4C).

3.7. Fine-tuned modulation of altered splicing elements in monocytes from SLE-LN(+) patients re-established its pathological phenotype

Lastly, to test the potential therapeutic strategy of the direct restoration of altered expression of SMC on monocyte activity from SLE-LN (+) patients, we performed transfection studies to overexpress PTB or inhibit the expression of RBM17 (Fig. 5A), and assess their influence on inflammatory activity and adhesion capacity. Both SMC were selected since they were deeply altered in monocytes from SLE-LN(+) patients showing opposite directions.

The results of this mechanistic study showed that the fine-tuned modulation of these molecules promoted a reduction in the adhesion capacity of SLE-LN(+) monocytes, thus limiting their migratory ability (Fig. 5 B). Likewise, this modulation caused a significant reduction in the secretion of numerous inflammatory mediators by monocytes, thus promoting the restoration of its pathological phenotype (Fig. 5C).

4. Discussion

The present study demonstrates, for the first time, that the splicing machinery is significantly and specifically altered in primary APS, SLE and APS plus SLE leukocytes and distinctly linked to the pathophysiology of these closely related autoimmune disorders.

Importantly, we observed that this alteration was associated with disease activity scores, relevant clinical features (i.e. CV risk and renal involvement) and autoimmunity. Besides, we extended these observations by identifying in SLE patients with nephropathy the relationship among altered levels of SMC and those of genes belonging to the IFN signature, the main pathway altered in this disease and tightly related with renal involvement. Moreover, in renal biopsies, on which we evidenced the presence of macrophage infiltration associated with the severity of renal damage in SLE patients, data obtained from a parallel study identified a simultaneous alteration of genes belonging to both, the splicing machinery and the IFN signature, which further correlated each other. In addition, connections among the serum inflammatory profile in SLE-LN(+) patients and both the leukocytes' IFN signature and the altered SMC were demonstrated. Lastly, *ex vivo* and *in vitro* studies identified potential mechanisms underlying these processes and assessed the effects of either silencing or overexpressing key SMC on leukocyte activity.

We previously reported that the SM is profoundly altered in Rheumatoid Arthritis leukocytes and linked to the pathophysiology of the disease [20]. However, this is the first study focused on a comparative analysis of this alteration in primary APS, SLE and APS plus SLE patients and its influence on the inflammatory, cardiovascular, and renal damage that mainly characterize these patients.

Several studies in other pathological conditions, have established relevant (dys)functions of some of these factors in leukocytes, including an aberrant expression of MAGOH, RBM17, KHDRBS1, TRA2B, and PTB. MAGOH is a core component of the splicing-dependent multiprotein exon junction complex (EJC) deposited at splice junctions on mRNAs, with a key role as regulator of cell cycle and apoptosis progress [21], being essential for cell viability. In addition, it has been shown that MAGOH negatively regulated IL-6-induced STAT activation via

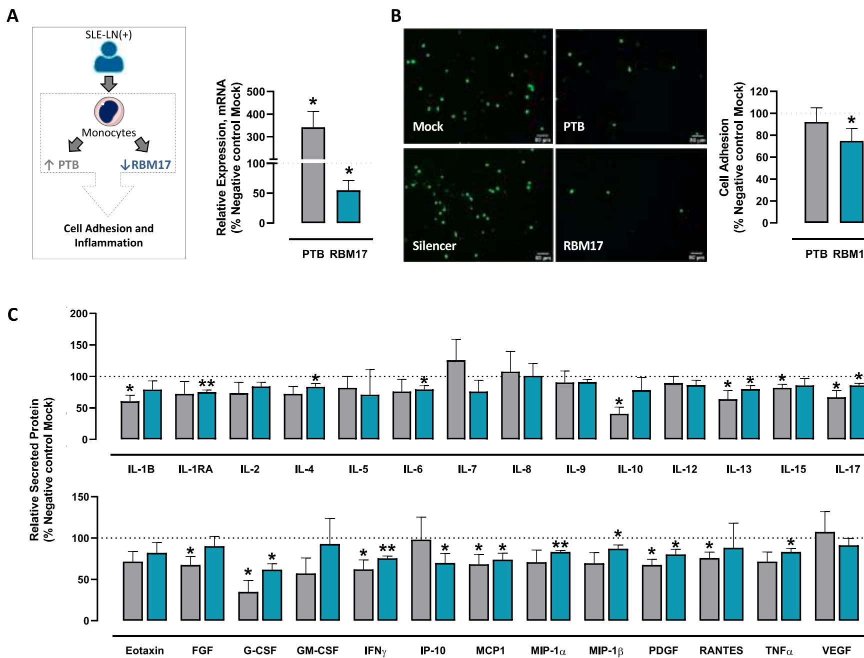

Fig. 5. Modulation of splicing elements reduces both, adhesion and proinflammatory cytokine secretion from monocytes of SLE-LN(+) patients. (A) Peripheral blood monocytes from SLE-LN(+) patients were transiently transfected to promote the overexpression of PTB or the inhibition of RBM17. Effectiveness of transfections was assessed by RT-PCR. (B) Downregulation of RBM17 reduced the adhesion capacity in monocytes. (C) Likewise, these alterations caused a significant reduction in the secretion by monocytes of numerous inflammatory mediators. All experiments were compared with mock transfected cells, which was used as control and set at 100% in each panel. (*$p < 0.05$, **$p < 0.01$).

interfered the binding between Y14 (a MAGOH partner in the EJC) and STAT3. Furthermore, small-interfering RNA-mediated reduction of MAGOH expression enhanced IL-6-induced gene expression [22].

Likewise, RBM17 has been identified as modulator of apoptosis, proliferation, and cell adhesion [23]. Equally, it has recently been demonstrated that KHDRBS1 has a bimodal regulatory role in controlling the activation of NFkB and MAPK pathways, so that although the deficiency of KHDRBS1 decreases toll-like receptors-induced NFkB activation, it increased the activation of ERK and JNK pathways, thus maintaining the proinflammatory status of cells [24]. Likewise, the transformer 2β (Tra2β) protein [25] is an important RNA-binding protein involved in alternative splicing. Tra2β is closely related to the PI3K/Akt signalling pathway. Although no study has investigated their relevance in autoimmune disorders, it has been shown, in the setting of other inflammatory and chronic disease, the Osteoarthritis, that TRA2B inhibited the inflammation levels in chondrocytes through activation of the PI3K/Akt signalling pathway [26,27].

Lastly, polypyrimidine tract-binding protein 1 (PTBP1) encodes a 57-kDa RRM containing protein, also called heterogeneous nuclear ribonucleoprotein type I. There is evidence to suggest that PTB regulates the stability of CD154 (CD40 ligand) mRNA. As CD154 is expressed by activated T cells and is necessary for the T-cell help signal, which drives B cell-mediated antibody and cytotoxic T cell responses, PTB may contribute to cell-mediated and humoral immunity [28]. Altogether, given the relevance of immune cells' activity and proinflammatory mediators in autoimmune disorders, the reduced levels of these SMC observed in APS and lupus patients might be linked to their enhanced inflammatory profile and related CV-risk or renal involvement. Indeed, both, *in vivo* and *in vitro* approaches in the present study seem to support this hypothesis. Nevertheless, further studies will be required to precisely dissect and define the role of these SMC in the setting of APS, SLE and APS plus SLE patients.

Compared with alterations seen in a recent study developed in RA patients, we observed that several SMC were similarly altered in different leukocyte subsets of RA, SLE and primary APS patients, including KHDRBS1, RBM17, RBM3, SNRNP70 and SRSF10, all of them involved in autoimmune and inflammatory processes that characterize all these diseases, thus suggesting their relevance in autoimmunity [29–34].

In the search for potential mechanisms underlying the SMC modulation, we evaluated whether restoration of altered levels of SMC might have a positive impact in the altered activity of leukocytes. Specifically, we assessed the effects of *MAGOH, TRA2B* and *KHDRBS1* overexpression based on their low expression in the three evaluated pathologies and their relevant clinicals' associations. Our data indicated a direct influence of the overexpression of these SMC on the leukocyte's activity, thus supporting the idea that their altered expression in these autoimmune disorders might influence their pathogenic profiles.

It is currently accepted that altered splicing is linked to several downstream pathologic mechanisms in numerous diseases, such as increased RNA degradation, accumulation of unspliced RNA and/or generation of truncated or altered proteins of some genes [9,35,36]. All these mechanisms might be also responsible for inducing aberrant immune cell activities involved in clinical features of APS and SLE. Future studies in this area will contribute to test this hypothesis.

Previous studies have demonstrated the relevance of monocytes in the increased CV-risk present in APS and SLE patients [13,37]. Accordingly, in the present study, levels of several altered SMC in monocytes significantly correlated with markers of atherothrombotic risk in APS, SLE and APS + SLE patients. Besides, among the three diseases evaluated, we found in the most prevalent of them, the SLE patients, that numerous dysregulated SMC, particularly in monocytes, were associated with the presence of nephropathy, the highest relevant comorbidity in this autoimmune disorder.

With this premise, and to gain insight in the molecular mechanisms underlying the renal involvement in SLE, we centered our study in the evaluation of the contribution of SMC dysregulation in SLE monocytes to the development of lupus nephritis.

Immunohistochemical analysis of renal biopsies from SLE-LN(+) patients showed macrophage infiltration in all samples, further linked with renal damage, thus reflecting the potential pathological role of the myeloid compartment in the development of lupus nephritis. Accordingly, transcriptomic analysis of monocytes from SLE-LN(+) patients versus SLE-LN(−) identified, among a number of differentially expressed genes, those belonging to the IFN pathway, which further correlated negatively with several SMC, thus pointing at a direct link among alterations in the splicing machinery and the IFN signature in the setting of lupus nephropathy in SLE patients.

To further interrogate the relevance of our results, we used publicly available datasets, were whole blood, monocytes, and renal biopsies of SLE patients were analysed. Despite being slightly different samples, involving not only immune cells but the whole blood cell population and renal tissue, we observed a clear correspondence with our results in both, the number and type of dysregulated SMC and genes belonging to the IFN signature, which further correlated each other. Thus, our results, in conjunction with the newly evaluated databases, support the presence of wide and simultaneous alterations of SMC and key genes in leukocytes and renal tissue associated with the development of lupus nephropathy.

Then, given the relationship observed *in vivo* in SLE-LN(+) patients among serum inflammatory mediators, SMC and the IFN pathway in monocytes, we evaluated *in vitro* the potential influence of biomolecules present in the serum of SLE patients on the expression of dysregulated SMC and inflammatory mediators in monocytes. This treatment promoted a significant dysregulation in both, various SMC and several IFN-related genes, suggesting that the inflammatory/immune profile present in SLE serum might contribute to the dysregulation of SMC linked to the pathogenesis of this chronic disorder.

It is well known that IFNs critically contribute to the clinical manifestations in SLE. Importantly, some investigators reported that type I (IFNα) and type II (IFNγ) signatures may overlap in the induction of distinct SLE-clinical profiles [38]. Accordingly, in this study we showed that both IFNs promoted significant and specific alterations in the SM of HD monocytes, revealing a new mechanism underlying the effects of these key cytokines in the activation of SLE-leukocytes.

Dysregulation of cytokines is a hallmark of SLE. Targeting the downstream signalling pathways mediated by the JAK-STAT proteins allows simultaneous suppression of multiple cytokines and is therefore an attractive therapeutic option for SLE [39]. Previous studies in animal models suggest that inhibition of the JAK-STAT pathway is promising in ameliorating murine lupus nephritis and dermatitis through modulation of the type I IFN downstream signalling [40]. As IFN-pathway is over-expressed in human SLE, JAK-STAT inhibition might prove to be an attractive therapeutic target of SLE.

With this premise, we developed *in vitro* studies on which HD-monocytes were treated with serum from SLE-LN(+) patients combined with two JAK inhibitors: Tofacitinib (a pan-JAK inhibitor) and Baricitinib (JAK1/2 inhibitor). We found similar although specific efficacy among them in the reversion of serum-induced alterations of SMC and IFN-related genes, thus exhibiting the specificity in the molecular mechanisms of these JAKinibs in lymphocytes activity and advising the potential efficacy of this new therapeutic approach in the treatment of SLE.

Lastly, we evaluated the potential involvement of some dysregulated SMC in the aberrant activity of SLE-LN(+) monocytes. The overexpression or inhibition of PTB and RBM17 (found to be reduced and overexpressed in SLE monocytes, respectively), promoted a reduction in their capacity of secretion of inflammatory mediators. In addition, the inhibition of RBM17, promoted a reduction in their adhesion capacity. These effects were acuter after silencing of RBM17, probably due to its central function in the core of the spliceosome, essential for survival and cell maintenance, causing significant gene expression changes [23], while the functional role of PTB might be limited to the splicing of

certain genes. Overall, these results demonstrated for the first time that the modulation of the splicing machinery in leukocytes from SLE-Ln(+) patients directly impacts relevant pathogenic functions associated with the disease.

This study has some limitations. First, new extensive cohorts of APS, SLE and APS plus SLE patients should be evaluated to further confirm the alterations observed in the splicing machinery, and their influence in the activity of these diseases. In addition, although our study provides several mechanistic insights related to the regulation of the SMC, detailed understanding of the mechanisms underlying their pathogenic role and modulation in disease context is still to be fully characterized, which warrants further investigation.

In sum, our results reveal, for the first time, a specific alteration of SMC in leukocytes from patients with APS, SLE and APS plus SLE, which might have widespread effects on the transcription of multiple genes. Their specific and coordinated altered expression on each disease would be responsible for the development of distinctive clinical profiles. The involvement of specific SMC on the pathogenesis of these closely related autoimmune disorders jointly invite to further explore the targeting of altered splicing as a novel source of therapeutic tools.

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Author statement

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Lopez-Pedrera had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: Carlos Perez Sanchez, Alejandra Maria Patiño-Trives, Tomás Cerdó, M[a] Angeles Aguirre and Chary Lopez-Pedrera. Acquisition of data: Alejandro Escudero-Contreras, Rafaela Ortega-Castro, Pedro Segui Azpilcueta, Mario Espinosa, Desirée Ruiz-Vilchez, Rosa Ortega, Ismael Sanchez-Pareja, Alejandro Ibañez-Costa, Laura Muñoz-Barrera, M[a] Carmen Abalos-Aguilera. Analysis and interpretation of data: Nuria Barbarroja, Justo P. Castaño, Raul Miguel Luque, Carlos Pérez-Sánchez, Alejandra M[a] Patiño-Trives, Tomás Cerdó, M[a] Angeles Aguirre and Chary Lopez-Pedrera.

Ethics approval

The study was approved by the medical ethics committee of the Reina Sofia Hospital (Cordoba, Spain). All patients gave written informed consent in accordance with the declaration of Helsinki.

Expression levels of major and minor spliceosome and associated splicing factors were quantified through a 48.48 Dynamic Array (Fluidigm) in monocytes, lymphocytes and neutrophils from 22 APS patients, 23 APS plus SLE patients, 35 SLE patients, and 50 HD.

Volcano plots showing the expression levels of SMC in monocytes, lymphocytes and neutrophils from (A) APS vs HD, (B), APS plus SLE vs HD and (C), SLE vs HD.

(D, E and F) Heat maps showing differential expression in the splicing machinery components from different leukocyte subsets between APS, APS plus SLE and SLE patients and HD (log10 fold change). Green and red colours represent downregulated and upregulated splicing machinery elements, respectively, showing significant differences ($p < 0.05$).

Levels of 27 cytokines/chemokines/adhesion molecules in the plasma of SLE-LN(+) vs SLE-LN(−) patients. Bar graphs show levels of the inflammatory mediators displaying differential expression. Boxes indicate the interval between the 25th and 75th percentiles and horizontal bars inside boxes indicate median. Whiskers indicate the interval of data within 1.5 x interquartile ranges(IQR). Black circles indicate data points outside 1.5 x IQR)(*$p < 0.05$, **$p < 0.01$, ***$p < 0.00$). (B) Diagram showing the correlation analysis between the relative expression of SMC and IFNs' genes signature with serum levels of inflammatory mediators differentially expressed between SLE-LN(+) and SLE-LN(−) patients. Red or blue circles in each square represent positive or negative Spearman correlation coefficients respectively. Color and size scales are defined for the ranges delimited by the correlation coefficients, where darker colour and greater size represent higher correlation coefficients, while lighter color and lower size indicate lower correlation coefficients. (C) 500,000 cells/well from HD peripheral blood monocytes were treated with IFN-alfa or IFN-gamma for 24 h. The expression levels of SMCs were analysed by RT-PCR. Data obtained was compared with untreated cells, which was used as control and set at 100% in each panel. (*$p < 0.05$)

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2022.102990.

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