Arteriosclerosis, Thrombosis, and Vascular Biology

ORIGINAL RESEARCH

Anti-dsDNA Antibodies Increase the Cardiovascular Risk in Systemic Lupus Erythematosus Promoting a Distinctive Immune and Vascular Activation

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OBJECTIVE: Systemic lupus erythematosus (SLE) is associated to boosted atherosclerosis development and a higher cardiovascular disease risk. This study aimed to delineate the role of anti-double stranded DNA (anti-dsDNA) antibodies on the molecular profile and the activity of immune and vascular cells, as well as on their enhanced cardiovascular risk.

APPROACH AND RESULTS: Eighty SLE patients were included. Extensive clinical/analytical evaluation was performed, including cardiovascular disease parameters (endothelial function, proatherogenic dyslipidemia, and carotid intima-media thickness). Gene and protein expression profiles were evaluated in monocytes from patients diagnosed positive or negative for anti-dsDNA antibodies by using NanoString and cytokine arrays, respectively. NETosis and circulating inflammatory profile was assessed in both neutrophils and plasma. Positivity and persistence of anti-dsDNA antibodies in SLE patients were associated to endothelial dysfunction, proatherogenic dyslipidemia, and accelerated atherosclerosis. In parallel, anti-dsDNA antibodies were linked to the aberrant activation of innate immune cells, so that anti-dsDNA(+) SLE monocytes showed distinctive gene and protein expression/activity profiles, and neutrophils were more prone to suffer NETosis in comparison with anti-dsDNA(–) patients. Anti-dsDNA(+) patients further displayed altered levels of numerous circulating mediators related to inflammation, NETosis, and cardiovascular risk. In vitro, Ig-dsDNA promoted NETosis on neutrophils, apoptosis on monocytes, modulated the expression of inflammation and thrombosis-related molecules, and induced endothelial activation, at least partially, by FcR (Fc receptor)-binding mechanisms.

CONCLUSIONS: Anti-dsDNA antibodies increase the cardiovascular risk of SLE patients by altering key molecular processes that drive a distinctive and coordinated immune and vascular activation, representing a potential tool in the management of this comorbidity.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: anti-dsDNA antibodies ■ comorbidity ■ lupus erythematosus, systemic ■ plasma ■ risk factors

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Nonstandard Abbreviations and Acronyms

ApoA	apolipoprotein A
ApoB	apolipoprotein B
CIMT	carotid intima-media thickness
CVD	cardiovascular disease
FcR	Fc receptor
FITC	fluorescein isothiocyanate
HD	healthy donor
HDL	high-density lipoprotein
HUVEC	human umbilical vein endothelial cell
IL	interleukin
MPO	myeloperoxidase
NE	neutrophil elastase
NET	neutrophil extracellular trap
SLE	systemic lupus erythematosus

Highlights

- By using high throughput–based technological approaches, we have unraveled the role of anti-double stranded DNA antibodies on the gene profile and activity of immune and vascular cells of systemic lupus erythematosus, as well as on their enhanced cardiovascular risk.
- Positivity and persistence of anti-double stranded DNA antibodies in systemic lupus erythematosus patients are associated to endothelial dysfunction, proatherogenic dyslipidemia, and accelerated atherosclerotic process.
- Anti-double stranded DNA antibodies increase the cardiovascular risk of systemic lupus erythematosus patients by altering key biological mechanisms, that is, inflammation, NETosis, apoptosis, that drive a distinctive and coordinated immune and vascular activation.

Systemic lupus erythematosus (SLE) is a serious multisystemic autoimmune disease that involves dysregulation of the immune system, production of pathogenic autoantibodies, and numerous immune-mediated damages.

SLE is further associated to boosted atherosclerosis development and a higher risk of cardiovascular disease (CVD), estimated to be higher in SLE patients, from 2.6 to 10×, than in the general population.[1–3] The development of different manifestations of SLE CVD (ie, myocardial infarction, stroke, thrombosis) involves a variety of predisposing genetic factors, as well as traditional cardiovascular risk factors (such as hyperlipidemia, hypertension, obesity, or drug therapy), autoimmune elements (mainly autoantibodies and immune cells), complement activation, oxidative stress, dysfunctional lipids, endothelial dysfunction, and numerous inflammatory molecules.[4–10]

There is a growing evidence of the relevant role of immune cells in the development of the CVD present in autoimmune disorders.[4,11,12] The key role of the myeloid compartment (monocytes and neutrophils) in the development of inflammation and CVD in SLE patients was recently highlighted in a study where we evaluated key aspects of their activity, including inflammation and oxidative stress. In these patients, changes in the expression of cytokines, chemokines, cell surface receptors, endothelial cell receptors, and a number of markers of autoimmunity and oxidation were found closely related and seemed to coordinate the mechanism underlying the development of CVD in SLE.[13] Our microarray study further allowed the identification of specific gene signatures in monocytes of antiphospholipid syndrome and SLE patients explaining the proatherosclerotic, prothrombotic, and inflammatory states in these autoimmune disorders.[14]

Neutrophils have recently been demonstrated to act as proatherogenic cells, through their capacity to release neutrophil extracellular traps (NETs).[15] Uncontrolled NET production, as well as the impairment of their degradation by nucleases, induces tissue damage, activates the coagulation system to promote thrombus formation, induces endothelial dysfunction, and represents a source of autoantigens.[16]

Anti-double stranded DNA (anti-dsDNA) antibodies—the key sign of SLE—constitute an effective parameter for the diagnosis and classification of SLE. Moreover, their oscillating titers during the evolution of the disease reflect its activity in many patients, and even a titer increase may predict a disease relapse.[17]

The production of anti-dsDNA antibodies is caused by a combination of multiple factors, including abnormalities of dendritic cells, B cells, or T cells, and lack of DNase, which makes it impossible to clean the released nuclear materials.[18] Accordingly, a variety of self-antigens can be recognized by anti-dsDNA antibodies, which then trigger inflammatory responses, apoptosis, and tissue fibrosis and destroy target cells and organs.

The involvement of anti-dsDNA antibodies in the injury of kidneys, skin, and brain in SLE has been fully demonstrated.[19,20] Yet, to date, the role of anti-dsDNA antibodies in the accelerated onset of atherosclerosis and CVD in SLE patients and the underlying molecular mechanisms that regulate these processes remains unknown. Thus, we undertook this study to identify the mechanisms involved in the onset of atherosclerosis and CVD in SLE patients and the specific role of anti-dsDNA autoantibodies in these processes. By using in vivo and in vitro genomic and protein-based technological approaches, we have unraveled the role of anti-dsDNA antibodies on the alterations observed in the gene profile and activity of immune and vascular cells, as well as in the inflammatory status and the endothelial dysfunction present in SLE patients with a higher cardiovascular risk.

METHODS

All data and materials supporting the findings of this study are available within this article and in Materials in the Data Supplement or from the corresponding authors on reasonable request. In addition, the expanded Methods section is available in Materials in the Data Supplement.

Patients

Eighty consecutive patients with SLE, who met the American College of Rheumatology criteria, were recruited from the Rheumatology Department of the Reina Sofía University Hospital (Spain), Córdoba, Spain, over a period of 24 months. Experimental protocols were approved by the Ethics Committee of the Reina Sofia Hospital in Cordoba, and written informed consent was obtained. The study was conducted according to the principles of the Declaration of Helsinki.

For this cross-sectional study, extensive clinical and demographic data collection was carried out for all patients, including analytical and clinical parameters (Table). On average, all study participants presented mild or inactive activity showing a low score on the Lupus Erythematosus Activity Index. Patients were classified for anti-dsDNA antibody negativity or positivity at the time of the visit, resulting in 2 groups with no statistical differences in terms of age, sex, and time of evolution and traditional cardiovascular risk factors. All lupus nephropathies were in remission except for 2 recruited patients with kidney failure.

Blood samples were obtained from all patients, and serum/plasma and leucocytes were purified for later analyses. Overview of the clinical and molecular parameters assessed in those samples is displayed in Figure I in the Data Supplement (flowchart). In addition, retrospective analysis of clinical and analytical parameters was performed, involving 7 years before sample collection.

See the Data Supplement for details.

Subclinical Measures of CVD: Microvascular Endothelial Function, Carotid Intima-Media Thickness, and Proatherogenic Dyslipidemia

The study of microvascular function was performed by the postocclusive reactive hyperemia test, using the Periflux 5010 linear Doppler laser (Perimed, Sweden).

The thickness of the carotid intima-media thickness (CIMT) in SLE patients was measured by Supra-aortic trunk Doppler. The B-mode high-resolution ultrasound test was performed by a single experienced vascular sonographer using Toshiba (Aplio platform) equipment with 7- to 10-MHz linear transducers.

Plasma atherogenic index was calculated as the ratio between triglycerides and HDL (high-density lipoprotein; plasma atherogenic index [mg/dL]=triglyceride/HDL). The Apo ratio was calculated as the ratio between ApoB (apolipoprotein B) and ApoA (apolipoprotein A; Apo ratio [mg/dL]=ApoB/ApoA).

See the Data Supplement for details.

Blood Sample Collection

Whole blood was collected in tubes by direct venipuncture using VACUETTE TUBE 9NC Coagulation 3.2% sodium citrate of 3.5 mL to obtain plasma, BD Vacutainer SST II Advanced tubes 8.5 mL to obtain serum, and BD Vacutainer 0.105M Sodium Citrate 6 mL tubes for cell purification. A total volume of 60 mL was obtained from each patient. To avoid changes promoted by diet and circadian rhythms, blood samples were always collected in the early hours of the morning and after an 8-hour fasting period.

A selected volume of blood samples was processed for routine clinical blood analysis (Table), and the rest was used for the purification of plasma, serum, and white cells for molecular analysis. See the Data Supplement for details.

Isolation of White Blood Cells

Neutrophils were isolated from patients by density centrifugation over Dextran-Ficoll Hypaque (Lymphoprep; StemCell Technology, Oslo, Norway), which allowed cells to keep a non-activated state. Thereafter, the separation of monocytes and lymphocytes from the mononuclear layer was performed by the immunomagnetic depletion of nonmonocytes using a commercially available kit (Monocyte Isolation Kit II; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the different cell types was evaluated by flow cytometry (FACScalibur single laser cytometer; BD Biosciences) by analyzing the size and complexity and the labeling with the following antibodies: anti-CD14 conjugated with fluorescein isothiocyanate (FITC), anti FITC-conjugated CD15, FITC-conjugated anti-CD19 (Miltenyi Biotec), and FITC-conjugated anti-CD3 (BD Biosciences). Isolation purity was ≥95% for all cell types.

Extraction of RNA and Protein

RNA and protein samples from leucocytes of both patients and in vitro experiments were processed with a DNA/RNA/Protein Purification Plus Kit (Norgen Biotek Corporation) following the manufacturer's instructions.

NanoString Array and RT-PCR

The nCounter human autoimmune profiling panel (NanoString Technologies) was used to identify changes in the gene expression profile of monocytes between SLE patients positive and negative for anti-dsDNA antibodies according to the manufacturer's protocol. A pool of 5 SLE patients of each group was used as exploratory cohort, and differentially expressed genes were validated by quantitative RT-PCR (real-time polymerase chain reaction) in the whole cohort of patients. Moreover, changes in the gene expression profile promoted by the in vitro incubation of healthy monocytes with either anti-dsDNA or IgG-control were also analyzed. The autoimmune profiling panel included 770 genes that covered the central pathways and processes that define autoimmune and inflammatory diseases. See the Data Supplement for details.

Array of Intracellular Signaling Proteins and Intracellular Cytokine Array

Proteome Profiler Human XL Cytokine Matrix Kit (ARY022B; R&D Systems, Inc, Abingdon, United Kingdom) and PathScan Intracellular Signaling Array Kit (Cell Signaling Technology, MA) were carried out for the analysis of intracellular proteins and pathways differentially regulated in the same samples used for the transcriptomic analysis. See the Data Supplement for details.

Table. Clinical and Laboratory Parameters of Patients With SLE

Parameter, mean±SD or n (%)	Total SLE patients, n=80		*P* value
	SLE anti-dsDNA(−), n=38	SLE anti-dsDNA(+), n=42	
Demographic and clinical characteristics			
Women/men, n (%)	34/4 (88%/12%)	38/4 (89%/11%)	0.970
Age, y	39±9	39±9	0.901
Time of evolution	15±8	12±7	0.204
SLEDAI	0.8±1.2	2.7±1.6	0.000
Lupus nephritis, n (%)	16/38 (42%)	26/42 (62%)	0.120
Pregnancy morbidity, n (%)	1/38 (3%)	10/42 (24%)	0.050
Hypertension, n (%)	6/38 (15%)	10/42 (24%)	0.414
Hyperlipemia, n (%)	9/38 (24%)	11/42 (27%)	0.437
Obesity, n (%)	2/38 (6%)	7/42 (16%)	0.263
Diabetes, n (%)	1/38 (3%)	2/42 (5%)	0.223
Smoker, n (%)	6/38 (15%)	15/42 (35%)	0.118
Medications			
Corticosteroids, n (%)	24/38 (63%)	30/42 (71%)	0.430
Antimalarials, n (%)	31/38 (81%)	30/42 (71%)	0.286
Immunosuppressant, n (%)	11/38 (29%)	15/42 (36%)	0.518
Anticoagulants/antiplatelets, n (%)	16/38 (42%)	19/42 (46%)	0.471
Statins, n (%)	11/38 (29%)	16/42 (38%)	0.387
Laboratory features			
Total cholesterol, mg/dL	188.8±35.0	176.7±33.9	0.178
Cholesterol HDL, mg/dL	57.8±15.9	55.6±15.7	0.601
Cholesterol LDL, mg/dL	109.7±28.7	105.3±27.7	0.547
Triglycerides, mg/dL	105.5±44.9	75.5±24.9	0.002
ApoA, mg/dL	156.2±32.3	146.4±24.5	0.210
ApoB, mg/dL	77.6±15.4	74.0±21.9	0.510
CRP, mg/L	4.1±8.1	2.9±4.9	0.527
ESR, mm/h	16.9±21.9	19.1±17.4	0.717
C3, mg/dL	109.5±30.1	95.5±26.4	0.063
C4, mg/dL	19.1±8.5	17.7±8.7	0.530
ANA, n (%)	30/38 (79%)	42/42 (100%)	…
Anti-dsDNA Ab, n (%)	0/38 (0%)	42/42 (100%)	…
Anti-SSA-Ro Ab, n (%)	10/38 (26%)	12/42 (28%)	0.821
Anti-SSB-La Ab, n (%)	1/38 (2%)	5/42 (12%)	0.115
Anti-SM Ab, n (%)	4/38 (10%)	9/42 (21%)	0.186
Anti-RNP Ab, n (%)	4/38 (10%)	9/42 (21%)	0.186
aCL IgG Ab, n (%)	1/38 (3%)	3/42 (8%)	0.479
aCL IgM Ab, n (%)	1/38 (3%)	1/42 (3%)	0.777
Anti-β2GP IgG Ab, n (%)	0/38 (0%)	4/42 (10%)	0.088
Anti-β2GP IgM Ab, n (%)	2/38 (6%)	2/42 (5%)	0.683

Values are mean±SD or n (%). Ab indicates antibody; aCL, anticardiolopin; ANA, antinuclear antibodies; anti-β2GP, anti-beta2-glycoprotein I; anti-dsDNA, anti-double stranded DNA; anti-RNP, anti-ribonucleoprotein; anti-SSA-Ro, anti-Sjögren's syndrome related antigen A; anti-SSB-La, anti-Sjögren's syndrome related antigen B; ApoA, apolipoprotein A; ApoB, apolipoprotein B; C3, complement component 3; C4, complement component 4; CRP, C-reactive protein; ESR, globularsedimentation rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SLE, systemic lupus erythematosus; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; and SM, anti-Smith.

Plasma Inflammatory Profile

The secreted levels of 27 cytokines, chemokines, and adhesion molecules in plasma from SLE patients were determined using the Bio-Plex Pro 27-plex Assay for Human Cytokines (Bio-Rad Laboratories SA, CA). The multiplex assay was performed according to the manufacturer's protocol, and the fluorescence measurement was detected in a Bio-Plex 200 Systems system (Bio-Rad, CA). Cytokine concentrations expressed in picograms

per milliliter were determined using standard curves generated in the Bio-Rad Bio-Plex Manager software (Bio-Rad, CA).

Characterization of Biological Functions and Pathways of Altered Genes and Proteins

Genes and proteins differentially expressed in monocytes were functionally classified and used for identifying enriched pathways and functional interconnected networks using both the genetic ontology enrichment analysis (http://www.geneontology.org/) and the STRING platform (https://string-db.org/).

Integrated analysis between altered genes and proteins identified through in vivo and in vitro analysis was carried out. Briefly, the differentially expressed genes and proteins were uploaded to the IPA software. Then, by using the tool My Pathway, a molecular network of interaction was built where only molecules interconnected were represented. Finally, canonical pathways mapping more molecules within the network were identified.

Determination of Oxidative Stress Biomarkers in Plasma

The total plasma nonenzymatic antioxidant capacity of SLE patients was determined by a colorimetric assay, using the TAC assay kit (BioVision, CA). Lipid hydroperoxides were quantified in plasma using a specific TBARS assay kit (Canvax, Córdoba, Spain) following the manufacturer's instructions. The signals from both assays were detected spectrophotometrically on a BioTek-PowerWave XS plate reader (Winooski, VT).

NETosis-Associated Proteins (NE and MPO) and Derived Products (Cell-Free Nucleosomes and Elastase)

Intracellular NE (neutrophil elastase) protein and MPO (myeloperoxidase) were analyzed by flow cytometry after incubation of the cells with FITC anti-human MPO antibody (BD Biosciences) or NE anti-human antibody (1:20; RbmAb to NE; Abcam, Cambridge, United Kingdom), followed for Alexa Fluor conjugated secondary antibody. Then cells were acquired in the FACS Calibur cytometer (BD Biosciences). See the Data Supplement for details.

Nucleosomes were measured in the plasma of SLE patients using the ELISAPLUS kit for detection of human cell death (Roche Diagnostics) according to the manufacturer's instructions. NE was also measured in SLE plasma using the Human Elastase PMN Sandwich ELISA Kit (Abcam) following the manufacturer's recommendations. The signals from both tests were detected spectrophotometrically in a BioTek-PowerWave XS plate reader (Winooski, VT).

Quantification and Purification of Anti-dsDNA Antibodies

Anti-dsDNA antibody levels were determined in the serum of SLE patients using the QUANTA Lite dsDNA assay (Quanta Lite-INOVA Diagnostics, CA) following the manufacturer's instructions. For in vitro studies, total IgG antibodies were purified from a pool of serum from 5 SLE patients (all positive for anti-double stranded DNA antibody and negative for the remaining ANA antibodies [anti-RNP, anti-SM, anti-Ro, and anti-LA and antiphospholipid antibodies]) and 5 healthy donors (HDs; IgG-control), using the MAbTrap Kit-GE Healthcare (Amersham Biosciences), based on high-affinity chromatography. Next, purification of anti-dsDNA antibodies was performed as described previously.[21–23] See the Data Supplement for details.

In Vitro Studies

Monocytes and neutrophils purified from 5 HDs were treated for 24 and 6 hours, respectively, with either IgG-control or IgG-dsDNA (40 µg/mL). In monocytes, both transcriptomic and protein analysis were performed as described previously. Apoptosis was also analyzed in monocytes by transmission electron microscopy and Western blot. In neutrophils, NETosis and its derived products were determined by scanning electron microscopy and specific commercial kits, respectively.

To evaluate the effects of FcR (Fc receptor) blockage, monocytes and neutrophils were preincubated with FcR Blocking Reagent (Miltenty) for 15 minutes at 4 °C before the treatment with IgG-control or IgG-dsDNA.

To mimic the interaction between the immune and vascular system in the presence of anti-dsDNA antibodies, 2 sets of cocultures experiments were performed.

Briefly, monocytes, neutrophils, and lymphocytes isolated from 5 HDs were used to perform coculture assays with human umbilical vein endothelial cells (HUVECs), either using transwells or directly, allowing a direct interaction among cells.

Effects of IgG-dsDNA on endothelial cell activation were assessed. See the Data Supplement for details.

Confocal Microscopy

Both IgG-control and IgG-dsDNA antibodies were conjugated with DyLight 488 dyes (ThermoFisher Scientific; green channel) following the manufacturer's instructions. Then, monocytes purified from HDs were incubated with both conjugated antibodies to analyze the surface binding and cellular uptake. After 6 hours of treatment, monocytes were stained with CellMask Plasma Membrane Stains (ThermoFisher Scientific; red channel) and DAPI (4',6-diamidino-2-phenylindole; blue channel) to determine the distribution of the antibodies in the plasma membrane and nucleus, respectively. Finally, cells were fixated with 4% paraformaldehyde and moved to microscope slides using mounting medium (Aqua-Poly/Mount; Polysciences).

Cells were imaged by confocal fluorescence microscopy under exactly identical instrument settings. Images were acquired at magnification ×63 in a Zeiss LSM710 Confocal Microscope.

The degree of colocalization of fluorescence signal from conjugated antibodies with the plasma membrane (red versus green channel) and nucleus (blue versus green channel) was quantified by calculating Manders distinct colocalization coefficients[24] for the confocal image material using the software Fiji via the JACoP plugin. Ten aleatory images containing 15 to 20 monocytes were analyzed in each condition (150–200 cells in total), and the mean and SD of Manders coefficients were obtained.

Statistical Analysis

Statistical analysis and graphical representation of results were performed using the GraphPad Prism 8 software (San Diego,

CA). The normal distribution of the variables to characterize the differences in the analyzed parameters was assessed using the Kolmogorov-Smirnov test.

Based on this test, comparisons between quantitative and qualitative variables were made using the Student *t* test, or alternatively, using nonparametric tests (Mann-Whitney *U* test). The relationship analyses between the studied parameters were performed using the Pearson and Spearman correlations.

The independent association between different variables in univariate analysis was determined by multivariate regression analysis using SPSS, version 25.0, for Windows statistical software.

Differences were considered significant at $P<0.05$.

The detailed design of the study is detailed in Figure I in the Data Supplement.

RESULTS

Positivity and Persistence of Anti-dsDNA Antibodies Are Associated With Endothelial Dysfunction, Proatherogenic Dyslipidemia, and Atherosclerosis in SLE Patients

The microvascular endothelial function in SLE patients positive for anti-dsDNA antibodies was altered in comparison with anti-dsDNA negative patients, as demonstrated by a significant reduction in both the area of hyperemia and peak flow minus rest flow after release of arterial blood flow occlusion (Figure 1A).

Among SLE patients retrospectively analyzed for persistence of anti-dsDNA positivity (n=58), 36% displayed constant positivity for anti-dsDNA antibodies in the last 8 years, while 31% had always been negative, and 33% of patients had changed between positivity and negativity (Figure IIA in the Data Supplement). Persistency of positivity for these autoantibodies was associated with that of moderate disease activity, altered kidney function, and higher inflammation (Figure IIB in the Data Supplement), as well as with the continued treatment with immunosuppressors in the retrospective period evaluated (Figure IIC in the Data Supplement).

In addition, both the positivity and the persistence of these antibodies were linked to increased CIMT. A direct relationship between positivity and persistence of anti-dsDNA antibodies with CIMT was also demonstrated (Figure 1B). Moreover, both titers and frequency of anti-dsDNA antibodies correlated with parameters related to endothelial function such as hyperemic area and peak flow minus rest flow (Figure 1C). Lastly, anti-dsDNA titers were linked to proatherogenic markers such as the atherogenic index and the apoB/A ratio (Figure 1D).

In SLE patients, the association between the positivity for anti-dsDNA and both endothelial dysfunction parameters and increased CIMT was independent from the presence of other cardiovascular risk factors (ie, hypertension, obesity, smoking habit, renal damage, and corticosteroid treatment; Table III in the Data Supplement).

Monocytes From SLE Patients Positive for Anti-dsDNA Antibodies Show a Distinctive Gene Expression Profile Related to Inflammatory and CVDs

The gene expression array identified 51 significantly altered genes in monocytes from anti-dsDNA positive SLE patients relative to anti-dsDNA negative (Figure 2A through 2C). To validate the data obtained, we analyzed in the whole cohort of SLE patients the expression of 18 selected genes, including those most significantly altered and a number of them known to be linked to the physiopathology of CVD in SLE.[14] These analyses confirmed the data of the mRNA array (Figure IIIA in the Data Supplement).

Monocytes From SLE Patients Positive for Anti-dsDNA Antibodies Display Distinct Profiles of Cytokines/Chemokines and Intracellular Signaling Related to Inflammation and CVD Compared With Anti-dsDNA Negative SLE Patients

Intracellular cytokine array showed that levels of 50 cytokines, chemokines, and growth factors were significantly deregulated between monocytes from SLE patients positive and negative for anti-dsDNA (Figure 2D and 2E). All of these mediators showed direct interactions with each other, suggesting a coordinated alteration (Figure IIIB and IIIC in the Data Supplement).

Several intracellular kinases involved in the translation of signals from said mediators were found more activated in monocytes from anti-dsDNA positive SLE patients, including proteins involved in apoptosis (caspase-3, BAD [BCL2-associated agonist of cell death]), and regulatory proteins of inflammation, thrombosis, and oxidative stress (ERK [extracellular signal-regulated kinases], p38 [p38 MAP kinase], GSK-3b [glycogen synthase kinase 3 beta], RPS6 [ribosomal protein S6], PRAS40 [proline-rich Akt substrate of 40 kDa], and JNK [c-Jun N-terminal kinases]; Figure 2F).

To have a full picture of the main molecular alterations identified in vivo in monocytes, we performed an integrated analysis between the genes and proteins differentially expressed in anti-dsDNA positive patients. The network created among the most interconnected molecules revealed that the main canonical pathways that mapped those molecules were NFKB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling, IFN signaling, atherosclerosis signaling, agranulocyte adhesion and diapedesis, and SLE signaling (Figure IVA in the Data Supplement).

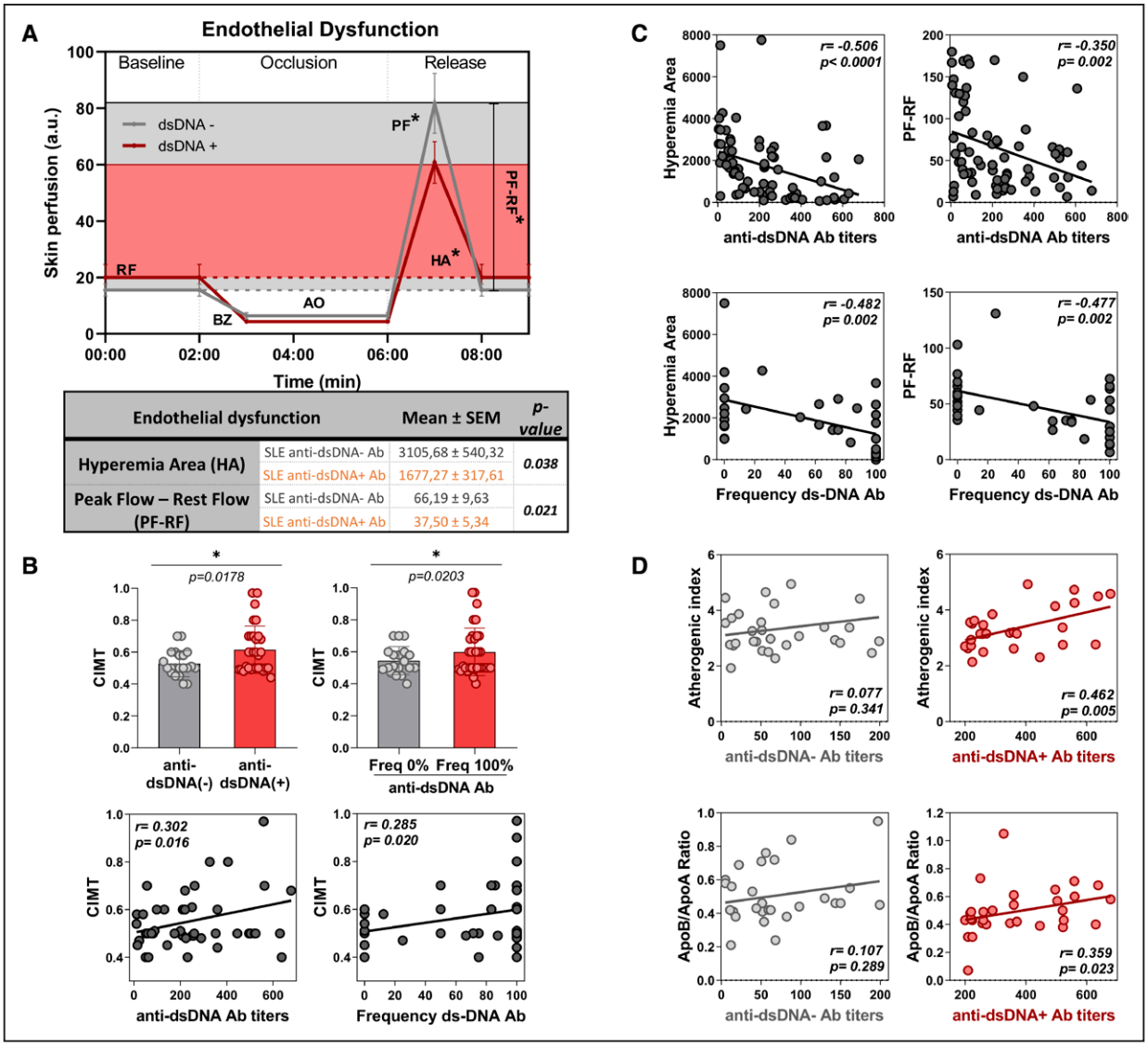

Figure 1. Different subclinical measures of cardiovascular disease: endothelial dysfunction, proatherogenic dyslipidemia, and atherosclerosis are associated with positivity and persistence of anti-double stranded DNA antibodies (anti-dsDNA Abs) in systemic lupus erythematosus (SLE) patients.

A, Microvascular endothelial function of SLE patients positive and negative for anti-dsDNA Abs determined with Periflux 5010 linear Doppler. Graphs and table show the mean±SEM. **B**, Relationship between the value of the carotid intima-media thickness (CIMT) and the levels and frequency of anti-dsDNA Abs (mean±SEM) and positive correlation between the values of the CIMT and the levels and frequency of anti-dsDNA Abs. **C**, Negative correlations among anti-dsDNA Ab titers and persistence in SLE patients and endothelial dysfunction parameters (hyperemia area [HA] and peak flow [PF] minus rest flow [RF]). **D**, Positive correlation between anti-dsDNA Ab levels and the plasma atherogenic index and the apoB/A. AO indicates occlusion area; and BZ, basal zero.

The Presence of Anti-dsDNA Antibodies Is Associated With Increased Levels of Circulating Inflammatory Mediators and Oxidative Stress Markers

Increased levels of cytokines and chemokines related to the macrophage response (IL [interleukin]-1β, IL-6, IL-8, and CCL2 [C-C motif chemokine ligand 2]), leukocyte chemotaxis (CCL5 and IP-10 [interferon gamma-induced protein 10]), activation by growth factors (VEGF [vascular endothelial growth factor]), and lymphocyte activity (IFNγ [interferon gamma] and IL-17) were demonstrated (Figure VA through VD in the Data Supplement). Accordingly, an alteration of the oxidative status, with increased production of lipoperoxides, was revealed in patients positive for anti-dsDNA in relation to those negative (Figure VE in the Data Supplement).

Anti-dsDNA Antibody Positivity Is Related to Increased Levels of NETosis-Related Products Both in Neutrophils and Plasma

Neutrophils from positive anti-dsDNA SLE patients exhibited increased NETs formation in relation to those negative for anti-dsDNA, demonstrated by higher levels

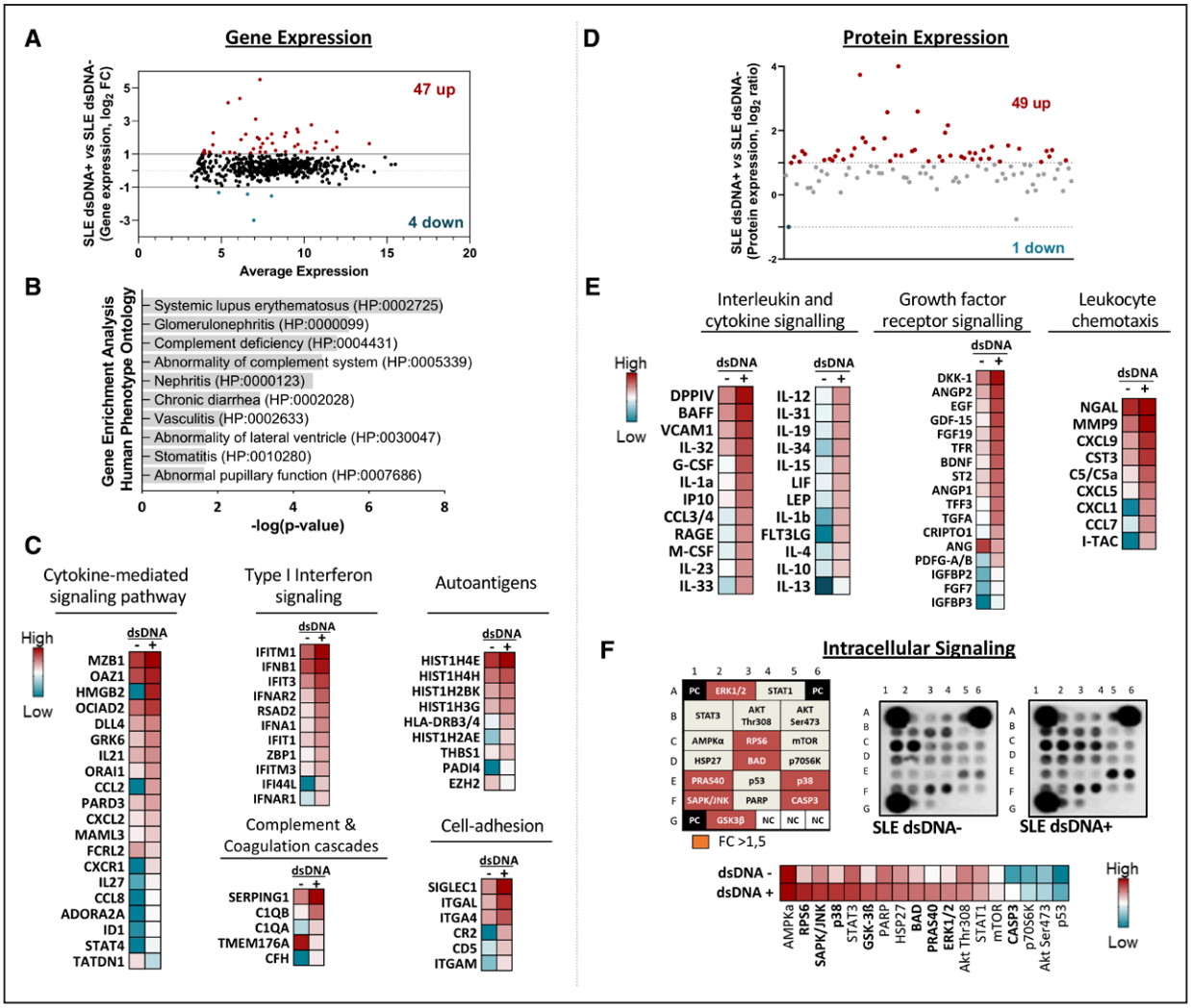

Figure 2. Gene expression and inflammatory protein profiles in monocytes from systemic lupus erythematosus (SLE) patients positive and negative for anti-double stranded DNA (anti-dsDNA) antibodies and functional categorization.
A, Scatter plot of the gene expression profile in monocytes from SLE patients positive for anti-dsDNA antibodies vs negative. The expression levels of 47 genes were upregulated in anti-dsDNA antibody positive SLE patients (fold change [FC], >2), 4 were downregulated (FC, <2). **B**, Main diseases enriched by gene ontology (GO) enrichment analysis of the gene expression signature in monocytes from SLE patients positive for anti-dsDNA antibodies. **C**, Heat map representing the expression data of the genes, their functional categorization, and the interaction network derived from the STRING k-clustering analysis of the gene expression signature in monocytes from SLE patients positive and negative for anti-dsDNA antibodies. **D**, Scatter plot of the cytokine/chemokine expression profile in monocytes from SLE patients positive for anti-dsDNA antibodies vs negatives. The expression levels of 49 proteins were upregulated in anti-dsDNA antibody positive SLE patients, whereas 1 was downregulated. **E**, Heat map representing the expression data of cytokines/chemokines and their functional categorization in monocytes from SLE patients positive and negative for anti-dsDNA antibodies. **F**, Analysis of the phosphorylation/activation status of intracellular kinases in monocytes from SLE patients positive and negative for anti-dsDNA antibodies. Heat map representing the phosphorylation data of intracellular proteins expressed in monocytes between SLE patients positive and negative for anti-dsDNA antibodies.

of intracellular elastase and MPO. Cell-free nucleosomes and cell-free elastase in plasma were also increased in positive anti-dsDNA SLE patients in relation to those negative for anti-dsDNA (Figure VI in the Data Supplement).

Clinical Cardiovascular Risk Profile of SLE Patients Positive for Anti-dsDNA Is Directly Associated With Its Altered Molecular Signatures

Correlation studies between the distinctive clinical and molecular profiles of anti-dsDNA positive SLE patients showed a relationship among altered transcriptomic signatures in monocytes, NETosis-derived products and plasma inflammatory profile, and subclinical measures of CVD (endothelial dysfunction, proatherogenic dyslipidemia, and atherosclerosis; Figure VII in the Data Supplement). Moreover, when patients positive for anti-dsDNA antibodies were divided into groups with low and high anti-dsDNA titers, we observed significant changes in a number of circulating inflammatory mediators (Table IV in the Data Supplement).

Anti-dsDNA Antibodies Modulate the Molecular Profile of Monocytes

Gene expression array (NanoString) identified 50 significantly altered genes in healthy monocytes treated with IgG-dsDNA antibodies relative to those treated with IgG-control (Figure VIIIA and VIIIB in the Data Supplement). Likewise, the IgG-dsDNA antibodies promoted, in vitro, an altered expression of numerous inflammatory proteins (Figure VIIIC through VIIIE in the Data Supplement).

The overall molecular alterations at gene and protein levels promoted by anti-dsDNA antibodies were characterized through an integrated analysis where differentially expressed genes and proteins after the treatment with anti-dsDNA were interconnected in a molecular network.

The main canonical pathways overrepresented by those molecules were similar to those identified in the in vivo analysis including NFKB signaling, apoptosis signaling, atherosclerosis signaling, agranulocyte adhesion and diapedesis, and SLE signaling (Figure IVB in the Data Supplement). Moreover, a panel of key altered molecules involved in these pathways, which are node of multiple connections, was commonly identified in vivo and in vitro (Figure IVA and IVB in the Data Supplement, highlighted in black circles). Thus, these molecules might exemplify main drivers of the pathophysiological effects promoted by these autoantibodies.

Anti-dsDNA Antibodies Modulate Apoptosis in Monocytes and NETosis in Neutrophils

In vitro treatment of healthy monocytes with anti-dsDNA antibodies induced apoptosis, as demonstrated by transmission electron microscopy analyses (Figure 3A). Similarly, Western blot analysis of apoptosis-associated proteins demonstrated altered expression/activity of various pro- and antiapoptotic proteins (Figure 3B).

Besides, in vitro, these autoantibodies promoted an increased phosphorylation of several intracellular proteins that govern the expression of numerous proinflammatory/prothrombotic molecules associated with the development of CVD (p38, ERK, JNK, and GSK-3; Figure 3C). Hence, treatment of HD-neutrophils with IgG-dsDNA induced NET extrusion, identified by fluorescence microscopy and scanning electron microscopy studies (Figure 3D). Increased levels of intracellular elastase and MPO and PADI-4 (peptidyl arginine deiminase, type IV), as well as elastase and DNA, in the supernatants of said cultures were also observed (Figure 3E). Consistently, IgG-dsDNA promoted an oxidative status (increased peroxides and mitochondrial damage and reduced GSH [glutathione] levels; Figure 3F) and enlargement in the expression of various mediators of atherosclerosis and CVD (IL-6, IL-8, CCL2, CCL3, and TF [tissue factor]; Figure 3G).

FcR Binding Plays a Mechanistic Role Associated With the Activation of the Myeloid Cells by Anti-dsDNA Antibodies

To get insight in the mechanisms related to the activation of myeloid cells by anti-dsDNA, we performed in vitro studies involving blocking the FcR.

First, we evaluated the binding capacity and location of anti-dsDNA in monocytes by confocal microscopy. Both, IgG-control and IgG-anti-dsDNA were conjugated with a fluorescent dye (green channel). Conjugated IgG-anti-dsDNA antibodies showed an increased binding capacity to healthy monocytes compared with conjugated IgG-control, evidenced by a higher fluorescence intensity (Figure 4A and 4B). This result was further validated by flow cytometry (Figure IXA and IXB in the Data Supplement).

Regarding the location in the cell, conjugated IgG-anti-dsDNA antibodies were mainly attached to the plasma membrane, demonstrated by the colocalization with the CellMask Plasma Membrane Staining (red versus green channel; Figure 4A and 4C). Moreover, the presence of anti-dsDNA antibodies in the nucleus was also identified by colocalization with DAPI staining (blue versus green channel) although with less extent than the one exhibited with plasma membrane (Figure 4A and 4D). The degree of colocalization analyzed using Manders coefficients in both plasma membrane and nucleus was significantly higher compared with IgG-control (Figure 4C and 4D).

When monocytes were preincubated with FcR blocking, the intensity of the binding, surface distribution, and nucleus presence were significantly reduced (Figure 4A through 4D), highlighting the role of this receptor in the physical interaction and internalization of anti-dsDNA antibodies in monocytes.

Second, we analyzed the functional implication of the blockage of FcR in the activation of myeloid cells by anti-dsDNA antibodies, in which a dose-dependent pattern was further demonstrated (Figure X in the Data Supplement). The induction of the apoptotic and proinflammatory status promoted by IgG-anti-dsDNA antibodies in monocytes, including the increased expression of a panel of inflammatory mediators associated with CVD, was prevented by the blockage of FcR (Figure IXC and IXD in the Data Supplement). Similarly, in neutrophils, the increased NETotic, oxidative, and proinflammatory status promoted by anti-dsDNA antibodies was also prevented by the blockage of FcR (Figure IXF through IXH in the Data Supplement). These results were in line with the reduced fluorescent intensity showed by the conjugated IgG-anti-dsDNA antibodies in neutrophils in the presence of FcR blocking (Figure IXE in the Data Supplement).

Anti-dsDNA Antibodies Promote Endothelial Dysfunction

Coculture experiments leukocytes/HUVECS using trans wells showed limited direct activating effects of IgG-dsDNA on HUVEC, promoting only increased

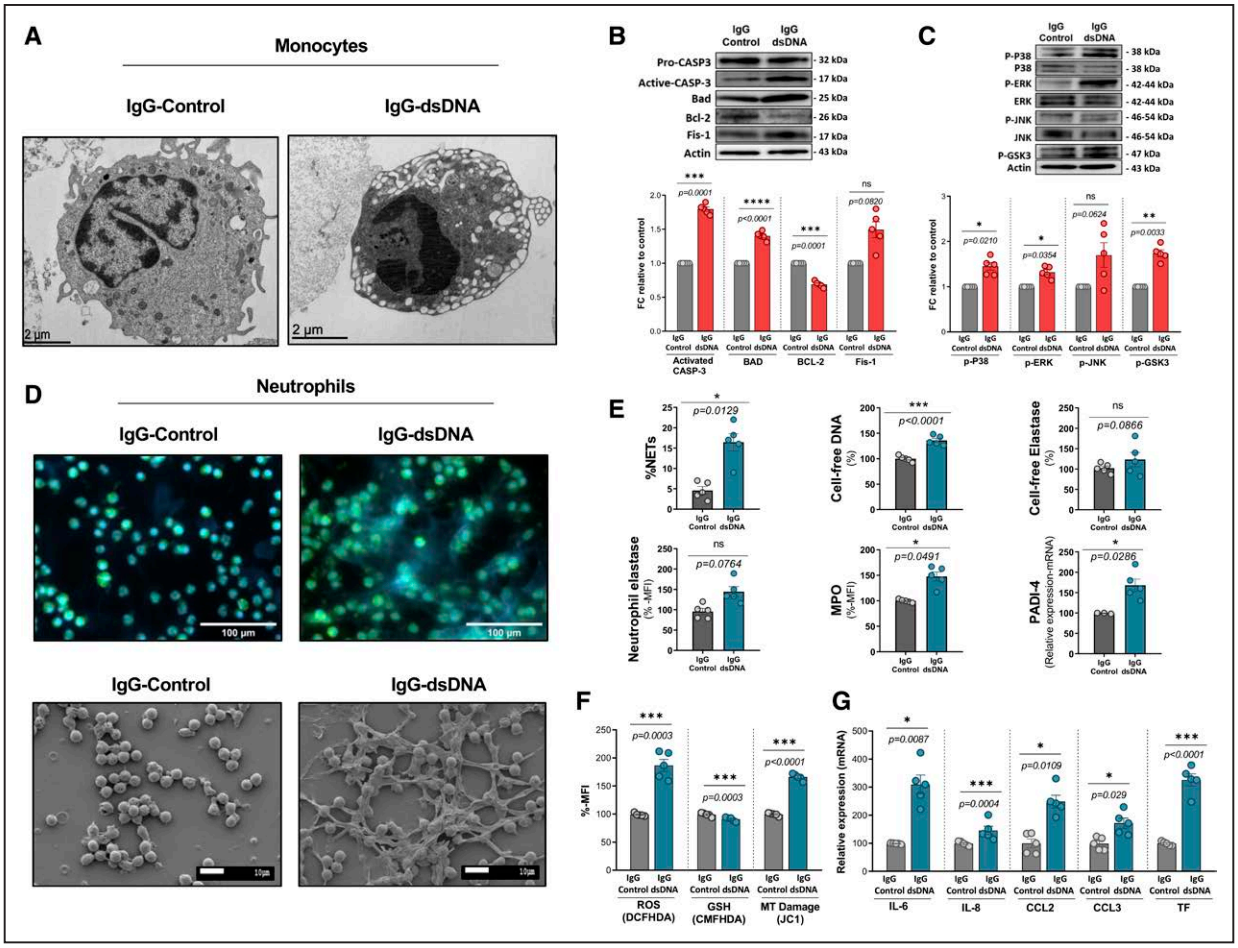

Figure 3. Anti-double stranded DNA (anti-dsDNA) antibodies modulate apoptosis in monocytes and NETosis in neutrophils. **A**, Representative transmission electron microscopy images of monocytes treated with IgG-control and IgG-dsDNA. Results are representative of 4 independent experiments. **B**, Western blot detection of proteins that control the apoptotic pathway (mean±SEM). **C**, Western blot detection of proteins that control signal translation, regulating the expression of numerous inflammatory mediators (mean±SEM). **D**, Representative images of fluorescence microscopy (elastase and DAPI are shown in green and blue, respectively) and scanning electron microscopy of neutrophils treated with IgG-control and IgG-dsDNA. **E**, Analysis of products derived from NETosis: the results of the analysis of NETosis by fluorescence microscopy were represented as %NETs±SEM. The expression levels of neutrophil elastase, MPO (myeloperoxidase), and PADI-4 and the levels of elastase and free DNA in the supernatants (mean±SEM) are also represented. **F**, Anti-dsDNA antibody effects in the oxidative and proinflammatory status (**G**) of neutrophils analyzed by flow cytometry and RT-PCR (reverse transcription polymerase chain reaction), respectively. Bar graphs represent the mean±SEM. DAPI indicates 4′,6-diamidino-2-phenylindole; FC, fold change; IL, interleukin; MFI, mean fluorescence intensity; NET, neutrophil extracellular trap; and PADI-4, peptidyl arginine deiminase, type IV. *P<0.05.

expression of 2 markers associated with endothelial function (eNOS [endothelial NOS] and ICAM-1 [intercellular adhesion molecule 1] at mRNA and protein levels, respectively; Figure 5A). Coculture with purified leukocytes potentiated the effects of IgG-dsDNA antibodies on HUVEC, promoting an increase in the expression of several proteins associated with endothelial activation, oxidative stress, inflammation, and thrombosis, both at mRNA and protein levels, most probably promoted by its interaction with mediators secreted by leukocytes (Figure 5B through 5D). Similar results were obtained on coculture experiments leukocytes/HUVECs allowing a direct interaction among cells (Figure XIA through XID in the Data Supplement). No significant differences on the degree of activation of endothelial cells among leukocyte subtypes were identified with any of the experimental approaches used.

DISCUSSION

This study proved for the first time the relevant role of anti-dsDNA antibodies as modulators of the enhanced cardiovascular risk in SLE patients. By using in vivo and in vitro genomic and protein-based technological approaches, we have unraveled the role of anti-dsDNA antibodies on the alterations in the molecular profile and activity of immune and vascular cells, as well as in the inflammatory status and the endothelial dysfunction present in SLE patients with a higher risk of CVD.

We have further characterized the molecular mechanisms underlying the effects of anti-dsDNA antibodies on endothelial and myeloid cells.

The prevalence of atherosclerosis and CVD in SLE patients is not entirely attributable to traditional risk factors,

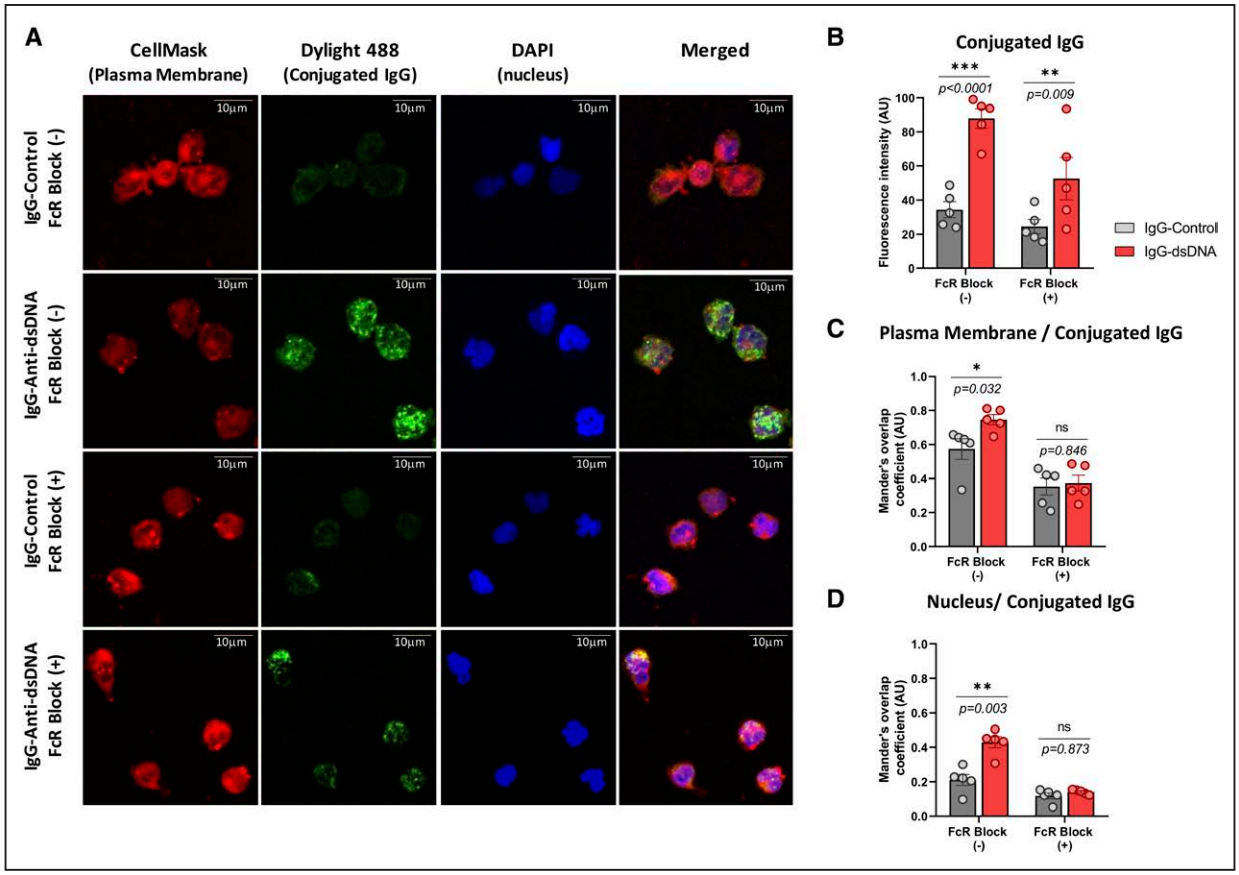

Figure 4. Confocal microscopy analysis.
Binding of anti-double stranded DNA (anti-dsDNA) antibodies and FcR (Fc receptor) blockage effects in monocytes. **A**, Representative confocal microscopy images of monocytes purified from healthy donors treated with either IgG-control or IgG-dsDNA (40 µg/mL) in the presence or absence of FcR blocking reagent (magnification ×63; scale bar, 100 µm). **B**, Fluorescence intensity of conjugated IgG (Dylight 488). **C**, Colocalization analysis of plasma membrane (CellMask) and conjugated IgG (Dylight 488) using Manders coefficients. **D**, Colocalization analysis of nucleus (DAPI [4′,6-diamidino-2-phenylindole]) and conjugated IgG (Dylight 488) using Manders coefficients. **P*<0.05, ***P*<0.001, ****P*<0.0001.

so that new findings argue in favor of an enlarged incidence of cardiovascular events in patients with higher disease activity and longer course of the disorder, which support a chronic inflammatory status.[1–3] In line with this, in our patients' cohort, the presence and titers of anti-dsDNA antibodies were linked to several parameters related to the increased cardiovascular risk, including the CIMT, the microvascular endothelial dysfunction, the atherogenic risk index, and the ApoB/ApoA ratio. Moreover, this relationship was independent of the presence of other cardiovascular risk factors, thus reinforcing the idea that positivity for anti-dsDNA might directly influence CVD development in SLE patients.

Serum levels of anti-dsDNA antibodies fluctuate with disease activity and are even modulable by therapies. Moreover, their titers may predict a disease relapse.[17] Thus, their relevance in the development of atherosclerosis and CVD, both progressive processes that develop slowly over time, should be evaluated over several years. Accordingly, we estimated the persistence of these autoantibodies 7 years before the study on SLE patients. The persistence of anti-dsDNA was found associated with both, the high thickness of the carotid intima-media and the altered microvascular endothelial dysfunction displayed by these patients, as well as to the persistence of clinical features such as moderate disease activity, kidney function and inflammation, and the continued treatment with immunosuppressants. These results indicate that the persistence of anti-dsDNA antibodies might have a significant impact on the clinical development of lupus pathology and related comorbidities, contributing to an accelerated atherosclerotic process.

Monocytes/macrophages play roles of major significance in the pathogenesis of atherosclerosis, including lipid metabolism alterations, increased adhesive properties, phagocytosis, and secretion of proinflammatory and prothrombotic mediators that drive CVD.[12] In preceding transcriptomic studies, we found that SLE monocytes exhibit an altered expression of many cytokines, chemokines, and inflammatory factors compared either with HD and with primary antiphospholipid syndrome, which were linked to disease status, early atherosclerosis development, and increased cardiovascular risk.[13,14] Remarkably, the present study validates that data and further delineates the role of anti-dsDNA antibodies as potential regulators of that alteration.

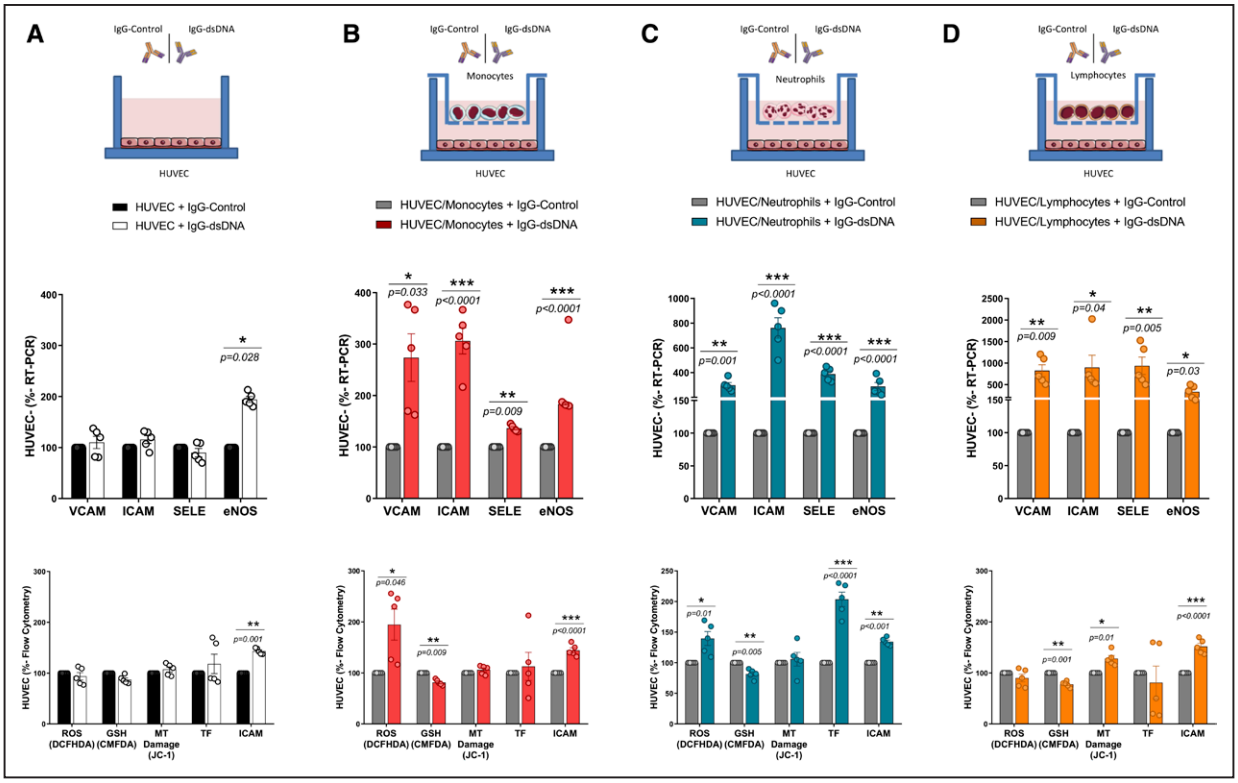

Figure 5. Endothelial activation by anti-double stranded DNA (anti-dsDNA) antibodies in coculture experiments with immune cells using transwells.

A, Expression levels of mRNA by RT-PCR (reverse transcription polymerase chain reaction) and specific proteins by flow cytometry in human umbilical vein endothelial cells (HUVECs) treated with IgG-control and IgG-dsDNA. **B**, Expression levels of mRNA and proteins in HUVECs cocultured with monocytes, (**C**) neutrophils, and (**D**) lymphocytes and treated with IgG-control and IgG-dsDNA. Bar graphs represent the mean±SEM from 5 independent experiments. *P<0.05. eNOS indicates endothelial NOS; GSH, glutathione; ICAM, intercellular adhesion molecule 1; MT, mitochondrial; ROS, reactive oxygen species; SELE, selectin E; TF, tissue factor; and VCAM, vascular cell adhesion molecule 1.

Monocytes from SLE patients positive for anti-dsDNA antibodies showed distinctive gene and protein expression profiles in relation to patients negative for anti-dsDNA, further related to inflammation and CVD, including IFN and molecules involved in hematopoietic hemostasis, complement coagulation cascades, immune cell activation, synthesis of inflammatory cytokines, and leukocyte chemotaxis and migration, among others. In parallel, also supporting previous studies in SLE,[13] a number of circulating molecules related to macrophage response, leukocyte activity and chemotaxis, growth factor activity, and oxidative stress were found significantly altered in anti-dsDNA positive SLE patients in comparison with those negative for anti-dsDNA.

While the precise pathogenic mechanisms of anti-dsDNA antibodies in SLE remain unknown, multiple studies, mainly developed on B/T lymphocytes and animal models, have suggested that pathology may be caused by several nonexclusive mechanisms, including apoptosis induction by binding cellular DNA, and the stimulated IFNα secretion induced by Fc-mediated uptake by bringing dsDNA ligand to TLR9 (Toll-like receptor 9).[25]

To assess the anti-dsDNA antibody effects on SLE monocytes, we first evaluated, in vivo, the intracellular signaling proteins activated in anti-dsDNA positive SLE patients versus patients negative for these autoantibodies. Intracellular kinases regulating apoptosis signaling along with proteins related to inflammation and thrombosis, such as ERK, p38, GSK-3β, and JNK, displayed a higher phosphorylation status in monocytes from SLE patients positive for anti-dsDNA, and proapoptotic factors such as BAD and cleaved caspase-3 were activated as well.

It has been demonstrated that SLE patients display a higher percentage of leukocyte apoptosis than HDs. Moreover, the percentage of apoptosis among the innate immune cells correlates with autoantibodies levels (eg, anti-dsDNA, anti-LA, and anti-Ro).[26] In our hands, the analyses by electron microscopy, along with that of proteins related to apoptosis, demonstrated the presence of numerous monocytes showing the characteristic apoptotic morphology in conjunction with the altered expression of several pro- and antiapoptotic proteins, thus supporting the role of anti-dsDNA antibodies inducing monocyte apoptosis.

Besides, mechanistic in vitro studies showed that blocking Fc receptors prevented the anti-dsDNA–induced apoptosis in monocytes, thus suggesting that the induction of apoptosis in monocytes by anti-dsDNA antibodies is mediated, at least partially, by its binding to FcR. These results are in agreement with precedent studies, showing that specific FcRs trigger apoptosis in human monocytes, being involved in controlling inflammation.[27]

In addition, combined flow cytometry and confocal microscopy analyses in these cells demonstrated that anti-dsDNA antibodies were also internalized in monocytes, so that it was identified in the nucleus of these cells. These results are in accordance with previous reports proving their penetration in cells. This process might further contribute to apoptosis, as it has been established that in the nucleus these antibodies cause DNA fragmentation and induce apoptosis.[28]

Likewise, the incubation of HD monocytes with purified IgG-dsDNA promoted a parallel gene alteration to that observed in vivo in SLE monocytes, including the activation of the IFN signaling and the overexpression of several interrelated proinflammatory cytokines and chemokines. Moreover, levels of these deregulated genes and proteins were linked with the cardiovascular risk factors found aberrantly altered in our cohort of patients positive for anti-dsDNA antibodies, such as the CIMT, the microvascular endothelial function, or the atherogenic index, thus linking the induced activation of monocytes by anti-dsDNA in SLE patients.

NETosis, considered a main source of modification and externalization of autoantigens in the production of SLE autoantibodies[29,30] and known to trigger the immune and inflammatory responses in autoimmune diseases, was found significantly increased in our cohort of SLE patients positive for anti-dsDNA in relation to those negative. Moreover, we have demonstrated for the first time that anti-dsDNA is closely related to a significant alteration of several inflammatory and oxidative stress molecules in immune and endothelial cells (ie, type I interferon signaling molecules, cytokines/chemokines, and peroxides), which are well-known inductors of NETosis.

In the present study, we have demonstrated that anti-dsDNA antibodies directly induce NETosis in neutrophils by promoting an increase in known triggers of this process, such as PADI-4, reactive oxygen species, and several cytokines.[31] Moreover, in our hands, blocking of FcRs, which avoided the binding of anti-dsDNA antibodies to cultured neutrophils, significantly inhibited these inductors and thus prevented NETosis. Thus, our data suggest that the induction of NETosis by anti-dsDNA antibodies is mediated, at least partially, by its binding to FcR. This mechanism is in line with previous findings suggesting the involvement of these receptors in NET formation.[32]

Studies have shown that the NETolitic activity is lower in SLE patients and that this incorrect elimination of NETs contributes to generate new epitopes for autoantibody production, including anti-dsDNA, whose levels further correlate with those of several NETosis-derived products.[33,34] That overall data suggest that in SLE patients, it can be identified a continuous loop on which continuous anti-dsDNA generation directly or indirectly supports NETosis production, and the inappropriate elimination of NETs favors the continuous presence of autoantigens for anti-dsDNA production.

Endothelium is a major regulator of vascular homeostasis. Persistent systemic inflammation associated with lupus may impair endothelium function and trigger a series of events. These events work in concert with traditional CVD risk factors to promote the occurrence and development of atherosclerosis.[35] In line with this evidence, we demonstrated that the presence, titers, and persistence of anti-dsDNA antibodies are responsible for the endothelial dysfunction observed in SLE patients. Our in vitro studies further support this hypothesis, showing that anti-dsDNA antibodies promote the deregulation of endothelial cell activation markers, including several adhesion molecules, along with procoagulant factors and oxidative stress molecules, when cocultured with immune cells, thus contributing to the establishment of a proatherosclerotic and prothrombotic status in SLE patients.

In conclusion, our results support that (1) positivity and persistence of anti-dsDNA antibodies in SLE patients contribute to endothelial dysfunction, proatherogenic dyslipidemia, and atherosclerosis development. (2) Molecularly, anti-dsDNA antibodies are linked to the aberrant activation of endothelial and innate immune cells, so that anti-dsDNA positive SLE monocytes show distinctive gene and protein expression/activity profiles, and neutrophils are more prone to suffer NETosis. (3) Anti-dsDNA positive SLE patients further display altered levels of numerous circulating mediators related to inflammation and enhanced cardiovascular risk. (4) Anti-dsDNA antibodies promote, in vitro, neutrophil NETosis and monocyte apoptosis, regulate the expression of molecules related to inflammation and thrombosis, and induce endothelial cell activation. (5) FcR-binding mechanisms mediate myeloid cell activation by anti-dsDNA antibodies (Figure VII in the Data Supplement).

Hence, anti-dsDNA antibodies might increase the cardiovascular risk of SLE patients by altering key molecular features that drive a distinctive and coordinated immune and vascular activation.

ARTICLE INFORMATION

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and suggestions; P. Font and N. Barbarroja performed statistical analysis and discussed the results; J. Antonio González-Reyes and J. Manuel Villalba performed some experiments and analyzed the data. C. López-Pedrera, C. Pérez-Sánchez, A.M. Patiño-Trives, and M.A. Aguirre-Zamorano formed the hypothesis, directed and coordinated the project, designed the experiments, analyzed the data, and wrote the manuscript.

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Disclosures

None.

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