



Immune mitochondrial complex I mediates vascular damage in toll-like receptor 7 (TLR7)-induced lupus

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ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by immune dysregulation and high cardiovascular risk, including hypertension and endothelial dysfunction. Metabolic reprogramming of immune cells, particularly CD4 + T cells, contributes to SLE pathogenesis. We investigated the role of mitochondrial metabolism, specifically the NDUF54 subunit of complex I, in immune cells during lupus induced by toll-like receptor (TLR)7 activation with imiquimod (IMQ). We analyzed transcriptomic data from peripheral blood mononuclear cells obtained from SLE patients and their matched healthy controls showing that dendritic cells and undetermined cells exhibited the highest mean expression levels of NDUF54. Using bone marrow chimeric mice, we demonstrate that NDUF54 deficiency in hematopoietic cells prevents IMQ-induced hypertension, reduces autoantibody production, and limits vascular oxidative stress, inflammation, and kidney injury. These effects are associated with a shift in CD4 + T cell polarization toward regulatory T cells and a reduction in Th1 and Th17 subsets in the spleen and blood. Pharmacological dual inhibition of glycolysis with 2-deoxy-D-glucose and mitochondrial metabolism with metformin further supports the therapeutic potential of metabolic targeting, improving vascular function and preventing aortic remodeling, characterized by proteoglycan and collagen accumulation, increased aortic stiffness, and secretory and profibrotic phenotypes. These interventions reduce oxidative stress and immune cell infiltration in the vascular wall, mitigating endothelial dysfunction and the shift to a synthetic phenotype. Our findings highlight mitochondrial complex I as a critical modulator of immune-mediated vascular damage in lupus induced by TLR7 activation and propose metabolic reprogramming as a promising therapeutic strategy for lupus-associated cardiovascular complications.

1. Introduction

Systemic lupus erythematosus (SLE) is a multiorgan autoimmune disease characterized by excessive production of autoantibodies,

triggering the formation and deposition of immune complexes, leading to damage to various organs and tissues. This autoimmune disease primarily affects young women, particularly those of childbearing age, with a female-to-male ratio of approximately 9:1. The interaction of

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genetic, environmental, hormonal, and metabolic factors contributes to the susceptibility to SLE [1]. This disease is associated with an increased risk of developing renal and cardiovascular diseases [2], which are the leading causes of mortality in SLE patients [3]. Additionally, there is a higher prevalence of hypertension in individuals with SLE compared with age-matched healthy individuals [4]. However, the pathophysiological mechanisms behind SLE-associated hypertension remain poorly understood. Inflammatory cytokines and reactive oxygen species (ROS) are known to contribute to the development of SLE hypertension by promoting local inflammation and causing renal and vascular dysfunction, which are likely consequences of the initial immune system dysregulation [5].

Toll-like receptor (TLR)7 signaling is a recognized pathogenic pathway in development and progression of SLE [6]. In this process, immune complexes are captured by plasmacytoid dendritic cells (pDCs), activating endosomal TLRs, which leads to the overproduction of type I interferons (IFNs) and an elevated IFN signature, observed in approximately half of SLE patients [7,8]. These cytokines subsequently activate various immune cells, including T cells, B cells, natural killer (NK) cells, and conventional dendritic cells (cDCs) [9]. Immune cells are highly adaptable, quickly shifting from a quiescent state to various effector functions in response to specific stimuli. This adaptability depends on the availability and utilization of specific metabolic substrates, a process known as metabolic reprogramming [10]. There is increasing evidence that cellular metabolism, particularly in T cells, is altered in lupus [11].

Autoreactive CD4 + T cells play a crucial role in SLE, with an increased number of activated CD4 + T cells and a rise in T helper (Th)1 and Th17 lymphocytes observed in both SLE patients and lupus-prone mice [12,13]. Th17 cells are associated with the cardiovascular complications of SLE [14]. Therefore, targeting Th1 and Th17 cells by reducing CD4 + T-cell activation, proliferation, and differentiation could be a promising strategy for treating SLE-associated hypertension. CD4 + T cells require substantial energy and biosynthesis to support these functions, with T-cell receptor activation leading to increased glycolysis and mitochondrial metabolism [15]. Quiescent T cells, which have lower energy demands, maintain their oxidative metabolism using glucose, lipids, and amino acids. The importance of glucose metabolism in autoimmunity is underscored by studies in which overexpression of the glucose transporter GLUT1 in mice led to immune complex-mediated nephritis, hypergammaglobulinemia, and CD4 + T cell hyperactivation [16]. While Th1, Th2, and Th17 cells primarily rely on glycolysis, regulatory T (Treg) cells and long-lived memory T cells exhibit higher rates of lipid oxidation [17]. Thus, CD4 + T lymphocytes depend on glycolysis to perform their inflammatory functions. Compared to non-autoimmune controls, CD4 + T cells from lupus-prone B6.Sle1.Sle2.Sle3 (TC) mice have shown increased glycolysis and mitochondrial oxidative metabolism [11,18]. However, the role of these immune metabolic mechanisms in lupus hypertension remains unclear.

Lupus pathogenesis is associated with heightened metabolism in immune cells; therefore, it has been suggested that metabolic inhibitors could be beneficial in treating lupus [19]. Interestingly, blocking glucose metabolism through the inhibition of glucose uptake with 2-deoxy-D-glucose (DG), combined with the inhibition of mitochondrial complex I and oxidative phosphorylation with metformin (Met), prevented disease in lupus-prone mice. However, this combination was ineffective in mice with established disease, indicating that glucose oxidation is critical for CD4 + T cell activation in SLE [11,18].

Our recent studies have shown that TLR7 activation can induce hypertension, vascular remodeling, and endothelial dysfunction in BALB/c mice, which are associated with increased vascular inflammation and oxidative stress [14]. We hypothesize that the mitochondrial hyperactivity observed in lupus T cells from lupus models may contribute to TLR7-induced hypertension, a relation not thoroughly investigated yet. Optimizing mitochondrial function can inhibit immune pathways in SLE; therefore, our study aims to examine the role of mitochondrial complex I (NDUFS4 subunit) in immune cells and its relation to

endothelial dysfunction and the development of hypertension in a mouse model of TLR7-induced lupus triggered by the TLR7 agonist imiquimod (IMQ).

2. Material and methods

2.1. Bioinformatics processing of transcriptomic data

For human transcriptomic studies, single-cell RNA sequencing (scRNA-seq) data were obtained from the publicly available dataset GSE174188, which is part of the California Lupus Epidemiological Study (CLUES) [20]. This dataset includes transcriptomic profiles of 162 peripheral blood mononuclear cell (PBMC) samples from SLE patients and 99 matched healthy controls collected from the UCSF Rheumatology Clinic. Preprocessing and quality control of raw sequencing reads were conducted using standard bioinformatics pipelines. Data were processed using Scanpy [21] in Python, and integration was performed with single-cell variational inference (scVI) package [22]. Cell clustering and annotation were performed using gene expression profiles and marker-based classification. Expression levels of NDUFS4 across immune cell populations were quantified and visualized using dot plots, where dot color intensity represents mean expression levels, and dot size indicates the percentage of cells expressing NDUFS4 within each immune subset.

2.2. Animals and experimental groups

Mouse strains used in this study included *Ndufs4*^{-/-} mice on a B6 background (JAX stock #027058) [23], generously provided by Dr. R. Palmiter (University of Washington, Seattle, Washington, USA), as well as B6/SJL (*Ptpcr^a Pepc^b*/BoyJ) mice expressing the CD45.1 allele (JAX stock #002014) and C57BL/6 J mice expressing the CD45.2 allele (JAX stock #005652), both obtained from the Jackson Laboratory. The procedures followed in the present study adhered to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines [24]. We specifically chose to use female mice due to their higher susceptibility to TLR7-driven functional responses and autoimmunity [25], which aligns with the higher prevalence of lupus in women compared to men.

All animal procedures and experiments adhered to ethical regulations and were approved by the University of Granada Ethics Committee and the Andalusian regional authorities (ref. 16/02/2022/013/A). These procedures complied with the EU Directive 2010/63/EU for the protection of animals used for scientific purposes and the EU Commission Recommendation 2007/526/EC on guidelines for the accommodation and care of animals used for experimental and other scientific purposes, as implemented in Spanish law under Real Decreto 1201/2005.

Mouse health was monitored daily for signs of discomfort, weight loss, and changes in behavior, mobility, feeding, or drinking habits. The mice were housed in a specific pathogen-free facility at the University of Granada's Biological Services Unit, where temperature and humidity were controlled, and a 12-hour light/dark cycle was maintained. All mice had *ad libitum* access to standard rodent chow and water.

For **Experiment 1**, we investigated the role of the mitochondrial complex I (NDUFS4 subunit) in immune cells by performing bone marrow (BM) transplantation, following a previously described protocol [26]. C57BL/6NRj mice, aged eight weeks, were irradiated with two doses of 550 rads (5.5 Gy), separated by at least three hours, for a total radiation dose of 1100 rads. Two hours later, the irradiated mice received BM cells extracted from either wild-type (WT) or *Ndufs4*^{-/-} mice. The deficiency of the NDUFS4 subunit results in a non-functional complex I [27]. Grafted mice received at least 2×10^6 BM cells in 100 μ L of phosphate-buffered saline (PBS) by intravenous injection, and an antibiotic (25 μ g/g of cefovecin) was administered subcutaneously. Blood samples were collected from mice three weeks after transplantation, and mononuclear cells were analyzed by flow cytometry for

CD45.1 (recipient) and CD45.2 (BM donor) to assess transplantation efficiency.

Eight weeks after transplantation, the mice were divided into four groups: 1) BM^{WT} (n = 10), 2) BM^{WT}-IMQ (n = 10), 3) BM^{Ndufs4^{-/-}} (n = 9), and 4) BM^{Ndufs4^{-/-}}-IMQ (n = 10). The IMQ-treated mice received a total of 1.25 mg of IMQ (Zyclara® 3.75 % cream from Laboratories Meda Pharma S.A.U., Madrid, Spain) applied to their right ears three times per week on alternate days for eight weeks. This topical IMQ application effectively induces the development of a systemic autoimmune disease [28], justifying its use in creating a murine model resembling SLE.

For **Experiment 2**: We used a combination of the metabolic modulators, Met and DG, administered via drinking water, to validate the results obtained in the previous experiment and further investigate the therapeutic target. As in the previous experiment, SLE was induced by the topical application of IMQ. Female C57BL/6Nj mice, aged 16 weeks, were randomly divided into four groups (n = 10): 1) Control (Ctrl), 2) Ctrl+Met+DG (Met, 3 mg/mL + DG 5 mg/mL in drinking water), 3) IMQ, and 4) IMQ+Met+DG (Met, 3 mg/mL + DG, 5 mg/mL in drinking water). The treatment lasted eight weeks.

2.3. Blood pressure measurements, organ weight indices, and renal injury

Systolic blood pressure (SBP) was measured in conscious mice that were pre-warmed to 35°C for 10–15 min and gently restrained for tail-cuff plethysmography (Digital Pressure Meter, LE 5001; Leticia S.A., Barcelona, Spain). The mice underwent familiarization, and the replication count was consistent with prior reports [29]. Body weights across all groups were recorded in grams. At the end of the experimental periods, the mice were killed by exsanguination under intraperitoneal anesthesia with ketamine, atropine, and diazepam. The weights of the left ventricle, liver, spleen, and kidneys were normalized to tibia length to obtain their respective weight indices. After the measurements, the samples were quickly cryopreserved in liquid nitrogen and stored at –80°C until further use.

For morphological evaluation, kidneys from all experimental groups were fixed in 10 % buffered formalin, embedded in paraffin, and cut into transverse sections. These tissue sections were stained using hematoxylin and eosin, Masson's trichrome, and periodic acid–Schiff (PAS) stains. A blinded observer analyzed 4-μm sections under a light microscope, selecting the most suitable stain for identifying each specific lesion. Renal abnormalities were scored semi-quantitatively as binary outcomes (0 or 1) and presented as the percentage of kidneys showing lesions. Glomerular alterations, including glomerulosclerosis, mesangial proliferation, endocapillary proliferation, expansion of the mesangial matrix, and deposition of immune complexes, were evaluated and quantified as the number of affected glomeruli out of 50 examined ones. Tubulointerstitial changes, such as tubular casts and inflammatory cell infiltration, were graded on a semi-quantitative scale ranging from 0 to 3. In addition, the number of nuclei per glomerular cross-section was recorded in 50 non-sclerotic glomeruli per mouse [14].

2.4. Plasma and spleen parameters

Blood samples were collected from the heart, kept on ice, and centrifuged for 10 min at 3500 rpm at 4°C. The resulting plasma samples were then frozen at –80°C. Levels of anti-dsDNA antibodies were measured using an Alpha Diagnostic ELISA Kit (Alpha Diagnostic International, San Antonio, Texas, USA) according to the manufacturer's instructions, as previously documented [14].

For the ELISA assay, flat-bottom 96-well polystyrene plates were coated overnight at 4 °C with either cardiolipin (100 ng per well; Sigma) or beta-2 glycoprotein I (β2GPI, also known as apolipoprotein H, ApoH; 100 pg per well; R&D Systems). Both antigens were prepared in 0.01 M sodium bicarbonate buffer (pH 9.55). Following incubation, plates were washed six times with PBS containing 0.1 % Tween-20 (PBS-T) and then blocked for one hour at room temperature using 10 % normal goat

serum in PBS-T. Mouse plasma samples were diluted 1:100 in PBS-T, and 200 μL of each diluted sample was added per well. Plates were incubated for one hour at room temperature. Afterward, another six washes with PBS-T were performed. A horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (specific for heavy and light chains; Jackson Immuno Research) was added at 1:2,000 dilution for one hour. Following a further six washes, the assay was developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Alpha Diagnostic International). Absorbance was measured at 450 nm (end point reading) and 620 nm (background reading) using a Sunrise microplate reader (Tecan Ibérica, Barcelona, Spain). Background correction was performed by subtracting the 620 nm readings from the 450 nm readings [30].

The ATP assay kit was used to detect ATP levels in spleen. Briefly, spleens were homogenized with lysis buffer to release cellular ATP. Then, the homogenate was centrifuged at 12000 g for 10 min at 4°C. Then supernatant was collected and assayed using the ATP Determination Kit (Molecular Probes, Invitrogen, A22066) according to the manufacturer's protocol. ATP levels were normalized to protein concentrations.

2.5. Vascular reactivity studies

The descending thoracic aortic segment was mounted onto a wire myograph (model 610 M, Danish Myo Technology, Aarhus, Denmark) containing Krebs solution. They were kept at 37°C, gassed with 95 % O₂ and 5 % CO₂ (pH = 7.4), and subjected to standard conditions for isometric tension measurement, as detailed in prior publications from our research group [31]. Pre-contraction was induced using thromboxane A₂ analog U46619 (3 nM) before the construction of a concentration-relaxation curve in response to acetylcholine (10⁻⁹-10⁻⁵ M). In some segments, serial relaxation curves were generated over 30 min, alternating between washes in the absence and presence of the specific phospho-AMP-activated protein kinase (AMPK)-α inhibitor compound C (5 μM) or the Rho kinase inhibitor Y27632 (0.5 μM). Results were expressed as relaxation levels relative to pre-contraction tone.

2.6. Vascular ROS content

To quantify vascular ROS, the American Heart Association recommends fluorescence analysis with dihydroethidium (DHE) as a useful approach to measure and image intracellular O₂⁻ production from tissues because of its high sensitivity, which should be complemented with a second independent assay such as lucigenin chemiluminescence assay with appropriate controls [32].

Aortic segments were embedded in optimal cutting temperature (OCT) compound and stored at –80°C. Cross-Section (5 μm) were incubated for 15 min at 37°C with DHE (1 μM) (Life Technologies S.A., Madrid, Spain), a red fluorescent dye that serves as a ROS sensor, as previously described [33]. The samples were then washed with HEPES buffer and incubated for 15 min at room temperature with 4',6-diamidino-2-phenylindole (DAPI, 500 nM, Thermo Fisher Scientific, Massachusetts, USA) for nuclear staining. Images were captured using a Leica DMI 3000B microscope (Leica Microsystems GmbH, Wetzlar, Germany), analyzed with ImageJ software, and processed for presentation using Adobe Photoshop. ROS production was quantified by calculating the ratio of ethidium to DAPI fluorescence. In some experiments, before DHE incubation, serial sections were treated with pegylated superoxide dismutase (PEG-SOD, 250 U/mL), or catalase (100 U/mL) for 30 min at 37°C to confirm the specificity of the reaction, or with the selective NADPH oxidase inhibitor VAS2870 (10 μM) for 30 min, or the mitochondrial ROS scavenger MitoQ (0.1 μM) for one hour to explore the source of ROS.

A lucigenin-enhanced chemiluminescence assay was conducted in intact aortic segments to measure NADPH oxidase activity, following established methods [34]. Aortic segments from all experimental groups were incubated at 37°C in a HEPES-based solution for 30 min, and

NADPH (100 μ M) was added to stimulate the enzymatic activity. Measurements were recorded using a scintillation chamber (Lumat LB 9507, Berthold, Germany) in the presence of lucigenin (5 μ M). NADPH oxidase activity was defined as the difference between the values obtained in the absence and those obtained in the presence of VAS2870. NADPH oxidase activity was quantified as relative luminescence units (RLU) per minute per milligram of dry aortic tissue.

2.7. Real-time quantitative PCR

Total RNA was extracted from mouse aortas and BM cells using PRiMeZOL™ reagent (Canvax Biotech, S.L., Córdoba, Spain). cDNA was synthesized via reverse transcription using standard RT-PCR protocols. The resulting cDNA was analyzed by quantitative real-time PCR (qPCR) using a PCRMax Eco 48 thermal cycler (PCRMax, Stone, Staffordshire, UK) and SYBR Green master mix (Promega). The forward and reverse primers used for the PCR reactions are listed in Table S1. The relative mRNA levels of target genes were quantified using the 2- $\Delta\Delta$ CT method and normalized to the expression of ribosomal protein L13a (*Rpl13a*) mRNA.

2.8. Immunoblot analysis

Samples from mouse aortas were isolated, frozen in liquid nitrogen, and then homogenized in RIPA buffer (150 mM NaCl, 50 mM Tris HCl pH8, 1 % NP-40, 0.1 % SDS, and 0.5 % sodium deoxycholate). BM cells were washed with ice-cold PBS and lysed in ice-cold total lysis buffer (20 mM Tris-HCl, pH 7.5; 1 % Triton X-100; 50 mM NaF; 5 mM MgCl₂; 500 mM NaCl; 10 mM EDTA), both supplemented with protease, phosphatase, and kinase inhibitors (100 μ M benzamidin, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin, 1 μ g/mL aprotinin, 1 μ M dithiothreitol, 1 mM PMSF, and 3 mM EGTA). Protein extracts were separated under reducing conditions on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and detected using the following antibodies: rabbit monoclonal anti-phospho-AMPK α (1:1000, #4188, Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-AMPK α (1:1000, #2532, Cell Signaling Technology), rabbit polyclonal anti-RhoA (1:500, #ab86297, Abcam, Cambridge, UK), mouse monoclonal anti-NDUFS4 (1:1000, #ab87399, Abcam), and mouse monoclonal anti- β -Actin (1:5000, #A5441, Sigma-Aldrich, St. Louis, MO, USA) antibodies. Then, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:10,000, Santa Cruz Biotechnology, Santa Cruz, USA) or goat anti-mouse (1:5000, #A4416, Sigma-Aldrich) secondary antibodies, as appropriate. Bound antibodies were detected with enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia Biotech, Amersham, UK), and densitometric analysis was performed by ImageJ software. Samples were normalized to β -actin. The phospho-AMPK α /AMPK α abundance ratio was calculated, and data are expressed as a fold change of the values from the control aorta or spleen on the same gel. Samples were re-probed for expression of β -actin.

2.9. Flow cytometry

Aortas, spleens, and blood were collected from all animals. Then, aortas were digested with ADES solution (activity units (U)/mL): collagenase type XI 125, hyaluronidase type 1 60, collagenase type 1 450, and DNase I 60 (Merck, Darmstadt, Germany) at 37°C for one hour. Spleens and aortas were homogenized and filtered to eliminate tissue debris. Erythrocytes were lysed using Gey's solution. For optimal detection of intracellular cytokines via flow cytometry, a protein transport inhibitor (BD GolgiPlug™) was applied following the manufacturer's instructions, along with 50 ng/mL phorbol 12-myristate 13-acetate and 1 μ g/mL ionomycin. All samples were first incubated with a viability dye (LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, Molecular Probes, Oregon, USA) in PBS for 20 min at 4°C in the dark. Then, cells were stained with FITC-conjugated CD45 (1:100, #130-116-500,

Miltenyi, Bergisch Gladbach, Germany), PE-conjugated CD3 (1:100, #130-121-133, Miltenyi), PerCP-Cyanine5.5-conjugated CD4 (1:100, #45-0042-82, Thermo Fisher), PE/Cyanine7-conjugated IL17A (1:100, #506921, BioLegend, San Diego, CA, USA), PE-Vio770-conjugated CD25 (1:100, #130-120-746, Miltenyi), APC-conjugated IFN- γ (1:100, #130-123-283, Miltenyi), APC-conjugated CD45R/B220 (1:100, #553092, BD Biosciences, East Rutherford, NJ, USA) antibodies. Flow cytometric analysis was conducted on a BD FACSymphony™ A5 Cell Analyzer (BD Biosciences, New Jersey, USA), outlining the gating strategy in Figs. S1–2.

2.10. Aortic histology

Aortic OCT cross-Section (5 μ m) were stained with hematoxylin and eosin, Sirius Red-Fast green, or Alcian Blue, or processed for immunofluorescence. Images were captured using a Leica DMI 3000B microscope. Collagen content and proteoglycan accumulation were quantified from Sirius Red and Alcian Blue stains, respectively, using ImageJ software.

For F-actin visualization, aortic OCT samples were thawed, fixed for 30 min in 4 % PFA, and 4- μ m cross-sections were permeabilized with 0.3 % Triton X-100 in PBS for 15 min. Sections were then blocked for one hour in a solution containing 5 % goat serum, 5 % horse serum, and 2 % BSA in PBS with 0.01 % Triton X-100, followed by overnight incubation at 4°C with Texas Red-X-conjugated phalloidin (1:2000, T7471; Thermo Fisher Scientific, Bremen, Germany). Nuclei were stained with DAPI, and sections were mounted in Citifluor AF4 mounting medium (Anamed, 17973). Images were acquired using a Leica DMI 3000B microscope with a 40 $\times$ objective.

Image processing, analysis, and quantification were performed using ImageJ software, with an intensity threshold set to include only specific signals. Total intensity was corrected for the medial aortic area and normalized to control mice.

2.11. Statistical analysis

Statistical analysis was performed with GraphPad Prism 8. The ROUT method was first used to identify and exclude outliers. Data normality was assessed using the D'Agostino-Pearson and Shapiro–Wilk normality tests, and appropriate tests were chosen according to the data distribution. Differences were analyzed using the Student's *t*-test; one-way, two-way, or repeated-measures two-way analysis of variance (ANOVA) with the Tukey post hoc test, or the Dunnett's multiple comparison test, when appropriate. Statistical significance was assigned at **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

The sample size was empirically chosen based on our previous experience with calculating experimental variability. The number of animals used is indicated in the corresponding figure legends. Investigators were not blinded to group allocation during *in vivo* experiments. The experimental groups were balanced by animal age and were randomly assigned to experimental treatments. Animals were genotyped before the experiments, caged together, and handled in the same manner.

3. Results

3.1. Lack of NDUFS4 in BM-derived cells protects against lupus-induced hypertension and autoantibody generation, associated with a change in the T cell profile

The link between an overactive metabolism in immune cells and lupus pathogenesis has led to the hypothesis that metabolic inhibitors could offer therapeutic benefits for lupus [35,36]. The activity of NDUFS4, a key subunit of complex I, is closely associated with redox homeostasis in immune cells, a crucial factor in the pathogenesis of autoimmune diseases [37]. To investigate the clinical relevance of

NDUFS4, we analyzed publicly available transcriptomic datasets from PBMCs obtained from SLE patients and their matched healthy controls [20]. Notably, dendritic cells and undetermined cells exhibited the highest mean expression levels of NDUFS4, as indicated by the darker blue coloration in the dot plot (Fig. 1a). Additionally, monocytes and platelets/megakaryocytes displayed moderate expression levels, while other immune cell types showed minimal or negligible expression. Although NDUFS4 expression tends to be higher in SLE compared to healthy controls, the difference is modest. Similar expression levels may not directly reflect functional or pathological consequences because upstream regulatory mechanisms or downstream pathway dynamics may differ, potentially leading to divergent outcomes even with comparable NDUFS4 expression levels. We conducted the following experiment to further explore the specific role that immune *Ndufs4* plays in cardiovascular complications in lupus mice. CD45.1 WT mice were irradiated and transplanted from CD45.2 WT or *Ndufs4*^{-/-} BM. After eight weeks of reconstitution period, mice were treated with topical IMQ (Fig. 1b). Efficient deletion of *Ndufs4* was confirmed by RT-qPCR and immunoblot analysis of BM cell extracts from these mice (Fig. S3). IMQ-treated groups (BM^{WT}-IMQ and BM^{*Ndufs4*^{-/-}}-IMQ) showed higher mortality than untreated mice (BM^{WT} and BM^{*Ndufs4*^{-/-}}) (Fig. 1c). Interestingly, NDUFS4-deficiency in donor BM was sufficient to prevent the IMQ-induced rise in SBP (Fig. 1d). In contrast, no significant differences in left ventricle weight/tibia length, left kidney weight/tibia length, and liver weight/tibia length ratios were found between IMQ-treated and untreated mice (Fig. S4).

IMQ-induced TLR7 signaling pathway is associated with increased plasma levels of autoantibodies, splenomegaly, and an imbalance of T cells [38] and B cell proliferation in lymph organs [14,39–41], all of which contribute to the development of SLE. Given these observations, we assessed for the first time the role of *Ndufs4* in BM cells in the signature of SLE disease. We found that, compared to untreated mice, IMQ-treated mice exhibited a higher spleen weight/tibia length (Fig. 1e), elevated plasma levels of anti-dsDNA (Fig. 1f), anti-cardiolipin and anti-apoH (Fig. 1g), and an increased percentage of B cells in the spleen (Fig. 1h) but not in blood (data not shown). *Ndufs4* depletion in BM cells did not influence the proportion of splenic B cells in lupus mice (Fig. 1g) nor splenomegaly (Fig. 1e). Of note, BM^{*Ndufs4*^{-/-}}-IMQ group showed a lower plasma levels of anti-dsDNA (Fig. 1f) and anti-cardiolipin compared to BM^{WT}-IMQ group, without affecting anti-apoH (Fig. 1g). Our findings show that the total amount of B cells is not involved in the protective effects of BM NDUFS4 deficiency in autoantibody synthesis in the TLR7-induced lupus model. This suggests that defective CD4⁺ T cell metabolism could play a crucial role in the autoimmune phenotype in lupus mice. We evaluated the polarization of T cells in the spleen and blood after TLR7 activation to test this hypothesis. IMQ mice transplanted with *Ndufs4*^{-/-} BM (BM^{*Ndufs4*^{-/-}}-IMQ group) promote Treg expansion (Fig. 2a) and a significant decrease in Th17 cell populations (Fig. 2b) in both spleen and blood compared to IMQ hosts grafted with WT BM (BM^{WT}-IMQ). In addition, IMQ increased the number of Th1 cells, which were selectively reduced in the spleen by *Ndufs4* deficiency in BM cells (Fig. 2c). Considering that Tregs decreased ds-DNA antibody in SLE [42], the increased Tregs differentiation found in IMQ mice with *Ndufs4*^{-/-} BM transplantation might be responsible for reduced SLE activity.

We evaluated the histopathological status of renal tissue because of the deleterious role of autoantibodies in the progression of kidney damage. No glomerulosclerosis was observed; however, BM^{WT}-IMQ mice exhibited enlarged glomeruli with an increased number of cells per glomerulus compared to control mice, suggesting mesangial cell proliferation. Additionally, mild mesangial matrix expansion and inflammatory infiltrates were evident in lupus-prone mice. Interestingly, the lack of NDUFS4 in BM-derived cells protects against IMQ-induced cell proliferation in the glomerulus, without affecting BM^{WT} (Fig. S5)

In both SLE patients and murine models, B cells and CD4⁺ T helper cells demonstrate heightened glycolytic activity and mitochondrial

metabolism, processes that are critical for their proliferation and differentiation [43,44]. In mice stimulated via TLR7, spleen tissue exhibited increased expression of glycolysis-related genes, including *Hk2* (hexokinase 2) and *Slc16a3* (solute carrier family 16 member 3) (Fig. S6a). Additionally, mRNA levels of *Pdk1* (pyruvate dehydrogenase kinase 1), a known inhibitor of pyruvate oxidation, were elevated in the IMQ-treated group, implying enhanced lactate production in lupus-afflicted spleens (Fig. S6a). The expression of *Cpt1a* (carnitine palmitoyltransferase 1a), a key enzyme involved in fatty acid oxidation, was also upregulated in IMQ mice (Fig. S6b), pointing to increased mitochondrial metabolism fueled by fatty acids. Notably, despite elevated glycolysis and mitochondrial activity, ATP concentrations in spleen samples from IMQ-treated mice remained similar to their respective controls (Fig. S6c), suggesting that enhanced metabolic activity supports increased energy expenditure associated with immune effector functions. Interestingly, these metabolic shifts induced by IMQ were reversed in BM chimeric mice lacking *Ndufs4* in hematopoietic cells (Fig. S6a, b). Nevertheless, interpretations should be made with caution, as the data were derived from total splenocyte populations. Variations in immune cell subset composition, particularly the increased proportion of metabolically active cells in IMQ-treated mice, may have influenced the observed outcomes.

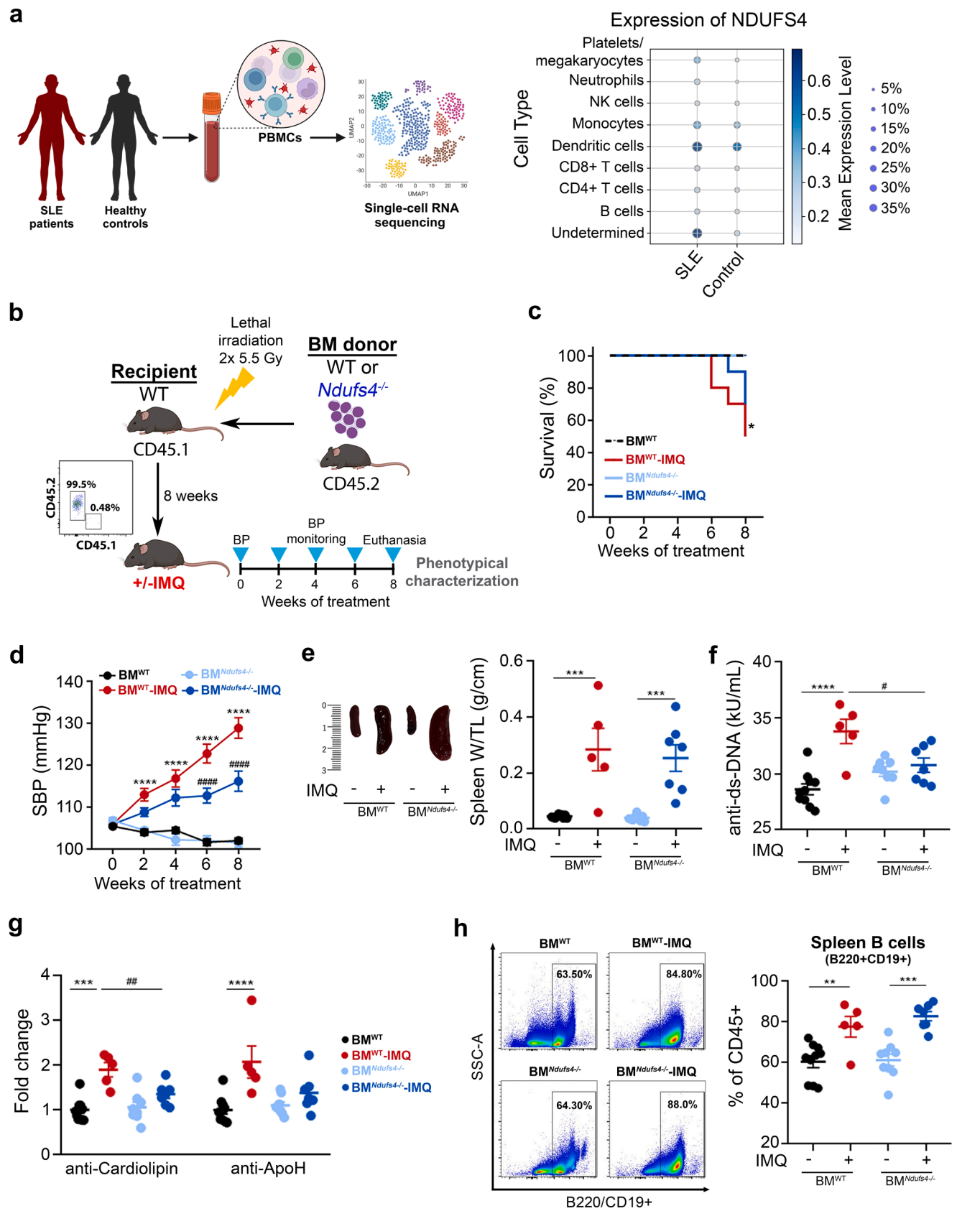
Overall, these results indicate that NDUFS4 is essential as a lupus disease biomarker and for lymphocyte functions, playing a crucial role in differentiating CD4⁺ T lymphocytes into inflammatory cell types, such as Th1 and Th17.

3.2. Loss of NDUFS4 in BM-derived cells prevents lupus-induced endothelial dysfunction, oxidative stress, and proinflammatory T cell infiltration in the vascular wall

Lupus is accompanied by impaired vascular function [14,45,46]; therefore, we inquired whether NDUFS4 absence in BM cells could restore aortic homeostasis in TLR7 activation-induced lupus mice. To address this, we analyzed their impact on the key mechanism involved in the impaired endothelium-dependent relaxant response to acetylcholine, which involves a high NADPH oxidase activity [14] (Fig. 3a). Notably, NDUFS4 deficiency in BM-derived cells reversed the IMQ-induced impairment of endothelium-dependent vasorelaxation responses to acetylcholine in WT mice reconstituted with WT BM (Fig. 3b). Since endothelial dysfunction was reversed following NADPH oxidase inhibition in the aorta of TLR7-induced SLE mice [14,47], we measured NADPH oxidase activity in aorta from all experimental groups. Our findings indicate that NDUFS4 ablation in BM prevented IMQ-induced NADPH oxidase activation (Fig. 3c). Since NADPH oxidase-driven ROS production has been linked to activation of RhoA/Rho kinase by IL-17 [48], we assessed endothelial function after incubation with the Rho kinase inhibitor Y27632. Notably, the acetylcholine-induced response also improved in the BM^{WT}-IMQ group after incubation with Y27632 (Fig. 3d), suggesting that the impaired acetylcholine-induced relaxation is, at least in part, due to Rho kinase activation. Moreover, IL-17 producing cells, such as Th17 cells, were predominantly infiltrated in IMQ group transplanted with WT BM (Fig. 3e), without affecting Th1 cell content (Fig. 3f). In contrast, knockout of *Ndufs4* in BM-derived cells in IMQ-treated mice polarized CD4⁺ T cells toward a Treg phenotype, which infiltrate in aorta (Fig. 3g).

In addition to endothelial dysfunction, we found a lower contractile response to U46619 (3 nM) in aorta of the BM^{WT}-IMQ group (Fig. 3h), which was associated with lower mRNA levels of contractility-related genes, such as *Tagln2* (transgelin 2), or *Acta2* (actin alpha 2, smooth muscle) but no *Myh11* (myosin heavy chain 11) (Fig. 3i). Interestingly, the lack of *Ndufs4* in BM-derived cells reversed all IMQ-induced changes.

Taken together, our data support the hypothesis that *Ndufs4* in immune cells plays a crucial role in regulating oxidative stress and inflammation through the IL-17/Rho-kinase pathway, contributing to



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Fig. 1. NDUFS4 deficiency prevents lupus-induced hypertension and autoantibody production. (a) Analysis of human peripheral blood mononuclear cells (PBMCs) from systemic lupus erythematosus (SLE) patients and control donors (left panel). Schematic representation of a dot plot illustrating the expression levels of *NDUFS4* across different cell types under two conditions: SLE and healthy individuals. The y-axis represents various immune cell types, including platelets, neutrophils, NK cells, monocytes, dendritic cells, CD8 + T cells, CD4 + T cells, B cells, and undetermined cells. The x-axis distinguishes between the SLE and healthy conditions. The color intensity of the dots represents the mean expression level of *NDUFS4*, as indicated by the color scale on the right, where darker shades of blue correspond to higher expression levels. The dot size denotes the percentage of cells expressing *NDUFS4* within each cell type, following the reference scale in the legend on the right. The legend indicates that dot sizes range from 5 % to 35 % of expressing cells. (b) Experimental design: 8-week-old female C57BL/6NRj CD45.1 mice were irradiated with two doses of 550 rads (5.5 Gy) for a total radiation dose of 1100 rads. After irradiation, the mice received bone marrow (BM) cells extracted from the BM of either CD45.2 wild-type (WT) or CD45.2 *Ndufs4*^{-/-} mice. Blood samples were collected from mice three weeks after transplantation, and mononuclear cells were analyzed by flow cytometry for CD45.1 (recipient) and CD45.2 (BM donor) to assess transplantation efficiency. After eight weeks, mice were divided into four groups: 1) BM^{WT} (n = 10), 2) BM^{WT}-IMQ (n = 10), 3) BM^{Ndufs4}^{-/-} (n = 9), and 4) BM^{Ndufs4}^{-/-}-IMQ (n = 10). IMQ treatment was applied to their right ears three times per week on alternate days for eight weeks. Blood pressure (BP) was measured at the indicated time points (blue triangles). Mice were killed after eight weeks of treatment. (c) Survival curves. Percentage of live animals in BM^{WT} (n = 10), BM^{WT}-IMQ (n = 10 at day 0, n = 5 at week 8), BM^{Ndufs4}^{-/-} (n = 9), and BM^{Ndufs4}^{-/-}-IMQ (n = 10 at day 0, n = 7 at week 8) at the indicated time points. Data are shown as the percentage of surviving animals relative to day 0 (Log Rank test). (d) Time-course of systolic blood pressure (SBP) measured using tail-cuff plethysmography. Data are shown as mean ± s.e.m. Each data point represents the group average. ****P < 0.0001 compared to the BM^{WT} group, and ###P < 0.0001 compared to the BM^{WT}-IMQ (repeated-measurements two-way ANOVA with Tukey's post hoc test). (e) Splenomegaly: spleen length (cm) and spleen weight were normalized to tibia length in all experimental groups. Data are shown as mean ± s.e.m. Each data point denotes an individual mouse (n = 5–10). ***P < 0.001 compared to the BM^{WT} or BM^{Ndufs4}^{-/-} group (one-way ANOVA with Dunnett's post hoc test). (f) Circulating double-stranded DNA (anti-ds-DNA) levels, and (g) antiphospholipid (aPL) (anti-cardiolipin autoantibody and beta-2-glycoprotein 1 or apolipoprotein H (Apo-H)) in BM^{WT}, BM^{WT}-IMQ, BM^{Ndufs4}^{-/-}, and BM^{Ndufs4}^{-/-}-IMQ groups. Data are shown as mean ± s.e.m. Each data point denotes an individual mouse (n = 5–10). ***P < 0.001 and ****P < 0.0001 compared to the BM^{WT} group, and #P < 0.05 and ##P < 0.01 compared to the BM^{WT}-IMQ group (one-way ANOVA with Dunnett's post hoc test or Student's *t*-test). (h) Splenic B cells populations were measured by flow cytometry and expressed as percentage of CD45 + parent population. Data are shown as mean ± s.e.m. Each data point represents an individual (n = 5–10). **P < 0.01 and ***P < 0.001 compared to the BM^{WT} or BM^{Ndufs4}^{-/-} group (one-way ANOVA with Dunnett's post hoc test).

vascular dysfunction in lupus-related aortopathy.

3.3. Restoring glycolysis and mitochondrial metabolism ameliorates hypertensive and SLE disease phenotypes

Next, we investigated whether targeting glycolysis and mitochondrial metabolism could optimize blood pressure, SLE manifestation, and progression. We treated female C57BL/6NRj mice with a combination of Met, a mitochondrial chain complex I inhibitor, and DG, a glucose metabolism inhibitor, in drinking water for eight weeks (Fig. 4a). The mortality rate in each group was as follows: Ctrl group, 0 %; Ctrl+Met+DG group, 0 %; IMQ group, 20 % (2 dead mice out of 10); and IMQ+Met+DG group, 10 % (1 dead mouse out of 10) (Fig. 4b). Daily treatment of the IMQ group with Met+DG significantly prevented the increase in SBP induced by IMQ (Fig. 4c). However, we observed a significant increase in the left ventricle weight/tibia length and left kidney weight/tibia length ratios in Met+DG treated mice compared to Ctrl animals, regardless of IMQ treatment (Fig. S7). At the end of the experiment, IMQ-induced clinical manifestations included hepatomegaly (Fig. S7), splenomegaly, splenic B cell accumulation, and anti-autoantibodies production [49] (Figs. 4d–4g). Met+DG treatment significantly reduced splenomegaly (Fig. 4d) in IMQ mice but had minimal effect on the liver weight/tibia length ratio (Fig. S7). In consistency with the results from Experiment 1, pharmacological inhibition of glycolysis and mitochondrial metabolism markedly decreased anti-dsDNA (Fig. 4f) and anti-cardiolipin production (Fig. 4g), without affecting splenic B cell content (Fig. 4e), suggesting that the reversal of disease features in this lupus model is independent of the total B cell content. Moreover, IMQ-treated mice exhibited three evident renal lesions compared to Ctrl mice: mild mesangial thickening, increased glomerular size and cellularity, and foci of chronic inflammatory infiltrate. Treatment with Met+DG in IMQ mice significantly reduced cellular proliferation and inflammation. Paradoxically, this intervention exacerbated renal damage in Ctrl mice, as indicated predominantly by glomerular hypertrophy (Fig. S8).

Given the potentially heightened involvement of B cells in lupus, particularly in activating CD4 + T cells and facilitating their differentiation into Th1 and T follicular helper (Tfh) cells, while limiting the expansion of Treg cells [50], we examined the effects of pharmacological metabolic modulation on the T cell imbalance in lupus. Lupus mice treated with Met+DG showed an increased percentage of splenic and circulating Tregs (Fig. 5a) and a decrease in Th17 cells (Fig. 5b).

Additionally, Met+DG treatment reduced Th1 polarization induced by IMQ (Fig. 5c).

When we explore the metabolic status of spleen tissue, we found in mice stimulated via TLR7 an increased expression of glycolysis-related genes, including *Hk1* and *Slc16a3*, *Pdk1* (Fig. S9a) and the fatty acid oxidation pathway gene *Cpt1a* (Fig. S9b), which were normalized by Met+DG treatment, confirming inhibition of glycolysis and mitochondrial metabolism. ATP concentrations in spleen samples remained similar among all experimental groups (Fig. S9c).

Together, these results indicate that rewiring immune cell metabolism mitigates tissue injury associated with lupus-like disease induced by TLR7 activation and underscores the potential of this approach as a therapeutic target for managing vascular alterations in lupus patients.

3.4. Dual inhibition of glycolysis and mitochondrial metabolism protects against lupus-like aortic pathology

Our data align with previous findings regarding the protective effects of metabolic modulators in reducing autoimmunity and preserving renal function in SLE. However, evidence for their effects on the vasculature remains limited. Therefore, we explored the therapeutic potential of Met+DG treatment to prevent or reverse vascular dysfunction in lupus mice by modulating T cell metabolism (Fig. 6a). Met+DG treatment improved the lower aortic endothelium-dependent relaxation in IMQ-treated mice (Fig. 6b). Met improves vascular endothelial function in diabetes mellitus through AMPK activation [51,52]. However, the improvement of the relaxant response to acetylcholine induced by Met+DG was not affected by the presence of the AMPK inhibitor, compound C, suggesting the involvement of AMPK-independent mechanisms (Fig. S10). Considering that compound C is not totally specific for AMPK, we studied AMPK phosphorylation at the activation site Thr172 in aortas from all experimental groups, as a marker of AMPK activation (Fig. S10). However, AMPKα phosphorylation was similar among all experimental groups, ruling out AMPK activation as a mechanism involved in the protective effects of endothelial dysfunction induced by Met+DG. Furthermore, this impaired endothelial function may be caused by excessive ROS production induced by NADPH oxidase [14]. Increased DHE staining was observed in the aortas of IMQ-treated mice compared to the Ctrl group (Fig. 6d). This aortic fluorescent signal was reduced by incubation with PEG-SOD, while catalase had no effect, indicating that the signal specifically reflects superoxide rather than hydrogen peroxide. Interestingly, VAS2870 and MitoQ partially reduced

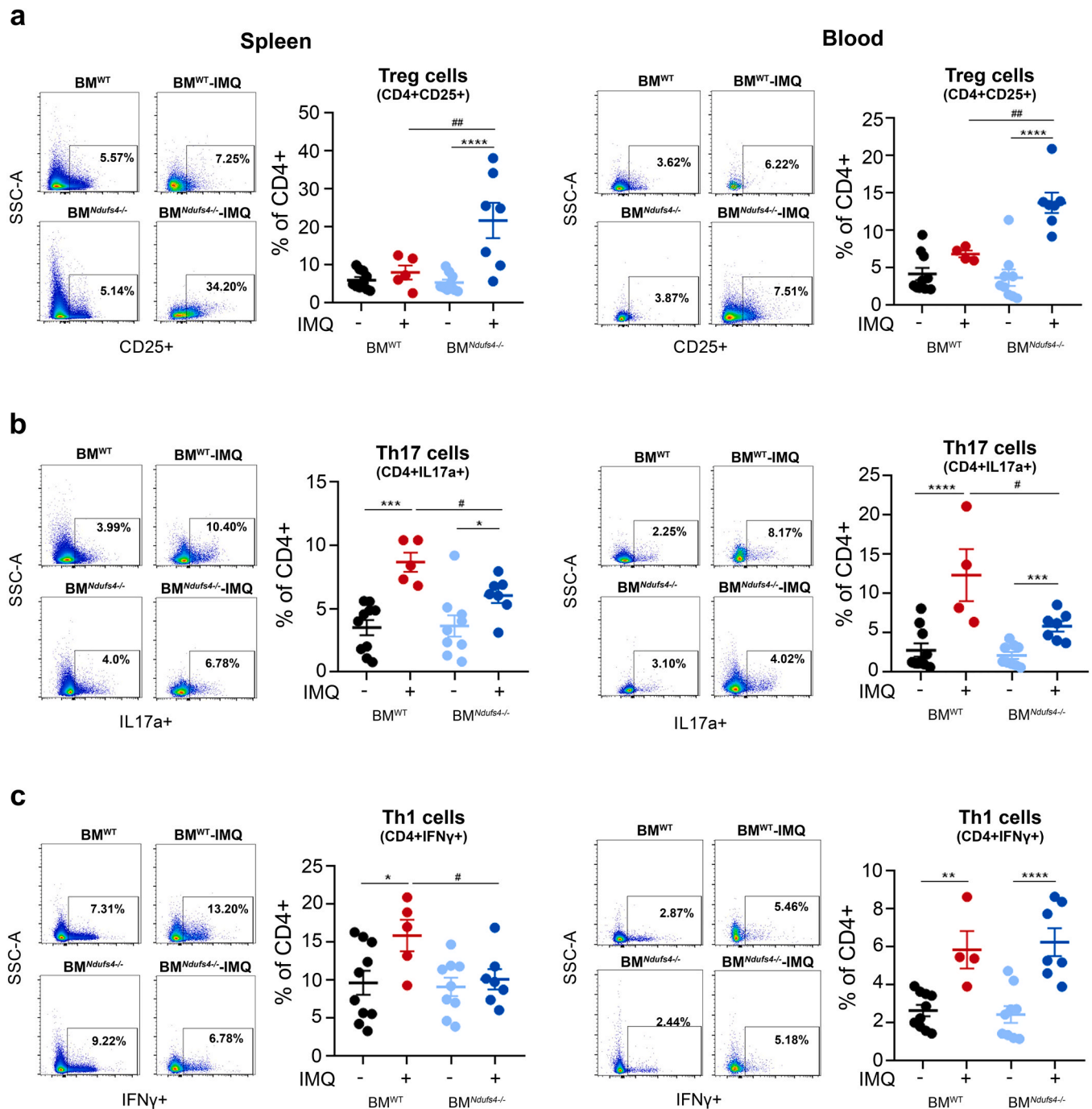
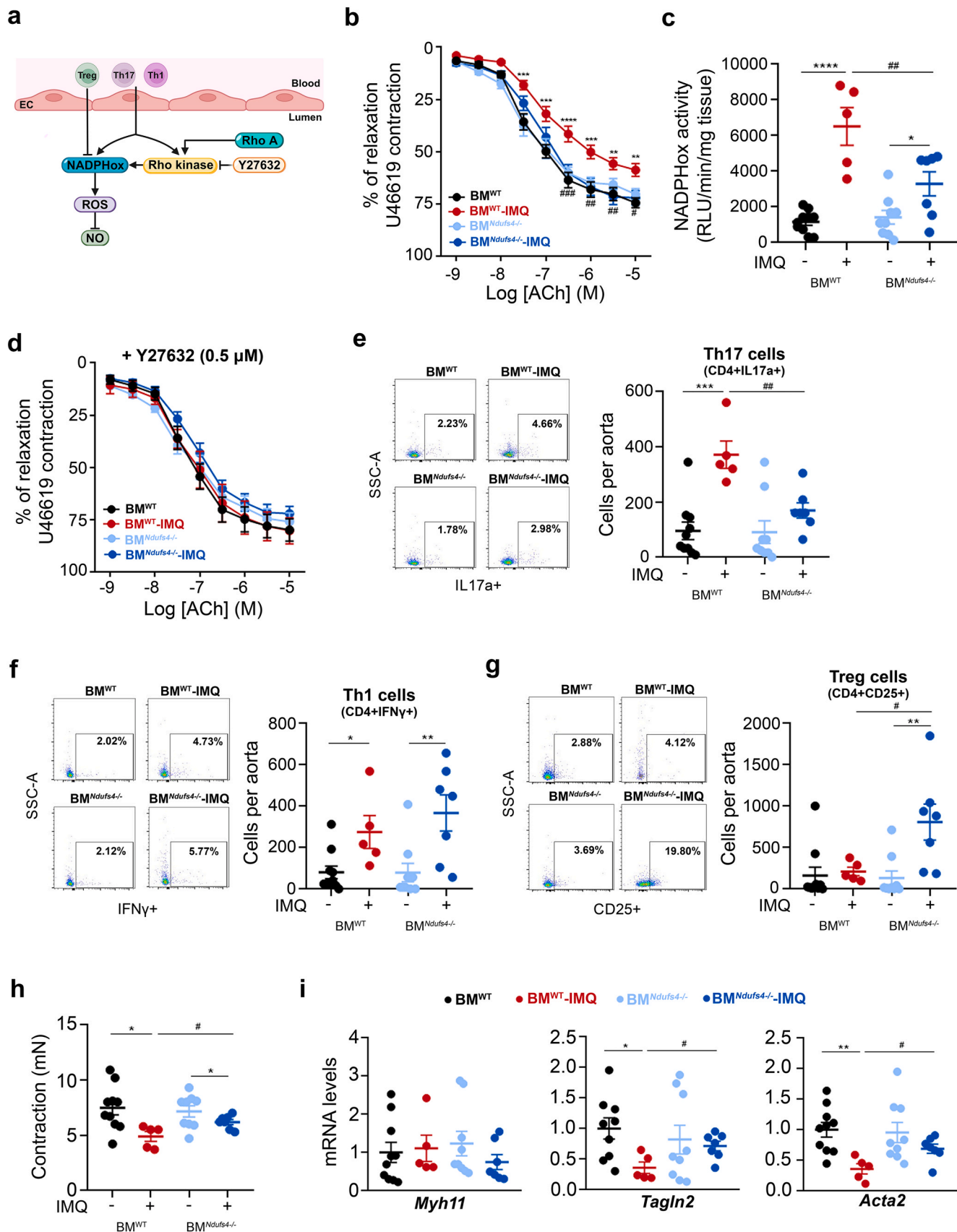


Fig. 2. Loss of NDUFS4 in bone marrow (BM)-derived cells prevents proinflammatory T cell polarization in lupus-associated hypertension. (a) Total Regulatory T (Treg) cells, (b) T helper 17 (Th17) cells, and (c) Th1 cells in spleen and blood from all experimental groups (Experiment 1). A representative plot (left) and dot plot (right). All data are expressed as percentage of the parent population. Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse ($n = 5-10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared to the BM^{WT} group or BM^{Ndufs4-/-} group. # $P < 0.05$ and ## $P < 0.01$ compared to the BM^{WT} IMQ group (one-way ANOVA with Dunnett's post hoc test or Student's t -test).

the DHE signal, suggesting that NADPH oxidase and mitochondria contribute to increased aortic superoxide levels (Fig. S11a). Notably, the IMQ increase in aortic superoxide generation and NADPH oxidase activity, both of which were prevented by Met+DG administration (Fig. 6c-d). The higher NADPH oxidase was linked with increased mRNA levels of the catalytic subunit *Nox4*, which were reduced by Met+DG treatment (Fig. S11b). Additionally, the acetylcholine induced response was improved in the IMQ group after incubation with the Rho kinase inhibitor Y27632 (Fig. 6e), suggesting that the impaired acetylcholine-induced relaxation is, at least in part, due to Rho kinase

activation. Consistent with these data, we found that the protein expression of RhoA (an upstream activator of Rho kinase) was higher in IMQ mice than in Ctrl mice, and Met+DG significantly decreased RhoA expression (Fig. 6f). These vasculoprotective effects were associated with a reduced infiltration of Th17 (Fig. 6g) and Th1 cells (Fig. 6h), along with an increased presence of Treg cells (Fig. 6i) and a lower gene expression of apoptosis regulator *Bcl2* (Fig. 6j) in the aorta.

The vascular wall can undergo a reversible transition from a contractile to a secretory phenotype (Fig. 7a). This shift to the secretory phenotype leads to pathological changes, such as an increased cell



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Fig. 3. NDUFS4 deficiency in bone marrow (BM)-derived cells improves endothelial function, NADPH oxidase activity, and immune cell infiltration in TLR7-induced lupus. (a) Schematic representation of the pathways regulating NO production in the vascular wall in a hypertensive lupus environment: T helper (Th)17 and Th1 cells induce NADPH oxidase, leading to reactive oxygen species (ROS) production, which activates RhoA/Rho kinase. (b) Percentage of vascular relaxation responses induced by acetylcholine (ACh) in endothelium-intact aortas pre-contracted by U46619 (3 nM) in all experimental groups. Data are shown as mean $\pm$ s.e.m. Each data point represents the group average. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to the BM^{WT} group, and # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared to the BM^{WT}-IMQ (repeated-measurements two-way ANOVA with Tukey's post hoc test) (n = 5–10). (c) Aortic NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence. Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 5–10). * $P < 0.05$ and **** $P < 0.0001$ compared to the BM^{WT} group or BM^{Ndufs4^{-/-}} group. ## $P < 0.01$ compared to the BM^{WT}-IMQ (one-way ANOVA with Dunnett's post hoc). (d) Percentage of vascular relaxation responses induced by ACh in endothelium-intact aortas pre-contracted by U46619 (3 nM) in the presence of the Rho kinase inhibitor Y27632 (0.5 μ M) in all experimental groups. Data are shown as mean $\pm$ s.e.m., with each data point representing the group average (repeated-measurements two-way ANOVA with Tukey's post hoc test) (n = 5–10). (e) Aortic infiltration of T helper 17 (Th17) cells, (f) Th1 cells, and (g) Total Regulatory T (Treg) cells by flow cytometry. A representative plot (left) and dot plot (right). Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 5–10). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the BM^{WT} or BM^{Ndufs4^{-/-}} groups. # $P < 0.05$ and ## $P < 0.01$ compared to the BM^{WT}-IMQ (one-way ANOVA with Dunnett's post hoc test or Student's *t*-test). (h) Aortic contractile responses to U46619 (3 nM) in aortas from all experimental groups. Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 5–10). * $P < 0.05$ compared to the BM^{WT} group or BM^{Ndufs4^{-/-}} group, and # $P < 0.05$ compared to the BM^{WT}-IMQ (one-way ANOVA with Dunnett's post hoc test or Student's *t*-test). (i) RT-qPCR analysis of Myosin heavy chain 11 (*Myh11*), Transgelin 2 (*Tagln2*) and Actin alpha 2, smooth muscle (*Acta2*) mRNA expression in aortas from all experimental groups. mRNA levels were normalized to ribosomal protein L13a (*Rpl13a*) expression (mean $\pm$ s.e.m.). Data are shown relative to BM^{WT} group, and each data point denotes an individual mouse (n = 5–10). * $P < 0.05$, ** $P < 0.01$ compared to the BM^{WT} group and # $P < 0.05$ compared to the BM^{WT}-IMQ (one-way ANOVA with Dunnett's post hoc test or Student's *t*-test).

proliferation and an excessive extracellular matrix (ECM) accumulation, contributing to medial degeneration in response to vessel injury or during various vascular diseases [53,54]. While research has begun to uncover the link between ECM, cytoskeletal reorganization, and vascular smooth muscle cell (VSMC) metabolism [55], the role of immune system metabolism in the development and progression of aortic disease in lupus remains unclear.

We examined the expression of genes involved in contractile, secretory, and ECM-related proteins to investigate this hypothesis. Interestingly, aortas from SLE mice induced by TLR7 activation showed increased expression of ECM genes *Col1a1* (Collagen type 1a1), and the metalloproteinases *Mmp2* and *Mmp9*, which was reduced by Met+DG treatment (Fig. 7b). Histological analysis of aortic cross-section in IMQ mice revealed proteoglycan and collagen accumulation, as shown by Alcian Blue, and Sirius-Red staining, respectively (Fig. 7c). In addition, IMQ-induced ECM remodeling of the aortic wall also increased aortic stiffness, characterized by lower thickness and media/lumen ratio (Fig. 7d) accompanied by elevated mRNA expression of genes linked to the secretory phenotype, including *Spp1* (secreted phosphoprotein 1), and the profibrotic factors *Tgfb1* (transforming growth factors β 1) and *Ccn2* (connective tissue growth factor) (Fig. 7e). Notably, Met+DG treatment inhibited the medial degeneration of the aortic wall (Figs. 7c, 7e).

Additionally, aortas from IMQ mice exhibited impaired vascular contractility in response to the thromboxane receptor A₂ agonist U46619 (Fig. 7f). This was associated with reduced mRNA expression of contractility-related genes, such as *Tagln2*, and *Acta2* (Fig. 7g). Actin cytoskeleton dynamics are crucial for effective cell contraction, and phalloidin staining showed a significant decrease in filamentous actin (F-actin) in untreated lupus mice (Fig. 7h). Moreover, Met+DG treatment in IMQ mice significantly increased vascular contractility, restored contractility marker expression (Fig. 7f-g), and enhanced F-actin formation to levels comparable to those in Ctrl mice (Fig. 7h), highlighting the importance of immune regulation for actin cytoskeleton dynamics in SLE.

Overall, these findings suggest that dual inhibition of glycolysis and mitochondrial metabolism results in changes in the T cell profile that significantly restore vascular contractility and function in lupus.

4. Discussion

Our study demonstrates that mitochondrial complex I activity, specifically mediated by the Ndufs4 subunit in immune cells, plays a critical role in vascular dysfunction associated with TLR7-driven lupus, which mimicked SLE patients with high IFN signature (>80 % patients). By integrating transcriptomic data from lupus patients with mechanistic studies in murine models, we have identified a novel

immunometabolism axis linking NDUFS4 expression to CD4⁺ T cell polarization, oxidative stress, and vascular pathology. Pharmacologic targeting of glycolysis and mitochondrial metabolism further supports the translational potential of metabolic reprogramming strategies in autoimmune cardiovascular disease.

Recent studies have begun to shed light on how immune reprogramming influences autoimmunity and contributes to lupus-associated renal and cardiovascular pathology. Beyond vascular involvement, lupus myocarditis should be considered as a differential diagnosis in patients presenting with new-onset heart failure; notably, treatment with immunosuppressive therapy may lead to favorable clinical outcomes [56]. Additionally, the m6A methyltransferase METTL3 has been shown to regulate CD4⁺ T-cell activation and effector T-cell differentiation in SLE [57], while METTL3 upregulation has been implicated in plasma cell infiltration and subsequent kidney damage in lupus [58]. Glycolysis and mitochondrial activity are involved in mesangial cell proliferation, a process that, when excessive in lupus nephritis, contributes to glomerular capillary injury and a decline in glomerular filtration rate. Recent findings indicate that inhibiting glycolysis can suppress mesangial cell proliferation [59]. In line with this, our results show that treatment with Met+DG reduced glomerular cell number, glomerular size, and inflammatory infiltrates in TLR7-induced lupus mice.

Previous studies have highlighted the relevance of immunometabolism in autoimmune disease progression, while our results extend this knowledge by demonstrating that selective deletion of Ndufs4 in hematopoietic cells is sufficient to mitigate hypertension, vascular remodeling, and inflammatory polarization of CD4⁺ T cells *in vivo*. Notably, NDUFS4 expression was highest in dendritic cells and undertermined immune cells in SLE patients, suggesting the involvement of NDUFS4 in disease pathophysiology. Our transplantation model confirmed that NDUFS4 deficiency in BM cells reduced lupus-related mortality and prevented the increase in SBP and vascular remodeling induced by IMQ treatment, aligning with previous findings linking immune cell metabolism to autoimmune pathogenesis [35,36]. These data were also confirmed through pharmacological inhibition of NDUFS4 with Met in combination with the inhibitor of glycolysis DG.

Despite these protective effects, no effects of the loss of NDUFS4 in BM cells on splenomegaly or the overall proportion of splenic B cells were found in lupus mice. The differentiation of B cells into antibody-secreting plasma cells is central to the adaptive immune system because it drives protective and specific antibody responses [60]. Interestingly, mice transplanted with *Ndufs4^{-/-}* BM cells exhibited reduced levels of anti-dsDNA antibodies -a key biomarker of SLE-following IMQ treatment, suggesting a role for Ndufs4 in the regulation of plasma cell differentiation. In fact, NDUFS4 is a critical regulator of mitochondrial metabolism in B cells. Its deficiency impairs ATP

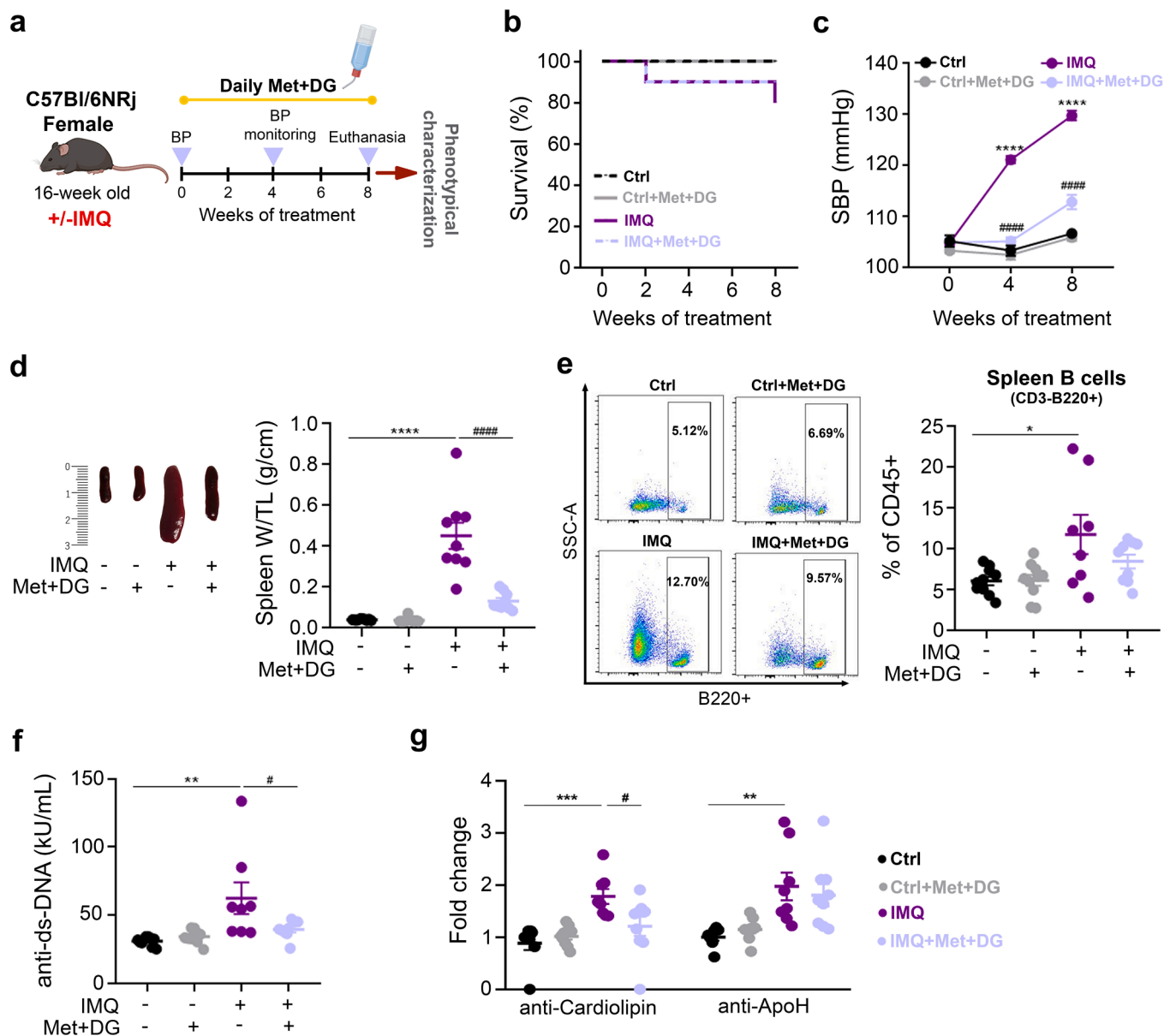


Fig. 4. Inhibition of glycolysis and mitochondrial metabolism prevents hypertension and lupus phenotype. (a) Experimental design: Female 16-week-old C57BL/6NRj mice were randomly divided into 4 groups (n = 10): 1) Control (Ctrl), 2) Control-treated with metformin (Met, 3 mg/mL) and 2-deoxy-D-glucose (DG, 5 mg/mL) in drinking water (Ctrl+Met+DG), 3) Lupus induced by TLR7 activation (IMQ), and 4) IMQ-treated with Met (3 mg/mL) and DG (5 mg/mL) in drinking water (IMQ+Met+DG). IMQ treatment was applied to the right ear three times per week on alternate days for eight weeks. Blood pressure (BP) was measured at the indicated time points (purple triangles). Mice were killed after eight weeks of treatment. (b) Survival curves. Percentage of surviving animals in Ctrl (n = 10), Ctrl+Met+DG (n = 10), IMQ (n = 10 at day 0, n = 8 at week 8), and IMQ+Met+DG (n = 10 at day 0, n = 9 at week 8) at the indicated time points. Data are shown as the percentage of surviving animals relative to day 0 (Log Rank test). (c) Time-course of systolic blood pressure (SBP) measured using tail-cuff plethysmography. Data are shown as mean $\pm$ s.e.m. Each data point represents the group average (n = 8–10). ****P < 0.0001 compared to the Ctrl group, and ####P < 0.0001 compared to the untreated IMQ group (repeated-measurements two-way ANOVA with Tukey's post hoc test). (d) Splenomegaly represented as spleen length (cm) and spleen weight normalized to tibia length in all experimental groups. Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 8–10). ****P < 0.0001 compared to the Ctrl group and ####P < 0.0001 compared to the untreated IMQ group (one-way ANOVA with Tukey's post hoc test). (e) B cell populations were measured by flow cytometry in the spleen from all groups. Data are expressed as % of grandparent population (% of CD45⁺ cells). Data are shown as mean $\pm$ s.e.m. Each data point represents an individual (n = 8–10). *P < 0.05 compared to the Ctrl group (Student's *t*-test). (f) Circulating double-stranded DNA (anti-ds-DNA) levels and (g) antiphospholipid (aPL) (anti-cardiolipin autoantibody and beta-2-glycoprotein 1 or apolipoprotein H (Apo-H)). Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 8–10). **P < 0.01 and ***P < 0.001 compared to the Ctrl group, and #P < 0.05 compared to the untreated IMQ group (one-way ANOVA with Tukey's or Dunnett's post hoc test).

production and disrupts ROS homeostasis, potentially interfering with B cell differentiation into plasma cells and reducing antibody production [61]. Unfortunately, plasma cell quantification was not performed in our study. Another possibility is that CD4⁺ T cell metabolism participated in antibody production. This is consistent with previous studies showing that metabolic reprogramming affects CD4⁺ T cell differentiation and

function in autoimmunity [62,63]. Importantly, our data suggest that NDUFS4 deficiency or its pharmacological inhibition promotes Treg expansion while reducing Th1 and Th17 populations in the spleen and blood. Moreover, Tregs suppress autoantibody production [42], therefore, their increased presence likely contributes to the reduced disease activity observed in our model. Notably, a strong inverse correlation has

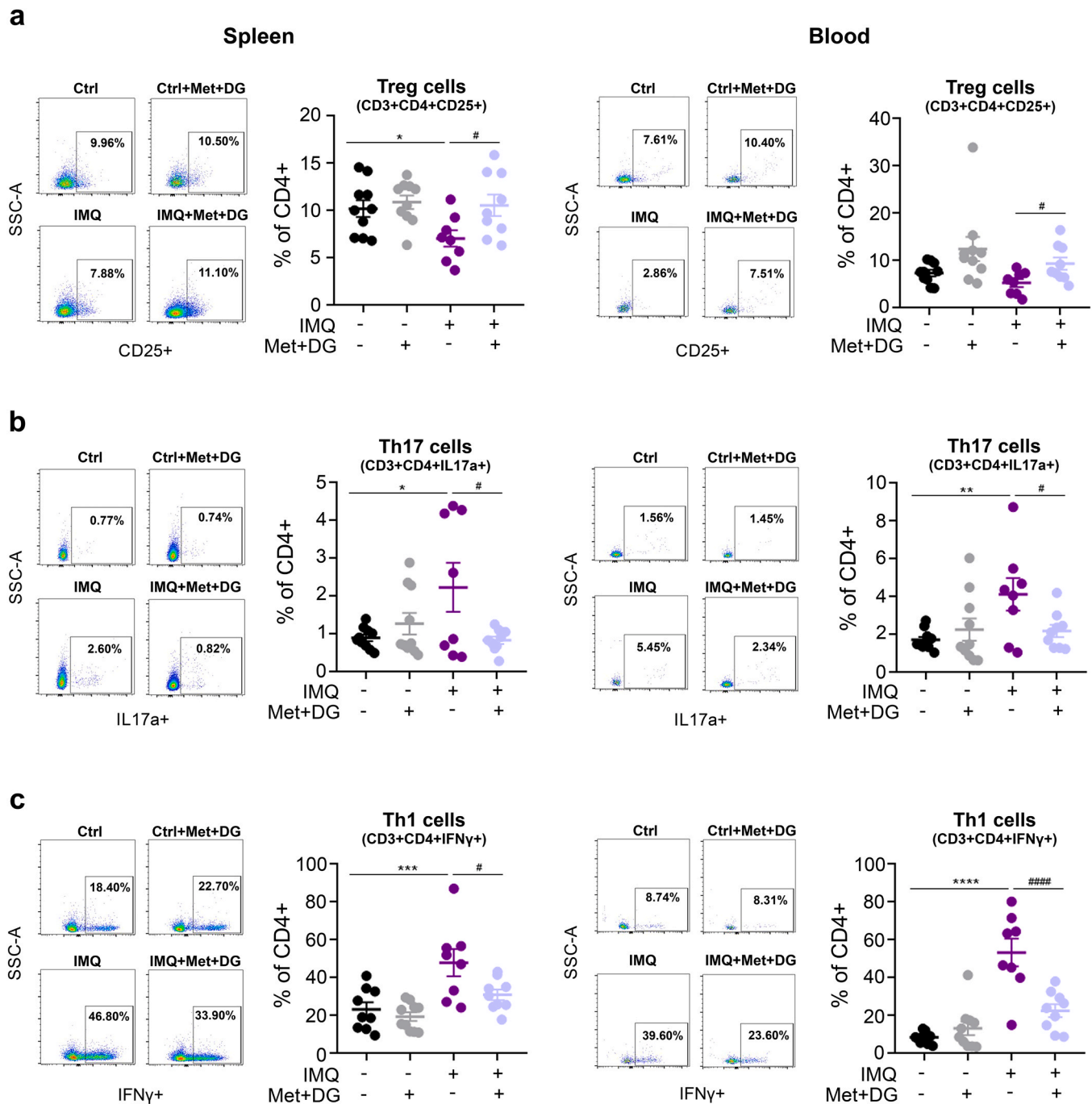


Fig. 5. Restoring glycolysis and mitochondrial metabolism prevents proinflammatory T cell polarization in TLR7-driven lupus. (a) Total Regulatory T (Treg) cells, (b) T helper 17 (Th17) cells, and (c) Th1 cells in spleen and blood from all experimental groups. All data are expressed as percentage of the parent population. Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse ($n = 8-10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared to the Ctrl group. # $P < 0.05$ and #### $P < 0.0001$ compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test or Student's t -test).

been reported between Treg levels and anti-dsDNA antibody levels [64]. Although the precise role of Tregs in SLE pathogenesis remains to be fully elucidated, their expansion could present a potential challenge in SLE treatment, particularly regarding the induction of B cell tolerance.

Animal models of SLE are essential for understanding the relation between autoantibodies and hypertension. Long-term B cell depletion using anti-CD20 [65], selective B cell reduction through the immunosuppressive drug mycophenolate mofetil [66], and plasma cell depletion via the proteasome inhibitor bortezomib [67] have all been shown to effectively reduce autoantibody synthesis and prevent the onset of hypertension in SLE mice. These findings suggest that the antihypertensive

effect observed with *Ndufs4* loss in BM may be linked to its role in reducing autoantibody production.

Endothelial dysfunction is a hallmark of SLE, and our findings highlight the pivotal role of *Ndufs4* in maintaining vascular health. IMQ treatment impaired endothelium-dependent vasorelaxation to acetylcholine, which was abolished by *Ndufs4* deficiency in BM-derived cells. High NADPH oxidase ROS synthesis can be linked to the loss of endothelial function and the increase in blood pressure in female IMQ-treated animals [14]. Furthermore, T cell-driven oxidative stress is a key mechanism of endothelial dysfunction in SLE mice [68]; therefore, we analyzed NADPH oxidase activity and found that its activation was

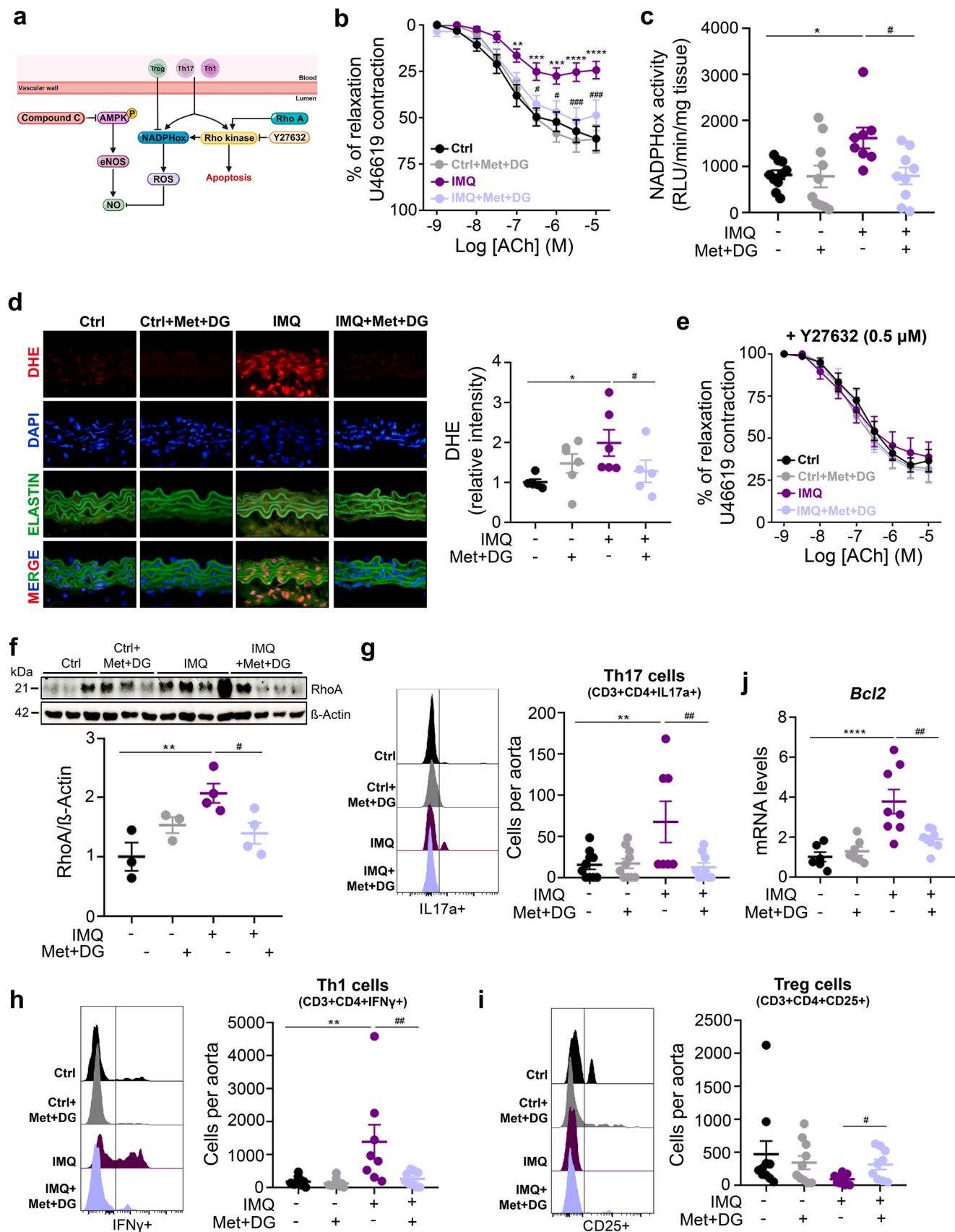


Fig. 6. Dual inhibition of glycolysis and mitochondrial metabolism mediates aortic pathology in SLE via the T helper (Th)17-Rho kinase-NADPHox-NO pathway. (a) Schematic representation of the pathways regulating NO production in the vascular wall. AMPK promotes eNOS phosphorylation, enhancing NO synthesis. Th17 cells induce NADPH oxidase, leading to ROS production, which reduces NO bioavailability and activates RhoA/Rho kinase. Rho kinase further suppresses NO production and activates NADPH oxidase, creating a feedback loop that exacerbates oxidative stress, endothelial dysfunction, and apoptosis. (b) Percentage of vascular relaxation responses induced by acetylcholine (ACh) in endothelium-intact aortas pre-contracted with U46619 (3 nM) in all experimental groups. Data are shown as mean $\pm$ s.e.m. Each data point represents the group average. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ compared to the Ctrl group, and $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ compared to the untreated IMQ group (repeated-measurements two-way ANOVA with Tukey's post hoc test). (c) Aortic NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence. Data are shown as mean $\pm$ s.e.m. Each data point represents the group average. Each data point denotes an individual mouse ($n = 8-10$). $^{*}P < 0.05$ compared to the Ctrl group, and $^{\#}P < 0.05$ compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test). (d) Representative images (left panel) and quantification (right panel) of dihydroethidium (DHE) immunofluorescence (red), DAPI-stained nuclei (blue), elastin auto-fluorescence (green) in aortic sections from all groups. Scale bar, 50 μ m. Data are shown relative to Ctrl mice as mean $\pm$ s.e.m., Each data point denotes an individual mouse ($n = 5-6$). $^{*}P < 0.05$ compared to the Ctrl group and $^{\#}P < 0.05$ compared to the untreated IMQ group (Student's *t*-test). Percentage of vascular relaxation responses induced by ACh in endothelium-intact aortas pre-contracted with U46619 (3 nM) after 30 min incubation with the Rho kinase inhibitor Y27632 (0.5 μ M). Data are shown as mean $\pm$ s.e.m. Each data point denotes the average of each group (repeated-measurements two-way ANOVA with Tukey's post hoc test). (f) Representative immunoblot analysis and quantification of the immunoblot signal of RhoA and β -Actin (loading control) in aortic protein extracts. Data are shown relative to Ctrl mice as mean $\pm$ s.e.m. Each data point denotes an individual mouse ($n = 3-4$). $^{**}P < 0.01$ compared to the Ctrl group, and $^{\#}P < 0.05$ compared to the untreated IMQ group (one-way ANOVA with Tukey's post hoc test or Student's *t*-test). Aortic infiltration of (g) T helper 17 (Th17) cells, (h) Th1 cells, and (i) Total Regulatory T (Treg) cells by flow cytometry. Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse ($n = 8-10$). $^{**}P < 0.01$ compared to the Ctrl group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test or Student's *t*-test). (j) RT-qPCR analysis of Apoptosis regulator Bcl-2 (*Bcl2*) mRNA expression in aortic tissue from all experimental groups. mRNA amounts were normalized to *Rpl13a* expression (mean $\pm$ s.e.m.). Data are shown relative to Ctrl mice and each data point denotes an individual mouse ($n = 8-10$). $^{****}P < 0.0001$ compared to the Ctrl group and $^{\#}P < 0.01$ compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test).

prevented by *Ndufs4* deletion. Since IL-17 has been involved in NADPH oxidase activation and Rho kinase signaling [69], we further examined endothelial function following Rho kinase inhibition. Notably, Y27632 improved acetylcholine-induced relaxation in IMQ-treated mice, supporting the idea that Rho kinase activation contributes to endothelial dysfunction. Moreover, Th17 cells infiltrated the aorta of IMQ-treated mice, whereas *Ndufs4* deletion promoted a shift toward a Treg phenotype, further reinforcing the role of immune metabolism in vascular homeostasis. Interestingly, the systemic administration of Met+DG significantly improved endothelial dysfunction. This improvement was associated with a reduction in Th1 and Th17 infiltration in the aorta, along with an increased accumulation of Tregs. These findings confirm that drug-induced modulation of immune metabolism protects against vascular dysfunction in lupus triggered by TLR7 activation.

Resistance arteries primarily regulate blood pressure, whereas large arteries also play a crucial role by modulating pulse wave pressure throughout the cardiac cycle. Aortic stiffness is an independent predictor of hypertension, linked to alterations in ECM proteins and endothelial dysfunction [70]. One of the key findings of the present study is the influence of metabolic modulation on aortic pathology in lupus. Our results reveal that lupus-induced vascular remodeling is marked by increased ECM deposition, proteoglycan accumulation, and a shift toward a secretory vascular phenotype. To our knowledge, this is the first study to establish a direct connection between glycolysis, mitochondrial metabolism, and ECM remodeling in lupus-related vascular disease. Similar vascular shift toward secretory phenotype contributes to aortic aneurysm development in Marfan syndrome [71]. Patients with SLE are known to have a higher risk of aortic aneurysm and dissection compared to the general population, although the underlying mechanisms remain unclear [72]. Notably, treatment with Met+DG not only mitigated ECM remodeling but also restored vascular contractility, as demonstrated by increased expression of contractile genes and enhanced F-actin formation. These protective effects on vascular remodeling may contribute to reduced blood pressure and suggest a lower risk of aortic aneurysm and dissection in SLE.

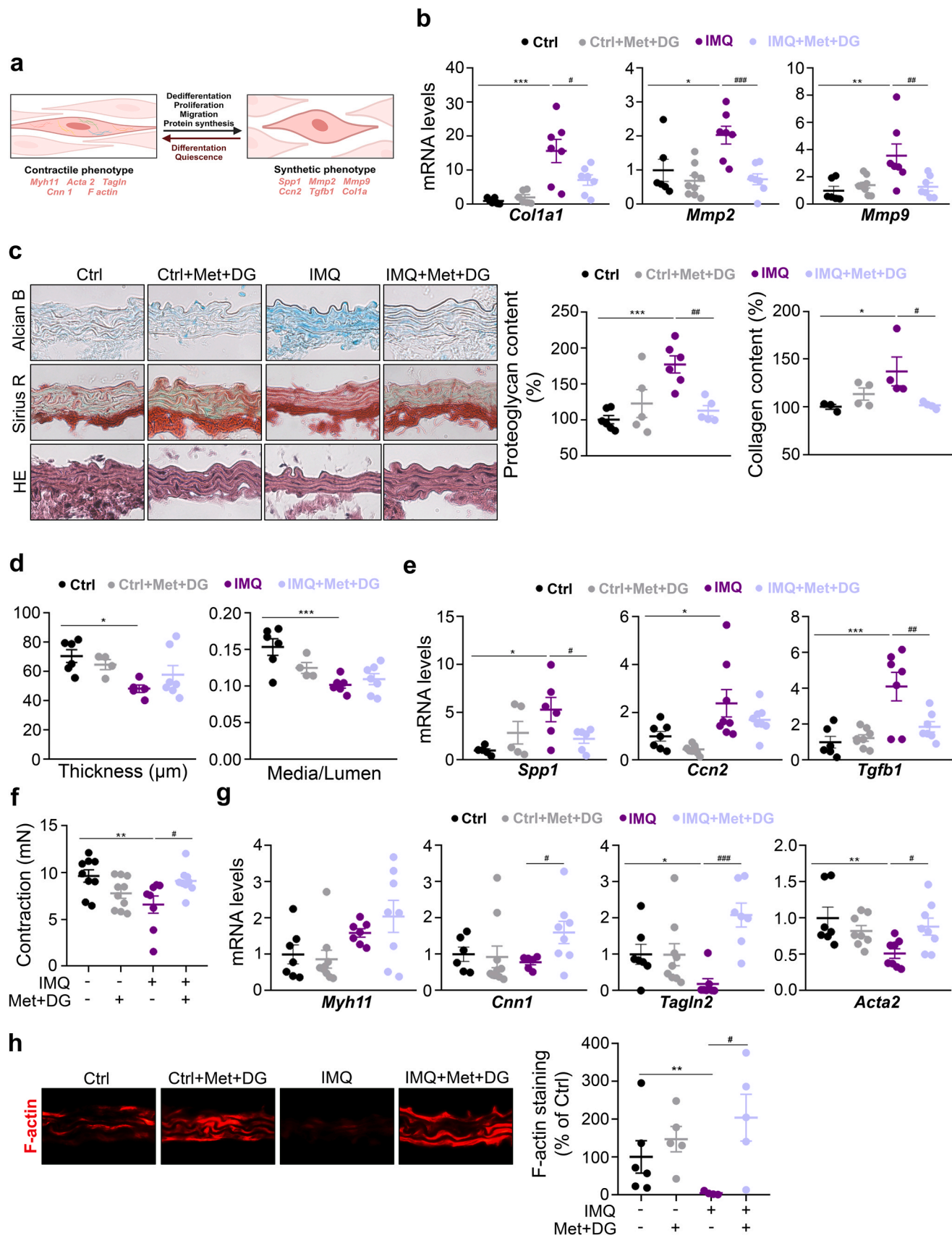
Several strengths and limitations of this study should be acknowledged. Among the key strengths are the integration of both human and murine data, which enhances the translational relevance and mechanistic depth of the findings. The use of BM chimeras, despite inherent limitations, allowed us to dissect hematopoietic contributions to SLE disease, while the convergence of genetic and pharmacological approaches strengthens causal inference. Furthermore, immunophenotyping of CD4⁺ T-cell subsets across lymphoid and vascular compartments provides mechanistic insight into how *Ndufs4* deficiency

confers vascular protection. However, the study also has important limitations. Firstly, the BM chimera model may introduce confounding factors unrelated to immune cell-intrinsic effects. Although we carefully matched donor and host genotypes and confirmed immune reconstitution, variables such as irradiation and host microenvironment could influence outcomes, potentially leading to a modest over- or underestimation of *Ndufs4*'s role in immune cells. Secondly, although our transcriptomic analysis of lupus patients revealed elevated *NDUFS4* expression in dendritic and undetermined immune cell populations, we did not perform direct functional assays in human immune cells, which limits the immediate translational applicability of our findings. Thirdly, further in-depth characterization of mitochondrial respiration and metabolic flux in specific immune cell subsets is still needed. Fourthly, although our study primarily focused on vascular outcomes, we did not perform specific measurements of mitochondrial ROS to further dissect their role in the observed phenotypes or assess vascular oxidative DNA damage. We found reductions in autoantibody levels, splenomegaly, and renal injury; however, a more comprehensive assessment of systemic lupus manifestations is warranted. This does raise the possibility that targeting immunometabolism may offer therapeutic benefits beyond vascular protection. Finally, metabolic profiling has revealed female SLE-specific alterations in steroid hormones, which are known to modulate immune responses in lupus patients [73]. Although we did not assess steroid hormone profiles in our female C57BL/6NRj mice, significant variability is not expected because all animals share the same genetic background.

In conclusion, our study provides strong evidence that *NDUFS4* is a key regulator of immune cells differentiation and vascular dysfunction in lupus. The loss of *NDUFS4* in BM-derived cells protects against hypertension, vascular impairment, and immune dysregulation by shifting the CD4⁺ T cell balance toward a regulatory phenotype. Current treatments remain inadequate for addressing hypertension and endothelial dysfunction in SLE, which significantly increase the risk of stroke and myocardial infarction [74]. Furthermore, pharmacological targeting of glycolysis and mitochondrial metabolism recapitulates these protective effects, highlighting metabolic reprogramming as a promising therapeutic strategy for SLE.

CRedit authorship contribution statement

Iñaki Robles-Vera: Visualization, Methodology, Investigation, Conceptualization. **Cristina González-Correa:** Writing – original draft, Visualization, Methodology, Investigation. **Juan Duarte:** Writing – review & editing, Writing – original draft, Visualization, Supervision,



(caption on next page)

Fig. 7. Restoring glycolysis and mitochondrial metabolism reverses aortic pathology in lupus-induced mice. (a) Schematic representation of the phenotypic transition of vascular smooth muscle cells (VSMCs) in lupus-associated hypertension. The contractile phenotype is characterized by elevated expression of markers such as myosin heavy chain 11 (Myh11), transgelin 2 (Tagln2) and actin alpha 2, smooth muscle (Acta2), and calponin 1 (Cnn1), facilitating actin (F-actin) polymerization and maintaining vascular integrity. In contrast, the synthetic phenotype is associated with increased expression of secreted phosphoprotein 1 (Spp1), matrix metalloproteinase (Mmp)2, Mmp9, cellular communication network factor 2 (Ccn2), transforming growth factor beta 1 (Tgfb1), and collagen type I alpha 1 chain (Col1a1), contributing to extracellular matrix remodeling, endothelial dysfunction, and aortic damage. (b) RT-qPCR analysis of *Col1a1*, *Mmp2*, and *Mmp9* mRNA expression in aortic tissues from all experimental groups. mRNA amounts were normalized to ribosomal protein L13a (*Rpl13a*) expression (mean $\pm$ s.e.m.). Data are shown relative to Ctrl mice, and each data point denotes an individual mouse (n = 7–10). *P < 0.05, **P < 0.01, ***P < 0.001 compared to the Ctrl group, and #P < 0.05, ##P < 0.01, ###P < 0.001 compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test or Student's *t*-test). (c) Representative staining images (left panel) of Alcian Blue (Alcian B), Sirius Red (Sirius R), and hematoxylin and eosin (HE) in aortic section from all groups. Scale bar, 50 μ m. Quantification of proteoglycan and collagen content (right panel). Data are shown relative to Ctrl mice as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 5–6). *P < 0.05, ***P < 0.001 compared to the Ctrl group, and #P < 0.05, ##P < 0.01 compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test). (d) Quantification of aortic thickness (μ m) and media/lumen ratio in aortic sections. Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 4–7). *P < 0.05, and ***P < 0.001 compared to the Ctrl group (one-way ANOVA with Dunnett's post hoc test). (e) RT-qPCR analysis of *Spp1*, *Ccn2*, and *Tgfb1* mRNA expression in aortas from all groups. mRNA amounts were normalized to *Rpl13a* expression (mean $\pm$ s.e.m.). Data are shown relative to Ctrl mice and each data point denotes an individual mouse (n = 5–8). *P < 0.05, and ***P < 0.001 compared to the Ctrl group, and #P < 0.05, and ##P < 0.01 compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test or Student's *t*-test). (f) Aortic contraction in response to U46619 (3 nM). Data are shown as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 8–10). **P < 0.01 compared to the Ctrl group and #P < 0.05 compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test). (g) RT-qPCR analysis of *Myh11*, *Cnn1*, *Tagln2* and *Acta2* mRNA expression in aortic tissues. mRNA amounts were normalized to *Rpl13a* expression (mean $\pm$ s.e.m.). Data are shown relative to Ctrl mice, and each data point denotes an individual mouse (n = 6–10). *P < 0.05, and **P < 0.01 compared to the Ctrl group, and #P < 0.05, and ##P < 0.01 compared to the untreated IMQ group (one-way ANOVA with Dunnett's post hoc test or Student's *t*-test). (h) Representative images (left panel) of F-actin staining (red) and quantification (right panel) in aortic sections. Scale bar, 50 μ m. Data are shown relative to Ctrl mice as mean $\pm$ s.e.m. Each data point denotes an individual mouse (n = 4–6). **P < 0.01 compared to the Ctrl group and #P < 0.05 compared to the untreated IMQ group (Mann-Whitney test).

Project administration, Funding acquisition, Conceptualization. **Javier Moledo**: Writing – original draft, Visualization, Methodology, Investigation. **Marta Toral**: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Sofía Miñano**: Writing – original draft, Visualization, Methodology, Investigation. **David Sancho**: Supervision, Funding acquisition. **de la Visitación Nestor**: Methodology, Investigation. **Semih Bayraktar**: Methodology, Investigation. **Manuel Sánchez**: Methodology, Investigation. **Manuel Gómez-Guzmán**: Methodology, Investigation, Funding acquisition. **Natividad Martín-Morales**: Methodology, Investigation. **Miguel Romero**: Methodology, Investigation. **Francisco O'Valle**: Supervision.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.phrs.2025.107910](https://doi.org/10.1016/j.phrs.2025.107910).

Data availability

Data will be made available on request.

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