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RESEARCH PAPER

Gut microbiota contributes to the development of hypertension in a genetic mouse model of systemic lupus erythematosus

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Background and Purpose: Hypertension is an important cardiovascular risk factor that is prevalent in the systemic lupus erythematosus patient population. Here, we have investigated whether intestinal microbiota is involved in hypertension in a genetic mouse model of systemic lupus erythematosus.

Experimental Approach: Twenty-six-week-old female NZW/LacJ (control) and NZBWF1 (F1 hybrid of New Zealand Black and New Zealand White strains; systemic lupus erythematosus) mice were treated for 6 weeks with a broad-spectrum antibiotic mixture or with vancomycin. Faecal microbiota transplantation was performed from donor systemic lupus erythematosus group to recipient to germ-depleted or germ-free mice.

Key Results: Antibiotic treatment inhibited the development of hypertension and renal injury, improved endothelial dysfunction and vascular oxidative stress, and decreased aortic Th17 infiltration in NZBWF1 mice. High BP and vascular complications found in systemic lupus erythematosus mice, but not autoimmunity, kidney inflammation and endotoxemia, were reproduced by the transfer of gut microbiota from systemic lupus erythematosus donors to germ-free or germ-depleted mice. Increased proportions of *Bacteroides* were linked with high BP in these mice. The reduced endothelium-dependent vasodilator responses to acetylcholine and the high BP induced by microbiota from hypertensive systemic lupus erythematosus mice were inhibited after IL-17 neutralization.

Conclusion and Implications: Changes in T-cell populations, endothelial function, vascular inflammation and hypertension driven by a genetic systemic lupus erythematosus background can be modified by antibiotic-induced changes in gut

Abbreviations: anti-ds-DNA, anti-double-stranded DNA; BIPES, barcoded Illumina paired-end sequencing; MCP-1, monocyte chemoattractant protein 1 (CCL2); NZBWF1, F1 hybrid of New Zealand Black and New Zealand White strains; SBP, systolic BP; Tregs, regulatory T cells.

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microbiota. The vascular changes induced by hypertensive systemic lupus erythematosus microbiota were mediated by Th17 infiltration in the vasculature.

KEYWORDS

endothelial dysfunction, gut dysbiosis, hypertension, immune system, oxidative stress, systemic lupus erythematosus

1 | INTRODUCTION

Systemic lupus erythematosus is considered as one of the most detrimental autoimmune inflammatory diseases. It is characterized by the development of autoantibodies that prompt the formation and precipitation of immune complexes, which, in turn, damage many organs and tissues. Systemic lupus erythematosus has been associated with a high risk of renal and cardiovascular disease development (Frostegård, 2008), the major causes of mortality in systemic lupus erythematosus patients (Bartels et al., 2014). It predominantly affects young women of child-bearing age, a population that is at lowest relative risk of atherosclerotic heart disease. This disease has also been linked to a high incidence of hypertension (Al-Herz et al., 2003). Genetic, environmental, hormonal and metabolic factors contribute to systemic lupus erythematosus susceptibility, while promoting chronic inflammation that can lead to alterations in BP (Wolf & Ryan, 2019). Nevertheless, the pathophysiological mechanisms promoting systemic lupus erythematosus hypertension are not fully understood. Several studies have shown that numerous mnediators have a role in the pathogenesis of systemic lupus erythematosus hypertension, such as inflammatory cytokines and oxidative stress. These mediators are known to contribute to local inflammation and the resulting renal and vascular dysfunction and are likely downstream of the initial immune system dysregulation (Taylor & Ryan, 2016). In fact, an anti-CD20 antibody treatment, which lowers the relative populations of B cells in spleen and the quantity of anti-double-stranded DNA (anti-ds-DNA) antibodies in plasma, prevents the onset of hypertension (Mathis et al., 2014). This underlines the relevance of B cells in the development of systemic lupus erythematosus hypertension. Nonetheless, the precise role of hyperactive T and B lymphocytes, both central to the progression of autoimmune diseases, in the genesis of systemic lupus erythematosus hypertension is still not well understood.

Several recent studies reported that the intestinal microbiota is involved in the genesis of the pathology. The published data indicates that the gut microbiota might promote the onset of symptoms and progression of this autoimmune disease in both human and mouse models of systemic lupus erythematosus (Hevia et al., 2014; Katz-Agranov & Zandman-Goddard, 2017; Li et al., 2017; López et al., 2016; Luo et al., 2018; Ma et al., 2019; Manfredo Vieira et al., 2018; Mu, Tavella, et al., 2017; Mu, Zhang, et al., 2017; Zegarra-Ruiz et al., 2019; Zhang et al., 2014). Despite this strong linking gut dysbiosis and autoimmunity in systemic lupus erythematosus, a few investigations are available which focus on the role of microbiota in the development of systemic lupus erythematosus hypertension (de la Visitación et al., 2019; Small et al., 2018). As in humans with systemic lupus erythematosus, the NZBWF1 (F1 hybrid of New Zealand Black and New Zealand White strains) mice produce anti-ds-DNA antibodies, develop immune complex glomerulonephritis and, crucially, develop hypertension (Ryan & McLemore, 2007). Further similar to humans, the primary causes of systemic lupus erythematosus in this model are thought to be polygenic and the females are more affected than males. Recently, we described that the modulation of the gut microbiota through the administration of the probiotic *Lactobacillus fermentum* prevented the development of hypertension in female NZBWF1 mice (Toral, Robles-Vera, Romero, et al., 2019), suggesting that gut microbiota plays a role in BP control. However, it is unclear whether microbiota is involved in the raise of BP in NZBWF1 mice and what the underlying mechanisms are.

A growing body of evidence supports the role of the immune system in the development of hypertension. The role of Th17 cells in hypertension was first demonstrated by Harrison's team (Madhur et al., 2010). The gut microbiome has been described as a modulator

What is already known

- Gut microbiota are involved in the control of BP.
- Excess dietary salt alters the gut microbiome and activates dendritic cells, leading to hypertension.

What does this study add

- Gut microbiota drives the BP changes in NZBWF1 mice by activation of pro-inflammatory Th17 cells.
- Gut microbiota transfers the hypertensive phenotype to mice without genetic background of systemic lupus erythematosus.

What is the clinical significance

- Changing gut microbiota composition may reduce cardiovascular complications associated with systemic lupus erythematosus.

of hypertension progression. The mechanisms by which gut dysbiosis influences BP are not exactly known, but modulation of the immune system through the control of Th17 polarization in gut lymph nodes has been proposed as a key mechanism. Recently, it has been described that gut microbiota facilitate angiotensin II-induced vascular dysfunction and hypertension, at least in part by supporting a monocyte chemoattractant protein 1 (MCP-1/CCL2)/IL-17-driven vascular immune cell infiltration and inflammation (Karbach et al., 2016), suggesting that the microbiota is primarily detrimental in the setting of hypertension. However, metabolomics data obtained in the presence/absence of gut microbiota (Cheema & Pluznick, 2019) suggest that although the microbiota have a negative effect on balance, some actions of the microbiota may serve to mitigate the effects of angiotensin II. In fact, down-regulation of the plasma metabolite *N*,*N*,*N*-trimethyl-5-aminovalerate with angiotensin II treatment may be reno-protective. In contrast, several indoles produced by the gut microbiota were down-regulated in plasma with angiotensin II treatment (indoleacetylglycine and indoleacetate), which is interesting in light of the 2017 study, which reported a decrease in faecal indoles on a high-salt diet associated with high BP (Wilck et al., 2017). Moreover, high dietary salt-induced dendritic cell activation underlies microbial dysbiosis-associated hypertension (Ferguson et al., 2019). These dendritic cells showed increased expression of B7 (CD80) ligand and maturation/activation marker CD86, which was previously implicated in the genesis of hypertension induced by microbiota from spontaneously hypertensive rats, in part mediated by Th17 cell infiltration in the vasculature (Toral, Robles-Vera, de la Visitación, et al., 2019). In the present study, we tested the hypothesis that gut microbiota might increase the susceptibility to disease activity and T-cell activation in secondary lymph organs in the gut, leading to endothelial dysfunction and hypertension in female NZBWF1 mice.

2 | METHODS

The data, methods and materials that support the findings of this study are available from the corresponding author on reasonable request.

2.1 | Animals and experimental groups

Our research complies with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and is approved by the Ethics Committee of Laboratory Animals of the University of Granada (Spain) (Ref. 12/11/2017/164). In addition, the experiments conform to the Guidelines for Transparency on Gut Microbiome Studies in Essential and Experimental Hypertension (Marques et al., 2019). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). Taken into account that oestrogens play a role in the pathogenesis of both systemic lupus erythematosus disease and the associated hypertension in human and animals (Taylor & Ryan, 2016), we only used female mice. Female NZBWF1 (systemic lupus erythematosus) and NZW/LacJ (control) mice from The Jackson Laboratory (RRID: SCR_004633, Bar Harbor, ME, USA) were randomly assigned to four different groups: control (CTR) ($n = 10$), systemic lupus erythematosus ($n = 10$), systemic lupus erythematosus-treated with a broad-spectrum antibiotic mixture (systemic lupus erythematosus-MIX) ($n = 10$) and systemic lupus erythematosus-treated with vancomycin (2 $g{\cdot}L^{-1}$) (systemic lupus erythematosus-VANCO) ($n = 8$). NZW/LacJ mice display limited autoimmunity, but in contrast to NZBWF1 mice they do not develop a severe lupus-like phenotype and they are classically used as control, but this is a limitation of the present study. Vancomycin is a non-absorbable antibiotic that kills primarily Gram-positive organisms. Broad-spectrum antibiotic administration consisted of metronidazole (1 $g{\cdot}L^{-1}$; Sigma), neomycin (1 $g{\cdot}L^{-1}$; Fisher Scientific), ampicillin (1 $g{\cdot}L^{-1}$; Sigma) and vancomycin (0.5 $g{\cdot}L^{-1}$; Pfizer) in the drinking water (Manfredo Vieira et al., 2018). For broad-spectrum antibiotic experiments, sweetener (Equal, 4 $g{\cdot}L^{-1}$) was added to both the antibiotics and control water to overcome the metallic taste of metronidazole. Treatments started when some mice had sign of kidney damage (increased protein excretion) but were normotensive (at 26 weeks old) and were maintained for 6 weeks.

To explore whether microbiota from hypertensive systemic lupus erythematosus mice is involved in BP regulation, a faecal microbiota transplantation experiment was performed as previously described (Toral, Robles-Vera, de la Visitación, et al., 2019). Faecal contents were collected fresh and pooled from individual systemic lupus erythematosus and separately from control mice at the experimental endpoint. Faecal contents were diluted 1:20 in sterile PBS and centrifuged at 60× *g* for 5 min. The supernatant was aliquoted and stored at −80°C. Normotensive 10-week-old C57Bl/6J female mice (Janvier, Saint-Berthevin Cedex, France) were used as recipient mice. These mice were administered with 0.1 ml ceftriaxone sodium (400 $mg{\cdot}ml^{-1}$) once daily for five consecutive days by oral gavage to decrease the pre-existing microbiota and to facilitate the recovery of the population and diversity of intestinal microbiota from donor mice after faecal microbiota transplantation. Forty-eight hours after the last antibiotic treatment, recipient mice were given donor stools by gavage (0.1 ml) for three consecutive days and then once every 3 days for a total period of 3 weeks. Animals were randomly sorted into two groups: - control with control microbiota (CTR-CTR) ($n = 8$) and control with systemic lupus erythematosus microbiota (CTR-systemic lupus erythematosus) ($n = 9$). In a different experiment, we aimed to determine whether IL-17 is involved in the development of hypertension induced by microbiota from systemic lupus erythematosus mice. Recipient control mice were assigned to two groups: - control with systemic lupus erythematosus microbiota (CTR-systemic lupus erythematosus) ($n = 6$) and control with systemic lupus erythematosus microbiota plus IL-17-neutralizing antibody (CTR-systemic lupus erythematosus + nIL-17) ($n = 8$). Intraperitoneal injections of either IL-17-neutralizing antibody (RRID:AB_10891109,

10 μg per mouse; R&D Systems) or the isotype control (10 μg per mouse; R&D Systems) were given on Days 7 and 11 after faecal microbiota transplantation.

In these experiments, mice were housed in specific pathogen-free facilities at University of Granada Biological Services Unit. All animals were maintained under standard laboratory conditions (12-h light/dark cycle, temperature 21–22°C and 50–70% humidity). Mice were housed in Makrolom cages (Ehret, Emmendingen, Germany), with dust-free laboratory bedding and enrichment. To avoid horizontal transmission of the microbiota among animals, each mouse was housed in a separate cage. Mice were provided with water and standard laboratory diet (SAFE A04, Augy, France) ad libitum. Water was changed every day and both water and food intake were recorded daily for all groups. Studies were designed to generate groups of equal size and sufficient statistical power. Animals were randomly assigned to treatment groups and the experimenter was blinded to drug treatment until data analysis was performed.

In another set of experiments, normotensive 10-week-old C57Bl/6J female germ-free mice (University of Granada, Granada, Spain) were used as recipient mice. Animals were randomly sorted into two different groups: - control with control microbiota (germ-free-CTR) ($n = 8$) and control with systemic lupus erythematosus microbiota (germ-free-systemic lupus erythematosus) ($n = 8$). Stool inoculation was performed for two consecutive days and animals were maintained for 3 weeks. All germ-free mice were kept under sterile conditions at a gnotobiotic facility.

2.2 | BP measurements, physical characteristics, heart and kidney weight indices and renal injury

Systolic BP (SBP) was measured in conscious, prewarmed for 10–15 min at 35°C and restrained mice by tail-cuff plethysmography (Digital Pressure Meter, LE 5001; Letica S.A., Barcelona, Spain). Mice were trained for 2 weeks for tail-cuff measurements of SBP. At least seven replicates (per mouse) of the SBP were recorded every session and the mean of the lowest three values within 5 mmHg was considered the SBP level (Gómez-Guzmán et al., 2014).

Body weight (in grams) was measured for all mice. The hearts were excised; the atria and the right ventricle were then removed and the remaining left ventricle was weighted. The left ventricle, liver, spleen and kidney weight indices were calculated by dividing their weights by the tibia length. Samples were snap frozen in liquid nitrogen and then stored at −80°C.

For conventional morphology, kidneys from all groups were buffered, 10% formaldehyde fixed and paraffin embedded, and transversal sections in horizontal plane were stained with haematoxylin–eosin, Masson's trichrome and periodic acid–Schiff stain. The morphological study was performed in blinded fashion on 4-μm sections with light microscopy, using the most appropriate stain for each lesion. In kidneys, the presence of lesions was calculated semi quantitatively as dichotomous variables (0–1) expressed as percentage of affected kidneys. In glomeruli, glomerulosclerosis, mesangioproliferation, endocapillary proliferation, mesangial matrix expansion and glomerular immune deposits were evaluated and expressed as number of glomeruli affected per 50 glomeruli. In the tubulointerstitial area, the tubular casts and the inflammatory infiltrate were assessed in a semiquantitative scale (0–3). Finally, the number of nuclei per glomerular cross-section (50 glomeruli without sclerosis per mouse) was also determined (Romero et al., 2017).

2.3 | Plasma and urine parameters

At the end of all experimental periods, mice were killed under isoflurane anaesthesia. Blood was collected from the heart, cooled on ice and centrifuged for 10 min at 1096× *g* at 4°C. The resulting plasma was frozen at −80°C. Plasma anti-ds-DNA antibodies were measured using a mouse Anti-dsDNA IgA ELISA Kit (Alpha Diagnostic International, San Antonio, TX, USA) according to the manufacturer's instructions, as previously described (Toral, Robles-Vera, Romero, et al., 2019). Plasma **LPS** levels were measured using the Limulus Amebocyte Lysate chromogenic endotoxin quantitation Kit (Lonza, Valais, Switzerland), following the manufacturer's instructions. Plasma cytokine-level assessment was conducted by a multiplex assay using Luminex technology (Merck Millipore, Darmstadt, Germany). To determine proteinuria, we used the Combur-Test strips (Roche Diagnostics, Mannheim, Germany).

2.4 | Intestinal permeability measures

Intestinal permeability was assessed *in vivo* by FITC-dextran in an independent experiment ($n = 5$ animals per group), as described previously (Fujisaka et al., 2016). After an overnight fast, mice were given by gavage fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, Madrid, Spain) (5 mg per mice dissolved in 100 μl of water). After 4 h, all animals were anaesthetized and blood was collected from the heart. Plasma was diluted with PBS and fluorescence was measured (excitation 492 nm and emission 525 nm) using a spectrophotofluorometer.

2.5 | Vascular reactivity studies

Descending thoracic aortic rings were resected from animals and were suspended in a wire myograph (Model 610M, Danish Myo Technology, Aarhus, Denmark) for isometric tension measurement as previously described (Toral et al., 2018). The organ chamber was filled with Krebs solution (composition in mM:- 118 NaCl, 4.75 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 2 $CaCl_2$, 1.2 KH_2PO_4 and 11 glucose) at 37°C and gassed with 95% O_2 and 5% CO_2 (pH = 7.4). Length–tension characteristics were obtained via the myograph software (Myodaq 2.01) and the aortas were loaded to a tension of 5 mN.

In endothelium-intact aorta, cumulative concentration–response curves to **acetylcholine** (1 nM to 10 μM) were constructed in intact

segments precontracted by the **thromboxane A_2** analogous **U46619** (10 nM). A second concentration–relaxant response curve to ACh was performed in each sample in the absence or in the presence of the specific pan-**NOX** inhibitor VAS2870 (5 μM) or the **Rho kinase** inhibitor **Y27632** (5 μM). To study the involvement of IL-17, we incubated the aortic rings for 6 h with anti-**IL-17a** antibody (10 $\mu g \cdot ml^{-1}$) prior to construct the relaxant response curve to **ACh**. Relaxation responses to ACh were expressed as percentages of precontraction induced by **U46619**.

2.6 | NADPH oxidase activity

In order to determine **NADPH oxidase** activity, we performed a lucigenin-enhanced chemiluminescence assay was performed in intact aortic segments as previously described (Romero et al., 2017). Aortic segments from all groups were incubated for 30 min at 37°C in HEPES-containing physiological saline solution (pH 7.4) of the following composition (in mM): NaCl 119, HEPES 20, KCl 4.6, $MgSO_4$ 1, Na_2HPO_4 0.15, KH_2PO_4 0.4, $NaHCO_3$ 1, $CaCl_2$ 1.2 and glucose 5.5. Aortic production of O_2^- was stimulated by the addition of NADPH (100 μM). The samples were subsequently placed in tubes with the physiological saline solution and with or without NADPH. Lucigenin was injected automatically at a final concentration of 5 μM to avoid artefacts occurring at higher concentrations. NADPH oxidase activity was determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals and was calculated by subtracting the basal values from those found in the presence of NADPH. Finally, all aorta rings were dried and dry weight was determined. NADPH oxidase activity is expressed as relative luminescence units $min^{-1} \cdot mg^{-1}$ dry aortic tissue.

2.7 | RT-PCR analysis

Total RNA was extracted from colon, renal cortex and faeces by homogenization and retrotranscribed into cDNA by standard methods to carry out RT-PCR determinations. Tissues were dissociated in 1 ml of TRI Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA isolation was performed with a well-established methodology using sequential washes with bromochloropropane, isopropanol and ethanol 75%. RNA concentration levels were determined with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). PCR was carried out with a Techgene thermal cycler (Techne, Cambridge, UK). A quantitative real-time RT-PCR technique was used to analyse mRNA expression. Sense and antisense primer sequences used for amplification are included in Table S1. Preliminary titrations were performed with different levels of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was performed using the RT-PCR method. The efficiency of the PCR reaction was determined through titration of a standard tissue sample. Quantification was carried out through the ΔΔCt method. The expression of GAPDH was used as housekeeping for internal normalization (Romero et al., 2017).

2.8 | Flow cytometry

Mesenteric lymph nodes, spleens, blood and aorta were collected from all animals. These tissues were adequately mashed with wet slides to reduce friction. Then the solutions were filtered using 40-μm cell strainers to isolate the cells. Next, the red blood cells were lysed with Gey's solution. To improve intracellular staining, 1×10^6 cells were counted and incubated with a protein transport inhibitor (BD GolgiPlug™) for an optimum detection of intracellular cytokines by flow cytometry and cells were stimulated with 50-$ng \cdot ml^{-1}$ **phorbol 12-myristate 13-acetate** plus 1-$\mu g \cdot ml^{-1}$ ionomycin. After 4.5 h, cell aliquots from each sample were blocked with anti-Fc-γ receptor antibodies to reduce non-specific binding (Miltenyi Biotec) and were stained with the live/dead stain as a viability dye (LIVE/DEAD® Fixable Aqua Dead Cell Stain, Thermo Fisher), incubating for 30 min at 4°C. After that, cells were processed for surface staining for 20 min at 4°C in the dark with anti-**CD45** (RRID:AB_2727597, FITC, clone 30-F11, Miltenyi), anti-**B220** (RRID:AB_398531, APC, clone RA3-6B2, BD Biosciences), anti-**CD3** (RRID:AB_2801803, PE, clone REA641, Miltenyi), anti-**CD4** (RRID:AB_1107001, PerCP-Cy5.5, clone RM4-5, Invitrogen) and anti-**CD25** (RRID:AB_2784091, PE-VIO770, clone 7D4, Miltenyi). Cells were then fixed, permeabilized and underwent intracellular staining for 30 min at 4°C in the dark with anti-iIL-17a (RRID:AB_1073235, PE-Cy7, clone eBio17B7, eBioscience, San Diego, USA) and anti-IFN-γ (RRID:AB_2738165, PE-VIO770, clone XMG1.2, eBioscience, San Diego, USA). Finally, cells were transferred to polystyrene tubes. Samples were run and data were recorded with a flow cytometer Canto II (BD Biosciences) as previously described (Romero et al., 2017; Toral et al., 2018). The gate strategy for flow cytometry is shown in Figure S1.

2.9 | DNA extraction, 16S rRNA gene amplification and bioinformatics

At the experimental endpoint of every procedure, we collected faecal samples from 6–8 animals per group. DNA was extracted from all samples with G-spin columns (iNtRON Biotechnology) starting from 30 mg of samples in PBS with proteinase K and RNAses (Dole et al., 2013). DNA concentration was determined using Quant-IT PicoGreen reagent (Thermo Fischer). Aliquots (about 3 ng) were used to amplify the V3–V4 region of the 16S rRNA gene (Caporaso et al., 2011). PCR products (approx. 450 bp) included extension tails, which allowed sample barcoding and the addition of specific Illumina sequences in a second low-cycle number PCR. Individual amplicon libraries were analysed using a Bioanalyzer 2100 (Agilent) and a pool of samples was made in equimolar amounts. The pool was further cleaned and quantified, and the exact concentration was determined by real-time PCR (Kapa Biosystems). Finally, DNA samples were

sequenced on an Illumina MiSeq instrument with 2 × 300 paired-end read sequencing at the Unidad de Genómica (Parque Científico de Madrid, Madrid, Spain).

In order to process raw sequences, the barcoded Illumina paired-end sequencing (BIPES) pipeline was used (Zhou et al., 2011). First, as per the BIPES protocols, the barcode primers were trimmed and filtered if they contained ambiguous bases or mismatches in the primer regions. Second, any sequences with more than one mismatch within the 40- to 70-bp region at each end were removed. Third, we used 30 Ns to concentrate the two single-ended sequences for the downstream sequence analyses. Previous more detailed protocols for these methods have already been published (Liu et al., 2017). Fourth, we used UCHIME (implemented in USEARCH, Version 6.1) to screen out and remove chimeras in the *de novo* mode (using-minchunk 20-xn 7-noskipgaps 2) (Edgar & Flyvbjerg, 2015).

Between 90,000 and 220,000 sequences were identified in each sample. The rest of analyses were carried out with 16S Metagenomics (Version 1.0.1.0) from Illumina. The sequences were subsequently clustered to an operational taxonomic unit (OTU) with USEARCH default parameters (USERACH61). The threshold distance was set to 0.03. Consequently, when similarities between 16S rRNA sequences were 97%, the sequences were classified as the same operational taxonomic unit. Quantitative Insights Into Microbial Ecology-based alignments of representative sequences were carried out with PyNAST and the Greengenes 13_8 database was used as the template file. The Ribosomal Database Project algorithm was used to classify the representative sequences into specific taxa with the default database (Zeng et al., 2019). The Taxonomy Database (National Center for Biotechnology Information) was used for classification and nomenclature.

2.10 | Statistical analysis

Shannon diversity, Chao richness and Pielou evenness and observed species indexes were calculated with the PAST4.02 Palaeontological Statistics (PAST 3×). Reads in each operational taxonomic unit were normalized to total reads in each sample. Only taxa with a percentage of reads >0.001% were used for the analysis. Principal component analysis was also used on these data to determine significant differences among all experimental groups in each treatment, with PAST 4.02 and SSPS. Linear discriminant analysis scores above 2 were displayed. Taxonomy was uploaded to the Galaxy platform (Segata et al., 2011) to generate LEfSe/cladogram enrichment plots considering significant enrichment at a $P < 0.05$. All data were analysed with GraphPad Prism 7 (RRID:SCR_000306). Results are expressed as means ± SEM of measurements. The evolution of tail SBP and the concentration–response curves to ACh were analysed by two-way repeated-measures ANOVA with the Bonferroni post hoc test. Post hoc tests were conducted only if F in ANOVA achieved $P < 0.05$. The remaining variables were tested on normal distribution using Shapiro–Wilk normality test and compared using an unpaired Student's *t*-test or one-way ANOVA and Tukey's post hoc test in case of normal distribution, or Mann–Whitney test or Kruskal–Wallis with Dunn's multiple comparison test in case of abnormal distribution; $P < 0.05$ was considered statistically significant. Sample sizes subjected to statistical analysis at least 5 animal per group ($n = 5$), where n = number of independent values. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.11 | Materials

All chemicals were obtained from Sigma-Aldrich (Barcelona, Spain), unless otherwise stated.

2.12 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the concise guide to PHARMACOLOGY 2019/20 (Alexander et al., 2019).

3 | RESULTS

3.1 | Changes in microbiota composition with the development of hypertension in systemic lupus erythematosus mice

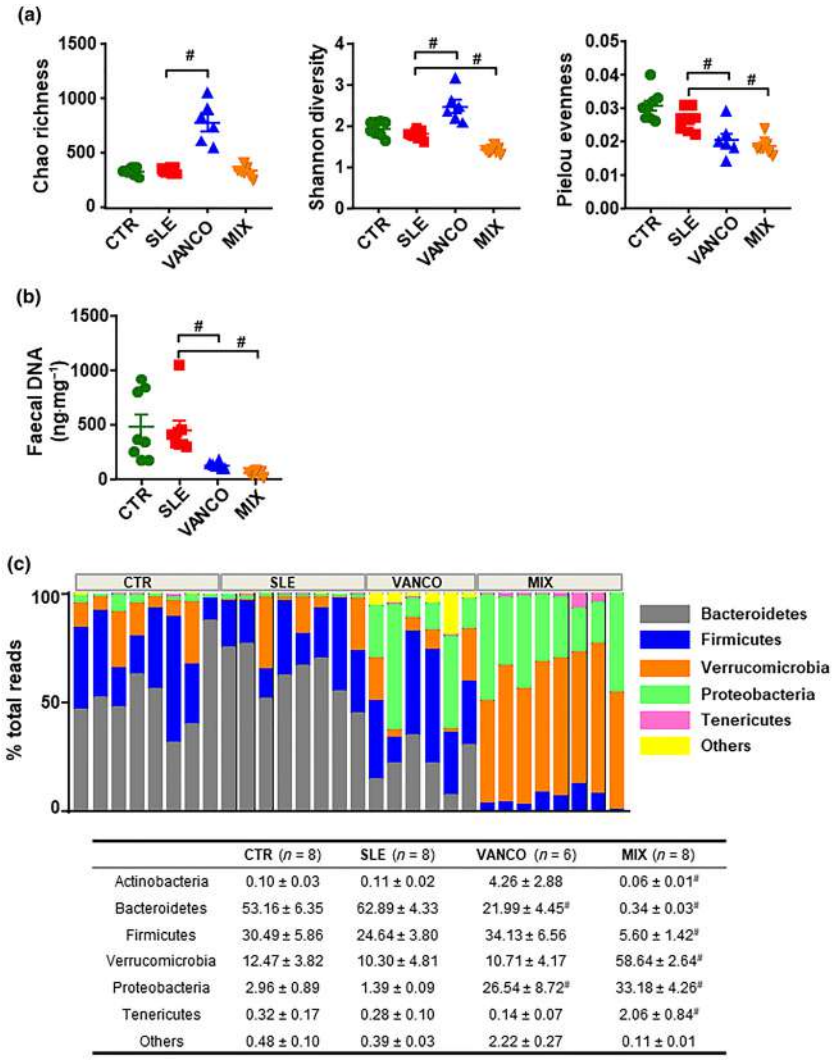
To assess the dynamics of the gut microbiota during BP increase associated to lupus progression in female NZBWF1 mice, we analysed faecal pellets collected at 26 weeks (pre-hypertensive) and at 32 weeks of age (hypertensive) and compared them with aged-matched control mice. At 26 weeks, the bacterial taxa (class, order, family and genus) that were altered in systemic lupus erythematosus mice, according to the linear discriminant analysis, showed that the relative abundance of 23 taxa was elevated (green) and 18 taxa were reduced (red) as compared with control (Figure S2A,B). Principal component analysis, at the genus levels, showed a clear separation between the two clusters, indicating two different gut environments (Figure S2C). The Kaiser–Meyer–Olkin (KMO) test, which measures sampling adequacy, was 0.858, indicating a meritorious sampling. Barlett's test of sphericity was $P < 0.05$. The key bacterial population that is responsible for discriminating between groups was the genus *Oscillospira* (loading 0.78). When we evaluated the phyla composition, faecal samples were dominated by Firmicutes and Bacteroidetes, with smaller proportions of Verrucomicrobia, Proteobacteria, Tenericutes and Actinobacteria (Figure S3A,B). We showed increased Firmicutes and reduced Bacteroidetes proportions in systemic lupus erythematosus mice as compared with control. At 32 weeks of age, both control and systemic lupus erythematosus mice showed differences at the phyla proportion of the microbiota. As compared with 26-week-old mice, a significant increase in the bacteria belonging to Verrucomicrobia and

Proteobacteria was detected in control mice, whereas in systemic lupus erythematosus, the dynamic is different, with significant increases in Bacteroidetes and Proteobacteria and the reduction of Firmicutes (Figure S2B). When we compared normotensive control with hypertensive systemic lupus erythematosus at 32 weeks of age, no significant differences in the proportion of bacteria belonging to each phylum were observed, as previously described (Toral, Robles-Vera, de la Visitación, et al., 2019). Moreover, no significant changes were observed between control and systemic lupus erythematosus at 32 weeks of age in the three major ecological parameters, including Chao richness (an estimate of a total number of operational taxonomic units present in the given community), Shannon diversity (the combined parameters of richness and evenness) and Pielou evenness (to show how evenly the individuals in the community are distributed over different operational taxonomic units) (Figure 1a). However, at family level, a significant increase of bacteria from Porphyromonadaceae and Sphingobacteriaceae was detected in hypertensive systemic lupus erythematosus as compared with normotensive aged-matched control mice (Figure S4). At genus levels, the principal component analysis also showed a clear separation between the two clusters and the proportions of several genera, such as *Parabacteroides*, *Pedobacter*, *Olivibacter* and *Clostridium* were also increased in systemic lupus erythematosus (Figure S5). It is interesting to note that at 26 weeks of age, no significant differences were found in the abundance of *Parabacteroides* and *Clostridium* between systemic lupus erythematosus and control mice (not shown).

The mRNA levels of the bacterial enzyme bile acid 7α-dehydratase, which is involved in 7-α-dehydroxylation of **taurocholic acid** and **cholic acid** to the production of inflammatory bile acids (Ridlon et al., 2006), were increased in faeces from hypertensive systemic lupus erythematosus as compared with control (Figure S6).

3.2 | Antibiotic treatments induced changes in gut microbiota and inhibited the increment of BP, target organ hypertrophy, renal injury and disease activity in lupus-prone mice

Quantitative PCR (qPCR) analysis showed that vancomycin or a broad-spectrum antibiotic mixture (MIX) treatment reduced



	CTR (n = 8)	SLE (n = 8)	VANCO (n = 6)	MIX (n = 8)
Actinobacteria	0.10 ± 0.03	0.11 ± 0.02	4.26 ± 2.88	0.06 ± 0.01#
Bacteroidetes	53.16 ± 6.35	62.89 ± 4.33	21.99 ± 4.45#	0.34 ± 0.03#
Firmicutes	30.49 ± 5.86	24.64 ± 3.80	34.13 ± 6.56	5.60 ± 1.42#
Verrucomicrobia	12.47 ± 3.82	10.30 ± 4.81	10.71 ± 4.17	58.64 ± 2.64#
Proteobacteria	2.96 ± 0.89	1.39 ± 0.09	26.54 ± 8.72#	33.18 ± 4.26#
Tenericutes	0.32 ± 0.17	0.28 ± 0.10	0.14 ± 0.07	2.06 ± 0.84#
Others	0.48 ± 0.10	0.39 ± 0.03	2.22 ± 0.27	0.11 ± 0.01

FIGURE 1 Effects of antibiotic treatments in microecological parameters and phyla composition of gut microbiota in systemic lupus erythematosus (SLE) mice. (a) Ecological parameters, (b) bacterial charge and (c) proportion of bacterial phyla in the gut microbiota in control (CTR), SLE and SLE groups treated with vancomycin (VANCO) or a broad-spectrum antibiotic mixture (MIX). Values are expressed as means ± SEM. $N = 6–8$ mice per treatment group for each comparison. # $P < 0.05$ compared with the untreated SLE group

total DNA levels in the faeces by ≈72% and ≈87%, respectively (Figure 1b). Vancomycin treatment of systemic lupus erythematosus mice increased richness and diversity but decreased evenness, whereas the broad-spectrum antibiotic mixture treatment reduced diversity and evenness. Profound changes in phyla composition were observed, especially with the MIX treatment (Figure 1c). The proportion of bacteria belonging to Bacteroidetes and Proteobacteria were reduced and increased, respectively, in the systemic lupus erythematosus-vancomycin group as compared with systemic lupus erythematosus, whereas bacteria belonging to Verrucomicrobia and Proteobacteria dominated the composition of gut microbiota in systemic lupus erythematosus-MIX, with intense reductions of Bacteroidetes and Firmicutes. Vancomycin normalized the proportion of the bacteria from Porphyromonadaceae and Sphingobacteriaceae, whereas systemic lupus erythematosus-MIX almost suppressed these bacteria. Interestingly, vancomycin increased by ≈2.7-fold the Lactobacillaceae family (Figure S4). Although they are Gram-positive organisms, lactobacilli are often phenotypically resistant to the action of vancomycin (Robinson & Young, 2010). At genus level, principal component analysis showed that vancomycin separated its cluster from systemic lupus erythematosus, which remains closer than control, whereas bacteria from systemic lupus erythematosus-MIX were far from the rest of the groups. The Kaiser–Meyer–Olkin test was 0.726, indicating a middling sampling. Barlett's test of sphericity was $P < 0.05$. The key bacterial population that is responsible for discriminating among groups was the genus *Bacteroides* (loading 0.85). In fact, the proportion of *Parabacteroides*, *Pedobacter*, *Olivibacter* and *Clostridium* was normalized by vancomycin and almost abolished by MIX. *Bacteriodes* were also almost suppressed by both groups of treatment with antibiotics (Figure S5). Interestingly, both antibiotic treatments normalized the increased expression of bile acid 7α-dehydratase in faeces from systemic lupus erythematosus mice (Figure S6), suggesting a reduction of the pro-inflammatory bile acids.

At 26 weeks of age, SBP values were similar for all experimental groups. At 32 weeks of age, SBP was significantly elevated in systemic lupus erythematosus mice in comparison with control mice by approximately 55 mmHg and this change was partially prevented by both vancomycin (≈40%) and MIX (≈44%) (Figure 2a). At 26 weeks of age, systemic lupus erythematosus mice showed higher protein excretion than control mice, despite similar SBP (Figure 2b). Protein excretion increased in untreated systemic lupus erythematosus mice with ageing, whereas 6 weeks of treatment with vancomycin or MIX reduced by ≈45% and ≈63%, respectively, this parameter. At the end of the experiment there was a significant increase in body weight of systemic lupus erythematosus mice (35.8 ± 2.3 g, $n = 10$) in comparison with control animals (30.2 ± 1.3 g, $n = 10$) and neither vancomycin (35.8 ± 1.6 g, $n = 8$) nor MIX (32.0 ± 1.5 g, $n = 10$) significantly changed the body weight in systemic lupus erythematosus mice. Anatomical analysis revealed that left ventricle weight/tibia length and right and left kidney weight/tibia length indices were significantly higher (≈47%, ≈49% and ≈49%, respectively) in systemic lupus erythematosus mice than in control mice (Figure 2c). Both antibiotic treatments prevented the increase of cardiac index but were unable to change the renal indices found in systemic lupus erythematosus mice. We measured systemic lupus erythematosus disease activity at the end of the experiment by plasma levels of autoantibodies and we found a significant rise in systemic lupus erythematosus mice in relation to control mice (Figure 2d), as previously reported (Toral, Robles-Vera, Romero, et al., 2019) Vancomycin did not change disease activity, but MIX treatment reduced plasma anti-dsDNA levels. Besides, disease progression has been linked to splenomegaly, most probably due to a lymphoproliferative disorder (Wofsy et al., 1988). We have also observed splenomegaly in lupus mice, which was abolished by MIX, whereas vancomycin was unable to change spleen weight (Figure 2e).

The main morphological results of comparative study of renal injuries in different mice groups and representative photomicrographs are shown in Figure S6A. Systemic lupus erythematosus group showed diffuse and segmental endocapillary, and mesangial hypercellularity, matrix expansion with hyalinosis, capillary wall thickening with wire-loop lesions, scan hyaline thrombi in lumen of tuft capillary and moderate extra-capillary proliferation (crescent in 23.7% of glomeruli). Scattered complete glomerular sclerosis or cystic could also be observed. Cortical tubules dilated with brush border loss showed numerous hyaline casts inside and moderate/severe clusters of renal papillae and tubulointerstitial chronic inflammatory infiltrate. The percentage of glomeruli exhibiting a severe mesangial sclerosis area and immune complex deposits in systemic lupus erythematosus mice was 50.4% and 34%, respectively. In contrast, we observed a statistically significant decrease in systemic lupus erythematosus-vancomycin and systemic lupus erythematosus-MIX groups for kidney glomerular and tubulointerstitial lesions when were compared with mice from the systemic lupus erythematosus group. In the systemic lupus erythematosus-MIX group, we observed the greatest reduction in injuries and the involvement in a lower percentage of mice with lesions. Mesangial hyalinization, crescent, wire loop, immune complex deposits, hyaline casts, glomerular cellularity and inflammatory infiltrate were reduced in systemic lupus erythematosus-MIX mice not being statistically significant the difference with the control mice (all renal lesion, except for the mesangial sclerosis and inflammatory infiltrate). In addition, mRNA levels of the pro-inflammatory cytokines (Figure S6B), **IL-6**, **IL-1β** and **TNF-α**, in renal cortex were increased by ≈2.8-fold, 5.8-old and 5.9-fold in systemic lupus erythematosus mice compared with control. Both vancomycin and MIX administration significantly decreased the expression levels of these genes.

3.3 | Antibiotic treatments induced changes in intestinal integrity and permeability

Gut barrier integrity was analysed by the colonic mRNA expression of barrier-forming junction transcripts (Figure 3a), such as occludin

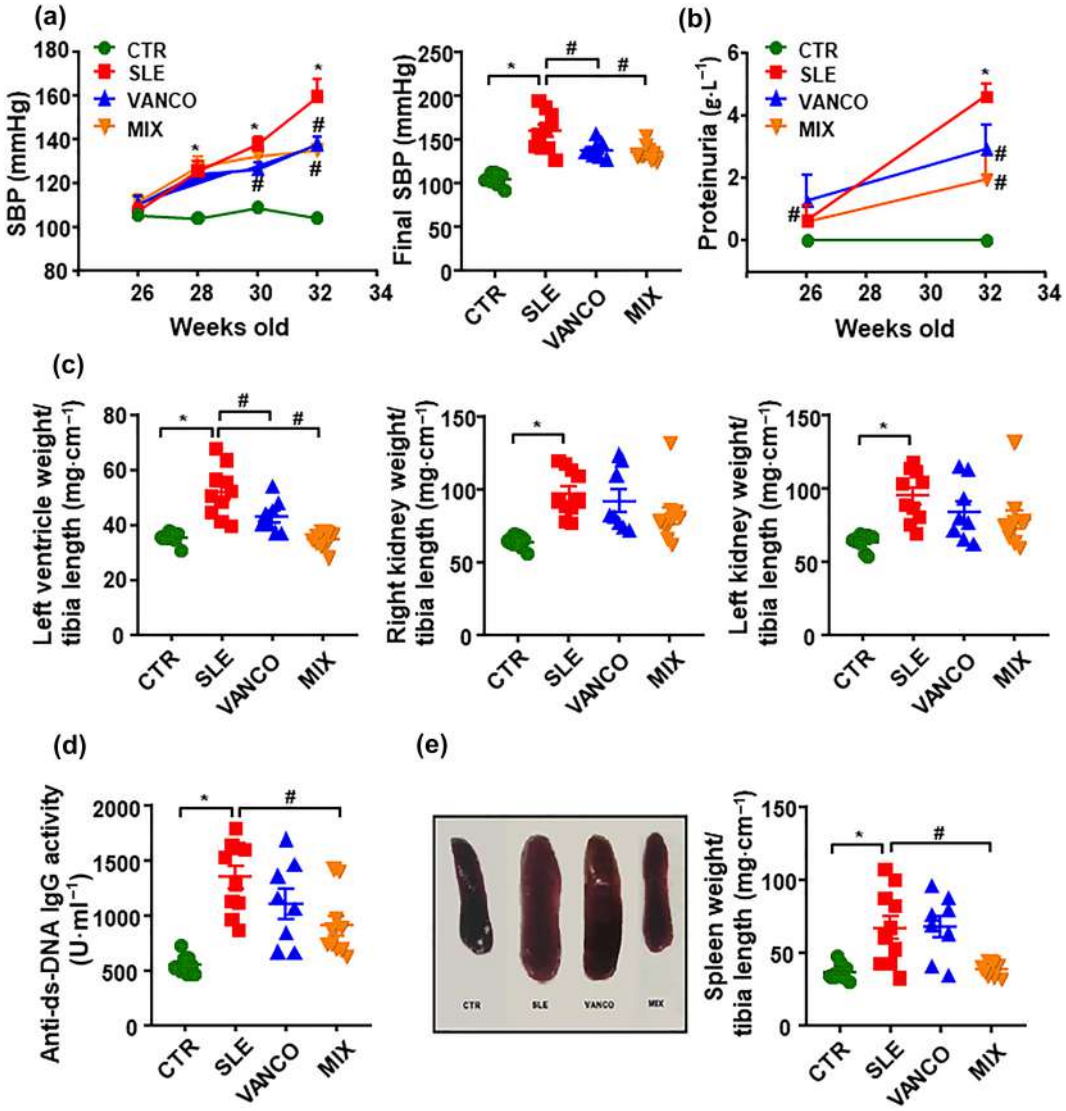


FIGURE 2 Antibiotic treatments inhibited the increase of BP, target organ hypertrophy and disease activity in systemic lupus erythematosus (SLE) mice. (a) Systolic BP (SBP) measured by tail-cuff plethysmography, (b) proteinuria, (c) morphological parameters, (d) circulating double-stranded DNA autoantibody levels and splenomegaly in control (CTR), SLE and SLE groups treated with vancomycin (VANCO) or a broad-spectrum antibiotic mixture (MIX). Values are expressed as means ± SEM ($n = 8–10$). $^{*}P < 0.05$ compared with the CTR group; $^{\#}P < 0.05$ compared with the untreated SLE group

and zonula occludens-1) and the mucins (Figure 3b), mucin-2 and mucin-3. No significant changes in these parameters were observed between control and systemic lupus erythematosus groups. However, vancomycin increased zonula occludens-1 and mucin-2, by ≈2.2-fold and 3.6-fold, respectively. The improvement in gut barrier integrity induced by vancomycin was accompanied by a 76% reduction in gut permeability measured by FITC-dextran (Figure 3c). The plasma endotoxin levels were significantly higher (≈1.9-fold) in systemic lupus erythematosus mice than in the control group (Figure 3d). Surprisingly, the long-term treatment with vancomycin or MIX did not change endotoxemia in systemic lupus erythematosus mice.

3.4 | Antibiotic treatments attenuate T-cell imbalance

High levels of autoantibodies and progressive lupus-like autoimmune disease are linked to an imbalance of T cells (Talaat et al., 2015) and increased B cells (Dar et al., 1988). We measured the levels of B and T cells in mesenteric lymph nodes, spleen and blood for all groups. Relative populations of B were higher in both secondary lymphoid organs from systemic lupus erythematosus mice in relation to control group populations, whereas the proportion of Th cells was only increased in spleen (Figure 4a,b). Vancomycin treatment of systemic lupus erythematosus mice did not change the levels of B

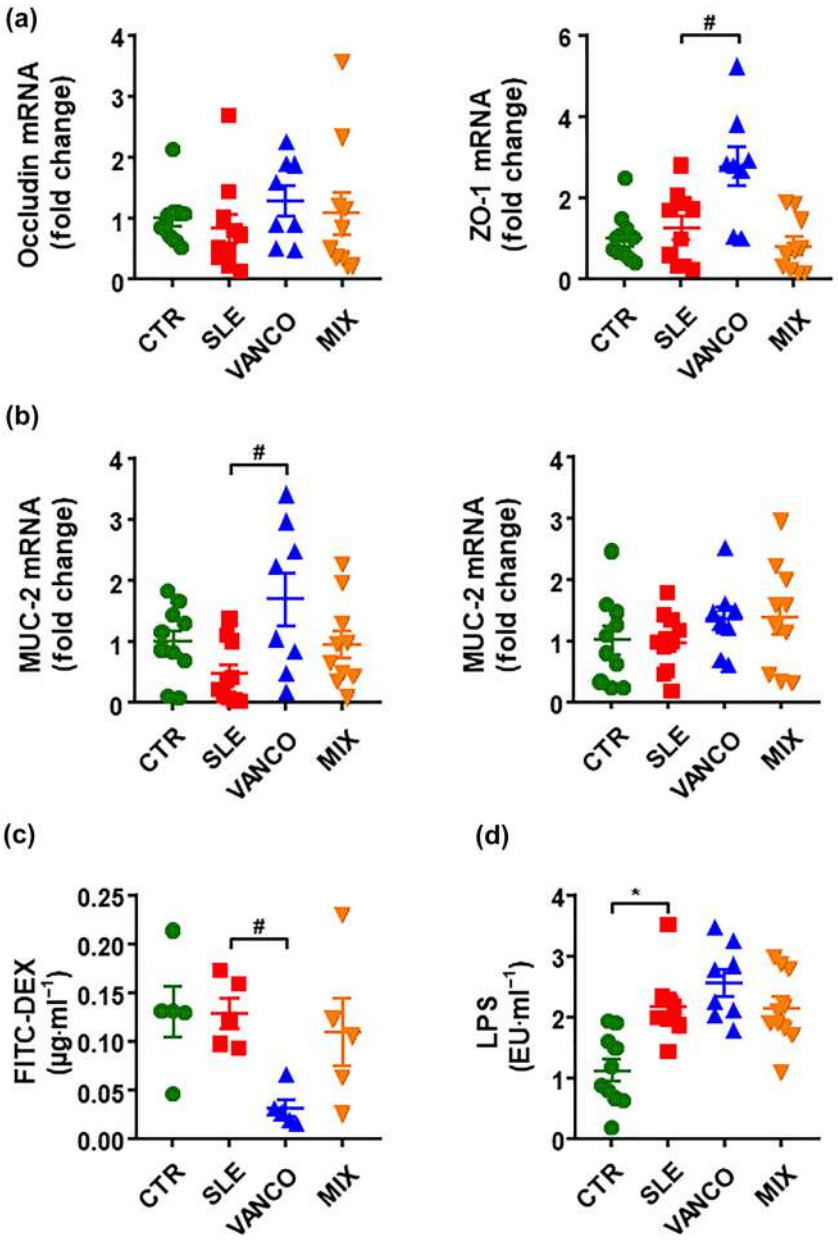


FIGURE 3 Effects of antibiotic treatments on epithelial integrity markers and permeability in systemic lupus erythematosus (SLE) mice. Colonic mRNA expression levels of (a) barrier-forming proteins occludin and zonula occludens-1 (ZO-1) and (b) mucin (MUC)-2 and MUC-3. (c) Intestinal permeability measured by FITC-dextran. (d) Plasma LPS levels in control (CTR), SLE and SLE groups treated with vancomycin (VANCO) or a broad-spectrum antibiotic mixture (MIX). Values are expressed as means ± SEM. $^{*}P < 0.05$ compared with the CTR group; $^{\#}P < 0.05$ compared with the untreated SLE group

and T cells in both lymphatic organs, whereas MIX decreased Th cells (Figure 4a,b). The percentages of regulatory T (Treg, $CD4^{+}/CD25^{+}$) cells, Th17 cells ($CD4^{+}$/IL-$17a^{+}$) and Th1 ($CD4^{+}$/IFN-γ^{+}) cells increased in systemic lupus erythematosus mice in mesenteric lymph nodes (Figure 4a), whereas only Treg and Th1 increased by lupus disease in the spleen (Figure S7). Vancomycin and MIX treatments prevented the increase of Th17 cell content induced by systemic lupus erythematosus only in mesenteric lymph nodes (Figure 4a).

Circulating B, Treg and Th17 cells were higher in systemic lupus erythematosus than in control mice (Figure S8A). In concordance with changes induced by antibiotic treatments in mesenteric lymph nodes, vancomycin and MIX reduced the proportion of circulating Th17 cells. Plasma levels of IL-6, IL-17a and IFN-γ were increased in systemic lupus erythematosus mice compared with control mice (Figure S8B). Both antibiotic treatments normalized plasma levels of pro-inflammatory cytokines IL-6 and IL-17a, being without significant effects in IL-10 and IFN-γ.

3.5 | Antibiotic treatments prevent endothelial dysfunction, vascular oxidative stress and Th17 infiltration in aorta

Aortas from systemic lupus erythematosus mice had markedly and significantly decreased endothelium-dependent vasorelaxant responses to ACh compared with aortas from the control group ($E_{max} = 26.9 \pm 5.6\%$ and $73.2 \pm 1.4\%$; Figure 5a). The treatment of systemic lupus erythematosus mice with both vancomycin and MIX improved the impairment of ACh-induced relaxation. This response induced by ACh was also improved in aortic rings from systemic lupus erythematosus mice after incubation with the Rho kinase

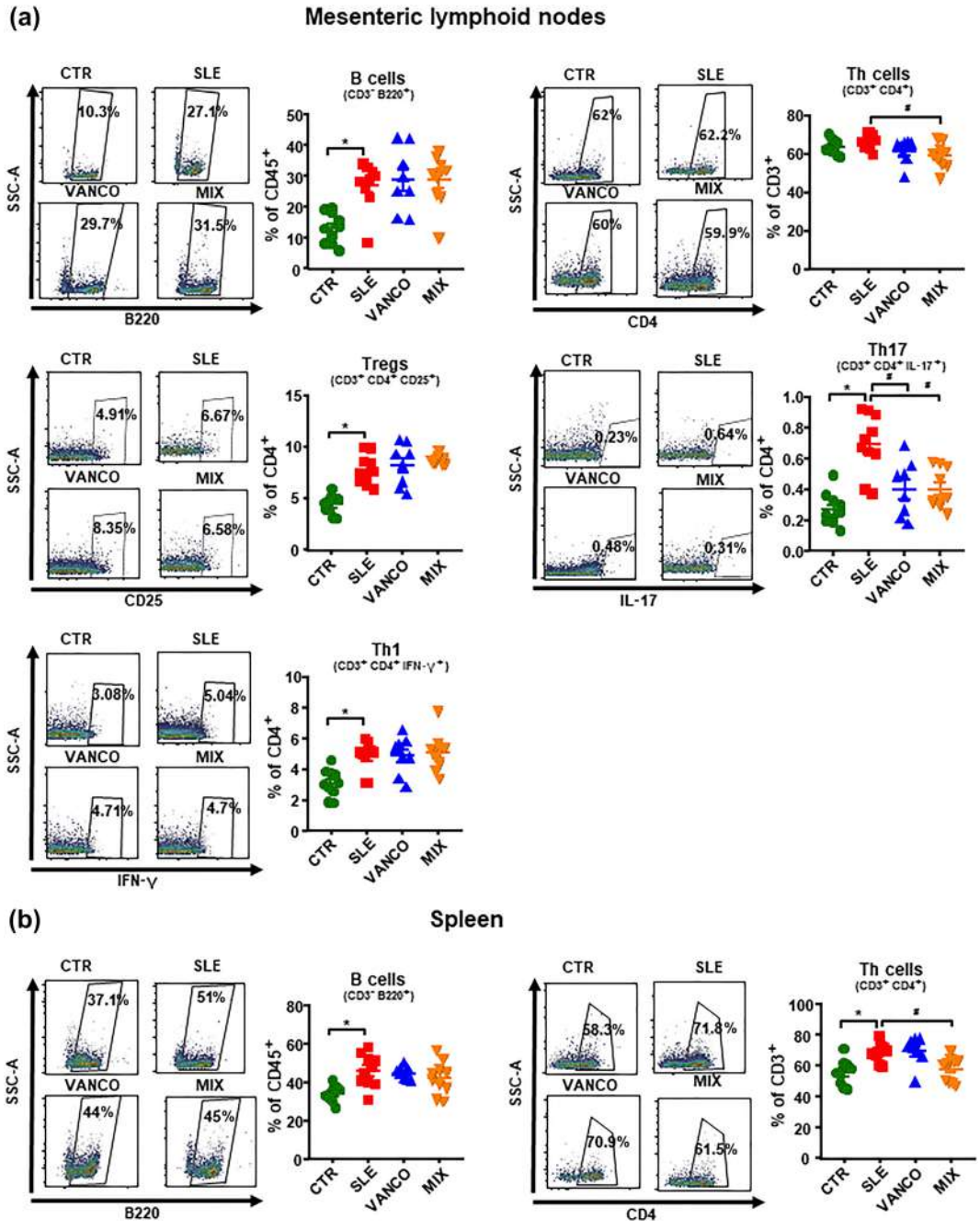


FIGURE 4 Effects of antibiotic treatments on lymphocytes populations in systemic lupus erythematosus (SLE) mice. (a) Total B lymphocytes, Th cells, regulatory T cells (Tregs), Th17 cells and Th1 cells measured by flow cytometry in mesenteric lymphoid nodes and (b) B and Th cells in spleen from control (CTR), SLE and SLE groups treated with vancomycin (VANCO) or a broad-spectrum antibiotic mixture (MIX). All data are expressed as % of parent, except for B cells, which are represented as % of grandparent (% of $CD45^{+}$). Values are expressed as means ± SEM ($n = 8–10$). * $P < 0.05$ compared with the CTR group; $^{\#}P < 0.05$ compared with the untreated SLE group

inhibitor Y27632 (Figure 5a), showing that the impaired ACh-induced relaxation of aorta is mediated, at least in part, by Rho kinase activation. ROS-dependent activation of RhoA/Rho kinase has been previously described (MacKay et al., 2017). Because NADPH oxidase is the major source of ROS in the vascular wall, we measured NADPH oxidase activity in all experimental groups (Figure 5b). NADPH oxidase activity was ≈2.8-fold higher in aortic rings of systemic lupus erythematosus mice than in aortic rings of control mice and both antibiotic treatments inhibited this activity. Taking into account that inflammatory cells increased vascular ROS production, we measured T-lymphocyte infiltration in aorta form all experimental groups. Th17 cells were increased in aorta from systemic lupus erythematosus mice as compared with control mice, being without change Treg and Th1 cells (Figure 5c). Both vancomycin and MIX reduced the infiltration on Th17 in aorta.

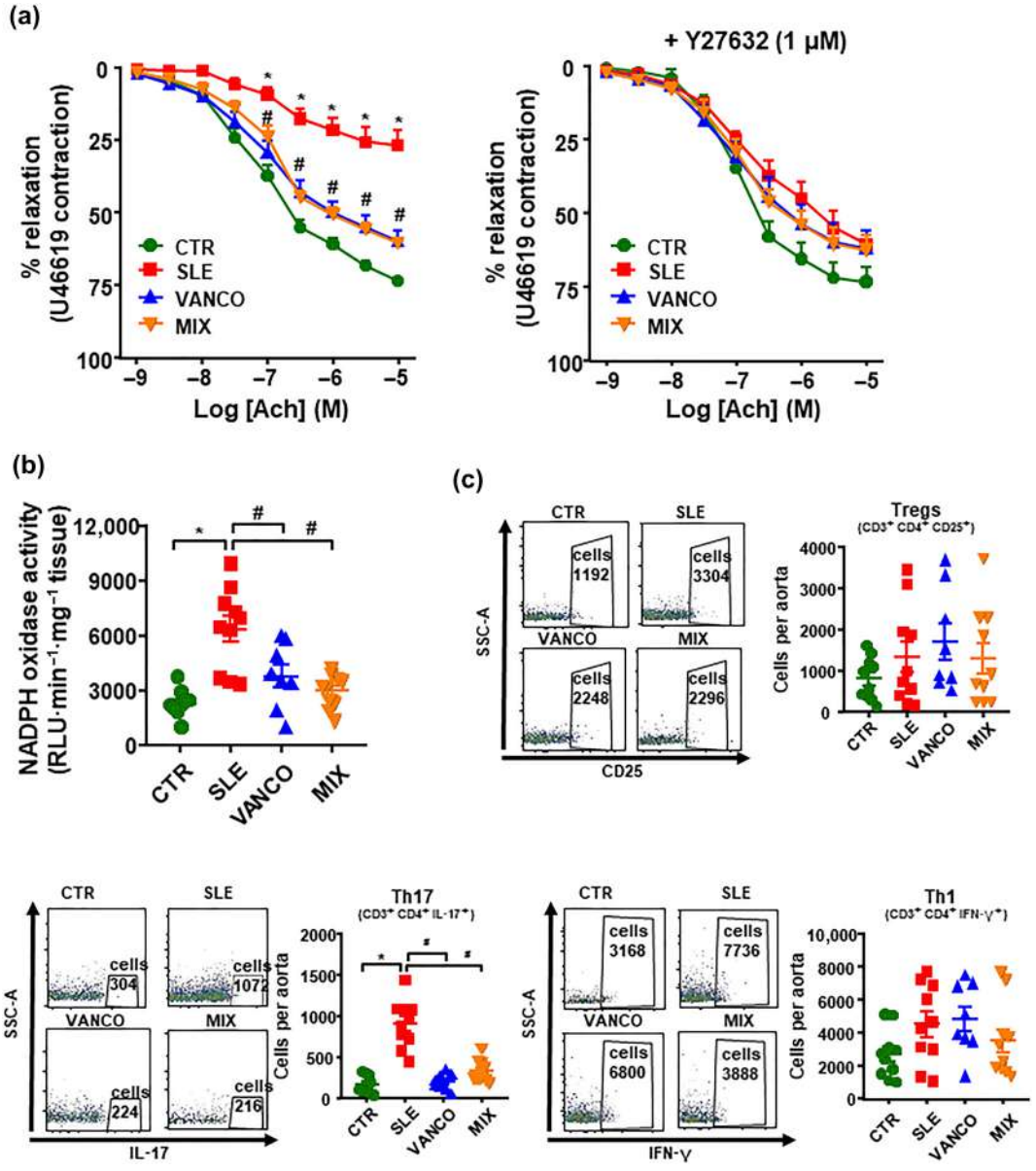


FIGURE 5 Effects of antibiotic treatments on endothelial function, NADPH oxidase activity and aortic infiltration of immune cells in systemic lupus erythematosus (SLE) mice. (a) Vascular relaxation responses induced by ACh in endothelium-intact aortas precontracted by U46619 (10 nM), in the absence or in the presence of the rho kinase inhibitor Y27632 (1 μM) in all experimental groups: Control (CTR), SLE and SLE groups treated with vancomycin (VANCO) or a broad-spectrum antibiotic mixture (MIX). (b) Aortic NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence. (c) Aortic infiltration of immune cells measured by flow cytometry. Values are expressed as means ± SEM ($n = 8–10$). * $P < 0.05$ compared with the CTR group; # $P < 0.05$ compared with the untreated SLE group

3.6 | Transplantation of gut microbiota from hypertensive NZBWF1 mice transfers increased BP and impaired endothelial function in mice without lupus genetic background

To address the question whether lupus mice microbiota could potentially regulate BP and endothelial function, we transferred microbiota from hypertensive NZBWF1 mice or from normotensive NZW/LacJ control mice to recipient C57Bl/6J female mice, during 3 weeks. In addition, C57Bl/6J germ-free mice were also inoculated with microbiota from systemic lupus erythematosus or control mice and were maintained for 3 weeks. Systemic lupus erythematosus microbiota raised SBP to a maximum of ≈16 mmHg, while faecal microbiota transplantation from control mice was ineffective (Figure 6a). However, systemic lupus erythematosus microbiota transplantation for 3 weeks was unable to induce morphological changes in left ventricle (Figure 6b) or evoke protein excretion (no proteins were detected in urine in both CTR-CTR and CTR-systemic lupus erythematosus groups) and renal cortex inflammation (mRNA levels of the pro-inflammatory cytokines, IL-6, IL-1β and TNF-α, were similar

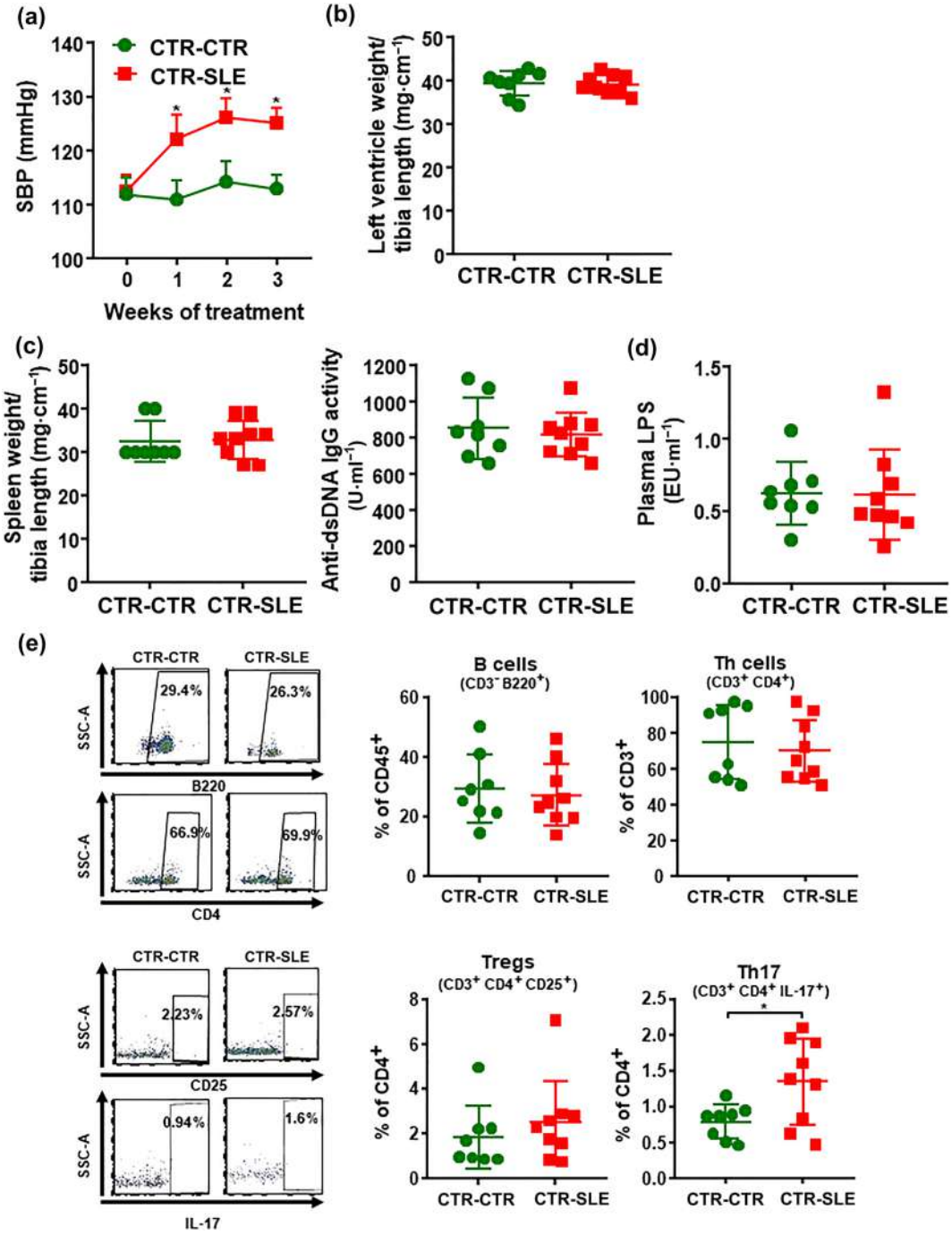


FIGURE 6 Effects of faecal microbiota transplantation on the evolution of systolic BP (SBP), disease activity, endotoxemia and lymphocytes populations in mesenteric lymph nodes. (a) SBP measured by tail-cuff plethysmography. (b) Left ventricular weight/tibia length ratio was measured as morphological parameter in the heart. (c) Spleen weight/tibia length ratio and autoantibody levels were measured as markers of the pathology. (d) Plasma LPS levels. (e) Proportion of different immune cell types in mesenteric lymph nodes: Total B lymphocytes, Th cells, regulatory T cells (Tregs) and Th17 cells measured by flow cytometry. All data are expressed as % of parent, except for B cells, which are represented as % of grandparent (% of $CD45^+$). Values are expressed as means ± SEM ($n = 8–9$). *$P < 0.05$ compared with the CTR-CTR group. CTR-CTR, C57Bl/6J mice transplanted with faeces from control mice; CTR-SLE, C57Bl/6J mice transplanted with faeces from hypertensive systemic lupus erythematosus mice

between both groups, Figure S11). Interestingly, no significant changes either in spleen weight or plasma levels of anti-dsDNA were observed between CTR-CTR and CTR-systemic lupus erythematosus groups (Figure 6c), showing no change in lupus activity induced by microbiota from systemic lupus erythematosus mice. Plasma LPS levels were unchanged between groups (Figure 6d). Microbiota from systemic lupus erythematosus increased the Th17 proportion in mesenteric lymph nodes, without changes in B cells, Th cells, Treg and

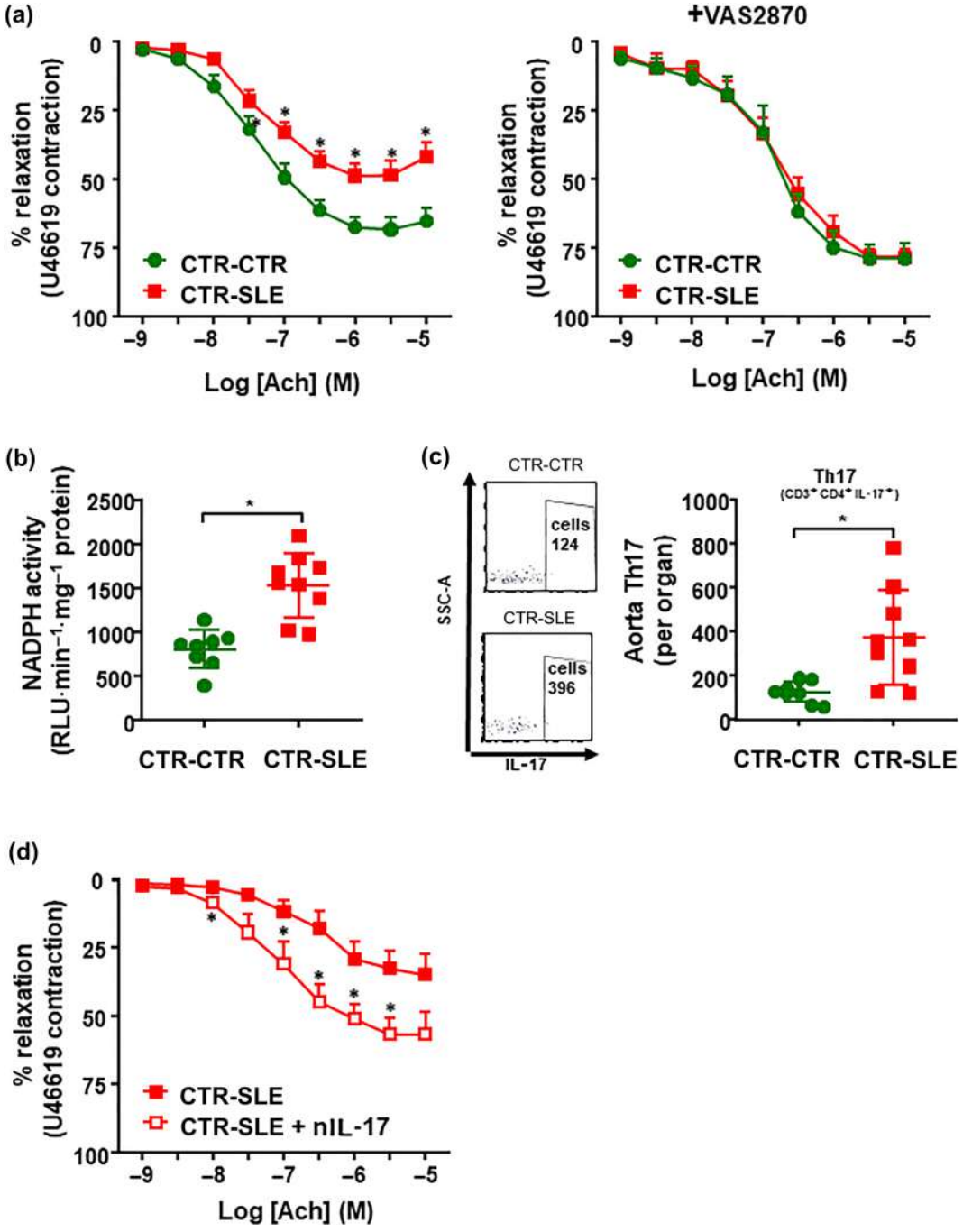


FIGURE 7 Effects of the faecal transplantation on endothelial function, NADPH oxidase activity and aortic infiltration of T cells. (a) Vascular relaxation responses induced by ACh in endothelium-intact aortas precontracted by U46619 (10 nM) in the absence or in the presence of the specific pan-NOX inhibitor VAS2870 (5 μM) in all experimental groups: Control–control (CTR-CTR) and control–SLE (CTR-SLE). (b) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence. (c) Aortic infiltration of Th17 measured by flow cytometry. (d) Vascular relaxation responses induced by ACh in endothelium-intact aortas precontracted by U46619 and preincubated for 6 h with a neutralizing agent of IL-17a (+ nIL-17). Values are expressed as means ± SEM ($n = 8–9$). * $P < 0.05$ compared with the CTR-CTR group. CTR-CTR, C57Bl/6J mice transplanted with faeces from control mice; CTR-SLE, C57Bl/6J mice transplanted with faeces from hypertensive systemic lupus erythematosus mice

Th1 populations (Figure 6e). In addition, endothelium-dependent relaxant responses to ACh in U46619-contracted aortic rings from the CTR-systemic lupus erythematosus group were significantly lower than in CTR-CTR group (E_{max}: 48.7 ± 4.6% vs. 67.7 ± 4.2 Figure 7a). Incubation for 30 min with the pan-NOX inhibitor VAS2870 abolished differences between groups in relaxation to ACh, showing the involvement of NADPH oxidase in this impaired relaxant response induced by systemic lupus erythematosus microbiota. Moreover, the

stool transfection from hypertensive systemic lupus erythematosus mice increased aortic NADPH oxidase activity (Figure 7b), as compared with CTR-CTR group. Interestingly, Th7 infiltration in aorta was also increased in CTR-systemic lupus erythematosus (Figure 7c) without changes in Tregs and Th1 (not shown). When we incubated the aortic rings from CTR-systemic lupus erythematosus for 6 h with nIL-17 or vehicle, neutralization of IL17 improved relaxation to ACh (Figure 7d) showing the key role of Th17 infiltration in endothelial dysfunction induced by systemic lupus erythematosus microbiota. Similarly, a higher SBP (≈10 mmHg), Th17 proportion in mesenteric lymph nodes (≈4.7-fold), aortic NADPH oxidase activity (≈2-fold) and impaired ACh-induced relaxation were found in germ-free-systemic lupus erythematosus as compared with germ-free-CTR, without splenomegaly induction (Figure S9).

We next examined the microbiota composition 3 weeks after faecal microbiota transplantation. Microbiota from donor control and systemic lupus erythematosus mice changes after transplantation in recipient C57Bl/6J, showing a significant increase in the proportion of Actinobacteria (≈8.1-fold and 12.3-fold, respectively) and a reduction of Bacteroidetes (≈33.1% and 39.8%, respectively) in faeces from recipient mice as compared with donors (Figure S10). We found a large variability in the Firmicutes and Verrucomicrobia phyla in mice from CTR-systemic lupus erythematosus group, without significant change in phyla proportion between CTR-CTR and CTR-systemic lupus erythematosus groups (Figures 8a and S10). Linear discriminant analysis score showed minor changes after transplantation with systemic lupus erythematosus microbiota, where the relative abundance of two taxa (Bacteroidaceae and *Bacteroides*) was increased and two taxa (*Incertae* and *sedis*) were decreased, when compared with control stool transplantation (Figure 8b). Principal component analysis plot illustrates a little separation between groups at genus level (Figure 8c). The Kaiser–Meyer–Olkin test was 0.803, indicating a meritorious sampling. Barlett's test of sphericity was $P < 0.05$. The key bacterial population that is responsible for discriminating between groups was the genus *Akkermansia* (loading 0.92). There was an increase in the genera *Bacteroides* and *Parasutterella* in mice transplanted with systemic lupus erythematosus faeces as compared with CTR-CTR mice (Figure 8d). Interestingly, faecal bile acid 7α-dehydratase expression was also increased in the CTR-systemic lupus erythematosus group as compared with CTR-CTR (Figure 8e).

3.7 | Role of IL-17 in hypertension and vascular dysfunction induced by faecal microbiota transplantation from hypertensive NZBWF1 mice

To further analyse the involvement of IL-17 in the hypertensive effects of systemic lupus erythematosus microbiota, we administered nIL-17a to hypertensive mice from the CTR-systemic lupus erythematosus group. Treatment of these mice with nIL-17 significantly reduced SBP (≈12 mmHg) (Figure 9a) as well as improved both aortic endothelium-dependent relaxation to ACh (Figure 9b) and the activity of NADPH oxidase (≈28%) (Figure 9c). No significant differences were found in the ACh-induced relaxation between both groups when aortic rings were incubated with VAS2870 or Y27632 (Figure 9b).

4 | DISCUSSION

Interactions of gut microbiota and host genetics play important roles in the development of autoimmune diseases, such as systemic lupus erythematosus. We have demonstrated for the first time that gut microbiota is involved in the regulation of endothelial function and BP in female NZBWF1 mice. This is mainly supported by the improvement of endothelial dysfunction, vascular oxidative stress and the inhibition of the SBP increase induced by an important reduction in faecal biomass resulting from the chronic vancomycin or MIX treatments. In addition, morphological alterations of target organs in hypertension, such as left ventricle or kidney, were also prevented.

Previous studies have described that the oral administration of antibiotics attenuates BP in angiotensin II-induced hypertension and in spontaneously hypertensive rats (Yang et al., 2015). In our experiment, MIX or vancomycin administered to mice with genetic background of systemic lupus erythematosus partially prevented, to a similar amount, the raise in BP, suggesting that vancomycin-sensitive bacteria were the main responsible for BP increase. It is interesting to note that the principal component analysis of gut microbiota in normotensive (at 26 weeks) NZBWF1 mice and age-matched control mice showed clear differences in the microbial communities, suggesting that these changes could be involved in the increased protein excretion found in systemic lupus erythematosus mice at 26 weeks. This microbiota in normotensive NZBWF1 mice might induce changes in the host which starts the increase in BP. However, increased percentage of vancomycin-sensitive bacteria, such as *Parabacteroides*, *Pedobacter*, *Olivibacter* and *Clostridium*, were positively linked to high BP in systemic lupus erythematosus mice (at 32 weeks). Surprisingly, *Clostridium* correlates negatively with BP in Chinese patients (Zeng et al., 2019) and suppresses systemic inflammatory responses (Van den Abbeele et al., 2013). However, *Clostridium* XI and *Clostridium* XIVa express the bacterial enzyme bile acid 7α-dehydratase (Ridlon et al., 2006), involved in 7-α-dehydroxylation of taurocholic acid and cholic acid involved in the production of inflammatory bile acids (taurodeoxycholic acid and deoxycholic acid), which have been implicated in the impaired glucose metabolisms in mice on a high-fat diet (Fujisaka et al., 2016). We found increased bile acid 7α-dehydratase mRNA levels in faeces from hypertensive systemic lupus erythematosus mice, suggesting increased inflammatory bile acid production by systemic lupus erythematosus microbiota, which was abolished by both antibiotic treatments. Moreover, bile acid metabolites control host immune responses by directly modulating the balance of Th17 and Treg cells in the intestinal lamina propria (Hang et al., 2020), which have been described to be involved in BP regulation (Toral, Robles-Vera, de la Visitación, et al., 2019). Meanwhile, *Parabacteroides* produced metabolites to promote Treg

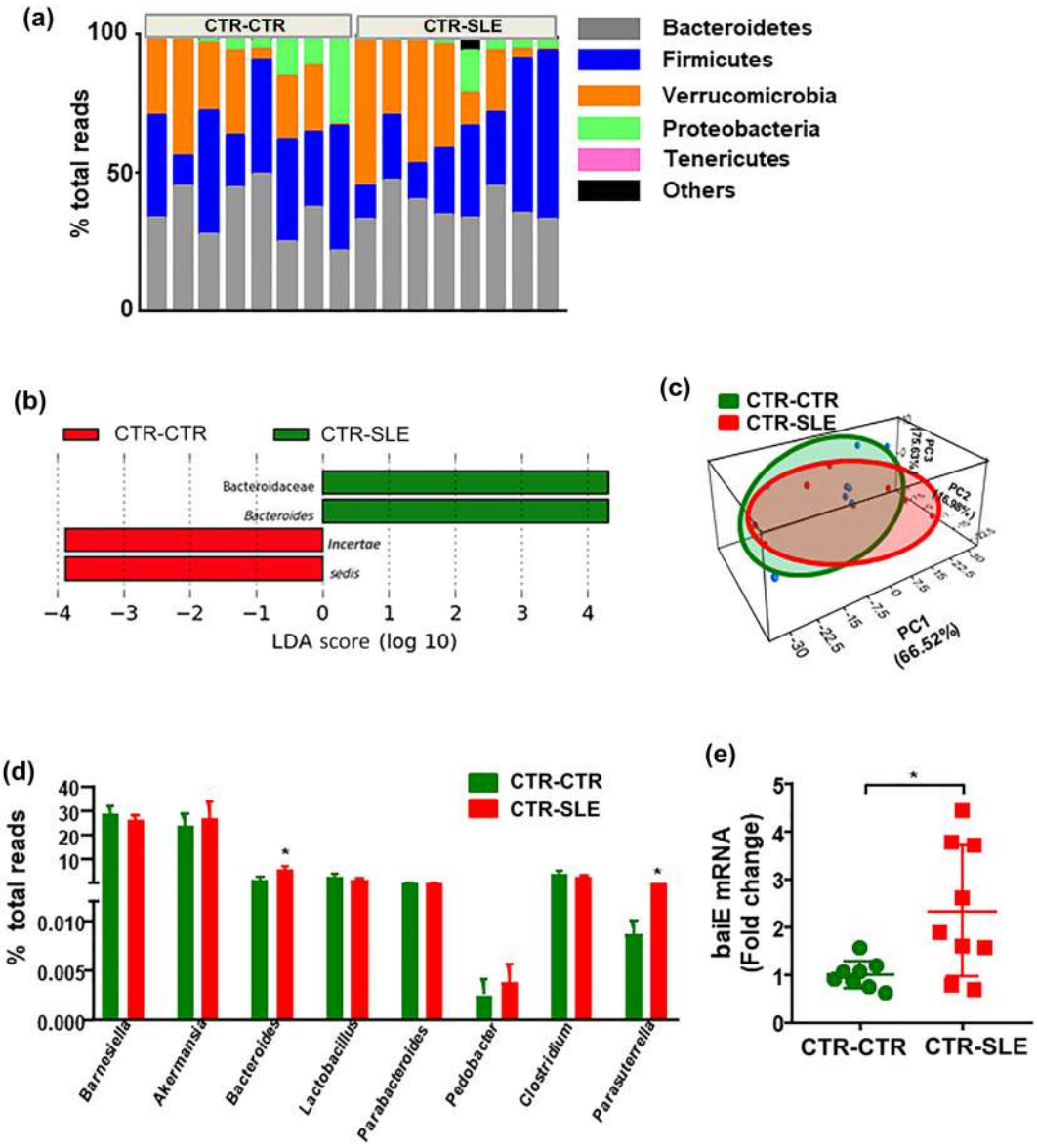


FIGURE 8 Composition of gut microbiota in mice when transplanted with control (CTR-CTR) and systemic lupus erythematosus (CTR-SLE) stools. (a) Proportion of bacterial phyla as percentages of the total population. (b) Linear discriminant analysis (LDA) effect size (LEfSe) identified significantly different bacterial taxa enriched in each cohort at LDA score >2, $P < 0.05$ (red bars, CTR-CTR enriched; green bars, CTR-SLE enriched). (c) Principal component analysis in the gut microbiota from all experimental groups. (d) Proportion of key bacterial species. (e) Bile acid 7α-dehydratase (baiE) mRNA expression levels in faeces. Values are expressed as means ± SEM. * $P < 0.05$ compared with the CTR-CTR group. CTR-CTR, C57Bl/6J mice transplanted with faeces from control mice; CTR-SLE, C57Bl/6J mice transplanted with faeces from hypertensive systemic lupus erythematosus mice

cell generation (Arpaia et al., 2013), which is in agreement with our results from mesenteric lymph nodes and spleen. Interestingly, vancomycin almost abolished *Bacteriodes* but increased Lactobacillaceae and *Lactobacillus* proportions in faeces, as compared with untreated systemic lupus erythematosus. *Lactobacillus* spp. could inhibit inflammation and decrease BP through its indole metabolites, which reduced Th17 populations in gut lymphoid organs (Wilck et al., 2017). However, we did not measure the bile acid profile and the content of indole metabolites in faeces, which limit the role of these mediators in the hypertensive effects of systemic lupus erythematosus microbiota. Furthermore, in faeces from hypertensive systemic lupus erythematosus, the proportion of *Lactobacillus* was similar to normotensive controls and MIX did not change it, despite reduced BP. This suggests that the effect of the gut microbiota on systemic lupus erythematosus hypertension is not the consequence of the change in the proportion on a specific bacterium.

The transplantation of intestinal microbiota from hypertensive systemic lupus erythematosus mice to recipient normotensive mice without genetic background of systemic lupus erythematosus disturbs the gut microbiota balance, possibly increasing the harmful bacteria

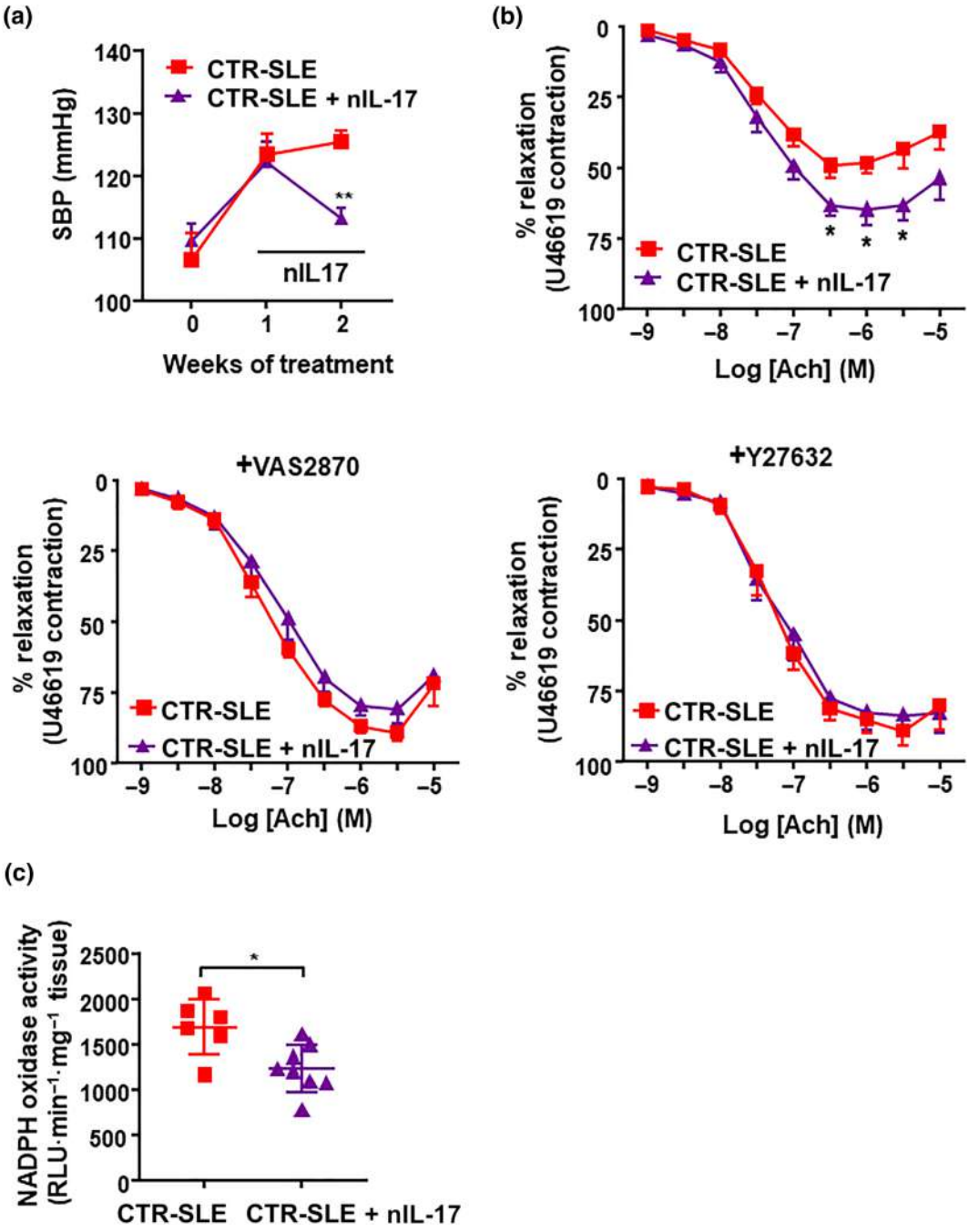


FIGURE 9 Effects of the neutralization of IL-17a (nIL-17) on systolic BP (SBP), endothelial function and NADPH oxidase activity in control mice when transplanted with systemic lupus erythematosus (CTR-SLE) stools. (a) SBP measured by tail-cuff plethysmography. (b) Vascular relaxation responses induced by ACh in endothelium-intact aortas precontracted by U46619 (10 nM) in all experimental groups: Control–SLE (CTR-SLE) and control–SLE treated with nIL-17 (CTR-SLE + nIL-17) and preincubated with VAS2870 (5 μM) or Y27632 (5 μM) 30 min before U46619 addition. (c) Aortic NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence. Values are expressed as means ± SEM ($n = 6–8$). * $P < 0.05$ compared with the CTR-SLE group

with prohypertensive effect, resulting in BP elevation. These results implied that the abnormal gut microbiota plays a key role in the development of hypertension and is one of the causes of systemic lupus erythematosus hypertension, rather than being the result and accompanying phenomena of lupus progression. The main harmful bacteria involved in BP elevation were *Bacteroides* and *Parasutterella*. Interestingly, an increased bile acid 7α-dehydratase expression was also found in control mice transplanted with systemic lupus erythematosus microbiota associated with high BP.

Several studies using female NZBWF1 mice have shown that a high number of factors play a role in the onset of hypertension besides B-cell hyperactivity and autoantibody production, such as

pro-inflammatory cytokines and oxidative stress (Taylor & Ryan, 2016). These mediators, which contribute to local inflammation and the associated renal and vascular dysfunction, are likely downstream of the initial immune system dysregulation (de la Visitación et al., 2019). In agreement with these data, we found in hypertensive systemic lupus erythematosus mice increased plasma levels of pro-inflammatory cytokines (IL-6, IL-17a and IFN-γ), vascular oxidative stress, increased circulating B cells and plasma anti-ds-DNA. Interestingly, both vancomycin and MIX reduced plasma levels of these cytokines and vascular ROS content, involving these mediators in the prohypertensive effects of microbiota in systemic lupus erythematosus mice. In our experiments, B-cell populations were higher in mesenteric lymph nodes and spleen from systemic lupus erythematosus mice as compared with control, but neither vancomycin nor MIX treatments reduced B-cell generation and circulating B cells, ruling out the involvement of B cells in the BP regulation induced by microbiota. Furthermore, stool transplantation from systemic lupus erythematosus mice to control mice did not increase the proportion of B cells in mesenteric lymph nodes. A direct function of autoantibodies in systemic lupus erythematosus was shown in patients with refractory hypertension, in which depletion of these autoreactive antibodies against the **α_1-adrenoceptor** was sufficient to decrease mean arterial pressure (Wenzel et al., 2008). Anti-ds-DNA production is regulated by microbiota in female NZBWF1 mice, as we showed that MIX reduced plasma levels of this autoantibody, similarly to chronic *L. fermentum* CECT5716 consumption (Toral, Robles-Vera, Romero, et al., 2019). However, in the present study the pathogenic role of anti-ds-DNA, as mediator of BP increase induced by microbiota, was not sufficiently supported by the data obtained. First, vancomycin reduced faecal mass and BP but did not modify plasma anti-ds-DNA. Second, stool transplantation from hypertensive systemic lupus erythematosus mice to control increased BP but did not increased circulating anti-ds-DNA.

Due to the importance of the kidney in the long-term control of BP, impaired renal function is certain to contribute to the prevalent hypertension in systemic lupus erythematosus patients and mice. In fact, in our study both antibiotic treatments reduced renal injury and BP simultaneously. Nevertheless, systemic lupus erythematosus hypertension can be present independently of nephritis (Petrin et al., 1993; Ward & Studenski, 1992). For instance, Shaharir et al. (2015) proved that 53% of systemic lupus erythematosus patients in one cohort were hypertensive even in the absence of nephritis. In agreement with these data, we found that urinary protein excretion was higher at 26 weeks in female NZBWF1 mice relative to control mice, but SBP was similar between both groups. Similarly, MRL/lpr mice present vasculitis and arthritis in addition to severe lupus nephritis, although they do not develop hypertension (Rudofsky et al., 1984). Interestingly, stool transplantation from hypertensive systemic lupus erythematosus mice increased BP without signs of renal inflammation or protein excretion, showing that effect in BP induced by this microbiota was independent of renal injury.

Hypertension is usually linked to endothelial function impairment, but whether this is causative in the progression of hypertension is difficult to demonstrate. Recently, we have shown an impaired aortic endothelium-dependent relaxation response to ACh in NZBWF1 mice (Gómez-Guzmán et al., 2014; Romero et al., 2017; Toral, Robles-Vera, Romero, et al., 2019) and a reduction in **NO** production induced by plasma from systemic lupus erythematosus patients with active nephritis in human endothelial cells (Toral et al., 2017). Increased NADPH oxidase-driven ROS production has been involved in both endothelial dysfunction and the raise of BP in female NZBWF1 mice (Gómez-Guzmán et al., 2014; Toral, Robles-Vera, Romero, et al., 2019). Consistent with this observation, in the present study, we also found that reduced ACh induced relaxation and increased NADPH oxidase activity in aorta from hypertensive systemic lupus erythematosus mice compared to normotensive control mice. Interestingly, chronic antibiotic treatments prevented the altered responses to ACh and the higher NADPH oxidase activity, indicating the involvement of gut microbiota in oxidative stress and endothelial function regulation. In agreement with this, stool transfer of microbiota from systemic lupus erythematosus to control mice or germ-free mice impaired ACh relaxation and increased NADPH oxidase activity. NADPH oxidase-driven ROS production is a highly relevant element in endothelial dysfunction induced by microbiota because incubation with the selective NADPH oxidase inhibitor VAS2870 suppressed the impairment of aortic endothelium-dependent relaxation to ACh. Circulating pro-inflammatory cytokines or that produced locally at vascular tissues, as consequence of inflammatory cell infiltration, regulate NADPH oxidase activity (Kelley & Wüthrich, 1999; Ryan, 2013; Toral, Robles-Vera, Romero, et al., 2019). Antibiotic treatments reduced Th17 differentiation in mesenteric lymph nodes, circulating Th17 and Th17 infiltration in aorta. The pro-inflammatory cytokine IL-17 causes Rho kinase-mediated endothelial dysfunction in the vascular wall (Nguyen et al., 2013), at least in part, by increasing ROS generation by NADPH oxidase activation (Pietrowski et al., 2011). In fact, the Rho kinase inhibitor Y27632 restored ACh relaxation to levels similar to those induced by the antibiotic, suggesting that the IL-17–Rho kinase pathway is controlled by gut microbiota in NZBWF1 mice. In agreement with this, faecal microbiota transplantation from systemic lupus erythematosus to control mice increased Th17 populations in mesenteric lymph nodes and Th17 infiltration in aorta. Moreover, incubation with nIL-17 improved ACh relaxation in control mice with systemic lupus erythematosus microbiota, showing that regulation of naïve T-cell differentiation towards Th17 in secondary lymph organs at the gut, with the subsequent Th17 infiltration at vascular tissues, is a key mechanism in the endothelial dysfunction induced by systemic lupus erythematosus microbiota. Interestingly, *in vivo* IL-17 neutralization in mice transplanted with systemic lupus erythematosus microbiota improved endothelial function and reduced BP, showing that IL-17 plays a key role in the prohypertensive effects induced by systemic lupus erythematosus microbiota.

In the vasculature, the activation of TLR-4 by the bacterial products, such as LPS, results in increased NADPH oxidase dependent O_2^- production and inflammation (Liang et al., 2013). In fact,

L. fermentum administration reduced plasma LPS levels with the subsequent reduction in the mRNA levels of TLR-4 and, consequently, improvement of vascular oxidative stress and inflammation in systemic lupus erythematosus (Toral, Robles-Vera, Romero, et al., 2019). We also found higher circulating levels of LPS in systemic lupus erythematosus mice, despite no increased gut permeability. Paradoxically, both vancomycin, which reduced gut permeability, and MIX were unable to reduce endotoxemia. These data could be related to the increased relative abundance of LPS-containing Gram-negative bacteria (Proteobacteria), induced by vancomycin and MIX treatments. Further studies are required to explain endotoxemia regulation in systemic lupus erythematosus mice. Interestingly, our results suggest that higher plasma LPS levels are not a key event in endothelial dysfunction and BP regulation induced by dysbiotic microbiota in NZBWF1 mice. This was also supported by stool transplantation from systemic lupus erythematosus mice to normotensive mice, which increased BP but not plasma LPS.

In conclusion, our study demonstrated that (i) gut microbiota is different in hypertensive systemic lupus erythematosus mice compared with age-matched controls, (ii) this gut microbiota drives a change in BP in the NZBWF1 as evidenced by antibiotic treatment and faecal microbiota transplantation and (iii) this is due to activation of pro-inflammatory Th17 cells. Neither plasma anti-ds-DNA nor plasma LPS were key mediators of the BP regulation induced by gut microbiota in systemic lupus erythematosus mice. Systemic lupus erythematosus is a female-biased disease with women getting disease nearly 9:1 over men. The results shown so far were obtained from female mice. Taken into account that there is some evidence to support that the gut microbiota is different according to sex (Beale et al., 2019), the involvement of gut microbiota in BP control in male mice should be analysed. The present results open new possibilities to the prevention of cardiovascular complications associated with systemic lupus erythematosus by the modulation of the gut microbiota composition. Nevertheless, caution should be taken when extrapolating these findings to humans due to the potential disparities between the features of animal and human microbiota.

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AUTHOR CONTRIBUTIONS

J.D. conceived and designed the research; N.d.l.V., I.R.-V, M.T., M.S., M.G.-G., R.J., J.M., C.G.-C., N.M.-M. and M.R. performed the experiments and analysed the data; M.R. and J.D. interpreted the results; I.R.-V, M.T., F.O. and R.J. prepared the figures; M.R. and J.D. drafted the manuscript; N.d.l.V., I.R.-V., M.T., R.J., F.O. and J.D. edited and revised the manuscript; and all authors approved the final version of manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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SUPPORTING INFORMATION

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