Title: Toll-like receptor 7 **(**TLR7)-driven lupus autoimmunity induces hypertension and vascular alterations in mice.

Short title: TLR7 activation promotes vascular damage.

Authors: Iñaki ROBLES-VERA[a,*], Néstor DE LA VISITACIÓN[a,*], Marta TORAL[b,c], Manuel SÁNCHEZ[a,d], Manuel GÓMEZ-GUZMÁN[a,d], Francisco O'VALLE[d,e], Rosario JIMÉNEZ[a,c,d], Juan DUARTE[a,c,d,f], Miguel ROMERO[a,d]

[a] Department of Pharmacology, School of Pharmacy, University of Granada,

[b]Gene Regulation in Cardiovascular Remodeling and Inflammation Group, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain.

[c]CIBER-Enfermedades Cardiovasculares (CiberCV), Spain.

[d] Instituto de Investigación Biosanitaria de Granada (ibs.GRANADA), Granada, Spain

[e] Department of Pathological Anatomy, School of Medicine, University of Granada, Granada, Spain.

[f] Centro de Investigaciones Biomédicas (CIBM), Granada. Spain.

* I.R.-V and N.d.l.V. contributed equally as first authors.

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Corresponding author: Juan Duarte. Department of Pharmacology, School of Pharmacy, University of Granada, 18071 Granada, Spain. Tel: (+34)-958241791, Fax: (+34)-958248264, Email: jmduarte@ugr.es

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Abstract

Objective: To investigate whether toll-like receptor 7 (TLR7) activation induces an increase in blood pressure and vascular damage in wild-type mice treated with the TLR7 agonist imiquimod (IMQ).

Methods: Female BALB/c mice (7-9 week-old) were randomly assigned to 2 experimental groups: an untreated control group and a group treated topically with IMQ (IMQ-treated) for 4 or 8 weeks. A group of IMQ-treated mice that take a combination of the antioxidants tempol and apocynin, and another treated IL-17-neutralizing antibody were also performed.

Results: TLR7 activation gradually increased blood pressure, associated with elevated plasma levels of anti-dsDNA autoantibodies, splenomegaly, hepatomegaly, and severe expansion of splenic immune cells with an imbalance between proinflammatory T cells and regulatory T cells. TLR7 activation induced a marked vascular remodeling in mesenteric arteries characterized by an increased media-lumen ratio (≈ 40%), and an impaired endothelium-dependent vasorelaxation in aortas from wild-type mice after 8 weeks of treatment. In addition, an increased ROS production, as a result of the upregulation of NADPH oxidase subunits, and an enhanced vascular inflammation were found in aortas from IMQ-treated mice. These functional and structural vascular alterations induced by IMQ were improved by antioxidant treatment. Anti-IL-17 treatment reduced blood pressure and improved endothelial dysfunction in IMQ-treated mice.

Conclusion: Our results demonstrate that TLR7 activation induces the development of hypertension and vascular damage in BALB/c mice, and further underscore the increased vascular inflammation and oxidative stress, mediated

in part by IL-17, as key factors contributing to cardiovascular complications in this TLR7-driven lupus autoimmunity model.

Key Words: vascular remodeling, endothelial dysfunction, hypertension, systemic lupus erythematosus, TLR7 activation.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a heterogeneous systemic autoimmune disease characterized by a dysregulation of innate and adaptive immune responses, with a loss of tolerance to nuclear self-antigens and an anomalous production of proinflammatory cytokines and multiorgan damage [1]. SLE is associated with an increased prevalence of kidney and cardiovascular diseases, which are the major causes of mortality in these patients, even after correction of the traditional risk factors [2, 3]. Hypertension is a well-recognized risk factor for the development of cardiovascular disease in SLE, as evidenced by the contribution of hypertension to the acceleration of atherosclerosis and arterial stiffening [4, 5]. In addition, numerous studies suggest direct detrimental effects on the endothelium during SLE, beyond the concomitance with traditional cardiovascular risk factors, including obesity, hypertension, dyslipidemia and diabetes mellitus [6-8]. Vascular inflammation and inflammatory cells infiltration are known to contribute to the pathogenesis of endothelial dysfunction in SLE, largely driven by immune dysregulation [9, 10]. Despite the high incidence of hypertension and vascular dysfunction in SLE patients, there are few studies directed at understanding the mechanisms of hypertension and endothelial dysfunction during SLE, due to the diversity in patient populations, severity of SLE and therapeutic strategies used to treat SLE [11, 12]. Currently available data regarding the pathophysiological mechanisms promoting the development of hypertension during SLE have focused on female NZBWF1 mice, a genetic mouse model of lupus nephritis accompanied with hypertension that develops similar features to human SLE [13-15]. Multiple lupus susceptibility loci have been identified in these mice, but

the contribution of these loci to hypertension in NZBWF1 mice is still not well understood.

Toll-like receptors (TLRs) are a family of innate pattern recognition receptors recognizing a wide range of pathogen-associated molecular patterns (PAMPs) and initiate innate immune responses [16]. Accumulating evidence supports the pathological role of TLRs signaling dysfunction in the induction and development of human and spontaneous murine models of SLE [17, 18]. Specifically, several recent studies have suggested that activation of endosomal TLR7 or TLR9 on dendritic cells and B lymphocytes is a critical step for lupus disease development in both humans and murine models; this process occurs through the induction of type I interferons (IFN) and other proinflammatory cytokines [19, 20]. However, an opposing relationship between TLR7 and TLR9 has been suggested as a potential mechanism regulating autoimmunity in SLE. In this sense, TLR7 activation has proven to be a key accelerator of lupus disease onset, whereas TLR9 plays a protective role in lupus-associated autoimmunity [21, 22].

Recently, a lupus model induced by epicutaneous application of the TLR7 agonist imiquimod (IMQ) has been described in several genetic backgrounds, including BALB/c mice [23]. TLR7 activation leads to several phenotypic and functional changes characteristic of human SLE, including elevated levels of autoantibodies and multiple organ involvement [23]. Although several studies have used TLR7 transgenic (TLR7tg) lupus-prone mice to elucidate the pathogenesis of SLE [24, 25], these genetically modified lupus models have disadvantages because of their polygenic (disease is driven by

multiple alleles, such as B6.RIIB(-/-)/yaa mice) or monoallelic nature (disease is driven by single alleles, such as B6. Yaa), thereby adding additional confounding factors to the effect of TLR7 activation alone. To date, no previous studies have evaluated changes in blood pressure in murine lupus induced by TLR7 activation. In addition, no studies have directly assessed whether alterations in vascular structure and function may contribute to raise blood pressure during lupus disease progression in this model. Therefore, the aim of the present study was to examine whether chronic topical application of IMQ in wild-type female BALB/c mice would induce an increase in blood pressure. We also aim to analyze whether these changes in blood pressure are associated with vascular alterations.

METHODS

Animals and Experimental Groups

Seven to nine weeks old female BALB/c mice, obtained from JANVIER (Le Genest, France), were randomly assigned to two experimental groups of 8-10 animals each: an untreated control (Ctrl) and a group treated with IMQ (IMQ-treated) for 4 or 8 weeks. A total of 1.25 mg of 5% IMQ cream (Aldara®) from Laboratories MEDA PHARMA SALU (Madrid, Spain) was administered topically on the right ears of IMQ-treated mice three times per week. We used female mice because their higher predisposition to TLR7-driven functional responses and autoimmunity [26]. Animals were maintained at a constant temperature (24º ± 1) with a 12-hour light/dark cycle in a specific pathogen-free environment and were provided with water and a standard laboratory diet (SAFE A04, Augy,

France) *ad libitum*.

In order to study the role of reactive oxygen species (ROS) we performed an experiment with two groups of IMQ-treated mice: a group taking drinking water (IMQ) and a group that take a combination of the antioxidants tempol (2.0 mmol/L) and apocynin (1.5 mmol/L) in the drinking water (IMQ+Antiox) [14].

In a separate experiment, we sought to determine whether IL-17 plays a role in hypertension induced by IMQ. Mice were assigned to 2 groups: IMQ-treated, and IMQ-treated plus IL-17-neutralizing antibody (IMQ+nIL-17). IP injections of either IL-17–neutralizing antibody (10 μg/rat; R&D Systems) or the isotype control (10 μg/rat; R&D Systems) were started on week 6 after IMQ treatment, and every 3 days [27].

All experimental protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animal and were approved by the Animal Care and Ethics Committee of the University of Granada (Spain; reference number: 68-CEEA-OH-2015).

Morphological variables

Body weight of mice was measured at the beginning and end of the treatment. The heart, kidney, spleen, and liver were collected and weighted. Then, the atria and the right ventricle were removed and the remaining left ventricle was also weighed. Finally, the cardiac, left ventricular, kidney, splenic and hepatic weight indices were calculated by dividing the weight of each organ by length of tibia. All tissue samples were frozen in liquid nitrogen and then stored at -80 ºC.

Blood pressure measurements

Systolic blood pressure (SBP) was measured every two weeks at room temperature, in the morning, in nonanesthetized, restrained mice (prewarmed for 10 to 15 min at 35°C) by tail-cuff plethysmography (digital pressure meter, LE5002, Letica SA, Barcelona, Spain). Briefly, animals were held in a plastic tube, and theirs tails were put through a rubber cuff, and the cuff was inflated with air. At least 7 determinations were made in every session, and the mean was taken as the SBP level. The animals were trained to stay in the tube for thirty minutes with the cuff and the transducer placed as shown, one week before the experiment [14]. At the end of the experimental period, mean arterial blood pressure (MABP) and heart rate (HR) were measured using continuous intra-arterial recording (MacLab; ADInstruments, Hastings, UK) in conscious mice under unrestrained conditions. MABP and HR values obtained during the last 30 min were averaged for intergroup comparisons [14].

Vascular reactivity studies

Descending thoracic aortic rings were mounted in a wire myograph (model 610M, Danish Myo Technology, Aarhus, Denmark) for isometric tension measurement as previously described [15]. The organ chamber was filled with Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2, KH_2PO_4 1.2 and glucose 11) at 37 ºC and gassed with 95% O_2 and 5% CO_2 (pH 7.4). Based on the length–tension characteristics obtained via

the myograph software (Myodaq 2.01), aorta arteries were maintained at a resting tension of 0.5 g.

Endothelium-dependent relaxation to acetylcholine (Ach, 1 nM -10 μM) was investigated in intact rings precontracted with the thromboxane A_2 analog U46619 (10 nM) in the absence or in the presence of the endothelial nitric oxide (NO) synthase (eNOS) inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME, 100 μM), or the non-selective nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor apocynin (10 μM). Both L-NAME and Apo were added 30 min before of U46619. In another experimental set, endothelium-independent relaxant responses to sodium nitroprusside (SNP, 0.1 nM - 1 μM) were studied in the dark in endothelium-denuded vessels precontracted with U46619 (10 nM). Relaxant responses to Ach and SNP are expressed as a percentage of precontraction induced by U46619.

In situ detection of vascular ROS levels and NADPH oxidase activity

Ex vivo vascular ROS content was measured using the oxidative fluorescent dye dihydroethidium (DHE) in aortic segments as previously described [14]. Briefly, unfixed thoracic aortic rings were cryopreserved (0.1 M PBS plus 30% sucrose for 1-2 h), embedded in optimum cutting temperature compound (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan) and frozen (-80 ºC) until use. Cross-sections of 10 μm thickness were obtained in a cryostat (Microm International Model HM500 OM) and incubated for 30 min in HEPES buffer solution, containing DHE (10 μM), counterstained with the nuclear stain 4,6-diamidino-2-phenylindoledichlorohydrate (DAPI, 30 nM). In the following 24 h,

sections were examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany) and photographed. Ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/). ROS production was estimated from the ratio of ethidium/DAPI fluorescence.

NADPH oxidase activity in intact aortic rings was determined using the lucigenin-enhanced chemiluminescence assay as previously described [14]. Briefly, aortic rings from all experimental groups were incubated for 30 minutes at 37 °C in HEPES containing a physiological salt solution (pH 7.4) with 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. Then, rings were placed in tubes containing the physiological salt solution, with or without NADPH (100 μM) to stimulate aortic production of superoxide anions (O_2^-) and lucigenin was injected automatically at a final concentration of 5 μM. 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 calculated by subtracting the basal values from those values found in the absence of NADPH. The vessels were then dried, and the dry weight was determined. NADPH oxidase activity was expressed as relative luminescence units (RLU) per min per mg of dry aortic tissue. In another set of experiments, the NADPH stimulated ROS production in homogenates from mesenteric arteries was also measured by DHE fluorescence in a microplate reader as previously described [28].

Histopathological analysis

Sections of the kidney and the superior mesenteric artery were obtained from each mouse by dissection. Sections were immersed in free-calcium Krebs solution for 30 min. Then, sections were fixed in 10% buffered formalin for 24 hours, dehydrated in graded ethanol solutions and embedded in paraffin.

For each artery, a series of four 5 μm cross sections were obtained with a precision microtome (Microm International Model HM500 OM). These sections were stained with hematoxylin-eosin to visualize the structure of the vascular wall. Arterial media thickness (MT), lumen diameter (LD), media cross-sectional area (MCSA) and media-lumen ratio (M/L) were measured as previously described [29] using a computer equipped with a Leica Q500MC image analyzer connected to a video camera of a Leica Leitz DMRB microscope (Leica, Wetzlar, Germany). For the evaluation of kidney histopathology, 4 μm sections were cut along the central axis of the biopsies. Then, samples were dewaxed and rehydrated for staining with hematoxylin-eosin, periodic acid-Schiff and Masson's trichrome. A morphological study on light microscopy was performed in a blinded fashion (MR and FO) and the presence of SLE-like lesions was studied. Glomerular cellularity (proliferation) was evaluated by counting the number of nuclei per glomerular cross section (20 glomerular cross sections per mouse) [30]. We considered proliferative glomeruli when the number of cells were >30.

Evaluation of kidney injury

Protein determination in urine samples collected for 24 hours was used to assess kidney injury [31]. For this purpose, mice were housed in metabolic

cages at the end of the experimental period to collect urine samples. Proteinuria was determined using bovine serum albumin as standard. Results were expressed as mg of protein excreted, normalized by body weight (per 100 g of mice) and time (during 24 h). The mean values obtained during the 2 experimental days were used for statistical comparisons between groups.

Plasma determinations

Blood samples were cooled on ice and centrifuged for 10 min at 3,500 rpm at 4 ºC, and the resulting plasma was frozen at -80 °C. Plasma anti-double-stranded DNA (anti-dsDNA) autoantibodies and IFN-α levels were determined using a commercial ELISA kit according to the manufacturer's instructions (Alpha Diagnostic International, San Antonio, TX, and Thermo Fisher Scientific, Madrid, Spain, respectively). Plasma cytokines were measured using a multiplex assay using Luminex ™ xMAP technology (Thermo Fisher Scientific, Madrid, Spain).

Flow Cytometry

Spleens were collected from all groups. The tissues were smashed very well with wet slides to decrease friction. Then the obtained solutions were filtered through a 70 µm cell strainer. Cells were isolated. Then, red blood cells were lysed with Gey's solution. A total of 10^6 cells were counted and blocked with anti-CD3 (clone17A2, eBioscience) and anti-CD28 (clone 37.51, eBioscience) for 30 minutes at 37°C to avoid non-specific binding to Fc-gamma receptors. After 4 hours, cells were incubated with a protein transport inhibitor (BD GolgiPlugTM)

for an optimum detection of intracellular cytokines by flow cytometry. Then, the cells were transferred to polystyrene tubes for the surface staining with mAbs anti-CD4 (PerCP-CyTM, clone RM4-5 BD PharmigenTM), anti-B220 (APC, clone RA3-6B2, BD PharmigenTM) and with viability dye (LIVE/DIED® Fixable Aqua Dead cell Sain Kit, Molecular Probes, Oregon, USA) for 20 min at 4°C in the dark. The lymphocytes were then fixed, permeabilized with the Fix/Perm Fixation/Permeabilization kit (eBioscience, San Diego, USA) and intracellular staining was achieved with mAbs anti-Foxp3 (PE,clone FJK-16s, eBioscience, San Diego, USA), anti-IL-17A (PE-Cy7, clone eBio17B7, eBioscience, San Diego, USA) and anti-IFN-γ (Alexa Fluor® 647, DB-1, 6B2 BD PharmigenTM, New Jersey, USA) for 30 min at 4°C in the dark. All samples were analyzed using a flow cytometer CANTO II (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR, USA) [15].

Gene expression analysis

The analysis of gene expression in aorta and mesenteric arteries was performed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, as previously described [14, 32]. For this purpose, total RNA was extracted by homogenization using TRI Reagent® following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) and 2 µg of RNA were reverse transcribed using oligo(dT) primers (Promega, Southampton, UK). Polymerase chain reaction was performed with a Techne Techgene thermocycler (Techne, Cambridge, UK). The sequences of the sense and antisense primers

used for amplification are described in **Table S1.** The efficiency of the PCR reaction was determined using a dilution series of standard vascular samples. To normalize mRNA expression, the expression of the housekeeping genes GAPDH and RPL13 were used. The mRNA relative quantification was calculated using the $\Delta\Delta$Ct method.

Western blotting analysis

A total of 40 µg of protein per lane from aortic homogenates were run on a sodium dodecyl sulphate (SDS)-polyacrylamide electrophoresis. Then, proteins were transferred to polyvinylidene difluoride membranes (PVDF), incubated overnight with primary mouse monoclonal anti-eNOS antibody (Transduction Laboratories, San Diego, California, USA) and with the correspondent secondary peroxidase conjugated antibody. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK). A densitometric analysis was performed using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com) as previously described [14, 32]. Samples were re-probed for expression of smooth muscle α-actin.

Reagents

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

Statistical analysis

Results are expressed as means ± SEM of measurements. The evolution of tail SBP over time was compared using the nested design. The fixed factors were treatment and number of weeks, while the mouse was the random factor. When the overall difference was significant, Bonferroni's method with an appropriate error was used for comparisons. Analysis of the nested design was also performed with groups and concentrations to compare the concentration-response curves to ACh. The remaining variables were tested on normal distribution using Shapiro-Wilk normality test and compared using one-way ANOVA and Tukey *post hoc* test. Differences of at least $P<0.05$ were considered statistically significant. Graph Pad Prism 7 software was used for the statistical analyses.

RESULTS

Blood pressure, target organs damage and systemic inflammation are increased in lupus induced by TLR7 activation.

BALB/c mice exposed to IMQ showed a high incidence of death rate (approximately 20%, **Fig. 1A)** and a profound weight gain compared to control animals (final weight: 24.10 ± 0.62 g and 21.89 ± 0.54, respectively, $p<0.05$) at 8 weeks of treatment, as previously reported in the original description of this model [23]. The gonadal and mesenteric fat weight indices were reduced in IMQ-treated mice **(Table 1)** in spite of the weight gain observed which appears to be related to the development of edema and swelling instead. IMQ-treated mice also displayed a progressive increase in SBP **(Fig. 1B)** and in the final MABP **(Fig. 1C)** measured by tail-cuff plethysmography and direct recordings,

respectively, being approximately 20 mmHg higher in IMQ-treated mice than in control mice at the end of the experiment. No significant changes in HR were induced by IMQ treatment (**Fig. 1D)**. In addition, both heart weight/tibia length and left ventricular weight/tibia length were higher in IMQ-treated mice than in control mice at 8 weeks of treatment **(Table 1)**, whereas renal hypertrophy and hepatomegaly were found from 4 weeks of IMQ treatment **(Table 1)**.

The IMQ-induced lupus model is characterized by kidney injury associated with increased plasma levels of autoantibodies in wild-type mice [22, 23]. As expected, we found significant higher plasma levels of anti-dsDNA autoantibodies (**Fig 2A)** and kidney injury (**Fig. Suppl. 1)** in IMQ-treated mice compared with control mice. We also found a marked splenomegaly in IMQ-treated mice (**Fig 2B)**, which was associated with autoimmune disease progression. In addition, a reduced clearance of apoptotic cells is associated with progressive lupus-like autoimmune disease and increased production of autoantibodies [33, 34]. Opsonins, such as C1q, thrombospondin-1 and milk fat globule-epidermal growth factor-8, are proteins released by macrophages enhancing the recognition and phagocytosis of apoptotic cells by macrophages. The primary source of circulating opsonins in the serum is the liver resident macrophages, Kupffer cells [35]. Thereby, we investigated the opsonin expression in the livers of mice from all experimental groups. We found that activation of TLR7 by a topical administration of IMQ reduced hepatic gene expression of opsonins only in IMQ-treated mice compared with control mice only after 8 weeks of treatment (**Fig. 2C)**.

Finally, we evaluated the immunomodulatory actions of TLR7 activation

by measuring the number of total cells and the levels of B and T cells in spleens from all experimental groups. IMQ treatment led to an increase in splenocyte numbers and in the percentages of both splenic B cells and T cells from 4 weeks of IMQ treatment compared to the control group **(Fig. 3A-C).** Specifically, the percentages of Th1 and Th17 cells were significantly increased only at 8 weeks of treatment, whereas percentage of Treg cells was reduced in splenocytes from all experimental groups **(Fig. 3D-F)**. Representative flow cytometry of spleens is shown in the supplemental data (**Fig. Suppl. 2)**. Besides, plasma levels of IFN-α, IFN-γ, IL-21, TNF-α, IL-6, and IL-17 were also increased in IMQ-treated mice at 8 weeks compared to control group (**Fig. Suppl. 3)**. Taken together, these results suggest that TLR7 activation by topical application of IMQ results in systemic inflammation at 8 weeks of treatment, being these results consistent with the previously described findings [22, 23, 36].

Lupus-induced by TLR7 activation promotes vascular remodeling and endothelial dysfunction.

Structural alterations in resistance arteries may be considered an important contributing factor to the pathogenesis of hypertension in humans and animal models [37, 38]. Here, we found that topical administration of IMQ is associated with structural alterations in superior mesenteric arteries characterized by a significant smaller LD and an increase in MT and M/L (≈ 40%) in BALB/c mice after 8 weeks (**Fig. 4A-D**). However, non-significant changes in MCSA were found in arteries from IMQ-treated mice at any time

(**Fig. 4E**).

Similarly, SLE hypertension is known to be associated with an impaired vascular function [13, 15, 39]. For that reason, we determine whether endothelial-dependent relaxation and contraction are altered in this model. We found that aorta from IMQ-treated mice showed strongly reduced endothelium-dependent vasodilator responses to ACh only after 8 weeks of topical treatment (maximal effect, 60.35 ± 2.69% *versus* 79.34 ± 1.79% in the control group; $P<0.001$) (**Fig. 5A**). The incubation for 30 minutes with the eNOS inhibitor L-NAME abolished the relaxant response induced by ACh in all experimental groups, involving NO in this relaxation (**Fig. 5B**). Moreover, no differences were found in the endothelium-independent relaxant response to the NO donor sodium nitroprusside in aortic rings from both control and IMQ-treated groups, excluding changes in the sensitivity of the NO-cGMP pathway in vascular smooth muscle cells (**Fig. 5C**). In addition to examining endothelial-dependent relaxation, we also tested whether there are changes in vessel contractility that might contribute to endothelial dysfunction. No differences were found among all experimental groups in the contractile response induced by U46619 in both intact aortic rings and in the presence of L-NAME (**Table S2**). In addition, since endothelial-dependent relaxation was progressively impaired in IMQ-treated mice, we only evaluated whether eNOS expression was altered at 8 weeks of IMQ treatment. Aortic eNOS mRNA levels (**Fig. 5D**) and protein expression (**Fig. 5E**) were reduced in IMQ-treated mice compared to control mice.

Finally, we also found that these changes in vascular structure and

function are associated with a marked increase in vascular expression of TLR7 mRNA in aorta and mesenteric arteries from IMQ-treated mice, whereas vascular TLR9 mRNA expression was unaffected (**Fig. Suppl. 4**).

Vascular oxidative stress and inflammation are increased in lupus induced by TLR7 activation.

Reactive oxygen species (ROS), particularly O_2^-, play an important role in vascular tone and structure, contributing to pathological mechanisms related to endothelial dysfunction, arterial remodeling and vascular inflammation [40]. Our results showed that aortic rings from IMQ-treated mice display a marked increase in red ethidium fluorescence staining of vascular wall compared to the control group (**Fig 6A**), suggesting an increased vascular ROS production. Moreover, the activity of the NADPH oxidase, considered the major source of O_2^- in the vascular wall, was also markedly increased in aorta from IMQ-treated mice at 8 weeks of treatment compared to the control mice (**Fig. 6B**), which was correlated with significant mRNA increase of its catalytic subunits NOX2, $p22^{phox}$ and $p47^{phox}$ (**Fig 6C**). Likewise, the effects of the non-selective NADPH oxidase inhibitor apocynin in endothelium-dependent relaxation to ACh were analyzed to evaluate the role of NADPH oxidase-driven ROS production in the impaired relaxation to ACh in aorta from IMQ-treated mice. No significant differences were found between groups after incubation with apocynin, suggesting that an increased NADPH oxidase activity is involved, at least in part, in the endothelial dysfunction found in aorta from IMQ-treated mice (**Fig 6D**).

Additionally, we measured the activity of the NADPH oxidase and the mRNA expression of its catalytic subunits in mesenteric arteries to determine the role of NADPH oxidase-derived ROS on vascular remodeling. A significant increased NADPH oxidase activity and NOX2, $p22^{phox}$ and $p47^{phox}$ mRNA overexpression were found in mesenteric arteries homogenates from IMQ-treated mice compared to the control group **(Fig. Suppl. 5)**. Incubation with polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) or Tiron for 30 min abolished this increased NADPH oxidase activity, involving O_2^- as the main ROS contributing to the vascular remodeling in these mice.

Furthermore, given the key role played by proinflammatory cytokines and chemokines in the pathogenesis of vascular remodeling and endothelial dysfunction in SLE [9, 41], we also analyzed the transcript level of vascular adhesion molecules and proinflammatory cytokines in aorta and mesenteric arteries homogenates from control and IMQ-treated mice. We found a higher mRNA expression of vascular cell adhesion molecule-1 (VCAM-1) and the proinflammatory cytokines IFN-α, IFN-γ, IL1β, IL-6 and IL-17, whereas mRNA expression of IL-10 and TFG-β were reduced in aorta (**Fig. 7**) and mesenteric artery **(Fig. Suppl. 6)** homogenates from IMQ-treated mice compared to control mice. These results are correlated with the increased systemic inflammation and the changes in splenic lymphoid cell populations induced by IMQ described above.

Role of ROS in cardiovascular alterations induced by TLR7 activation

Antioxidant treatment prevented the raise of SBP (**Fig. 8A**), and the

vascular remodeling, reducing M/L ratio, MT, and MCSA, and increasing LD in mesenteric arteries from IMQ-treated mice (**Fig. 8B-F**). In addition, both the impaired endothelium-dependent relaxation to Ach (**Fig. 8G**) and the higher NADPH oxidase activity (**Fig. 8H**) found in aorta from IMQ-treated mice, were also improved by antioxidant treatment.

Involvement of IL-17 in hypertension and vascular dysfunction induced by TLR7 activation

To further analyse the participation of IL-17 in the hypertensive effects of IMQ, we administered nIL-17a to hypertensive mice. Treatment of these mice with nIL-17 significantly reduced SBP (**Fig. 9A**) as well as improved both aortic endothelium-dependent relaxation to acetylcholine (**Fig. 9B**) and the activity of NADPH oxidase (**Fig. 9C**).

DISCUSSION

We demonstrate, for the first time to our knowledge, that TLR7 activation by epicutaneous application of IMQ results in a gradual increase in arterial blood pressure, which is likely associated with autoimmune disease progression as evidenced by elevated plasma levels of anti-dsDNA autoantibodies, splenomegaly and hepatomegaly, and severe expansion of splenic immune cells with enhancement of lymphocyte polarization towards a proinflammatory phenotype. Moreover, our present results show a marked hypertrophic effect on target organs for high blood pressure, such as heart and kidney. Additionally,

and as a novel finding, the activation of TLR7 by IMQ also induces vascular remodeling in resistance arteries and endothelial dysfunction in aortas from wild-type mice. These vascular alterations seem to be related to a loss of NO bioavailability and to an increase in ROS production, as a result of enhanced NADPH oxidase activity and an enhanced vascular inflammation.

Recently, TLR7 activation by topical application of IMQ has shown to induce severe glomerulonephritis and kidney injury associated with elevated autoantibodies generation [22, 23, 36]. In our experiment, we found increased plasma levels of anti-dsDNA autoantibodies in IMQ-treated mice, which are correlated with albuminuria and morphological alterations in kidney cortex from these mice, which were significant after 4 weeks of IMQ treatment. Interestingly, our results showed a severe expansion of splenic immune cells in association with lymphocyte polarization to a proinflammatory phenotype. Of note, we observed an imbalance between T helper cell subtypes (Th1 and Th17) and regulatory T cells (Treg) in spleen from IMQ-treated mice, with a predominance of the former. These results are consistent with previous evidences showing that TLR7 activation by IMQ is associated with autoimmune disease progression because of a lymphoproliferative disorder as a result of abnormalities in B-cell activation and enhanced T helper-1 (Th1)-type immune responses, at least partially attributable to a defect in immunosuppressive Treg function [22, 42].

Furthermore, a defective clearance of apoptotic cells is also associated with a progressive lupus-like autoimmune disease and an increased production of autoantibodies [33, 34]. Notably, an impaired ability to engulf apoptotic cells

has been demonstrated in both C1q-deficient humans and mice, which was related to a higher risk to develop SLE [43, 44]. Here, we found a reduced expression of hepatic opsonins only in IMQ-treated mice compared with control mice. This result suggests that the activation of TLR7 causes a deficiency in the clearance of apoptotic cells which, together with the increased percentage of splenic B cells and plasma levels of IL-21, might contribute to the exacerbation of the anti-dsDNA autoantibodies found in IMQ-treated mice, being these results consistent with those previously described [34, 45].

Previous studies have shown that autoimmune disease progression and the resultant increase in systemic inflammation and kidney damage are important underlying factors involved in the development of hypertension and vascular dysfunction associated with SLE [2, 3, 46]. In fact, it has been shown that preventing autoimmunity with immunosuppressive therapy attenuates lupus disease progression and protects against the development of hypertension [47, 48]. Our results demonstrate for the first time a progressive increase in blood pressure induced by TLR7 activation in IMQ-treated mice, without changes in HR. Moreover, hypertensive IMQ-treated mice developed cardiac and renal hypertrophy. However, currently available data do not allow establishing a direct relationship between glomerulonephritis and increased blood pressure in both murine models and patients with SLE [46, 49, 50]. In our study, we found that the increase in blood pressure begins after the development of proteinuria, suggesting that early kidney injury may contribute to the evolution of hypertension during lupus disease progression induced by TLR7 activation.

Numerous studies suggest that the presence of vascular remodeling in resistance arteries and endothelial dysfunction are crucial in the pathogenesis of SLE hypertension in humans and animal models [13, 14, 39, 41]. In the present study, we found that TLR7 activation induced a significant M/L ratio increase as a result of a reduced LD and an increased MT in superior mesenteric arteries from IMQ-treated mice, observed only at 8 weeks of treatment.

Recently, Liu et al. (2018) have also found endothelial dysfunction triggered by IMQ treatment in wild-type mice [22]. Importantly, we also demonstrate that IMQ-treated mice display significant impaired endothelium-dependent vasorelaxation compared to control mice. Endothelial dysfunction is characterized by an impaired NO availability and a concomitant increase in the generation of ROS [51]. It is well known that NO secretion is required for normal endothelium-dependent vasodilatation, and a deficiency of eNOS function in endothelial cells have been reported to happen in both murine models and humans with SLE [15, 52]. We found that endothelium-dependent relaxations induced by ACh were abolished by eNOS inhibition with L-NAME, suggesting a defect in NO pathway in IMQ-treated mice. However, no significant changes in vasodilator response to the NO donor nitroprusside were found in all experimental groups, suggesting that sensitivity to the NO-cGMP pathway in vascular smooth muscle cells was unaltered.

It is well established that vascular inflammation and inflammatory cells infiltration are contributing factors to the pathogenesis of vascular dysfunction in SLE, largely driven by immune dysregulation [9, 10]. Previous studies have

shown that TLR7 activation causes an increase in circulating proinflammatory cytokines in both wild-type and TLR9-deficient mice. These cytokines are potentially involved in the pathogenesis of lupus and its deleterious effects in the vasculature [22, 23]. Accordingly, we also found increased plasma levels of the proinflammatory cytokines IFN-α, IFN-γ, IL-21, TNF-α, IL-6 and IL-17 in IMQ-treated mice, which were correlated with changes in lymphoid cell populations found in the spleen of these mice. High concentrations of proinflammatory cytokines downregulate eNOS and induce oxidative stress [53, 54]. Interestingly, we found a progressive decrease in eNOS mRNA levels and protein expression in aorta from IMQ-treated mice. Although our study is limited due to the lack of measurements of direct NO release and eNOS activity, our results suggest that changes in eNOS expression might contribute, at least partially, to the loss of NO production. In fact, a recent study have described that IFN-α negatively regulates the expression of eNOS and NO production in endothelial cells [55].

Oxidative stress is an important contributor to vascular remodeling and endothelial dysfunction associated with SLE, acting as mediator of the link between autoimmune responses and vascular inflammation [41, 54]. Our results show an increased production of ROS in both aorta and mesenteric arteries from IMQ-treated mice. This ROS production is correlated with significant NADPH oxidase activity as a result of the overexpression of its catalytic subunits NOX2, $p22^{phox}$ and $p47^{phox}$. Our results clearly demonstrated that this exacerbated NADPH oxidase-driven ROS production seems to be a key event in the development of vascular structural alterations and endothelial dysfunction

induced by TRL7 activation. This was supported by i) the incubation with the NADPH oxidase inhibitor apocynin increased the aortic endothelium-dependent relaxation to ACh in IMQ-treated mice up to a level similar to that found in the control group, and ii) chronic antioxidant treatment prevented vascular remodeling and endothelial dysfunction in IMQ-treated mice. Furthermore, NADPH oxidase-derived ROS may be a key component in the upregulation of TLRs in the vasculature [56]. Accordingly, our results show a marked increase in vascular TLR7 mRNA expression in aortas from IMQ-treated mice, while vascular TLR9 mRNA expression was unaffected.

In addition, TLR7 activation by IMQ stimulates the release of proinflammatory cytokines and chemoattractant proteins, which have an impact on the development of premature atherosclerosis in SLE [57]. According to these previous results, we observe a marked increase in the mRNA expression of VCAM-1 and proinflammatory cytokines IFN-α, IFN-γ, IL-6, IL1β and IL-17 in homogenates of aortic and mesenteric arteries from IMQ-treated mice, whereas the mRNA expression of IL-10 and TFG-β is reduced. These cytokines regulate vascular ROS levels, vascular structure and tone and blood pressure. In fact, low splenic Treg and low vascular IL-10 level were associated with angiotensin II-induced endothelial dysfunction and hypertension in mice [58, 59], similarly to that found in IMQ-treated mice. IL-17 plays a key role in vascular dysfunction induced by TLR7 activation since nIL-17 injection decreased aortic NADPH oxidase activity, improved endothelial dysfunction and reduced SBP in IMQ-treated mice. Postnatal smooth muscle cells-specific deletion of TGF-β type II receptor is reported to cause the rapid thickening of the thoracic aorta [60].

Thus, reduced TFG-β mRNA levels found in mesenteric arteries from IMQ-treated mice might be also involved in the structural modification induced by TLR7 activation. Therefore, the detrimental vascular effects of TLR7 activation by IMQ might be the result of the increase in plasma cytokines and direct effects on the vasculature through increasing oxidative stress and vascular inflammation via an imbalance between proinflammatory and inhibitory cytokines.

In conclusion, our results demonstrate for the first time that TLR7 activation by chronic topical application of IMQ induces a progressive increase in blood pressure and vascular structural and functional changes in wild-type mice. In addition, our results highlight the increased vascular oxidative stress, mediated at least in part by IL-17, as key molecular mechanisms contributing to cardiovascular complications in this TLR7-driven lupus autoimmunity model. Therefore, our findings have highlighted the potential importance of TLR7 signaling in SLE hypertension and vascular dysfunction, future studies should focus on TLR7 as a therapeutic target. However, we did no use male mice, which limit the pathophysiological relevance of our study.

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Authors' contributions

M.R. and J.D. participated in the research design; M.R., I.R.-V., N.d.l.V., M.T., M.S., M.G.-G. and R.J. performed most of the experiments; F.O.; M.R. and J.D. performed the histopathological analysis; M.R., I.R.-V., N.d.l.V., M.T., R.J., and J.D. contributed to data analysis and interpretation. M.R. and J.D. wrote or contributed to the writing of the manuscript.

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TABLES

Table 1. Morphological parameters.

Variables	Ctrl 4W (n=8)	IMQ 4W (n=8)	Ctrl 8W (n=8)	IMQ 8W (n=8)
BW (g)	21.54±0.61	21.75±0.64	21.89±0.54	24.10± 0.62 ** †
TL (mm	20.61±0.19	21.50±0.40	21.11±0.14	21.13± 0.30
HW/TL (mg/cm)	4.56±0.16	4.71±0.14	4.61±0.11	5.25± 0.24 * †
LVW/TL (mg/cm)	2.93±0.07	2.91±0.08	2.80±0.06	3.17± 0.12 * †
KW/TL (mg/cm)	5.36±0.06	5.89±0.21 *	5.20±0.08	6.83± 0.43 ** †
LW/TL (mg/cm)	42.35±2.49	67.15±2.29 **	39.29±1.42	86.26± 5.88 ** ††
Spleen/TL (mg/cm)	4.50±0.17	24.04±2.00 **	4.70±0.29	36.18± 3.63 ** †
Mesenteric fat/BW (%)	0.42±0.03	0.39±0.04	0.38±0.02	0.21±0.03 ** ††
Gonadal fat/BW (%)	2.04±0.18	1.89±0.18	1.80±0.16	0.89±0.07 ** ††

Results are shown as mean ± SEM. *P < 0.05 and **P < 0.01 *vs*. control group; †P < 0.05 and † † P < 0.01 *vs*. IMQ 4 weeks group. BW, Body weight; TL, Tibia length; HW, Heart weight; LVW, Left ventricular weight; KW, Kidney weight; LW, Liver weight.

FIGURE LEGENDS

Figure 1. TLR7 activation promotes blood pressure increase in imiquimod (IMQ)-induced autoimmunity. (A) Cumulative survival rate of BALB/c mice following topical application of IMQ on their right ears three times per week for 8 weeks. (B) Time course of systolic blood pressure (SBP) measured by tail-cuff plethysmography was determined in control (Ctrl) and IMQ-treated mice. (C) Mean arterial blood pressure (MABP) and (D) heart rate (HR) measured by direct recording in left carotid artery at the end of the experimental period. Experimental groups: Ctrl 4 weeks (W) (n=8), Ctrl 8W (n=8), IMQ 4W (n=8), IMQ 8W (n=8). Values are represented as Mean ± SEM. Results were compared by 1-way ANOVA and Tukey *post hoc* test. **P<0.01, ***P<0.001 compared to their respective control groups.

Figure 2. TLR7 activation leads to higher levels of serum autoantibodies anti-dsDNA, marked splenomegaly and altered clearance of apoptotic cells in imiquimod (IMQ)-treated mice. (A) Circulating double-stranded DNA autoantibodies, (B) splenomegaly, and (C) mRNA levels of hepatic opsonins measured by reverse transcriptase-polymerase chain reaction were assessed in control (Ctrl) and IMQ-treated mice. Experimental groups: Ctrl 4 weeks (W) (n=8), Ctrl 8W (n=8), IMQ 4W (n=8), IMQ 8W (n=8). Results were compared by 1-way ANOVA and Tukey *post hoc* test. Values are represented as Mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to their respective control groups.

Figure 3. TLR7 activation induces an increase in splenic B cells and T cells and promotes T-cell polarization to proinflammatory phenotype. (A) Number of total cells, (B) percentage of B cells and (C) percentage of T cells were measured by flow cytometry in spleens from imiquimod (IMQ)-treated mice. (D) Percentage of Th1, (E) Th17 and (F) Treg cells measured by flow cytometry were assessed in control (Ctrl) and IMQ-treated mice. Experimental groups: Ctrl 4 weeks (W) (n=8), Ctrl 8W (n=8), IMQ 4W (n=8), IMQ 8W (n=8). Results were compared by 1-way ANOVA and Tukey *post hoc* test. Values are represented as Mean ± SEM. *P<0.05, ***P<0.001 compared to their respective control groups.

Figure 4. TLR7 activation promotes vascular remodeling in imiquimod (IMQ)-treated mice. Effects of TLR7 activation on structural modifications induced in superior mesenteric arteries from IMQ-treated mice. (A) Representative histological sections of paraffin-embedded tissues stained with hematoxylin-eosin. Morphometric analysis of media:lumen ratio (M/L) (B), medial thickness (C), lumen diameter (D) and media cross-sectional area (MCSA) (E). Experimental groups: Ctrl 4 weeks (W) (n=8), Ctrl 8W (n=8), IMQ 4W (n=8), IMQ 8W (n=8). Results were compared by 1-way ANOVA and Tukey *post hoc* test. Values are represented as Mean ± SEM. *P<0.05 compared to their respective control groups.

Figure 5. TLR7 activation leads to marked impairment of endothelium-dependent vasorelaxation in imiquimod (IMQ)-treated mice. Vascular

relaxant responses induced by acetylcholine (ACh, 1nM–10μM), in endothelium-intact aortas precontracted using U46619 (10nM) in the absence (A) or in the presence of the endothelial nitric oxide synthase (eNOS) inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME, 100μM) (B). (C) Endothelium-independent relaxant responses to sodium nitroprusside (SNP, 0,1nM–10μM) in endothelium-denuded vessels precontracted using U46619 (10nM). Relaxant responses to Ach and SNP were expressed as a percentage of precontraction induced by U46619. (D) mRNA expression and (E) protein expression of eNOS in aorta homogenates from all experimental groups. Data are calculated using the $2^{-\Delta\Delta Ct}$ method or eNOS/actin ratio compared to the control group. Experimental groups: Ctrl 4 weeks (W) (n=8), Ctrl 8W (n=8), IMQ 4W (n=8), IMQ 8W (n=8). Results were compared by 1-way ANOVA and Tukey *post hoc* test. Values are represented as Mean ± SEM. **P<0.01 compared to their respective control groups.

Figure 6. TLR7 activation promotes a significant increase in vascular reactive oxygen species and NADPH oxidase activity in aorta from imiquimod (IMQ)-treated mice. (A) Representative pictures of arteries incubated in the presence of dihydroethidium (DHE), which produces a red fluorescence when oxidized to ethidium by O_2^-, and the nuclear stain 4,6-diamidino-2-phenylindoledichlorohydrate (DAPI), which produces a blue fluorescence (magnification X400), and averaged values of the red ethidium fluorescence normalized to the blue DAPI fluorescence, Mean ± SEM (n= 8). (B) NADPH oxidase activity measured by lucigenin-enhanced

chemiluminescence. (C) mRNA expression of the NADPH oxidase subunits NOX-2, p22phox and p47phox in aorta homogenates from all experimental groups. Data are represented as a ratio of arbitrary units of mRNA ($2^{-\Delta\Delta Ct}$). (D) Endothelium-dependent vasodilator responses to acetylcholine (ACh) in intact aortic rings precontracted using U46619 (10 nM) in the presence of apocynin (10 μM). Experimental groups: Ctrl 8 weeks (W) (n=8), IMQ 8W (n=8). Results were compared by 1-way ANOVA and Tukey *post hoc* test. Values are represented as Mean ± SEM. **P<0.01 and ***P<0.001 compared to the control group.

Figure 7. TLR7 activation promotes a higher gene expression of vascular adhesion molecules and proinflammatory cytokines in aorta from imiquimod (IMQ)-treated mice. mRNA expression of vascular cell adhesion molecule-1 (VCAM-1) (A), proinflammatory cytokines IFN-α (B), IFN-γ (C), IL-1β (D), IL-6 (E) and IL-17 (F), and anti-inflammatory cytokine IL-10 (G) and TFG-β (H) in aorta homogenates from IMQ-treated mice at 8 weeks of treatment (8W). Data are presented as a ratio of arbitrary units of mRNA ($2^{-\Delta\Delta Ct}$). Experimental groups: Ctrl 8W (n=8), IMQ 8W (n=8). Results were compared by 1-way ANOVA and Tukey *post hoc* test. Values are represented as Mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 compared to the control group.

Figure 8. Antioxidant treatment prevents hypertension, vascular remodeling, endothelial dysfunction and increased NADPH oxidase activity induced by TLR7 activation in imiquimod (IMQ)-treated mice.

Effects of chronic antioxidant (Antiox; tempol+apocynin) treatment on (A) time course of systolic blood pressure (SBP) measured by tail-cuff plethysmography, and structural modifications induced in superior mesenteric arteries: (B) Representative histological sections of paraffin-embedded tissues stained with hematoxylin-eosin, morphometric analysis of media:lumen ratio (M/L) (C), medial thickness (D), lumen diameter (E) and media cross-sectional area (MCSA) (F). Effects of chronic antioxidant (Antiox; tempol+apocynin) treatment on (C) vascular endothelium-dependent vasorelaxation induced by acetylcholine (ACh, 1nM–10μM) in endothelium-intact aortas precontracted using U46619 (10nM), and (D) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence in aorta from IMQ-treated mice. Experimental groups: IMQ (n=8) and IMQ+Antiox (n=8). Results were compared by 1-way ANOVA and Tukey post hoc test. Values are represented as Mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 compared to the IMQ group.

Figure 9. Role of IL-17a in hypertension, endothelial dysfunction and increased NADPH oxidase activity induced by TLR7 activation in imiquimod (IMQ)-treated mice. Effects of IL-17a-neutralizing antibody (n-IL-17, 10μg/mouse) or the isotype control (10μg/mouse, start of n-IL-17administration) on (A) time course of systolic blood pressure (SBP) measured by tail-cuff plethysmography, (B) vascular endothelium-dependent vasorelaxation induced by acetylcholine (ACh, 1nM–10μM) in endothelium-intact aortas precontracted using U46619 (10nM), and (C) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence in aorta from

IMQ-treated mice. IL-17a-neutralizing antibody or the isotype control were intraperitoneally injected from the sixth week of imiquimod treatment every three days until the end of the experiment. Experimental groups: IMQ (n=8) and IMQ+n-IL17 (n=8). Results were compared by 1-way ANOVA and Tukey post hoc test. Values are represented as Mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 compared to the IMQ group