IMMUNOSUPPRESSION-INDEPENDENT ROLE OF REGULATORY T CELLS AGAINST HYPERTENSION-DRIVEN RENAL DYSFUNCTIONS

by

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ABSTRACT

Hypertension-associated cardiorenal diseases represent one of the heaviest burdens for current health systems. In addition to hemodynamic damage, recent results have revealed that hematopoietic cells contribute to the development of these diseases by generating proinflammatory and profibrotic environments in heart and kidney. However, the cell subtypes involved remain poorly characterized. Here, we report that CD39+ regulatory T (T\text{REG}) cells utilize an immunosuppression-independent mechanism to counteract renal and possibly cardiac damage during angiotensin II (AngII)-dependent hypertension. This mechanism relies on the direct apoptosis of tissue-resident neutrophils by the ecto-adenosine triphosphate diphosphohydrolase activity of CD39. Consistent with this, experimental and genetic alterations in T\text{REG}/T\text{H} cell ratios have a direct impact on tissue-resident neutrophil numbers, cardiomyocyte hypertrophy, cardiorenal fibrosis and, to a lesser extent, in arterial pressure elevation during AngII-driven hypertension. These results indicate that T\text{REG} cells constitute a first protective barrier against hypertension-driven tissue fibrosis and, in addition, suggest new therapeutic avenues to prevent hypertension-linked cardiorenal diseases.
INTRODUCTION

Foxp3^{+}CD4^{+} T_{REG} cells are primarily involved in the negative control of conventional T cell-dependent immune processes. To this end, they utilize a number of effector mechanisms including cytokine-dependent paracrine signaling events, interleukin 2 consumption, presentation of immunosuppressive ligands, cytolysis of target cells, and the modification of cell responses through the degradation of extracellular ATP. This latter regulatory mechanism is mediated by CD39, an ectoenzyme that displays adenosine triphosphate diphosphohydrolase activity (1, 2). In addition, they can promote immunomodulation through the regulation of other hematopoietic cells such as B lymphocytes, dendritic cells, and macrophages (1, 2). Recent observations have revealed that tissue-specific T_{REG} subtypes can also exert immunosuppression-independent functions. The best-characterized examples are T_{REG} cells present in adipose tissue and injured skeletal muscles that control metabolic indexes and muscle repair, respectively. These T_{REG} subsets are distinct from those involved in immunosuppression according to T cell receptor repertoire and transcriptomal features (3, 4).

Hypertension and associated cardiovascular diseases represent nowadays one of the heaviest burdens for our health systems (5, 6). In addition to the hemodynamic-related damage inflicted by hypertension itself, a number of pathophysiological circuits that change the inflammatory, fibrotic, and functional status of peripheral tissues also influence the progression of these dysfunctions. If untreated, these processes eventually lead to end organ disease and failure (7, 8). Extensive data indicates that T_{REG} cells play protective roles against high arterial pressure, cardiovascular remodeling, and heart damage (9-11). The
The exact nature of such protective action is unknown, although it has been commonly assumed that it was primarily associated with immunosuppression-linked mechanisms. Consistent with this, a large number of studies have shown that conventional T lymphocytes, the main cellular targets of T_{REG} cells, do play proactive roles during both the initiation and progression of hypertension-related pathophysiological events (8, 12-22). The exact T cell subpopulation(s) involved in those processes is still under debate. Thus, some studies have proposed the implication of different helper T (T_H{17}, T_H{1}, T_H{2}) subtypes in the engagement of these pathophysiological responses (13, 16, 17). By contrast, other analyses have postulated the control of the extent of the hypertensive response is under the regulation of nonconventional CD3^+CD4^-CD8^- T cell subpopulation that are specifically localized in perivascular adipose tissue (15). It is possible that these divergent results could reflect the implication of different T cell subsets in tissue specific pathophysiological responses of the vasculature, heart, and kidney. Settling this issue is of paramount importance to design new approaches to combat the inflammatory processes priming cardiorenal fibrosis and, eventually, end organ disease. In the same context, it is important to clarify the specific role of T_{REG} cells in the regulation of this complex pathophysiological program and the cellular targets that are controlled by them.

The Vav family is a group of phosphorylation-dependent GDP/GTP exchange factors involved in the activation step of Rho proteins. This family has three members in mammalian species that have been designated as Vav1 (formerly known as Vav or p95^-ve^), Vav2, and Vav3. Vav1 is primarily expressed in most hematopoietic lineages whereas Vav2 and Vav3 show more widespread expression patterns (23-25). Vav1 and, to a lesser
extent the other family members are important for lymphocyte development, selection, and
efferent functions (23-25). Consistent with this, single Vav1−/− and triple Vav1−/−;Vav2−/−;
Vav3−/− knockout mice exhibit both peripheral T cell lymphopenia and deficient, T cell
receptor-dependent antigenic responses (26-28). Vav2 and Vav3, but not Vav1, are also
involved in signaling routes contributing to cardiovascular homeostasis. As a consequence,
the single and compound elimination of these two proteins causes hypertension,
cardiovascular remodeling, cardiorenal fibrosis, and renal dysfunctions in mice (29-32).
Based on those observations, we believed that this collection of Vav family knockout mice
could represent a useful tool to clarify obscure aspects of the interplay between the immune
and cardiovascular systems during hypertension. The use of those animals, together with
other experimental mouse models for immunodeficiency and hypertension, allowed us to
discover a hitherto unknown T_{REG} cell-dependent mechanism that controls the extent of the
overall arterial pressure response and end organ dysfunctions during AngII-dependent
hypertension conditions. As we show here, this mechanism is unexpectedly mediated by
the direct, CD39- and apoptosis-mediated homeostatic control of tissue-resident neutrophil
numbers rather than on standard T cell immunosuppression processes. Hence, it is fully
operative even in complete absence of conventional T lymphocytes. This new mechanism
offers potentially interesting therapeutic avenues to prevent cardiorenal fibrosis and ensuing
end-organ disease in patients with long-term, AngII-dependent types of hypertension.
MATERIALS AND METHODS

Animals. 

Vav1−/− and Vav2−/−;Vav3−/− mice have been previously described (27, 29, 30). All Vav family knockout mice were homogenized in the C57BL/10 genetic background. Ly5.1+ mice were originally obtained from The Jackson Laboratories and maintained at the animal facility of the Centro de Biología Molecular Severo Ochoa (CSIC, Madrid, Spain). For the experiments, animals were transferred to the animal facility of the Centro de Investigación del Cáncer. BALB/c, SCID/Beige (C.B-17/lcrHsd-Prkdc<sup>scid</sup>Lyst<sup>bg-1j</sup>), and Foxn<sup>1nu</sup> mice were obtained from Harlan Laboratories. For all in vivo studies, animals of the same genotype were randomly assigned to the different experimental groups. Animals used were either four- (in the case of analysis of basal conditions) or three-month (in the case of analyses using osmotic pumps) old. All animal work has been done in accordance with protocols approved by the Bioethics committees of the University of Salamanca, CSIC, and Centro Nacional de Investigaciones Cardiovasculares.

Blood pressure-related analyses. Mean blood pressure was recorded in conscious mice with the non-invasive tail cuff method (CODA, Kent Scientific). Mice were familiarized with the procedure during the week previous to data recording to minimize stress-related blood pressure variations. Blood pressure measurements were always done in the afternoon.

Histological analyses. Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline solution, paraffin-embedded, cut in 2-3 μm sections and stained with hematoxylin.
and eosin (Sigma). Aorta media walls and cardiomyocyte areas were quantified with MetaMorph software (Universal Imaging).

**Tissue fibrosis determinations.** Fibrosis was quantified both immunohistochemically and biochemically. In the former case, paraffin-embedded tissue sections were stained with Sirius Red (Fluka). In the latter case, we measured the amount of hydroxyproline present in tissue lysates. Data from those analyses were converted into amount of collagen by considering the presence of 12.7% of hydroxyproline residues in that protein. These two procedures were done as indicated before (29, 30).

**Analysis of kidney function.** Mice were placed in metabolic cages and daily urine production was collected and measured, as indicated before (30). Protein content in urine was detected with Bradford Protein Assay Reagent (Bio-Rad) using a standard curve of bovine serum albumin (Sigma). To calculate creatinine clearance rates, we collected urine from individual mice for a 24 hour-long period using metabolic cages. In addition, we took blood samples from animals under study by cardiac puncture to measure the creatinine concentration in plasma. Urine and plasma creatinine concentrations were determined by a kinetic colorimetric method using a creatinine assay kit (QuantiChrom™ Creatinine Assay Kit, BioAssay Systems) according to the manufacturer’s protocol. Creatinine clearance rate (CCR) was calculated taking into account the urine flow (uf) and concentrations of creatinine in both urine ([Cre]u) and plasma ([Cre]p) using the formula $\text{CCR} = \frac{uf \times [\text{Cre}]_u}{[\text{Cre}]_p}$. Values were further normalized taking into account the weight of kidneys.
**Determination of mRNA abundance.** RNA was extracted with TRIzol (Sigma) and quantitative RT-PCR performed with ScriptOne-Step RT-PCR Kit (Bio-Rad) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Data were analyzed with the StepOne software v2.1 (Applied Biosystems). Expression of the endogenous mouse *P36b4* transcript was used as normalization control. Primers used were 5’-TTG ATG ATG GAG TGT GGC ACC-3’ (forward for mouse *P36b4* cDNA), 5’-GTG TTT GAC AAC GGC AGC ATT-3’ (reverse for mouse *P36b4* cDNA), 5’-CAC CAC GGA CTA CAA CCA GTT CGC-3’ (forward for mouse *Lcn2* cDNA), and 5’-TCA GTT GTC AAT GCA TTG GTC GGT G-3’ (reverse for mouse *Lcn2* cDNA).

**Pharmacological mouse treatments.** AngII (1.44 mg/kg/day, Sigma) and ARL67156 (1.1 μg/kg/day, Sigma) were administered for 14 days using osmotic delivery pumps (Model 1002, Alzet) inserted subcutaneously in back of animals. L-NAME (70 mg/100 ml, Sigma) was administered in the drinking water for four weeks (32). MMF (CellCept, Roche Farma) was prepared as previously described (33) and administered as indicated in experiments.

**Isolation of hematopoietic cells from tissues.** To isolate kidney-infiltrating cells, anesthetized mice were infused phosphate-buffered saline solution through the heart left ventricle to eliminate kidney-resident circulating cells. The two kidneys of each mouse were then collected, decapsulated, cut into small pieces with a sterile scalpel, and incubated in 10 ml of RPMI containing 10% fetal bovine serum and collagenase (5 mg, Sigma) 20 min at 37 °C. After filtering tissue debris, cells were washed twice with phosphate-buffered...
saline solution and resuspended in 8 ml of Roswell Park Memorial Institute (RPMI) media plus 10% fetal bovine serum. Upon addition of a 5 ml lower layer of Ficoll (Sigma) with a pipette, samples were centrifuged at 2,000 rpm at room temperature for 10 min. Cells in the medium/Ficoll interface were collected using a pipette and counted. To obtain cells from spleen and thymus, single cell suspensions were obtained by mechanical homogenization of tissues in 1 ml of phosphate-buffered saline solution supplemented with 2% bovine serum albumin and 0.5 mM EDTA. Bone marrow neutrophils were obtained by flushing the medullar cavity of isolated femoral bones with phosphate-buffered saline solution. In the case of circulating cells, blood samples were collected from hearts with the aid of heparinized syringes. Cell suspensions obtained in the above conditions were washed once in phosphate-buffered saline solution, subjected to 0.17 M NH₄Cl lysis to eliminate erythrocytes, and finally washed twice. Final cell pellets were resuspended in phosphate-buffered saline solution before the immunostaining step.

**Characterization and isolation of hematopoietic cell percentages.** Cell suspensions obtained as indicated above were stained with combinations of fluorescein isothiocyanate (FITC)-, allophycocyanin (APC)-, APC-Cy7- or V500-labeled antibodies to CD4 (BD Biosciences); FITC-, Pacific blue (PB)- or phycoerythrin (PE)-labeled antibodies to CD8 (BD Biosciences); APC- or PE-Cy7-labeled antibodies to CD25 (BD Biosciences); FITC- or APC-labeled antibodies to Gr1 (eBioscience and BD Biosciences, respectively); APC- or PE-Cy7-labeled antibodies to F4/80 (eBioscience and BD Biosciences, respectively); PE- or V500-labeled antibodies to CD11b (BD Biosciences); PE- or FITC-labeled antibodies to CD11c.
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193 (BD Biosciences); biotin- or APC-labeled antibodies to B220 (BD Biosciences) or PE-Cy7
194 labeled anti-CD39 (eBioscience). Cell immunolabeling with either FITC- or PE-labeled
195 antibodies to Foxp3 was done using the Foxp3 Staining Buffer Set (eBioscience). For data
196 acquisition, samples were collected using a FACSaria III flow cytometer (BD Biosciences)
197 and blindly analyzed using the FlowJo software (Tree Star). For in vitro studies, samples
198 were sorted in sterile condition using a FACSaria III system, collected in phosphate-
199 buffered saline solution supplemented with 50% fetal bovine serum, centrifuged,
200 resuspended in RPMI (Gibco Life Technologies) supplemented with 10% fetal bovine
201 serum, 2 mM L-glutamine and antibiotics (100 units/ml Penicillin and 100 µg/ml
202 Streptomycin, Invitrogen) (referred hereafter as complete media), and cultured in complete
203 RPMI media as indicated.

205 iT<sub>REG</sub> cell generation. Freshly sorted splenic, CD4<sup>+</sup>CD25<sup>+</sup> T cells were plated (1 × 10<sup>6</sup>
206 cells/ml) onto culture dishes coated with antibodies to mouse CD3 (5 µg/ml, BD
207 Biosciences) and containing complete RPMI media supplemented with antibodies to CD28
208 (5 µg/ml, BD Biosciences), transforming growth factor β<sub>1</sub> (5 ng/ml, R&D Systems) and
209 recombinant mouse interleukin 2 (100 IU/ml, Peprotech) for four days. Cells were then
210 centrifuged, washed twice with phosphate-buffered saline solution, stained with FITC-
211 labeled anti-CD4 (BD Biosciences), APC-labeled anti-CD25 (BD Biosciences), and PE-
212 Cy7-labeled anti-CD39. The iT<sub>REG</sub> cell population was separated either as a whole
213 CD4<sup>+</sup>CD25<sup>+</sup> population or fractionated into CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>+</sup> and CD4<sup>+</sup>CD25<sup>+</sup>CD39<sup>−</sup>
populations before being injected into animals. Cells were collected for in vivo experiments using a FACSria cell sorter as above.

\( T_{\text{REG}} \) injections in mice. iT\( T_{\text{REG}} \) generated as above and freshly sorted splenic CD4\(^+\)CD25\(^+\) nT\( T_{\text{REG}} \) cells were resuspended in 100 \( \mu \)l of phosphate-buffered saline solution at a cell density of \( 2 \times 10^6 \) cells/ml and injected into the lateral tail vein one day after the implantation of the drug delivery pumps. Unless otherwise stated, \( 2 \times 10^5 \) cells were injected per animal in these experiments. When indicated, injections were performed with splenic-derived CD4\(^+\)CD25\(^-\) T cells as above.

Immunodepletion of nT\( T_{\text{REG}} \) cells. 250 \( \mu \)g of either a monoclonal rat antibody to mouse CD25 (clone PC61.5, eBioscience) or an IgG1 isotype negative control (eBioscience) were injected intraperitoneally one day before the implantation of drug delivery osmotic pumps. Extent of immunodepletion achieved in each animal was assessed weekly by flow cytometry. To that end, blood from anesthetized mice was drawn, cells collected, washed in phosphate-buffered saline solution, subjected to 0.17 M NH\( _4 \)Cl-mediated erythrocyte lysis, washed twice with phosphate-buffered saline solution, stained with both FITC-labeled CD4 and APC-labeled CD25, and analyzed by flow cytometry.

Immunodepletion of neutrophils. WT mice were intraperitoneally injected with either 500 \( \mu \)g of a rat monoclonal antibody to the Ly6G surface marker (clone 1A8, Biolegend) or the same amount of an IgG1K isotype negative control (eBioscience) one day before
implantation of AngII-delivery osmotic pumps. Percentages of neutrophils in blood were assessed every three days by flow cytometry using FITC-labeled antibodies to Gr1. At the end of experiments, percentages of neutrophils, macrophages, and CD4+ T cells in kidneys were determined by labeling cells with antibodies to Gr1 (FITC-labeled, eBiosciences), CD11b (PE-labeled, BD Biosciences), F4/80 (APC-labeled, BD Biosciences), and CD4 (V500-labeled, BD Biosciences).

Neutrophil recruitment assays. When indicated, conditioned cell culture supernatants were obtained from fresh cultures of indicated splenic T cells at $1 \times 10^6$ cells/ml. After 72 hours, cells were centrifuged and supernatants collected. For chemotactic assays, $1 \times 10^5$ of freshly purified bone marrow neutrophils were placed onto the upper chamber of a transwell plate (Corning HTS 24 well transwell, 3.0 µm pore size) that contained in the lower chamber complete RPMI, cell-conditioned supernatants from indicated cell types and, when indicated complete RPMI supplemented with 1 µM angiotensin II.

Determination of ROS production and apoptosis in neutrophil cultures. Freshly sorted bone marrow neutrophils were cultured in complete RPMI and treated with 1 µM AngII II for 20 min. When indicated, cells were pretreated with either 10 µM losartan (Merck) or 10 µM PD123319 (Sigma) for 30 min. 10 µM MMF (CellCept, Roche Farma) was included in cultures for 120 min before the angiotensin II stimulation. For neutrophil/nT_{REG} cell coculture experiments, cells sorted as indicated above, treated or untreated with 250 µM ARL67156 in complete RMP for 30 min, and then cultured together in same culture media.
for 24 hours always using 1:10 nTREG cell:neutrophil ratios. In some of these experiments, nTREG cells were either substituted or cocultured with other T cells following the same experimental procedure. Neutrophil ROS production and apoptosis were assessed using lucigenine-based chemiluminescence (34) and annexin V/propidium iodide flow cytometry determinations (35), respectively. The latter assay was done using the Apoptosis Detection kit from Immunostep.

Statistics. Data were analyzed with either a two-tailed Student’s t test or a one-way ANOVA with Bonferroni post hoc test in the case of multiple comparisons.
RESULTS

Vav proteins are important for hypertension-driven cardiorenal fibrosis

We have reported before that $Vav2^{-/-}$, $Vav3^{-/-}$, and $Vav2^{-/-};Vav3^{-/-}$ (DKO) knockout mice develop hypertension in an age- and AngII-dependent manner (Fig. 1A) (29, 30, 32).

Further analyses indicated that DKO mice also develop hypertension-linked dysfunctions similar to those present in the single $Vav2^{-/-}$ and $Vav3^{-/-}$ knockout animals, including the thickening of the aorta media wall (Fig. 1, B [left panels] and C), hypertrophy of left ventricle cardiomyocytes (Fig. 1, B [second column from left] and D) and interstitial fibrosis in both heart (Fig. 1, B [third column from left] and E) and kidney (Fig. 1, B [right panels] and F). Signs of the latter condition included the detection of collagen with Sirius red staining (Fig. 1B, third and fourth panels from left) and hydroxyproline tissue content (Fig. 1, E and F) in both heart and kidney tissue samples. Furthermore, the histological analyses of kidneys revealed the presence of extensive glomerular sclerosis, necrosis of distal tubules, and protein casts in kidney sections from DKO mice (Fig. 1G). Consistent with this, we observed that DKO mice also exhibit alterations in renal physiology such as reduced flow of urine (Fig. 1H), increased protein content in urine (proteinuria, Fig. II), reduced creatinine clearance rates (Fig. 1J) and increased amounts of the transcript for lipocalin 2 (Lcn2, Fig. 1K), a well-known early biomarker for both acute and chronic nephropathies (36). These results led us to investigate the effect of the additional inactivation of the $Vav1$ gene in this cardiovascular phenotype using the triple $Vav1^{-/-};Vav2^{-/-};Vav3^{-/-}$ knockout (TKO) mouse strain. We found that these mice exhibit hypertension (Fig. 1A) and vascular remodeling (Fig. 1, B [left panels] and C), although at
However, we unexpectedly found that they show no obvious signs of the cardiomyocyte hypertrophy ([Fig. 1, B] [second column from left] and D) and cardiorenal fibrosis ([Fig. 1, B] [third and fourth columns from left], E and F). They also show normal rates of urine production ([Fig. 1H]), urine protein content ([Fig. 1I]), creatinine clearance ([Fig. 1J]) and intrarenal amounts of Lcn2 transcripts ([Fig. 1K]), further indicating that these mice do not have any significant signs of heart remodeling and cardiorenal fibrosis in the kidneys.

We surmised that the mild hypertension present in TKO mice could be the cause of the protection exhibited by these animals against cardiorenal fibrosis and cardiovascular remodeling. To explore this possibility, we decided to compare the foregoing pathophysiological parameters in wild-type (WT), DKO and TKO mice under systemic administration conditions of AngII, a well-known vasoconstriction agent (37). We speculated that if the above idea were correct, then the chronic delivery of this vasoconstriction agent had to restore cardiorenal fibrosis and heart remodeling in TKO mice. To this end, we implanted AngII-delivery osmotic pumps on the backs of WT, DKO and TKO animals and, subsequently, followed the evolution of blood pressure during a 14 day-long period. In addition, we monitored the rest of cardiovascular and renal parameters using samples from mice euthanized at the end of AngII treatment. As expected, such conditions promote the development of high blood pressure ([Fig. 1L]), renal fibrosis ([Fig. 1M]), and increased amounts of Lcn2 transcripts in kidneys ([Fig. 1N]) in WT mice. Likewise, they exacerbate the hypertensive state of DKO mice ([Fig. 1L]). However, this change in arterial blood pressure does not lead to further increases in the already high levels
of collagen deposition (Fig. 1M) and Lcn2 mRNA expression (Fig. 1N) present in the kidneys of these mice. The systemic infusion of AngII also aggravates the hypertension state of TKO mice, although to a lower extent to those found in AngII-infused DKO and WT mice (Fig. 1L). However, arterial blood pressure does reaches under those conditions levels comparable to those exhibited by the untreated DKO mice (Fig. 1L) that, as shown above, do develop cardiorenal fibrosis (Fig. 1, C to K). Despite this, the AngII-treated TKO mice still do not show any change in renal fibrosis-related parameters (Fig. 1, M and N). These results suggest that the differences in overall arterial blood pressures could not be the main leading cause of the different cardiorenal phenotypes exhibited by DKO and TKO mice. In addition, they indicate that Vav proteins play either direct or indirect roles in hypertension-driven cardiorenal fibrosis.

The Vav1 gene deficiency is sufficient to confer protection against tissue fibrosis

To investigate whether the protection exhibited by TKO mice against heart remodeling and cardiorenal fibrosis was due to the elimination of the three Vav family genes or to the single inactivation of the Vav1 gene, we next compared the response of control and single Vav1−/− knockout mice to the systemic administration of AngII. With the exception of the normal development of vascular remodeling (Fig. 2A), AngII-infused Vav1−/− mice develop no obvious signs of cardiomyocyte hypertrophy (Fig. 2B), cardiorenal fibrosis (Fig. 2, C and D) or changes in the expression of the Lcn2 mRNA in kidneys (Fig. 2E). Furthermore, although AngII-infused Vav1−/− mice initially show elevations in blood pressure similar to controls (Fig. 2F), they eventually undergo smaller hypertensive responses than WT
animals at later periods of the systemic administration of AngII (Fig. 2, F and G). The time of the differential response takes place between the 6th and the 10th day of AngII infusion, which roughly corresponds to the time in which the cardiorenal fibrosis reaches a plateau in control mice (Fig. 2H). Thus, the elimination of the Vav1 gene fully recapitulates the effects derived from the removal of the three Vav family members in this pathophysiological scenario. Unlike the case of AngII-driven hypertension, we observed that the Vav1−/− mice are not protected against the dysfunctions under study when treated with the nitric oxide synthase inhibitor Lω-Nitro-L-arginine methyl ester (L-NAME) (Fig. 2, I to N). This compound promotes hypertension through the blockage of the nitric oxide-mediated dilatation of resistance blood vessels (38). These results suggest that the antifibrotic protection induced by the Vav1 gene deficiency is not probably due to a nonspecific, buffering effect on the damaging hemodynamic effects of hypertension on peripheral tissues.

Immunosuppressed mice show no resistance to hypertension-linked tissue fibrosis

Vav1−/− and TKO mice, unlike the case of DKO animals, have reduced numbers of peripheral T cells that, in addition, cannot adequately respond to antigens (26-28). Based on previous reports indicating that conventional T cells are implicated in the development of hypertension, tissue fibrosis and end-organ disease (7, 8, 12, 13, 20), we surmised that the lymphopenic status of those two mouse strains could be the main cause of the resistance shown to AngII-triggered heart remodeling and cardiorenal fibrosis. If that were the case, we assumed that a similar phenotype had to be found in other immunocompromised mice.
In agreement with both this idea and published results (12, 13), we initially found that the treatment of AngII-infused WT mice with the T cell immunosuppressant mycophenolate mofetil (MMF) (39) does lead to a hypertensive buffering effect and protection against cardiorenal fibrosis (SF, data not shown). As an additional control to corroborate these results, we next decided to analyze the response of Foxn1nu mice, a strain that cannot generate T lymphocytes due to problems in the development of the thymus, to the infusion of AngII. Unexpectedly, we found in this case no significant protection against the cardiorenal fibrosis (Fig. 3A and B) and a total lack of the hypertension buffering effect (Fig. 3C) that had been previously seen in Vav1−/− mice. Similar results were obtained with AngII-treated severe combine immunodeficiency (SCID)/Beige mice that lack both T and B lymphocytes (SF, data not shown, see also Fig. 7 below). We inferred from these experiments that T cell immunosuppression per se cannot explain the results obtained with Vav1−/− and MMF-treated WT mice and, therefore, that other cell types had to be involved in the development of cardiorenal fibrosis during AngII-triggered hypertension. Concurring with this idea, we found that MMF also promotes resistance to both cardiorenal fibrosis (Fig. 3A and B) and excessive elevations of blood pressure (Fig. 3C) when administered to AngII-infused Foxn1nu mice.

Taking into account that the Vav1 gene is predominantly expressed in hematopoietic cells (23), we hypothesized that alterations in cells belonging to either the adaptative or innate immune had to be involved in the protection against fibrosis exhibited by Vav1−/− mice. As a first approximation to identify them, we next carried out flow cytometry determinations to analyze the distribution of a large variety of hematopoietic cell lineages
in the kidneys, blood, spleens, and thymi from DKO (which showed no cardiorenal protection), TKO (which exhibited cardiorenal protection), Vav1\textsuperscript{−/−} (normotense and immunodeficient), and normotense WT mice. We excluded the heart from these studies due to the difficulty in avoiding crosscontamination from the large numbers of circulating cells present in that tissue. We found that TKO and Vav1\textsuperscript{−/−} mice have reduced numbers of both neutrophils and CD4\textsuperscript{+} cells in kidneys when compared to the other mouse strains (Table 1). This neutropenia is kidney-specific, because no statistically significant differences are detected in circulating neutrophils between TKO and DKO mice (Table 1). However, these two mouse knockout strains do exhibit neutrophilia in the blood when compared to WT and Vav1\textsuperscript{−/−} animals (Table 1). Whether this is due to their common hypertensive condition remains unknown. Unlike the case of neutrophils, the CD4\textsuperscript{+} T cell lymphopenia found in kidneys of TKO and Vav1\textsuperscript{−/−} mice is also seen in the rest of tissues surveyed (Table 1), a result consistent with the known intrathymic T cell selection defects present in those mice (27). Unexpectedly, further flow cytometry analyses revealed that the CD4\textsuperscript{+} T cell lymphopenia of TKO and Vav1\textsuperscript{−/−} mice does not apply to CD4\textsuperscript{+}Foxp3\textsuperscript{+} T\textsubscript{REG} cells, because the percentage of these immunoregulatory cells is comparable to those present in WT and DKO animals in all tissues surveyed (Table 1). By contrast, we observed no statistically significant variations in the percentages of other hematopoietic lineages surveyed, including macrophages, CD11c\textsuperscript{+} and CD11c\textsuperscript{−} dendritic cells, CD8\textsuperscript{+} T cells, and B lymphocytes in kidneys of control, DKO, TKO, and Vav1\textsuperscript{−/−} mice (Table 1). These results suggested that T\textsubscript{REG} cells, neutrophils or both cell lineages combined could be potentially
involved in the protection against cardiorenal protection seen in TKO, AngII-infused TKO, and AngII-infused Vav1−/− mice.

**T\textsubscript{REG} cells promote protection against hypertension-driven cardiorenal fibrosis**

Owing to the above results, we decided to investigate the possible implication of T\textsubscript{REG} cells in the protection against hypertension-driven cardiorenal fibrosis shown by Vav1−/− mice. As a first approximation, we decided to analyze whether the artificial increase in the T\textsubscript{REG}/T\textsubscript{H} ratio in WT mice could reproduce the protection offered by the Vav1 gene deficiency against cardiorenal fibrosis. To this end, we generated in vitro T\textsubscript{REG} cells (referred hereafter as iT\textsubscript{REG}) by culturing splenic WT CD4+CD25− lymphocytes in the presence of a differentiation cocktail and, upon purification by flow cytometry (Fig. 4A, second and third panels from left), injected them into recipient WT mice. We carried out two independent control experiments to make sure that this approach led to effective changes in the T\textsubscript{REG}/T\textsubscript{H} cell ratio in the recipient mice. Firstly, we confirmed by flow cytometry that we were actually injecting T\textsubscript{REG} cells, since ≈80% of the cells differentiated in vitro were positive for the T\textsubscript{REG} cell-specific Foxp3 transcriptional factor (Fig. 4A, right panel). Secondly, by using pilot injection experiments of iT\textsubscript{REG} cells generated from C57BL/6 Ly5.1 mice (whose hematopoietic cells are CD45.1+) into WT C57BL/10 mice (whose hematopoietic cells are CD45.2+), we could estimate that the chosen experimental protocol does promote a three- and two-fold increase in the total amount of T\textsubscript{REG} cells in the recipient mice during the first six and late postinjection days, respectively (Fig. 4B, see plot with closed boxes). These variations are directly derived from the iT\textsubscript{REG} cells (CD45.1+) that
had been injected into mice (Fig. 4B, see plot with open boxes). Moreover, we found that
the injected iT\textsubscript{REG} population maintains the CD4\textsuperscript{+}CD25\textsuperscript{+} status during an extended period of
time, although it undergoes a two-fold decrease at the latest time point analyzed (Fig. 4C,
see lanes with light blue boxes). As a result, we calculated that the injected animals
maintain high T\textsubscript{REG}/nonT\textsubscript{REG} CD4\textsuperscript{+} cell ratios during most of the time course used in these
studies (Fig. 4C, compare lanes with light blue and light red boxes). When we repeated
these injection experiments with standard CD57BL/10 iT\textsubscript{REG} cells, we observed that the
recipient WT mice exhibit no cardiorenal fibrosis (Fig. 4, D and E) and a buffered
hypertensive response (Fig. 4F) upon the systemic administration of AngII. Based on the
results obtained in the analyses of hematopoietic cells present in Vav family knockout mice
(Table 1), we also decided to include as an additional read-out in these studies the
evolution of neutrophil numbers present in the kidneys of iT\textsubscript{REG}-injected mice. We observed
that these mice show a significant reduction in the percentage of neutrophils in the kidneys
when compared to controls (Fig. 4, G and H), thus suggesting a possible direct link
between the elevated T\textsubscript{REG}/T\textsubscript{H} ratios and the kidney neutropenia previously inferred from
the experiments with TKO mice (see above, Table 1). Interestingly, this latter effect is also
observed in mice not treated with AngII (Fig. 4I), possibly indicating a hypertension-
independent crosstalk between T\textsubscript{REG} cells and neutrophils in this tissue. Using a similar
experimental approach, we demonstrated that the expression of Vav1 is not important for
the efficient generation (Fig. 4J) and functionality (Fig. 4K) of iT\textsubscript{REG} cells in this
experimental setting. Thus, it is unlikely that the phenotype observed in Vav1\textsuperscript{+/−} mice could
be directly caused by the presence of nonfunctional T\textsubscript{REG} cells in kidneys.
To further confirm the implication of $T_{\text{REG}}$ cells in this process, we next assessed the effect of eliminating $T_{\text{REG}}$ cells in $Vav1^{-/-}$ mice. According to our hypothesis, such a procedure had to restore fibrosis in the kidneys of these animals and induce a concomitant increase in the number of intrarenal neutrophils. To achieve this end, we carried out an immunodepletion strategy consisting in injecting the animals under study with an antibody to the high affinity interleukin 2 receptor (CD25). As shown in Fig. 5A, this procedure promotes the efficient and long-term elimination of $T_{\text{REG}}$ cells in mice. When infused with AngII, we observed that $T_{\text{REG}}$-immunodepleted $Vav1^{-/-}$ mice behave like WT mice in terms of cardiorenal fibrosis development (Fig. 5, B and C) and elevation in blood pressure (Fig. 5D). Importantly, we found that these mice also recover WT-like percentages of kidney-resident neutrophils both under AngII administration (Fig. 5, E and F) and basal (Fig. 5G) conditions. In addition to further confirm the negative crosstalk between $T_{\text{REG}}$ and neutrophils, these results suggest that the intrakidney neutropenia previously detected in both AngII-treated and untreated $Vav1^{-/-}$ mice (see above, Table 1 and Fig. 4) cannot be caused by intrinsic signaling dysfunctions of the $Vav1^{-/-}$ neutrophils. Collectively, these findings support the idea of a direct functional link between $T_{\text{REG}}/T_H$ ratios and neutrophil numbers that, eventually, modulate AngII-driven tissue fibrosis in hypertensive mice.

Neutrophils contribute to hypertension-driven cardiorenal fibrosis

Given the direct correlation between effective fibrosis development and neutrophil numbers observed in the foregoing experiments, we next decided to address the direct contribution of these myeloid cells to this process. To this end, we immunodepleted them in WT mice.
using injections with an antibody (1A8) to the Ly6G subunit of the Gr1 antigen. The efficient depletion of these cells was confirmed by detecting the percentage of Gr1$^+$ cells in the blood of injected animals using flow cytometry analyses (Fig. 6A). Using this approach, we observed that neutrophil-depleted WT mice phenocopy the Vav1 gene deficiency in terms of protection against cardiorenal fibrosis (Fig. 6, B and C) and development of mild hypertensive responses (Fig. 6D) when systemically infused with AngII. This effect is neutrophil-dependent, because the kidneys of the mice pretreated with the 1A8 antibody exhibit percentages of macrophage (Fig. 6E) and CD4$^+$ T cells (Fig. 6F) similar to those found in control mice. However, under the same conditions, they show the expected lack of neutrophils in this organ (Fig. 6, G and H). These results indicate that neutrophils are critical for the induction of all those AngII-triggered pathophysiological responses even in fully immunocompetent mice.

$T_{\text{REG}}$ cells protect against hypertension-driven fibrosis in immunodeficient mice

To further rule out that the antifibrotic effect of iT$_{\text{REG}}$ cells was independent on their roles in general T cell immunosuppression, we decided to repeat some of the above experiments in AngII-infused SCID/Beige (C.B-17/lcrHsd-Prkdc$^{\text{scid}}$Lyst$^{bgL}$) and Foxn1$^{nu}$ mice. SCID/Beige mice are totally devoid of T and B lymphocytes due to a SCID mutation that impairs the V(D)J recombination of antigen receptor genes in these two cell lineages. They also have diminished natural killer cell and macrophage activity owing to the presence of the beige mutation. As controls for these animals, we used immunocompetent mice of the same genetic background (BALB/c). Similarly to our results with WT C57BL/10 animals (see
above, Fig. 4), we observed that the injection of iT\textsubscript{REG} cells promotes protection against AngII-induced renal fibrosis in SCID/Beige mice (Fig. 7, A to C), BALB/c (Fig. 7, A to C), and Foxn\textsuperscript{nu} mice (Fig. 7, D and E; red bars). These effects are associated with parallel reductions in the normal percentages of neutrophils present in the kidneys of those animals (Fig. 7, F to H). As negative control, we did not observe any significant protection upon the injection of equal numbers of CD4\textsuperscript{+}CD25\textsuperscript{−} lymphocytes into Foxn\textsuperscript{nu} mice (Fig. 7, D, E and H; blue bars). Conversely, a similar protection is obtained upon the immunodepletion of neutrophils (Fig. 7, F and G) in both SCID/Beige and BALB/c mice (Fig. 7, A to C).

Taking into account the above results, we decided to investigate whether the antifibrotic effects previously observed with the MMF immunosuppressant in the T cell immunodeficient Foxn\textsuperscript{nu} strain (Fig. 3) could also involve alterations in either the number or functionality of neutrophils. We observed that the treatment with this drug does not induce any significant effect in the neutrophils of Foxn\textsuperscript{nu} mice under normal conditions. However, MMF does promote a severe and systemic neutropenic effect when combined with the administration of AngII (Fig. 7I). Taken together, these findings indicate that the protective effects observed with the Vav\textsubscript{I} deficiency, iT\textsubscript{REG} cells, and MMF against tissue fibrosis can be all probably integrated in a common mechanistic framework whose final end-point is the control of either tissue-resident (in the case of Vav\textsubscript{I}-deficient and iT\textsubscript{REG}-injected WT mice) or systemic (in the case of MMF treatments) neutrophil numbers. Furthermore, they indicate that this T\textsubscript{REG}/neutrophil axis confers cardiorenal protection using immunosuppression-independent mechanisms.
T\textsubscript{REG} cells promote direct apoptosis of neutrophils in a CD39-dependent manner

Although our results with both Foxn1\textsuperscript{nu} and SCID/Beige mice excluded the participation of conventional T cells in this response, they could not formally ruled out the possibility that other T\textsubscript{REG} cell partners such as macrophages or dendritic cells could indirectly mediate the negative effects of T\textsubscript{REG} cells on neutrophil numbers. Such an idea is, in fact, consistent with previous findings indicating that T\textsubscript{REG} cells can mediate both acute lung injury-induced fibrosis and some pathogen-triggered inflammatory responses by influencing the activity of non T cell hematopoietic lineages (40, 41). To tackle this issue, we decided to investigate the effect of T\textsubscript{REG} cells, AngII and MMF on both neutrophil viability and function using cell culture experiments. To this end, we first analyzed the effect of coculturing primary bone marrow neutrophils with splenic CD4\textsuperscript{+}CD25\textsuperscript{+} T cells that were directly obtained from mice using flow cytometry purification procedures. These latter cells will be referred to hereafter as “natural” T\textsubscript{REG} (nT\textsubscript{REG}) cells in order to distinguish them from the in vitro-differentiated iT\textsubscript{REG} cells used in previous experiments. An example of the purification of these cells can be found in Figure 8A. These experiments revealed that neutrophils exhibit higher apoptotic rates when cultured in the presence of nT\textsubscript{REG} cells (Fig. 8B), an effect that was independent on the presence of AngII in the culture media (SF, data not shown). Probably as a result of this apoptotic effect, we also found that the presence of nT\textsubscript{REG} cells leads to the elimination of the production of reactive oxygen species (ROS) that is stimulated by AngII in neutrophils (Fig. 8C). ROS production is mediated by the AngII AT\textsubscript{1} receptor in neutrophils, because this response can be blocked by AT\textsubscript{1} (losartan), but not AT\textsubscript{2} (PD123319), receptor antagonists (Fig. 8C). Additional experiments indicated that nT\textsubscript{REG}
cells are not exclusively involved in the negative regulation of neutrophils. For example,
we observed that the supernatants from nT_{REG} cultures can promote the chemotaxis of
primary neutrophils (Fig. 8D). All these actions seem to be nT_{REG}-specific, because no
significant effects were observed when using T_{H} (CD4^+) and cytotoxic (CD8^+) T
lymphocytes (Fig. 8, B to D) in these experiments. Thus, similarly to the observations
made in vivo, the present results indicate that T_{REG} cells control in an AngII-independent
manner the viability of primary neutrophils.

T_{REG} cells can inhibit other cell types using a number of signaling mechanisms,
including CTLA4-mediated immunosuppression, granzyme and perforin-dependent
cytotoxicity, cytokine-based paracrine signaling, and the degradation of extracellular ATP
using the ecto-adenosine triphosphate diphosphohydrolase activity of plasma membrane-
localized CD39 molecules (1, 2). We ruled out the three former mechanisms, because our
results indicated that the T_{REG}/neutrophil interconnection was probably associated with an
immunosuppression-independent proapoptotic mechanism (see above, Fig. 7 and Fig. 8B).
We also excluded a paracrine-dependent proapoptotic effect, given our observations
indicating that the supernatants obtained from nT_{REG} cell cultures promote the chemotaxis
rather than the apoptosis of neutrophils (Fig. 8D). The foregoing observations led us to
consider the possible implication of CD39, a surface molecule expressed by 50 ± 7% and
58 ± 6 % of the iT_{REG} and splenic nT_{REG} cells used in this work, respectively \((n = 4
independent experiments)\). This percentage is further expanded up to 71.7 ± 4.1% of all the
iT_{REG} population when AngII is added to the in vitro T_{REG} differentiation cocktail \((n = 4
independent experiments, \(P \leq 0.05\)). To test this idea, we purified and separated away
splenic CD39+ and CD39− nT\textsubscript{REG} cells by flow cytometry (Fig. 8E) and, subsequently, cultured them with primary bone marrow neutrophils. Using this approach, we observed that the CD39+ nT\textsubscript{REG} cell population, but not the CD39− one, prompts the apoptosis of neutrophils (Fig. 8F). This proapoptotic effect is eliminated when the ATPase activity of this ectoenzyme is blocked in those cultures with either a CD39 drug inhibitor (ARL67156; Fig. 8F) or excess of its normal catalytic substrate (ATP; Fig. 8G). As control, no rescue is observed when the T\textsubscript{REG}/neutrophil cultures are supplemented with a nonhydrolyzable form of ATP (adenosine 5'-[γ-thio]triphosphate, Fig. 8G). Consistent with these results, we found that ARL67156 eliminates the negative effect of nT\textsubscript{REG} cells on ROS production by AngII-stimulated neutrophils (Fig. 8C). By contrast, ARL67156 does not block other T\textsubscript{REG} cell activities, such as neutrophil chemotaxis (Fig. 8D). ARL57156 (Fig. 8F) and ATP (Fig. 8G) cannot prevent either the basal apoptotic rates typically exhibited by neutrophils in cell culture, further confirming that their effects are CD39-dependent.

We next investigated whether the neutropenic effect found in MMF-treated mice could be the result in some direct action of the drug on primary neutrophils. As shown in Fig. 8H, MMF has no effect on the viability of these cells under normal culture conditions. However, it promotes enhanced apoptotic rates when used in AngII-stimulated neutrophil cultures (Fig. 8H). This goes in parallel with the elimination of ROS production by the stimulated neutrophils (Fig. 8I). In agreement with the stimulation-dependent effect of this drug, its proapoptotic effect is lost when the stimulated neutrophils are pretreated with losartan but not PD123319 (Fig. 8H). Taken together, these results indicate that the neutropenia observed in TKO, Vav1−/−, and AngII plus MMF-treated nude mice is probably
due to a direct proapoptotic cell autonomous effect of both CD39+ T\textsubscript{REG} cells and MMF on neutrophils.

**CD39+ T\textsubscript{REG} cells protect against AngII-driven hypertension and its comorbidities**

To corroborate the physiological relevance of the in vitro culture experiments described in the previous section, we investigated the actual contribution of CD39+ T\textsubscript{REG} cells to the cardiovascular and renal parameters under study in this work. To this end, we first decided to analyze the effect of injecting flow cytometry-purified CD39+ and CD39− iT\textsubscript{REG} cells (Fig. 9A) in the AngII-mediated fibrosis of WT mice. We observed that the CD39+ iT\textsubscript{REG} subset does promote kidney neutropenia (Fig. 9B), protection against cardiorenal fibrosis (Fig. 9, C and D), and the expected buffering effect on arterial blood pressure elevation (Fig. 9E) in the recipient mice. However, such an activity was not observed when using CD39− iT\textsubscript{REG} cell-injected WT mice (Fig. 9, B to E). Further underscoring the role of the CD39 ectoenzyme in this process, we found that the effect induced by the injected CD39+ iT\textsubscript{REG} cells is abolished when the recipient mice are treated with ARL57156 (Fig. 9, B to E). Confirming the implication of these cells in the phenotype shown by knockout mice, we found that the administration of this CD39 inhibitor to AngII-treated Vav1−− mice mimicked the effects of the T\textsubscript{REG} immunodepletion previously seen in those mice, including the restoration of increased numbers of kidney-resident neutrophils (Fig. 9F), development of fibrosis in both the heart (Fig. 9G) and kidneys (Fig. 9H), elevated amounts of Lcn2 mRNA expression in kidneys (Fig. 9I), reductions in creatinine clearance by kidneys (Fig. 9J), and the restoration of a full hypertensive response (Fig. 9K). Collectively, these results
indicate that $T_{\text{REG}}$ cells are involved in the protection against hypertension-driven cardiorenal fibrosis using a nonconventional, CD39- and neutrophil-dependent mechanism (Fig. 9L).

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DISCUSSION

Our results have unveiled a hitherto unknown CD39+ T_{REG} cell/neutrophil-based mechanism that probably represents a first trench of protection against AngII-elicited heart remodeling and cardiorenal fibrosis (Fig 9L). This mechanism has been found operative under both normotensia and hypertension conditions, suggesting that it may be involved in the homeostatic control of neutrophil numbers in some specific tissues. The utilization of a CD39+ T_{REG} cell/neutrophil axis seems prima facie a good functional strategy to control these pathophysiological processes, because evidence from other fibrosis-associated kidney dysfunctions indicates that neutrophils act as critical “decision-shapers” during the inflammatory-based priming phase of the fibrotic process (8). Furthermore, they are highly dependent on both extracellular ATP and ADP availability for a large variety of functions, including survival, chemotaxis, and other proinflammatory-connected functions (42). Interestingly, this antifibrotic mechanism seems to be quite specific, as it does not confer protection under L-NAME-triggered hypertension conditions. Since Vav1+/− mice show increased T_{REG}/T_{H} cell ratios and neutropenia prior to the hypertension step, it is likely that this differential response could be due to the engagement of different proinflammatory or profibrotic routes upon the systemic administration of AngII and L-NAME. Likewise, we have observed that this antifibrotic mechanism does not prevent the arterial remodeling that typically develops under AngII-driven hypertension conditions. This is consistent with previous results indicating that this response may involve either macrophages or a nonconventional CD3+CD4-CD8- T cell subpopulation (15, 20).
It is worth noting that this new regulatory layer involved in fibrotic responses does not exclude the participation of T<sub>H</sub> cells and other myeloid cells further downstream in the fibrotic pathophysiological cascade. On the contrary, our observations suggest that this first T<sub>REG</sub>-dependent barrier of defense against AngII-driven tissue fibrosis is probably overcome under chronic hypertension conditions by the subsequent recruitment and/or activation of either T<sub>H</sub> cell subtypes or downstream proinflammatory responses providing that normal T<sub>REG</sub>/T<sub>H</sub> ratios are preserved in mice (Fig. 9L). In any case, the protection shown by T<sub>REG</sub> cell-transplanted WT mice suggests that therapies based on either alterations of T<sub>REG</sub>/T<sub>H</sub> cell ratios (i.e., via either allogenic T<sub>REG</sub> cell transfer or in vivo expansion of T<sub>REG</sub> cells) or ATP/ADP depletion-dependent reduction of neutrophil numbers (i.e., using recombinant CD39 delivery techniques) could be valuable, either singlehandedly or in combination with AngII route inhibitors, to prevent cardiorenal fibrosis and the ensuing end-organ disease in patients with AngII-dependent hypertension.

Our results also suggest that previous findings regarding the positive effect of MMF and other immunosuppressants in hypertension-driven fibrosis (12, 13, 21) can be explained by the enhancement of this T<sub>REG</sub>-dependent mechanism. Consistent with this, it has been shown that some of those immunosuppressants promote the peripheral expansion of T<sub>REG</sub> cells (43, 44). The mechanism reported here could be also involved in both the fibrotic and hypertensive effects induced by either the transplantation and depletion of both conventional T and T<sub>REG</sub> cells due to the changes in T<sub>REG</sub>/T<sub>H</sub> ratios intrinsically associated with these treatments (9, 10, 15-17). Interestingly, it has been reported that the MMF treatment promotes neutropenia in some tissue-transplanted patients (45-47). Based on the
present results, it would be interesting to verify whether this collateral effect could be caused by a similar mechanism to the one described here. It would be also worth pursuing a better understanding of the proapoptotic effect of MMF on neutrophils. Given that this effect is only observed in an AngII- and AT$_1$ receptor-dependent manner, we surmise that MMF must create some metabolic imbalance in stimulated neutrophils. Whether this is done via in-target (inhibition of inosine monophosphate dehydrogenase) or off-target effects of the drug, remains to be determined. Effects of MMF in non lymphoid cells have been previously described (39).

Interestingly, we have observed that the lack of tissue fibrosis found in TKO and $Vav1^{-/-}$ mice is always associated with the development of milder hypertensive states during AngII-driven hypertension conditions (Fig. 9L). Our interpretation is that this latter effect is a downstream consequence of the lack of fibrosis and the subsequent preservation of normal renal functions in these mice. However, given the highly intertwined connections between these two pathophysiological programs, it could be also argued that the mild hypertensive conditions are in fact responsible for the antifibrotic protection exhibited by these two mouse strains. Although it is difficult to formally exclude this possibility at the experimental level, we do not believe this alternative scenario is possible because: (i) We have observed that the arterial blood pressure of AngII-infused WT and $Vav1^{-/-}$ mice is rather similar during the early phases of the AngII infusion (0 to six weeks) and only become statistically different upon the long-term exposure of animals to this vasopressor (10-15 weeks). Thus, from a kinetic point of view, it does not seem that the differential hypertension values could be the original cause of the lack of fibrosis in $Vav1$-deficient
mice. (ii) We have found that the overall blood pressure levels shown by AngII-infused
Vav1\textsuperscript{−/−} and TKO mice are similar to those present in animals that do develop a full fibrotic
response (i.e., untreated DKO mice). Consistent with this, we also have found that TKO
(both untreated and AngII-treated) and Vav1\textsuperscript{−/−} (AngII treated) mice do develop other
hypertension-associated dysfunctions such as, for example, the thickening of the arterial
media wall. (iii) We have seen that the time of divergence of blood pressure values between
AngII-infused WT and Vav1\textsuperscript{−/−} mice matches the time when the controls have already
developed cardiorenal fibrosis (Fig. 2H), thus suggesting that the milder hypertension
shown by untreated TKO, AngII-treated TKO, and AngII-infused Vav1\textsuperscript{−/−} mice could be an
effect derived from the lack of fibrosis development in these animals. This could originate
from either a direct pressor effect of the lack of fibrosis or by the collateral engagement of
other hypertension-linked processes that contribute to the pressure-natriuresis mechanism
involved in long-term pressure control (i.e., production of aldosterone and vasopressin)
(48). Regardless of the initial cause of the event, it is clear that the renal dysfunctions and
the hypertensive state will eventually collaborate in a mutually reinforcing loop to
ultimately determine the final arterial pressure and fibrosis state found in each of the
control and test strains analyzed in the present study.

Outside the cardiovascular field, this work has also given information about the role
of Vav proteins in lymphocyte subpopulations and neutrophils. Thus, we have observed
that Vav proteins seem to be dispensable for both T\textsubscript{REG} cell development and effector
functions at least in the pathophysiologial context studied in this work. This is in sharp
contrast to the critical roles played by these proteins during the stepwise development,
selection, and final effector stages of conventional CD4+ and CD8+ T cells (27, 28). Given
that T_{REG} cells require low T cell receptor signals to undergo effective positive selection
(49), this result suggests that Vav proteins probably contribute to establish optimal
thresholds of T cell receptor signaling during the intrathymic selection process rather being
involved in yes/no digital decisions. The increased T_{REG}/T_{H} cell ratios present in these mice
may also explain the lack of autoimmunity present in Vav family deficient mice despite
their defects in negative selection (50). Such property is specific for Vav proteins, because
the elimination of other proximal T cell receptor signaling elements such as Zap70 and Lat
does block T_{REG} cell development in the thymus (51, 52). We have also seen that the lack of
Vav1 does not seem to significantly affect the overall functionality of neutrophils.
Consistent with this, we have observed that the renal neutropenia found in these mice can
be restored upon the immunodepletion of iT_{REG} cells. Conversely, a similar neutropenic
effect can be induced when WT mice are injected with iT_{REG} cells. Additional experiments
have also revealed that the AngII-stimulation of neutrophil migration and ROS production
is Vav1-independent (SF, unpublished data). These data are in agreement with previous
results from H. Welch’ lab indicating no major defects in the in vivo biological properties
of Vav1−/− neutrophils (53, 54). It would be important, in any case, to continue the analyses
of Vav1−/− T_{REG} cells and neutrophils to assess whether this protein plays some catalysis-
dependent or independent roles in any of these cell types.

Taken together, these results provide a new biological perspective to understand the
early steps involved in the ontogeny of cardiorenal fibrosis, one of the most fatal
comorbidities associated with essential hypertension (5, 6). Given that other fibrotic- or
$T_{REG}$ cell-dependent diseases in liver, lung, and muscle are influenced by AngII (8, 37), it would be interesting to investigate whether the $T_{REG}$ cell/neutrophil axis reported here could also play protective roles against those conditions.
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COMPETING INTERESTS

None.

AUTHOR CONTRIBUTIONS

SF participated in all the experimental work, analyzed data, and contributed to artwork design and manuscript writing. MM-M performed animal-based work, designed experiments, and analyzed data. JR-V analyzed flow cytometry data and carried out histochemical analyses. MP and JML-N performed experiments related to in vivo renal functions. MAS and MJA collaborated in blood pressure determination experiments. AM-
M and PM carried out work related to the characterization of kidney-resident hematopoietic cells. CG-M performed and analyzed immunohistochemical analyses in tissue sections. BA helped in cell transplantation experiments with Ly5 mice. XRB designed the work, analyzed data, wrote the manuscript, and carried out the final editing of figures.
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<td>70.25±1.19</td>
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<td>Thymus</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>9.71±0.53</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;Foxp3&lt;sup&gt;+&lt;/sup&gt; T&lt;sub&gt;REG&lt;/sub&gt; cells</td>
<td>0.49±0.04</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>6.57±2.03</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; thymocytes</td>
<td>75.3±1.96</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD8&lt;sup&gt;+&lt;/sup&gt; thymocytes</td>
<td>8.24±0.86</td>
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* Percentage (in %) of indicated cells in the total population analyzed were measured by flow cytometry as indicated in Experimental Procedures. Statistically significant variations are shown using red bold type. * and †, P ≤ 0.05 relative to WT and DKO mice, respectively (n = 6 mice/genotype). ND, not determined.
FIGURE 1. Vav family TKO mice show protection against heart hypertrophy and cardiorenal fibrosis. (A) Mean arterial pressure evolution in indicated mice. *, $P \leq 0.05$; ***, $P \leq 0.001$ ($n = 8$). (B) Examples of aorta (left panels), heart ventricles (second panels from left), left ventricle (third panels from left) and kidney (right panels) sections obtained from four-month-old mice of indicated genotypes (left). Sections were stained as indicated (bottom). Sirius-red-stained fibrotic deposits in interstitial areas are seen as dark pink areas (fourth panel from left). amw, aorta media wall; lv, left ventricle; rv, right ventricle. Scale bars, 100 µm. Similar results were obtained using serial sections and independent mice ($n = 4$). (C to F) Status of cardiovascular (C and D) and cardiorenal (E and F) parameters in mice of indicated genotypes. LV, left ventricle. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ relative to either control or indicated experimental pair (in brackets) ($n = 4$). (G) Example of kidney sections from mice of indicated genotypes (top). WT mice exhibit normal glomeruli (g) and tubules (t) whereas DKO animals display typical signs of glomerular sclerosis, cytoplasmic vacuolar accumulation (v) in necrotic tubules, and protein casts (c). Erithrocytes (e) are seen as red spots in all sections. Scale bars, 50 µm. (H to J) Daily urine production ($H, n = 6$), urine protein content ($I, n = 6$), and creatinine clearance rates ($J, n = 4$) in mice of indicated genotypes. *, $P \leq 0.05$ relative to either control or indicated experimental pair (in brackets). (K) Levels of Lcn2 transcript in kidneys of indicated animals. *, $P \leq 0.05$ relative to either control or indicated experimental pair (in brackets) ($n = 4$). (L to N) Area under the curve (a.u.c.) values (L) and renal parameters (M and N) in mice of indicated genotypes after being infused with (+) or without (−) AngII. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ relative to either control or indicated experimental pairs (in
(n = 4). NS, not statistically significant. Error bars in panels of this figure and subsequent ones represent the S.E.M.
FIGURE 2. The Vav1 gene deficiency protects against AngII-triggered cardiorenal dysfunctions. (A to E) Status of indicated cardiovascular (A to C) and renal (D and E) parameters in mice of indicated genotypes after being infused with (+) or without (−) AngII. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 (n = 10). (F and G) Mean arterial pressure evolution (F) and a.u.c. values (G) in indicated mouse strains and treatments. *, P ≤ 0.05; ***, P ≤ 0.001 (n = 6). (H) Kinetics of cardiorenal fibrosis development in AngII-treated WT mice. *, P ≤ 0.05 (n = 4). (I and J) Evolution (I) and a.u.c. (J) of mean arterial pressure in mice of indicated genotypes in the presence or absence of L-NAME. ***, P ≤ 0.001 (n = 4). (K to N) Status of indicated vascular (K), heart (L,M) and renal (N) parameters in WT and Vav1−/− mice at the end of L-NAME treatments. Please note that, unlike the case of AngII infusion, the oral administration of L-NAME does not induce aorta remodeling even in WT mice (K). *, P ≤ 0.05; ***, P ≤ 0.001 (n = 4). Statistical values are given relative to either untreated WT controls or indicated experimental pairs (in brackets).
FIGURE 3. MMF protects immunodeficient mice against cardiorenal fibrosis. (A to B) Cardiorenal fibrosis (A and B) and a.u.c. blood pressure levels (C) developed by Foxn1nu mice after a 14-day-long treatment with indicated agents (bottom) *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ relative to either control or indicated experimental pairs (in brackets) ($n = 6$).
**FIGURE 4.** Increased T<sub>REG</sub>/T<sub>H</sub> cell percentages protect against AngII-triggered cardiorenal fibrosis. (A) Example of purified iT<sub>REG</sub> cells used in these experiments. CD4<sup>+</sup> cells obtained from homogenized spleens and separated by flow cytometry (two left panels) were cultured with antibodies to CD3 and CD28, interleukin 2 and transforming growth factor β1. After
four days, CD25 expression was checked by flow cytometry (third panel from left) and injected into mice. Aliquots of cells were in some cases checked for Foxp3 expression (right). (B) Ratio of indicated cell populations in kidneys from animals that, upon injection of 2 x 10^5 Lyt.1^+ T_{REG} cells, were infused 24 hours later with AngII for the indicated periods of time. (C) Mean number of indicated cell populations found in kidneys from mice used in panel B (n = 4). (D to F) Cardiorenal fibrosis (D and E) and a.u.c. mean arterial pressure levels (F) present in WT mice that had been maintained under the indicated conditions for 14 days, *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 relative to either control or indicated experimental pairs (in brackets) (n = 4). (G and H) Representative example (G) and quantification (H) of kidney-resident neutrophils in kidneys from WT mice at the end of the above experiments. **, P ≤ 0.01 relative to either control or indicated experimental pairs (in brackets) (n = 4). (I) Quantification of kidney-resident neutrophils present in WT mice under the indicated treatments. *, P ≤ 0.05 (n = 4). (J) Percentage of iT_{REG} cells generated from splenic WT and Vav1^-/- CD4^+CD25^- T cells in cell culture (n = 4). (K) Extent of kidney fibrosis in AngII-infused Foxn1^-/- mice injected with the indicated iT_{REG} cells as shown in insets. *, P ≤ 0.05; **, P ≤ 0.01 relative to either control or indicated experimental pairs (in brackets) (n = 4).
FIGURE 5. The immunodepletion of T\textsubscript{REG} cells restores renal fibrosis in Vav1-deficient mice. (A) Evolution of the CD4\textsuperscript{+}CD25\textsuperscript{+} cell population in Vav1\textsuperscript{−/−} mice upon immunodepletion with antibodies to CD25. Values are given relative to cell percentages present in the same experimental time point in mice injected with a control antibody. The time of injection (t\textsubscript{0}) is indicated with an arrow. ***, P ≤ 0.001 (n = 4). (B to D) Cardiorenal fibrosis (B and C) and a.u.c. mean arterial pressure levels (D) in Vav1\textsuperscript{−/−} mice subjected to indicated experimental conditions. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 relative to either control or indicated experimental pairs (in brackets) (n = 4). (E and F) Representative example (E) and quantification (F) of neutrophil numbers present in kidneys
from \( VavI^{+/-} \) mice used in above experiments. **, \( P \leq 0.01 \); relative to either control or indicated experimental pair (in brackets) \((n = 4)\). (G) Quantification of kidney-resident neutrophils in \( VavI^{+/-} \) mice under indicated conditions. *, \( P \leq 0.05 \) \((n = 4)\).
**Figure 6**

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**FIGURE 6.** Neutrophils are involved in AngII-driven cardiorenal fibrosis. (A) Impact of the immunodepletion step in the neutrophil numbers present in WT mice. Values are given relative to numbers obtained in mice treated with a control antibody. ***, P ≤ 0.001 (n = 4).**
The time points of antibody and implantation of osmotic pumps are indicated by arrows. (B and C) Extent of cardiorenal fibrosis obtained in WT animals injected with either control or anti-Ly6G antibodies and, subsequently, infused with AngII as indicated. *, $P \leq 0.05$ relative to either control or indicated experimental pairs (in brackets) ($n = 4$). (D) a.u.c. values of mean arterial pressure increase obtained in the above experiment. *, $P \leq 0.05$; ***, $P \leq 0.001$ relative to either control or indicated experimental pairs (in brackets) ($n = 4$). (E and F) Quantification of kidney-resident macrophages (E) and CD4+ T cells (F) at the end of the above experiments. (G and H) Representative dot-plots (G) and quantification (H) of neutrophil numbers present in kidneys at the end of the above experiments. **, $P \leq 0.01$ relative to either control or indicated experimental pair (in brackets) ($n = 4$).
**FIGURE 7.** T\(_{\text{REG}}\) cells protect against hypertension-driven fibrosis in an immunosuppression-independent manner. (A to E) Renal fibrosis (A and D), kidney abundance of *Lcn2* transcripts (B), and a.u.c mean arterial pressure levels (C and E) in indicated mouse strains and treatment conditions. *, *P* ≤ 0.05; **, *P* ≤ 0.01; ***, *P* ≤ 0.001
relative to either control or indicated experimental pairs (in brackets) \((n = 4)\). (F and G) Representative dot-plots (F) and quantification (G) of kidney-resident neutrophils in mouse strains and treatment conditions. *, \(P \leq 0.05\); **, \(P \leq 0.01\); ***, \(P \leq 0.001\) relative to either control or indicated experimental pairs (in brackets) \((n = 4)\). (H and I) Number of kidney-resident and circulating neutrophils present in \(F_{oxn1}\) mice under indicated experimental conditions. *, \(P \leq 0.05\) relative to control \((n = 4)\).
**FIGURE 8.** T<sub>REG</sub> cells induce CD39-dependent neutrophil apoptosis. (A) Example of the flow cytometry purification step to obtain splenic CD4<sup>+</sup>CD25<sup>+</sup> nT<sub>REG</sub> cells used in experiments shown in this figure. (B and C) Apoptotic response (A) and ROS production (B) of neutrophils under indicated in vitro conditions. ARL, ARL67156; CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>−</sup> T cells; CD8<sup>+</sup>, CD8<sup>+</sup> T cells; CD39<sup>+</sup>, CD4<sup>+</sup>CD25<sup>−</sup>CD39<sup>+</sup> nT<sub>REG</sub> cells; CD39<sup>−</sup>, CD4<sup>+</sup>CD25<sup>−</sup>CD39<sup>−</sup> nT<sub>REG</sub> cells; Los, losartan; PD, PD123319. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001 relative to either control or indicated experimental pairs (in brackets) (n = 4). (D) Effect of T<sub>REG</sub> cell-derived culture supernatants on neutrophil chemotaxis (values obtained with media alone were given an arbitrary value of 1). *, P ≤ 0.05 relative to control (n = 4). (E) Example of the purification step used to obtain splenic CD39<sup>+</sup> and CD39<sup>−</sup> nT<sub>REG</sub> cells used in this figure. (F to H) Apoptotic response of neutrophils under indicated culturing conditions. α-CD3 and α-CD28, antibodies to mouse CD3 and CD28, respectively. **, P ≤ 0.01; ***, P ≤ 0.001 relative to either control or indicated experimental pairs (in brackets) (n = 4). (I) AngII-stimulated ROS production of neutrophils under indicated culturing conditions. **, P ≤ 0.01 relative to control (n = 4).
**FIGURE 9.** CD39\(^+\) T\(_{REG}\) cells are involved in the protection against AngII-driven cardiorenal dysfunctions. (A) Example of the flow cytometry-mediated purification of CD39\(^+\) and CD39\(^-\) iT\(_{REG}\) cells used in these experiments. (B to E) Percentage of kidney-infiltrating neutrophils (B), extent of cardiorenal fibrosis (C and D), and blood pressure levels (E) in WT mice under indicated experimental conditions. *, \(P \leq 0.05\); **, \(P \leq 0.01\); ***, \(P \leq 0.001\) relative to either control or indicated experimental pairs (in brackets) \((n = 4)\). (F to K) Percentage of kidney-infiltrating neutrophils (F, \(n = 4\)), cardiorenal fibrosis (G and H, \(n = 4\)), abundance of Lnc2 transcripts in kidney (I, \(n = 4\)), creatinine clearance rates (J, \(n = 6\)), and overall blood pressure levels (K, \(n = 4\)) in indicated mice and experimental conditions. *, \(P \leq 0.05\); **, \(P \leq 0.05\); ***, \(P \leq 0.001\) relative to either control or indicated experimental pairs (in brackets). (L) The new mechanism (green) described in this work. Inhibitors tested exclusively in vitro and in vivo are in red and blue color, respectively. Those used in both conditions are in light brown. The T\(_{REG}\)-T\(_{H}\)-neutrophil connection is proposed based on previously published data. RAS, renin-angiotensin system.