Abstract—The immunosuppressive calcineurin inhibitors cyclosporine A and tacrolimus alter T-cell subsets and can cause hypertension, vascular dysfunction, and renal toxicity. We and others have reported that cyclosporine A and tacrolimus decrease anti-inflammatory regulatory T cells and increase proinflammatory interleukin-17–producing T cells; therefore, we hypothesized that inhibition of these effects using noncellular therapies would prevent the hypertension, endothelial dysfunction, and renal glomerular injury induced by calcineurin inhibitor therapy. Daily treatment of mice with cyclosporine A or tacrolimus for 1 week significantly decreased CD4+/FoxP3+ regulatory T cells in the spleen and lymph nodes, as well as induced hypertension, vascular injury and dysfunction, and glomerular mesangial expansion in mice. Daily cotreatment with all-trans retinoic acid reported to increase regulatory T cells and decrease interleukin-17–producing T cells, prevented all of the detrimental effects of cyclosporine A and tacrolimus. All-trans retinoic acid also increased regulatory T cells and prevented the hypertension, endothelial dysfunction, and glomerular injury in genetically modified mice that phenocopy calcineurin inhibitor–treated mice (FKBP12-Tie2 knockout). Treatment with an interleukin-17–neutralizing antibody also increased regulatory T-cell levels and prevented the hypertension, endothelial dysfunction, and glomerular injury in cyclosporine A–treated and tacrolimus-treated mice and FKBP12-Tie2 knockout mice, whereas an isotype control had no effect. Augmenting regulatory T cells and inhibiting interleukin-17 signaling using noncellular therapies prevents the cardiovascular and renal toxicity of calcineurin inhibitors in mice. (Hypertension. 2017;70:183-191. DOI: 10.1161/HYPERTENSIONAHA.117.09374.) • Online Data Supplement

Key Words: antibodies, neutralizing ■ calcineurin inhibitors ■ hypertension ■ inflammation ■ lymphocytes

Calcineurin inhibitors (CNIs) are extremely important for maintenance of immunosuppression in numerous conditions including solid organ transplant and autoimmune disorders. However, a limiting factor in their use is the development of cardiovascular and renal toxicity leading to organ dysfunction and hypertension.1-4 Although new and potentially safer immunosuppressive drugs are being developed, the CNIs cyclosporine A (CsA) and tacrolimus remain the most widely used drugs for maintenance immunosuppression. Thus, it is important to rapidly develop ways to ameliorate the toxic cardiovascular and renal effects in patients on chronic CNI therapy.

One of the mechanisms by which CNIs cause cardiovascular and renal toxicity and hypertension is by altering T-cell subsets. Previous reports have described how CNIs reduce regulatory T cells (Tregs) but increase IL-17 (interleukin-17)–producing T-helper cells (Th17) despite reducing the overall number of T cells.5-12 The loss of Tregs, which secrete the anti-inflammatory cytokines TGF-β (transforming growth factor β) and IL-10, kill proinflammatory immune cells and promote immune tolerance, and the augmentation of Th17 cells that produce the potent proinflammatory and prohypertensive cytokine IL-17 together lead to organ dysfunction and hypertension.8,9 However, it is unknown whether targeting these effects can have vascular and renal protective effects and attenuate the hypertension caused by CNIs.

Autologous Treg therapy is in clinical trials and has been reported to be effective in some conditions; however, there are still major issues that need to be addressed including off-target effects, dosing, etc.13,14 Novartis’ Cosentyx (secukinumab) is the first IL-17 inhibitor to receive US Food and Drug Administration approval; however, the 2 indications are ankylosing spondylitis and psoriatic arthritis and are not cardiovascular related. Despite these promising therapies, it is unclear how noncellular therapies that affect Tregs and Th17 cells influence CNI-induced toxicity and hypertension. Therefore,
we tested whether all-trans retinoic acid (RA), reported to both increase Tregs and decrease Th17 cells,15–19 would be effective in preventing CNI-induced hypertension. We also tested whether neutralizing IL-17 could indirectly increase Tregs and have the same beneficial effects as RA. We examined whether these strategies would also be beneficial in transgenic mice that phenocopy chronic CNI treatment. These mice lack FKBP12 (FK506-binding protein 12) in endothelial and hematopoietic cells (Tie2 promoter), leading to activation of TGF-β receptors, which in T cells controls the polarization to either Tregs or Th17 cells.8,20,21 Mice were trained for this procedure for IP; Alomone, Israel), tacrolimus (10 mg/kg/d, IP; LC Laboratories) at baseline and on day 7 of daily treatment with CsA (50 mg/kg/d, IP; R&D Systems) or diluent (saline and dimethyl sulfoxide, 0.2% final concentration) as described previously.20,21 Mice were trained for this procedure on days 1, 4, and 7. Animals were anesthetized on day 8 with isoflurane and euthanized by cervical dislocation. All procedures were approved by the Institutional Animal Care and Use Committees in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Methods

Animals and In Vivo Measures and Treatments

Male C57Bl/6J mice (Jackson Laboratory) aged 10 to 18 weeks were used for the CNI treatment studies and controls in all experiments. Male FK12Tie2 KO mice were generated as described previously and were used between the ages of 10 to 18 weeks.8 All mice were maintained on a 12:12 light/dark cycle and had access to standard chow ad libitum. Tail-cuff systolic blood pressures (ITC, Inc) were measured at baseline and on day 7 of daily treatment with CsA (50 mg/kg/d, IP; Alomone, Israel), tacrolimus (10 mg/kg/d, IP; LC Laboratories) or diluent (saline and dimethyl sulfoxide, 0.2% final concentration) as described previously.20,21 Mice were trained for this procedure for 3 days before baseline measurements. Some mice were given daily, IP injections of RA (300 µg/mouse; Sigma). Other control, CsA-treated, tacrolimus-treated, and FK12Tie2 KO mice were given IP injections of either an IL-17–neutralizing antibody (100 µg/mouse; R&D Systems) or the isotype control (100 µg/mouse; R&D Systems) on days 1, 4, and 7. Animals were anesthetized on day 8 with isoflurane and euthanized by cervical dislocation. All procedures were approved by the Institutional Animal Care and Use Committees in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Flow Cytometry

Spleens, lymph nodes (inguinal, cervical, axillary, and mediastinal), and blood were harvested from the mice. Single-cell suspensions were generated from the spleen and lymph nodes by homogenization using a 40 µm sterile strainer on a Petri dish in 10 mL of phosphate buffered saline containing 1% fetal bovine serum and then treated with a red blood cell lysis buffer according to the manufacturer’s protocol (BD Pharm Lyse, BD Pharmingen). Cells were washed with phosphate buffered saline+1% fetal bovine serum, counted, and resuspended in phosphate buffered saline+1% fetal bovine serum. Lymphocytes were isolated from heparinized blood using Lympholyte M Cell Separation Media (Cedar Lane) according to the manufacturer’s protocol. One million cells from the spleen, lymph nodes, or blood were stained with antimonode CD3e (BD Pharmingen), antimouse CD4 (BD Pharmingen), or isotype controls for 1 hour at 4°C in the dark. The cells were then fixed and permeabilized with the BD CytoFix/Cytoperm Fixation/Permeabilization Kit (BD Pharmingen). Intracellular staining was performed using antimouse FoxP3 (eBioscience). Flow cytometry was performed on a BD FACS Canto II and analyzed using FlowJo software. A negative control along with an isotype-matched control was used to determine the positive and negative cell populations for each sample. CD3+, CD3+/CD4+, CD3+/CD8+, and CD4+/FoxP3+ lymphocytes were quantified and averaged as described previously.8 Data are expressed as % of lymphocytes for spleen and lymph node and % of leukocytes for blood.

Vascular Reactivity

Vascular reactivity was measured in endothelium-intact aortas as described previously.3,9,21–22 Concentration-force curves were generated for the endothelium-dependent dilator acetylcholine and the endothelium-independent dilator sodium nitroprusside after contraction with an EC50 concentration of phenylephrine.

Immunoblotting

For protein analyses from whole aortic tissue, individual mouse aorta were powdered using a mortar and pestel and homogenized in Cell Lysis Buffer (Cell Signaling) containing 1 mmol/L phenylmethanesulfonyl fluoride (Sigma). The lysates were then collected by pelleting the cell debris at 14,000 rpm for 10 minutes at 4°C. Total protein concentration was determined by Bradford Assay (BioRad). Protein lysates (50 µg per lane) were separated by 4% to 12% SDS–PAGE (Invitrogen) and transferred onto a nitrocellulose membrane (BioRad). The membranes were blocked at room temperature with Blocking Buffer (LI-COR Biosciences) for 1 hour, incubated with appropriate antibodies (in LI-COR blocking buffer) overnight at 4°C, and washed 3 times with tris-buffered saline with Tween 20. Secondary antibodies (1:10000 in 50% blocking buffer/50% TBST) consisted of antirabbit and antimouse IgGs conjugated to Alexa-Fluor 680 and IR800Dye (LI-COR Biosciences). The blots were probed for fibronectin (1:1000; Abcam) and β-actin (1:5000; Sigma) and were identified simultaneously (800 and 700 nm wavelengths, respectively) using near-infrared visualization (Odyssey System; LI-COR Biosciences). Densitometry was performed using the Odyssey software.

Histology and Morphometric Analysis

The mice were anesthetized by isoflurane and euthanized by cervical dislocation. Left kidneys were isolated, decapsulated, and placed in 10% formalin. Sections (3–5 µm thick) were obtained on a microtome, deparaffinized, and stained with hematoxylin and eosin. Three blinded investigators quantitated glomerular injury. The investigators scored 15 glomeruli per tissue section on a 0 to 4 scale, where 0=normal size, normal mesangium, and no congestion; 1=minimal hypertrophy, mesangial expansion, and congestion; 2=mild hypertrophy, mesangial expansion, and congestion; 3=moderate hypertrophy, mesangial expansion, and congestion; and 4=severe hypertrophy, mesangial expansion, and congestion. A consensus score was recorded for each glomeruli, and statistics were run on the mean glomerular injury index for each group as calculated by [(0×N0)+(1×N1)+(2×N2)+(3×N3)+(4×N4)]/15, where N0 to N4 are numbers of glomeruli with scores of 0 to 4, respectively, as described previously.23

Statistical Analyses

Results are presented as mean±SEM or mean±SEM. The 2-tailed Student t test was used to compare variables between 2 groups. For multiple comparisons, either a 2-way ANOVA or a repeated-measures 2-way ANOVA (main effects: group [Con, CsA, tacrolimus, and FK12Tie2 KO] and treatment [Veh, RA, isotype neutralizing antibody [nAb], and IL-17nAb]) was used following the Student–Newman–Keuls post hoc test. The significance level was set at 0.05. All analyses were performed using SigmaStat 3.5 software.

Results

Retinoic Acid Prevents the Decrease in Regulatory T Cells in CNI-Treated and FKBP12-Tie2 KO Mice

Mice treated daily with CsA or tacrolimus for 1 week, and untreated FKBP12-Tie2 KO mice, had significantly decreased levels of CD4+/FoxP3+ Tregs in the spleen (Figure 1A) and lymph nodes (Figure 1B) compared with vehicle-treated mice (all P<0.05 versus controls). There were no significant group×treatment interactions. Daily
treatment with RA for 7 days prevented the significant decrease in CD4+/FoxP3+ Treg levels in both the spleen (Figure 1A) and lymph nodes (Figure 1B) of CsA-treated, tacrolimus-treated, and FKBP12-Tie2 KO mice (all \( P > 0.05 \) versus control+RA). Representative dot plots for each group are presented in Figure 1A and 1B.

To confirm that the dosages of CsA and tacrolimus were immunosuppressive and thus clinically relevant, we measured CD3+, CD3+/CD4+, and CD3+/CD8+ T cells in the blood by flow cytometry. Circulating CD3+ T cells were decreased significantly in CsA-treated mice and tacrolimus-treated mice compared with vehicle-treated mice (% of leukocytes: control=50±1%, CsA=25±3%, tacrolimus=27±3%; both \( P < 0.05 \) versus control; Figure S1 in the online-only Data Supplement). FKBP12-Tie2 KO mice on the contrary had normal levels of circulating CD3+ T cells (45±6%; \( P > 0.05 \) versus control; Figure S1). With respect to CD3+/CD4+ T cells, CsA-treated and tacrolimus-treated mice had significantly reduced levels in their circulation while FKBP12-Tie2 KO mice had normal levels (% of leukocytes: control=30±1%, CsA=18±2%, tacrolimus=18±1%, FKBP12-Tie2 KO=32±5%; CsA and tacrolimus \( P < 0.05 \) versus control; Figure S1). Finally, circulating CD3+/CD8+ T cells were decreased significantly in CsA-treated, tacrolimus-treated, and FKBP12-Tie2 KO mice compared with control mice (% of leukocytes: control=16±1%, CsA=7±1%, tacrolimus=6±1%, FKBP12-Tie2 KO=9±2%; all \( P < 0.05 \) versus control; Figure S1).

**Retinoic Acid Prevents the Development of Hypertension and Endothelial Dysfunction in CNI-Treated and FKBP12-Tie2 KO Mice**

Daily treatment of control mice with either CsA or tacrolimus for 1 week significantly increased systolic blood pressure (SBP) compared with vehicle-treated mice (control=98±2 mmHg, CsA=129±3 mmHg, tacrolimus=145±3 mmHg; all \( P < 0.05 \) versus control; Figure 2A). Untreated FKBP12-Tie2 KO mice also exhibited hypertension (FKBP12-Tie2 KO=140±2 mmHg; Figure 2A), confirming our previous report.\(^8\) Daily RA treatment for 7 days prevented the development of hypertension in CsA-treated and tacrolimus-treated mice (SBP: CsA+RA=101±2 mmHg, tacrolimus+RA=98±1 mmHg; both \( P > 0.05 \) versus control+RA; Figure 2A). The same RA treatment also normalized SBP in FKBP12-Tie2 KO mice (103±3 mmHg; \( P > 0.05 \) versus control+RA) while having no effect on SBP in control mice (105±4 mmHg; Figure 2A). There were no significant group×treatment interactions.

CNIs are known to induce endothelial dysfunction, and this was evident in CsA-treated and tacrolimus-treated mice and in FKBP12-Tie2 KO mice.\(^8\) CNI treatment significantly decreased aortic endothelium-dependent relaxation responses (maximal acetylcholine-induced relaxation: control=67±3%, CsA=25±2%, tacrolimus=22±4%; both \( P < 0.05 \) versus control; Figure 2B), and decreased aortic relaxation responses were confirmed in untreated FKBP12-Tie2 KO mice (30±4%; \( P < 0.05 \) versus control; Figure 2B).\(^8\)

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**Figure 1.** Retinoic acid prevents decreased regulatory T cells in calcineurin inhibitor (CNI)–treated and FKBP12-Tie2 knockout (KO) mice. Spleens and lymph nodes were isolated from vehicle-treated (CON), cyclosporine A–treated (CsA), tacrolimus-treated (TAC), and FKBP12-Tie2 KO (FK12Tie2 KO) mice and the same groups given retinoic acid (RA) daily and processed for flow cytometry. Splenic (A) and lymph node (B) CD4+/FoxP3+ regulatory T cells (Tregs) were measured as a percentage of live lymphocytes based on isotype gating. Results expressed as mean±SEM. *\( P < 0.05 \) vs CON and \( n=4 \) to 8 mice in each group. There were no significant group×treatment interactions as determined by 2-way ANOVA.
There were no differences in aortic endothelium-independent relaxation responses to sodium nitroprusside in any of the groups (Figure 2C). Daily RA treatment prevented the endothelial dysfunction in all groups of mice (maximal acetylcholine-induced relaxation: control+RA=68±2%, CsA+RA=69±4%, tacrolimus+RA=70±5%, FKBP12-Tie2 KO=64±3%; all \( P > 0.05 \) versus control+RA; Figure 2B) while having no effect on aortic endothelium-independent relaxation responses (Figure 2C). There were no significant group×treatment interactions.

Figure 2. Retinoic acid ameliorates the hypertension, endothelial dysfunction, and vascular injury in calcineurin inhibitor (CNI)-treated and FKBP12-Tie2 knockout (KO) mice. A, Systolic blood pressures were measured before injections on day 7 of treatment in vehicle-treated (CON), cyclosporine A–treated (CsA), tacrolimus-treated (TAC), and FKBP12-Tie2 KO (FK12Tie2 KO) mice and the same groups given retinoic acid (RA) daily. Aortic relaxation responses to acetylcholine (B) and sodium nitroprusside (C) after contraction to phenylephrine were measured in the same groups of mice. D, Aortas were isolated from vehicle-treated (CON), cyclosporine A–treated (CsA), and tacrolimus-treated (TAC) mice and the same groups given retinoic acid (RA) daily and processed. Western blots for fibronectin and actin (loading control) were imaged from the same membrane using a LiCor Odyssey. Representative blot and densitometry are presented. Results expressed as mean+SEM. *\( P < 0.05 \) vs CON, and \( n = 4 \) to 8 mice in each group. There were no significant group×treatment interactions as determined by 2-way ANOVA or repeated-measures 2-way ANOVA.

Figure 3. Retinoic acid prevents glomerular injury (glomerular hypertrophy, mesangial expansion, and congestion) in kidneys from calcineurin inhibitor (CNI)-treated and FKBP12-Tie2 knockout (KO) mice. Left kidneys were isolated from vehicle-treated (CON), cyclosporine A–treated (CsA), tacrolimus-treated (TAC), and FKBP12-Tie2 KO (FK12Tie2 KO) mice and the same groups given retinoic acid (RA) daily, formalin fixed, and paraffin embedded. Hematoxylin and eosin staining was performed and DAPI (4',6'-diamidino-2-phenylindole; blue) was used to denote nuclei. Representative light microscopy images magnified at \( \times 20 \). Scoring on a 0 to 4 scale was performed on 15 glomeruli per tissue section by 3 blinded investigators. \( n = 3 \) mice in each group. There were no significant group×treatment interactions as determined by 2-way ANOVA.
Retinoic Acid Prevents Vascular Injury in CNI-Treated Mice

CNI treatment for 1 week caused a significant 3- to 4-fold increase in the expression of fibronectin, a cellular injury marker, in aortas from CsA-treated and tacrolimus-treated mice (Figure 2D). RA treatment of CsA-treated and tacrolimus-treated mice for 1 week ameliorated the vascular injury in all groups as aortic fibronectin levels were not significantly different compared with control mice treated with RA (Figure 2D). There were no significant group×treatment interactions.

Retinoic Acid Ameliorates Renal Glomerular Injury in CNI-Treated and FKBP12-Tie2 KO Mice

CsA and tacrolimus treatment significantly increased glomerular injury as evidenced by glomerular hypertrophy, mesangial expansion, and congestion, which were also evident in untreated FKBP12-Tie2 KO mice (Figure 3). RA treatment for 1 week significantly decreased glomerular injury as it reduced glomerular size and ameliorated glomerular mesangial expansion and congestion (Figure 3). There were no significant group×treatment interactions.

IL-17 Inhibition Prevents the Decrease in Regulatory T Cells in CNI-Treated and FKBP12-Tie2 KO Mice

In addition to decreased Tregs, we have previously demonstrated that CNI-treated mice, and FKBP12-Tie2 KO mice, have increased IL-17–producing T cells. Given that levels of Tregs and Th17 cells are for the most part inversely related and that IL-17 signaling inhibits Treg polarization, we hypothesized that sequestering IL-17 and inhibiting downstream signaling would prevent the CNI-induced decrease in Tregs. Mice treated with CsA or tacrolimus daily for 1 week along with FKBP12-Tie2 KO mice were given either an IL-17–neutralizing antibody or an isotype control on days 1, 4, and 7. CsA-treated, tacrolimus-treated, and FKBP12-Tie2 KO mice given the isotype control antibody still had significantly decreased levels of Tregs in their spleens (Figure 4A) and lymph nodes (Figure 4B) compared with vehicle-treated mice given the isotype antibody. However, IL-17–neutralizing antibody treatment prevented the decrease in splenic (Figure 4A) and lymph node (Figure 4B) levels of Tregs. There were no significant group×treatment interactions.

IL-17 Inhibition Prevents the Development of Hypertension and Endothelial Dysfunction in CNI-Treated and FKBP12-Tie2 KO Mice

Isotype control treatment had no effect on the development of hypertension in CsA-treated, tacrolimus-treated, and FKBP12-Tie2 KO mice (control+Iso=98±4 mm Hg, CsA+Iso=137±3 mm Hg, tacrolimus+Iso=152±4, FKBP12-Tie2 KO+Iso=140±3 mm Hg; all P<0.05 versus control; Figure 5A). However, treatment with an IL-17–neutralizing
antibody on days 1, 4, and 7 prevented the development of hypertension in CsA-treated and tacrolimus-treated mice (SBP: CsA+IL-17nAB=107±5 mmHg, tacrolimus+IL-17nAB=96±1 mmHg; both P>0.05 versus control+IL-17nAB). Additionally, IL-17–neutralizing antibody treatment was able to normalize SBP in FKBP12-Tie2 KO mice (106±4 mmHg; P>0.05 versus control+IL-17nAB) while having no effect on SBP in control mice (93±3 mmHg; Figure 5A). There were no significant group×treatment interactions.

Treatment with the isotype control antibody had no effect on the development of endothelial dysfunction induced by CNIs and in FKBP12-Tie2 KO mice (aortic maximal acetylcholine-induced relaxation: control+Iso=67±2%, CsA+Iso=33±4%, tacrolimus+Iso=28±5%, FKBP12-Tie2 KO+Iso=18±4%; all P>0.05 versus control+Iso; Figure 5B). In contrast, treatment with the IL-17–neutralizing antibody prevented the endothelial dysfunction in all groups of mice (aortic maximal acetylcholine-induced relaxation: control+IL-17nAB=68±4%, CsA+IL-17nAB=65±4%, tacrolimus+IL-17nAB=65±2%, FKBP12-Tie2 KO+IL-17nAB=67±2%; all P>0.05 versus control+IL-17nAB; Figure 5B). Neither the isotype nor the IL-17–neutralizing antibody had any effect on aortic endothelium-independent relaxation responses because no differences were observed between any of the groups (Figure 5C). There were no significant group×treatment interactions.

IL-17 Inhibition Prevents Vascular Injury in CNI-Treated Mice

Isotype antibody treatment had no effect on the increased aortic levels of fibronectin induced by CsA treatment (Figure 5D) or tacrolimus treatment (Figure 5E). Treatment with the IL-17–neutralizing antibody, however, normalized aortic fibronectin levels in CsA-treated (Figure 5D) and tacrolimus-treated
IL-17 Inhibition Ameliorates Renal Glomerular Injury in CNI-Treated and FKBP12-Tie2 KO Mice

A significant increase in glomerular injury as evidenced by glomerular hypertrophy, mesangial expansion, and congestion was still observed in kidneys from CsA-treated, tacrolimus-treated, and FKBP12-Tie2 KO mice treated with an isotype antibody on days 1, 4, and 7 (Figure 6). However, IL-17–neutralizing antibody treatment significantly decreased glomerular injury as it reduced glomerular size and ameliorated glomerular mesangial expansion and congestion in all groups (Figure 6). There were no significant group×treatment interactions.

Discussion

The use of CNIs is necessary for immunosuppression in a variety of conditions including their lifelong use in allograft recipients. In T cells, inhibition of the calcium-dependent phosphatase calcineurin keeps it from dephosphorylating NFAT (nuclear factor of activated T cells), thus preventing NFAT from entering the nucleus where it transcribes IL-2, the self-proliferation signal needed for T-cell expansion and adaptive immune responses. However, in addition to reducing circulating T cells, CNIs also decrease the Treg/Th17 ratio and promote endothelial dysfunction, renal toxicity, and hypertension. Our objective was to determine whether augmenting Tregs or inhibiting IL-17 using noncellular approaches could prevent CNI-induced cardiovascular and renal toxicity. The main findings of the current study are that treatment with either RA or an IL-17–neutralizing antibody increased levels of Tregs and were able to prevent the hypertension, endothelial dysfunction, and vascular and glomerular injury caused by CNIs.

TGF-β receptor activation and subsequent SMAD2/3 signaling in CD4+ T cells polarizes them into Tregs via STAT5 activation and FoxP3 expression. However, if the proinflammatory cytokines IL-6 and IL-21 are present, then TGF-β receptor activation on CD4+ T cells induces STAT3 activation and SOCS3 inhibition leading instead to Th17 cell polarization. Both CsA and tacrolimus increase TGF-β and IL-6 levels and inflammation leading to decreased Tregs. This mechanism is supported by our findings in FKBP12-Tie2 KO mice that also exhibit increased TGF-β receptor activation, increased IL-6, decreased Tregs, endothelial dysfunction, renal injury, and hypertension. Furthermore, calcineurin A-α–deficient mice have increased TGF-β levels and develop CNI-like nephrotoxicity, whereas calcineurin A-β–deficient mice have normal levels of TGF-β and do not develop hypertension. Together, these findings suggest that both CsA and tacrolimus reduce Tregs by inducing activation of TGF-β receptors and increasing IL-6 and that augmenting Tregs may prevent the toxicity induced by CNIs.

Until autologous Treg therapy is optimized and tested in CNI-treated patients, alternative noncellular strategies that modulate T-cell polarization to increase Tregs and decrease Th17 cells may be beneficial in reducing CNI toxicity. In 2007, the vitamin A metabolite RA was reported to affect this T-cell polarization resulting in augmented Tregs and reduced Th17 cells. Since then, other groups have confirmed these effects along with the mechanisms involved. In the current study, daily RA treatment was able to fully prevent the decrease in Tregs and development of hypertension, endothelial dysfunction, and glomerular injury induced by CNIs and in transgenic mice that phenocopy CNI therapy but have normal levels of TGF-β. The beneficial effects are likely not because of improvements in calcineurin/NFAT activity because these are not inhibited in FKBP12-Tie2 KO mice, evident by their normal circulating levels of CD3+ and CD4+ T cells. RA has...
been reported to be beneficial in numerous diseases (ie, colitis, type 1 diabetes mellitus, and atherosclerosis), including various nephropathies (allograft, acute kidney injury, glomerulonephritis).18-29,33 Given its US Food and Drug Administration approval, large animal studies and clinical trials should be initiated to determine whether RA has the same beneficial effects in CNI-treated animals and humans.

CNI therapy also leads to increased Th17 cells as a result of TGF-β receptor and STAT3 activation in the presence of high levels of IL-6 and IL-21 despite a reduction in overall T cells. In support, mice with deficient TGF-β receptor activity have decreased Th17 cells.24 IL-17 is known to directly reduce endothelial NO production leading to reduced vasodilation and hypertension.3 We determined that neutralizing IL-17 in CNI-treated mice and FKBP12-Tie2 KO mice was able to, like RA, prevent the decrease in Tregs and the development of hypertension, endothelial dysfunction, and glomerular injury. Inhibition of IL-17 has been reported to be beneficial in other models of hypertension including deoxycorticosterone acetate–salt hypertension and angiotensin II hypertension.34,35 IL-17 inhibitors, receptor antagonists, and neutralizing antibodies should also be tested to determine whether they can ameliorate the cardiovascular and renal toxicity in CNI-treated large animals and humans.

We acknowledge that RA and IL-17 inhibition have effects on other cell types including vascular endothelial cells and myeloid cells, and the beneficial in vivo effects may not all be because of the augmentation of Tregs. It is also possible that both lead to decreased IL-6 levels, and a reduction in IL-6 would favor Treg polarization. Previous reports demonstrate that RA can upregulate NO production and reduce endothelin-1 gene expression in endothelial cells, which would also contribute to the improvement in endothelial function and blood pressure in CNI-treated mice and FKBP12-Tie2 KO mice.36-38 RA was also reported to be renoprotective by suppressing proinflammatory macrophage polarization after acute kidney injury.39 Nonetheless, RA’s beneficial effects on both Tregs and endothelial cells, both targets of CNIs, make it a great candidate to reduce CNI toxicity. Another limitation is that sequestering IL-17 in vivo does not imply that the sole source of IL-17 comes from CNI-induced Th17 cells as many cell types can produce IL-17. Other effects of IL-17 inhibition aside from improving vascular relaxation, such as reducing renal sodium retention,40,41 may also account for the beneficial in vivo effects. However, the focus of the current study was to determine whether treatment with an IL-17–neutralizing antibody was sufficient to prevent CNI toxicity and hypertension, which it was able to do in all 3 groups of mice.

In conclusion, noncellular therapies that alter T-cell polarization and result in increased levels of Tregs are able to prevent the hypertension, endothelial dysfunction, and glomerular injury caused by CNIs or genetic activation of TGF-β receptors in endothelial and immune cells. RA and IL-17 inhibitors reduce the cardiovascular and renal toxicity of CNIs and may be beneficial in patients treated with CNIs.

**Perspectives**

The ability to normalize Treg and IL-17 levels by noncellular therapies and fully prevent the vascular and renal toxicity, and hypertension induced by CNIs suggests that the current US Food and Drug Administration–approved RA and IL-17 inhibitor should be tested in patients undergoing chronic CNI therapy. This could be accomplished relatively quickly and may provide beneficial cardiovascular and renal effects in these patients until new immunosuppressive drugs become available.

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**Disclosures**

None.

**References**


**Novelty and Significance**

**What Is New?**

- Existing noncellular therapies are able to increase regulatory T cells and prevent the hypertension and vascular and glomerular injury caused by calcineurin inhibitors.

**What Is Relevant?**

- Calcineurin inhibitors are widely used to suppress the immune system for a variety of conditions, but their use is limited by their propensity to cause hypertension and cardiovascular and renal toxicity.

**Summary**

Therapies that result in increased levels of beneficial regulatory T cells are able to prevent the hypertension and injury to blood vessels and kidneys caused by calcineurin inhibitors.
Regulatory T-Cell Augmentation or Interleukin-17 Inhibition Prevents Calcineurin Inhibitor–Induced Hypertension in Mice

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REGULATORY T CELL AUGMENTATION OR INTERLEUKIN-17 INHIBITION PREVENTS CALCINEURIN INHIBITOR-INDUCED HYPERTENSION IN MICE

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Short Title – CNIs, Immune cells, and Hypertension

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METHODS

Animals and In vivo measures and treatments
Male C57Bl/6J mice (Jackson Laboratory; Bar Harbor, ME) aged 10-18 weeks were used for the CNI treatment studies as well as controls in all experiments. Male FK12Tie2 KO mice were generated as described previously and were used between the ages of 10-18 weeks. All mice were maintained on a 12:12 light/dark cycle and had access to standard chow ad libitum. Tail-cuff systolic blood pressures (IITC, Inc.; Woodland Hills, CA) were measured at baseline and on day 7 of daily treatment with CSA (50 mg/kg/day, i.p.; Alamone, Israel), TAC (10 mg/kg/day, i.p.; LC Laboratories; Woburn, MA) or diluent (saline and DMSO, 0.2% final concentration) as described previously. Mice were trained for this procedure for 3 days prior to baseline measures. Some mice were given daily i.p. injections of RA (300 ug/mouse/day; Sigma; St. Louis, MO). Other control, CSA-treated, TAC-treated, and FK12Tie2 KO mice were given i.p. injections of either an IL-17 neutralizing antibody (100 ug/mouse; R&D Systems; Minneapolis, MN) or the isotype control (100 ug/mouse; R&D Systems; Minneapolis, MN) on days 1, 4, and 7. Animals were anesthetized on day 8 with isoflurane and euthanized by cervical dislocation. All procedures were approved by the Institutional Animal Care and Use Committees in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Flow Cytometry
Spleens, lymph nodes (inguinal, cervical, axillary, and mediastinal), and blood were harvested from the mice. Single cell suspensions were generated from the spleen and lymph nodes by homogenization using a 40 µm sterile strainer on a petri dish in 10ml of PBS containing 1% FBS and then treated with a red blood cell lysis buffer according to the manufacturer’s protocol (BD Pharm Lyse, BD Pharmingen; San Jose, CA). Cells were washed with PBS+1% FBS, counted, and resuspended in PBS+1% FBS. Leukocytes were isolated from heparinized blood using Lympholyte M Cell Separation Media (Cedar Lane) according to the manufacturer’s protocol. One million cells from the spleen, lymph nodes, or blood were stained with anti-mouse CD3e (BD Pharmingen; San Jose, CA), anti-mouse CD4 (BD Pharmingen; San Jose, CA), or isotype controls for 1 hour at 4°C in the dark. The cells were then fixed and permeabilized with the BD CytoFix/CytoPerm™ Fixation/Permeabilization Kit (BD Pharmingen; San Jose, CA). Intracellular staining was performed using anti-mouse FoxP3 (eBioscience; San Diego, CA). Flow cytometry was performed on a BD FACS Canto II and analyzed using FlowJo software. A negative control along with and isotype-matched control was used to determine the positive and negative cell populations for each sample. CD3+, CD3+/CD4+, CD3+/CD8+, and CD4+/FoxP3+ lymphocytes and leukocytes were quantified and averaged. Data are expressed as % of total lymphocytes or leukocytes for spleens/lymph nodes and blood, respectively.

Vascular Reactivity
Vascular reactivity was measured in endothelium-intact aortas as described previously. Concentration-force curves were generated for the endothelium-dependent dilator acetylcholine (ACh) and the endothelium-independent dilator sodium nitroprusside following contraction with an EC70 concentration of phenylephrine (PE).
Immunoblotting
For protein analyses from whole aortic tissue, individual mouse aorta were powdered using a mortar and pestal and homogenized in Cell Lysis Buffer (Cell Signaling; Beverly, Massachusetts) containing 1 mM phenylmethansulfonylfluoride (Sigma; St. Louis, MO). The lysates were then collected by pelleting the cell debris at 14,000 rpm for 10 minutes at 4°C. Total protein concentration was determined by Bradford Assay (BioRad; Hercules, CA). Protein lysates (50 µg per lane) were separated by 4-12% SDS PAGE (Invitrogen) and transferred onto a nitrocellulose membrane (BioRad; Hercules, CA). The membranes were blocked at room temperature with Blocking Buffer (LI-COR Biosciences; Lincoln, NE) for 1 hour, incubated with appropriate antibodies (in LI-COR Blocking Buffer) overnight at 4°C, and washed three times with TBST. Secondary antibodies (1:10,000 in 50% Blocking Buffer/50% TBST) consisted of anti-rabbit and anti-mouse IgGs conjugated to Alexa-Fluor 680 and IR800Dye (LI-COR Biosciences; Lincoln, NE). The blots were probed for Fibronectin (1:1,000; Abcam; Cambridge, MA) and β-actin (1:5,000, Sigma; St. Louis, MO) and were identified simultaneously (800 nm and 700 nm wavelengths, respectively) using near-infrared visualization (Odyssey System, LI-COR Biosciences; Lincoln, NE). Densitometry was performed using the Odyssey software.

Histology
The mice were anesthetized by isoflurane and euthanized by cervical dislocation. Left kidneys were isolated, decapsulated, and placed in 10% formalin. Sections (3-5 µm thick) were obtained on a microtome, deparaffinized, and stained with hematoxylin and eosin.

Statistical Analyses
Results are presented as mean ± SEM. The two-tailed Student’s t-test was used to compare variables between 2 groups. For multiple comparisons, an analysis of variance was used followed by the Student's-Newman-Keuls post hoc test. The significance level was set at 0.05. All analyses were performed using SigmaStat 3.5 software.

References

Figure S1. CNI treatment decreased circulating leukocytes. Blood was obtained from control, CSA-treated, TAC-treated, and FK12Tie2 KO mice by cardiac puncture at the time of euthanization and processed for flow cytometry. CD3+, CD3+/CD4+, and CD3+/CD8+ T cells were measured and are expressed as mean + SEM as a percent of leukocytes. *p<0.05 vs. CON and n=4-8 mice in each group.