Fk506 Binding Protein 12 Deficiency in Endothelial and Hematopoietic Cells Decreases Regulatory T Cells and Causes Hypertension

Valorie L. Chiasson, Deepa Talreja, Kristina J. Young, Piyali Chatterjee, Amy K. Banes-Berceli, Brett M. Mitchell

See Editorial Commentary, pp 1058–1060

Abstract—Patients treated with the immunosuppressive drug tacrolimus (FK506), which binds FK506 binding protein 12 (FKBP12) and then inhibits the calcium-dependent phosphatase calcineurin, exhibit decreased regulatory T cells, endothelial dysfunction, and hypertension; however, the mechanisms and whether altered T-cell polarization play a role are unknown. Tacrolimus treatment of mice for 1 week dose-dependently decreased splenic CD4+FoxP3+ (regulatory T cells), increased splenic CD4+IL-17+ (T-helper 17) cells, and caused endothelial dysfunction and hypertension. To determine the mechanisms, we crossed floxed FKBP12 mice with Tie2-Cre mice to generate offspring lacking FKBP12 in endothelial and hematopoietic cells only (FKBP12EC knockout [KO]). Given the role of FKBP12 in inhibiting transforming growth factor-β receptor activation, Tie2-Cre–mediated deletion of FKBP12 increased transforming growth factor-β receptor activation and SMAD2/3 signaling. FKBP12EC KO mice exhibited increased vascular expression of genes and proteins related to endothelial cell activation and inflammation. Serum levels of the proinflammatory cytokines IL-2, IL-6, interferon-γ, IL-17a, IL-21, and IL-23 were increased significantly, suggesting a T-helper 17 cell-mediated inflammatory state. Flow cytometry studies confirmed this, because splenic levels of CD4+IL-17+ cells were increased significantly, whereas CD4+/FoxP3+ cells were decreased in FKBP12EC KO mice. Furthermore, spleens from FKBP12EC KO mice showed increased signal transducer and activator of transcription 3 activation, involved in T-helper 17 cell induction, and decreased signal transducer and activator of transcription 5 activation, involved in regulatory T-cell induction. FKBP12EC KO mice also exhibited endothelial dysfunction and hypertension. These data suggest that tacrolimus, through its activation of transforming growth factor-β receptors in endothelial and hematopoietic cells, may cause endothelial dysfunction and hypertension by activating endothelial cells, reducing regulatory T cells, and increasing T-helper 17 cell polarization and inflammation. (Hypertension. 2011;57:1167-1175.) • Online Data Supplement

Key Words: endothelium ■ hypertension ■ experimental ■ inflammation ■ lymphocytes ■ T cells ■ FK506 (tacrolimus)

Tacrolimus (FK506) is a macrolide used for maintenance immunosuppression in organ transplant recipients. Tacrolimus first binds to its intracellular target, FK506 binding protein 12 (FKBP12), and, together, they form a complex that binds and inhibits the calcium-dependent phosphatase calcineurin. The inhibition of calcineurin in T cells suppresses the immune system by preventing the gene transcription of proinflammatory cytokines and T cell proliferation; however, all of the cells express FKBP12 and calcineurin. Inhibition of these molecules in other cell types may mediate the development of endothelial dysfunction and hypertension in a majority of patients, which negatively affects allograft function and patient survival.1–7

Because tacrolimus and another calcineurin inhibitor, cyclosporine, both cause hypertension, the cellular mechanism was thought to be the inhibition of calcineurin in the kidney and the vasculature. However, FKBP12 plays an important role in a variety of cellular functions, including the isomerization of numerous proteins, regulation of intracellular calcium channel gating, and growth factor receptor inhibition.8–12 FKBP12 binds transforming growth factor-β (TGF-β) receptor I, and removal of FKBP12 from the TGF-β...
receptor leads to activation and downstream signaling mediated by SMAD2/3. In T cells, TGF-β receptor activation and SMAD2/3 signaling polarize naïve CD4+ T cells into either anti-inflammatory regulatory T cells (Tregs) or pro-inflammatory, interleukin (IL) 17-producing T cells (Th17 cells), depending on the cytokine milieu. A pro-inflammatory state is in which increased levels of IL-6, which can be produced by activated endothelial cells, coupled with TGF-β receptor and signal transducer and activator of transcription (STAT) 3 activation shifts naïve T-cell polarization to Th17 cells instead of Tregs, which are induced by TGF-β receptor and STAT5 activation. Thus, cross-talk between activated endothelial cells and T cells involving various cytokines plays a large role in Treg/Th17 cell polarization, immune responses, and possibly the development of hypertension.

Patients treated with tacrolimus exhibit decreased levels of Tregs, which may result from altered CD4+ T cell polarization mediated by TGF-β receptor activation and subsequent SMAD2/3 signaling in hematopoietic cells. Although tacrolimus reduces the overall number of T cells because of the inhibition of calcineurin, it is possible that the remaining T cells differentiate into Th17 cells, which release the potent pro-inflammatory cytokine IL-17, which is elevated in various forms of hypertension. Whether this occurs in vivo is unknown. We hypothesized that tacrolimus decreases Tregs and increases Th17 cell polarization, which contribute to the development of endothelial dysfunction and hypertension. If this is true, then in the absence of calcineurin inhibition, one would expect that conditional TGF-β receptor activation in endothelial and hematopoietic cells would lead to increased levels of Th17 cells, inflammation, endothelial dysfunction, and hypertension. To test this hypothesis we generated mice lacking FKBP12 in endothelial and hematopoietic cells to determine the role of TGF-β receptor activation in endothelial function, T-cell polarization, and blood pressure regulation.

Methods

An expanded Materials and Methods section can be found in an online Data Supplement, available at http://hyper.ahajournals.org.

Animals and In Vivo Measures and Treatments

Male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) aged 10 to 18 weeks were used for the tacrolimus treatment studies, as well as controls, in all of the experiments. Male Fkbp12 (lox) mice (generously provided by Dr Susan Hamilton, Baylor College of Medicine, Houston, TX, and described previously) were crossed with Tie2-Cre mice obtained from the Jackson Laboratory. Tie2 expression is restricted to endothelial and hematopoietic cells. Male Tie2-Cre+;Fkbp12lox/lox (FKBP12EC knockout [KO]) mice were used between the ages of 10 to 18 weeks. Tail-cuff systolic blood pressures were measured at baseline and on day 7 of daily treatment with tacrolimus (1 or 10 mg/kg per day, IP; LC Laboratories, Woburn, MA) or diluent (saline and dimethyl sulfoxide, 0.2% final concentration), as described previously. Animals were anesthetized with isoflurane and euthanized by cervical dislocation. All of the 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

Splenocytes were isolated followed by lysis of red blood cells according to the manufacturer’s protocol (BD Pharm Lyse, BD Pharmingen). The splenocytes were then fixed, permeabilized with the BD CytoFix/CytoPerm Fixation/Permeabilization kit (BD Pharmingen), and stained according to the manufacturer’s protocol. Flow cytometry and data analysis were performed using an Accuri C6 flow cytometer and C-Flow Plus software (Ann Arbor, MI). Data are expressed as the percentage of total splenocytes.

Serum Cytokine Array

Sera was obtained from control and FKBP12EC KO mice at the time of euthanization and used to measure 12 different proinflammatory and anti-inflammatory cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-23, interferon-γ, tumor necrosis factor-α, and TGF-β) according to the manufacturer’s protocol (SABiosciences, Frederick, MD). Serum IL-21 was measured using an ELISA kit according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN).

Vascular Reactivity

Vascular reactivity was measured in endothelium-intact aortas as described previously.

Immunoblotting

Immunoblotting was performed on aortas and spleens from control and FKBP12EC KO mice as described previously.

Real-Time Quantitative PCR

Aortic cDNA from control and FKBP12EC KO mice was used in a Mouse Endothelial Cell Biology RT2 Profiler PCR Array (SABiosciences), whereas blood cDNA was used in a Mouse Th17 for Autoimmunity and Inflammation RT2 Profiler PCR Array (SABiosciences). Both arrays were performed according to the manufacturer’s protocol and used the housekeeping genes heat shock protein 90, GAPDH, and β-actin for normalization, as well as 3 reverse-transcription controls and 3 positive PCR controls.

Pulmonary Endothelial Cells

Pulmonary endothelial cells were isolated from control and FKBP12EC KO mice, purified using PECAM antibodies (BD Biosciences) conjugated to magnetic beads, and then cultured for 2 passages. Protein levels of Fkbp12 and Fkbp12.6 were measured as described above.

Statistical Analyses

Results are presented as mean±SEM. The 2-tailed Student t test was used to compare variables between 2 groups. For multiple comparisons, an ANOVA was used, followed by the Student-Newman-Keuls post hoc test. The significance level was set at 0.05. All of the analyses were performed using SigmaStat 3.5 software.

Results

Endothelial Dysfunction, Hypertension, and Altered T-Cell Polarization in Tacrolimus-Treated Mice

Daily treatment of control mice with either low-dose (1 mg/kg) or high-dose (10 mg/kg) tacrolimus for 1 week dose-dependently decreased aortic endothelium-dependent relaxation responses (maximal acetylcholine-induced relaxation: control, 74±4% versus low dose, 47±5% versus high dose, 17±2%; Figure 1A) but had no effects on endothelium-independent relaxation responses (Figure 1B). Consistent with the known hypertensive effects of tacrolimus, systolic blood pressure increased significantly in a dose-dependent manner (control, 103±3 mm Hg versus low dose, 133±5 mm Hg versus high dose, 149±6 mm Hg; Figure 1C).
mice compared with controls (Figure 2). In addition, tacrolimus treatment dose-dependently increased the percentage of CD4\(^+\)/IL-17\(^+\) splenocytes in mice (Figure 2). There were no differences in the percentages of Th1 (CD4\(^+\)/interferon-\(\gamma\)\(^+\)) or Th2 (CD4\(^+\)/IL-4\(^+\)) cells in the spleens of tacrolimus-treated mice compared with controls (data not shown).

### SMAD2/3 and Endothelial Cell Activation in FKBP12EC KO Mice

To determine the mechanisms by which tacrolimus causes endothelial dysfunction and hypertension, we generated mice lacking FKBP12 in endothelial and hematopoietic cells. FKBP12EC KO mice exhibited markedly decreased FKBP12 protein expression in pulmonary endothelial cells (Figure S1A, available in the online Data Supplement at http://hyper.ahajournals.org). The residual FKBP12 observed may be attributed to a small amount of contamination by other cell types, because the pulmonary endothelial cells were purified 2 times, as well as the <100% efficiency of Tie2-Cre recombinase activity. The genetic deficiency of FKBP12 increased TGF-\(\beta\) receptor activation demonstrated by increased SMAD2/3 phosphorylation and a marked increase in SMAD2/3 expression (Figure S1B). Protein expression of intercellular adhesion molecule, as well as STAT3 Tyr phosphorylation, were also increased significantly in aortas from FKBP12EC KO mice compared with controls (Figure S2A and S2B), which suggests that FKBP12 removal from TGF-\(\beta\) receptors leads to endothelial cell activation.

### Increased Inflammation in FKBP12EC KO Mice

Because tacrolimus increased Th17 cells and FKBP12 deficiency increased endothelial cell activation, we hypothesized that these would be associated with increased inflammation. Aortic expression of proinflammatory genes Bcl-like 1, Birc2, Cxcl2, and Cx3c11 (fractalkine) were increased significantly in FKBP12EC KO mice compared with controls (Figure S3). However, there was no difference in inducible NO synthase mRNA expression between FKBP12EC KO mice and controls (FKBP12EC KO: -1.25-fold versus controls; \(P>0.05\)). Serum levels of the proinflammatory cytokines IL-2, IL-6, and interferon-\(\gamma\) were increased significantly in FKBP12EC KO mice compared with controls (Figure 3A). In addition, serum levels of the Th17 cell-related cytokines IL-17a, IL-21, and IL-23 were also increased significantly in FKBP12EC KO mice (Figure 3B). There were no differences in serum levels of the proinflammatory cytokines IL-5, IL-12, and tumor necrosis factor-\(\alpha\) or in serum levels of the anti-inflammatory cytokines IL-4, IL-10, IL-13, and TGF-\(\beta\) between FKBP12EC KO and control mice.

The proinflammatory response includes increased angiogenesis to augment the number of immune cells to the site of infection or injury. In support of the proinflammatory status in FKBP12EC KO mice, aortic gene expression of the vascular endothelial growth factor family members Flt-1, Plgf, Kdr, and Tek were increased significantly in FKBP12EC KO mice compared with controls (Figure S4A). In addition, there was an \(\approx\)2-fold increase in aortic protein expression of vascular endothelial growth factor-A in FKBP12EC KO mice compared with controls (Figure S4B).
Figure 2. Altered T-cell polarization in tacrolimus-treated mice. Mice treated with tacrolimus for 1 week exhibited a dose-dependent decrease in splenocyte CD3⁺/CD4⁺ T cells and regulatory T cells (Tregs; CD4⁺/FoxP3⁺). Th17 T cells (CD4⁺/interleukin 17a⁺) increased in a dose-dependent manner in spleens of tacrolimus-treated mice. **A**, Density plots from flow cytometry studies and (**B**) mean data for each group. Results are expressed as mean±SEM. *P<0.05 vs control, #P<0.05 vs FK506-LD. LD indicates low dose (1 mg/kg per day); HD, high dose (10 mg/kg per day); and n=6 to 10 in each group.
Altered T-Cell Polarization in FKBP12EC KO Mice

Based on our findings that tacrolimus alters Treg/Th17 cell polarization, together with elevated Th17-related serum cytokines in FKBP12EC KO mice, we examined whether FKBP12EC KO mice also exhibit Treg/Th17 cell imbalance. Figure 4A demonstrates that CD3+/CD4+ T cells were increased significantly in spleens from FKBP12EC KO mice compared with controls. In FKBP12EC KO mice, the percentage of CD4+/FoxP3+ splenocytes was decreased significantly, whereas the percentage of CD4+/IL-17+ splenocytes was increased significantly compared with controls (Figure 4B). There were no differences in the percentages of Th1 (CD4+/ interferon-γ+) or Th2 (CD4+/IL-4+) cells in the spleens of FKBP12EC KO mice compared with controls (data not shown). STAT5 phosphorylation, which aids in the induction of Tregs, was decreased significantly (Figure 4C), whereas STAT3 Tyr phosphorylation, which aids in the induction of Th17 cells, was increased significantly (Figure 4D) in spleens from FKBP12EC KO mice compared with controls. To examine changes in Th17-related genes in circulating immune cells, we performed a PCR array using blood from FKBP12EC KO and control mice. The Table displays the 10 highest Th17-related genes that were differentially expressed. The only gene of these listed that was decreased significantly in the blood of FKBP12EC KO mice compared with controls was SOCS3, which is known to negatively affect Th17 polarization.16

Endothelial Dysfunction and Hypertension in FKBP12EC KO Mice

Consistent with previous findings in humans and animals treated with tacrolimus, FKBP12EC KO mice had significantly decreased aortic endothelium-dependent relaxation responses (maximal acetylcholine-induced relaxation: wild type, 77±6% versus FKBP12EC KO, 31±6%; Figure 5A) but no differences in endothelium-independent relaxation responses (Figure 5B). To determine whether the decreased endothelium-dependent relaxation responses in FKBP12EC KO mice were associated with changes in NO, cyclooxygenase-derived metabolites, and/or endothelium-derived hyperpolarizing factor, we measured relaxation responses following N\textsuperscript{G}-nitro-L-arginine methyl ester (NO synthase inhibitor), indomethacin (cyclooxygenase inhibitor), and both N\textsuperscript{G}-nitro-L-arginine methyl ester and indomethacin. Figure S5A demonstrates that aortic relaxation responses were completely abolished by N\textsuperscript{G}-nitro-L-arginine methyl ester (please see the online Data Supplement). Indomethacin had no significant effects on aortic relaxation responses in control mice but tended to mildly increase relaxation in aortas from FKBP12EC KO mice, suggesting increased thromboxane production; however, this did not reach statistical significance (Figure S5B). Aortas tended to contract to acetylcholine in the presence of both N\textsuperscript{G}-nitro-L-arginine methyl ester and indomethacin, which supports a lack of an endothelium-derived hyperpolarizing factor component in the aorta (Figure S5C). Overall, it appears that NO production is decreased, and this is likely the main cause of the endothelial dysfunc-

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**Figure 3.** Increased proinflammatory cytokine levels in serum from FKBP12EC knockout (KO) mice. A, Serum levels of interleukin (IL) 2, IL-6, and interferon (IFN)-γ were increased significantly in FKBP12EC KO mice compared with controls. B, Serum levels of IL-17A, IL-21, and IL-23 were also increased significantly in FKBP12EC KO mice. Results are expressed as mean±SEM as a percentage of control (n=5 in each group). \( ^* P<0.05 \) vs control.
tion in FKBP12EC KO mice; however, studies are needed in other vascular beds to determine whether this holds true. Furthermore, to determine whether a decrease in NO leads to increased superoxide and oxidative stress, we examined whether the decreased aortic relaxation responses of aortas from FKBP12EC KO mice could be restored by polyethylene glycol superoxide dismutase. Figure S5D demonstrates that polyethylene glycol superoxide dismutase restored aortic relaxation responses of FKBP12EC KO aortas to that of controls while having no effect in control aortas.

In support of decreased NO bioavailability in FKBP12EC KO mice, aortas from FKBP12EC KO mice had significantly decreased levels of endothelial NO synthase expression (Figure 5C) compared with controls, as well as decreased endothelial NO synthase Ser1177 phosphorylation (Figure S6), a measure of endothelial NO synthase activity. Lastly, FKBP12EC KO mice had significantly increased systolic blood pressures compared with controls (wild-type, $106\pm0.2$ mm Hg versus FKBP12EC KO, $136\pm0.2$ mm Hg; Figure 5D).

**Discussion**

Tacrolimus is one of the most widely used drugs for maintenance immunosuppression in allograft recipients; however, major adverse effects, such as endothelial dysfunction and hypertension, limit its efficacy. A common finding in
patients treated with tacrolimus is a decrease in levels of anti-inflammatory Tregs, which may contribute to these adverse effects. It was previously unknown how tacrolimus decreases anti-inflammatory Tregs and whether the inhibition of FKBP12 in endothelial and hematopoietic cells plays a role. Our current findings that treatment of mice with tacrolimus, at concentrations that caused endothelial dysfunction and hypertension, dose-dependently decreased Tregs and increased Th17 cells suggest that TGF-β receptor activation in endothelial and hematopoietic cells plays a role in the etiology of tacrolimus-induced hypertension. In support, mice genetically deficient of FKBP12 in endothelial and hematopoietic cells, which causes TGF-β receptor activation, also exhibit decreased Tregs and increased Th17 cells, as well as endothelial dysfunction and hypertension. These effects are likely attributed to SMAD2/3 and endothelial cell activation and the release of proinflammatory cytokines at the vascular level, coupled with increased proinflammatory Th17 cell polarization in T cells.

Tacrolimus causes hypertension in a majority of allograft recipients, and posttransplant blood pressure is negatively associated with allograft function. One mechanism by which tacrolimus causes hypertension is via inhibition of FKBP12/12.6 in blood vessels leading to reduced vasodilation and/or increased vasoconstriction. Supportive evidence comes from the finding that FKBP12/12.6-deficient mice

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<td>Tumor necrosis factor receptor-associated factor 6</td>
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Table. Differential Expression of T-Helper 17 Cell-Related Genes in Blood of FKBP12EC Knockout vs Control Mice

n=3 mice in each group. *P<0.05.

Figure 5. Endothelial dysfunction and hypertension in FKBP12EC knockout (KO) mice. FKBP12EC KO mice exhibited decreased aortic endothelium-dependent relaxation responses (A) but no difference in endothelium-independent relaxation responses (B). C, Aortic endothelial NO synthase (eNOS) expression was decreased significantly in FKBP12EC KO mice. Representative Western blot image (top) and densitometry (bottom) are presented. D, Systolic blood pressure was increased significantly in FKBP12EC KO mice. Results are expressed as mean±SEM. C, control; F, FKBP12EC KO. *P<0.05 vs control.
develop hypertension and our previous work, which shows that genetic and pharmacological removal of FKBP12/12.6 from intracellular calcium channels dose-dependently decreases NO production and endothelial function.10–12 In addition to its negative effects on endothelial NO production, tacrolimus also increases TGF-β levels, and elevated levels of TGF-β are associated with hypertension. Calcineurin inhibition does not directly affect vascular reactivity, because we have reported previously that direct inhibition of calcineurin, without FKBP12 inhibition, had no acute effect on endothelial function in isolated blood vessels.12 However, Gooch et al26 showed that calcineurin Aa deficiency in mice increased TGF-β levels, suggesting that calcineurin inhibition may contribute to the development of hypertension by TGF-β receptor activation and SMAD2/3 signaling. Cyclosporine, another immunosuppressive drug that inhibits calcineurin and causes hypertension, also increases TGF-β levels. Although cyclosporine does not bind FKBP12, the increased TGF-β levels may contribute to the endothelial dysfunction and hypertension by activating SMAD2/3, leading to endothelial cell activation and altered Treg/Th17 cell imbalance. Furthermore, calcineurin Aβ-deficient mice, which do not exhibit increases in TGF-β levels, do not develop hypertension.26 Together these data suggest a predominant role for TGF-β receptor activation in the development of tacrolimus- and cyclosporine-induced hypertension.

It is becoming more evident that immune cells can affect vascular reactivity and blood pressure regulation.7,27 Several clinical studies have reported that Treg levels are decreased in patients treated with tacrolimus, and high levels of IL-17 are associated with hypertension.17–20 Tregs and Th17 cells both originate from naïve CD4+ T cells, and the polarization into one or the other is regulated by the presence of various cytokines. Common to both pathways is the activation of the TGF-β receptor by TGF-β and SMAD2/3 signaling. Thus, tacrolimus-mediated inhibition of FKBP12 from TGF-β receptors in T cells and/or increase in TGF-β levels may favor Th17 cell polarization in the presence of other proinflammatory cytokines, including IL-6 and IL-21. In tacrolimus-treated, hypertensive mice, we saw that Tregs were decreased in a dose-dependent manner, similar to findings in patients treated with tacrolimus.17–20 In addition, there was a significant increase in the percentage of splenic Th17 cells. Although tacrolimus decreased the number of CD4+ T cells, the composition of these T cells was altered dramatically. Tacrolimus treatment in patients should theoretically reduce the number of CD4+ T cells, including both Tregs and Th17 cells. However, serum IL-17 levels are typically not different in tacrolimus-treated patients compared with controls, despite a reduction in the number of T cells.29 We propose that this imbalance of Tregs and Th17 cells caused by tacrolimus contributes to the development of endothelial dysfunction and hypertension. This may also partially explain why chronic tacrolimus treatment impedes the induction of tolerance, of which Tregs play a crucial role.30 in allograft recipients.

At the vascular level, TGF-β receptor activation and increased SMAD2/3 signaling lead to endothelial cell activation and the release of the proinflammatory cytokine IL-6. Increased circulating levels of IL-6, together with IL-21 and TGF-β receptor activation in CD4+ T cells, induce the development of Th17 cells and prevent Treg differentiation. This is mediated by increased STAT3 activity and SOCS3 inhibition.16–21 In naïve CD4+ T cells, TGF-β receptor activation in the absence of IL-6 and IL-21 increases STAT5 activity, leading to FoxP3 expression and Treg induction. Therefore, given the importance of TGF-β receptor activation and SMAD2/3 signaling in endothelial function and Treg versus Th17 cell polarization, we generated mice lacking FKBP12 only in endothelial and hematopoietic cells. Results obtained from these mice demonstrate that a genetic deficiency of FKBP12 in endothelial and hematopoietic cells, which leads to SMAD2/3 activation in the absence of increased TGF-β levels or decreased calcineurin activity (data not shown), is sufficient to cause endothelial cell activation, Treg downregulation, Th17 cell-mediated inflammation, endothelial dysfunction, and hypertension. These effects are similar to those found in tacrolimus-treated humans and animals. In support, Qin et al26 found decreased Th17 cell development in mice with deficient TGF-β receptor activity. Our FKBP12EC KO mice represent a novel system to study these interactions in vivo, because they demonstrate SMAD2/3 activation, endothelial cell activation, increased IL-6 and IL-21 levels, decreased splenic SOCS3 gene expression, increased splenic STAT3 activity, and decreased splenic STAT5 activity, without changes in the anti-inflammatory cytokine TGF-β.

**Perspectives**

Our data demonstrate that endothelial and hematopoietic TGF-β receptor activation and SMAD2/3 signaling play important roles in endothelial cell activation in blood vessels and Treg:Th17 cell imbalance in T cells, which, together, converge to cause inflammation, endothelial dysfunction, and hypertension after tacrolimus treatment or conditional FKBP12 gene deletion. These findings may explain in part why patients treated with tacrolimus exhibit decreased Tregs, endothelial dysfunction, and hypertension. Immunosuppressive drugs that do not affect TGF-β or FKBP12 may help prevent the development of hypertension and potentiate the development of functional tolerance in organ transplant recipients.

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**Disclosures**

None.

**References**


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FK506 BINDING PROTEIN 12 DEFICIENCY IN ENDOTHELIAL AND HEMATOPOIETIC CELLS DECREASES REGULATORY T CELLS AND CAUSES Th17 CELL-MEDIATED HYPERTENSION

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Short Title – FKBP12, Tregs, and Hypertension

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EXPANDED 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 tacrolimus treatment studies as well as controls in all experiments. Male Fkbp12 (lox) mice (generously provided by Dr. Susan Hamilton, Baylor College of Medicine, and described previously1) were crossed with Tie2-Cre mice obtained from the Jackson Laboratory. Tie2 expression is restricted to endothelial and hematopoietic cells. Male Tie2-Cre+−Fkbp12lox−/− (FKBP12EC KO) mice, which exhibited a 97% C57Bl/6 background, 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 tacrolimus (1 or 10 mg/kg/day, i.p.; LC Laboratories, Woburn, MA) or diluent (saline and DMSO, 0.2% final concentration) as described previously.2 Animals were anesthetized 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
Splenocytes were isolated by passing the spleen through a 70 µm sterile strainer followed by lysis of red blood cells according to the manufacturer’s protocol (BD Pharm Lyse, BD Pharmingen). Cells were washed with PBS/1% BSA, counted, and resuspended in PBS/1% BSA. One million cells from each spleen were stained with PE-Cy™7 hamster anti-mouse CD3e (T cell marker; clone 145-2c11, BD Pharmingen), FITC rat anti-mouse CD4 (T helper cell marker; clone GK1.5, BD Pharmingen), or isotype controls for 30 minutes at 4°C in the dark. The splenocytes were then fixed and permeabilized with the BD CytoFix/CytoPerm™ Fixation/Permeabilization Kit (BD Pharmingen). Intracellular staining was performed using either PE rat anti-mouse FoxP3 (Treg marker; clone MF23, BD Pharmingen), PE rat anti-mouse IL-17A (Th17 marker; BD Pharmingen), PE rat anti-mouse IFN-γ (Th1 marker; BD Pharmingen), or PE rat anti-mouse IL-4 (Th2 marker; BD Pharmingen). Flow cytometry and data analysis was performed using an Accuri C6 flow cytometer and C-Flow Plus software (Ann Arbor, MI). Compensation and quadrants were set based on the isotype control data and negative staining controls and lymphocyte gating in the forward x side scatter plot. The percent of CD3+/CD4+, CD4+/IL-17A+, CD4+/FoxP3+, CD4+/IFN-γ+, and CD4+/IL-4+ lymphocytes were quantified and averaged. Data are expressed as % of total splenocytes.

Serum Cytokine Array
Sera was obtained from blood taken from control and FKBP12EC KO mice at the time of sacrifice and used to measure 12 different pro-inflammatory and anti-inflammatory cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-23, IFN-γ, TNF-α, and TGF-β) according to the manufacturer’s protocol (SABiosciences; Frederick, MD). Serum IL-21 was measured using an ELISA kit according to the manufacturer’s protocol (R&D Systems, Minneapolis, MN).
Vascular Reactivity
Vascular reactivity was measured in endothelium-intact aortas as described previously. Relaxation responses were measured in the absence and presence of indomethacin (10 μmol/L, 45 minutes), L-NAME (100 μmol/L, 45 minutes), and/or PEG-SOD (100 Units/L, 45 minutes). 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
Immunoblotting was performed on aortas and spleens from control and FKBP12EC KO mice as described previously. Primary antibodies for FKBP12/12.6 (Santa Cruz Biotechnology), phospho-SMAD2/3 (Santa Cruz Biotechnology), SMAD2/3 (Cell Signaling), ICAM (R&D Systems), phospho-STAT3 Ser727 (Cell Signaling), phospho-STAT3 Tyr705 (Cell Signaling), STAT3 (BD Biosciences), phospho-STAT5 Tyr694 (Abcam), STAT5 (Abcam), phospho-eNOS Ser1177 (Millipore), eNOS (BD Biosciences), VEGF (Cell Signaling), and β-actin (loading control; Sigma) were used and secondary antibodies consisted of anti-rabbit and anti-mouse IgGs conjugated to Alexa-Fluor 680 and IR800Dye (LI-COR Biosciences). The blots were probed first for phospho-proteins followed by the corresponding total proteins. Bands were identified simultaneously (800 nm and 700 nm wavelengths, respectively) using near-infrared visualization (Odyssey System, LI-COR Biosciences; Lincoln, NE) and densitometry was performed using the Odyssey software.

Real-Time qPCR
Aortic cDNA from control and FKBP12EC KO mice was used in a Mouse Endothelial Cell Biology RT² Profiler™ PCR Array (SABiosciences), while blood cDNA was used in a Mouse Th17 for Autoimmunity & Inflammation RT² Profiler™ PCR Array (SABiosciences). Both arrays were performed according to the manufacturer’s protocol and used the 3 housekeeping genes Hsp90, GAPDH, and β-actin for normalization as well as 3 reverse transcription controls and 3 positive PCR controls.

Pulmonary Endothelial Cells
Pulmonary endothelial cells were isolated from control and FKBP12EC KO mice, purified using PECAM antibodies (BD Biosciences) conjugated to magnetic beads, then cultured for 2 passages. Protein levels of FKBP12 and FKBP12.6 were measured as described above with β-actin serving as a loading control.

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. SMAD2/3 activation in FKBP12EC KO mice. (A) Pulmonary endothelial cells from FKBP12EC KO mice exhibited significantly decreased levels of FKBP12. (B) Aortas from FKBP12EC KO mice exhibited markedly increased levels of SMAD2/3 phosphorylation and expression. Representative Western blot images (top) and densitometry (bottom) are presented. Densitometry expressed as mean ± SEM (n = 5 independent experiments). C=Control, F=FKBP12EC KO. *p<0.05 vs. Control.
Figure S2. Endothelial cell activation in FKBP12EC KO mice. (A) Aortic expression of the endothelial cell activation marker ICAM was increased significantly in FKBP12EC KO mice. (B) Aortas from FKBP12EC KO mice exhibited significantly increased levels of STAT3-Tyr phosphorylation. Representative Western blot images (top) and densitometry (bottom) are presented. Densitometry expressed as mean ± SEM (n = 5 independent experiments). C=Control, F=FKBP12EC KO. *p<0.05 vs. Control.
Figure S3. Increased pro-inflammatory gene expression in FKBP12EC KO mice. Aortic mRNA expression of the pro-inflammatory genes Bcl2-like 1, Birc2, Cxcl2, and Cx3cl1 (fractalkine) were increased significantly in FKBP12EC KO mice. Results are expressed as mean ± SEM (n = 3 independent experiments). Dashed line equals a significant 2-fold difference compared to controls.
**Figure S4**

(A) Aortic mRNA expression of the VEGF family members Flt-1, Plgf, Kdr, and Tek were increased significantly in FKBP12EC KO mice. Dashed line equals a significant 2-fold difference compared to controls. (B) Protein expression of VEGF-A was increased significantly in aortas from FKBP12EC KO mice compared to controls as demonstrated by Western blot (top) and densitometry for the ratio of VEGF-A to actin as a percent of control (bottom). Results are expressed as mean ± SEM (n = 5 independent experiments). C=Control, F=FKBP12EC KO. *p<0.05 vs. Control.
Figure S5. Decreased NO-mediated, but not prostacyclin- or EDHF-mediated, relaxation responses in FKBP12EC KO mice. (A) The NOS inhibitor L-NAME completely abolished relaxation responses in aortas from both control and FKBP12EC KO mice. (B) The cyclooxygenase inhibitor indomethacin had no significant effects on relaxation responses in aortas from control or FKBP12EC KO mice. (C) Inhibition of both NOS and cyclooxygenase tended to cause contractions to acetylcholine in aortas from both control and FKBP12EC KO mice. (D) Scavenging of superoxide with PEG-SOD restored relaxation responses in aortas from FKBP12EC KO mice. Results are expressed as mean ± SEM (n = 4-6 animals for each). *p<0.05 vs. Control.
Figure S6. Decreased eNOS activity in FKBP12EC KO mice. eNOS Ser1177 phosphorylation, a measure of eNOS activity, was decreased significantly in aortas from FKBP12EC KO mice compared to controls as demonstrated by Western blot (top) and densitometry for the ratio of p-eNOS Ser1177/eNOS to actin as a percent of control (bottom). Results are expressed as mean ± SEM (n = 4 independent experiments). C=Control, F=FKBP12EC KO. *p<0.05 vs. Control.