RESEARCH ARTICLE

Endothelial Dysfunction

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Lactobacillus fermentum Improves Tacrolimus-Induced Hypertension by Restoring Vascular Redox State and Improving eNOS Coupling

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Scope: The aim is to analyze whether the probiotic *Lactobacillus fermentum* CECT5716 (LC40) can prevent endothelial dysfunction and hypertension induced by tacrolimus in mice.

Methods and results: Tacrolimus increases systolic blood pressure (SBP) and impairs endothelium-dependent relaxation to acetylcholine and these effects are partially prevented by LC40. Endothelial dysfunction induced by tacrolimus is related to both increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) and uncoupled endothelial nitric oxide synthase (eNOS)-driven superoxide production and Rho-kinase-mediated eNOS inhibition. LC40 treatment prevents all the aortic changes induced by tacrolimus. LC40 restores the imbalance between T-helper 17 (Th17)/regulatory T (Treg) cells induced by tacrolimus in mesenteric lymph nodes and the spleen. Tacrolimus-induced gut dysbiosis, that is, it decreases microbial diversity, increases the Firmicutes/Bacteroidetes (F/B) ratio and decreases acetate- and butyrate-producing bacteria, and these effects are prevented by LC40. Fecal microbiota transplantation (FMT) from LC40-treated mice to control mice prevents the increase in SBP and the impaired relaxation to acetylcholine induced by tacrolimus.

Conclusion: LC40 treatment prevents hypertension and endothelial dysfunction induced by tacrolimus by inhibiting gut dysbiosis. These effects are associated with a reduction in vascular oxidative stress, mainly through NOX2 downregulation and prevention of eNOS uncoupling, and inflammation possibly because of decreased Th17 and increased Treg cells polarization in mesenteric lymph nodes.

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1. Introduction

Transplants require life-long immunosuppressive treatment, exposing patients to significant side effects. The immunosuppressive treatment is detrimental for the cardiovascular system, cardiovascular diseases being the main cause of mortality among renal transplant recipients and the leading cause of nonallograft-related death in liver and heart transplantation.[1–5] In fact, calcineurin and mammalian target of rapamycin (mTOR) inhibitors have been associated with increased cardiovascular risk due to endothelial dysfunction, hyperlipidemia, and diabetes in transplant patients.[6] Tacrolimus (FK506) is a macrolide used for maintenance of immunosuppression in organ transplant recipients that have been shown to affect blood pressure and immunologic memory. The increases in blood pressure and the reduction of relaxation of blood vessels have been associated with a shift in the balance of proinflammatory versus anti-inflammatory T-cell subsets.[7] Tacrolimus limits the immune response via inhibition of the activity of calcineurin. Calcineurin signaling regulates the expression of interleukin IL-2, IL-3, IL-4, and interferon-γ, as well as expression of the IL-2 receptor, which promotes the expansion of cytotoxic T cells, for example, proinflammatory response. Although tacrolimus reduces the overall number of helper T (Th) cells because of its inhibitory effect on calcineurin, the remaining Th cells differentiate into IL17-producing T cells (Th17), which release the potent proinflammatory cytokine IL-17, leading to endothelial dysfunction and hypertension.[7] Tacrolimus produces a strong inhibition of endothelial function by reducing endothelial nitric oxide synthase (eNOS) expression and/or activity,[8] which leads to hypertension.[9] Thus, elucidation of mechanisms that contribute to posttransplant hypertension has major clinical implications. In fact, no effective and safe immunosuppressive treatment has been established so far.[10] However, cardiovascular complications induced by tacrolimus might be prevented by shifting naïve T-cell polarization to anti-inflammatory regulatory T (Treg) cells instead of proinflammatory Th17 cells.

The intestinal microbiota composition has a marked role in host immune functions,[11] and its modulation can result in beneficial effects. In fact, we recently demonstrated that the administration of probiotic bacteria, such as *Lactobacillus fermentum* CECT5716 (LC40), improves endothelial dysfunction in spontaneously hypertensive rats.[12] In addition, LC40 can modulate the human immune system, increasing Treg cells.[13,14] Therefore, we hypothesize that the changes in T-cell polarization induced by LC40, through increasing Treg, can prevent endothelial dysfunction and hypertension produced by tacrolimus. Thus, the aim of this study was to analyze the preventive effects of the probiotic LC40 in the hypertension and endothelial dysfunction induced by tacrolimus in mice. We have also analyzed if these effects are linked to changes in the gut microbiota and T-cell populations.

2. Experimental Section

All procedures conform to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85-23, revised 2011) and approved by the University of Granada Institutional Committee for the ethical care of animals (Ref No. 03-CEEA-OH-2013). This is a short version of the protocols used, further details are provided in the Supporting Information.

2.1. Animals and Experimental Groups

Ten-week-old male C57Bl/6J mice were divided into four groups: control ($n = 9$), control treated ($n = 6$), tacrolimus ($n = 8$), and tacrolimus treated ($n = 6$). Treated mice received the probiotic LC40 (Biosearch, S.A., Granada, Spain) incorporated in drinking water and prepared daily, at a dose of 5×10^8 colony-forming units (CFU) per day per mouse, 7 days before tacrolimus (1 mg kg^{-1} per day, i.p) or vehicle (saline and DMSO, 0.2% final concentration) administration. Probiotic and tacrolimus treatments were followed for 7 days. At the end of the treatment, mice were killed by decapitation under isoflurane anesthesia.

2.2. Blood Pressure Measurements

Systolic blood pressure (SBP) evolution was measured by tail-cuff plethysmography, and final mean arterial blood pressure (MABP) by intra-arterial register.[15]

2.3. Flow Cytometry

Mesenteric lymph nodes and spleen were collected from mice for flow cytometry studies and for lymphocyte conditioned media preparation. Flow cytometry was used to measure T cells (positive to anti-CD4+, total T; anti-CD4+ FOXP3+, Treg; anti-CD4+ IL17+, Th17; and anti-CD4+ IFNγ+, Th1).[16]

2.4. Lymphocyte Conditioned Media Preparation

The nodes were mashed with wet slides to decrease friction and then the solutions were filtered through a cell strainer of 70 μm. Lymphocyte conditioned media was prepared according to a previous study.[17]

2.5. Vascular Reactivity Studies

Descending thoracic aortic rings were mounted in a wire myograph for isometric tension measurement as previously described.[18]

2.6. In Situ Detection of Vascular Reactive Oxygen Species Content

Vascular reactive oxygen species (ROS) levels were estimated from the ratio of ethidium/4,6-diamidino-2-phenylindole dichlorohydrate (DAPI) fluorescence in sections of unfixed thoracic aortic rings incubated with dihydroethidium (DHE) and counterstained with the nuclear stain DAPI.[15]

2.7. NADPH Oxidase Activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described.[16] In another set of experiments, the NADPH-stimulated ROS production in homogenates from aortas was also measured by DHE fluorescence assay in the microplate reader, as described previously.[19]

2.8. Gene Expression Analysis

Protein and mRNA expression were measured by Western blotting analysis and RT-PCR analysis, respectively, as described previously.[15]

2.9. DNA Extraction and 454/Roche Pyrosequence Analysis

DNA from fecal content was isolated using phenol:chloroform following a protocol modified from Sambrook and Russell.[20]

2.10. Analysis of 16S rRNA Sequences

MG-RAST metagenomics analysis server with the Ribosomal Database Project (RDP) was used for analyses of all sequences.[21]

2.11. Primary Culture of Mouse Aorta Endothelial Cells

Mouse aorta endothelial cells (MAECs) were isolated from mouse thoracic aortas as described previously.[22]

2.12. Quantification of Nitric Oxide Release by Diaminofluorescein-2

Quantification of NO released by MAECs was performed using the NO-sensitive fluorescent probe diaminofluorescein-2 (DAF-2) as described previously with several modifications.[23]

2.13. Measurement of Intracellular ROS Concentrations in MAECs

Endothelial ROS production was measured using the fluorescent probe 5-(and-6-) chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA).[23]

2.14. Fecal Microbiota Transplantation

Fecal microbiota transplantation (FMT) into recipient mice were carried out following a previously reported method with several modifications.[24]

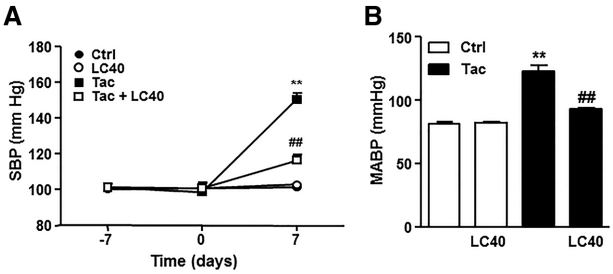


Figure 1. *Lactobacillus fermentum* CECT5716 (LC40) administration prevents tacrolimus (Tac)-induced hypertension in mice. Systolic blood pressure (SBP) was measured by A) tail-cuff plethysmography and B) mean arterial blood pressure (MABP) by direct recording in mice. Values are expressed as mean ± SEM ($n = 6$–9). ** indicates $p < 0.01$ compared with the control (Ctrl) group. ## $p < 0.01$ compared with the Tac group (two-way ANOVA with post hoc Sidak test).

2.15. Statistical Analysis

Results are expressed as means ± SEM. Statistical analyses were performed using Graph Pad Prism 7 software. A two-way ANOVA (with Sidak's correction for comparison of multiple means) was used for comparisons of four groups with two variables (tacrolimus and LC40 treatments). Two-way ANOVA with repeated measurements approach (with Tukey's correction for comparison of multiple means) was used for comparisons of vasodilator potency. *t*-test was performed for comparing two groups. Significance was accepted at $p < 0.05$.

3. Results

3.1. LC40 Treatment Improves Hypertension and Endothelial Dysfunction

Tail SBP increased significantly in mice treated with tacrolimus, by approximately 52 mmHg (**Figure 1**A). The administration of LC40 dose dependently (10^8, 5×10^8, and 10^{10} CFU per day) prevented the rise in SBP induced by tacrolimus (Figure S1A, Supporting Information), being the maximum inhibitory effect (70%) at 5×10^8 CFU per day, without significant effects in control mice. Similar changes were found in final MABP measured by direct recordings (Figure 1B). In addition, when LC40 treatment, at 5×10^8 CFU per day, started 7 days after tacrolimus treatment, the probiotic was able to reduce SBP significantly (Figure S1B, Supporting Information). Daily treatment of control mice with tacrolimus for 1 week decreased endothelium-dependent relaxation responses in phenylephrine-contracted aortic rings (maximal acetylcholine-induced relaxation, Emax: 82 ± 3% vs 60 ± 4%, $p < 0.05$, control and tacrolimus, respectively; **Figure 2**A), but had no effects on endothelium-independent relaxation responses to sodium nitroprusside (Figure S2, Supporting Information). Similarly, endothelium-dependent relaxation to acetylcholine in U46619-precontracted small mesenteric arteries was also reduced in the tacrolimus group as compared to control (Figure 2D). LC40 treatment improved the aortic and mesenteric artery endothelium-dependent relaxation in tacrolimus-treated mice (Emax: 75 ± 4%). The relaxant response

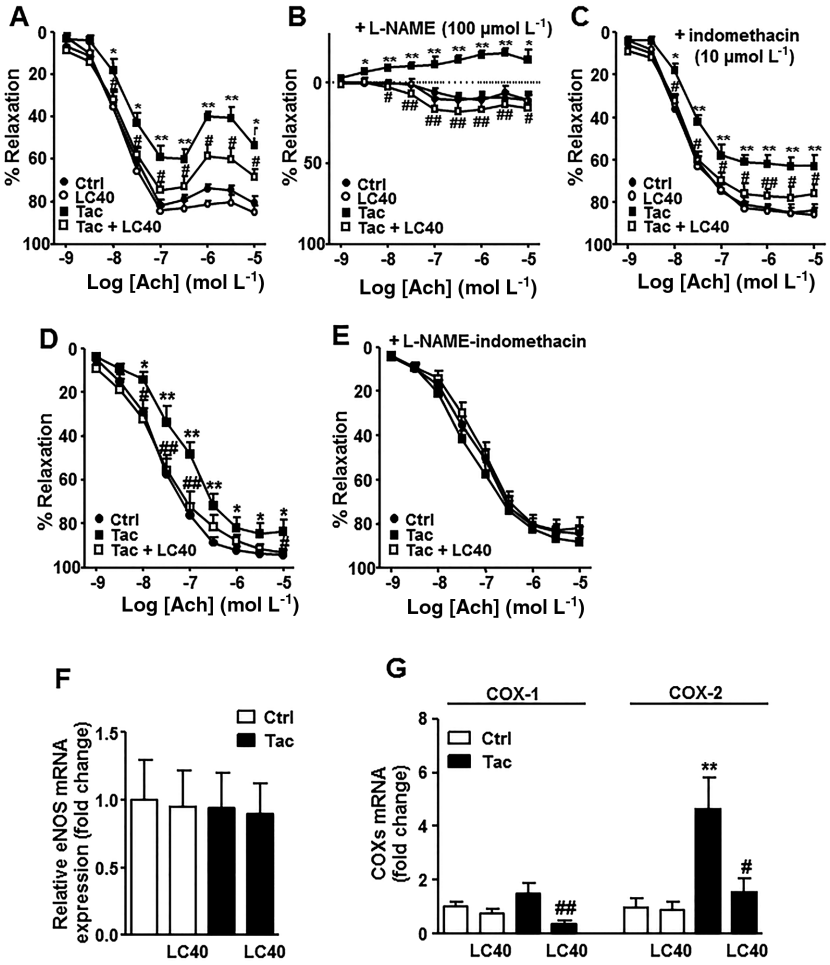


Figure 2. *Lactobacillus fermentum* CECT5716 (LC40) administration prevents tacrolimus (Tac)-induced aortic endothelial dysfunction in mice. Vascular relaxant responses induced by acetylcholine (ACh) in aortas precontracted by phenylephrine in the A) absence and B) presence of L-NAME (100 μmol L^{-1}) or C) indomethacin (10 μmol L^{-1}), and in small mesenteric arteries precontracted by U46619 (10^{-8} mol L^{-1}) in the D) absence and E) presence of L-NAME (100 μmol L^{-1}) plus indomethacin (10 μmol L^{-1}). Values are expressed as mean ± SEM ($n = 6$–9; two-way ANOVA with post hoc Tukey test). F) Aortic eNOS and G) COX-1 and COX-2 mRNA levels ($n = 5$–9). Values are expressed as mean ± SEM (two-way ANOVA with post hoc Sidak test). * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the control (Ctrl) group. # and ## indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the Tac group.

induced by acetylcholine in the aorta was almost fully inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) in all experimental groups (Figure 2B), showing that in these vessels, acetylcholine-induced relaxation in both control and tacrolimus groups was almost entirely dependent on endothelium-derived NO. However, in mesenteric arteries incubated with L-NAME plus indomethacin no significant differences among groups were observed (Figure 2E), showing no alteration of endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation. In addition, in aortas from the tacrolimus group, acetylcholine induced a contractile response under acute eNOS inhibition, suggesting the release of endothelial-derived vasoconstrictor factors, which was also suppressed by LC40. In the presence of indomethacin, a nonselective cyclooxygenase (COX) inhibitor, the contractile response induced by high concentrations of acetylcholine in phenylephrine-contracted rings from tacrolimus group was suppressed, confirming the release of prostanoids (Figure 2C). LC40 treatment also improved the acetylcholine-induced relaxation in the aorta from tacrolimus-treated mice in the presence of L-NAME (Figure 2B) and indomethacin (Figure 2C), suggesting that tacrolimus inhibited prostanoid release and increased NO effects, respectively. No significant changes were observed in the aortic mRNA levels of eNOS (Figure 2F). However, both COX-1 and COX-2 mRNA levels were increased in the tacrolimus group and they were normalized by treatment with LC40 (Figure 2G).

3.2. LC40 Administration Reduces Vascular Oxidative Stress and Inflammation

ROS levels within the vascular wall were characterized and localized by ethidium red fluorescence in sections of aorta incubated with DHE. Positive red nuclei could be observed in adventitial, medial, and endothelial cells from sections of aorta

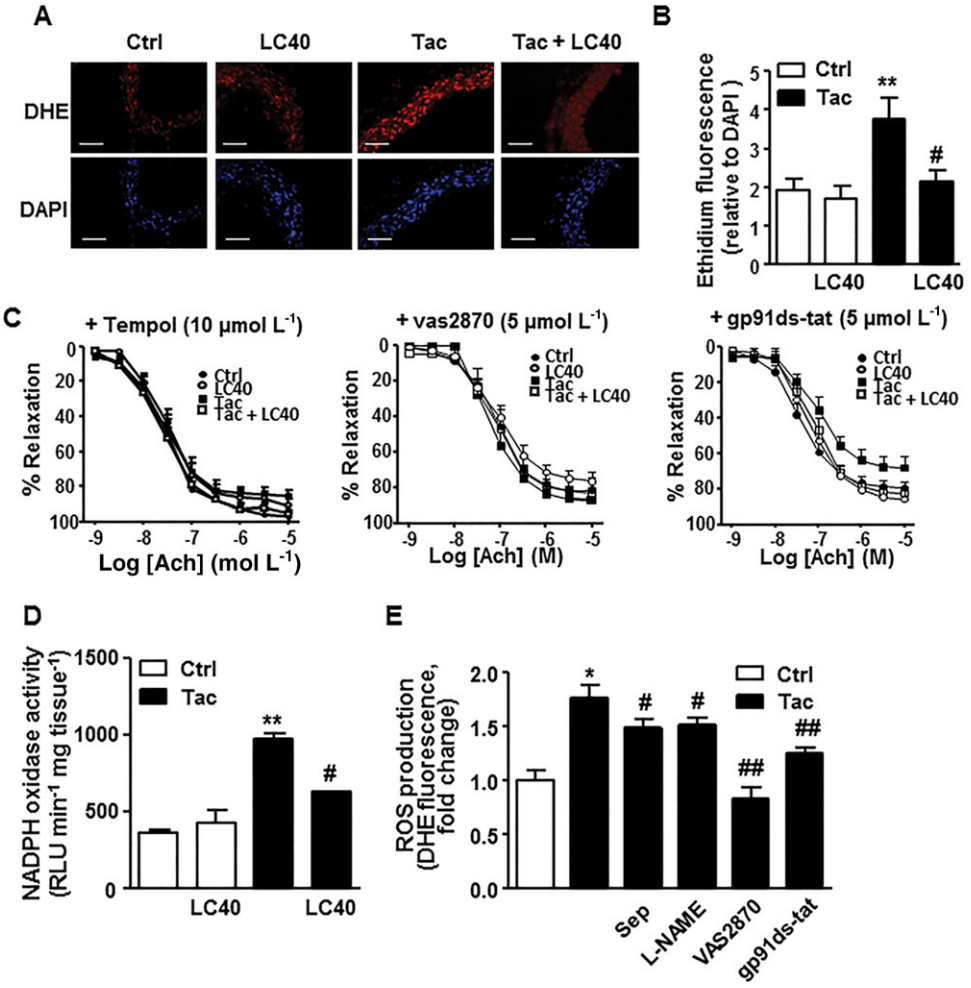


Figure 3. *Lactobacillus fermentum* CECT5716 (LC40) administration prevents tacrolimus (Tac)-induced endothelial dysfunction by reducing vascular oxidative status. A) Pictures show aortas incubated in the presence of DHE which produces a red fluorescence when oxidized to ethidium by O_2^- and blue fluorescence of the nuclear stain DAPI. Scale bars = 50 μm. B) Averaged values, mean ± SEM (n = 6–9 rings from different mice) of the red ethidium fluorescence normalized to the blue DAPI fluorescence (two-way ANOVA with post hoc Sidak test). C) Vascular relaxant responses induced by acetylcholine (ACh) in aortas precontracted by phenylephrine in the presence of tempol (10 μmol L^{-1}), vas2870 (5 μmol L^{-1}), or gp91ds-tat (5 μmol L^{-1}). Values are expressed as mean ± SEM (n = 5–9 rings from different mice; two-way ANOVA with post hoc Tukey test). D) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence in intact aortas from all experimental groups, expressed as mean ± SEM (n = 5–7 rings from different mice, two-way ANOVA with post hoc Sidak test). E) NADPH-stimulated ROS production, measured by DHE fluorescence in microplate reader, in homogenate of aortas from control (Ctrl) or Tac group incubated with sepiapterin (sep, 100 μmol L^{-1}), L-NAME (100 μmol L^{-1}), vas2870 (5 μmol L^{-1}), or gp91ds-tat (5 μmol L^{-1}). Values are expressed as mean ± SEM (n = 6–8; t-test). ** indicates $p < 0.01$ compared with the control (Ctrl) group. #$p < 0.05$, and ##$p < 0.01$ compared with the Tac group.

incubated with DHE (**Figure 3**A). Nuclear red ethidium fluorescence was quantified and normalized to the blue fluorescence of the nuclear stain DAPI, allowing comparisons between different sections. Rings from tacrolimus-treated mice showed a marked staining in adventitial, medial, and endothelial cells, which was higher than in control mice. These effects were prevented by LC40 administration (Figure 3A,B). To examine whether ROS are involved in endothelial dysfunction induced by tacrolimus in the mouse aorta, we analyzed the endothelium-dependent relaxant response to acetylcholine in the presence of the superoxide dismutase (SOD) mimetic—tempol. This agent significantly improved the impaired aortic relaxation to acetylcholine induced by tacrolimus (Figure 3C). Since NADPH oxidase is the major source of ROS in the vascular wall, we investigated the effect of NADPH oxidase inhibition by the pan-NOX inhibitor vas2870, and the NOX2 inhibitor gp91dstat in the relaxant response to acetylcholine. No significant differences among groups were observed in this response in the presence of both agents, suggesting the involvement of NOXs and NOX2 in the impaired endothelium-dependent relaxation induced by tacrolimus (Figure 3C). We also measured the effects of LC40 treatment on NADPH-stimulated ROS production, as a marker of NADPH oxidase activity, and gene expression of its main subunits in aorta from all experimental groups. Tacrolimus increased aortic NADPH oxidase activity (Figure 3D) and the mRNA levels of its catalytic subunits (Figure S3, Supporting Information) NOX1,

NOX4, and NOX2 as compared to the control group. It had no effect in the regulatory subunits $p22^{phox}$ and $p47^{phox}$ (Figure S3, Supporting Information). Again, LC40 administration reduced both NADPH oxidase activity and upregulation of the catalytic subunits in tacrolimus-treated mice. In addition, we also found increased $p47^{phox}$ and $p67^{phox}$ translocation from the cytosol to the membrane in the tacrolimus group, which was reduced by LC40 treatment (Figure S4, Supporting Information). Taking into account that NADPH can also stimulate a variety of ROS generating enzymes, such as uncoupled eNOS, we measured NADPH-stimulated ROS production by DHE fluorescence in aortic homogenates incubated in the presence of the eNOS inhibitor L-NAME, or the tetrahydrobiopterin (BH4) precursor sepiapterin. We confirm increased ROS production stimulated by NADPH in the tacrolimus group, as compared to control, which was inhibited by L-NAME, and sepiapterin, involving uncoupled eNOS as a source of ROS in aorta from tacrolimus group. In addition, vas2870 and gp91dstat also inhibited ROS production, confirming that tacrolimus increased NADPH oxidase activity (Figure 3E).

In vascular cells, the nuclear factor-κB (NF-κB) is an important redox-sensitive transcriptional factor that regulates transcription of genes encoding inflammatory cytokines, which, in turn, can activate the production of ROS and other proinflammatory cytokines.[25] We found that in aortic homogenates the phosphorylation of IκBα (Figure S5A, Supporting Information), an indirect marker of NF-κB, and the expression of TNFα (Figure S5B, Supporting Information) and IL-6 (Figure S5C, Supporting information) were higher in tacrolimus group when compared to the control group. LC40 administration significantly reduced NF-κB activity and mRNA levels of these proinflammatory cytokines in tacrolimus-treated mice.

3.3. LC40 Administration Induces Changes in T-Cell Polarization

To determine whether these vascular effects were associated with changes in T-cell polarization in lymphoid organs, we measured the levels of Treg and Th17 cells in mesenteric lymph nodes and spleens from all experimental groups (**Figure 4**A–C). As expected, daily tacrolimus treatment decreased the percentage of $CD4^+$ cells, as compared to control mice in both organs. The percentages of Treg ($CD4^+$/FoxP3$^+$) and Th1 ($CD4^+$/interferon-γ^+) lymphocytes were decreased in tacrolimus-treated mice when compared with controls. In addition, tacrolimus treatment increased the percentage of Th17 ($CD4^+$/IL-17$^+$) lymphocytes in mice. LC40 treatment prevented the altered T-cell polarization induced by tacrolimus. To analyze whether these changes in lymphoid organs induced changes in the circulating levels of cytokines we measured plasma levels of IL-7 and IL-10. We found increased IL-17 levels and reduced IL-10 levels in plasma from the tacrolimus group, which were restored by treatment with LC40 (Figure 4D).

As expected, the mRNA level of IL-10, the cytokine released by Treg, was also reduced by tacrolimus and restored by pretreatment with LC40 (**Figure 5**A). Th17 cell infiltration in aorta was measured by both RORγ, which induces Th17, and IL-17a mRNA levels. Aortic RORγ (Figure 5B) and IL-17a levels (Figure 5C) were markedly increased by tacrolimus treatment and reduced by LC40 administration. To determine whether changes in T-cell polarization in mesenteric lymph nodes are involved in the endothelial dysfunction, we first examined endothelium-dependent relaxation to acetylcholine in aorta from control mice incubated for 4 h with conditioned media of lymphocytes from the experimental groups. The relaxation induced by acetylcholine in U46619-contracted aortic rings was impaired after incubation with conditioned media of lymphocytes from the tacrolimus group, as compared to that obtained from the control group (**Figure 6**A). This alteration was suppressed when the rings exposed to conditioned medium from lymphocytes of tacrolimus group were also coincubated with the Rho-kinase inhibitor Y-27632 (Figure 6B), confirming the involvement of Rho-kinase in this endothelial dysfunction. In addition, when relaxation to acetylcholine was tested in the presence of tempol, no significant differences were observed between rings incubated with conditioned medium of lymphocytes from tacrolimus and control groups (Figure 6C), indicating the involvement of ROS in endothelial dysfunction induced by tacrolimus. Furthermore, aortic NADPH oxidase activity was also increased by lymphocyte conditioned medium from the tacrolimus-treated mice as compared to the control group, and effect that was again reduced by treatment with LC40 (Figure 6D). Aortas incubated with lymphocyte conditioned medium from tacrolimus-LC40 group showed similar endothelium-dependent relaxation and NADPH oxidase activity than control aortas. In MAECs obtained from control mice, the incubation with lymphocyte condition media from tacrolimus group reduced the calcium ionophore A23187-stimulated NO production (Figure 6E) and increased the intracellular ROS levels (Figure 6F). Again, both NO and ROS production were normalized in MAECs incubated with lymphocyte conditioned media from tacrolimus-LC40 group.

The Rho-kinase pathway-mediated endothelial dysfunction has also been reported in response to Angiotensin II, a key factor in hypertension. Moreover, the Treg and Th17 lymphocytes also play a pivotal role in the induction of hypertension in response to increased angiotensin II. Taking into account that some *Lactobacillus* strains reduce blood pressure as a result of angiotensin converting enzyme (ACE) inhibition,[26,27] we measured ACE activity in aorta from all experimental groups. We found increased ACE activity in aorta from tacrolimus group, which was unchanged by LC40 treatment (Figure S5, Supporting Information), ruling out the involvement of inhibitory ACE activity in the protective effects induced by this probiotic.

3.3.1. LC40 Administration Improves Intestinal Tight-Junction Integrity and Inflammation

Gut permeability is controlled by several specific tight-junction proteins, such as occludins, that have been proposed as key markers of tight-junction integrity.[28] LC40 consumption increased occludin mRNA expression in the colonic segment of tacrolimus-treated mice, whereas no significant effects were observed in control mice (Figure S7A, Supporting Information). However, the transcription of the mucins, mucin-2 (Figure S7B, Supporting Information) and mucin-3 (Figure S7C, Supporting Information) genes, which are involved in mucus production, were unaltered

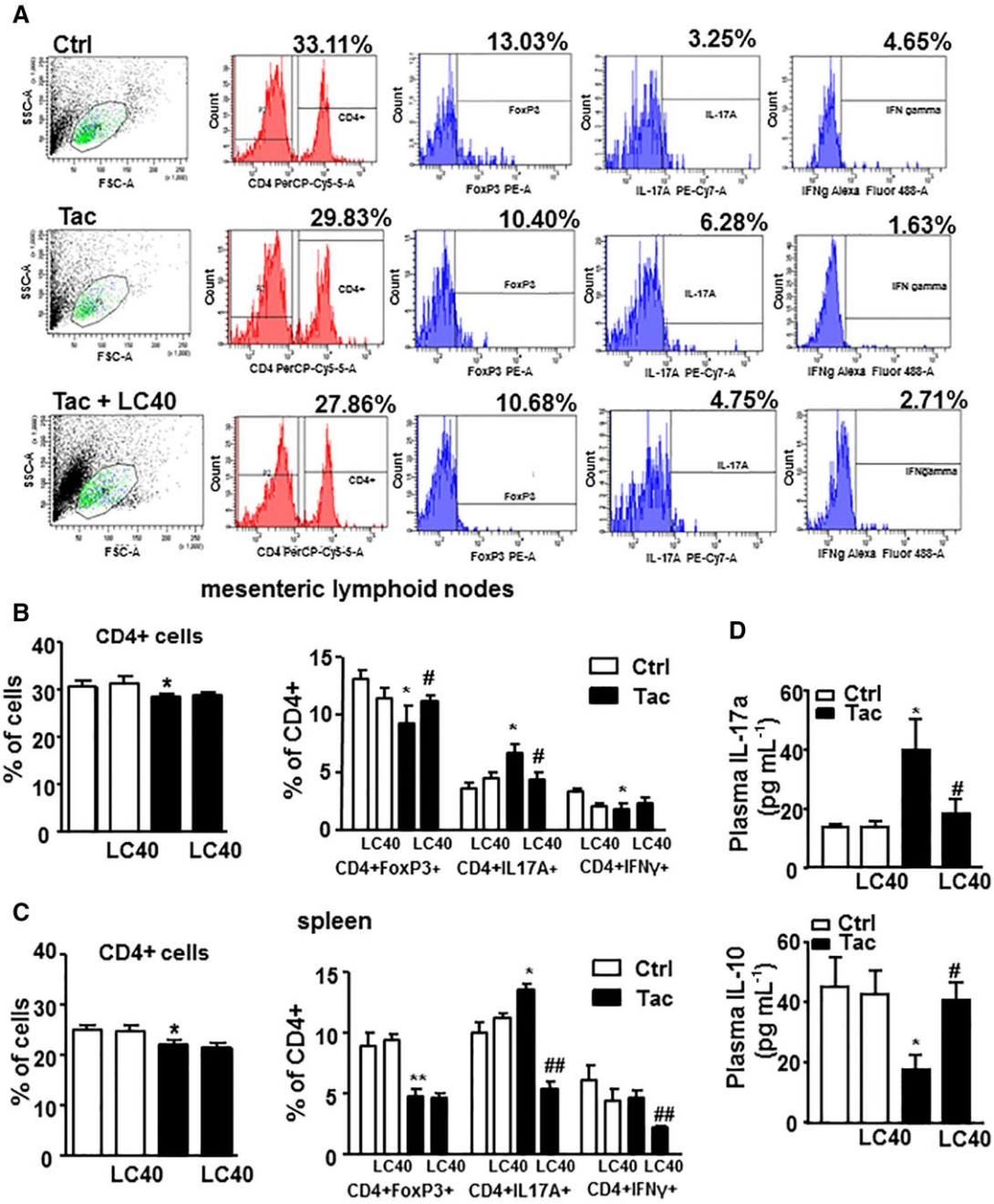


Figure 4. *Lactobacillus fermentum* CECT5716 (LC40) administration prevents tacrolimus (Tac)-induced T-cells polarization in mesenteric lymph nodes and spleen. Representative gating strategy used to identify A) different T populations in mesenteric lymph nodes (MLNs), B) altered T-cell polarization in MLNs, and C) spleen from Tac-treated mice characterized by a decrease in CD4+ T cells, regulatory T cells (Treg; CD4+/FoxP3+), and Th1 (CD4+/interferon-γ+) and an increase in Th17 T cells (CD4+/interleukin 17a+). D) Levels of IL-17a and IL-10 measured in plasma from all experimental groups. Values are expressed as mean ± SEM ($n = 5$–6; two-way ANOVA with post hoc Sidak test). * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the control (Ctrl) group. # and ## indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the Tac group.

by the probiotic. Furthermore, the increased mRNA levels of the colonic proinflammatory cytokines TNFα (Figure S7D, Supporting Information), IL-1β (Figure S7E, Supporting Information), and IL-6 (Figure S7F, Supporting Information) in tacrolimus-treated mice were significantly reduced by LC40 administration.

3.4. Gut Microbial Changes Induced by Tacrolimus Were Prevented by LC40 Administration

Fecal DNA was isolated from all experimental mice groups. The compositions of bacterial community were evaluated by

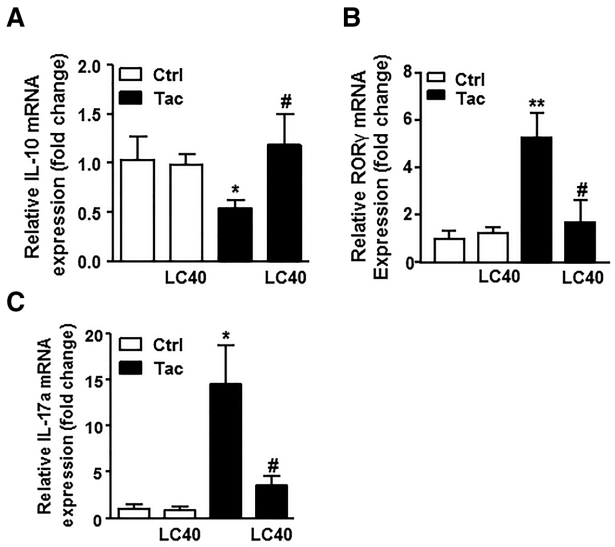


Figure 5. *Lactobacillus fermentum* CECT5716 (LC40) administration prevents tacrolimus (Tac)-induced aortic T-cells infiltration. A) IL-10 mRNA levels (n = 5–7). Aortic Th17 infiltration, measured by mRNA levels of B) RORγ and C) IL-17a (n = 4–6). Values are expressed as mean ± SEM (two-way ANOVA with post hoc Sidak test). * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the control (Ctrl) group. # indicates $p < 0.05$ compared with the Tac group.

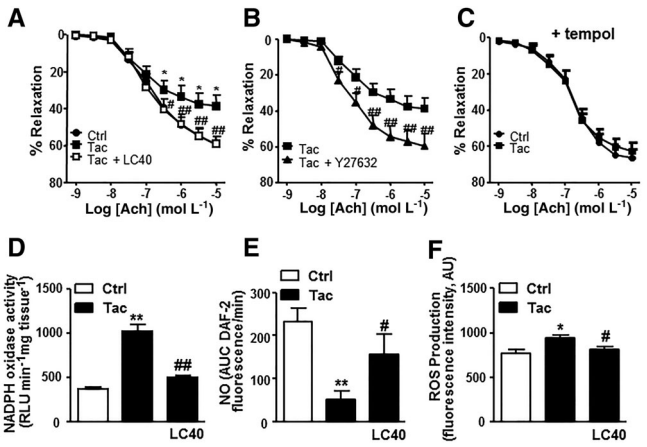
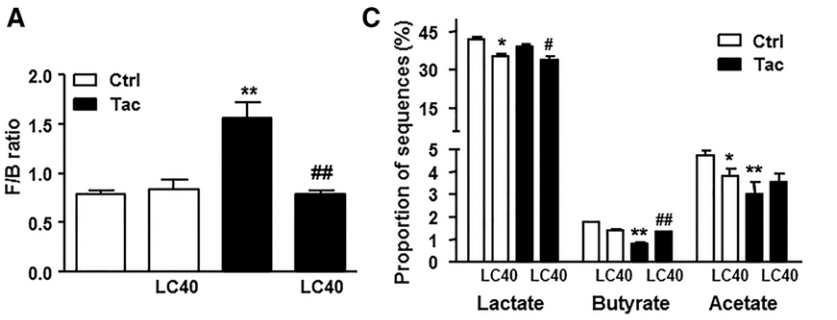


Figure 6. T-cells polarization in mesenteric lymph nodes induced by *Lactobacillus fermentum* CECT5716 (LC40) administration is involved in the prevention of tacrolimus (Tac)-induced endothelial dysfunction. A–C) Vascular relaxant responses induced by acetylcholine (ACh), in aortas precontracted by U46619 (10^{-8} mol L^{-1}) in the (A) absence and (C) presence of the SOD mimetic tempol (10^{-5} mol L^{-1}) for 30 min, after incubation for 4 h of control rings with lymphocyte conditioned media from the experimental groups in the (B) absence or in the presence of the Rho-kinase inhibitor Y27632 (50 μmol L^{-1}). Values are expressed as mean ± SEM (n = 6–9 rings from different mice; t-test). D) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence after incubation during 4 h of control rings with lymphocyte conditioned media from the experimental groups. Values are expressed as mean ± SEM (n = 6–9; t-test). E) NO release, estimated from the area under the curve (AUC) of the fluorescent signal of DAF-2 for 30 min in MAECs stimulated with the calcium ionophore calcimycin (A23187, 1 μmol L^{-1}). Results are mean ± SEM (n = 6–9; t-test). F) ROS production, measured by fluorescence in CM-H2DCFDA-loaded MAECs. Results are mean ± SEM (n = 6–9; t-test). MAECs were incubated for 4 h with lymphocyte conditioned media from the experimental groups. Values in (E) and (F) were obtained in three different determinations. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the control (Ctrl) group. # and ## indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the Tac group.

calculating three major ecological parameters, including Chao richness, Pielou evenness, and Shannon diversity. No significant changes among groups were observed in microbial richness and evenness (Figure S8A,B, Supporting Information). However, microbial diversity was found to be decreased in mice that received tacrolimus as compared to the control group, being restored in those mice treated with tacrolimus and LC40 (Figure S8C, Supporting Information). Furthermore, when 2D scatterplots were generated by principal coordinate analysis (PCoA) to visualize whether the experimental groups in the input phylogenetic tree have significantly different microbial communities, we found that the composition of fecal microbial communities was distinct among experimental groups (Figure S8D, Supporting Information).

When the phyla composition was evaluated in the different experimental groups, a significant increase in the ratio of Firmicutes (F) and Bacteroidetes (B; F/B), a signature of gut dysbiosis, was observed in the tacrolimus-treated group in comparison with the control group. Of note, the administration of the probiotic LC40 to the tacrolimus-treated group was able to normalize this ratio (**Figure 7**A). Fecal samples were dominated by Firmicutes and Bacteroidetes and with smaller proportions of Actinobacteria and Proteobacteria (Figure 7B). The increase induced by tacrolimus in F/B was caused by an expansion of Firmicutes and a contraction of Bacteroidetes. We also found a trend toward lower ($p = 0.0658$) proportion of Actinobacteria in the tacrolimus group as compared to controls, indicating a less diverse microbiota, which is consistent with the decrease in Shannon index.

We also evaluated what genera of bacteria contributed to the alteration of microbiota composition. At the genus level, butyrate- (including *Butyricimonas*) and acetate-producing bacteria were found reduced in tacrolimus group, without changes in lactate-producing bacteria (Figure 7C). LC40 treatment prevented the reduction in butyrate-producing bacteria, and reduced lactate-producing bacteria, without significant changes in acetate-producing bacteria, as compared to tacrolimus-treated mice. However, LC40 significantly reduced acetate-producing bacteria as compared to control group. Interestingly, when other genera were considered, a significant depletion of *Bifidobacterium* was found in the tacrolimus group (Figure S9A, Supporting Information). The genus *Bifidobacterium,* which belongs to the Actinobacteria phylum, is commonly considered as a beneficial bacterial genus, and plays a critical role in the maturation and regulation of the immune system.[29,30] In fact, *Bifidobacterium infantis* effectively attenuates TNBS-induced colitis by decreasing Th1 and Th17 responses and increasing FoxP3(+) Treg response in the colonic mucosa.[31] LC40 treatment increased the accumulation of *Bifidobacterium* in both control and tacrolimus-treated mice, which would be involved in the change in T cells polarization in mesenteric lymph nodes and spleen induced by this probiotic. In addition, bacteria belonging to the order Clostridiales were relatively increased after tacrolimus, whereas LC40 treatment prevented this increase (Figure S9B, Supporting Information).



Phylum	Mean value (%) ± SEM			
	Ctrl (n=9)	LC40 (n=6)	Tac (n=8)	Tac+LC40 (n=6)
Actinobacteria	4.200 ± 0.615	5.187 ± 1.034	2.983 ± 0.466	3.900 ± 0.628
Bacteroidetes	49.778 ± 1.867	49.375 ± 3.262	31.833 ± 2.414**	49.333 ± 2.076##
Firmicutes	37.500 ± 1.599	39.000 ± 3.479	50.167 ± 3.380**	38.333 ± 1.764##
Proteobacteria	0.787 ± 0.089	0.614 ± 0.190	0.545 ± 0.118	0.695 ± 0.234
Others	7.735 ± 1.112	5.824 ± 1.647	14.472 ± 2.012**	7.738 ± 1.783#

Figure 7. *Lactobacillus fermentum* CECT5716 (LC40) administration prevents tacrolimus (Tac)-induced phyla changes in the gut microbiota composition. A) The Firmicutes/Bacteroidetes ratio (F/B ratio) was calculated as a biomarker of gut dysbiosis. B) Phylum breakdown of the four most abundant bacterial communities in the fecal samples from all experimental groups. C) Relative proportions of lactate-, butyrate-, and acetate-producing bacteria in the gut microbiota from all experimental groups. Values are expressed as mean ± SEM ($n = 6$–9; two-way ANOVA with post hoc Sidak test). * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the control (Ctrl) group. # and ## indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the Tac group.

3.5. Altered Gut Microbiota by LC40 Regulates Hypertension and Endothelial Dysfunction Induced by Tacrolimus

To address the question whether microbiota from LC40 mice could potentially regulate the changes in blood pressure and endothelial function induced by tacrolimus, we transferred microbiota from 7 days in treatment with LC40 or vehicle hosts into control mice, and both groups were then treated with tacrolimus for 7 days (**Figure 8**A). Tacrolimus increased SBP by ≈43 mmHg in FMT control mice, whereas this increase was lower (≈54%) in FMT LC40 mice (Figure 8B). This protective effect was corroborated by direct blood pressure measurements of MABP (Figure 8C). In addition, endothelium-dependent relaxant responses to acetylcholine in U46619-contracted aortic rings from both experimental groups were significantly higher in FMT LC40 than in FMT control mice (Emax: 90 ± 3% vs 70 ± 4%, $p < 0.01$, respectively; Figure 8D). Moreover, the stool transfection from LC40-treated mice inhibited the increase in aortic NADPH oxidase activity (Figure 8E) and mRNA level of proinflammatory cytokines (Figure 8F) induced by tacrolimus.

To study if changes induced by tacrolimus in the gut microbiota are involved in their effects on blood pressure and endothelial function, we performed FMT from tacrolimus and tacrolimus + LC40-treated mice to control recipient mice (Figure 8G). Fecal transfection from tacrolimus group increased SBP by ≈37 mmHg, whereas the increase induced by FMT from tacrolimus + LC40-treated mice was lesser than in the aforementioned case (≈18 mmHg, $p < 0.05$; Figure 8H). In addition, the relaxant responses to acetylcholine were significant higher in this group as compared to mice transplanted with feces from tacrolimus group (Figure 8I).

4. Discussion

The effect of immunosuppressant drugs on long-term patient cardiovascular outcome has become as much a clinical issue as has their effect on acute transplant rejection. Herein, we report that oral administration of the probiotic LC40 can prevent the vascular alterations induced by tacrolimus without reducing its immunosuppressant effect, that is, the decrease in total CD4+ cells.

One of the mechanisms by which tacrolimus causes hypertension is via inhibition of FK506 binding protein 12 (FKBP12/12.6) in blood vessels, leading to reduced vasodilation and/or increased vasoconstriction.[8,32–34] Also, tacrolimus exerts direct detrimental vascular effects by inducing phosphorylation of the inhibitory eNOS residue Thr495, which decreases endothelium-dependent relaxant responses.[32–35] Our data showed that tacrolimus reduced aortic endothelium-dependent relaxation to acetylcholine,

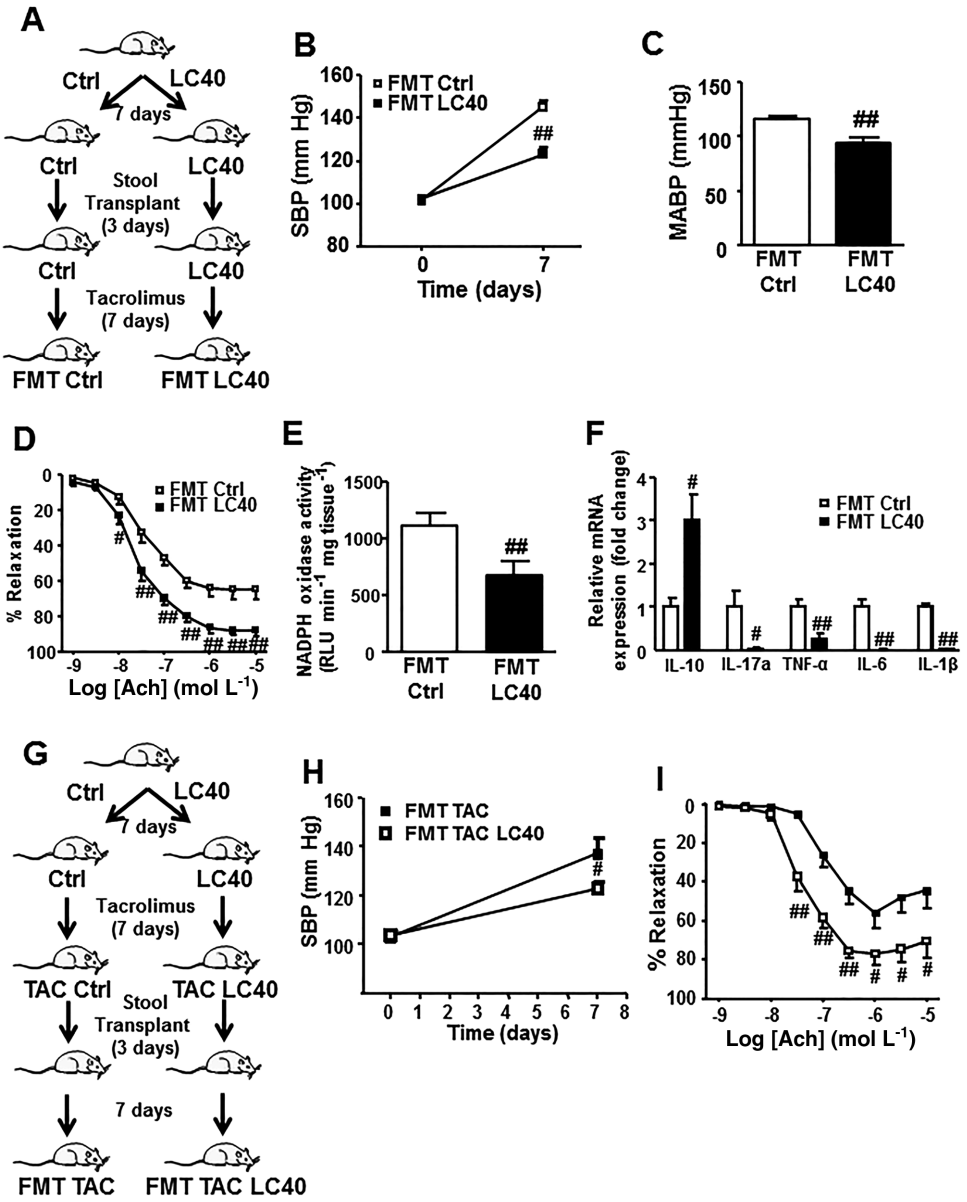


Figure 8. Fecal microbiota transplantation (FMT) from *Lactobacillus fermentum* CECT5716 (LC40)-treated mice to control mice prevents both hypertension and endothelial dysfunction and inflammation induced by tacrolimus (Tac). A) Schema of stool transplantation from donor mice taking vehicle (Ctrl) of LC40 treatment in the drinking water for 7 day to control recipient mice, then treated with Tac for 7 days. B) Time course of systolic blood pressure (SBP), measured by tail-cuff plethysmography, in control mice with stool transplant from Ctrl or LC40-treated mice, at time 0 and 7 days after Tac treatment ($n = 7$). C) Mean arterial blood pressure (MABP), measured by intra-arterial recording into left carotid artery, at the end of the experimental period ($n = 4$–6). D) Vascular relaxant responses induced by acetylcholine (ACh) in aortas precontracted by U46619 (10^{-8} mol L^{-1}; $n = 7$). E) NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence in aorta ($n = 5$–6). F) Aortic mRNA levels of cytokines ($n = 5$–7). Values are expressed as mean ± SEM (*t*-test). G) Schema of stool transplantation from donor mice treated with Tac that received vehicle or LC40 to control mice. H) Time course of SBP, measured by tail-cuff plethysmography, in control mice with stool transplant, at time 0 and 7 days after FMT. I) Vascular relaxant responses induced by ACh in aortas precontracted by U46619 (10^{-8} mol L^{-1}). Values are expressed as mean ± SEM ($n = 7$; *t*-test). # and ## indicate $p < 0.05$ and $p < 0.01$, respectively.

without altering the relaxant response to the activator of soluble guanylyl cyclase nitroprusside in endothelium-denuded vessels, indicating that alterations in responsiveness to cyclic GMP do not seem to contribute to endothelial dysfunction in tacrolimus-treated mice. NO is the major factor accounting for endothelium-dependent relaxation as denoted by the almost full inhibitory action of L-NAME in the aorta from control mice. However, under these eNOS inhibiting conditions, acetylcholine induced a significant increase in vascular tone in aortas from tacrolimus-treated mice, showing the endothelial release of vasoconstrictor factors. The contractile response induced by high concentrations of acetylcholine in phenylephrine-contracted rings was absent

when TP receptors were activated by the thromboxane A_2 analog U-46619, and by COX inhibition with indomethacin, suggesting the release of vasoconstrictor prostanoids. In fact, COX expression (COX-1 and COX-2) was higher in aortas from tacrolimus-treated mice as compared to control mice. Thus, the diminished acetylcholine-induced relaxation indicates an impaired agonist-induced NO bioactivity, attributed to an alteration in NO synthesis or its bioavailability, and/or increased prostanoids release. Probiotic administration prevented this tacrolimus-induced endothelial dysfunction, protecting agonist-induced NO bioactivity and reducing COX-derived vasoconstriction. In addition, the eNOS expression was similar among all experimental groups, whereas COX-1 and COX-2 mRNA levels were normalized by LC40. Similarly, LC40 treatment also improved the impaired acetylcholine relaxation induced by tacrolimus in small mesenteric arteries. This impaired relaxation was also suppressed when these resistance arteries, involved in the control of blood pressure, were incubated in the presence of L-NAME plus indomethacin, showing that EDHF-mediated relaxation is preserved by tacrolimus and that LC40 improved NO-mediated relaxations.

A key mechanism of endothelial dysfunction involves the vascular production of ROS, particularly O_2^-, which reacts rapidly with NO and inactivates it.[36] The impairment of endothelium-dependent relaxation seen in tacrolimus-treated mice involves inactivation of NO by O_2^-, because the SOD mimetic tempol abolished this endothelial dysfunction. Moreover, tempol also abolished endothelium-dependent contractions induced by high concentrations of acetylcholine in phenylephrine-contracted rings, suggesting the involvement of ROS in this component of acetylcholine response. In addition, aortas from tacrolimus-treated mice showed higher ROS levels and, again, LC40 administration abolished this increase. The NADPH oxidase, a multi-enzymatic complex formed by $gp91^{phox}$ or its vascular homologous NOX-1, NOX-2, NOX-4, rac, $p22^{phox}$, $p47^{phox}$, and $p67^{phox}$, is considered the major source of O_2^- in the vascular wall.[37] We found a marked increase in both the NADPH oxidase activity and the expression of the catalytic NADPH oxidase subunits NOX1, NOX-2, and NOX4 in aortas from tacrolimus-treated mice, which were prevented by LC40 administration. Interestingly, the NOX2 inhibitor gp91dstat abolished both the increased ROS production and the impaired relaxant response induced by tacrolimus, which strongly suggest that NOX2 is the most important NOX isoform involved in these effects. All of the cytosolic subunits assemble on the membrane upon activation, allowing the enzyme to generate ROS. We also found that tacrolimus increased membrane translocation of the cytosolic subunits $p47^{phox}$ and $p67^{phox}$, and this effect was inhibited by LC40. It is well established that NOX activation increased the local production of ROS, which oxidizes BH4 to induce BH4 deficiency. In these conditions, eNOS produces superoxide rather than NO, leading to an uncoupling eNOS status.[38] We found that aorta from tacrolimus-treated mice showed increased NADPH-stimulated ROS production which was inhibited by both eNOS blockade with L-NAME, and by the BH4 precursor sepiapterin, suggesting the involvement of uncoupled eNOS as an ROS-producing enzyme in tacrolimus group. So, reduced NADPH oxidase activity and eNOS uncoupling status could be involved in the reduction of vascular ROS level induced by this probiotic. All the results suggest that the reduction of O_2^- levels in the vascular wall, and the subsequent prevention of NO inactivation, constitute a key mechanism involved in the LC40 protective effects on endothelial function.

The enhancement of ROS bioavailability, in particular O_2^-, affects endothelial function not only by reducing NO bioavailability, but also by promoting inflammation.[24,39] Our data are in agreement with this oxidative-proinflammatory vascular status via NF-κB activation, since both IκBα phosphorylation and the mRNA expression of the proinflammatory cytokines TNFα and IL-6 were higher in aortas from the tacrolimus-treated versus the control mice. Moreover, LC40 administration, which reduced ROS levels, also prevented the NF-κB activation and the subsequent raise in the mRNA levels of TNFα and IL-6 induced by tacrolimus.

It is becoming more and more evident that immune cells can affect vascular reactivity, renal function, and blood pressure regulation.[40,41] Several clinical studies have reported that Treg levels are decreased in patients treated with tacrolimus,[42,43] and high levels of IL-17 are associated with hypertension.[44,45] Our data are in agreement with the study of Chiasson et al.[35] They suggested that the ability of tacrolimus to promote spleen T-cell proliferation toward the expansion of Th17 cells, together with a reduction in Treg cell number, provides an additional mechanism that may contribute to the hypertension induced by tacrolimus, in addition to its direct deleterious effects on blood vessels. In fact, we found that tacrolimus reduced Treg and increased Th17 cells in mesenteric lymph nodes and spleen, and the circulating levels of IL-10 and IL-17 reflect the modifications seen in these lymphoid organs. In addition, preventing T-cell polarization by LC40 administration improved endothelial dysfunction induced by tacrolimus. Several data support this finding. First, LC40 treatment prevented Th17/Treg polarization induced by tacrolimus in lymphoid organs. Second, the antioxidant cytokine released by Treg, IL-10[17] were increased by LC40 in the vascular wall. Third, the level of the proinflammatory cytokine IL-17, which causes Rho-kinase-mediated endothelial dysfunction by increasing phosphorylation of the inhibitory eNOS residue Thr495,[44] was reduced by LC40 in tacrolimus mice. Fourth, the incubation of control mice aortas with the conditioned media of cultured lymphocytes of mesenteric nodes from tacrolimus group impairs endothelium-dependent relaxation to acetylcholine, whereas this relaxation was preserved when aortas were incubated with conditioned media of lymphocytes from tacrolimus-LC40-treated mice. The impaired relaxation to acetylcholine was suppressed by the presence of Y27632 or tempol, involving Rho-kinase activation and ROS production in this endothelial dysfunction. Taking into account that tempol completely abolished this endothelial dysfunction and IL17 promotes ROS generation by NOX,[46] it is reasonable to suggest that the effects on RhoA/Rho kinase are mediated via the increased ROS, rather than directly by IL-17. In fact, ROS-dependent activation of RhoA/Rho kinase has been previously described.[47] In addition, similarly to what we found in aorta in vivo, the NADPH oxidase activity in aorta incubated with the conditioned media of cultured lymphocytes from mesenteric nodes from the tacrolimus group was higher than those from the control group. Again, the NADPH oxidase activity was restored to control values when the aorta was incubated with the conditioned media of cultured lymphocytes of

mesenteric nodes from the tacrolimus-LC40 group. Furthermore, the incubation of MAECs with the conditioned media of cultured lymphocytes of mesenteric nodes from tacrolimus-LC40 group restored the reduced NO production and the elevated ROS production induced by conditioned media of cultured lymphocytes of mesenteric nodes from tacrolimus group. Taken together, these data suggest that the changes in T-cell populations in peripheral lymphoid organs may guide the alterations found in endothelial function, and the changes in T-cell polarization induced by the probiotic LC40 seem to play a key role in the prevention of endothelial dysfunction induced by tacrolimus in mice. However, the identity of the active component in mesenteric lymph nodes after LC40 treatment is unknown.

The intestinal microbiota has a marked effect on host immune functions.[11] In our experimental conditions, tacrolimus-induced gut microbial dysbiosis characterized by low microbial diversity and increased F/B ratio, caused by an expansion of Firmicutes and a contraction of Bacteroidetes. Interestingly, gut dysbiosis induced by tacrolimus contributes to hypertension and endothelial dysfunction, since FMT from tacrolimus-treated mice to control recipient mice increased SBP and impaired acetylcholine relaxation. The mechanism of tacrolimus inducing gut dysbiosis remains to be determined. It has been reported that the stimulation of the sympathetic nervous system in the gut compromises its barrier function,[48] and it is capable of altering the microbiota.[49] Interestingly, tacrolimus induces sympathetic activation[50] and this may result in dysbiosis, and subsequently in intestinal inflammation. In fact, we found increased levels of intestinal proinflammatory cytokines and occludins in mice treated with tacrolimus. In addition, tacrolimus induces an antibiotic effect,[51] which can modify gut microbiota.

The reduction in acetate- and butyrate-producing bacteria can be linked to hypertension. Butyrate plays a regulatory role in the epithelial cell differentiation, barrier function, stimulation of Treg cells, and amelioration of mucosal inflammation.[52–54] In consequence, the observed reduction in butyrate-producing bacteria induced by tacrolimus in mice might be involved in the damage of gut integrity and development of a colonic inflammatory status, associated to the reduction of Treg population in lymph mesenteric nodes, and the subsequent endothelial dysfunction and increased SBP. In fact, the administration of LC40 to mice prevented most of the changes in the microbiota induced by tacrolimus, that is, increased microbial diversity, reduced F/B ratio, and increased butyrate-producing bacteria, which was associated to a reduction of intestinal inflammatory markers, increased Treg cells, and improvement of endothelial function and SBP. In addition, LC40 reduced the population of lactate-producing bacteria, which also might collaborate in its protective effects because plasma lactate levels have been associated with increased SBP.[55]

Interestingly, blood pressure was reduced in mice receiving LC40 microbiota as compared to control. Moreover, LC40 microbiota transplantation also led to an increased aortic endothelium-dependent relaxation to acetylcholine, to NADPH oxidase activity reduction, and to reduced levels of proinflammatory cytokines and increased IL-10 compared to mice receiving control microbiota. These data support the hypothesis that modification in the gut microbiota, favoring butyrate-producing bacteria, can play an essential role in the antihypertensive effects of LC40 in tacrolimus-treated mice.

In conclusion, our data showed that LC40 administration leads to improvement of endothelial dysfunction and hypertension induced by tacrolimus independently of ACE inhibition, at least in part, through an improvement in the composition of the microbiota. These effects seem to be associated with reduction of vascular oxidative stress, mainly through NOX2 downregulation and improvement of eNOS uncoupling, and vascular inflammation, possibly as a result of a decreased Th17 and increased Treg cells polarization in mesenteric lymph nodes (Figure S10, Supporting Information). These results open new possibilities to increase the benefit–risk ratio of this immunosuppressor drug by modulation of microbiota with safe probiotic bacteria such as LC40. However, caution should be taken when extrapolating these findings to humans because of the possible differences in the behavior of the animal and human gut microbiota when exposed to tacrolimus, which might alter the possible applicability to clinical practice in humans of LC40 treatment.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

endothelial dysfunction, gut dysbiosis, probiotics, tacrolimus, T cells

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