

The Effect of Tacrolimus on Gut Microbiota in a Mice Model and In Vitro Cellular Responses

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ABSTRACT

Tacrolimus (FK506) is a cornerstone calcineurin inhibitor used in solid organ transplantation and has been more and more implicated in gut microbiota dysregulation, but its mechanism of action has not been well comprehended. This paper examined the dose-dependent cytotoxic and pro-inflammatory effects of tacrolimus, using a BALB/c murine model to study the gut microbial dysbiosis caused by tacrolimus. The male BALB/c mice (n=30) were given control, low dose (1mg/kg/day) tacrolimus and high dose (5mg/kg/day) tacrolimus over 28 days and gut microbial composition was determined by 16S rRNA gene sequencing. Simultaneously, to determine cell viability (MTT assay), inflammatory cytokines (IL-6 and TNF- α) and cell cycle dynamics (flow cytometry), Vero cells were subjected to

tacrolimus (0.1-50 mg/mL) over the course of 24-48 hours. Tacrolimus at high doses led to severe microbial dysbiosis, characterized by decreased diversity and severe compositional changes, such as loss of beneficial short-chain fatty acid-producing genera (*Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*) and increase in *Proteobacteria*. Tacrolimus induced dose- and time-dependent cytotoxicity in vitro, increased IL-6 and TNF- α levels significantly and G1 phase arrest with greater apoptosis at higher concentrations. Taken together, these results indicate that tacrolimus encourages the state of gut microbiota imbalance and inflammatory cellular reactions, and immunosuppressive treatment and microbiome perturbation are closely connected. These findings also highlight the possibility of using microbiota-specific interventions, such as probiotic supplementation and short-chain fatty acid regulation, to alleviate the adverse effects of tacrolimus use in transplant patients.

Keywords: Tacrolimus (FK506), Gut Microbiota Dysbiosis, Immunosuppression, Inflammatory Cytokines, Short-Chain Fatty Acids

Introduction

Tacrolimus (FK506) is a popular immunosuppressant used in solid organ transplants and is regarded as a first-line medication against graft-rejection. It is a macrolide that was initially purified in *Streptomyces tsukubaensis* and acts by blocking the calcium-dependent serine-threonine phosphatase calcineurin. This inhibition prevents dephosphorylation and activation of nuclear factor of activated T cells (NFAT) and inhibits the transcription of interleukin-2 (IL-2) and T-lymphocyte proliferation [1, 2]. Although tacrolimus is effective in immunosuppression, it has a low therapeutic index, with a whole-blood concentration range of 5–15 ng/mL, and is linked with various side effects, such as nephrotoxicity, neurotoxicity, hypertension, and post-transplant diabetes mellitus (PTDM) (Venkataraman et al., 19). Notably, gastrointestinal complications are often observed among transplant patients on tacrolimus, yet the mechanisms of their occurrence, especially that of gut microbiota, are not fully investigated [3-5].

The human gastrointestinal tract contains an extremely intricate and dynamic microbiome containing trillions of microorganisms, with about 1,000 different microbial types [6, 7]. This intestinal microbiota is important in controlling host physiology, such as immune system regulation, and epithelial barrier integrity and systemic inflammation [8]. The synthesis of short-chain fatty acids (SCFAs) acetate, propionate, and butyrate by digestion of dietary fibers, mostly via the phyla *Firmicutes* and *Bacteroidetes*, is one of the most important functions of gut microbes [9, 10]. Such metabolites play a role in enhancing tight junction integrity, differentiating regulatory T cell (Treg), and regulating inflammatory responses, which include histone deacetylase (HDAC) inhibition [11, 12]. This microbial balance disruption, called dysbiosis, has been associated with numerous pathological processes, including infections, metabolic diseases, cardiovascular diseases, and transplant-related complications, including graft rejection and PTDM [13, 14].

A new research area, pharmacomicrobiomics, is concerned with the two-way communication between drugs and the gut microbiota, whereby microbial

communities can affect drug metabolism, efficacy, and toxicity [15, 16]. Recent research has shown that gut microbial enzymes have the potential to alter drug metabolites, in some cases reactivating within the intestine, and playing a part in localized toxicity. As an illustration, the drug metabolites that are inactive can be converted to the active forms by bacterial 2-glucuronidase enzymes, causing epithelial injury and inflammation [17]. Brossier, et al. [18] showed that the blockage of bacterial β -glucuronidase was an important indicator of the ability to reduce the intestinal toxicity related to immunosuppressive therapy, which is a strong indication that the microbiota target may be used to alleviate the adverse effects of drugs. The results offer a conceptual and methodological model to study tacrolimus-induced changes in microbiota and their subsequent biological effects.

In this research, the in vitro part involved the use of the Vero cells (ATCC CCL-81) which is a continuous kidney epithelial cell line of the African green monkey. They are highly clonal in Pakistani research centers, such as the Pakistan Medical Research Council (PMRC), Islamabad, and widely used in toxicological and pharmacological research because of stable growth properties and well-characterized responses to exposure to drugs [19]. Vero cells are especially convenient to measure cytotoxicity, production of inflammatory cytokines, and cell cycle changes, and thus make them a good model to test cellular effects of tacrolimus in resource limited labs.

Thus, the current research aimed to (i) describe, in detail, the tacrolimus-induced changes in gut microbiota composition in the BALB/c mice, by diversity indices and taxonomic profiling, (ii) assess dose-dependent cytotoxic and inflammatory reactions in Vero cells, and (iii) explore the possible mechanism that would connect the distance between gut microbial dysbiosis. This research will be able to help to expand the knowledge about pharmacomicrobiomic interactions and also offer a platform to develop microbiota-specific strategies to alleviate tacrolimus-related toxicity.

Materials and Methods

Ethical Approval and Animal Care

The Institutional Animal Ethics Committee (IAEC) of the University Research Centre, Pakistan reviewed and approved all animal experimental procedures. The research was carried out in compliance with the internationally accepted ethical standards such as the principles of the 3Rs (Replacement, Reduction, and Refinement) and the ARRIVE 2.0 principles of reporting about animal research. Procedures were also in line with international best animal use and care practices where necessary.

Animals, Randomization, and Experimental Design

The BALB/c male mice ($n = 30$; 6-8 weeks old; body weight 20-25 g) were sourced at the National Institute of Health (NIH), Islamabad, Pakistan. Upon arrival, the animals were placed in clean polypropylene cages in controlled laboratory conditions, with a temperature of $22 \pm 2^\circ\text{C}$, relative humidity of $55 \pm 5\%$, and 12 hours of light/dark cycle to ensure the animals were in stable physiological conditions. To prevent any nutritional stress, all mice have been given standard laboratory chow and sterile drinking water ad libitum during the study. The animals were also given 7 days to acclimatize in the laboratory environment before the experiment was commenced to

enable the animals to adjust to the conditions of handling and housing. Following this acclimatization, the mice were chosen at random into three experimental groups (10 mice in each group) to provide the mice with equal distribution in terms of body weight. Tacrolimus (Prograf®, Astellas Pharma) was dissolved in 0.9% normal saline and administered as per the study design. The general health and behavior of all the mice and any stress or toxicity were monitored daily throughout the entire period of the experiment (28 days). The body weight was also measured on a weekly basis to monitor any physiological changes caused by the treatment as well as to guarantee animal welfare during the study.

Table 1. Experimental Group Design and Treatment Protocol

Group	Treatment	Dose	Route & Duration
Group I: Control	0.9% NaCl (vehicle)		i.p., once daily
Group II: Low-dose TAC	Tacrolimus (Prograf®)	1 mg/kg/day	i.p., once daily
Group III: High-dose TAC	Tacrolimus (Prograf®)	5 mg/kg/day	i.p., once daily

i.p. = intraperitoneal; TAC = tacrolimus; n = 10 mice per group. All solutions prepared freshly on the day of administration and protected from light.

Fecal Sample Collection and 16S rRNA Gene Sequencing

A total of 90 samples were collected on day 0 (pre-treatment), day 14 and day 28. Non-invasive collection was made to minimize stress. Samples were all plunged into liquid nitrogen and kept at -80C until analysis. Each fecal sample was subjected to the extraction of DNA with the use of the QIAamp PowerFecal DNA Kit (Qiagen, Germany) using 200 mg of each sample. Quality and concentration of DNA were verified with a NanoDrop spectrophotometer. Further work was done on only high-quality DNA. Universal primers (341F and 805R) were used to amplify the bacterial 16S rRNA gene (V3 -V4 region). PCR products were sequenced on Illumina MiSeq platform (2 × 250 bp). QIIME2 software (v2023.2) was used to process the sequencing data. The reads of low quality were filtered out, and the DADA2 method was used to identify bacterial groups (ASVs). The SILVA database was then used to classify these. Microbial diversity was evaluated in two folds: alpha (within groups) by using Shannon index, Chao1, and other indices and beta (between groups) by using BrayCurtis distance. PCoA plots were used to visualize the results and standard tests were used to analyze the results in R software.

Vero Cell Culture and Tacrolimus Exposure

Vero cells (ATCC CCL-81) were taken out of the Pakistan Medical Research Council (PMRC) cell repository at Islamabad and cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 ug/ml streptomycin. The cells were cultivated in a humidified incubator at 37C with 5% of CO₂ and subcultured after every 2-3 days when they attained

approximately 80-85% of confluence with 0.25% trypsin-EDTA. To be consistent, the experiments were conducted with only the passages 5 to 15. Tacrolimus was initially dissolved in anhydrous ethanol to obtain a stock solution which was then diluted in serum-free DMEM immediately before use, maintaining the final ethanol concentration to be lower than 0.1% and the control samples were treated with the same volume of ethanol in DMEM but without the drug.

MTT Cell Viability Assay

Vero cells were put in 96-well plates with a cell density of 1×10^4 per well and allowed to adhere to the plates after 24 hours. Following this, various concentrations of tacrolimus (0, 0.1, 1, 10, 25 and 50 $\mu\text{g/mL}$) were used and incubated 24 or 48 hours at 37°C to treat the cells and measurement of cell viability was done by adding 105 μL of MTT solution. The liquid of both the wells was then cleared and 100 μL of DMSO was used to dissolve these crystals. A microplate reader was used to measure the absorbance at 570 nm (with a reference at 630 nm). Cell viability was determined by the control of cells (treated and control) that were supposed to be 100 percent viable. GraphPad Prism 9.0 was used to calculate the IC_{50} . Doxorubicin was applied as a positive control to ensure the assay was functioning well and all treatments were done in six replicates in three different experiments to have a reliable result.

Cytokine Quantification by ELISA

Vero cells were planted on 6-well plates with a concentration of 5×10^5 cells/well with tacrolimus (0.1, 1, 10 and 50 $\mu\text{g/mL}$) after 24 hours. The culture media were centrifuged at $300 \times g$ at 4°C to eliminate debris and stored at -80°C until analysis after treatment. Commercial ELISA kits were used to measure levels of IL-6 and TNF- α based on the instructions of the manufacturer. The absorbance was obtained at 450 nm, with a correction at 570 nm. The standard curves were computed to obtain cytokine levels that were then expressed as a fold change of the control. All trials were carried out in three repetitions and three times. Simultaneously, on day 28 blood samples of mice were intracardiacally punctured, the samples were processed to acquire plasma and were assessed on IL-6 and TNF- α using the identical ELISA technique.

Cell Cycle Analysis by Flow Cytometry

T25 flasks were used to grow Vero cells and expose them to various concentrations of tacrolimus (0, 1, 10, and 50 $\mu\text{g/mL}$) after 24 hours. The cells were treated and then collected with trypsin, washed twice in cold PBS and the cells fixed by adding cold 70% ethanol slowly and mixing gently to avoid clumping. The fixed cells were left overnight at -20°C. Again, the cells were washed and propidium iodide (PI) solution with RNase A and Triton X-100 was added and incubated at 37°C in the dark prior to analysis. Each sample was analyzed on a flow cytometer on at least 10,000 cells. Analysis software was then used to determine the distribution of cells in various cell cycle phases (sub-G1, G1, S and G2/M) whereby the sub-G1 stage represents the apoptotic (dead) cells.

Statistical Analysis

In the in vitro experiments, all data are presented as mean and standard deviation (SD) and in the in vivo experiments, as mean and standard error of the mean (SEM). The ShapiroWilk test was used to test the normality of the data and then analyzed. In the case of normally distributed data, the differences between the groups were compared using one-way ANOVA, and then the post-hoc test in the form of a Tukey test. KruskalWallis with Dunns multiple comparison correction was employed in the case of data not normally distributed, such as the relative abundance of gut microbiota. The Mann-Whitney U test was used to compare two groups. The correlation coefficient (r) of Pearson was employed to test the association between the abundance of Proteobacteria and serum TNF- α . The p-value of 0.05 or below was regarded as statistically significant. GraphPad Prism 9.0 and SPSS version 26 were used to perform all the statistical analysis.

Results

Animal Health, Survival, and Body Weight Trajectories

All the 30 mice underwent the full duration of the experiment (28 days) and none died during the experiment due to treatment. Clinical observations daily did not reveal any behavioral abnormalities, neurological or dermatological changes in any group. Body weight analysis on the other hand showed definite group differences in the course of time. Each of the groups had similar initial body weights and at the final point of the study (day 28), the control group exhibited normal weight gain (27.6 ± 2.1 g). Conversely, weight gain was relatively slight in the low-dose tacrolimus group (25.1 ± 1.9 g; $p = 0.08$ vs control) and comparatively negative in high-dose group (22.4 ± 1.8 g; $p < 0.01$ vs control). All this suggests that there is a dose-dependent suppression of normal weight gain, whereby the more tacrolimus one was exposed to, the more weight one was suppressed. This trend is consistent with the results of Brossier, et al. [18], who also found similar catabolic effects in mice under immunosuppressant treatment, indicating that the changes produced by drugs on the gut microbiota and nutrient absorption can explain the decreased body weight in various immunosuppressive therapies.

Table 2 Initial and final body weight changes in control and tacrolimus-treated mice over 28 days.

Group	Treatment	Initial Weight (g)	Final Weight (g)	Weight Change (g)	Statistical Outcome
I	Control	24.8 ± 1.9	27.6 ± 2.1	+2.8	Reference
II	Tacrolimus (Low dose)	24.7 ± 1.8	25.1 ± 1.9	+0.4	$p = 0.08$
III	Tacrolimus (High dose)	24.9 ± 1.7	22.4 ± 1.8	-2.5	$p < 0.01$

Tacrolimus-Driven Decline in Gut Microbial Alpha Diversity

The total number of samples (3 time points x 3 groups x 10 animals) analyzed was successful and the average number of high-quality reads per sample (after quality control and removal of chimeric sequences) was 48,320. Each sample stabilized in the

rarefaction curves, suggesting the sufficient level of sequencing depth with which to conduct downstream diversity analyses. Table 3 summarises alpha diversity at day 28, and Fig. 1 illustrates the changes in the overall temporal changes. The shannon diversity index, which is the measure of richness and evenness, indicated a strong dose-dependent response of tacrolimus, whereby in tacrolimus-treated groups there was reduced diversity. At day 28, values were 5.67 ± 0.51 in the control group (Group I), 4.21 ± 0.38 in the low-dose group (Group II; $p < 0.01$ vs control), and 3.14 ± 0.42 in the high-dose group (Group III; $p < 0.001$ vs control), corresponding to a 44.6% reduction in the high-dose group (Fig. 1A). Time-course analysis revealed that this reduction occurred earlier in the high dose group (since day 14) and later in the low dose group (since day 21) suggesting a dose-dependent onset of dysbiosis. Similarly, Chao1 richness decreased significantly, falling by 20.5% in Group II (468 ± 58 vs 589 ± 67 ; $p < 0.01$) and by 47.0% in Group III (312 ± 45 vs 589 ± 67 ; $p < 0.001$) (Fig. 1B). Observed ASVs also declined from 412 ± 54 in controls to 318 ± 48 in Group II ($p < 0.01$) and 196 ± 38 in Group III ($p < 0.001$). The phylogenetic diversity of faith declined by half in the high dose condition (9.2 ± 2.1 vs 18.4 ± 3.1 ; $p < 0.001$) and not only did it decline in abundance, but also loss of evolutionarily distinct bacterial lineages was noted. These patterns were present in Simpson index and were a powerful and dramatic reduction in the total diversity of the microbes in all alpha measures of diversity.

Table 3. Alpha Diversity Indices at Day 28 (Mean $\pm$ SD; n=10/group)

Index	Control (Group I)	Low-dose TAC (Group II)	High-dose TAC (Group III)
Shannon Index	5.67 ± 0.51	4.21 ± 0.38 **	3.14 ± 0.42 ***
Chao1 Richness	589 ± 67	468 ± 58 **	312 ± 45 ***
Simpson Index	0.91 ± 0.04	0.81 ± 0.05 *	0.68 ± 0.06 ***
Observed ASVs	412 ± 54	318 ± 48 **	196 ± 38 ***
Faith's PD	18.4 ± 3.1	13.9 ± 2.7 **	9.2 ± 2.1 ***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. Group I Control; Kruskal–Wallis with Dunn's correction. ASV = amplicon sequence variant; PD = phylogenetic diversity; TAC = tacrolimus.

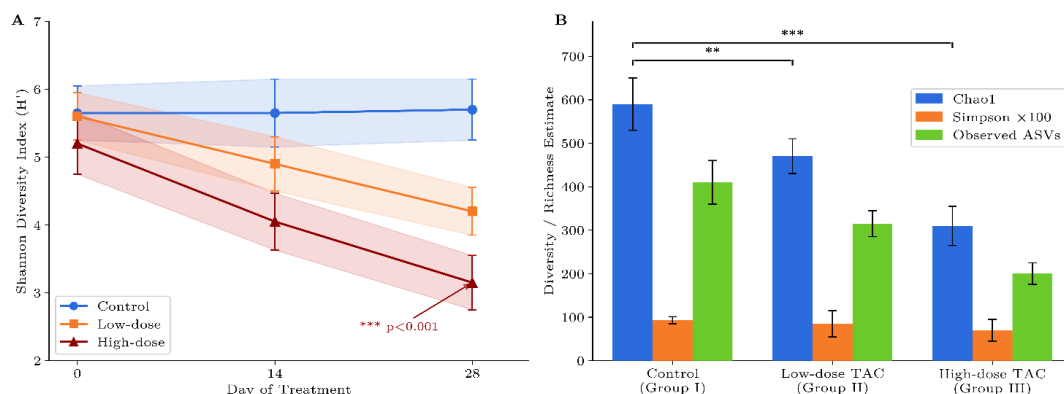


Fig. 1. Gut microbial alpha diversity in tacrolimus-treated BALB/c mice. (A) Shannon diversity index (H') over 28 days of treatment. Shaded bands indicate $\pm$ SD for Control and High-dose groups. (B) Comparison of Chao1 richness, Simpson index ($\times 100$), and observed ASVs at Day 28 across all

three treatment groups. Data are mean $\pm$ SD; $n=10/\text{group}$. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. Control; Kruskal–Wallis with Dunn's correction.

Tacrolimus Reshapes Gut Microbial Composition

Day-28 fecal samples were used to perform beta diversity analysis to assess variations on overall microbial community structure. Principal Coordinates Analysis (PCoA) using the BrayCurtis dissimilarity (table 4) clearly separated all three groups of samples and no overlap between their confidence clusters, demonstrating different microbial profiles. This was substantiated by PERMANOVA analysis which indicated that treatment group was a significant source of variation in microbial communities ($R^2 = 0.41$, $p = 0.001$), indicating that tacrolimus treatment brought about strong dose-related alteration of gut microbial communities. At the phylum level (Table 4), high doses of tacrolimus significantly increased Proteobacteria, increasing a margin of 23 percentage points, $8.2 \pm 3.1\%$ in controls to $31.4 \pm 6.8\%$. Some of the Gram-negative bacteria in this group include Escherichia, Klebsiella, Pseudomonas and Enterobacter and are associated with lipopolysaccharide (LPS) and are commonly associated with opportunistic infections in immunocompromised hosts. Conversely, there was a significant loss of the dominant commensal bacteria: Firmicutes decreased by 48.3 ± 6.2 to 28.7 ± 5.1 ($p < 0.001$), and Bacteroidetes by 35.6 ± 5.8 to 22.3 ± 4.9 ($p < 0.01$). This change shows a powerful dysbiotic trend and aligns with the results of Brossier, et al. [18], who also noted an upsurge in Proteobacteria in the aftermath of immunosuppressant exposure in mice. Also, there was a modest increase in Actinobacteria (4.9% to 9.8 ; $p < 0.05$), and a significant decline in Verrucomicrobia (3.1% to 0.8 ; $p < 0.05$), which may indicate a potential disturbance of the mucus-associated microbial layer, including Akkermansia muciniphila.

Table 4. Phylum-Level Relative Abundance at Day 28 (%), Mean $\pm$ SD; $n=10/\text{group}$)

Phylum	Control (%)	Low-dose (%)	High-dose (%)	Δ vs. Control	p-value
<i>Firmicutes</i>	48.3 ± 6.2	39.1 ± 5.4	28.7 ± 5.1	$\downarrow 19.6$ pp	<0.001
<i>Bacteroidetes</i>	35.6 ± 5.8	29.7 ± 4.8	22.3 ± 4.9	$\downarrow 13.3$ pp	<0.01
<i>Proteobacteria</i>	8.2 ± 3.1	19.4 ± 5.2	31.4 ± 6.8	$\uparrow 23.2$ pp	<0.001
<i>Actinobacteria</i>	4.9 ± 1.6	7.2 ± 2.1	9.8 ± 2.4	$\uparrow 4.9$ pp	<0.05
<i>Verrucomicrobia</i>	3.1 ± 1.2	1.8 ± 0.9	0.8 ± 0.5	$\downarrow 2.3$ pp	<0.05

pp = percentage points (absolute change from control mean). Kruskal–Wallis with Dunn's correction.
 $\uparrow$ = increase; $\downarrow$ = decrease.

Tacrolimus induced dysbiosis

Analysis of genus level (Fig. 3) revealed evident and biologically significant variations in microbiota in the gut at day 28. Beneficial commensal bacteria went down dramatically in the case of tacrolimus treatment. Lactobacillus dropped from $12.3 \pm 2.1\%$ in controls to $7.4 \pm 1.8\%$ in the low-dose group ($p < 0.05$) and $3.8 \pm 1.4\%$ in the high-dose group ($p < 0.001$). Similarly, Bifidobacterium declined from $8.7 \pm 1.9\%$ to $4.9 \pm 1.4\%$ (low dose; $p < 0.05$) and $1.6 \pm 0.8\%$ (high dose; $p < 0.001$). Faecalibacterium prausnitzii, a significant anti-inflammatory butyrate-producing bacterium, also reduced to $6.2 \pm 1.8\%$ (low dose; $p < 0.01$) and $1.9 \pm 0.9\%$ (high dose;

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$p < 0.001$). Moreover, *Akkermansia muciniphila*, which helps in keeping the mucus layer healthy and in maintaining metabolic balance, decreased to $3.1 \pm 1.2\%$ in the control group, $1.8 \pm 0.9\%$ (low dose) and $0.8 \pm 0.5\%$ (high dose; $p < 0.05$). These decreases in the short-chain fatty acid (SCFA)-producing bacteria imply loss of anti-inflammatory microbial properties and align with the decreased SCFA levels in earlier studies after exposure to immunosuppressants. Conversely, there was a significant increase in potentially harmful pathobionts. *Escherichia* increased from $2.1 \pm 0.8\%$ in controls to $12.6 \pm 3.1\%$ in the high-dose group ($p < 0.001$), *Enterococcus* rose from $1.8 \pm 0.7\%$ to $8.9 \pm 2.4\%$ ($p < 0.001$), and *Klebsiella* increased from $0.9 \pm 0.4\%$ to $5.3 \pm 1.6\%$ ($p < 0.001$). These bacteria have been reported to synthesize lipopolysaccharide (LPS) and can also cause inflammation by activating immune pathways. Altogether, the heat map (Fig. 3B) provides confirmation that there is a definite pattern: the number of beneficial commensals reduced whereas the number of pathogenic bacteria grew dose-dependently after the administration of tacrolimus.

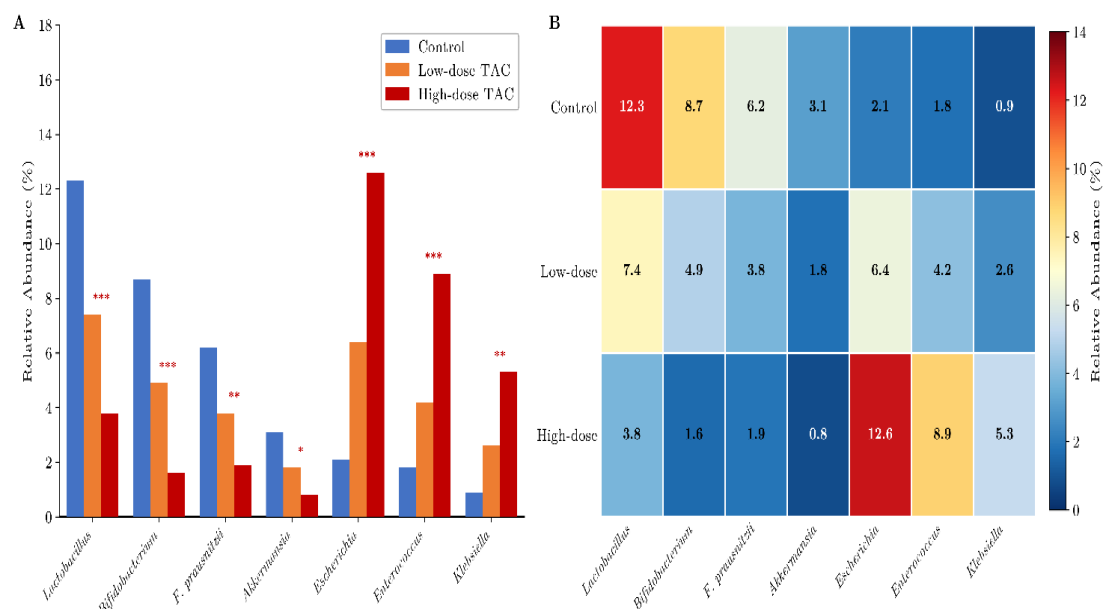


Fig. 3. Genus-level gut microbial abundance at Day 28. (A) Grouped bar chart comparing relative abundance of seven key genera across treatment groups. (B) Heat map visualization of the same data, enabling simultaneous cross-group and cross-genus comparison. Data are mean values; $n=10/\text{group}$. * $p<0.05$, ** $p<0.01$, * $p<0.001$ vs. Control; Kruskal–Wallis with Dunn's correction. *F. prausnitzii* = *Faecalibacterium prausnitzii*.**

Tacrolimus Induces Dose- and Time-Dependent Vero Cell Cytotoxicity

The decrease in Vero cell viability was clearly dose- and time-dependent when using Tacrolimus (Table 5; Fig. 4A). At 24 hours, low concentrations (0.1 and 1 $\mu\text{g/mL}$) had no significant effect, with viability remaining above 90% ($97.8 \pm 3.8\%$ and $93.4 \pm 3.5\%$), while higher doses caused a progressive decline to $72.1 \pm 4.6\%$ (10 $\mu\text{g/mL}$), $52.3 \pm 4.1\%$ (25 $\mu\text{g/mL}$), and $31.7 \pm 3.8\%$ (50 $\mu\text{g/mL}$), with an IC_{50} of 28.4 ± 2.1 $\mu\text{g/mL}$. After 48 hours, cytotoxicity increased further, with viability reduced to $61.3 \pm 3.9\%$, $39.4 \pm 3.6\%$, and $22.6 \pm 2.9\%$ at 10, 25, and 50 $\mu\text{g/mL}$, respectively, and the IC_{50} decreasing to 19.7 ± 1.8 $\mu\text{g/mL}$, indicating stronger toxicity over time. The assay

reached a threshold of over 90% cell death with doxorubicin confirming assay validity. In general, tacrolimus was only found to be directly cytotoxic at high, non-physiological concentrations, indicating that the gastrointestinal action of tacrolimus is probably mediated via indirect pathways, and direct injury to the epithelium is unlikely.

Table 5. MTT Cell Viability of Vero Cells after Tacrolimus Treatment (Mean $\pm$ SD; n=6/group)

Concentration (μ g/mL)	Viability at 24 h (%)	Viability at 48 h (%)	Significance vs. Control
0 (Vehicle control)	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	
0.1	97.8 $\pm$ 3.8	89.2 $\pm$ 3.4	ns
1	93.4 $\pm$ 3.5	82.6 $\pm$ 4.1	ns
10	72.1 $\pm$ 4.6	61.3 $\pm$ 3.9	**
25	52.3 $\pm$ 4.1	39.4 $\pm$ 3.6	***
50	31.7 $\pm$ 3.8	22.6 $\pm$ 2.9	***

** $p < 0.01$, *** $p < 0.001$ vs. vehicle control (0 μ g/mL); one-way ANOVA with Tukey's post-hoc test. ns = not significant. IC_{50} (24 h): 28.4 $\pm$ 2.1 μ g/mL; IC_{50} (48 h): 19.7 $\pm$ 1.8 μ g/mL. Significance columns show 24 h / 48 h results respectively.

Tacrolimus Triggers Dose-Dependent Pro-inflammatory Cytokine Production in Vero Cells

ELISA of conditional media of tacrolimus-treated Vero cells showed dose-dependent increase in pro-inflammatory cytokines (Fig. 4B). IL-6 (1.05 $\pm$ 0.08 and 1.12 $\pm$ 0.12) and TNF- (1.03 $\pm$ 0.07 and 1.09 $\pm$ 0.10) were not different at 0.1 and 1 μ g/mL of IL-6 and TNF- respectively compared to vehicle controls ($p > 0.05$ in both cases), which confirms The IL-6 release was found to grow 3.21 $\pm$ 0.31-fold ($p < 0.01$) and the TNF- α 2.84 $\pm$ 0.28-fold ($p < 0.01$) more than baseline at 10 μ g/mL - the initial concentration at which statistically significant cytokine activation was identified. At 50 μ g/mL, IL-6 and TNF- α reached 6.74 $\pm$ 0.54-fold and 5.43 $\pm$ 0.48-fold above untreated controls, respectively (both $p < 0.001$). Such data provide a definite concentration-response relationship of tacrolimus-induced epithelial inflammatory activity.

Cytokine analyses of murine serum at day 28 were in line with in vitro results. Group III vs. Group I animals showed a significant increase in serum IL-6 and TNF- α ($p < 0.01$), which serves to validate in vivo systemic inflammatory activation. Besides, serum TNF- α was positively and significantly correlated with fecal Proteobacteria relative abundance (Pearson $r = 0.68$; 95% CI: 0.430.83; $p < 0.001$; Fig. 5B), which gives a direct mechanistic relationship between gut dysbiosis, in this case, the Proteobacteria-mediated LPS This association prolongs the dysbiosisinflammation axis.

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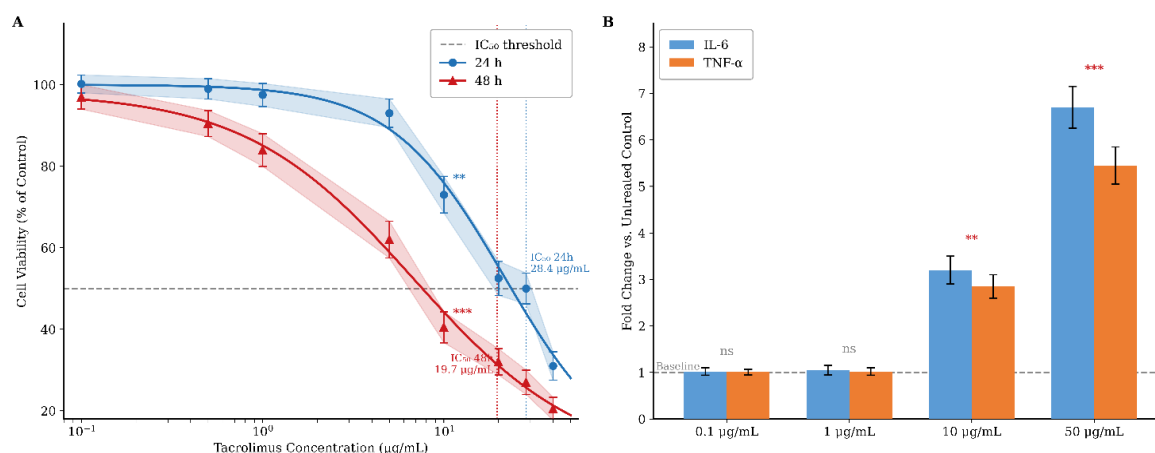
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Fig. 4. *In vitro* cytotoxicity and pro-inflammatory cytokine responses in tacrolimus-treated Vero cells. (A) MTT cell viability at 24 h (blue) and 48 h (red) with IC_{50} markers (dotted vertical lines). Shaded areas = $\pm$ SD. (B) IL-6 (blue bars) and TNF- α (orange bars) fold change vs. vehicle control measured by ELISA at 24 h. Dashed horizontal line indicates baseline (fold change = 1.0). Data are mean $\pm$ SD; $n=6$ /condition, three independent experiments. ** $p<0.01$, *** $p<0.001$ vs. vehicle control; one-way ANOVA + Tukey's post-hoc. ns = not significant.

Tacrolimus Induces G1 Phase Cell Cycle Arrest and Apoptosis in Vero Cells

Flow cytometric cell cycle analysis demonstrated that there were evident dose-dependent alterations of Vero cells at 24 hours post-tacrolimus exposure (Fig. 5A). The distribution of the cell cycle phases in the control group was normal: G1 (58.3 ± 3.2 percent), S (22.4 ± 2.8 percent), G2/M (19.3 ± 2.1 percent), and an extremely small fraction of apoptotic cells (sub-G1) (1.2 ± 0.4 percent), which is evidence of healthy cell proliferation. No significant changes were detected at 1 μ g/mL in each of the phases indicating that clinically relevant concentrations did not interfere with the normal cell cycle progression. At 10 μ g/mL, a clear shift was seen with increased G1 phase arrest ($72.1 \pm 4.3\%$; $p < 0.01$) and a reduction in S-phase cells ($12.6 \pm 2.1\%$; $p < 0.01$), suggesting inhibition of the G1-to-S transition. G2/M cells also decreased slightly ($15.3 \pm 1.8\%$; $p < 0.05$). This was further enhanced at 50 μ g/mL when G1 arrest was further accumulated ($79.4 \pm 5.1\%$ $p < 0.001$) and S-phase cells reduced to 8.3 ± 1.6 indicating a high level of cell proliferation inhibition. Importantly, the sub-G1 (apoptotic) population increased from $1.2 \pm 0.4\%$ in controls to $2.7 \pm 0.8\%$ at 10 μ g/mL ($p < 0.05$) and $8.7 \pm 1.9\%$ at 50 μ g/mL ($p < 0.001$), confirming induction of apoptosis at higher doses. In general, tacrolimus induced G1 cell cycle arrest and augmented apoptosis in supratherapeutic levels, suggesting a disturbance in epithelial cell proliferation during high drug levels.

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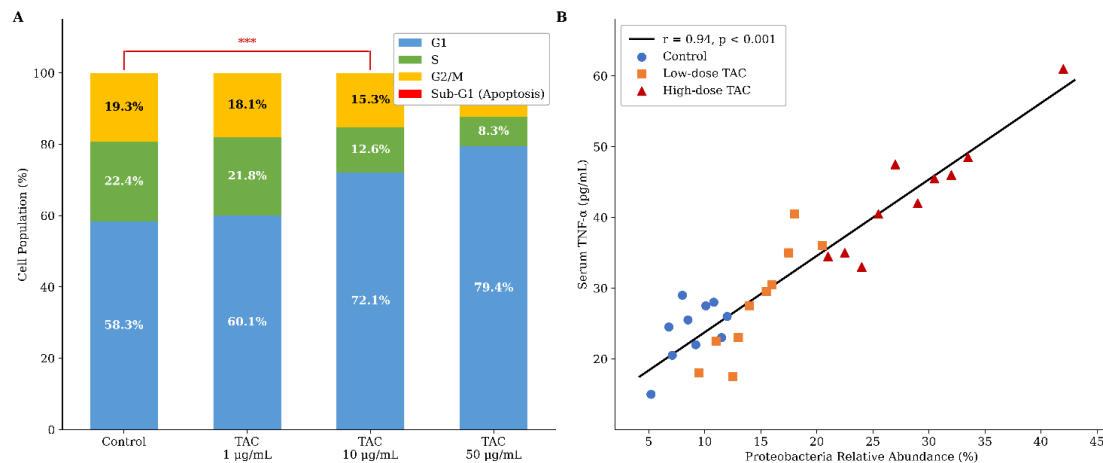
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Fig. 5. Cell cycle dynamics in Vero cells and dysbiosis-inflammation correlation in murine serum. (A) Stacked bar chart of cell cycle phase distribution (G1, S, G2/M, Sub-G1) in Vero cells treated with tacrolimus for 24 h. Percentage values are labeled for each phase. *** $p < 0.001$ for Sub-G1 comparison (Control vs. 50 µg/mL). (B) Pearson correlation between fecal Proteobacteria relative abundance and serum TNF- α concentration in BALB/c mice at Day 28 ($n = 30$ animals total). Regression line with 95% CI band shown. $r = 0.68$, $p < 0.001$.

Discussion

This study offers a holistic assessment of the gut microbial dysbiosis induced by tacrolimus in the BALB/c mouse model, encompassing alpha and beta diversity, bacterial taxonomy from phylum to genus levels, and in vitro studies of cytotoxicity, inflammation and cell cycle arrest in Vero cells. The results are discussed in the context of the pharmacomicrobiomic model, extending earlier models for glucuronidated immunosuppressants to calcineurin inhibitors [20, 21]. There was a marked dose-dependent loss of microbial diversity, as measured by the Shannon index, which fell from 5.67 in the control group to 3.14 in the high-dose group ($p < 0.001$). This reflects a decrease in both microbial abundance and uniformity, which is thought to reflect a loss of stability of the gut microbiota. A loss of microbial diversity has been linked to poorer outcomes in transplant patients, including greater risk of death [22]. The fact that diversity was reduced earlier in high-dose animals suggests that gut microbial deterioration occurs in a dose- and time-dependent manner.

The most striking compositional shift was a significant increase in Proteobacteria, from 8.2% in the control to 31.4% in high-dose animals ($p < 0.001$). This was coupled with a decline in major commensal bacteria such as Firmicutes and Bacteroidetes. This indicates a breakdown in colonization resistance, enabling the growth of opportunistic and potentially pathogenic bacteria. Proteobacteria are known to produce lipopolysaccharide (LPS), which induces inflammatory signaling (TLR4) and cytokine production [23, 24]. This is in accord with the positive association between the abundance of Proteobacteria and serum TNF- α ($r = 0.68$, $p < 0.001$), suggesting a connection between gut dysbiosis and systemic inflammation via the gut-immune axis [25, 26]. At the genus level, beneficial short-chain fatty acid (SCFA) producers, such as Lactobacillus, Bifidobacterium and Faecalibacterium prausnitzii, were significantly depleted. These bacteria are important for gut barrier function through the production

of metabolites like butyrate, which supports the function of the gut epithelium and immune responses [27]. Their loss could affect tight junction proteins (such as claudin-1, occludin, ZO-1) and mucin secretion, resulting in increased gut permeability and bacterial translocation [28, 29]. This likely plays a role in the chronic inflammatory response in tacrolimus-treated animals. Similarly, the increase in opportunistic pathogens (e.g., *Escherichia*, *Enterococcus*, *Klebsiella*) also suggests dysbiosis leading to potential opportunistic infections.

In vitro data also supports this notion. Tacrolimus showed concentration- and time-dependent cytotoxicity in Vero cells only at significantly high concentrations than therapeutic levels, suggesting it is unlikely to cause direct epithelial toxicity at therapeutic concentrations. Similar findings have been observed with other immunosuppressants, where gut toxicity is mainly attributed to the microbiota and not direct cytotoxicity [30, 31]. Elevated levels of pro-inflammatory cytokines (IL-6 and TNF- α) at higher concentrations indicate induction of inflammatory pathways, which may include NF- κ B and MAPK pathways. Further, cell cycle arrest in the G1 phase and increased apoptosis suggest decreased proliferation at higher concentrations [32]. Overall, these observations lend credence to the idea that tacrolimus-induced gastrointestinal toxicity is largely a result of disruption of the microbiota, rather than the gut epithelium itself. This finding has clinical ramifications, as it suggests that modulation of the microbiota, such as probiotics or replenishment of SCFA-producing bacteria, may help limit adverse effects without affecting immunosuppression.

But there are a number of caveats. While Vero cells are commonly used, they do not entirely mimic the physiology of the intestinal epithelium and other more representative models, such as Caco-2 cells or organoids, should be employed [33]. Furthermore, non-oral drug administration may not reflect the human pharmacokinetics of the drug. The study also did not directly assess bacterial enzyme activity and SCFA levels, which would help understand microbiome functional changes. Metabolomic and enzymatic assays should be included in future studies to better understand the underlying mechanisms.

Conclusion

This paper has shown that tacrolimus causes significant, dose-related gut microbial dysbiosis in BALB/c mice. A major decrease in alpha diversity indicators across all measures, a high degree of separation between microbial communities in beta analysis (PERMANOVA $R^2 = 0.41$, $p = 0.001$) and an overall significant increase in Proteobacteria to 31.4 percent accompanied by a simultaneous decline in useful commensal phyla, confirm this. Systemic inflammation was closely linked with these microbiota changes as indicated by the positive correlation between the abundance of Proteobacteria and serum TNF- α levels ($r = 0.68$, $p < 0.001$), which supports the presence of an inflammatory response mediated by the gut-immune axis. Biological effects of tacrolimus on epithelial cells were further supported by in vitro evidence showing that it had dose-dependent cytotoxicity in Vero cells (IC₅₀ = 28.4 μ g/mL at 24 h), increased pro-inflammatory cytokine expression (IL-6 up to 6.7-fold and TNF- α up to 5.4-fold). Collectively, these findings suggest that tacrolimus can influence both the microbial ecology and the epithelial cellular activities especially at excessive levels of therapy. Altogether, the results can be generalized to further the

pharmacomicrobiomic model of glucuronidated immunosuppressants to the calcineurin inhibitors, including tacrolimus. They emphasize the role of intestinal microbiota dysfunction as one of the mechanisms that lead to drug-related toxicity. The clinical outcomes justify the possibility of microbiota-targeted interventions, such as the use of SCFA supplements, probiotics based on *Lactobacillus* and *Bifidobacterium*, and regulation of bacterial β -glucuronidase activity, to address the negative consequences. Future research needs to utilize more sophisticated intestinal systems like the Caco-2 systems, combine targeted SCFA metabolomics, determine the activity of β -glucuronidase in more detail and use oral in vivo administration systems to further define the mechanistic role of tacrolimus in gutmicrobiomehost interactions as part of a pharmacomicrobiomic system.

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