**Original Research**

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# Cyclosporin, Tacrolimus, Mycophenolic Acid, and Dexamethasone Suppress Peripheral Blood Mononuclear Cell Proliferation and Inhibit Interferon-Gamma, IL-10 and IL-13 Production *In Vitro*

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## Abstract

**Objective:** The primary goals of immunosuppressive therapy are to prevent graft rejection and to improve graft and patient survival. Long-term maintenance therapy after kidney transplantation includes cyclosporine A (CsA), tacrolimus (FK506), mycophenolic acid (MPA), and dexamethasone (Dex). This study aimed to determine the effects of these immunosuppressants on the proliferation of human peripheral blood mononuclear cells (PBMC) and the production of interferon-gamma (IFN-γ), interleukin (IL)-10, and IL-13 *in vitro*.

**Materials and Methods:** Human PBMC were isolated and stimulated with an immobilized anti-CD3 (OKT3) monoclonal antibody in the absence (control) or presence of immunosuppressants. Additionally, unstimulated cells were cultured without anti-CD3 or immunosuppressants. On day 3 of cell culture, PBMC proliferation was assessed by 3H-thymidine incorporation, and cytokine production of IFN-γ, IL-10, and IL-13 was measured by ELISA.

**Results:** CsA, FK506, MPA, and Dex inhibited PBMC proliferation and the production of IFN-γ, IL-10, and IL-13. CsA (100-1000 ng/mL) and FK506 (1-10 ng/mL) inhibited PBMC proliferation significantly ($p<0.05$). All concentrations of MPA (0.01-10 µg/mL) and Dex ($10^{-4}$-$10^{-8}$ M) inhibited PBMC proliferation ($p<0.05$). High doses of CsA (100 ng/mL), MPA (1000 ng/mL), FK506 (10 ng/mL), and Dex ($10^{-7}$ M) significantly inhibited IFN-γ, IL-10, and IL-13 significantly inhibited ($p<0.05$), while low-dose MPA (10 ng/mL) significantly increased IL-10 and IL-13 levels ($p<0.05$).

**Conclusion:** The maintenance immunosuppressive regimen at therapeutically relevant concentrations including CsA (100 ng/mL), FK506 (10 ng/mL), MPA (1 µg/mL), and Dex ($10^{-6}$-$10^{-7}$ M) suppressed PBMC proliferation and inhibited IFN-γ, IL-10, and IL-13 production *in vitro*.

**Keywords:** Cyclosporin, tacrolimus, mycophenolic acid, dexamethasone, PBMC proliferation, IFN-γ, IL-10, IL-13

## Introduction

Acute allograft rejection is initiated by an immune response against the allograft and occurs days to months after transplantation. It starts with a nonspecific innate response, followed by a donor-specific adaptive immune response. T-cells recognize alloantigens, migrate into the graft, and induce the necrosis of transplant tissue (1). The primary goals of immunosuppressive therapy are to prevent acute allograft rejection and improve graft and patient survival. Immunosuppressive treatment after kidney transplantation can be classified as intense induction therapy (lasting up to 2 weeks following the transplant) or long-term maintenance therapy, which starts immediately following the transplant and continues for life. Maintenance immunosuppressive treatments include calcineurin inhibitors (CNIs), corticosteroids, and either azathioprine or mycophenolate mofetil (MMF) (2).

Kidney transplant immunosuppression relies greatly on CNIs, including cyclosporine A (CsA) and tacrolimus (FK506) (3). CsA and FK506 inhibit calcineurin, thereby

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blocking the transcription of nuclear factor of activated T-cells (NFAT) and preventing T-lymphocyte activation by inhibiting calcium-associated signaling pathways that regulate T-cell activation and the gene expression of cytokines, such as interferon-gamma (IFN-γ), interleukin (IL)-2, IL-4, IL-5, and IL-13 (4-7).

Mycophenolic acid (MPA) functions as an antiproliferative agent and is present in the form of MMF, which is a prodrug, and mycophenolate sodium (MPS). It acts as a non-competitive, selective, and reversible inhibitor of inosine monophosphate dehydrogenase, which is vital for the production of guanosine nucleotides in T- and B-lymphocytes (8). The impact of MPA on T-cell proliferation is particularly relevant in the context of homeostatic proliferation, where it has been shown to inhibit lymphocyte proliferation and plays an important role in the prevention of solid organ transplant rejection (9-11).

Corticosteroids, which are steroid hormones produced by the adrenal cortex, can be categorized into glucocorticoids and mineralocorticoids. Dexamethasone (Dex) is a strong synthetic member of the glucocorticoid category with extensive anti-inflammatory and immunosuppressive effects (12). It induces immunosuppression by inhibiting T-cell proliferation by reducing CD28 co-stimulatory signals and inhibiting Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling by IL-2 and related cytokines (13,14).

Maintenance immunosuppressive regimen including corticosteroids, MPA, CsA, and FK506 has been demonstrated to be effective in preventing allogeneic transplant rejection (10,11,15,16). Triple therapy with CNIs (tacrolimus or CsA), plus an antiproliferative agent (MPA), azathioprine (AZA), sirolimus, and corticosteroids, is the cornerstone of post-transplant immunosuppression (17). According to a recent study by Arnol et al. (18), the most popular immunosuppressive regimen used in kidney transplant recipients (KTRs) in South-Eastern Europe was triple therapy, which consists of CNIs (CNI; tacrolimus or cyclosporine), antiproliferative medications (MPS or MMF), and corticosteroids.

Understanding the mechanisms by which immunosuppressive agents and cytokines influence T-cell proliferation and cytokines production is crucial for developing targeted therapeutic interventions in conditions where immune response modulation is required. However, the literature regarding concurrent assessment of these immunosuppressive drugs in human peripheral blood mononuclear cells (PBMC) proliferation and Th1/Th2 cytokines production is scarce. Therefore, this study aimed to evaluate the effects of CsA, FK506, MPA, and Dex on the proliferation of the primary human PBMC and the production of IFN-γ, IL-10, and IL-13 *in vitro* simultaneously.

## Materials and Methods

This study was approved by Umm Al-Qura University Biomedical Research Ethics Committee (approval no: HAPO-02-K-012-2024-05-2130, date: 03.05.2024). The volunteers were informed, and consent forms were obtained.

### PBMC Isolation

Heperinized human peripheral blood was collected from adult healthy individuals following the local guidelines. PBMC were isolated using a Histopaque-1077 density gradient (Sigma Aldrich, UK). The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 20 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid buffer, 1% L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Sigma Aldrich, UK) and incubated in a humidified atmosphere containing 5% $CO_2$ at a temperature of 37°C.

### Thymidine Incorporation Assay and ELISA

The proliferative capacity of PBMC is most frequently assessed via tritiated thymidine incorporation (19). To examine the effect of immunosuppressive drugs on the proliferation of PBMC, the cells were seeded at a density of $1 \times 10^5$ cells/well in triplicate in 96-well plates pre-coated with mouse anti-human CD3 monoclonal antibody (OKT3) at 1 µg/mL (Thermo Fisher Scientific, USA). All immunosuppressive drugs were purchased from Sigma-Aldrich, UK. FK506 and CsA were dissolved in DMSO whereas MPA and Dex were dissolved in ethanol.

The immunosuppressive drugs were added to the cell culture at different concentrations. CsA (1000, 100, and 10 ng/mL), FK506 (10, 1, and 0.1 ng/mL), MPA (10, 1, 0.1 and 0.01 µg/mL) and Dex ($10^{-4}$-$10^{-9}$ M) were used in proliferation and cytokines assays. The dosing of the MPA, FK506 and CsA was based on trough levels and literature (20-24). The trough level is 1-3.5 mg/L for MPA (25), 8-10 ng/mL for FK506 in the first 3 months and 5-8 ng/mL beyond 3 months and 250-350 ng/mL for CsA up to 3 months after transplant (20,21,26). The dosing of Dex was based on pharmacological concentrations of corticosteroids (22). The control cells were stimulated with the anti-CD3 antibody but not treated with drugs. Additionally, unstimulated cells were cultured without OKT3 or drugs. On day 3 of the culture, the cells were pulsed with 3H-thymidine (0.5 acid/well) for 6 hours.

Day 3 was chosen because the activated T-cells require up to 3 days to divide and exhibit the highest levels of cytokine production at 3 days post-activation (27-29). 3H-thymidine, which is incorporated into newly

dividing DNA during mitosis, was measured using a liquid scintillation beta-counter (1450 Microbiota, LKB Wallac, Turku, Finland). Thymidine incorporation was expressed as count per minute (CPM) of triplicate cultures, reflecting the extent of cell proliferation in response to the immunosuppressive drugs. Additionally, triplicate samples of the cell culture supernatants were collected on day 3 to measure IFN-γ, IL-10, and IL-13 levels using ELISA kits (Bioscience, UK) according to the manufacturer's instructions.

### Statistical Analysis

GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analyses and graphs. The samples used in the proliferation and cytokine assays were set up in triplicate, and the results were expressed as mean ± standard error of the mean and analyzed using the unpaired t-test. Differences were considered significant if the p-value was <0.05.

## Results

### Effect of Immunosuppressants on PBMC Proliferation

The count and percentage of inhibition of PBMC proliferation after treatment with immunosuppressive drugs compared to the control are summarized in Table 1. As shown in Table 1 and Figure 1, CsA, FK506, MPA, and Dex inhibited PBMC proliferation in a dose-dependent manner. CsA at a concentration of 100-1000 ng/mL inhibited PBMC proliferation significantly ($p<0.05$) while CsA at 10 ng/mL was similar to the control ($p=0.9$). FK506 at a concentration of 1-10 ng/mL reduced PBMC proliferation significantly ($p<0.05$) while FK506 0.1 µg/mL did not ($p=0.7278$). All concentrations of MPA (0.01-10 ug/mL) and Dex ($10^{-4}$-$10^{-8}$ M) inhibited PBMC proliferation significantly inhibited ($p<0.05$). The therapeutic dose of MPA ranges from 1 to 3.5 µg/mL (25). MPA at concentrations 10, 1, and 0.1 µg/mL had a dramatic inhibitory effect on PBMC proliferation ($p<0.05$). Dex inhibited PBMC proliferation at concentrations that corresponded to physiologic ($10^{-8}$) and therapeutic ($10^{-6}$-$10^{-7}$ M) corticosteroid concentrations ($p<0.05$) (22,24). The therapeutic dose of MPA ranges from 1 to 3.5 µg/mL (25). MPA at concentrations 10, 1, and 0.1 µg/mL had a dramatic inhibitory effect on PBMC proliferation.

### Effect of Immunosuppressants on IFN-γ, IL-10, and IL-13 Levels

The effects of immunosuppressive drugs on IFN-γ production by PBMC are shown in Figure 2. CsA, FK506, and MPA inhibited IFN-γ in a dose-dependent manner. CsA (100 ng/mL), FK506, MPA and Dex ($5x10^{-7}$ M) inhibited IFN-γ significantly ($p<0.05$). However, Dex at a concentration of $10^{-9}$ M physiologic dose increased IFN-γ slightly ($p<0.05$). As can be seen in Figure 3, CsA,

**Table 1.** The percentage of inhibition of PBMC proliferation after treatment with cyclosporine A (CsA), and tacrolimus (FK506) dexamethasone (Dex) and mycophenolic acid (MPA) compared to the controls

| Drug | CPM ± SD | of inhibition % | p-value |
|---|---|---|---|
| Control for CsA | 110724 ± 1054 | | |
| CsA 1000 ng/mL | 36946 ± 5377 | 66.6 | <0.0001 |
| CsA 100 ng/mL | 71884 ± 11403 | 35 | 0.0042 |
| CsA 10 ng/mL | 109752 ± 3318 | 0.9 | 0.6541 |
| Control for FK506 | 117053 ± 19399 | | |
| FK506 10 ng/mL | 35103 ± 2319 | 70 | 0.0019 |
| FK506 1 ng/mL | 75064 ± 4708 | 35.9 | 0.0219 |
| FK506 0.1 ng/mL | 112862 ± 1301 | 3.6 | 0.7278 |
| Control for MPA | 119618 ± 20216 | | |
| MPA 10 µg/mL | 95.67 ± 15 | 99.9 | 0.0005 |
| MPA 1 µg/mL | 201.7 ± 83 | 99.8 | 0.0005 |
| MPA 0.1 µg/mL | 8987 ± 1938 | 92.5 | 0.0007 |
| MPA 0.01 µg/mL | 82891 ± 6825 | 30.7 | 0.0407 |
| Control for Dex | 114128 ± 614 | | |
| Dex $10^{-4}$ M | 57683 ± 2007 | 49.5 | <0.0001 |
| Dex $10^{-5}$ M | 77193 ± 3482 | 32.4 | <0.0001 |
| Dex $10^{-6}$ M | 78749 ± 412 | 31 | <0.0001 |
| Dex $10^{-7}$ M | 87825 ± 729 | 23 | <0.0001 |
| Dex $10^{-8}$ M | 82531 ± 4304 | 27.7 | 0.0002 |

PBMC: Peripheral blood mononuclear cells, CPM: Conditioned pain modulation, SD: Standard deviation

FK506, and high-dose MPA (1000 ng/mL) and Dex ($5x10^{-7}$ M) inhibited IL-10 significantly ($p<0.05$), while low-dose MPA (10 ng/mL) increased IL-10 production slightly ($p<0.05$). As shown in Figure 4, IL-13 was inhibited by high doses of CsA (100 ng/mL), FK506 (10 ng/mL) and MPA (1000 ng/mL) and by Dex significantly ($p<0.05$). However, low doses of FK506 (0.1 ng/mL) and MPA (0.01 ug/mL) significantly increased IL-13 levels ($p<0.05$). The summary of the p-values of IFN-γ, IL-10, and IL-13 levels by PBMC after treatment with immunosuppressive drugs versus control are shown in Table 2.

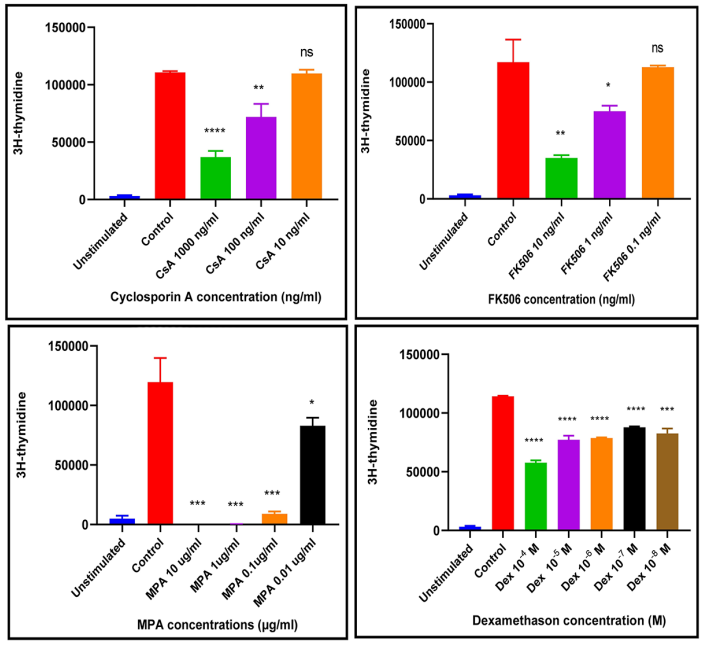


**Figure 1.** The effect of immunosuppressants on PBMC proliferation. PBMC were stimulated with immobilized anti-CD3 antibody (OKT3) in the absence (control) or presence of cyclosporine A (CsA) or tacrolimus (FK506) or dexamethasone (Dex) or mycophenolic acid (MPA). The unstimulated cells were cultured without CD3 or immunosuppressants. PBMC proliferation was determined on day 3 of cell culture by 3H-thymidine incorporation and compared to the control. Results are represented as means ± SEM of triplicate samples.
*PBMC: Peripheral blood mononuclear cells, SEM: Standard error of mean, unpaired t-test was used to determine the significance (*: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001 and ns is p>0.05)*

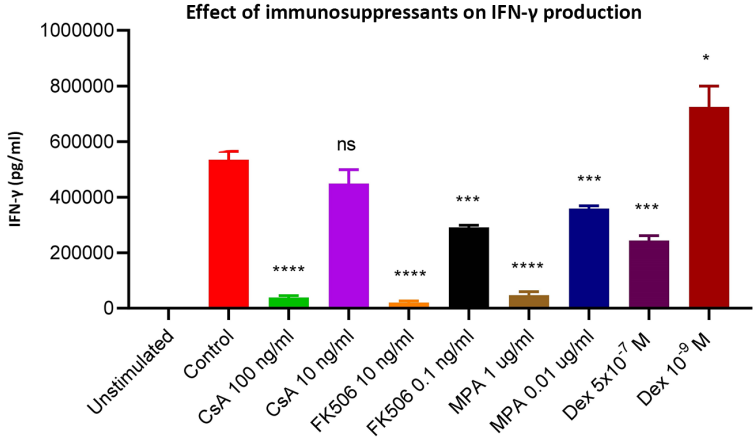


**Figure 2.** IFN-γ production by human PBMC after stimulation with immobilized anti-CD3 antibody (OKT3) in the absence (control) or presence of cyclosporine A (CsA) or tacrolimus or mycophenolic acid (MPA) or dexamethasone (Dex). The unstimulated cells were cultured without anti-CD3 or immunosuppressants. The effect of immunosuppressants on cytokines levels was measured on day 3 of cell culture by ELISA and compared to the control. Results are represented as means ± SEM of triplicate samples.
*IFN-γ: Interferon-gamma, PBMC: Peripheral blood mononuclear cells, ELISA: Enzyme-linked immunosorbent assay, SEM: Standard error of mean, FK506: Tacrolimus, unpaired t-test was used to determine the significance (*: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001 and ns is p>0.05)*

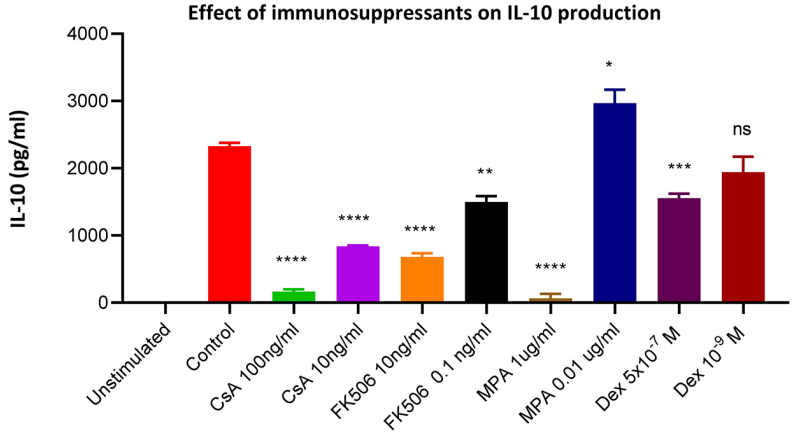


**Figure 3.** IL-10 production by human PBMC after stimulation with immobilized anti-CD3 antibody (OKT3) in the absence (control) or presence of cyclosporine A (CsA) or tacrolimus or mycophenolic acid (MPA) or dexamethasone (Dex). The unstimulated cells were cultured without anti-CD3 or immunosuppressants. The effect of immunosuppressants on cytokines levels was measured on day 3 of cell culture by ELISA and compared to the control. Results are represented as means ± SEM of triplicate samples.
*IL: Interleukin, PBMC: Peripheral blood mononuclear cells, ELISA: Enzyme-linked immunosorbent assay, SEM: Standard error of mean, FK506: Tacrolimus, unpaired t-test was used to determine the significance (*: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001 and ns is p>0.05).*

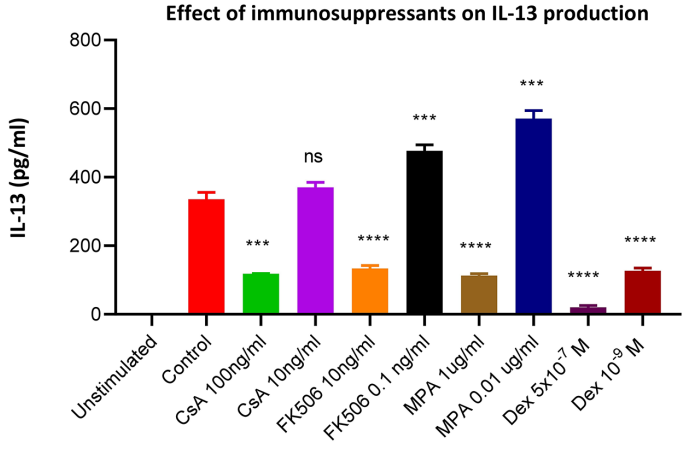


**Figure 4.** IL-13 production by human PBMC after stimulation with immobilized anti-CD3 antibody (OKT3) in the absence (control) or presence of cyclosporine A (CsA) or tacrolimus or mycophenolic acid (MPA) or dexamethasone (Dex). The unstimulated cells were cultured without anti-CD3 or immunosuppressants. The effect of immunosuppressants on cytokines levels was measured on day 3 of cell culture by ELISA and compared to the control. Results are represented as means ± SEM of triplicate samples.
*IL: Interleukin, PBMC: Peripheral blood mononuclear cells, ELISA: Enzyme-linked immunosorbent assay, SEM: Standard error of mean, FK506: Tacrolimus, unpaired t-test was used to determine the significance (*: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001 and ns is p>0.05).*

**Table 2.** The summary of the p-values of IFN-γ, IL-10, and IL-13 cytokines levels by PBMC after treatment with immunosuppressive drugs versus control

| Drug | p-value | | |
|---|---|---|---|
| | IFN-γ | IL-10 | IL-13 |
| CsA 100 ng/mL | <0.0001 | <0.0001 | 0.0007 |
| CsA 10 ng/mL | 0.0628 | <0.0001 | 0.0703 |
| FK506 10 ng/mL | <0.0001 | <0.0001 | <0.0001 |
| FK506 0.1 ng/mL | 0.0002 | 0.0013 | 0.0008 |
| MPA 1 µg/mL | <0.0001 | 0.0007 | <0.0001 |
| MPA 0.01 µg/mL | 0.0006 | 0.0243 | 0.0002 |
| Dex $5x10^{-7}$ M | 0.0001 | 0.0009 | <0.0001 |
| Dex $10^{-9}$ M | 0.0154 | 0.1441 | <0.0001 |

IFN-γ: Interferon-gamma, IL: Interleukin, PBMC: Peripheral blood mononuclear cells, CsA: Cyclosporine A, FK506: Tacrolimus, MPA: Mycophenolic acid, Dex: Dexmedetomidine

## Discussion

Choosing the appropriate doses of immunosuppressive drugs is crucial in the field of organ transplantation, but their dosing needs to be carefully tailored to achieve the delicate balance between adequate immunosuppression and avoiding adverse effects (30). These drugs often have a narrow therapeutic index, necessitating individualized dosing based on factors such as drug concentrations in the blood or plasma (26). It has been demonstrated that immunosuppressive medications such as corticosteroids, MPA, FK506, and CsA can successfully prevent allogeneic transplant rejection. (10,11,15,16).

During acute renal allograft rejection, the PBMC of KTRs exhibited elevated transcription of the IL-2, IL-4, and IL-15 genes and lowered the expression of the *IL-10* gene (31). In the present study, different doses of CsA and FK506, MPA and Dex were cultured with PBMC to assess PBMC proliferation and cytokines production. The results of this study demonstrated that therapeutic doses of CsA, FK506, MPA and Dex inhibited PBMC proliferation and suppressed the production of IFN-γ, IL-10, and IL-13 cytokines. These results are in harmony with the previous studies. CsA and FK506 have been shown to suppress T-cell activation and proliferation (32) and inhibit IL-2, IL-4, and IFN-γ mRNA expression in PHA-stimulated canine PBMC (23). Additionally, CsA and FK506 have been demonstrated to induce apoptosis and modulate the activation and effector function in T-cells (33).

MPA is the active ingredient of the immunosuppressant MMF, which is effective in preventing acute and chronic allograft rejection by inhibiting PBMC proliferation and suppressing the production of various cytokines such as IFN-γ (8). This study has shown that MPA inhibited PBMC proliferation and the production of IFN-γ, IL-10, and IL-13. This is similar to previous reports that found MPA to suppress the production of T-cell IL-17, IFN-γ, and tumour necrosis factor alpha (TNF-α) and to upregulate the expression of negative co-stimulators programmed death 1, cytotoxic T-lymphocyte antigen 4 (34,35). Additionally, MPA has been found to suppress dendritic cell maturation, inhibit human T- and B-lymphocytes proliferation, and induce T-lymphocyte apoptosis (36). Furthermore, it has been shown that long-term MMF therapy, which inhibits the proliferation of T- and B-cells, was linked with a lower risk of primary and recurrent late acute renal rejection episodes (37).

In the present study, Dex reduced PBMC proliferation inhibited IFN-γ, IL-10 and IL-13. These findings are in agreement with previous studies, which reported that Dex inhibited T-cell activation in human PBMC and reduced T-cell proliferation and cytokine production such as IFN-γ, IL-10, IL-4, IL-6, and TNF (38,39). Interestingly, the lowest concentration of Dex that was used ($10^{-9}$ M) caused small but significant increase in IFN-γ. This result is in partial agreement with a study by Haczku et al. (22) who found that $10^{-9}$ M Dex enhanced T-cell proliferation. IL-2 is a crucial cytokine for T-cell proliferation and survival and cytokine production of effector T-cells. The inhibition of IL-2-mediated Stat5 activation correlates with the inhibition of T-cell proliferation (40). Dex can inhibit the IL-2-induced JAK-STAT signaling pathway and suppress the expression of the IL-2 receptor (IL-2R) and consequently block signaling by other cytokines that use IL-2R such as IL-4, IL-7, and IL-15 (14).

It was recently shown that the triple combination of tacrolimus with MPA and prednisolone dramatically decreased the frequencies of IFN-$\gamma^+$, TNF-$\alpha^+$, and IL-$2^+$cells within $CD4^+$ T-cells. Furthermore, tacrolimus and prednisolone dramatically decreased the frequencies of IL-$2^+$ cells inside $CD4^+$ T-cells as well as the IFN-$\gamma^+$ cells within $CD4^+$ T-cells. Furthermore, the triple combination of tacrolimus, sirolimus, and prednisolone in the presence of MPA significantly decreased T-cell proliferation (41).

### Study Limitations

The current *in vitro* study has some limitations that must be acknowledged. One notable constraint is the discrepancy between *in vitro* conditions and *in vivo* environment. Additionally, applying the findings of *in vitro* studies to humans is complex, as tissue-specific responses may not fully represent the complexities of human biological systems.

## Conclusion

The maintenance immunosuppressive regimen at therapeutically relevant concentrations including CsA (100 ng/mL), FK506 (10 ng/mL), MPA (1 µg/mL) and Dex ($10^{-6}$ -$10^{-7}$ M) suppressed PBMC proliferation and inhibited IFN-γ, IL-10, and IL-13 production *in vitro*. These findings highlight the importance of understanding the specific mechanisms through which immunosuppressants influence PBMC functions to tailor therapeutic strategies effectively.

### Ethics

**Ethics Committee Approval:** This study was approved by Umm Al-Qura University Biomedical Research Ethics Committee (approval no: HAPO-02-K-012-2024-05-2130, date: 03.05.2024).

**Informed Consent:** The volunteers were informed, and consent forms were obtained.

### Footnotes

**Financial Disclosure:** The authors declare that they have no relevant financial.

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