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Impact of immunosuppression on the immune response to SARS-CoV-2 infection: A mechanistic study

Victoria G. Hall | **Victor Ferreira** | **Deepali Kumar** | **Atul Humar**

Transplant Infectious Diseases and Multi-Organ Transplant Program, University Health Network, Toronto, Ontario, Canada

Correspondence
Atul Humar, Transplant Infectious Diseases and Multi-Organ Transplant Program, University Health Network, PMB 11–175, 585 University Avenue, Toronto, Ontario M5G 2N2, Canada.
Email: Atul.humar@uhn.ca

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the Ajmera Transplant Centre

Abstract

The optimal management of immunosuppression in transplant patients infected with COVID-19 is unknown. We performed an in vitro study to determine the effect of individual immunosuppressive agents on SARS-CoV-2-specific T-cell cytokine expression. Convalescent peripheral blood mononuclear cells from eleven non-immunosuppressed patients with COVID-19 were preincubated with clinically relevant concentrations of immunosuppressive drugs (tacrolimus, mycophenolate, sirolimus, prednisone) and then stimulated with a SARS-CoV-2 peptide pool. Supernatants were analyzed by 14-plex high sensitivity T-cell cytokine array. With increasing concentrations of tacrolimus, there was a trend to reduction in the release of IL-2 ($p = .0137$), and IFN-γ ($p = .0147$) in response to peptide stimulation. There was also a subsequent trend toward a Th2 phenotype, indicated by lower IFN-γ:IL-13 ratio ($p = .0663$) and IFNγ:IL-4 ratio ($p = .0176$). Sirolimus appeared to be associated with a proinflammatory cytokine release, including TNF-α ($p = .0027$) and IL-1β ($p = .0016$), in response to SARS-CoV-2 peptides. In contrast, mycophenolate and prednisone did not influence the SARS-CoV-2-specific cytokine response. These are preliminary findings only, with larger studies required to inform clinical recommendations.

KEYWORDS
COVID-19, cytokines, immunosuppression, SARS-CoV-2, transplant

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), has had significant impact on solid organ transplantation; with mortality rates in transplant recipients ranging from 10% to 20%.[1,2] SARS-CoV-2 is a spherical, single-stranded RNA beta-coronavirus, composed of four structural proteins including the spike glycoprotein (S), membrane (M), envelope (E), and nucleocapsid protein (N) that binds viral RNA.[2–4]

The transition to adaptive immune response is crucial to the clinical progression of SARS-CoV-2 infection.[1] A protective response is T-cell dependent; with $CD4^+$ T lymphocytes helping B cells to produce specific neutralizing antibodies, and cytotoxic $CD8^+$ cells capable of eliminating infected cells with minimal tissue damage.[1] Overall, current data show that both $CD4^+$ T-cell and $CD8^+$ T-cell responses occur in most patients infected by SARS-CoV-2 within 1–2 weeks after symptom onset and are skewed toward the production of mainly T-helper 1 (Th1) cytokines; namely interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin-2 (IL-2), and a subsequent Th1 immune response.[2] This is in contrast to a dysfunctional immune response, characterized by uncoordinated or partially neutralizing antibodies, uncontrolled viral replication, and elimination of infected cells, which may result in an exacerbated inflammatory response and eventual cytokine storm, acute respiratory distress syndrome and multiorgan dysfunction.[1,2] In contrast to other respiratory viruses, there is significant induction of proinflammatory cytokines. Severe COVID-19 has been associated with high IL-2R, interleukin-6 (IL-6), interleukin-10 (IL-10), and TNF-α serum levels with conflicting data on interleukin-1 β (IL-1β), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-17 (IL-17), IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[5–8]

In transplant patients with acute COVID-19 infection, a major unanswered clinical question is how to adjust immunosuppression. Decisions around immunosuppression are often formulated based on patient symptoms, the risk of rejection and estimation of how to improve patient outcomes. Reduction in calcineurin inhibitors or antimetabolite doses is performed in order to enhance immune responses, but there are minimal data to guide decisions.[3,9,10] We performed an in vitro study to determine the effect of clinically relevant, differing concentrations of individual immunosuppressive agents (tacrolimus, mycophenolate, sirolimus, and prednisone) on SARS-CoV-2-specific T-cell cytokine expression.

2 | MATERIALS AND METHODS

2.1 | Patient cohort

A total of 1890 cytokine measurements were performed after stimulation of peripheral blood mononuclear cells (PBMCs) from 11 nonimmunosuppressed patients with a prior diagnosis of COVID-19. Inclusion criteria were adults ≥18 years of age, with a diagnosis of COVID-19 via polymerase chain reaction on appropriate clinical sample, who were nonimmunosuppressed and not in receipt of dexamethasone at the time of PBMCs collection.

Median age of the cohort was 56 years (range 34–94 years old) and predominantly male (10 out of 11 participants) (Supplementary data, Table S1). At the peak of disease acuity, 4 out of 11 participants (36.3%) required oxygenation and 3 out of 11 participants (27.2%) were admitted to ICU. Only two participants were treated with dexamethasone at least 4 days prior to PBMC collection. Median time from diagnosis to sample collection was 24 days (range 14–49 days). PBMCs were isolated using standard methods described previously and stored in liquid nitrogen until needed.[11,12] Ethics approval was granted by the University Health Network Research Ethics Board.

2.2 | Immunosuppressive drugs

PBMCs were incubated with various concentrations of four immunosuppressive drugs commonly used in transplant recipients: prednisone, mycophenolate, tacrolimus, and sirolimus. Briefly, all immunosuppressive drugs were dissolved in dimethylsulfoxide (Fisher Scientific, Toronto, Ontario, Canada) except prednisone 1 mg/ml, which was obtained in liquid form (PediaPred™, Sanofi Aventis Canada). Tacrolimus was dissolved to a stock concentration of 5 mg/ml (Astellas Pharma Canada, Markham, Ontario), mycophenolate mofetil (MPA) to 250 mg/ml (Hoffman-La Roche, Mississauga, Ontario), and sirolimus to 0.5 mg/ml (Pfizer Canada, Kirkland, Quebec). The final concentration of DMSO in all compounds was less than 1%. We prepared a serial twofold or fourfold dilution series in complete growth medium (RPMI supplemented with 10% FBS, 1% glutamate, 1% penicillin-streptomycin, 1% HEPES, and 1% nonessential amino acids—all from Sigma, St. Louis, MO) to achieve clinically relevant, differing concentrations of the immunosuppressive agents. Immunosuppressive drug concentrations were selected based on previously published data[13] and the recommended therapeutic range of the drugs.[11] Tacrolimus concentrations ranged from 1.5 to 24 ng/ml, MPA from 0.25 to 4 μg/ml, sirolimus from 1 to 16 ng/ml, and prednisone from 1 to 16 ng/ml. These concentrations are well in the range of either trough or peak concentrations seen in clinical practice and have been used in previous studies evaluating the in vitro immune response to cytomegalovirus.[11,13,14]

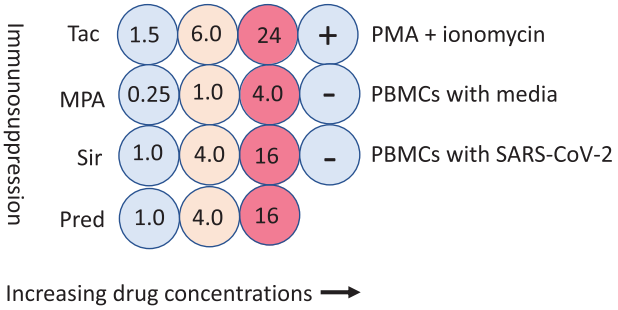

FIGURE 1 Experimental layout. PBMCs with increasing concentrations of immunosuppressive drugs were preincubated at 37°C, 5% CO_2 for 4 h. Tacrolimus dose ranged from 1.5 to 24 ng/ml, mycophenolate from 0.25 to 4 μg/ml, sirolimus from 1 to 16 ng/ml, and prednisone from 1 to 16 ng/ml. Each analyte had its own positive (PMA and ionomycin) and negative (PBMCs with media alone and PBMCs with SARS-CoV-2 stimulation) controls. Abbreviations: PBMCs = peripheral blood mononuclear cells, Tac = tacrolimus, MPA = mycophenolate, Sir = sirolimus, Pred = prednisone

2.3 | Stimulation of COVID-19-specific cytokines

PBMCs (0.5×10^6) were preincubated at 37°C for 4 h in the presence of increasing concentrations of immunosuppressant agents for a total volume of 500 μl with growth medium. The preincubation step is similar to that used in previously published studies.[13,15] After 4 h, PBMCs were washed with phosphate-buffered saline and centrifuged at 350 *g* for 5 min at room temperature. PBMCs were then transferred into a 24-well plate with a total volume of 200 μl/well and stimulated. PMA and ionomycin (final concentrations of 1 and 0.5 μg/ml; eBioscience, ThermoFisher) were used as a positive control. For each participant's experiment, media alone with PBMCs served as a negative control. Another control, with PBMCs and SARS-CoV-2 stimulation, was included to determine the amount of cytokine production with no immunosuppression. These two negative controls were used as the negative controls for all immunosuppressant medication concentrations. A cocktail made of SARS-CoV-2 spike and nucleoprotein peptides (SARS-CoV-2 PepTivator® Peptide Pools, Miltenyi Biotec) was used to stimulate the PBMCs, and incubation continued for a total of 24 h. Peptides were 15mers with 11 amino acid overlaps. Final concentration of all peptides was 2.5 μg/ml. After 24 h, the content of each well (500 μl) was transferred to a corresponding 1.5 ml tube. The tubes were then centrifuged at 12 000 RCF for 3 min. Supernatant was collected and frozen at −20°C for batch analysis. Figure 1 shows the experimental layout.

2.4 | Analysis of cytokine production

Simultaneous analysis of 14 cytokines was performed using a Human High Sensitivity T-Cell Discovery Array 14-plex by Eve Technologies (Calgary, Alberta, Canada). Cytokines measured included granulocyte-macrophage colony stimulating factor (GM-CSF), IFN-γ, IL-1β, IL-2, interleukin-4 (IL-4), interleukin-5 (IL-5), IL-6, IL-8, IL-10, interleukin-12 (IL-12p70), interleukin-13 (IL-13), IL-17a, interleukin-23 (IL-23), and TNFα. Cytokine production was expressed in pg/ml. The quantification limit (QL) was 0.15 pg/ml for IFN-γ, 0.10 pg/ml for IL-2, 0.06 pg/ml for IL-6, 0.11 pg/ml for TNF-α, 0.12 pg/ml for IL-1β and 0.33 pg/ml for IL-10. For the purposes of statistical analysis, values below the QL were given a value of half the QL.

2.5 | Statistical analysis

For each individual and each immunosuppressant, a separate plot of the specific cytokine production against the dosage of immunosuppression was performed. An analysis of the T helper 1 (Th1) to T helper 2 (Th2) cytokines was performed by plotting the IFN-γ:IL-13 and IFN-γ:IL-4 ratios for each concentration of immunosuppression and compared with the baseline response (PBMCs with SARS-CoV-2 peptide stimulation alone). A similar analysis was also conducted to examine a proinflammatory versus anti-inflammatory response by plotting the IL-6:IL-10, TNF-α:IL-10, and IL-1β:IL-10 ratio for differing concentrations of immunosuppression and compared with the baseline response (PBMCs with SARS-CoV-2 peptide stimulation alone). Statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad, San Diego, CA) and SPSS version 19.0 (Chicago, IL). The measured cytokine concentrations were expressed as median values, in pg/ml, with interquartile ranges because data were nonnormally distributed. The Friedman test was utilized to calculate the differences in cytokine expression between the three differing concentrations of each immunosuppressant medication (i.e., >2 cytokine values) and the baseline response (PBMCs with SARS-CoV-2). Statistical significance was defined at $p = .05$ with Holm–Bonferroni used to control for multiple comparisons and to reject or accept the null hypothesis. For representative Th1 and Th2 cytokines, using Holm–Bonferroni, there were 11 comparisons. On ranking the comparisons, it was found that $p = .0125$ was the first p value to be exceeded by the test p value; therefore, if the p value was > .0125, the null hypothesis was accepted, if the p value was < .0125, then the null hypothesis was rejected.

3 | RESULTS

3.1 | Effect of immunosuppression on cytokine profiles

PBMCs stimulation with SARS-CoV-2 peptides resulted in broad cytokine responses in the context of differing concentrations of the standard immunosuppressive agents. All positive controls were significantly and appropriately induced for each cytokine response. Figure 2A and B shows IFN-γ and IL-2 release, respectively, for the 11 participants plotted against differing concentrations of tacrolimus. There was a trend to reduction in IFN-γ release in the presence of tacrolimus ($p = .0147$). A similar finding was observed for IL-2 release with tacrolimus ($p = .0137$). IFN-γ and IL-2 release for tacrolimus, mycophenolate, sirolimus, and prednisone is displayed in Table S2.

Sirolimus was found to be associated with a proinflammatory cytokine release in response to SARS-CoV-2 peptides; including IL-1β ($p = .0016$), TNF-α ($p = .0027$), with a possible trend with GM-CSF ($p = .0143$). There was no significant association with IL-6 ($p = .1968$). Figure 3A–D represents IL-6, TNF-α, IL-1β, and GM-CSF. Only 10 participants were included in the sirolimus analysis due to lack of PBMCs for one participant. Participant 6 appeared to exhibit a greater release of proinflammatory cytokines compared to others, most evident in Figure 3B and C. This patient had no clear discerning clinical features and was largely representative of the overall cohort. He was a 56-year-old male with moderate COVID-19 who required hospitalization and ward-level care.

The differing concentrations of mycophenolate and prednisone did not appear to influence cytokine response postpeptide stimulation. There was also no significant induction between immunosuppression doses or agents for the remaining cytokines including IL-10, IL-17a, IL-4, IL-23, IL-13, IL-12p70, IL-8, and IL-5 (Supplementary data, Table S2). Of these, only IL-17 and IL-8 appeared to respond to stimulation with SARS-CoV-2 peptides compared with the negative control of PBMCs alone.

3.2 | T helper 1 (Th1) to T helper 2 (Th2) cytokine ratio and the effect of immunosuppression

Based on the hypothesis that a predominant Th1 cytokine response is important in a protective immune response to SARS-CoV-2 infection, we analyzed two separate Th1:Th2 ratios: IFN-γ:IL-13 ratio and IFN-γ:IL-4 ratio (Figure 4A and B, respectively).[16,17] In the presence of higher concentrations of tacrolimus, there was a possible trend toward a Th2 phenotype as indicated by a reduced IFN-γ: IL-13 ratio ($p = .0663$) and IFN-γ: IL-4 ratio ($p = .0176$) when compared to the baseline response without immunosuppression (PBMCs + SARS-CoV-2 peptides).

3.3 | Proinflammatory versus anti-inflammatory cytokine release

Given the importance of significant induction of proinflammatory cytokines associated with COVID-19 infection, in particular, the proinflammatory state observed in severe COVID-19, we analyzed the proinflammatory cytokines TNF-α, IL-1β, and IL-6 as a ratio to IL-10 (anti-inflammatory cytokine) expression, to assess proinflammatory to anti-inflammatory cytokine release and how it is affected by immunosuppression (Figure 5A–C). Ten participants were included in this

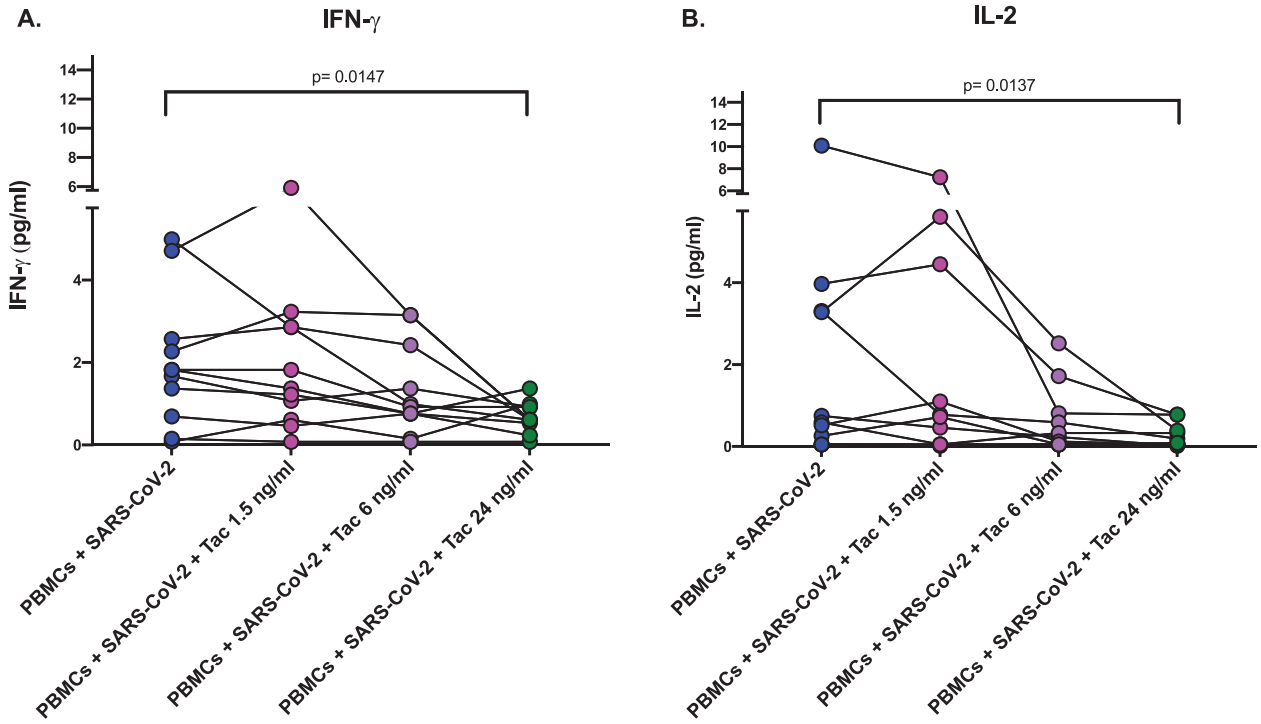

FIGURE 2 Impact of tacrolimus at increasing concentrations on IFN-γ (A) and IL-2 (B) cytokine release. Individual points represent each participant's cytokine value with a line connector for each experimental condition. Cytokine values for increasing concentrations of tacrolimus and the baseline value without immunosuppression (PBMCs + SARS-CoV-2 stimulation) were analyzed by the Friedman test. Using Holm–Bonferroni correction for multiple comparisons, a p value $< .0125$ indicates statistical significance. Abbreviations: PBMCs = peripheral blood mononuclear cells, Tac = tacrolimus, IFN-γ = interferon-gamma and IL-2 = interleukin-2

analysis due to lack of PBMCs for one participant. In the presence of increasing concentrations of sirolimus, there was a significant increase in the TNF-α:IL-10 ratio ($p = .0056$), IL-6:IL-10 ratio ($p = .0062$), and IL-1β:IL-10 ratio ($p = .0005$), suggesting a proinflammatory phenotype in the context of sirolimus. These cytokine ratios were not significantly altered in the presence of tacrolimus, mycophenolate, and prednisone (Supplementary data, Table 2).

4 | DISCUSSION

Our study provides novel preliminary insights into the SARS-CoV-2-specific T-cell cytokine profile and how it is influenced by standard immunosuppressive medications used in solid organ transplantation. Several important findings were observed: (1) higher concentrations of tacrolimus were associated with trend toward a reduction in SARS-CoV-2-specific IFN-γ and IL-2 expression and Th2 phenotype by differential pattern of cytokine expression; (2) sirolimus was associated with a proinflammatory cytokine release in response to SARS-CoV-2 stimulation; and (3) different mycophenolate doses and low-dose prednisone were neutral in terms of their effect on SARS-CoV-2-specific responses. These findings are hypothesis generating, and further exploration is required in a larger, clinical cohort study.

Tacrolimus competitively binds to calcineurin and inhibits the translocation of transcription factors, leading to reduced transcriptional activation of cytokine genes, including IL-2 and IFN-γ, among others.[19] Ultimately, the proliferation of T lymphocytes is reduced.[10,19] There was a trend toward reduction in IL-2 and IFN-γ release in our study and a Th2 phenotype in the presence of increasing concentrations of tacrolimus; however, these did not reach statistical significance. Although trough concentrations in SOT are generally kept under 15 ng/ml, a 2-h postdose level of >20 ng/ml can be achieved and has been suggested to be a better correlate for toxicity and efficacy.[14] Thus, the highest concentration of tacrolimus in our study approximates peak concentrations in vivo. IL-2 is the predominant Th1 cytokine that results from stimulating PBMCs of patients previously diagnosed with COVID-19; this is different from other infections, including influenza where IFN-γ is predominant.[20] IL-2 also plays a key role in the generation of effector and memory T cells, and proliferation of T cells.[21] Elevated levels of IL-2 or its receptor IL-2R have been detected in patients with COVID-19 and other coronaviruses.[1] The production of IFN-γ by activated T lymphocytes stimulates innate macrophage-mediated immunity and is critical in the immune response to SARS-CoV-2.[22,23] The importance of both of these cytokines in the immunopathogenesis of COVID-19 highlights the delicate balance required in a regulated cytokine response, which is seen to enhance pathogen clearance but potentially is attached to an increased risk of immunopathology.[24]

In response to SARS-CoV-2 peptides, we unexpectedly observed that sirolimus was significantly associated with a proinflammatory

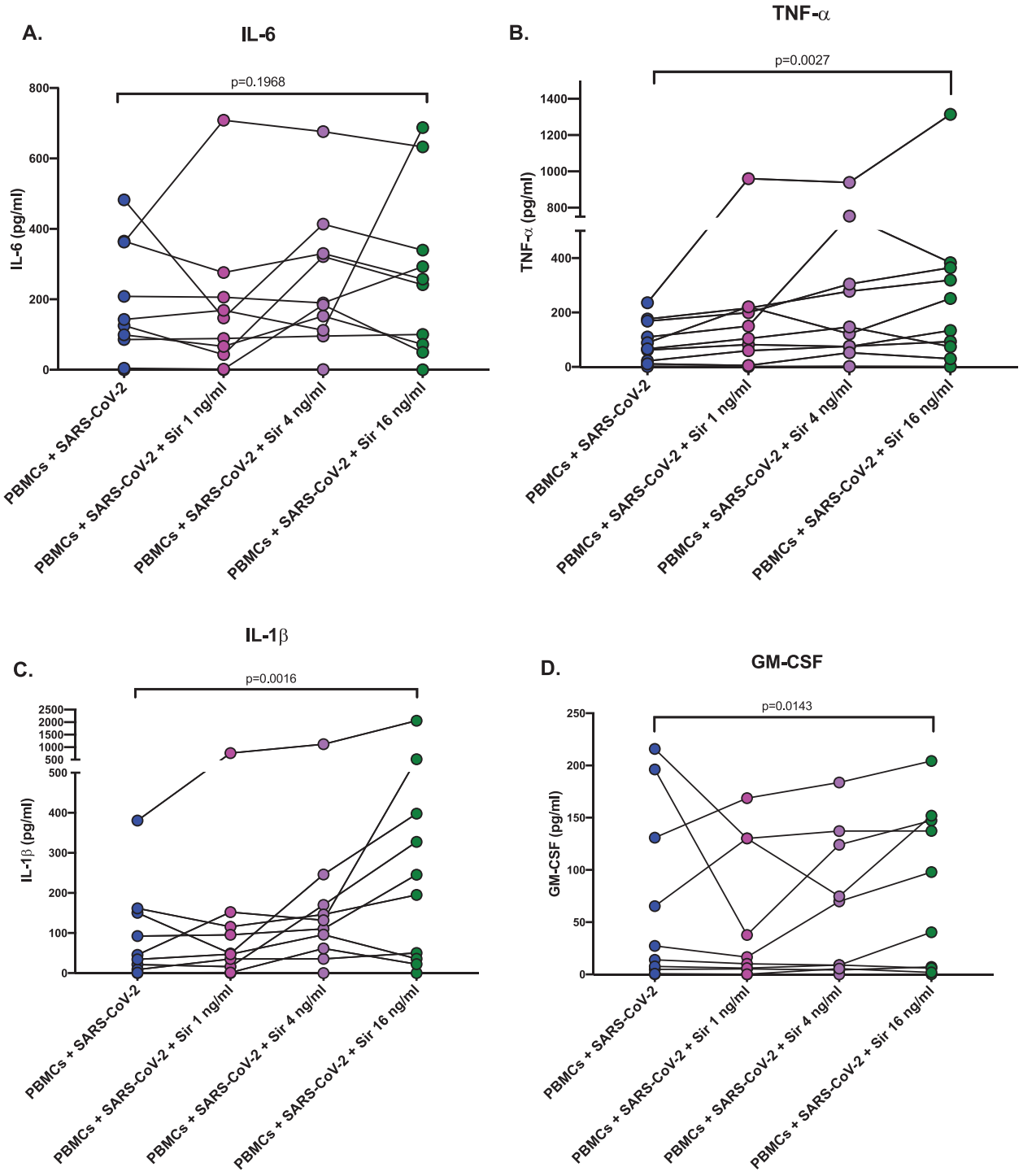

FIGURE 3 Impact of sirolimus at increasing concentrations on IL-6 (A), TNF-α (B), IL-1β (C), and GM-CSF (D). Individual points represent each participant's cytokine value with a line connector for each experimental condition. Statistical analysis was performed between cytokine expression and the baseline value without immunosuppression (PBMCs + SARS-CoV-2 stimulation) by the Friedman test. Using Holm–Bonferroni correction for multiple comparisons, a p value $< .0125$ indicates statistical significance. Abbreviations: PBMCs = peripheral blood mononuclear cells, Sir = sirolimus, IL-6 = interleukin-6, TNF-α = tumor necrosis factor-α, IL-1β = interleukin-1β, GM-CSF = granulocyte-macrophage colony stimulating factor

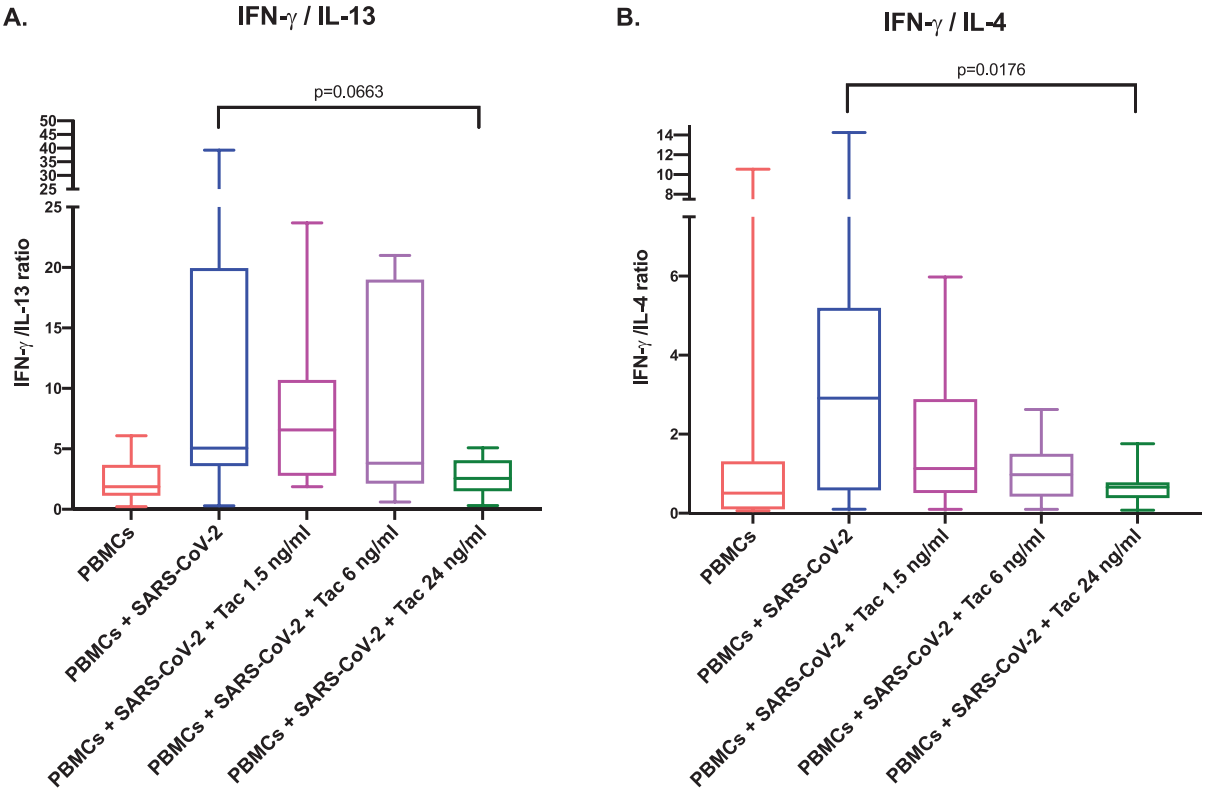

FIGURE 4 Impact of tacrolimus on Th1:Th2 cytokine ratio. IFN-γ:IL-13 ratio (A) and IFN-γ:IL-4 ratio (B) as demonstrated by a box and whisker plot, with maximum and minimum values. Statistical analysis was performed between cytokine expression and the baseline value without immunosuppression (PBMCs + SARS-CoV-2) by the Friedman test. Using Holm–Bonferroni correction for multiple comparisons, a p value $< .0125$ indicates statistical significance. Abbreviations: PBMCs = peripheral blood mononuclear cells, Tac = tacrolimus, IFN-γ = interferon-gamma, IL-13 = interleukin-13, IL-4 = interleukin-4

cytokine release including higher levels of TNF-α and IL-1β. This is important in the context of moderate-severe COVID-19, which is characterized by a proinflammatory state and high levels of inflammatory cytokines.[1,2,24] This finding may be explained by the mechanism of action of sirolimus or a potential drug toxicity at higher concentrations. Sirolimus inhibits the mammalian target of rapamycin and subsequent IL-2-, IL-4-, and IL-15-driven T-cell proliferation.[19,25] Its mechanism of action is to block the response of T- and B-cell activation by cytokines, preventing cell cycle progression and proliferation, distinct from tacrolimus and prednisone, which are both able to inhibit the production of cytokines.[19] Therefore, we may be witnessing unchecked cytokine release in response to SARS-CoV-2 stimulation rather than a specific effect related to sirolimus. The other potential reason is drug toxicity, which has been observed at higher concentrations of sirolimus. However, the concentrations we used are well within trough and peak concentrations commonly seen in transplant patients.[25] Also the effect was not seen in all patient PBMCs tested, arguing against simple drug toxicity. These findings are important to reflect upon in the context of COVID-19 infection in the inflammatory phase.

This study has some limitations, including a small sample size with varied cytokine response observed. We acknowledge the heterogeneity in this cohort in terms of age range and clinical severity, and this should be taken into consideration when interpreting the study findings and limits clinical extrapolation. Only a small number of discrete patient PBMCs were analyzed; therefore, the true effect size may be under or overestimated. However, the analysis is similar to previous studies evaluating immunosuppression and CMV-specific T-cell response.[11] While the dose concentrations of prednisone aimed to mimic usual dosing for solid organ transplant patients, it was not equivalent to the recommended dose of dexamethasone administered in the RECOVERY trial.[18] This was not a specific aim of our study however and may account for the neutral response seen with prednisone; higher concentrations may have been required to influence cytokine response. The incubation time of 24 h may have not been suitable for all immunosuppressive medications, in particular mycophenolate, and limited the effect observed on cytokine release. In addition, we did not analyze cyclosporine or combinations of immunosuppression together and we acknowledge this as a limitation, primarily due to insufficient PBMC numbers from patient clinical samples. This would be of interest for a future study. Only one arm of the immune response to SARS-CoV-2 was assessed; other aspects including innate immunity and antibody generation were not included, and it is possible that the immunosuppression medications included in this study may have significant influence here that has not been captured. Further studies are required to explore the findings in our study, in particular, in larger, prospective, clinical cohorts.

We performed an exploratory in vitro study of SARS-CoV-2-specific T-cell cytokine release in the presence of clinically relevant, differing

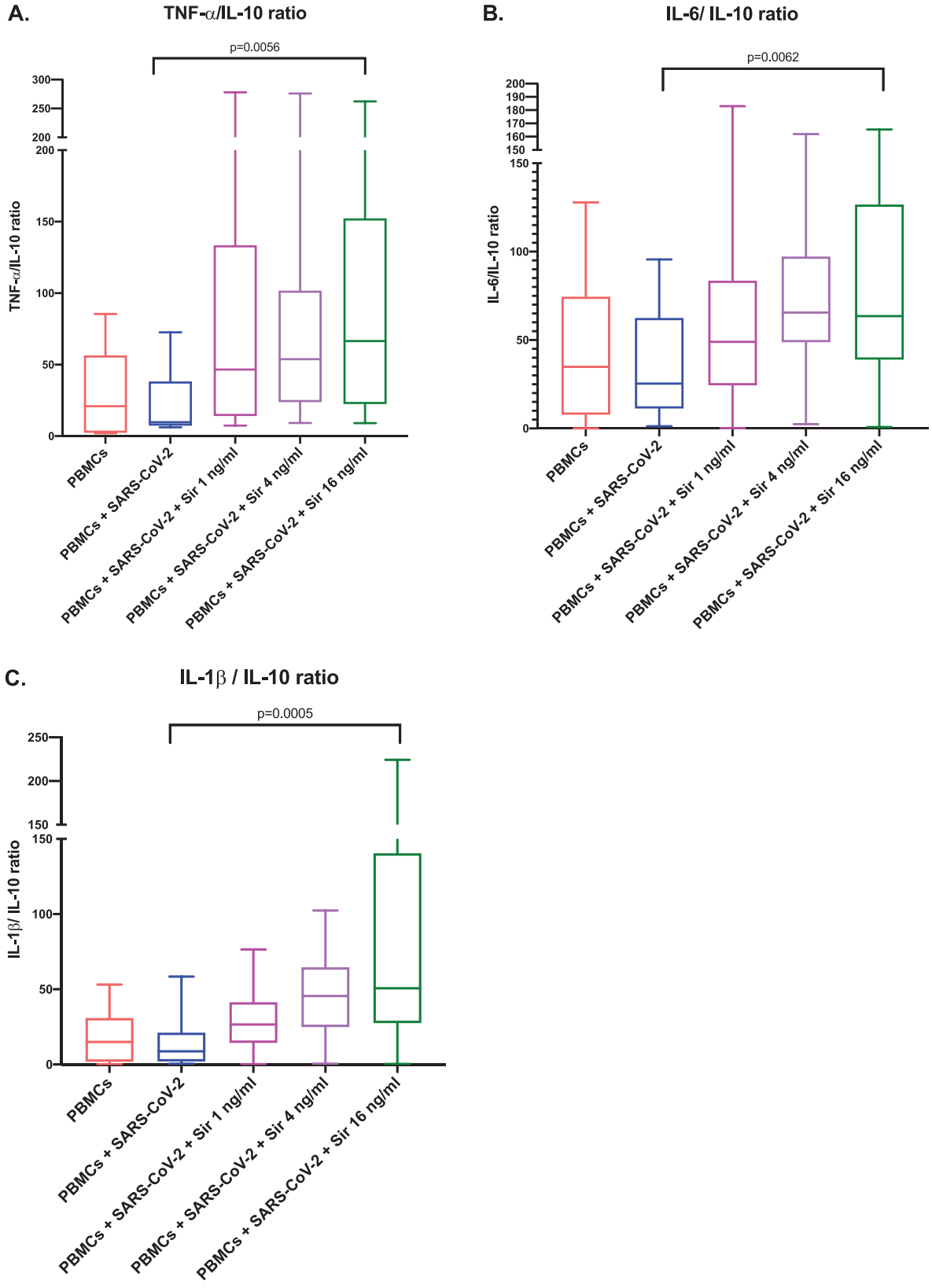

FIGURE 5 Impact of sirolimus at increasing concentrations on proinflammatory: anti-inflammatory cytokine ratio. TNF-α/IL-10 (A), IL-6/IL-10 ratio (B), IL-1β/IL-10 ratio (C) as demonstrated by a box and whisker plot, with maximum and minimum values. Statistical analysis was performed between cytokine expression and the baseline value without immunosuppression (PBMCs + SARS-CoV-2) by the Friedman test. Using Holm–Bonferroni correction for multiple comparisons, a p value $< .0125$ indicates statistical significance. Abbreviations: PBMCs = peripheral blood mononuclear cells, Sir = sirolimus, TNF-α = tumor necrosis factor-alpha, IL-10 = interleukin-10, IL-6 = interleukin-6, IL-1β = interleukin-1 beta

concentrations of standard immunosuppressive agents used in SOT. There was a suggested trend toward the inhibition of Th1 responses with greater concentrations of tacrolimus, whereas sirolimus was associated with an unexpected proinflammatory response, and mycophenolate was neutral. Our research is hypothesis generating and descriptive; translation to clinical recommendations, including dose reduction of specific immunosuppressive agents requires further study with a larger sample size.

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CONFLICT OF INTEREST

DK has received advisory board fees from Pfizer. AH has received honoraria and research grant from Astellas. The remaining authors have no conflicts of interest.

ORCID

Victoria G. Hall https://orcid.org/0000-0001-9353-0059
Victor Ferreira https://orcid.org/0000-0002-1472-9711
Deepali Kumar https://orcid.org/0000-0003-1961-0477
Atul Humar https://orcid.org/0000-0002-1751-7159

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