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Pharmacodynamic Effect of mTOR Inhibition-based Immunosuppressive Therapy on T- and B-cell Subsets After Renal Transplantation

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Background. The mammalian target of rapamycin inhibitor (mTORi) therapy after kidney transplantation is solely monitored pharmacokinetically, not necessarily reflecting PI3K-Akt-mTOR pathway blockade efficacy leading to potential under- or overimmunosuppression. **Methods.** In this cross-sectional study, phosphoflow cytometry was used to determine the efficacy of mTOR inhibition in peripheral T- and B-lymphocyte subsets by assessing p70S6 kinase (p70S6K) phosphorylation in renal transplant recipients upon treatment with a combination of either mTORi and calcineurin inhibitors (n = 18), or mTORi with mycophenolic acid (n = 9). Nine dialysis patients with end-stage renal disease and 17 healthy age-matched volunteers served as controls. **Results.** mTORi treatment reduced p70S6K phosphorylation in $CD4^+$, $CD8^+$ T, and $CD19^+$ B cells compared with healthy controls (HCs). Subpopulation analysis of $CD4^+$ T cells and $CD19^+$ B cells revealed a significant reduction of p70S6K phosphorylation in $CD4^+CD45RA^-CD25^-$ Th cells ($P < 0.05$), $CD24^{hi}CD38^{hi}$ transitional B cells ($P < 0.001$), $CD24^+CD38^-$ memory B cells ($P < 0.001$), and $CD24^{int}CD38^{int}$-naive B cells ($P < 0.05$) upon mTORi treatment, whereas $CD4^+CD45RA^-CD25^{++}CD127^-$ regulatory T cells and $CD24^-CD38^{hi}$ plasmablasts were not affected. Compared with mTORi + mycophenolic acid therapy, mTORi + calcineurin inhibitor treatment exhibited an even stronger inhibition of p70S6K phosphorylation in $CD4^+CD45RA^-CD25^-$ Th cells and $CD8^+$ T cells. However, trough levels of mTORi did not correlate with p70S6K phosphorylation. **Conclusions.** mTORi selectively inhibited p70S6K phosphorylation in select lymphocyte subtypes. Assessing p70S6K phosphorylation by phosphoflow cytometry may serve as an approach to understand cell subset specific effects of mTORi providing detailed pharmacodynamic information for individualizing immunosuppression.

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The success of kidney transplantation critically depends on effective immunosuppression to prevent acute and chronic allograft rejection.[1,2] mTOR inhibitors (mammalian target of rapamycin inhibitor [mTORi]) suppress the immune response by blocking immune cell proliferation, differentiation and function.[3] mTORi display different side effects as calcineurin inhibitors (CNIs).[4–6] Importantly, mTORi lead to less nephrotoxicity compared with CNIs.[7,8] Therefore, their

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A.H., S.W., and M.W. designed the study. S.W. and A.H. conducted blood sampling and collected patients' informed consents. S.W. and I.A. carried out the experiments. X.W., S.W., D.Y., and A.H. analyzed the data. S.W., T.J.-S., and X.W. collected and analyzed patients' clinical data from the digital medical files. X.W., D.Y., and A.H. wrote the article. J.W., M.W., M.S., D.D., and C.H.K.L. critically revised the article and provided important input for the study. All authors contributed to the article and approved the submitted version.

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application allows for substitution or dose reduction of CNIs, thus preserving renal function while maintaining immunosuppression.[9] mTORi therapy after kidney transplantation is solely monitored pharmacokinetically, not necessarily reflecting PI3K-Akt-mTOR pathway blockade efficacy leading to potential under- or overimmunosuppression.[10–12] p70S6 kinase (p70S6K) is located downstream of mTOR. mTOR inhibition reduces phosphorylation and thereby inactivates the downstream effector kinase p70S6.[13] Therefore, p70S6K phosphorylation might serve as parameter for sensitive and reliable detection of the mTORi pharmacodynamic effects.[14]

Previously, we have established the determination of p70S6K phosphorylation in $CD3^+$ T cells by phosphoflow cytometry as valid and reliable tool to monitor the pharmacodynamic effects of mTORi. As the mTORi mediated reduction in p70S6K phosphorylation did not exhibit a correlation with mTORi trough levels, we suggested a phosphoflow cytometric quantification of p70S6K phosphorylation as an adjunct tool for individualizing mTORi therapy. Furthermore, we observed a selective reduction of the p70S6K phosphorylation in $CD4^+CD25^-$ T cells, while $CD4^+CD25^{hi}$ regulatory T cells (Treg) remained unaffected. This indicated that mTORi treatment exerts distinct effects on different T-cell subsets.[14]

In this study, we extend our previous findings demonstrating that a therapeutic regimen combining mTORi with different immunosuppressive agents elicits varying changes of p70S6 phosphorylation within peripheral blood lymphocytes after kidney transplantation. This variability may modulate the proliferation and activation of specific immune cell subsets, subsequently impacting allograft rejection and thus graft survival rates. These findings may enable a further refinement of a personalized posttransplant immunosuppression.

MATERIALS AND METHODS

Patients and Interventions

This was a cross-sectional study comparing 2 mTORi-based treatment regimens, one including CNI and the other mycophenolic acid (MPA). A total of 27 adult renal transplant recipients immunosuppressed with mTORi in the Department for Nephrology and Hypertensiology at the University Hospital Erlangen were enrolled. Seventeen healthy age-matched individuals and 9 patients with end-stage chronic kidney disease regularly undergoing hemodialysis served as controls. All subjects provided informed consent before study inclusion and the study protocol was approved by the Ethics Committee of the Medical Faculty of the Friedrich Alexander University Erlangen-Nuremberg (Ethic vote 207_16B). The patients' enrollment and allocation within this study are summarized in Figure 1. Transplant patients were categorized into subgroups based on their type of immunosuppression: 18 patients receiving maintenance immunosuppression consisting of an mTORi (everolimus or sirolimus) combined with a CNI (cyclosporine A or tacrolimus) were assigned as mTORi+CNI group and 9 patients receiving an mTORi combined with MPA (mycophenolate sodium or mycophenolate mofetil) as mTORi+MPA. The mycophenolate sodium dose was 180–540 mg twice per day, whereas the mycophenolate mofetil dose was 250–750 mg twice per day. Information on immunosuppression at study inclusion, including mean serum drug trough levels, is provided in Table 1. Heparinized blood was collected at various timepoints beginning 6 wk posttransplantation (mean 3–181 mo). Along with the blood samples, clinical data including age, sex, and clinical chemistry were analyzed.

Antibodies

Surface staining for flow cytometry was conducted utilizing fluorochrome-conjugated antibodies: $CD25^-$PE-Cy7 (M-A251), $CD25^-$APC (M-A251), $CD4^-$PerCP-Cy5.5 (RPA-T4), $CD19^-$BV421 (J3-119), $CD3^-$FITC (SK7), $CD8^-$BV510 (SK1), $CD24^-$FITC (SN3 A5-2H10), $CD38^-$PE-Cy7 (HB-7), $CD45^-$RA-BV510 (HI100), $CD127^-$BV510 (HIL-7R-M21), and $CD127^-$BV421 (HIL-7R-M21). Intracellular staining of p70S6K was performed with a primary anti-p70S6K

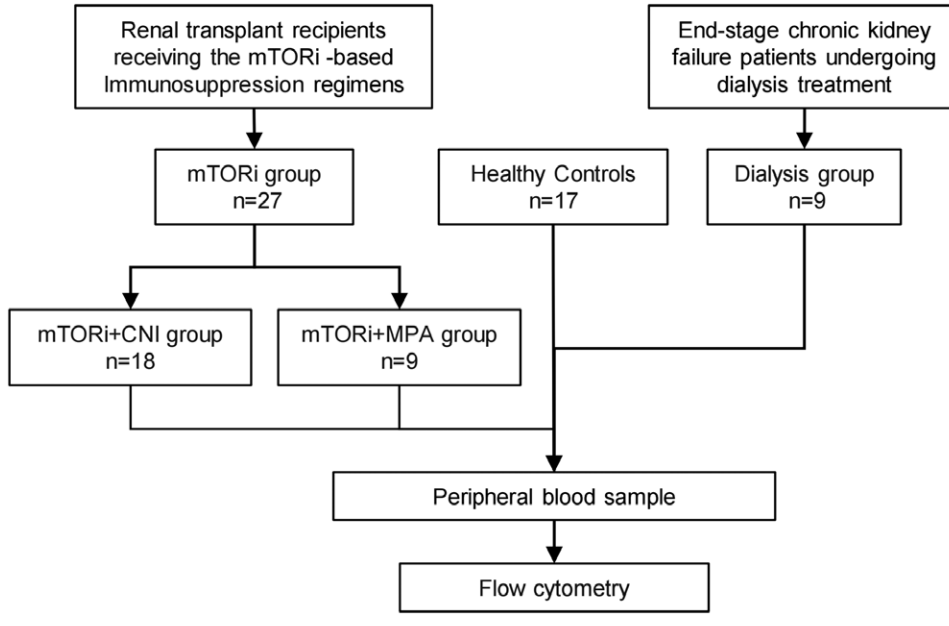

FIGURE 1. Overview of the patient cohort. CNI, calcineurin inhibitor; MPA, mycophenolate sodium; mTORi, mammalian target of rapamycin inhibitor.

TABLE 1.

Immunosuppression treatment of renal transplant recipients at inclusion

	mTORi+CNI group (n = 18)	mTORi+MPA group (n = 9)	*P*
Immunosuppression			
Everolimus	10 (56)	3 (33)	0.42
Sirolimus	8 (44)	6 (66)	0.42
Tacrolimus	16 (88)	0 (0)	
Cyclosporine A	2 (11)	0 (0)	
Mycophenolate sodium	0 (0)	2 (22)	
Mycophenolate mofetil	0 (0)	7 (77)	
Prednisolone	16 (88)	5 (55)	0.14
Trough level			
Everolimus (ng/mL)	5.9 ± 2.8	7.0 (4.6–7.3)	0.82
Sirolimus (ng/mL)	5.6 (4.3–8.0)	5.1 (3.9–7.9)	0.86
Tacrolimus (ng/mL)	6.0 ± 1.5		
Cyclosporine A (ng/mL)	43.5 (38.0–49.0)		

Qualitative data are presented as frequency and percentage. The values of quantitative data are presented as median and interquartile ranges or mean and SD
CNI, calcineurin inhibitor; MPA, mycophenolic acid; mTORi, mammalian target of rapamycin inhibitor.

monoclonal antibody (1A5, Cell Signaling) and a secondary antimouse IgG2a-PE monoclonal antibody (m2a-15F8, eBioscience).

Peripheral Blood Mononuclear Cells Isolation and Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) isolation and flow cytometry were performed within 3–4 h after sample collection as previously described.[14] In brief, PBMCs were isolated by gradient density centrifugation (Lymphoprep, Axis Shield), immediately fixed with 1× PhosFlow Fix buffer (BD Bioscience) for 10 min at 37 °C, washed, and stained with directly conjugated fluorescent antibodies as listed for 20 min at room temperature in the dark. Cells were washed and permeabilized using Phosflow Perm-Wash Buffer I (BD Bioscience) on ice for 15 min for subsequent staining of p70S6K.[14] Immunophenotypes and gating of each cell subpopulation are shown in Figure 2. Data acquisition was conducted using an FACS Canto II flow cytometer (BD Biosciences); data analysis was performed with FlowJo (version 10; BD Biosciences). To identify the cell subsets of interest, gates were set based on fluorescence minus one and/or unstained controls. The level of phosphorylated p70S6K was calculated using the formula: Mean fluorescence intensity index (MFIx) = $([MFI_{p70S6K} - MFI_{IgG2a}]/MFI_{IgG2a})$.[14]

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism V9.5. Comparisons between two groups were assessed using the Fisher exact test. According to the results of the normality test, comparisons between 2 groups were performed using T or Mann-Whitney U tests. Comparisons of >2 groups were analyzed by ANOVA followed by Tukey post hoc test or Kruskal-Wallis followed by the Dunn post hoc test. Correlation analysis was carried out using the Pearson test for parametric or Spearman test for nonparametric data distribution, respectively.

RESULTS

Clinical and Demographic Characteristics of Renal Transplant Recipients

To investigate the effects of mTORi on PBMCs in renal transplant recipients, we divided the mTORi-treated patients into 2 groups: the mTORi+CNI group (n = 18) and the mTORi+MPA group (n = 9). CNI+MPA represents the preferred initial standard treatment after kidney transplantation at our institution. Discontinuation of the CNI and initiation of an mTORi therapy was performed in those patients presenting complications such as liver or kidney function impairment and/or neurotoxicity. This explains why the duration from transplantation to sampling in the mTORi+CNI group was significantly shorter compared with the mTORi+MPA group. The clinical characteristics and laboratory data are displayed in Table 2. No statistically significant differences concerning other clinical parameters were detected.

mTORi-based Immunosuppression Reduced p70S6K Phosphorylation and CD4⁺ T-cell Frequency, but Increased CD8⁺ T-cell Frequency

Frequency and p70S6K phosphorylation status of T-cell subsets were assessed according to the gating strategy presented in Figure 3A. As previously shown, we found significantly lower p70S6K phosphorylation in CD4⁺ T cells upon mTORi treatment compared with HCs ($P = 0.02$, Figure 3B). Furthermore, p70S6K phosphorylation in mTORi+CNI was significantly reduced compared with mTORi+MPA ($P = 0.02$, Figure 3C). This indicated a stronger reduction of p70S6K phosphorylation by the mTORi+CNI combination. Regression analysis showed no correlation between p70S6K phosphorylation in CD4⁺ T cells and mTORi serum levels ($P = 0.33$, $r = 0.21$; Figure 3D).

The frequency of CD4⁺ T cells among lymphocytes was significantly lower upon mTORi treatment compared with HCs ($P = 0.0001$, Figure 3E) and was clearly correlated with p70S6K phosphorylation ($P = 0.005$, $r = 0.38$; Figure 3F). However, patients receiving mTORi+MPA and those receiving mTORi+CNI displayed no difference in CD4⁺ T-cell frequencies (Figure 3G). This suggests that the observed correlation was not substantially influenced by CNI.

We observed no differences in the frequencies of CD45RA⁺CD4⁺ T naive ($CD4^+T_{naive}$) and CD45RA⁻CD4⁺ T memory ($CD4^+T_{mem}$) cells among the mTORi, dialysis, and HCs groups (Figure 3H and I). Furthermore, frequencies of CD45RA⁺CD4⁺ T_{naive} and CD45RA⁻CD4⁺ T_{mem} cells did not differ between mTORi+CNI and mTORi+MPA (Figure 3J and K). These findings indicated that the mTORi-based regimen exerts no discernible impact on the balance of naive/memory CD4⁺ T cells.

When examining CD8⁺ T cells (**Figure S1A, SDC,** http://links.lww.com/TXD/A672), no differences in p70S6K phosphorylation levels were observed between mTORi, dialysis, and HCs groups ($P = 0.20$, **Figure S1B, SDC,** http://links.lww.com/TXD/A672). Similar to the CD4⁺ T cells, mTORi+CNI treatment demonstrated a lower p70S6K phosphorylation

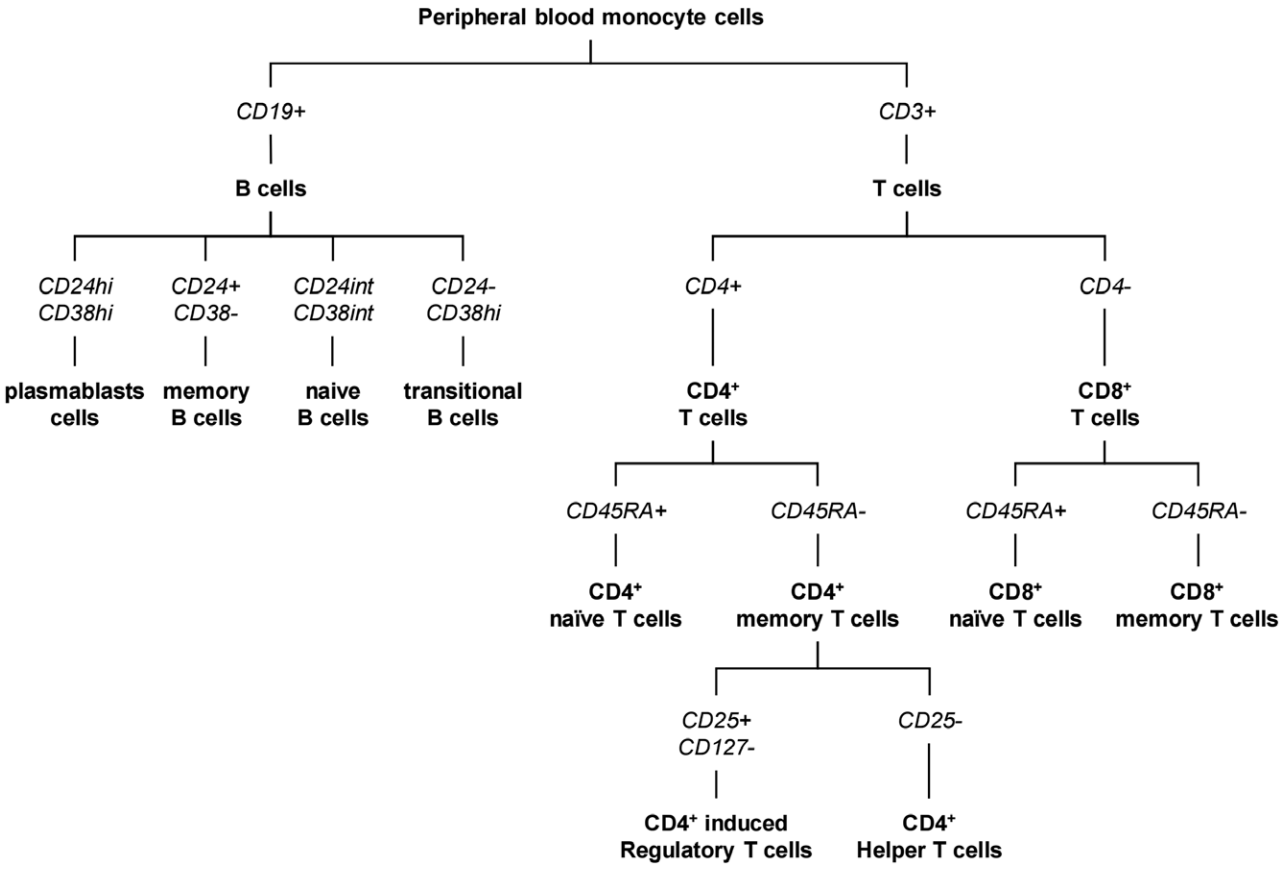

FIGURE 2. Fourteen subpopulations were identified using previously published combinations of surface markers for each subject's PBMCs sample. PBMC, peripheral blood mononuclear cell.

compared with mTORi+MPA ($P = 0.03$, **Figure S1C, SDC**, http://links.lww.com/TXD/A672). Furthermore, there was no correlation between p70S6K phosphorylation and mTORi trough levels ($P = 0.17$, $r = 0.29$, **Figure S1D, SDC**, http://links.lww.com/TXD/A672).

However, $CD8^+$ T-cell frequency among lymphocytes was higher upon mTORi treatment compared with HCs ($P = 0.02$, **Figure S1E, SDC**, http://links.lww.com/TXD/A672), with no difference between the mTORi+CNI and mTORI+MPA groups (**Figure S1F, SDC**, http://links.lww.com/TXD/A672). This indicates differences in mTORi response for $CD4^+$ and $CD8^+$ T-cell subsets in vivo, as $CD8^+$ T-cell population was expanded while $CD4^+$ T-cell population was reduced.

Although we found no differences in the frequencies of $CD45RA^+CD8^+T_{naive}$ cells and $CD45RA^-CD8^+T_{mem}$ cells among the mTORi, dialysis, and HCs groups (**Figure S1G–I, SDC**, http://links.lww.com/TXD/A672), comparison of mTORi+CNI and mTORi+MPA revealed a higher percentage of $CD45RA^+CD8^+T_{naive}$ cells ($P = 0.03$, **Figure S1G and J, SDC**, http://links.lww.com/TXD/A672) and a lower percentage of $CD45RA^-CD8^+T_{mem}$ cells ($P = 0.03$, **Figure S1G and K, SDC**, http://links.lww.com/TXD/A672). This may demonstrate an inhibition of maturation of $CD45RA^-CD8^+T_{mem}$ cells by mTORi+CNI compared with mTORi+MPA, thereby impacting the naive/memory $CD8^+$ T-cell balance. Furthermore, p70S6K phosphorylation was negatively correlated with $CD8^+$ T_{naive}-cell frequency ($P = 0.04$, $r = -0.30$, **Figure S1L, SDC**, http://links.lww.com/TXD/A672), but positively with $CD8^+$ T_{mem}-cell frequency ($P = 0.04$, $r = 0.30$, **Figure S1M, SDC**, http://links.lww.com/TXD/A672).

mTORi-based Immunosuppression Reduced p70S6K Phosphorylation in $CD4^+CD25^-$ Th Cells Without Impacting $CD4^+CD127^-CD25^{++}$Treg Cells

We subsequently investigated the impact of mTORi on p70S6K phosphorylation in $CD4^+CD45RA^-CD25^-$ T helper (Th) cells and $CD4^+CD45RA^-CD25^{++}CD127^-$ Treg (Figure 4A). Consistent with our previous results, a notably reduced phosphorylation was observed in Th cells compared with Treg within the HCs ($P = 0.003$; Figure 4B). Next, the evaluation of mTORi-treated transplant recipients revealed a significantly lower p70S6K phosphorylation in Th cells compared with the HCs ($P = 0.03$, Figure 4C). Subgroup analysis indicated an even lower p70S6K phosphorylation upon mTORi+CNI treatment compared with mTORi+MPA treatment ($P = 0.03$, Figure 4D). In contrast, p70S6K phosphorylation in Treg upon mTORi treatment showed no differences in both, HCs and dialysis patients ($P > 0.99$, Figure 4E) and no difference between mTORi+CNI and mTORi+MPA treatment ($P = 0.12$, Figure 4F).

These results suggest suppression of p70S6K phosphorylation in Th cells by mTORi, whereas CNI co-administration exerted an amplifying effect. However, neither mTORi nor mTORi+CNI had a discernible impact on p70S6K phosphorylation in Treg.

Given the distinct effects of mTORi on p70S6K phosphorylation in Th cells and Treg, we further explored the

TABLE 2.

Demographics and clinical parameters of renal transplant recipients

Parameter	mTORi+CNI (n = 18)	mTORi+MPA (n = 9)
Age (y)	56.3 ± 9.4	53.9 ± 18.1
Male sex, n (%)	13 (72)	6 (67)
Duration from Tx to sampling (m)	27.2 ± 27.4*	120.6 ± 45.1*
Infection, n (%)	17 (94)	6 (67)
CMV	7 (39)	2 (22)
BV	9 (50)	2 (22)
Other viral infections	2 (11)	2 (22)
Other bacterial infections	3 (17)	0 (0)
DSAs	3 (17)	2 (22)
Rejection reaction	6 (33)	4 (44)
Humoral rejection	2 (11)	2 (22)
Cellular rejection	4 (22)	2 (22)
Creatinine (mg/dL)	1.1 (1.3–2.6)	1.7 (1.1–2.0)
Uric acid (mg/dL)	7.4 ± 1.5	7.0 ± 2.0
Urea (mg/dL)	77.1 ± 37.0	74.1 ± 45.0
CRP (mg/L)	5.0 ± 3.6	6.6 (0.7–15.2)
Triglyceride (mg/dL)	220.6 ± 80.2	202.0 (172.0–258.0)
Cholesterin (mg/dL)	252.6 ± 38.6	212.8 ± 42.7
eGFR (mL/min)	34.3 ± 15.2	39.0 (23.0–44.0)
Hematocrit (%)	34.8 (31.8–37.8)	37.7 ± 4.1
WBCs (10^3/μL)	6.7 ± 2.7	8.0 ± 3.3
Platelets (10^3/μL)	215.6 ± 57.6	266.4 + 107.5
Albumin (g/L)	40.7 ± 2.8	39.8 ± 2.9

*$P < 0.0001$; mTORi, mammalian target of rapamycin inhibitor.

Qualitative data are presented using frequency and percentage. The values of quantitative data are presented as median and interquartile ranges or mean and SD.

Cutoff levels: creatinine: female 0.51–0.95 mg/dL, male: 0.67–1.17 mg/dL; uric acid: female:2.4–5.7 mg/dL, male: 3.4–7.0 mg/dL; urea: 17–43 mg/dL; CRP: <5 mg/L; triglyceride: 50–200 mg/dL; cholesterin: <200 mg/dL; eGFR: >60 mL/min; hematocrit: female:35%–46%, male: 39%–51 %; WBCs: female:4–11 × 10^3/μL, male: 4–10 × 10^3/μL; platelets: female:160–400 × 10^3/μL, male:140–350 × 10^3/μL; albumin: 35–55 g/L.

CNI, calcineurin inhibitor; CRP, C-reactive protein; DSA, donor-specific antibody; eGFR, estimated glomerular filtration rate; MPA, mycophenolic acid; mTORi, mammalian target of rapamycin inhibitor; Tx, transplantation; WBC, white blood cell.

influence of mTORi on the Th/Treg ratio within $CD4^+$ T_{mem} cells. We found no differences in Th cell and Treg frequencies among the mTORi, dialysis, and HC groups (Figure 4G and H). Notably, mTORi+CNI exhibited a higher percentage of Th cells ($P = 0.01$, Figure 4I) and a lower percentage of Treg ($P = 0.02$, Figure 4J) within the $CD4^+$ T_{mem} cells compared with mTORi+MPA. The Th/Treg ratio upon mTORi+CNI treatment was significantly higher compared with mTORi+MPA treatment ($P = 0.009$, Figure 4K and L), indicating a shift from Treg to Th cells upon mTORi+CNI treatment.

mTORi-based Immunosuppression Led to a Pronounced Reduction of p70S6K Phosphorylation and $CD19^+$ B-cell Frequency

Frequency and p70S6K phosphorylation status of B-cell subsets were assessed according to the gating strategy presented in Figure 5A. $CD19^+$ B-cells displayed a significantly lower p70S6K phosphorylation upon mTORi treatment compared with HCs ($P = 0.01$, Figure 5B), whereas p70S6K phosphorylation in B cells was comparable upon mTORi+CNI and mTORi+MPA treatment (Figure 5C). This suggests no synergistic inhibition of p70S6K in B lymphocytes by co-administration of CNI. Again, and consistent with the effects observed in $CD4^+$ and $CD8^+$ T cells, p70S6K phosphorylation in B cells showed no correlation with mTORi trough levels (Figure 5D).

Studying the B-cell frequencies among lymphocytes demonstrated a significantly lower frequency of B cells upon mTORi treatment compared with HCs ($P = 0.0002$, Figure 5E) and a correlation with p70S6K phosphorylation levels ($P = 0.008$, $r = 0.38$, Figure 5F). However, B-cell frequencies were comparable in the mTORi+CNI and mTORi+MPA groups ($P = 0.07$, Figure 5G). Of note, in 6 patients within the mTORi+CNI group, almost no B cells could be detected. Among them, only 2 patients had undergone B-cell depletion therapy by rituximab before sample collection (8 or 37 mo before, respectively), while the others had not. A correlation analysis between Tacrolimus trough levels and B-cell frequencies revealed a significant negative correlation ($P = 0.02$, $r = -0.59$, Figure 5H). This underlines the potential dose-dependent impact of CNIs on the frequency of B lymphocytes when used in combination with mTORi.

Next, we examined the B-cell subsets based on CD24 and CD38 expression (Figure 5A). Similar to $CD4^+$ T cells, distinct B-cell subsets exhibited significant differences: The p70S6K phosphorylation in $CD24^-CD38^{hi}$ plasmablasts was significantly lower than in $CD24^{hi}CD38^{hi}$ transitional B cells, $CD24^+CD38^-$ B memory (B_{mem}) cells, and $CD24^{int}CD38^{int}$ B naive (B_{naive}) cells among the HC subjects ($P = 0.0005$, $P < 0.0001$, and $P < 0.0001$, respectively, Figure 6A). Upon mTORi treatment, the p70S6K phosphorylation in $CD24^{hi}CD38^{hi}$ transitional B cells, B_{mem} cells, and B_{naive} cells was significantly decreased compared with HCs ($P = 0.0005$, $P = 0.0007$, and $P = 0.02$, respectively, Figure 6B–D), whereas $CD24^-CD38^{hi}$ plasmablasts were not affected ($P = 0.65$, Figure 6E). These findings indicate that mTORi targets p70S6K activity in $CD24^{hi}CD38^{hi}$ transitional, B_{mem}, and B_{naive} cells. Given their high baseline mTOR activity, these subsets appeared particularly susceptible to mTORi, potentially impacting their proliferation and alloactivation. Subgroup analysis revealed no differences in p70S6K phosphorylation within B-cell subsets upon mTORi+CNI or mTORi+MPA treatment (Figure 6F–I).

When investigating the frequency of B cell subpopulation, the proportion of B_{mem} and B_{naive} cells, as well as $CD24^-CD38^{hi}$ plasmablasts among total $CD19^+$ B lymphocytes showed no differences across all groups (**Figure S2, SDC**, http://links.lww.com/TXD/A672), whereas the proportion of $CD24^{hi}CD38^{hi}$ transitional B cells was significantly lower upon mTORi treatment compared with HCs and patients with dialysis ($P = 0.0016$, Figure 7A and B). Further analysis indicated a positive correlation between the frequency of transitional B cells and their p70S6K phosphorylation ($P = 0.0006$, $r = 0.51$, Figure 7C). Furthermore, transitional B-cell frequencies were reduced upon mTORi+CNI treatment compared with mTORi+MPA treatment ($P = 0.04$, Figure 7D), and the frequency of transitional B cells upon mTORi+CNI treatment negatively correlated with Tacrolimus trough levels ($P = 0.03$, $r = -0.74$, Figure 7E).

p70S6K Phosphorylation and the Occurrence of Donor-specific Antibodies and Allograft Rejection

We further investigated whether p70S6K phosphorylation in T- and B-cell subsets was associated with production of donor-specific antibodies (DSAs), allograft rejection, and polyoma virus (BK)/cytomegalovirus (CMV) infections. Among all post-transplant patients receiving an mTORi-based treatment, a total of 6 patients displayed DSA. The time interval between DSA

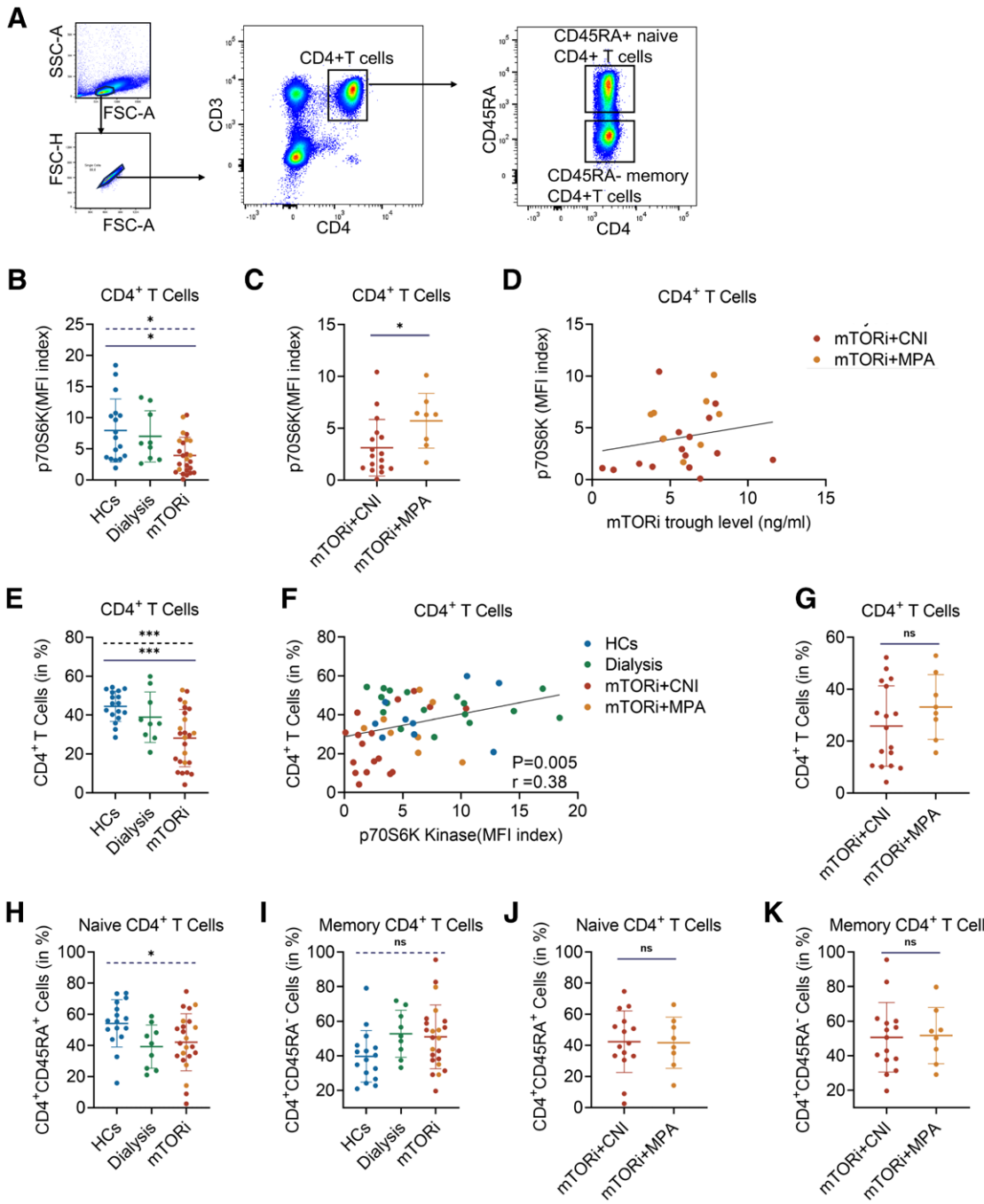

FIGURE 3. p70S6K phosphorylation levels and frequency in CD4⁺ T-cell subsets. A, Gating strategy for CD4⁺ T-cell populations. B, The scatter plot shows the phosphorylation levels of p70S6K in CD4⁺ T cells among HC subjects, dialysis patients, and renal transplant patients receiving mTORi-based immunosuppression. C, mTORi subgroup analysis reveals a significantly lower p70S6K phosphorylation in mTORi+CNI-treated patients. D, The correlation analysis shows no association of p70S6K phosphorylation in CD4⁺ T cells with mTORi trough levels. E, The scatter plot shows the frequencies of CD4⁺ T cells among healthy subjects, dialysis patients, and renal transplant patients receiving mTORi-based immunosuppression. F, The frequencies of CD4⁺ T cells positively correlate with their p70S6K phosphorylation levels. G, The frequency of CD4⁺ T cells is significantly reduced in the mTORi+CNI group cohort. H–K, The scatter plots depict the frequency of CD45RA⁺-naive CD4⁺ T cells and of CD45RA⁻ memory CD4⁺ T cells among HC subjects, dialysis subjects, and renal transplant patients receiving mTORi-based immunosuppression. ***$P < 0.001$; The dashed lines represent the ANOVA results for comparisons among 3 groups, while the solid lines represent the results of multiple comparison tests between 2 groups. CNI, calcineurin inhibitor; HC, healthy control; MPA, mycophenolic acid; mTORi, mammalian target of rapamycin inhibitor.

detection and p70S6K assessment was 3.00 ± 4.35 mo in the DSA (n = 6) and 5.40 ± 4.98 mo in the non-DSA (n = 21) group, respectively ($P = 0.26$). Our analysis revealed no significant differences in p70S6K phosphorylation in B and T cells between the DSA and non-DSA groups (**Figure S3, SDC,** http://links.lww.com/TXD/A672). However, as the time interval between DSA and p70S6K assessment was long and samples were not assessed simultaneously, a clear interpretation is difficult.

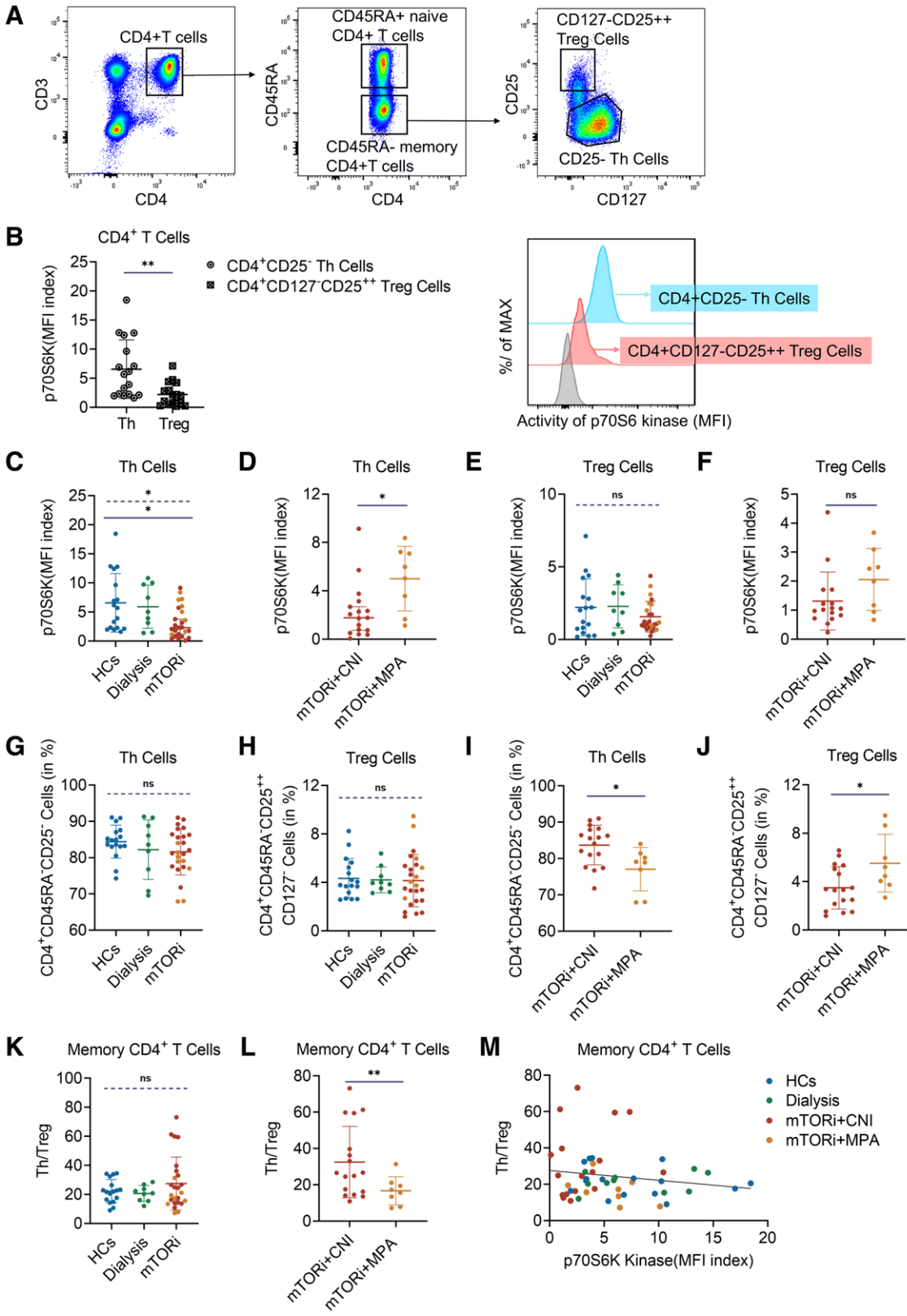

FIGURE 4. p70S6K phosphorylation levels and frequency of $CD4^+CD25^-$ Th cells and $CD4^+CD127^-CD25^{++}$Treg cells. A, Gating strategy for $CD4^+CD25^-$ Th cells and $CD4^+CD127^-CD25^{++}$Treg cell populations. B, The scatter plot and histograms in B depict differential phosphorylation levels of p70S6K in $CD4^+CD25^-$ Th cells and in $CD127^-CD25^{++}$Treg cells among HC subjects. Phosphorylation levels of p70S6K in $CD4^+CD25^-$ Th cells (C and D) and in $CD127^-CD25^{++}$Treg cells (E and F) among HC subjects, dialysis subjects, and renal transplant patients receiving mTORi-based immunosuppression. G and I, Scatter plots show the frequency of $CD4^+CD25^-$ Th cells and (H and J) $CD4^+CD127^-CD25^{++}$Treg cells, respectively. The ratio of Th/Treg cells among the $CD4^+$ T_{mem} cell subset is shown in (K) and (L), whereas there was no correlation between the Th/Treg ratio and p70S6K phosphorylation levels in $CD4^+$ T_{mem} cells (M). $^*P < 0.05$, $^{**}P < 0.01$; The dashed lines represent the ANOVA results for comparisons among 3 groups, whereas the solid lines represent the results of multiple comparisons tests between 2 groups. CNI, calcineurin inhibitor; HC, healthy control; MPA, mycophenolic acid; mTORi, mammalian target of rapamycin inhibitor; Th cells, T helper cells; T_{mem} cells, T memory cells; Treg, induced regulatory T cell.

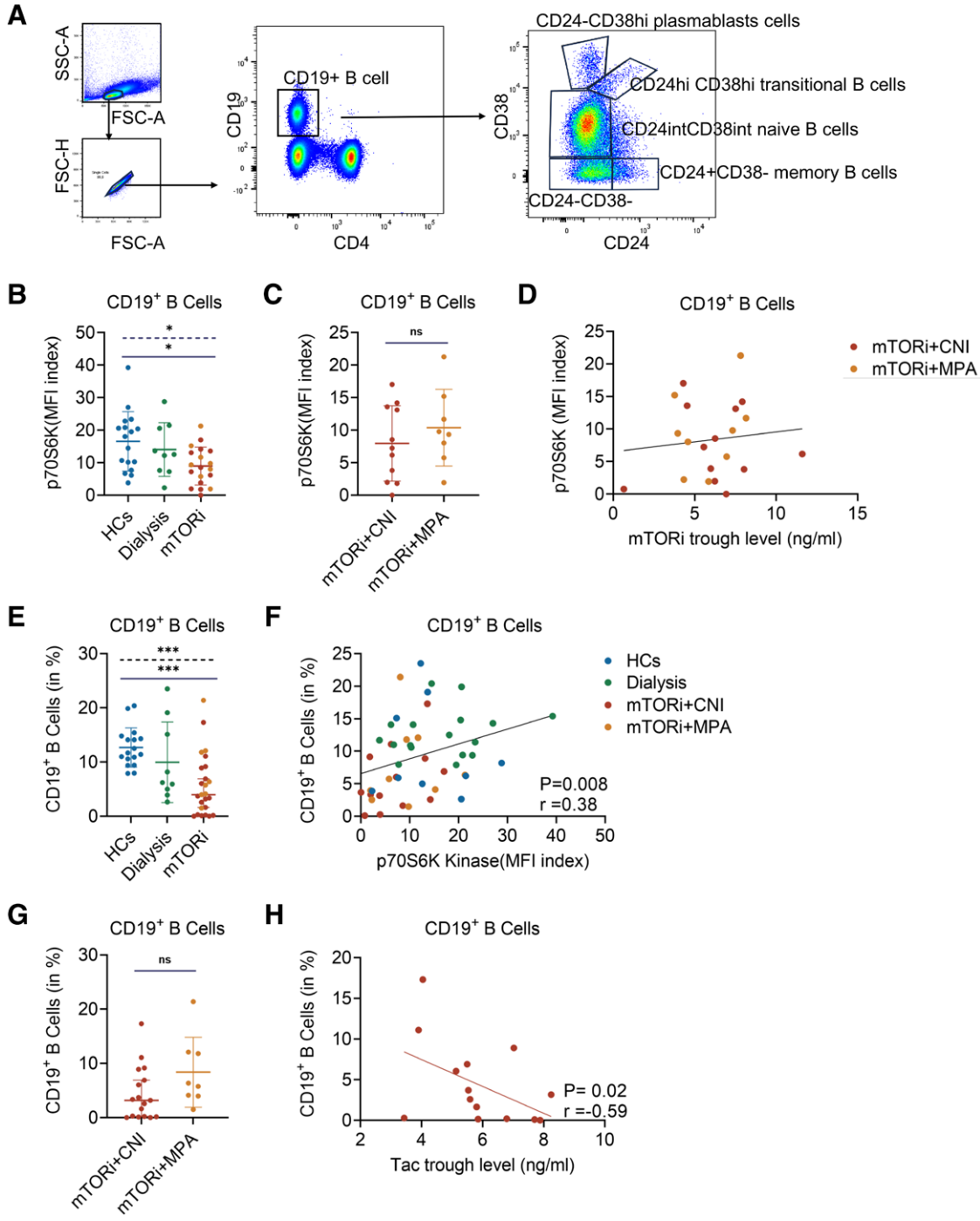

FIGURE 5. p70S6K phosphorylation level and frequency of CD19⁺ B-cell subset. A, Gating strategy for CD19⁺B-cell subsets. B and C, The scatter plots show the phosphorylation levels of p70S6K in CD19⁺ B cells. D, There was no correlation between p70S6K phosphorylation of CD19⁺ B cells and mTORi trough levels in renal transplant patients. E, The frequencies of CD19⁺ B-cell subsets among HC subjects, dialysis patients, and renal transplant patients receiving mTORi-based immunosuppression. F, Frequencies of CD19⁺ B cells positively correlate with their p70S6k phosphorylation levels. G, Frequencies of CD19⁺ B-cell subsets did not differ between the mTORi+CNI and the mTORi+MPA group. H, The frequencies of CD19⁺ B cells negatively correlate with the Tac trough levels after transplantation. *$P < 0.05$, ***$P < 0.001$; The dashed lines represent the ANOVA results for comparisons among 3 groups, whereas the solid lines represent the results of multiple comparison tests between 2 groups. CNI, calcineurin inhibitor; HC, healthy control; MPA, mycophenolic acid; mTORi, mammalian target of rapamycin inhibitor; Tac, tacrolimus.

When focusing on allograft rejection events among patients receiving a mTORi-based treatment, we found a significantly stronger p70S6K phosphorylation in the rejection group (n = 8), in $CD24^{-}CD38^{hi}$ plasmablasts compared with the non-rejection group ($P = 0.01$, **Figure S4, SDC,** http://links.lww.com/TXD/A672). However, no statistically significant differences were observed in p70S6K phosphorylation in other B and T lymphocyte subsets between the rejection and nonrejection groups (Figure S5 http://links.lww.com/TXD/A672).

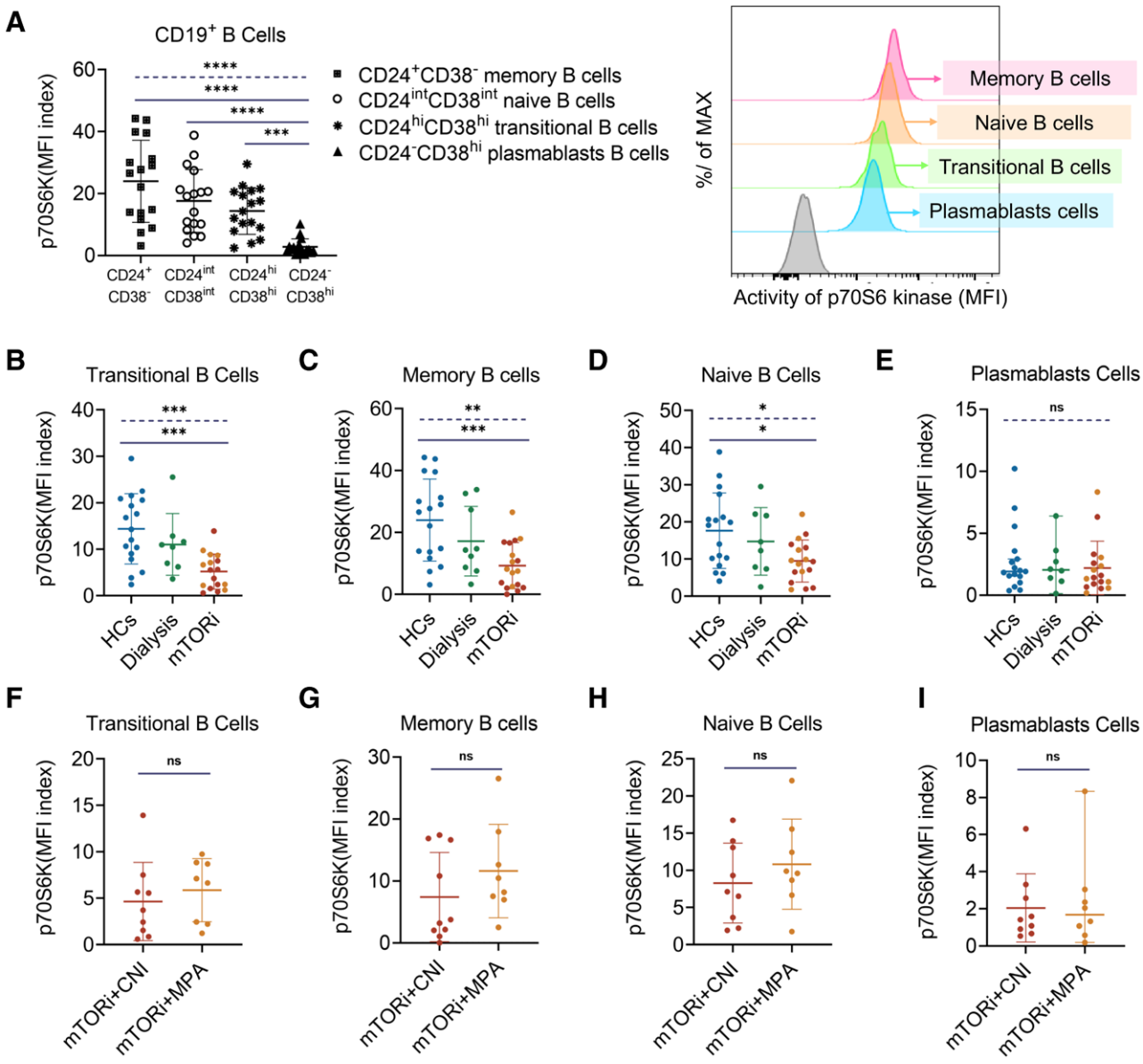

FIGURE 6. Phosphorylation levels of p70S6K in transitional B cells, memory B cells, naive B cells, and plasmablasts. A, The scatter plot and histograms depict the p70S6K phosphorylation levels of B-cell subtypes among HC subjects. Note that especially transitional B cells and plasmablasts exhibit a physiologically reduced p70S6K phosphorylation. B–E, The phosphorylation levels of p70S6K in $CD24^{hi}CD38^{hi}$ transitional B cells (B), $CD24^{+}CD38^{-}$ memory B cells (C), $CD24^{int}CD38^{int}$-naive B cells (D), and $CD24^{-}CD38^{hi}$ plasmablasts (E) are shown. F–I, Phosphorylation levels of p70S6K in $CD24^{hi}CD38^{hi}$ transitional B cells (F), $CD24^{+}CD38^{-}$ memory B cells (G), $CD24^{int}CD38^{int}$-naive B cells (H) and $CD24^{-}CD38^{hi}$ plasmablasts (I) did not differ in respective comparisons of mTORi+CNI and the mTORi+MPA groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; The dashed lines represent the ANOVA results for comparisons among 3 groups, whereas the solid lines represent the results of multiple comparison tests between 2 groups. CNI, calcineurin inhibitor; HC, healthy control; MPA, mycophenolic acid; mTORi, mammalian target of rapamycin inhibitor.

p70S6K Phosphorylation Was Not Associated With CMV or BK Virus Infections

Among all post-transplant patients receiving the mTORi-based treatment, 9 patients experienced a CMV infection. We found no difference of p70S6K phosphorylation in B and T cells between the CMV (n = 9) and non-CMV (n = 18) groups (**Figure S6, SDC,** http://links.lww.com/TXD/A672).

Furthermore, 11 of all posttransplant recipients undergoing an mTORi-based therapy experienced a BK virus infection. Again, no significant differences of p70S6K phosphorylation were observed in B and T cells between the BK (n = 11) and the non-BK (n = 16) virus groups (**Figure S7, SDC,** http://links.lww.com/TXD/A672). This indicates that the p70S6K phosphorylation had neither a promoting nor an inhibiting impact on the likelihood to acquire a CMV or BK virus infection in mTORi-treated transplant recipients.

DISCUSSION

mTORi treatment play a pivotal role in immunosuppression after kidney transplantation.[3] As p70S6K is downstream of mTOR, it offers a reliable target for the detection of effects of mTORi on immune cell activity.[14] In this study, we found a selective inhibition of p70S6K phosphorylation in lymphocyte subsets upon mTORi-based immunosuppression. Assessing p70S6K phosphorylation by phosphoflow cytometry may thus

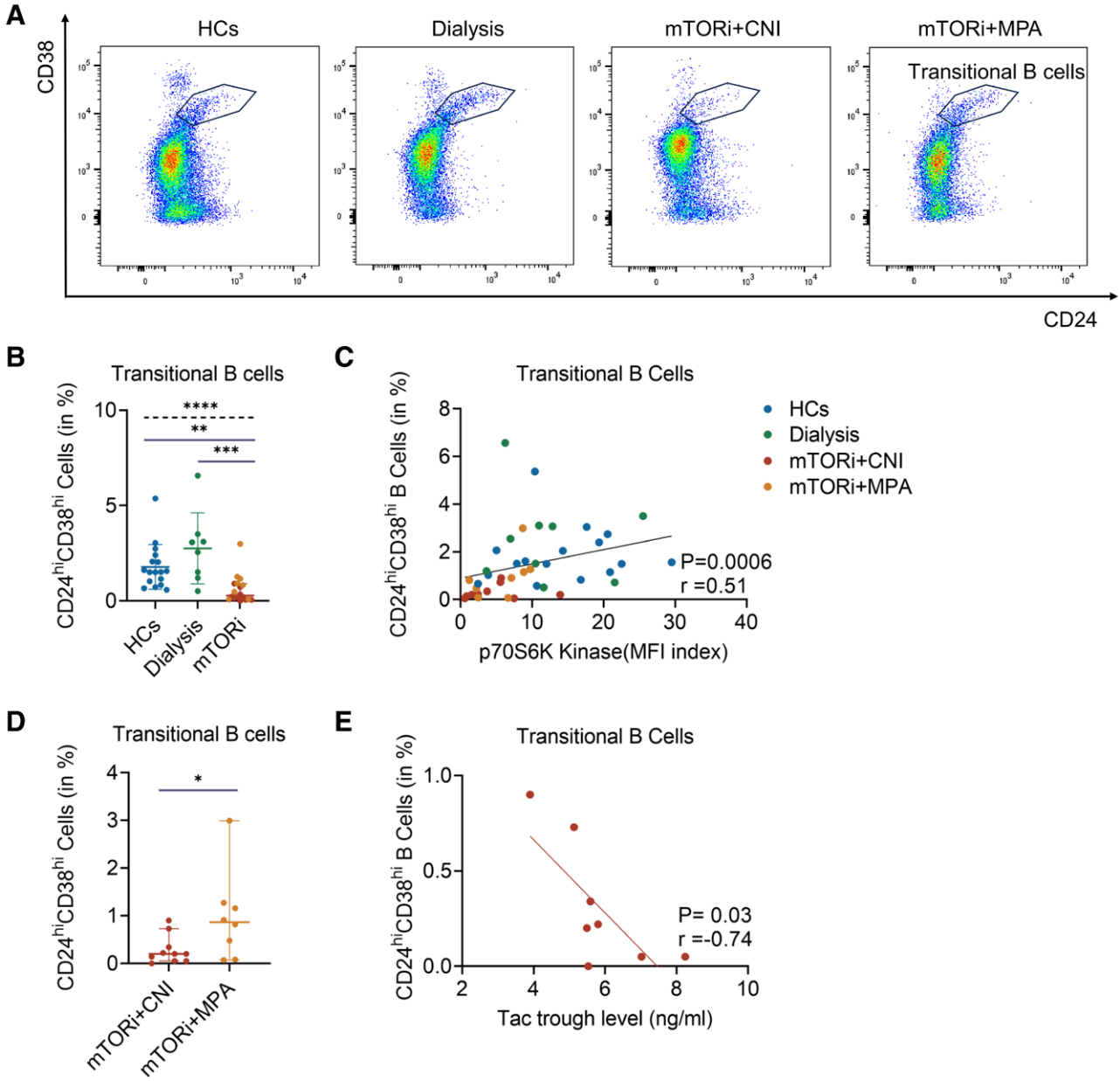

FIGURE 7. Frequency of CD24^{hi}CD38^{hi} transitional B cells is impaired in mTORi receiving transplant patients. A, Gating strategy for analysis of CD24^{hi}CD38^{hi} transitional B cells. B, CD24^{hi}CD38^{hi} transitional B cells are reduced in patients receiving a mTORi-based immunosuppression and (C) frequencies of CD24^{hi}CD38^{hi} transitional B cells positively correlate with their p70S6k phosphorylation levels. D, Frequencies of CD24^{hi}CD38^{hi} transitional B cells are significantly reduced in the mTORi+CNI group cohort and (E) negatively correlate with Tac trough levels. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; The dashed lines represent the ANOVA results for comparisons among 3 groups, whereas the solid lines represent the results of multiple comparison tests between 2 groups. CNI, calcineurin inhibitor; HC, healthy control; MPA, mycophenolic acid; mTORi, mammalian target of rapamycin inhibitor; Tac, tacrolimus.

serve as approach to understand cell subset–specific effects of mTORi, providing detailed pharmacodynamic information to individualize mTORi therapy.

Our previous studies showed a reduced p70S6K phosphorylation in CD4$^+$CD25^{hi} Treg cells compared with CD4$^+$CD25$^-$ Th cells.[14] Here, we reaffirmed these results with additional Treg-specific markers, for example, the lack of CD127 expression (Figure 4).[15–17] mTOR signaling impacts Th cell homeostasis, activation, and differentiation,[13,18] whereas it controls differentiation and function of Treg.[18–20] These distinct roles of mTOR may be attributed to differential signaling pathway:[21] One is the differential response to interleukin (IL)-2R signaling as it triggers multiple pathways, including STAT5 and PI3K/Akt/mTOR pathways. Zeiser et al[22] found a predominant STAT5 phosphorylation rather than PI3K/Akt/mTOR activation by IL-2 in Treg cells, compared with Th cells. Specifically, in the presence of IL-2, mTORi enabled a constant activation and expansion of Treg via STAT5, whereas CD4 Th cells were impaired. Consistently, studies demonstrated inhibition of proliferation and induction of apoptosis of Th cells as well as a selectively expansion of Treg upon mTORi treatment in vivo or in vitro.[22–24] In line, we here demonstrated a suppression of p70S6K phosphorylation in CD4$^+$CD45RA$^-$CD25$^-$Th cells by mTORi, while CD4$^+$CD45RA$^-$CD25^{++}CD127$^-$Treg remained unaffected. The latter reflect their independence from mTOR signaling (Figure 4).[22] This finding further supports previous

findings of a selective inhibitory on specific T-cell subsets upon mTORi treatment, indicating a potential resistance of Treg to mTORi. This may also be attributed to PTEN (phosphatase and tensin homolog), a crucial negative regulator within the PI3K/Akt/mTOR pathway, and the FOXP3-regulated Pim 2 kinase.[23] A previous study[22] demonstrated a per se elevated expression of PTEN in Treg cells compared with Th cells. Conversely, targeted removal of PTEN enhances the sensitivity of Treg to mTORi by rapamycin in a mouse model of acute graft-versus-host disease.[22]

With respect to B cells, we observed a selective suppression of p70S6K phosphorylation by mTORi in transitional, memory, and naive B cells, whereas no difference was observed in plasmablasts (Figure 6). Studies on the mTORi effects on B cells, particularly plasmablasts and plasma B cells, are limited.[25] Hence, this might be important to better understand the production and prevention of DSAs after kidney transplantation upon mTORi-based immunosuppression.[26–28] Notably, p70S6K phosphorylation in plasmablasts was significantly stronger in the rejection group compared with the nonrejection group (**Figure S4, SDC,** http://links.lww.com/TXD/A672). However, further investigation is needed to determine the effect of mTORi on plasmablast maturation and function, the clinical effect of co-immunosuppression with CNI/MPA in preventing humoral allograft rejection, and basically whether the distinct responses of different B cell subsets to mTORi are due to the differences in their PTEN expression.

Cherneha et al[29] identified a negative correlation of tacrolimus serum levels and p70S6K phosphorylation in $CD4^+$T cells of kidney transplant patients undergoing CNI- or mTORi-based treatments, indicating a dose-dependent inhibition on p70S6K phosphorylation by Tacrolimus. In this study, we found a synergy of mTORi and CNI for a reduction of p70S6K phosphorylation in Th cells (Figure 4) and $CD8^+$ T cells (**Figure S1, SDC,** http://links.lww.com/TXD/A672), when compared with mTORi+MPA. This implies an additional inhibition of the mTOR pathway by CNI in Th cells and $CD8^+$ T cells, thus augmenting the inhibitory effect of mTORi. In addition, rapamycin and tacrolimus are chemically similar and interact both with the common immunophilin FKBP, which is pivotal role for the efficacy of both drugs.[30–32] Previous studies employing Rat insulinoma INS-1β cells demonstrated the viability of the Tac/FKBP12/mTOR complex by docking and immunoprecipitation experiments. These findings suggest the potential of Tacrolimus to inhibit the mTOR pathway by binding to FKBP12.[33] Subsequently, this study unveiled that Tacrolimus significantly suppressed the mTOR pathway in INS-1β-cells, evident in decreased mTOR, p70S6K, and S6 phosphorylation.[33] This may contribute to the synergistic effect of CNI with mTORi to reduce p70S6K phosphorylation. Alternatively, CNI treatment might reduce IL-2 production, crucial for $CD4^+$ and $CD8^+$ T-cell activity and function, due to the inhibition of NFAT (nuclear factor of activated T cells).[34] IL-2 is also crucial for activating select signaling pathways, including the PI3K-Akt-mTOR pathway, resulting in an increase of mTOR activity.[35] Therefore, mTORi+CNI therapy may suppress the PI3K-Akt-mTOR pathway by inhibiting the production of IL-2, resulting in decreased p70S6K phosphorylation.

Currently, mTORi dose adjustment after solid organ transplantation relies on assessing trough levels.[10] However, our data revealed no correlation between mTORi serum trough levels and p70S6K phosphorylation in $CD4^+$ T cells (Figure 3), $CD8^+$ T cells (**Figure S1, SDC,** http://links.lww.com/TXD/A672), and B cells (Figure 5). Similar findings have been reported by Maxim et al and our group.[14,29] This lack of correlation may be attributed to individual variations in drug sensitivity, suggesting that mTORi trough levels may not adequately reflect the individual response or drug impact on the immune response due to interindividual variability in pharmacokinetics results.

A sufficient inhibition of p70S6K phosphorylation and thus adequate mTOR inhibition should impede lymphocyte development. Accordingly, there was a significant correlation of immune cell subset frequencies with p70S6K phosphorylation in kidney transplant recipients, including $CD4^+$ T cells (Figure 3), $CD8^+$ T_{mem} cells (**Figure S1, SDC,** http://links.lww.com/TXD/A672), B cells (Figure 5), and transitional B cells (Figure 7). Considering the close relationship between mTOR activity and its downstream target p70S6K, we believe that p70S6K phosphorylation is a promising parameter for providing valuable additional information on pharmacodynamic effects of mTORi.[36] Phosphoflow cytometry as a multiparametric approach allows for the simultaneous detection of phosphorylation status of signaling molecules in various cell types on a single-cell level.[37–39] Therefore it is the preferable method to determine phosphorylation status at Thr389 of p70S6K as less cells than for Western blots are needed and, as demonstrated here, the p70S6K phosphorylation is dependent on the immune cell type investigated. Of note, there was no significant difference in p70S6K phosphorylation among all investigated T- and B-cell subsets between HCs and patients with dialysis. This suggests that the phosphoflow cytometry method is less influenced by nondrug factors, such as patients' biochemical conditions, making it a stable and reliable tool for clinical routine applications.

Studies demonstrates that CMV and BKV are strongly dependent on mTOR pathway activation and have developed mechanisms to guarantee mTOR activity.[40–42] Therefore, we investigated whether alterations in p70S6K phosphorylation upon mTORi therapy affects CMV and BKV infections. Our study revealed no significant differences in p70S6K phosphorylation in B and T lymphocyte subsets from CMV and non-CMV infected individuals (**Figure S6, SDC**, http://links.lww.com/TXD/A672) or from BKV- and non-BKV-infected patients (**Figure S7, SDC**, http://links.lww.com/TXD/A672). This suggests that p70S6K phosphorylation neither enhanced nor reduced the likelihood to acquire a CMV or BKV infection.

Our study has some limitations. The sample size is relatively small and a cross-sectional design was used. Thus, the patient groups were not randomized, making it challenging to eliminate potential confounding factors that might have affected our results. For instance, the time from kidney transplant surgery to sampling was significantly shorter in the mTORi+CNI compared with the mTORi+MPA group as CNI+MPA represents the standard initial immunosuppressive treatment during the first months and years after kidney transplantation. Although the correlation analysis indicated no significant difference between phosphorylation of p70S6K among the investigated immune cell subsets and time to sampling (**Figures S8 and S9, SDC,** http://links.lww.com/TXD/A672), this may still diminish our ability to interpret intergroup differences. Second, the cross-sectional design constrained the consistency of the groups concerning other treatments, such as prednisolone. However, there was no statistically significant difference in prednisolone use and dosage between these 2 groups ($P = 0.14$, Table 1). Further standardization and interlaboratory cross-validation, as well as randomized controlled

studies, are necessary to better evaluate the clinical applicability of this method in independent cohorts of multicenter transplant clinical trials.

In summary, our data demonstrate that mTORi not only selectively reduces p70S6K phosphorylation in Th cells, transitional, memory, and naive B cells but also impacts their frequencies in peripheral blood. These findings provide insights into the potential modulation of the immune system by mTORi-based immunosuppression after kidney transplantation. The assessment of p70S6K phosphorylation may hence serve as an approach providing pharmacologic and pharmacodynamic information to enable an individual tailoring of mTORi therapy.

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