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# Oscillatory mTOR inhibition and $T_{reg}$ increase in kidney transplantation

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## Summary

**Intracellular metabolic pathways dependent upon the mammalian target of rapamycin (mTOR) play a key role in immune-tolerance control. In this study, we focused on long-term mTOR-dependent immune-modulating effects in kidney transplant recipients undergoing conversion from calcineurin inhibitors (CNI) to mTOR inhibitors (everolimus) in a 1-year follow-up. The conversion to everolimus is associated with a decrease of neutrophils and of $CD8^+$ T cells. In addition, we observed a reduced production of interferon (IFN)-γ by $CD8^+$ T cells and of interleukin (IL)-17 by $CD4^+$ T lymphocytes. An increase in $CD4^+CD25^+$ forkhead box protein 3 $(FoxP3)^+$ [regulatory T cell [($T_{reg}$)] numbers was also seen. $T_{reg}$ increase correlated with a higher proliferation rate of this regulatory subpopulation when compared with the $CD4^+FoxP3^-$ effector counterpart. Basal phosphorylation level of S6 kinase, a major mTOR-dependent molecular target, was substantially maintained in patients treated with everolimus. Moreover, oscillations in serum concentration of everolimus were associated with changes in basal and activation-dependent S6 kinase phosphorylation of $CD4^+$ and $CD8^+$ T cells. Indeed, T cell receptor (TCR) triggering was observed to induce significantly higher S6 kinase phosphorylation in the presence of lower everolimus serum concentrations. These results unveil the complex mTOR-dependent immune-metabolic network leading to long-term immune-modulation and might have relevance for novel therapeutic settings in kidney transplants.**

**Keywords:** everolimus, immunosuppression, kidney transplants, mTOR

## Introduction

Calcineurin inhibitors (CNI) are the standard treatment in kidney transplantation [1,2]. Chronic use of CNI is associated with graft dysfunction, increased risk of cardiovascular disorders and malignancies [3,4]. The key challenge in the management of renal transplants is to reduce adverse effects while maintaining a low acute rejection rate. New therapeutic protocols have been proposed [5,6], aimed at limiting CNI use from early post-transplant phases up to their discontinuation. Inhibitors of mammalian target of rapamycin (mTOR) have been found to represent a viable alternative to CNI [7–9].

  

The serine/threonine kinase mTOR, which belongs to the phosphatidylinositol kinase-related kinase (PIKK) family, regulates cell growth and metabolism in response to environmental cues [10]. The mTOR structure provides binding sites for multiple proteins that regulate its activity. It associates with *raptor* and *rictor* to form mammalian target of rapamycin complex 1 (mTORC1) and mTORC2, respectively, with different regulatory activities [10].

Survival of a transplanted kidney is mainly dependent upon immune tolerance *versus* allogeneic cells [11]. Immune-regulatory systems include accessory signals intrinsic to antigen recognition and those mediated by suppressor subsets, represented primarily by $CD4^+CD25^+$ regulatory T cells ($T_{reg}$) expressing the forkhead box protein 3 (FoxP3) transcription factor [12–14].

Cross-talk between immune response and metabolism is still largely undefined. Defective mTOR activity severely impairs T helper type 1 (Th1), Th2 and Th17 cell differentiation [15,16] and induces the $T_{reg}$ [17,18]. $T_{reg}$ availability and proliferation depend specifically upon mTOR oscillatory activity [19], while FoxO1, a major transcriptional regulator of CD8 differentiation, also depends upon mTORC1 [20].

The mTORC1 inhibitor everolimus, a synthetic derivative of rapamycin, shows high oral bioavailability, stability and solubility [9,21]. Beyond its use as immunosuppressor, everolimus has been approved for treatment of solid [22,23] and haematological malignancies [24]. Its dosage is higher (by six to 10-fold) in an oncology setting than in transplantation [22,23,25]. The difference in both the dosage and administration schedule of the drug could be relevant to induce immune tolerance rather than inhibition of cancer cell growth. In order to address this issue, we investigated the immune profile of kidney-transplanted patients undergoing conversion from CNI to everolimus. We performed an *ex-vivo* analysis of leucocyte number, T cell cytokine profile, $T_{reg}$ number and proliferation, as well as evaluation of the major mTOR-dependent molecular pathway (S6 kinase) in $CD4^+$ and $CD8^+$ T cells before (T0) and after drug conversion, throughout a 1-year follow-up (T12). A better understanding of the mTOR-dependent immune metabolic network is expected to favour manipulation of specific adaptive effectors, hopefully improving the survival of functional graft in kidney transplant recipients.

## Material and methods

### Study population

The study was carried out on 19 renal transplant recipients, all first transplant from cadaver donors. Inclusion criteria were aged 18–65 years; transplant vintage $> 3$ years; plasma creatinine $< 2$ mg/dl, with stable estimated glomerular filtration rate (eGFR) in the previous 3 months; haemoglobin value $> 10$ g/dl; white cell count $> 3000/\mu l$ (neutrophils $> 1500/\mu l$); platelets $> 75{\cdot}000/\mu l$; and absence of rejection signs or infectious episodes in the previous 3 months.

Exclusion criteria included previous or combined transplantation; panel-reactive antibodies (PRA) $> 25\%$ and/or the presence of donor-specific antibodies (DSA) at transplantation; the presence of proteinuria exceeding 300 mg/day on 24-h samples; hyperlipidaemia (baseline cholesterol and/or tryglicerides values exceeding 220 and 200 mg/dl, respectively); and evidence of autoimmune diseases or of viral infections.

### Study protocol

At baseline (T0), dosage of CNI was reduced empirically by 50% and everolimus was introduced at a starting dosage of 0·50 mg/twice a day (b.i.d.). This initial dose of everolimus, lower than that (0·75 mg/b.i.d.) suggested by the ZEUS study [26], was chosen because of the difference in the clinical features of the cohort we enrolled, characterized by a long transplant vintage, stable renal function and no immunological/infectious problems in the 3 months preceding enrolment. Plasma levels of both drugs were checked after 1 week, and everolimus dosage was modified opportunely to reach trough levels (TL) of 5–8 ng/ml (with further dose modifications, if necessary). After a 4-week stabilization period, CNI dose was reduced further by 25% and finally withdrawn (within the fourth month), whereas everolimus TL were increased up to 6–10 ng/ml. After 6 months all the patients were on everolimus alone; they were evaluated again at 1 year from baseline (T12). Dosage of steroids was never altered throughout the study. Six of the enrolled patients continued mycophenolic acid (MFA) co-treatment that was associated with everolimus. These patients, whose immune-modulating regimen included MFA co-administration, were analysed independently throughout the study.

At each study step (T0 and T12), all the patients were scheduled in clinical visits; samples were withdrawn to determine the main laboratory data, including TL of immunosuppressive drugs.

The study, conducted in agreement with good clinical practice guidelines, was approved by the Ethics Committee of Federico II University of Naples (protocol number: 66/11). All the procedures were in accordance with the Declaration of Helsinki, as revised in 2008. All the patients signed their informed consent to the study. Twelve healthy blood donors, age- and sex-matched with the patients, were enrolled into the study as controls.

### Immunofluorescence, cell sorting and T cell activation

Blood samples were analysed by immunofluorescence and flow cytometry by using a two-laser equipped fluorescence activated cell sorter (FACS)Calibur apparatus and CellQuest analysis software (Becton Dickinson, San Jose, CA, USA). Fluorescein isothiocyanate (FITC), phycoerythrin (PE),

cychrome and allophycocyanin (APC)-labelled monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD56, invariant natural killer T (NKTi), CD25, FoxP3, Ki67, interferon (IFN)-γ, interleukin (IL)-4, IL-17 and isotype-matched controls were purchased from Becton Dickinson. APC-labelled anti-phospho S6 kinase mAb was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA).

To analyse cytokine production, peripheral blood mononuclear cells (PBMC) were cultured overnight in the presence of phorbol myristate acetate (PMA), ionomycin and brefeldin-A (Sigma-Aldrich, St Louis, MO, USA), as described previously [27].

All phenotypes referred to flow cytometry analysis of the lymphocyte population gated using forward- (FSC) and side-scatter (SSC) parameters. Intracellular cytokine profile, FoxP3, Ki67 and phospho S6 kinase staining were performed with a fixation-permeabilization buffer (Becton Dickinson), following the manufacturer's instructions.

$CD4^+$ and $CD8^+$ T cells were sorted by FACSJazz (Becton-Dickinson). To mimic antigen-dependent T cell activation, sorted $CD4^+$ and $CD8^+$ cells were incubated for 1 h with anti-CD3/anti-CD28 mAb-coupled microbeads (Life Technologies AS, Oslo, Norway) at the cell/bead ratio of 1 : 0.2, as described previously [18].

To evaluate some possible oscillation in the results, two independent samples obtained for each patient at T0 and T12 were analysed at 1-week intervals and produced substantially comparable results (not shown).

### Molecular signalling analysis

Independent total cell lysates, obtained from $CD4^+$ and $CD8^+$ sorted T cells, were incubated as indicated previously, and 30 μg of total proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions; proteins were transferred onto a nitrocellulose filter membrane (Protan; Schleicher & Schuell, Dassel, Germany) with a Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA, USA). Filters were then incubated with specific mAbs (anti-phospho-S6 Ser240/244 and anti-S6 5G10 clone, from Cell Signaling Technology; anti-extracellular-regulated kinase (ERK)1/2 (clone H72; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and with a peroxidase-conjugated secondary antibody (Amersham Biosciences, Picaraway, NJ, USA). Peroxidase activity was detected with the enhanced chemiluminescence (ECL) system (Amersham Biosciences) or Femto (Pierce, Rockford, IL, USA). Normalization was performed against total ERK1/2. All filters were quantified by band densitometry analysis using the ScionImage version 1·63 software (Scion Corporation, Frederick, MD, USA).

### Statistical analysis

Statistical evaluation of the data using InStat version 3·0 software (GraphPad Software Inc., San Diego, CA, USA), was performed by Mann–Whitney test or Wilcoxon's matched-pairs signed-rank test, as indicated. Two-sided *P*-values less than 0·05 were considered significant. The corrected *P*-value ($P_c$) was calculated by applying Bonferroni adjustment for multiple comparisons, as described previously [28].

## Results

### The clinical evaluation of patients during the study

The demographic data of patients are reported in Table 1. One patient left the study after the sixth month because of the occurrence of an acute antibody-mediated rejection. The main laboratory data are presented in Table 2. Cholesterol was higher at T12 than at T0 (+19·8%; $P < 0{\cdot}001$) and haemoglobin concentration was lower at T12 than at T0 (−6%; $P < 0{\cdot}001$). No modification was detected in urinary protein excretion. Glomerular filtration rate was increased slightly, although not significantly, after CNI withdrawal.

The mean everolimus TL, effective in maintaining adequate immunosuppression, were at the lower step of the desired range (6–10 ng/ml). The six enrolled patients receiving CNI/MFA also continued MFA co-administration after everolimus conversion. No dose adjustment in statins or in erythropoiesis-stimulating factors was performed during the study. All the patients remained under 4 mg/day of methylprednisolone.

Side effects after the everolimus switch were mild and transient (headache, pruritus, joint pain). One patient complained of persisting aphthous stomatitis, requiring therapy and a small reduction of the everolimus dosage.

### Effect of conversion from CNI to everolimus on leucocyte number and cytokine production profile

As shown in Fig. 1a, the conversion from CNI to everolimus was able to reduce the number of total leucocytes to T12 ($7099 \pm 476$) compared to T0 ($8443 \pm 690$). This reduction trend was observed specifically in patients undergoing everolimus treatment without MFA association ($9457 \pm 770$ at T0 *versus* $7261 \pm 628$ at T12; $P_c < 0{\cdot}05$).

**Table 1.** Demographic and laboratory data of the patients enrolled into the study.

| Patients ($n = 19$) | |
|---|---|
| Sex (M/F) | 13/6 |
| Age (years) | $49{\cdot}6 \pm 11{\cdot}7$ |
| Weight (kg) | $74{\cdot}1 \pm 13{\cdot}2$ |
| Transplant vintage (years) | $3{\cdot}5 \pm 1{\cdot}0$ |
| CNI (Cya) | 19/19 |
| MPA derivatives (yes/no) | 6/13 |
| Anti-hypertensive drugs (*n*) | $2{\cdot}3 \pm 0{\cdot}8$ |
| Statins (yes/no) | 14/5 |

CNI = calcineurin inhibitors; Cya = cyclosporin; MPA = mycophenolic acid; M/F = male/female.

**Table 2.** Main laboratory data throughout the study.

| | T0 | T6 | T12 |
|---|---|---|---|
| eGFR (m/min) | 65·9 ± 20·1 | 70·3 ± 20·8 | 72·3 ± 27·6 |
| Plasma creatinine (mg/dl) | 1·30 ± 0·47 | 1·32 ± 0·83 | 1·27 ± 0·47 |
| Plasma haemoglobin (g/dl) | 13·8 ± 1·4 | 13·3 ± 1·5 | 13·0 ± 1·7* |
| Plasma albumin (g/dl) | 4·7 ± 0·3 | 4·50 ± 0·3 | 4·6 ± 0·3 |
| Plasma cholesterol (mg/dl) | 176·2 ± 29·3 | 211·6 ± 45·3 | 211·3 ± 38·4* |
| Plasma triglycerides (mg/dl) | 126·0 ± 58·6 | 134·3 ± 54·0 | 141·2 ± 58·8 |
| 24-h urinary protein excretion (g) | 0·10 ± 0·13 | 0·19 ± 0·24 | 0·18 ± 0·24 |
| Everolimus trough levels (ng/ml) | 0 | 6·47 ± 3·4 | 7·0 ± 2·1 |

T0 = baseline (under calcineurin inhibitors); T6 = 6 months from baseline (under everolimus); T12 = 12 months from baseline (under everolimus); eGFR = glomerular filtration rate (MDRD equation). *Indicates significant difference from T0 value.

Similar behaviour was seen for neutrophils (Fig. 1b). Indeed, the significant reduction observed at T12 (6192 ± 509 at T0 *versus* 3958 ± 406 at T12; $P_c < 0{\cdot}05$) specifically involved the subgroup of patients treated with everolimus without MFA (5776 ± 660 at T0 *versus* 3833 ± 596 at T12; $P_c < 0{\cdot}05$). Analysis of $CD8^+$ T cell number (Fig. 1d) also revealed a significant reduction of this cell subset in patients receiving the everolimus treatment without MFA (743 ± 135 at T0 *versus* 471 ± 58 at T12; $P_c < 0{\cdot}05$). No differences were observed in total lymphocyte count (Fig. 1c) or in $CD3^+CD4^+$ T cell number (data not shown) when comparing T0 *versus* T12 data. Percentage analysis confirmed this trend (data not shown).

Therefore, long-term everolimus-dependent mTORC1 inhibition reduced significantly the number of neutrophils and CD8 T cells in kidney transplant recipients. These changes were hampered by MFA co-administration.

To evaluate the effect of everolimus conversion on cytokine production, we analysed IFN-γ, IL-4 and IL-17 in NKTi lymphocytes, a major player in cytokine profile polarization [29]. IFN-γ in $CD8^+$ T cells and IFN-γ and IL-17 in $CD4^+$ T lymphocytes were also evaluated. Notably, NKTi lymphocyte numbers in controls were similar to those in patients, regardless of their immunosuppressive treatment (data not shown). Comparison with healthy controls has been included in order to underline, when present, the persistent reduced cytokine production observed in our patient cohort after conversion.

As shown in Fig. 2a, no significant changes in IFN-γ production were observed after the conversion from CNI to everolimus. Indeed, the percentage of IFN-γ-producing NKTi cells remained significantly lower in patients than in healthy donors both at T0 (12·68 ± 2·65 at T0 *versus* 22·79 ± 1·25 in controls; $P_c < 0{\cdot}05$) as well as at T12 (6·96 ± 1·74; $P_c < 0{\cdot}005$ *versus* controls), regardless of their immune-modulating regimen.

In contrast, the high IL-4 production in MFA-CNI-treated patients at T0 (21·51 ± 8·35 *versus* 5·51 ± 0·90 in controls; $P_c < 0{\cdot}005$) was reduced at T12, so that it became similar to healthy donors (Fig. 2b).

The frequency of IL-17-producing NKTi cells reduced significantly compared to controls at T0, and remained substantially unchanged at T12 (Fig. 2c).

The production of IFN-γ by $CD8^+$ T cells (Fig. 2d) decreased significantly after the everolimus conversion (30·88 ± 5·12 at T0 *versus* 9·14 ± 1·76 at T12; $P_c < 0{\cdot}001$),

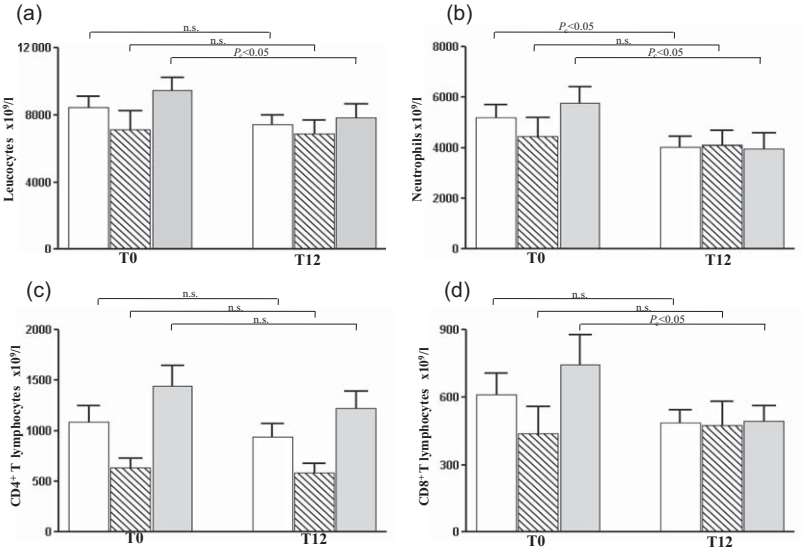


**Fig. 1.** Leucocytes, neutrophils and T cell subsets in kidney transplanted patients undergoing calcineurin inhibitors (CNI) to everolimus conversion. (a–d) Evaluation of leucocytes, neutrophils, CD4 and CD8 T cell subsets as indicated at T0 before therapy conversion and T12 1 year after everolimus conversion. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Wilcoxon's matched-pairs signed-rank test is reported. The corrected *P*-value ($P_c$) was calculated by applying Bonferroni adjustment for multiple comparisons.

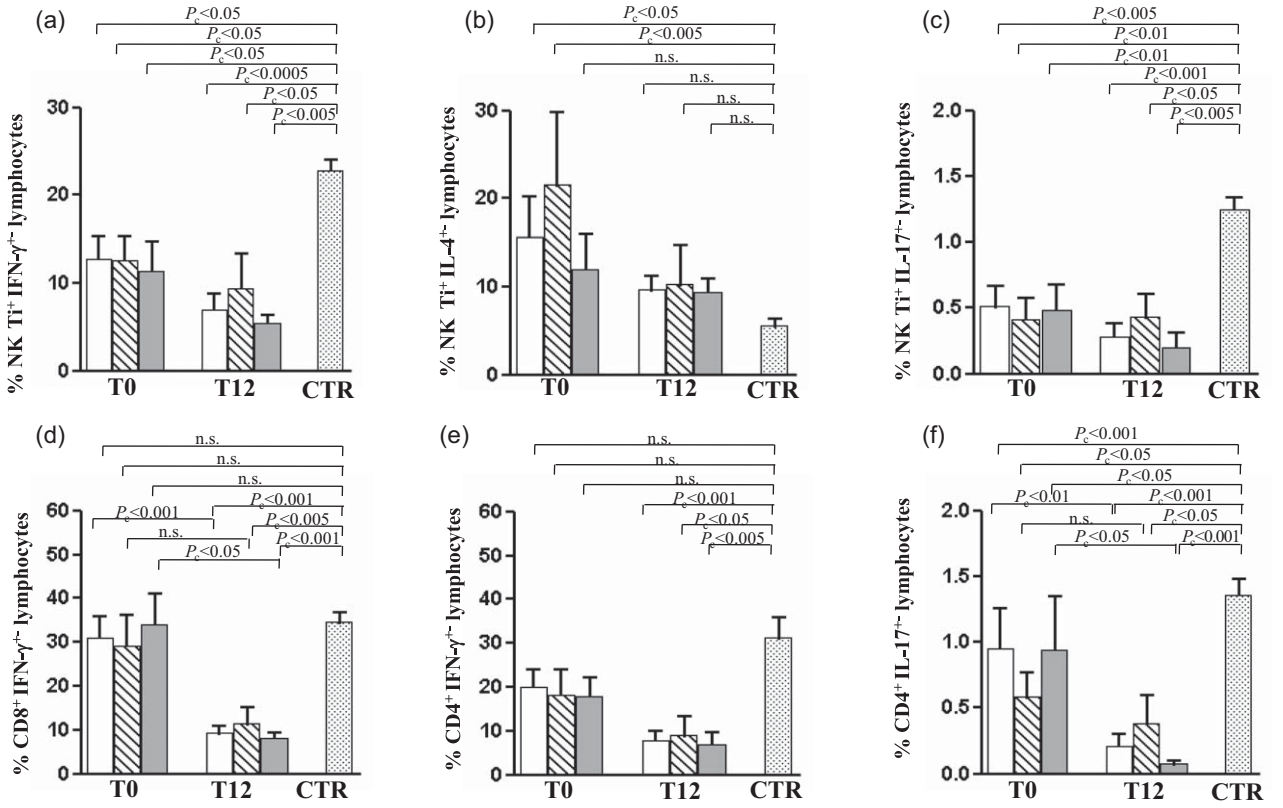


**Fig. 2.** Cytokine secretion profile of invariant natural killer T (NKTi), $CD8^+$ T and $CD4^+$ T lymphocytes in kidney-transplanted patients undergoing conversion from calcineurin inhibitors (CNI) to everolimus. (a–c) Interferon (IFN)-γ, interleukin (IL)-4 and IL-4 production by NKTi cells at T0 and T12 after CNI to everolimus conversion. (d) IFN-γ production in $CD8^+$ T cells and (e,f) IFN-γ and IL-17 production by $CD4^+$ lymphocytes at T0 and T12. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Dotted columns indicate data obtained in healthy controls (CTR). Mann–Whitney test is reported. For T0–T12 comparison of paired samples, Wilcoxon's matched-pairs signed-rank test has been performed. The corrected *P*-value ($P_c$) was calculated by applying Bonferroni adjustment for multiple comparisons.

significantly lower than controls at T12 ($P_c < 0{\cdot}001$). Notably, the reduction trend was observed to involve preferentially the group of patients whose treatment did not include MFA association ($34{\cdot}06 \pm 7{\cdot}01$ at T0 *versus* $8{\cdot}08 \pm 1{\cdot}25$ at T12; $P_c < 0{\cdot}05$). Therefore, MFA co-administration specifically hampered everolimus-dependent modulation of IFN-γ production in $CD8^+$ T lymphocytes. This observation suggests the relevance for mTOR-dependent mechanisms in proinflammatory cytokine production by $CD8^+$ T lymphocytes.

As shown in Fig. 2e, the production of IFN-γ by $CD4^+$ T cells was significantly lower than controls at T12 ($7{\cdot}70 \pm 2{\cdot}32$ *versus* $31{\cdot}10 \pm 4{\cdot}75$; $P_c < 0{\cdot}001$), regardless of the immune-modulating regimen.

IL-17 producing $CD4^+$ T lymphocytes (Fig. 2f) were reduced significantly in patients than in controls at T0 ($0{\cdot}95 \pm 0{\cdot}29$ *versus* $1{\cdot}36 \pm 0{\cdot}12$; $P_c < 0{\cdot}001$) and at T12 ($0{\cdot}2 \pm 0{\cdot}09$; $P_c < 0{\cdot}001$). Moreover, comparison of T0 and T12 values in paired samples revealed that everolimus treatment without MFA co-administration was able to mediate complete inhibition of IL-17 producing $CD4^+$ T cells ($0{\cdot}94 \pm 0{\cdot}4$ at T0 *versus* $0{\cdot}086 \pm 0{\cdot}02$ at T12; $P_c < 0{\cdot}05$). Thus, MFA association seemed to affect significantly everolimus-dependent modulation of IL-17 production by $CD4^+$ T lymphocytes.

No change was observed in NK-dependent IFN-γ production between controls and patients, regardless of their immunosuppressive treatment (not shown).

### $T_{reg}$ numbers and proliferation in kidney transplant recipients undergoing everolimus conversion

Because mTOR-dependent pathways affect $T_{reg}$ homeostasis [17–19], we analysed the effect of everolimus conversion on number and suppressor activity of $T_{reg}$ in our patients. No difference in the $T_{reg}$ suppressor activity was observed in any of the patients, regardless of the treatment group (not shown). As shown in Fig. 3, the comparison of paired samples revealed a significant increase of $T_{reg}$ after CNI to everolimus conversion ($89 \pm 16{\cdot}5$ at T0 *versus* $129{\cdot}9 \pm 22{\cdot}87$ at T12; $P_c < 0{\cdot}05$) only in those patients

  

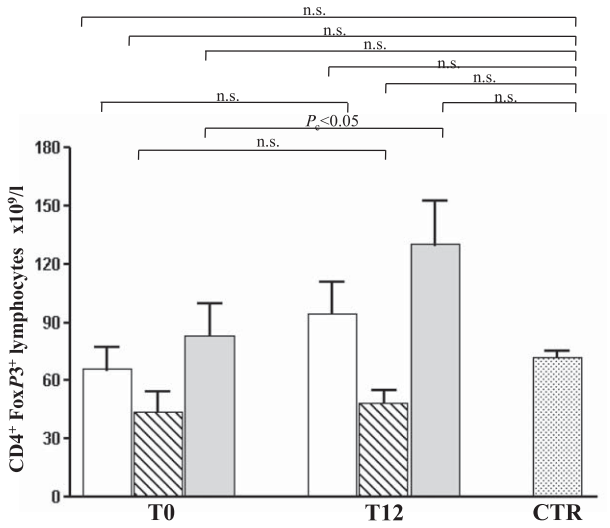


**Fig. 3.** Regulatory T cell ($T_{reg}$) population in kidney-transplanted patients following calcineurin inhibitors (CNI) to everolimus conversion. Number of $T_{reg}$, gated as $CD4^+$forkhead box P3 (FoxP3)$^+$ T cells at T0 and T12 were reported. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Dotted columns indicate data obtained in healthy controls (CTR). For comparison with CTR the Mann–Whitney test is reported. For T0–T12, comparison of paired-samples Wilcoxon's matched-pairs signed-rank test was performed. The corrected *P*-value ($P_c$) was calculated by applying Bonferroni adjustment for multiple comparisons.

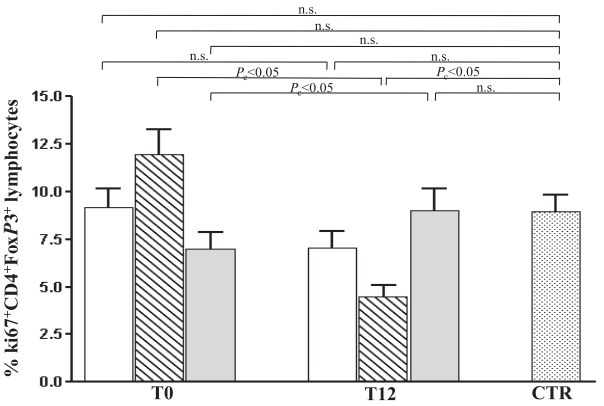


**Fig. 4.** Ki67 expression on regulatory T cells ($T_{reg}$) gated as $CD4^+$forkhead box P3 (Foxp3)$^+$ T cells in kidney-transplanted patients. Percentage is given for Ki67 expression in $T_{reg}$, gated as $CD4^+FoxP3^-$ T cells, at T0 and T12 after calcineurin inhibitors (CNI) to everolimus conversion. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Dotted columns indicate data obtained in healthy controls (CTR). For comparison with CTR, the Mann–Whitney test is reported. For T0–T12, comparison of paired-samples Wilcoxon's matched-pairs signed-rank test was performed. The corrected *P*-value ($P_c$) was calculated by applying Bonferroni adjustment for multiple comparisons.

whose treatment did not include MFA association. Percentage data confirmed this trend (not shown). Notably, $T_{reg}$ numbers always remained similar to controls.

To investigate the growth ability of $T_{reg}$, we analysed their *ex-vivo* expression of the proliferation marker Ki67 [30]. As shown in Fig. 4, CNI to everolimus conversion associated with a significant increase of Ki67 expression on the $T_{reg}$ population (6·99 ± 0·89 at T0 *versus* 8·99 ± 1.15 at T12; $P_c < 0{\cdot}05$). Conversely, everolimus-MFA co-treatment was observed to mediate reduction of this proliferation marker in the $T_{reg}$ subset (11.43 ± 1·23 at T0 *versus* 4·53 ± 0·52 at T12; $P_c < 0{\cdot}05$). Therefore, MFA association hampered the positive effect of everolimus significantly on $T_{reg}$ growth ability. As already stated, no difference in $T_{reg}$ suppressor activity was observed between T0 and T12, regardless of the immunosuppressive treatment of the patients (not shown).

As the growth ability of T cell effectors is crucial for T cell activation, we also analysed the *ex-vivo* Ki67 expression of $CD4^+FoxP3^-$ T cells supposed to include preferentially the CD4 effector population. As shown in Fig. 5, Ki67 expression in $CD4^+FoxP3^-$ T lymphocytes was significantly higher in T0 patients than in controls (7·07 ± 0·83 *versus* 1·26 ± 0·12; $P_c < 0{\cdot}0005$), regardless of the immune-modulating regimen. A significant decrease in growing $CD4^+FoxP3^-$ T cells was observed at T12 (2·81 ± 0·47; $P_c < 0{\cdot}01$ *versus* T0; NS *versus* controls). This decreased trend was observed to be very strong in T12 patients treated with MFA–everolimus association [7·32 ± 0·94 at T0 *versus* 1·74 ± 0·33 at T12; $P_c < 0{\cdot}05$; not significant (n.s.) *versus* controls]. Indeed, the reduced level of Ki67 expression in $CD4^+FoxP3^-$ T cells of T12 patients treated with everolimus alone remained significantly higher than in controls (6·57 ± 1·30 at T0 *versus* 3·48 ± 0·66 at T12; $P_c < 0{\cdot}05$; $P_c < 0{\cdot}01$ *versus* controls). The MFA–everolimus association was observed to mediate a strong decrease of cell growth in both $T_{reg}$ and $CD4^+FoxP3^-$ T cells. Conversely, a preferential effect on $T_{reg}$ growth ability was observed in everolimus-treated patients. Notably, the CNI–everolimus conversion always restored the physiological difference in growing ability between $T_{reg}$ and T cell effector subset, lost at T0 in our cohort.

### Everolimus serum concentration associates with different levels of mTOR-dependent S6 kinase phosphorylation in CD4 and CD8 T cells of kidney transplant recipients

To investigate the molecular mechanisms underlying immune modulation in kidney transplant recipients shifted from CNI to everolimus, we analysed the phosphorylation

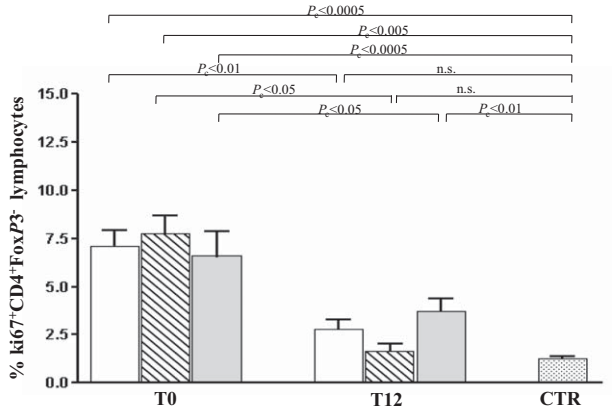


**Fig. 5.** Ki67 expression on $CD4^+$forkhead box P3 (Foxp3)$^-$ T cells in kidney-transplanted patients. Percentage is given for Ki67 expression in $CD4^+$ T cell effectors, gated as $CD4^+$FoxP3$^-$ T cells at T0 and T12 after calcineurin inhibitors (CNI) to everolimus conversion. Data refer to mean ± standard error of the mean (s.e.m.). White columns indicate data obtained in all the patients enrolled into the study ($n = 18$); striped columns indicate patients whose treatment included mycophenolic acid (MFA) co-administration ($n = 6$). Grey columns indicate patients treated with immune-modulating regimens not including MFA ($n = 12$). Dotted columns indicate data obtained in healthy controls (CTR). For comparison with CTR, the Mann–Whitney test is reported. For T0–T12, comparison of paired-samples Wilcoxon's matched-pairs signed-rank test was performed. The corrected *P*-value ($P_c$) was calculated by applying Bonferroni adjustment for multiple comparisons.

of S6 kinase as a major downstream target of mTORC1 activity [10]. In order to avoid MFA-dependent interference, we analysed only samples obtained from patients undergoing immune-modulating regimens not including MFA administration.

To ascertain whether phosphorylation of mTOR-dependent targets might be conditioned by drug serum concentration, we evaluated S6 kinase phosphorylation (p-S6) levels in $CD4^+$ and $CD8^+$ T cells of T12 patients after 3 (T12–3 h) and 12 h (T12–12 h) from everolimus administration, reflecting maximal and minimal TL of the drug (data not shown). T cell receptor (TCR) triggering was mimicked by incubation with anti-CD3/anti-CD28 beads, as reported [18]. Figure 6 shows Western blot analysis of sorted $CD4^+$ and $CD8^+$ T cells after a 1-h culture in the presence of medium alone or with anti-CD3/anti-CD28 beads. As shown, no difference was observed in p-S6 levels in medium-cultured samples obtained from T0 and T12 patients. Conversely, a tremendous increase of TCR-dependent p-S6 up-regulation was observed in the samples obtained from T12 patients after 12 h from everolimus administration (minimal drug serum concentration) in both the $CD4^+$ and $CD8^+$ T cells (Fig. 6a,b). As shown in Fig. 6c,d, strongly reduced pS6 up-regulation was observed upon TCR triggering in the samples obtained from the same patients 3 h after everolimus administration (maximal drug serum concentration). It should be noted that *ex-vivo* TCR triggering of the cells was always performed in the absence of the drug.

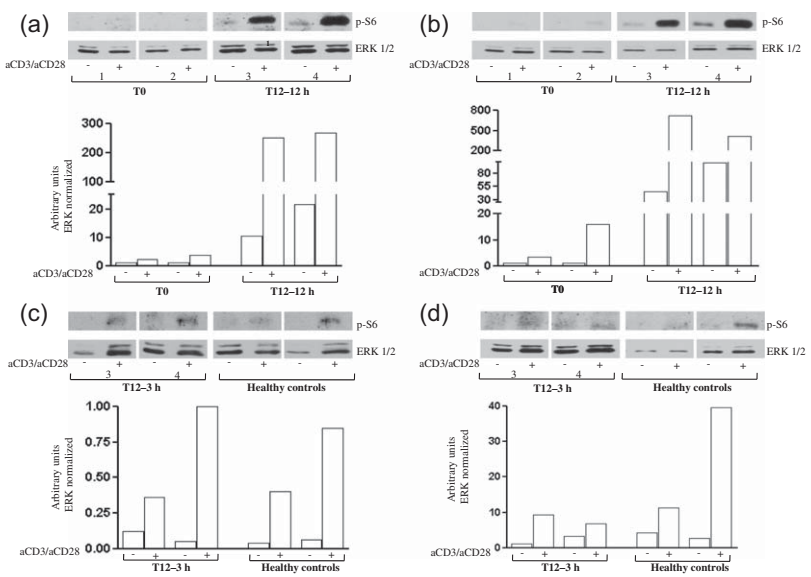


**Fig. 6.** Analysis of S6 kinase phosphorylation level as a measure of mammalian target of rapamycin complex 1 (mTORC1) activity in $CD4^+$ and $CD8^+$ T cells after calcineurin inhibitors (CNI) to everolimus conversion. (a–d) Comparative analysis of phospho-S6 kinase in all four patients analysed. As indicated, samples from T12 patients were obtained 12 h (T12–12 h) after everolimus administration (minimal drug TL) and 3 h (T12–3 h) after drug administration (maximal drug TL). Western blot analysis for p-S6 kinase from protein lysates of sorted $CD4^+$ and $CD8^+$ T cells from two representative T0 and T12 patients are reported; as indicated, data refer to 1 h culture with medium or anti-CD3/anti-CD28 monoclonal antibody (mAb)-coupled microbeads. As shown, a tremendous increase of pS6 levels was observed in T12–12 h patients after T cell receptor (TCR) triggering. As indicated (c,d) comparable up-regulation of pS6 was observed in T12–3 h patients and controls. Arabic numbers (1–4) identify samples obtained from single patients. Data are representative of two concordant experiments.

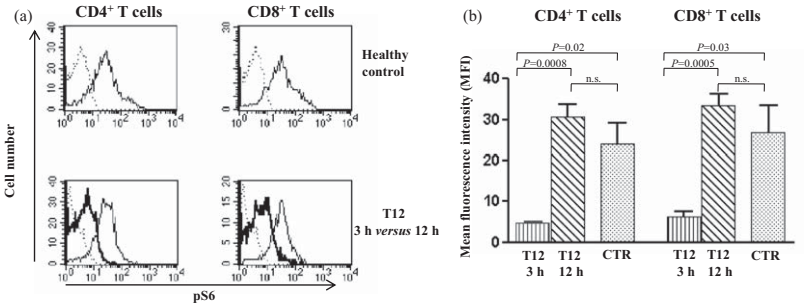


**Fig. 7.** p-S6 kinase analysis 3 and 12 h after everolimus administration in T12 patients. (a) Flow cytometry comparative evaluation of p-S6 kinase levels in $CD4^+$ and $CD8^+$ T cells obtained from one representative T12 patient 3 h and 12 h after everolimus administration (T12–3 h and T12–12 h) and in one healthy control. As indicated, upper histogram plots in (a) show the p-S6 kinase level in $CD4^+$ and $CD8^+$ T cells of one healthy donor (plane line); isotype matched control binding is indicated as dashed line. Lower histogram plots in (a) refer to the p-S6 kinase evaluation in cells of one representative patient 3 h (bold line) and 12 h (plane line) after everolimus administration; dashed line indicates isotype matched control binding. (b) Comparative analysis of p-S6 phosphorylation levels of $CD4^+$ and $CD8^+$ T cells after 3 h (vertical depicted column) and 12 h (oblique depicted column) in all four T12 patients analysed. Dotted columns indicate healthy controls (CTR). Statistical analysis was performed by Mann–Whitney test.

Comparative analysis by immune fluorescence of basal p-S6 levels in $CD4^+$ and $CD8^+$ T cells from samples obtained from T12 patients 3 and 12 h after everolimus administration (highest and lowest drug TL, respectively) confirmed the occurrence of an oscillatory inhibition of mTORC1 kinase activity (Fig. 7a,b). Indeed, a significant decrease of p-S6 kinase was observed in $CD4^+$ and $CD8^+$ T cells obtained after 3 h, compared with those obtained after 12 h from everolimus administration ($P < 0{\cdot}001$). Moreover, p-S6 kinase levels were significantly lower than controls in samples after 3 h ($P < 0{\cdot}05$), but not after 12 h from everolimus (Fig. 7a,b).

Thus, no significant difference in basal S6 phosphorylation levels was mediated by CNI compared with the everolimus-based immune-modulating regimen. Moreover, changes in everolimus serum concentration, due probably to the dosage and administration schedule of the drug, were observed to associate with oscillatory basal and TCR-dependent activation of mTORC1 kinase in both $CD4^+$ and $CD8^+$ T cells. This behaviour might be of particular relevance in the presence of chronic TCR stimulation, as represented by the allotransplantation setting.

## Discussion

mTOR targeting was proposed as an immunosuppressor to limit CNI adverse effects in renal transplant recipients [5,6]. This condition represents a unique opportunity to study the effect of long-term mTOR inhibition therapy in a transplantation model. With this purpose in mind, we focused on mTOR-dependent immune modulation, also addressing the hypothesis that targeting the immune metabolic network could optimize clinical manipulation of specific adaptive immune effectors.

Our data indicate that an immune-modulating regimen based on everolimus administration was associated specifically with a significant decrease in leucocyte and neutrophil numbers. This effect might be accounted for by mTOR-dependent effects on cellular survival, migration and proliferation, as well as by activation of the CD11b/CD18 complex, which alters granulocyte adhesion to endothelial cells [31–34].

Neutrophils play a key role in inflammation [35]. They release proinflammatory, angiogenic and anti-inflammatory mediators and can interfere with the development of intimal hyperplasia and transplant vasculopathy [36–38]. *Ex-vivo* everolimus treatment of isolated neutrophils mediates inhibition of their vascular endothelial growth factor (VEGF) and IL-8 release, also increasing the anti-inflammatory IL-1RA [34]. Therefore, the ability of everolimus to specifically modulate such a population might be of some relevance to optimize clinical management of kidney transplants.mTOR has been described to play a key role in regulating antigen-independent proliferation of $CD8^+$ T cells [39] and in maintaining homeostasis of effector lymphocytes [40–42]; accordingly, CNI to everolimus conversion was observed to mediate a significant reduction of $CD8^+$ T cells in our cohort. The MFA–everolimus association was revealed to hamper such effects significantly.

mTORC1 is a central regulator of adaptive immunity [43,44]. It affects Th1 and Th17, while mTORC2 is required for Th2 differentiation [15–18]. Our data confirm such effects, showing that everolimus-based immune-modulating therapy is able to decrease the proinflammatory activity of adaptive effectors significantly in kidney transplant recipients. Accordingly, CNI to everolimus conversion maintained a reduced proinflammatory activity (IFN-γ and IL-17 production) in the absence of significant modification of IL-4 secretion by NKTi cells, the key regulators of cytokine polarization [29]. Moreover, everolimus treatment modulates specifically IFN-γ-producing $CD8^+$ and $CD4^+$ T cells, while maintaining the decreased IL-17

production by $CD4^+$ T lymphocytes from patients treated with CNI. These effects seemed to be favoured by immune-modulating protocols not including MFA administration (Fig.2a,f). Moreover, the inhibition of IFN-γ production by $CD8^+$ T cells without effect on NK effectors confirms the role of mTORC1-dependent pathways in T cell response [17,44] and $CD8^+$ T cell homeostasis regulation [39–42]. The major role of mTOR in regulating FoxO1-dependent $CD8^+$ T cell differentiation [20] might also account for such effects.

A number of data have suggested the critical involvement of $T_{reg}$-dependent immune modulation in mTOR inhibition-based immunosuppression regimens [17,45]. Moreover, both $T_{reg}$-hampering [46,47] as well as enhancing activity [48] has been referred for MFA-based treatment. Here, we describe that $T_{reg}$ increase, coupled with significant effects on their growth rate, characterizes everolimus-based immunosuppression in kidney transplant recipients. Moreover, opposite effects on Ki67 expression were observed by mTORC1 inhibition alone (T12 patients treated with everolimus without MFA association) compared with immune-modulating regimens, including MFA/everolimus co-administration. The analysis of the growing ability of $CD4^+Foxp3^-$ T cell subset, probably representing the effector T lymphocyte population, allowed better characterization of MFA and everolimus-dependent effects in our cohort. As shown, a preferential effect on growing lymphocytes, regardless of the subset to which they belong, has been observed for MFA co-administration, while a specific increase of Ki67 expression in the $T_{reg}$ subset, coupled with a significant modulation of $Ki67^+CD4^+FoxP3^-$ T cells, was observed to characterize mTORC1 inhibition. Specific amplification of $T_{reg}$ cells able to modulate alloreactive T cell effectors might be hypothesized to account for everolimus-dependent immune-suppression in kidney transplant recipients. Conversely, preferential modulation of growing immune effectors represents a key element for immunosuppression regimens, including MFA administration. Moreover, mTOR-dependent immune-modulating effects are hampered by MFA co-administration. Thus, the inhibition of purine biosynthesis pathways, key targets of MFA, has been observed to severely impair mTOR-dependent immune modulation.

We observed that changes in everolimus serum levels correlate with oscillation in basal and activation-dependent phosphorylation of S6 kinase, a major target of mTORC1 kinase. As expected in a transplantation setting, continuous antigen stimulation is an activation trigger for T cells. Our data indicate that mTOR inhibition therapy is able to mediate significant oscillation, probably depending on drug serum concentration, in the mTOR kinase activity. This effect relates to a significant increase of $T_{reg}$ numbers and growing ability, and decreases Ki67 expression by the $CD4^+FoxP3^-$ counterpart.

The possibility that the effects observed on $T_{reg}$ and T cell effector homeostasis might affect selected cell clones preferentially, probably involved in alloantigen recognition, needs to be investigated. In this context, our data confirm and extend our previous *in-vitro* observations indicating that oscillatory inhibition of mTOR activity induces robust proliferation of TCR triggered $T_{reg}$, also inhibiting T cell effector growth [18,19]. Thus, a key role for $T_{reg}$ modulation coupled with a decrease of neutrophils and $CD8^+$ T cells and reduced proinflammatory activity might be hypothesized to underlie mTOR inhibition in a transplantation setting.

Two opposite therapeutic indications are currently proposed for everolimus: immunosuppression [8,9,21] and cancer control [22,23,25]. In this context, we might hypothesize the possibility that dosage level (lower by six to 10-fold than in the oncology setting) and administration schedule (twice *versus* once a day in cancer therapy) could represent a therapeutic strategy to regulate mTOR-dependent intracellular pathways differentially and target immune tolerance or cancer control preferentially.

Taken together, our results shed light on the complex mTOR-dependent immune metabolic network, and propose that oscillatory inhibition of TCR-dependent mTORC1 activity might represent a therapeutic strategy to optimize targeted manipulation of specific adaptive effectors in kidney transplant recipients.

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## Author contribution

A. T. P., V. R., M. G. and G. M. participated in the design, interpretation and analysis of data as well as in writing the paper; S. T. and L. A. participated in the clinical management of the patients and in acquisition and evaluation of data; M. S. and A. G. participated in acquisition and evaluation of data and contributed to paper writing; M. S., G. R. and G. T. planned, directed and co-ordinated the research and revised the paper. M. S. also co-ordinated the clinical management of patients.

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