Differential regulation of Tregs and Th17/Th1 cells by a sirolimus-based regimen might be dependent on STAT-signaling in renal transplant recipients☆

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ABSTRACT

Background: Sirolimus (SRL), a mammalian target of rapamycin inhibitor, has been used as a de novo base therapy with steroids and mycophenolate mofetil to avoid the use of calcineurin inhibitors. Our aim was to determine whether immunoregulation is promoted after conversion from tacrolimus (TAC) to SRL.
Methods: The study included 24 renal transplant recipients who converted from TAC to SRL therapy and 24 normal controls. The frequency of T helper (Th) cells and the presence of signal transducer and activator of transcription (STAT) proteins in peripheral blood were analyzed by flow cytometry before conversion and at 3 and 6 months after conversion. Plasma levels of interleukin (IL)-1β, interferon-γ (IFN-γ), IL-17, IL-6, and IL-10 were analyzed by the Bio-Plex® suspension array system before and at 3 months after conversion.
Results: Renal transplant recipients who switched to SRL showed a significant increase in regulatory T cell (Treg) frequencies and better renal function compared with preconversion ($P < 0.05$). The plasma concentrations of inflammatory cytokines IL-1β, IL-6, IL-17, and IFN-γ were significantly decreased after conversion to SRL. Furthermore, recipients who switched to SRL showed an increase in STAT5 activation and a decrease in STAT3 activation compared with the TAC group.
Conclusion: Our results indicate that conversion to SRL might both minimize calcineurin inhibitor toxicity and promote immune tolerance.

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1. Introduction

Lifelong immunosuppression is generally required after renal transplantation. Immunosuppressive regimens based on calcineurin inhibitors (CNIs) such as tacrolimus (TAC) have significantly reduced acute rejection after transplantation. However, long-term survival is still very low because CNIs induce various complications, including renal function impairment, neurotoxicity, de novo diabetes mellitus and hypertension.

Early conversion from a CNI to a mammalian target of rapamycin (mTOR) inhibitor is one of the immunosuppressive strategies that have been investigated to prevent progression in chronic kidney disease following organ transplantation, providing an alternative immunosuppression regimen [1–3].

Recently, many studies have reported that interleukin (IL)-2 plays an important role in maintaining immune tolerance by promoting regulatory T cells (Tregs) and negatively regulating T helper (Th)17 cells. Laurence et al. [4] concluded that IL-2 promotes the generation of Tregs and inhibition of Th17 cell polarization by the transcription factor signal transducer and activator of transcription (STAT) 5, whereas TAC hinders immunoregulation by inhibiting IL-2 production. Our previous study [5] showed that a CNI inhibits IL-2, which is associated with a Th17/Treg imbalance in the peripheral blood and underlies subsequent renal dysfunction after renal transplantation.

In contrast, the mTOR inhibitor sirolimus (SRL) might facilitate immunoregulation without blocking IL-2 production by increasing Treg percentages [6–10]. In recent studies, Delgoffe [11,12] demonstrated that mTOR promotes the differentiation of inflammatory Th1 and Th17 cells. As an inhibitor of mTOR, whether SRL can suppress the production of inflammatory cells and increase Tregs in renal transplant recipients is not well understood. We hypothesized that SRL can antagonize the induction of Th1 and Th17 cells and skew cells down the Treg pathway, and thus may have potential immunoregulatory effects and reduce chronic allograft dysfunction compared with a TAC-based regimen in renal transplant recipients.

2. Materials and methods

2.1. Renal transplant recipients

This study was approved by the Ethics Committees of the Chinese Human Genome and West China Hospital. Written informed consent was obtained from all recipients. We included 24 first kidney transplant recipients who had converted from a TAC-based regimen to an SRL-based regimen because of clinical indications at West China Hospital. All participants had received a living related donor transplant. Before conversion, a triple immunosuppression regimen consisting of TAC, mycophenolate mofetil (MMF), and steroids was administered to the 24 recipients for more than half a year. The TAC dose was adjusted to maintain a trough blood concentration of between 5 and 10 ng/ml. MMF was administered at 750 mg twice daily. The steroid regimen was tapered from 10 to 20 mg per day until 3 months after transplantation and then reduced to a maintenance dose of 10 mg daily.

All recipients underwent CNI withdrawal in the first evening and SRL was then administered in the following morning at 2–4 mg/day. All 24 transplant recipients received an SRL-based regimen (SRL + MMF + steroids) for more than half a year. The SRL dose was adjusted to maintain a trough blood concentration of 5–10 ng/ml. MMF and steroid therapy was not changed during conversion. No recipient had undergone acute rejection. Twenty-four age and sex-matched healthy subjects were included as normal controls. The normal controls had not received any medical treatment.

The reasons for the recipient conversion from a TAC-based regimen to an SRL-based regimen were as follows: (i) progressive decline in renal function (increasing serum creatinine, 18 recipients, 75%; biopsy results showed that nine of these recipients had chronic allograft nephropathy); (ii) CNI toxicity (two recipients, 8.3%), as shown by renal allograft biopsy; and (iii) post-transplantation diabetes mellitus (four recipients, 16.7%). The demographic and clinical characteristics of the recipients and normal controls are listed in Table 1.

2.2. Cell preparation and Th cell analysis

Th cells were analyzed once before conversion and twice after conversion at 3 and 6 months respectively. Whole blood (500 μl) was cultured in complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum) for 5 h at 37 °C with 5% CO_2 in the presence of phorbol myristate acetate (PMA, 50 ng/ml), ionomycin (1 μg/ml), and monensin (1 μg/ml). To analyze Tregs, we collected 50 μl whole blood and aliquot into tubes for further staining.

For Th17 and Th1 cell analyses, 50 μl stimulated whole blood was incubated with PerCP-labeled anti-human CD3 (SK7) (BD Bioscience, San Diego, CA, USA) and fluorescein isothiocyanate (FITC)-labeled anti-human CD8 (SK2) (BD Bioscience) antibodies at 4 °C for 30 min. Because CD4-positive cells become negative after PMA stimulation, we used anti-human CD3 and CD8 antibodies for surface staining of Th17 cells and analyzed CD3 + CD8 − cells instead of CD3 + CD4 + cells [13,14]. For Treg analysis, 50 μl whole blood without stimulation was incubated with an FITC/allophycoerythrin (APC)-labeled anti-human CD4/CD25 cocktail (RPA-T4/BC96) (eBioscience, San Diego, CA, USA) at 4 °C for 30 min. After surface staining, the whole blood was stained with phycoerythrin (PE)-labeled anti-human IL-17A antibody (SCPL1362) (eBioscience) for Th17 cell detection, an APC-labeled anti-human interferon-γ (IFN-γ) antibody (eBioscience) for Th1 cell detection, or a PE-labeled anti-human Foxp3 antibody (PCH101) (eBioscience) for Treg detection following fixation and permeabilization according to the manufacturer's instructions. Isotype controls were included for correcting fluorescence compensation and confirmation of antibody specificity. Stained cells were assessed on a FACS Canto cytometer (BD Bioscience), and the data were analyzed using FACS Diva software (BD Bioscience).

2.3. Analysis of phosphorylated (p)-STAT5 (pY694) and p-STAT3 (pY705) in lysed whole blood

For p-STAT5 analysis, blood was collected in the presence of heparin, and 50 μl unstimulated whole blood was incubated with APC-labeled anti-human CD8 (BD Bioscience) and FITC-labeled anti-human CD4 (SK2) (BD Bioscience) antibodies at 4 °C for 15 min. The whole blood was then coped with 100 ng/ml recombinant human IL-2 (Cat. No. 554603) for 15 min at 37 °C and was protected from light. The samples were lysed and fixed with 1× BD™ Phosflow Lyse/Fix buffer (Cat. No. 558049) for 10 min at 37 °C and then permeabilized with BD™ Phosflow Perm Buffer III (Cat. No. 558050) on ice for 30 min. The cells were then washed twice in BD Pharmingen™ Stain Buffer (Cat. No. 554656) and stained with PE-labeled mouse anti-Stat5 (pY694) (BD Bioscience) and PerCP-labeled anti-human CD3 (SK7) antibodies. Stained cells were assessed on a FACS Canto II cytometer (BD Bioscience), and the data were analyzed using FACSDiva software.

For p-STAT3 analysis, blood was collected in the presence of heparin, and 50 μl unstimulated whole blood was incubated with APC-labeled anti-human CD8 and FITC-labeled anti-human CD4 (SK2) antibodies at 4 °C for 15 min. Then, the blood was stimulated with 100 ng/ml recombinant human IL-6 (MN 550071) for 15 min at 37 °C while

Table 1
Clinical and histological outcomes of preconversion and postconversion.

Clinical characteristics	Preconversion (TAC)	Postconversion (SRL)	Normal control
Age (years)	39(32–45)		37(29–48)
Sex (M/F)	17/7		17/7
Months on TAC/SRL therapy	8(6–16)	9(7–10)	/
Trough TAC/SRL level (ng/ml)	4.5(3.9–5.8)	5.9(3.5–8.3)	/
Fasting blood glucose (mmol/l)	4.81(4.60–5.20)	5.09(4.86–5.77)	4.51(4.12–5.41)
Systolic pressure (mm Hg)	134(126–147)	126(110–138)	112(95–125)
Diastolic pressure (mm Hg)	91(67–117)	87(65–106)	74(68–98)
ALT (IU/l)	18.5(14.0–25.8)[a]	26(18.0–37.5)[b]	19.5(19.0–28.0)
AST(IU/l)	23(16.3–26.0)[a]	25.5(20.5–36.2)[b]	16(16.8–26.5)
Cholesterol (mmol/l)	4.67(3.97–5.51)[a]	5.4(4.92–5.77)[b]	3.1(2.73–4.55)
Triglycerides (mmol/l)	1.23(0.98–2.14)	1.68(1.09–2.21)	1.14(0.56–2.23)
SCr (mg/dl)	1.67 (1.28–2.15) [ac]	1.37(1.26–1.76)	1.03(0.87–1.35)
Random proteinuria (g/l)	0.2(0.1–0.5)	0.3(0.2–0.7)	/
Platelet count (10^9/l)	226(156–347)	234(178–289)	180(169–256)
Chronic allograft nephropathy	9/20	8/20	/

Values are medians (range). TAC, tacrolimus. SRL, sirolimus. Alt, alanine aminotransferase. AST, aspartate aminotransferase. SCr, serum creatinine.

[a] P < 0.05 for TAC vs. SRL.
[b] Means SRL vs. normal control.
[c] P < 0.05 for TAC vs. normal control.