Th1, Th2, Th17 cell subsets in two different immunosuppressive protocols in renal allograft recipients (Sirolimus vs mycophenolate mofetil): A cohort study

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ABSTRACT

Long-term use of calcineurin inhibitors (CNI) is associated with nephrotoxicity, which is an important cause of renal dysfunction. Therefore, CNI-minimization strategies which decrease the CNI nephrotoxicity under the protection of additional immunosuppressant drugs have been developed. The aim of current cohort study was to compare the effect of two immunosuppressive protocols [tacrolimus (TAC) in combination with mycophenolate mofetil (MMF) and prednisolone (PRED) versus TAC in combination with sirolimus (SRL) and prednisolone] on the frequency of T helper cell subsets (Th1, Th2 and Th17 cells) and their associated cytokine (IFN-γ, IL-4 and IL-17A) levels in renal allograft recipients.

In this study, renal transplant recipients who received induction therapy (Antithymocyte globulin) and were also on triple immunosuppressive therapy were included and divided in to two groups: Group A was comprised 14 patients who received TAC, MMF and PERD whereas group B was composed of 10 patients who received TAC, SRL and PERD. The frequency of Th1, Th2 and Th17 cells in the peripheral blood mononuclear cells (PBMCs) of the patients was analyzed by flow cytometry before and 4 months after transplantation. In addition, IFN-γ, IL-4 and IL-17A concentrations in PBMC culture supernatants of patients before and 4 months after transplantation were quantified by ELISA.

The results of our study showed that TAC, MMF and PRED protocol did not diminish the frequency of Th17 cells at 4 months post-transplantation (5% ± 2.5) compared with pre-transplantation (2.3% ± 1; P < 0.05). However, Th1 (3.6% ± 1.5 pre-transplantation vs 2.2% ± 0.9 at 4 months post-transplantation; P < 0.05), Th2 (1.4% ± 0.3 pre-transplantation vs 0.8% ± 0.4 at 4 months post-transplantation; P < 0.05) cell subsets and IL-4 concentration (71.5 pg/ml ± 12 pre-transplantation vs 62.5 pg/ml ± 4.4 at 4 months post-transplantation; P < 0.05) were significantly decreased after transplantation in patients who had received SRL, TAC and PRED.

In conclusion, the data of the current study suggest that using reduced dose of TAC in SRL, TAC and PRED protocol is in favor of allograft survival; however a cohort study with larger sample size is needed for confirming our results.

1. Introduction

Recent improvement in immunosuppressive medications and therapy, make kidney transplantation as a standard treatment for end-stage renal disease (ESRD). However, these drugs cannot prevent the chronic rejection of the transplantation, and also prolonged use of these drugs has been associated with emergence of various types of malignancies and infections. Therefore avoiding long-term immunosuppression with the goal of achieving immunological tolerance can be considered as a final solution for long-term survival of allograft. One of the

Abbreviations: SRL, sirolimus; mTOR, mammalian target of rapamycin; CNI, calcineurin inhibitors; TAC, tacrolimus; PRED, prednisolone
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main obstacles in kidney transplantation is allograft-immune response by T helper cell subsets especially Th1 and Th17 and their associated inflammatory cytokines (i.e. IFN-γ and IL-17) through activation of endothelial cells, macrophages and Th2 cells and their related cytokine IL-4 through activation of B cells [1]. Until now, an Immunosuppressive regimen containing TAC, MMF and PRED has been widely used. This triple immunosuppressive regimen has been able to decrease the incidence of rejection rates down to 20% and increase the graft 1 year survival rate up to 90%. However, long-term graft survival (10 years) and graft function improvements are still low (below 60%) [2].

Immunosuppressive therapeutic protocols with TAC-based calcineurin inhibitors may result in serious side effects, as indicated by nephrotoxicity and subsequently fibrosis which could be led to long-term renal dysfunction/damage, malignancy, diabetes mellitus and hypertension in renal allograft patients [3]. Furthermore, TAC cannot suppress Th17 cells effectively since it is positively correlated with chronic allograft injury, fibrosis and chronic allograft dysfunction (CAD) development which subsequently lead to chronic rejection [4].

TAC is a macrolide antibiotic with the calcineurin inhibitor activity which can bind to calcineurin and inhibit its function resulting in the suppression of T cell activation. Through nuclear factor of activated T cells (NFAT) activation, calcineurin, a serine threonine phosphatase, mediates IL-2 production [5]. IL-2 is a fundamental cytokine that controls proliferation and differentiation of T cells to Th1 and Th2 but not Th17 cell subsets [6]. By inhibiting IL-2 synthesis, TAC blunts T cell differentiation to Th1 and Th2 cell subsets, however, this antibiotic may increase Th17 differentiation rate and it may, thereby, lead to increased risk of renal dysfunction and rejection [7].

It should be noted that calcineurin inhibitor (CNI) based-therapeutic regimens (e.g. Tacrolimus (TAC)), may significantly decrease the rate of acute rejection. Once long-term treatment with CNI is associated with some adverse side effects such as nephrotoxicity and vascular disease, therefore, an alternative immunosuppressive protocol containing mTOR inhibitors (e.g. Sirolimus (SRL)) and antimetabolite (e.g. Mycophenolate Mofetil (MMF)) are used in combination with CNI to minimize the CNI dose and hence its related adverse side effects. SRL, a non-nephrotoxic immunosuppressant not only can reduce differentiation of Th1 and Th17 cells but also, through suppressing mTOR (mammalian Target of rapamycin), can inhibit T cell proliferation [8,9]. However, it has been reported that patients who were in the second year of conversion from TAC to SRL have not shown significant decrease in CAD and an increased rate of acute rejection has been also evident [10–12]. Development of adverse side effects such as wound healing failure, hyperlipidemia and oral mucositis are the main cause of SRL withdrawal followed by using an alternative protocol [13]. The concomitant use of SRL and reduced-dose of TAC, instead of using TAC or SRL alone, can ameliorate TAC associated adverse events, while preserving therapeutic efficacy and safety [2], amplifying TAC’s immunosuppressive functions [14], lowering infection rates of BK virus (BKV), cytomegalovirus (CMV), and Epstein-Barr virus (EBV) and reducing malignant potential [15].

Although different clinical trials have shown that combined use of TAC/SRL decreases the acute rejection rate and increases the graft survival rate [16–18], to date, there has not been a report that aimed to study the effects of this protocol on immune cells subsets in vivo. Finally, in the present study, our aim was to evaluate the effect of two immunosuppressive protocols (TAC/MMF/PRED vs. TAC/SRL/PRED) on the frequency of T helper cell subsets (Th1, Th2 and Th17 cells) in peripheral blood and their associated cytokine (IFN-γ, IL-4 and IL-17A) levels in PBMC culture supernatant of patients before and four months after transplantation.

### Table 1
Demographic data of patients in the two studied groups.

<table>
<thead>
<tr>
<th></th>
<th>TAC/MMF/PRED (n = 14)</th>
<th>TAC/SRL/PRED (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34.58 ± 11.83</td>
<td>31.33 ± 6.67</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>9/5</td>
<td>6/4</td>
</tr>
<tr>
<td>Donor type (n)</td>
<td>Living donor 9</td>
<td>Deceased donor 5</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD. P < 0.05 considered as significant and is marked with an asterisk (*).

### Table 2
Clinical outcomes of two studied groups at 4 months after transplantation.

<table>
<thead>
<tr>
<th></th>
<th>TAC/MMF/PRED</th>
<th>TAC/SRL/PRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.7 ± 0.8</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>36.1 ± 12.9</td>
<td>39 ± 11.6</td>
</tr>
<tr>
<td>GFR (ml/min/1.73 m²)</td>
<td>51.6 ± 6.4</td>
<td>70.94 ± 5.7</td>
</tr>
<tr>
<td>FBS (mg/dl)</td>
<td>93.1 ± 12.6</td>
<td>96.8 ± 19.0</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>6.6 ± 1.9</td>
<td>5.3 ± 2.2</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>21.3 ± 9.2</td>
<td>22.8 ± 4.8</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>37.7 ± 22.1</td>
<td>50.2 ± 15.6</td>
</tr>
<tr>
<td>ALP (IU/l)</td>
<td>240.1 ± 79.6</td>
<td>245.4 ± 147.4</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>13.1 ± 1.8</td>
<td>13.2 ± 1.2</td>
</tr>
<tr>
<td>WBC (×10³/µl)</td>
<td>4.5 ± 1</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>WBC (×10⁵/µl)</td>
<td>6 ± 2</td>
<td>7 ± 2</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD. P < 0.05 considered as significant and is marked with an asterisk (*).
2.1. TAC/MMF /PRED (group A) TAC/SRL/PRED (group B) Isotype control

CD4+

2.2.2. SRL group

The initial dose of TAC in this group was 0.08 mg/kg/day, and target trough levels were 6–7 ng/ml during the first 6 months and 4–5 ng/ml afterward. The dose of Sirolimus was 2 mg for 96 h during the surgery and it was then decreased to 1 mg/day to reach a plasma level of 3–5 ng/ml in the first 6 months followed by an increase to reach plasma levels of 6–8 ng/ml.

2.3. Human PBMC isolation and cell culture

The peripheral blood mononuclear cells (PBMCs) were isolated from whole blood with Ficoll gradient (Inno-Train, Germany) according to the manufacturer's instructions. 1×10⁶ PBMCs were cultured in a complete culture medium including medium 1640 enriched by 10% heat inactivated fetal Bovine serum. PBMCs were then stimulated for 5 h with PMA (50 ng/ml) and ionomycin (1 μg/ml) and incubated at 37°C and 5% CO₂ atmosphere in the presence of monensin.

2.4. Intracellular staining and flow cytometric analysis

After PBMCs stimulation, the supernatant of the culture medium was collected and centrifuged at 250g at room temperature (RT) for 10 min and aliquoted in microtubes and stored at −70°C.

The cells were then washed with Staining Buffer (SB) containing PBS. The isolated PBMCs were frozen in a cryoprotective media containing 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS) and stored at −196°C.

For analysis of T helper cell subsets, after thawing of PBMCs, the cells were washed twice with phosphate-buffered saline (PBS) containing 0.3% fetal bovine serum.

To determine surface and intracellular markers, the cells were first stained with the following fluorochrome-conjugated monoclonal antibodies (all from BD Bioscience, USA).

For T helper cell subsets analysis, cells were fixed with 1 ml BD Cytofix Fixation Buffer, incubated for 10 min at RT followed by washing with SB. Cells were then permeabilized with BD Perm/Wash buffer 1x (900μL distilled water + 100μL BD Perm/Wash buffer 10×) and incubated at RT for 15 min followed by a centrifugation at 250g for
10 min. The fixed permeabilized cells, were incubated for 1 h with BD Pharmingen mAb cocktail containing surface anti-Human CD4 PerCP-Cy5.5 mAb (clone: SK3), intracellular mAbs including anti-Human IL-17A PE (clone: N49-653), anti-Human IFN-γ FITC (clone: B27) and anti-Human IL-4 APC (clone: MP4-25D2). Appropriate gating was conducted using suitable matched isotype control antibodies (BD Pharmingen three-color fluorescent Ig Isotype Cocktail with Human CD4+ PerCP-CyTM5.5). Flow cytometric data were obtained using a FACSCalibur flow cytometer system and analyzed by CellQuest software (BD Biosciences).

2.5. Cytokine assay

$1 \times 10^6$ PBMCs/ml were stimulated with PHA 1% in complete culture medium containing 10% FBS for 48 h. Next, supernatant was collected by spinning down of the cells at 500 g for 5 min. The cell free supernatant was aliquoted and stored at $-70 \, ^\circ\text{C}$. The concentrations of IFN-γ, IL-4, IL-17A were measured by sandwich ELISA according to manufacturer’s instructions (Tecan Trading AG, Switzerland).

2.6. Statistical analysis

The data were gathered in Microsoft Excel and the analysis was performed SPSS (Version 21, Chicago, IL, USA) software. Paired t-test was performed to compare cell frequency and cytokine changes before transplantation and four months after transplantation. ANCOVA analyses (analysis of covariance) were performed to comparing cell frequency and cytokine levels changes in two immunosuppressive treatment protocols, adjusting for baseline values. P values $< 0.05$ were considered statistically significant.

3. Result

3.1. Patients’ demographic data in two studied groups

Comparative assessments have shown that the distribution of gender, age and donor type was almost equal in two studied groups (Table 1).
PRED vs. TAC/SRL/PRED). Cell subsets between two immunosuppressive treatment protocols (TAC/MMF/PRED vs. TAC/SRL/PRED) were evaluated at four months after transplantation vs. baseline (before transplantation) in each group. However, GFR rate (estimated via Modification of Diet in Renal Disease (MDRD)) in group B patients was higher (70.94 ± 5.7) than that in group A patients (51.6 ± 6.4) at 4 months post-transplantation (P > 0.05). Also, variations of lymphocytes and CD4+ cells over time showed no significant differences between two groups (P < 0.05).

3.4. Comparison of the frequency of Th cell subsets (Th1, Th2 and Th17 cells) before and four-months after transplantation in two studied groups

There was not a significant decrease in the frequency of Th1 cells neither at 4 month post-transplantation compared with pre-transplantation in each group nor between two groups (Table 3, Fig. 1A and D). The percentage of Th2 cells in group B was 1.4% ± 0.3 before transplantation and significantly decreased to 0.8% ± 0.4 at 4 month (P < 0.05) post-transplantation. However, there was not a significant decrease in the frequency of Th2 cells in the group A over time (Table 3, Fig. 1A and E). Also, variations of Th2 cells over time showed no significant differences between two groups (P > 0.05).

There was a significant increase in the frequency of Th17 cells in group A four-months after transplantation (5% ± 2.5) compared with pre-transplantation (2.3% ± 1; P < 0.05). In group B, four-months after transplantation, the percentage of Th17 cells was significantly reduced (2.2% ± 0.9) when compared with pre-transplantation (3.6% ± 1.5; P < 0.05). Also, variations of Th17 cells over time showed significant differences between two studied groups (P < 0.05) (Table 3 and Fig. 1A and F).

3.5. Alteration of IFN-γ, IL-4 and IL-17A levels before and four-months after transplantation in two studied groups

As shown in Table 4 and Fig. 2, in each group, IFN-γ, IL-4, IL-17A levels were decreased 4-months after transplantation compared to pre-transplantation. However, this change was statistically significant for IL-4 (71.5% ± 12 pre-transplantation vs 62.5% ± 4.4 at 4 months post-transplantation; P < 0.05) in group B and IL17A in group A (24.5% ± 5.3 pre-transplantation vs 20.7% ± 3.6 at 4 months post-transplantation; P < 0.05). Also, variations of IFN-γ, IL-4 and IL-17A cytokine levels over time showed no significant differences between two groups (P > 0.05).

4. Discussions

Different studies on kidney transplant recipients are demonstrated the pivotal role of effector T cells and their inflammatory cytokines in acute and chronic rejection events [19]. Th1 secreting IFN-γ and Th17 secreting IL-17A activate endothelial cells and macrophage for inducing immediate inflammatory damage to transplanted organ. In addition, by activating the Th2 and secretion of IL-2 and IL-4, the B lymphocytes will expand and the class switching for production of anti-HLA IgG antibody is occurred. Allantoi-HLA antibodies, through binding to HLA antigens which are expressed on surface of vascular endothelial cells, can induce endothelial damage and thrombus generation leading to graft destruction and thereby transplant rejection. Therefore development and/or utilizing a potent and appropriate immunosuppressive protocol for suppressing function of the effector T helper subsets in preventing allograft rejection is necessary.

Multiagent immunosuppressive treatment protocol containing a calcineurin inhibitor like TAC and an anti-proliferative drug like mycophenolate mofetil for suppressing T cells responses, has improved 1-year graft survival up to 95% but long term graft survival is still a clinical challenge. In the current protocol, one of the most important adverse events for long-term graft survival is the nephrotoxicity of calcineurin inhibitor like TAC. Other adverse side effects such as diabetes and malignancies have also been reported [2]. Therefore, utilizing an appropriate immunosuppression protocol with lowest-dose of calcineurin inhibitor to improve long-term graft survival and to reduce associated adverse side effects is needed. TAC is a macrolide

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Frequency of circulating Th1, Th2 and Th17 cells before and four-months after transplantation in two studied groups.</th>
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<tbody>
<tr>
<td></td>
<td>TAC/MMF/PRED</td>
</tr>
<tr>
<td></td>
<td>(Group A)</td>
</tr>
<tr>
<td>Th1 (IFN-γ + CD4 +) (%)</td>
<td>14.4 ± 9.9</td>
</tr>
<tr>
<td>Base line</td>
<td>11.6 ± 6.8</td>
</tr>
<tr>
<td>4 months</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Th2 (IL-4 + CD4 +) (%)</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>Base line</td>
<td>2.3 ± 1.0</td>
</tr>
<tr>
<td>4 months</td>
<td>5.0 ± 2.5-⁎</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD. Th, T helper cells.

* P < 0.05 is representing a significant difference between the frequency of Th cell subsets four-months after transplantation vs. base line (before transplantation) in each group.

# P < 0.05 is representing a significant difference for the frequency of Th cell subsets between two immunosuppressive treatment protocols (TAC/MMF/PRED vs. TAC/SRL/PRED).

<table>
<thead>
<tr>
<th>Table 4</th>
<th>IFN-γ, IL-4 and IL-17A cytokine levels before and four-months after transplantation in two studied groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAC/MMF/PRED</td>
</tr>
<tr>
<td></td>
<td>(Group A)</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>5.6 ± 4.3</td>
</tr>
<tr>
<td>Base line</td>
<td>4.3 ± 2.2</td>
</tr>
<tr>
<td>4 months</td>
<td>67.5 ± 10.8</td>
</tr>
<tr>
<td>IL-4 (pg/ml)</td>
<td>63.5 ± 5.2</td>
</tr>
<tr>
<td>Base line</td>
<td>24.5 ± 5.3</td>
</tr>
<tr>
<td>4 months</td>
<td>20.7 ± 3.6</td>
</tr>
</tbody>
</table>

Values were expressed as mean ± SD.

* P < 0.05 is representing a significant difference between cytokine level four months after transplantation vs. base line (before transplantation) in each group.

3.2. Patients’ clinical data in two studied groups

Renal function was evaluated by measurement of serum creatinine, BUN, uric acid levels and GFR rate. There were no significant differences between the two groups regarding mentioned analysis at 4 months post-transplantation. However GFR rate (estimated via modification of diet in renal disease (MDRD)) in group B patients was higher (70.94 ± 5.7) than that in group A patients (51.6 ± 6.4) at 4 month post-transplantation but this did not reach statistical significance (P > 0.05). Also, significant differences were not found between the two groups regarding other laboratory tests at 4 months post-transplantation (Table 2).

3.3. Comparison of the frequency of lymphocytes and CD4+ cells before and four-months after transplantation in two studied groups

As shown in Fig. 1B, the percentage of lymphocytes in group A was significantly decreased at 4 months post-transplantation compared to pre-transplantation (P < 0.05). Also, there was a decrease in the frequency of lymphocytes in group B at 4 months post-transplantation when compared with pre-transplantation, nevertheless this did not reach statistical significance (P > 0.05). A significant decrease of CD4+ cells at 4 months post-transplantation compared to pre-transplantation in both groups was evident (P < 0.05) (Fig. 1C). Also, variations of lymphocytes and CD4+ cells over time showed no significant differences between two groups (P > 0.05).
immunosuppressant that binds to a cytoplasmic protein, FK binding protein, and inhibits calcineurin. This drug is frequently used after kidney, liver and heart transplantations. The nuclear factor of activated T cells (NFAT) proteins are a family of transcription factors whose activation is controlled by calcineurin, a Ca²⁺-dependent phosphatase. TAC, through NFAT inactivation, subsequently leads to suppression of IL-2 transcription and other cytokine genes. Thus, TAC by controlling calcineurin and IL-2 production, induces Th17 differentiation and inhibits Th1 and Th2 differentiation [7,20].

Recent studies have shown failure of TAC/MMF/PRED protocol in controlling Th17 cells responses [9,21–23]. Chung and colleagues have confirmed that this protocol is not adequate for decreasing Th17 abundance which is related to allograft dysfunction, fibrosis and, thereby, chronic transplant rejection [4]. Valorie et al., also showed that tacrolimus can result in Th17/Treg imbalance and increase in the serum level of cytokines including IL-6, IL-17a, and IL-21 in the mice.

Similar to our study, Li and colleagues have shown that increased abundance of Th17 cells was led to reduction of GFR. The authors have also demonstrated that the frequency of Th17 cells was higher in renal transplant recipients treated by TAC compared to SRL group [24]. Our data have also revealed that in the treated recipients with TAC/MMF/PRED (group A), the abundance of Th17 cells was increased significantly four months after transplantation compared with pre-transplantation (P < 0.05), whereas, the IL-17A level which is related to the functions of Th17 cells, was significantly decreased (P < 0.05) in 4-month samples when compared with before transplantation. These data are also in line with Liu findings where they showed that corticosteroids nonspecifically and dose-dependently inhibited the production of inflammatory cytokines [21]. Furthermore, Abadja et al., have shown that MMF, through strong inhibition of IL-17A production, may aid to better blunt the Th17-related allogeneic immune response in the context of calcineurin inhibitor-minimizing protocol [25].

Due to the adverse side effects of calcineurin inhibitors and poor long-term graft survival, different trials have been conducted aiming either to minimize the dose or to convert to sirolimus-based protocol. SRL (also known as an mTOR inhibitor) binds to the immunophilin FK506 binding protein (FKBP) to create an immunosuppressive complex that attaches to inhibit the mTOR, an important regulatory kinase which is required for translation of proteins associated with cell growth, proliferation and survival. mTOR induces differentiation of naïve T cells to Th1, Th2 and Th17 cells through activation of signal transducer and activator of transcription (STAT) proteins [26]. By inhibiting mTOR, sirolimus can blunt differentiation of naïve T cells to Th1, Th2 and Th17 cells. However, SRL adverse side effects such as hands and feet swelling, wound healing failure, and oral mucositis are the main cause of SRL protocol withdrawal [13]. Therefore, this study was aimed to investigate and compare the effects of two triple immunosuppressive protocols and to find out a more appropriate treatment protocol. In this study, decreased doses of sirolimus, tacrolimus plus prednisolone (SRL/TAC/PRED) (group B) were utilized to reduce the adverse side effects of sirolimus and tacrolimus and to enhance TAC/SRL immunosuppressive functions.

The data of the current study showed that there was a decrease in the frequency of Th1, Th2 cells and IFN-γ, IL-4 cytokine levels in the group B (SAL/TAC/PRED) four months after transplantation versus pre-transplantation. This reduction was only significant for Th2 cells and IL-4 (P < 0.05). Also, the frequency of Th17 cells was decreased in the group B (P < 0.05) and it was increased in the group A (P < 0.05) at 4 months post-transplantation compared with pre-transplantation. These results were in agreement with the study of Li et al., where they have demonstrated that SRL strongly reduced the frequency of Th1 and Th17 cells in kidney transplant recipients [27]. Kim and colleagues have also demonstrated that unlike TAC, the SRL could suppress the frequency of Th17 cells. They also found that there was a significant decrease in the abundance of Th17 cells after conversion from TAC to SRL in KTRs [28]. Moreover, Gallon et al. showed that SRL, in
combination with TAC, could amplify TACs immunosuppressive functions on the Th1 and Th17 cells in vitro [14].

It should be noted that, lymphocytes have multiple subsets and TH2, TH17 frequency rate in lymphocytes population is so low. Although decrease of lymphocytes in group A was significant but not in group B, its mainly because of other subsets variation effect in decrease of lymphocytes frequency.

In conclusion, although TAC/MMF/PRED treatment protocol could significantly decrease acute rejection, it was not able to hinder chronic rejection. The data of the present study showed that the TAC/MMF/PRED protocol was not able to decrease the abundance of Th17 cells which may lead to chronic allograft. The amount of GFR which is one of the main factor of kidney performance was low in this group. Based on our data, the frequency of Th17 and Th2 cells and the production level of IL-4 cytokine were also significantly decreased in the patients who received TAC/SRL/PRED protocol (group B). Moreover, GFR proved the clear benefit of this protocol (group B) in allograft survival. These data indicated that utilizing SRL/TAC/PRED protocol is not only in favor of allograft survival in terms of immunological factors but also this protocol apparently improves renal function because of TAC dosage reduction.

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Declaration of interest
None.

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