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Systemic Immunoregulatory and Proteogenomic Effects of Tacrolimus to Sirolimus Conversion in Liver Transplant Recipients

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Abstract

Immunosuppression withdrawal from calcineurin inhibitors is only possible in ~20% of liver transplant recipients. However, mTOR inhibitors (sirolimus) appear to be more immunoregulatory and might promote a tolerant state for withdrawal. Our purpose was to determine if systemic (blood, marrow, allograft) signatures of immunoregulation are promoted by conversion from tacrolimus to sirolimus. We therefore performed the following serial assays before and after sirolimus conversion in liver transplant recipients to test for enhanced markers of immunoregulation: 1) Flow cytometry immunophenotyping of PBMC and bone marrow aspirates for regulatory T cells (Tregs: CD4⁺CD25⁺FOXP3⁺) and regulatory dendritic cells (DCregs: ILT3⁺/4⁺); 2) liver biopsy immunohistochemical staining (FOXP3:CD3, CD4:CD8 ratios) and immunophenotyping of biopsy-derived Tregs after growth in culture; 3) effects of pre- vs. post-conversion sera on Treg generation in mixed lymphocyte reactions; 4) peripheral blood non-specific CD4 responses (Cylex[®] ImmuKnow); 5) peripheral blood gene transcripts and proteomic profiles. We successfully converted 20 non-immune, non-viremic recipients (age 57.2±8; 3.5±2.1 years post-LT) from tacrolimus to sirolimus for renal dysfunction. Our results demonstrated significant increases in Tregs in PBMC and marrow and DCregs in PBMC (p<0.01) following

conversion. In biopsy immunohistochemistry, FOXP3:CD3 and CD4:CD8 ratios were significantly higher after conversion and a number of biopsy cultures developed new or higher FOXP3⁺ cell growth. Non-specific CD4 responses (Cylex[®] ImmuKnow) did not change. Both pre- and post-conversion sera inhibited mixed lymphocyte reactions, although only tacrolimus sera suppressed Treg generation. Finally, 289 novel genes and 22 proteins, several important in immunoregulatory pathways, were expressed after conversion.

Conclusions—Tacrolimus to sirolimus conversion increases systemic Tregs, DCregs and immunoregulatory proteogenomic signatures in liver transplant recipients and may therefore facilitate immunosuppression minimization or withdrawal.

Keywords

immunosuppression; tolerance; regulatory T cells; dendritic cells; mTOR inhibition

Life-long immunosuppression (IS) is generally required after liver transplantation (LT). With the advent of calcineurin inhibitors (CNI), rejection rates have declined, yet toxicity due to CNI therapy has led to long term adverse outcomes (1). Complete IS withdrawal (operational tolerance) would be ideal, although this has thus far been possible in only ~20% of LT recipients (2). The inability to immunologically predict successful IS withdrawal has obligated long term CNI maintenance at therapeutic doses, despite toxicities.

The identification of specific cell populations and pathways responsible for immunoregulation may give clues towards achieving tolerance in LT. Tolerance develops initially by the interaction of antigen-specific T cells with unique thymic antigen-presenting cells (APC) or regulatory dendritic cells (DCregs), respectively resulting in either clonal deletion, anergy or an active immunoregulatory process (3). Such DCregs are characterized by high surface expression of CD123 and/or immunoglobulin-like transcripts ILT3 or ILT4 that inhibit antigen presentation, i.e. reflecting immunoregulation (4, 5). As mentioned, this interaction can lead to the generation of Tregs (CD4⁺CD25^{high}) that migrate peripherally to control immune responses. These Tregs typically express an intracellular protein FOXP3 which blocks transcription of T cell activation molecules such as IL-2 and the expression of CD127 (6, 7). Moreover, gene transcripts and protein expression patterns (antibodies, circulating and cell proteins) as markers for immunoregulation may also provide a window into the tolerant state.

There is thus strong interest in cellular (Treg, DCreg), genomic and proteomic assays to assess immunoregulation and predict more reliably who might achieve IS withdrawal (8, 9). As the liver appears to be the most tolerogenic transplanted organ, high numbers of such cells residing in the liver may be regulatory and protect against rejection. Similarly, the bone marrow, as a formative organ of the immune system may also contain localized Treg niches that enhance immunoregulation (10–12). Thus, assessing tolerogenic signatures not only in the blood but also in the allograft and bone marrow may further differentiate regulatory from alloreactive states.

In addition to the need for predictive assays, there have been few efforts to modulate the immune system to induce tolerance other than simultaneous hematopoietic cell and solid organ transplantation (13), a risky approach in patients with severe end stage liver disease undergoing LT. One potential reason for the low efficacy of IS withdrawal is CNI therapy itself, as most patients in such studies were being treated with CNIs at withdrawal (2). CNIs may hinder immunoregulation by inhibiting IL-2 production needed for Treg development and also by *not* inhibiting DC maturation (14, 15). In contrast, mTOR inhibitors such as sirolimus (SRL) might facilitate immunoregulation by increasing Treg percentages (does not block IL-2 production) and inhibiting DC maturation and function (16–18). We previously

demonstrated that LT recipients on SRL monotherapy had significantly higher Treg percentages than those on tacrolimus (TAC) monotherapy (19). Thus, conversion to SRL may both minimize CNI toxicity (20) and facilitate IS withdrawal by promoting immunoregulation.

We therefore hypothesized that *prospectively studied* LT recipients converted from CNI to SRL monotherapy would display enhanced systemic cellular, functional and proteogenomic immunoregulatory markers. This would support larger scale studies utilizing SRL conversion as an intermediate step toward more clinically successful IS withdrawal in LT.

EXPERIMENTAL PROCEDURES

Clinical Protocol and Study Procedures

The study was a prospective investigation of the changes in immunoregulatory markers in the blood, bone marrow, and liver allograft in recipients converted from TAC monotherapy to SRL monotherapy for clinical indications (TAC toxicity). Inclusion Criteria: Age 18 years; 6 months post-LT; TAC monotherapy 1 month before SRL monotherapy conversion for nephrotoxicity (GFR 30 – 60 cc/min by MDRD) or other indication; 6 months without a rejection episode; no lymphocyte depletion therapy for 1 year; normal liver function tests; no rejection or fibrosis on pre-conversion liver biopsy. Exclusion Criteria: Prior liver or multi-organ transplant; prior immune or viral liver disease unless HCV RNA was undetectable; proteinuria (> 0.5 g/day), eGFR < 30 cc/min; 2 rejections post-LT; history of hepatic artery thrombosis; hematological abnormalities, severe hypertriglyceridemia; active infection or malignancy; inadequacy for follow-up. All patients signed informed consent and were followed for seven months after SRL conversion. The protocol conformed to the Declaration of Helsinki guidelines and was approved by the Northwestern Institutional Review Board.

History and physical exams, complete blood counts, comprehensive metabolic panels, fasting lipids, hemoglobin A1C tests, and spot urine protein:creatinine ratios were performed before, 3 and 6 months after conversion. Bone marrow aspirations and percutaneous liver biopsies were performed once before and six months after conversion. For conversion, SRL 2 or 3 mg (< 100 kg body weight) daily was initiated with subsequent weekly SRL trough level monitoring. When these reached 5 ng/mL, TAC was discontinued followed by weekly laboratory tests and SRL trough levels (goal 5–8 ng/mL) for one month, then monthly. Prospective liver/renal function tests, lipid levels, urine protein:creatinine ratios and any new SRL toxicities were recorded.

Assay Methods

1. Peripheral Blood

a. Regulatory T cell Immunophenotyping (twice before conversion and 3, 4, 6 and 7 months after conversion): PBMC were isolated from heparinized samples on Ficoll-Hypaque gradients. Tregs were enumerated utilizing extracellular immunofluorescent staining with CD3-FITC, CD4-PerCP, CD8-PerCP, CD25-APC, CD127-FITC (Becton-Dickinson, San Diego, CA). Following fixation and permeabilization, the cells were washed and incubated with anti-human FOXP3-PE or rat IgG2a-PE isotype control (eBioscience, San Diego, CA) (21, 22). Samples were acquired using the FACS-Calibur flow cytometer and analyzed by gating based on CD4⁺CD127⁻CD25⁺ expression against FOXP3 to calculate the percentage of Tregs.

b. Cell Subset analysis: Peripheral blood was also labeled with monoclonal antibodies for T cell subsets (CD3, CD4, CD8, CD25), B cells (CD19), monocytes (CD14) and natural killer

cells (CD56). After red blood cell lysing, samples were acquired on the FACS-Calibur and the absolute cell subset numbers/ μ L blood were calculated.

c. Dendritic cell surface markers (twice before conversion and 3, 4, 6 and 7 months after conversion): PBMC isolated by Ficoll-Hypaque gradients were incubated with FITC-labeled monoclonal antibodies to lineage markers (CD3, CD14, CD16, CD19, CD20, CD56) for negative selection and markers to distinguish between monocytoïd (CD11c) and plasmacytoïd (CD123) DCs (Beckman-Coulter, Miami, FL) (19, 23). Cells were further stained with CD205-PE and CD83-PC5 as DC antigen uptake/presentation markers or ILT3-PC5 and ILT4-PE as tolerogenic DC markers. Four-color multi-parameter flow cytometry was performed using a Coulter FC500 instrument compensated with single fluorochromes.

d. Functional assays (once before and once 6 months after conversion): 1) *The Immune Cell Function Assay*® (Cylex Inc., Columbia, MD) assay was performed per the manufacturer's instructions, detecting CD4 responses by ATP production in whole blood after 18 hours of incubation with phytohemagglutinin stimulation. 2) *The Treg-MLR assay*: Using healthy HLA typed volunteer PBMCs, responding cells were stimulated with x-irradiated HLA 2 DR matched stimulating allogeneic cells (21, 22). To these cultures, LT recipient sera containing trough levels of TAC (pre-conversion) vs. SRL (post-conversion) were added and compared with addition of similar volumes of human AB sera (Invitrogen, Carlsbad, CA) vs. media controls. After 7 culture days, lymphoproliferation was assessed by 3 H-TdR incorporation and immunophenotyping was performed for CD3, CD4, CD8, CD25 and FOXP3 markers. Stimulation indices were calculated from the counts/minute measured with a beta counter (24).

f. Gene Expression Microarrays and Protein Multi-Analyte Profiles (MAPs) (once before and once six months after conversion): Peripheral blood was collected in PaxGene tubes (Qiagen, Valencia, CA) for gene microarrays and Baxter PPT tubes for MAPs (25, 26). RNA was extracted using the PaxGene blood RNA kit. Whole blood human genome profiling was performed with Affymetrix GeneChip 1.0 ST arrays (Santa Clara, CA) following standard protocols. Plasma proteomics were performed using a proprietary Luminex Bead technology testing the 189 protein Human DiscoveryMAP® v1.0 (Rules Based Medicine®, Austin, TX).

2. Bone Marrow Aspirates

Regulatory T cell and DC Immunophenotyping (once before and once six months after conversion): Under local anesthesia as outpatients, 10 cc of marrow was aspirated from the posterior iliac crest using a 16 gauge needle. After five minutes of pressure, normal daily activities resumed. The leukocytes were isolated by Ficoll-Hypaque gradients and characterized by flow cytometry for Treg and DC subsets identical to the peripheral blood methods (above).

3. Liver Biopsy

Routine Histology; Regulatory T cell Immunophenotyping by Immunohistochemical (IHC) Staining and After Culture (once before and once six months after conversion):

A 2 cm core was obtained for H&E, trichrome, and IHC. Immunohistochemical staining of formalin-fixed tissue was performed with streptavidin-biotin-peroxidase using dual staining antibodies to FOXP3, CD3 and CD4, CD8 (27). The number of CD3 and FOXP3 positive and CD4 and CD8 positive lymphocytes were counted in a 400X power field. The ratios of FOXP3:CD3 and CD4:CD8 were calculated and an average of three portal tract ratios was recorded. A second core was obtained for flow immunophenotyping after 14 days of culture

in media (50U/ml r-IL2 + 50% MLR supernatant) that reliably expands cells already activated *in vivo* (28, 29).

Statistical Analysis

Pre- vs. post-conversion measurements of immune assays (PBMC, marrow, biopsies) and clinical outcomes were performed using the appropriate paired analysis (paired t-test; Wilcoxon-Signed Rank) or the Chi-squared/Fisher exact test for continuous or categorical measures, respectively. For microarray and multi-analyte profile comparisons, the p-values were calculated using a 2-way ANOVA model by the Method of Moments (30), using the Partek Genomics Suite (St. Louis, MO). A false discovery rate correction of 10% (q-values) was used for the proteomic data. A paired ANOVA was used for the gene expression changes since the samples represented two time points from the same individual. Analyses were performed using SAS 9.2 software (SAS Inc., Cary, NC).

RESULTS

Patient Characteristics

Twenty-seven LT recipients were initially considered candidates for TAC to SRL conversion because of renal dysfunction. Two were excluded before conversion, one due to elevated ALT at screening and one with interface hepatitis on the pre-conversion biopsy. Five were excluded as they were converted back to TAC within one month after SRL conversion due to cost (n=1), SRL intolerability (1 foot ulcer, 1 nausea), or mild rejection on biopsy (n=2, each resolved with TAC reversion). Other than biopsy IHC staining in the two with rejection, these five patients were withdrawn from the study and followed clinically as it was not considered necessary (i.e. no longer on SRL) or ethical to continue the serial sample collections. Thus, 20 were successfully converted and completed the study (Table 1).

Clinical and Histological Outcomes

Sirolimus was generally well tolerated. There were no infectious complications. Side effects (4 diarrhea, 3 anemia, 4 lower extremity edema, 1 rash) resolved by targeting lower SRL troughs (~5 ng/mL). Although renal parameters (eGFR by MDRD equation, BUN, creatinine, potassium levels) improved, the urine protein:creatinine ratios increased with SRL conversion (Table 2). Biochemical changes included minor decreases in bilirubin and increases in ALT. Significant increases in low density lipoproteins and triglycerides occurred. Liver biopsies did not demonstrate significant histological changes, other than mild steatosis and increased portal lymphocytes later characterized as staining FOXP3+ (Figure 2 below).

Immunophenotyping: Peripheral Blood, Bone Marrow, and Allograft

To emphasize the rationale for these assays, changes in Treg and DCreg percentages following SRL conversion were assessed because high percentages of these cells were formerly reported in tolerant LT recipients (5, 8, 9, 31). Also, in previous studies, we have safely and repeatedly performed outpatient marrow aspirations, demonstrating the role of bone marrow cells in controlling anti-donor immune responses (10, 11, 32). Recently, bone marrow Tregs have been shown to establish an immunoregulatory niche supporting stem cells and protecting against immune injury (12). As SRL inhibits DC function *in vitro*, it was also questioned whether SRL conversion might affect the percentage of ILT3, ILT4, and CD123, all markers of regulatory DCs (5, 18, 33). We therefore measured bone marrow immunophenotypes (Treg, DCreg) before and after conversion, to determine if changes similar to those observed in PBMC occurred. In addition, liver biopsy IHC staining has been utilized in prior studies demonstrating high Treg numbers in tolerant LT recipients (8, 27).

We therefore performed both liver biopsy IHC staining and allograft culture immunophenotyping, previously validated approaches (8, 27–29), to characterize the percentage of Tregs residing within the graft before and after conversion.

a. Peripheral Blood and Bone Marrow Immunophenotyping—In both the PBMC and marrow aspirates, percentages of CD4⁺CD25⁺FOXP3⁺ and CD4⁺CD25^{high}FOXP3⁺ phenotypic Tregs significantly increased after SRL conversion (Figure 1A, Suppl. Table 1). The PBMC CD3⁺ (total T cells), CD14⁺ (monocytes) and CD56⁺ (natural killer cells) cell numbers all statistically decreased following conversion, although the absolute changes in number/uL whole blood were minor (Suppl. Table 1). Also, the percentage of DC (CD123⁺ and CD11c⁺) expressing ILT3 and ILT4 increased significantly in the peripheral blood ($p<0.01$, Figure 1B) but not in the bone marrow (Suppl. Table 2). Other than decreased total HLA-DR⁺ cells and DCs and increased CD11c⁺/11c⁺83⁺ cell percentages, no differences were seen in other DC subsets (Suppl. Table 2).

b. Allograft Immunophenotyping—The ratio of FOXP3:CD3 positive cells on IHC slide staining increased significantly after SRL conversion (0.19 ± 0.1) compared to either pre-conversion (0.11 ± 0.1 , $p=0.01$) or rejection controls (two from this study and five randomly selected from our pathology database: 0.09 ± 0.01 , $p=0.005$). A representative example is shown in Figure 2. The ratio of CD4:CD8 positive cells also increased significantly (1.2 ± 0.5) compared to pre-conversion (0.86 ± 0.2 , $p=0.02$) and those with rejection (0.9 ± 0.1 , $p=0.01$). For the liver biopsy cultures, there was significant variability in cell growth, precluding an appropriate pre- and post-conversion statistical analysis (Suppl. Table 3). Of the biopsies that had growth pre- and post-conversion, two had decreases, six had no change, and three had increases in Treg percentages. In the others, two lost growth but seven had new Treg growth following conversion, perhaps suggesting a trend toward increased intra-graft Tregs after culture. However, given the variability and problems with culture growth, these data are not as conclusive as the IHC staining results.

Functional Assays

There has been recent interest in functional assays (Cylex[®] ImmuKnow) assessing non-specific CD4 responses to distinguish alloreactive from immunosuppressed states (34). In the present study, the mean ATP values did not change after SRL conversion (266 ± 132 to 274 ± 149 ng/ml, $p=0.15$), suggesting that SRL conversion and Treg generation did not appear to lead to non-specific over-immunosuppression.

We have also recently reported on a novel *in vitro* immune monitoring assay in humans, the Treg MLR, demonstrating favorable immunoregulatory effects of SRL vs. TAC when added directly to MLR cultures (21, 22). As another functional measure, we therefore questioned if addition of patient sera containing TAC vs. SRL and possibly other resulting regulatory molecules might suppress lymphoproliferation and enhance Treg generation (21, 22). Both pre- and post-conversion sera equally suppressed MLR lymphoproliferation (stimulation indices) below media controls ($n=13$, $p<0.05$) (Figure 3A). However, TAC sera also suppressed CD4⁺CD25⁺FOXP3⁺ cell generation ($n=13$, $p<0.01$) (Figure 3B), while SRL sera did not.

Proteogenomic Signatures

Genomic, proteomic and cytokine signatures may have the potential to predict tolerance (9, 35). In previous reports, transcripts for cell proliferation arrest proteins and $\gamma\delta$ T and NK cell receptors have been identified as putative LT tolerance signatures, correlating with increased circulating Tregs. We examined whether similar signatures of immunoregulation might be also seen after SRL conversion. In the present study, several gene transcripts

(n=288, Suppl. Table 4) and plasma proteins (n=22, Table 3), many involved in immunoregulatory pathways, were found to be significantly different after SRL conversion ($p<0.005$). Within the heat map displayed in Figure 4 were up-regulated transcripts of FOXP3, CD25, and CTLA-4, TGF- β , and CD4 and down-regulated transcripts of CCR3, Apo C-IV, and collagen type IV (Suppl. Table 4). Also, a number of proteins known to be involved in lymphocyte and DC activation (IL-3, IL-7, IL-13, MIP-1 alpha, CD40), lymphocyte trafficking (VCAM-1, PARC), and renal injury (TRAIL-R3, MIP-1 alpha, TFF3) were down-regulated after conversion (Table 3). The relevance of the changes in the remaining proteins (Table 3) to the present study is not known.

DISCUSSION

This study complements previous reports supporting the immunoregulatory properties of mTOR inhibitor therapy (16–18, 22, 36). First, Treg populations increased prospectively in all key immune compartments studied (blood, marrow, allograft) following SRL conversion. This provides a more robust classification of patients that might be ‘tolerance-prone’, rather than single time-point analysis of peripheral blood (8, 19). Speculatively, these intra-graft FOXP3⁺ cells promoted by SRL might regulate immune responses and facilitate tolerance (8, 37). Second, the serial paired data on monotherapy conversion provides strong support that these changes were directly due to SRL. Third, to our knowledge, this study is the first to analyze serial changes in DC profiles and supports SRL therapy promoting tolerogenic DCs (18, 38, 39). Finally, the functional and proteogenomic regulatory signatures coincided with the phenotypic cellular markers of immunoregulation after SRL conversion. Sera from patients on SRL inhibited lymphoproliferation alloreactivity but did not inhibit Treg generation like TAC sera, possibly due to the action of SRL itself or other regulatory serum proteins (40). Many of the genes/proteins were up-regulated (immunoregulatory pathways) or down-regulated (kidney injury pathways) by conversion, supporting their combined use as surrogate tolerance signatures (9, 26).

Previous reports have demonstrated differences in the effects of CNIs vs. mTOR inhibitors on Tregs and DCregs (14, 16, 17, 41). Tacrolimus inhibits cytokines (IL-2) important in FOXP3 expression and Treg function (7). In contrast, SRL inhibits post-activation signaling (PI3K/mTOR pathways) and does not block IL-2 production or other cascades (STAT5) involved in Treg generation (14, 17, 39, 42). (17, 42). Likewise, we demonstrated allo-specific inhibition and Treg generation by SRL vs. TAC *in vitro* (22) and reported higher blood Tregs in LT recipients on SRL vs. TAC (19).

Moreover, while CNIs have little impact on DC function, SRL impairs DC maturation, generation, and co-stimulation (18). The resulting immature DCs are less capable of allo-stimulation and more proficient in generating allo-specific Tregs (39, 43). Alternatively, the reverse regulatory process might occur, given that FOXP3⁺ suppressor T cells can induce ILT3/4 on DCs, rendering them tolerogenic (44). While our study did not demonstrate an increase in plasmacytoid (CD123⁺) to myeloid (CD11c⁺) DC ratios seen previously in tolerant LT patients (5), an increase in negative co-stimulatory molecules ILT3 and ILT4 occurred following conversion. In addition, the plasma co-stimulatory CD40 protein was reduced, which might also signify impairment of DC allo-activation and enhanced immunoregulation.

Supporting the immunophenotyping, SRL conversion led to enhanced peripheral blood transcript expression of known Treg markers (CD4, FOXP3, CD25, CTLA-4) and Treg-enhancing cytokines (TGF- β). In addition, proteins involved in lymphocyte responses (IL-3, IL-7, IL-13), trafficking/adhesion (VCAM-1, PARC) and DC development and co-stimulation (MIP-1 alpha, CD40) were down-regulated (Table 3). Interestingly, other genes

(Apo C-IV, collagen type IV) and proteins (TFF3, factor VII, TRAIL-R3, MIP-1 alpha) often associated with renal dysfunction were down-regulated, correlating clinically with eGFR improvement. Two (TFF3, factor VII) were associated with CKD after liver transplantation in our recent report (26). This might be merely related to CNI withdrawal, although SRL has known anti-fibrotic effects (45) that might improve renal function.

Several limitations in this report need to be mentioned. First, our final enrollment was only 20 patients, although our tolerability was somewhat better than trials in which ~30% could not tolerate SRL. This is possibly due to our monotherapy patients being further removed from LT and targeted for lower trough levels. Second, many of the biopsies did not grow in culture media designed to expand Tregs, precluding a pre- and post-conversion statistical analysis. However, several biopsies had new or higher Treg percentages following conversion. Also, the increased CD4+ and FOXP3+ cells (putative Tregs) on IHC staining might be more directly indicative of the regulatory cell changes within the allograft. Third, as donor cells were not available, we could not assess the impact of SRL conversion on donor-specific immunoregulation seen *in vitro* (21). Finally, our preliminary results, particularly the proteogenomic analysis, need to be validated in larger, prospective patient cohorts.

In conclusion, this study is consistent with the notion that CNI to SRL conversion after LT could take advantage of the regulatory properties of SRL and allow more successful subsequent IS minimization and/or full withdrawal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ALT	alanine aminotransferase
APC	antigen presenting cell
APO	apolipoprotein
CD	cluster differentiation
CKD	chronic kidney disease
CNI	calcineurin inhibitors
DC	dendritic cell
eGFR	estimated glomerular filtration rate
FOXP3	forkhead box P3
HCV	hepatitis C virus
HLA	human leucocyte antigen

IHC	immunohistochemistry
IL	interleukin
ILT	immunoglobulin-like transcript
IS	immunosuppression
LT	liver transplantation
MAP	multi-analyte profiles
MDRD	Modified Diet in Renal Disease
MIP	macrophage inflammatory protein
MLR	mixed lymphocyte reaction
mTOR	molecular target of rapamycin
PARC	pulmonary and activation-regulated chemokine
PBMC	peripheral blood mononuclear cell
PI3K	phosphatidylinositol 3-kinase
SRL	sirolimus
STAT	signal transducers and activators of transcription
TAC	tacrolimus
TB	total bilirubin
TCR	T-cell receptor
TFF	trefoil factor 3
Treg	regulatory T cell
TRAIL	TNF-related apoptosis-inducing ligand
VCAM	vascular cell adhesion molecule

References

1. Ojo AO, Held PJ, Port FK, Wolfe RA, Leichtman AB, Young EW, Arndorfer J, et al. Chronic renal failure after transplantation of a nonrenal organ. *N Engl J Med*. 2003; 349:931–940. [PubMed: 12954741]
2. Levitsky J. Operational tolerance: past lessons and future prospects. *Liver Transpl*. 2011; 17:222–232. [PubMed: 21384504]
3. Sykes M. Chimerism and central tolerance. *Curr Opin Immunol*. 1996; 8:694–703. [PubMed: 8902396]
4. Chatzipetrou MA, Mathew JM, Kenyon NS, Esquenazi V, Miller J, Ricordi C, Tzakis AG. Analysis of post-transplant immune status in recipients of liver/bone marrow allografts. *Hum Immunol*. 1999; 60:1281–1288. [PubMed: 10626743]
5. Mazariegos GV, Zahorchak AF, Reyes J, Ostrowski L, Flynn B, Zeevi A, Thomson AW. Dendritic cell subset ratio in peripheral blood correlates with successful withdrawal of immunosuppression in liver transplant patients. *Am J Transplant*. 2003; 3:689–696. [PubMed: 12780560]
6. Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*. 2006; 203:1701–1711. [PubMed: 16818678]
7. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science*. 2003; 299:1057–1061. [PubMed: 12522256]

8. Li Y, Zhao X, Cheng D, Haga H, Tsuruyama T, Wood K, Sakaguchi S, et al. The Presence of Foxp3 Expressing T Cells Within Grafts of Tolerant Human Liver Transplant Recipients. *Transplantation*. 2008; 86:1837–1843. [PubMed: 19104431]
9. Martinez-Llordella M, Lozano JJ, Puig-Pey I, Orlando G, Tisone G, Lerut J, Benitez C, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest*. 2008; 118:2845–2857. [PubMed: 18654667]
10. Mathew JM, Fuller L, Carreno M, Garcia-Morales R, Burke GW 3rd, Ricordi C, Esquenazi V, et al. Involvement of multiple subpopulations of human bone marrow cells in the regulation of allogeneic cellular immune responses. *Transplantation*. 2000; 70:1752–1760. [PubMed: 11152108]
11. Miller J, Mathew J, Garcia-Morales R, Zucker KE, Carreno M, Jin Y, Fuller L, et al. The human bone marrow as an immunoregulatory organ. *Transplantation*. 1999; 68:1079–1090. [PubMed: 10551630]
12. Fujisaki J, Wu J, Carlson AL, Silberstein L, Putheti P, Larocca R, Gao W, et al. In vivo imaging of Treg cells providing immune privilege to the haematopoietic stem-cell niche. *Nature*. 2011; 474:216–219. [PubMed: 21654805]
13. Kawai T, Cosimi AB, Spitzer TR, Tolkoff-Rubin N, Suthanthiran M, Saidman SL, Shaffer J, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med*. 2008; 358:353–361. [PubMed: 18216355]
14. Gao W, Lu Y, El Essawy B, Oukka M, Kuchroo VK, Strom TB. Contrasting effects of cyclosporine and rapamycin in de novo generation of alloantigen-specific regulatory T cells. *Am J Transplant*. 2007; 7:1722–1732. [PubMed: 17511761]
15. Szabo G, Gavalá C, Mandrekar P. Tacrolimus and cyclosporine A inhibit allostimulatory capacity and cytokine production of human myeloid dendritic cells. *J Investig Med*. 2001; 49:442–449.
16. Battaglia M, Stabilini A, Roncarolo MG. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood*. 2005; 105:4743–4748. [PubMed: 15746082]
17. Baan CC, van der Mast BJ, Klepper M, Mol WM, Peeters AM, Korevaar SS, Balk AH, et al. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation*. 2005; 80:110–117. [PubMed: 16003241]
18. Hackstein H, Taner T, Zahorchak AF, Morelli AE, Logar AJ, Gessner A, Thomson AW. Rapamycin inhibits IL-4--induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo. *Blood*. 2003; 101:4457–4463. [PubMed: 12531798]
19. Levitsky J, Miller J, Wang E, Rosen A, Flaa C, Abecassis M, Mathew J, et al. Immunoregulatory profiles in liver transplant recipients on different immunosuppressive agents. *Hum Immunol*. 2009; 70:146–150. [PubMed: 19141306]
20. Morard I, Dumortier J, Spahr L, Hadengue A, Majno P, Morel P, Mentha G, et al. Conversion to sirolimus-based immunosuppression in maintenance liver transplantation patients. *Liver Transpl*. 2007; 13:658–664. [PubMed: 17457887]
21. Levitsky J, Mathew JM, Flaa CW, Rosen A, Tambur AR, Miller J. The human “Treg MLR”: Immune monitoring for FOXP3+ T Regulatory Cell Generation. *Transplantation*. 2009 Dec. 88:1303–1311. [PubMed: 19996930]
22. Levitsky J, Gallon L, Miller J, Tambur AR, Leventhal J, Flaa C, Huang X, et al. Allospecific regulatory effects of sirolimus and tacrolimus in the human mixed lymphocyte reaction. *Transplantation*. 2011; 91:199–206. [PubMed: 21239962]
23. Wertheimer AM, Bakke A, Rosen HR. Direct enumeration and functional assessment of circulating dendritic cells in patients with liver disease. *Hepatology*. 2004; 40:335–345. [PubMed: 15368438]
24. Fathman CG, Collavo D, Davies S, Nabholz M. In vitro secondary MLR. I. Kinetics of proliferation and specificity of in vitro primed responder cells. *J Immunol*. 1977; 118:1232–1238. [PubMed: 66282]
25. Nakorchevsky A, Hewel JA, Kurian SM, Mondala TS, Campbell D, Head SR, Marsh CL, et al. Molecular mechanisms of chronic kidney transplant rejection via large-scale proteogenomic analysis of tissue biopsies. *J Am Soc Nephrol*. 2010; 21:362–373. [PubMed: 20093355]

26. Levitsky J, Salomon DR, Abecassis M, Langfelder P, Horvath S, Friedewald J, Wang E, et al. Clinical and plasma proteomic markers correlating with chronic kidney disease after liver transplantation. *Am J Transplant*. 2011; 11:1972–1978. [PubMed: 21794091]
27. Oo YH, Weston CJ, Lalor PF, Curbishley SM, Withers DR, Reynolds GM, Shetty S, et al. Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. *J Immunol*. 2010; 184:2886–2898. [PubMed: 20164417]
28. Zeevi, A. Propagation of Lymphoid Cells from Biopsies. In: Hahn, A.; Land, GA.; Rosemarie, MS., editors. *American Society of Histocompatibility Laboratory Manual*. Vol. 1. 2000.
29. Poindexter NJ, Steward NS, Shenoy S, Brennan DC, Lowell J, Singer G, Howard T, et al. Renal allograft infiltrating lymphocytes: frequency of tissue specific lymphocytes. *Hum Immunol*. 1997; 55:140–147. [PubMed: 9361965]
30. Eisenhart C. The assumptions underlying the analysis of variance. *Biometrics*. 1947; 3:1–21. [PubMed: 20240414]
31. Li Y, Koshiba T, Yoshizawa A, Yonekawa Y, Masuda K, Ito A, Ueda M, et al. Analyses of peripheral blood mononuclear cells in operational tolerance after pediatric living donor liver transplantation. *Am J Transplant*. 2004; 4:2118–2125. [PubMed: 15575917]
32. Mathew JM, Carreno M, Fuller L, Ricordi C, Tzakis A, Esquenazi V, Miller J. Modulatory effects of human donor bone marrow cells on allogeneic cellular immune responses. *Transplantation*. 1997; 63:686–692. [PubMed: 9075839]
33. Manavalan JS, Rossi PC, Vlad G, Piazza F, Yarilina A, Cortesini R, Mancini D, et al. High expression of ILT3 and ILT4 is a general feature of tolerogenic dendritic cells. *Transpl Immunol*. 2003; 11:245–258. [PubMed: 12967778]
34. Kowalski RJ, Post DR, Mannon RB, Sebastian A, Wright HI, Sigle G, Burdick J, et al. Assessing relative risks of infection and rejection: a meta-analysis using an immune function assay. *Transplantation*. 2006; 82:663–668. [PubMed: 16969290]
35. Cookson S, Doherty DG, Todryk S, Gibbs P, Portmann B, O'Grady J, Rela M, et al. Hepatic expression of IL-15 mRNA is associated with liver graft acceptance. *Transpl Immunol*. 2003; 11:39–48. [PubMed: 12727474]
36. Nikolaeva N, Bemelman FJ, Yong SL, van Lier RA, ten Berge IJ. Rapamycin does not induce anergy but inhibits expansion and differentiation of alloreactive human T cells. *Transplantation*. 2006; 81:445–454. [PubMed: 16477233]
37. Bestard O, Cunetti L, Cruzado JM, Lucia M, Valdez R, Olek S, Melilli E, et al. Intra-graft Regulatory T Cells in Protocol Biopsies Retain Foxp3 Demethylation and Are Protective Biomarkers for Kidney Graft Outcome. *Am J Transplant*. 2011; 11:2162–2172. [PubMed: 21749644]
38. Chiang PH, Wang L, Bonham CA, Liang X, Fung JJ, Lu L, Qian S. Mechanistic insights into impaired dendritic cell function by rapamycin: inhibition of Jak2/Stat4 signaling pathway. *J Immunol*. 2004; 172:1355–1363. [PubMed: 14734710]
39. Turnquist HR, Raimondi G, Zahorchak AF, Fischer RT, Wang Z, Thomson AW. Rapamycin-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific Foxp3+ T regulatory cells and promote organ transplant tolerance. *J Immunol*. 2007; 178:7018–7031. [PubMed: 17513751]
40. Creput C, Le Friec G, Bahri R, Amiot L, Charpentier B, Carosella E, Rouas-Freiss N, et al. Detection of HLA-G in serum and graft biopsy associated with fewer acute rejections following combined liver-kidney transplantation: possible implications for monitoring patients. *Hum Immunol*. 2003; 64:1033–1038. [PubMed: 14602232]
41. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, Contag CH, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood*. 2006; 108:390–399. [PubMed: 16522809]
42. Lim DG, Koo SK, Park YH, Kim Y, Kim HM, Park CS, Kim SC, et al. Impact of immunosuppressants on the therapeutic efficacy of in vitro-expanded CD4+CD25+Foxp3+ regulatory T cells in allotransplantation. *Transplantation*. 89:928–936. [PubMed: 20305583]

43. Taner T, Hackstein H, Wang Z, Morelli AE, Thomson AW. Rapamycin-treated, alloantigen-pulsed host dendritic cells induce ag-specific T cell regulation and prolong graft survival. *Am J Transplant*. 2005; 5:228–236. [PubMed: 15643982]
44. Manavalan JS, Kim-Schulze S, Scotto L, Naiyer AJ, Vlad G, Colombo PC, Marboe C, et al. Alloantigen specific CD8+CD28- FOXP3+ T suppressor cells induce ILT3+ ILT4+ tolerogenic endothelial cells, inhibiting alloreactivity. *Int Immunol*. 2004; 16:1055–1068. [PubMed: 15226269]
45. Patsenker E, Schneider V, Ledermann M, Saegesser H, Dorn C, Hellerbrand C, Stickel F. Potent antifibrotic activity of mTOR inhibitors sirolimus and everolimus but not of cyclosporine A and tacrolimus in experimental liver fibrosis. *J Hepatol*. 2011; 55:388–398. [PubMed: 21168455]

Figure 1. Increase in Phenotypic Regulatory T Cells and Regulatory Dendritic Cells with Tacrolimus to Sirolimus Conversion

Immunophenotyping flow cytometry was performed on peripheral blood and bone marrow aspirate specimens before (0, horizontal axis) and after (means of 12 and 14 weeks and of 24 and 26 weeks for PBMC, horizontal axis) conversion from tacrolimus to sirolimus. Note the significant increase in the percentage of phenotypic Tregs (CD4⁺CD25⁺FOXP3⁺, CD4⁺CD25^{high}FOXP3⁺) in the peripheral blood and marrow (A), as well as the number of peripheral blood CD123⁺ILT3⁺ILT4⁺ and CD11c⁺ILT3⁺ DCregs (B), following sirolimus conversion. * p<0.05 and ** p<0.01 compared to time point 0 (pre-conversion on TAC). More details are given in Supplemental Tables 1 and 2.

Figure 2. Increased FOXP3⁺ cells in Liver Biopsies after Tacrolimus to Sirolimus Conversion
Immunohistochemical staining was performed on liver biopsy specimens before and after conversion from tacrolimus to sirolimus (representative example shown). Note the increase in the number of FOXP3⁺ Cells (in dashed-line circles) following sirolimus conversion.

Figure 3. Effect of Patient Pre-conversion (TAC) and Post-conversion (SRL) Sera on Lymphoproliferation and Treg Generation in MLR

5×10^5 CFSE labeled responding PBMC from healthy volunteers were cultured with 5×10^5 PKH26 labeled x-irradiated HLA-2DR matched stimulator cells in the presence of the indicated heat inactivated serum samples (final 50%). Standard ^3H thymidine incorporation assays were also performed with 1×10^5 each of the responders and stimulators in presence of indicated sera. The results from peak responses on day 7 are depicted in (A). In parallel, flow cytometric analysis was also performed on day 7 and depicted in (B). Both pre- (TAC) and post- (SRL) conversion sera inhibited lymphoproliferation by ^3H -thymidine incorporation (A; $*=p<0.05$), but only the pre-conversion sera (TAC) significantly inhibited the generation of new $\text{CD4}^+\text{CD127}^-\text{CD25}^{\text{High}}\text{FOXP3}^+$ Tregs ($**=p<0.01$) (B). To minimize variation between experiments, the data are depicted as percentage of medium control. Only

13 MLRs were performed because the amount of pre- and post-conversion sera available from the remaining seven patients was inadequate to conduct these experiments.

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Table 1

Pre-Conversion Clinical Characteristics (n=20)

	Mean $\pm$ SD	Median (Range)
Age (years)	59.2 $\pm$ 7.9	58 (44–71)
Gender	13 M; 7F	
Caucasian Race	19 (95%)	
Cause of Liver Disease:		
HCV (non-viremic)	4 (20%)	
Alcohol	7 (35%)	
Cryptogenic/Fatty Liver	8 (40%)	
α -1 antitrypsin deficiency	1 (5%)	
Months post-LT	42.8 $\pm$ 25.1	40.8 (9.6–104.6)
Months on TAC monotherapy before conversion	24.4 $\pm$ 26.7	39.6 (1.5–100.1)
Mean trough TAC level at conversion (ng/ml)	6.3 $\pm$ 1.5	5.6 (2.3–7.7)
Other immunosuppressive therapy*		
Induction at LT (alemtuzumab)	5 (25%)	
Number on prior prednisone post-LT	15 (75%)	
Months on prednisone post-LT	5.4 $\pm$ 3.2	6 (3–12)
Number on mycophenolate mofetil post-LT	14 (70%)	
Months on mycophenolate mofetil post-LT	20.7 $\pm$ 16	16.9 (6.1–59.4)

* Other immunosuppressive therapy (prednisone, mycophenolate mofetil) was discontinued three months prior to study enrollment (see inclusion criteria)

Table 2

Clinical and Histological Outcomes of TAC to SRL conversion (n=20)

	Pre-conversion (TAC)	Post-conversion (SRL)	p value
Blood Pressure (mmHg)			
Systolic	130.9 ± 12.8	135.4 ± 20.7	0.15
Diastolic	76.5 ± 8.4	78.8 ± 10.8	0.25
Complete Blood Count			
White count (10 ³ /μL)	5.0 ± 1.8	4.7 ± 1.8	0.26
Hemoglobin (g/dL)	12.9 ± 1.4	12.9 ± 1.7	0.49
Platelet (10 ³ /μL)	159.7 ± 56.1	164.3 ± 58.7	0.46
Renal/Electrolyte			
eGFR (ml/min/1.73 m ²) by MDRD	45.9 ± 7.9	49.5 ± 10.8	0.02
BUN (mg/dL)	26.6 ± 11.3	22.9 ± 9.2	0.001
Creatinine (mg/dL)	1.49 ± 0.3	1.42 ± 0.2	0.03
Urine protein:creatinine ratio	0.08 ± 0.04	0.29 ± 0.46	0.05
Serum potassium (mEq/L)	4.6 ± 0.6	4.3 ± 0.46	0.03
Liver function			
Total bilirubin (mg/dL)	0.76 ± 0.23	0.6 ± 0.25	0.0001
ALT (units/L)	25.8 ± 11.4	31.4 ± 14.6	0.001
Alkaline phosphatase (units/L)	73.2 ± 33.0	76.7 ± 30.1	0.16
Hemoglobin A1c (%)	5.4 ± 0.6	5.6 ± 0.6	0.15
Mean trough SRL level (ng/mL)	n/a	6.4 ± 2.1	n/a
Cholesterol (mg/dL)			
Total	157 ± 34.1	179.2 ± 40.8	0.07
Low density lipoprotein	92.8 ± 32.8	114.5 ± 34.5	0.03
High density lipoprotein	39 ± 8.9	40.5 ± 11.9	0.51
Triglycerides	126.1 ± 73.8	180.2 ± 98.7	0.0003
Body Mass Index (kg/m ²)	29.9 ± 5.7	30.6 ± 5.3	0.62
Liver Histology			
Normal	10 (50%)	6 (30%)	0.33
Steatosis <30%	5 (25%)	5 (25%)	0.1
Mild portal lymphocytes	5 (25%)	7 (35%)	0.73
Steatosis <30% + mild portal lymphocytes	0	4 (20%)	0.11

Table 3

Proteomic Profile Changes with Tacrolimus to Sirolimus Conversion

	Fold Change (Post vs. Pre)	p-value
Interleukin-7	-8.83	0.0037
Interleukin-3	-3.49	0.0053
Interleukin-13	-2.24	0.0026
Pulmonary and Activation-Regulated Chemokine	-1.82	2.15E-06
Ferritin	-1.66	0.0115
Trefoil Factor 3	-1.43	0.0020
CD5	-1.43	0.0104
Macrophage Inflammatory Protein-1 alpha	-1.38	9.14E-05
Sex Hormone-Binding Globulin	-1.36	0.0066
Interleukin-18	-1.32	0.0049
CD 40 antigen	-1.31	0.0095
TRAIL-R3	-1.25	0.0008
AXL Receptor Tyrosine Kinase	-1.20	0.0097
Vascular Cell Adhesion Molecule-1	-1.16	0.0107
Complement C3	1.22	0.0022
Haptoglobin	1.37	0.0057
Serum Amyloid P-Component	1.38	0.0001
Cancer Antigen 125	1.40	0.0050
Factor VII	1.43	0.0011
Matrix Metalloproteinase-3	1.43	6.42E-05
Glutathione S-Transferase alpha	2.07	0.0020
Pancreatic Polypeptide	2.65	0.0068