Published in final edited form as:
Hum Immunol. 2009 March ; 70(3): 146–150. doi:10.1016/j.humimm.2008.12.008.

Immunoregulatory profiles in liver transplant recipients on different immunosuppressive agents

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Abstract

We compared peripheral blood immunophenotyping in 31 adult liver transplant recipients on differing long-term immunosuppressive (IS) monotherapy with and without peri-transplantation alemtuzumab (AL) induction. All patients had been stable on monotherapy with either sirolimus (SRL) (n = 10) or without SRL (tacrolimus (TAC) (n = 10), mycophenolate mofetil (MMF) (n = 11)) for more than 6 months. Five-color flow cytometry for putative "regulatory" T and dendritic cells as well as serum assays for soluble HLA-G (sHLA-G) were performed. The SRL monotherapy group had significantly higher percentages of $CD4+CD25^{high+}$ Foxp3+ T cells (1.3 ± 1.0) compared with the non-SRL group (0.7 ± 0.6) (p = 0.04). The SRL effect was even higher in a subset with prior AL induction and no prior hepatitis C or rejection (1.7 ± 0.2) compared with all other subgroups (0.7 ± 0.6) (p = 0.02). TAC patients showed significantly higher "regulatory" DC2:DC1 ratios (10 ± 7.6) compared with non-TAC patients (4.1 ± 2.3) (p = 0.04). Although sHLA-G levels appeared higher in TAC patients, the differences were not statistically significant. In conclusion, IS monotherapy provides an opportunity to investigate regulatory roles of individual agents. SRL maintenance and prior AL induction in subsets of patients appeared to show a regulatory T cell immunophenotype. However, TAC patients may have other regulatory characteristics, supporting the need for larger, prospective studies to clarify differences.

Keywords

Regulatory T cells; Dendritic cells; Immunophenotyping; Liver transplantation; Immunosuppression

1. Introduction

A small number of centers in the past have reported some success with withdrawal of immunosuppression (IS) in recipients after liver transplantation (LT) [1–9]. There are also a few more recent reports of successfully withdrawn (operationally tolerant) patients

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associated with a genomic or immunophenotypic signature in peripheral blood [10,11]. However, only approximately 20% of patients in these weaning protocols were able to successfully withdraw from IS. Ideally, specific assays testing immunoregulation would be performed before withdrawal as a predictive measure of successful withdrawal, although these have been imprecise [12]. Some recently include immunophenotyping of "regulatory" dendritic cells (*i.e.*, DCregs; high plasmacytoid (DC2): myeloid (DC1) ratio, ILT3 and ILT4 expression), regulatory T cells (*i.e.*, CD4+CD25^{high+}FOXP3+), donor-specific cytokine production, measurement of soluble HLA-G (a nonclassical regulatory HLA), microchimerism testing, genomic arrays, and characterization of liver tissue immunocytes [10,11,13–16].

Previous laboratory studies have more definitively demonstrated a difference in specific maintenance IS agents in promoting an immunoregulatory, unresponsive, or tolerant state. In some studies, it was proposed that regulatory T cells require interleukin (IL)–2 for their activation [17]. Inhibition of IL-2 production by calcineurin inhibitors (CNIs) such as tacrolimus (TAC) may adversely affect this process. Indications of the counterregulatory effects of CNI therapy include inhibition of FOXP3 expression, lack of preservation of the CD27+ subset of CD4+CD25+ T cells, and lack of inhibition of dendritic cell maturation [18–20]. In contrast, sirolimus (SRL) and mycophenolate mofetil (MMF), the former specifically inhibiting the downstream effects of IL-2, appear to be associated with a more immunoregulatory state, either alone or in the context of costimulatory blockade or IL-10 treatment. This is characterized by decreased IFN-γ–producing CD4+ and CD8+ cells, increased percentages of CD4+CD25^{high+} and CD8+ FOXP3+ T regulatory cells, and inhibition of the maturation and function of DCs [21–26]. In addition, some peri-transplantation induction antibodies such as alemtuzumab (AL) are also thought to promote an immunoregulatory state [27,28].

This suggests that selection of a specific IS agent (induction or maintenance) may be important in the development of immuno-regulation and in the future potential for IS minimization or withdrawl, especially in patients who have been clinically immunoquiescent, although few previous clinical studies are available. Patients receiving IS monotherapy are ideal candidates for investigating the role of each specific agent. Therefore, the purpose of this pilot study was to examine the difference in immunoregulatory profiles in LT recipients receiving IS monotherapy (SRL, MMF, or TAC) with or without AL induction at the time of LT.

2. Subjects and methods

2.1. Patients, treatment modalities, and sample collection

Liver transplant recipients stable on IS monotherapy (SRL, MMF, or TAC) were identified from the organ transplant database at Northwestern. Inclusion criteria were as follows: 1) age ≥18 years; 2) orthotopic deceased or living related LT recipients; 3) more than 6 months with stable graft function on current monotherapy (SRL, MMF, or TAC); 4) more than 1 year post-LT without an acute rejection episode; and 5) normal liver function tests (no evidence of recurrent viral infection, chronic rejection, or hepatitis). Patients were excluded if they had received more than one LT or other organ, had graft dysfunction of any etiology,

or had inadequate data or follow-up. The protocol was approved by the Institutional Review Board at Northwestern.

Before 2003, all of the patients received TAC and prednisone (with or without MMF) therapy immediately after transplantation. Between 2003 and 2006, non-HCV recipients by protocol (nonrandomized) received a steroid-free regimen of AL induction (30 mg IV immediately postoperatively) followed by TAC and MMF therapy. Eventual conversion to monotherapy occurred at a mean of 2.7 ± 1.3 years post-LT as described below. Conversion to SRL and MMF monotherapy was performed for nephrotoxicity related to TAC.

Peripheral blood was collected and PBMCs were immunophenotyped as described below. In addition, serum concentrations of sHLA-G (units/ml) were determined. Updated clinical history, laboratory values, appropriate 12-hour TAC and SRL trough levels, and MMF dosing were recorded at the time of this collection.

2.2. Testing procedures

2.2.1. T lymphocyte immunophenotyping—Five-color flow cytometry was performed on PBMCs isolated by Ficoll Hypaque gradient centrifugation. Using the Coulter 5×500 Flow Cytometer (Beckman Coulter, Fullerton, CA), T cells were enumerated by extracellular staining for CD4 FITC and CD25 APC or CD8 FITC, CD28 APC, and CD127 PC7 (E-Bioscience, San Diego, CA) After fixation and permeabilization, the cells were washed and blocked for nonspecific binding sites using normal rat serum. Anti-human FOXP3 PE (E-Bioscience) or rat IgG2a PE isotype control (E-Bioscience) was added for additional incubation. Samples were analyzed by gating based on CD4+ (or CD8+) expression and then extracting a dot plot of CD25 (or CD28) against CD127. FOXP3 expression was then calculated as the percentage of CD4+ $CD25^{high}CD127^{low}$FOXP3+ (or CD8+CD28-$CD127^{low}$FOXP3+) cells. In most cases, FOXP3+ cells corresponded to the CD4+ $CD25^{high}CD127^{low}$ cell population [29]. Histograms of FOXP3 were plotted to monitor for the level of expression (median channel fluorescence) and to compare differences in positive T-cell numbers among different patients.

2.2.2. Dendritic cell immunophenotyping—From 1×10^6 Ficoll-Hypaque isolated PBMC, dendritic cells were identified as lineage negative (CD3 (Tcell), CD14 (monocyte), CD19 (B cell), and CD56 (NK cell); all were FITC labeled (E-Bioscience). HLA-DR–positive cells, ECD labeled (Beckman Coulter), were stained for CD123 or CD11c, both PC7 labeled (E-Bioscience). The latter were used to distinguish between plasmacytoid versus myeloid dendritic cells. The different cell subsets were further stained for CD205 PE and CD83 APC (E-Bioscience) as putative markers for DC functional capacity; or with ILT4 PE (R&D systems, Minneapolis, MN) and ILT3 PC5 (Beckman Coulter) as markers of putative regulatory DCs. Isotype control cocktails were used in parallel. Data were collected and analyzed using the CXP analysis software. A minimum of 10,000 DCs were counted to ensure statistical significance at the 100-cell/specimen level (giving an accuracy of 1 cell per 1500 at the 95% confidence level) [30].

2.2.3. Soluble HLA-G—Serum samples were incubated in microtiter wells coated with mouse monoclonal anti-human sHLA-G antibody (HLA-G1 and HLA G5) using a

commercially available kit (Biovendor, Modrice, Czech Republic) and following the manufacturer's recommendations. Mouse monoclonal anti-human beta-2 microglobulin antibody conjugated to horseradish peroxidase was used as the secondary antibody. The addition of H_2O_2 tetramethylbenzidine allowed a color reaction to take place, with absorbance measured spectrophotometrically at 450 nm. Values (units/ml) were compared with a standard curve, and the concentration of the unknown samples was determined.

2.3. Statistical analysis

Because our data illustrated non-Gaussian distribution resulting from the small sample size, we used nonparametric statistics for the data analysis. The Kruskal-Wallis test was used to determine differences in immunoregulatory profiles among the TAC, SRL, and MMF groups. For the *post hoc* analysis, the Wilcoxon rank sum test was used to conduct pairwise comparisons. A separate subanalysis was performed in patients receiving prior AL induction therapy without hepatitis C or prior rejection, as early post-transplantation IS management strategies differed in these latter subpopulations before monotherapy was introduced. Three patients were excluded from lymphocyte immunophenotyping and one patient from dendritic cell immunophenotyping because of improper blood processing. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Patient demographics

Of the 31 LT recipients enrolled (Table 1), compared with patients receiving MMF ($n = 11$) and TAC ($n = 10$), those receiving SRL ($n = 10$) had a significantly shorter interval after LT ($p = 0.01$) and less time on monotherapy ($p = 0.0002$). Patients who received AL induction ($n = 10$) also had a shorter interval after LT ($p = 0.008$), less time on monotherapy ($p = 0.0005$), and fewer months on prednisone ($p = 0.0008$) (see above). Overall, no other significant differences in patient demographic and clinical characteristics were found between study groups.

3.2. Lymphocyte immunophenotyping

Table 2 displays full lymphocyte immunophenotyping information comparing the monotherapy groups and patients who did or did not receive AL per transplant induction therapy. Overall, the percentage of CD4+CD25^{high+}FOXP3+ T cells trended toward being higher in the SRL group (1.3 ± 1.0) compared with the TAC (0.5 ± 0.6) or MMF groups (0.8 ± 0.7) ($p = 0.08$), but was significantly higher than the non-SRL group (TAC and MMF combined) (0.7 ± 0.6) ($p = 0.04$). No differences were seen among the mono-therapy and AL groups in other markers associated with regulatory profiles (CD28 ^{low}, CD127^{low}).

Even years after AL induction, the percentage of CD4+ cells was significantly lower in this group (24.9 ± 17 vs. 35.8 ± 13, $p = 0.04$), suggesting a prolonged effect of this induction agent. In AL-induced patients without a history of HCV or rejection ($n = 6$), there was a trend toward a higher percentage of CD4+CD25^{high+}FOXP3+ cells compared with the non-AL group ($n = 10$) (1.2 ± 0.6 vs. 0.7 ± 0.6, $p = 0.09$). Within this small group, the AL-induced patients on SRL monotherapy ($n = 3$), however, had a significantly higher

percentage of CD4+CD25^{high+}FOXP3+ cells compared with all other groups (n = 13) (1.7 ± 0.2 vs. 0.7 ± 0.3, p = 0.02).

3.3. Dendritic cell immunophenotyping

The ratio of CD123+ to CD11c (DC2:DC1) trended toward being higher in the TAC group (10.0 ± 7.6) compared with those on either SRL (3.5 ± 1.1) or MMF monotherapy (4.6 ± 2.9) (p = 0.08) (Table 2) but was significantly higher than the non-TAC group (SRL and MMF combined) (4.1 ± 2.3) (p = 0.04). When patients without a history of HCV or rejection were separately analyzed, a similar trend in the DC2:DC1 ratios in the TAC (9.3 ± 3.9, n = 4) group compared with the SRL (3.7 ± 0.8, n = 8) and MMF (4.1 ± 2.1, n = 6) groups was seen (p = 0.08).

3.4. Soluble HLA-G comparisons

Although patients on TAC monotherapy appeared to have higher sHLA-G values (1.03 ± 1.0 units/ml) compared with those on SRL (0.45 ± 0.43 units/ml) or MMF monotherapy (0.53 ± 0.49 units/ml) or both (the latter groups combined), statistically significant differences were not present (p = 0.2) (Table 2). No differences in sHLA-G values were seen in patients with or without prior AL induction therapy.

4. Discussion

Our pilot study suggests that LT recipients on maintenance SRL monotherapy have a higher percentage of CD4+CD25^{high+}FOXP3+ T cells in the peripheral blood compared with recipients on non-SRL monotherapy. While other differences in patient demographics, such as multiple previous immunosuppressive regimens, might have influenced these findings, we consider it more likely that the long-standing nature of monotherapy overrode these variables. This supports prior nonclinical studies that have demonstrated an increase in regulatory T cells with the use of SRL [20–23], although no functional assessment was performed in the present report. While the data are limited to a small subset of patients, the highest percentage of CD4+CD25^{high+}FOXP3+ T cells was seen in the non-HCV non-rejection SRL monotherapy group with prior AL induction having lower total CD4+ cells, although these patients had less time from LT and on monotherapy. These preliminary findings lend support to the previously reported regulatory properties of AL observed *in vitro* and in other clinical studies, which may be enhanced by SRL [27,31,32].

Mechanistically, it is not surprising that both SRL and AL appear to have such effects on T cells. FOXP3 expression in T cells requires IL-2, and transcription is blocked by CNIs but not by SRL. In addition, CD25+ cells may be more resistant to AL-induced lymphodepletion [31]. Although CD25+ cells are also activated effectors and, when present early after transplantation, may be associated with a predisposition to rejection, the combination of AL induction and later conversion to SRL therapy from CNI may allow regulatory T cells eventually to predominate.

Interestingly, and somewhat unexpectedly, TAC monotherapy patients appeared to have higher DC2:DC1 ratios and (speculatively) sHLA-G levels. In laboratory studies, SRL and MMF are thought to inhibit the maturation of dendritic cells compared with CNIs, allowing

for regulatory immature dendritic cells (DC2) to develop [33–35]. In clinical studies, however, patients receiving low-dose TAC in a weaning protocol were found to have high regulatory dendritic (DC2) cell populations compared with those on higher dose maintenance TAC [36], although comparisons with other IS agents (SRL, MMF) were not performed. Regarding sHLA-G, one study noted a higher level in kidney recipients on TAC with a lower rate of rejection compared with patients receiving other IS (cyclosporine, azathioprine, MMF, SRL) [37]. Certainly, elevated DC2:DC1 ratios and sHLA-G levels might be associated with other regulatory effects of TAC that are not observed with SRL or MMF.

The strengths of our study include the analysis of LT recipients with stable graft function on monotherapy (thus potentially isolating the regulatory properties of IS agents). However, there are also several study limitations. First, serial samples were not performed from the time of transplantation, during or after IS changes, or even after the first blood collection. This allows a cross-sectional analysis of these profiles of only limited robustness. Second, our small sample size and associated large standard deviations limit the power to detect differences in other assay measurements and in the subgroup analyses; a number of demonstrated trends are not of statistical significance, perhaps related to the sample size. Third, patient characteristics and immunologic factors may have affected the comparability of the monotherapy groups and possibly the immunoregulatory profiles, including differences in time from LT, length of monotherapy, and corticosteroid use. Finally, other assays not performed, such as functional (donor-specific or non–donor-specific) or genomic assays, might have provided further information on the immunoregulatory status of these patients. Therefore, although our pilot study does not settle the controversy over which IS agent might promote immunoregulation, it points to the promise that further, prospectively obtained data will clarify these discrepancies.

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Table 1

Patient characteristics

Monotherapy agent	**TAC (*n* = 10)**	**SRL(*n* = 10)**	**MMF (*n* = 11)**	**AL (*n* = 10)**	**No AL(*n* = 21)**
Age (years)	57.8 ± 8.2	56 ± 15.9	63.3 ± 8.2	59.5 ± 10.7	61.9 ± 7.6
Gender	4M;6F	3M;7F	11M;0F	5M;5F	13M;8F
Cause of liver disease					
HCV	3	0	1	0	4
ETOH	0	2	5	2	5
Immune	2	3	0	3	2
Cryptogenic	4	2	3	3	7
HBV	0	1	2	1	2
Other	1	2	0	1	1
Years post LT	4.5 ± 2.4	3.6 ± 1.9*	6.4 ± 2.8	3.3 ± 0.8*	5.8 ± 2.9
Years on monotherapy	3.9 ± 1.3	1.5 ± 0.7*	2.7 ± 0.9	2.0 ± 0.9*	3.2 ± 1.5
Prior alemtuzumab induction	2	5	3	10	n/a
No. with prior rejection	2	2	4	2	6
Years from last rejection[a]	5.1 ± 0.9	3.0 ± 0.9†	8.1 ± 1.3	2.3 ± 1.0†	7.6 ± 1.9
Mean trough level orMMFdose (g/day)	6.3 ± 2.4	6.2 ± 1.8	2.0 ± 0.4	TAC 4.1 ± 0.7 SRL 6.2 ± 1.9 MMF 1.8 ± 0.3	TAC 6.6 ± 2.3 SRL 7.0 ± 1.7 MMF 2.1 ± 0.5
Prior therapy[b]					
Cyclosporine	0	2	7	3	6
TAC	n/a	9	6	10	17
SRL	2	n/a	1	4	6
MMF	3	8	n/a	10	12
Months on prednisone	12.9 ± 7.3	5.7 ± 4.6‡	20.5 ± 20.6	3.8 ± 4.6‡	18.2 ± 14.2

[a] Data refer only to those patients who had prior rejection in the row above.

[b] Until monotherapy was established, multiple maintenance agents were utilized.

* $p = 0.0005$ SRL vs. MMF; $p = 0.001$ AL vs. no AL.

** $p = 0.0008$ SRL vs. MMF; $p = 0.0001$ SRL vs. TAC; $p = 0.01$ AL vs. no AL.

† $p = 0.02$ SRL vs. MMF; $p = 0.01$ AL vs. no AL.

‡ $p = 0.04$ SRL vs. MMF; $p = 0.0005$ AL vs. no AL.

Table 2

Lymphocyte/dendritic cell immunophenotyping and soluble HLA-G: monotherapy and alemtuzumab comparisons

Lymphocyte	TAC (*n* = 9)	SRL(*n* = 9)	MMF (*n* = 9)	AL (*n* = 9)	No AL(*n* = 18)
%$CD4^+$	39.0 ± 14.8	25.5 ± 11.8	32.0 ± 16.4	24.9 ± 17	35.8 ± 12.9†
% $CD4^+$ $CD127^{lo}$ CD25+ Foxp3+ (from all lymph)	1.4 ± 0.6	1.3 ± 0.8	1.9 ± 1.8	1.5 ± 1.2	1.6 ±1.2
% $CD4^+$ $Foxp3^+$ (from all lymph)	4.6 ± 5.4	2.5 ± 1.9	2.9 ± 2.8	2.4 ± 1.9	3.8 ± 4.2
% $CD4^+$ $CD25^{high}$ Foxp3+ (from $CD4^+$ only)	0.5 ± 0.6	1.3 ± 1.0*	0.8 ± 0.7	1.0 ± 0.6	0.8 ± 1.0
$Foxp3^+$ (from all lymph)	5.0 ± 45	2.9 ± 2.1	3.4 ± 2.8	2.6 ± 2.0	4.3 ± 4.3
%CD8+ CD28-$Fox3^+$	0.2 ± 0.4	0.2 ± 0.2	0.1 ± 0.2	0.1 ± 0.2	0.2 ± 0.3
Dendritic cell	TAC (*n* = 9)	SRL(*n* = 10)	MMF (*n* = 11)	AL (*n* = 10)	No AL(*n* = 20)
%of$CD123^+$ Lin neg cells	17.7 ± 15.6	9.9 ± 11.6	14 ± 12.9	15.8 ± 16.4	12.7 ± 11.8
% of $CD11c^+$ Lin neg cells	2.5 ± 1.7	2.8 ± 2.8	3.8 ± 3.9	3.4 ± .0	2.9 ± 3.1
DC2:DC1 ratio	10.0 ± 7.6**	3.5 ± 1.1	4.6 ± 2.9	4.2 ± 2.1	6.7 ± 6.1
% of $CD123^+$, $CD83^+$/$CD123^+$	2.2 ± 3.2	2.3 ± 3.4	4.2 ± 4.9	2.1 ± 2.1	3.4 ± 4.6
% of $CD11c^+$, $CD83^+$/$CD11^+$	5.6 ± 6.6	7.5 ± 12.4	13.3 ± 16.5	6.8 ± 8.9	10.3 ± 14.5
% of $CD123^+$, $CD205^+$/$CD123^+$	0.9 ± 1.1	1.2 ± 1.8	1.2 ± 2.2	1.8 ± 2.6	0.8 ± 0.8
% of $CD11c^+$, $CD205^+$/$CD11^+$	0.3 ± 0.5	2.4 ± 2.4	0.5 ± 0.7	0.9 ± 1.2	1.1 ± 2.0
% of $CD123^+$, $1LT3^+$/$CD123^+$	28.4 ± 11.5	34.9 ± 14.2	20.3 ± 9.5	24.1 ± 12.0	29.3 ± 13.5
% of $CD123^+$, 1LT4+/$CD123^+$	3.6 ± 4.5	5.2 ± 4.9	2.9 ± 2.1	3.8 ± 2.6	3.9 ± 4.5
%of $CD11c^+$, $1LT3^+$/$CD11c^+$	54.2 ± 19.7	52.7 ± 15.7	35.6 ± 29.8	36.3 ± 24.3	52.2 ± 22.3
	TAC (*n* = 10)	SRL(*n* = 10)	MMF (*n* = 11)	AL (*n* = 10)	No AL(*n* = 21)
sHLA-G (units/mL)	1.03 ± 1.0‡	0.45 ± 0.43	0.53 ± 0.49	0.56 ± 0.39	0.69 ± 0.79

* p = 0.03 SRL vs. TAC; *p* = 0.08 SRL vs. MMF.

** *p* = 0.02 TAC vs. MMF; *p* = 0.007 TAC vs. SRL.

† p = 0.04 AL vs. no AL.

‡ TAC vs. SRL; *p* = 0.06 TAC vs. MMF.