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Ex vivo generation of regulatory T cells from liver transplant recipients using costimulation blockade

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Abstract

The potential of adoptive cell therapy with regulatory T cells (Treg) to promote transplant tolerance is under active exploration. However, the impact of specific transplant settings and protocols on Treg manufacturing is not well-delineated. Here, we compared the use of peripheral blood mononuclear cells (PBMC) from patients before or after liver transplantation to the use of healthy control PBMC to determine their suitability for Treg manufacture using *ex vivo* costimulatory blockade with belatacept. Despite liver failure or immunosuppressive therapy, the capacity for Treg expansion during the manufacturing process was preserved. These experiments did not identify performance or quality issues that disqualified use of post-transplant PBMC, the currently favored protocol design. However, as Treg input correlated with output, significant CD4-lymphopenia in both pre- and post-transplant patients limited Treg yield. We therefore turned to leukapheresis post-transplant to improve absolute yield. To make deceased donor use feasible, we also developed protocols to substitute splenocytes for PBMC as allostimulators. In addition to demonstrating that this Treg expansion strategy works in a liver transplant context, this preclinical study illustrates how characterizing cellular input populations and their performance can both inform and respond to clinical trial design and Treg manufacturing requirements.

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Supporting Information: Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Allogeneic liver transplantation outcomes have improved significantly, due in part to more efficacious immunosuppressive therapy (IST). However, pharmacologic IST utilization is accompanied by substantial rates of cardiovascular and renal damage, diabetes, infection, and therapy-related malignancies as well as socioeconomic costs of chronic medication dependence.^{1–6} Graft survival remains a problem.^{4,7} Therefore, alternatives producing similarly low rates of acute and/or chronic graft rejection while minimizing toxicities of chronic pharmacologic IST are desirable.⁸

Interest in using immunosuppressive immune populations to induce or support transplant tolerance^{8–12} has encouraged development of methods for expanding regulatory T cells (Treg), generally defined by a CD4⁺CD25^{hi}CD127^{lo}Foxp3⁺ phenotype, for administration to organ transplant recipients.^{9,10,12–25} We and others have demonstrated that: 1) potent, alloantigen-specific Treg from healthy donors can be generated using antibodies to CD80 and CD86 or first (abatacept) or second (belatacept) generation CTLA4Ig fusion proteins to induce costimulatory blockade (CSB) during an *ex vivo* mixed lymphocyte reaction (MLR)^{14,20,23}; and 2) CSB-MLR supports Treg expansion using peripheral blood mononuclear cell (PBMC) responders from end-stage renal failure patients and healthy control (HC) or HLA-mismatched healthy donor PBMC stimulators.^{13,15,18,26}

Liver transplantation (LTx) presents an attractive option for Treg administration.^{8–10,17,19,27,28} The liver itself supports an immunosuppressive state,^{29–31} and LTx recipients can achieve persistent operational tolerance, at low frequency but higher than with other solid organs.^{8,27,31–34} Nonetheless, translational development of Treg adoptive transfer for LTx has been challenging. In comparison to HC donors of PBMC used to develop cell manufacturing strategies, end-stage liver failure (ESLD) patients are frequently leukopenic, lymphocytopenic, and demonstrate immunologic abnormalities potentially influenced by ESLD etiology and duration.^{35–41} Conditions accompanying ESLD, such as coagulopathy and ascites, can complicate PBMC collection, adversely affecting both patient safety and product characteristics.^{42,43} The unknowns of donor availability and timing pose relevant issues for study procedures, including consent, cell collection and manufacturing. Post-LTx patients, whose donor type, ESLD etiology, IST, alloantigen exposure and infections can modulate immune function,^{1–3,5–7,44–52} also present challenges related to collection and storage of liver donor stimulators for subsequent manufacturing, PBMC collection, and potential intercurrent rejection.

In summary, context-specific medical issues, responder and stimulator cell properties, and procedures for cell collection and storage have potential to impact the outcome of cell manipulation strategies established using healthy volunteers. We therefore investigated the suitability of PBMC collected from patients prior to (pre) or post-LTx as substrates for manufacturing Treg using *ex vivo* CSB and examined what methodologic modifications could address the challenges of deceased donor use and improving Treg yield.

Material and Methods

Study Participants.

Individuals with ESLD, pre- or post-LTx, were recruited at Massachusetts General Hospital (MGH) from 02/2016 to 05/2019 and 02-06/2021. To support an Investigational New Drug application for an Immune Tolerance Network study “A Phase I/II Drug Withdrawal Study of Alloantigen-Specific Tregs in Liver Transplantation” (LITTMUS, ITN073ST), eligibility largely paralleled that protocol, prioritizing individuals meeting standard LTx criteria, aged 18–70 years, seronegative for HIV-1/2, within 8 months post-LTx (the anticipated manufacturing window) and excluded those with autoimmune etiology. During the COVID-19 pandemic, this was relaxed to accommodate decreased clinic visits by post-LTx patients. All participants provided written informed consent (Partners Human Research Committee Institutional Review Board (IRB)-approved study 2016P000214).

In total, 122 patients enrolled and provided peripheral blood (PB) samples. Nineteen with inadequate cell yield and 5 post-LTx patients beyond the 8-month window were excluded. As only 4 post-LTx patients received IST including sirolimus (RAPA), their results were excluded from overall results and reported separately. Table 1 shows characteristics of the remaining 94 patients. The single patient with both pre- and post-LTx samples collected is counted in each group. Because of cytopenia and/or protocol limitations on sample volume, some samples contained insufficient PBMC for all determinations. Rejection episodes had occurred post-LTx in 20% (10/49). HC PBMC were obtained from deidentified volunteer PB or platelet-donor pheresis collars (DF/Partners Cancer Center IRB approved protocol 05–321). Spleens were obtained from deceased organ donors (MGH IRB approved protocol 2013P000392/1). Except for the clinical trial patient, the spleen donor differed from the future (for pre-LTx) or actual (for post-LTx) liver donor.

Cell collection and preparation.

PBMC were isolated by gradient centrifugation using Ficoll-Paque (GE Healthcare, Piscataway, NJ), as previously described^{15,23} and splenocytes (SPL) were isolated from deceased donor spleens. Details are provided in Methods S1.

Ex vivo belatacept-mediated costimulatory blockade (CSB) during MLR.

MLRs with belatacept-mediated CSB were established as described previously^{15,23} with minor modifications as described in Methods S2.

Flow Cytometry

Measurement of Treg frequency.—Treg frequencies were determined in unmanipulated PBMCs (baseline) and after 3 days in CSB-MLR by flow cytometry (gating strategy, Fig. S2; antibodies, Table S1) as described previously¹⁵ and detailed in Methods S3.

Intracellular cytokine staining.—Interleukin-2 (IL-2) response to T cell stimulation was assessed using flow cytometry as described in Methods S4.

TCR signaling assessment by phospho-flow cytometry.—PBMC were stimulated with 1 µg/mL anti-CD3 (clone UCTH1) and anti-CD28 (clone CD28.2) and then processed following BD Phosflow protocol for human PBMCs (BD Bioscience) as detailed in Methods S5.

FOXP3 Treg-specific demethylated region (TSDR) methylation analysis.—Treg were isolated from HC and post-LTx responder PBMC at baseline (unstimulated) or derived from primary CSB-MLRs. Untouched CD4⁺T cells were pre-enriched using EasySep Human CD4⁺ T cell negative selection kit (StemCell Technologies, St. Louis, MO) then stained with CD4, CD25, and CD127 antibodies and a 7-AAD viability dye. Viable Treg (7AAD-CD4⁺CD25^{hi}CD127⁻) and control conventional (CD4⁺CD25⁻) T cells (Tconv) were sorted (Sony SH800z Cell Sorter, Sony Biotechnology, San Jose, CA), flash frozen in liquid nitrogen and submitted to EpigenDx Inc. (Hopkinton MA) for TSDR assay (Assay ID: ADS783FS2). For female samples, values were corrected by a factor of 2 as one TSDR allele is methylated due to X-inactivation.⁵³

Real-time PCR.—Total RNA was isolated from PBMC and analyzed as detailed in Methods S6.

Treg-mediated Suppression.—Treg-mediated suppression was assessed by adding purified CSB-Treg to a fresh *in vitro* MLR followed by flow cytometry analysis. CSB-Treg were purified from new (modified) CSB-MLRs using HC or post-LTx patient responder PBMC with EasyStep Human CD4+CD127low CD25+Regulatory T cell Isolation kit (StemCell Technologies, Vancouver, USA), per manufacturer's instructions. By flow cytometry, isolated Treg purity was always above 90%. Fresh untreated autologous responder PBMCs were labeled with CellTrace™ (CTV) Proliferation Kit (Life Technologies) prior to co-culture, per manufacturer's instructions. Purified CSB-Treg were added to an *in vitro* MLR containing 10⁵ CTV-labeled autologous responder PBMCs and equal numbers of γ-irradiated stimulators (i.e. same stimulator used to generate the CSB-Treg) in U-bottom 96-well plates. Treg:responder ratios of 1:5 and 1:10 were evaluated. Triplicate wells were set up for each condition. After 7–8 days, cells were stained and analyzed using flow cytometry (antibodies, Table S1) to assess CD8⁺ T cell proliferation (CTV dilution), activation (CD25 and/or HLA-DR expression) and cytotoxicity (perforin+granzymeB expression). Preliminary experiments using HLA-A2 discordant responder:stimulator pairs confirmed the absence of live stimulators at day 7–8. Percentage suppression was calculated as $100 \times [1 - (\text{untreated responders} + \text{Treg}) / (\text{untreated responders alone})]$.

Statistics

Statistical analysis was performed using GraphPad Prism v8 (GraphPad, La Jolla, CA). Two-tailed unpaired or paired t tests were performed throughout. Unpaired t tests did not assume equal variance. Welch's correction was applied where appropriate. F test was used to compare variances. Pearson's r was calculated to assess correlation. A p value of <0.05 was used to reject the null hypothesis.

Results

Quantitative and qualitative characteristics of PB lymphocytes and Treg in individuals with before and after liver transplantation

Our prior Treg expansion studies mostly used responder PBMC from healthy (stem cell transplant or HC) donors unlikely to have significant T cell defects. Studies report quantitative and qualitative immune abnormalities in individuals with ESLD.^{29,35–41} Post-LTx patients experience immunomodulation via IST, alloantigen exposure, potential rejection and/or infection.^{1,6,7,44,46,47,50,54,55} To determine whether T cells from individuals pre- and/or post-LTx were suitable for our manufacturing strategy, we first evaluated lymphocyte yield, phenotype and several relevant parameters of T cell immunity.

Pre- and post-LTx patients have decreased T cell and Treg counts.—Pre- and post-LTx populations had similar median PBMC concentrations falling within expected HC range (Fig. 1A).⁵⁶ In comparison to HC (median $\times 10^6$ cells/mL 0.862 and 95% CI 0.751–1.103), pre- (0.403, 0.356–0.560) and post-LTx (0.465, 0.396–0.648) samples exhibited significant lymphopenia (Fig. 1B). All groups had similar CD19⁺B cell concentrations (Fig. 1C). However, in comparison to HC (median $\times 10^6$ cells/mL 0.593, 95% CI 0.519–0.792), pre- (0.231, 0.199–0.338) and post-LTx (0.230, 0.194–0.348) samples had significantly decreased CD3⁺T cell concentrations (Fig. 1C). Relative to HC (median $\times 10^6$ cells/mL 0.371, 95% CI 0.302–0.441), pre- (0.160, 0.143–0.247) and post-LTx (0.130, 0.129–0.219) samples had decreased CD4⁺T cells (Fig. 1D). Relative to HC (median $\times 10^6$ cells/mL 0.191, 95% CI 0.159–0.237), pre- (0.038, 0.041–0.082) and post-LTx (0.055, 0.050–0.125) samples also had lower CD8⁺T cell concentrations. Pre- and post-LTx concentrations of CD3⁺, CD4⁺ and CD8⁺T cells were similar.

Treg frequency (Fig. 1E) was significantly greater in pre- (median 4.47, 95% CI 4.029–5.181) and lower in post-LTx (median, 2.29, 95% CI 2.032–2.896) samples than in HC (median 3.085, 95% CI 2.642–3.998). Post-LTx samples also had lower Treg frequency than pre-LTx. However, reflecting their CD4⁺ lymphopenia, median Treg concentration ($\times 10^3$ cells/mL, Fig. 1F) was decreased in pre- (median 5.67, 95% CI 5.93–10.55) and post-LTx (median 2.920, 95% CI 2.747–5.025) compared to HC (median 10.75, 95% CI 8.774–15.59). This was significant for post-LTx samples with a trend observed for pre-LTx. Post-LTx Treg concentration was decreased in comparison to pre-LTx. Notably, the variance in the Treg frequency (Fig. 1E) was greater in pre-LTx (1.917) than post-LTx (1.351) samples ($p=0.0285$) as was the variance in Treg concentration (Fig. 1F) (pre-LTx 7.855 vs post-LTx 3.738, $p<0.0001$).

T cell receptor (TCR) signaling and IL-2 production are preserved in PBMC from pre- and post-LTx patients.—As MLR depends on TCR and costimulatory signaling,^{57,58} we used anti-CD3/CD28 antibodies to provide stimulation and assessed several key signaling molecules by phospho-flow cytometry. T cells in all groups responded with increased phosphorylation of proximal (Akt) and distal (Erk and S6) TCR pathway signaling molecules (Fig. 2). Results in HC and post-LTx were similar with a median 2–3-fold increase in phosphorylation in CD4⁺ and CD8⁺T cells. Median increase in

phosphorylation was greater (2–6-fold) in pre-LTx samples. This was significant for all comparisons in CD4⁺T cells. In CD8⁺T cells, there was no significant difference between pre-LTx and HC, while the pre- vs post-LTx difference reached significance for pAkt and pErk but not pS6.

IL-2 production is critical to Treg expansion and function.^{58–64} By RT-qPCR, baseline IL-2 expression by PBMC (median, range: HC 0.33, 0.11–1.01; pre-LTX 0.33, 0.08–8.23; post-LTx 0.30 0.14–1.52) showed no significant differences (HC vs pre-LTx $p=0.3516$, HC vs post-LTx $p=0.2164$, pre-LTx vs post-LTx $p=0.4796$). IL-2 expression rose in response to anti-CD3/anti-CD28 antibody stimulation in all groups (median, range: HC 2.06, 0.17–6.42; pre-LTX 4.31, 0.25–18.39; post-LTx 1.097, 0.26–12.25). The fold increase (Fig. 2C) was similar overall with trend for higher response in pre-LTx samples. We also evaluated the frequency of IL-2 producing cells after PMA/ionomycin stimulation. As CD4⁺ Treg do not produce IL-2,^{65,66} we assessed non-Treg CD4⁺ and CD8⁺T cells (Fig. 2D). IL-2 producing non-Treg CD4⁺T cell frequency (median, 95%CI) was similar in all groups: HC (33.70, 24.00–53.70), pre- (48.60, 39.8–61.80) and post-LTx (44.30, 23.91–55.29). IL-2 producing CD8⁺T cell frequency was also similar: HC (24.80, 14.68–35.49), pre- (30.20, 17.27–46.96) and post-LTx (19.20, 10.77–46.28). There were no significant differences in pre- vs post-LTx non-Treg CD4⁺ or CD8⁺ IL-2 producing cell frequency.

Belatacept-mediated CSB-MLR reduced alloproliferation and supported Treg expansion by both HC and post-LTx PBMC responders

The most likely LITTMUS trial design favored Treg production from recipient PBMC collected post-LTx. As no contraindications to using post-LTx responders for Treg manufacturing had emerged, we next compared effects of belatacept-mediated CSB-MLR on HC and post-LTx PBMC responders. Notably, despite post-LTx IST, alloproliferation of post-LTx PBMC responders in MLRs without CSB was very similar to HC ($p=0.7508$). Addition of belatacept reduced alloproliferation in both HC and post-LTx responders (Fig. 3A) with significantly greater inhibition using post-LTx (median 82.7%, range 66.2–95.6) than HC (median 71.0%, range 39.4–97.95) PBMC.

Treg frequency at baseline (Fig. 3B,C) was lower in post-LTx (median 2.04%, range 0.24–7.86) than HC (median 2.92%, range 1.56–4.75) CD4⁺ PBMC. After CSB-MLR, Treg frequency increased significantly in both groups (Fig. 3B,C) with no significant difference in final frequency between HC (median 4.04%, range 2.54–7.81) and post-LTx (median 5.07%, range 1.6–7.32). Baseline Treg frequency correlated with the frequency after CSB-MLR in both HC and post-LTx responders ($r=0.8308$ with $p<0.001$, and $r=0.6236$ with $p=0.0228$, respectively). There was no correlation between baseline values and fold-change (HC: $r=0.1153$ with $p=0.6093$; post-LTx: $r=-0.2861$ with $p=0.3674$). Treg from both groups expressed increased surface CD25 and intracellular Foxp3 (Fig. 3D). Pre-LTx PBMC produced similar results (Fig. S3), although median (1.27-fold) Treg expansion by pre-LTx responders was significantly less than HC (1.53, $p=0.015$) or post-LTx (2.38, $p=0.047$).

Adapting and optimizing belatacept-mediated CSB as a manufacturing strategy for Treg generation in the setting of liver transplantation

Most LTx are performed using deceased donor organs, making collection of PBMC stimulators for Treg manufacturing problematic. We therefore evaluated thawed allogeneic splenocytes (SPL, processed and cryopreserved after organ procurement) as stimulators. We also determined whether altering CSB-MLR conditions could enhance Treg production.

SPL can be substituted for PBMC stimulators in CSB-MLR generation of Treg.

—Primary MLRs and CSB-MLRs were set up using HC PBMC responders and either HC PBMC or SPL as stimulators (stimulators came from different individuals as paired acquisition was not feasible from HC or deceased donors). CSB-mediated inhibition of proliferation using SPL or HC PBMC was comparable (Fig. 4A: SPL median 79.23%, range 55.97–92.30 vs PBMC median 70.39%, range 39.40–108.0). SPL stimulators also supported similar increases in Treg frequency (Fig. 4B: SPL median 1.96-fold increase (range 0.77–2.430) vs PBMC median 1.54-fold increase (range 1.11–3.50) ($p=0.207$)).

Modified CSB-MLR conditions augment Treg yield.—Production of sufficient Treg in a feasible, cost-effective manner required reduction of CSB-MLR coculture volume and increased yield from a given cell input. Increases in cell density (from 1×10^6 to 5×10^6 cells/mL) and responder:stimulator ratio (1:1 to 2:1) were screened to determine the most promising (“new”) conditions (1.5×10^6 total cells/mL; 2:1). No significant difference in CSB-mediated inhibition of MLR proliferation was observed using standard or new conditions with PBMC or SPL stimulators (Fig. 5A).

Treg expansion under new conditions (Fig. 5B) was comparable using either PBMC or SPL stimulators (median 1.61-fold increase [range, 2.03–1.40] vs 1.51 [range, 2.37–0.89], respectively, $p=0.36$). Treg produced under both conditions using either stimulator had similar Foxp3 MFI, a correlate of Treg suppressive capacity (Fig. 5C).⁶⁷ Moreover, high TSDR demethylation status was sustained in Treg isolated after CSB-MLR under both conditions and using either stimulator (Fig. 5D). Natural Treg isolated before CSB-MLR had highly demethylated FOXP3 TSDR (median 93.6%, range 78.5–93.9, $n=5$). The TSDR in CSB-Treg remained highly demethylated (condition:stimulator cell type: Standard:PBMC [98.4%, range 94.9–101.8 ($n=2$)]; Standard:SPL [94.8%, range 88.9–100.7 ($n=2$)]; New:PBMC [95.0%, range 88.0–100.3 ($n=5$)]; New:SPL [90.4.0%, range 61.1–102.3 ($n=5$)]).

New CSB-MLR coculture conditions support Treg expansion in post-LTx

PBMC.—To evaluate these modifications in the trial context, CSB-MLRs using new conditions were conducted with three post-LTx PBMC responders. CSB produced consistent inhibition of MLR proliferation (Fig. 6A: median 74%, range 66–88%), increased CD25^{hi}Foxp3⁺ Treg frequency (Fig. 6B: median increase 1.74-fold, range 1.15–2.32) and increased Treg surface CD25 (Fig. 6C). As observed in HC (Fig. 5C), intracellular Foxp3 MFI in CSB-Treg from post-LTx PBMC responders was increased (Fig. 6D). These CSB-Treg also demonstrated high TSDR demethylation (Fig. 6E) comparable to CSB-Treg from

HC [post-LTx 94.0%, range 86.7–102.0 (n=3) vs HC 90.4.0%, range 61.1–102.3 (n=5); p-value = 0.3977].

More limited characterization of CSB-Treg generation using PBMC from 4 patients on RAPA-containing IST (Fig. S4) was consistent with the larger dataset.

CSB-Treg from HC and post-LTx PBMC effectively suppress autologous CD8⁺ T cells.

—Alloreactive T cells, particularly cytotoxic CD8⁺ T cells, are implicated in immune-mediated tissue damage and graft rejection and can be modulated by Treg.^{68–71} To assess the functionality of CSB-Treg generated from post-LTx (using the new conditions), we evaluated their capacity to suppress autologous CD8⁺ T using an *in vitro* MLR between the original (autologous) responder and γ -irradiated stimulator SPL pair. We observed negligible proliferation of CTV-labelled CD8⁺ T cells within post-LTx responder PBMC alone whereas brisk proliferation of CD8⁺ T cells was observed in MLR co-cultures with corresponding increases in the proportion expressing activation (CD25⁺ and/or HLA-DR⁺) and cytotoxicity-associated (granzymeB⁺perforin⁺) markers (Fig. 7A, columns R, RS). Similar results were observed using HC PBMC responders and stimulators (Fig. 7B, columns R, RS).

Addition of post-LTx CSB-Treg to MLRs at a ratio of 1_{Treg}:5_{Responders} results in robust suppression of autologous responder CD8⁺ T cells (Fig. 7A) with decreases (median, range) in proliferation (74.7%, 63.4–85.9%) and the percent expressing activation (73.0%, 73.0–73.1%) and cytotoxic (66.4%, 60.7–72.1%) markers. These results were comparable to those observed with addition of HC CSB-Treg to HC responders [decreases in proliferation (78.6%, range 38.4–88.8%) and the percent expressing activation (84.4%, range 66.4–86.4%) and cytotoxic (49.6%, range 23.3–75.2%) markers] (Fig 7B). Similar but slightly decreased suppression was observed when responder PBMC from LTx patients or HCs were cultured at 1_{Treg}:10_{Responders}, suggesting a dose-response effect (Fig. 7 A,B). These data show that in the *in vitro* setting CSB-Treg derived from post-LTx PBMC provided potent suppression of autologous alloreactive cytotoxic CD8⁺ T cell responses.

Use of oxygen permeable tissue culture bags permits scaled up manufacture of CSB-MLR Treg.—In all studies above, CSB-MLRs were performed in culture flasks or plates.¹⁵ As considerable volume is required to manufacture Treg in greater numbers, we evaluated use of oxygen permeable tissue culture bags with 32–510 ml/bag capacity. CSB-MLR were conducted in either flasks or bags (Fig. S5) using same HC responder:stimulator pairs and standard conditions (1×10⁶ cells/mL; 1:1 responder:stimulator ratio; PBMC stimulators). Pair-to-pair comparison resulted in similar CD4⁺ responder cell viability, Treg frequency within CD4⁺, as well as Foxp3 MFI expression. We next evaluated the new conditions (1.5×10⁶ cells/mL; 2:1 responder:stimulator ratio; SPL stimulators) in 32 ml tissue culture bags, using HC PBMC responders. Frequencies of Treg of expected phenotype increased during CSB-MLR.

Optimized CSB-MLR with post-LTx leukapheresis PBMC produces a Treg product suitable for clinical administration.—When using post-LTx responders, we of necessity relied on stimulator PBMC or SPL that were not identical to the liver donor.

Here, we briefly describe the manufacturing outcome of the first patient enrolled in the LITTMUS trial, which used post-LTx PBMC responders from the organ recipient and banked SPL stimulators from the organ donor. Recipient responder PBMC were obtained 5 months post-LTx by leukapheresis, isolated by density gradient in CMCF, and used fresh. Using 17 510C culture bags, 9×10^9 recipient PBMC were cocultured with 4×10^9 40 Gy-irradiated donor SPL (total 1.5×10^6 /mL; 1.7:1 responder:stimulator ratio) in the presence of belatacept. 1.3-fold Treg expansion was measured in the pooled washed CSB-MLR product (Fig. 8). Surface CD25 and intracellular Foxp3 MFI increased, and 93.6% of expanded CD25^{hi}CD127⁻ Treg expressed Foxp3. Despite significant losses during column-based Treg selection, final Treg yield increased 1–2 log compared to standard conditions using venipuncture-acquired PBMC in a renal transplant setting.¹⁸

Discussion

Studies detailing variable abnormalities in PBMC from ESLD patients pre- and post-LTx^{1–3,5–7,35–41,44–52,72} provide insufficient basis for predicting whether these characteristics would compromise CSB-MLR-mediated Treg manufacturing. Therefore, we evaluated pre- and post-LTx PBMC, from baseline characteristics through CSB-MLR manufacturing conditions, to assess feasibility and then improve outcome of CSB-MLR as a Treg production strategy for LTx.

Many animal models and *in vitro* studies describe IST effects, but fewer detail effects of *in vivo* chronic post-transplant IST exposure on human PBMC.^{3,32,46,50,54,55,72–75} We therefore used pre/post-LTx PBMC to examine immune functions, including response to activation and IL-2 expression, present in HC cells in which our strategy was developed and related to mechanisms by which: 1) CSB exerts specificity and engenders energy, and 2) Treg expand and persist.^{57–64} We found that individuals pre-LTx or on IST post-LTx could respond to anti-CD3/anti-CD28 or PMA/ionomycin stimulation and proliferate to alloantigen. Indeed, responses closely resembled those of HC. Our IL-2 expression data is consistent with Hartel et al⁷⁶ who found PBMC from HC and post-kidney transplant patients on IST demonstrated similar IL-2 mRNA expression at baseline and after anti-CD3/anti-CD28 stimulation. Notably, peak IL-2 expression was delayed in post-transplant samples, an analysis we could not perform as our samples were a volume-limited convenience sample whereas they acquired larger samples timed to IST administration. However, their work suggests additional timepoints should be adopted for future studies.

Our short duration CSB-MLR manufacturing process has many benefits – as previously described, it is fast, simple, produces Treg with significant allospecificity^{14,23} and is relatively economical as it requires few special reagents or tools. However, unlike longer *ex vivo* expansion approaches,^{9,10,12,13,16–19,21,22,24,25} the shorter process provides less time for Treg expansion and likely underlies the direct correlation between baseline values and final Treg yield. Lower baseline Treg concentrations were not associated with decreased relative expansion. This suggested greater input could compensate for the lower expansion, prompting a decision to obtain responder PBMC by leukapheresis rather than phlebotomy. During CSB-MLR, Treg expansion from post-LTx samples was significantly greater than observed with pre-LTx and comparable to HC. As persuasive clinical considerations favored

use of post-LTx PBMC, and absent clear contraindications to their use, we prioritized examination of post-LTx samples in process modification. Interestingly, while the trial and manufacturing strategy are very different, Sanchez-Fueyo et al¹⁷ recently reported improved Treg production using PBMC obtained by leukapheresis from patients on IST post-LTx rather than by venipuncture pre-LTx. Notably, we observed multiple pre-LTx assessments were more variable than were post-LTx, seemingly reflecting the inconsistent Treg quantitation reported in ESLD.^{35,39–41} Pursuing this observation was lateral to our aims but may require investigation in some manufacturing settings.

The decision to use leukapheresis engendered manufacturing changes, including transitions to large oxygen-permeable culture bags and moderate increments in cell concentration and ratio. Larger deviations resulted in increased cell death and decreased Treg yield. Similarly, use of deceased donors required rethinking the stimulator population and evaluating how SPL preparation processes affected manufacturing outcome. While selection or depletion of SPL subpopulations might improve results, the goal was to design a rapid and simple process that could minimize cost and effort, avert manufacturing failures potentiated by longer, more complex approaches and facilitate dissemination. Modified CSB-MLR conditions reproduced prior results¹⁵ with both HC and post-LTx samples yielding Treg with increased Foxp3 MFI and highly demethylated TSDR signatures, characteristics associated with suppressive activity and functional stability. HC and post-LTx CSB-Treg also demonstrated significant capacity to suppress autologous responder proliferation and expression of activation and cytotoxic molecules.

In summary, together with many other considerations favoring their use, these studies provide support for use of post-LTx PBMC as a manufacturing substrate to generate Treg via CSB-MLR. Moreover, this procedure can be adapted to accommodate the practical demands of using deceased donors and improving Treg yield without compromising its attractive simplicity and rapidity. This stepwise translational process illustrates the importance of assessing the demands of each cell therapy setting and reconfiguring manufacturing processes to address the identified limitations and opportunities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability:

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Abbreviations:

Az	azathioprine
CM	complete media
CsA	cyclosporine
CSB	costimulatory blockade
ESLD	end-stage liver disease
Evr	everolimus
HC	healthy control
IL-2	interleukin-2
IST	immunosuppressive therapy
LTx	liver transplantation
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
MTX	methotrexate
P	prednisone
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
RAPA	sirolimus
SPL	splenocyte
TAC	tacrolimus
Tconv	conventional T cells
TCR	T cell receptor
Treg	regulatory T cells
TSDR	Treg-specific demethylated region

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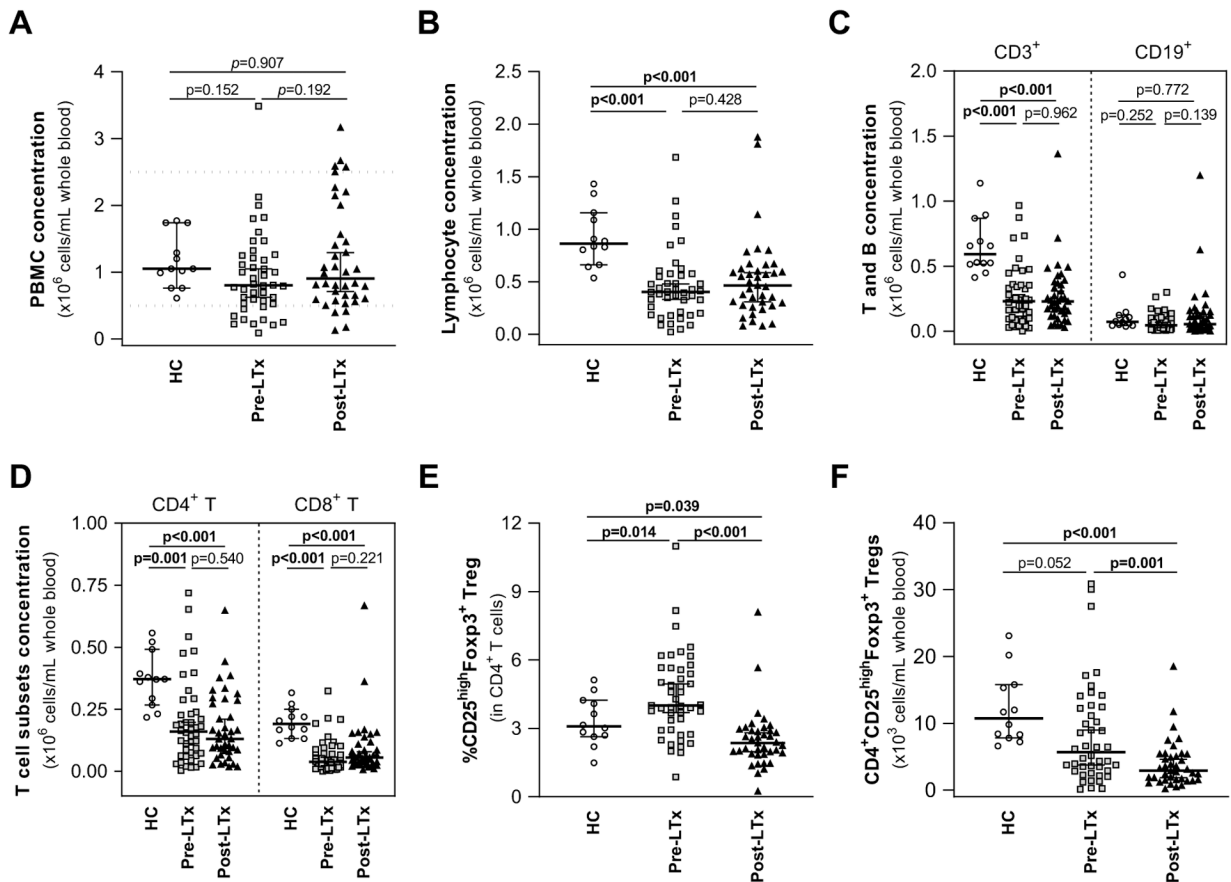


Figure 1. Pre- and post-LTx patients show T cell lymphopenia and decreased absolute numbers of Treg.

PBMC were isolated from whole blood of pre-LTx (n=44) and post-LTx patients (n=39), and HC donors (n=12).

(A) The absolute number of PBMC/mL was calculated using trypan blue staining and manual cell count. Values were similar for all groups. Dotted lines represent the normal range of healthy adult subjects.⁷²

(B-F) Flow cytometry was used to assess the concentration (cells/ml of whole blood) of (B) lymphocytes, (C) CD3⁺ and CD19⁺ lymphocytes, (D) CD4⁺ and CD8⁺ T cells. Pre- and post-LTx patients demonstrated significant lymphopenia, decreased CD3⁺, CD4⁺ and CD8⁺ T cells in comparison to HC. Differences in (E) CD25^{high}Foxp3⁺ Treg frequency were observed. (F) The concentration (cells/ml of whole blood) of CD25^{high}Foxp3⁺ Treg within CD3⁺CD4⁺ T cells in pre- and post-LTx patients was decreased in comparison to HC. Data show the median \pm 95% CI. P-values were calculated using unpaired t-test with Welch's correction. Significant p-values are highlighted in bold. LTx: liver transplant; HC: healthy controls; PBMC: peripheral blood mononuclear cells; Treg: regulatory T cell.

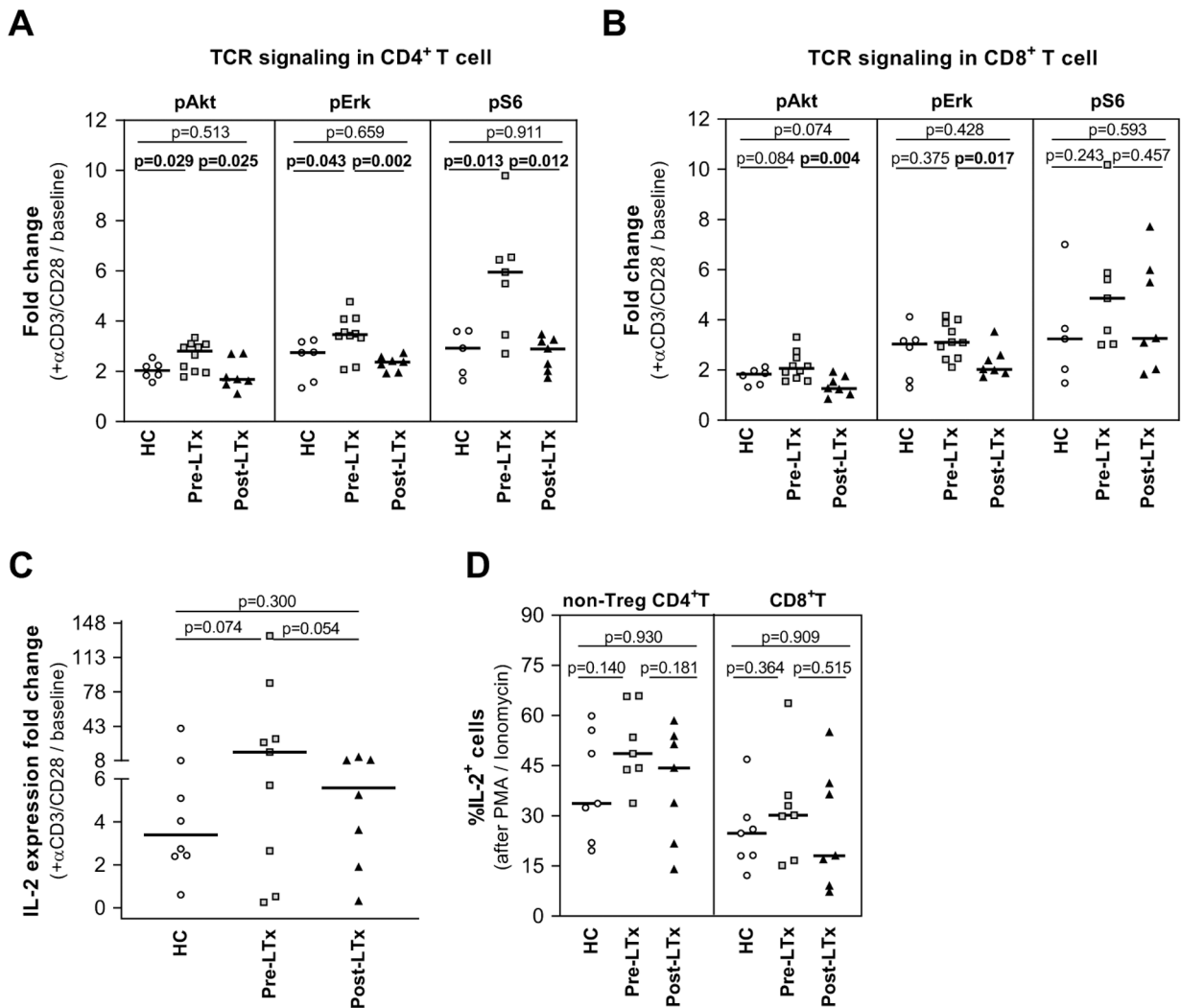


Figure 2. T cell receptor signaling, and IL-2 production are preserved in PBMC from pre- and post-LTx patients.

(A-C) PBMC were stimulated with anti-CD3/CD28 antibodies for 6h. (A-B) The integrity of TCR signaling was studied using phospho-flow cytometry to assess phosphorylated Akt, Erk, and S6 signaling molecules, in (A) CD4⁺ and (B) CD8⁺ T cells. In all cases, HC and post-LTx cells generated similar results whereas values in pre-LTx were consistently higher in CD4⁺, but not CD8⁺, pre-LTx cells. Values represent the fold change to baseline of each stimulated responder (HC=6, pre-LTx=10, post-LTx=7). (C) IL-2 production was evaluated using real time PCR. The IL-2 fold change vs unstimulated cells demonstrated similar results for all groups, with a trend to greater increase in pre-LTx (HC=8, pre-LTx=9, post-LTx=7). (D) PBMC stimulated for 4–6h with PMA/Ionomycin were analyzed by flow cytometry to determine the frequency of IL-2⁺ in non-Treg CD4⁺ and CD8⁺T cells. Results were equivalent in all groups (n=7/group).

Results represent the median. P-values were calculated using unpaired t-test with Welch's correction. Significant p-values are highlighted in bold. HC: healthy controls; PBMC: peripheral blood mononuclear cells; TCR: T cell receptor; LTx: liver transplant.

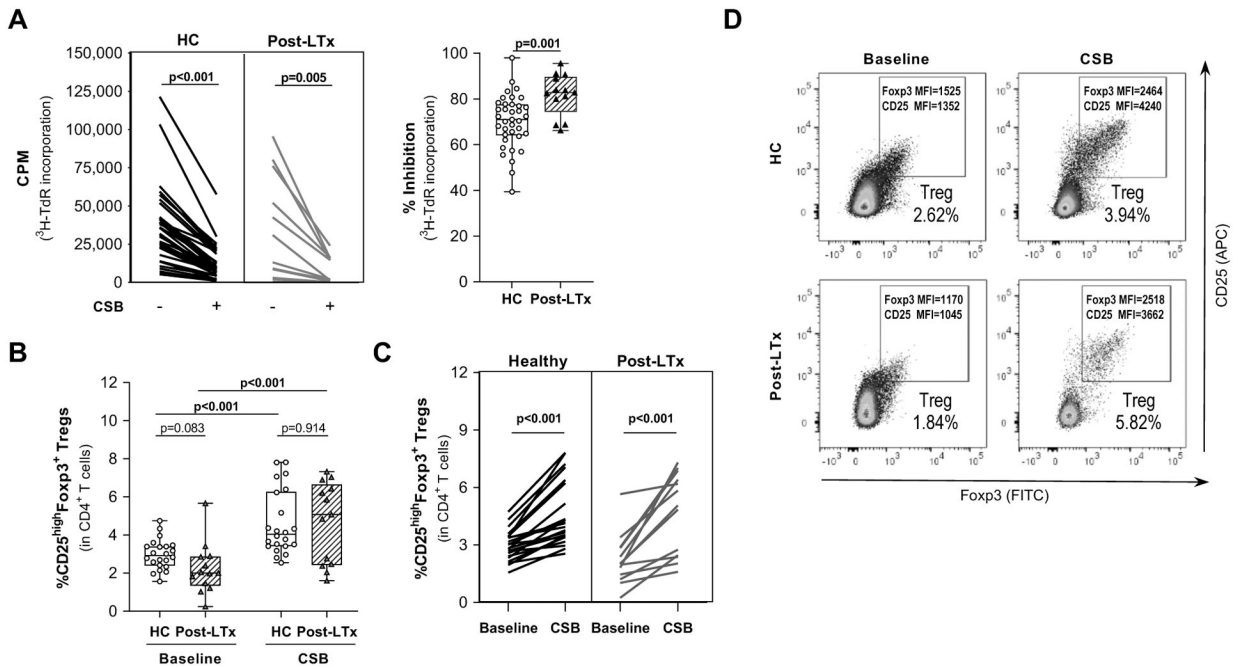


Figure 3. Belatacept-mediated CSB during primary MLR reduces alloproliferation and supports Treg expansion by post-LTx PBMC responders.

Responder PBMC from HC and post-LTx patients were co-cultured with irradiated PBMC stimulators in the presence or absence of belatacept-mediated CSB.

(A) Proliferation was measured by ^3H -thymidine incorporation on day 6 of primary MLR. Left panel shows pairwise proliferation data for each sample in the presence (+) or absence (–) of belatacept-mediated CSB. Right panel shows aggregate data with median % inhibition of proliferation. CSB inhibited proliferation in both groups. The median degree of inhibition was greater in post-LTx samples (HC=34, post-LTx=22).

(B–D) Frequency of Treg (CD25^{high}Foxp3⁺) within CD3⁺CD4⁺ T cells was assessed by flow cytometry prior to MLR and after primary CSB-MLR (denoted CSB) All panels: HC=22, post-LTx n=13. (B) The median Treg increase was similar for HC and post-LTx samples as was the final proportion of Treg in CD4⁺ cells. (C) The expansion of Treg for each pair is shown. (D) Representative flow cytometry plots demonstrated the marked increase in Foxp3 and CD25 MFI after CSB.

Data show the median \pm maximum/minimum range. P-values were calculated using paired t-test or unpaired t-test with Welch's correction. Significant p-values are highlighted in bold. HC: healthy controls; PBMC: peripheral blood mononuclear cells; CSB: costimulatory blockade; MLR: mixed lymphocyte reaction; LTx: liver transplant; cpm: counts per minute; Treg: regulatory T cell; MFI: mean fluorescence intensity

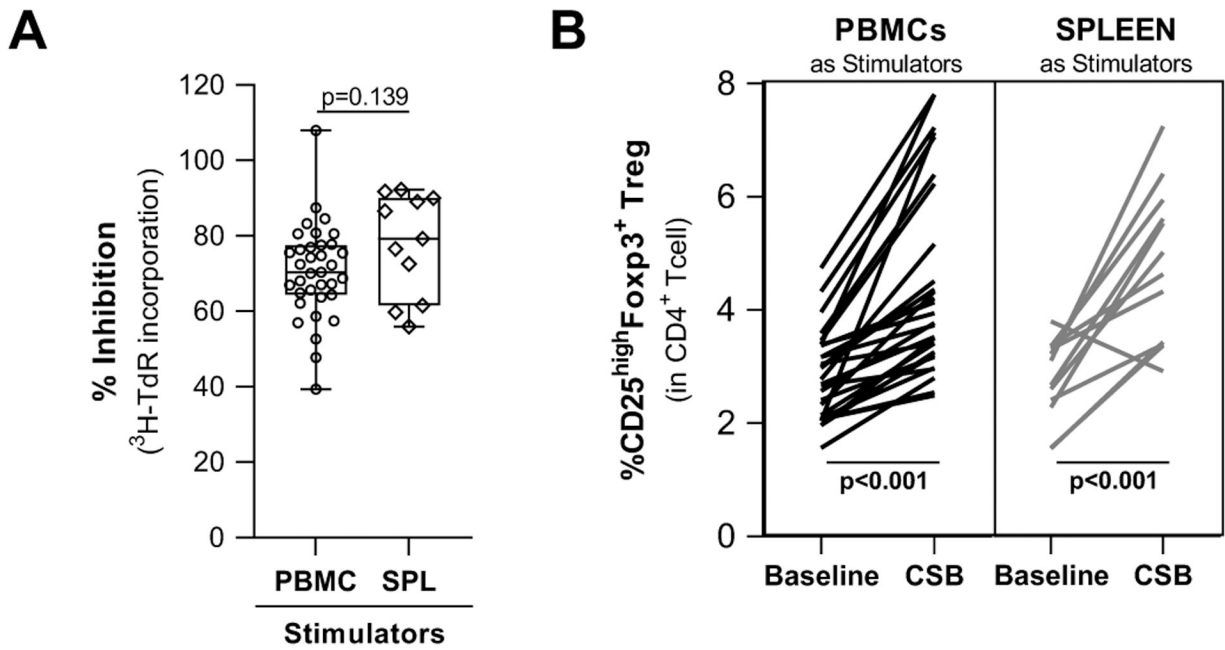


Figure 4. The use of splenocytes as stimulators supports CSB-mediated expansion of Treg in healthy PBMCs.

HC were co-cultured with irradiated PBMC or SPL as stimulators in the presence of belatacept-mediated CSB.

(A) Percent inhibition of proliferation (measured by ^3H -thymidine incorporation) on day 6 of primary CSB-MLR was comparable using PBMCs or SPL as stimulators. (HC=35, post-LTx=11)

(B) Frequencies of CD25^{high}Foxp3⁺ Treg expressed as percentage of CD3⁺CD4⁺ cells prior to (baseline) and after primary CSB-MLR (denoted CSB) indicated similar Treg expansion using PBMC or SPL as stimulators. (HC=28, post-LTx=12)

Horizontal lines are medians, boxes are 25th and 75th percentiles, and whiskers are maximum and minimum values. P-values were calculated using unpaired t-test with Welch's correction (A) and paired-t test (B) with significant p-values highlighted in bold. HC: healthy controls; MLR: mixed lymphocyte reaction; CSB: costimulatory blockade; SPL: splenocytes; Treg: regulatory T cell; MFI: mean fluorescence intensity

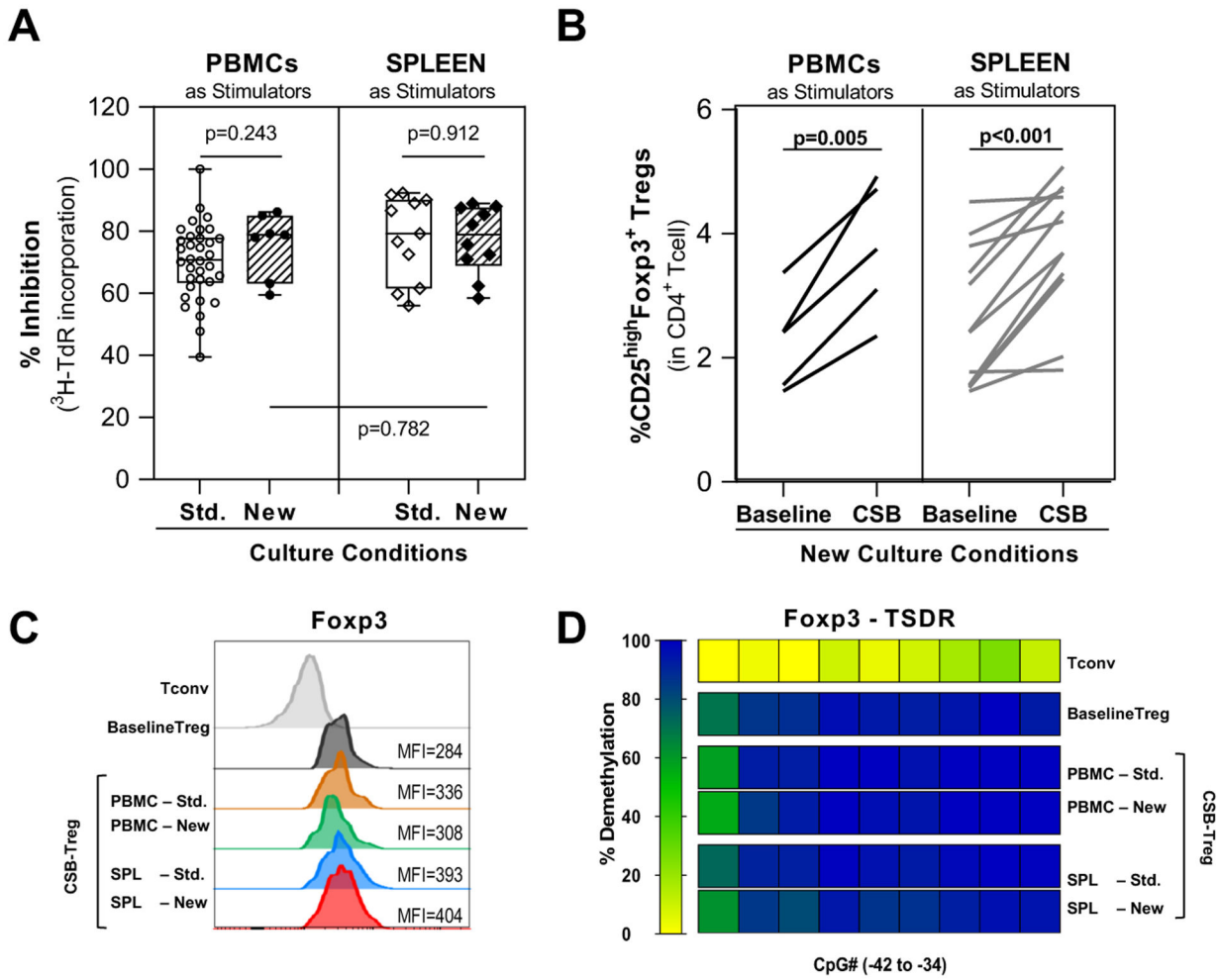


Figure 5. New CSB-MLR coculture conditions support scale up of Treg expansion in healthy PBMC

HC PBMC were co-cultured with irradiated PBMC or SPL stimulators in the presence of belatacept-mediated CSB using standard culture conditions (1×10^6 cells/mL and 1R:1S ratio) or new optimized conditions (1.5×10^6 cells/mL and 2R:1S ratio)

(A) Percent inhibition of proliferation (measured by ^3H -thymidine incorporation) on day 6 of primary CSB-MLR was not significantly different using standard or new CSB-MLR co-culture conditions with either PBMC (left) or SPL (right) as stimulators. (n=10–34)

(B) Under new CSB-MLR culture conditions, measurement of CD25^{high}Foxp3⁺ Treg frequency, expressed as percentage of CD3⁺CD4⁺ T cells, prior to MLR (baseline) and after primary CSB-MLR (CSB) demonstrated similar Treg expansion using PBMCs (n=5) or SPL (n=10) as stimulators.

(C-D) Characterization of CSB-MLR Treg manufactured using different culture conditions. Conventional CD4 T cells (Tconv) and natural Treg without CSB (baseline Treg) were used as controls. (C) Representative histograms and MFI values of the Treg marker Foxp3 in CSB-Treg (CD4⁺CD25^{high}Foxp3⁺) showed no differences in Foxp3 expression.

(D) TSDR demethylation in CSB-Tregs isolated by multicolor fluorescence-activated cell sorting (CD4⁺CD25^{high}CD127⁻). The demethylation rates were translated into a color code

from yellow (0%) through green (50%) up to blue (100%). Each rectangle represents the demethylation of one CpG motif. All CSB-Tregs showed comparable high demethylation pattern. (Tconv n=1; baseline Treg n=5; CSB-Treg n=2-5)

Horizontal lines are medians, boxes are 25th and 75th percentiles, and whiskers are maximum and minimum values. The p-values were calculated using unpaired T test with Welch's correction (A) and paired t-test (B) with significant p-values highlighted in bold. HC: healthy controls; MLR: mixed lymphocyte reaction; CSB: costimulatory blockade; SPL: splenocytes; Std: standard culture conditions; New: new optimized culture conditions; MFI: mean fluorescence intensity; Tconv: conventional CD4 T cells; TSDR: Treg-specific demethylated region; Treg: regulatory T cell

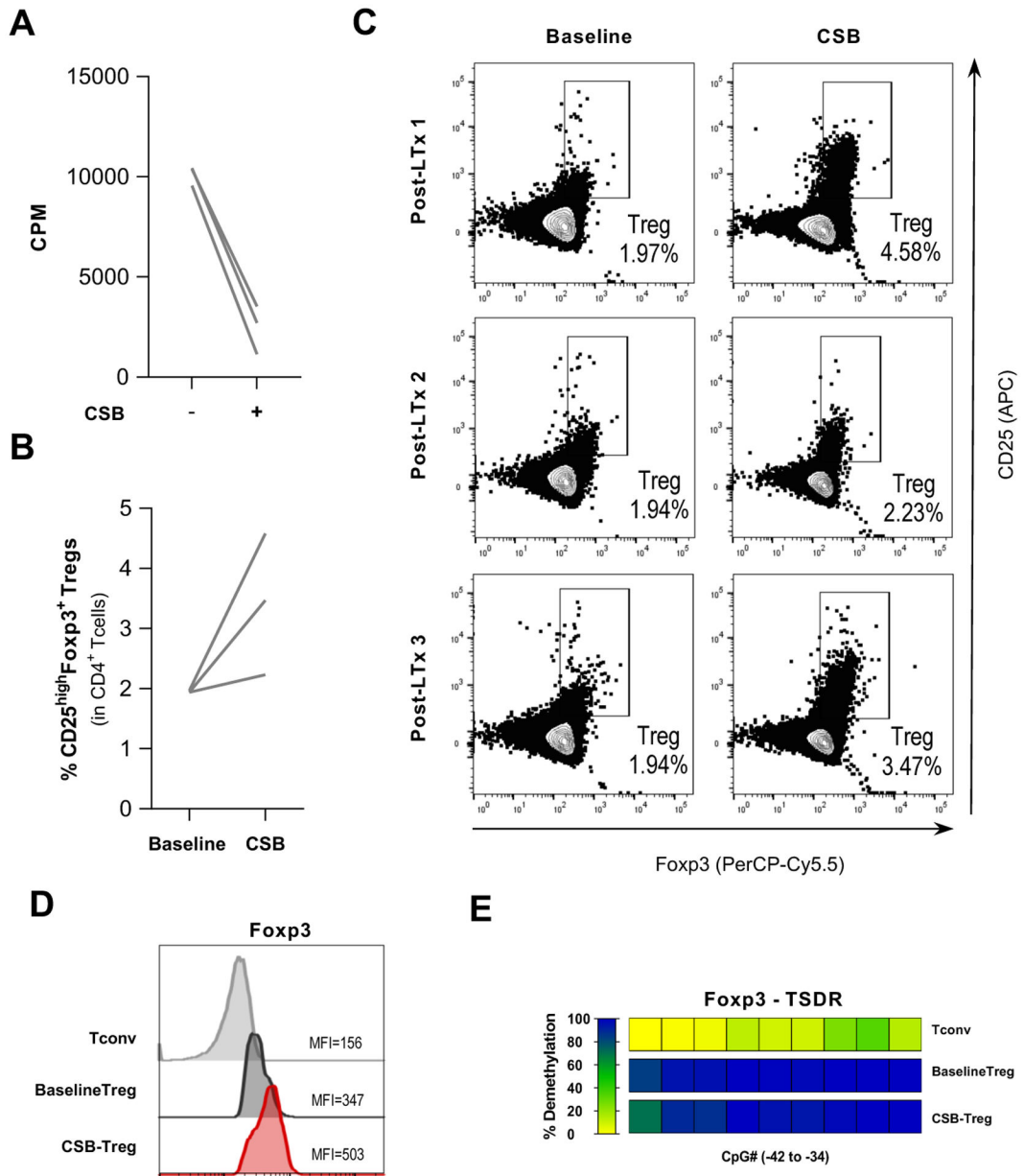


Figure 6. New CSB-MLR co-culture conditions with SPL as stimulators support Treg expansion in post-LTx PBMCs

PBMCs from post-LTx patients were co-cultured using new MLR conditions (1.5×10^6 cells/mL and 2R:1S ratio) with irradiated SPL as stimulators in the presence of belatacept-mediated CSB.

(A) CSB-MLR demonstrated expected decreases in proliferation, measured by ^3H -thymidine incorporation on day 6, showed an overall median percentage inhibition of 76% (range, 88 – 66%).

(B) Frequencies of CD25^{high}Foxp3⁺ Tregs expressed as percentage of CD3⁺CD4⁺ T cells at day 0 (baseline) and day 3 CSB-MLR (CSB) showed an overall 1.9-fold median Treg expansion.

(C) Individual flow cytometry plots demonstrate the increase in CD25 expression and the frequency of CD25^{high}Foxp3⁺ Tregs within CD3⁺CD4⁺ gate prior to MLR (baseline) and at day 3 after primary CSB-MLR culture (CSB).

(D-E) Characterization of CSB-MLR Treg from post-LTx PBMC samples. Conventional CD4⁺ T cells (Tconv) and natural Tregs without CSB (baseline Treg) from post-LTx patients were used as controls. (D) Representative histograms and MFI values of the Treg marker Foxp3 in post-LTx CSB-Tregs (CD4⁺CD25^{high}Foxp3⁺) showed high Foxp3 expression. (E) TSDR demethylation in CSB-Treg isolated by multicolor fluoresce-activated cell sorting (CD4⁺CD25^{high}CD127⁻). The demethylation rates were translated into a color code from yellow (0%) through green (50%) up to blue (100%). Each rectangle represents the demethylation of one CpG motif. Post-LTx CSB-Treg showed retention of high TSDR demethylation. (Tconv n=2; baseline Treg n=3; CSB-Treg n=3).

HC: healthy controls; PBMC: peripheral blood mononuclear cells; CSB: costimulatory blockade; MLR: mixed lymphocyte reaction; post-LTx: post liver transplant; SPL: splenocytes; cpm: counts per minute; Treg: regulatory T cell. MFI: mean fluorescence intensity; Tconv: conventional CD4⁺ T cells; TSDR: Treg-specific demethylated region; Treg: regulatory T cell

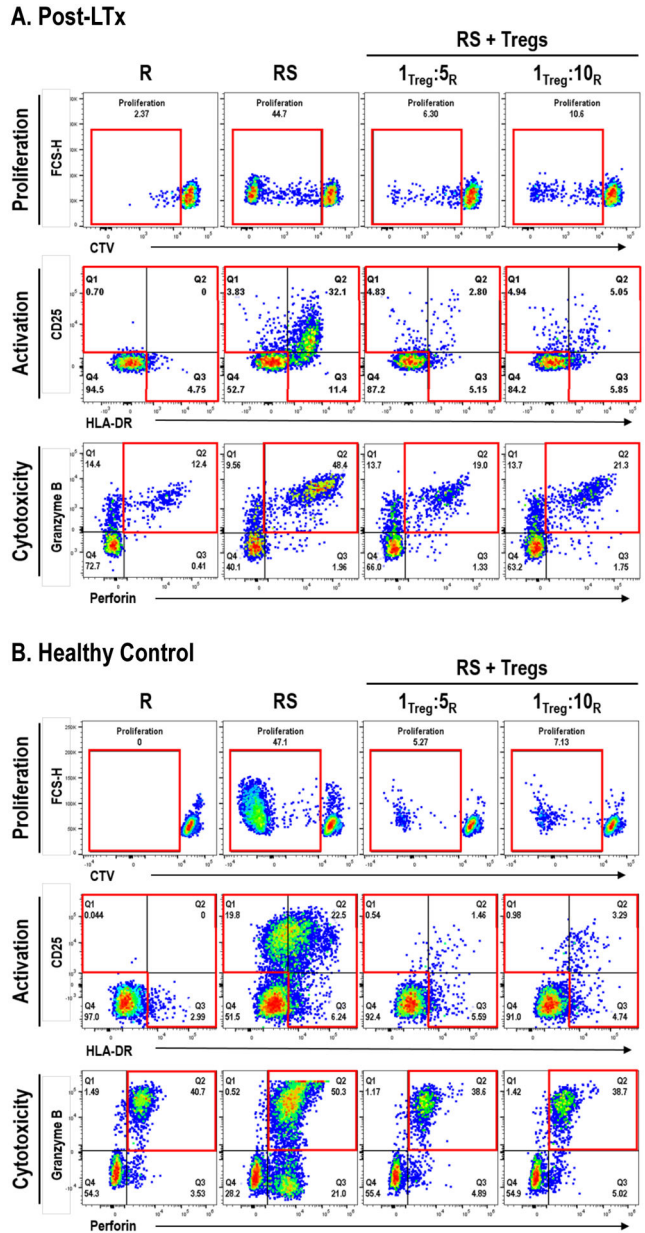


Figure 7. CSB-Treg from post-LTx and HC PBMC effectively suppress autologous CD8⁺ T cells. CSB-Treg from post-LTx patients (A) or HC samples (B) generated using the new conditions were evaluated for their capacity to suppress autologous CD8⁺ T cells using an *in vitro* MLR between the original (autologous) CTV-labeled responder PBMCs and the γ -irradiated stimulator pair. Different ratios of Treg:Responder were evaluated. At day 7, autologous responder PBMC cells were stained and analyzed using flow cytometry to assess CD8⁺ T cell proliferation (CTV dilution), as well as frequency of CD8⁺ T cells expressing activation (CD25⁺ and/or HLA-DR⁺) and cytotoxic markers (granzyme B⁺perforin⁺). Data is shown in representative flow cytometry plots from single experiments (independent triplicate experiments were performed for each condition, using n=3 post-LTx and n=3 HC samples). All samples were gated within CD3⁺CD8⁺ viable lymphocytes. Gates of interest

are highlighted in red. Resting responder cells (R) cultured for the same period were used as a control.

(A) CD8⁺ T cells from post-LTx samples showed little proliferation whereas they demonstrated significant proliferation, and an increased proportion expressed activation markers and cytotoxic markers after MLR (RS). Addition of CSB-Tregs to the MLR (Treg + RS) was associated with robust suppression of autologous responder CD8⁺ T cell proliferation and a decrease CD8⁺ T cells expressing activation and cytotoxic molecules. (B) Similar results to (A) were obtained using HC PBMC responders, stimulators and HC CSB-Treg.

R: responder PBMC; S: Stimulator SPL or PBMCs; RS: responder PBMCs cocultured with stimulator cells (SPL cells for post-LTx and PBMC for HC); RS+Tregs: RS plus autologous Treg (isolated from a CSB-MLR using the same R and S) at either 1_{Treg}:5_R or 1_{Treg}:10_R ratios. HC: healthy controls; PBMC: peripheral blood mononuclear cells; CSB: costimulatory blockade; post-LTx: post liver transplant; SPL: splenocytes; Treg: regulatory T cell; CTV: CellTrace™ dye; MLR: mixed lymphocyte reaction

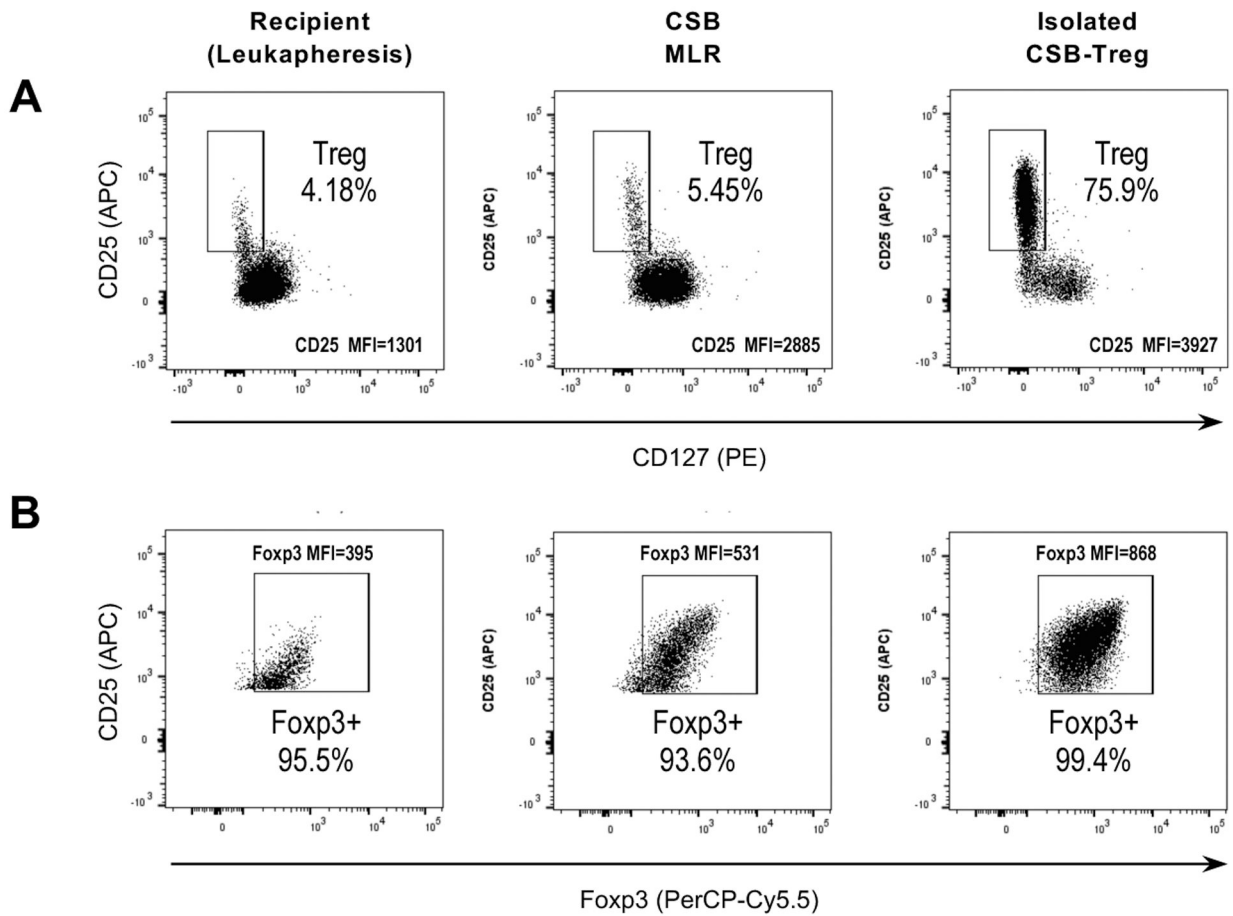


Figure 8. CSB-Treg manufacturing

Flow cytometry plots show the frequency of CD25^{high}CD127⁻ Tregs within CD3⁺CD4⁺ T cells (A) and the frequency of Foxp3⁺ cells within CD25^{high}CD127⁻ Treg (B), in the organ recipient PBMCs prior to MLR (baseline), after primary CSB-MLR culture (CSB), and in the final Treg product after Treg isolation using depletion and selection columns and magnetic beads. MLR: mixed lymphocyte reaction; CSB: costimulatory blockade; MFI: mean fluorescence intensity; Treg: regulatory T cell.

Table 1.

Demographic and clinical characteristics of study patients

	Pre-LTx patients*	Post-LTx
Number of patients	45	49
Age [Median \pm SD (range)]	58 \pm 9 (35–69)	57 \pm 11.5 (21–69)
Gender (Male : Female)	32 : 13	35 : 14
Race (% Caucasian)	93%	92%
Diagnosis		
Alcoholic cirrhosis/hepatitis	15	6
NASH	6	5
Viral hepatitis	2	5
PSC/PBC	3	2
HCC [#]	11	25
Other ^{##}	8	6
Immunosuppressive Therapy		
Tac + MMF + P		32
Tac + MMF		6
Tac + Evr		1
Tac + P		4
Tac		2
CsA + MMF + P		3
CsA + Az + P		1
Time from LTx		
1 month (30 days)		15
> 1 – 3 months (31–91 days)		17
3 – 8 months (92–244 days)		14
> 8 months		3

* One patient provided a pre and post-LTx sample.

[#]HCC patients' second diagnoses (if any): pre-LTx - NASH 5, alcoholic cirrhosis/hepatitis 3, viral hepatitis 1; post-LTx NASH 8, alcoholic cirrhosis/hepatitis 5, viral hepatitis 8, congenital biliary atresia 1

^{##}Other diagnosis: pre-LTx - cryptogenic 4, and 1 each lipodystrophy plus alpha1 antitrypsin deficiency, nodular regenerative hyperplasia, neuroendocrine tumor, substance abuse; post-LTx - 1 each cryptogenic, autoimmune hepatitis, Wilson's disease, common variable immunodeficiency, cholangiocarcinoma, Waldenstrom's macroglobulinemia

Abbr: Pre-LTx: pre liver transplant, post-LTx: post liver transplant, NASH: non-alcoholic steatohepatitis, PSC: primary sclerosing cholangitis; PBC: primary biliary cholangitis, HCC: hepatocellular carcinoma, Tac: tacrolimus, CsA: cyclosporine A, MMF: mycophenolate mofetil; P: prednisone, Az:azathioprine; Evr: everolimus