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Abbreviations: AKI, acute kidney injury; ATG, anti-thymocyte globulin; BK, BK virus; BM(T), bone marrow (transplantation); CKBMT, combined kidney and bone marrow transplant; CNI, calcineurin inhibition; DCreg, regulatory dendritic cell; DLI, donor lymphocyte infusion; FC, facilitating cell; GVHD, graft-versus-host disease; HSC, hematopoietic stem cell; IS, immunosuppressive/ion; NHP, nonhuman primate; SOC, standard of care; TBI, total body irradiation; TCR, T cell receptor; TLI, total lymphoid irradiation; Tmem, memory T cell; Tn, naïve T cell; Treg, regulatory T cell.

ABSTRACT

The International Workshop on Clinical Transplant Tolerance is a biennial meeting that aims to provide an update on the progress of studies of immunosuppression minimization or withdrawal in solid organ transplantation. The Fourth International Workshop on Clinical Tolerance was held in Pittsburgh, Pennsylvania, September 5-6, 2019. This report is a summary of presentations on the status of clinical trials designed to minimize or withdraw immunosuppressive drugs in kidney, liver and lung transplantation, without subsequent evidence of rejection. All protocols had in common the use of donor or recipient cell therapy combined with organ transplantation. The Workshop also included presentations of mechanistic studies designed to improve understanding of the cellular and molecular basis of tolerance and to identify potential predictors/biomarkers of tolerance. Strategies to enhance the safety of hematopoietic cell transplantation and to improve patient selection/risk stratification for clinical trials were also discussed.

1. INTRODUCTION

The International Workshop on Clinical Transplant Tolerance is held every two years with the aim of providing a broad update on the progress of studies of immunosuppression withdrawal in solid organ transplantation. Discussions are led by investigators who are either leading or planning clinical trials in the field, identified from all known programs through prior attendance, publication, or registration on clinical trial registries internationally. The purpose of the current Workshop was (i) to review and discuss updated information from actively engaged investigators of trials of the safe minimization or withdrawal of immunosuppressive (IS) agents in kidney, liver and lung transplantation, (ii) to discuss underlying mechanisms and immunologic monitoring, and (iii) to discuss prospects for future strategies. This report details the current state of the field as conveyed by the investigators leading these studies and includes information on successes, failures and the design of ongoing or planned studies.

2. TOLERANCE/IS WITHDRAWAL TRIALS: KIDNEY

Details of hematopoietic chimerism-based tolerance trials in kidney transplantation are summarized in **Table 1**. Adverse events are summarized in **Table 2**. The **Stanford** experience was presented by **Dr. Samuel Strober**. The approach is to establish persistent mixed chimerism by performing combined hematopoietic cell and kidney transplantation from the same donor ¹⁻³. Mixed chimerism reduces the risk of graft-versus-host disease (GVHD) and immunodeficiency as compared to full chimerism. The Stanford trial enrolled 29 fully HLA-matched and 27 HLA haplotype-matched patients. There has been no graft loss due to rejection, disease relapse or infection in 51 of the 56 patients enrolled during the last 14 years. None of the 56 patients developed severe or chronic infections or GVHD. Maintenance IS drugs were withdrawn completely from 80% of the fully-matched patients, without subsequent evidence of rejection with follow up of up to 14 years ¹⁻³.

In order to establish persistent mixed chimerism, recipients were given post-transplant conditioning with 10 doses of total lymphoid irradiation (TLI) and 5 doses of anti-thymocyte globulin (ATG) over 11 days. A combination of enriched CD34⁺ hematopoietic progenitors and a defined number of donor T cells were injected immediately after completion of TLI. Donors were given granulocyte colony-stimulating factor to mobilize progenitors into the blood such that collection was performed via apheresis. Fully-matched patients were given 1x10⁶ T cells/kg along with the CD34⁺ cells. Of the 29 fully-matched patients enrolled, 24 had persistent mixed chimerism for at least 1 year. The latter patients were withdrawn from mycophenolate mofetil (MMF) after 1 month and from calcineurin inhibitor (CNI) at around 1 year, as long as they had persistent mixed chimerism and no evidence of rejection or GVHD. Twenty three of these patients had no rejection episodes thereafter.

HLA-haplotype-matched recipients were given the same conditioning regimen, and escalating doses of T cells in order to overcome greater barriers to establishment of mixed chimerism compared to fully matched patients. Recipients who developed chimerism were withdrawn from prednisone and MMF during the first year, then maintained on tacrolimus monotherapy during the second year. Patients with persistent chimerism on monotherapy showed no histological evidence of rejection on protocol biopsies. Tacrolimus was gradually tapered to discontinuation during the second year to determine whether chimerism would persist after complete IS drug withdrawal without evidence of rejection. However, tapering tacrolimus to subtherapeutic blood levels resulted in loss of chimerism and in some instances, rejection episodes. Patients who were maintained on therapeutic blood levels

of tacrolimus monotherapy continued to have persistent mixed chimerism. T cell chimerism was uniformly low at 1 year (<20% donor type cells) in the haplotype-matched patients with persistent chimerism.

In order to increase the levels of T cell chimerism in the haplotype-matched patients, the Stanford group is continuing to escalate the dose of donor T cells and CD34⁺ cells injected. They will determine whether IS drugs can be withdrawn completely without loss of chimerism in those patients with higher levels of T cell chimerism at the end of year 1. Thus, the Stanford group has achieved complete IS drug withdrawal and tolerance in fully-matched patients, and partial withdrawal with maintenance of mixed chimerism in haplotype-matched patients ¹⁻³.

Interim results of a phase III prospective, randomized, multi-center, open label, controlled trial performed by Medeor Therapeutics Inc to assess whether tolerance could be induced in HLA-matched patients given combined kidney and hematopoietic progenitor cell transplants were summarized by **Dr. Dixon Kaufman, University of Wisconsin**. The trial was developed based on a conditioning regimen of TLI and ATG and infusion of a donor cell product (MDR-101), composed of enriched CD34⁺ cells with a defined dose of T cells, as reported previously by the Stanford group. The goal was to establish mixed chimerism for at least 6 months, and to wean and withdraw all IS drugs thereafter.

The trial involves 33 centers with the primary objective of achieving freedom from IS drugs for 24 months without evidence of rejection, and comparison of safety and tolerability to standard-of-care (SOC) therapy. The goal is to enroll 30 patients with a 2:1 randomization of experimental and control patients. Interim results indicate 18 patients enrolled with 7 controls and 11 investigational subjects. Seven of the latter subjects have received combined kidney and hematopoietic cell transplants. All have developed mixed chimerism and 3 are in the midst of weaning after 6 months. Donor cell infusions were well-tolerated, none of the patients developed evidence of rejection, and none had IS withdrawn at the time of the interim analysis.

Dr. Joseph Leventhal summarized the outcome of a tolerance trial at **Northwestern and Duke Universities**, using combined kidney and hematopoietic cell transplants in HLA-mismatched and unrelated living donor kidney transplants. The goal was to establish durable chimerism, such that IS drugs could be withdrawn completely, starting 1 year post-transplant without subsequent rejection.

Thirty-seven pts have been transplanted since 2009 in this phase II trial to induce tolerance in recipients of living donor renal allografts. The protocol is based upon tolerogenic CD8⁺/TCR-facilitating cells (FC) and nonmyeloablative conditioning. Subjects were conditioned with fludarabine (30mg/m²/dose, days -5,-4,-3), cyclophosphamide (Cy; 50mg/kg/dose, day-3 and +3), 200 cGy total body irradiation (TBI; day-1) followed by kidney transplantation (day 0). A G-CSF-mobilized apheresis product was collected from the donor >2 weeks pre-transplant, processed to remove (GVHD-producing cells, yet retain CD34⁺ cells and FC, and cryopreserved until administration on day +1 post-transplant.⁴⁻⁶

All subjects (ages 18-65 years) have reached ≥ 3 years of follow-up (range 36-129 months). Donor-recipient HLA match ranged from 6/6 matched,-related to 0/6 matched,- unrelated; 17 were unrelated and 20 were related. Two subjects were re-transplants. MMF and tacrolimus-based IS was weaned and discontinued 1 year post-transplant if chimerism, normal renal function and normal renal biopsy were present. Thirty-five of 37 patients exhibited peripheral blood donor chimerism at 1 month. Durable chimerism, allowing full IS withdrawal developed in 26 patients (time off IS 24-112 months); the majority of these subjects (23/26) showed full (>95%) donor whole blood and T cell chimerism. All stable chimeric subjects retained chimerism after removal of IS and remain rejection-free. Long-term chimeric subjects off IS have shown no evidence of immune defects: they show robust lineage reconstitution and can be safely and effectively vaccinated. Two subjects off IS have had successful pregnancies. Transiently chimeric subjects resumed endogenous hematopoiesis and were maintained on low dose IS with stable renal function. Late (> 4 yrs post-transplant) acute rejection occurred in 2 subjects with transient chimerism who became noncompliant with IS.

There have been 2 cases of GVHD, - one serious that was associated with death of the subject within 1 year of transplant. There have been two graft losses, both related to infections. Two additional subject deaths have occurred: one in a heavy smoker, who developed advanced stage lung cancer 4.5 years post-transplant, and the second in a chimeric subject 3.5 years post-transplant who developed pneumococcal sepsis – notably, he did not comply with recommended vaccinations. Overall survival is 91.8% and death censored graft survival 94.1%. Tolerant subjects in the FC trial off IS have significantly better renal function than comparable kidney recipients on SOC IS within 2

years following transplant. Treatment for hypertension and hyperlipidemia has been more common in SOC than in tolerant FC trial subjects

Updated results of the **Massachusetts General Hospital (MGH)/Immune Tolerance Network (ITN)** clinical trial ⁷⁻⁹ and new MGH pilot trials were summarized by **Dr. Tatsuo Kawai**; The initial conditioning regimen for 10 HLA haplotype-mismatched kidney transplant recipients included Cy, humanized anti-CD2 monoclonal (m)Ab (MEDI-507) and local thymic irradiation (700 cGy). On day 0, kidney transplantation was performed, followed by i.v. infusion of whole donor BM cells. CNi was administered postoperatively and then slowly tapered after 6 months and completely discontinued by 9-14 months (MGH/ITN regimen) ⁷. Following significant humoral responses in the initial recipients, the regimen was subsequently modified by adding two pretransplant or four peri-transplant rituximab (anti-CD20) injections. Transient mixed chimerism for up to 3 weeks was induced in all recipients, without evidence of GVHD. IS-free renal allograft survival was achieved for 5 years or more in seven of the 10 recipients enrolled in this trial. This greatly exceeded the primary endpoint, which was 24 month graft survival in the absence of maintenance IS. Four of the recipients remain off IS with normal kidney function, more than 10 to 17 years post-transplant. In the other 3, IS was resumed after 5, 7 and 8 years to treat either recurrence of the original kidney disease or development of chronic rejection ⁸. Despite ongoing maintenance IS, these three subjects eventually lost graft function at 12.5, 10.5 and 10 years, but all subsequently underwent successful kidney re-transplantation with conventional IS.

To address a major adverse event, transient acute kidney injury (AKI) with capillary endothelial injury, which was observed 2-3 weeks post-transplant in 9 of the 10 study subjects ¹⁰, a pilot clinical trial was conducted more recently in 2 subjects. In this pilot trial, Cy was replaced with low dose TBI (1.5 Gy x 2) without changing any of other components of the MGH/ITN conditioning regimen. The rationale for this modification was based on nonhuman primate (NHP) studies, in which no AKI was observed in recipients after conditioning with TBI rather than Cy ¹¹⁻¹³. The first human recipient of this revised regimen developed mixed chimerism between days 13-23 and all IS agents were discontinued successfully by 9 months. He continued IS-free normal kidney function for the subsequent 3.75 years. However, IS was resumed at 4.6 years when a protocol biopsy showed

evidence of Ab-mediated rejection. He is currently stable at 6 years on conventional IS. The second recipient failed to develop chimerism and IS was never discontinued.

When anti-CD2 mAb (Medi-507) subsequently became unavailable, another pilot clinical trial was initiated in 2 subjects using low dose TBI in place of Cy, and with ATG and Belatacept (CTLA4Ig) (TBI/Bela) in place of Medi-507. This regimen (TBI/Bela) had been shown to induce mixed chimerism and renal allograft tolerance in NHPs ¹⁴. The first recipient of the TBI/Bela regimen, however, was not withdrawn from IS due to insufficient chimerism, as well as biopsy evidence of IgA recurrence. The second recipient showed evidence of chimerism on day 6 which disappeared rapidly with severe AKI on day 9. Although he recovered completely from AKI, IS was not withdrawn due to insufficient evidence of chimerism. These pilot trials have demonstrated an apparent discrepancy between NHPs and humans in chimerism induction. In particular, humans appear to be much more resistant to establishment of hematopoietic chimerism than NHPs using low dose TBI. Since anti-CD2 mAb will soon be available again as Siplizumab®, produced by ITBMed (New York, NY), a new clinical trial with a revised conditioning regimen (ITB-01) is planned. In this trial, MMF and additional doses of Siplizumab will be added in an attempt to avoid AKI by suppressing the effector cells that are a likely cause of AKI.

Comparable approaches to mixed chimerism induction for renal allograft tolerance have been studied at the **Samsung Medical Center** (Seoul, S. Korea). A clinical trial update was provided by **Dr. Kyo Won Lee**. This group performed 9 combined kidney and BM transplants (CKBMTs) from HLA mismatched living donors between December 2011 and July 2019. Anti-CD2 mAb was initially replaced by thymoglobulin (1.5 mg/kg, -1, 0, 1 days) as anti-CD2 mAb was not available in Korea. Although one of two patients treated with the initial regimen achieved IS-free survival for 1 year, both suffered AKI and Cy toxicity. Therefore, the regimen was revised by adding fludarabine (15 mg/m², -6, -5, -4, -3 days) and one additional dose of thymoglobulin to reduce the dose of Cy (22.5mg/kg, -5 and -4 days). One of three recipients of this modified regimen achieved long-term IS-free survival, but all suffered from BK nephritis. Therefore, the ATG dose was reduced to 3 administrations and the fludarabine dose was reduced from 15 mg/m² to 10 mg/m² in the most recent modification. In addition, tacrolimus was replaced by rapamycin 1 month after CKBMT. All 3 recipients tolerated this

modification well, with only minor or no BK nephritis, and have been off IS for 4, 3 and 1 years, with stable kidney function.

3. TOLERANCE/IS WITHDRAWAL TRIALS: LIVER

The feasibility of IS drug withdrawal in selected adult and pediatric liver transplant recipients has been demonstrated over several decades¹⁵. Recently published or completed studies are summarized in **Supporting Table 1**. Several investigators reported results from ongoing trials, as well as long-term outcomes from previously published studies¹⁵.

Dr Abhinav Humar, University of Pittsburgh, described a first-in-human, single center, open label, non-controlled, non-randomized phase I/II trial of donor-derived regulatory dendritic cells (DCreg) in low risk, living donor liver transplantation. The objectives of the trial are (i) to determine the safety of donor-derived DCreg infusion one week before transplant, followed by IS weaning and withdrawal, (ii) to determine preliminary efficacy of DCreg infusion in achieving drug withdrawal that can currently be achieved in only 10-15% of liver recipients in the first 2 years post-transplant¹⁶ and (iii) to conduct a preliminary assessment of the fate of the infused DCreg product and the impact of its infusion and IS withdrawal on host anti-donor immunity. Major exclusion criteria include an etiology of autoimmune liver disease, or HBV or CMV viral load positivity at the time of transplant. Donor-derived DCreg are generated over 1 week beginning 14 days before transplant, from elutriated monocytes of non-mobilized leukapheresis products. A half dose of MMF is administered between the time of cell infusion (7 days before transplant) and the day of transplant surgery. Thereafter, the patients receive SOC IS that comprises a rapid steroid taper, MMF and tacrolimus. MMF is weaned per SOC, but must be discontinued prior to tacrolimus weaning. A pre-weaning protocol biopsy is performed at 1 year. If permissive, the anticipated weaning schedule is to withdraw tacrolimus over 6 months, but withdrawal may extend beyond 18 months post-transplant. There have been no cell manufacturing issues and DCreg have been infused safely into 13 patients in the target cell dose range of $2.5-10 \times 10^6$ DCreg/kg. The incidence of early (1 month) de novo DSA is similar to that observed in matched, SOC living donor liver recipients at the University of Pittsburgh.

Dr Sandy Feng, University of California, San Francisco, reported on withdrawal of IS for pediatric parental living donor liver transplant recipients (WISP-R), a single arm, 3 center pilot trial in

20 patients, and on IS withdrawal (iWITH) for stable pediatric liver transplant candidates, a trial involving 12 centers. In the WISP-R study, histology of 12 operationally tolerant patients' grafts did not deteriorate over 8 years^{17, 18}. In the iWITH study, the primary endpoint of operational tolerance was defined per protocol by stable ALT, γ GT and histology 1 year after the last dose of IS, compared with study entry 2 years earlier. Of 88 patients that initiated IS withdrawal, 53 were off IS at 1 year; following liver biopsy analysis by a central pathologist, 33 patients were classified as tolerant, 16 as non-tolerant and 4 as rejectors. In both the WISP-R and iWITH trials, absence of portal inflammation was considered a predictor of tolerance. Increased APC/leukocyte pairs, MAC387⁺ and CD8⁺ cells were also considered predictors of tolerance, while de novo DSA was negatively associated with tolerance. Feng et al¹⁹ also performed a transcriptional and cytometric analyses of graft biopsies from 157 stable, long-term pediatric liver transplant recipients with consistently normal liver test results,- a systems biology analysis that incorporated clinical, serologic, histologic, and transcriptional data. Evidence was found of chronic graft injury (inflammation and/or fibrosis). Biopsies with interface activity had a gene expression pattern associated with T cell-mediated rejection (TCMR). This study highlighted the heterogeneity of patients deemed to be stable and the importance of considering such differences in the outcomes of withdrawal trials. Separately, alloreactive Treg therapy is being evaluated at UCSF in liver transplantation in combination with depleting agents (thymoglobulin or Cy) or without depletion. A 3-center trial (ARTEMIS) is being conducted of the safety of donor alloantigen-reactive (dar) Tregs infused without use of a depleting agent, 2-6 years post-transplant, to facilitate minimization and/or discontinuation of IS in adult liver transplantation. Treg manufacturing failures have been encountered for some recipients. Cell therapy manufacturing failures in this and other studies are often a result of inadequate expansion or a poor starting population number, – this is an active area of investigation^{20, 21}.

Dr Josh Levitsky (Northwestern University, Chicago) reported on several trials, including the ITN-supported A-WISH multi-center study of early, staged withdrawal (up to 2 years) of IS versus maintenance IS in deceased donor liver recipients¹⁶. Of 275 recipients enrolled before transplantation, 95 were randomly assigned 4:1 to withdrawal (n = 77) or maintenance (n = 18) 1- to 2-years post-transplant. Fifty-two of 77 subjects (67.5%) reduced to $\leq 50\%$ of baseline dose, and 10 of 77 (13.0%) discontinued all IS for ≥ 1 year. In a prospective trial of sirolimus monotherapy

withdrawal in non-immune, non-viremic liver recipients, >3 years post-transplant ²², sirolimus was weaned over ~6 months and biopsies performed 12 months post-weaning, or at concern for acute rejection (AR). Twenty-one patients were consented; 6 were excluded due to subclinical AR on baseline biopsy, or other reasons; 15 underwent weaning (age 61.3±8.8 yrs; time of transplant to sirolimus weaning 6.7±3 yrs). Eight patients (53%) achieved operational tolerance. Ongoing multi-center, prospective trials of IS drug withdrawal in adult liver transplantation include the ITN-supported OPTIMAL study that is evaluating donor-specific immune senescence and exhaustion as biomarkers of operational tolerance in liver recipients 18-50 years old and >6 years post-transplant, or >50 years old and >3 years post-transplant. As of august 2019, 61 of 100 patients screened initiated IS withdrawal; of these, 27 completed withdrawal and 12 remained off IS. Dr Levitsky also described an ongoing ITN-supported single center (MGH; PI Dr James Markmann), phase I/II non-randomized trial of donor alloantigen-reactive Treg (darTreg) therapy to facilitate IS withdrawal in liver recipients. Nine participants will receive 2.5-500 x 10⁶ total darTreg. Study interventions include everolimus conversion, leukapheresis followed by administration of Cy and Mesna prior to a single iv dose of darTregs, and tacrolimus discontinuation followed by complete everolimus withdrawal.

Dr Alberto Sanchez-Fueyo (King's College, London) described a prospective, open label, single arm trial designed to assess the activity, safety and clinical efficacy of low dose IL-2 in expanding Tregs and promoting complete discontinuation of IS in adult liver transplantation. Ten patients (>50 years old), enrolled 2-6 years post-transplant, agreed to participate and 6 initiated treatment with daily s.c. IL-2. Although low dose IL-2 expanded the circulating Treg pool in patients on tacrolimus, it failed to preferentially expand Tregs in the graft and promoted T cell-mediated rejection before IS was reduced.

Dr Satoru Todo gave a follow-up report of the **Hokkaido University** pilot study ²³ conducted in 10 consecutive, adult living donor liver transplant patients given an ex vivo-generated, autologous Treg-enriched cell product early post-transplant. The cells were generated following 2-week coculture of recipient lymphocytes with irradiated donor cells in the presence of anti-CD80/86 mAbs. Patients were splenectomized and received post-transplant Cy (day 5), followed by Treg (day 13), in conjunction with steroid, MMF and CNI. IS agents were tapered from 6 months, reduced every 3 months, and completely discontinued by 18 months. Seven patients completed successful weaning

and cessation of all IS agents (those that failed IS withdrawal had autoimmune disorders as their primary disease). The patients have now been IS drug free for 5.2-6.8 years; 6.9-8.6 years post-transplant. Development of DSA has not compromised these clinically tolerant patients.

4. TOLERANCE/IS WITHDRAWAL TRIAL: LUNG

BM transplantation (BMT) can be a curative therapy for patients with primary immunodeficiency (PID) syndromes, however those patients with end-stage lung disease are ineligible for either BMT or bilateral orthotopic lung transplantation (BOLT). Thus, definitive treatment for patients with PID/end-stage lung disease represents an unmet clinical need. A first, single center, phase I/II tandem BOLT/BMT trial in 8 patients with PID/end-stage lung disease is being conducted at the **University of Pittsburgh** and was presented by **Dr. Paul Szabolcs**. The trial evaluates the safety and efficacy of BOLT, followed by CD3⁺/CD19⁺-depleted BMT from the same deceased, partially HLA-matched donor^{24, 25}. The hypothesis being tested is that tandem BOLT/BMT in patients with PID/end-stage lung disease will result in correction of the underlying PID, normalization of lung function, restoration/preservation of pathogen-specific T cell mucosal immunity, and a level of chimerism sufficient to establish long-term lung allograft/BMT tolerance that will allow withdrawal of IS. Success in this study would represent a paradigm shift in the fields of lung transplantation and BMT, and proof-of-concept for tandem BOLT/BMT could potentially be extended to other solid organ candidates in an effort to achieve tolerance.

5. REGULATORY CELL THERAPY FOR IS MINIMIZATION IN KIDNEY TRANSPLANTATION

(i) The ONE Study,- Continental Update

The *ONE Study* was initiated in 2011 as a large-integrated project funded by the European Union with the aim of testing different immunoregulatory T cell, dendritic cell and macrophage-based products in a parallel series of 6 single arm trials conducted in living-donor kidney transplant recipients at centers in Europe and the United States. Each early phase trial has been conducted with only one of 6 cell products, but in the same well-defined patient population and using identical background IS. In addition, a separate multi-center control (reference) group has been conducted whereby each of the

same participating centers has enrolled the same subgroup of kidney transplant recipients treated with SOC IS. Critically, patients in this reference group received the same dosing/IS regimen (steroids, MMF and tacrolimus) as the cell therapy patients; the only difference being that reference group received basiliximab (anti-CD25 [IL-2R α] mAb) induction, whereas the cell therapy patients received a cell product instead of basiliximab and had the option to be weaned off MMF after 9 months.

Dr. Ed Geissler, University of Regensburg, reported that the reference group and 6 cell therapy group trials had all reached completion and that the *ONE Study* group is now able to: 1) directly compare the results of the individual cell trials to each other, 2) compare the individual cell therapy trials to a SOC treatment, 3) perform a first evaluation as to whether patients on cell therapy could be weaned successfully to tacrolimus monotherapy, and 4) pool results from the individual cell therapy trials as a group and to compare “immune cell therapy” to SOC treatment. The extensive centralized and standardized immune monitoring performed in these trials is enabling an early evaluation of potential immunological effects of each cell therapy, and of immune cell therapy as a whole, in kidney transplant recipients. (Sawitzki et al, *Lancet*, accepted)

(ii) Oxford/London ONE and TWO Studies

The UK (Oxford/London) arm of the ONE Study (NCT02129881) has investigated the feasibility and safety of autologous expanded polyclonal Tregs in living donor renal transplantation. The trial was a standard 3+3 dose escalation design, with 12 patients recruited and treated with a single dose infusion of cells 5 days post-transplant. Tregs were isolated from blood by magnetic bead separation followed by anti-CD3/anti-CD28-coated bead-driven expansion with IL-2 and sirolimus ²⁶. **Dr. Fadi Issa, University of Oxford**, reported that 16 cell therapy batches were manufactured at doses of 1, 3, 6 and 10x10⁶ cells/kg, with 4 batches failing due to insufficient cell numbers or bacterial contamination. Successful batches were cryopreserved for later use. Interestingly, expansion kinetics differed between patients, although the starting population appeared critical to achieving the required dose. The final patient received the maximum dose in January 2016. This trial demonstrated feasibility and safety of autologous polyclonal Treg therapy, laying the foundations for the next phase trial, the UK Medical Research Council-funded TWO Study, the aim of which is to assess treatment efficacy, with the goal of facilitating minimization of IS to a single agent by 6 months post-transplant. The TWO

Study differs from the ONE Study in several crucial areas. Firstly, patients will receive alemtuzumab (anti-CD52) induction, based on encouraging data from the 3C Trial ²⁷. Secondly, Tregs will be infused at 6 months, just after MMF minimization, to avoid the risk of early rejection and encourage Treg survival. Thirdly, the TWO Study has been designed as a randomized controlled trial with patients in the control arm receiving SOC IS. It aims to enroll up to 68 patients randomized equally between each treatment arm.

(iii) U.S. Treg Trials

The phase I Treg Adoptive Cell Therapy (TRACT) trial (NCT 02145325), run through Northwestern University, Chicago, has similarly assessed autologous polyclonal expanded Treg therapy in living donor renal transplant recipients. **Dr. Joseph Leventhal** reported that the trial was designed as a non-randomized, dose-ranging study with doses of 0.5, 1 and 5×10^9 cells/recipient ²⁸. Nine patients were recruited, with 3 patients at each tier. Unlike the ONE Study, Tregs were isolated from a leukapheresis product rather than peripheral blood. The expansion process was similar and included IL-2, sirolimus and transforming growth factor β in the cell culture, although sirolimus was excluded from day 9 onwards. Treg were cryopreserved and infused as a single dose, 60 days after transplantation. Patients received alemtuzumab induction, followed by tacrolimus and MMF, with conversion from tacrolimus to sirolimus at day 30. Interestingly, compared to historical controls, patients receiving Treg had elevated levels of $CD4^+CD25^{hi}CD127^-(IL-7R\alpha)^+Foxp3^+$ cells in peripheral blood, approaching or even exceeding pre-transplant levels. The study has demonstrated safety, with no episodes of infusion-related adverse events or rejection up to 2 years post-transplant. On the basis of its success, a phase II trial at Northwestern University is planned. In this trial, 120 patients will be recruited over the next 2 years, with 74 patients receiving Treg therapy. Enrolled patients will receive either SOC IS or Treg infusion 60 days post-transplant. The aim is to withdraw tacrolimus IS in a subset of enrolled patients.

Overall, the strategy taken by the reported cell therapy trials in kidney transplantation has been conservative, with a progression from safety through to early efficacy trials and controlled IS withdrawal.

6. MAKING CLINICAL HSCT SAFER

Approaches were presented to reduce adverse events after allogeneic hematopoietic stem cell (HSC) transplantation to treat hematologic malignancies. **Dr. Samuel Strober** discussed the **Stanford** approach to reducing the incidence and severity of GVHD and non-relapse mortality (NRM) after allogeneic HSC transplantation based on preclinical models that separate GVHD from graft anti-tumor activity ²⁹⁻³⁴. Two strategies were developed ; the first was to use the TLI conditioning regimen instead of TBI to establish complete chimerism and protect against GVHD. The second was to use a donor lymphocyte infusion (DLI) composed of CD8⁺ memory T cells (Tmem) instead of infusion of total T cells to prevent or treat tumor relapse, without inducing GVHD ²⁹.

TLI protects against GVHD by promoting the dominance of donor Tregs in recipient lymphoid tissues.³⁰⁻³⁵ These Tregs suppress the ability of conventional donor T cells in the transplant to mediate acute and chronic GVHD. CD8⁺ Tmem produce little or no IL-2 after infusion and fail to expand in recipient lymphoid tissues ²⁹. Robust expansion is a necessary condition for the induction of GVHD ²⁹. Nevertheless, these cells can convert mixed to complete chimerism and kill tumor cells.

The outcome of 625 patients at Stanford with lymphoma or leukemia given an allogeneic, mobilized blood hematopoietic progenitor transplant using the TLI conditioning regimen was presented. The large majority of patients (85%) were fully HLA-matched; 44% had related donors, whereas 41% had unrelated donor transplants obtained from the national marrow transplant registry. Donor transplants with one HLA Ag mismatch (15%) were also obtained from the registry. The incidence of severe acute GVHD (grade III-IV) at 100 days was 4% and the NRM at 1 year 7%. About 25% of recipients developed mixed chimerism and the remainder complete chimerism. The incidence of tumor relapse was increased significantly in patients with mixed chimerism ³⁶. In order to convert mixed chimeras to complete chimeras after transplantation, the mixed chimeras were given a DLI composed of CD8⁺ Tmem obtained from the donors by collecting an apheresis product without mobilization, and processing the cells to enrich CD8⁺ T cells and deplete CD62L naïve T cells ³⁷. In the Stanford preclinical models, the infusion of CD8⁺ Tmem converted mixed chimeras to complete chimeras without inducing GVHD, and allowed graft anti-tumor activity that eliminated tumor cells

In phase 1 safety and feasibility studies, the CD8⁺ Tmem were infused into 16 mixed chimeras with post-transplant tumor relapse ³⁷. One of the patients developed grade II acute GVHD that resolved after treatment. Ten of the patients developed durable tumor responses after the DLI extending to 2 years.

A follow-up study was initiated in which patients were assessed for mixed versus complete chimerism 28 days post-transplant. Those with mixed chimerism were given a CD8⁺ Tmem DLI as a prophylactic regimen to prevent tumor relapse. Sixteen patients have been treated, of which 10 converted from mixed to complete chimerism. Six failed to convert. One of the 10 patients who converted developed severe acute GVHD. The study continues to monitor these patients and to enroll additional patients. A second study is testing the ability of CD8⁺ Tmem from EBV and CMV seropositive donors to reduce the incidence of EBV and CMV post-transplant in patients with AML who are conditioned with myeloablative therapy and infused with T cell-depleted hematopoietic progenitor cell transplants.

Dr. Everett Meyer summarized another approach used at **Stanford** for prevention of GVHD after HSC transplantation while allowing graft anti-tumor activity,- the infusion of Tregs ³⁸. He reviewed results of an ongoing phase II trial using Tregs to prevent GVHD in patients undergoing myeloblastic HSC transplantation for high risk hematologic malignancy or myelodysplasia ³⁸. Donor-derived CD4⁺CD25⁺CD127^{lo} cells were selected by bead enrichment followed by flow sorting to >95% purity. These primary cells were then administered fresh with CD34-selected hematopoietic cells on day 0 of transplantation. Two days later, up to 3.0 x 10⁶/kg CD3⁺ cells were administered. Early results suggest a very low incidence of GVHD and comparable relapse rates. Strategies being considered to augment Treg function include transient genetic modification with chimeric antigen receptors (CAR). In rodents, CAR Tregs significantly prolonged islet allograft survival ³⁹. Strategies to use CAR Tregs targeted to the BM niche to promote donor hematopoietic engraftment were also presented.

Dr. Warren Shlomchik, University of Pittsburgh summarized the outcome of a clinical trial of BM transplantation to treat patients with hematologic malignancies conducted in collaboration with the **Fred Hutchinson Cancer Research Center** in Seattle ⁴⁰. The goal was to reduce GVHD by depleting CD45RA⁺ naïve T cells (Tn) from the donor cell transplant, while maintaining graft anti-

tumor activity. The trial was based on preclinical studies that showed marked reduction in GVHD after Tn depletion. In a single-arm trial, 35 patients with high-risk leukemia were transplanted with Tn-depleted PBSC grafts following conditioning with TBI, thiotepa, and fludarabine ⁴⁰. Prophylactic management of GVHD was with tacrolimus alone. Subjects received CD34-selected peripheral blood stem cells and a defined dose of Tmem depleted of CD45RA⁺ cells. The incidence of acute GVHD was not reduced; however, GVHD was universally corticosteroid responsive. Chronic GVHD was infrequent (9%; median follow-up 932 days) compared with historical rates of approximately 50% with T cell-replete grafts. The grafts resulted in rapid T cell recovery and transfer of protective virus-specific immunity. Safety was improved, since excessive rates of infection or relapse did not occur and overall survival was 78% at 2 years.

7. MECHANISTIC STUDIES AND IMMUNE MONITORING

A summary of presentations related to the role of exploratory immune monitoring, biomarker discovery and exploration of tolerogenic mechanisms is available as **Supporting Information 1**.

8. TISSUE TYPING AND PATHOLOGY

(i) Better Patient Selection Through Improved Typing and Matching

A clear unmet need for the kidney transplant recipient is to improve individual alloimmune risk assessment with a precise prognostic value that would allow personalized IS. An enhanced understanding of alloimmune risk is key to the design of trials of IS withdrawal to ensure an appropriate balance is achieved. **Dr. Peter Nickerson, University of Manitoba**, discussed how HLA molecular mis-match (mMM),- a measure of the degree of dissimilarity between donor and recipient HLA molecules at the structural level, is emerging as a tool to provide more precise assessment of risk for primary alloimmunity ⁴¹. Work conducted by the Transplant Manitoba research group supports the following: (i) class II (DR and DQ) HLA eplet mMM score is a potential prognostic biomarker for primary alloimmunity (i.e. T cell-mediated rejection [TCMR], de novo DSA, and Ab-mediated rejection [ABMR]),- further validation is required before full adoption into clinical practice;⁴² (ii) HLA eplet mMM evaluation of each individual HLA-DR/DQ molecule provides a more precise assessment of the risk of de novo DSA development against that unique molecule

compared to approaches that sum all HLA mMM scores at a given HLA loci ⁴²; (iii) patients can be assigned to a low, intermediate, or high primary alloimmune risk category using HLA eplet mMM thresholds derived for all HLA-DR $\beta_{1/3/4/5}$ and HLA-DQ α_1/β_1 molecules ⁴²; (iv) class II (DR and DQ) HLA eplet mMM score is potentially a predictive biomarker of CNi therapy requirements,- if validated prospectively it could lead to personalized IS therapy ⁴³.

Based on this work, the FDA Center for Drug Evaluation and Research (CDER) has accepted HLA-DR/DQ eplet mMM score into its Biomarker Qualification Program (<https://www.fda.gov/downloads/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/BiomarkerQualificationProgram/UCM628985.pdf>). Once fully validated and qualified by the FDA as a prognostic biomarker for primary alloimmunity, then HLA-DR/DQ eplet mMM score could be used for enrichment, or risk stratification in phase II and III clinical trials.

(ii) The Importance of Tissue Pathology in Tolerance Trials

Dr. Jake Demetris, University of Pittsburgh, discussed how, although imperfect, traditional histopathology offers the most granular evaluation of allograft inflammation, providing spatial and inferred temporal contextual data with respect to inflammation, injury, fibrosis, and architectural distortion that facilitates pathophysiological insights ^{44, 45}. Liver biopsies have been used in native chronic hepatitis to document the presence, gauge the severity, and monitor the progression or effect on therapy of chronic HBV, HCV,⁴⁴ steatohepatitis, and autoimmune hepatitis, and predict the ability to successfully stop IS in patients with established autoimmune hepatitis under therapy ⁴⁴⁻⁴⁶. Legacy lessons from native liver diseases have been applied successfully to liver allografts to show that low-grade portal inflammation accompanied by interface activity: (i) portends future fibrosis development ^{47, 48}; (ii) is associated with a mRNA signature typically associated with TCMR; and (iii) even portal inflammation alone, without interface activity, predicts the development of TCMR after lowering of IS ^{17, 49}. This has led to development of Banff consensus liver histopathological criteria for determining eligibility for IS minimization and tolerance assessment after IS withdrawal ⁵⁰. In essence, operationally “tolerant” liver allografts appear similar to normal, native livers, with absence of portal inflammation associated with interface activity and substantial fibrosis.

Unfortunately, no such consensus criteria have been proposed for kidney allografts, despite the fact that the controversial “borderline change”, is observed in some “tolerant” kidney allograft

recipients in tolerance induction or IS minimization trials ⁵¹. In other cohorts, however, borderline change has been associated with progressive decline in renal allograft function ⁵². ‘Next generation pathology’, defined as multiplex staining (protein, mRNA, and DNA)/labeling techniques, coupled with whole slide digital imaging and automated image analysis, enhances traditional evaluation of graft biopsies. It increases the value of traditional assessments ⁴⁵ by retaining spatial context and inferred temporal relationships in tissue sections that is not possible with “grind and bind” approaches, while enabling application of complex spatial context algorithms (e.g. detecting virtual immunological synapses), that enable investigation of immunological mechanistic insights.

9. NIH UPDATES

Updates on NIAID Clinical Trials in Organ Transplantation (CTOT), NHLBI Clinical Trials in HSCT, The Immune Tolerance Network (ITN) and the Nonhuman Primate Transplantation Tolerance Cooperative Study Group (NHPCSG) were presented and are available as **Supporting Information 2**.

10. Workshop Summary and future directions

In a discussion at the end of the meeting led by **Dr. Fadi Lakkis, University of Pittsburgh**, a consensus emerged that (a) clinical transplantation tolerance trials have made significant inroads into establishing the feasibility (proof of concept that tolerance can be attained) and relative safety of chimerism induction approaches; (b) likewise, regulatory cell therapies appear to be safe and feasible, but it remains unclear whether they impart clinical benefit in the form of improvement in graft outcome or reduction in conventional IS; and (c) continued development and validation of biomarkers that risk-stratify patients or measure the tolerance state are clearly warranted, with tangible progress already made in the areas of histocompatibility and graft histopathology. Naturally, the perennial question that followed was how to make transplantation tolerance a clinical reality. In other words, what additional steps are needed before tolerance induction regimens can be presented to patients as a safe and effective alternative to conventional IS?

One idea that was proposed at the meeting is the design and initiation of multi-center, randomized, controlled trials that compare tolerance induction regimens to SOC IS, with traditional graft and patient outcomes (e.g., patient survival, graft survival, and/or graft function) as primary endpoints. The principal objective of such studies would be to determine whether a given tolerance induction therapy is non-inferior to SOC IS. If that turns out to be the case, one could then imagine tolerance offered to suitable patients as an alternative to chronic IS. Conducting these studies, however, would require prior agreement on which tolerance induction protocols are sufficiently feasible, safe, and promising to move forward and how to circumvent the need to recruit very large numbers of patients to achieve statistical power. The reality on the ground is that certain chimerism-induction trials have already progressed to the randomized, albeit small, trial stage and that risk-stratification methods could very well be used soon to identify patients in whom tolerance is more likely to be achieved, or who are most likely to benefit from it. The same is true for regulatory cell therapies. Randomized trials, particularly in the Treg arena, are already on their way or in the planning stages in different parts of the world. Ultimately, the rigor of the scientific method will be the final arbiter of whether tolerance regimens or cell therapies will become viable options for transplant recipients.

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Disclosure

The authors of this manuscript have conflicts to declare as described by the *American Journal of Transplantation*. Samuel Strober: Co-founder and Consultant, Medeor Therapeutics Inc.; Joseph Leventhal: Financial Relationship with TRACT Therapeutics (Founder); Megan Sykes: received sponsored research funding from ITBMed; Peter Nickerson: Consultant for Vitaeris Inc and Renalytix AI Inc., and honoraria from Astellas and Thermo Fisher Scientific.

Data availability statement

No data were created or analyzed in this report.

Future Workshops

The 5th International Workshop on Clinical Transplant Tolerance is planned for New York City in 2021 and will be hosted by Dr Megan Sykes at Columbia University. This workshop will report final data from completed studies, long-term outcomes of past studies, as well as interim data from the planned studies described in this report. A new section of the meeting will be dedicated to discussing the design of new trials in preparation for future funding applications.

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TABLE 1 Regimens of hematopoietic-chimerism-based trials in kidney transplantation

| Patients and outcomes | Medical Center | | | | |
|--|--|---|---|---------------------------------------|-------------------------------|
| | Northwestern University^a | Stanford University | Medeor Trial^c (MDR-101) | Massachusetts General Hospital | Samsung Medical Center |
| HLA matched or mismatched | mismatched | matched/mismatched | matched | mismatched | mismatched |
| Recipient conditioning regimen | TBI+CY+Flu | TLI+ATG/TLI+ATG | TLI+ATG | LTI+CY+anti-CD2 | LTI+CY+Flu+ATG |
| Donor cell composition | mobilized blood CD34 cells + T cells + facilitator cells | mobilized blood CD34 cells + T cells | mobilized blood CD34 cells + T cells | whole bone marrow | whole bone marrow |
| No. patients enrolled and given kidney transplants | 37 | 29/27 | 11 | 10 | 8 |
| No. patients with withdrawal of IS drugs attempted | 26 | 24/6 ^b | 4 ^d | 7 | 6 |
| No. patients | 26 | 21/0 | 4 | 4 | 4 |

| | | | | | |
|--|------------|-------------------|---------|-------------|-----------|
| continuously off IS drugs | | | | | |
| Duration | 29-117 mos | 12-135 mos/- | 2-6 mos | 129-204 mos | 18-61 mos |
| continuously off IS drugs | | | | | |
| No. patients off IS drugs with return to IS drugs due to rejection or relapse | 0 | 3/2 | 0 | 3 | 2 |
| Duration off IS drugs before return to IS drugs | - | 12-63 mos/3-5 mos | NA | 2-96 mos | 2-16 mos |

TBI, 200 cGy total body irradiation; TLI, 80-120 cGyx10 doses total lymphoid irradiation; CY, cyclophosphamide; ATG, anti-thymocyte globulin; Flu, fludarabine; CD34, enriched CD34 hematopoietic progenitor cells from G-CSF mobilized blood; G-CSF, granulocyte-colony stimulating factor; mos, months; mAb, monoclonal antibody; LTI-700cGy, local thymic irradiation; HLA, human leukocyte antigen; IS, immunosuppressive.

^aOne of 37 recipients was enrolled and transplanted at Duke University.

^b4 patients returned to therapeutic levels of Tacrolimus after loss of chimerism.

^cInvolves 33 centers

^dEnrollment is active; IS being weaned and numbers may increase

TABLE 2 Adverse events in hematopoietic chimerism-based tolerance trials in kidney transplantation

| Patients and outcomes | Medical Center | | | | |
|---|--------------------------------------|---------------------|-------------------------------------|--------------------------------|------------------------|
| | Northwestern University ^a | Stanford University | Medeor Trial ^c (MDR-101) | Massachusetts General Hospital | Samsung Medical Center |
| HLA matched or mismatched | mismatched | matched/mismatched | matched | mismatched | mismatched |
| No. patients given transplants | 37 | 29/27 | 11 | 10 | 8 |
| No. with BK viremia/nephropathy | 0 | 0/0 | 0 | 0 | 7 |
| No. with GVHD | 2 | 0/0 | 0 | 0 | 0 |
| No. with engraftment syndrome | 0 | 0/3 | 0 | 9 | 2 |
| No. with graft loss (rejection or disease relapse or infection) | 2 ^b | 2/3 | 0 | 6 ^d | 2 ^b |
| No. deaths | 3 | 3/1 | 0 | 0 | 0 |

GVHD, graft-versus-host-disease.

^aOne of 37 recipients was enrolled and transplanted at Duke University.

^bGraft loss due to infection.

^cInvolves 33 centers

^d Three recipients lost their grafts after 10 years (chronic rejection), 10.5 years (chronic rejection) and 12.5 years (recurrence)