Pretransplant Infusion of Donor Stem Cells Open Gateway to Tolerance through Induction of Regulatory T Cells and Activation of Other Allosuppressive Immune Mechanisms – Single Centre Experience in Living Donor Renal Transplantation

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Keywords: Spinal cord injury (SCI), Human umbilical cord blood-derived mononuclear cells (hUCMNCs), Mesenchymal stem cells (MSCs), Human umbilical cord blood stem cells (hUCBSCs).

Abstract

Introduction: Data regarding stem cell infusion (SCI) for tolerance induction in renal transplantation (RT) is sparse. We designed a prospective trial in a cohort of live-donor RT (LDRT) patients subjected to pre-transplant SCI to test its tolerance promoting effects.

Patients and Methods: Ninety patients were subjected to pretransplant donor SCI of hematopoietic stem cells (HSC) and adipose tissue derived mesenchymal stem cells under non-myeloablative conditioning of Cyclophosphamide, rabbit-antithymocyte globulin, Rituximab and total lymphoid irradiation (TLI)/Bortezomib. Patients with diabetes, unwillingness, hepatitis C/B were excluded. Immune monitoring included donor specific antibodies (DSA) and peripheral regulatory T-cell (pTregs) [CD127low/CD25high/CD4⁺]. Initial maintenance immunosuppression was calcineurin-inhibitor based to be discontinued with stable graft function and absence of rejection episodes. Protocol biopsy was performed after 100 days of immunosuppression withdrawal in willing patients, for graft dysfunction. Rejections were treated by anti-rejection therapy followed by rescue immunosuppression.

Results: All immunosuppression except Prednisone has been withdrawn for mean 2.8 years in 90 patients with mean age 32.2 years and donor-recipient HLA match, 2.81. Mean serum creatinine of 1.4 mg/dL and p-Tregs, 3.63% has remained stable after withdrawal. Rescue was required in 6 patients. DSA were absent in 37 patients and present in 53 patients. Protocol biopsies in all 26 willing patients were unremarkable.

Conclusions: This is the first clinical report showing induction of T-regs with SCI leading to stable graft function in LDRT on low dose steroid monotherapy. MSC may serve as novel, safe and effective immunomodulators in clinical transplantation.

Abbreviations

AD-MSC: adipose tissue derived mesenchymal stem cells; AR: acute rejection; BM: bone marrow; DSA: donor specific antibodies; HSC: hematopoietic stem cells; LDRT: living donor renal transplantation; pTregs: peripheral T regulatory cells; r-ATG: rabbit anti-thymocyte globulin; RT: renal transplantation; SCT: stem cell transplantation; SCr: serum creatinine.

Introduction

Transplantation is now a well accepted therapeutic option compared to dialysis for patients with end stage renal disease (ESRD). However,
patients are required to be administered immunosuppressants life-long to prevent the graft rejection. Unfortunately these immunosuppressants have been able to control acute rejections partially; however, the problem of chronic rejections remains unanswered. In addition to causing financial burden to the individual/family/social system (depending upon the geographical location of the suffering patient) the recipient is prone to infections/malignancy which are detrimental to the graft and often responsible for causing morbidity and mortality. Thus transplanters have been caught in the vicious circle of their own success. The only answer to this problem is transplantation tolerance or transplantation with minimum/no immunosuppression rendering safe and meaningful healthy life to the transplant recipients. Tolerance may be defined as normal functioning graft in absence of immunosuppression while keeping third party immune response of the recipient intact. The true test for establishment of clinical tolerance in the transplant setting, the complete and successful withdrawal of immunosuppressive medications, has been achieved anecdotally and experimentally in rare renal transplant recipients. Although such withdrawal was not planned in most cases, a number of trials are currently underway that include withdrawal of immunosuppression as part of the protocol. Salvateira et al showed that DST can improve the renal graft survival. Taking lessons from this work, we started using stem cells (SC) to achieve transplantation with minimum/no immunosuppression. In furthermore of our objective; we designed a prospective trial to minimize immunosuppression to monotherapy of Prednisone, 5-10 mg/day in a cohort of willing living donor renal transplant (LDRT) recipients who had undergone pre-transplant SC therapy (SCT).

**Patients and Methods**

Ninety patients (81 males, 9 females) with mean age 32.2 ± 10.9 years and donor-recipient HLA match, 2.81 ± 1.33, were subjected to immunosuppression minimization. There were 5 patients with 0/6 HLA match, 10 patients with 1/6 match, 15 patients with 2/6 match, 38 patients with 3/6 match, 14 patients with 4/6 match, 5 patients with 5/6 match and 3 patients with full (6/6) HLA match. Donors were parents for 50 patients, spouses for 16, siblings for 16, children for 2 patients and unrelated (cross-over) in 6 patients. Original disease causing renal failure was unknown in 53 patients, hypertensive nephropathy in 11, chronic tubulointerstitial nephritis in 8, reflux nephropathy in 6, obstructive uropathy in 3, vasculitis in 2, Alport’s syndrome in 2, lupus nephritis in 2 and autosomal dominant polycystic kidney disease, focal segmental glomerulosclerosis and membranous nephropathy in 1 each.

All these patients underwent transplantation using pre-transplant SCT between September ’98 and November ’11. Inclusion criteria were stable graft function for ≥ 1 year with SCr < 2.0 mg/dL and absence of rejections. Unwilling patients, diabetics and hepatitis C/B were excluded from the trial because we believe that immunological status of these patients is different from others and we have separate protocols for such patients.

All patients received donor hematopoietic stem cells (HSCT) (mean, 71.3 ± 24.14 ml, with 0.26 ± 0.16 x 10⁸ nucleated cells/kgBW of recipient with mean CD34+ count, 0.18 ± 0.22 x 10⁶/kgBW). In addition, donor adipose tissue derived mesenchymal stem cells (AD-MSC) (total 1.98 ± 0.97 x10⁴ cells/kgBW) with mean CD45-/90+ cells, 0.64 ± 0.8 x 10⁴/kgBW and CD45-/73+ cells, 0.24 ± 0.34 x 10⁴/kgBW) were infused in 68 patients. Non-myeloablative conditioning of Cyclophosphamide, 20 mg/kgBW, rabbit-antithymocyte globulin (r-ATG), 1.5 mg/kg BW and Rituximab, 375 mg/m² was given to all patients. In addition 42 patients had undergone total lymphoid irradiation (TLI), (200 CgY x 5) as a part of conditioning and 39 patients received Bortezomib, 1.3 mg/m² x 4 along with methylprednisone, 125 mg intravenously instead of TLI prior to transplantation. (Bortezomib became available later on, hence it was replaced by TLI). Donor specific antibodies (DSA) (luminex single antigen assay) and Peripheral T-regulatory cells (p-Tregs) (CD127low/-/CD4+/CD25high) (flow cytometry) were tested before minimization of immunosuppression and three months after withdrawal of principal immunosuppressants.

**DSA Measurement**

All Class I and Class II antibody specificity screening was performed with Single Antigen Beads (One Lambda, Canoga Park, CA, USA). Screening tests for anti-HLA-specific IgG antibodies was performed using LABScreen® Single Antigen beads, class I and II (One Lambda Inc., Canoga Park, CA, USA). The assays were performed on
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**STEM CELL LAB PROTOCOLS**

**IN VITRO EXPANSION OF HSC**

BM collected from donor was transferred in self designed medium composed of Dulbecco’s Modified Eagle’s Medium (DMEM) with antibiotics and then immediately shifted to culture medium composed of DMEM with high glucose, essential amino acids, albumin, growth factors and antibiotics. Medium was replenished every other day for 8-10 days, the supernatant was removed on 7th/8th day and cultured marrow was mixed with AD-MSC after testing for viability, sterility, staining and quantification.

**IN VITRO GENERATION OF AD-MSC**

Adipose tissue was resected from anterior abdominal wall of kidney donor under LA after making a small incision on left lateral side below umbilicus. Sutures were taken after hemostasis was secured. This adipose tissue collected in self designed medium containing α-MEM, 20% human albumin and antibiotics, and was taken to the lab and minced with knife into tiny pieces. Then it was transferred in to the above medium with addition of collagenase type I, incubated at 37°C for 1 hour on self-designed shaker at 35-40 RPM for digestion. The contents of the medium processed in Petri dish were transferred to centrifuge tubes, centrifuged at 780 RPM for 8 minutes. The supernatant and pellets were separately cultured in the medium with same composition on 100 sq. cm and 25 sq. cm cell+ culture dishes (Sarsted, USA) respectively, at 37°C with 5% CO2 for 8-10 days. The medium was replenished every other day and then harvested by trypsinization. Collected cells after being tested were subjected to flow cytometric analysis. Cells were mixed with cultured BM and infused in portal circulation of patients.

**PROCUREMENT OF PERIPHERAL BLOOD STEM CELLS (PBSC)**

PBSC was collected from cytokine-stimulated donors for 2 days. They were subjected to leucopheresis on stem cell separator (Cobe Spectra version 7-Gambro, China). GCSF, 300 microgram, twice a day for 2 days, was used as cytokine for BM stimulation and mobilization before procurement.

**BONE MARROW (BM) ASPIRATION AND PROCESSING PROCEDURE**

A total quantity of 100 ml BM was aspirated from posterior superior iliac crest of donor under local anesthesia (LA) and sedation (if donor was apprehensive) after cytokine stimulation for 2 days (as mentioned above). The marrow was collected in transfer medium and transferred for culturing to stem cell lab to increase the yield of CD34+ cells by in vitro expansion and fortifying un-fractionated BM with stromal cells.

**PORTAL INFUSION OF STEM CELLS**

Under general anesthesia, a midline incision of approximately 3-5 cm length was made above the umbilicus by laparotomy, omental vein was identified and canulated with 2 guaze intracath. Stem cell bag was connected and they were infused directly without using any filters, at the rate of 6-8 ml/min. After infusion, omental vein was ligated with silk and hemostasis was checked. The wound was closed with vicryl 2/0, and subcuticular stitches were taken using 3/0 monocryl.

**CELL COUNTS, VIABILITY AND STERILITY**

Total nucleated cell counts, viability and sterility tests were performed by standard lab techniques. SC were analysed using FACScan (BD Biosciences, US). CD34+/CD45+/CD33+/- cell lines were measured using CD33 mAb (PE-conjugated), CD34 mAb (FITC-conjugated) and CD45 mAb (PerCP-conjugated) (B.D. Biosciences, USA). For AD-MSC, CD 45-/90+ and CD 73+, CD 73 mAb (PE-conjugated), CD90 mAb (FITC-conjugated) and CD45 mAb (PerCP-conjugated) were used.

Luminex platform following the manufacturer’s protocol. Trimmed mean fluorescence intensity (MFI) values were obtained from the output file generated by the flow analyzer, and normalized using the formula: [(Sample #N beads – Sample negative control (NC) beads) – (Negative control serum #N beads – Negative control serum NC beads)]. Any normalized MFI value over 2,000 were considered positive.

**MEASUREMENT OF pTREGS**

Measurement of pTregs was performed from peripheral blood of patients of all groups using CD127 mAb (PerCP-Cy), CD4 mAb (phycoerythrin [PE]), and CD25 mAb (fluorescein isothiocyanate [FITC]) (Becton Dickinson (BD) Biosciences, USA) according to manufacturer’s protocol using FACScan (BD Biosciences, USA).

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Peripheral blood and BM samples were collected in EDTA. In FACS tubes 20 µl of appropriate antibody was taken and 100 µl of blood was added. After vortexing for 5 seconds, the tubes were incubated in dark for 30 minutes. Then 2 ml of 10x lysis solution was added followed by centrifuging at 1000 RPM for 5 minutes. Supernatant was discarded and 2 ml of sheath fluid was added. The tubes were again centrifuged at 1000 RPM x 5 minutes. Supernatant was discarded. Finally 500 µl of sheath fluid was added for blood/AD-MSC samples and 1 ml was added for BM samples and subjected to data acquisition. Unstained blood samples were used as negative controls.

Initial maintenance immunosuppression consisted of calcineurin inhibitor (CNI) [Cyclosporin (3 mg/kgBW/day)/Tacrolimus (0.05 mg/kgBW/day)] or Sirolimus (1-2 mg/day) and/or mycophenolate sodium (360 mg BD)/Azathioprine (50-100 mg/day) and Prednisone (5-10 mg/day).

CNI levels and Sirolimus were measured at weekly intervals for first 2 months, fortnightly for the next 2 months and subsequently whenever indicated clinically, using Siemens reagent flex kit (Siemens RxL Max) according to manufacturer’s protocol with the aim of maintaining trough levels of CsA between 100-150 ng/ml and that of Tacrolimus and Sirolimus between 4-7 ng/ml. Mycophenolate measurement was not performed.

Immunosuppression withdrawal was started with CNI followed by anti-proliferative agents. Prednisone was continued. Rejections were planned to be treated by anti-rejection therapy followed by rescue immunosuppression. Protocol biopsies were planned after 100 days of steroid monotherapy whenever patients gave their written informed consent. Trials were approved by the Institutional Review Board.

RESULTS

All immunosuppression except prednisone has been withdrawn in all 90 patients. Totally 6 (6.7%) patients developed acute rejections after mean 11.13 ± 5.18 months withdrawal and hence were rescued with mycophenolate. Their mean SCr of 1.71 ± 0.28 mg/dL has increased to 1.85 ± 0.53 mg/dL. Mean posttransplant follow-up is 6.23 ± 2.98 years and mean follow-up since steroid monotherapy is 2.8 ± 1.85 years. DSA-class-1 were present in 25 (27.8%), class-2 in 12 (13.3%), both in 16 (17.8%) and both were absent in 37 (41.1%) patients. Interestingly the same status remained even with steroid monotherapy. Protocol biopsies performed in all 26 patients who gave their consent, were unremarkable (Figure 1). Mean p-Tregs, 3.63 ± 1.55% and mean SCr of 1.4 ± 0.24 mg/dL at the time of immunosuppression withdrawal has remained at that level (Figure 2).

DISCUSSION

At least three major mechanisms for tolerance induction have been proposed: clonal deletion, clonal anergy, and regulation/suppression. Most experts in the field agree that any durable tolerogenic therapy will involve manipulation of more than one mechanism, with the goal of profound reduction in clonal T-cell expansion accompanied by active immune regulation. Multiple receptors, ligands, and signaling intermediates have been identified that are instrumental to these processes and now serve as therapeutic targets for tolerance induction strategies, including co-stimulatory blockade, T-cell receptor targeting, and profound T-cell depletion. Extensive animal and human data suggest that the administration of donor antigen concurrent with these immunomodulatory agents may be an important adjunctive therapy for the success of any clinical tolerance strategies. With the understanding of these concepts, anecdotal reports have been published on tolerance induction however no definite reproducible model of deliberately induced and sustained clinical tolerance has evolved.

Our journey to the promised land of tolerance began in 1998 with megadose of HSC5. We kept on modifying our tolerance induction protocols with improvement in our understanding. The reason for using peripheral blood stem cells (PBSC) was that PBSC is chiefly composed of donor T-cells along with a small dose of CD34+ SC. Double negative (CD3- CD4- CD8-) putative T-regs in PBSC may counteract anti-donor T-cells, both systematically and locally as well as infiltrate the graft, thereby facilitating graft and donor cell survival. Portal infusion of SC was planned because liver is the most tolerogenic organ. It helps in achieving prope' tolerance with low-grade lymphohematopoietic chimerism. We have observed contrary to the current understanding about thymic attrition with advancement in age, that thymus remains active in controlling central tolerance, and to be more definite about the SC reaching thymus, we directly injected them in to
the thymus which preserves substantial numbers of T-regs in the recipient lymphoid repertoire as well as seeking apoptosis of activated T cells\textsuperscript{29,30}. We added nonmyeloablative conditioning treatment to create space in the marrow and reticuloendothelial systems, using cyclophosphamide and rabbit antithymocyte globulin to delete stimulated T-cell clones and rituximab to delete stimulated B-cell clones and control antibody response\textsuperscript{31}. However, our patients still required conventional immunosuppression. Hence we modified the regimen by adding irradiation\textsuperscript{32,33}. Radio-resistant NKT (natural killer T-cells) cells were thus able to interact with antigen presenting cells. At one stage we omitted thymic inoculation, which was deemed not acceptable. At this stage, we began to culture BM to generate a larger yield of CD34+SC. Subsequently we stimulated major histocompatibility complex (MHC)-restricted T- and B-cell clones and then deleted them. We then developed our own technique of generating human adipose tissue-derived mesenchymal stem cells (ad-MSC) in the lab and we fortified our SC with AD-MSC and HSC\textsuperscript{34,35}. By that time proteasome inhibitor bortezomib became available to delete plasma cells hence we replaced it with TLI\textsuperscript{36,37}. However
with long experience we have realised that TLI before transplantation and Bortezomib after organ transplantation is still the most effective and safe conditioning.

MSC have immunomodulatory, immunosuppressive, and tolerogenic effects and also enhance the action of HSC\textsuperscript{38,39}. T-regs are involved in maintenance of tolerance and also dampen immune responses against cancer and allogeneic organs. MSC have been considered as a potential “homeostatic niche” for Tregs and play role in Treg recruitment and regulation and maintenance in vitro\textsuperscript{40-42}. Immunoregulatory functions of MSC are not fixed but rather the result of microenvironment they encounter in vivo\textsuperscript{43}. We believe that p-Tregs have protected the grafts of our patients from chronic rejections. SC, especially MSCs exhibit their genetically unrestricted immunosuppressive effects by inhibition of proliferation and function of T-cells, B-cells and NK cells in dose-dependent manner. MSCs also have tolerogenic effect by which they prolong survival of organ grafts and prevent graft versus host disease. MSCs avoid allogenic rejection by being hypoinmunogenic, modulating T-cell phenotype and by creating an immunosuppressive local milieu. Thus MSC exhibit immunogenicity, “tolerogenicity”, and immunosuppressive effects\textsuperscript{44-52}. Control of chronic rejection which occurs through the indirect pathway has been achieved in our model with sustained presence of p-Tregs.

**Shortcomings of the present study**

This study shows promising clinical results in achieving successful minimization of immunosuppression in LDRT to low dose steroid monotherapy. In this study we have not carried out chimerism studies since our previous experience indicated that peripheral blood chimerism may not be associated with absence of rejection episodes and vice versa\textsuperscript{53,54}. Protocol biopsies are also performed in limited number of patients since it is not easy to procure consent from patients. Financial constraints were the major shortcomings for performing more frequent immunologic monitoring. However we have already started performing DSA at 3 monthly intervals. Multi-centre trial will prove the beneficial effects mentioned here. However the major problem could be in replicating the in-vitro generation of adipose tissue derived MSC and a protocol which may require longer hospital stay.

In conclusion, we have achieved successful minimization of immunosuppression to low dose steroid monotherapy in LDRT using pre-transplant SCT with generation and recruitment of p-Tregs (CD127low/-/CD4+/CD25high).

**Acknowledgements:**

The authors thank staff and technicians of IKDRC-ITS, India for all the technical help. We also thank Priyadarshini Shah, Shobhna Sengunthar and Yazdi Wadia from IKDRC-ITS who have maintained and provided the data on all the patient charts, follow-up and statistical analysis.

**Conflict of Interest:**

The Authors declare that they have no conflict of interests.

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