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ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION INDUCED MIXED CHIMERISM ACROSS MHC BARRIERS CAN ACHIEVE PROPE TOLERANCE IN LIVING RELATED RENAL TRANSPLANTATION -AHMEDABAD EXPERIENCE

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ABBREVIATIONS

BM : Bone marrow
ESRD : End stage renal disease
GVHD : Graft versus host disease
HSCT : Hematopoietic stem cell transplantation
NSSC : Natural suppressor cell chimerism
CsA : Cyclosporin
G-CSF : Granulocyte colony stimulating factor
HSC : Hematopoietic stem cells
NSS : Natural suppressor cells
SCr : Serum creatinine

KEY WORDS

hematopoietic stem cell transplantation, tolerance, renal transplantation

ABSTRACT

Aims : We designed prospective randomized-control trial to evaluate efficacy of tolerance induction protocol in living-related renal transplantation(LRDRTx) using hematopoietic stem cell transplantation(HSCT), low intensity conditioning-induced natural suppressor cell chimerism(NSCC). Reproducible cytoanalytical method to identify and sequentially monitor subset of donor NSCC in periphery correlating their presence with tolerance was devised.

Methods : 410 patients divided in 2 equal groups were enrolled: treated(Tn) and control(Cn), were balanced in demographics + HLA match profile. Tn received high dose HSCT ($20x10^8$ cells/kg), conditioning with low dose irradiation, anti-T-cell antibodies, Treosulfan, cyclophosphamide and Cyclosporin(CsA). Donor BM was infused in thymus, BM, portal, peripheral circulation, supplemented by 2 infusions of peripherally mobilized HSCs. RTx was performed after negative lymphocytotoxicity cross-match. Donor NSCC was monitored by flow cytometry at 3 monthly intervals post Tx. Cn received triple drug immunosuppression. Tn received Pred, 0.2 mg/kg/day and CsA, 3 ± 0.5 mg/kg/day. Trough CsA levels were maintained at 120 ±20 ng/ml. Immunosuppression was discontinued 1 year postTx subject to cytoanalysis in Tn.

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Results

Over mean follow-up of 35.8 months, Tn had stable graft function with mean serum creatinine(SCr),1.3 mg%, low rejection, no patient/graft loss. $CD3^{+dim}CD4^{-}CD8^{-}$ cell levels were 3.8 ±1.4 %. Robust tolerance was achieved in 12.2 %, prope tolerance in 84.9%.

Over mean follow-up of 36.2 months, mean SCr of Cn was 2.8 mg%, $CD3^{+dim}CD4^{-}CD8^{-}$ cell levels were 2.1 ±1.4 %; 68.8 % had rejection episodes; 35.1 % grafts and 23.4 % patients were lost.

Conclusion : We achieved prope tolerance across MHC barriers in LRDRTx associated with NSCC in periphery.

INTRODUCTION

Tolerance was conceptually envisioned by Peter Medawar in 1953 in a neonatal mouse model by creating mixed hematopoietic chimerism. Immunology of a neonatal mouse is quite different from the advanced immune environment of humans and therefore it has not been easy to translate tolerance in clinic.

We developed a protocol to induce allogeneic hematopoietic stem cell (HSC) grafting resulting in mixed hematopoietic chimerism associated tolerance across MHC barriers in humans which would be safe and efficacious. The principal theme was donor specific alloreactive lymphocyte depletion followed by establishment of mixed chimerism. The clinical effectiveness of this protocol depended upon stimulation-depletion of peripheral T-B cell repertoire and establishment of permanent mixed chimerism particularly in thymus.

PATIENTS AND METHODS

Study Design

We carried out a prospective, randomized, open labeled, simultaneous control clinical trial in our living-related donor renal allograft recipients from September 1998 to July 2005 at the Institute of Transplantation Sciences and Institute of Kidney Diseases and Research Centre, Ahmedabad, India, to evaluate the efficacy and safety of tolerance induction protocol in inducing mixed hematopoietic chimerism associated donor-specific tolerance. We also devised a cyto-analytical method to identify and correlate with clinical tolerance, and sequentially monitor a subset of donor natural suppressor cell chimerism (NSCC) in bone marrow (BM) and periphery of study group (Tn) and compared with controls (Cn). The clinical trial was conducted in accordance with the revised Declaration of Helsinki. Institutional Ethics Committee approved the study protocol and consent forms of clinical trial.

Selection of patients

Four hundred and ten consecutive patients admitted with end stage renal disease (ESRD) for transplantation were approached with intent to treat and their informed consent was obtained. They were distributed in two equal groups of 205 patients each: Tn and Cn. Patient demographics and baseline characteristics are summarized in tables 1 and 2.

PATIENTS	Tn (n=205)	Cn (n=205)
Gender – M:F	178 : 27	177 : 28
Mean Age (range) (Yrs)	31 (10 -51)	34 (8 -55)
Average 3rd party infusions	14	16
Etiology of ESRD		
Chronic Glomerulonephritis	80 (39 %)	83 (40.5 %)
Obstructive Uropathy	19 (9.3 %)	23 (11.2 %)
Reflux Nephropathy	15 (7.3 %)	18 (8.8 %)
Hypertensive Nephropathy/ CIN(each)	26 (12.7 %)	18 (8.8 %)
Chronic Pyelonephritis	10 (4.9 %)	09 (4.4 %)
Diabetic Nephropathy	06 (2.9 %)	07 (3.4 %)
MPGN	08 (3.9 %)	09 (4.4 %)
FSGS	09 (4.4 %)	08 (3.9 %)
Primary IgA Nephropathy	06 (2.9 %)	07 (3.4 %)
Alport Syndrome	08 (3.9 %)	07 (3.4 %)
Miscellaneous	18 (8.8 %)	16 (7.8 %)

Table1 Patient Demographics

n=205	0/6	1/6	2/6	3/6	4/6	5/6	6/6
Tn	29	35	29	100	10	2	0
Cn	34	37	34	97	3	0	0

Table 2 Donor -Recipient HLA match profile

Legends for table:

CIN: Chronic interstitial nephritis
FSGS: Focal segmental glomerulosclerosis
ESRD : End stage renal disease
MPGN : Membranoproliferative glomerulonephritis

The 3 most common etiologies of ESRD were chronic glomerulonephritis, obstructive uropathy and reflux nephropathy. Patients with hepatitis B/C virus infections were excluded from this study. All patients were followed-up at the same out-patient clinic at weekly intervals for the first 3 months, fortnightly for the next 3 months, monthly intervals till 1 year post-transplant and 3 monthly thereafter. In every visit their renal, liver functions and complete blood counts were monitored, and cyclosporine A (CsA) levels were monitored using EMIT 2000 assay (Syva Co., Dade Behring, USA, with trough levels of 50 to176 ng/ mL). HIV, HBsAg, HCV and CMV (IgG/ IgM antibodies) infection status was monitored using ELISA technique at monthly intervals in the first 6 months and every 3 months for the next 6 months. Tn were monitored for development of skin rashes, fever and gastrointestinal symptoms of graft versus host disease (GvHD) for 1 year post-transplant. Their liver and BM functions were also monitored at monthly intervals during the same period.

Natural suppressor cell (NSC) analysis and fluorescent in-situ hybridization studies (FISH)

NSCC was studied using FACScan (Becton Dickinson, U.S.A.) and FISH using Nikon micro-station (Japan). In flow cytometry, we analyzed $CD3^{dim}CD4^{-}CD8^{-}$, $CD34^{+}$ $CD45^{weak}CD33^{+}$ cell lines every 3 months posttransplant in peripheral blood and at 6 monthly intervals in BM samples collected from posterior superior iliac crest of all recipients. We used CD3 mAb(PerCP conjugated), CD4 mAb (fluorescein isothiocynate (FITC) conjugated) and CD8 mAb (phycoerythrin(PE) conjugated). For the CD 33/ 34/45 cell line analysis, we used CD33 mAb (PE conjugated), CD34 mAb (FITC conjugated) and CD45 m Ab (Per CP conjugated).

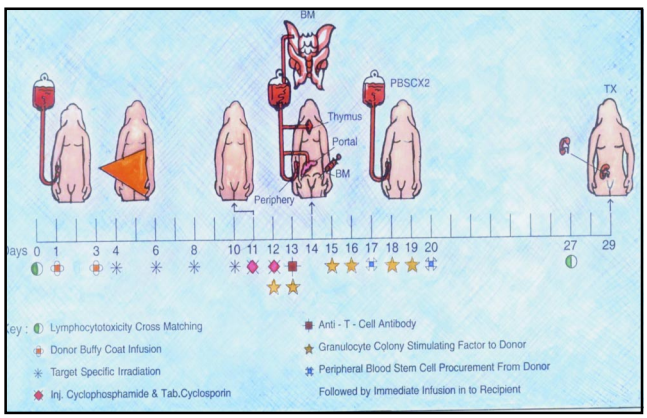

Figure 1 Ahmedabad Tolerance Induction Protocol

FISH was used as a confirmatory test in gender mismatched transplantation. We looked for donor cells which were of opposite gender in a subset of the samples collected above, using Vysis (XX/XY) probes.

Tolerance induction protocol (figure 1)

Step 1: Donor leucocyte infusions were carried out twice, on days 1 and 3.

Step 2: Target specific low dose fractionated irradiation (500 cGy x alternate days) to sub-diaphragmatic lymph nodes, spleen, vertebral bodies and part of pelvic bones on days 4, 6, 8, 10.

Step 3: Cyclosporine (CsA), 3 mg/kg BW/ day, was started on day 11 after irradiation and continued posttransplant. Simultaneous non-myeloablative low intensity conditioning was done with cyclophosphamide, 10 mg/kg BW/day, on days 11 and 13, and polyclonal anti-rabbit T-cell antibody, 1.5 mg/Kg BW and Treosulfan, 15 ng / Kg BW each, in a single dose on day 13.

Step 4: High dose HSC administration in unmodified form with target of = 20 x 10^8 nucleated cells / kg BW was performed on day 14. This was administered in thymus, intra-marrow, portal and peripheral circulation.

HSC mobilization, collection, infusion and inoculation techniques

Donors received Granulocyte-Colony Stimulating Factor (G-CSF), 10 *u*g /kg BW /day subcutaneously for two days. BM aspiration (400 ml) was performed under sedation and local anesthesia from their posterior superior iliac crest after cytokine stimulation and mobilization (G-CSF, 10 µg/Kg BW/ day x 2 days). We inoculated approximately 1 ml of concentrated donor BM in recipient thymus. Four cm long incision was made in to the right second intercostal space of recipient under general anesthesia. After cutting all the muscles, mediastinal fascia was opened and thymus was identified in the retrosternal space. Then 1 ml of concentrated marrow was inoculated with 20 gauze needle. Hemostasis was checked and wound closed. Mini laparatomy was performed simultaneously for portal infusion. Omental vein was identified and canulated. Unmodified BM (200 ml) was infused using IV set through this canula, which was removed after infusion, hemostasis was checked and the wound closed. Forty ml of the above marrow was infused in to bilateral anterior superior iliac crests. The remaining 200 ml was infused in peripheral circulation using IV set.

Step 5: Subsequently G-CSF, 10 *u*g/ kg BW/ day, was continued to stimulate and mobilize HSCs. Donors were then subjected to leucophoresis on days 17 and 20, on stem cell separator (Hemonetics, MCS 3p, USA and Cobe Spectra version 7, Gambro, China) and peripheral blood stem cells (PBSC) were collected and immediately infused in unmodified form in the periphery of recipients.

Each stem cell inoculum was subjected to cell count for total nucleated, differential and CD34+ cell counts.

Tolerance induction protocol was completed in 21 days and patients underwent kidney transplantation after negative lymphocyte-toxicity cross-matching (LCM) performed about 1 week after the last PBSC infusion.

HLA typing and LCM

HLA typing and LCM were performed at the beginning and end of tolerance induction protocol using conventional serological techniques (one-Lambda pre-dot trays, U.S.A.) using auto cross-match, DTT and standard cytotoxicity methods with mixed-cell population. T and B lymphocytes were each separately utilized for cross-matching and donor-specific positivity was found with mixed-cell population.

Desensitization protocol for sensitized host following stem cell infusion

Lymphoid irradiation to spleen (100 cGy x 3), plasmapheresis (1-3 procedures) and administration of MMF, 2 gm /day x 2 weeks was employed in the event of occurrence of donor-specific cytotoxic allo-antibodies. Renal transplantation was performed as soon as LCM negativity was achieved.

Recipient immunosuppression

CsA was the principal immunosuppressant for Tn. The doses were adjusted with an intention to maintain trough blood levels of 120 ± 20 ng/ ml in the first 6 months and 60 ± 20 ng/ ml subsequently. Prednisolone, 0.5 mg/kg BW /day was administered for the first 3 months post-transplantation followed by 0.2 mg/kg BW/ day subsequently. Azathioprine, 2 mg/kg BW/day/ mycofenolate mofetil, 2 gm/ day, was added as a third drug following acute rejection (AR) episodes. Cn were treated with standard triple drug immunosuppression.

Tolerance classification and immunosuppression withdrawl

In Tn, at the end of 1 year posttransplant, immunosuppression was gradually withdrawn over a period of 3 months with peripheral $CD3^{dim}$ cell levels of >3 %. It was initiated by discontinuing Prednisolone followed by CsA withdrawl. Prednisolone was initially tapered to 0.1 mg/kg BW/day followed by administering it on alternate days, then on every third day and then was discontinued. CsA was also gradually tapered to 1 mg/kg BW/day and the discontinued. The patients with no rejection episodes, who had stable, adequate graft function for >100 days of complete immunosuppression withdrawl were classified as having robust tolerance. Their biopsies were performed at the end of 100 days of drug withdrawl to rule out sub-clinical rejection.

Patients with peripheral $CD3^{dim}$ cell levels of 3 ± 0.5 %, were continued on CsA, 1.5 ± 0.5 mg/Kg BW/day and Prednisolone, 5 mg/ day. These patients with no rejection episodes and stable graft function were classified as patients with prope tolerance. Patients with peripheral $CD3^{dim}$ cell levels of 2.3 ± 0.3 %, steroid responsive rejection episodes and subsequent stable, adequate graft function on low dose CsA (1.5 ± 0.5 mg/Kg BW/day) and Prednisolone (0.2 mg/Kg BW/day) were classified as patients having metastable tolerance.

Diagnosis of rejection and its treatment

All recipients were biopsied whenever clinically suspected for rejection, which was diagnosed according to the modified Banff criteria[1] and treated with intravenous methylprednisolone (MP), 250 mg/day for three consecutive days. FK 506 (Tacrolimus) was used as rescue therapy in MP-resistant rejections.

RESULTS (Table 3)

Tn with an average follow-up of 35.8 months (range: 7-83 months) showed significantly better graft function and had very low incidence of steroid-responsive acute rejection (AR) episodes. There was acute borderline tubulo-interstitial rejection, and acute vascular rejection, type IIA, each, in 2 (0.98 %) patients. No CMV infection was seen. There was no graft/ patient loss in Tn. Mean serum creatinine (SCr) was 1.3 mg % at the end of 6 years post-transplantation.

Mean nucleated cell count of HSC was 23.3 x 10^8 cells/ kgBW. Mean CD34 + counts of BM infusion was 0.9 ± 0.2 % and of peripheral blood stem cells was 0.7 ± 0.2 %.

Parameters	Tn (n=205)	Cn (n=205)
Rejection	Borderline ATIR-2(0.98 %)	AVR type IIA- 6 (2.9 %)
	C4d- AVR type I – 2(0.98 %)	AVR type IIB- 5 (2.4 %)
		ATIR type IB – 48 (23.4 %)
		ATIR type IB + AVR type IIA - 13 (6.34 %)
		ATIR type IA + AVR type II B - 19 (9.3 %)
		ATIR, type IB + AVR type IIA +C.R.- 50 (24.4 %)
Graft loss	0	72 (35.1 %)
Patient loss	0	48 (23.4 %)
Present mean SCr (mg %) (range)	1.33 (0.7- 1.95)	2.8 (0.9- 5.8)

Table 3 Comparative data of allograft function in Tn Vs. Cn

TOLERANCE

Three types of tolerance were observed in Tn. Robust tolerance (stable graft function for > 100 days without any immunosuppression and unremarkable biopsy) was achieved in 25 (12.2 %) patients, prope tolerance (early, adequate, stable graft function with no rejection episodes and minimum immunosuppression) was achieved in 174 (84.9 %) patients. Minimum immunosuppression was defined as CsA, 1.5 ± 0.5 mg/ kgBW/day ± Prednisolone, 0.1 mg/kg BW daily or alternate days. Meta-stable tolerance (adequate graft function on minimum immunosuppression with single episode of steroid responsive acute rejection episode) was observed in 6 (2.9 %) patients.

In Cn with an average follow-up of 36.2 months (range:7-83 months), 141 (68.8 %) patients had single AR episodes. Out of these, 6(2.9 %) patients had acute vascular rejection (AVR)-type IIA, 5 (2.4 %) had AVR IIB, 48 (23.4 %) had acute tubulo-interstitial rejection (ATIR) type IB, 13(6.3 %) had ATIR type IB+ AVR type IIA, 19 (9.3 %) had ATIR type 1A +AVR type IIB and 50 (24.4 %) patients had ATIR, type IB +AVR type II A with chronic transplant glomerulopathy, arteriopathy as well as rejection which ultimately led to graft loss. Forty eight (23.4 %) patients and 72 (35.1 %) grafts were lost to steroid-resistant rejections and failure of rescue therapy. The remaining patients have an average SCr of 2.8 mg % (range: 0.9-5.8 mg %). CMV infection was noted in 85 (41.5 %) patients out of whom 63 recovered after adequate treatment with Gancyclovir.

A comparative study of SCr values of all the groups was undertaken at 6 months and yearly intervals respectively using student's paired t test and Tn had significantly better graft function ($p<0.001$) (fig 2).

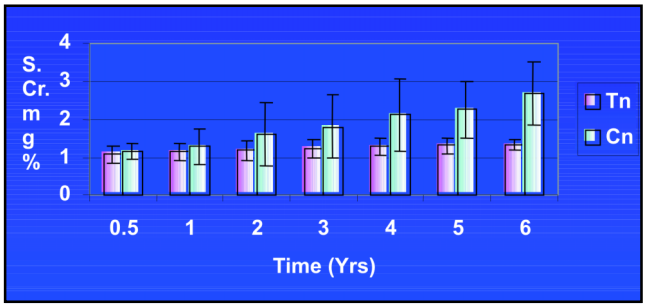

Figure 2 Allograft function over different time intervals post-transplant in Tn Vs. Cn In terms of serum creatinine (S.Cr.)

None of the patients from Tn had GvHD. Transient rise of donor specific cytotoxic allo-antibodies was noted in all patients of Tn at the end of tolerance induction protocol. Desensitization protocol was effectively used in 5(2.4 %) patients to return them to transplantable range in 3 weeks, and the remaining patients returned to transplantable range of donor-specific cytotoxic antibody levels within 2 weeks.

The side effects of G-CSF injection to the donors were malaise (93 %), mild pyrexia (89 %) and occasional skin rashes which responded to anti-pyretic and anti-histaminic agents. None of the donors had serious or life-threatening reactions. Portal, thymic and intra-marrow inoculation were uneventful.

Legends for table:

ATIR : Acute tubulo-interstitial rejection AVR : Acute vascular rejection SCr : Serum creatinine

CELL VALUES (%)	6 MTHS	1 YR	2 YR	3 YR	4 YR	5 YR
$CD3^{dim}$						
PB/BM	3.78 ± 2.3/	3.5 ± 1.8/	3.5 ± 1.4/	3.4 ± 2.2/	3.6 ± 1.1/	3.5 ± 1.7/
Tn	5.23 ± 2.5	3.6 ± 2.1	7.8 ± 0.1	4.5 ± 0.5	5.6 ± 2.1	4.8 ± 1.8
Cn	1.42 ± 0.3/	1.6 ± 0.5/	2.2 ± 0.8/	1.7 ± 0.2/	1.9 ± 0.6/	1.8 ± 0.6/
	3.6 ± 1.3	3.5 ± 1.1	3.1 ± 0.9	2.8 ± 0.6	2.5 ± 0.5	2.4 ± 0.5

Table 4 Comparative natural suppressor cell chimerism analysis in Tn Vs. Cn

NSCC analysis

We observed that out of two cell lineages, $CD3^{dim}$ $CD4^-$ $CD8^-$ and $CD34^+$ CD 33 $^+$ $CD45^{weak}$ in peripheral blood nucleated cells and bone marrow, the former were significantly higher in Tn. $CD34^+$ subset natural suppressor cells were not statistically different at different time intervals post-transplant (table 4).

We have found persistent, stable levels of peripheral blood chimerism for > 5 years in a subset of our patients in whom $CD3^{dim}$ $CD4^-$ $CD8^-$ levels in peripheral blood were 3.5 ± 1.7 % and in BM were 4.8 ± 1.8 %. In Cn, $CD3^{dim}$ $CD4^-$ $CD8^-$ levels in peripheral blood were 1.8 ± 0.6 % and in BM were 2.4 ± 0.5 %.

The mean peripheral $CD3^{dim}$ levels were 5.3 %(range: 3.7 to 8 %) in robust tolerance, they were 2.9 % (range:2.8 to 3.6 %) in prope tolerance and 2.2 % (range: 2.1 to 2.7 %) in metastable tolerance. FISH studies revealed 0.05 ± 0.02 % donor nucleated cells in peripheral blood and 0.6 ± 0.2 % in BM in Tn. These findings were persistent and stable.

DISCUSSION

Organ transplantation is now an acceptable mode of therapy for patients with end stage organ failure however tolerance (transplantation with minimum/ no drugs) has still remained an elusive goal. R.D. Owen described naturally occurring hematopoietic chimerism in freemartin cattle twins who shared each other's red cells for long time after birth[2]. Anderson et al further established chimerism associated tolerance by observing that bovine fraternal twins permanently accepted each other's skin grafts [3]. Billingham, Brent and Medawar created the first experimental tolerance model in neonatal mice where splenic and HSCs from adult mice were injected in neonatal mice. The infused cells when engrafted produced mixed chimerism, and skin graft from adult mice to neonatal mice survived indefinitely. This seminal work of cell transplantation in a defenseless host became the base for BM transplantation of the future[4]. Slavin demonstrated that tolerance in this model cannot be induced in adult mice without irradiation [5].

We have achieved prope tolerance in renal transplantation using HSCT [6-9]. We achieved the benefits of activation induced cell death leading to depletion of potentially rejecting T- cell repertoire, using megadose allo-HSCs utilizing Zinkernagel's concept of MHC restriction [10]. We administered donor leucocyte infusions to stimulate donor specific $CD4^+$ clones in recipient peripheral T-cell repertoire. The next step of non-myeloablative low intensity conditioning with target specific irradiation was taken to create space. Polyclonal anti-T-cell antibodies were used to achieve significant depletion of recipient $CD4^+$ $CD8^+$ cell population to less than 10 % of their baseline levels. Waldmann has established the critical dose and time factor for this infusion [11]. CsA was administered to protect the chimeric cell population. Cyclophosphamide was used to create space in thymus [12]. Treosulfan along with irradiation was used to delete cobblestone-like stem cell colonies which create allo-resistance to grafting [13]. We adopted the portal route for administration of HSC, based on Gorczynski's demonstration of hepatic chimerism associated donor specific tolerance in mouse model [14]. This techniques of mini-laparotomy was developed by us and successfully used in all our patients since 1998 [15]. Posselt has created donor-specific unresponsiveness by intrathymic islet cell transplantation [16]. We therefore carried out thymic inoculation of donor HSC to achieve central tolerance by creating thymic chimerism and augmenting apoptotic donor specific clonal deletion. We preferred intra-marrow administration of HSC for better grafting, since the marrow provides the ideal microenvironment for HSC grafting. This method of intra-marrow inoculation was also devised by us and has been regularly used in all our patients. In majority of our patients we achieved early, adequate stable graft function with almost zero rejection on low dose CsA and steroids, which has been described by Sir Roy Calne as prope tolerance [17].

We were in search of a biological signature of tolerance in the lab. Naturally occurring nonspecific suppressor cells are thought to regulate the generation of antigen-specific/ nonspecific suppressor cells which maintain tolerance to foreign allografts [18]. We decided to measure two lineages of natural suppressor cells in periphery and BM to find out if there was any correlation of tolerance with chimerism established by circulating levels of these cells. We observed $CD3^{dim}$ cell levels in periphery were correlating with clinical tolerance and also noted that higher the levels of $CD3^{dim}$ cells in peripheral blood, better is the graft function without immunosuppression (unpublished data). The levels of tolerant $CD33^{+}/34^{+}$ cell levels were not statistically significant in our studies in comparison to $CD\ 3^{dim}$ cell levels. Presence of such donor cell lineages leads to a state of mixed hematopoietic chimerism synonymous with unresponsiveness to donor tissue allo-antigens. This is the mechanism of tolerance in our depletion- chimerism human model.

CONCLUSION

Ahmedabad tolerance induction protocol is safe, effective and reproducible in inducing prope tolerance in 83.4 % patients and robust tolerance has been achieved in 12.2 % patients across MHC barriers in LRD renal transplantation. Robust tolerance is achieved with persistent peripheral chimerism of >3.6 % $CD3^{dim}$ NSC levels.

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