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# MICROCHIMERISM IN PROMOTING GRAFT ACCEPTANCE IN CLINICAL TRANPLANTATION[1]

**James M. Mathew**[2,3,4], **Joseph R. Leventhal**[2], and **Joshua Miller**[2,4]

[2]Comprehensive Transplant Center, Department of Surgery, Northwestern University Feinberg School of Medicine, Chicago, Illinois, 60611, United States

[3]Department of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, 60611, United States

[4]Jesse Brown VA Medical Center, Chicago, Illinois, 60643, United States

## Abstract

**Purpose of the Review—**Infusions of bone marrow derived cells together with "space making" continue to be tested in clinical organ transplant tolerance protocols. These trials are based on the hypothesis that this might produce initial multilineage chimerism. There is some evidence that this in turn induces regulatory cells which control alloimmunity. Although a wealth of knowledge is available from animal models, this review deals with what we know or can speculate about donor bone marrow cells and chimerism in human organ transplantation.

**Recent Findings—**Calcineurin inhibitors are employed in most of these protocols to blunt the initial immune response. One protocol also has a stepwise regulatory cell generating treatment with sirolimus before total withdrawal. A number of donor chimeric lineages including stem cells, dendritic cells, myeloid precursors and various lymphoid subpopulations cells have been described. Currently, it is recognized that the nature of cells that make up the chimerism could influence graft rejection vs. acceptance. Tolerogenic donor chimeric cells may also generate regulatory subsets thus controlling alloimmunity on two fronts.

**Summary—**It might be speculated that prolonged and sustained regulation or possible anergy induced by chimerism may eventually lead to clonal deletion, thereby bringing about classical immunologic tolerance.

### Keywords

Microchimerism; donor stem cells; immune regulation; graft acceptance; clinical organ transplants

## Introduction

Infusions of bone marrow derived cells continue to be tested in clinical protocols intended to induce specific immunologic tolerance of solid organ transplants. This is aside from their

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Address correspondence to James M. Mathew, PhD, Comprehensive Transplant Center, Northwestern University Feinberg School of Medicine, 300 E. Superior St.; Tarry Bldg. 11-727, Chicago, IL 60611, United States. james-mathew@northwestern.edu, TEL: 001-312-908-5180; Fax: 001-312-503-3366.

more conventional use in conferring engrafted immune and myelopoietic systems into ablated individuals. A wealth of knowledge from experimental animal models has associated chimerism and organ transplant tolerance [1–3]. However, this review deals with what we know or can speculate about donor bone marrow derived cells in human organ transplant recipients, with an emphasis on our own work.

The seminal observations of Billingham, Brent and Medawar [4] in 1953, that H-2 disparate donor bone marrow derived cells infused into fetal or new-born murine recipients could bring about life long specific acquired immunologic tolerance to skin allografts laid the foundation of establishing clinical donor specific tolerance. It was over 20 years later that nonspecific and subsequently donor specific blood transfusions were described to improve human kidney transplant acceptance [5, 6]. The first clinical attempt to use iliac crest donor bone marrow cells (iDBMC) was by Monaco et al. in kidney transplantation [7]. Subsequently, Barber et al. reported initial encouraging results [8], but later observed no significant difference with the control group [9]. However, observations of microchimerism of bone marrow derived cells in several transplant recipients who had stopped immunosuppression (IS) for several years with functioning grafts [10, 11] added impetus to these protocols. In 1994, Fontes et al. [12] reported preliminary clinical results in recipients of several types of organ allografts using vertebral body donor bone marrow cells (vDBMC). Our own clinical studies were performed between 1994 and 2000, in over 350 deceased donor liver (or liver/intestinal), 111 kidney, 25 kidney/pancreas, and 5 kidney/islet transplants accompanied by vDBMC [13–19] as well as 47 living-related-donor (LRD) haploidentical kidney recipients infused with iDBMC [15, 18, 20, 21]. In deceased donor kidney transplant recipients higher graft survival was observed compared to (non-randomized) non-infused controls [17, 22]. Similar observations were also made by others [23–25]. However, in none was immunosuppression withdrawn.

Donor bone marrow cell infusions can bring about a number of immunological effects [21]. These included the infused cells functioning as 1) down-regulators of anti-donor immunity, 2) stimulators that might sensitize, 3) responders that could cause GvHD, and 4) autologous inhibitors of these GvH responses. These theoretical immune effects were studied using non-chimeric marrow from deceased donors *in vitro* [26–32] suggesting strong inhibitory properties for a number of vDBMC sub-populations that could overcome both responding and stimulatory effects, thereby promoting unresponsiveness [21].

## Operational tolerance by Donor Bone Marrow Cell Infusions in Clinical Transplantation

Operational tolerance, i.e. maintenance of the allograft in the absence of immunosuppressive treatment, can be spontaneously achieved in about 20% of liver transplant recipients. The liver contains enormous quantities of passenger leukocytes which generate donor microchimerism in the recipient [33–35]. In contrast, documented occurrences of operational tolerance in kidney transplants are fewer, other than those deliberately induced through donor bone marrow derived cellular infusions involving more potent (ablative or lympho-depleting) induction regimens than in conventional transplants [36–47].

The first deliberate successful clinical attempt was made at Massachusetts General Hospital. HLA-identical (HLA-Id) LRD-kidney transplants were performed accompanied by DBMC infusions, in patients who had received previous chemotherapy for multiple myeloma, the cause of their end-stage renal disease [36, 37]. Thymic x-irradiation (7 gy) was administered, together with (equine) anti-thymocyte globulin induction therapy (ATGAM®; Upjohn, Kalamazoo, MI), and a short course of cyclosporine, which was then totally withdrawn [36]. These studies were then extended to haploidentical renal transplant

recipients [38, 39]. The details are reviewed in this issue of the journal, and hence are not further discussed.

Strober and colleagues [40, 41] initiated a protocol in HLA-identical (HLA-Id) kidney transplant recipients (n=12) by conditioning with ten doses of total lymphoid irradiation (TLI) and five infusions of rabbit anti-thymocyte globulin. This was followed by granulocyte-colony stimulating factor (GCSF) mobilized and purified $CD34^+$ Donor Hematopoetic Stem Cells (DHSC) with low numbers of T cells. Criteria for withdrawal of immunosuppression at ≥ six months were stable chimerism and absence of rejection or graft versus host disease (GVHD). Some patients developed rejection (3/12). In 6 others immunosuppression was withdrawn [41]. Nonetheless, there has been subsequent loss of donor chimerism without deleterious effects.

Trivedi et.al initiated somewhat similar clinical procedures evolving over time [42–44]. They first used GM-CSF mobilized but unpurified DHSC in high dose infusions intraportally, systemically and into the thymus. The majority of recipients were either fully or haplomismatched with the donors. More recently, donor specific blood transfusions to stimulate allospecific immunity have been followed by “deletion” of responding cells with cyclophosphamide, ATG and TLI resulting in 16/69 (23%) patients immunosuppression free or on low dose of steroids at 13–23 months post-operatively. Another modification included the use of rituximab and Bortezomib to eliminate B cells and plasma cells respectively [44].

Currently we are conducting clinical trials using 2 different approaches. In the first, HLA-Id LRD renal transplant recipients are given 4 infusions of $CD34^+$ DHSC, the first purified from iliac crest marrow and the others from GCSF mobilized DHSC in peripheral blood. The infusions extend from day +5 to +270 post-operatively, with alemtuzimab induction on days 0 and +4. Maintenance immunosuppression with tacrolimus is converted to sirolimus by day +80. Mycophenolate, also started at surgery, is discontinued between 12 and 18 months and finally sirolimus withdrawn by 24 months [45]. Chimerism has never reached above 3% and became lower than the detection level of 0.01% in both the peripheral blood and the bone marrow usually after 1 year. Five of 7 recipients are >2 years with immunosuppression withdrawn upto 12 months, thus far with normal renal biopsies and function.

In the second, we have explored combined DHSC and kidney transplantation in HLA mismatched living related and unrelated transplant recipients in collaboration with University of Louisville [46, 47]. This was based on observations that a subpopulation of bone marrow derived cells, the $CD8^+TcR\text{-}\alpha\beta^{negative}$ facilitating cells (FC), significantly increased DHSC engraftment without GVHD in a mouse model [48] as well as in HLA mismatched leukemia [49, 50] and sickle-cell disease patients [51]. A subsequent Phase 1 study of FC-enriched DHSC in renal transplant recipients established the safety of the protocol, although durable chimerism was never achieved [51]. The current study involves nonmyeloablative conditioning pre- and peri-transplant (fludarabine, cyclophosphamide, 200cGy TBI), and infusion of FC-enriched DHSC on Day +1 [46, 47]. Maintenance immunosuppression is with tacrolimus and MMF, with planned total elimination by one year. All initial 8 patients entered into this Phase 2 trial have demonstrated macrochimerism post-transplant, ranging from 6 to 100% at 1 month. Chimerism was lost in 2 subjects due to suboptimal cell dosing and more limited conditioning (less cyclophosphamide was used). However, durable full (100% donor) chimerism has developed in the others, along with evidence of donor-specific hyporesponsiveness. Three patients have been successfully weaned from immunosuppression thus far, one for over 10 months. They are immunocompetent responding to mitogen (PHA) and MHC-disparate third party alloantigens; none have developed donor-specific antibodies using flow crossmatch

techniques. Most notably, none have developed GVHD, in spite of high levels of chimerism. These encouraging early results suggest that nonmyeloablative conditioning in conjunction with FC-enriched DHSC preparations can safely achieve durable donor macrochimerism in mismatched kidney transplant recipients, allowing for immunosuppression withdrawal.

## Role of chimerism

The term chimerism was popularized in transplantation biology by Medawar [52] based on the observations of Owen [53] in freemartin calf dizygotic twins describing a mixture of blood cells due to cross-circulation in the common placenta *in utero*. This type of chimerism, established during the fetal or newborn stages, has been synonymous with a state of lifelong unresponsiveness to donor alloantigens. All the afore-mentioned clinical trials have in common the intent to generate donor chimerism in the adult, some with the additional hypothesis that even transient microchimerism might be sufficient to induce donor specific immune tolerance. Therefore, it is important to analyze the immune reactions that can occur due to chimerism.

## Detection of chimerism

Differences between donor and recipient gene polymorphisms or their products have been used to detect chimerism in a variety of fluorescent and molecular methods. These include HLA polymorphism, gender differences (XX-XY chromosome), variable number of tandem repeat sequences (VNTR) and other cytogenetic markers including ABO blood group antigens. Specific methods include polymerase chain reaction (PCR) [54–56], fluorescent *in situ* sequence hybridization (FISH) [57], flow cytometry (>0.1%) [58] and a combination of PCR and flow cytometry called PCR-Flow. [14, 59]. However, the most widely used and FDA approved method is PCR amplification of short tandem repeats and single-nucleotide polymorphism-specific quantitative real-time PCR (reviewed in [60]*).

## Chimerism: friend or foe

There is controversy about the role and the extent of chimerism needed, especially in humans to be associated with drug-free organ transplant acceptance [61, 62]. Microchimerism mediated by blood transfusions, organ transplantation, or pregnancy has even been associated with allo-sensitization and rejection [55, 56, 63, 64] as well as the development of GVHD in liver and small bowel transplant recipients [65, 66]. Conversely, other studies describe microchimerism as either only an epiphenomenon derived from the vascularized organ or helping to induce allograft acceptance. Although we had reported clinical evidence linking increasing microchimerism in the bone marrow compartment with the absence of graft loss [15, 17, 21], it was with *ex vivo* experiments that we have clarified a role for chimeric cells in amplifying donor-specific unresponsiveness in renal transplant recipients [21, 67, 68]*. Currently, it is recognized that the nature of cells that make up the chimerism could influence rejection vs. graft acceptance.

## Distribution and phenotype of chimeric cells

Passenger leukocytes that migrate from the vascularized transplant in non-immunosuppressed rodent recipients were found to first circulate through the blood stream and rapidly disappear [69, 70] possibly into central and secondary lymphoid organs. In immunosuppressed patients given DBMC infusions, chimeric cell numbers were highest in the peripheral circulation during first 3 months and then gradually decreased until they approached minimal detection levels by 1 year post-transplantation [10, 38] with few exceptions [21, 46, 47, 71]. However, cells of donor origin have been detected long-term in bone marrow, skin and lymph nodes of kidney and liver transplant recipients [10, 11, 72,

73]. These belong to a number of lineages including stem cells, dendritic cells, some myeloid precursors and various lymphoid subpopulations, i.e., T, B and NK cells [14, 15, 18, 21, 74, 75]. When isolated using anti-HLA antibodies to the donor mismatched antigens and with magnetic microbeads, a substantial percentage of the recipient derived donor (RdD) chimeric cells were found to be $CD3^+$, TcR-$\alpha\beta^+$ and $CD28^+$ T cells with markedly decreased CD40L, CD80 and CD86 receptors [21, 67]. However, a significant proportion of RdD cells remained undetermined (lineage negative). Thus, it appears that the chimerism generated in DBMC infused recipients is of multiple lineages.

## Regulatory functions of chimeric cell of donor origin

Very few studies directly demonstrated regulatory functions of chimeric cells of donor or even recipient origin post-transplant. In 1995, Burlingham et al. [76] observed that removal of the donor chimeric cells failed to reverse CTL unresponsiveness in a “chimeric” patient functionally tolerant to a maternal kidney allograft without immunosuppression. However, although restimulation of primary cultures with donor cells plus exogenous IL-2 completely reversed unresponsiveness, addition of fresh patient PBMC subsets to tertiary MLR cultures, inhibited the generation of anti-donor CTL. In our own studies, in addition to phenotypically characterizing RdD cells from the iDBMC LRD-renal transplant recipients (see above), we have tested them in functional *ex vivo* assays. In the recipients with residual anti-donor responses, depletion of cells of donor phenotype allo-specifically increased donor-specific mixed lymphocyte reactions (Figure 1A&B) and addition of these cells back into the culture inhibited them more potently than freshly isolated non-chimeric iDBMC from the non-immunosuppressed LRD volunteers (Figure 1C) [67, 68]*. This inhibition was quasi-antigen-specific, in that at higher doses RdD cells inhibited non-specifically but as the doses decreased non-specific inhibition disappeared while donor-specific inhibition still occurred (Figure 1C vs D) [68]*. Analogously, Demirkiran et.al. observed that up to 5% of $CD4^+CD25^+CTLA4^+$ T cells in liver transplant recipient blood were derived from the donor liver within the first weeks, and that when purified using monoclonal antibodies specific to the donor, these cells inhibited recipient’s anti-donor MLRs [77].

## “Regulation Recruitment”: of cells that develop in the recipient

Microchimerism may have its greatest and long-lasting effect by inducing a regulatory profile within the recipient. Initially in parallel to the immunoregulatory studies with donor chimeric RdD cells described above [67, 68]*, we also tested purified recipient-derived recipient (RdR) “chimeric” cells from the peripheral blood and bone marrow of iDBMC infused LRD-kidney transplant recipients for donor specific regulatory functions [18]. When used as modulators, the RdR cells also inhibited the recipient anti-donor MLR (and CML) responses. In a number studies, depletion of $CD25^+$ cells from recipient responding PBMC increased their donor specific MLR or CTL responses [41, 68]*; but there were exceptions [37, 78]. Conversely, addition of purified $CD4^+CD25^+$ cells from the post-transplant PBMC inhibited recipient’s responses in a dose dependent manner [68, 79–81]*.

Recently, we have approached this as an ancillary study to our HLA-identical renal transplant DHSC infusion trial. The percentages of $CD4^+CD127^-CD25^{high}FOXP3^+$ cells in the PBMC of all patients increased by 10-fold from the pre-operative values during the first 6 months and remained >4-fold even after 24 months. When these post-op recipient PBMC containing these high percentages of putative Tregs were added as third component modulators, they inhibited the donor-specific proliferation of cryopreserved pre-op recipient CFSE-labeled PBMC responders. Noteworthy is the post-op PBMC modulators enhanced the newly generated $CD4^+CD127^-CD25^{high}FOXP3^+$ cells in the CFSE labeled proliferating responders [45, 82]. We described this generation of additional Tregs *ex vivo* as “regulation

recruitment" [82, 83]. These inhibition and recruitment effects identified donor-specific Tregs operating in HLA-id renal transplant recipients undergoing thus far successful immunosuppression withdrawal.

## Conclusions and Synthesis

Infusions of bone marrow derived cells together with "space making" continue to be tested in clinical protocols to induce specific immunologic tolerance in solid organ transplants [36–47]. These trials are based, among other possible mechanisms, on an hypothesis that this might produce initial multilineage chimerism which in turn induces regulatory cells controlling alloimmunity [21]. Conventional immunosuppression with calcineurin inhibitors is employed to blunt the initial immune response amplified by inflammation [36–41, 45–47]. In addition stepwise regulatory cell generating immunosuppression with sirolimus may be beneficial before total withdrawal [45].

A variety of regulatory cell subsets have been described in transplantation and a number of studies including our own have demonstrated regulatory roles played by chimeric cells of both donor and recipient origin [21, 67, 68]*. We introduce the terminology "regulation recruitment" to describe the latter phenomenon. It might be speculated that regulation by donor chimeric cells may also involve the induction of anergy [76] possibly by incomplete antigen presentation in the absence of costimulatory molecules [84], tolerogenic allopeptides [85] or by the transduction of an as yet undefined negative signal, perhaps even involving B cells [86, 87] with a memory and inhibitory phenotype [88]. Prolonged and sustained regulation or anergy may eventually lead to the clonal deletion, thereby bringing about classical immunologic tolerance [89].

These clinical studies have generated more questions than answers. Is "operational tolerance" a ticking time bomb, i.e., a balancing act that can easily be tipped over by an immune stimulus as "mundane" as a viral infection or is it long-lasting? More definition of mechanisms is needed. Are anergy or deletion eventually involved? Does the thymus and central tolerance play a role? Answers are essential before these protocols can be routine in clinical transplantation.

KEY POINTS

- Infusions of bone marrow derived cells together with "space making" continue to be tested in clinical organ transplant protocols with one hypothesis being that this might produce initial multilineage chimerism which in turn induces regulatory cells controlling alloimmunity.
- Conventional immunosuppression with calcineurin inhibitors is employed to blunt the initial immune response.
- Stepwise treatment with sirolimus may augment regulatory subsets before total withdrawal.
- Chimeric cells of various subsets of both donor and recipient origin have been shown to play regulatory roles
- It might be speculated that prolonged and sustained regulation or possible anergy induced by chimerism may eventually lead to clonal deletion, thereby bringing about classical immunologic tolerance.

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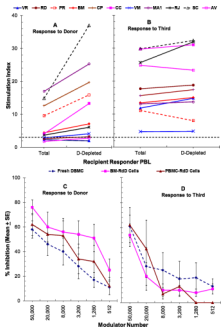


**Figure 1. Role of chimeric cells of donor phenotype present in DBMC recipients at 1 year post-transplantation on MLR regulation**

(A & B): Recipient PMBC were used as responders in MLR either before (Total) or after the depletion of donor cells (D-Depleted) using monoclonal antibodies to mismatched HLA-Class I and Miltenyi magnetic microbeads. Statistically significant differences were obtained in the MLR responses to the donor between Total versus D-depleted recipient responders ($p<0.01$).

(C & D): $1\times10^5$ PMBC from renal transplant recipients depleted of donor chimeric cells were stimulated with $1\times10^5$ irradiated PMBC from the living related donors (C) or third Party (D) in presence of the indicated number of donor modulator cells and standard $^3$H-thymidine incorporation assays were performed on day 7. Data are shown as percentage inhibition ± SE (n=6). Statistically significant differences were obtained in the inhibition of anti-donor MLR between fresh DBMC versus RdD cells from the bone marrow (BM-RdD; $p< 0.001$) and fresh DBMC versus RdD cells from the peripheral blood (PBL-RdD; $p<0.01$). [Previously published in *Human Immunology: 2010; 71(6): 566–576*]