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ORIGINAL ARTICLE

# Multidonor bone marrow transplantation improves donor engraftment and increases the graft versus tumor effect while decreasing graft-versus-host disease

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## Summary

In partially matched donor transplantation, mandatory T-cell depletion (TCD) increases the risks of rejection/graft failure, relapse, and post-transplant infections. A multi-donor approach was offered to resolve some of these drawbacks. This hypothesis was previously tested in a TCD fully mismatched murine model. However, the effect of multi-donor transplantation (MDT) on graft-versus-host disease (GVHD) and graft versus tumor (GVT) effect were never tested. To assess the safety and efficacy of MDT, we used it in non-TCD transplantation and murine breast carcinoma model. We found that when transplanting non-TCD MDT composed by C57Bl/6 and C3H cells into BALB/c, a consistent trichimerism is established, dominated by C57Bl/6 cells. Following MDT the study animals experienced reduced GVHD compare with those transplanted from C57Bl/6 alone, while the GVT effect was superior. We conclude that MDT may serve as a technique that suppresses GVHD while maintaining the GVT effect.

## Introduction

In the absence of a fully matched family donor for stem cell transplantation (SCT), the options for an alternative donor are few and may include a matched unrelated donor, unrelated cord blood transplantation (UCBT) and haploidentical SCT (haplo-SCT). The paucity of stem cells in cord blood graft, which is a cause for delayed engraftment and increased rate of graft failure, initiated an interest in transplanting two or more cord blood units simultaneously. This approach was initially tested in animal models [1], and later on in humans [2,3] and resulted in a shorter time to engraftment [4] and perhaps improved graft versus leukemia effect [5]. Multi-donor transplantation (MDT) from MHC-mismatched allogeneic donors was tested in a murine model [6] showing that the addition of a second T-cell depletion (TCD) bone marrow graft doubled the white blood, platelets, and T-cell counts. We speculated that MDT from an MHC-mismatched allogeneic donor will also improve the graft versus tumor (GVT) effect. We also wanted to examine the effect of such procedure on graft-versus-host disease (GVHD).

To assess the safety and efficacy of MDT, animal studies were performed in the C57Bl/6, C3H, and BALB/c mouse model.

## Materials and methods

### Animals

Two- to 4-month-old inbred male and female C57Bl/6, C3H/HeNHsd (C3H) and BALB/c mice purchased from Harlan Breeding Facility (Jerusalem, Israel) were used as donors and recipients. Mice were kept under clean, specific pathogen free (SPF) conditions with autoclaved cages and sawdust. Food and acidified water were supplied *ad libitum*. All animal protocols were approved by the institutional committee for animal experimentation.

### Total body irradiation (TBI)

Recipient mice were placed in radiation chambers on day −1 and exposed to a submyeloablative TBI dose of 5 or 7.5 Gy (Figs 1a and 2a, respectively) delivered by a linear accelerator (Varian Clinac 6×, Palo Alto, CA, USA) at a dosage rate of 1.79 Gy/min, at a source-to-skin distance of 80 cm.

### Chemotherapy

Cyclophosphamide (Cy) was used in some of the experiments and was injected intraperitoneally (IP) at a single dose of 200 mg/kg (Fig. 1a).

### Bone marrow graft

Bone marrow cells were obtained by flushing the long bones of donor mice with RPMI (Biological Industries, Beit Haemek, Israel) supplemented with 10% bovine calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (Biological Industries).

### Bone marrow transplantation (BMT)

The totally mismatched C57Bl/6 and/or C3H into BALB/c transplantation model is aimed at looking into host versus graft and GVH reactions. Using this model we can also assess GVT effect against BALB/c tumors. To assess the safety of MDT which may theoretically increase the risk of GVHD, we have tested all transplants without TCD thus loading the recipient animals with graft(s) which contain 12–18% T-cell lymphocytes [7,8]. When transplanting a non-TCD graft from C57Bl/6 into BALB/c, the result will always be high GVHD rate with prominent GVHD-associated mortality rate. C3H transplant into BALB/c mice usually results with chronic GVHD like syndrome, usually without high mortality rate (most probably because of inherent immune deficiency in the C3H mice).

Recipient mice were conditioned with TBI on day −1 and on day 0 transplanted with $10 \times 10^6$ bone marrow cells (Fig. 2a). In some experiments (Fig. 1a), we have used the selective depletion of activated donor-reactive host cells method [9], initiated a day after 5 Gy TBI. In this method described by Prigozhina *et al.*, the recipient is exposed to donor bone marrow cells ($20–30 \times 10^6$) for 1 day. A day following the injection of the bone marrow cells, IP 200 mg/kg Cy is given to the recipient, thus killing most of the proliferating recipient cells that responded to the donor cells in attempt to reject them [9]. This was followed a day later by grafts from 1 or 2 donors (again $20–30 \times 10^6$). With either above described

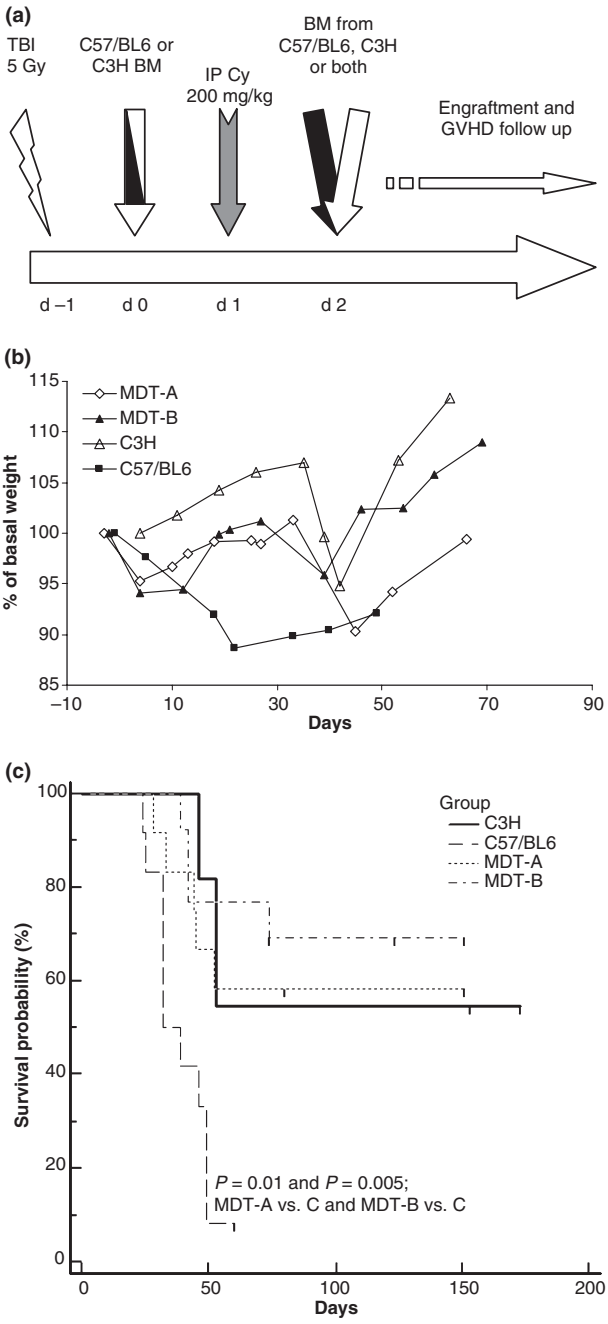


**Figure 1** Multi-donor transplantation (MDT) in a non-T-cell depleted totally mismatched significantly reduced graft-versus-host disease (GVHD) despite C57Bl/6 engraftment. (a) On day −1, recipient mice (11–13 animals per group) were conditioned with 5 Gy total body irradiation followed a day later with exposure to donor bone marrow. A day following the injection of the bone marrow cells, intraperitoneal (IP) 200 mg/kg cyclophosphamide (Cy) is given to the recipient and a day later followed by grafts from 1 or 2 donors. Follow-up of recipient BALB/c weight (b) and survival (c) as markers for the appearance of GVHD following transplant with either C3H, C57Bl/6 or both (MDT-A/B).

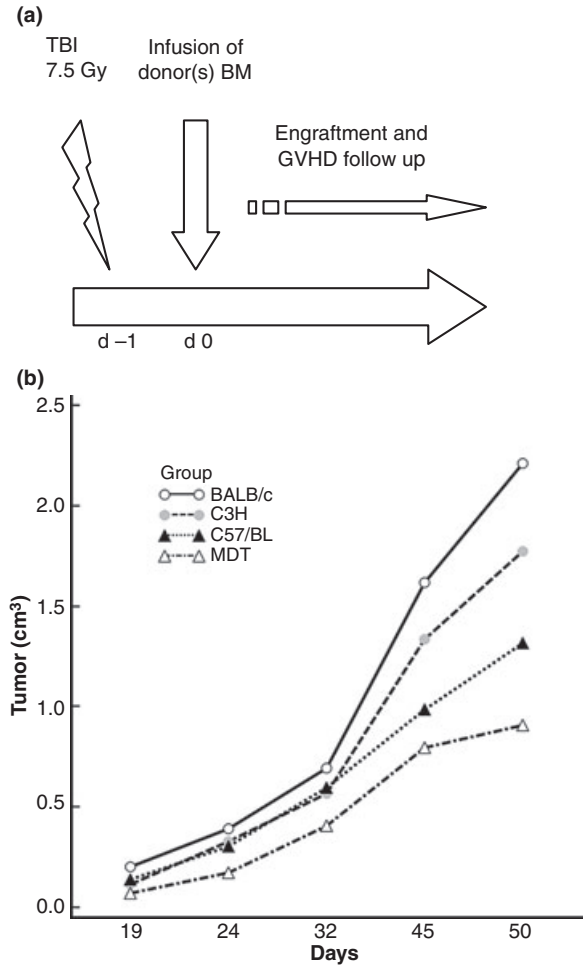


**Figure 2** 4T1 breast carcinoma subcutaneous tumor growth. (a) Recipient BALB/c mice were conditioned with total body irradiation 7.5 Gy followed by $10 \times 10^6$ bone marrow (BM) cells from C3H, C57Bl/6 or both [multi-donor transplantation (MDT), receiving a total of $20 \times 10^6$ BM cells]. On the day of transplant, all animals were also inoculated subcutaneously with $10^4$ 4T1 cells. The animals were followed up for local tumor development and tumor-related death. (b) Tumor growth was inhibited in all allogeneic groups. Despite of graft-versus-host disease suppression in the MDT group, the stronger tumor growth inhibition was found in the MDT group ($P < 0.0001$ and 0.003; MDT vs. C3H and MDT vs. C57Bl/6 respectively).

protocols, cells were injected into the lateral tail vein of the recipient mice to a total volume of 0.2 ml.

Study animals were followed for the hematological reconstitution, development of clinical signs of GVHD including hunched back, diarrhea and weight loss, GVHD-related mortality and donors-recipient chimerism.

### 4T1 cells

The 4T1 breast cancer cell line was originally derived from a breast carcinoma, which arose spontaneously in BALB/c mice [10]. It is a transplantable tumor cell line that is highly tumorigenic and invasive and, unlike most tumor models, can spontaneously metastasize from the primary tumor to multiple distant sites including lymph nodes, blood, liver, lung, brain, and bone [11].

The 4T1 mouse mammary tumor cell line was cultured in high glucose RPMI supplemented with 10% FCS, 1% sodium pyrovate, 1% L-Glutamin and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere containing 5% $CO_2$. On day 0, recipient mice receive $10^4$ 4T1 cells subcutaneously. Other than the above mentioned transplant related events, the mice were followed once weekly for tumor size. The major and minor axes of the tumor mass were measured, using a digital gauge to calculate the tumor volume using the following formulas: (large diameter × short diameter$^2$/2) [12].

### Assay for chimerism

Characterization of the phenotype of mononuclear blood and spleen cells obtained from the surviving recipients was carried out as follows: cells ($10^6$) were incubated with fluorescein isothiocyanate monoclonal antibodies against mouse antigens (H-2$^d$, H-2$^b$, H-2$^k$; PharMingen, San Diego, CA, USA) at 4 °C for 30 min, followed by washing with ice-cold PBS containing 1% sodium azide. Freshly stained cells were analysed by fluorescence-activated cell sorting (FACStar plus; Becton Dickinson, San Jose, CA, USA).

### Statistical analysis

Significance was determined using the Student's *t*-test (Excel; Microsoft, Redmond, WA, USA), Kaplan–Meier survival analysis and analysis of variance (ANOVA) test (MedCalc, Mariakerke, Belgium); $P \leq 0.05$ was considered statistically significant. Studies were repeated twice.

## Results

### MDT from two totally mismatched donors is feasible, safe and reduces GVHD

In this study of complete MHC mismatched allogeneic transplantation, BALB/c served as the recipient and C57Bl/6 and C3H were the donors (11–13 animals per group). The recipients received 5 Gy TBI followed by either C57Bl/6 or C3H bone marrow used for a selective depletion of activated donor-reactive host cells [9] (groups MDT-A and MDT-B respectively; Fig. 1a) followed by a single IP 200 mg/kg Cy injection followed by grafts from both donors. These were compared with controls with selective depletion and transplant from the

same single donor (either C57Bl/6 or C3H before and after Cy).

Hematological reconstitution was achieved in all animals and engraftment was stable. GVHD-associated weight loss (Fig. 1b) was more prominent in animals transplanted solely from C57Bl/6 (group C). MDT was protective against GVHD-related weight loss and most animals regained weight even with C57Bl/6 engraftment ($P = 0.01$ and $P = 0.0015$; MDT-A vs. C57Bl/6 and MDT-B vs. C57Bl/6). As expected, GVHD-associated death occurred in most of the animals transplanted from C57Bl/6 alone (11/12 animals) and in 5/11 animals transplanted from C3H alone. However, transplantation from both C57Bl/6 and C3H (groups MDT-A and MDT-B) again showed protection against GVHD despite C57Bl/6 engraftment, with only 5/12 and 4/13 animals suffering from GVHD-associated mortality, respectively ($P = 0.01$ and $P = 0.005$; MDT-A vs. C57Bl/6 and MDT-B vs. C57Bl/6; Fig. 1c). GVHD-associated mortality was not statistically different between groups MDT-A, MDT-B, and C3H.

### Despite decrease of GVHD, the graft versus tumor effect is augmented

In view of the suppression of GVHD, we evaluated the GVT effect using the 4T1 model. Recipient BALB/c received TBI 7.5 Gy followed by transplantation with $10 \times 10^6$ BM cells from C3H, C57Bl/6 or both (Fig. 2a) while control animals were transplanted from syngeneic BALB/c (14 animals per group). On day 0, recipient animals were injected subcutaneously with $10^4$ 4T1 cells.

4T1 tumors rapidly developed in BALB/c transplanted by syngeneic BM and reached a volume of 2.46 $cm^3$ at 50 days whereas all allogeneic transplantations induced inhibition to tumor growth. The allogeneic inhibitory effect was induced in a stronger extent in those transplanted with C57Bl/6 ($P = 0.028$). However, it was further pronounced in the animal of the MDT group. As mentioned above, the median subcutaneous tumor size on day +50 in the control BALB/c group was 2.46 $cm^3$. At the same timing, tumor size in the C57Bl/6 and C3H groups was significantly lower compared with the control group with a median of 1.47 and 1.57 $cm^3$ respectively. The median tumor size was furthermore decreased in the MDT group to 0.84 $cm^3$ (Fig. 2b. $P < 0.0001$ and 0.003; MDT vs. C3H and MDT vs. C57Bl/6 respectively). Tumor follow-up was not continued as all animals died in due course from autopsy proven metastatic disease or GVHD.

We therefore have a proof of concept that MDT is feasible and safe. It is furthermore shown that MDT may reduce transplant toxicity (i.e. GVHD), with improvement of the GVT effect and accordingly could improve transplant success.

### The effect of MDT on chimerism

To evaluate post-transplant chimerism, recipient BALB/c received TBI 7.5 Gy followed by transplantation with $10 \times 10^6$ BM cells from C3H, C57Bl/6 or both (Fig. 2a). There was high proportion of donor cell chimerism in all experimental groups as assessed by FACS analysis of the peripheral blood mononuclear cells. The median combined donors' chimerism (±SE) in the MDT group was 91.2 ± 1.1% and 95.3 ± 0.8% at 30 days and 60 days respectively. Donor chimerism of animals transplanted with C57Bl/6 and C3H was 85.6 ± 1.8% on day 30 and 93.8 ± 0.4% on day 60, and 83.6 ± 3.3% on day 30 and 96.2 ± 0.5% on day 60, respectively (Fig. 3, showing significantly better donor chimerism in the MDT group

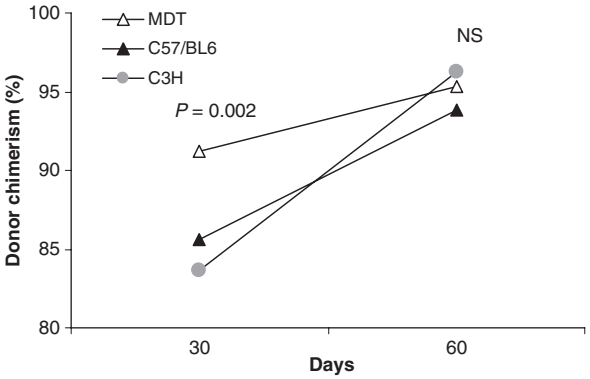


**Figure 3** Multi-donor transplantation (MDT) speeds donor engraftment. Median peripheral blood mononuclear cells donor chimerism was significantly higher in animals receiving MDT compare with those treated with a single donor at day +30 (all conditioned with 7.5 Gy). The donor chimerism equalized at day +60.

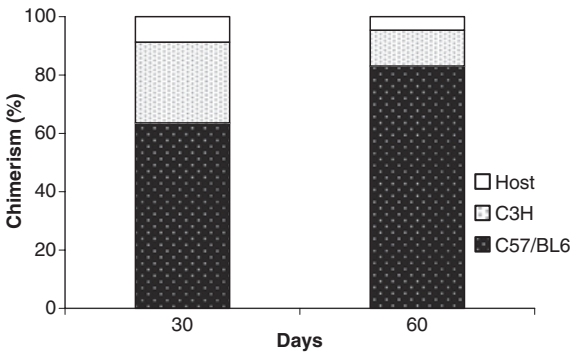


**Figure 4** C57Bl/6 consistently contributed more to the double donor chimerism. Both at day +30 and day +60 post double donor transplant following 7.5 Gy conditioning, C57Bl/6 cells (in black) level of chimerism was significantly higher compared with C3H cells (in gray).

compared with both C57Bl/6 and C3H groups at 30 days; $P = 0.0018$ and 0.0017 respectively).

In the MDT group, we saw at both time points, that C57Bl/6 cells were significantly more dominant with 63.4% and 84.2% of C57Bl/6 donor cells chimerism respectively ($P = 0.004$ and 0.0001 at day 30 and day 60 respectively; Fig. 4), although one of the 14 mice showed consistent C3H engraftment (87.8 and 94.5% at day 30 and 60 respectively).

## Discussion

For patients with no matched sibling available for SCT (consisting approximately 70% of patients) a matched unrelated donor or allogeneic UCBT may provide an alternative treatment option. However, frequently, a donor cannot be found and/or the search process is too long [13,14]. The transplantation of SC from a parent, a sibling, or a child of a patient with only one identical HLA haplotype may provide a donor for almost every patient in need at an optimal timing. The use of a mismatched donor usually necessitates TCD or positive selection of SC to prevent uncontrolled GVHD [15], which increases the risk of graft rejection because of the loss of engraftment facilitating lymphocytes and decreases the intensity and efficacy of graft-versus-leukemia (GVL) effect. In UCBT, to overcome the delayed engraftment, the possibility of using several cord blood units to increase the stem cell dose was investigated [1]. This enabled the use of double cord blood units in adults. In the small number of dual cord transplants reported, it was found that usually only one unit engrafted faster, while the other was rejected [2–4,16] at the price of increase GVHD rate. It was also shown in a single report that the transplantation of two cord blood units can potentially augment the GVL effect [5]. This effect was most prominent in patients undergoing transplantation in their first or second complete remission. It is not known whether this enhanced GVL effect is mainly the result of increased HLA disparity from the use of two mismatched units or if it is attributed to the presence of additional graft–graft or graft–patient immune effects. Chen *et al.* [6], evaluated the kinetics of hematological engraftment and immune reconstitution following transplantation of T-cell depleted bone marrow cells from a single donor (C57Bl/6 or SJL/J) and 2 units from different donors (C57Bl/6 + SJL/J) into lethally irradiated (8.5 Gy) BALB/c recipients. They found that the addition of TCD bone marrow from an MHC-mismatched allogeneic donor doubled the white blood counts compared with recipients of one single unit on days +10 and +14. Similar effects were also observed on platelets. The beneficial effect of additional cells on peripheral T-cell counts were first observed on day +14 and cells both from donors and recipient contributed to the myeloid and lymphoid reconstitution. Thus, it was shown in animals and in humans that the combination of two donors in SCT is beneficial for engraftment and the GVL effect and for immune recovery in animals.

Based on this information, we wanted to assess the GVHD/GVT effect in a model similar to the one used by Chen *et al.* [6] using the 4T1 murine breast carcinoma model. We hypothesize that enhanced immune reconstitution (earlier recovery of lymphocytes and NK cells of both donor origin facilitated by the used of higher doses of stem cells) will improved GVT. To confront MDT, all our studies were without TCD as was previously reported in the NOD/SCID models [1] and in contrary to the only reported study performed using mismatched bone marrow [6] in which the grafts were TCD. As our main concern with transplanting two donors simultaneously was safety (e.g. GVHD), the experiments described in the first paragraph were designed to assess this issue. We therefore used the selective depletion protocol [9] with two aims: (i) infusion of megadoses of stem cells (achieved by using $20–30 \times 10^6$ bone marrow cells per donor and (ii) No TCD; both known to increase GVHD tendency. In the GVT studies, we have used a lesser amount of bone marrow cells to prevent early death, and decrease overall GVHD-associated death that will prevent us from drawing any conclusions.

Surprisingly, we found that opposed to our preliminary expectations, the use of two mismatched donors decreased rather than increased GVHD in a consistent manner as judged by clinical GVHD symptoms, weight loss and GVHD-associated mortality (Fig. 1). We further show that the transplantation of C57Bl/6 together with C3H into BALB/c animals that were inoculated with syngeneic 4T1 breast carcinoma cells resulted in the strongest delay of tumor growth (Fig. 2b). Namely, at 50 days post-inoculation, median tumor size of single transplant control animals was 1.45 and 1.42 $cm^3$, whereas in the MDT group it was 0.94 $cm^3$.

We also found that donors' engraftment and corresponding recipient displacement was better with MDT (Fig. 3). We appreciate that the enhancement of GVT effect and donor engraftment might be a consequence of higher dose of BM cells rather than two different sources of BM cells and further studies are planned to assess these assumptions. However, in clinical setting we expect that double dose of cells will be given rather than same dose from two donors. Additionally, there are no concrete data showing that more stem cells or T cells induce better GVT. There are, however, data showing the in patients with acute leukemia transplanted with two UCBT (most of which mismatched to the recipient) had significantly lower

relapse risk compared with patients transplanted with a single unit [5] with elevated GVHD rates. One striking feature of double UCBT is that almost all cases show rapid skewing of donor engraftment with long-term hematopoiesis derived from one UCB donor [3]. Such findings may suggest an immune-based, graft-versus-graft interaction that ends with rejection of one unit by the other and as a consequence enhanced GVL effect [albeit the mechanism and effector cell population(s) remain unclear]. This might be the explanation of the GVT phenomenon described in the current study, but does not seem related to the reduction of GVHD. As the current study was designed to assess the safety and efficacy of MDT, we did not measure the levels of T-cells' sub-populations ($CD4^+$, $CD8^+$, NK, NK-T, $CD4^+/25^+$, etc.). It is, however, possible that the GVHD is reduced because of a higher level of regulatory cells ($CD8^+$ T-cells, $T_{regs}$ or others). Further studies are planned to assess whether a change in these subpopulations is related to either decreased GVHD and/or increased GVT. If this is indeed the mechanism, it might be that these regulatory cells decrease the GVHD through interference of the graft versus graft interaction without interfering in the GVT process. We plan to repeat this study with myeloablative conditioning, different cell doses and as this study was performed without T-cell manipulation, we also want to evaluate the GVT effect with two TCD grafts to supplement the Chen study [6].

These findings, in combination with the safety results presented by Chen [6] and our earlier report of the safety and efficacy of bi-parental MDT regardless of the conditioning dose used (myeloablative or non-myeloablative) [17], lead us to progress into human studies and a clinical phase I/II study has been initiated using two haploidentical T-cell depleted grafts (registered at the PRS, NCT00716690).

Our method may also set ground for the induction of wide range solid organ allograft tolerance. The paucity of organs for solid organ transplantation (SOT) and the delay in their achievement increased the use of a living family member as donor. It is long known that lympho-hematopoietic chimerism is associated with donor-specific allograft tolerance [18]. In this study, we showed that that bi-donor chimerism can be initiated. If these donors would be both parents, the induction of persistent mixed parental chimerism would lead into pan-familial tolerance thus creating an immunological platform for SOT from father, mother or other siblings. Further studies are warranted to prove this assumption.

## Authorship

YZ and HE: performed the research and collected the data; WL: performed the research; GO: analyzed the data; SS: designed the research; SM: designed the research, analyzed the data and wrote the article.

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