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Investigation of immunological approaches to enhance engraftment in a 1 Gy TBI canine haematopoietic stem cell transplantation model

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Abstract

Objective—Stable mixed haematopoietic chimerism can be established in a canine stem cell transplantation model using a conditioning consisting of total body irradiation (TBI, 2Gy) and postgrafting immunosuppression with mycophenolate mofetil (MMF) and cyclosporin (CSA). Reduction of TBI had resulted in graft rejection in this model previously. We investigated whether postgrafting stimulation of donor T-cells against recipient's haematopoietic antigens or graft augmentation with donor monocyte-derived dendritic cells (MoDC) promote engraftment following 1Gy TBI.

Methods—All dogs received dog leukocyte-antigen-identical bone marrow transplantation. Dogs were conditioned with either 2Gy of TBI (group 1) or 1Gy of TBI followed by repetitive recipient haematopoietic cell lysate vaccinations (group 2) or graft augmentation with MoDC (group 3). Immunosuppression consisted of CSA and MMF.

Results—In group 1 four animals remained stable chimeras >wk110, and 3 rejected their grafts (wk10, wk14, wk16). All dogs in groups 2 and 3 rejected their graft (median: wk 10 and 11, respectively). Peak chimerism and engraftment duration was shorter in the 1Gy groups ($p < 0.05$) compared to group 1.

Conclusion—Neither postgrafting vaccination nor graft augmentation with MoDC were effective in supporting durable engraftment. Additional modifications are necessary to improve potential strategies aimed at establishment of early tissue specific graft-versus-host reactions.

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Keywords

bone marrow transplantation; chimerism; vaccination; host vs. graft reaction; graft vs. host reaction; dendritic cells

Introduction

Haematopoietic stem cell transplantations (HSCT) remain the only curative treatment for a number of malignant and nonmalignant haematological diseases such as acute leukemias and hemoglobinopathies. Modalities of HSCT have changed significantly over recent years due to better understanding of graft composition, graft-host interactions and medications available. One of the most important advances was the development of toxicity reduced conditioning regimen. These regimen were initially developed in preclinical models [1-3] and thereafter transferred rapidly into clinical settings [4-8].

The Seattle group has used the canine allogeneic HSCT model in order to develop a conditioning regimen that is based on 2 Gy total body irradiation (TBI) in combination with mycophenolate mofetil (MMF)/cyclosporin A (CSA) during the pre- and posttransplantation period. Using this regimen > 90 % of dog leukocyte-antigen (DLA)-identical littermate HSCT recipients developed stable mixed haematopoietic chimerism [1,9]. TBI dose reduction to 1 Gy led to an initial engraftment and transient mixed chimerism in 4 of 6 dogs, but all grafts were rejected eventually [1]. Subsequently the Seattle group used this model to evaluate the efficiency of new transplantation proceedings using the engraftment as read out parameter. Thereby, modification of graft composition as well as changes in the pre- and/or posttransplantation immunosuppression allowed prolonged engraftment in some settings [6,9-12]. These studies demonstrate the sensitive balance between donor and recipient cells and, hence, the suitability of this model for evaluating new approaches for adoptive immunotherapy. Here, we used this model in order to address the question whether in vivo graft stimulation by vaccination strategies or graft augmentation with dendritic cells (DC) might allow to direct the graft reaction early against recipient haematopoietic cells. If so, stable engraftment following 1 Gy TBI should be possible.

Materials and Methods

All experiments were approved by the review board of the state Mecklenburg-Vorpommern (Landesveterinär-/Lebensmitteluntersuchungsamt M-V, Germany).

Laboratory animals

Litters of random-bred beagles were obtained from commercial kennels licensed by the German Department of Agriculture. All dogs were dewormed and immunized against rabies, parainfluenza, leptospirosis, distemper, hepatitis, and parvovirus. DLA-identical donor/recipient sibling pairs were selected on the basis of matching for highly polymorphic DLA class I and class II microsatellite markers [1]. At study entry dogs weighed a median of 13.6 kg (range 10.4-19.0 kg) and were a median of 14 months (range 7-14 mths) old.

Haematopoietic stem cell transplantation

The study design is displayed in Figure 1. Characteristics of HSCT recipients and graft composition are depicted in Table I.

HSCT was performed as described previously [1]. Briefly, conditioning of recipient dogs consisted of nonmyeloablative TBI at single doses of 2 Gy (control group 1, n = 7) and 1 Gy

(experimental groups 2 (n = 7) and 3 (n = 6)) at a dose rate of 0.25 Gy/min from a high-energy linear accelerator (Siemens Primus; 10 MV X-ray). Bone marrow (BM) of DLA-identical littermates was collected under general anesthesia from the humeri, femora, and iliac crest. Marrow grafts containing a median of 3.5×10^8 total nucleated cells (TNC) per kg (range $1.6\text{--}11.4 \times 10^8$ TNC/kg) and a median CD34⁺ cell content of 0.5×10^6 cells/kg (range $0.2\text{--}2.2 \times 10^6$ cells/kg) were infused intravenously within 24 h of TBI. The day of marrow infusion was designated as day 0. All recipients received immunosuppression consisting of CSA at 15 mg/kg orally 2 times daily on days -1 to +35 in combination with MMF at 10 mg/kg orally 2 times daily on days 0 to +27. Assessment of clinical status of recipients was done twice daily. The end point of the study was the incidence and duration of mixed haematopoietic chimerism.

Assessment of haematopoietic chimerism

Before and after HSCT peripheral blood of the recipients was obtained every 1-2 weeks for analyses of the donor/recipient haematopoietic chimerism. Granulocyte and peripheral blood mononuclear cell (PBMC) fractions were separated by standard Ficoll-Hypaque density gradient centrifugation (density 1.074 g/mL) and genomic DNA of both cell fractions were isolated (Nucleobond CB 100; Macherey-Nagel, Düren, Germany). (GAAC)_n nucleotide repeats that were polymorphic between donors and recipients were amplified and quantified as described previously [13]. Engraftment was defined as detection of > 2 % of donor derived DNA. Graft rejection was defined as detection of no donor derived DNA in two subsequent chimerism analyses of the PB and once of the BM.

Vaccination of the recipient with haematopoietic cell lysate

After HSCT the donor marrow graft was sensitized in vivo to recipient haematopoietic antigens by vaccination with recipient blood cell lysates (group 2). PBMC and granulocytes were isolated from recipient PB before transplantation. After washing with phosphate buffered saline PBMC and granulocytes were diluted in 0.9 % NaCl and mixed at a ratio of 1:1 for a final concentration of 1×10^5 cells/kg body weight in 1 mL. Cells were 5 times frozen in liquid nitrogen and thawed in a waterbath at 37 °C for lysis. Animals were vaccinated intradermally and subcutaneously with 1 mL lysate applied bilateral in the groin 3 times per week (wks 1-5) and weekly during weeks 6-9 after HSCT. At the time of vaccination granulocyte macrophage-colony stimulating factor (GM-CSF, 125 µg/ vaccination; Berlex Laboratories, Inc., Richmond, CA) was applied at the site of vaccination subcutaneously.

Isolation of monocytes and generation of monocyte-derived DC (MoDC)

Five days before HSCT 300 mL of heparinized blood of the donor was collected and PBMC were isolated. Cells were incubated with a FITC-conjugated canine anti-CD3 monoclonal antibody (Serotec, Düsseldorf, Germany) and subsequently labeled with anti-FITC immunomagnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells were run through an AutoMACS device (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instruction. In a second step, the CD3-negative fraction was incubated with anti-CD14 microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and sorted by using the AutoMACS. Monocytes were grown in 24-well plates (Costar, Corning Inc., NY, USA) for 5 days in RPMI-1640 medium supplemented with 10 % heat-inactivated pooled dog serum, 1 % penicillin/streptomycin (10000 U/mL penicillin, 10000 µg/mL streptomycin), 500 U/mL canine Interleukin-4 (R&D Systems, Wiesbaden, Germany), and 100 ng/mL GM-CSF (R&D Systems, Wiesbaden, Germany). Medium was renewed on day 2. On the day of HSCT MoDC were harvested and added to the marrow graft at a median of 9.8×10^5 cells/kg (range $6.4\text{--}23.3 \times 10^5$ cells/kg).

Mixed leukocyte reactions

Mixed leukocyte reactions were performed to investigate the alloreactivity of PBMC before and after HSCT. PBMC were isolated pretransplant (recipient cells only) and on days +14, +28, and +91 after transplantation (i.e. cells of recipient and donor origin in chimeric animals). Triplicates of cells were cultured in Quantum 007 medium (PAA Laboratories, Cölbe, Germany) at 1×10^5 cells/well in 96-well U-bottom cell culture plates together with 1×10^5 irradiated (25 Gy) PBMC of the same dog (recipient cells only) that had been collected and cryopreserved before HSCT as stimulator cells. Cells were cultured in a total volume of 200 μ L/well at 37 °C and 5 % CO₂ for 9 days. For positive control, 2 μ g/mL of the lymphocyte mitogen concanavalin A (ICN Biomedicals, Aurora, Ohio) was added to responder cells on day 3. Cells were restimulated on day 6 with 1×10^5 irradiated stimulator cells/well and 100 IU/mL IL-2 (Chiron BV, München, Germany). On day 8 after primary stimulation cells were pulsed with 37 kBq [methyl-³H]-thymidine (Amersham Biosciences, Freiburg, Germany) per well for 18 h and afterwards harvested using the Harvester Filtermate 196 (Packard Instrument Company, Meriden, Connecticut). Counts per minute (cpm) were measured in a β -scintillation counter (Packard Instrument Company, Meriden, Connecticut).

Intracellular cytokine staining

To assess the intracellular cytokine production before and after transplantation the “Intracellular Cytokine Staining Starter Kit-human” (BD Pharmingen, Heidelberg, Germany) was used as specified by the manufacturer. Briefly, PBMC were isolated pretransplant and at days +14, +28, +56, and +91 after transplantation from heparinized blood. Cells were cultured in IMDM medium (PAA Laboratories, Cölbe, Germany) supplemented with 10 % pooled dog serum, 1 % sodium pyruvate, and 1 % non-essential amino acids in 96-well U-bottom cell culture plates at a density of 3×10^5 cells/well. Cells were stimulated with 2 μ L/mL Leukocyte Activation Cocktail for 6 h at 37 °C and 5 % CO₂. After fixation and permeabilization with Cytofix/Cytoperm Buffer cells were stained with 4 μ g/mL of an APC-conjugated anti-human tumor necrosis factor alpha monoclonal antibody (TNF- α , clone Mab11, BD Pharmingen, Heidelberg, Germany) and 0.02 μ g/mL of a FITC-conjugated anti-bovine interferon gamma monoclonal antibody (IFN- γ , clone CC302, Serotec, Düsseldorf, Germany) in the dark at room temperature for 20 min. Isotype matched control antibodies (BD Pharmingen, Heidelberg, Germany) labeled with APC and FITC, respectively, were used as controls. Subsequently, cells were washed two times in Perm/Wash Buffer, resuspended in Staining Buffer, and kept at 4 °C until flow cytometric analysis.

Delayed-type hypersensitivity (DTH)

DTH skin test was performed with recipient cell lysate consisting of 1×10^6 PBMC per 0.5 mL NaCl 0.9 % that were lysed by 5 freeze-thawing cycles as described above. 0.5 mL of the lysate was injected intradermally and subcutaneously into the upper foreleg before as well as 14 and 28 days after HSCT. A positive skin reaction was defined as erythema > 3 mm and/or induration of the skin 48 h after application.

Statistics

The distribution of data was described using medians and ranges. Statistical analyses between treatment groups were performed by using the Mann-Whitney U-Test. Within the treatment groups data of different days were analysed by the Wilcoxon matched-pairs signed rank test. Probability of $p < 0.05$ was considered significant.

Results

A total of 20 HSCT were performed. Data on graft composition are displayed in Table I.

Engraftment

Donor BM engrafted in all animals initially. Results of chimerism analyses are summarized graphically in Figure 2. Earliest detection of chimerism was on median day +14 in granulocyte compartment and day +7 in the PBMC compartment in all three groups, respectively. Compared to the 2 Gy TBI group (control) engraftments at 4-weeks of groups 2 and 3 were at significantly lower donor chimerism levels. No differences in week 4 chimerism patterns between groups 2 and 3 were observed. The maximum median chimerism of the 2 Gy group was 75 and 41 % for granulocytes and PBMC, respectively. The 1 Gy groups had significantly lower maximum granulocytes and PBMC donor chimerism with medians of 26 and 13 % in group 2 as well as 27 and 14 % in group 3, respectively. The engraftment duration was significantly reduced in the 1 Gy groups compared to controls (Table I, Fig. 2). However, if graft rejection occurred time courses were similar between dogs of the 2 Gy group (median time of rejection: 14 weeks, range 10-16 weeks) and the 1 Gy groups (median time of rejection: 10 weeks, range 8-16 weeks).

Haematological toxicity

Within all three groups a time-dependent decrease in leukocyte and platelet counts was observed. Animals of the 2 Gy TBI group had significant lower leukocyte nadirs (median $2.3 \times 10^9/l$ [range $1.1-2.7 \times 10^9/l$]; day 8) compared to animals of the 1 Gy TBI conditioning groups (group 2: $4.9 \times 10^9/l$ [$2.9-8.3 \times 10^9/l$], day 5; group 3: $5.0 \times 10^9/l$ [$4.0-5.9 \times 10^9/l$], day 8; $p < 0.05$ both). Similarly, platelet nadirs were significantly lower in the 2 Gy group (median $35 \times 10^9/l$ [range $7-56 \times 10^9/l$]; day 11) compared to the 1 Gy groups (group 2: $111 \times 10^9/l$ [$43-157 \times 10^9/l$], day 11; group 3: $80 \times 10^9/l$ [$14-131 \times 10^9/l$], day 12; $p < 0.05$ both).

Proliferation assay and intracellular cytokine staining

None of the animals developed a graft versus host disease (GvHD) or showed a positive DTH reaction.

In order to determine alloreactivity over time [3H]-thymidine proliferation assays as well as intracellular cytokine stainings for TNF- α and IFN- γ were performed in dogs of the 1 Gy groups. At day +91 a median 2.6-fold increase in proliferation rate could be observed compared to pretransplantation when recipient PBMC were used as stimulators (Fig. 3). In general, stimulation of posttransplantation mixed chimeric PBMC with both recipient PBMC cell lysate and Leukocyte Activation Cocktail did not demonstrate significant changes in the percentage of TNF- α and IFN- γ producing T-cells at days +14, +28, +56, and +91, possibly due to low numbers (data not shown). However, splitting the data in subgroups in regards to the time point of rejection indicated the presence of more cytokine producing cells during graft rejection. An example is given in Figure 4, which shows the cytokine data of a dog that was rejecting starting on day +84.

Discussion

Allogeneic effects following HSCT are potent immunological reactions that allow tumor eradication if directed against tumor antigens but may also cause GvHD. Nonmyeloablative conditioning regimen were developed that allow stable donor haematopoietic engraftment with reduced toxicity. Here, we asked whether modification of the graft or in vivo graft

stimulation early after transplantation allows stable engraftment in a canine nonmyeloablative HSCT model that has demonstrated invariably graft rejection otherwise.

Our results demonstrate that haematopoietic cell lysate vaccinations as well as enrichment of BM grafts with donor-derived immature MoDC failed to promote stable engraftment after 1 Gy conditioning. Using proliferation assays and intracellular cytokine staining, we showed a significant increase in T-cell proliferation 91 days after HSCT and a tendency to higher TNF- α and IFN- γ production at the time of graft rejection in dogs treated with a 1 Gy conditioning.

Nonmyeloablative HSCT with 2 Gy TBI for conditioning was performed according a previously described protocol [1]. In their studies 92 % of the transplanted animals (11/12) developed a stable mixed chimerism [1,9]. In our setting we modified the application route of MMF (oral instead of subcutaneously) and the dose rate of TBI (0.25 versus 0.07 Gy/min). Using this regimen we achieved in 57 % of the animals (4/7 dogs) a prolonged mixed haematopoietic chimerism. Since quantities of TNC were comparable between both studies the most likely explanation for the difference in engraftment is a reduced postgrafting immunosuppression by oral MMF in our study. Oral MMF has been reported to cause significant interdose variability that might result in a reduced immunosuppression [14]. Irradiation with a higher dose rate should enhance recipient immunosuppression rather than lower it [15] and, therefore, may not account for a more frequent graft rejection.

Data of our control group indicate that the amplitude of engraftment at four weeks post transplantation may be critical for long-term engraftment. At this time MMF was discontinued and one week later CSA was discontinued as well. In our study dogs with a mean granulocyte/PBMC donor chimerism < 50 % at the time of MMF withdrawal rejected their grafts whereas in dogs with a mean granulocyte/PBMC donor chimerism > 50 % stable chimerism could be established. This data is in line with clinical data that showed that early chimerism is predictive for engraftment [16,17]. Considering this delicate immunologic balance, in the present study efforts were made to stimulate the incoming graft immediately after transplantation.

Stem cell transplantation in combination with both administration of the vaccine and graft enrichment with MoDC was safe and well tolerated. One of the seven dogs in our control group and none of the 13 animals of the 1 Gy groups required blood cell transfusion. No clinical signs of GvHD or other adverse events were observed.

The current failure of durable engraftment is likely due to insufficient T-cell activation of the graft achieved with vaccination or graft modification to overcome the suboptimal recipient immunosuppression i.e. 1 Gy conditioning. Engraftment kinetics in both 1 Gy treatment groups seemed to be similar with results of a previous study in which dogs that were conditioned with 1 Gy TBI only rejected their allografts after 3 to 12 weeks [1]. It has been reported that immune reconstitution is critical for effective antitumor vaccination after HSCT. Transplantation settings using myeloablative/lethal conditioning are characterized by pronounced pancytopenia in the immediate posttransplant period accompanied by significant immunosuppression. Previous studies reported, that vaccination failed to induce antitumor effects 1 to 2 weeks after HSCT [18,19] but induced an substantial antitumor response 3 to 6 weeks post HSCT [18,20]. Hence, the ability of vaccines to enhance antitumor immunity seems to correlate with the state of immune reconstitution in the early phase post transplant. On the other hand these studies indicate that recovery of normal numbers of lymphocytes is not a prerequisite for the generation of effective responses to tumor cell vaccines. Moreover, a study by Ma *et al* [21] demonstrated that lymphopenia-driven T-cell proliferation improves the therapeutic efficacy of the vaccine. In the present study nonmyeloablative

conditioning caused only a moderate reduction in leukocyte counts and therefore lymphopenia driven T-cell proliferation might have been reduced.

Since the reduced conditioning by TBI required intensification of pharmacological immunosuppression after HSCT, the potent postgrafting immunosuppression with CSA/MMF up to five weeks after transplantation used in our model may account for the absence of sufficient immunostimulatory effects as well. Pharmacokinetic measurements revealed a high exposure to CSA with median C0 and C2 levels of 450 ng/mL and 1,500 ng/mL, respectively (data not shown). Although initial engraftment during the first 4 weeks after HSCT confirmed the occurrence of an immune reaction *in vivo*, no immune response was detectable in our *in vitro* studies during this period. However, at later time points numbers of TH1 cytokine producing T-cells seemed to correlate with graft rejection. These results support the assumption of suppression of alloreactivity by vigorous postgrafting immunosuppression elicited by CSA/MMF. Corresponding, previous studies demonstrated an inhibition of alloreactive capacity and cytokine production of DC following CSA treatment even at lower CSA concentrations [22-24].

Another aspect may be a suboptimal stimulation through alloantigens of the recipients by virtue of MHC-identity. A higher degree of MHC-matching correlates generally with a lower GvH reaction [25]. In our MHC-identical setting, only donor-derived, minor histocompatibility antigen (mHAg)-specific T-cells mediate GvH effects. In a MHC-identical but mHAg-mismatched mouse model early vaccination after HSCT with tumor-associated antigen-loaded DC caused a significant reduction in tumor progression [26]. However, in contrast to our study recipients were conditioned with 10 Gy TBI and received no posttransplant immunosuppression.

Efficacy of vaccination seems to depend further on vaccine dose. Ito *et al* [27] described a dose-dependent increase in the percentage of tumor-free survival in mice after vaccination with tumor cell lysate at concentrations of 5×10^5 up to 5×10^6 cells/lysate. In the present study dogs were treated with haematopoietic cell lysates that were prepared using 1×10^5 cells/kg. If doses are adjusted to body weight, calculations reveal that in mouse models generally higher vaccination doses are used that are hardly achievable in the canine setting.

The role of DC in the graft is discussed controversially. Some studies have shown that DC counts in the early phase after allogeneic HSCT seem to correlate with transplant outcome. In a clinical study patients with high DC counts at engraftment had a significantly lower risk for relapse and death [28]. Though, authors concluded from their data that the number of DC in the graft has no impact, whereas the number of DC ultimately reconstituted in the recipient is decisive. In the present study, addition of donor MoDC to the graft did not promote engraftment as well. In contrast, enrichment of stem cell graft with plasmacytoid precursor DC effectively facilitated HSC engraftment [29]. However, Waller *et al* [30] suggested that a higher DC count in donor BM is associated with increased relapse following HSCT. One reason for these controversial results may be the heterogeneity of the DC population and the use of different DC subpopulations in these studies [31]. Different states of differentiation, maturation, or activation of the DC may also account for variable immunologic response [32].

In conclusion, both vaccination with haematopoietic cell lysate and enrichment of graft with donor MoDC did not avoid graft rejection following conditioning with 1 Gy TBI in a canine MHC-identical transplantation model. Stronger stimulation of donor T-cells seems to be necessary to induce stable long-term engraftment.

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Figure 1.

Study design. Three HSCT groups were investigated. Group 1 (control) received unmodified DLA-identical littermate bone marrow (BM) for HSCT following 2 Gy TBI conditioning. Group 2 received unmodified DLA-identical littermate BM for HSCT following 1 Gy TBI conditioning and received recipient haematopoietic cell lysate vaccinations (1×10^5 lysed cells/kg + 125 μ g GM-CSF; 3x week [wk 1-5], 1x week [wk 6-9]) for in vivo graft stimulation thereafter. Group 3 received DLA-identical littermate BM enriched with donor monocyte derived dendritic cells (MoDC_{Donor}) for HSCT following 1 Gy TBI conditioning. All dogs received for immunosuppression CSA (days -1 to +35) and MMF (days 0 to +27).

Figure 2.

Development of haematopoietic donor chimerism following HSCT. Dogs received 2 Gy conditioning (group 1; a, b; n = 7), 1 Gy conditioning in combination with vaccination (group 2; c, d; n = 7), and 1 Gy TBI plus enrichment of graft with MoDC (group 3; e, f; n = 6). Analyses were performed within the granulocyte (a, c, e) as well as within the PBMC (b, d, f) compartment.

Figure 3.

Recipient T-cell proliferation response at different times after HSCT. T-cells were stimulated with irradiated (25 Gy) PBMC of the same dog collected and cryopreserved before transplantation. Proliferation was measured using a [^3H]-thymidine incorporation assay and has been illustrated as counts per minute. Data are representative of 5 independent experiments done in triplicates ($^+p < 0.05$ compared to pretransplantation [pre Tx]).

Figure 4.

Flow cytometric 4 quadrant analyses of the intracellular cytokine production by lymphocytes. Percentages of positive cells are given for each quadrant. The dog received a DLA-identical HSCT but lost its graft eventually on day +98. TNF- α and IFN- γ producing cells seem to increase during the period of graft rejection.

Table 1
Composition of marrow grafts and duration of mixed chimerism after conditioning with sublethal TBI.

	Cell dose		PBMC chimerism, week 4 (%)	Granulocytes chimerism, week 4 (%)	Sustained engraftment	Chimerism duration (weeks)
	TNC (10 ⁸ /kg)	CD34+(10 ⁶ /kg)				
group 1 (2 Gy TBI)						
median	2.4	0.6	32	68	4/7	69
range	1.6-11.4	0.2-2.2	5-44	10-74		10.0-148.0
group 2 (1 Gy TBI, vaccination)						
median	3.9	0.5	12*	19*	0/7	10.3*
range	2.5-4.6	0.2-0.9	10-19	11-24		8.0-11.0
group 3 (1 Gy TBI, MoDC)						
median	3.6 [†]	0.5	13*	19*	0/7	11.4*
range	3.3-5.9	0.2-1.1	8-16	16-27		8.1-16.0

[†] 9.8 × 10⁵/kg (6.4-23.3 × 10⁵/kg) MoDC were added to the bone marrow graft

* p < 0.05 compared to control (group 1)