

# Human mesenchymal stem cells suppress donor CD4<sup>+</sup> T cell proliferation and reduce pathology in a humanized mouse model of acute graft-versus-host disease

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## Summary

Acute graft-versus-host disease (aGVHD) is a life-threatening complication following allogeneic haematopoietic stem cell transplantation (HSCT), occurring in up to 30–50% of patients who receive human leucocyte antigen (HLA)-matched sibling transplants. Current therapies for steroid refractory aGVHD are limited, with the prognosis of patients suboptimal. Mesenchymal stem or stromal cells (MSC), a heterogeneous cell population present in many tissues, display potent immunomodulatory abilities. Autologous and allogeneic *ex-vivo* expanded human MSC have been utilized to treat aGVHD with promising results, but the mechanisms of therapeutic action remain unclear. Here a robust humanized mouse model of aGVHD based on delivery of human peripheral blood mononuclear cells (PBMC) to non-obese diabetic (NOD)-severe combined immunodeficient (SCID) interleukin (IL)-2 $\gamma^{\text{null}}$  (NSG) mice was developed that allowed the exploration of the role of MSC in cell therapy. MSC therapy resulted in the reduction of liver and gut pathology and significantly increased survival. Protection was dependent upon the timing of MSC therapy, with conventional MSC proving effective only after delayed administration. In contrast, interferon (IFN)- $\gamma$ -stimulated MSC were effective when delivered with PBMC. The beneficial effect of MSC therapy in this model was not due to the inhibition of donor PBMC chimerism, as CD45<sup>+</sup> and T cells engrafted successfully in this model. MSC therapy did not induce donor T cell anergy, FoxP3<sup>+</sup> T regulatory cells or cause PBMC apoptosis in this model; however, it was associated with the direct inhibition of donor CD4<sup>+</sup> T cell proliferation and reduction of human tumour necrosis factor- $\alpha$  in serum.

**Keywords:** acute graft-versus-host disease, humanized mouse model, mesenchymal stem cell, xenogeneic

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## Introduction

Allogeneic haematopoietic stem cell transplantation (HSCT) has become widely used for the treatment of haematological malignancies and inherited blood disorders [1]. However, the development of acute graft-versus-host disease (aGVHD) is a life-threatening complication following allogeneic HSCT. GVHD can occur in up to 30–50% of patients who receive human leucocyte antigen (HLA)-matched sibling transplants and more frequently in HLA-mismatched, unrelated donor transplants [2]. In order for GVHD to occur, the donor graft must contain immune-competent T cells, be transplanted into a recipi-

ent unable to mount a successful immune response against the graft, and the recipient must express tissue antigens not present in the donor transplant [3]. The standard first-line therapy for aGVHD focuses on the suppression of donor T cells through the administration of glucocorticosteroids combined with immunosuppressive drugs, such as cyclosporin A or tacrolimus [4]. Steroid therapies have improved the outcome and increased survival of many patients with aGVHD [5–7]. Nevertheless, the prognosis for steroid refractory aGVHD patients remains very poor, with a 5-year survival rate as low as 30% [2,8]. In these cases, a second-line therapy is required.

Mesenchymal stem or stromal cells (MSC) are a heterogeneous pericyte-like cell population present in bone marrow, adipose, cord blood and other tissues [9,10]. MSC form plastic adherent colonies *in vitro* and are capable of osteocyte, adipocyte and chondrogenic differentiation [11,12]. These cells are potential agents for regenerative medicine [13], and act through the secretion of 'trophic factors' that promote repair through the recruitment and activation of other reparative cells. MSC may also act through cytoprotective mechanisms or by immune suppression [13,14]. *In vitro*, MSC have a direct suppressive effect on T and B lymphocytes, natural killer (NK) cells and supporting dendritic cell (DC) functions [15–21]. The combination of immunoregulatory and regenerative properties suggest a potential role for MSC in the therapeutic induction of immune tolerance. To this effect, there has been interest in the use of MSC as a cell therapy for a number of inflammatory conditions, such as Crohn's disease, multiple sclerosis and aGVHD [22–25].

Autologous and allogeneic *ex-vivo* expanded human MSC have been utilized in studies of haematological disorders, with promising results. Le Blanc *et al.* demonstrated the potential for MSC infusion to treat steroid-refractory GVHD of the gut and liver, showing no reactivity between the haploidentical MSC and recipient lymphocytes [26], and this was extended to MSC from mismatched unrelated donors [24]. However, the initial optimism for MSC as a cell therapy for aGVHD has become tempered by recent clinical trials. While MSC proved safe and beneficial following infusion to patients with aGVHD in a Phase II trial [25], a Phase III trial for steroid-refractory aGVHD demonstrated no statistical difference between MSC or the placebo groups in relation to achievement of complete response within 28 days of initiating treatment [27,28]. However, it is important to note that beneficial effects were observed in this Phase III study for the treatment of aGVHD of the gut and liver, but not of the skin. These perplexing data highlight the need for more refined models for examining the exact mechanisms of disease modulation by human MSC.

The use of mouse models offers a feasible alternative to human observations, when hypothesis-driven studies are needed, but mouse-in-mouse systems do not always reflect the pathology of human diseases. In many aGVHD models, the effector cell is based on infusion of murine splenocytes which may behave differently to human effector cells; furthermore, conventional mice are not well aligned to the study of human cell therapy products. The introduction of the interleukin (IL)-2 receptor gamma mutation onto the non-obese diabetic (NOD)-severe compromised immunodeficient (SCID) background has allowed for the development of refined mouse models. NOD-SCID IL-2 $\gamma^{\text{null}}$  (NSG) mice are deficient for T, B and NK cell activity and allow engraftment of high levels of human peripheral blood mononuclear cells (PBMC) [29].

The NSG model offers an opportunity to examine human donor cells in combination with clinical cell therapeutics.

Using a humanized NSG mouse model of aGVHD, this study sought to examine the effect of human MSC cell therapy, and to investigate the possible therapeutic mechanisms involved. Human MSC cell therapy significantly prolonged the survival of NSG mice with aGVHD, reducing target organ pathology. MSC therapy did not interfere with donor PBMC engraftment or involve the induction of donor T cell apoptosis, anergy or regulatory cell expansion, but rather the direct inhibition of both donor CD4<sup>+</sup> T cell proliferation and tumour necrosis factor (TNF)- $\alpha$  production.

## Materials and methods

### A xenogeneic aGVHD model

All procedures involving animals or human material were carried out by licensed personnel according to approved guidelines. Ethical approval for all work was received from the ethics committee of National University of Ireland (NUI) Maynooth. A humanized mouse model of aGVHD was adapted and optimized from a protocol described by Pearson *et al.* [29]. NOD.Cg-Prkdc<sup>scid</sup>IL2<sup>tm1Wjl</sup>/Szj mice (NOD-SCID IL-2 $\gamma^{\text{null}}$ ) (NSG) (Jackson Laboratories, Bar Harbour, ME, USA) were exposed to a conditioning dose of 2.4 Gray (Gy) of whole-body gamma irradiation. Human PBMC from healthy volunteer donors were isolated by Ficoll-density centrifugation and administered intravenously (i.v.) to NSG mice ( $6.3 \times 10^5 \text{ g}^{-1}$ ) via the tail vein 4 h following irradiation. Negative control mice received a sham infusion of phosphate-buffered saline (PBS) alone. Signs of aGVHD occurred typically between days 12 and 15 post-PBMC transfusion. In some mice, conventional human mesenchymal stem cell (MSC) ( $4.4 \times 10^4 \text{ g}^{-1}$ ) therapy was administered on day 7 post-PBMC transfusion. In other groups, interferon (IFN)- $\gamma$  stimulated MSC ( $4.4 \times 10^4 \text{ g}^{-1}$ ) were administered concurrent with PBMC on day 0. The level of human cell chimerism was analysed by flow cytometry (days 4, 8 and 12), examining the expression of CD45<sup>+</sup> cells and the ratios between human CD4 and CD8 T cells. aGVHD development was determined by examining features daily including body weight, ruffled fur, locomotor activity, posture and diarrhoea. Animals that displayed greater than 15% total body weight loss or a pathological score of 8 were killed humanely according to local ethical committee guidelines. Upon aGVHD development in the group of mice receiving PBMC alone (positive control) (days 12–15), target organs and sera were harvested from all groups for histological analysis, serum analysis and cell characterization. All experiments were repeated two or more times with five to seven mice per group on each occasion.

### Histopathological analysis and scoring

Target organs (lung, liver and gut) were recovered from mice (days 12 or 15) and fixed in 10% (v/v) buffered formalin, processed for histology and embedded in paraffin wax. Five- $\mu\text{m}$  tissue sections were stained by haematoxylin and eosin (H&E) and coded without reference to prior treatment, blinded and then examined by two independent observers. A semi-quantitative scoring system was used to assess abnormalities in the lung, liver and gastrointestinal tract (GI) tract [30–32].

### Human mesenchymal stem cell isolation and culture

Human bone marrow mesenchymal stem cells were generated as previously described [33] in collaboration with the Regenerative Medicine Institute (REMEDI, NUI Galway, Ireland). Briefly, bone marrow aspirates were taken from the iliac crest of healthy consenting adult donor patients according to an approved clinical protocol [34]. Human MSC batches used in this study conformed to the International Society for Cellular Therapy (ISCT) criteria [16] and were capable of differentiation to adipocytes, osteocytes and chondrocytes and were only used at low passage (3–8). Human MSC were cultured in complete Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Dublin, Ireland) supplemented with 10% (v/v) fetal bovine serum (FBS), 200 U/ml penicillin and 200  $\mu\text{g}/\text{ml}$  streptomycin. In some instances, MSC were stimulated with recombinant human IFN- $\gamma$  (500 U/ml) (Peprotech, London, UK) for 48 h and washed extensively with PBS prior to their use *in vitro* or *in vivo*.

### Assessment of MSC-induced T cell apoptosis *in vitro* and *in vivo*

For *in-vitro* apoptosis, PBMC ( $0.5 \times 10^6/\text{ml}$ ) were co-cultured with MSC ( $1.5 \times 10^5/\text{ml}$ ) in complete RPMI (cRPMI) in the presence or absence of 500  $\mu\text{g}/\text{ml}$  cisplatin (control) (Sigma-Aldrich, Arklow, Ireland). After 24 h, PBMC were recovered by gentle aspiration from adherent MSC and apoptosis was detected by annexin V/propidium iodide (PI) staining (BD Biosciences, Oxford, UK), measured by flow cytometry using a BD fluorescence activated cell sorter (FACS) Calibur cytometer with CellQuest software (BD Biosciences).

For *in-vivo* apoptosis, in order to optimize, first, the detection of apoptosis FAM-FLIVO™ green dye (Immunochemistry Technologies, Bloomington, MN, USA) was used. As a control for the detection of FLIVO *in vivo*, BALB/c mice were irradiated lethally with 12 Gy gamma irradiation. After 24 h, 8  $\mu\text{g}$  (100  $\mu\text{l}$ ) of FAM-FLIVO™ green dye was injected per mouse and left to circulate for 1 h. After 1 h (or other times, not shown), the liver was harvested and

isolated cells were analysed by flow cytometry to verify detectability of apoptosis *in vivo*. To assess potential MSC-induced apoptosis following cell therapy, PBMC ( $6.3 \times 10^5/\text{g}^{-1}$ ) and/or IFN- $\gamma$  stimulated MSC ( $4.4 \times 10^4/\text{g}^{-1}$ ) were delivered via the tail vein to 2.4 Gy-irradiated NSG mice on day 0. On day 12 (or days 1 and 5, data not shown), 8  $\mu\text{g}$  (100  $\mu\text{l}$ ) of FAM-FLIVO™ green dye (Immunochemistry Technologies) was injected per mouse and left to circulate for 1 h. The lungs and livers were harvested and cells isolated following collagenase (300 U/ml) (Sigma-Aldrich) and DNase I (10 mg/ml) digestion (Roche Diagnostics, West Sussex, UK). Cells were counterstained with anti-human CD4 allophycocyanin (APC) (eBioscience, San Diego, CA, USA) and analysed by flow cytometry.

### Assessment of human MSC-induced lymphocyte energy and proliferation

Bone marrow-derived dendritic cells (DC) were isolated from BALB/c mice and cultured in cRPMI supplemented with 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech) for 8 days. Human CD4<sup>+</sup> T cells were isolated from PBMC by magnetic bead separation following the manufacturer's guidelines (R&D Systems, Minneapolis, MN, USA). Murine DC ( $1.5 \times 10^5/\text{ml}$ ) were matured following stimulation with polyinosinic-polycytidylic acid (polyIC) (20  $\mu\text{g}/\text{ml}$ ), as described previously [35], and co-cultured with human CD4<sup>+</sup> T cells ( $1 \times 10^6/\text{ml}$ ) in the presence or absence of human MSC ( $1 \times 10^5/\text{ml}$ ) in cRPMI supplemented with 0.1% (v/v) beta-mercaptoethanol. After 5 days, human CD4<sup>+</sup> T cell were repurified from co-cultures by CD4<sup>+</sup> magnetic bead separation and allowed to rest for 24 h in cRPMI. Repurified human CD4<sup>+</sup> T cells ( $1 \times 10^6/\text{ml}$ ) were then co-cultured with irradiated BALB/c DC ( $1 \times 10^5/\text{ml}$ ) and stimulated with polyIC (20  $\mu\text{g}/\text{ml}$ ) in the presence or absence of recombinant human IL-2 (rhIL-2) (100 U/ml) for 72 h and proliferation was assessed.

*In-vitro* proliferation was determined by culture of human PBMC ( $1 \times 10^6$  cells/ml) in the presence or absence of human MSC ( $1 \times 10^5$  cells/ml) in cRPMI. In mitogen-driven assays, cultures were stimulated with phytohaemagglutinin (PHA) (Sigma-Aldrich) at 5  $\mu\text{g}/\text{ml}$ . Cell culture supernatants were sampled for the presence of human TNF- $\alpha$  and IFN- $\gamma$  by enzyme-linked immunosorbent assay (ELISA) (R&D Systems). After 72 h, [<sup>3</sup>H]-thymidine (Amersham Biosciences, Buckinghamshire, UK) at 0.5  $\mu\text{Ci}/\text{ml}$  was added. Cultures were harvested 6 h later using an automatic cell harvester and radioactive incorporation, assessed as previously described [16,36].

*In-vivo* proliferation was measured by labelling human PBMC with 10  $\mu\text{M}$  carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen), washed twice with PBS and administered at  $6.3 \times 10^5/\text{g}^{-1}$  to irradiated NSG mice on day 0. IFN- $\gamma$ -stimulated MSC ( $4.4 \times 10^4/\text{g}^{-1}$ ) were delivered

concurrently with PBMC on day 0. After 5 days the lungs, livers and spleens were harvested from each mouse. A single-cell suspension of  $1 \times 10^6$  cells/ml was counterlabelled with anti-human CD4 APC for 15 min at 4°C. Cells were analysed for CFSE staining and the expression of human CD4 by flow cytometry.

### Detection of human FoxP3 expression

Forkhead box protein 3 (FoxP3) expression *in vitro* was assessed using whole unsorted PBMC ( $0.5 \times 10^6$ /ml), or with CD4<sup>+</sup> CD25<sup>-</sup> or CD4<sup>+</sup> CD25<sup>+</sup> sorted T cells (FACS Aria BD). These populations were then co-cultured with MSC ( $1.5 \times 10^5$ /ml) for 72 h in cRPMI. PBMC or sorted CD4<sup>+</sup> T cells were recovered from culture by gentle aspiration from adherent MSC and examined by flow cytometry. Cells were washed in PBS, surface-stained for CD4 APC and CD25 phycoerythrin (PE) where required. Cells were then fixed in 2% (v/v) paraformaldehyde, permeabilized in PBS/Tween and blocked using normal rat serum. Following this, cells were incubated with anti-human FoxP3 fluorescein isothiocyanate (FITC) (eBioscience) for 30 min at 4°C. Cells were washed, fixed in 1% (v/v) formaldehyde/PBS and analysed by flow cytometry within 4 h.

Regulatory T cell (T<sub>reg</sub>) induction *in vivo* was examined in the aGVHD model described above with either IFN- $\gamma$ -stimulated MSC ( $4.4 \times 10^4$  g<sup>-1</sup>) administered i.v on day 0 or non-stimulated MSC ( $4.4 \times 10^4$  g<sup>-1</sup>) on day 7 post-PBMC transfusion. On day 12, the day of aGVHD pathology manifestation, the lungs, livers and spleens of NSG mice were harvested and a single-cell suspension prepared. The surface expression of human CD4 APC, CD25 PE and intracellular expression of human FoxP3 FITC was determined by flow cytometry.

### Statistical methods

Statistical analysis was performed using GraphPad Prism™ software (GraphPad, San Diego, CA, USA). The Student's paired *t*-test was used when statistical analysis was required between two experimental groups. One-way analysis of variance (ANOVA) was used to test for statistically significant difference when multiple experimental groups were compared. Kaplan–Meier curves (log-rank test) were used to compare survival between treatment groups. Data are presented as  $\pm$  standard error of the mean (s.e.m.). *P*-values of  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) or  $P < 0.001$  (\*\*\*) were considered statistically significant.

## Results

### Human MSC reduce aGVHD pathology and prolong survival in a humanized mouse model

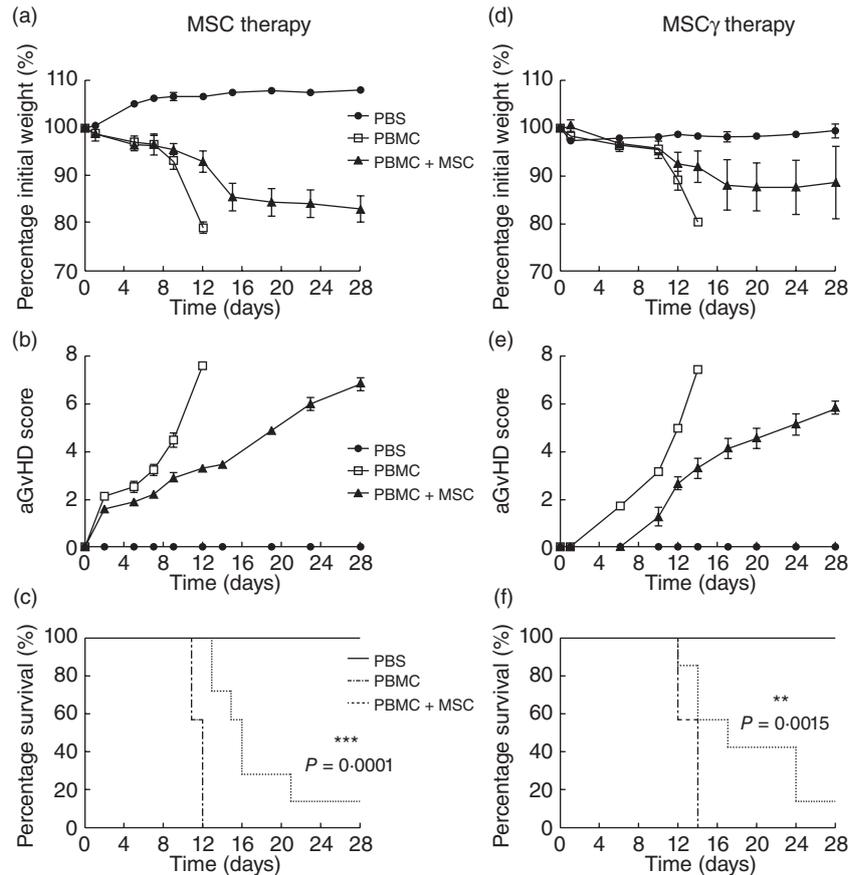
A robust and reproducible model of aGVHD was established in NSG mice by delivery of human PBMC.

This was adapted from Pearson *et al.* [29], and reproducibility achieved by (i) normalizing PBMC dose to murine body weight, (ii) use of freshly isolated PBMC from healthy donors and (iii) preconditioning of mice by exposure to 2.4 Gy irradiation prior to PBMC delivery. On day 7 post-PBMC transfusion, human MSC allogeneic to the PBMC donor were given i.v. as a cell therapy. NSG mice that received PBS alone did not develop any signs of aGVHD, whereas mice that received PBMC developed aGVHD consistently between days 12 and 15, with weight loss, hunched posture, ruffled fur and reduced locomotion (Fig. 1a,b). Delivery of non-stimulated human MSC on day 0 had no detectable beneficial effect (data not shown); however, MSC therapy on day 7 significantly extended the survival of NSG mice with aGVHD ( $P < 0.0001$ ), with some mice surviving for more than 30 days (Fig. 1c).

The livers of NSG mice that received PBS as a control appeared normal, with no cell infiltration, tissue fibrosis or endothelialitis (histopathology score of 0) (Fig. 2a). Mice receiving PBMC displayed a significant mononuclear cell infiltration, especially surrounding the hepatic ducts with endothelialitis ( $P < 0.0001$ ) (Fig. 2a). MSC therapy on day 7 reduced liver pathology ( $P < 0.0086$ ), with decreased cell infiltration and reduced endothelialitis (Fig. 2a). Similarly, the small intestines of PBS-treated control mice appeared normal, with no sloughing of villi and no accumulation of infiltrating cells into the lamina propria (Fig. 2b). In comparison, NSG mice that received PBMC displayed blunting of villi with cell infiltration into the lamina propria and intestinal crypts (Fig. 2b) ( $P < 0.0001$ ). This was reduced significantly by human MSC therapy at day 7 ( $P < 0.0249$ ). Control NSG mouse lungs appeared normal, but PBMC delivery provoked cellular infiltration/inflammation (Fig. 2c) ( $P < 0.0002$ ). In contrast to the protective effects in the liver and gut, treatment with MSC on day 7 did not ameliorate pathology in the lungs compared to aGVHD mice (Fig. 2c).

Stimulation of MSC with proinflammatory cytokines such as IFN- $\gamma$  promotes the immunosuppressive capacity *in vitro* and enhances their beneficial role in treating aGVHD *in vivo* [32,36], a phenomenon termed 'licensing'. Therefore, MSC were stimulated *in vitro* with IFN- $\gamma$  (MSC $\gamma$ ) for 48 h prior to administration to NSG mice on day 0 in the aGVHD model. MSC $\gamma$  therapy reduced aGVHD-related weight loss and pathology (Fig. 1d,e), while significantly increasing the survival time of mice with aGVHD ( $P < 0.0015$ ) in comparison to mice that had not received MSC therapy (Fig. 1f). MSC $\gamma$  therapy on day 0 reduced aGVHD pathology of the liver significantly ( $P < 0.0163$ ), reducing cell infiltration and endothelialitis (Fig. 2a). IFN- $\gamma$  stimulated MSC also reduced gut pathology with reduced cell infiltration and significantly less tissue damage to villi ( $P < 0.0142$ ) (Fig. 2b), similar in extent to non-stimulated MSC therapy at day 7. However, as seen earlier, MSC $\gamma$

**Fig. 1.** Mesenchymal stem or stromal cell (MSC) therapy significantly prolongs the survival of non-obese diabetic (NOD) severe combined immunodeficient (SCID) interleukin (IL)-2 $\gamma^{\text{null}}$  (NSG) mice with acute graft-versus-host disease (aGVHD). NSG mice received 2.4 Gy low-dose irradiation; 4 h later peripheral blood mononuclear cells (PBMC) ( $6.3 \times 10^5 \text{ g}^{-1}$ ) were administered intravenously via the tail vein. Non-stimulated MSC were administered on day 7 post-PBMC transfusion or interferon (IFN)- $\gamma$ -prestimulated MSC (MSC $\gamma$ ) ( $4.4 \times 10^4 \text{ g}^{-1}$ ) were delivered on day 0, concurrent with PBMC. (a,d) Weight loss, (b,e) aGVHD score and (c,f) survival were recorded daily. Both non-stimulated and IFN- $\gamma$ -stimulated MSC significantly prolonged the survival of NSG mice with aGVHD ( $P < 0.0001$  and  $P < 0.0015$ , respectively), limiting weight loss and reducing the clinical signs of aGVHD development ( $n = 7$  mice per group). Data shown are representative of at least two experiments.



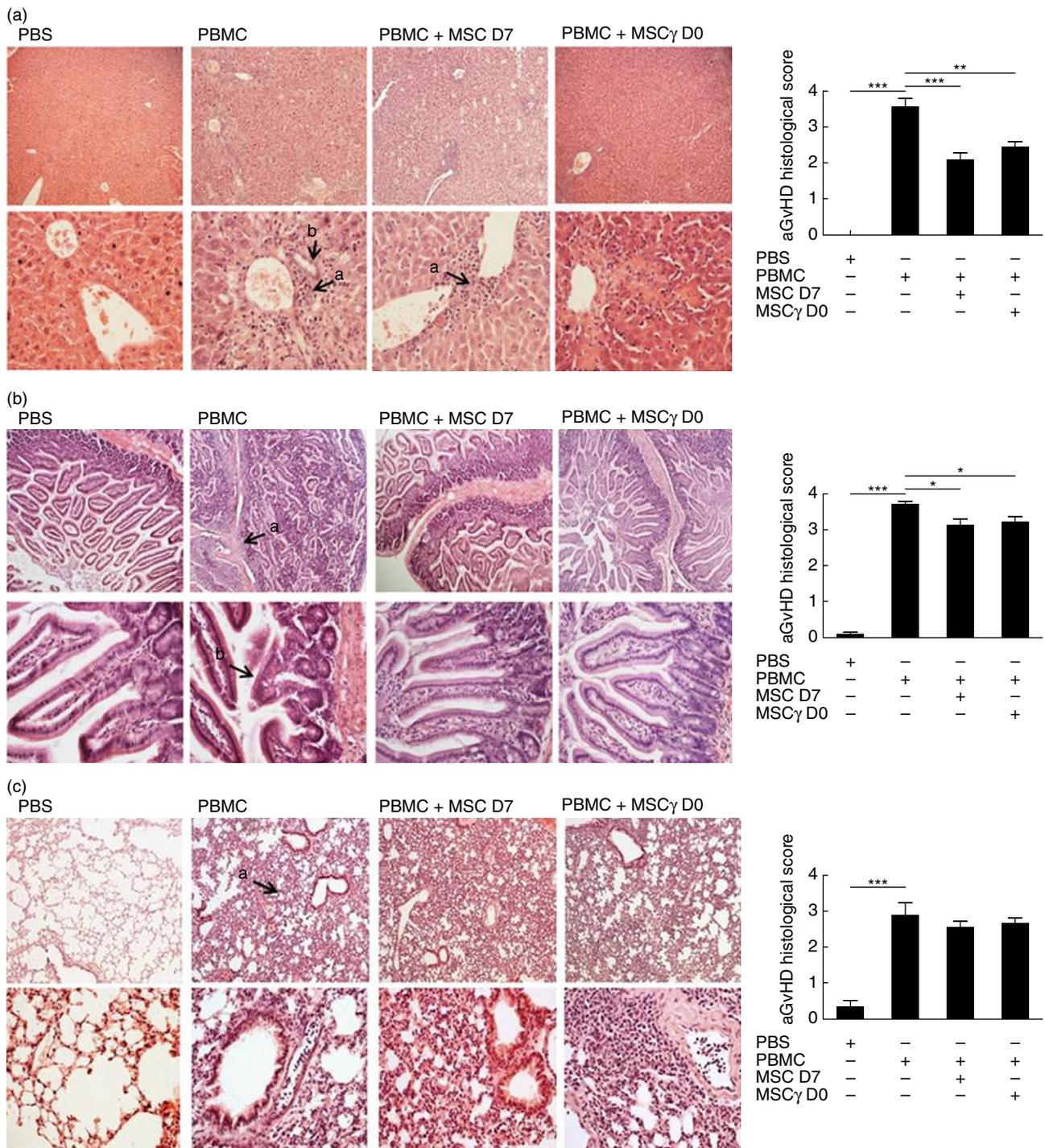
therapy did not ameliorate the pathology observed in the lung (Fig. 2c).

### Reduction of aGVHD following human MSC therapy was not due to impaired engraftment or through the induction of donor PBMC apoptosis or anergy

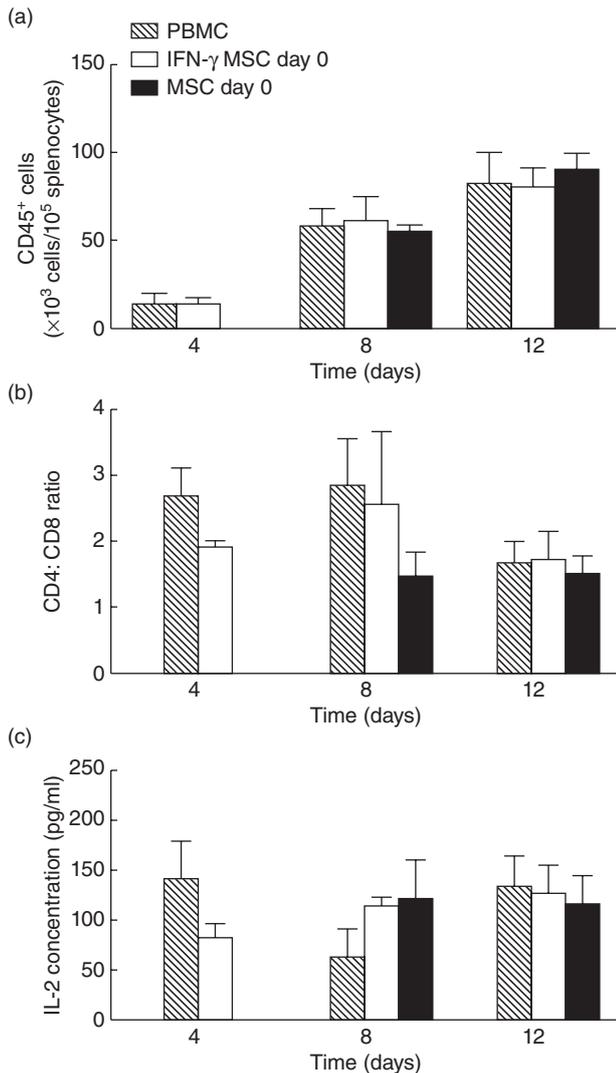
A simple explanation for the observation above could be that human MSC therapy reduces human PBMC engraftment in the NSG model. To exclude this possibility, the numbers of human CD45 $^+$  cells and the ratios of CD4/CD8 T cells were investigated in the above model. IFN- $\gamma$ -stimulated human MSC therapy on day 0 or non-stimulated MSC therapy on day 7 did not affect the engraftment of human CD45 $^+$  cells (Fig. 3a). Human CD4 and CD8 T cells were detectable in the spleens of NSG mice following human PBMC infusion, but MSC therapy (IFN- $\gamma$ -stimulated or not) did not prevent the engraftment of human T cells or significantly alter the CD4:CD8 ratio (Fig. 3b). In support of this observation, the levels of human IL-2 in the sera of NSG mice following PBMC infusion was not significantly altered by MSC therapy (Fig. 3c), indicating that MSC therapy did not hinder effector cell engraftment.

The mechanism by which MSC therapy limited aGVHD in this humanized mouse model might involve immuno-

logical tolerance, such as the induction of either donor lymphocyte apoptosis or anergy. The ability of MSC to induce apoptosis of T cells was investigated, both *in vitro* and *in vivo*. The induction of PBMC apoptosis *in vitro* by human MSC was examined using an MSC/PBMC co-culture model. A known inducer of PBMC apoptosis, cisplatin, caused significant apoptosis of PBMC (Fig. 4a), whereas allogeneic human MSC did not ( $P < 0.0001$ ) (Fig. 4a). However, the lack of apoptosis *in vitro* might not reflect the *in vivo* situation, therefore the NSG model was adapted to detect apoptotic cells. NSG mice were treated with PBS or PBMC, with or without MSC $\gamma$  cell therapy on day 0. FLIVO (a reagent which detects active caspases of apoptotic cells *in vivo*) was administered i.v. 12 days later and allowed to circulate for 1 h. After 1 h, the lungs (Fig. 4b) and livers (Fig. 4c) were harvested and analysed for FLIVO/CD4 staining by two-colour flow cytometry. Although CD4 $^+$  T cells were detected, there was no increase in apoptotic CD4 $^+$  T cells following MSC $\gamma$  therapy in either organ sampled on day 12 (Fig. 4b,c) or at other times prior to day 12 (days 1 or 5, data not shown). These data suggested that MSC did not induce detectable apoptosis of donor human CD4 $^+$  T cells *in vivo* or *in vitro* and that this was unlikely to be the mechanism involved in the beneficial effect mediated by MSC in this model.



**Fig. 2.** Mesenchymal stem or stromal cells (MSC) cell therapy significantly reduces pathology in the liver and gut of non-obese diabetic (NOD) severe combined immunodeficient (SCID) interleukin (IL)-2 $\gamma^{\text{null}}$  (NSG) mice with acute graft-*versus*-host disease (aGVHD). aGVHD was established in NSG mice (as Fig. 1) and treated with non-stimulated MSC (day 7) or interferon (IFN- $\gamma$ )-stimulated MSC (day 0). The livers, small intestine and lungs were harvested on day 12. (a) The livers of NSG mice displayed a significant increase in mononuclear cell infiltration (denoted by arrow and letter a) and increased endothelialitis, especially around hepatic ducts (denoted by arrow and letter b). Both MSC and interferon (IFN- $\gamma$ )-prestimulated MSC (MSC $\gamma$ ) treatment significantly reduced this pathology. (b) aGVHD of the small intestine resulted in the accumulation of infiltrating cells into the lamina propria (denoted by arrow and letter a) and increased blunting of the villi (denoted by arrow and letter b). Similar to the liver, MSC or MSC $\gamma$  cell therapy resulted in a significant decrease of cell infiltration and villous blunting. (c) aGVHD development in the lungs manifested by a significant increase of mononuclear cells into alveolar air spaces (denoted by arrow and letter a). Unlike the liver and small intestine, treatment of aGVHD NSG mice with MSC or MSC $\gamma$  did not alleviate the symptoms of airway aGVHD. Bar charts in the right-hand panel summarize histological scores. Data are representative of a minimum five mice per treatment group and at least two independent experiments.



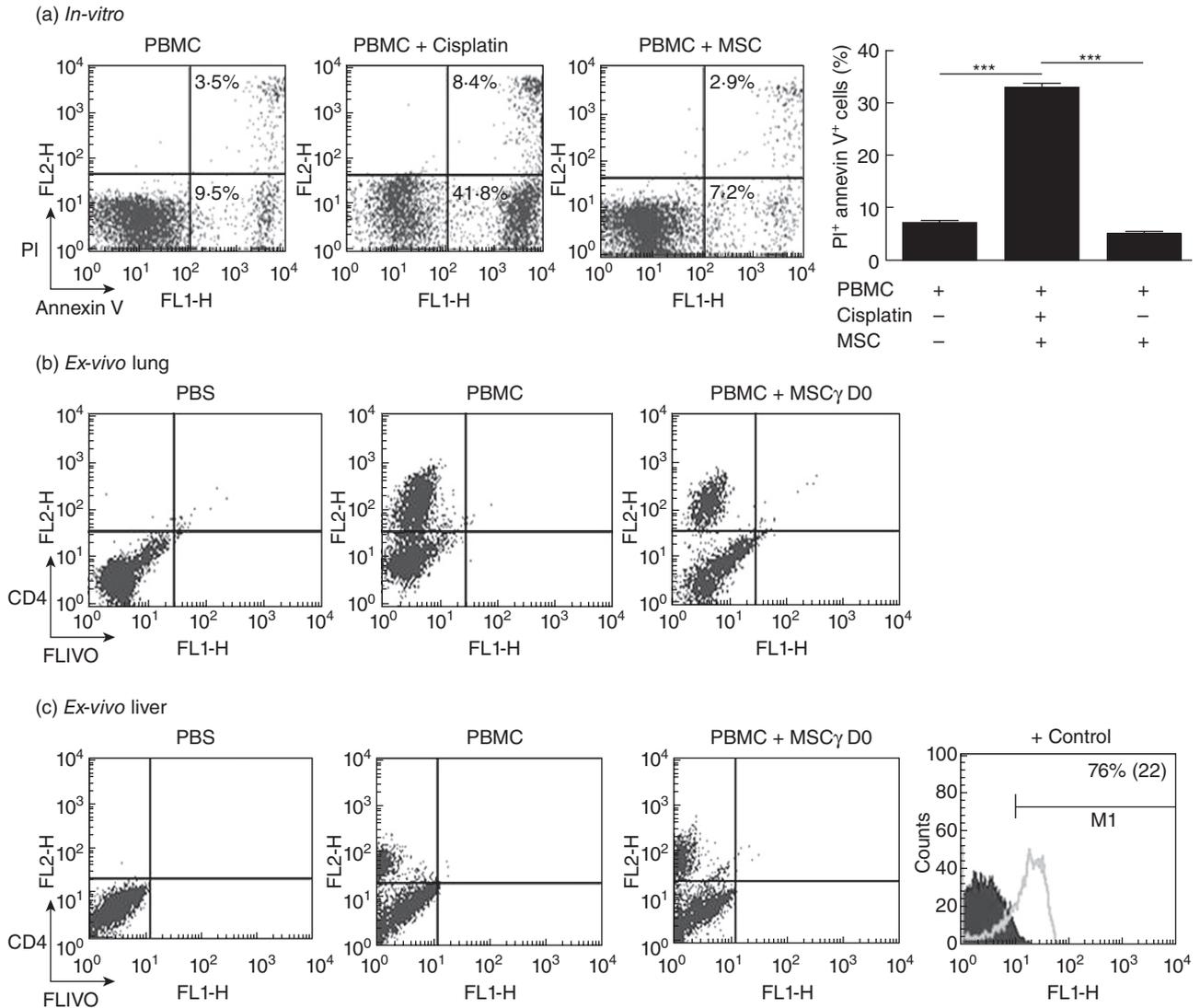
**Fig. 3.** Mesenchymal stem or stromal cells (MSC) cell therapy did not interfere with donor peripheral blood mononuclear cells (PBMC) engraftment. Interferon (IFN)- $\gamma$ -stimulated (day 0) or non-stimulated MSC (day 7) ( $4.4 \times 10^4 \text{ g}^{-1}$ ) were administered to irradiated non-obese diabetic (NOD) severe combined immunodeficient (SCID) interleukin (IL)-2 $\gamma^{\text{null}}$  NSG mice that had received PBMC ( $6.3 \times 10^5 \text{ g}^{-1}$ ) on day 0 ( $n = 5$  per group). The spleens of the NSG mice from each treatment group were harvested on days 4, 8 and 12 and analysed for the expression of (a) human CD45<sup>+</sup> and (b) the ratio of human CD4 to CD8 T cells by flow cytometry. Human PBMC engrafted readily, with a significant increase in CD45<sup>+</sup> cells from days 4 to 8 ( $P < 0.05$ ) and 12 ( $P < 0.002$ ) compared to controls. MSC therapy had no significant effect on the engraftment of human PBMC. The number of CD45<sup>+</sup> cells per  $10^5$  total splenocytes was enumerated using counting beads. (c) The level of human IL-2 present in the sera of mice from each treatment group was analysed by cytokine bead array. MSC therapy had no effect on the amount of human IL-2 detectable in sera.

An alternative hypothesis for the beneficial effect of MSC cell therapy was formulated around the induction of donor T cell anergy. To examine this, an *in vitro* two-step proliferation assay was designed which would closely mimic *in vivo*

circumstances. First, murine DC isolated from the bone marrow of BALB/c mice were used to mimic the murine (host) antigen-presenting cell. These were matured using polyIC as a stimulus and co-cultured with human CD4<sup>+</sup> T cells for 5 days in the presence or absence of MSC. After 5 days, the proliferation of human CD4<sup>+</sup> T cells was analysed. Human CD4<sup>+</sup> T cells proliferated strongly when cultured with mature murine DC ( $P < 0.0001$ ); however, allogeneic human MSC significantly reduced this effect ( $P < 0.05$ ) (Fig. 5a). These data showed that MSC were capable of inhibiting T cell proliferation in a xenogeneic setting, analogous to that found in the aGVHD NSG model. To examine if the reduction in T cell proliferation by MSC was due to the induction of T cell anergy, a two-stage assay was then performed. Human CD4<sup>+</sup> T cells were co-cultured with mature murine DC and/or MSC for 5 days; human CD4<sup>+</sup> T cells were re-isolated from cultures by magnetic bead isolation. Re-isolated CD4<sup>+</sup> T cells were allowed to rest overnight then cultured for a second time with irradiated BALB/c DC stimulated with or without polyIC/IL-2. Following the second-stage co-culture, human CD4<sup>+</sup> T cells proliferated in response to irradiated mature DC (Fig. 5b). The proliferation of CD4<sup>+</sup> T cells that had been previously cultured in the presence of MSC was not significantly different. Furthermore, the addition of IL-2 did not alter the proliferation of human CD4<sup>+</sup> T cells, suggesting that MSC did not induce T cell anergy *in vitro* (Fig. 5c). These data suggested that the beneficial effects seen *in vivo* following MSC therapy were not due to donor T cell apoptosis or anergy but to some other mechanism.

#### Allogeneic human MSC-induced regulatory-like T cells expressing FoxP3 *in vitro*, but not *in vivo*, in the NSG aGVHD model

Previous studies of cell therapy in other models have shown that the MSC-driven induction of FoxP3-expressing T<sub>reg</sub> cells are responsible for some of the beneficial effects of MSC *in vivo* [22,37]. The induction/expansion of T<sub>reg</sub> following MSC therapy was therefore examined as a possible mechanism involved in the therapeutic effect. First, human MSC were tested for the ability to expand FoxP3<sup>+</sup> T<sub>reg</sub> cells *in vitro* from a whole population of allogeneic PBMC (Fig. 6a). After co-culture with MSC for 72 h *in vitro*, PBMC were analysed for the co-expression of CD4, CD25 and intracellular FoxP3. MSC expanded a CD4<sup>+</sup> T<sub>reg</sub>-like cell population expressing FoxP3 and CD25 *in vitro* (Fig. 6a), in agreement with our previous work [16]. An examination of sorted CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells showed that MSC did not induce FoxP3<sup>+</sup> populations *de novo* from CD4<sup>+</sup>CD25<sup>-</sup> cells, but rather expanded a pre-existing population of FoxP3<sup>+</sup> T<sub>reg</sub> cells (Fig. 6b). Following this observation, T<sub>reg</sub> cell expansion by MSC and MSC $\gamma$  was explored in the NSG model of aGVHD. On day 12 (the typical onset day of aGVHD pathology), the lungs, livers and spleens



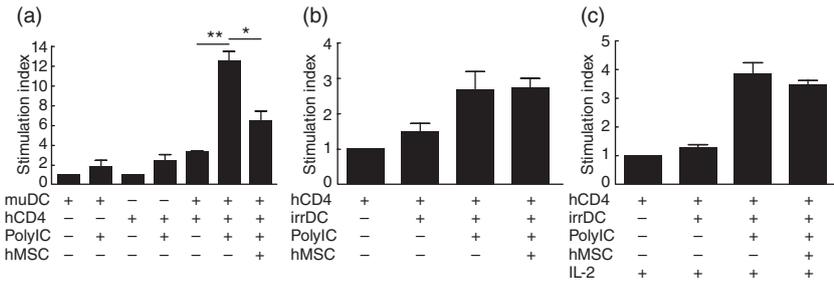
**Fig. 4.** Mesenchymal stem or stromal cells (MSC) did not induce T cell apoptosis *in vitro* or *in vivo*. (a) Peripheral blood mononuclear cells (PBMC) were cultured in the presence of cisplatin or MSC for 24 h. PBMC cultured alone or with cisplatin at 250 µg/ml were used as controls. The presence of MSC did not induce apoptosis of PBMC *in vitro* ( $P < 0.0002$ ). The percentages of positive cells within the lower region are represented in the bar chart. Data are representative of two studies. Irradiated non-obese diabetic (NOD) severe combined immunodeficient (SCID) interleukin (IL)-2 $\gamma^{\text{null}}$  NSG mice received phosphate-buffered saline (PBS) or PBMC ( $6.3 \times 10^5 \text{ g}^{-1}$ ) on day 0. Interferon (IFN)- $\gamma$ -prestimulated MSC (MSC $\gamma$ ) ( $4.4 \times 10^4 \text{ g}^{-1}$ ) were given on day 0, concurrent with PBMC. On day 12, FLIVO green dye (8 µg/mouse) was injected intravenously and left to circulate for 1 h. (b) The lungs and (c) livers were harvested from each mouse and analysed for FLIVO staining and human CD4 phycoerythrin (PE) labelling by flow cytometry. MSC cell therapy did not induce CD4 T cell apoptosis *in vivo*. Liver cells from lethally irradiated BALB/c were used as a positive control for FLIVO detection *in vivo*.

were harvested and analysed for the presence of human cells expressing CD4, CD25 and/or Foxp3 by flow cytometry (Fig. 6c–e). There was no evidence of expansion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cell populations *in vivo* (Fig. 6c–e), even though we have detected MSC expansion of T<sub>reg</sub> previously using these methods [37]. T<sub>reg</sub> expansion could not be detected following treatment with either non-stimulated MSC on day 7 or MSC $\gamma$  on day 0 in the lungs (Fig. 6c), livers (Fig. 6d) or spleens (Fig. 6e). These data suggested that in this model, MSC expansion of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>

T<sub>reg</sub>-like cells was unlikely to be the mechanism involved in prolonged survival following cell therapy.

#### Allogeneic MSC directly inhibited the proliferation of donor CD4<sup>+</sup> T cells *in vivo*

It is well documented that MSC have the ability to directly suppress T cell proliferation *in vitro* [16,20,36,38]. Therefore, it was possible that the beneficial effect of MSC therapy in the NSG model of aGVHD could be attributed



**Fig. 5.** Mesenchymal stem or stromal cells (MSC) did not induce T cell anergy *in vitro*. (a) Human CD4<sup>+</sup> T cells ( $1 \times 10^6$ /ml) were co-cultured with or without BALB/c bone marrow-derived dendritic cells (muDC) ( $1 \times 10^5$ /ml) matured using polyinosinic-polycytidylic acid (polyIC) (20  $\mu$ g/ml) in the presence or absence of human MSC (hMSC) ( $1 \times 10^5$ /ml) for 5 days. [<sup>3</sup>H]-thymidine was then added to cultures for the final 6 h and proliferation was measured. PolyIC matured murine dendritic cells (DC) induced significant human CD4<sup>+</sup> T cell proliferation ( $P < 0.0001$ ). In the presence of hMSC, the proliferation of CD4<sup>+</sup> T cell proliferation was significantly reduced ( $P < 0.05$ ). Following co-culture, CD4<sup>+</sup> T cells were repurified and co-cultured in a second-stage experiment with irradiated mature DC (irrDC/polyIC) for 72 h in the (b) absence or (c) presence of recombinant human interleukin (rhIL)-2 to assess anergy. T cell proliferation was analysed by [<sup>3</sup>H]-thymidine incorporation.

to a direct anti-proliferative effect on donor T cells *in vivo*. To explore this, MSC were first examined to verify the *in vitro* suppression of PBMC proliferation. Human MSC inhibited the proliferation of alloantigen-driven and mitogen-driven proliferation of PBMC (Fig. 7a,b) ( $P < 0.0001$ ). This inhibition was associated with a significant decrease in both IFN- $\gamma$  (Fig. 7c,d) ( $P < 0.0001$ ) and TNF- $\alpha$  (Fig. 7e,f) ( $P < 0.0201$  and  $P < 0.0001$ , respectively) present in culture supernatants. These data suggested that MSC might have a similar effect *in vivo*, suppressing the development of aGVHD.

To investigate the influence of MSC on proliferation of donor PBMC *in vivo*, conditioned NSG mice received CFSE-labelled PBMC with or without MSC $\gamma$  treatment concurrently on day 0. In this instance, MSC $\gamma$  therapy was chosen in preference to MSC therapy to allow a directly aligned comparison on T cell proliferation over time. Mice were left for 5 days before analysing the effect of MSC $\gamma$  treatment on PBMC proliferation. Lungs, livers and spleens were harvested and the fluorescence of CFSE<sup>+</sup> labelled CD4<sup>+</sup> T cells was analysed by flow cytometry (Fig. 8a). CFSE-labelled PBMC were detected in the lungs of NSG on day 5, but sufficient cells could not be recovered from other organs at this time-point, consistent with the cell infiltration evident in this model (Fig. 2c and data not shown). MSC $\gamma$ -treated mice had significantly fewer CD4<sup>+</sup> T cells progressing to division ( $P < 0.0041$ ) when compared to mice that received PBMC alone on day 0 (Fig. 8a,b). MSC $\gamma$  therapy also significantly reduced the absolute number of divisions underwent by human CD4<sup>+</sup> T cells ( $P < 0.0037$ ) (Fig. 8b). This reduction in T cell proliferation could not be due to the inhibition of human T cell chimerism within the model following MSC therapy, as not only did human T cells readily engraft, but MSC therapy did not prevent this T cell engraftment (Fig. 3). Interestingly, these data also revealed that aGVHD development in this humanized mouse model was associated with CD4<sup>+</sup> rather than CD8<sup>+</sup> T cell expansion *in vivo* (Fig. 8).

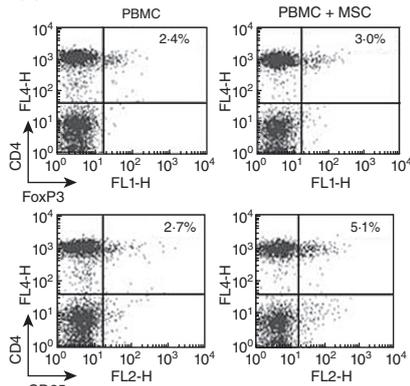
Serum was harvested from all NSG mice at the time of aGVHD development (day 12) and analysed for the presence of human IFN- $\gamma$  and TNF- $\alpha$ . As expected, NSG mice that received PBMC had significantly more human TNF- $\alpha$  present in the serum after 12 days when compared to PBS controls (Fig. 8c,  $P < 0.0027$ ). MSC $\gamma$  cell therapy significantly reduced human TNF- $\alpha$  (Fig. 8c,  $P < 0.0197$ ), but had no significant effect on the presence of human IFN- $\gamma$  in the serum of NSG mice (Fig. 8d). Collectively, these data suggest that MSC cell therapy in this model acts through the direct suppression of donor T cell proliferation, limiting aGVHD pathology *in vivo* and reducing TNF- $\alpha$ , a key CD4<sup>+</sup> T cell-derived effector molecule in aGVHD [2,39].

## Discussion

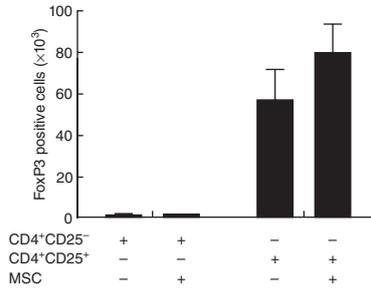
In this study, a humanized mouse model of aGVHD was developed that allowed the reproducible assessment of human cell therapeutics. Allogeneic human MSC therapy given on day 7 or IFN- $\gamma$  stimulated MSC on day 0 increased the survival of NSG mice with aGVHD. Therapeutic effects of MSC were significant in the liver and gut of mice with aGVHD, but were not effective in the lung. Examinations of the mechanisms of therapeutic action by MSC in this model revealed that protection was not associated with MSC induction of donor T cell apoptosis, the induction of donor T cell anergy or prevention of donor cell engraftment. Unlike other models, protection against aGVHD was not linked to MSC-driven expansion of T<sub>reg</sub> populations, but rather the direct suppressive effect on donor T cell proliferation and reduction of T cell-derived human TNF- $\alpha$ . This model mimics closely the data seen from recent clinical trials and offers a system in which mechanisms of action may be explored.

The key to improving current cell therapies for aGVHD is an understanding of the mechanisms of cell action. The humanized mouse model described here provides a refined tool to test human cell therapies and their mechanisms of

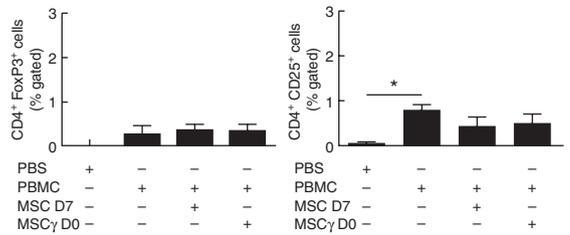
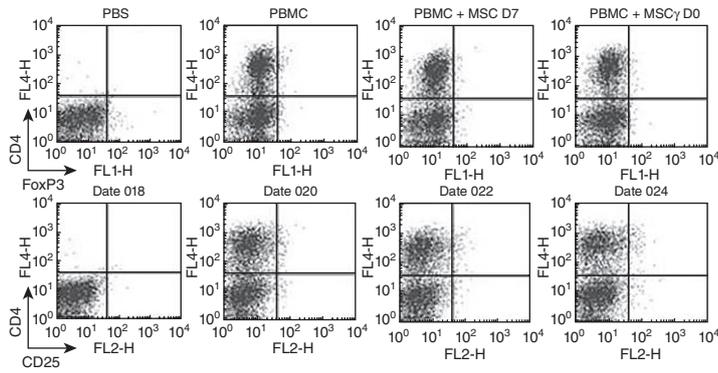
(a) *In-vitro* PBMC



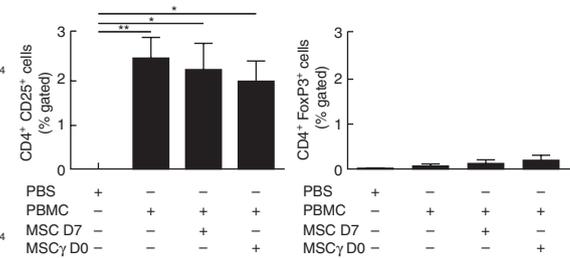
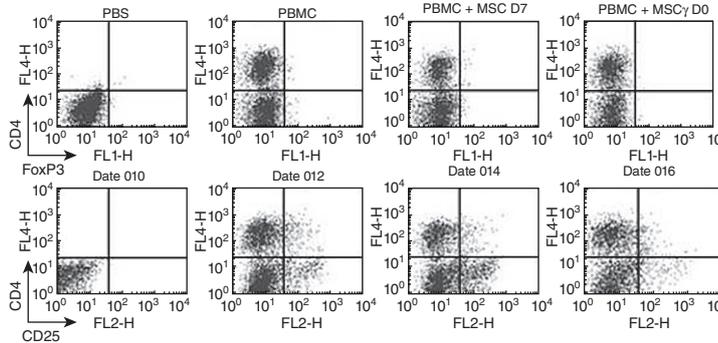
(b) *In-vitro* purified CD4<sup>+</sup> T cells



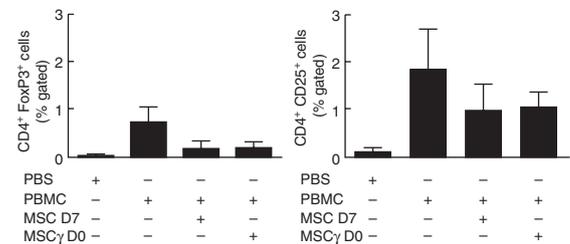
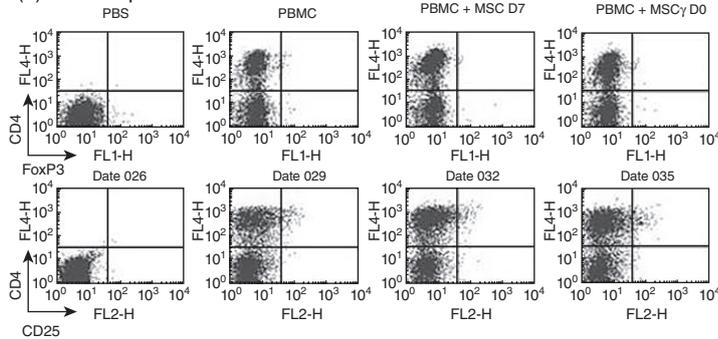
(c) *In-vitro* lung



(d) *In-vivo* liver



(e) *In-vivo* spleen

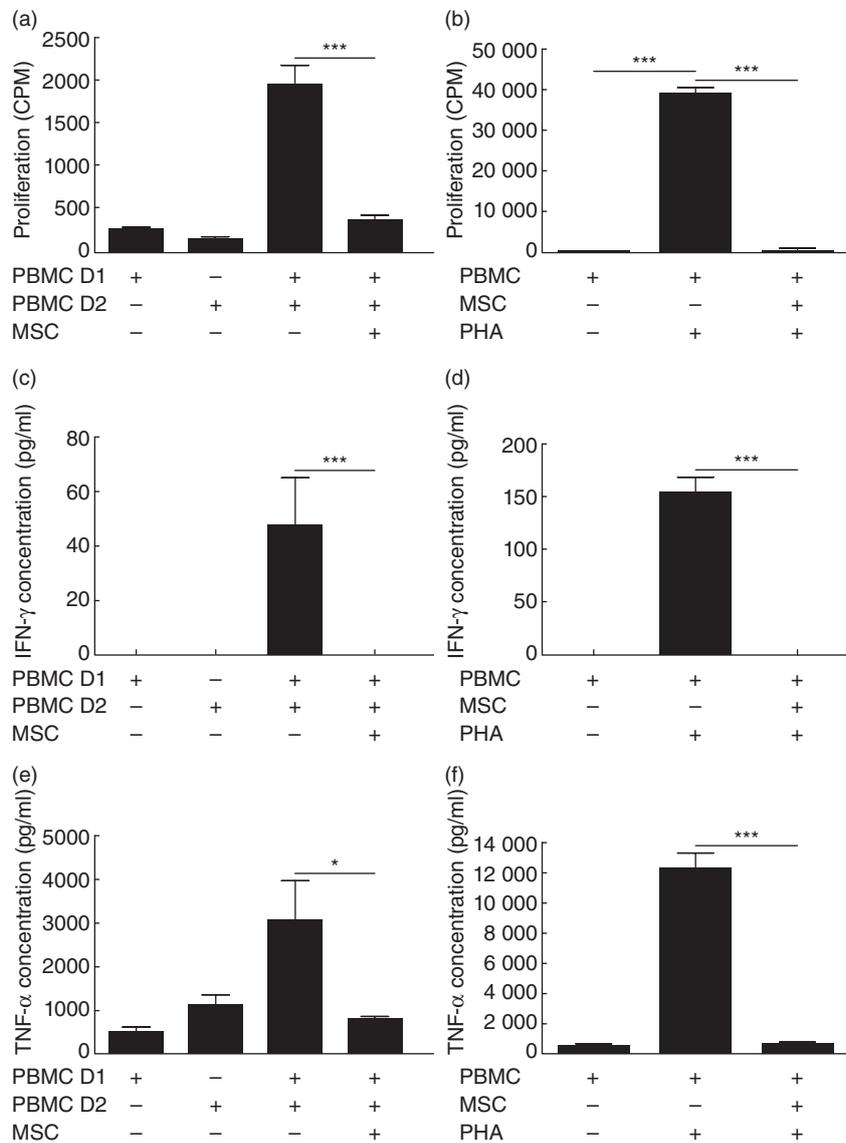


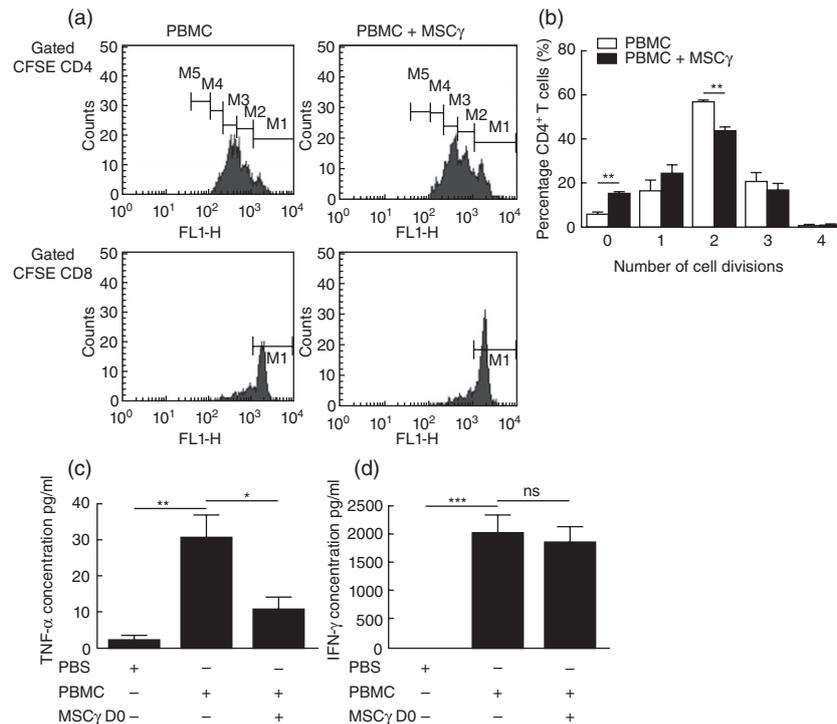
**Fig. 6.** Human mesenchymal stem or stromal cells (hMSC) supported the expansion of CD4<sup>+</sup> CD25<sup>+</sup> forkhead box protein 3 (FoxP3)<sup>+</sup> T cells *in vitro* but not *in vivo*. Expression of FoxP3 was measured *in vitro* (a,b) and *in vivo* (c–e). *In vitro*, (a) peripheral blood mononuclear cells (PBMC) or (b) sorted human CD4<sup>+</sup> CD25<sup>+</sup> or CD4<sup>+</sup> CD25<sup>-</sup> T cells ( $0.5 \times 10^6$ /ml) were co-cultured with or without MSC ( $1.5 \times 10^5$ /ml) for 72 h. Cells were recovered and examined for the expression of CD4, CD25 (PBMC population) and FoxP3 by intracellular flow cytometry. (a) The percentage of double-positive PBMC expressing CD4/CD25 or CD4/FoxP3 is given in the upper quadrant. (b) The number of FoxP3-expressing CD4 cells sorted from whole PBMC is represented in the bar chart. MSC did not induce a population of FoxP3<sup>+</sup> regulatory T cells (T<sub>reg</sub>) cells, but rather expanded an already existing population *in vitro* (data are representative of three independent experiments). *In-vivo* non-obese diabetic (NOD) severe combined immunodeficient (SCID) interleukin (IL)-2r<sup>null</sup> NSG mice received phosphate-buffered saline (PBS) or PBMC ( $6.3 \times 10^5$  g<sup>-1</sup>) on day 0 with or without non-stimulated MSC on day 7 or interferon (IFN)- $\gamma$ -prestimulated MSC (MSC $\gamma$ ) on day 0 ( $4.4 \times 10^4$  g<sup>-1</sup>). On day 12, the (c) lungs, (d) livers and (e) spleens were harvested and analysed for the expression of human CD4, CD25 and FoxP3 by flow cytometry. Data are summarized in bar charts in the right-hand panels. CD4<sup>+</sup> FoxP3<sup>+</sup> T<sub>reg</sub>-like cells were not detected *in vivo* following MSC therapy. In this instance data are representative of six mice per group ( $n = 6$ ).

action. Animal models of GVHD have well-known limitations, especially with regard to assessment of human cell therapies. For example, Sudres *et al.*, using a model where C57BL/6 bone marrow cells were injected into lethally irradiated BALB/c mice, found that murine MSC therapy had

no beneficial effect on survival [40]. Jeon *et al.* found that human MSC were unable to prevent GVHD development and the symptoms of GVHD were not alleviated *in vivo* [41], the drawback of the latter system being the mismatch between human MSC and murine effector cells (murine as

**Fig. 7.** Mesenchymal stem or stromal cells (MSC) inhibit peripheral blood mononuclear cell (PBMC) proliferation and suppress interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$  production *in vitro*. (a) PBMC ( $1 \times 10^6$ /ml) from two major histocompatibility complex (MHC) mismatched donors (D1 or D2) were cultured in the presence or absence of MSC ( $1 \times 10^5$ /ml) in a mixed lymphocyte reaction (MLR). MSC inhibited alloantigen-driven proliferation significantly ( $P < 0.0001$ ). (b) Human MSC also suppressed mitogen [phytohaemagglutinin(PHA)]-driven proliferation of allogeneic human PBMC ( $P < 0.0001$ ) *in vitro*. The inhibition of proliferation correlated with a significant decrease in the production of (c,d) IFN- $\gamma$  ( $P < 0.0001$ ) and (e,f) TNF- $\alpha$  ( $P < 0.0201$ ,  $P < 0.0001$ , respectively), as measured by enzyme-linked immunosorbent assay. Data are representative of three experiments, each performed in triplicate.





**Fig. 8.** Mesenchymal stem or stromal cells (MSC) reduced the proliferation of CD4<sup>+</sup> T cells and suppressed tumour necrosis factor (TNF)- $\alpha$  production *in vivo*. Peripheral blood mononuclear cells (PBMC) labelled with 10  $\mu$ M carboxyfluorescein succinimidyl ester (CFSE) were administered to conditioned non-obese diabetic (NOD) severe immunodeficient interleukin (IL)-2 $\gamma^{\text{null}}$  NSG mice with or without interferon (IFN)- $\gamma$ -prestimulated MSC (MSC $\gamma$ ) on day 0. After 5 days, the lungs, livers and spleen were harvested. (a) The level of CFSE in CD4<sup>+</sup> T cells was analysed by flow cytometry. MSC reduced the proliferation of CD4<sup>+</sup> T cells in the lung at 5 days. Sufficient CFSE-stained CD4<sup>+</sup> T cells were not recoverable from the livers or spleen after 5 days. (b) The percentage of CD4<sup>+</sup> cells present in the lung at each division *in vivo*. Serum was taken from NSG mice on day 12 and analysed for the presence of (c) human TNF- $\alpha$  and (d) human IFN- $\gamma$  by bead array. Prestimulated MSC $\gamma$  reduced significantly the level of (c) human TNF- $\alpha$  ( $P < 0.0267$ ) in the sera of NSG mice with acute graft-versus-host disease (aGVHD). Human MSC (hMSC) therapy had no significant effect on (d) human IFN- $\gamma$  production in sera. Data are representative of five mice per group ( $n = 5$ ).

opposed to human graft). In the model described here, the effector cells are those deployed in human recipients and the MSC may be sourced from production batches intended for clinical use. Thus, this model offers a system to evaluate batches of MSC therapeutics against the donor lymphocytes to be used clinically.

The observation that the kinetics of therapeutic delivery had a profound outcome on survival was not surprising. Polchert *et al.* found no significant improvement in aGVHD-related mortality when murine MSC were given as a therapy on day 0, but treatment with MSC on days 2 or 20 post-bone marrow transplantation prolonged the survival of mice with aGVHD [32]. In order for human MSC cell therapy to be beneficial at day 0, MSC required stimulation or activation with IFN- $\gamma$  (Fig. 1). These results were similar to those of other studies [32,42,43], suggesting that MSC require prestimulation or 'licensing' with IFN- $\gamma$  at the earliest time-points [32]. The failure of non-stimulated MSC to treat aGVHD when delivered concurrently with donor PBMC is interesting. Normally, IFN- $\gamma$  enhances allogenicity; however, MSC stimulated with IFN- $\gamma$  show enhanced immunosuppressive ability [36,44,45]. As

GVHD develops in this model, the levels of IFN- $\gamma$  increase. It may be that sufficient levels of IFN- $\gamma$  are required for the activation of non-stimulated MSC [32]. Therefore, MSC administered after the development of a proinflammatory environment *in vivo* are more successful in prolonging the survival of mice with GVHD than those delivered at day 0. These data highlight the importance of cell manipulation as well as timing in designing MSC therapeutic protocols.

The humanized model used here allowed for the successful engraftment of human cells (Fig. 3). This engraftment of human CD45<sup>+</sup> cells was not hindered by MSC therapy, but both non-stimulated (at day 7) and IFN- $\gamma$ -stimulated MSC therapies significantly reduced the severity of aGVHD pathology in the small intestines and livers of NSG mice after 12 days (Fig. 2). Human MSC therapy reduced villous blunting and lymphocyte infiltration into the lamina propria of the small intestine, while reducing vascular endothelialitis and lymphocyte infiltration into the parenchyma of the liver. These data were similar to Polchert *et al.* and Joo *et al.*, where murine MSC therapy significantly improved the histological score of the intestine and liver of mice with GVHD [32,42]. Unlike Polchert *et al.*, human

MSC therapy did not improve the histological analysis of the lung in NSG mice with aGVHD, as there was a significant amount of cell infiltration in all treatment groups (Fig. 2). Importantly, the histological results herein mirrored those of a recent Phase III human clinical trial [27]. This trial set out to examine the effects of human MSC, Prochymal®, in the treatment of patients with steroid-refractory aGVHD. Although Prochymal® cell therapy was well tolerated in patients with no adverse effects in a Phase II trial [25], findings of a Phase III trial have been difficult to interpret mechanistically. In the Phase III clinical trial, patients who presented with aGVHD manifesting in the liver and the gut showed significant improvement following treatment, similar to that seen here. However, cell therapy had no beneficial effect on skin manifestations. Although histological analysis of the skin was not examined in the humanized model, the beneficial effect of MSC-based cell therapy here was also target organ-dependent. This might be linked to MSC localization to different target organs, a hypothesis testable in the model we describe.

The major benefit of this model is that it allows a mechanistic exploration of MSC therapy not possible in patients, and specifically the link between MSC therapy and immunological tolerance. The induction of immune tolerance involves a precise balance between activation and inhibition of T cell responses, which is important in the development of GVHD. Tolerance can occur through the induction of lymphocyte apoptosis, anergy, regulatory cell induction/expansion or the direct inhibition of lymphocyte proliferation. Several studies have given contradictory evidence in relation to the induction of T cell apoptosis by MSC [46,47]. In this study, MSC did not induce apoptosis of PBMC *in vitro* (Fig. 4) or suppress engraftment (Fig. 3). MSC therapy to NSG mice with aGVHD did not increase the number of detectable apoptotic cells after 12 days (Fig. 4). These data are in line with other groups reporting that MSC play no role in the induction of T cell apoptosis [17,18,47,48], but are in contrast to Plumas *et al.*, who found that human MSC induced the induction of apoptosis of activated T cells through the production of indoleamine-2,3-dioxygenase (IDO) [46]. Despite the contradictory literature, the data herein indicated that the induction of T cell apoptosis by MSC was unlikely to be the mechanism by which MSC prolonged the survival of NSG mice with aGVHD.

The concept that MSC induce T cell anergy has also been controversial [47,49]. Studies of bone marrow-derived murine MSC co-cultures have resulted in T cells that did not regain their ability to proliferate in response to the cognate antigen, reversible by the addition of IL-2, suggesting the induction of T cell anergy [47,49]. The findings here suggested that MSC did not induce CD4<sup>+</sup> T cell anergy *in vitro*. Using a classical two-step assay, human MSC inhibited the proliferation of allogeneic human CD4<sup>+</sup> T cells following stimulation by murine DC. Upon restimulation of purified

CD4<sup>+</sup> T cells (with irradiated murine DC in the presence or absence of IL-2), T cell proliferation was unaltered (Fig. 5). This suggested that MSC did not induce an antigen-specific anergic T cell population. In other murine and human studies, T cell unresponsiveness was shown as transient and reversible if MSC were removed from cultures, suggesting a more direct suppressive effect than classical anergy [17,50]. While it is difficult to make comparisons across diverse experimental systems, the data from this system do not support an interpretation that MSC evoke classical T cell anergy in this model.

CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells play a role in the induction and maintenance of immune tolerance [51]. Many murine studies have identified a correlation between T<sub>reg</sub> cells and the induction, acceleration and treatment/prevention of aGVHD [52–54]. It is well documented both here (Fig. 6) and by others that MSC are capable of expanding T<sub>reg</sub>-like cell populations *in vitro* [16,55,56]. The deletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells from bone marrow grafts prior to transplantation dramatically accelerates aGVHD development in other murine models [52,57,58]. Additionally, the infusion of *ex-vivo*-expanded CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells prevents aGVHD development, while preserving graft-versus-leukaemia (GvL) activity [53,54,58–60]. This inverse correlation between T<sub>reg</sub> cells and aGVHD has also been seen in patients with aGVHD [61]. We were surprised to find that non-stimulated or IFN- $\gamma$ -stimulated MSC cell therapy did not result in increased CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells in the lung, liver or spleens of NSG mice with aGVHD, especially as we have detected these cells in other disease systems [37]. These findings are also in contrast with work published by other groups in different systems [42,62]. The data here may have multiple causes. It may be that as MSC expand but do not induce T<sub>reg</sub>, the lack of such populations here reflects the low frequency of T<sub>reg</sub> in the initial donor PBMC populations. Thus, the numbers of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells present in the donor PBMC were too low for their expansion following MSC transfusion *in vivo*. Alternatively, it may reflect a more fundamental issue with NSG mice and a limitation of our model. It could be that the absence of human stromal factors to support the expansion of human T<sub>reg</sub> cells in the NSG mouse model of aGVHD or that other non-conventional FoxP3 T<sub>reg</sub> populations are involved. A related feature is that in NSG mice (even with aGVHD), lymph nodes are vestigial and the stromal compartment may not be sufficient for T<sub>reg</sub> cells to develop effectively in this system. Interestingly, MSC therapy prolonged the survival of NSG mice with aGVHD but did not prevent aGVHD development in the longer term (as seen in clinical trials also) [25,27]. If T<sub>reg</sub> cells had been induced or expanded a more permanent suppression might be expected, which would suggest that MSC therapy as a single dose has a more transient/limiting effect on aGVHD development, rather than induction of immune tolerance, as has been suggested previously [43].

MSC inhibition of T cell proliferation *in vitro* is well documented [16,17,47,49], but there are contradictory data available for the inhibition of T cell proliferation by MSC *in vivo* [40,47]. Sudres *et al.* found that although murine MSC inhibited the proliferation of T cells *in vitro*, administration on day 1 to treat GVHD had no effect on the proliferation of CFSE-labelled T cells *in vivo* [40], others have also shown that although murine MSC could inhibit T cell proliferation *in vitro*, this was not detectable *in vivo* [43]. We could not detect suppression in the liver or spleen in the NSG model of aGVHD due to the very low recovery of T cells from MSC-treated mice. However, in the lungs, the organ with the greatest inflammatory manifestation, IFN- $\gamma$  stimulated MSC therapy resulted in the reduction of CD4<sup>+</sup> T cell proliferation in NSG mice after 5 days (Fig. 8). These data showed that MSC inhibition of T cell proliferation and reduction in serum TNF- $\alpha$  are features of MSC-mediated immune suppression *in vivo*. Although these data suggest that the suppression of T cell proliferation/activation is the primary mechanism of human MSC $\gamma$  therapy, it is important to note that stimulated and non-stimulated MSC may work in different ways, and this requires further investigation. None the less, these data highlighted a possible mechanism by which MSC cell therapy prolonged the survival of NSG mice with aGVHD and suggests that improvements to MSC therapy are amenable to exploration in the model described herein.

### Acknowledgements

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### Disclosure

The authors declare no conflict of interests.

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