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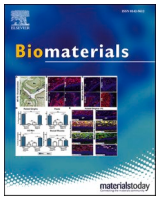

Bone formation by human paediatric marrow stromal cells in a functional allogeneic immune system

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ABSTRACT

Allogeneic stem-cell based regenerative medicine is a promising approach for bone defect repair. The use of chondrogenically differentiated human marrow stromal cells (MSCs) has been shown to lead to bone formation by endochondral ossification in immunodeficient pre-clinical models. However, an insight into the interactions between the allogeneic immune system and the human MSC-derived bone grafts has not been fully achieved yet. The choice of a potent source of MSCs isolated from pediatric donors with consistent differentiation and high proliferation abilities, as well as low immunogenicity, could increase the chance of success for bone allografts. In this study, we employed an immunodeficient animal model humanised with allogeneic immune cells to study the immune responses towards chondrogenically differentiated human pediatric MSCs (ch-pMSCs). We show that ch-differentiated pMSCs remained non-immunogenic to allogeneic CD4 and CD8 T cells in an *in vitro* co-culture model. After subcutaneous implantation in mice, ch-pMSC-derived grafts were able to initiate bone mineralisation in the presence of an allogeneic immune system for 3 weeks without the onset of immune responses. Re-exposing the splenocytes of the humanised animals to pMSCs did not trigger further T cell proliferation, suggesting an absence of secondary immune responses. Moreover, ch-pMSCs generated mature bone after 8 weeks of implantation that persisted for up to 6 more weeks in the presence of an allogeneic immune system. These data collectively show that human allogeneic chondrogenically differentiated pediatric MSCs might be a safe and potent option for bone defect repair in the tissue engineering and regenerative medicine setting.

1. Introduction

Unresolved bone defects caused by congenital malformations, trauma or tumors remain a challenging problem in the clinic nowadays [1]. The use of allogeneic chondrogenically primed human marrow stromal cells (MSCs) has been previously described as a potentially attractive 'off-the-shelf' solution to repair large bone defects [2], being much less invasive for the patient than the current gold standard of autologous bone grafting. Several studies have reported successful bone formation in pre-clinical models upon implantation of transforming growth factor beta (TGF-β)-primed human MSCs [3,4], which form a cartilage template that is eventually replaced by bone through the endochondral ossification process. In this context, the use of allogeneic MSCs rather than autologous MSCs is desirable due to the possibility of testing, expanding and differentiating cells from healthy donors [5] in advance of their requirement. An ideal source of allogeneic MSCs to follow this approach would be one that allows for rapid expansion, consistent chondrogenic differentiation and low immunogenicity [6].

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We have previously *in vitro* characterised pediatric human MSC (pMSCs) [7], and demonstrated their capacity to meet these requirements, showing increased proliferation capabilities and more reliable differentiation than adult MSCs, as well as immunomodulatory properties towards T and B cells [8].

MSCs have traditionally been described as having low *in vitro* immunogenicity due to their low expression of co-stimulatory molecules, and are immunomodulatory towards a wide range of cells from both the innate and the adaptive immune system, including T cells [9,10]. However, the immunogenicity of allogeneic MSCs in an *in vivo* situation might differ [11]. Emerging proof suggests that when systemically administered *in vivo*, undifferentiated MSCs can induce immune reactions [12,13,14], such as anti-allogeneic immune responses mediated by allo-antibodies when used as a cell therapy [15,16]. On the other hand, other authors have claimed that only a transient immune response that is resolved after 10 days occurs when using undifferentiated allogeneic MSCs [17]. Although the behavior of undifferentiated MSCs towards the immune system seems to be well characterised, whether chondrogenic differentiation could alter their interaction with immune cells remains controversial. Some studies have shown that in an *in vitro* co-culture model, MSCs induce allogeneic T cell proliferation after chondrogenic differentiation [18], as well as systemic and local allo-immune responses upon subcutaneous implantation of chondrogenically differentiated MSC [19]. In contrast, other studies have reported no significant immune responses from chondrogenically differentiated MSCs when co-cultured with allogeneic T cells [20], and suggest they retain their ability to suppress T cell proliferation in these circumstances [21], as well as skew the ratio of specific helper CD4 T cell subsets rather than triggering cytotoxic responses [22]. A recent study has also proven that the conversion of allogeneic rat MSC-derived cartilage implants to bone can take place even in the presence of an immune system in an bone defect model [23], evidencing only a low to moderate immune reaction. Despite these advances, an animal model recapitulating the process of allogeneic human MSCs to form bone within a functional immune system is still missing.

In addition to the potential to persist in a allogeneic setting, further research suggests that MSC-mediated bone regeneration is directed by the host CD4 and CD8 T cells [24]. Schlundt et al. showed that a higher CD4/CD8 T cell ratio improved bone repair [25]. Bone mineralisation has also been correlated with T cell presence in a bone defect animal model, proving earlier mineralisation in the fracture of T cell-defective mice compared to immunocompetent animals [26]. The role of T cells as a main player in endogenous bone repair processes has been demonstrated, showing an association between higher amounts of effector CD8 T cells with an increased inflammatory reaction and a delay in bone repair [27]. Altogether, these studies highlight the importance of understanding the donor/host immune cell interaction to achieve successful bone formation.

Thus, investigating adaptive immune responses in the recipients is key to ensure successful stem cell-derived bone repair. Therefore, in this study we successfully proved for the first time that allogeneic chondrogenically differentiated MSCs from a pediatric origin were able to form mature bone grafts in a fully functional humanised animal model that persisted for at least 14 weeks without triggering significant immune responses.

2. Materials and methods

2.1. Study design

The aim of this study was to analyse the capacity of human chondrogenically differentiated pMSCs (ch-pMSCs) to form bone in an allogeneic recipient. To this objective, we carried an initial *in vitro* experiment where we analysed the immunogenicity and immunomodulation of pMSCs under pro-inflammatory conditions. T cell proliferation was assessed by flow cytometry, and interactions between T cells and ch-pMSCs were analysed by immunohistochemistry and PCR.

Subsequently, we utilised an allogeneic *in vivo* model to provide a mismatched immune setting by injecting allogeneic human PBMCs in immunodeficient IL2rg−/−RAG2−/− mice. Three different experimental designs were followed for the animal experiments. In an initial study to analyse the survival of subcutaneously-implanted ch-pMSCs, as well as their ability to initiate bone formation in an allogeneic immune setup, mice received an injection of human PBMCs (humanisation) for three weeks, and upon that time subcutaneous implantations were performed, up to week 6 (Fig. S1). In the same study, splenocytes from the humanised animals were isolated and re-exposed *ex vivo* to the same (pre-exposed) and a different (non-pre-exposed) pMSC donor for 3 days, and the T cell responses were analysed by FACS. Additionally, we studied the ability of mature bone derived from ch-pMSCs to persist in our animal model. Ch-pMSCs were implanted in the immunodeficient animals and at week 8 an injection of human PBMCs was administered (Fig. S3). As a way of evaluating the persistence of mature ch-pMSC grafts for a longer period of time, we made use of a serial transplantation model. Hereby, 12-week old implanted constructs were harvested from 4-week humanised or non-humanised mice (donors) and retransplanted in a second group of non-humanised or 2-week-humanised mice (recipients) up to week 14 (Fig. S5). Bone formation was evaluated by measurements of the mineralisation on ch-pMSC grafts by Micro-Computer Tomography (μCT). The interactions of the ch-pMSCs grafts with the allogeneic immune system were analysed by PCR, flow cytometry and immunohistochemistry, and the degree of human immune cell engraftment and the systemic responses were assessed by flow cytometry.

Sample sizes were estimated using power analyses for the analysis of variance (ANOVA) test to determine sample size with a significance of alpha = 0.05 and a statistical power of 80 %. An additional 10 % of animals to account for potential losses due to surgery-related complications was included. The endpoints of the animal experiments were determined based on previous observations to avoid the onset of GvHD in this humanised animal model. Animals were housed under similar conditions and randomly assigned to the different experimental groups. For the first animal study, the data of one animal in the Graft + PBMCs condition was excluded due to failure of humanisation.

2.2. Isolation and culture of human pediatric bone marrow derived MSCs

Cells were obtained with the approval of the medical ethics committee at Erasmus Medical Centre (Erasmus MC, The Netherlands). pMSCs were isolated from leftover iliac crest bone chips of pediatric patients undergoing alveolar bone graft surgery (MEC-2014-16) as previously described [7,8].

2.3. Isolation of peripheral blood mononuclear cells (PBMCs) from peripheral blood

Peripheral blood from healthy male donors was obtained from Sanquin Blood Bank (Rotterdam, the Netherlands), and PBMCs were isolated as previously described [7,8].

2.4. Chondrogenic differentiation of pMSCs

pMSCs were chondrogenically differentiated in a three-dimensional culture following our previous protocol [20]. Briefly, pMSCs were detached with 0.05 % w/v trypsin and resuspended in standard chondrogenic medium (high glucose Dulbecco's Modified Eagle's Medium, DMEM supplemented with 1 mM sodium pyruvate (Sigma-Aldrich), 1:100 insulin-transferrin-sodium selenite (ITS+) (BD Biosciences) 50 μg/mL of gentamycin, 1.5 μg/mL Amphotericin B (Sigma-Aldrich) at a concentration of 0.2×10^6 cells per 0.5 mL of medium in polypropylene tubes (Sarstedt). For chondrogenesis induction, 100 nmol/L of dexamethasone (Sigma-Aldrich), 10 ng/mL of TGF-β3 (R&D) and 0.1 mM

fresh L-ascorbic acid 2-phosphate was added to high-glucose DMEM for 21 days, refreshing the medium every 3 or 4 days.

2.5. Co-culture of chondrogenic pellets with PBMCs and pro-inflammatory cytokines

After 21 days of chondrogenesis cell pellets were washed twice in PBS and transferred to 1.5 mL tubes, where 1×10^6 either Carboxyfluorescein succinimidyl ester (CFSE)-labeled unstimulated (for immunogenicity experiments) or CD3/CD28 stimulated (1 mg/mL, BD Biosciences) (for immunomodulation experiments) PBMCs were added in PBMC medium consisting on RPMI-1640 medium with 1 % v/v GlutaMAX, 1.5 μg/mL Amphotericin B, 50 μg/mL gentamycin and 10 % v/v heat inactivated human serum (Thermo Fischer) for 5 days. To mimic pro-inflammatory conditions, PBMC medium was supplemented with 10 ng/mL of IFN-γ and 10 ng/ml TNF-α (Peprotech).

2.6. Analysis of T cell proliferation

Upon 5 days of co-culture with pellets, PBMCs in suspension were collected, washed three times in FACS Flow and stained for flow cytometric analysis. In the last washing step, cells were resuspended in 100 μL of FACS flow containing a master mix of antibodies against CD3 (PerCP), CD4 (PE), CD8 (APC), and CD25 (PE-Cy7) (all from BD Biosciences). Samples were analysed using FACS Jazz (BD Biosciences) and Flowjo v.10. Results were individually plotted per round of experiment, but analysed together. As a measurement of proliferation, the Mean Fluorescence Intensity (MFI) of the CFSE incorporation in the cells was analysed, and the 1/MFI was plotted to represent a loss of the CFSE as the cells proliferate more.

2.7. Mice

Animal experiments were approved by the National Animal Experiments Committee at the Erasmus Medical Centre, Rotterdam, The Netherlands (animal license AVD101002015114, protocol numbers 127-54-01, 15-114-01, 15-114-02 and 15-114-10). Balb/c IL2Rγ$^{-/-}$ RAG2 $^{-/-}$ were bred and housed under specific pathogen free (SPF) conditions, being between 8 and 16 weeks old at the beginning of the experiments. Animals were socially housed in groups of 2–4 animals per cage, with *ad libitum* access to water and food and a 12 h light/dark cycle. At the harvest timepoint (week 3, 12 or 14 post-implantation), animals were euthanised by cardiac puncture and cervical dislocation under general anaesthesia, and bone constructs, blood, spleens, and femurs were retrieved.

2.8. Human immune cell injection

At the specified timepoint of each experimental design, human allogeneic PBMCs were thawed, resuspended in Phosphate-Buffered Saline (PBS) and counted for viability with trypan blue. 5×10^6 viable PBMCs in 0.2 mL of PBS per animal were then intraperitoneally (IP) injected.

2.9. Subcutaneous implantations of ch-pMSC pellets

In vitro 21 day chondrogenically differentiated pellets (grafts) were subcutaneously implanted in Balb/c IL2Rγ$^{-/-}$ RAG2 $^{-/-}$ mice. Animals were anaesthetised with 2–3.5 % isofluorane and pre-operative pain medication was administered (buprenorphine, 0.05 mg/kg). Under general anaesthesia, four parallel incisions were created at the back of the animals (two at the top, between the shoulder blades, and two between the hip bones), and one pocket per incision was made by blunt dissection. Three pellets per pocket were then (re-) implanted for 3, 8 or 14 weeks, depending on the experimental setup (Figure S1, S3 and S5)

2.10. Micro-computed tomography (CT) imaging

Micro CT scans were performed either *ex vivo* or on living animals at weeks 6, 8, 12, and 14 using the Quantum-FX or the Quantum-GX2 (PerkinElmer, Groningen, the Netherlands) at the Applied Molecular Imaging of the Erasmus MC facility. For the 6 and 12 week studies, imaging was performed using a 30 mm field of view for 3 min (90kV/160 μA) at the Quantum-FX. For evaluating the long-term allogeneic immune responses, calcification was detected using a 36 mm field of view for 4 min (90 kV/160 μA) at the Quantum-GX2.

Bone mineralisation was quantified using two phantoms with known density (0.25 g/cm^3 and 0.75 g/cm^3; Bruker MicroCT) under identical conditions. Calcification was determined in the scans using the software Analyse 11.0 (AnalyzeDirect)

2.11. Flow cytometric analyses of blood, spleens and femurs

Blood was harvested by intracardiac puncture and kept on ice in EDTA lined tubes until processing of the samples. Plasma was removed after centrifugation of the samples at 400 g for 5 min, and PBMC staining was performed directly in the rest of the blood sediment after red blood cell lysis using FACS lyse (3 mL per sample).

Spleens were weighed and mechanically dissociated using a 70 μm strainer to obtain a single cell suspension. Cells were spun down at 400g for 5 min and 3 mL of pre-diluted ACK Lysis buffer (8 g/L ammonium chloride, 1 g/L potassium bicarbonate 200uL/L 05 M EDTA) was added per spleen and incubated for 10 min. After that time, 20 mL of PBS was added and cells were centrifuged for 5 min at 400 g. A cell count was then performed.

Femurs were cleaned from any remaining tissue and the proximal and distal ends of the bone were cut. Then they were flushed through twice with RPMI-1640 with 1 % v/v GlutaMAX, 1.5 μg/mL Amphotericin B, 50 μg/mL gentamycin and 10 % v/v heat inactivated human serum (Sigma-Aldrich) on top of a 100 μm strainer. The resulting cells were centrifuged at 500 g for 5 min and washed with supplemented RPMI-1640 once again before counting.

PBMCs from both blood and splenocytes were stained with anti-human CD3 (FITC), CD4 (PE), CD8 (APC), CD45 (PerCP), and CD25 (PE-Cy7) and anti-mouse CD45 (FITC) (all from BD Biosciences), and femurs using anti-mouse CD45 (PE-Cy7) (Sony). Samples were analysed using FACS Jazz (BD Biosciences) and FlowJo v.10.

2.12. Flow cytometric analysis of immune cell infiltration in the grafts

Retrieved bone grafts were placed in 10 mL of pre-cooled RPMI-1640 medium containing 5 % FBS and kept on ice or at 4 °C until adding 1.5 mL/construct of a digestion solution, consisting of 1.5 mL of RPMI-1640 medium containing 5 % FBS, 3 mg/mL collagenase A (Roche) and 10 μL/mL of DNase. Samples with the digestion solution were then transferred to a 5 ml polypropylene FACS tube and rotated at 37 °C for 90 min. Following the incubation, the solution was poured through a 100 μm cell strainer and FACS tubes were washed with 3 mL of RPMI-1640 medium containing 5 % FBS. The remaining parts of the graft on top of the strainer were then manually crushed, and the strainer was washed again with 10 mL of RPMI-1640 medium containing 5 % FBS. The resulting solution was then spun down at 400 g for 5 min, supernatant was removed and the pellet was resuspended in 500 μl of FACS flow buffer. Cells were counted and stained with anti-human CD3 (PerCP), CD4 (PE), CD8 (APC), CD45 (PerCP) (BD Biosciences), and anti-mouse CD45 (PE-Cy7) (Sony). Samples were further analysed using the FACS Jazz and FlowJo v.10.

2.13. Ex vivo analysis of splenocyte responses

Graft-matched (pre-exposed) and graft-mismatched (non-pre-exposed) pMSCs were pre-treated with 50 ng/mL of IFN-γ (Peprotech)

for 3 days. 24 h before the harvest of the mice, cells were seeded in a U-bottom low evaporation 96 well plate at a density of 0.2×10^6 cells per well in 100 μL of PBMC medium. For heat inactivation of the pMSCs, cells were heated to 50 °C for 30 min and seeded in a 96 well plate, which was kept in the fridge overnight.

On the harvest day, splenocytes were isolated as previously described in section 5 and labeled with CFSE. Cells were then co-cultured with graft-matched and mismatched pMSCs on a 1:2 pMSC: splenocyte ratio for 3 days. After that time, samples were harvested and stained with human anti-CD3 (PE-CF594), CD4 (PE), CD8 (BV786), CD45 (PerCP). Analysis was performed using the BD Fortessa cytometer and Flowjo v10.

2.14. Histology

pMSC pellets in co-culture with PBMCs and pro-inflammatory cytokines were retrieved, washed twice in PBS and fixed in 4 % formalin. Grafts and femurs were fixed in 4 % formalin and decalcified in 10 % ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) prior to embedding. Small samples (pellets and small bone constructs) were embedded in 2–3 % w/v agarose (Eurogentec) prior to the paraffin embedding. Sections were deparaffinised and 6 μm thick sections were cut and prepared for histochemistry and immunohistochemistry. Hematoxylin-Eosin staining and TRAP stainings were then performed as previously described.

2.15. Immunohistochemistry

To stain for human-specific GAPDH, a heat induced antigen retrieval was performed for 20 min at 95 °C in 10 mM Sodium Citrate at pH 6.0 and 0.05 % Tween-20. For human CD3, CD4 and CD8 stainings, heat induced antigen retrieval was performed for 20 min at 95 °C in 10 mM Tris at pH 9.0, 1 mM EDTA and 0.05 % v/v Tween-20. Non-specific binding was blocked by incubation of the slides with 10 % v/v normal goat serum in either Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.5, 150 mM NaCl; Sigma Aldrich) for the human GAPDH, or PBS for the other immunohistochemical stainings containing 1 % w/v BSA and 1 % w/v milk (Elk, Campina).

Slides were then stained for 1 h at room temperature (CD3 and GAPDH) or overnight at 4 °C (CD4 and CD8) using either a human GAPDH specific antibody (0.2 μg/ml rabbit-*anti*-human GAPDH, Abcam) in TBS +1 % BSA; or a CD3 specific antibody (1:100 v/v rabbit-*anti*-CD3, Abcam), a CD4 specific antibody (0.33 μg/ml rabbit-*anti*-human CD4 Abcam), a CD8 specific antibody (0.5 μg/ml Rabbit-*anti*-human CD8, Abcam), a or Rabbit IgG isotype control (X0903, Dako) diluted in PBS + 1 % w/v BSA + 1 % w/v milk.

Thereafter, sections were incubated with a biotinylated anti-Rabbit IgG link, (HK-326-UR, Biogenex, 2 %), followed by a streptavidin-alkaline phosphatase label, (HK-321-UK, Biogenex. 2 %). Staining was visualised using 0.01 % w/v Neu Fuchsin, 0.01 % $NaNO_2$, 0.3 mg/ml Naphthol AS-MX (Sigma) and 0.25 mg/ml levamisole (Sigma) in 0.2 M Tris-HCl. After staining, samples were counterstained with hematoxylin. Finally, samples were air-dried and covered with a coverslip and Vectamount (Vector Laboratories, Burlingame, USA) and dried at 37 °C.

Ranking of the immunohistochemical sections for TRAP was performed once blindly by counting the positive cells for each of the evaluated stainings and assigning each section a score.

2.16. mRNA gene expression analysis

Samples were homogenised in 350 μl Trizol, then 70 μl chloroform was added and samples were agitated and incubated for 10 min at room temperature, followed by phase separation at 12 000 g for 10 min. The aqueous phase was transferred to a new tube, mixed with an equal volume of 70 % Ethanol and loaded on RNeasy® micro kit columns. RNA was purified using RNeasy® microkit (Qiagen; Venlo, Netherlands) according to the manufacturer instructions and cDNA was reverse transcribed using a First Strand cDNA Synthesis Kit (RevertAid; Thermo Fischer) as per manufacturer's instructions. Real-time PCR was performed using 5 ng of cDNA. Samples were amplified using either SYBR Green I dye (Eurogentec) or TAQman 2xReagent (Thermo Fischer) in 10 μL PCR mix reactions containing assay on demand (all from Thermo Fischer) for CD3E (Hs00167894), CD4, CD8, IL-2RA (CD25) (Hs00166229), CD69 (Hs00934033), Perforin (PRF1; Hs00169473), granzyme (GZMB; Hs01554355), FOXP3 (Hs00169473), IDO (Hs 00158027.m1) or GAPDH (Fwd 5′-GTCAACGGATTTGGTCGTATTGGG-3′, Rev 5′-TGCCATGGGTGGAATCATATTGG-3′ and FAM-TAMRA probe: 5′-TGGCCCCAACCAGCC-3′) for 40 cycles in a CFX96 real time PCR detection system (Biorad).

2.17. Statistical analysis

Samples were analysed using IBM SPSS version 27 using a linear mixed model with Bonferroni post-correction test. For multivariate analyses, a 2-way ANOVA on GraphPad Prism version 10 was used. To establish correlations, a Spearman's correlation analysis was performed in GraphPad Prism. Values are represented as the mean ± standard deviation (SD), and $P < 0.05$ was considered statistically significant. For the *in vitro* experiments, N = 3 donors in triplicate; for the *in vivo* experiments, N = 6–11 animals per group and N = 6 = 13 samples per condition, with N = 1–2 PBMC donors and N = 3–4 pMSC donors per experimental design.

3. Results

3.1. Allogeneic ch-pMSCs remain non-immunogenic under pro-inflammatory conditions

It has been previously reported that the immune status of MSCs can be affected by factors such as pro-inflammatory cytokines [28,29], as well as chondrogenic differentiation [18]. Therefore, to clarify their effect on the immunogenicity and immunomodulatory capacities of pMSCs, cell pellets made of ch-pMSCs were co-cultured with either unstimulated, or CD3/CD28 stimulated allogeneic PBMCs in the presence of 10 ng/mL of IFN-γ and TNF-α for 5 days.

When co-culturing ch-pMSCs with unstimulated PBMCs, no significant differences were detected in the proliferation rates of both the CD4 and CD8 T cell fractions (Fig. 1A and B) regardless of the presence of the pro-inflammatory cytokines. To determine whether allogeneic T cells were detectable within the pellets, we analysed the cell pellets for CD3 gene expression, showing low but detectable expression in pellets cultured with unstimulated PBMCs, also under inflammatory conditions (Fig. 1C).

CD3/CD28 stimulation induced a significant increase in the proliferation of CD4 and CD8 T cells compared to unstimulated controls and this proliferation was unaffected by the ch-pMSCs (Fig. 1D and E). No significant increase was observed in the proliferation rates of CD4 and CD8 T cells cultured in the presence of ch-pMSCs, but a significant reduction in the proliferation of the $CD4^+$ T cell subset occurred in the presence of IFN-γ and TNF-α co-cultured with ch-pMSCs. To examine whether activated T cells migrate into the pellets, the gene expression levels (Fig. 1F) as well as the protein expression of CD3 (Fig. 1G) were analysed. No significant differences were detected in terms of CD3 expression or presence in the pellets that were co-cultured with stimulated PBMCs compared with the ones in which the pro-inflammatory cytokines were present. These results suggest that, despite being in close contact, ch-pMSCs are non-immunogenic towards T cells even in an inflammatory microenvironment *in vitro*.

3.2. Allogeneic ch-pMSC-derived grafts persist after 3 weeks in vivo

In order to determine the ability of ch-pMSCs to form bone *in vivo* in

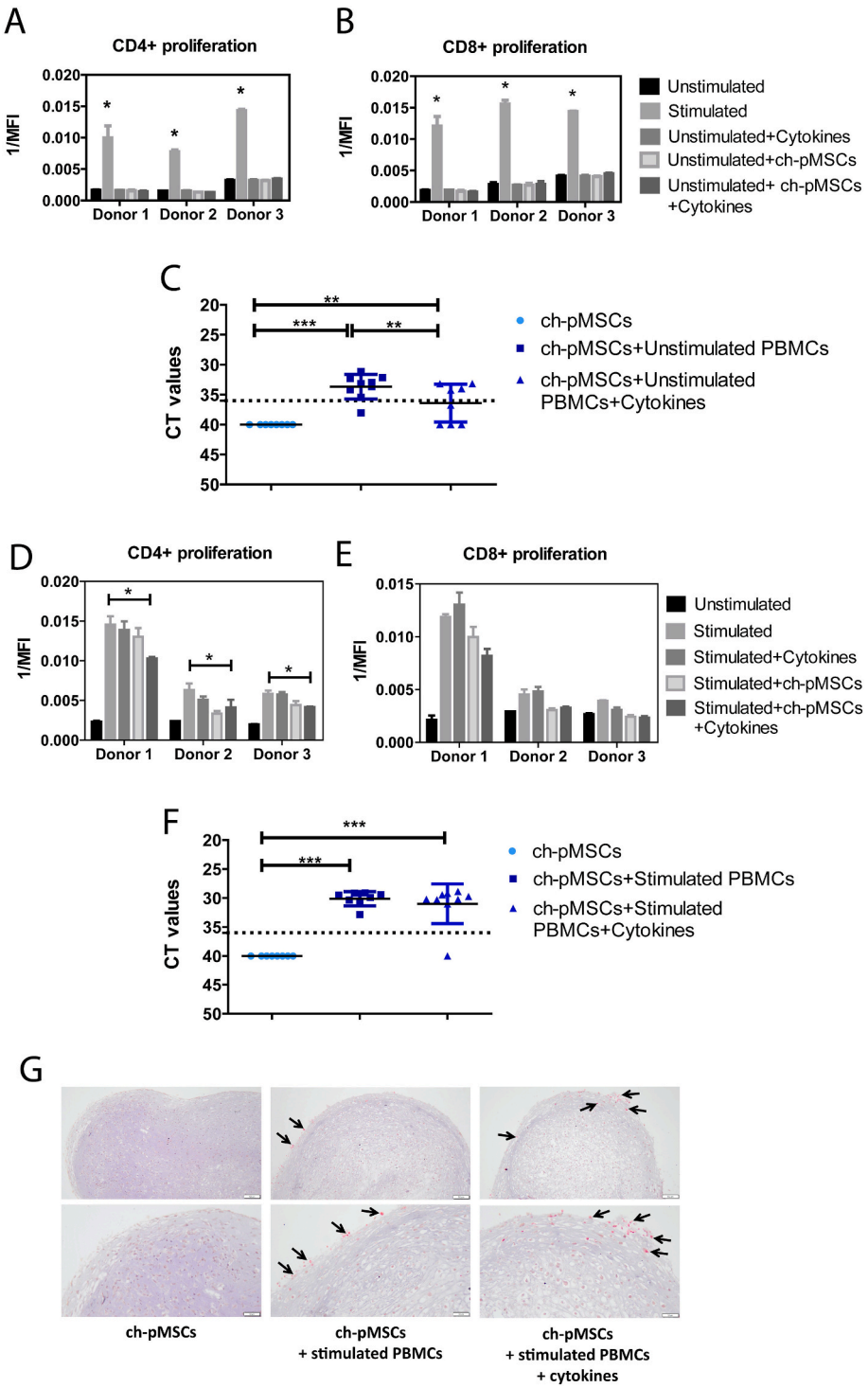

Fig. 1. ***In vitro*** **evaluation of the immunogenicity and immunomodulatory abilities of ch-pMSCs under pro-inflammatory conditions.** Flow cytometric analyses of the $CD4^+$ **(A)** and $CD8^+$ **(B)** T cell proliferation rates of unstimulated PBMCs in co-culture with ch-pMSCs and 10 mg/mL of IFN-γ and TNF- α expressed as the 1/Mean Fluorescence Intensity (MFI) of CFSE. **(C)** CD3 mRNA expression in the ch-pMSC pellets with unstimulated PBMCs+10 mg/mL of IFN-γ and TNF-α. (N = 3 pMSC donors and 3 PBMC donors in triplicates). Dotted line indicates the selected Ct threshold above which gene expression is considered to be present. Flow cytometric analyses of the $CD4^+$ **(D)** and $CD8^+$ **(E)** T cell proliferation rates of CD3/CD28 stimulated PBMCs in co-culture with ch-pMSCs and 10 mg/mL of IFN-γ and TNF-α expressed as the 1/MFI CFSE. **(F)** CD3 mRNA expression in the ch-pellets with CD3/CD28 stimulated PBMCs and 10 mg/mL of IFN-γ and TNF-α. Dotted line indicates the selected threshold above which gene expression is considered to be detectable. Unstimulated and Stimulated labels represent negative and positive controls in each experiment. **(G)** Immunohistochemical staining for CD3 expressing T cells in the ch-pMSCs pellets. Arrows indicate positively stained cells. (N = 3 pMSC donors and 3 PBMC donors in triplicates). *P < 0.05, linear mixed model with Bonferroni post-correction.

the presence of an allogeneic immune system, a group of three ch-pMSC cell pellets (grafts) was subcutaneously implanted for 3 weeks in host animals 3 weeks after generation of a humanised immune system with an intraperitoneal injection of human allogeneic PBMCs (Fig. S1). The human immune cell engraftment in the animals in blood and femurs was assessed after the total 6 weeks of humanisation (Fig. S2). The results showed similar levels of circulating human $CD45^+$ cells in blood in the humanised animals with and without chondrogenic grafts (Fig. S2A). In the femurs, the percentage of human immune cells remained below 25 % in all cases (Fig. S2C). The CD4/CD8 T cell ratio correlated between immune compartments (Fig. S2B and 2D). Taken together, these results suggest an absence of an immune response towards the ch-pMSC grafts after 3 weeks.

The grafts persisted after 3 weeks indicating the absence of resorption by the recipient. Importantly, there was no evidence of an inflammatory response or the presence of a large amount of mononuclear cell infiltrate in any of the conditions, with active cartilage matrix remodeling observed in samples that had received the graft with and without humanisation (Fig. 2A–C). This tissue becomes mineralised by week 3 but is not yet remodelled into bone. The μCT scans after 3 weeks of implantation revealed variations in the mineralised tissue volume of the grafts that belonged to humanised animals, however this was not significant compared with the graft only group (Fig. 2B). Evidence of $TRAP^+$ osteoclastic presence was detectable in both conditions (Fig. 2D). A human-specific GAPDH immunohistochemical staining revealed donor-derived human cells (Fig. 2E) in both conditions throughout the grafts. We did not find a significant correlation between the amount of $TRAP^+$ cells and the bone volumes of the grafts (Fig. 2F, $R = 0.31$).

3.3. Allogeneic human $CD3^+$ T cells are detected in ch-pMSC-derived grafts after 3 weeks

To determine the interaction of the allogeneic immune system with the pMSC-derived grafts in the early stages of bone formation, the presence of allogeneic human T cells in the grafts was analysed at week 6 post-humanisation (Fig. S1). Humanised animals showed human $CD3^+$ staining predominantly at the edges of the graft structure, indicating many of the allogeneic T cells surround the graft at this stage (Fig. 3A). This data was supported by the presence of $CD45^+$ human cells by flow cytometry (Fig. 3B). To further characterise the human T cells detected in the graft, a CD4 vs CD8 analysis was carried out (Fig. 3C), showing a general predominance of the helper T cells (CD4) compared with the cytotoxic (CD8) T cells (71.2 ± 16.79 % vs 28.7 ± 16.82 %) in the allografts. This data was correlated by immunohistochemical stainings, showing a higher presence of $CD4^+$ cells compared with the $CD8^+$, mainly at the periphery of the grafts (Fig. 3D). A significantly inverse correlation was found between the amount of $CD3^+$ human cells and the $TRAP^+$ osteoclastic cells in the graft ($R = -0.725$), but not between the amount of mineralised tissue volume and the $CD3^+$ human cells ($R = -0.104$) (Fig. 3E and F). This data suggests a role for the allogeneic human immune system in the initial stages of graft recognition.

3.4. pMSCs do not elicit immunogenic responses from pre-exposed allogeneic splenocytes

To determine whether repeated exposure to allogeneic ch-pMSCs triggers secondary effector responses, the spleens of animals reconstituted with human immune cells for 6 weeks and that carried a ch-pMSC graft for the last 3 weeks of humanisation were retrieved. Spleen cell suspensions were re-exposed *ex vivo* to the same pMSCs donor used in the grafts (pre-exposed) or to a different pMSC donor to which the PBMCs had not been exposed (non-pre-exposed) for 3 days. As it has been previously reported that IFN-γ priming can enhance the expression of costimulatory molecules, as well as antigen-presenting molecules such as HLA-II [30], IFN-γ primed pMSCs were used as a positive control of immunogenicity.

Our results revealed that viable graft-matched or mismatched pMSCs support splenocyte T cell survival *in vitro* after 3 days (9.35 ± 2.64 and 9.30 ± 2.65 %), in contrast to heat-inactivated pMSCs and splenocytes on their own (2.029 ± 0.817 and 2.369 ± 1.489) (Fig. 4A). Importantly, $CD3^+$ T cell proliferation was also unaffected by the presence of pMSCs, regardless of their pre-exposed or non-pre-exposed origin or IFN-γ pre-activation status (Fig. 4B). In the conditions where T cell survival was promoted by pMSCs, we found a predominance of CD4 T cell survival compared with the CD8 (Fig. 4C and D) and no significant differences were detected in the percentages of these cells regardless of the pMSC source. These results demonstrate that ch-pMSCs are unlikely to trigger a secondary T cell-mediated effector response.

3.5. Allogeneic human T cells are detected in the mature ch-pMSC-derived grafts

To assess the survival of mature ch-pMSC derived bone grafts within an allogeneic immune seting in the medium to long term and their immunogenicity, cell pellets were implanted for 8 weeks in immunodeficient animals, during which time they underwent endochondral ossification. After 8 weeks a PBMC injection was given for a further 4 weeks (Fig. S3). A similar percentage of circulating human $CD45^+$ cells were detected in the blood of the humanised animals, without a difference in the animals that received the grafts, and similar levels of spleen human immune engraftment were observed (Fig. S4A and 4C). Similar CD4/CD8 ratios were observed in the blood and spleens of both of the humanised groups, with a higher engraftment of the human $CD4^+$ T cells (Fig. S4B and 4D).

In order to clarify the interactions of the immune system with the mature bone grafts, human specific immunohistochemistry was performed to detect allogeneic T cells. The results revealed $CD3^+$ human cells throughout the marrow of the bone grafts in the animals that received a PBMC injection, in contrast with the non-humanised mice (Fig. 5A). When further analysing the $CD3^+$ allogeneic human T cells subsets, both $CD4^+$ and $CD8^+$ were detected within the graft-derived marrow of the humanised animals, without a predominance of one or another in a certain region.

Gene expression (Fig. 5B), further supported human T cell presence in the grafts of animals that received a PBMC injection. In order to look into specific T cell processes, we analysed a panel of genes associated with T cell functionality. Both CD4 and CD8 expression was detected in the grafts of animals that received a humanisation. Cytotoxic-related proteins, such as perforin (PRF1) and granzyme B (GZMB) were also expressed in these samples. Expression of early (CD69) and intermediate (CD25) T cell activation markers were detected close to the detection threshold, whereas varying levels of regulatory-associated genes, such as FoxP3 and IDO were present in the grafts of the humanised animals. Altogether, these results indicate the presence of T cells with cytotoxic and regulatory function. Flow cytometry results supported the presence of $CD45^+$ human cells within the grafts of the humanised group (Fig. 5C).

3.6. Allogeneic bone derived from ch-pMSCs persists after 4 weeks

μCT analysis revealed the presence of ectopic mineralised tissue at both 8 weeks (before the start of the humanisation) and 12 weeks (4 weeks post-humanisation) (Fig. 6A). Quantification of the μCT data showed no significant differences in the mineralised tissue volume in the grafts regardless of the humanisation (Graft only versus Graft + PBMCs) at either of the timepoints (Fig. 6B), with no decrease in bone volume between pre and post humanisation. The retrieval of the grafts revealed a structure comprised of an outer bone ring, a bone marrow and some remains of calcified cartilage (Fig. 6C and D). Blood vessels were also observed, as identified by the presence of erythrocytes within a lumen. The marrow of the samples showed a variety of structures, ranging from more adipose-like marrows to more cellular ones. No significant

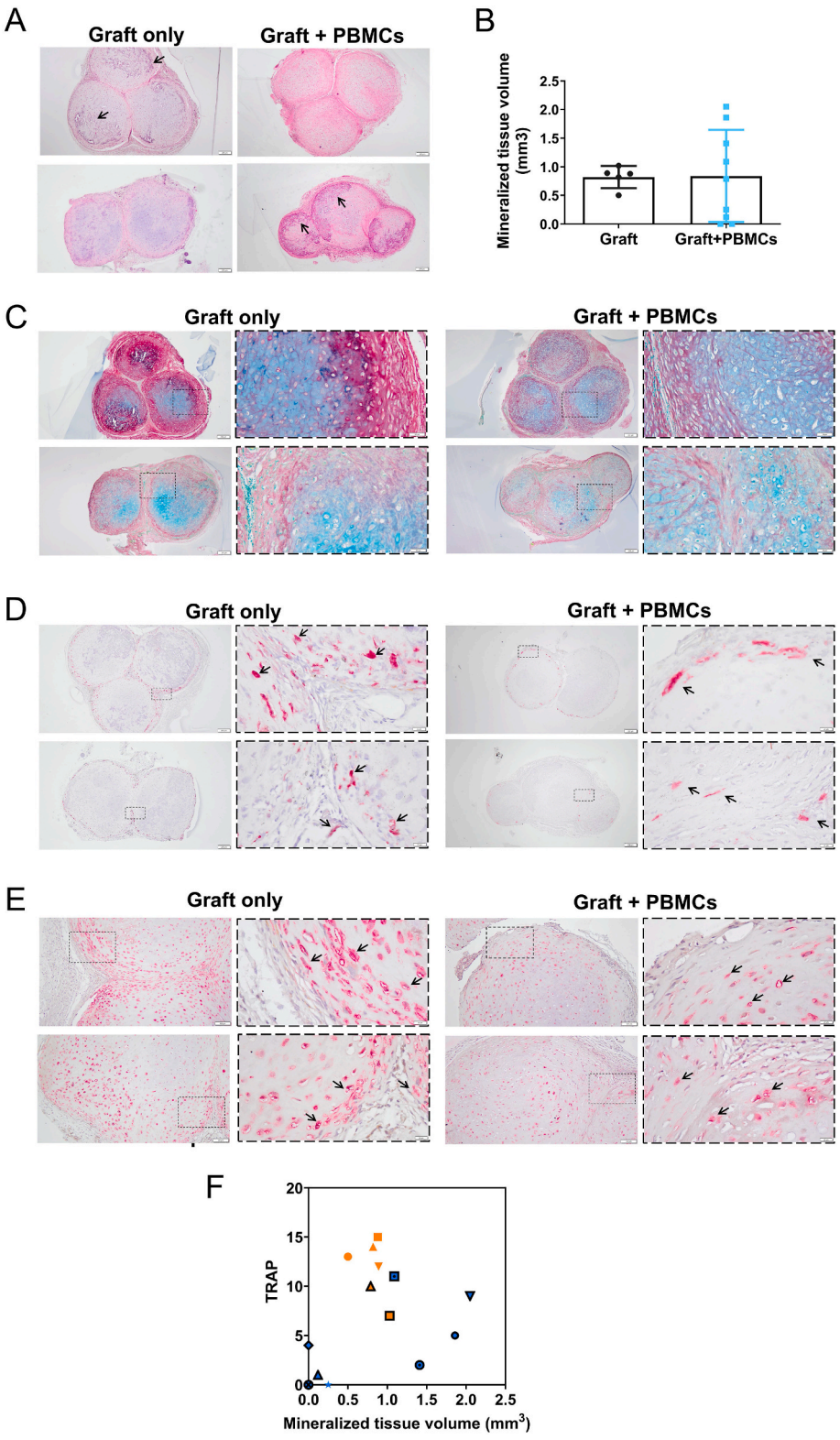

Fig. 2. Analysis of the potential of ch-pMSCs to persist *in vivo* in the presence of a pre-existing allogeneic human immune system. (A) Hematoxylin and eosin staining of ch-pMSC derived grafts implanted for 3 weeks in 3 week-humanised mice. Arrows indicate areas of active matrix mineralisation and remodeling. **(B)** Quantification of the μCT scan data showing mineralisation (mm3, n = 5–9 grafts per group). A linear mixed model with Bonferroni post-correction was used for this analysis. **(C)** Picrosirius Red, fast Green, and alcian Blue (RGB) staining and **(D)** TRAP histochemical staining. Arrows indicate areas of positive staining. **(E)** Human GAPDH histochemical staining. **(F)** Spearman's correlation analysis of the TRAP + cells per sample versus the bone volumes (R = 0.31). Each symbol represents an individual sample. Graft only samples are indicated in orange and Graft + PBMC samples in blue. No correlation was observed.

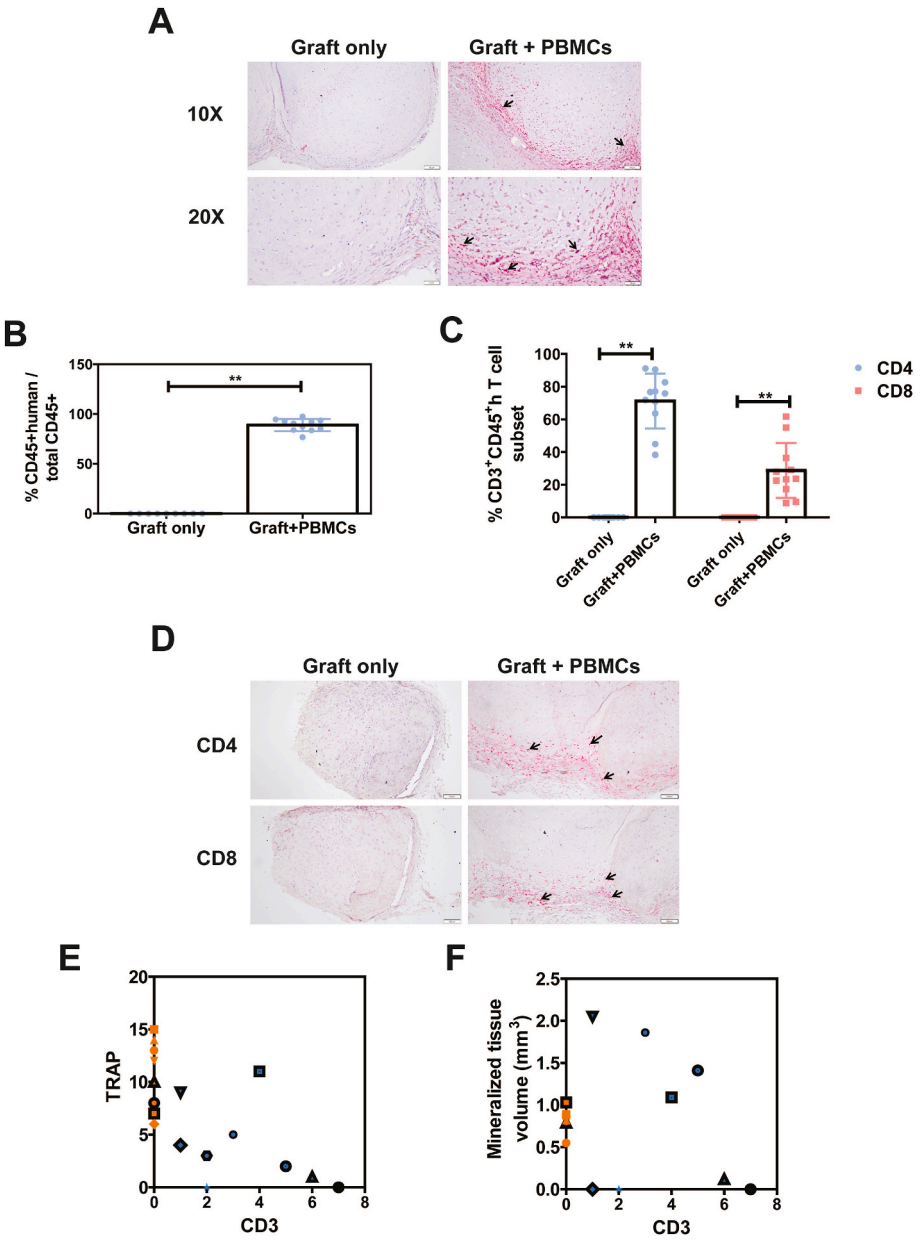

Fig. 3. Allogeneic human T cells are detected in the grafts after 3 weeks of implantation in a 6- week humanised animal model. (A) Immunohistological staining of human CD3 indicates their presence in 3-week ch-pMSC grafts. Arrows point out areas of positive staining. Flow cytometric analysis of the ratio of $CD45^+$ human cells **(B)** and the $CD4^+$ versus $CD8^+$ human T cells **(C)** in the 3 week grafts. $^{**}P < 0.01$, linear mixed model with Bonferroni post-correction. **(D)** Immunohistochemical staining of human $CD4^+$ and $CD8^+$ T cells. Arrows indicate positive staining. **(E)** Spearman's correlation of the TRAP + cells in the 3-week grafts versus the $CD3^+$ human T cells, and the bone volumes versus the $CD3^+$ human T cells **(F)**. Each symbol represents an individual sample. Graft only samples are indicated in orange and Graft + PBMC samples in blue.

structural differences of the grafts or marrows were observed between the animals that received a PBMC injection versus the graft only controls. TRAP staining demonstrated the presence of osteoclasts lining the remnants of the calcified cartilage and newly formed bone tissue in both conditions, with no clear difference in number or location observed between conditions (Fig. 6E). A human-specific GAPDH immunohistochemical staining revealed the presence of human (donor-derived) cells after 12 weeks in graft samples from both animals that received PBMCs and in the ones that only received the graft (Fig. 6F), both in the edges of the newly-formed bone and in the bone marrow. In addition, human $GAPDH^+$ cells were observed in remnants of cartilage matrix.

3.7. No significant immune responses against mature ch-pMSC-derived bone grafts after 6 weeks

Due to the risk of graft versus host disease after 5–6 weeks of humanisation, which prevented long-term graft survival experiments, we employed a re-transplantation model. Hence, a second group of recipient mice, that were humanised for 2 weeks, received mature 12-week grafts (Figure S5). During the 4 weeks prior to the re-transplantation, the donor mice had been humanised with the same allogeneic PBMC donor as the recipients, and the grafts contained allogeneic T cells. Humanisation was assessed in the blood and spleens of the donors (Figure S6), showing human immune engraftment after 4 weeks. We observed no difference in the levels of human immune cells in the blood of the humanised recipients that received grafts coming from

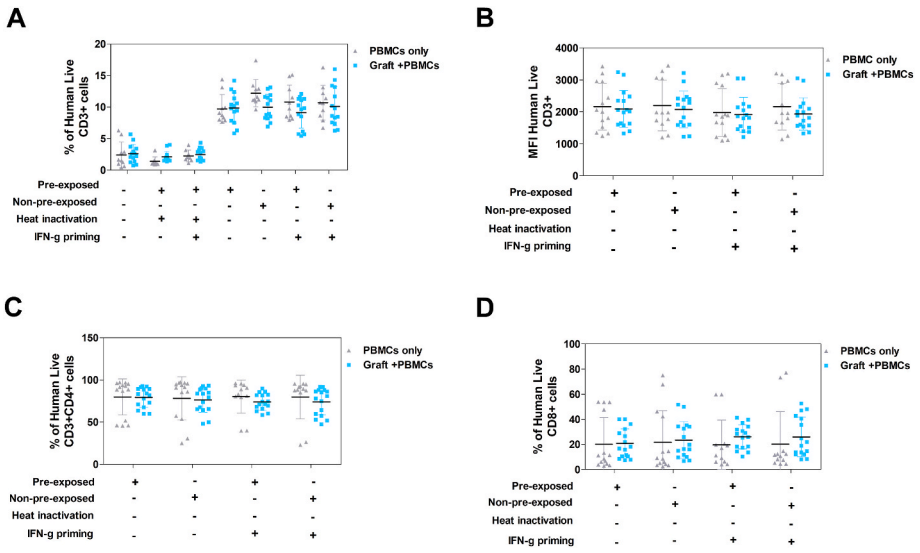

Fig. 4. pMSCs do not elicit proliferation from pre-sensitised splenocytes regardless of the degree of mismatch. Flow cytometric analyses of graft-matched pre-exposed and mismatched (non-pre-exposed) pMSCs in co culture with splenocytes from humanised mice for 5 days revealed that pMSCs promote allogeneic T cell survival (% live cells) (A). pMSCs or IFN-g primed pMSCs did not trigger a significant increase of the proliferation of $CD3^+$, as measured by the MFI of CFSE **(B)**, nor changes in the percentages of live $CD4^+$ **(C)**, and $CD8^+$ **(D)** human T cells. N = 12–16 samples per group. Data was analysed using a linear mixed model with Bonferroni post-correction.

humanised animals (H to H) compared with the ones that received non-humanised grafts (KO to H) (Fig. 7A). A significant increase in the ratio of $CD8^+$ cells versus $CD4^+$ cells was found in the in the H to H group in comparison to the KO to H group (Fig. 7B and C).

When looking at the presence of the immune cells in the grafts, we reported a higher infiltration of $CD45^+$ human cells in the non-humanised animals that received a humanised graft (H to KO) in comparison with the humanised animals that received a humanised graft (H to H) (Fig. 7D). However, similar percentages of $CD4^+$ and $CD8^+$ human immune cells were found in both of the groups (Fig. 7E and F). These results were further confirmed by immunohistochemical staining for human CD3, CD4 and CD8 (Fig. 7G).

3.8. pMSC-derived bone grafts persist in the long-term in an immune allogeneic milieu

Histological analysis of the retrieved grafts at 14 weeks revealed a similar structure to the previous study from 12 weeks (Fig. 8A), showing a bone marrow and an outer bone ring. A quantification analysis of the grafts by μCT at 12 weeks in the donor group and at 14 weeks in the recipients showed no significant variations in terms of the bone volumes for in any of the groups (Fig. 8B and C), with a tendency towards an increase in the mineralised bone volume observed in all groups, suggesting the absence of an allogeneic immune response interfering with bone formation. Human $GAPDH^+$ cells were detected in the bone structures of all the groups, indicating a sustained role for the donor (human) cells in the process of bone formation and remodeling even in the later stages of endochondral ossification (Fig. 8A). Human cells were only detected in the marrow of those constructs in which animals also received PBMCs.

4. Discussion

In this study we have investigated the ability of chondrogenically primed pediatric MSCs to form bone via endochondral ossification and to maintain that bone in the long term in the presence of an allogeneic humanised immune system. We demonstrate that, despite the presence of allogeneic T cells, these processes are not negatively impacted. These results can have implications for the development of *off-the-shelf* allogeneic cell therapies aimed at large bone defect repair via MSC mediated endochondral ossification.

We previously described the immunomodulatory abilities of undifferentiated pMSCs to modulate the allogeneic B and T cell immune responses under inflammatory conditions [8]. However, a number of studies have reported a loss of the MSC immunoprivileged status when these cells are chondrogenically differentiated [18,19]. In contrast, other studies have stated an absence of MSC-derived immunogenicity upon T cells after chondrogenesis [20]. Here, we show that chondrogenically differentiated pMSCs retain their immunoprivileged status and form and maintain bone in the presence of an allogeneic immune system. During the process of bone repair, a number of immune cells are recruited to the injury site. The adaptive immune response, comprised of B and T cells, plays an important role in the modulation of bone formation through the production of pro-inflammatory cytokines. *Dighe* et al. reported an inhibition of bone formation in allogeneic MSC-derived implants modulated by a T helper 1-mediated response by producing IFN-γ [31]. Moreover, the presence of IFN-γ and TNF-α is known to impair bone repair, acting as MSC apoptosis mediators [24]. Here, we show that chondrogenically differentiated pMSCs are non-immunogenic towards $CD4^+$ and $CD8^+$ T cells in the presence of IFN-γ and TNF-α, and exhibit similar immunomodulatory abilities in the absence of inflammatory stimuli. Hence, we hypothesised that, even under the inflammatory conditions that can accompany an injury during bone repair or a surgical procedure, allogeneic chondrogenically differentiated pMSCs are able to retain their immune privileged status.

To determine the ability of ch-pMSCs to form and maintain bone *in vivo* we performed three distinct studies. First, we investigated the early immune reaction to chondrogenically primed grafts actively remodeling for 3 weeks. At this stage the grafts became invaded with host vasculature and began to form the marrow cavity. Despite this active phase in the bone formation, where most cells are still of human origin, we did not observe a significant difference in the quantity of mineralised tissue or the phenotype of the retrieved grafts following 3 weeks in animals. To assess the sensitivity of the allogeneic immune system towards possible pMSC-derived immune responses, we studied the behaviour of pre-sensitised T cells of humanised animals that had received the grafts towards pMSCs to which they had been pre-exposed, or third party pMSCs. Crucially, we found no significant immune responses in terms of the T

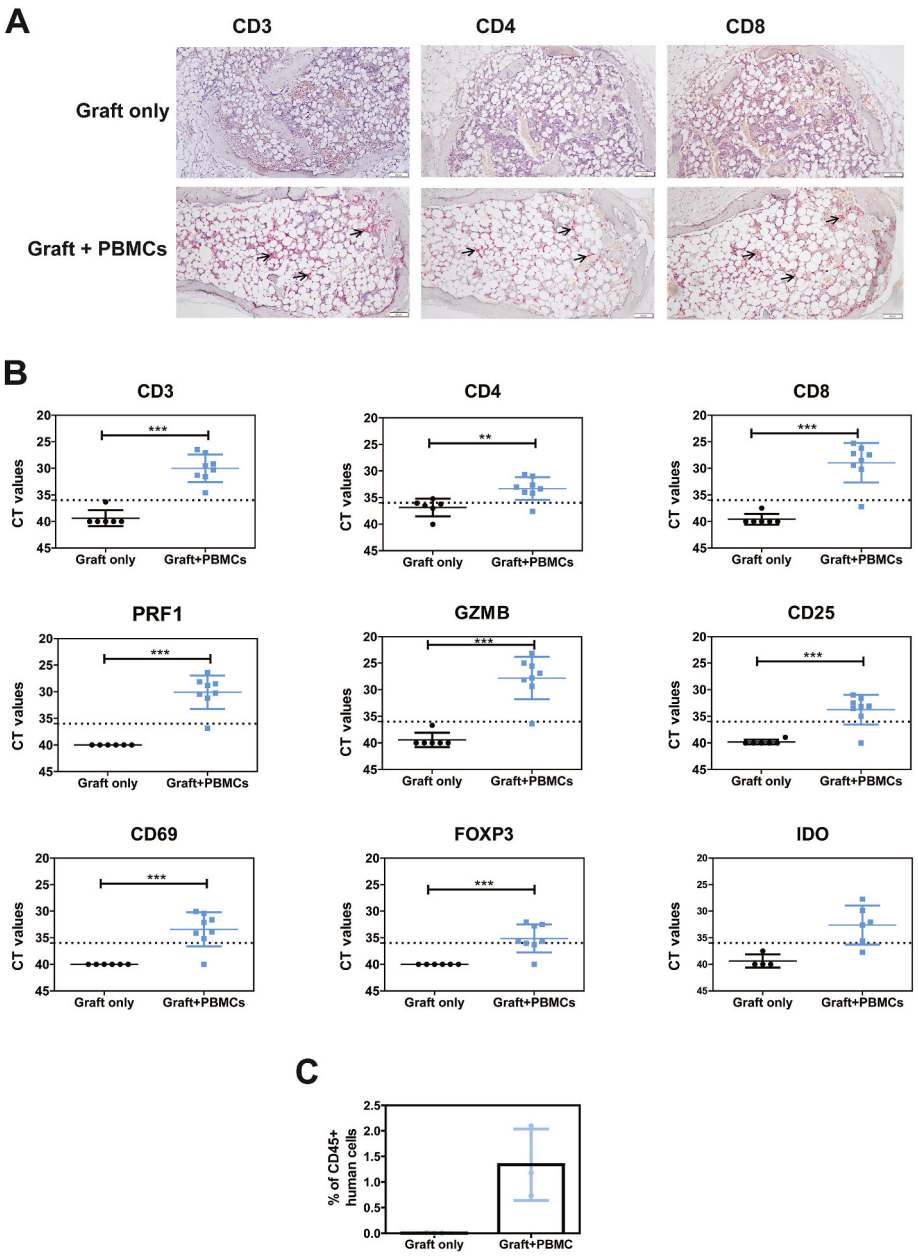

Fig. 5. Allogeneic human T cells and T-cell related markers were detected in the bone grafts after 12 weeks. An immunohistochemical staining of human CD3, CD4 and CD8 as indicated by the black arrows **(A)** revealed the presence of allogeneic T cells in the 12-week grafts of the humanised animals (n = 8–10 per group). **(B)** The gene expression of human CD3, CD4,CD8, perforin, granzyme, CD25, CD69, FOXP3 and IDO was analysed (n = 6–8 per group). A flow cytometric analysis of the grafts showed the presence of $CD45^{+}$ human and mouse cells **(C)** (n = 3). Data was analysed using a linear mixed model with Bonferroni post-correction, $^{**}P < 0.01$, $^{***}P < 0.001$.

cell proliferation of splenocytes co-cultured with pMSCs compared to the splenocytes on their own. This would suggest that pre-sensitised splenocytes of animals that received the grafts would not be more prone to trigger an allogeneic immune response to a secondary exposure of pMSCs, regardless of their degree of mismatch. When it comes to evaluating the stability of an allograft, in particular bone grafts, it is important to take into account the possibility of the need of a second graft within the same patient. In line with our previous hypotheses, it is likely that the allo-recognition of the effector T cells decreases over time due to a decrease in the presence of human pMSC-derived chondrocytes. This could mean that, with this model, allograft-responsive memory T cells might not develop and suggests that this type of response is not likely to occur in the long term even upon receiving a second pMSC-derived bone graft. This would appear to indicate a degree of immune privilege conferred to bone forming ch-pMSCs in the early stages of endochondral ossification.

Subsequently, we implanted ch-pMSC grafts in immunodeficient animals for 8 weeks, followed by humanisation of the recipient mice for further 4 weeks. We aimed to determine whether mature bone, comprised of both human and mouse cells, could persist in the presence of a humanised immune system. We observed complete bone formation via endochondral ossification process in all conditions. Despite detectable levels of genes important in the cytotoxic T cell response within the grafts, no damage to the newly formed bone was observed, nor was there any reduction in the mineralised tissue volume. This indicated to us that up to 4 weeks exposure to an allogeneic immune system was not detrimental to the survival and maturation of newly formed human MSC derived bone. However, we did observe the presence of a significant

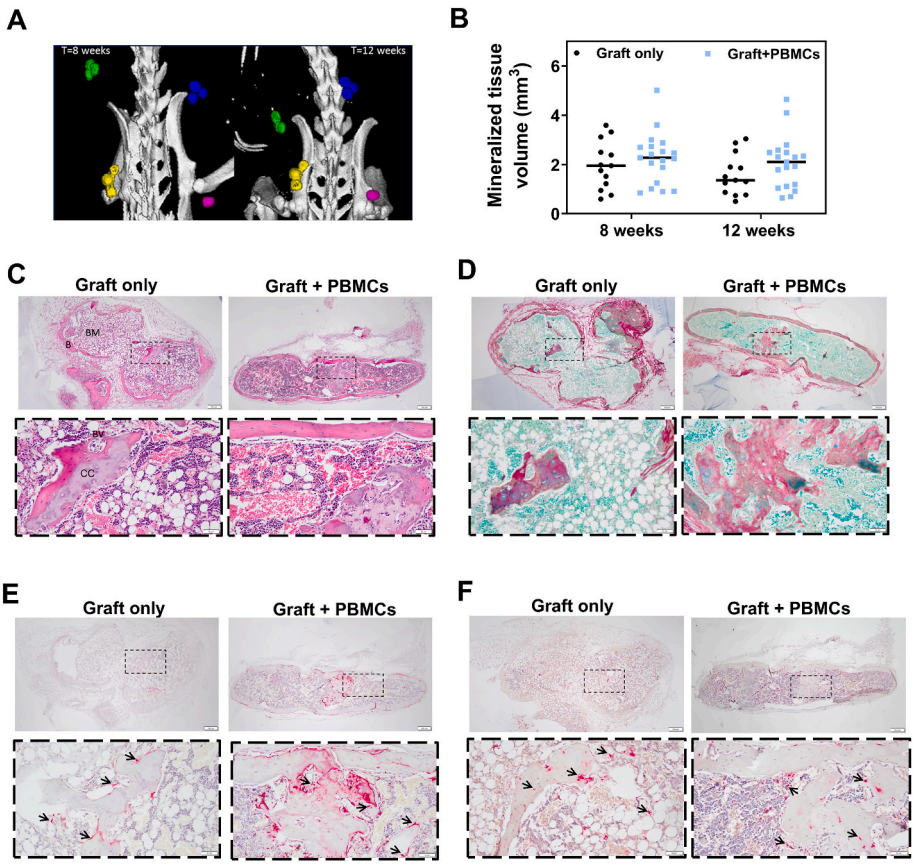

Fig. 6. ch-pMSC-derived bone formation occurs in an allogeneic immune system after 12 weeks. (A) Representative μCT scans of the mineralised grafts at 8 weeks and 12 weeks. Each color represents an individual graft. **(B)** μCT scan quantification of the mineralised tissue volume at week 8 (before humanisation) and week 12 (4 weeks after humanisation) (n = 12–19 grafts per group). A two way ANOVA was used for the statistical analysis. **(C)** Representative H&E staining of the 12-week grafts. A bone (B) ring surrounds a bone marrow (BM) cavity with remaining calcified cartilage (CC) and Blood Vessels (BV). Magnified parts show a higher detail of these structures. **(D)** Representative RGB staining of the 12-week grafts and (E) TRAP histochemical staining. (F) A human GAPDH staining reveals the presence of human cells in the grafts after 12 weeks. Arrows indicate exemplary areas of positive staining (n = 7–10 samples per group)

number of CD4 and CD8 T cells within the newly formed marrow. This led us to question whether there were was a negative immune reaction to the bone beginning to take place.

Therefore, in our third *in vivo* study we wanted to confirm that these newly formed bone constructs could persist even following a further challenge. We serially transplanted our newly formed bone grafts into new recipients with two aims: firstly, if the immune cells within the grafts would attack the graft given more time to do so and secondly, whether a local or systemic immune response to these grafts would take place upon a secondary humanisation with the same PBMC donor. Interestingly, we observed fewer human cells in the grafts of the animals that received PBMCs (H to H) compared to those that did not (H to KO), and bone formation was unaffected. This again suggests that ch-pMSCs can form and maintain bone in the presence of an allogeneic immune system. After 6 weeks of an immune reconstitution with the immune system, at week 14 post-implantation, we were still able to detect these mature bone constructs. Interestingly, we did not find a significant variation in the bone volumes of the constructs of the animals that received allogeneic immune cells compared to the ones that did not, nor between any of the groups at 12 or 14 weeks. This suggests that the presence of the allogeneic immune system does not impact the survival and formation of new bone for up to 6 weeks.

A common problem in bone regeneration when using cell-derived allografts is the lack of vascularisation [32], resulting in allograft death due to lack of nutrients and therefore considerably impacting the long-term survival of the grafts. Upon retrieval of the pMSC-derived bone constructs at week 12 in our model, we observed bone-like structures, including a bone lining of human (donor) and mouse (host) cells, a marrow, and abundant blood vessels and sinusoids. Therefore, our approach demonstrated a complete integration of the newly formed bone constructs in the allogeneic host, including *de novo* vascularisation that could be a good indicator for the long-term stability of the grafts, as ensured by a stable blood supply. In our initial study, we detected allogeneic T cell presence in the edges of the graft after 3 weeks of implantation, and in the later timepoints, at 12 and 14 weeks post-implantation, we observed human $CD3^+$ T cells in the newly formed bone marrow of the bone graft. Earlier studies have shown infiltration of $CD8^+$ T cells when subcutaneously implanting MSC grafts in allogeneic mice after 2 weeks [15]. Another study showed that implantation from MSCs for bone formation purposes led to transplant rejection when using xenogeneic models [23]. Hence, it has been suggested that MSCs lose their immunosuppressive abilities in an *in vivo* mismatched setting, preventing bone formation [33]. However, despite observing T cell presence in the grafts, we did not observe any structural differences nor alterations in the quality of the bone constructs retrieved from the humanised animals compared to the non-humanised controls at either of the analysed timepoints. A pro-inflammatory response, marked by higher numbers of $CD8^+$ T cells, has been previously described to play a predominant role in the initial stages upon allograft transplantation, leading to graft rejection after only 3 weeks [34]. Nevertheless, we did not observe any structural alterations that could suggest rejection of the grafts after 4 and 6 weeks of humanisation, indicating that they could successfully persist in the long term without evidence of significant T-cell derived immune rejection.

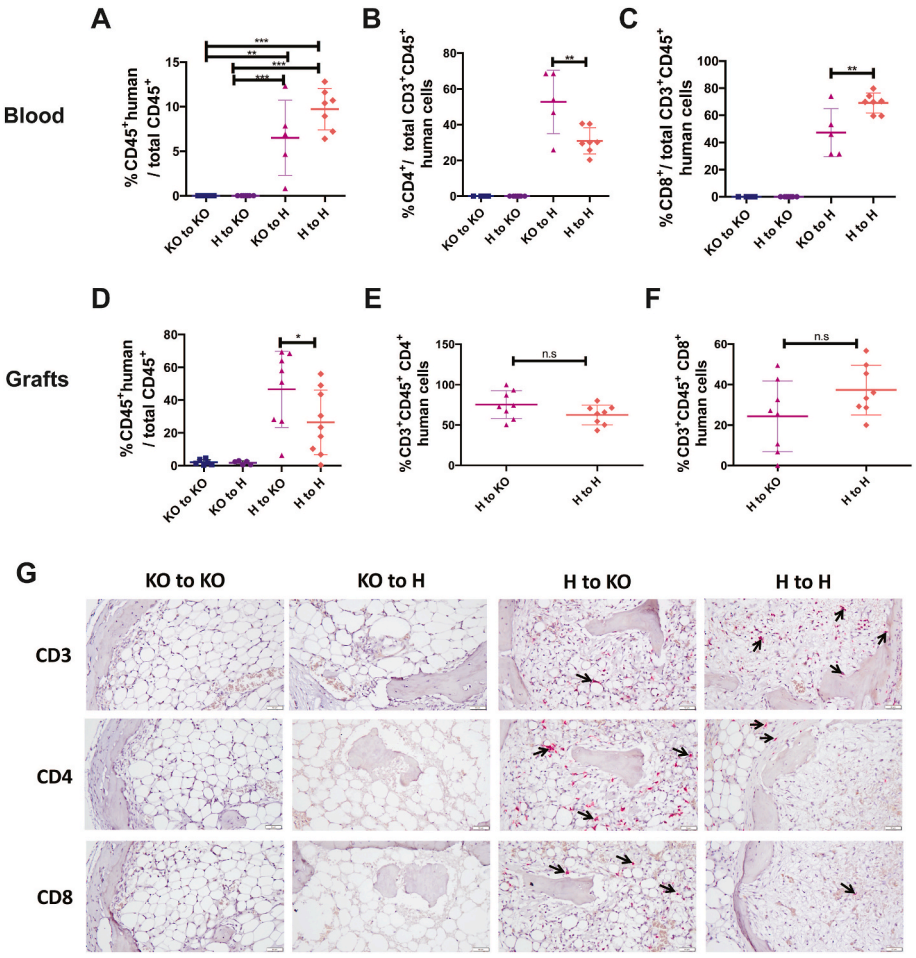

Fig. 7. No significant graft-directed allogeneic immune responses are detected in the long term. 12-week grafts from 4-week humanised animals were serially transplanted in 2-week humanised animals for a further 2 weeks. The percentage of circulating $CD45^+$ **(A)**, $CD4^+$ **(B)** and $CD8^+$ **(C)** human T cells was quantified in the blood of the recipient animals, as well as the presence of human $CD45^+$ **(D)**, human $CD4^+$ **(E)** and human $CD8^+$ **(F)** cells in the grafts by flow cytometry, and correlated by immunohiostochemical stainings **(G)**. Different groups of recipient animals were analysed: immunodeficient recipients that received grafts from immunodeficient donors (KO to KO), immunodeficient recipients that received grafts from humanised donors (H to KO), humanised recipients that received grafts from immunodeficient donors (KO to H), and humanised recipients that received grafts from humanised donors (H to H). N = 5–9 animals per group, or 5–9 grafts per group. Data was analysed using a linear mixed model with Bonferroni post-correction, *P < 0.05 **P < 0.01, ***P < 0.001, n.s = non significant.

Endochondral bone formation is a process that relies on the conversion of a cartilage template into bone, where hypertrophic chondrocytes are known to produce a variety of factors that mediate the recruitment of cells from the host, promoting bone formation [2]. In our studies, we observed a small number of human cells still present in the allogeneic bone at week 12 post-implantation. In comparison to the overall cell number in the graft, donor-derived human pMSC numbers decrease as a consequence of the endochondral ossification and remodeling process. Therefore, it is likely that eventually a majority of host cells will replace the bone structure. Furthermore, those that are still present are potentially shielded from immune rejection by the dense bony matrix in which they reside. Zhou et al. and others have previously showed that chondrocytes can transdifferentiate into osteoblasts *in situ* [35]. Since MSC-derived osteogenic cells have been proven to retain similar immunomodulatory abilities to MSC-derived chondrocytes *in vitro,* particularly by suppressing T cell proliferation [36], it is possible that these donor cells that remain in the newly-formed bone will turn into osteoblasts eliciting no further reaction from the immune system. Taken together, these facts would suggest that a strong immune reaction at an even later stage is also quite unlikely. One area of interest that we have not focused on in this study is the crucial role the innate immune system plays in bone formation and repair. Future studies should also focus on assessing the response of innate immune cells in a transplanted allogeneic tissue engineered construct setting.

A drawback of our study is that the use of an immunodeficient mouse model engrafted with human cells such as this one is known to lead to xenogeneic graft-versus-host disease (GvHD), due to the presence of the host innate immune fraction [37]. Hence, this poses a limitation when it comes to assessing the stability of the allogeneic bone constructs in the longer-term. In addition, despite the fact that the choice of a double knockout in *Rag2* and *IL2Rγ* is known to improve human immune engraftment upon PBMC injection over other models, a poor engraftment of the human innate immune fraction, as well as the mature B cells [38], and consequently a limited antibody-mediated response [39] is known to occur. Hence, this humanised model might only provide a partial understanding of the allogeneic immune responses, mostly focused on the T cell activity. New humanised models that facilitate a decrease or elimination of the mouse innate response, as well as enhance the production of human-specific cytokines that might be important for the recruitment and development of other immune cell subsets, such as cells from the innate immune response [40], might provide an even more complete picture of the allogeneic immune responses in the complex process of bone regeneration.

The use of potent paediatric MSCs as an allogeneic source of cells represents a promising approach to the treatment of aged multi-morbid patients. However, it also has its challenges with regard to the regulation

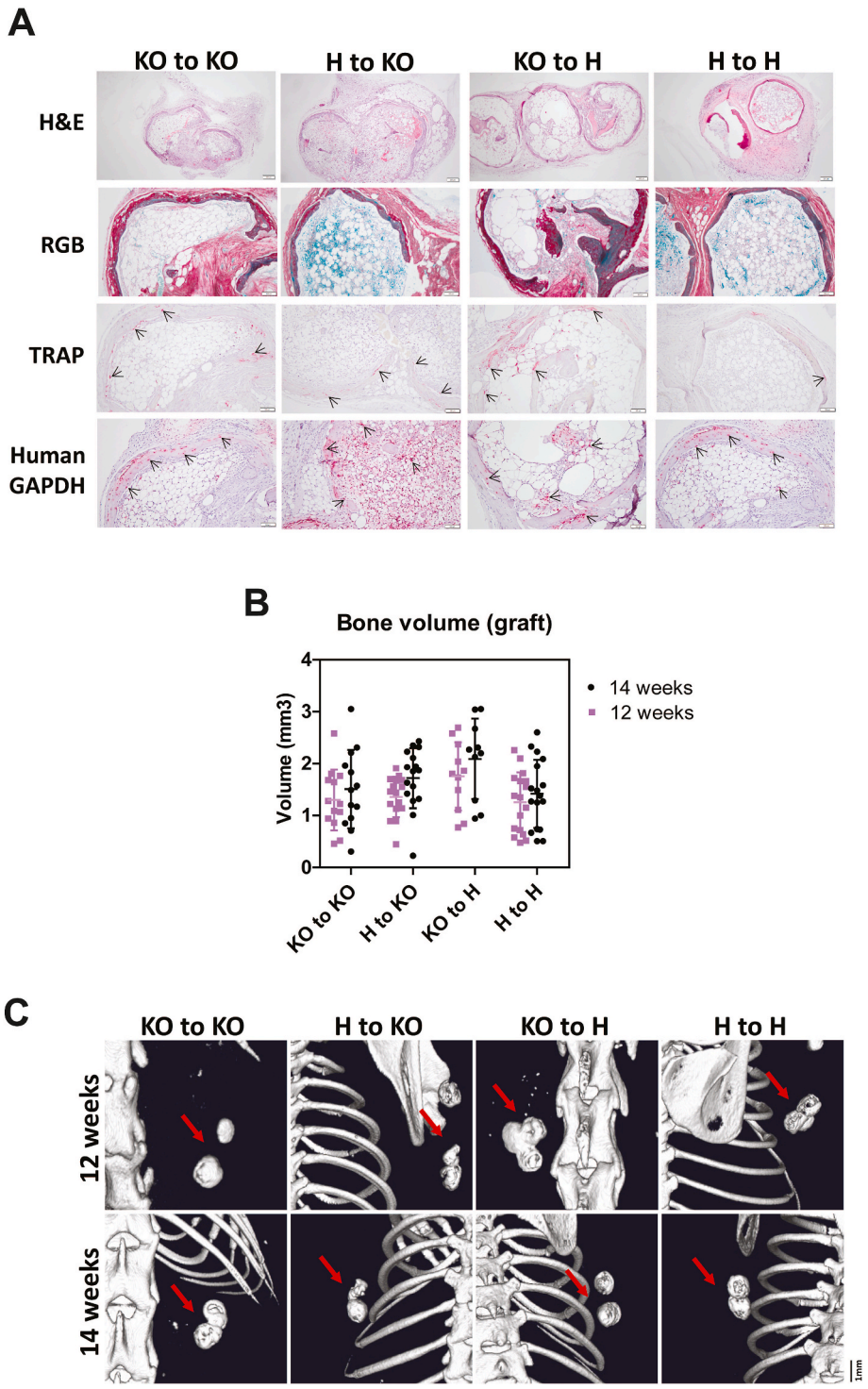

Fig. 8. Mature allogeneic bone persists in the recipient animals. (A) Representative images of H&E, RGB, TRAP and human GAPDH histological stainings showing the structure and the human GAPDH presence in the 12-week grafts from 4-week humanised animals that were re-transplanted in 2-week humanised animals for 2 more weeks. **(B)** Quantification of the bone volumes of the grafts by μCT scans, analysed with a two-way ANOVA statistical test. Groups included: immunodeficient recipients that received grafts from immunodeficient donors (KO to KO), immunodeficient recipients that received grafts from humanised donors (H to KO), humanised recipients that received grafts from immunodeficient donors (KO to H), and humanised recipients that received grafts from humanised donors (H to H). N = 5–9 animals per group, or 13–17 grafts per group. (C) 3D reconstruction of μCT scans of representative constructs from each experimental group at 12 weeks and at 14 weeks following 2 weeks in the second recipient mice.

of such an approach. In order to scale up such an approach, several hurdles will need to be overcome, including the expansion and priming of cells in a GMP-compliant and cost effective manner and also with approval from parents/guardians and medical ethical committees. While these are not trivial, the fact that these cells have excellent expansion potential and would otherwise be discarded, certainly lowers the bar with regard to feasibility of such an approach. In previous work, we have investigated the differences between adult and paediatric MSCs and showed that pMSCs are more reproducible with regard to multi-lineage differentiation potential and could proliferate twice as fast as

their adult counterparts [7]. They also contained less senescent cells but had a similar cell surface marker expression, with the exception of CD271, which was significantly lower in our expanded pMSCs. As we and others showed (discussed in Ref. [7]), the general consensus is that cells isolated from younger donors (children vs adult) are more potent and thus represent a better source fur use in cell therapy based applications.

In conclusion, we show for the first time that human chondrogenically primed pMSCs can generate bone constructs after 8 weeks, that then persist for up to 6 more weeks in the presence of an allogeneic immune system without significant immune responses. Additionally, we have developed a pre-clinical model that can be used to study the role of allogeneic T cell responses in bone regeneration. This study offers a promising approach to bone repair by using a potent allogeneic source of non-immunogenic MSCs.

CRediT authorship contribution statement

Virginia Palomares Cabeza: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Niamh Fahy:** Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Caoimhe H. Kiernan:** Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Andrea Lolli:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Janneke Witte-Bouma:** Data curation, Methodology, Writing – original draft, Writing – review & editing. **Shorouk Fahmy Garcia:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Ana Merino:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Nicole Kops:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Yanto Ridwan:** Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Eppo B. Wolvius:** Resources, Supervision, Writing – original draft, Writing – review & editing. **Pieter A.J. Brama:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **Martin J. Hoogduijn:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. **Eric Farrell:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Eric Farrell reports financial support was provided by AO Foundation.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2024.122471.

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