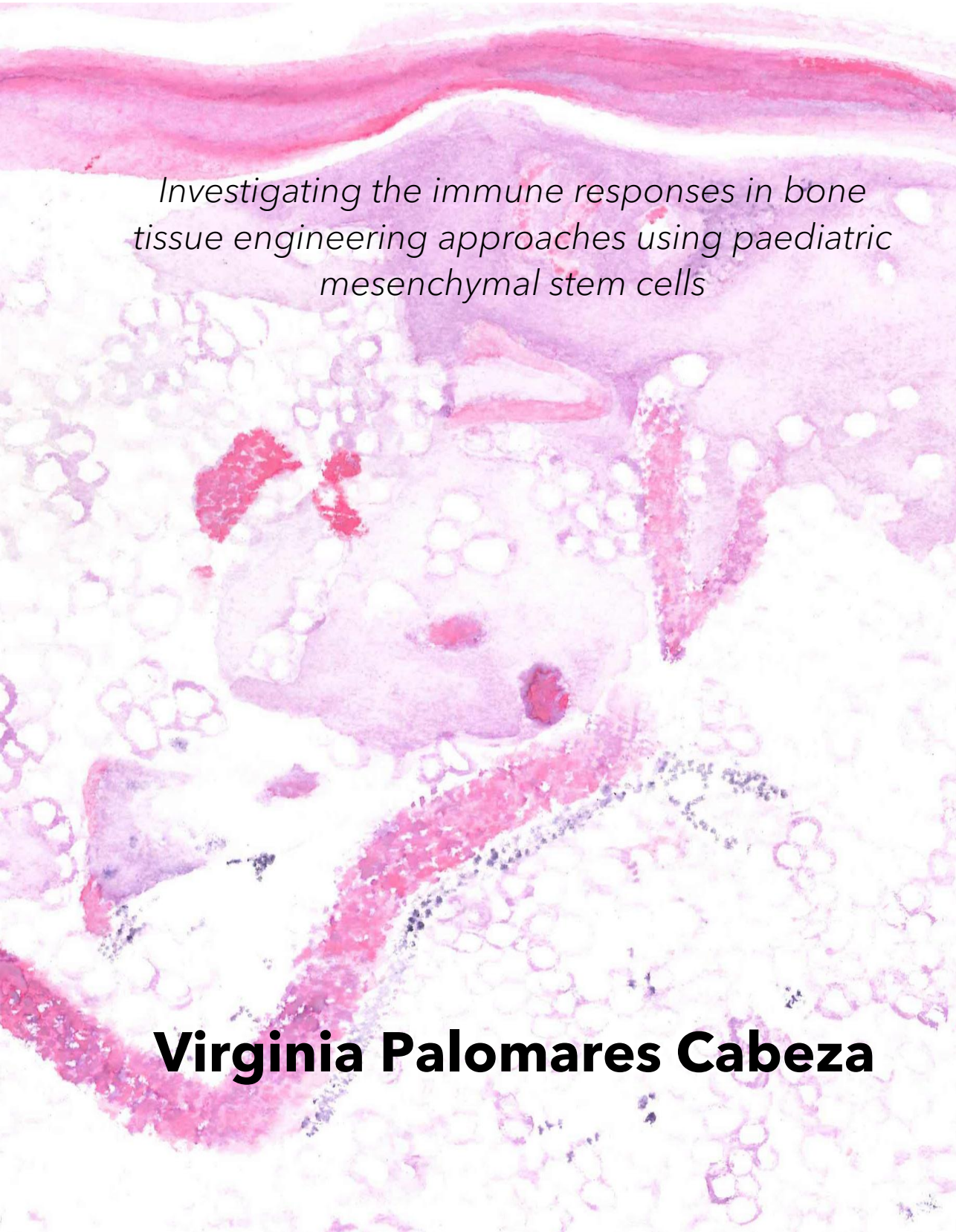
DOWN TO THE BONE

Investigating the immune responses in bone tissue engineering approaches using paediatric mesenchymal stem cells



Virginia Palomares Cabeza

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Investigating the Immune Responses In Bone Tissue Engineering Approaches Using Paediatric Mesenchymal Stem Cells

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Colophon

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Down To The Bone

Investigating the immune responses in bone tissue engineering approaches using paediatric mesenchymal stem cells

Tot op het bot

Het onderzoeken van de immuunresponses van botweefselmanipulatie door pediatrische mesenchymale stromale cellen

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
rector magnificus

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and in accordance with the decision of the Doctorate Board.
The public defence shall be held on

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Copromotors:	Dr. E.J. Farrell Dr. M.J. Hoogduijn

"I hope you know
Between the shots
Of poison,
Indifference
And rage
That were darted at it,
Underneath the shadow
Of fear
That darkens everything
Behind the mist
Of doubt
And regret
Which seems to fill it all,
Your heart is still beating.
Pure,
Soft
And Loving.
Unconditional.
As it once was,
As it will ever be."

"So into the woods you go again
You have to every now and then
Into the woods, no telling when
Be ready for the journey"
Stephen Sondheim.

A mi familia, que me ha dado alas para volar en este mundo.

To my family, who's given me my wings to soar across this world.

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1

General Introduction

BONE DEVELOPMENT AND BONE HEALING

Bone is a highly specialized and crucial tissue for supporting and protecting our body. In the natural process of bone development, mesenchymal stem cells, also known as marrow stromal cells (MSCs) that are recruited by cytokines and growth factors to the site of bone formation play a key role in the generation of new bone [1].

MSCs can differentiate either osteogenically (intramembranous ossification) or chondrogenically (endochondral ossification)[2]. In the intramembranous ossification route, MSCs directly differentiate into osteoblasts, a cell type that lays down the new bone [3]. In the endochondral ossification pathway, however, an intermediate cartilage template is produced before bone formation takes place (Figure 1). MSCs condense and undergo chondrogenic differentiation, turning into chondrocytes that initially proliferate and form cartilage [4]. At a later stage, they become hypertrophic and secrete a mineralised matrix, that is invaded by blood vessels recruited by the release of pro-angiogenic factors[5]. Along with blood vessels, osteoblasts are recruited to the bone formation site. A part of the hypertrophic chondrocytes become apoptotic, and the cartilage matrix serves as a scaffold for the invading osteoblasts to create *de novo* bone[6]. The remaining matrix is then degraded by monocyte-derived osteoclasts, responsible for bone resorption, which are recruited through the blood supply. Another part of the hypertrophic chondrocytes transdifferentiates directly into osteoblasts [7].

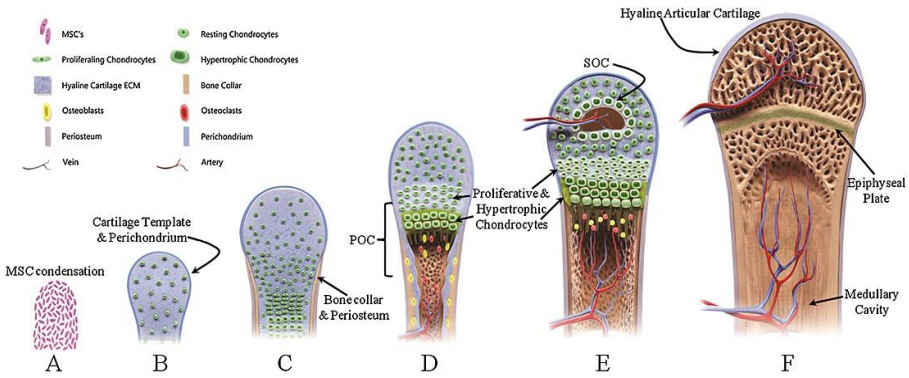

Figure 1. The process of endochondral ossification. Bone formation through the endochondral ossification process relies on an intermediate cartilage template generated by mesenchymal stem cell (MSCs)-derived chondrocytes that is eventually replaced by bone. *Thompson, E. M., Matsiko, A., Farrell, E., Kelly, D. J. & O'Brien, F. J. Recapitulating endochondral ossification: a promising route to in vivo bone regeneration*. J. Tissue Eng. Regen. Med. **9**, 889–902 (2015).

Under normal circumstances, bone has the ability to renew itself upon damage, following the endochondral ossification pathway in a similar process to bone development [8]. During bone repair, initial tissue damage marks the beginning of the inflammatory phase, in which the vascular supply is disrupted. A haematoma then forms, causing the production of specific bone growth factors that recruit mesenchymal stem cells to the injury site, as well as other pro-angiogenic factors that promote re-vascularisation [9]. From then on, a cartilage template is created and eventually replaced by new bone tissue via the endochondral ossification process described above [10]. During the whole process of bone repair the immune system plays an important role in the different stages, from the pro-inflammatory phase until the last phase of cartilage conversion into bone [11].

THE INTERPLAY BETWEEN THE SKELETAL AND THE IMMUNE SYSTEM

The immune system: a brief introduction

The immune system is a complex and intricate network that controls the destruction of invading pathogens and toxins through the development of immune responses. These can be carried out by the innate immune system, composed of monocytes, dendritic cells, macrophages, neutrophils and natural killer (NK) cells, which are the initial line of defense; and the adaptive immune system, which comprises the T and the B lymphocytes and is responsible for enabling immunological memory for a faster and more specific response to a certain pathogen [12]. This process takes place through the presentation of a foreign molecule, or antigen, carried out by antigen presenting cells (APCs), such as monocytes and

dendritic cells. These cells can process and present the antigen to the T cells via their Major Histocompatibility Complex (MHC) molecules [13]. The antigen-MHC complex is recognized by the T cells and leads to their activation. In order for this process to occur, three signals are required (Figure 2) [14]. The first signal is provided by the interaction of the T cell receptor (TCR) on the T cell membrane with the antigen bound to the MHC on the surface of the APC. This signal provides T cell activation, but it does not trigger proliferation and differentiation of the T cells. This requires the interaction between co-stimulatory molecules such as CD80 and CD86 on the APC, and the coreceptor CD28 on the T cell surface that leads to signal 2. Lastly, this co-stimulation induces the expression of cytokines, such as interleukin-2 (IL-2) on the T cell, which in turn influences T cell differentiation and proliferation towards a specific T cell subset.

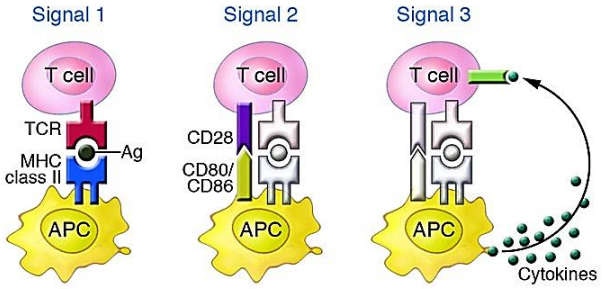

Figure 2. **The three-signal model that leads to T cell activation by APCs.**
Gutcher I. and Becher B., J Clin Invest. 2007;117(5):1119-1127

Upon activation, T cells become effector cells. There are two main subsets of T cells, which are characterised by a distinct phenotype through their membrane expression of either the CD4 or the CD8 co-receptor. $CD8^+$ T cells, also known as cytotoxic lymphocytes (CTLs) or T cytotoxic (Tc) lymphocytes, are responsible for the cytotoxic-mediated responses in viral infections, as well as playing a role in anti-tumour activity. They interact with antigens expressed on the MHC class I molecules of the target cells. Upon activation, $CD8^+$ T cells produce proteins named perforins that create pores in the target cell. Through these pores, granules containing

other proteins named granzymes cause apoptosis of the cell, leading to its destruction [15].

$CD4^+$ T cells are traditionally known as helper T cells (Th), and can be grouped into different subsets according to their cytokine production pattern [16]. $CD4^+$ T cells become activated through the recognition of antigens presented on MHC class II molecules, and their main function is to orchestrate the immune response to activate other immune cells, such as B cells. Classically, $CD4^+$ T cells have been grouped into the Th1 $CD4^+$ T cells, known to produce IL-2 and IFN-γ and control intracellular pathogen infections, and the Th2 $CD4^+$ T cells, which fight extracellular pathogens through the production of IL-4, IL-5, IL-6 and IL-10, that are also involved in B-cell mediated antibody production. However, other $CD4^+$ subsets, such as the Th17 and the regulatory $CD4^+$ T cells also play an important role in the mounting and regulation of the immune responses [17]. Th17 cells are involved in the immune responses against parasites and extracellular bacteria, and a role for them in autoimmunity has also been described. Regulatory $CD4^+$ T cells (Tregs), on the other hand, are responsible for the maintenance of immunological tolerance, by negatively regulating the immune system after the destruction of a pathogen, and in the distinction between self and non-self antigens. Moreover, Tregs are also involved in modulating inflammation during tissue injury [18].

B cells are responsible for the humoral or antibody-mediated branch of the adaptive immune response. B cells can act as APCs through the recognition of antigens via the B Cell Receptor (BCR), also known as surface immunoglobulin M (IgM). Upon antigen recognition, they process and present it on their MHC class II molecules to T cells [19]. In turn, cytokines such as IL-21 are produced by the T cells, stimulating B cell proliferation and differentiation into antibody-producing cells or plasmablasts [20]. The interaction between the co-receptor CD40 on B cells and the CD40 ligand (CD40L) on T cells induces Ig class switching towards the soluble form of IgG, which enables antibody secretion.

The immune system in bone homeostasis and bone healing

The interaction between bone cells and the immune system plays a central role in bone maintenance and healing, and osteoimmunology research has gained relevance in the past years [21] [22].

Bone cells maintain bone homeostasis through cytokines such as TNF-α and IFN-γ. IL-17-α controls bone resorption, whereas IL-1R, IL-4 and IL10 or TGF -β3 promote bone formation.
Since the immune system is responsible for orchestrating the production of these cytokines, it is clear there is an interdependence between the immune and the skeletal system.

The immune system is also involved in the process of bone healing. Upon bone damage, the immune cells that are recruited to the injury site seem to be in control of the differentiation of the osteoprogenitor cells. In this process, both cells from the innate and the adaptive immune response play a role. It has been shown that an impaired macrophage function severely delays endochondral ossification in the process of bone regeneration. T and B cells also play a key role, as proven by their recruitment to the fracture site 3 days post-injury [23]. Moreover, T and B cell depletion has been reported to lead to delayed healing and repair due to impaired osteoblastic maturation in an osteotomy model [24], and these cells have also shown to be in close contact with osteoclasts and osteoblasts during bone repair [25]. $CD8^+$ T cells can impede bone repair through the production of the pro-inflammatory cytokines IFN-γ and TNF-α [26]. On the other hand, a role for $CD4^+$ Treg in regulating osteoclast activity and promoting bone formation has been described [27] [28]. B cells can also control the differentiation of osteoclasts in the injury site, as well as induce their apoptosis [29]

UNRESOLVED BONE HEALING: BONE GRAFTING

Despite bone's intrinsic ability to heal when a fracture occurs, extensive unresolved defects or insufficient bone healing following trauma, surgery

or injury still represents a significant challenge for clinical practitioners [30]. In fact, bone transplantation is one of the most common transplantation procedures in the world, being second after blood [31 32]. The current gold standard for large bone defects is autologous bone grafting, which involves harvesting a piece of bone from a secondary anatomical location of the patient to fill in the defect [33]. However, donor site morbidity [34] and the limited amount of bone that can be obtained through this method are disadvantageous [35].

Alternatively, bone substitutes from human allogeneic sources have been investigated as a possibility for replacement. The most commonly used allogeneic bone grafts are generally obtained from cadavers. The possibilities of a high supply and a tailor-made graft approach for each defect could be an advantage for this approach [36]. However, the risks of immune rejection due to the allogeneic nature of the graft and the transmission of disease raises concerns for this approach. In addition, the ability to recruit cells to promote bone formation on the injury site by the allogeneic grafts seem to be variable and highly dependent on the donor and the processing method, which can compromise its stability [37]. Artificially-produced or synthetic bone grafts composed of calcium, silicon or aluminum have also been under investigation for these purposes [38 39]. Nevertheless, a major disadvantage of these materials is that they do not induce new bone formation.

The lack of a satisfactory bone graft option that satisfies all the requirements has brought regenerative medicine to investigate the use of tissue-engineered grafts, that mimic the natural-occurring process of bone formation by mesenchymal stem cells during bone development.

MESENCHYMAL STEM CELLS

Many therapeutic strategies have been researched over the years to improve bone regeneration in a variety of disorders and injuries. Among

them, the use of MSCs mimicking the natural occurring processes of bone formation has been widely investigated [40] [41].

Marrow stromal cells, also known as mesenchymal stromal cells or mesenchymal stem cells, were first described in the 1960s by Friedenstein as plastic-adherent cells with a fibroblast-like morphology within the bone marrow [42]. They are a source of self-renewing multipotent cells that can differentiate along the adipogenic, osteogenic and chondrogenic lineages [43]. Even though they can be obtained from a number of tissues, including the umbilical cord and dental pulp [44], the most common sources of human MSCs are the bone marrow and the adipose tissue [45]. In addition to the previous characteristics, a series of markers were later on defined by the International Society for Cellular Therapy to standardise the definition of MSCs [46]. These included the expression of CD105, CD73 and CD90, and the lack of CD45, CD34, CD14 or CD11b, CD79α or CD19 and MHC class II molecules.

In addition to their potent differentiation capacities, MSCs have also been shown to interact with the immune system, exerting potent anti-inflammatory and immunomodulatory effects, through the secretion of a variety of cytokines and other soluble factors [47]. Moreover, MSCs express low to intermediate levels of MHC class I, whereas co-stimulatory molecules such as CD40, CD80 and CD86 are absent [48] [49]. These phenotypic characteristics make them unlikely to activate the immune system and trigger a response. For these reasons, MSCs have been widely researched as potential therapeutic candidates for bone tissue regeneration [50].

The use of mesenchymal stem cells for bone regeneration

Bone tissue engineering approaches have traditionally focused on the osteogenic differentiation of MSCs. A number of studies have shown bone formation by *in vitro* differentiating these cells prior to implantation to form a bone-like matrix [51] [52]. However, problems such as the lack of vascularisation, resulting in poor nutrient distribution and core necrosis [30],

have shifted this approach towards the use of chondrogenically differentiated MSCs. Through the addition of transforming growth factor beta 3 (TGF-β3) *in vitro*, MSCs can form bone from a cartilage precursor without the need of scaffolds in immunodeficient animals [53]. Following the naturally occurring process of endochondral ossification, MSCs condense and form a transient cartilage template that is eventually replaced by bone tissue. The bone constructs retrieved present a bone ring, marrow cavities, and vascular invasion, which could definitively overcome the previously mentioned limitations of osteogenically differentiated MSCs [54 55 56].

The quality, as well as the differentiation potential of MSCs from the different sources for bone repair purposes are still under debate [57]. Bone-marrow derived MSCs have been most investigated, due to their natural tendency to become hypertrophic [55] and the existence of consistent protocols that allow for chondrogenic differentiation [43]. Diverse reports have shown that the age and disease status of the MSC donor seem to impact the quantity as well as the quality of bone formation [58]. For example, cell attachment, as well as proliferation and differentiation seem to be negatively affected with increasing donor age [59]. In addition, age increases senescence [60], reducing their chondrogenic differentiation [61] and bone formation capacities [62].

For this reason, more and more MSC-based approaches for tissue regeneration rely on the use of allogeneic cells from younger and healthier donors [63], rather than using the own patient MSCs. However, when it comes to employing a source of allogeneic MSCs, the potential risk of an immune reaction in the allograft recipient needs to be taken into account [64]. Little is known about the success of using MSC-based allografts for bone regeneration approaches in the presence of a fully functional immune system, though. In a recent study using rat allogeneic chondrogenically differentiated MSCs for bone repair in a femoral defect, no strong allogeneic immune reactions were detected [65]. However, only a limited amount of animals showed full bone bridging, in contrast to syngeneic approaches. The immune response towards human allogeneic

MSC-derived constructs is also unclear [66]. The majority of studies using human chondrogenically differentiated MSCs have been performed in nude animals that lack a fully functional allogeneic immune system. For all these reasons, further research is needed to clarify the feasibility of an allogeneic MSC source for bone regeneration purposes, as well as investigating the interactions of the donor MSCs with the host immune cells.

Undifferentiated MSCs and their interaction with the immune system

Undifferentiated MSCs are well known for their abilities to inhibit the proliferation and differentiation of a number of immune cells, ranging from the innate immune response, such as Natural Killer Cells (NKs) [67] and dendritic cells (DCs) [68], to the T [69] [70] and B cells [71] [72]. This immunomodulation seems to take place in the form of cell contact-dependent mechanisms, as well as through the secretion of soluble mediators [73] [74].

MSCs can suppress T cell proliferation of both $CD4^+$ and $CD8^+$ T cell subsets in mixed lymphocyte reactions in a dose-dependent manner [75]. In this process, pro-inflammatory cytokines, such as IFN-γ, IL-1α, IL-1β or TNF-α, have been shown to impact the immunomodulatory properties of MSCs [76] [77]. Upon IFN-γ stimulation, the constitutive production of the enzyme indoleamine 2,3-dioxygenase (IDO) on MSCs is increased, leading to tryptophan depletion [78], and potentiating their anti-proliferative effect on T cells [79]. In addition, during this process IL-4 production on anti-inflammatory Th2 cells is stimulated and IFN-γ production by the pro-inflammatory Th1 is inhibited [80] [81].

MSCs can also exert their immunomodulatory activities on B cells inhibiting their proliferation through soluble factors [82] and abrogating the antibody-producing B cell differentiation independently of T cells [71]. Moreover, upon IFN-γ stimulation they significantly decrease B cell maturation and proliferation following an IDO-dependent mechanism [72].

Chondrogenically differentiated MSCs and their interaction with the immune system

Despite numerous reports on the immunomodulatory abilities of undifferentiated MSCs, whether they retain their immune-privileged status upon TGF-β3-mediated chondrogenic differentiation is still contradictory. On the one hand, some authors claim that chondrogenically differentiated MSCs are non-immunogenic and do not induce T cell proliferation [48]. In another study by Kiernan *et al*. [83], an absence of immunogenicity when co-culturing these cells with allogeneic Peripheral Blood Mononuclear Cells (PBMCs) was observed, however no immunomodulation was reported. In addition, Zheng *et al*. observed that chondrogenically primed MSCs were able to reduce the proliferation rates of T cells in a dose-dependent fashion, as well as inhibiting the production of the pro-inflammatory cytokines IFN-γ and TNF-α [84]

Other authors have claimed that MSCs trigger immunogenic reactions upon their chondrogenic differentiation. Ryan *et al*. reported an induced T cell proliferation when co-culturing chondrogenically primed MSCs with PBMCs in a 1:5 ratio [85]. Another study using chondrogenically differentiated MSCs concluded as well that they were prone to trigger lymphocyte proliferation in co-culture [86]. However, these two studies were not performed using human cells. In addition, an enzymatic digestion of the differentiated MSCs was performed prior to the co-culture experiments to remove the matrix, which is unknown if it could affect their immune privileged properties. Therefore, the different methods of chondrogenic differentiation, as well as the processing and the source of the MSCs, could impact their immune status [64].

THE ALLOGRAFT-IMMUNE SYSTEM INTERACTION: A KEY FACTOR TO SUCCESS

In the context of an allogeneic transplantation, the immune system plays a central role when it comes to determining the fate of the allograft. The innate immune system will initially present allo-antigens that can activate

the adaptive immune system [87]. In particular, mature DCs can act as allo-antigen presenters and become potent stimulators of T cells, increasing their pro-inflammatory cytokine production, and upregulating the expression of co-stimulatory molecules [88]. Macrophages can as well act as antigen presenters to effector T cells, in addition to producing pro-inflammatory cytokines and reactive oxygen and nitrogen species that can contribute to rejection [89]. Furthermore, Natural Killer (NK) cells can amplify early graft inflammation, as well as support the activity of T cells [90].

Upon allo-antigenic recognition through a donor-derived (direct presentation) or host-derived (indirect presentation) APC, T cells become activated and transition to effector cells. Stimulated T cells can then activate B cells, which orchestrate humoral rejection through the production of allo-antibodies, as well as provide additional co-stimulation to T cells [91]. Importantly, after a primary exposure, memory T and B cells can develop a stronger and faster immune response upon secondary antigen stimulation [92].

TOWARDS THE IDEAL MSC-DERIVED GRAFTS FOR BONE FORMATION

As potential candidates for bone tissue engineering, investigating the MSC's ability to trigger immune responses is key to avoid short or long-term graft rejection. Since the immune response to chondrogenically differentiated MSCs could differ among patients undergoing bone graft transplantation, a 'universal' source of MSCs with low immunogenic properties, as well as consistent proliferation and differentiation abilities would be advantageous (Figure 3). From this source, *ready-to-go* batches of cells could be created, allowing for rapid expansion and manufacture of the bone grafts, and considerably reducing waiting times for the patients. In addition, as previously mentioned, several studies have reported that increasing age and disease status can negatively impact the bone formation capacities of adult MSCs [93] [59] [60] [61], so ideally a young healthy source would be required. In this line, the use of paediatric MSCs (pMSCs) is a promising alternative. Hence, investigating their potential for bone

regeneration purposes in the context of an allogeneic transplantation, as well as addressing the possible graft-derived immune responses upon chondrogenic differentiation, is essential to consider them candidates for a potential *off-the-shelf* therapy in regenerative medicine.

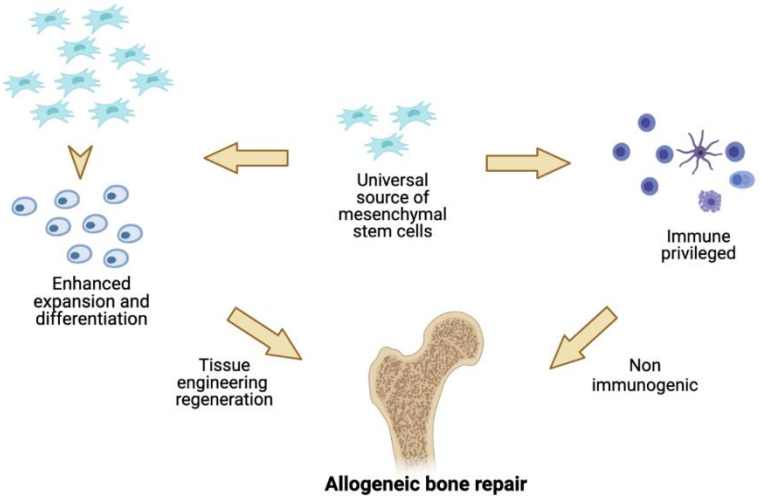

Figure 3. Characteristics of an ideal source of mesenchymal stem cells (MSCs) for bone repair. The ability of a young and healthy source of MSCs to be immune privileged and not trigger immune responses, as well as have higher proliferation and consistent differentiation capabilities would allow for a universal source that could be employed for tissue engineering regeneration purposes, generating allogeneic bone that could considerably improve the current bone repair strategies.

AIMS AND OUTLINES OF THIS THESIS

In this thesis, we aimed to look at the feasibility of the use of paediatric mesenchymal stem cells for bone regeneration in the presence of a functional allogeneic immune system.

As a first step, in **Chapter 2** we obtained pMSCs from leftover surgical material, and we show their superior proliferation and differentiation abilities compared with adult MSCs, as well as less senescence, making them attractive candidates for stem-cell based bone tissue engineering.

Subsequently, in **Chapter 3** we analyse the immunological profile of undifferentiated pMSCs, and their behaviour towards T and B cells upon pro-inflammatory stimulation. Due to their ability to modulate the B cell responses, including antibody production, and their immunomodulatory abilities towards T cells even upon IFN-γ stimulation, in **Chapter 4** we demonstrate the potential use of these pMSCs for bone formation in an allogeneic pre-clinical model. To do so, an *in vitro* study was initially carried out to model the interactions between allogeneic immune cells and pMSCs. There, the immunomodulatory and immunogenic abilities of pMSCs were studied upon chondrogenic differentiation in a pro-inflammatory microenvironment with IFN-γ and TNF-α. Once proven that pMSCs maintain their immunoprivileged status under these conditions and do not trigger T cell proliferation, we proceeded with a first *in vivo* study that analysed the allogeneic response in a 3-week humanised immune system towards chondrogenically differentiated pMSCs (grafts) in the short-term. We found a persistence of the grafts in this setup, as well as the absence of a systemic immune response. Some indications of early bone mineralisation after 3 weeks of implantation were demonstrated. We also showed that no immune responses occurred after the exposure of pre-sensitized immune cells to graft-matched and mismatched pMSCs.

In the second part of chapter 4, we investigated the alloimmune reaction towards 8-week mature bone constructs after 4 weeks in an allogeneic immune system. There, we show that mineralised grafts with a complete bone-like structure, including bone marrow and blood vessels, are able to

persist for 4 more weeks in the presence of an allogeneic immune system without significant immune responses.

Finally, in the last part of chapter 4, we conclude with a third *in vivo* model to demonstrate the potential of use of pMSC derived mature bone allografts in the longer term. There, we show the persistence of 8-week mature bone after 6 weeks in the presence of an immune system.

In **Chapter 5**, we take a next step and investigate the potential of pMSCs for bone formation in an immunocompetent mouse model, by implanting chondrongenically primed pMSC grafts in *wild type* mice. There, we reported indications of bone mineralisation and we showed no systemic responses after 2 and 12 weeks of implantation.

Chapter 6 provides a general discussion, conclusion and future perspectives outlining the implications of this thesis for the use of allogeneic paediatric mesenchymal stem cells for bone repair, and **Chapter 7** contains a summary.

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2

Isolating Pediatric Mesenchymal Stem Cells with Enhanced Expansion and Differentiation Capabilities

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ABSTRACT

Mesenchymal stem cells/marrow stromal cells (MSCs) are attractive for applications ranging from research and development to use in clinical therapeutics. However, the most commonly studied MSCs, adult bone marrow MSCs (A-MSCs), are limited by significant donor variation resulting in inconsistent expansion rates and multilineage differentiation capabilities. We have recently obtained permission to isolate pediatric MSCs (PMSCs) from surplus iliac crest bone chips. Here, we developed a simple and easily replicable isolation protocol yielding P-MSCs, which adhere to MSC defining guidelines. After confirming immunophenotypic marker expression, we compared expansion rates, senescence, morphology, and trilineage differentiation of P-MSCs to A-MSCs for multiple donors. We found P-MSCs have faster *in vitro* replication, consistently show significantly lower senescence, and are capable of more reproducible multilineage differentiation than A-MSCs. We, therefore, believe P-MSCs are a promising candidate for use in research applications and potentially as part of an allogeneic therapeutic treatment.

INTRODUCTION

Over the past decade, interest in mesenchymal stem cells/marrow stromal cells (MSCs) has grown; many have recognized their potential to advance scientific discovery and improve clinical treatment options[1-3] .MSCs unlike other lineage-committed progenitors or terminally differentiated cells are capable of multilineage differentiation, which is desirable for a number of applications ranging from developmental research to use in advanced therapeutic medicinal products (ATMPs)[4-7].MSCs are attractive for these applications as they can be easily isolated, cultured, and expanded *in vitro*.[2,8] They have been found in a variety of tissues, blood, and even urine[9-11]. Regardless of their point of isolation, MSCs must adhere to criteria determined by the International Society for Cellular Therapy as outlined by Dominici et al[12].In brief, cells must (i) be plastic adherent, (ii) retain their multipotent differentiation capacity, and (iii) express a panel of surface antigens. Although a diverse variety of MSCs meet these criteria, there are still numerous differences between populations depending on the method and tissue they are isolated from, including variability in *in vitro* expansion, differentiation capability, and cell surface marker expression[8,13–16]. These differences and the inherent donor variation observed between MSCs make clinical translation and their use in ATMPs challenging.

In order for MSCs to be used as part of AMTPs, a sufificient quantity of cells must be obtained that are capable of producing consistent outcomes that satisfy the regulatory requirements set by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA)[17–19].When removed from the environment of their *in vivo* niche and expanded *in vitro*, MSCs rapidly lose their ability to replicate and differentiate, meaning their characteristics change npredictably over time in culture[16,20–22].This variation is often observed in bone marrow (BM) MSC populations, which as of now is still considered the gold standard when it comes to MSCs[23,24].Other MSC sources such as umbilical cord and adipose tissue are being actively characterized with promising but conflicting results[11,25–27]. An ideal MSC source would allow isolation with minimal patient discomfort and yield

cells capable of reproducibly meeting EMA/ FDA regulatory requirements[28]. Although BM MSCs are currently the gold standard for MSCs, their isolation is associated with a painful procedure and harvesting of such material results in substantial patient discomfort and recovery time when used[27,29]. Many researchers, us among them, use surplus clinical material obtained from patients undergoing surgical procedures (total hip or knee replacement, for example). However, the age and disease status of the donors often negatively influence MSC performance[20]. Kretlow *et al*. found that cell attachment, proliferation, and differentiation were all affected as donor age increased[30,31]. MSCs from aged donors were less capable of secreting and maintaining a chondrogenic matrix[32], and had a decreased bone-forming potential *in vivo*[33]. It has also been reported that cells from the elderly often exhibit cellular dysregulation, which negatively impacts stem cell populations[31,34,35]. In addition, MSCs from elderly patients have been shown to have age-induced gene expression changes and earlier replicative senescence that further negatively effects MSC performance[31,36]. Cellular dysregulation in aging populations has also been hypothesized to add to the pathogenesis of these diseases, which results in a damaged stem cell population[31,34]. As neither cell dysregulation nor senescence is useful for research or the clinics, MSCs isolated from adult or geriatric populations are not an ideal cell source. MSCs isolated from younger patients have shown promise[11].

Recently we have gained access to small quantities of surplus bone from pediatric patients undergoing craniofacial reconstruction surgery from which we can easily isolate pediatric MSCs (P-MSCs). The resulting MSCs are plastic adherent, maintain MSC-related immunophenotype, and are capable of consistent differentiation. Here we outline how these cells are obtained, isolated, and cultured as well as describe the morphological and phenotypic characteristics of these novel MSCs to allow others in the scientific community to utilize them for their own applications. We compare P-MSCs with adult MSCs (A-MSCs) isolated from BM and find P-MSCs to be capable of more consistent multilineage differentiation. We believe P-MSCs to be a promising candidate for use in both research and clinical applications.

MATERIALS AND METHODS

Mesenchymal stem cell isolation and expansion

All samples were harvested with the approval of the medical ethics committee at Erasmus Medical Centre (Erasmus MC, The Netherlands). A-MSCs were isolated and expanded as previously described (MEC-2004-142 and MEC-2015-644)[13]. P-MSCs were isolated from leftover iliac crest bone chip material obtained from patients undergoing alveolar bone graft surgery (MEC-2014-16; 9–13 years). P-MSCs were isolated by gently swirling 10 mL of expansion medium (aMEM containing 10% serum [lot no. 41Q204K; Gibco], 50 mg/mL gentamycin, 1.5 mg/mL fungizone, 25 mg/mL l-ascorbic acid 2-phosphate, and 1 ng/mL fibroblast growth factor-2 [Instruchemie]) with iliac crest bone chips. The medium was removed and the process was repeated with an additional 10 mL expansion medium. The cell suspension from the combined medium of both washes was plated in a T75 flask and iliac crest chips were processed for histology. Flasks were washed 24 h after plating with phosphate-buffered saline (PBS) to remove non-adherent cells and debris. Cells were cultured at 37°C and 5% carbon dioxide (CO_2). Expansion medium was refreshed twice a week. P-MSCs were passed at 80–90% confluency using 0.05% trypsin and replated at 2300 cells/ cm^2 . After passages 2–4, A-MSCs were used for trilineage differentiation and after passage 5 for FACS analysis, immunocharacterization, and β-galactosidase stainings. PMSCs were always used after passage 5 unless otherwise noted (β-galactosidase staining, passage 8).

FACS analysis

A-MSCs and P-MSCs were trypsinised at passage 5 and rinsed in FACS flow. Cells were incubated for 30 minutes in 100 µL FACS buffer (BD Biosciences) containing antibodies against CD90 (APC), CD105 (FITC), CD73 (PE), CD271 (APC), CD166 (PE), HLA-DR (PerCP), HLA-ABC (FITC) or CD45 (PerCP). MSCs were washed with FACS flow, centrifuged at 689 g for 5 minutes, resuspended in 200 µL of FACS flow and analysed on a FACS Jazz flow cytometer (all antibodies BD Biosciences). Post-analysis was completed using FlowJo software version 10.0.7 (Treestar Inc.).

Senescence staining and quantification

The percentage of senescent cells was determined by staining for senescence-associated lysosomal β -galactosidase using a modification of Debacq-Chainiaux *et al*.'s protocol[37]. A-MSCs and P-MSCs were seeded at 2300 cells/cm^2 in complete expansion medium (as described in Mesenchymal stem cell isolation and expansion). On day 3, cells were refreshed and after 6 days, cells were fixed in 1% (v/v) formaldehyde (Sigma) and 0.5% glutaraldehyde (v/v) (Sigma) in PBS for 15 min at 4°C. After washing with distilled water, cells were incubated for 24 h at 37°C with 250 mL/cm^2 staining solution (1 mg X-gal [5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside, Roche Diagnostics; 1.64 mg potassium hexacyanoferrate (III), Sigma; 2.1 mg potassium hexacyanoferrate (II) trihydrate, Sigma; 2 mmol magnesium chloride hexahydrate, Sigma; 150 mmol sodium chloride and 7.3 mmol monohydrous citric acid, Sigma; 25.3 mmol disbasic sodium phosphate dihydrate, (Sigma) per mL distilled water; pH 6.0). After rinsing in distilled water, cells were counterstained with 1 g/L neutral red (Sigma) in a solution of 0.2% acetic acid. The number of positive cells was quantified and plotted relative to total number of cells.

Chondrogenic differentation

A total of 2 x10^5 A-MSCs or P-MSCs were suspended in 500 µL of chondrogenic medium (high-glucose Dulbecco's modified Eagle's medium [DMEM] supplemented with 50 µg/ mL gentamycin [Invitrogen], 1.5 µg/mL fungizone [Invitrogen], 1 mM sodium pyruvate [Invitrogen], 40 mg/mL proline [Sigma], 1:100 v/v insulin-transferrin-selenium [ITS$^+$; BD Biosciences], 10 ng/mL transforming growth factor 3 [Peprotech], 25 µg/mL l-ascorbic acid 2-phosphate [Sigma], and 100 nM dexamethasone [Sigma]) in 15 mL polypropylene tubes. Samples were centrifuged at 200 g for 8 min. The medium was replaced twice weekly for 21 days (P-MSCs) or for 28–35 days (A-MSCs). Samples were formalin fixed for histology (4% [w/v] formaldehyde in PBS for 2 h).

Osteogenic differentiation

A-MSCs or P-MSCs were plated at a density of $3x10^3$ cells/cm^2 in expansion medium (as described in Mesenchymal stem cell isolation and expansion). Twenty-four hours after seeding, medium was replaced with osteogenic induction medium (high-glucose DMEM supplemented with 10% serum, 50 µg/mL gentamycin, 1.5 mg/mL fungizone, 10 mM glycerol phosphate [Sigma], 0.1µM dexamethasone [Sigma], and 0.1 mM L-ascorbic acid 2-phosphate [Sigma]). Medium was refreshed as previously described for 14–21 days, depending on when cell sheets began to pull away from the outer perimeter of the well or when calcium deposition was observed macroscopically, at which point the culture was ended. Cells were cultured at 37°C and 5% CO_2. Samples were prepared for histology (fixed in 70% EtOH at 4°C) after the end of culture.

Adipogenic differentiation

A-MSCs or P-MSCs were plated at a density of $2.1x10^4$ cells/cm^2 in adipogenic induction medium (high-glucose DMEM supplemented with 10 % serum, 50 µg/mL gentamycin, 1.5 mg/mL fungizone, 0.2 mM indomethacin [Sigma], 0.01 mg/mL insulin [Sigma], 0.5 mM 3 iso-butyl-1-methyl-xanthine [Sigma]). Medium was refreshed as previously described and cells were maintained at 37°C and 5% CO_2. Samples were prepared for histology (fixed in 4% (w/v) formalin) following harvest.

Haematoxylin-eosin staining

Paediatric bone chips were fixed for 24 hours in 4% (w/v) formalin, decalcified in 10% EDTA (w/v) for 30 days and paraffin embedded. Chondrogenic MSC pellets were fixed in 4% (w/v) formalin for 2 hours and paraffin embedded. 6 mm-thick sections were cut, deparaffinised and stained with haematoxylin-eosin (H&E). H&E staining was performed by incubating deparaffinized samples in Gill's haematoxylin (Sigma) for 5 minutes, washed in none distilled water for 5 minutes, washed in distilled water, and counterstained for 45 seconds with 2% Eosin (Merck; in 50% ethanol, 0.5% acetic acid). Samples were fixed in 70% EtOH for 10 seconds

and rehydrated (sequentially in 96% EtOH, 100% EtOH, and xylene for 1 minute). Samples were mounted in Entellan (Depex).

Thionine staining

Deparaffinized samples were incubated in 0.04% thionine (prepared in 0.01M sodium acetate, pH 4.5) for 5 min, differentiated in 70% EtOH for 10 s, and then rehydrated as previously described. Samples were mounted in Entellan (Depex).

von Kossa staining

Osteogenically differentiated MSCs were fixed in 4% (w/ v) formalin for 1 h. After a rinse with ultrapure water, samples were incubated in 5% w/v silver nitrate (Sigma) for 30 min under direct light provided by a light box. After incubation, samples were washed in ultrapure water and counterstained with nuclear fast red (Merck) for 5 min. Samples were dehydrated in 70% EtOH for 10 s followed by 96% EtOH and 100% EtOH for 1 min. Samples were imaged in 100% EtOH directly after staining.

Oil red staining

After a 15-min fixation in 4% (w/v) formalin, samples were rinsed in distilled water for 10 min. Then 0.5% w/v Oil-red O (in 2-propanol; Sigma) was added to samples for 10 min followed by further rinsing with distilled water. Samples were imaged in distilled water.

P-MSC-PBMCs co-culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy male donors (Sanquin, Rotterdam) by a Ficoll-Paque PLUS gradient separation as previously described[38] (GE Healthcare). PBMCs were resuspended in human serum conditioned medium (HCM) (RPMI-1640 medium, 1% GlutaMAX (Life Technologies), 50 µg/mL gentamycin, 1.5 µg/mL fungizone, 10% human serum (Sigma-Aldrich)) and stored at -80°C until use. P-MSCs were trypsinised as previously described and seeded in low-evaporation round bottom 96 well plates. 24 hours

following seeding, $1x10^7$ PBMCs were labelled with 20 µL of CFSE. T cells were stimulated by adding anti-CD3/CD28 antibodies (1 µL/10^6 cells, 1 mg/mL) to PBMC suspension with an anti- goat linker antibody (2 µl/10^6 cells, 0.5 mg/mL) (BD Biosciences). Stimulated 100,000 PBMCs were co-cultured with P-MSCs at a P-MSC:PBMC ratios of 1:2.5, 1:5, 1:10 or 1:20., PBMCs were harvested 5 days later and stained with CD3-PerCP (clone SK7), CD8- PE-Cy7 and CD4-APC (BD Biosciences). Samples were fixed in 3.6% paraformaldehyde and analysed using a FACS Jazz flow cytometer (BD Biosciences) and post-analysis was completed using FlowJo software version 10.0.7 (Treestar Inc.). Data is represented as reciprocal of the mean fluorescence intensity (MFI).

Statistical analysis

Mann Whitney U analysis was performed using SPSS (Ver 21. SPSS Inc, Chicago, USA) on data used in figures 1, 2, and 3. Kruskal-Wallis analysis with Dunn's multiple comparison was performed on figure 4. Data are shown as mean ± SD, P-values under 0.05 were considered significant.

RESULTS

P-MSCs were isolated from small quantities of surplus bone biopsies from patients undergoing cleft pallet reconstruction surgery. The environment from which the P-MSCs are isolated contains both bone and BM elements (Figure 1).

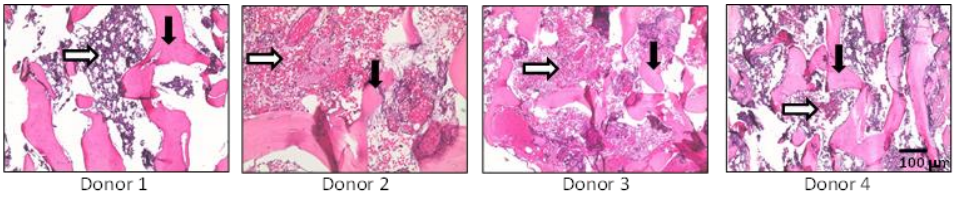


Figure 1. P-MSCs are isolated from illiac crest rest material containing both bone and bone marrow. Hematoxylin and eosin-stained sections of the illiac crest chips from which P-MSCs are isolated. The cell source environment is rich in

both bone marrow elements and bone (black arrows indicate bone, white arrows indicate bone marrow). MSC, mesenchymal stem cells/marrow stromal cells; P-MSCs, pediatric MSCs. Color images available online at www.liebertpub.com/tec

To prove that isolated cells were indeed true MSCs, we characterized the immunophenotypic expression of common MSC markers. These markers included a panel that are known to be expressed on MSCs, including CD105, CD90, CD73, CD271, CD166, and HLA-ABC as well as a commonly used negative marker, lymphocyte-associated CD45[12,39]. Both A-MSCs and P-MSCs were analyzed after five passages. MSC markers were expressed at a similar level in P-MSCs and A-MSCs (Figure 2).

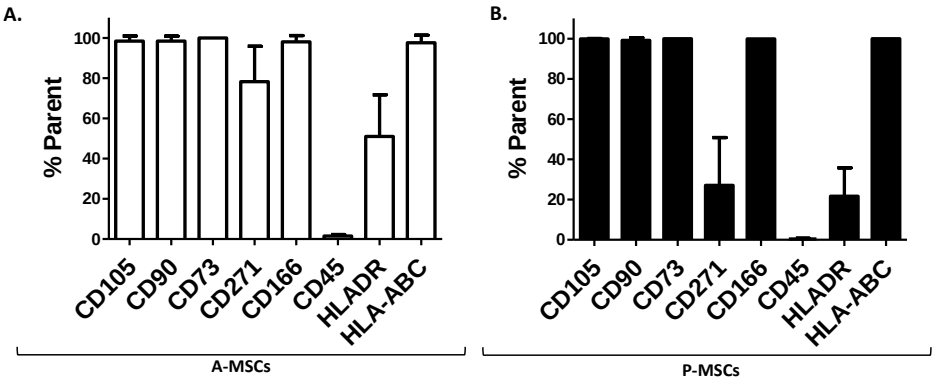


Figure 2. P-MSCs and AMSCs express similar levels of general stem cell markers. General MSC-related markers that are commonly expressed on A-MSCs **(A)** are also exppressed at a similar level in PMSCs **(B)** (N.S. differences between A-MSCs and PMSCs; Mann–Whitney U test). Both populations are negative for hematopoietic marker CD45; however, they are positive for a panel of other immune-related markers.

Both populations were negative for CD45 and positive HLA-ABC. About half the P-MSCs and A-MSCs population were positive for HLA-DR, which was not surprising as HLA-DR expression can increase with *in vitro* culture of MSCs[40]. We found no significant difference in HLA-DR expression between P-MSCs and A-MSCs (Fig. 2)

P-MSCs have enhanced expansion properties compared with A-MSC

During expansion, P-MSCs exhibit a typical MSC morphology similar to that observed in A-MSCs (Fig. 3A). Although the total number of days A-MSCs and P-MSCs took to establish the initial culture (Fig. 3B) and reach passage 3 (Fig. 3C) did not change, P-MSCs expanded significantly faster than A-MSCs, yielding more cells after the same time in culture (Fig. 3D; $p < 0.02$). This difference in cell number could be contributed to cell size. A-MSCs enlarged the longer they were in culture, whereas P-MSCs remained small (Figs. 3A and 4A).

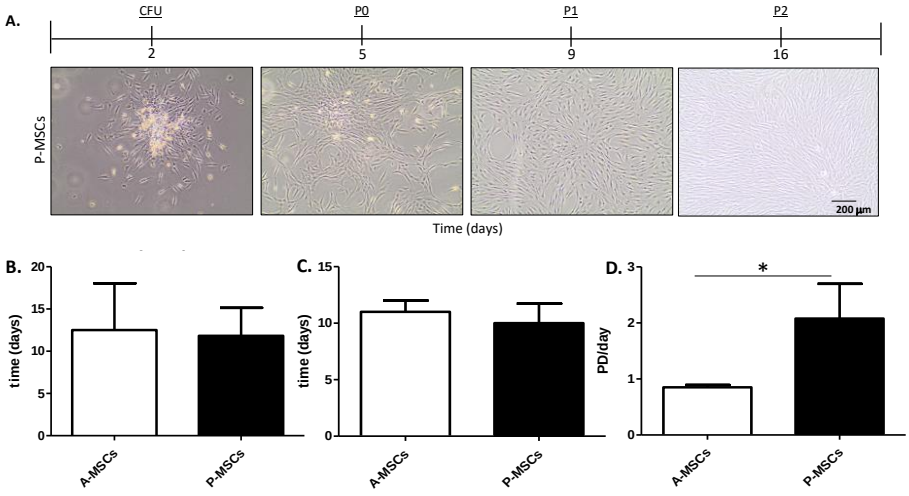


Figure 3. P-MSCs and A-MSCs have a similar rate of expansion but P-MSCs undergo more population doublings. **(A)** Cell morphology typically observed during expansion of P-MSCs (representative donor). **(B)** Total days taken to establish culture from plating initial cell suspension to passage 0 do not differ between P-MSCs and A-MSCs. (C) Expansion time from passage 0 to passage 3 does not differ between A-MSCs and P-MSCs. (D) Total number of population doublings between passage 1 and passage 3 is greater in P-MSCs than in A-MSCs *$p < 0.05$ ($p < 0.0238$; Mann–Whitney U test). AMSCs, adult MSCs; CFU, colony-forming units; P0, passage 0; P1, passage 1; P2, passage 2; P3, passage 3; PD, population doublings. Color images available online at www.liebertpub.com/tec

P-MSCs are a less senescent cell source compared with A-MSCs

As increased cell size is a hallmark of senescence, a permanent cell cycle arrest that A-MSCs have been shown to undergo *in vitro*, we compared senescence between AMSCs and P-MSCs[41,42]. We observed cell enlargement of A-MSCs compared with P-MSCs (Fig. 4A). Senescenceassociated lysosomal β-galactosidase staining showed that P-MSCs, even after extensive passage (p8; Fig. 4B, C), contained significantly less senescent cells than A-MSCs at an earlier passage (p5; Fig. 4B, C)

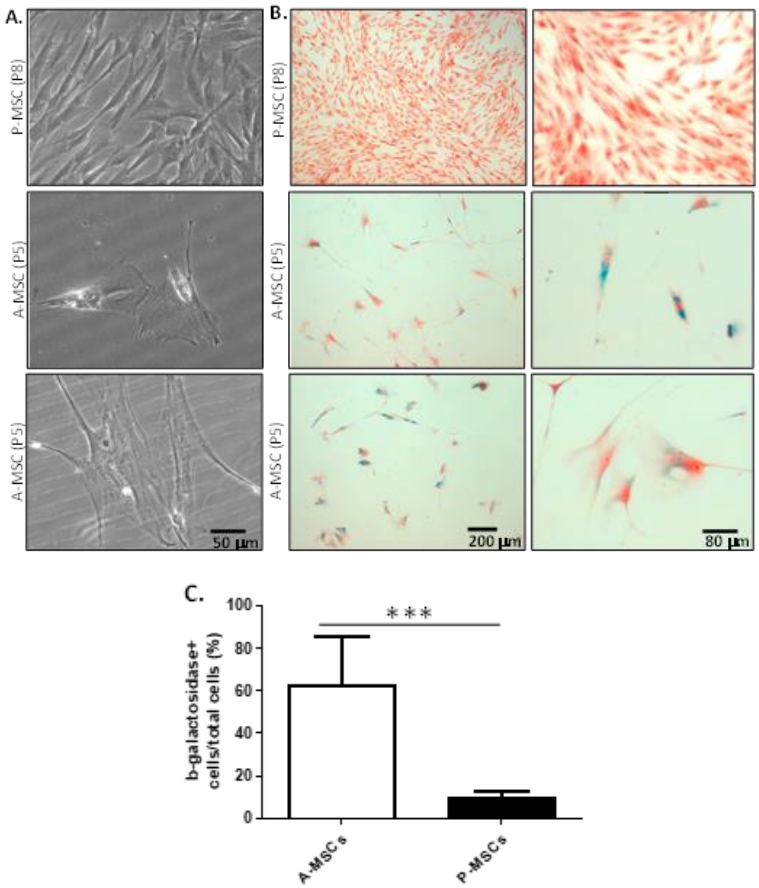


Figure 4. P-MSCs contain significantly less senescent cells than A-MSCs. (A) Cell morphology observed during expansion of P-MSCs (p8) and of A-MSCs (p5). A-MSCs display a larger cell morphology compared to P-MSCs even though they are an earlier passage. Both A-MSCs and P-MSCs were seeded at the 2,300

cells/cm^2 and expanded under normal conditions for 6 days. (**B)** b-galactosidase staining of both P-MSCs (p8) and A-MSCs (p5). P-MSCs contain far less positively b-galactosidase stained cells than A-MSCs. (**C)** Percentage of senescent cells counted in P-MSCs is significantly lower than that of A-MSCs (n=4; p<0.000; Mann Whitney test). (Abbreviations: P5: passage 5, P8: passage 8)

In monolayer, P-MSCs reduce T cell proliferation at a similar level as A-MSCs

Our laboratory has previously shown that A-MSCs repress the proliferation of allogeneic T cells, a feature essential for many anti-inflammatory MSC-based therapeutics[38]. To examine whether P-MSCs repress T cell proliferation, allogeneic T cells from PBMC fractions were CD3/CD28 stimulated and added in suspension to P-MSC monolayers. In both CD4$^+$ and CD8$^+$ T cell subsets, P-MSCs inhibited T cell proliferation in a dose-dependent manner and to a similar extent as we have previously reported for A-MSCs (Fig. 5). Here we found that P-MSCs exhibited a similar level of inhibition to what was reported by A-MSCs[38].

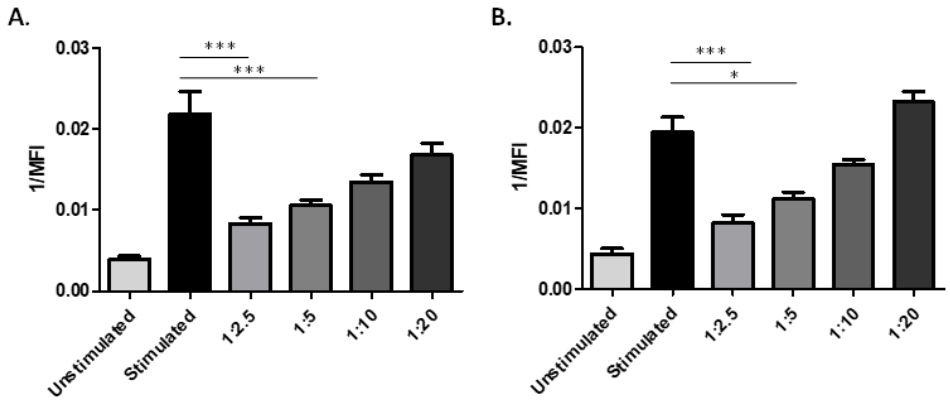
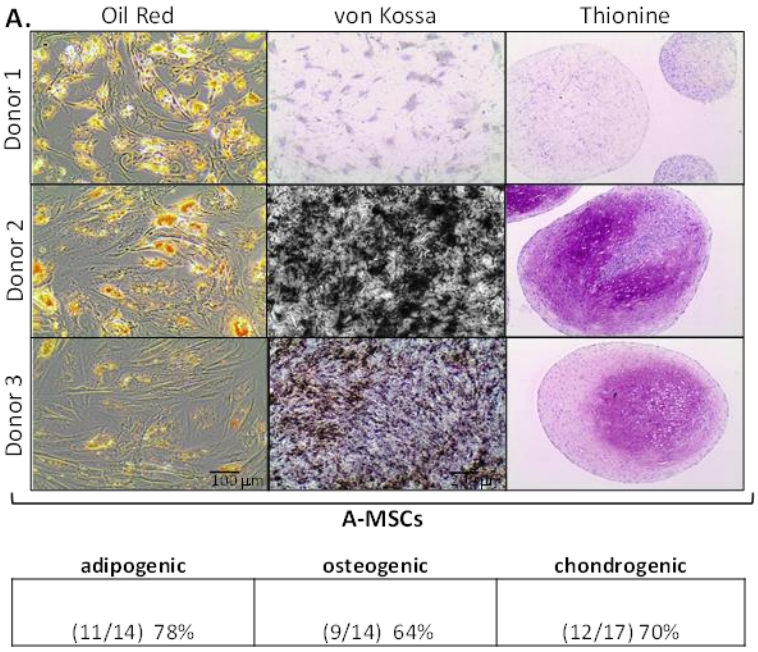


Figure 5. P-MSCs reduce allogeneic CD4+ and CD8+ T cell proliferation in monolayer. Stimulated (+CD3/CD28) PBMCs cocultured with P-MSCs at different MSC:PBMC ratios (1:2.5, 1:5, 1:10, 1:20). CD4$^+$ **(A)** and CD8$^+$ **(B)** proliferation rates were found to decrease in a dose-dependent manner after 5 days in culture. (n = 3 P-MSC donors; n = 2 PBMC donors; Kruskal–Wallis with Dunn's post hoc correction; ***p > 0.001, *p > 0.05). MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells.

P-MSCs exhibit more consistent multilineage differentiation capacity compared with adult donors

A-MSCs are known to exhibit inconsistent differentiation capabilities that vary greatly between donors. This severely limits their use in applications wherein consistency is essential[43]. Compared with A-MSCs donors (passages 2–5; Fig. 6A), the trilineage differentiation potential of P-MSCs (passage 5; Fig. 6B) was found to be more consistent. Even though P-MSCs were used several passages beyond that used for the A-MSCs, P-MSCs more consistently underwent adipogenesis, osteogenesis, and chondrogenesis, with only 1 out of 12 donors not being able to make bone or cartilage after treatment (Fig. 6B). A-MSCs showed much more variability in their differentiation potential. Out of the 14 AMSC donors tested, 3 donors failed to undergo adipogenesis, 5 donors failed to osteogenically differentiate, and 5 donors were unable to chondrogenically differentiate (Fig. 6A).



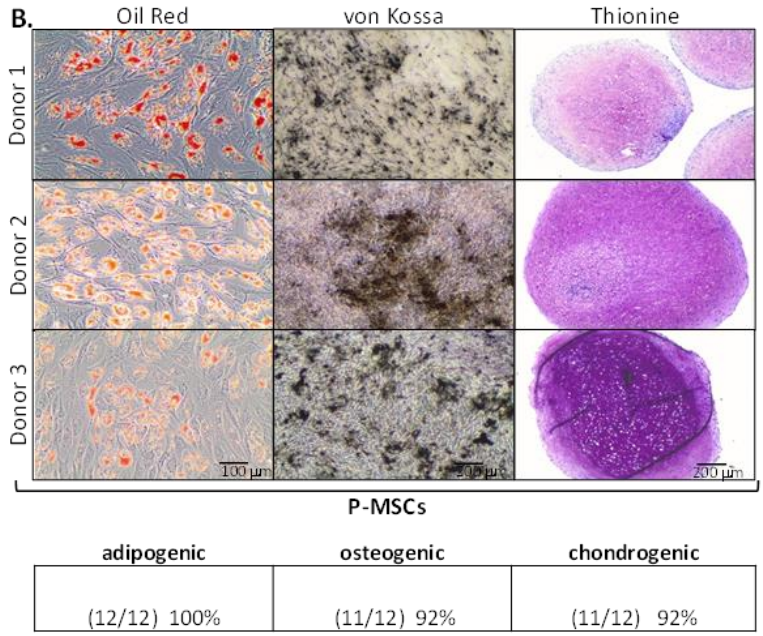


Figure 6. P-MSCs show more consistent capacity for multilineage differentiation. (A) A-MSCs (passage 2-4) show great variation in differentiation capacity. A-MSCs are known to exhibit far greater variation in differentiation capacity (Chamberlain, Fox *et al*. 2007; Noort, Scherjon *et al*. 2003). (**B)** P-MSCs (passage 5-6) are capable of tri-lineage differentiation with all donors but one being capable of osteogenic and chondrogenic differentiation. All P-MSCs tested could adipogenically differentiate.

DISCUSSION

Identifying cell sources with enhanced and reliable differentiation capabilities and expansion properties is necessary in order for MSCs to be more readily utilized both in research and in ATMPs. Here we have established a simple isolation protocol to obtain P-MSCs from surplus iliac crest bone chip material (Fig. 1). We confirmed P-MSCs expressed cell surface markers typically used to properly identify MSC populations. P-MSCs showed similar expression levels of general stem cell markers CD105, CD90, CD73, and CD166 compared with AMSCs, as well as the

absence of the hematopoietic marker CD45 (Fig. 2). As these markers are conventionally used to identify MSCs,[44,45] we are confident that this marker expression in combination with other results confirms their phenotype. In this study, A-MSCs displayed a higher expression of CD271 and HLA-DR than P-MSCs. It has been previously reported that in MSCs, CD271 as well as HLA-DR expression decrease over time in culture[46,47]. As P-MSCs undergo more population doublings than A-MSCs at the same passage (Fig. 3D), this increased cellular division could have contributed to the decreased expression of both CD271 and HLA-DR we observe in P-MSCs.

P-MSCs expanded more rapidly than A-MSC donors, which might be attributed to the relatively low senescence in P-MSCs (Fig. 4). Senescent cells are much larger than nonsenescent cells[48]. A-MSCs have more enlarged senescent cells that do not divide[49,50], making it easy to understand why the population doublings in A-MSCs are effected. In addition, by preventing proliferation, senescence can also block differentiation pathways requiring proliferation, such as chondrogenic differentiation.[51] Being able to obtain cells with higher proliferation and differentiation capacity in a shorter time than is possible with A-MSCs makes P-MSCs an attractive cell source.

If P-MSCs are to be utilized in an allogeneic setting, it is important to show P-MSCs maintain immunomodulatory capabilities typically observed in A-MSCs[38,52]. MSCs are known to be able to manipulate T cell proliferation and phenotypic behavior, and their immunosuppressive nature makes them an interesting candidate from a clinical perspective[52–54]. In this study, we found P-MSCs were capable of inhibiting T cell proliferation at a level similar to what we previously reported with A-MSCs using a 1:5 ratio (MSC:PBMCs)[38]. For use in an allogenic model, it is advantageous for P-MSCs to inhibit T cell proliferation to prevent an unwanted immune reaction after transplantation. However, how P-MSCs interact with other immune cell types including antigen presenting cells needs to be determined to further understand how they would respond to a fully functional immune system.

A-MSCs have been reported to have inconsistent multilineage differentiation capabilities[15,20,55]. P-MSCs were capable of more consistent multilineage differentiation than the A-MSCs in this study. Senescence could have contributed, in part, to the difference we observed here as senescent MSC populations undergo phenotypic changes[56,57] and exhibit chromosomal abnormalities,[50,58] which ultimately could influence their differentiation capacity[51]. In this study, it is plausible that a combination of factors influenced the differentiation capacity of these cell populations. It is logical that cells from a younger, healthier patient who contain less senescent cells would be capable of better multilineage differentiation than senescent cells obtained from elderly patients. Having a cell source with more consistent differentiation capacities is ideal as it allows for more reproducible results.

Here we have described an easy isolation protocol that allows access to a P-MSC population with enhanced expansive and differentiation potential compared with AMSCs. P-MSCs with their rapid expansion, low senescence, and consistent multilineage differentiation are, therefore, prime candidates for applications from drug screening and development to use in ATMPs.

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DISCLOSURE STATEMENT

No competing financial interests exist.

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3

Pediatric Mesenchymal Stem Cells exhibit immunomodulatory properties towards allogeneic T and B cells under inflammatory conditions

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ABSTRACT

Mesenchymal stem cells from pediatric patients (pMSCs) are an attractive cell source in regenerative medicine, due to their higher proliferation rates and better differentiation abilities compared to adult MSCs (aMSCs). We have previously characterized the immunomodulatory abilities of pMSCs on T cells under co-culture. It has also been reported that aMSCs can inhibit B cell proliferation and maturation under inflammatory conditions. In this study, we therefore aimed to clarify the immunomodulatory effect of pMSCs towards T and B cells in an inflammatory microenvironment. Bone marrow derived pMSCs were primed to simulate inflammatory conditions by exposure with 50 ng/mL of IFN-γ for 3 days. To analyze the interaction between pMSCs and T cells, CD3/CD28 stimulated peripheral blood mononuclear cells (PBMCs) were co-cultured with primed or unprimed pMSCs. To investigate B cell responses, quiescent B cells obtained from spleens by CD43 negative selection were stimulated with anti-IgM, anti-CD40, IL-2 and co-cultured with either IFN-γ primed or unprimed pMSC. pMSC phenotype, B and T cell proliferation, and B cell functionality were analyzed. Gene expression of indoleamine 2,3-dioxygenease (IDO), as well as the expression of HLA-ABC, HLA-DR and the co-stimulatory molecules CD80 and CD86 was upregulated on pMSCs upon IFN-γ priming. IFN-γ did not alter the immunomodulatory abilities of pMSCs upon $CD4^+$ nor $CD8^+$ stimulated T cells compared to unprimed pMSCs. IFN-γ primed pMSCs but not unprimed pMSCs strongly inhibited naïve ($CD19^+CD27^-$), memory ($CD19^+CD27^+$), and total B cell proliferation. Antibody-producing plasmablast ($CD19^+CD27^{high}CD38^{high}$) formation and IgG production were also significantly inhibited by IFN-γ primed pMSCs compared to unprimed pMSCs. Collectively, these results show that pMSCs have immunomodulatory effects upon the adaptive immune response which can be potentiated by inflammatory stimuli. This knowledge is useful in regenerative medicine and allogeneic transplantation applications towards tailoring pMSCs function to best modulate the immune response for a successful implant engraftment and avoidance of a strong immune reaction.

INTRODUCTION

Mesenchymal stem cells (MSCs) are a source of self-renewing multipotent stem cells that are capable of differentiating along adipogenic, osteogenic and chondrogenic lineages [1, 2]. MSCs are also known to exert potent immunomodulatory effects upon a wide range of immune cells, both of the innate and the adaptive immune system [3]. This effect seems to be dependent upon the inflammatory conditions of the micro-environment in which these MSCs are found[4, 5]. Particularly for the adaptive immune response, pro-inflammatory cytokines, such as IFN-γ, seem to potentiate the anti-proliferative effect of MSC on T cells by inducing an increase in the activity of the enzyme indoleamine 2,3-dioxygenease (IDO)[6]. Regarding their interaction with B cells, MSCs have been described to suppress B cell proliferation through soluble factors [7] and abrogate plasmablast formation independently of T cells [8]. It has also been shown that upon IFN-γ stimulation, MSCs significantly inhibit B cell proliferation and maturation by upregulating IDO expression [9].

Due to their multipotent differentiation and immune modulation properties, MSCs have been successfully investigated for their use in several diseases such as ischemia[10], autoimmune diseases [11, 12], as well as in solid organ transplantation [13]. Other applications of MSCs include their potential use in tissue engineering and regenerative medicine [14, 15]. In particular in this field, bone marrow derived MSCs (BM-MSCs) have been successfully differentiated towards a chondrogenic phenotype and used for *in vivo* bone formation following the process of endochondral ossification[16, 17]. Nevertheless, the high variability between BM-MSC donors as a result of age and disease status has been shown to have an increasing importance by negatively influencing their bone formation potential in the case of elderly donors[18, 19]. Hence, a source of BM-MSCs with less age related variations are potentially more promising candidates for these applications[18]. Pediatric BM-MSCs (pMSCs) obtained from iliac crest bone chips from individuals between 7 and 13 years old have increased differentiation and proliferation capacities compared to adult BM-MSCs (aMSCs)[20]. pMSCs have been described to maintain an

immunophenotype identical to aMSCs and are significantly less senescent [20].

In the context of an allogeneic transplantation, the adaptive immune response plays an important role in determining the outcome of the engraftment of the allograft [21]. Naïve and memory $CD4^+$ and $CD8^+$ alloreactive T cells mediate rejection and graft-versus-host disease processes [21, 22]. The cross-talk between B and T cells is critical in these immune responses, since B cells are known to be the mediators of humoral rejection by producing donor-specific human leukocyte antigen (HLA) antibodies upon activation by T cells [23].

We have previously shown that pMSCs can exert an immunomodulatory effect on T cells by reducing their proliferation rates in an *in vitro* co-culture model [20]. Since in an allogeneic transplantation setting pMSCs might be subjected to an inflammatory microenvironment their immune properties might also be altered, affecting their success for clinical uses. Hence, to characterize how the inflammatory microenvironment can affect their immune status, in this study we investigated the effect of IFN-γ priming of a novel source of pMSCs on their immunomodulatory functionality towards B and T cells.

METHODS

Isolation and culture of human pediatric Bone Marrow Derived MSCs (pMSCs)

pMSCs were isolated from leftover iliac crest bone chips of pediatric patients undergoing alveolar bone graft surgery. Written consent was not required according to institutional guidelines for the use of waste surgical material but an opt out was available. This was approved by the Erasmus Medical Ethical Committee (MEC-2014-16). The age of the patients ranged between 9 and 13 years old Detailed information about age and sex of the donors can be found in Table 1.

Donor	Age (years)	Sex
Donor 1	12	Male
Donor 2	12	Female
Donor 3	10	Female
Donor 4	10	Male
Donor 5	Between 9 and 13	Male
Donor 6	9	Male

Table 1. Details of age and sex of the pMSC donors used in the study.

Briefly, pMSCs were obtained by washing the iliac crest chips twice with 10 mL of αMEM expansion medium supplemented with 10% heat inactivated fetal bovine serum, 1.5 µg/mL Amphotericin B, 25 µg/mL L-ascorbic acid 2-phosphate, 50 µg/mL gentamycin (all from Invitrogen) and 1 ng/mL fibroblast growth factor-2 (BioRad). The medium from the two washes containing the pMSCs was then plated in T75 flasks which were washed twice 24h after with phosphate-buffered saline (PBS) supplemented with 2% v/v heat inactivated fetal bovine serum to remove non-adherent dead cells. Viable pMSCs were at all times cultured at 37°C and 5% carbon dioxide (CO_2) in a humidified atmosphere. Expansion medium was replaced at least twice a week and the cells were firstly passaged when several visible colonies were detected using 0.05% trypsin-EDTA (Invitrogen). Upon the second passage, cells were always trypsinized at 70-80% of confluency. The cells showed an MSC characteristic morphology and were used between passages three and five for all experiments. Their phenotypic characteristics were previously extensively described by our group [20].

Isolation of PBMCs from peripheral blood

Peripheral blood from healthy male donors was obtained from Sanquin Bloedvoorziening (Rotterdam, the Netherlands). Samples were

centrifuged at 388 g for 7 min to remove the top layer of plasma and washed at a 1:2 dilution in wash medium (RPMI-1640 supplemented with 1.5 µg/mL Amphotericin B and 50 µg/mL gentamycin). The remaining cell suspension was then transferred to Ficoll-Paque PLUS (density 1.077 g/mL; GE Healthcare) containing tubes and centrifuged at 690 g for 20 minutes with the brake turned off. The plasma was removed and the layers above the filter were then washed with wash medium up to a total volume of 50 mL. Samples were then washed three times in wash medium as in the previous step, and then finally cells were counted and resuspended in human serum conditioned medium (PBMC medium) composed of 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 (Sigma-Aldrich). Cells were then resuspended in PBMC medium supplemented with a 10% of dimethylsulphoxide in appropriate numbers for optimal conservation and stored in liquid nitrogen until used for the experiments.

IFN-γ pre-stimulation of pMSCs

Based on previous optimization experiments, pMSCs were pre-treated for 3 days using IFN-γ (50 ng/mL, Peprotech) prior to co-cultures [9]. 24 hours before the co-culture day, cells were detached with 0.05% w/v trypsin-EDTA, washed with PBS and seeded in a 96 well plate at a density of $0.2x10^6$ cells per well in either 100 µL of Iscove's Modified Dulbecco's Medium (IMDM, Lonza) supplemented with a 10% v/v heat inactivated FBS (for B cell co-cultures), or in 100 µL of PBMC medium (for PBMC co-cultures).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

After 3 days of IFN-γ stimulation, $0.3x10^6$ pMSCs were placed in 300 µL of RLT buffer and snap frozen. RNA was isolated using a RNeasy micro kit (QIAGEN), and complementary cDNA was synthetized using a first strand cDNA kit (RevertAid cDNA kit, Thermo Scientific). qRT-PCR was performed using a 2x TaqMan Universal PCR master mix (Applied Biosystems), according to manufacturer's instructions and assay on demand primers

(Thermoscientific) for IDO (Hs 00158027.m1) and for GAPDH (forward: 5'-ATGGGGAAGGTGAAGGTCG-3', reverse: 5'-TAAAAGCAGCCCTGGTGACC-3', probe (FAM-TAMRA): 5' CGCCCAATACGACCAAATCCGTTGAC-3'). TAQ DNA polymerase (Hot Start) was activated for 10 min at 95°C, then DNA was amplified following 40 cycles of 15 seconds at 95°C, and 1 min at 60°C. TAQman was analyzed on a CFX-96 thermal cycler (BioRad). Results are expressed as relative copy number of PCR products in respect to the housekeeper GAPDH.

pMSC phenotyping by flow cytometry

pMSCs were immunophenotypically analyzed with and without IFN-γ pre-stimulation by assessing the expression of surface markers: HLA-ABC FITC, HLA-DR PerCP (clone G46.6), CD80 PE-Cy7 (clone L307.4), CD86 PE (clone 2331 FUN-1), all from BD Biosciences, San Jose, CA, USA by Flow Cytometry (FACS Jazz, BD Biosciences, San Jose, CA, USA). A total of N=3 different pMSC donors in triplicates were analyzed.

T cell proliferation analysis

Isolated PBMCs were thawed in 10 mL of pre-warmed PBMC medium and centrifuged at 248 g for 8 min. Cells were counted and in order to track proliferation, they were resuspended to a concentration of 10^7 cells/mL, and 20 µL of carboxyfluorescein succinimidyl ester (CFSE, 5µM) were added per 0.980 µL of cell suspension for 7 min at 37°C. After that time, cell suspensions were topped up to a 10 mL volume of cold PBMC medium, and centrifuged 10 min at 690 g. T cell proliferation was stimulated using antibodies against CD3 and CD28 (1 mg/mL,1 µL each per 10^6 cells, BD Biosciences) and a Goat linker antibody (0.5 mg/mL, 2 µL per 10^6 cells, BD Biosciences). Stimulated PBMCs were then added at 1:2.5, 1:5, 1:10 and 1:20 ratios to previously seeded pMSCs and co-cultured for 5 days.

PBMCs were removed by careful aspiration from the wells and washed with FACSflow. Cells were resuspended in 100 µL of FACSflow containing antibodies and fixed overnight in 4.6% paraformaldehyde. Prior to the

analysis, samples were washed and resuspended in 100 µL of FACSflow. To identify T cells and specific subsets, antibodies against CD3 PerCP (clone SK7), CD4 APC (SK3) and CD8 PE-Cy7 (SK1) were used (all from BD Biosciences, San Jose, CA, USA). T cell proliferation was tracked by flow cytometry using FACS Jazz. Samples were analyzed using the software FlowJo V10.07 (BD Biosciences). N=3 different pMSC donors with N=3 different PBMC donors in triplicates were analyzed.

Isolation of B cells from spleens

Human splenocytes were obtained from the spleens of deceased kidney donors (The Netherlands Law of organ donation [Wet op Orgaandonatie, WOD], article 13). Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient separation was performed on spleens segments that were previously mechanically disrupted and filtered with a 70 µM cell strainer. Mononuclear cells were stored at -150°C until the date of use. On the co-culture day, quiescent B cells were isolated from thawn splenocytes by using anti-CD43 magnetic beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). $CD19^+$ purity was determined on the $CD43^-$ fraction by flow cytometry (FACS Canto II, BD Biosciences, San Jose, CA, USA) and cells suspensions with a >97% purity were used.

B cell proliferation and subset analysis

Purified $CD43^-$ quiescent B cells were labelled with CFSE (Molecular Probes Invitrogen, Karlsruhe, Germany) as described previously for PBMC proliferation for 7 min at 37°C. After labeling, B cells were resuspended in IMDM with 10% v/v heat inactivated FBS supplemented with a cocktail to mimic T cell activation, composed of 1000 UI/mL IL-2 (Proleukin, Novartis, Prometheus laboratories Inc., San Diego, CA, USA), 10 µg/mL Goat anti-human IgM (Jackson Immunoresearch, Cambridgeshire, UK), and 5 µg/mL soluble recombinant human CD40L (Biolegend, San Diego, CA, USA). 100 µL per well of the previously described cell suspension were added to the previously seeded pMSCs at a 1:5 MSC:B cell ratio and co-cultured for 7 days.

B cell proliferation was characterized by flow cytometry (FACS Canto II) and analyzed with FlowJo V10.07 (BD Biosciences). Samples were collected by careful aspiration and centrifuged for 5 min at 690 g, and supernatants were stored at -80°C for IgG quantification. Cells were stained using the following flow cytometry antibodies: CD19-BV512 (clone HIB19), CD27 PE-Cy7 (clone 0323), CD38-PE (clone HB7), Viaprobe (BD Biosciences, San Jose, CA, USA). A total of N=3 different B cell donors co-cultured with one pMSC donor in duplicates or triplicates were analyzed.

IgG ELISA

Human IgG present in the supernatants was quantified by using a Human total IgG Ready SET Go ELISA kit (Thermofisher) following the manufacturer's instructions. Briefly, ELISA plates were coated overnight in coating buffer. The next day, samples were thawed in ice and diluted 1:2 in Assay buffer. Plates were washed twice with 400 µL per well of Wash Buffer, and blocked with 250 µL of Blocking buffer. 100 µL of either 1:2 diluted samples or standard IgG were added to the wells and incubated at room temperature for 2 hours with 400 rpm agitation. Upon incubation, wells were washed four times and 100 µL of detection antibody was added for 1 hour at 400 rpm. After 4 washes, 100 µL per well of substrate solution were added and incubated for 15 min, upon when the reaction was stopped by pipetting 100 µL of stop solution to each well. Absorbance was read at 450 nm using a Versamax plate reader.

Statistical Analysis

Data is expressed as mean ± Standard Deviation (SD), n=3 (experimental replicates) in duplicates or triplicates unless otherwise stated, where $p<0.05$ values were considered as statistically significant. For all B and T cell proliferation analysis, N=3 PBMC/B cell donors and a minimum of N=1 pMSC donors were used. Statistical analysis was performed by the software IBM SPSS Version 24 using a linear mixed model with Bonferroni post-correction test for all figures, except for Figure 1A for which a Mann-Whitney test was used. p values are represented as ***$p < 0.001$, ** $p<0.01$, *$p <0.05$.

RESULTS

IFN-γ pre-treatment upregulates the expression of IDO and immune related markers on pMSCs

To study the effect of IFN-γ on the expression of immunomodulatory and co-stimulatory molecules on pMSCs, 50 ng/mL of IFN-γ was added to undifferentiated pMSCs for 3 days. After that time, the expression of IDO was quantified by qRT-PCR, and CD80, CD86, HLA-ABC and HLA-DR surface levels were measured by FACS (Figure 1). Stimulation of pMSCs with IFN-γ significantly upregulated the gene expression levels of IDO (Figure 1A). Unprimed pMSC of all donors were highly positive for HLA-ABC, whereas for the rest of markers (CD80, CD86 and HLA-DR levels) unprimed cells expressed less than 50% of positive cells. Upon IFN-γ stimulation the percentage of positive cells of CD80, CD86 and HLA-DR was increased for all donors.. We also found an increase in the expression levels per cell, expressed as the Mean Fluorescence Intensity (MFI) of HLA-ABC and HLA-DR for all donors (Figures 1B and 1C).

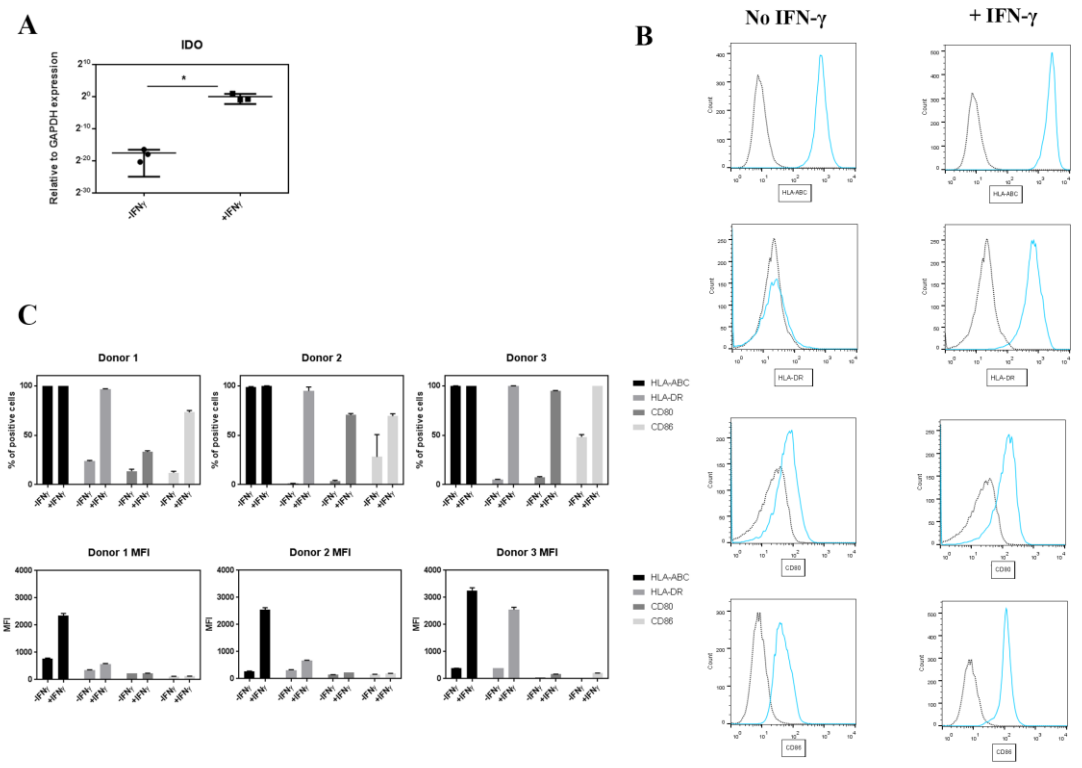

Figure 1. Effect of IFN-γ on the expression of immune regulatory and co-stimulatory markers on pMSCs upon IFN-γ stimulation. pMSCs treated with 50 ng/mL IFN-γ for 3 days or unprimed (-IFN-γ) pMSCs were analysed for IDO gene expression by qRT-PCR, and stained for HLA-ABC, HLA-DR, CD80, and CD86 expression and analyzed by FACS. **(A)** Expression of gene encoding for IDO relative to GAPDH. N=3 pMSCs donors, single replicates **(B)** Representative flow cytometry histograms of HLA-DR, HLA-ABC, CD80 and CD86 expression (blue line) on pMSCs based on the unstained control (dot line) with and without the addition of IFN-γ. **(C)** Percentage of positive cells and Mean Fluorescence Intensity (MFI) for HLA-ABC, HLA-DR, CD80 and CD86 markers. N=3 different pMSC donors in triplicates. Results are shown as means ± SD.

These results imply that IFN-γ pre-conditioning increases the expression of the immunomodulatory related enzyme IDO, but also the levels of HLA and co-stimulatory molecules involved in the activation of B and T cells on pMSCs.

IFN-γ pre-treatment on pMSCs does not affect their immunomodulatory properties upon T cells

To assess the immunomodulatory abilities of pMSCs towards T cells under inflammatory conditions, IFN-γ primed or unprimed pMSCs were co-cultured with stimulated PBMCs (+CD3/CD28 antibodies) at 1:2.5, 1:5, 1:10 and 1:20 pMSC:PBMC for 5 days (Figure 2). The proliferation of $CD4^+$ and $CD8^+$ T cells was tracked using CFSE. The reduction in T cell proliferation for both $CD4^+$ (Figure 2B) and $CD8^+$ (Figure 2C) T cell subsets induced by pMSCs was dose dependent for IFN-γ primed or unprimed pMSCs co-cultures. However, there was not a significant difference between the IFN-γ primed or unprimed conditions for any of the doses tested. Hence, we concluded that pMSCs are able to exert their immunomodulatory abilities towards T cells regardless of the addition of IFN-γ.

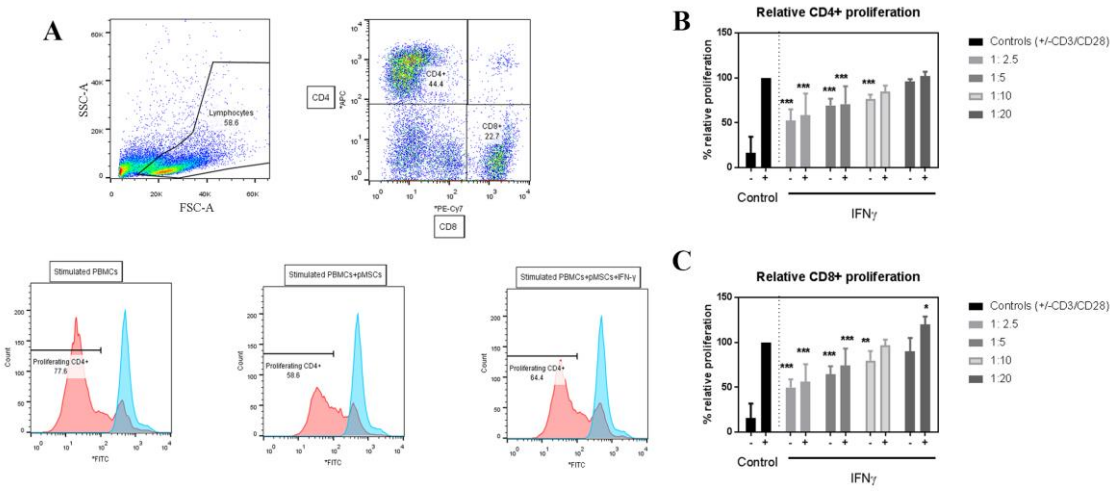

Figure 2. pMSCs are immunomodulatory towards T cells in a dose-dependent manner. CD3/CD28 stimulated PBMCs were co-cultured with IFN-γ primed (+IFN-γ) or unprimed (-IFN-γ) pMSCs at 1:2.5, 1:5, 1:10 and 1:20 pMSCs:PBMCs ratios. Flow cytometric analysis was performed after 5 days of co-culture, and $CD4^+$ and $CD8^+$ proliferating T cells were detected by CFSE. **(A)** Representative FACS plots and histograms showing the gating strategy for stimulated $CD4^+$ and $CD8^+$ T cells alone or in co-culture with unprimed or IFN-γ primed pMSCs. **(B)** CD4+ and **(C)** CD8+ T cell proliferation in co-culture with unprimed or IFN-γ primed pMSCs. Results are expressed as the relative proliferation measured as the 1/Mean Fluorescence Intensity (MFI) of CFSE normalized to the (+CD3/CD28) stimulated control. N=3 different pMSC donors with N=3 different PBMC donors in triplicates. Results are represented as means ± SD.

IFN-γ primed pMSCs significantly reduce the proliferation rates of B cells

To examine whether inflammatory conditions could influence the anti-proliferative capacities of pMSCs on B cells, we co-cultured IFN-γ primed pMSCs with stimulated B cells for 7 days. We hypothesized that IFN-γ primed pMSCs would have similar immunomodulatory capacities on B cells compared to aMSCs [9].

B cells were stimulated with anti-CD40, anti-IgM, and IL-2 to mimic T cell activation in the absence of T cells. Viable B cells (Viaprobe^{-} CD19^{+}) were analyzed by FACS and classified into CD27^{+} (memory) and CD27^{-} (naïve) B cells or plasmablasts (CD27^{high} CD38^{high}) (Figure 3A).

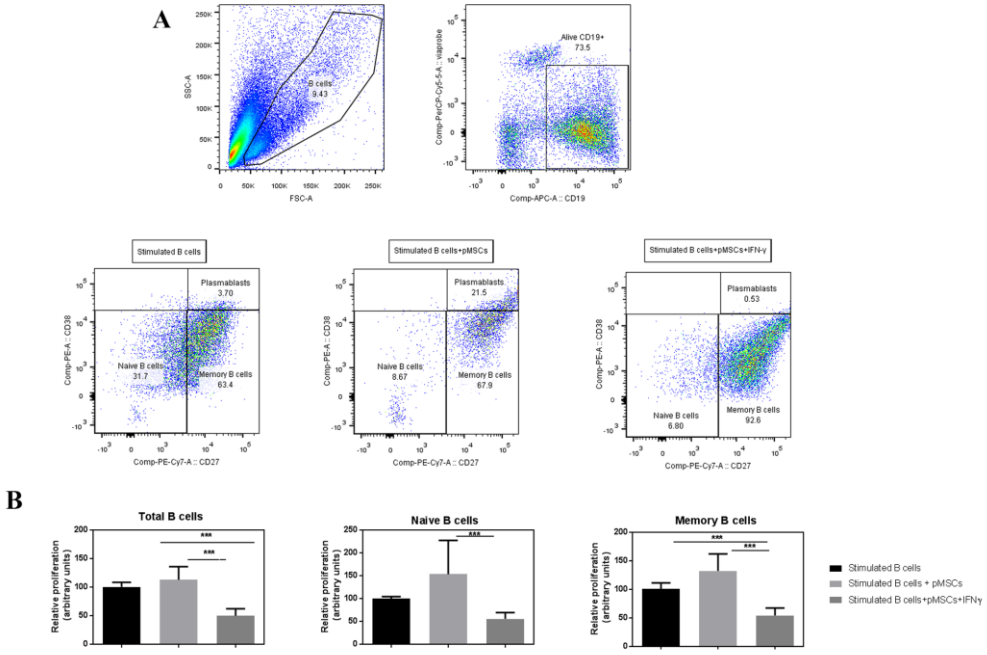

Figure 3. IFN-γ primed pMSCs significantly reduce the proliferation rates on naïve, memory and total numbers of B cells compared to unprimed cells. Stimulated (+anti-CD40, anti-IgM, and IL-2) B cells were co-cultured with either IFN-γ primed or unprimed pMSCs. B cells were retrieved and analyzed by flow cytometry after 7 days. **(A)** FACS gating strategy of viable CD19^{+} B cells that were classified according to the expression of CD27 into memory (CD27^{+}), naïve (CD27^{-}) B cells, or plasmablasts (CD27^{high} CD38^{high}). **(B)** Relative proliferation of B cells when co-cultured with IFN-γ primed or unprimed pMSCs. Results are shown as the 1/MFI (CFSE) of CD19^{+} viable cells (total B cells), CD27^{+} cells (memory), and CD27^{-} cells (naïve) relative to the B cell stimulated condition. N=3 B cell donors co-cultured with 1 pMSC donor in duplicates or triplicates. Figures show means ± SD.

Co-culture of unprimed pMSCs with B cells did not reduce proliferation rates of total B cells, naïve or memory B cells, but a tendency towards an increased proliferation was observed compared to stimulated B cells on their own. However, IFN-γ pre-conditioning of pMSCs decreased the proliferation rates of naïve, memory and total B cells in co-cultures compared to stimulated B cells. Moreover, IFN-γ primed pMSCs significantly decreased the proliferation rates of all B cell subsets when compared to unprimed pMSCs. (Figure 3B).

IFN-γ primed pMSCs abrogate plasmablast differentiation and IgG production

After 7 days of co-culture with IFN-γ primed or unprimed pMSCs, we determined the frequencies of $CD19^{+}CD27^{high}CD38^{high}$ plasmablast cells by FACS (Figure 3A). In order to characterize B cell functionality, we quantified the amount of antibody released by B cells by measuring the IgG production in the supernatants of the co-cultures by ELISA.

Unprimed pMSCs did not significantly reduce the number of plasmablasts when co-cultured together with stimulated B cells (Figure 4A). However, there was a statistically significant reduction in the percentage of plasmablasts detected when co-cultured with IFN-γ primed pMSCs (8.9% of plasmablasts versus 1.8%, respectively). A significant reduction in the amount of IgG present in the supernatants was also found when B cells were co-cultured with IFN-γ primed pMSCs compared to unprimed pMSCs, from 43 ng/mL to 14 ng/mL (Figure 4B)

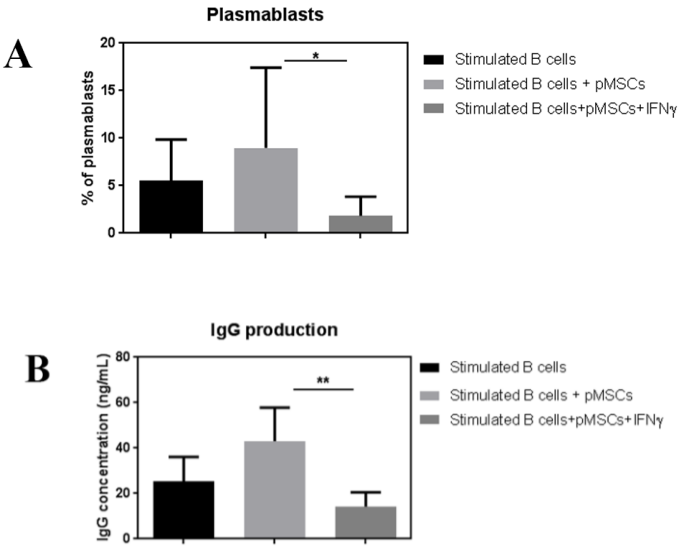

Figure 4. IFN-γ primed pMSCs but not unprimed significantly decrease plasmablast differentiation and IgG production. (A) Plasmablast frequencies were measured upon 7 days of co-culture of stimulated B cells with IFN-γ primed or unprimed pMSCs. Results are expressed as the % of viable $CD19^{+}$ $CD27^{high}$ $CD38^{high}$ cells **(B)** IgG concentration (ng/mL) was detected in the supernatants of stimulated B cells and IFN-γ primed or unprimed pMSCs by ELISA. N=3 different B cell donors co-cultured with 1 pMSC donor Results are shown as means ± SD.

DISCUSSION

The immunomodulatory abilities of MSCs upon the adaptive immune response have been previously investigated in a number of studies [8, 9, 24, 25]. Moreover, MSCs immunomodulatory properties upon T and B cells have been shown to be highly influenced by the local inflammatory microenvironment[4]. These studies have reported some promising alternatives to tailor the immunosuppressive abilities of MSCs, by subjecting them to certain pro-inflammatory cytokines such as IFN-γ [9, 26]. However, they were performed using MSCs derived from diverse sources of aMSC donors, which can lead to high variability and unpredictable results due to a large age and origin variation among donors [27], as well as reduced immunomodulatory abilities associated to age [28]. We have previously established that the use of a potent source of pMSCs with enhanced multilineage differentiation and expansion abilities, as well as reduced senescence, could make them attractive candidates for regenerative medicine [20]. Moreover, in the same study pMSCs were shown to reduce T cell proliferation in an allogeneic *in vitro* co-culture model. Here, we aimed to further investigate the immunomodulatory abilities of pMSCs towards T and B cells under inflammatory conditions as a promising MSC source for regenerative medicine and allogeneic transplantation with more consistent differentiation abilities and increased expansion properties.

Inflammatory signals have been suggested to alter the immunomodulatory functionality of MSCs towards B cells [4]. Luk *et al*. [9] previously showed that IFN-γ primed adipose derived aMSCs inhibited naïve ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cell proliferation. In this study we clarified the immunomodulatory abilities of pMSCs upon stimulated B cells. Our findings suggest that IFN-γ priming is crucial for pMSCs in order to exert their immunomodulatory functionality upon B cells. IFN-γ primed pMSCs but not unprimed pMSCs showed a significant reduction in B cell proliferation for all different B cell subsets.

Franquesa *et al*. (2015) [8] previously reported that in the presence of adipose-derived MSCs, antibody producing plasmablast

($CD19^{+}CD27^{high}CD38^{high}$) formation was inhibited. Hence, we hypothesized that pMSCs would also reduce plasmablast differentiation and that this effect would be enhanced by IFN-γ pre-priming. Our results showed that pMSCs reduced the numbers of plasmablasts only upon priming them with IFN-γ. We also showed that antibody production was significantly decreased when IFN-γ primed pMSCs were present and not in the presence of unprimed pMSCs. Since plasmablasts possess an antibody-producing functionality, these results suggest that in the case of an inflammatory microenvironment pMSCs significantly suppress humoral responses mediated by B cells. Moreover, since IFN-γ can be produced by T cells upon activation, these results suggest that the cross-talk between B and T cells might be of importance as a potential source of IFN-γ that can promote pMSC's immunosuppressive mechanisms upon B cells. In the situation of an allogeneic transplant, these properties of pMSCs might be advantageous to avoid allo-antibody formation against the allograft, one of the principal consequences of rejection and graft versus host disease (GvHD) [29]. This knowledge might also be useful in the clinical context where an acute or chronic B cell mediated inflammation is present, such as autoimmune diseases or osteoarthritis.

MSCs are also well known to express major histocompatibility complex (MHC) class I molecules on their surface, but to have a minimal expression of MHC class II [30] and co-stimulatory molecules such as CD80 and CD86 [1]. Addition of IFN-γ has been reported to enhance the expression of MHC class I and II [31], but it does not seem to increase the levels of CD80 and CD86 [32]. Here, we show that unprimed pMSCs express high levels of MHC class I (HLA-ABC) and low levels of MHC class II (HLA-DR), CD80 and CD86. However, IFN-γ stimulation upregulated the levels of HLA-DR and both co-stimulatory molecules on pMSCs. This increase could mean that pMSCs would be more prone to act as antigen presenting cells and activate T cells under inflammatory conditions [33]. Many studies have discussed the crucial role of IFN- γ on MSCs immunomodulatory abilities towards T cells [4, 34]. Since an increased immunosuppressive effect from MSCs upon T cells under inflammatory conditions has been linked to a higher IDO activity [6], we examined the gene expression of IDO on pre-primed pMSCs. Our

results showed that, in contrast to previous studies [34, 35], IFN-γ priming did not affect pMSCs immunomodulatory abilities upon T cells. However, IFN-γ significantly upregulated the gene expression of IDO in all pMSCs donors. Therefore, it is possible that the upregulation of HLA-DR, CD80 and CD86 triggered by IFN-γ on pMSCs counteracted the IDO mediated enhancement on their immunomodulatory abilities upon T cells. The extent of this upregulation was observed to be donor dependent. This indicates that intrinsic variation among pMSCs donors might impact the immunomodulatory abilities of pMSCs towards T cells according to the degree of expression of co-stimulatory molecules in an inverse manner. Despite this, under inflammatory conditions, pMSCs were still able to suppress T cell proliferation in a similar trend than unprimed pMSCs. Previous studies have reported as well that the immunomodulatory potency of MSCs is highly related to the inflammatory milieu created by T cells [36]. Hence, the cytokine profile of T cells might in turn affect the immunomodulatory effect of MSCs on immune cells.

Differences in the immunomodulatory abilities of IFN- γ primed pMSCs compared to other sources of aMSCs may depend on multifactorial pathways that have been described to play a role in the degree of immunomodulation on MSCs towards T and B cells. Luk *et al.*[9], previously reported that upon IFN-γ addition, MSCs inhibited B cell proliferation as well as IgG production and regulatory B cells (B_{reg}) formation through the tryptophan depleting activity of the enzyme IDO. Some studies have reported the involvement of other immunomodulatory mechanisms, such as Galectin-9 (Gal-9) which significantly reduced IgG titers in an *in vivo* murine model [37].The Cyclooxygenase 2 (COX-2) pathway has also been indicated to play a role in Breg suppression through IL-10 depletion [38]. Signaling pathways involving the release of metalloproteinase-processed CC-chemokine ligand 2 (CCL-2) by MSCs controlled by the downregulation of olfactory 1/early B cell factor–associated zinc-finger protein (OAZ) have been proposed as a mechanism of MSC inhibition of IgG synthesis on B cells [39].

Moreover, it is also important to remark that different extents in the increase of co-stimulatory molecules on pMSCs surface, which seems to be donor-dependent as presented in this study, might entail as well differences in IFN- γ primed pMSCs immunomodulatory abilities .This is due to the fact that a higher upregulation of these co-stimulatory molecules, such as CD80 and CD86, in certain donors may negatively impact the immunosuppressive potential of IFN- γ primed pMSCs. Hence, this intrinsic donor variability on the immune profile of pMSCs needs to be taken into account when considering using pMSCs for immune therapies.

Together, we show that IFN-γ priming exerts an impact on the expression of immune markers and co-stimulatory molecules on a novel source of pediatric MSCs. In this context, pMSCs maintain their immunomodulatory abilities upon T cells, significantly suppress B cell proliferation, as well as plasmablast differentiation and antibody production. *In vivo*, this might mean that upon receiving certain inflammatory signals, such as IFN-γ produced by activated T cells, pMSCs safeguard their immunomodulatory status towards T cells, and gain anti-proliferative abilities upon B cells, avoiding an allo-antibody immune response. This knowledge is useful for the design of novel immunotherapies in several types of diseases where a chronic inflammation is present, as well as in the areas of regenerative medicine and solid organ transplantation by using a novel MSC source with enhanced differentiation abilities and an immune privileged condition.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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4

Bone formation by human pediatric 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 humanized 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 mineralization in the presence of an allogeneic immune system for 3 weeks without the onset of immune responses. Re-exposing the splenocytes of the humanized 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.

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-ß)-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]. We have previously *in vitro* characterized 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 characterized, 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 mineralization has also been correlated with T cell presence in a bone defect animal model, proving earlier mineralization 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 humanized animal model that persisted for at least 14 weeks without triggering significant immune responses.

MATERIALS AND METHODS

Study design

The aim of this study was to analyze 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 analyzed 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 analyzed by immunohistochemistry and PCR.

Subsequently, we utilized 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 analyze 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 (humanization) for three weeks, and upon that time subcutaneous implantations were performed, up to week 6 (Fig. S1). In the same study, splenocytes from the humanized animals were isolated and re-exposed *ex vivo* to the same (matched) and a different (mismatched) pMSC donor for 3 days, and the T cell responses were analyzed 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 humanized or non-humanized mice (donors) and re-transplanted in a second group of non-humanized or 2-week-humanized mice (recipients) up to week 14 (Fig. S5). Bone formation was evaluated by measurements of the mineralization on ch-

pMSC grafts by Micro-Computer Tomography (μCT). The interactions of the ch-pMSCs grafts with the allogeneic immune system were analyzed 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 humanized 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 humanization.

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].

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].

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 $0.2x10^6$ 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-ß3 (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.

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 $1x10^6$ either Carboxyfluorescein succinimidyl ester (CFSE)-labeled unstimulated (for immunogenicity experiments) or CD3/CD28 stimulated (1mg/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 (Sigma-Aldrich) 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).

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 analyzed using FACS Jazz (BD Biosciences) and Flowjo v.10. Results were individually plotted per round of experiment, but analyzed together. As a measurement of proliferation, the Mean Fluorescence Intensity (MFI) of the CFSE incorporation in the cells was analyzed, and the 1/MFI was plotted to represent a loss of the CFSE as the cells proliferate more.

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 hour light/dark cycle. At the harvest timepoint (week 6, 12 or 14 post-implantation), animals were euthanized by cardiac puncture and cervical dislocation under general anaesthesia, and bone constructs, blood, spleens, and femurs were retrieved.

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. $5x10^6$ viable PBMCs in 0.2 mL of PBS per animal were then intraperitoneally (IP) injected.

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 2, 3 or 12 weeks, depending on the experimental setup (Figure. S1, S3 and S5)

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 (Perkin-Elmer, 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 uA) at the Quantum-FX. For evaluating the long-term allogeneic immune responses, calcification was detected using a 30 mm field of view for 4 min (90 kV/160 uA) at the Quantum-GX2.

Bone mineralization 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 Analyze 11.0 (AnalyzeDirect)

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 minutes, 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 minutes and 3mL of pre-diluted 1X RBC lysis buffer (eBioscience) was added per spleen and incubated for 10 minutes. After that time, 20 mL of PBS was added and cells were centrifuged for 5 minutes 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 analyzed using FACS Jazz (BD Biosciences) and Flowjo v.10.

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 1.5mL 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 minutes. 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 minutes, supernatant was removed and the pellet was resuspended in 500 ul 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 analyzed using the FACS Jazz and Flowjo v.10.

***Ex vivo* analysis of splenocyte responses**

Graft-matched and graft-mismatched pMSCs were pre-treated with 50 ng/mL of IFN-γ (Peprotech) for 3 days. 24 hours 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 minutes 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 labelled 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.

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-Aldricht) 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 deparaffinized and six micrometer thick sections were cut and prepared for histochemistry and immunohistochemistry. Hematoxylin-Eosin staining and TRAP stainings were then performed as previously described.

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, 1mM 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, 150mM NaCl; Sigma Aldrich) for the human GAPDH, or PBS for the rest of immunohistochemical stainings with 1% w/v BSA and 1% w/v milk (Elk, Campina).

Slides were then stained for 1 hour at room temperature (CD3 and GAPDH) or overnight at 4°C (CD4 and CD8) using either a human GAPDH specific antibody (0.2 ug/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 ug/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 visualized using 0.01% w/v Neu Fuchsin, 0.01% $NaNO_2$, 0.3 mg/ml Naphtol AS-MX (Sigma) and 0.25 mg/ml levamisole (Sigma) in 0.2 M Tris-HCl. After staining, samples were counterstained with haematoxylin. 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.

mRNA Gene expression analysis

Samples were homogenized 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 000g 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 5ng 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).

Statistical analysis

Samples were analyzed 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.

RESULTS

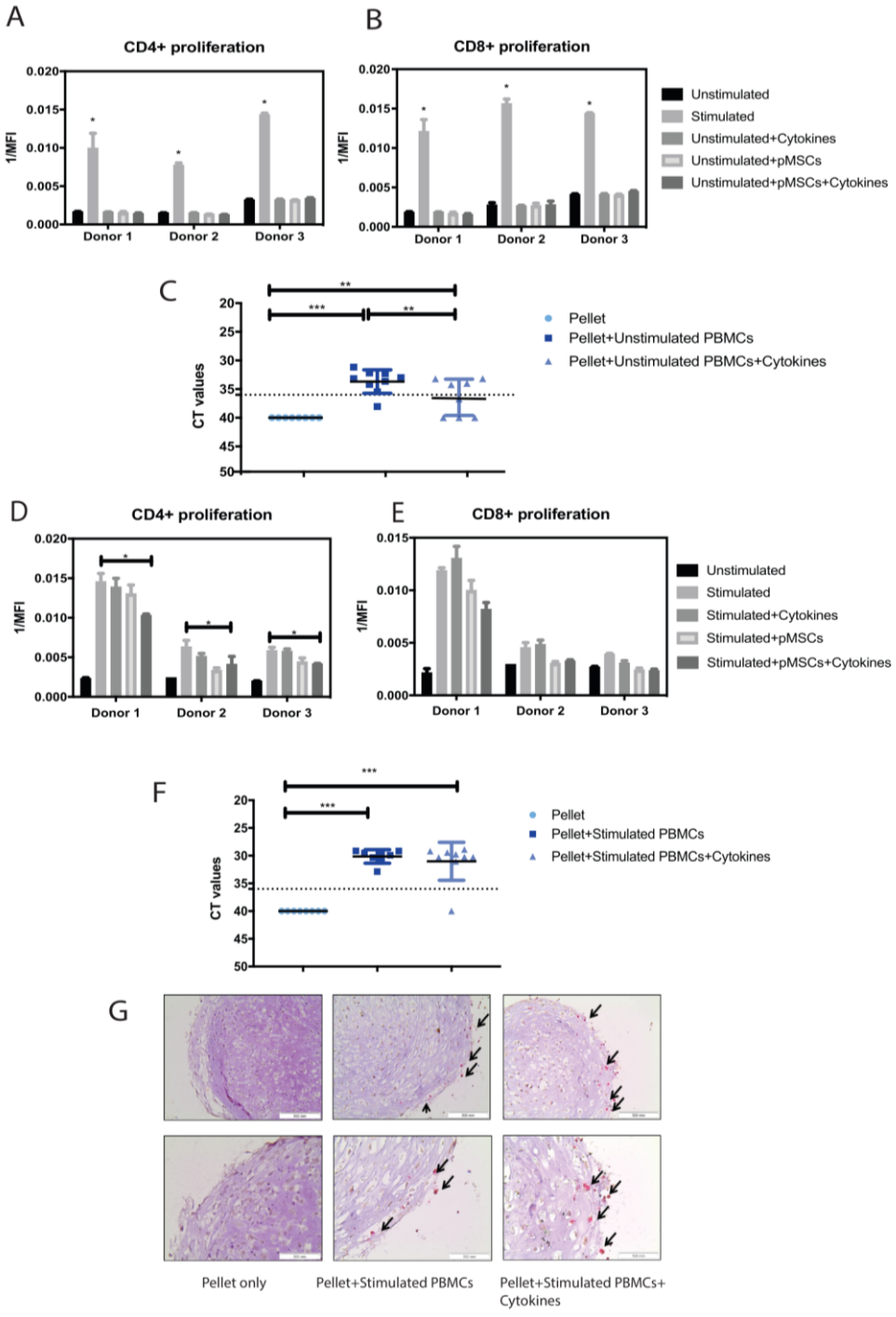
Allogeneic ch-pMSCs remain non-immunogenic under pro-inflammatory conditions

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 1B) regardless of the presence of the pro-inflammatory cytokines. To determine whether allogeneic T cells were detectable within the pellets, we analyzed 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 (Figures 1D and 1E). 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. To examine whether activated T cells migrate into the pellets, the gene expression levels (Figure 1F) as well as the immunohistochemical levels of CD3 (Figure 1G) were analyzed. 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.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Unstimulated+Cytokines
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Unstimulated+pMSCs+Cytokines
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Pellet+Unstimulated PBMCs+Cytokines
D
CD4+ proliferation
E
CD8+ proliferation
Stimulated+Cytokines
Stimulated+pMSCs
Stimulated+pMSCs+Cytokines
F
Pellet+Stimulated PBMCs
Pellet+Stimulated PBMCs+Cytokines
G
Pellet only
Pellet+Stimulated PBMCs
Pellet+Stimulated PBMCs+ Cytokines

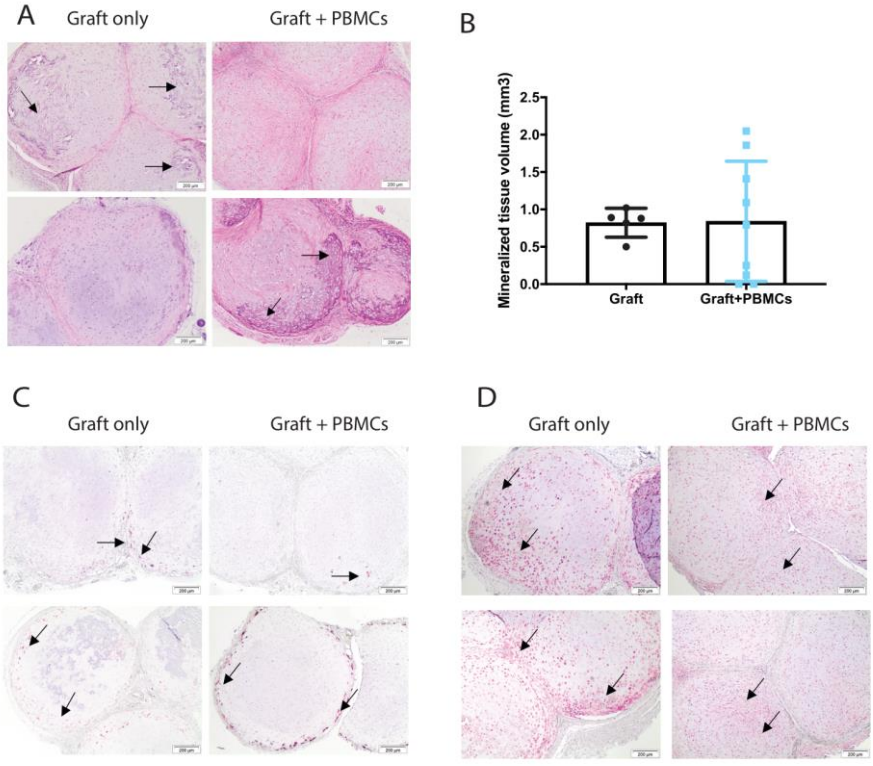
Figure 1. ***In vitro*** **evaluation of the immunogenicity and immunomodulatory abilities of ch-pMSCs under pro-inflammatory conditions.** Flow cytometric analyses of the CD4+ **(A)** and CD8+ **(B)** T cell proliferation rates of unstimulated PBMCs in co-culture with ch-pMSCs and 10 mg/mL of IFN-γ 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 **(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.

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 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 humanization (Fig. S2). The results showed similar levels of circulating human $CD45^+$ cells in blood in the humanized 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 remodelling observed in samples that had received the graft with and without humanization (Figure 2A). The μCT scans after 3 weeks of implantation revealed variations in the mineralized tissue volume of the grafts that belonged to humanized animals, however this was not significant compared with the graft only group (Figure 2B). Evidence of $TRAP^+$ osteoclastic presence was detectable in both conditions (Figure 2C). Despite this, we did not find a significant correlation between the amount of $TRAP^+$ cells and the bone volumes of the grafts (Figure 2E, R=0.31). A human-specific GAPDH immunohistochemical staining revealed donor-derived human cells (Figure 2D) in both conditions throughout the grafts, suggesting an active role of the donor cells as well as the host cells in the early stages of bone formation.



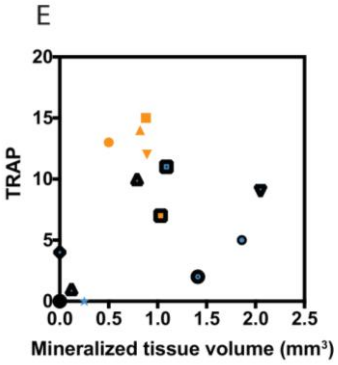


Figure 2. Analyses of the potential of ch-pMSCs to persist *in vivo* in the presence of a pre-existing allogeneic human immune system. (A) Hematoxylin and eosin staining of ch-pMSC derived grafts implanted for 3 weeks in 3 week-humanized mice. Arrows indicate areas of active matrix mineralization and remodelling. **(B)** Quantification of the µCT scan data showing mineralization (mm^3, n=5-9 grafts per group). A linear mixed model with Bonferroni post-correction was used for this analysis. **(C)** TRAP histochemical staining. Arrows indicate areas of positive staining. **(D)** Human GAPDH histochemical staining. **(E)** 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.

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 analyzed at week 6 post-humanization (Fig. S1). Humanized 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 (Figure 3A). This data was supported by the presence of $CD45^+$ human cells by flow cytometry (Figure 3B). To further characterize the human T cells detected in the graft,

a CD4 vs CD8 analysis was carried out (Figure 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 (Figure 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 mineralized tissue volume and the $CD3^+$ human cells (R=-0.104) (Figures 3E and 3F). This data suggests a role for the allogeneic human immune system in the initial stages of graft recognition.

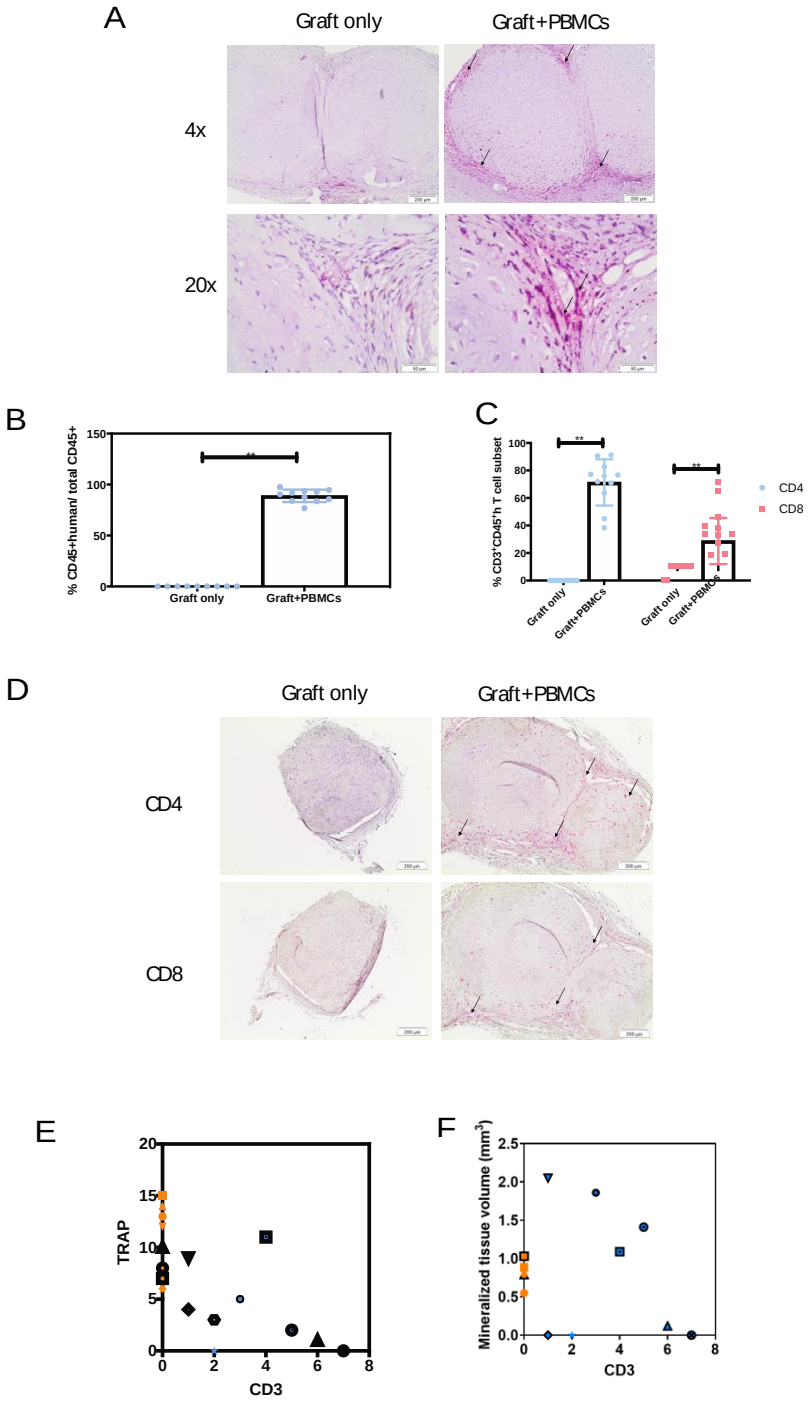

A
Graft only
Graft+PBMCs
4x
20x
B
% CD45+human/ total CD45+
Graft only
Graft+PBMCs
C
% CD3+CD45+h T cell subset
CD4
CD8
D
Graft only
Graft+PBMCs
CD4
CD8
E
TRAP
CD3
F
Mineralized tissue volume (mm3)
CD3

Figure 3. Allogeneic human T cells are detected in the grafts after 3 weeks of implantation in a 6-week humanized 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.

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 humanization were retrieved. Spleen cell suspensions were re-exposed *ex vivo* to the same pMSCs donor used in the grafts (matched) or to a different pMSC donor to which the PBMCs had not been exposed (mismatched) for 3 days. Heat-inactivated matched pMSCs were used as a negative control of immunogenicity. 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) (Figure 4A). Importantly, $CD3^+$ T cell proliferation was also unaffected by the presence of pMSCs, regardless of their matched or mismatched origin or IFN-γ preactivation status (Figure 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

(Figures 4C and 4D) 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.

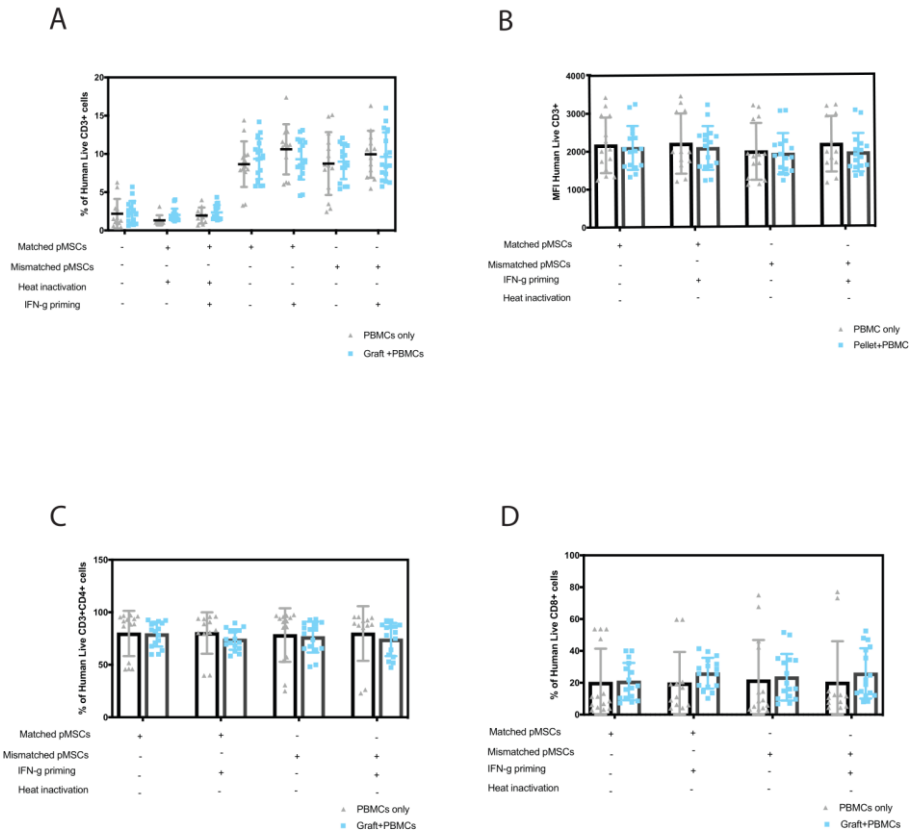


Figure 4. pMSCs do not elicit proliferation from pre-sensitized splenocytes regardless of the degree of mismatch. Flow cytometric analyses of graft-matched and mismatched pMSCs in co-culture with splenocytes from humanized mice for 5 days revealed that pMSCs promote allogeneic T cell survival (% live 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 analyzed using a linear mixed model with Bonferroni post-correction.

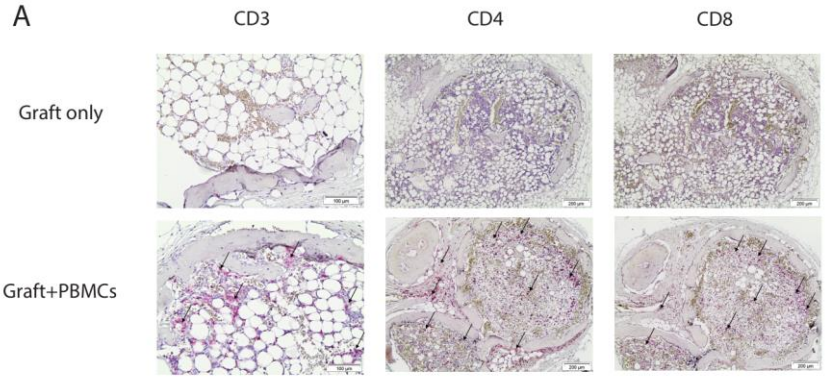
Allogeneic human T cells are detected in the mature ch-pMSC-derived grafts

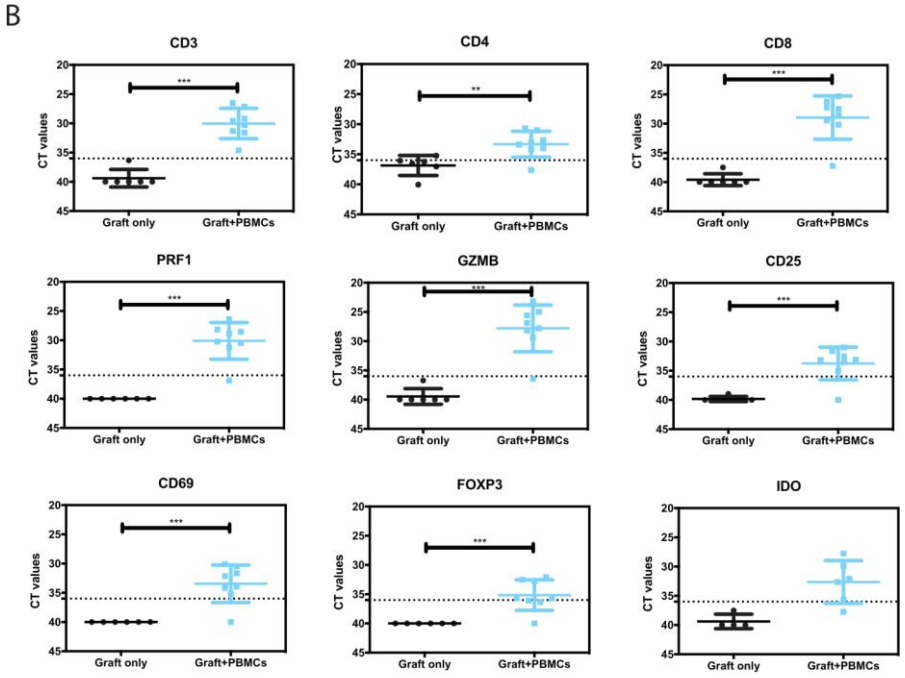
To assess the survival of mature ch-pMSC derived bone grafts within an allogeneic immune seting in the medium to long term and their immunogenicity, cell pellets were implanted for 8 weeks in immunodeficient animals, during which time they underwent endochondral ossification. After 8 weeks a PBMC injection was given for a further 4 weeks (Fig. S3). A similar percentage of circulating $CD45^+$ levels of human cells were detected in the blood of the humanized 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 humanized 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-humanized mice (Figure 5A). When further analyzing the $CD3^+$ allogeneic human T cells subsets, both $CD4^+$ and $CD8^+$ were detected within the graft-derived marrow of the humanized animals, without a predominance of one or another in a certain region.

Gene expression (Figure 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 analyzed a panel of genes associated with T cell functionality. Both CD4 and CD8 expression was detected in the grafts of animals that received a humanization. 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 humanized 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 humanized group (Figure 5C).


A
CD3
CD4
CD8
Graft only
Graft+PBMCs


B
CD3
CD4
CD8
PRF1
GZMB
CD25
CD69
FOXP3
IDO
CT values
Graft only
Graft+PBMCs

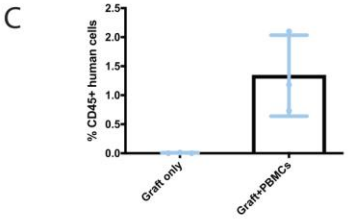
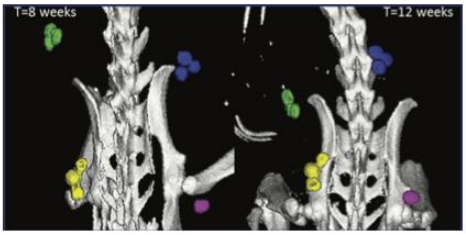

C
% CD45+ human cells
Graft only
Graft+PBMCs

Figure 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 humanized 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 analyzed using a linear mixed model with Bonferroni post-correction, **P<0.01, ***P<0.001

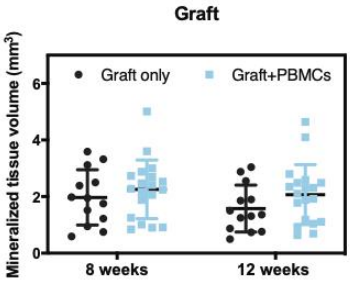
Allogeneic bone derived from ch- pMSCs persists after 4 weeks

μCT analysis revealed the presence of ectopic mineralized tissue at both 8 weeks (before the start of the humanization) and 12 weeks (4 weeks post-humanization) (Figure 6A). No significant differences in the mineralized tissue volume were detected in the grafts regardless of the humanization (Graft only versus Graft+PBMCs) at neither of the timepoints (Figure 6B), with no decrease in bone volume between pre and post humanization. The retrieval of the grafts revealed a structure comprised of an outer bone ring, a bone marrow and some remains of calcified cartilage (Figure 6C). 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 structural differences of the grafts or marrows were observed between the animals that received a PBMC injection versus the graft only controls. 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 (Figure 6D), 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.

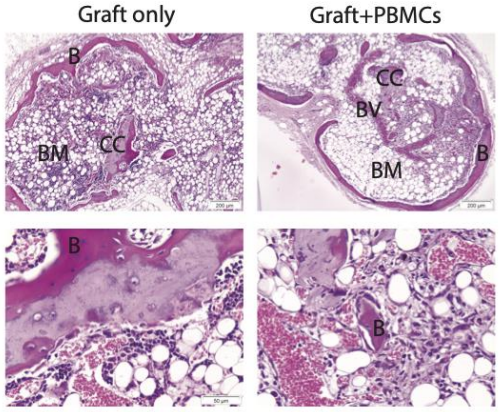
A


T=8 weeks
T=12 weeks

B


Graft
Graft only
Graft+PBMCs
Mineralized tissue volume (mm³)
0
2
4
6
8 weeks
12 weeks

C


Graft only
Graft+PBMCs
10x
4x
B
BM
CC
BV

D

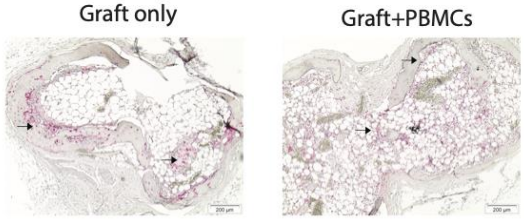

Graft only
Graft+PBMCs

Figure 6. ch-pMSC-derived bone formation occurs in an allogeneic immune system after 12 weeks. (A) Representative μCT scans of the mineralized grafts at 8 weeks and 12 weeks. Each color represents an individual graft. **(B)** μCT scan quantification of the mineralized tissue volume at week 8 (before humanization) and week 12 (4 weeks after humanization) (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)** 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)

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 humanization, which prevented long-term graft survival experiments, we employed a re-transplantation model. Hence, a second group of recipient mice, that were humanized for 2 weeks, received mature 12-week grafts (Figure S5). During the 4 weeks prior to the re-transplantation, the donor mice had been humanized with the same allogeneic PBMC donor as the recipients, and the grafts contained allogeneic T cells. Humanization 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 humanized recipients that received grafts coming from humanized animals (H to H) compared with the ones that received non-humanized grafts (KO to H) (Figure 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 (Figures 7B and 7C).

When looking at the presence of the immune cells in the grafts, we reported a higher infiltration of $CD45^+$ human cells in the non-humanized animals that received a humanized graft (H to KO) in comparison with the humanized animals that received a humanized graft (H to H) (Figure 7D). However, similar percentages of $CD4^+$ and $CD8^+$ human immune cells

were found in both of the groups (Figures. 7E and 7F). These results were further confirmed by a histological staining of human CD3, CD4 and CD8 (Figure 7G).

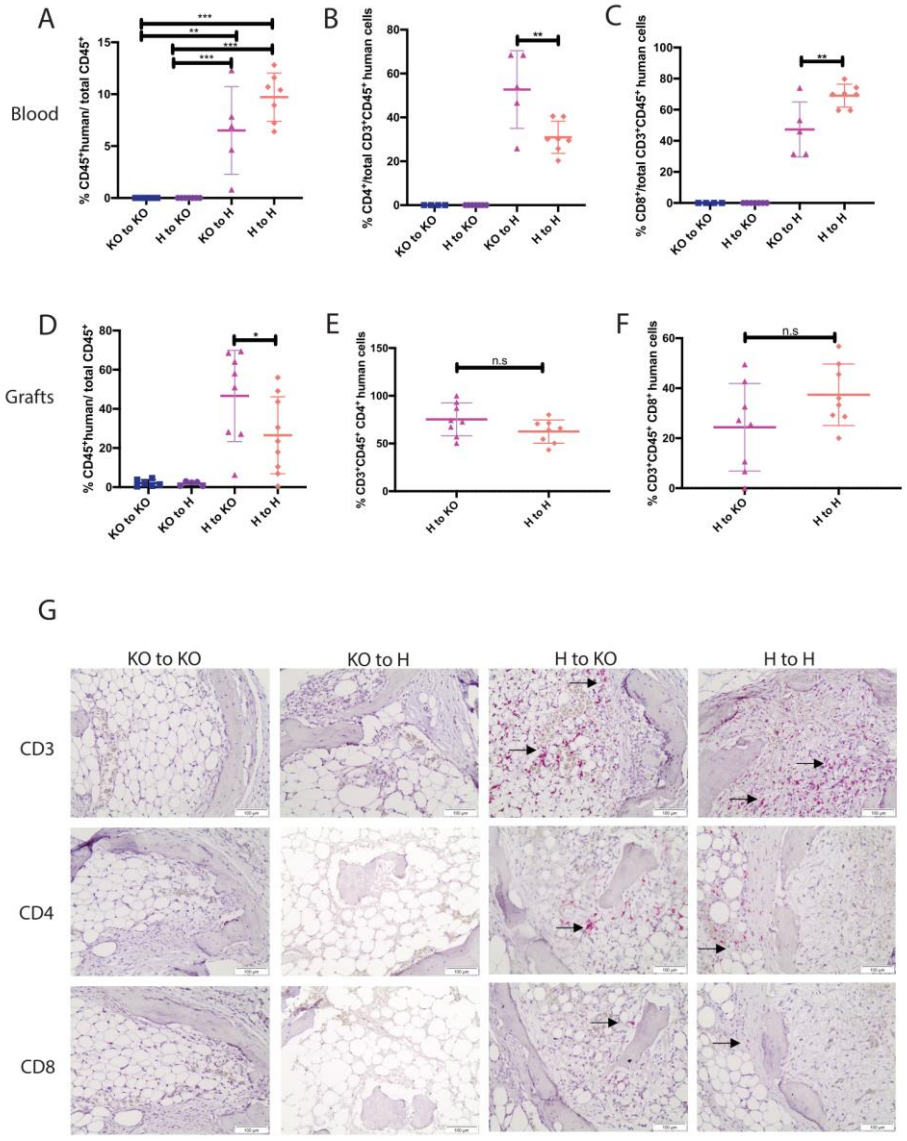
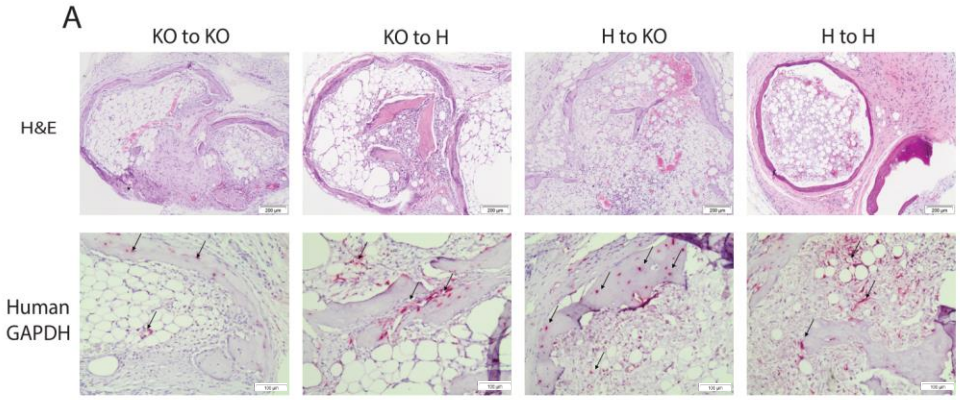


Figure 7. No significant graft-directed allogeneic immune responses are detected in the long term. 12-week grafts from 4-week humanized animals were serially transplanted in 2-week humanized animals for a further 2 weeks. The percentage of circulating $CD45^+$ **(A)**, $CD4^+$ **(B)** and $CD8^+$ **(C)** 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 analyzed: immunodeficient recipients that received grafts from immunodeficient donors (KO to KO), immunodeficient recipients that received grafts from humanized donors (H to KO), humanized recipients that received grafts from immunodeficient donors (KO to H), and humanized recipients that received grafts from humanized donors (H to H). N=5-9 animals per group, or 5-9 grafts per group. Data was analyzed using a linear mixed model with Bonferroni post-correction, *P<0.05 **P<0.01, ***P<0.001, n.s=non significant.

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 (Figure 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 (Figure 8B), with a tendency towards an increase in the mineralized 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 (Figure 8A).



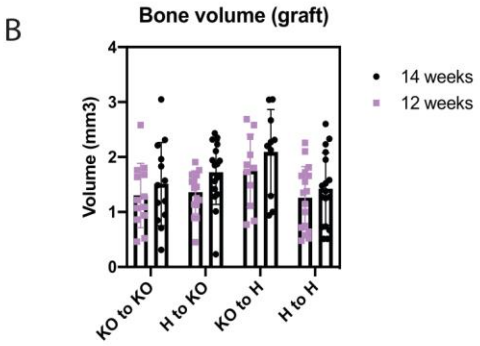


Figure 8. Mature allogeneic bone persists in the recipient animals. (A) Representative images of the histological stainings showing the structure and the human GAPDH presence in the 12-week grafts from 4-week humanized animals that were re-transplanted in 2-week humanized animals for 2 more weeks. **(B)** Quantification of the bone volumes of the grafts by μCT scans, analyzed 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 humanized donors (H to KO), humanized recipients that received grafts from immunodeficient donors (KO to H), and humanized recipients that received grafts from humanized donors (H to H). N=5-9 animals per group, or 13-17 grafts per group

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 humanized 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[19] [18]. 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 hypothesized 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 mineralized 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-sensitized T cells of humanized animals that had received the grafts towards matched and mismatched pMSCs. Crucially, we found no significant immune responses in terms of the T cell proliferation of splenocytes co-cultured with pMSCs compared to the splenocytes on their own. This would suggest that pre-sensitized splenocytes of animals that received the grafts would not be more prone to trigger an allogeneic immune response to a secondary exposition 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, following by humanization 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 humanized 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 mineralized 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 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 to 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 humanization 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 vascularization[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* vascularization 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, in contrast to other *in vitro* studies, 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 humanized animals compared to the non-humanized controls at either of the analyzed 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 humanization, indicating that they could successfully persist in the long term without evidence of significant T-cell derived immune rejection.

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.

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 *IL2R*γ 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 humanized model might only provide a partial understanding of the allogeneic immune responses, mostly focused on the T cell activity. New humanized 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.

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.

SUPPLEMENTARY FIGURES

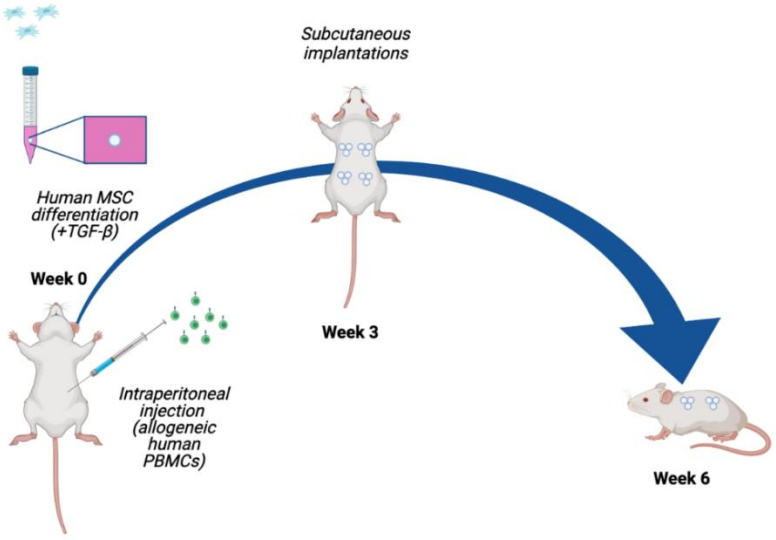


Figure S1. Schematic experimental design 1

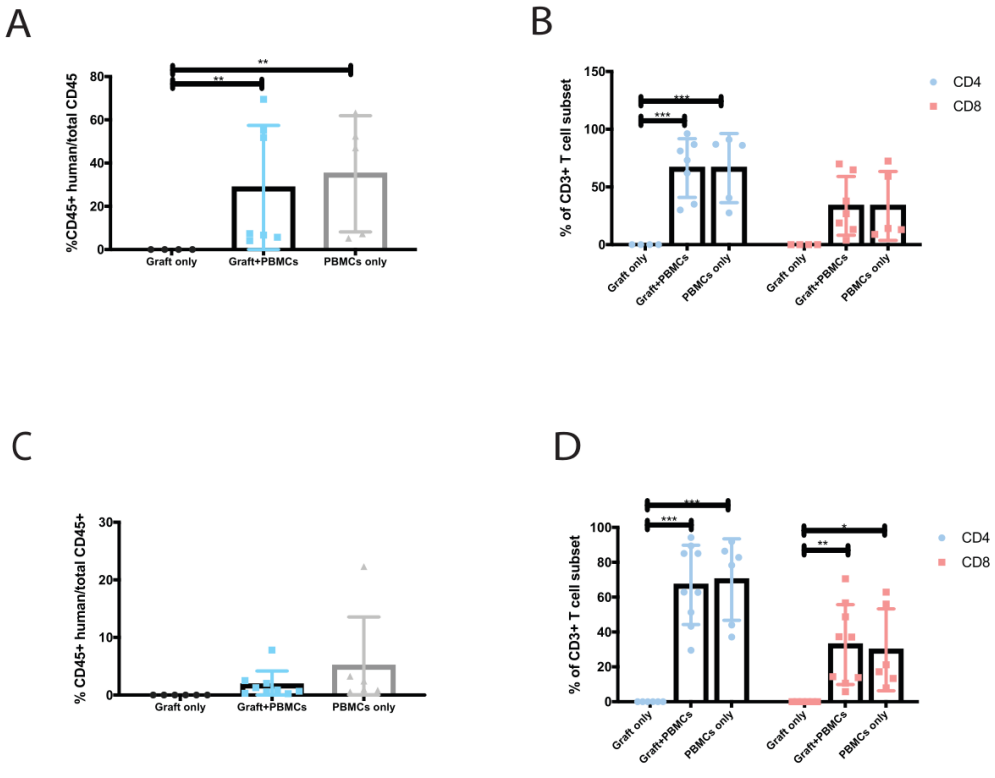


Figure S2. Flow cytometric analyses of the human immune T cell presence in blood **(A)** and femurs **(C)** after 6 weeks of humanization. **(B)** Analyses of the CD4/CD8 T cell ratio in blood and femurs **(D)**. N= 4-7 animals per group. Data was analyzed using a linear mixed model with Bonferroni post-correction; *P<0.05, **P<0.01, ***P<0.001

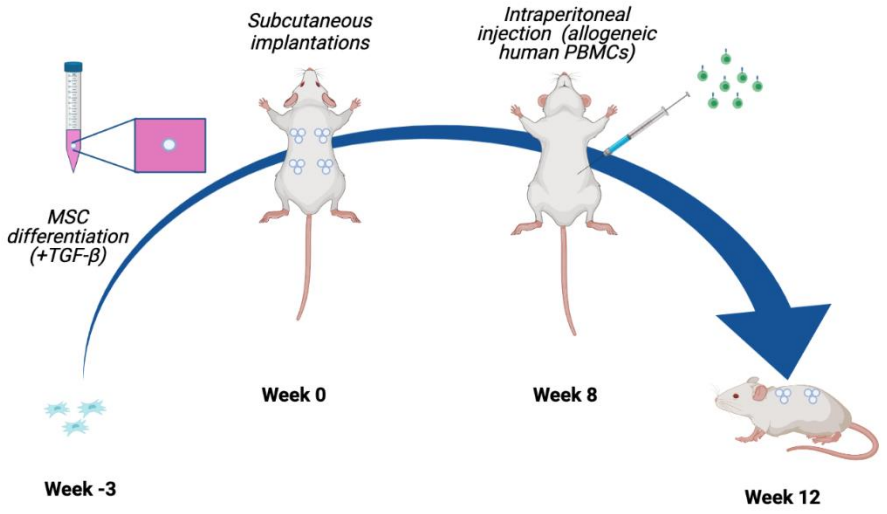


Figure S3. Schematic experimental design 2

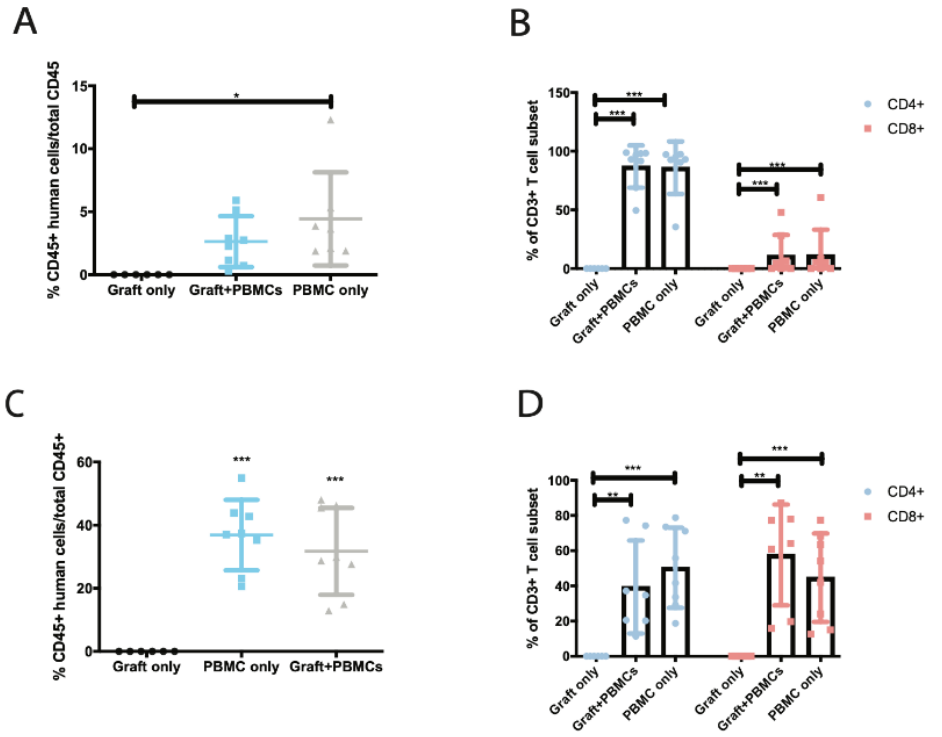


Figure S4. Flow cytometric analyses of the human immune engraftment in blood **(A)** and spleens **(C)** after 4 weeks of humanization **(B)** Analyses of the CD4/CD8 T cell ratio in blood and spleens **(D)** N= 6-7 animals per group. Data was analyzed using a linear mixed model with Bonferroni post-correction; *P<0.05, **P<0.01, ***P<0.001

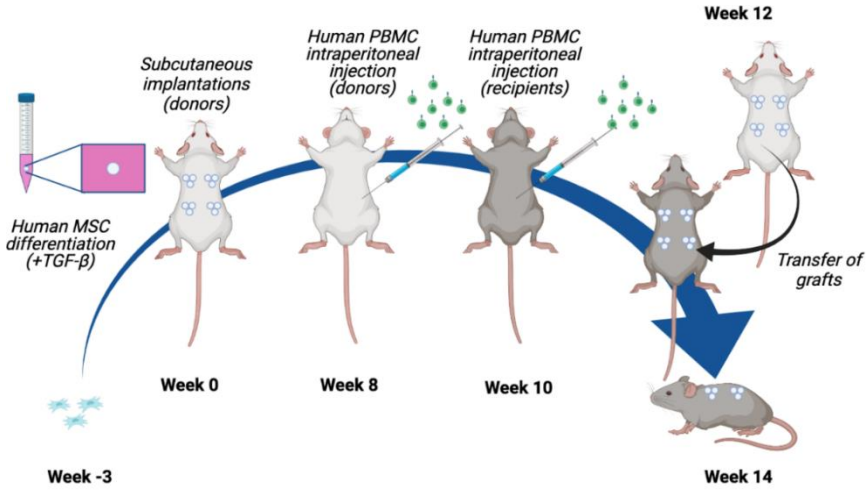


Figure S5. Schematic experimental design 3

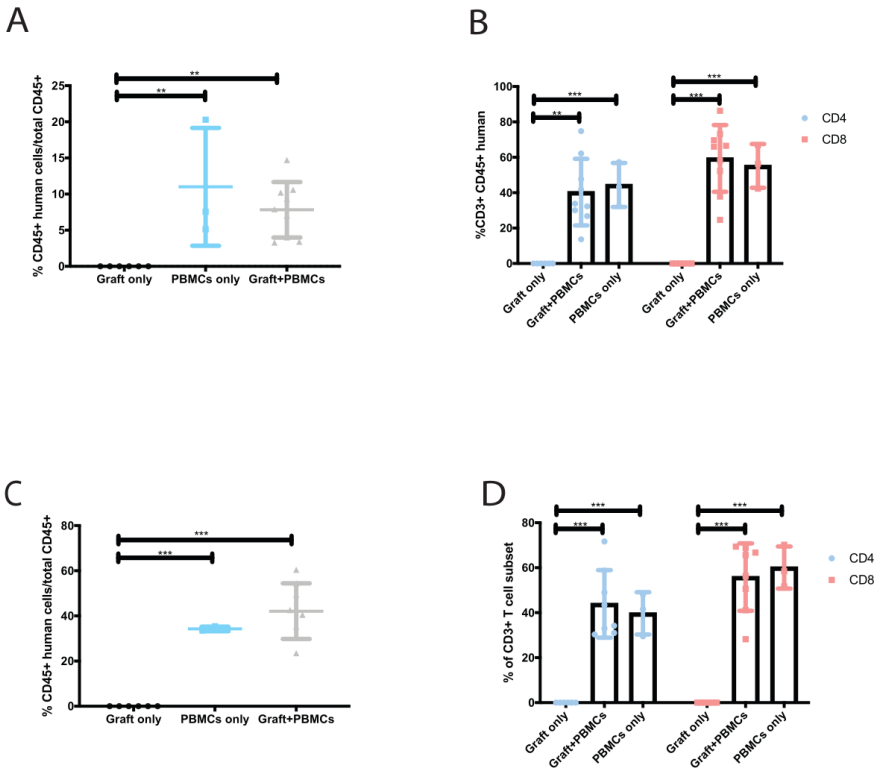


Figure S6. Flow cytometric analyses of the human immune engraftment in blood **(A)** and spleens **(C)** after 4 weeks of humanization in the donor groups **(B)** Analyses of the CD4/CD8 T cell ratio in blood and spleens **(D)** N= 3-9 animals per group. Data was analyzed using a linear mixed model with Bonferroni post-correction; *P<0.05, **P<0.01, ***P<0.001

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5

Chondrogenically-primed human mesenchymal stem cells persist and undergo early stages of endochondral ossification in an immunocompetent xenogeneic model

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ABSTRACT

Tissue engineering approaches using progenitor cells such as mesenchymal stromal cells (MSCs), represent a promising strategy to regenerate bone. Previous work has demonstrated the potential of chondrogenically-primed human MSCs to recapitulate the process of endochondral ossification and form mature bone *in vivo*, using immunodeficient xenogeneic models. To further the translation of such MSC-based approaches, additional investigation is required to understand the impact of interactions between human MSC-constructs and host immune cells upon the success of MSC-mediated bone formation. Although human MSCs are considered hypoimmunogenic, the potential of chondrogenically-primed human MSCs to induce immunogenic responses *in vivo*, as well as the efficacy of MSC-mediated ectopic bone formation in the presence of fully competent immune system requires further elucidation. Therefore, the aim of this study was to investigate the capacity of chondrogenically-primed human MSC constructs to persist and undergo the process of endochondral ossification in an immune competent xenogeneic model. Chondrogenically differentiated human MSC pellets were subcutaneously implanted to wild type BALB/c mice and retrieved at 2 and 12 weeks post-implantation. The percentages of $CD4^+$ and $CD8^+$ T cells, B cells and classical/non-classical monocyte subsets were not altered in peripheral blood of mice that received chondrogenic-MSC constructs compared to sham-operated controls at 2 weeks post-surgery. However, MSC-implanted mice had significantly higher levels of serum total IgG compared to sham-operated mice at this timepoint. Flow cytometric analysis of retrieved MSC-constructs identified the presence of T cells and macrophages at 2 and 12 weeks post-implantation, with low levels of immune cell infiltration to implanted MSC constructs detected by CD45 and CD3 immunohistochemical staining. Despite the presence of immune cells in the tissue, MSC constructs persisted *in vivo* and were not degraded/resorbed. Furthermore, constructs became mineralised, with longitudinal micro-computed tomography imaging revealing an increase in mineralised tissue volume from 4 weeks post-implantation until the

experimental endpoint at 12 weeks. These findings indicate that chondrogenically differentiated human MSC pellets can persist and undergo early stages of endochondral ossification following subcutaneous implantation in an immunocompetent xenogeneic model. This scaffold-free model may be further extrapolated to provide mechanistic insight to osteoimmunological processes regulating bone regeneration and homeostasis.

INTRODUCTION

Tissue engineering approaches using progenitor cells, such as mesenchymal stromal cells (MSCs) represent a promising strategy to generate bone graft substitutes for the repair of bone defects [1, 2]. Previous work has highlighted the potential of chondrogenically-primed human MSCs to recapitulate the natural process of endochondral ossification and form mature bone *in vivo*, following subcutaneous implantation in immunodeficient animal models after 8 to 12 weeks [3-8]. Implantation of chondrogenic-MSC constructs in immunodeficient animals is known to lead to the maturation of hypertrophic cartilage, followed by blood vessel invasion, remodelling of the cartilaginous template and eventual conversion to bone [3, 4, 9]. In these studies, chondrogenically-primed MSC constructs were found to form a bone ossicle containing a bone marrow cavity with evidence of vascularisation, indicating full integration with the host [4]. Upon translation of an MSC based approach for large bone defect repair to the patient, potential interactions between MSC-constructs and immune cells of the host may be key in determining the success of MSC-mediated bone formation [10]. Therefore, new models of bone formation, such as humanised or immune competent mouse models, that are more relevant to the clinical situation with regard to osteoimmunology are required. Human MSCs are considered to be hypoimmunogenic [11], with chondrogenically-primed human MSCs previously shown to not induce immunogenic responses *in vitro* [12, 13]. However, the potential of chondrogenically-primed human MSCs to induce immunogenic responses *in vivo*, as well as the efficacy of MSC-mediated ectopic bone formation in the presence of fully competent immune system requires further elucidation.

MSCs are considered to have low immunogenic properties, due to their low expression of Major Histocompatibility Complex (MHC) class I, and a lack of MHC class II and other co-stimulatory molecules required for recognition by immune cells of the host [11, 14]. Additionally, MSCs have been shown to have an immunomodulatory capacity towards cells of both the innate [15, 16] and adaptive immune system [17-19]. Undifferentiated human MSCs have been previously shown to suppress immune responses in

xenogeneic models utilising immunocompetent mice [20]. However, current reports on the potential of chondrogenically differentiated MSCs to modulate host immune responses are conflicting. Some authors claim that chondrogenically primed human MSCs retain their immunosuppressive properties, and can modulate allogeneic T cell proliferation [11, 12], dendritic cell (DC) maturation [13] and natural killer (NK) mediated cytotoxicity [21] *in vitro*. On the other hand, others have reported immunogenic reactions when co-culturing chondrogenically differentiated MSCs with allogeneic peripheral blood mononuclear cells (PBMCs) [22]. In addition, Chen *et al*. showed an increase in DC maturation and T cell proliferation when co-culturing chondrogenically primed rat-derived MSCs with human PBMCs *in vitro* [23]. In light of these findings, the potential of chondrogenically differentiated human MSCs to persist and form bone *in vivo* in the presence of the host immune system in a xenogeneic model remains unclear.

Given the central role played by the immune system during the natural process of bone homeostasis [24] and fracture healing [25, 26], additional investigation of the potential interaction between chondrogenically-primed human MSCs and the immune system of an immune competent host may further our current understanding of these mechanisms [27]. Also, increasing interest in the potential ability to use allogeneic cells in various regenerative medicine approaches further necessitates the development of new models of bone formation encompassing a functional immune system. Hence, the aim of this study was to determine to what extent chondrogenically-primed human MSC constructs elicit host immune responses, persist and recapitulate the process of endochondral ossification following subcutaneous implantation in an immune competent xenogeneic model.

MATERIALS AND METHODS

Isolation and expansion of human MSCs

Human MSCs were isolated from surplus iliac crest bone chip material harvested from pediatric patients undergoing alveolar bone graft surgery

(Donor 1: female, < 18 years old; donor 2: female, 10 years old; donor 3: male, 10 years old). All human samples were obtained with the approval of the Erasmus University Medical Center Medical Research Ethics Committee (MEC-2014-16). Written consent was not required in accordance with institutional guidelines for the use of waste surgical material, and an opt-out option was available. Iliac crest bone chips were washed with expansion medium composed of Minimum Essential Medium (MEM)-α (Containing nucleosides) supplemented with heat inactivated 10% v/v fetal bovine serum (FBS), 1.5 µg/ml fungizone, 50 µg/ml gentamicin (All Thermo Fisher Scientific, Waltham, MA, USA), 25 µg/ml L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA) and 1 ng/ml fibroblast growth factor-2 (Instruchemie, Delfzijl, the Netherlands), and the resulting cell suspension was seeded in T75 flasks. Cells were washed twice with phosphate buffered saline (Thermo Fisher Scientific) supplemented with 2% v/v heat inactivated FBS 24 hours following seeding to remove non-adherent cells. MSCs were cultured at 37°C and 5% carbon dioxide under humidified conditions, with expansion medium refreshed every 3-4 days. MSCs were subcultured upon reaching 80 - 90% confluency using 0.25% w/v trypsin-EDTA (Thermo Fisher Scientific) and reseeded at a cell density of 2300 cells/cm^2. MSCs were used at passage 3 for chondrogenic pellet cultures.

Chondrogenic differentiation of MSCs

For chondrogenic differentiation, 2 x 10^5 MSCs were suspended in 500 µl of chondrogenic differentiation medium composed of high glucose Dulbecco's Modified Eagle Medium supplemented with 1.5 µg/ml fungizone, 50 µg/ml gentamicin, 1 mM sodium pyruvate (All Thermo Fisher Scientific), 1% v/v Insulin-Transferrin-Selenous acid (ITSTM+ Premix, Corning, Bedford, MA, USA), 40 µg/ml proline (Sigma-Aldrich), 25 µg/ml L-ascorbic acid 2-phosphate, 100 nM Dexamethasone (Sigma-Aldrich) and 10 ng/ml transforming growth factor-β3 (R&D systems, Minneapolis, MN, USA). The cell suspension was added to 15 ml conical polypropylene tubes (VWR, Radnor, PA, USA) and centrifuged at 300 g for 8 min to facilitate pellet formation. Chondrogenic MSC-pellets were cultured at 37°C and 5% carbon dioxide in a humidified atmosphere, and culture

medium was refreshed every 3-4 days for 21 days. Chondrogenic differentiation of MSCs *in vitro* following 21 days of culture was confirmed histologically by thionine staining (Supplementary figure S1).

Subcutaneous implantation model

Animal experiments were conducted with the approval of the Animal Ethical Committee of the Erasmus University Medical Center (Licence number AVD101002015114, work protocol number 15-114-101). Male BALB/c mice (BALB/cAnNCr, 8-9 weeks old, 24.6 ± 2.2g; Charles River Laboratories, Wilmington, MA, USA) were housed in groups of 3 under a standard 12 hour light-dark cycle with water and standard chow ad libitum. Mice were anaesthetised with 3% isoflurane, 0.8 L/min O_2 (Pharmachemie BV, Haarlem, the Netherlands), and 0.05 mg/kg buprenorphine (Temgesic, RB Pharmaceuticals Limited, Slought, UK) was injected subcutaneously 30 min prior to the procedure as analgesic. Four incisions were made dorsally, bilateral at the level of shoulders and hips, and four subcutaneous pockets were created. 3 MSC pellets were implanted per subcutaneous pocket, with pellets of one MSC donor implanted per mouse. At 2 and 12 weeks post-implantation, peripheral blood was harvested for flow cytometric analysis by cardiac puncture of mice under general anaesthesia at each experimental time point. Mice were euthanised by cervical dislocation and MSC constructs were retrieved for histological and flow cytometric analysis. As a reference point to compare early endochondral ossification in immunodeficient mice, tissue sections resulting from a separate study were included in the present study for histological evaluation of MSC-mediated endochondral ossification after 4 weeks *in vivo*. In this separate study (Conducted with the approval of the Animal Ethical Committee of the Erasmus University Medical Center, licence number AVD101002015114 and work protocol, number 18-6166-01), male BALB/c nude mice (CAnN.Cg-Foxn1nu/Crl, 8 weeks old; Charles River Laboratories) were subcutaneously implanted with human MSC pellets from 1 donor as already described. Mice were euthanised by cervical dislocation and MSC constructs were retrieved for histological analysis at 4 weeks post-implantation.

Flow cytometric analysis (Peripheral blood and pellet digest)

100 µl of whole blood was centrifuged at 400 g for 5 min, following which the sera was removed and replaced with an equal volume of PBS. Diluted blood was subsequently stained for CD19 and CD138 to identify B cells, CD3, CD4 and CD8 for T cells, and CD11b, CD115, Ly6G, Ly6C and CD62L for monocyte subsets (Table 1). Staining with a Via-probe™ (T cells, B cells; BD Biosciences, San Jose, CA, USA) or LIVE/DEAD™ Fixable Dead Cell Stain (Macrophages; Thermo Fisher Scientific) was included for dead cell exclusion. Blood was stained for 10 min, and subsequently lysed for 10 mins with 3 mL of FACS Lysis Solution (BD Biosciences) in the dark and washed twice with FACSFlow buffer (BD Biosciences). Samples were resuspended in FACSFlow buffer and stored at 4°C prior to analysis. For analysis of immune cell subsets within MSC constructs, retrieved pellets were subjected to enzymatic digestion. MSC pellets were incubated with 3 mg/ml collagenase A (Sigma-Aldrich) and 1.5 mg DNase I (Sigma-Aldrich) in RPMI-1640 media (Thermo Fisher Scientific) containing 5% FBS, at 37 C for 90 min. Following incubation, the resulting cell suspension was filtered through a 100 µm cell strainer and pelleted by centrifugation at 400 g for 5 min. Cells were washed and resuspended in FACSFlow buffer, and stained for the expression of CD3, CD4, and CD8 for the identification of T cells, and F4/80, CD11b, CD86, CD206, CD163 for macrophages (Table 2). Staining with Via-probe™ (T cells; BD Biosciences) or LIVE/DEAD™ Fixable Dead Cell Stain (Macrophages; Thermo Fisher Scientific) was performed to facilitate the exclusion of dead cells. Cells were incubated in the dark at 4°C for 30 min, washed with FACSFLow buffer, and fixed with 2% paraformaldehyde (Sigma-Aldrich) in PBS. Finally, samples were washed twice with FACSFlow buffer and stored at 4°C prior to analysis. All samples were analysed using a BD FACS Canto II cytometer (BD, Franklin Lakes, NJ, USA) and data were analysed using FlowJo software version 10.0.7 (FlowJo LLC, Ashland, OR, USA). The gating strategies applied for flow cytometric analysis are presented in supplementary figure S2 and S3.

Table 1. Panel of antibodies used to detect T and B cell responses by flow cytometry

Antibody	Clone	Fluorochrome	Company
CD19	6D5	APC-Cy7	BioLegend, San Diego, CA, USA
CD138	281-2	APC	BD Biosciences, San Jose, CA, USA
CD3	17A2	FITC	Thermo Fisher Scientific, Waltham, MA, USA
CD4	RPA-T4	V450	BD Biosciences, San Jose, CA, USA
CD8a	53-6.7	PE-Cy7	BD Biosciences, San Jose, CA, USA

Table 2. Panel of antibodies used to detect monocyte and macrophage responses by flow cytometry

Antibody	Clone	Fluorochrome	Company

Monocyte analysis panel			
Anti-mouse/human CD11b	M1/70	PerCP-Cy5.5	BioLegend, San Diego, CA, USA
Anti-mouse CD115	AFS98	PE	BioLegend, San Diego, CA, USA
Anti-mouse Ly6C	HK1.4	FITC	BioLegend, San Diego, CA, USA
Anti-mouse CD62L	MEL-14	APC	BioLegend, San Diego, CA, USA
Anti-mouse Ly6G	1A8	PE-Cy7	BioLegend, San Diego, CA, USA
Macrophage analysis panel			
Anti-mouse F4/80	BM8	FITC	BioLegend, San Diego, CA, USA
Anti-mouse/human CD11b	M1/70	PerCP-Cy5.5	BioLegend, San Diego, CA, USA

Histological analysis

In vitro chondrogenically differentiated MSC pellets were fixed for 2 hours in 4% formaldehyde (BoomLab, Meppel, the Netherlands). A fixation period of 2 hours was previously determined to be adequate for the fixation of MSC pellets that were chondrogenically-primed *in vitro* for 21 days [17, 28]. Due to the potential of chondrogenically-primed MSC pellets to form mineralised tissue following subcutaneous implantation *in vivo* [28],

MSC pellets that were retrieved from mice at 2 and 12 weeks post-implantation were fixed for 24 hours in 4% formaldehyde to ensure adequate fixation, and subsequently decalcified for 10 days in 10% w/v ethylenediaminetetraacetic acid (Sigma-Aldrich) in deionised water. Following embedding in paraffin, sections of 6 µm thickness were cut from all samples. Sections from *in vitro* chondrogenically differentiated MSC pellets were deparaffinised and stained with thionine as described previously [29]. Sections of MSC pellets retrieved from mice were deparaffinised and staining with haematoxylin & eosin (H&E) was performed as previously described [28].

For human specific Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) staining, antigen retrieval was achieved by heat-induced epitope retrieval (HIER) in citrate buffer (10 mM tri-sodium citrate dihydrate, 0.05% Tween 20; pH 6.0; Sigma-Aldrich) for 25 min at 95 °C. Slides were then rinsed with Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.5, 150mM NaCl; Sigma-Aldrich)/0.025% v/v Triton X-100 (Sigma-Aldrich), and sections pre-incubated with 10% v/v normal goat serum (NGS; Southern Biotech, Birmingham, USA) in TBS/1% w/v bovine serum albumin (BSA; Sigma-Aldrich) + 1% w/v Elk milk powder (Campina, Amersfoort, the Netherlands) for 60 min. Following the blocking of non-specific binding sites, sections were incubated with a primary antibody against human GAPDH (Rabbit monoclonal; Abcam, Cambridge UK, ab128915; 0.2 µg/ml) or rabbit IgG (Dako, Glostrup, Denmark, X0903) in TBS/1% w/v BSA for 1 hour at room temperature. Next, sections were incubated with a biotinylated anti-rabbit Ig link (Biogenex, Fremont, CA, USA, HK-326-UR; 2% v/v) followed by a streptavidin-alkaline phosphatase label (Biogenex, HK-321-UK; 2% v/v), and staining was visualised by Neu Fuchsin substrate (Chroma, Köngen, Germany). Slides were mounted with VectaMount (Vector Laboratories, Burlingame, CA, USA).
For CD3 staining, antigen retrieval was performed by HIER in Tris-EDTA buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20; pH 9.0; Sigma-Andrich) at 95°C for 20 min. Sections were rinsed with phosphate buffered saline (PBS; Sigma-Aldrich) and pre-incubated with 10% v/v NGS in PBS/1% w/v BSA/1% w/v Elk milk powder for 60 min. Next, sections were

incubated with a primary antibody against CD3 (Rabbit monoclonal, Abcam, ab16669; 1:100) or rabbit IgG in PBS/1% w/v BSA for 1 hour. Sections were incubated with a biotinylated anti-rabbit Ig link (2% v/v) followed by a streptavidin-alkaline phosphatase label (2% v/v), and staining was visualised by Neu Fuchsin substrate. Slides were mounted with VectaMount.

For CD45 staining, antigen retrieval was achieved by HIER in citrate buffer for 25 min at 95°C. Following rinsing with PBS, sections were pre-incubated with 5% v/v rabbit serum (Jackson ImmunoResearch laboratories, PA, USA) and 5% v/v mouse serum (Jackson ImmunoResearch laboratories) in PBS/1% w/v BSA/1% w/v Elk milk powder for 30 min. Sections were then incubated with a primary antibody against CD45 (Rat monoclonal; Biolegend, San Diego, CA, USA, 103101; 1 µg/ml) or rat IgG2a (eBioscience, San Diego, CA, USA, 14432182) in PBS/1% w/v BSA/1% w/v Elk milk powder for 1 hour. Endogenous peroxidase was blocked with 1% v/v H_2O_2 (Sigma-Aldrich) in PBS and sections were incubated with a biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, BA-4000; 6µg/ml) in PBS/1% w/v BSA with 5% v/v Mouse/5% v/v human serum (CLB, The Netherlands) for 30 min. Next, sections were incubated with a streptavidin-peroxidase label (Biogenex, HK-320-UK; 2% v/v) and staining visualised using a DAB substrate solution (0.05% DAB, 0.015% v/v H_2O_2, 0.01M PBS, pH 7.2; Sigma-Aldrich). Finally, sections were dehydrated and slides mounted with Depex (Merck, Darmstadt, Germany).

Micro-Computed Tomography imaging

Micro-Computed Tomography (µCT) scanning was performed every two weeks starting from week 4 post-implantation up to week 12, at the Applied Molecular Imaging Erasmus MC facility using the Quantum-GX2 (Perkin-Elmer, Groningen, the Netherlands). Scanning was performed using a 30 mm field of view for 4 min (90 kV/160 uA), voxel size of 72 µm, and X ray filter Cu 0,06 mm + Al 0,5 mm. An automated reconstruction utilising the Quantum-GX2 software was performed after imaging. Scans were quantified using two phantoms with a known mineral density (0.25 and 0.75 g/cm^3), under the same scan conditions. Bone mineralisation was

assessed using the software Analyze 11.0 (AnalyzeDirect, Overland Park, KS, USA).

Total IgG ELISA analysis

Levels of IgG in the sera of MSC-implanted and sham-operated mice were quantified utilising a commercially available mouse total IgG ELISA kit (Thermo Fisher Scientific) according to manufacturer's instructions.

Statistical analyses

Analyses were performed using IBM SPSS version 24 (IBM, Armonk, NY, USA). For the comparison of sham-operated and MSC-implanted mice at 2 weeks post-implantation, normality testing was performed using a Shapiro-Wilk test and data analysed using an independent T test. Analysis of repeated measures data was performed using a linear mixed model with Bonferroni post-correction. Values are plotted as the mean +/- the standard deviation (SD), and a p value <0.05 was considered to be statistically significant. N=6 MSC-implanted mice per experimental time point, with 4 subcutaneous pockets per mouse and N=3 human MSC donors. N=3 mice per sham-operated group.

RESULTS

Chondrogenically differentiated human MSC pellets do not induce systemic monocyte and T cell-mediated immune responses following subcutaneous implantation in immune competent mice

In order to determine the potential of chondrogenically differentiated MSCs to initiate systemic innate or adaptive immune responses which may lead to acute rejection of implanted constructs, peripheral blood levels of monocyte subsets, T cells and B cells were analysed following MSC pellet implantation. The distribution of classical, intermediate and non-classical monocyte subsets in the blood of MSC-implanted mice did not significantly differ compared to sham-operated control mice at two weeks post-implantation (Figure 1A). Furthermore, the percentage of circulating

$CD3^+$ T cells (Figure 1B), as well as the ratio of $CD4^+$ to $CD8^+$ T (Figure 1C) cells in the peripheral blood of MSC-implanted mice did not significantly differ compared to sham-operated control mice at this time point. Levels of $CD19^+CD138^+$ B cells present in peripheral blood were also not altered in response to the subcutaneous implantation of chondrogenically differentiated MSC pellets at two weeks post-implantation (Figure 1D). The proportion of T cell subsets and B cells present in the peripheral blood of mice at 12 weeks post-implantation, was in line with levels observed in the blood of MSC-implanted and sham-operated mice at 2 weeks post-implantation (Figure 1B,C & D). However, serum concentrations of total IgG were significantly higher in MSC-implanted mice compared to control animals at 2 weeks (Figure 1E; $p=0.001$). Interestingly, serum levels of total IgG of mice at 12 weeks post-implantation were lower than concentrations detected in the serum of MSC-implanted mice at 2 weeks (363.60 ± 80.67 µg/ml vs 451.27 ± 64.03 µg/ml at week 2), although still higher than levels detected in sham-operated control mice (206.29 ± 48.21 µg/ml; Figure 1D).

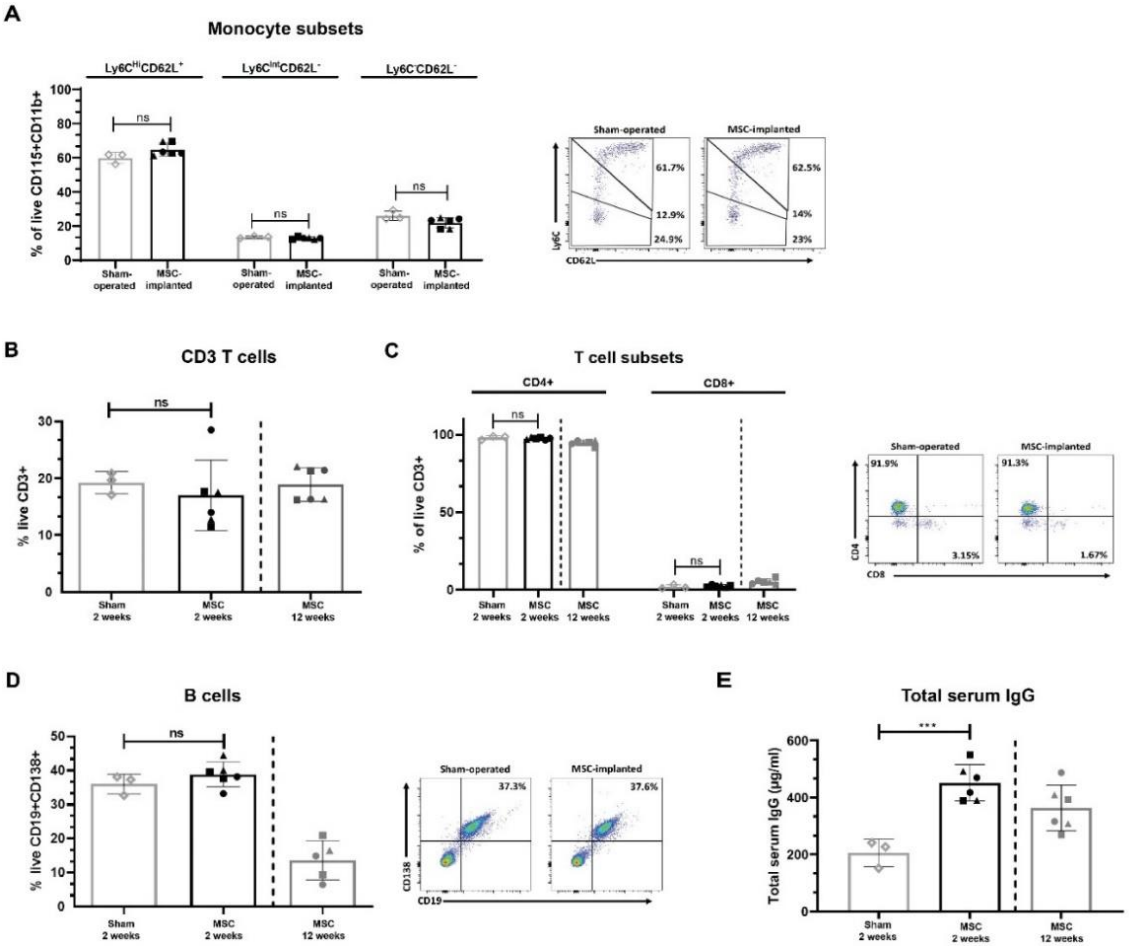

Figure 1. Subcutaneous implantation of chondrogenically differentiated human MSC pellets does not alter the percentage of innate or adaptive immune cell subsets systemically. Proportion of monocyte subsets present in peripheral blood of implanted mice compared to sham-operated controls at 2 weeks, as determined by flow cytometry **(A)**. $CD3^+$ **(B)** and $CD4^+$/$CD8^+$ T cells **(C)**, and B cells **(D)** present in the peripheral blood of sham-operated control and MSC-implanted mice at 2 and 12 weeks, as determined by flow cytometry. **(E)** Total serum IgG levels of human MSC-implanted mice compared to sham-operated controls at 2 weeks post implantation, and levels detected at 12 weeks post-MSC implantation. Data represent mean ± standard deviation, n=3 sham-

operated mice and n=6 for MSC-implanted mice. N=5 MSC-implanted mice for B cell analysis at 12 weeks post-implantation, due to loss of blood sample during handling. *** p=0.001, data analysed using an independent T test. Filled symbols of MSC-implanted groups represent different MSC donors.

Chondrogenically differentiated MSC constructs persist with cells of the innate and adaptive immune system present at 2 weeks post-subcutaneous implantation

Chondrogenically-differentiated human MSC constructs were found to persist at 2 weeks post-implantation, with the presence of human cells within the construct detected by human-specific GAPDH immunohistochemical staining (Figure 2A). Furthermore, the majority of cells within the core of retrieved MSC pellets were human GAPDH$^+$. Expression of CD45 was detected within adjacent tissue surrounding MSC constructs as well as the border of MSC pellets, with no staining observed within the cartilaginous matrix of retrieved pellets. Similarly, expression of CD3 as determined by immunohistochemistry was primarily localised to the periphery of MSC constructs and surrounding tissue, with a similar pattern of staining localisation observed in constructs of all three MSC donors. No immune cell infiltrate indicative of rejection was observed. Flow cytometric analysis of MSC constructs that were retrieved with a surrounding layer of subcutaneous tissue attached to pellets, confirmed the presence of CD3$^+$ T cells and additionally identified the presence of CD4$^+$ and CD8$^+$ T cell subsets, as well as F4/80$^+$ macrophages within tissue digests (Figure 2B).

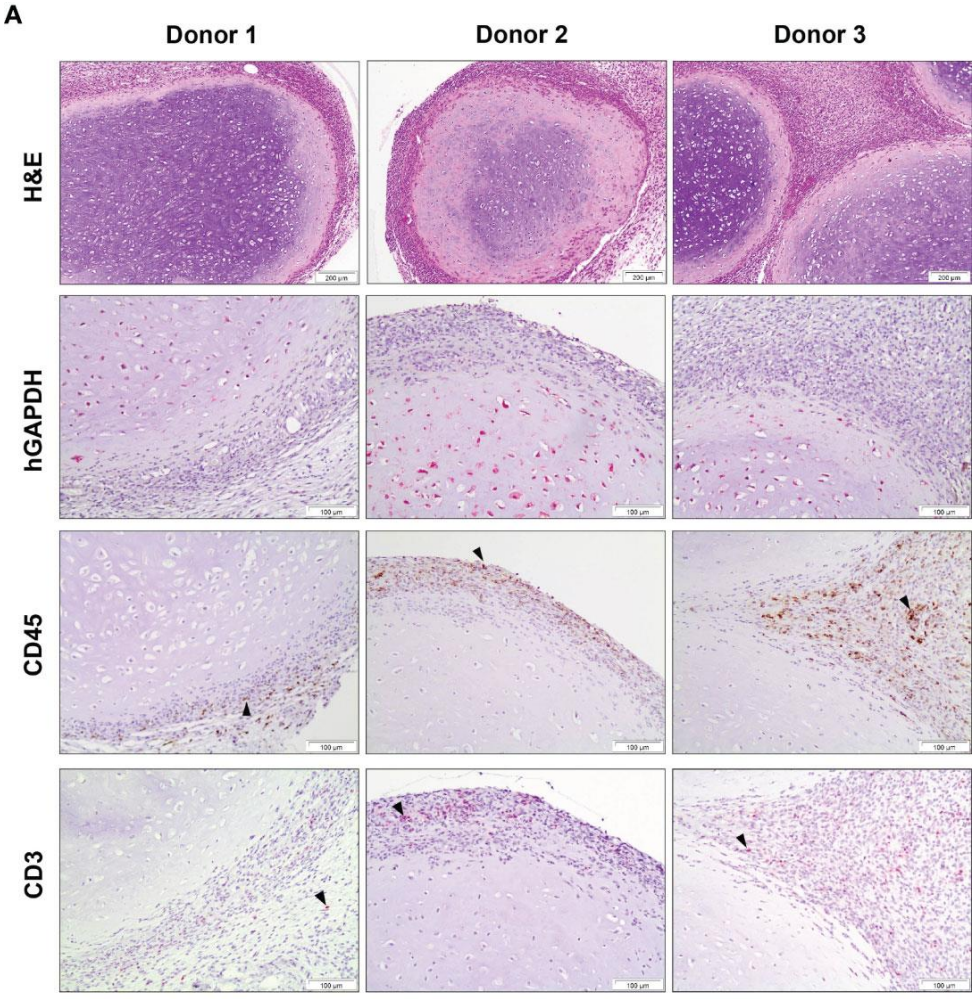
A
Donor 1
Donor 2
Donor 3
H&E
hGAPDH
CD45
CD3
B
% live cells from digested tissue
20
15
10
5
0
CD3+
CD3+CD4+
CD3+CD8+
CD11b+F4/80+

Figure 2. Chondrogenically differentiated MSC pellets persist with cells of the innate and adaptive immune system present at 2 weeks post-subcutaneous implantation. **(A)** H&E, human specific GAPDH, CD45 and CD3 immunohistochemical staining of human MSC constructs retrieved at 2 weeks post-implantation. Images are representative of 3 individual mice and 3 human MSC donors, black arrowheads indicate positive staining. **(B)** Detection of T cells ($CD3^+$, $CD4^+$, $CD8^+$) and macrophages ($CD11b^+F4/80^+$) within digested MSC constructs retrieved at 2 weeks post-implantation. Data represent mean ± standard deviation, n=6 mice and n=3 human MSC donors (2 constructs per donor).

No signs of pellet rejection are observed in MSC pellets at 12 weeks post implantation

Chondrogenically differentiated human MSC constructs had the capacity to survive in an immune competent xenogeneic host following 12 weeks of subcutaneous implantation, and were not associated with dense immune cell infiltration (Figure 3). Human $GAPDH^+$ cells were observed in all samples, highlighting the persistence of human cells at 12 weeks post implantation (Figure 3). The presence of $CD45^+$ cells within the constructs, as detected by immunohistochemical staining, was mainly localised to the periphery of pellets, with some expression detected within the matrix. Additionally, a low number of $CD3^+$ cells was observed throughout the constructs by immunohistochemistry, with CD3 expression primarily detected at the MSC pellet margin and surrounding subcutaneous tissue. In accordance with histological analysis, a low percentage of CD3+ T cells (Figure 3B; 3.32% of total cells ± 1.18%) were detected within digested constructs by flow cytometry, with lower levels of $CD3^+$ T cells observed in comparison with digested constructs analysed at 2 weeks post-implantation (Figure 2B; 10.48% ± 2.84%). Furthermore, $CD11b^+F4/80^+$ macrophages were also detected by flow cytometry within tissue digests at 12 weeks (Figure 3B; 9.78% ± 6.74%), at levels comparable with constructs retrieved at 2 weeks post implantation (Figure 2B; 6.91% ± 3.48%).

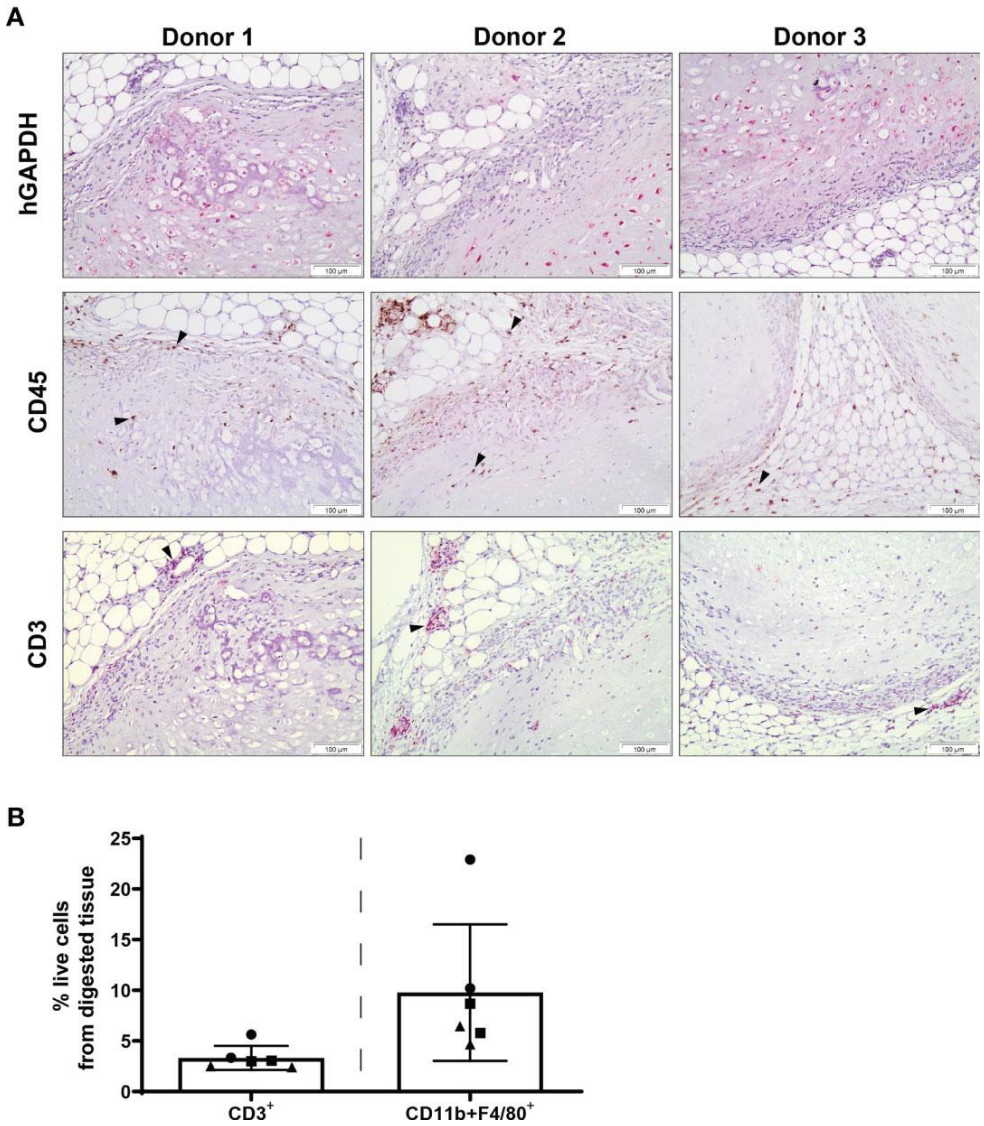

Figure 3. Innate and adaptive immune cell subsets are present at the site of human MSC constructs following 12 weeks of subcutaneous implantation. **(A)** Human specific GAPDH, CD45 and CD3 immunohistochemical staining of MSC constructs retrieved at 12 weeks post-implantation. Images are representative of 3 individual mice and 3 human MSC donors, black arrowheads indicate positive staining. **(B)** Detection of T cells ($CD3^+$) and macrophages ($CD11b^+F4/80^+$) within digested MSC constructs retrieved at 12 weeks post-

implantation. Data represent mean ± standard deviation, n=6 mice and n=3 human MSC donors (2 constructs per donor).

Chondrogenically-primed MSCs generate mineralised constructs that persist after 12 weeks in an immune competent animal model

Upon retrieval of the constructs after 12 weeks of implantation, haematoxylin and eosin staining revealed a chondrogenic structure with abundant extracellular matrix (Figure 4A). This staining further revealed some differences across donors in their levels of chondrogenesis, with an altered appearance of the cartilage extracellular matrix observed in some of the samples, indicating active remodelling, consistent with ongoing endochondral ossification. For comparison, supplementary figure S4 demonstrates the degree of bone formation in an immune compromised mouse after only 4 weeks *in vivo*. Furthermore, calcified cartilage was observed in all samples. Mineralisation of constructs was observed by μCT scans from 4 weeks post-implantation in all donors (Figure 4B), increasing in volume in all donors every two weeks up to week 12 (Figure 4C, supplementary figure S5).

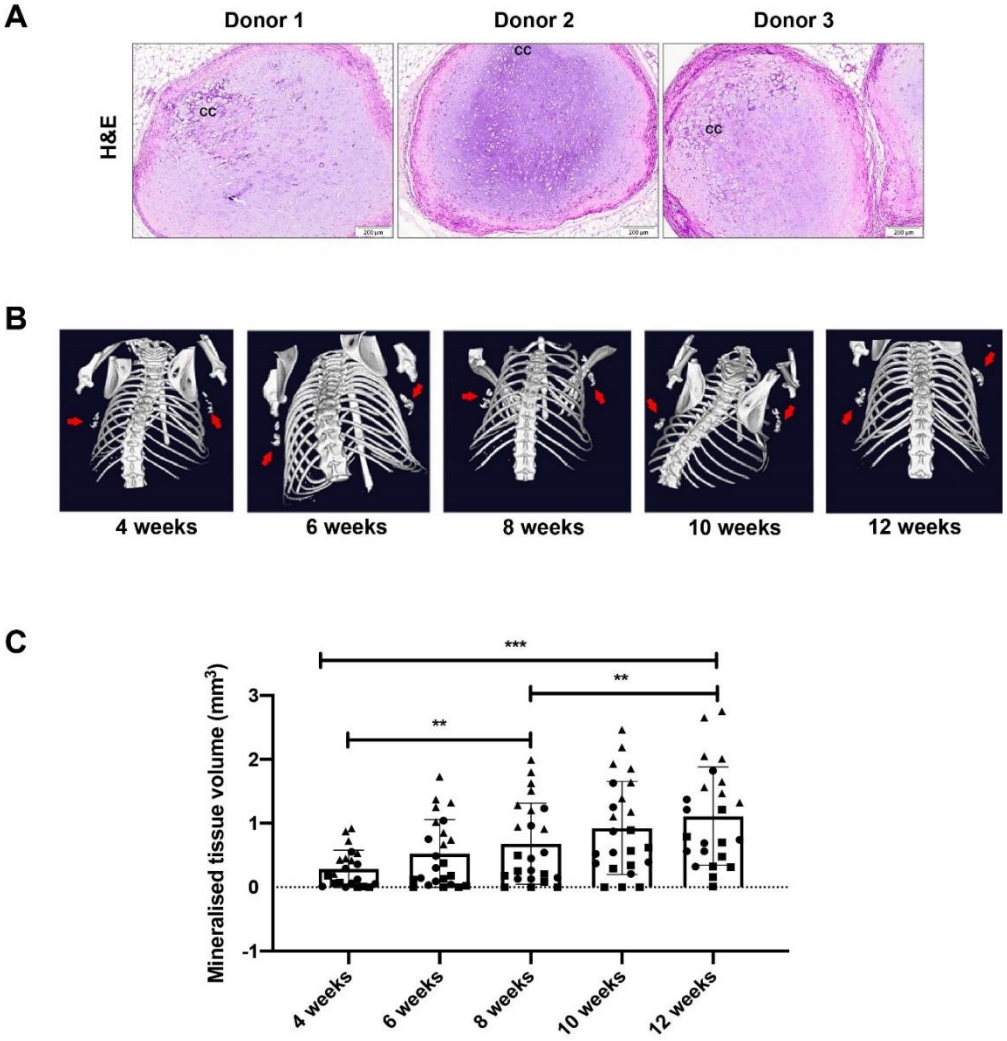

Figure 4. Chondrogenically differentiated human MSC constructs persist and become mineralised at 12 weeks post-implantation. **(A)** Representative images of H&E staining of 3 individual mice and 3 MSC donors. **(B)** Representative images by μCT showing mineralised tissue volume and **(C)** quantification. Data represent mean ± standard deviation, with n=6 mice and n=3 human MSC donors (8 constructs per donor). Each datapoint represents one MSC construct, with 4 MSC constructs implanted per mouse and symbols representing different MSC donors. CC= calcified cartilage.

DISCUSSION

The use of human MSCs for bone regeneration has been previously studied in immune deficient mouse models. In order to further determine the underlying mechanisms governing the process of MSC-mediated ectopic bone formation, as well as other mechanisms in bone related diseases and development, new models of bone formation in a functioning immune system are required. In this context, we sought to determine the potential of human chondrogenically differentiated MSCs to persist and recapitulate endochondral bone formation in the presence of a xenogeneic immune system. Our findings indicate that the proportion of monocyte subsets, $CD4^+$ and $CD8^+$ T cells and $CD19^+CD138^+$ B cells are not altered systemically following 2 weeks of implantation of human MSC-derived chondrogenic constructs, and highlight prolonged persistence of MSC-derived pellets until 12 weeks following implantation. These pellets are mineralised at 4 weeks, and progress along the endochondral ossification pathway, albeit at a slower rate than is usual in immunocompromised animals [3, 7], having not formed the marrow cavity by 12 weeks. These findings highlight the potential of immunocompetent xenogeneic models as a tool to assay human MSC-mediated tissue formation and examine the role of host immune responses during this process.

Previously it has been shown that systemic infusion of undifferentiated human MSCs to immune competent mice can suppress innate and adaptive immune responses in inflammatory disease models [30-32]. Such studies have demonstrated the potential of xenogeneic models as a useful tool to investigate the immune suppressive activity of human MSCs[20]. Although MSCs are considered hypoimmunogenic, they have been reported to have the potential to generate immune responses following *in vivo* implantation in animal models [33,34]. Additionally, it has been proposed that host rejection of MSCs may be determined by the balance between their immunosuppressive and immunogenic activity[35]. Reports to date on the potential of differentiated human MSCs to evade host immune responses and survive in an immunocompetent xenogeneic model, have not fully determined the suitability of this model system to examine MSC-

mediated tissue formation. The findings of our study highlight prolonged persistence and low immune cell infiltration of chondrogenically-differentiated human MSC pellets in immune competent mice, which are in line with previous findings demonstrating the capacity of human MSCs to retain their immunosuppressive activity following chondrogenic priming [11-13]. Interestingly, the dense extracellular matrix of intact cartilage has been previously postulated to play a role in providing protection against the recognition of chondrocytes by the host immune system following allogeneic transplantation [36-38]. However, in contrast to our results with chondrogenically-primed human MSCs, the xenotransplantation of cartilage explants has been reported to result in chronic rejection and destruction of implanted grafts [39,40]. Additional investigation at later time points is required to determine potential immune responses towards human MSC constructs following further tissue remodelling and vascularisation. Niemeyer *et al*. have previously reported decreased survival of osteogenically differentiated MSCs seeded on mineralised collagen scaffolds, following 8 weeks of subcutaneous implantation in immunocompetent xenogeneic hosts [41]. Furthermore, higher levels of macrophage and T cell infiltration of osteogenic-MSC seeded biomaterial scaffolds were also observed in this study in comparison to implanted scaffolds containing undifferentiated MSCs [41]. In contrast to our findings, Longoni *et al*. have observed local innate and adaptive immune cell responses following the implantation of chondrogenically differentiated human MSCs embedded in a collagen carrier to the site of a critical sized femoral defect in immunocompetent rats [42]. However, in a similar manner to the present study, mineralised tissue volumes were observed by 12 weeks post-human MSC implantation which were comparable to the size of implanted constructs, although no full bridging of the defect was observed in this model [42]. Further investigation is required to determine whether these observed differences in immunogenic responses of chondrogenically differentiated human MSCs, may be attributable to the ectopic versus orthotopic implantation sites, as well as the use of a biomaterial.

In addition to their potential to modulate cell-mediated immune processes [14,16,43], MSCs may also alter humoral immune responses following implantation in the host. Allogeneic undifferentiated MSCs have been reported to induce the formation of allo-antibodies in various immune competent animal models [44-46], highlighting the capacity of MSCs to stimulate an active humoral response. In the present study, although we did not observe expansion of circulating $CD19^+CD138^+$ B cells at 2 weeks post-implantation of chondrogenically differentiated human MSC constructs, the concentration of total IgG in the sera of MSC implanted mice was increased compared to sham-operated control animals. Interestingly, Longoni and colleagues have detected the production of anti-human antibodies following implantation of chondrogenically-primed human MSCs in a rat femur defect model [42]. Although we found human MSC constructs to persist and remain intact following 12 weeks of subcutaneous implantation, further investigation is required to fully confirm a lack of long-term systemic effects and antibody-mediated destruction of subcutaneously implanted human MSC constructs in immune competent mice. Furthermore, we did not evaluate local humoral immune responses at the site of implanted MSC constructs, which is a limitation of our study. Future studies performing an in-depth analysis of humoral immune responses locally at the site of implanted human MSC constructs as well as systemically, are required to further develop the present findings. Additionally, the long-term presence of implanted chondrogenically-primed MSC constructs and the potential of the dense extracellular matrix to protect such constructs against immune rejection requires further investigation. Interestingly, a low percentage of circulating $CD8^+$ T cells were detected in the peripheral blood of both sham-operated and MSC-implanted mice at both experimental time points in our study. BALB/c mice have been previously reported to have a higher predominance of circulating $CD4^+$ versus $CD8^+$ T cells compared to other mouse strains [47,48], and T cell subsets distribution may fluctuate with ageing [49]. However, whether such factors may have contributed to the low levels of circulating $CD8^+$ T cells observed in our study also requires additional investigation.

Previous work investigating ectopic endochondral bone formation by chondrogenically primed human MSCs has implemented the use of immunodeficient athymic mouse models, and demonstrated the formation of a bone ossicle containing a bone marrow cavity by 8 weeks following subcutaneous implantation [3]. In the presence of a fully functional host immune system, we have observed the initiation and progression of mineralisation by chondrogenically differentiated MSC pellets during the 12 week period following subcutaneous implantation, recapitulating early phases during the process of endochondral ossification. This progression of cartilage calcification and mineralisation which we have observed, is known to precede bone and marrow cavity formation during MSC-mediated endochondral bone formation in immunodeficient animals [3,28]. However, in the present study the progression of this process appears to be slower compared with immune compromised mice, given that previous studies have observed the progression of chondrogenically-primed MSCs towards a hypertrophic phenotype and onset of mineralisation at 4 weeks post-implantation in immunodeficient animals [50]. In a separate unpublished dataset, we have also observed a comparable degree of cartilage matrix remodelling at 4 weeks post implantation of chondrogenically differentiated human MSCs pellets in immunodeficient BALB/c nude mice (Supplementary figure S4), which is known to progress to form bone and bone marrow at 12 weeks post-implantation [3,28]. This data further corroborates our present findings suggesting delayed but ongoing MSC-mediated endochondral ossification in an immunocompetent xenogeneic model. However, we cannot rule out that the bone formation process is proceeding at a normal rate in the presence of a complete immune system and it is simply accelerated in immune compromised animals. Future experiments should take a later time point as the endpoint (16+ weeks) to confirm that complete endochondral ossification occurs in these animals. Interestingly, athymic mice have been previously reported to have elevated natural killer cell and macrophage activity levels compared to their wildtype counterparts [51,52]. Furthermore, *recombination activating gene 1* knockout mice which lack an adaptive immune system, have been reported to have accelerated endochondral ossification and remodelling during fracture healing compared to wild-

type mice [53]. In addition, El Khassawna and colleagues have identified a critical role of T and B cells in the regulation of mineralisation, matrix formation and subsequent quality of bone formed during fracture healing [54], highlighting the importance of experimental models encompassing a complete immune system to examine bone formation processes. Whether the differences in immune system composition between immunocompetent and immunodeficient mouse models may potentially play a role in determining the rate of extracellular matrix remodelling and progression of MSC-mediated endochondral bone formation, requires further elucidation. Additionally, further investigation is required to determine the impact of a potential foreign body reaction following the subcutaneous implantation of human chondrogenically-primed MSCs pellets, which may also influence MSC-mediated endochondral bone formation [55].

In conclusion, the findings of the present study indicate that chondrogenically differentiated human MSC pellets can persist, eliciting only a minor immune response and undergo the early stages of endochondral ossification following subcutaneous implantation in an immunocompetent xenogeneic model. However, the nature of the differences in the speed of the endochondral ossification process in an immune competent scenario, compared with immunodeficient mouse models, needs to be further investigated. This scaffold-free model may be further extrapolated to provide mechanistic insight into the underlying mechanisms by which the immune system might influence the process of MSC-mediated endochondral ossification.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FUNDING

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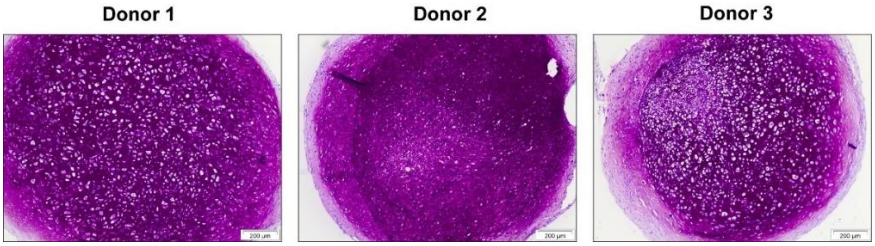
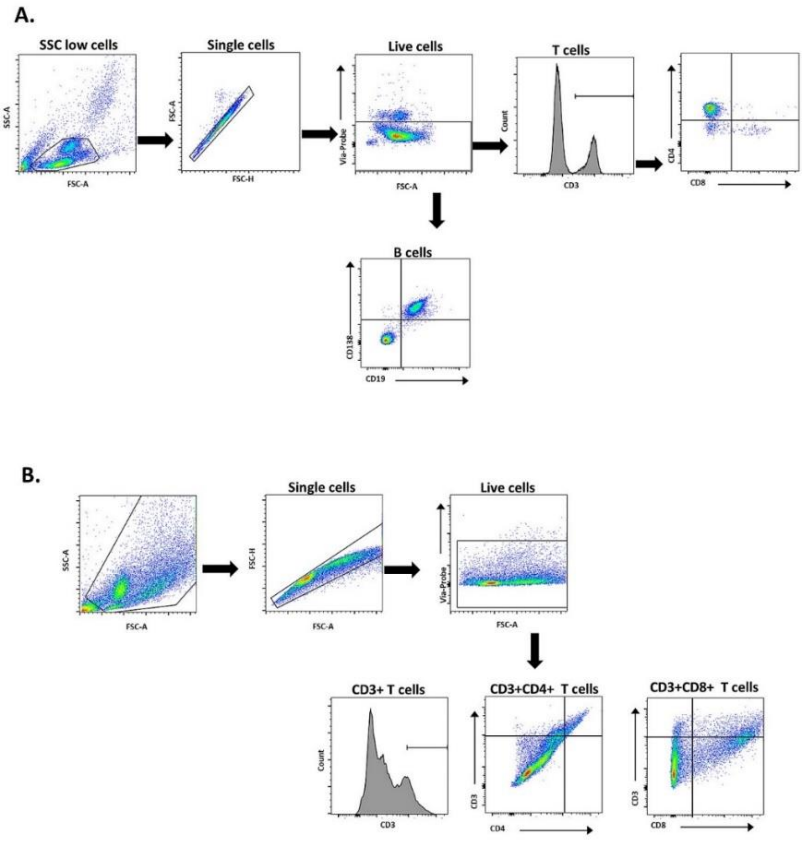
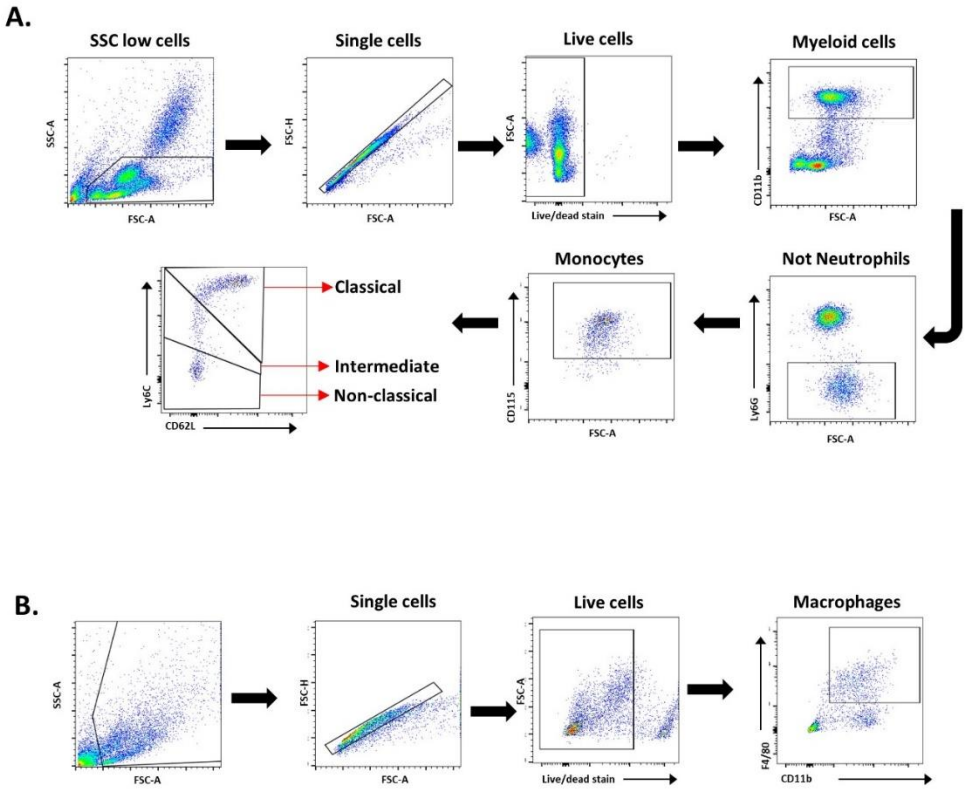
SUPPLEMENTARY FIGURES



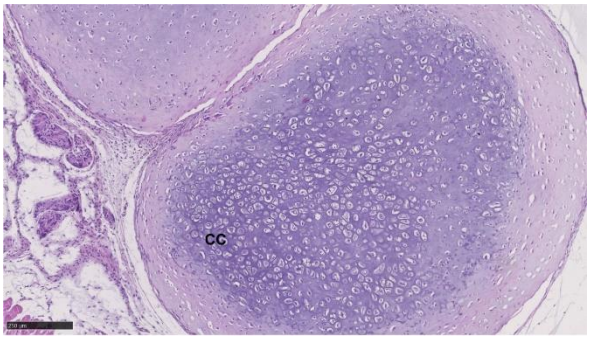
Supplementary Figure S1. Chondrogenic differentiation of human MSC donors *in vitro*. Thionine staining indicates GAG deposition following 21 days of culture in the presence of TGF- β3 (10 ng/ml).



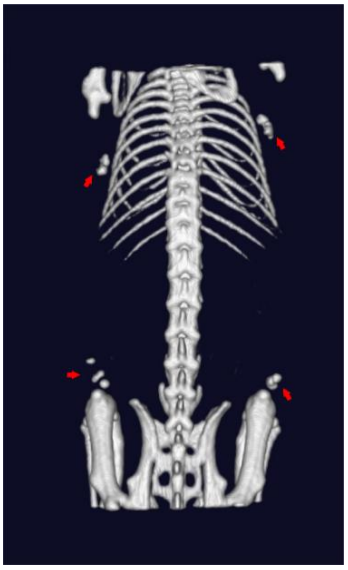
Supplementary Figure S2. Flow cytometric gating strategy for T and B cell analysis. Representative plots showing the gating strategy applied to detect T and B cells in peripheral blood (A), and T cells in digested MSC-constructs retrieved following subcutaneous implantation (B).



Supplementary Figure S3. Flow cytometric gating strategy for monocyte and macrophage analysis**.** Representative plots showing the gating strategy applied to detect peripheral blood monocyte subsets (A), and macrophages in digested MSC-constructs retrieved following subcutaneous implantation (B).



Supplementary Figure S4. Cartilage matrix remodelling at 4 weeks post-implantation of chondrogenically-primed human MSCs pellets in immunodeficient mice. Representative image of H&E staining of human MSC pellets retrieved at 4 weeks following subcutaneous implantation in an immunodeficient BALB/c nude mouse. Scale bar = 250 μm.



Supplementary Figure S5. Representative image by μCT at 12 weeks following MSC implantation showing mineralised tissue volume at all 4 MSC implantation sites.

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6

Discussion and future perspectives

From the beginning of the 20th century, the use of allogeneic MSCs to achieve bone formation through an intermediate temporary cartilage anlage has been investigated as a therapy for bone defects[1]. When mimicking this naturally-occurring process in pre-clinical models, challenges such as obtaining a sufficient amount of newly formed bone and guaranteeing the absence of immune responses in the host when using cells derived from a second-party donor still represent important limitations. In this thesis, we suggest the use of a potent source of non-immunogenic human paediatric MSCs with enhanced proliferation and differentiation capacities for allogeneic bone formation that overcomes these obstacles.

The aim of this thesis was to investigate the feasibility of using potent paediatric mesenchymal stem cells for bone formation in the presence of an immune system. To do so, we performed several *in vitro* and *in vivo* studies whose main findings will be discussed in the following sections.

ONE STEM CELL TO RULE THEM ALL: TOWARDS AN IMPROVED SOURCE OF MESENCHYMAL STEM CELLS FOR BONE REPAIR

Bone marrow-derived MSCs have been pointed out as remarkable candidates for tissue engineering approaches due to their regenerative properties and the possibility of differentiating into several cell types [2]. However, intrinsic donor variation and age-related factors, as well as disease status of the donor, [3 4 5] are known to impact their abilities for bone repair.

Some authors have looked into the use of autologous MSCs due to the absence of a risk for a potential graft-derived immune rejection [6], which could make this approach initially advantageous. However, a high percentage of patients with impaired bone healing are elderly adults, in some cases with additional co-morbidities [7]. It is known that the functionality and ability of regeneration of human cells and organs decay over the years[8], so it comes at no surprise that scientific reports show that adult MSCs lose their differentiation capacities as the individual ages,

potentiated by an age-dependent increased senescence of these cells [4]. In addition, a high number of cells are required to make a bone graft, so the use of autologous MSCs would involve a waiting time of approximately 4-6 weeks for the patient in order to extract, purify, expand and achieve the required number of chondrogenically differentiated cells.
For this reason, characterising in advance a source of potentially healthy and young MSCs that can be expanded consistently is highly advantageous. We demonstrated that paediatric MSCs are less senescent than adult MSCs, able to proliferate twice as fast and differentiate with a better consistency rate. Therefore, using paediatric MSCs for bone tissue engineering purposes might alleviate donor-related issues associated with the use of autologous MSCs, setting the basis for potentially creating a one-cell-fits-all approach where no pre-testing of MSCs would be needed.

When scaling up the culture of these cells, we could create *ready-to-go* batches of pMSCs that could be easily expanded and differentiated, shortening the waiting time for the patients with 3 weeks compared to employing adult MSCs and ensuring a better differentiation. Since these cells are obtained from an ethically approved surplus, this would technically mean a steady supply that would not require additional pain and discomfort for the patient.

Moreover, these cells could potentially be applied into other fields of regenerative medicine due to their multilineage differentiation capacity, such as fat grafts for reconstruction upon breast cancer surgery[9] or articular cartilage[10], where extensive amounts of tissue might be required.

ARE PAEDIATRIC MSCs IMMUNOPRIVILEGED IN AN ALLOGENEIC SETUP?

As previously mentioned, another important factor to take into account when considering the use of allogeneic or third-party sources of MSCs for clinical purposes is the potential for undesired immune responses. Traditionally, MSCs have been considered to present immunomodulatory capacities [11], and due to their low expression of co-stimulatory and MHC molecules, to not promote immunogenic responses. Investigating the

immune behaviour in the context of an allogeneic setup of pMSCs is therefore a central factor to address possible allogeneic derived immune responses in the process of bone formation.

In this thesis, we show that undifferentiated pMSCs can exert dose-dependent immunomodulatory effects towards T cells in monolayer, and towards T and B cells even upon IFN-γ stimulation). The T cell-mediated response plays a central role in driving the mechanisms of allo-immunity in graft rejection, as well as in the activation of the antibody-mediated responses by B cells [12]. Hence, these findings could mean that, even in the onset of a pro-inflammatory microenvironment created by a bone defect, in which T cells are shifted towards a Th1 response, these pro-inflammatory signals would maintain the ability of pMSCs to modulate the T cell response. In addition, as their immunomodulatory capacities towards B cells are also increased in these circumstances, the onset of allo-antibody production would be impeded.

Whether the chondrogenic differentiation of MSCs alters their immune privileged status still remains controversial and source-dependent. In this thesis, we showed infiltration of the allogeneic PBMCs into the chondrogenically differentiated pMSCs and grafts, however we did not find indications of immune cell activation. This suggested that likely the chondrogenic matrix that surrounds the pMSCs exerts a protective effect, shielding them from the T cell immune responses and would allow the process of endochondral ossification to progress. Therefore, we proceeded with implanting our chondrogenically primed pMSCs in an allogeneic animal model.

MORE HASTE, LESS SPEED: PAEDIATRIC MSCs AND THEIR BONE FORMATION CAPACITIES IN A MISMATCHED IMMUNE SYSTEM

In this thesis, we demonstrate that *in vivo* persistence and mineralisation of chondrogenically primed human pMSCs can be achieved both in an allogeneic immune setup and in immune competent animals. As previously mentioned, when using a third-party source of MSCs, investigating whether the host immune system would recognise these as

foreign cells and proceed to their clearance is key. A recent study employing rat allogeneic MSCs for bone repair in an allogeneic setup proved the presence of these cells in the host after 12 weeks [13]. Here, we proved the persistence of mature bone derived from human pMSCs within an allogeneic immune system for up to 14 weeks, indicating that the destructive responses against the mature bone graft at later time-points did not occur.

When implanting our pMSC grafts in immunocompetent animals (**Chapter 5**), we observed indications of mineralisation and presence of bone lacunae, however, no proper bone structures were detected at 12 weeks in this xenogeneic model. Since bone formation was observed at the same timepoint in our allogeneic animal model (**Chapter 4**), we hypothetise that the absence of a completely functional immune system -as will be discussed later- might accelerate the speed of the endochondral ossification process. Hence, since no significant immune responses that led to clearance of the pMSC grafts were detected in the xenogeneic scenario, this might simply mean that longer timepoints are required to achieve pMSC-mediated bone formation. Whether this is a more realistic situation of the actual speed of the actual endochondral ossification process, rather than in the case of our allogeneic studies, still needs further elucidation, with experiments that look at longer timepoints in immune competent animals.

Translating these findings towards a clinical prospect for bone regeneration purposes still has to be done with caution. Despite having set the basis for a potential universal and unlimited source of MSCs that could pave the way for better tissue-engineering approaches, further timepoints (12+ weeks) should be studied in an allogeneic animal model to establish the long-term stability of these bone grafts. As it will be discussed later, it can be challenging to find an animal model that allows for a good engraftment of allogeneic immune cells for more than 6 weeks. In addition, a wider testing including more donors should be done to study possible differences between the immune status of different pMSCs donors, their bone forming capacities and their behaviour in diverse

allogeneic environments. Different patients also mean different immune systems, which in turn can have different intrinsic immune statuses at different times [14]; as well as other factors such as disease or immune suppression. Aging of the human immune system also means a gradual decrease in the immune functionality and hematopoiesis, and the participation of the innate and the adaptive immune system on the different stages of the naturally-occurring bone formation process is known to play a role[15] [16]. Therefore, further studies that include humanisation of animal models with a wider range of allogeneic immune cells to account for these circumstances, such as geriatric or immune suppressed patients, might be required before clinically extending the use of a universal source of pMSCs for bone tissue engineering purposes in more cohorts of patients.

T CELLS IN THE HOUSE: HELP OR HINDRANCE?

T cells have been described as the main mediators of allorejection in solid organ transplantation. These mechanisms are mainly driven by allospecific memory $CD4^+$ Th1 cells, which, in addition to becoming re-activated upon a secondary antigenic exposure, act as helpers for providing a potent allospecific $CD8^+$ reactive response [17] [18].

We found allogeneic human T cell present in our pMSC allografts (**Chapter 4**). Upon 3 weeks of implantation, these cells were predominantly $CD4^+$, while at later timepoints more $CD8^+$ presence was observed. These findings are in line with previous allogeneic responses, in which $CD4^+$ T cells function initially as activators for the $CD8^+$ branch[17]. In other models of solid organ transplantation, such as renal grafts, T cell infiltration is generally accompanied with other signs of pathological rejection. In this case, uniform criteria to define rejection to different degrees has been established, and it is classified based on interstitial fibrosis, athropy and loss of tissue[19]. However, despite reporting T cell presence, no significant histological alterations that resembled graft rejection were reported in the pMSC-derived bone grafts. This phenomenon might be explained by the activation status and the specificity of the T cells in the graft. As we did not perform analyses to study

the phenotypic profile of these T cells, their allospecificity, as well as well as their activation levels, were unknown. It has been recently reported in a study of allograft T cell analyses that nonactivated or bystander T cells, that are not antigen specific, might play a role in the immunosuppresion of allospecific T cells by diverting cytokines and growth factors from them[20]. This hypothesis is supported by the fact that, when we exposed the splenocytes from the animals that had received the pMSCs derived bone grafts to the same pMSC donors *in vitro* no further T cell activation or proliferation was observed, indicating that they were not sensitized to pMSCs antigens. This suggests that these T cells were not specific for pMSCs, as they did not respond to their re-stimulation, and that they were not alloreactive. Furthermore, this experiment also suggested that no memory responses had developed from an initial exposure to the pMSC grafts, which might explain the absence of T cell-derived alloresponses.

This hypothesis is also supported by the well-known immune status of pMSCs to not increase the proliferation of T cells, even upon chondrogenic differentiation, as shown in this thesis (**Chapters 2, 3 and 4**). A study suggested that MSCs induce a state of division arrest of T cells, which would make them prone to tolerance[21]. These MSCs can, therefore, inhibit T cell proliferation whilst supporting their survival, albeit in a quiescent state[22]. Furthermore, in a separate study it was shown that MSCs can suppress differentiation of the cytotoxic T cells into cytotoxic T cell effectors [23], hence impeding a cytotoxic response. Therefore, as the allogeneic T cells enter into contact with the pMSC derived grafts, it is likely that these cells prevent T cell-mediated responses through the stated mechanisms. Overall, further studies need to focus on more specific phenotypic analyses of the T cells that are detected in the graft, in order to clarify their allospecificity, clonality and activation status.

TOWARDS THE PERFECT MOUSE MODEL TO STUDY ALLOGENEIC IMMUNE RESPONSES: A SCIENTIFIC FICTION?

The generation of immunodeficient strains of mice as candidates for engraftment with human immune cells has been extensively investigated

over the past 30 years [24]. It is clear that these models constitute an important tool to study the potential mechanisms of allograft rejection, hence giving an insight into the immune behaviour and mechanisms of rejection and permitting the development of therapies to guarantee the safety and long-term stability of the allograft.

By choosing a *Balb/c IL2Rg-/- RAG2-/- KO* strain, lacking NK, T and B cells [25] [26] and humanised with human PBMCs as our allogeneic animal model (**Chapter 4)**, we could mimic the initial allograft-mediated immune responses that could be triggered in a human host upon receiving our pMSC-derived allograft. However, this model poses some important limitations. First, the degree of engraftment variability among human PBMC donors. Despite achieving a medium to high engraftment of human $CD45^+$ $CD3^+$ T cells in the spleen in all experiments (20-70% of the total of PBMCs) this was highly PBMC donor-dependent. This limitation could be seen as an advantage when it comes to mimicking as many diverse patient immune profiles as possible. Since the immune activation status of the individuals could intrinsically vary due to factors such as age and disease [27], by achieving different degrees of engrafted and circulating allogeneic immune cells we were able to get a more diverse immune representation.

Another important limitation of this humanisation model is the onset of intrinsic model-derived xenogeneic graft-versus-host disease (GvHD) [28], associated to the recognition of the mouse MHC class I by the engrafted human T cells. Therefore, only relatively short-term allogeneic immune responses could be addressed. In addition, the lack of memory T cells in our humanised mouse model, which could be potentially alloreactive, also represents an important downside. We tried to overcome both of these limitations by re-implanting the T-cell loaded pMSC bone grafts in humanised secondary hosts, therefore probing the T cells to a second antigenic exposure, and achieving a longer timespan of our mature bone grafts in a humanised immune system. A study using *IL2R-/-RAG2-/-CD47-/-* models suggest that loss of CD47, a transmembrane protein that leads to phagocytosis, led to decreased GvHD responses in these animals [29]. Hence, this and other models that target CD47 or MHC molecules [30] could

potentially open the door for the investigation of allogeneic responses in the longer term (14+ weeks).

Lastly, a weak point of our humanised animal model was the lack of engraftment of certain immune subsets due to the method of humanization, such as APCs, NK cells, mature B cells and T regs [31] [32]. The immune response relies on a complex interaction that comprises many different celullar and extracelullar factors, by losing these cells we also lose an important part of the immune network, such as antibody-mediated mechanisms. In addition, allograft infiltrating regulatory T cells have been shown to exert a protective effect in renal grafts [33], so a key role for these cells in allograft tolerance might have been lost when employing this model. Hence, it is clear that some of the steps of the ladder that orchestrates the human allogeneic immune responses are missing in our investigations of the allogeneic responses, which might explain the absence of a robust graft response. In order to gain an additional perspective on the complex network of immune responses towards mismatched pMSC-derived bone grafts, in **Chapter 5** we implanted chondrogenically primed pMSCs in immune competent animals, which permitted us to analyse potential B cell-related responses. However, these xenogeneic responses might not be totally indicative of the actual situation in an allogeneic setup, as it is known that intra-species variations between the mouse and human immune system exists, such as in the higher amount of ciculating lymphocytes in mice[34]. In addition, allograft and xenograft rejection studies have reported before that the onset of an immune response and rejection mechanisms can take place at different speeds depending on the allogeneic or xenogeneic setup [35]. Therefore, although they can provide an insight on the pMSC-derived bone formation process, extrapolating the xenogeneic immune responses to pMSCs to an allogeneic scenario might not be precise enough.

Hence, despite representing an invaluable preclinical tool to study and mimic the allogeneic immune responses without putting patients at risk, further improvements such as an optimisation of the B-cell mediated responses, as well as memory T cell formation and a reduction of the

xenogenic GvHD response could lead to better humanised mouse models [36 37]. Nevertheless, it is important to keep in mind that humanised mouse models might be more useful to address specific questions, rather than offer a full recapitulation of a certain homeostatic process or pathology, for which immune competent animals as pre-clinical models might be a more potent tool. Therefore, despite offering an insight into the understanding of certain biological processes, such as allogeneic transplantation, interpreting the data obtained from experiments utilizing these animal models should be done complementarily.

CONCLUSIONS

In the last years regenerative medicine approaches have gained relevance through the use of stem cells that can give rise to different tissues and organs. In particular, MSCs for bone regeneration have been extensively investigated as they can mimic the naturally-occurring process of endochondral ossfication to generate bone. However, the lack of understanding about the allogeneic immune responses towards MSCs in a pre-clinical model is an important obstacle. In addition, finding a steady and potent source of MSCs also poses a limitation. To overcome this, we extensively characterise the properties of a relatively novel source of paediatric MSCs with improved proliferation and differentiation capacities, and we proved their immune privileged status towards T and B cells *in vivo*. Furthermore, we analysed their remarkable ability for bone formation in an allogeneic animal model without provoking significant immune responses. Lastly, we offer perspectives into improving tissue-engineered mediated approaches and the study of allograft rejection by suggesting donor pre-screening and indications for the improvement of relevant animal models. Altogether, these findings pave the way for the use of allogeneic paediatric mesenchymal stem cells for a universal strategy for bone repair towards clinical translation.

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7

Summary

Bone defects that remain unresolved represent a clinical challenge. Ideally, the use of the own patient's bone to fill in the defect (autologous transplantation) would be the gold standard. However, the use of this approach is, in reality, not ideal, as it requires an extra surgical procedure and poses the limitation of the amount of bone that can be harvested.

Tissue engineering approaches aim to overcome this problem, by employing marrow stromal cells (MSCs). These cells are able to differentiate into bone through an intermediate cartilage anlage when chondrogenically stimulated with TGF- β3. This laboratory-induced process mimics the endochondral ossification route that occurs naturally in the human body when bone formation takes place. The choice of cells to follow this approach remains crucial, as creating a bone 'mould' requires a high number of cells that are able to differentiate into the desired tissue upon the right stimuli. However, in aged patients this might be not an option, as these abilities decay with age. Prior evidence suggests that MSCs derived from young, healthy sources outperform adult cells for differentiation purposes. Therefore, finding the best source of cells that is able to be expanded and differentiated into a bone graft rapidly would be the starting point to make this approach a reality. In addition, guaranteeing the absence of a potential immune response that might arise when using an allogeneic source of cells would be key in order to guarantee the long-term stability of the bone allograft. These topics are discussed **in chapter 1,** which provides a general introduction of this thesis.

In **chapter 2**, we focus on investigating the differentiation and proliferation properties of a source of paediatric MSCs (pMSCs) in comparison with adult MSCs (aMSCs). Here we find that, under the same conditions, paediatric MSCs are more suitable than adult MSCs for tissue engineering purposes in terms of faster proliferation, more consistent multilineage differentiation, including chondrogenesis, and less senescent. Similar to adult MSCs, these cells did not seem to be immunogenic in an initial co-culture assay with T cells. Hence, they were chosen as the ideal candidates for the rest of the studies.

As previously mentioned, investigating the behaviour of MSCs in an allogeneic immune system is essential to avoid a potential immune rejection process that could destroy the bone allograft. In particular, the adaptive fraction of the immune response composed of T and B cells has been shown to play a fundamental role in allogeneic-derived rejection processes. In **chapter 3**, the adaptive immune response towards undifferentiated pMSCs is investigated. To mimic the most realistic scenario that would occur when implanting these bone allografts in a surgery, we incorporated the pro-inflammatory cytokine IFN-γ. Our results show that IFN-γ does not alter the immunomodulatory abilities of pMSCs towards T cells. However, IFN-γ primed pMSCs but not unprimed pMSCs strongly inhibited B cell proliferation, as well as antibody-producing plasmablast formation and immunoglobulin G (IgG) antibody production. Therefore, in the case of pro-inflammatory signals, such as IFN-γ produced by activated T cells, pMSCs safeguard their immunomodulatory status towards T cells, and gain anti-proliferative abilities upon B cells. This could indicate that an allo-antibody immune response would therefore not develop *in vivo*, even under inflammatory conditions.

It is to mimic a more *in vivo* realistic situation that in **chapters 4 and 5** we move towards mouse models. In **chapter 4**, we used an immunodeficient animal model humanised with allogeneic immune cells to study the immune responses towards chondrogenically differentiated pMSCs (ch-pMSCs). These ch-pMSC-derived grafts did not show immunogeneic responses *in vitro*. Upon subcutaneous implantation, they were able to mineralise in the presence of an allogeneic immune system for 3 weeks without the onset of immune responses. Moreover, ch-pMSCs generated mature bone after 8 weeks of implantation. This bone persisted for up to 6 more weeks in the presence of an allogeneic immune system, suggesting that these bone allografts do not get cleared by the host. However, the intrinsic limitations of the humanised animal model, namely the lack of engraftment of certain immune subsets such as antigen-presenting cells (APCs) or B cells, made us look for other potential animal models. In

chapter 5, we made use of an immunocompetent mouse model to study the potential of our ch-pMSCs to form bone in the presence of a fully functional xenogeneic immune system. In that scenario, we found that the percentages of $CD4^+$ and $CD8^+$ T cells, B cells and classical/non-classical monocyte subsets were not systemically altered in these animals after two weeks. However, ch-pMSC-implanted mice had significantly higher levels of serum total IgG compared to sham-operated mice at this timepoint. After 12 weeks of implantation, we found low levels of T cell and macrophage infiltration in the xenografts. Despite this, ch-pMSC constructs persisted and did not show signs of resorption. In addition, constructs became mineralised, and their bone volume increased from 4 weeks post implantation to 12 weeks.

The findings of this thesis are discussed in **chapter 6**. We also suggest ways of improving the existent animal models towards mimicking more realistic biological situations for bone repair and osteoimmunology. All in all, our results indicate that pMSCs are excellent candidates for bone repair, proving that initiation and maintenance of bone formation occurs without a substantial immune response in both a xenogeneic and allogeneic scenario.

8

Appendices

Nederlandse Samenvatting
Resumen en español
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Nederlandse Samenvatting

Niet genezende botdefecten zijn nog steeds een grote klinische uitdaging in de patiëntenzorg. Het gebruik van eigen bot van de patiënt om het defect op te vullen (autologe transplantatie) is nog steeds de gouden standaard. Echter, het gebruik van deze benadering is niet ideaal. Het vereist een extra chirurgische ingreep, die pijn en ongemak voor de patiënt met zich meebrengt. Daarnaast is de beschikbare hoeveelheid bot in het lichaam van de patiënt beperkt.

Weefseltechnologie is bedoeld om dit probleem op te lossen door gebruik te maken van mesenchymale stromale cellen (MSC's). Deze cellen zijn in staat om te differentiëren tot bot via een tussenliggend kraakbeen sjabloon tijdens chondrogene stimulatie met TGF-β3. Dit door het laboratorium geïnduceerde proces bootst de endochondrale ossificatie route na die van nature in het menselijk lichaam voorkomt wanneer botvorming via een kraakbeen sjabloon plaatsvindt. De keuze van deze cellen (MSC's) blijft cruciaal, omdat het creëren van een bot sjabloon een groot aantal cellen vereist die in staat moeten zijn om op de juiste stimuli te differentiëren in het gewenste weefsel. Bij oudere patiënten is dit echter geen optie, omdat deze capaciteit met leeftijd afneemt. Eerder bewijs suggereert dat MSC's die zijn afgeleid van jonge, gezonde bronnen beter presteren dan volwassen cellen voor differentiatie doeleinden. Daarom zou het vinden van de beste bron van cellen die in staat is om snel te groeien en te differentiëren tot een bottransplantaat, het startpunt zijn om deze benadering te realiseren. Bovendien zou het garanderen van de afwezigheid van een potentiële immuunreactie die zou kunnen optreden bij het gebruik van een celbron van iemand anders (allogeen), van cruciaal belang zijn om de stabiliteit van het bot op lange termijn te garanderen. Deze onderwerpen worden besproken in **hoofdstuk 1**, waarin een algemene introductie van dit proefschrift gegeven wordt.

In **hoofdstuk 2** richten we ons op het onderzoeken van de differentiatie- en proliferatie-eigenschappen van een bron van pediatrische MSC's

(pMSC's) in vergelijking met volwassen MSC's (aMSC's). Hier ontdekken we dat pediatrische MSC's onder dezelfde omstandigheden geschikter zijn dan volwassen MSC's voor weefselmanipulatie doeleinden op het gebied van snellere proliferatie, meer consistente multilineage differentiatie inclusief chondrogenese, en minder veroudering. Net als bij volwassen MSC's waren deze niet immunogeen in een initiële co-cultuurtest met T-cellen. Vandaar dat zij werden gekozen als de ideale kandidaten voor de rest van de onderzoeken in dit proefschrift.

Zoals eerder vermeld, is het onderzoeken van het gedrag van MSC's in een allogeen immuunsysteem essentieel om een mogelijk immuun afstotingsproces te voorkomen dat het bot allograft zou kunnen vernietigen. In het bijzonder is aangetoond dat de adaptieve fractie van de immuunreactie bestaande uit T- en B-cellen een fundamentele rol speelt in allogene afstotingsprocessen. In **hoofdstuk 3** wordt de adaptieve immuunreactie op ongedifferentieerde pMSCs onderzocht. Om het meest realistische scenario na te bootsen dat zich zou voordoen bij het implanteren van deze allografts in een operatie, hebben we het ontstekingsbevorderende cytokine IFN-γ opgenomen. De resultaten laten zien dat IFN-γ de immunomodulerende vermogens van pMSC's naar T-cellen niet verandert. IFN-γ-geprimede pMSC's, maar niet niet-geprimede pMSC's, remden echter sterk de proliferatie van B-cellen af, net als de antilichaam producerende plasmablast vorming en immunoglobuline G (IgG) -antistoffen productie. Daarom beschermen pMSC's in het geval van ontstekingsbevorderende signalen, zoals IFN-γ geproduceerd door geactiveerde T-cellen, hun immunomodulerende status ten opzichte van T-cellen en verkrijgen ze anti-proliferatieve vermogens op B-cellen. Dit zou erop kunnen wijzen dat een allo-antilichaam-immuunrespons zich daarom niet *in vivo* zou ontwikkelen, zelfs niet met ontstekingsbevorderende factoren.

Om een meer *in vivo* realistische situatie na te bootsen zijn we in **hoofdstuk 4 en 5** overgegaan op muismodellen. In **hoofdstuk 4** gebruikten we een immunodeficiënt diermodel gehumaniseerd met allogene immuuncellen om de immuunresponsen tegen chondrogeen

gedifferentieerde pMSCs (ch-pMSCs) te onderzoeken. Deze van ch-pMSC afgeleide transplantaten vertoonden *in vitro* geen immunogene reacties. Na subcutane implantatie waren ze in staat om gedurende 3 weken te mineraliseren in aanwezigheid van een allogeen immuunsysteem zonder het begin van immuunreactie. Bovendien genereerden ch-pMSC's rijp bot na 8 weken implantatie. Dit bot hield nog tot 6 weken aan in aanwezigheid van een allogeen immuunsysteem, wat suggereert dat deze allografts niet door de muizen worden afgestoten. De intrinsieke beperkingen van het gehumaniseerde diermodel, namelijk het ontbreken van enting van bepaalde immuun subsets zoals antigeen presenterende cellen (APC's) of B-cellen, deden ons echter op zoek gaan naar andere potentiële diermodellen. In **hoofdstuk 5** hebben we gebruik gemaakt van een immunocompetent muismodel om het potentieel van onze ch-pMSCs om bot te vormen in aanwezigheid van een volledig functioneel xenogeen immuunsysteem te bestuderen. Hier zagen we dat de percentages $CD4^+$ en $CD8^+$ T-cellen, B-cellen en klassieke/niet-klassieke monocyten-subsets na twee weken niet systemisch veranderd waren bij deze dieren. Echter, ch-pMSC-geïmplanteerde muizen hadden op dit tijdstip significant hogere niveaus van serum totaal IgG vergeleken met schijn-geopereerde muizen. Na 12 weken implantatie vonden we lage niveaus van infiltratie van T-cellen en macrofagen in de xenotransplantaten. Desondanks bleven ch-pMSC-constructen bestaan en vertoonden ze geen tekenen van uitschakeling. Bovendien werden zij gemineraliseerd en nam hun botvolume toe van 4 weken na implantatie tot 12 weken.

De resultaten van dit proefschrift worden besproken in **hoofdstuk 6**. We stellen ook manieren voor om de bestaande diermodellen te verbeteren om meer realistische biologische situaties voor botherstel en osteo-immunologie na te bootsen. Al met al geven onze resultaten aan dat pMSC's uitstekende kandidaten zijn voor botherstel, wat aantoont dat initiatie en instandhouding van botvorming plaatsvindt zonder een substantiële immuunreactie in zowel een xenogeen als een allogeen scenario.

Resumen en español

Los defectos óseos que no se resuelven por sí mismos constituyen un desafío clínico. De manera ideal, el uso de hueso del propio paciente para cubrir el defecto (trasplante autólogo) sigue siendo el método de referencia. Sin embargo, el empleo de este método no es, en práctica, lo ideal, ya que requiere un procedimiento quirúrgico adicional, que implica dolor y molestias para el paciente, y plantea la limitación de la cantidad de hueso que se puede extraer.

Los métodos de ingeniería de tejidos pretenden superar este problema, empleando células madre estromales (MSCs del inglés Mesenchymal Stromal Cells). Estas células son capaces de diferenciarse en el hueso cuando son estimuladas condrogénicamente con TGF- β3, formando una plantilla de cartílago antes de convertirse en hueso. Este proceso inducido en el laboratorio imita la ruta de osificación endocondral que se produce de forma natural en el cuerpo humano cuando se forma el hueso. La elección de las células para seguir este método sigue siendo crucial, ya que la creación de un "molde" óseo requiere un elevado número de células que sean capaces de diferenciarse en el tejido deseado ante los estímulos adecuados. Sin embargo, en pacientes de edad avanzada esto podría no ser una opción, ya que ha sido demostrado que estas capacidades decaen con la edad. Los estudios previos sugieren que las MSCs obtenidas de fuentes jóvenes y sanas superan a las células adultas a efectos de diferenciación. Por lo tanto, encontrar la mejor fuente de células que sea capaz de expandirse y diferenciarse en un injerto óseo rápidamente sería el punto de partida para poner en práctica este método. Además, garantizar la ausencia de una posible respuesta inmune que pudiera surgir al utilizar una fuente alogénica de células sería clave para garantizar la estabilidad a largo plazo del aloinjerto óseo. Estas cuestiones se analizan en el **capítulo 1,** el cual ofrece una introducción general de esta tesis.

En **el capítulo 2**, nos centramos en investigar las propiedades de diferenciación y proliferación de una fuente de células madre estromales

pediátricas (pMSCs) en comparación con las de adulto (aMSCs). Aquí descubrimos que, bajo las mismas condiciones, las células estromales pediátricas son más adecuadas que las de un adulto para fines de ingeniería de tejidos en cuanto a una proliferación más rápida, una diferenciación multilineaje más consistente, incluyendo la condrogénesis, y menor senescencia. Al igual que las MSCs adultas, las pMSCs no mostraron reacciones inmunogénicas en un ensayo inicial de co-cultivo con células T. Por lo tanto, fueron elegidas como las candidatas ideales para el resto de los estudios de esta tesis.

Como se mencionó anteriormente, investigar el comportamiento de las MSCs en un sistema inmune alogénico es fundamental para evitar para evitar un potencial proceso de rechazo inmunológico que podría destruir el aloinjerto óseo. En concreto, se ha demostrado que la fracción adaptativa de la respuesta inmune, compuesta por células T y B, desempeña un papel fundamental en los procesos de rechazo alogénico. En el **capítulo 3**, investigamos la respuesta inmune adaptativa hacia las pMSCs no diferenciadas. Para imitar el escenario más realista que se produciría al implantar estos aloinjertos óseos durante una cirugía, incorporamos la citoquina proinflamatoria IFN-γ. Nuestros resultados muestran que el IFN-γ no altera la capacidad inmunomoduladora de las pMSCs hacia las células T. Sin embargo, las pMSCs estimuladas con IFN-γ, pero no las no estimuladas inhibieron en gran medida la proliferación de células B, así como la formación de plasmablastos productores de anticuerpos y la producción de inmunoglobulina G (IgG). Por lo tanto, en el caso de las señales proinflamatorias, como el IFN-γ producido por las células T activadas, las pMSCs mantienen su capacidad inmunomoduladora hacia las células T, y adquieren capacidades antiproliferativas sobre las células B. Esto podría indicar que, por tanto, no se desarrollaría una respuesta inmunitaria mediada por aloanticuerpos *in vivo*, incluso bajo condiciones inflamatorias.

Para imitar una situación más realista a la que ocurriria *in vivo*, en los **capítulos 4 y 5** pasamos a utilizar modelos animales de ratones. En el **capítulo 4,** empleamos un modelo animal inmunodeficiente humanizado

con células inmunes de origen alogénico para estudiar la respuesta inmune hacia las pMSCs previamente diferenciadas condrogénicamente (ch-pMSCs). Estos injertos de ch-pMSCs no desencadenaron respuestas inmunes *in vitro*. Tras su implantación subcutánea, fueron capaces de mineralizarse en presencia de un sistema inmune alogénico durante 3 semanas sin que aparecieran respuestas inmunes. Además, las ch-pMSCs generaron hueso maduro tras 8 semanas de implantación. Este hueso persistió hasta 6 semanas más en presencia de un sistema inmune alogénico, lo que sugiere que estos aloinjertos óseos no son eliminados por el huésped. Sin embargo, las limitaciones intrínsecas del modelo animal humanizado, tales como la dificultad de ciertas células del sistema inmune como las células presentadoras de antígenos (APC) o las células B para sobrevivir tras la humanización, nos hicieron buscar otros posibles modelos animales. En el **capítulo 5**, utilizamos un modelo de ratón inmunocompetente para estudiar la capacidad de nuestras ch-pMSC para formar hueso en un sistema inmunitario xenogénico plenamente funcional. En ese escenario, descubrimos que los porcentajes de células T $CD4^+$ y $CD8^+$, células B y subconjuntos de monocitos clásicos/no clásicos no presentaban una alteración sistémica en los animales después de dos semanas. Sin embargo, los ratones implantados con ch-pMSC tenían niveles significativamente más altos de IgG total en suero en comparación a los ratones que no recibieron ch-pMSCs. Tras 12 semanas de implantación, encontramos bajos niveles de infiltración de células T y macrófagos en los xenoinjertos. A pesar de ello, los implantes de ch-pMSC persistieron y no mostraron signos de reabsorción. Además, los injertos se mineralizaron y su volumen óseo aumentó desde las 4 semanas posteriores a la implantación hasta las 12 semanas.

Los resultados de esta tesis se analizan en el **capítulo 6**. Además, sugerimos formas de mejorar los modelos animales existentes para que imiten situaciones biológicas más realistas en los campos de de ingeniería de tejidos y osteoinmunología. En definitiva, nuestros resultados indican que las pMSCs son excelentes candidatas para la regeneración ósea, demostrando que la iniciación y el mantenimiento de formación de hueso

se producen sin una respuesta inmune sustancial tanto en un escenario xenogénico como alogénico.

Translated by Celia Palomares Cabeza

Résumé en français

Les défauts osseux qui restent non résolus représentent un défi clinique. Dans l'idéal, l'utilisation de l'os du patient pour combler le défaut (autogreffe) reste la solution de référence. Cependant, l'utilisation de cette approche n'est en réalité pas idéale, car elle exige une procédure chirurgicale supplémentaire, impliquant douleur et inconfort pour le patient, il pose également la limitation de la quantité d'os qui peut être prélevée.

Les approches d'ingénierie tissulaire visent à surmonter ce problème, en utilisant des cellules stromales de moelle (CSM). Ces cellules sont capables de se différencier formant un modèle de cartilage avant de devenir un os lorsqu'elles sont stimulées par la chondrogenèse avec du TGF- β3. Ce processus induit en laboratoire imite la voie de l'ossification endochondrale qui se produit naturellement dans le corps humain lors de la formation osseuse. Le choix des cellules pour suivre cette approche reste essentiel, car la création d'un "moule" osseux exige un nombre élevé de cellules capables de se différencier en tissu souhaité sous l'effet des bons stimuli. Toutefois, chez les patients âgés, cette possibilité n'est pas envisageable, car ces capacités diminuent avec l'âge. Des recherches antérieures suggèrent que les CSM dérivées de sources jeunes et saines sont plus performantes que les cellules adultes en termes de différenciation. Par conséquent, trouver la meilleure source de cellules capable de se développer et de se différencier rapidement en un greffon osseux serait le point de départ pour faire de cette approche une réalité. En outre, il est essentiel de garantir l'absence d'une réponse immunitaire potentielle qui pourrait survenir lors de l'utilisation d'une source allogénique de cellules, afin de garantir la stabilité à long terme de l'allogreffe osseuse. Ces sujets sont abordés dans le chapitre 1, qui constitue une introduction générale de cette thèse.

Dans le chapitre 2, nous nous concentrons sur l'étude des propriétés de différenciation et de prolifération d'une source de CSM pédiatriques (pMSCs en anglais) par rapport aux CSM adultes (aMSCs en anglais). Nous constatons ici que, dans les mêmes conditions, les CSM pédiatriques sont

plus adaptées que les CSM adultes à des fins d'ingénierie tissulaire en termes de prolifération plus rapide, de différenciation multilinéaire plus cohérente, y compris la chondrogenèse, et de sénescence moindre. Comme les CSM adultes, elles n'étaient pas immunogènes lors d'un premier essai de co-culture avec des cellules T. C'est pourquoi elles ont été choisies comme modèle idéal. Elles ont donc été choisies comme les candidates idéales pour le reste des études de cette thèse.

Comme il a été mentionné auparavant, l'étude du comportement des CSM dans un système immunitaire allogénique est essentielle pour éviter un processus potentiel de rejet immunitaire qui pourrait détruire l'allogreffe osseuse. En particulier, il a été démontré que la fraction adaptative de la réponse immunitaire composée de cellules T et B joue un rôle fondamental dans les processus de rejet d'origine allogénique. Dans le chapitre 3, la réponse immunitaire adaptative envers les pMSCs indifférenciées a été étudiée. Pour imiter le scénario le plus réaliste qui se produirait lors de l'implantation de ces allogreffes osseuses dans une opération chirurgicale, nous avons incorporé la cytokine pro-inflammatoire IFN-γ. Nos résultats montrent que l'IFN-γ n'altère pas les capacités immunomodulatrices des pMSCs vis-à-vis des lymphocytes T. Cependant, l'IFN-γ a amorcé le processus d'immunisation des pMSCs. Cependant, les pMSC amorcées par l'IFN-γ, mais pas les pMSCs non amorcées, ont fortement inhibé la prolifération des cellules B, ainsi que la formation de plasmablastes producteurs d'anticorps et la production d'immunoglobuline G (IgG). Par conséquent, dans le cas de signaux pro-inflammatoires, tels que l'IFN-γ produit par des cellules T activées, les pMSCs préservent leur statut immunomodulateur vis-à-vis des cellules T, et acquièrent des capacités anti-prolifératives sur les cellules B. Cela pourrait indiquer qu'une allo-régulation de la prolifération des cellules B est nécessaire. Cela pourrait indiquer qu'une réponse immunitaire par allo-anticorps ne se développerait donc pas in vivo, même dans des conditions inflammatoires.

Afin d'imiter une situation plus réaliste in vivo, nous nous sommes tournés vers des modèles de souris dans les chapitres 4 et 5. Dans le chapitre 4,

nous avons utilisé un modèle animal immunodéficient humanisé avec des cellules immunitaires allogéniques pour étudier les réponses immunitaires envers les pMSCs différenciées par la chondrogénie (ch-pMSCs). Ces greffons dérivés de ch-pMSCs n'ont pas montré de réponses immunogéniques in vitro. Après l'implantation sous-cutanée, ils ont pu se minéraliser en présence d'un système immunitaire allogène pendant 3 semaines sans apparition de réponses immunitaires. De plus, les ch-pMSCs ont généré de l'os mature après 8 semaines d'implantation. Cet os a persisté jusqu'à 6 semaines supplémentaires en présence d'un système immunitaire allogénique, ce qui suggère que ces allogreffes osseuses ne sont pas éliminées par l'hôte. Cependant, les limites intrinsèques du modèle animal humanisé, à savoir l'absence de greffe de certains sous-ensembles immunitaires tels que les cellules présentatrices d'antigènes (CPA) ou les cellules B, nous ont incités à rechercher d'autres modèles animaux potentiels. Dans le chapitre 5, nous avons utilisé un modèle de souris immunocompétente pour étudier le potentiel de nos ch-pMSC à former des os en présence d'un système immunitaire xénogénique pleinement fonctionnel. Dans ce scénario, nous avons constaté que les pourcentages de cellules T CD4+ et CD8+, de cellules B et de sous-ensembles de monocytes classiques/non classiques n'étaient pas altérés de manière systémique chez ces animaux après deux semaines. Cependant, les souris ayant reçu une implantation de ch-pMSC présentaient des taux d'IgG totales sériques significativement plus élevés que les souris ayant subi une opération fictive à ce moment-là. Après 12 semaines d'implantation, nous avons constaté de faibles niveaux d'infiltration de cellules T et de macrophages dans les xénogreffes. Malgré cela, les constructions ch-pMSC ont persisté et n'ont pas montré de signes de résorption. De plus, les constructions se sont minéralisées et leur volume osseux a augmenté de 4 semaines après l'implantation à 12 semaines.

Les résultats de cette thèse sont discutés dans le chapitre 6. Nous suggérons également des moyens d'améliorer les modèles animaux existants afin de reproduire des situations biologiques plus réalistes pour la réparation osseuse et l'ostéoimmunologie. Dans l'ensemble, nos

résultats indiquent que les pMSCs sont d'excellents candidats pour la réparation osseuse, prouvant que l'initiation et le maintien de la formation osseuse se produit sans une réponse immunitaire substantielle dans un scénario xénogénique et allogénique.

Translated by Celia Palomares Cabeza

List of Publications

Knuth, C.A, Kiernan C.H, **Palomares Cabeza V.**, Lehmann J., Witte-Bouma J. , Ten Berge D., Brama P.A, Wolvius E.B., Strabbing E.M, Koudstaal M.J, Narcisi R., Farrell E. *Isolating Pediatric Mesenchymal Stem Cells with Enhanced Expansion and Differentiation Capabilities*. Tissue Eng Part C Methods 2018; 24(6):313-321.

Palomares Cabeza V., Hoogduijn M.J, Kraaijeveld R., Franquesa M., Witte-Bouma J., Wolvius E.B., Farrell E., Brama P.A. *Pediatric Mesenchymal Stem Cells Exhibit Immunomodulatory Properties Toward Allogeneic T and B Cells Under Inflammatory Conditions*. Frontiers in Bioengineering and Biotechnology 2019; 7.

Delcassian D., Malecka A.A., Opoku D., **Palomares Cabeza V**., Merry C., Jackson A.M.
Primary human macrophages are polarized towards pro-inflammatory phenotypes in alginate hydrogels. bioRxiv 2019; 824391

Fahy N.*, **Palomares Cabeza V**.*, Lolli A., Witte-Bouma J., Merino A., Ridwan Y., Wolvius E. B., Hoogduijn M. J., Farrell E., Brama P. A. J. *Chondrogenically Primed Human Mesenchymal Stem Cells Persist and Undergo Early Stages of Endochondral Ossification in an Immunocompetent Xenogeneic Model*. Frontiers in Immunology 2021;2.

Palomares Cabeza V., Fahy N., Kiernan C.H., Lolli A., Witte-Bouma J., Fahmy García S., Merino A., Kops N., Ridwan Y.R, Wolvius E., Brama P.A., Hoogduijn M.J., Farrell E. *Bone formation by human pediatric marrow stromal cells in a functional allogeneic immune system*. Manuscript in preparation for submission in 2022.

Palomares Cabeza V.*, Kummer L.Y.L*, Wieske L. , Hagen R.R, Konijn V.A.L., Duurland M.C.,. van Dam P.J; Stalman E.W., van de Sandt C.E., Boekel L., Verstegen N.J.M. , Steenhuis M., Rispens T., Tas S.W,Wolbink G.J., Killestein J., Kuijpers T.W., van Ham S.M, Eftimov F., ten Brinke A., van

Kempen Z.L.E. on behalf of the T2B! SARS-CoV-2 study group. *Longitudinal T-cell responses after a third SARS-CoV-2 vaccination in MS patients on ocrelizumab or fingolimod*. Accepted. Neurology: Neuroimmunology and Neuroinflammation (N2), 2022.

Freen-van Heeren J.J., **Palomares Cabeza V**., Cobeta López D., Kivits D., Rensink I., Turksma A.W., ten Brinke A. *Assessing antigen-specific T cell responses through IFN--γ enzyme-linked immune absorbent spot (ELISpot).* Manuscript in preparation for submission in 2022

**Shared first authorship*

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PhD portfolio

Personal details

Name PhD Student: Virginia Palomares Cabeza

Erasmus MC department(s): Departments of Oral and Maxillofacial Surgery and Internal Medicine
PhD period: September 2016 – April 2021
Research School: Postgraduate School Molecular Medicine
Promotors: Prof. Eppo B. Wolvius, and Prof. Pieter A. Brama

Co-Promotors: Eric Farrell, PhD, and Martin Hoogduijn, PhD

PhD training

Year	Courses/Workshop	Workload (ECTS)
2016	Introduction to laboratory Animal Science	4
2016	Species-specific: small rodents	0.5
2017	Scientific Integrity	0.3
2017	Biomedical Research Techniques	1.5
2017	Monocytes: origins, destinations, functions and diagnostic targets	0.2

2018	Research Management for PhD students and Postdocs	1
2018	Personal Leadership and Communication	1
2018	Personal and Career Development	0.15
2019	Advanced course on Applications in flow cytometry	1
2019	Presentation Skills	1
2019	Biomedical English Writing and Communication	3
2019	Medicine by Design Summer School, Toronto	5

Year	International Conferences	Workload (ECTS)
2016	25th Annual NBTE meeting, Lunteren	1
2017	21st Molecular Medicine Day. Poster presentation	0.5
2018	22nd Molecular Medicine Day. Poster presentation	0.5
2018	7th International Conference in Osteoimmunology,	1.5

	Chania. Poster presentation	
2019	TERMIS EU Conference, Rhodes. Oral and Poster Presentation	1
2019	23rd Molecular Medicine Day. Poster presentation	0.5
2019	ACE Score and Systems Biomedicine Day	0.5

Year	Teaching Activities	Workload (ECTS)
2019	Minor Biomedical Science lectures	0,5

Year	Department Meetings and Presentations	Workload (ECTS)
2016-2021	Research Meetings Department Orthopaedics (weekly)	2
2016-2021	Research Meetings Department Oral and Maxillofacial Surgery	2
2016-2021	Research Meetings Department Internal Medicine	2
2016-2020	Journal Club meetings	1

Year	Miscellaneous	Workload (ECTS)
2016-2021	Oral and Maxillofacial Surgery Department Day (annually)	0,5
2019	Organisation Lab Day Orthopaedics/Oral and Maxillofacial Sugery	0,5
2016-2018	Dutch Language Courses, Baay School Rotterdam levels A1-A2.1	3

Year	Awards and Grants
2018	23rd Molecular Medicine Day, Best Poster Award
2018	Summer by Design School Award Top 3 Best Pitch Presentation

Total ECTS: 35,65

Curriculum vitae (about the author)

Virginia Palomares Cabeza (she/her) was born on 12th of August 1993 (Leo Sun/Virgo rising) in a small Spanish town geographically located in the African continent called Ceuta. After finishing her scientific education in high school, she decided to pursue a Bachelor (BSc.) in Biotechnology at the University of Salamanca (Spain) in 2011.

Since back then she considered herself a plant biotechnologist, she obtained her BSc. Thesis *'Biotechnological Function of Nitric Oxide During Biotic and Abiotic Stress'* in 2015 at the Centro Hispano Luso de Investigaciones Agrarias (Salamanca), under the supervision of Dr. Luis Sanz and Dr. Dolores Rodriguez, with whom she also carried out curricular and extracurricular internships in 2014 and 2015. Despite enjoying her days in the greenhouse, in the summer of 2015 she decided to steer her scientific career in a new direction and began her Masters of Science (MSc.) in Cancer Immunology and Biotechnology at the University of Nottingham (United Kingdom), of which she graduated in 2016 with Distinction. She obtained her thesis titled *'The impact of three dimensional culture on dendritic cells function*' the same year, under the supervision of Dr. Andrew Jackson and Dr. Derfogail Delcassian at the Nottingham City Hospital and the Centre for Biomolecular Sciences. Looking for brighter skies and warmer temperatures, she made the decision of moving to the Netherlands in 2016, in order to start her joint PhD project between the departments of Oral and Maxillofacial Surgery and Internal Medicine at the Erasmus MC, and the School of Veterinary Medicine at the University

College Dublin in Ireland. During the following four and a half years, she carried out the research compiled in the present thesis under the supervision of prof. Eppo Wolvius, Dr. Eric Farrell, Dr. Martin Hoogduijn and prof. Pieter Brama. Somehow still not tired of research and in the midst of a pandemic, in 2021 she embarked on her postdoctoral research journey at the Department of Immunopathology of Sanquin Bloedvoorziening (Amsterdam), supervised by prof. Marieke van Ham and Dr. Anja ten Brinke. She currently works on various projects, focusing on clarifying the T cell responses towards SARS-CoV-2 in immunosuppressed patients, and the T cell responses in patients with multiple sclerosis after tolerogenic dendritic cell therapy.

Virginia currently lives in her *decorated-to-perfection* dream apartment in Amsterdam, with a walk-in closet and a wide range of surfaces that serve as practice props for her flexibility tricks. When she is not doing science-related stuff, she enjoys dancing ballet in pointe shoes designed by Satan, catching flights, and eating any kind of good food.