Intra-Articular Application of Mesenchymal Stem Cell Therapy for Osteoarthritis — The Next Step

Optimization of Therapeutic Capacity and Applicability

Maarten Leijs

Intra-Articular Application of Mesenchymal Stem Cell Therapy for Osteoarthritis — The Next Step

Maarten Leijs

Financial support for publication and dissemination of this thesis was provided by:

• Department of Orthopedics, Erasmus MC University Medical Center, Rotterdam
• Centrum Orthopedie Rotterdam b.v., Rotterdam
• FS Fysio, Capelle aan den Ijssel
• BÜCHI Labortechnik GmbH, Hendrik-Ido-Ambacht
• Chipoft b.v., Amsterdam
• Nederlandse Orthopaedische Vereniging (NOV), 's Hertogenbosch
• Erasmus University Rotterdam, Rotterdam
• Anna Fonds | NOREF, Leiden

The research described in this thesis was financially supported by the NIRM (Netherlands Institute of Regenerative Medicine) and the Dutch Organisation for Science, division Applied and Engineering Sciences (technical foundation STW, grant number 12898).

Cover design: Maarten Leijs en Erwin Timmerman (Optima Grafische Communicatie, Rotterdam)
Lay-out and printing: Optima Grafische Communicatie (www.ogc.nl), Rotterdam, the Netherlands
ISBN: 978-94-6361-615-7

De digitale versie van dit proefschrift is online te vinden via onderstaande QR-code.

Intra-Articular Application of Mesenchymal Stem Cell Therapy for Osteoarthritis — The Next Step

Optimization of Therapeutic Capacity and Applicability

Intra-articulaire mesenchymale stamceltherapie bij artrose — de volgende stap

Optimalisatie van therapeutische capaciteit en toepasbaarheid

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof. dr. A.L. Bredenoord

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
Donderdag 2 december 2021 om 10:30 uur

door

Maarten Johannes Cornelis Leijs
geboren te Soest, Nederland

Erasmus University Rotterdam

PROMOTIECOMMISSIE

Promotor:	Prof. dr. G.J.V.M. van Osch
Overige leden:	Prof. dr. M.E.J. Reinders Prof. dr. G.J. Strijkers Dr. P.J. Emans
Copromotoren:	Dr. M.R. Bernsen Dr. P.K. Bos

TABLE OF CONTENTS

Chapter 1

General introduction

Aims and outline of the thesis

GENERAL INTRODUCTION

Osteoarthritis

Osteoarthritis (OA) is a progressive degenerative joint disease, causing pain, stiffness and impaired movement. OA is one of the most common causes of disability in adults older than 60 years of age, especially in developed countries. Between 10% to 18% of all adults aged over 60 years have some degree of OA with prevalence higher among women[1-5]. The prevalence of OA will increase in the future due to ageing of the population and rising obesity rates[6]. The prevalence of OA in the Dutch population is estimated to increase from 7% in 2011, to 12% in 2040[7,8]. In **figure 1** the estimated patient increase in absolute numbers between 2015 and 2040 is depicted. The United Nations estimates that people aged over 60 years will account for more than 20% of the world's population in 2050[3]. This will result in a high prevalence of people that suffer from OA worldwide in 2050 and this will be accompanied by high medical expenses, increasing disability-adjusted life years (DALYs) and high costs considering time off at work[4,9].

OA is a disease with a multifactorial etiology. Risk factors to develop OA are ageing, obesity, joint trauma, genetic predisposition, physical load and female sex at older age[10,11]. OA is characterized by inflammation and catabolic processes causing cartilage degeneration, subchondral bone alterations and osteophyte formation (**figure 2,B**). OA is accompanied by pain, stiffness, reduced load capacity and impaired movement due to the affected articular tissues. Degeneration of articular cartilage is one of the main problems in the pathophysiology of OA. Cartilage is a highly specialized connective tissue which consists of chondrocytes (1-5%) and a complex network of macromolecules, mainly collagen and proteoglycan (PG), that form the extracellular matrix (ECM) and ensure the function of the cartilage[12]. Hyaline cartilage is the type of cartilage that covers the articular surface. The function of this cartilage is to reduce friction and absorb peak loading and to distribute the load over the underlying bone and thereby increasing wear-resistance in the joint. Articular cartilage can fulfil this function due to the complex composition of the ECM with its negatively charged glycosaminoglycans that make it possible to maintain the large amount of water[13,14]. The chondrocytes in cartilage are the architects of the ECM and maintain the ECM. Chondrocytes are dependent on oxygen and nutrient diffusion from the synovial fluid and subchondral bone. Cartilage itself is avascular tissue[15] and due to this avascularity, and its complex ECM structure, the intrinsic repair capacity of cartilage is very limited[16].

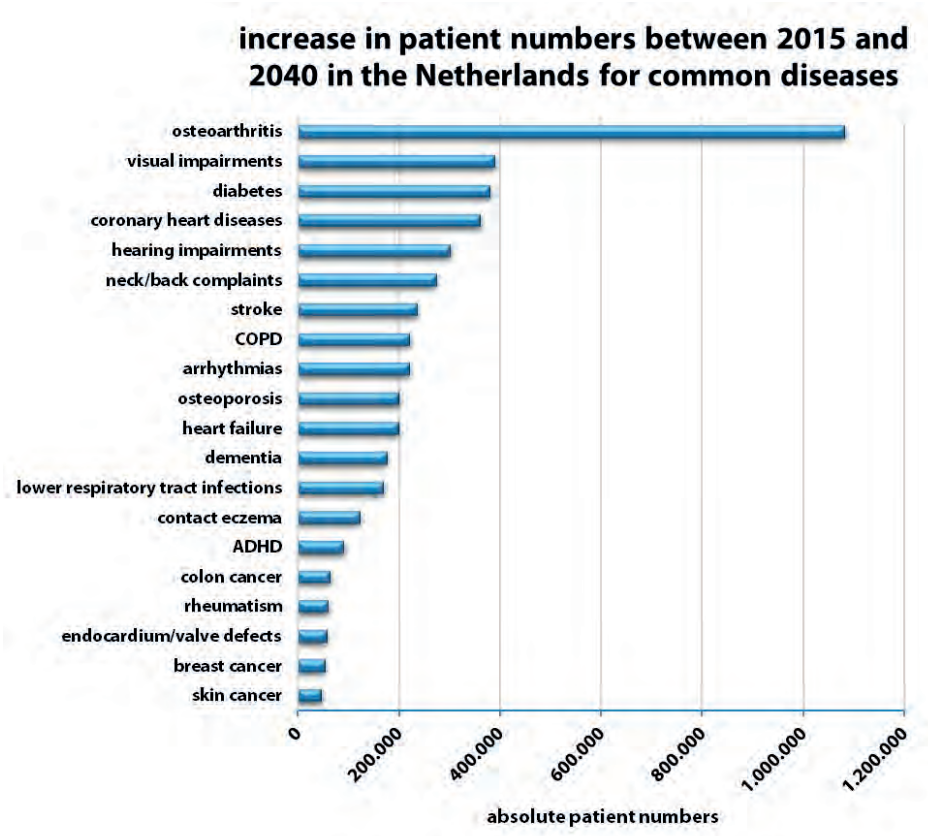


***Figure 1**. Estimated increase in patient numbers between 2015 and 2040 in the Netherlands for common diseases. Reproduced from: National Institute for Public Health and the Environment, Netherlands. Public Health Foresight Study 2018 (VTV-2018): diseases[8].*

Inflammation and catabolic processes that exist in OA, affect joint tissues including cartilage, where it results in cartilage damage with ECM degradation, chondrocyte phenotype changes and chondrocyte proliferation[17-19]. These processes will stimulate the inflammatory and catabolic processes leading to a self-retaining situation of progressive joint degeneration resulting in cartilage erosion and exposure of the subchondral bone[14,17,20]. This leads to (severe) pain, stiffness and impaired movement.

Eventually, surgical options are available to relieve the pain. Depending on the extent and location of the degeneration an operation can be performed. In joints with less severe degeneration, joint preserving operations can be performed like an osteotomy and joint distraction. Alternative operation options are resection arthroplasty or an arthrodesis. Ultimately unicompartimental or total joint re-

placement can be performed[16,20]. Many studies have been performed to elucidate the disease mechanism(s) of OA and to find a non-surgical treatment for OA[18,21,22]. However, to date the complete etiology of OA remains unclear and no drugs or therapies are available to cure OA. Current treatments for OA have the ability to temporarily reduce and control the symptoms of OA[16,18,20]. The non-surgical, also referred to as conservative, standard treatment options are: lifestyle changes, physical therapy, analgesics like paracetamol and non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular injections with corticosteroids. The various common treatment options now available to reduce symptoms in OA are shown in **figure 2**. Non-standard care treatments and treatments in experimental setting are for instance: hyaluronic acid, platelet rich plasma, disease modifying osteoarthritic drugs (DMOADs) like matrix-metalloproteinase (MMPs) inhibitors, bone marrow aspiration concentrate, bisphosphonates, glucosamine, calcitonin, cytokine blockers, inducible nitric oxide synthase (iNOS) inhibitors and Wnt pathway inhibitors[2,20,21,23,24]. There is a wide variation in success, invasiveness, complication risk, costs and durability of all treatments, with the aim to reduce pain and regain mobility.

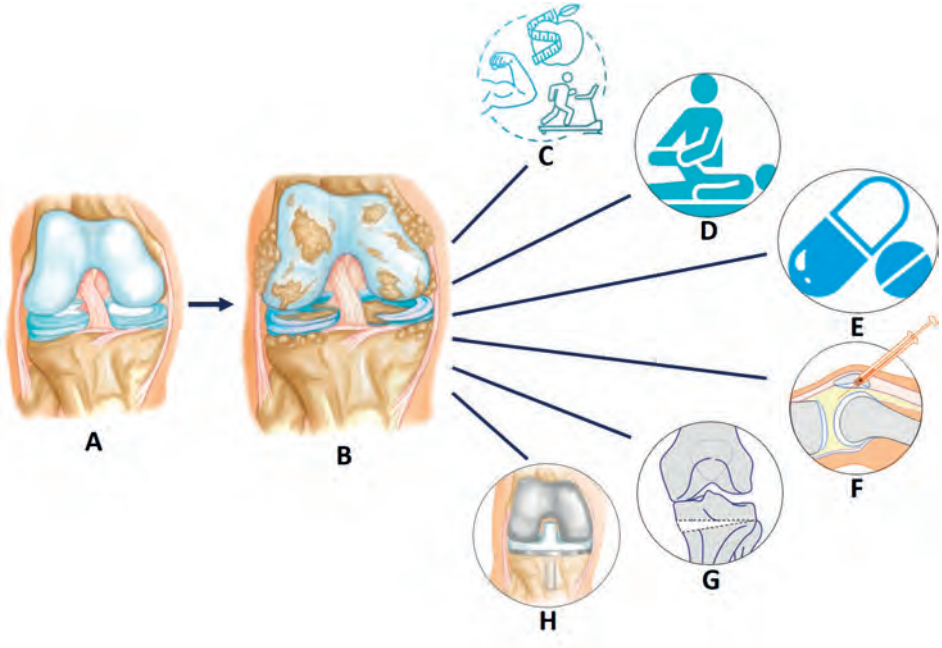


***Figure 2**. Various treatment options available to reduce symptoms in OA. Normal knee joint (**A**). Knee with OA, with cartilage degeneration and osteophyte formation (**B**). Different conservative standard treatment options: lifestyle changes (**C**), physical therapy (**D**), analgesics like paracetamol NSAIDs (**E**), intra-articular injections with corticosteroids (**F**). Operation options: osteotomy (**G**) and total joint replacement (**H**). NSAIDs (Non-steroidal anti-inflammatory drugs), OA (osteoarthritis).*

Treatment with Mesenchymal stem cells

In the search for effective non-surgical treatments for OA, different biological treatment possibilities such as platelet-rich plasma (PRP), bone marrow aspiration concentrate and cytokine blockers for OA are under investigation. Another biological treatment option is the use of cell therapy. Cell therapy with mesenchymal stem cells (MSCs) for OA is promising. MSCs are the progenitors of connective tissue cells and are able to differentiate into different cell types of the mesenchymal lineages[25]. The term mesenchymal stem cell was first used by Caplan in 1991. The first descriptions of research with these cells date back to the 19th century (Goujon 1869), to Tavassoli, Crosby and Friedenstein 1960s-1970s. They all showed osteogenic potential of bone marrow cells and Fiedenstein showed that it was a small subpopulation of the bone marrow cells that had osteogenic potential[26,27]. MSCs rapidly gained popularity in science, because of their multilineage differentiation capacities, the fact that MSCs are relatively easy to culture and expand, and MSCs can be harvested from multiple sources[27,28]. For research and treatment purposes, MSCs are harvested most often from bone marrow or adipose tissue. Other possible sources are for instance fetal/cord blood, dental pulp and synovium[29,30]. Cells need to meet specific criteria in order to be regarded as mesenchymal stem cell. These criteria have been established by the ISCT (international society for cell and gene therapy) in 2006 by Dominici et al[31]. Cells should at least adhere to plastic culture flasks and have fibroblast-like morphology with colony forming capabilities. MSCs should have a multilineage differentiation capacity, and should have an expression profile containing a typical set of surface markers such as CD73, CD90, and CD105, and should be negative for lineage-specific markers CD34, CD14, CD45. One of the reasons why MSCs are promising candidates for cell therapy for OA is because of their chondrogenic potential, and their ability to produce extracellular matrix molecules[28,32-34]. Besides their differentiation capacity, MSCs have immunomodulatory and trophic capacities by secreting anti-inflammatory factors and growth factors, which could possibly inhibit and change the inflammatory and catabolic environment of OA (**figure 3**)[28,35-43]. MSCs are considered to be immuneprivileged which would make them even more attractive for cell therapy. Theoretically this allows the use of allogeneic MSCs which will reduce costs and enable off-the-shelf therapy. MSCs have already been injected intra-articularly in preclinical and clinical studies as a treatment for cartilage damage and OA, showing promising results[44-49].

Current state of the art of application of MSCs in OA treatment

There is an increasing number of preclinical and clinical studies with mesenchymal stem-cell-based therapy for OA throughout the world[22,50-58]. Although the initial

results of these studies are promising considering pain and function outcome, the results on the effect on structural changes are contradictory. The majority of these studies are of limited value due to the concerns of risk of bias and quality of evidence. There is a great heterogeneity in the way MSCs are harvested and cultured, and in the way MSCs are applied. Furthermore, it is important that the effect of MSC treatment should be at least as successful or even better in reducing pain and increasing mobility, compared to current standards of conservative treatment with painkillers (NSAIDs) and exercise therapy or (total) joint replacement. The final and ultimate goal is that MSCs will cure OA.

The most important goal for the patient is pain reduction and functional improvement. In one of our own studies the effects of intra-articularly injected, culture-expanded rat MSCs and rat bone marrow-derived mononuclear cells (BMMNCs) on pain, cartilage damage, bone changes and inflammation were studied. OA was chemically induced in rat knees *in vivo* to evaluate multiple OA pathology aspects and to explore possibilities to enhance clinical translatability[18]. The therapy was well tolerated by all animals and only the injected MSCs gave significant pain relieve. There was no effect on inflammation or cartilage degeneration. MSCs were labeled and tracked by MRI and bioluminescence imaging, by which we could monitor the location and metabolic activity of the intra-articularly injected MSCs over time. In this way we observed total cell death/cell loss intra-articular within 3 weeks after injection (**figure 4**).

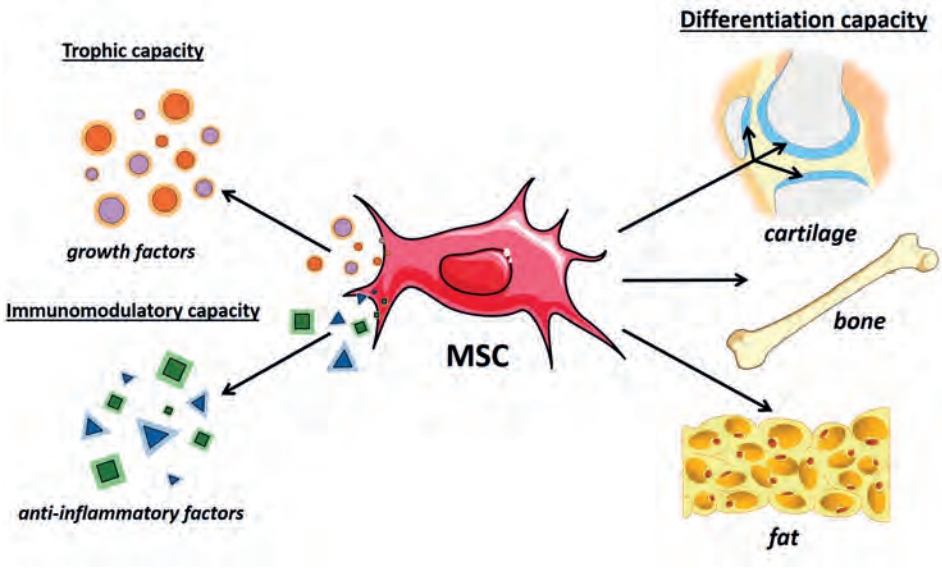


***Figure 3**. MSCs are promising candidates for cell therapy for OA because of their different capacities.*

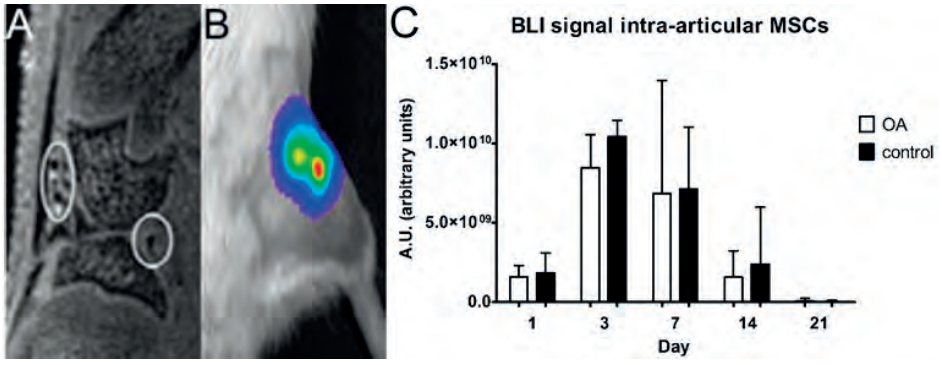


Figure 4. *Cell tracking experiments with labeled MSCs were found in the joint space by MRI (**A**). BLI signal of injected MSCs showed the presence of viable cells in the knee joints in a 3-week time course (**B-C**). N=3 per group, mean (SD) is shown. BLI (Bioluminescence imaging)*[18].

In literature there are inconsistent results about cell survival after intra-articular injection. Some studies reported a longer survival of intra-articular injected MSCs, compared to our results, with a survival of up to 6 months while others could not find any residual MSCs after two weeks[46,59-62]. The time that MSCs remain intra-articularly will be important for their therapeutic function in OA. The assumption is that the longer they can exert their therapeutic capacity near the degenerative tissues, the better the therapeutic effect will be. Imaging can be a useful tool in studying the fate of intra-articular injected MSCs. There are different labeling and imaging techniques available to evaluate cell survival and cell residence time. By using and adjusting techniques to quantify imaging results, the location and activity of MSCs can be evaluated over time.

Although the MSCs might disappear relatively fast[18,63-65], cell-based therapy with MSCs showed promising results, pre-clinically as well as clinically, in different diseases such as interstitial lung diseases, glomerulonephritis, graft versus host disease, solid organ transplantation rejection, rheumatoid arthritis (RA), Crohn's disease and heart failure[63,66-77]. Despite these promising results, a significant part of the potential OA disease modifying mechanism is still unclear. In particular we do not know much about the behaviour of MSCs in the osteoarthritic joint and the interactions between MSCs and the OA joint environment. Moreover, further optimization of the effects of MSCs is needed[78].

AIM AND OUTLINE OF THE THESIS

The aim of my research is to study factors that may be important in increasing the efficacy/effect of MSCs for cell-therapy for OA.

The specific research objectives that are covered in this thesis are:

1. To evaluate the safety of the use of culture expanded mesenchymal stem cells for intra-articular cell treatment in OA.
2. To study the effect of the osteoarthritic environment on the therapeutic capacities of MSCs.
3. To develop and study different approaches to improve the therapeutic potential of MSCs for OA.

To provide a framework from which further developments of intra-articular cell-therapy for OA can be undertaken in a safe manner, we first provide an overview about the safety of cultured mesenchymal stem cells for intra-articular use in humans via a systematic review of the literature that evaluated adverse events in studies about intra-articular treatment with culture expanded MSCs in humans in **chapter 2**.

By injecting MSCs intra-articularly as cell-therapy for OA, MSCs will be introduced to the OA environment. The diseased environment is very likely to have an effect on the therapeutic capacities of the MSCs and therefore we study this effect, as described in **chapter 3**, by exposing MSC *in vitro* to synovial fluid of OA patients we evaluate the expression of genes involved in immunomodulation by MSCs and the effect on lymphocyte proliferation. The diseased environment might not only affect the therapeutic capacities, but it could also influence the migration or adherence of MSCs intra-articularly, since different intra-articular tissues are affected in an OA joint. These tissues might have a different effect in terms of cell attraction and adhesion. In **chapter 4** the effects of factors secreted by OA tissues on attraction/migration and adherence of MSCs are studied.

MSCs are promising as cell-therapy due to their immunomodulatory and trophic capacities. However, their therapeutic effect in OA is not satisfying yet, considering conflicting results on pain relieve and effect on structural changes. This might be explained by the short intra-articular presence after injection. In order to become clinically successful, further improvement of the therapeutic potential of MSCs in OA is important and therefore different strategies to prolong the therapeutic properties of MSCs are investigated. In **chapter 4** we evaluate whether MSCs can

be stimulated in culture with different stimulation methods (i.e. hypoxia, inflammatory factors, factors secreted by different OA tissues and OA synovial fluid) to increase their migration/adhesion capacity. We evaluate this in three different ways: by gene expression of 12 genes encoding for chemokine or adhesion receptors, by the migration of MSCs toward factors secreted by OA tissues *in vitro*, and by the attachment of injected MSCs in healthy and OA rat knees *in vivo*. In **chapter 5** a new strategy is used to retain the therapeutic properties of MSCs for a long period while protecting them against the immune system by developing a MSC encapsulation construct with alginate. This could even allow the use of allogeneic cells thereby offering the possibility to develop an "off-the-shelf" therapy. The preservation of the integrity of the MSC-alginate construct is essential for its function. Therefore, an imaging method with quantitative MRI is created to evaluate the integrity of MSC-alginate constructs over time. This is possible since the constructs can be labelled with gadolinium, a contrast agent which is widely used in Radiology. The methods and technical aspects of integrity imaging by MRI are described in **chapter 6**. In **chapter 6** two different, clinical grade alginates are used in the development and optimization of an injectable MSC-alginate construct for intra-articular use. By using quantitative MRI we can evaluate the construct integrity of both alginates and by using bioluminescence we can evaluate cell activity over time in these constructs *in vivo*. Imaging outcomes are correlated with histological findings.

Chapter 7 presents overall conclusions and we discuss the position of our findings in the research field and elaborate on future perspectives of MSC cell-therapy for OA. In **chapter 8** the findings of this thesis are summarized.

REFERENCES

1. Leyland KM, Gates LS, Sanchez-Santos MT, et al. Osteoarthritis: A Serious Disease. *Osteoarthritis Cartilage.* 2016, December 1:103.
2. Nelson AE. Osteoarthritis year in review 2017: clinical. *Osteoarthritis Cartilage.* 2017.
3. Wittenauer R, Smith L, Aden K. Priority Medicines for Europe and the World "A Public Health Approach to Innovation". *WHO Priority Medicines for Europe and the World 2013 Update.* 2013, januray 28th 32.
4. Vos T, Flaxman AD, Naghavi M, et al. Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* 2012;380(9859):2163-2196.
5. Cisternas MG, Murphy L, Sacks JJ, Solomon DH, Pasta DJ, Helmick CG. Alternative Methods for Defining Osteoarthritis and the Impact on Estimating Prevalence in a US Population-Based Survey. *Arthritis Care Res (Hoboken).* 2016;68(5):574-580.
6. Vina ER, Kwoh CK. Epidemiology of osteoarthritis: literature update. *Curr Opin Rheumatol.* 2018;30(2):160-167.
7. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. *Lancet.* 2019;393(10182):1745-1759.
8. National Institute for Public Health and the Environment, Netherlands. Public Health Foresight Study 2018 (VTV-2018): diseases. 2018. https://www.vtv2018.nl/en/diseases (accessed April 7, 2019).
9. Cross M, Smith E, Hoy D, et al. The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study. *Ann Rheum Dis.* 2014;73(7):1323-1330.
10. Blagojevic M, Jinks C, Jeffery A, Jordan KP. Risk factors for onset of osteoarthritis of the knee in older adults: a systematic review and meta-analysis. *Osteoarthritis Cartilage.* 2010;18(1):24-33.
11. Srikanth VK, Fryer JL, Zhai G, Winzenberg TM, Hosmer D, Jones G. A meta-analysis of sex differences prevalence, incidence and severity of osteoarthritis. *Osteoarthritis Cartilage.* 2005;13(9):769-781.
12. Muiznieks LD, Keeley FW. Molecular assembly and mechanical properties of the extracellular matrix: A fibrous protein perspective. *Biochim Biophys Acta.* 2013;1832(7):866-875.
13. Hardingham T, Heng BC, Gribbon P. New approaches to the investigation of hyaluronan networks. *Biochem Soc Trans.* 1999;27(2):124-127.
14. Felson DT. An update on the pathogenesis and epidemiology of osteoarthritis. *Radiol Clin North Am.* 2004;42(1):1-9, v.
15. Sophia Fox AJ, Bedi A, Rodeo SA. The basic science of articular cartilage: structure, composition, and function. *Sports Health.* 2009;1(6):461-468.
16. Tuan RS, Chen AF, Klatt BA. Cartilage regeneration. *J Am Acad Orthop Surg.* 2013;21(5):303-311.
17. Houard X, Goldring MB, Berenbaum F. Homeostatic mechanisms in articular cartilage and role of inflammation in osteoarthritis. *Curr Rheumatol Rep.* 2013;15(11):375.
18. van Buul GM, Siebelt M, Leijs MJ, et al. Mesenchymal stem cells reduce pain but not degenerative changes in a mono-iodoacetate rat model of osteoarthritis. *J Orthop Res.* 2014;32(9):1167-1174.
19. Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res Ther.* 2009;11(3):224.

20. Bijlsma JW, Berenbaum F, Lafeber FP. Osteoarthritis: an update with relevance for clinical practice. *Lancet.* 2011;377(9783):2115-2126.
21. Karsdal MA, Michaelis M, Ladel C, et al. Disease-modifying treatments for osteoarthritis (DMOADs) of the knee and hip: lessons learned from failures and opportunities for the future. *Osteoarthritis Cartilage.* 2016;24(12):2013-2021.
22. Lopa S, Colombini A, Moretti M, de Girolamo L. Injective mesenchymal stem cell-based treatments for knee osteoarthritis: from mechanisms of action to current clinical evidences. *Knee Surg Sports Traumatol Arthrosc.* 2018.
23. Qvist P, Bay-Jensen AC, Christiansen C, Dam EB, Pastoureau P, Karsdal MA. The disease modifying osteoarthritis drug (DMOAD): Is it in the horizon? *Pharmacol Res.* 2008;58(1):1-7.
24. Yazici Y, McAlindon TE, Fleischmann R, et al. A novel Wnt pathway inhibitor, SM04690, for the treatment of moderate to severe osteoarthritis of the knee: results of a 24-week, randomized, controlled, phase 1 study. *Osteoarthritis Cartilage.* 2017;25(10):1598-1606.
25. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999;284(5411):143-147.
26. Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell.* 2008;2(4):313-319.
27. Bianco P, Robey PG, Saggio I, Riminucci M. "Mesenchymal" stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease. *Hum Gene Ther.* 2010;21(9):1057-1066.
28. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *J Cell Biochem.* 2006;98(5):1076-1084.
29. Berebichez-Fridman R, Montero-Olvera PR. Sources and Clinical Applications of Mesenchymal Stem Cells: State-of-the-art review. *Sultan Qaboos Univ Med J.* 2018;18(3):e264-e277.
30. Klingemann H, Matzilevich D, Marchand J. Mesenchymal Stem Cells - Sources and Clinical Applications. *Transfus Med Hemother.* 2008;35(4):272-277.
31. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006;8(4):315-317.
32. Caplan AI. Mesenchymal stem cells. *J Orthop Res.* 1991;9(5):641-650.
33. Caplan AI. The mesengenic process. *Clin Plast Surg.* 1994;21(3):429-435.
34. Solchaga LA, Welter JF, Lennon DP, Caplan AI. Generation of pluripotent stem cells and their differentiation to the chondrocytic phenotype. *Methods Mol Med.* 2004;100:53-68.
35. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol.* 2000;28(8):875-884.
36. Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. *Exp Biol Med (Maywood).* 2001;226(6):507-520.
37. Kim DH, Yoo KH, Choi KS, et al. Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell. *Cytokine.* 2005;31(2):119-126.
38. Chen X, Armstrong MA, Li G. Mesenchymal stem cells in immunoregulation. *Immunol Cell Biol.* 2006;84(5):413-421.
39. Schinkothe T, Bloch W, Schmidt A. In vitro secreting profile of human mesenchymal stem cells. *Stem Cells Dev.* 2008;17(1):199-206.
40. Hoogduijn MJ, Popp F, Verbeek R, et al. The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy. *Int Immunopharmacol.* 2010;10(12):1496-1500.

41. Meisel R, Brockers S, Heseler K, et al. Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. *Leukemia.* 2011;25(4):648-654.
42. Landgraf K, Brunauer R, Lepperdinger G, Grubeck-Loebenstein B. The suppressive effect of mesenchymal stromal cells on T cell proliferation is conserved in old age. *Transpl Immunol.* 2011;25(2-3):167-172.
43. Pers YM, Ruiz M, Noel D, Jorgensen C. Mesenchymal stem cells for the management of inflammation in osteoarthritis: state of the art and perspectives. *Osteoarthritis Cartilage.* 2015;23(11):2027-2035.
44. Mardones R, Jofre CM, Tobar L, Minguell JJ. Mesenchymal stem cell therapy in the treatment of hip osteoarthritis. *J Hip Preserv Surg.* 2017;4(2):159-163.
45. Agung M, Ochi M, Yanada S, et al. Mobilization of bone marrow-derived mesenchymal stem cells into the injured tissues after intraarticular injection and their contribution to tissue regeneration. *Knee Surg Sports Traumatol Arthrosc.* 2006;14(12):1307-1314.
46. Murphy JM, Fink DJ, Hunziker EB, Barry FP. Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum.* 2003;48(12):3464-3474.
47. Toghraie FS, Chenari N, Gholipour MA, et al. Treatment of osteoarthritis with infrapatellar fat pad derived mesenchymal stem cells in Rabbit. *Knee.* 2011;18(2):71-75.
48. Vangsness CT, Jr., Farr J, 2nd, Boyd J, Dellaero DT, Mills CR, LeRoux-Williams M. Adult human mesenchymal stem cells delivered via intra-articular injection to the knee following partial medial meniscectomy: a randomized, double-blind, controlled study. *J Bone Joint Surg Am.* 2014;96(2):90-98.
49. Mamidi MK, Das AK, Zakaria Z, Bhonde R. Mesenchymal stromal cells for cartilage repair in osteoarthritis. *Osteoarthritis Cartilage.* 2016;24(8):1307-1316.
50. Roffi A, Nakamura N, Sanchez M, Cucchiarini M, Filardo G. Injectable Systems for Intra-Articular Delivery of Mesenchymal Stromal Cells for Cartilage Treatment: A Systematic Review of Preclinical and Clinical Evidence. *Int J Mol Sci.* 2018;19(11).
51. Emadedin M, Labibzadeh N, Liastani MG, et al. Intra-articular implantation of autologous bone marrow-derived mesenchymal stromal cells to treat knee osteoarthritis: a randomized, triple-blind, placebo-controlled phase 1/2 clinical trial. *Cytotherapy.* 2018;20(10):1238-1246.
52. McKinney JM, Doan TN, Wang L, et al. Therapeutic efficacy of intra-articular delivery of encapsulated human mesenchymal stem cells on early stage osteoarthritis. *Eur Cell Mater.* 2019;37:42-59.
53. Hatsushika S, Funasaka S. [Estimation of surviving auditory nerve by electrically evoked auditory brainstem response (FABR)--comparison between extra-cochlear stimulation and intra-cochlear stimulation]. *Nihon Jibiinkoka Gakkai Kaiho.* 1989;92(7):1005-1011.
54. Kim JE, Lee SM, Kim SH, et al. Effect of self-assembled peptide-mesenchymal stem cell complex on the progression of osteoarthritis in a rat model. *Int J Nanomedicine.* 2014;9 Suppl 1:141-157.
55. Pigott JH, Ishihara A, Wellman ML, Russell DS, Bertone AL. Investigation of the immune response to autologous, allogeneic, and xenogeneic mesenchymal stem cells after intra-articular injection in horses. *Vet Immunol Immunopathol.* 2013;156(1-2):99-106.
56. Saulnier N, Viguier E, Perrier-Groult E, et al. Intra-articular administration of xenogeneic neonatal Mesenchymal Stromal Cells early after meniscal injury down-regulates metal-

loproteinase gene expression in synovium and prevents cartilage degradation in a rabbit model of osteoarthritis. *Osteoarthritis Cartilage.* 2015;23(1):122-133.

57. Iijima H, Isho T, Kuroki H, Takahashi M, Aoyama T. Effectiveness of mesenchymal stem cells for treating patients with knee osteoarthritis: a meta-analysis toward the establishment of effective regenerative rehabilitation. *NPJ Regen Med.* 2018;3:15.
58. Xia P, Wang X, Lin Q, Li X. Efficacy of mesenchymal stem cells injection for the management of knee osteoarthritis: a systematic review and meta-analysis. *Int Orthop.* 2015;39(12):2363-2372.
59. Toupet K, Maumus M, Peyrafitte JA, et al. Long-term detection of human adipose-derived mesenchymal stem cells after intraarticular injection in SCID mice. *Arthritis Rheum.* 2013;65(7):1786-1794.
60. ter Huurne M, Schelbergen R, Blattes R, et al. Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. *Arthritis Rheum.* 2012;64(11):3604-3613.
61. Horie M, Choi H, Lee RH, et al. Intra-articular injection of human mesenchymal stem cells (MSCs) promote rat meniscal regeneration by being activated to express Indian hedgehog that enhances expression of type II collagen. *Osteoarthritis Cartilage.* 2012;20(10):1197-1207.
62. Sato M, Uchida K, Nakajima H, et al. Direct transplantation of mesenchymal stem cells into the knee joints of Hartley strain guinea pigs with spontaneous osteoarthritis. *Arthritis Res Ther.* 2012;14(1):R31.
63. Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet.* 2004;363(9419):1439-1441.
64. Galleu A, Riffo-Vasquez Y, Trento C, et al. Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. *Sci Transl Med.* 2017;9(416).
65. Hu J, Yu X, Wang Z, et al. Long term effects of the implantation of Wharton's jelly-derived mesenchymal stem cells from the umbilical cord for newly-onset type 1 diabetes mellitus. *Endocr J.* 2013;60(3):347-357.
66. Kunter U, Rong S, Djuric Z, et al. Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. *J Am Soc Nephrol.* 2006;17(8):2202-2212.
67. Lee RH, Seo MJ, Reger RL, et al. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc Natl Acad Sci U S A.* 2006;103(46):17438-17443.
68. Minguell JJ, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. *Exp Biol Med (Maywood).* 2006;231(1):39-49.
69. Ortiz LA, Dutreil M, Fattman C, et al. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci U S A.* 2007;104(26):11002-11007.
70. Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet.* 2008;371(9624):1579-1586.
71. Reinders ME, Bank JR, Dreyer GJ, et al. Autologous bone marrow derived mesenchymal stromal cell therapy in combination with everolimus to preserve renal structure and function in renal transplant recipients. *J Transl Med.* 2014;12:331.

72. Reinders ME, de Fijter JW, Roelofs H, et al. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. *Stem Cells Transl Med.* 2013;2(2):107-111.
73. Bouffi C, Bony C, Courties G, Jorgensen C, Noel D. IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis. *PLoS One.* 2010;5(12):e14247.
74. Forbes GM, Sturm MJ, Leong RW, et al. A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol.* 2014;12(1):64-71.
75. Panes J, Garcia-Olmo D, Van Assche G, et al. Long-term Efficacy and Safety of Stem Cell Therapy (Cx601) for Complex Perianal Fistulas in Patients With Crohn's Disease. *Gastroenterology.* 2018;154(5):1334-1342 e1334.
76. Panes J, Garcia-Olmo D, Van Assche G, et al. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet.* 2016;388(10051):1281-1290.
77. Bartolucci J, Verdugo FJ, Gonzalez PL, et al. Safety and Efficacy of the Intravenous Infusion of Umbilical Cord Mesenchymal Stem Cells in Patients With Heart Failure: A Phase 1/2 Randomized Controlled Trial (RIMECARD Trial [Randomized Clinical Trial of Intravenous Infusion Umbilical Cord Mesenchymal Stem Cells on Cardiopathy]). *Circ Res.* 2017;121(10):1192-1204.
78. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. *Front Immunol.* 2014;5:148.

Chapter 2

Safety of intra-articular cell-therapy with culture-expanded stem cells in humans: a systematic literature review

Osteoarthritis Cartilage. 2013 Oct;21(10): 1465-73

Charles M.M. Peeters
Maarten J.C. Leijs
Max Reijman
Gerjo J.V.M. van Osch
Pieter K. Bos

ABSTRACT

Background: An important goal of stem cell research in orthopaedics is to develop clinically relevant techniques that could be applied to heal cartilage or joint pathology. Stem cell treatment in orthopaedics for joint pathology is promising since these cells have the ability to modulate different processes in the various tissues of the joint simultaneously. The non-life-threatening nature of musculoskeletal system disorders makes safety of stem cell therapy a necessary prerequisite.

Objective: To systematically review the literature and provide an overview of reported adverse events (AEs) of intra-articular treatment with culture-expanded stem cells in humans.

Design: A systematic literature search was performed in Pubmed, EMBASE, Web of Science and CINAHL in February 2013. AEs were reported into three categories: local/systemic, serious adverse event or adverse event (SAE/AE), related/unrelated.

Results: 3039 Potentially eligible articles were identified of which eventually eight fulfilled our inclusion criteria. In total, 844 procedures with a mean follow-up of 21 months were analysed. Autologous bone marrow-derived mesenchymal stem cells (BM-MSCs) were used for cartilage repair and osteoarthritis treatment in all included studies. Four SAEs were reported by the authors. One infection following bone marrow aspiration (BMA) was reported as probably related and resolved with antibiotics. One pulmonary embolism occurred 2 weeks after BMA and was reported as possibly related. Two tumours, both not at the site of injection, were reported as unrelated. Twenty-two other cases of possible procedure-related and seven of possible stem cell-product related AEs were documented. The main AEs related to the procedure were increased pain/swelling and dehydration after BMA. Increased pain and swelling was the only AE reported as related to the stem cell-product.

Conclusions: Based on current literature review we conclude that application of cultured stem cells in joints appears to be safe. We believe that with continuous caution for potential side effects, it is reasonable to continue with the development of articular stem cell therapies.

INTRODUCTION

Stem cell therapies are rapidly emerging as a potential strategy for tissue repair and regeneration in many fields of medicine[1]. The use of autologous or allogenic stem cells is very promising for biological modulation and repair of various disease processes of the musculoskeletal system. In the field of orthopaedics, cartilage repair has played a pioneering role in the translational application of cell therapy. Autologous chondrocyte implantation (ACI) and derivative techniques such as matrix-induced chondrocyte implantation (MACI) have been employed and evaluated in the last two decades. Generally good to excellent results have been reported for these cell transplantation techniques, without significant safety problems for this intra-articular use of differentiated cells[2,3]. The Use of differentiated cells leads to several limitations in number of cells available, choice of cell and donor-site morbidity[4-6]. Stem cells, on the contrary, are multipotent, can be harvested from many different cell sources and have a high proliferation potential[7]. Stem cells have already been used in orthopaedic applications, although experimentally, in the treatment of avascular bone necrosis, osteochondral defects, pseudoarthrosis and traumatic cartilage defects[8-11]. Recently, Pastides et al. provided an overview of the effectivity of the clinical application of stem cells in cartilage defects[12].

Safety is an important prerequisite for translational application of stem cell therapies. Unlike for life- threatening diseases where stem cell therapy is used for heart failure following myocardial infarction[13], severe graft vs host disease (GVHD)[14], Crohn's disease[15] or leukaemia, diseases in the orthopaedic field eligible for stem cell therapy are generally not life-threatening. For this reason, intensive monitoring of the safety of intra-articular use of culture-expanded stem cells in musculoskeletal diseases is even more important. This systematic literature review provides an overview of reported AEs based on all published studies with human cases of intra-articular treatment with culture-expanded stem cells.

METHODS

Search strategy

A comprehensive search of the literature was carried out in February 2013. Electronic databases – PubMed, EMBASE, Web of Science and CINAHL – were used to identify relevant studies since their inception up to February 2013. An overview of the complete search strategy is shown in **table 1**.

Table 1
Search strategy in Medline

(stem cells[mesh] OR stemcell*[tw] OR stem cell*[tw] OR progenitor cell*[tw] OR nucleated cell*[tw] OR bone marrow cell*[tw])
AND
(joints[mesh] OR joint*[tw] OR articul*[tw] OR intraarticul*[tw] OR cartilag*[tw] OR chondrocyt*[tw])
AND
(inject*[tw] OR admin*[tw] OR treat[tw] OR treated[tw] OR treatment*[tw] OR therapy[tw] OR therapies[tw] OR therapeut*[tw] OR implant*[tw] OR transplant*[tw] OR repair*[tw] OR reconstruct*[tw])
NOT
((animals[mesh] OR animal*[tw]) NOT (humans[mesh] OR human*[tw] OR patient[tw] OR patients[tw] OR people*[tw] OR men[tw]))
AND
(dut[la] OR eng[la] OR ger[la] OR spa[la] OR ita[la] OR fre[la])

Inclusion and exclusion criteria

The articles retrieved from the search were included in this systematic review according to the following inclusion and exclusion criteria:

1. Subjects were treated with culture-expanded stem cells in joints. To obtain an extensive perspective on safety, studies with the use of all sorts of stem cells were included, as long as the cells were culture-expanded and applied in joints. No restriction was made on joint disease.
2. Human subjects.
3. Full text of the article available.
4. Study published in English, Dutch, German, Italian, French or Spanish.
5. Study had to report information about AEs.

In vitro studies and animal studies were considered ineligible for inclusion. Comments, editorials, reviews, letters, guidelines and protocols were also excluded. In case of potential duplicate studies or studies with overlap (i.e., dynamic cohorts) we contacted the senior authors. In those cases, the study with the longest follow-up time and/or more detailed presentation of relevant outcomes was included in the review. Of the included studies, only intra-articular procedures with culture-expanded stem cells were considered eligible for analysis, procedures in vertebral discs and other treatment sites were excluded.

Study selection

Two reviewers (PB and CP) independently examined article titles and abstracts for eligibility. Subsequently, full-text reports of potential studies were screened to determine final eligibility for inclusion in this review. Disagreements concerning the inclusion of the studies were solved by consensus. A third reviewer (MR) was

consulted when disagreement persisted. Disagreements were solved in a single consensus meeting without the help of the third reviewer. In addition, the reference lists of the selected papers were screened with the intention to add eligible studies that were not found with the search. The selection of articles is shown schematically in **figure 1**.

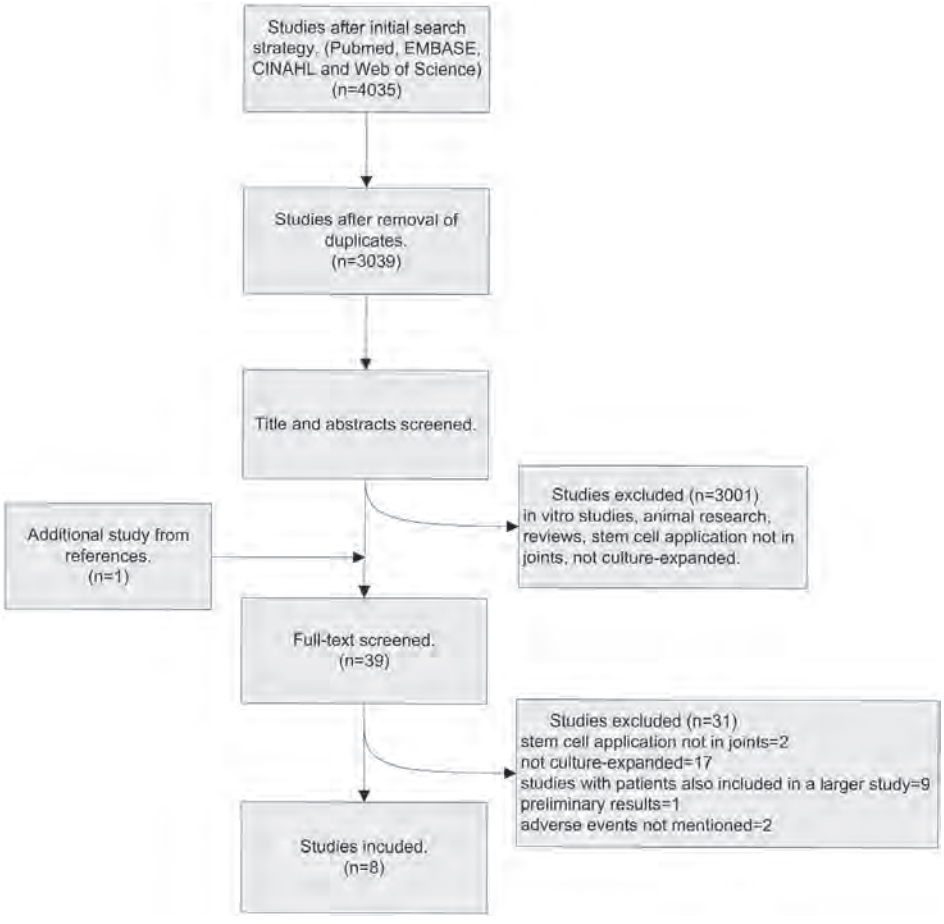


Figure 1. *Study selection.*

Data extraction and presentation

One author (CP) extracted the data of the finally included studies. Information was collected on study design, study population, origin stem cells, procedure, outcome measures, duration of follow-up and results. All reported AEs are listed in the results. The AEs are subdivided in three tables: complications reported as possibly related to the procedure, stem cell product complications reported as possibly related and AEs reported as unrelated to the procedure or stem cell

Table 2
Methodological quality criteria

Item	Judgement
1. Did the authors state a prospective evaluation of AEs? (= active mode of AEs collection)	Yes/No/Unable to determine
2. Were adequate methods for monitoring AEs reported?	
A) MRI, arthroscopy	Yes/No/Unable to determine
B) Prospective checklist and/or patient questionnaire or patient diary	Yes/No/Unable to determine
3.	
A) Was the number of participants that withdraw or were lost to follow-up specified?	Yes/No/Unable to determine
B) Were patients excluded from the AEs analysis because of an AE?	Yes/No/Unable to determine
4.	
A) Were all categories of AEs reported on (all AEs)?	Yes/No/Unable to determine
B) Did the study specify whether AEs related to harvesting, application procedure or cell product?	Yes/No/Unable to determine
5.	
A) Were SAE precisely defined?	Yes/No/Unable to determine
B) For SAE were all categories of AEs reported on?	Yes/No/Unable to determine
6. Did the study specify who collected the AEs?	Yes/No/Unable to determine
7. Did the study specify the timing and frequency of collection of the AEs?	Yes/No/Unable to determine
8. Was the follow-up of AEs evaluation at least 1 year after the last administration of stem cells?	Yes/No/Unable to determine

Item 4 was scored yes when the following 2/3 categories were reported: local/systemic, serious/nonserious, related/unrelated. Our definitions: local AE, AE limited to the joint; systemic AE, AE unrelated to the joint; SAEs includes death, neoplasms, infections, pulmonary embolisms, anaphylactic shock and leukaemia.

product. Reported AEs are once more subdivided in local or systemic and serious adverse events (SAE) or other AE. Since no definition of SAE for this application was available, we defined SAE as death, neoplasms, infections, pulmonary embolisms, anaphylactic shock and haematological neoplasms.

Quality assessment

Two authors (CP and ML) independently assessed the methodological quality of AEs collection of each included study, using questions from the McHarm quality assessment scale for AEs[16] (the eight quality criteria are listed in **table 2**). Each item was scored as a 'yes' 'no' or 'unable to determine'. Disagreements were resolved by consensus. Consultation of a third reviewer (GO) when disagreement persisted, appeared unnecessary.

RESULTS

Study inclusion and characteristics

A total of 4035 records were found after the electronic search (**figure 1**). After the removal of duplicates, 3039 potentially eligible articles were identified. Finally, eight articles fulfilled our inclusion criteria and are included in this systematic review[9,17-23] (**table 3**).

Wakitani and Centeno were contacted for potential duplicate or near duplicate studies. Wakitani reported that all subjects reported in his six papers were included in Wakitani et al., 2011[22,24-28]. Centeno reported that his last safety article[9] included all subjects reported in his five papers[9,29-32].

The prospective cohort study of Centeno et al., 2010 has been updated in 2011[9,32]. The study of 2011 reports changes and AEs since the last reporting in 2010, which made it difficult to obtain a complete overview of the AEs in numbers and details. Therefore, Centeno was requested for a complete overview of their reported AEs, which made enumeration and classification possible. We report the AEs based on the acquired list.

From a total of 904 procedures in 470 individuals, 844 were intra-articular procedures (789 injections and 55 cell constructs or sheets) and were analysed with a mean follow-up of 21 months. All included studies used autologous bone marrow-derived mesenchymal stem cells (BM-MSCs). The MSCs were implanted in the knee joint (503 procedures), hip joint (219), foot/ankle joint (55), shoulder joint

Table 3

First author, year of publication	Design	Study population	Number of procedures (joints) [individuals]*	Origin stem cells	Serum used for expansion in culture	Number of passages in culture	Amount of intra-articular injected cells (mean)	Composition of injected/ implanted MSCs	Different joint types (%)	Mean age at surgery in years ± SD (range)	% Male	Mean follow-up period ± SD (range)
Centeno et al. 2011[9]	Prospective cohort study	Chronic or degenerative joint disease	709, (NA), [>279]†	Autologous iliac crest BM-MSCs	PL	2 – 7	NA	MSCs in Autologous PL10-20% diluted in PBS or conditioned serum of PRP	Knee (52.8%) Hip (30.7%) Foot/ankle (7.6%) Shoulder (6.8%) Hand/wrist (2.1%)	±53 ± 13.85	±63.1	±435 days ± 261 days
Davatchi et al. 2011[17]	Pilot study	OA	4, (4), [4]	Autologous iliac crest BM-MSCs	FBS	1	± 8-9 x 10⊠	Normal saline supplemented with 2% human serum albumin	Knee (100%)	57.6 (54 – 65)	50	1 year
Emadedin et al. 2012[18]	Clinical trial	OA	6, (6), [6]	Autologous iliac crest BM-MSCs	HBS	2	± 20-24 x 10⊠	physiological serum	Knee (100%)	54.6	0	1 year
Haleem et al. 2010[19]	Pilot study	OD, OCD	5, (5), [5]	Autologous iliac crest BM-MSCs	FBS	1	±2 x 10⊠/ cm^2	MSC mixed with allogenous PR-FG and left to gelate	Knee (100%)	25.7 (21 – 37)	80	14.2 months
Kasemkijwattana et al.. 2011[20]	Case serie	CD	2, (2), [2]	Autologous iliac crest BM-MSCs	FBS	2	NA	MSCs seeded in collagen scaffolds fixed with FG	Knee (100%)	24.5 (24 – 25)	100	30.5 months (30 – 31)

Table 3 (continued)

Lee et al. 2012[23]	Prospective cohort study	CD	70, (70), [70]	Autologous iliac crest BM-MSCs	FBS	1	±10 x 10⊠	In autologous serum followed by 2 ml HA injection	Knee (100%)	44	51.4	24.5 months
Teo BJ et al. 2012[21]	Case serie	OD	3, (3), [3]	Autologous iliac crest BM-MSCs	FBS	NA	±10-15 x 10⊠	Cell sheets in autologous serum. Fixed with FG	Knee (100%)	NA	NA	NA
Wakitani et al. 2011[22]	Prospective cohort study	OA, CD	45, (45), [41]‡	Autologous iliac crest BM-MSCs	FCS (until 2003) AS (since 2003)	2	NA	Until 2003: embedded in 0.25% acid-soluble type I collagen from porcine tendon and gelated or placed on a collagen sheet and gelated in DMEM + 15% autologous serum. Since 2003: embedded in 1% acid-soluble type I collagen from bovine skin, gelated on porcine tendon collagen sheet in aMEM + 15% autologous serum	Knee (86.7%) Ankle (2.2%) Hip (2.2%) Elbow (8.9%)	50	NA	75 months (5 - 137)

Characteristics of the included studies

OA: osteoarthritis, OD: Osteochondritis dissecans, OCD: osteochondral defect, CD: chondral defect, PL: platelet lysate, FBS: fetal bovine serum, HBS: hyclone bovine serum, FCS: fetal calf serum, AS: autologous serum, NA: not available, PR-FG: platelet-rich fibrin glue, FG: fibrin glue, HA: hyaluronic acid.

* Joints: total number of joints injected, procedures: total joint procedures.

† Including three patients of three case reports of Centeno *et al.*, 2008 and 222 procedures of Centeno *et al.*, 2010. The exact number of patients is not determinable after exclusion of disc procedures and other site treatments.

‡ Including 12 patients of Wakitani *et al.*, 2002, two patients of Wakitani *et al.*, 2004, three patients of Wakitani *et al.*, 2006, one patient of Kuroda *et al.*, 2007 and three patients of Wakitani *et al.*, 2007.

(48), hand/wrist joint (15) and elbow joint (4). According to our inclusion criteria, we have excluded 34 vertebral disc procedures and 26 procedures in various other treatment sites reported in the study of Centeno et al[9].

Quality assessment

According to the quality assessment (**table 4**), the study of Centeno et al. 2011 described an adequate method regarding the AEs collection[9]. All other included studies reported information on AEs, but did not use a standardized method for AE monitoring.

Table 4
Quality assessment

Item	Centeno *et al.* 2011[9]	Davatchi *et al.* 2011[17]	Emadedin *et al.* 2012[18]	Haleem *et al.* 2010[19]	Kasemkijwattana *et al.*, 2011[20]	Lee *et al.* 2012[23]	Teo *et al.* 2012[21]	Wakitani *et al.* 2011[22]
1	Yes	No	No	No	No	No	No	Yes
2. A)	Yes	No	No	No	No	No	No	No
B)	Yes	No	No	No	No	No	No	No
3. A)	No	No	No	No	No	No	Yes	Yes
B)	Un	No	No	No	No	No	No	No
4. A)	Yes	No	No	No	No	No	No	No
B)	Yes	No	Un	Un	Un	Un	Un	Un
5. A)	Yes	No	No	No	No	No	No	Yes
B)	Yes	No	No	No	No	No	No	No
6.	Yes	No	No	No	No	No	No	No
7.	Yes	No	No	No	No	No	No	Yes
8.	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

For the description of items 1-8 see **table 2**. Un: unable to determine.

SAEs

Four SAEs have been reported: one infection, one pulmonary embolism and two tumours[9]. The infection was at the bone marrow aspiration (BMA) site. It was listed by the authors as a probable procedural related complication and was successfully treated with oral antibiotics[9]. The onset of the pulmonary embolism was 2 weeks after the BMA before the initiation of any stem cell therapy and was documented as a possible procedural related complication. The patient was successfully treated in hospital[9]. Two tumours were detected in the period after the stem cell procedure in two separate patients. One patient was diagnosed with a benign schwannoma from T12-L2 and another patient was diagnosed with prostate carcinoma. The stem cell implantations were in the hip and knee joint respectively. Both tumours were not at the site of injection and were reported by the authors as unrelated[9].

Table 5
Procedure-related complications

First author, year of publication	Number of procedures [subjects]	Mean follow-up period ± SD (range)	SAE/ AE	Procedure location	Procedure complications reported as probably/possibly related	Loc	Sys
Centeno *et al.* 2011[9]	709, [>279]†	±435 days ± 261 days	SAE	Crista iliaca	Infection at bone marrow draw site: 1	X	
				Crista iliaca	Pulmonary embolism 2 weeks after BMA: 1		X
			AE	Crista iliaca (2)	Increased pain at site of BMA: 2	X	
				Crista iliaca (2)	Dehydration after BMA: 2		X
				Crista iliaca	Recurrence of herpes zoster after BMA: 1	X	
				Ankle	Urticaria after procedure: 1		X
				Knee(8), hip(4), facet(1)	Increased pain/swelling at implantation site: 13	X	
				Knee	Laboratory abnormalities: Transient elevation of LFT: 1.		X
				Hand	Transient numbness and tingling in the arm used for blood draw: 1		X
Davatchi *et al.* 2011[17]	4, [4]	14.2 months	SAE		None		
			AE	Knee	Mild swelling: 1	X	
Emadedin et al. 2012[18]	6, [6]	1 year			None		
Haleem *et al.* 2010[19]	5, [5]	1 year			None		
Kasemkijwattana *et al.* 2011[20]	2, [2]	30.5 months (30 – 31)			None		
Lee *et al.* 2012[23]	70 [70]	24.5 months			None		
Teo *et al.* 2012[21]	3, [3]*	NA			None		
Wakitani *et al.* 2011[22]	45, [41]	75 months (5 - 137)			None		

Our definitions: SAE included death, neoplasms, infections, pulmonary embolisms, anaphylactic shock and leukaemia. Loc: local AE, Sys: systemic AE, NA: Not available.

* These three patients are part of a larger group, of which 20 received ACI. Therefore, follow-up cannot be reduced.

† Including three patients of three case reports of Centeno *et al.*, 2008 and 222 procedures of Centeno *et al.*, 2010. The exact number of patients is not determinable after exclusion of disc procedures and other site treatments.

Table 6
Stem cell-product related complications

First author, year of publication	Number of procedures [subjects]	Mean follow-up period ± SD (range)	SAE/ AE	Procedure location	Stem cell product complications reported as probably/possibly related	Loc	Sys
Centeno *et al.* 2011[9]	709, [>279]†	±435 days ± 261 days	SAE		None		
			AE	Knee (6), Ankle (1)	Increased pain/ swelling: 7	X	
Davatchi *et al.* 2011[17]	4, [4]	1 year			None		
Emadedin *et al.* 2012[18]	6, [6]	1 year			None		
Haleem *et al.* 2010[19]	5, [5]	14.2 months			None		
Kasemkijwattana *et al.* 2011[20]	2, [2]	30.5 months (30 – 31)			None		
Lee *et al.* 2012[23]	70, [70]	24.5 months			None		
Teo *et al.* 2012[21]	3, [3]*	NA			None		
Wakitani *et al.* 2011[22]	45, [41]	75 months (5 - 137)			None		

Our definitions: SAE included death, neoplasms, infections, pulmonary embolisms, anaphylactic shock and leukaemia. Loc: local AE, Sys: systemic AE, NA: Not available.

* These three patients are part of a larger group, of which 20 received ACI. Therefore, follow-up cannot be reduced.

† Including three patients of three case reports of Centeno *et al.*, 2008 and 222 procedures of Centeno *et al.*, 2010. The exact number of patients is not determinable after exclusion of disc procedures and other site treatments.

Table 7
Reported unrelated AEs

First author, year of publication	Number of procedures [subjects]	Mean follow-up period ± SD (range)	SAE/ AE	Procedure Location	Adverse events reported as unrelated
Centeno *et al.* 2011[9]	709, [>279]†	±435 days ± 261 days	SAE AE	Knee (1), Hip (1)	Tumour: 2
				Hip	TIA: 1
				Hip	MRSA infection: 1
				Knee (7), Hip (11), ankle (2), hand (1), shoulder (1)	Increased pain and swelling, in most cases: most likely progression of underlying disease: 22
				Knee (4), Hip (5)	Increased pain outside re-implant area: 9
				Hip	Cardiac problems: 1
				Knee (1), Hip (3), Hand (1)	Laboratory abnormalities: 5
				Knee, hip	Low grade fever: 1
				Knee	Adrenal gland on unrelated lumbar MRI: 1
				Hip	Popping sensation in mouth with numbness and drooling: 1
				Hip	Involuntary tremors in treated leg: 1
				Knee	Arm swelling after peripheral blood collection: 1
				Knee	Kidney stone pain: 1
				Hip	Sore throat and congestion: 1
				Knee	Tightness in hamstrings and gluteus: 1
				Knee	Polymyalgia rheumatica: 1
				Knee	Bronchitis: 1
				Knee	Excema and Barrack's disease: 1
				Hip	Dermatomyositis: 1
				Knee (2)	Osteoporosis: 2
				Hip	Shingles after BMA: 1

Table 7 (continued)
Reported unrelated AEs

First author, year of publication	Number of procedures [subjects]	Mean follow-up period ± SD (range)	SAE/ AE	Procedure Location	Adverse events reported as unrelated
Davatchi *et al.* 2011[17]	4, [4]	1 year			None
Emadedin *et al.* 2012[18]	6, [6]	1 year			None
Haleem *et al.* 2010[19]	5, [5]	14.2 months			None
Kasemkijwattana *et al.* 2011[20]	2, [2]	30.5 months (30 – 31)			None
Lee *et al.* 2012[23]	70 [70]	24.5 months			None
Teo *et al.* 2012[21]	3, [3]*	NA			None
Wakitani *et al.* 2011[22]	45, [41]	75 months (5 - 137)			None

Our definitions: SAE included death, neoplasms, infections, pulmonary embolisms, anaphylactic shock and leukaemia. NA: Not available.
* These three patients are part of a larger group, of which 20 received ACI. Therefore, follow-up cannot be reduced.
† Including three patients of three case reports of Centeno *et al.*, 2008 and 222 procedures of Centeno *et al.*, 2010. The exact number of patients is not determinable after exclusion of disc procedures and other site treatments.

AEs

All complications related to the harvesting procedure of stem cells or to the administration procedure were self-limited or were remedied with simple therapeutic therapies[9,17].

Seven complications were reported as related to the stem cell product. All these patients reported increased pain and swelling. In four cases, drainage via arthrocentesis was required to resolve swelling and pain and in one case, an injection of corticosteroids was administered besides drainage. One stem cell treatment was cancelled due to an insidious onset of knee swelling, 2 weeks after procedure. This patient was eventually treated with a total knee arthroplasty (**tables 5-7**).

DISCUSSION

The aim of this systematic review is to provide an overview of AEs of the application of culture-expanded stem cells in joints. All published studies with human cases were included to provide an extensive overview of reported AEs. Of the 844 intra-articular implantations with MSCs, four individuals were presented with a serious complication. Two of the four serious complications were probably related to the procedure: an infection at the BMA site and a pulmonary embolism 2 weeks after BMA. Two tumours reported were regarded unrelated[9].

The eight included studies showed no safety issues regarding the MSC-product. The only reported stem cell-product related AEs were increased pain and swelling. These were mild and transient. It is difficult to attribute these stem cell-product related AE to one cause. Prerequirements for stem cell therapy are suitable cell counts and culture passages and applicable compositions of MSC solutions or constructs for injection and implantation. Different cell counts, passages and compositions of MSC solutions or constructs are used in the included studies. These factors can all potentially affect the occurrence of AE. For each individual AE information regarding these factors would be of great interest. However, our included studies did not provide this AE information specifically per patient.

Other clinical studies using culture-expanded MSCs for other applications also did not show any safety problems[15,33,34]. In Duijvestein et al. administration of autologous MSCs in nine patients appeared to be safe in the treatment of refractory Crohn's disease[15]. Likewise, Karamouzian et al. concluded that transplantation of

culture-expanded MSCs via lumbal puncture in 11 complete spinal cord injured patients at thoracic level is a safe technique[33].

To provide an impression of the number of AE in relation to other intra-articular treatments, we have compared stem cell injections with hyaluronic acid and high molecular hylan injections. For this comparison we have used the data on stem cell injections from the study of Centeno et al., because this is the only study with an adequate method for AEs collection. In this study 23 (3.2%) AE related to the intra-articular injection with stem cells or the stem cell product were reported[9]. The systematic review and meta-analysis of Reichenbach et al. with a total of 890 hyaluronic acid and 650 hylan treatments reported 42 (4.7%) local AEs in the hyaluronic acid group and 50 (7.7%) in the high molecular hylan injection group[35]. This would mean that intra-articular treatments with culture-expanded stem cells have at least a comparable number of AE with hyaluronic acid and hylan treatments.

The follow-up period differed greatly among the included studies. Six studies reported a mean follow-up period between 12 and 31 months[9,17-20,23]. The group of Wakitani reported a follow-up range from 2 to 11 years, with a mean follow-up of 6 years[22]. One year of follow-up will probably not be sufficiently long enough to detect all SAEs such as neoplasms. However, many animal studies showed no evidence of neoplasms at stem cell re-implantation sites[36-39]. Of two studies that did show spontaneous malignant transformation of human tissue-derived culture-expanded MSCs, following extended culture and implantation in mice, one was retracted[40,41] and the other discussed[42,43] by the authors in a later paper based on suspected cross-contamination with human fibrosarcoma or osteosarcoma cell lines.

All included studies used autologous bone marrow-derived MSCs. Companies such as Mesoblast are developing off-the-shelf adult stem cell products that are obtained from a single donor, commercially expanded and frozen, and subsequently used in allogeneic recipients. However, knowledge about the safety of the use of allogeneic MSCs is limited. Of the studies included, seven used fetal calf or bovine serum for cell culturing/expansion (**table 1**)[17-23]. The use of animal-based serum during the expansion of the stem cells could increase the risks of possible disease transmission and reactions of the immune system[44-47]. To assure maximal safety during the period of culture, contact of MSCs with animal-derived supplementary products must be minimal. Therefore, the use of alternative methods of cell culturing such as autologous serum and platelet lysate increases. Each change or difference in the culture procedure can influence cell population, cell phenotype

and consequently cell behavior. Therefore, this review is only the beginning of exploring the safety of intra-articular treatment with culture-expanded stem cells.

A limitation of our review is that most studies did not classify the observed AEs. Well-described AE collections lacked in all included studies except in the study of Centeno et al. 2011[9]. Furthermore, unpublished studies with detrimental results and studies which did not mention AEs, may have caused publication bias. In this review there were two studies with a total of 37 patients excluded because they did not give information on presence or absence of AEs[48,49].

We have extracted the data and subdivided the AEs into three categories: local/systemic, SAE/AE, related/unrelated. Centeno et al. 2011 reported two of the three categories[9], the other studies did not categorise the AEs. Clear classification of AEs for orthopaedic applications of stem cells is warranted in future study reports.

Furthermore, it is not unlikely that uncommon side effects are not reported yet or which may arise after a longer and accurate follow-up. Future studies should include adequate methods regarding the AEs collection using prospective checklists or patient questionnaires/patient diaries for symptoms, (non)invasive techniques for evaluation for structural changes such as enhanced MRI or arthroscopy, and laboratory controls.

In conclusion, intra-articular cell-therapy with culture-expanded MSCs appears to be safe based on 844 treatments in eight studies. Based on the reported AEs and their classification in this systematic literature review we conclude that there are no compelling arguments against proceeding with intra-articular stem cell application in human cases.

REFERENCES

1. Wagner J, Kean T, Young R, Dennis JE, Caplan AI. Optimizing mesenchymal stem cell-based therapeutics. *Curr Opin Biotechnol.* 2009;20(5):531-536.
2. Moseley JB, Jr., Anderson AF, Browne JE, et al. Long-term durability of autologous chondrocyte implantation: a multicenter, observational study in US patients. *Am J Sports Med.* 2010;38(2):238-246.
3. Peterson L, Vasiliadis HS, Brittberg M, Lindahl A. Autologous chondrocyte implantation: a long-term follow-up. *Am J Sports Med.* 2010;38(6):1117-1124.
4. Pelttari K, Lorenz H, Boeuf S, et al. Secretion of matrix metalloproteinase 3 by expanded articular chondrocytes as a predictor of ectopic cartilage formation capacity in vivo. *Arthritis Rheum.* 2008;58(2):467-474.
5. Matricali GA, Dereymaeker GP, Luyten FP. Donor site morbidity after articular cartilage repair procedures: a review. *Acta Orthop Belg.* 2010;76(5):669-674.
6. Liu G, Kawaguchi H, Ogasawara T, et al. Optimal combination of soluble factors for tissue engineering of permanent cartilage from cultured human chondrocytes. *J Biol Chem.* 2007;282(28):20407-20415.
7. Bos PK, Melle ML, Osch GJVM. Articular cartilage repair and the evolving role of regenerative medicin. *Open Access Surgery.* 2010;2010:3:109-122.
8. Pak J. Autologous adipose tissue-derived stem cells induce persistent bone-like tissue in osteonecrotic femoral heads. *Pain Physician.* 2012;15(1):75-85.
9. Centeno CJ, Schultz JR, Cheever M, et al. Safety and complications reporting update on the re-implantation of culture-expanded mesenchymal stem cells using autologous platelet lysate technique. *Curr Stem Cell Res Ther.* 2011;6(4):368-378.
10. Gangji V, De Maertelaer V, Hauzeur JP. Autologous bone marrow cell implantation in the treatment of non-traumatic osteonecrosis of the femoral head: Five year follow-up of a prospective controlled study. *Bone.* 2011;49(5):1005-1009.
11. Giannotti S, Bottai V, Ghilardi M, et al. Treatment of pseudoarthrosis of the upper limb using expanded mesenchymal stem cells: a pilot study. *Eur Rev Med Pharmacol Sci.* 2013;17(2):224-227.
12. Pastides P, Chimutengwende-Gordon M, Maffulli N, Khan W. Stem cell therapy for human cartilage defects: a systematic review. *Osteoarthritis Cartilage.* 2013.
13. Clifford DM, Fisher SA, Brunskill SJ, et al. Stem cell treatment for acute myocardial infarction. *Cochrane Database Syst Rev.* 2012;2:CD006536.
14. Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet.* 2008;371(9624):1579-1586.
15. Duijvestein M, Vos AC, Roelofs H, et al. Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut.* 2010;59(12):1662-1669.
16. Santaguida PL, Raina P. The Development of the McHarm Quality Assessment Scale for adverse events: Delphi Consensus on important criteria for evaluating harms. 2008. http://hiru.mcmaster.ca/epc/mcharm.pdf. Accessed May 14, 2008.
17. Davatchi F, Abdollahi BS, Mohyeddin M, Shahram F, Nikbin B. Mesenchymal stem cell therapy for knee osteoarthritis. Preliminary report of four patients. *Int J Rheum Dis.* 2011;14(2):211-215.

18. Emadedin M, Aghdami N, Taghiyar L, et al. Intra-articular Injection of Autologous Mesenchymal Stem Cells in Six Patients with Knee Osteoarthritis. *Arch Iran Med.* 2012;15(7):422-428.
19. Haleem AM, Singergy AA, Sabry D, et al. The Clinical Use of Human Culture-Expanded Autologous Bone Marrow Mesenchymal Stem Cells Transplanted on Platelet-Rich Fibrin Glue in the Treatment of Articular Cartilage Defects: A Pilot Study and Preliminary Results. *Cartilage.* 2010;1(4):253-261.
20. Kasemkijwattana C, Hongeng S, Kesprayura S, Rungsinaporn V, Chaipinyo K, Chansiri K. Autologous bone marrow mesenchymal stem cells implantation for cartilage defects: two cases report. *J Med Assoc Thai.* 2011;94(3):395-400.
21. Teo BJ, Buhary K, Tai BC, Hui JH. Cell-based Therapy Improves Function in Adolescents and Young Adults With Patellar Osteochondritis Dissecans. *Clin Orthop Relat Res.* 2012.
22. Wakitani S, Okabe T, Horibe S, et al. Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. *J Tissue Eng Regen Med.* 2011;5(2):146-150.
23. Lee KB, Wang VT, Chan YH, Hui JH. A novel, minimally-invasive technique of cartilage repair in the human knee using arthroscopic microfracture and injections of mesenchymal stem cells and hyaluronic acid–a prospective comparative study on safety and short-term efficacy. *Ann Acad Med Singapore.* 2012;41(11):511-517.
24. Kuroda R, Ishida K, Matsumoto T, et al. Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells. *Osteoarthritis Cartilage.* 2007;15(2):226-231.
25. Wakitani S. Bone Marrow transplantation: New Research. In: Davidson. DF, ed. *autologous culture expanded bone marrow stromal cell transplantation for cartilage repair* 2006.
26. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M. Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. *Osteoarthritis Cartilage.* 2002;10(3):199-206.
27. Wakitani S, Mitsuoka T, Nakamura N, Toritsuka Y, Nakamura Y, Horibe S. Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: two case reports. *Cell Transplant.* 2004;13(5):595-600.
28. Wakitani S, Nawata M, Tensho K, Okabe T, Machida H, Ohgushi H. Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees. *J Tissue Eng Regen Med.* 2007;1(1):74-79.
29. Centeno CJ, Busse D, Kisiday J, Keohan C, Freeman M. Increased knee cartilage volume in degenerative joint disease using percutaneously implanted, autologous mesenchymal stem cells, platelet lysate and dexamethasone. *Am J Case Rep.* 2008;9:246-251.
30. Centeno CJ, Busse D, Kisiday J, Keohan C, Freeman M, Karli D. Regeneration of meniscus cartilage in a knee treated with percutaneously implanted autologous mesenchymal stem cells. *Med Hypotheses.* 2008;71(6):900-908.
31. Centeno CJ, Busse D, Kisiday J, Keohan C, Freeman M, Karli D. Increased knee cartilage volume in degenerative joint disease using percutaneously implanted, autologous mesenchymal stem cells. *Pain Physician.* 2008;11(3):343-353.
32. Centeno CJ, Schultz JR, Cheever M, Robinson B, Freeman M, Marasco W. Safety and complications reporting on the re-implantation of culture-expanded mesenchymal stem cells using autologous platelet lysate technique. *Curr Stem Cell Res Ther.* 2010;5(1):81-93.

33. Karamouzian S, Nematollahi-Mahani SN, Nakhaee N, Eskandary H. Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients. *Clin Neurol Neurosurg.* 2012.
34. Bernardo ME, Fibbe WE. Safety and efficacy of mesenchymal stromal cell therapy in autoimmune disorders. *Ann N Y Acad Sci.* 2012;1266:107-117.
35. Reichenbach S, Blank S, Rutjes AW, et al. Hylan versus hyaluronic acid for osteoarthritis of the knee: a systematic review and meta-analysis. *Arthritis Rheum.* 2007;57(8):1410-1418.
36. Lee KB, Hui JH, Song IC, Ardany L, Lee EH. Injectable mesenchymal stem cell therapy for large cartilage defects--a porcine model. *Stem Cells.* 2007;25(11):2964-2971.
37. Horie M, Sekiya I, Muneta T, et al. Intra-articular Injected synovial stem cells differentiate into meniscal cells directly and promote meniscal regeneration without mobilization to distant organs in rat massive meniscal defect. *Stem Cells.* 2009;27(4):878-887.
38. Agung M, Ochi M, Yanada S, et al. Mobilization of bone marrow-derived mesenchymal stem cells into the injured tissues after intraarticular injection and their contribution to tissue regeneration. *Knee Surg Sports Traumatol Arthrosc.* 2006;14(12):1307-1314.
39. Frisbie DD, Kisiday JD, Kawcak CE, Werpy NM, Mcllwraith CW. Evaluation of adipose-derived stromal vascular fraction or bone marrow-derived mesenchymal stem cells for treatment of osteoarthritis. *J Orthop Res.* 2009;27(12):1675-1680.
40. Rubio D, Garcia-Castro J, Martin MC, et al. Spontaneous human adult stem cell transformation. *Cancer Res.* 2005;65(8):3035-3039.
41. de la Fuente R, Bernad A, Garcia-Castro J, Martin MC, Cigudosa JC. Retraction: Spontaneous human adult stem cell transformation. *Cancer Res.* 2010;70(16):6682.
42. Rosland GV, Svendsen A, Torsvik A, et al. Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res.* 2009;69(13):5331-5339.
43. Torsvik A, Rosland GV, Svendsen A, et al. Spontaneous malignant transformation of human mesenchymal stem cells reflects cross-contamination: putting the research field on track - letter. *Cancer Res.* 2010;70(15):6393-6396.
44. Kuznetsov SA, Mankani MH, Robey PG. Effect of serum on human bone marrow stromal cells: ex vivo expansion and in vivo bone formation. *Transplantation.* 2000;70(12):1780-1787.
45. Selvaggi TA, Walker RE, Fleisher TA. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. *Blood.* 1997;89(3):776-779.
46. Mackensen A, Drager R, Schlesier M, Mertelsmann R, Lindemann A. Presence of IgE antibodies to bovine serum albumin in a patient developing anaphylaxis after vaccination with human peptide-pulsed dendritic cells. *Cancer Immunol Immunother.* 2000;49(3):152-156.
47. Tuschong L, Soenen SL, Blaese RM, Candotti F, Muul LM. Immune response to fetal calf serum by two adenosine deaminase-deficient patients after T cell gene therapy. *Hum Gene Ther.* 2002;13(13):1605-1610.
48. Nejadnik H, Hui JH, Feng Choong EP, Tai BC, Lee EH. Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study. *Am J Sports Med.* 2010;38(6):1110-1116.
49. Adachi N, Ochi M, Deie M, Ito Y. Transplant of mesenchymal stem cells and hydroxyapatite ceramics to treat severe osteochondral damage after septic arthritis of the knee. *J Rheumatol.* 2005;32(8):1615-1618.

Chapter 3

Effect of arthritic synovial fluids on the expression of immunomodulatory factors by mesenchymal stem cells: an explorative in vitro study

Front Immunol. 2012 Aug 2;3:231

Maarten J.C. Leijs
Gerben M. van Buul
Erik Lubberts
Pieter K. Bos
Jan A.N. Verhaar
Martin J. Hoogduijn
Gerjo J.V.M. van Osch

ABSTRACT

Background: In diseased joints, the catabolic environment results in progressive joint damage. Mesenchymal stem cells (MSCs) can have immunomodulatory effects by secreting anti-inflammatory factors. To exert these effects, MSCs need to be triggered by proinflammatory cytokines. To explore the potential of MSCs as a treatment for diseased joints, we studied the effect of synovial fluid (SF) from donors with different joint diseases and donors without joint pathology on the immunomodulatory capacities of human MSCs *in vitro*. We hypothesized that SF of diseased joints influences the immunomodulatory effects of MSCs.

Materials and Methods: MSCs were cultured in medium with SF of six osteoarthritis (OA) or six rheumatoid arthritis (RA) donors and three donors without joint pathology were used as control. Gene expressions of IL-6, HGF, TNFα, TGFβ1, and indoleamine 2,3-dioxygenase (IDO) were analyzed. L-kynurenine concentration in conditioned medium (CM) by MSCs with SF was determined as a measure of IDO activity by MSCs. Furthermore, the effect of CM with SF on proliferation of activated lymphocytes was analyzed.

Results: Addition of SF significantly up-regulated the mRNA expression of IL-6 and IDO in MSCs. SF (OA) induced significantly higher expression of IDO than SF (control), although no difference in IDO activity of the MSCs could be shown with a L-kynurenine assay. Medium conditioned by MSCs with SF (OA or RA) suppressed activated lymphocyte proliferation *in vitro* more than medium conditioned by MSCs without SF or with SF (control).

Discussion: SF can influence the expression of genes involved in immunomodulation by MSCs and the effect on lymphocyte proliferation. We found indications for disease-specific differences between SFs but the variation between donors, even within one disease group was high. These data warrant further research to examine the potential application of MSC therapy in arthritic joints.

Keywords: MSC, osteoarthritis, rheumatoid arthritis, synovial fluid, immunomodulation

INTRODUCTION

Osteoarthritis (OA) and rheumatoid arthritis (RA) are high prevalent forms of arthritis. OA is mainly characterized by progressive functional loss and cartilage degeneration. Main factors involved in cartilage degeneration are a variety of matrix degrading enzymes and pro-inflammatory cytokines[1,2]. It is possible to treat the symptoms of OA with lifestyle changes, analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), or intra-articular injections with corticosteroids or hyaluronic acid and the ultimate treatment for end stage OA is joint replacement. A treatment to cure OA, however, is still not available. RA is an auto-immune disease initiated by immune complexes that together with cytokines, complement, and metalloproteinases[3] cause an inflammatory and catabolic environment in the joint[2]. It is a systemic disease characterized by persistent synovitis, systemic inflammation, and auto-antibodies which eventually cause joint damage with progressive cartilage degeneration and bone alterations. There is a wide range of therapeutic options for RA like analgesics, NSAIDs, disease-modifying anti rheumatic drugs (DMARDs), and biologicals[4,5]. However, to date there is no treatment available to cure RA. Human mesenchymal stem cells (MSCs), the progenitors of connective tissue cells, are able to differentiate into different cell types including chondrocytes[6-9]. This has attracted the interest of many people working in the area of cartilage repair. Besides the ability to reconstruct tissues, MSCs also have the ability to modulate the environment by secreting many immunomodulating and trophic factors like cytokines, chemokines, and growth factors[8,10-16]. These factors have potent immunomodulatory capacity as demonstrated *in vitro* by inhibition of T lymphocyte proliferation after adding MSCs in mixed lymphocyte reactions[12,17]. MSCs also inhibit the antibody production of B lymphocytes and inhibit the generation and function of antigen presenting cells[12,18,19]. The stimulation of MSC by pro-inflammatory cytokines like TNFα and IFNγ strongly enhances the immunosuppressive function of MSCs[12,16,20-22].

In a healthy joint environment, a balance exists between an anabolic and catabolic state. In a situation of inflammation or chronic damage, i.e., OA or RA, the environment becomes more catabolic[2,23]. All joint tissues are exposed to synovial fluid (SF) and in OA and RA inflammatory factors are secreted into the SF. The aim of the present study was to investigate whether SF of donors with OA, RA, or no joint pathology triggers MSCs to become immunomodulatory. Since inflammation plays a large role in RA and OA, we hypothesized that MSCs will be triggered to become immunomodulatory. We explored this by studying the effect of SF of OA and RA patients as well as SF of non-pathological (control) donors on MSCs. Our hypoth-

esis was that MSCs conditioned in SF (RA) will express a large anti-inflammatory effect compared to SF (control) due to the high inflammation state of RA patients and MSCs conditioned with SF (OA) will express a mild anti-inflammatory effect compared to SF (control) as a reaction to a less inflamed environment in joints of OA patients.

We evaluated the effect of SF on expression of genes of MSCs for immunomodulatory factors. Furthermore, we performed a functional assay to study the capacity of factors secreted by MSCs in response of SF to inhibit proliferation of activated lymphocytes.

MATERIALS AND METHODS

Synovial fluids

Fifteen SF samples were obtained from six OA patients, six RA patients, and three donors without any joint pathology. SFs (OA) were obtained from patients undergoing total knee replacement surgery. All patients implicitly consented to the use of these fluids for scientific research (with approval by Erasmus MC medical ethical committee protocol #MEC-2004-322). SFs (RA) were obtained from RA patients with active inflammation of the knee during consultation at the rheumatology outpatient clinic (with approval by Erasmus MC medical ethical committee protocol #MEC-236.904-2003-255). SFs (control) were purchased from SF donors without joint diseases, post mortem within 24 h of death (Articular Engineering, Northbrook, IL, USA). After aspiration, all SF samples from the joints of all donors were centrifuged to remove debris. Supernatant was stored at -80°C.

To evaluate the inflammatory aspects of the different SFs we did amplify enzyme linked immunosorbent assays (ELISA) to quantify cytokines IL-6, TNFα (R&Dsystems, Minneapolis, MN, USA), and IFNγ (Invitrogen, Carlsbad, CA, USA). Measurements of IL-6, TNFα, and IFNγ were performed in duplicate. All SFs were treated with 1:3 hyaluronidase (1000 U/ml PBS, 10 min at 37°C) prior to ELISA measurements. ELISAs were carried out according to the manufacturer's instructions by means of a multilabel plate reader (VersaMax™, Molecular Devices, Sunnyvale, CA, USA).

MSC isolation

Mesenchymal stem cells were isolated from heparinized femoralshaft marrow aspirate of patients undergoing total hip arthroplasty (with informed consent

after approval by Erasmus MC medical ethical committee protocol #MEC-2004-142). About 5–10 ml marrow was harvested with a sterile Jamshidi needle into sterile 10 ml syringes containing 0.5 ml of heparin (1000U/ml). About 30–100 x 10^6 mononuclear cells were plated in a T175 flask in 25 ml expansion medium (Dulbecco's Modified Eagle Medium (DMEM) low glucose (Invitrogen, Carlsbad, CA, USA) containing 15% heat inactivated fetal calf serum (Lonza, Verviers, Belgium, selected batch), 1.5 μg/ml fungizone (All Invitrogen, Carlsbad, CA, USA), 50 μg/ml gentamicin (Invitrogen, Carlsbad, CA, USA), 1 ng/ml fibroblast growth factor-2 (Instruchemie B.V., Delfzijl, The Netherlands),and 0.1 mM of L-ascorbicacid 2-phosphate (vitamin C; Sigma, St.Louis, MO, USA). After 24 h, non-adherent cells and erythrocytes were removed by washing three times with 2% FCS in 1xPBS (Invitrogen, Carlsbad, CA, USA). Remaining adherent cells were cultured in expansion medium at 37°C and 5% carbondioxide (CO_2). Expansion media were renewed twice a week. At subconfluent cells were trypsinized with a 0.25% trypsin solution containing 0.01% EDTA (Invitrogen, Carlsbad, CA, USA) and plated at a density of 2300 cells/cm^2.

MSC culture with SF

Cryopreserved MSCs of passage two were used for the experiments. After thawing, MSCs were seeded in a T175 flask at a density of 2300 cells/cm^2, expanded for one passage and subsequently plated in six well plates at a density of 4000 cells/cm^2 for the experimental conditions. At 70% confluence the existing medium was discarded and the cells were washed three times using PBS (Invitrogen, Carlsbad, CA, USA). Subsequently 0.8 ml of DMEM low glucose containing 9 μg/ml fungizone and 50 μg/ml gentamicin, was applied per well. The different SFs (OA, RA, and control) were added in triplicates to the media in a concentration of 20%. In preliminary tests MSCs were cultured in 0, 10, or 25% SF of four OA donors, gene expression was not significantly different in 10 and 25% SF. Based on this and taking into account the availability of the SF (from SF (control) we obtained maximal 1 ml per donor) we decided to use 20% SF for all further experiments. All conditions contained a total concentration of 1% ITS (BD Bioscience, Bedford, MA, USA). Nine wells with only medium plus 1% ITS were used as negative controls for unstimulated MSCs. After 48 h of incubation, MSCs were harvested for gene expression analyses and the conditioned medium (CM) was harvested and stored at –80°C.

Gene expression analysis

After 48 h of incubation total RNA from MSCs was isolated using Rneasy® microkit (Qiagen, Hilden, Germany) with Rneasy MinElute spin columns. After quantifica-

tion of nucleic acids by spectrophotometry (NanoDrop 2000, Thermo Scientific, Isogen Life Science, Ijsselstein, The Netherlands) the RNA was reverse transcribed using a First Strand cDNA Synthesis kit (RevertAid™; MBI Fermentas, St. Leon-Rot, Germany). Amplifications were performed as 20 µl reactions with real-time PCR. Thermocycler conditions comprised an initial holding at 95˚C for 10 min, followed by one step at 95˚C for 15 s and 60˚C for 60 s for 40 cycles. A dissociation stage was added at the end using 95˚C for 15 s, 60˚C for 20 s, and 95˚C for 15 s. For UBC, IL-6, HGF, TNFα, qPCR™ Mastermix Plus for SYBR® Green I (Eurogentec, Nederland B.V., Maastricht, The Netherlands) was used. For GAPDH, HPRT, IDO, and TGFβ1 TaqMan Master Mix (ABI, Branchburg, NJ, USA) was used. Sets of primers and probes used in this study: GAPDH (NM_002046.3) Fw: ATGGGGAAGGTGAAGGTCG Rv: TAAAAGCAGCCCTGGTGACC Probe: Fam-CGCCCAATACGACCAAATCCGTTGAC; HPRT (NM_000194.2) Fw: TATGGACAGGACTGAACGTCTTG Rv: CACACAGAGGGCTACAATGTG Probe: Fam-AGATGTGATG AAGGAGATGGGAGGCCA; UBC (NM_021009.5) Fw: ATTTGG GTCGCGGTTCTTG Rv: TGCCTTGACATTCTCGATGGT; IL-6 (NM_000600.3) Fw: TCGAGCCCACCGGGAACGAA Rv: GCAGGGAAGGCAGCAGGCAA; HGF (NM_000601.4) Fw: GGCTGGGGCTACACTGGATTG Rv: CCACCATAATCCCCCTCACAT; TNF-aplha (NM_000594.2) Fw: GCCGCATCGCCGTCTCCTAC Rv: AGCGCTGAGTCGGTCACCCT; TGF-beta1 (NM_000660.4) Fw: GTGACAGCAGGGATAACACACTG Rv: CATGAATGGTGGCCAGGTC Probe: Fam-ACATCAACGGGTTCACTACCGGC. IDO was detected using a taqman assay on demand (Applied Biosystems, Capelle a/d Ijssel, The Netherlands) of which the primer sequence is not known to us. Data were collected and quantitatively analyzed on an ABI Prism 7000 Sequence Detection System (SDS) with SDS software, version 1.2.3 (Applied Biosys- tems, Capelle a/d Ijssel, The Netherlands). Gene expressions of the cytokines and IDO in MSCs were calculated by cycle threshold (CT) values. CT values of 36 and higher were considered as non- expressed and set to 100 for further calculations. The CT values of the housekeeper genes GAPDH, HPRT, and UBC were averaged by using geometric averaging of every sample. This average is the best keeper index (BKI) for every single sample. All separate CT values were corrected to the BKI by using the $2^{-\Delta CT}$ formula.

L-kynurenin assay

In order to evaluate whether SF influenced IDO activity in MSCs, we measured the concentration of L-kynurenine in the CM and SFs. To correct for possible L-kynurenine in SF, the SFs were diluted in the same concentration and the same media as the CM and values were subtracted from the CM values. Values of one of the OA donors could not be used since no remaining SF was available for correction. Thirty percent trichloroacetic acid was added to the samples in a 1:3 ratio

and after 30 min incubation at 50˚C the samples were centrifuged at 12000 rpm for 5 min. Supernatant of all conditions were diluted 1:1 in Ehrlich reagent (200 µg 4-dimethylaminobenzaldehyde (Sigma, St. Louis, MO, USA) in 10 ml of glacial acetic acid) in duplicate in a 96-wells flat bottom plate and absorbance was determined at 490 nm in a multilabel plate reader (VersaMax™, Molecular Devices, Sunnyvale, CA, USA). L-kynurenine (Sigma, St. Louis, MO, USA) was used as standard.

PBMC proliferation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin, Rotterdam, The Netherlands) of healthy volunteers using Ficoll-Paque™Plus (GE Healthcare, Uppsala, Sweden) separation and stored at 135˚C until use. PBMCs were thawed and centrifuged at 2000 rpm for 5 min. Viable cells were counted using trypan blue exclusion test. PBMCs were seeded in alpha-modified Minimum Essential Medium (aMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 20% heat inactivated FCS (Lonza, Verviers, Belgium, selected batch), 2% pen-strep (Penicillin 10,000 UI/ml, Streptomycin 10,000 UI/ml, Lonza, Verviers, Belgium), and 2% L-glutamine (200 mM, Lonza, Verviers, Belgium) and activated with anti-CD3 and anti-CD28 linked with linker goat-anti-mouse antibody (BD Pharmingen, San Diego, CA, USA). 5 x 10^4 PBMCs in 100 µl expansion medium were seeded per well in round-bottom 96-well plates (Nunc, Roskilde, Denmark) and incubated for 5 days. The immunosuppressive capacity of factors secreted by MSCs was evaluated by using the CM from the MSC culture conditions described earlier. One hundred microliters of CM of MSCs incubated with each of the SFs, except one of the OA donors where no SF was left, was added in triplicate to the PBMCs for 5 days. CM of MSCs without SF and unconditioned medium, identical to the medium used in the CM except for the fact that it had not been in contact with MSCs, were added in triplicate as a control. To correct for direct effects of the SF present in the CM on the PBMCs we added controls of medium not conditioned by MSCs with similar concentration of SF of each of the donors. At day four of incubation, ^{3}H-thymidine (0.5 µCi/well; Perkin Elmer, Inc., San Jose, CA, USA) was added. At day five, after 16 h of incorporation of ^{3}H-thymidine, PBMCs were harvested, and ^{3}H-thymidine incorporation measured using a β-plate reader (Wallac 1450 MicroBeta TriLux Liquid Scintillation Counter and Luminometer, Perkin Elmer, Inc., San Jose, CA, USA).

Data analysis

Statistical difference in gene expression by MSCs conditioned with SF (OA), SF (RA), and SF (control) was analyzed by using a mixed linear model in which condition (SF of OA, RA, or no joint pathology donors) was considered a fixed factor and the different SF donors for all conditions a random factor. Values for the genes

IDO, TNFα, and TGFβ were log-transformed to approach a normal distribution. Statistical differences of inhibitory capacity of the different CM was analyzed by using a mixed linear model in which condition (CM by MSCs incubated with OA, RA, or control SF) was considered a fixed factor, different donors a random factor and Sidak was used as adjustment for multiple comparisons. Inhibitory effects of CM with SF compared to SF only were explored by statistical analyses with the Wilcoxon signed ranks test.

Data are presented as the mean standard deviation and 2.5 – 97.5 percentile. P-value of 0.05 was considered statistical significant; *P < 0.05, **P < 0.001. Analyses were performed using SPSS 17.0 Statistics (SPSS, Inc., Chicago, IL, USA).

RESULTS

Effect of SF on gene expression of MSCs

To evaluate the effect of SF on mRNA expression of IL-6, HGF, IDO, TNFα, and TGFβ1 by MSCs, MSCs were cultured in medium containing 20% SF of each of the 15 different donors. Medium with 1% ITS was used as SF free culture control and represented as a dotted line in **figure 1**. Addition of SF significantly up-regulated IL-6 (2.43 ± 0.22-fold; P < 0.001) and IDO (1.72 ± 0,17-fold; P = 0.007) expression. There is a trend of down-regulation of TNFα albeit not significant. Gene expressions of HGF, TNFα and TGFβ1 were not significantly affected by SF compared to the SF free control (**figure 1**). Next, we explored the effect of three different types of SF separately. MSCs cultured in SF (OA) expressed IDO 1.69-fold (P = 0.048) higher than MSCs cultured in SF (control). For SF (RA) we also found an up-regulation in gene expression for IDO, albeit not significant which is probably caused by the large variation between the six different RA donors. No further significant differences in gene expression of IL-6, TNFα, TGFβ1, and HGF were found between MSCs cultured in the three different SFs (**figure 1**). IDO activity of MSCs was analyzed by an L-kynurenine assay on all different CM with SF corrected for L-kynurenine content in SF of that donor. No significant differences of IDO activity by MSCs cultured in SF of different donors were found (data not shown).

Effect of conditioned medium on lymphocyte proliferation

Conditioned medium harvested after culturing MSCs in 20% SF was used to analyze the effect of secreted factors of MSCs on the proliferation of CD3/CD28 activated PBMCs (**figure 2**). The CM was mixed 1:1 with fresh medium and added to PBMCs. CM of MSCs without SF (CM control) did not influence PBMC proliferation. There

was no difference in PBMC proliferation between CM of MSCs without SF and CM of MSCs incubated in SF (control). There was significantly more inhibition of PBMC proliferation by CM with SF (OA) compared to CM with SF (control; $P < 0.001$) and by CM with SF (RA) compared to CM with SF (control; $P < 0.001$).

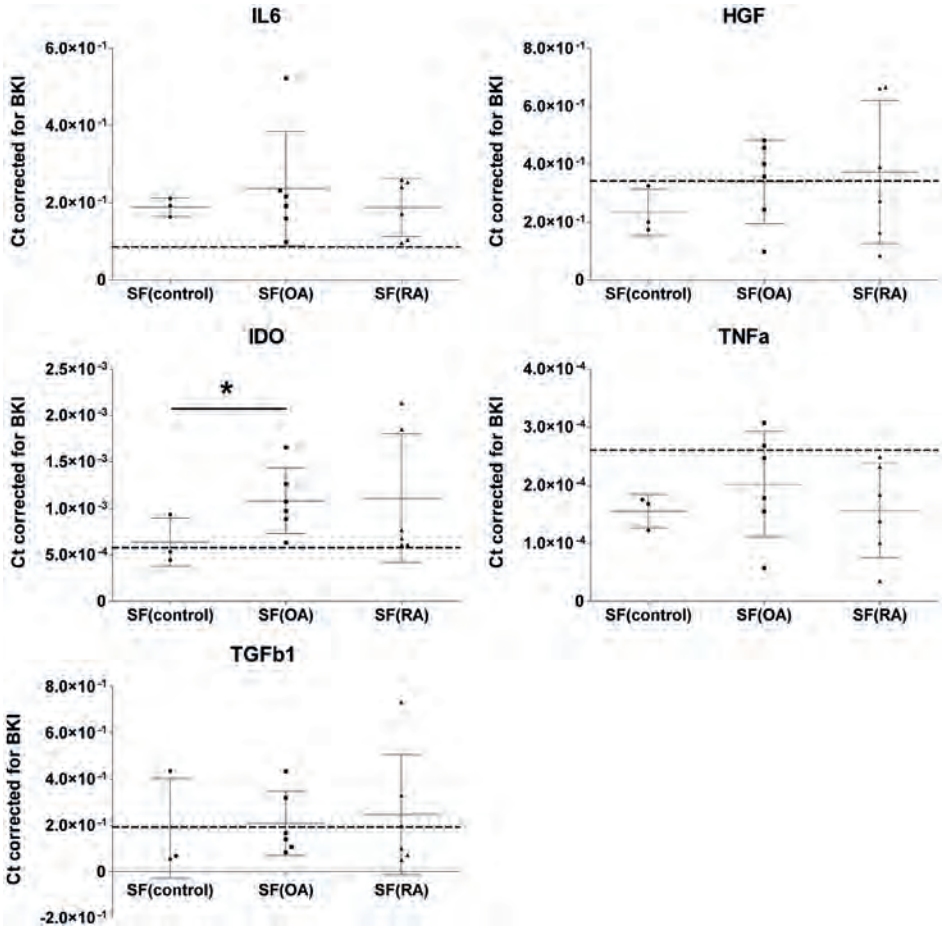


Figure 1. Effect of different synovial fluids on gene expression of immunomodulatory factors by MSCs. *Gene expressions expressed in cycle thresholds (ct) normalized to BKI in every sample. MSCs were cultured in 20% SF of six OA and six RA donors and three donors without joint pathology. Dotted lines indicate the average gene expression in MSCs cultured in medium without SF. The data are presented as median scatterplots, each point represents an average of three measurements per donor (Mean ± SD). SF, synovial fluid; SF (control, OA and RA) culture media of the MSCs supplemented with respectively non-pathological SF, OA SF and RA SF. IDO, indoleamine 2,3-dioxygenase; IL-6, interleukin-6; TNFα, tumor necrosis factor-alpha; TGFβ1, transforming growth factor-beta 1; HGF, hepatocyte growth factor; BKI, Best Keeper Index consisting of: GAPDH, UBC and HPRT. *Expression in MSCs after culture in SF (OA) different from SF (control) by mixed linear test of these two conditions, $P < 0.05$.*

To correct for direct effects of SF on PBMCs we added controls with unconditioned medium with SF. The SF appeared to inhibit PBMC proliferation, independent of disease state. A preliminary experiment with different concentrations of SF (OA) indicated that the effect of SF on lymphocyte proliferation is dose dependent (data not shown).

Conditioned medium of MSCs incubated in SF (control) caused significantly less inhibition of PBMC proliferation than unconditioned medium (medium that was not in contact with MSCs but contained SF (control)). No significant differences in proliferation inhibition were found between OA and RA CM (**figure 2**).

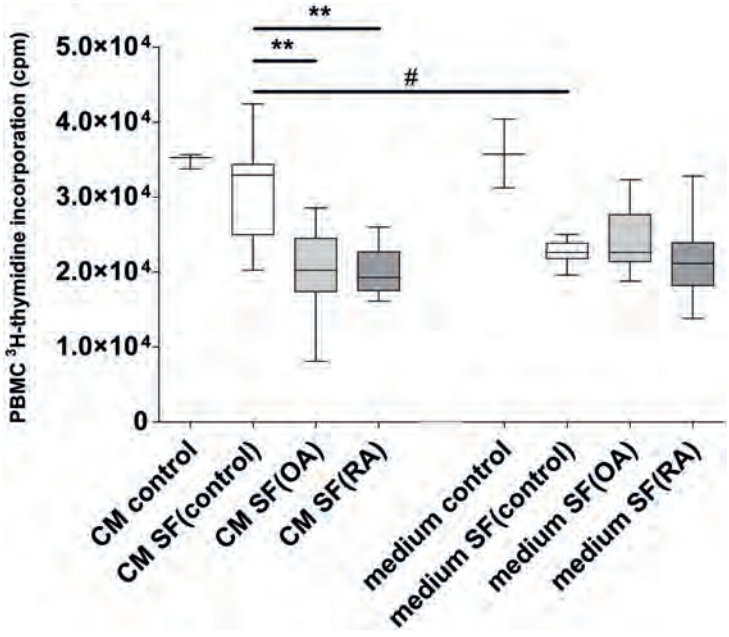


Figure 2. Effect of conditioned medium of MSCs with different types of synovial fluid (SF) on proliferation of CD3/28 activated PBMCs. *Box-and-Whisker plot 2.5–97.5 percentile; * P <0.05; ** P <0.001; # P <0.05 between CM and SF effect. CM, conditioned medium; SF, synovial fluid; (medium) culture medium with ITS; (CM control) MSC conditioned medium consisting of only culture medium with ITS; (Medium control) Only culture medium with ITS; SF (control) synovial fluid of donors without joint pathology; SF (OA) synovial fluid of patients with osteoarthritis; SF (RA) synovial fluid of patients with rheumatoid arthritis.*

DISCUSSION

The aim of the study was to evaluate to what extent SF influences the immunomodulation of MSCs. This study indicates that SF can influence the expression of genes in MSCs that are involved in immunomodulation. Moreover, factors secreted

by MSCs incubated with SF (OA and RA) inhibited the proliferation of activated lymphocytes significantly more than factors secreted by MSCs incubated without SF or with SF (control). This indicates that factors in diseased SF stimulate MSCs to secrete anti-inflammatory factors.

To our knowledge this is the first report on the effect of SF on the expression and secretion of immunomodulatory factors in MSCs. This information is important for the application of MSCs in joints of patients with joint diseases. Upon injection or implantation in the joint the MSCs will be exposed to SF. SF is known to contain a mix of factors secreted by the tissues of the joint. In the current study we investigated whether SF from non-diseased and OA and RA donors triggers MSCs to have an immunomodulatory effect. We demonstrated that SF (OA) can upregulate MSC gene expression of IDO. In addition, MSCs treated with SF up-regulated expression of IL-6, a pleiotropic cytokine with pro-inflammatory functions, but also involved in regenerative processes and regulation of metabolism[24]. Which factor(s) secreted by MSCs cause the immunomodulatory effects cannot be concluded from our study and deserves further investigation.

We hypothesized that MSCs will be triggered by a catabolic environment in the joint to become immunomodulatory and that SF (RA) will induce large anti-inflammatory and SF (OA) will induce mild anti-inflammatory effects compared to SF (control). Our data could partly confirm this hypothesis. Diseased SF triggered MSCs to become immunomodulatory but we did not find any differences between the effects of SF (OA and RA) on gene expression of MSCs and PBMC proliferation. Whereas we assumed SF (control) would be immunological quiescent and diseased SF inflammatory, surprisingly we found inhibited lymphocyte proliferation by all SFs. This inhibition further increased by secreted factors of MSCs cultured with addition of SF (OA or RA), albeit non-significant. Surprisingly in the presence of SF (control), the inhibition of lymphocyte proliferation by SF was significantly reduced. This unexpected outcome suggests different effects of non-pathologic SF on excretion of factors by MSCs. Since the composition of healthy or diseased SF is not precisely known, it is difficult to explain the effects of SFs on MSCs and on PBMCs.

To provide a relatively clean way to study the effect of factors secreted by MSCs on lymphocyte proliferation, we used CM of MSCs exposed to SF. Different durations of exposure to SF and direct interactions between lymphocytes and MSCs in the presence of SF can play a role as well and this should be investigated in the future.

We here demonstrate that MSCs can be differently influenced by exposure to SF from diseased and non-pathological joints, but the effects were small compared to commonly used stimulation with TNFα and IFNγ[12,25,26]. This might explain why resident MSCs in joints cannot prevent disease development; they might not be properly activated by the environment. It can also be regarded as somewhat disappointing in respect to the application of MSCs in the diseased joint since exposure to SF might not be sufficient to stimulate the healing activity of the MSCs.

Although it is unknown which factor in SF does stimulate MSCs, we performed ELISA on SFs. SF (RA) has a higher concentration of IL-6 compared to SF (OA; 7380 vs. 525.4 pg/ml; P = 0.009) and SF (control; 7380 vs. 22.4 pg/ml; P = 0.024), confirming previous reports[27]. TNFα was measurable in only one OA donor and two RA donors and IFNγ was measurable in only one OA and one RA SF donor (data not shown). Neither of these cytokines correlated with the effects of SF on MSCs or PBMCs but we cannot exclude that other factors evoke an effect on MSCs. Moreover, *in vivo*, direct contact with MSCs and inflamed synovial tissue, immune cells in the synovium, or degenerated cartilage might, however, activate the MSCs. Finally, it should be noted that we have selected a limited number of immunomodulatory factors to evaluate the effect on MSCs and we cannot exclude that SF stimulates other processes in MSCs that can affect healing of the diseased joint.

Since the SFs were considered as redundant materials, ethical regulations preclude the availability of patient-specific information. It is very likely that the OA and RA patients used medication that might have influenced the compositions of the SFs. It has been demonstrated that analgesic drugs, NSAIDs, and DMARDs can change concentrations of immunomodulatory factors in SF[28-30]. Use of different types of medication within donor groups could be a cause for the high variations within the groups.

Moreover, this explorative study was performed with SF of six OA donors, six RA donors, and three donors without joint pathology. To gain sufficient power the study should be repeated with larger numbers of pathological and non-pathological SFs. SF was used in a concentration of 20% for 48 h in analyses on MSCs. It remains unknown how MSCs will react on 100% SF over a longer period of time, which eventually will be the environment for MSCs when they are injected in a joint.

Although MSCs appear a promising therapy for degenerative joint diseases, the working mechanisms are not entirely clear. In animal studies it is possible to track MSCs injected in the joint. It was demonstrated that some of the injected MSCs

stayed in the joint and adhered to the synovium or affected areas[31,32] from where they could exert a modulating effect and decrease the inflammatory or catabolic environment in diseased joints. The immunomodulatory capacity of MSCs can be useful for patients with OA and RA. Good therapeutic options for RA are already available, such as DMARDs and biologicals. However, MSCs are capable of secreting many different factors, possibly for a prolonged time, which can influence many different mechanisms and are not restricted to one single target, unlike for example anti-TNFα. This explorative study shows that (1) SF can influence the expression of genes by MSCs involved in immunomodulation and (2) factors in CM by MSCs cultured with arthritic SF inhibit lymphocyte proliferation more than factors in CM by MSCs cultured without SF or with SF (control). These results warrant further research to examine the potential application of MSC therapy in arthritic joints.

REFERENCES

1. Goldring MB. The role of the chondrocyte in osteoarthritis. *Arthritis Rheum.* 2000;43(9):1916-1926.
2. Goldring MB, Marcu KB. Cartilage homeostasis in health and rheumatic diseases. *Arthritis Res Ther.* 2009;11(3):224.
3. Weissmann G. The pathogenesis of rheumatoid arthritis. *Bull NYU Hosp Jt Dis.* 2006;64(1-2):12-15.
4. Lee DM, Weinblatt ME. Rheumatoid arthritis. *Lancet.* 2001;358(9285):903-911.
5. Scott DL, Wolfe F, Huizinga TW. Rheumatoid arthritis. *Lancet.* 2010;376(9746):1094-1108.
6. Caplan AI. Mesenchymal stem cells. *J Orthop Res.* 1991;9(5):641-650.
7. Caplan AI. The mesengenic process. *Clin Plast Surg.* 1994;21(3):429-435.
8. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *J Cell Biochem.* 2006;98(5):1076-1084.
9. Solchaga LA, Welter JF, Lennon DP, Caplan AI. Generation of pluripotent stem cells and their differentiation to the chondrocytic phenotype. *Methods Mol Med.* 2004;100:53-68.
10. Chen X, Armstrong MA, Li G. Mesenchymal stem cells in immunoregulation. *Immunol Cell Biol.* 2006;84(5):413-421.
11. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol.* 2000;28(8):875-884.
12. Hoogduijn MJ, Popp F, Verbeek R, et al. The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy. *Int Immunopharmacol.* 2010;10(12):1496-1500.
13. Kim DH, Yoo KH, Choi KS, et al. Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell. *Cytokine.* 2005;31(2):119-126.
14. Meisel R, Brockers S, Heseler K, et al. Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. *Leukemia.* 2011;25(4):648-654.
15. Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. *Exp Biol Med (Maywood).* 2001;226(6):507-520.
16. Schinkothe T, Bloch W, Schmidt A. In vitro secreting profile of human mesenchymal stem cells. *Stem Cells Dev.* 2008;17(1):199-206.
17. Landgraf K, Brunauer R, Lepperdinger G, Grubeck-Loebenstein B. The suppressive effect of mesenchymal stromal cells on T cell proliferation is conserved in old age. *Transpl Immunol.* 2011;25(2-3):167-172.
18. Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One.* 2008;3(4):e1886.
19. Sze SK, de Kleijn DP, Lai RC, et al. Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. *Mol Cell Proteomics.* 2007;6(10):1680-1689.
20. Eggenhofer E, Steinmann JF, Renner P, et al. Mesenchymal stem cells together with mycophenolate mofetil inhibit antigen presenting cell and T cell infiltration into allogeneic heart grafts. *Transpl Immunol.* 2011;24(3):157-163.
21. Klyushnenkova E, Mosca JD, Zernetkina V, et al. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *J Biomed Sci.* 2005;12(1):47-57.

22. Siegel G, Schafer R, Dazzi F. The immunosuppressive properties of mesenchymal stem cells. *Transplantation.* 2009;87(9 Suppl):S45-49.
23. Findlay DM, Haynes DR. Mechanisms of bone loss in rheumatoid arthritis. *Mod Rheumatol.* 2005;15(4):232-240.
24. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. *Biochim Biophys Acta.* 2011;1813(5):878-888.
25. Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR. Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. *Am J Physiol Cell Physiol.* 2008;294(3):C675-682.
26. Hemeda H, Jakob M, Ludwig AK, Giebel B, Lang S, Brandau S. Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine expression and migration properties of mesenchymal stem cells. *Stem Cells Dev.* 2010;19(5):693-706.
27. Kokebie R, Aggarwal R, Lidder S, et al. The role of synovial fluid markers of catabolism and anabolism in osteoarthritis, rheumatoid arthritis and asymptomatic organ donors. *Arthritis Res Ther.* 2011;13(2):R50.
28. Alvarez-Soria MA, Largo R, Santillana J, et al. Long term NSAID treatment inhibits COX-2 synthesis in the knee synovial membrane of patients with osteoarthritis: differential proinflammatory cytokine profile between celecoxib and aceclofenac. *Ann Rheum Dis.* 2006;65(8):998-1005.
29. Bianchi M, Broggini M, Balzarini P, et al. Effects of tramadol on synovial fluid concentrations of substance P and interleukin-6 in patients with knee osteoarthritis: comparison with paracetamol. *Int Immunopharmacol.* 2003;3(13-14):1901-1908.
30. Bianchi M, Broggini M, Balzarini P, Franchi S, Sacerdote P. Effects of nimesulide on pain and on synovial fluid concentrations of substance P, interleukin-6 and interleukin-8 in patients with knee osteoarthritis: comparison with celecoxib. *Int J Clin Pract.* 2007;61(8):1270-1277.
31. Qi Y, Feng G, Yan W. Mesenchymal stem cell-based treatment for cartilage defects in osteoarthritis. *Mol Biol Rep.* 2012;39(5):5683-5689.
32. Sato M, Uchida K, Nakajima H, et al. Direct transplantation of mesenchymal stem cells into the knee joints of Hartley strain guinea pigs with spontaneous osteoarthritis. *Arthritis Res Ther.* 2012;14(1):R31.

Chapter 4

Pre-treatment of human mesenchymal stem cells with inflammatory factors or hypoxia does not influence migration to osteoarthritic cartilage and synovium

Am J Sports Med. 2017 Apr;45(5): 1151-1161

Maarten J.C. Leijs
Gerben M. van Buul
Jan A.N. Verhaar
Martin J. Hoogduijn
Pieter K. Bos
Gerjo J.V.M. van Osch

ABSTRACT

Background: Mesenchymal stem cells (MSCs) are promising candidates as a cell-based therapy for osteoarthritis (OA), although current results are modest. Pre-treatment of MSCs before application might improve their therapeutic efficacy.

Hypothesis: Pre-treatment of MSCs with inflammatory factors or hypoxia will improve their migration and adhesion capacities toward OA-affected tissues.

Study Design: Controlled laboratory study.

Methods: We used real-time polymerase chain reaction to determine the effects of different fetal calf serum (FCS) batches, platelet lysate (PL), hypoxia, inflammatory factors, factors secreted by OA tissues, and OA synovial fluid (SF) on the expression of 12 genes encoding chemokine or adhesion receptors. Migration of MSCs toward factors secreted by OA tissues was studied *in vitro*, and attachment of injected MSCs was evaluated *in vivo* in healthy and OA knees of male Wistar rats.

Results: Different FCS batches, PL, or hypoxia did not influence the expression of the migration and adhesion receptor genes. Exposure to inflammatory factors altered the expression of CCR1, CCR4, CD44, PDGFRα, and PDGFRβ. MSCs migrated toward factors secreted by OA tissues *in vitro*. Neither pre-treatment with inflammatory factors nor the presence of OA influenced MSC migration *in vitro* or adhesion *in vivo*.

Conclusion: Factors secreted by OA tissues increase MSC migration *in vitro*. *In vivo*, no difference in MSC adhesion was found between OA and healthy knees. Pre-treatment with inflammatory factors influenced the expression of migration and adhesion receptors of MSCs but not their migration *in vitro* or adhesion *in vivo*.

Clinical Relevance: To improve the therapeutic capacity of intra-articular injection of MSCs, they need to remain intra-articular for a longer period of time. Pre-treatment of MSCs with hypoxia or inflammatory factors did not increase the migration or adhesion capacity of MSCs and will therefore not likely prolong their intra-articular longevity. Alternative approaches to prolong the intra-articular presence of MSCs should be developed to increase the therapeutic effect of MSCs in OA.

Keywords: mesenchymal stem cells; migration; osteoarthritis; cell therapy; inflammation

INTRODUCTION

Osteoarthritis (OA) is characterized by an inflammatory and catabolic intra-articular environment, which causes progressive degeneration and inflammation of multiple intra-articular tissues. Sport-related injuries like anterior cruciate ligament ruptures or meniscal tears increase the incidence of OA[2,50]. To date, only symptomatic treatments are available for OA. A successful strategy to cure OA would consist of long-term modulation of the degenerative joint environment by simultaneously reducing inflammation and promoting tissue regeneration. Mesenchymal stem cells (MSCs) are promising candidates for this therapeutic approach to OA. They have been shown capable of suppressing immune-mediated cartilage destruction while contributing to cartilage repair via their chondrogenic differentiation and paracrine stimulation of endogenous repair processes[8,16,27,33]. The use of MSCs as cell therapy for OA by intra-articular injection has been reported to be safe[4,34]. However, clinical trials and preclinical studies did not show consistent data on healing or regeneration of dam- aged tissues after MSC treatment[4,48]. A possible explanation for these inconsistent results could be the fast decrease in the number of viable MSCs after injection in the joint; no viable MSCs were detected in the joint 1 to 2 weeks after injection[44,46]. A small proportion of intra-articularly injected MSCs attached to synovial surfaces after these injections, but almost none attached to the affected cartilage[32]. During their rather short local intra-articular presence, MSCs can have only a small effect. In cardiology, it has been shown that providing a longer interplay between the diseased tissue and applied MSCs increased the therapeutic effect[31,49,53]. We hypothesized that pre-treatment of MSCs with inflammatory factors or hypoxia would improve their migration and adhesion capacities toward OA affected tissues.

The migration and adhesion of injected MSCs in OA joints will depend on multiple considerations, including the chemotactic factors secreted by OA joint tissues, the expression of chemotactic receptors for these factors, and the expression of cell adhesion receptors by the MSCs[3,13,14,16,21,23,24]. In OA joints, the presence of multiple chemokines that can attract MSCs has been described[3,6]. Furthermore, multiple chemokine receptors and adhesion receptors have already been indicated as possible migration receptors and adhesion receptors of MSCs[3,16,19,35,39,43,51,54]. Previous studies showed that the expression of these migration receptors can be influenced by exposure to inflammatory factors[16,18,35,37] or hypoxia[10,22], but none of these studies evaluated the effect on intra-articular migration or adherence. Moreover, exposure of MSCs to inflammatory factors increases their immunomodulatory capacities[15,28,38,41] and was previously shown by us to increase the

anti-inflammatory and anticatabolic effects of MSCs on inflamed osteoarthritic joint tissues in culture[47]. In the current study, we evaluated whether OA synovium and cartilage produce chemotactic factors that can stimulate MSCs to migrate and adhere to the OA-affected tissues after injection and whether we could influence MSC migration capacities *in vitro* and adhesion capacities *in vivo*. Abbreviations used in the article are defined in **table 1**.

METHODS

Harvesting OA synovial fluid and conditioned medium of OA synovium and cartilage

Human SF, cartilage explants, and synovial explants were obtained as surgical waste material from patients undergoing total knee replacement surgery for OA. All patients implicitly consented to the use of these tissues for scientific research (with approval of medical ethical committee, MEC-2004-322). Schematic overviews of all methods used are depicted in **appendix figures A3** and **A4**.

To remove debris, SF samples were centrifuged (1600 rpm, 5 minutes), and supernatant was subsequently stored at -80°C. Full-thickness layers of uncalcified cartilage of macroscopically unaffected areas were taken from weightbearing and nonweightbearing parts of the tibial plateau and femoral condyles.

The synovial layer was carefully dissected from Hoffa's fat pad, with care taken to include as little fat tissue as technically possible. The cartilage and synovium explants were cut in 1-4 mm^2 pieces. To generate CM, 1 g of tissue was cultured per 5 ml for 24 hours in medium we used previously[5], which consisted of low-glucose DMEM (Invitrogen) containing 1% ITS (BD Bioscience), 1.5 µg/ml Fungizone (Invitrogen), 50 µg/ml gentamicin (Invitrogen), and 0.1 mM L-ascorbic acid 2-phosphate (vitamin C) (Sigma). CM was harvested after 24 hours and centrifuged (1200 rpm, 8 minutes), and the supernatant was stored at -80°C.

Chemokine detection in OA synovial fluid and conditioned media of OA tissues

Selected chemokines in SF of OA patients (n = 6 patients; 2 male and 4 female; age 65.5 years [range, 59-73 years]), OA synovium CM (n = 6 patients; 1 male and 5 female; age 63.3 years [range, 59-73 years]), and OA cartilage CM (n = 6 patients; 2 male and 4 female; age 62.7 years [range, 54-70 years]) were measured by use of a custom-made 10-plex Milliplex multiplex assay (Merck Millipore). Technical dupli-

Table 1
Abbreviations used

Abbreviation	Definition
aMEM	Minimum Essential Medium Eagle–alpha modification
CCR1	C-C chemokine receptor type 1
CCR4	C-C chemokine receptor type 4
CCR5	C-C chemokine receptor type 5
cDNA	complementary DNA
CM	conditioned medium
CT	cycle threshold
CXCR1	CXC chemokine receptor 1
CXCR3	CXC chemokine receptor 3
CXCR4	CXC chemokine receptor 4
CX3CL1	fractalkine
CX3CR1	CX3C chemokine receptor 1/fractalkine receptor
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
FGF2	fibroblast growth factor 2
GAG	glycosaminoglycan
HPRT	hypoxanthine phosphoribosyltransferase
IFN-γ	interferon gamma
IP-10 (CXCL10)	interferon gamma–induced protein 10
IL-8 (CXCL8)	interleukin 8
ITGβ1	integrin beta-1
ITGβ2	integrin beta-2
ITS	insulin-transferrin-selenium
MCP-1 (CCL2)	monocyte chemotactic protein 1
MCP-3 (CCL7)	monocyte chemotactic protein 3
MDC (CCL22)	macrophage-derived chemokine
MIA	Monoiodoacetate
MIP-1α (CCL3)	macrophage inflammatory protein 1 alpha
MIP-1β (CCL4)	macrophage inflammatory protein 1 beta
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSC	mesenchymal stem cell
OA	osteoarthritis
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGFRα	platelet-derived growth factor receptor alpha
PDGFRβ	platelet-derived growth factor receptor beta
PL	platelet lysate
SF	synovial fluid
SPIO	superparamagnetic iron oxide
TNF-α	tumor necrosis factor alpha

cates of the samples were used for the measurements. Ten factors were selected by matching known chemokine and adhesion receptors of MSCs with chemokines known to be present in OA SF[3,6,18,23,35,39]. The multiplex assay was performed according to the manufacturer's protocol, with the human chemokine magnetic bead panel kit for CX3CL1, MCP-3 (CCL7), MDC (CCL22), PDGF-AA, PDGF-BB, IL-8 (CXCL8), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α (CCL3), and MIP-1β (CCL4) (EMD Millipore Corporation). Assay readout was performed by use of the Bio-Plex 200 multiplex array reader and Bio-Plex software (Bio-Rad Laboratories). Values below the minimum detectable concentration were set at 50% of the minimum detectable concentration.

Isolation and culture of MSCs

MSCs were isolated from heparinized femoral-shaft marrow aspirate of patients undergoing total hip arthroplasty, after signed informed consent and with approval of the local ethical committees. Five to 10 ml of marrow was harvested with sterile Jamshidi needles and sterile 10-ml syringes containing 0.5 ml of heparin (1000 U/ml). Mononuclear cells (30 - 100 x 10^6) were plated in T175 flasks in 25 ml of expansion medium consisting of aMEM (Invitrogen) containing 10% heat inactivated FCS (Lonza; selected batch), 1.5 µg/ml Fungizone, 50 µg/ml gentamicin, 1 ng/ml FGF2 (Instruchemie B.V.), and 0.1 mM vitamin C. After 24 hours, nonadherent cells and erythrocytes were removed by washing 3 times with 2% FCS in x1 PBS (Invitrogen). Remaining adherent cells were cultured in expansion medium at 37°C and 5% carbon dioxide. Expansion media were renewed twice a week. Cells were trypsinized at subconfluency with 0.25% trypsin solution containing 0.01% EDTA (Invitrogen) and were replated at a density of 2300 cells/cm^2.

To evaluate whether different FCS batches or PL, generally used for MSC culturing, influence the migration or adhesion factor expression in MSCs, cryopreserved passage 2 MSCs (n = 3 donors) were used. MSCs were expanded for 1 passage in 3 different 10% FCS batches with 3 different lot numbers of the same company (Lonza; selected batches) and PL (>50 pooled human donors prepared as described previously[33]) in expansion medium. Before use, PL was thawed and centrifuged at 2000g for 10 minutes to remove remaining platelet fragments. After centrifugation, PL was stored at 4°C and used for up to 1 week. After passaging, the MSCs remained in their specific serum condition for another 72 hours, with a final medium change 24 hours before harvesting for gene expression analysis.

To study the effect of OA SF and the effect of exposure to inflammatory factors and hypoxia, passage 2 MSCs were thawed and expanded for 1 passage. MSCs

(n = 3 donors) were plated in 6-well plates and at 70% confluence were washed with PBS and cultured for 48 hours in low-glucose DMEM containing 20% OA SF, 1% ITS, 1.5 µg/ml Fungizone, 50 µg/ml gentamicin, and 0.1 mM vitamin C.[29] After 48 hours, MSCs were harvested for gene expression analyses. To evaluate the effect of hypoxia and inflammatory factors, MSCs were plated in 6-well plates (2 x 10^5 cells/well) and cultured in expansion medium for 48 hours under 20% of oxygen (normoxic) or 1% of oxygen (hypoxic) conditions. After 48 hours, cells were washed with PBS and cultured for 24 hours in low-glucose DMEM containing 1% ITS, 1.5 µg/mL Fungizone, 50 µg/mL gentamicin, and 0.1 mM vitamin C with an additional 1 ng, 20 ng, or 50 ng IFNγ and TNFα (PeproTech) per milliliter. MSCs were kept in their normoxic or hypoxic state and subsequently were harvested for gene expression analyses; CM was harvested, centrifuged, and stored at -80°C. The choices for concentrations of inflammatory factors and exposure time to hypoxia and inflammatory factors were made based on the literature and our own experiments[10,11,40,47].

Gene expression analysis

Total RNA from MSCs was isolated by use of the RNeasy microkit with RNeasy MinElute spin columns (Qiagen). After nucleic acid quantification by spectrophotometry (NanoDrop 2000; Thermo Scientific), RNA was reverse transcribed by means of a first-strand cDNA synthesis kit (RevertAid; MBI Fermentas). Amplifications were performed as 10-µL reactions in a CFX96 real-time thermal cycler (Bio-Rad Laboratories). Thermocycler conditions comprised an initial holding at 95°C for 10 minutes, followed by one step at 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. A dissociation stage was added at the end using 95°C for 15 seconds, 60°C for 20 seconds, and 95°C for 15 seconds. For *HPRT*, TaqMan Master Mix (ABI) was used. For receptor expression for chemotactic factors, TaqMan universal Master Mix (Life Technologies) was used for receptors *CCR1, CCR4, CCR5, PDGFRα, PDGFRβ, CXCR1, CXCR3, CXCR4, CX3CR1*, and adhesion factors *ITGβ1, ITGβ2*, and homing cell adhesion molecule *CD44*. Primers and probes used in this study are depicted in **table 2**. Genes were detected using TaqMan assays on demand (Life Technologies). Data were quantitatively analyzed on CFX manager software (version 3.1, Bio-Rad). Gene expressions in MSCs were calculated by CT values. CT values of 36 or higher were considered nonexpressed and were set to 100 for further calculations. CT values were corrected for housekeeper gene *HPRT* by using the $2^{-\Delta CT}$ formula.

Table 2
Primers and probes used for Real-Time polymerase chain reaction

Genes	Gene Accession Number	Predesign Assay Number	Primer/Probe
HPRT (housekeeper)	NM_000194.2	-	Fw:TATGGACAGGACTGAACGTCTTG Rv:CACACAGAGGGCTACAATGTG FamAGATGTGATGAAGGAGATGGGAGGCCA
CCR1	NM_001295.2	Hs00928897_s1	Assay on demand
CCR4	NM_005508.4	Hs00747615_s1	Assay on demand
CCR5	NM_001100168.1	Hs99999149_s1	Assay on demand
CD44	NM_000610.3	Hs01075861_m1	Assay on demand
PDGFRα	NM_006206.4	Hs00998018_m1	Assay on demand
PDGFRβ	NM_002609.3	Hs01019589_m1	Assay on demand
ITGβ1	NM_002211.3	Hs00559595_m1	Assay on demand
ITGβ2	NM_000211.3	Hs00164957_m1	Assay on demand
CXCR1	NM_000634.2	Hs01921207_s1	Assay on demand
CXCR3	NM_001142797.1	Hs01847760_s1	Assay on demand
CXCR4	NM_001008540.1	Hs00607978_s1	Assay on demand
CX3CR1	NM_001171171.1	Hs01922583_s1	Assay on demand

Migration assay

The migration assay was performed with 24-well-plate transwell inserts (Millicell, inserts 8.0 µm PET, Cat. No. PIEP12R48; Merck Millipore). OA cartilage CM (pooled from 6 donors), OA synovium CM (pooled from 6 donors), and control medium consisting of low-glucose DMEM, 1% ITS, 1.5 µg/ml Fungizone, 50 µg/ml gentamicin, and 0.1 mM vitamin C were placed in the wells (600 µl/well). Each condition was performed in 6 wells. Transwell inserts were placed in the wells, and 6 x 10^3 passage 2 MSCs in 200 µl of control medium were plated in the transwell insert and incubated for 18 hours. Then, transwell inserts were harvested and the medium was discarded. The bottoms of the wells were checked for adherent MSCs to exclude missed migrated cells. The inserts were washed with PBS, and cells on the insert membrane (inside and outside) were fixed with 10% formalin for 30 minutes, permeabilized with 0.1% Triton X-100 for 5 minutes, and washed with Milli-Q (Millipore). Cells were stained by placing the inserts in toluidine blue for 60 seconds followed by a quick rinse in Milli-Q. The inner side of the membrane was swabbed with a cotton wool swab to remove non migrated MSCs. Microscopic pictures of MSCs that migrated to the outer side of the transwell membrane were taken directly after the swab. Migrated cells were counted at a X200 magnification by using an inlay raster and 9 preset standardized fields for the membranes. Based on these counts, the total number of cells per square centimetre could be calcu-

lated and extrapolated to the total surface of the membrane. With this method, the number of migrated cells per condition could be determined.

In vivo cell adhesion in OA knees and healthy rat knees

The animal experiments were approved by the animal ethical committee (EMC2285 116-11-03). The animals were housed in groups of 2 animals per individually ventilated cage (21 x 37 cm) in sawdust that was enriched with nesting material. The animals had food and water ad libitum with a light-dark cycle of 12 hours. Discomfort and welfare were evaluated and scored daily by the animal caretakers. Animals were allocated to the OA or control group at random. OA was induced bilaterally in the knees of 13-week-old male wild-type Wistar rats (n = 5) (Harlan Netherlands BV) by an intra-articular injection of 20 µl of saline containing 1 mg of MIA. Healthy controls, 13-week-old male wild-type Wistar rats (n = 5), were bilaterally injected intra-articularly in the knee with 20 µl of saline. All injections and imaging procedures were applied under isoflurane inhalation anesthesia (Pharmachemie BV) while monitoring cardiac function, with additional subcutaneous injections of 0.01 mg/kg buprenorphine during and 1 day after intra-articular injections. MIA injections cause structural damage after 3 weeks[42], and at that point MSCs were injected via the intra-articular route.

Passage 3 MSCs were labelled at 80% to 90% confluency with SPIO by incubating the MSCs for 24 hours in aMEM with 5% FCS, ferumoxides 100 µg/ml (11.2 mg Fe/ml; Endorem, lot/batch 08GE601A), and protamine sulphate 5 µg/ml (LEO Pharma)[45]. Labelled cells were trypsinized and plated at a density of 2 x 10^4 MSCs/cm^2. After 48 hours, MSCs were exposed to 50 ng/ml IFNγ and 50 ng/ml TNFα (IFNγ/TNFα) in expansion medium or cultured in normal expansion medium (nonactivated) for 24 hours. After 24 hours, MSCs were washed with PBS and trypsinized. A 50-µl syringe (Hamilton) was used for intra-articular injection of 1 x 10^6 MSCs in 20 µl of saline. This number of MSCs (1 x 10^6) was selected based on literature that reported the effect of this cell number and side effects of higher numbers of cells[1,20,46]. At 24 hours after injection, SPIO-MSCs were visualized with a preclinical 7.0-T MRI scanner (MR 901 Discovery; Agilent/GE Healthcare). Cell attachment and migration were evaluated after 24 hours of injection since we expected attachment and migration processes to take place in the first 24 hours. Directly after the scan, the rats were sacrificed by a pentobarbital overdose, and the knees were harvested for histologic evaluation.

Tissue harvest and histologic evaluations

All knees (n = 20 knees) were fixed in formalin 4% (vol/vol) at room temperature for 1 week, decalcified in 10% EDTA at room temperature for 3 weeks, and embedded in paraffin. Coronal sections were stained with thionin to evaluate cartilage damage[46]. Perls iron staining was done for localization of SPIO-MSCs. All samples were blinded before scoring, and 2 different histologic sections of each knee were scored by 2 researchers. Disagreements of greater than 1 point were resolved by consensus. Consultation of a third researcher when disagreement persisted appeared unnecessary. The average score of MSC attachment of both researchers is presented. To confirm OA, cartilage quality was scored on the patella, trochlea, tibial plateau, and femoral condyles with a modified Pritzker score[36,46]. An average score for GAG loss and structural damage was calculated. To score the number of injected MSCs that adhered to different tissues in the joint, we applied a semi-quantitative scoring system to the Perls iron histologic results: (1) <10 MSCs, (2) 10-50 MSCs, (3) 50-250 MSCs, (4) 250-1000 MSCs, (5) >1000 MSCs. Locations of the injected MSCs were divided among synovium, cartilage, and free cells in the joint space.

Statistics

Effects of different FCS batches, PL, inflammatory factors, hypoxia, and OA SF on gene expression of chemotactic receptors genes were analyzed with a mixed-model analysis of variance (ANOVA), which takes into account correlation within donors. Cell migration was analyzed with a separate ANOVA linear regression model for MSCs with and without exposure to inflammatory factors. Post hoc, independent, 2-tailed *t* tests were performed to compare conditions within both groups. The effect of MIA on MSC adhesion to different OA tissues *in vivo* was determined by independent 2-tailed *t* tests. *P* values of ≤.05 were considered statistically significant. Analyses were performed using SPSS 21.0 (SPSS Inc).

RESULTS

Chemokines in the synovial fluid and secreted by OA tissues *in vitro*

To evaluate chemokine secretion by cartilage and synovium, we conducted a 10-plex Milliplex assay on CM of OA synovium and cartilage and on OA SF. The 10 selected factors were detected in all conditions (**table 3**). High levels of MCP-1 and IL-8 were secreted by synovium and cartilage. Furthermore, synovium secreted high amounts of MIP-1α and MIP-1β, whereas cartilage secreted high amounts of PDGF-AA and IP-10. The mRNA of the receptors for these chemokines was ex-

pressed in cultured MSCs. *CD44, PDGFR*α, and *PDGFR*β were highly expressed (CT ≤ 25); *CCR1, CCR4, CCR5, CXCR3*, and *CX3CR1* were intermediately expressed (CT = 26-36); and *CXCR1* was not expressed (CT ≥ 36).

Table 3
Chemokine secretion by osteoarthritic synovium and cartilage[α]

chemokine	OA synovium CM Mean (range)	OA cartilage CM Mean (range)	OA synovial fluid Mean (range)	Minimum DC (pg/ml)
MCP-1	31,753 (15,689 – 63,264)	3,499.4 (1,344.6 – 6,040.8)	3,276.6 (847.4 – 4,935.5)	1.9
IL-8	116,848 (63,634 – 197,030)	3,548.3 (114.0 – 11,826)	170.2 (27.4 – 463.5)	0.4
MIP-1α	309.1 (48.6 – 1,077.4)	12.7 (3.2 – 23.9)	7.6 (1.5 – 11.8)	2.9
MIP-1β	367.2 (73.4 – 718.0)	29.7 (5.8 – 57.1)	72.6 (24.8 – 197.1)	3.0
PDGF-AA	14.7 (4.9 – 32.2)	258.3 (115.1 – 513.5)	104.9 (26.7 – 229.0)	0.4
IP-10	139.1 (4.3 – 425.5)	1,014.0 (171.5 – 3,297.1)	4,661.8 (2,223.9 – 7,907.7)	8.6
MDC	74.7 (29.4 – 197.4)	146.4 (104.7 – 168.4)	234.8 (137.0 – 369.5)	3.6
MCP-3	27.7 (1.9 – 47.2)	6.7 (1.9 – 14.7)	66.4 (1.9 – 263.6)	3.8
PDGF-AB/BB	97.6 (1.1 – 318.9)	3.3 (1.1 – 6.1)	799.0 (417.4 – 1,706.2)	2.2
Fractalkine	434.8 (213.9 – 795.2)	19.8 (11.4 – 34.8)	111.6 (11.4 – 208.3)	22.7

α Chemokine secretion by osteoarthritic synovium and cartilage for 24 hours in medium and chemokine content in osteoarthritic synovial fluid in pg/ml. Undetectable and below-minimum detectable concentrations values were set at 50% of the minimum detectable concentration value. n = 6 donors.

Expression of migration and adhesion factors in MSCs is independent of serum used in culture

During culture, the MSCs are generally exposed to serum. Different laboratories use different batches of FCS. Moreover, the use of pooled batches of human PL has become increasingly popular. Serum could potentially influence cell behaviour and could make comparison between results obtained in different laboratories difficult. We evaluated the expression of known migration receptors *PDGFR*α*, PDGFR*β*, CCR1*, and *CCR4* and adhesion receptors *ITG*β*1, ITG*β*2*, and *CD44* in MSCs expanded in 3 different batches of FCS and a batch of pooled human PL. Different culture sera did not significantly affect the expression of migration receptors *CCR1, CCR4, PDGFR*α, and *PDGFR*β or the expression of the adhesion receptors *CD44* and *ITG*β*1* in MSCs (**figure 1**).

Influence of inflammation, hypoxia and OA synovial fluid on MSC migration/adhesion receptors

To evaluate whether a short treatment in culture could increase expression of migration or adhesion receptor genes in MSCs, we exposed the cells to the combination of inflammatory factors IFNγ/TNFα or cultured the cells in hypoxia (1% oxygen). Furthermore, we determined whether OA SF would influence expression

of the aforementioned genes. Exposure of MSCs to different concentrations of IFNγ/TNFα upregulated the expression of *CCR1, CCR4* and *CD44*, whereas *PDGFR*α and *PDGFR*β were downregulated by the inflammatory factors (**figure 2**). The other migration receptor genes, *CCR5, CXCR1, CXCR3, CXCR4* and *CX3CR1*, and the adhesion receptor genes, *ITG*β*1* and *ITG*β*2*, were not influenced by inflammatory factors (data not shown). Hypoxia did not have any effect on the gene expression

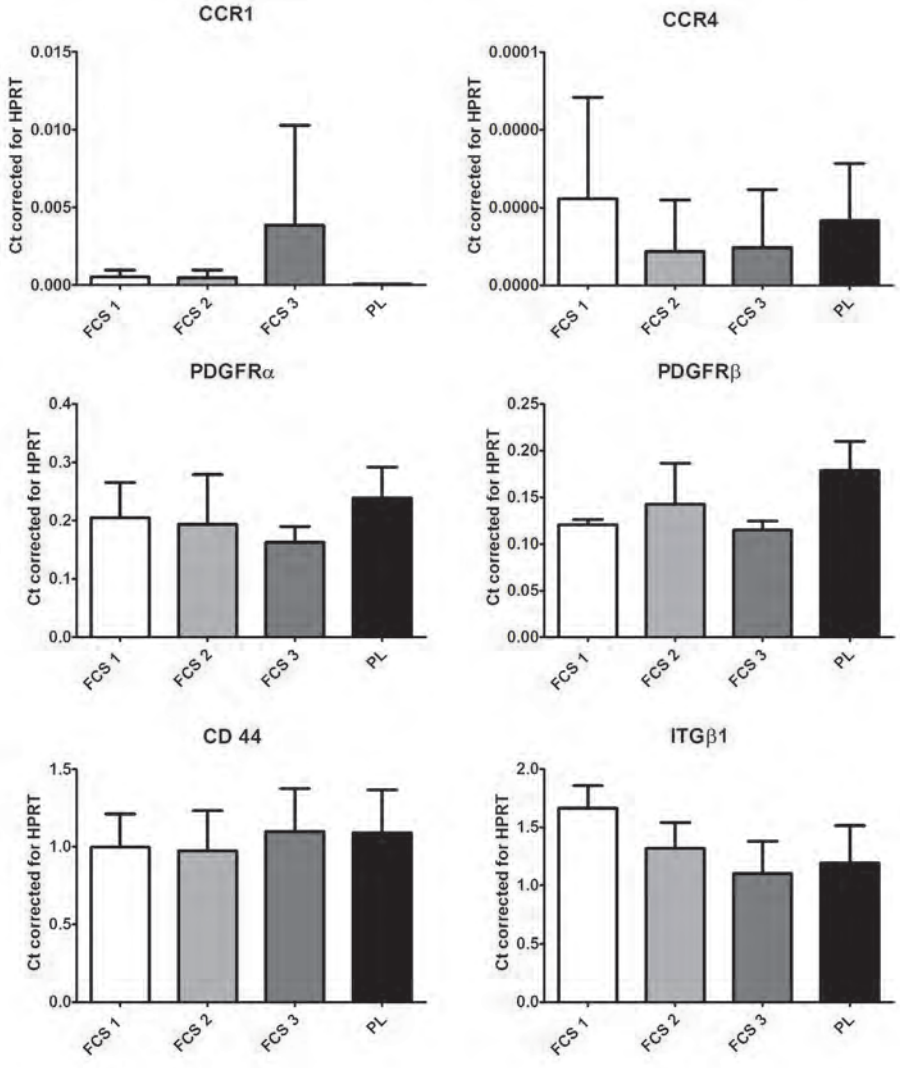


***Figure 1**. The expression of migration and adhesion receptors in MSCs is independent of culture serum. MSCs were cultured (n = 3 donors with triplicate samples for each donor) with 3 different batches of FCS in the expansion medium or with a batch of pooled human PL (n > 50 donors) in the expansion medium. Data are shown as mean ± SD.*

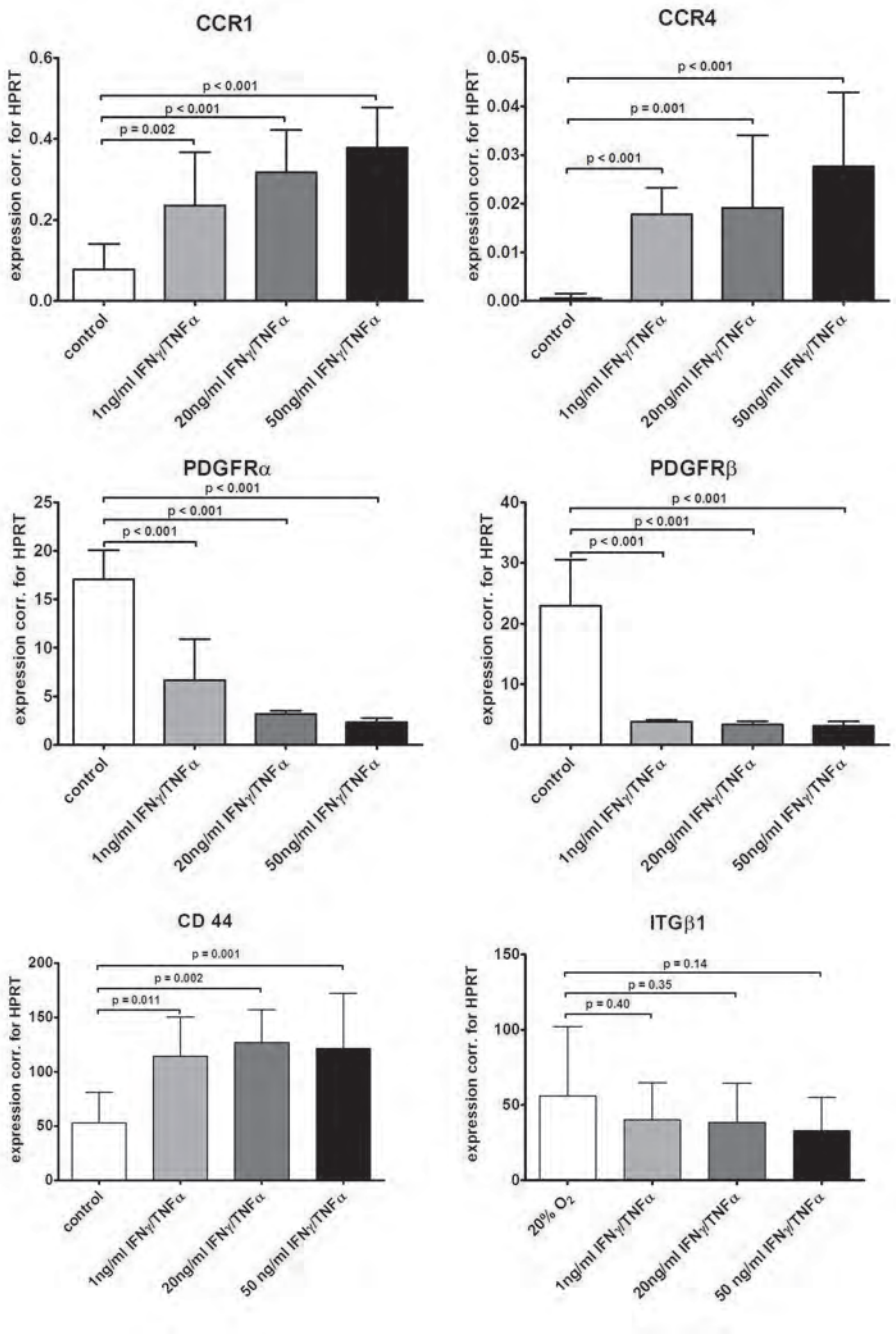


***Figure 2**. Gene expression of migration and adhesion receptors in MSCs after exposure to the combination of inflammatory factors IFNγ and TNFα for 24 hours. N = 3 MSC donors with triplicate samples for each donor. Data are shown as mean ± SD.*

of the migration receptors and adhesion receptors studied, either in the presence or absence of IFNγ/TNFα (**appendix figure A1**, available online). Finally, we observed an upregulation of gene expression of only *PDGFR*β (P =.006) in MSCs by OA SF (**appendix figure A2**).

MSC migration toward factors secreted by OA tissues

We evaluated whether MSCs would migrate to factors secreted by OA synovium or cartilage and whether this was influenced by pre-treatment of MSCs with the combination IFNγ/TNFα. MSCs migrated more toward OA synovium CM and OA cartilage CM compared with control medium for both nonactivated (P ≤.001) and activated MSCs (P <.001), indicating that OA cartilage and synovium secreted factors that stimulated MSC migration (**figure 3**). No significant effect of pre-treatment of MSCs was found (P =.060). Nonactivated MSCs migrated more toward synovium CM compared with cartilage CM (P =.031), and a comparable trend was observed for MSCs activated by inflammatory factors (P =.065).

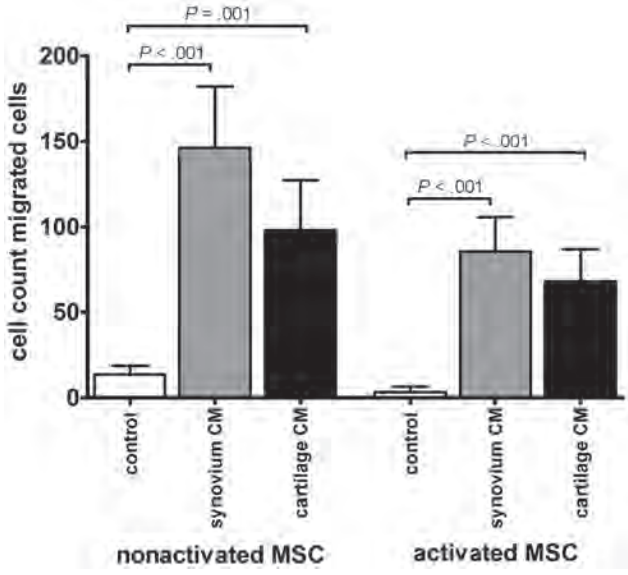


***Figure 3**. MSC migration toward factors secreted by OA synovium and OA cartilage in a transwell migration assay. Data are shown as mean ± SD. "Nonactivated" MSCs were cultured in normal expansion medium for 24 hours; "activated" MSCs were exposed to the combination of 50 ng/ml IFNγ/50 ng/ml TNFα in medium for 24 hours.*

In vivo adhesion of nonactivated MSCs and activated MSCs in healthy knees or OA knees

An *in vivo* experiment was performed to study MSC migration and attachment to synovium and cartilage. All animals used were wild-type male Wistar rats that were healthy before the study, and no side effects or health problems were found during the study. We evaluated whether there was a difference between activated and nonactivated MSCs in their migration and adherence patterns and whether this was influenced by the presence or absence of OA. MSCs were found attached to synovium or cartilage or were present as free cells in the joint space (**figure 4E**). Cartilage damage and synovial inflammation were clearly visible after OA induction by the MIA model (modified Pritzker score 20.4 ± 4.84) and were absent in control knees (modified Pritzker score 4.4 ± 1.27) (P <.001) (**figure 4A, 4B**). At 24 hours after injection, MSCs were present in all control and OA knees. MSCs were mostly found attached to synovium (semiquantitative score 3.13 ± 1.19) or located freely in the joint space (semiquantitative score 3.95 ± 1.25); this was approximately equally distributed based on our semiquantitative scoring system. Since very small amounts of cells were found attached to cartilage (semi- quantitative score 1.03 ± 0.11) (**figure 4D**), we evaluated only MSC attachment to synovium to compare healthy versus OA knees and to evaluate the effect of MSC pre-treatment with inflammatory factors. We could not detect significant differences in MSC attachment to synovium between OA and healthy knees or an effect of pre-treatment with inflammatory factors (**figure 4F, 4G**).

DISCUSSION

We investigated whether MSCs migrate to OA tissues and whether this migration could be influenced by adapting the culture conditions before application of MSCs. Both OA synovium and OA cartilage secreted substantial amounts of chemokines. The profile of the secreted chemokines varied between both tissues. We showed that MSCs express migration and adhesion receptor genes and migrate to factors secreted by both OA synovium and cartilage *in vitro*. *In vivo*, more MSCs attached to synovium than to cartilage. The expression of migration and adhesion receptors was altered by adding inflammatory factors during culture, although this did not influence migration *in vitro* or adhesion *in vivo*.

Throughout the world, laboratories use different batches of FCS to culture MSCs. Human PL is often used and is described to have a positive effect on proliferation and differentiation of MSCs[25]. However, contradictory results have been published

regarding the effect of culturing with PL on the migration/adhesion capacity of MSCs and the migration of MSCs toward PL[17,33]. We compared the effects of 3 different batches of FCS and a batch of pooled PL on gene expression of migration and adhesion receptors in MSCs. Our results indicate that different FCS batches or PL will not likely have a differential effect on migration and attachment of MSCs, such that the use of different sera in expansion culture is less likely to be a confounder for migration and adhesion in the comparison of different studies with intra-articularly injected MSCs.

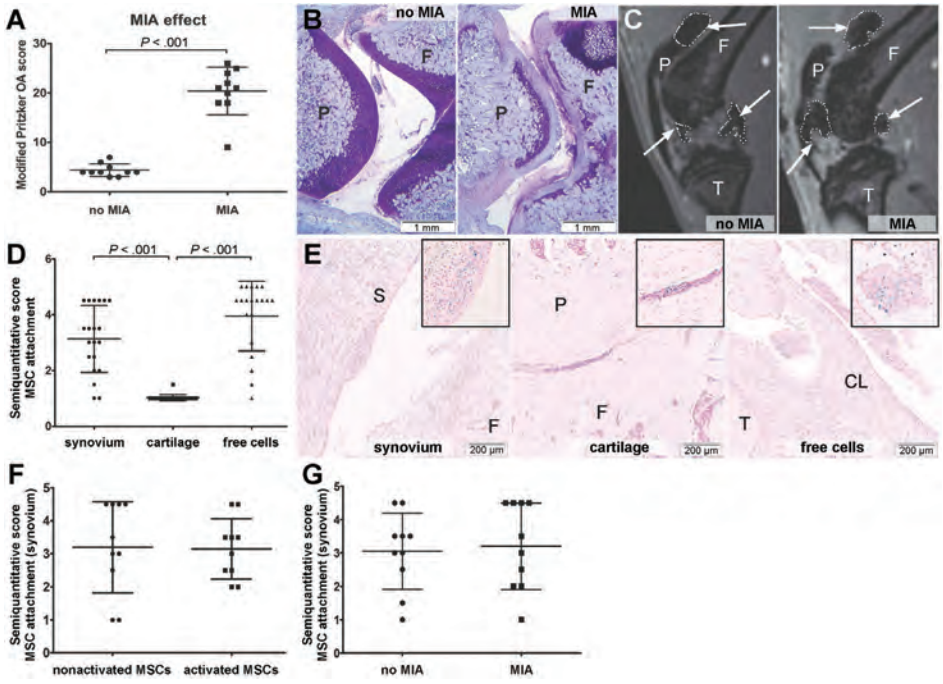


Figure 4*. In vivo adherence of nonactivated MSCs and activated MSCs in healthy knees or knees with OA. OA was bilaterally induced in rat knees (n = 10 knees) with an MIA injection. Healthy rat knees (n = 10 knees) were used as control. (**A**) Modified Pritzker OA score on histologic results of 20 rat knees. (**B**) Thionin staining on coronal knee histologic sections with a control knee (left side) and MIA-induced OA knee (right side). (**C**) Arrows indicate SPIO-labelled MSC on MRI in a control knee (left side) and OA knee (right side). (**D**) Semiquantitative score of MSC attachment on synovium and cartilage (control and OA knees combined). (**E**) Perls iron staining on coronal knee histologic results was performed to localize intra-articularly injected MSCs. From left to right: histological samples of MSCs attached to synovium, cartilage, and free cells in the joint space. The upper right corner shows an enlargement of the attached cells. (**F**) Comparison between the attachment of activated and nonactivated MSCs to synovium in all knees. (**G**) Difference between control and OA knees in the migration of MSCs toward synovium. Data are shown as mean ± SD. CL, cruciate ligament; F, femur; P, patella; T, tibia; S, synovium. "Nonactivated" MSCs were cultured in normal expansion medium for 24 hours; "activated" MSCs were exposed to the combination of 50 ng/ml IFNγ/50 ng/ml TNFα in medium for 24 hours.*

Next to the capacities of MSCs to differentiate and regenerate damaged tissue, their immunomodulatory and trophic properties are very important for their therapeutic function in OA. MSCs need to be activated with inflammatory factors like IFNγ and TNFα to secrete immunomodulatory and trophic factors[15,28,38,41]. This immunomodulatory and trophic capacity is maintained under hypoxic conditions[30,40], which is important because MSCs used as cell therapy for OA will be injected in a hypoxic joint environment. We showed no effect of hypoxia on the expression of migration or adhesion receptor genes in MSCs. It is not clear what the levels of hypoxia are in joints, but for culture, hypoxia levels range from 1% to 5% oxygen. We chose 1% oxygen based on previous studies and experiments in our laboratory[10,40]. A different oxygen level might have different effects. It is known that inflammatory factors can upregulate chemokine receptors in MSCs *in vitro*[16,18,35,37]. We hypothesized that these inflammatory factors could also improve the migration and adhesion of MSCs to OA tissues.

Indeed, exposure to a combination of inflammatory factors IFNγ and TNFα upregulated gene expression of *CCR1* and *CCR4*, although *PDGFR*α and *PDGFR*β were downregulated. Chemokines that bind to CCR1 and CCR4 (ie, MIP-1α, MIP-1β, MCP-1, MCP-3) were found in a higher concentration in synovium CM compared with cartilage CM. These chemokines can increase MSC migration[7]. Since the receptors that bind these chemokines are upregulated in MSCs by pre-treatment with IFNγ/TNFα, we would expect better attachment and migration to synovium. Nevertheless, this was not confirmed *in vitro* nor *in vivo*, where cell pre-treatment with IFNγ and TNFα did not improve functional migration of MSCs to synovium. PDGFβ, a chemokine that binds to PDGFRβ and indirectly interacts with PDGFRα, was more abundantly found in cartilage CM. There was less MSC attachment and migration to cartilage, although pre-treatment with IFNγ and TNFα did not decrease the cell attachment and migration. These results are in line with a recent report on MSC homing in a mouse hypoxic gut model[26]. We cannot exclude that longer or shorter exposure to culture conditions or different concentrations of inflammatory factors or oxygen might have had an effect. In our study, the absolute migration was even slightly decreased *in vitro*, although the activated cells still migrated toward factors secreted by OA tissues. We excluded that cell death due to treatment with the combination of 50 ng/mL IFNγ/50 ng/mL TNFα could explain the decreased absolute migration found, since exposure to these factors did not decrease cell survival after 24 hours. However, we have not evaluated whether the immunomodulatory properties of MSCs pre-treated with IFNγ/TNFα were indeed increased *in vivo*. This will need careful consideration before future applications,

since Kavanagh et al[26] recently found a decreased vasculoprotective effect *in vivo* of MSCs pre-treated with IFNγ or TNFα.

There are several limitations to our study. We used human MSCs from relatively aged OA donors who underwent total hip arthroplasty. Although these MSCs might be considered less potent *in vivo* compared with rat MSC from young donors, we consider this cell type to be more clinically relevant, being better predictive of expected results in future clinical application. We used cartilage and synovium of 6 different donors and MSCs of 3 donors. Donor variability should always be taken into account. The level and the variation we have seen in the chemokine data are comparable to the variation we have seen in previous studies[6,9]. The effect of inflammatory factors or hypoxia on chemokine and adhesion receptor expression was not large on all MSC donors. Therefore, we consider our conclusions valid. Furthermore, cells were labelled using SPIO for tracking purposes by MRI and histologic evaluation. SPIO labelling could have an effect on cell migration and adhesion capacities[12,52]. Although in previous studies we did not find effects of our labelling procedure on cell viability, differentiation, and secretion of immunomodulatory factors[47], and we confirmed that the cells were viable and adherent when cultured on tissue culture plastic, we cannot completely rule out an effect. Migration and adherence of the cells were evaluated 24 hours after injection. These processes are expected to take place in the first 24 hours. We realize that this time window is not sufficient to study the many processes that might happen afterward, such as cell proliferation, cell invasion into tissues, and cell assume that cells will not attach when they have died during injection or during the first hours after injection. The attached cells were scored on histologic evaluation by a semiquantitative score, which can be not accurate enough to quantify eventual differences between the groups.

MSCs migrated more toward factors secreted by OA synovium compared with cartilage. In line with this, more cells attached to synovium than to cartilage *in vivo*. This could be explained by both chemoattractive and mechanical processes. Overall, chemotactic factors were found in a higher concentration in synovial CM compared with cartilage CM. Care should be taken, however, to directly compare the levels of factors secreted by synovium and cartilage in our studies. Although synovium CM and cartilage CM were made in a standardized way, based on wet weight of tissue per volume, synovial tissue contains substantially more cells per gram of wet weight than cartilage tissue. Nevertheless, we can conclude that both synovium and cartilage secrete high amounts of chemokines, and the profile of the secreted chemokines seems to differ between synovium and cartilage. The factors

secreted by cartilage and synovium were also found in synovial fluid. In a previous study we found that the levels of MDC, PDGF-AA, IP-10, CCL5, and MIP-1β were higher in OA than in control SF[6], which would indicate higher chemotactic effects of osteoarthritic tissues. Nevertheless, no differences in migration or adhesion were seen between rat knees with and without OA. The synovial fluid did not affect the receptor gene expression in MSCs (**appendix figure A2**), but we did not evaluate whether the secreted factors individually could affect the gene expression or function of MSCs. Another explanation for better adhesion of MSCs to synovium compared with cartilage could be the continuous movements of the joint that might evoke shear stresses that MSCs cannot resist, especially those that primarily attached to cartilage. Furthermore, cells are more likely to attach to a rough surface like synovium than to a smooth surface like cartilage. Nevertheless, in our OA model with marked cartilage damage, no improvement of cell adhesion to cartilage was seen. We assume that intra- articular cell attachment is influenced by a combination of chemoattractive processes, tissue surface characteristics and biomechanics.

CONCLUSION

Intra-articular tissues affected by OA secrete chemotactic factors that can attract MSCs. Exposure to inflammatory factors influenced MSC gene expression of several chemokine receptors, although these inflammatory factors did not affect the migration of MSCs *in vitro* or MSC attachment *in vivo*.

REFERENCES

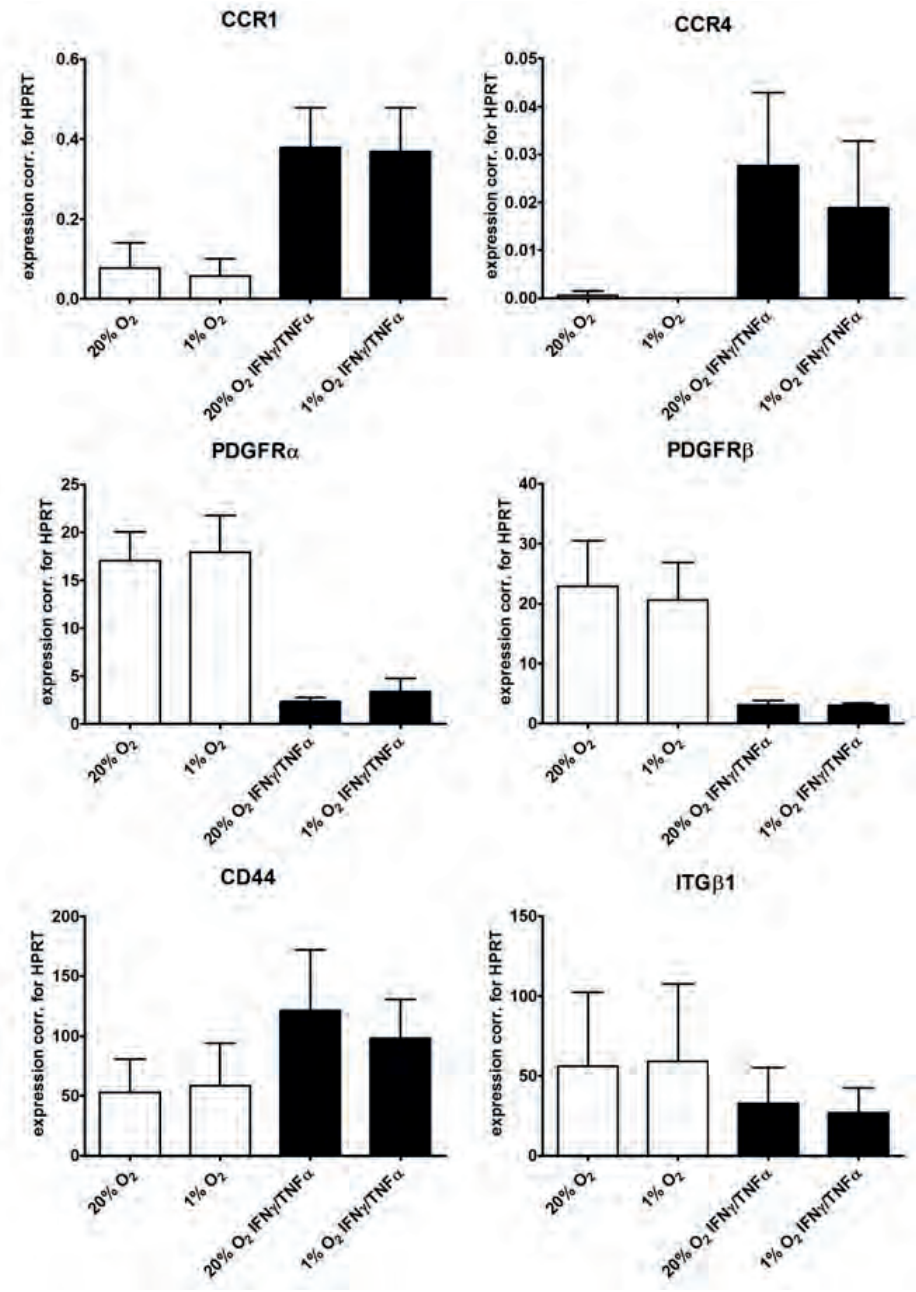
1. Agung M, Ochi M, Yanada S, et al. Mobilization of bone marrow-derived mesenchymal stem cells into the injured tissues after intraarticular injection and their contribution to tissue regeneration. *Knee Surg Sports Traumatol Arthrosc.* 2006;14(12):1307-1314.
2. Ajuied A, Wong F, Smith C, et al. Anterior cruciate ligament injury and radiologic progression of knee osteoarthritis: a systematic review and meta-analysis. *Am J Sports Med.* 2014;42(9):2242-2252.
3. Augello A, Kurth TB, De Bari C. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. *Eur Cell Mater.* 2010;20:121-133.
4. Barry F, Murphy M. Mesenchymal stem cells in joint disease and repair. *Nat Rev Rheumatol.* 2013;9(10):584-594.
5. Beekhuizen M, Bastiaansen-Jenniskens YM, Koevoet W, et al. Osteoarthritic synovial tissue inhibition of proteoglycan production in human osteoarthritic knee cartilage: establishment and characterization of a long-term cartilage-synovium coculture. *Arthritis Rheum.* 2011;63(7):1918-1927.
6. Beekhuizen M, Gierman LM, van Spil WE, et al. An explorative study comparing levels of soluble mediators in control and osteoarthritic synovial fluid. *Osteoarthritis Cartilage.* 2013;21(7):918-922.
7. Boomsma RA, Geenen DL. Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. *PLoS One.* 2012;7(4):e35685.
8. Caplan AI. Why are MSCs therapeutic? New data: new insight. *J Pathol.* 2009;217(2):318-324.
9. Clockaerts S, Bastiaansen-Jenniskens YM, Feijt C, et al. Cytokine production by infrapatellar fat pad can be stimulated by interleukin 1beta and inhibited by peroxisome proliferator activated receptor alpha agonist. *Ann Rheum Dis.* 2012;71(6):1012-1018.
10. Das R, Jahr H, van Osch GJ, Farrell E. The role of hypoxia in bone mar- row-derived mesenchymal stem cells: considerations for regenerative medicine approaches. *Tissue Eng Part B Rev.* 2010;16(2):159-168.
11. de Witte SF, Franquesa M, Baan CC, Hoogduijn MJ. Toward development of mesenchymal stem cells for immunomodulatory therapy. *Front Immunol.* 2015;6:648.
12. Diana V, Bossolasco P, Moscatelli D, Silani V, Cova L. Dose dependent side effect of superparamagnetic iron oxide nanoparticle labeling on cell motility in two fetal stem cell populations. *PLoS One.* 2013;8(11):e78435.
13. Docheva D, Popov C, Mutschler W, Schieker M. Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system. *J Cell Mol Med.* 2007;11(1):21-38.
14. Dong F, Harvey J, Finan A, Weber K, Agarwal U, Penn MS. Myocardial CXCR4 expression is required for mesenchymal stem cell mediated repair following acute myocardial infarction. *Circulation.* 2012;126(3):314-324.
15. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett.* 2007;110(2):91-100.
16. Eseonu OI, De Bari C. Homing of mesenchymal stem cells: mechanistic or stochastic? Implications for targeted delivery in arthritis. *Rheumatology (Oxford).* 2015;54(2):210-218.

17. Goedecke A, Wobus M, Krech M, et al. Differential effect of platelet- rich plasma and fetal calf serum on bone marrow-derived human mesenchymal stromal cells expanded in vitro. *J Tissue Eng Regen Med.* 2011;5(8):648-654.
18. Hemeda H, Jakob M, Ludwig AK, Giebel B, Lang S, Brandau S. Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine expression and migration properties of mesenchymal stem cells. *Stem Cells Dev.* 2010;19(5):693-706.
19. Honczarenko M, Le Y, Swierkowski M, Ghiran I, Glodek AM, Silberstein LE. Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. *Stem Cells.* 2006; 24(4):1030-1041.
20. Horie M, Sekiya I, Muneta T, et al. Intra-articular Injected synovial stem cells differentiate into meniscal cells directly and promote meniscal regeneration without mobilization to distant organs in rat massive meniscal defect. *Stem Cells.* 2009;27(4):878-887.
21. Huang J, Zhang Z, Guo J, et al. Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. *Circ Res.* 2010;106(11):1753-1762.
22. Hung SC, Pochampally RR, Hsu SC, et al. Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment in vivo. *PLoS One.* 2007;2(5):e416.
23. Ip JE, Wu Y, Huang J, Zhang L, Pratt RE, Dzau VJ. Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. *Mol Biol Cell.* 2007;18(8):2873-2882.
24. Ji JF, He BP, Dheen ST, Tay SS. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells.* 2004;22(3):415-427.
25. Jonsdottir-Buch SM, Sigurgrimsdottir H, Lieder R, Sigurjonsson OE. Expired and pathogen inactivated platelet concentrates support differentiation and immunomodulation of mesenchymal stromal cells in culture. *Cell Transplant.* 2015;24(8):1545-1554.
26. Kavanagh DP, Suresh S, Newsome PN, Frampton J, Kalia N. Pre-treatment of mesenchymal stem cells manipulates their vasculoprotective potential while not altering their homing within the injured gut. *Stem Cells.* 2015;33(9):2785-2797.
27. Kim YS, Kwon OR, Choi YJ, Suh DS, Heo DB, Koh YG. Comparative matched-pair analysis of the injection versus implantation of mesenchymal stem cells for knee osteoarthritis. *Am J Sports Med.* 2015;43(11):2738-2746.
28. Krampera M, Cosmi L, Angeli R, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells.* 2006;24(2):386-398.
29. Leijs MJ, van Buul GM, Lubberts E, et al. Effect of arthritic synovial fluids on the expression of immunomodulatory factors by mesenchymal stem cells: an explorative in vitro study. *Front Immunol.* 2012;3:231.
30. Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR. Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. *Cell Transplant.* 2010;19(1):29-42.
31. Mathieu E, Lamirault G, Toquet C, et al. Intramyocardial delivery of mesenchymal stem cell-seeded hydrogel preserves cardiac function and attenuates ventricular remodeling after myocardial infarction. *PLoS One.* 2012;7(12):e51991.
32. Murphy JM, Fink DJ, Hunziker EB, Barry FP. Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum.* 2003;48(12): 3464-3474.

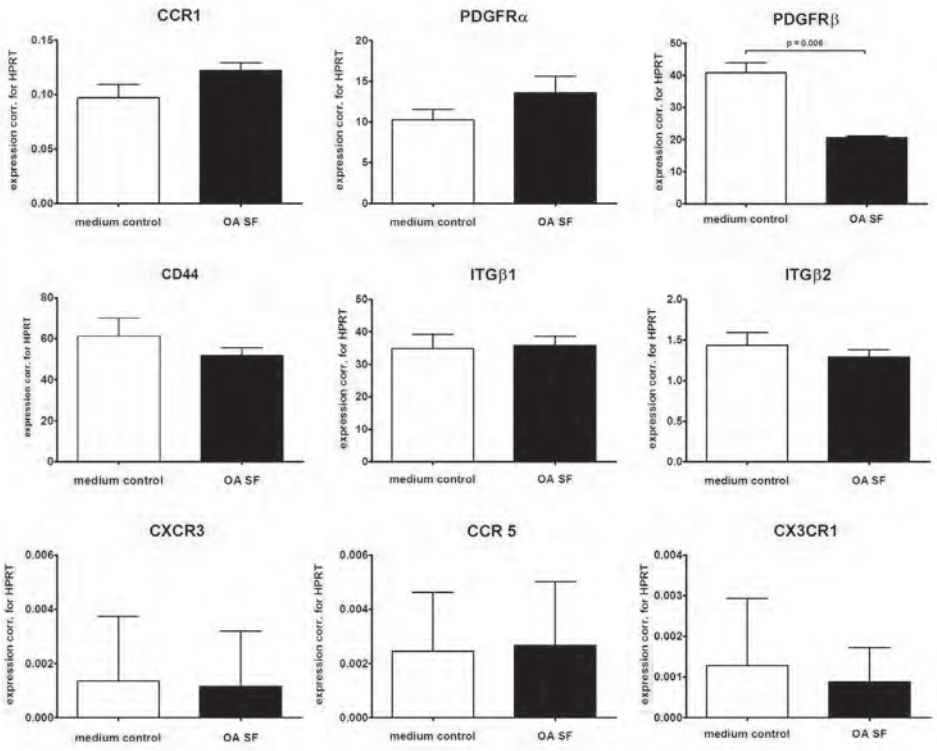
33. Naaijkens BA, Niessen HW, Prins HJ, et al. Human platelet lysate as a fetal bovine serum substitute improves human adipose-derived stromal cell culture for future cardiac repair applications. *Cell Tissue Res.* 2012;348(1):119-130.
34. Peeters CM, Leijs MJ, Reijman M, van Osch GJ, Bos PK. Safety of intra-articular cell-therapy with culture-expanded stem cells in humans: a systematic literature review. *Osteoarthritis Cartilage.* 2013;21(10):1465-1473.
35. Ponte AL, Marais E, Gallay N, et al. The in vitro migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. *Stem Cells.* 2007;25(7):1737-1745.
36. Pritzker KP, Gay S, Jimenez SA, et al. Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage.* 2006; 14(1):13-29.
37. Ren G, Roberts AI, Shi Y. Adhesion molecules: key players in mesenchymal stem cell-mediated immunosuppression. *Cell Adh Migr.* 2011;5(1):20-22.
38. Ren G, Zhang L, Zhao X, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell.* 2008;2(2):141-150.
39. Ringe J, Strassburg S, Neumann K, et al. Towards in situ tissue repair: human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. *J Cell Biochem.* 2007;101(1):135-146.
40. Roemeling-van Rhijn M, Mensah FK, Korevaar SS, et al. Effects of hypoxia on the immunomodulatory properties of adipose tissue derived mesenchymal stem cells. *Front Immunol.* 2013;4: 203.
41. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin Exp Immunol.* 2007;149(2):353-363.
42. Siebelt M, Waarsing JH, Kops N, et al. Quantifying osteoarthritic cartilage changes accurately using in vivo microCT arthrography in three etiologically distinct rat models. *J Orthop Res.* 2011;29(11):1788- 1794.
43. Sordi V, Malosio ML, Marchesi F, et al. Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood.* 2005;106(2):419-427.
44. ter Huurne M, Schelbergen R, Blattes R, et al. Antiinflammatory and chondroprotective effects of intraarticular injection of adipose- derived stem cells in experimental osteoarthritis. *Arthritis Rheum.* 2012;64(11):3604-3613.
45. van Buul GM, Farrell E, Kops N, et al. Ferumoxides-protamine sulfate is more effective than ferucarbotran for cell labeling: implications for clinically applicable cell tracking using MRI. *Contrast Media Mol Imaging.* 2009;4(5):230-236.
46. van Buul GM, Siebelt M, Leijs MJ, et al. Mesenchymal stem cells reduce pain but not degenerative changes in a mono-iodoacetate rat model of osteoarthritis. *J Orthop Res.* 2014;32(9):1167-1174.
47. van Buul GM, Villafuertes E, Bos PK, et al. Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. *Osteoarthritis Cartilage.* 2012;20(10):1186-1196.
48. Vega A, Martin-Ferrero MA, Del Canto F, et al. Treatment of knee osteoarthritis with allogeneic bone marrow mesenchymal stem cells: a randomized controlled trial. *Transplantation.* 2015;99(8): 1681-1690.

49. Wang T, Jiang XJ, Tang QZ, et al. Bone marrow stem cells implantation with alpha-cyclodextrin/MPEG-PCL-MPEG hydrogel improves cardiac function after myocardial infarction. *Acta Biomater.* 2009;5(8):2939-2944.
50. Whittaker JL, Woodhouse LJ, Nettel-Aguirre A, Emery CA. Outcomes associated with early post-traumatic osteoarthritis and other negative health consequences 3-10 years following knee joint injury in youth sport. *Osteoarthritis Cartilage.* 2015;23(7):1122-1129.
51. Wu Y, Zhao RC. The role of chemokines in mesenchymal stem cell homing to myocardium. *Stem Cell Rev.* 2012;8(1):243-250.
52. Yang JX, Tang WL, Wang XX. Superparamagnetic iron oxide nanoparticles may affect endothelial progenitor cell migration ability and adhesion capacity. *Cytotherapy.* 2010;12(2):251-259.
53. Zhang X, Wang H, Ma X, et al. Preservation of the cardiac function in infarcted rat hearts by the transplantation of adipose-derived stem cells with injectable fibrin scaffolds. *Exp Biol Med (Maywood).* 2010;235(12):1505-1515.
54. Zwolanek D, Flicker M, Kirstatter E, Zaucke F, van Osch GJ, Erben RG. Beta1 integrins mediate attachment of mesenchymal stem cells to cartilage lesions. *Biores Open Access.* 2015;4(1):39-53.

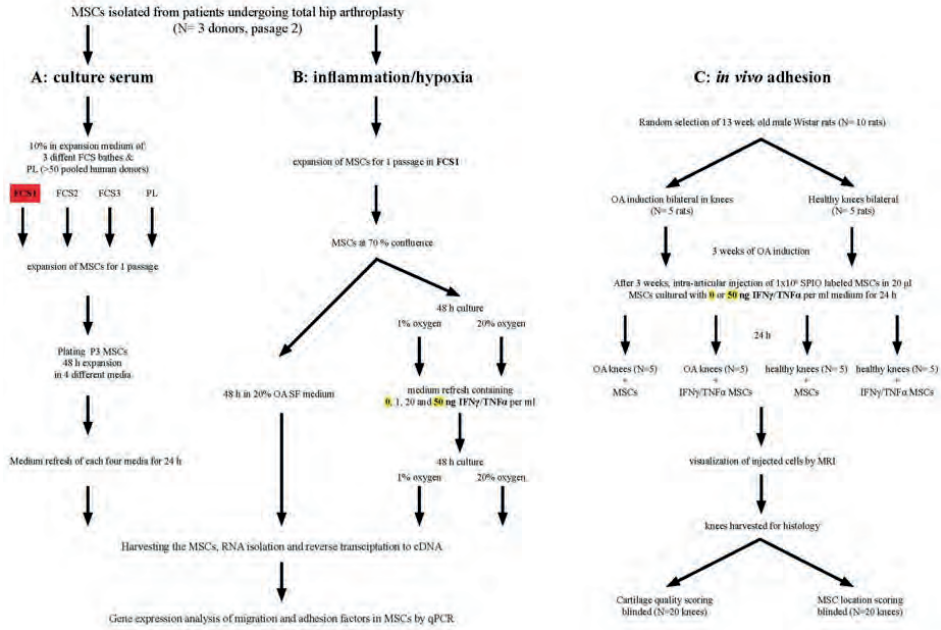
SUPPLEMENTARY DATA



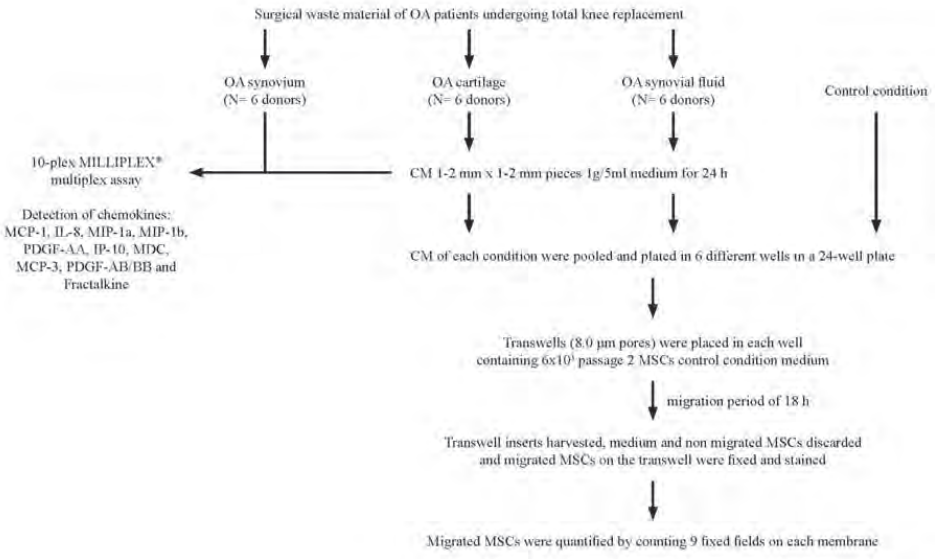
***Supplementary figure A1**. MSC culture under hypoxia and the effect on expression of migration and adhesion receptors. MSCs (N=3 donors with triplicate samples for each donor) were cultured for 48 h under 20 % of oxygen (normoxic) or 1 % of oxygen (hypoxic) conditions with additional 24 h culturing in combination with or without 50 ng/ml of IFNγ/TNFα. Data is shown as mean ± SD.*



***Supplementary figure A2**. MSC culture with OA SF and the effect on expression of migration and adhesion receptors. MSCs (N=3 donors) were cultured for 48 h with 20% OA SF in serum free medium. Data shown as mean ± SD.*



Supplementary figure A3. *Schematic overview of the methods used. (**A**) The effect of different culture sera on the expression of migration and adhesion factors in MSCs. The FCS marked in red is the serum we used in the rest of our experiments. (**B**) The effect of MSC pre-treatment by inflammatory factors and hypoxia on the expression of migration and adhesion factors in MSCs. The yellow marked concentrations of IFNγ/TNFα were used in the other experiments. (**C**) Schematic overview of the in vivo adhesion experiment.*



***Supplementary figure A4**. Schematic overview of the methods used for chemokine detection in conditioned medium of different OA tissues and the methods used to evaluate migration of MSCs towards the secreted factors of the different OA tissues.*

Chapter 5

Encapsulation of allogeneic mesenchymal stem cells in alginate extends local presence and therapeutic function

Eur Cell Mater. 2017 Jan 30;33: 43-58

Maarten J.C. Leijs
Esther Villafuertes
Joost C. Haeck
Wendy J.L.M. Koevoet
Benjamin Fernandez-Gutierrez
Martin J. Hoogduijn
Jan A.N. Verhaar
Monique R. Bernsen
Gerben M. van Buul
Gerjo J.V.M. van Osch

ABSTRACT

Bone marrow derived mesenchymal stem cells (MSCs) have immunomodulatory and trophic capacities. For therapeutic application in local chronic inflammatory diseases, MSCs, preferably of allogeneic origin, have to retain immunomodulatory properties. This might be achieved by encapsulation of MSCs in a biomaterial that protects them from the host immune system. Most studies investigating the properties of MSCs for therapeutic application use short term cultures of cells in monolayer. Since the physical environment of MSCs can influence their functionality, we evaluated the feasibility of preserving the immunomodulatory properties of MSCs encapsulated in a three-dimensional alginate construct.

After 5 weeks of implantation in immunocompetent rats, active allogeneic MSCs encapsulated in alginate were still detectable by Bio Luminescence Imaging and Magnetic Resonance Imaging of luciferase transduced and superparamagnetic iron oxide labelled MSCs. MSCs injected in saline were only detectable up to 1 week after injection. Moreover, the MSCs encapsulated in alginate responded to inflammatory stimuli similarly to MSCs in monolayer culture. In addition, MSC-alginate beads secreted immunomodulatory and trophic factors and inhibited T cell proliferation after 30 d of *in vitro* culture. Our data indicate that allogeneic MSCs encapsulated in alginate persist locally and could act as an interactive immunomodulatory or trophic factor release system for several weeks, making this an interesting system to investigate for application in inflammatory disease conditions.

Keywords: Mesenchymal stem cells, alginate, construct, encapsulation, cell therapy, immunomodulation.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent cells that can be found in several tissues such as bone marrow, adipose tissue, synovium, deciduous teeth, umbilical cord blood and blood vessels[1-4]. As they are promising candidates for cell therapy, they are used as therapeutic agents in experimental models of tissue diseases such as osteoarthritis (OA), interstitial lung diseases, glomerulonephritis, graft versus host disease (GvHD) and myocardial infarction[5-10]. MSCs are a promising cell type for therapy, because they have the potential to differentiate into repair tissue and also have trophic and immunomodulatory capacities. While they have been shown to be capable of improving damaged tissue, their contribution does not seem to originate from long-term engraftment and differentiation[11,12]. This suggests that MSCs can also stimulate endogenous tissue repair, in addition to their ability to differentiate into cells of the mesoderm lineage. The immunomodulatory property of MSCs is very useful when inflammation is a major contributor to the pathophysiology such as OA[13], rheumatoid arthritis[14] or GvHD[6]. Immune suppression by MSCs needs to be induced by pro-inflammatory cytokines such as interferon (IFN)γ, tumour necrosis factor (TNF)α, interleukin (IL)-1α or IL-1β[15-17]. *In vitro*, MSCs can inhibit B cell derived antibody production[18], generation and function of antigen presenting cells[19] or T lymphocyte proliferation and pro-inflammatory cytokine production[20,21]. *In vivo*, MSCs were able to reduce tissue degradation and inflammation in OA[13], reduce immune activity in autoimmune enteropathy[22], prolong heart and skin allograft survival[23,24] and improve experimental colitis[25].

The mechanisms responsible for MSCs immune modulation have been acknowledged to be based on paracrine activity. Although it is not precisely known which factors cause the effects, it was demonstrated that prostaglandin E2 (PGE2) production, indoleamine 2,3-dioxygenase (IDO) activity, suppression of nitric oxide (NO) production and secretion of cytokines such as IL-6[19,26,27] can be (partly) responsible. In addition, growth factors such as transforming growth factor β1 (TGF-β1) and vascular endothelial growth factor (VEGF)[28], secreted by MSCs, have been shown to influence tissue repair and immunological processes[29]. Most studies have considered the immunomodulatory properties of MSCs in 2-dimensional (2D) monolayer culture[30], as cell expansion is necessary to obtain the cell numbers required for therapeutic applications. MSCs in monolayer can differ considerably in morphology, cell adhesion, cell cycle and differentiation from those in 3-dimensional (3D) environments *in vitro* or *in vivo*[31-36]. Most of these studies performed short-term 3D experiments. However, for clinical applications, where MSCs therapy is aimed at modulating chronic inflammatory reactions, their

immunomodulatory properties have to be guaranteed for at least several weeks in order to improve the pathology. The use of alginate might be interesting to retain cells at the desired location. Alginate, a natural polysaccharide isolated from brown seaweed, is the most common used gel for cell encapsulation due to its biocompatibility and stability *in vivo*[37-40]. Moreover, alginate has the capacity to protect encapsulated cells against recognition by the immune system[31,41-44], which will enable the use of allogeneic cells. This would greatly enhance the clinical translatability of MSCs-based therapies.

In this study using longitudinal imaging, we evaluated whether encapsulation in alginate would prolong the local presence of allogeneic MSCs in an immunocompetent rat and if the cells would maintain their immunomodulatory and trophic function for a prolonged period after encapsulation.

MATERIALS AND METHODS

In vivo evaluation of MSCs activity and localisation after encapsulation in alginate

Isolation, encapsulation and culture of MSCs

Animal experiments were performed with prior approval of the ethics committee for laboratory animal use (protocol # EMC116-12-07,5,1).

MSC-alginate mix was polymerised in 102 mM $CaCl_2$ and washed two times in saline. Constructs were subcutaneously implanted on the back of sixteen weeks old immunocompetent male Wistar rats (Harlan Netherlands BV, Horst, The Netherlands). Allogeneic bone marrow MSCs from F344 rats (purchased from Millipore, Billerica, MA, USA) and xenogeneic human bone marrow MSCs were used. Human bone marrow derived MSCs were isolated by means of heparinised femoral shaft marrow aspirate from patients undergoing a total hip arthroplasty (after written informed consent with approval of the Medical Ethical Committee of Erasmus MC, protocol #MEC-2004-142). Bone marrow aspirates were plated in low glucose culture medium (DMEM, Dulbecco's Modified Eagle's Medium; Gibco, Carlsbad, CA, USA) with heat inactivated 15 % foetal calf serum (FCS; selected batch Lonza, Verviers, Belgium), 50 µg/ml gentamycin (Invitrogen, Carlsbad, CA, USA), 1.5 µg/ml Fungizone (Invitrogen), 1 ng/ml FGF2 (Instruchemie B.V., Delfzijl, The Netherlands), 0.1 mM vitamin C (Sigma, St. Louis, MO, USA) and after 24 h non-adherent cells were removed by washing with 2 % FCS in PBS. Adherent cells were cultured and upon passaging seeded at a density of 2,300 MSCs/cm^2 and

trypsinised (Invitrogen) at sub-confluence. MSCs from the third to fourth passage were used for experiments.

Activity evaluation of long-term encapsulated allogeneic MSCs in vivo

To evaluate the retention of viable, encapsulated allogeneic MSCs *in vivo*, we performed bioluminescence imaging (BLI) of luciferase transduced F344 MSCs (Fluc-MSCs) as described before[45] (Guenoun *et al.*, 2013). 3D MSC-alginate constructs with 4×10^6 Fluc-MSCs/ml filter sterilised 1.2 % low viscosity alginate (Keltone LV, Kelco, Surrey, UK) were prepared. The MSC-alginate mix was polymerised in a sterilised, custom-designed mould consisting of two Durapore membranes (5 µm pore size; Millipore) at both sides of a 3 mm thick metal ring (Wong *et al.*, 2001). The final cylindrical constructs of 8 mm diameter, containing a volume of approximately 151 µll and an estimated cell number of 6×10^5 MSCs were made with sterile dermal punches (8 mm; Spengler, Hannover, Germany). We compared subcutaneous implantation of six allogeneic MSC-alginate constructs in one rat with six subcutaneous injections of 6×10^5 allogeneic MSCs in physiological saline in another rat. The subcutaneous immunocompetent rat model is well accepted to study biocompatibility and tissue reactions. Moreover, it allows reliable longitudinal imaging of luminescence and iron oxide particles. Longitudinal cell viability was measured by luciferase activity of the transplanted and injected MSCs 30 min after intraperitoneal (i.p.) injection of 100 µg/kg of D-luciferine (Promega Benelux B.V., Leiden, The Netherlands) using the Xenogen IVIS spectrum (Caliper LS, Hopkington, MA, USA) for an emission detection time of 10 min. Sensitivity of our BLI was evaluated by BLI signal measurements of different amounts of Fluc-MSCs *in vitro* (data not shown). Minimum detection limit was 50,000 cells/well in monolayer in a 48-well plate. *In vivo* the optimal BLI signal over time was evaluated by repeated 10 min imaging after admission of D-luciferine up to 1 h. Optimal bioluminescence signal detection with BLI was 30 min after admission of D-luciferine. BLI signal increased the first 30 min after D-luciferine admission; after 30 min the signal remained constant up to at least 1 h. Best results were obtained with 10 min BLI measurements (integration time 600 s; f/stop 1; binning medium; FOV C). *In vivo* scans were performed 1 d after implantation and weekly thereafter for a total of 5 weeks. Optical intensity is reported as arbitrary units. Data were analysed using the Living Image version 3.2 software (Caliper LS).

Localisation of long-term encapsulated allogeneic MSCs in vivo

To localise the allogeneic MSCs precisely, MSCs were labelled with superparamagnetic iron oxide (SPIO) 1 d prior to injection/implantation by using ferumoxides 100 µg/ ml medium (Endorem™, Guerbet S.A., Paris, France) complexed to prot-

amine sulphate 5 µg/ml medium (LEO Pharma N.V., Wilrijk, Belgium) as described previously[46]. Magnetic resonance imaging (MRI) was performed directly after the cell implantation/injection to confirm the subcutaneous localisation of the SPIO-Fluc-MSCs. MR imaging was performed on a preclinical 7.0T MRI scanner (MR 901 Discovery, Agilent/GE Healthcare, Milwaukee, WI, USA) equipped with a 72 mm transmit/receive body coil. A fast spoiled gradient echo sequence was performed with the following settings: TE/ TR = 1.1/7.3 ms, NEX = 4, FOV = 8 × 6 cm^2, acquisition matrix = 256 × 192, slice thickness = 1 mm, bandwidth= 60 kHz, flip angle = 150°. Sagittal and coronal scans were performed to localise the hypo-intense SPIO deposits.

Histological evaluation of the implanted allogeneic MSC-alginate constructs

5 weeks after implantation/injection, rats were euthanised directly after the last scans. The subcutaneous layer of the back of the rats, containing all transplanted/injected regions, was harvested, separated and scanned individually in the MRI to localise the correct injection/transplantation regions using the SPIO signal. Samples were fixed in 0.05 M TRIS buffered saline with 10 % formalin and 15 mM $CaCl_2$ for 24 h. All samples were embedded in paraffin. Sections of 6 µm were sliced and deparaffinised before staining. Perl's iron staining (Klinipath BVBA, Duiven, The Netherlands) was performed, according to the manufacturer's protocol, to locate the SPIO-labelled MSCs. Perl's iron staining, stained iron particles blue. CD68 staining was performed on the same sections to identify macrophages. Antigen retrieval for CD68 was performed through incubation in citrate buffer (10 mM citric acid, 0.05 % Tween 20, pH 6.0) for 20 min at 90-95 °C. Sections were incubated for 1 h with primary antibodies for CD68 (#BM4000; OriGene Europe-Acris Antibodies, Herford, Germany) diluted to a concentration of 5 µg/ml in PBS/1 % BSA (#A7284; Sigma) after blocking of nonspecific binding sites with 10 % goat serum (#0060-01; Southern Biotech, Birmingham, AL, USA) in PBS/1 % BSA. A secondary biotinylated antibody goat-anti-mouse 1:50 (HK-325-UM; Biogenex, Fremont, CA, USA) was used, followed by incubation with the third antibody streptavidin-AP 1:50 (HK-321-UK; Biogenex). Staining was then visualised using an alkaline-phosphate substrate followed by counter staining with haematoxylin. CD68 positive cells stained pink.

Evaluation of implanted xenogeneic MSC-alginate constructs

To evaluate reproducibility and to improve clinical translatability, we encapsulated human bone marrow derived MSCs in alginate. 4 × 10^6 MSCs were mixed in 1 ml filter-sterilised 1.2 % low viscosity alginate and constructs were created by dripping the MSC-alginate mixture through a 23-gauge needle in a 102 mM $CaCl_2$ solution. Five constructs were subcutaneously implanted in two different rats for

5 weeks. Additionally, five alginate constructs without MSCs were subcutaneously implanted in two different rats to evaluate the effect of the alginate on the host immune system. 5 weeks after implantation, rats were euthanised and transplanted regions were harvested. Localisation of the constructs, human bone marrow MSCs and host immune reaction were evaluated with histological analysis by haematoxylin and eosin (HE) and CD68 staining.

In vitro evaluation of MSCs function and survival after encapsulation in alginate

Stimulation of immunomodulatory properties

Encapsulated human MSCs, isolated as described above, were cultured in DMEM low glucose medium with 2 % FCS, 50 µg/ml gentamycin and 1.5 µg/ml Fungizone for 48 h. To activate the immunomodulatory properties, MSC-alginate constructs were incubated 24 h in DMEM low glucose medium with 1 % ITS (BD Bioscience, Bedford, MA, USA), 50 µg/ml gentamycin and 1.5 µg/ ml Fungizone supplemented with IFNγ and TNFα (50 ng/ ml each; PeproTech, London, UK), designated as cytokine medium. As control, MSCs were cultured in monolayer until subconfluency and then incubated with control or cytokine medium for 24 h.

To evaluate long-term function, MSC-alginate constructs were pre-cultured for 30 d and subsequently stimulated with cytokine medium. Constructs were harvested directly after the 24 h stimulation for gene expression analyses and medium was harvested and stored at – 80°C for analyses of secreted factors.

Gene expression analyses

Alginate was dissolved in 55 mM sodium citric acid (Sigma) and spun down for 8 min at 175 × *g* at 4 °C. Cell pellets were resuspended in 1 ml RNABee (Tel-test, Firendswood, TX, USA) for RNA isolation. The monolayer MSCs cultures in 6-well plates were resuspended in 1 ml RNABee. After addition of 0.2 ml chloroform, samples were spun down for 15 min at 12,000 × *g*. Total RNA was isolated from the supernatant using the Qiagen RNA Micro Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) and nucleic acid content was determined spectrophotometrically (NanoDrop ND1000, Isogen Life Science, IJsselstein, The Netherlands). cDNA was generated according to manufacturer's instructions using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). Gene expression analysis was performed using ABI7000 cycle. Cycle threshold (Ct) values were corrected by the best housekeeper index (BKI), which was calculated by the average of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fw: ATGGGGAAGGTGAAGGTCG; Rv: TAAAAGCAGCCCTGGTGACC;

Probe: Fam-CGCCCAATACGACCAAATCCGTTGAC), ubiquitin C (UBC; Fw: ATTTGGGTCGCGGTTCTTG; Rv: TGCCTTGAC ATTCTCG ATGGT) and hypoxanthine phosphoribosyltransferase (HPRT; Fw: TATGGACAGGACTGAACGTCTTG; Rv: CACACAGAGGGCTACAATGTG; Probe: Fam-AGATGTGATGAAGGAGATGGGAGGCCA).

RT-PCR primer nucleotide sequences used for IL-6, tissue inhibitor of metalloproteinases (TIMP-1), TIMP-2, IDO, TGF-β1 and VEGF were described previously[47]. Relative expression levels were calculated using the $2^{-\Delta Ct}$ method[48].

Enzyme-Linked Immuno Sorbent Assay

TIMP-2 and IL-6 protein levels were measured in stimulated and non-stimulated MSC-conditioned media from three donors by means of ELISA assay according to the manufacturer's protocol (R&D systems, Abingdon, UK). All factors were corrected for the amounts present in standard MSC culture medium. To determine the amount of IDO enzymatic activity in MSC media, the level of its metabolite L-kynurenine was measured spectrophotometrically as described previously[49].

Immunomodulation by MSC-alginate constructs

MSC-alginate constructs were co-cultured with activated lymphocytes to study their immunosuppressive capacity. Therefore, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy blood donors (Sanquin, Rotterdam, The Netherlands) using Ficoll-Paque™ PLUS (density 1.077 g/ml; GE Healthcare, Uppsala, Sweden) separation. Cells were frozen at –150 °C until further use in RPMI-1640 medium containing 1 % GlutaMAX™-I (Life Technologies, Waltham, MA, USA) supplemented with 1 % P/S (Penicillin 10,000 UI/ml, Streptomycin 10,000 UI/ml; Lonza), 10 % human serum (Sanquin) and 10 % dimethylsulphoxide (DMSO, Merck, Hohenbrunn, Germany).

MSC-alginate constructs containing 4×10^6 cells/ml resulted in approximately 3×10^4 MSCs per construct. 2 d and 30 d after encapsulation, MSCs were either stimulated with cytokine medium or cultured in control medium (150 µl/bead) for 24 h. After 24 h constructs were washed two times with PBS and transferred per four, two, and one bead in a 48-wells plate in triplicate.

PBMCs were thawed and washed extensively with PBS to remove FCS and brought to a concentration of 1×10^7 cells/ml in PBS and labelled with 1 µM carboxyfluorescein succinimidyl ester (CFSE) by quickly mixing followed by 7 min incubation at 37 °C. Cells were resuspended in RPMI medium with 1 % P/S and 10 % FCS and, to stimulate T cell activation, antibodies against CD3 and CD28 (1 µl per 1×10^6 cells

in 1 ml) were added to the suspension with a cross-linking antibody (2 µl per 1×10^6 cells in 1 ml) (all from BD Biosciences, Bedford, MA, USA). 3×10^5 stimulated CFSE-PBMCs in 600 µl were added to the alginate beads in a 48-well plate. As positive and negative T lymphocyte proliferation control, 3×10^5/600 µl stimulated and non-stimulated CFSE-PBMCs were cultured in triplicate. Stimulated CFSE-PBMCs with 4 empty alginate beads (not containing MSCs) were used as baseline control. As a positive control for the proliferation inhibitory effect, MSCs in monolayer were plated at a density of 1.2×10^5 MSCs/well in a 48-well plate. After 24 h of attachment MSCs were stimulated or non-stimulated with cytokine medium for 24 h. After stimulation, cells were washed two times with PBS, and 3×10^5 stimulated CFSE-PBMCs suspended in 600 µl were added. After 5 d of co-culture, PBMCs were retrieved, labelled for 30 min with CD8/CD4 (BD Biosciences). Cells were analysed by fluorescence-activated cell sorting (FACS) on a FACS Canto II flow cytometer (BD Biosciences). After initial broad selection of lymphocytes based on forward and side scatter to exclude dead cells and debris, we selected the single CD4+ and single CD8+ cells. Proliferation of these cells was analysed by decrease of CFSE label.

Characterisation of the encapsulated MSCs

To evaluate if the MSCs maintained their multilineage differentiation capacity after 2 and 30 d of encapsulation in alginate, the alginate structure was disrupted using sodium citrate/ethylene diamine tetra acetate (EDTA, Sigma) to release the cells and osteogenic and adipogenic differentiation was performed for 21 d. For osteogenic differentiation, MSCs were plated at a density of 3×10^3 cells/cm^2 and cultured in high glucose DMEM (Gibco) containing 10 % FCS, 10 mM β-glycerophosphate (Sigma), 0.1 µM dexamethasone (Sigma) and 0.5 mM L-ascorbic acid 2 phosphate (Sigma). For adipogenic differentiation, MSCs were plated at a density of 2×10^4 cells/cm^2 and cultured in high glucose DMEM containing 10 % FCS, 1 µM dexamethasone, 0.2 mM indo-methacin (Sigma), 0.01 mg/ml insulin (Sigma) and 0.5 mM 3-isobutyl-l- methyl-xanthine (Sigma). All media contained 50 µg/ ml gentamycin and 1.5 µg/ml Fungizone. Histological evaluation was performed with Von Kossa staining (Sigma) for osteogenic differentiation and Oil Red O (Sigma) for adipose differentiation.

DNA Content

To determinate cell survival after one month of encapsulation in alginate we measured DNA content weekly (t = 0, 7, 14, 21 and 30 d, n = 4 donors). Beads were digested overnight at 56 °C in papain digestion buffer (250 µg/ml papain in 50 mM EDTA and 5 mM l-cysteine hydrochloride; all from Sigma). The amount of DNA

in each papain-digested sample was analysed in the Wallac 1420 victor2 (Perkin-Elmer, Wellesley, MA, USA) using an extinction filter of 340 nm and an emission filter of 590 nm by means of an ethidium bromide assay (Sigma) with calf thymus DNA as a standard[50].

Statistics

Statistical analysis of the *in vivo* BLI data was performed by Mann-Whitney U tests. The *in vitro* T cell proliferation data were analysed in SPSS 21.0 (IBM) by ANOVA with Dunnett's correction using empty beads as control group. Analyses of the *in vitro* data were performed by two way ANOVA test using GraphPad Pris 5.00. $p < 0.05$ was considered statistically significant.

RESULTS

Alginate encapsulated allogeneic MSCs were retained in immunocompetent rats for at least 5 weeks

To evaluate long-term cell activity, we subcutaneously implanted allogeneic rat SPIO-Fluc-MSCs encapsulated in alginate and compared this to subcutaneously injected SPIO-Fluc-MSCs suspended in saline. MSCs encapsulated and MSCs injected were well tolerated without any macroscopic sign of inflammation in the immunocompetent animals. Clear BLI signal of SPIO-Fluc-MSCs was observed directly after both implantation of alginate-cell constructs and injection of cells suspended in saline (**figure 1A,B**). From 2 weeks onward, injected cells could no longer be detected, whereas BLI signal of the encapsulated SPIO-Fluc-MSCs in alginate constructs remained clearly visible till the end of the study, 5 weeks after implantation (**figure 1 C-E**).

MRI images confirmed the subcutaneous location of the six SPIO-Fluc-MSC-alginate constructs and the six subcutaneously SPIO-Fluc-MSCs injected regions directly after implantation or injection (**figure 2A,B**). For all time points, hypointense signal from SPIO remained visible on MR images. The SPIO signal voids created by encapsulated F344 SPIO-Fluc-MSCs became less hypointense over time but remained comparable in size. The injected cell-suspension on the other hand generated smaller signal voids over time, although intensity remained approximately the same (**figure 2C**,**D**). Histology of the subcutaneous transplantation regions was performed at the end of the study to confirm presence of implanted cells. Perl's iron staining confirmed the presence of SPIO containing cells in subcutaneous regions of encapsulated and injected MSCs. In the injected MSCs condition,

cells were positive for CD68 and Perl's iron staining, indicating death of the MSCs and uptake of the released iron by macrophages (**figure 3A**), corresponding to the deceased BLI signal of the injected SPIO-Fluc-MSCs. Encapsulated SPIO-Fluc- MSCs had a viable appearance, were found isolated in the alginate and positive for Perl's iron and negative for CD68 (**figure 3B**).

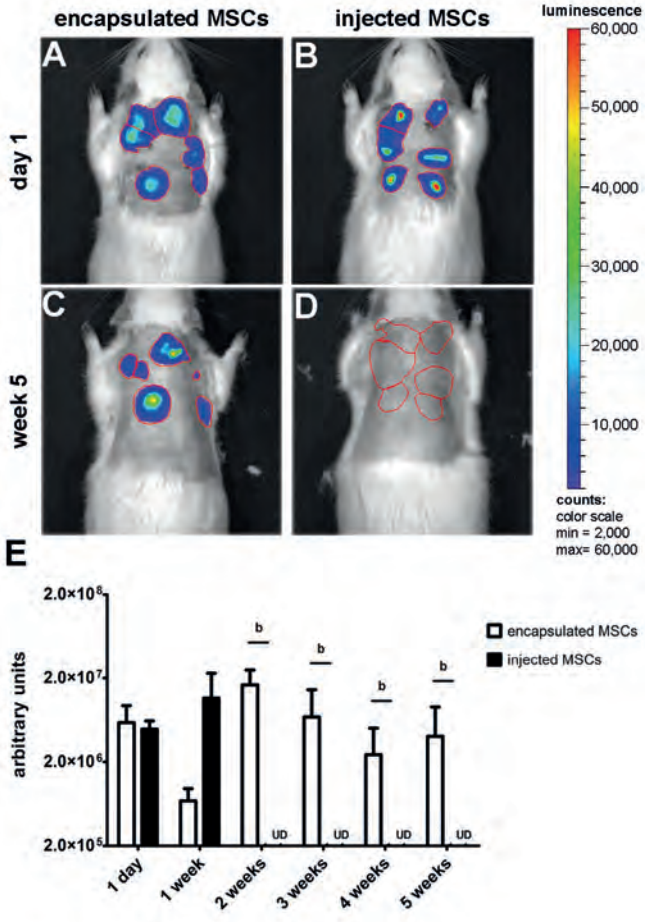


***Figure 1**. Long-term cell activity of allogeneic rat SPIO-Fluc-MSCs in vivo in an immunocompetent rat. BLI signal of encapsulated allogeneic rat MSCs and injected allogeneic rat MSCs subcutaneously in immunocompetent rats (**A**,**B**) 1 d and (**C**,**D**) 5 weeks after implantation/injection. (**E**) Quantification of BLI signal generated by viable rat SPIO- Fluc-MSCs up to 5 weeks depicted as arbitrary units: white bars represent encapsulated allogeneic rat MSCs, black bars represent injected allogeneic rat MSCs. The 1 week time point was excluded due to a technical failure during luciferin injection. 6 × 10^5 rat MSCs were implanted/injected per location with a total of 6 locations per rat. Mean ± SD is shown, ^{b} $p < 0.01$. UD= undetectable.*

To improve clinical translatability of the allogeneic rat MSCs results *in vivo*, we implanted encapsulated human bone marrow MSCs subcutaneously in rats for 5 weeks. Transplantation of these xenogeneic MSCs was well tolerated without any macroscopic sign of inflammation in the immunocompetent rats. Histology confirmed the presence of alginate encapsulated MSCs with a viable aspect at HE staining (**figure 3C**), without signs of macrophage infiltration (**figure 3D**,**E**). Alginate constructs without MSCs did not show ingrowth of host cells or macrophage infiltration (**figure 3F**,**G**). By encapsulating allogeneic and xenogeneic MSCs in alginate it was possible to retain MSCs in one location for at least 5 weeks in an *in vivo* setting.

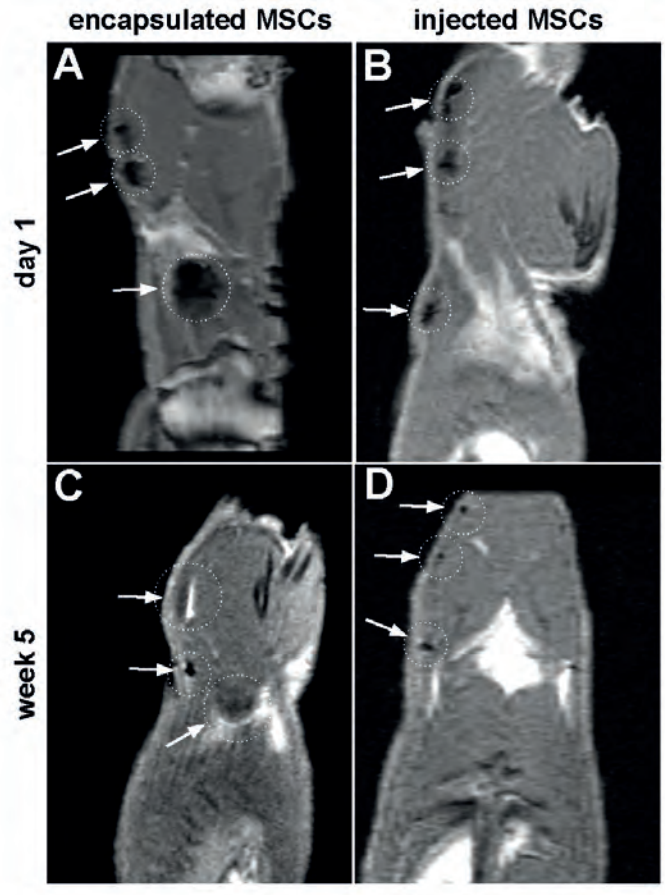


***Figure 2**. Long-term localisation of allogeneic rat SPIO-Fluc-MSCs applied subcutaneously in immunocompetent rats by MRI. Oblique views of MR images confirmed the subcutaneous location of the encapsulated allogeneic rat MSCs and injected allogeneic rat MSCs (**A**,**B**) 1 d after implantation/injection and (**C**,**D**) 5 weeks after implantation/injection. Due to the different subcutaneous implantation/injection locations, only three pockets can be displayed in a plane image.*

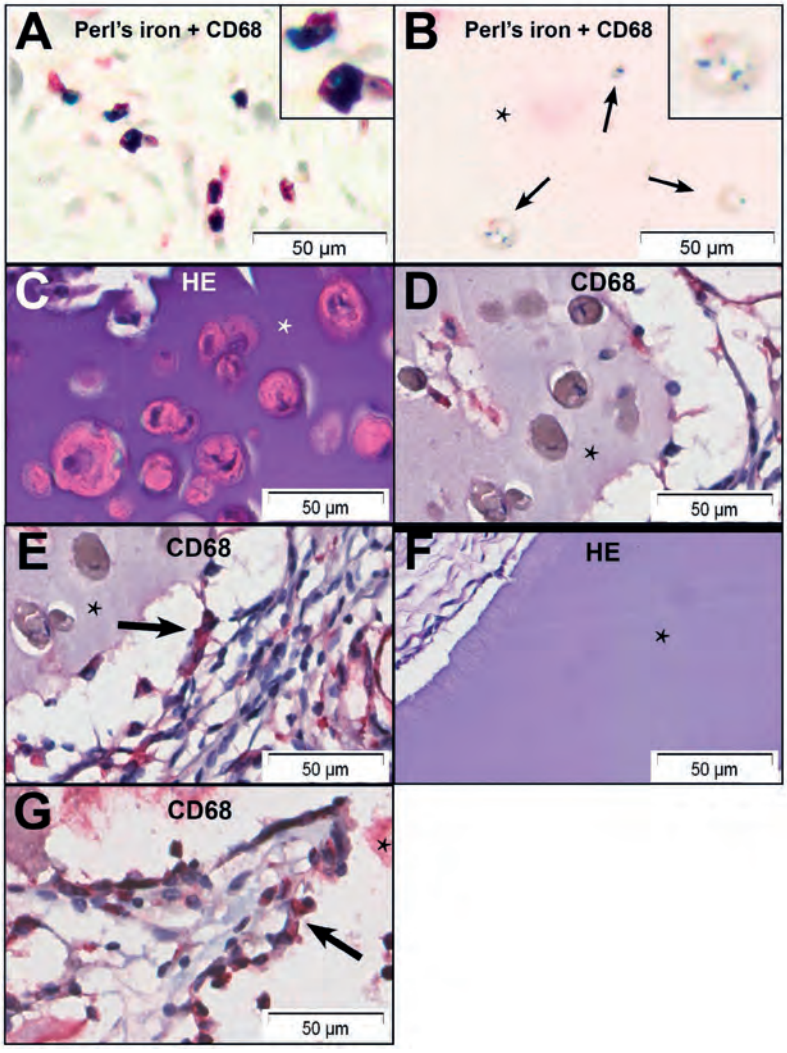


***Figure 3**. Histology of the subcutaneous implanted/injected allogeneic and xenogeneic MSCs in immunocompetent rats, 5 weeks after implantation/injection. (**A**,**B**) Implanted/injected allogeneic rat SPIO-Fluc-MSCs were stained with Perl's iron staining (blue), which stains SPIO. CD68 staining (pink) was used to stain macrophages. (**A**) Injected cells were positive for Perl's iron and CD68 staining suggesting macrophage phagocytosis of SPIO and MSC death, while (**B**) the Perl's iron and CD68 staining in combination confirmed SPIO labelled MSCs (arrows) encapsulated in alginate without CD68 staining. (**C**) Implanted encapsulated xenogeneic human bone marrow derived MSCs were stained with haematoxylin and eosin (HE) staining, which shows isolated MSCs in the alginate with a viable appearance. (**D**) CD68 staining was used to identify macrophages and the encapsulated MSCs were negative for CD68. (**E**) Some macrophages were identified in the host tissue around the construct (arrow) without evident macrophage infiltrations. (**F**) Alginate constructs without MSCs were implanted as a control. HE staining showed no cell infiltration in the constructs. (**G**) CD68 staining showed some macrophages in the host tissue surrounding the alginate construct without MSCs (arrow), but no evident macrophage infiltrations were found. Haematoxylin and eosin staining (HE), *asterisk indicates alginate in B, C, D and F and indicates the location where the alginate was located before processing the sample for CD68 staining in E and G.*

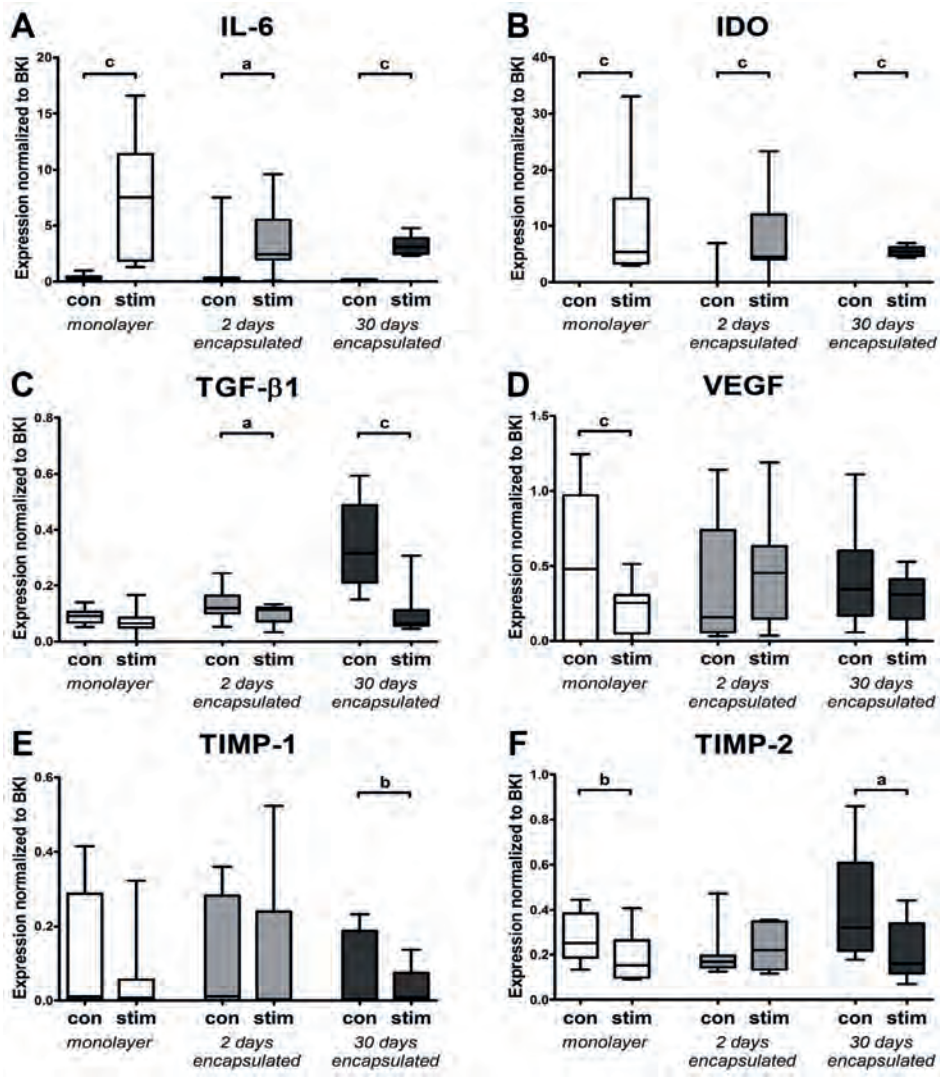


Figure 4. *Gene expression of immunomodulatory and trophic genes by MSCs with or without stimulation by inflammatory cytokines IFNγ/TNFα for 24 h. MSCs were cultured in monolayer for 2 d (control condition) or encapsulated in alginate and cultured for 2 d and 30 d before 24 h of stimulation. Box and whisker plot 2.5-97.5 percentile is shown. n = 4 donors with duplicate or triplicate samples per donor. BKI (best housekeeper index) as average of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin C (UBC) and hypoxanthine phosphoribosyltransferase (HPRT); Interleukin 6, IL-6; Indoleamine 2,3-dioxygenase, IDO; Transforming growth factor β1, TGF-β1; Vascular endothelial growth factor, VEGF; Tissue inhibitor of metalloproteinases 1, TIMP-1; Tissue inhibitor of metalloproteinases 2, TIMP-2; non-stimulate cells with IFNγ/TNFα as control, con, stimulated cells with IFNγ/TNFα for 24 h, stim.* ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$.

MSC-alginate constructs retain long-term immunomodulatory capacity *in vitro*

Expression of immunomodulatory and trophic genes in encapsulated MSCs

We evaluated the effect of alginate-encapsulation on several properties of human bone marrow derived MSCs. To study the immunomodulatory and trophic capabilities after encapsulation, MSCs were stimulated for 24 h with IFNγ/TNFα after 2 d of culture in alginate. Encapsulated MSCs were compared to monolayer cultured cells.

MSCs encapsulated in alginate constructs responded to IFNγ/TNFα very similarly to MSCs cultured in monolayer (**figure 4**). Gene expression of IL-6 and IDO ($p < 0.05$) was up-regulated in response to IFNγ/TNFα in both monolayer and alginate culture. TGF-β1 was down-regulated in all conditions but only reached significance in alginate cultures ($p < 0.05$), whereas VEGF was only significantly down-regulated in monolayer cultures ($p < 0.05$). To check whether MSCs in alginate maintained their immunomodulatory and trophic response capacity for a longer period, we performed 24 h stimulation with control and cytokine medium on encapsulated MSCs, which had been in culture for 30 d. MSCs retained a response to IFNγ/TNFα similar to the 2 d cultured alginate beads with up-regulated IL-6 and IDO expression ($p < 0.05$) and down-regulated TGF-β1, TIMP-1 and TIMP-2 expression ($p < 0.05$) while VEGF was not significantly altered (**figure 4**).

Secretion of immunomodulatory factors by MSC-alginate constructs

MSCs cultured in monolayer secreted IL-6 and TIMP-2, and expressed IDO activity. MSCs encapsulated in alginate for 2 and 30 d secreted IL-6 and TIMP-2, and expressed IDO activity as well (**table 1**). IDO activity and IL-6 secretion were increased by IFNγ/TNFα at all time points, in accordance with gene expression analysis (albeit IL-6 did not reach statistical significance in alginate cultures due to high donor variation in absolute secretion levels. Nevertheless, in all donors IL-6 secretion increased after IFNγ/TNFα treatment). The absolute IDO activity and IL-6 secretion diminished between 2 d and 30 d pre-culture. TIMP-2 secretion was not affected by cytokine treatment.

Table 1
Immunomodulatory factors in conditioned medium of MSCs.

	2 d monolayer		2 d encapsulation in alginate		30 d encapsulation in alginate	
	Control	**Cytokine**	**Control**	**Cytokine**	**Control**	**cytokine**
IL-6 pg/ml	5495 ± 553	53594±3335[a]	1650 ± 1271	36044±23304	UD	4148 ± 2439
TIMP2 pg/ml	26181±5099	27750±3917	15541±8027	15291±7669	9483 ± 6913	8635 ± 5518
Kynurenine ng/ml	440 ± 158	13069±1012[b]	690 ± 631	11531 ± 102[a]	1430 ± 1430	7661 ± 1548[a]

Measurement of immunomodulatory factors in conditioned medium of MSCs with or without stimulation by inflammatory cytokines IFNγ and TNFα for 24 h. MSCs were cultured in monolayer for 2 d (control condition) or encapsulated in alginate and cultured for 2 d and 30 d before 24 h of stimulation. IL-6 (pg/ml) and TIMP-2 (pg/mL) secretion were measured in the conditioned medium by ELISA. IDO enzymatic activity was measured spectrophotometrically by means of L-kynurenine level (ng/ml) in the conditioned medium. Mean ± SD is shown. $n = 3$ donors with triplicate samples per donor. [a] $p < 0.05$, [b] $p < 0.01$ comparing control and cytokine medium. Under detection limit, UD.

Immunomodulatory effect of MSC-alginate constructs

Immunomodulation by MSC-alginate constructs was evaluated by the effect on T lymphocyte proliferation. MSC-alginate constructs that were cultured for 2 d significantly inhibited proliferation of stimulated CD4+ and CD8+ T lymphocytes in a dose-dependent manner (**figure 5**). Four MSC-alginate constructs (approximately 3×10^4 cells/bead) 2 d after encapsulation had similar inhibitory effect on stimulated T lymphocytes (68 % inhibition for CD4+ cells; 52 % for CD8+ cells compared to the control with 4 empty constructs; $p < 0.05$) as the MSCs monolayer control (approximately 1.2×10^5 cells per well). This effect diminished after 30 d of encapsulation, albeit all conditions still inhibited CD4+ T lymphocyte proliferation and four MSC-alginate constructs still significantly inhibit CD8+ T lymphocyte proliferation (**figure 5**; inhibition CD4+ proliferation 30 % and CD8+ proliferation 12 % with 4 constructs). Pre-stimulation of MSC-alginate constructs with IFNγ/TNFα for 24 h did not influence the inhibitory effects on T lymphocyte proliferation (data not shown).

Long-term retention of MSC properties in alginate

To evaluate their multilineage differentiation capacity after encapsulation in alginate, MSCs were released after 2 and 30 d of encapsulation. Adipogenic and osteogenic differentiation assays were performed and compared with MSCs that were not encapsulated in alginate. The released MSCs could still differentiate adipogenically and osteogenically, although these differentiation capacities were diminished by encapsulation in alginate (**figure 6A-F**).

Since we observed a decrease in absolute levels of secreted factors and in the inhibition of lymphocyte proliferation after 30 d in alginate, we evaluated the

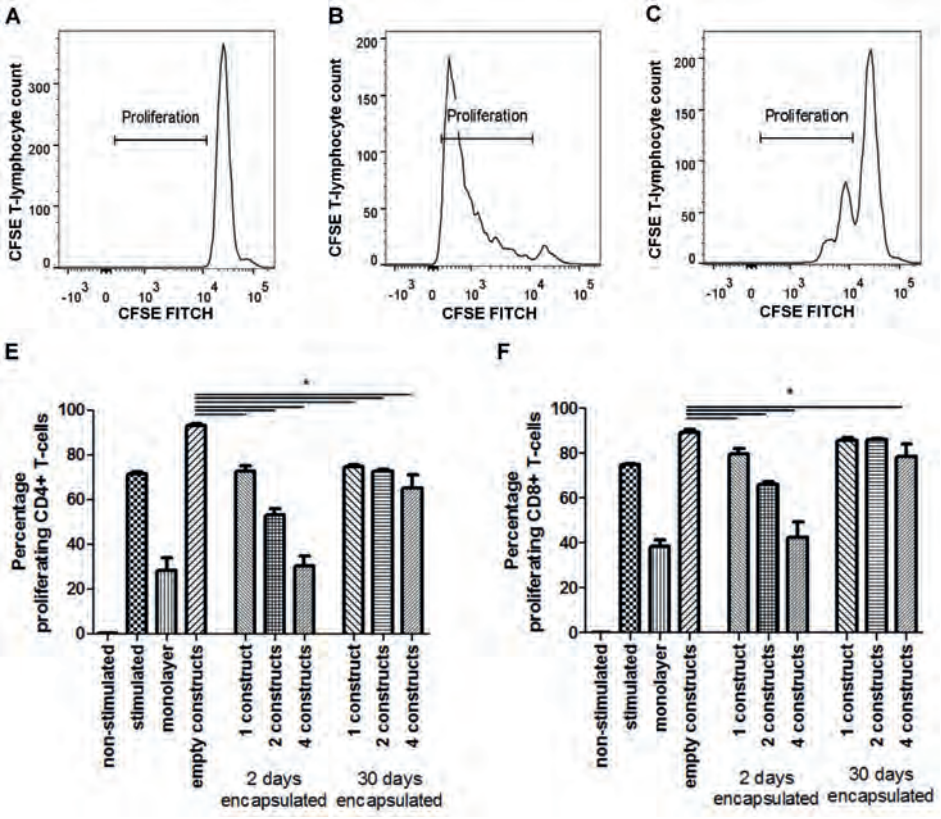


***Figure 5**. Inhibition of T lymphocyte proliferation by alginate encapsulated MSCs (approximately 3 × 10^4 cells/construct). Examples of FACS histograms show the fluorescence intensity of (**A**) non-stimulated CD4+ T lymphocytes stained with CFSE; (**B**) anti-CD3/anti-CD28 stimulated CD4+ T lymphocytes stained with CFSE in the presence of empty alginate constructs and (**C**) anti-CD3/anti-CD28 stimulated CD4+ T lymphocytes co-cultured with 4 MSC-alginate constructs. FACS histograms of stimulated (**D**) CD4+ T lymphocytes and (**D**) CD8+ T lymphocytes co-cultured with one, two and four constructs 2 d and 30 d after encapsulation of MSCs. Mean ± SD is shown. Fluorescence-activated cell sorting, FACS; carboxyfluorescein succinimidyl ester, CFSE. Negative control T lymphocyte proliferation, non-stimulated; positive controls T lymphocyte proliferation, stimulated; proliferation inhibition control, monolayer (approximately 1.2 × 10^5 cells per well, same cell count as 4 constructs); empty constructs, control (stimulated T lymphocytes co-cultured with 4 empty alginate constructs). * indicates statistical significance.*

effect of the alginate encapsulation on MSCs survival by measurements of DNA content of the alginate constructs after 0, 7, 14, 21 and 30 d of culture. DNA content diminished after culture of encapsulated MSCs in alginate ($p < 0.001$), reducing viable MSC numbers by approximately 50 % after 30 d (**figure 6G**).

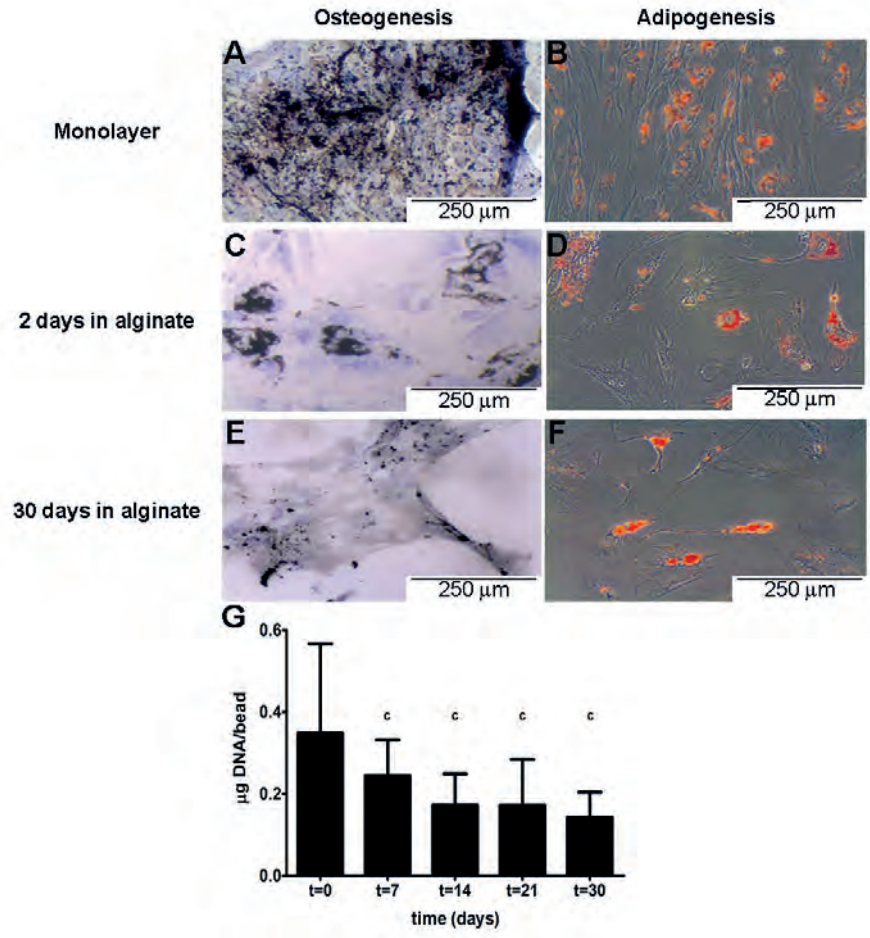


***Figure 6**. Osteogenic and adipogenic differentiation of MSCs after encapsulation. Representative pictures are shown. (**A**,**C**,**E**) Von Kossa staining was used for osteogenic differentiation with black indicating calcium-phosphate crystals. (**B**,**D**,**F**) Oil Red O staining was used for adipogenic differentiation with red indicating lipid drops. (**G**) Cell survival over time in vitro of encapsulated MSCs is measured by DNA content of the beads. Mean ± SD is shown. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$ compared to DNA content at $t = 0$.*

DISCUSSION

MSCs are known to have both trophic and immunomodulatory properties, which can be used for therapeutic applications in regenerative medicine. The possibility of using allogeneic cells would reduce costs and make it more feasible to guarantee certain qualities of the used product, thus increasing the clinical applicability. For a potent effect, in particular in chronic inflammatory diseases, cells should remain present locally over a certain period of time. Previous studies using stem cell tracking methods could not demonstrate the long-term presence of these

cells after administration in various applications[51-54]. We demonstrated that by encapsulation in alginate, active allogeneic and xenogeneic MSCs were retained over a period of 5 weeks at the implanted location in immunocompetent animals. Moreover, MSC-alginate constructs expressed multiple immunomodulatory properties and trophic properties *in vitro*.

Protection of allogeneic cells against the host immune system is important since we aim to use allogeneic MSCs to create an "off-the-shelf" therapy. Despite the belief that MSCs are immune evasive or privileged[51], MSCs viability is reduced when used in inflammatory and immunocompetent environments. Furthermore, it is known that cultured MSCs or MSCs exposed to inflammatory environments can express major histocompatibility complex (MHC)-I and MHC-II. To protect allogeneic cells from fast rejection by the immune system of the immunocompetent animals, we encapsulated them in alginate. Alginate has been used before to protect cells against the host immune system[41,42,44]. Although in our *in vitro* assay, alginate appeared to stimulate the proliferation of stimulated T cells, when MSCs were encapsulated, the alginate constructs inhibited T cell proliferation. After *in vivo* implantation in an immunocompetent animal, encapsulated allogeneic MSCs were detectable with BLI for up to 5 weeks, indicating local presence and stable viability of the cells. Since no BLI signal of the injected SPIO-Fluc-MSCs was detected from 2 weeks onward, while the hypo-intense SPIO signal remained visible on MRI, we hypothesise that our labelled allogeneic cells died and the SPIO was taken up by macrophages. Histology showed double stained cells for CD68 and Perl's iron staining, thus endorsing this hypothesis. Similarly, the fact that the BLI signal of encapsulated cells remained present throughout the study implies that alginate inhibited cell death and migration.

Human MSC-alginate constructs expressed immunomodulatory and trophic properties *in vitro,* even after a culture period of 30 d. Although donor variation was found, each donor consistently showed upregulation of gene expression and secretion of these factors after stimulation of the MSCs with inflammatory cytokines. The variation between different MSCs cultures could be due to differences between donors or between isolated subpopulations or as a result of differences introduced during the culture process to expand the cells. MSCs were isolated from patients undergoing total hip replacement surgery by plating out bone marrow on plastic and washing away non-adherent cells after 24 h. This isolation protocol is extensively used in our laboratory, leading to cell populations with multiple MCS characteristics[55,56], but still to a heterogeneous population. These effects will be less relevant for application if we can use allogeneic cells. In this case, the MSC-

alginate constructs can be extensively tested before application in patients and the most effective MSCs will be selected. For the purpose of selection it would be beneficial to know the effectors of the MSC-alginate constructs. For this study we chose to measure IDO, IL-6, TIMPs, TGF-β1 and VEGF as factors secreted by MSCs, since these factors are known to play a role in modulating inflammation and tissue repair. IDO in MSCs promotes immunosuppression[57-59]. IL-6 has been reported to have anti-inflammatory characteristics[60,61], as well as pro-inflammatory characteristics[62,63]. The maintenance of TIMP-1 and TIMP-2 gene expression we observed may also act locally and control the MMP-induced breakdown of extracellular matrix[64]. TGF-β1 has been associated with immune surveillance and immune suppression mechanisms[65,66]. Its long-term overexpression leads to severe hyperplasia in normal epidermis or oral mucosa[67-69]. VEGF has been reported to have beneficial effects in certain diseases, such as myocardial infarction, by contributing as a growth factor in the angiogenesis required for tissue repair[47,70].

Those molecules are amongst the most frequently reported. However, this is only a small fraction of the biologically active factors that are secreted by MSCs, either soluble or in extracellular vesicles[71,72]. It is, therefore, important to test the functionality of the secreted factors. T cell proliferation tests are the most commonly used for this purpose. For the MSC-alginate construct used in this study, we found that 2 d after alginate encapsulation, the inhibition of CD4+ and CD8+ T lymphocyte proliferation was similar to MSCs in monolayer. This indicates that alginate does not prevent the secretion of factors important for inhibition of T cell proliferation. 30 d after encapsulation in alginate, the MSCs were still able to inhibit T cell proliferation. The effect was reduced, but still statistically significant, which we find encouraging. The data indicate that encapsulated in alginate, MSCs have the potential to inhibit HLA class I (by CD8+) and class II (by CD4+) mediated T cell responses. Our results are in agreement with data showing that MSCs suppress the proliferation of CD4+ and CD8+ T cells in cell contact but also transwell conditions[73]. The inhibition of CD8+ cells can have important implications for the use of MSC encapsulated cells in immune suppression therapy after organ transplantation. It is relevant to mention that pre-stimulation of MSCs with IFNγ/TNFα for 24 h did not influence the inhibitory effects on T lymphocyte proliferation, which might be explained by the fact that stimulated T lymphocytes produce IFNγ and TNFα that will stimulate the co-cultured MSCs. These findings can have implications for the use of alginate encapsulated MSCs in chronic inflammation. In these conditions, factors such as IFNγ and TNFα will be present and in particular CD4+ T cells have been demonstrated to play an important role.

For this study we encapsulated MSCs in alginate using a method we have described previously to generate cartilage from long-term culture[74]. The MSC-alginate constructs so generated, expressed immunomodulatory capacity. This confirms the recent studies of Stucky and co-workers, demonstrating an inhibition of neuro-inflammation by MSCs that were encapsulated in alginate for 1 d, and Gray and co-workers, demonstrating secretion of factors in reaction to inflammatory cytokines after 2 d of encapsulation in alginate[75,76]. We demonstrate, in this study, that MSC encapsulated in alginate can inhibit T cell proliferation. This capacity, however, was reduced after 30 d of encapsulation. This can be partly explained by the difference in number of viable cells present at 2 d and 30 d after encapsulation. Moreover, we cannot exclude that some of cells have changed their phenotype. We confirmed whether the MSCs still had their multilineage differentiation potential after encapsulation. Since our aim was to retain the immunomodulatory capacity of the encapsulated MSCs and, therefore, cell differentiation in our construct was not preferred, we evaluated extracellular matrix production of the encapsulated MSCs *in vivo* at the end of the experiments by picrosirius red staining. No positive staining for collagen was found in the constructs (data not shown) suggesting that there was no differentiation of encapsulated MSCs in the constructs. Although, we did not evaluate the formation of glycosaminoglycans, we consider it very unlikely that these would be retained in the absence of a collagen matrix. Nevertheless, in the long-term small quantities of matrix formed by MSCs could possibly influence the secretion of immunomodulatory factors and could be playing a role in the diminished immunomodulatory effects of our constructs observed after 30 d.

Immediately after encapsulation we typically find around 30 % loss of cells. In this study, 50 % of the primary encapsulated cells remained viable and the cells did not proliferate. Other studies showed higher cell survival after encapsulation[31,77], which might – among other factors – be explained by differences in cell-type used, the amount of serum in culture medium or the type of alginate used. Whereas most studies used 10 % foetal calf serum for culture, the concentration usually used to expand MSCs, we maintained the MSCs in low serum medium (2 %), which we considered more comparable with the clinical situation after implantation, where the availability of nutrients is likely to be restricted. The alginate environment and the specific medium used might have selected a subpopulation of cells able to survive under these conditions. This population however, retained the capacity to secrete factors in response to inflammatory conditions. Different construct adjustments can be made by adjusting the type of alginate, the alginate concentration and the construct size, which will influence the integrity of the construct and thereby the survival of the MSCs. RGD-alginate is known as a good environment for MSC

survival but since it will stimulate cell attachment it is less favourable for preventing host-donor interactions in case of allogeneic cells[77,78]. Binder and colleagues[79] proposed the co-delivery of alginate with Lysophosphatidic acid (LPA) to rescue undifferentiated MSCs from serum deprivation and hypoxia-induced apoptosis and thus to improve the persistence of undifferentiated MSCs *in vivo*. Although LPA should be used with care, since it is a potent mitogen and may contribute to oncogenesis, this indicates possibilities for further improvement of the function of MSC-alginate constructs.

The beads can easily be applied during surgical procedures. Moreover, it has been suggested that alginate beads can be used safely and effectively to deliver stem cells percutaneously with minimal loss of viability[31,77,80]. This holds great promise for use in osteoarthritis, tendinopathy, myocardial infarction or acute spinal cord injury, amongst others. The construct might require further optimisation depending on the final application. For application in osteoarthritis, for instance, where the MSC-alginate beads should be delivered in the joint, the ability to withstand mechanical forces in the joint is an important prerequisite that needs attention. Further fine tuning the viscoelasticity of the beads (alginate concentration, alginate type, crosslinking) and the size of the beads are important material properties to consider in this respect.

CONCLUSION

In vivo allogeneic and xenogeneic MSC-alginate constructs remained locally present at the site of implantation, subcutaneously in an immunocompetent rat for at least 5 weeks. After long-term culture, MSC-alginate beads expressed the ability to interactively modulate their microenvironment by IDO activity and secreting several immunomodulatory and trophic factors such as IL-6, TIMP-1, TIMP-2, TGF-β1 and VEGF. MSC-alginate constructs are therefore an interesting system for application in various musculoskeletal diseases with an inflammatory component, such as osteoarthritis, tendinopathy or acute spinal cord injury. Future studies are needed to show how long cells remain encapsulated, to optimise the constructs for specific locations of application and to evaluate what the effect will be in diseased conditions.

REFERENCES

1. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials.* 2004;25(16):3211-3222.
2. De Angelis L, Berghella L, Coletta M, et al. Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. *J Cell Biol.* 1999;147(4):869-878.
3. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood.* 2004;103(5):1669-1675.
4. Sottile V, Halleux C, Bassilana F, Keller H, Seuwen K. Stem cell characteristics of human trabecular bone-derived cells. *Bone.* 2002;30(5):699-704.
5. Kunter U, Rong S, Djuric Z, et al. Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. *J Am Soc Nephrol.* 2006;17(8):2202-2212.
6. Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet.* 2004;363(9419):1439-1441.
7. Lee RH, Seo MJ, Reger RL, et al. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc Natl Acad Sci U S A.* 2006;103(46):17438-17443.
8. Minguell JJ, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. *Exp Biol Med (Maywood).* 2006;231(1):39-49.
9. Ortiz LA, Dutreil M, Fattman C, et al. Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci U S A.* 2007;104(26):11002-11007.
10. Rodriguez-Merchan EC. Intra-articular injections of mesenchymal stem cells for knee osteoarthritis. *Am J Orthop (Belle Mead NJ).* 2014;43(12):E282-291.
11. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation.* 2002;105(1):93-98.
12. Zhang J, Huang X, Wang H, et al. The challenges and promises of allogeneic mesenchymal stem cells for use as a cell-based therapy. *Stem Cell Res Ther.* 2015;6:234.
13. Daghestani HN, Pieper CF, Kraus VB. Soluble Macrophage Biomarkers Indicate Inflammatory Phenotypes in Patients with Knee Osteoarthritis. *Arthritis Rheumatol.* 2014.
14. Komatsu N, Takayanagi H. Arthritogenic T cells in autoimmune arthritis. *Int J Biochem Cell Biol.* 2015;58:92-96.
15. Groh ME, Maitra B, Szekely E, Koc ON. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Exp Hematol.* 2005;33(8):928-934.
16. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol.* 2003;57(1):11-20.
17. Ren G, Zhang L, Zhao X, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell.* 2008;2(2):141-150.
18. Comoli P, Ginevri F, Maccario R, et al. Human mesenchymal stem cells inhibit antibody production induced in vitro by allostimulation. *Nephrol Dial Transplant.* 2008;23(4):1196-1202.

19. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R, Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. *J Immunol.* 2006;177(4):2080-2087.
20. Di Nicola M, Carlo-Stella C, Magni M, et al. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood.* 2002;99(10):3838-3843.
21. Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood.* 2003;101(9):3722-3729.
22. Parekkadan B, Tilles AW, Yarmush ML. Bone marrow-derived mesenchymal stem cells ameliorate autoimmune enteropathy independently of regulatory T cells. *Stem Cells.* 2008;26(7):1913-1919.
23. Bartholomew A, Sturgeon C, Siatskas M, et al. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol.* 2002;30(1):42-48.
24. Popp FC, Eggenhofer E, Renner P, et al. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. *Transpl Immunol.* 2008;20(1-2):55-60.
25. Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut.* 2009;58(7):929-939.
26. Jiang XX, Zhang Y, Liu B, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood.* 2005;105(10):4120-4126.
27. Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood.* 2004;103(12):4619-4621.
28. Wang XJ, Dong Z, Zhong XH, et al. Transforming growth factor-beta1 enhanced vascular endothelial growth factor synthesis in mesenchymal stem cells. *Biochem Biophys Res Commun.* 2008;365(3):548-554.
29. Mallat Z, Tedgui A. The role of transforming growth factor beta in atherosclerosis: novel insights and future perspectives. *Curr Opin Lipidol.* 2002;13(5):523-529.
30. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy.* 2006;8(4):315-317.
31. Barminko J, Kim JH, Otsuka S, et al. Encapsulated mesenchymal stromal cells for in vivo transplantation. *Biotechnol Bioeng.* 2011;108(11):2747-2758.
32. Birgersdotter A, Sandberg R, Ernberg I. Gene expression perturbation in vitro–a growing case for three-dimensional (3D) culture systems. *Semin Cancer Biol.* 2005;15(5):405-412.
33. Cukierman E, Pankov R, Yamada KM. Cell interactions with three-dimensional matrices. *Curr Opin Cell Biol.* 2002;14(5):633-639.
34. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Biol.* 2006;7(3):211-224.
35. Hansen LK, Mooney DJ, Vacanti JP, Ingber DE. Integrin binding and cell spreading on extracellular matrix act at different points in the cell cycle to promote hepatocyte growth. *Mol Biol Cell.* 1994;5(9):967-975.
36. Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. *Cell.* 2007;130(4):601-610.

37. de Vos P, Hoogmoed CG, Busscher HJ. Chemistry and biocompatibility of alginate-PLL capsules for immunoprotection of mammalian cells. *J Biomed Mater Res.* 2002;60(2):252-259.
38. Murua A, Portero A, Orive G, Hernandez RM, de Castro M, Pedraz JL. Cell microencapsulation technology: towards clinical application. *J Control Release.* 2008;132(2):76-83.
39. Shoichet MS, Rein DH. In vivo biostability of a polymeric hollow fibre membrane for cell encapsulation. *Biomaterials.* 1996;17(3):285-290.
40. Wu Y, Wang J, Scott PG, Tredget EE. Bone marrow-derived stem cells in wound healing: a review. *Wound Repair Regen.* 2007;15 Suppl 1:S18-26.
41. Herrero EP, Del Valle EM, Galan MA. Immobilization of mesenchymal stem cells and monocytes in biocompatible microcapsules to cell therapy. *Biotechnol Prog.* 2007;23(4):940-945.
42. Kang KS, Lee SI, Hong JM, et al. Hybrid scaffold composed of hydrogel/3D-framework and its application as a dopamine delivery system. *J Control Release.* 2014;175:10-16.
43. Trouche E, Girod Fullana S, Mias C, et al. Evaluation of alginate microspheres for mesenchymal stem cell engraftment on solid organ. *Cell Transplant.* 2010;19(12):1623-1633.
44. Zanotti L, Sarukhan A, Dander E, et al. Encapsulated mesenchymal stem cells for in vivo immunomodulation. *Leukemia.* 2013;27(2):500-503.
45. Guenoun J, Ruggiero A, Doeswijk G, et al. In vivo quantitative assessment of cell viability of gadolinium or iron-labeled cells using MRI and bioluminescence imaging. *Contrast Media Mol Imaging.* 2013;8(2):165-174.
46. van Buul GM, Kotek G, Wielopolski PA, et al. Clinically translatable cell tracking and quantification by MRI in cartilage repair using superparamagnetic iron oxides. *PLoS One.* 2011;6(2):e17001.
47. Yuan Q, Sun L, Li JJ, An CH. Elevated VEGF levels contribute to the pathogenesis of osteoarthritis. *BMC Musculoskelet Disord.* 2014;15:437.
48. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008;3(6):1101-1108.
49. Kang JW, Kang KS, Koo HC, Park JR, Choi EW, Park YH. Soluble factors-mediated immunomodulatory effects of canine adipose tissue-derived mesenchymal stem cells. *Stem Cells Dev.* 2008;17(4):681-693.
50. Royce PM, Lowther DA. Fluorimetric determination of DNA in papain digests of cartilage, using ethidium bromide. *Connect Tissue Res.* 1979;6(4):215-221.
51. Ankrum JA, Ong JF, Karp JM. Mesenchymal stem cells: immune evasive, not immune privileged. *Nat Biotechnol.* 2014;32(3):252-260.
52. Fischer UM, Harting MT, Jimenez F, et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev.* 2009;18(5):683-692.
53. Gholamrezanezhad A, Mirpour S, Bagheri M, et al. In vivo tracking of 111In-oxine labeled mesenchymal stem cells following infusion in patients with advanced cirrhosis. *Nucl Med Biol.*38(7):961-967.
54. Harting MT, Jimenez F, Xue H, et al. Intravenous mesenchymal stem cell therapy for traumatic brain injury. *J Neurosurg.* 2009;110(6):1189-1197.
55. Farrell E, van der Jagt OP, Koevoet W, et al. Chondrogenic priming of human bone marrow stromal cells: a better route to bone repair? *Tissue Eng Part C Methods.* 2009;15(2):285-295.
56. Hellingman CA, Koevoet W, Kops N, et al. Fibroblast growth factor receptors in in vitro and in vivo chondrogenesis: relating tissue engineering using adult mesenchymal stem cells to embryonic development. *Tissue Eng Part A.* 2010;16(2):545-556.

57. Gerdoni E, Gallo B, Casazza S, et al. Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. *Ann Neurol.* 2007;61(3):219-227.
58. Matysiak M, Stasiolek M, Orlowski W, et al. Stem cells ameliorate EAE via an indoleamine 2,3-dioxygenase (IDO) mechanism. *J Neuroimmunol.* 2008;193(1-2):12-23.
59. Ryan JM, Barry FP, Murphy JM, Mahon BP. Mesenchymal stem cells avoid allogeneic rejection. *J Inflamm (Lond).* 2005;2:8.
60. Djouad F, Charbonnier LM, Bouffi C, et al. Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells.* 2007;25(8):2025-2032.
61. Xing Z, Gauldie J, Cox G, et al. IL-6 is an antiinflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest.* 1998;101(2):311-320.
62. Alonzi T, Fattori E, Lazzaro D, et al. Interleukin 6 is required for the development of collagen-induced arthritis. *J Exp Med.* 1998;187(4):461-468.
63. Yamamoto M, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. *J Immunol.* 2000;164(9):4878-4882.
64. Vaalamo M, Leivo T, Saarialho-Kere U. Differential expression of tissue inhibitors of metalloproteinases (TIMP-1, -2, -3, and -4) in normal and aberrant wound healing. *Hum Pathol.* 1999;30(7):795-802.
65. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol.* 2006;24:99-146.
66. Gorelik L, Flavell RA. Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. *Nat Med.* 2001;7(10):1118-1122.
67. Li AG, Wang D, Feng XH, Wang XJ. Latent TGFbeta1 overexpression in keratinocytes results in a severe psoriasis-like skin disorder. *EMBO J.* 2004;23(8):1770-1781.
68. Liu X, Alexander V, Vijayachandra K, Bhogte E, Diamond I, Glick A. Conditional epidermal expression of TGFbeta 1 blocks neonatal lethality but causes a reversible hyperplasia and alopecia. *Proc Natl Acad Sci U S A.* 2001;98(16):9139-9144.
69. Lu SL, Reh D, Li AG, et al. Overexpression of transforming growth factor beta1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation. *Cancer Res.* 2004;64(13):4405-4410.
70. Angelo LS, Kurzrock R. Vascular endothelial growth factor and its relationship to inflammatory mediators. *Clin Cancer Res.* 2007;13(10):2825-2830.
71. Bruno S, Deregibus MC, Camussi G. The secretome of mesenchymal stromal cells: Role of extracellular vesicles in immunomodulation. *Immunol Lett.* 2015;168(2):154-158.
72. Lavoie JR, Rosu-Myles M. Uncovering the secretes of mesenchymal stem cells. *Biochimie.* 2013;95(12):2212-2221.
73. DelaRosa O, Lombardo E, Beraza A, et al. Requirement of IFN-gamma-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. *Tissue Eng Part A.* 2009;15(10):2795-2806.
74. Pleumeekers MM, Nimeskern L, Koevoet WL, et al. The in vitro and in vivo capacity of culture-expanded human cells from several sources encapsulated in alginate to form cartilage. *Eur Cell Mater.* 2014;27:264-280; discussion 278-280.
75. Gray A, Marrero-Berrios I, Ghodbane M, et al. Effect of Local Anesthetics on Human Mesenchymal Stromal Cell Secretion. *Nano Life.* 2015;5(2):1550001-1550014.

76. Stucky EC, Schloss RS, Yarmush ML, Shreiber DI. Alginate micro-encapsulation of mesenchymal stromal cells enhances modulation of the neuro-inflammatory response. *Cytotherapy.* 2015;17(10):1353-1364.
77. Duggal S, Fronsdal KB, Szoke K, Shahdadfar A, Melvik JE, Brinchmann JE. Phenotype and gene expression of human mesenchymal stem cells in alginate scaffolds. *Tissue Eng Part A.* 2009;15(7):1763-1773.
78. Markusen JF, Mason C, Hull DA, et al. Behavior of adult human mesenchymal stem cells entrapped in alginate-GRGDY beads. *Tissue Eng.* 2006;12(4):821-830.
79. Binder BY, Genetos DC, Leach JK. Lysophosphatidic acid protects human mesenchymal stromal cells from differentiation-dependent vulnerability to apoptosis. *Tissue Eng Part A.* 2014;20(7-8):1156-1164.
80. Abruzzo T, Cloft HJ, Shengelaia GG, et al. In vitro effects of transcatheter injection on structure, cell viability, and cell metabolism in fibroblast-impregnated alginate microspheres. *Radiology.* 2001;220(2):428-435.

Chapter 6

MSC encapsulation in alginate microcapsules prolongs survival after intra-articular injection, a longitudinal in vivo cell and bead integrity tracking study

Cell Biol Toxicol. 2020 Dec;36(6): 553-570

Sohrab Khatab
Maarten J.C. Leijs
Gerben M. van Buul
Joost Haeck
Nicole Kops
Michael Nieboer
Pieter K. Bos
Jan A.N. Verhaar
Monique R. Bernsen
Gerjo J.V.M. van Osch

ABSTRACT

Mesenchymal stem cells (MSC) are promising candidates for use as a biological therapeutic. Since locally injected MSC disappear within a few weeks, we hypothesize that efficacy of MSC can be enhanced by prolonging their presence. Previously, encapsulation in alginate was suggested as a suitable approach for this purpose. We found no differences between the two alginate types, alginate high in mannuronic acid (High M) and alginate high in guluronic acid (High G), regarding MSC viability, MSC immunomodulatory capability, or retention of capsule integrity after subcutaneous implantation in immune competent rats. High G proved to be more suitable for production of injectable beads. Firefly luciferase-expressing rat MSC were used to track MSC viability. Encapsulation in high G alginate prolonged the presence of metabolically active allogenic MSC in immune competent rats with monoiodoacetate-induced osteoarthritis for at least 8 weeks. Encapsulation of human MSC for local treatment by intra-articular injection did not significantly influence the effect on pain, synovial inflammation, or cartilage damage in this disease model. MSC encapsulation in alginate allows for an injectable approach which prolongs the presence of viable cells subcutaneously or in an osteoarthritic joint. Further fine tuning of alginate formulation and effective dosage might be required in order to improve therapeutic efficacy depending on the target disease.

Keywords Cell encapsulation, Cell therapy, Alginate, Mesenchymal stem cells, *In vivo* longitudinal imaging.

Statement of significance

We describe the evaluation of a method to encapsulate human mesenchymal stem cells in small, injectable hydrogel beads. Alginate hydrogel is used as a carrier and protective barrier for stem cells, thus improving the therapeutic use of (allogeneic) stem cells—based on their known capacity to secrete factors that modulate the diseased environment. The work contains extensive *in vitro* and *in vivo* evaluations of survival and functionality of the encapsulated cells. With a novel *in vivo* imaging approach, we longitudinally followed the fate of the beads. Next to their use in osteoarthritis, which we evaluated in our final tests, this can be used for other local degenerative diseases such as myocardial infarction, macular degeneration, or diabetic ulcers.

INTRODUCTION

Application of mesenchymal stem cells (MSC) is promising due to their ability to influence their (micro-)environment by secreting trophic mediators[1-6]. These secreted factors have been demonstrated to counteract inflammatory and catabolic processes and attract endogenous repair cells in various pathological conditions[6-9]. MSC-secreted factors have been shown to improve cardiac function after myocardial infarction in pigs[6], improve lower limb movement after spinal cord ischemia in rats[10], ameliorate limb ischemia in mice[11], and reduce pain in a murine osteoarthritis (OA) model[12]. Previously, it was demonstrated that although injection of MSC has beneficial effects, the MSC themselves are no longer detectable 3 weeks after intra-articular injection[13,14]. We hypothesize that the efficacy of MSC can be enhanced by prolonging their local presence by enabling longevity through encapsulation in a biomaterial.

Alginate is widely used in tissue engineering and drug delivery because of its biocompatibility, stability, non-antigenicity, and chelating ability[15,16]. This commonly used gel for cell encapsulation provides protection of the encapsulated cells against the host's immune system, and at the same time retains cells at the desired location, by acting as a mechanical barrier. The increased cell retention and cell survival can result in an enhanced therapeutic efficacy at the local site of the disease[17,18]. Besides providing a barrier for cells, alginate allows for the release of growth factors and cytokines produced by the encapsulated cells to the microenvironment and vice versa. Cytokines from the microenvironment can reach the encapsulated cells. This provides a setting for dynamic cross talk between cells and their environment[16,19,20]. Furthermore, by encapsulating cells in alginate, we may create a safer way for using allogeneic cells as an alternative to autologous grafts by shielding them from the host's immune system[21-23]. This would greatly enhance the clinical translatability of MSC-based therapies. We have previously shown that allogenic MSC encapsulated in alginate could survive locally after subcutaneous implantation *in vivo* and could act as an interactive immunomodulatory release system for at least 5 weeks *in vitro*, hereby emphasizing the possible advantages of this approach[23].

The variety in composition and production methods of different alginates has a major effect on its biocompatibility, stability, non-antigenicity, and chelating ability[15]. Therefore, the first objective of this work was to find the most suitable clinical grade alginate for MSC encapsulation to enable their longevity *in vivo*, while maintaining anti-inflammatory and tissue-modulating capacities. Alginate consists

of a combination of β-D-mannuronic acid and α-L- guluronic acid. We compared two alginates, one consisting of a high concentration of β-D-mannuronic acid (High M alginate) and the other with high concentration of α-L-guluronic acid (High G alginate). The alginates were evaluated regarding their effect on cell survival, preservation of immunomodulatory function of the MSC, and histocompatibility using a set of *in vitro* assays and *in vivo* tests. One alginate formulation was selected to reproducibly produce small beads of injectable size. Then, we tested the prolonged presence of MSC and alginate microcapsules as well as their therapeutic efficacy in a local disease model.

Injection of MSC has been shown to diminish several features of osteoarthritis (OA) in pre-clinical and some initial clinical studies[24-29]. OA is a degenerative disabling joint disease, characterized by loss of cartilage integrity, subchondral bone changes, formation of osteophytes, and inflammation of the synovial membrane[30]. Unfortunately, to this date, no curative treatment for OA exists, while OA is a growing problem in society, already affecting over 10% of individuals aged 60 years or older[30]. We evaluated whether encapsulation in alginate could prolong the local presence of allogeneic MSC in an immunocompetent rat OA model, using longitudinal bioluminescence imaging (BLI) and we followed the structural integrity of the alginate beads after injection in the knee of rats via longitudinal MRI. Since pain and functional disability are the main reasons for patients to seek medical treatment, we evaluated the efficacy of encapsulation of MSC in alginate beads to reduce pain as well as cartilage damage and synovial inflammation in a rat model of OA.

MATERIALS AND METHODS

Expansion of rat and human mesenchymal stem cells

Allogeneic rat MSC (rMSC) were used for cell tracking experiments *in vivo*. rMSC were isolated (with ethical approval under animal ethical no. EMC 116-12-08) from 3 to 4 months old male Lewis rats (Janvier labs) as described elsewhere and expanded up to passage 3[31], to be used for subcutaneous *in vivo* experiments. For *in vivo* cell tracking experiment in the joint, we used allogeneic F344 rat MSC (Millipore, Billerica, MA) that were transduced to express firefly luciferase (r(Fluc) MSCs) as described before[29,32].

Human bone marrow MSC (hMSC) were used to evaluate therapeutic efficacy *in vitro* and *in vivo*. Cells were derived from 6 patients undergoing total hip replace-

ment (mean age 49 ± 11.2 years; F:M ratio, 1:1) by needle aspiration after written informed consent and approval by the medical ethical committee (Erasmus MC protocol METC-2004-142 and Albert Schweizer Hospital protocol 2011-07). Bone marrow cells were plated at 50,000 cells/cm^2 and after 24 h flasks were washed to remove non-adherent cells and cells were further cultured and expanded as described below for a maximum of 4 passages.

For cell expansion, both rat and human MSC were seeded at a density of 2300 cells/cm^2 in cell culturing flasks, in expansion medium consisting of Minimal Essential Medium Alpha (αMem; Gibco, Rockville, USA), 10% heat-inactivated Fetal Calf Serum (FCS; Gibco, Rockville, USA), 1.5 µg/ml fungizone (Invitrogen, Carlsbad, USA), 50 µg/ml gentamicin (Invitrogen, Carlsbad, USA), 25 µg/ml ascorbic acid-2-phosphate (Sigma-Aldrich, Saint Louis, USA), and 1 ng/ml Fibroblast Growth Factor 2 (FGF2; AbD Serotec, Oxford, UK). Cells were cultured in an incubator at 37 °C, 5% CO_2, and 90% humidity. Medium was renewed twice a week. When MSCs were approximately 70% confluent, they were passaged by trypsinization of cells with a 0.25% trypsin/EDTA solution (Life Technologies, Waltham, USA).

Preparation of MSC-alginate constructs

Clinical-grade high mannuronate (M) alginate (*Laminaria pallida*) and high guluronate (G) alginate (*Laminaria hyperborea*) (respectively; Lot no. E01 AAL- 070912 and Lot no. C01 AAL-110808 both kind gifts of BTG/CellMed AG, Alzenau, Germany) were used. Both alginates were diluted in a 0.5%, 1.1%, and 2.5% concentration in NaCl 0.9% and filter-sterilized afterwards. The shear-dependent viscosity of the solutions was measured by a rheometer Physica MCR301 (Anton Paar GmbH, Ostfildern, Germany) at room temperature (20 °C). The viscosity was measured in a shear rate range of 1–5000 s^{-1} by increasing the shear rate every 5 s for a duration of 2 min and 45 s. Data were analyzed with Rheoplus Software version 3.4 (Anton Paar GmbH, Ostfildern, Germany). For 1.1% High M alginate, the low shear viscosity at 20 °C was found to be 1320 mPa s; for 1.1% High G alginate, the low shear viscosity at 20 °C was 274 mPa s. The effect of shear stress on the viscosity was similar for both alginates.

Prior to encapsulation, MSC were washed with saline. A homogeneous solution of 4.0×10^6 MSC per 1 ml filter-sterilized 1.1% High M alginate or 1.1% High G alginate was prepared. This cell density was selected after a series of tests comparing 0.4, 4, and 20 million cells/ml, indicating that 4 million cells/ml was the most efficient cell number in terms of cell viability and immunomodulatory properties during 2 weeks encapsulation in alginate *in vitro* (data not shown).

Beads of approximately 2 mm in diameter were created by manually dripping the MSC-alginate mixture through a 23-gauge needle in 102 mM $CaCl_2$ solution for 10 min. After incubation, beads were washed two times for 5 min with saline before further use in *in vitro* experiments.

For subcutaneous implantation, alginate disks were created by polymerization of the rMSC-alginate solution which took place in a sterilized, custom-designed mold consisting of two durapore membranes (5-µm pore size, Millipore) at both sides of a 3-mm thick metal ring[33]. After 30 min in 102 mM $CaCl_2$, the construct was washed two times in saline and 8-mm diameter constructs were made with sterile dermal punches (Spengler, Hanover, Germany).

To produce smaller beads in a more reproducible way, we used the Buchi Encapsulator B-395 Pro (Buchi Labortechnik AG, Flawil, Switzerland). After optimizing the settings, beads of approximately 300 µm in diameter were made from 1.1% High G alginate with the following machine settings: flow rate 3 ml/min, nozzle size 150 µm, frequency 1600 Hz, voltage 730 V, stir-rate 30% speed. To be able to track the alginate beads using MRI *in vivo*, we solidified the alginate solution with 102 mM $CaCl_2$ with 20 mM gadolinium (III) chloride hexahydrate (Lot no. MKBJ3153V, Sig- ma-Aldrich, St. Louis, USA). Beads were kept in this solution for 10 min, then washed twice with saline solution, and kept for a maximum of 4 h in saline prior to injection.

In vitro characterization of MSC-alginate constructs

Three hMSC-alginate beads were placed in 24-well plates in 900 µl of medium consisting of αMem with fungizone (1.5 µg/ml), gentamicin (50 µg/ml), 1% insulin-transferrin-selenium (ITS; Biosciences, New Jersey, USA), and 0.1 mM vitamin C (Sigma, St. Louis, MO). Medium was refreshed twice a week. Beads were harvested directly after encapsulation and washing with saline (T = 0), after 1 week (T = 1), and 2 weeks of culture (T = 2) to determine cell viability and immunomodulatory capacity.

Cell viability

Survival of encapsulated hMSC was measured by the amount of DNA and LIVE/DEAD® assay at T = 0 and T = 2 weeks (using cells from 2 different bone marrow donors). For DNA analyses, six beads were harvested at each time point and dissolved in 150 µl/bead. Sodiumcitrate buffer (150 mM NaCl (Sigma-Aldrich, St. Louis, 55 mM Na-citrate (Sigma-Aldrich), 20 mMEDTA (Sigma-Aldrich)) for half an hour at 4 °C. Samples were centrifuged at 180×g for 8 min and pellets were stored

at – 80 °C. Standard curves were made with DNA of hMSC of the same donor before encapsulation. DNA was determined with the CyQUANT® Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The fluorescence measurements were performed on a microplate reader with excitation at 480 nm and emission detection at 520 nm (Spectramax Gemini, Molecular Devices, Sunnyvale, CA, USA).

LIVE/DEAD® assay (Invitrogen, Carlsbad, CA, USA) was performed by incubating MSC-alginate beads for 30 min in 100 µl labelling solution with 1.0 µl/ml green-fluorescent calcein-AM and 1.5 µl/ml red fluorescent ethidium homodimer-1, at 37 °C. Z-stacks were made using an Axiovert 200 MOT fluorescent microscope (Carl Zeiss microscopy, Thornwood, NY, USA) with a thickness of 200 µm per slide. Viable and dead cells were counted in two Z-stacks on two areas of 0.25 mm^2 per Z-stack using ImageJ 1.48 (Java, Redwood Shores, CA, USA).

Immunomodulatory capacity

First, immunomodulatory capacity of the encapsulated hMSC (using cells from 2 different bone marrow donors) was determined by measuring interleukin-6 (IL-6) protein levels and IDO activity. After 2 weeks of culture, hMSC were stimulated with 50 ng/ml IFNγ and 50 ng/ ml TNFα (Peprotech, London, UK). For control, medium without IFNγ and TNFα was added to encapsulated hMSC. After 24 h, conditioned medium was harvested and stored at -80 °C until analyses. IL-6 levels in the stimulated and non-stimulated hMSC conditioned media were measured by ELISA (R&D systems, Abingdon, UK) according to the manufacturer's instructions. IDO activity was determined in the stimulated and non-stimulated MSC conditioned media by the level of its metabolite L-kynurenine. This was measured spectrophotometrically as described previously[34].

The immunosuppressive capacity of encapsulated hMSC was determined in a co-culture with activated lymphocytes. The MSC-alginate beads (using MSC from 1 bone marrow donor) were cultured for 2 days and 29 days and then were stimulated with 50 ng/ml IFNγ and 50 ng/ml TNFα for 24 h. The MSC-alginate beads were washed two times with saline and 4, 2, or 1 bead (approx. 3.0×10^4 hMSC per bead) was transferred in a 48-well plate to obtain a 1:2.5, 1:5, and 1:10 MSC/ peripheral blood mononuclear cells (PBMCs) ratio. PBMCs were isolated with Ficoll-Paque™ PLUS (density 1.077 g/ml; GE Healthcare, Uppsala, Sweden) from buffy coats of healthy blood donors (Sanquin, Rotterdam, The Netherlands) and frozen at – 150 °C until further use. In total, 1.0×10^6 PBMCs/ml were labelled with 1 µM carboxyfluorescein succinimidyl ester (CFSE) and activated with antibodies against CD3

and CD28 (1 µl per 1×10^6 cells in 1 ml, BD Biosciences). As positive and negative lymphocyte proliferation control, activated and non-activated CFSE-PBMCs were used. As a positive control for immunomodulatory capacity of hMSC, 1.2×10^5 hMSC in monolayer were used. After 5 days of co-culture, PBMCs were retrieved, and incubated with CD4 (APC-A; BD Biosciences) and CD8 (PE-CY7-A; BD Biosciences). Proliferation was determined from dilution of CFSE (FITC) staining using 8 colors FACSCANTO-II with FACSDIVA Software (BD Bio- sciences) and FlowJo Software (Tree Star Inc., Palo Alto, CA).

Animal experiments

We performed three separate animal experiments to assess influence of MSC encapsulation on cell longevity and effect of encapsulation on treatment efficacy. These experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. First, we implanted rMSC-alginate (High G and High M) constructs subcutaneously in rats to asses construct integrity and rMSC survival *in vivo* (**experiment A, figure 1**). In the second *in vivo* experiment, we moved to the joint and traced intra-articularly injected r(Fluc)MSC and r(Fluc)MSC-alginate High G beads cross-linked in the presence of gadolinium, over time to prove that we can prolong the presence of rMSC at the desired location (**experiment B, figure 1**). In the third experiment, we studied the therapeutic efficacy of intra-articularly injected hMSC either free or encapsulated in beads (**experiment C, figure 1**). All experiments are explained in further detail below. All experiments were performed on 16-week-old male Wistar rats, weighing 250–300 g (Harlan Netherlands BV, The Netherlands), with approval of the animal ethics committee (protocol no. EMC116-15-02). Rats were housed in groups of two per cage, under 12 h light-dark cycle at a temperature of 24 °C, and had access to water and food ad libitum at the animal testing facilities of the Erasmus MC, University Medical Center. Before the start of the experiments, rats were allowed to acclimatize for a week. All procedures involving subcutaneous implantations, intra-articular injections, or scanning were applied under 2.5% isoflurane anesthesia.

Subcutaneous implantation

The constructs of High G alginate and High M alginate with rMSC were placed in saline and subcutaneously implanted on the back of three rats. Each rat received two constructs of High G alginate with rMSC and one without cells and two constructs of High M alginate with rMSC and one without cells. Directly and 12 h after the operation, the rat got a subcutaneous injection with buprenorphine (Temgesic) 0 .01 mg/kg bodyweight. To track the subcutaneously implanted rMSC, they were labelled 1 day prior to encapsulation in alginate with superparamagnetic iron

oxide (SPIO) using ferumoxides 100 µg/mL medium (Endorem™, Guerbet S.A., Paris, France) complexed to protamine sulphate 5 µg/ml medium (LEO Pharma N.V., Wilrijk, Belgium) as described previously[35]. Imaging of the MSC constructs was done by MR imaging directly after implantation and thereafter weekly up to 5

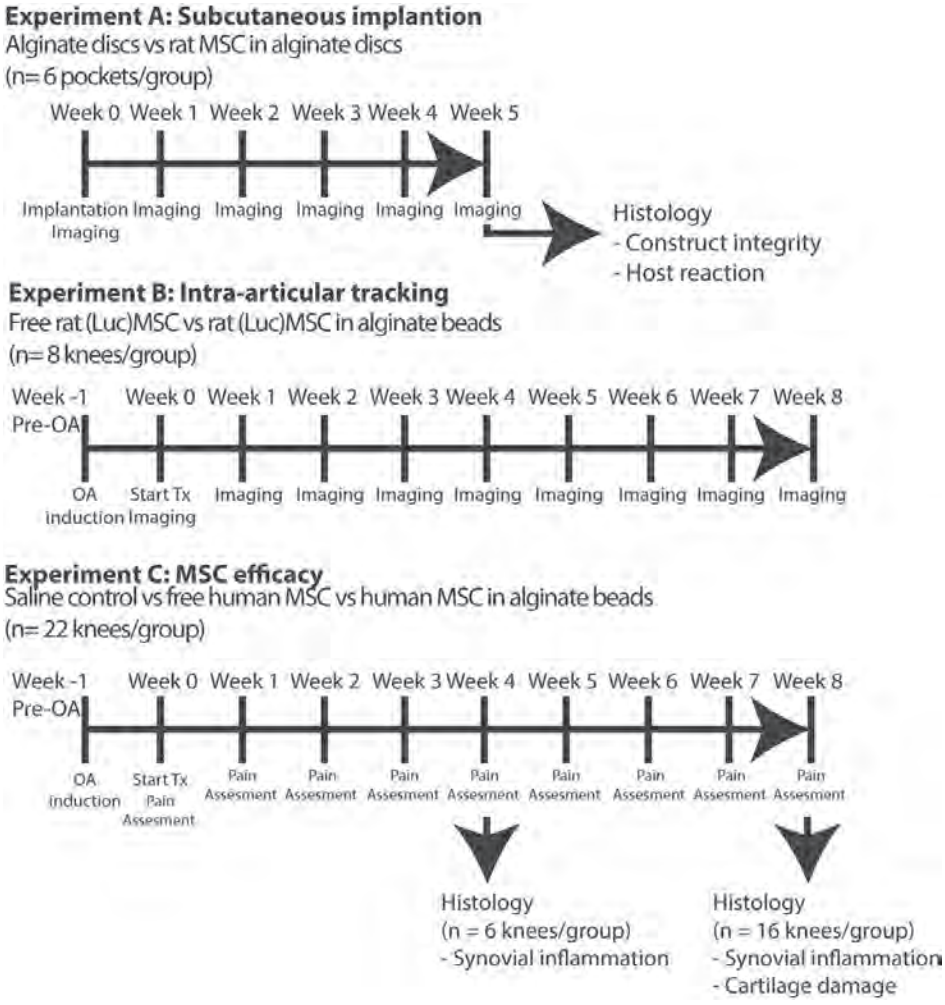


***Figure 1**. Experimental set up of in vivo experiments to evaluate the effect of encapsulation of MSC in alginate on cell viability and efficacy to treat OA. In experiment A, allogeneic rMSC-alginate constructs and empty alginate constructs were implanted subcutaneously in rats to assess construct integrity and MSC survival in vivo. In experiment B, longevity of MSC in an OA knee joint was tested using allogenic r(Fluc) MSC either free or encapsulated in alginate beads. Weekly imaging with MRI for construct integrity and BLI for cell viability followed until the end of the experiment at week 8. In experiment C, the therapeutic efficacy of hMSC in an OA knee joint was studied. hMSC were injected intra-articularly either free or encapsulated in alginate beads and compared with saline control. The effect on pain was measured weekly and knees were harvested for histology at week 4 (synovial inflammation) and week 8 (synovial inflammation and cartilage damage)*

weeks. Five weeks after implantation, the rats were euthanized. The subcutaneous implantation regions were harvested, fixed in 0.05 M Tris-buffered saline with 10 % formalin and 15 mM CaCl for 24 h and embedded in paraffin.

Intra-articular cell tracking experiments

To evaluate bead integrity and the retention of encapsulated cells in a diseased environment that is mechanically loaded, we induced knee OA in rats. Monoiodoacetate (MIA; 300 µg) was intra-articularly injected bilaterally in 25 µl of saline[29] using a 50-µl glass syringe (Hamilton Company, Ghiroda, Romania) and a 27-G needle (Becton, Dickinson and Company, Benelux N.V. Belgium). One week after OA induction (referred to as day 0), rats were randomly divided into two treatment groups: (1) freely injected 1.0×10^5 r(Fluc)MSC (n = 8 knees), and (2) approx. 1.0×10^5 r(Fluc)MSC encapsulated in gadolinium-labelled High G alginate beads (n = 8). Beads and loose cells were both injected in a total volume of 25 µl saline. These injections were done with a 250-µl glass syringe and a custom made 23-G needle (Hamilton Company, Ghiroda, Romania). The choice of this relatively low cell number was based on the assumption that with longer presence of cells the number of cells needed for a therapeutic effect would be lower. In a previous study, we found an analgesic effect of 1.0×10^6 freely injected cells in the same OA model[29].

To follow up cell viability and alginate bead integrity, weekly bioluminescence and MR imaging were performed (methods see below). Animals were scanned once directly after injection of the cells and hereafter once a week for a total of 8 weeks. Animals were euthanized 8 weeks after treatment.

Intra-articular hMSC efficacy experiment

Bilateral OA was induced as described above. One week after OA induction (referred to as day 0), rats were randomly divided into three treatment groups, and rats received in both knees the same treatment, except one animal which received free hMSC in one knee and saline control in the contralateral knee resulting in three groups: (A) saline control (n = 19); (B) 1.0×10^5 freely injected hMSC (n = 19); (C) $0.8 \times 10^5 \pm 0.1 \times 10^5$ hMSC encapsulated in alginate beads (n = 22). MSC from 3 human donors were pooled to take into account the inter-donor variability. Four weeks after treatment, the animals were euthanized to assess the effects of our treatments on synovial inflammation and knee joints were prepared for histological evaluation (n = 6 knees/ group). The remaining animals were euthanized 8 weeks after start of treatment and knee joints were harvested for histological

analysis (n = 16 knees/group). In the latter group, pain was evaluated weekly with mechanical allodynia tests (method see below).

Imaging

Bioluminescence imaging (BLI)

To evaluate the presence of living cells over time, luciferase activity of injected r(Fluc)MSC was measured using the Xenogen IVIS Spectrum (PerkinElmer, Hopkington, MA), 15 min after intra-peritoneal injection of 50 µg beetle luciferin in 150 µl saline (Promega Benelux B.V., Leiden, the Netherlands). Optical intensity is reported as arbitrary units. Data were analyzed using the software Living Image version 3.2 (Caliper LS).

Magnetic resonance imaging (MRI)

MR imaging was performed on a preclinical 7.0-T MRI scanner (MR 901 Discovery, Agilent/GE Healthcare, Milwaukee, WI). For imaging SPIO-labelled rMSC, a 72-mm transmit/receive body coil was used. Image acquisition was performed using a fast spoiled gradient echo sequence with the following parameter settings: TE/TR = 1.1/7.3 ms, NEX = 4, FOV 8 × 6 cm^2, acquisition matrix 256 × 192, slice thickness = 1 mm, bandwidth = 60 kHz, 16 degrees. Sagittal and coronal scans were performed to localize the hypointense SPIO deposits.

For intra-articular localization of alginate beads and to follow up the presence of these beads *in vivo*, we used gadolinium in the alginate beads and scanned with a 150-mm body coil for transmission, and a four-channel cardiac coil (Rapid BiomedGmbH, Rimpar, Germany) for signal reception. A 3D, fast spoiled gradient echo sequence was used to scan the injected rat knees (TE/TR 10.0/30.0 ms, NEX 2, FOV 6.00 × 4.50 cm^2, acquisition matrix 512 × 512, slice thickness 0.50 mm, bandwidth 31.25 kHz, flip angle 16°). The number of beads per knee was counted manually using the built-in DICOM viewer on the scanner (Software build 1094.1, General Electric Healthcare, Milwaukee, WI).

Pain assessment

Hind paw withdrawal reflex was measured with von Frey filaments (Bioseb, France) as an indicator of pain[36]. Animals were habituated to measuring cages and handling by the examiner starting 2 weeks prior to OA induction. The hind paws of the rats were stimulated using a series of von Frey filaments, increasing in strength starting at 0.2 to a maximum of 26 g. If the paw was withdrawn after the administration of the von Frey filament for a minimum of 4/5 times, the strength of the filament was noted. If no reaction was seen after 5 attempts, for a maximum of

3 s each, a stronger filament was used until a response was measured. A baseline measurement was performed after the rats were habituated and just before OA induction. Follow-up measurements were performed 7 days after OA induction, which was just before therapy administration, and thereafter once weekly until the end of the experiment at 8 weeks. All measurements were performed by the same examiner, blinded for the treatment groups, in the same room, with temperature set at 18–20°C and the same background noises present at time of measurement. Measurements were performed at the same time of day.

Histology

Evaluation of subcutaneously implanted MSC-alginate constructs

Paraffin sections (6 µm) were deparaffinised and stained for Perls' iron according to the manufacturer's protocol (Klinipath BVBA, Duiven, The Netherlands) to locate the SPIO-rMSC. SPIO-labelled rMSC are stained blue with Perls'. CD68 and CD3 staining was performed to identify macrophages and T lymphocytes as an indication of a local inflammatory response. Antigen retrieval for CD68 and CD3 was performed through incubation in citrate buffer (10 mM citric acid, 0.05% Tween20, pH 6.0) for 20 min at 90–95 °C. Sections were incubated for 1 h with primary antibodies for CD68 (BM4000 5 µg/ml; OriGene Technologies, Herford) or CD3 (Ab16669, dilution 1:100; Abcam Cambridge, UK) diluted in PBS/1 %BSA (Sigma no. A7284) after blocking of non-specific binding sites with 10 % goat serum (Southern Biotech no. 0060-01) in PBS/1%BSA. A secondary antibody biotinylated goat anti-mouse 1:50 (Biogenex, HK-325-UM) was used, followed by incubation with streptavidin-AP 1:50 (Biogenex, HK-321- UK). Staining was then visualized using an alkaline-phosphatase substrate followed by counterstaining with hematoxylin.

Evaluation of knee joints after MSC-alginate bead injection

Knees were fixed in formalin 4% (v/v) for 1 week, decalcified in 10% EDTA for 2 weeks, and embedded in paraffin, and coronal sections of 6 µm were cut. Sections were collected anterior to posterior every 300 µm to give a good overview of the damage throughout the entire knee. Cartilage damage was evaluated on Safranin O-stained sections, with a scoring system described by Pritzker et al.[37]. Scoring was done on three sections aiming around the midportion of the joint. The Pritzker score ranges from 0–6 for structural damage and 0–4 for GAG staining intensity. These scores were multiplied with a factor 1–4 to account for the percentage of surface affected (factor 1, 0–25%; 2, 26–50%; 3, 51–75%; 4, 76–100% surface area). This led to a maximum score of 24 for structural damage and a maximum of 16 for GAG loss, as described previously by van Buul et al.[29]. The scoring of two blinded observers was averaged and used for data analyses.

Synovial inflammation was evaluated on sections stained with hematoxylin–eosin. The sections were imaged using NanoZoomer Digital Pathology program (Hamamatsu Photonics, Herrsching am Ammersee, Germany), and synovial thickness was measured from the capsule to the superficial layer of the synovial membrane in the parapatellar recesses at the medial and the lateral sides at three positions per section, as previously described Khatab et al. 2018a; Khatab et al. 2018b[12,38]. These measurements were performed on three sections per knee, with 300 µm between the sections. The thickness measurements were averaged to obtain a single value per knee joint.

Statistical analysis

Data was analyzed with IBM SPSS statistics 24 (SPSS, Chicago, IL). To evaluate the *in vitro* data of DNA, live/ dead cell count, IL-6 secretion, IDO activity, and lymphocyte proliferation of MSC-alginate beads, Mann-Whitney *U* tests were performed. To evaluate the number of alginate beads on MRI scans of rat joints over time, a Wilcoxon signed-rank test was performed, since data did not meet requirement for normality with the Shapiro-Wilk test. To compare fluorescence intensity of r(Flu) MSC in the free MSC group vs. the MSC-alginate group, a Mann-Whitney *U* test was performed, since data did not meet the requirement of equal distribution and normality with the Shapiro-Wilk test. To evaluate the fluorescence intensity within groups over time, a Wilcoxon signed-rank test was performed. For treatment effects on pain, all groups were compared using a linear mixed model in which measurement time point and treatment were considered fixed factors and withdrawal threshold a dependent factor. After significance was confirmed, a one-way ANOVA was performed to determine differences between groups. To determine differences over time per treatment, a linear mixed model analysis was performed in which measurement time point was considered a fixed and withdrawal threshold a dependent factor. Post hoc analysis using Bonferroni correction was performed.

For synovial inflammation, homogeneity of variances and normality were confirmed with a Shapiro-Wilk test. Next an one-way ANOVA was performed; post hoc analyses were performed and Bonferroni correction was applied.

For non-parametric cartilage scoring data, Mann-Whitney *U* tests were used to assess MIA or measurement time point effects. Kruskal Wallis tests were used for treatment effects within time points. Post hoc analyses were performed by Bonferroni correction. For all tests, p values < 0.05 were considered statistically significant.

RESULTS

MSC remain viable and immunomodulatory active in both clinical grade High M alginate and High G alginate

The amount of DNA measured in the beads after 2 weeks was 45.4% in High M alginate (p = 0.01) and 57.4% in High G alginate (p = 0.04) of the amount at the moment of encapsulation (**figure 2a**). No significant difference was found in the amount of DNA between High M alginate and high G alginate constructs. The number of viable cells was not significantly different between High M and High G alginates directly after encapsulation or after 2 weeks in culture (**figure 2b**). hMSC encapsulated in either alginate retained their immunomodulatory capacities when stimulated with IFNγ and TNFα. This stimulation induced IL-6 secretion (**figure 2c**) and IDO activity (**figure 1d**) from the encapsulated MSC irrespective of the type of alginate used. Alginate-encapsulated hMSC significantly inhibited proliferation of stimulated CD4+ and CD8+ T lymphocytes. Three days after encapsulation hMSC encapsulated in High G and High M alginates (**figure 2e,f**) significantly inhibit T lymphocyte proliferation in a dose-dependent manner (all p = 0.024). Thirty days after encapsulation, inhibition was reduced but in particular still present in High G alginate when four and two beads were used (p = 0.024) (**figure 2f**). Empty constructs of alginate had no effect on T cell proliferation. The inhibition by 1.2 × 10^5 hMSC in monolayer was similar to the inhibition of 4 alginate constructs, containing a similar number of MSC on day 0.

No difference in construct integrity and MSC retention after *in vivo* implantation of encapsulated allogeneic MSC in High M alginate and High G alginate

Subcutaneously implanted alginate-encapsulated SPIO-MSC remained clearly visible on MR images over 5 weeks (**figure 3a,b**) and where clearly visible macroscopically upon explantation (**figure 3c**) without noticeable differences between high M and high G alginate constructs. As observed in histological sections, there was good integrity of the constructs (**figure 3d–g**) and a homogenous distribution of SPIO-labelled cells in alginate constructs (**figure 3h–k**). The rat tissue surrounding the constructs showed very limited foreign body reaction without cell infiltration of macrophages (CD68; **figure 3h–k**) or T lymphocytes (CD3; **figure 3l–o**). No differences in construct morphology or foreign body reaction were observed between High M alginate and High G alginate.

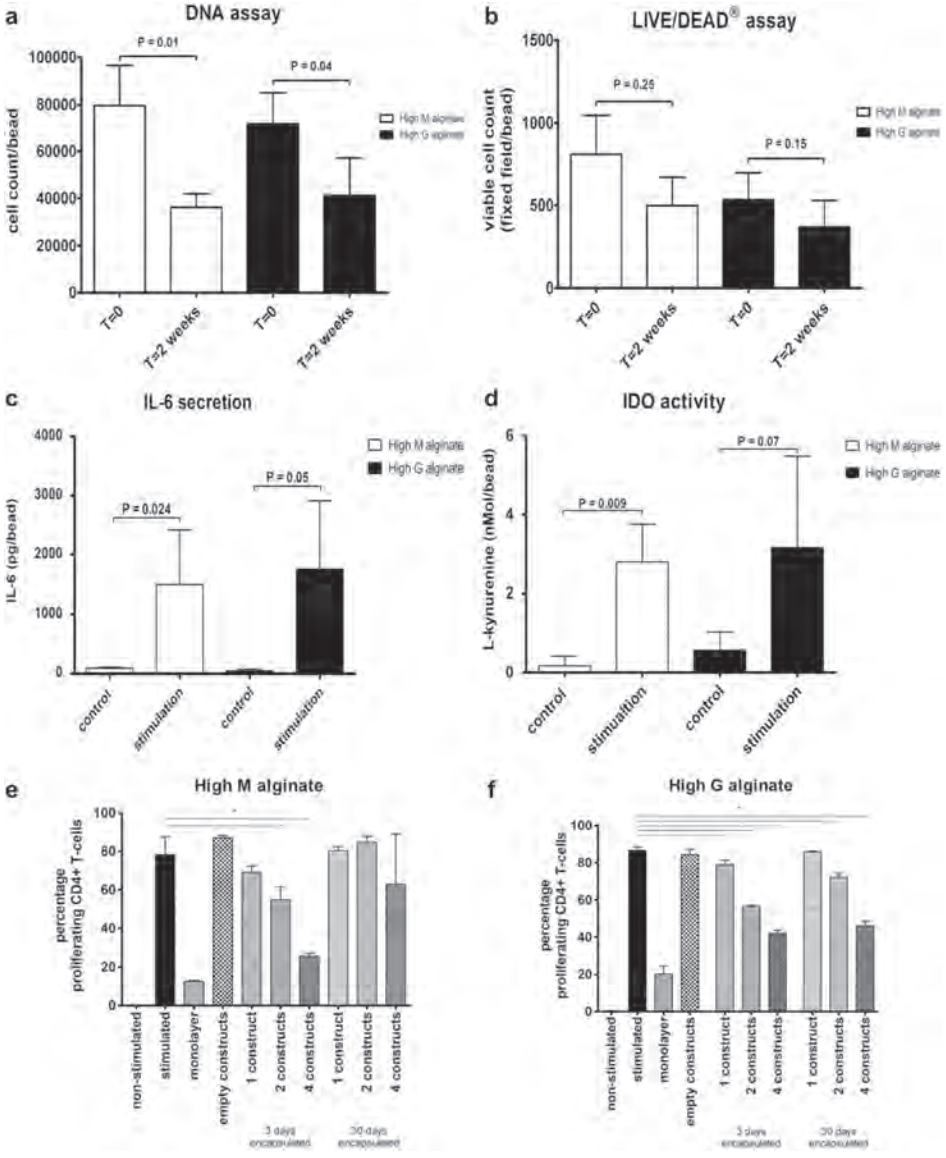


***Figure 2**. Viability and immunomodulatory capacity of encapsulated MSC in High G alginate and High M alginate. **a** DNA amount directly after encapsulation or after 2 weeks. **b** Number of viable cells directly after encapsulation and after 2 weeks. **c** IL-6 secretion and **d** IDO activity measured as concentration of L-kynurenine in the medium after stimulation with IFNγ/TNFα (**a–d** all performed with hMSC of 2 different donors with 3 samples per donor). Activated CD4+/CD8+ T lymphocytes co-cultured with one, two, and four hMSC-alginate constructs with **e** High M or **f** High G alginate, 3 days and 30 days after encapsulation of hMSC (performed in triplicate with samples of 1 hMSC donor and 1 PBMC donor). First bar: non-stimulated PBMCs; positive control. Second bar: stimulated PBMC without alginate constructs. Third bar: stimulated PBMC in the presence of 1.2 × 10^5 hMSC in monolayer. Fourth bar: stimulated PBMC in the presence of empty alginate constructs. Mean ± SD is shown * indicates statistical significance.*

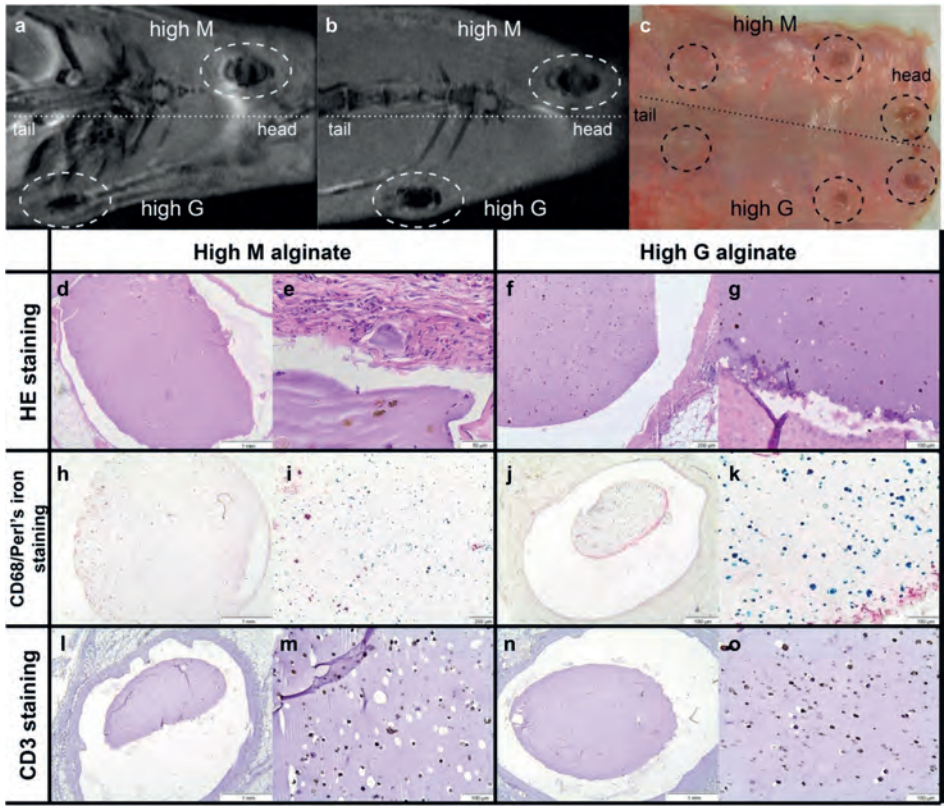


***Figure 3**. Subcutaneous implanted allogeneic rMSC in immunocompetent rats. **a** MRI image directly after implantation and **b** MRI image of the same animal 5 weeks after implantation. Alginate constructs are visible due to the labelled SPIO cells in the constructs. **c** After 5 weeks, the constructs were clearly visible after removal of the skin postmortem. **d–g** Hematoxylin and eosin-stained tissue sections of biopsies taken at the site of the SPIO-labelled-MSC-containing construct implants of high M and high G alginate constructs. **h–k** Staining of corresponding sections shown in **d–g** with Perl's iron staining (blue), which stains SPIO combined with CD68 staining (red) to stain macrophages. **l–o** CD3 staining (red) to stain T lymphocytes (black dots in cells represent SPIO particles).*

Alginate encapsulation using a micro-encapsulator results in small injectable beads with vital MSC

After optimizing the settings of the encapsulator device, we were able to produce homogenous beads of 0.3 mm using High G alginate. With the more viscous High M alginate, the beads were larger and the size was less homogenous. We decided to continue with High G alginate. The average bead size produced with High G alginate was 284 ± 28 µm, with each bead containing 112 ± 32 MSC (**figure 4a,b**). To confirm that the anti-inflammatory capacity of the hMSC was not affected by the procedure with the micro-encapsulator, we performed an IDO assay on the secretome of the stimulated hMSC. We compared hMSC in monolayer vs. hMSC encapsulated in alginate beads (n = 3 donors). Encapsulated hMSC displayed simi-

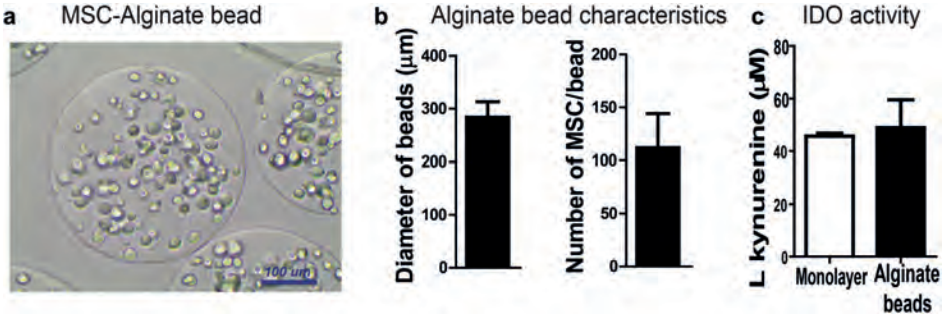


Figure 4. *Characteristics of MSC-alginate beads produced with micro-encapsulator device.* ***a*** *hMSC-alginate beads prepared with the micro-encapsulator.* ***b*** *The average diameter of the alginate beads and number of hMSC/bead.* ***c*** *Concentration of L-kynurenine as measure of IDO activity corrected for the number of cells in the secretome of MSC stimulated with TNFα/IFNγ.*

lar IDO activity compared with hMSC in monolayer (L-kynurenine concentration; 48.91 ± 10.67 µM vs. 45.63 ± 1.17 µM respectively using equivalent numbers of cells) (**figure 4c**). This indicates that after cell encapsulation, hMSC maintained anti-inflammatory capacities.

Intra-articularly injected MSC-alginate beads remain present and metabolically active in the joint for at least 8 weeks *in vivo*

Unfortunately, one rat of the group with alginate died during imaging at day 0 probably due to anesthesia related issue and the results of these knees were excluded from analyses. The other animals were longitudinally followed by imaging in MRI and BLI during 8 weeks.

To track the MSC-alginate beads *in vivo*, alginate was cross-linked with gadolinium ions which are visible on MRI. At baseline, the number of alginate beads per knee was 73 ± 36 (**figure 5a,b**). A majority of the alginate beads were located in the suprapatellar pouch. On follow-up scans, the alginate beads appeared more dispersed throughout the joint. The number of beads decreased to 46 ± 34 per knee at week 4 (p = 0.028 compared with week 0), and remained stable afterwards until the end of the experiment at week 8 (37 ± 20). To track long-term cell activity after intra-articular injection, we used bioluminescence (BLI) scanning of allogeneic r(Fluc)MSC that were either encapsulated in alginate beads before injection (n = 6 knees) or freely injected in the knee (n = 8). The first scan was preformed immediately after injection (**figure 5c**) and subsequently scanned repeatedly until week 8. The BLI signal in the r(Fluc)MSC-alginate group was lower than expected based on cell number at day 0, most likely due to impaired metabolic activity of the cells shortly after encapsulation in alginate which is supported by a higher BLI signal after 2 weeks. BLI signal decreased significantly from week 2 to week 3 (p =

0.028) but remained stable hereafter ($p > 0.293$). From week 3 on, the fluorescence in the r(Fluc)MSC-alginate group was significantly stronger than that in the free r(Fluc)MSC group ($p < 0.04$ for all time points (**figure 5c,d**).

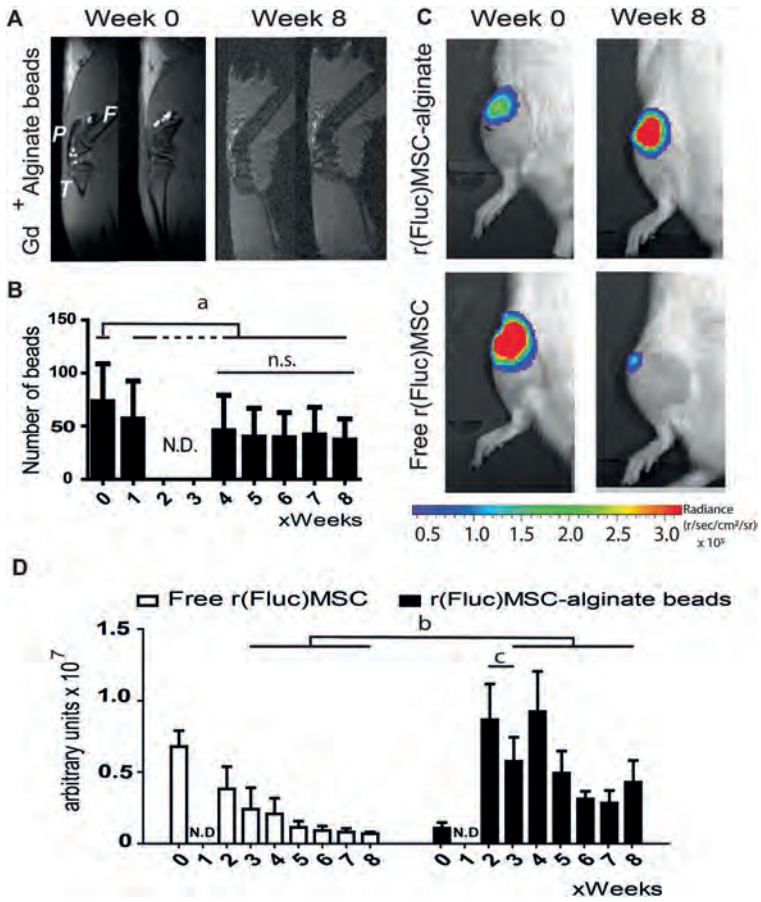


***Figure 5**. In vivo cell tracking. **A** MRI of rat knee joints injected with gadolinium-labelled alginate beads, directly after intra-articular injection and after 8 weeks. **B** Quantification of the number of alginate beads per joint over time. (Due to technical problems with the MRI scanner, week 2 and 3 scans were not available). **C** BLI of free r(Fluc)MSC and r(Fluc)MSC-alginate bead directly after injections and after 8 weeks. **D** Quantification of BLI signal over time (due to technical problems with the IVIS, week 1 scans were not available). The images shown in **A** and **C** are representative animals for each group. In **A**: P = patella, F= femur, T = tibia. In **D**: white bars = free (Fluc)MSC and black bars = r(Fluc)MSC-alginate beads. (**b**, p< 0.04; **c**, p = 0.028). n = 8 knees for free r(Fluc)MSC and n = 6 knees for r(Fluc)MSC-alginate group. N.D. = not determined due to technical error. n.s. = not significant.*

Encapsulation in alginate did not improve effect of hMSC on pain, cartilage damage, or synovial inflammation

To test the efficacy of the hMSC-alginate beads as therapy for osteoarthritis, we assessed the effect on pain reduction, cartilage damage, and synovial inflammation in a rat OA model. Pain was assessed by means of tactile allodynia using the von Frey filaments. Prior to MIA injection, all the animals had comparable withdrawal thresholds. One week after MIA injection and before treatment, all three treatment groups (saline control, free hMSC, and hMSC-alginate beads) showed a significant decrease in withdrawal threshold ($p < 0.02$), indicating pain as a result of MIA injection. One week after treatment, only the animals in the saline control group showed an additional significant decrease in withdrawal threshold compared with the time point just before treatment ($p = 0.001$), indicating exacerbating pain over time. No increase in sensitivity to pain stimulus was observed in the free hMSC or hMSC-alginate beads group. Although rats in the free hMSC group showed a trend toward less pain in time, a significant difference compared with the saline-treated group was only reached at the end of the experiment at week 8 (saline control vs. free hMSC, p=0.036).The hMSC-alginate beads group was not significantly different from saline control or free hMSC at week 8 (resp. p=0.404 and p = 0.722), or any other week (**figure 6a,b**).

Cartilage damage was scored 8 weeks after treatment on the femorotibial compartment of the joint as well as the patella using a modified Pritzker score method. Mild osteoarthritic changes were present in all groups. There were no significant differences in cartilage damage or GAG loss between treatment groups (**figure 6c–f**).

As an indicator of inflammation, we performed thickness measurements of the synovial membrane at the parapatellar recesses at 4 and 8 weeks after start of treatment (**figure 6g,h**). No significant differences between groups were found at week 4 ($p= 0.198$). The hMSC-alginate group showed a trend toward a thicker membrane at week 8 ($p = 0.058$) and more infiltration of inflammatory cells next to encapsulation of alginate remnants (black arrows in **figure 6i,j**) compared with the saline control and free hMSC group. To examine if alginate would induce inflammation in the joint, we injected empty alginate beads intra-articularly in 2 additional healthy rat knees. One week after injection, synovial inflammation was seen on histology, characterized by synovial hypercellularity and encapsulation of the alginate beads, indicating a mild foreign body reaction against the alginate (**figure 7**).

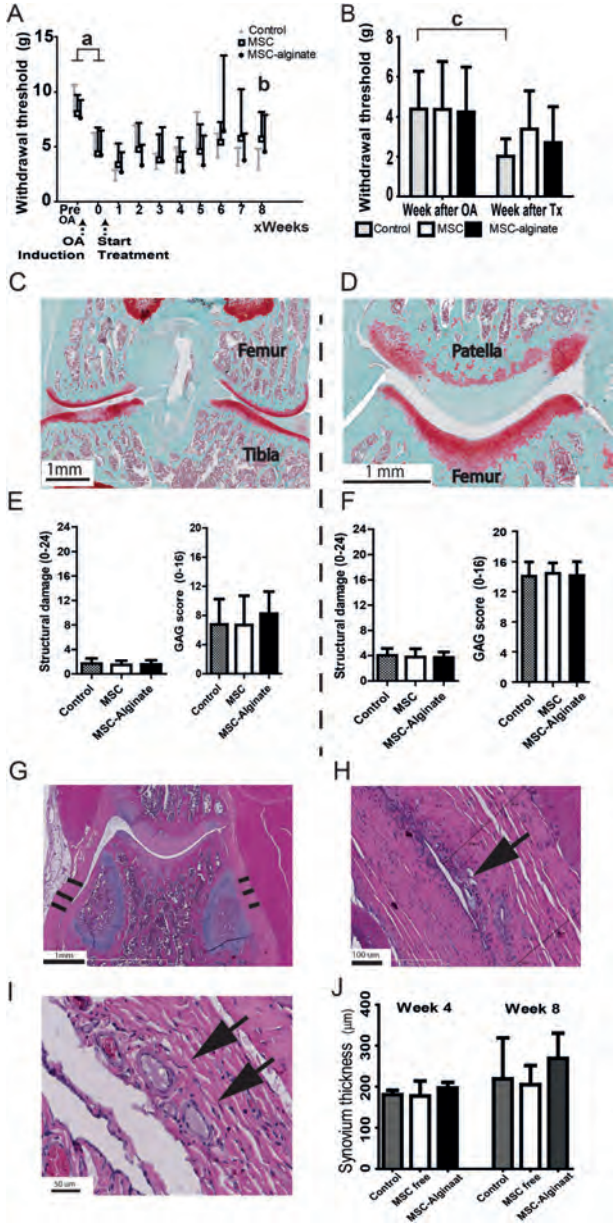


Figure 6. *Therapeutic efficacy of MSC-alginate beads in a rat OA model.* ***A*** *Hind limb withdrawal threshold as measure of pain over time.* ***B*** *Withdrawal threshold 1 week after treatment.* ***C*** *A representative example of the Safarin-O staining at the femorotibial compartment and* ***D*** *at the patellofemoral compartment.* ***E*** *The structural damage according to the Prizker score and GAG loss for femorotibial;* ***F*** *structural damage and GAG loss in patella. The maximum score for structural damage was 24 and for GAG loss 16, in which a higher score represents more damage.* ***G*** *HE staining of parapatellar recesses and indication of synovial membrane thickness.* ***H–I*** *Some degradation and encapsulation of alginate was observed (black arrows).* ***J*** *Quantification of synovial thickness over time (**a** $p < 0.02$, **b** $p = 0.036$, **c** $p = 0.001$). All data shown as mean ± SD. At week 4, n = 5 knees/group; week 8, n = 16 knees/group.*

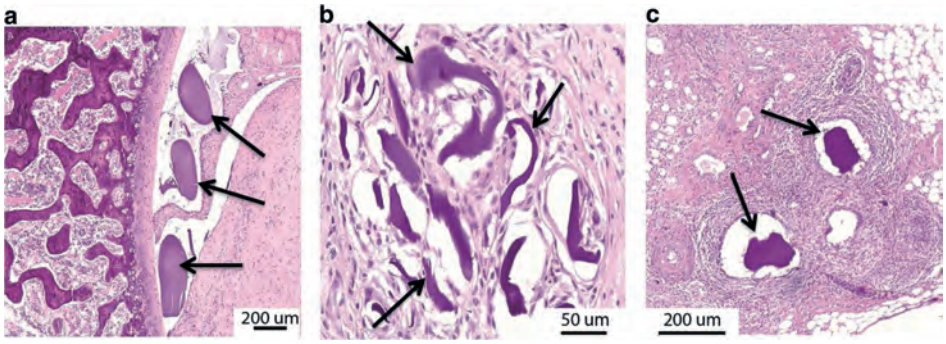


***Figure 7**. Empty alginate microbeads in healthy rat knees. HE staining one week after injection. (**a+b**) synovial thickening, encapsulation of the alginate beads. (**c**) hypercellularity in the synovium. Arrows indicate alginate. The shape of the beads may have changed due to the processing for histological analysis.*

DISCUSSION

MSC have previously been described to have a beneficial effect in regenerative medicine, both in preclinical and some initial clinical studies, although evidence for long-term engraftment is low[4,13,14,29,39]. This led Prockop et al.[4] and von Bahr et al.[39] to postulate the "hit-and-run" mechanism which proposes the cells to only have a short interaction with the micro-environment. The design of the current study is based on the idea that the therapeutic efficacy of MSC could be enhanced by prolonging the local presence of MSC and their secreted factors at the desired location. To achieve this purpose, we encapsulated MSC in alginate and demonstrate that the cells remained viable in this carrier, and are protected against the allogeneic immune system and retained immunomodulatory capacity when stimulated by external cytokines or immune cells. Moreover, we demonstrate retention of construct integrity *in vivo* over time by longitudinal MRI. For this purpose, gadolinium was used to cross-link the alginate. By combining MRI with BLI of constructs that contained luciferase transfected cells, we showed that encapsulation of MSC is beneficial for *in vivo* cell survival and that it prolonged their local presence in a diseased and inflamed environment.

We used two types of alginate to encapsulate cells, both were clinical grade but differed in composition with respect to the ratio of guluronate and mannuronate. With both alginate types, MSC retained their immunomodulatory capacity *in vitro*. The results are similar to our previous study where we used a different type of alginate that had a low viscosity and less well-defined composition[23]. As there is a great variability in the ratio of mannuronate and guluronate between different types of alginate that are (commercially) available, our work in which we used high-quality GLP produced High G and High M alginates demonstrates that a wide

range of alginates might be suitable for encapsulation. Different alginates have different viscosities which can greatly influence the mechanical properties of the construct and thus its integrity and the infiltration of cells. After subcutaneous implantation in immune competent rats, constructs of High G and High M alginates remained intact with a thin capsule formed around the construct. There was no infiltration of immune cells in the alginate. We took the alginate-encapsulated MSC a step further by evaluating them in a diseased situation: in our case, in rat knees after induction of osteoarthritis. To provide an injectable therapy, we optimized a protocol using a machine for encapsulation that enabled reproducible generation of a homogeneous population of MSC-alginate microbeads, with an average diameter below 300 µm. The size of these constructs contributes to easy clinical application since they are small enough to pass through a 23G needle that can be used for most clinical applications.

The use of gadolinium, with its contrast properties in MR imaging[40], made it possible to monitor localization and integrity of the alginate constructs over time. Gadolinium was incorporated in the guluronate or mannurate molecules upon polymerization, and loss of gadolinium signal was attributed to loss of construct integrity. Quantification with MRI of the Gd-labelled beads indicated an initial loss of some beads with subsequent retained visible presence of approximately half of the alginate beads up to the end of the experiments at 8 weeks post-injection. Although we cannot exclude that the loss of Gadolinium signal is caused partly by diffusion of gadolinium out of the bead, under *in vitro* conditions leakage of gadolinium out of the alginate beads was not seen at all during a 3-week follow-up period (data not shown). Therefore, we assume that lessening of the number of visible beads is due to disintegration of the beads with concomitant release and loss of hMSC. The latter is confirmed by the BLI data that showed a matching decrease in cell signal over time. A substantial part of the cells, however, remained present until the end of the study. Possibly, some beads are lost due to mechanical forces in the joint during movement of the animal. We speculate that this problem might be less in a larger joint where the beads have more space to be distributed to a relatively sheltered position, such as in the suprapatellar pouch, where high loading that occurs between cartilage surfaces can be avoided. The unique option to follow bead integrity on MRI, while having the anatomy of the joint visible in the same image, provides a safe and helpful tool to follow alginate constructs, also in a clinical setting in human, equine, or canine patients. The method might be useful for *in vivo* tracking of other materials that polymerize with divalent cations such as fibrin.

Besides bead and cell tracking to demonstrate prolonged cell presence, we tested therapeutic efficacy of the encapsulated MSC in a rat model for OA. Although we have previously shown MSC retain osteogenic and adipogenic differentiation capacity after 30 days of alginate encapsulation[23], we hypothesize that therapeutic effect of MSCs is mainly by secretion of factors. In previous work, we have shown that multiple intra-articular injections of MSC secretome can inhibit pain and have a protective effect on cartilage damage in a mouse OA model[12]. This confirms that MSC-based treatments can exert their effects *in vivo* by their secretome and do not rely solely on cell–cell contact or their differentiation capacity. In this study, we quantified the stimulation-induced IL-6 secretion and IDO activity from the encapsulated hMSC. This is, however, only a small fraction of the biologically active factors that are secreted by MSC, either soluble or in extracellular vesicles. It is, therefore, important to test the functionality of the secreted factors, which we did by demonstrating that these encapsulated hMSC significantly inhibited proliferation of stimulated CD4+ and CD8+ T lymphocytes in a dose-dependent manner.

Preferably, a continuous interaction and feedback loop between the diseased tissue and the exogenous MSC is created, in order to produce cytokines and growth factors at the right time and in the right concentration. Based on the longer presence, we chose to inject 1×10^5 cells per joint. This number is ten times lower than what we injected previously in the same rat OA model[29]. Possibly as a consequence of that, in our study a therapeutic effect of freely injected human MSC was not detectable. The encapsulated MSC, however, did not do better than the freely injected MSC. This absence of improved therapeutic effect by encapsulation could be due to an insufficient number of cells. Maybe, initially a larger cell number is needed to reduce the inflammation. The small size of the rat joint, however, did not allow injection of more beads. Because preliminary experiments had indicated the density of 4 million cells/ml to be a good balance between concentration of secreted factors and stability of the gel construct, we have not considered using higher cell numbers per bead. Furthermore, we have chosen to use human MSC for this study to increase the clinical translatability of a human allogeneic MSC-alginate construct. A disadvantage of the use of xenogeneic MSC in this setup could be that some important factors and cytokines might not be interspecies conserved. This can cause *in vivo* miscommunication between xenogeneic MSC and the diseased environment. Since we and others have seen anti-inflammatory effects of xenogeneic MSC secretome alone, we can conclude that the secreted factors of xenogeneic MSC are capable of at least achieving anti-inflammatory and chondroprotective effects in OA[38]. Nevertheless, it is still possible that the use of xenogeneic MSC depreciates the full potential of MSC therapy, an issue that could

be tackled by using allogenic MSC. The use of xenogeneic MSC could also explain the discrepancy between our work and the recently published work of Choi et al., showing promising results using allogenic encapsulated MSC in a rabbit OA model, although in that study no cell or construct tracking was performed[19].

The use of alginate encapsulation is promising in the field of regenerative medicine, but it might bring safety and regulatory issues. Although, the fibrous capsule formed around the alginate implants when implanted subcutaneously was very thin and the constructs remained completely intact, upon injection in the joint, we noticed a trend to synovial thickening and the alginate beads were encapsulated in the synovial membrane. This reaction, even though it was not a strong foreign body response, might have dampened the anti-inflammatory effect of MSC and in extension its effect on pain. Since this reaction seemed less strong after subcutaneous implantation of MSC-alginate or empty alginate constructs, it might be caused by mechanical damage to the constructs or the presence of local inflammation in the osteoarthritic joint. If the alginate is compromised and starts to slowly release the xenogeneic hMSC, an adaptive immune response can be initiated, further reducing the therapeutic potential. Although immune privileged, MSC do maintain a degree of immunogenicity[41]. This foreign body reaction might lead to a slow release of xenogeneic MSC out of the alginate, possibly causing a chronic local inflammation. Thus, to limit this reaction, two factors play an important role: the biomaterial (the alginate) and the MSC. Focusing on the biomaterial, it is possible that a different type of alginate could be more resistant to damage in the osteoarthritic joint. This would prevent the release of xenogeneic hMSC, thus the adaptive immune response, and decrease the fibrous tissue formation as seen in our experiments. Another way to decrease this reaction is to use autologous MSC: this would further inhibit the graft vs. host disease. Of course, extensive *in vitro* and *in vivo* experiments are needed to investigate these hypotheses.

In conclusion, we have provided a method to produce a homogenous gadolinium-labelled cell-alginate construct combined with imaging techniques that are suitable for minimal invasive longitudinal follow-up studies in patients. We showed that non-autologous MSC can survive longer and remain metabolically active *in vivo* up to at least 8 weeks when encapsulated in alginate. The possibility to retain non-autologous cells and the production of standardized small beads greatly increased the feasibility of producing cell-alginate microcapsules in a standardized safe way and, on a large scale, giving it the potential of an “off-the- shelf” biological therapeutic option. These are both important additional steps toward clinical applicability. Unfortunately, the overall treatment effect on pain, synovial

inflammation, and cartilage quality in this study could not be confirmed in our *in vivo* OA model, possibly due to specific local tissue responses to the alginate beads or a suboptimal cell number. Our results encourage further development of this strategy to provide an injectable therapy by cell encapsulation that greatly prolongs the interplay between the therapeutic cells and their diseased target tissues, taking into account specific local and disease requirements.

REFERENCES

1. Caplan AI. Mesenchymal Stem Cells: Time to Change the Name! *Stem Cells Transl Med.* 2017;6(6):1445-1451.
2. Kinnaird T, Stabile E, Burnett MS, et al. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation.* 2004;109(12):1543-1549.
3. Le Blanc K, Mougiakakos D. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol.* 2012;12(5):383-396.
4. Prockop DJ. Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. *Mol Ther.* 2009;17(6):939-946.
5. Ren G, Zhang L, Zhao X, et al. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell.* 2008;2(2):141-150.
6. Timmers L, Lim SK, Hoefer IE, et al. Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem Cell Res.* 2011;6(3):206-214.
7. Estrada R, Li N, Sarojini H, An J, Lee MJ, Wang E. Secretome from mesenchymal stem cells induces angiogenesis via Cyr61. *J Cell Physiol.* 2009;219(3):563-571.
8. Prockop DJ, Oh JY. Medical therapies with adult stem/progenitor cells (MSCs): a backward journey from dramatic results in vivo to the cellular and molecular explanations. *J Cell Biochem.* 2012;113(5):1460-1469.
9. van Buul GM, Villafuertes E, Bos PK, et al. Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. *Osteoarthritis Cartilage.* 2012;20(10):1186-1196.
10. Takahashi S, Nakagawa K, Tomiyasu M, et al. Mesenchymal Stem Cell-Based Therapy Improves Lower Limb Movement After Spinal Cord Ischemia in Rats. *Ann Thorac Surg.* 2018;105(5):1523-1530.
11. Huang WH, Chen HL, Huang PH, et al. Hypoxic mesenchymal stem cells engraft and ameliorate limb ischaemia in allogeneic recipients. *Cardiovasc Res.* 2014;101(2):266-276.
12. Khatab S, van Osch GJ, Kops N, et al. Mesenchymal stem cell secretome reduces pain and prevents cartilage damage in a murine osteoarthritis model. *Eur Cell Mater.* 2018;36:218-230.
13. Diekman BO, Wu CL, Louer CR, et al. Intra-articular delivery of purified mesenchymal stem cells from C57BL/6 or MRL/MpJ superhealer mice prevents posttraumatic arthritis. *Cell Transplant.* 2013;22(8):1395-1408.
14. Mak J, Jablonski CL, Leonard CA, et al. Intra-articular injection of synovial mesenchymal stem cells improves cartilage repair in a mouse injury model. *Sci Rep.* 2016;6:23076.
15. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. *Prog Polym Sci.* 2012;37(1):106-126.
16. Sun J, Tan H. Alginate-Based Biomaterials for Regenerative Medicine Applications. *Materials (Basel).* 2013;6(4):1285-1309.
17. Levit RD, Landazuri N, Phelps EA, et al. Cellular encapsulation enhances cardiac repair. *J Am Heart Assoc.* 2013;2(5):e000367.
18. Serra M, Correia C, Malpique R, et al. Microencapsulation technology: a powerful tool for integrating expansion and cryopreservation of human embryonic stem cells. *PLoS One.* 2011;6(8):e23212.

19. Choi S, Kim JH, Ha J, et al. Intra-Articular Injection of Alginate-Microencapsulated Adipose Tissue-Derived Mesenchymal Stem Cells for the Treatment of Osteoarthritis in Rabbits. *Stem Cells Int.* 2018;2018:2791632.
20. Shoichet MS, Li RH, White ML, Winn SR. Stability of hydrogels used in cell encapsulation: An in vitro comparison of alginate and agarose. *Biotechnol Bioeng.* 1996;50(4):374-381.
21. de Vos P, Faas MM, Strand B, Calafiore R. Alginate-based microcapsules for immunoisolation of pancreatic islets. *Biomaterials.* 2006;27(32):5603-5617.
22. Duvivier-Kali VF OA, Parent RJ, O'Neil JJ, Weir GC. . ion of islets against allorejection and autoimmunity by a simple barium-alginate membrane. . *Diabetes.* 2001;2001;50:1698–1705.
23. Leijs MJ, Villafuertes E, Haeck JC, et al. Encapsulation of allogeneic mesenchymal stem cells in alginate extends local presence and therapeutic function. *Eur Cell Mater.* 2017;33:43-58.
24. Gupta PK, Chullikana A, Rengasamy M, et al. Efficacy and safety of adult human bone marrow-derived, cultured, pooled, allogeneic mesenchymal stromal cells (Stempeucel(R)): preclinical and clinical trial in osteoarthritis of the knee joint. *Arthritis Res Ther.* 2016;18(1):301.
25. Lamo-Espinosa JM, Mora G, Blanco JF, et al. Intra-articular injection of two different doses of autologous bone marrow mesenchymal stem cells versus hyaluronic acid in the treatment of knee osteoarthritis: multicenter randomized controlled clinical trial (phase I/II). *J Transl Med.* 2016;14(1):246.
26. Murphy JM, Fink DJ, Hunziker EB, Barry FP. Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum.* 2003;48(12):3464-3474.
27. Pers YM, Rackwitz L, Ferreira R, et al. Adipose Mesenchymal Stromal Cell-Based Therapy for Severe Osteoarthritis of the Knee: A Phase I Dose-Escalation Trial. *Stem Cells Transl Med.* 2016;5(7):847-856.
28. ter Huurne M, Schelbergen R, Blattes R, et al. Antiinflammatory and chondroprotective effects of intraarticular injection of adipose-derived stem cells in experimental osteoarthritis. *Arthritis Rheum.* 2012;64(11):3604-3613.
29. van Buul GM, Siebelt M, Leijs MJ, et al. Mesenchymal stem cells reduce pain but not degenerative changes in a mono-iodoacetate rat model of osteoarthritis. *J Orthop Res.* 2014;32(9):1167-1174.
30. Zhang Y, Jordan JM. Epidemiology of osteoarthritis. *Clin Geriatr Med.* 2010;26(3):355-369.
31. Farrell E, O'Brien FJ, Doyle P, et al. A collagen-glycosaminoglycan scaffold supports adult rat mesenchymal stem cell differentiation along osteogenic and chondrogenic routes. *Tissue Eng.* 2006;12(3):459-468.
32. Guenoun J, Ruggiero A, Doeswijk G, et al. In vivo quantitative assessment of cell viability of gadolinium or iron-labeled cells using MRI and bioluminescence imaging. *Contrast Media Mol Imaging.* 2013;8(2):165-174.
33. Wong M, Siegrist M, Wang X, Hunziker E. Development of mechanically stable alginate/chondrocyte constructs: effects of guluronic acid content and matrix synthesis. *J Orthop Res.* 2001;19(3):493-499.
34. Kang JW, Kang KS, Koo HC, Park JR, Choi EW, Park YH. Soluble factors-mediated immunomodulatory effects of canine adipose tissue-derived mesenchymal stem cells. *Stem Cells Dev.* 2008;17(4):681-693.
35. van Buul GM, Farrell E, Kops N, et al. Ferumoxides-protamine sulfate is more effective than ferucarbotran for cell labeling: implications for clinically applicable cell tracking using MRI. *Contrast Media Mol Imaging.* 2009;4(5):230-236.

36. Koda M, Furuya T, Kato K, et al. Delayed granulocyte colony-stimulating factor treatment in rats attenuates mechanical allodynia induced by chronic constriction injury of the sciatic nerve. *Spine (Phila Pa 1976).* 2014;39(3):192-197.
37. Pritzker KP, Gay S, Jimenez SA, et al. Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage.* 2006;14(1):13-29.
38. Khatab S, van Buul GM, Kops N, et al. Intra-articular Injections of Platelet-Rich Plasma Releasate Reduce Pain and Synovial Inflammation in a Mouse Model of Osteoarthritis. *Am J Sports Med.* 2018;46(4):977-986.
39. von Bahr L, Batsis I, Moll G, et al. Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. *Stem Cells.* 2012;30(7):1575-1578.
40. Lux J, Sherry AD. Advances in gadolinium-based MRI contrast agent designs for monitoring biological processes in vivo. *Curr Opin Chem Biol.* 2018;45:121-130.
41. Schu S, Nosov M, O'Flynn L, et al. Immunogenicity of allogeneic mesenchymal stem cells. *J Cell Mol Med.* 2012;16(9):2094-2103.

Chapter 7

General discussion and future perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Mesenchymal stem cells (MSCs) are considered a promising option for cell therapy in osteoarthritis (OA), which have gained considerable interest since there is currently no effective treatment that can cure OA. MSCs are promising, because they have the potential to modulate the different OA disease processes simultaneously by their immunomodulatory capacity, trophic capacity and differentiation capacity[1-15]. MSCs are relatively easy to harvest from different sources, they can be expanded in culture and they are immunoprivileged initially[1,16-22]. Intra-articular injection of MSCs has been applied as potential therapy for osteoarthritis in several clinical studies over the past years. We have systematically reviewed the literature in **chapter 2** and concluded that MSCs can be safely used intra-articularly; our findings were confirmed in some more recent systematic reviews[23,24]. Clinical studies in other application fields also confirm the safe use of MSCs, without any severe adverse events documented[25-29].

Promising results of MSCs as cell therapy for other areas within medicine are being reported in literature[17]. In particular the immunomodulatory/trophic function of autologous and allogeneic MSCs are proven to be effective in the field of cardiology, graft-versus-host disease (GvHD), Crohn's disease and organ transplantation[28-35]. The use of both autologous and allogeneic MSCs appeared to be safe in a clinical trial[29]. These promising outcomes of MSC therapies in other fields could offer a good prospect for the use of MSCs to treat OA. To be effective, MSCs should be able to slow down, stop or cure OA, instead of only treating the symptoms. Currently there is no such therapy for OA. In theory there are two main therapeutic mechanisms of MSCs that could work to treat OA: 1) MSCs have the capacity to differentiate into cartilage tissue[2-5,22,36] and thereby addressing the cartilage loss, which is a main characteristic of OA. The great challenge here is the complex interplay of factors to initiate chondrogenic differentiation of MSCs[2,37-39] and the generation of functional articular cartilage in humans; 2) MSCs may improve or cure OA by secretion of immunomodulatory and/or trophic factors. The immunomodulatory factors can inhibit the inflammatory reaction in OA and improve the catabolic-anabolic imbalance of the intra-articular homeostasis[40]. The immunomodulatory capacity of MSCs can be useful for patients with OA, because they secrete various immunomodulatory factors that can influence many different pathways and are thus not restricted to one single target, unlike for example anti-TNFα[41]. Moreover, the trophic factors may also stimulate endogenous repair mechanisms. In preclinical and clinical studies promising results of MSC therapy in OA have been shown[42-46]. However, current results described in literature are con-

tradictory regarding cartilage regeneration via therapy with MSCs in humans[22,36,47]. Significant improvement in treated OA patients was limited to pain and functional outcome[42-44]. MSC therapy in other orthopaedic diseases (osteonecrosis, fracture non-union) and as an additional treatment to surgical intervention (high tibia osteotomy, anterior cruciate ligament reconstruction, partial meniscectomy and lumbar fusion) show promising results as well[48-57]. In my thesis I have focused on the immunomodulatory and trophic capacity of MSCs as a treatment for OA.

It is well known that MSCs need to be stimulated to execute their immunomodulation or trophic capacities. To stimulate immunomodulation and secretion of trophic factors by MSCs, MSCs are typically exposed *in vitro* to inflammatory factors or hypoxia[11,40,58-61]. In **chapter 3** we evaluated to what extent the OA joint environment influences the immunomodulation of MSCs. We exposed MSCs to synovial fluids (SF) from human knee joints with OA or rheumatoid arthritis. We demonstrated that SF can influence the expression of genes in MSCs that are involved in immunomodulation and can upregulate the expression of anti-inflammatory genes (**figure 1**)[62]. The effects of SF were smaller compared to commonly used stimulation with TNFα and IFNγ[10,11,40,58,60,63]. This might explain why resident MSCs are not activated to prevent disease development. This result strengthens the idea that we have to improve therapies to be effective and may explain why the body itself fails to stop or reverse the OA process, once started. Preconditioning MSCs before injection might optimize the expression of their therapeutic capacities as suggested by other studies[64-66].

Preconditioning of MSCs can be interesting in an even broader sense, since long-term results with consistent cartilage healing and long-term local function of MSCs still lack[67]. In **Chapter 1 and 5** we showed imaging results of our studies, in which intra-articularly injected MSCs could be tracked for up to two weeks after injection, after which they became undetectable[41,68]. A successful strategy to cure OA would consist of long-term modulation of the degenerative joint environment by simultaneously reducing inflammation and promoting tissue regeneration. Therefore, we hypothesized that the therapeutic functions of MSCs for OA would be optimized by retaining the MSCs intra-articular for a long period of time near the OA affected tissues. By using different stimuli *in vitro* (among which exposure to inflammatory factors, hypoxia and platelet lysate), we studied in **chapter 4** whether we could precondition MSCs to optimize their expression of migration and adhesion factors and their function (**figure 1)**. By preconditioning we tried to improve their migration and adhesion to degenerated tissues in an OA environment with the idea that this would prolong and improve the therapeutic function of MSCs. We showed

that MSCs express migration and adhesion receptor genes and migrate to factors secreted by both OA synovium and cartilage *in vitro*. By preconditioning the MSCs *in vitro*, the expression of migration and adhesion receptors were altered. However, this did not influence migration *in vitro* or adhesion *in vivo* compared to non-preconditioned MSCs[69].

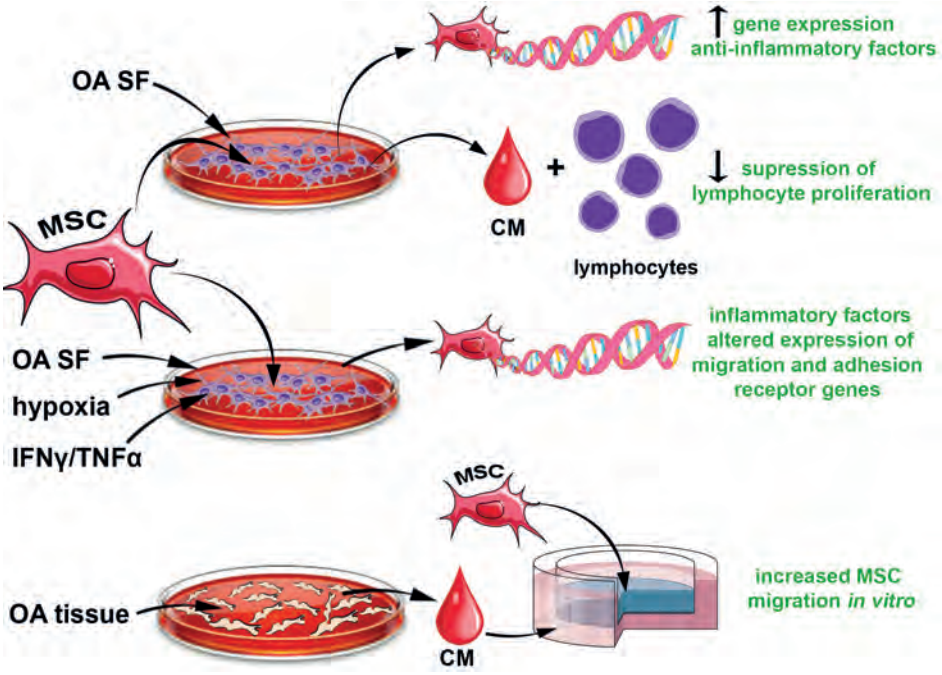


Figure 1. *Schematic overview of the main study approach and outcomes of chapter 3 and chapter 4 combined. MSC (mesenchymal stem cell), OA (osteoarthritis), SF (synovial fluid), CM (conditioned medium), IFNγ (interferon-gamma), TNFα (Tumor Necrosis Factor-alfa).*

This outcome made us use another approach to keep the MSCs in the intra-articular space for a longer period of time by encapsulating the cells in biocompatible constructs as described in **chapter 5** and **chapter 6**[70,71]. A schematic representation of this approach is shown in **figure 2**.

Encapsulation is a strategy to implant allogeneic MSCs by incorporating the cells in a semipermeable membrane. This membrane protects the MSCs from the host immune system while their therapeutic capacity by secreting factors is maintained[72]. Biomaterials have been well established in biomedical applications nowadays with functions ranging from isolating cells or their secretome, to matrices with biological signals for tissue engineering[73]. Alginate is one of these biomaterials

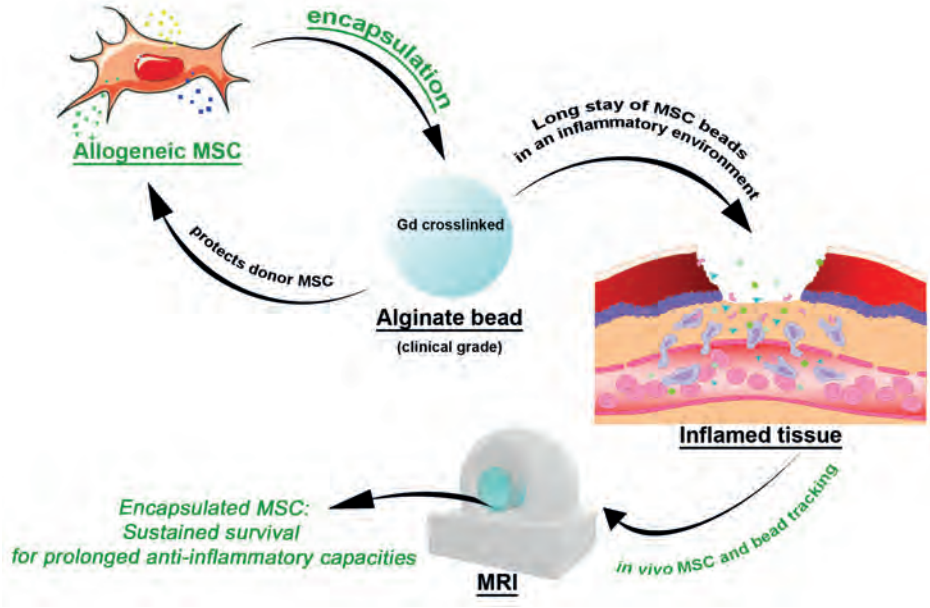


Figure 2. *Schematic overview of the study approach of chapter 5 and chapter 6 combined. MSC (mesenchymal stem cell), Gd (gadolinium), MRI (magnetic resonance imaging).*

and has been used for encapsulation of cells since 1980[74]. Alginate was discovered in 1881 by E.C.C. Stanford. By developing and using alkali extraction of viscous 'algin' he was the first person who isolated alginate from algae[75]. In 1929 Kelco Co. in California produced the first commercial alginate[42,75,76]. Different alginates are available for experimental as well as clinical use with variations in composition and production methods which among others things makes a difference in biocompatibility, gelation, and stability. Variety in composition is achieved by differences in ratio of β-D-mannuronic acid and α-L-guluronic acid[77-80]. The clinical grade alginate we used in our experiments should be suitable for medical use. Common clinical applications of alginate are in wound healing, drug delivery and tissue engineering. In **chapter 5** and **chapter 6** we used different types of alginate to encapsulate MSCs. MSCs remained viable and retained their immunomodulatory capacity and trophic capacity *in vitro* in the two different alginates tested, which have different ratios of mannuronic acid and guluronic acid[68,80]. This suggests that a wide range of alginates may be suitable for MSC encapsulation. *In vivo* the local retention of MSCs could be enhanced by encapsulation; MSCs remained viable and were protected against the host's immune system[80]. Alginate constructs implanted subcutaneously remained intact over time and a thin fibrous capsule was formed around the constructs without infiltration of immune cells in the alginate[80]. Another study with implantation of alginate constructs containing cells,

reported on a gradual invasion by granulation tissue containing multinucleated giant cells, lymphocytes and fibroblasts[77]. In **chapter 6** we produced MSC- alginate microbeads and injected these constructs in rat knees. In contrast to the subcutaneously implanted constructs, these constructs showed some integrity loss over time; possibly due to mechanical forces in the joint. However, a substantial part of the cells remained present until the end of the study at 8 weeks after injection[80]. The therapeutic effect of MSC-alginate microbeads injected intra-articularly was evaluated in an OA model in rats. There was no improved therapeutic effect by the encapsulated MSCs compared to freely injected MSCs and the control group with saline injection. This might be due to a relatively low number of cells injected in total. Also, the use of xenogeneic cells, incorporation of some constructs in the synovial membrane causing shielding of the secreted factors, or maybe even the loss of construct integrity, allow the cells to release intra-articularly and to quickly disappear from the joint. Other studies with encapsulated MSCs have shown more promising results with respect to the therapeutic effect[81]. Choi et al. used a two and a half times higher concentration of MSCs in their alginate and performed three periodic injections[81]. Preliminary experiments in our laboratory had indicated the density of $4x10^6$ cells/ml alginate to be a good balance between concentration of secreted factors and stability of the gel construct. Therefore, we did not use higher cell numbers per bead. In our study, rat knees contained approximately $0.8x10^5$ encapsulated MSCs. This number is more than ten times lower than the $1x10^6$ freely injected MSCs in OA rat knees in previous research[41]. The most effective amount of MSCs is still open for debate considering the heterogeneity in the amount of MSCs used in preclinical and clinical trials[29,42,43,67]. In **chapter 5** and **chapter 6** all constructs were made of alginate. Other biomaterials with the ability to encapsulate cells in a semipermeable membrane and thereby retaining the therapeutic capacity of the cells are available as well[82,83]. Considering the possible integrity problems of the constructs, single-cell microgels could be a solution. Encapsulation of single cells in microgels provides exquisite control over the culture and analysis of cells *in vitro*, as well as the fate of cell-based therapies *in vivo*[84]. The Single-cell microgel technology is increasingly emerging as biotechnology tool and is likely to have an increasing role in tissue engineering and regenerative medicine because of the possibility of stem cell injection and immunoprotection[84]. By injecting single-cell microgels we might avoid construct integrity problems while prolonging the MSC retention time and retaining their therapeutic capacity, as well as improving cell survival due to a protective microenvironment[84].

FUTURE PERSPECTIVES

It is of great importance to fully understand the disease mechanism of OA as well as the full mechanism of action of MSCs in order to develop the best and most endurable cell therapy for OA. To date neither mechanism is fully understood and there is much on-going research trying to reveal the mechanisms.

The research performed in this thesis is focused on cell therapy for OA with MSCs. The disease osteoarthritis is a multifactorial disease; the disease is heterogeneous in nature with its different grades and intermittent symptoms[85]. Most likely it is important to assess each OA patient as an individual with a different phenotype and severity grade of OA. Based on the heterogeneity of OA and inflammation severity we think that timing of intra-articular administration of MSCs will be of importance for the treatment effect. Osteoarthritis patients are seen by their doctors based on the severity of their complaints. Imaging studies at this stage will determine the radiological gradation of OA. The processes that have led to these complaints started much earlier, since metabolic changes of the joint tissues started long before structural degeneration exists[86]. A great number of studies are performed to find biomarkers to identify and grade different OA stages[86-92]. Biomarkers can potentially help in early diagnosing OA and subsequently developing OA therapy strategies. Imaging is one of the keystones of diagnosing OA, including so called imaging biomarkers, yet validated predictive abilities of imaging biomarkers for clinical outcomes in OA are still lacking[92]. However, nuclear scintigraphy, CT and especially MRI, with advanced quantitative techniques, have good potential in detecting early OA and may be used for OA phenotype stratification in clinical research. Quantitative MRI, for instance via dGEMRIC imaging with gadolinium, is a promising contrast-based technique to measure and evaluate cartilage matrix quality[92]. Other studies show that semiquantitative MRI biomarkers including cartilage thickness and surface area, meniscal morphology, synovitis and bone marrow lesions are significantly associated with clinically relevant OA progression[93,94]. MRI analysis of bone shapes can even predict risks for future arthroplasty, also in knees free of radiographic OA at baseline[95]. However, until now, there is no validated radiological biomarker available for OA disease onset and progression, or that can be used to monitor treatment effects[86,91,92]. Cell therapy with MSCs in OA is promising, but timing of the MSC admission in the OA process of the individual patient based on biomarkers, will most likely be important to evaluate and show the therapeutic effectiveness.

Different sources of MSCs are being explored for osteoarthritis in pre-clinical and clinical studies, such as bone marrow, adipose tissue, synovial membrane and umbilical cord blood[1,18,42,43,96]. For this thesis I only used bone marrow derived MSCs (bMSCs), which have been one of the most frequently used types of MSCs[29]. A type that is currently receiving increasing attention are adipose tissue derived MSCs (aMSCs). Some of the advantages of aMSCs over bMSCs are: the easier and less invasive way of harvesting and their abundance that leads to a greater yield of MSCs[96-98]. aMSC and bMSC have good trophic/immunomodulatory capacity, and both MSCs show promising results as cell therapy in OA in clinical studies[21,97-100]. Clinical studies show a variation in level of evidence with additional risks of bias. Selection and detection bias for example, due to quasi-randomization procedures or follow-up procedures, including imaging. It is difficult to properly identify the cause of beneficial outcomes of MSC therapy in clinical studies. Different MSC administration methods are used with different biological adjuncts like platelet rich plasma, hyaluronic acid and fibrin[21,97,98,100-102]. These biological adjuncts cloud the effect of administered MSCs and create a performance bias. Furthermore, different doses of MSCs and different harvesting methods for both bMSCs and aMSCs are used. The cost considerations, problems with standardization, fear for graft versus host reaction/foreign body reaction and the associated regulatory hurdles involved with therapy with MSCs, raise the question whether a cell-free MSC therapy could be a good alternative.

In recent years new cell-free treatment options are being studied. Cell-free treatment options that can be derived from MSCs are: cell secretome, microvesicles and exosomes. These products play a role in immunomodulatory and remodelling processes. Cell secretome contains immunomodulatory factors and trophic factors secreted by MSCs, and have proven anti-osteoarthritic effects[40,67,103-106]. The mode of action is related to the "hit and run" principle, in which further presence of the cells may not be necessary for therapeutic effect of MSCs after their initial release of therapeutic factors[67,103]. Advantages of cell-free MSC therapy over MSC therapy are: the possibility for easier mass production under optimized *in vitro* conditions; cell-free products can be easily dry-freezed and transported; production of a single preparation for multiple injections of OA joints; possibly less problems of antigenicity/rejection of the treatment[105,107]. Besides these advantages there are disadvantages: MSCs need to be subjected to *in vitro* preconditioning regimens to increase production of therapeutic factors with possible influence of this precondition on the cell function/survival and residues of these preconditioning regimens may also be present in the cell solution being injected[107]. Cell-free bioactive formulations intended for cartilage regeneration are tested in pre-clinical

studies with promising results[108,109]. Preclinical studies show early pain reduction and a protective effect for cartilage degeneration[103,106]. However, proteomic studies are needed to standardize the secretome and elucidate the working mechanisms.

In contrast to secretome, a part of the protein content and active components from MSCs are encapsulated in extracellular vesicles. These vesicles act as vehicles for intercellular communication and consist of microvesicles and exosomes[110,111]. Microvesicles are heterogeneous spheres released by ectocytosis of the plasma membrane. Microvesicles are approximately 100 – 1000 nm in diameter and have been demonstrated to contain around 365 types of proteins[110]. Exosomes are membrane vesicles arising from endocytosis and are stored intracellularly until endosomal structures fuse with the plasma membrane after which the exosomes are secreted[110,111]. Exosomes are approximately 40 – 100 nm in diameter and can contain about 217 types of proteins[110]. Both vesicle types contain unique proteins that play a role in anti-inflammatory processes. Microvesicles and exosomes can influence target cells trough binding to their receptors. Exosomes can intergrade their own content with the content of a target cell directly or by endocytosis, resulting in numerous biological processes[109,112]. Microvesicles and exosomes exert similar anti-inflammatory and chondroprotective effects in OA, consistent with conditioned medium and MSCs[109-111,113]. Tofino-Vian et al. state that microvesicles provide even better anti-inflammatory action and chondroprotection compared to exosomes, and conditioned medium[110]. Microvesicles and exosomes are natural carriers with specific advantages and disadvantages for drug delivery compared to synthetic carriers. Advantages include: less toxicity and immune reactivity, better longevity and better stability. A disadvantage is that microvesicles and exosomes need to be harvested from a suitable cell source that should meet the following requirements: large quantities of cells should be available with good isolation and expansion possibilities[111,112]. Even though extracellular vesicles seem to have great advantages, more studies are needed to optimize cell sources and to optimize/standardize the biological containment/dosages of microvesicles and exosomes before they can be used in clinical applications as a suitable treatment alternative for OA.

Cell therapy, including MSC treatment, has been studied in the last two decades, with progressively promising preclinical and clinical results. In this thesis we contributed to this research field by studying various characteristics of MSCs and optimization methods for cell therapy with MSCs in OA. By encapsulation of MSCs we have taken a step in improving the delivery to the joint environment by reduced immune reactivity, improved localization and retention of therapeutic

activity. Future cell therapy options should aim to modulate immune reactivity and regenerative processes simultaneously. The continuous growth of the orthobiological research field, with cell therapy and therapeutic delivery mechanisms of cell products, provides more and more insight into the possible mechanisms of action. Furthermore, the potency of these therapies is increasingly validated in preclinical and in clinical trials. As with all pharmaceutical, biological and surgical treatments, the effectiveness and safety of cell therapy must be continuously substantiated and observed. When these issues are clear and positive, given the promising results of current preclinical studies, I expect more clinical implementations in the coming years with great potential for an effective therapy in OA.

REFERENCES

1. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006;98(5):1076-1084.
2. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411):143-147.
3. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991;9(5):641-650.
4. Caplan AI. The mesengenic process. Clin Plast Surg. 1994;21(3):429-435.
5. Solchaga LA, Welter JF, Lennon DP, Caplan AI. Generation of pluripotent stem cells and their differentiation to the chondrocytic phenotype. Methods Mol Med. 2004;100:53-68.
6. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol. 2000;28(8):875-884.
7. Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. Exp Biol Med (Maywood). 2001;226(6):507-520.
8. Chen X, Armstrong MA, Li G. Mesenchymal stem cells in immunoregulation. Immunol Cell Biol. 2006;84(5):413-421.
9. Kim DH, Yoo KH, Choi KS, et al. Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell. Cytokine. 2005;31(2):119-126.
10. Schinkothe T, Bloch W, Schmidt A. In vitro secreting profile of human mesenchymal stem cells. Stem Cells Dev. 2008;17(1):199-206.
11. Hoogduijn MJ, Popp F, Verbeek R, et al. The immunomodulatory properties of mesenchymal stem cells and their use for immunotherapy. Int Immunopharmacol. 2010;10(12):1496-1500.
12. Meisel R, Brockers S, Heseler K, et al. Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. Leukemia. 2011;25(4):648-654.
13. Landgraf K, Brunauer R, Lepperdinger G, Grubeck-Loebenstein B. The suppressive effect of mesenchymal stromal cells on T cell proliferation is conserved in old age. Transpl Immunol. 2011;25(2-3):167-172.
14. Pers YM, Ruiz M, Noel D, Jorgensen C. Mesenchymal stem cells for the management of inflammation in osteoarthritis: state of the art and perspectives. Osteoarthritis Cartilage. 2015;23(11):2027-2035.
15. Galipeau J, Sensebe L. Mesenchymal Stromal Cells: Clinical Challenges and Therapeutic Opportunities. Cell Stem Cell. 2018;22(6):824-833.
16. Berebichez-Fridman R, Montero-Olvera PR. Sources and Clinical Applications of Mesenchymal Stem Cells: State-of-the-art review. Sultan Qaboos Univ Med J. 2018;18(3):e264-e277.
17. Klingemann H, Matzilevich D, Marchand J. Mesenchymal Stem Cells - Sources and Clinical Applications. Transfus Med Hemother. 2008;35(4):272-277.
18. Bianco P, Robey PG, Saggio I, Riminucci M. “Mesenchymal” stem cells in human bone marrow (skeletal stem cells): a critical discussion of their nature, identity, and significance in incurable skeletal disease. Hum Gene Ther. 2010;21(9):1057-1066.
19. Xing D, Wang Q, Yang Z, et al. Mesenchymal stem cells injections for knee osteoarthritis: a systematic overview. Rheumatol Int. 2018;38(8):1399-1411.
20. Peeters CM, Leijs MJ, Reijman M, van Osch GJ, Bos PK. Safety of intra-articular cell-therapy with culture-expanded stem cells in humans: a systematic literature review. Osteoarthritis Cartilage. 2013;21(10):1465-1473.

21. McIntyre JA, Jones IA, Han B, Vangsness CT, Jr. Intra-articular Mesenchymal Stem Cell Therapy for the Human Joint: A Systematic Review. Am J Sports Med. 2018;46(14):3550-3563.
22. Harrell CR, Markovic BS, Fellabaum C, Arsenijevic A, Volarevic V. Mesenchymal stem cell-based therapy of osteoarthritis: Current knowledge and future perspectives. Biomed Pharmacother. 2019;109:2318-2326.
23. Borakati A, Mafi R, Mafi P, Khan WS. A Systematic Review And Meta-Analysis of Clinical Trials of Mesenchymal Stem Cell Therapy for Cartilage Repair. Curr Stem Cell Res Ther. 2018;13(3):215-225.
24. Peeters CMM, Leijs MJC, Reijman M, van Osch GJVM, Bos PK. Safety of intra-articular cell-therapy with culture-expanded stem cells in humans: a systematic literature review. Osteoarthr Cartilage. 2013;21(10):1465-1473.
25. Lalu MM, Mazzarello S, Zlepnig J, et al. Safety and Efficacy of Adult Stem Cell Therapy for Acute Myocardial Infarction and Ischemic Heart Failure (SafeCell Heart): A Systematic Review and Meta-Analysis. Stem Cells Transl Med. 2018;7(12):857-866.
26. Lalu MM, McIntyre L, Pugliese C, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. PLoS One. 2012;7(10):e47559.
27. Reinders ME, Dreyer GJ, Bank JR, et al. Safety of allogeneic bone marrow derived mesenchymal stromal cell therapy in renal transplant recipients: the neptune study. J Transl Med. 2015;13:344.
28. White IA, Sanina C, Balkan W, Hare JM. Mesenchymal Stem Cells in Cardiology. Methods Mol Biol. 2016;1416:55-87.
29. Rodriguez-Fuentes DE, Fernandez-Garza LE, Samia-Meza JA, Barrera-Barrera SA, Caplan AI, Barrera-Saldana HA. Mesenchymal Stem Cells Current Clinical Applications: A Systematic Review. Arch Med Res. 2021;52(1):93-101.
30. Ciccocioppo R, Bernardo ME, Sgarella A, et al. Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. Gut. 2011;60(6):788-798.
31. Forbes GM, Sturm MJ, Leong RW, et al. A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. Clin Gastroenterol Hepatol. 2014;12(1):64-71.
32. Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet. 2008;371(9624):1579-1586.
33. Le Blanc K, Rasmusson I, Sundberg B, et al. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 2004;363(9419):1439-1441.
34. Reinders ME, de Fijter JW, Roelofs H, et al. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. Stem Cells Transl Med. 2013;2(2):107-111.
35. Tan J, Wu W, Xu X, et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. JAMA. 2012;307(11):1169-1177.
36. De Bari C, Roelofs AJ. Stem cell-based therapeutic strategies for cartilage defects and osteoarthritis. Curr Opin Pharmacol. 2018;40:74-80.

37. Bae HC, Park HJ, Wang SY, Yang HR, Lee MC, Han HS. Hypoxic condition enhances chondrogenesis in synovium-derived mesenchymal stem cells. Biomater Res. 2018;22:28.
38. Ronziere MC, Perrier E, Mallein-Gerin F, Freyria AM. Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells. Biomed Mater Eng. 2010;20(3):145-158.
39. Uzieliene I, Bernotas P, Mobasheri A, Bernotiene E. The Role of Physical Stimuli on Calcium Channels in Chondrogenic Differentiation of Mesenchymal Stem Cells. Int J Mol Sci. 2018;19(10).
40. van Buul GM, Villafuertes E, Bos PK, et al. Mesenchymal stem cells secrete factors that inhibit inflammatory processes in short-term osteoarthritic synovium and cartilage explant culture. Osteoarthr Cartilage. 2012;20(10):1186-1196.
41. van Buul GM, Siebelt M, Leijs MJ, et al. Mesenchymal stem cells reduce pain but not degenerative changes in a mono-iodoacetate rat model of osteoarthritis. J Orthop Res. 2014;32(9):1167-1174.
42. Roffi A, Nakamura N, Sanchez M, Cucchiarini M, Filardo G. Injectable Systems for Intra-Articular Delivery of Mesenchymal Stromal Cells for Cartilage Treatment: A Systematic Review of Preclinical and Clinical Evidence. Int J Mol Sci. 2018;19(11).
43. Lopa S, Colombini A, Moretti M, de Girolamo L. Injective mesenchymal stem cell-based treatments for knee osteoarthritis: from mechanisms of action to current clinical evidences. Knee Surg Sports Traumatol Arthrosc. 2018.
44. Emadedin M, Labibzadeh N, Liastani MG, et al. Intra-articular implantation of autologous bone marrow-derived mesenchymal stromal cells to treat knee osteoarthritis: a randomized, triple-blind, placebo-controlled phase 1/2 clinical trial. Cytotherapy. 2018;20(10):1238-1246.
45. Iijima H, Isho T, Kuroki H, Takahashi M, Aoyama T. Effectiveness of mesenchymal stem cells for treating patients with knee osteoarthritis: a meta-analysis toward the establishment of effective regenerative rehabilitation. NPJ Regen Med. 2018;3:15.
46. Xia P, Wang X, Lin Q, Li X. Efficacy of mesenchymal stem cells injection for the management of knee osteoarthritis: a systematic review and meta-analysis. Int Orthop. 2015;39(12):2363-2372.
47. Vega A, Martin-Ferrero MA, Del Canto F, et al. Treatment of Knee Osteoarthritis With Allogeneic Bone Marrow Mesenchymal Stem Cells: A Randomized Controlled Trial. Transplantation. 2015;99(8):1681-1690.
48. Wong KL, Lee KB, Tai BC, Law P, Lee EH, Hui JH. Injectable cultured bone marrow-derived mesenchymal stem cells in varus knees with cartilage defects undergoing high tibial osteotomy: a prospective, randomized controlled clinical trial with 2 years' follow-up. Arthroscopy. 2013;29(12):2020-2028.
49. Tabatabaee RM, Saberi S, Parvizi J, Mortazavi SM, Farzan M. Combining Concentrated Autologous Bone Marrow Stem Cells Injection With Core Decompression Improves Outcome for Patients with Early-Stage Osteonecrosis of the Femoral Head: A Comparative Study. J Arthroplasty. 2015;30(9 Suppl):11-15.
50. Sen RK, Tripathy SK, Aggarwal S, Marwaha N, Sharma RR, Khandelwal N. Early results of core decompression and autologous bone marrow mononuclear cells instillation in femoral head osteonecrosis: a randomized control study. J Arthroplasty. 2012;27(5):679-686.

51. Hauzeur JP, De Maertelaer V, Baudoux E, Malaise M, Beguin Y, Gangji V. Inefficacy of autologous bone marrow concentrate in stage three osteonecrosis: a randomized controlled double-blind trial. Int Orthop. 2018;42(7):1429-1435.
52. Mao Q, Wang W, Xu T, et al. Combination treatment of biomechanical support and targeted intra-arterial infusion of peripheral blood stem cells mobilized by granulocyte-colony stimulating factor for the osteonecrosis of the femoral head: a randomized controlled clinical trial. J Bone Miner Res. 2015;30(4):647-656.
53. Liebergall M, Schroeder J, Mosheiff R, et al. Stem cell-based therapy for prevention of delayed fracture union: a randomized and prospective preliminary study. Mol Ther. 2013;21(8):1631-1638.
54. Zhang H, Xue F, Jun Xiao H. Ilizarov method in combination with autologous mesenchymal stem cells from iliac crest shows improved outcome in tibial non-union. Saudi J Biol Sci. 2018;25(4):819-825.
55. Silva A, Sampaio R, Fernandes R, Pinto E. Is there a role for adult non-cultivated bone marrow stem cells in ACL reconstruction? Knee Surg Sports Traumatol Arthrosc. 2014;22(1):66-71.
56. Vangsness CT, Jr., Farr J, 2nd, Boyd J, Dellaero DT, Mills CR, LeRoux-Williams M. Adult human mesenchymal stem cells delivered via intra-articular injection to the knee following partial medial meniscectomy: a randomized, double-blind, controlled study. J Bone Joint Surg Am. 2014;96(2):90-98.
57. Hart R, Komzak M, Okal F, Nahlik D, Jajtner P, Puskeiler M. Allograft alone versus allograft with bone marrow concentrate for the healing of the instrumented posterolateral lumbar fusion. Spine J. 2014;14(7):1318-1324.
58. Crisostomo PR, Wang Y, Markel TA, Wang M, Lahm T, Meldrum DR. Human mesenchymal stem cells stimulated by TNF-alpha, LPS, or hypoxia produce growth factors by an NF kappa B- but not JNK-dependent mechanism. Am J Physiol Cell Physiol. 2008;294(3):C675-682.
59. Krampera M, Cosmi L, Angeli R, et al. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells. 2006;24(2):386-398.
60. English K, Barry FP, Field-Corbett CP, Mahon BP. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. Immunol Lett. 2007;110(2):91-100.
61. Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. Clin Exp Immunol. 2007;149(2):353-363.
62. Leijs MJ, van Buul GM, Lubberts E, et al. Effect of Arthritic Synovial Fluids on the Expression of Immunomodulatory Factors by Mesenchymal Stem Cells: An Explorative in vitro Study. Front Immunol. 2012;3:231.
63. Hemeda H, Jakob M, Ludwig AK, Giebel B, Lang S, Brandau S. Interferon-gamma and tumor necrosis factor-alpha differentially affect cytokine expression and migration properties of mesenchymal stem cells. Stem Cells Dev. 2010;19(5):693-706.
64. Rodriguez LA, 2nd, Mohammadipoor A, Alvarado L, et al. Preconditioning in an Inflammatory Milieu Augments the Immunotherapeutic Function of Mesenchymal Stromal Cells. Cells. 2019;8(5).

65. Philipp D, Suhr L, Wahlers T, Choi YH, Paunel-Gorgulu A. Preconditioning of bone marrow-derived mesenchymal stem cells highly strengthens their potential to promote IL-6-dependent M2b polarization. Stem Cell Res Ther. 2018;9(1):286.
66. Lin T, Pajarinen J, Nabeshima A, et al. Preconditioning of murine mesenchymal stem cells synergistically enhanced immunomodulation and osteogenesis. Stem Cell Res Ther. 2017;8(1):277.
67. Mancuso P, Raman S, Glynn A, Barry F, Murphy JM. Mesenchymal Stem Cell Therapy for Osteoarthritis: The Critical Role of the Cell Secretome. Front Bioeng Biotechnol. 2019;7:9.
68. Leijs MJ, Villafuertes E, Haeck JC, et al. Encapsulation of allogeneic mesenchymal stem cells in alginate extends local presence and therapeutic function. Eur Cell Mater. 2017;33:43-58.
69. Leijs MJ, van Buul GM, Verhaar JA, Hoogduijn MJ, Bos PK, van Osch GJ. Pre-Treatment of Human Mesenchymal Stem Cells With Inflammatory Factors or Hypoxia Does Not Influence Migration to Osteoarthritic Cartilage and Synovium. Am J Sports Med. 2017;45(5):1151-1161.
70. Su P, Tian Y, Yang C, et al. Mesenchymal Stem Cell Migration during Bone Formation and Bone Diseases Therapy. Int J Mol Sci. 2018;19(8).
71. Desando G, Bartolotti I, Cavallo C, et al. Short-Term Homing of Hyaluronan-Primed Cells: Therapeutic Implications for Osteoarthritis Treatment. Tissue Eng Part C Methods. 2018;24(2):121-133.
72. Ashimova A, Yegorov S, Negmetzhanov B, Hortelano G. Cell Encapsulation Within Alginate Microcapsules: Immunological Challenges and Outlook. Front Bioeng Biotechnol. 2019;7:380.
73. Langer R, Vacanti JP. Tissue engineering. Science. 1993;260(5110):920-926.
74. Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science. 1980;210(4472):908-910.
75. A. N. Hydrocolloid Applications. Boston, MA, USA: Springer, Boston, MA; 1997.
76. Gasperini L, Mano JF, Reis RL. Natural polymers for the microencapsulation of cells. J R Soc Interface. 2014;11(100):20140817.
77. Heiligenstein S, Cucchiarini M, Laschke MW, et al. In vitro and in vivo characterization of nonbiomedical- and biomedical-grade alginates for articular chondrocyte transplantation. Tissue Eng Part C Methods. 2011;17(8):829-842.
78. Bidarra SJ, Barrias CC, Granja PL. Injectable alginate hydrogels for cell delivery in tissue engineering. Acta Biomater. 2014;10(4):1646-1662.
79. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog Polym Sci. 2012;37(1):106-126.
80. Khatab S, Leijs MJ, van Buul G, et al. MSC encapsulation in alginate microcapsules prolongs survival after intra-articular injection, a longitudinal in vivo cell and bead integrity tracking study. Cell Biol Toxicol. 2020.
81. Choi S, Kim JH, Ha J, et al. Intra-Articular Injection of Alginate-Microencapsulated Adipose Tissue-Derived Mesenchymal Stem Cells for the Treatment of Osteoarthritis in Rabbits. Stem Cells Int. 2018;2018:2791632.
82. Kamperman T, Henke S, Zoetebier B, et al. Nanoemulsion-induced enzymatic crosslinking of tyramine-functionalized polymer droplets. J Mater Chem B. 2017;5(25):4835-4844.
83. Portalska KJ, Teixeira LM, Leijten JC, et al. Boosting angiogenesis and functional vascularization in injectable dextran-hyaluronic acid hydrogels by endothelial-like mesenchymal stromal cells. Tissue Eng Part A. 2014;20(3-4):819-829.

84. Kamperman T, Karperien M, Le Gac S, Leijten J. Single-Cell Microgels: Technology, Challenges, and Applications. Trends Biotechnol. 2018;36(8):850-865.
85. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. Lancet. 2019;393(10182):1745-1759.
86. Saberi Hosnijeh F, Bierma-Zeinstra SM, Bay-Jensen AC. Osteoarthritis year in review 2018: biomarkers (biochemical markers). Osteoarthritis Cartilage. 2019;27(3):412-423.
87. Chou CH, Attarian DE, Wisniewski HG, Band PA, Kraus VB. TSG-6 - a double-edged sword for osteoarthritis (OA). Osteoarthritis Cartilage. 2018;26(2):245-254.
88. Wisniewski HG, Colon E, Liublinska V, et al. TSG-6 activity as a novel biomarker of progression in knee osteoarthritis. Osteoarthritis Cartilage. 2014;22(2):235-241.
89. Amano K, Huebner JL, Stabler TV, et al. Synovial Fluid Profile at the Time of Anterior Cruciate Ligament Reconstruction and Its Association With Cartilage Matrix Composition 3 Years After Surgery. Am J Sports Med. 2018;46(4):890-899.
90. Blanco FJ, Camacho-Encina M, Gonzalez-Rodriguez L, et al. Predictive modeling of therapeutic response to chondroitin sulfate/glucosamine hydrochloride in knee osteoarthritis. Ther Adv Chronic Dis. 2019;10:2040622319870013.
91. Lotz M, Martel-Pelletier J, Christiansen C, et al. Value of biomarkers in osteoarthritis: current status and perspectives. Ann Rheum Dis. 2013;72(11):1756-1763.
92. McIlwraith CW, Kawcak CE, Frisbie DD, et al. Biomarkers for equine joint injury and osteoarthritis. J Orthop Res. 2018;36(3):823-831.
93. Collins JE, Losina E, Nevitt MC, et al. Semiquantitative Imaging Biomarkers of Knee Osteoarthritis Progression: Data From the Foundation for the National Institutes of Health Osteoarthritis Biomarkers Consortium. Arthritis Rheumatol. 2016;68(10):2422-2431.
94. Damman W, Liu R, Bloem JL, Rosendaal FR, Reijnierse M, Kloppenburg M. Bone marrow lesions and synovitis on MRI associate with radiographic progression after 2 years in hand osteoarthritis. Ann Rheum Dis. 2017;76(1):214-217.
95. Neogi T, Felson DT. Osteoarthritis: Bone as an imaging biomarker and treatment target in OA. Nat Rev Rheumatol. 2016;12(9):503-504.
96. Kozlowska U, Krawczenko A, Futoma K, et al. Similarities and differences between mesenchymal stem/progenitor cells derived from various human tissues. World J Stem Cells. 2019;11(6):347-374.
97. Di Matteo B, El Araby MM, D'Angelo A, et al. Adipose-Derived Stem Cell Treatments and Formulations. Clin Sports Med. 2019;38(1):61-78.
98. Zhang R, Ma J, Han J, Zhang W, Ma J. Mesenchymal stem cell related therapies for cartilage lesions and osteoarthritis. Am J Transl Res. 2019;11(10):6275-6289.
99. Jayaram P, Ikpeama U, Rothenberg JB, Malanga GA. Bone Marrow-Derived and Adipose-Derived Mesenchymal Stem Cell Therapy in Primary Knee Osteoarthritis: A Narrative Review. PM R. 2019;11(2):177-191.
100. Jevotovsky DS, Alfonso AR, Einhorn TA, Chiu ES. Osteoarthritis and stem cell therapy in humans: a systematic review. Osteoarthritis Cartilage. 2018;26(6):711-729.
101. Hurley ET, Yasui Y, Gianakos AL, et al. Limited evidence for adipose-derived stem cell therapy on the treatment of osteoarthritis. Knee Surg Sports Traumatol Arthrosc. 2018;26(11):3499-3507.
102. Gomez-Aristizabal A, Kim KP, Viswanathan S. A Systematic Study of the Effect of Different Molecular Weights of Hyaluronic Acid on Mesenchymal Stromal Cell-Mediated Immunomodulation. PLoS One. 2016;11(1):e0147868.

103. Khatab S, van Osch GJ, Kops N, et al. Mesenchymal stem cell secretome reduces pain and prevents cartilage damage in a murine osteoarthritis model. Eur Cell Mater. 2018;36:218-230.
104. Platas J, Guillen MI, del Caz MD, Gomar F, Mirabet V, Alcaraz MJ. Conditioned media from adipose-tissue-derived mesenchymal stem cells downregulate degradative mediators induced by interleukin-1beta in osteoarthritic chondrocytes. Mediators Inflamm. 2013;2013:357014.
105. Chen YC, Chang YW, Tan KP, Shen YS, Wang YH, Chang CH. Can mesenchymal stem cells and their conditioned medium assist inflammatory chondrocytes recovery? Plos One. 2018;13(11).
106. Chen W, Sun Y, Gu X, et al. Conditioned medium of mesenchymal stem cells delays osteoarthritis progression in a rat model by protecting subchondral bone, maintaining matrix homeostasis, and enhancing autophagy. J Tissue Eng Regen Med. 2019;13(9):1618-1628.
107. Pereira T, Ivanova G, Caseiro AR, et al. MSCs conditioned media and umbilical cord blood plasma metabolomics and composition. PLoS One. 2014;9(11):e113769.
108. Delgado-Enciso I, Paz-Garcia J, Valtierra-Alvarez J, et al. A phase I-II controlled randomized trial using a promising novel cell-free formulation for articular cartilage regeneration as treatment of severe osteoarthritis of the knee. Eur J Med Res. 2018;23(1):52.
109. Patel JM, Saleh KS, Burdick JA, Mauck RL. Bioactive factors for cartilage repair and regeneration: Improving delivery, retention, and activity. Acta Biomater. 2019;93:222-238.
110. Tofino-Vian M, Guillen MI, del Caz MDP, Silvestre A, Alcaraz MJ. Microvesicles from Human Adipose Tissue-Derived Mesenchymal Stem Cells as a New Protective Strategy in Osteoarthritic Chondrocytes. Cell Physiol Biochem. 2018;47(1):11-25.
111. Mianehsaz E, Mirzaei HR, Mahjoubin-Tehran M, et al. Mesenchymal stem cell-derived exosomes: a new therapeutic approach to osteoarthritis? Stem Cell Res Ther. 2019;10(1):340.
112. Pourakbari R, Khodadadi M, Aghebati-Maleki A, Aghebati-Maleki L, Yousefi M. The potential of exosomes in the therapy of the cartilage and bone complications; emphasis on osteoarthritis. Life Sci. 2019;236:116861.
113. Wang Y, Yu D, Liu Z, et al. Exosomes from embryonic mesenchymal stem cells alleviate osteoarthritis through balancing synthesis and degradation of cartilage extracellular matrix. Stem Cell Res Ther. 2017;8(1):189.

Chapter 8

Summary

SUMMARY

Osteoarthritis (OA) is a progressive degenerative joint disease that causes pain, stiffness and impaired movement. OA is one of the most common causes of disability in the world in adults over 60 years of age and is characterised by cartilage degeneration, subchondral bone changes and synovitis. Cartilage is avascular tissue with a complex extracellular matrix structure. As a result, cartilage has very limited intrinsic repair capacity. To date there is no therapy to cure OA. In this thesis we described studies on potential curative treatment strategies for OA in consideration of the complex nature of the disease.

The general introduction (**chapter 1**) provides an introduction to OA, its etiology, pathophysiology, and current treatment options. Mesenchymal Stromal Cells, also referred to as Mesenchymal Stem Cells (MSCs) are promising as cell therapy for OA, because these cells can influence different disease processes of osteoarthritis. MSCs have capabilities to reduce inflammation and stimulate regeneration in OA joints. This is due to their immunomodulatory, trophic and differentiation capacities; the first two functions mainly being carried out through the secretion of factors that reduce inflammation or stimulate tissue repair. Since MSCs are promising as cell therapy for OA, many preclinical and clinical studies have been carried out with these cells. In literature, conflicting results have been reported on cell survival after intra-articular injection. The period of time that MSCs remain intra-articularly after injection will be important for their therapeutic function in OA. The general aim of this thesis was to study factors that can increase the efficacy of MSCs as cell-therapy for the treatment of OA.

In **chapter 2** we evaluated the safety of cultured MSCs for intra-articular use in human subjects, by performing a systematic literature review. In the 8 studies included, no safety concerns with injected MSC products were found. The only reported adverse reactions from stem cell products were mild pain and transient swelling. We concluded that intra-articular cell therapy with culture-expanded MSCs was shown to be safe based on 844 intra-articular treatments performed in 8 separate studies. Considering this outcome, we had no compelling arguments against proceeding with intra-articular MSC application developments in humans.

Studies show promising results of MSC therapy in OA, including pain relief, and function recovery. However, in clinical studies that show clinical improvement there is no or only limited structural change as revealed by Magnetic Resonance Imaging (MRI) follow up. When MSCs are applied in joints, the OA environment

will most likely have an effect on the therapeutic capabilities of the MSCs. Synovial fluid (SF) contains many factors that are released by tissues affected by the OA process and therefore, provides a good representation of the OA environment of a joint. In **chapter 3**, we studied the effect of SF of OA patients on the expression and secretion of immunomodulatory factors by MSCs. SF could affect the expression of genes encoding for immunomodulatory factors by MSCs. Incubation of MSCs with SF from osteoarthritis patients as well as SF from rheumatoid arthritis patients resulted in secretion of anti-inflammatory factors by MSCs that significantly inhibited the proliferation of activated lymphocytes. We concluded that factors in the OA joint environment most likely stimulate the anti-inflammatory effect of MSCs. In addition to the effect on the anti-inflammatory capacity of MSCs, the factors secreted by the affected tissues in OA can potentially have an effect on the attraction/migration of MSC and their attachment to joint tissues. In **chapter 4** we investigated whether OA tissues have this effect. Furthermore, we studied whether MSCs could be stimulated in culture to increase their capacity to migrate and to attach with the aim to prolong the presence of the MSCs in the joint after injection. By using conditioned medium of OA synovium and OA cartilage of 6 different patients, we found that factors secreted by arthritic tissues could attract MSCs in a migration assay *in vitro*. Stimulating MSCs with inflammatory factors affected the expression of various migration and adhesion receptors in MSCs. However, stimulation of MSCs did neither affect their *in vitro* migration nor their attachment to joint tissues *in vivo*.

To prolong the intra-articular presence of MSCs and improve their therapeutic capabilities, a different approach was required. In **chapter 5**, we encapsulated MSCs in alginate and implanted the encapsulated cells subcutaneously in immune competent rats. We imaged/analyzed the presence of the cell constructs and demonstrated presence of active allogeneic and xenogeneic MSCs at the implanted site for at least 5 weeks after implantation. Under *in vitro* conditions, MSCs in alginate constructs still displayed multiple immunomodulatory and trophic properties, and were able to inhibit T cell proliferation. MSC-alginate constructs are therefore an interesting cell therapy system for use in OA treatment.

The concept of MSC-alginate constructs was further optimized and explored in **chapter 6** by encapsulating MSCs in two different types of alginate that are suitable for use in humans. MSCs remained viable and retained their immunomodulatory activity in both types of alginate. With the use of a micro-encapsulator, we were able to reproducibly generate very small MSC-alginate constructs suitable for needle injection into the joint. These constructs could be labelled with gadolinium.

Gadolinium is a contrast agent which is widely used in Radiology. Gadolinium was incorporated in alginate. This way the constructs could be followed over time by MRI, demonstrating effective delivery of the capsules into the joint and prolonged presence of the capsules in the joint. The survival of non-autologous MSCs was enhanced by encapsulation and the MSCs remained metabolically active *in vivo* for at least 8 weeks. However, no effect was found on pain relief, synovial inflammation or improvement of cartilage quality in rats with OA. This may be due to specific local tissue responses to the alginate constructs or due to a suboptimal cell number per construct. Nonetheless, the possibility to preserve non-autologous MSCs locally for a longer period of time by encapsulation, and the possibility of standardized production of micro-constructs has significantly increased the feasibility of producing MSC-alginate constructs for clinical application.

In conclusion, the research described in this thesis is a step forward towards a feasible cell therapy for OA. We have established that culture-expanded MSCs appear to be safe for intra-articular injection and the OA environment primarily stimulates the immunomodulatory activity of MSCs. Encapsulation of non-autologous MSCs in combination with imaging techniques opens new possibilities for minimal invasive longitudinal follow-up studies in patients for the treatment of OA. This work stimulates further development of therapeutic delivery systems in OA, which is not limited to cell therapy, but also includes new cell-free constructs with for example, cell secretome/conditioned medium, microvesicles and exosomes of MSCs. Further research into the effects of these treatments in different stages of osteoarthritis will have to take place in order to ultimately arrive at an effective injectable therapy for osteoarthritis.

NEDERLANDSE SAMENVATTING

Artrose is een progressieve degeneratieve gewrichtsaandoening die gepaard gaat met pijn, stijfheid en bewegingsstoornissen. Artrose is één van de meest voorkomende oorzaken van invaliditeit bij volwassenen ouder dan 60 jaar en wordt gekenmerkt door kraakbeen slijtage, veranderingen van het bot onder het kraakbeen en ontsteking van het gewrichtskapsel. Kraakbeen is weefsel met een complexe structuur zonder eigen bloedvoorziening. Hierdoor heeft kraakbeen een zeer beperkt intrinsiek herstelvermogen wanneer het beschadigd is. Tot op heden is er geen therapie om artrose te genezen. In dit proefschrift beschrijven we studies naar mogelijke curatieve behandelingsstrategieën voor artrose, rekening houdend met de complexe aard van de ziekte.

De algemene inleiding (**hoofdstuk 1**) geeft een introductie tot de ziekte artrose met betrekking tot de ontstaanswijze en de behandelingsmogelijkheden. Door de complexiteit van de ziekte artrose is er tot op heden geen therapie die genezing biedt. Mesenchymale stromale cellen (MSCs), ook wel mesenchymale stamcellen genoemd, zijn veelbelovend voor celtherapie bij artrose, omdat deze cellen in theorie verschillende ziekteprocessen van artrose tegelijk kunnen beïnvloeden. De MSCs hebben capaciteiten waarmee ze ontsteking in artrose gewrichten zouden kunnen remmen en waarmee ze herstel van beschadigd kraakbeen zouden kunnen verbeteren. Dit doen ze vooral door het uitscheiden van stoffen, maar MSCs zijn ook in staat om te differentiëren naar kraakbeencellen en zo mogelijk kraakbeenherstel te bevorderen. Omdat MSCs veelbelovend zijn als celtherapie voor artrose wordt er veel preklinisch en klinisch onderzoek gedaan met deze cellen. In de literatuur zijn tegenstrijdige resultaten beschreven over cel overleving na injecties in gewrichten. De duur van aanwezigheid van MSCs na injectie in een aangedaan gewricht zal belangrijk zijn voor hun therapeutische functie bij artrose. Het algemene onderzoeksdoel van mijn proefschrift was het bestuderen van factoren die van belang kunnen zijn bij het verhogen van de werkzaamheid van MSCs als celtherapie voor de behandeling van artrose.

Door middel van een systematisch literatuuronderzoek hebben we in **hoofdstuk 2** de veiligheid van gekweekte MSCs voor het gebruik in gewrichten bij mensen onderzocht. In de 8 geïncludeerde studies werden geen veiligheidsproblemen met geïnjecteerde MSC-producten gevonden. De enige gemelde bijwerkingen van stamcelproducten waren milde pijn en zwelling van voorbijgaande aard. We concludeerden dat celtherapie met in kweek vermenigvuldigde MSCs, veilig bleek te zijn op basis van 844 behandelingen in gewrichten binnen 8 studies. Gezien deze

uitkomst waren er geen bezwaren tegen verdere studies naar ontwikkelingen van MSC-toepassingen in gewrichten bij mensen.

In de literatuur worden veelbelovende resultaten getoond van MSCs op het gebied van pijnverlichting en functieherstel bij artrose. De beschreven effecten op structurele schade aan het kraakbeen zijn echter tegenstrijdig. Wanneer MSCs in gewrichten worden toegepast, zal de artrotische omgeving hoogstwaarschijnlijk een effect hebben op de therapeutische capaciteiten van de MSCs. De gewrichtsvloeistof, ook wel synoviale vloeistof (SV), bevat stoffen uitgescheiden door de weefsels die betrokken zijn bij het artrose proces en geeft een goede representatie van de artrose omgeving in een gewricht. In **hoofdstuk 3** hebben we het effect van SV van artrose patiënten op de aanmaak van ontstekingsremmende stoffen in MSCs bestudeerd. SV kon de aanmaak van ontstekingsremmende stoffen door MSCs beïnvloeden. Incubatie van MSCs met SV van artrose en van reumatoïde artritis patiënten resulteerde in de uitscheiding van stoffen door MSCs, die de vermenigvuldiging van geactiveerde lymfocyten (ontstekingscellen) significant remden.

De stoffen die worden uitgescheiden door de aangedane weefsels bij artrose kunnen, naast hun effect op de aanmaak en uitscheiding van stoffen door MSCs, mogelijk een effect hebben op de aantrekking/migratie van MSCs en hun binding aan weefsels in het gewricht. In **hoofdstuk 4** onderzochten we of dit het geval was en bestudeerden we of MSCs in kweek gestimuleerd konden worden om hun migratie- en bindingscapaciteit te vergroten. Het doel hierbij was om de aanwezigheidsduur van de MSCs in het gewricht na injectie te verlengen. Het bleek dat stoffen die worden uitgescheiden door artrotische weefsels, MSCs kunnen aantrekken. Het stimuleren van MSCs met ontstekingsstoffen beïnvloedde de expressie van verschillende migratie- en bindingsreceptoren in MSCs. Stimulatie van MSCs had echter geen invloed op hun migratie *in vitro* of op hun binding aan weefsels *in vivo*.

Om de aanwezigheidsduur van MSCs in het gewricht te verlengen en hun therapeutische capaciteiten gedurende een langere periode te behouden, was een andere aanpak nodig. In **hoofdstuk 5** hebben we MSCs ingekapseld in alginaat. Hierdoor konden zelfs allogene en xenogene MSCs ten minste 5 weken actief aanwezig blijven op de geïmplanteerde locatie in immuun competente ratten, omdat alginaat de cellen beschermt tegen het immuunsysteem. MSCs in alginaat constructen waren nog steeds in staat ontstekingsremmende en herstelbevorderende stoffen aan te maken en remden de deling van lymfocyten *in vitro*. MSC-alginaat constructen zijn daarom een interessant celtherapie systeem voor toepassing bij artrose.

De toepassingsmogelijkheden van MSC-alginaat constructen werden verder geoptimaliseerd en onderzocht in **hoofdstuk 6** door MSCs in te kapselen in twee verschillende soorten alginaat die geschikt zijn voor toepassing in mensen. Ook in deze alginaat formuleringen bleven de MSCs levensvatbaar en behielden hun ontstekingsremmende activiteit. Door het gebruik van een geautomatiseerde micro-encapsulator konden we hele kleine MSC-alginaat bolletjes produceren die geschikt waren voor injectie met een naald in het gewricht. Deze bolletjes konden ook gelabeld worden middels het gebruik van gadolinium. Gadolinium is een contrastmiddel dat veelvuldig gebruikt wordt in de radiologie. Omdat gadolinium wordt gebonden in alginaat was het mogelijk de bolletjes middels Magnetic Resonance Imaging (MRI) scans in de tijd te volgen en aan te tonen dat de constructen tenminste 8 weken aanwezig bleven in het gewricht. De overleving van MSCs nam toe door inkapseling en de cellen bleven actief *in vivo* voor ten minste 8 weken. Er werd echter geen effect gevonden op pijnvermindering, vermindering van gewrichtskapsel ontsteking of verbetering van kraakbeenkwaliteit in ratten met artrose. Dit is mogelijk te wijten aan specifieke lokale weefselreacties op de alginaat constructen of aan een suboptimaal cel aantal per construct. De mogelijkheid om lichaamsvreemde MSCs langer lokaal te kunnen behouden door ze in alginaat in te kapselen en de mogelijkheid van gestandaardiseerde productie van micro bolletjes, heeft de haalbaarheid van het produceren van MSC-alginaat bolletjes voor klinische toepassing vergroot.

Concluderend, met het onderzoek beschreven in dit proefschrift, is een verdere stap gezet in de richting naar een realiseerbare celtherapie voor artrose met MSCs. Toepassing van in kweek vermenigvuldigde MSCs blijkt vooralsnog veilig te zijn. De artrotische omgeving stimuleert vooral de ontstekingsremmende activiteit van MSCs. Inkapseling van lichaamsvreemde MSCs in combinatie met beeldvormende technieken opent nieuwe mogelijkheden voor minimaal invasieve longitudinale follow-up studies bij patiënten naar de behandeling van artrose. Dit werk stimuleert de verdere ontwikkeling van een therapeutisch afgiftesysteem voor artrose; niet alleen voor de celtherapie met MSCs, maar ook voor nieuwe, cel-vrije therapiesystemen van bijvoorbeeld door MSCs uitgescheiden stoffen en kleine blaasjes gevuld met werkzame stoffen. De effecten van deze behandelingen in verschillende stadia van artrose zullen verder onderzocht moeten worden om uiteindelijk tot een effectieve injecteerbare therapie voor artrose te komen.

Chapter 9

Appendices

List of abbreviations
PhD portfolio
Curriculum vitae
List of publications
Dankwoord

LIST OF ABREVIATIONS

2D	2-dimensional
3D	3-dimensional
ACI	autologous chondrocyte implantation
AEs	adverse events
aMEM	alpha-modified Minimum Essential Medium
ANOVA	analysis of variance
AS	autologous serum
BKI	best keeper index
BLI	bioluminescence imaging
BMA	bone marrow aspiration
BMMNCs	bone marrow mononuclear cells
BM-MSCs	bone marrow-derived mesenchymal stem cells
BSA	bovine serum albumin
CCL5	Chemokine ligand 5
CCR1	C-C chemokine receptor type 1
CCR4	C-C chemokine receptor type 4
CCR5	C-C chemokine receptor type 5
cDNA	complementary DNA/copy DNA
CFSE	carboxyfluorescein succinimidyl ester
CL	cruciate ligament
CM	conditioned medium
CO_2	carbon dioxide
CT	cycle threshold
CX3CL1	fractalkine
CX3CR1	CX3C chemokine receptor 1/fractalkine receptor
CXCR1	CXC chemokine receptor 1
CXCR3	CXC chemokine receptor 3
CXCR4	CXC chemokine receptor 4
DALYs	disability-adjusted life years
DMARDs	disease-modifying anti rheumatic drugs
DMEM	Dulbecco's Modified Eagle Medium
DMOADs	disease modifying osteoarthritic drugs
DMSO	dimethylsulphoxide
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assays
FACS	fluorescence-activated cell sorting

FBS	fetal bovine serum
FCS	fetal calf serum
FG	fibrin glue
FGF2	fibroblast growth factor 2
Fluc	firefly luciferase
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gd	gadolinium
GLP	good laboratory practice
GvHD	graft versus host disease
HA	hyaluronic acid
HBS	hyclone bovine serum
HE	haematoxylin and eosin
HGF	hepatocyte growth factor
High G	high concentration of α-L-guluronic acid
High M	high concentration of β-D-mannuronic acid
HLA	human leukocyte antigen
hMSC	human bone marrow mesenchymal stem cells
HPRT	hypoxanthine phosphoribosyltransferase
IDO	indoleamine 2,3-dioxygenase
IFNγ	interferon γ
IL-1α	interleukin -1 alpha
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-8	Interleukin-8
iNOS	inducible nitric oxide synthase
IP-10	interferon γ–induced protein 10
ITGβ1	integrin β-1
ITGβ2	integrin β-2
ITS	insulin-transferrin-selenium
LPA	Lysophosphatidic acid
MACI	matrix-induced chondrocyte implantation
MCP-1	monocyte chemotactic protein 1
MCP-3	monocyte chemotactic protein 3
MDC	macrophage-derived chemokine
MHC	major histocompatibility complex
MIA	monoiodoacetate
MIP-1α	macrophage inflammatory protein 1alpha
MIP-1β	macrophage inflammatory protein 1beta

MMPs	matrix-metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSCs	mesenchymal stem cells
NA	not available
NIRM	Netherlands Institute of Regenerative Medicine
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
OARSI	Osteoarthritis Research Society International
OCD	osteochondral defect
OD	osteochondritis dissecans
P/S (pen-strep)	Penicillin/Streptomycin
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDGFRα	platelet-derived growth factor receptor α
PDGFRβ	platelet-derived growth factor receptor β
PGE2	prostaglandin E2
PG	proteoglycan
PL	platelet lysate
PR-FG	platelet-rich fibrin glue
PRP	platelet-rich plasma
RA	rheumatoid arthritis
rMSC	rat mesenchymal stem cells
RNA	ribonucleic acid
SAE	serious adverse event
SD	standard deviation
SDS	Sequence Detection System
SF	synovial fluid
SPIO	superparamagnetic iron oxide
TE/TR	echo time/ repetition time
TGF-β1	transforming growth factor β1
TIMP	tissue inhibitor of metalloproteinase
TNFα	tumor necrosis factor-alpha
UBC	ubiquitin C
UD	undetectable
VEGF	vascular endothelial growth factor

PHD PORTFOLIO

Name PhD student: Maarten Johannes Cornelis Leijs
Erasmus MC department: Orthopedics and Radiology & Nuclear medicine
Research school: Postgraduate School Molecular Medicine (Mol-Med)
PhD period: January 2012 – December 2015
Promotor: Prof. dr. G.J.V.M. van Osch
Co-promotoren: Dr. M.R. Bernsen, Dr. P.K. Bos

1. PhD training

	Year	workload (ECTS)
General courses		
- Animal course article 9	2012	3.0
- Biomedical English writing course	2014	4.0
Specific courses (e.g. Research school, Medical training)		
- Molecular and cellular basis of Regenerative Medicine	2011	1.5
- Stralingsbescherming niveau 5B	2012	1.0
- MRI veiligheid niveau 2	2015	0.1
Workshops and journal clubs		
- Journal clubs (every first Monday of the month)	2011-2015	2.0
- The basic introduction course on SPSS	2012	0.8
- Photoshop en illustrator CS6	2013	0.3
- InDesign CS6	2013	0.15
Presentations		
- Oral presentations at research meetings at the department of Orthopaedics, Radiology and meetings with Internal medicine	2011-2015	4.0
- Oral presentations at research project meetings	2011-2015	1.0
(inter)national conferences		
- 16th molecular medicine day, Rotterdam, The Netherlands (Poster presentation)	2012	1.0
- 17th molecular medicine day, Rotterdam, The Netherlands (Poster presentation)	2013	1.0
- eCM XIV Meeting, Davos, Switzerland (Oral presentation and poster presentation)	2013	1.0
- NIRM consortium meeting, Utrecht, The Netherlands	2013	1.0

- 22nd annual meeting NBTE (Netherlands society for biomaterials and tissue engineering), Lunteren, the Netherlands (oral presentation)	2013	1.0
- 18th molecular medicine day, Rotterdam, The Netherlands (Poster presentation, poster award)	2014	1.0
- OARSI world congress, Paris, France (Poster presentation)	2014	1.0
- TERMIS EU congress, Genova, Italy (two oral presentations)	2014	2.0
- NOV-voorjaarsvergadering 2015, Utrecht, the Netherlands (oral presentation)	2015	1.0
- TERMIS world congress, Boston, MA, USA (poster presentation)	2015	1.0
- AAV wetenschapsmiddag, Rotterdam, the Netherlands (oral presentation, presentation award)	2015	1.0
- Wetenschapsdag Orthopedie Erasmus MC Rotterdam, the Netherlands (Oral presentation)	2018	1.0
- ROGO dag Rotterdam, the Netherlands (Oral presentation)	2018	1.0

Other

- Handelingen met dieren gehuisvest in IVC's	2013	0.2

2. Teaching

Courses

- Omgaan met groepen	2013	0.1
- Cursus Coaching studenten (BKO)	2015	0.2

Lecturing

- Lecturing 3rd year medical students attending the minor "Orthopaedic Sports Traumatology"	2011-2015	2.0
- Tutor 1st year medicine students 2013-2014 and 2014-2015	2013-2015	2.0
- Coach of 6 medical students (first year)	2015-2017	0.5

Supervising practicals

- Vaardigheidsonderwijs histologie botpathologie 1e jaar geneeskunde studenten en minor studenten TU Delft.	2014-2015	1.0
- supervising minor research project two master students Biomedical engeneering TU Delft	2015	1.0

Supervising master research		
- Alginate-MSC constructs; finding the best construct for cell-based therapy in osteoarthritis, Michael Nieboer, medicine, Erasmus university Medical Center, Rotterdam, the Netherlands	2014	2.0
Total		**40,85**

CURRICULUM VITAE

Maarten Leijs werd geboren op 6 november 1986 te Soest. Hij groeide op in Soest en haalde in 2005 zijn VWO-diploma op het Baarnsch Lyceum. Aansluitend startte hij met de studie geneeskunde aan het Erasmus MC te Rotterdam. Dit combineerde hij met werkzaamheden als medisch student op de afdeling urologie en gynaecologie, en als onderzoeksassistent op de afdeling maatschappelijke gezondheidszorg. Tijdens de coschappen werd duidelijk dat hij het meest enthousiast werd van de orthopedie en traumatologie. Het afstudeeronderzoek vond plaats in het laboratorium van de afdeling orthopedie in het Erasmus MC, waar een fundering werd gelegd voor het latere onderzoek. Na een korte klinische stage bij de kinderchirurgie in Benin (Afrika) en het volbrengen van het keuze en oudste coschap orthopedie in het Reinier de Graaf Gasthuis, behaalde hij in 2011 zijn artsendiploma.

In 2021 startte hij met het promotieonderzoek, getiteld: “Intra-articular application of mesenchymal stem cell therapy for osteoarthritis — the next step”. Het promotietraject werd uitgevoerd onder begeleiding van zijn promotor prof. dr. G.J.V.M. van Osch en copromotoren dr. M.R. Bernsen en dr. P.K. Bos.

In 2016 startte hij met de anderhalf jaar durende vooropleiding chirurgie in het Amphia ziekenhuis te Breda onder begeleiding van prof. dr. L. van der Laan. Het perifere deel van zijn opleiding orthopedie werd gevolgd in het Reinier de Graaf Gasthuis te Delft en het Reinier Haga orthopedisch centrum te Zoetermeer onder leiding van dr. R.M. Bloem en dr. G.A. Kraan. Het academische deel wordt gevolgd in het Erasmus MC onder leiding van dr. P.K. Bos. In 2020 kreeg hij naast zijn opleiding ook een aanstelling als clubarts bij voetbalclub Excelsior Rotterdam.

Maarten is woonachtig in Berkel en Rodenrijs samen met Karen en hun twee zoons Daan (2016) en Tim (2019).

LIST OF PUBLICATIONS

S. Khatab, **M.J.C. Leijs**, G.M. van Buul, J.C. Haeck, N. Kops, M. Nieboer, P.K. Bos, J.A.N. Verhaar, M.R. Bernsen, G.J.V.M. van Osch. MSC encapsulation in alginate microcapsules prolongs survival after intra-articular injection, a longitudinal in vivo cell and bead integrity tracking study. Cell Biol Toxicol. 2020 Dec;36(6):553-570.

M.J.C. Leijs, E. Villafuertes, J.C. Haeck, W.J. Koevoet, B. Fernandez-Gutierrez, M.J. Hoogduijn, J.A.N. Verhaar, M.R. Bernsen, G.M. van Buul, G.J.V.M. van Osch. Encapsulation of allogeneic mesenchymal stem cells in alginate extends local presence and therapeutic function. Eur Cell Mater. 2017 Jan 30;33: 43-58.

M.J.C. Leijs, G.M. van Buul, J.A.N. Verhaar, M.J. Hoogduijn, P.K. Bos, G.J.V.M van Osch. Pre-Treatment of Human Mesenchymal Stem Cells With Inflammatory Factors or Hypoxia Does Not Influence Migration to Osteoarthritic Cartilage and Synovium. Am J Sports Med. 2017 Apr;45(5):1151-1161.

S. Lopa, **M.J.C. Leijs**, M. Moretti, E. Lubberts, G.J.V.M van Osch, Y.M. Bastiaansen-Jenniskens. Arthritic and non-arthritic synovial fluids modulate IL10 and IL1RA gene expression in differentially activated primary human monocytes. Osteoarthritis Cartilage. 2015 Nov;23(11):1853-7

G.M. van Buul, M. Siebelt, **M.J.C. Leijs**, P. K. Bos, J.H. Waarsing, N. Kops, H. Weinans, J.A.N. Verhaar, M.R. Bernsen, G.J.V.M. van Osch. Mesenchymal stem cells reduce pain but not degenerative changes in a mono-iodoacetate rat model of osteoarthritis. J Orthop Res. 2014 Sep;32(9): 1167-74.

C.M.M. Peeters, **M.J.C. Leijs**, M. Reijman, G.J.V.M. van Osch, P.K. Bos. Safety of intra-articular cell-therapy with culture-expanded stem cells in humans: a systematic literature review. Osteoarthritis Cartilage. 2013 Oct;21(10): 1465-73.

M. Roemeling-van Rhijn, F.K.F. Mensah, S.S. Korevaar, **M.J.C. Leijs**, G.J.V.M. van Osch, J.N.M. Ijzermans, M.G.H. Betjes, C.C. Baan, W. Weimar, M.J. Hoogduijn. Effects of Hypoxia on the Immunomodulatory Properties of Adipose Tissue-Derived Mesenchymal Stem cells. Front Immunol. 2013 Jul 18;4: 203.

M.J.C. Leijs, G.M. van Buul, E. Lubberts, P.K. Bos, J.A.N. Verhaar, M.J. Hoogduijn, G.J.V.M. van Osch. Effect of Arthritic Synovial Fluids on the Expression of

Immunomodulatory Factors by Mesenchymal Stem Cells: An Explorative in vitro Study. Front Immunol. 2012 Aug 2;3: 231.

DANKWOORD

Mijn laatste stelling is: “For the strength of the pack is the wolf, and the strength of the wolf is the pack”. Met name het tweede deel van de stelling is erg toepasselijk voor het ontstaan van dit werk. Deze thesis was namelijk nooit tot stand gekomen zonder alle fijne collega’s, de goede samenwerking met andere disciplines/afdelingen en alle dierbare mensen in mijn naaste omgeving. Ik ben daarom een hoop mensen dank verschuldigd, waarbij ik in het bijzonder een aantal personen hieronder wil bedanken.

Prof. dr. G.J.V.M. van Osch, beste Gerjo, in 2010 kwam ik tijdens mijn co-schap in aanraking met Dr. Marijnissen die ons in contact bracht voor mijn keuze-onderzoek. Geen ervaring op gebied van fundamenteel wetenschappelijk onderzoek en toch durfde je het aan met mij. Na een half jaar intensieve begeleiding van jou en Gerben voltooide ik mijn keuze-onderzoek. Je hebt me daarna de kans geboden om een promotietraject te starten. Ik ben je zeer dankbaar voor de intensieve begeleiding die je mij gaf tijdens mijn wetenschappelijke ontwikkeling, ondanks jouw drukke agenda. Daarnaast zijn jouw kwaliteiten als people manager/motivator bewonderenswaardig. Nu terugkijkend ga je pas beseffen hoeveel iemand voor een bepaald deel in je leven heeft betekend en voor de rest van je leven zal betekenen. Dank voor alles wat je mij tot nu toe gebracht hebt!

Dr. M.R. Bernsen, beste Monique, als co-promotor was jij al snel nauw betrokken bij mijn onderzoek. Altijd enthousiast over de ideeën en het onderzoek en een positieve kijk, wanneer ik het zelf misschien even niet zag. De vele meetingen, de begeleiding en de goede kritische schrijf-aanwijzingen waren zeer waardevol en hebben voor verbetering van de kwaliteit van de publicaties gezorgd. Dank daarvoor.

Dr. P.K. Bos, beste Koen, jouw visie als co-promotor was vanaf het begin ook erg belangrijk voor mij om het onderzoek goed in het perspectief van klinische translatie te kunnen blijven plaatsen. Ik bewonder jouw basale wetenschap kennis en klinische kennis en dit is ook tijdens besprekingen van grote waarde geweest voor mij. Voor mij ben je natuurlijk niet alleen mijn co-promotor, maar ook mijn opleider. Dank dat je het vertrouwen in mij had als AIOS orthopedie, ik ben dan ook erg trots inmiddels in mijn laatste fase van de opleiding te zitten onder jouw vleugels. Dank voor alles wat jij hebt bijgedragen in mijn wetenschappelijke en klinische ontwikkeling.

Prof. dr. M.E.J. Reinders, prof.dr. G.J. Strijkers en dr. P.J. Emans, leden van de kleine commissie. Hartelijk dank voor het kritisch lezen en beoordelen van dit proefschrift.

Prof. dr. C.C. Baan en dr. M.N. Helder, dank voor uw bereidheid om plaats te nemen in de grote commissie.

Prof. dr. J.A.N. Verhaar, beste professor, de besprekingen met u en de “meet de professor” dagen brachten altijd nieuwe inzichten en kritiekpunten. Uw uitgebreide kennis, de manier waarop u kliniek, wetenschap en management/ bestuurschap weet te combineren is bewonderenswaardig. Dank voor alles wat u mij geleerd heeft en voor de mogelijkheden die u mij geboden heeft. Geniet van uw wel verdiende pensioen.

Dr. G.M. van Buul, beste Gerben, jouw werk vormde de basis voor mijn proefschrift. Dank voor je voorwerk zodat ik direct door kon pakken. Jouw bevlogenheid en enthousiasme voor fundamenteel wetenschappelijk onderzoek werkte zeer aanstekelijk. Dank ook voor je kritische blik en schrijf-aanwijzingen waardoor de publicaties in kwaliteit stegen. Ik heb je naast het onderzoek ook als collega mee mogen maken op de ziekenhuis vloer. Ondanks zware tijden bleef je altijd door gaan en je in mijn onderzoek interesseren. Ik heb veel respect voor jou hoe je door hebt weten te zetten. Dank voor al jouw inzet.

Prof. dr. Krestin, dank voor het goedkeurende oordeel van u en de radiologie wetenschapscommissie 1 jaar na het starten van mijn promotie, wat mij de rest van het traject enorme steun en vertrouwen heeft gegeven.

Ik wil alle senior onderzoekers en stafleden van de afdeling orthopedie bedanken. Jullie waren altijd bereid kritisch naar mijn onderzoek te kijken en weefsel te verzamelen. Alle orthopedisch chirurgen en AIOS van het Erasmus MC dank voor de leerzame en plezierige tijd tijdens de opleiding.

Yvonne en Eric wil ik in het bijzonder bedanken voor ondersteuning bij specifieke lab testen. Daarnaast ook Erwin, bedankt voor de hulp van enkele statistische vraagstukken. Roberto, I am grateful for a great friendship that originated in the lab. We will continue to see each other often, making new experiences and we will join new sports events together.

Graag wil ik alle co-auteurs bedanken voor hun expertise en inzet, waarbij ik de volgende speciaal wil benoemen. Martin Hoogduijn, dank dat ik gebruik mocht maken van jullie lab, voor al je expertise en hulp bij de lymfocyten. Carlo Peeters, ik mocht jou begeleiden bij het maken van de review tijdens je afstudeeronderzoek. Nu zie ik je nog regelmatig op meetings in de orthopedische wereld. Wie weet werken we elders nog een keer samen. Max Reijman, dank voor de begeleiding bij het tot stand komen van de review en voor jouw kritische blik en inzichten bij de wetenschappelijke meetings. Erik Lubberts, dank voor de benodigde onderzoeksmaterialen en voor je visie, inzichten en kritische beoordeling vanuit de reumatologische hoek. Joost Haeck, al vroeg in mijn onderzoekscarrière kwam ik bij jou op de kamer terecht, hoog in de ivoren toren. Het was altijd fijn even te praten met jou. Tijdens het promotie traject heb ik veel van jouw expertise gebruik mogen maken op imaging gebied. Dit ging altijd in alle rust en je nam de tijd voor uitleg van technische onderwerpen die mij wel eens deden duizelen. Dank voor al je ondersteuning. Harm Nieuwstadt dank voor al je hulp. Ik heb het tot mijn spijt nooit gemerkt, maar ik hoop dat je op een betere plek bent. Michael Nieboer, ik mocht jouw afstudeeronderzoek begeleiden, waarvan een onderdeel in dit proefschrift is opgenomen. Nu samen oudste assistent in opleiding tot orthopedisch chirurg in het Erasmus MC. Het is een mooie rit geweest om elkaar te zien groeien. Sohrab Khatab, partner in crime voor de laatste fase van mijn onderzoeksactiviteit op het lab en het begin van het jouwe. Samen in de kamer boven het helikopterplatform, waardoor de gevleugelde uitspraak bij elke landing door de kamer vloog: “get down, get to the chopper!”. Zet hem op met de laatste loodjes van jouw PhD traject!

Nicole, Wendy en Janneke, de analisten op het lab. De vaste waarden, de steunpilaren, waarvan ik veel ondersteuning heb gehad. Altijd even fijn om langs te lopen in jullie kamer voor een goed en/of gezellig gesprek en nooit te beroerd wanneer er iets gedaan moest worden. Dank voor alle gezellige momenten en ondersteuning.

Sandra, belangrijk voor Gerjo, maar ook voor ons. Dank voor alle dingen die je hebt geregeld, ook in de laatste fase en voor alle gesprekken en belangstelling. Je bent een topper.

Alle collega’s in het lab op de 16e, in de kliniek in ‘het onderzoekshok’ en bij de radiologie: Stefan, Johan, Anna, Rintje, Marloes, Nienke, Mieke, Jasper, Michiel, Marjan, Marianne, Harry, Holger, Esther, Femke, Lizette, Panithi, Mairéad, Caoimhe, Andrea, Niamh, Callie, Silvia, Johannes, Shorouk, Simone, Laurie, Kavitha, Mathijs, Job, Belle, Tijs, Vincent, Mark, Joost, Eline, Suus, Sandra, Gaby en Yanto

en alle anderen, heel veel dank voor jullie samenwerking, koffie, lab days, cakes (van de week), borrels, festivals, congressen enzovoorts.

Chirurgen en AIOS van het Amphia ziekenhuis in Breda, en in het bijzonder mijn eerste opleider prof. dr. van der Laan. Dank voor de fijne en goede basis die ik bij jullie in mijn vooropleiding heb mogen leggen.

Orthopedisch chirurgen van het RdGG en RHOC. Dr. Kraan dank voor de fijne opleidingstijd in Delft/Zoetermeer. Dr. Bloem dank voor uw vertrouwen in mij als AIOS orthopedie. Het was een hele ervaring om de overgang naar uw nieuwe orthopedische centrum te mogen meemaken. Alle geweldige collega's van ROGO Rotterdam en ROGO Leide!, dank voor jullie interesse en onuitputtelijke werklust iedere dag weer, wat aanstekelijk is en zorgt voor die fijne werksfeer!

Mijn paranimfen, niet zomaar hier aan mijn zijde. Twee goede vrienden, voorbeelden na ook aan hun zijde te hebben gestaan op hetzelfde podium. Joris jou ken ik al vanaf het eerste studiejaar en vanaf dat moment heb ik samen met jou elke stap mogen maken in onze carrière. Jij bent inmiddels PhD en dermatoloog in Delft. Ik kijk dus inmiddels op naar jou ;). Heerlijk was en is het om met jou in Zoutelande te zijn, de vele sport momenten samen te beleven en vrije tijd met jou en Marlot te besteden. Dank dat je mijn paranimf wilt zijn.

Wu, partner in crime, als Gerjo had geweten wat voor dynamisch duo ze aangenomen had, weet ik niet of ze dat weer zou doen ;). Ook wij kennen elkaar al van vroeg in de studie, maar ons contact is geïntensiveerd op het lab. We hebben zelfs nog even samengewoond bij jou. Ik heb een hele harde schijf vol met prachtige momenten en mijlpalen. Ik hoop dat we nog vele harde schijven vol gaan maken. Dank dat je mijn paranimf wilt zijn.

Alle lieve vrienden, familie en buren. Dank voor jullie interesse, support en luisterend oor. Jullie zijn niet alleen voor dit traject, maar altijd belangrijk.

Lieve Henk, Annemiek en Nicole. Ik ben gezegend met zo'n geweldige schoonfamilie. Ook jullie inzet voor het voltooien van dit werk, met het zorgdragen voor de jongens, heeft mij erg geholpen. Jullie zijn altijd belangstellend en zorgen voor een warm bad en de nodige afleiding met mooie zeilreisjes door het hele land. Dank jullie wel.

Lieve Mama, Papa, Bart, Nicole, Willemijn, Koen en Floor. Wat ben ik toch blij dat ik deel uitmaak van zo'n gezellig en liefdevol gezin. Een basis die belangrijk is en waarvan je weet dat je onvoorwaardelijke steun hebt. Mama en papa jullie hebben vanaf het begin veel interesse getoond, jullie luisterend oor was altijd fijn. Mam dank voor je hulp en samen met pap ben je er altijd voor me, dank daarvoor. Lieve Bart, Nicole en Floor, helaas in het zuidelijkste puntje woonachtig en ik zie jullie dus te weinig, ik hoop dat dit meer zal worden. Gelukkig bellen/facetimen we veel en zijn de updates met jou, lieve broer en kleine Floor altijd erg fijn. Col, de slimme van ons twee medici als AIOS hematologie, de volgende promotie is van jou, zet hem op! Willemijn en Koen, altijd een luisterend oor en bereid om zorg te dragen voor de jongens wanneer nodig. Zelfs een hele snoeppizza, voor bij het voltooien van dit proefschrift, kan ik van jullie verwachten via de brievenbus. Dank voor jullie steun.

Lieve Daan en Tim, mijn kleine boefjes. De tijd gaat veel te snel, dat voelde nooit zo, maar nu begrijp ik het wel. Ik zal minder boven gaan zitten en meer tijd stoppen in het voetballen en verstoppertje spelen met jullie. Ik ben een trotse vader en hou ontzettend veel van jullie!

Lieve Kaar, wat ben ik blij met jou aan mijn zijde. Je bent mijn toverfee, want ik begrijp af en toe niet hoe jij zoveel ballen in de lucht weet te houden. Zonder jou was dit niet gelukt. Dank je wel voor je onvoorwaardelijke steun, liefde en geduld. Ik hou ongelooflijk veel van je en hoop de rest van mijn leven met je door te brengen.