Utilization of Immune Cells to Aid in Bone Regeneration

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There is only one happiness in life: To love and be loved.

-- George Sand
## Contents

**Chapter 1**  General introduction  11

**Chapter 2**  Intraoperative construct preparation: a practical route for cell-based bone tissue engineering  21

**Chapter 3**  Macrophage type modulates osteogenic differentiation of adipose tissue MSCs  47

**Chapter 4**  Toward intra-operative preparation of cell-based constructs for bone regeneration  69

**Chapter 5**  Biomaterial property effects on platelets and macrophages: an in vitro study  89

**Chapter 6**  Physical and chemical characteristics of titanium surfaces modulate macrophage polarization  111

**Chapter 7**  Osteoclast-based constructs induce ectopic bone formation  129

**Chapter 8**  Combinatorial surface roughness effects on osteoclastogenesis and osteogenesis  149

**Chapter 9**  Summary, closing remarks and future perspectives

**Acknowledgements**  194

**List of Publications**  201

**Curriculum Vitae**  203
Chapter 1
General introduction
Chapter 1

Background

Bone is a dynamic and highly vascularized tissue, which is continuously being remodeled throughout an individual’s lifetime. As a part of the skeletal system, it is responsible for mechanical support for the soft tissues and muscles, body shape and movement, and provides protection for the internal organs of the body. In addition, bone is closely associated with mineral and blood homeostasis through its storage of mineral components and with blood production by regulating the bone marrow microenvironment. When normal bone damage occurs after traumatic injuries, its high regenerative capacity ensures the majority of defects to heal spontaneously with or without minor (surgical) intervention. However, large bone defects resulting from trauma, tumor resection, infection or nonunion represent a significant clinical problem for over 1 million people in Europe each year [1, 2]. This incidence is expected to grow given increasing life expectancy and larger numbers of patients with compromised medical conditions and inferior bone healing capacity in aging individuals.

Current approaches in bone regeneration

Autografts, which are often harvested from the iliac crest, are currently used as the “gold standard” option for clinical treatment of bone defects. However, complications including infection, bleeding, and additional chronic pain at the harvest site exist after harvesting autografts. More crucially, the supply of autografts within the human body is limited. Allografts, which are acquired from cadaveric bone and xenografts from non-human species, can solve the problem of supply, but are restricted by immunological and disease transmission issues and lack osteoinductive potential [3, 4]. As an alternative to tissue-based bone grafts, synthetic biomaterial developments over the last decades have resulted in many bone substitute materials available for clinical applications. However, their biological performance remains to be inferior to autografts [5]. Toward improving the biological performance of bone substitute materials, the use of cell-based constructs, in which autologous cells are combined with a (synthetic) biomaterial scaffolds [6], are being explored.

Most cell-based constructs focus on the use of (adult) stem cells, generally isolated as so-called mesenchymal stromal cells (MSCs) from either bone marrow or adipose tissue because of their biological characteristics [6]. This idea appears to be promising since it allows MSCs to directly generate new bone tissue and contribute to improving bone healing [7, 8]. However, this approach is restricted by its time-consuming and costly in vitro expansion procedure. Furthermore, signaling molecules and cells involved in the processes initiated prior to bone healing are largely ignored in MSCs-based approaches, which frequently has resulted in unsatisfactory performance due to donor variation, limited vascularization, and uncontrolled inflammation. From this perspective, the optimal strategy for cell-based bone regenerative approaches should be practical in the clinic and follow the natural chronological order of bone healing.
Involvement of the innate immune system in bone regeneration

In view of the natural bone healing process, three distinct but overlapping stages occur from a few hours to several weeks [9]: (1) the early inflammatory stage; (2) the repair stage; and (3) the late remodeling stage. It is tenable to speculate that the inflammatory response, which is evoked by the host immune system, initiates and primes the later bone repair process. In fact, the immune and skeletal systems have been reported to share a number of signaling molecules and regulatory networks [10]. Research on the topic of bone remodeling has shown the influence of the immune system on bone healing success and has led to the emergence of “osteoimmunology” [10], which identifies the immune system as a potential tool for new therapeutic approaches to bone healing.

The innate immune system in particular is rapidly activated within a few hours after bone injury, with platelets, neutrophils, and monocytes being the first components present at injured sites. They release a wide variety of inflammatory cytokines, growth factors, and chemokines to further promote leukocyte recruitment, modulate the immune response and activate MSCs [9]. During this procedure, monocytes, and the descendant macrophages, multinuclear giant cells (MNGCs), and osteoclasts, are recognized as key elements for the orchestration of the bone healing processes [11-13] (Figure 1).

1. Monocytes
Monocytes are the largest type of leukocytes circulating in the blood, bone marrow, and spleen and are the precursor of many other immune cells, such as macrophages and dendritic cells. After bone injury, monocytes are recruited to the injury site to clear cell debris and to destroy any pathogen or foreign body by secreting molecules such as reactive oxygen species (ROS) [14]. Monocytes also secrete cytokines and chemokines, which can recruit additional immune cells to the injured sites and stimulate endothelial progenitor cells, fibroblasts, and MSCs to orchestrate the bone repair response [13]. Monocytes per se secrete several osteogenic cytokines and growth factors, such as bone morphogenetic protein 2 (BMP-2) [15], tumor necrosis factor-α (TNFα) [16], and oncostatin (OSM) [17], to promote the osteogenic differentiation of bone forming cells (e.g. MSCs and osteoblastic progenitors) present at the injury sites. Furthermore, monocytes are well-known to regulate bone turnover by controlling the differentiation and activity of osteoclasts (i.e. the bone resorbing cells). This information suggests that monocytes are key players in bone homeostasis, acting both in osteoblast mineralization through cytokine secretion and in bone resorption by regulating osteoclast activity.

2. Macrophages
Macrophages are heterogeneous myeloid lineage cells, the majority of which differentiate from bone marrow-derived monocytes circulating in the blood. Two types of macrophages were identified to be involved in bone remodeling and regeneration according to their positions: OsteoMacs and inflammatory macrophages [18]. OsteoMacs were present in resting osteal tissues and increased at sites undergoing active
bone anabolism. In contrast, inflammatory macrophages are derived from a distinct population of blood monocytes that rapidly infiltrate tissues after bone damage. Depending on the micro-environment, macrophages also display distinct polarization states and give rise to two populations with distinct receptor expression, cytokine secretion, and functions: the “classically activated” pro-inflammatory M1 type macrophages and the “alternatively activated” anti-inflammatory M2 type macrophages [19]. M1 macrophages exert pro-inflammatory activities to lead to immune stimulation; in contrast, M2 macrophages are involved in debris scavenging, inflammation resolution, and tissue remodeling. After bone damage, inflammatory macrophages and OsteoMacs coexist within the injury site and start a cascade of events that facilitate the recruitment of MSCs and initiate bone healing [20]. Recent studies depicted a

Figure 1. Schematic representation of the involvement of monocytes, macrophages and osteoclasts in bone regeneration. Monocytes extravasate from peripheral blood vessels and migrate to bone damage sites, where they differentiate into macrophages. These macrophages are further polarized into different phenotypes i.e. pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages to modulate the bone healing process. Macrophages can also fuse into multinucleated giant cells (MNGCs) in a certain microenvironment. Furthermore, both monocytes and macrophages can develop into mature osteoclasts, which resorb the damaged bone and secrete several stimulatory signals (e.g. matrix-derived TGF-β and IGF-1, secreted collagen triple helix repeat containing 1 (CT-1), and sphingosine 1-phosphate (S1P)) to stimulate new bone formation. It needs to be emphasized that cell-cell interactions also exist between different cell types and that osteoblastic cells also reversely regulate the behavior of immune cells.
switch in macrophage subtype from the M1 subtype to the M2 subtype during the bone healing process [21], suggesting differential roles of these macrophage subtypes and their secreted cytokines on the behavior of MSCs. However, how different types of macrophages contribute to the osteogenic differentiation of MSCs and which factors affect macrophage subtypes remains to be investigated, which represent an important step to develop new bone regenerative strategies.

3. Osteoclasts

Osteoclasts, which are the only cells capable of resorbing bone, are multinucleated giant cells formed from hematopoietic precursors of monocytes and macrophages. After bone damage, osteoclasts attach to the bone surface, seal and acidify a resorbing compartment by secreting H⁺ ions, facilitate dissolution of the bone mineral and thereby expose the organic matrix to proteolytic enzymes to degrade it [22]. The resorption of bone matrix releases several osteogenic factors, such as BMP-2, Transforming growth factor β (TGF-β), platelet-derived growth factor-BB (PDGF-BB) or epitopes, and some ions such as Ca²⁺, which can promote migration and osteogenic differentiation of MSCs [22, 23]. However, recently emerging data suggest that osteoclasts affect bone formation by secreting bone anabolic signals independent of their bone resorption activity [24, 25]. With the findings of different types of osteoclasts in physiological and pathological conditions [26], osteoclasts seem to carry out more crucial functions to affect bone formation and bone quality than previously thought. This information, however, is largely ignored in the previous bone graft design and gained much attention in recent studies on the mechanism of bone formation [27, 28].

Objective of this thesis

Overall, during bone regeneration, innate immune cells not only directly differentiate into bone-resorbing cells to get involved in bone remodeling, but also affect the osteoblastic cell recruitment, migration and osteogenic differentiation to establish bone formation. This information encourages researchers to consider immune cell implementation for bone regeneration. On the one hand, immune cells are plastic and sensitive to the local environment, which provides the potential to modulate the microenvironment, such as changing material properties, adding particular cells or cytokines to manipulate the immune system in a favorable manner for enhanced bone repair and regeneration [29]. On the other hand, due to the easy isolation procedure and high yield of immune cells, particularly monocytes compared to MSCs, immune cells have great practical potential to be utilized in cell-based approaches given reduced cost and (absence or short) time of in vitro expansion procedures.

In view of this, the overall aim of this thesis was to contribute to a better understanding and application of using immune cells to aid in bone regeneration. More specifically, this thesis aimed to 1) investigate the role of different macrophage subtypes on osteogenic differentiation of osteoblast progenitors, 2) exploit the potential to promote osteogenic differentiation and bone formation by co-culturing and co-seeding MSCs
Chapter 1

with monocytes/macrophages, 3) modulate the macrophage phenotype by material properties, 4) elucidate the function of osteoclasts in bone formation, and 4) utilize material properties to affect osteoclast formation and characteristics for functional steering of osteogenic differentiation.

As a scientific approach for these (sub)aims, we addressed the following research questions:

1. What is the state of and problem with current cell-based bone regenerative approaches and how can immune cells and other easily accessible cells be utilized for bone regeneration in an intraoperative manner? (Chapter 2)
2. To what extent do different macrophage subtypes modulate osteogenic differentiation of adipose tissue MSCs (Chapter 3)?
3. Does co-seeding stromal vascular fraction or adipose MSCs with monocytes/macrophages enhance bone regeneration compared to conventional MSC-based approaches in orthotopic bone defects (Chapter 4)?
4. Do material surface properties differentially activate platelets and affect macrophage behavior (Chapter 5)?
5. Does material roughness modulate macrophage activation and polarization? (Chapter 6)
6. As the derivative of monocytes/macrophages, what is the role of osteoclasts in ectopic bone formation? (Chapter 7)
7. Does material roughness affect osteoclast formation and characteristics, and hence have an effect on coupling osteoclastic with osteoblastic activity (Chapter 8)?
General introduction

References


Chapter 2

Intraoperative construct preparation: a practical route for cell-based bone tissue engineering

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Chapter 2

Introduction

Large bone defects and fractures are still clinical problems that require surgical intervention, especially in a condition where the regenerative process is compromised [1]. Although autografts are currently considered as the gold standard for such treatment, their application is restricted by the limited supply and associated complications [2]. Besides, allografts from cadaveric bone and xenografts from non-human species, are considered as the other alternative for reconstructing bone defects but are restricted by immunological rejection and disease transmission issues [3, 4]. Attempts to find superior substitutes for bone graft have resulted in the emergence of bone constructs based on tissue engineering principles. The term Bone Tissue Engineering is defined to develop biologic bone graft substitutes with a synthetic scaffold, osteogenic cells or growth factors to repair damaged bone or regenerate new bone [5]. In particular, cell-based bone tissue engineering has gained much attention due to the vast investigation on stem cells and controlled bone regeneration over grows factor based strategy.

The classical procedure of cell-based bone tissue engineering consists of isolating MSCs from a patient, which, after expansion and differentiation in culture, are seeded onto a suitable scaffold before implantation back into the same patient [6] (Figure 1a). MSCs from different sources, such as bone marrow, adipose tissue, peripheral blood, and dental pulp, have been explored extensively and have demonstrated favorable bone regenerative capacity in many animal studies (reviewed by Ma et al.) [7]. Furthermore, several clinical trials with cell-based constructs for bone regeneration have been conducted and also showed feasibility and efficacy regarding bone formation (reviewed by Grayson et al.) [8]. Despite great advances, several limitations of bone tissue engineering have been brought forward. Firstly, the routine bone tissue engineering needs expansion and osteogenic priming of the isolated cells in vitro to obtain sufficient cell numbers and to foster the osteogenic potential of seeded cells. This procedure, which takes several days to weeks, is time-consuming and costly (Figure 1a). In this perspective, the clinical application of tissue-engineered bone constructs is not a cost-effective alternative for auto-/allo-/xenografts. Secondly, the ex vivo cell manipulation increases the risk of pathogenic contamination and immune rejection related to the use of animal serum and the risk of genetic alterations during cell passages. Lastly, two invasive procedures, one to harvest autologous cells and the other to implant constructs, is not patient-friendly and convenient. These problems hinder the practical application of bone tissue engineering and delay their approval by regulatory authorities for clinical applications. This has encouraged researchers to consider the possibility to prepare tissue-engineered bone constructs during the time of surgery without cumbersome in vitro manipulation, which is defined as intraoperative preparation of tissue-engineered bone constructs.

The intraoperative preparation of tissue-engineered bone constructs implicates that the manufacturing process, including harvest of autologous cells, generation of constructs, and finally implantation, can be combined with the surgical procedure to treat bone defects (Figure 1b). This method entails several benefits compared to the
conventional bone tissue engineering strategies: 1) without *in vitro* cell manipulation and expansion procedure, surgery can be performed within several hours, which would dramatically reduce the cost and labor and is more patient-friendly; 2) without using exogenous components such as animal serum for several passages expansion, the risk of gene mutation and pathogenic contamination likely become negligible; 3) due to the manipulation and surgery performed entirely within one operating room and the minimal manipulation of cells or tissues, this approach would ease the route to regulatory approval.

In the following sections, we will review current approaches to intraoperatively prepare tissue-engineered constructs, with which cells can be harvested, minimally manipulated, and implanted to bone defect areas during the surgery. Based on the reviewed articles, we here discuss the cells that are intraoperatively available to prepare such constructs and provide an overview of the available data of preclinical and clinical studies using intraoperatively prepared tissue-engineered bone constructs. Furthermore, the therapeutic efficiency and future perspectives and technologies to develop such intraoperatively prepared tissue-engineered bone constructs are discussed.

**Available cell sources**

Osteogenic cells are the most essential component to intraoperatively prepare tissue-engineered bone constructs, which endow grafts with high osteogenic activity. Although cells with osteogenic potential are present in many tissues, to meet the requirements for intraoperative preparation, only few tissues have been explored, namely, bone marrow, adipose tissue, and peripheral blood. The advantages and disadvantages of utilization of these tissues for cell isolation are displayed in Table 1.

1. **Bone marrow**

Bone marrow (BM) is to date the most common tissue source applied both experimentally and clinically for preparing intraoperative tissue-engineered bone constructs, which mainly relates to its high osteogenic capacity and relatively easy access. The firstly developed strategy was the direct use of bone marrow aspiration concentrate cells (BMACs) [9, 10], which are a mixture of erythrocytes, granulocytes, hematopoietic cells (HCs), endothelial progenitor cells (EPCs), MSCs, lymphocytes and immature monocytes amongst others. This approach can be performed in a short time and at relatively low cost, and few studies reported positive effects of using these cells for bone regeneration [11-13]. However, the majority of studies involving BMACs demonstrated a lack of stimulatory effects on bone regeneration [9, 14, 15], which can likely be attributed to the relatively low numbers of osteogenic cells and a high number of non-effective cells.

After aspiration of bone marrow and elimination of erythrocytes and granulocytes, a heterogeneous cell population, i.e. bone marrow-derived mononuclear cells (BMNCs), can be achieved. This fraction includes a higher number of HCs (around 4%), MSCs (around 0.1%), and EPCs (around 5%), from which MSCs play a major
role in bone healing [16-18]. Multiple animal studies have demonstrated beneficial effects of BMNCs on bone healing [14, 19-21]. A large-scale clinical report also demonstrated the safety and efficacy of BMNCs for intraoperatively prepared tissue-engineered bone constructs [22]. However, due to the limited volume of bone marrow in one patient (maximal ~300 ml) and the relatively low yield of MSCs in bone marrow (maximum 100 colony-forming unit fibroblasts (CFU-f)/million MNCs in young people), only 1500-3000 MSCs/ml in healthy human bone marrow can be obtained [23] cannot provide the desired or required numbers of MSCs, especially in elderly or otherwise compromised (e.g. osteoporotic) patients.

2. Adipose tissue
In contrast to the limited supply of bone marrow, adipose tissue is largely available and has recently attracted much attention as a promising alternative source for osteogenic cells. Fat tissue can be collected through a less invasive method with minimal morbidity upon harvest and in larger quantities of effective stem cells than bone marrow. The numbers of MSCs in adipose tissue (i.e. 1-3×10^4 CFU-f/million MNC) is 100 to 300 times higher compared to bone marrow [24], and the number of stem cells that can be isolated per unit volume of lipoaspirate is approximately 10-fold greater than that from bone marrow (i.e. 3×10^4 MSCs/ml of lipoaspirate). Consequently, small adipose tissue reservoirs can already provide sufficient numbers of MSCs for clinical applications.
Additionally, perivascular stem cells (PSCs), another stem cell type that is abundant in fat tissue [25], have been demonstrated to have a mesenchymal potential equal or even superior to conventional MSCs in some cases [26, 27]. James et al. demonstrated the efficacy of using PSCs for the intraoperative preparation of tissue-engineered bone constructs.

Stromal vascular fraction (SVF), the freshly isolated fraction from the lipoaspirate or fat patch, is a mixture of several cell types, including MSCs, EPCs, pericytes, and monocytes [28]. Since the isolation of SVF can be achieved within a few hours, it is suitable for a one-step surgical procedure to prepare tissue-engineered bone constructs. One additional important advantage of SVF is the presence of endothelial lineage cells among the heterogeneous cell mixture. These cells, which are typically lost during prolonged monolayer cultures, but preserved upon direct construct preparation, have shown to contribute to the formation of blood vessels in recipient sites, which is critical for the survival and function of cells in implanted tissue-engineered bone constructs [29]. Nevertheless, few studies have postulated concerns that the osteogenic capacity of SVF is significantly lower than that of MSCs isolated from bone marrow [30, 31].

3. Peripheral blood

As blood is more easily accessible than bone marrow and adipose tissue, the advantages of using peripheral blood as a source of MSCs is emerging. Mononuclear cells (MNCs) from PB contain both hematopoietic stem cells (HSCs) and EPCs [32]. The osteogenic potential of PB-derived MSCs (PBMSCs) has been suggested by accumulating evidence from both in vitro and in vivo experiments [33, 34]. However, only small numbers of stem cells exist in PB (less than 20 CFU-f/million MNCs) compared to numbers in BM [35], which is a major obstacle for an intraoperative approach using cells derived from PB. Several steps have been taken to enrich stem cells in PB, such as mobilizing them from BM with granulocyte colony-stimulating factor (G-CSF) [36, 37], chemotherapeutic agents [38], or using CD133 selection [39]. This can concentrate PBMSCs over 20 times (to reach levels comparable to those in fresh bone marrow). However, this CFU-f value is still far from the effective implantation threshold (≥ 1000 cells/cm³ for BMSCs) [40]. Therefore, the application of PBMSCs for intraoperatively prepared tissue-engineered bone constructs has not been reported to date. Other cell types, e.g. CD31⁺ EPCs [41] and CD34⁺ endothelial/hematopoietic progenitor cells [42], are present in large quantities in PB, independent of the individual’s age and gender. When applied to rat femoral bone fractures, advanced bone tissue restoration was observed [41, 42]. Given its availability (between 70-80%) and general leukocyte enrichment efficiency (4×10⁶ cells/ml), 5 ml of peripheral blood would provide 1×10⁷ CD31⁺ cells, which is sufficient to prepare tissue-engineered bone constructs without expansion cultures.

Cell isolation approaches

Isolation or concentration of desired cells is needed to prepare tissue-engineered bone constructs. This step can select a certain cell mixture or even a specific type of cells
and remove detrimental cells. For bone marrow-derived cells, density separation (DS) and selective retention (SR) are mostly adapted methods for isolating desired cells. DS involves the use of a centrifuge to concentrate nucleated cells and connective tissue progenitors while SR involves adsorbing connective tissue progenitor cells through a porous substrate. Recently, a method based on red cell lysis to isolate BMNCs was established to concentrate BMNCs over 50 times [11]. This method is more efficient, faster and more easily standardized compared to DS and SR, showing promise in clinical applications for preparing bone marrow-derived intraoperative bone constructs.

To obtain SVF for intraoperative preparation of tissue-engineered bone constructs, adipose tissue is generally harvested via minimally invasive techniques, and processed by enzymatic digestion and centrifugal enrichment. This can be achieved with a single surgical procedure lasting less than 2 hours. To meet the requirement from regulatory authorities for ‘less than minimally manipulated’ cells, a non-enzymatic method was recently developed to obtain highly enriched adipose tissue-derived elements by mild mechanical forces [43], showing another option to intraoperatively obtain desired cells from lipoaspirate. To further select a subpopulation from SVF, such as CD31+ or CD34+ cells, cell sorting by flow cytometry is mostly used based on the surface markers of desired cells. Due to the special format of PB, the isolation of blood-derived cells is generally easily performed by a concentration of the buffy coat with a sedimentation agent such as hydroxyethyl starch, and sorting by flow cytometry afterwards.

Preclinical studies with intraoperative tissue-engineered bone constructs

Following the above-mentioned screening procedure, 33 preclinical studies meeting the criteria were identified (Table 2): 7 animal studies using ectopic implantation models and 27 animal studies using an orthotopic model. Among these preclinical studies, 12 studies used bone marrow-derived cells, 19 studies used adipose tissue-derived cells and 4 studies used peripheral blood-derived cells to generate bone with an intraoperative preparation concept. 1 study utilized cells from different sources (i.e. adipose tissue and bone marrow) to evaluate their bone healing capacity.

To prepare tissue-engineered bone constructs, normal tissue aspiration combined with either SR, a centrifugation step, or cell sorting (e.g. flow cytometry (FACS) or magnetic sorting) is the common approach to get “easily available cells”. To support these intraoperatively isolated cells, different cell carriers were utilized, including allografts, xenografts, collagen, synthetic bioceramics, and polymers. Additionally, platelet-rich plasma (PRP), which can be intraoperatively obtained as well, was used as a ‘glue’ to entrap cells or as an inducer in 8 studies. The effect of PRP addition on bone formation, however, was investigated in none of these studies.

Regarding the mode of action of these intraoperatively prepared tissue-engineered bone constructs, there are a few possibilities. Bone regeneration could be established through direct differentiation of intraoperatively grafted cells [44, 45]. Also, one study indicated that potential paracrine effects, including modulatory effects on vascularization, osteogenesis and inflammatory responses of host tissue, play a more important
Intraoperative construct preparation: a practical route for cell-based bone tissue engineering

role than the intrinsic cell differentiation potential [46]. It is also possible that both mechanisms act simultaneously based on the type of cells and the conditions in which cells are grafted. For instance, when PB-derived CD34+ cell-based bone constructs were applied to femoral fractures, direct vasculogenesis and osteogenesis by transplanted PB-derived CD34+ cells were detected. Moreover, grafted cells significantly enhanced the intrinsic angiogenesis and osteogenesis of the recipient cells by upregulating VEGF, Angiopoietin-1 (Ang-1), and bone morphogenetic protein-2 (BMP-2) gene expression at the fracture sites [44, 45]. Therefore, it seems likely that these “easily available cells” act using both direct and indirect mechanisms to aid in bone healing by promoting neovascularization and osteogenesis.

Clinical studies with intraoperative tissue-engineered bone constructs

Among the 29 clinical studies (Table 3), 21 publications dealt with cells obtained from bone marrow, 7 from adipose tissue, and 1 from peripheral blood. Bone marrow-derived cells, especially enriched BMNCs, dominated the clinical trials involving intraoperative preparation of tissue-engineered bone constructs. This is different from what was reviewed for preclinical studies, where adipose tissue was the main source for usage. This discrepancy probably results from differences in the moment of discovery of the presence of adult stem cells in these tissues and the concomitant amount of (in vitro and preclinical) evidence regarding the bone regenerative potential of isolated cells from these tissues.

One interesting observation from all clinical studies is that researchers used medical devices to intraoperatively isolate cells in half of the retrieved clinical studies. This would assumedly improve the reproducibility of intraoperatively prepared tissue-engineered bone constructs. Moreover, these isolation devices are closed systems and can be used in the operating room without cell laboratory support, which makes intraoperative preparation more feasible and applicable. To isolate bone marrow-derived cells, DS is more frequently used than SR. Although these two methods have been compared in preclinical studies, their effect on bone formation has not been reported in any clinical study. For adipose tissue-derived cell isolates, tissue aspiration combined with a collagenase digestion step was generally used to isolate SVF. Only one study utilized a filtration device to obtain ADMSCs without enzymatic collagenase treatment [47]. With regard to cell carriers, auto-/allo-/xenografts (11 of 29 studies) were more frequently used in clinical compared to preclinical studies. Further, bioceramics and collagen are the second most commonly used cell carriers in the retrieved clinical studies. When these two cell carriers were compared in the same condition, the postoperative bone formation appeared 7 weeks earlier and bone healing was 5 weeks faster for bioceramics versus collagen [48]. Cells without a carrier, but with native fibrin were applied in 5 studies [49-53]; however, its efficacy was not compared to constructs with a certain cell carrier. Notably, PRP, which acts as a cell carrier as well as a cytokine reservoir, was widely used in these clinical studies.
Chapter 2

Efficiency of intraoperative approach

The literature search results show that using ‘easy access cells’ to intraoperatively prepare tissue-engineered bone constructs has been explored in numbers of pre- and clinical studies so far. These studies used different cells, isolation procedures and seeding strategies, which led to dissimilar therapeutic effects. It is valuable to compare these studies and draw some conclusions to aid the future design of intraoperatively prepared tissue-engineered bone constructs.

1. Does the intraoperative approach improve bone healing compared to acellular controls?

From 28 animal studies using an orthotopic model, 24 studies compared intraoperative tissue-engineered bone constructs to cell-free constructs. Amongst these, 12 studies used intraoperatively available sorted cells. Irrespective of the source (e.g. from bone marrow (BMNCs), adipose tissue (PSCs) or peripheral blood (PB-derived EPCs and ECs)), these cells enhanced bone formation compared to cell-free constructs [10, 11, 14, 15, 22, 42, 44, 54-57]. In contrast, unsorted cells (e.g. BMACs, SVF, and PBMNCs) appeared inconclusive regarding their regenerative capacity. 14 studies demonstrated a promotive effect on bone healing [9, 11, 14, 15, 45, 56-64], while 4 studies showed comparable bone healing compared to cell-free constructs [15, 54, 65, 66]. This discrepancy may be associated with the cell density used to make these constructs. A high density of desired cells (10⁶/cm³ and 10⁵/cm³) seeded on the scaffold generally led to the bone formation, while a low density (10⁴/cm³) did not [44, 45]. Still, the optimal number of seeded cells required in a graft site remains unknown.

Due to the difficulty to set up a control group in clinical cases, comparison of these intraoperatively prepared tissue-engineered bone constructs with cell-free constructs is not practical. Only 3 studies reported the comparison between SVF- and BMNCs-based with cell-free constructs in a clinical setting and demonstrated superior bone healing for SVF- and BMNCs-based constructs [44, 67, 68]. 2 studies used autografts as controls and demonstrated comparable bone formation of constructs prepared with bone marrow-derived cells [21, 69]. Cell density similarly appears to play an important role in the clinical outcome as in animal studies, as indicated by the superior bone healing outcome for constructs with higher numbers of progenitor cells (6000 CFU-f/cm³) [49, 50].

2. Do the intraoperatively prepared bone constructs performance superior to conventional bone tissue engineering approach?

The aim of the intraoperative construct preparation is to overcome limitations of conventional cell-based bone tissue engineering for bone regeneration. However, how efficient of intraoperative approach compared to conventional approach is rarely reported. In all searched publications, only 4 studies compared therapeutic effects of intraoperatively prepared constructs to conventional tissue-engineered constructs (Table 4). All studies showed an inferior capacity of intraoperatively prepared constructs
in promoting bone healing [9, 58, 65, 66], which indicates the necessity to improve the osteogenic potential of seeding cells. Besides, limitations of this comparison exist as none of these studies used human-derived cells and donor-matched comparison.

3. Is sorted cell-based approach superior to unsorted cell-based approach?
In the retrieved publications, both sorted and unsorted cell populations for intraoperative preparation of tissue-engineered bone constructs were used. The use of sorted homogeneous cells, such as ADMSCs and PSCs, facilitates improved standardization and greater control on the outcome of bone healing. The disadvantages include longer processing times and a requirement of extra instruments. In contrast, unsorted heterogeneous populations, such as BMNCs, SVF, and PBMNCs, contain multiple cell types (e.g. MSCs, ECs, pericytes, and monocytes) and may enable accelerated bone regeneration through heterotypic interactions among endogenous cell populations. The drawback of this method is the uncertain composition of the heterogeneous cell isolate. 7 studies compared the therapeutic efficiency of sorted and unsorted cell-based intraoperative approach, enriching cell isolates showed superior bone healing capacity in 6 studies compared to constructs prepared using unsorted cells [20, 25, 41, 42, 56, 70], except for one study that demonstrated similar bone healing results [12]. This information further suggests the importance to enrich desired cells during the intraoperative preparation of tissue-engineered bone constructs.

4. Which tissue-derived cells have superior bone healing effects for intraoperatively prepared tissue-engineered bone constructs?
Respecting the bone formation capacity of cells from different tissues, only one study compared effects of SVF (adipose tissue) with BMAC (bone marrow), which showed the superiority of BMAC over SVF regarding bone formation [54]. More comprehensive, donor-matched comparison is desired to draw a clear conclusion.

Future perspectives

Both preclinical and clinical results showed a large potential of intraoperatively prepared tissue-engineered bone constructs for repairing damaged bone. However, further improvements in their therapeutic efficacy are suggested, which can likely be achieved by taking into consideration the following issues:

1. Enrichment of desired cells
Previous studies revealed a positive correlation between bone regeneration and the number of osteoprogenitors in BMNC-based constructs [11, 50]. Except for the reported DS and SR methods to isolate BMNCs, cell sorting methods based on cell surface markers of specific cell types have been developed to select the target, and remove unwanted cell populations. For instance, a highly homogenous CD34+ cell population can be concentrated from peripheral blood by immunoselection in short time by flow cytometry [38]. This was also applied to select CD34+ MSCs from adipose tissue, which
Chapter 2

exhibited 5 times more bone formation after 8 weeks compared to unsorted SVF cells [71]. With the same principle, magnetically activated cell sorting has been used to isolate bone marrow-derived connective tissue progenitors on the basis of their hyaluronan antigens [20]. Based on this idea, an easier, inexpensive, and more feasible method was invented to use DNA aptamers, which are single-stranded DNA or RNA with high specificity and affinity to membrane proteins of target cells [72]. Notably, these aptamers can be customized by labeling them with functional groups and retain their activities after binding on cell carriers and after implantation [73]. So far, this method has only been conducted to isolate bone marrow cells. It seems worthwhile to assess effects of cells isolated by this strategy on bone healing and its application on adipose or other tissue in the future. Of note, all these immunoselection-based cell sorting methods are premised on the basis of a well-defined marker panel of desired cells.

2. Co-culture methods

Except for the interaction between unfractionated cells, whose mechanism and benefits on bone formation has not been well illustrated and proved, the reported cell-cell interactions between purified cells can be utilized to potentially improve bone healing. More importantly, this strategy can use intraoperatively available cells from different tissue(s), which will fully take advantage of various cell types. For instance, cells from bone marrow have high osteogenic potential while CD34+ cells from fat tissue have high vascular potential. This gives rise to the possibility to intraoperatively use cells from bone marrow and adipose tissue to generate vascularized tissue-engineered bone constructs, which not only keep the advantages of intraoperative preparation but also improve the outcome of these tissue-engineered bone constructs. Another example is PB-derived cell subsets, such as monocytes and circulating EPCs. All these cells can be easily obtained and have been proved to be involved in endogenous tissue healing, which in certain cases can promote bone regeneration [45, 74]. Therefore, these PB-derived subpopulations have the potential to improve adipose tissue-, peripheral blood-derived stem cell-based bone healing, which has an insufficient osteogenic potential. Future studies can also focus on the use of macrophages [75], regulatory T cells [76, 77] and haematopoietic cells [78], together with certain easily available cells to promote desired regeneration events, as all of them have been shown to be essential for bone formation and widely present in peripheral blood and adipose tissue.

3. Cell carrier design

To date, intraoperative preparation of tissue-engineered bone constructs is rather simplistic, typically seeding cells on the carriers without control over cell composition, phenotype, and function in vivo. However, based on the aforementioned clinical evidence, bone healing following treatment with intraoperatively prepared tissue-engineered bone constructs depends on cell carrier characteristics [67, 79]. An ideal design of a carrier is able to concentrate and sort progenitor populations and to cause more rapid attachment of specific cell types during seeding, to promote cell survival
Intraoperative construct preparation: a practical route for cell-based bone tissue engineering

and direct cell fate toward desired differentiation after seeding and to improve bone and blood vessel formation after implantation. This design includes, but is not limited to chemical, physical and mechanical properties. Future technologies in material science and chemistry will greatly aid the development of optimized cell carriers, which certainly requires a fundamental biological understanding of cell-material interactions.

Conclusion

From a practical view, intraoperative bone construct preparation will dramatically decrease the time, costs, safety concerns and favoring patient comfort. Cells from bone marrow, adipose tissue, peripheral blood provide the possibility to utilize this approach toward effective bone healing. Both preclinical and clinical studies displayed promising outcome when the intraoperative preparation concept is applied, especially with the benefits from new technologies, such as cell isolation devices, co-culture method and advanced design of cell carriers. Collectively, the intraoperative approach demonstrate the tremendous promise to advance stem cell therapies for bone repair and will serve as a new trend to make cell-based regenerative transplants.
Chapter 2

References


Intraoperative construct preparation: a practical route for cell-based bone tissue engineering


Chapter 2


Intraoperative construct preparation: a practical route for cell-based bone tissue engineering


[92] F. Duttenhoefer, S.F. Hieber, A. Stricker, R. Schmelzeisen, R. Gutwald, S. Sauerbier, Follow-up of implant survival comparing ficoll and bone marrow aspirate concentrate methods for hard tissue regeneration with mesenchymal stem cells in humans, BioResearch open access 3(2) (2014) 75-76.
Intraoperative construct preparation: a practical route for cell-based bone tissue engineering


### Table 1. The advantages and disadvantages of using easily available cells from different tissues to make intraoperative concept based bone constructs

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Cell type</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>BMACs</td>
<td>high osteogenic potential; endothelial sub-population</td>
<td>donor variation; no minimal invasive harvest</td>
</tr>
<tr>
<td></td>
<td>BMNCs</td>
<td>high osteogenic potential; extensively studied</td>
<td>low yield; no minimal invasive harvest</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>SVF</td>
<td>abundant tissue; easy harvest procedure; endothelial sub-population</td>
<td>donor variation; multi-step isolation procedure; low osteogenic potential</td>
</tr>
<tr>
<td></td>
<td>PSCs</td>
<td>high yield from adipose tissue; homogenous cell population</td>
<td>low osteogenic potential; loss of endothelial sub-population</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>PBMNCs</td>
<td>non-invasive harvest procedure; endothelial sub-population</td>
<td>low yield of osteogenic cells; presence of undesired cells</td>
</tr>
<tr>
<td></td>
<td>PB-derived EPCs/ECs</td>
<td>non-invasive harvest procedure; easy isolation method</td>
<td>low yield of osteogenic cells; not widely studied</td>
</tr>
</tbody>
</table>

BMACs: bone marrow aspiration concentrate cells; BMNCs: bone marrow derived mononuclear cells; SVF: stromal vascular fraction; PSCs: perivascular stem cells; PBMNCs: peripheral blood mononuclear cells; PB: peripheral blood; EPCs: endothelial progenitor cells; ECs: endothelial cells.
<table>
<thead>
<tr>
<th>Cell source</th>
<th>Cell isolation / treatment</th>
<th>Scaffold</th>
<th>Seeding density</th>
<th>In vivo time</th>
<th>Species and implantation site</th>
<th>Evaluation method</th>
<th>Efficacy of bone formation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ectopic studies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat BMACs</td>
<td>Aspirate</td>
<td>HA/TCP</td>
<td>Unclear</td>
<td>8 weeks</td>
<td>Rat subcutaneous</td>
<td>Histology</td>
<td>Negligible osseous tissue</td>
<td>(9)</td>
</tr>
<tr>
<td>Human SVF</td>
<td>Centrifuge</td>
<td>PRP</td>
<td>2 × 10⁶/cm³</td>
<td>8 weeks</td>
<td>Nude mouse subcutaneous</td>
<td>Histology</td>
<td>No frank bone tissue</td>
<td>(80)</td>
</tr>
<tr>
<td>Human SVF</td>
<td>Centrifuge</td>
<td>HA</td>
<td>1.4 × 10⁷/construct</td>
<td>8 weeks</td>
<td>Nude mouse subcutaneous</td>
<td>Fluorescence microscopy</td>
<td>No frank bone tissue</td>
<td>(31)</td>
</tr>
<tr>
<td>Human SVF</td>
<td>Centrifuge</td>
<td>PRP allogenic cartilage</td>
<td>0.24-1 × 10⁶/construct</td>
<td>12 weeks</td>
<td>Nude mouse subcutaneous</td>
<td>μCT, histology</td>
<td>SVF (5 × 10⁶/construct) formed more bone than cell-free and other cell density constructs</td>
<td>(62)</td>
</tr>
<tr>
<td>Murine SVF</td>
<td>Centrifuge</td>
<td>PRP BMM</td>
<td>5 × 10⁴/cm³</td>
<td>8 weeks</td>
<td>Mouse subcutaneous</td>
<td>Histology</td>
<td>No bone formation</td>
<td>(81)</td>
</tr>
<tr>
<td>Human SVF, PSCs</td>
<td>FACS</td>
<td>DBM</td>
<td>2.5 × 10⁷/ construct</td>
<td>4 weeks</td>
<td>SCID mouse intramuscular</td>
<td>μCT, histomorphometry</td>
<td>PSC formed more bone than SVF</td>
<td>(25)</td>
</tr>
<tr>
<td>Human PSCs</td>
<td>FACS</td>
<td>DBM</td>
<td>2.5 × 10⁷/ construct</td>
<td>4 weeks</td>
<td>SCID mouse intramuscular</td>
<td>μCT, histomorphometry</td>
<td>PSC formed more bone than cell-free control</td>
<td>(82)</td>
</tr>
<tr>
<td><strong>Orthotopic studies</strong></td>
<td></td>
<td></td>
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<tr>
<td>Rat BMACs</td>
<td>Aspirate</td>
<td>HA/TCP</td>
<td>Unclear</td>
<td>8 weeks</td>
<td>Rat femoral segmental defect</td>
<td>Histomorphometry</td>
<td>No difference between BMACs and cell-free control</td>
<td>(9)</td>
</tr>
<tr>
<td>Canine BMNCs</td>
<td>SR</td>
<td>Cancellous BMM</td>
<td>Unclear</td>
<td>12 weeks</td>
<td>Canine spinal fusion</td>
<td>CT</td>
<td>BMNCs formed more bone than cell-free control</td>
<td>(10)</td>
</tr>
<tr>
<td>Canine BMACs, BMNCs</td>
<td>SR</td>
<td>DBM</td>
<td>122, 269 × 10⁶/ construct</td>
<td>12 weeks</td>
<td>Canine spinal fusion</td>
<td>CT</td>
<td>Enriched BMNCs had greater fusion volume and area than BMACs and cell-free</td>
<td>(56)</td>
</tr>
<tr>
<td>Canine BMACs, BMNCs</td>
<td>SR</td>
<td>PRP DBM</td>
<td>Unclear</td>
<td>16 weeks</td>
<td>Canine segmental femoral defect</td>
<td>Histology</td>
<td>BMNC/PRP healed a higher percentage of bone defects than BMACs and cell-free</td>
<td>(14)</td>
</tr>
<tr>
<td>Ovine BMACs, BMNCs</td>
<td>SR</td>
<td>β-TCP</td>
<td>Unclear</td>
<td>6 months</td>
<td>Ovine spinal fusion</td>
<td>μCT, histology</td>
<td>Denser bone formation in the BMNCs than BMACs and cell-free</td>
<td>(15)</td>
</tr>
<tr>
<td>Cell source</td>
<td>Cell isolation / treatment</td>
<td>Scaffold</td>
<td>Seeding density</td>
<td>In vivo time</td>
<td>Species and implantation site</td>
<td>Evaluation method</td>
<td>Efficacy of bone formation</td>
<td>Reference</td>
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</tr>
<tr>
<td>Canine BMACs, BMNCs</td>
<td>Magnetic sorting</td>
<td>Allografts</td>
<td>16.1, 20.5 × 10^6/construct</td>
<td>4 weeks</td>
<td>Canine femoral defect</td>
<td>Histomorphometry</td>
<td>BMNCs had more bone formation than BMACs</td>
<td>(20)</td>
</tr>
<tr>
<td>Canine BMACs, BMNCs</td>
<td>Red blood cell lysis</td>
<td>β-TCP</td>
<td>3.1, 169.3 × 10^6/construct</td>
<td>24 weeks</td>
<td>Canine orbital bone defect</td>
<td>Histology</td>
<td>BMNCs had more bone formation than BMACs and cell-free</td>
<td>(11)</td>
</tr>
<tr>
<td>Ovine BMACs, BMNCs</td>
<td>FICOLL centrifuge</td>
<td>PRP</td>
<td>5.34, 44.5 × 10^4/construct</td>
<td>16 weeks</td>
<td>Ovine sinus augmentation</td>
<td>Histomorphometry</td>
<td>BMNCs formed more bone than BMACs</td>
<td>(70)</td>
</tr>
<tr>
<td>Human BMNCs</td>
<td>FICOLL centrifuge</td>
<td>β-TCP</td>
<td>1 × 10^7/defect</td>
<td>8 weeks</td>
<td>Nude rat femoral defect</td>
<td>Histomorphometry</td>
<td>BMNCs formed more bone than cell-free</td>
<td>(17)</td>
</tr>
<tr>
<td>Canine BMACs, BMNCs</td>
<td>DS, SR, DS+PRP</td>
<td>Allografts</td>
<td>58, 110, 76, 276 × 10^6/construct</td>
<td>4 weeks</td>
<td>Canine femoral multidefect model</td>
<td>μCT</td>
<td>No difference in bone formation between BMACs and DS-BMNCs, SR-BMNCs and DS+PRP-BMNCs</td>
<td>(12)</td>
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<tr>
<td>Rat BMNCs, SVF</td>
<td>Centrifuge</td>
<td>BCP</td>
<td>2.5, 5 × 10^6/construct</td>
<td>3 weeks</td>
<td>Rat irradiated tibial and femoral defect</td>
<td>SEM</td>
<td>BMNCs had more bone formation than SVF, no difference between SVF and cell-free</td>
<td>(54)</td>
</tr>
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<td>Equine SVF</td>
<td>Centrifuge</td>
<td>Collagen sponge</td>
<td>5 × 10^5/construct</td>
<td>12 weeks</td>
<td>Nude rat calvarial defect</td>
<td>μCT</td>
<td>No difference between SVF and cell-free</td>
<td>(65)</td>
</tr>
<tr>
<td>Rat SVF</td>
<td>Centrifuge</td>
<td>PRP</td>
<td>1 × 10^7/construct</td>
<td>8 weeks</td>
<td>Rat calvarial defect</td>
<td>Radiodensitometry</td>
<td>SVF showed more abundant bone formation than cell-free</td>
<td>(63)</td>
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<td>Goat SVF</td>
<td>Centrifuge</td>
<td>PLCL</td>
<td>5 × 10^6/construct</td>
<td>6 months</td>
<td>Goat spinal fusion</td>
<td>Radiography</td>
<td>SVF showed more spinal fusion than cell-free</td>
<td>(64)</td>
</tr>
<tr>
<td>Rabbit SVF</td>
<td>Centrifuge</td>
<td>PLGA</td>
<td>1 × 10^6/construct</td>
<td>8 weeks</td>
<td>Rabbit ulna defect</td>
<td>μCT</td>
<td>SVF formed more bone than cell-free</td>
<td>(58)</td>
</tr>
<tr>
<td>Human SVF, PSCs</td>
<td>FACS</td>
<td>PLGA</td>
<td>2.5 × 10^5/construct</td>
<td>8 weeks</td>
<td>SCID mouse calvarial defect</td>
<td>μCT</td>
<td>PSC formed more bone than SVF, no difference between SVF and cell-free</td>
<td>(57)</td>
</tr>
<tr>
<td>Cell source</td>
<td>Cell isolation / treatment</td>
<td>Scaffold</td>
<td>Seeding density</td>
<td>In vivo time</td>
<td>Species and implantation site</td>
<td>Evaluation method</td>
<td>Efficacy of bone formation</td>
<td>Reference</td>
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<tr>
<td>Human PSCs</td>
<td>FACS</td>
<td>DBM</td>
<td>0.15, 0.5, 1.5 x 10⁶/construct</td>
<td>4 weeks</td>
<td>Nude rat spinal fusion</td>
<td>µCT, histomorphometry</td>
<td>PSC formed more bone than cell-free</td>
<td>(55)</td>
</tr>
<tr>
<td>Rat SVF</td>
<td>Centrifuge</td>
<td>Type I collagen gel</td>
<td>1 x 10⁶/defect</td>
<td>6 weeks</td>
<td>Rat femoral callus distraction</td>
<td>Radiography</td>
<td>SVF had more bone formation compared to cell-free</td>
<td>(59)</td>
</tr>
<tr>
<td>Goat SVF</td>
<td>Centrifuge</td>
<td>PLCL</td>
<td>5 x 10⁶/construct</td>
<td>6 months</td>
<td>Goat spinal fusion</td>
<td>Radiography</td>
<td>No difference between SVF and cell-free</td>
<td>(66)</td>
</tr>
<tr>
<td>Rat SVF</td>
<td>Centrifuge</td>
<td>Chitosan</td>
<td>2.19 x 10⁸/construct</td>
<td>28 days</td>
<td>Rat mandibular defect</td>
<td>Histochemistry</td>
<td>SVF formed more bone than cell-free</td>
<td>(60)</td>
</tr>
<tr>
<td>Murine SVF, PSCs</td>
<td>Magnetic sorting</td>
<td>Allografts</td>
<td>1 x 10⁶/construct</td>
<td>12 weeks</td>
<td>Murine segmental femoral defect</td>
<td>µCT</td>
<td>PSCs based constructs formed more bone than cell-free</td>
<td>(83)</td>
</tr>
<tr>
<td>Human SVF</td>
<td>Centrifuge</td>
<td>PRP Silicated-HA</td>
<td>8 x 10⁶/construct</td>
<td>8 weeks</td>
<td>Nude rat segmental femoral defect</td>
<td>Histochemistry</td>
<td>SVF formed more bone than cell-free</td>
<td>(61)</td>
</tr>
<tr>
<td>Human SVF</td>
<td>Centrifuge</td>
<td>PRP alogenic cartilage</td>
<td>0.24-1 x 10⁶/construct</td>
<td>4 weeks</td>
<td>Nude rat calvarial defect</td>
<td>Histology</td>
<td>SVF formed more bone than cell-free</td>
<td>(62)</td>
</tr>
<tr>
<td>Human PB-derived EPCs</td>
<td>FACS</td>
<td>Atelocollagen</td>
<td>1 x 10³, 1 x 10⁴/construct</td>
<td>8 weeks</td>
<td>Nude rat femoral defect</td>
<td>Radiography</td>
<td>More fracture healing in 10⁴ and 10⁵ group than 10³ and cell-free group, no difference between 10³ and cell-free</td>
<td>(44)</td>
</tr>
<tr>
<td>Human PBMCNs</td>
<td>FACS</td>
<td>Atelocollagen</td>
<td>1 x 10⁶, 1 x 10⁷/construct</td>
<td>8 weeks</td>
<td>Nude rat femoral nonunion</td>
<td>Histology</td>
<td>10⁵ group showed superior bone healing than 10⁶ and cell-free, no difference between 10⁶ and cell-free</td>
<td>(45)</td>
</tr>
<tr>
<td>Human PB-derived EPCs and PBMCNs</td>
<td>FACS</td>
<td>Atelocollagen</td>
<td>1 x 10⁵, 1 x 10⁷/construct</td>
<td>12 weeks</td>
<td>Nude rat femoral nonunion</td>
<td>Radiography</td>
<td>EPCs formed more bone than PBMCNs and cell-free</td>
<td>(42)</td>
</tr>
<tr>
<td>Rat PB-derived EPCs, PBMCNs</td>
<td>Magnetic sorting</td>
<td>Autologous blood clot</td>
<td>Unclear</td>
<td>6 weeks</td>
<td>Rat femoral defect</td>
<td>µCT, histomorphometry</td>
<td>EPCs advanced bone tissue restoration than PBMCNs</td>
<td>(41)</td>
</tr>
</tbody>
</table>

BMACs: bone marrow aspiration concentrate cells; BMNCs: bone marrow derived mononuclear cells; SVF: stromal vascular fraction; PSCs: perivascular stem cells; PB: peripheral blood; PBMCNs: peripheral blood mononuclear cells; EPCs: endothelial progenitor cells; ECs: endothelial cells. HA: hydroxyapatite; β-TCP: β-tricalcium phosphate; PRP: platelet-rich plasma; BMM: Bone mineral matrix; DBM: Demineralized bone matrix; BCP: biphasic calcium phosphate; PLA: Poly(lactic acid); PLCL: Poly (L-lactide co ε-caprolactone); PLGA: Poly(Lactide-co-Glycolide); DS: density separation; SR: selective retention.
Table 3. Clinical studies with intraoperatively prepared tissue-engineered bone constructs

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Cell isolation</th>
<th>Cell treatment</th>
<th>Cell carrier</th>
<th>Number of patients</th>
<th>Repair site</th>
<th>Evaluation method(s)</th>
<th>Reported outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMACs</td>
<td>Aspirate</td>
<td>Local injection</td>
<td>60</td>
<td>Femur osteonecrosis</td>
<td>MRI</td>
<td>Both methods improved hip function, BMNCs was superior to BMACs to treat avascular necrosis of femoral head.</td>
<td>(84)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>Aspirate</td>
<td>Local injection</td>
<td>11</td>
<td>Distal tibial metaphysis nonunion</td>
<td>CT</td>
<td>9/11 patients attained bony union within 6 months of bone marrow injection. 6/9 patients reported improved bone healing.</td>
<td>(85)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS (device)</td>
<td>Local injection</td>
<td>116</td>
<td>Osteonecrotic femoral head</td>
<td>Radiography MRI</td>
<td>Low incidence of complications and effective in earlier stages of osteonecrosis; greater number of progenitor cells had superior outcomes</td>
<td>(49)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS (device)</td>
<td>Local injection</td>
<td>13</td>
<td>Osteonecrotic femoral head</td>
<td>Radiography MRI</td>
<td>1/10 hips in the BMNCs treated group showed progression to stage III compared to 5/8 hips in the control group after 24 months</td>
<td>(51)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS (device)</td>
<td>Local injection</td>
<td>60</td>
<td>Atrophic tibial nonunion</td>
<td>Radiography</td>
<td>Bone union obtained in 53 patients after an average of 12 weeks; bone formation correlated with MSCs number and concentration</td>
<td>(40)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS</td>
<td>PRP</td>
<td>β-TCP</td>
<td>1</td>
<td>Cleft lip and alveolus</td>
<td>CT</td>
<td>Sufficient bone regeneration shown after 6 months</td>
<td>(86)</td>
</tr>
<tr>
<td>BMACs</td>
<td>Aspirate</td>
<td>Allograft</td>
<td>13</td>
<td>Aneurysmal bone cyst</td>
<td>Radiography</td>
<td>Healing obtained in 11 patients after a mean follow-up duration of 3.9 years</td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>BMACs</td>
<td>Aspirate</td>
<td>Allograft, β-TCP</td>
<td>5</td>
<td>Sinus floor augmentation</td>
<td>Histology</td>
<td>Graft sites healed for 4-7 months; bone regeneration depended on graft materials</td>
<td>(79)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS (device)</td>
<td>β-TCP +/- local bone chips</td>
<td>41</td>
<td>Posterior spinal fusion</td>
<td>CT</td>
<td>Positive bony fusion was observed within 12 months postoperatively</td>
<td>(19)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>SR (device)</td>
<td>Calcium phosphate</td>
<td>101</td>
<td>Bone healing/disturbances</td>
<td>No complications were reported and only 2/101 need further surgery within an average follow-up period of 14 months; bone formation not evaluated</td>
<td>(87)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>Blood cell lysis</td>
<td>Local injection</td>
<td>342</td>
<td>Osteonecrotic femoral head</td>
<td>MRI</td>
<td>440/534 treated hips showed either complete resolution or a decreased osteonecrotic volume at an average follow-up duration of 12 years</td>
<td>(52)</td>
<td></td>
</tr>
<tr>
<td>Cell source</td>
<td>Cell isolation</td>
<td>Cell treatment</td>
<td>Cell carrier</td>
<td>Number of patients</td>
<td>Repair site Evaluation method(s)</td>
<td>Reported outcome</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>------------</td>
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<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>SR (device)</td>
<td>Collagen sponge</td>
<td>10</td>
<td>Volumetric bone deficiencies</td>
<td>Radiography</td>
<td>All patients showed bony healing and/or sufficient new bone formation within an average follow-up period of 8.3 months</td>
<td>(88)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>SR (device)</td>
<td>Allograft</td>
<td>3</td>
<td>Osteonecrotic femoral condyle</td>
<td>Radiography</td>
<td>Excellent graft integration and no further disease progression or subchondral collapse at 2 years</td>
<td>(89)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>SR (device)</td>
<td>Autograft</td>
<td>1</td>
<td>Atrophic mandibular fracture nonunion</td>
<td>Radiography</td>
<td>Excellent integration of the bone graft at 4 month follow-up</td>
<td>(90)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>SR (device)</td>
<td>PRP</td>
<td>Xenograft</td>
<td>Maxillary sinus augmentation</td>
<td>Histomorphometry</td>
<td>Biopsies obtained after 3 months indicated no difference in new bone formation between BMACs and BMNCs treated sinus</td>
<td>(91)</td>
<td></td>
</tr>
<tr>
<td>BMNCs/BMACs</td>
<td>DS in FICOLL (device)</td>
<td>PRP</td>
<td>Xenograft</td>
<td>Atrophied maxillary sinus</td>
<td>Histomorphometry</td>
<td>Biopsies obtained after an average of 3.41 months indicated no difference in new bone formation between BMNCs treatment and autografts</td>
<td>(69)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS</td>
<td>Collagen sponge, HA</td>
<td>39</td>
<td>Volumetric bone deficiencies</td>
<td>Radiography</td>
<td>New bone was detected in all patients whereas complete bone healing occurred in 36 cases within a follow-up period of 6 months; postoperative bone formation was significantly earlier in the HA compared to collagen treated group</td>
<td>(48)</td>
<td></td>
</tr>
<tr>
<td>BMSCs</td>
<td>Magnetic sorting</td>
<td>PRP</td>
<td>Allograft</td>
<td>Distal tibial fractures</td>
<td>Radiography</td>
<td>All fractures healed within 12 month follow-up; significant faster fusion rate in the BMNCs treated compared to control group</td>
<td>(68)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS</td>
<td>Local injection</td>
<td>62</td>
<td>Osteonecrotic femoral head</td>
<td>Radiography CT</td>
<td>72/78 hips achieved a satisfactory clinical result while only 6 hips required replacement after a follow-up period of 5 years</td>
<td>(53)</td>
<td></td>
</tr>
<tr>
<td>BMNCs/BMACs</td>
<td>DS in FICOLL (device)</td>
<td>PRP</td>
<td>Xenograft</td>
<td>Maxillary sinus augmentation</td>
<td>Clinical evaluation</td>
<td>Implant survival was not different between BMACs and BMNCs loaded grafts up to 2.5 years after prosthetic loading</td>
<td>(92)</td>
<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS</td>
<td>Allograft</td>
<td>18</td>
<td>Bone fracture nonunion</td>
<td>Radiography</td>
<td>All bone gaps were filled and faster healing in cell-based group compared to autografts</td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>Cell source</td>
<td>Cell isolation</td>
<td>Cell treatment</td>
<td>Number of patients</td>
<td>Repair site</td>
<td>Evaluation method(s)</td>
<td>Reported outcome</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>------------</td>
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<td></td>
</tr>
<tr>
<td>BMNCs</td>
<td>DS</td>
<td>β-TCP</td>
<td>10</td>
<td>Humerus fracture</td>
<td>Radiography</td>
<td>In all cases bone fracture healed within observation time of 12 weeks</td>
<td>(22)</td>
<td></td>
</tr>
<tr>
<td>SVF</td>
<td>Centrifuge</td>
<td>PRP Autografts</td>
<td>1</td>
<td>Calvarial defect</td>
<td>CT</td>
<td>Marked ossification in 120 cm² defect area 3 months after surgery</td>
<td>(93)</td>
<td></td>
</tr>
<tr>
<td>SVF</td>
<td>Centrifuge</td>
<td>PRP Hyaluronic acid</td>
<td>2</td>
<td>Osteonecrotic femoral head</td>
<td>MRI</td>
<td>Significant filling of bone defects 12 weeks after treatment</td>
<td>(94)</td>
<td></td>
</tr>
<tr>
<td>SVF</td>
<td>Centrifuge</td>
<td>PRP Hyaluronic acid</td>
<td>2</td>
<td>Osteonecrotic femoral head</td>
<td>MRI</td>
<td>Persistent bone regeneration sustained for at least 7 and 16 months after treatment</td>
<td>(95)</td>
<td></td>
</tr>
<tr>
<td>SVF</td>
<td>Centrifuge</td>
<td>PRP Hyaluronic acid</td>
<td>91</td>
<td>Osteonecrotic femoral head, osteoarthritic knee/ankle, spinal disc herniation</td>
<td></td>
<td>No neoplastic complications; bone formation not evaluated</td>
<td>(96)</td>
<td></td>
</tr>
<tr>
<td>ADMSCs</td>
<td>Filtration (device)</td>
<td>Allograft</td>
<td>8</td>
<td>Maxillary cysts and/or mandibular odontogenic</td>
<td>Histology</td>
<td>Biopsy obtained 3 months after treatment showed compact bone</td>
<td>(47)</td>
<td></td>
</tr>
<tr>
<td>SVF</td>
<td>Unclear (device)</td>
<td>β-TCP, BCP</td>
<td>6</td>
<td>Maxillary cysts and/or mandibular odontogenic</td>
<td>Histology</td>
<td>Biopsy obtained 3 months after treatment showed compact bone</td>
<td>(67)</td>
<td></td>
</tr>
<tr>
<td>SVF</td>
<td>Centrifuge</td>
<td>PRP Silicated-HA</td>
<td>8</td>
<td>Humerus fracture</td>
<td>µCT and histology</td>
<td>In 5/6 biopsies de novo bone formation in the implant within 12 months</td>
<td>(61)</td>
<td></td>
</tr>
<tr>
<td>PB CD34+ cells</td>
<td>Magnetic sorting</td>
<td>Atelocollagen, autograft</td>
<td>7</td>
<td>Femoral and tibial nonunion</td>
<td>Radiography</td>
<td>In 5/7 cases fracture healing was achieved after 12 weeks</td>
<td>(97)</td>
<td></td>
</tr>
</tbody>
</table>

BMNCs: bone marrow derived mononuclear cells; BMACs: bone marrow aspiration concentrate cells; SVF: stromal vascular fraction; ADMSCs: adipose tissue derived mesenchymal stem cells; PB: peripheral blood; β-TCP: β-tricalcium phosphate; HA: hydroxyapatite; BCP: biphasic calcium phosphate; PRP: platelet-rich plasm DS: density separation; SR: selective retention.
Table 4. Comparison of therapeutic efficiency of intraoperative approach with conventional bone tissue engineering

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Scaffold</th>
<th>Seeding density</th>
<th>Sample size</th>
<th>In vivo time</th>
<th>Species and repair site</th>
<th>Evaluation method</th>
<th>Efficacy of bone formation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat BMACs, Cultured BMSCs</td>
<td>HA/TCP</td>
<td>Unclear</td>
<td>6</td>
<td>8 weeks</td>
<td>Rat femoral defect</td>
<td>Histomorphometry</td>
<td>43.3% bone fill in cultured BMSCs compared to 17.2% in BMACs</td>
<td>(9)</td>
</tr>
<tr>
<td>Equine SVF, cultured ADMSCs</td>
<td>Collagen sponges</td>
<td>5 x 10⁵ / construct</td>
<td>6</td>
<td>12 weeks</td>
<td>Rat calvarial defect</td>
<td>CT</td>
<td>25% new bone in cultured ADMSCs compared to 10% in SVF</td>
<td>(65)</td>
</tr>
<tr>
<td>Rabbit SVF, Cultured ADMSCs</td>
<td>PLGA</td>
<td>1 x 10⁶ / construct</td>
<td>5</td>
<td>8 weeks</td>
<td>Rabbit segmental ulna defect</td>
<td>μCT</td>
<td>50% new bone in cultured ADMSCs compared to 30% in SVF</td>
<td>(58)</td>
</tr>
<tr>
<td>Goat SVF, cultured ADMSCs</td>
<td>PLCL</td>
<td>5 x 10⁶ SVF / construct</td>
<td>5</td>
<td>6 months</td>
<td>Goat spinal fusion</td>
<td>Radiography</td>
<td>43% fusion rate in ADMSCs compared to 30% in SVF</td>
<td>(66)</td>
</tr>
</tbody>
</table>

BMACs: bone marrow aspiration concentrate cells; BMSCs: bone marrow derived mesenchymal cells; SVF: stromal vascular fraction; ADMSCs: adipose tissue derived mesenchymal stem cells; HA: hydroxyapatite; TCP: tricalcium phosphate; PLGA: Poly(lactic-co-glycolic acid); PLCL: Poly (l-lactide co ε-caprolactone).
Chapter 3

Macrophage type modulates osteogenic differentiation of adipose tissue MSCs

Yang Zhang, Thomas Böse, Ronald E Unger, John A Jansen, C James Kirkpatrick, Jeroen JJP van den Beucken

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Chapter 3

Introduction

Bone defects resulting from trauma, cancer and fractures represent a significant clinical problem for over 9 million people worldwide each year [1]. The treatment of these bone defects relies predominantly on transplantation of autografts or allografts, and to a lesser extent on the use of synthetic biomaterial scaffolds. In order to improve the efficacy of synthetic biomaterial scaffolds, major efforts have focused on cell-based constructs that combine such synthetic biomaterial scaffolds with cells from the patients [2].

Most cell-based constructs focus on the use of (adult) stem cells, generally isolated as so-called mesenchymal stromal cells (MSCs) from either bone marrow or adipose tissue. It is remarkable that in view of the chronological order of wound and bone healing, signaling molecules and cells involved in the processes prior to wound healing are largely ignored. In the natural healing process of damaged tissue, three distinct but overlapping stages occur from a few hours to several weeks [3]: 1) the early inflammatory stage; 2) the repair stage; and 3) the late remodeling stage. It is reasonable to speculate that the inflammatory response, which is evoked by the host immune system, initiates and primes the later bone repair process. In fact, the immune and skeletal systems have been reported to share a number of signaling molecules and regulatory networks [4].

Research on the topic of bone remodeling has shown the influence of the immune system on bone healing success [5] and has led to the emergence of “osteoimmunology” [4], which identifies the immune system as a potential tool for new therapeutic approaches to bone healing. Among the cells of the innate immunity, macrophages are recognized as key elements for the orchestration of the processes to re-establish tissue integrity and function after damage [6, 7]. Thus, for instance, inflammatory bone disorders generally resulted in increased bone resorption and decreased bone formation [8] and ablation of macrophages has shown to inhibit intramembranous bone healing [9]. Nevertheless, the fundamentals of macrophage involvement in the behavior of osteoprogenitor cells and bone formation remain unclear.

It is well documented that following bone injury, monocytes are rapidly recruited to the injury site and differentiate into macrophages, where they persist throughout the bone repair process [10, 11]. In supporting the multiple events occurring during the healing process, versatile subtypes of macrophages have been distinguished depending on the environmental stimuli [12]. M0 macrophages, after in vitro differentiation from monocytes by macrophage-colony-stimulating factor (M-CSF) or phorbol-12-myristate-13-acetate (PMA), are mature macrophages with larger and more flattened morphology compared to monocytes [13]. Further, two macrophage phenotypes are present as extremes of a continuum of functional states. M1 macrophages, in vitro classically polarized by lipopolysaccharide (LPS) and interferon gamma (IFN-γ), present a pro-inflammatory profile with high antigen-presenting capacity and increased secretion of pro-inflammatory cytokines (e.g. interleukin 1β, IL-1β), and tumor necrosis factors alpha, TNF-α). In contrast, M2 macrophages, alternatively polarized by interleukin 4 (IL-4) and interleukin 13 (IL-13), secrete high levels of anti-inflammatory cytokines (e.g. transforming growth factor beta, TGF-β and interleukin 10, IL-10),
regulate and scavenge debris, and promote angiogenesis and tissue remodeling [12]. Recent studies depicted a switch in macrophage subtype from the pro-inflammatory M1 subtype to the anti-inflammatory M2 subtype during the bone healing process [14, 15], suggesting differential roles of these macrophage subtypes, and their secreted cytokines on the recruitment, proliferation and differentiation of MSCs. However, more detailed information is required to clarify macrophage contribution to the osteogenic differentiation of MSCs.

The objective of this study was to elucidate the effect of different macrophage subtypes on the osteogenic differentiation of MSCs in co-culture models. We hypothesized that M2 macrophages are able to promote the osteogenic differentiation of MSCs, while M0 and M1 macrophages inhibit this process. Therefore, human monocytes were activated and/or polarized into M0, M1 and M2 macrophages and then directly co-cultured with human MSCs at different ratios to determine their osteogenic capacities. Further studies on the mechanism by which these macrophages affect MSCs were performed by an indirect co-culture set-up to reveal paracrine effects of macrophage subtypes on the behavior of MSCs and to identify the involved signaling molecules.

**Materials and methods**

1. **Reagents and cells**

Dulbecco’s Modified Eagle Medium (α-MEM), RPMI-1640 medium, 1% penicillin-streptomycin (1% P/S) was purchased from Gibco (GrandIsland, USA). Fetal bovine serum (FBS), bovine serum albumin (BSA), trypsin, basic fibroblast growth factor (bFGF), PMA, LPS, IFN-γ, IL-4, IL-13, glycerol 2-phosphate disodium salt hydrate (β-glycerophosphate), dexamethasone, and ascorbic acid were purchased from Sigma-Aldrich (St. Louis, USA). Collagenase was purchased from Roche Diagnostics (Mannheim, Germany). TNF-α and TGF-β ELISA kits were purchased from eBioscience (San Diego, USA). BMP-2 and OSM ELISA kits were purchased from R&D systems (San Diego, USA). Monoclonal anti-human CCR7 antibody was purchased from Abcam (Cambridge, UK), mouse purified anti-human CD36 was from Biolegend (San Diego, USA) and mouse anti-human CD68 was from Dako (Heverlee, Belgium). All secondary antibodies and 4, 6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen (Waltham, USA). All cell culture flasks and plates were purchased from Greiner Bio-one (Frickenhausen, Germany).

Human adipose tissue MSCs were isolated and expanded as previously described [16]. In brief, human subcutaneous adipose tissue was obtained from the Department of Plastic Surgery (Radboudumc, Nijmegen, the Netherlands) after informed consent. Obtained adipose tissue was minced using surgical scalpels and washed with PBS. The aspirated lipid fraction was diluted with an equal volume of 0.1% collagenase digestion solution and then incubated at 37 °C for 60 min under rotation (250 rpm). After Ficoll density centrifugation (600g for 10 min), the cell pellet was resuspended and filtered through a 100 µm cell strainer. Mononuclear cells were adjusted to 1x10^7 cells per 15 ml and then cultured in 10% FBS, 1% PS, and 1 ng/ml of bFGF supplemented α-MEM. The attached cells are designated as adipose tissue MSCs and characterized by positive
expression of CD73, CD90 and CD105 and negative expression of CD45 [17]. Cells in passage 3-5 from three donors were used in this study. The human monocytic cell line (THP-1) was purchased from the American Type Culture Collection (Manassas, USA) and cultured in RPMI-1640 medium supplemented with 10% heat inactivated FBS and 1% P/S.

2. Polarization and characterization of macrophages
THP-1 cells were differentiated and polarized according to established protocols [18, 19]. Briefly, 1×10^6 cells were plated in 6-well plates with 3 ml culture medium plus 50 ng/ml PMA for 48 hours to activate monocytes into M0 macrophages. For polarization, M0 macrophages were treated for another 48 hours, either with an addition of 20 ng/ml IFN-γ and 100 ng/ml LPS to obtain M1 macrophages or with 20 ng/ml IL-4 and 20 ng/ml IL-13 to obtain M2 macrophages. Conditioned medium from polarized macrophages was used for measuring TNF-α, TGF-β and IL-10 via ELISAs following the instructions of the manufacturer. Activated and polarized macrophages were fixed with 4% paraformaldehyde and then subjected to immunocytochemistry. The M1 macrophage marker CCR7 [18] and M2 macrophage marker CD36 [18] were stained with the primary antibodies, rabbit monoclonal anti-human CCR7 and mouse purified anti-human CD36, respectively, for 2 h in PBS with 1% BSA. Cells were then washed and incubated for 1h with goat anti-mouse Alexa-488 labeled IgG and donkey anti-rabbit Alexa-568 labeled IgG in the dark. After washing, cells were stained with DAPI for 5 min. Immunofluorescence images were acquired with a fluorescence microscope (Zeiss AxioCam MRc5, Carl Zeiss Microimaging GmbH, Germany) and the relative intensity of fluorescence was analyzed using ImageJ (U.S. National Institutes of Health, Bethesda, USA). The values of red (Alexa-568) and green (Alexa-488) fluorescence of each sample were further normalized to the value of blue fluorescence (DAPI).

3. Direct co-culture of macrophages and MSCs

3.1 Direct co-culture of macrophages and MSCs at different ratios
THP-1 cells were differentiated and polarized into the various subtypes of macrophages (i.e. M0, M1, and M2) as described above. Macrophages were detached by trypsin and counted by a hemocytometer (LO-Laboroptik GmbH, Friedrichsdorf, Germany). Then, 8×10^4, 2×10^4 and 5×10^3 M0, M1 or M2 macrophages were plated into 24-well plates, in which 2×10^4 adipose tissue MSCs had been seeded 6 hours before. A mixture of THP-1 cell culture medium and osteogenic medium (mixture medium, 10% heat-inactivated FBS with 10 nM dexamethasone, 100 μM ascorbic acid, and 10 mM β-glycerophosphate) was used and refreshed every 3 days.

3.2 Immunostaining of direct co-culture
Cells were seeded on plastic coverslips (Thermanox, 13mm; MA, USA) in 24 well plates. After 4 weeks, the coverslips with attached cells (8×10^4 macrophages groups) were washed with PBS and then fixed with 4% paraformaldehyde, followed by blocking with 1% BSA. Cells were then stained with mouse anti-human CD68 and goat anti-mouse
Alexa-488 labeled IgG and DAPI. After staining and mounting, coverslips were imaged with a fluorescence microscope (Keyence International, Mechelen, Belgium). The number of macrophages and MSCs were counted based on nuclear staining (shape; macrophage nuclei were round; MSC nuclei were elongated) and CD68 positive cells (macrophages) by counting four random fields per well (magnification 400x). Cell densities were then normalized to the area of the fields.

3.3 Mineralization of direct co-culture
For mineralization tests, direct co-cultures were maintained for 4 weeks, washed twice with PBS, and incubated overnight with 1 ml 0.5 N acetic acid on a shaking table at room temperature. The calcium content of each well was quantified by a calcium assay as described previously [16].

4. Indirect co-culture of macrophages and MSCs

4.1 Indirect co-culture of macrophages and MSCs by a transwell system
8×10⁴ macrophages were plated into 0.4-µm pore inserts of 24-well transwell plates in 200 µl of mixture medium (1:1 THP-1 cell culture medium and osteogenic medium), with 800 µl mixture medium containing 2×10⁴ MSCs added to the bottom of the well. Medium was changed on day 3, day 7 and then twice a week. After 2 weeks and 4 weeks, MSCs were stained with Alizarin Red or quantified by calcium test. In parallel samples, MSCs were collected for DNA content and ALP activity test and stained with ALP dyes after 7, 14, and 28 days. Additionally, medium from each group was collected at days 3, 7, 14, and 28 for protein analysis and MSCs were homogenized with 350 µl lysis buffer and then stored at -80°C for RT-PCR.

4.2 DNA content of MSCs
Cell proliferation for MSCs was assessed using the PicoGreen DNA quantification assay kit, (Invitrogen, Waltham, USA). Cell layers were washed with PBS twice, after which 1 ml MilliQ water was added. Following two freeze-thaw cycles, samples were used for DNA quantification according to the instructions of the manufacturer.

4.3 ALP activity of MSCs
The ALP activity was measured using the same samples as used for cellular DNA content. A p-nitrophenyl phosphate (4-NP) method was adapted as developed previously [16]. ALP activity results were normalized for DNA (expressed as nmol 4-NP/ng DNA/h). In addition, 2 parallel samples from each group were fixed with 4% paraformaldehyde and then histochemically stained in methanol using the Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich, St. Louis, USA) per the manufacturer’s protocol.

4.4 Mineralization of MSCs
The calcium content for indirect co-cultures was quantified with the same method as described for direct co-cultures. In parallel, 2 samples from each group were fixed in 4% paraformaldehyde at indicated time points and then stained with 1 ml/well alizarin red
solution for 15 min at room temperature using an osteogenesis quantification kit (EMD Millipore, Billerica USA). Stained samples were then photographed with a microscope (Keyence International, Mechelen, Belgium).

4.5 Osteogenic gene expression of MSCs
mRNA of cells was extracted using the RNeasy Mini Kit (Qiagen, Valencia, USA) per the manufacturer’s protocol. After isolation, RNA was quantified using a Nanodrop ND1000 Spectrophotometer (Thermo Scientific, Hudson, USA). cDNA was generated from 1 µg of RNA using the SuperScript III reverse transcription kit (Invitrogen, Waltham, USA). For the RT-PCR reaction, 2 µl cDNA, 12.5 µl Mastermix (Life Technologies, Waltham, USA) and 3 µl primer mix with specific forward and reverse primers (Table S1) and 7.5 µl RNase free water was mixed. PCR reactions were performed and monitored using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer/Applied Biosystems, Rotkreuz, Switzerland). The level of gene expression was calculated via the ∆∆Ct method [20]. Four independent samples were used for each gene of interest.

5. Osteogenic factors involved in macrophage and MSC interaction

5.1 Protein quantification of osteogenic factors
Medium collected from indirect co-culture at indicated time points and conditioned medium from polarized macrophages were used for BMP-2 and OSM ELISAs following the manufacturer’s instructions. Colorimetric changes were measured using a multi-mode spectrophotometer (Biotek, Winooski, USA).

5.2 Gene expression of osteogenic factors in MSCs and polarized macrophages
MSCs and different types of macrophages were homogenized with lysis buffer at indicated time points and mRNA was isolated as described above. RT-PCR was conducted with the same protocol and gene expression of BMP-2, OSM and OSMR were quantified using primers listed in Table S1.

6. Statistical analysis
Data are expressed as the mean and standard deviation (± SD). Statistical analysis was performed by GraphPad Prism version 5 (GraphPad software, San Diego, USA) using one-way analysis of variance (ANOVA) with Dunnett post-test where multiple results were compared against a control, or with Bonferroni’s test for multiple comparisons. Two-way ANOVA with Bonferroni’s test was performed where two independent variables were present. Probability (p) values of p < 0.05 were considered statistically significant.

Results

1. Characterization of polarized macrophages
THP-1 monocytes were activated with PMA to generate M0 macrophages, which made the cells adherent to plastic. M0 macrophages were further polarized into M1 and
Macrophage type modulates osteogenic differentiation of adipose tissue MSCs

M2 macrophages in the presence of the appropriate cytokines (Figure 1a). Cytokine secretion profiles for TNF-α, TGF-β, and IL-10 depended on macrophage subtype (Figure 1b). Significantly higher TNF-α secretion was observed for M1 macrophages (565.74 ± 17.58 ng/ml) compared to M0 macrophages (3.52 ± 1.55 ng/ml; P < 0.001) and M2 macrophages (3.06 ± 0.74 ng/ml; P < 0.001). In contrast, significantly higher TGF-β secretion was observed for M2 macrophages (499.32 ± 69.48 ng/ml) compared to M0 macrophages (193.56 ± 68.74 ng/ml; P < 0.001) and M1 macrophages (151.69 ± 66.49 ng/ml; P < 0.001). Similarly, IL-10 secretion was significantly higher for M2 macrophages (0.82 ± 0.06 ng/ml) compared to both M0 macrophages (0.28 ± 0.20 ng/ml; P < 0.01) and M1 macrophages (0.35 ± 0.14 ng/ml; P < 0.01).

Immunostaining for macrophage subtype markers showed mixed populations of M1 and M2 macrophages after polarization procedures (Figure 1c, d & e). M1 polarization showed macrophages with positive staining for the M1 marker, CCR7, and slightly less positive staining for the M2 marker, CD36. In contrast, M2 macrophages showed enhanced positive staining for CD36 and less positive staining for CCR7. Quantification of the fluorescent signal from macrophage polarization marker immunoreactions showed obvious differences between the three macrophage phenotypes (Figure 1f). CCR7 was significantly higher expressed in M1 macrophages (29.44 ± 3.55; P < 0.05), compared to M0 (7.96 ± 0.57) and M2 macrophages (14.27 ± 3.61). In contrast, CD36 was significantly higher expressed in M2 macrophages (40.94 ± 5.81) compared to M0 (12.70 ± 1.22) and M1 macrophages (17.72 ± 3.05).

2. Direct co-culture of macrophages and MSCs
Experiments were performed with MSCs isolated from three different donors. As similar results were obtained from these experiments, the results below mainly describe the data from donor 3 as representative experiment.

2.1 Cell distribution
After 4 weeks of direct co-culture, pan-macrophage staining (CD68) combined with nuclear staining (DAPI) showed a homogeneous distribution of both cell types (Figure 2a). Although an equal number of macrophages were initially seeded for the co-cultures, higher numbers of M0 macrophages (592 ± 101/mm²; P < 0.05) were observed during the co-culture compared to both M1 (368 ± 45/mm²) and M2 (351 ± 27/mm²) macrophages with MSCs (Figure 2b & c). Simultaneously, MSCs co-cultured with M0 (681 ± 57/mm²) and M2 (418 ± 13/mm²) macrophages showed significantly higher number compared to MSCs monoculture (327 ± 17/mm²; P < 0.05), while MSCs number decreased with M1 macrophages (204 ± 22/mm²; P < 0.05).

2.2 Mineralization
Calcium content measurements were used to determine the mineralization capacity of MSCs co-cultured with macrophages and MSCs monoculture controls. Different ratios of MSCs to macrophages and two co-culture methods, namely direct co-culture and
Figure 1. The polarization method and cytokine expression of M0, M1 and M2 macrophages derived from THP-1 monocytes. The schematic figure shows the differentiation of monocytes into polarized macrophages and their morphological appearance (a). TNF-α, TGF-β and IL-10 production in M0, M1 and M2 macrophages conditioned medium were assessed by ELISA (b). M0 (C), M1 (d) & M2 (e) macrophages were stained with M1-marker CCR7 (red), M2-marker CD36 (green) and DAPI (blue), respectively. The relative fluorescence intensity of CCR7 and CD36 were quantified by ImageJ (f). Statistical analysis was performed by One-Way ANOVA with Bonferroni’s test. * p < 0.05; ** p < 0.01, *** p < 0.001.
indirect co-culture by using transwell system, were used here to investigate this effect (Figure 3a). The calcium content from direct co-cultures showed macrophage subtype-dependent promoting effects on MSCs, irrespective of donors (Figure 3b). Taking donors 3 for example, M0 macrophages decreased the mineralization of co-cultured MSCs to around 40 µg/ml, irrespective of the macrophage to MSC ratio. M1 macrophages, however, showed different effects on the mineralization of MSCs depending on their ratio. M1 macrophages and MSCs at a ratio of 1:1 also enhanced the mineralization (72.75 ± 4.06 µg/ml) compared to MSCs controls (63.26 ± 1.77 µg/ml; P < 0.01), while this effect was not obvious for the ratios 1:4 and 4:1. In contrast, M2 macrophages significantly increased the mineralization of co-cultured MSCs, and this effect was proportional to the ratio of macrophages to MSCs. M2 macrophages to MSCs at 4:1 and 1:1 ratios reached significantly higher mineralization of 146.84 ± 12.31 µg/ml and 131.38 ± 10.94 µg/ml respectively, compared to MSCs monoculture (63.26 ± 1.77 µg/ml; P < 0.001). At a ratio of 1:4, M2 macrophages reached a similar mineralization level (68.01 ± 6.59 µg/ml) compared to MSC monoculture (p > 0.05).

3. Indirect co-culture of macrophages and MSCs

Experiments were performed with MSCs isolated from three different donors. As similar results were obtained from these experiments, the data below describe the data of donor 3 as representative experiment. More data from donor 1 and donor 2 was showed in Supplementary Figure S1.

3.1 Cell proliferation of MSCs

DNA content of co-cultured MSCs was assessed to study effects of different types of macrophages on the growth of MSCs during co-culture. As shown in Figure 4a, after 7 days, all of three types of macrophages increased the cell number of MSCs, with the effect of M2 macrophages on MSC proliferation being the highest (913.93 ± 334.15 ng/ml; P < 0.001), followed by MSCs with M1 macrophages (585.49 ± 74.20 ng/ml; P < 0.05) and M0 macrophages (547.22 ± 27.90 ng/ml; P < 0.05) compared to MSC monoculture (229.44 ± 21.42 ng/ml). After 14 days, although DNA content was increased in all groups, no difference was observed between MSC monoculture and co-cultures with macrophages. However, on day 28, M2 macrophages still significantly stimulated the growth of co-cultured MSCs (614.27 ± 36.53 ng/ml) compared to MSC monoculture (454.29 ± 26.63 ng/ml; P < 0.05) and MSCs with M1 macrophages (453.85 ± 27.04 ng/ml; P < 0.05).

3.2 ALP activity of MSCs

The activity of alkaline phosphatase in co-cultured MSCs was assessed as a marker of osteogenic differentiation. Generally, the level of endogenous ALP activity increased and peaked around 14 days and then decreased for all experimental groups (Figure 4b & c). In view of different macrophage subtype effects, the co-culture of MSCs with M0 and M1 macrophages increased ALP activity to 0.42 ± 0.07 nmol/ng DNA/h and 0.54 ± 0.08 nmol/ng DNA/h, respectively, measured at day 7, compared to MSC monoculture.
Figure 2. Immunostaining of different types of macrophages (MΦ) co-cultured with MSCs. MSCs were monocultured or co-cultured with M0, M1 and M2 macrophages (1:4 ratio) for 4 weeks and stained with DAPI (blue) and pan-macrophage marker CD68 (green) (a). The number of macrophages and MSCs was counted based on shape of nuclei (b) and quantified (c). White arrow indicates the MSC while yellow arrow indicates the macrophage. Statistical analysis was performed by One-Way ANOVA with Bonferroni’s test. N= 4, * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 3. Mineralization of MSCs directly co-cultured with M0, M1 and M2 macrophages. The schematic diagram shows the set-up of MSCs monoculture, direct co-culture (at different ratios) or indirect co-culture (at ratio of 1:4) with M0, M1 and M2 macrophages respectively (a). Mineralization capacity of monoculture and direct co-culture from 3 donors was assessed by calcium content after 4 weeks in osteogenic medium (b). Statistical significance relative to controls (MSCs monoculture) and between groups was determined by Two-Way ANOVA with Bonferroni’s test correction, N = 5, * * p < 0.01, *** p < 0.001. Asterisks placed directly on the columns indicate significant differences from the MSCs control.
Macrophage type modulates osteogenic differentiation of adipose tissue MSCs

3.3 Mineralization of MSCs

In indirect co-cultures using a transwell system (Figure 3a), MSCs co-cultured with M2 macrophages attained the apparent highest mineralization compared to other experimental groups, based on alizarin red staining. In contrast, the effect from M0 and M1 macrophages on the mineralization of MSCs was not obvious compared to MSC monoculture (Figure 5a & b). Quantitatively, 27.3 ± 3.26 μg/ml calcium was obtained after 2 weeks of indirect co-culture of M2 macrophages and MSCs (donor 3), which obtained similar levels (p > 0.05) as the other experimental groups (less than 20 μg/ml; Figure 5c). After 4 weeks indirect co-culture, the calcium content of MSCs co-cultured with M2 macrophages reached 120.55 ± 10.09 μg/ml compared to MSC monoculture (71.30 ± 17.11 μg/ml; P < 0.001), MSCs with M0 (48.46 ± 16.70 μg/ml; P < 0.001) and M1 macrophages (67.03 ± 18.39 μg/ml; P < 0.001) (Figure 5d).
3.4 Gene expression of osteogenic markers of MSCs in indirect co-culture

Osteogenic differentiation of MSCs occurs along with an increase in the expression of osteogenesis-related genes, which were examined by RT-PCR analysis. After 3 days co-culture, the gene expression levels of runt-related transcription factor 2 (Runx2), collagen I, ALP, and osteocalcin (OCN) were 2~3 times increased in MSCs indirectly co-cultured with M1 macrophages, in comparison to MSC monoculture (P < 0.001). M0 macrophages increased the expression of two osteogenic markers (Runx 2 and OCN), but to a lesser extent (P < 0.05). For co-cultures with M2 macrophages, this stimulating effect was not observed on day 3 (Figure 6a).

On day 7, MSCs along with M0 and M1 macrophages inhibited the gene expression of Runx2, OCN and bone sialoprotein II (BSP II) (P < 0.001). By contrast, all osteogenesis-related genes, except for ALP, showed enhanced expression in MSCs co-cultured with M2 macrophages (Figure 6b).

After 14 days, Runx2, ALP and OCN, were still more highly expressed in MSCs co-cultured with either M1 or M2 macrophages, compared to MSC monoculture (P < 0.05). Notably, BSP II expression was significantly enhanced (approximately 10-fold) for MSCs with M2 macrophages (P < 0.001), but not for MSCs co-cultured with M0 or M1 macrophages (P > 0.05) (Figure 6c).
4. Osteogenic factors involved in macrophage and MSC interaction

Two osteogenesis-related soluble proteins, OSM and BMP-2, were analyzed in the co-culture medium to evaluate potential involvement in the effects of macrophages on the osteogenic induction of MSCs (Figure 7). Higher concentrations of OSM were found in the co-culture medium from M0-MSCs (15.02 ± 4.06 pg/ml) and M1-MSCs (17.63 ± 3.09 pg/ml) on day 3 compared to MSC monoculture (2.11 ± 1.52 pg/ml; P < 0.01) and M2-MSCs (4.01 ± 2.39 pg/ml; P < 0.01) (Figure 7a). We further assessed OSM gene expression and protein secretion in three types of macrophages and their corresponding conditioned medium. M0 and M1 macrophages expressed almost twice to four times more OSM than M2 macrophages at the gene level (Figure 7b). Secretion of OSM was also higher at the protein level in the conditioned medium of M1 macrophages (14.38 ± 3.42 pg/ml) compared to M2 macrophages (6.20 ± 3.06 pg/ml; P < 0.001) (Figure 7c). The expression of the receptor of OSM (OSMR) was found to be enhanced in MSCs co-cultured with M0 (around 2-fold) and M1 macrophages (around 7-fold) after 3 days and 2-fold enhanced with M0 and M1 macrophages after 7 days (Figure 7d) compared to MSC monoculture controls and MSCs with M2 macrophages.
Figure 7. Secretion of key osteogenesis-related proteins in MSCs monoculture, MSCs and macrophages indirect co-cultures and macrophages conditioned medium. OSM secretion in macrophages and MSCs co-culture medium was assessed by ELISA (a). OSM gene expression of M0, M1 and M2 macrophages was determined by RT-PCR (b) and its protein secretion in conditioned medium was determined by ELISA (c). OSM receptor (OSMR) gene expression in MSCs was determined by RT-PCR (d). BMP-2 secretion in macrophages and MSCs co-culture medium was assessed by ELISA (e). BMP-2 gene expression of M0, M1 and M2 macrophages was determined by RT-PCR (f) and its protein secretion in conditioned medium was determined by ELISA (g). BMP-2 gene expression in MSCs was determined by RT-PCR (h). One-Way ANOVA with Bonferroni’s correction was performed for comparison of OSM and BMP-2 gene expression and protein secretion between different types of macrophages. One-Way ANOVA with Dunnett post-test correction was performed for comparison of OSM and BMP-2 concentration, OSMR and BMP-2 gene expression in MSCs. N= 4, * p < 0.05, ** p < 0.01, *** p < 0.001.
Regarding BMP-2, higher concentrations of BMP-2 in M2 macrophages and MSC co-culture medium were observed after 7 days (1209.85 ± 156.74 pg/ml) and 14 days (497.88 ± 61.80 pg/ml) compared to conditioned medium from MSC monoculture (748.03 ± 66.49 pg/ml at day 7 (P < 0.001) and 247.52 ± 19.95 pg/ml at day 14 (P < 0.05), respectively) (Figure 7e). No significant differences were observed regarding BMP-2 secretion in conditioned medium between different phenotypes of macrophages, even though significantly higher BMP-2 gene expression in M2 macrophages was observed (Figure 7f & g). BMP-2 expression in macrophage-MSC co-culture showed significantly higher values (around 4-fold) with M2 macrophages on day 7 and day 14 compared to MSC monoculture (Figure 7h).

Discussion

The bone healing process after injury involves interactions of multiple cell types, including osteoprogenitor cells, such as MSCs, and inflammatory cells, such as monocytes/macrophages. Although this interaction has been indicated to be critical for bone formation and related to a macrophage phenotype switch, the mechanisms involved still remain unclear. In the present study, we generated and characterized three types of macrophages and demonstrated their differential effects on the behavior of MSCs. M2 macrophages increased the mineralization of co-cultured MSCs, and this effect was proportional to the ratio of macrophages to MSCs. In contrast, M0 and M1 macrophages showed opposite effects at certain ratios. Furthermore, several potential osteogenic factors were proved to be involved in interaction between diverse macrophage subtypes and MSCs, which stimulated their osteogenic differentiation.

Macrophage phenotypes ranging from M0 to M1 and M2 were generated and characterized before co-culture. The selected cytokine secretion profiles (TNF-α, TGF-β, and IL-10), which were previously shown to discriminate M0, M1, and M2 macrophages [19], demonstrated the successful polarization of different types of macrophages. However, unambiguous classification of macrophage phenotypes is problematic due to non-specific staining of different types of macrophages for M1 (CCR7) and M2 (CD36) markers. The quantification of relative fluorescence intensity facilitated the assignment of markers toward M1 or M2 predominance and provided an additional method to interpret macrophage subtypes. With this macrophage subtype characterization, we initiated macrophage/MSC co-cultures, during which macrophages were shown to influence MSC behavior and vice versa. As a result of these dynamics, fluctuations in macrophage subtypes can occur during co-culture, and hence maintenance of the condition at cell seeding over the course of 4 weeks cell culture is unlikely. Consequently, the pan-macrophage marker, CD68, was used to assess the distribution and viability of seeded macrophages due to the difficulty of using specific markers for different subtypes of macrophages. A larger number of macrophages were observed for M0 macrophages and MSCs in direct co-culture after 4 weeks. Additionally, different proliferation rate and osteogenic behavior of MSCs were found with three types of macrophages in direct
and indirect co-cultures. These findings indicate dynamic interactions between MSCs and different types of macrophages over the co-culture period.

M2 macrophages were shown to promote osteogenic differentiation of MSCs isolated from three different donors, evidenced by the significantly higher mineralization capacity compared to MSC monoculture, both in direct and indirect co-cultures. This finding corroborates data from previous studies, which indicated that M2 macrophages stimulate the mineralization capacity of MSCs [21-25]. However, several pieces of contrasting data on M1 macrophage effects on MSC mineralization have been reported [26, 27]. This discrepancy probably relates to experimental design differences. For instance, these previous studies did not use actual co-cultures but macrophage-conditioned medium for the culture of MSCs. Consequently, the dynamic bi-directional cellular interactions between MSCs and macrophages were not operative. Furthermore, characterization of macrophages was not reported in some studies to insure certain subtype. Finally, the used cells for co-culture studies were dissimilar from several perspectives influencing mineralization, including MSC differentiation status (i.e. osteoprogenitors versus mature osteoblasts), origin (i.e. adipose tissue versus bone marrow) [28] and donor characteristics [29], monocytes/macrophage characteristics (e.g. CD16 surface marker expression) [30], and co-culture cell ratios [31]. This study used THP-1 monocytes activated by PMA and then polarized by LPS and IFNγ or IL4 and IL13 to reflect the M0, M1 and M2 macrophages that may occur during the bone healing in vivo. As an immortalized human cell line, THP-1 cells are characterized to retain all necessary markers and morphologic features to be qualified as a monocyte cell population [32]. Under certain microenvironments, they can undergo differentiation and polarization into functional, mature macrophages. A cell line is needed here to address multivariate research questions that require large numbers of cells and also for high reproducibility of results, which was not practical and ideal for primary monocytes isolated from peripheral blood. In addition, two-dimensional co-culture was utilized in the present study to simplify the experimental complexity. A three-dimensional co-culture is undergoing to more closely mimic the real cell-cell interaction microenvironment. In the following study, an ectopic and an orthotopic in vivo model will be conducted as well to assess the clinical relevance of our findings in the context of bone formation and bone repair capacity. The ratio of M2 macrophages to MSCs in the direct co-culture showed a correlation with the extent of mineralization, and an imbalance in favor of M2 macrophages even significantly increased mineralization compared to MSC monoculture controls. In our preliminary experiments (data not shown), a 10:1 ratio of M2 macrophages to MSCs showed significantly lower mineralization compared to MSC monoculture controls. Given our experimental set-up for direct co-cultures with equal numbers of MSCs for all experimental groups, this ratio-dependency suggests an optimally effective (in vitro) cytokine secretion profile. In addition, it is worthy of notice that M1 macrophages with MSCs at the ratio of 1:1 slightly promoted the mineralization capacity of MSCs as well. During this co-culture process, all types of macrophages promoted the proliferation of MSCs in the first week. M0 and M2 macrophages showed this beneficial effect even
Macrophage type modulates osteogenic differentiation of adipose tissue MSCs

after 4 weeks. On the other hand, M0 and M1 macrophages significantly promoted the osteogenic differentiation of co-cultured MSCs in the early and middle stage of osteogenesis, evidenced by high ALP activity and high gene expression of early-stage osteogenic markers such as Runx2, ALP, Col I at the early time points. In contrast, M2 macrophages showed delayed stimulatory effects on the osteogenic gene expression profile of co-cultured MSCs, with a 10-fold increased expression of BSP II on day 14. This finding corroborates the results of Omar et al. and Loi et al., who showed that pro-inflammatory macrophages could promote the early osteogenic differentiation of bone marrow derived MSCs and this effect was further enhanced by macrophage phenotype modulation from M1 to M2 via IL-4 treatment 72 hours after seeding [27, 33]. Considering these findings, it could be speculated that an optimized timing of M1 and M2 macrophage appearance exists to achieve the maximum osteogenesis of co-cultured MSCs. This hypothesis was also postulated by other researchers, but needs further investigation [5, 10, 22].

A vital role of soluble factors in the osteoinductive effects of M2 macrophages on MSCs was found because the calcium content from indirect co-culture (120.55 ± 10.09 µg/ml) was comparable to that from MSC and M2 macrophages direct co-culture (146.84 ± 12.31 µg/ml) at the same ratio. BMP-2 and OSM are most likely stimulatory molecular candidates based on previous studies [21, 24, 26, 31, 34]. Several studies have found that monocytes/macrophages enhanced osteogenic differentiation of MSCs in a manner dependent on an OSM signaling pathway [21, 26, 31]. In this study, M0 and M1 macrophages, but not M2 macrophages, were found to express and secrete higher OSM levels at the early time points to drive the osteogenic differentiation of co-cultured MSCs through the OSM-OSMR signaling pathway. On the other hand, higher BMP-2 secretion was observed only for MSC and M2 macrophage co-cultures, whereas BMP-2 secretion in the three types of macrophages was not significantly different. We further confirmed the increased endogenous BMP-2 secretion of MSCs when co-cultured with M2 macrophages. Two studies also proved monocytes/macrophages acting via exosomes or soluble factors on MSCs to induce autologous BMP-2 production [33, 34]. This finding, however, was contradictory to few previous report, which demonstrated the beneficial effect of exogenous BMP-2 [24, 35]. However, it needs to be emphasized that only conditioned medium was used and endogenous BMP-2 from MSCs was not tested in these studies. Nonetheless, in the present study, the possibility that BMP-2 expression in M2 macrophages was enhanced when co-cultured with MSCs cannot be excluded.

In the present study, all macrophage subtypes promoted the osteogenic differentiation of MSCs, albeit to a different extent and at different stages during co-culture. This finding challenges the traditional knowledge about macrophages and inflammation, in which macrophages were generally considered to adversely affect the bone healing process. Since at an organismal physiological level inflammatory signals resulting from bone tissue injury or surgery, and the implanted biomaterial mediate the differentiation of monocytes into different types of macrophages, our findings provide new impetus for the future design of supporting scaffolds and cell selection for treatment of bone
defects. For instance, hydrophilic nanostructured surfaces have been shown to drive M2 macrophage polarization and improve osseointegration [36], and enhancement of M2 phenotype in bone defects further improved bone healing [37]. Furthermore, MSCs, irrespective of their origin, have been indicated to function as immunomodulators to macrophages beyond their differentiation potential in tissue regeneration [38, 39]. Their effect on bone regeneration through immunoregulation mechanisms is worthy to be investigated in the future.

Conclusion

This work systematically studied the effects of different macrophage subtypes on the osteogenic differentiation of adipose tissue MSCs. We found that M2 macrophages had a beneficial effect on ADMSCs mineralization by promoting their proliferation and osteogenic differentiation. In contrast, this enhanced mineralization effect was not observed for ADMSCS co-cultured with M0 and M1 macrophages at certain ratios, although both of them were able to promote the early osteogenic process. Furthermore, indirect co-cultures demonstrated that the stimulatory effect was mediated by soluble factors, in which autocrine BMP-2 and OSM osteogenic factors were involved. Our findings not only elucidate the critical role of macrophages in the osteogenic differentiation process of osteoprogenitor cells, but also provide important considerations for the implementation of macrophage-osteoprogenitor cell interactions in the development of bone regenerative treatments.
References


Chapter 3


Macrophage type modulates osteogenic differentiation of adipose tissue MSCs
Chapter 4

Toward intra-operative preparation of cell-based constructs for bone regeneration

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Submitted
**Chapter 4**

**Introduction**

Bone tissue engineering and regenerative medicine concepts have been proposed and conducted for decades to meet the increasing need for bone regenerative therapies in clinics and to overcome the limitations of allografts and autografts [1, 2]. In view of the use of cell-based constructs, mesenchymal stromal cells (MSCs) [3] have been mostly used as an autologous cell source to boost the bone regeneration process. Generally, these procedures involved bone marrow isolation, MSC expansion *in vitro*, MSC seeding on a scaffold material and priming osteogenic differentiation *in vitro*, and finally implantation of MSC-based constructs into the bone defect of the patient [4]. This conventional approach has severe drawbacks regarding tissue harvest via bone marrow aspiration [5], for which an alternative in the form of liposuction has become available after the first reports on the existence of MSCs within adipose tissue with osteogenic differentiation capacities [6, 7]. The advantages of adipose tissue as a source for MSCs include the easy access via liposuction/-aspiration, high cell yield, and rapid *in vitro* expansion [4]. Although recent work has clearly demonstrated the feasibility and efficacy of using human adipose tissue MSCs to generate cell-based constructs that promote bone formation in pre-clinical animal studies [8, 9], this approach still is limited for clinical applications due to the disadvantages of the generation process. To generate MSC-based constructs, the harvested heterogeneous cell population from fat tissue, known as stromal vascular fraction (SVF), requires purification and expansion *in vitro* to obtain sufficiently high cell numbers of MSCs, which is generally performed via gradient centrifugation, cell selection based on plastic adherence, and prolonged cell culture in a specific medium. All these *in vitro* procedures are time-consuming, costly, prone to decreases in cell bone forming capacity due to passaging, and risk of contamination and mix-up [10]. In addition, inconvenience from two surgical procedures, for respectively cell harvest and construct implantation, is an obstacle as well. In order to attain simplicity, practicality, and cost-efficiency, the manufacturing process of cell-based constructs should ideally be compatible with the timeline of a single surgical procedure. In this perspective, tissue harvest, cell isolation, cell seeding onto a scaffold and subsequent implantation of a construct should occur within a few hours in an intraoperative manner, without ex vivo cell culture.

SVF, the primary isolate from a lipoaspirate, is a heterogeneous mixture of stromal cells, endothelial cells, pericytes, lymphocytes, mast cells, and pre-adipocytes [7, 11, 12]. Recent work has already demonstrated the feasibility of using this SVF for a one-step surgical approach to generate cell-based constructs and showed formation of bone tissue and vascular structures upon implantation in femoral factures and calvarial defects in rats [13, 14]. From preparation to implantation, this process can be finalized in several hours and has shown superior bone formation compared to cell-free scaffolds after 8 and 12 weeks. To date, however, no scientific data report on the performance of SVF-based constructs compared to MSC-based constructs.
Upon implantation of cell-based constructs via a surgical procedure, circulating peripheral blood monocytes are recruited to the injury site and differentiate into activated macrophages participating in the bone healing [15, 16]. Activated macrophages are further transformed into polarized macrophages of either the M1 (pro-inflammatory) or M2 (pro-tissue repair) type depending on the environmental cues [17]. In that role, they affect the behavior of other cells by secreting factors with chemotactic, mitogenic and/or morphogenic activity to respectively attract other cells, stimulate cells to proliferate and/or induce cells to undergo differentiation [17]. This concept is currently being extended to generate a prevascular network in engineered bone constructs to accelerate vascularization and increase cell survival upon implantation of cell-based constructs [18-20]. Furthermore, several studies have shown that monocytes stimulate the osteogenic differentiation of adipose tissue MSCs [21, 22]. This observation justifies further translational research on the applicability of co-culture strategies using these two types of ‘easy access cells’, i.e. SVF and monocytes, to intra-operatively prepare bone constructs with superior osteogenic potential.

Here, we isolated both SVF and ADMSCs and prepared ADMSC-based constructs in the conventional manner (i.e. within vitro expansion culture) as well as SVF-based constructs in an intra-operative manner by combining either cell type with ceramic granulate, after which we comparatively evaluated their bone regenerative performance in a rat femoral condyle bone defect model. Additionally, we added human monocytes to these constructs to analyze potential beneficial effects on bone regeneration. We firstly evaluated the cellular interaction of human SVF and ADMSCs with human monocytes in vitro. To demonstrate feasibility and efficacy of the intra-operatively available constructs, we then comparatively treated bone defects with these constructs and evaluated bone regeneration histologically and histomorphometrically.

Materials and methods

1. Human cell isolation and culture

Human subcutaneous adipose tissue was obtained in the form of a fat patch from the Department of Plastic Surgery (Radboudumc) after ethical approval (Commissie Mensgebonden Onderzoek; dossier number #3252) and informed consent. The adipose tissue was excised into pieces and then minced in a 0.1% collagenase type II (Mannheim, Germany) solution with 1% bovine serum albumin (BSA, Sigma, St. Louis, USA) for 1 hour at 37°C under shaking conditions, as described previously [23]. After centrifugation at 600g for 10 min at room temperature, the supernatant together with the fat layer was discarded. The resulting cell pellet was washed twice with phosphate buffered solution (PBS; Gibco, Merelbeke, Belgium) with subsequent filtration through a polypropylene membrane with a pore size of 100 µm. After centrifugation at 600g for 10 min at room temperature, the supernatant together with the fat layer was discarded. The resulting cell pellet was washed twice with phosphate buffered solution (PBS; Gibco, Merelbeke, Belgium) with subsequent filtration through a polypropylene membrane with a pore size of 100 µm. After centrifugation, the cell pellet was suspended in proliferation medium, consisting of α-MEM (Gibco, Merelbeke, Belgium) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, USA). Red blood cells were lysed by incubation for 2 min in a solution of 7.49 g/L NH₄Cl (2.06 g/L Tris Base, pH 7.2). Following centrifugation, the deposited cell aggregates were denoted as...
Chapter 4

SVF, suspended in proliferation medium, and counted using a Neubauer chamber. This SVF was either frozen as $3 \times 10^6$ cells per tube in the liquid nitrogen or used for further culturing to obtain a homogeneous population of ADMSCs.

For culturing, SVF containing $3 \times 10^6$ cells was plated in 75 cm$^2$ tissue culture flasks (Greiner Bio-one, Frickenhausen, Germany) in proliferation medium supplemented with 1 ng/ml of bFGF (Sigma-Aldrich, St. Louis, USA). Cells were grown in a humidified incubator at 37°C in an atmosphere of 5% CO$_2$. Two days after seeding, the adherent cells were thoroughly rinsed with PBS to remove non-adherent cells from the culture. In this way, a homogeneous population of ADMSCs was obtained. When reaching around 80% confluency, cells were detached with 0.05% trypsin in 0.5mM EDTA (Sigma-Aldrich, St. Louis, USA) and were passaged. SVF and ADMSCs were characterized for surface marker expression by flow cytometry for the mesenchymal markers CD73 (APC-conjugated, BD Bioscience), CD90 (PE-Cy5-conjugated, BD Bioscience), CD105 (PE-conjugated, BD Bioscience, Franklin Lakes, USA), and negatively against CD45 (PE-conjugated, BD Bioscience). Osteogenic differentiation capacity of ADMSCs was confirmed by 4-week cultures in osteogenic medium (proliferation medium supplemented with 10 nM dexamethasone, 100 μM ascorbic acid, and 10 mM β-glycerophosphate).

The human monocytic cell line THP-1 was purchased from the American Type Culture Collection (ATCC; Manassas, USA) and cultured in RPMI-1640 medium (Gibco, Carlsbad, USA) supplemented with 10% FBS.

2. Scaffold for constructs preparation

Tricalcium phosphate (β-TCP) granules were kindly provided by Cam Bioceramics (Leiden, the Netherlands). Pore sizes of TCP granules varied from 500 μm - 2 mm and the granules had an overall porosity of 84%. After sterilization by gamma irradiation (SynergyHealth, Ede, the Netherlands), 11 mg (total volume around 21 mm$^3$ corresponding to the bone defect size for in vivo experimental work) of these granules per sample were placed into individual wells of a non-adherent well plate.

3. Procedure for construct preparation

Before construct preparation, $3 \times 10^6$ SVF cells in the liquid nitrogen were unfrozen and suspended in 300 μl proliferation medium to be loaded onto TCP granule. This group named as SVF groups. Purified ADMSCs from SVF were expanded in proliferation medium with bFGF as described above. Osteogenic medium was applied for ADMSCs 3 days before preparation. $1 \times 10^6$ ADMSCs suspended in 300 μl proliferation medium were then loaded onto the same scaffold and nominated as ADMSCs group. All wells were then supplemented with 300 μl proliferation medium and incubated at 37°C for 2 hours. Non-attached cells were collected and loaded onto granules again every hour. Cell attachment was confirmed through 4', 6-diamidino-2-phenylindole (DAPI) staining and was quantified by DNA content to ascertain the comparable number of attached cells. After 2h incubation, $1 \times 10^6$ monocytes suspended in 200 μl proliferation medium were added to the SVF or ADMSCs constructs to make SVF+MO and ADMSCs+MO group. Same constructs for animal study were achieved by directly adding monocytes...
to the defect sites during the surgery. TCP granules supplemented with the same amount of proliferation medium or $1 \times 10^6$ monocytes only were set as control and MO group respectively (Figure 1A). Then all constructs were ready either for in vitro study or for implantation.

4. In vitro assessment

4.1 cell adhesion and viability on constructs

Cell attachment and cellular morphology on constructs were checked before implantation by identifying nuclei and actin of attached cells. The scaffolds were incubated in 20 μM Alexa Fluor® 568 Phalloidin (Thermofisher Scientific, Breda, Netherlands) for 15 min and in 300 nM DAPI stain for 5 min and then quantified with a Zeiss AxioPlan immunofluorescence microscope (Carl Zeiss Microimaging GmbH, Gottingen, Germany). Additionally, cell viability was assessed using a LIVE/DEAD® viability/cytotoxicity kit for mammalian cells (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer instructions.

4.2 cell proliferation and osteogenic differentiation on constructs

Constructs from above 6 groups were cultured in osteogenic medium up to 4 weeks. After washing with PBS once, constructs were placed in 1 ml MilliQ and subjected to two cycles of freezing and thawing. Cell proliferation was evaluated by assessing the DNA content using a DNA quantification kit (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Osteogenic differentiation of all constructs was analyzed by quantification of alkaline phosphatase (ALP) activity of cell lysates using a 4-nitrophenyl phosphate based method [23] after 1, 2 and 4 weeks of culture. To test mineralization, same type and number of cells without TCP granules were seeded in 24-wells cell culture plates. After 4 weeks, mineralization was evaluated by deposit calcium as described earlier [23].

4.3 effect of SVF or ADMSCs on macrophages polarization

To assess the effect of SVF or ADMSCs on macrophages polarization, $1 \times 10^6$ ADMSCs or $3 \times 10^6$ SVF were seeded on 11 mg TCP granules for 2 h as described above. These cell-loaded constructs and TCP granules were then transferred into 0.4-mm-pore size Corning transwell inserts (Sigma-Aldrich, Zwijndrecht, the Netherlands) and placed into the 24-well plates where $1 \times 10^6$ THP-1 monocytes were seeded with 250 nM PMA (monocytes were activated into macrophages) for 48 hours in advance. Co-cultures were incubated for another 48 h. Wells supplemented with 100 ng/ml LPS (Sigma-Aldrich, St Louis, MO, USA) and 30 ng/ml IFN-g (Sigma-Aldrich, St Louis, MO, USA) or 20 ng/ml IL-4 (Sigma-Aldrich, St Louis, MO, USA) and 20 ng/ml IL-13 (Sigma-Aldrich, St Louis, MO, USA) were set as control. The secreted TNF-α and TGF-β in the supernatants were evaluated using ELISA kits (e-Bioscience, San Diego, CA, USA) according to the manufacturer’s instructions.
5. Animal surgery
The study was approved by the Centrale Commissie Dierproeven (CCD; project 2015-137), national guidelines for animal care and welfare were obeyed, conforming to the ARRIVE guidelines. A total of 48 nude rats (Crl:NIH-Foxn1\textsuperscript{nu}, Charles River) weighing between 250g and 300g were used in the present study. All rats were pre-medicated with an intramuscular injection (5 mg/kg) of carprofen. General anesthesia with nitrous oxide, oxygen and isoflurane were applied during the surgery. Bone defects were surgically created (3 mm diameter, 3 mm depth) bilaterally in the femoral condyles using a 3-mm trephine burr (Hager & Meisinger GmbH, Neuss, Germany) at low rational speed. The implants were randomly placed into defects and the soft tissue incision was closed with superficial sutures. In all cases, the transplantation was performed within 4 hours. After surgery, 150 mg/kg buprenorphine (Temgesic, Schering-plough, Amstelveen, the Netherlands) was subcutaneously given for 2 days to reduce postoperative pain. 2 rats died in the surgery. 4 and 10 weeks after implantation, 46 rats were sacrificed by inhalation of CO\textsubscript{2}. The constructs with surrounding tissue were harvested and fixed in a 4% formalin buffer for 48 hours. One sample from each group was randomly selected and then dehydrated and embedded in paraffin for histochemistry and immunohistochemistry process. The rest of samples were randomly distributed, dehydrated and embedded in polymethyl methacrylate (PMMA) for the histological process.

6. Histological and histomorphometric analysis
After fixation, certain specimens were dehydrated in a graded series of ethanol and embedded in PMMA without decalcification. Specimens were sectioned longitudinally (335 μm thick) using a diamond saw (Leica Microsystems SP 1600, Nussloch, Germany). Three sections representative of the center of the defect and subcutaneous sample from each specimen were then stained with methylene blue/basic fuchsin to evaluate new bone formation. Stained samples were further photographed with Zeiss Imager Z1 microscope equipped with the Axiocam camera using AxioVision 4.8 software. After that, the bone formation percentage (BF) was determined in stained sections using an automated image-analysis system (Leica Qwin Proimage analysis system, Wetzlar, Germany) which recognize bone tissue based on different RGB values from highly magnified images. Minor manual corrections were made to ensure the precise selection of newly formed bone in the defects. The percentage of new bone formation was measured by normalized to initial defect area measured from the cross-section.

7. Immunohistochemical and histochemical staining
After fixation, one sample from each group was decalcified in an EDTA solution, dehydrated in a series of alcohol and embedded in paraffin. The specimens were sectioned in at a thickness of 5 μm and stained with hematoxylin and eosin for conventional, qualitative bright field light microscopy analysis. Sections were further stained with Masson’s trichrome and observed by light microscopy as well. For immunostaining, paraffin sections were rehydrated inserials of ethanol and antigen was retrieved in
Toward intra-operative preparation of cell-based constructs for bone regeneration

sodium citrate buffer (PH 6.0) at 70°C for 10 min. Subsequently, slides were blocked with 10% normal donkey serum (NDS) and then incubated with the primary antibody overnight at 4°C. Slides were then treated with a biotin-conjugated secondary antibody (Chemicon, Temecula, USA) for 1h at room temperature, followed by counterstaining with hematoxylin. Negative controls using 2% NDS instead of the primary antibody were generated in parallel to ensure that the staining was specific. Finally, the sections were dehydrated and mounted. Stained sections were photographed with the same Zeiss Imager Z1 microscope. To detect human cells in the constructs implanted in rats, immunostaining was performed using human specific anti-mitochondria antibody (EMD Millipore, No. MAB1273, Amsterdam-Zuidoost; diluted 1:200) as protocol indicated. Human and rat skin sections were used as positive and negative controls, respectively. The presence of human origin macrophages was assessed by using human specific mouse anti-human CD68 (Dako, Leuven, Belgium; diluted 1:200). For blood vessel staining, monoclonal anti-actin, α-smooth muscle antibody (α-SMA, Sigma-Aldrich, No. A2547, Billerica, USA; diluted 1:128,000) were performed. For Tartrate-resistant acid phosphatase (TRAP) staining, sections were deparaffinized, rinsed in PBS and incubated with a solution containing 50 mM sodium acetate (pH 5.2), 0.15% Naphtol-AS-TR-phosphate, 50 mM sodium tartrate, and 0.1% Fast Red T.R. (Sigma-Aldrich Chemie Gmbh, Taufkirchen, Germany) for 30 - 40 min at room temperature. Subsequently, the sections were rinsed in PBS and counterstained.

8. Statistical analysis
Data are presented as means ± standard deviations (SD). Statistical analysis was performed using a one-way ANOVA with Graphpad Prism 5 software. When ANOVA indicated a significant difference between different groups, Tukey Post-hoc Multiple Comparisons were performed. The unpaired test was used to compare the calcium content between SVF and ADMSCs. P values < 0.05 were regarded as significant.

Results

1. Comparative characterization of human ADMSCs and SVF
After regular digestion, filtration and centrifugation steps of a fat patch, we obtained 260 × 10^6 SVF cells from 400 ml liposapirate. To obtain purified ADMSCs, we seeded 6 × 10^6 SVF cells per tissue culture flask for cell selection based on adherence and culture with proliferation medium. To characterize the SVF population, we performed cytofluorimetric analysis in comparison with conventionally purified ADMSCs. The analysis of stromal cell markers (CD73, CD90, and CD105) showed consistent presence of stromal cells in SVF and stromal cells took up around 1/3 of the SVF population (Figure S1).
Figure 1. Construction of intraoperative preparation and conventional strategy based bone grafts. (A) Cell number used for each construct preparation. (B) Schematic structure of the experimental design. PM, proliferation medium; OM, osteogenic medium; EPCs, endothelial progenitor cells; ECs, endothelial cells; MCs, mast cells; HPSCs, hematopoietic stem/progenitor cells. (C) Time line toward to the intraoperative preparation of cell-based bone grafts. These constructs can be made within 4 hours. Cell attachment was confirmed by DAPI staining. Monocytes were added to the defect sites when SVF-based constructs were loaded into femoral defects (yellow dashed circle).
2. Preparation of constructs and viability assessment
To prepare SVF constructs, we seeded $3 \times 10^6$ SVF cells on 21 mm$^3$ TCP granules and incubated these in proliferation medium for 2 hours to allow for cell attachment. Similarly, we seeded $1 \times 10^6$ ADMSCs similarly on TCP granules to obtain a comparable number of stromal cells on each construct. Subsequently, we added $1 \times 10^6$ monocytes to the SVF and ADMSC constructs in the wells in vitro or to the constructs in the defects in vivo (Figure 1A). Based on the design, from the isolation of SVF cells and peripheral blood monocytes till implantation of SVF-based constructs with monocytes, this procedure can be performed within 4 hours (Figure 1B & C). In contrast, the conventional ADMSC-based approach takes at least 10 days. To assess cell attachment to the prepared constructs, we performed nuclei and actin staining. Cells showed homogeneous distribution over the surface of granules (Figure S2). Cell viability after 2h in vitro incubation demonstrated that the majority of cells attached to the scaffold were viable, without apparent differences in dead cells between the experimental groups (Figure S2).

3. Monocytes promote osteogenic differentiation of SVF and ADMSCs
To study cellular behavior upon effect of cell-cell interactions between monocytes and SVF or ADMSCs, we cultured cell-loaded constructs in osteogenic medium for up to 4 weeks and assessed cell proliferation (DNA content; Figure 2A), osteogenic differentiation (ALP-activity; Figure 2B), and mineralization (calcium assay; Figure 2C). SVF and ADMSCs cultures showed similar cell proliferation profiles. In contrast, cultures containing monocytes showed a significantly increased proliferation. Additionally, whilst monocyte monocultures did not show ALP-activity, monocytes slightly increased ALP-activity in co-cultures with either SVF or ADMSCs. Monocytes further significantly increased mineralization in co-cultures with either SVF (~2-fold) or ADMSCs (~1.5-fold) compared to SVF and ADMSCs monocultures, respectively.

4. SVF cells and ADMSCs induce distinct polarization of monocytes/macrophages
In parallel with our experiments focused on the effect of monocyte addition to either SVF or ADMSC cultures, we oppositely focused on effects of SVF or ADMSCs on monocytes activation and polarization. Therefore, we set up an in vitro culture model [24, 25] and analyzed macrophage polarization via cytokine expression profiles of the pro-inflammatory M1 macrophage cytokine TNF-$\alpha$ and the pro-wound healing M2 macrophage cytokine TGF-$\beta$ (Figure 3A), using conventional induction into M1 macrophage polarization via LPS+IFN-$\gamma$ and into M2 macrophage polarization via IL-4+IL-13 stimulation as controls. TCP granules used as cell carrier material in our experiments induced polarization into a pro-inflammatory M1 macrophage, while SVF and ADMSCs induced macrophage polarization toward the pro-wound healing M2 type (Figure 3B & C). The ratio TGF-$\beta$/TNF-$\alpha$ (Figure 3D) clearly shows the overall M2 macrophage polarization stimulation, particularly by SVF.
5. Cell-based constructs enhance early bone healing

To assess the bone regeneration capacity, we implanted control, MO, SVF, SVF+MO, ADMSCs, and ADMSCs+MO constructs (n ≥ 7 for each group) into rat femoral defects (3 mm in diameter, 2.8 mm in depth) and evaluated bone regeneration histologically and histomorphometrically after 4 and 10 weeks. Methylene blue/basic fuchsin staining (Figure 4A) displayed substantial amounts of newly formed bone at the defect margins.
in between the voids of TCP granules in all SVF and ADMSC constructs after 4 weeks, irrespective of monocyte addition. In contrast, control and MO constructs showed limited amounts of newly formed bone. Histomorphometrically, SVF (34.5 ± 8.6%) showed similar amounts of new bone formation to ADMSCs (27.1 ± 6.0%; P < 0.05), both of which regenerated approximately 3 times more bone compared to control and MO (Figure 4B).

We further assessed the contribution of SVF and ADMSCs to bone formation using specific anti-human mitochondrial staining to detect human cells (i.e. SVF, ADMSCs and monocytes) in the bone defect (Figure 4C and Figure S3). SVF and SVF+MO constructs clearly showed the presence of human cells in the vicinity of the TCP granulate and newly formed bone, but not within the newly formed bone. This positive staining for human cells originated from SVF, as we observed no positive staining for the human monocyte/macrophage marker CD68 (Figure S4) nor for the osteoclastic marker TRAP (Figure S5). In contrast, ADMSCs and ADMSCs+MO constructs showed no human cells within any of the histological sections.
With vascularization as an essential part of bone formation [26, 27], we used α-SMA staining to evaluate blood vessel formation. SVF and ADMSCs constructs showed apparently larger vessel-like structures compared to control and MO constructs, which suggests beneficial effects of SVF and ADMSCs on the vascularization of implanted constructs (Figure 4C).

6. SVF constructs show complete bone defect healing

At 10 weeks post implantation, SVF constructs (w/- MO) exhibited complete healing of the defect site with newly formed bone present throughout the entire defect area (Figure 5A). In contrast, ADMSCs (w/- MO) showed substantial bone ingrowth centripetally from the original defect edge toward the defect center without presence of newly formed bone in the center. Control and MO constructs still showed large defect areas containing fibrous connective tissue with only sparse bone formation at the edge of the original defect. Residual TCP granulate was still present in non-healing defects and integrated with new bone in SVF and ADMSCs constructs. Quantitative analysis of new bone formation by histomorphometry (Figure 5B) revealed superior amounts of newly formed bone.
form bone for SVF and SVF+MO constructs (64.9 ± 9.1% and 66.3 ± 6.0%, respectively) compared to ADMSCs (45.4 ± 3.7%; P < 0.05), ADMSCs+MO (55.2 ± 10.6%; P < 0.05), MO (17.3 ± 8.0%; p < 0.01), and control (17.2 ± 9.0%; p < 0.01) constructs. Further, ADMSC and ADMSC+MO constructs showed significantly higher bone formation compared to MO (p < 0.01) and control constructs (p < 0.01).

Anti-human mitochondrial staining to detect cells of human origin demonstrated absence of human cells for any type of implanted construct after 10 weeks (Figure 5C). Based on α-SMA staining to assess vascularization, constructs with SVF or ADMSCs apparently gained more organized and elongated vessel-like structures (Figure 5C). Different to our observations after 4 weeks of implantation, osteoclastic activity was abundantly present in all defects containing cell-based constructs and only to a limited extent in control constructs (Figure S5).

Discussion

Cell-based bone tissue engineering, especially with the application of adipose tissue derived MSCs, represents an appealing alternative for the current best practice treatments using auto-/allografts for bone regeneration. This approach, however, is challenged by complex, impractical and expensive in vitro cell handling. Aiming to circumvent these, we followed an intra-operative approach based on stromal vascular fraction from fat tissue, as described recently [13, 14, 28]. However, the efficacy of this intra-operative approach has not been directly compared to conventional ADMSC-based method. As another type of ‘easy access’ cells with potential stimulatory effects on wound healing and stromal cell differentiation [21, 22], we explored the addition of monocytes to both ADMSC- and SVF-based constructs both in vitro and in vivo using a rat femoral condyle bone defect model.

SVF is reported to be a heterogeneous mixture of MSCs, endothelial progenitor cells, pericytes, mast cells, pre-adipocytes, and other cell types [7, 12]. From flow cytometry and in vitro cell culture results, we proved that MSCs take up around 1/3 of the whole SVF population, which corroborates previous reports [29-31]. Consequently, we prepared constructs containing 3 million SVF cells or 1 million purified ADMSCs to compare their osteogenic potential in vitro and bone regeneration capacity in vivo. In vitro mineralization demonstrated a similar osteogenic potential between SVF and ADMSCs. In contrast, although both SVF and ADMSCs improved bone defect healing compared to cell-free controls, data from our in vivo work demonstrated superiority of SVF-based constructs over ADMSC-based constructs regarding bone regenerative capacity. This difference between in vitro and in vivo results suggests that in vitro osteogenic differentiation capacity is not predictive for in vivo bone healing capacity.

The synergetic effect of endothelial progenitor cells and pericytes contained in SVF probably plays an important role in the superiority of SVF-based bone healing [30, 32]. An important observation, likely related to the superior bone regeneration for SVF and SVF+MO constructs, is the prolonged presence of SVF cells in the bone defect region compared to ADMSCs.
Speculating about the mechanism of SVF- and ADMSCs-based bone regeneration, our results and those of others [33] indicate that direct differentiation of grafted cells into bone forming osteoblasts or eventually embedded osteocytes seems very unlikely. Further, it has been postulated that the grafted cells provide osteoinductive signals or act as a source of trophic factors to modulate microenvironments [34-36]. Here, we did not observe grafted human cells within the newly formed bone, ruling out the mechanism of direct differentiation of SVF or ADMSCs into bone forming osteoblasts or osteocytes. Consequently, we speculate that SVF and ADMSCs exerted a certain paracrine effect on surrounding host cells to stimulate bone regeneration. The exact paracrine factors secreted by these implanted cells responsible for bone healing are largely unknown yet due to the complexity of in vivo conditions. Still, few pioneering reports indicated several possible roles of grafted cells, such as promoting progenitor cells migration [15, 16], benefiting vascularization [34, 37] and modulating immune response [38-40], to stimulate bone formation. In this study, SVF and ADMSCs activated and polarized monocytes/macrophages into anti-inflammatory phenotype as proved in in vitro co-culture experiment, which correspondingly led to more profound vascularization and bone formation compared to constructs without SVF and ADMSCs in vivo. The coincidence of superior immunoregulatory effect and more remarkable bone regeneration capacity by grafted SVF warrants further investigations into the mechanisms underlying the immunoregulatory capacity of grafted cells in bone healing.

Once a bone fracture occurs, circulating monocytes travel to the injured sites within several hours, become activated macrophages and contribute to various aspects of the healing process [41, 42]. Due to their reported beneficial effects on osteogenic differentiation [21, 22] and the fact that monocytes are easily accessible via a venous puncture, the concept of harnessing the power of monocytes/macrophages for bone tissue engineering has been proposed by several researchers [19, 38], but has never been applied for cell-based bone regeneration. Our study also confirmed their beneficial effects on in vitro osteogenic differentiation, evidenced by significantly higher ALP-activity and mineralization in co-cultures with SVF or ADMSCs. Interestingly, monocytes exerted a more obvious effect on SVF compared to ADMSCs, which indicates the different cell status (undifferentiated and differentiated), cell components (heterogeneous and homogenous), and cell-cell interaction of SVF compared to ADMSCs. Nonetheless, in our orthotopic animal model, we did not observe this favorable effect on bone regeneration following monocyte addition. As we did not observe specific immunostaining for human monocytes/macrophages in any of our histological sections (Figure S3), this might be explained by the short duration of exogenously added monocytes present in the defects, the relatively limited half-live of this cell life, and less adhesive properties compared to stromal cells [43, 44].

Our study also has several limitations. First, we used human monocytes from a cell line rather than human primary monocytes considering the experimental feasibility and reproducibility. Although THP-1 has been proven as an ideal research cell culture model for primary monocytes [45], further studies are needed to analyze the behavior of SVF and ADMSCs co-cultured with primary monocytes. Secondly, SVF- and ADMSC-based
constructs enhanced bone formation after 4 weeks, which suggests that the seeded cells function in the early post-implantation stage. Therefore, observation of host response (i.e. cell infiltration, vascularization and macrophage phenotype) at earlier time points would likely provide more clarity on the potential immunoregulatory role of grafted cells in bone healing. In addition, due to the component similarity and close distribution between TCP and newly formed bone, it is not practical to use regular X-ray or Micro CT to provide additional information on bone mineralization and defect healing. The advanced technology such as Nano CT and contrast reagents can be applied in the future study to analyze new bone formation in real-time.

Compared to simply focusing on outcome of cell-based bone tissue engineering in most studies, our study put more emphasis on viewing their potential for clinical applications. Since both SVF and monocytes are easily accessible and isolated during the surgical procedure itself and can be isolated via standard procedures, this strategy meets the requirements of “good manufacturing practice” and “during the same visit” defined by the concept of intraoperative preparation, making this approach more feasible for clinical applications and permissible from a regulatory perspective. In addition, an intra-operative method likely reduces the high-cost associated to ex vivo cell expansion and its safety was confirmed in various clinical applications [13, 46]. Although further experiments are needed, for instance to precisely define the composition of SVF and to apply this approach in more bone defect models, this ‘easy access cells’ based intra-operative approach and its superior bone regenerative capacity will greatly innovate the concept, design, and development of cell-based bone tissue engineering and regenerative medicine.
Chapter 4

References

Toward intra-operative preparation of cell-based constructs for bone regeneration

Chapter 4


Toward intra-operative preparation of cell-based constructs for bone regeneration
Chapter 5

Biomaterial property effects on platelets and macrophages: an in vitro study

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Chapter 5

Introduction

Implants for the replacement and fixation after bony fractures have become a general treatment modality in the current dental and mainly orthopedic clinic, respectively. Generally, these bone implants are made from metallic materials that allow to withstand mechanical forces. Among the metallic materials used for bone implant preparation, titanium (Ti) presents excellent biocompatibility and high mechanical strength [1, 2]. Also, from the biological perspective, orthopedic Ti implants can be improved by combining different surface modifications [3]. In view of this, research efforts have focused on the effect of surface modifications for implant surfaces to promote implant integration within the native bone tissue [2, 4-6].

The nature of these surface modifications can be categorized as either physical (e.g. roughness or topography) or chemical (e.g. coatings). In this way, titanium implants coated with different physiochemical properties can stimulate the osseointegration ability, directly affecting the performance and clinical success of the implant [3]. In this context, calcium phosphate ceramics (CaPs) are the appealing group of materials for the deposition of coatings for bone implants. Many studies have shown that CaP coatings, particularly those based on hydroxyapatite (HA), β-tricalciumphosphate (β-TCP), or combinations thereof, provide a favorable microenvironment for the interaction of the implant surface with the bone tissue, allowing an accelerated and more effective integration of such implants in the surrounding bone tissue [7-9].

Upon implantation, any biomaterial induces a cascade of events initiated by the activation of platelets and inflammatory cells (e.g. macrophages and foreign body giant cells) [10-13]. Within this cascade of events, the challenge for the biomedical engineering field is to optimize the initial inflammatory events toward an effective regenerative phase, and hence achieve an improved performance of biomedical devices [10, 14]. In the initial inflammatory events, platelets play an active role in the immunological and inflammatory aspect of tissue healing in normal hemostasis as well as in host defense. Platelets can be directly involved in the inflammatory response by the production and release of several inflammatory mediators, including a variety of cytokines and chemokines. Platelet activation results in stimulation of various leukocytes, including macrophages [15]. Beside platelets, macrophages also contribute to tissue homeostasis by clearance of injured host components and to defense against infection. Once out of the circulation and in the tissue at a wounded site, macrophages can acquire different morphologies and functionalities in response to pathogens and local environment stimuli. Two major macrophage subpopulations have been defined in vitro, designated as either classically activated macrophages (M1) or alternatively activated macrophages (M2) [16]. Classically activated M1 induced by lipopolysaccharide (LPS), interferon (IFN)-γ or tumor necrosis factor alpha (TNF) are associated with the first phases of acute inflammation. This macrophage subtype is characterized by the secretion of pro-inflammatory cytokines, Tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS or NOS2), reactive oxygen species (ROS), reactive nitrogen intermediates (RNI), promotion of Th1 responses, and strong microbicidal and tumoricidal activity.
Biomaterial property effects on platelets and macrophages: an in vitro study

[17]. In contrast, alternatively activated M2 macrophages are characterized by increased phagocytic activity, high expression of scavenging, mannose and galactose receptors, production of ornithine and polyamines through the arginase pathway, a distinct chemokine repertoire (e.g., CCL17, CCL18 and CCL22) and an IL-12lo, IL-10hi, IL-1, decoyRhi, IL-1RAhi phenotype [18]. The role of implant surface properties in the initial phase after implantation is largely unknown. However, it is generally accepted that the physical-chemical properties of a biomaterial surface (e.g. microporosity, surface roughness, coating chemistry and solubility), the inflammatory response evoked in the tissue (i.e. platelet adhesion and activation and inflammatory responses), the design and size of the medical device, and the anatomical site in which it will be inserted [8, 13, 19] are major determinants for implant success. There is a vast amount of evidence that CaP biomaterials with osteogenic properties have enormous potential in bone healing, reducing the chances of complications and time to repair. Urquia Edreira et al. [20] evaluated the effect of CaP sputter-coatings with different phase composition in an in vitro and in vivo study. Their data demonstrated that the differences in physico-chemical properties of the coatings affect both in vitro and in vivo results. One of the CaPs, the hydroxyapatite (HA), has been widely used to improve the bioactivity and osseointegration of metallic implants since it is an osteoconductive material capable of enhancing the bond with the surrounding bone tissue [21]. Several studies show positive effects on osseointegration of HA-coated metallic implants [21-23]. In addition, β-TCP is another widely used CaP that has osteoconductivite properties to be used as a coating for Ti implants. Several studies demonstrated greater ability to form new bone after being implanted in the body [24-26]. Although several studies have shown the effects of HA and TCP as coatings of metallic implants, the initial response of blood-born components (i.e. platelets and monocytes/macrophages) are largely neglected by many researchers evaluating bone response after implantation of a biomaterial. Consequently, we here evaluated biomaterial surface property effects on the response of blood-born components that account for the initial biological cascade of events following implantation. We used in vitro experiments with human platelets and monocytes/macrophages to investigate (i) platelet adhesion, and (ii) monocyte/macrophage morphology, proliferation, cytokine secretion and polarization, using either unmodified or CaP-coated Ti discs. We hypothesized that CaP-coated discs (HA or β-TCP) would alter the initial response of blood-born components and platelet adhesion and macrophage secretion/polarization in favor of wound healing.

Materials and methods

1. Material preparation and characterization
Commercially available pure Ti discs (99.9 wt% Ti, thickness 1.5 mm, diameter 12 mm) were Al₂O₃ grit-blasted before deposition. The target materials used in the deposition process for the coating were hydroxyapatite (HA; Ca₁₀(PO₄)₆(OH)₂) granulated powder
obtained from CAMCERAM® (CAM Bioceramics, Leiden, the Netherlands; low porous granules 500-1000µm) and β-tricalcium phosphate (β-TCP; Ca₃(PO₄)₂) (CAM Bioceramics, Leiden, the Netherlands). The solubility product constant (Ksp) of HA and TCP is 10⁻¹₁⁶.₈ mol/L and 10⁻₂₈.₉ mol/L respectively. As a control, Ti discs and Thermanox coverslip (Diameter: 13 mm; thickness: 0.2 mm; use with a 24-well multi-dish; Thermo Scientific Nunc®) were used. The coatings for this study were deposited using RF magnetron sputtering equipment (Edwards High Vacuum ESM 100 system, Crawford, England) as described previously [20, 27-29]. Before deposition, the discs were cleaned ultrasonically in acetone and propanol to remove impurities. Subsequently, the discs were placed on a rotating holder and the coating deposition process with HA and β-TCP were initiated (sputtered target: HA and β-TCP; distance between target and implants: 80 mm; power: 400W; working gas: Argon; pressure: 5.0 x 10⁻³ Pa; treatment time: 10 hours for HA deposition and 13 hours to β-TCP deposition). After sputtering, the discs received a heat-treatment of 15s in air at final heating temperature of up to 600°C using an infrared furnace (Quad Ellipse Chamber, Model E4- 10-P, Research, MN) [20]. Infrared irradiation was carried out under pure argon flow as described by Yoshinari et al. [30].

2. Surface analysis
Ti discs, HA and β-TCP coated discs were morphologically inspected by field emission scanning electron microscopy (FE-SEM; JEOL 6310, Nieuw-Vennep, the Netherlands). Additionally, thickness and roughness of the coatings (quadruplicate samples, n=4) were measured using a Universal Surface Tester (UST®, Würzburg, Germany).

3. Physico-chemical characterization and stability of the coatings
The crystal structure of each specimen was determined by X-ray diffraction (XRD, Phillips, PW3710, Eindhoven, the Netherlands) using CuKα radiation (power: 40 kV; current: 30 mA). In addition, infrared spectra of the coatings were obtained by a reflection Fourier transform infrared spectrometer (FTIR, Perkin Elmer, Spectrum Two, Groningen, the Netherlands).

For analyzing coatings stability, discs (triplicate samples; n=3) were placed in 4 mL of Milli-Q water and incubated at 37°C in a water bath on a shaker table (70 rpm) for 4 weeks. After 7, 14, 21 and 28 days of incubation, MilliQ water was refreshed completely and used for the calcium assay based on orthocresolphtalein complexone (OCPC). At the end of the experimental period, the coated discs were incubated overnight in 1 ml 0.5 N acetic acid on a shaker table to dissolve remaining calcium phosphate on the discs. For analysis, 300 µl working reagent was added to 10 µl sample or standard in a 96 wells plate. The well plates were incubated for 10 min at room temperature. The absorbance was measured using a microplate spectrophotometer at 570 nm (Bio-Tech Instruments, Winooski, VT, USA). The total calcium content within the coatings (µg/cm²) was determined by cumulating total calcium release and remaining calcium on the discs, normalized for disc surface area.
4. Platelet experiments

Human platelet-rich plasma (hPRP) was obtained from Sanquin (Nijmegen, the Netherlands) with cell counts of 4-5 x 10^8 platelets/mL. Platelets counts were obtained with a particle count and size analyzer according to the instructions of the manufacturer (Beckman Coulter, Z2, Florida, USA).

The experimental discs (quadruplicate samples; n=4) were placed in 24-well plates with custom-made Teflon molds surrounding each disc and a cylindrical volume above the disc (1 ml volume) as described previously [31]. These molds restrict platelet adherence to only the disc surface. A platelet solution containing 200 µl of the freshly prepared PRP and EDTA solution with a final concentration of 10 mm EDTA (Sigma, MO, USA) was added to each well containing one disc. Thereafter, the well plate was centrifuged at 150g for 10 minutes to achieve platelet adherence to the disc surface. After centrifugation, the 24-well plates were incubated at 37°C on a horizontal shaker (70 rpm) for 30 minutes. After that, the experimental discs were washed with PBS in order to remove non-adherent platelets and prepared for (qualitative) SEM examination of adherent platelets as well as (quantitative) lactate dehydrogenase (LDH) activity.

4.1 Scanning electron microscopy of platelets

SEM (JEOL 6310, Nieuw-Vennep, the Netherlands) was used for qualitative analysis of adherent platelets. After 3 hours of incubation, platelets were washed twice with PBS and subsequently fixed for 15 min in 2% glutaraldehyde in 0.1M sodium cacodylate buffered solution. Then, samples were rinsed twice with cacodylate buffered solution and dehydrated using a graded series of ethanol. Finally, samples were dried with tetramethylsilane. The samples were sputter-coated with gold prior to SEM examination.

4.2 Platelet adhesion

The quantification of platelet adherence was determined using a photospectrometric measurement based on kinetic determination of lactate dehydrogenase (LDH) activity [7, 32, 33]. Adherent platelets were lysed by adding 200 µL of 1% Triton buffer (Triton X-100, Sigma) to each well. The wells were incubated for 3 hours at room temperature. After the incubation, 100 µL of each lysate was collected and mixed with 100 µL of reaction solution (LDH measurement kit, Roche Life Science, Almere, the Netherlands). Per manufacturer’s instructions, LDH-activity was determined by recording absorbance at 490 nm.

5. Monocytes/macrophage experiments

The human monocytic cell line (THP-1; ATCC, LGC Standards GmbH, Germany) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and streptomycin at 37°C in a 5% CO₂ and 95% atmospheric air. THP-1 cells were activated into macrophages by 50 ng/ml phorbol-12-myristate-13-acetate (PMA) as previously reported [34]. 1 x 10⁵ cells/cm² were seeded on the experimental discs in culture medium (quadruplicate samples; n = 4) and cultured for 1, 4 and 7 days.
5.1 Morphological analysis
Cell morphology was assessed by SEM (JEOL 6310, Nieuw-Vennep, the Netherlands). After 1, 4 and 7 days, the cells were washed twice with PBS and subsequently fixed for 15 min in 2% glutaraldehyde in 0.1M sodium cacodylate buffered solution. Then, samples were rinsed twice with cacodylate buffered solution and dehydrated using a graded series of ethanol. Finally, samples were dried with tetramethysilane and sputter coated with gold prior to SEM analysis.

5.2 Cellular DNA content
After day 1, 4 and 7 of cell culture, total DNA content was determined to obtain information about cellular proliferation. Cellular DNA content was measured using Quantifluor™ dsDNA System (Promega Benelux BV, Leiden, the Netherlands) according to the instructions of the manufacturer. Medium was removed and the cell layer was washed twice with PBS, after which 1 mL of MilliQ was added to each well and the samples were stored at -80°C until further use. For the standard curve, serial dilutions of dsDNA stock were prepared to final concentrations of 0-2000 ng/mL. Next, 100 µL of each sample and 100 µL freshly made 1x Quantifluor™ dye working solution were added to a 96-wells plate in duplicate. The plates were incubated at room temperature in the dark for 5 min, after which the fluorescence excitation/emission at 480/520 nm was read.

5.3 Cytokine measurements by ELISA
The secretion of Tumor Necrosis Factor-alpha (TNF-α) and Transforming Growth Factor-beta (TGF-β) was measured in the cell culture media. After 1, 4 and 7 days of culture, the culture medium was aspirated and stored frozen at -80°C until analysis. The concentrations of TNF-α (pro-inflammatory) and TGF-β (pro-wound healing) were determined using ELISA kits (Bioscience, USA), according to the manufacturer’s instructions. Relative values of cytokine secretion were obtained by normalization to DNA quantification data (i.e. results are expressed as pg/mL/ng DNA content).

5.4 Macrophage polarization assessment
For immunostaining, THP-1 was cultured on the experimental discs for 1, 4 and 7 days. After that, the cells were fixed with 500 uL of 3.7% paraformaldehyde (PFA) for 10 min at room temperature and blocked with 500 uL of 1% bovine serum albumin (BSA) during 15 min. After blocking, the discs were incubated with primary antibodies CCR7 (M1 macrophage marker; Abcam, Cambridge MA, USA) and CD36 (M2 macrophage marker; Biolegend, San Diego CA, USA) overnight at 4°C. The sections were washed three times with PBS, incubated for 1 h with Alexa-Fluor 488 or 568 secondary antibodies and then incubated with DAPI (nucleus; Thermo Fisher Scientific, Waltham MA, USA) for 5 min. Microscopy images were obtained using a fluorescent microscope equipped with a digital camera (Carl Zeiss, Göttingen, Germany). The exposure time of each light channel was kept the same for all samples. The relative intensity of each fluorescence after images collection was analyzed using ImageJ (U.S. National Institutes of Health,
The values of red (Alexa-568) and green (Alexa-488) fluorescence of each sample were further normalized to the value of blue fluorescence (DAPI).

5.5 Quantitative PCR
Quantitative PCR (qPCR) was performed to detect the macrophage polarization markers indoleamine 2,3-dioxygenase (INDO) (M1); CXCL11 (M1); MCR-1 (M2) and CCL13 (M2). Total RNA was isolated from the cells using TRizol reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions. In brief, after removing the culture medium, 1 mL TRizol reagent was added to each well. The cell extract was mixed vigorously with 0.2 mL of chloroform and centrifuged at 12,000 g for 15 min at 4°C. The aqueous phase of the sample was collected and mixed with 0.5 mL of 100% isopropanol. After incubation at room temperature for 10 min, the extract was centrifuged and then washed with 75% ethanol. Successively, the RNA pellet was dissolved in RNase-free water and concentration and purity was determined using the NanoDrop (ND-2000; Thermo Scientific, Waltham MA, USA).

For real-time PCR, total RNA (1 ug) was applied as template for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) following the manufacturer’s instructions. The cDNA samples were subjected to quantitative real time polymerase chain reaction (qRT-PCR) using a BIORAD CFX96 real-time system. Oligonucleotide primers were designed for human β-actin, INDO; CXCL11; MCR-1 and CCL13 (Table 1). All real-time primers were initially tested against standards and a standard curve was generated. The optimized PCR conditions were: initial denaturation at 94°C for 10 min, followed by 40 cycles consisting of denaturation at 94°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 60 s. Negative control reactions with no template (deionized water) were also included in each run. For each gene, all samples were amplified simultaneously in duplicate in one assay run. Analyses of relative gene expression were performed using the 2^ΔΔCT method. Human β-actin was used as a housekeeping gene to normalize gene expression data.

6. Statistical analysis
Data are presented as mean ± standard deviation of the mean. The normality of all variables was verified using the Shapiro-Wilk W-test. For coating thickness and roughness analysis, Student’s t-tests were used. For the multiple analyses of variables that exhibited normal distribution (i.e. cytokine secretion; macrophage proliferation, polarization and gene expression), comparisons among the groups were made using one-way analysis of variance (ANOVA) with a post-hoc Tukey Multiple Comparisons test. For variables that exhibited non-normal distribution (i.e. roughness analysis, coating stability, platelet adhesion, and monocyte/macrophage proliferation), Kruskal Wallis tests were used with post-hoc Dunn tests. GraphPad Prism version 6.01 (Software Mackiev, Boston, MA, United States) was used to perform statistical analysis. Values of p < 0.05 were considered statistically significant.
Chapter 5

Results

1. Material preparation and characterization

1.1. Surface analysis
SEM was used to morphologically examine the topography of the different material surfaces. SEM results of Thermanox, grit-blasted Ti, HA, and β-TCP are depicted in Figure 1. Thermanox showed a smooth appearance, whereas grit-blasted Ti, Ti-HA, and Ti-β-TCP exhibited apparent roughness (Figure 1A).

Table 2 depicts the results of thickness and roughness analyses of grit-blasted Ti, HA, and Ti-β-TCP. The average thickness of HA and β-TCP coatings was 1.423 ± 0.229 µm and 1.688 ± 0.130 µm, respectively (p>0.05). The roughness of grit-blasted Ti, Ti-HA, and Ti-β-TCP was similar with average Ra values of 2.598 ± 0.074 µm, 2.425 ± 0.049 µm, and 2.538 ± 0.092 µm, respectively (p>0.05).

Figure 1. Surface characterization of different surfaces. (A) SEM micrographs of Thermanox, grit-blasted Ti, Ti-HA and Ti-β-TCP surfaces; (B) X-ray diffraction spectra of grit-blasted Ti and as-deposited and heat treated Ti-HA and Ti-β-TCP coatings; (C) Fourier transforms infrared spectra of as-deposited and heat treated Ti-HA and Ti-β-TCP.
1.2. Physico-Chemical Characterization

Figure 1B shows the XRD patterns of grit-blasted Ti, HA and β-TCP (as-deposited and with heat treatment). The XRD patterns of grit-blasted Ti showed characteristic titanium peaks at 35.04°, 38.40° and 40.10° (ICDD 5-682). The XRD pattern of as-deposited HA showed 2 characteristic peaks at 25.9° and 32° that can be attributed to apatite. The XRD patterns of as-deposited β-TCP presented peaks (26°, 32°, 34°) with low intensity. However, heat treatment increased the intensity of peaks in the XRD pattern at 34° and 49° for HA (ICDD 9-0432) and 26°, 32°, 34° for β-TCP (ICDD 9-169). These results indicate that heat treatment evoked a more crystalline structure in the ceramic coatings. These findings were confirmed by FTIR spectra of HA and β-TCP as-deposited and after heat treatment (Figure 1C). HA coating showed bands at 575, 670, 970, 1050 and 1090 cm⁻¹ (stretching and bending motion of phosphate) [20]. These P-O bonds of calcium phosphate appear sharper after heat treatment. Furthermore, the β-TCP coating spectrum presented bands in the same regions as HA coatings (575, 670, 970, 1050 and 1090 cm⁻¹) characteristics of stretching and bending motion of phosphate [20]. After heat treatment can be observed sharper bands revealing that this treatment was effective to make the coating more crystalline. Therefore, both spectrum showed similar characteristic absorption bands at 500-650 cm⁻¹ (P-O bending) and 900-1200 cm⁻¹ (P-O stretching), which increased after heat treatment and match with crystalline structure of HA and β-TCP.

### Table 1
Primer sequence for each gene analyzed in the present study.

<table>
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<tr>
<th>Gene</th>
<th>Forward primer</th>
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### Table 2
Coating thickness and roughness.

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<th>Experimental group</th>
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<td>Grit-blasted Ti</td>
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<td>2.598 ± 0.074</td>
</tr>
<tr>
<td>Ti-HA</td>
<td>1.423 ± 0.229</td>
<td>2.425 ± 0.049</td>
</tr>
<tr>
<td>Ti-β-TCP</td>
<td>1.688 ± 0.130</td>
<td>2.538 ± 0.092</td>
</tr>
</tbody>
</table>

1.3 Coating stability

Table 3 shows the total amount of calcium on the surface of the coated discs (µg/cm²). Ti-HA and Ti-β-TCP coatings showed similar calcium amounts within the coating of 177.12 ± 14.47 µg/cm² (as-deposited Ti-HA), 191.67 ± 14.70 µg/cm² (heat-treated Ti-HA), 186.80 ± 6.82 µg/cm² (as-deposited Ti-β-TCP), and 182.06 ± 24.97 µg/cm² (heat-treated Ti-β-TCP). Figure 2A presents cumulative calcium release from the Ti-HA and
Chapter 5

Ti-β-TCP coatings after 7, 14, 21 and 28 days incubation in MilliQ. Cumulative calcium release was significantly higher for Ti-β-TCP coatings compared to Ti-HA coatings after 7 days (p=0.036) and after 21 days (p=0.037). No significant differences between as-deposited and heat treated coatings were observed, irrespective of CaP coating type (p>0.05). After 28 days, the calcium remaining on the discs was measured (Figure 2B). Calcium remaining for Ti-HA heat (130.73 µg/cm²) was higher when compared to Ti-β-TCP heat (58.15 µg/cm²) (p=0.011).

2. Platelet activation

2.1 Platelet morphology

Qualitative analyses of platelet adhesion and morphology were performed with SEM (Figure 3A). On Thermanox, numbers of platelets with spherical shape and some platelets with changes in their shape (development of tiny pseudopodia) were observed. On grit-blasted Ti, Ti-HA and Ti-β-TCP, the platelets were more difficult to identify because of the roughness of the discs. However, on grit-blasted Ti, Ti-HA and Ti-β-TCP, the appearance of adherent platelets was similar, showing spherical platelets spread and aggregated on the disc surface.

![Figure 2. Calcium release of calcium phosphate coatings.](image)

- A) Calcium release from as-deposited and heat-treated Ti-HA and Ti-β-TCP coatings over a 28-day incubation period in MilliQ water (n=3). * p = 0.036, Ti-β-TCP heat vs. Ti-HA heat; ≠ p = 0.037, Ti-β-TCP heat vs. Ti-HA heat. (B) Calcium remaining on the coated discs after 28 days in MilliQ water soaking experiment for as-deposited and heat treated HA and β-TCP coatings (n=3). * p = 0.011.

2.2 Platelet adhesion (LDH-activity assay)

Quantitative analysis of platelets adhesion based on LDH-activity assay (Figure 3B) showed a significant increase in platelet adherence to grit-blasted Ti (p=0.0024), Ti-HA (p=0.0007) and Ti-β-TCP (p=0.0011) compared to Thermanox (p=0.0024, 0.0007 and 0.0011, respectively).
3. Macrophage polarization

3.1 Cell morphology
Morphology of macrophages after 1, 4 and 7 days of culture on the different experimental surfaces was observed (Figure 4). Spherical macrophages were observed at the first day on Thermapox. Interestingly, after four and seven days morphological changes were observed in the form of prolongations. In contrast, macrophages showed a faster transition to a flattened morphology on rough surfaces of grit-blasted Ti, Ti-HA and Ti-β-TCP.

3.2 Cell proliferation
The DNA content of macrophages seeded on the different experimental substrates after culture periods of 1, 4 and 7 days are presented in Figure 5A. Both grit-blasted Ti and Thermapox showed an increase in DNA content values from day 1 to day 4, and a decrease thereafter to day 7. In contrast, Ti-HA and Ti-β-TCP showed similar DNA content values over time, both of which were significantly lower compared to those of grit-blasted Ti and Thermapox.
3.3 Cytokine secretion
Secretion levels for the cytokines TNF-\(\alpha\) and TGF-\(\beta\) are presented in Figure 5B and C. After 1 day, TNF-\(\alpha\) secretion was significantly higher for Ti-\(\beta\)-TCP compared to Thermanox \(p=0.008\). After 4 days, Ti-HA showed significantly higher secretion of TNF-\(\alpha\) compared to Thermanox \(p=0.0318\). At day 7, both Ti-HA (0.178 pg/mL/ng DNA) and Ti-\(\beta\)-TCP (0.422 pg/mL/ng DNA content) showed an increased TNF-\(\alpha\) secretion compared to Day 1 and Day 4. Additionally, in this period, both Ti-HA and Ti-\(\beta\)-TCP showed a significantly higher secretion of TNF-\(\alpha\) compared to Thermanox \(p=0.019\) and grit-blasted Ti \(p=0.019\).

TGF-\(\beta\) levels (Figure 5C) showed no statistically significant differences during the first 4 days of macrophages culture (range: 0.006– 0.156 pg/mL/ng DNA content). At day 7, Ti-HA showed a significantly higher secretion of TGF-\(\beta\) levels (0.920 pg/mL/ng DNA content) compared to Thermanox (0.377 pg/mL/ng DNA content) \(p=0.014\), grit-blas-
3.4 Macrophage polarization on experimental surfaces

Representative fluorescent images of the experimental groups at their corresponding time points are shown in Figure 6. The images demonstrated that all surfaces presented the M1 (CCR7) and M2 (CD36) macrophages markers across all time points. The images also suggest that grit-blasted Ti, Ti-HA and Ti-β-TCP are effective at maintaining the CD36 staining (M2 macrophages) especially after 4 days.

Quantification of the immunofluorescent staining was performed. Figures 7A, B and C shows the immunostaining for CCR7 and CD36 after 1, 4 and 7 days. After 1 day, M1 macrophage marker CCR7, can be observed a decreased of labeling in the Ti and an increase in the labeling in the HA (Figure 7A). No significant differences were observed between the Thermanox and Ti-β-TCP in this experimental period. For CD36, an increase of immunostaining was observed in the grit-blasted Ti, Ti-HA and Ti-β-TCP compared to Thermanox (Figure 7A). On day 4, no statistically significant was observed for CCR7 between the groups analyzed. For CD36, similar results were observed the analysis after 1 day, with increased immunostaining for CD36 in the Ti, Ti-HA and Ti-β-TCP groups when compared to Thermanox (Figure 7B). Finally, as shown in Figure 7C, CCR7 and CD36 immunostaining labeling in all groups after 7 days. For CCR7, can be observed an increase in the Ti-HA compared to Thermanox and grit-blasted Ti and Ti-β-TCP. The lowest values for the CCR7 immunostaining were observed in Thermanox. No significant differences were observed between the grit-blasted Ti and Ti-β-TCP in this experimental period. For CD36, the grit-blasted grit-blasted Ti and Ti-HA demonstrated the higher values of immunostaining compared to Thermanox and Ti-β-TCP.

3.5 Quantitative PCR

The macrophage polarization was also assessed by RT-PCR, in which INDO and CXCL11 were considered as M1 macrophage markers and MCR-1 and CCL13 were considered as M2 macrophage markers. After 1 day, grit-blasted Ti, Ti-HA and Ti-β-TCP showed more obvious biological activity compared to Thermanox controls, which promoted both M1 macrophage and M2 macrophage polarization process, evidenced by higher INDO, CXCL11, MCR-1 and CCL13 gene expression. Amongst these, Ti and Ti-HA showed significantly higher M1 and M2 macrophage marker expression than β-TCP (Figure 7D). After 4 days, grit-blasted Ti, Ti-HA and Ti-β-TCP still showed more obvious biological activity compared to Thermanox controls as the trend on day 1. However, the difference is that significantly higher expression of INDO, CXCL11, MCR-1 and CCL13 in the Ti-β-TCP was found compared to grit-blasted Ti. Still, Ti-HA displayed the highest INDO, CXCL11, MCR-1 and CCL13 gene expression in the Ti-HA (Figure 7E).

After 7 days, intriguingly, Ti-β-TCP significantly enhanced the M1 macrophage polarization, evidenced by INDO and CCL11 gene expression and decreased M2 macrophage gene expression MCR-1 and CCL13 compared to Ti-HA and grit-blasted Ti and Ther manox controls (Figure 7F).
Chapter 5

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Figure 6. Immunostaining of macrophage cultured on different surfaces. Immunostaining for DAPI (nuclei), CCR7 (M1-marker), CD36 (M2-marker) and merged images for macrophages cultured on grit-blasted Ti, Ti-HA, and Ti-β-TCP disc after cultures of 1 (A), 4 (B) and 7 (C) days.

Figure 7. Specific gene expression of macrophage cultured on different surfaces. Quantitative immunostaining for CCR7 (M1-marker) and CD36 (M2-marker) for macrophages cultured on Theranox, grit-blasted Ti, Ti-HA, and Ti-β-TCP disc after cultures of 1 day (A), 4 days (B) and 7 days (C) (n=4). Quantitative PCR for INDO and CXCL11 (M1-markers) and MCR-1 and CCL13 (M2-markers) for macrophages cultured on Theranox, grit-blasted Ti, Ti-HA, and Ti-β-TCP disc after cultures of 1 day (D), 4 days (E) and 7 days (F) (n=4). *p<0.05; **p<0.01 and ***p<0.0001.
Chapter 5

Discussion

Dental and orthopedic bone implants should provide a complete host tissue integration and moreover, prevent an exacerbated immune response. Upon implantation, such biomaterial devices can activate many cells, and hence the secretion of cytokines and factors by members of the hematological and immune system [10-12]. This activation is the initial step for bone healing, and hence represents a challenge for the medical field in terms of modulating this foreign body response toward functional performance of implanted biomaterials [10, 14]. Here, we evaluated bone implant surface effects on the response of blood-borne components that account for the initial biological cascade of events following implantation, using in vitro experiments with human platelets and macrophages. The characterization of the different coatings showed that the heat treatment was effective in increasing coating crystallinity, but not coating stability. For platelets, higher numbers of adherent platelets were observed for grit-blasted Ti, Ti-HA and Ti-β-TCP compared to Thermanox. Macrophage experiments showed decreased cell proliferation on CaP-coated Ti-discs (Ti-HA and Ti-β-TCP) compared to Thermanox and grit-blasted Ti. However, the coated CaP seems more biological active than Thermanox, proved by upregulated pro-inflammatory and anti-inflammatory cytokine secretion, M1/M2 macrophage marker and M1/M2 macrophage gene expression. Additionally, Ti-β-TCP demonstrated a more pro-inflammatory function than Ti-HA.

In this study, two calcium phosphate ceramics (CaPs) were used as a coating, the HA and β-TCP. These coatings are considered a class of bioactive materials, which have properties that affect the adhesion and proliferation of immune and bone cells and induce bone formation [8, 20]. HA is an osteoconductive biomaterial similar to natural bone mineral both from a chemical and a structural point of view [35, 36]. In contrast, β-TCP is a biomaterial used in biomedical applications mainly due to its mechanical performance, chemical stability, solubility and reabsorption rate [20, 36]. These CaP biomaterials were used to provide the Ti discs a thin coating deposition using the RF sputtering, which has been shown to be useful to control ceramic coating properties and the adhesion between the substrate and the coating [30, 37, 38] besides to permit a uniform and continuous deposition coatings [20, 27]. After the sputtering, the discs were heat treated in an infrared furnace. This treatment is a necessary post-annealing treatment to crystallize the coating. Yoshinari et al. [30] demonstrated that the heat treatment with infrared radiation around 600° was the best treatment for RF magnetron sputtered coatings, which was used in this study [30]. The physical-chemical characterization analyzed by XRD and FTIR of the CaP coatings used in this study showed that the heat treatment with infrared radiation around 600° was the best treatment for RF magnetron sputtered coatings, which was used in this study [30]. The physical-chemical characteristics of CaPs coatings and their link with the Ti discs is crucial for the first host body response and can affect the success of the implantation. Many studies on bone implants, however, neglect the importance of physicochemical characteristics such as dissolution, crystallinity, corrosion among others and how they alter the microenvironment around the implant.

Platelets play an active role in the immunological and inflammatory aspect of tissue
healing in normal hemostasis as well as in host defense. Platelets can be directly involved in the inflammatory response by the production and release of several inflammatory mediators, including a variety of cytokines and chemokines. Furthermore, the ions and/or particles release as calcium release can affect the behavior of the platelets and macrophages [7]. In this study, the calcium assay showed that the β-TCP coatings can permit more calcium release and transform the microenvironment around the implant. It is known that the release of calcium ions plays an essential role in several steps of the bone repair process, from platelet activation to biomineralization and bone remodeling [39]. In the process of osseointegration of implants, calcium establishes electrostatic bridges between the surface of the implant and several proteins, modifying them and allowing a better integration of the bone tissue with the implant. In addition, after implantation, the platelets need to be activated to perform their functions properly [40]. In this context, calcium participates in the binding of platelet membrane phospholipids to Factor Xa and Factor IXa, which are necessary for the tenase and prothrombinase complexes, which convert prothrombin to thrombin (Factor IIa), polymerizing fibrin [39, 40]. Thus, calcium actively participates in platelet adherence and activation and exocytosis of their granules to perform its function. In addition, platelets secrete cytokines, generated from platelets in contact especially with calcium ions (Ca) in the surface can upregulate neutrophil activation and consequently stimulate osteogenic cell proliferation in vivo [7]. Kikuchi et al. [7] observed that the Ca and phosphate (PO4\(^-\)) in the coatings surface can increase the microtopographical complexity resulting in an increase in the platelets activation levels. The findings of this work demonstrated that there was no significant difference between Ti-HA and Ti-β-TCP groups on platelet adherence in the discs. It is known that platelets adherence and activation generally occur at the same time when contact with materials and they are closely combined. Additionally, platelet activation results in stimulation and behavior of various leukocytes, including macrophages [15]. Beside platelets, the other important immune cells as to macrophages migrate to the local of the implantation site and produce many chemokines, cytokines and growth factors to play an important tissue remodeling response after an injury or a host defense. In the present study, a significant decrease in the DNA content of macrophages when in contact with CaP coated discs was observed. Besides macrophage adhesion, macrophage polarization has been proved to play crucial roles in bone-implant interaction and further its osseointegration [41]. Therefore, we investigated effects of different surface chemical properties on macrophage polarization by assessing M1/M2 macrophage cytokine secretion, marker gene expression based on our previous work [42]. We demonstrated a significantly upregulated pro-inflammatory and anti-inflammatory cytokine secretion and marker gene expression of macrophages on HA and β-TCP coatings. This hybrid macrophage phenotype with simultaneously M1 and M2 markers was also reported in previous data [13]. However, its action of mode and functions in in vivo performance remains to be decoded. Furthermore, HA induced an earlier M1 macrophage polarization than β-TCP since M1 macrophage markers CCR7 and CXCL11 and CCR7 immunostaining showed
Chapter 5

higher expression than those on β-TCP after 1 and 4 days. During this time period, M2 macrophage polarization was also enhanced on HA coated discs. However, after 7 days, β-TCP showed more obvious M1 macrophage polarization than HA. These results can be attributed to the over-physiological calcium content in the medium. From the clinical review, HA coating is superior than β-TCP since it is more biological bioactive and macrophages around HA will be converted into M2 macrophages after 7 days, which is beneficial for the ending of inflammation and tissue remodeling.

Conclusion

This study demonstrated that the biomaterial surface property of HA and β-TCP coatings induce different responses to blood-born components that account for the initial biological cascade of events following implantation. Grit-blasted Ti, Ti-HA and Ti-β-TCP did not display significant difference for platelet adhesion. However, for macrophages, both types of coatings (Ti-HA and Ti-β-TCP) decreased more than twice of macrophage proliferation compared to grit-blasted Ti. Furthermore, Ti-β-TCP significantly upregulated pro-inflammatory cytokine TNF-α secretion while Ti-HA significantly upregulated anti-inflammatory cytokine TGF-β secretion after 7 days of macrophages culture on these surfaces. Immunostaining and gene expression of M1/M2 macrophages further revealed a hybrid macrophage phenotype with simultaneous M1 and M2 markers induced by Ti-HA and Ti-β-TCP compared to grit-blasted Ti. In addition, Ti-HA induced an earlier M1 macrophage polarization and earlier M1-M2 macrophage transformation compared to Ti-TCP. Further studies are required to verify the clinical significance of our findings and evaluate effects of biomaterial surface properties on multiple cell types in suitable in vitro coculture models and in vivo models with appropriate post-implantation time points.
Biomaterial property effects on platelets and macrophages: an in vitro study

References

Biomaterial property effects on platelets and macrophages: an in vitro study


Chapter 6

Physical and chemical characteristics of titanium surfaces modulate macrophage polarization

Yang Zhang, Xian Cheng, John A Jansen, Fang Yang, Jeroen JJP van den Beucken

Submitted
Chapter 6

Introduction

Different metals and metallic alloys have been utilized as dental and orthopedic implant materials, because of their acclaimed mechanical strength, stability, and biocompatibility. In particular, titanium implants with a surface roughness (e.g. as created by grit-blasting and acid etching) or a bioceramic coating are widely used to favor the bone response based on many years’ clinical experience [1]. In a systematic review, smooth and minimally rough surfaces (Ra < 0.5 µm) were demonstrated to show less bone formation and bone-implant contact than rougher surfaces while moderately rough surfaces (Ra 1 ~ 2 µm) showed more bone formation and bone-implant contact than excessively rough surfaces (Ra > 2 µm) [2]. Thereafter, implant surfaces with roughness value between 1 and 1.5 µm were indicated as the optimal surface to achieve superior bone formation [3, 4]. On the other hand, ceramic coatings, such as hydroxyapatite (HA) layer, also show enhanced and favored initial bone response and bone ingrowth compared to uncoated implants [5]. These findings suggest the importance of implant surface properties to orchestrate the bone healing process in the close vicinity of metallic implants. Due to the increase of patients with unfavorable bone healing conditions such as diabetes and osteoporosis, more knowledge about the healing mechanism is required to optimize implant surface properties for the safe and predictable installation of implants in such patients.

Given that osteoblasts play a major role in the osseointegration process, the majority of previous studies focused on the response of mesenchymal cell types to titanium implants. Inconsistencies between in vitro and in vivo data regarding effects of a physical or chemical surface on the osteogenic differentiation and bone healing capacity, however, are commonly found [6, 7], which emphasizes the need to unravel further the mechanisms that govern the capacity of material surface properties to mediate osseointegration.

When a bone implant is installed, a proteinaceous layer is immediately adsorbed on the implant surface and a blood clot is formed. Subsequently, inflammatory cells including peripheral monocytes that give rise to macrophages and multinucleated cells populate the blood clot and simultaneously elicit an inflammatory response. Then, this blood clot remodels, neovascularization occurs and at later times, induction and commitment of osteogenic precursors is achieved [8]. During this process, bone healing cannot occur or is significantly reduced without the presence of inflammation or with extravagant inflammation [9, 10]. As monocytes/macrophages are one of the main cell types associated with the inflammatory response around implants and they secrete various cytokines and growth factors relevant for the differentiation of mesenchymal cells (MSCs), it can be hypothesized that monocytes/macrophages play an important role in the final outcome of the bone healing process. Consequently, some researchers have proposed the novel concept of “osteoimmunomodulation”, which refers to the modulation of bone immune response by implant materials [11]. This concept implies that macrophages exert either inhibitory or stimulatory effects on osteogenesis due to the heterogeneity and plasticity of macrophages, which are governed by implant surface properties.
Indeed, recent studies pinpointed the versatility of the macrophage phenotype, classically activated M1 types and alternatively activated M2 types [12]. M1 macrophages predominantly secrete pro-inflammatory cytokines (e.g. TNF-α, interleukin-1β (IL-1β) and IL-6) involved in chronic inflammation as well as in recruitment of polymorphonuclear neutrophils. In contrast, M2 macrophages predominantly secrete anti-inflammatory mediators (e.g. TGF-β, IL-10, and IL-1ra) to promote the resolution of inflammation and benefit tissue regeneration [12]. M2 macrophages also support the migration, growth, and osteogenic differentiation of MSCs [13]. Recent work has shown that prolonged M1 polarization can lead to the perpetuation of chronic inflammation, which results in eventual osteolytic loosening of implants [14]. In contrast, an effective and timely switch toward M2 macrophage phenotype can result in an osteogenesis-enhancing and tissue remodeling cytokine secretion profile and subsequent osseointegration [15, 16]. Thus, engineering a microenvironment around implanted devices that would convert macrophages to a more M2 phenotype seems a promising approach to attenuate adverse immune reactions and improve the bone formation around enossal implants [17, 18].

The effect of material roughness and CaP coatings on macrophage activation and cytokine secretion has been studied in several previous studies [19-22]. The majority of these investigations, however, considered macrophages as conventional inflammatory cells and consequently to avoid the macrophage-material interaction. The newly identified pro-healing characteristic of macrophages is lack of consideration. Additionally, murine macrophages rather than human macrophages were commonly used in these reported data. Although macrophages from mouse and human share some common characteristics, an apparent difference in their proliferation, spreading and cytokine secretion has been demonstrated, especially when assessing effects of surface physical characteristics on cell behaviors [23, 24]. Furthermore, amongst these studies, effects of smooth surface and (a certain of) rough surface on macrophage secretion of inflammatory cytokines were compared, while comparison of different degrees of roughness were rarely reported and the combined effect of roughness and HA coating was not depicted. Therefore, the current study aimed to assess the effect of titanium surfaces provided with a series of roughness and with/without HA coating on adherent human monocyte/macrophage behaviors. Specifically, macrophage polarization into M1/M2 phenotype was examined by quantification of secreted pro- and anti-inflammatory cytokines, cytokine gene expression, and immunostaining. M1/ M2 macrophages conventionally induced with specific cytokines on glass slides were set as controls. We hypothesized that both physical (roughness) and chemical (HA coating) characteristics would influence human macrophage adhesion and polarization on titanium surface.

Materials and methods

1. Preparation of titanium surfaces
Commercially pure titanium disks (Purity > 99.9 wt%; diameter 12 mm; thickness 1.5 mm; Ti) were purchased from Machinefabriek G Janssen, the Netherlands. Disks were
sandblasted with 50 µm, 110 µm and 250 µm Al₂O₃ particles for 30 seconds from a distance of approximately 10 mm to obtain titanium surfaces with low roughness (Ti-LR), medium roughness (Ti-MR) and high roughness (Ti-HR), respectively. After that, all disks were subjected to ultrasonic cleaning in four successive baths of 10% nitric acid solution, acetone, ethanol, deionized distilled water for 10 min in each bath. Ti-MR disks were then provided with a 0.3 µm thick hydroxyapatite (HA) \( \text{Ca}_{10}\text{(PO}_4\text{)}_{6}\text{(OH)}_2 \); TiHA-MR) coating using radiofrequency (RF) magnetron sputtering and subjected to heat treatment (600 °C) as previously reported [25]. Glass slides (diameter 13 mm) were commercially obtained from VWR, Germany and were used as control substrates. All samples were then autoclaved, dried at 37°C and ready for characterization and cell culture.

2. Surface characterization of titanium surfaces
The different surfaces were characterized for morphology, surface roughness, wettability, and crystal structure. The morphology of these surfaces was characterized with scanning electron microscopy (SEM; Zeiss, Germany). The crystalline phase of the deposited HA coating was analyzed by X-ray diffraction (XRD; Phillips PW3710, the Netherlands) as described previously [25]. The surface profile and roughness of the titanium disks were measured using a Universal Surface Tester (UST) (Innowep GmbH, Germany) and a surface topography scanner (Proscan 2100, Scantron, Germany) under the ISO standards settings for scanning (ISO 4288-1996). The 2D Roughness Average (Ra), the arithmetic average of the 3D roughness (Sa), the average space between the irregularities (Scx) parameter were used as a previously reported method to numerically characterize the surface roughness [26]. All samples were measured for water contact angles using Surface Tension (Biolin scientific, Finland) equipped with a digital camera and image analysis software.

3. Culture of human THP-1 monocytic cells
Human THP-1 cells (ATCC, USA) were cultured in suspension in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, USA) and incubated at 37°C, 5% CO₂ in a 95% humidified atmosphere. The cell density was kept at 0.2 ~ 1.2 × 10⁶ cells/ml.

4. Cell seeding
All disks were put in 24-well non-adherent plates (Greiner-one, Germany) before cell seeding. 5 × 10⁵ monocytes suspended in 1 ml medium were added to the different surfaces in each well with phorbol myristate acetate (PMA; Sigma-Aldrich, USA) at a concentration of 50 ng/ml. M0, M1 and M2 phenotype controls were prepared by unstimulating macrophages on glass for M0, stimulation with 240 ng/ml LPS (Sigma-Aldrich, USA) and 20 ng/ml INF-γ (Sigma-Aldrich, USA) for M1, or stimulation with 20 ng/ml IL-4 (Sigma-Aldrich, USA) and 20 ng/ml IL-13 (Sigma-Aldrich, USA) for M2 as previously developed [13]. The plates were incubated for different time periods until analyses.
5. Cell adhesion
Macrophages were cultured for 2 and 4 days. The medium was changed every 2 days. The supernatant was stored for cytokine quantification. Cell monolayers were then washed twice with PBS, immersed in 1 ml MilliQ and went through two freeze and thaw cycles. DNA content was quantified in cell lysates using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, the Netherlands) per the manufacturer’s protocol.

6. Cytokine secretion
The supernatant from all wells was harvested after 2 and 4 days of incubation. The media were aspirated and transferred to Eppendorf tubes and centrifuged at 400 g for 5 min. TNF-α, IL1-β, TGF-β (eBioscience, USA) and CXCL18 (R&D Systems, USA) ELISA analyses were performed using commercially available kits with a multi-mode spectrophotometer (Biotek, USA).

7. RT-PCR for gene expression analysis
After 2 days of culture, wells were washed twice with PBS and total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA quality and quantity were assessed using a Nanodrop ND-1000 Spectrometer (Thermo Scientific). A total of 800 ng RNA was transcribed to cDNA using TaqMan Reverse Transcription kit (Applied Biosystems). RT-PCR reactions of INDO, CXCL11, MCR-1, CCL13 were performed using Fast SYBR Green Master Mix Kit (Applied Biosystems, CA, USA) by Applied Biosystems PRISM 7500 sequence amplification system with the primers listed in Table 1. Each PCR reaction consisted of 1 µl of the cDNA as template, 2 µl (10 pmol) of forward and reverse primers, 12.5 µl Mastermix, and 9.5 µl RNAnase-free water. Thermal cycle conditions consisted of denaturation at 95 ºC for 40 s, annealing at 60 ºC for 1 min. After 40 cycles, the reaction was completed with a final extension step at 72 ºC for 5 min. The expression of target genes was normalized to the relative abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold induction was calculated using the 2^{-ΔΔCt} method.

Table 1. Primer sequence used for RT-PCR in the present study.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>CTCTGCTCTCTCTGTTGACA</td>
<td>ACGACCAAATCCGTGACTC</td>
</tr>
<tr>
<td>INDO</td>
<td>CTTAGGAGCTACCATCTGC</td>
<td>TCAAGTCCTCCAGTTCTTC</td>
</tr>
<tr>
<td>CXCL11</td>
<td>AGTCTCTGGAAAGAGCAGTCT</td>
<td>TCAACCTCTTTTCTC</td>
</tr>
<tr>
<td>MCR-1</td>
<td>GGTATATGAGCCAGGTTGAA</td>
<td>AAACCTGAACGGGAATGCAC</td>
</tr>
<tr>
<td>CCL13</td>
<td>ATCTTCTGGAGAGGCTGAA</td>
<td>ACTTCTCTTTTGGTACGCA</td>
</tr>
</tbody>
</table>

8. Immunostaining for macrophage phenotype markers
After 3 days, all samples were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100 diluted in PBS for 5 min and then incubated with 1% BSA in PBS for 20 min. After that, cells were incubated with rabbit anti-human CCR7 (1:2000; abcam, No. ab32527) and
mouse anti-human CD36 (1:200; Biolegend, No.336202) primary antibodies in 1% BSA in PBS at 4 °C overnight. After washing twice with PBS, Alexa Fluor-546 donkey anti-rabbit IgG, Alexa Fluor-488 goat anti-mouse IgG (all Life Technologies, USA) secondary antibodies diluted in 1% BSA in PBS were added and incubated with cells at room temperature for 2 h. The wells were washed twice with PBS and nuclei was then stained with 5 µg/ml 4, 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Samples were then mounted with the mounting medium and fluorescent images were acquired using an inverted fluorescence microscope (Zeiss AxioCam MRC5, Germany). Each fluorescence was kept on the same exposure time for all samples and images were further analyzed using ImageJ software (NIH, USA). The values of red (Alexa-568) and green (Alexa-488) fluorescence of each sample were quantified and further normalized to the value of blue fluorescence (DAPI) to measure the relative fluorescence intensity.

9. Statistical analysis
Experiments were performed twice with consistent results. Presented data are from one experiment. All data were expressed as mean values ± standard deviation. Statistical analysis was performed using Prism Software (GraphPad, USA) via one-way-ANOVA followed by a Tukey’s Multiple Comparison Test. A Student’s t-test was used for comparing TiMR and TiHA-MR. Results were considered significant at p < 0.05.

Results

1. Surface characterization
The surface morphology of as-received smooth, sandblasted rough, and HA-coated titanium disks was characterized by SEM, together with the glass substrate (Figure 1A). Glass slides exhibited a smooth surface. In contrast, as-received Ti discs showed a typical concentric pattern due to use of a milling machine during their production, while sandblasted titanium surfaces showed crack structures and blasting scars. XRD patterns of the HA coating displayed a peak at 2θ = 26° and 32°, indicating the presence of HA on top of TiHA-MR surface (Figure 1B). When the 2D surface profile is tested by UST, glass surfaces showed a smooth line and as-received Ti discs showed a slightly fluctuant line. The fluctuating extent of this line gradually increased for Ti-LR, Ti-MR, and Ti-HR while TiMR-HA displayed a similar extent as TiMR (Figure 1C). With further characterization of the surfaces with a 3D surface scanner, glass displayed a smooth surface and as-received titanium surfaces. In contrast, the rest surfaces displayed varying degrees of ragged surfaces and irregular space between peaks (Figure 1D).

The quantitative roughness measurements showed glass slides a smooth surface (Ra = 0.02 µm; Sa = 0.02 µm), while titanium disks showed a gradually increasing surface roughness ranging from Ra = 0.20 µm; Sa = 0.33 µm for Ti to Ra = 2.60 µm; Sa = 4.12 µm for Ti-HR (Figure 2A). Ti-MR and
Physical and chemical characteristics of titanium surfaces modulate macrophage polarization

Figure 1. Preparation of glass, titanium surfaces with different roughness with or without HA coating. The morphology of glass control and different titanium surfaces prepared in this study were observed with SEM (A; the scale bar is 20 \( \mu \)m). XRD patterns of the HA coatings displayed the typical peak of HA at \( 2\theta = 26^\circ \) and \( 32^\circ \), which are indicated by arrowheads (B). The surface profile was further assessed with UST (C) and Surface Scanner (D).

117
TiHA-MR displayed no significant difference in 2D surface roughness (Ra = 1.36 µm and 1.41 µm respectively; Figure 2B). However, the 3D surface roughness of TiHA-MR (Sa = 2.59 µm) is lower than Ti-MR (Sa = 2.91 µm). Water contact angle (WCA) data for the different surface are shown in Figure 2B. Glass showed WCA at 55° while all other titanium surfaces showed WCA ranging from 70° to 80°, indicating moderate hydrophilicity of these surfaces. Differences in titanium surface roughness and with/without HA coating did not significantly alter the surface hydrophilicity after the autoclave sterilization.

2. Macrophage adhesion
The adhesion of PMA activated macrophages to the different sandblasted surfaces was significantly lower compared to smooth Ti after 2 days of culture. In contrast, macrophage adhesion to amongst these rough surfaces did not display significantly difference. In contrast, TiHA-MR significantly increased macrophage adhesion compared to Ti-MR (Figure 3A). After 4 days, total cell adhesion values revealed a reduction compared to day 2 and were similar for all experimental surfaces, except for TiHA-MR which displayed significantly higher macrophage adhesion compared to Ti-MR (Figure 3B).

3. Pro- and anti-inflammatory cytokine secretion
To assess the effect of roughness and HA coating on macrophage polarization, we investigated the secretion of pro- and anti-inflammatory mediators (Figure 4). Macrophage responses elicited by all titanium surfaces appeared to be predominantly anti-inflammatory because the cytokine secretion profile was relatively more similar to that of M2 macrophages on glass controls, showing low amounts of typical inflammatory mediators (TNF-α and IL-1β) and high amounts of anti-inflammatory cytokines (TGF-β and CCL18). Specifically, after 2 days, TNF-α secreted by macrophages cultured on the Ti-LR and Ti-MR surface decreased significantly compared to Ti surfaces and Ti-HR. Macrophages cultured on HA coated surfaces, in contrast, significantly increased TNF-α secretion compared to Ti-MR surfaces (Figure 4A). Additionally, macrophages on all rough titanium surfaces showed significantly lower levels of IL-1β compared to those cultured on Ti. In contrast, IL-1β secreted by the macrophages cultured on HA coated surfaces increased significantly compared with Ti-MR surfaces (Figure 4B). In view of anti-inflammatory cytokines, CCL18 and TGF-β secretion were greatly increased on all rough surface compared to Ti, in particular on Ti-MR (Figure 4C & D). Increased secretion of CCL18 was found on TiHA-MR compared to Ti-MR, but not of TGF-β. A similar trend was observed after 4 days of culture, when the level of TNF-α secretion was significantly lower on Ti-LR and Ti-MR compared to Ti and Ti-HR. The presence of the HA coating significantly increased TNF-α secretion on Ti-MR compared to day 2 (Figure 4E). Further, secretion of the inflammatory cytokine IL-1β significantly decreased at day 4 compared to that at day 2. Only on TiHA-MR a significantly increased IL-1β secretion was observed compared to TiMR (Figure 4F). In view of anti-inflammatory cytokines, significantly increased levels of CCL18 secretion were detected on all rough surfaces.
Physical and chemical characteristics of titanium surfaces modulate macrophage polarization

Chapter 6

compared to the smooth Ti surface. The presence of an HA coating also significantly promoted CCL18 secretion compared to Ti-MR (Figure 4G). Regarding TGF-β secretion at day 4, its level was significantly higher on Ti-LR and Ti-MR surfaces than on Ti and Ti-HR. Further, TiHA-MR induced significantly higher TGF-β secretion compared to Ti-MR (Figure 4H).

4. Pro- and anti-inflammatory cytokine gene expression

To further investigate macrophage polarizing nature on surfaces with different roughness and HA coating, we performed gene expression analysis of selected marker genes, consisting of M1 macrophage markers (INO, CXCL11) and M2 macrophage markers (MCR-1 and CCL13). All titanium surfaces showed extremely low pro-inflammatory markers gene expression compared to M1 macrophages and similar anti-inflammatory markers gene expression as M2 macrophages (Figure 5). Rough surfaces, especially Ti-LR and Ti-MR, significantly downregulated M1 macrophage surface markers INO and CXCL11 compared to Ti and Ti-HR (Figure 5A & B). In contrast, the presence of
an HA coating significantly increased the expression of both these markers compared to Ti-MR (Figure 5A & B). Additionally, the relative mRNA expression levels of MCR-1 and CCL13 were significantly increased for macrophages cultured on Ti-LR and Ti-MR compared to Ti and Ti-HR (Figure 5C & D). The presence of an HA coating induced a macrophage polarization tendency toward the M2 phenotype, but without a significant difference compared to Ti-MR (Figure 5C & D).

Figure 4. Quantification of pro-inflammatory cytokines TNF-α (A & E; n=4) and IL-1β (B & F; n=4) and anti-inflammatory cytokines CCL18 (C & G; n=4) and TGF-β (D & H; n=4) in supernatant harvested from THP-1 derived macrophages cultured on glass and different titanium surfaces for 2 days and 4 days respectively. A significant difference between Ti, TiLR, TiMR and TiHR was indicated by a, b & c (p < 0.05). Groups with different letters are significantly different and groups sharing the same letter are not significantly different. A significant difference between TiMR and TiHA-MR was indicated by * (p < 0.01), ** (p < 0.05), *** (p < 0.001) and ns (no significant difference).
5. M1 and M2 macrophage immunostaining
To further determine macrophage phenotype upon culture on the surfaces with
different roughness and HA coating, macrophages were stained with M1 macrophage
surface marker CCR7 and M2 macrophage surface marker CD36, respectively. Control
macrophage phenotypes M0, M1 and M2 all showed some expression of CCR7 and
CD36 (Figure 6A), which correlates with previous reports about the unexclusiveness of
markers for M1 and M2 human macrophages [13]. However, M1 macrophages showed
relatively higher CCR7 expression, while M2 macrophages showed relatively higher CD36
expression (Figure 6B). Upon culture on surfaces with different roughness, relatively
higher CCR7 expression was observed on Ti and TiHA-MR surfaces, followed by Ti-MR,
Ti-HR. The least CCR7 positive signal was found on the Ti-LR surface. In contrast, CD36
positive signal intensity appeared considerably more on Ti-LR, Ti-MR than on Ti and
TiHR. In addition, TiHA-MR surfaces displayed significantly higher intensity than Ti-MR
(Figure 6C).

![Image of bar charts showing mRNA expression levels for INDO, CXC11, MCR-1, and CCL13](figure5.png)

Figure 5. Quantitative RT-PCR analysis of the levels of M1/M2 macrophage marker genes in THP-1
derived macrophages on the glass and different titanium surfaces after 2 days of culture. INDO and
CXC11 mRNA expression levels represent for surface marker genes of the M1 phenotype (A & B; n =
4). MCR-1 and CCL13 mRNA expression levels represent for surface marker genes of the M2 phenotype
(C & D; n = 4). A significant difference between Ti, TiLR, TiMR and TiHR was indicated by a, b, c & d (p <
0.05). Groups with different letters are significantly different and groups sharing the same letter are not
significantly different. A significant difference between TiMR and TiHA-MR was indicated by *** (p < 0.001)
and ns (no significant difference).
Figure 6. THP-1 cells were activated and cultured on glass and different titanium surfaces for 3 days, then (A) stained with CCR7 (M1 phenotype; red), CD36 (M1 phenotype; green), and nucleus (DAPI, blue). The scale bar is 50 µm. The relative fluorescence intensity was quantified by ImageJ and analyzed by Graphpad prism (B & C; n =3). A significant difference between Ti, TiLR, TiMR and TiHR was indicated by a, b & c (p < 0.05). Groups with different letters are significantly different and groups sharing the same letter are not significantly different. A significant difference between TiMR and TiHA-MR was indicated by * (p < 0.01) and ** (p < 0.05).

Discussion

The aim of this study was to investigate the effect of physical and chemical surface properties of titanium implants on the function of human macrophages. More specifically, this investigation focused on the effect of surface roughness and the presence of a HA coating on macrophage adhesion and polarization into different phenotypes in the absence of any cytokines, using the human macrophage cell line THP-1 as a model. The results demonstrated that roughness did not remarkably affect macrophage adhesion while the presence of an HA coating significantly increased macrophage adhesion. More notably, low and medium roughness (i.e. Ra=0.51~1.36 µm; Sa=0.66~2.91 µm) had more potency to polarize macrophages into the M2 phenotype, while the presence of an HA coating increased both M1 and M2 macrophage characteristics. These findings prove that macrophage polarization in response to implants are influenced by surface physical and chemical properties.

Upon in vivo implantation of a material, macrophage migration and adhesion occur after the fibrin clot is formed. The physical and chemical properties of the material surface have a direct effect on this event. Previous studies have shown that macrophage adhesion to titanium surface was not dependent on surface roughness rather on the
Physical and chemical characteristics of titanium surfaces modulate macrophage polarization

chemical properties [20, 27, 28], which correlates with our findings that cell adhesion did not display significant difference to different rough surfaces while coated HA significantly increased the macrophage adhesion. A slight discrepancy observed in this study is an increased macrophage adhesion on smooth Ti surface compared to rough surfaces after 2 days, which is likely due to its slightly higher hydrophilicity.

After adhesion to an implant, macrophage polarization in response to microenvironmental cues is the main driver orchestrating further physiological processes and ultimately bone healing [17, 18]. Although this polarization into M1 or M2 phenotype has been widely demonstrated in cell biology [12] and is proposed to be of great importance in determining the material suitability and bone healing capacity for metallic implants [15, 29], effects of material properties on macrophage polarization are not well characterized for human macrophages due to the lack of exclusive markers [13]. Thus, using multiple cellular characteristics and cytokine secretion profiles are recommended to characterize a macrophage population. Thereafter, cytokine secretion, certain gene expression, and surface markers were adopted in the present study to define the macrophage phenotype on different surfaces. Low and medium rough titanium surfaces (Ra=0.51~1.36 µm; Sa=0.66~2.91 µm) downregulated the secretion and gene expression of pro-inflammatory cytokines and upregulated the secretion and gene expression of anti-inflammatory markers compared to as-received smooth surfaces (Ra=0.20 µm; Sa=0.33 µm), indicating more M2 phenotype induction upon macrophage attachment to these surfaces. We further applied the internal comparison of effects of different rough surfaces on macrophage behaviors, which was rarely reported before. High rough surface (Ra=2.60 µm; Sa=4.11 µm) displayed significantly upregulated M1-related cytokine secretion including TNF-α, INDO, and CXCL11 and downregulated M2-related cytokine secretion including TGF-β, CCL18, MCR-1, and CCL13. To sum up, we here revealed the optimal roughness of titanium surface with a Ra value from 0.51~1.36 µm for M2 macrophage polarization after comparing a series of titanium surfaces. Indeed, previous observations from an animal study also showed that this range of roughness (1~1.5 µm) leads to superior osseointegration compared to a smooth surface and higher roughness [3, 4, 30]. However, whether this improvement of osseointegration is directly associated with macrophage phenotype switch in vivo needs more pre-clinical and clinical evidence. In addition, TiHA-MR enhanced both the secretion of pro- and anti-inflammatory cytokines compared to the Ti-MR surface, indicating the complex role of HA with medium roughness on macrophage polarization. Above all, these findings demonstrate that the macrophage polarization is strongly dependent on the combination of physical (roughness) and chemical (Ti or HA) surface properties.

Here, titanium surfaces influence the polarization of macrophages to a different extent. However, none of material-induced macrophages fall distinctly into the M1 or M2 phenotypes as cytokines induced. HA coating even induced a macrophage phenotype with both M1 and M2 characteristics. This agrees with recent claims that material-activated macrophage phenotypes are not identical with conventionally cytokine-activated states [31-33]. It articulates the complexity of macrophages and indicates the
conventional classification into M1 or M2 probably outdated. The phenomenon that a
decrease of M1 characteristics does not correlate with an increase of M2 characteristics
and vice versa indicates the distinct signaling pathways of M1 and M2 macrophage
activation.
The underlying mechanism of surface roughness regulating the polarization of
macrophages is incompletely understood. Based on studies about roughness with
other cell types, it is hypothesized to be related to two aspects. The first one is that
roughness affects protein adsorption, such as complement components, fibrinogen,
fibronectin and vitronectin and their kinetics, binding strengths, activities, and
structures. Upon contact, adsorbed proteins may change these properties to be
recognized by macrophages and further alter macrophage phenotypes [34, 35]. The
second aspect is regulation of macrophage polarization by the molecules involved in
focal contact or focal adhesion formation, which in turn is determined by the nature
of the implant roughness [19, 36]. HA coating influencing macrophage polarization
is more complicated. It can be attributed to specific HA-absorbing proteins or ions
released from the HA coatings during macrophage culture or synergetic effects of
protein adsorption, ions, and medium roughness. Several studies have indicated
effects of HA on protein adsorption, macrophage cytoskeleton structure and activation
[37, 38]. Furthermore, Ca$^{2+}$ as one of the major components released from HA is well
documented to be involved in certain inflammatory signaling pathways [39, 40]. These
different signaling pathways probably synergistically resulted in the specific material-
activated phenotype with both M1 and M2 macrophage characteristics. This specific
phenotype was surprisingly observed reproducibly on other rough implant surfaces
coated with divalent cations [41]. Additional work is needed to confirm this phenotypic
change at a functional level.

**Conclusion**

We here demonstrate that titanium surface modifications via roughness or HA-coating
affect the polarization of human macrophages into different phenotypes. Rough(er)
surfaces displayed decreased initial macrophage adhesion and low and medium rough
surfaces (Ra=0.51~1.36 µm; Sa=0.66~2.91 µm) seems to show more tendency to
polarize macrophages into M2 phenotype than smooth and high rough surfaces did.
The presence of an HA-coating elicited a non-typical, hybrid macrophage phenotype
with both increased pro- and anti-inflammatory cytokine profiles. These results confirm
that surface roughness and chemistry differentially affect macrophage polarization and
force macrophages into a specific material-activated state. Knowledge on this surface
physical and chemical characteristics-induced M1/M2 paradigm might facilitate
the design of biomaterial surfaces capable of generating desired inflammatory and
immunological responses. In view of the potential impact of this phenomenon on
the bone response to endossal implants, more research is necessary to unravel the
underlying biological mechanisms, which remain elusive.
Physical and chemical characteristics of titanium surfaces modulate macrophage polarization

Reference


Chapter 6


Physical and chemical characteristics of titanium surfaces modulate macrophage polarization
Chapter 7

Osteoclast-based constructs induce ectopic bone formation

Yang Zhang, Jinling Ma, Femke van den Tillaart, Jeroen JJP van den Beucken

In Preparation
Chapter 7

Introduction

Bone is a dynamic tissue that undergoes continuous remodeling and can regenerate upon tissue damage. This continuous remodeling occurs through a dynamic process of breakdown by osteoclasts and rebuilding by osteoblasts. Under normal circumstances, more than 97% resorbed bone is precisely replaced in location and amount by new bone [1]. This tightly balanced process is referred to as “coupling of bone formation and resorption”. Uncoupling occurs when the balance between resorption and formation is disrupted, which often leads to pathological situations, such as osteoporosis or osteopetrosis. However, uncoupling also occurs under physiological conditions, i.e. during skeletal growth, when bone formation exceeds bone resorption. In clinical practice, there are often situations, in which major injuries, diseases or birth defects cause serious bone damage or large bone defects, which also need the remodeling cycle uncoupled in favor of bone formation. Thus far, the majority of studies tried to induce bone formation by grafting bone forming cells, such as MSCs and osteoblasts [2]. The outcome of these attempts, however, are not consistent, reliable, and satisfactory. Also, the mechanism of exogenous MSCs and osteoblasts contributing to bone regeneration is controversial to date [3-5]. Furthermore, cost and safety issues behind these approaches have been postulated [2]. More advanced strategies to induce new bone formation are urgently needed, which are expected to be premised on the basic knowledge of the initiation of bone formation.

According to the theory on coupling bone formation and resorption, in bone remodeling, osteoclasts start to resorb a package of bone due to microtrauma or other local inductive phenomena [6]. The resorbed bone matrix releases osteogenic factors, such as insulin-like growth factor (IGF) and transforming growth factor (TGF-β), which simultaneously induce osteogenic differentiation of surrounding mesenchymal stem cells [6]. More recently, it has become clear that osteoclasts, irrespective of their bone resorption activity, are able to secrete bone anabolic signals (clastokines) that affect the behavior of osteoblasts in vitro [7, 8] and in vivo [9, 10]. To explain these observations, several clastokines of interest, including sphingosine 1-phosphate (S1P), bone morphogenetic protein 6 (BMP-6), Wnt10b, platelet-derived growth factor-BB (PDGF-BB) and collagen triple helix repeat containing 1 (CTHRC1) have been identified as regulators of bone formation through complex activities on the osteoblast lineages [6]. Amongst these, S1P is well-documented to promote the recruitment, survival, and osteogenic differentiation of osteoblast precursors at the site of damaged bone [11, 12]. Furthermore, another cell-cell interaction between osteoclasts and osteoclast was discovered through the EphrinB2-Eph4 signaling pathway [6].

The finding of clastokines changed the conventional understanding of osteoclasts and promoted more researchers to investigate the role of osteoclasts in bone formation. Colnot et al. recently investigated the time-course of natural bone healing to detect reparative osteogenesis. They found osteoclasts appeared as early as day 5 compared to bone matrix on day 7. Osteoclast deficiency gives rise to a reduced osteoblast population and activity and defective mineralization [13]. When a bone substitute
Osteoclast-based constructs induce ectopic bone formation

β-TCP was used to heal rat and rabbit bone defects, the appearance of osteoclasts still occurred earlier than that of new bone formation [14, 15]. The attachment and activity of osteoclasts functioned not only to resorb substitute material but also as an essential preparatory step to be replaced by new bone. Furthermore, local application of alendronate reduced the number of osteoclasts on the surface of β-TCP and subsequent new bone formation [16]. In MSC-based bone regeneration, a similar finding was observed: TRAP and cathepsin K-positive positive-multinucleated cells appeared after day 7 and day 14 and directly attached to MSC-based scaffolds. Scaffolds were resorbed by osteoclasts and this event promoted ectopic bone formation [4, 17]. Interestingly, for osteoinductive materials, which have the capacity to induce ectopic bone formation without exogenous growth factor loading or cell grafting, the appearance of a large number of active osteoclasts also precedes new bone formation. This phenomenon was firstly reported for β-TCP by Kondo et al. [18] and thereafter confirmed by several research groups [19, 20]. The osteoinductive capacity was further attributed to the submicron-scale surface architecture of the implanted materials and correlated to the material capacity to induce osteoclastogenesis [21, 22]. Complete depletion of osteoclasts but not macrophages showed to block ectopic bone formation [23, 24], while stimulating osteoclastogenesis with EP4 agonist accelerated osteoinduction [19]. All these observations suggest that the osteoclastogenesis plays a key role in the subsequent cellular events to initiate ectopic bone formation.

In view of suggested anabolic effects of osteoclasts in vitro and a pivotal role in bone formation in vivo, we speculated that osteoclasts might have the capacity to initiate bone formation. To test this, we prepared osteoclast-based constructs using RAW264.7 and primary mouse macrophages on commercially available bone substitute material and subcutaneously implanted these constructs into mice; BMP-2 loaded and acellular bone substitute material served as positive (+Control) and negative controls (-Control), respectively. Ectopic bone induction and osteoclast formation were histologically and histomorphometrically evaluated.

Materials and Methods

1. Cell isolation and culture

RAW264.7 cells were obtained from China Infrastructure of Cell Line Resources (Beijing, China) and cultured in α-MEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (RAW proliferation medium).

Primary mouse bone marrow derived-macrophages were isolated as previously developed with minor modifications [25]. Briefly, 4-6 week old BALB/c nude mice were obtained from Institute of Animal Science of Vital River Co., Ltd., Beijing, China after the approval from the local ethical committee (Capital Medical University C4417). After euthanasia, the mice were sprayed with 70% ethanol and the femurs were dissected using scissors. Muscles connected to the femur bone were removed using plastic gauze with Iodine, and then femurs were placed into a polypropylene tube containing RPMI 1640 medium with 1 ug/ml Fungizone. The bones were sterilized with 70% ethanol in
cotton ball in the cell culture hood and washed twice with medium. The bones were then cut with scissors and flushed with a syringe filled with RPMI 1640 to extrude bone marrow into a 15 mL sterile polypropylene tube. Bone marrow was gently homogenized with a pipette and then filtered through a cell strainer. Bone marrow cells were counted using a cytometer, centrifuged at 200×g for 5 min and adjusted to the concentration of 4 × 10⁶/ml with RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (primary proliferation medium). These cells were then transferred to a 100 × 20 mm petri dish (Corning, China) and incubated at 37°C in a 5% CO₂ atmosphere. After overnight incubation, non-adherent monocytes were collected and suspended in above primary proliferation medium.

2. Osteoclast-based construct preparation
Four experimental groups were used throughout this work:
- control (acellular constructs)
- +control (BMP-2 loaded constructs)
- OC-R (constructs containing osteoclasts derived from RAW264.7 cell line)
- OC-P (constructs containing osteoclasts derived from primary mouse monocytes)

For OCs-R, 3 × 10⁵ RAW264.7 cells were suspended in 2 ml proliferation medium and dropped on 0.5 g Bio-Oss granules, which were kept in a 50 ml tube and were pre-treated with FBS for 24 hours. The tube was then transferred to a rotation system (hl-2000 hybrilinker hybridization oven, UVP) at 10 RPM at 37°C for 2 h. After cell attachment, 13 ml proliferation medium was added to the tube and tilted the tube in the incubator to make largest interface of medium and air. The lid of the tube was kept loosen to allow for air and oxygen exchange. After one day, 15 ml medium supplemented with 50 ng/ml RANKL was used to replace the medium. Part of cell-containing granules (around 10 mm³) were placed in the 24-well plates. When loading RAW264.7 cells onto Bio-Oss granules, 2 × 10³ RAW264.7 cells/cm² without granules were directly seeded in 24-well tissue culture plates (2D) and cultured in the presence/absence of RANKL as an osteoclastic differentiation control to 3D osteoclast culture onto Bio-Oss granules. The constructs and TCP were culture for 5 days until implantation. Medium with cytokines was refreshed every 2 days.

For OCs-P, 6 × 10⁶ primary mouse monocytes were suspended in 2 ml proliferation medium supplemented with 30 ng/ml M-CSF and dropped onto 0.5 g Bio-Oss granules (around 1.5 cm³), which were kept in a 50 ml tube and were pre-treated with FBS for 24 h to increase cell attachment. Next, the tube was transferred to a rotation system (hl-2000 hybrilinker hybridization oven, UVP) at 10 RPM at 37°C for 2 hours. Thereafter, 13 ml proliferation medium supplemented with 30 ng/ml M-CSF were added to the tube and tilted the tube in the incubator and loosened the lip as above. After 2 days, the medium was changed to proliferation medium supplemented with 30 ng/ml M-CSF and 50 ng/ml RANKL. Part of cell-containing granules (around 10 mm³) were placed in the 24-well tissue culture plates (TCP). When loading monocytes onto Bio-Oss granules, 2 ×
10^4 monocytes were seeded in 24-well plates and cultured in the presence of 30 ng/ml M-CSF and +/- 50 ng/ml RANKL as an osteoclastic differentiation control as above. The constructs and TCP were cultured for 6 days until implantation. Medium was changed and cytokines were refreshed every 2 days. Control constructs were prepared via incubation of 0.5 g Bio-Oss granules in proliferation medium supplemented with M-CSF and RANKL (incubation for 6 days; -Control) or loading 0.5 g Bio-Oss granules with BMP-2 via an incubation procedure (2 ml of BMP-2 solution containing 200 µg BMP-2; incubation for 24 hours; +Control).

3. Osteoclastogenesis quantification in vitro
DNA content and TRAP activity were assessed for evaluation of cell proliferation and osteoclastogenesis, respectively. Cell layers or cell-seeded granules were rinsed with PBS three times before adding 1 mL of MilliQ and two freeze-thaw cycles (-80°C and room temperature). DNA content was measured using Quant-iTTM Picogreen Kit (Invitrogen; Breda, the Netherlands) and TRAP-activity was determined using a TRAP assay kit (Sigma-Aldrich, the Netherlands) as per the recommendation of the manufacturer. TRAP activity was normalized for DNA content and expressed as mM pNP/ng DNA.

4. Animal studies
11 BALB/c nude mice obtained from Institute of Animal Science of Vital River Co., Ltd. were used for implantation surgery under the approval form ethical committee (Capital Medical University #15JL72). Mice were premeditated by subcutaneous injection of zoletil (10 mg/kg; Virbac, France) to reduce operative pain. Mice were immobilized and placed in a dorsal position. Two longitudinal incisions were made on each side of the vertebral column and four subcutaneous pockets were created using blunt dissection. Each animal received one sample of each experimental construct (~40 mg granules) inserted into the pockets and then the skin was closed using resorbable sutures. Six weeks after implantation, animals were euthanized using CO2 suffocation. The specimens with the surrounding tissue were retrieved and fixed in 10% neutral formalin for 24 h and transferred into 70% ethanol.

5. Histology analysis
Five samples from each group were dehydrated and embedded in poly(methylmethacrylate) (PMMA) before cross-sectioning with a diamond saw (10 µm thick sections). All sections were stained with methylene blue and basic fushin (MB-BF) as described previously [26]. Six samples from each group were decalcified in 10% EDTA, dehydrated, and embedded in paraffin before cross-sectioning with a microtome (5 µm thick sections). All sections were then stained with hematoxylin and eosin (HE) and tartrate-resistant acid phosphatase (TRAP).

6. Ectopic bone quantification
All MB-BF sections were imaged with the automated Axio Imager Z1 microscope equipped with the AxioCam MRC5 camera and AxioVision 4.8 software (Carl Zeiss)
Micro Imaging GmbH, Göttingen, Germany). The paraffin sections were photographed with the Panoramic Scanner 250 Flash III (3D Histech, Budapest) and Panoramic Viewer (3D Histech, Budapest). All sections were then evaluated for the incidence of the new bone formation based on the morphology. The area with Bio-Oss materials (M), new bone (B), and TRAP-positive stain (T) was selected in a region of interest (ROI; area with residual materials and new bone) using ImageJ. The percentage of bone in each sample was determined as $B\% = B \times 100/(ROI-M)$. The percentage of TRAP-positive area (T) in each sample was similarly determined as $T\% = T \times 100/(ROI-M)$.

7. Statistical analysis
All data are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) with the aid of GraphPad Prism 7 was conducted to compare the means among the groups. If the ANOVA was applicable, then a Tukey HSD test was used as a post-hoc test. A student’s T-test was performed to compare new bone formation between the +control and OC-P constructs. P-values less than 0.05 were considered statistically significant.

Results

1. Osteoclasts formed on constructs after osteoclastogenic induction in vitro
We quantified the osteoclastogenesis of RAW264.7 and primary mouse macrophages cultured on TCP (2D) and Bio-Oss scaffold (3D) after osteoclastogenic induction by assessing TRAP activity (Figure 1). Induction with RANKL significantly increased TRAP activity for both primary monocytes and Raw264.7 cells. For cells cultured without RANKL, only negligible TRAP activity was measured. Especially for OCs-P constructs, the 3D affected the extent of TRAP activity: on Bio-Oss, OCs-P showed the highest TRAP activity ($5.52\pm0.85$ mM pNP/ng DNA/h), which was approximately twice higher than OCs-P on TCP ($2.98\pm0.11$ mM pNP/ng DNA/h; $p < 0.001$) and was almost 10 times higher than RAW264.7 cells on TCP and Bio-Oss granules (around $0.6\pm0.02$ mM pNP/ng DNA/h).

Figure 1. TRAP activity of induced osteoclasts. RAW264.7 and primary mouse BM macrophages were cultured in tissue culture plates (TCP; 2D) and Bio-Oss granules (Bio-Oss; 3D) with and without osteoclastogenic induction factors for 6 days. The TRAP activity of RAW264.7 derived osteoclasts (OCs-R) and primary mouse osteoclasts (OCs-P) was quantified in the cell lysate ($n = 4$).
Osteoclast-based constructs induce ectopic bone formation

Figure 2. In vivo ectopic bone formation. Histological images of MB-BF stained PMMA sections showing ectopic bone formation, fibrous tissues and residual materials within the (a) -Control, (c) +Control, (e) OCs-R, and (g) OCs-P based constructs after 6 weeks implantation. Images at higher magnification was showed for (b)-Control, (d) +Control, (f) OCs-R, and (h) OCs-P based constructs. Histological images of HE stained paraffin sections showing ectopic bone formation, bone marrow, fibrous tissues and residual materials within the (i) -Control, (j) +Control, (k) OCs-R, and (l) OCs-P based constructs after 6 weeks implantation. New bone was observed in +Control and OCs-P based constructs. Asterisk indicates residual Bio-Oss granules, Black thick arrow indicates the new bone area, blue arrow indicate the osteoid tissue and yellow think arrow indicates the bone marrow. Scale bar = 200 µm.

2. Ectopic bone formation is induced by OCs-P
After 6 weeks, typical fibrous tissues attached to the implanted constructs was observed in all collected specimens (Figure 2a-l). None of the specimens for -control, +control, and OCs-P showed an inflammatory response (Figure 2a, c, g, i & l). In contrast, OCs-R based constructs showed a severe inflammatory response with massive cell infiltration (Figure 2e & k). Newly formed bone, evidenced by typical bone matrix and osteocytes,
was reproducibly observed for OCs-P (11 out of 11) and +control constructs (Tab. 1). Furthermore, bone marrow tissue was detected in the majority of OCs-P (8 out of 11) and +control constructs (10 out of 11; Figure 2j & l). In contrast, few osteoid tissue (immature bone) was found in the -control group (3/11). OCs-R based constructs did not show any presence of newly formed bone in any region of the implanted area (Figure 2e & k).

3. Distinct spatial patterns of new bone formation

New bone was formed both in +Control and OCs-P based constructs (Figure 3a-d). However, two major differences between +Control and OCs-P were observed. Firstly, in +controls, new bone was mainly formed adjacent to fibrous tissue surrounding the total granulate material (Figure 3b & e). In OCs-P constructs, however, bone mainly formed adjacent to the granulate material and within the intergranular spaces bridging the neighboring granules (Figure 3d & f). Secondly, the interface between new bone and the Bio-Oss granules surface in +Control had a clear demarcation line. In contrast, new bone formed in OCs-P was mostly closely adjacent to Bio-Oss granule surfaces.

Table 1. The incidence of ectopic bone formation in all constructs after 6 weeks implantation.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>-Control</th>
<th>+control</th>
<th>OCs-R</th>
<th>OCs-P</th>
</tr>
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<tr>
<td>Incidence of ectopic bone</td>
<td>0/11</td>
<td>11/11</td>
<td>0/10</td>
<td>11/11</td>
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<tr>
<td>Incidence of bone marrow</td>
<td>0/11</td>
<td>10/11</td>
<td>0/10</td>
<td>8/11</td>
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4. Quantitative analysis of new bone formation

We further quantified the new bone in all MB-BF stained sections, +Control had a significantly higher amount of bone formation (4.54% ± 2.20%) than OCs-P (2.32% ± 1.00%; p = 0.03) (Figure 4a). When this measurement was applied to HE sections (Figure 4b; n = 6), +Control (3.73% ± 2.86%) similarly displayed significantly higher bone formation than OCs-P constructs (1.32% ± 0.44%; p = 0.0009).

5. Quantitative analysis of osteoclastic activity

To examine the cellular events of monocyte-macrophage lineage cells, we performed TRAP immunohistochemistry staining. TRAP-positive cells were absent in -Control group while quite few TRAP-positive multinucleated cells were present in OCs-R specimens. In contrast, abundant TRAP-positive multinucleated cells appeared in +Control and OCs-P constructs (Figure 5a-d). When further determine the location of TRAP-positive area, it was mainly distributed adjacent to residual Bio-Oss granules (Figure 5a, c, e, & f). However, part of TRAP-positive area also distributed between fibrous tissue and new bone in +Control based constructs (Figure 5e). We further quantified the TRAP-positive area in these two constructs (Figure 5g; n=6), a significantly higher amount of TRAP-stained area was present in +Control constructs (2.27% ± 0.30%) than OCs-P constructs (1.12% ± 0.27%; p = 0.003). This value was almost negligible in the OCs-R based constructs and -controls.
Figure 3. Location of ectopic bone formation in all constructs. Histological images of H&E stained paraffin sections showing the position of ectopic bone formation within the (a) -Control, (b) +Control, (c) OCs-R, and (d) OCs-P based constructs after 6 weeks implantation. New bone was not observed in any position of -control and OCs-R based constructs while a large amount of new bone was observed adjacent to fibrous tissue in +Control (b & e) and adjacent to Bio-Oss granules in OCs-P based constructs (c & f). Asterisk indicates residual Bio-Oss granules, Black thick arrow indicates the new bone area. Scale bar = 200 µm.

Figure 4. Quantification of ectopic bone formation in +Control and primary osteoclasts based constructs after 6 weeks implantation. The percentage of newly formed bone per unit area of +Control and OCs-P based constructs were quantified based on (a) MB-BF stained PMMA sections (n = 5) and (b) H&E stained paraffin sections (n = 6).
Figure 5. Osteoclastic activity in all constructs demonstrated by TRAP staining. Representative histological images showed TRAP activities (red staining) within the (a) -Control, (b) +Control, (c) OCs-R, and (d) OCs-P based constructs after 6 weeks implantation. TRAP-positive signals were identified in +Control and OCs-P based constructs. Representative histological images showed different distribution of TRAP-positive area in (b & e) +Control and (c & f) and OCs-P based construct. TRAP-positive signals were observed mainly adjacent to fibrous tissue and Bio-Oss in +Control (b & e). In contrast, these signals were mainly adjacent to Bio-Oss granules in OCs-P based constructs (c & f). TRAP activity was quantified and compared in +Control and OCs-P based constructs (n = 6). Asterisk indicates residual Bio-Oss granules, Blue arrow head indicates the TRAP-positive area. Scale bar = 200 µm.
Discussion

Bone remodeling continues to occur throughout lifetime and involves a continuous balance between bone formation and bone resorption. However, how new bone formation is initiated remains unclear due to this close coupling between bone resorption and bone formation. Here, we evaluated the capacity of osteoclasts to induce bone formation in a subcutaneous implantation model. Our results demonstrate that primary osteoclasts seeded on commercially available, devitalized bovine bone induce the initiation of ectopic bone formation. To the best of our knowledge, thus far no data have described this phenomenon of osteoinductive capacity for osteoclasts. This finding greatly supplements the theory of bone formation in a broader biological context and is of utmost importance for the future application of bone tissue engineering and regenerative medicine in clinical practice.

Over the past several decades, osteoinductive capacity has been merely ascribed to cells with bone forming capacity (e.g. osteoblastic cells [27] and mesenchymal stromal cells [3]), growth factor members of the TGF-b-like superfamily (i.e. BMP-2 [28] and BMP-7 [29]), as well as few biomaterials (e.g. calcium phosphates [20] and titanium [30]). After the finding of adult stem cell presence in the body [31], it was straightforward to assume bone marrow-derived MSCs to migrate to bone defect/fracture sites and differentiate into osteoblasts, which subsequently form new bone. Based on this hypothesis, MSC-based approaches have been widely used to directly provide bone-forming cells for the treatment of bone defects [2, 32]. However, the pre-clinical and clinical performance of this approach has not been proven consistently satisfactory [2, 33]. Further, MSCs have recently been found to only slightly or even not directly contribute to the formation of bone tissue [3, 4], suggesting other unrevealed modes of action pivotal in the initiation of bone formation. Furthermore, the hypothesis of MSCs initiating bone formation is contradictory to the findings in the natural bone remodeling process, in which bone resorption precedes bone formation [1, 6]. Especially related to the osteoinductive capacity of few biomaterials, emerging data suggest a dominant role of osteoclasts in the initiation of bone formation [18-23], but thus far no direct evidence has proven this concept. Therefore, with the aid of the well-established cell-based approaches and osteoclast differentiation methods, we prepared osteoclast-based constructs and implanted thes in a subcutaneous model to evaluate the osteoinductive capacity of osteoclasts.

The osteoinductive capacity of BMP-2 has been widely demonstrated [34], as well as the lack thereof for Bio-Oss granules [35]. Consequently, we used this knowledge to include positive and negative controls for osteoinductive capacity in the present study. BMP-2 loaded BioOss reproducibly induced bone formation, while bone formation was absent for Bio-Oss granules after 6 weeks of subcutaneous implantation in mice. Remarkably, bone formation was observed in the primary osteoclasts-based constructs with a 100% incidence (11 out of 11 retrieved specimens). This finding is direct proof for our hypothesis that osteoclasts can initiate the bone formation process. In contrast, osteoinductive capacity was not observed for any of the RAW264.7-derived osteoclast-
based constructs (0 out of 9 specimens). Instead of bone formation, these constructs induced a severe inflammatory response with massive inflammatory cell infiltration. Indeed, macrophages have also been proven crucial for osteogenic differentiation of MSCs, both dependent and independent of osteoclasts [36, 37]. On the one hand, macrophages can directly fuse into osteoclasts upon supplementation of a cytokine cocktail and/or a surface microenvironments [38, 39] and regulate differentiation of osteoblast progenitor cells by secreting various cytokines known as clastokines [40, 41]. On the other hand, macrophages, independent of osteoclasts, can affect osteogenic differentiation of osteoblastic cells and further bone formation [36, 42, 43]. However, monocytes/macrophages alone cannot initiate ectopic bone formation [44, 45]. As such, despite an important role for macrophages (via osteoclastogenesis or inflammation) in the initiation of bone formation, osteoclasts seem the decisive cell type responsible for initiation of bone formation. Furthermore, the OC-R based constructs failing to induce ectopic bone formation was likely ascribed to the extravagant inflammation arising from RAW264.7 derived macrophages, which suggests that osteoclast based ectopic bone formation is crucially on the surrounding microenvironment such as controlled inflammation.

Although both BMP-2 and primary osteoclast-based constructs induced the formation of ectopic bone, the spatial pattern and amount of the newly formed bone was distinct. For BMP-2, a band of bone was formed at the periphery of the subcutaneous pocket as recently demonstrated [46], close to the fibrous tissue, and only limitedly adjacent to the Bio-Oss granules. In contrast, primary osteoclasts induced bone formation predominantly adjacent to Bio-Oss granules. This discrepancy may indicate a difference in the mechanism of initiating bone formation. It is likely that BMP-2 release from BioOss leads to entrapment of this growth factor in the fibrous capsule surrounding the subcutaneous pocket, which may subsequently induce osteogenic differentiation, and hence peripheral bone formation, of osteoprogenitor cells embedded in the nearby soft tissue. In contrast, osteoclast-based constructs likely induced bone formation through anabolic effects of osteoclasts via paracrine actions adjacent to BioOss granules. Furthermore, we observed a significantly higher TRAP activity and higher bone formation in BMP-2 constructs than osteoclast based constructs. Consequently, we cannot rule out that BMP-2 induced bone formation also follows an osteoclastic path, which was suggested in previous work but requires more evidence [47, 48]. Although we firstly demonstrated the finding that osteoclasts are able to initiate bone formation, several relevant questions require further investigations:

1) What is the biological mechanism by which osteoclasts initiate ectopic bone formation? Based on previous knowledge and our findings, we proposed three potential mechanisms: Firstly, Bio-Oss granules as a deproteinized xenograft can release ionic species (e.g. Ca$^{2+}$ and PO$_4^{3-}$) upon osteoclastic activity that may further promote migration and osteogenic differentiation of osteoprogenitor cells [5, 49]. Secondly, seeded osteoclasts secrete anabolic factors, including S1P, BMP6, Wnt10b, THRC1, which initiate osteoprogenitor cells recruitment and osteogenic differentiation [11]. Thirdly, osteoclastic resorption forms a specific topography on granule surfaces during
Osteoclast-based constructs induce ectopic bone formation

the culture and after implantation, which activates the osteogenesis process [50].

Although the third hypothesis is relatively novel, previous work with nano- and micro-
sized material surface features for cultures of osteoprogenitor cells and osteoclasts
already suggested a material-driven mechanism [51, 52]. Additionally, the possible full
biological mechanism of osteoclast-induced bone formation may not follow one single
proposed mechanism, but also a combination of aforementioned possibilities.

2) What is the origin of osteoblastic cells that form bone tissue? It was long thought that
osteoblasts to form ectopic bone are derived from bone marrow MSCs [53]. This idea
is being challenged by emerging evidence. Actually, upon a construct implantation, a
highly vascular connective tissue and capillary sprouting are generally initiated and
invade the construct [46]. Compelling evidence indicates that pericytes, satellite cells
or endothelial cells, which locate around the smallest vessels and in the mussels, seem
to transform into bone-forming cells in response to the microenvironment [54].

3) Following what pathway is the (ectopic) bone formed in osteoclast-based constructs?
Bone formation can occur via the endochondral or intramembranous pathway. BMP-2
is well-known to induce bone formation through the intramembranous pathway [55].

How the ectopic bone is formed in osteoclast-based constructs remains unknown,
although no histological signs of cartilaginous tissue were observed. In view of this
and the temporal similarity of bone formation induced by BMP-2 loaded controls,
this strongly suggests an intramembranous pathway for bone formation induced by
osteoclasts.

Here, we demonstrate that osteoclast-based constructs have the capacity to initiate
bone formation at an ectopic location. This finding is a valuable supplement to the
existing knowledge on the role of osteoclasts in bone resorption and the coupling of
bone resorption and formation in bone remodeling. Our findings also suggest powerful
potential to develop bone regenerative constructs based on osteoclastic activity. One
strategy is to make osteoclast based bone constructs as we used here. Compared to
currently developed bone morphogenetic protein based constructs, which have safety
concerns due to uncontrolled bone formation and other life-threatening side effects
[56, 57], osteoclasts based constructs can precisely formed bone adjacent to the seeded
scaffolds. Also, this approach is superior to the conventional MSC-based approach in
many aspects such as more convenient isolation procedure, less induction time. The
other strategy is to prepare special scaffold materials which are ready to be resorbed
by osteoclasts and simultaneously induce bone formation after implantation by
modulating surface properties. This strategy is more advantageous due to the unlimited
supply and greatly reduced cost and time when circumnavigating cell expansion and
differentiation in vitro. For instance, Synthetic carbonate-substituted biomimetic
hydroxyapatite showed increased osteoclastogenesis and deeper resorption pits from
osteoclasts in vitro [58] and demonstrated more bone formation compared to pure HA
scaffold in vivo [59].
Chapter 7

Conclusion

We demonstrated here that primary mouse osteoclast-based constructs have the capacity to induce ectopic bone formation. The new bone evoked by osteoclasts had less amount and distinct pattern compared to BMP-2 induced new bone, which further correlated with the amount and location of host osteoclasts. Although more evidence and the mechanism of initiation of bone formation by osteoclasts need to be discovered and decoded, our finding greatly innovates the conventional cognition of bone formation process and aids the understanding of the cellular basis of this event, which can provide valuable information for improving existing treatment approaches of bone disease and novel strategies to develop bone substitute to repair damaged bone in the clinic.
References

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Osteoclast-based constructs induce ectopic bone formation


Chapter 7


Osteoclast-based constructs induce ectopic bone formation
Chapter 8

Combinatorial surface roughness effects on osteoclastogenesis and osteogenesis

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Submitted
Chapter 8

Introduction

Metallic biomaterials and devices are widely used in dental, orthopedic, and spinal surgery to facilitate replacement and repair of damaged bone due to their robust mechanical properties and their ability to integrate into bone (osseointegration) [1, 2]. Amongst them, titanium and its alloys have been developed as most common bone implant materials and as a model substrate for studying cell and tissue responses to biomaterials, because of their clinical relevance, suitable biocompatibility and the diverse possibilities for surface modifications [3, 4]. Still, clinical work has reported an implant failure incidence of up to 20% [5], the majority of which was caused by deficient or poor early bone healing at the bone/implant interface in the early post-implantation period [6, 7]. Consequently, it is of crucial importance to improve the early bone healing of an implant for its long-term performance.

The biomaterial surface properties of an implanted medical device have demonstrated to contribute to the host cellular and tissue response and play a significant role in determining overall implant success or failure [8, 9]. Therefore, manipulating surface physical or chemical properties offers an effective and straightforward strategy to improve the biological performance of implant materials. For titanium implants installed in bone defects in animal studies, multiple studies have shown superior bone-to-implant contact and peri-implant bone formation when the surface roughness (arithmetical mean deviation of the surface profile, Ra) is between 1-1.5 \( \mu \text{m} \) [10]. Clinical studies also demonstrated that the osseointegration rate of rough implants was significantly higher than that of machined smooth implants [11-13]. To elucidate the mechanism responsible for these observations from (pre-) clinical studies, previous work has extensively studied how micron and submicron scale roughness contributes to osteoblastic cell attachment, spreading and differentiation, in which these cells originated from mesenchymal stem cells (MSCs) [14-17]. Remarkably, the other cell type involved in bone remodeling and osseointegration, i.e. osteoclasts, have to date been largely ignored. Interestingly, osteoclasts have been observed to appear earlier than osteoblastic cells around bone implants and seem to initiate the remodeling process to form new bone tissue in the peri-implant region [18-20]. Furthermore, the preferred roughness of bone implant surfaces, consisting of a combination of micron scale roughness created by sandblasting and submicron scale roughness generated by acid etching, was found to be strikingly similar to osteoclast resorption pit dimensions on bone wafers [21-23]. All this evidence suggests that biomaterial surface roughness modulates the behavior of osteoclasts, which further affect the bone formation process. Osteoclasts are giant multinucleated bone-resorbing cells differentiated from precursors of the monocyte/macrophage lineage. Polarized osteoclasts form sealing zones, detectable as actin rings and ruffled borders, containing protons and catabolic enzymes such as TRAP, to resorb bone [24, 25]. Osteoclast functions, however, have been widely recognized not to be limited to their ability to resorb bone only. In the context of bone remodeling, osteoclasts also contribute to bone formation by communicating with osteoblastic cells in a process called “coupling of the bone formation to resorption”
In this process, osteoclasts locally promote osteoblastic cell recruitment and osteogenic differentiation through the secretion of coupling factors, known as clastokines, which include sphingosine-1-phosphate (S1P), bone morphogenetic protein 6 (BMP-6), Wnt10b, collagen triple helix repeat containing 1 (Cthrc1) and complement component 3a (C3a) [26, 27]. In contrast, Semaphorin4D (Sema4D), another clastokine secreted by osteoclasts, inhibits osteoblastic cell differentiation [28]. Taken together, these findings indicate that coupling factors released by osteoclasts play an important role in the local regulation of bone formation by influencing recruitment, osteogenic differentiation, and activity of osteoblastic cells.

Above all, the combined activity of osteoblastic cells and osteoclasts and their bidirectional interactions are relevant for bone remodeling and regeneration. Regarding the chronological order in presence at the site of bone regeneration and the stimulatory effects of roughness, we hypothesized that surface roughness influences osteoclastogenesis and the behavior of formed osteoclasts in terms of clastokine secretion. Subsequently, these secreted clastokines then orchestrate osteoblastic cell behavior. To test this hypothesis, we prepared and characterized titanium surfaces with a series of roughness ranging from submicron to micron levels and evaluated their effects on osteoclast behavior (i.e. morphology, growth, and differentiation) using both the murine RAW264.7 cell line and primary mouse macrophages. To evaluate the coupling function of surface roughness induced osteoclast effects on osteoblastic cell differentiation, murine MC3T3 osteoprogenitor cell line and primary rat bone marrow MSCs were cultured in the conditioned medium of these osteoclasts and their osteogenic differentiation was evaluated by mineralization and osteogenic marker analysis and then correlated with osteoclast parameters.

Materials and methods

1. Materials and reagents

Receptor activator for NF-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) were purchased from Peprotech (Rocky Hill, USA). Acid Phosphatase Leukocyte Kit and acid phosphatase activity assay kit were obtained from Sigma (St. Louis, USA). RNA Isolation Kit was obtained from Qiagen (Venlo, Netherlands). ELF97 dye, Alexa Fluor 568 labeled phalloidin, 4’, 6-Diamidino-2-Phenyindole (DAPI), mounting medium, PicoGreen DNA quantification assay kit, TaqMan Reverse Transcription kit and Fast SYBR Green Master Mix Kit were obtained from Thermo Fisher Scientific (Breda, Netherlands). Osteogenesis quantification kit was obtained from EMD Millipore (Darmstadt, Germany). Minimum Essential Medium α (α-MEM), fetal bovine serum (FBS), antibiotics, and Phosphate Buffered Saline (PBS) were obtained from Gibco (Delft, Netherlands). Titanium (Ti) disks (1.5 mm in thickness, 12 mm in diameter; 99.9 wt% purity) were purchased from Machinefabriek G Janssen (Valkenswaard, Netherlands). Glass coverslips with 12 mm diameter were obtained from VWR (Renswoude, Netherlands).
2. Preparation and characterization of titanium (Ti) surfaces

Ti surfaces with different roughness were made through grit blasting with Al₂O₃ using different particle size (50 µm and 250 µm) and pressure (1, 2 bar and 3 bar). Each group of disks was consecutively washed with 10% nitric acid, acetone and ethanol in and ultrasonic bath for 10 minutes, respectively. The disks were then sterilized by autoclaving. The roughness of prepared disks was tested with Universal Surface Tester (UST; Innowep GmbH, Germany). Contact angle measurements were obtained using Surface Tension (Biolin scientific, Finland) equipped with a digital camera and image analysis software. Four disks from each titanium surface were utilized for each test.

3. RAW264.7 derived osteoclasts

RAW264.7 was obtained from Sigma-Aldrich® and cultured in α-MEM supplemented with 10% FBS. Cells were seeded at 2 × 10³ cells/cm² and passaged until 80% confluence using a plastic scraper (Greiner Bio-One, Netherlands). For osteoclastic differentiation, 2 × 10³ cells/cm² RAW264.7 were seeded on the surface of titanium disks and glass slides. The medium was changed to differentiation medium (α-MEM supplemented with 10% FBS supplemented with 50 ng/ml of murine sRANK ligand) after 24h. The medium was then refreshed every 2 days. Cells and conditioned medium were collected after 4 days of the osteoclasts induction.

4. Mouse bone marrow macrophages derived osteoclasts

Mouse bone marrow mononuclear cells were isolated from 6- to 8-week-old male C57Bl/6 mice by flushing femurs and tibia with α-MEM medium supplemented with 0.5 mg/ml gentamycin and 3 µg/ml fungizone. Cells were cultured in α-MEM containing 10% FBS and the non-adherent cells were collected after 24h. Cells were then seeded in flasks with 30 ng/mL human M-CSF for 2 days. Attached cells were detached and seeded on different surfaces at 2 × 10⁴ cells/cm² and cultured in α-MEM containing 10% FBS supplemented with 30 ng/ml M-CSF and 50 ng/ml of murine RANKL for osteoclast induction. The medium was then changed every 2 days. Cells and conditioned medium were collected after 4 days.

5. Characterization of osteoclasts behaviors on different roughness

5.1 Cell morphology

Macrophage morphology on the tested surfaces was examined by scanning electron microscopy (SEM, Jeol SEM6310, Nieuw-Vennep, Netherlands) at indicated time points. Cells were fixed with 2% glutaraldehyde in cacodylate buffer, dehydrated in a sequential series of ethanol followed by tetramethylsilan, coated with gold and then observed using SEM.

5.2 TRAP staining

Osteoclasts cultured on different surfaces were fixed with 4% of paraformaldehyde (PFA) for 10 mins and stained using Acid Phosphatase Leukocyte Kit per the manufacturer’s
instructions. Briefly, 10 ml Acid Phosphatase solution consisting of 9 ml pre-warmed MilliQ, 400 µl acetate solution, 100 µl Naphthol AS-BI Phosphoric Acid, 200 µl Tartrate Solution and 200 µl Diazotized Fast Garnet GBC was prepared and cells were stained with this solution for 20 min at 37°C. TRAP positive multinucleated cells on these surfaces were observed using a light microscope (Leica, Germany).

5.3 DNA content
Cell growth on different surfaces was assessed using the PicoGreen DNA quantification assay kit. Cell layers were washed twice with PBS, after which 1 ml MilliQ was added. Following two freeze-thaw cycles, samples were aspirated several times and used for DNA quantification per the instructions of the manufacturer.

5.4 TRAP activity assay
Cell layers were washed twice with PBS, rinsed with 1 ml of MilliQ and lysate with two freeze-thaw cycles. TRAP activity was tested using acid phosphatase activity assay kit per the manufacturer’s instructions. Briefly, 50 µl sample was mixed with 50 µl substrate dissolved in citrate buffer and incubated at 37°C for 30 minutes. The reactions were stopped by adding 200 µl of Stop Solution (0.5 N NaOH). A blank control (citrate buffer) and standard solutions were made in parallel. The absorption was measured at 405 nm with a multi-mode spectrophotometer (Biotek, Winooski, USA). The value of TRAP activity was then normalized to DNA content.

5.5 Gene expression of osteoclast markers
RNA was isolated from osteoclasts on different surfaces using RNA Isolation Kit per the manufacturer’s protocol. Reverse transcription was performed using TaqMan Reverse Transcription kit. RT-PCR expression analysis was performed using a Fast SYBR Green Master Mix Kit and PRISM 7500 sequence amplification system (Applied Biosystems, USA). TRAP, Cathepsin K (CTSK), Receptor activator of nuclear factor kappa-B (RANK), Matrix metallopeptidase 9 (MMP-9) were tested with GAPDH as housekeeping gene. The primer sets used are shown in Supplementary Table 1. The level of gene expression was calculated via the $2^{-\Delta\Delta CT}$ method. Three independent samples were used for each gene of interest.

5.6 ELF97 staining
Samples were washed twice with PBS and fixed with 4% of paraformaldehyde (PFA). After fixation, samples were washed twice with PBS. ELF97 dye was diluted 50 times with above Acid Phosphatase solution, added to each well and incubated for 15 minutes at 37°C in the dark. Samples were then washed twice with PBS and images were taken using a microscope (Zeiss AxioCam MRC5; Carl Zeiss Microimaging, Germany).

5.7 Actin and nuclei staining
For actin and nuclei staining, cells were rinsed twice with PBS and then fixed with 4% PFA in PBS for 10 min. After washing twice, samples were incubated with Alexa
Fluor 568 labeled phalloidin and (1:50 dilution) in PBS for 15 mins. After washing with PBS, samples were incubated with DAPI (1:500 dilution) for 5 mins and mounted with mounting medium. Cells were imaged with a Zeiss microscope. Osteoclast number (more than three nuclei), actin ring perimeter, nuclei per osteoclast and osteoclast area were quantified using Image J. Cell area was categorized into one of the following classes: ≤25 × 10³ µm² per cell, 25-50 × 10³ µm² per cell, 50-100 × 10³ µm² per cell, 100-200 × 10³ µm² per cell, 200-400 × 10³ µm² per cell, ≥400 × 10³ µm² per surface. Nuclei number of each osteoclast was counted for four disks. If the number of osteoclasts was more than 100 per disk, the measurements were performed at random 100 osteoclasts on each specimen.

6. Effect of osteoclast medium on osteogenic differentiation of osteoblastic cells

6.1 Mouse osteoprogenitor and rat primary MSCs culture

The mouse osteoblastic precursor cell line MC3T3 was purchased from Sigma-Aldrich®. Rat bone marrow-derived MSCs were isolated from 6-week-old male Fischer rats. All animal procedures were approved by the Radboud University Nijmegen Animal Ethics Committee. Both MC3T3 cells and MSCs were cultured in growth medium (α-MEM with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin) at 37°C in humidified 5% CO₂.

For osteogenic differentiation assays, 2 × 10⁴ cells were seeded in 48 well plates. After 24 hours, medium was changed with 1:1 conditioned medium from RAW264.7-derived osteoclast cultures (for MC3T3 cells) or primary mouse osteoclast cultures (for primary MSCs) on Ti disks with different roughness and osteogenic medium (growth medium supplemented with 10 nM dexamethasone, 100 µM ascorbic acid, and 10 mM β-glycerophosphate). Medium was changed twice per week. These cultures were used to obtain samples for alizarin red staining and mineralization quantification and for gene expression analysis.

6.2 Alizarin red staining and quantification

After 14 days, cell layers were washed twice with PBS and then fixed with 4% PFA for 10 mins and then washed twice with PBS. Cell were then stained with 1 ml/ well alizarin red solution for 15 min at room temperature using an osteogenesis quantification kit. Stained samples were photographed with a light microscope. The quantification of alizarin red of cell layer was conducted based on the protocol. Briefly, cell layers were dissolved in 10% acetic acid, vortexed, heated at 85 °C and then centrifuged. The supernatant was neutralized and then colorimetric changes were measured at the absorbance of 405 nm using a multi-mode spectrophotometer (Biotek, Winooski, USA). To reduce the internal errors, alizarin red values from each sample were normalized to cells cultured in 1:1 of osteogenic medium and growth medium.
6.3 Gene expression of osteogenic markers
Osteoprogenitor cells cultured in conditioned medium of osteoclasts for 7 days, RNA was isolated, transcribed as aforementioned. Gene expression of RUNX2, Collagen I, ALP and osteocalcin (OCN) was analyzed by RT-PCR using the primers listed in Supplementary Table 1. GAPDH was used as housekeeping gene and the level of gene expression was calculated via the $2^{\Delta\Delta Ct}$ method. Four independent samples were used for each gene of interest.

7. Regression analysis of osteoclast subtype and its anabolic activity
Size of actin ring, number of osteoclasts per cm$^2$, number of nuclei per osteoclast and TRAP activity were averaged for each surface roughness group. These values were then correlated with the alizarin red value of osteoblastic cells cultured in each type of osteoclasts on different surfaces. Scatter diagrams were made with Excel, regression analyses were conducted and the most accurate trend lines were displayed based on $R^2$ value.

8. Statistical analysis
All experiments were repeated three times and figures showed the representative data of a single representative experiment. All results are expressed as the mean ± standard deviation (SD) from multiple samples per experimental group (see figure captions for exact sample numbers) and $P < 0.05$ was considered statistically significant. One-way analysis of variance (ANOVA) was used for each experiment to compare the means among the groups. Where applicable, a Tukey HSD test was used as a post-hoc test.

Results

1. Preparation and characterization of titanium disks with different surface roughness
Four types of titanium disks were prepared with significantly different surface roughness (Figure 1); Ra values were 0.24 µm for machined (Ti), 0.81 µm for low roughness (TiLR), 2.30 µm for medium roughness (TiMR), and 3.63 µm for high roughness (TiHR). Glass slides with a smooth surface (Ra = 0.02 µm) served as controls (Figure 1A). To exclude effects of surface wettability on cell behavior, contact angles were measured using the sessile drop method. All surfaces had a similar wettability value (range: 64° - 84°; Figure 1A). SEM assessment of the surface morphology showed that control and Ti surfaces were apparently smooth. TiLR displayed a low roughness and uniform topography compared to TiMR and TiHR, which showed crack structures and blasting scars with a higher roughness (Figure 1B).
Chapter 8

1. Surface characterization of titanium disks with different surface roughness.
Machined titanium disks were sand-blasted to create different roughness and smooth glass slides were used as control. (A) The roughness of glass and different rough titanium surfaces was determined with Universal Surface Tester and the contact angle was measured with Surface Tension. (B) The morphology of different rough surfaces was observed by SEM. Original magnification for SEM ×500; Scale bar is 10 µm in all panels.

2. Osteoclast morphology on different surface roughness
The morphology of macrophages after osteoclastogenic induction on different surfaces was observed with SEM (Figure S1; RAW264.7 macrophages were representatively displayed). Both large giant cells and smaller, undifferentiated macrophages were visible on all surfaces. Osteoclast morphology was greatly affected by the surface roughness. Osteoclasts present on the smooth surfaces of controls and Ti showed a more spread shape and more clear cell fusion than cells on rougher surfaces (TiLR, TiMR and TiHR), on which cells were smaller and separately distributed.

3. Osteoclast growth and differentiation with different surface roughness
To determine the osteoclastic nature of the formed multinucleated cells, TRAP staining was performed. Different sizes and numbers of TRAP-positive cells originating from RAW264.7 macrophages were observed on different surfaces (Figure 2A). TRAP-positive cells were bigger and present in lower numbers on smoother disks (Ctrl and Ti) than on rougher disks. A difference between TiLR, TiMR and TiHR was not obvious. To assess cell numbers on these different surfaces, DNA content was measured after 4 days of osteoclastogenic induction. DNA content from RAW264.7 cells gradually increased with surface roughness. Significantly higher cell growth was observed for rougher surfaces (TiMR and TiHR) compared to smoother titanium surfaces (controls, Ti and TiLR; Figure 2B). Quantitative assessment of osteoclastic TRAP activity on different surfaces (Figure 2C) showed a tendency of smooth surfaces to have higher TRAP activity than rougher surfaces. Specifically, the highest TRAP activity was found on Ti, followed by control and TiLR, which were significantly higher than TiMR and TiHR. The negative control without RANKL showed negligible TRAP activity (data not shown). To further confirm osteoclastic differentiation, a series of specific markers were analyzed by RT-PCR (Figure 2D, E, F & G). Osteoclastogenic markers were generally expressed at significantly higher levels on control and Ti than on rougher surfaces. Among roughened surfaces, TiLR had significantly higher RANK and MMP-9 gene expression than TiMR and TiHR.
Figure 2. Osteoclastogenic differentiation of RAW264.7 derived osteoclasts on different rough surfaces. RAW264.7 derived macrophages were cultured on glass control and different rough titanium with RANKL for 4 days. (A) Osteoclasts were then stained with TRAP biochemical staining (n = 4). (B) Their growth were quantified by DNA content (n = 4) and their osteoclastogenic differentiation were determined by (C) TRAP activity (n = 4) and gene expression of osteoclast makers including (D) TRAP (n = 3), (E) RANK (n = 3), (F) MMP-9 (n = 3) and (G) CTSK (n = 3). A significant was indicated by a, b, c d. Groups with different letters mean significant difference and groups sharing the same letter are not significantly different.
Chapter 8

For primary mouse osteoclasts, TRAP biochemical staining and TRAP activity displayed similar responses to the different surfaces (Figure 3). More specifically, with increasing surface roughness, primary mouse osteoclasts decreased in size on TiLR, TiMR and TiHR compared to control and Ti. The number of TRAP positive cells was generally higher on rougher surfaces (Figure 3A). Cell number evaluated with DNA content was also significantly higher on rougher surfaces and TRAP activity declined with increasing roughness (Figure 3B & C). However, TRAP activity was highest on controls for primary mouse osteoclasts (in contrast to Ti for RAW264.7 derived osteoclasts).

4. Osteoclasts number, size and cytoskeletal organization on different surface roughness

When osteoclast precursors differentiate into mature osteoclasts, they form clusters of dynamic, F-actin-rich adhesion structures enriched in integrin receptors called podosome that self-organize into actin rings at the cytomembrane periphery. We investigated effects of surface roughness on actin ring formation by analysis of F-actin (phalloidin stain), nuclei (DAPI stain) and endogenous phosphatase activity (ELF97 stain) organization. On all surfaces, osteoclasts from RAW264.7 exhibited multiple nuclei, a typical F-actin ring and endogenous phosphatase positivity (Figure 4A). Actin rings were typically big and heterogeneous on smoother surfaces. In contrast, osteoclasts cultured on rougher surfaces displayed small but homogeneous F-actin ring organization and cluster structure. To be specific, osteoclasts on control (average 1100 µm) and Ti (average 906 µm) exhibited significantly bigger actin rings than osteoclasts on TiLR (average 566 µm) and TiMR (average 491µm) surfaces, with TiHR having the lowest size (average 358 µm; Figure 4B). The number of osteoclasts was quantitatively determined on the different surfaces with the aid of F-actin ring and endogenous phosphatase staining. A significantly larger number of osteoclasts was found on rougher surfaces, especially on TiHR (37 ± 3.2 osteoclasts/cm²) and TiMR surfaces (26 ± 2.3 osteoclasts/cm²), compared to the Ti (3.5 ± 0.5 osteoclasts/cm²) and control (2.7 ± 0.8 osteoclasts/cm²) smoother surfaces (Figure 4C). The average nuclei number in each osteoclast, however, was significantly higher on smoother surfaces, especially on Ti (average 87 nuclei/osteoclast). This was approximately 3 times more than TiLR, 4 times more than TiMR, and 8 times more than TiHR (Figure 4D). Osteoclast size, evaluated by cell area, was also analyzed based on F-actin ring and endogenous phosphatase staining. Both large and small osteoclasts were observed and measured on all surfaces, where the largest osteoclast areas were found on smoother surfaces (control and Ti; Figure 4E). More specifically, on smoother surfaces (control and Ti), large osteoclasts with an area >50 × 10³ µm² were predominant, constituting more than 71% of all osteoclasts, while small osteoclasts with a typical area of less than 25 × 10³ µm² constituted only 15% of the population. On rougher surfaces (TiLR, TiMR and TiHR) an opposite trend was observed: osteoclasts with an area <25 × 10³ µm² were the predominant phenotype, constituting more than 51% of total osteoclast population, whereas large osteoclasts with an area >50 × 10³ µm² constituted less than 17%.

Compared to RAW264.7 derived osteoclasts, primary mouse osteoclasts also
generally exhibited bigger F-actin ring-like structures on smoother surfaces than rougher surfaces, except for that on controls, these cells did not display phalloidin-labeled F-acting ring formation in three independent experiments (Figure 5A). When F-actin rings were measured on other surfaces for quantitative comparison, F-actin ring sizes of primary mouse osteoclasts on Ti (average 1746 µm) similarly displayed significantly larger size than those on TiLR (average 493 µm), TiMR (average 207 µm) and TiHR (average 196 µm; Figure 5B). The number of osteoclasts was also highest on TiHR (111±12 osteoclasts/mm²), followed by TiMR (65±4.1 osteoclasts/mm²) and TiLR (33±4.3 osteoclasts/mm²), all of which were significantly higher than on Ti and control (around 2 osteoclasts/mm²; Figure 5C). Also, primary mouse osteoclasts on Ti (average 198 nuclei/osteoclast) and control (average 32 nuclei/osteoclast) had significantly more nuclei per osteoclast than those on rougher surfaces (all fewer than 19 nuclei/osteoclast; Figure 5D). Regarding cell area, primary mouse osteoclasts displayed less diversity compared to RAW264.7 derived osteoclasts on all surfaces. Osteoclast areas <25 × 10³ µm² predominated for primary mouse osteoclasts on TiLR, TiMR and TiHR. However, primary mouse osteoclasts on control and Ti showed larger area, with an area <25 × 10³ µm² taking up less than 17% of the osteoclasts and an area >50 × 10³ µm².
approximately constituting 60% of the osteoclasts (Figure 5E).

Figure 4. Number, size and cytoskeletal organization of RAW264.7 derived osteoclasts on different rough surfaces. RAW264.7 derived macrophages were cultured on glass control and different rough titanium with RANKL for 4 days. (A) Osteoclasts were then stained with DAPI (blue), Alexa Fluor 568 Phalloidin (red) and ELF 97 (green). (B) The perimeter of actin rings, (C) Osteoclasts density, (D) nuclei number per osteoclast and (E) osteoclast area on these different surfaces were determined to quantify the osteoclastogenic differentiation. Scale bar = 50 µm. A significant difference was indicated by a, b, c d. Groups with different letters mean significant difference and groups sharing the same letter are not significantly different.

5. Effect of osteoclast medium on osteogenic differentiation of osteoblastic cells
Given that osteoclast differentiation and cytoskeletal organization are affected by surface roughness, we hypothesized that these osteoclasts further differentially regulate the behavior of osteoblastic cells, possibly by expressing and/or secreting coupling factors. To this end, both mice osteoprogenitor cells and primary rat MSCs were cultured in medium collected from the osteoclasts cultured on the surface with different roughness (50% v/v; Figure 6). Osteoblastic cells subjected to conditioned medium harvested from osteoclasts displayed significantly higher alizarin red positive bone nodule formation than osteoblastic cells in normal osteogenic medium (Figure 6A). More specifically, when MC3T3 osteoblastic cells were cultured with RAW264.7 derived osteoclast conditioned medium, medium from osteoclasts cultured on control, Ti and TiLR showed 3 times more alizarin red than osteoblast control cultures.
Further, osteoclast conditioned medium from TiMR and TiHR showed twice more alizarin red than osteoblast control cultures and this effect was more obvious on TiMR (Figure 6B). This anabolic effect on osteoblastic cells was also observed for conditioned medium from primary osteoclasts on primary rat MSCs (Figure 6C). Conditioned medium from osteoclasts cultured on all surfaces except TiHR exhibited significantly higher alizarin red nodule formation than osteoblast control cultures in osteogenic medium. Particularly, conditioned medium from Ti induced approximately 5 times higher mineralization than osteoblast control cultures and 2.5 times higher than TiLR and TiMR. In agreement with our observations on mineralization, MC3T3 osteoblastic cells cultured in conditioned medium collected from RAW264.7 derived osteoclasts on control and Ti had significantly higher osteogenic marker gene expression than those from TiMR and TiHR, including ALP, Collagen I and OCN (Figure S2).

Figure 5. Number, size and cytoskeletal organization of primary osteoclasts on different rough surfaces. Primary mouse macrophages were cultured on glass control and different rough titanium with M-CSF and RANKL for 4 days. (A) Osteoclasts were then stained with DAPI (blue), Alexa Fluor 568 Phalloidin (red) and ELF 97 (green). (B) The perimeter of actin rings, (C) osteoclasts density, (D) nuclei number per osteoclast and (E) osteoclast area on these different surfaces were determined to quantify the osteoclastogenic differentiation. Scale bar = 100 µm. A significant difference was indicated by a, b, c d. Groups with different letters mean significant difference and groups sharing the same letter are not significantly different.
Figure 6. The osteogenic differentiation of osteoblastic cells in conditioned medium of osteoclasts cultured on different rough surfaces. Mouse osteoprogenitor cells (MC3T3) and primary rat MSCs were cultured in the conditioned medium of osteoclasts cultured on glass control and different titanium rough surfaces. (A) The mineralization nodules were stained with alizarin red and quantified with alizarin red in mouse osteoprogenitor cells in (B) conditioned medium from RAW264.7 derived osteoclasts and (C) in primary rat MSCs in conditioned medium from primary mouse osteoclasts. A significant difference was indicated by a, b, c d, e. Groups with different letters mean significant difference and groups sharing the same letter are not significantly different.

6. Regression analysis between osteoclast characteristics and their anabolic effects on osteoblastic cells

In order to further reveal the dependent relationship of osteoclast anabolic effects and osteoclast subtypes induced by roughness, we correlated the main differentiation parameters of osteoclast subtypes (i.e. average osteoclast number, average nuclei number per osteoclast, average osteoclast perimeter and TRAP activity) with the mineralization content of osteoblastic cells cultured in conditioned medium from these different osteoclast subtypes. Both for RAW264.7 derived osteoclasts and primary mouse osteoclasts, the closest correlation of osteoclast anabolic effects was found to be with osteoclast average nuclei number (correlation coefficient $R^2 \geq 0.95$; Figure 7A & B). An increasing nuclei number per osteoclast was depicted to be logarithmically correlated with an increasing anabolic effect. The number of osteoclast per cm$^2$ also showed correlation with osteoblastic cell mineralization (correlation coefficient of $R^2 \geq 0.74$; Figure S3A & B). Nonetheless, the correlation of primary mouse osteoclast actin ring size and TRAP activity with its osteoclast anabolic effects was not obvious (Figure S3C & D).

162
Figure 7. The regression analysis between osteoclast phenotype and its anabolic effects and the schematic of the hypothesis of interactions between osteoblastic cells, osteoclasts and surface roughness. The average nuclei number of each osteoclast on different surfaces was correlated with its anabolic effects on osteoblastic cells. Both (A) average nuclei number of osteoclasts from RAW264.7 and their anabolic effects on mouse osteoprogenitor cells and (B) average nuclei number of primary mouse osteoclasts and their anabolic effects on primary rat MSCs osteoblastic cells from displayed a logarithmic correlation respectively. (C) Based on our findings of different osteoclast phenotypes and their anabolic effects, the hypothesis of the role of roughness on osteoclastogenic differentiation and further osteogenic differentiation was proposed. On smoother surfaces, osteoclasts exhibit larger size and more nuclei in each osteoclast and secrete more anabolic clastokines to promote osteogenic differentiation of MSCs. In contrast, on rougher surfaces, osteoclasts exhibit smaller size and fewer nuclei in each osteoclast and secrete less anabolic clastokines to promote osteogenic differentiation. Yellow dots indicate clastokines secreted from osteoclasts.

Discussion

Surface properties play an important role in regulating cell and tissue responses to bone and dental implants and further determine the bone formation and osseointegration [9, 29]. The cross-talk between surface roughness and bone resorption cell osteoclasts and subsequent bone forming osteoblastic cells is of great importance but remains unclear. In this study, we demonstrated that osteoclasts are sensitive to the specific surface roughness and exhibit various phenotypes, characterized by different morphology, growth and differentiation capacity. Conditioned medium from these different osteoclastic phenotypes further promoted the osteogenic differentiation of osteoblastic cells to different degrees. The tight correlation of osteoclast nuclei number
with its anabolic effect of conditioned medium from osteoclasts with this phenotype was identified. This information highlights the importance of surface properties toward osteoclast phenotype and function, which differentially orchestrates osteogenic differentiation of osteoblastic cells via secreted clastokines. Additionally, the formation of osteoclasts with specific phenotypes on surfaces with different physical properties may provide a potential guide for developing biomedical devices with an optimal surface to stimulate osseointegration.

Both RAW264.7 and primary bone marrow derived macrophages formed osteoclasts upon stimulation with RANKL, evidenced by the presence of a cell population displaying TRAP activity, the presence of multinucleated cells with well-defined actin rings, and the expression of the osteoclast-related genes TRAP, RANK, CATK and MMP-9. Another multinucleated cell type, so-called foreign body multinucleated giant cells (FBGCs), are frequently formed at implant surfaces and related to foreign-body reactions. Similar to osteoclasts, FBGCs arise from the fusion of monocytes/macrophages, and hence share several characteristics with osteoclasts, such as multinuclearity, TRAP expression, and actin rings [30]. An important distinction between these two cell types on non-mineral surfaces is that only osteoclasts express the matrix degrading enzyme CTSK [30]. The high gene expression of CTSK here indicates the formation of osteoclasts rather than FBGCs on all surfaces in this work.

After cell seeding, significantly higher osteoclast cell growth was observed for rougher surfaces than for smoother surfaces, both for RAW264.7 derived osteoclasts and primary mouse macrophage derived osteoclasts. This finding is consistent with previous reports, in which rougher surfaces had higher osteoclast cell adhesion and growth than smoother surfaces [31, 32]. Regarding osteoclast differentiation on different surfaces, smoother surfaces generally more robustly induced osteoclast differentiation than rougher surfaces, evidenced by significantly higher TRAP activity and higher gene expression of osteoclastogenic markers. This is in line with previous studies reporting that the increase in surface roughness (1~2 µm) decreased osteoclast-associated features [33, 34], including TRAP activity and resorption capacity. However, it has also been observed that osteoclasts display an increased differentiation on increasing surfaces (0.1~0.5 µm), measured as TRAP activity and specific gene expression [31]. The different roughness range, high variety of osteoclast cellular origins, osteoclast culture protocols, and induction times may attribute to these conflicting conclusions.

For example, primary osteoclasts were utilized and collected after a 21-day induction period by Costa-Rodrigues et.al [31]. However, as reported and proven in our study, osteoclasts appeared as early as 4 days after induction. Additionally, a difference between primary and RAW264.7 derived osteoclasts existed as primary osteoclasts could be grown for 7 days while apoptosis was apparent in RAW264.7 derived osteoclasts after 5 days of culture due to extremely high cell density. This is one of the reasons why we used two cell types to obtain osteoclasts, a cell line and primary cells, to obtain a reliable conclusion here.

Osteoclast functionality depends on its tight adhesion to the bone surface, which is mediated via an actin-rich integrin adhesion structure known as the podosome belt.
Podosome belt formation and turnover are highly sensitive to the local environment [24]. Generally, larger actin rings were found on smoother surfaces and small actin rings were found on rougher surfaces. One interesting finding is that primary osteoclasts on glass controls did not display this featured structure, perhaps due to a short-lived actin ring formation [35]. Actin ring on glass probably early appeared and disappeared after 4 days of osteoclastogenic induction. This finding further emphasizes the importance of culture protocols and treatment time when studying osteoclasts. Except for the difference in actin ring formation, we also found different phenotypes of osteoclasts on surfaces with different roughness. Generally, larger sized, a higher number of nuclei per osteoclast but lower osteoclast numbers were found on smoother surfaces than on rougher surfaces. This difference plausibly can be attributed to the effects of roughness on the cell fusion process required for osteoclast formation. On smoother surfaces, no topographical features are present to hinder this cell fusion process. Hence, cells effectively fuse to form fewer osteoclasts with a larger size on these surfaces. In contrast, on the rough surfaces, the topographic features might hinder cell fusion process leading to more osteoclasts with a smaller size [36]. Intriguingly, our findings of different osteoclast morphology correspond with some observations of osteoclasts in vivo. Two types of morphologically different osteoclasts are observed in mice [37]. One type exhibits an abundant “foamy”, acidophilic staining of the cytoplasm and large oval vesicular nuclei. This type of osteoclast is abundant in young mice and at fracture sites in both young and old mice. The second osteoclast type exhibits considerably altered morphological characteristics and is smaller in size. These cells are found to be more abundant at the metaphysis of older animals. Furthermore, a significant shift from the former type to latter type occurs with increasing age. Therefore, it is tenable to conclude that different types may represent different functional states of osteoclasts. The osteoclasts with a larger size in young mice may have stronger anabolic but less resorption activity, while osteoclasts with smaller size may have weaker anabolic but stronger resorption activity. This idea is reinforced by the finding that osteoclasts with large surface area correspond to non-resorbing osteoclasts, while smaller osteoclasts correspond to actively resorbing osteoclasts [38-40]. This hypothesis then promoted us to further explore the effect of roughness induced osteoclast phenotypes on their anabolic activity.

Despite the observation that resorption and bone formation are coupled and implants induce the formation of multinuclear osteoclast-like cells around the new bone area [41, 42], the relation between osteoclasts and bone regeneration using bone implants is rarely reported. The conventional consensus regarding the relation between bone resorption and bone formation is that specific factors such as TGF-β and IGF-1 stored in the bone matrix are released and activated during bone resorption and then promote the osteogenic differentiation [43, 44]. This hypothesis is now being challenged by findings that non-resorbing osteoclasts still possess the ability to support bone formation [45-47], suggesting that osteoclast anabolic effects might not correlate directly with osteoclast resorption activity, but merely with the presence of osteoclasts. Several ‘clastokines’ that regulate osteoblastic cell behavior have been identified, including Insulin-like Growth Factor (IGF-1), Transforming Growth Factor-β (TGF-β), and S1P [26,
The data in present study corroborate the concept of non-resorbing osteoclasts on titanium and glass to have the potential to secrete certain clastokines that promote osteogenic differentiation. We also observed that surface roughness affects osteogenic differentiation through a cross-talk between osteoclast activity and osteoblastic cell activity. Smoother surfaces promoted osteoclastogenic differentiation and conditioned medium from osteoclast cultures thereon highly promoted osteogenic differentiation of osteoblast progenitors. In contrast, rougher surface evoked less osteoclastic differentiation and conditioned medium from osteoclast culture thereon promoted osteogenic differentiation of osteoblast progenitors to a lesser extent (Figure 7C). Since osteoclasts have shown to appear much earlier than osteoblastic cells around implants [19, 20], it is reasonable to hypothesize that effects of bone implant surface properties on osteoclasts and then on osseointegration overweighs effects of surface roughness directly on osteoblastic cells. Another hypothesis is that roughness modulates osteogenic differentiation through two separate ways: indirect effects on osteoblastic cells through osteoclasts and direct effects on osteoblastic cells. In this perspective, both roughness and osteoclast phenotype affect osteoblastic cell behavior, and in turn, both roughness and osteoblastic cell differentiation affect osteoclast behavior [48, 49].

The optimized roughness for osteogenic differentiation should be the sum effect from these two. Osteoblastic cell differentiation has shown to be enhanced on micro-rough surfaces (Ra=2 µm) compared to smooth surfaces while osteoclast activity was higher on smooth than on micro-rough surfaces [33]. Therefore, the optimal roughness for osseointegration was proposed to be 1~1.5 µm [10]. However, this hypothesis should be validated through well-designed animal studies. Whatever the mechanism is, our information further strengthens the previously proposed idea that maintaining non-resorbing osteoclasts, as opposed to reducing osteoclast numbers, leads to increased bone formation, bone volume, and ultimately higher bone strength in vivo as sources of anabolic molecules for the osteoblasts [50].

The most innovative finding in this study was the identification of the different osteoclasts phenotypes and their differential anabolic effects, which we observed via osteoblastic cell cultures in conditioned medium. Various types of osteoclasts have been observed in physiological and pathological situations in vivo [51]; their bone catabolic and anabolic functions, which crucially determine the bone quality and treatment effects, remains unclear and debatable. We firstly revealed that osteoclast nuclei number positively correlates with these anabolic effects. This information might greatly aid future bone implant design, which can optimize physical surface properties to induce desired osteoclast phenotypes and enhance osseointegration. In addition, given the reported heterogeneity of osteoclasts under different physiological conditions (e.g. osteopetrosis, osteoporosis, osteonecrosis, and (rheumatoid) arthritis) and their differential effects on the balance between bone resorption and bone formation, the finding that anabolic effects are associated with osteoclast heterogeneity and nuclei number would help to elucidate the function of observed osteoclast types in these diseases and contribute to the design and development of specific therapeutic drugs for the treatment of these diseases [51].
Conclusion

We here examined combinatorial effects of surface roughness on osteoclast behavior and anabolic effects on osteogenic differentiation of osteoblast progenitors. Osteoclasts cultured on smoother surfaces showed less growth, larger size and actin rings, and more nuclei per osteoclast compared to osteoclasts cultured on rougher surfaces. All conditioned medium from these different osteoclast phenotypes significantly promoted osteogenic differentiation of osteoblastic cells compared to conventional osteogenic medium and this effect was far more obvious for conditioned medium from osteoclasts cultured on smoother surfaces. This anabolic effect of conditioned medium on osteogenic differentiation was further revealed to logarithmically correlate with the number of nuclei per osteoclast. These results suggest that surface roughness is an important factor in mediating osteoclast-material interactions, which might determine osteogenic differentiation of osteoblast progenitor cells, and hence the process of osseointegration.
Chapter 8

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Combinatorial surface roughness effects on osteoclastogenesis and osteogenesis


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Chapter 8


Combinatorial surface roughness effects on osteoclastogenesis and osteogenesis
Chapter 9
Summary, closing remarks and future perspectives

Samenvatting, Conclusie en Toekomstperspectieven
1. Summary
To overcome the drawbacks of current bone graft materials, bone tissue engineering (BTE) using autologous stem cells has been proposed as a promising technique for treating bone defects. Indeed, various pre-clinical [1] and clinical studies [2] have shown the capacity of cell-based BTE constructs to heal bone defects and repair the damaged bone. On the other hand, there are also multiple studies that do not show bone formation or regeneration using cell-based BTE constructs [3, 4] and maybe even many more unpublished data with these results. Finally, the discrepancy in bone regenerative efficacy between cell-based BTE applications in preclinical versus clinical studies is puzzling and indicates substantial differences in bone healing and regeneration using human cells in human patients compared to using animal or human cells in available animal models.

Several severe challenges are hypothesized to limit the efficacy of cell-based BTE for bone repair in the clinic, including the adverse immune response and insufficient vascularization after bone graft implantation [5, 6]. On the one hand, immune cells are involved in the inflammatory response that is an essential stage in tissue regeneration, but might affect the viability of transplanted cells [7]. On the other hand, these immune cells secrete various types of chemokines, cytokines, and growth factors to affect the migration, proliferation and osteogenic differentiation of transplanted and host stem cells [8]. As the main cellular component of the innate immune system, monocytes and their successors macrophages and osteoclasts, have shown to play a pivotal role in orchestrating the inflammatory response, mesenchymal stromal cell (MSC) differentiation and further bone formation [9]. With recent findings of heterogeneous macrophage phenotypes and anabolic functions of osteoclasts, utilization of immune cells, in particular monocytes, macrophages and osteoclasts in BTE has become an appealing approach. From a practical view, monocytes/macrophages are one of the most abundant cells in patients and have a high proliferative ability and great phenotypic plasticity. Also, monocytes/macrophages are an accessible type of endogenous cells as their isolation is possible from blood, which is a standard procedure in and even outside hospitals. As such, using these cells is relatively less invasive, time-saving and more patient-friendly than harvesting other cell types. Further, monocytes, macrophages, and osteoclasts naturally appear around bone graft materials to become involved in the inflammatory response and bone healing process and display distinct pro-osteogenic effects on MSCs [9, 10]. Taken together, these aspects seem appealing for an exploration into the role of these immune cells in therapeutic strategies for bone regenerative applications.

This thesis aimed to exploit the potential of cells of the innate immune system, in particular monocytes and macrophages, to aid in bone regeneration and the development of cell-based construct for BTE. Specifically, the applicability, heterogeneity, and effects on osteogenic cell differentiation in vitro and bone formation in vivo, were explored in a series of studies. Chapter 1 provides a general introduction to the challenges of current cell-based approaches in bone regeneration, functions of monocytes, macrophages, and osteoclasts in bone regeneration, and their potential for bone regeneration.
Each chapter after presents a separate study to address the research (sub)questions described in the last paragraph of Chapter 1 in a successive order.

(i) What is the state of and problem with current cell-based bone regenerative approaches and how can immune cells and other easily available cells be utilized for bone regeneration in an intraoperative manner?

Cell-based BTE has been developed in the last decades to overcome limitations associated with the use of auto- and allografts, which represent the current state-of-the-art clinical treatment for bone defects and fractures. However, numerous problems were encountered upon translation from proof-of-principle in the laboratory to clinical practice. In particular, the procedure of construct preparation by combining a scaffold with (expanded) autologous MSCs appeared to be impractical and not cost-effective compared to autologous bone transplantation. Consequently, an engineering strategy circumventing any ex vivo cell manipulation or additional cumbersome surgery for the patient would be advantageous. Hereto, intraoperative preparation of cell-based BTE constructs, in which cell-based constructs are prepared with easily accessible autologous cells within the same surgical procedure as the bone regenerative treatment itself, was recently proposed [11]. In Chapter 2, we evaluated the concept of intraoperative BTE construct preparation and highlighted the available cellular options, including bone marrow aspiration concentrate cells (BMACs), stromal vascular fraction (SVF), and immune cells, such as monocytes, macrophages, and lymphocytes. Furthermore, we summarized methods to prepare intraoperative constructs and reviewed data of currently available preclinical and clinical studies on this topic for efficacy in bone regenerative applications. Based on this review, we concluded that intraoperative preparation of cell-based BTE constructs is promising for (future) clinical applications, although several technical issues and regulatory hurdles need to be addressed. New technologies and methods, such as the invention of optimized cell isolation devices, the introduction of immune cells and utilization of advanced cell carriers, would simplify and broaden the opportunities to translate this concept for cell-based construct preparation into clinical treatments.

(ii) How do different macrophage subtypes modulate osteogenic differentiation of adipose tissue MSCs?

More knowledge about the biology of bone healing is desirable to develop novel strategies to improve bone defect treatment. In bone regeneration, macrophages are one of the central components of physiological response in the early stage and bone remodeling in the late stage. During this process, a switch of macrophage phenotype from pro-inflammatory (M1) to pro-wound healing (M2) is observed [12]. An appealing option for bone regeneration would be to exploit this regulatory role for the benefit of osteogenic differentiation of MSCs and to eventually utilize this knowledge to improve therapeutic options for bone defect treatment. As such, Chapter 3 focused on the in vitro interaction of differently activated and/or polarized macrophages with adipose tissue MSCs (ADMSCs) to monitor the behavior (i.e. proliferation, differentiation, and
Chapter 9

mineralization) of the latter in dedicated co-culture models. We found that co-culture of ADMSCs with M2 macrophages, but not with M1 macrophages or M0 macrophages, resulted in significantly higher mineralization, and that this effect resulted from cell-cell interactions via soluble factors. Specifically, M2 macrophages promoted the proliferation and osteogenic differentiation of ADMSCs, while M0 and M1 macrophages solely stimulated the osteogenic differentiation of MSCs in the early stage. These effects were attributed to oncostatin M (OSM) and endogenous bone morphogenetic protein 2 (BMP-2) signaling pathways. The observed effects of different macrophage types on osteogenic differentiation of MSCs extend the recognition of the role of macrophages from adverse effects in inflammation to beneficial effects in osteogenic differentiation, and can be capitalized for further improvements in the treatment of bone defects.

(iii) Does combining stromal vascular fraction or adipose MSCs with monocytes/macrophages enhance bone regeneration compared to conventional MSC-based approaches in a rat bone defect model?

Due to the beneficial roles of monocytes/macrophages and their different phenotypes in the osteogenic differentiation of ADMSCs demonstrated in vitro in chapter 3, we used stromal vascular fraction (SVF) and ADMSCs from human adipose tissue and human monocytes to intra-operatively prepare bone constructs and evaluated their bone regenerative capacity in vivo in Chapter 4. Compared to conventionally expanded ADMSCs, SVF displayed equal in vitro osteogenic differentiation, which for both was significantly enhanced upon co-culture with monocytes. Upon implantation in rat femoral condyle bone defects, constructs with grafted SVF demonstrated a significantly higher bone formation compared to constructs grafted with ADMSCs and cell-free controls, which was attributed to distinct differences between immunoregulatory effects of SVF and ADMSCs. Whereas in vitro cultures showed profound effects of monocytes on osteogenic differentiation of SFV and ADMSCs, in vivo addition of monocytes to SVF- or ADMSC-grafted constructs did not further enhance bone regeneration.

(iv) Can material physical and chemical properties affect macrophage phenotype?

As macrophage phenotype plays a crucial role in the osteogenic differentiation of MSCs in vitro and bone formation in vivo, we aimed to investigate to what extent physical and chemical surface properties affect the macrophage phenotype, with the ultimate aim to facilitate the design of biomaterial surfaces capable of generating desired inflammatory and immunological responses that enhance bone healing. In Chapter 5 & 6, we created and characterized a series of titanium surface, which differed in surface roughness at the submicron to micron level and was used without or with hydroxyapatite (HA) and β-TCP coating. Human THP-1 derived macrophages were cultured on these different surfaces next to conventional M1 and M2 macrophage controls on glass slides. Our data demonstrated that chemistry rather than roughness remarkably affected macrophage adhesion. Compared to control M1 and M2 macrophages, the presence of differential roughness or a coating led to macrophage polarization into a specific surface-associated state. Interestingly, arithmetic average surface roughness (Ra) from 0.51 to
1.36 μm revealed a tendency to polarize adherent macrophages toward M2 phenotype by downregulating pro-inflammatory and upregulating anti-inflammatory cytokine secretion, gene expression and surface marker expression. In contrast, the presence of an HA- and β-TCP- coating resulted in a hybrid macrophage subtype with both M1 and M2 characteristics and HA induced an earlier M1-M2 macrophage transformation than β-TCP. In conclusion, our data prove that adhesion and polarization of macrophages are critically influenced by surface physical and chemical properties.

(v) As the derivative of monocytes/macrophages, what is the role of osteoclasts in ectopic bone formation?
Except for differentiation into macrophages and polarization into different macrophage phenotypes, monocytes can fuse to form multinucleated osteoclasts. These osteoclasts represent one of the most important cells in regulating bone remodeling. Thus far, the majority of studies tried to induce bone formation by grafting MSCs, but these attempts resulted in unreliable and unsatisfactory bone forming and regenerative effects, particularly in clinical applications. Emerging data suggest that osteoclasts carry out crucial functions to initiate bone formation more than the intrinsic catabolic regulation of bone resorption [10, 13]. To prove this, in Chapter 7, we prepared constructs containing osteoclasts originating from either RAW264.7 or primary mouse macrophage by grafting these cells on bone substitute granulate. These osteoclast-based constructs were confirmed to have osteoclastic activity in vitro and were subsequently subcutaneously implanted to assess their capacity to induce bone formation in vivo. After 6 weeks, we observed new bone formation in 100% of the constructs containing osteoclasts originating from primary mouse macrophages, and in positive control constructs loaded with BMP-2. In contrast, both negative controls (i.e. bone substitute granulate only) and constructs containing osteoclasts originating from RAW264.7 macrophages did not show bone formation. Further histological and histomorphometrical evaluation demonstrated lower amounts and distinct bone patterns of newly formed bone in osteoclast-based constructs compared to positive controls. These data offer subversive insights into functions of osteoclasts in the biological context of bone initiation processes and open up a new dimension for designing and preparing BTE constructs for bone regenerative treatment.

(vi) How can we use material roughness to modulate osteoclast formation and characteristics to contribute to osteogenic differentiation?
Our finding in chapter 5 challenges the concept that osteoclasts only resorb mature bone during bone remodeling as bone-resorbing cells. Osteoclasts likely not only initiate the new bone formation in an ectopic environment, but also contribute to bone formation by communicating with osteoblastic cells in a process called “coupling of the bone formation to resorption” in the context of bone remodeling [13]. In this process, osteoclasts locally promote osteoblastic cell recruitment and osteogenic differentiation through the secretion of coupling factors. However, to what extent osteoclasts respond to material surface roughness and subsequently affect
osteoblastic cell behavior remains unclear. Therefore, in Chapter 8, we prepared and characterized titanium surfaces with a series of roughness ranging from submicron to micron levels and evaluated their effects on osteoclastogenesis (i.e. morphology, growth, and differentiation) using both the murine RAW264.7 cell line and primary mouse macrophages. We further evaluated the coupling function of surface roughness induced osteoclast effects on osteoblastic cell differentiation and then correlated in vitro mineralization with osteoclast characteristics. We found that surfaces with different roughness induced the formation of osteoclasts with distinct phenotypes, based on total osteoclast numbers, morphology, size, cytoskeletal organization, nuclei number per osteoclast, and osteoclastic gene expression. Furthermore, these different osteoclast phenotypes displayed differential anabolic effects toward the osteogenic differentiation of osteoblastic cells, which was revealed to logarithmically correlate with the nuclei number per osteoclast. Our results demonstrate the existence of a combinatorial effect of surface roughness, osteoclastogenesis, and osteogenic differentiation. These insights open up a new dimension for designing bone grafts by considering the material characteristics to locally regulate bone formation through coupling osteoclastogenesis with osteogenesis.

2. Closing remarks and future perspectives

During bone regeneration, innate immune cells not only directly differentiate into bone-resorbing cells to get involved in bone remodeling, but also affect osteoblastic cell recruitment, migration, and osteogenic differentiation to establish bone formation. This knowledge encourages us to make use of immune response-based therapeutic strategies in bone regeneration. Although several challenges still remain before our approaches can be applied in the clinic, this thesis contributes to a better understanding and application of using immune response-based strategies in bone regeneration to overcome the current limitations of tissue-engineered bone grafts. Based on obtained data, several major conclusions can be drawn:

(i) Different phenotypes of macrophages have distinct cytokine profiles and particularly exert differential effects on the behavior of adipose tissue MSCs. M2 macrophages can increase the osteogenic differentiation of co-cultured MSCs, and this effect is proportional to the ratio of M2 macrophages to MSCs. In contrast, M0 and M1 macrophages showed opposite effects at certain ratios.

(ii) SVF has equal in vitro osteogenic differentiation compared to expanded MSCs, which for both can be enhanced upon co-seeding with monocytes. However, although SVF is superior to adipose tissue MSCs in bone regeneration, the addition of monocytes to SVF- or ADMSC-grafted constructs does not further enhance bone regeneration in rat femoral bone defects. Furthermore, SVF has superior immunoregulatory effects to polarize M2 macrophages over osteogenically primed adipose tissue MSCs.

(iii) Both physical and chemical properties of material surfaces and stromal cell status affect the macrophage polarization. Rough surfaces ranging from 0.51 to 1.36 µm have a tendency to polarize macrophages into M2 phenotype, but not smoother and rougher surfaces. The presence of HA and β-TCP elicits a decreased proliferation of
macrophages and a non-typical, hybrid macrophage phenotype with both M1 and M2 profiles compared to grit-blasted Ti. Furthermore, HA induced an earlier M1 macrophage polarization and an earlier M1-M2 macrophage transformation than β-TCP.

(iv) Osteoclasts, beyond their recognized bone-resorbing capacity, have the capacity to initiate ectopic bone formation. Additionally, osteoclast behavior and their anabolic effects can be modulated by the material physical properties, i.e. surface roughness. Surface roughness differences led to the formation of distinct types of osteoclasts, and their anabolic effects on the osteogenic differentiation of MSCs logarithmically correlate with the nuclei number per osteoclast.

Although our results provide a wealth of information for functions of cells of the innate immune system (i.e. monocytes, macrophages and osteoclasts) in BTE based bone regeneration, a number of limitations and challenges need to be addressed and investigated in the future for the ultimate success of using immune response-based approaches for bone regeneration.

First of all, as we observed, monocytes, macrophages, and osteoclasts are sensitive to the microenvironment they reside in. Both material physical and chemical properties affect monocyte activation, macrophage polarization and osteoclast behavior [14]. Furthermore, the surrounding cells, including MSCs and endothelial cells, can exert differential effects on these cells [15, 16]. This highlights the importance of incorporating rational control and modulation, and importantly not elimination, of immune cell behavior into the design of BTE graft materials. In addition, it reminds us that a comprehensive characterization of material properties and cell status is urgently required when we carry out studies on monocytes, macrophage, and osteoclasts. For example, modifying the material surface roughness probably also changes the surface hydrophilicity, which also affects (immune) cell adhesion and activation. In addition, even the source of culture medium used in (immune) cell culture was reported to affect the behavior of monocytes and macrophages [17]. In addition, we currently draw the conclusion of macrophage polarization based on the conventional classification of cytokine-induced macrophage phenotypes. However, specific material-activated macrophage phenotypes were observed in our and other studies, indicating the conventional classification of macrophages probably to be outdated. In the future, a detailed characterization of macrophage and osteoclast profiles and functions facing to different material properties would greatly aid our understanding of roles of immune cells in bone regeneration, but also aid in the development of favorable scaffolds to promote desired immune responses for bone regeneration.

Second, according to our discrepant findings of effects of monocytes/macrophages on the osteogenic differentiation of MSCs in vitro and bone formation in vivo, it is important to keep in mind that gaps exist between lab work, animal studies, and clinical application. In our studies, the question whether material properties and MSCs similarly affect macrophage polarization and osteoclast behavior in vivo remains unanswered. New technologies such as nonlinear intravital microscopy [18] and nano-labeling [19] would characterize the participating macrophage populations, identify their origin and clarify their contributions to the decision of what repair mechanism
is initiated during bone regeneration. Such information will greatly contribute to the development of BTE constructs using immune cells. Similarly, in chapter 6, we found primary mouse osteoclasts rather than RAW264.7 derived osteoclasts to induce new bone formation when implanted ectopically in mice. This highlights the difference between cell lines and primary cells and the importance of choosing animal models. Given this information, follow-up studies with human cells in an orthotopic model will strengthen our demonstrated role of osteoclasts in bone formation.

Third, as a crucial part of the immune system, the adaptive immune response is not considered here due to the complexity of adaptive system and utilization of human cells in animal studies. Indeed, several studies have shown the various effects of adaptive immune cells during the healing process of a fractured bone. CD8+ T cells as part of the adaptive immune response were found to negatively influence wound healing. In contrast, CD4+ T cells can increase the osteogenic differentiation of MSCs [7]. The latter could hence be a promising therapeutic target in bone healing, because the bone marrow microenvironment has a higher percentage of CD8+ and CD4+ T cells compared to other tissues. When applying immune cell-based bone regeneration in the clinic, these beneficial or detrimental effects need to be taken into consideration. Additionally, modulating the T-cell response away from Th1 effector function and toward a Th2-tolerant phenotype by harnessing the macrophage M2 phenotype is becoming appealing and worthy of further investigation.

Last, when considering and using advantageous effects of innate immune cells on bone regeneration, the inflammatory response, also evoked by innate immune cells may exert negative effects on the survival and performance of BTE-based constructs. Previous studies have demonstrated that the inflammatory response and insufficient vascularization are the main limitations of cell-based BTE approaches for bone regeneration after constructs implantation [5]. Here, we also found that RAW264.7 derived macrophages evoked an intense inflammation when subcutaneously implanted in nude mice. All this information indicates that immune cells are a double-edged sword and a balanced inflammatory response is desired for the superior bone regeneration capacity. In future studies, we can focus on finding the optimal inflammatory environment for bone regeneration and controlling the inflammatory response after construct implantation. All these targets depend on a deep understanding of the bone healing process and function of immune cells in bone formation and also the advanced techniques to control cell behavior, bone remodeling microenvironment in vivo. Therefore, scaffolds for bone regeneration should shift from traditionally immune-inert to immunomodulatory, which allows for desired cell-specific responses that accelerate bone regeneration.

In summary, bone regeneration via modulating the immune response and using innate immune cells involves many factors, including cell types, scaffold surface properties, animal models and interaction of these factors. Given the present time, abundance and versatile functions, manipulation of immune cells as an apex regulator appears to be a more successful approach in bone regeneration compared to conventional approaches that target downstream effector cells and/or molecular mediators. Although several
Summary, closing remarks and future perspectives

Limitations and challenges exist for the translation of this approach toward the clinic, immune cell-based bone regeneration holds great promise for the future treatment of bone fracture and defects. To achieve this, an increasing knowledge of stem cells and immune cells, a profound advance of materials science, a thorough understanding of cell-material interactions, and a comprehensive investigation of this approach in pre-clinical and clinical studies is required. This needs collaboration with many disciplines including physiology, cell biology, immunology, material science, chemical engineering and clinical feedbacks in an iterative manner.
References


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Summary, closing remarks and future perspectives

1. Samenvatting

Om de nadelen van de huidige botvervangers tegen te gaan, maakt bot tissue engineering (BTE) gebruik van lichaamseigen stamcellen en wordt derhalve gezien als een veelbelovende techniek voor de behandeling van botdefecten. Meerdere pre-klinische [1] en klinische [2] studies hebben aangetoond dat celgebaseerde BTE constructen daadwerkelijk botdefecten en beschadigd botweefsel kunnen regenereren. Daarentegen zijn er ook verschillende studies die geen botvorming of -regeneratie laten zien met het gebruik van celgebaseerde BTE constructen [3,4]; mogelijk zijn er daarnaast veel ongepubliceerde data met gelijkaardige resultaten. Tenslotte is de tegenstelling m.b.t. botregeneratieve capaciteit tussen pre-klinische en klinische studies lastig te verklaren en duidt dit op substantiële verschillen in botgenezing en -regeneratie met het gebruik van humane cellen tussen patiënten en beschikbare proefdiermodellen. Enkele ernstige uitdagingen beperken de werkzaamheid van celgebaseerde BTE constructen voor klinische botregeneratie, waaronder immunologische reacties en gebrek aan vascularisatie na implantaat [5,6]. Enerzijds zijn immuuncellen betrokken bij de ontstekingsreactie die essentieel is voor weefselregeneratie, maar deze cellen tasten mogelijk tevens de levensvatbaarheid van de getransplanteerde cellen aan [7]. Anderzijds scheiden immuuncellen veel chemokines, cytokines en groeifactoren uit die migratie, proliferatie en osteogene differentiatie van getransplanteerde eigen cellen beïnvloeden [8]. Als belangrijkste cellulaire component van het aangeboren immuunsysteem hebben monocyten (en daaruit voortkomende macrofagen en osteoclasten) aangetoond een centrale rol te spelen in het verloop van ontstekingsreacties, de differentiatie van mesenchymale stromale cel (MSC) differentiatie en botvorming [9]. Vanwege het recente bewijs voor heterogeniteit binnen macrofaag fenotype en de anabole functies van osteoclasten, is het gebruik van immuuncellen, met name monocyten, macrofagen en osteoclasten, in BTE interessant. Vanuit praktisch oogpunt zijn monocyten/macrofagen een veelvoorkomend celtype in patiënten met een hoog proliferatief vermogen en aanzienlijke fenotypische plasticiteit. Daarnaast zijn monocyten/macrofagen redelijk eenvoudig te winnen omdat ze uit bloed geïsoleerd kunnen worden, waaraan de standaard procedure van bloedafname voor nodig is binnen of eventueel buiten ziekenhuizen. Hierdoor vereist het gebruik van deze cellen relatief minimaal invasieve procedures, kost het weinig tijd en is het patiënt-vriendelijk in vergelijking met andere celltypes. Bovendien komen monocyten, macrofagen en osteoclasten van nature voor in de nabijheid van botvervanginge materialen tijdens de ontstekingsreactie en botregeneratie, waarbij ze specifieke pro-osteogene effecten hebben op MSCs [9,10]. Samenvattend is er daarmee rechtvaardiging voor het onderzoeken van de rol van deze immuuncellen t.b.v. therapeutische strategieën voor botregeneratie.

Dit proefschrift had als doel gebruik te maken van de potentie van cellen van het aangeboren immuunsysteem, specifiek monocyten en macrofagen, om bij te dragen aan botregeneratie en de ontwikkeling van cel-gebaseerde constructen voor BTE. Meer specifiek werd de toepasbaarheid, heterogeniteit en de effecten van deze cellen op osteogene differentiatie van cellen in vitro en botvorming in vivo onderzocht in...
verschillende experimentele studies. **Hoofdstuk 1** geeft een algemene introductie m.b.t. de uitdagingen van huidige cel-gebaseerde ontwikkelingen voor botregeneratie, de functie van monocyten/macrofagen/osteoclasten in botregeneratie en de potentie daarvan voor botregeneratie. Ieder volgend hoofdstuk beschrijft vervolgens een separate studie met een onderzoeksvraag zoals omschreven in de laatste paragraaf van **Hoofdstuk 1**.

(i) *Wat is de status van en problematiek met huidige cel-gebaseerde botregeneratie en hoe kunnen immuuncellen (en andere eenvoudig te winnen cellen) gebruikt worden voor botregeneratie op een intra-operatieve manier?*

Cel-gebaseerde BTE is ontwikkeld in de laatste decennia om de tekortkomingen m.b.t. het gebruik van autoloog en allogeen bot te ondervangen, welke de huidige state-of-the-art zijn voor klinische behandeling van botdefecten en -fracturen. Echter, veel problemen komen kijken bij de translatie van experimentele proof-of-principle (binnen het laboratorium) naar de klinische praktijk. Vooral de procedure om een construct te maken middels de combinatie van een scaffold met (vermeerderde) autologe MSCs blijkt niet praktisch en kosteneffectief t.o.v. autologe bottransplantatie. Een engineering strategie zonder ex vivo manipulatie of additionele chirurgische ingrepen bij de patiënt is derhalve gewenst. De intra-operatieve preparatie van cel-gebaseerde BTE constructen, waarbij de constructen gemaakt worden met eenvoudig te winnen autologe cellen binnen dezelfde chirurgische procedure als de botregeneratieve ingreep zelf, is recent voorgesteld [11]. In **Hoofdstuk 2** hebben we het concept van intra-operatieve BTE construct preparatie geëvalueerd en de cellulaire opties, inclusief bone marrow aspiration concentrate cells (BMACs), stromal vascular fraction (SVF) en immuuncellen (bijv. Monocyten, macrofagen en lymfocyten), aangegeven. Daarnaast hebben we 2 methoden van intra-operatieve BTE construct preparatie samengevat en een overzicht gegeven van beschikbare data op dit gebied betreffende pre-klinische en klinische studies naar werkzaamheid voor botregeneratieve toepassingen. Op basis van dit overzicht hebben we geconcludeerd dat intra-operatieve preparatie van cel-gebaseerde BTE constructen veelbelovend is voor (toekomstige) klinische toepassingen, maar dat enkele technische en regulatoire aspecten opgelost moeten worden. Nieuwe technologieën en methodes, bijv. de ontwikkeling van verbeterde cel-isolatie apparatuur, het introduceren van immuuncellen en het gebruik van geavanceerde scaffolds, zouden de mogelijkheden vergroten om dit concept te transleren naar klinische behandeling.

(ii) *Hoe moduleren verschillende macrofaag subtypes de osteogene differentiatie van vetweefsel MSCs?*

Meer kennis over de biologie van botgenezing is gewenst om nieuwe strategieën te ontwikkelen ter verbetering van behandelingen voor botdefecten. Tijdens botregeneratie zijn macrofagen een van de centrale componenten binnen de fysiologische reactie in een vroeg stadium en botremodelering in een laat stadium. Tijdens dit proces vindt een omschakeling in macrofaag fenotype plaats van pro-inflammatoir (M1) naar
Summary, closing remarks and future perspectives

Pro-wondgenezing (M2) [12]. Een interessante mogelijkheid voor botregeneratie is het gebruik van deze omschakeling t.b.v. osteogene differentiatie van MSCs en hiermee uiteindelijk de therapeutische opties voor de behandeling van botdefecten te verbeteren. **Hoofdstuk 3** richt zich op de in vitro interactie van verschillend geactiveerde/gepolariseerde macrofagen met vetweefsel MSCs om het gedrag (proliferatie, differentiatie en mineralisatie) ervan te bestuderen in cokweek-modellen. Onze resultaten lieten zien dat cokweek van MSCs met M2-macrofagen, maar niet met M1 of M0 macrofagen, resulteert in significant verhoogde mineralisatie, en dat dit effect wordt veroorzaakt door cel-cel interacties middels oplosbare factoren. M2 macrofagen bevorderden de proliferatie en osteogene differentiatie van MSCs, terwijl M0 en M1 macrofagen enkel de osteogene differentiatie in een vroeg stadium stimuleerden. Deze effecten werden toegeschreven aan oncostatin M (OSM) en endogene BMP2 signaling. De waargenomen effecten van verschillende typen macrofaag op osteogene differentiatie van MSCs versterkt de erkenning van macrofagen als belangrijke cellen voor osteogene differentiatie en kunnen gebruikt worden voor verbeteringen t.b.v. de behandeling van botdefecten.

(iii) Verbeter de combinatie van SVF of vetweefsel MSCs met monocyten/macrofagen botregeneratie in vergelijking met conventionele MSC-gebaseerde constructen in een rat botdefect model?
Vanwege de gunstige effecten van monocyten/macrofagen en hun verschillende fenotypes op de osteogene differentiatie van vetweefsel MSCs zoals aangetoond in hoofdstuk 3, hebben we SVF en vetweefsel MSCs gebruikt samen met humane monocyten voor intra-operatieve preparatie van cel-gebaseerde constructen en het evalueren van de botregeneratieve capaciteit daarvan in vivo in **Hoofdstuk 4**. Vergelijken met conventioneel vermeerderde vetweefsel MSCs vertoonde SVF een gelijke in vitro osteogene differentiatie, welke voor beide celtypen werd gestimuleerd door cokweek met monocyten. In vivo implantatie in kniedefecten van ratten toonde dat SVF-constructen significant meer botvorming opleveren dan MSC-constructen en celvrije controles, hetgeen werd toegeschreven aan specifieke verschillen tussen immunoregulatoire effecten van SVF en MSCs. Hoewel in vitro het effect van monocyten op osteogene differentiatie overduidelijk was, bleek in vivo toevoeging van monocyten aan SVF- of MSC-constructen geen verdere verbetering van de botregeneratie op te leveren.

(iv) In hoeverre beïnvloeden fysische en chemische materiaaleigenschappen het fenotype van macrofagen?
Gezien de belangrijke rol van macrofaag fenotype in de osteogene differentiatie van MSCs in vitro en botvorming in vivo stelden wij ons tot doel om te onderzoeken in hoeverre fysische en chemische materiaaleigenschappen het macrofaag fenotype beïnvloeden; het uiteindelijke doel hiervan is gericht op het ontwikkelen van biomateriaal oppervlakken met het vermogen om gewenste ontstekings- en immuunreacties op te wekken teneinde botgenezing te bevorderen. In **Hoofdstukken 5 & 6**
hebben we verschillende titaanoppervlakken gemaakt en gekarakteriseerd door ruwheid te variëren op submicron en micron niveau en het al dan niet deponeren van een keramische coating. Humane THP-1-afgeleide macrofagen werden hierop gekweekt naast conventionele M1 en M2 macrofagen controles op glas. Onze data tonen dat vooral chemische eigenschappen de adhesie van macrofagen beïnvloedt. T.o.v. controle M1 en M2 macrofagen liet ruwheid of een coating een specifiek effect zien op macrofaag polarisatie. Een ruwheid Ra van 0.51-1.36 µm induceerde polarisatie in de richting van M2 vanwege het verminderen van pro-ontstekings- en verhoging van anti-ontstekingsfactoren, gen expressie en expressie van oppervlakte-receptoren. Anderzijds zorgde een keramische coating voor een hybride macrofaag fenotype met zowel M1 als M2 kenmerken, waarbij HA eerder een M1-M2 transformatie induceerde dan β-TCP. Samenvattend lieten onze data zien dat adhesie en polarisatie van macrofagen sterk afhankelijk zijn van fysische en chemische eigenschappen van het oppervlak waarop ze gekweekt worden.

(v) Als een afgeleide van monocyten/macrofagen, wat is de rol van osteoclasten in ectopische botvorming?
Naast differentiatie tot macrofaag en polarisatie tot een specifiek macrofaag fenotype, kunnen monocyten ook fuseren tot meerkernige osteoclasten. Deze osteoclasten zijn een van de belangrijke celtypen in de regulatie van botremodelering. Tot dusver heeft veel onderzoek zich gericht op het gebruik van MSCs om botvorming te induceren, maar deze inspanningen zijn veelal onbetrouwbaar gebleken met onbevredigende botvormings- en regeneratieve effecten, vooral in klinische toepassingen. Recent onderzoek heeft suggereerd dat osteoclasten naast de catabolische functie in botresorptie ook een belangrijke rol spelen bij de initiatie van botvorming [10,13]. Om dit te bewijzen hebben we in Hoofdstuk 7 constructen gemaakt met osteoclasten afkomstig van RAW264.7 cellijn of primaire muis macrofagen en botvervangend granulaat. Deze osteoclast-constructen vertoonden osteoclastische activiteit in vitro en werden vervolgens onderhuids geïmplanteerd in naakte muizen om hun osteoinductieve capaciteit te bepalen. Na 6 weken zagen we de novo botvorming in constructen met primaire osteoclasten en positieve controles geladen met BMP2. Echter, botvorming was niet aanwezig in de negatieve controles (alleen granulaat), noch constructen met osteoclasten afkomstig van RAW264.7 cellijn. Histologische en -morfometrische evaluatie toonde verder verschillen aan in hoeveelheid de novo botweefsel en het patroon van het gevormde botweefsel tussen primaire osteoclasten constructen en positieve controles. Deze bevindingen ondermijnen de huidige inzichten betreffende de functie(s) van osteoclasten in de context van biologische initiatie van botvorming en geven een nieuwe dimensie aan het design en de preparatie van BTE-constructen voor botregeneratieve behandelingen.

(vi) Kan biomateriaal ruwheid het gedrag van osteoclasten beïnvloeden en bijdragen aan botregeneratie?
Onze resultaten van hoofdstuk 5 tonen dat osteoclasten meer doen dan alleen botweefsel
resorberen gedurende botremodelering. Behalve de novo botvorming induceren op ectopische locaties, dragen ze ook bij aan de botvorming via communicatie met botvormende cellen [13]. In dit proces promoten osteoclasten lokaal de rekrutering en osteogene differentiatie van botvormende cellen via de secretie van factoren. Echter, in hoeverre osteoclasten reageren op oppervlakteruwheid en daaropvolgend gedrag van botvormende cellen reguleren is onbekend. Daartoe hebben we in Hoofdstuk 8 titaanoppervlakken ruw gemaakt op submicron en micron schaal en bestudeerd wat het effect daarvan is op osteoclastogenese (morfologie, groei en differentiatie) van RAW264.7 cellijn en primaire muis macrofagen als voorlopers van osteoclasten. Vervolgens hebben we gekeken naar de correlatie tussen afgegeven factoren met osteogene differentiatie stimulatie en osteoclast kenmerken. Onze data toonden dat ruwheidsafhankelijke fenotypes van osteoclasten gevormd werden met verschillen in totaal aantal osteoclasten, morfologie, grootte, celskelet organisatie, aantal kernen per osteoclast, en genexpressie. Bovendien zorgden deze verschillende osteoclast fenotypes voor verschillende anabole effecten op de osteogene differentiatie van botvormende cellen, hetgeen een correlatie liet zien met het aantal kernen per osteoclast. Daarmee tonen onze data het bestaan van een gecombineerd effect van ruwheid, osteoclastogenese en osteogene differentiatie. Deze data verschaffen nieuwe inzichten voor de ontwikkeling van botvervangende materialen gebaseerd op materiaalkenmerken als regulerende factor voor botvorming via de koppeling van osteoclastogenese met botvorming.

2. Afsluitende opmerkingen en toekomstperspectieven
Tijdens botregeneratie differentiëren cellen van het aangeboren immuunsysteem niet alleen naar botresorberende cellen als speler in botremodelering, maar deze cellen beïnvloeden tevens de rekrutering, migratie en osteogene differentiatie van botvormende cellen om botvorming tot stand te brengen. Deze kennis stimuleert ons om therapeutische, botregeneratieve strategieën na te streven o.b.v. immuno-modulering. Hoewel meerdere uitdagingen overwonnen moeten worden voordat toepassing ervan in de kliniek mogelijk is, draagt dit proefschrift bij aan het begrijpen en toepassen van dit nieuwe concept voor botregeneratie om de beperkingen van huidige tissue engineered constructen te verminderen. Onze data leiden tot de volgende conclusies:

(i) Verschillende fenotypen van macrofagen hebben verschillende cytokineprofielen en oefenen in het bijzonder verschillende effecten uit op het gedrag van vetweefsel MSCs. M2-macrofagen kunnen de osteogene differentiatie van gelijktijdig gekweekte MSCs verhogen, en dit effect is evenredig met de verhouding van M2-macrofagen tot MSCs. Daarentegen toonden M0- en M1-macrofagen tegengestelde effecten bij bepaalde verhoudingen.
(ii) SVF heeft een gelijke in vitro osteogene differentiatie in vergelijking met vermeerdere MSCs, die voor beide kunnen worden verbeterd via cokweek met monocyten. Hoewel SVF superieur is aan MSCs van vetweefsel in botregeneratie, verbetert de toevoeging
van monocyten aan SVF- of ADMSC-geënte constructen niet verder de botregeneratie bij femorale botdefecten in de rat. Verder heeft SVF superieure immunoregulatoire effecten om M2-macrofagen te polariseren in vergelijking met MSCs uit vetweefsel.

(iii) Zowel fysische als chemische eigenschappen van materiaaloppervlakken en de stromale celstatus beïnvloeden de macrofaagpolarisatie. Ruwe oppervlakken van 0,51 tot 1,36 µm hebben de neiging om macrofagen in het M2-fenotype te polariseren, maar gladdere en ruwere oppervlakken niet. De aanwezigheid van HA en β-TCP roept een verminderde proliferatie van macrofagen en een niet-typerend hybride macrofagen fenotype met zowel M1- als M2-profielen op in vergelijking met met gestraald Ti. Verder induceerde HA een vroegere M1-macrofaagpolarisatie en een vroegere M1-M2-macrofaagtransformatie dan β-TCP.

(iv) Osteoclasten hebben, buiten hun erkende botresorptiecapaciteit, het vermogen om ectopische botvorming te initiëren. Bovendien kunnen osteoclastgedrag en hun anabole effecten worden gemoduleerd door de materiële fysische eigenschappen, d.w.z. oppervlakteruwheid. Verschillen in oppervlakteruwheid leiden tot de vorming van verschillende soorten osteoclasten en hun anabole effecten op de osteogene differentiatie van MSCs correleren logaritmisch met het aantal kernen per osteoclast. Hoewel onze resultaten een schat aan informatie bieden voor functies van cellen van het aangeboren immuunsysteem (bijv. monocyten, macrofagen en osteoclasten) in BTE-gebaseerde botregeneratie, moeten een aantal beperkingen en uitdagingen in de toekomst worden aangepakt en onderzocht voor het uiteindelijke succes van het gebruik van op het immuunsysteem gebaseerde conceptuele benaderingen voor botregeneratie.

Allereerst, zoals we hebben gezien, zijn monocyten, macrofagen en osteoclasten gevoelig voor de micro-omgeving waarin ze zich bevinden. Zowel materiële fysische als chemische eigenschappen van materiaaloppervlakken beïnvloeden monocytenactivering, macrofaagpolarisatie en osteoclastgedrag [14]. Bovendien kunnen de omliggende cellen, inclusief MSCs en endotheelcellen, differentiële effecten op deze cellen uitoefenen [15, 16]. Dit benadrukt het belang van het opnemen van rationele controle en modulatie, en vooral niet eliminatie, van immunocelgedrag in het ontwerp van BTE-transplantaatmaterialen. Bovendien herinnert het ons eraan dat een uitgebreide karakterisering van materiaaleigen schappen en celstatus dringend vereist is wanneer we onderzoeken uitvoeren op monocyten, macrofagen en osteoclasten. Het modificeren van de oppervlakteruwheid van het materiaal verandert bijvoorbeeld waarschijnlijk ook de hydrofilititeit van het oppervlak, die ook de (immuun) cellhechting en -activering beïnvloedt. Bovendien werd gerapporteerd dat zelfs de bron van het kweekmedium gebruik in (immuun) celweek het gedrag van monocyten en macrofagen beïnvloedde [17]. Bovendien trekken we momenteel de conclusie van macrofaagpolarisatie op basis van de conventionele classificatie van cytokine-geïnduceerde fenotypes van macrofagen. Specifieke, door materiaal geactiveerde fenotypes van macrofagen werden echter waargenomen in onze en andere onderzoeken, hetgeen aangeeft dat de conventionele indeling van macrofagen waarschijnlijk verouderd is. In de toekomst zou een gedetailleerde karakterisering van macrofaag- en osteoclastprofielen en -functies tegenover ver-
Summary, closing remarks and future perspectives

schillende materiaaleigenschappen ons begrip van de rol van immuuncellen bij de botregeneratie ten zeerste helpen, maar ook helpen bij de ontwikkeling van effectieve scaffolds om de gewenste immuunresponse voor botregeneratie te bevorderen.


Ten derde, als een cruciaal onderdeel van het immuunsysteem, wordt de adaptieve immuunrespons hier niet beschouwd vanwege de complexiteit van het adaptieve systeem en het gebruik van menselijke cellen in dierstudies. Inderdaad, verschillende onderzoeken hebben de verschillende effecten van adaptieve immuuncellen aangetoond tijdens het genezingsproces van een bot. CD8+ T-cellen als onderdeel van de adaptieve immuunrespons blokken de genezing van wonden negatief te beïnvloeden. CD4+ T-cellen daarentegen kunnen de osteogene differentiatie van MSCs verhogen [7]. Het laatste kan daarom een veelbelovend therapeutisch doel zijn bij botgenezing, omdat de micro-omgeving van het beenmerg een hoger percentage CD8+ en CD4+ T-cellen heeft in vergelijking met andere weefsels. Bij het toepassen van onze op het immuunsysteem gebaseerde botregeneratie in de kliniek, moeten deze gunstige of nadelige effecten in aanmerking worden genomen. Bovendien wordt het moduleren van de T-celreactie weg van de Th1-effectorfunctie en naar een Th2-tolerant fenotype door het macrofagen M2-fenotype te gebruiken aantrekkelijk en verdient verder onderzoek de voorkeur.

Als laatste, bij het overwegen en gebruiken van voordelige effecten van aangeboren immuuncellen op botregeneratie, kan de ontstekingsreactie, ook opgewekt door aangeboren immuuncellen, negatieve effecten hebben op de overleving en prestaties van op BTE-gebaseerde constructen. Eerdere studies hebben aangetoond dat de inflammatoire respons en onvoldoende vascularisatie de belangrijkste beperkingen zijn van cel-gebaseerde BTE-benaderingen voor botregeneratie na implantaatie van constructen [5]. Hier vonden we ook dat RAW264.7-afgeleide osteoclasten een intense ontsteking opriepen na subscutane implantaatie in naakte muizen. Al deze informatie
geeft aan dat immuuncellen een tweesnijdend zwaard zijn en een uitgebalanceerde ontstekingsreactie gewenst is voor de superieure botregeneratiecapaciteit. In toekomstige studies kunnen we ons concentreren op het vinden van de optimale ontstekingsomgeving voor botregeneratie en het beheersen van de ontstekingsreactie na constructimplantatie. Al deze doelen zijn afhankelijk van een diep begrip van het botgenezingproces en de functie van immuuncellen in botvorming en ook de geavanceerde technieken om celgedrag, botremodellerende micro-omgeving in vivo te controleren. Daarom moeten scaffolds voor botregeneratie verschuiven van traditioneel immuun-inert naar immuunmodulerend, wat zorgt voor gewenste celspecifieke responsen die de botregeneratie versnellen.

Samenvattend, botregeneratie door het moduleren van de immuunrespons en het gebruik van aangeboren immuuncellen omvat vele factoren, waaronder celtypes, oppervlakte eigenschappen van scaffolds, diermodellen en interactie van deze factoren. Gezien de huidige tijd, overvloed en veelzijdige functies lijkt manipulatie van immuuncellen als een apex-regulator een meer succesvolle benadering in botregeneratie vergeleken met conventionele benaderingen die stroomafwaartse effectorcellen en/of moleculaire mediatoren als doel hebben. Hoewel er verschillende beperkingen en uitdagingen bestaan voor de vertaling van deze benadering naar de kliniek, vormen immuuncel-gebaseerde concepten voor botregeneratie een grote belofte voor de toekomstige behandeling van botbreuken en -defecten. Om dit te bereiken is een toenemende kennis van stamcellen en immuuncellen, een grondige vooruitgang van materiaalwetenschap, een grondig begrip van cel/materiaal interacties en een uitgebreid onderzoek van deze benadering in preklinische en klinische studies vereist. Dit vereist samenwerking tussen vele disciplines, waaronder fysiologie, celbiologie, immunologie, materiaalwetenschap, chemische technologie en klinische feedback op een iteratieve manier.
Summary, closing remarks and future perspectives

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Acknowledgements

List of publications

Curriculum Vitae
Chapter 9

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Summary, closing remarks and future perspectives

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**List of Publications**

**Related to this thesis:**
6. Zhang Y, Chen ES, Shao J, van den Beucken JJJP. Combinatorial surface roughness effects on osteoclastogenesis and osteogenesis. (submitted to *ACS Applied Materials & Interface*).

**Related to PhD projects:**
1. Zhang Y, Yang W, Devit A, van den Beucken JJJP. Efficiency of coculture with angiogenic cells or physiological BMP-2 administration on in vitro osteogenic differentiation and ectopic bone formation of MSCs. (submitted to *Stem Cell Research*).
Other Publications:
Summary, closing remarks and future perspectives

Curriculum Vitae

Yang Zhang was born on August 18, 1987 in Shiyan, Hubei Province, China. In 2010, he received the B.Sc. degree in Biotechnology with honors from Southwest University (Chongqing, China). In the same year, he was admitted as a recommended postgraduate in the State Key Laboratory of Silkworm Genome Biology (Chongqing, China) focusing on creating novel biomedical fibers by the transgenic technology and the self-assembly of natural fibroin polymers. After three years, he received the M.Sc. Degree in Cell Biology with honors from Southwest University (Chongqing, China).

In October 2013, he started his Ph. D. research at the Department of Biomaterials, Radboud University Medical Center (Radboudumc, Nijmegen, the Netherlands) under the supervision of Professor John Jansen and Dr. Jeroen van den Beucken. His project is mainly about unraveling interactions between immune cells, stem cells and materials to aid in bone regeneration. During his PhD studies, he was a visiting researcher at Johannes Gutenberg University Mainz (Mainz, Germany) and Capital Medical University (Beijing, China). Besides, he had the collaboration with Federal University of Sao Paulo (Brazil), Huazhong University of Science and Technology (China), University of Twente (the Netherlands) and University of Jaume I (Spain). The results of his PhD projects were described in this thesis and will be published as separate research articles in several scientific journals. Part of the results was presented in several international conferences, including 3rd Belgium Symposium on Tissue Engineering (Belgium, 2015), 10th World Biomaterials Congress (Canada, 2016), and the 6th International Conference on Tissue Engineering (Greece, 2017) where he received several Travel Awards. In 2018, he started working as a postdoctoral associate in Radboudumc and continued his research on stem cells and immunoengineering in regenerative medicine.
Never forget why you started, and your mission can be accomplished.

-- Avatamsaka Sutra