Modulation of the Immune Response in Strategies for Bone Regeneration

Michiel Croes

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INTRODUCTION

Regeneration of bone

The intrinsic healing capacity of bone

The bony skeleton supports the movement and mechanical stability of the body, protects the vital organs, and is also involved in different biological processes such as mineral metabolism and blood cell production. To perform these vital functions, bone has evolved into a tissue with a remarkable intrinsic capacity to regenerate after damage[1]. Unlike most other tissues, bone tissue is able to regenerate completely without the formation of scar tissue, so that the newly formed bone is normally indistinguishable from existing healthy bone[2].

Bone fracture healing is the most widely used model to study the biology of bone regeneration, and shows that this process largely involves the same well-orchestrated biological events as seen during development of the skeleton in the embryo[3]. Fractures most often heal in an indirect manner, whereby a fracture callus of provisional repair tissue is deposited first. Driven by the local and systemic factors expressed after injury, progenitor cells are recruited from various tissues to the injury site and differentiate into specialized matrix-producing chondrocytes and osteoblasts[4,5]. Chondrocytes replace the initial granulation tissue with a cartilaginous soft callus, which then calcifies, and functions to quickly stabilize the defect. After hypertrophy and mineralization of the cartilage template, infiltrating osteoblasts deposit an organic matrix composed of type I collagen, proteoglycans, and bone-specific proteins, that promotes formation of phosphate crystals that constitute the mineralized matrix of bone[6]. In addition to the indirect pathway of bone formation, resident cell populations that are already disposed to form bone can directly contribute to bone formation without generation of a cartilage precursor phase[1,6,7].

The mechanically-weak woven bone in the fracture callus provides initial stability to the defect, and is replaced into more highly organized and superior lamellar bone, whereby the bone density and architecture will adapt to the mechanical loads under which it is formed[8]. During adult life, this bone remodeling is a continuous process and involves a tight coordination between bone-forming osteoblasts and bone-resorbing osteoclasts. It has become well established that the immune system, from which the osteoclasts originate, is a critical regulator in the crosstalk between osteoblasts and osteoclasts during development, repair, and disease[9,10]. The interactions between the immune system and bone are studied in a scientific field coined ‘osteoimmunology’[11].

Failure of bone healing and the need for bone transplantations

Despite the intrinsic healing capacity of bone, failure of bone healing can require a surgical intervention to stabilize the defect and re-establish the mechanical and biological conditions needed for bone repair[12]. Incomplete bone fracture healing is observed in 5-15% of trauma patients, and is associated with risk factors such as comorbidities, high age, and unfavorable characteristics of the injury[13,14]. Augmentation of bone repair is also required in conditions where the amount of bone tissue formation needed simply exceeds the self-healing potential. In animal model research, such a lack of bone stock is mimicked with so called ‘critical size defects’, which are defined as the smallest size defect that will not naturally heal irrespective of elapsed time. For example, it is presumed that defects

in the long bones do not regenerate when their size is larger than roughly twice the bone diameter[15]. In addition to the repair of large bone defects, the surgical induction of bone formation outside the original bone margins may also be necessary, as is common in spinal fusion surgery[16].

Treatments that aim to restore non-healing bone defects or create new bone, mostly include the use of some form of bone graft[12]. Bone grafts can comprise autologous bone transplants or off-the-shelf substitutes such as fresh-frozen or processed allogeneic bone, natural or synthetic bone substitutes, or any combination of these with or without specific bone-stimulating factors[17]. They can possess different biological properties that contribute to bone formation[16,18]. Transplanted autologous bone is considered optimal since it firstly acts as a structural matrix for the migration, attachment and matrix formation by invading bone cells, a process called osteoconduction. Autologous grafts also contain native growth factors that regulate the recruitment of endogenous bone progenitor cells to the surgical site and the induction of differentiation into osteoblasts, a process called osteoinduction[19,20]. Viable bone (progenitor) cells present in fresh bone transplants likely also have paracrine effects such as the production of growth factors, cytokines or hypoxia-related factors supporting osteogenesis and/or angiogenesis[21,22]. Finally, the cells isolated from autograft bone also have an osteogenic potential when cultured *in vitro*[23], but the significance of this property for autografts' clinical effect is not yet established.

Current grafting options and the need for substitutes

Autologous bone surgically obtained from the patient's iliac crest (i.e. iliac crest bone graft, ICBG) is a widely used autologous bone graft due to its ease of harvesting and relative abundance[18]. It is estimated that more than a million ICBG procedures are performed each year worldwide[18]. Despite the effectiveness of ICBG, the amount of harvestable bone can be inadequate to meet the demand[24]. Additionally, there is an increased operation time and morbidity related to the harvesting procedure[25]. Locally-harvested autologous bone can be a replacement for ICBG in some cases, but is limited by the smaller volume[26].

There have been longstanding efforts to develop bone substitutes with similar or better biological properties than autologous bone, in particular for the use in spinal fusion surgeries. Since bone grafting can be considered a basic component of these surgeries, they account for half of all bone grafting procedures[27]. The aim of spinal fusions is to create a solid bridge between two or more vertebrae in order to stabilize the spinal column in case of an excessive motion in a spine segment or in spinal deformities at risk for progression[28]. The spinal fusion environment is a challenging bone healing environment, with a non-union rate of 5% to 45% even when ICBG is used as a graft[29]. Of the different surgical approaches (e.g. anterior, posterior) or fusion techniques (e.g. between or adjacent to the vertebral bodies)[28], posterolateral spinal fusions show the highest non-union rate. As the bone graft is placed adjacent to the vertebral bodies, the bridging area comprises a central zone that has limited or no contact with the underlying bone[29]. As a result, the posterolateral spine fusions generally fail to heal with osteoconductive materials alone[30].

Currently, different biological and synthetic off-the-shelf replacements of autograft are available for spinal fusions and other large defects, but are all considered inferior to ICBG (Figure 1). A popular alternative to autograft is allograft bone, which shares many characteristics and is widely available through the establishment of tissue banks[31]. As a

limitation however, the bone-inductive properties of allograft are reduced due to the absence of viable cells and diminished levels of bioactive growth factors[32,33]. Also, no donor matching is performed for allograft procedures, and it is uncertain how the remaining antigenicity affects the incorporation of the graft[34]. For large bone defects, allografts can be effective, but usually only when an osteoinductive stimulus such as local bone graft can be provided[35]. Allograft remains the most widely used bone substitute due to its off-the-shelf nature, good osteoconductive properties, and availability in various shapes and sizes[33]. The demineralization of allograft bone may render it more osteoinductive, presumably because growth factors present in the extracellular matrix become bioavailable. The osteoinductivity between demineralized bone matrix (DBM) products is however highly variable, and affected by the source of the bone and processing methods[19]. This tempers its use despite the clinical evidence for several indications[36].

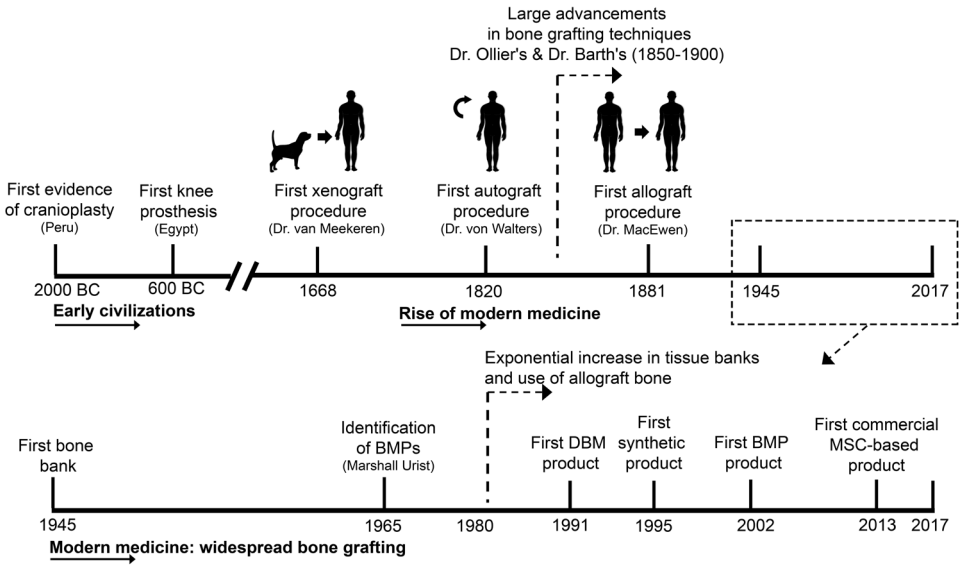

Figure 1. Timeline showing the history of different bone grafting techniques used in spinal fusion surgery.
BMP: bone morphogenetic protein, DBM: demineralized bone matrix, MSC: mesenchymal multipotent stromal cell.

The pillars of bone regenerative medicine

The use of osteoconductive biomaterials, osteoprogenitor cells, and osteoinductive stimuli, or the generation of the optimal mechanical environment, can all lead to a more favorable situation for bone formation[37]. These critical conditions for bone healing are described as the 'diamond concept'[12]. The next sections summarize the state of the art in these elements in the context of bone regenerative medicine. The role of mechanical stability is beyond the scope of this thesis and not further discussed.

Biomaterials

Numerous synthetic biomaterials have been developed that can be used as off-the-shelf bone graft[38,39]. Materials that mimic the inorganic phase and architecture of bone such as calcium sulphates /phosphates, or materials that can induce a beneficial cellular

response (e.g. bioactive glass or hyaluronic acid) have demonstrated the ability to augment bone formation[40]. This mainly occurs through an osteoconductive mechanism and their behavior can be addressed accordingly by changing material parameters such as chemical composition, macrostructure and surface (micro)structure[40].

There has been only a slight shift in the use of autologous bone towards the use of such bone substitutes, despite the widespread introduction of these products into clinical practice[27]. This is explained by the fact that the benefit of synthetic bone substitutes is controversial. The current clinical evidence only supports synthetic bone graft substitutes as autologous bone graft extenders[29,39].

As the role of synthetic grafts has changed from a more passive load-bearing role to a role where the material actively contributes to tissue healing by inducing a specific cellular response, several materials have been identified that have the capacity to induce heterotopic bone formation in selective animal models of subcutaneous or intramuscular implantation[41]. This material-associated osteoinduction is most frequently observed for calcium phosphate ceramics and may be relevant, since in preclinical models usually more bone formation is seen in bone defects treated with ceramics with an osteoinductive capacity compared to non-osteoinductive ceramics[42]. Nevertheless, the clinical effectivity of osteoinductive ceramics has yet to be established.

Current biomaterials require the use of additional osteoinductive components in large bone defects[43]. Until the exact properties are identified that can maximize a biomaterials' ability to induce bone formation, their further enhancement with bioactive agents is likely needed for them to have a merit as true off-the-shelf autograft replacement in a large healing environment. Traditionally, the performance of synthetic bone grafts is enhanced with cells or growth factors[44,45]. To retain the synthetic character of these biomaterials, and possibly avoid the costs and complications associated with cell-or growth factor-based treatments, more simple compounds such as bioinorganics are also being explored[46]. Furthermore, mediators that modulate the early immune response could enhance the bone-forming ability of synthetic bone grafts[47]. As the key parameters for biomaterial-associated osteoinduction are being investigated, there is increasing evidence that the early immune response to biomaterials is important for their *in vivo* ability to induce bone formation[48,49].

Cells

The different cell sources that contribute to osteogenesis and chondrogenesis during fracture healing include resident osteoblasts and tissue-specific progenitor cells derived from bone marrow, bone connective tissue linings and surrounding muscles[4,5]. *In vivo* tracking studies furthermore show that bone progenitor cells recruited from the circulation also engraft in the fracture callus[50]. Of the different bone progenitor cells, mesenchymal multipotent stromal cells (MSCs) are the most studied cell type in the field of bone regenerative medicine. MSCs form a major fraction of the heterogeneous population of plastic-adherent cells that can be isolated from various tissues - predominantly bone marrow and adipose tissue, and differentiate into osteoblasts under appropriate stimuli[51]. Through this process, native MSCs can contribute to bone fracture healing, with increasing evidence showing that MSCs mostly reside in the periphery of the vasculature and enter the injured site to facilitate regeneration[52].

MSCs can very effectively recreate bone tissue in small animal models following their implantation[53,54]. The mechanism of action of MSC-induced bone regeneration in tissue engineering is still under debate, and the original paradigm that transplanted cells undergo differentiation and morphogenesis to form new tissue has shifted towards a model where the function of MSCs in regeneration is complemented by their paracrine and immunomodulatory effects[55-57]. Clinical trials are currently investigating the efficacy of adipose tissue or bone-marrow derived MSCs from both allogeneic and autologous cell sources for various orthopedic applications[58,59]. Although a limited number of allogeneic MSC-based products are already commercially available[58], the ideal cell source and its efficacy still needs to be established[55,60]. Furthermore, the difficulty to obtain sufficient amount of MSCs remains a limiting factor for MSC-based therapies[58]. Since many of the factors expressed in the bone healing environment are known to affect the recruitment and differentiation of MSCs[61], endogenous progenitor cells can possibly be targeted *in vivo* to promote bone formation.

Growth factors

The signaling cascades regulating chondrogenesis, osteogenesis and angiogenesis in the normal bone-healing environment are activated by various growth factors that are expressed in a well-orchestrated manner[45]. In the initial phase, transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF) are released by platelets as soon as a blood clot is formed and provide migrational and mitogenic signals for leukocytes and MSCs[62]. Chondrogenic and osteogenic differentiation processes are subsequently regulated by TGF-β superfamily members, of which the bone morphogenetic proteins (BMPs) are crucial[6]. In addition, various growth factors mediate the necessary revascularization steps after disruption of the vasculature. In the first phase, Angiopoietin (Ang)-1, fibroblast growth factor (FGF), and PDGF act together with pro-inflammatory cytokines to re-establish normoxic conditions, remove debris and supply the fracture zone with cells. In a second phase, vascular endothelial growth factor (VEGF) expression plays a crucial role in the angiogenic response needed for endochondral bone formation[61,63,64].

The aforementioned growth factors have all been studied for their ability to augment bone formation processes in pre-clinical models[45]. In many cases, a combined growth factor delivery is superior to the delivery of a single factor[45], which seems to recapitulate the multitude of signalling cues present in the bone fracture healing environment[62]. Angiogenic factors such as PDGF, VEGF, or FGF show stimulatory effects on the healing of drill hole defects, fractures, and in ectopic bone formation models in rodents, but only when co-delivered with osteoinductive growth factors[45]. Similarly, chondrogenic growth factors such as TGF-β3 likely need a second osteogenic signal to increase their bone-enhancing efficacy[65,66].

To date, no growth factors have been able to match the BMPs in terms of bone induction. BMPs are key mediators in skeletal development, regeneration and disease by orchestrating tissue architecture, including the formation of cartilage and bone[67]. Of the more than 20 different BMPs identified, BMP-2 and 7 have shown to induce the differentiation of progenitor cells towards osteoblasts in isolated cell cultures[68]. BMP-2 is currently regarded as the single factor with the strongest pro-osteogenic effects. While most BMPs are not indispensable for fracture healing, even the early steps of fracture healing are already blocked in BMP-2 deficient mice[69]. BMP-2 also shows the largest

stimulatory effect on the osteogenic differentiation of osteoprogenitor cells[68], emphasizing the importance of osteoinductive stimuli for bone regeneration.

Following the discovery that recombinant human BMP-2 (rhBMP-2) produced by mammalian cells could induce ectopic bone formation in rodents[70], rhBMP-2 quickly made its way into clinical practice. In 2002, rhBMP-2 was approved as an autograft substitute for the treatment of anterior lumbar spinal fusion. The subsequent exponential increase in clinical rhBMP-2 use clearly illustrates the huge need for substitute grafts that can lower the reliance on autograft and potentially improve the fusion rates. During its peak in 2007, rhBMP-2 was already used in half of anterior lumbar spinal fusions in the USA[71]. Moreover, 85% of principal procedures using rhBMP-2 were for off-label applications in the spine[72]. Since a decade, there has been a decline in the clinical use of rhBMP-2 in indications such as the cervical spine, due to reports about serious side effects[73,74], including ectopic bone formation, inflammatory reactions, osteolysis, and even urogenital or neurological complications[75]. Furthermore, a discrepancy is observed between the effectiveness of rhBMP-2 when comparing pre-clinical and clinical data. In contrast to many animal studies that show superior spinal fusion rates with BMP-2 in a direct comparison to autologous bone[76,77], improved fusion rates with rhBMP-2 compared to ICBG is usually not observed in patients[25,78,79]. Finally, the high cost of the rhBMP-2 product relative to other bone substitutes can be a limiting factor to its use[80].

BMP-2 research is focused on its improved delivery, since the single ultra-high dose delivery that is currently applied is considered to be inefficient and related to complications[81,82]. The current dose is 1-2 mg/cc carrier based on studies in non-human primates[83,84]. This concentration highly exceeds the native concentration of BMPs in bone (in the order of ng/cc bone)[84,85], and furthermore, different cell types are already responsive to BMP-2 *in vitro* in this ng/ml range. Animal studies show that the incidence of side effects is decreased when lowering the BMP-2 concentrations[86,87]. However, an attempt to lower the BMP-2 dose from 1.5 mg/cc to 0.75 mg/ cc in patients resulted in a significant decrease in bone formation[88]. To induce the appropriate cellular responses, alternative strategies such as carriers for sustained release or gene therapy are explored[84]. The use of anti-inflammatory drugs or bisphosphonates may also further reduce the side effects and increase the efficacy of BMP-2[75]. In addition, their combined delivery with secondary stimuli needed for bone formation (i.e. cells, cytokines, growth factors) may be required, as a biological effect of BMP-2 can already be seen for a concentration as low as 25 ng/ml in co-delivery with MSCs in mice[89,90].

Based on the experience with BMPs, it can be questioned whether the use of a single growth factor in an ultra-high dose is a safe and reliable way to recapitulate the complex cascades involved in normal bone regeneration. The sequential or simultaneous delivery of growth factors such as BMPs with other factors expressed in the bone healing environment should therefore be investigated.

Harnessing inflammation for bone regeneration

Acute inflammation in bone regeneration

The healing of damaged bone largely recapitulates the process of skeletogenesis in the embryo[3,7]. The key processes involved are chondrogenesis, osteogenesis, angiogenesis, and remodeling. In contrast to the systemic osteogenic signaling seen during skeletogenesis, fracture healing is a process which depends on the local recruitment and differentiation of competent cells for healing[3]. Bone healing therefore relies on an acute inflammatory response to provide the essential local cues to initiate bone formation.

Several observations point to the fact that the acute inflammation established after bone damage holds strong regenerative effects. The initial fracture hematoma is a source of inflammatory cells and cytokines which subsequently interact with resident cells and infiltrating bone progenitor cells[1]. The finding that removal of the hematoma leads to delayed or hypertrophic non-unions[91], or that intramuscular implantation of the fracture hematoma induces bone formation[92], holds proof that the initial fracture hematoma contains essential bone-regenerative signals. The balanced inflammatory response after bone injury generally does not last more than 7 days and strongly contributes to bone formation[1,93]. Specific inflammatory signals are required, which is illustrated by the finding that suppression of the immune response with anti-inflammatory drugs hampers the bone healing process[1]. Systemic inflammatory diseases such as rheumatoid arthritis or temporary hyper-inflammation after polytrauma can lead to a reduced bone healing[1], showing the importance of a balanced local inflammatory response for new bone formation.

Inflammatory processes can also trigger excessive (usually clinically detrimental) bone formation in a pathological context[94-96]. Studying these conditions could lead to the identification of pro-osteogenic mediators that are either or not involved in physiological bone healing. Heterotopic ossification (HO), i.e. the abnormal formation of mature trabecular bone in soft tissues, can manifest after traumatic events including neurological injury, burns, and hip arthroplasties[97,98]. Animal models of HO show that the ossification can be a surprisingly robust and quick process[99,100]. The underlying mechanism of HO is poorly understood, but there is considerable evidence that local inflammation contributes to the process. For example, trauma-induced HO is enhanced when soft tissue inflammation is aggravated by bacterial inoculation[101]. Furthermore, the inhibition of the pro-inflammatory lipid prostaglandin E2 (PGE2) prevents HO in the so-called Michelsson model of forced joint mobilization[97]. In line, PGE2 is upregulated by macrophages and epithelial cells during fracture healing and modulates the inflammatory cascade and the activity of bone cells[102,103]. Finally, bone formation has been linked to the increased expression of pro-inflammatory mediators in systemic conditions such as ankylosing spondylitis and fibrodysplasia ossificans progressiva (FOP)[104,105].

Immune cells as regulators in bone formation

The immune system generally employs two mechanisms: 1) the innate response, which is more specialized in providing non-specific immediate action, and 2) the adaptive response, which takes a specific action but usually has a later onset. In most responses however, there is a dependent relationship between the cells of the innate and adaptive immune system[106]. Following soft tissue injury for instance, both innate and adaptive

immunity are involved and complement each other to mediate tissue regeneration[107]. Similarly, in fracture healing, innate and adaptive immune cell subsets can be found at the fracture site during acute inflammation (Figure 2)[108]. Studies using knockout mice have confirmed their functional roles in fracture healing[109,110]. In agreement, both the innate and adaptive immune systems have a role in HO[111].

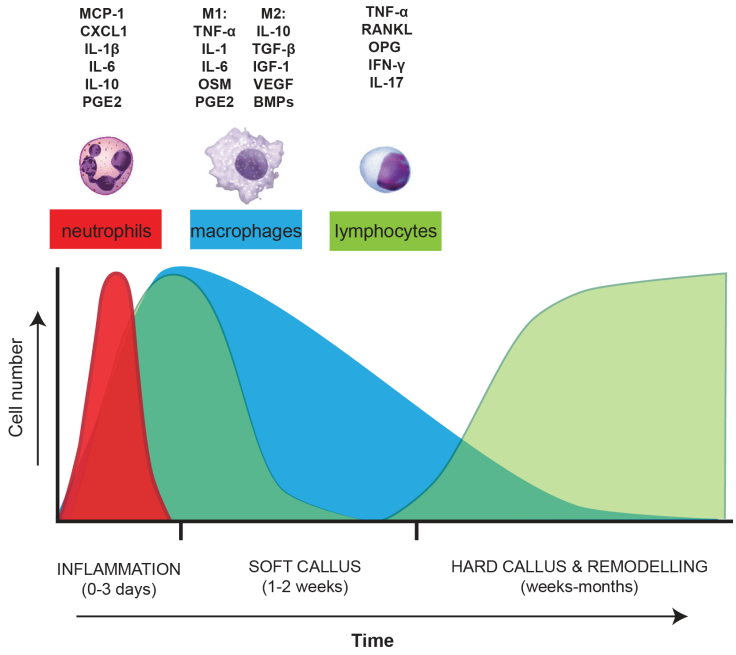

Figure 2. The presence of different immune cell subsets during various stages of fracture healing.
The secreted factors are listed for which a demonstrated effect on bone healing exists. Neutrophils mainly contribute to bone regeneration through the modulation of the immune response and the chemoattraction of macrophages. Activated macrophages stimulate the migration/differentiation of MSCs and are involved in angiogenesis. More recent evidence suggests that different T lymphocytes subpopulations are also involved in the early stage of healing, but the mechanism of action is unknown.

Innate immunity in bone formation

Neutrophils enter the damaged site within minutes and are only present in the early fracture hematoma, i.e. before day 3[112,113]. Inhibition of neutrophils in fracture studies leads to a reduced bone content and impaired mechanical properties of the callus[114]. Their regenerative functions are likely mediated through their attractive effects on monocytes and the synthesis of extracellular matrix[114,115].

In response to tissue damage and the chemoattractants produced by neutrophils, inflammatory macrophages are the next major innate immune cell type to appear in the fracture hematoma. In addition to the resident bone-lining macrophages[110], inflammatory macrophages which infiltrate the defect site, are characterized by their higher degree of plasticity[116]. The selective depletion of macrophages at different time points after the bone fracture has shown that macrophages contribute to bone regeneration throughout the various stages of healing[117]. Macrophages are also important mediators of bone formation

in models of HO. In a model combining spinal cord injury and localized inflammation, bone formation in the muscles is abolished after selective depletion of macrophages[99]. In a model of FOP, BMP-4-induced HO seems mediated by activated macrophages that stimulate local stem/progenitor cells to differentiate along osteogenic pathways[118].

Macrophages contribute to bone formation through multiple actions, including the production of cytokines or growth factors favoring osteogenesis[93,119], and through the secretion of factors promoting angiogenesis and vascular remodeling[120]. The macrophage-derived pro-inflammatory cytokines TNF-α, IL-6, IL-1β and Oncostatin M are upregulated during acute inflammation[93,119]. Each of these cytokines have a functional role during bone healing as shown by knockout mouse studies[119,121-123], and may have different actions on chemotaxis, osteoblast differentiation, angiogenesis or cartilage production[7,93]. Particularly TNF-α has important contributions in these different processes[93,124]. While pro-inflammatory cytokine-secreting macrophages (i.e. M1 macrophages) predominate during the acute phase of inflammation, the phenotype changes towards a pro-regenerative cell type (i.e. M2 macrophages) expressing several pro-angiogenic growth factors such as VEGF, PDGF, bFGF and insulin-like growth factor (IGF)-1[116,120]. It has been suggested that a disturbance in this polarization process is the reason for impaired osteogenesis and angiogenesis in models of delayed bone healing[120].

Adaptive immunity in bone formation

It is well established that lymphocytes have a critical function in bone remodeling through the expression of receptor activator of NF-κB ligand (RANKL) and its decoy receptor osteoprotegerin (OPG), which together determine the local activity of osteoclasts[125]. Lymphocytes consequently can be an important effector of inflammatory bone loss under uncontrolled inflammatory conditions, when unbalanced expression of these factors is seen[9].

The pro-regenerative effects of adaptive immune cells in bone healing have more recently also received interest. Mice lacking functional lymphocytes demonstrate abundant callus formation, but their fracture calluses exhibit decreased bone quality and lower levels of bone markers[109,126]. When the individual role of each T and B cells is investigated, the negative changes in matrix deposition can be linked to the selective role of T cells[126]. In line with these findings, T cells are involved in the progression of HO in animal models[118]. This possibly also explains how changes in T cell composition reduce the development of HO in patients after prophylactic irradiation[127].

Distinct T lymphocyte subsets and their associated cytokines likely have specific roles in osteogenesis[71,128]. For example, a suppressive effect of CD8+ cytotoxic T cells on bone repair has been suggested[129]. Furthermore, the decreased bone healing in the absence of T cells is accompanied by a shift in the local cytokine profile from the expression of proinflammatory cytokines, including IFN-γ and TNF-α, towards the expression of anti-inflammatory cytokines such as IL-10 and IL-4. Together, this suggests that only selective pro-inflammatory T lymphocyte subsets contribute to bone healing[109]. In addition to the activity of pro-inflammatory T cell subsets, the timely suppression of adaptive immune responses in the fracture callus by induced regulatory T cells seems equally important for the normal healing response[7,71,109,130].

It is currently unknown which T lymphocyte subsets hold pro-regenerative effects and which process of bone repair (i.e. chondrogenesis, osteogenesis or angiogenesis) is

modulated by these cells. Moreover, it is unclear whether T lymphocytes and their secreted factors can directly affect the osteogenic response in MSCs.

DAMPS, PAMPs, and their impact on bone

Following acute injury, the immune system is rapidly activated by signals that are released at the site of tissue damage. For this purpose, the immune system is equipped with receptors that allow a quick response to a wide range of 'danger signals'[106]. After a traumatic event such as a bone fracture, necrotic and apoptotic cells expose damage-associated molecular patterns (DAMPs), which activate neutrophils to initiate the inflammatory cytokine cascade, clear cellular debris, and subsequently attract monocytes[106,114] . During an infectious inflammatory response, the recognition of pathogen-associated molecular patterns (PAMPs) present on different classes of microbes is the first step in the immune response[131]. In this respect, toll-like-receptors are the most important class of receptors involved in the recognition and reaction towards endogenous (i.e. DAMPs) and exogenous (i.e. PAMPs) danger signals and therefore play a critical role in the early immune response[132].

DAMPs and PAMPs largely activate the same set of receptors on immune cells[133]. The response to bacteria-derived PAMPs has a particular drastic impact on bone and bone cells[106,131]. Generally, a steep rise in the number of bone-resorbing osteoclasts is seen during bacterial bone infections. In response to bacteria and subsequent tissue inflammation, osteoblasts and activated T lymphocytes produce high levels of RANKL, which leads to a high activity of osteoclasts at the infection site[134-136]. At the same time, the compression of vascular channels and programmed cell death may cause a lack of sufficient matrix-depositing cells at the location of the bacterial burden[137-139]. Together, this leads to the resorption of bone[140]. Furthermore, the process of osteoblast formation seems impaired, as implant osteointegration, spinal arthrodesis and fracture healing are all negatively affected by bacterial infection in animal models[76,141,142].

Paradoxically, bacterial bone infections are occasionally also associated with new bone formation[101,143-145]. Although the bone formation is often only reported in a qualitative manner in patients (Figure 3)[145], a direct correlation between *Staphylococcus aureus* (*S. aureus*) infection and a bone anabolic response has been made in a limited number of animal studies.

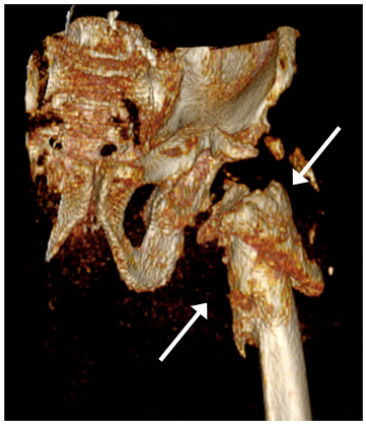

Figure 3. CT image showing new bone formation in a patient suffering from an infection around a hip prosthesis which has been removed (image courtesy: dr. H. C. Vogely, UMC Utrecht)

Cassat et al. showed that *S. aureus* infection impaired femoral defect healing, but also caused a 30-50% higher cortical volume peripheral to the infection site[143]. Stadelmann et al. used a micro-CT algorithm to track the volume of resorbed and newly formed bone adjacent to *S. aureus*-contaminated screws placed in the tibia[146]. Although bone resorption was measured at the surface of the contaminated screws, steady new bone formation was measured more distant from the screws. Finally, it was reported that methicillin-resistant *S. aureus* causes a 4-fold increase in ectopic bone formation in a rat trauma model[101].

The prominent effects of bacterial infections on bone leads to the suggestion that the response to bacteria-derived PAMPs can be a model to identify factors with pro- or anti-osteogenic effects. Moreover, it can be hypothesized that certain bacteria-derived PAMPs may even be used to induce osteogenesis when applied under controlled conditions.

Research aims, approach, and thesis outline

Aim

Study of the normal bone healing process shows that local factors expressed during the acute inflammatory response contribute to different processes needed for new bone formation. These include the recruitment of competent cells, osteogenesis, chondrogenesis and/or angiogenesis. This generates the hypothesis that the modulation of the early inflammatory environment could lead to bone-promoting processes.

The aim of this thesis is to identify and test the feasibility of immunomodulatory strategies to enhance new bone formation.

Approach

It is currently unknown which inflammation-associated factors have the largest impact on the process of new bone formation. Innate immune cells, mainly macrophages, and their soluble factors have been extensively studied for their *in vitro* and *in vivo* pro-osteogenic effects. More recent evidence points to the fact that T lymphocytes and their cytokines are also important regulators, but it remains unknown through which mechanism T lymphocytes can affect the bone formation process and which soluble factors are involved.

In a pathological context, inflammatory processes are also known to result in excessive new bone formation. It is therefore of interest to elucidate which immune cell subsets or mediators are involved in conditions leading to clinically-unwanted new bone formation. This pathological bone formation usually involves heterotopic bone formation, either local (e.g. periarticular ossification), or systemic (e.g. ankylosing disorders). New bone formation following bacterial infections, however, is often seen as a localized orthotopic response and therefore is an interesting phenomenon to further investigate. This could lead to the identification of local stimuli with bone-promoting action.

Based on these observations, it seems of interest to explore different classes of pro-inflammatory stimuli for their possible pro-osteogenic effects *in vitro* and *in vivo*. These pro-inflammatory stimuli could first comprise cytokines, which seem to have a contributing role in bone healing, and can be derived from either innate or adaptive immune systems.

Since these cytokines can likely target bone (progenitor) cells directly, their feasibility can be first tested in *in vitro* cell differentiation assays, followed by their *in vivo* validation (i.e. bottom-up approach). In addition, the study of whole bacteria, selective bacterial components, or downstream soluble factors expressed in response to bacteria, could all lead to avenues to harness their bone-inductive properties in the service of bone tissue engineering. Since the mechanisms behind bacteria-induced new bone formation are poorly understood, the feasibility of these pro-inflammatory stimuli should first be characterized in an established *in vivo* infection model, followed by their validation in more suitable models of bone formation (i.e. top-down approach).

Thesis outline

This thesis is divided into two sections. The first section (*chapters 2-5*) follows a bottom-up approach to investigate if, and when, specific pro-inflammatory mediators have bone stimulatory effects. This is first investigated on the osteogenic differentiation of human MSCs, and subsequently on bone formation in *in vivo* ectopic models. The second section (*chapters 6 and 7*) follows a top-down approach. First, we aim to quantify the pro-osteogenic effect of bacterial infection in an established *in vivo* model. Then, the pro-osteogenic effect is studied after the bacterial infection is confined to a sterile inflammation using killed bacteria or bacterial components. Furthermore, parameters such as the choice of bacterial antigen, dose-dependency, and delivery methods are investigated in multiple ectopic implants.

In *chapter 2*, we investigate the influence of selective pro-inflammatory mediators on the osteogenic differentiation of human MSCs. To identify conditions that favor osteogenesis, a number of parameters are investigated in parallel, such as the stage of lineage commitment of the MSCs and the interaction of pro-inflammatory mediators with different osteogenic inducers. TNF-α is tested, since this cytokine is involved in various processes contributing to bone fracture healing. A toll-like-receptor (TLR) agonist is investigated, as TLR signaling plays a major role in the early tissue response to bacteria and might therefore have modulatory effects on osteogenesis.

In *chapter 3*, we determine the influence of pro-inflammatory stimuli on the amount of new bone formation in ectopic ceramic constructs. We focus on the possible osteoinductive behaviour of these stimuli, and on their synergy with BMP-2. In the same chapter, the feasibility of an ectopic 'biomembrane model' is evaluated as an *in vivo* screening model to study new bone formation in conjunction with an induced localized inflammatory response. The goal is to determine whether the prior induction of confined biological pockets in the biomembrane model enables the localized delivery of pro-inflammatory mediators. Further, it is our goal to establish whether the biomembrane model affects new bone formation.

In *chapter 4*, we aim to determine how activated T lymphocytes influence the osteogenic differentiation of human MSCs in co-cultures, as there is little understanding of the direct effects of T lymphocytes on osteoprogenitor cells. The role of cell-cell contact and the effect of activated T lymphocyte-associated cytokines in their interaction is investigated. A distinction is made between pro- and anti-inflammatory T lymphocyte subsets to take into account possible specific roles in osteogenesis within the heterogeneous T lymphocyte population.

In *chapter 5*, we study if the diverse responses mediated by the T lymphocyte-derived cytokine IL-17 also lead to enhanced bone formation. For this purpose, ceramic constructs either with or without IL-17, are implanted ectopically. Furthermore, we determine if bone formation is superior when using an IL-17/BMP-2 co-delivery strategy in comparison to the delivery of these components alone.

In *chapter 6*, we assess how the response to bacterial infection correlates with bone formation in an established animal model of periprosthetic infection. In this model, the amount of bone is quantified and the onset, localization and progression of bone formation are assessed with fluorochrome labeling.

In *chapter 7*, we investigate whether the pro-osteogenic response seen in the infection model can be recapitulated without the use of viable bacteria. To decrease the risks for clinical application, we assess the osteogenic response to different strains of killed bacteria. Furthermore, we investigate whether these different strains of killed bacteria can affect bone formation in ectopic ceramic constructs.

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PART I

The effect of proinflammatory stimuli on osteogenesis

Chapter 2

Proinflammatory mediators enhance the osteogenesis of human mesenchymal stem cells after lineage commitment

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ABSTRACT

Several inflammatory processes underlie excessive bone formation, including chronic inflammation of the spine, acute infections, or periarticular ossifications after trauma. This suggests that local factors in these conditions have osteogenic properties. Mesenchymal stem cells (MSCs) and their differentiated progeny contribute to bone healing by synthesizing extracellular matrix and inducing mineralization. Due to the variation in experimental designs used *in vitro*, there is controversy about the osteogenic potential of proinflammatory factors on MSCs. Our goal was to determine the specific conditions allowing the pro-osteogenic effects of distinct inflammatory stimuli. Human bone marrow MSCs were exposed to tumor necrosis factor alpha (TNF-α) and lipopolysaccharide (LPS). Cells were cultured in growth medium or osteogenic differentiation medium. Alternatively, bone morphogenetic protein 2 (BMP-2) was used as osteogenic supplement to simulate the conditions *in vivo*. Alkaline phosphatase activity and calcium deposition were indicators of osteogenicity. To elucidate lineage commitment-dependent effects, MSCs were pre-differentiated prior treatment. Our results show that TNF-α and LPS do not affect the expression of osteogenic markers by MSCs in the absence of an osteogenic supplement. In osteogenic differentiation medium or together with BMP-2 however, these mediators highly stimulated their alkaline phosphatase activity and subsequent matrix mineralization. In pre-osteoblasts, matrix mineralization was significantly increased by these mediators, but irrespective of the culture conditions. Our study shows that inflammatory factors potently enhance the osteogenic capacity of MSCs. These properties may be harnessed in bone regenerative strategies. Importantly, the commitment of MSCs to the osteogenic lineage greatly enhances their responsiveness to inflammatory signals.

INTRODUCTION

The autologous bone graft is currently considered the gold standard for bone repair and regeneration, but is associated with disadvantages such as limited availability and donor-site morbidity. Therefore, alternative approaches are being studied[1]. As a bone replacement therapy, osteoconductive materials lack the osteogenic and osteoinductive properties of autografts. They are therefore often combined with mesenchymal stem cells (MSCs) in an experimental setting[2,3]. Bone stimulating factors such as the bone morphogenetic proteins (BMPs) are also effective in enhancing bone regeneration, but it is uncertain if the delivery of BMPs alone induces the optimal pro-osteogenic environment required for bone regeneration[4,5]. To better mimic the physiological environment, a new approach is to identify the critical pro-osteogenic factors involved in inflammation and to harness them to promote bone formation in bone replacement strategies[6].

While uncontrolled inflammation often has destructive effects on bone[7], at the same time, there are numerous examples of how inflammatory processes trigger new bone formation. A classical example is found in the tightly controlled inflammatory phase after fracture, which initiates repair and is required for adequate bone remodeling[8,9]. Transgenic animal models have shown the pivotal role of tumor necrosis factor-alpha (TNF-α) and Interleukin-6 (IL-6) in fracture healing[10,11]. In line with these observations, pharmacological agents with an anti-inflammatory action impair proper healing[6,12]. Moreover, heterotopic ossifications and several diseases of the vertebral column associated with excessive bone formation are preceded by an inflammatory phase[13,14].

In vitro, inflammatory mediators control the proliferation, migration and differentiation of MSCs. TNF-α, IL-6 and IL-1β have been mainly studied due to their critical role in fracture repair[6,15]. The effects of these cytokines on MSC osteogenic differentiation have been contradictory[16-20]. As a strong activator of the immune system, the endotoxin lipopolysaccharide (LPS) has also been investigated. In MSCs, it induces cell migration and the production of several cytokines and chemokines[21]. On osteogenic differentiation, both stimulatory and inhibitory effects of LPS have been reported[22-25].

Due to the discordance in *in vitro* experimental designs used to study their osteogenic potential, there is still debate on whether proinflammatory mediators can be harnessed for bone regeneration strategies. The aim of the current study was therefore to assess the effects of the proinflammatory cytokine TNF-α and the endotoxin LPS on the osteogenicity of human bone progenitor cells. We sought to compare a number of parameters in parallel, i.e. the stage of lineage commitment, the timing of delivery, and the use of different osteogenic inducers. Under specific conditions, strong pro-osteogenic effects of these inflammatory stimuli were observed.

MATERIALS AND METHODS

Experimental design

Recombinant human tumor necrosis factor-alpha (TNF-α, 0.5-50 ng/mL; eBioscience, San Diego, CA, USA) and lipopolysaccharide (LPS, 0.05-5 µg/mL; from *E. coli* O55:B5, Sigma-Aldrich, St. Louis, MO, USA) were used as mediators. They were freshly added in the medium at each change.

In the first experimental design (Figure 1), MSCs were continuously exposed to TNF-α and LPS. To induce differentiation, osteogenic medium was used, while growth medium served as a negative control. Enzymatic alkaline phosphatase (ALP) activity was measured at day 10 as the levels of this marker generally peak around this time point[26]. Calcium deposition was analyzed at day 22.

Next, the effect of a brief exposure of these mediators on osteogenesis was studied. This would elucidate if short stimulation of MSCs was sufficient to achieve an osteogenic response, similar to the conditions *in vivo*[27]. Cells were only exposed to the mediators during the initial 2 days (Figure 2). Osteogenic medium was added after the initial 2 days or from the start of the experiment, while growth medium served as a negative control. ALP activity in cultures was measured at day 10.

To study the effects of inflammatory signals on osteogenic-committed cells, MSCs were pre-differentiated for 12 days in osteogenic medium. This yields cells that are committed to the osteogenic lineage but not yet undergoing middle or end-stage osteoblastic differentiation[28]. From day 12, these pre-osteoblasts received TNF-α or LPS. This was done in the presence or absence of the osteogenic stimulus they had been exposed to (Figure 3). ALP activity was measured at day 20. At day 26, quantification of mineralization was done using xylenol orange fluorescence staining or Alizarin Red S staining. Furthermore, osteocalcin immunocytochemistry was performed, as this marker is only expressed in osteoblasts/cytes during mineralization[26,29].

Ethics Statements

Bone marrow was obtained from patients at our institute who had given written informed consent, according to the guidelines of the local medical ethical committee (University Medical Center Utrecht). The ethical committee approved all experiments.

Human MSC isolation and expansion

MSCs were isolated from bone marrow obtained from patients undergoing arthroplasty at our institute. Male and female donors of different ages were included to ascertain that findings were not unique to a specific source. The mononuclear cell fraction was isolated by centrifugation on Ficoll-paque and plated in expansion medium: growth medium [α-MEM (Invitrogen, Carlsbad, CA, USA), 10% (v/v) heat-inactivated fetal bovine serum (Cambrex, East Rutherford, NJ, USA), 100 units/mL penicillin/streptomycin (Invitrogen)] supplemented with 1 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN, USA). The cultures were washed after three days to remove non-adherent cells. Cells were always replated below 70% confluency and used between passages 3 and 7. Culture was performed at 37 °C in a humidified atmosphere containing 5% $C0_2$. The cells from different donors were never pooled.

The multipotency of MSCs using this isolation method has been established previously by standard differentiation assays along osteogenic, adipogenic and chondrogenic lineages[30]. To confirm their phenotype, cells were characterized for the expression of specific surface antigens defining hMSCs[31]. As such, >95% of cells were negative for CD14 and CD45, and >99% of cells were negative for CD19 and CD34. In addition, >95% were positive for CD73, CD105 and CD90 (Supplementary Figure 1).

Osteogenic differentiation assay

MSCs were plated in flat-bottom plates in triplicates at 6000 or 1000 cells/cm^2 for early and late analyses, respectively. Cells were grown until confluency and then exposed to different experimental conditions. For osteogenic differentiation, growth medium (see above) was supplemented with 10 mM of β-glycerophosphate (Sigma-Aldrich) and rhBMP-2 (750 ng/mL, InductOS, Wyeth/Pfizer, New York, NY, USA). In addition, osteogenic differentiation medium (ODM) was used, consisting of 10 mM of β-glycerophosphate and 10 nM dexamethasone/0.2 mM L-ascorbic acid 2-phosphate (Sigma-Aldrich). Medium was changed every 3-4 days.

Alkaline phosphatase activity

For ALP staining, cells were fixed in 4% (w/v) paraformaldehyde and permeabilized in 0.2% (v/v) Triton X-100 in phosphate buffered saline (PBS). The cell monolayer was incubated for 1 h with the Fuchsin and Chromogen-Substrate system (Dako) and then examined by light microscopy.

For quantitative ALP determination, cells were lyzed in 0.2% (v/v) Triton X-100 in PBS for 30 min. ALP activity was measured by conversion of the p-nitrophenyl phosphate Liquid Substrate System (Sigma-Aldrich). The absorbance was measured at 405 nm and corrected at 655 nm (Bio-rad, Hercules, CA, USA). Values were normalized to a standard ALP measurement using serial dilutions of calf intestinal ALP (Sigma-Aldrich) in 0.2% (v/v) Triton X-100 in PBS.

The same cell lysate used to measure ALP was stored at -80 °C and subsequently used to determine the DNA content with the Quant-It PicoGreen kit (Invitrogen) according to the manufacturer's instructions.

Calcium deposition

For qualitative assessment of matrix mineralization, the cell monolayer was fixed in 4% (w/v) paraformaldehyde, stained for 10 minutes with 2% (w/v) Alizarin red S solution (pH 4.2, Sigma-Aldrich) and examined by light microscopy. To quantify the calcium deposition, samples were incubated with 0.2 % (w/v) Alizarin Red S for 60 minutes. Subsequently 10% cetylpyridinium was added for 60 minutes to extract the calcium-bound Alizarin. Absorbance was measured at 595 nm and corrected at 655 nm.

To quantify the amount of calcium present per cell, the calcium-chelating fluorochrome xylenol orange was used. Samples were fixed in 4% (w/v) paraformaldehyde and a solution of 40 µM xylenol orange (Sigma-Aldrich) was added overnight at 37 °C in the dark. Prior to fluorescence imaging, cell nuclei were stained with Hoechst (2 µg/mL, bisBenzimide H 33258, Sigma-Aldrich) for 30 min. A total of 25 fluorescence images were collected for each individual well using the ArrayScan XTI (Thermo Scientific, Waltham, MA, USA). Masks were applied around cell nuclei with Cellomic VHS software (Thermo Scientific). The mean fluorescence signal in the xylenol orange channel was determined per nucleus. A threshold value was set to discriminate between calcium-positive and negative cells.

Osteocalcin immunocytochemistry

Cell monolayers were cultured on glass chamber slides (Lab-Tek, Sigma-Aldrich) and fixed for 15 min in 80% (v/v) methanol. The cell membranes were permeabilized for 10 min by incubation with 0.2% (v/v) Triton X-100 in PBS. After a blocking step of 30 min with 5%

(v/v) bovine serum albumin/PBS, samples were incubated overnight at 4 °C with 10 µg/mL mouse monoclonal antibody recognizing human osteocalcin (clone OCG4, cat num ALX-804-537, ID AB_2064910, Enzo Life Sciences, Farmingdale, NY). The monoclonal mouse IgG1 antibody (X0931, Dako) was used as isotype-matched control at the same concentration. This was followed by incubation with 10 µg/mL goat-anti-mouse polyclonal antibody conjugated to Alexa Fluor 488 (cat num A-11001, ID AB_141367, Invitrogen). Samples were finally enclosed with VectaShield containing DAPI counterstain (Vector Laboratories, Burlingame, CA, USA) and evaluated on a fluorescence microscope (Olympus BX51; Olympus DP70, Olympus, Shinjuku, Tokyo, Japan).

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Results for individual donors were based on triplicate measurements. Differences between the groups with TNF-α/LPS were considered significant versus the groups without TNF-α/LPS in the same medium when $P<0.05$ using one-way ANOVA with Bonferroni post-hoc analysis.

RESULTS

Early osteogenic differentiation of MSCs

Human MSCs were subjected to proinflammatory stimuli during their early osteogenic differentiation. To examine the mechanism by which these stimuli are involved in lineage determination, we made a distinction between ODM or osteogenic medium supplemented with BMP-2. TNF-α or LPS did not affect the ALP activity in undifferentiated MSCs. Together with an osteogenic inducer, TNF-α or LPS significantly enhanced the ALP activity in MSCs (Figure 1A). In ODM, stimulation with TNF-α (5 ng/mL) or LPS (0.5 µg/mL) increased the ALP activity 3.5-fold versus the group without mediator. In BMP-2 differentiating cells, this induction was 1.5 to 2-fold, respectively. These observations suggest that proinflammatory signals alone do not induce MSC osteogenic lineage commitment but may contribute to the early osteogenic differentiation of hMSCs together with an osteogenic stimulus.

In addition to the early ALP activity, calcium deposition was measured as a late indicator of osteogenic differentiation. We detected limited calcium nodules in the absence of an inflammatory mediator after 22 days. Calcium deposition however markedly increased in a dose-dependent way in the presence of an inflammatory signal (Figure 1B, Supplementary Figure 2B). As for ALP activity, we observed the highest calcium deposition levels at 5 ng/mL TNF-α and 0.5 µg/mL LPS. For higher concentrations, the amount of calcium declined (Supplementary Figure 2B). Persistent exposure of MSCs to TNF-α or LPS for 22 days did not induce any calcium deposition by cells in growth medium. To elucidate if inflammatory factors direct the commitment of MSCs specifically to the osteogenic lineage, MSCs were treated with the mediators in adipogenic differentiation medium. Although TNF-α or LPS tended to have both inhibitory and stimulatory effects on the adipogenic differentiation of MSCs respectively, the effect sizes were moderate compared to changes in ALP (Supplementary Figure 3). These data collectively show that these inflammatory factors together act with osteogenic inducers to potently stimulate osteogenesis in hMSCs. This is independent of the osteogenic stimuli used, i.e. BMP-2 or dexamethasone/ascorbic acid in ODM.

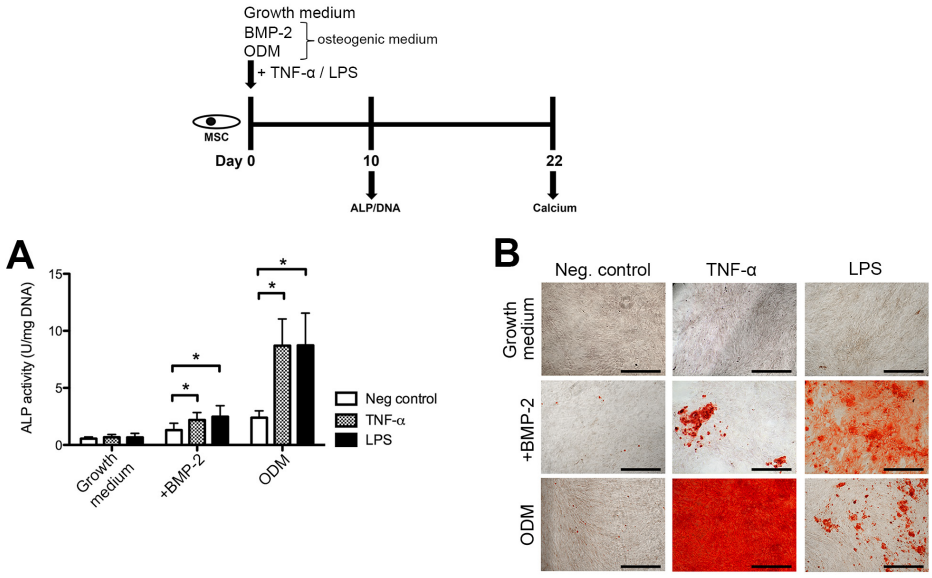

Figure 1. Expression of osteogenic markers by MSC treated with proinflammatory mediators. MSCs were exposed to TNF-α (5 ng/mL) or LPS (0.5 µg/mL). (**A**) ALP normalized for DNA content was measured at day 10. Bars represent the means ± SD (n = 6). *P<0.05 compared to untreated cells cultured in the same medium. (**B**) Alizarin Red S staining was performed to demonstrate matrix mineralization (representative for 4 donors). Scale bar: 500 µm. See Supplementary Figs. 2A and 2B for all concentrations.

ALP activity in MSCs after short exposure

To examine at which stage of lineage commitment proinflammatory stimuli mediate their effect, MSCs were exposed to the mediators during the initial two days only (Figure 2). We found that short TNF-α or LPS treatment significantly increased the ALP activity in differentiating cells, but only if co-delivered with BMP-2 or ODM from the start of culture. The responses were more pronounced in ODM than in BMP-2 differentiating cells (Supplementary Figure 2C). Although higher concentrations of these mediators were needed to induce this response during brief exposure, i.e. 50 ng/mL TNF-α and 5 µg/mL LPS, the effect sizes were similar compared to their continuous exposure. These observations suggest that osteogenic commitment primes MSCs for their responsiveness to inflammatory signals.

Late osteogenic differentiation in pre-osteoblasts

To determine the role of proinflammatory stimuli in late osteoblast differentiation, we performed a series of experiments on pre-osteoblasts. Similar to the MSCs, we cultured pre-osteoblasts in growth medium or in osteogenic medium. TNF-α or LPS treatment only moderately enhanced the ALP activity in pre-osteoblasts (Figure 3A). We found the highest ALP activities following LPS treatment in BMP-2 pre-cultured cells. In BMP-2 pre-treated cells, an inhibitive action was observed for high TNF-α concentrations (Supplementary Figure 4A).

As a decline in ALP activity can be a sign of end-stage osteogenic differentiation at this time point[28], we studied the matrix mineralization in parallel. TNF-α or LPS treatment of pre-osteoblasts increased the amount of deposited calcium (Figure 3B). This was more pronounced for pre-osteoblasts obtained in ODM. Signficant increases in the calcium deposition by these cells were found after treatment with the proinflammatory mediators, even in growth medium (Figure 3B, right panel). In addition, short stimulation of pre-osteoblasts with TNF-α or LPS was sufficient to induce a strong osteogenic response when pre-osteoblasts were cultured in ODM (Figure 3C). Quantification of the amount of calcium per cell by xylenol orange staining demonstrated that the observed increases in matrix mineralization were not clouded by changes in cell proliferation (Supplementary Figure 4B). The highest matrix mineralization was seen at 5 or 50 ng/mL TNF-α and 0.5 µg/mL LPS (Supplementary Figs. 4B, 4C). Taken together, these data suggest that inflammatory stimuli not only promote the early osteogenic differentiation, but also the late osteoblast differentiation. Furthermore, the strong osteogenic response to the mediators by pre-osteoblasts in the absence of BMP-2 or ODM strengthens the observation that osteogenic commitment primes hMSCs for their responsiveness to inflammatory signals.

We furthermore performed an immunocytochemical staining for osteocalcin as this marker is only expressed by cells committed to the osteogenic lineage. We observed an increased expression of osteocalcin in MSCs cultured in growth medium following treatment with an inflammatory stimulus from day 12 to day 26 (Figure 3D, upper panel). This is in line with the finding that TNF-α or LPS can induce matrix mineralization by MSCs cultured in growth medium for a long period (Supplementary Figure 4B). Although BMP-2 and ODM culture both induced intracellular osteocalcin expression (Figure 3D, left panel), we found an inhibitory effect of inflammatory stimuli on their osteocalcin expression (Figure 3D, middle and right panels). This was most pronounced for LPS, where we observed almost complete loss of staining. Our data agree with that of others, showing that the osteocalcin expression in MSCs does not predict their matrix mineralization *in vitro*[19].

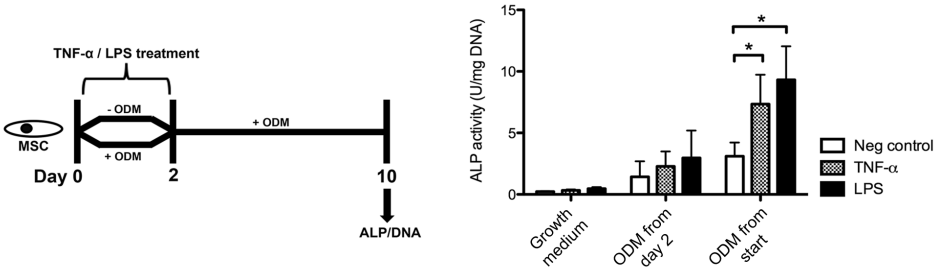

Figure 2. ALP expression by MSCs after short TNF-α or LPS treatment. Cells were exposed to TNF-α (50 ng/mL) or LPS (5 µg/mL) for 2 days, after which the mediators were withdrawn. At day 10, ALP activity levels were measured and normalized for DNA. Data represent the means ± SD (n = 4). *P<0.05 compared to untreated cells cultured in the same medium. See Supplementary Figure 2C for all experimental conditons.

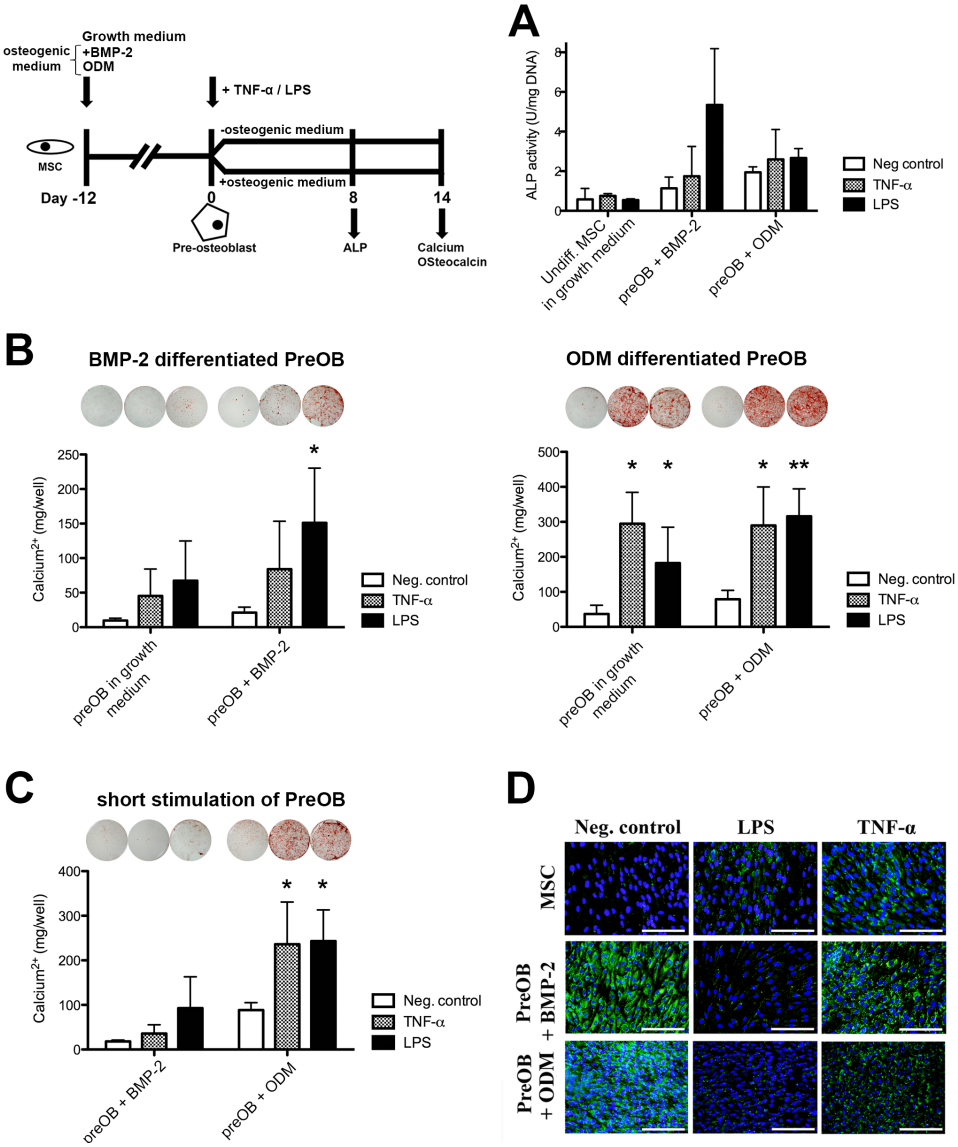

Figure 3. Late osteogenic differentiation in pre-osteoblasts. (**A**) Pre-osteoblasts were exposed to TNF-α (50 ng/mL) or LPS (5 µg/mL). The ALP activity was measured after 8 days and normalized for DNA content. The total calcium deposition was quantified at day 14 after continuous (**B**) and short (**C**) stimulation with TNF-α (5 ng/mL) or LPS (0.5 µg/mL). (**D**) Cells were exposed to LPS (0.5 µg/mL) or TNF-α (5 ng/mL) from days 12 to 26 of culture. Immunocytochemical staining for intracellular osteocalcin was performed as a marker of osteogenic differentiation. Osteocalcin and Hoechst are shown in green and blue, respectively (representative for 2 donors). Scale bar: 500 µm. See Supplementary Figure 4 for a dose response. Data represent the means ± SD (n = 4). *P<0.05/**P<0.005 compared to untreated cells cultured in the same medium.

DISCUSSION

In this study, we showed that the proinflammatory agents TNF-α and LPS potently enhanced BMP-2 and ODM-induced osteogenic differentiation of human bone marrow MSCs. We characterized the biological mineralization *in vitro* as the main parameter to assess osteogenic differentiation. The ALP activity in MSCs was also quantified to confirm these results, ruling out the possibility of dystrophic mineralization in the cultures. Early ALP activity was preferred over the quantification of osteogenic markers on the RNA level, as it may be more predictive for the *in vivo* bone-forming capacity of human MSCs[32]. Pro-osteogenic effects of these factors alone were only observed in pre-osteoblasts, suggesting that these mediators only stimulated osteogenesis after lineage commitment of bone progenitor cells. In therapies aimed at bone healing and regeneration, the co-delivery of inflammatory mediators may therefore potentiate the effect of bone-promoting factors *in vivo* similar to the early inflammatory response after injury. Alternatively, osteogenic priming of MSCs may be a strategy to increase the efficacy of inflammatory factors in bone regeneration strategies.

Thus far there have been conflicting reports on the effects of TNF-α. Many studies using rodent cells found an inhibitive action of TNF-α on MSC osteogenic differentiation[20,33]. As the few studies using human MSCs as starting cells have yielded comparable results as described here[17,34], species-specific differences likely exist in the action of TNF-α on MSC osteogenic differentiation[35]. Our results on MSCs are furthermore in line with the findings of those who demonstrate stimulatory effects of LPS on the matrix mineralization in human bone marrow- and adipose tissue-derived MSCs[23,36].

We aimed to investigate timing effects of inflammatory stimuli during very initial lineage commitment. We found similar effects following brief exposure to the mediators, although a 10-fold higher concentration was necessary to achieve this effect. Furthermore, early effects were only observed when MSCs were simultaneously treated with an osteogenic stimulus. To our knowledge, only a single study has obtained results that mirrored the *in vivo* response to TNF-α[18], where brief signaling stimulates bone regeneration and prolonged signaling has destructive effects on bone[6]. These experiments were however performed on primary osteoblasts from bone digests and different timing effects may exist for early MSC osteogenic differentiation.

We further show that the pre-differentiation of MSCs further potentiated the effects of the inflammatory stimuli on matrix mineralization. Interestingly, TNF-α or LPS mediated a stimulatory effect on pre-osteoblast differentiation even without an osteogenic stimulus. This supports the concept that inflammatory stimuli only affect the differentiation of cells committed to the osteogenic lineage. As an exception, inflammatory stimuli also induced an osteoblast phenotype in cells never exposed to an osteogenic supplement. This was demonstrated by a limited matrix mineralization and elevated intracellular osteocalcin levels in growth medium. It has been reported before that MSCs exhibit basal signs of osteogenic differentiation in growth medium when high cell densities are reached[26].

Interestingly, the present data suggest that proinflammatory signals may target downstream regulators of osteogenesis together with osteogenic inducers in bone progenitor cells. Moreover, this action is synergistic, as the effect of TNF-α or LPS with an osteogenic stimulus is larger than the sum of their individual effects. Indeed, some members of the TGF-β superfamily, including BMP-2, are locally expressed immediately

after injury during the inflammatory phase of healing[37]. A crosstalk is therefore thought to exist between signaling systems from growth factors and inflammatory mediators to recruit MSCs and guide their differentiation. Future studies should further elucidate the molecular mechanisms by which proinflammatory signals act in synergy with BMP-2 or dexamethasone/ascorbic acid to drive their differentiation.

We compared the responses to the inflammatory stimuli for different-acting osteogenic inducers. Dexamethasone is thought to antagonize signalling by inflammatory cytokines, possibly masking their true effects[6]. Our report is the first to use BMP-2 as an alternative to dexamethasone to induce osteogenesis in the context of inflammatory mediators. The use of BMP-2 mimics the physiological bone-healing environment and may therefore better simulate the conditions *in vivo*[38]. The outcomes in our experiments were the same following induction with BMP-2 or the synthetic glucocorticoid, even though dexamethasone had a more pronounced effect on the ALP activity in our MSC cultures. The different effects of dexamethasone and BMP-2 on ALP activity in hMSCs *in vitro* have been reported before[39]. Several lines of evidence point uniquely to the fact that their osteogenic effects are mediated by different signaling pathways. BMP-2 induced differentiation primarily involves the Smad proteins. Pathways involving mitogen-activated protein (MAP) kinases, PI3-K, Wnt and NF-κB can further interfere with Smad signaling or induce responses independent of Smads[40-42]. Alternatively, dexamethasone-induced differentiation occurs entirely independent of Smads[28,41,43].

Of the many signaling pathways, modulation of the nuclear factor kappa B (NF-κB) activity by inflammatory mediators is the most likely mechanism by which they exert an effect on the osteogenic differentiation of bone cells. Indeed, the activity of NF-κB is not only enhanced by TNF-α or LPS, but by a wide range of cell harming stimuli[17,22]. Moreover, NF-κB signaling is involved in osteoblast differentiation, demonstrated by the fact that overactivation of NF-κB results in enhanced BMP-2, Runx2 and Osx expression in hMSCs[17]. Importantly, one of the anti-inflammatory mechanisms of dexamethasone is the inactivation of NF-κB in almost all cell types. In MSCs, dexamethasone may therefore interfere with TNF-α/LPS induced signaling[6,44]. In none of the experiments did we observe such antagonizing effects of TNF-α or LPS with dexamethasone. On the contrary, the combination of ODM and a proinflammatory mediator induced a synergistic response. Possibly, anti-inflammatory effects of dexamethasone are achieved at different target cells and only for higher concentrations, ruling out its interfering effects in our osteogenic differentiation assay[44].

In conclusion, the present study shows that TNF-α and LPS directly enhance the differentiation of human bone marrow MSCs, in particular after osteogenic commitment. Moreover, proinflammatory signals likely interplay with common downstream regulators of osteogenesis in bone cells, as we observed a similar outcome for the differentially acting osteogenic inducers BMP-2 and dexamethasone.

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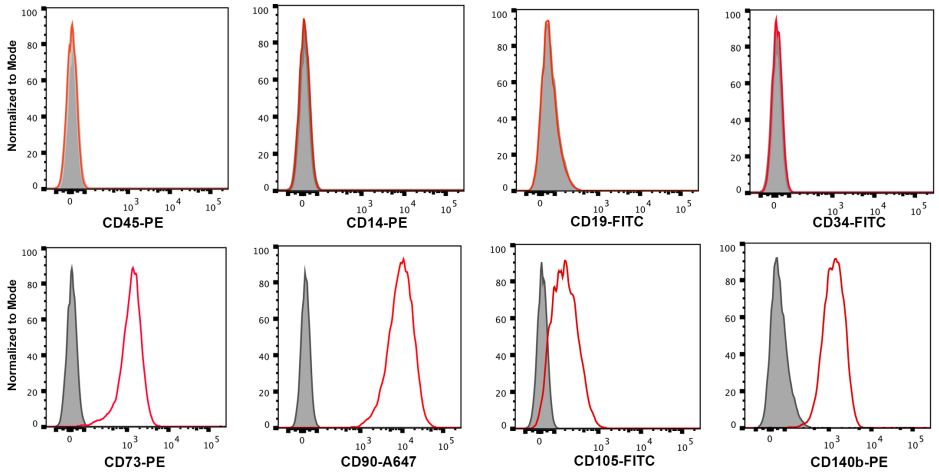
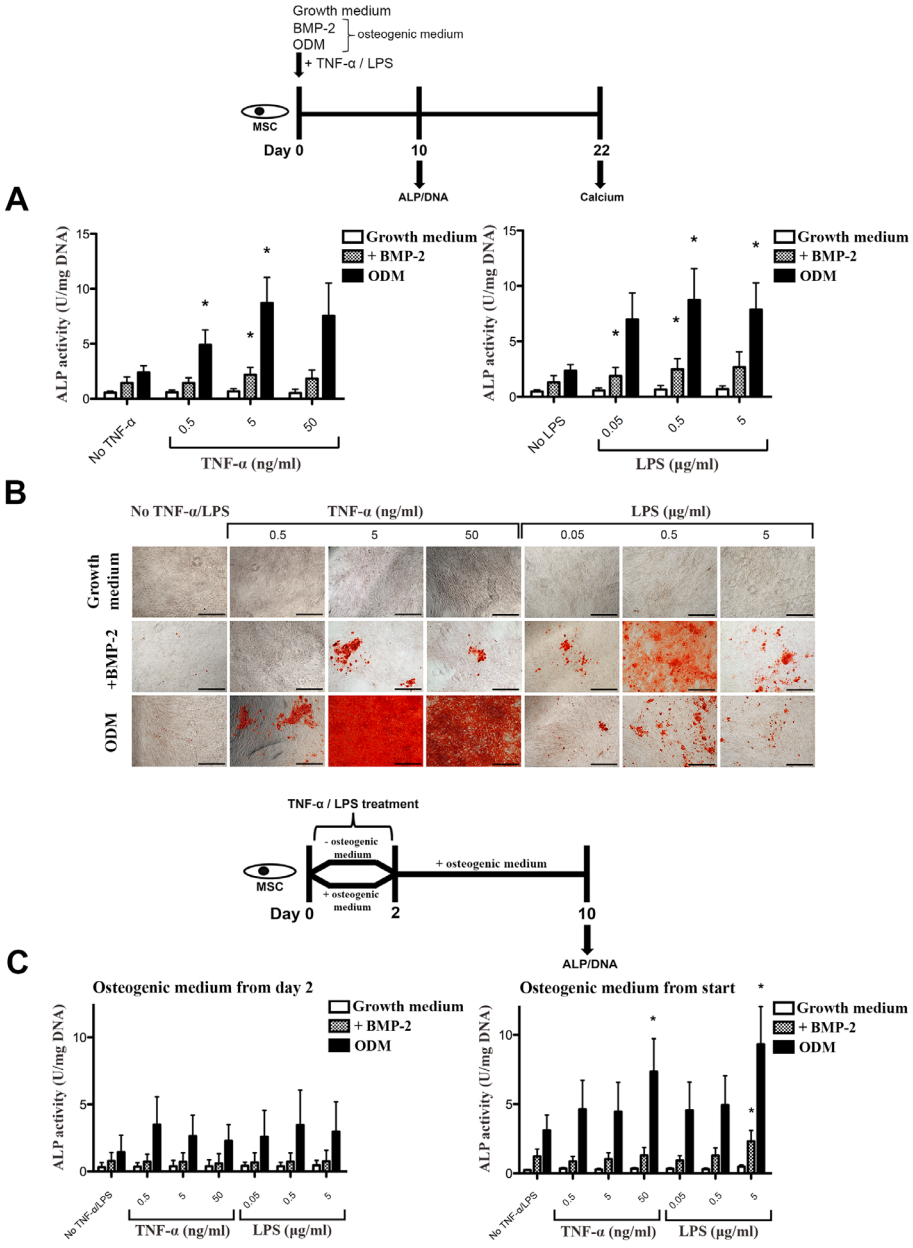
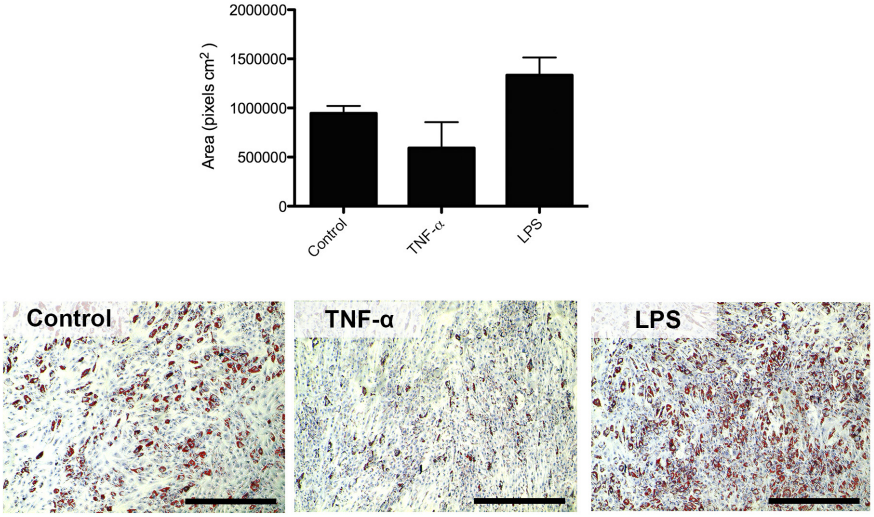
SUPPLEMENTARY DATA



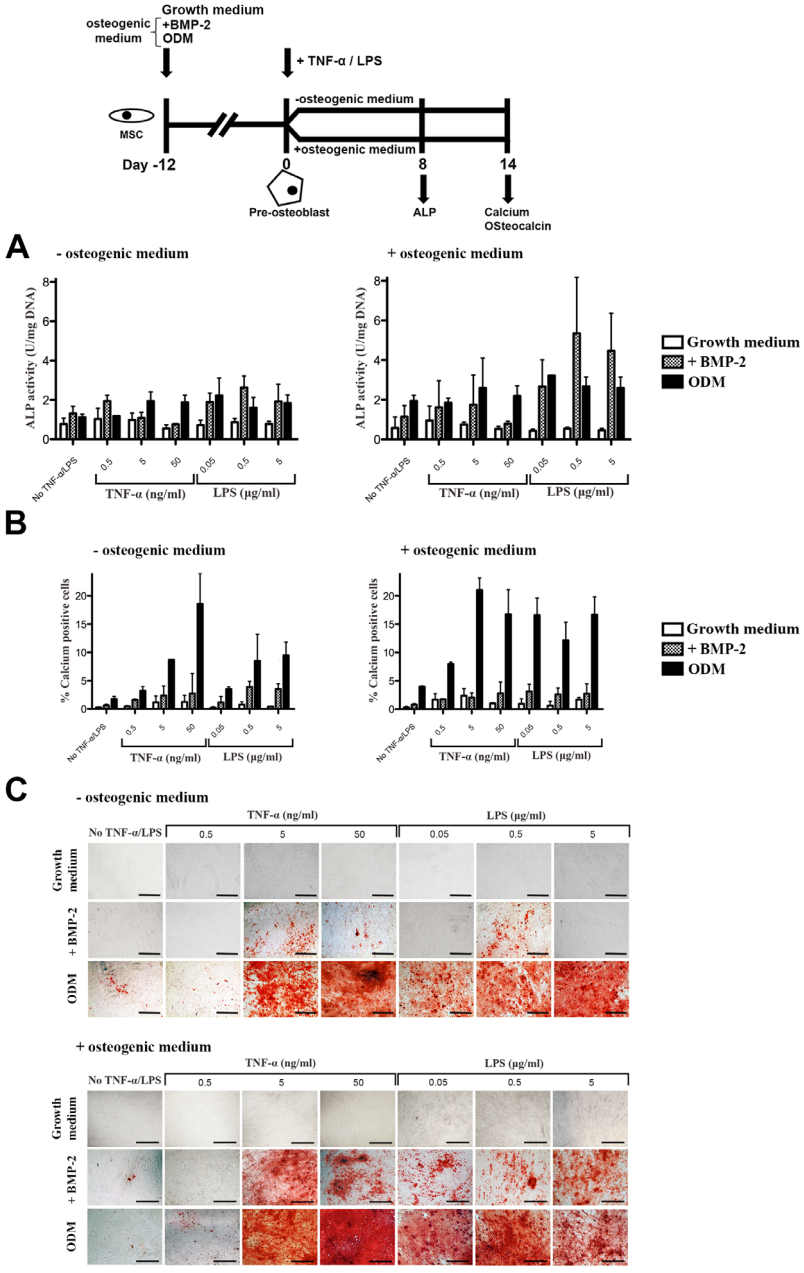
Supplementary Figure 1. Characterization of MSCs by flow cytometry. MSCs were stained with the fluorochrome-conjugated antibodies listed under each graph (red histograms), or with the isotype controls for the fluorophore (grey histograms). Cells were incubated for 30 min at 4 °C with human FcR blocking reagent (Miltenyi Biotec) and the following monoclonal mouse-anti-human antibodies: CD45 (cat num 560975, ID AB_2033960, BD Biosciences), CD14 (cat num R08641, ID AB_579551, Dako), CD19 (cat num 130-091-328, ID AB_244222, Miltenyi), CD34 (cat num 555821, ID AB_396150, BD), CD73 (cat num 550257, ID AB-393561,BD), CD90 (cat num 328118, ID AB_2303335, Biolegend) and CD105 (cat num FAB10971F, ID AB-356989, R&D Systems). Cell fluorescence was measured in viable cells using a BD FACSCanto II flow cytometer (BD). SytoxBlue (Invitrogen) was used for exclusion of dead cells. Cells were positive for CD90, CD105, CD14b, and CD73, but negative for CD14, CD45, CD19, and CD34.



Supplementary Figure 2. Stimulation of early MSC osteogenesis by TNF-α/LPS. MSCs were cultured in osteogenic medium consisting of BMP-2 or ODM. Growth medium served as a negative control for osteogenic differentiation. Cells were continuously exposed to TNF-α or LPS. **(A)** ALP activity in MSCs was measured after 10 days and normalized for DNA content (n = 6). (**B**) At day 22, Alizarin Red S staining was performed to demonstrate matrix mineralization. Scale bar: 500 μm. (**C**) MSCs were exposed to TNF-α or LPS for 2 days, after which the mediators were withdrawn. Early TNF-α/LPS treatment was performed in the absence (left panel) or presence (right panel) of osteogenic medium. At day 10, ALP activity levels were measured and normalized for DNA content (n = 4). Data represent the means ± SD. * P<0.05 versus the group without TNF-α/LPS in the same medium.



Supplementary Figure 3. Effect of inflammatory mediators on the adipogenic differentiation of MSCs. Cells were grown until confluency and then differentiated for 10 days using the StemPro Adipogenesis Differentiation Kit (Gibco), according to the manufacturer's instructions. Differentiation was performed with or without TNF-α (5 ng/mL) or LPS (0.5 µg/mL). Lipid droplets were stained with an Oil Red O solution and counterstained with hematoxylin. The histogram represents the total area of Oil red O staining as determined by histomorphometry (mean ± SD, n = 3). Scale bar: 500 µm.



Supplementary Figure 4. Effects of proinflammatory mediators on late osteogenic differentiation of pre-osteoblasts. MSCs were cultured in normal growth medium or osteogenic medium consisting of BMP-2 or ODM. Following 12 days of pre-differentiation, cells were exposed to TNF-α and LPS, either in the absence (-osteogenic medium) or presence (+osteogenic medium) of the primary osteogenic stimulus. (**A**) ALP activity levels were measured after 8 additional days of culture and normalized for DNA content (n = 2). (**B**) At day 14, calcium expression was measured following the binding of xylenol orange (n = 2). (**C**) Alizarin Red S staining confirmed the calcium deposition by cells following TNF-α/LPS treatment, during culture without (-osteogenic medium) or with (+osteogenic medium) an osteogenic stimulus. Scale bar: 500 μm. Data represent the means ± SD.

Chapter 3

Proinflammatory T cells and IL-17 stimulate osteoblast differentiation

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ABSTRACT

The local immune response is important to consider when the aim is to improve bone regeneration. Recently T lymphocytes and their associated cytokines have been identified as regulators in fracture callus formation, but it is not known whether T cells affect bone progenitor cells directly. The goal of this *in vitro* study was to investigate the role of different T cell subsets and their secreted factors on the osteogenic differentiation of human mesenchymal stem cells (MSCs). Significant increases in the alkaline phosphatase activity and the subsequent matrix mineralization by MSCs were found after their exposure to activated T cells or activated T cell-derived conditioned medium. Blocking IFN-γ in the conditioned medium abolished its pro-osteogenic effect, while blocking TGF-β further enhanced osteogenesis. The relative contribution of an anti- or proinflammatory T cell phenotype in MSC osteogenic differentiation was studied next. Enrichment of the fraction of anti-inflammatory regulatory T cells had no beneficial osteogenic effect. In contrast, soluble factors derived from enriched T helper 17 cells upregulated the expression of osteogenic markers by MSCs. IL-17A and IL-17F, their main proinflammatory cytokines, similarly exhibited strong osteogenic effects when exposed directly to MSCs. IL-17A in particular showed a synergistic action together with bone morphogenetic protein 2. These results indicate that individual T cell subsets, following their activation, affect osteoblast maturation in a different manner through the production of soluble factors. From all T cells, the proinflammatory T cells, including the T helper 17 cells, are most stimulatory for osteogenesis.

INTRODUCTION

Bone tissue normally has the capacity to fully regenerate in response to injury[1]. Healing can however be impaired under certain conditions such as severe trauma, increased age, metabolic disease, mechanical instability, or the use of nonsteroid anti-inflammatory drugs[2-4]. It has become increasingly clear that a balanced immune response is a prerequisite for successful bone regeneration[5-7]. After injury, proinflammatory cytokines act in concert with bone-promoting factors to initiate repair by regulating angiogenesis and the migration and differentiation of bone progenitor cells[8]. In particular TNF-α, IL-6 and IL-1β show a strong biphasic expression profile following bone injury, coinciding with the inflammatory and remodeling phases of bone regeneration[5,9]. The subsequent upregulation of anti-inflammatory cytokines is suggested to ensure a balanced inflammatory response and optimal regeneration[10]. In a pathological context, unregulated expression of inflammatory cytokines is thought to underlie excessive new bone formation[11-13], suggesting that these factors can stimulate osteoblast maturation of bone progenitor cells.

Although the innate inflammatory response is a key component during early bone repair[14], the adaptive immune system and its associated cytokines has recently also received attention as a regulator in bone regeneration. In the field of osteoimmunology, T lymphocytes are mainly studied for their effects on osteoclast activation and bone resorption in conditions such as inflammatory arthritis and periodontitis[15]. Following the observation that T cells are regulators in soft tissue healing[16], their contribution has also been studied in fracture healing. Studies performed in mice lacking functional lymphocytes, i.e. RAG-1-deficient mice, indicate that T cells may indeed play a role in bone regeneration. As such, the fracture calluses of RAG-1-deficient mice exhibit lower levels of bone markers which leads to poor bone quality. This is accompanied by a shift in the local cytokine profile from the expression of proinflammatory cytokines, including IFN-γ and TNF-α, towards the expression of anti-inflammatory cytokines such as IL-10 and IL-4[17,18].

It is likely that distinct lymphocyte subsets and their associated cytokines have specific roles in osteogenesis[19,20]. Although B cells are more likely involved in the later remodeling phase of bone regeneration[21,22], in contrast, T cells may be more important during the acute phase of bone healing[21]. A stimulatory role for the proinflammatory T helper 17 (T_H17) cell subset in osteoblast maturation has been suggested[17], whereas improved bone formation was demonstrated after the delivery of anti-inflammatory CD4+CD25+Foxp3+ regulatory T (T_{REG}) cells during mesenchymal stem cell (MSC)-based bone regeneration[23]. Finally, a negative correlation has been reported for the number of CD8+ cytotoxic T cells in peripheral blood and the outcome of fracture healing in otherwise healthy patients[24].

The modulation of the adaptive immune response has proven to be a promising approach to stimulating bone regeneration. The local depletion or systemic delivery of different T cell populations has resulted in improved fracture healing or MSC-based bone regeneration[23,24]. It is currently unknown whether T cells are directly involved in osteoblast maturation. The goal of this study was therefore to investigate how different T cell subsets have an effect on the osteogenic differentiation of MSCs. The approach chosen involves coculturing these purified cell populations or exposing MSCs to T cell-secreted factors, followed by monitoring of cell performance and differentiation.

MATERIALS AND METHODS

MSC isolation and culture

Bone marrow was obtained from four patients undergoing orthopedic procedures at our institute (University Medical Center Utrecht, Utrecht, The Netherlands). Different bone marrow sources were included to ascertain that the findings were not unique to a specific source (female, 53 yr, iliac crest; male, 62 yr, iliac crest; male, 77 yr, vertebra; adolescent female, calcaneus). All patients gave written informed consent, with approval of the local medical ethical committee. The mononuclear cell fraction was isolated by Ficoll-paque centrifugation and plated in growth medium [α-MEM (Invitrogen, Carlsbad, CA), 10% (v/v) heat-inactivated fetal bovine serum (Cambrex, East Rutherford, NJ), 100 units/ml penicillin/streptomycin (Invitrogen)] supplemented with 1 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN). Adherent cells were expanded and cryopreserved for use between passage 3 and 6. Cells derived from different donors were never pooled. Culture was performed at 37 °C in a humidified atmosphere containing 5% CO_2.

The multipotency of MSCs using this isolation method has been established previously by our group using standard differentiation assays along osteogenic, adipogenic and chondrogenic lineages[25]. Furthermore, cells were characterized for the expression of specific surface antigens defining human MSCs, according to the Mesenchymal and Tissue Stem Cell Committee of the ISCT[26]. Based on FACS analyses, >95% of cells were negative for CD14 and CD45, and >99% of cells were negative for CD19 and CD34. In addition, >95% were positive for CD73, CD105 and CD90[27].

T cell isolation and culture

Blood from healthy male and female controls was requested from the blood bank Mini Donor Dienst of the UMC Utrecht after written informed consent and with approval of the local medical ethical committee. The donors were between 25 and 55 years old (mean age 40 yr). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll-paque centrifugation. CD3+ T cells and CD4+ T helper cells were isolated by positive selection using MicroBeads according to the manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany). To further differentiate the T cells, CD45RO-positive cells were first depleted with MicroBeads. To enrich CD8+ cytotoxic T cells from the PBMCs, CD4 depletion and CD3 positive selection were performed accordingly. The purity of the cell isolates was confirmed by FACS analysis using the following antibodies: CD3 (clone BW264/56, Miltenyi), CD4 (clone VIT4, Miltenyi) and CD45RO (clone UCHL1, BD, Franklin Lakes, NJ). The percentage of CD3+ and CD4+ T cells after selection ranged between 90-99% (Supplementary Figure 1A). CD45RO depletion resulted in 80-90% CD45RO- cells (Supplementary Figure 1B). T cells were cultured in RPMI 1640 (Sigma-Aldrich, St. Louis, MO), supplemented with 100 units/ml penicillin/streptomycin, 10% (v/v) heat-inactivated fetal bovine serum and 2 mM L-glutamine (Invitrogen).

T cell activation and differentiation

For T cell activation, cells were cultured at $1x10^6$ cells/ml in plates coated with anti-CD3 mAb (5 µg/ml, clone CLB-T3/4.E, 1XE, Sanquin Reagents, Amsterdam, The Netherlands), and with soluble anti-CD28 mAb (2 µg/ml, clone CLB-CD28/1,15E8, Sanquin Reagents) and rhIL-2 (20 ng/ml, Thermo Fisher Scientific, Carlsbad, CA) for 4 days. As a control,

resting T cells were obtained by treatment with IL-2 only. The activation of cells was quantified by FACS analysis for CD25 (clone 4E3, Miltenyi), which was increased from 20% in controls to 60-70% after stimulation (Supplementary Figure 1C).

The survival and activation status of the T cells was also studied by FACS analysis. For this purpose, CD4+ T cells were activated as described above and subsequently cultured for 10 days in 24-well plates (750.000 cells/well) on a layer of MSCs (initial density 8500 cells/cm^2). In the control group, the T cells were cultured in the absence of MSCs for 10 days. At different time points, the cells were detached (Accutase, Sigma) and double-stained with CD4 (clone VIT4, Miltenyi) and CD25 (clone 4E3, Miltenyi). To assess the T cell viability, cells were stained with 7-AAD (10 µg/ml in PBS, Sigma) for 30 minutes.

For T cell differentiation experiments, CD4+CD45RO- naïve T cells were stimulated with anti-CD3/CD28 mAbs and IL-2. For the enrichment of the Foxp3-expressing regulatory T cell (T_{REG}) fraction, the cultures were supplemented with 20 ng/ml TGF-β1 (R&D) for 5 days. For T helper 17 cell (T_H17) differentiation, cultures were supplemented with TGF-β (10 ng/ml), IL-6 (30 ng/ml, R&D), IL-1β (25 ng/ml, Sigma) and IL-23 (75 ng/ml, eBioscience, San Diego, CA) in the absence of IL-2 for 5 days. T cells were replated after 5 days and cultured for an additional 5 days with these antibodies and IL-2 (20 ng/ml). The enrichment of T_{REG} and T_H17 cell fractions was determined by FACS analysis using anti-human Foxp3 eFluor 450 (eBioscience) and anti-human IL-17A-APC (Miltenyi). T_{REG} polarization resulted in a purity of 40-50% Foxp3-positive cells compared to approximately 20% in the undifferentiated control (Supplementary Figure 1D). T_H17 polarization similarly resulted in a doubling of the number of IL-17A-producing cells to about 40% (Supplementary Figure 1E).

Osteogenic differentiation assay

MSCs were seeded at 8500 cells/cm^2. Two types of osteogenic differentiation medium (ODM), containing either dexamethasone or BMP-2, were used for experiments. Although dexamethasone is a strong inducer of the osteogenic differentiation of MSCs *in vitro*, ODM with BMP-2 is thought to better simulate the conditions *in vivo*[28]. As such, growth medium (see section 2.1) was supplemented with a combination of 10 mM β-glycerophosphate (Sigma) and 10 nM dexamethasone/0.2 mM L-ascorbic acid 2-phosphate (Sigma), or a combination of 10 mM β-glycerophosphate and 500 ng/ml rhBMP-2 (InductOS, Wyeth/Pfizer, NY). Ascorbic acid was not used in cocultures due to their reported effects on T cells[29].

For coculture experiments (Figure 1), T cell activation/polarization was always performed prior to culture with MSCs. T cells were subsequently added to the MSCs in the different media at different concentrations. Half of the medium was replaced every 3 days. To study the role of cell-to-cell contact, T cells were separated from the MSCs in Transwell supports (6.5 mm, 0.4 µm pores, Sigma).

To obtain conditioned medium (CM), T cells were reseeded at 5x10^5 cells/ml after activation/polarization in medium supplemented with only anti-CD3/CD28 mAbs (Figure 1). The cell suspensions were harvested after 24 hours, centrifuged, and the supernatant was collected for storage at -80 °C until use. The CM was added to the MSCs at different concentrations (10-50%), and fresh CM was added at each medium change. In ODM culture, the concentration of osteogenic stimuli was corrected for the total volume of medium. To study the effect of T_H17-specific cytokines on osteogenesis, MSCs were cultured with 5-500 ng/ml rhIL-17A or rhIL-17F (R&D). The following mouse anti-human monoclonal antibodies were added to the CD4+ T cell CM to neutralize the activity of

these cytokines (all purchased from R&D Systems): IL-17 (IgG2B, 1.75 µg/ml), TNF-α (IgG1, 1 µg/ml), IFN-γ (IgG2B, 0.85 µg/ml), IL-10 (IgG2B, 0.75 µg/ml) and TGF-β (IgG1, 0.75 µg/ml). The manufacturer's recommended concentrations were used. Mouse IgG1 (2 µg/ml) and mouse IgG2B (2 µg/ml) antibodies were used as controls.

The ALP activity in MSCs was measured at day 10, as we and others have shown that the levels generally peak between day 10 and 14[30]. For ALP determination, cells were lysed in 0.2% (v/v) Triton X-100 in PBS for 30 min. ALP activity was measured by conversion of the p-nitrophenyl phosphate Liquid Substrate System (Sigma). The absorbance was measured at 405 nm and corrected at 655 nm (Bio-rad, Hercules, CA). Values were normalized to a standard ALP measurement using serial dilutions of calf intestinal ALP (Sigma) in 0.2% (v/v) Triton X-100 in PBS. The same cell lysate used to measure ALP was stored at -80 °C and used to determine the DNA content with the Quant-It PicoGreen kit (Invitrogen), according to the manufacturer's instructions. In coculture experiments, MSC numbers were quantified by FACS analysis using counting beads (Countbright, Life Technologies), according the manufacturer.

For the qualitative assessment of matrix mineralization, the cell monolayer was fixed after 16 days culture in 4% (w/v) paraformaldehyde, stained for 10 minutes with 2% (w/v) Alizarin Red S solution (pH 4.2, Sigma) and examined by light microscopy. For quantification, samples were incubated with 0.2 % (w/v) Alizarin Red S for 60 minutes, washed extensively, and treated with 10% cetylpyridinium to extract the calcium-bound Alizarin. Absorbance was measured at 595 nm and corrected at 655 nm (Bio-rad).

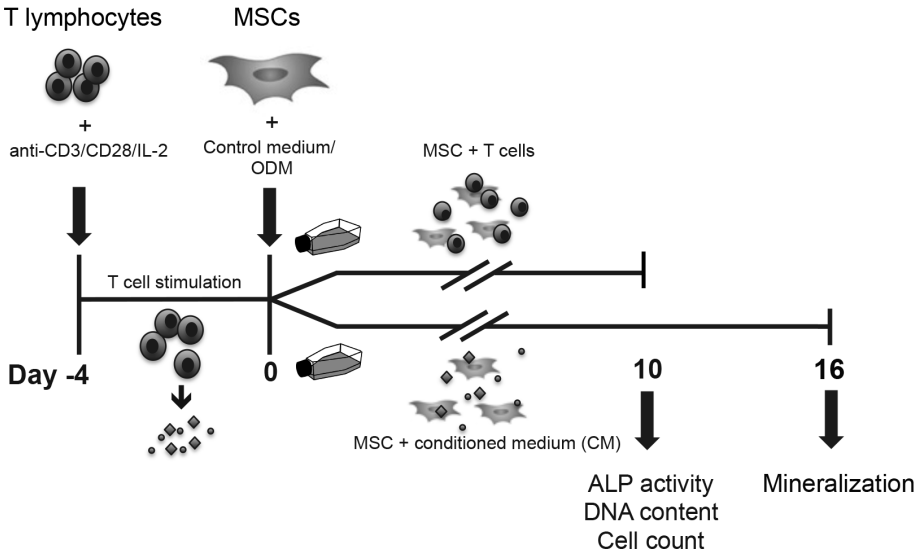

Figure 1. Experimental design. T cells purified from peripheral blood were activated. MSCs were isolated from bone marrow and cultured in growth medium (control) or osteogenic differentiation media (ODM). Two types of ODM were used, containing either dexamethasone or BMP-2. To study the effect of T cell-secreted factors on osteogenesis, MSCs were cocultured with T cells directly or incubated with T cell-derived conditioned media (CM). Alkaline phosphatase (ALP) activity and MSC proliferation were quantified after 10 days. T cell CM was used to study the effect of T cell cytokines on the matrix mineralization after 16 days.

Statistical Analysis

All data are expressed as the mean ± standard deviation for the different lymphocyte donors. When indicated in the captions, experiments were performed in multiple MSC donors. For statistical analysis, one-way analysis of variance (ANOVA) and Bonferroni post-hoc correction were applied. One-way ANOVA with LSD post-hoc correction was performed to study the effect of the neutralizing antibodies in the conditioned medium experiments.

RESULTS

Activated T cells stimulate the osteogenic differentiation of MSCs

A possible effect of T cells on MSC osteogenesis was first tested in direct cocultures. The MSCs were cultured in growth medium or in medium supplemented with BMP-2, with or without different lymphocyte subsets. Compared to cultures in the absence of any lymphocytes, MSCs exhibited a higher ALP activity when cocultured with PBMCs or with purified CD4+ and CD8+ cells (Figure 2A). The observation that the ALP activity was also enhanced in MSCs by lymphocytes in cultures without BMP-2 suggests that T cells alone may have the ability to induce osteogenic differentiation.

We furthermore studied the effect of the different T lymphocyte populations on the matrix mineralization by MSCs. Due to the practical limitations of the longer culture time that is required, T cell conditioned media (CM) was used for this purpose. A significantly higher calcium deposition was measured when MSCs were incubated with the CM derived from activated PBMCs or T lymphocytes during their osteogenic differentiation (Figure 2B). In line with the early ALP activity, the highest matrix mineralization was found after incubation of MSCs with CM collected from the CD4+ T cell population. It appeared that individual T cell subsets, i.e. CD4+ and CD8+ T cells, had a higher stimulatory effect compared to the entire T cell population (Figure 2A, 2B).

CD4+ T cells secrete factors that promote osteoblast maturation

We studied the CD4+ T cell population in more detail to extend our previous findings. In coculture experiments, we found a dose-dependent significant increase in the ALP activity of MSCs after coculture with the entire T cell pool (Figure 2C). A higher ALP induction was however found when the fraction of CD4+ T cells was increased by cell sorting, suggesting that the CD4+ population has the most beneficial effects on osteogenesis. No significant changes in the ALP activity were observed when MSCs were cocultured with resting CD4+ T cells, showing that only T cells with an activated phenotype affect MSC osteogenic differentiation.

FACS analysis demonstrated that the CD4+ T cells gradually lost their activation status during the 10-day culture period, as shown by their decrease in CD25 expression (Supplementary Figure 2A). The MSCs had an inhibitory effect on the CD25 expression, depending on the donor. In addition, there was a profound loss in T cell viability in the second half of the culture period. The presence of MSCs in the coculture resulted in a significant higher number of non-viable T cells at day 10 compared to T cells which were cultured alone (Supplementary Figure 2B). These data together suggest that CD4+ T cells mediate most of their beneficial effects on osteogenesis during initial coculture with the MSCs.

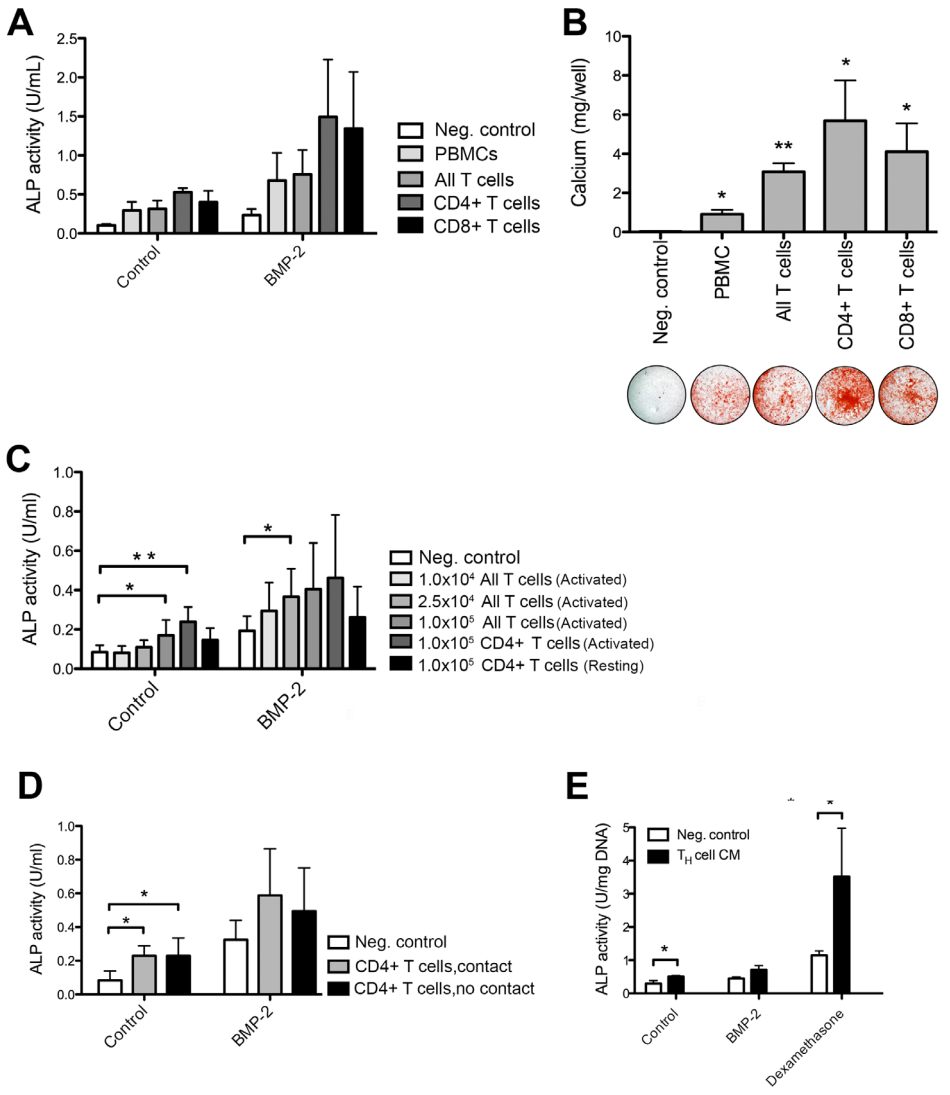

Figure 2. The effect of T cells and their secreted factors on the osteogenic differentiation of MSCs. (**A**) Day 10 ALP activity in MSCs cultured in the presence of $1x10^5$ activated allogeneic T cells in 96-well plates, n = 2 blood donors and one allogeneic MSC donor. (**B**) Day 16 matrix mineralization by MSCs following osteogenic induction with dexamethasone and treatment with 25% CM from PBMCs and activated lymphocytes. Calcium deposition at day 16 was quantified after Alizarin Red S staining, n = 3 blood donors and one MSC donor. * p<0.05/** p<0.005 compared to the negative control (**C**) Day 10 ALP activity in MSCs cultured with different amounts of allogeneic T cells in 96-well plates, n = 5 blood donors for independent experiments using two MSC donors (**D**) Day 10 ALP activity in MSCs cultured in growth medium (control) or BMP-2 osteogenic medium in the presence of 2.5 x 10^5 activated allogeneic CD4+ T cells in 24-well plates. Transwell inserts were used to inhibit cell-to-cell contact, n = 4 blood donors for independent experiments using two MSC donors. (**E**) Day 10 ALP activity/DNA in MSCs cultured in 25% CM collected from activated CD4+ T cells, n = 4 blood donors for independent experiments using two MSC donors. Histograms show the mean ± SD. * p<0.05/** p<0.005 compared to the negative control in the same medium.

The importance of direct cell contact in the MSC-T cell interactions was explored by culturing T cells separated from MSCs in permeable Transwell inserts. This allowed paracrine signaling between the different cell types in the absence of direct cell-to-cell contact. This did not change the CD4+ T cell-mediated effects on the ALP activity in MSC compared to direct cocultures (Figure 2D). CM derived from activated CD4+ T cells also significantly enhanced the ALP activity in MSCs (Figure 2E). This finding suggests that T cell-mediated effects on MSC osteogenic differentiation are largely caused by soluble factors.

To ascertain that the observed T cell-mediated effects on MSCs were not obscured by changes in MSC proliferation, we routinely measured the number of MSCs in parallel to the differentiation assays. The presence of T cells (Figure 3A) or their soluble factors (Figure 3B) decreased the proliferation of MSCs modestly. A significant decline in DNA content was observed when MSCs were cultured in high concentrations of T cell CM. These data indicate that changes in ALP activity measured in coculture experiments reflect an increase in osteogenesis and not a change in MSC proliferation.

To identify the factors that contributed to the pro-osteogenic effect of the T cell CM, several cytokines associated with T helper and regulatory T cells were blocked in the cultures (Figure 4). Two cytokines were found that significantly affected the ALP activity in MSCs when blocked during treatment with CM. Blocking IFN-γ resulted in a 80% decrease in the pro-osteogenic effect of the CM. In contrast, blocking TGF-β further enhanced the effect of the CM on the ALP activity more than two-fold. IFN-γ and TGF-β seemed to have an opposing effect on osteogenesis, as no change in the ALP activity was seen compared to the control when these two cytokines were neutralized simultaneously in the CM.

Pro- and anti-inflammatory CD4+ T cell subsets differently affect osteoblast maturation

To further establish the contribution of differentiated CD4+ T cell subpopulations to MSC osteogenesis, naïve CD4+ T cells were differentiated into anti-inflammatory T_{REG} or proinflammatory T_H17 cells. In direct cocultures, an enrichment of the T_{REG} cell fraction had no additional effect on the ALP activity in MSCs compared to the undifferentiated CD4+ T cell population (Figure 5A). In contrast, enrichment of the T_H17 cell fraction resulted in a loss of the osteogenic effects.

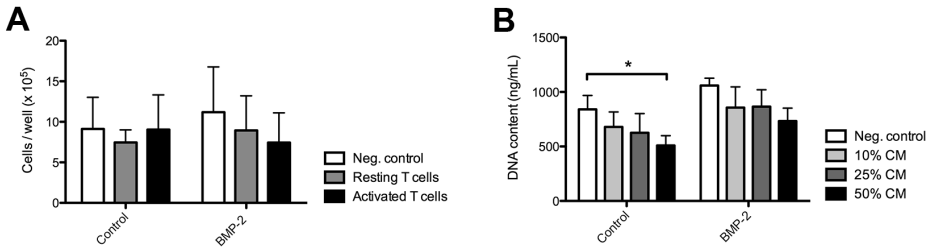

Figure 3. The effect of T cells on MSC proliferation. (**A**) MSCs were cultured with $2.5x10^5$ activated allogeneic T cells in 24-well plates for 10 days. The MSCs were counted by flow cytometry, n = 4 blood donors for independent experiments using two MSC donors. (**B**) MSCs were cultured in different dilutions of activated T cell CM. DNA levels were measured at day 10, n = 6 T cell donors for independent experiments using two MSC donors. Histograms show the mean ± SD. * $p<0.05$.

As the MSCs are likely only exposed to a short T cell stimulus in direct cocultures (Supplementary Figure 2), we also exposed the MSCs to the CM derived from the enriched T_{REG} or T_H17 subsets. This furthermore allowed us to gain insight in the effects of T_{REG}/T_H17-derived factors alone (Figure 5B, 5C). The CM of activated T_{REG} cells exhibited no additional effect on ALP activity in MSCs compared to CM from undifferentiated CD4+ T cells (Figure 5B). Although MSCs stimulated with BMP-2 and 10% T_{REG} CM showed a tendency towards increased ALP activity, the opposite effect existed for culture in a higher concentration T_{REG} CM. In contrast, MSCs dose-dependently exhibited an increase in ALP activity after treatment with soluble factors from enriched T_H17 cells (Figure 5C). T_H17-derived CM was more osteogenic than the CM from non-differentiated cells, resulting in a 7-fold increase in the ALP activity in MSCs.

T_H17-specific cytokines stimulate osteoblast maturation

To evaluate the direct effects of T_H17 cell-specific cytokines on osteoblast differentiation[31], MSCs were treated with recombinant human IL-17A or IL-17F. In ODM, we found significant increases in the ALP activity in MSCs after treatment with these proinflammatory factors (Figure 6A). IL-17A stimulated the ALP activity in MSCs similarly for all concentrations tested, whereas treatment with IL-17F resulted in a dose-dependent increase in the ALP activity. MSCs treated with IL-17 similarly exhibited a marked increase in the mineralized matrix deposition, but only in ODM (Figure 6B). In particular, BMP-2 and IL-17A had marked synergistic effects on the calcium deposition by MSCs.

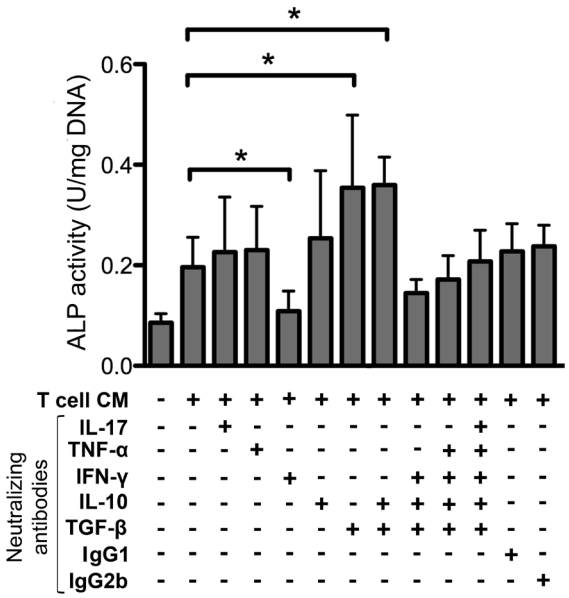

Figure 4. The contribution of individual cytokines in CD4+ T cell CM-induced osteogenesis. Day 10 ALP activity/DNA in MSCs cultured in growth medium supplemented with 25% CM collected from activated CD4+ T cells. The activity of a number of T cell-derived cytokines was blocked by adding neutralizing antibodies to the medium, either alone or in combinations. The possibility of non-specific binding of the neutralizing antibodies was excluded using isotype-matched control antibodies. The histogram shows the mean ± SD for n=4 blood donors and one MSC donor. * $p<0.05$ compared to the T cell CM group without antibodies.

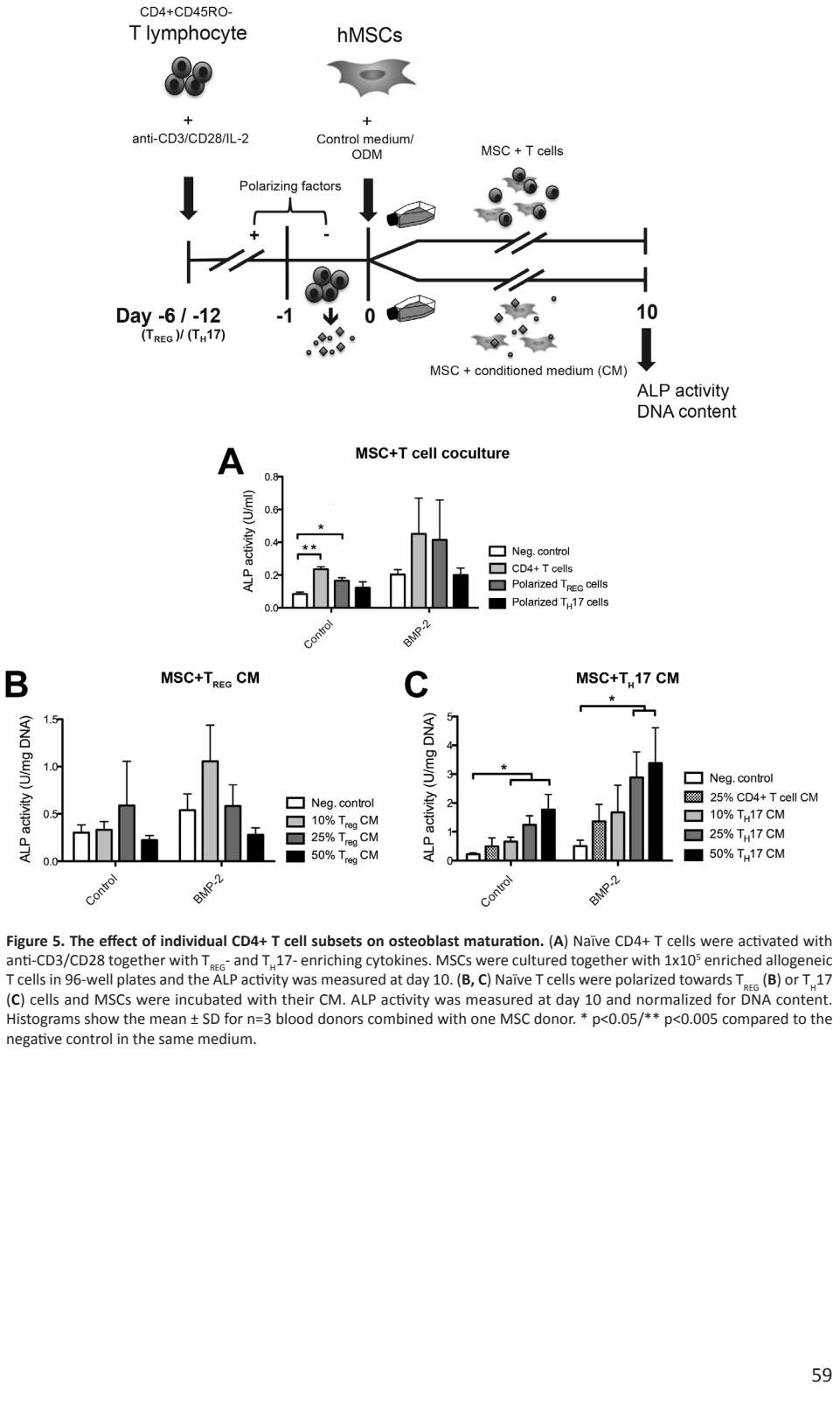

Figure 5. The effect of individual CD4+ T cell subsets on osteoblast maturation. (**A**) Naïve CD4+ T cells were activated with anti-CD3/CD28 together with T_{REG}- and T_H17- enriching cytokines. MSCs were cultured together with $1x10^5$ enriched allogeneic T cells in 96-well plates and the ALP activity was measured at day 10. (**B, C**) Naïve T cells were polarized towards T_{REG} (**B**) or T_H17 (**C**) cells and MSCs were incubated with their CM. ALP activity was measured at day 10 and normalized for DNA content. Histograms show the mean ± SD for n=3 blood donors combined with one MSC donor. * p<0.05/** p<0.005 compared to the negative control in the same medium.

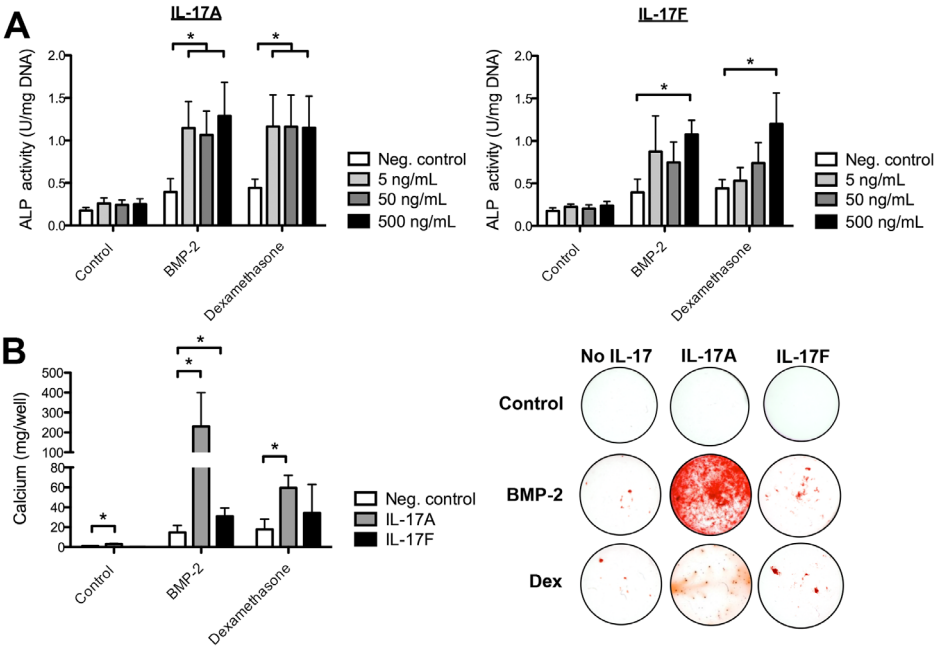

Figure 6. The effect of T helper 17-specific cytokines on the osteogenic differentiation of MSCs. (**A**) MSCs were cultured with different concentrations of IL-17A or IL-17F for 10 days in growth medium (control) or ODM (BMP-2 or dexamethasone). ALP activity/DNA was measured at day 10. (**B**) MSCs were cultured in growth medium (control) or ODM for 16 days during treatment with IL-17 cytokines. Alizarin Red S staining was performed to quantify the calcium deposition (representative images in right panel). Histograms show the mean ± SD for n=4 MSC donors. * p<0.05 compared to the negative control in the same medium.

DISCUSSION

In addition to their role in pathological bone resorption[15,32], T lymphocytes may also exert anabolic effects on bone tissue[21]. The interplay between MSCs and T cells has been investigated extensively to assess the immunomodulatory role of MSCs[33], but little is reported on the effects of T cells on MSCs. Although the interaction between MSCs and T cells partially occurs through macrophages as a third cell type *in vivo*[34-37], we used a simplified human MSC-T cell coculture model to study the direct effect of activated T cells on osteogenesis. Due to the practical and ethical limitations of combining MSCs and freshly-isolated lymphocytes from the same human donor, the MSC-T cell interactions studied here were of allogeneic nature. To eliminate possible confounding effects arising from MSC-T cell HLA mismatching, conditioned media (CM) experiments were performed in parallel. The similar outcomes observed for the CM experiments suggests that the difference in donor source did not have a profound effect on the MSC-T cell interactions in the context of osteogenesis. However, the allogeneic MSCs did have an inhibitory effect on the activation status and the viability of the T cells. As a result, the T cells likely only mediated a short-lasting effect on the MSCs in the coculture experiments. In contrast, MSCs were exposed to a continuous stimulus in the CM experiments.

The ALP activity was measured as an early osteogenic marker as it is highly predictive for the *in vivo* bone-forming capacity of human MSCs[38]. After treatment of MSCs with

activated T cells or their CM, MSCs exhibited a significantly higher early ALP activity. This was even the case in non-osteogenic medium, which suggests that certain T cell cytokines contain strong osteogenic potency. Although osteocalcin is an important bone-specific marker[39], it was not measured in the current study as its expression does not seem to coincide with the osteoblast differentiation of human MSCs after stimulation with proinflammatory factors[40,41]. In line with the early ALP activity, MSCs treated with soluble factors from activated T cells subsequently exhibited an increase in their ability to mineralize the matrix, as a sign of terminal osteoblast differentiation. Although recipient T cells are thought to inhibit the bone formation induced by allogeneic MSCs[23,42], this may be specifically related to the negative effects of proinflammatory T cell cytokines on the survival of exogenously delivered MSCs. Our results furthermore show that the pro-osteogenic effects of T cells require their activated phenotype. It has previously been shown that after bone injury, lymphocytes quickly migrate towards the site of damage[21]. Based on our data, it can be speculated that bone injury induces the activation of T cells, which then participate in the healing process by stimulating osteoblast development[43]. The activation and recruitment of CD4+ T helper cells during repair has already been established in soft tissue injury[44].

The stimulatory effects of activated T cell-derived CM on osteogenesis were reported before[45]. Here, we further discriminated between T cell subpopulations and established that CD4+ T cells were more stimulatory for osteogenesis than CD8+ cytotoxic (T_{CTX}) T cells. This is in line with the finding that CD4+ T cells may be more beneficial for bone regeneration than their CD8+ T_{CTX} cell counterpart[24,46]. When studying the secreted factors of the CD4+ T cell population more closely[47,48], IFN-γ was found to be an important proinflammatory cytokine contributing to the pro-osteogenic nature of the CM. While the high levels of IFN-γ trigger bone loss under chronic inflammatory conditions[49], it is also suggested that IFN-γ plays a role in the commitment of MSCs into the osteoblastic lineage both *in vitro* and *in vivo*[50]. Furthermore, a loss in IFN-γ signaling may be one of the reasons for the impaired bone healing observed in mice lacking functional T cells[17]. We also demonstrated a negative association between the activity of the anti-inflammatory cytokine TGF-β and osteoblast differentiation. Although similar inhibitory effects *in vitro* have also been reported by others[17], it contradicts the general assumption that TGF-β signaling is beneficial for osteogenesis in an early stage of differentiation[51,52]. There are data to support that the contradicting roles of IFN-γ and TGF-β may be related to their different actions in endogenous BMP-2 signaling in MSCS[45,53].

The opposite effects observed for IFN-γ and TGF-β in T cell-induced osteogenesis led to the hypothesis that pro- and anti-inflammatory T cell subsets within the CD4+ T population have different effects on osteoblast maturation. We studied the T_H17 and T_{REG} subsets more extensively, because they are recognized as the archetypal proinflammatory and anti-inflammatory CD4+ T cell phenotypes, respectively. Considering the low occurrence of these cells in peripheral blood[54,55], we enriched these cells *in vitro* following polarization protocols. Although the enriched fractions never contained more than 50% T_{REG} or T_H17 cells, the increase in differentiated cells was sufficient to cause significant changes in the ALP activity of MSCs compared to unpolarized cells. T_{REG} cells are known inhibitors of osteoclast formation *in vitro* and *in vivo*[32,56,57], but little is known about their role in osteoblast maturation. The results of our T_{REG} experiments showed that the enriched cells and their soluble factors are likely not important regulators in osteoblast

maturation. Although this contradicts the observation that the delivery of T_{REG} cells enhances MSC-based bone regeneration in mice, this effect is possibly indirect[23]. In line with our current findings for TGF-β, mostly inhibitory effects have been demonstrated when studying the main T_{REG} anti-inflammatory cytokines directly for their role on osteoblast maturation[17,58].

In contrast to our observations for T_{REG} cells, our results show that proinflammatory T cell cytokines have profound stimulatory effects on the osteogenic differentiation of bone progenitor cells. First, the CM derived from enriched T_H17 cells induced osteogenesis significantly more than that derived from undifferentiated T cells. Second, T_H17 cell-derived cytokines showed significant pro-osteogenic effects when tested directly on MSCs. As IFN-γ-producing T_H17 cells can only be found under rare conditions[59], it is unlikely that IFN-γ contributed to the beneficial effects of the T_H17 cell-derived CM in our experiments[60]. Therefore, we studied the IL-17 family of cytokines directly for their role in osteoblast maturation. During fracture healing, BMP-2 is one of the growth factors that is up-regulated together with proinflammatory factors[9,61]. To mimic a more physiological environment for the MSCs, BMP-2 was therefore used as an alternative to dexamethasone in the osteogenic differentiation medium. IL-17A and IL-17F, the two predominant isoforms produced by T_H17 cells, were both strong pro-osteogenic stimuli for MSCs together with BMP-2.

IL-17A in particular, showed a synergistic effect together with BMP-2. This novel finding requires more investigation in a preclinical setting, but is in line with the observation that IL-17A protects against bone loss in mice[62]. In contrast to IL-17A[63,64], little is yet known about the effects of IL-17F on osteoblast maturation. Nam et al. only recently proposed an important role for IL-17F in bone repair. Our data obtained with IL-17F in human MSCs agree with the finding from these same authors that IL-17F treatment increases the expression of a panel of bone markers in mouse MSCs[17]. In discordance to the direct effects found for IL-17 and TNF-α on MSCs[40], blocking these cytokines did not reduce the pro-osteogenic effect of the CD4+ T cell CM on the ALP activity. A first explanation is the missing second osteogenic stimulus required for their efficacy[40]. Alternatively, TNF-α and IL-17 may induce different effects on the MSCs in the context of the cocktail of cytokines present in the CM. Based on these results, we hypothesize that the exogenous delivery of IL-17 cytokines, in combination with well-known osteoinductive growth factors, may create a physiological stimulus for bone regeneration. Importantly, IL-17 signaling is well-known for its detrimental effects on bone tissue through the stimulation of osteoclastogenesis[65]. Therefore, a bone anabolic therapy using IL-17 should also rely on methods to directly[66] or indirectly[67,68] suppress the action of osteoclasts.

A contradictory finding was that T_H17 cells did not show an effect on osteoblast differentiation in direct coculture with MSCs. MSCs are known to suppress T_H17 cell differentiation while driving T_{REG} differentiation in coculture models[69,70], which might influence the studied MSC-T cell interaction. However, other studies show that these immunomodulatory effects are less profound in our experimental design, i.e. the scenario where the T_H17 cells are exposed to the MSCs after their initial activation and differentiation[71,72]. It is thus more likely that the different outcomes observed for the T_H17 cells between the CM and coculture experiments are related to the duration of the stimulus to which the MSCs are exposed. In the CM experiments, the MSCs are exposed to a continuous stimulus during the entire culture period, while the T cells likely only

induce a short-lasting effect on the MSCs in the cocultures (Supplementary Figure 2) .

Our results indicate that activated T lymphocytes can contribute to osteoblast maturation through the production of soluble factors. Although the CD4+ T cell population demonstrates the highest osteogenic effects as a whole, individual CD4+ T cell subsets can still contribute to MSC osteogenesis in different ways. The beneficial role of IFN-γ in osteoblast differentiation suggests that the T helper 1 subset of the CD4+ population should be further elucidated. Our current polarization experiments show that proinflammatory T cell populations, including the T_H17 cells, are most stimulatory on bone progenitor cells. Our current *in vitro* findings are in agreement with the *in vivo* results of Nam et al., who proposed that the local balance between anti-inflammatory T_{REG} and proinflammatory T_H17 cells affects the outcome of fracture healing[17]. In agreement with this hypothesis, we show that different T cell subsets and their secreted factors may differently affect the outcome of bone regeneration. For instance, the relative occurrence of different CD4+ T cell subsets may be predictive for the success of bone healing. This is in line with data showing that the systemic presence of a subset of CD8+ effector memory T cells is associated with decreased fracture healing[24]. Alternatively, bone-stimulating T cell cytokines may be harnessed to promote bone regeneration in tissue engineering strategies[73].

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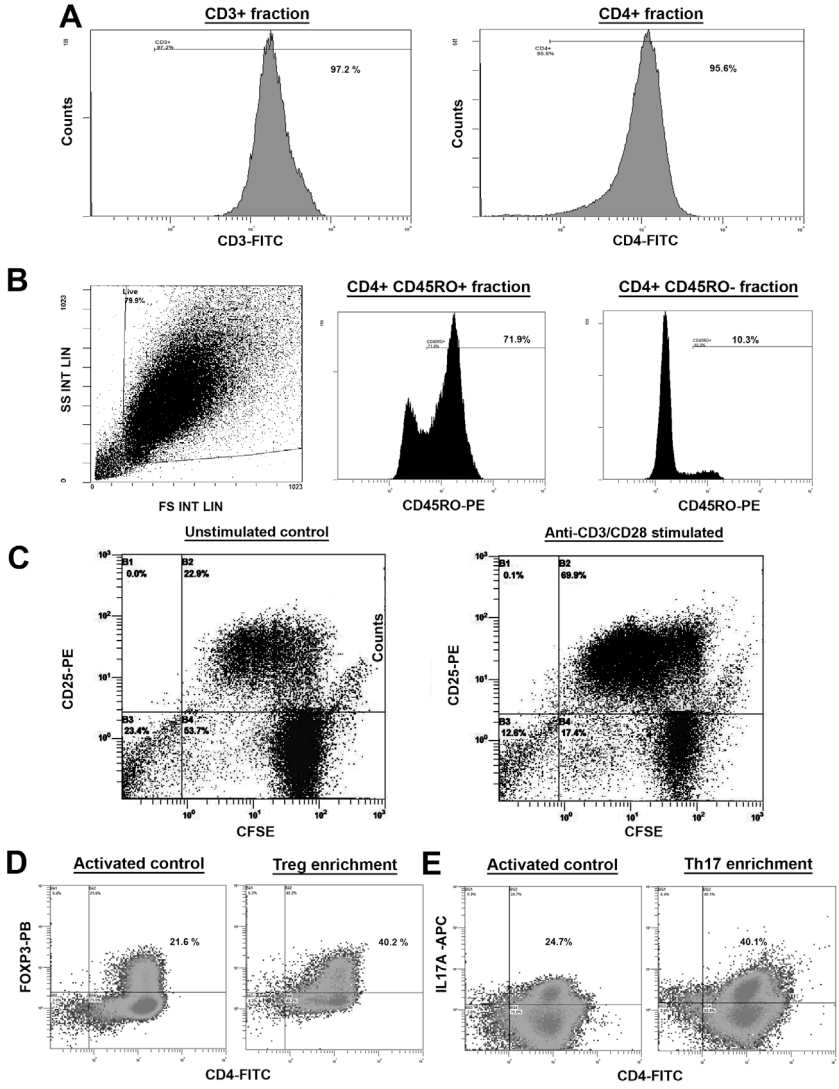
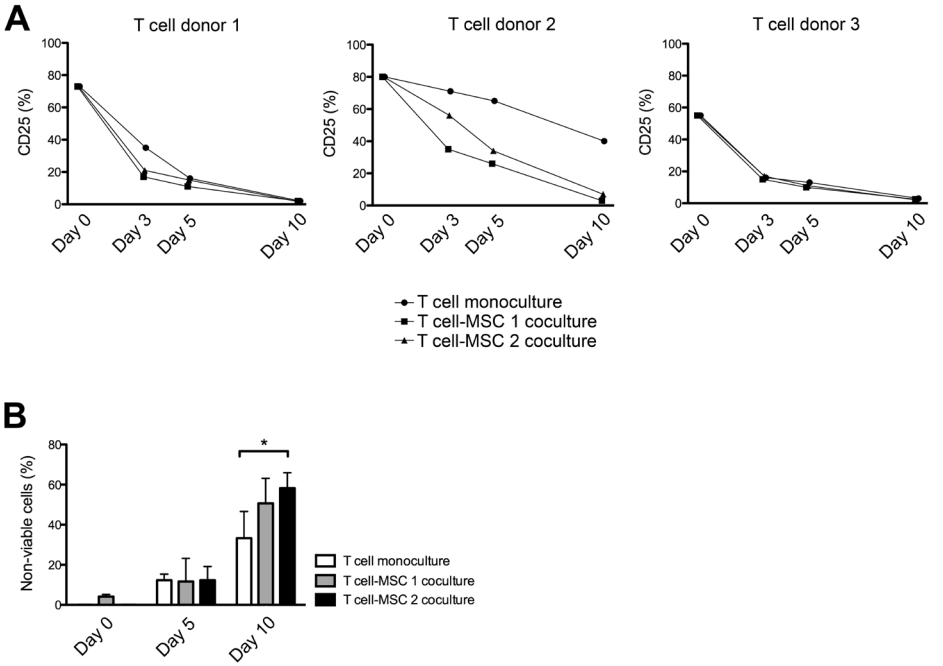
SUPPLEMENTARY DATA



Supplementary Figure 1. FACS analysis after T cell isolation and differentiation. (**A**) PBMCs were isolated from human blood. CD3 or CD4 positive cells were isolated by positive selection using MicroBeads. Histograms show the cell counts after staining with CD3-FITC (left) and CD4-FITC (right), gated on the live cell population. The purity of cells ranged from 90-99%. (**B**) CD3+ T cells were isolated, labeled with carboxyfluorescein succinimidyl ester (CFSE), and stimulated with anti-CD3/CD28 and IL-2 for 4 days. The expression of the activation marker CD25 was approximately 20% in controls and 60-70% in stimulated cells (live gate). (**C**) CD4+CD45RO- naïve T cells were obtained for differentiation experiments by CD45 depletion followed by CD4 positive selection with MicroBeads. Plots show the CD45RO-positive and negative fractions on the live CD4+ population. 80-90% of starting cells were negative for CD45RO. (**D**) CD4+CD45RO- naïve T cells were stimulated for 5 days with anti-CD3/CD28 and IL-2 without (left) or with (right) rhTGF-β1. Intracellular staining for the transcription factor Foxp3 was performed as a marker of induced regulatory T cells. Treatment with rhTGF-β1 resulted in a doubling of the number of CD4+Foxp3+ cells to 40-50%. (**E**) CD4+CD45RO- naïve T cells were stimulated with anti-CD3/CD28 for 5 days. Cells were restimulated for 5 days in the presence of IL-2 and treated with PMA/ionomycin overnight in the presence of protein transport inhibitor Brefedin A. Intracellular staining was performed for IL-17A cytokine as a marker for T_H17 cells. Treatment of cells with polarizing antibodies resulted in approximately 40% IL-17A-producing cells.



Supplementary Figure 2. T cell activation status (A) and viability (B) during coculture with MSCs. (**A**) CD4+ T cells were isolated from three PBMC donors using MicroBeads and stimulated with anti-CD3/CD28 and IL-2 for 4 days. After activation (day 0), the cells were cocultured with two allogeneic MSC donors for 10 days. At day 3, 5, and 10 of the coculture the cells were subjected to FACS analysis. The percentage of CD25-expressing cells was determined after gating on the CD4+/7AAD- population. As a control, T cells were cultured in the absence of MSCs (T cell monoculture). (**B**) The number of 7AAD+/CD4+ T cells before (day 0) and after (day 5 and 10) coculture with allogeneic MSCs was quantified by FACS analysis. The histogram shows the mean ± SD for n=3 blood donors. * $p<0.05$.

Chapter 4

Local induction of inflammation affects bone formation

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ABSTRACT

To explore the influence of inflammatory processes on bone formation, we applied a new *in vivo* screening model. Confined biological pockets were first created in rabbits as a response to implanted bone cement discs. These biomembrane pockets were subsequently used to study the effects of inflammatory stimuli on ectopic bone formation within biphasic calcium phosphate (BCP) constructs loaded with TNF-α, lipopolysaccharide (LPS) or lipoteichoic acid (LTA), all with or without bone morphogenetic protein (BMP)-2. Analysis of bone formation after 12 weeks demonstrated that the inflammatory mediators were not bone-inductive in combination with the BCP alone, but inhibited or enhanced BMP-induced bone formation. LPS was associated with a strong inhibition of bone formation by BMP-2, while LTA and TNF-α showed a positive interaction with BMP-2. Since the biomembrane pockets did not interfere with bone formation and prevented the leakage of pro-inflammatory compounds to the surrounding tissue, the biomembrane model can be used for *in vivo* approaches to study local inflammation in conjunction with new bone formation. Using this model, it was shown that the modulation of the inflammatory response could be beneficial or detrimental to the subsequent bone formation process. The co-delivery of inflammatory factors and bone-related growth factors should be further explored as a strategy to enhance the bone-forming efficacy of bone substitutes.

INTRODUCTION

New strategies are needed to reduce the widespread use of bone transplants for the regeneration and restoration of skeletal defects. Considering the complication rate of 10-40%[1,2], the limited supply of donor bone[3], and the significant increase in surgery time associated with bone harvesting[4], different approaches are being explored to create bone substitutes which can perform similar or better than autograft bone[1,5]. The use of stromal or stem cells[6], growth factors[7], and various biomaterials[8] have shown successful outcomes in animal studies. However, these approaches have not yet reached the clinical practice for widespread use, due to regulatory issues and the difficulty to demonstrate the efficacy of these treatments in clinical studies[9]. Currently, the bone morphogenetic proteins (BMPs) are the only osteoinductive alternatives for bone grafting in the treatment of large bone defects[10,11]. However, their current dosage and delivery method is associated with serious adverse effects[12,13], high costs[14], and unpredictable outcomes[15-17].

Modulation of the inflammatory response is a relatively new strategy that is being explored to enhance the pro-osteogenic effects of bone graft substitutes[18-20]. As bone has the unique capacity to fully repair under normal conditions, the process of bone fracture healing has been studied as a model for bone regeneration[21]. After injury, the initial inflammatory phase is essential for optimal fracture healing to occur, as this triggers a complex interaction between infiltrating immune cells, resident cells and bone progenitor cells[19,22-24]. During this phase, proinflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-17 act together with BMPs and other transforming growth factor family members to induce processes leading to repair and restoration[21,22,25-30]. Several of these cytokines have stimulatory effects on the osteogenic differentiation of mesenchymal stem cells (MSCs) *in vitro*[19,31-33]. A balanced expression of these cytokines seems to be required for optimal fracture healing, since this process may be impaired in patients with chronic inflammation[19,21], or patients with systemic and serious acute inflammation such as polytrauma[34,35]. In a pathological context, inflammatory processes can also result in excessive new bone formation. Some typical examples include ankylosing spondylitis[36], soft tissue trauma[37,38], neurogenic trauma[39], fibrodysplasia ossificans progressiva[40], and osteomyelitis[41]. In these conditions, the same cytokines are expressed that are known to be crucial in bone fracture healing and MSC osteogenic differentiation[36,38,42,43].

In addition to being progenitors for the osteogenic lineage, MSCs exert immunomodulatory functions and express various receptors that are involved in inflammatory processes[44-46]. These include the toll-like-receptors (TLRs), which are most widely expressed on innate immune cells[45,47,48]. TLR signaling involves similar cascades of signal transduction and transcription regulation as TNF-α signaling[49], and both can target downstream regulators of osteogenesis in bone progenitor cells[31,45,50]. While several lines of evidence show that TNF-α stimulates the osteoblast differentiation in human MSCs, this effect seems receptor-specific for the TLR agonists[25,50,51]. Furthermore, *in vitro*, pro-inflammatory mediators only promote osteoblast differentiation in the presence of an osteoinductive factor like dexamethasone or BMP-2, suggesting that the commitment of bone progenitor cells to the osteogenic lineage enhances their responsiveness to inflammatory signals[25,45,52].

In order to create localised tissue responses, a biological membrane (biomembrane) pocket model was tested. This model may particularly serve as a tool for osteoimmunological

research. The biomembrane is formed as a response to a polymethylmethacrylate (PMMA) spacer and provides a confined space for the implantation of biomaterials together with various stimuli[53-55]. This model shows similarities with the air pouch model, where a subcutaneous cavity is generated by the repeated injection of air. This provides a localised environment for the delivery of inflammatory compounds, and the subsequent tracking of the infiltration of inflammatory cells and their produced soluble factors by harvesting the inflammatory exudate[56,57]. As additional advantages, the biomembrane model allows for multiple identical compartments to be created within the same animal, while the membrane also provides growth factors and cytokines supporting bone formation[54,58,59].

We hypothesise that certain pro-inflammatory signals act in synergy with osteoinductive signals to induce bone formation by promoting osteoblast differentiation. To test this, the pro-inflammatory factors TNF-α, lipopolysaccharide (LPS) or lipoteichoic acid (LTA) were either or not combined with BMP-2 on porous biphasic calcium phosphate (BCP) scaffolds and implanted in subcutaneous or intramuscular biomembrane pockets in rabbits. The possible synergy between the pro-inflammatory signals and BMP-2 was investigated in the subcutaneous location, where bone induction is dependent on exogenous BMP-2 delivery. In the intramuscular environment however, the BCP material is bone-inductive without exogenous growth factors[60], allowing us to study the interaction between pro-inflammatory signals and osteoinductive signals. Furthermore, to assess the potential of the biomembrane model for osteoimmunological research, we aimed to investigate if the biomembrane environment affects the bone formation in established osteogenic constructs of ceramics loaded with MSCs or BMP-2.

MATERIALS AND METHODS

Study design

First, an assessment study was performed to verify the potential for ectopic bone formation in the biomembrane pocket model. Established constructs for ectopic bone formation, i.e. porous BCP scaffolds loaded with MSCs or BMP-2, were implanted intramuscularly and subcutaneously in rats (n=4) and rabbits (n=4), inside and outside the biomembrane pockets. The newly formed bone (bone area%) in the BCP constructs was the primary outcome after 8 weeks (rats) or 12 weeks (rabbits). Subsequently, the rabbit model (n=11) was used to 1) screen for the potential of inflammatory stimuli to induce bone formation intramuscularly and to 2) screen for the modifying effects of inflammatory stimuli on BMP-induced bone formation subcutaneously. The bone volume (bone area%) in the BCP constructs was the primary outcome after 12 weeks.

Materials

Polymethylmethacrylate (PMMA, Simplex P, Stryker, Kalamazoo, USA) discs were produced using custom-made silicone molds. The PMMA discs were sterilised in a 1 M NaOH solution and extensively washed with PBS. Biphasic calcium phosphate (BCP) blocks were made with dimensions of 3.5x3.5x3 mm (rats) or 6x6x3 mm (rabbits). These BCP blocks consisted of 20±5% β-tricalcium phosphate and 80±5% hydroxyapatite by weight, and had a total porosity of 75±5%[61]. The BCP blocks were autoclaved at 121 °C for 30 min and dried at 60 °C.

The following inflammatory mediators were tested for their effect on bone formation: human tumor necrosis factor alpha (TNF-α, 14-8329, Ebioscience, San Diego, USA), lipopolysaccharide (LPS, *E. coli*, L5418, Sigma, St. Louis, USA), and lipoteichoic acid (LTA, *S. aureus*, L2515, Sigma). These mediators were tested with or without recombinant human BMP-2 (InductOS, Wyeth/Pfizer, New York, USA).

Mesenchymal stem cell isolation and culture

For syngeneic rat MSC transplantations, one rat was killed using an overdose of CO_2. Using a sterile technique, the bone marrow was flushed from the femurs. For autologous rabbit MSC transplantation, bone marrow was harvested from each animal under general anesthesia by aspiration from the iliac crest using an 18G needle. The mononuclear cell fraction was isolated by Ficoll-paque centrifugation and plated in expansion medium, consisting of α-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Cambrex, East Rutherford, NJ, USA), 0.2 mM L-ascorbic acid 2-phosphate (Sigma), and 100 units/ml penicillin/streptomycin (Invitrogen). The cell cultures were kept in a humidified incubator at 37 °C and 5% CO_2. The adherent cells were expanded to passage 4 and cryopreserved. The cells were replated and cultured for two days before *in vivo* implantation.

Construct preparation

For the preparation of MSC or BMP-2 loaded BCP constructs (assessment study, Table 1), solutions of 10^7 cells/ml or 150 µg/ml BMP-2 in PBS were prepared. Subsequently, 25 µl (rats) or 70 µl (rabbits) of this solution, or empty PBS (controls), was pipetted onto the scaffolds. After two hours in a humidified incubator, the constructs were submerged in α-MEM medium and stored overnight at 37 °C and 5% CO_2.

For the comparative study (Table 2), inflammatory stimuli were added to the BCP, alone or in combination with BMP-2, as follows: either 15 µl PBS (intramuscular samples) or 15 µl of a 100 µg/ml BMP-2 solution in PBS (subcutaneous samples) was pipetted onto the scaffolds. Subsequently, 55 µl empty fibrin glue (controls, Tissucol 500®, Baxter, Deerfield, IL, USA) or 55 µl fibrin glue containing the proinflammatory mediators was pipetted onto the scaffolds. For this purpose, the mediators were resuspended in 27.5 µl of the thrombin component (diluted 1:50 in PBS), mixed with 27.5 µl of the fibrinogen component (diluted 1:30 in PBS) and immediately pipetted onto the BCP scaffolds. The constructs were prepared on the day of surgery and stored in a humidified environment at 37 °C until implantation. To determine the release profile, BCP scaffolds were loaded with LPS in fibrin glue. As a control, LPS was loaded onto the scaffolds in PBS. The constructs were kept in PBS at 37 °C. At different time points, samples were taken from the PBS to determine the LPS concentration using a Limulus Amebocyte Lysate assay according to the manufacturer's protocol (Genscript, Piscataway, NJ, USA). It was found that the LPS was better retained using the fibrin glue method during the first hours, although the LPS was completely released from the constructs within 24 hours in both conditions (Figure 1).

Table 1. Conditions implanted in pre-induced biomembrane pockets and in fresh pockets (assessment study in rats and rabbits).

Group	MSCs/BMP-2		n	PMMA/BCP size
	(Dose)	(Conc)		
Rat subcutaneous and intramuscular groups				
Empty control	-	-	4	6 mm ø x 4 mm L / 3.5x3.5x3 mm
Syngeneic MSCs	$2.5x10^5$	10^7/ml	4	6 mm ø x 4 mm L / 3.5x3.5x3 mm
BMP-2	3.75 µg	150 µg/ml	4	6 mm ø x 4 mm L / 3.5x3.5x3 mm
Rabbit subcutaneous and intramuscular groups				
Empty control	-	-	4	10 mm ø x 4 mm L / 6 x6x3 mm
Autologous MSCs	$7x10^5$	10^7/ml	4	10 mm ø x 4 mm L / 6 x6x3 mm
BMP-2	10.5 µg	150 µg/ml	4	10 mm ø x 4 mm L / 6 x6x3 mm

BMP=bone morphogenetic protein, BCP=biphasic calcium phosphate, MSC=mesenchymal stem cell, PMMA=polymethylmethacrylate.

Table 2. Conditions implanted in pre-induced biomembrane pockets (comparative study in rabbits).

Group	BMP-2		Mediator		n
	(Dose)	(Conc)	(Dose)	(Conc)	
Intramuscular groups					
Control		-		-	11
TNF-α		-	1, 10, 100 ng	14-1430 ng/ml	8
LPS		-	0.1, 1, 10 µg	1-143 µg/ml	8
LTA		-	0.5, 5, 50 µg	7-710 µg/ml	8
Subcutaneous groups					
Control	1.5 µg	22 µg/ml		-	11
TNF-α	1.5 µg	22 µg/ml	1, 10, 100 ng	14-1430 ng/ml	8
LPS	1.5 µg	22 µg/ml	0.1, 1, 10 µg	1-143 µg/ml	8
LTA	1.5 µg	22 µg/ml	0.5, 5, 50 µg	7-710 µg/ml	8

BMP=bone morphogenetic protein, BCP=biphasic calcium phosphate, TNF-α=tumor necrosis factor alpha, LPS=lipopolysaccharide, LTA=lipoteichoic acid.

Animals

Animal experiments were performed after approval of the local Ethics Committee for Animal Experimentation and in compliance with the Institutional Guidelines on the use of laboratory animals (Utrecht University, Utrecht, The Netherlands). A total of fifteen male New Zealand white rabbits (14 weeks old, 2.5-3.0 kg, Crl:KBL, Charles River, L'Arbresle, France) and five male Fischer rats (14 weeks old, 300-350 g, F344/IcoCrl, Charles River) were used for the experiments. Four rabbits and five rats were used to evaluate the ectopic bone formation within the induced biomembrane (assessment study). Eleven rabbits were used to study the effect of inflammatory stimuli on ectopic bone induction

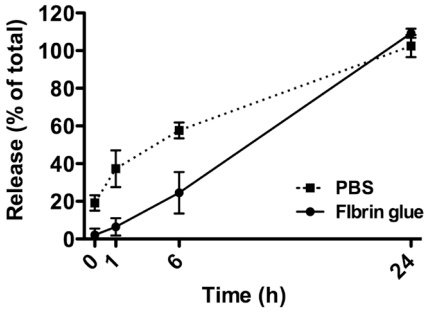


Figure 1. *In vitro* release of LPS from the BCP constructs. LPS was loaded in fibrin glue or in PBS, and its concentration was measured in the supernatant at different time points.

and formation (comparative study). All animals were housed at the Central Laboratory Animal Research Facility, Utrecht University. They were allowed to acclimatise for at least two weeks before the surgery. Food and water were given *ad libitum*.

Surgical procedure

The animals underwent two surgeries under general anesthesia as part of the two-step biomembrane pocket model (Figure 2). In general, the same surgical methods were applied for the assessment and comparative study. As a difference, the two surgeries were separated by a period of six weeks in the assessment study, and five weeks in the comparative study. Rats received 3% isoflurane, while rabbits received Ketamine (15 mg/kg i.m.; Narketan®, Vétoquinol BV, 's-Hertogenbosch, The Netherlands) and Glycopyrrolate (0.1 mg/kg i.m.; Robinul, Riemser Arzneimittel AG, Greifswald, Germany) pre-operatively, and Medetomidine (0.25 mg/kg s.c.; Dexdomitor®, Orion Corporation, Espoo, Finland) peri-operatively. Anesthesia was reversed with Atipamezole hydrochloride (0.5-1.0 mg/kg i.v., Atipam® (Eurovet Animal Health B.V., Bladel, The Netherlands). The rabbits received antibiotic prophylaxis with Enroflaxicine (10 mg/kg sc; Baytril®, Bayer animal health, Leverkusen, Germany) once daily for three days peri-operative during the first surgery, and Penicillin ($3x10^4$ IE benzylpenicilline/kg, Duplocilline®, Merck Animal Health, Madison, USA) once during the second surgery. Animals were given pain relief pre-operatively, and post-operatively every 8 hours for 2 days with Buprenorphine (0.03 mg/kg s.c.; Temgesic®, RB Pharmaceuticals Limited, Slough, UK).

After shaving and disinfecting the skin with povidone-iodine (Betadine), a midline incision was made to expose the paraspinal muscles. In the rabbits, four intramuscular pockets were created on each side by blunt dissection for implantation of the PMMA discs (Figure 2B). The same approach was used for the subcutaneous pockets. In the assessment study, each animal received two additional subcutaneous PMMA implantations for histological analyses of the biomembrane. Pockets were closed with a non-resorbable suture (Prolene®, Ethicon, USA), followed by closure of the skin (Monocryl®, Ethicon). At the second surgery, an incision was made in the membrane surrounding the PMMA disc, the disc was replaced by a BCP construct, and the opening was sutured (Prolene®, Ethicon). For the assessment study, duplicate constructs were also implanted in freshly prepared intramuscular and subcutaneous pockets during the second surgery. Furthermore, as part

of the assessment study, two subcutaneous biomembranes were explanted from each animal during the second surgery and fixed in 4% formalin for histology.

Fluorochrome labels were injected subcutaneously to determine the onset and location of new bone formation[62]: calcein green at week 4 (10 mg/kg s.c. in 0.2 M $NaHCO_3$, Sigma), oxytetracycline at week 8 (25 mg/kg s.c. in 50/50 PBS/demineralised water, Merck Millipore, Billerica, USA) and xylenol orange at week 11 (30 mg/kg s.c. in 0.12 M $NaHCO_3$, Sigma). The incorporation of fluorochromes in the bone was examined by fluorescence microscopy on methyl methacrylate-embedded sections. The rats were killed with CO_2 8 weeks after the second surgery. The rabbits were killed 12 weeks after the second surgery under general anesthesia, by an intravenous Pentobarbital injection (Euthanimal®, Alfasan Nederland BV, Woerden, The Netherlands). In the assessment study, an additional procedure was performed five hours prior to termination. In each animal, 100 µl of a 175 µg/ml LPS solution was injected through the skin into one of the biomembrane pockets using a 27 gauge needle, while the injection of PBS in another pocket served as a negative control. The presence of phagocytes at the BCP-biomembrane interface was determined by immunohistochemical staining.

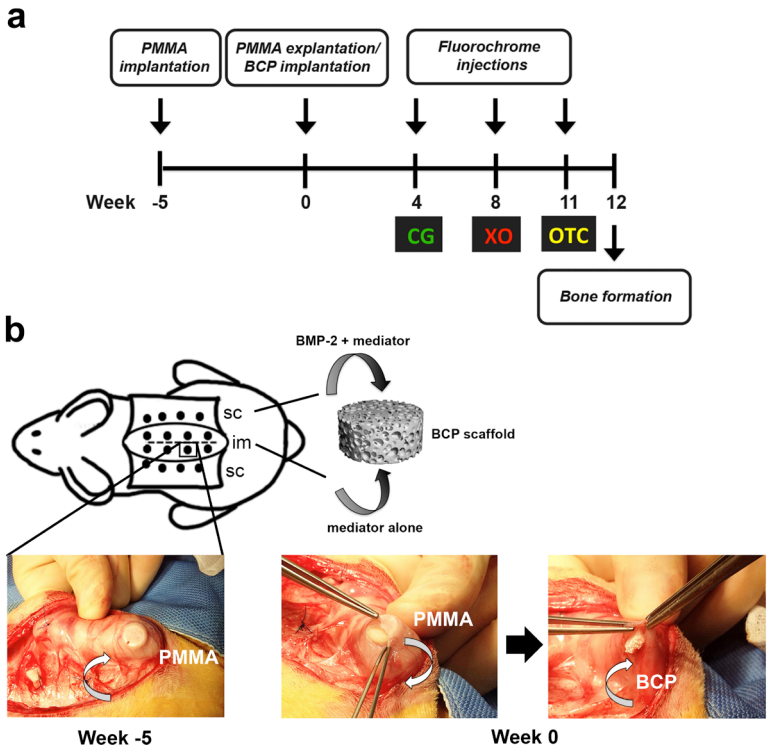


Figure 2. Design of the comparative study to investigate local bone formation in conjunction with inflammatory stimuli. (**A**) Timeline showing the relative time points for the different interventions. Bone formation was the primary outcome after 12 weeks. (**B**) Schematic representation of the subcutaneous (sc) en intramuscular (im) implantations in the rabbits. The mediators were loaded onto biphasic calcium phosphate (BCP) scaffolds and studied alone, or in combination with bone morphogenetic protein (BMP)-2. These constructs were implanted into 5-week old polymethylmethacrylate (PMMA)-induced biomembrane pockets.

Bone histomorphometry

After retrieval of the constructs, a quarter of each sample was removed for decalcification and paraffin embedding using a rotary tool with circular saw attachment (Dremel 4000, Mount Prospect, IL, USA). The remaining material was fixed in 4% formaldehyde, dehydrated by an ethanol series and embedded in methyl methacrylate (MMA, Merck Millipore). Subsequently, 35 µm-thick sections were cut using a sawing microtome (Leica, Nusslochh, Germany) and stained with basic fuchsin and methylene blue. The samples were completely sectioned and scored for the presence of bone. Two mid- sections were pseudo-coloured in Adobe Photoshop CS6 (Adobe Systems, San Jose, USA) to quantify the percentage of bone in the available pore space (bone area%). The mean value of two sections per sample was used for further statistical analyses. For four rabbits, one MMA section per BCP sample was left unstained for fluorochrome detection by fluorescence microscopy (Olympus BX51 with DP70 camera, Olympus, Shinjuku, Tokyo, Japan).

Immunohistochemical stainings

Sections of decalcified (0.3 M EDTA) BCP samples were embedded in paraffin and cut into 6 µm sections. Antigen retrieval was performed with 0.1% (w/v) proteinase K for 15 min at 37 °C, followed by blocking steps with 0.3% (v/v) H_2O_2 in PBS for 10 min and 5% bovine serum albumin (BSA) for 30 min at room temperature. For staining of macrophages and neutrophils, sections were incubated with the mouse-anti-human calprotectin antibody (5 µg/ml, MCA874A488, clone MAC387, Hercules, CA, USA) for 2 h at room temperature. A mouse IgG1 antibody (X0931, Dako) was used as an isotype-matched control. Sections were treated with the secondary antibody (1:200, RPN1001, sheep anti-mouse IgG-Biotin, GE healthcare, Chicago, IL, USA) for 30 min at room temperature. To enhance the signal, samples were incubated with streptavidin-HRP (2 µg/ml, FP0397, Dako) for 30 min. A rabbit spleen was used as a positive control.

For the detection of T lymphocytes, sections were incubated with a mouse-anti-human CD3 antibody (0.7 mg/ml, M7254, clone F7.2.38, Dako) for 2 h at room temperature. A mouse IgG1 antibody (X0931, Dako) was used as an isotype-matched control. Sections were incubated with the secondary goat-anti-mouse-HRP (5 µg/ml, P0447, Dako) for 30 min at room temperature. A rabbit lymph node was used as a positive control. Immunostainings were developed with 3,3'-diaminobenzidine tetrahychloride hydrate (DAB, D5637, Sigma) and counterstained with Mayer's hematoxylin.

The absolute number of CD3-positive cells in the entire cross-section was quantified. Furthermore, the fraction of T lymphocytes within the lymphoid clusters was determined by counting the CD3-positive and CD3-negative nuclei within three clusters for three different samples from each group. The number of lymphoid clusters per H&E stained section was scored by three researchers on blinded samples. A low interobserver variation in the counts was noted and therefore the average of the counts was used for further analyses.

Statistical analysis

For the assessment study, an arbitrary sample size of 4 was chosen. All conditions could be implanted in the same animal, thus requiring 4 animals for this study. For the comparative study, a sample size calculation was performed for the bone area% as main outcome parameter. This showed that a sample size of 8 was needed, based on an

estimated effect size of 30% with a standard deviation of 15%[63], using a power of 80% and an alpha of 1.7% for multiple pairwise comparisons. Since not all conditions could be implanted in the same animal, 11 rabbits were required for this experiment. All results are shown as the mean ± standard deviation. Statistics were performed using SPSS version 20.0 (IBM, Chicago, USA). Differences in bone area% were analysed using a linear mixed-model approach. One-way ANOVA was used to analyse differences in the average number of lymphoid clusters or CD3-positive cells. Bonferroni correction was used for multiple comparisons. Mixed model regression analysis was used to determine dose response relationships. The significance of intramuscular bone induction was analysed with a linear mixed-model approach with binary outcome measure (i.e. bone or no bone).

RESULTS

Biomembrane pocket characteristics and influence on bone formation

A clear biomembrane had formed around the implanted PMMA discs in a period of 6 weeks (Figure 3A). The biomembrane pockets had similar characteristics in rats and rabbits. They appeared well vascularised by macroscopic evaluation, while microscopically, H&E staining showed the formation of a 300-400 µm thick membrane. The membranes consisted of a cell-rich inner connective tissue layer with a thickness of 20-50 µm. The outer part of the biomembranes consisted of a thicker layer of loose connective tissue, containing larger blood vessels. Histological evaluation showed no signs of inflammation or multinucleated giant cells at the PMMA-biomembrane interface. The biomembrane had appropriate biomechanical properties for handling and suturing, and provided enough space for the implantation of the BCP scaffolds.

To determine if the biomembrane supported the localised delivery of mediators inside of the pocket, a 1% methylene blue solution was injected into the biomembrane *ex vivo*. No leakage of the solution out of the membrane was observed (Figure 3B). Furthermore, the *in vivo* transcutaneous injection of LPS into the subcutaneous biomembrane on the final day of the experiment resulted in a localised inflammatory response, i.e. presence of phagocytes within the biomembrane pocket but absent outside of the pocket (Figure 3C). This tissue response was LPS-specific, since no phagocyte infiltrations were observed in the pockets injected with PBS. The biomembrane-implanted constructs were always covered by a thicker fibrous capsule compared to directly-implanted constructs (Figure 3D).

To assess bone formation within the biomembrane, osteogenic constructs were implanted within the induced biomembrane pockets, or in fresh pockets (Table 1). After 8 weeks (rats, Figure 3E) or 12 weeks (rabbits, Figure 3F), the bone area% did not obviously differ for any of the conditions when comparing the biomembrane pockets to the fresh pockets. Whereas empty BCP constructs failed to induce any bone formation ectopically in rats, minute spots of bone were present in 3/4 empty BCP samples in rabbits implanted in the fresh muscle pockets.

Effects of inflammatory stimuli on bone induction

The intramuscularly-implanted constructs showed no signs of scaffold degradation. Scoring of the empty control scaffolds showed no bone formation after 12 weeks (Figure 4A). In addition, none of the constructs loaded with LPS demonstrated new bone formation.

Although TNF-α induced bone formation in 2 constructs, no concentration dependence was observed. LTA at the dose of 5 μg/sample was associated with bone formation in 2/8 rabbits (Figure 4A, B). No bone tissue was seen for the other LTA concentrations. The area of this newly formed bone was always less than 1% and the presence of bone was not significantly associated with one of the conditions.

Effects of inflammatory stimuli on BMP-2-induced bone formation

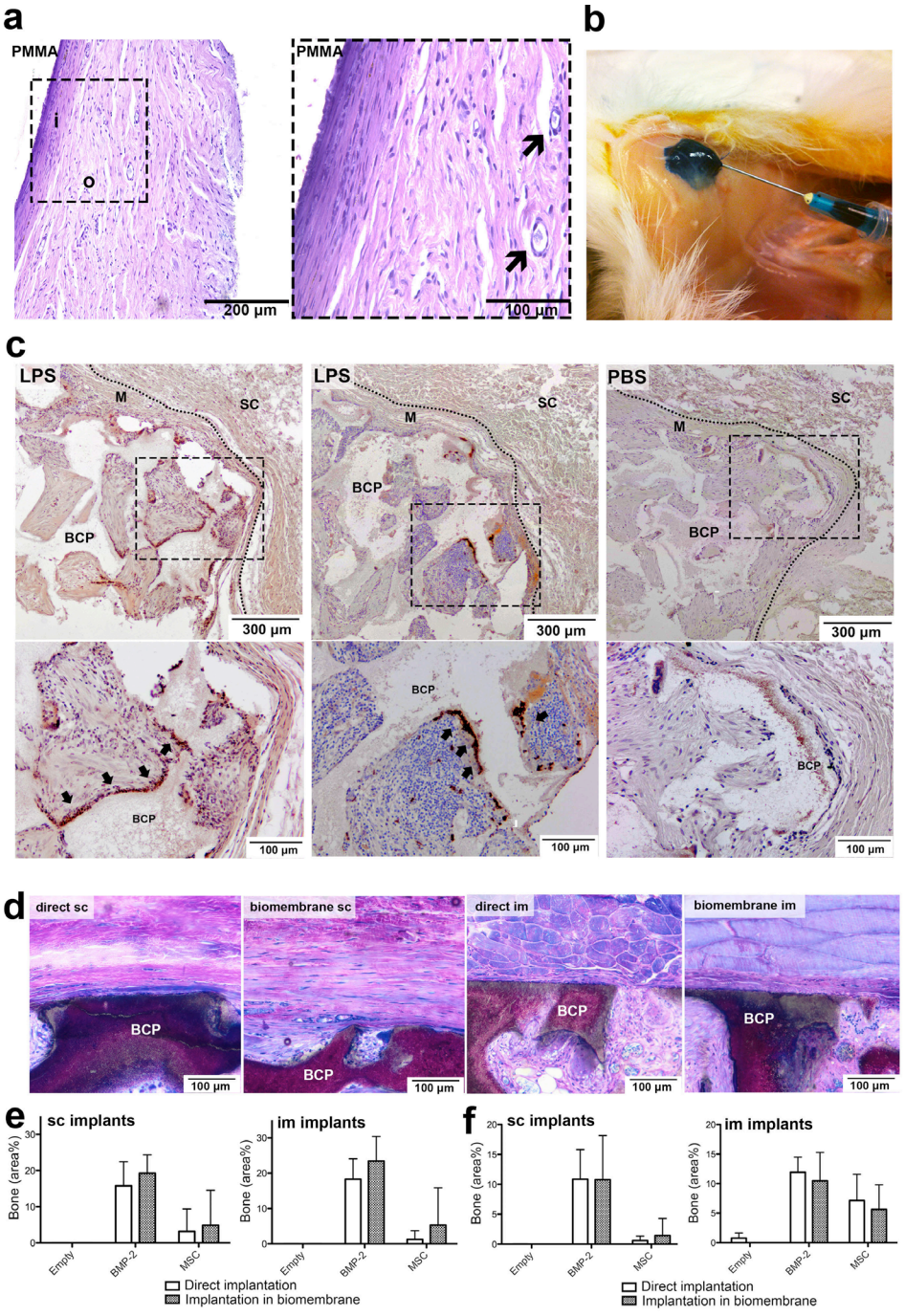
There were no signs of scaffolds degradation seen for the subcutaneously implanted constructs. These constructs, which all contained BMP-2, demonstrated the presence of bone in various amounts in almost all groups. Only the condition loaded with the high dose (10 μg/construct) of LPS did not show any bone. In regions where bone tissue was found, fat tissue was also present in varying amounts. The rest of the BCP pore spaces were filled with fibrous tissue (Figure 5A).

A relatively low dose of BMP-2 (1.5 μg/construct) was chosen to discriminate the effects of the inflammatory stimuli studied. As a result, there was a large variation in the amount of bone in the samples of the control group (5.7±5.3 area%, Figure 5B). In agreement with the histological observations, LPS was associated with a dose-dependent decrease in bone formation. Estimates based on mixed-model regression showed a 3.5-point drop in bone area% ($p=0.010$) for each 10-fold increase in the LPS concentration (Figure 5C). TNF-α on the other hand, had a stimulatory effect on bone formation, which was also concentration-dependent. Constructs containing 10 ng TNF-α were associated with the most prominent bone formation seen in this study. In this group, the bone area% was doubled (13.6±9.9, $p=0.005$) compared to the control (5.7±5.3 area%). A higher concentration of TNF-α did not result in a further increase in bone formation. Although statistical analysis did not show a difference in the average amount of bone between the control group and the LTA-loaded groups, a borderline linear correlation existed between the LTA concentration and the bone area% ($P=0.074$) (Figure 5C). For the lowest LTA concentration tested, a modest decrease in bone volume was seen, while an increase was found for the highest LTA concentration.

The fluorochrome labels revealed that the dynamics of new bone formation were independent of the bone volume in the different groups (Figure 6). As such, the constructs co-loaded with 10 μg TNF-α and BMP-2 showed the same fluorochrome incorporation as the samples loaded with BMP-2 alone. In all samples associated with bone formation, the 4-week label was located against the border of the BCP, indicating that bone formation had started before week 4. Furthermore, the 8-week label was present in the different groups throughout the bone indicating that mineralisation continued between week 4 and week 8. The 11-week label was only seen sporadically, suggesting that there was less active bone mineralisation around this time point.

Local immune response

The only immune response present at 12 weeks consisted of dense lymphoid cell clusters (Figure 7A). After quantifying them in the different groups, it appeared that the constructs loaded with LTA had the lowest average number of lymphoid clusters, while the highest number was found for LPS-loaded constructs (Figure 7B). Staining for CD3 revealed that a fraction of the cells in the clusters had a T cell phenotype (Figure 7A). Quantitative analysis showed that 15-20% of cells within the clusters were CD3-positive in all groups.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Since the BMP-2 loaded samples demonstrated significant differences in bone volume between groups, we determined the number of CD3-positive cells in them (Figure 7C). No significant differences were found in the absolute number of CD3-positive cells between the inflammatory mediator-loaded constructs and the controls. However, the CD3-positivity correlated with the number of lymphoid clusters in the different conditions. There was no association between the presence of lymphoid cells and the bone volume at the level of individual samples.

◀ **Figure 3. Results of the assessment study to evaluate the ectopic biomembrane pocket model.**
(**A**) Histological appearance of the polymethylmethacrylate (PMMA)-induced biomembrane pockets created subcutaneously in rabbits. H&E staining shows the formation of a thick outer membrane (O) of connective tissue with an endothelium-like inner lining (i) after 6 weeks. Blood vessels were observed in the outer membrane (arrows). (**B**) *Ex vivo* injection of methylene blue into the subcutaneous biomembrane pocket in rabbits. (**C**) Immunohistochemical staining for calprotectin to demonstrate the presence of phagocytes after the injection of LPS (left and middle figure) or PBS (right figure) into the biomembrane. Calprotectin-positive cells (arrows) were observed at the BCP-biomembrane (M) interface, while they were absent in the subcutaneous tissue (sc) outside of the biomembrane pocket. (**D**) Fibrous capsules observed around directly or biomembrane-implanted BCP constructs in the subcutaneous (sc) and intramuscular (im) locations after 12 weeks in rabbits as shown on methylene blue/basic fuchsin-stained MMA sections. (**E,F**) Bone formation in the constructs following direct implantation in fresh pockets, or implantation in the pre-induced biomembrane pockets. The pockets were created subcutaneously (sc) and intramuscularly (im) in rats (**E**) and rabbits (**F**). Constructs containing BMP-2 (3.75 µg in rats, 10.5 µg in rabbits) or MSCs ($2.5x10^5$ in rats, $7x10^5$ cells in rabbits) were compared to empty constructs. The results are represented as the mean ± standard deviation (n=4).

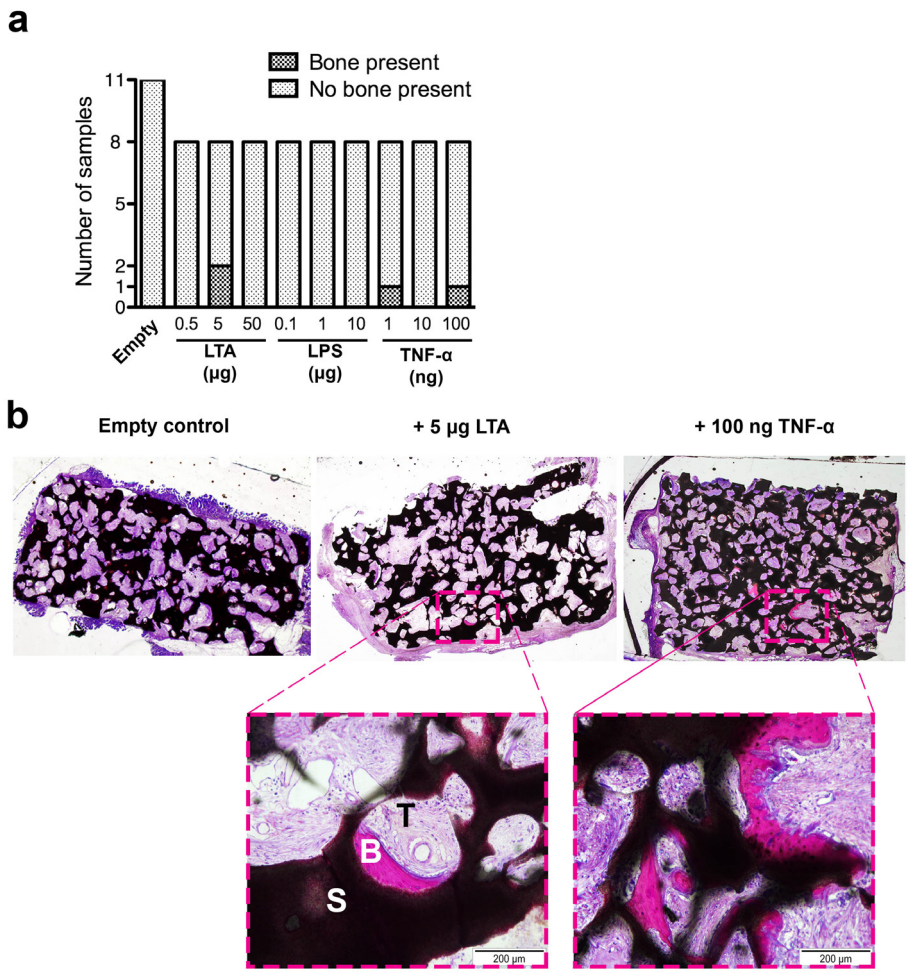

Figure 4. Bone induction in rabbits in the absence of BMP-2. (**A**) Scoring for the presence of bone tissue in intramuscularly-implanted empty BCP scaffolds and constructs loaded with pro-inflammatory mediators. (**B**) Histology of sections stained with methylene blue and basic fuchsin reveals only limited spots of bone (bright pink). B=bone, S=scaffold material, T=fibrous tissue ingrowth.

DISCUSSION

Since the modulation of the inflammatory response may be a way to improve the performance of bone substitutes[18-20,64,65], there is need for animal models to screen for immune-modifying strategies with stimulatory effects on bone formation. We propose that the creation of biomembrane pockets can serve as a practical tool to investigate the local response to different inflammatory stimuli in conjunction with new bone formation[53,54]. Based on the clinical Masquelet technique[66], the biological membrane is formed as a response to a PMMA spacer. This model has a number of characteristics making it particularly useful for osteoimmunological research. First, the biomembrane

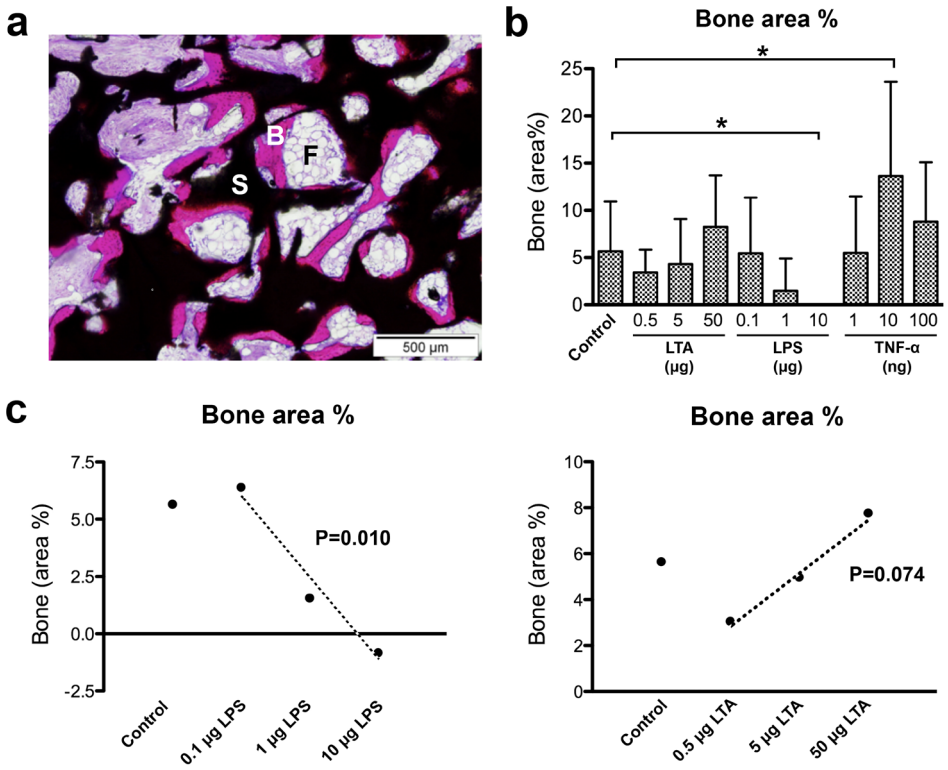

Figure 5. Bone formation in BMP-2 loaded constructs implanted subcutaneously in rabbits. (**A**) Histology of methylene blue and basic fuchsin-stained sections, representative for the control samples with 1.5 µg BMP-2 alone. Bone tissue is stained bright pink. B=bone, S=scaffolds material, F=fat tissue. (**B**) Quantification of bone formation in constructs loaded with BMP-2 alone (control) or BMP-2 in combination with pro-inflammatory mediators. The results are represented as the mean ± standard deviation. * p<0.05 compared to the control with 1.5 µg BMP-2 alone. (**C**) Dose-dependent effects of LPS and LTA on new bone formation. Regression analyses shows the linear relationship between the mediator concentration and the amount of bone in subcutaneously implanted constructs. The estimated means are shown together with the trend line.

can hold synthetic granules or particles of graft material in a confined space[59,67]. Furthermore, multiple identical cavities can be created in the same animal. Moreover, we demonstrate here with local LPS injections that the biomembrane prevents the leakage of immunomodulatory agents to the surrounding tissue. Similar to the air pouch model in rodents[68], the subcutaneous biomembrane pockets in theory allow for the repeated injections of inflammatory compounds, together with the evaluation of the inflammatory exudate. In comparison to the air pouch membrane, the PMMA-induced biomembrane is even 3 to 4- fold thicker[54,56,57]. We believe that these practical advantages outweigh the undesired need for a second surgery in this model.

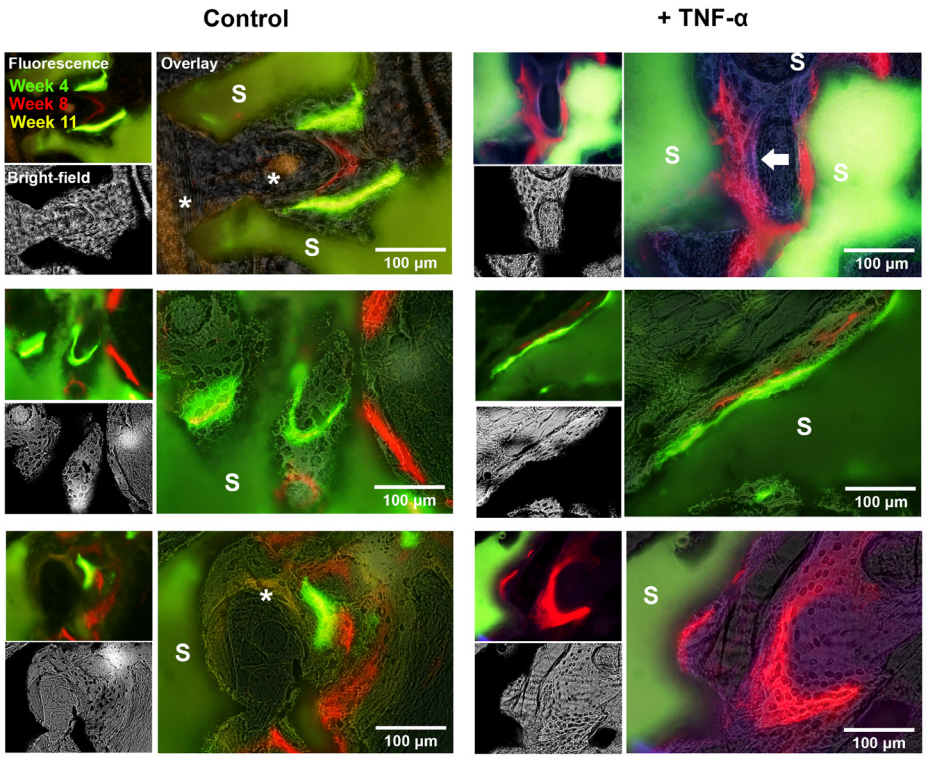

Figure 6. Fluorochrome incorporation in subcutaneously implanted samples in rabbits. Fluorochromes were injected at 4 (calcein, green), 8 (xylenol orange, red), and 11 (oxytetracycline, yellow) weeks. Their incorporation in new bone was assessed by fluorescence microscopy. Left panel: 1.5 µg BMP-2 alone. Right panel: 1.5 µg BMP-2 and 10 ng TNF-α. The upper, middle and lower panels are representative for three rabbits. Oxytetracycline incorporation (arrow) was only occasionally seen as a thin line, not to be confused with the background as a result of image merging (asterisks). S=scaffold.

An assessment study was first performed to compare the ectopic bone formation within osteogenic constructs in the biomembrane pocket environment to that of directly implanted constructs (i.e. fresh pockets). Although the group sizes were not chosen to perform powered statistical analyses, we did not observe gross differences in the ectopic bone formation induced by constructs with mesenchymal stem cells or growth factors implanted in a biomembrane pocket relative to directly implanted constructs. Moreover, considering the importance of species-dependency in ectopic bone formation[60], we showed this was true in both rat and rabbit models.

Since the biomembrane environment did not interfere with ectopic bone formation, a comparative study was performed using this model in which distinct inflammatory stimuli were studied for their effect on bone induction and formation. TNF-α, LPS and LTA were compared based on their different abilities to affect osteogenic processes *in vitro*[25,51]. It is often postulated that a short inflammatory response is more beneficial for bone healing[19,69] or ectopic bone formation[70] compared to a sustained response. We therefore studied the inflammatory stimuli using a burst release approach (i.e. released within 24 h *in vitro*). Since the mediators significantly affected the bone volume after 12 weeks, it

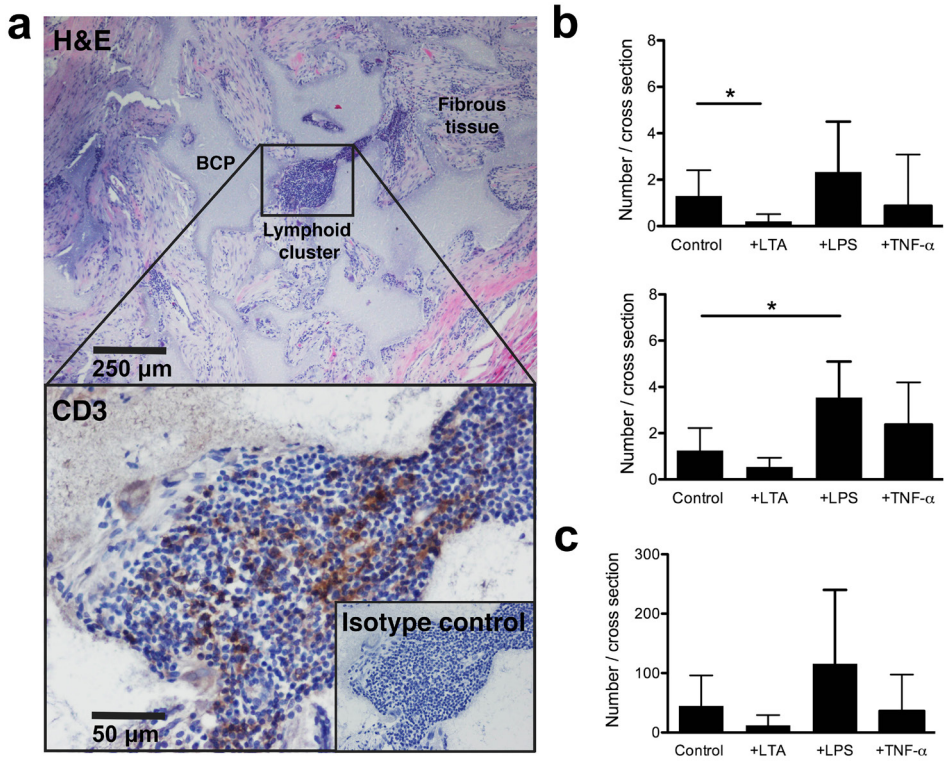

Figure 7. Presence of lymphoid cell clusters in the constructs after 12 weeks in rabbits. (**A**) Immunohistochemistry for CD3 shows the presence of T cells within the lymphoid clusters (inset stained with isotype matched control antibody). (**B**) Quantification of lymphoid clusters in subcutaneous (with BMP-2, upper panel) or intramuscular (without BMP-2, lower panel) samples. (**C**) Quantification of CD3-positive cells in subcutaneous (with BMP-2) samples loaded with 10 ng TNF-α, 10 µg LPS or 50 µg LTA. The results are represented as the mean ± standard deviation (n=8). * $p<0.05$ compared to the control group.

is unlikely that their local action was affected by the surgical procedure. In agreement, measurements performed during more invasive surgical procedures show a relatively mild response at the surgical site, with low levels of IL-6 and IL-8 in relative to the concentrations of TNF-α, LPS and LTA in the BCP constructs[71-73].

In the current study, the incorporation of a single inflammatory mediator was not an effective strategy to induce a significant amount of new bone formation in the intramuscular location. Although BCP-binding factors induce the formation of matrix-depositing osteoblasts in this location[60], the pro-inflammatory mediators did not further enhance this process. Contrary to this, the same mediators caused profound, but different outcomes when combined with BMP-2. In the optimal condition, the concurrent treatment with TNF-α and BMP-2 resulted in doubling of the bone volume. There have been conflicting reports on the effects of TNF-α on osteogenic differentiation *in vitro*, i.e. an inhibitive action on rodent MSCs[74,75] and a stimulatory action on human MSCs[25,31,50]. Although there are no other reports of the effects of TNF-α on ectopic bone formation, in an orthotopic model, local TNF-α treatment augmented fracture healing in rats, but only when administered during the first 24 h after injury[32,76]. In addition to its pro-

osteogenic effects[25], TNF-α may also be involved in cell migration[32] or may contribute to angiogenesis[77,78].

Since TLR agonists can affect downstream regulators of osteogenesis in a similar way as TNF-α[25,31,45,49-51], LTA and LPS were also studied for their effect on bone formation. Despite a lacking effect of LTA on MSC osteogenesis *in vitro*[51], a correlation was found between the LTA concentration and subsequent bone formation. It should be investigated whether a further increase in LTA concentration results in significant effects. In contrast, although LPS promotes the osteoblast differentiation of human MSCs in the presence of BMP-2 *in vitro*[25,79], it impaired the effects of BMP-2 in a dose-dependent manner. Considering the important role of TLR receptor signaling in the innate immune response[47], the direct effects of LPS and LTA on MSC osteogenesis[25,51] were likely overshadowed by the cytokine response to these mediators *in vivo*. As such, macrophages produce various cytokines in response to TLR agonists[80-82]. LPS induces an exaggerated secretion of TNF-α, IL-1 and IL-6 in comparison to LTA or TNF-α[49,80-83]. Of these abovementioned cytokines, TNF-α and IL-6 contribute to bone healing[30,64] and enhance the osteogenic differentiation of MSCs[32,84]. Although the conditioned medium derived from LPS-stimulated monocytes has shown to induce osteoblast differentiation of MSCs without exogenous growth factors[85], LPS-stimulated macrophages can also produce IFN-γ and NO[80,81]. The synergistic effects of IFN-γ and TNF-α may explain the inhibitory effects of LPS on bone induction[18,65]. NO production after LPS stimulation may also have an inhibitory effect on osteoblast growth and differentiation, partly through a pro-apoptotic effect[81,86]. Macrophages may be targeted to increase the efficacy of bone substitutes. They play an important role in the bone formation by bioceramics, since their blockade inhibits *de novo* bone formation in bone-inductive β-tricalcium phosphate[87]. Moreover, scaffold vascularisation may be improved by inducing a specific macrophage phenotype[20]. A better understanding of the involvement of macrophages in the bone-modifying effects of different TLR agonists is therefore highly interesting.

Based on the above, the combination of inflammatory stimuli with bone-related growth factors is expected to better approach physiological bone regeneration compared to their single delivery. During normal bone fracture healing, bone-promoting growth factors are supported by factors associated with the inflammatory response[19,22,28]. Similarly, we previously observed *in vitro* that proinflammatory mediators require a second, osteogenic stimulus in order to contribute to osteogenesis[25,52]. This agrees with the current finding that the co-delivery of these factors creates a potent stimulus for new bone formation. The current clinical practice of delivering single supra-physiological doses of BMP-2 could be the reason for its unpredictable induction of new bone formation[15-17]. Therefore, it is of great interest to elucidate if this co-delivery approach may indeed result in a reduction of the minimal effective BMP dose, considering the high costs[14] and side effects[12,13] associated with its current use. Furthermore, it is of importance to investigate if pro-inflammatory stimuli also interact with other BMPs or growth factors associated with bone regeneration[88].

As a limitation of this study, we did not investigate the early inflammatory response. However, at the time of explantation, we found a negative correlation between the presence of lymphoid cell clusters and the amount of bone tissue formation caused by BMP-2. In agreement with others[89], we show that these cell infiltrations are likely a mixed population of B and T cells. Interestingly, LTA-loaded constructs presented the lowest

number of lymphoid cells, while the opposite association was seen for LPS-loaded constructs. T lymphocytes express the TLRs for these antigens, and therefore can play a direct role in osteogenesis. Alternatively, their activation could also occur secondary to the innate immune response[90,91]. The local presence of adaptive immune cells can be both stimulatory[27,33] or inhibitory[18,65,89] for bone regeneration, and is likely dependent on the contribution of specific T cell subsets. For example, IL-17-producing T cells contribute to bone healing and MSC osteogenesis[27,33,52], while others have shown that T cells inhibit bone formation[18,65]. In a follow-up, a functional assay should elucidate whether lymphocytes are actually involved in the bone formation process in this model.

CONCLUSIONS

The ectopic biomembrane pocket model is a suitable *in vivo* model to study local inflammation in conjunction with new bone formation. The biomembrane environment does not interfere with cell- or growth factor-induced ectopic bone formation, and allows a localised response after the delivery of immunomodulatory agents. In this model, we demonstrated that the local delivery of inflammatory stimuli can either be beneficial or detrimental to the subsequent bone formation process. Pro-inflammatory stimuli by themselves do not induce profound de novo bone formation, but modify the degree of BMP-2-induced bone formation. The appropriate modulation of the early inflammatory response therefore forms an attractive approach to enhance the bone-inductive properties of bone substitutes.

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Chapter 5

Interleukin 17 enhances bone morphogenetic protein-2-induced ectopic bone formation

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ABSTRACT

Pro-inflammatory cytokines contribute to the healing response after bone injury, and may therefore be harnessed to promote bone regeneration. Interleukin 17 (IL-17) stimulates the osteogenic differentiation of progenitor cells *in vitro* through a synergy with bone morphogenetic protein (BMP)-2. This study investigates whether the diverse responses mediated by IL-17 *in vivo* also lead to enhanced BMP-2-induced bone formation. Since IL-17 is a strong inducer of osteoclastogenesis, we studied the influence of locally applied zoledronic acid (ZOL) on the interactions between IL-17 and BMP-2. Synthetic scaffolds, either or not carrying a ZOL coating, were loaded with a suboptimal dose BMP-2 and a dose-range of IL-17. Histological evaluation revealed that IL-17 alone did not induce any osteoclasts at day 10. On the other hand, BMP-2 clearly stimulated early tissue ingrowth and osteoclastogenesis in the scaffolds. Both of these processes were blocked in presence of ZOL. IL-17 signaling restored early vascularized connective tissue formation and osteoclastogenesis induced by BMP-2 in ZOL-coated scaffolds. After 12 weeks, the bone volume induced by co-delivery of BMP-2 and IL-17 was doubled as compared to that induced by BMP-2 alone. We conclude that IL-17 has osteo-stimulatory effects through a synergy with bone-inductive BMP-2. Local and single application of IL-17 does not mediate osteoclast formation. In addition to enhanced osteogenic differentiation, IL-17 could promote other processes involved in bone formation such as connective tissue ingrowth. The use of IL-17 may contribute to the development of improved bone graft substitutes.

INTRODUCTION

There is a need for bone substitutes that can decrease the reliance on autologous bone grafts for repair of bone defects[1]. An effective bone substitute consists of an osteoconductive matrix incorporated with biological factors that maximize the osteogenic response[2]. Selective growth factors such as bone morphogenetic protein 2 (BMP-2) can provide bone-inductive properties to synthetic grafts, however their clinical use needs to be improved in terms of efficacy, safety, and costs[3,4]. Consequently, there is an ongoing search for strategies that can strongly enhance the osteoinductive effects of BMP-2.

Pro-inflammatory cytokines modulate bone regeneration, likely in part through their interaction with BMP-2[5,6]. Interleukin 17 (IL-17) is mainly produced by T lymphocytes and neutrophils, and is an important mediator of the inflammatory response in conditions such as trauma, infection and autoimmunity[7]. In bone research, IL-17 has been implicated in the progression of inflammation-mediated bone loss[8,9]. On the other hand, IL-17 may also show pro-osteogenic effects. For example, IL-17 promotes bone healing and is a mediator of pathological bone formation in conditions such as spondyloarthritis[10-13].

The mechanisms responsible for the pro-osteogenic effects of IL-17 are largely unknown. To our knowledge, only a single study has investigated the effect of exogenous IL-17 delivery on bone regeneration. This study showed attenuated calvarial defect healing when IL-17 was added to ceramic bone grafts[14]. Although this suggests that IL-17 exerts a negative effect on the osteogenic response, its influence on bone regeneration could be heavily dependent on the context of IL-17 signaling. First, IL-17 alone is not osteoinductive in MSC cultures, yet it dramatically increases MSC matrix mineralization mediated by BMP-2[13]. The investigation of possible synergistic actions between IL-17 and BMP-2 on *in vivo* osteogenesis is therefore of interest. Second, IL-17 signaling could be a double-edged sword in bone formation due to its stimulatory effects on osteoclast differentiation[8,5,16]. While a balanced osteoclast activity is normally coupled to osteoblast formation[17,18], exaggerated osteoclastogenesis results in bone resorption and has a negative impact on new bone formation[9,19]. Therefore it is of relevance to determine if suppression of osteoclasts could skew IL-17-mediated effects towards enhanced bone formation. In the context of immunomodulatory strategies, the use of bisphosphonates such as zoledronic acid (ZOL) may be feasible, since they are widely used in clinical practice and have shown to enhance bone mass also when applied under inflammatory conditions[19,20].

In the present study, we investigate how IL-17 affects ectopic bone formation in ceramic scaffolds in rabbits. Following the observation that IL-17 acts in a synergistic manner with osteoinductive factors *in vitro*, it was hypothesized that IL-17 could potentiate new bone formation induced by BMP-2 under suboptimal conditions. Effects of IL-17 were studied with respect to early tissue response and subsequent bone formation. To assess the role of osteoclasts in IL-17 mediated responses, scaffolds were used that were either or not coated with ZOL.

MATERIALS AND METHODS

Preparation of *in vivo* implants

Porous biphasic calcium phosphate (BCP) blocks were used with a dimension of 10x10x10 mm. A larger dimension was used relative to a previous study[5], in order to more easily discriminate possible additive effects mediated by ZOL and/or IL-17 on bone formation. The material consisted of 20±5% β-tricalcium phosphate (TCP) and 80±5% hydroxyapatite (HA) by weight, and had a total porosity of 75±5%[21]. The blocks were autoclaved at 121 °C and dried at 60 °C. To prepare scaffolds coated with zoledronic acid (ZOL, Zometa®, Novartis Pharma, Switzerland), 110 µl of a 228 µg/ml stock solution in MilliQ was pipetted onto the BCP blocks. This volume was completely absorbed by the scaffold. This resulted in an end concentration of 33.4 µg/ml, considering that 0.75 cm^3 is the available space in the scaffolds. Control constructs received 110 µl MilliQ. The samples were air dried for 48 h at room temperature.

Non-precoated and ZOL-precoated scaffolds were loaded with rhIL-17 (R&D Systems, MN, USA), alone or in combination with rhBMP-2 (InductOS®, Wyeth/Pfizer, NY, USA), according to Table 1. For the constructs containing BMP-2, first 30 µl of a 100 µg/ml BMP-2 stock solution in PBS was pipetted onto the scaffolds, resulting in an end concentration of 4 µg/ml in the scaffolds. The other constructs received 30 µl PBS. Subsequently, 80 µl of an IL-17 stock solution (12.5-1250 ng/ml) in PBS was pipetted onto the scaffolds to an end concentration of 1.3-133 ng/ml. The controls received 80 µl PBS. Implants were made on the day of surgery and stored in a humidified environment at 37 °C.

ZOL *in vitro* release experiment

Non-coated and ZOL-coated scaffolds were incubated in 700 µl MilliQ in eppendorf tubes at 37 °C. At different time points, the scaffolds were removed and the supernatant was stored at -20 °C. Separate tubes were made for each time point in duplicate. The ZOL concentration in the supernatant was measured by High Performance Liquid Chromatography (HPLC) as described previously[22]. A mobile phase mixture was used consisting of methanol:water (10:90), with 6 mM tetrabutylammonium hydrogen as an ion pair for ZOL at pH 2.6. Samples were injected into the HPLC system (Alliance 2695, Waters Corporation, MA, USA) coupled with LiChrospher® C-18 column (Merck Millipore, MA, USA). The absorption was measured at 208 nm (UV detector 2478, Waters) at an elution speed of 0.8 ml/min. Results were analyzed with Waters Empower 3 Pro software.

Animal study

Animal experiments were performed after approval of the local Ethics Committee for Animal Experimentation (Utrecht University, Utrecht, The Netherlands). A total of twelve male New Zealand White rabbits (14 weeks old, 2.5-3.0 kg, Crl:KBL, Charles River, France) were used, and were housed at the Central Laboratory Animal Research Facility, Utrecht University. One rabbit died a day after surgery due to unknown reasons, and was replaced. Three rabbits were euthanized after 10 days to study the early tissue response. Eight rabbits were euthanized after 12 weeks to study bone formation (Table 1).

The rabbits received ketamine (15 mg/kg i.m.; Narketan®, Vétoquinol, The Netherlands) and glycopyrrolate (0.1 mg/kg i.m.; Robinul®, Riemser Arzneimittel, Germany) pre-operatively, and medetomidine (0.25 mg/kg s.c. ; Dexdomitor®, Orion Corporation,

Table 1. Composition of the constructs for the *in vivo* assessment of 10-day tissue response and 12-week bone formation

	Group	BMP-2 (Dose)	IL-17 (Dose)	ZOL (Dose)	n
	10-day study				
Non-coated scaffolds	Empty	-	-	-	3
	IL-17 high	-	100 ng	-	3
	BMP-2	3 µg	-	-	3
	BMP-2+IL-17 high	3 µg	100 ng	-	3
ZOL-coated scaffolds	Empty	-	-	25 µg	3
	IL-17 high	-	100 ng	25 µg	3
	BMP-2	3 µg	-	25 µg	3
	BMP-2+IL-17 high	3 µg	100 ng	25 µg	3
	12-week study				
Non-coated scaffolds	Empty	-	-	-	4
	IL-17 high	-	100 ng	-	8
	BMP-2	3 µg	-	-	8
	BMP-2 + IL-17 low	3 µg	1 ng	-	8
	BMP-2 + IL-17 med	3 µg	10 ng	-	8
	BMP-2 + IL-17 high	3 µg	100 ng	-	8
ZOL-coated scaffolds	BMP-2	3 µg	-	25 µg	8
	BMP-2 + IL-17 low	3 µg	1 ng	25 µg	8
	BMP-2 + IL-17 med	3 µg	10 ng	25 µg	8
	BMP-2 + IL-17 high	3 µg	100 ng	25 µg	8

BMP-2= bone morphogenetic protein 2, IL-17=interleukin 17, ZOL=zoledronic acid

Finland) peri-operatively. Anesthesia was reversed with atipamezole hydrochloride (0.5-1.0 mg/kg i.v., Atipam®, Eurovet Animal Health, The Netherlands). Antibiotic prophylaxis (Penicillin $3x10^4$, IE benzylpenicilline/kg, Duplocilline®, Merck Animal Health, WI, USA) was given once peri-operatively. Animals received pain relief pre-operatively, and post-operatively every 12 hours for 2 days with buprenorphine (0.03 mg/kg s.c.; Temgesic®, RB Pharmaceuticals Limited, UK).

After shaving and disinfecting the skin, subcutaneous pockets were created in the dorsum by incision of the skin and blunt dissection of the subcutaneous tissue. Ten constructs were implanted in each animal in a randomized order. The rabbits were euthanized after 10 days (n=3) or 12 weeks (n=8) with sodium pentobarbital injection (Euthanimal®, Alfasan, The Netherlands) after inducing same general anesthesia as the surgery.

Histological processing
Constructs were fixed in 4% formaldehyde. For the 12-week study, a third of each sample was removed and decalcified in 0.3 M EDTA for paraffin embedding. The remaining material was embedded in methyl methacrylate (MMA, Merck Millipore). The constructs from the 10-day study were entirely decalcified and embedded in paraffin.

Goldner's trichrome staining
To demonstrate connective tissue formation, sections were deparaffinized and incubated with Weigert's hematoxylin for 5 min, followed by Goldner's solution [0.2% (v/v) glacial acetic acid, 0.033% (w/v) acid fuchsin, 0.13% (w/v) Ponceaux de xilidine] for 45 s, 2% (w/v) Orange G for 7 min and 0.15% (w/v) Light green for 7 min. Differentiation was done with 1% (v/v) acetic acid for 5 s after each staining. Sections were dehydrated and mounted in Depex. Slides were scored by two blinded observers for connective tissue presence, in either 0, 25, 50, 75 or 100% of the available space. Data are presented as the average of the two scores.

Osteoclast detection
For staining of tartrate-resistant acid phosphatase (TRAP) activity, samples were incubated with 0.2 M acetate buffer-tartaric acid for 20 min at 37 °C. Naphtol AS-MX phosphate (0.5 mg/ml, Sigma, MO, USA) and Fast red TR salt (1.1 mg/ml, Sigma) were added for another 2 h. Sections were counterstained with Mayer's hematoxylin. Osteoclasts were defined as multinucleated TRAP-positive cells lining the surface of bone or scaffold. The absolute number of osteoclasts was counted in one cross-section from each construct and averaged per group.

Immunohistochemistry
Stainings for calprotectin-expressing phagocytes (clone MAC387, Bio-Rad, CA, USA) or T lymphocytes (CD3, clone F7.2.38, Dako, Denmark) were performed as described previously[5]. To stain blood vessels, samples were incubated with anti-CD31 (clone JC70A, 1:50, Dako) for 1 h at room temperature following heat-induced antigen retrieval with EDTA (1 mM, pH 9.0). Blocking and staining was performed with a kit, according to the manufacturer's protocol (Dako EnVision+ System HRP, Dako). Using the same kit, cytoplasmic staining for connective tissue macrophages (clone RAM11, 1:100, Dako) was performed with overnight incubation of the primary antibody at 4 °C. Sections were counterstained with Mayer's hematoxylin.

Bone histomorphometry
For undecalcified histology, 35 µm-thick sections were cut with a saw microtome (Leica, Germany) and stained with basic fuchsin/methylene blue. Two midsections of each construct were pseudo-colored in Adobe Photoshop (Adobe Systems, CA, USA) to quantify the percentage of bone in the available pore space (bone area%) or the surface of BCP in contact with bone (bone contact%). The mean value of two sections per sample was used for further analyses.

Statistical analyses

A sample size calculation was performed for the 12-week study, using the bone area% as main outcome parameter. A power of 80% was used, with an alpha of 5% that was adjusted for multiple comparisons. This indicated that a sample size of 8 was needed to detect a 50% bone volume change given a 30% standard deviation. Changes in bone volume were analyzed using a linear mixed-model approach in SPSS (IBM, IL, USA). For the non-coated and ZOL-coated samples, pairwise comparisons were made between each IL-17 concentration and the control without IL-17. For *post hoc* analysis, Bonferroni correction was used. An arbitrary sample size of 3 was used for the 10-day study. All results are shown as the mean ± standard deviation (SD).

RESULTS

***In vitro* ZOL release from ZOL-coated scaffolds**

HPLC measurements showed that the scaffolds retained approximately half of the loaded ZOL after an initial burst release, without any additional release at later time points (Figure 1). Hence, the ZOL was strongly bound to the BCP, which corresponds with its high affinity for HA[23].

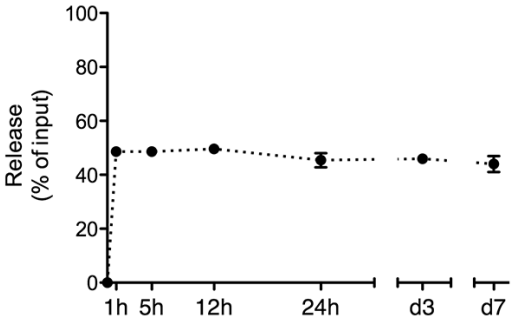

Figure 1. ***In vitro*** **release of zoledronic acid (ZOL) from precoated BCP scaffolds.**

Clinical results

One rabbit from the 10-day study died the day after surgery and the cause of death could not be identified by necropsy. The implants from this rabbit were retrieved within an hour after its death for histological analyses ('24 h constructs'). The missing rabbit was replaced to match the group sizes. All other animals recovered well from the surgery and implanted samples were all retrieved.

Early tissue response

The 24 h constructs contained erythrocytes within a fibrin matrix, whereas the inflammatory response was characterized by numerous calprotectin-expressing neutrophils (Figure 2). In comparison, there were no obvious signs of active inflammation

in the 10-day constructs, since calprotectin-expressing phagocytes were only scarcely found. The few calprotectin-positive cells were localized at the periphery of the scaffolds and appeared to be macrophages based on their morphology. Staining for CD3 showed absence of lymphocytes.

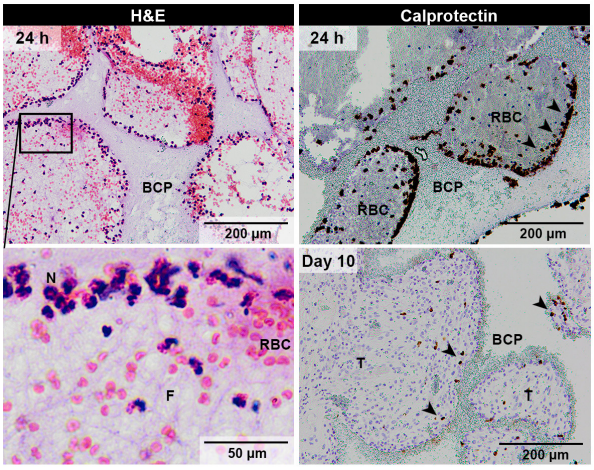

Figure 2. Inflammatory response after 24 hours and 10 days. Empty BCP scaffolds were implanted subcutaneously and retrieved after 24 or 10 days. Calprotectin staining (arrowheads) was performed to demonstrate activated phagocytes in the constructs. BCP=biphasic calcium phosphate, F=fibrin matrix, N=neutrophils, RBC=red blood cells, T=connective tissue.

The combination of Goldner's trichrome and CD31 stainings showed that the ingrowth of vascularized connective tissue was not completed in all constructs by day 10, and instead, remnants of the hematoma and necrotic cells were prominent in the central regions of many constructs (Figure 3A). Connective tissue formation was enhanced by BMP-2 and IL-17, and was abrogated in ZOL-coated constructs (Figure 3B, 3C). This points towards an inhibitory effect of ZOL on early soft tissue ingrowth. Co-administration of IL-17 and BMP-2 was not sensitive to the inhibitory effect of ZOL. Goldner's trichrome staining indicated no formation of osteoid or bone in any of the constructs at this time point.

The 10-day time point coincides with the initial peak in osteoclast formation in osteogenic constructs[18,24]. Analysis of the number of osteoclasts showed a complete absence in the empty controls. Osteoclasts were neither found in samples with IL-17 alone. In contrast, many osteoclasts were present in BMP-2-loaded scaffolds without ZOL, which were preferentially localized at the periphery of the scaffolds (Figure 4A). The induction of osteoclasts by BMP-2 was lost in ZOL-coated scaffolds (Figure 4B), suggesting a high efficacy of ZOL. Similar to the established trend for connective tissue ingrowth, co-administration of IL-17 and BMP-2 was insensitive to ZOL-coating, and resulted in a large presence of osteoclasts.

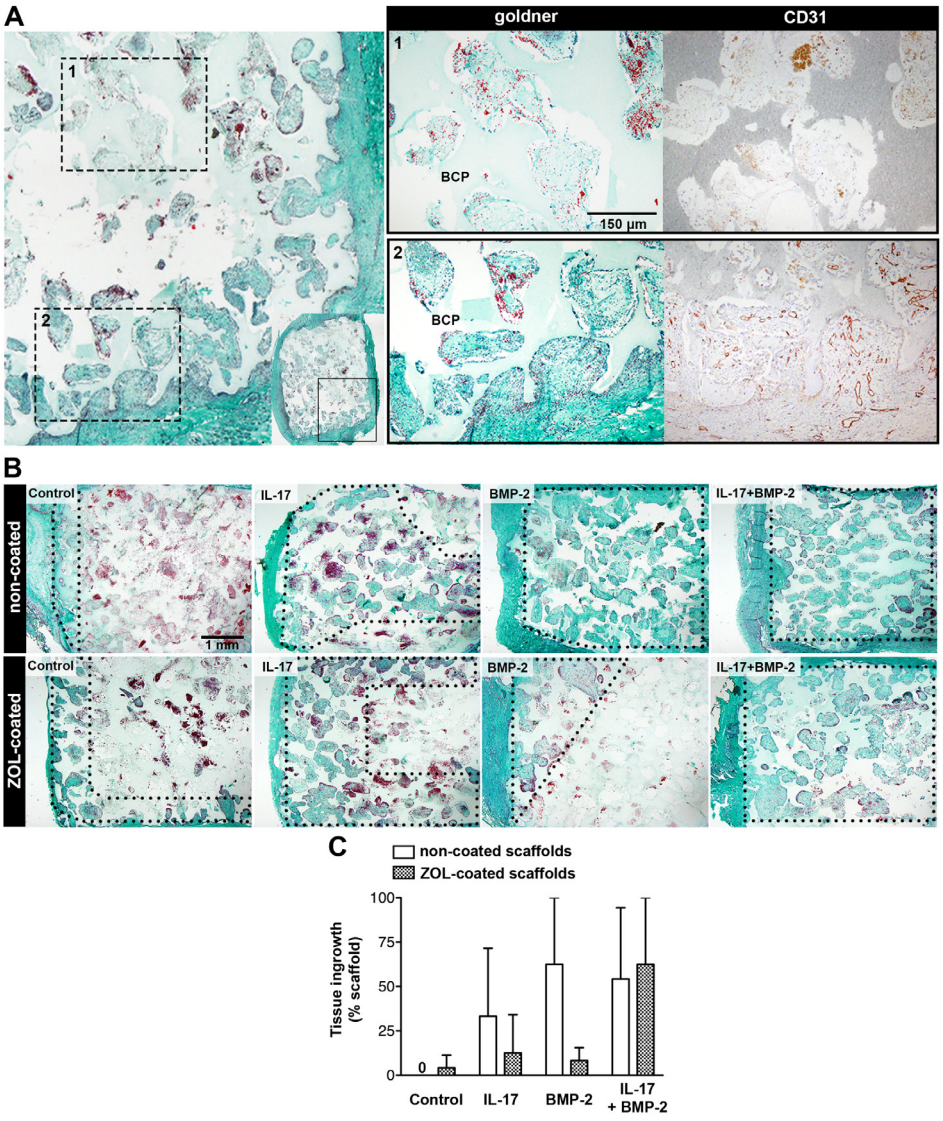

Figure 3. Connective tissue ingrowth in the 10-day study. (**A**) Goldner's trichrome staining was indicative of collagen in connective tissue (in green); note the differences in collagen between the periphery and central regions in a BMP-2-loaded construct without ZOL. CD31-positive blood vessels (in brown) were found in regions where connective tissue had formed. (**B**) Goldner's trichrome staining showing differences in connective tissue formation between groups. A region of interest is shown which covers half of the construct's total area from the periphery (left) to the center (right). The areas within the dotted lines indicate regions of vascularized connective tissue formation. All images are representative for the group. (**C**) Quantification of connective tissue ingrowth, represented as the mean ± SD (n=3).

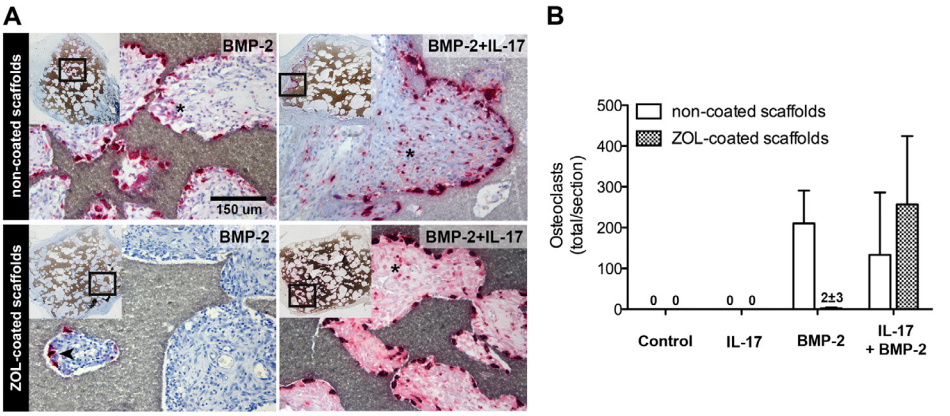

Figure 4. Role of osteoclasts during the early tissue response. (**A**) Tartrate-resistant acid phosphatase (TRAP) staining indicates the presence of osteoclasts (in red) at day 10 in ZOL-coated and non-coated scaffolds with BMP-2 or BMP-2+IL-17. (**B**) Osteoclast counts are represented as the mean ± SD (n=3). Mononucleated TRAP-positive cells (indicated by asterisks) were not included in the osteoclast counts.

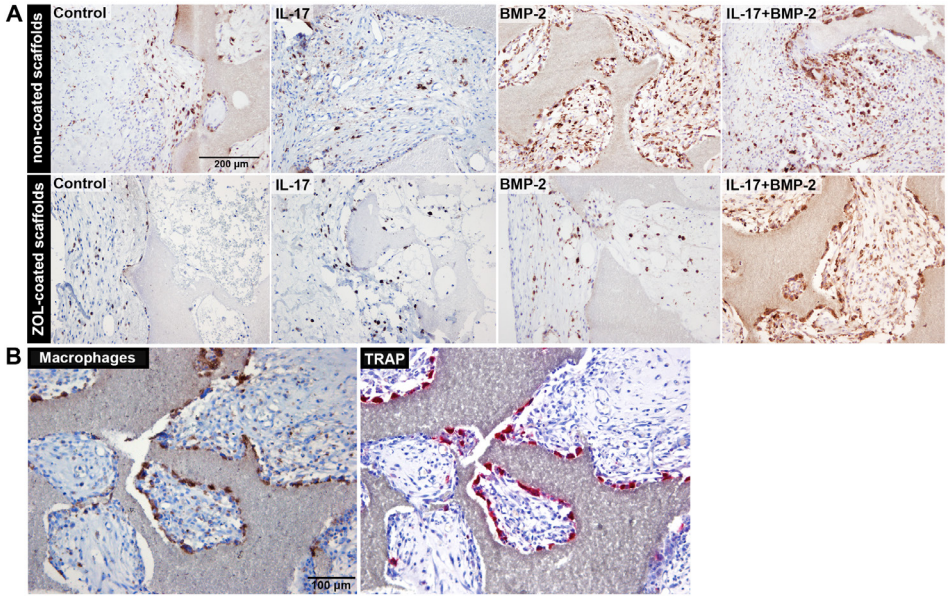

Figure 5. Presence of macrophages at day 10. Zoledronic acid (ZOL)-coated and non-coated scaffolds were loaded with BMP-2 (3 µg/construct) and/or IL-17 (100 ng/construct) and implanted subcutaneously for 10 days. (**A**) Images taken from the edge of the constructs show the presence of connective tissue macrophages stained with the RAM11 antibody (brown). (**B**) Consecutive sections showing the co-localization of calprotectin- and tartrate-resistant acid phosphatase (TRAP)-positive cells. Images are representative for BMP-2-loaded constructs without ZOL.

A staining for connective tissue macrophages was performed to determine whether the ZOL coating also affected TRAP-negative phagocytes. Macrophages were found in the periphery of the constructs in all conditions, irrespective of ZOL-coating, but were more frequent in samples demonstrating enhanced connective tissue ingrowth (Figure 5A). The highest density of macrophages was seen in regions demonstrating increased osteoclastogenesis (i.e. BMP-2+IL-17 conditions), with co-localization of TRAP and macrophage stainings in serial sections (Figure 5B).

The effect of IL-17 on new bone formation

There were no signs of BCP degradation during the 12-week implantation. All constructs showed excellent connective tissue ingrowth. Empty BCP constructs or constructs that contained IL-17 alone (100 ng/construct) did not contain any bone. The groups that contained BMP-2 demonstrated the presence of bone and fatty tissue (Figure 6A). BMP-2 was chosen at the suboptimal dose of 3 μg/construct to enable detection of IL-17-mediated effects in both directions. A dose-dependent increase in the amount of bone was seen when IL-17 was co-administered (Figure 6B). The optimal condition with 10 ng IL-17/construct significantly enhanced the bone area% from 11.6 ± 7.4 to 20.3 ± 7.1 ($p=0.042$). For BCP constructs coated with ZOL, increased bone formation with IL-17 was not significant. Analysis of bone contact% showed the same trend as the area% (Figure 6C): with only BMP-2 it was 23.0 ± 16.2, and increased to 35.7 ± 9.8 when 10 ng IL-17/construct was co-administered ($p=0.016$).

Immune response at week 12

TRAP-positive osteoclasts were only observed in BMP-2-loaded constructs, and were consistently localized at the bone surface (Figure 7). Their absolute numbers were higher at week 12 than at day 10, and comparable in the different groups, namely 300-400/cross-section on average in all groups. Calprotectin staining demonstrated an absence of activated phagocytes at this time point.

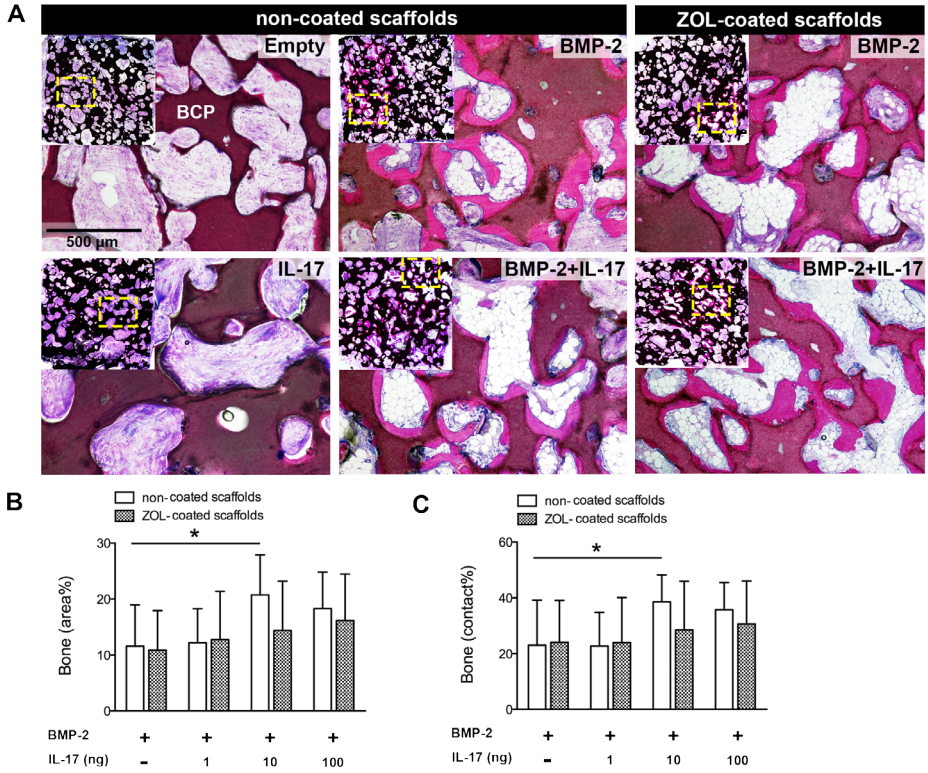

Figure 6. Bone formation in scaffolds with suboptimal BMP-2 dose after 12-week implantation. (**A**) Methylene blue/basic fuchsin staining of bone (pink). Quantification of bone formation as bone area% (**B**) and bone contact% (**C**). Data are represented as the mean ± SD (n=8). * p<0.05.

DISCUSSION

The objective of this study was to explore the possible role of IL-17 in the early and/or late osteogenic response. BCP scaffolds with suboptimal BMP-2, either or not combined with IL-17, were investigated in subcutaneous pockets in rabbits. In this location, IL-17-mediated effects can be detected on osteoinductive pathways leading to bone formation. In line with its promotive effect on MSC osteogenic differentiation[13], IL-17 significantly enhanced BMP-2-induced new bone formation. We furthermore showed that IL-17 does not induce early osteoclastogenesis, but supports other processes involved in bone formation such as connective tissue ingrowth.

In the current study, the combined delivery of BMP-2 and IL-17 led to a twofold greater bone volume as compared to BMP-2 alone. Our results contradict those from Kim et al., who found an inhibitory effect of IL-17 on calvarial bone defect healing in rats. They, and others, supported this *in vivo* finding by showing decreased osteogenic marker expression in calvarial osteoblasts *in vitro*[14,25]. Here, a stimulatory effect of IL-17 on ectopic new bone formation was found, a process that relies on the migration and differentiation of inducible cells[26]. In agreement, IL-17 potently enhances the further osteogenic differentiation of

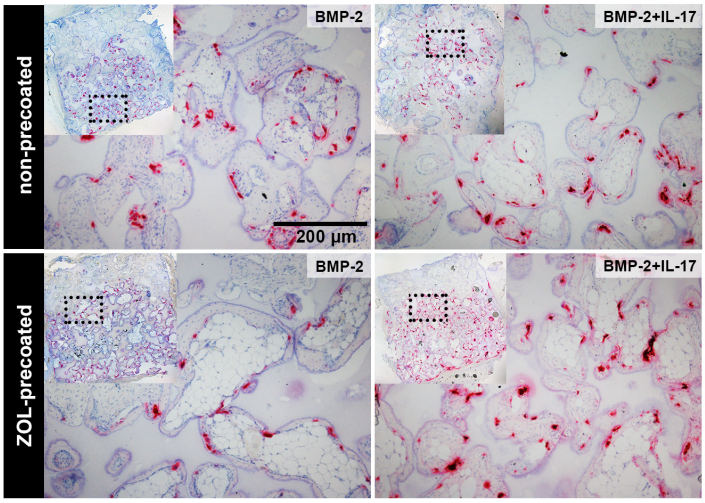

Figure 7. Presence of osteoclasts in the newly formed bone after 12-week implantation. ZOL- coated and non-coated scaffolds were loaded with BMP-2 (3 µg/construct) and/or IL-17 (100 ng/construct) and implanted subcutaneously. Tartrate-resistant acid phosphatase (TRAP) staining (in red) shows the presence of bone-lining osteoclasts.

undifferentiated MSCs and other immature cells[10,13,27,28]. Thus, IL-17 may have different effects on bone cells depending on their differentiation stage. Furthermore, the IL-17 receptor expression levels may vary in bone cells isolated from various sources, which could explain variable efficacy of IL-17 depending on the target tissue[14,27]. In the current study, only IL-17A was investigated, commonly referred to as IL-17[29]. Nevertheless, other IL-17 family members may also be interesting to study, since we already confirmed a pro-osteogenic role for IL-17F *in vitro*[13].

The observed synergistic interaction between IL-17 and suboptimal dose BMP-2 in terms of bone formation may recapitulate the natural crosstalk between pro-inflammatory and osteogenic signaling pathways during bone regeneration[6]. IL-17 was effective in the ng/ml range, resembling its expression levels during inflammation[30]. Furthermore, an *in vivo* burst release of IL-17 was expected, which mimics its short-lived action in the bone healing response[10]. The timely delivery of a physiological amount of IL-17 could be a potentially feasible method, as it circumvents the destructive effects of chronic IL-17 signaling[9,29]. Consequently, the co-delivery approach should be explored as a strategy to

improve the efficacy of BMP-2. This is of great importance, since the high costs and side effects of BMP-2 are likely related to its inefficient use[31]. To further establish the potential of IL-17, it is of relevance to determine if it also enhances bone substitutes in an orthotopic environment, where osteoinductive cues are naturally present or surgery-induced[32].

A prominent role for osteoclasts was observed at early time points in conditions carrying BMP-2. In line with this observation, previous reports show that a first phase of osteoclastogenesis precedes new bone formation in cell or growth factor-based osteogenic constructs[18,24]. Furthermore, the selective inhibition of osteoclastogenesis impairs new bone formation[18,33-35], which is likely due to uncoupling of osteoclast-osteoblast signaling[17]. We hypothesized that IL-17-mediated effects on osteoclastogenesis could hamper its pro-osteogenic effects[19], and therefore also studied IL-17 in combination with the osteoclast-inhibitor ZOL. In ZOL-coated scaffolds, early BMP-2-induced osteoclast formation *in vivo* was effectively blocked. As an unexpected finding, BMP-2 induced similar amounts of bone in ZOL-coated scaffolds as compared to non-coated scaffolds. This is in agreement with other reports showing that, at long term, a burst release of BMP-2 can overcome the early inhibitory effects of ZOL[20,36,37]. The BCP scaffold could act as a carrier to retain BMP-2 *in vivo* through the high binding affinity of BMP-2 to β-TCP and HA[38,39]. On the other hand, the *in vivo* half-life of BMP-2 is limited to 4-8 days when used locally[40]. Hence, the sustained osteogenic response in the BCP constructs in absence of exogenous BMP-2 signaling could also be attributed to paracrine/autocrine effects of inducible osteogenic cells[41].

Despite the inhibition of BMP-2-mediated osteoclastogenesis by ZOL, high numbers of osteoclasts were found in ZOL-coated scaffolds with co-administration of BMP-2 and IL-17. As there was no obvious stimulatory effect of IL-17 on the number of osteoclasts in the other conditions, it is unlikely that direct inductive effects of IL-17 on osteoclastogenesis played a role in this model. Alternatively, we provide evidence that IL-17 could be involved in other early regenerative processes. As part of the TGF-β superfamily, BMP-2 plays a role in tissue repair by inducing chemotaxis, MSC proliferation/differentiation and extracellular matrix production[6,42]. The connective tissue ingrowth seen at day 10 in constructs with BMP-2 was strongly impaired by ZOL. Several mechanisms could be responsible for this inhibitory effect, depending on the ZOL concentration and timing. For instance, ZOL inhibits blood vessel formation induced by vascular endothelial growth factor (VEGF) and has pro-apoptotic effects on endothelial cells. Equally important, ZOL has negative influences on the survival and function of bone (precursor) cells depending on the applied concentration[43,44].

The negative impact of ZOL on BMP-2-induced vascularization and connective tissue formation were counteracted by IL-17. Therefore, it is possible that processes critical for bone formation, other than osteoclastogenesis, may be positively influenced by IL-17. For example, in wound healing, IL-17 contributes to tissue regeneration through the recruitment of other inflammatory cells and the modulation of angiogenesis[45]. MSCs could be a particularly important target, showing changes in migration, proliferation/differentiation and cytokine expression in response to IL-17[27,46-48]. Hence, the identification of the different targets of IL-17 in the osteogenic response is an important focus of further investigation.

In the present study, we identify a novel role of IL-17 in potentiating bone substitutes. As an underlying mechanism, IL-17 acts synergistic with BMP-2 to enhance new bone

formation. The mechanism by which IL-17 mediates this remains elusive. In addition to its stimulatory effect on MSC osteogenic differentiation, we showed that IL-17 may promote soft tissue ingrowth. On the other hand, IL-17 does not promote osteoclastogenesis. Together, these findings suggest that the induction of IL-17 signaling is a feasible strategy to promote bone regeneration.

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PART II

Bacterial antigens for bone regeneration

Chapter 6

Inflammation-induced osteogenesis in a rabbit tibia model

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ABSTRACT

Pathologic conditions associated with bone formation can serve as models to identify bone-promoting mediators. The inflammatory response to bacterial infections generally leads to osteolysis and impaired bone healing, but paradoxically, it can also have pro-osteogenic effects. As a potential model to investigate pro-osteogenic stimuli, this study characterizes the bone formation in an established rabbit tibia model of periprosthetic infection. Our hypothesis was that the infection with *Staphylococcus aureus* (*S. aureus*) correlates with bone formation as a response to local inflammation. Fluorochromes showed excessive subperiosteal bone formation in infected tibiae, starting the first week and continuing throughout the study period. Despite the observed cortical lysis on micro-CT after 28 days, infection resulted in a 2-fold higher bone volume in the proximal tibiae compared to uninfected controls. The ipsilateral fibulae, nor the contralateral fibulae or tibiae, were affected by infection. Next, we sought to confine the cause of stimulated bone formation to the isolated *S. aureus* cell wall. In absence of virulent bacterial infection, the *S. aureus* cell wall extract induced bone in a more favorable way without cortical lysis. This suggests that the sterile inflammatory reaction to bacterial antigens may be harnessed for bone regenerative purposes. Future investigations in this rabbit tibia model can lead to further identification of effective stimuli for clinical application.

INTRODUCTION

New strategies to induce local bone formation are of great interest[1-3] considering the limitations of the autologous bone graft (autograft)[4]. Various synthetic grafts, particularly the calcium phosphates, hold a great potential to treat various bone defects due to their biocompatibility and osteoconductive properties[5]. However, they are not suitable as stand-alone graft in larger defects due to their limited bone-inductive capacity[6]. Traditionally, the performance of synthetic bone grafts is therefore enhanced with cells or growth factors[2,7].

More recently, the incorporation of immunomodulatory factors is also being investigated as a way to enhance the efficacy of bone substitutes[8,9]. After tissue injury, the inflammatory response initiates the reparative cascade[10]. During this phase, the close interaction between bone cells and immune cells is a prerequisite for normal bone healing[11]. In line, macrophages appear to be involved in the bone formation induced by certain calcium phosphates[12,13]. These bone graft substitutes therefore are a potential target in immunomodulatory strategies.

Studying pathologic conditions associated with bone formation can be helpful to identify pro-osteogenic mediators during inflammation[14-16]. To facilitate this, a reproducible and quantifiable animal model is needed. This model should allow easy induction of a local and orthotopic inflammation in an immune competent animal. Although several animal models are known in which bone formation is linked to an underlying inflammatory process, they involve heterotopic bone formation or systemic disease[17-19]. In contrast, new bone formation during osteomyelitis seems to be a localized orthotopic response and therefore may be a clinically interesting model to investigate bone-promoting stimuli[20,21]. Although virulent bacteria are likely unfavorable for any future therapies, investigating the phenomenon of bone formation in osteomyelitis can potentially lead to the identification of bacteria-derived components or downstream secreted cytokines which can be harnessed as a safer alternative for new bone formation.

Bacterial infections are known to drastically affect bone tissue. The inflammatory response to bacterial antigens causes a misbalance between the number of osteoclasts and osteoblasts[22-24], which generally leads to bone loss, impaired fracture healing and failure of spinal fusion[23,25,26]. Paradoxically, bacterial infections are occasionally also linked to new bone formation, suggesting that the local response to bacteria can stimulate osteogenic pathways. For example, subperiosteal bone formation is commonly observed in osteomyelitis of the long bones[15,27], and bacterial infection is a potential risk factor for the development of heterotopic ossifications after soft tissue trauma[28,29]. Although the underlying mechanism of bacteria-induced osteogenesis has not been fully established, several observations point to the fact that local inflammatory factors may stimulate the process[30-33]. Furthermore, the activation of receptors important in the innate immune response, i.e. toll-like-receptors, affects osteoblast activity and bone formation[34-36].

This study characterizes a rabbit tibia model as a potential model to investigate bone-promoting mediators. This well-established model was originally developed to study periprosthetic infection and shows gross signs of both osteolysis and new bone formation[37,38]. To test the hypothesis that *Staphylococcus aureus* infection induces a localized pro-osteogenic response in this model, we studied (1) how infection affects the bone morphology and histology; (2) how infection locally and systemically affects the

bone volume; and (3) what the onset is of bone formation during infection. To test the hypothesis that an inflammatory response induces bone formation in this model in absence of virulent bacterial infection, we studied (4) what the effect is of the *S. aureus* cell wall extract on the bone volume and its histological appearance.

MATERIALS AND METHODS

Design

In the first part of this study ('Infection study'), the osteogenic response to *Staphylococcus aureus* (*S. aureus*) infection was investigated in the tibiae of adolescent rabbits[37]. The animals underwent unilateral treatment and the bone response with or without infection was compared (n=6 per group). In the second part of this study ('Cell wall extract study'), the osteogenic response to *S. aureus* cell wall was investigated in the same model without an implant. For this study, the untreated contralateral tibia served as the control (n=4).

Sample preparation

S. aureus (Wood 46, ATCC 10832) was cultured in LB medium at 37 °C to mid-log phase. The bacteria were aliquoted and stored at -80 °C in PBS with 20% (v/v) glycerol. The number of colony forming units (CFU) was determined by plating on blood agar plates. The bacteria were washed with PBS before *in vivo* use.

For the preparation of *S. aureus* cell wall extract (CWE), the bacteria were resuspended in lysis buffer containing 300 mg raffinose (Sigma-Aldrich, Saint Louis, MO, USA) and 100 µg lysostaphin (Sigma) per ml of Tris/$MgCl_2$ solution (50mM Tris-HCl pH 7.5; 20 mM $MgCl_2$), at a concentration of 10^{10} CFU *S. aureus*/mL buffer. An EDTA-free protease inhibitor (200 µl/mL buffer, Roche, Basel, Switzerland) was added. After 2 h at 37 °C, the samples were centrifuged, and the supernatant containing the cell wall extract was passed through a 0.4 µm syringe filter. This protocol yielded a total concentration of 800 µg/mL of *S. aureus*-associated proteins (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Waltham, MA, USA).

Animal surgery

The animal experiments were performed after approval of the local Ethical Committee for Animal Experimentation (Utrecht University, The Netherlands). Animals were housed at the Central Laboratory Animal Institute (Utrecht University). The rabbits' daily diet consisted of 100 g of pellet food (Stanrab, SDS, Essex, UK). Water was available *ad libitum*. As part of the surgery, animals received ketamine (15 mg/kg i.m.; Narketan®, Vétoquinol, 's-Hertogenbosch, The Netherlands) and glycopyrrolate (0.1 mg/kg i.m.; Robinul®, Riemser Arzneimittel AG, Greifswald, Germany) pre-operatively, and medetomidine (0.25 mg/kg s.c.; Dexdomitor®, Orion Corporation, Espoo, Finland) peri-operatively. Anesthesia was reversed with atipamezole hydrochloride (0.5-1.0 mg/kg i.v., Atipam®, Eurovet Animal Health, Bladel, The Netherlands). Buprenorphine (0.03 mg/kg s.c.; Temgesic®, RB Pharmaceuticals Limited, Slough, UK) was given for 2 days to relieve pain.

The left hind limbs were shaven and disinfected with povidone-iodine (Betadine). The stifle joint was opened through a medial para-patellar incision. In the infection study (female New Zealand White, 16 weeks old, 3.0-3.5 kg, Charles River, L' Arbresle, France), an opening was created in the tibial canal with a drill (4.1 mm Ø). After rinsing and

draining, a cylindrical sandblasted titanium rod (4 mm Ø x 25 mm, roughness 5.6 µm, Adler Ortho SRL, Milan, Italy) was inserted. For the infection study 10^5 CFU of *S. aureus* in 50 µl PBS were pipetted in the canal before implantation (n=6). The control group received PBS (n=6). The right limbs were left untreated. In the cell wall extract study (n=4, female New Zealand White, 12-15 weeks old, 2.5-3.0 kg, Charles River), the same method was used to create a 1.0 mm Ø opening. After draining, 250 µl of the cell wall extract was injected into the tibial canal and the opening was immediately closed with 200-300 µl fibrin glue (Tissucol 500®, Baxter, Deerfield, IL, USA). The tissues and skin were closed (Monocryl®, Ethicon, Somerville, NJ, USA).

The body weight was measured pre-operatively on the day of the surgery and every week thereafter. Blood was collected from the central auricular eir vein weekly (Infection study) or at day 7 (Cell wall extract study) to measure systemic markers of inflammation (Laboratory of Clinical Chemistry and Hematology, UMC Utrecht).

Fluorochrome labels were injected to trace new bone formation[39]. In the infection study, xylenol orange (30 mg/kg s.c. in 1% w/v $NaHCO_3$, Sigma) and calcein green (10 mg/kg s.c. in 2% w/v $NaHCO_3$, Sigma) were injected on days 3 and 7 in half of the animals, and on days 7 and 21 in the other animals. In the cell wall extract study, all animals received fluorochromes on days 14 and 28. In these animals, *in vivo* micro-CT scans of the limbs were also made on day 28 under the same anesthesia protocol as described above.

The animals were euthanized with pentobarbital (i.v. Euthanimal®, Alfasan, Woerden, The Netherlands), after using the same anesthesia as the surgery. In *S. aureus* contaminated tibiae, samples of the anterior tibiae were collected under sterile conditions and used for bacterial counting (see below). A distal 1 cm-thick cross-sectional bone sample was harvested for paraffin embedding and histological assessment (Dremel rotary saw, model 300, Breda, The Netherlands)(Figure 1A). The remaining tissue was fixed in 4% (v/v) formaldehyde for micro-computed tomography (micro-CT) scanning and methyl methacrylate (MMA) sectioning.

Bacterial count

Bone samples harvested from the tibiae of *S. aureus*-contaminated animals (Figure 1A) were weighed and homogenized (Polytron PT3100; Kinetica Benelux, Best, The Netherlands). Serial dilutions were cultured on blood agar plates. The CFU was counted and normalized to the weight of the fragments ($6.3 \times 10^5 \pm 1.2 \times 10^6$ CFU/g bone).

Micro CT analysis

Reconstructed images were acquired with a tube voltage of 90 kV and a tube current of 180 µA (Quantum FX, PerkinElmer, Waltham, MA), and represented as a stack of 2D TIFF images (Analyze, version 11.0, AnalyzeDirect Inc, Overland Park, KS, USA). All analyses were performed with the BoneJ plugin (version 1.3.12) in ImageJ freeware version 1.48[40,41]. The intramedullary implant was first excluded from the data set using a customized macro that uses a global threshold to identify the implant. An adaptive threshold was applied based on the mean local greyscale distribution to precisely segment the bone for volume and porosity measurements[42].

In the infection study (voxel size 60 µm^3), several parameters were calculated on the proximal 300 cross-sectional slices (18 mm section) containing the implant: tibial and fibular volume (mm^3), peri-implant volume (mm^3), tibial porosity (%) and density (g/cm^3). Cortical segmentation was performed to measure the total bone volumes. For infected tibiae, this volume was corrected for the tissue removed for bacterial culture (calculation in Figure 1B). The right tibiae served as a within-subject control to determine the left-to-right ratio in total volume. The tibial bone volumes were calculated based on the product of their total volume and porosity (Figure 1C). The peri-implant bone volume was defined as the bone volume measured within a defined Region Of Interest (ROI). This ROI was a cylinder (5 mm Ø x 18 mm) selected around the center of the (4 mm Ø x 18 mm) implant (Figure 1D). For bone density calculation, the average grey values were related to a calibrated series of samples, assuming a linear relationship between grey value and bone density[43].

In the cell wall extract study (voxel size 120 µm^3), the bone volume measurement was performed on a tibial section of 250 slices (30 mm section) distal to the proximal tibiofibular joint. The right tibiae served as a within-subject control.

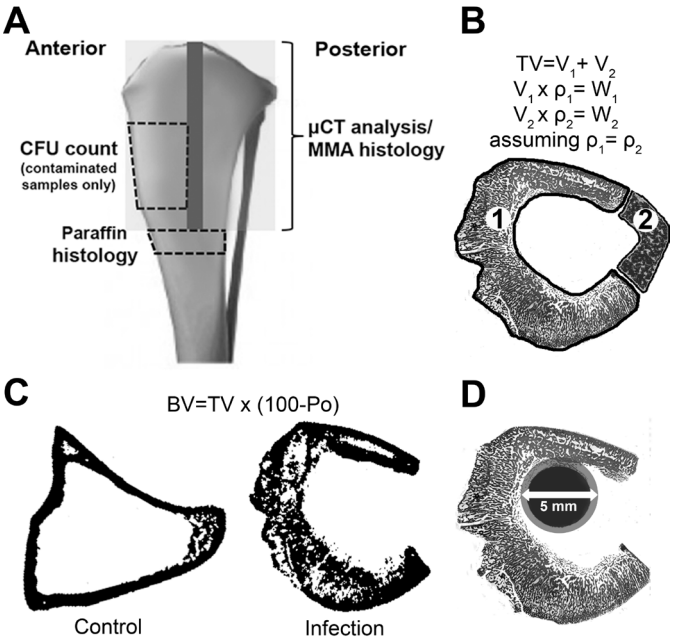


Figure 1. (**A**) Schematic representation showing the analyses performed on different tissue regions of the tibia (Infection study). (**B-D**) Bone volume calculations. (**B**) In infected tibiae, the volume of the removed anterior part (V_2) was extrapolated by dividing the known weight (W_2) by the density (ρ_2). The density was assumed to be the same as the determined bone density (ρ_1). This calculated volume V_2 was added to the scanned volume (V_1) to yield the total volume (TV). (**C**) A local threshold was applied to obtain the exact bone architecture and the bone porosity (Po). The bone volume was determined using the TV and Po values. (**D**) A peri-implant Region Of Interest (ROI) was defined as a 0.5 mm layer of bone tissue around the 4 mm diameter implant (grey region).

Histology

Undecalcified bones were fixed in 4% (v/v) formaldehyde, dehydrated in an ethanol series, and embedded in methyl methacrylate (MMA) containing 20% (v/v) plastoid N (Sigma) and 2.8% (w/v) benzoylperoxide (Luperox, Sigma). Transverse 40 µm-thick sections (Leica microtome, Nussloch, Germany) were stained with basic fuchsin/methylene blue for global histological assessment, or left unstained for fluorochrome detection (Olympus BX51 with DP70 camera, Olympus, Shinjuku, Tokyo, Japan).

For stainings on paraffin sections, the bone samples were decalcified in 0.5 M EDTA for a total of three weeks. The EDTA was replaced by formaldehyde for 2 d halfway, and for 2 d at the end of the decalcification procedure. Samples were embedded in paraffin and cut into 6 µm-thick sections. A modified Gram stain was performed to localize Gram-positive bacteria (Department of Pathology, UMC Utrecht). In short, samples were stained with Crystal Violet (Sigma, St. Louis, MO, USA) solution for 2 min and Lugol's solution (Merck, Billerica, MA, USA) for 1 min. Sections were then decolorized with acetone and counterstained with 0.05% (w/v) Light Green SF (Sigma). To demonstrate the presence of osteoclasts, a staining for tartrate-resistant acid phosphatase (TRAP) activity was performed. Samples were pre-incubated with 0.2 M acetate buffer-tartaric acid for 20 min at 37 °C. Naphtol AS-MX phosphate (0.5 mg/mL, Sigma) and Fast red TR salt (1.1 mg/mL, Sigma) were then added for another 2 h. Samples were counterstained with Mayer's hematoxylin.

Immunohistochemical staining for type II collagen was performed to identify cartilaginous tissue. Sections were treated with 1 mg/mL pronase for 30 min and 10 mg/mL hyaluronidase for 30 min at 37 °C. Following a blocking step with 0.3% (v/v) H_2O_2 in PBS, the sections were incubated with an antibody specific for type II collagen (1:100 from ascites, II-II6B3, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) overnight at 4 °C. Incubation with the secondary goat-anti-mouse antibody conjugated to horse-radish peroxidase (HRP, 1:200, P0447, Dako) was done for 1 h at room temperature. Detection was performed by incubation with 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB, D5637, Sigma). Sections were counterstained with Mayer's hematoxylin.

Macrophages and activated neutrophils were stained with an antibody specific for calprotectin. The same blocking and detection steps were used as described above. Antigen retrieval was performed with 0.1% (w/v) proteinase K for 15 min. Sections were incubated with the primary antibody (5 µg/mL, mouse-anti-human, MAC387, Abd Serotec, Kidlington, UK) for 2 h at room temperature. A mouse IgG1 monoclonal antibody (X0931, Dako) served as an isotype-matched control. This was followed by incubation with the secondary antibody (1:200, RPN1001, sheep anti-mouse IgG-biotin, GE healthcare) for 30 min at room temperature. To enhance the signal, samples were incubated with streptavidin-HRP (2 µg/mL, FP0397, Dako) for 30 min. Sections were counterstained with Mayer's hematoxylin.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using SPSS version 20.0 (IBM, Chicago, IL, USA). One-way ANOVA was used to analyze the micro-CT results. One-way ANOVA with Bonferroni post-hoc correction was used to analyze the changes in haematological parameters. Differences were considered

significant for $p < 0.05$. For the infection group, the Spearman correlation coefficient (ρ) with the two-tailed probability was calculated to assess the relationship between the bacterial load at day 28 (CFU/g bone) and the relative difference in tibial volume (left-to-right ratio).

RESULTS

Clinical signs of infection

All rabbits completed the 4-week follow-up. In all animals, weight loss was seen in the first week after surgery. In the control group, the animals regained their weight during follow-up, while this was not the case in the *S. aureus*-contaminated group (Figure 2A). Furthermore, only the control animals recovered to full weight bearing of the treated limb during the study. The erythrocyte sedimentation rate (ESR) and leukocyte counts were significantly higher in the *S. aureus*-contamined group at day 7 compared to the control group (Figure 2B). Together, these data suggest that infection was successfully established in all contaminated animals. The bacterial count showed that two out of six bone samples from the infection group were negative for bacteria, indicating a low-grade, or absence of active infection at the end of the study (Table 1).

Table 1. Bacterial count and tibial volume increase at day 28 in the infection group

Rabbit	Bacterial count	Group designation	Bone volume increase
	(CFU/g bone)	(CFU)	(Left-right ratio)
1	0	No	1.2
2	0	No	1.2
3	320	Low	1.6
4	$6.1x10^4$	Low	2.1
5	$6.7x10^5$	High	2.1
6	$3.1x10^6$	High	3.3

Histological assessment of infected bones

Basic histology showed several morphological changes in the infection group (Figure 3A), including thickening of the cortex, bone resorption, enlarged Haversian canals in the inner cortex, and new bone formation. Micro-abscesses were occasionally seen in the tibial canal. Gram-positive bacteria were encountered in infected specimens, but only in the original cortex and never in the newly formed bone tissue (Figure 3B). In sections of infected tibiae, numerous osteoclasts were found in the enlarged Haversian canals of the original bone and the mesenchymal stroma of the new bone (Figure 3C). In non-infected animals, osteoclasts were only observed in the endosteal and periosteal lining.

Immunohistochemical staining for type II collagen did not show evidence of increased endochondral bone formation in the infection group (Figure 4A-C). First, type II collagen was seen in all samples in the original cortex, but never in newly formed bone tissue.

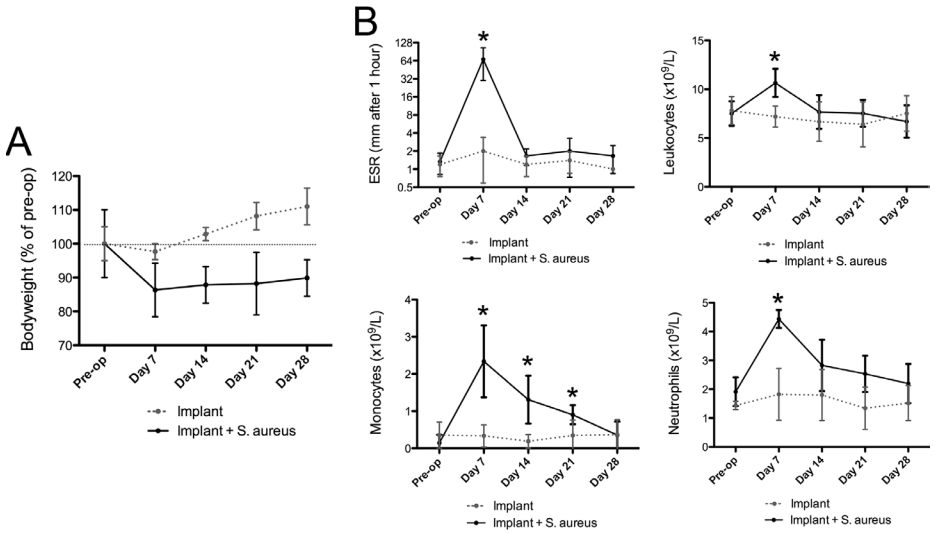


Figure 2. (**A**) Bodyweight changes in the infection study. (**B**) The erythrocyte sedimentation rate (ESR) and leukocyte counts were measured in blood samples. Mean ± SD, * $p < 0.05$ using one-way ANOVA with Bonferroni post-hoc test for comparison between non-contaminated and contaminated animals (n=6 per group).

Second, the collagen II matrix never contained cells. Staining for activated phagocytes with calprotectin was negative in tibiae of non-infected animals, while the treated tibiae of the infection group showed positive staining indicating the presence of active inflammation (Figure 4D-I). In these samples, staining was always observed in the medullary cavity (Figure 4D-F). Furthermore, the staining was localized to the inner bone cortex (Figure 4G-I), but only in tibiae associated with a bacterial burden at day 28 (Low and High CFU, Table 1).

Quantitative bone changes in infected tibiae

Micro-CT images showed clear differences between tibiae treated with an implant in the control and infection group. Mild to extensive thickening of the cortex was found in all samples in the infection group with occasional subperiosteal extrusions. This was associated with an increased porosity of the bone structure (Figure 5A). The quantified data confirmed the macroscopic observations (Figure 5B-F). Tibiae of the infection group showed a more than 2.5-fold higher total volume (p=0.01) and a more than 2-fold higher bone volume (p=0.01) compared to tibiae of the control group (Figure 5B). Infected tibiae furthermore demonstrated a more than 3-fold higher bone porosity (p=0.01) with a 10% lower bone density (p=0.01) compared to the control group (Figure 5C). There was a strong correlation (ρ=0.94, p=0.01) between the bacterial load at day 28 and the relative increase in tibial volume compared to the contralateral sides (Table 1). *S. aureus*-contaminated implants had a lower (p=0.04) peri-implant bone volume (Figure 5D). In the control group, the presence of the titanium implant alone did not affect the tibial volume when comparing the implant and non-implant side (Figure 5E). Although the tibial infection did not affect the ipsilateral fibulae, the bone volume of the fibula was significantly lower in the treated limb compared to the untreated limb in both groups (Figure 5F).

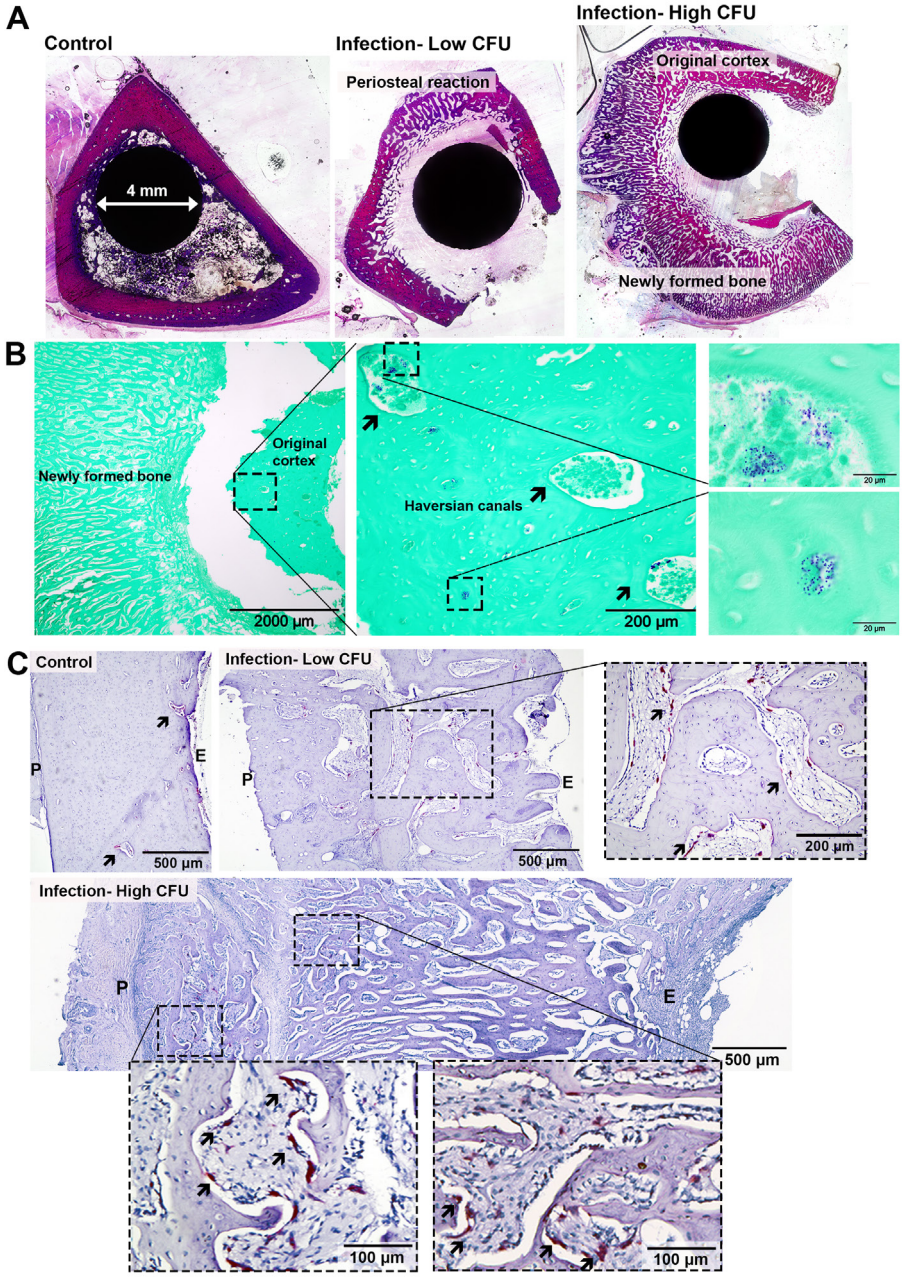


Figure 3. Histological differences between treated tibiae from the control and infection group (Infection study). (**A**) MMA samples were stained with methylene blue/basic fuchsin to examine gross morphological differences. Infected tibiae were associated with bone resorption, cortical thickening and subperiosteal bone apposition. (**B**) A Gram stain occasionally showed bacterial colonies (arrows) in the original bone tissue. A modified Gram stain was used (blue); samples were counterstained with Light Green SF. (**C**) In infected samples, TRAP staining demonstrated high osteoclast presence (arrows) within Haversian canals (original bone) and mesenchymal stroma (new bone). The representative images for each group are shown according to Table 1.

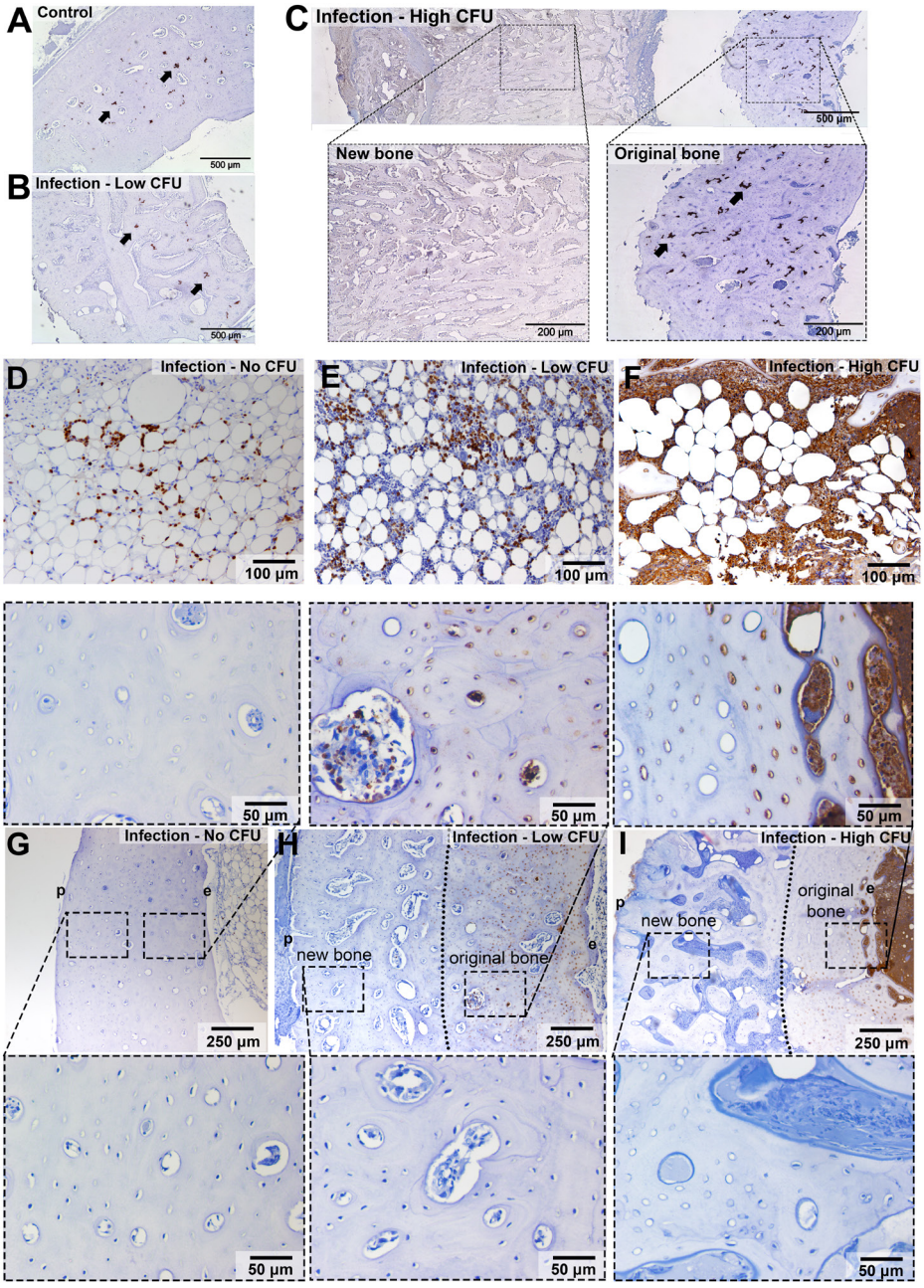


Figure 4. Immunohistochemical stainings performed on the treated tibiae from the control and infection group (Infection study). (**A-C**) Staining for collagen II. Patches of collagen II-positive tissue were observed in all samples (arrows), but never in newly formed bone tissue. Representative images are shown for the control group (**A**) and samples associated with a low (**B**) or high bacterial load (**C**) after 28 days according to Table 1. (**D-F**) The distribution of calprotectin in the medullary cavity of infected tibiae. Representative images are shown for samples associated with a low (**D**), average (**E**), or high (**F**) bacterial load after 28 days according to Table 1. (**G-I**) The distribution of calprotectin in the cortex of infected tibiae. Representative images are shown for samples associated with no bacteria (**G**), or a low (**H**) or high (**I**) bacterial load after 28 days according to Table 1. p: periosteum, e: endosteum.

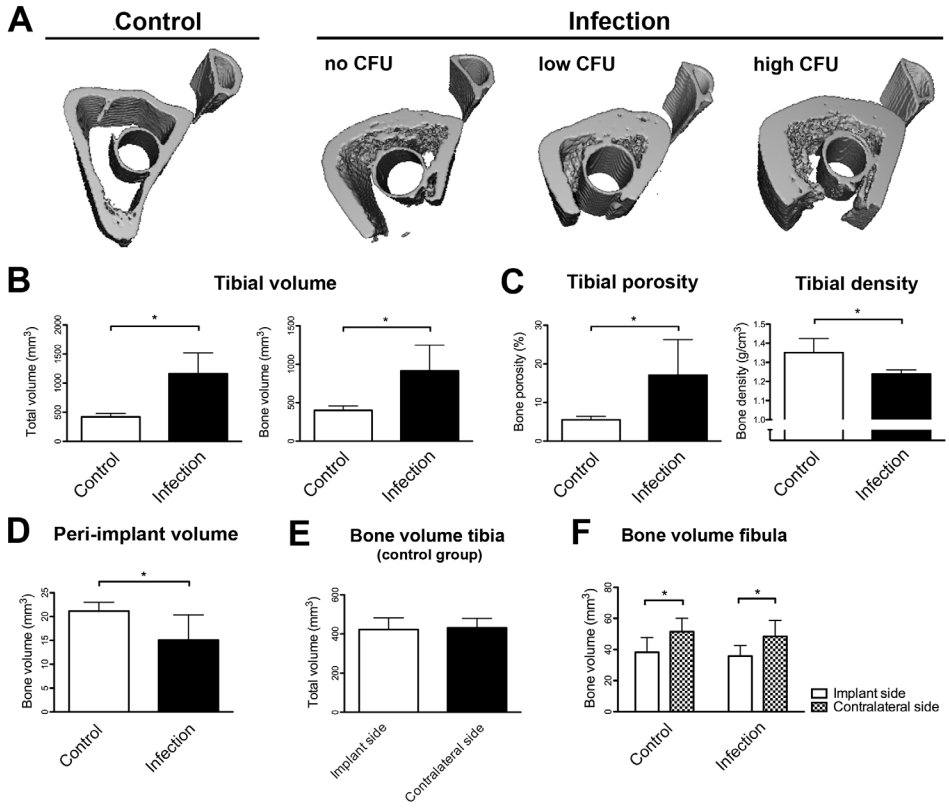


Figure 5. Micro-CT analyses of the treated tibiae from the control and infection group (Infection study). (**A**) 3D-reconstructed images of infected tibiae demonstrated increased thickness of the cortex. (**B-D**) *S. aureus*-infection affected the tibial volume (**B**), morphological parameters (**C**), and the bone apposition around the implant (**D**). (**E**) The implant alone did not change the tibial bone volume. (**F**) In all animals, a lower fibular volume was measured in implant side compared to the contralateral side. Mean ± SD, * $p < 0.05$ using one-way ANOVA for comparison between the control and infection group (n=6 per group).

Onset of bone formation

Incorporation of fluorochrome labels at predetermined time intervals was used to determine the onset and location of bone deposition (Figure 6). Although subperiosteal bone formation was seen in both groups, the extensive incorporation of fluorochromes suggested increased bone apposition in the infection group (Figure 6C, 6D) compared to the control group (Figure 6A, 6B). Bone formation had an onset between day 3 and 7 and continued throughout the study period. In the infection group, two distinct layers with high fluorochrome incorporation were found, resembling an ongoing process of bone deposition and remodeling (Figure 6C, 6D). In the control group (Figure 6E), endosteal bone formation towards the implant was always observed, while this was only seen in one tibia in the infection group (Figure 6F).

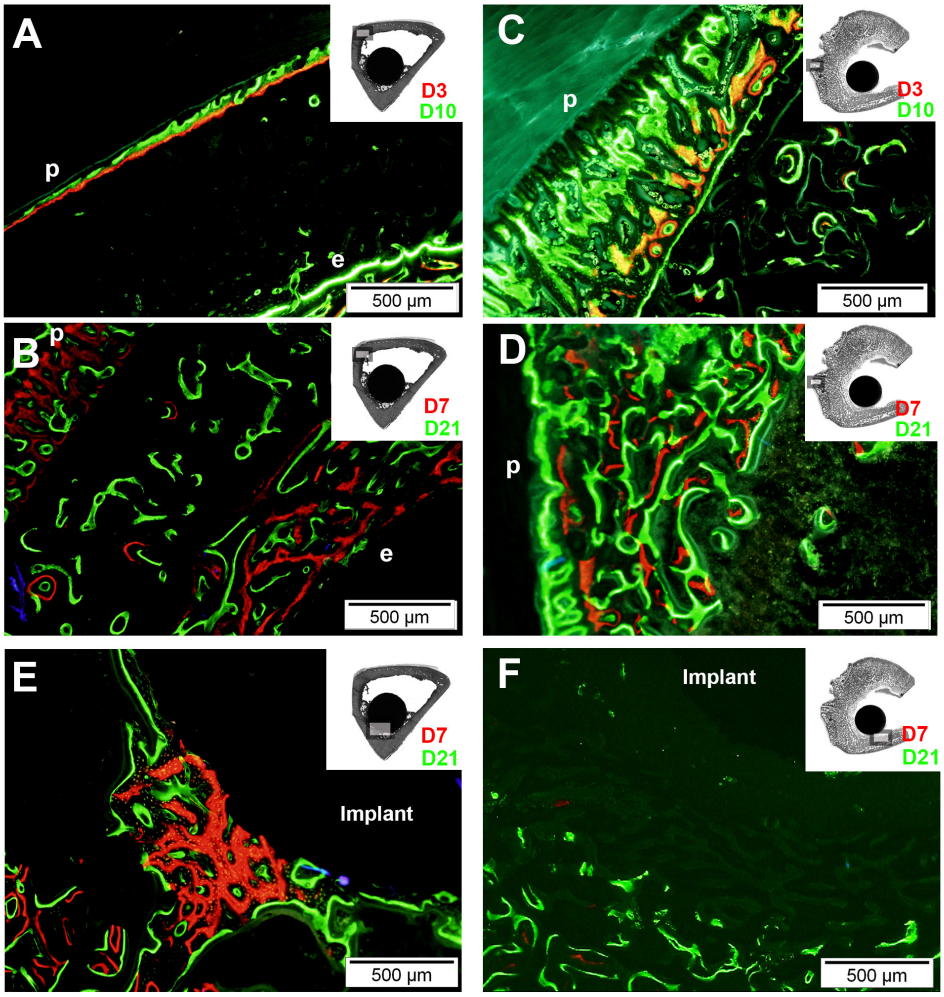


Figure 6. The incorporation of xylenol orange (red) and calcein (green) flurochromes showing the onset and progression of bone deposition (Infection study); Dx represents the day of fluorochrome injection. Periosteal bone formation in the tibiae of control (**A, B**) and infection animals (**C, D**). Peri-implant bone formation in the tibiae of control (**E**) and infection (**F**) animals. p: periosteum, e: endosteum.

S. aureus cell wall-induced bone changes

The effect of a sterile inflammation on the bone parameters was studied by inoculating the rabbit tibiae with an *S. aureus* crude cell wall extract (CWE). The animals increased in bodyweight after the first post-operative week (Figure 7) and returned to full weight bearing of the treated limb either in the second or third week after surgery. In the CWE-treated animals, the systemic markers of inflammation were analyzed at day 7, since they were found to peak at this time point (Figure 2B). Their ESR was the same at day 7 compared to the pre-operative value. Furthermore, their leukocyte counts only showed subtle increases compared to sham-treated controls (Table 2).

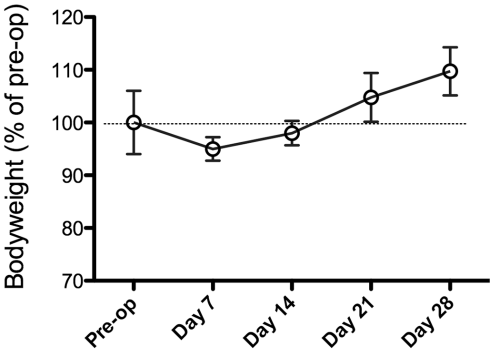


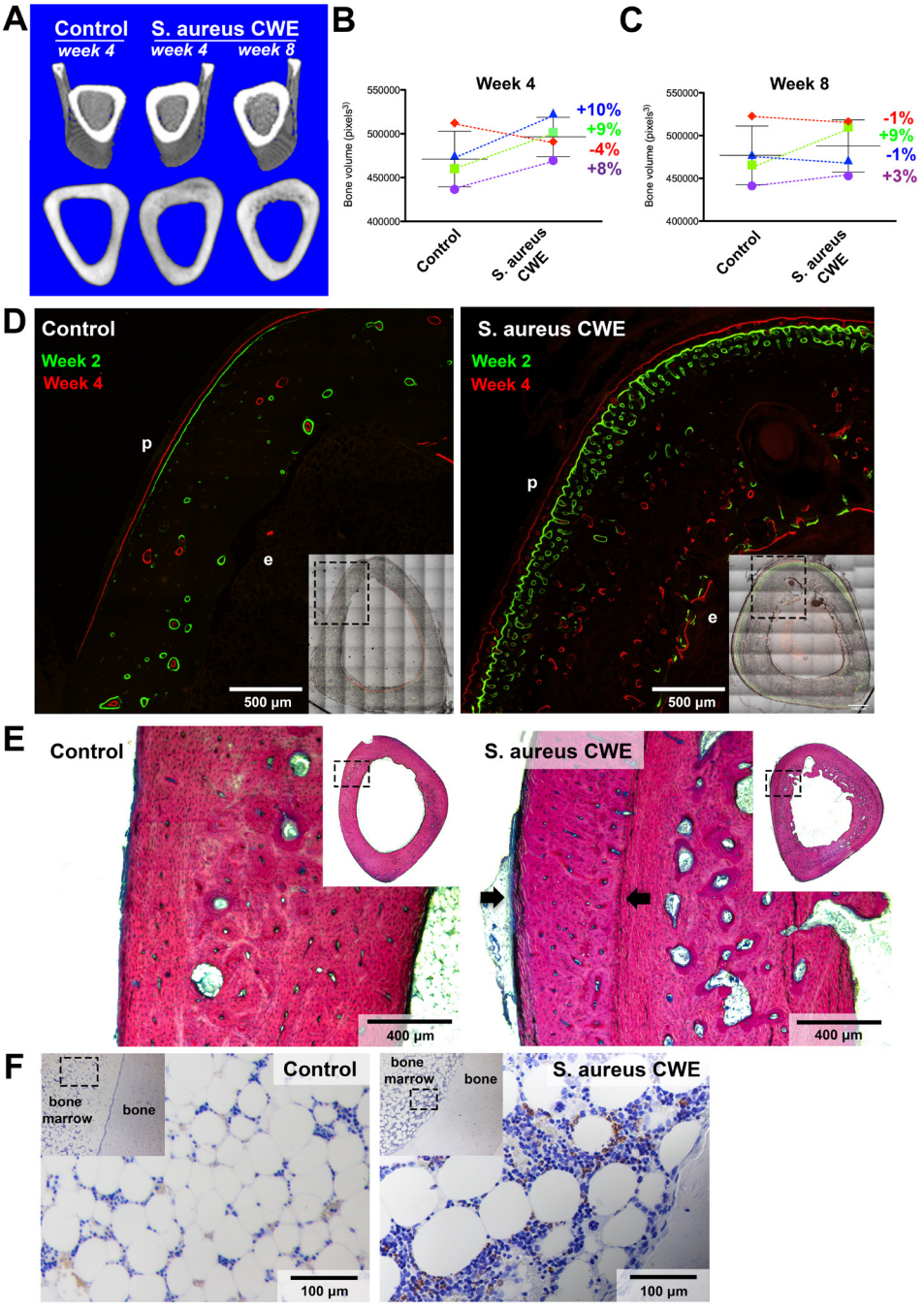
Figure 7. Change in bodyweight in rabbits treated with *S. aureus* cell wall extract.

Table 2. Non-specific markers of inflammation measured at day 7

	Implant		**Implant + *S. aureus***		***S. aureus* CWA**	
	(mean±sd)	(change[a])	(mean±sd)	Change[a])	(mean±sd)	Change[a])
ESR (mm/h)	2.0±1.4	2	67.5±37.4	54	1.0±0.0	1
Leukocytes (x10^9/l)	7.2±1.1	1	10.7±1.4	1	9.0±1.4	1
Monocytes (x10^9/l)	0.3±0.3	1	2.3±1.0	9	0.4±0.3	2
Neutrophils (x10^9/l)	1.8±0.9	1	4.4±0.3	3	3.1±0.4	2

[a] Fold-change at day 7 vs pre-operative. ESR=erythrocyte sedimentation rate, CWA=cell wall extract

Figure 8. Bone characteristics following a sterile inflammation (*S. aureus* cell wall extract study). ▶
(**A**) Representative micro-CT images from one rabbit, showing tibial cortical thickening after inoculation with *S. aureus* cell wall extract (CWE), together with the untreated contralateral tibia (control). Quantitative measurement of the tibial bone volume by micro-CT at week 4 (**B**) and week 8 (**C**). Each rabbit is represented by a unique color. The percentages indicate the relative bone volume increases within each animal. Mean ± SD (n=4). (**D**) The incorporation of calcein (green, week 2) and xylenol orange (red, week 4) fluorochromes showing the dynamics of bone deposition. p: periosteum, e: endosteum. (**E**) MMA samples were stained with methylene blue/basic fuchsin to examine the tissue morphology. A representative image is shown of a tibia with cortical thickening 8 weeks after inoculation with *S. aureus* cell wall extract, together with the untreated contralateral tibia (control). The arrows indicate the area of newly formed bone tissue. (**F**) The distribution of calprotectin in the medullary cavity. A representative image is shown of a tibia 8 weeks after inoculation with *S. aureus* cell wall extract, together with the untreated contralateral tibia (control).


A
Control
week 4
S. aureus CWE
week 4
week 8
B
Week 4
Bone volume (pixels³)
550000
500000
450000
400000
+10%
+9%
-4%
+8%
Control
S. aureus CWE
C
Week 8
-1%
+9%
-1%
+3%
D
Control
Week 2
Week 4
p
e
500 µm
S. aureus CWE
E
400 µm
F
bone marrow
bone
100 µm

Qualitative micro-CT assessment of *S. aureus* CWE-inoculated tibiae showed cortical thickening and osteolytic bone changes in comparison to the contralateral tibiae (Figure 8A). In contrast to an *S. aureus* infection, there was no cortical bone loss. Quantitative assessment showed a transient bone volume increase in 3 out of 4 treated tibiae (Figure 8B, 8C). The relative bone volume increase compared to the contralateral tibia was 6 ± 7% at week 4 (Figure 8B), and 2 ± 5% at week 8 (Figure 8C).

An increased amount of fluorochrome markers were incorporated in CWE-treated tibiae compared to untreated tibia (Figure 8D). In addition, the week 2 label was more abundant than the week 4 label. Similar to the infected tibiae, the localization of the fluorochromes indicated that subperiosteal bone apposition was the mechanism behind the bone volume increase. Basic fuchsin/methylene blue staining on MMA-embedded samples showed mild morphological bone changes characteristic of a bacterial infection (Figure 8E), i.e. thickening of the cortex, new bone formation and enlargement of the Haversian canals. Micro-abscesses were not observed. Staining for calprotectin after 8 weeks showed the occasional presence of activated phagocytes in the medullary cavity in 3 out of 4 tibiae treated with *S. aureus* CWE, while the bone marrow had a normal appearance (Figure 8F). TRAP staining showed the sporadic presence of osteoclasts after 8 weeks, with no differences between *S. aureus* CWE-treated and control tibiae (not shown).

DISCUSSION

This study characterizes the osteogenic response in an established rabbit model of periprosthetic *S. aureus* infection. As for many other infection models, there was an inconsistency in the current study to induce the same severity of infection in all animals. Despite this variation, all *S. aureus*-contaminated tibiae showed pronounced bone formation leading to a net bone volume increase. This pro-osteogenic effect could be measured in terms of bone volume changes on micro-CT, since the intramedullary implant alone did not affect the bone volume in the control condition. The response appeared to be local, as the volumes of the contralateral tibiae and even the ipsilateral fibulae remained unaffected in the infection group.

Fluorochrome analysis showed that bone apposition mostly occurred in the subperiosteal region. Since the periosteum can react to mechanical changes[44], the induced osteogenesis in the periosteum may have occurred as an adaptive response to maintain the bone strength following endocortical bone resorption[45,46]. This however is unlikely, considering the early onset of bone formation (i.e. before 7 days), the substantial net bone increase in infected tibiae, and the absence of an osteogenic response in the ipsilateral fibulae. Alternatively, periosteal bone formation can also be a direct response to inflammatory stimuli[47]. In agreement, we observed a strong correlation between the bacterial load at the study endpoint and the bone volume increase despite the relative small sample size. Also, there was a trend towards a higher local presence of activated phagocytes in the tibiae associated with most bone formation.

The use of virulent bacteria as bone-stimulating agent is unfavorable due to the uncontrolled expansion, systemic effects, and/or severe osteolysis associated with infection[23,35,48]. Therefore, we hypothesized that a transient inflammatory response to

isolated *S. aureus* cell wall components could also induce new bone formation. The same tibia location was used to allow a close comparison between the inflammation-induced effects during infection and the *S. aureus* cell wall-induced effects. There were only small changes in the experimental design. First, the *S. aureus* cell wall isolate could be delivered into the tibial canal in a less invasive approach without an implant. Furthermore, the untreated tibia served could serve as a within-subject control, since even the sham implants without infection did not affect the bone. In line with the hypothesis, bone changes were observed with an obvious pro-osteogenic response. Although the net bone volume change was less pronounced, the overall response was more favorable due to the absence of the severe osteolysis as observed in the infected tibiae. This finding further suggests that bone apposition in this model occurs as a direct response to inflammation, and uncoupled from osteolysis. This in agreement with other observations. For instance, the induction of inflammation with oncostatin M induces periosteal bone formation without increased osteoclast activity[49], while inflammation in the spine can lead to ankylosis without higher bone resorption[50].

Although more detailed analyses are needed in future studies, the use of bacteria-derived products could be a potentially safe strategy, as shown by the minimal effect of the *S. aureus* cell wall extract on systemic inflammation markers, bodyweight and general performance compared to sham-treated animals. Future research is needed to identify which clinical conditions (e.g. type of defect or patients) will benefit most from inflammation-induced bone formation. Considering the importance of the periosteum in callus formation and its responsiveness to inflammatory signals,[47,51] it can be speculated that such a strategy is particularly effective in defects which rely on periosteal-mediated bone regeneration.

These findings allow future studies to identify and characterize the most favorable stimuli for bone formation in this model. For example, different toll-like-receptor (TLR) agonists should be investigated since they play an important role in the response to bacterial antigens[52], and could promote bone formation through different mechanims[36,53]. The bone stimulatory or resorptive effects of proinflammatory mediators is likely dependent on their local concentrations. In the current model, we never observed bacteria or activated phagocytes in newly formed bone, which is in line with other studies showing that bone apposition in response to an *S. aureus* infection is usually not observed in direct vicinity of the bacteria, or within a subperiosteal abcess, but further away from the bacterial burden[20,21]. The rabbit tibia model can be used to study inflammation-induced bone formation as a phenomenon, but the actual therapeutic use of bone-inductive mediators should be studied in a more translational approach, i.e. in functional models such as critical size defects or spinal fusion models, and as part of bone tissue engineering constructs. These studies should also include a more detailed assessment of adverse reactions and mechanical performance of the treated defects to verify their true therapeutic potential.

In conclusion, the rabbit tibia model is suitable to study the osteogenic response to local inflammation. After inoculation with *S. aureus*, bone formation started within a week and was localized to the contaminated tibiae, suggesting a direct effect of inflammatory stimuli on osteogenesis. Since the *S. aureus* cell wall extract induced a more favorable osteogenic response, the sterile inflammatory reaction to bacterial antigens may be harnessed for bone regenerative therapies.

ACKNOWLEDGEMENTS

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Chapter 7

Bacterial antigens for skeletal tissue regeneration

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ABSTRACT

Immune cells and their soluble factors tightly regulate skeletal cells during bone healing and remodeling. Modulation of their interaction therefore is a potential strategy to enhance new bone formation. Bacterial infections can have pro-osteogenic effects, which are usually overshadowed by the severe osteolysis and systemic concerns they cause. Here, we show that a controlled inflammation induced by killed bacteria leads to new bone formation in the rabbit tibia, without the detrimental effects of active infection. Killed bacteria also promoted regenerative processes in ectopic bone scaffolds where osteoinduction was exclusively provided by bone morphogenetic protein-2. Pro-osteogenic effects of killed bacteria were species-dependent, and the purified bacterial cell wall constituent lipoteichoic acid was identified as an osteo-stimulatory factor. Our data indicate that bacterial components stimulate osteogenesis through their synergy with osteoinductive stimuli. This finding holds promise for the use of bacterial antigens as immuno-modulatory tools for bone regeneration.

INTRODUCTION

Transplantation of autologous bone is the gold standard in the repair of large bone defects, however its limited availability and the increased surgery time are major drawbacks[1,2]. Several biological and synthetic off-the-shelf bone substitutes are available, but are still considered inferior to autologous bone[3]. As the role of biomaterials for orthopedic applications is evolving from a structural role to a bioactive role to actively promote regeneration, the properties of these biomaterials are fine-tuned to direct the optimal tissue response[4,5]. Factors that enhance the differentiation of progenitor cells to the osteogenic lineage, such as bone morphogenetic proteins (BMPs), can contribute to repair, although it is generally accepted that their *in vivo* delivery is still suboptimal in terms of timing and dosing[6]. The immune system plays an essential role during bone healing[7,8], and there is growing evidence that the immuno-modulatory properties of biomaterials can help regulate the osteogenic response[5,9]. As a result, incorporation of immuno-modulating drugs or pro-inflammatory cytokines into bone graft substitutes are currently being explored as strategies to provide the appropriate pro-osteogenic environment needed for bone regeneration[10,11].

Generally, a balanced inflammatory response coordinates osteogenic and angiogenic processes during bone healing[12]. Certain pathological conditions are also thought to involve the activation of inflammatory pathways leading to osteogenesis[13,14]. For example, bacterial infections induce inflammatory responses that mostly have detrimental effects on bone formation due to uncontrolled receptor activator of nuclear factor kappa-B ligand (RANKL)-induced osteoclast formation[15,16]. Paradoxically, bacterial infections are also associated with periosteal bone formation or heterotopic ossifications via yet unknown pathways[17,18]. These profound effects of bacterial infections on bone suggest that bacteria-induced inflammatory cues can harbor an osteogenic response under some conditions. It has previously been demonstrated that a localized *Staphylococcus aureus* (*S. aureus*) infection leads to both osteolysis and significant osteogenesis in an established rabbit tibia model[19]. In this model, it was shown as a proof of principle that inoculation with isolated *S. aureus* cell wall fragments also results in substantial new bone formation.

The bacterial components responsible for osteo-stimulatory effects still have to be identified. The host immune system detects and responds to bacteria by sensing pathogen-associated molecular patterns (PAMPs) predominantly in the bacterial cell wall[20,21]. Hence, a comparison of different bacterial cell wall components in the form of intact killed bacteria provides a means to study how different PAMPs affect bone anabolic and catabolic pathways. As Gram-positive and Gram-negative bacteria have distinct cell wall properties with respect to e.g. thickness of the peptidoglycan layer and presence of teichoic acids, lipoproteins, and lipopolysaccharides, their differential stimulation of the host immune system could subsequently affect the osteogenic response[22]. Elucidating the mechanism through which bacterial components or immune cell-secreted factors affect new bone formation is clearly of most interest. To date, some studies point to possible interactions between inflammation-induced signals and osteoinductive factors in directing the differentiation of bone progenitor cells, leading to enhanced new bone formation[10,23]. The contribution of osteoinductive pathways is best studied in an ectopic implantation model outside of the native bone environment, where bone formation relies on recruitment and differentiation of osteoprogenitor cells[24].

In this study, the potential of bacterial components to enhance bone formation is explored in rabbits. We demonstrate that the localized inflammatory response that is thought to occur in response to various species of killed bacteria, has profound effects on the osteogenic response. In the tibia, this led to an increase in bone mass along with a reduced systemic response compared to groups receiving viable bacteria. In the ectopic location, killed bacteria enhanced the bone-inductive effects of BMP-2 in ceramic scaffolds, demonstrating that bacteria-induced inflammatory signals and osteogenic pathways interact. Together, these data imply that the immuno-modulatory properties of bacterial components can be used to stimulate new bone formation.

MATERIALS AND METHODS

Bacterial culture

Bacteria were cultured to mid-log phase in liquid media. *Escherichia coli* (*E. coli*, MG1656) and *S. aureus* (Wood 46) were cultured in LB medium. *Haemophilus influenzae* (*H. influenzae*, NCTC 8468) was cultured in Brain Heart Infusion (BHI) broth supplemented with X & V factors. *Mycobacterium marinum* (*M. marinum*, E11) was grown in Middlebrook 7H9 medium supplemented with Middlebrook oleic acid-albumin-dextrose-catalase (BD Biosciences, CA, USA) and 0.05% Tween -80. *Bacillus cereus* (*B. cereus*, ATCC 14579) was cultured in BHI medium. Bacteria were stored at -80 °C and extensively washed in PBS before *in vivo* use.

Bacterial inactivation

Freshly-cultured bacteria were killed by gamma irradiation (γi) at 25 kGy. For heat inactivation, *S. aureus* were incubated in PBS at 60 °C for 30 min under gentle shaking. For formalin inactivation, *S. aureus* were incubated overnight in 4% formaldehyde under rotation. The absence of any viable bacteria was confirmed by plate cultures. The killed bacterial suspensions were stored overnight at 4 °C and extensively washed in PBS before *in vivo* use.

Bacterial count

To quantify *S. aureus* during infection, sterile harvested bone samples were weighed and homogenized (Polytron PT3100; Kinetica, The Netherlands). The number of colony forming units (CFU) on blood agar plates was normalized to the weight of the bone fragments.

Preparation of ectopic constructs

Biphasic calcium phosphate (BCP) scaffolds consisted of 20±5% β-tricalcium phosphate and 80±5% hydroxyapatite by weight, and had a total porosity of 75±5% (Xpand, The Netherlands). The blocks had a dimension of 6x6x3 mm. A total available pore space of 70 mm^3 was considered for concentration calculations, since 70 µl was the maximal volume that was completely absorbed by the scaffolds. For intramuscular samples, a volume of 70 µl PBS containing killed bacterial strains or lipoteichoic acid (LTA, *S. aureus*-derived, L2515, Sigma, MO, USA) was pipetted onto the scaffolds. For subcutaneous samples, the mediators were studied in combination with a minimal dose of recombinant human BMP-2 (InductOS, Wyeth/Pfizer, NY, USA). For this purpose, 15 µl of a 100 µg/mL

BMP-2 suspension was first pipetted onto the scaffolds. Subsequently, 55 µl of a PBS suspension containing the killed bacterial strains or LTA was pipetted onto the scaffolds. This resulted in a BMP-2 end concentration of 21.5 µg/mL.

Animal procedures

Animal experiments were performed after approval of the local Ethics Committee for Animal Experimentation. A total of forty-four female New Zealand White rabbits (12-16 weeks old, 2.5-3.5 kg, Charles River, France) were used. Surgery was performed as described before[19]. In short, the left stifle joint was opened through a medial para-patellar incision. In animals receiving an implant, the tibial canal was opened using a 4.1 mm Ø drill. After draining, 10^5 viable bacteria or 10^9 γi bacteria were pipetted in the canal in 50 µl PBS and a cylindrical sandblasted titanium rod (4 mm Ø x 25 mm) was inserted. In the model without implant, the tibial canal was drilled with a 1.0 mm Ø drill. After draining, bacteria were injected in a volume of 50 µl PBS and the opening was closed with 200-300 µl fibrin glue (Tissucol 500®, Baxter, IL, USA) to prevent leakage of the bacterial suspension. The patella and skin were closed with resorbable sutures. *In vivo* microCT scans of the hind limbs were made on day 28 under anesthesia. For the tibia model, the right limbs served as the negative control for all analyses.

For the ectopic model (Figure 3), the same animals were used as for the second orthotopic study (Figure 2), and received the ectopic implants as part of the same surgery. A dorsal midline incision was made to expose the para-spinal muscles and 5 BCP blocks were implanted on each side of the spine in intramuscular pockets (samples without BMP-2). After closure of the midline skin incision, the same number of BCP blocks was implanted into subcutaneous pockets (samples with BMP-2). A total of 20 BCP blocks was implanted per animal in a randomized way.

The animals' bodyweight was measured weekly. Blood was collected pre-operatively, and on days 3, 7, 14, and 28 to measure systemic markers of inflammation. To trace new bone formation, the fluorochrome labels Xylenol orange (30 mg/kg s.c. in 1% w/v $NaHCO_3$, Sigma) and Calcein green (10 mg/kg s.c. in 2% w/v $NaHCO_3$, Sigma) were injected at time points indicated in the figures.

Animals were euthanized with pentobarbital (i.v. Euthanimal®, Alfasan, The Netherlands) under the same anesthesia as the surgery. The hind limbs were harvested and immediately scanned by microCT. To determine the presence of colony-forming bacteria, a bone sample of the anterior tibiae (1.4 ± 0.6 g) was removed with a sterile rotary saw. The remaining tissue was used for histological analyses.

MicroCT analysis

Scans were acquired with a tube voltage of 90 kV and tube current of 180 µA (Quantum FX, PerkinElmer, MA, USA). Images were represented as a stack of 2D TIFF images (Analyze, version 11.0, AnalyzeDirect Inc, KS, USA). All analyses were performed with the BoneJ plugin (version 1.3.12) in ImageJ freeware version 1.48 (U.S. National Institutes of Health). The intramedullary implant was excluded using a customized macro in ImageJ based on a global threshold. An adaptive threshold was applied based on the mean local gray scale distribution to segment the bone for volume and porosity measurements.

For the tibia implant model (voxel size 60 μm^3), analyses were performed separately on the proximal implant-containing (280 slices, 16.8 mm) and distal-to-implant (220 slices,

13.2 mm) tibial sections. The proximal and distal fusion points between tibia and fibula were used as reference to cover the same bone area. In the model without implant (voxel size 120 μm^3), analyses were performed on a tibial section (250 slices, 30 mm) proximal of the distal fusion between tibia and fibula. Gray value profile plots of the middle slices were used to assess changes in bone density.

Tissue processing
For the tibia implant model, a bone sample immediately distal to the implant was used for decalcified histology. In the model without implant, a bone sample was taken just proximal to the fusion point between tibia and fibula. These samples where fixed in 4% formaldehyde, decalcified in 12.5% (w/v) EDTA and embedded in paraffin. Subsequently, 6 µm-thick cross-sections were cut for histological stainings. The rest of the tibia was fixed in 4% formaldehyde and embedded in methyl methacrylate (MMA), and 35 µm-thick sections were cut using a saw microtome. For histological analyses of BCP scaffolds, a quarter of each construct was used for paraffin embedding and sectioning, while the remaining construct was used for MMA histology.

Scoring of bone changes
Periosteal bone formation, enlargement of Haversian canals, and cortical bone loss were scored on basic fuchsin/methylene blue-stained MMA sections. The parameters were scored by two observers (MC and SAP) on blinded samples as 0 (absent), 1 (moderate), or 2 (severe) according to the portion of the cortical circumference that was affected relative to untreated contralateral tibiae. The average value of the two observers was used for further analyses. Measurements in the untreated tibiae showed that Haversian canals should be defined as enlarged for a diameter exceeding 100 µm.

Bone histomorphometry
MMA sections were stained with basic fuchsin and methylene blue. Two midsections were pseudo-colored in Adobe Photoshop CS6 (Adobe Systems, CA, USA) to quantify the percentage of bone tissue in the available pore space (bone area%). The mean value of two midsections per sample was used for statistical analyses.

Fluorochrome detection
Two blinded observers (MC and SAP) scored the presence of fluorochromes in unstained MMA sections. Fluorochrome overview images were taken with a Laser scanning confocal microscope (SP8X, Leica). Sections were imaged with a 10x dry objective using a white light laser (470-670 nm). Hybrid detectors collected fluorescence signal from calcein (494/509-550 nm) and xylenol orange (570/610-780 nm), which were given the pseudo-colors green and red. All images were processed using Leica LASX acquisition software.

Histological stainings
For Gram staining, decalcified bone samples were treated with 10% (w/v) Crystal Violet followed by a diluted Lugol's solution. Differentiation was performed with acetone, following by staining with 10% (v/v) Ziehl-Neelsen's carbol fuchsin. Picric acid was used as a counterstain (VWR, PA, USA). To localize Gram-positive bacteria, a modified Gram

stain was used in which samples were counterstained with 0.05% (w/v) Light Green SF after the differentiation step. To identify mycobacteria, an acid-fast bacteria stain kit was used according to the manufacturer's instructions (Dako, Denmark). Tartrate-resistant acid phosphatase (TRAP) activity was visualized to demonstrate the presence of osteoclasts, as described before[19]. Immunohistochemical stainings were performed as described in detail previously[10]. For each sample, the total number positively-stained cells in one cross section was counted. A mouse-anti-human calprotectin antibody (5 µg/mL, MCA874A488, clone MAC387, Bio-rad, CA, USA) was used to detect activated macrophages and neutrophils. For detection of T lymphocytes, a mouse-anti-human CD3 antibody (0.7 mg/mL, M7254, clone F7.2.38, Dako) was used.

Statistical analyses

All data are presented as mean ± standard deviation (SD). SPSS version 20.0 was used (IBM, IL, USA). To analyze changes in bodyweight or the levels of systemic inflammation markers, repeated measures ANOVA was performed. One-way ANOVA was performed to compare bone changes on MMA samples. To analyze differences in the bone volume between treated and untreated limbs on microCT, a paired sample T-test was used. The group sizes are shown in the figure legends (n = 6-8). Differences in ectopic bone volume (bone area%) were analyzed using a linear mixed-model approach. Pairwise comparisons were made between γi bacteria (n = 7-9) or LTA-loaded (n = 10) scaffolds and the control sample in each animal (n = 24). Within this model, the TEST subcommand was used to test the hypothesis that the highest concentration of bacteria results in a lower bone volume compared to the other concentrations. A *post hoc* test with Bonferroni correction was used. The group sizes used in the ectopic study were based on sample size analyses, using a power of 80% and an alpha of 5% that was adjusted for multiple comparisons. An effect size of 30% and standard deviation of 20% was used.

RESULTS

Local delivery of killed bacteria confines the inflammatory response

It has previously been shown that a virulent *S. aureus* peri-prosthetic infection increases the bone mass in the rabbit tibia despite the mixed osteoblastic-osteolytic reaction[19]. To characterize the role of the host immune response in this pro-osteogenic response, we studied the effect of *S. aureus* on bone formation under different scenarios (Figure 1A). First, we hypothesized that a relatively mild infection in this model would limit the osteolysis without necessarily affecting the increased osteoblast activity, and therefore allow the pro-osteogenic response to prevail. The presence of a foreign body is thought to promote infections by supporting bacterial adhesion and biofilm formation[25, 26]. Therefore, to establish infection conditions with relatively high and low bacterial burden, we compared *S. aureus* inoculation with (SA+) or without (SA-) metal implant, respectively. Second, we hypothesized that the use of gamma irradiation-killed (γi) *S. aureus* could provoke the inflammatory response needed for bone induction[27], while limiting systemic inflammation due to absence of bacterial spread and exotoxin secretion[28, 29]. Animals receiving γi *S. aureus* also received the metal implant to provide the same conditions as the peri-prosthetic infection group.

We found considerable differences in systemic and inflammatory responses between the three groups (Figure 1B-D). A significant reduction in bodyweight was observed in all groups in the first week. This was maintained in four out of eight animals from the SA+ group whereas the other animals had regained their normal weight at day 28 (Figure 1B). All groups showed increased levels of systemic inflammation in the first 3 days after surgery (Figure 1C), which normalized by day 7 in the γi SA+ group and by day 14 in the viable *S. aureus* groups. All tibiae contaminated with viable *S. aureus* were found to be

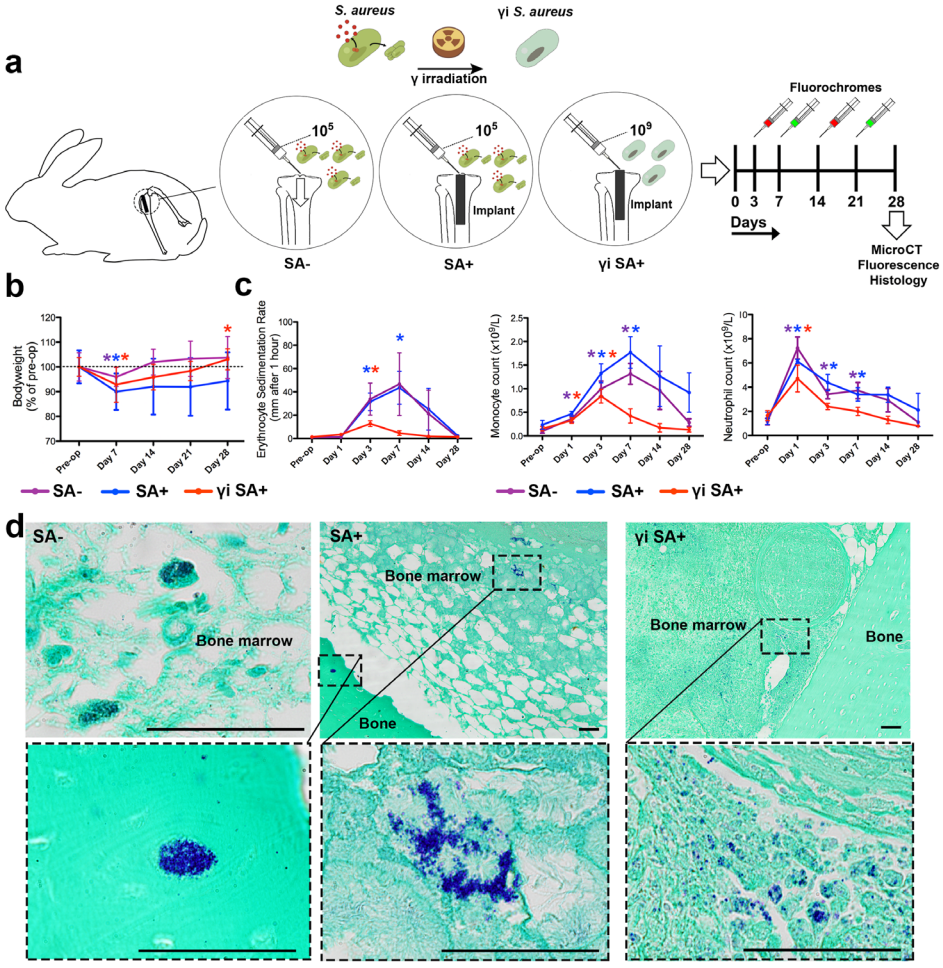


Figure 1. Systemic responses induced by viable and killed *S. aureus*. (**A**) Study design used to investigate the effects of *S. aureus* on the inflammatory response and local bone parameters in the rabbit tibia. Viable *S. aureus* were studied either in absence (SA-) or presence (SA+) of a metal implant. Gamma irradiation-killed *S. aureus* were also studied together with an implant (γi SA+). Fluorochromes were injected to determine the onset and dynamics of bone formation. The primary outcome parameter was the tibial bone volume measured by microCT after 28 days. (**B**) Changes in bodyweight relative to the pre-operative value. (**C**) Systemic inflammation, as determined by erythrocyte sedimentation rate and peripheral blood monocyte and leukocyte counts. For **B-C** data are presented as mean ± SD for the SA- (n = 6), SA+ (n = 6), and γi SA+ (n = 8) groups. *p<0.05 compared to pre-operative value, repeated measures ANOVA with Bonferroni correction for *post hoc* analysis. (**D**) Representative cross-sections of tibia stained for Gram-positive bacteria or cell walls (blue). Scale bar=50 µm.

infected at day 28. Quantification of the number of bacterial colonies demonstrated that the bacterial amount was 100-fold higher in the SA+ group (1.9 ± 2.1x10^6 CFU/g bone) than the SA- group (1.9 ± 2.1x10^4 CFU/g bone). This shows that the implant was not obligatory to establish *S. aureus* bone infection, although the implant clearly promoted bacterial expansion and development of a chronic infection. No colonies could be cultured from any of the bones from the γi SA+ group, but diffuse Gram-positive staining indicated the presence of bacterial cell walls or their remnants in the bone marrow (Figure 1D). In agreement with the CFU counts, numerous Gram-positive colonies were stained in the bone and bone marrow in the SA+ group.

These results indicate that the different *S. aureus* treatment scenarios provided a range of systemic responses of the host. High dose γi *S. aureus* resulted in its sustained presence in the tibial bone marrow. Despite the 10^4 times higher inoculation dose, a less pronounced systemic inflammation was seen for γi *S. aureus* compared to the viable *S. aureus* groups.

Killed *S. aureus* induce a favorable net increase in bone mass

It was determined how the inflammatory response induced by the different *S. aureus* groups described above is associated with changes in cortical bone volume and structure. The untreated contralateral tibia served as a within-subject control for microCT measurements in the tibia model, as it was previously shown that sham treatment with implant alone does not affect the bone volume or appearance[19].

Qualitative microCT analysis demonstrated mild cortical osteolysis in the endosteal region in the SA- group (Figure 2A). In the SA+ and γi SA+ groups, local cortical osteolysis was accompanied by areas of new bone formation, as indicated by cortical thickening. Cortical resorption was more profound in the SA+ group, and also resulted in a separation of the periosteal layer from the cortex. Quantitative microCT analyses showed a significant increase in the cortical bone porosity in all treated groups compared to the contralateral tibia (Figure 2B). Nevertheless, significant bone volume increases were measured in the SA+ and the γi SA+ groups (Figure 2C). Bone volume measurements were performed separately for the proximal (peri-prosthetic) and distal tibial regions to establish the role of the implant in the osteogenic response. In the SA+ group, a significant bone volume increase was only measured in the proximal tibia (+89%, p=0.047), likely because the bacterial burden was confined to this region. In contrast, γi bacteria induced the osteogenic response in a larger bone region, as a bone volume increase was measured in both the proximal (+18%, p=0.019) and distal (+28%, p=0.003) tibia. These results are in agreement with a previous study where disrupted *S. aureus* fragments were found to cause new bone formation in the rabbit tibia[19]. In the current study, we found a larger relative increase in bone volume for intact γi *S. aureus* compared to disrupted *S. aureus* cell wall fragments, which can be attributed to their slower clearance and more sustained and diffuse response.

The incorporation of injected fluorochrome markers at sites of mineralization was assessed to trace the location and progression of bone formation in time (Figure 2D). In comparison to untreated contralateral tibiae, fluorochromes were abundant in the periosteal region in groups with increased bone volume (i.e. SA+ and γi SA+ groups). Bone apposition had an onset before day 3 and was visible throughout the entire study period. The prominent subperiosteal bone apposition observed here possibly shows similarity with the high responsiveness of periosteal osteoblast precursors to inflammatory cues

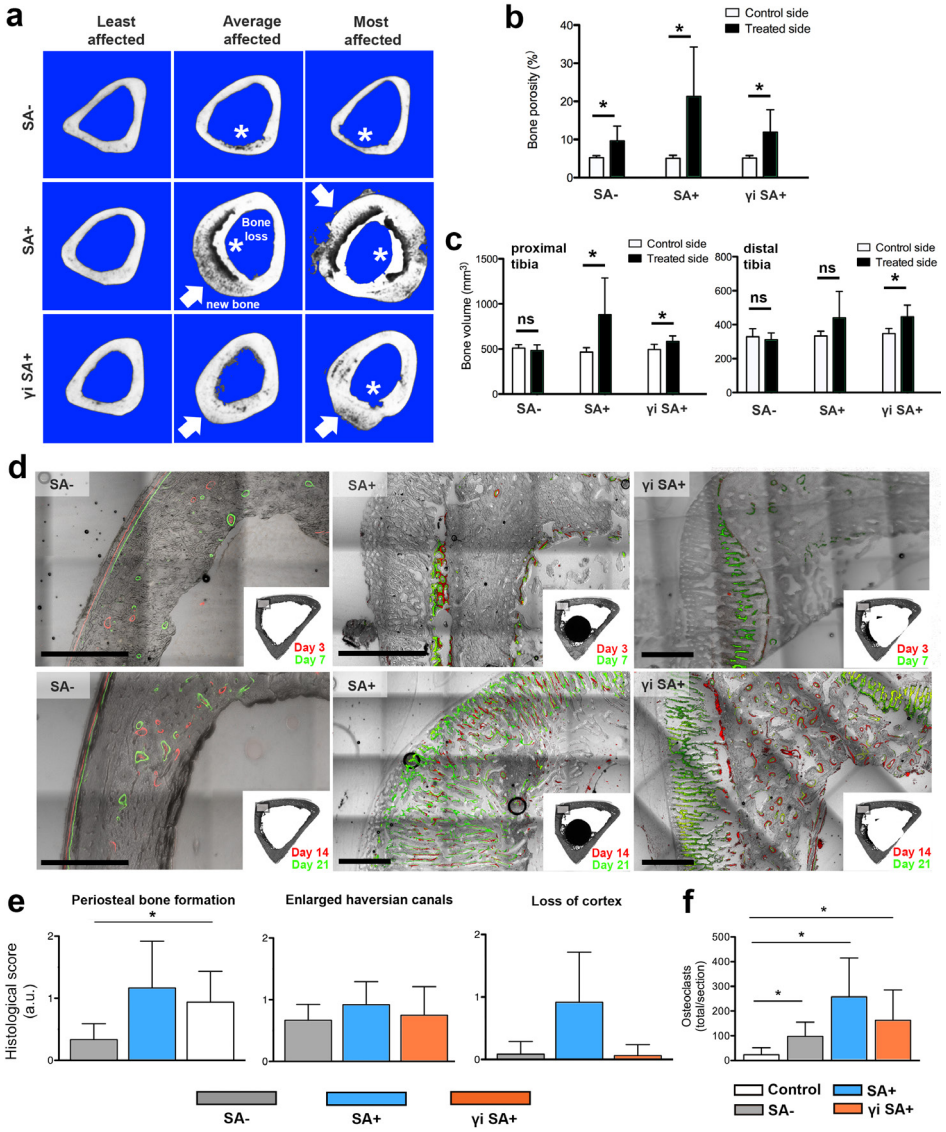


Figure 2. Effect of viable and killed *S. aureus* on the tibial bone volume. (**A**) Representative microCT images of middle sections of tibiae at day 28. Arrows: periosteal bone formation. Asterisks: osteolysis. (**B**) Cortical bone porosity measured at day 28 by microCT. (**C**) Tibial bone volume in peri-prosthetic bone region (left) and distal-to-implant bone region (right) at day 28 measured by microCT. For **B-C** data are shown as the mean ± SD for the SA- (n = 6), SA+ (n = 6), and γi SA+ (n = 8) groups. *p<0.05; paired sample T test with Bonferroni *post hoc* test. (**D**) Overlay images of brightfield and fluorescence channels demonstrating incorporation of xylenol orange (red) and calcein (green) during bone deposition in tibiae treated with viable or γi killed *S. aureus* on days 3/7 (upper panel) and days 14/21 (lower panel). (**E**) Scoring of regional bone changes in the rabbit tibiae. Data are shown as the mean ± SD for the SA- (n = 6), SA+ (n = 6), and γi SA+ (n = 8) groups. *p<0.05; one-way ANOVA with Bonferroni correction for *post hoc* analysis. Scorings: 0=absent, 1=moderate, 2=severe. (**F**) Osteoclasts detected by tartrate-resistant acid phosphatase (TRAP) staining on sections from untreated contralateral tibiae (control) or tibiae treated with viable or γi bacteria. Data are shown as mean ± SD (n = 6-8), *p<0.05; one-way ANOVA with Bonferroni correction for *post hoc* analysis. Scale bar=1 mm.

during bone healing[30]. Histology showed that γi *S. aureus* induced bone changes typical for osteomyelitis (Figure 2E, Supplementary Figure 1A). As a difference, cortical bone resorption was more prominent in the SA+ group. Treated tibiae showed a higher density of osteoclasts compared to the contralateral tibiae in all three groups (Figure 2F). Together, these data show that the sterile inflammatory response to γi *S. aureus* stimulates bone formation in the tibia over a period of at least three weeks, without extensive osteolysis.

The pro-osteogenic response to killed bacteria is species dependent

In a first step towards pinpointing which bacterial components have an osteo-stimulatory effect in the tibia model, we compared the effect of γi bacterial species that have distinct cell wall compositions based on their Gram classification (Figure 3A)[31, 32]. In addition to γi *S. aureus* (Gram-positive), γi *E. coli* (Gram-negative) was studied as this species is also associated with osteomyelitis[16]. In addition, γi *M. Marinum* was investigated. Mycobacteria are neither considered Gram-positive nor Gram-negative, and have a complex cell envelope with unique antigens for which the bone-modifying effects are unknown[33]. We investigated the γi bacteria without metal implant to reduce the invasiveness of the procedure, as the previous experiment showed that the implant was unlikely involved in the pro-osteogenic response towards γi *S. aureus* (Figure 2C). MicroCT analyses were extended to day 56 to assess the progression of osteolysis and osteogenesis in time.

The animals loaded their treated limb already from the second post-operative week onward, and there was no apparent influence on their bodyweight (Figure 3B). The systemic responses of the animals were mostly characterized by increased leukocyte numbers at day 7, while the erythrocyte sedimentation rate was not elevated compared to animals previously undergoing sham operation with implant alone (Supplementary Table 1)[19]. These results are similar to other reports showing relatively mild systemic effects in rabbits after local delivery of a high dose of killed bacteria[28].

MicroCT at day 28 showed consistent cortical thickening in tibiae of the γi *S. aureus* and γi *E. coli* groups, but this was highly variable in the γi *M. marinum* group (Figure 3C). Bacterial species affected the tibial bone volume differently (Figure 3D). γi *S. aureus* induced an average 5% bone volume increase with a high inter-individual variation. In comparison, six out of seven animals of the γi *E. coli* group demonstrated an increased bone volume of the treated tibia (16% increase on average, P=0.028). The effect of γi *M. marinum* was variable and did not affect the average bone volume. There were no significant bone volume differences between treated and contralateral tibiae at day 56, demonstrating that the bone volume increases had largely regressed (Figure 3E). We measured the microCT gray values to determine possible changes in density of the bone. In order to describe both location and direction of these changes, the gray values along the cortex were plotted. Cortical thickening was associated with a modest decrease in the bone density, even in the most affected samples (Figure 3F, Supplementary Figure 1B). In line with the previous findings, fluorochrome incorporation showed that the pro-osteogenic response was characterized by bone apposition at the periosteal surface, and was most pronounced in the γi *E. coli* group (Supplementary Figure 1C). In addition, enhanced bone porosity and periosteal new bone formation always coincided and were most prominent in the γi *S. aureus* and γi *E. coli* groups (Supplementary Figure 1D).

Bacterial stainings provided no evidence of remaining bacterial components in any of the groups after 56 days. Nevertheless, an ongoing inflammatory response was seen in

two out of seven γi *M. marinum* and three out of seven γi *E. coli* samples, characterized by the presence of numerous giant cells with granuloma formation, fibrosis, and infiltrations of phagocytes and lymphocytes. Immunohistochemistry showed that immune cell infiltrations had a mixed innate (i.e. macrophages/neutrophils) and adaptive (i.e. T lymphocytes) immune cell phenotype (Supplementary Figure 2A, B). The γi *E. coli*–treated bones with active inflammation at the time of explantation (day 56) were associated with the largest bone mass increases. In these samples, endosteal bone apposition and the formation of lamellar bone nodules in the medullary canal was also seen (Supplementary Figure 2C). Only few osteoclasts were observed, localized at the periosteal lining (Supplementary Figure 2D).

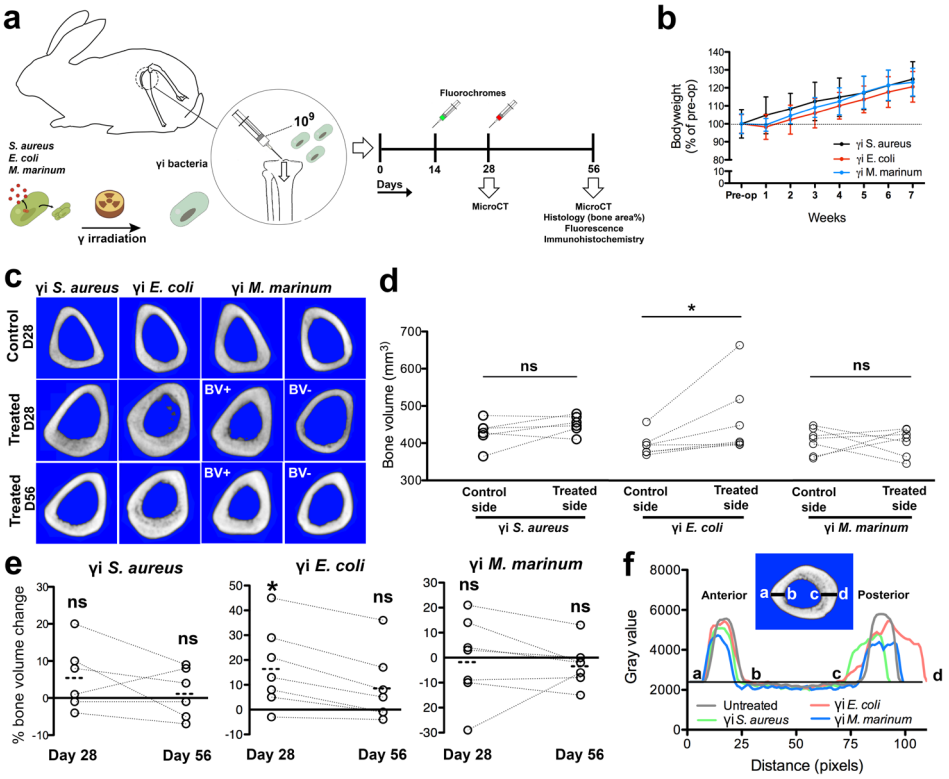


Figure 3. Species-related effects of killed bacteria on the pro-osteogenic response. (**A**) Gamma irradiation-killed (γi) bacteria were injected into the tibial medullary cavity. The primary outcome parameter was the tibial bone volume measured by microCT after 28 and 56 days. Fluorochromes were injected to determine the onset and dynamics of bone formation. (**B**) Bodyweight was measured and normalized to the pre-operative value. (**C**) MicroCT images of the middle section of the tibiae shows changes in cortical thickness in untreated tibiae, or in tibiae inoculated with γi bacteria. Images are shown for the most affected sample in each group. For γi *M. marinum*, tibiae with increased (BV+) or decreased (BV-) bone volume are shown. (**D**) Quantitative micro-CT measurement of the tibial bone volume on day 28 in control limbs or after treatment with γi *S. aureus*, *E. coli* or *M. marinum*. (**E**) Quantitative microCT measurement of the tibial bone volume on day 28 and day 56 after treatment with γi S. aureus, γi *E. coli* or γi *M. marinum*. The fold bone volume increase is shown for each rabbit when comparing treated to untreated tibiae. For **D-E** data are shown as mean ± SD for γi *S. aureus* (n = 6), γi *E. coli* (n = 7), and γi *M. marinum* (n = 7). *p<0.05; paired sample T test with Bonferroni correction for *post hoc* analysis. (**F**) Profile plot showing microCT gray values along a line extending the cortex in anterior to posterior direction. Gray values are shown for a single representative contralateral tibia (untreated), or single tibiae for which the largest changes in bone volume were measured after treatment with γi *S. aureus*, γi *E. coli* or γi *M. marinum*.

These results show clear differences in the bone-stimulatory effect of bacterial species. New bone formation was induced by different bacteria irrespective of Gram-status, therefore it is likely that pro-osteogenic responses can occur for a wide range of bacterial species. The bone mass increase appeared to be greatest in samples with sustained active inflammation based on histology, suggesting that osteo-immunomodulatory effects are related to the duration of the inflammatory response.

Killed bacteria promote synergistic bone formation with minimal dose BMP-2

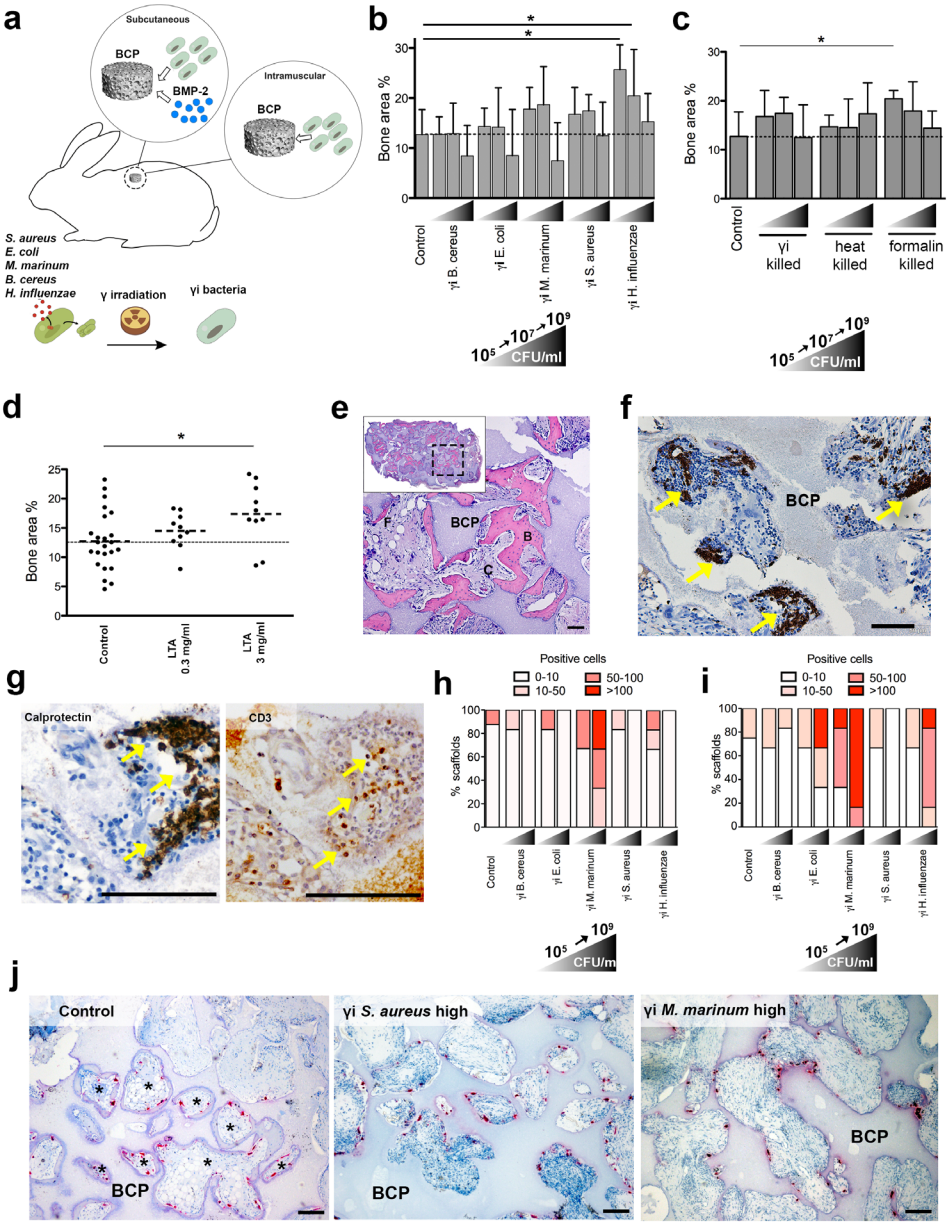
There is evidence that bacterial components, either directly or indirectly, affect the osteogenic differentiation of multipotent mesenchymal stromal cells (MSCs)[10,22]. Therefore, the immuno-modulatory effects of killed bacteria on new bone formation was investigated in ectopic ceramic scaffolds (Figure 4A). In the absence of periosteum or bone, the process of ectopic bone formation relies solely on the migration and differentiation of osteoprogenitors[24]. We found that none of the ceramic scaffolds induced a relevant amount of bone (i.e. >1% bone area%) ectopically, either alone or after incorporation with any of the γi bacteria. As a potential strategy to better target the formation of osteoblasts[23,34], γi bacteria were also co-administered with a suboptimal BMP-2 dose in the ceramic scaffolds. A promotive effect of γi bacteria on BMP-2-mediated new bone formation was observed. In the control group with low dose BMP-2, the average bone volume was 12.7 ± 5 area% (Figure 4B). In experimental groups co-loaded with 10^5-10^7 γi bacteria/mL, there was a trend towards a higher bone volume irrespective of the bacterial species. Only γi *H. influenzae* was found to induce a significant increase in the bone volume. A high load of bacteria (10^9/mL) was associated with a significantly lower ($P<0.05$) bone volume compared to the constructs loaded with a low (10^5/mL) or medium (10^7/mL) γi bacterial load.

It has previously been suggested that the *S. aureus* cell wall possesses pro-osteogenic mediators[19]. We therefore aimed to address how changes in *S. aureus* cell wall characteristics affect its pro-osteogenic properties. Commonly used bacterial inactivation methods differently affect protein degradation or cross-linking, with subsequent changes in immunogenicity of bacterial cell wall components[35]. Since it is unknown if different inactivation methods also lead to changes in the osteogenic response *in vivo*, we investigated if our results with γi *S. aureus* could be reproduced with heat- or formalin-killed *S. aureus*. A trend towards increased bone was seen for all inactivation methods, but only formalin-killed *S. aureus* significantly enhanced the bone volume in the ectopic model (Figure 4C). Furthermore, heat-killed *S. aureus* appeared to induce a reversed concentration-response curve as compared to the other inactivation methods. This is most likely explained by the destructive effects of high temperatures on the bacterial membrane and cell wall proteins, leading to altered immune stimulation[36]. However, larger sample sizes are need to more firmly establish this finding.

The immuno-modulatory properties of killed bacteria are for a large part attributed to the effect of bacterial cell wall components that target toll-like-receptors (TLRs) in various cell types[20]. We therefore explored the role of *S. aureus*-derived TLR ligands in the observed osteogenic response. As an important activator of the TLR2 pathway[21], lipoteichoic acid (LTA) was found to dose-dependently enhance BMP-2-induced bone formation (Figure 4D). This confirmed that not only killed *S. aureus* as a whole, but also selective bacterial cell wall components of *S. aureus* can modulate the osteogenic

response. Furthermore, this finding provides evidence that TLR2, and possibly also other TLR signaling pathways, can be targeted to direct osteogenesis.

Histological evaluation showed that BMP-2 control samples were filled with bone, fibrous connective tissue and fat tissue (Figure 4E). In samples loaded with γi bacteria, there was no evidence for remaining bacterial components, although immune cell infiltrations were apparent particularly in the γi *M. marinum* group (Figure 4F).



The immune cell clusters were characterized by a mixed population of T lymphocytes and calprotectin-expressing phagocytes (Figure 4G). The leukocytes in the BMP-2 constructs loaded with BMP-2 and a low (10^5/mL) or a high (10^9/mL) dose of γi bacteria were characterized, since these groups consistently showed a difference in the amount of new bone formation. The high dose of bacteria generally coincided with a higher number of T lymphocytes, while such a correlation was less evident for the phagocytes. The presence of these immune cell subsets was highest in samples loaded with γi *M. marinum* (Figure 4H, I). These findings are in line with the generally accepted view that bacterial ligands differently affect T lymphocyte activation and differentiation depending on the TLR signaling pathway that is targeted[37]. Osteoclasts were generally only localized at the bone surface, however, in samples loaded with a high number of γi *M. marinum* or γi *S. aureus*, osteoclasts were occasionally lining the scaffold surface in absence of any bone tissue (Figure 4J).

Our results agree with previous reports that indicate that pro-inflammatory stimuli alone cannot induce new bone formation, but enhance bone formation through a synergism with bone-inductive cues such as BMPs[10,34]. Bacterial cell wall ligands including, but not limited to LTA, can enhance the osteogenic response, suggesting an involvement of TLR signaling pathways.

◀ **Figure 4: Effect of killed bacteria on new bone formation in bone scaffolds.** (**A**) Porous biphasic calcium phosphate (BCP) scaffolds were loaded with gamma irradiation-killed (γi) bacteria and studied for bone formation in ectopic locations, with or without a suboptimal dose bone morphogenetic protein-2 (BMP-2). (**B**) Bone volume after 8 weeks in constructs implanted in subcutaneous pockets in rabbits. Samples were loaded with 21.5 µg/mL BMP-2 alone (control) or 21.5 µg/mL BMP-2 in combination with different γi bacteria ranging from 10^5-10^9/mL. (**C**) Bone volume in constructs implanted in subcutaneous pockets in rabbits, loaded with 21.5 µg/mL BMP-2 alone (control) or 21.5 µg/mL BMP-2 in combination with *S. aureus* killed by different methods. (**D**) Bone volume after 8 weeks in constructs implanted in subcutaneous pockets in rabbits, loaded with 21.5 µg/mL BMP-2 alone or BMP-2 in combination with lipoteichoic acid (LTA, 0.3-3.0 mg/mL). For **B-D** data are presented as mean ± SD for the control group (n = 24), LTA groups (n = 10) and γi bacteria groups (n = 7-9). *p<0.05; linear mixed-model with Bonferroni correction for *post hoc* analysis. (**E**) The pores of the BMP-2 control samples were filled with B=bone, C=connective tissue and,F= fat tissue after 8 weeks. (**F**) Calprotectin-expressing phagocytes (arrows) in a representative sample loaded with the highest concentration (10^9/mL) γi M. marinum. (**G**) Representative immunostaining for calprotectin-expressing phagocytes and CD3-positive T lymphocytes on consecutive BCP sections loaded with BMP-2 and the highest concentration (10^9/mL) γi M. marinum. (**H,I**) Scorings of macrophages/neutrophils (**H**, Calprotectin) and T lymphocytes (**I**, CD3) after 8 weeks in BCP constructs loaded with BMP-2 alone (control) or BMP-2 and γi bacteria, implanted in subcutaneous pockets. (**J**) Tartrate-resistant acid phosphatase (TRAP) staining to demonstrate osteoclasts (red) in samples loaded with BMP-2 alone, or BMP-2 in combination with 10^9/mL γi bacteria. The asterisks indicate the areas of bone formation in the control group. Scale bar=100 µm.

DISCUSSION

Immune modulation is being studied as a strategy to enhance the osteogenic response for bone repair[5,38]. In this context, the use of bacterial components as pro-inflammatory mediators is an unexplored field. We hypothesized that bacteria-induced inflammation could activate bone regenerative processes under controlled conditions. Gamma irradiation-killed (γi) bacterial species were locally applied to eliminate the hazards of infection in terms of uncontrolled spread and systemic interference. Despite the 10^4-fold higher number of γi *S. aureus* used compared to viable *S. aureus*, the reaction confined to the bacterial cell wall led to a clear osteogenic response with limited cortical osteolysis. This was an important finding, since active infection is known to strongly induce osteolysis through exotoxins that target host cells or factors that degrade the extracellular matrix[16]. Equally important, the activation of anabolic or catabolic pathways is affected by the relative contribution of systemic inflammation, which was more obvious and prolonged in groups receiving viable *S. aureus*. In contrast to our local approach, systemic delivery of killed *S. aureus* was found to activate the formation of osteoclasts without an osteogenic response, leading to reduced bone volume[27]. Furthermore, rat models have been used previously to study fracture healing under septic conditions. In these studies, intraperitoneal delivery of single bacterial cell wall molecules decreased callus formation, but their local application led to the formation of a hypertrophic callus[39,40]. Despite the bone volume increase induced by local application of γi bacteria, the stimulatory effects of pro-inflammatory signaling on osteoclastogenesis were also apparent[41]. Hence, it should be determined if inflammation-induced osteogenesis benefits from factors that inhibit selective pathways involved in bone erosion, such as the use of bisphosphonates or the timely blockage of osteolysis-inducing RANKL or pro-inflammatory cytokines[15,42].

The pro-osteogenic response induced by bacteria has mostly been reported and quantified for *S. aureus*[17,18,43], which is by far the most studied infectious organism in bone infection models. The few comparative studies suggest a difference in osteogenic response between Gram-positive and Gram-negative bacteria. For example, bacterial colonization with methicillin-resistant *S. aureus* (Gram-positive) increases ectopic bone formation in a rat trauma model, while this is not the case for *Acinetobacter baumannii* (Gram-negative)[18]. Moreover, archetypical cell wall immune stimulants derived from Gram-positive and Gram-negative bacteria have clear opposite effects on ectopic new bone formation[10]. In the tibia model however, we observed a similar pro-osteogenic response for γi *S. aureus* and γi *E. coli*. A same trend was also found for γi bacterial species with different Gram classifications in the ectopic location, although the effect sizes seemed to be species-dependent. The finding that Gram-negative bacteria stimulated bone formation in both orthotopic (i.e. *E. coli*) and ectopic (i.e. *H. influenzae*) locations was unexpected, since their major cell wall constituent lipopolysaccharide (LPS) is detrimental for new bone formation[10]. Hence, mutant bacterial strains[44] could be used to investigate whether LPS deficiency in Gram-negative bacteria further increases their osteo-stimulatory effect.

We further explored the conditions needed for inflammation-induced osteogenesis and found that immune modulation by killed bacteria holds regenerative properties outside the native bone environment. This was a significant finding considering the presumed importance of the periosteum in inflammation-induced bone formation[30]. Although killed bacteria alone never induced new bone formation in the ectopic location,

their presence significantly potentiated scaffolds with suboptimal osteoinductive capacity. This agrees with previous findings that, under certain circumstances, pro-inflammatory signals act in synergy with BMP-2 in bone formation[10]. This is at least partially mediated by an enhanced osteogenic differentiation in MSCs[23]. The interaction between pro-inflammatory stimuli and bone-inductive growth factors is important to further investigate, since this could lead to a higher efficacy in their clinical use[45]. Although BMP-2 is the most potent bone-inductive agent known, the current use of a supraphysiological dose BMP-2 is associated with high costs and occasionally severe complications[46]. Others have reported that the effective BMP-2 dose can be lowered when secondary osteogenic stimuli are applied[47,48]. Here, we provide evidence that co-delivery of selected killed bacteria also enhances the response to BMP-2. The coinciding changes in local inflammation elicited by different bacterial dosages and/or species, infers that there is a cross-talk between inflammatory and BMP signaling pathways in osteogenic (precursor) cells, similar to that observed in early bone healing[7]. With the co-delivery approach, the upper limit in bone that can form within the scaffolds (i.e. 20-30 bone area%) was reached. To determine the actual potential of the co-delivery method, BMP-2 concentration-response curves could be established using carrier materials that allow larger effect sizes to be measured[49]. Alternatively, it is of interest to investigate whether this approach can cause a desired shift in the BMP-2 dose-response curve and thus lower the effective BMP-2 concentration for bone healing in a clinically-relevant bone environment.

We furthermore identified that the pro-osteogenic effects of killed bacteria were dose-dependent. The finding that the highest bacterial dosage was associated with reduced bone formation indicates that only mild inflammatory conditions are favorable for new bone formation. In agreement, fracture healing studies show that a timely resolution of the inflammatory response usually leads to the most optimal healing[7,12]. After bone injury, acute inflammation normally only persists for several days, after which anti-inflammatory cytokines and molecules are upregulated[12]. When inflammation persists, overexpression of pro-inflammatory cytokines can have a negative impact on the osteogenic and angiogenic response[12,38]. In the orthotopic tibia model, bone induction was largest in samples with active inflammation at the time of explantation. However, bone formation occurred further away from the inflammatory burden in the subperiosteal bone, even up to the distal tibia, where there may be a lower concentration of proinflammatory mediators. In the ectopic model, the highest concentration of killed bacteria induced the highest T cell numbers, indicating exaggerated inflammation[10]. Depending on the context, T cells can have dual effects on osteogenesis. T cell cytokines like IL-17 are pro-osteogenic under acute conditions[34,50], but cytokines such as IFN-γ and TNF-α induce cell apoptosis and impair osteoblast differentiation during prolonged inflammation[11,51]. As a limitation of this study, the immune response at an earlier time point was not determined. Therefore, a characterization of early immune cell activity playing a possible role in bone formation could not be done. It is of particular interest to evaluate the role of different macrophage subsets in the first days after implantation, since osteogenesis and angiogenesis within bone tissue engineered constructs relies heavily on the activity of these cells[5,9,52]. Moreover, it has been shown that activation of macrophages by bacterial ligands leads to secretion of cytokines stimulating bone formation[53].

In order to translate these findings to a clinical setting, the bacterial components with largest bone-stimulatory effects and minimal side effects should be identified. Immuno-

stimulation by intact γi bacteria is largely mediated by bacterial cell wall components, although intracellular components such as bacterial DNA can also activate the immune system following bacterial internalization[33]. It was previously found that *S. aureus* fragments extracted by lysostaphin digestion induced new bone formation when applied in the rabbit tibia[19]. Since lysostaphin digestion intends to extract cell wall fragments while keeping the bacterial protoplast intact, contamination by intracellular proteins or DNA is minimized. Therefore, it is likely that some pro-osteogenic mediators are localized in the bacterial cell wall. In line with this finding, differences were found in *S. aureus* osteogenicity for different killing methods, which is likely explained by the relative sensitivity of specific cell wall components to the different inactivation methods[36].

Upon infection, the host recognizes and responds to the various bacterial components for a large part through TLRs[20]. The pro-osteogenic effect found for the TLR agonist LTA provides evidence that this class of receptors can be targeted for downstream bone-stimulatory responses[37]. This is supported by the finding that TLR ligands can activate pathways involved in osteogenesis in MSCs[23,54]. Alternatively, activation of TLRs can produce pro-inflammatory cytokines which affect MSC differentiation[53]. Moreover, TLR signaling induced by endogenous danger signals plays a role in tissue regeneration after injury[55]. In terms of versatility, specific TLRs can be targeted to establish the desired immune cell recruitment and cytokine responses[37]. In terms of safety, the use of TLR ligands as immune modulators also offers the advantage that small synthetic TLR ligands with increased safety profile have already been developed for clinical use[37]. It was demonstrated that the pro-osteogenic effects of killed bacteria are not dependent on Gram-status. Therefore, it is of particular interest to elucidate how TLR ligands common to both Gram-positive and Gram-negative species, such as bacterial peptidoglycan, affect osteogenesis[27,40].

In conclusion, the data presented here show that bacteria-induced inflammatory signals stimulate bone formation through a synergy with bone-inductive BMP-2. The optimal bacterial components in terms of safety and efficacy should be identified next, together with their downstream targets. The current work demonstrates that bacterial antigens hold promise for bone regeneration purposes.

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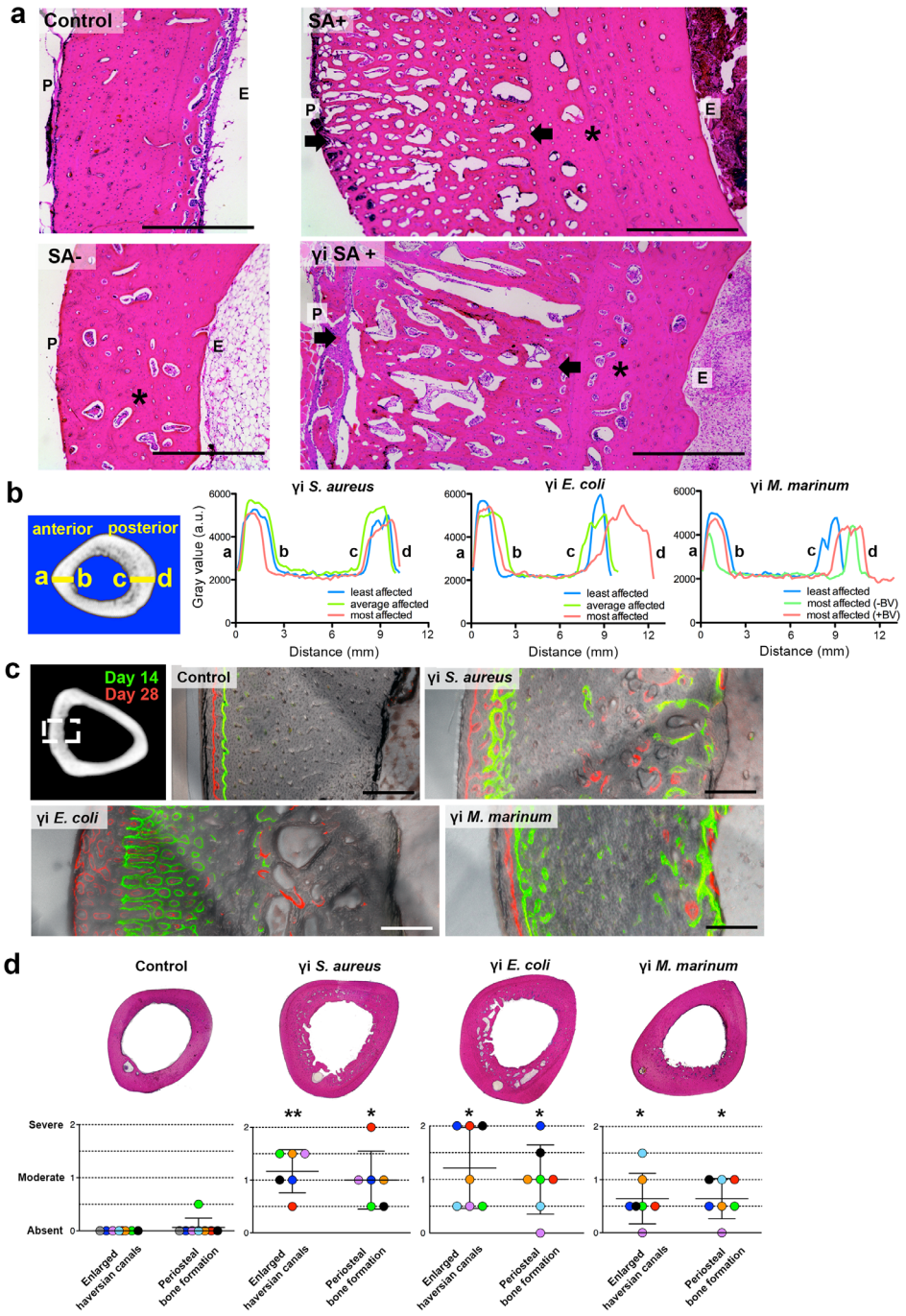
SUPPLEMENTARY DATA

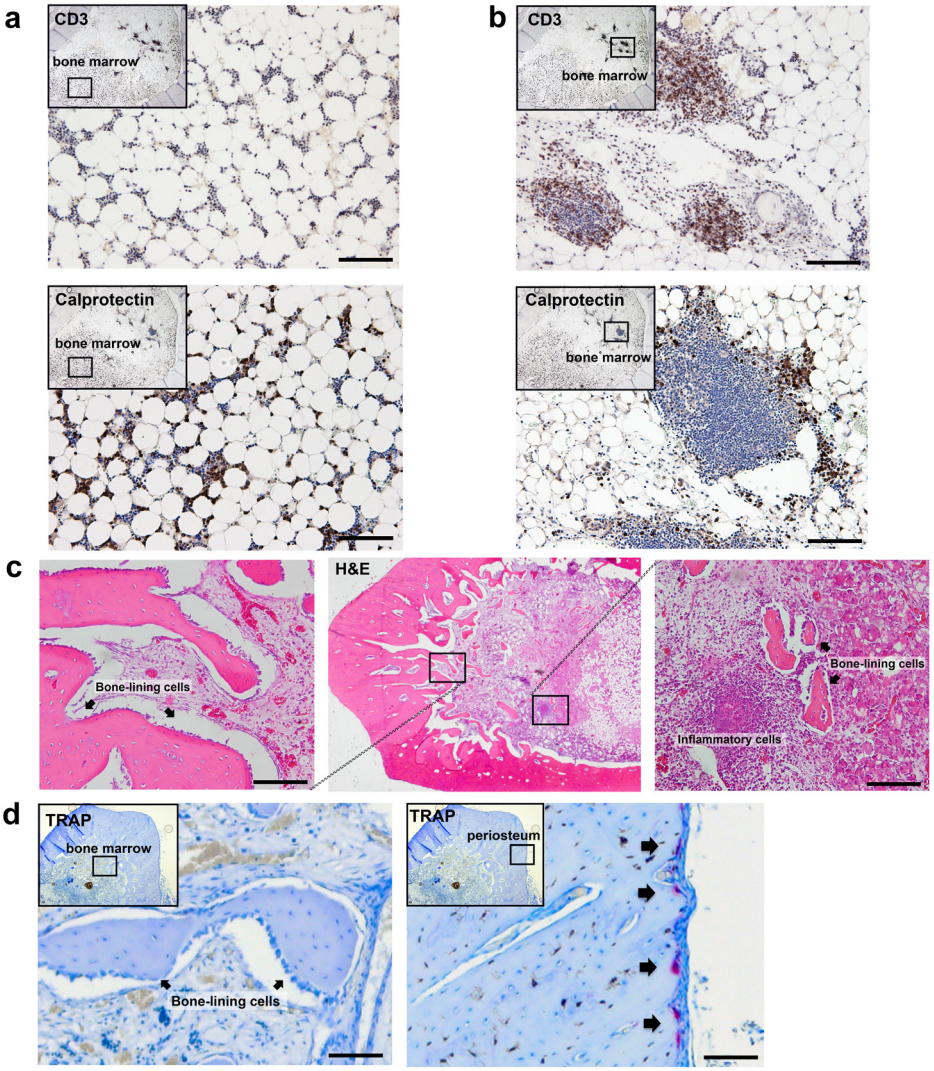


Supplementary Table 1. Non-specific markers of inflammation measured at day 7

	γi *S. aureus*	**γi *E. coli***	**γi *M. marinum***
ESR (mm/h)	1.7 ± 0.8	1.4 ± 0.5	2.2 ± 0.7
Leukocytes (x10^9/l)	8.2 ± 2.3	13.0 ± 3.7	10.0 ± 2.2
Monocytes (x10^9/l)	0.6 ± 0.2	1.1 ± 0.6	1.0 ± 0.4
Neutrophils (x10^9/l)	1.9 ± 0.6	4.4 ± 1.7	2.9 ± 1.3

ESR= erythrocyte sedimentation rate, γi= gamma irradiation-killed. Data are presented as the mean ± SD for γi *S. aureus* (n=6), γi *E. coli* (n=7),and γi *M. marinum* (n=7).

◀ **Supplementary Figure 1.** (**A**) H&E staining of an untreated contralateral tibia and the most affected tibiae at day 28 after treatment with viable *S. aureus* without (SA-) or with (SA+) implant, or gamma irradiation-killed *S. aureus* with implant (γi SA+). The regions between the arrows indicate newly formed bone. The asterisks indicate osteolysis. P=periosteum, E=endosteum. (**B**) Profile plot showing the cortical gray values after 28 days. Samples are shown with the least, average, or largest change in bone volume after treatment with γi *S. aureus*, γi *E. coli*, or γi *M. marinum* (+BV: sample with increased bone volume, -BV: sample with decreased bone volume). (**C**) Overlay images of brightfield and fluorescence channels demonstrating the incorporation of calcein (green, day 14) and xylenol orange (red, day 28) in tibia sections. The images are representative for the untreated contralateral tibiae (control), or tibiae treated with γi bacteria. (**D**) Bone changes were scored on MMA sections by two blinded observers. The dots represent the average score for a single tibia. In the different groups, each rabbit is represented by a color. Data are presented as the mean ± SD for γi *S. aureus* (n = 6), γi *E. coli* (n = 7), and γi *M. marinum* (n = 7). *p<0.05/**p<0.005 compared to control group, one-way ANOVA with Bonferroni correction for *post hoc* analysis. Scale bar=750 μm.



Supplementary figure 2. (**A, B**) Stainings for T lymphocytes (CD3) and activated macrophages/neutrophils (Calprotectin) after 56 days on tibia sections treated with gamma irradiated-killed (γi) *E. coli* (**A**) or γi *M. marinum* (**B**). (**C**) H&E staining of a tibia section 56 days after treatment with γi *E. coli*. (**D**) Staining for tartrate-resistant acid phosphatase (TRAP) activity to demonstrate the presence of osteoclasts. A representative section is shown from a tibia 56 days after treatment with γi *E. coli*. Scale bar=100 µm.

PART III

General discussion & Summary in Dutch

Chapter 8

General discussion

This thesis showed that modulation of the local inflammatory response can affect new bone formation, which is at least partly explained by the finding that certain pro-inflammatory cytokines direct MSC osteogenesis. *Chapter 2* demonstrated that the pro-inflammatory cytokine TNF-α, and the TLR ligand LPS, are both direct stimulators of MSC osteogenic differentiation. The responsiveness of MSCs to these stimuli is profoundly increased once they have committed to the osteogenic lineage. *Chapter 3* introduced an improved *in vivo* screening model for osteoimmunology research. The advantage of this model as compared to standard ectopic implantations, owes itself to the confined biological environments that are created in the form of biomembrane pockets. Using this model, it was found that the pro-inflammatory cytokine TNF-α, and the TLR ligands LPS and LTA, differently affect BMP-2-mediated bone formation. TNF-α demonstrated a high potential to enhance new bone formation. *Chapter 4* showed that T cells have a pro-osteogenic effect on MSCs in coculture assays. Analysis of the T cell subsets and their secreted factors, indicated that the IL-17 family of cytokines were an important osteo-stimulatory component. *Chapter 5* provided evidence that IL-17 enhances early and late osteogenic responses in synergy with BMP-2.

This thesis also reported the finding that bacteria, or specific components they hold, can induce immune responses that favor osteogenesis. *Chapter 6* used a rabbit tibia infection model to show that bacterial infection can lead to a net increase in local bone mass despite enhanced bone resorption. The results indicated that the prominent osteoblastic response was a result of local pro-inflammatory cues. *Chapter 7* established that various species of killed bacteria can either positively or negatively affect the osteogenic response in orthotopic and ectopic models. The direction of the response is determined by the nature of the inflammatory response, namely the activity of the specific bacterial antigen and severity and duration of inflammation.

The contribution of pro-inflammatory cytokines to osteogenesis

In the first part of this thesis, we studied possible mechanisms by which immune cells interact with skeletal cells in the osteogenic response. The production of various pro-inflammatory cytokines is locally upregulated during the acute inflammatory phase of bone fracture healing, and is thought to modulate angiogenesis and osteogenesis[1]. Considering the critical role of MSCs in bone induction[2], our aim was to determine whether pro-inflammatory cytokines directly target the osteogenic differentiation of MSCs, and if so, which circumstances enhance this process. In the literature, both stimulatory and inhibitory effects of pro-inflammatory cytokines on MSC osteogenic differentiation have been reported[3]. The data contradict each other, depending on the use of rodent or human MSCs[4, 5]. Furthermore, most experiments have been performed with the corticosteroid dexamethasone as an osteogenic supplement, which is known to suppress the action of pro-inflammatory mediators[6].

In *chapters 2 and 4*, we performed experiments with human bone marrow-derived MSCs. Pro-inflammatory cytokines were investigated in conjunction with BMP-2 as a physiological osteoinductive stimulus, alongside of dexamethasone. TNF-α and IL-17 were found to strongly enhance MSCs osteogenic differentiation in cells co-exposed to an osteoinductive stimulus. This suggests that there is some interplay between pro-inflammatory signals and regulators of osteogenesis. This line of reasoning is further strengthened by existing literature showing that pro-inflammatory cytokines alone do

not increase bone marker expression at the protein level[4, 7, 8], and that pro-inflammatory cytokines usually have more pronounced effects on pre-differentiated cells than fully naïve MSCs[9].

The pathways by which pro-inflammatory cytokines influence MSC osteogenic differentiation are largely unknown. NF-κB signaling is considered to be involved, since it regulates MSC osteogenesis and is activated by different classes of pro-inflammatory mediators[4, 10]. As another possible mechanism, pro-inflammatory cytokines might affect paracrine functions of MSCs, with subsequent changes in osteogenic differentiation. In agreement with our data, TNF-α or IL-17 induce the expression of BMP-2 in several cell types[11, 12]. The interactions are reciprocal, as in like manner, BMP signaling affects pro-inflammatory cytokine secretion in different cells including MSCs[13, 14].

In *chapters 3 and 5*, we investigated whether pro-inflammatory cytokines and BMP-2 also synergistically promote new bone formation in immunocompetent animals, taking into account the entire spectrum of interactions between immune cells and bone cells. The single delivery of TNF-α and IL-17 doubled the amount of newly formed bone in ectopic implants with BMP-2. Furthermore, their pro-osteogenic effects occurred at physiological concentrations, i.e. similar to that measured during inflammatory conditions (ng/ml range)[15, 16]. The co-delivery of a pro-inflammatory cytokine with BMP-2 therefore seems to resemble the early events in bone healing, when both signaling pathways are crucial[17, 18].

It is well established that macrophages, as a main producer of TNF-α, orchestrate regenerative processes in early bone healing[19, 20]. T cells are also major producers of pro-inflammatory cytokines, but in contrast to macrophages, their role in the acute inflammatory response has been relatively unexplored[8, 21]. In *chapter 4*, we described that T_H17 cells are highly stimulatory for osteogenesis. Likewise, their main cytokine IL-17 was shown to be an enhancer of BMP-2 osteoinductive activity both *in vitro* and *in vivo*. In addition to its stimulatory effect on MSC osteogenic differentiation, we found that co-delivery of IL-17 and BMP-2 could also promote other important processes, such as induction of connective tissue formation and osteoclastogenesis. In line with these findings, others recently reported that IL-17-producing T cells are recruited to sites of bone damage and contribute to the healing process[8]. Together, this identifies a novel role for T cells and IL-17 in bone regeneration[17, 22]. In *chapter 5*, only IL-17 (also called IL-17A) was studied as the prototypic member of the IL-17 family, which is composed of different cytokines[23]. Other IL-17 family members may also promote osteogenesis, like we already confirmed for IL-17F *in vitro*. Moreover, IL-17A and IL-17F naturally exist as a heterodimer, which could lead to even more effective targeting of IL-17 signaling in various cells[23]. When considering the application of IL-17 in bone regeneration strategies, it is important to take into account the broad range of functions of IL-17. For example, in wound healing, IL-17 contributes to tissue regeneration through the recruitment of other inflammatory cells and the modulation of angiogenesis[24].

Due to these findings, it can be hypothesized that a selective combination of pro-inflammatory cytokines can further optimize the osteogenic response. During inflammatory processes *in vivo*, pro-inflammatory cytokines including TNF-α and IL-17 are often co-expressed, and have synergistic effects on many cell types[25]. Similarly, the delivery of a single pro-inflammatory cytokine was never osteoinductive in our MSC cultures, while in *chapter 4*, the cocktail of various cytokines produced by activated T cells alone was capable

of inducing early osteogenic differentiation. In agreement, others have shown that a combination of pro-inflammatory cytokines (TNF-α, TGF-β, IFN-γ, and IL-17) can promote matrix mineralization in MSCs, even without osteogenic supplement[26]. Apart from TNF-α and IL-17, cytokines from the IL-6 family have pro-osteogenic effects on MSCs and are worthwhile to investigate in such future strategies[27]. Taken together, TNF-α and IL-17 can be pinpointed as osteo-stimulatory cytokines, but require further translation in more clinically relevant animal models.

Bacterial ligands for osteo-immunomodulation

It is clinically observed that bacterial infections can be associated with pathological new bone formation, which most frequently manifests itself during osteomyelitis in the long bones[28]. In more rare cases, infections are also thought to be involved in trauma-induced heterotopic ossifications or cochlear ossifications[29, 30]. In truth, the causal relationship between infection and new bone formation is speculative, as active bone infections are also known to induce osteolysis. In like manner, it is unknown if only viable pathogens, or also selective components they possess, can trigger new bone formation. Finally, it is uncertain if pathogens directly target the activity of bone cells, or whether indirect (immune-mediated) routes predominate. In part two of this thesis, we addressed these questions, as our aim was to determine whether bacteria-induced inflammatory processes can be harnessed for their bone-inductive effects.

We provided evidence that bacterial ligands can be used to modulate the osteogenic response. In *chapters 6 and 7*, we studied the phenomenon of new bone formation in the context of implant-associated infection in the rabbit tibia. Despite its uncontrolled nature, this model was found to recapitulate the reactive bone formation typically seen in osteomyelitis patients[28, 31]. The invasiveness of the approach was reduced by avoiding the use of the implant and by applying killed bacteria. In particular, it was thought that the use of killed bacteria would limit the activity of secreted virulence factors and enzymes that mostly induce pathological bone remodeling and osteolysis[32, 33, 34]. In agreement, the bone resorption was obviously reduced, thereby improving the overall osteogenic response. The finding that a crude *S. aureus* cell wall extract also is bone-inductive, suggests that at least some of the pro-osteogenic mediators are present in the bacterial cell wall.

The mechanisms by which bacterial ligands affect bone formation remain largely elusive. In *chapter 7,* we found that killed bacteria potentiate the osteoinductive effect of BMP-2, hinting towards the possibility that bacterial components affect MSC recruitment and/or differentiation. The host immune response is activated by pathogen-specific molecules, which act for a large part through TLRs. In fact, MSCs also express several TLRs, and TLR signaling could even activate similar osteogenic and immunomodulatory pathways in MSCs as pro-inflammatory cytokines do[35, 36]. In agreement, in *chapter 2* we observed that the archetypical TLR ligand LPS could directly target MSCs to promote osteogenesis. Despite the clear effects of LPS and other TLR ligands on isolated MSC cultures[37], *chapters 2 and 4* collectively showed that their *in vivo* effects on bone formation are much more complex. This is most likely explained by the abundant TLR expression in innate immune cells such as dendritic cells and macrophages[38]. Moreover, TLR ligands are known to induce their expression of a multitude of chemokines and cytokines[39]. Hence, the direct effects of TLR agonists on MSC osteogenesis are possibly overshadowed by the various activated

immune cells *in vivo*[40]. Indeed, we found that LPS and LTA influenced new bone formation in an opposite direction, and that the outcome was linked to their specific immune activation. As such, the exaggerated immune activation and pro-inflammatory cytokine signaling induced by LPS was obviously detrimental for bone induction, as reported earlier by others[41]. On the contrary, LTA induces lower levels of pro-inflammatory cytokines, which could be more favorable for MSC osteogenesis[42, 43]. Taken together, bacterial ligands can direct MSC osteogenesis *in vitro*, but their effects *in vivo* are for a large part determined by the activation of pro-inflammatory signaling pathways in various other cell types.

In *chapter 7*, various species of killed bacteria were compared in an attempt to identify their pro-osteogenic components. Enhanced new bone formation was apparent for a number of bacterial species, making it hard to pinpoint the single most effective species for future translational work. Likewise, a variety of bacterial components, either or not localized in the bacterial cell wall, could have unknown modulatory effects on bone formation. It should also be noted that the selection of bacterial species with osteogenic potential is complicated by the observation that many variables can affect immunity of a given bacterium. For example, the specific *in vitro* growth conditions already influence the expression of virulence factors by the same pathogen[44], which could then affect their pro-osteogenic activity. In addition, heterogeneity is found in isolated bacterial components derived from different bacterial species and substrains[45, 46].

To overcome these challenges, a systematic approach is needed to identify pro-osteogenic bacterial ligands. Instead of comparing a multitude of bacterial species, a comparison of the osteogenic response induced by a specific bacterial species in wild type and specific knockout mice can be a way of narrowing down the involvement of selective bacterial components[47, 48]. To illustrate, a reduction in osteogenic response to *S. aureus* in TLR-2 knockout mice compared to wild type mice implicates that TLR-2 ligands (e.g. LTA, peptidoglycan) are responsible for its pro-osteogenic effect. The role of single bacterial components can also be studied by comparing mutant bacteria lacking one or more virulence factors with their wild type counterpart. For example, mutant *E. coli* strains have been identified with strongly reduced immunogenic activity of their modified LPS[49, 50]. This provides a means of investigating whether LPS deficiency in Gram-negative bacteria like *E. coli* or *H. influenzae* can further increase their osteo-stimulatory effect, while also reducing possible side effects.

Pro-inflammatory stimuli as enhancer of osteoinductive proteins

Based on earlier *in vitro* results, one of the important aims was to establish a possible synergy between pro-inflammatory signals and BMP-2 *in vivo*. In order to do this, pro-inflammatory stimuli were studied with a suboptimal dose of BMP-2 in the BCP implants. Numerous studies show that the response to BMP-2 is increased when appropriate secondary stimuli are applied. These include other growth factors that regulate osteogenesis, chondrogenesis and/or angiogenesis[51], or osteogenic cells that produce secondary cues via a paracrine mechanism[52]. Throughout this thesis, we provide evidence that pro-inflammatory stimuli are also enhancers of BMP-2 osteoinductive activity. In an initial dose-response study (unpublished), a suboptimal BMP-2 concentration was first identified, allowing us to discriminate the effects of the mediators of interest. Using this methodology, the combined delivery of different pro-inflammatory stimuli with this BMP-2 dose lead to approximately twofold greater bone formation compared to BMP-2 alone.

It remains a matter of interest to determine if larger effect sizes can be obtained in other models, since the bone volume in optimal conditions seemed to reach the upper threshold of bone that can form in the specific ectopic model we used. This upper threshold was presumed to be around 30 % of total available volume in the BCP scaffolds based on own experiences with cell-based conditions or higher BMP-2 concentrations.

BMP-2 protein released from a collagen sponge is clinically used as a bone substitute in certain spine, trauma or maxillofacial procedures. However, high doses of this protein (1-2 mg/ml) are applied, leading to increased costs and potentially serious complications[53]. Therefore, it is of great relevance to determine if the co-delivery of selective pro-inflammatory stimuli will indeed result in a reduction of the effective BMP-2 dose. The orthotopic environment should be considered in these future studies, since different osteoinductive cues are present in the natural bone environment that could potentiate the effect of pro-inflammatory stimuli with even less or no exogenous BMP-2[54, 55]. In fact, we showed in *chapter 7* that killed bacteria alone stimulate new bone formation in the rabbit tibia, but not in the ectopic model without BMP-2. In the same line of reasoning, pro-inflammatory stimuli can possibly enhance naturally-available osteoinductive products including autologous growth factors (e.g. bone marrow aspirate, platelet gel) or allogeneic grafts such as demineralized bone matrix[56].

The synergistic effect of pro-inflammatory mediators and BMP-2 on new bone formation seems to mimic their natural crosstalk in the fracture hematoma, where cytokines such as TNF-α, IL-1β, IL-6 and Oncostatin M are co-expressed with BMP-2[8, 17, 57, 58]. It can be hypothesized that the actions of osteoinductive BMPs other than BMP-2 are also regulated by pro-inflammatory factors, since a second peak in pro-inflammatory cytokine expression coincides notably with the restricted expression pattern found for BMP-4 and BMP-7[22, 59]. In a pathological context, inflammatory and osteoinductive stimuli also interact in the progression of HO[60, 61]. This is most striking in the rare disease FOP, where pro-inflammatory cytokines aggravate the bone induction process by BMPs[62].

Inflammation as double-edged sword

Fracture healing studies show that a tightly regulated inflammatory reaction leads to optimal bone healing[63], whereas local hyperinflammation or systemic inflammation (e.g. polytrauma, diabetes mellitus, rheumatoid arthritis) are risk factors for delayed healing[18, 64]. Based on these observations, it is widely presumed that short-lived or prolonged duration of the inflammatory response may differently influence bone regeneration[63, 65]. One of our goals was to determine whether the contradicting effects of pro-inflammatory stimuli on new bone formation could be related to the severity and/or duration of the inflammatory response they elicited.

We found that the effect of pro-inflammatory stimuli on the osteogenic response depends on the applied dose. For TNF-α and IL-17, an optimal concentration could be identified. A more apparent dual effect was seen for killed bacteria, where bone formation was enhanced at lower concentrations, but generally reduced beyond the maximal effective concentration. We questioned whether dose-dependent effects of the mediators could be explained by the temporal context of the inflammatory response they induced. Immunohistochemical stainings showed no/mild active inflammation at day 10 in conditions that were stimulatory for bone formation, i.e. constructs loaded with TNF-α, IL-17, LTA, or low dose of killed bacteria. In contrary, TLR signaling mediated by LPS or

high dose of killed bacteria resulted in numerous activated phagocytes at day 10 and continued presence of lymphocytes at 8 or 12 weeks. A reduced bone formation under conditions of excessive inflammation was not entirely unexpected, as prolonged pro-inflammatory cytokine signaling is known to impair bone formation through several mechanisms, including decreased extracellular matrix production, delayed angiogenesis and/or uncontrolled cell death[63, 65, 66, 67].

These data indicate that the severity of immune activation, together with its timing, should be controlled for optimal new bone formation. In fact, it has even been suggested that the exogenous presentation of anti-inflammatory cytokines is needed to direct appropriate inflammatory responses[63, 68]. Pro-inflammatory cytokine signaling is normally tempered by the expression of anti-inflammatory cytokines (e.g. TGF-β, IL-10, IL-4 and IL-13) from specific macrophage and lymphocyte subsets[63, 69]. MSCs that infiltrate the damaged tissue can further suppress pro-inflammatory lymphocytes[70]. Finally, several biochemical factors expressed in damaged tissue (e.g. lipoxins, resolvins, and protectins) also have inhibitory effects on inflammation[65]. Hence, it is imaginable that this normal feedback system fails when the pro-inflammatory stimulus is not timely cleared from the local site, or when it induces a prolonged cytokine cascade.

Together with the applied dose and delivery method, the duration of the inflammatory response will be largely determined by the nature of the pro-inflammatory stimulus. In this respect, ligands of TLRs or other PAMPs could induce complex and long-lasting effects on immune activation. To illustrate this, TLRs are thought to link the innate and adaptive immune response through several mechanisms. These include their effect on specific cytokine production, activation of antigen presentation, and expression of costimulatory molecules needed for activation of adaptive immunity[71, 72]. Consequently, bacterial components that target different TLRs induce divergent immune responses with respect to cytokine and immune cell involvement, and duration of the response, which could also differently influence the osteogenic response. For example, TLR4 ligation (e.g. LPS) induces strong pro-inflammatory cytokine profiles from monocytes, dendritic cells, and T_H1 cells, which can lead to persisting inflammation in the form of a cytokine storm[42, 43]. TLR2 ligation (e.g. LTA) on the other hand, induces the release of T_H2 cytokines which are also important for resolution of inflammation[72]. In comparison to TLR ligands, the bolus delivery of cytokines like TNF-α or IL-17 will most probably induce a short-lived response, as they rely on a synergy with multiple cytokines over a prolonged period to aggravate inflammation[73, 74, 75]. In agreement, we did not observe any inhibitory effects of these cytokines on new bone formation within the tested concentration range. This agrees with the presumed higher safety profile that pro-inflammatory cytokines generally offer as compared to bacterial ligands. On the other hand, the short-lived action of cytokines *in vivo* could be a drawback when prolonged signaling is desired in a clinically relevant setting. For instance, the stress and tissue damage associated with orthopedic surgeries leads to changes in leukocyte trafficking and activation, together with local and systemic increases in chemokines and pro-inflammatory cytokines[76, 77]. Importantly, these changes can be sustained for at least 24 hours post-operatively[77]. In this setting, prolonged delivery of pro-inflammatory cytokines may be desired to overcome such masking-effects of surgery, especially considering their short half-lives[78, 79].

Models to study osteo-immunomodulatory strategies

In this thesis, new models were designed and tested to study the interaction between bone cells and immune cells. Likewise, new suggestions can be made for future improvements in in vitro and *in vivo* models for osteo-immunological studies. In the first part of this thesis, a straightforward approach was used that studied the differentiation of MSCs under pro-inflammatory conditions *in vitro*. Accordingly, direct effects of pro-inflammatory cues on bone cells could be easily identified. As a drawback, this model neglects the full spectrum of interactions between immune cells and bone cells that occur *in vivo*. The importance of this interaction was clearly demonstrated in this thesis. For example, bacterial LPS could directly target TLRs in MSCs to promote their osteogenic differentiation, while a strong inhibitory effect of LPS on new bone formation was found *in vivo*. In the complex *in vivo* environment, immune-mediated cytokine cascades likely influence the osteogenic differentiation pathway. Therefore, in addition to studying the effects of pro-inflammatory stimuli on MSC osteogenesis in monocultures, complementary in vitro models are needed. MSC-monocyte/macrophage cocultures are currently a frequently used model to study osteo-immunomodulatory strategies[80, 81], but do not recapitulate the tight link between the innate and adaptive immune response[81]. Alternatively, it can be postulated that a coculture model existing of MSCs and PBMCs better captures the important interactions between different immune cell types responsive to pro-inflammatory stimuli, such as monocytes and T cells[39]. A suitable bone substitute material intended as *in vivo* carrier can even be incorporated in the system of evaluation, taking into account the importance of the biomaterial-induced immune response in their performance[82, 83].

The feasibility of immunomodulatory strategies was next investigated in different *in vivo* models. An ideal *in vivo* model would allow the evaluation of multiple conditions in an orthotopic location. The spinal cage model is one of the few models that meets these requirements[84], but was deemed inappropriate to study pro-inflammatory agents due to likelihood of crossover effects and minimal soft tissue involvement. As an alternative, we followed two parallel approaches, i.e. a bottom-up and top-down method, to respectively 1) evaluate multiple pro-inflammatory stimuli in the ectopic location and 2) study the process of inflammation-induced osteogenesis in an orthotopic location. The rabbit model was preferred over a rodent model, as there is a larger difference between rodent and human immunology which can mask the effect of bacterial ligands[85].

To compare mediators and test dose-dependency in parallel, we performed numerous ectopic implants in the same animals. Using this methodology, there is a risk of systemic interference and local crossover effects between implants. Although, as far as we can judge, the actual impact of crossover effects on bone development has never been studied, researchers in this field usually try to minimize these risks by adjusting the number of implants and/or their implantation site[86, 87]. As a first step towards an improved ectopic model, we hypothesized that induced biological pockets would reduce the contamination of neighboring samples, hereby justifying the implantation of samples with different (pro-inflammatory) mediators in vicinity of each other. In *chapter 3*, we showed that standardized biological pockets could be created in response to PMMA bone cement discs. These biomembrane pockets can subsequently be used to study bone scaffolds in conjunction with a confined inflammatory response, either or not induced by repeated transcutaneous injections of the mediator. The benefits of this model would include a

better use of animals, both from an ethical and practical point of view, which outweighs the drawbacks of the biomembrane model (i.e. the extra time needed to develop the membrane and the requirement of a second surgery). At this moment, it is not fully clear whether the biomembrane pockets indeed minimize local crossover effects between adjacent implants. Equally important, it is uncertain whether the biomembrane pockets can facilitate the characterization of early immune reactions in terms of cellular and molecular content, which is a major advantage of the comparable intradermal air pouch model[88].

The tibia model used in *chapters 6 and 7*, involving intramedullary delivery of bacterial ligands, clearly demonstrated the principle of orthotopic inflammation-induced bone formation either or not in the context of active bacterial infection. Future work should elaborate on key findings in this model, such as the importance of osteoclast-osteoblast coupling in the osteogenic response[89, 90]. In addition, the role of periosteal cells in this model requires more investigation. Since new bone formation in the rabbit tibia was predominantly localized in the subperiosteal region, it can be hypothesized that the inflammatory response especially targets osteoblast differentiation in periosteum-specific cells. The periosteum contributes to bone fracture healing or pathological bone formation[91], and furthermore, periosteum-derived cells have demonstrated pro-osteogenic and pro-angiogenic features[92]. The influence of immunomodulatory strategies on the osteogenic capacity of these cells could provide a better understanding of the means by which inflammatory cues affect differentiation pathways in inducible cells.

For bone-healing related questions, a model based on intramedullary inoculation of pro-inflammatory agents is redundant. To study the effect of immunomodulatory components in synthetic bone grafts, functional models such as spinal fusion or segmental defect models are needed in which osteoconductive and osteoinductive properties of the graft both contribute to bone healing[55, 93]. However, an inherent drawback of these models is their inefficiency in terms of the number parameters that can be evaluated per animal. Hence, non-weightbearing models like the iliac wing model could be a practical model to study multiple immunomodulatory strategies in conjunction with synthetic bone grafts in the same animal[94].

It must be stressed that the choice of the animal model impacts the effectiveness of osteo-immunomodulatory strategies. While generally pathogen-free housing conditions are used in small animal research, it neglects the important role of natural variation in the individual's unique immune build-up in bone regeneration[95]. This suggests that the standardized *in vivo* conditions can mask the variation in the response, which should be taken into the account in the design of large animal studies.

Considerations in future translation

Local immunomodulation turned out to be a feasible strategy to promote new bone formation in the rabbit model. While this may be true, we only studied a select number of pro-inflammatory mediators, whereas a controlled pro-inflammatory response can in theory be realized by a broad array of pro-inflammatory stimuli: pathogen-derived components, endogenous PAMPs, cytokines, and possibly other chemical compounds that are known to elicit an inflammatory reaction[89, 96]. For translation of the current research towards clinical application, we propose careful selection and investigation of pro-inflammatory mediators that likely have high merit to reach clinical testing phase, for

instance based on their safety profile, the possibility for batch production, and the ability to incorporate the mediator into a chosen carrier material. For bacterial components, future studies should more firmly establish the potential of single isolated bacterial components relative to intact killed bacteria, as the chosen strategy will greatly impact the appeal of the therapy in terms of clinical translation. From a safety perspective, off-target effects can likely be minimized when using a single component compared to the intact or fragmented bacterial cell wall, considering that several bacterial cell wall components have been identified with bone-destructive or systemic effects[48, 97]. The exact mode-of-action is also more easily pinpointed for single molecules, which can facilitate regulatory approval. Of equal importance, batch-to-batch consistency of a final product is more easily safeguarded for single components. Following this reasoning, the use of small synthetic TLR ligands should be considered in future work to maximize safety of the treatment. A number of them have been developed to mimic the action of bacterial ligands, but with reduced toxicity allowing their use as vaccine adjuvants or cancer therapeutics[98]. Clinical evidence shows that TLR ligands can be rendered safer by redesign of the molecules and by local application[97]. On the other hand, adopting pro-inflammatory cytokines for an orthopedic application seems more straightforward. The technology of recombinant protein production is advanced, and as another potential advantage, various cytokines have already reached clinical testing phase for other purposes[99, 100].

To maximize the effectiveness of any osteo-immunomodulatory approach, the pro-osteogenic stimuli are preferably applied in conjunction with carriers that have a high intrinsic ability to promote bone healing. In comparison to the BCP used in our *in vivo* studies, ceramics with a faster resorption, such as specific tricalcium phosphate (TCP) formulations, have shown to be more effective in challenging large animal models such as posterolateral spinal fusion[101]. The ability of this material to promote bone healing is mainly attributed to its osteoconductive behavior, but is also linked to an *in vivo* osteoinductive capacity[101, 102]. In the ectopic model, we studied the effect of pro-inflammatory stimuli in the context of BMP-2-mediated bone formation, which primarily targets MSC osteogenic differentiation. However, it seems of equal importance to study whether material-induced bone formation can be potentiated with immunomodulatory agents, as this process could be driven by intrinsically different mechanisms involving material dissolution, the host response and/or the activation of phagocytic cells[86, 101]. Unfortunately, only a small number of rabbits demonstrated quantifiable amounts of material-induced bone formation in response to the BCP scaffolds. The ectopic implantation of osteoinductive TCP in larger animal models may be more suitable to identify strategies that improve material-associated osteoinduction[101, 103]. In parallel, the use of specific mouse models that demonstrate heterotopic bone formation in response to ectopically implanted TCP can facilitate mechanistic studies, considering the plethora of mouse research tools and the possibility to make knockouts.

The induction of the inflammatory response may have multiple effects on both osteoblast and osteoclast precursors in a clinical setting[104]. This assumption was confirmed by our observations in the rabbit tibia following inoculation of high dose killed bacteria. While a certain threshold in inflammatory response resulted in induction of new bone formation, bone histology also suggested a positive correlation between new bone formation and enhanced bone resorption. Thus, it is likely that the inhibition of catabolic pathways could protect bone in a clinically relevant location. In *chapter 5*, local depletion

of osteoclasts with a bisphosphonate was not a feasible anti-catabolic strategy in the context of BMP-2-mediated new bone formation. This may however be specifically related to the ectopic location or the method and/or the timing of bisphosphonate delivery[105, 106]. The co-delivery of bisphosphonates, or other applied methods to inhibit RANKL signaling or T cell-mediated bone destruction[104, 107], remain tempting strategies to investigate in the future.

Finally, it should be taken into account that many factors could potentially mask, potentiate, or exaggerate osteo-immunomodulatory strategies in a clinical setting by affecting patients' immune responses. Importantly, orthopedic operations are accompanied by local and systemic expression of cytokines/chemokines that could influence the action of pro-inflammatory agents[76, 77]. Moreover, surgical stress or systemic factors such as trauma can lead to changes in leukocyte distribution or activity[64, 108]. Finally, the use of inflammation-modifying drugs is not uncommon, and can interfere with the therapy[18]. Taken together, it can be hypothesized that the efficacy of an osteo-immunomodulatory strategy is dependent on optimization of the patient's immune status. When we gain more understanding in the role of the individual's immune build-up on the osteogenic response[95], osteo-immunomodulatory strategies would further benefit from patient-specific approaches.

Final remarks

This thesis showed that pro-inflammatory cytokines could enhance bone formation, mediated in part, by an interaction with BMP-2 to target osteoblast differentiation. The crosstalk between inflammatory and osteoinductive pathways likely resembles the natural cues bone progenitor cells are exposed to during bone healing. The local and short-lived delivery of pro-inflammatory cytokines also seems to be a potentially safe strategy.

It was also established that the complex, but unique inflammatory response induced by bacterial ligands could stimulate processes involved in new bone formation. Accordingly, bacterial ligands can serve as model compounds to advance our understanding of the immune reactions favoring osteogenesis, such as the relative importance of innate or adaptive immunity. Bacterial ligands would also have therapeutic merit in strategies for bone regeneration when they could be used to direct specific immune responses. The immunomodulatory properties of bacterial ligands are already being pursued in other fields, offering the advantage that downgraded analogues of bacterial ligands with increased safety profile are becoming increasingly available. In any case, the condition should be chosen in order to minimize systemic effects and allow controllable timing of the response.

New models were designed and tested to study the interaction between bone cells and immune cells. Several findings in this thesis also provide a basis for further improvements in models to investigate the crosstalk between skeletal cells and immune cells. The pro-inflammatory stimuli with osteo-stimulatory potential should next be confirmed in relevant locations and/or functional models.

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Chapter 9

Summary in Dutch
Nederlandse samenvatting

Transplantatie van lichaamseigen bot

Binnen de orthopedische chirurgie wordt vaak lichaamseigen bot getransplanteerd. De bottransplantaten worden gebruikt om bot afwijkingen te vullen en te genezen als dit niet op natuurlijke wijze kan plaatsvinden. Denk hierbij aan serieuze ongevalletsels, chronische pijn of deformiteiten in de wervelkolom, of afwijkingen die ontstaan als gevolg van bottumoren. Het is algemeen aanvaard dat vers-geïsoleerde bottransplantaten de nodige cellen en eiwitten bevatten die voor botgenezing zorgen. Daarnaast zijn afstotingsverschijnselen uitgesloten wanneer lichaamseigen bottransplantaten worden gebruikt.

Transplantatie van lichaamseigen bot heeft een aantal beperkingen. Het bottransplantaat wordt vaak weggehaald uit een ander gezond lichaamsdeel van de patiënt, zoals de bekkenkam, wat extra schade aanbrengt. Bovendien is de beschikbaarheid van het bottransplantaat soms beperkt, bijvoorbeeld bij oudere patiënten die al eerdere operaties hebben ondergaan. Tot slot gaat de isolatieprocedure gepaard met nóg langere operatietijden. Donor bot wordt ook frequent gebruikt, maar dit bot is van mindere kwaliteit dan lichaamseigen bot, omdat het bewerkt wordt om afstoting en ziekte-overdracht te vermijden.

Doel van het onderzoek

Binnen de regeneratieve geneeskunde is het doel gesteld om alternatieve producten ('bot substituten') te ontwikkelen voor het herstel van bot defecten, die wel alle voordelen, maar niet de nadelen van lichaamseigen bottransplantaten met zich mee brengen. Synthetische bot substituten die qua chemische samenstelling en structuur lijken op bot, zijn over het algemeen erg veilig en kunnen in grote schaal geproduceerd worden. De effectiviteit van dergelijke bot substituten kan verhoogd worden door er bot-stimulerende componenten aan toe te voegen.

Om bot-stimulerende componenten te identificeren, kan men het normale bot genezingsproces als uitgangssituatie nemen. Een gecontroleerde ontstekingsreactie is een natuurlijke reactie van het lichaam op weefselschade, en initieert het herstelproces. Tijdens de botgenezing werken immuuncellen en botcellen nauw samen om processen aan te sturen die nodig zijn voor botaanmaak. Er worden ontstekingsfactoren geproduceerd die onder andere de groei en differentiatie van bot- en kraakbeencellen stimuleren, of de vorming van bloedvaten bevorderen. De hypothese was daarom dat **lokale toediening van ontstekingsfactoren - onder de juiste omstandigheden - een gunstig effect heeft op de botvorming**. In dit proefschrift hebben we de bot-stimulerende werking van verschillende ontstekingsfactoren bestudeerd in zowel celkweek experimenten alsmede verschillende diermodellen.

Bevindingen

Bot-stimulerende eigenschappen van ontstekingseiwitten (hoofdstuk 2-5)

Door het effect van verschillende ontstekingsfactoren op botcellen te bestuderen in celkweek experimenten, is geprobeerd een beter begrip te krijgen van de mechanismen waarop ontstekingsfactoren de botaanmaak zouden kunnen beïnvloeden.

In *hoofdstuk 2* is het effect van ontstekingseiwitten en bacteriële componenten bestudeerd op de groei en functie van bot cellen. De uitkomsten van de experimenten wezen uit dat

ontstekingsfactoren de groei van bot voorloper cellen, zogenaamde mesenchymale stamcellen (MSCs), stimuleren richting functionele bot cellen. De ontstekingsfactoren versterken hierbij de werking van het bone morphogenetic protein 2 (BMP-2) eiwit, een groeifactor die een belangrijke rol vervult in de ontwikkeling en reparatie van het skelet. In *hoofdstuk 3* is in een konijnen model aangetoond dat gecombineerde toediening van het ontstekingseiwit TNF-α samen met de groeifactor BMP-2, leidt tot een verdubbeling van de hoeveelheid gevormd bot in vergelijking met het gebruik van alleen BMP-2. In de celkweek experimenten in *hoofdstuk 4* is onderzocht hoe een specifieke subpopulatie van immuuncellen, zogenaamde T lymfocyten, een interactie aangaan met MSCs. T lymfocyten vormen een belangrijk onderdeel van het verworven immuunsysteem, en spelen mogelijk een rol in de natuurlijke bot genezing. Uit de experimenten bleek dat de T lymfocyten eiwitten kunnen uitscheiden die bijdragen aan de groei van MSCs richting functionele bot cellen. Aansluitend is in *hoofdstuk 5* aangetoond dat IL-17 – een ontstekingseiwit met name geproduceerd door T lymfocyten - een bot-stimulerende werking heeft: in konijnen versterkte het IL-17 eiwit op een vroeg tijdstip de weefsel ingroei in bot substituten, terwijl het op langere termijn zorgde voor meer botaanmaak.

Effect van bacteriële componenten op de botaanmaak (hoofdstuk 6 & 7)
Ziekteprocessen in het skelet kunnen het botgenezingsproces ook sterk beïnvloeden. Een kenmerkend voorbeeld is dat botinfecties gepaard gaan met bot afbraak, maar ogenschijnlijk óók met (ongewenste) botaanmaak. De causale relatie tussen de bacteriën, de mate van ontsteking, en de hoeveelheid botaanmaak, is vaak speculatief. Één van de doelen was daarom om het fenomeen van botgroei bij infecties te onderzoeken in een konijnen model. Er is vervolgens getest of de lokale toediening van bacteriële componenten ook tot botaanmaak leidt.

In *hoofdstuk 6* hebben we in konijnen de veranderingen in botstructuur- en volume gemeten bij een lokale botinfectie. De belangrijkste bevinding was dat de infectie zorgde voor een verdubbeling van het botvolume, waarbij de hoeveelheid nieuw gevormd bot correleerde met de mate van de infectie. In *hoofdstuk 7* is in konijnen aangetoond dat het toedienen van dode bacterie fragmenten of specifieke componenten van de bacteriële celwand, óók botaanmaak stimuleert. In vergelijking met een ongecontroleerde infectie, zorgen deze steriele vormen van ontsteking voor minder nadelige systemische verschijnselen of veranderingen in botkwaliteit. Met behulp van histologische analyses is een verband aangetoond tussen het lokale ontstekingsproces en de hoeveelheid botaanmaak: een milde activiteit van ontstekingscellen heeft een gunstige werking op de botvorming, terwijl de overactivatie van immuuncellen de botvorming juist remt. De keuze van de bacteriële component, en de wijze/duur van toediening bepaalt dus het botgenezingsproces.

Slotopmerkingen
Er zijn een aantal typen ontstekingsfactoren geïdentificeerd die de botaanmaak versterken in diermodellen. Zodoende zijn er belangrijke stappen gezet in de ontwikkeling van verbeterde bot substituten, met als doel om het het aantal bottransplantaties nog verder te reduceren. Er is aangetoond dat ontstekingsfactoren de groei van stamcellen richting volwassen bot-vormende cellen kunnen stimuleren. Daarnaast versterken ze het effect van BMP-2, een groeifactor die een cruciale rol vervult in het natuurlijke botgenezingsproces.

Uit vervolgonderzoek moet blijken hoe effectief de beschreven ontstekingseiwitten of bacteriële componenten zijn in het genezen van grote bot defecten in diermodellen. Ontstekingseiwitten zoals TNF-α en IL-17 hebben mogelijk een klinische toepasbaarheid omdat ze bij een lage concentratie al effectief zijn en snel geklaard worden door het lichaam. Het gebruik van dode bacterie fragmenten of bacterie-specifieke componenten brengt logischerwijs meer risico's met zich mee, maar mogelijk is er in de toekomst tóch een rol voor ze weggelegd binnen de orthopedische chirurgie. Voorwaarde hiervoor is dat uitgezocht wordt welke specifieke bacteriële componenten een bot-stimulerend werking hebben, én hoe ze zo gecontroleerd en veilig mogelijk toegediend moeten worden.

APPENDICES

LIST OF ABBREVIATIONS

ALP	alkaline phosphatase
Ang-1	angiopoietin 1
ANOVA	one-way analysis of variance
BCP	biphasic calcium phosphate
BSA	bovine serum albumin
BMP	bone morphogenetic protein
ESR	erythrocyte sedimentation rate
CFU	colony forming units
CM	conditioned medium
CT	computed tomography
CWA	cell wall extract
DAB	diaminobenzidine tetrahychloride hydrate
DAMP	damage-associated molecular pattern
DBM	demineralized bone matrix
DEX	dexamethasone
ECM	extracellular matrix
FGF	fibroblast growth factor
FOP	fibrodysplasia ossificans progressiva
γi	gamma irradiated
HO	heterotopic ossification
HPLC	high Performance Liquid Chromatography
HRP	horse-radish peroxidase
ICBG	iliac crest bone graft
IFN-γ	interferon gamma
IGF	insulin-like growth factor
IL	interleukin
i.m.	intramuscular
i.v.	intravenous
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAP	mitogen-activated protein
MMA	methyl methacrylate
MSC	multipotent mesenchymal stromal cell
NF-kB	nuclear factor kappa B
ODM	osteogenic differentiation medium
OPG	osteoprotegerin
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
PMMA	polymethyl methacrylate
RANKL	receptor activator of nuclear factor kappa B ligand
rhBMP-2	recombinant human bone morphogenetic protein 2
s.c.	subcutaneous

SD	standard deviation
T_{CTX}	cytotoxic T cell
T_H17	T helper 17 cell
TNF-α	tumor necrosis factor alpha
TLR	toll-like-receptor
TRAP	tartrate-resistant acid phosphatase
T_{REG}	regulatory T cell
TGF-β	transforming growth factor beta
VEGF	vascular endothelial growth factor
ZOL	zoledronic acid

LIST OF PUBLICATIONS

M. Croes, F.C. Öner, M.C. Kruyt, T.J. Blokhuis, O. Bastian, W.J.A. Dhert, J. Alblas. Proinflammatory mediators enhance the osteogenesis of human mesenchymal stem cells after lineage commitment. PloS one 10, e0132781 (2015).

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F.C. Öner, J. Alblas, **M. Croes**. Composition for use in the treatment of an individual suffering a condition necessitating new bone formation. PCT/EP2016/073551. Application date October 3rd 2016.

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De paranimfen

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Mijn collega's en overige betrokkenen

Leden van de beoordelingscommissie. Prof. dr. J. de Boer, prof. dr. J.D. de Bruijn, prof. dr. L.P.H. Leenen, prof. dr. B.P. Meij, en de voorzitter prof. dr. L. Koenderman. Hartelijk dank voor het beoordelen van mijn proefschrift.

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CURRICULUM VITAE

Michiel Croes was born on October 18th 1986 in Taipei, Taiwan. He graduated from the Schola Europaea high school in Mol (Belgium) in 2005. To pursue his wide interest in both technology and biology, he started the bachelor's program Technical Medicine at the University of Twente in Enschede. He continued with the Technical Medicine master's track Reconstructive Medicine at the same university. Michiel obtained his master's degree in 2012 after completion of a one-year research project at the department of Rheumatology at the UMC Utrecht under supervision of Prof. dr. F.P.J.G. Lafeber and dr. S.C. Mastbergen.

Michiel followed-up on his interest in the musculoskeletal system in the UMC Utrecht and started as a PhD candidate at the department of orthopedics in 2012, under supervision of prof. dr. F.C. Öner, prof. dr. W.J.A. Dhert, dr. J. Alblas and dr. M.C. Kruyt. The results of this research are described in this thesis. For his research, Michiel was awarded with consecutive research grants from the Anna Fonds|NOREF foundation in 2014, 2015 and 2016. Michiel's educational responsibilities involved teaching of Medical students of Utrecht University and the supervision of students during their internships. He furthermore attended several national and international conferences, and completed the Graduate School of Life Sciences training on Regenerative Medicine.

Michiel worked as a research associate at the department of orthopedics in 2017, where he was involved in research on bone regeneration and infection-prevention. This was combined with his venture activities, for which he participated in YES!Delft's Launchlab and Health Holland's Venture Challenge with team Osteomore. Michiel is currently working as a post-doctoral researcher at the UMC Utrecht.