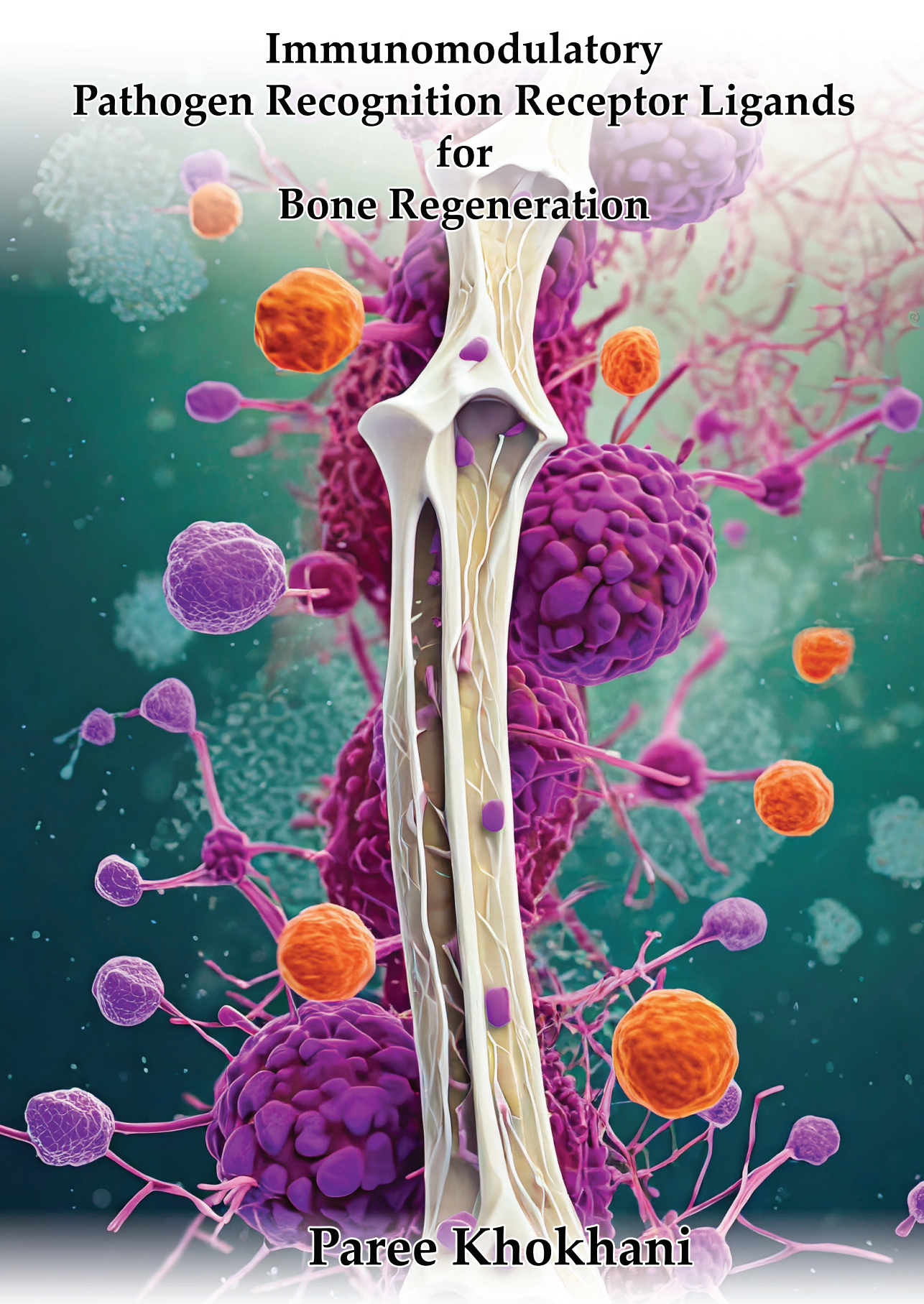
Immunomodulatory Pathogen Recognition Receptor Ligands for Bone Regeneration



Paree Khokhani

Immunomodulatory Pathogen Recognition Receptor Ligands for Bone Regeneration

Paree Khokhani

Immunomodulatory Pathogen recognition receptor ligands for bone regeneration

PhD thesis, Utrecht University, The Netherlands

ISBN: 978-94-6483-810-7

doi: https://doi.org/10.33540/2160

Author: Paree Khokhani

Cover design: Chirayu Gandhi

Layout: Chirayu Gandhi and Paree Khokhani

Printing: Ridderprint | www.ridderprint.nl

The printing of this thesis is kindly and partly financially supported by the EU's H2020 research and innovation program under the Marie S. Curie Cofund RESCUE grant agreement and the Netherlands Society for Biomaterials and Tissue Engineering (NBTE).

Immunomodulatory Pathogen Recognition Receptor Ligands for Bone Regeneration

Immunomodulerende Pathogeen Herkennings Receptor Liganden voor Bot Regeneratie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

dinsdag 12 maart 2024 des middags te 4:15 uur

door

Paree Kishor Khokhani

geboren op 16 september 1993
te Mumbai, India, India

Promotoren:

Prof. Dr. M.C Krujit
Prof. Dr. ir. H.H. Weinans

Copromotoren:

Dr. D. Gawlitta

Beoordelingscommissie:

Prof. dr. K. Ito
Prof. dr. F.P.J.G. Lafeber
Prof. dr. J. Klein Nulend
Prof. dr. F.C. Oner
Dr. S. Amin Yavari

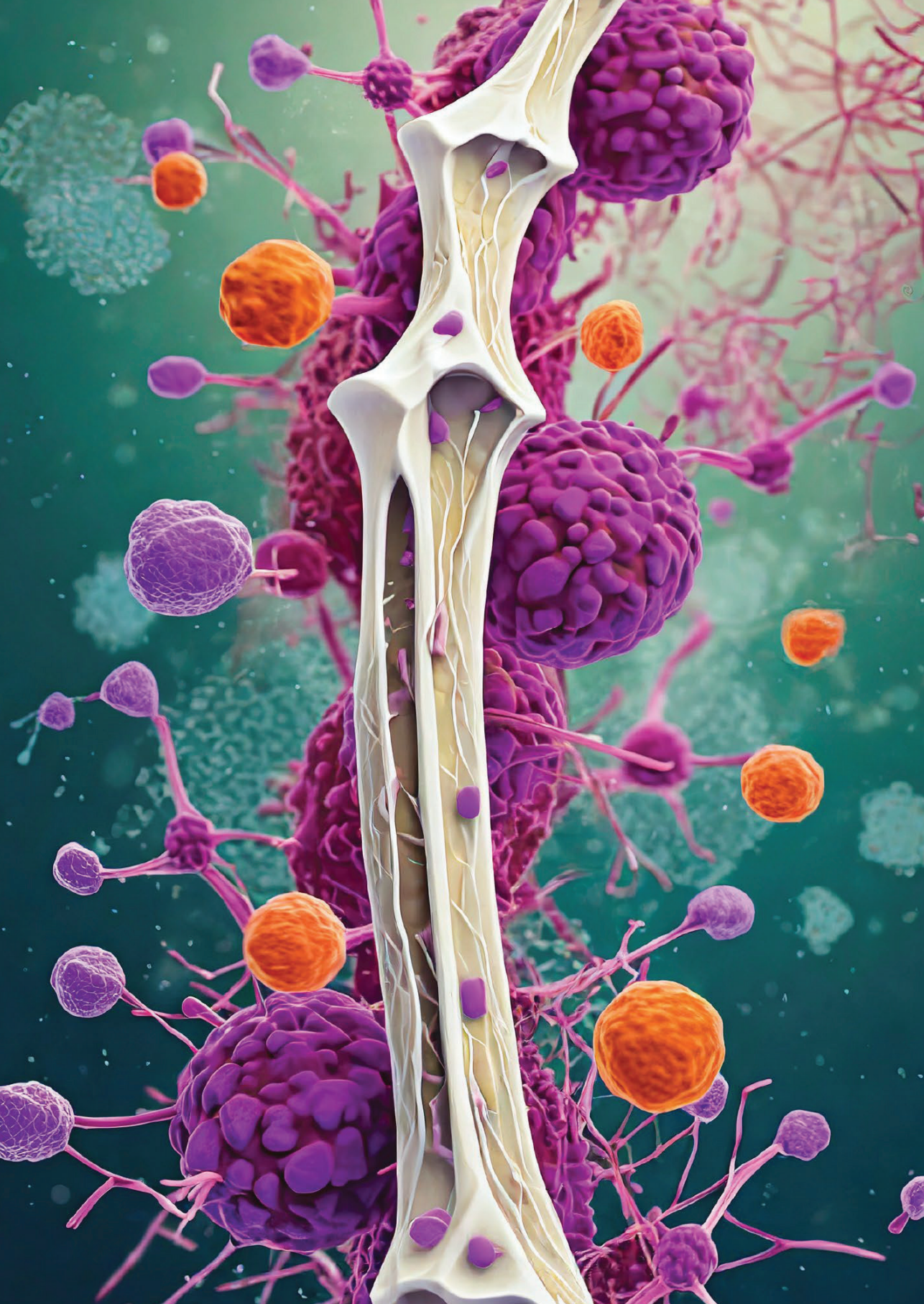
Paranimfen:

Dr. L. Cecotto
A.A.A. Duits

"Things work out best for those who make the best of how things work out."

\- John Wooden

Table of Contents



Chapter 1

General introduction

Bone Regeneration

Bone makes up the structural skeleton of the human body. It serves as a locomotory organ aiding in mobility. It acts as a primary site for hematopoiesis, storage of multiple growth factors and minerals that sustain other vital organs of the body, and also participates in various hormonal and metabolic functions such as regulation of glucose metabolism [1,2]. Unlike its stable appearance, bone is a self-regenerating organ as it undergoes a well-orchestrated complex process right from the skeletal development in the embryos to the continuous remodeling throughout adult life to maintain its structure and bone mass, repair microdamage, and adapt to mechanical loads [3].

The fracture healing mechanism is the most common form of bone regeneration in clinical settings, as it undergoes a complex cascade starting from early inflammation, going through the repair phase,, and eventually bone remodeling [4]. Bone fractures can heal via intramembranous healing or endochondral healing mechanisms [4]. During the renewal phase, the osteoprogenitors, namely, mesenchymal stem cells (MSCs), undergo differentiation. In intramembranous ossification, MSCs differentiate into cuboid-shaped osteoblasts under the influence of growth factors, bone morphogenetic proteins, cytokines, hormones, and cell-cell and cell-extracellular matrix interactions [5,6]. Osteoblasts represent only 4-6% of total resident cells in bone tissue and abundantly express alkaline phosphatase (ALP) in the plasma membrane [7]. ALP is used as a characteristic marker of osteoblasts. They then produce collagen type I and other bone matrix proteins bone sialoprotein and osteocalcin which stimulate bone mineralization [8]. This healing process occurs when fractures are surgically stabilized. In contrast, during endochondral ossification, MSCs differentiate into chondrocytes forming a cartilaginous intermediate, which is eventually mineralized and converted into bone. After bone deposition, osteoclasts and osteocytes kick off the remodelling phase of bone regeneration. Osteoclasts are giant multinucleated cells, which are differentiated from monocytes present in the blood, due to the activation of RANKL cytokines, initiating the process of bone resorption. Embedded within bones, osteocytes function as mechanical sensors and play a critical role in regulating bone turnover. They release signalling molecules that trigger bone formation or resorption, contributing significantly to this process [9].

Bone fractures and their impact

Most defects in the bone undergo scar-free healing due to the bone's dynamic self-renewal capabilities. Despite this, 5-10% of all fractures result in pathological conditions like delayed bony union, development of pseudoarthrosis, large traumatic bone defects, and extensive bone loss after tumor resection or revision surgeries after arthroplasties, representing a significant challenge in clinical practice [10,11]. Fracture non-unions pose a massive clinical problem as it debilitatingly affects patients' quality of life, affecting their mental well-being and causing an enormous impact on socio-economic aspects [12–15]. Fractures that fail to heal for over nine months or show no possibility of healing without intervention are defined as fracture non-unions. Fracture non-unions often show signs of persistent pain, deformity, and overall loss of function. Large bone defects greater than 2.5 cm or approximately twice the size of the bone diameter, especially in the long bones, show impaired healing [16]. Moreover, augmentation of bone i.e. bone grafting is also required in spinal fusion surgeries where bone formation outside the original bone margin is necessary [17].

Current treatments and their limitations

Bone grafting is a standard surgical procedure, in which missing parts of the bone are replaced or augmented using bone grafts to ensure bone repair. Various bone grafts, including autologous, allografts, and xenografts, can be sourced naturally [18]. The current clinical gold standard is to use autografts. While the autografts can be harvested from the tibia, fibula, ribs of the patient, it is mainly harvested from the patient's iliac crest due to its relatively high amount of available bone compared to other skeletal harvesting sites [19]. Allografts are bone transplants taken from another human while porcine or bovine = xenogenous grafts are popular alternatives [20]. Due to traditional harvesting and storage protocols, the risk of disease transmission is negligible. However, these grafts show a slower integration to the host site and, thus, perform inferior to autografts [21]. Autografts possess the same regenerative properties of the bone as that of the host and are capable of inducing new bone formation at the site of injury, avoiding the possibility of transmission of diseases and auto-immune rejection as observed in allografts and xenografts; however, their use comes with disadvantages of limited

availability and donor site pain [21,22]. In a challenging healing environment, like spinal fusion, where a solid bony bridge needs to be established , the performance of autografts has achieved a success rate of 58%-93% accompanied with significant morbidities [23–28]. Due to the limitations mentioned earlier, there has been a tremendous effort to develop synthetic bone substitutes with similar or better biological properties than autografts, particularly for spinal fusion applications.

Synthetic bone substitutes are biomaterials that can be categorized depending on their composition into metals, polymers, ceramics, and composites. The biomaterials as a substitute should be selected based on their intended application. For example, load-bearing applications utilize metals, while ceramics like hydroxyapatite, biphasic, and tricalcium phosphates are widely used as injectable bone cement and bone extenders in non-load bearing applications like spinal fusion surgeries [29,30]. Current biomaterials especially metals and polymers lack the capability of inducing bone on their own. Recently, it was shown that ceramics with altered surface topography possess osteo-inductive properties and were shown to have a noninferior performance compared to the autografts for spinal fusion; however, they do not outperform the current gold standard in spine surgery [31–34]. Thus, there is a clear need for enhancing these bone substitutes to achieve 100% spinal fusion. For this reason, they are often used with an additional osteo-inductive agent [18,35].

Traditionally, the addition of osteoprogenitor cells like MSCs from different sources or growth factors like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and bone morphogenetic proteins (BMPs) are used to enhance synthetic bone grafts [36–38]. Mesenchymal stromal cells are the most studied in bone regenerative medicine due to their self-renewal properties, multipotential differentiation capacity, and contribution to fracture healing. However, the ideal source to accommodate the number of cells needed for stem cell-based therapies and their survival after implantation remain a significant challenge [39,40]. Based on various tests conducted *in vitro* and in preclinical animal models, BMP-2 and BMP-7 have been identified as the most efficient growth factors for osteogenesis [41,42]. However, the clinical use of BMP-2 has been limited due to several reported side effects, including osteolysis, inflammatory reactions and

neurological complications [43,44]. Further, BMP-2 did not show improved fusion rates in patients compared to autografts and combined with high costs indicated that use of growth factors in its purest forms is an unreliable way to recapitulate bone regeneration [42]. Further investigation is needed to assess the different delivery methods encapsulating growth factors like BMPs [45,46]. Efforts at optimizing the delivery of BMP has yielded fruitful results in preclinical rat models. For example, Koolen et al. showed that fibrin gel containing BMP2 was able to achieve complete bone regeneration in a critically sized segmental defect rat model [47]. It would be interesting to see if this approach would yield positive results in a large animal model and eventually end up in the clinics as a treatment for large defects and fracture non-unions in the coming years. In conclusion, despite all the attempts to enhance bone substitutes, an ideal solution is yet to be found.

The importance of the initial inflammatory phase on fracture healing outcomes

The initial stage of fracture healing, known as the inflammatory or reactive phase, is a critical period after a bone fractures. This stage sets the foundation for the subsequent steps and involves a complex series of events [48,49]. Upon rupture, blood vessels involved in the injury are disrupted, leading to bleeding and the activation of the plasma coagulation cascade. Platelets from the blood accumulate and release clotting factors that ultimately form a fracture hematoma composed of a meshwork of fibrin [4]. During this initial phase, hypoxia due to the disruption of blood vessels results in the change of pH in the cellular microenvironment leading to a shift in cell metabolism [50,51]. On the other hand, immune cells are able to adapt to low-oxygen environment and secrete cytokines and chemokines to attract MSCs and pre-osteoblasts needed for the healing process [52,53]. This hematoma serves as a provisional scaffold that helps cellular infiltration and adhesion of inflammatory immune cells and somewhat stabilizes the fractured bone ends [54,55]. The findings that removing initial fracture hematoma leads to delayed or hypertrophic non-unions [56] or that the implantation of the fracture hematoma intramuscularly induces bone formation exhibits the initial inflammatory phase's impact on the bone regenerative process

[57]. The need for specific inflammatory signals in bone healing is evident from studies showing that inhibiting immune responses through anti-inflammatory drugs impairs bone healing [58]. These findings highlight the importance of a balanced and controlled inflammatory response for optimal bone regeneration.

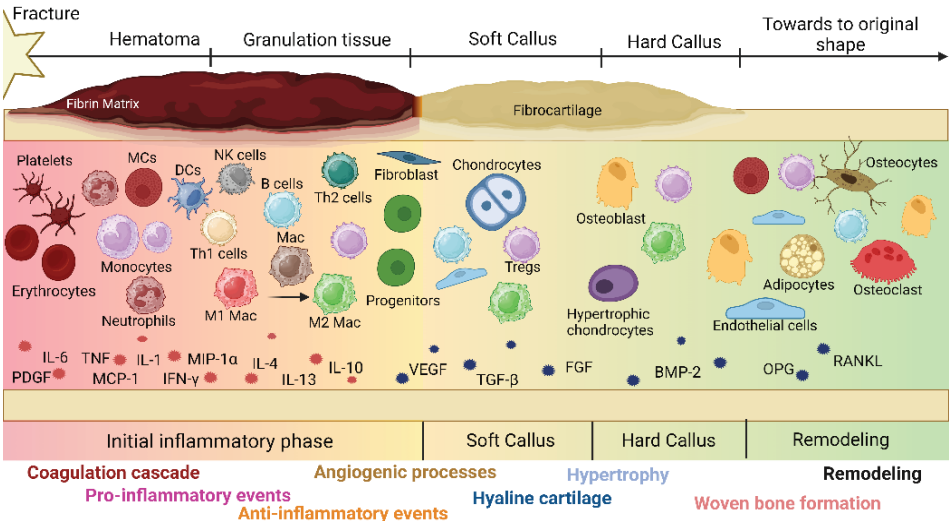


Figure 1. The interdependent phases and dynamic cellular environment of fracture healing. The fracture healing process is divided into four different stages [59]. Multiple events coincide throughout the stages, ensuring a well-orchestrated sequence for bone re-establishment. The cellular design in the fracture callus is constantly changing and adapting to the various stages. Various cells and their secreted factors play crucial roles in regulating the fracture healing process [53,60]. The image was created by author using biorender.com. MCs (Mast cells), NK cells (Natural killer cells), DCs (Dendritic cells), Mac (Macrophages), Th1 and Th2 cells (T helper cells), TNF (Tumor necrosis factor), PDGF (platelet derived growth factor), IL-6 (Interleukin-6), MCP-1 (Monocyte chemoattractant protein-1), MIP-1α (macrophage inflammatory protein- 1 alpha), IL-1 (Interleukin- 1), IL-4 (Interleukin-4), IL-13 (Interleukin-13), IL-10 (Interleukin-10), VEGF (vascular endothelial growth factor), TGF-β (transforming growth factor), FGF (fibroblast related growth factor), BMP-2 (bone morphogenetic protein), OPG (osteoprotegerin), RANKL (receptor activator of nuclear factor kappa beta ligand).

The impact of immune cells in the initial phase of fracture healing

The interplay between immune and bone cells is fundamental to fracture healing. Immune cells, particularly those of the innate immune system, contribute to inflammation and tissue repair, while the role of adaptive immune cells in fracture healing is complex and still an area of ongoing research. The involvement and phases of each cell type can be seen in the figure above (**Figure 1**).

Upon injury, local vasculature of the bone and surrounding tissues is disrupted giving rise to activation of plasma coagulation cascade and recruitment of platelets at the site of injury. Platelets and erythrocytes contribute in forming a fibrin network and that serves as a provisional matrix to attract influx of inflammatory cells like neutrophils [59] (**Figure 1**). Neutrophils are then recruited to the fracture site mainly by platelets and erythrocytes upon the release of multiple danger signals known as danger-associated molecular patterns (DAMPs) that derive from host cells, PDGF, IL-6, TNF and complement system [61]. Neutrophils are known to induce a strong inflammatory reaction and recruit monocytes/ macrophages by secreting cytokines like IL-6 and chemotactic attractants like MCP-1, MIP-1α [62,63]. To illustrate their role, neutrophil depletion reduced MSC infiltration in small rodents, resulting in delayed bone healing [64,65]. Studies *in vitro* suggest that neutrophils alter cytokine release and inhibit matrix formation by MSCs [65,66]. Neutrophils also release TNF, matrix metalloproteinase 9 (MMP9), and VEGF, which helps in wound healing and angiogenesis [67–69]. Neutrophils also form neutrophil extracellular traps (NETs) in response to trauma, and overshooting the NET formation leads to delayed healing [70,71]. Finally, neutrophils contribute to the inflammatory reaction after trauma; however, their exact role in bone healing is still being explored.

Other innate immune cells like mast cells (MCs) and dendritic cells (DCs), are also been recruited at the same time as the neutrophils and have also been implicated in fracture healing [60] (**Figure 1**). Mast cells (MCs) are present throughout the callus formation phase [72,73]. They are known to store numerous preformed cytokines and chemokines that are released upon activation. Factors secreted by MCs are known to regulate bone cell metabolism [72]. Delayed healing in MC-deficient mice further emphasized the importance of MCs in inflammation,

angiogenesis, and anabolic and catabolic processes of bone healing [74,75]. Furthermore, dendritic cells (DCs) were found to infiltrate the fracture site and regulate immune and inflammatory processes, as well as tissue repair and remodeling. In more detail, DCs secrete interleukin- 12 (IL-12), contributing to the inflammatory reaction by enabling the differentiation of CD4+ T cells into Th1 helper cells [76]. Furthermore, shortly after, the natural killer cells (NK) arrive at the site of injury (**Figure 1**). NK cells are known for their cytotoxic activity against infected or malignant cells and are attracted to the fracture site in response to TNF and IL-6 [77]. NK cells signal the clearance of injured tissue by recruiting inflammatory cells and osteoclasts due to their ability to produce IFN-γ and RANKL [78–80].

Next, monocytes migrate to the fracture site shortly after injury, where they differentiate into various cell types depending on the local environment. They can become pro-inflammatory M1 macrophages, immunosuppressive M2 macrophages, DCs, or bone-resorbing osteoclasts (**Figure 1**). During the inflammatory phase, cytokines and bacterial products can stimulate monocytes toward the pro-inflammatory M1 phenotype. These M1 macrophages phagocytose debris, produce inflammatory molecules such as nitric oxide, reactive oxidative species (ROS), interferon-γ (IFN-γ), TNF, IL-6, and facilitate the recruitment of MSCs to the fracture site [81,82]. As the inflammatory phase progresses, the macrophages phenotype switches to the anti-inflammatory M2 phenotype, influenced by changes in the microenvironment at the fracture site as well as presence of growth factors TGF-β, VEGF and SDF-1 and cytokines like interleukin-4 (IL-4) and interleukin-13 (IL-13) produced by M1 macrophages and MCs. These M2 macrophages then secrete, IL-4, IL-10 and various growth factors like BMP-2, TGF-β, VEGF that help in resolving the inflammation, stimulate neovascularization as well as facilitate ossification [83–85]. Macrophages play a crucial role in bone formation, as their depletion in animal models impairs healing and reduces callus size [86,87]. Furthermore, *in vitro* studies have demonstrated that macrophages can enhance bone formation by the secretion of factors like BMP-2 and oncostatin M (OSM) [88]. MSCs have shown the ability to shift macrophages from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype. This shift is accompanied by a decrease in the secretion of pro-inflammatory cytokines and an increase in the production of anti-inflammatory

cytokines. The underlying mechanisms of this phenotypic switch involve factors such as prostaglandin E2 (PGE2), interleukin receptor-1 agonist (IL1RA), IL-6, NF-κB, STAT-3, and IFN-γ-mediated indoleamine 2,3-dioxygenase activation secreted by the interaction between the MSCs and other innate cells like neutrophils, MCs and M1 macrophages [89–91].

Like innate immune cells, T-cells also participate in various stages of fracture healing, however their activation status influences their contribution to the fracture healing process (**Figure 1**). For example, terminally differentiated CD8+ effector cells were enriched in the fracture hematoma and were found to be related to delayed healing [92]. The depletion of CD8+ T cells in a mouse osteotomy model demonstrated enhanced fracture healing indicating the negative impact of these cells on the fracture healing process [49]. Naïve T cells upon antigen presentation differentiate into Th1 and Th2 cells. Th1 and Th2 cells appear during the granulation phase of the fracture healing process and were shown to inhibit osteoclast differentiation and promote bone formation by degradation of TRAF-6, however, several previous studies also showed Th1 cells mainly promote osteoclast precursor cell differentiation, making the roles of these subset controversial in bone regeneration [93,94]. Recent studies have identified IL-17-producing γδT-cells during callus formation as promotors of bone healing [95]. However, IL-17 producing cells are also shown to promote osteoclast differentiation by activating RANKL on the surface of osteoclast precursors [93]. Another subset of T cells known as regulatory T cells (Tregs), known for their role in preventing excessive inflammation, become prominent after the switch of M1 macrophage phenotype to M2 phenotype, exerting influence until the remodeling phase. They release anti-inflammatory cytokines like IL-10, contributing to a shift towards a immunosuppression after trauma [96,97]. Tregs secrete TGF-β and IL-4 that lead to cytokine dependent suppressive effects on osteoclast differentiation invitro are shown to regulate osteoblast and osteoclast function later in fracture healing [98,99]. Systematic injection of Treg cells effectively reduced levels of local inflammatory factors, improved the osteogenic differentiation of transplanted cells that lead to achieve bone regeneration in a calvarial defects in mice [94]. This shows that unlike other T cell subsets, Treg cells promote bone healing.

Similar to the antigen presenting T cells, B-cells exhibit crucial immunomodulatory roles in the fracture healing process (**Figure 1**). During the

healing process, IL-10 producing CD27+ B cells effectively suppressed IFN-γ, TNF, IL-2 expression leading to suppression in CD4+ T-cells proliferation and enhanced of Treg cells [100]. B-cells showed diminished IL-10 production over time, emphasizing the regulatory function of B-cells in successful bone healing in patients with delayed healing [101]. In an inflammatory environment, T cells promote B-cells to produce osteoprotegerin via CD40/CD40L signaling pathway, which promotes in bone tissue regeneration [102]. Although a clear role of B cells still remains elusive, it is shown that the differentiation and maturation of B-cells is directly influenced by osteoprogenitor cells, mainly MSCs. MSCs modulate B-cell activity by suppressing proliferation, activation, and antibody secretion through secreted factors like IFN-γ and cell-to-cell contact via interactions with programmed cell death ligands (PD-1/PD-L1) [103]. However, *in vitro* studies have yielded conflicting results, indicating that MSCs can promote or inhibit B-cell proliferation and differentiation [104].

In summary, bone healing is considered to be an osteoimmune system which encompasses complex interaction of bone cells and immune cells residing within the bone marrow. Both innate and adaptive immune cells influence the divergent phases of the fracture healing process starting right from the early inflammatory phase until the remodeling phase. Understanding the roles of not only the innate immune cells but also the adaptive immune cells can provide insights in developing new strategies targeting alterations in the immune response for fracture healing.

Modulation of the early inflammatory response as a strategy for bone formation

The initial pro-inflammatory reaction during fracture healing is crucial as it serves a dual purpose of initiating the defense mechanism and attracting necessary cells for subsequent stages of recovery. Modulating this early inflammatory response offers a promising avenue for enhancing bone formation by optimizing the microenvironment conducive to effective osteogenesis [59,105]. It can potentially restore endogenous cascades of fracture healing, preventing bone loss or non-unions and reducing the need for secondary surgeries. However, this approach

carries risks, including the possibility of systemic or chronic inflammation, which can be detrimental to many metabolic processes including bone healing.

Pro-inflammatory cytokines to enhance bone healing have garnered significant attention in regenerative medicine (**Figure 1**). One pro-inflammatory cytokine that has been extensively studied in the context of bone healing is TNF-α. TNF-α is involved in various cellular processes, including osteoclastogenesis, angiogenesis, and recruitment of immune cells to the site of injury [106]. Studies have shown that controlled administration of TNF-α can enhance bone formation and accelerate the healing process in animal models of fractures and bone defects [107–109]. TNF-α also stimulates osteoblast activity and the production of bone matrix components, ultimately leading to improved bone regeneration [107,109,110].

Another pro-inflammatory cytokine of interest is interleukin-1 (IL-1). IL-1 also promotes bone healing by stimulating osteoblast proliferation and differentiation and inducing the expression of osteogenic markers similar to TNF-α [106,108,111]. Additionally, IL-1 is crucial in angiogenesis, vital for delivering oxygen and nutrients to the healing site. Daily local injections of IL-1β for three days showed accelerated bone healing in vivo [112]. Through its pro-inflammatory properties, IL-1 contributes to the recruitment of immune cells that facilitate tissue repair and remodeling (**Figure 1**). IL-6 is another pro-inflammatory cytokine following the same signaling pathway as TNF and IL-1. To illustrate, *In vitro* studies investigating the role of IL-6 revealed an increase in RUNX2 and osteocalcin osteogenic markers [113,114] (**Figure 1**). Additionally, repeated sequential injections of parathyroid hormone (PTH) fragments and IL-6 in the fracture hematoma during the initial two weeks after the injury resulted in a significant enhancement of mechanical strength in the fracture callus and accelerated bone union *in vivo* [115].

While pro-inflammatory cytokines may hold the potential to enhance bone healing, it is essential to note that their application requires careful regulation and control. Excessive or prolonged inflammation can harm the healing process. Therefore, controlled release systems, using biomaterials or gene therapy approaches to precisely modulate the release and activity of pro-inflammatory cytokines are explored [116].

Our approach: use of bacterial antigens and their components

The clinical observation of new bone formation in patients with infections prompted the exploration of bacterial antigens as potential stimulators of bone formation [117]. It is generally acknowledged that bacterial infections harm bone formation, leading to osteolysis and bone loss. On the contrary, bacterial infections can also stimulate new bone formation, as seen in some cases of osteomyelitis [118]. Infections may also be the initiating factor in trauma-induced heterotopic ossifications, [119,120]. It is believed that the immune response triggered in an attempt to eliminate the bacteria, leads to the release of various inflammatory mediators like cytokines and chemokines that alter the status of bone-forming progenitor cells like MSCs towards immunosuppressive and regenerative phenotype. Other than eliminating the invading bacterial pathogen, the immune cells also contribute to new bone formation, especially in the case of heterotopic ossification.

It was demonstrated that whole viable bacteria, but also selective parts of bacteria are responsible for new bone formation. Croes et al. found that gamma-irradiated *Staphylococcus Aureus (S. Aureus)*, when injected in the medullary canal of rabbit tibia, resulted in thickening of the cortex without causing osteolysis as compared to live *S. Aureus* which caused osteolysis [121]. Furthermore, isolated cell wall extracts exhibit pro-osteogenic properties as shown by a net bone volume increase in the rabbit tibia when injected with crude *S. Aureus* cell wall extracts [121]. The killed bacteria or components thereof have the potential to regulate the immune response for bone regeneration. For clinical translation purposes, identifying a single or a group of synthetic bacterial components is a relatively more acceptable strategy. The challenges of using killed *S. Aureus* like off-target side effects and batch-to-batch inconsistencies can be avoided using particular bacterial cell wall components. The immunomodulatory effects of the cell wall extracts are primarily attributed to the activation of the toll-like receptors in various cell types. Thus, the impact of Lipoteichoic acid (LTA), an *S. Aureus*-derived TLR2 ligand, was investigated in a rabbit model of subcutaneous implantation [122]. When combined with BMP-2, LTA resulted in a dose-dependent increase in bone volume, indicating that selective bacterial components also could be used as adjuvants for bone formation [122]. These findings paved the way for assessing

the potential of synthetically available bacterial components known as pathogen recognition receptor PRR ligands for bone formation.

Pathogen recognition receptors (PRRs) act as pathogen sensors of the immune system

PRRs and the innate immune system

The innate immune system serves to protect the host from potential harm. Immune cells eliminate pathogens or damaged host material through prompt recognition and binding of the molecules present on the surface of the pathogens or at apoptotic and damaged cells [123,124]. The innate immune cells PRRs that enable them to continue the surveillance and provide protection against infections and tumors [52,125,126]. PRRs also initiate and participate in pathogen-tailored immune responses, triggering the adaptive immune response. Since the discovery of the Toll-like receptor (TLR) gene in Drosophila in 1994, scientists have identified numerous PRRs and their corresponding ligands [127,128]. PRRs are a family of specialized receptors expressed in innate immune cells like macrophages, dendritic cells, natural killer cells, and neutrophils (**Table 1**). To date, several PRRs have been identified such as Toll-like receptors (TLRs), Retinoic acid-inducible (RIG)-1-like (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs), each specialized in recognizing a signature pathogen structure (**Table 1**) [129].

Following an injury, local inflammation is triggered by danger signals released from damaged host tissue. Damage-associated molecular patterns (DAMPs) are secreted due to the damaged or necrotic cells owing to any potential presence of pathogens at the site of injury to the innate immune system [130,131]. DAMPs are host molecules explicitly released by dying host cells in situations like injury or chronic disease. On the other hand, pathogen associated molecular patterns (PAMPs) are molecules found on the surface of pathogens like viruses, fungi, and bacteria and have unique molecular or subcellular characteristics not present in

the host cells [131,132]. The innate immune cells, like macrophages, neutrophils, dendritic cells, eosinophils, and basophils, distinguish between the healthy host cells and PAMPs via the PRRs (**Table 1**) [133]. PRRs employ several mechanisms like recognizing subcellular and unique characteristics present displayed by PAMPs, crosstalk between PRRs or the presence of co-receptors and accessory proteins for PAMP recognition by PRR s[134,135]. Localization and differential expression of PRRs also enable effective recognition of PAMPs and help in discriminating them from host cells. They can detect self-derived molecules or altered self-structures, triggering tolerogenic responses and preventing excessive immune activation against self-antigens. PRRs are widely distributed in immune cells and can be found on the cell membrane, intracellular compartment membranes, and the cytoplasm (**Figure 2**) [136]. This thesis will only focus on TLRs NLRs and CLRs, while other PAMP binding molecules ALRs and RLRs will not be investigated.

PRRs and the adaptive immune system

PRRs play a crucial role in the adaptive immune system by bridging the innate and adaptive immune responses. While the adaptive immune system primarily relies on antigen-specific recognition by B and T lymphocytes, PRRs initiate and shape the adaptive immune responses. Upon encountering PAMPs or DAMPs, PRRs recognize and bind to these conserved molecular structures. When this recognition occurs, it sets off a series of signals that creates pro-inflammatory cytokines and activates antigen-presenting cells [132]. PRR signaling also promotes the upregulation of co-stimulatory molecules and major histocompatibility complex (MHC) molecules on APCs, facilitating efficient antigen presentation to T cells [137]. By linking innate and adaptive immunity, PRRs activate and mature antigen-presenting cells, promoting their ability to capture, process, and present antigens to T cells. This process enhances the priming of naive T cells and the subsequent activation of specific T cell responses. Also, PRR activation influences the differentiation of T helper cell subsets, directing the adaptive immune response towards appropriate effector functions [138]. Furthermore, PRRs regulate immune tolerance and maintain immune homeostasis [139].

PRR family	Pathogen Recongition Receptors (PRR)	PAMPs	Synthetic derivatives (PRR ligands)	Source	Cellular distribution
Toll-like receptors (TLRs)	TLR1 (TLR1-TLR2)	Triacyl lipoprotein	Pam3CSK4	Bacteria	Monocytes, Dendritic cells, Mast cells, Eosinophils, Basophils
	TLR2 (TLR1-TLR2) (TLR2-TLR6)	Lipoteichoic acid	Pam2CSK4	Bacteria	
		Arabinomannan Peptidoglycan Zymosan Lipoprotein Pore protein		Mycobacterium Bacteria Fungi Mycoplasma Neisseria	
	TLR3	dsRNA	Poly(I:C)	Virus	Macrophages, Dendritic cells, epithelial cells
	TLR4	Lipopolysacchride (LPS) Heat shock proteins	LPS	Bacteria Host	Macrophages, Dendritic cells, Mast cells, Eosinophils
	TLR5	Flagellin		Bacteria	Epithelial cells
	TLR6	Lipoteichoic acid (LTA) Peptidoglycan (PGN)	LTA PGN	Bacteria	Monocytes, Dendritic cells, Mast cells, Eosinophils, Basophils
	TLR7	ssRNA	Imiquimod/ Imidazoquinoline	Virus	plasmacytoid dendritic cells,macrophages, Eosinophils
	TLR8	ssRNA	Resiquimod	Virus	Macrophages, Neutrophils
	TLR9	bacterial CpG motifs	ODN (M362)/Tolamba	bacteria/ virus	plasmacytoid dendritic cells, Eosinophils, basophils
	TLR10	dsRNA		Virus	
Nucleotide binding oligomerization domain-like receptors (NLRs)	NOD-1	iE-DAP		Gram negative bacteria	cytosol of macrophages, epithelial cells
	NOD2	Muramyl Dipeptide (MDP)	Murabutide	Bacteria	
C-type lectin receptors (CLRs)	Dectin-1	β-Glucan	Curdlan	Fungus	Dendritic cells and macrophages
	Dectin-2	α-Mannan		Fungus	
RIG-I-like receptors (RLRs)	MDA5	Poly(I:C), long chain dsRNA		Virus	Cytosol
	LGP2 RIG-I	dsRNA short chain RNA		Virus Virus	
Absent in melanoma-2-like receptors (ALRs)	ALRs	dsDNA		Bacteria	Cytosol

Table 1. Overview of the common PRRs, and their activating ligands in the innate immune system. The synthetic PRR ligands tested in this thesis are highlighted in the red box. Adapted from [140]. Abbreviations: PRR (pathogen

recognition receptor), PAMPs (pathogen associated molecular pattern), MDA5 (melanoma differentiation associated gene 5), iE-DAP (γ-D-Glu-mDAP)

PRR-PAMP binding mechanisms

Toll-like receptors (TLRs)

TLRs are type I transmembrane glycoproteins that recognize specific ligands and induce signal transduction. As a result, immune cells are stimulated to secrete pro-inflammatory and antiviral factors [141–143]. To date, ten functional TLRs have been identified in humans. Some TLRs (TLR1, TLR2, TLR4, TLR5, TLR6) are found on the cell membrane in the form of heterodimers or homodimers, while TLR3, TLR7, TLR8, and TLR9 are homodimers expressed in the intracellular membranes. TLR10 on the other hand, is still being researched and hence, the location of this receptor is unknown. While cell surface-bound receptors mainly recognize lipids, lipoproteins, and proteins of the pathogen, nucleic acids of the pathogens are recognized by the intracellular receptors (**Table 1**) [144].

TLRs are composed of an extracellular region containing leucine-rich repeats (LRRs) and an intracellular transmembrane domain containing the Toll/IL-1R (TIR) domain (**Figure 2**) [145]. LRRs mediate the extracellular recognition of specific ligands while the intracellular TIR domain conducts signal by binding to myeloid differentiation primary response 88 (MyD88) adapter protein or TIR-domain-containing adapter-inducing interferon-β (TRIF) adapter protein in the cytoplasmic region (**Figure 2** & **Figure 3**) [146]. The study of the crystal structure of the TLR-PAMP complex helps in understanding the binding mechanism [147]. For example, **TLR2** forms heterodimers with **TLR1** or **TLR6** to recognize tri-acylated and di-acylated lipoproteins, synthetic **Pam3CSK4, Pam2CSK4**, and TLR2/TLR1-triacylated lipoproteins form M-type crystal structures (**Table 1**) [148]. Further, **TLR3** uses the N-terminus and C-terminus of the LRR domain to bind to double-stranded RNA (**dsRNA**) in its natural origin or to **Poly(I:C)** in synthetic form (**Figure 2**, **Table 1**) [149]. Similarly, **TLR5** recognizes **bacterial flagellin** in the form of a homodimer and thus plays a significant role in pathogen elimination and immune homeostasis regulation [150]. On the other hand,

lipopolysaccharide (LPS) is initially recognized by LRR structural protein CD14 on the surface of the monocytes and macrophages and later forms a complex with **TLR4** /myeloid differentiation 2 (MD2) (**Figure 2**) [151]. Intracellular **TLR8** and **TLR9** exist as preformed dimers to allow their effective recognition of **single stranded RNA** in its natural origin as well as **Resiquimod** in its synthetic form and **unmethylated CpG motifs (ODN M362)** ligands respectively (**Table 1**, **Figure 2**) [147].

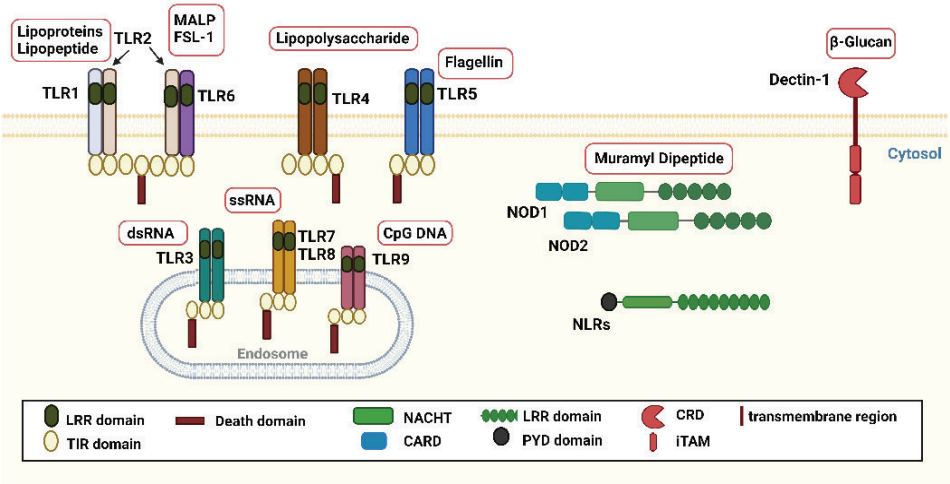


Figure 2. **The structure of the PRRs is displayed with their PRR-ligand binding complexes** (in red outlined boxes). Adapted from [140]. TLR (Toll-like receptors), NLR (NOD-like receptors), MALP (macrophage activating lipopeptide), FSL-1 (fibroblast stimulating lipopeptide), LRR (leucine-rich repeats), TIR (Toll/IL-1R domain), dsRNA(double stranded ribonucleic acid), ssRNA (single stranded ribonucleic acid), CARD (caspase activation and recruitment domain), PYD (pyrin domain), CRD (carbohydrate recognition domain), iTAM (immunoreceptor tyrosine/based activation motif).

NOD-like receptors (NLRs)

NOD-like receptors (NLRs) are intracellular PRRs in the cytoplasm that recognize the virus particles, some bacteria, and parasites that have entered the cytoplasm [152]. NLRs contain three domains: LRRs that identify the ligands, a central

nucleotide-binding domain (NBD) which plays a crucial role in exposing the NACHT domain for nucleic acid binding and oligomerization of NLRs and finally, a protein interaction domain such as caspase activation and recruitment domain (CARD) or pyrin domain (PYD) (**Figure 2**) [153]. Unlike TLRs, NLRs do not possess transmembrane domains. When ligands bind directly or indirectly to LRRs, NLRs undergo a conformational change, exposing the NACHT domain and activating NLRs [154]. Simultaneously, the CARD is also revealed, leading to homotypic interactions resulting in downstream adaptor molecules recruited to initiate signal transduction (**Figure 2**) [155].

Being the most studied NLR, **NOD1** recognizes diaminopimelic acid present on the cell wall of gram-negative bacteria (**Table 1**) [156]. At the same time, **NOD2** recognition ranges from the **muramyl dipeptide (MDP)** and **Murabutide** (synthetic form) in all bacterial cell walls to a complete viral single-stranded RNA (**Table 1**) [157,158]. For example**,** when macrophages engulf harmful bacteria, they form phagolysosomes. The bacteria's cell wall components are broken down into peptidoglycan by lysosomal enzymes. **Peptidoglycan** can be further degraded into a cell wall peptide with an immunomodulatory activity that enters the cytosol, activating **NOD2** (**Table 1**) [159].

C-type lectin receptors (CLRs)

C-type lectin receptor (CLRs) are phagocytic receptors that bind to Fungus and place pathogens in cytoplasmic vesicles for direct digestion and elimination [160]. CLRs detect the carbohydrates found in pathogenic microorganisms in the presence of calcium ions (Ca^{2+}) [161]. They can distinguish them from the host via a compact spherical structure called the carbohydrate recognition domain (CRD) (**Figure 2**) [162]. CRD is connected to the intracellular region the immunoreceptor tyrosine/based activation motif (iTAM) via a transmembrane region. iTAM is responsible for signal transduction (**Figure 2**) [163]. Among the CLR family members, dendritic cell-associated C-type lectins, Dectin-1 and Dectin-2 are important representatives. **Dectin-1** recognizes fungal pathogens such as ***Candida albicans***, *Pneumocystis carinii*, and yeast (**Table 1**) [164–166]. **β-1,3-glucan** in its natural form and **Curdlan** in its synthetic form serves as the ligand for **Dectin-1**,

which can trigger downstream signaling pathways through both tyrosine-kinase-dependent and tyrosine-kinase-independent mechanisms (**Table 1**) [167].

PRRs and immune cells - Signaling pathways

PRRs exhibit diverse activation mechanisms in response to their specific ligands. However, despite these variations, the three critical components involved in signal transduction—protein kinases, adaptor proteins, and transcription factors—serve similar functions. Consequently, these molecules contribute to the activation of shared signaling pathways, namely, **NF-κB, MAPK, and the interferon regulatory factor** signaling pathway (**Figure 3**). This activation, in turn, triggers the transcription of proinflammatory cytokines, type I interferons, and chemokines.

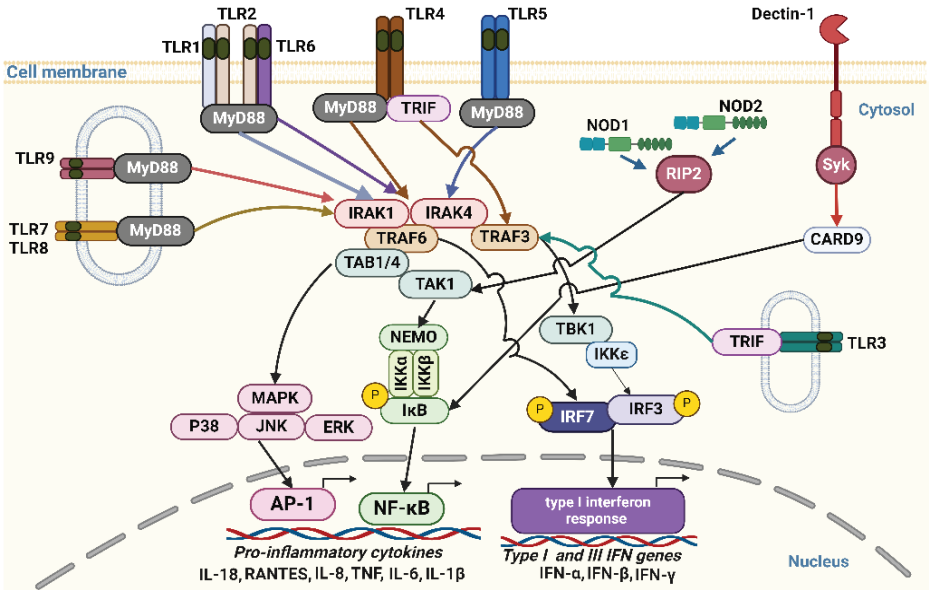


Figure 3. **Overview of the signaling pathways involved upon recognition of PRR ligands by various PRRs**. Adapted from [140]. MyD88 (myeloid differentiation primary response 88), (TRIF) TIR-domain-containing adapter-inducing interferon-β, IRAK (interleukin-1 receptor associated kinase), RIP2 (receptor-interacting serine-threonine protein 2), TRAF (TNF receptor associated

factor), TAK(TGF-β- activated kinase), IKK (inhibitor of nuclear factor-κB (IκB) kinase), Syk (spleen tyrosine kinase), MAPK (mitogen-activated protein kinase), JNK (c-Jun N-terminal kinase), (IRF) interferon regulatory factor.

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway

NF-κB is the most commonly activated signaling pathway in immune cells resulting in cellular inflammation and immune response[168]. It is activated via all TLRs, except TLR3 and activate the MyD88-dependent pathway. MyD88-dependent signaling leads to the secretion of pro-inflammatory cytokines TNF-α, IL-6, and chemokines [169,170]. MyD88's C-terminus interacts with TLRs' TIR domain inside the cell, which brings in interleukin-1 receptor associated kinase- 4 (IRAK4) to activate IRAK1 and IRAK2 (**Figure 3**) [171]. Conversely, NLRs (NOD1 and 2) recruit downstream receptor-interacting serine-threonine protein 2 (RIP2) through its CARD (**Figure 3**) [172]. These processes trigger the recruitment of TNF receptor associated factor (TRAF6), TGF-β- activated kinase (TAK1), two TAK binding proteins TAB1, and TAB4, which combine to form a complex. Because of this, the ubiquitination process leads to the degradation of TRAF6 [173,174]. The TAK1-TAB1-TAB4 complex activates the inhibitor of nuclear factor-κB (IκB) kinase **(**IKK) complex, which phosphorylates and degrades IκB via ubiquitination. As a result, NF-κB is released and translocated to the nucleus, regulating the transcription of inflammatory genes (**Figure 3**) [175,176].

CLR -mediated signaling pathways (Dectin-1) in NF-κB activation differ from other typical pathways. In these pathways, phosphorylated iTAM motifs on CLRs associate with and activate spleen tyrosine kinase (Syk). Syk, in turn, triggers downstream events by activating protein kinase C-δ, which leads to the phosphorylation of CARD9 [177]. The phosphorylated CARD9 forms a complex with B cell lymphoma and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (**Figure 3**) [178]. This three-molecule complex effectively activates NF-κB, facilitating the transcription of genes involved in inflammatory responses [179].

The mitogen-activated protein kinase (MAPK) signaling pathway

The MAPK pathway is a crucial intersection for multiple signal transduction pathways, playing essential roles in cell proliferation, stress response, inflammation, differentiation, synchronization, transformation, and apoptosis [180,181]. It acts as a vital mediator, transmitting signals from the cell surface to the nucleus. Within the MyD88-dependent Toll-like receptors (TLRs) pathway, IRAK-1 becomes activated through phosphorylation and interacts with TRAF6. Apart from activating the IKK complex, it can also activate MAPKs, namely c-Jun N-terminal kinase (JNK) and p38 MAPK [182]. Moreover, when bacterial components invade cells, NLRs are activated, recruiting downstream CARD9, which subsequently activates p38, and JNK, ultimately leading to the activation of the MAPK pathway (**Figure 3**) [183]. This activation contributes to the release of pro-inflammatory factors.

The interferon regulatory factor 3/7 (IRF3/7) signaling pathway

TRIF is the adaptor protein of the MyD88 independent pathway. Activation of TRIF mainly induces the production of type I interferons and plays a crucial role in antiviral responses (**Figure 3**) [184]. TLR3 and TLR4 recruit the TRIF, followed by TRAF3 activation, leading to the recruitment of IKKε/TANK-binding kinase 1 (TBK1)[185]. This complex then leads to the phosphorylation of IRF3 and activation of IFN genes leading to the release of IFN-α and IFN-β [186]. TLR7 and TLR9 ligands also induce type I IFNs through the activation of IRF7 [187,188]. MyD88 interacts with IRF7 along with IRAK1, IRAK4, IKKα, TRAF3, and TRAF6 (**Figure 3**).

PRRs in clinics

PRRs and their ligands have become a hot topic in immunology and drug research due to their essential role in innate immunity. These receptors and their various ligands offer potential drug targets for conditions such as inflammation, tumors, autoimmune diseases, microbial infections, and more, making them a promising avenue for immunotherapy [140,189]. Activating PRRs can have both positive and

negative effects. While it can stimulate innate and adaptive immunity, which helps defend against pathogens, it can also cause the release of many cytokines, leading to an inflammatory microenvironment and potential tissue damage [133,190].

Strategies for using PRRs often involve using ligand analogs to activate them or antagonists to inhibit their activation [191,192]. **Imiquimod**, a **TLR7** agonist, was the first PRR ligand that was approved for in the form of a cream for its use in treating Bowens disease, genital warts and basal cell carcinoma It has the potential to modulate the immune system and treat tumors by stimulating the production of cytokines like IFN-α, IL-6, and TNF-α that regulate the immune response [193]. Recent studies have demonstrated that **Selgantolimod**, a new **TLR8** agonist, triggers cytokine responses in individuals with chronic hepatitis B infection[194,195]. Nevertheless, additional research with extended treatment periods is required to evaluate its effectiveness. Studies have shown that **TLR9** agonist, CpG oligonucleotides (**CpG ODN**) can be a powerful antitumor treatment. Structurally modified CpG ODN mimics like IMO-2055, MGN-1703, and MGN-1704 have already demonstrated their effectiveness as a non-small cell lung cancer and sigmoid colon cancer respectively [196]. Clinical trials for MGN-1703 are currently being conducted to treat advanced colorectal cancer as it triggers a robust type I IFN response in the gut. **Resiquimod**, an **TLR7/TLR8** agonist is also currently being used as a treatment for cutaneous T cell lymphoma, skin lesions and as vaccine for influenza. Additionally, clinical trials are underway for non-metastatic sarcoma using NLR agonists such as Mifamurtide.

Clinically, PRR agonists can also be used as adjuvants in immunotherapy, as over period, hosts develop resistance towards existing drugs. One of the examples is recent FDA-approved CYFENDUS vaccine developed against anthrax disease. CYFENDUS consists of alum and **CpG 7909**, a **TLR9** agonist that is used as an adjuvant. Another example is the conjugated STING and **TLR1/2** agonist **Pam3CSK4**-CDGSF which is currently being investigated as an effective adjuvant for constructing vaccines to enhance antitumor immunotherapy. A **TLR3** ligand, **Poly(I:C)** along with a peptide is currently being investigated as a therapy for prostate cancer. It is worth mentioning that even with considerable number of clinical trials employing PRR ligands, advancements are still in progress. Extensive studies in the field of immunology focusing on PRR ligand- PRR

binding mechanisms will help identifying new targets and methods for disease treatments.

The research aims and thesis outline

This thesis explores the potential of pathogen recognition receptor (PRR) ligands

as immunomodulatory agents to enhance fracture repair, or

as an additive agent to improve the performance of the available off-the-shelf bone substitutes.

The present **Chapter 1** introduces an understanding of the stages of the fracture healing process and highlights the importance of the early inflammatory phase in fracture healing. The crosstalk between the immune cells and MSCs was also reviewed. This chapter also establishes the role of PRR ligands in the innate and adaptive immune systems, their structure and downstream signaling pathways along with their current clinical status paving the way for assessing the potential of PRR ligands (mentioned in **Table 1**, highlighted in the red box and in bold throughout the text) as immunomodulators in bone regeneration.

To evaluate the effects of PRR ligands for bone regeneration, in **Chapter 2** we first investigated their direct influence on bone cells progenitors namely, MSCs and monocytes using simple 2D invitro models. MSCs derived from bone marrow were differentiated into osteogenic lineage whereas monocytes were differentiated into bone resorbing osteoclasts. This simple invitro model was applied to screen for different individual PRR ligands targeting an array of PRRs in various concentration ranges. Since PRR ligands are shown to possess immunomodulatory effects, we investigated their immunomodulatory properties on human MSCs and human monocytes by characterizing the cytokines secreted upon stimulation.

In **Chapter 3**, we established a novel in vitro model to test the effect of the soluble factors secreted by the immune cells on the osteogenic differentiation of human MSCs. This model was developed to take into account the effects of the crosstalk

between the immune cells and MSCs. With this model, we first established the effects of non-stimulated whole peripheral blood mononuclear cells (immune cells) on MSC osteogenic differentiation in vitro. Further, we applied this model to evaluate the direct or via the immune cells' effects of the immunomodulatory PRR ligands for promoting MSC osteogenic differentiation.

In **Chapter 4**, we pointed out the positive effects of gamma-irradiated *S. aureus* on bone formation observed in various rabbit models. We determined the effect and the underlying mechanism of gamma-irradiated *S. aureus* stimulation on the osteogenic differentiation of human MSCs with the aim of replacing gamma-irradiated *S. aureus* by using a combination of PRR ligands (mixtures). We first evaluated the effect of gamma-irradiated *S. aureus* and the mixtures on osteogenic differentiation of MSCs by using previously established immune-cells- MSCs setup. We also compared the PRR activation pattern induced in the immune cells as well as the signaling pathways involved in gamma-irradiated *S. aureus* to different mixtures of PRR ligands. Further characterization of the secreted soluble factors by the immune cells upon stimulation with gamma-irradiated *S. aureus* and the mixtures was also investigated. We aimed at mimicking and identifying the mixture composition of the PRR ligands that most closely mimics the immune response as well as the osteogenic response of gamma-irradiated *S. aureus*.

After the in vitro evaluation of the PRR ligands on the immune cells and bone cells in vitro, in **Chapter 5,** we investigated their effects on fracture healing in an ovariectomized closed femur fracture model in rats. We delivered the PRR ligands at the fracture site through a local injection to elicit a local immune response. We also assessed the early immune response in fracture hematomas and monitored the fracture healing via radiographic analysis over four weeks.

Lastly, all findings presented in this thesis are summarized and discussed in a broader context in **Chapter 6**, indicating the limitations and future perspectives in the field.

References

1. Buck, D.W.; Dumanian, G.A. Bone Biology and Physiology: Part I. The Fundamentals. *Plast Reconstr Surg* **2012**, *129*, 1314–1320, doi: 10.1097/PRS.0b013e31824eca94.
2. Cipriani, C.; Colangelo, L.; Santori, R.; Renella, M.; Mastrantonio, M.; Minisola, S.; Pepe, J. The Interplay Between Bone and Glucose Metabolism. *Front Endocrinol (Lausanne)* **2020**, *11*, 122, doi:10.3389/fendo.2020.00122.
3. Rucci, N. Molecular Biology of Bone Remodelling. *Clin Cases Miner Bone Metab* **2008**, *5*, 49–56.
4. Loi, F.; Córdova, L.A.; Pajarinen, J.; Lin, T.; Yao, Z.; Goodman, S.B. Inflammation, Fracture and Bone Repair. *Bone* **2016**, *86*, 119–130, doi: 10.1016/j.bone.2016.02.020.
5. Caetano-Lopes, J.; Canhão, H.; Fonseca, J.E. Osteoblasts and Bone Formation. *Acta Reumatol Port* **2007**, *32*, 103–110.
6. Marie, P.J. Bone Cell–Matrix Protein Interactions. *Osteoporos Int* **2009**, *20*, 1037–1042, doi:10.1007/s00198-009-0856-7.
7. Nakamura, H. Morphology, Function, and Differentiation of Bone Cells. *Journal of Hard Tissue Biology* **2007**, *16*, 15–22, doi:10.2485/jhtb.16.15.
8. Gilbert, S.F. Osteogenesis: The Development of Bones. In *Developmental Biology. 6th edition*; Sinauer Associates, 2000.
9. Datta, H.K.; Ng, W.F.; Walker, J.A.; Tuck, S.P.; Varanasi, S.S. The Cell Biology of Bone Metabolism. *Journal of Clinical Pathology* **2008**, *61*, 577–587, doi:10.1136/jcp.2007.048868.
10. Wildemann, B.; Ignatius, A.; Leung, F.; Taitsman, L.A.; Smith, R.M.; Pesántez, R.; Stoddart, M.J.; Richards, R.G.; Jupiter, J.B. Non-Union Bone Fractures. *Nat Rev Dis Primers* **2021**, *7*, 1–21, doi:10.1038/s41572-021-00289-8.
11. Tzioupis, C.; Giannoudis, P.V. Prevalence of Long-Bone Non-Unions. *Injury* **2007**, *38 Suppl 2*, S3-9, doi:10.1016/s0020-1383(07)80003-9.
12. Ekegren, C.L.; Edwards, E.R.; De Steiger, R.; Gabbe, B.J. Incidence, Costs and Predictors of Non-Union, Delayed Union and Mal-Union Following Long Bone Fracture. *International Journal of Environmental Research and Public Health* **2018**, *15*, 2845, doi:10.3390/ijerph15122845.
13. Brinker, M.R.; Trivedi, A.; O'Connor, D.P. Debilitating Effects of Femoral Nonunion on Health-Related Quality of Life. *J Orthop Trauma* **2017**, *31*, e37–e42, doi:10.1097/BOT.0000000000000736.
14. Shapiro, J.A.; Stillwagon, M.R.; Tornetta, P.I.; Seaver, T.M.; Gage, M.; O'Donnell, J.; Whitlock, K.; Yarboro, S.R.; Jeray, K.J.; Obremskey, W.T.; et al. Serology and Comorbidities in Patients With Fracture Nonunion: A Multicenter

Evaluation of 640 Patients. *JAAOS - Journal of the American Academy of Orthopaedic Surgeons* **2022**, 10.5435/JAAOS, doi:10.5435/JAAOS-D-21-00366.

15. Nicholson, J.; Makaram, N.; Simpson, A.; Keating, J. Fracture Nonunion in Long Bones: A Literature Review of Risk Factors and Surgical Management. *Injury* **2021**, *52*, S3–S11, doi: 10.1016/j.injury.2020.11.029.

16. Nauth, A.; Schemitsch, E.; Norris, B.; Nollin, Z.; Watson, J.T. Critical-Size Bone Defects: Is There a Consensus for Diagnosis and Treatment? *Journal of Orthopaedic Trauma* **2018**, *32*, S7, doi:10.1097/BOT.0000000000001115.

17. Pneumaticos, S.G.; Triantafyllopoulos, G.K.; Chatziioannou, S.; Basdra, E.K.; Papavassiliou, A.G. Biomolecular Strategies of Bone Augmentation in Spinal Surgery. *Trends in Molecular Medicine* **2011**, *17*, 215–222, doi: 10.1016/j.molmed.2010.12.002.

18. Battafarano, G.; Rossi, M.; De Martino, V.; Marampon, F.; Borro, L.; Secinaro, A.; Del Fattore, A. Strategies for Bone Regeneration: From Graft to Tissue Engineering. *International Journal of Molecular Sciences* **2021**, *22*, 1128, doi:10.3390/ijms22031128.

19. Tuchman, A.; Brodke, D.S.; Youssef, J.A.; Meisel, H.-J.; Dettori, J.R.; Park, J.-B.; Yoon, S.T.; Wang, J.C. Iliac Crest Bone Graft versus Local Autograft or Allograft for Lumbar Spinal Fusion: A Systematic Review. *Global Spine Journal* **2016**, *6*, 592–606, doi:10.1055/s-0035-1570749.

20. Oryan, A.; Alidadi, S.; Moshiri, A.; Maffulli, N. Bone Regenerative Medicine: Classic Options, Novel Strategies, and Future Directions. *Journal of Orthopaedic Surgery and Research* **2014**, *9*, 18, doi:10.1186/1749-799X-9-18.

21. Betz, R.R. Limitations of Autograft and Allograft: New Synthetic Solutions. *Orthopedics* **2002**, *25*, S561–S570, doi:10.3928/0147-7447-20020502-04.

22. Marchand, L.S.; Rothberg, D.L.; Kubiak, E.N.; Higgins, T.F. Is This Autograft Worth It? *Journal of Orthopaedic Trauma* **2017**, *31*, 205–209, doi:10.1097/BOT.0000000000000811.

23. Buser, Z.; Brodke, D.S.; Youssef, J.A.; Meisel, H.-J.; Myhre, S.L.; Hashimoto, R.; Park, J.-B.; Tim Yoon, S.; Wang, J.C. Synthetic Bone Graft versus Autograft or Allograft for Spinal Fusion: A Systematic Review. *J Neurosurg Spine* **2016**, *25*, 509–516, doi:10.3171/2016.1.SPINE151005.

24. Lee, S.-C.; Chen, J.-F.; Wu, C.-T.; Lee, S.-T. In Situ Local Autograft for Instrumented Lower Lumbar or Lumbosacral Posterolateral Fusion. *J Clin Neurosci* **2009**, *16*, 37–43, doi: 10.1016/j.jocn.2008.02.009.

25. Sengupta, D.K.; Truumees, E.; Patel, C.K.; Kazmierczak, C.; Hughes, B.; Elders, G.; Herkowitz, H.N. Outcome of Local Bone versus Autogenous Iliac Crest Bone Graft in the Instrumented Posterolateral Fusion of the Lumbar Spine. *Spine (Phila Pa 1976)* **2006**, *31*, 985–991, doi: 10.1097/01.brs.0000215048.51237.3c.

26. Ohtori, S.; Koshi, T.; Yamashita, M.; Takaso, M.; Yamauchi, K.; Inoue, G.; Suzuki, M.; Orita, S.; Eguchi, Y.; Ochiai, N.; et al. Single-Level Instrumented Posterolateral Fusion versus Non-Instrumented Anterior Interbody Fusion for

Lumbar Spondylolisthesis: A Prospective Study with a 2-Year Follow-Up. *J Orthop Sci* **2011**, *16*, 352–358, doi:10.1007/s00776-011-0088-5.

27. Inage, K.; Ohtori, S.; Koshi, T.; Suzuki, M.; Takaso, M.; Yamashita, M.; Yamauchi, K.; Inoue, G.; Orita, S.; Eguchi, Y.; et al. One, Two-, and Three-Level Instrumented Posterolateral Fusion of the Lumbar Spine with a Local Bone Graft: A Prospective Study with a 2-Year Follow-Up. *Spine (Phila Pa 1976)* **2011**, *36*, 1392–1396, doi:10.1097/BRS.0b013e3181f40e69.

28. Han, S.; Park, B.; Lim, J.-W.; Youm, J.-Y.; Choi, S.-W.; Kim, D.H.; Ahn, D.K. Comparison of Fusion Rate between Demineralized Bone Matrix versus Autograft in Lumbar Fusion : Meta-Analysis. *J Korean Neurosurg Soc* **2020**, *63*, 673–680, doi:10.3340/jkns.2019.0185.

29. Nickoli, M.S.; Hsu, W.K. Ceramic-Based Bone Grafts as a Bone Grafts Extender for Lumbar Spine Arthrodesis: A Systematic Review. *Global Spine J* **2014**, *4*, 211–216, doi:10.1055/s-0034-1378141.

30. Nouri, A.; Wen, C. 5 - Noble Metal Alloys for Load-Bearing Implant Applications. In *Structural Biomaterials*; Wen, C., Ed.; Woodhead Publishing Series in Biomaterials; Woodhead Publishing, 2021; pp. 127–156 ISBN 978-0-12-818831-6.

31. van Dijk, L.A.; Barbieri, D.; Barrère-de Groot, F.; Yuan, H.; Oliver, R.; Christou, C.; Walsh, W.R.; de Bruijn, J.D. Efficacy of a Synthetic Calcium Phosphate with Submicron Surface Topography as Autograft Extender in Lapine Posterolateral Spinal Fusion. *Journal of Biomedical Materials Research - Part B Applied Biomaterials* **2019**, 1–11, doi:10.1002/jbm.b.34301.

32. van Dijk, L.A.; Barrère-de Groot, F.; Rosenberg, A.J.W.P.; Pelletier, M.; Christou, C.; de Bruijn, J.D.; Walsh, W.R. MagnetOs, Vitoss, and Novabone in a Multi-Endpoint Study of Posterolateral Fusion. *Clin Spine Surg* **2020**, *33*, E276–E287, doi:10.1097/BSD.0000000000000920.

33. Lehr, A.M.; Oner, F.C.; Delawi, D.; Stellato, R.K.; Hoebink, E.A.; Kempen, D.H.R.; van Susante, J.L.C.; Castelein, R.M.; Kruyt, M.C.; Dutch Clinical Spine Research Group Increasing Fusion Rate Between 1 and 2 Years After Instrumented Posterolateral Spinal Fusion and the Role of Bone Grafting. *Spine (Phila Pa 1976)* **2020**, *45*, 1403–1410, doi:10.1097/BRS.0000000000003558.

34. Lehr, A.M.; Oner, F.C.; Delawi, D.; Stellato, R.K.; Hoebink, E.A.; Kempen, D.H.R.; van Susante, J.L.C.; Castelein, R.M.; Kruyt, M.C. Efficacy of a Standalone Microporous Ceramic Versus Autograft in Instrumented Posterolateral Spinal Fusion. *Spine* **2020**, *45*, 944–951, doi:10.1097/BRS.0000000000003440.

35. Albrektsson, T.; Johansson, C. Osteoinduction, Osteoconduction and Osseointegration. *European Spine Journal* **2001**, *10*, S96–S101, doi:10.1007/s005860100282.

36. Lode, A.; Wolf-Brandstetter, C.; Reinstorf, A.; Bernhardt, A.; König, U.; Pompe, W.; Gelinsky, M. Calcium Phosphate Bone Cements, Functionalized with

VEGF: Release Kinetics and Biological Activity. *Journal of Biomedical Materials Research Part A* **2007**, *81A*, 474–483, doi: 10.1002/jbm.a.31024.
37. Wu, M.; Chen, G.; Li, Y.-P. TGF-β and BMP Signaling in Osteoblast, Skeletal Development, and Bone Formation, Homeostasis and Disease. *Bone Res* **2016**, *4*, 1–21, doi:10.1038/boneres.2016.9.
38. Davies, O.G.; Grover, L.M.; Lewis, M.P.; Liu, Y. PDGF Is a Potent Initiator of Bone Formation in a Tissue Engineered Model of Pathological Ossification. *J Tissue Eng Regen Med* **2018**, *12*, e355–e367, doi:10.1002/term.2320.
39. Ayala-Cuellar, A.P.; Kang, J.-H.; Jeung, E.-B.; Choi, K.-C. Roles of Mesenchymal Stem Cells in Tissue Regeneration and Immunomodulation. *Biomol Ther (Seoul)* **2019**, *27*, 25–33, doi:10.4062/biomolther.2017.260.
40. Giai Via, A.; Frizziero, A.; Oliva, F. Biological Properties of Mesenchymal Stem Cells from Different Sources. *Muscles Ligaments Tendons J* **2012**, *2*, 154–162.
41. Blokhuis, T.J.; Buma, P.; Verdonschot, N.; Gotthardt, M.; Hendriks, T. BMP-7 Stimulates Early Diaphyseal Fracture Healing in Estrogen Deficient Rats. *Journal of Orthopaedic Research* **2012**, *30*, 720–725, doi: 10.1002/jor.22013.
42. Fernandez, L.; Petrizzo, A. The Use of Bone Morphogenetic Protein 2 (BMP-2) in Spine Surgery: Is It Valuable? *Bulletin of the NYU Hospital for Joint Diseases* **2023**, *81*, 40–46.
43. James, A.W.; LaChaud, G.; Shen, J.; Asatrian, G.; Nguyen, V.; Zhang, X.; Ting, K.; Soo, C. A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2. *Tissue Engineering Part B: Reviews* **2016**, *22*, 284–297, doi: 10.1089/ten.teb.2015.0357.
44. Epstein, N.E. Complications Due to the Use of BMP/INFUSE in Spine Surgery: The Evidence Continues to Mount. *Surg Neurol Int* **2013**, *4*, S343–S352, doi:10.4103/2152-7806.114813.
45. Guillot, R.; Gilde, F.; Becquart, P.; Sailhan, F.; Lapeyrere, A.; Logeart-Avramoglou, D.; Picart, C. The Stability of BMP Loaded Polyelectrolyte Multilayer Coatings on Titanium. *Biomaterials* **2013**, *34*, 5737–5746, doi: 10.1016/j.biomaterials.2013.03.067.
46. Lee, J.S.; Lee, J.C.; Heo, J.S. Polydopamine-Assisted BMP-2 Immobilization on Titanium Surface Enhances the Osteogenic Potential of Periodontal Ligament Stem Cells via Integrin-Mediated Cell-Matrix Adhesion. *Journal of Cell Communication and Signaling* **2018**, *12*, 661–672, doi:10.1007/s12079-018-0468-0.
47. Koolen, M.; Longoni, A.; van der Stok, J.; Van der Jagt, O.; Gawlitta, D.; Weinans, H. Complete Regeneration of Large Bone Defects in Rats with Commercially Available Fibrin Loaded with BMP-2. *Eur Cell Mater* **2019**, *38*, 94–105, doi:10.22203/eCM.v038a08.
48. Schmidt-Bleek, K.; Kwee, B.J.; Mooney, D.J.; Duda, G.N. Boon and Bane of Inflammation in Bone Tissue Regeneration and Its Link with Angiogenesis. *Tissue Engineering Part B: Reviews* **2015**, *21*, 354–364, doi: 10.1089/ten.teb.2014.0677.

49. Bucher, C.H.; Schlundt, C.; Wulsten, D.; Sass, F.A.; Wendler, S.; Ellinghaus, A.; Thiele, T.; Seemann, R.; Willie, B.M.; Volk, H.-D.; et al. Experience in the Adaptive Immunity Impacts Bone Homeostasis, Remodeling, and Healing. *Frontiers in Immunology* **2019**, *10*.
50. Berkmann, J.C.; Herrera Martin, A.X.; Ellinghaus, A.; Schlundt, C.; Schell, H.; Lippens, E.; Duda, G.N.; Tsitsilonis, S.; Schmidt-Bleek, K. Early pH Changes in Musculoskeletal Tissues upon Injury—Aerobic Catabolic Pathway Activity Linked to Inter-Individual Differences in Local pH. *International Journal of Molecular Sciences* **2020**, *21*, 2513, doi:10.3390/ijms21072513.
51. Lu, C.; Saless, N.; Wang, X.; Sinha, A.; Decker, S.; Kazakia, G.; Hou, H.; Williams, B.; Swartz, H.M.; Hunt, T.K.; et al. The Role of Oxygen during Fracture Healing. *Bone* **2013**, *52*, 220–229, doi: 10.1016/j.bone.2012.09.037.
52. Gajewski, T.F.; Schreiber, H.; Fu, Y.-X. Innate and Adaptive Immune Cells in the Tumor Microenvironment. *Nat Immunol* **2013**, *14*, 1014–1022, doi:10.1038/ni.2703.
53. Baht, G.S.; Vi, L.; Alman, B.A. The Role of the Immune Cells in Fracture Healing. *Curr Osteoporos Rep* **2018**, *16*, 138–145, doi:10.1007/s11914-018-0423-2.
54. Shiu, H.T.; Leung, P.C.; Ko, C.H. The Roles of Cellular and Molecular Components of a Hematoma at Early Stage of Bone Healing. *Journal of Tissue Engineering and Regenerative Medicine* **2018**, *12*, e1911–e1925, doi:10.1002/term.2622.
55. Kolar, P.; Schmidt-Bleek, K.; Schell, H.; Gaber, T.; Toben, D.; Schmidmaier, G.; Perka, C.; Buttgereit, F.; Duda, G.N. The Early Fracture Hematoma and Its Potential Role in Fracture Healing. *Tissue Engineering Part B: Reviews* **2010**, *16*, 427–434, doi: 10.1089/ten.teb.2009.0687.
56. Park, S.-H.; Silva, M.; Bahk, W.-J.; McKellop, H.; Lieberman, J.R. Effect of Repeated Irrigation and Debridement on Fracture Healing in an Animal Model. *Journal of Orthopaedic Research* **2002**, *20*, 1197–1204, doi:10.1016/S0736-0266(02)00072-4.
57. Tsunoda, M.; Mizuno, K.; Matsubara, T. The Osteogenic Potential of Fracture Hematoma and Its Mechanism on Bone Formation--through Fracture Hematoma Culture and Transplantation of Freeze-Dried Hematoma. *Kobe J Med Sci* **1993**, *39*, 35–50.
58. Wheatley, B.M.; Nappo, K.E.; Christensen, D.L.; Holman, A.M.; Brooks, D.I.; Potter, B.K. Effect of NSAIDs on Bone Healing Rates: A Meta-Analysis. *JAAOS - Journal of the American Academy of Orthopaedic Surgeons* **2019**, *27*, e330, doi:10.5435/JAAOS-D-17-00727.
59. Mountziaris, P.M.; Spicer, P.P.; Kasper, F.K.; Mikos, A.G. Harnessing and Modulating Inflammation in Strategies for Bone Regeneration. *Tissue Engineering Part B: Reviews* **2011**, *17*, 393–402, doi: 10.1089/ten.teb.2011.0182.
60. Ehnert, S.; Relja, B.; Schmidt-Bleek, K.; Fischer, V.; Ignatius, A.; Linnemann, C.; Rinderknecht, H.; Huber-Lang, M.; Kalbitz, M.; Histing, T.; et al.

Effects of Immune Cells on Mesenchymal Stem Cells during Fracture Healing. *World J Stem Cells* **2021**, *13*, 1667–1695, doi:10.4252/wjsc.v13.i11.1667.

61. Opal, S.M. Phylogenetic and Functional Relationships between Coagulation and the Innate Immune Response. *Critical Care Medicine* **2000**, *28*, S77.

62. Hurst, S.M.; Wilkinson, T.S.; McLoughlin, R.M.; Jones, S.; Horiuchi, S.; Yamamoto, N.; Rose-John, S.; Fuller, G.M.; Topley, N.; Jones, S.A. IL-6 and Its Soluble Receptor Orchestrate a Temporal Switch in the Pattern of Leukocyte Recruitment Seen during Acute Inflammation. *Immunity* **2001**, *14*, 705–714, doi:10.1016/S1074-7613(01)00151-0.

63. Xing, Z.; Lu, C.; Hu, D.; Yu, Y.; Wang, X.; Colnot, C.; Nakamura, M.; Wu, Y.; Miclau, T.; Marcucio, R.S. Multiple Roles for CCR2 during Fracture Healing. *Disease Models & Mechanisms* **2010**, *3*, 451–458, doi:10.1242/dmm.003186.

64. Kovtun, A.; Bergdolt, S.; Wiegner, R.; Radermacher, P.; Huber-Lang, M.; Ignatius, A. The Crucial Role of Neutrophil Granulocytes in Bone Fracture Healing. *European cells & materials* **2016**, doi:10.22203/ECM.V032A10.

65. Chung, R.; Cool, J.C.; Scherer, M.A.; Foster, B.K.; Xian, C.J. Roles of Neutrophil-Mediated Inflammatory Response in the Bony Repair of Injured Growth Plate Cartilage in Young Rats. *J Leukoc Biol* **2006**, *80*, 1272–1280, doi:10.1189/jlb.0606365.

66. Grøgaard, B.; Gerdin, B.; Reikerås, O. The Polymorphonuclear Leukocyte: Has It a Role in Fracture Healing? *Arch Orthop Trauma Surg* **1990**, *109*, 268–271, doi:10.1007/BF00419942.

67. Feiken, E.; Rømer, J.; Eriksen, J.; Lund, L.R. Neutrophils Express Tumor Necrosis Factor-Alpha during Mouse Skin Wound Healing. *J Invest Dermatol* **1995**, *105*, 120–123, doi:10.1111/1523-1747.ep12313429.

68. Gong, Y.; Koh, D.-R. Neutrophils Promote Inflammatory Angiogenesis via Release of Preformed VEGF in an *in vivo* Corneal Model. *Cell Tissue Res* **2010**, *339*, 437–448, doi:10.1007/s00441-009-0908-5.

69. Christoffersson, G.; Vågesjö, E.; Vandooren, J.; Lidén, M.; Massena, S.; Reinert, R.B.; Brissova, M.; Powers, A.C.; Opdenakker, G.; Phillipson, M. VEGF-A Recruits a Proangiogenic MMP-9-Delivering Neutrophil Subset That Induces Angiogenesis in Transplanted Hypoxic Tissue. *Blood* **2012**, *120*, 4653–4662, doi:10.1182/blood-2012-04-421040.

70. Agarwal, S.; Loder, S.J.; Cholok, D.; Li, J.; Bian, G.; Yalavarthi, S.; Li, S.; Carson, W.F.; Hwang, C.; Marini, S.; et al. Disruption of Neutrophil Extracellular Traps (NETs) Links Mechanical Strain to Post-Traumatic Inflammation. *Front Immunol* **2019**, *10*, 2148, doi:10.3389/fimmu.2019.02148.

71. Bastian, O.W.; Koenderman, L.; Alblas, J.; Leenen, L.P.H.; Blokhuis, T.J. Neutrophils Contribute to Fracture Healing by Synthesizing Fibronectin+ Extracellular Matrix Rapidly after Injury. *Clin Immunol* **2016**, *164*, 78–84, doi: 10.1016/j.clim.2016.02.001.

72. Ragipoglu, D.; Dudeck, A.; Haffner-Luntzer, M.; Voss, M.; Kroner, J.; Ignatius, A.; Fischer, V. The Role of Mast Cells in Bone Metabolism and Bone Disorders. *Front Immunol* **2020**, *11*, 163, doi:10.3389/fimmu.2020.00163.
73. Banovac, K.; Renfree, K.; Makowski, A.L.; Latta, L.L.; Altman, R.D. Fracture Healing and Mast Cells. *J Orthop Trauma* **1995**, *9*, 482–490, doi:10.1097/00005131-199509060-00005.
74. Behrends, D.A.; Cheng, L.; Sullivan, M.B.; Wang, M.H.; Roby, G.B.; Zayed, N.; Gao, C.; Henderson, J.E.; Martineau, P.A. Defective Bone Repair in Mast Cell Deficient Mice with C-Kit Loss of Function. *Eur Cell Mater* **2014**, *28*, 209–221; discussion 221-222, doi:10.22203/ecm.v028a14.
75. Kroner, J.; Kovtun, A.; Kemmler, J.; Messmann, J.J.; Strauss, G.; Seitz, S.; Schinke, T.; Amling, M.; Kotrba, J.; Froebel, J.; et al. Mast Cells Are Critical Regulators of Bone Fracture-Induced Inflammation and Osteoclast Formation and Activity. *J Bone Miner Res* **2017**, *32*, 2431–2444, doi:10.1002/jbmr.3234.
76. Spaggiari, G.M.; Moretta, L. Interactions between Mesenchymal Stem Cells and Dendritic Cells. *Adv Biochem Eng Biotechnol* **2013**, *130*, 199–208, doi:10.1007/10_2012_154.
77. Brøchner, A.C.; Toft, P. Pathophysiology of the Systemic Inflammatory Response after Major Accidental Trauma. *Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine* **2009**, *17*, 43, doi:10.1186/1757-7241-17-43.
78. Almeida, C.R.; Caires, H.R.; Vasconcelos, D.P.; Barbosa, M.A. NAP-2 Secreted by Human NK Cells Can Stimulate Mesenchymal Stem/Stromal Cell Recruitment. *Stem Cell Reports* **2016**, *6*, 466–473, doi: 10.1016/j.stemcr.2016.02.012.
79. Hauser, C.J.; Joshi, P.; Jones, Q.; Zhou, X.; Livingston, D.H.; Lavery, R.F. Suppression of Natural Killer Cell Activity in Patients With Fracture/Soft Tissue Injury. *Archives of Surgery* **1997**, *132*, 1326–1330, doi:10.1001/archsurg.1997.01430360072013.
80. Söderström, K.; Stein, E.; Colmenero, P.; Purath, U.; Müller-Ladner, U.; de Matos, C.T.; Tarner, I.H.; Robinson, W.H.; Engleman, E.G. Natural Killer Cells Trigger Osteoclastogenesis and Bone Destruction in Arthritis. *Proc Natl Acad Sci U S A* **2010**, *107*, 13028–13033, doi:10.1073/pnas.1000546107.
81. Saldaña, L.; Bensiamar, F.; Vallés, G.; Mancebo, F.J.; García-Rey, E.; Vilaboa, N. Immunoregulatory Potential of Mesenchymal Stem Cells Following Activation by Macrophage-Derived Soluble Factors. *Stem Cell Res Ther* **2019**, *10*, 58, doi:10.1186/s13287-019-1156-6.
82. Pajarinen, J.; Lin, T.; Gibon, E.; Kohno, Y.; Maruyama, M.; Nathan, K.; Lu, L.; Yao, Z.; Goodman, S.B. Mesenchymal Stem Cell-Macrophage Crosstalk and Bone Healing. *Biomaterials* **2019**, *196*, 80–89, doi: 10.1016/j.biomaterials.2017.12.025.
83. Michalski, M.N.; McCauley, L.K. Macrophages and Skeletal Health. *Pharmacol Ther* **2017**, *174*, 43–54, doi: 10.1016/j.pharmthera.2017.02.017.
84. Murray, P.J.; Wynn, T.A. Protective and Pathogenic Functions of Macrophage Subsets. *Nat Rev Immunol* **2011**, *11*, 723–737, doi:10.1038/nri3073.

85. Schlundt, C.; El Khassawna, T.; Serra, A.; Dienelt, A.; Wendler, S.; Schell, H.; van Rooijen, N.; Radbruch, A.; Lucius, R.; Hartmann, S.; et al. Macrophages in Bone Fracture Healing: Their Essential Role in Endochondral Ossification. *Bone* **2018**, *106*, 78–89, doi: 10.1016/j.bone.2015.10.019.
86. Alexander, K.A.; Chang, M.K.; Maylin, E.R.; Kohler, T.; Müller, R.; Wu, A.C.; Van Rooijen, N.; Sweet, M.J.; Hume, D.A.; Raggatt, L.J.; et al. Osteal Macrophages Promote *in vivo* Intramembranous Bone Healing in a Mouse Tibial Injury Model. *J Bone Miner Res* **2011**, *26*, 1517–1532, doi:10.1002/jbmr.354.
87. Raggatt, L.J.; Wullschleger, M.E.; Alexander, K.A.; Wu, A.C.K.; Millard, S.M.; Kaur, S.; Maugham, M.L.; Gregory, L.S.; Steck, R.; Pettit, A.R. Fracture Healing via Periosteal Callus Formation Requires Macrophages for Both Initiation and Progression of Early Endochondral Ossification. *The American Journal of Pathology* **2014**, *184*, 3192–3204, doi: 10.1016/j.ajpath.2014.08.017.
88. Guihard, P.; Danger, Y.; Brounais, B.; David, E.; Brion, R.; Delecrin, J.; Richards, C.D.; Chevalier, S.; Rédini, F.; Heymann, D.; et al. Induction of Osteogenesis in Mesenchymal Stem Cells by Activated Monocytes/Macrophages Depends on Oncostatin M Signaling. *Stem Cells* **2012**, *30*, 762–772, doi:10.1002/stem.1040.
89. Nicolaidou, V.; Wong, M.M.; Redpath, A.N.; Ersek, A.; Baban, D.F.; Williams, L.M.; Cope, A.P.; Horwood, N.J. Monocytes Induce STAT3 Activation in Human Mesenchymal Stem Cells to Promote Osteoblast Formation. *PLOS ONE* **2012**, *7*, e39871, doi: 10.1371/journal.pone.0039871.
90. Lu, L.Y.; Loi, F.; Nathan, K.; Lin, T.; Pajarinen, J.; Gibon, E.; Nabeshima, A.; Cordova, L.; Jämsen, E.; Yao, Z.; et al. Pro-Inflammatory M1 Macrophages Promote Osteogenesis by Mesenchymal Stem Cells via the COX-2-Prostaglandin E2 Pathway. *Journal of Orthopaedic Research* **2017**, *35*, 2378–2385, doi:10.1002/jor.23553.
91. Omar, O.M.; Granéli, C.; Ekström, K.; Karlsson, C.; Johansson, A.; Lausmaa, J.; Wexell, C.L.; Thomsen, P. The Stimulation of an Osteogenic Response by Classical Monocyte Activation. *Biomaterials* **2011**, *32*, 8190–8204, doi: 10.1016/j.biomaterials.2011.07.055.
92. Reinke, S.; Geissler, S.; Taylor, W.R.; Schmidt-Bleek, K.; Juelke, K.; Schwachmeyer, V.; Dahne, M.; Hartwig, T.; Akyüz, L.; Meisel, C.; et al. Terminally Differentiated $CD8^+$ T Cells Negatively Affect Bone Regeneration in Humans. *Sci Transl Med* **2013**, *5*, 177ra36, doi:10.1126/scitranslmed.3004754.
93. Sato, K.; Suematsu, A.; Okamoto, K.; Yamaguchi, A.; Morishita, Y.; Kadono, Y.; Tanaka, S.; Kodama, T.; Akira, S.; Iwakura, Y.; et al. Th17 Functions as an Osteoclastogenic Helper T Cell Subset That Links T Cell Activation and Bone Destruction. *J Exp Med* **2006**, *203*, 2673–2682, doi:10.1084/jem.20061775.
94. Liu, Y.; Wang, L.; Kikuiri, T.; Akiyama, K.; Chen, C.; Xu, X.; Yang, R.; Chen, W.; Wang, S.; Shi, S. Mesenchymal Stem Cell–Based Tissue Regeneration Is

Governed by Recipient T Lymphocytes via IFN-γ and TNF-α. *Nature Medicine* **2011**, *17*, 1594–1601, doi:10.1038/nm.2542.

95. Ono, T.; Okamoto, K.; Nakashima, T.; Nitta, T.; Hori, S.; Iwakura, Y.; Takayanagi, H. IL-17-Producing Γδ T Cells Enhance Bone Regeneration. *Nat Commun* **2016**, *7*, 10928, doi:10.1038/ncomms10928.

96. Venet, F.; Chung, C.-S.; Monneret, G.; Huang, X.; Horner, B.; Garber, M.; Ayala, A. Regulatory T Cell Populations in Sepsis and Trauma. *J Leukoc Biol* **2008**, *83*, 523–535, doi:10.1189/jlb.0607371.

97. Marik, P.E.; Flemmer, M. The Immune Response to Surgery and Trauma: Implications for Treatment. *J Trauma Acute Care Surg* **2012**, *73*, 801–808, doi:10.1097/TA.0b013e318265cf87.

98. Zaiss, M.M.; Axmann, R.; Zwerina, J.; Polzer, K.; Gückel, E.; Skapenko, A.; Schulze-Koops, H.; Horwood, N.; Cope, A.; Schett, G. Treg Cells Suppress Osteoclast Formation: A New Link between the Immune System and Bone. *Arthritis & Rheumatism* **2007**, *56*, 4104–4112, doi:10.1002/art.23138.

99. Kim, Y.G.; Lee, C.-K.; Nah, S.-S.; Mun, S.H.; Yoo, B.; Moon, H.-B. Human CD4+CD25+ Regulatory T Cells Inhibit the Differentiation of Osteoclasts from Peripheral Blood Mononuclear Cells. *Biochem Biophys Res Commun* **2007**, *357*, 1046–1052, doi: 10.1016/j.bbrc.2007.04.042.

100. Sun, G.; Wang, Y.; Ti, Y.; Wang, J.; Zhao, J.; Qian, H. Regulatory B Cell Is Critical in Bone Union Process through Suppressing Proinflammatory Cytokines and Stimulating Foxp3 in Treg Cells. *Clinical and Experimental Pharmacology and Physiology* **2017**, *44*, 455–462, doi:10.1111/1440-1681.12719.

101. Yang, S.; Ding, W.; Feng, D.; Gong, H.; Zhu, D.; Chen, B.; Chen, J. Loss of B Cell Regulatory Function Is Associated with Delayed Healing in Patients with Tibia Fracture. *APMIS* **2015**, *123*, 975–985, doi:10.1111/apm.12439.

102. Könnecke, I.; Serra, A.; El Khassawna, T.; Schlundt, C.; Schell, H.; Hauser, A.; Ellinghaus, A.; Volk, H.-D.; Radbruch, A.; Duda, G.N.; et al. T and B Cells Participate in Bone Repair by Infiltrating the Fracture Callus in a Two-Wave Fashion. *Bone* **2014**, *64*, 155–165, doi: 10.1016/j.bone.2014.03.052.

103. Schena, F.; Gambini, C.; Gregorio, A.; Mosconi, M.; Reverberi, D.; Gattorno, M.; Casazza, S.; Uccelli, A.; Moretta, L.; Martini, A.; et al. Interferon-γ-Dependent Inhibition of B Cell Activation by Bone Marrow-Derived Mesenchymal Stem Cells in a Murine Model of Systemic Lupus Erythematosus. *Arthritis Rheum* **2010**, *62*, 2776–2786, doi:10.1002/art.27560.

104. Traggiai, E.; Volpi, S.; Schena, F.; Gattorno, M.; Ferlito, F.; Moretta, L.; Martini, A. Bone Marrow-Derived Mesenchymal Stem Cells Induce Both Polyclonal Expansion and Differentiation of B Cells Isolated from Healthy Donors and Systemic Lupus Erythematosus Patients. *Stem Cells* **2008**, *26*, 562–569, doi:10.1634/stemcells.2007-0528.

105. Maruyama, M.; Rhee, C.; Utsunomiya, T.; Zhang, N.; Ueno, M.; Yao, Z.; Goodman, S.B. Modulation of the Inflammatory Response and Bone Healing. *Front. Endocrinol.* **2020**, *11*, doi:10.3389/fendo.2020.00386.
106. Lorenzo, J. Cytokines and Bone: Osteoimmunology. In *Bone Regulators and Osteoporosis Therapy*; Stern, P.H., Ed.; Handbook of Experimental Pharmacology; Springer International Publishing: Cham, 2020; pp. 177–230 ISBN 978-3-030-57378-2.
107. Bastidas-Coral, A.P.; Bakker, A.D.; Zandieh-Doulabi, B.; Kleverlaan, C.J.; Bravenboer, N.; Forouzanfar, T.; Klein-Nulend, J. Cytokines TNF-α, IL-6, IL-17F, and IL-4 Differentially Affect Osteogenic Differentiation of Human Adipose Stem Cells. *Stem Cells International* **2016**, *2016*, doi:10.1155/2016/1318256.
108. Croes, M.; Oner, F.C.; Kruyt, M.C.; Blokhuis, T.J.; Bastian, O.; Dhert, W.J.A.; Alblas, J. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. *PLoS ONE* **2015**, *10*, 1–14, doi: 10.1371/journal.pone.0132781.
109. Ding, J.; Ghali, O.; Lencel, P.; Broux, O.; Chauveau, C.; Devedjian, J.C.; Hardouin, P.; Magne, D. TNF-Alpha and IL-1beta Inhibit RUNX2 and Collagen Expression but Increase Alkaline Phosphatase Activity and Mineralization in Human Mesenchymal Stem Cells. *Life Sci* **2009**, *84*, 499–504, doi: 10.1016/j.lfs.2009.01.013.
110. Huang, H.; Zhao, N.; Xu, X.; Xu, Y.; Li, S.; Zhang, J.; Yang, P. Dose-Specific Effects of Tumor Necrosis Factor Alpha on Osteogenic Differentiation of Mesenchymal Stem Cells. *Cell Proliferation* **2011**, *44*, 420–427, doi: 10.1111/j.1365-2184.2011.00769.x.
111. Lacey, D.C.; Simmons, P.J.; Graves, S.E.; Hamilton, J.A. Proinflammatory Cytokines Inhibit Osteogenic Differentiation from Stem Cells: Implications for Bone Repair during Inflammation. *Osteoarthritis and Cartilage* **2009**, *17*, 735–742, doi: 10.1016/j.joca.2008.11.011.
112. Lange, J.; Sapozhnikova, A.; Lu, C.; Hu, D.; Li, X.; Miclau III, T.; Marcucio, R.S. Action of IL-1β during Fracture Healing. *Journal of Orthopaedic Research* **2010**, *28*, 778–784, doi:10.1002/jor.21061.
113. Huh, J.-E.; Lee, S.Y. IL-6 Is Produced by Adipose-Derived Stromal Cells and Promotes Osteogenesis. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **2013**, *1833*, 2608–2616, doi: 10.1016/j.bbamcr.2013.06.025.
114. Li, Y.; Bäckesjö, C.-M.; Haldosén, L.-A.; Lindgren, U. IL-6 Receptor Expression and IL-6 Effects Change during Osteoblast Differentiation. *Cytokine* **2008**, *43*, 165–173, doi: 10.1016/j.cyto.2008.05.007.
115. Rozen, N.; Lewinson, D.; Bick, T.; Jacob, Z.C.; Stein, H.; Soudry, M. Fracture Repair: Modulation of Fracture-Callus and Mechanical Properties by Sequential Application of IL-6 Following PTH 1–34 or PTH 28–48. *Bone* **2007**, *41*, 437–445, doi: 10.1016/j.bone.2007.04.193.

116. Vo, T.N.; Kasper, F.K.; Mikos, A.G. Strategies for Controlled Delivery of Growth Factors and Cells for Bone Regeneration. *Advanced Drug Delivery Reviews* **2012**, *64*, 1292–1309, doi: 10.1016/j.addr.2012.01.016.
117. Croes, M.; Wal, B.C.H.; Vogely, H.Ch. The Impact of Bacterial Infections on Osteogenesis: Evidence From *In vivo* Studies. *Journal of Orthopaedic Research* **2019**, jor.24422, doi:10.1002/jor.24422.
118. Urish, K.L.; Cassat, J.E. Staphylococcus Aureus Osteomyelitis: Bone, Bugs, and Surgery. *Infect Immun* **2020**, *88*, doi:10.1128/IAI.00932-19.
119. Ramirez, D.M.; Ramirez, M.R.; Reginato, A.M.; Medici, D. Molecular and Cellular Mechanisms of Heterotopic Ossification. *Histol Histopathol* **2014**, *29*, 1281–1285.
120. Felix-Ilemhenbhio, F.; Pickering, G.A.E.; Kiss-Toth, E.; Wilkinson, J.M. Pathophysiology and Emerging Molecular Therapeutic Targets in Heterotopic Ossification. *International Journal of Molecular Sciences* **2022**, *23*, 6983, doi:10.3390/ijms23136983.
121. Croes, M.; Kruyt, M.C.; Boot, W.; Pouran, B.; Braham, M.V.; Pakpahan, S.A.; Weinans, H.; Vogely, H.C.; Fluit, A.C.; Dhert, W.J.; et al. The Role of Bacterial Stimuli in Inflammation-Driven Bone Formation. *European cells & materials* **2019**, *37*, 402–419, doi:10.22203/eCM.v037a24.
122. Croes, M.; Kruyt, M.C.; Loozen, L.; Kragten, A.H.; Yuan, H.; Dhert, W.J.; Öner, F.C.; Alblas, J. Local Induction of Inflammation Affects Bone Formation. *Eur Cell Mater* **2017**, *33*, 211–226, doi:10.22203/eCM.v033a16.
123. Kaur, B.P.; Secord, E. Innate Immunity. *Pediatr Clin North Am* **2019**, *66*, 905–911, doi: 10.1016/j.pcl.2019.06.011.
124. Riera Romo, M.; Pérez-Martínez, D.; Castillo Ferrer, C. Innate Immunity in Vertebrates: An Overview. *Immunology* **2016**, *148*, 125–139, doi:10.1111/imm.12597.
125. Demaria, O.; Cornen, S.; Daëron, M.; Morel, Y.; Medzhitov, R.; Vivier, E. Harnessing Innate Immunity in Cancer Therapy. *Nature* **2019**, *574*, 45–56, doi:10.1038/s41586-019-1593-5.
126. Janeway, C.A. Approaching the Asymptote? Evolution and Revolution in Immunology. *Cold Spring Harb Symp Quant Biol* **1989**, *54 Pt 1*, 1–13, doi:10.1101/sqb.1989.054.01.003.
127. Schneider, D.S.; Jin, Y.; Morisato, D.; Anderson, K.V. A Processed Form of the Spätzle Protein Defines Dorsal-Ventral Polarity in the Drosophila Embryo. *Development* **1994**, *120*, 1243–1250, doi:10.1242/dev.120.5.1243.
128. O'Neill, L.A.J.; Golenbock, D.; Bowie, A.G. The History of Toll-like Receptors — Redefining Innate Immunity. *Nature Reviews Immunology* **2013**, *13*, 453–460, doi:10.1038/nri3446.
129. ShaoPing, H.; JianHua, H.; Jiayi, C.; Fu, C.; ShuQi, O. Research advancement of innate immunity and pattern recognition receptors. *Chinese Journal of Animal Nutrition* **2017**, *29*, 3844–3851.

130. Gong, T.; Liu, L.; Jiang, W.; Zhou, R. DAMP-Sensing Receptors in Sterile Inflammation and Inflammatory Diseases. *Nat Rev Immunol* **2020**, *20*, 95–112, doi:10.1038/s41577-019-0215-7.
131. Herwald, H.; Egesten, A. On PAMPs and DAMPs. *J Innate Immun* **2016**, *8*, 427–428, doi:10.1159/000448437.
132. Zindel, J.; Kubes, P. DAMPs, PAMPs, and LAMPs in Immunity and Sterile Inflammation. *Annu Rev Pathol* **2020**, *15*, 493–518, doi:10.1146/annurev-pathmechdis-012419-032847.
133. Brubaker, S.W.; Bonham, K.S.; Zanoni, I.; Kagan, J.C. Innate Immune Pattern Recognition: A Cell Biological Perspective. *Annu Rev Immunol* **2015**, *33*, 257–290, doi:10.1146/annurev-immunol-032414-112240.
134. Tan, R.S.T.; Ho, B.; Leung, B.P.; Ding, J.L. TLR Cross-Talk Confers Specificity to Innate Immunity. *International Reviews of Immunology* **2014**, *33*, 443–453, doi:10.3109/08830185.2014.921164.
135. Anwar, M.A.; Basith, S.; Choi, S. Negative Regulatory Approaches to the Attenuation of Toll-like Receptor Signaling. *Exp Mol Med* **2013**, *45*, e11–e11, doi:10.1038/emm.2013.28.
136. Mortaz, E.; Adcock, I.M.; Tabarsi, P.; Darazam, I.A.; Movassaghi, M.; Garssen, J.; Jamaati, H.; Velayati, A. Pattern Recognitions Receptors in Immunodeficiency Disorders. *Eur J Pharmacol* **2017**, *808*, 49–56, doi: 10.1016/j.ejphar.2017.01.014.
137. Palm, N.W.; Medzhitov, R. Pattern Recognition Receptors and Control of Adaptive Immunity. *Immunological Reviews* **2009**, *227*, 221–233, doi:10.1111/j.1600-065X.2008.00731.x.
138. Johnson, S.; Zhan, Y.; Sutherland, R.M.; Mount, A.M.; Bedoui, S.; Brady, J.L.; Carrington, E.M.; Brown, L.E.; Belz, G.T.; Heath, W.R.; et al. Selected Toll-like Receptor Ligands and Viruses Promote Helper-Independent Cytotoxic T Cell Priming by Upregulating CD40L on Dendritic Cells. *Immunity* **2009**, *30*, 218–227, doi: 10.1016/j.immuni.2008.11.015.
139. Pasare, C.; Medzhitov, R. Toll-like Receptors: Balancing Host Resistance with Immune Tolerance. *Current Opinion in Immunology* **2003**, *15*, 677–682, doi: 10.1016/j.coi.2003.09.002.
140. Li, D.; Wu, M. Pattern Recognition Receptors in Health and Diseases. *Sig Transduct Target Ther* **2021**, *6*, 1–24, doi:10.1038/s41392-021-00687-0.
141. De Nardo, D. Toll-like Receptors: Activation, Signalling and Transcriptional Modulation. *Cytokine* **2015**, *74*, 181–189, doi: 10.1016/j.cyto.2015.02.025.
142. Lacagnina, M.J.; Watkins, L.R.; Grace, P.M. Toll-like Receptors and Their Role in Persistent Pain. *Pharmacol Ther* **2018**, *184*, 145–158, doi: 10.1016/j.pharmthera.2017.10.006.

143. Kumar, H.; Kawai, T.; Akira, S. Toll-like Receptors and Innate Immunity. *Biochemical and Biophysical Research Communications* **2009**, *388*, 621–625, doi: 10.1016/j.bbrc.2009.08.062.
144. Chuenchor, W.; Jin, T.; Ravilious, G.; Xiao, T.S. Structures of Pattern Recognition Receptors Reveal Molecular Mechanisms of Autoinhibition, Ligand Recognition and Oligomerization. *Curr Opin Immunol* **2014**, *26*, 14–20, doi: 10.1016/j.coi.2013.10.009.
145. Kawai, T.; Akira, S. The Role of Pattern-Recognition Receptors in Innate Immunity: Update on Toll-like Receptors. *Nat Immunol* **2010**, *11*, 373–384, doi:10.1038/ni.1863.
146. Barton, G.M.; Medzhitov, R. Toll-like Receptor Signaling Pathways. *Science* **2003**, *300*, 1524–1525, doi:10.1126/science.1085536.
147. Gao, D.; Li, W. Structures and Recognition Modes of Toll-like Receptors. *Proteins* **2017**, *85*, 3–9, doi:10.1002/prot.25179.
148. Farhat, K.; Riekenberg, S.; Heine, H.; Debarry, J.; Lang, R.; Mages, J.; Buwitt-Beckmann, U.; Röschmann, K.; Jung, G.; Wiesmüller, K.-H.; et al. Heterodimerization of TLR2 with TLR1 or TLR6 Expands the Ligand Spectrum but Does Not Lead to Differential Signaling. *J Leukoc Biol* **2008**, *83*, 692–701, doi:10.1189/jlb.0807586.
149. Liu, L.; Botos, I.; Wang, Y.; Leonard, J.N.; Shiloach, J.; Segal, D.M.; Davies, D.R. Structural Basis of Toll-like Receptor 3 Signaling with Double-Stranded RNA. *Science* **2008**, *320*, 379–381, doi:10.1126/science.1155406.
150. Yoon, S.; Kurnasov, O.; Natarajan, V.; Hong, M.; Gudkov, A.V.; Osterman, A.L.; Wilson, I.A. Structural Basis of TLR5-Flagellin Recognition and Signaling. *Science* **2012**, *335*, 859–864, doi:10.1126/science.1215584.
151. Park, B.S.; Song, D.H.; Kim, H.M.; Choi, B.-S.; Lee, H.; Lee, J.-O. The Structural Basis of Lipopolysaccharide Recognition by the TLR4-MD-2 Complex. *Nature* **2009**, *458*, 1191–1195, doi:10.1038/nature07830.
152. Motta, V.; Soares, F.; Sun, T.; Philpott, D.J. NOD-like Receptors: Versatile Cytosolic Sentinels. *Physiol Rev* **2015**, *95*, 149–178, doi:10.1152/physrev.00009.2014.
153. Meunier, E.; Broz, P. Evolutionary Convergence and Divergence in NLR Function and Structure. *Trends Immunol* **2017**, *38*, 744–757, doi:10.1016/j.it.2017.04.005.
154. Wang, J.; Hu, M.; Wang, J.; Qi, J.; Han, Z.; Wang, G.; Qi, Y.; Wang, H.-W.; Zhou, J.-M.; Chai, J. Reconstitution and Structure of a Plant NLR Resistosome Conferring Immunity. *Science* **2019**, *364*, eaav5870, doi:10.1126/science.aav5870.
155. Le, H.T.; Harton, J.A. Pyrin- and CARD-Only Proteins as Regulators of NLR Functions. *Front Immunol* **2013**, *4*, 275, doi:10.3389/fimmu.2013.00275.
156. Chamaillard, M.; Hashimoto, M.; Horie, Y.; Masumoto, J.; Qiu, S.; Saab, L.; Ogura, Y.; Kawasaki, A.; Fukase, K.; Kusumoto, S.; et al. An Essential Role for NOD1 in Host Recognition of Bacterial Peptidoglycan Containing Diaminopimelic Acid. *Nat Immunol* **2003**, *4*, 702–707, doi:10.1038/ni945.

157. Girardin, S.E.; Boneca, I.G.; Viala, J.; Chamaillard, M.; Labigne, A.; Thomas, G.; Philpott, D.J.; Sansonetti, P.J. Nod2 Is a General Sensor of Peptidoglycan through Muramyl Dipeptide (MDP) Detection. *J Biol Chem* **2003**, *278*, 8869–8872, doi:10.1074/jbc.C200651200.
158. Pashenkov, M.V.; Dagil, Y.A.; Pinegin, B.V. NOD1 and NOD2: Molecular Targets in Prevention and Treatment of Infectious Diseases. *Int Immunopharmacol* **2018**, *54*, 385–400, doi: 10.1016/j.intimp.2017.11.036.
159. Moreira, L.; Zamboni, D. NOD1 and NOD2 Signaling in Infection and Inflammation. *Frontiers in Immunology* **2012**, *3*.
160. Lepenies, B.; Lee, J.; Sonkaria, S. Targeting C-Type Lectin Receptors with Multivalent Carbohydrate Ligands. *Adv Drug Deliv Rev* **2013**, *65*, 1271–1281, doi: 10.1016/j.addr.2013.05.007.
161. Ebner, S.; Sharon, N.; Ben-Tal, N. Evolutionary Analysis Reveals Collective Properties and Specificity in the C-Type Lectin and Lectin-like Domain Superfamily. *Proteins* **2003**, *53*, 44–55, doi:10.1002/prot.10440.
162. Dam, T.K.; Brewer, C.F. Lectins as Pattern Recognition Molecules: The Effects of Epitope Density in Innate Immunity. *Glycobiology* **2010**, *20*, 270–279, doi:10.1093/glycob/cwp186.
163. Liu, B.; Li, P.; Yang, G. [The Immunomodulatory Role of C-type Lectin Receptors in Parasitic Infection]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **2015**, *33*, 228–232.
164. Saijo, S.; Fujikado, N.; Furuta, T.; Chung, S.; Kotaki, H.; Seki, K.; Sudo, K.; Akira, S.; Adachi, Y.; Ohno, N.; et al. Dectin-1 Is Required for Host Defense against Pneumocystis Carinii but Not against Candida Albicans. *Nat Immunol* **2007**, *8*, 39–46, doi:10.1038/ni1425.
165. Taylor, P.R.; Tsoni, S.V.; Willment, J.A.; Dennehy, K.M.; Rosas, M.; Findon, H.; Haynes, K.; Steele, C.; Botto, M.; Gordon, S.; et al. Dectin-1 Is Required for Beta-Glucan Recognition and Control of Fungal Infection. *Nat Immunol* **2007**, *8*, 31–38, doi:10.1038/ni1408.
166. Gow, N.A.R.; Netea, M.G.; Munro, C.A.; Ferwerda, G.; Bates, S.; Mora-Montes, H.M.; Walker, L.; Jansen, T.; Jacobs, L.; Tsoni, V.; et al. Immune Recognition of Candida Albicans Beta-Glucan by Dectin-1. *J Infect Dis* **2007**, *196*, 1565–1571, doi:10.1086/523110.
167. Zhou, M.N.; Delaveris, C.S.; Kramer, J.R.; Kenkel, J.A.; Engleman, E.G.; Bertozzi, C.R. N-Carboxyanhydride Polymerization of Glycopolypeptides That Activate Antigen-Presenting Cells through Dectin-1 and Dectin-2. *Angew Chem Int Ed Engl* **2018**, *57*, 3137–3142, doi:10.1002/anie.201713075.
168. DiDonato, J.A.; Mercurio, F.; Karin, M. NF-κB and the Link between Inflammation and Cancer. *Immunol Rev* **2012**, *246*, 379–400, doi:10.1111/j.1600-065X.2012.01099.x.

169. Gay, N.J.; Symmons, M.F.; Gangloff, M.; Bryant, C.E. Assembly and Localization of Toll-like Receptor Signalling Complexes. *Nat Rev Immunol* **2014**, *14*, 546–558, doi:10.1038/nri3713.
170. Kawai, T.; Akira, S. Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. *Immunity* **2011**, *34*, 637–650, doi: 10.1016/j.immuni.2011.05.006.
171. De Nardo, D.; Balka, K.R.; Cardona Gloria, Y.; Rao, V.R.; Latz, E.; Masters, S.L. Interleukin-1 Receptor-Associated Kinase 4 (IRAK4) Plays a Dual Role in Myddosome Formation and Toll-like Receptor Signaling. *J Biol Chem* **2018**, *293*, 15195–15207, doi:10.1074/jbc.RA118.003314.
172. Park, J.-H.; Kim, Y.-G.; McDonald, C.; Kanneganti, T.-D.; Hasegawa, M.; Body-Malapel, M.; Inohara, N.; Núñez, G. RICK/RIP2 Mediates Innate Immune Responses Induced through Nod1 and Nod2 but Not TLRs. *J Immunol* **2007**, *178*, 2380–2386, doi:10.4049/jimmunol.178.4.2380.
173. Häcker, H.; Redecke, V.; Blagoev, B.; Kratchmarova, I.; Hsu, L.-C.; Wang, G.G.; Kamps, M.P.; Raz, E.; Wagner, H.; Häcker, G.; et al. Specificity in Toll-like Receptor Signalling through Distinct Effector Functions of TRAF3 and TRAF6. *Nature* **2006**, *439*, 204–207, doi:10.1038/nature04369.
174. Verstak, B.; Nagpal, K.; Bottomley, S.P.; Golenbock, D.T.; Hertzog, P.J.; Mansell, A. MyD88 Adapter-like (Mal)/TIRAP Interaction with TRAF6 Is Critical for TLR2- and TLR4-Mediated NF-kappaB Proinflammatory Responses. *J Biol Chem* **2009**, *284*, 24192–24203, doi:10.1074/jbc.M109.023044.
175. Kawai, T.; Sato, S.; Ishii, K.J.; Coban, C.; Hemmi, H.; Yamamoto, M.; Terai, K.; Matsuda, M.; Inoue, J.; Uematsu, S.; et al. Interferon-Alpha Induction through Toll-like Receptors Involves a Direct Interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol* **2004**, *5*, 1061–1068, doi:10.1038/ni1118.
176. Cohen, P.; Strickson, S. The Role of Hybrid Ubiquitin Chains in the MyD88 and Other Innate Immune Signalling Pathways. *Cell Death Differ* **2017**, *24*, 1153–1159, doi:10.1038/cdd.2017.17.
177. Rogers, N.C.; Slack, E.C.; Edwards, A.D.; Nolte, M.A.; Schulz, O.; Schweighoffer, E.; Williams, D.L.; Gordon, S.; Tybulewicz, V.L.; Brown, G.D.; et al. Syk-Dependent Cytokine Induction by Dectin-1 Reveals a Novel Pattern Recognition Pathway for C Type Lectins. *Immunity* **2005**, *22*, 507–517, doi: 10.1016/j.immuni.2005.03.004.
178. Leibundgut-Landmann, S.; Osorio, F.; Brown, G.D.; Reis e Sousa, C. Stimulation of Dendritic Cells via the Dectin-1/Syk Pathway Allows Priming of Cytotoxic T-Cell Responses. *Blood* **2008**, *112*, 4971–4980, doi:10.1182/blood-2008-05-158469.
179. Ye, X.-C.; Hao, Q.; Ma, W.-J.; Zhao, Q.-C.; Wang, W.-W.; Yin, H.-H.; Zhang, T.; Wang, M.; Zan, K.; Yang, X.-X.; et al. Dectin-1/Syk Signaling Triggers Neuroinflammation after Ischemic Stroke in Mice. *J Neuroinflammation* **2020**, *17*, 17, doi:10.1186/s12974-019-1693-z.

180. Sakurai, K.; Dainichi, T.; Garcet, S.; Tsuchiya, S.; Yamamoto, Y.; Kitoh, A.; Honda, T.; Nomura, T.; Egawa, G.; Otsuka, A.; et al. Cutaneous P38 Mitogen-Activated Protein Kinase Activation Triggers Psoriatic Dermatitis. *J Allergy Clin Immunol* **2019**, *144*, 1036–1049, doi: 10.1016/j.jaci.2019.06.019.
181. Khorasanizadeh, M.; Eskian, M.; Gelfand, E.W.; Rezaei, N. Mitogen-Activated Protein Kinases as Therapeutic Targets for Asthma. *Pharmacol Ther* **2017**, *174*, 112–126, doi: 10.1016/j.pharmthera.2017.02.024.
182. Alexopoulou, L.; Holt, A.C.; Medzhitov, R.; Flavell, R.A. Recognition of Double-Stranded RNA and Activation of NF-kappaB by Toll-like Receptor 3. *Nature* **2001**, *413*, 732–738, doi:10.1038/35099560.
183. Hsu, Y.-M.S.; Zhang, Y.; You, Y.; Wang, D.; Li, H.; Duramad, O.; Qin, X.-F.; Dong, C.; Lin, X. The Adaptor Protein CARD9 Is Required for Innate Immune Responses to Intracellular Pathogens. *Nat Immunol* **2007**, *8*, 198–205, doi:10.1038/ni1426.
184. Ysebrant de Lendonck, L.; Martinet, V.; Goriely, S. Interferon Regulatory Factor 3 in Adaptive Immune Responses. *Cell Mol Life Sci* **2014**, *71*, 3873–3883, doi:10.1007/s00018-014-1653-9.
185. Wu, X.; Lei, C.; Xia, T.; Zhong, X.; Yang, Q.; Shu, H.-B. Regulation of TRIF-Mediated Innate Immune Response by K27-Linked Polyubiquitination and Deubiquitination. *Nat Commun* **2019**, *10*, 4115, doi:10.1038/s41467-019-12145-1.
186. Yamamoto, M.; Sato, S.; Hemmi, H.; Hoshino, K.; Kaisho, T.; Sanjo, H.; Takeuchi, O.; Sugiyama, M.; Okabe, M.; Takeda, K.; et al. Role of Adaptor TRIF in the MyD88-Independent Toll-like Receptor Signaling Pathway. *Science* **2003**, *301*, 640–643, doi:10.1126/science.1087262.
187. Chen, H.C.; Zhan, X.; Tran, K.K.; Shen, H. Selectively Targeting the Toll-like Receptor 9 (TLR9)--IRF 7 Signaling Pathway by Polymer Blend Particles. *Biomaterials* **2013**, *34*, 6464–6472, doi: 10.1016/j.biomaterials.2013.05.016.
188. Bender, A.T.; Tzvetkov, E.; Pereira, A.; Wu, Y.; Kasar, S.; Przetak, M.M.; Vlach, J.; Niewold, T.B.; Jensen, M.A.; Okitsu, S.L. TLR7 and TLR8 Differentially Activate the IRF and NF-κB Pathways in Specific Cell Types to Promote Inflammation. *Immunohorizons* **2020**, *4*, 93–107, doi:10.4049/immunohorizons.2000002.
189. Shekarian, T.; Valsesia-Wittmann, S.; Brody, J.; Michallet, M.C.; Depil, S.; Caux, C.; Marabelle, A. Pattern Recognition Receptors: Immune Targets to Enhance Cancer Immunotherapy. *Ann Oncol* **2017**, *28*, 1756–1766, doi:10.1093/annonc/mdx179.
190. Cao, X. Self-Regulation and Cross-Regulation of Pattern-Recognition Receptor Signalling in Health and Disease. *Nat Rev Immunol* **2016**, *16*, 35–50, doi:10.1038/nri.2015.8.
191. Aleynick, M.; Svensson-Arvelund, J.; Flowers, C.R.; Marabelle, A.; Brody, J.D. Pathogen Molecular Pattern Receptor Agonists: Treating Cancer by

Mimicking Infection. *Clin Cancer Res* **2019**, *25*, 6283–6294, doi: 10.1158/1078-0432.CCR-18-1800.

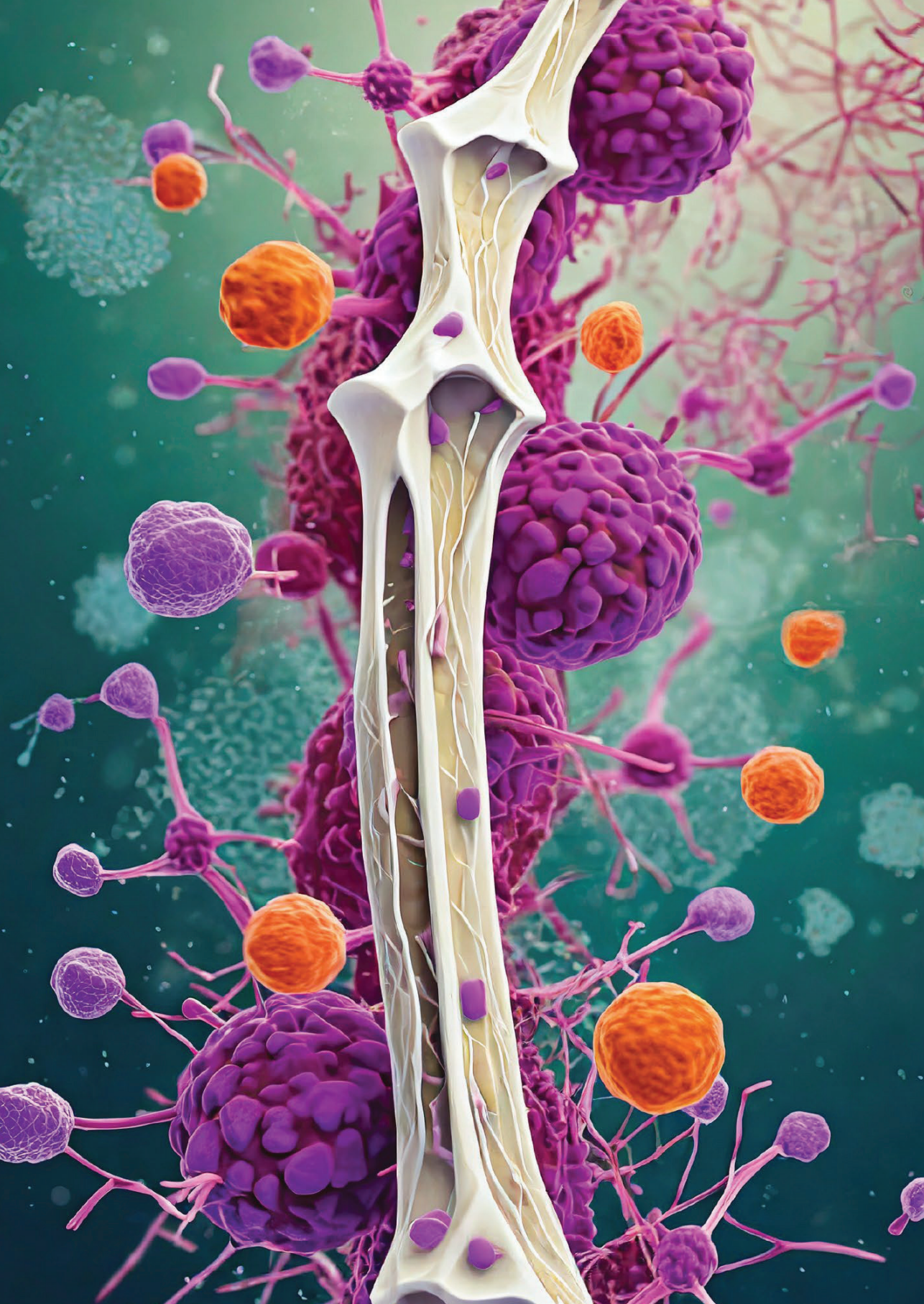
192. Pizzolla, A.; Smith, J.M.; Brooks, A.G.; Reading, P.C. Pattern Recognition Receptor Immunomodulation of Innate Immunity as a Strategy to Limit the Impact of Influenza Virus. *J Leukoc Biol* **2017**, *101*, 851–861, doi:10.1189/jlb.4MR0716-290R.

193. Hemmi, H.; Kaisho, T.; Takeuchi, O.; Sato, S.; Sanjo, H.; Hoshino, K.; Horiuchi, T.; Tomizawa, H.; Takeda, K.; Akira, S. Small Anti-Viral Compounds Activate Immune Cells via the TLR7 MyD88-Dependent Signaling Pathway. *Nat Immunol* **2002**, *3*, 196–200, doi:10.1038/ni758.

194. Gane, E.J.; Kim, H.J.; Visvanathan, K.; Kim, Y.J.; Nguyen, A.-H.; Wallin, J.J.; Chen, D.Y.; McDonald, C.; Arora, P.; Tan, S.K.; et al. Safety, Pharmacokinetics, and Pharmacodynamics of the Oral TLR8 Agonist Selgantolimod in Chronic Hepatitis B. *Hepatology* **2021**, *74*, 1737, doi:10.1002/hep.31795.

195. Amin, O.E.; Colbeck, E.J.; Daffis, S.; Khan, S.; Ramakrishnan, D.; Pattabiraman, D.; Chu, R.; Micolochick Steuer, H.; Lehar, S.; Peiser, L.; et al. Therapeutic Potential of TLR8 Agonist GS-9688 (Selgantolimod) in Chronic Hepatitis B: Remodeling of Antiviral and Regulatory Mediators. *Hepatology* **2021**, *74*, 55, doi:10.1002/hep.31695.

196. Smith, D.A.; Conkling, P.; Richards, D.A.; Nemunaitis, J.J.; Boyd, T.E.; Mita, A.C.; de La Bourdonnaye, G.; Wages, D.; Bexon, A.S. Antitumor Activity and Safety of Combination Therapy with the Toll-like Receptor 9 Agonist IMO-2055, Erlotinib, and Bevacizumab in Advanced or Metastatic Non-Small Cell Lung Cancer Patients Who Have Progressed Following Chemotherapy. *Cancer Immunol Immunother* **2014**, *63*, 787–796, doi:10.1007/s00262-014-1547-6.



Chapter 2

Use of Therapeutic Pathogen Recognition Receptors Ligands for Osteo-immunomodulation

Paree Khokhani[1], Nada R. Rahmani[1], Anne Kok[1], F. Cumhur Öner[1], Jacqueline Alblas[1], Harrie Weinans[1,2], Moyo C. Kruyt[1] and Michiel Croes[1]

1. Department of Orthopedics, University Medical Center Utrecht, Utrecht, the Netherlands
2. Department of Biomechanical Engineering, Technical University Delft, Delft, The Netherlands

**Published in Materials (Basel). 2021 Feb 27;14(5):1119.*

Abstract

Therapeutic pathogen recognition receptor (PRR) ligands are reaching clinical practice following their ability to skew the immune response in a specific direction. We investigated the effects of various therapeutic PRR ligands on bone cell differentiation and inflammation. Following stimulation, alkaline phosphatase (ALP) activity (Day 10), osteocalcin, osteonectin expression (Day 14), and calcium deposition (Day 21) were quantified in bone marrow-derived human mesenchymal stem cells (hMSCs). The osteoclastogenic response was determined by measuring tartrate-resistant acid phosphate (TRAP) activity in human monocytes. TNF-α, IL-6, IL-8, and IL-10 expressions were measured by enzyme-linked immunosorbent assay as an indicator of the ligands' inflammatory properties. We found that nucleic acid-based ligands Poly(I:C) and CpG ODN C increased early ALP activity in hMSCs by 4-fold without affecting osteoclast formation. These ligands did not enhance expression of the other, late osteogenic markers. MPLA, Curdlan, and Pam3CSK4 did not affect osteogenic differentiation, but inhibited TRAP activity in monocytes, which was associated with increased expression of all measured cytokines. Nucleic acid-based ligands are identified as the most promising osteo-immunomodulators, as they favor early osteogenic differentiation without inducing an exaggerated immune-cell mediated response or interfering in osteoclastogenesis and thus can be potentially harnessed for multifunctional coatings for bone biomaterials.

Keywords

multifunctional coatings; adjuvant; osteoimmunology; osteoblast; osteoclast; pathogen-recognition receptors; pathogen-associated molecular patterns

Introduction

Various off-the-shelf synthetic bone substitutes, such as biodegradable ceramics and polymers, are readily available in the clinic for use in orthopedic interventions [1–3]. Although the performance of certain synthetic replacements are even reported to be non-inferior to autograft in applications such as spinal fusion, the success rate is not optimal [4,5]. A challenge remains in their limited osteoinductive capacity, namely the ability to induce the differentiation of bone progenitor cells, during the critical early weeks following implantation [3,6,7]. To enhance the efficacy of synthetic bone biomaterials, one approach is the development of coatings that align with the biological process of bone formation.

Since the immune and skeletal systems are closely entangled, strategies are being explored to modulate the local host immune environment in favor of bone formation [6,7]. Referring to fracture healing as a model for efficient bone regeneration, the early inflammatory phase is proven to be a vital step in osteogenesis. During this initial phase, pro-inflammatory cytokines including TNF-α, IL-6, IL-8, IL-17 and anti-inflammatory cytokines such as IL-10 and IL-4 are being released at the site of injury and subsequently, recruit and differentiate progenitor cells towards the osteogenic lineage [8,9]. A balanced inflammatory reaction is key, as a sustained inflammatory response will mitigate the bone regenerative process [10–12]. Since microbial stimuli were found recently to be involved in osteo-immunomodulation, it is thought that the induction and transcription of pro-osteogenic cytokines can also result from the activation of pattern recognition receptor (PRR) signaling cascades present in immune or bone cells [11,13,14]. Indeed, the specific targeting of PRRs with ligands derived from intact or fragmented bacterial cell wall components has been demonstrated to induce local and transient inflammation that results in enhanced bone formation *in vivo* [15–17].

It is currently unknown which PRR ligands lead to the most optimal bone response, and which class of PRR ligands have highest clinical merit. The realization that PRR-targeting ligands can selectively initiate and propagate immune responses has provoked the pharmaceutical industry to develop synthetic immunological adjuvants that resemble microbial cell wall components or their nucleic acids, but with higher clinical merit due to high purity and stability, lower toxicity, and ability to resist rapid degradation *in vivo* [18–20]. This class of agents has shown success in the pre-clinical and clinical testing phase as vaccine adjuvants to enhance the adaptive immune response by antigen presentation or as stand-alone therapeutic agents to suppress or enhance the inflammatory response depending on the specific need, e.g., treatment of infections, the suppression of autoimmune responses, and the stimulation of anti-

tumor immunity [21]. In the context of orthopedic application, it is yet unknown if the immune modulating capacity of synthetic therapeutic PRR ligands can be employed to recruit and dictate the fate of the various cells involved in the bone healing process.

Mesenchymal stem cells (MSCs) are the progenitors of osteoblasts and therefore crucial target cells in nearly all clinical scenario's necessitating bone regeneration [22,23]. MSCs express a multitude of PRRs that are thought to regulate their proliferation, differentiation and inflammatory properties [24–26]. Until now, the bone-forming capacity of PRR ligands has been explored *in vivo* mainly in rodents, with inconsistent responses in comparison to human cells [27]. These inconsistencies are likely related to the difference in the expression of PRRs in different species and the generally lower sensitivity of rodents to microbial stimuli [24,25]. To better predict the possible clinical reaction, further investigation is required to explore the response of human MSCs (hMSCs) towards therapeutic PRR ligand stimulation.

Apart from hMSCs, the local activity of osteoclasts is also important for bone regenerative strategies. Osteoclasts derive from the hematopoietic cell line and are considered early responders to PRR ligands after *in vivo* delivery [26,28]. Local depletion of osteoclasts or their precursors during early inflammation is suggested to impair the onset of new bone formation in biomaterials [28,29]. Therefore, potential therapeutic PRR ligands used for orthopedic settings should not have inhibitory properties towards osteoclast formation during the early phases of inflammation.

In this study we investigate the potential use of synthetically developed therapeutic PRR ligands as immunomodulators for bone regeneration. Different classes of therapeutic PRR ligands are evaluated for their effect on osteogenic differentiation of hMSCs, osteoclast formation and cytokine expression by both hMSCs and monocytes.

Materials and Methods

Study Design

A set of therapeutic PRR ligands (**Table 1**) was evaluated for their effects on osteogenic differentiation, osteoclast formation, and pro-inflammatory activity in human cells. Bone-marrow derived hMSCs were stimulated with the PRR ligands in dexamethasone-based osteogenic differentiation medium to identify possible modulatory effects on osteogenic differentiation. Moreover, hMSCs were stimulated with PRR ligands in expansion medium to discriminate between

possible pro-osteogenic effect independent of osteogenic stimuli, namely osteoinduction. As a marker of early osteogenic differentiation, cells were assessed for the day 10 activity in alkaline phosphatase (ALP), an enzyme secreted by cells of the early osteoblast lineage and that plays a role in matrix calcification. As markers of late osteogenic differentiation, the day 14 expression of osteonectin and osteocalcin—non-collagenous proteins that regulate biological mineralization process—and day 21 biological mineralization were measured [27,30,31].

Therapeutic PRR Ligand	Receptor	Natural Ligand	Concentration
Pam3CSK4	TLR1/2 a	Bacterial lipoproteins	0.01–1 μg/mL
Curdlan	Dectin-1 a	n/a	0.1–10 μg/mL
MPLA	TLR4 a	Gram-negative bacterial Lipid A	0.01–1 μg/mL
Resiquimod	TLR7/8 b	Microbial single-stranded RNA	0.01–1 μg/mL
Murabutide	NOD2 b	Bacterial peptidoglycan	0.1–10 μg/mL
CpG ODN C	TLR9 b	Microbial DNA	0.01–1 μg/mL
Poly(I:C)	TLR3 b	Microbial double-stranded RNA	0.1–10 μg/mL

a Cell-surface, b intracellular, TLR = toll-like receptor, NOD2 = nucleotide-binding oligomerization domain-2.

Table 1. Overview of investigated pathogen recognition receptor (PRR) ligands and working concentrations.

Next, PRR ligands were investigated for their modulatory effects on monocyte-derived osteoclast differentiation. We investigated the effects of PRR stimulation on day 6 tartrate-resistant acid phosphatase (TRAP) activity, an enzyme secreted by resorptive osteoclasts, in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL), as the presence of both of these factors is a prerequisite for the differentiation of human monocytes into osteoclasts [26].

As the changes in local inflammatory milieu are thought to underlie the effects of PRR immunomodulation on osteoblast and osteoclast differentiation [32–34], the effects of PRR ligand stimulation were studied on the cytokine production by hMSCs and monocytes. The levels of pro-inflammatory (TNF-α, IL-6, IL-8) and anti-inflammatory (IL-10) cytokines were measured after 24 h stimulation, as they

provide a general depiction of the degree of inflammation induced by the different PRR ligands.

Reagents

Poly(I:C) high molecular weight, CpG oligodeoxynucleotide (ODN) type C (M362), Resiquimod, Pam3CSK4, Monophosphoryl Lipid A (MPLA), Curdlan (Beta-1,3-glucan from Alcaligenes faecalis), and Murabutide were obtained from InvivoGen (San Diego, CA, USA). These mediators were selected for their ability to activate different cell surface and intracellular PRRs (**Table 1**). Their final concentrations were based on the ability to induce TNF-α production in human monocytes (Resiquimod, Pam3CSK4, MPLA Curdlan, and Murabutide) (**Supplementary Figure S1**) or the manufacturer's data sheet (Poly(I:C) and CpG ODN). Recombinant human M-CSF and recombinant human RANKL were purchased from Peprotech (London, UK).

Cell Sources and Culture Conditions

Human material was obtained in accordance with the Declaration of Helsinki, with the approval of the local medical ethical committee (University Medical Center Utrecht, Utrecht, The Netherlands) under the protocols METC 08-001/K and METC 07-125/C, and with the written consent of the participants. Bone marrow was harvested from the vertebrae of female patients aged 15–30 years (n = 7), diagnosed with idiopathic scoliosis. These cell sources were selected because of the young donor age and the minimal probability of interfering factors like systemic diseases, co-morbidities, or use of medication.

hMSCs were isolated and cryopreserved as described in detail previously, as this method of MSC isolation yields multipotent cells as shown by standard differentiation assays along osteogenic, adipogenic, and chondrogenic lineages [35]. Cells below passage 7 were used for the experiments. Human peripheral blood from healthy volunteers aged 25-40 years (4 females, 1 male) was obtained from the Mini Donor Service (University Medical Center Utrecht, Utrecht, The Netherlands) in heparinized tubes. The mononuclear cell fraction was isolated by density centrifugation using Ficoll–Paque, followed by monocyte separation using positive CD14 magnetic-activated cell sorting (MACS), according to the manufacturer's instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). MSC expansion medium consisted of α-MEM (Invitrogen, Carlsbad, CA, USA) with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Hyclone CSG0412, GE Healthcare Life Sciences), 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco), and 0.2 mM L-ascorbic-acid-2-phosphate (Sigma, St Louis, MO, USA).

Monocyte medium consisted of RPMI (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) heat inactivated FBS and 100 units/mL penicillin and 100 μg/mL streptomycin. All cell culture experiments were performed at 37 °C in a humidified atmosphere containing 5% CO2.

hMSC Osteogenic Differentiation

To test the effect of PRR ligands on early and late osteogenic differentiation, hMSCs were seeded at a density of 15,000 cells/cm2 in a 96-well plate or 24 well-plates in technical triplicates and cultured in MSC expansion medium. Upon 100% confluency, cells were cultured in expansion medium or osteogenic differentiation medium (expansion medium supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone), in absence or presence of PRR ligands (final concentrations in **Table 1**). The medium and PRR ligands were refreshed twice a week for 10 days (early analyses), 14 days and 21 days (late analyses).

For ALP activity quantification, cells were lysed in 0.2% (v/v) Triton X-100/PBS for 30 min. ALP activity was measured by the conversion of the p-nitrophenyl phosphate liquid substrate system (pH = 9.6) (SigmaFast p-nitrophenyl phosphate tablets, Sigma-Aldrich). The absorbance was measured at 405 nm and corrected at 655 nm (Bio-Rad, Hercules, CA, USA). The cell lysate was also used to determine the DNA content with the Quant-It PicoGreen kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The ALP/DNA was normalized for the control not receiving any PRR ligands.

Osteocalcin (n = 3 MSC donors) and osteonectin (n = 2 MSC donors) expression were visualized using immunocytochemical staining. At day 14, cells were fixed in 4% (w/v) paraformaldehyde and permeabilized in 0.2% (v/v) Triton X-100/PBS. The cells were treated with 5% (w/v) bovine serum albumin (BSA)/PBS for 30 min in order to block non-specific binding of the antibody. Samples were incubated for 1 h at room temperature with 10 μg/mL mouse monoclonal antibody recognizing human osteocalcin (clone OCG4, Enzo Life Sciences, Zandhoven, Belgium) or 10 μg/mL anti-human osteonectin (AON-1, DSHB, Iowa City, IA, USA). The monoclonal mouse IgG1 antibody was used as isotype-matched control Ab. This was followed by 1 h incubation with 10 μg/mL goat-anti-mouse polyclonal antibody conjugated to Alexa Fluor 488 (Invitrogen). After washing with PBS, nuclei of the cells were stained with 1 μg/mL 4',6- diamidino-2-phenylindole (DAPI) for 10 min. The staining was visualized using a fluorescence microscope (Thunder, Leica microsystems, Wetzlar, Germany). Quantification was done by measuring the mean intensity of the binary image (area of the image = 0.6 mm2) obtained from experiments done in duplicates per donor using ImageJ

Freeware version 1.53e software (National Institutes of Health, Bethesda, MD, USA).

To assess and quantify the matrix mineralization after 21 days, samples were incubated with 0.2% Alizarin Red S (ARS) for 60 min (pH = 4.2, Sigma) and examined using light microscopy. In addition, Alizarin Red was extracted from the monolayer of the cells by incubating in 10% (w/v) cetylpyridinium dissolved in 10 mM sodium di-phosphate buffer solution (pH = 7.2) (Sigma Aldrich) for 60 min. Absorbance was measured at 595 nm and corrected at 655 nm. The amount of calcium deposited in each well (experiments done in triplicates) was then quantified using the standard curve obtained by dissolving known concentration of ARS and considering 2 mol of Ca2+/mol of dye in solution.

Osteoclast Differentiation Assay

CD14+ monocytes (n = 3–4 donors) were seeded at a density of 500,000 cells/cm2 in a 96-well plate in technical triplicates and cultured in osteoclast differentiation medium, consisting of α-MEM supplemented with 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin and 100 μg/mL streptomycin, 25 ng/mL M-CSF and 50 ng/mL RANKL. As a negative control for osteoclast differentiation, macrophage differentiation was induced using M-CSF medium in absence of RANKL. The effects of the PRR ligands were studied in osteoclast differentiation medium at a final concentration as mentioned in the figure legend. Culture was performed for a total of 6 days, with a medium/PRR ligand change on day 3. For TRAP staining, cells were fixed in 4% (w/v) paraformaldehyde and incubated for 20 min with 50 mM tartaric acid in 0.2 M acetate buffer (pH 5.0). Subsequently, 0.5 mg/mL naphtol AS-MX phosphate (Sigma-Aldrich) and 1.1 mg/mL fast red TR salt (Sigma-Aldrich) were added to the buffer and incubated for another 10 min at 37 °C. The samples were imaged on the IX53 Inverted Microscope (Olympus, Tokyo, Japan). Osteoclasts were defined as TRAP-positive cells (pink/red) with 3 or more nuclei [32] and were counted by a blinded observer (MC) in 4 predefined regions of interest (ROI) with a total surface area of 0.6 mm2. The osteoclast counts were normalized to the controls receiving only M-CSF and RANKL.

Cytokine Expression

hMSCs were seeded at a density of 62,500 cells/cm2 in MSC expansion medium in 96-well plates in technical triplicates. Monocytes were seeded at a density of 240,000 cells/cm2 in monocyte medium in 96-well plates in technical duplicates. Cells were stimulated for 24 h with PRR ligands according to the final concentrations mentioned in the figure legend and the supernatant was stored at

-20 °C for cytokine determination. The concentrations of TNF-α, IL-6, IL-8, and IL-10 were measured using commercially available ELISA kits (Duoset, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The results were normalized to the non-stimulated control.

Statistical Analysis

Data were tested for a normal Gaussian distribution using the Shapiro–Wilk normality test. Repeated measures ANOVA with Dunnett's post hoc correction or repeated measures mixed model with Sidak's post hoc correction were performed in SPSS (V24, IBM, Armonk, NY, USA using $p < 0.05$ as a threshold for significance. All data are presented as mean ± standard deviation, with the group sizes indicated in the figure legends.

Results

Effect of PRR Ligands on hMSC Osteogenic Differentiation

None of the cell surface PRR ligands affected the early osteogenic differentiation in terms of the ALP activity in hMSCs as compared to the control (**Figure 1A**). A significant 5-fold increase in ALP activity at day 10 was observed in the group stimulated with Poly(I:C), and a similar trend in increased ALP activity was observed in the group stimulated with CpG ODN C. On closer investigation of the responses per individual donor, it was observed that the effects of Poly(I:C) and CpG ODN C were both donor- and dose-dependent (**Figure 1B**). More specifically, only 4/7 donors were responsive to PRR stimulation in terms of significantly enhanced ALP activity. Poly(I:C) and CpG ODN C had a synergistic interaction with the osteogenic differentiation medium. No effects of these PRR ligands were observed when culturing the cells in expansion medium (**Supplementary Figure S2**), suggesting a lack of any osteoinductive effect.

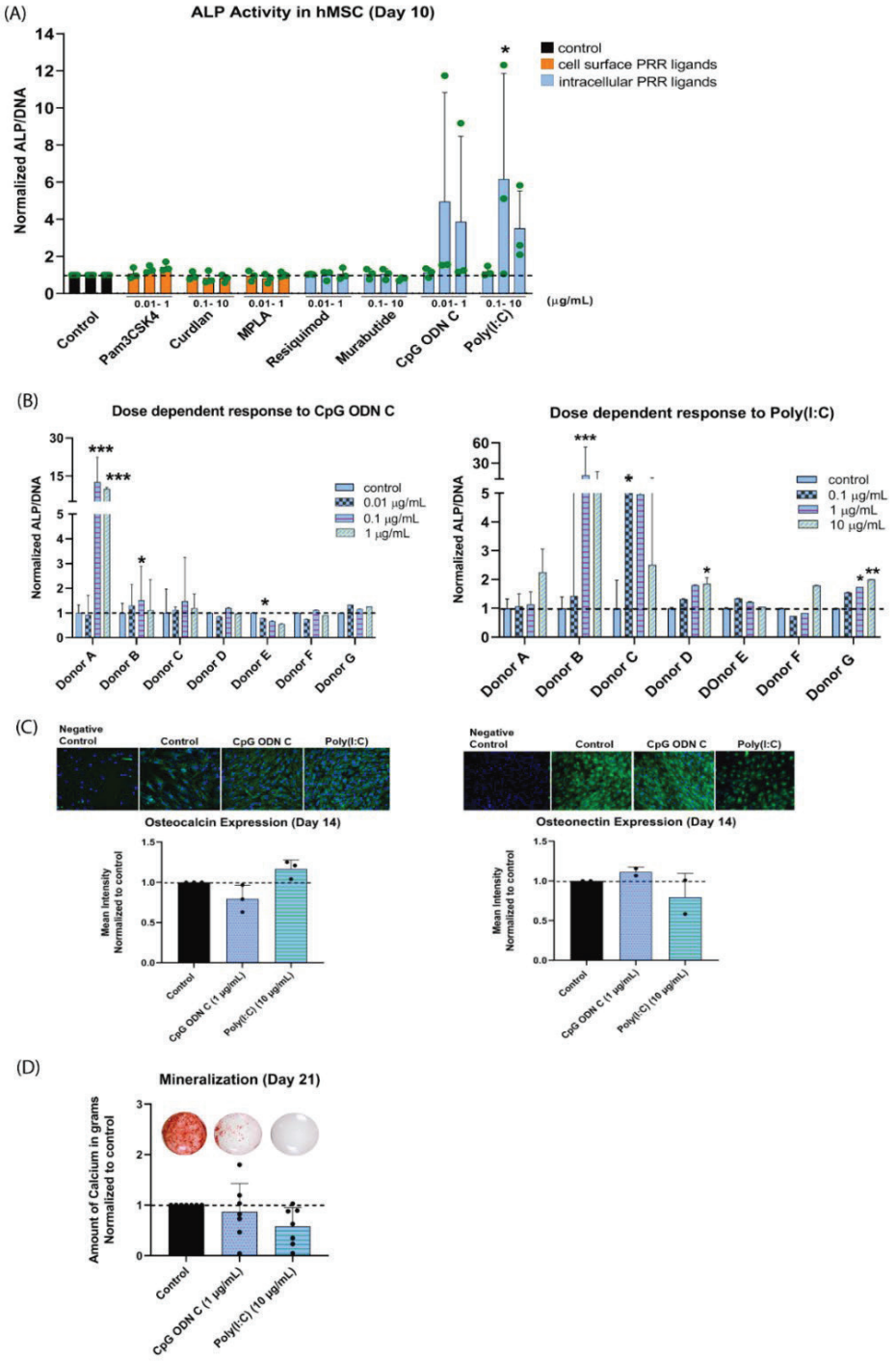

(A)
ALP Activity in hMSC (Day 10)
Normalized ALP/DNA
control
cell surface PRR ligands
intracellular PRR ligands
(μg/mL)
Control
Pam3CSK4
Curdlan
MPLA
Resiquimod
Murabutide
CpG ODN C
Poly(I:C)
(B)
Dose dependent response to CpG ODN C
Dose dependent response to Poly(I:C)
control
0.01 μg/mL
0.1 μg/mL
1 μg/mL
10 μg/mL
Donor A
Donor B
Donor C
Donor D
Donor E
Donor F
Donor G
(C)
Negative Control
Control
CpG ODN C
Poly(I:C)
Osteocalcin Expression (Day 14)
Osteonectin Expression (Day 14)
Mean Intensity Normalized to control
(D)
Mineralization (Day 21)
Amount of Calcium in grams Normalized to control

Figure 1. Effect of PRR ligands on human mesenchymal stem cells (hMSCs) osteogenic differentiation. (A) Alkaline phosphatase (ALP) activity in hMSCs after stimulation with PRR ligands in osteogenic differentiation medium for 10 days. hMSCs were stimulated with a concentration range of each PRR ligand (Table 1). ALP activity was corrected for DNA content of the cells and normalized to the control in osteogenic medium. Results show the mean ± standard deviation for 3 MSC donors. (B) ALP activity for 7 individual MSC donors after stimulation with CpG ODN C or Poly(I:C). Results show the mean ± standard deviation of the technical triplicate performed per donor. (C) Immunocytochemical staining for intracellular osteocalcin (representative for 3 MSC donors) and osteonectin (green) (representative for 2 MSC donors) was performed in hMSCs after stimulation with CpG ODN C (1 μg/mL) and Poly(I:C) (10 μg/mL) in osteogenic medium for 14 days. The staining was compared to the isotype control (negative control) to confirm the specificity of the positive signal. Cell nuclei were stained with DAPI (blue). Scale bar: 200 μm. Mean intensity of the images was calculated using ImageJ and normalized to the control in osteogenic medium. Results show the mean ± standard deviation. (D) Alizarin Red S staining was performed after stimulation with CpG ODN C (1 μg/mL) and Poly(I:C) (10 μg/mL) for 21 days (representative for 7 MSC donors). Total amount of calcium deposited per well was quantified and normalized to the control in osteogenic medium. Results show the mean ± standard deviation for 7 MSC donors. Significance was tested using repeated-measures one-way ANOVA with Sidak's post hoc test for multiple comparisons. All the treatment groups were compared to the control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In contrast to the findings regarding the early osteogenic marker ALP, groups stimulated with Poly(I:C) and CpG ODN C did not show an increased response to the late osteogenic differentiation markers, i.e., osteocalcin and osteonectin expression at day 14 (**Figure 1C**) and biological mineralization at day 21 (**Figure 1D**). An inhibitory effect of Poly(I:C) in 5/7 donors and CpG ODN C in 4/7 donors was even seen in terms of biological mineralization. Less variation was observed in their protein expression among the donors (**Supplementary Figure S3**).

Effect of PRR Ligands on Cytokine Expression of hMSCs

In hMSCs, generally low production of TNF-α and IL-10 was observed in comparison to the production of IL-6 and IL-8 (**Supplementary Table S1**). As compared to the non-stimulated control, a significantly increased production of IL-6 was observed following stimulation with Curdlan (7-fold), MPLA (7-fold), or Poly(I:C) (12-fold) (**Figure 2(c)**). Poly(I:C) stimulation resulted in a significantly

higher production of IL-8 (15-fold) as compared to the control. A trend towards increased production of IL-8 was seen following treatment with the cell surface PRR ligands.

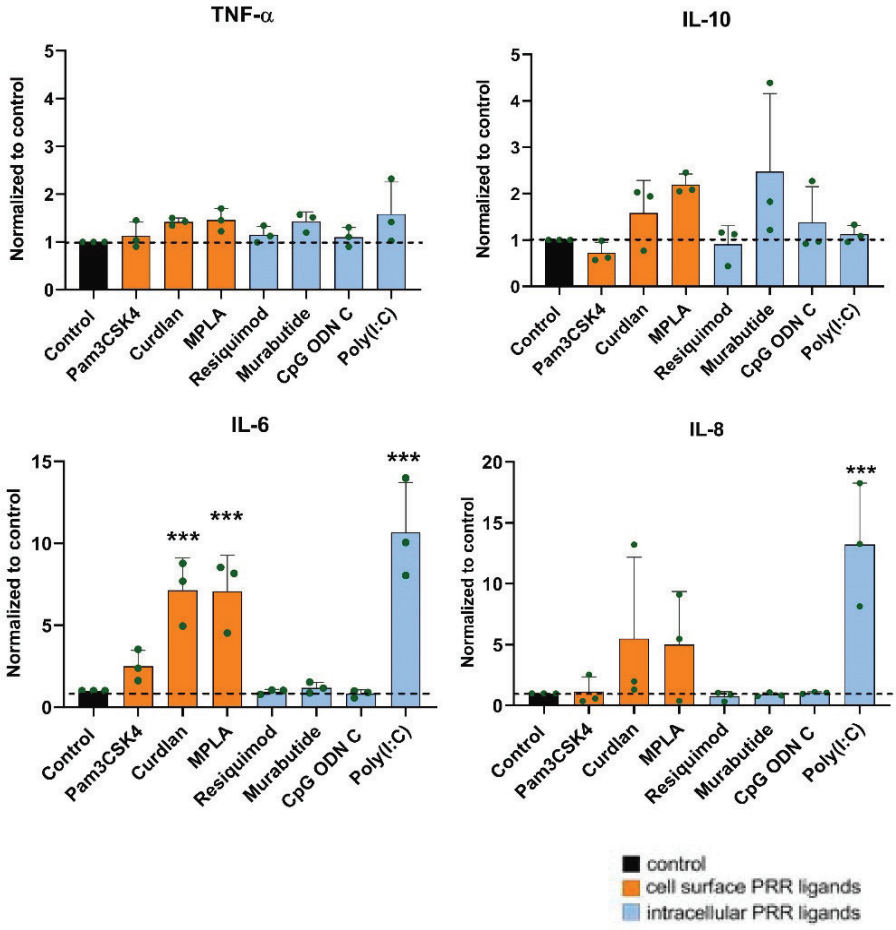


Figure 2. Cytokine production by hMSCs after stimulation with PRR ligands for 24 h. Cytokines measured were (a) TNF-α, (b) IL-10, (c) IL-6, and (d) IL-8. Concentrations used are the following: Pam3CSK4 (0.1 μg/mL), Curdlan (1 μg/mL), MPLA (10 μg/mL), Resiquimod (0.1 μg/mL), Murabutide (10 μg/mL), CpG ODN C (1 μg/mL), and Poly(I:C) (1 μg/mL). All results shown here are represented as mean ± standard deviation (n = 3 donors) and normalized to the control. Absolute values of the controls can be found in supplementary Table S1. Significance was tested using repeated-measures one-wayANOVA with Sidak's post hoc test for multiple comparisons. *** $p < 0.001$.

Effect of PRR Ligands on Human Osteoclast Differentiation

It was confirmed that the combination of M-CSF and RANKL in the osteoclastogenic medium led to the differentiation of monocytes into TRAP-positive multinucleated osteoclasts. In comparison, monocytes maintained their mono-nucleated phenotype in absence of RANKL (**Figure 3A**). Cell-surface PRR ligands had strong effect as compared to intracellular PRR ligands on the day 6 TRAP activity in osteoclastogenic medium. A significant 75% decrease in TRAP activity was observed following stimulation with the cell surface PRR ligands Pam3CSK4 or Curdlan, while MPLA completely inhibited the TRAP activity. On the other hand, stimulation with the intracellular PRR ligands Resiquimod, Murabutide, CpG ODN C, or Poly(I:C) did not affect osteoclast differentiation (**Figure 3B, C**).

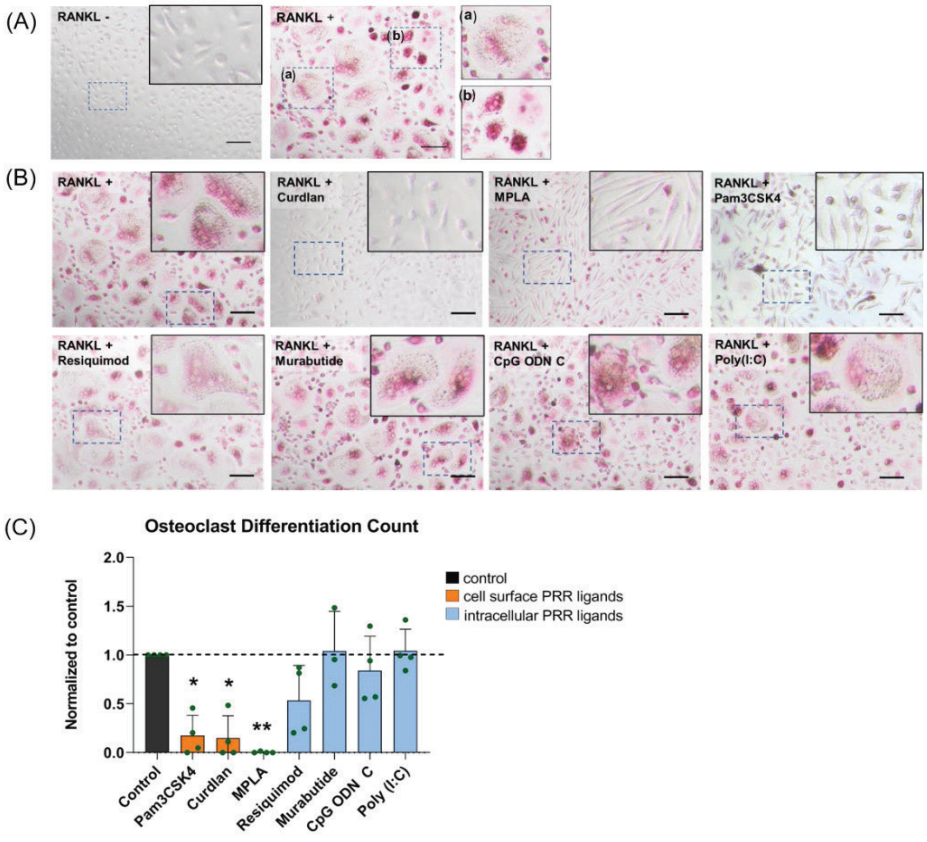


Figure 3. Effect of PRR ligands on osteoclast formation. (A) Tartrate-resistant acid phosphate (TRAP) staining performed on human monocytes cultured for 6 days +/- receptor activator of NF-κB ligand (RANKL). Osteoclasts are shown as TRAP-positive (red/pink) cells with ≥ 3 nuclei. (a) and (b) show variations in the shape and size of osteoclasts. (B) Representative images of monocytes stimulated with PRR ligands. Concentrations used are the following: Pam3CSK4 (0.1 μg/mL), Curdlan (1 μg/mL), Monophosphoryl Lipid A (MPLA) (10 μg/mL), Resiquimod (0.1 μg/mL), Murabutide (10 μg/mL), CpG ODN C (1 μg/mL), and Poly(I:C) (1 μg/mL). Scale bars correspond to 200 μm and 50 μm (inside images). (C) Osteoclast counts are represented as mean ± standard deviation (n = 3-4 donors) and normalized to the control. Significance was tested using a repeated measures mixed model approach with Sidak's post hoc test for multiple comparisons. All the treatment groups were compared to the control group. * $p < 0.05$, ** $p < 0.005$.

Effect of PRR Ligands on Cytokine Expression of Human Monocytes

Cell surface PRR stimulation in monocytes resulted in increased levels of cytokine production, whereas the intracellular PRR ligands had almost no effect. A significant increase in TNF-α production was observed following stimulation with Pam3CSK4 (20-fold), Curdlan (50-fold) or MPLA (50-fold) (**Figure 4 (a)**). IL-6 production increased even 500-fold after MPLA treatment (**Figure 4 (c)**). IL-8 production increased following stimulation with Pam3CSK4 (20-fold), Curdlan (25-fold), or MPLA (25-fold) (**Figure 4 (d)**). Only a trend towards increased IL-10 production was seen following stimulation with cell surface PRR ligands (**Figure 4 (b)**). Resiquimod, Murabutide, CpG ODN C, and Poly(I:C) had no significant effects on the cytokine production.

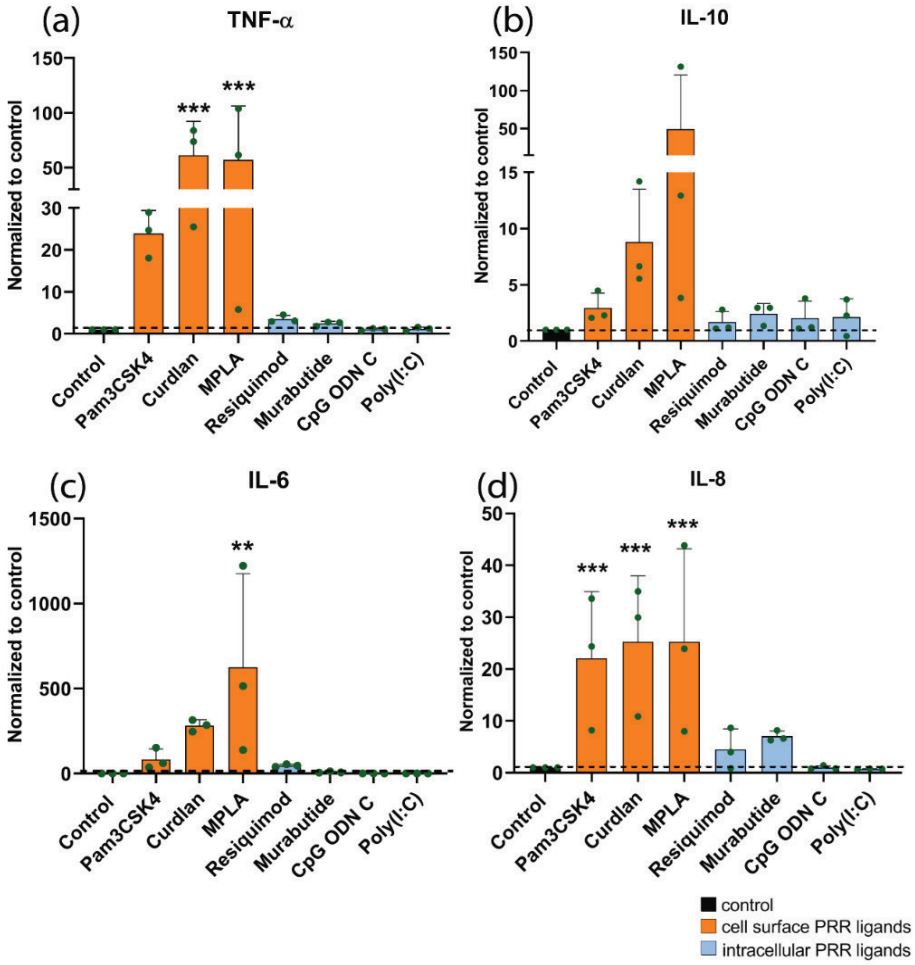


Figure 4. Cytokine production by human monocytes upon stimulation with PRR ligands for 24 h. Cytokines measured were (a) TNF-α, (b) IL-10, (c) IL-6, and (d) IL-8. Concentrations used are the following: Pam3CSKA (0.1 μg/mL), Curdlan (1 μg/mL), MPLA (10 μg/mL), Resiquimod (0.1 μg/mL), Murabutide (10 μg/mL), CpG ODN C (1 μg/mL), and Poly(I:C) (1 μg/mL). Results are represented as mean ± standard deviation (n = 3 donors) and normalized to the control. Absolute values of the controls can be found in supplementary Table S2. Normality of the data was tested using the Shapiro–Wilk test. Significance was tested using repeated-measures one-way ANOVA with Sidak's post hoc test for multiple comparisons. All treatment groups were compared to the control. ** $p < 0.01$, *** $p < 0.001$.

Discussion

The current study identifies nucleic acid-based PRR ligands as the most promising osteo-immunomodulators. Poly(I:C) and CpG ODN C comprise of synthetic microbial RNA and DNA, respectively, and are currently being investigated in clinical trials to be used as clinical adjuvants in asthma therapy and anti-tumor therapies [23,36]. Upon recognition by the intracellular receptors TLR3 and TLR9, Poly(I:C) and CpG ODN C induce the activation of the NF-κB and TIR domain-containing adapter-inducing interferon β (TRIF) signaling pathways, which results in the secretion of mostly Interferon (IFN)-α, IL-6, and IL-10 in immune cells [36,37]. In our current work, it was found that Poly(I:C) and CpG ODN C strongly enhance the hMSC early osteogenic differentiation capabilities. In line with previous studies for selective pro-inflammatory cytokines, Poly(I:C), and CpG ODN C also acted in synergy with the osteogenic inducer dexamethasone [9,31]. It can be inferred from the current results that PRR ligands are capable of inducing pro-osteogenic cytokines in hMSCs favorable for osteogenesis. It was observed that the specific micro-environment induced by Poly(I:C), characterized by high IL-6 and IL-8 production in hMSC, could in part underlie the enhanced osteogenic differentiation seen, as these cytokines have demonstrated pro-osteogenic effects [38,39]. In agreement with recent studies, upon continuous stimulation with Poly(I:C), hMSCs may switch to a pro-inflammatory phenotype [40]. Although CpG ODN C treatment did not promote the production of any of the tested cytokines in hMSCs, it is likely that CpG ODN C stimulation may favor their production of TRIF-related cytokines similar as in immune cells [36]. Hence, in the future, it will be of importance to investigate the production of type I IFNs in the supernatant to get a better insight into the pathways leading to enhanced osteogenic differentiation. Considering the response of human monocytes derived cells to be most important sign of acute inflammation, absence of pro-inflammatory cytokines in human monocytes upon stimulation with these nucleic acids indicates a lack of immune cell mediated inflammation *in vitro* [11]. However, a strong inflammatory environment induced by the MSC may contribute and modulate the immune response *in vivo*. In contrast to other reports [31,41], TNF-α was found not to be a critical regulator of inflammation-induced osteogenesis in hMSCs. Whereas TNF-α expression is increased in immune cells after PRR stimulation [42], the low levels of TNF-α secreted by hMSCs after PRR stimulation has been explained by an absence of TNF-α enhancer complexes needed for their efficient TNF-α gene expression [40]. As a strong MSC donor dependency was seen in the changes in ALP activity in response to Poly(I:C) and CpG ODN C, it is of interest to regard the large differences in responsiveness to PRR ligands between human individuals [43–45] and establish a possible

relationship between inter-individual differences in TLR expression by MSCs and the strength of the osteogenic responses.

Notably, there was an obvious discrepancy between the effects of CpG ODN C and Poly(I:C) on early and late osteogenic differentiation. Opposing the observed increase in ALP activity, Poly(I:C) and CpG ODN C did not increase the expression of late osteogenic markers, that is, osteocalcin, osteonectin, and matrix mineralization. *In vitro*, ALP activity in hMSCs is a far superior predictor of *in vivo* bone formation as compared to the late osteogenic markers osteocalcin and osteonectin [46]. Moreover, although biological mineralization is considered as functional end point of differentiation assays, there is evidence that ALP activity does not necessarily correlate with the ability of the cells to mineralize, and it is imperative to investigate the expression of Collagen type 1 and Bone sialoprotein along with biological mineralization [47]. Our *in vitro* findings are only partially predictive for *in vivo* bone formation, reason why the use of *in vivo* models is warranted to validate and verify whether nucleic acid-based ligands are indeed effective osteo-immunomodulators, considering that osteogenesis can only be determined *in vivo*.

To further determine the final role of nucleic acid based PRR ligands in osteogenic differentiation and their optimal delivery method, it can be hypothesized that timing effects (i.e., short, or continuous stimulation) may differently affect the expression of late bone markers. With this respect, shorter stimulation with CpG ODN C and Poly(I:C) may lead to more optimal upregulation of late osteogenic markers as compared to continuous stimulation to mimic the *in vivo* acute inflammatory response, as shown by many previous findings [48,49]. Moreover, given that innate immune cells mediate PRR ligand recognition and indirectly lead to cytokine-mediated regulation of hMSC functionality, the investigation of these PRR ligands in a co-culture setup comprising immune cells and hMSCs would provide additional insight into immune-mediated osteogenesis [50].

Considering that a normal osteoclast activity is a prerequisite for the onset of new bone formation around biomaterials [28,29], it was an important finding that, in addition to their pro-osteogenic effects, Poly(I:C) and CpG ODN C did not impair osteoclast formation. Although it was found that therapeutic PRR ligands could also profoundly affect human osteoclast formation, the outcome depends strongly on the cellular target[51–53]. Our study shows that in general, cell-surface PRR ligands (Curdlan, MPLA, and Pam3CSK4) markedly impaired osteoclast formation, whereas intracellular PRR-targeting ligands (Resiquimod, Murabutide, CpG ODN C, Poly(I:C)) did not influence osteoclastogenesis. In agreement, human monocytes have been reported to show higher expression of cell surface-bound TLR2 and TLR4 compared to intracellular TLR3, TLR7, and

TLR9 [54], although the expression of these PRRs can also change in response to different stimuli [48,55].

Following stimulation with the former PRR ligands, a negative association existed between the expression of NF-κB-related pro-inflammatory cytokines produced by monocytes and RANKL-mediated osteoclast formation. While PRR activation and subsequent pro-inflammatory cytokine induction is known to mediate osteoclast formation through the NF-κB signaling pathway [43,44], it reportedly leads to varying outcomes depending on the maturation stage of the osteoclast precursor cell [49,50,56]. Early myeloid precursors not yet primed with RANKL maintain their phagocytic phenotype when exposed to PRR ligands [48,57,58]. On the other hand, PRR stimulation of committed myeloid precursor cells further increases the differentiation of myeloid cells into osteoclasts and maintains their survival [44,51]. In the current study, the monocytes were simultaneously exposed to PRR ligands and RANKL from the start of culture, as this is thought to best represent the acute *in vivo* setting after biomaterial implantation [59,60]. As RANKL is also generated by osteoblasts under the stimulation of pro-inflammatory mediators [32], future studies investigating the effects of PRR ligands towards osteoblast-mediated osteoclast formation would also be of interest.

With the current novel findings, several future strategies could lead to improved bone biomaterials for clinical use. The successful application of PRR ligands ideally establishes a balanced local innate immune response without leading to sustained or systemic reactions [33,52]. Following this premise, the delivery of nucleic acid-based PRR ligands in the form of nano-particles or other delivery vehicles could be formulated to more effectively target hMSCs and the bone formation process around biodegradable bone substitutes, since they could otherwise lead to suboptimal effectiveness or cause systemic toxicities [53,56]. Various coating techniques like layer-by-layer or direct immobilization of the nucleic acids have already been employed to modify biomaterial surfaces for the controlled release and/or the intracellular delivery of nucleic acids [57,58]. Moreover, many biological effects of nucleic acid-based PRR ligands could potentially lead to their multi-purpose use in orthopedic biomaterials at high risk of bacterial colonization [61,62]. CpG ODN pretreatment confers protection against different types of infection, involving changes in both innate and adaptive host immune cells [63]. Consequently, local pretreatment with CpG ODN has protective effects against S. aureus infection in a rat bone infection model [64]. At the same time, the immune protective effect of CpG ODN follows the premise that it enhances bacterial phagocytosis and intracellular killing by various cell types, including osteoblasts [65–67]. As opposed to CpG ODN, Poly(I:C) is a booster of the antiviral response. Since viral infections are known to increase the susceptibility to bacterial infections [68], it is questionable whether Poly(I:C)

would have protective effects against bacteria commonly found in orthopedic infections. To illustrate, although Poly(I:C) is anti-infective towards Gram-negative bacteria [65], contrarily, mice pretreated with Poly(I:C) are more susceptible to Methicillin-resistant Staphylococcus aureus infection [69].

Conclusions

Therapeutic PRR ligands have immunomodulatory effects that exceed their current clinical indication, having different effects on bone-forming and bone-resorbing cell types. Most notably, the nucleic acid based PRR ligands Poly(I:C) and CpG ODN C may have potential to be applied as immunomodulators of bone regeneration. As favorable characteristics to support new bone formation, these PRR ligands had a direct stimulatory effect on the early differentiation of hMSCs into the osteogenic lineage but without interfering with osteoclast formation. Since late osteogenic markers were not upregulated in response to these PRR ligands, further investigation and verification is necessary to determine the final role of nucleic acid based PRR ligands in osteogenic differentiation and their optimal delivery strategy. The cytokine expression profiles of human MSCs and monocytes indicated that the early pro-osteogenic effects of Poly(I:C) and CpG ODN C were associated with a mild pro-inflammatory response. Since therapeutic PRR ligands have demonstrated anti-infective properties, it should be established whether nucleic acid-based ligands could be harnessed for multifunctional coatings that may enhance the bone forming capacity of synthetic biomaterial in orthopedic application, while mitigating the risk of orthopedic infections.

Funding

This work is funded by PPS allowance from the Health~Holland LSH-TKI (grant number: LSHM18011) and the EU's H2020 research and innovation programme under Marie S. Curie Cofund RESCUE (grant agreement No 801540).

Institutional Review Board Statement

Human material was obtained in accordance with the Declaration of Helsinki, with the approval of the local medical ethical committee (University Medical Center Utrecht, Utrecht, The Netherlands) under the protocols METC 08-001/K and METC 07-125/C.

Acknowledgments

We would like to thank Alasdair Irvine for his help with the English language

References

1. Campana, V.; Milano, G.; Pagano, E.; Barba, M.; Cicione, C.; Salonna, G.; Lattanzi, W.; Logroscino, G. Bone Substitutes in Orthopaedic Surgery: From Basic Science to Clinical Practice. J. Mater. Sci. Mater. Med. 2014, 25, 2445–2461, doi:10.1007/s10856-014-5240-2.
2. Kolk, A.; Handschel, J.; Drescher, W.; Rothamel, D.; Kloss, F.; Blessmann, M.; Heiland, M.; Wolff, K.-D.; Smeets, R. Current Trends and Future Perspectives of Bone Substitute Materials – From Space Holders to Innovative Biomaterials. J. Cranio-Maxillofac. Surg. 2012, 40, 706–718, doi: 10.1016/j.jcms.2012.01.002.
3. Barradas, A.M.C.; Yuan, H.; Blitterswijk, C.A.V.; Habibovic, P.; Medicine, T. Osteoinductive Biomaterials: Current Knowledge of Properties, Experimental Models and Biological Mechanisms. 2011, 21, 407–429.
4. Lehr, A.M.; Oner, F.C.; Delawi, D.; Stellato, R.K.; Hoebink, E.A.; Kempen, D.H.R.; van Susante, J.L.C.; Castelein, R.M.; Kruyt, M.C. Efficacy of a Standalone Microporous Ceramic Versus Autograft in Instrumented Posterolateral Spinal Fusion. Spine 2020, 45, 944–951, doi:10.1097/BRS.0000000000003440.
5. Morris, M.T.; Tarpada, S.P.; Cho, W. Bone Graft Materials for Posterolateral Fusion Made Simple: A Systematic Review. Eur. Spine J. 2018, 27, 1856–1867, doi:10.1007/s00586-018-5511-6.
6. Spiller, K.L.; Nassiri, S.; Witherel, C.E.; Anfang, R.R.; Ng, J.; Nakazawa, K.R.; Yu, T.; Vunjak-Novakovic, G. Sequential Delivery of Immunomodulatory Cytokines to Facilitate the M1-to-M2 Transition of Macrophages and Enhance Vascularization of Bone Scaffolds. Biomaterials 2015, 37, 194–207, doi: 10.1016/j.biomaterials.2014.10.017.
7. Mountziaris, P.M.; Spicer, P.P.; Kasper, F.K.; Mikos, A.G. Harnessing and Modulating Inflammation in Strategies for Bone Regeneration. Tissue Eng. Part B Rev. 2011, 17, 393–402, doi: 10.1089/ten.teb.2011.0182.
8. Bastidas-Coral, A.P.; Bakker, A.D.; Zandieh-Doulabi, B.; Kleverlaan, C.J.; Bravenboer, N.; Forouzanfar, T.; Klein-Nulend, J. Cytokines TNF-α, IL-6, IL-17F, and IL-4 Differentially Affect Osteogenic Differentiation of Human Adipose Stem Cells. Stem Cells Int. 2016, 2016, doi:10.1155/2016/1318256.
9. Croes, M.; Kruyt, M.C.; Groen, W.M.; van Dorenmalen, K.M.A.; Dhert, W.J.A.; Öner, F.C.; Alblas, J. Interleukin 17 Enhances Bone Morphogenetic Protein-2-Induced Ectopic Bone Formation. Sci. Rep. 2018, 8, 7269, doi:10.1038/s41598-018-25564-9.
10. Claes, L.; Recknagel, S.; Ignatius, A. Fracture Healing under Healthy and Inflammatory Conditions; 2012; Vol. 8;.
11. Pajarinen, J.; Lin, T.; Gibon, E.; Kohno, Y.; Maruyama, M.; Nathan, K.; Lu, L.; Yao, Z.; Goodman, S.B. Mesenchymal Stem Cell-Macrophage Crosstalk and

Bone Healing. Biomaterials 2019, 196, 80–89, doi: 10.1016/j.biomaterials.2017.12.025.
12. Croes, M.; Kruyt, M.C.; Loozen, L.; Kragten, A.H.; Yuan, H.; Dhert, W.J.; Öner, F.C.; Alblas, J. Local Induction of Inflammation Affects Bone Formation. Eur. Cell. Mater. 2017, 33, 211–226, doi:10.22203/eCM.v033a16.
13. O'Neill, L.A.J.; Golenbock, D.; Bowie, A.G. The History of Toll-like Receptors — Redefining Innate Immunity. Nat. Rev. Immunol. 2013, 13, 453–460, doi:10.1038/nri3446.
14. Hwa Cho, H.; Bae, Y.C.; Jung, J.S. Role of Toll-Like Receptors on Human Adipose-Derived Stromal Cells. Stem Cells 2006, 24, 2744–2752, doi:10.1634/stemcells.2006-0189.
15. Croes, M.; Kruyt, M.C.; Boot, W.; Pouran, B.; Braham, M.V.; Pakpahan, S.A.; Weinans, H.; Vogely, H.C.; Fluit, A.C.; Dhert, W.J.; et al. The Role of Bacterial Stimuli in Inflammation-Driven Bone Formation. Eur. Cell. Mater. 2019, 37, 402–419, doi:10.22203/eCM.v037a24.
16. Croes, M.; Wal, B.C.H.; Vogely, H.C. Impact of Bacterial Infections on Osteogenesis: Evidence From *In vivo* Studies. J. Orthop. Res. 2019, 37, 2067–2076, doi:10.1002/jor.24422.
17. Croes, M.; Boot, W.; Kruyt, M.C.; Weinans, H.; Pouran, B.; van der Helm, Y.J.M.; Gawlitta, D.; Vogely, H.C.; Alblas, J.; Dhert, W.J.A.; et al. Inflammation-Induced Osteogenesis in a Rabbit Tibia Model. Tissue Eng. Part C Methods 2017, 23, 673–685, doi: 10.1089/ten.tec.2017.0151.
18. Dowling, J.K.; Mansell, A. Toll-like Receptors: The Swiss Army Knife of Immunity and Vaccine Development. Clin Transl Immunol. 2016, 5, e85, doi:10.1038/cti.2016.22.
19. Hussein, W.M.; Liu, T.Y.; Skwarczynski, M.; Toth, I. Toll-like Receptor Agonists: A Patent Review (2011 - 2013). Expert Opin Ther Pat 2014, 24, 453–470, doi:10.1517/13543776.2014.880691.
20. Basith, S.; Manavalan, B.; Lee, G.; Kim, S.G.; Choi, S. Toll-like Receptor Modulators: A Patent Review (2006 - 2010). Expert Opin. Ther. Pat. 2011, 21, 927–944, doi:10.1517/13543776.2011.569494.
21. Kline, J.N. Eat Dirt: CpG DNA and Immunomodulation of Asthma. Proc. Am. Thorac. Soc. 2007, 4, 283–288, doi:10.1513/pats.200701-019AW.
22. Colnot, C. Skeletal Cell Fate Decisions within Periosteum and Bone Marrow during Bone Regeneration. J. Bone Miner. Res. 2009, 24, 274–282, doi:10.1359/jbmr.081003.
23. Zheng, C.; Chen, J.; Liu, S.; Jin, Y. Stem Cell-Based Bone and Dental Regeneration: A View of Microenvironmental Modulation. Int J Oral Sci 2019, 11, 23, doi:10.1038/s41368-019-0060-3.
24. Qi, C.; Xiaofeng, X.; Xiaoguang, W. Effects of Toll-like Receptors 3 and 4 in the Osteogenesis of Stem Cells. Stem Cells Int. 2014, 2014, 917168, doi:10.1155/2014/917168.

25. Ha, H.; Lee, J.-H.; Kim, H.-N.; Kwak, H.B.; Kim, H.-M.; Lee, S.E.; Rhee, J.H.; Kim, H.-H.; Lee, Z.H. Stimulation by TLR5 Modulates Osteoclast Differentiation through STAT1/IFN-β. J. Immunol. 2008, 180, 1382–1389, doi:10.4049/jimmunol.180.3.1382.
26. Boyle, W.J.; Simonet, W.S.; Lacey, D.L. Osteoclast Differentiation and Activation. Nature 2003, 423, 337–342.
27. Ducy, P.; Karsenty, G. Two Distinct Osteoblast-Specific Cis-Acting Elements Control Expression of a Mouse Osteocalcin Gene. Mol. Cell. Biol. 1995, 15, 1858–1869, doi:10.1128/MCB.15.4.1858.
28. Davison, N.L.; Gamblin, A.L.; Layrolle, P.; Yuan, H.; de Bruijn, J.D.; Barrère-de Groot, F. Liposomal Clodronate Inhibition of Osteoclastogenesis and Osteoinduction by Submicrostructured Beta-Tricalcium Phosphate. Biomaterials 2014, 35, 5088–5097, doi: 10.1016/j.biomaterials.2014.03.013.
29. Soto-Peñaloza, D., Martín-de-Llano, J. J., Carda-Batalla, C., Peñarrocha-Diago, M., & Peñarrocha-Oltra, D. Basic Bone Biology Healing During Osseointegration of Titanium Dental Implants. Atlas Immed. Dent. Implant Load. 2019, 17–28.
30. Akhavan, B.; Croes, M.; Wise, S.G.; Zhai, C.; Hung, J.; Stewart, C.; Ionescu, M.; Weinans, H.; Gan, Y.; Amin Yavari, S.; et al. Radical-Functionalized Plasma Polymers: Stable Biomimetic Interfaces for Bone Implant Applications. Appl. Mater. Today 2019, 16, 456–473, doi: 10.1016/j.apmt.2019.07.002.
31. Croes, M.; Oner, F.C.; Kruyt, M.C.; Blokhuis, T.J.; Bastian, O.; Dhert, W.J.A.; Alblas, J. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. PLoS ONE 2015, 10, 1–14, doi: 10.1371/journal.pone.0132781.
32. Terheyden, H.; Lang, N.P.; Bierbaum, S.; Stadlinger, B. Osseointegration - Communication of Cells. Clin. Oral Implants Res. 2012, 23, 1127–1135, doi:10.1111/j.1600-0501.2011.02327.x.
33. Souza, P.P.C.; Lerner, U.H. The Role of Cytokines in Inflammatory Bone Loss. Immunol. Invest. 2013, 42, 555–622, doi:10.3109/08820139.2013.822766.
34. Guihard, P.; Danger, Y.; Brounais, B.; David, E.; Brion, R.; Delecrin, J.; Richards, C.D.; Chevalier, S.; Rédini, F.; Heymann, D.; et al. Induction of Osteogenesis in Mesenchymal Stem Cells by Activated Monocytes/Macrophages Depends on Oncostatin M Signaling. Stem Cells 2012, 30, 762–772, doi:10.1002/stem.1040.
35. Pennings, I.; van Dijk, L.A.; van Huuksloot, J.; Fledderus, J.O.; Schepers, K.; Braat, A.K.; Hsiao, E.C.; Barruet, E.; Morales, B.M.; Verhaar, M.C.; et al. Effect of Donor Variation on Osteogenesis and Vasculogenesis in Hydrogel Cocultures. J. Tissue Eng. Regen. Med. 2019, 13, 433–445, doi:10.1002/term.2807.
36. Krug, A.; Rothenfusser, S.; Hornung, V.; Jahrsdörfer, B.; Blackwell, S.; Ballas, Z.K.; Endres, S.; Krieg, A.M.; Hartmann, G. Identification of CpG Oligonucleotide Sequences with High Induction of IFN-Alpha/Beta in

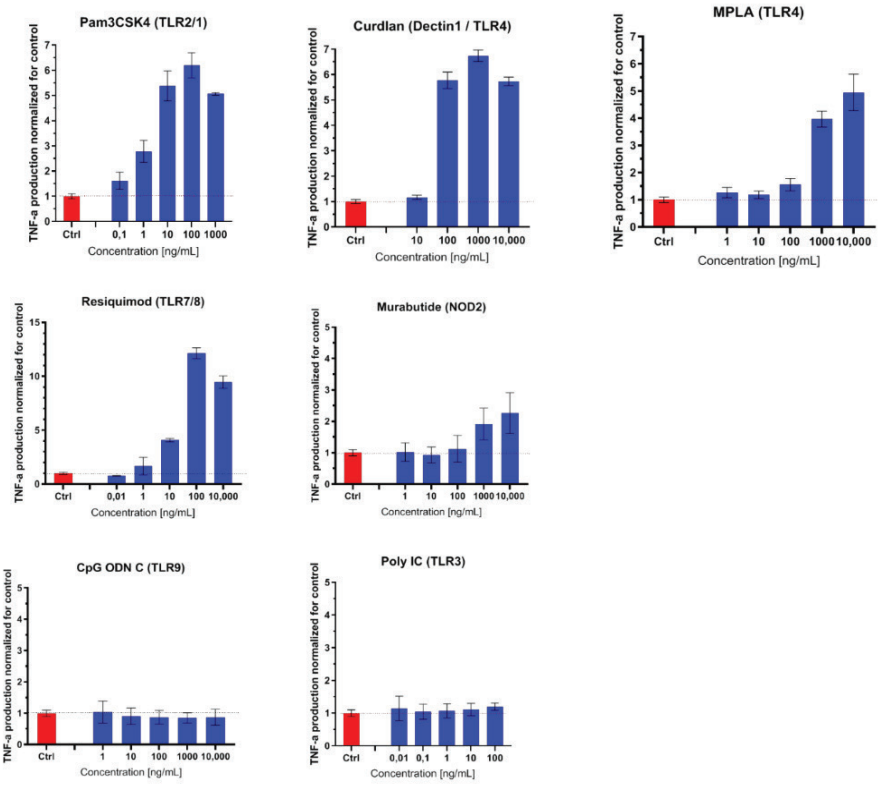
Plasmacytoid Dendritic Cells. Eur. J. Immunol. 2001, 31, 2154–2163, doi:10.1002/1521-4141(200107)31:7<2154::aid-immu2154>3.0.co;2-u.
37. Romieu-Mourez, R.; François, M.; Boivin, M.-N.; Bouchentouf, M.; Spaner, D.E.; Galipeau, J. Cytokine Modulation of TLR Expression and Activation in Mesenchymal Stromal Cells Leads to a Proinflammatory Phenotype. J. Immunol. 2009, 182, 7963–7973, doi:10.4049/jimmunol.0803864.
38. Yang, A.; Lu, Y.; Xing, J.; Li, Z.; Yin, X.; Dou, C.; Dong, S.; Luo, F.; Xie, Z.; Hou, T.; et al. IL-8 Enhances Therapeutic Effects of BMSCs on Bone Regeneration via CXCR2-Mediated PI3k/Akt Signaling Pathway. Cell. Physiol. Biochem. 2018, 48, 361–370, doi:10.1159/000491742.
39. Huh, J.-E.; Lee, S.Y. IL-6 Is Produced by Adipose-Derived Stromal Cells and Promotes Osteogenesis. Biochim. Biophys. Acta BBA - Mol. Cell Res. 2013, 1833, 2608–2616, doi: 10.1016/j.bbamcr.2013.06.025.
40. Dumitru, C.A.; Hemeda, H.; Jakob, M.; Lang, S.; Brandau, S. Stimulation of Mesenchymal Stromal Cells (MSCs) via TLR3 Reveals a Novel Mechanism of Autocrine Priming. FASEB J. 2014, 28, 3856–3866, doi:10.1096/fj.14-250159.
41. Glass, G.E.; Chan, J.K.; Freidin, A.; Feldmann, M.; Horwood, N.J. TNF- α Promotes Fracture Repair by Augmenting the Recruitment and Differentiation of Muscle-Derived Stromal Cells. 2011, 108, 1585–1590, doi:10.1073/pnas.1018501108.
42. Mayringer, I.; Reindl, M.; Berger, T. A Critical Comparison of Frequently Used Methods for the Analysis of Tumor Necrosis Factor-α Expression by Human Immune Cells. J. Immunol. Methods 2000, 235, 33–40, doi:10.1016/S0022-1759(99)00208-2.
43. Takami, M.; Kim, N.; Rho, J.; Choi, Y. Stimulation by Toll-Like Receptors Inhibits Osteoclast Differentiation. J. Immunol. 2002, 169, 1516–1523, doi:10.4049/jimmunol.169.3.1516.
44. Souza, P.P.C.; Lerner, U.H. Finding a Toll on the Route: The Fate of Osteoclast Progenitors after Toll-like Receptor Activation. Front. Immunol. 2019, 10, 1–12, doi:10.3389/fimmu.2019.01663.
45. Zou, W.; Bar-Shavit, Z. Dual Modulation of Osteoclast Differentiation by Lipopolysaccharide. J. Bone Miner. Res. 2002, 17, 1211–1218, doi:10.1359/jbmr.2002.17.7.1211.
46. Prins, H.-J.; Braat, A.K.; Gawlitta, D.; Dhert, W.J.A.; Egan, D.A.; Tijssen-Slump, E.; Yuan, H.; Coffer, P.J.; Rozemuller, H.; Martens, A.C. *In vitro* Induction of Alkaline Phosphatase Levels Predicts *in vivo* Bone Forming Capacity of Human Bone Marrow Stromal Cells. Stem Cell Res. 2014, 12, 428–440, doi: 10.1016/j.scr.2013.12.001.
47. Hoemann, C.D.; El-Gabalawy, H.; McKee, M.D. *In vitro* Osteogenesis Assays: Influence of the Primary Cell Source on Alkaline Phosphatase Activity and Mineralization. Pathol. Biol. 2009, 57, 318–323, doi: 10.1016/j.patbio.2008.06.004.

48. Dickie, L.J.; Church, L.D.; Coulthard, L.R.; Mathews, R.J.; Emery, P.; McDermott, M.F. Vitamin D3 Down-Regulates Intracellular Toll-like Receptor 9 Expression and Toll-like Receptor 9-Induced IL-6 Production in Human Monocytes. Rheumatol. Oxf. Engl. 2010, 49, 1466–1471, doi:10.1093/rheumatology/keq124.
49. Huang, H.; Zhao, N.; Xu, X.; Xu, Y.; Li, S.; Zhang, J.; Yang, P. Dose-Specific Effects of Tumor Necrosis Factor Alpha on Osteogenic Differentiation of Mesenchymal Stem Cells. Cell Prolif. 2011, 44, 420–427, doi: 10.1111/j.1365-2184.2011.00769.x.
50. Kovach, T.K.; Dighe, A.S.; Lobo, P.I.; Cui, Q. Interactions between MSCs and Immune Cells: Implications for Bone Healing. J. Immunol. Res. 2015, 2015, doi:10.1155/2015/752510.
51. Kobayashi, K.; Takahashi, N.; Jimi, E.; Udagawa, N.; Takami, M.; Kotake, S.; Nakagawa, N.; Kinosaki, M.; Yamaguchi, K.; Shima, N.; et al. Tumor Necrosis Factor α Stimulates Osteoclast Differentiation by a Mechanism Independent of the ODF/RANKL-RANK Interaction. J. Exp. Med. 2000, 191, 275–285, doi:10.1084/jem.191.2.275.
52. Hancock, R.E.W.; Nijnik, A.; Philpott, D.J. Modulating Immunity as a Therapy for Bacterial Infections. Nat. Rev. Microbiol. 2012, 10, 243–254, doi:10.1038/nrmicro2745.
53. Suzuki, Y.; Wakita, D.; Chamoto, K.; Narita, Y.; Tsuji, T.; Takeshima, T.; Gyobu, H.; Kawarada, Y.; Kondo, S.; Akira, S.; et al. Liposome-Encapsulated CpG Oligodeoxynucleotides as a Potent Adjuvant for Inducing Type 1 Innate Immunity. Cancer Res. 2004, 64, 8754–8760, doi: 10.1158/0008-5472.CAN-04-1691.
54. Hornung, V.; Rothenfusser, S.; Britsch, S.; Krug, A.; Jahrsdörfer, B.; Giese, T.; Endres, S.; Hartmann, G. Quantitative Expression of Toll-Like Receptor 1–10 MRNA in Cellular Subsets of Human Peripheral Blood Mononuclear Cells and Sensitivity to CpG Oligodeoxynucleotides. J. Immunol. 2002, 168, 4531–4537, doi: 10.4049/jimmunol.168.9.4531.
55. Muzio, M.; Bosisio, D.; Polentarutti, N.; D'amico, G.; Stoppacciaro, A.; Mancinelli, R.; van't Veer, C.; Penton-Rol, G.; Ruco, L.P.; Allavena, P.; et al. Differential Expression and Regulation of Toll-Like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells. J. Immunol. 2000, 164, 5998 LP – 6004, doi:10.4049/jimmunol.164.11.5998.
56. Gale, E.C.; Roth, G.A.; Smith, A.A.A.; Alcántara-Hernández, M.; Idoyaga, J.; Appel, E.A. A Nanoparticle Platform for Improved Potency, Stability, and Adjuvanticity of Poly(I:C). Adv. Ther. 2020, 3, 1900174, doi:10.1002/adtp.201900174.
57. Scharnweber, D.; Bierbaum, S.; Wolf-Brandstetter, C. Utilizing DNA for Functionalization of Biomaterial Surfaces. FEBS Lett. 2018, 592, 2181–2196, doi:10.1002/1873-3468.13065.

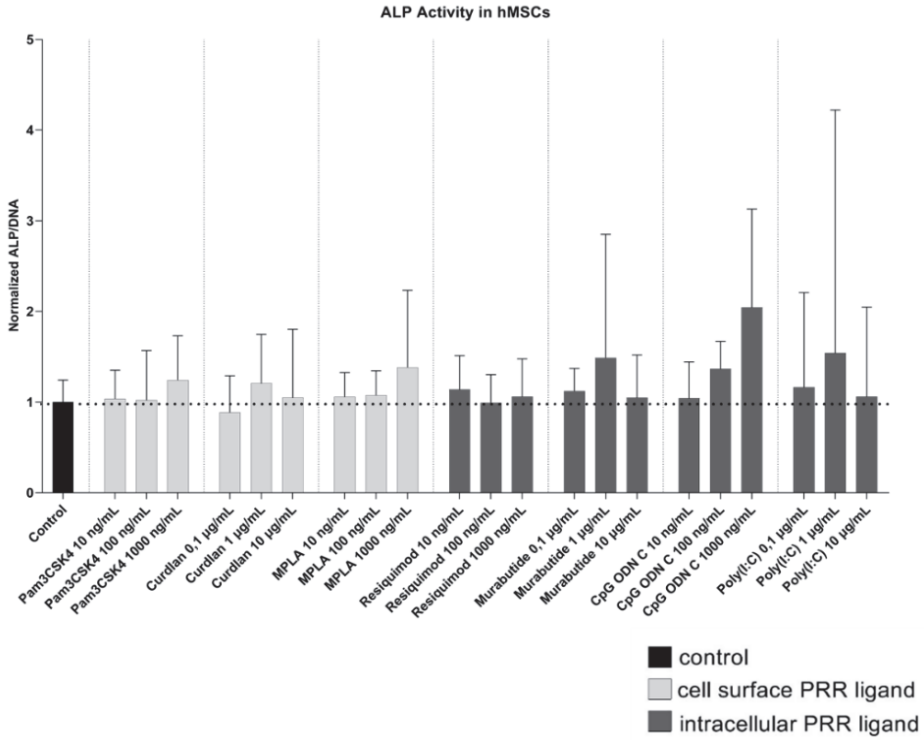
58. Amin Yavari, S.; Croes, M.; Akhavan, B.; Jahanmard, F.; Eigenhuis, C.C.; Dadbakhsh, S.; Vogely, H.C.; Bilek, M.M.; Fluit, A.C.; Boel, C.H.E.; et al. Layer by Layer Coating for Bio-Functionalization of Additively Manufactured Meta-Biomaterials. Addit. Manuf. 2020, 32, 100991, doi: 10.1016/j.addma.2019.100991.
59. Marsell, R.; Einhorn, T.A. The Biology of Fracture Healing. Injury 2011, 42, 551–555, doi: 10.1016/j.injury.2011.03.031.
60. Irie, K.; Alpaslan, C.; Takahashi, K.; Kondo, Y.; Izumi, N.; Sakakura, Y.; Tsuruga, E.; Nakajima, T.; Ejiri, S.; Ozawa, H.; et al. Osteoclast Differentiation in Ectopic Bone Formation Induced by Recombinant Human Bone Morphogenetic Protein 2 (RhBMP-2). J. Bone Miner. Metab. 2003, 21, 363–369, doi:10.1007/s00774-003-0430-x.
61. Inzana, J.A.; Schwarz, E.M.; Kates, S.L.; Awad, H.A. Biomaterials Approaches to Treating Implant-Associated Osteomyelitis. Biomaterials 2016, 81, 58–71, doi: 10.1016/j.biomaterials.2015.12.012.
62. Yavari, S.A.; Castenmiller, S.M.; Strijp, J.A.G. van; Croes, M. Combating Implant Infections: Shifting Focus from Bacteria to Host. Adv. Mater. 2020, 32, 2002962, doi: 10.1002/adma.202002962.
63. Weighardt, H.; Feterowski, C.; Veit, M.; Rump, M.; Wagner, H.; Holzmann, B. Increased Resistance Against Acute Polymicrobial Sepsis in Mice Challenged with Immunostimulatory CpG Oligodeoxynucleotides Is Related to an Enhanced Innate Effector Cell Response. J. Immunol. 2000, 165, 4537–4543, doi:10.4049/jimmunol.165.8.4537.
64. Sethi, S.; Thormann, U.; Sommer, U.; Stotzel, S.; Mohamed, W.; Schnettler, R.; Domann, E.; Chakraborty, T.; Alt, V. Impact of Prophylactic CpG Oligodeoxynucleotide Application on Implant-Associated Staphylococcus Aureus Bone Infection. Bone 2015, 78, 194–202, doi: 10.1016/j.bone.2015.04.030.
65. Ribes, S.; Arcilla, C.; Ott, M.; Schütze, S.; Hanisch, U.-K.; Nessler, S.; Nau, R. Pre-Treatment with the Viral Toll-like Receptor 3 Agonist Poly(I:C) Modulates Innate Immunity and Protects Neutropenic Mice Infected Intracerebrally with Escherichia Coli. J. Neuroinflammation 2020, 17, 24, doi:10.1186/s12974-020-1700-4.
66. Mohamed, W.; Domann, E.; Chakraborty, T.; Mannala, G.; Lips, K.S.; Heiss, C.; Schnettler, R.; Alt, V. TLR9 Mediates S. Aureus Killing inside Osteoblasts via Induction of Oxidative Stress. BMC Microbiol. 2016, 16, 230, doi:10.1186/s12866-016-0855-8.
67. Wu, H.-M.; Wang, J.; Zhang, B.; Fang, L.; Xu, K.; Liu, R.-Y. CpG-ODN Promotes Phagocytosis and Autophagy through JNK/P38 Signal Pathway in Staphylococcus Aureus-Stimulated Macrophage. Life Sci. 2016, 161, 51–59, doi: 10.1016/j.lfs.2016.07.016.
68. Sun, K.; Metzger, D.W. Inhibition of Pulmonary Antibacterial Defense by Interferon-γ during Recovery from Influenza Infection. Nat. Med. 2008, 14, 558–564, doi:10.1038/nm1765.

69. Ruiz, J.; Kanagavelu, S.; Flores, C.; Romero, L.; Riveron, R.; Shih, D.Q.; Fukata, M. Systemic Activation of TLR3-Dependent TRIF Signaling Confers Host Defense against Gram-Negative Bacteria in the Intestine. Front. Cell. Infect. Microbiol. 2016, 5, doi:10.3389/fcimb.2015.00105.

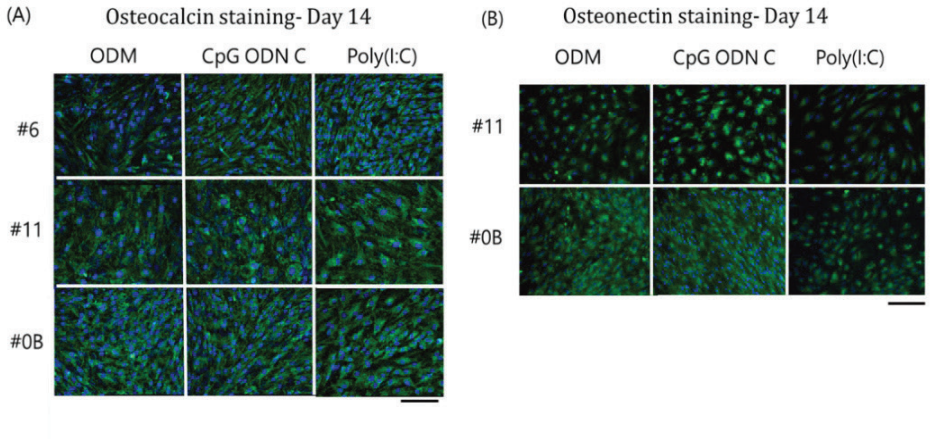
Supplementary Materials



Supplementary Figure S1. TNF-α production by human monocytes upon stimulation with PRR ligands for 24 hours (concentrations used in Table 1). Results are represented as mean ± standard deviation (n = 2 donors) and normalized to the control.



Supplementary Figure S2. Effect of PRR ligands on hMSCs on day 10 ALP activity in absence of dexamethasone. Results are represented as mean ± standard deviation (n = 3 donors) and normalized to the control.



Supplementary Figure S3. Effect of Poly(I:C) and CpG ODN C ligands on hMSCs on day 14 (A) osteocalcin ($n = 3$) and (B) osteonectin ($n = 2$) expression. Results are represented as mean ± standard deviation and normalized to the control (ODM) containing osteogenic medium. Scale bar = 200 μm.

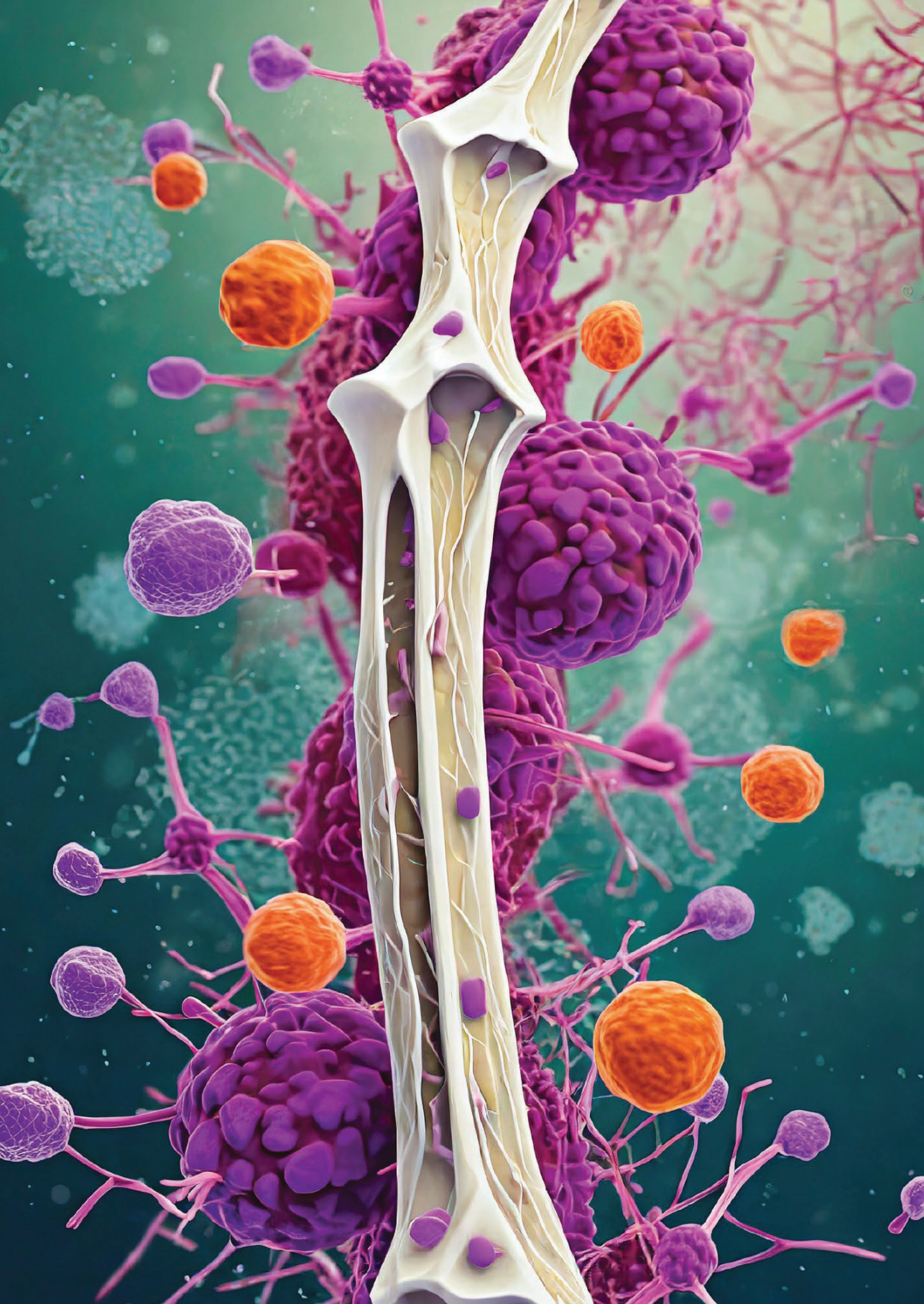
	TNF-α	IL-10	IL-6	IL-8
Donor A	17.70 ± 0.20	29.08 ± 21.06	1546.49 ± 59.58	2713.38 ± 225.14
Donor B	30.76 ± 3.41	73.52 ± 38.37	1497.58 ± 99.33	2776.52 ± 28.05
Donor C	32.42 ± 2.16	65.86 ± 8.00	1142.78 ± 83.51	2856.84 ± 41.84

Supplementary Table S1. Absolute values in cytokine expression (pg/mL) by hMSCs, expressed as mean ± SD for each donor.

	TNF- α	IL-10	IL-6	IL-8
Donor A	27.15 ± 0.00*	11.379 ± 0.00*	9.38 ± 0.00*	1207.63 ± 0.00*
Donor B	29.04 ± 0.48	9.14 ± 0.85	9.38 ± 0.00*	1193.14 ± 539.23
Donor C	66.59 ± 7.72	42.27 ± 3.24	36.31 ± 27.56	4582.08 ± 2696.00

* The lowest concentration in the standard curve was used for calculations when absorbance values were below that of the lowest concentration in the standard curve.

Supplementary Table S2. Absolute values in cytokine expression (pg/mL) by human monocytes, expressed as mean ± SD for each donor.



Chapter 3

An *in vitro* model to test the influence of immune cell secretome on MSC osteogenic differentiation

Paree Khokhani[1], Ruggero Bulloerumo[1], Michiel Croes[1], Debby Gawlitta[2], Moyo C. Kruyt[1] and Harrie Weinans[1]

1. Department of Orthopedics, University Medical Center Utrecht, Utrecht, the Netherlands
2. Department of Oral and Maxillofacial Surgery, Prosthodontics and Special Dental, University Medical Center (UMC), Utrecht, The Netherlands.

**Published in Tissue Eng Part C Methods. 2022 Aug;28(8):420-430.*

Abstract

Immune cells and their soluble factors have an important role in the bone healing process. Modulation of the immune response, therefore, offers a potential strategy to enhance bone formation. To investigate the influence of the immune system on osteogenesis, we developed and applied an *in-vitro* model that incorporates both innate and adaptive immune cells. Human peripheral blood mononuclear cells (PBMCs) were isolated and cultured for 24 hours and subsequently stimulated with immune-modulatory agents; C-class CpG oligodeoxynucleotide (CpG ODN C), Polyinosinic acid-polycytidylic acid Poly(I:C), and lipopolysaccharide (LPS); all pathogen recognition receptor agonists, and that target Toll-like receptors TLR9, -3, and -4, respectively. The conditioned medium obtained from PBMCs after 24 hours was used to investigate its effects on the metabolic activity and osteogenic differentiation capacity of human bone marrow-derived mesenchymal stromal cells (MSCs). Conditioned media from unstimulated PBMCs did not affect the metabolic activity and osteogenic differentiation capacity of MSCs. The conditioned medium from CpG ODN C and LPS stimulated PBMCs increased alkaline phosphatase activity of MSCs by approximately 3-fold as compared to the unstimulated control, whereas Poly(I:C) conditioned medium did not enhance ALP activity of MSCs. Moreover, direct stimulation of MSCs with the immune-modulatory stimuli did not result in increased alkaline phosphatase activity. These results demonstrate that soluble factors present in conditioned medium from PBMCs stimulated with immune-modulatory factors enhance osteogenesis of MSCs. This *in-vitro* model can serve as a tool in screening immune-modulatory stimulants from a broad variety of immune cells for (indirect) effects on osteogenesis and to identify soluble factors from multiple immune cell types that may modulate bone healing.

Impact Statement

In this study, an *in-vitro* model is introduced that consists of multiple immune cell types from PBMCs and evaluates the effect of their secretome on MSC osteogenesis after stimulation with pathogen recognition receptors ligands. This model can be used to develop a better understanding of the crosstalk between immune cells and bone forming MSCs, paving the way to improve strategies for bone healing.

Keywords

Osteoimmunology, pathogen recognition receptors, osteogenesis, bone healing, immune cells, inflammation

Introduction

Bone tissue has an intrinsic capacity to regenerate itself upon injury. However, the healing process of bone can be impaired under conditions like severe trauma, cancer (treatment), metabolic diseases, or mechanical instability[1,2]. Transplantation of autologous bone to the defect site is currently the gold standard despite its limitations like increased procedure time[3] and limited availability[4]. Various synthetic grafts, particularly ceramics, hold the potential to be used as bone substitutes due to their biocompatibility and osteoconductive properties. However, they generally lack bone-inducing capacity limiting their use as stand-alone grafts to treat large bone defects[5]. Even if ceramics perform as well as autografts, there still is a need for improvement to address the most challenging conditions such as spinal fusion[6,7]. Strategies involving the use of cells, biomaterials, and/or growth factors[8,9] are currently explored as a way to enhance the performance of synthetic grafts.

Due to their close relationship with osteoblasts and bone deposition in the body, human multipotent mesenchymal stromal cells (MSCs) are the most common cells employed to study the *in-vitro* osteogenic potential of bone grafts and therapeutics[10]. Studies have shown that MSCs are capable of differentiation into osteoblasts, the effectors of bone formation *in vivo*, hence many strategies to augment bone regeneration focus on increasing the osteogenic potential of MSCs[11]. However, therapeutic approaches targeting only MSCs usually fail when translating their application to *in vivo* settings, indicating that the mechanisms underlying bone formation are still poorly understood[10]. For this reason, several new strategies to achieve predictable and enhanced bone formation *in vivo* are under development, and osteo-immunomodulation is a promising candidate. Modulation of the local immune response as a strategy to enhance bone formation has gained a lot of interest, considering the emerging recognition of the pivotal role of a balanced early inflammatory phase in the bone healing process[12]. Research showed that administration of bacterial stimuli leads to increased bone formation in a rabbit tibia model[13]. The rationale behind this phenomenon is believed to be the release of cytokines upon the activation of pathogen recognition receptors in immune cells and bone cells. Recently, it was shown that specific targeting of pathogen recognition receptors (PRRs) can be harnessed to promote osteogenesis via immune-modulation [14]. PRRs are single-pass transmembrane proteins with an extracellular region (involved in recognition of the pathogens), and an intracellular region (involved in signal transduction). They are highly expressed in immune cells, and, upon stimulation, PRRs trigger the release of cytokines and soluble factors[15]. Hence, the stimulation of PRR ligands to trigger the release of pro-osteogenic cytokines

represents a valid therapeutic strategy to promote augmented osteogenesis in MSCs via the immune system.

Taking cues from the fracture healing process as an example of efficient bone regeneration, the crosstalk between the immune cells and bone cells or their precursors (MSCs) is a prerequisite for optimal bone healing[16]. Both innate and adaptive immune cells can be found at the fracture site during the early stage after fracture, which indicates that inflammation is the first action of repair[17,18]. After the injury, pro-inflammatory cytokines such as Tumor necrosis factor (TNF-α), Interleukins-1 (IL-1), Interleukin-6 (IL-6), Interleukin-17 (IL-17), and anti-inflammatory cytokines such as Interleukin-10 (IL-10), Interleukin-4 (IL-4) act in synergy with bone morphogenetic proteins (BMPs) and other transforming growth factors to facilitate infiltration of immune cells, regulate angiogenesis and induce migration and differentiation of bone progenitor cells (MSCs) at the site of injury[19,20]. Studies using knockout mice have demonstrated the functional role of these immune cells and related inflammatory factors during fracture healing. For example, the selective depletion of macrophages, dendritic cells, and T-, and B- cells at different time points after the fracture has confirmed their contribution to the fracture healing process [21–23]. The innate and adaptive immune cells secrete cytokines and growth factors that favour osteogenesis and angiogenesis. For example, it was demonstrated that administrating the conditioned medium obtained by stimulating human macrophages with LPS leads to increased alkaline phosphatase activity (ALP) in MSCs, indicating the presence of soluble pro-osteogenic factors in the medium[24]. Moreover, cytokines secreted by macrophages have shown to induce signal transducer and activator of transcription 3 (STAT3) signalling in MSCs leading to up-regulation of osteoblast-related genes, such as ALP and Runt-related transcription factor 2 (Runx2)[25]. Similarly, we have shown that soluble factors present in antibody-CD3/CD28 activated T-cell-derived conditioned medium led to an increase in MSC osteogenic differentiation[26]. These examples underline that both the innate and adaptive immune cells actively participate in the processes underlying bone regeneration, but the precise mechanisms are still poorly understood. The optimal inflammatory milieu needed to promote osteogenesis in MSCs is still elusive.

In this work, we introduce a model for the *in-vitro* evaluation of all blood-resident immune cell types simultaneously to discover the inflammatory milieu needed to promote bone formation. To this end, investigating peripheral blood mononuclear cells (PBMCs), which include circulating immune cells, represents a valid approach[27]. PBMCs are mononuclear cells consisting of lymphocytes (T cells, B cells, and NK cells) and monocytes, the precursors of macrophages. Since PBMCs can be easily collected, they are currently an attractive source of immune cells for molecular and immunologic studies. However, studies utilizing whole PBMCs in *in-vitro* models to screen potential osteo-immunomodulators and their effects on

the secretome of immune cells for bone regeneration are still lacking. The *in-vitro* model that we herewith introduce consists of both innate and adaptive immune cells from PBMCs and we investigate the effects of their indirect secretory factors on MSC differentiation into the osteogenic lineage. This model may be used as a screening tool for osteo-immunomodulation to improve bone healing.

Methods

In-vitro model set up

Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of five healthy donors using the density gradient centrifugation method. The PBMCs were seeded in 24 well tissue culture plates and cultured in PBMC culture medium in the presence and/or absence of immunomodulatory factors (PRR ligands). After 24 hours, the supernatant was collected and centrifuged at 1500 g for 5 minutes to pellet the cells. The cell-deprived supernatant, referred to as the conditioned medium, was then stored at -80ºC. The conditioned medium was analysed using a cytokine array kit for quantifying the secreted soluble factors. Further, the effect of the conditioned medium was investigated on MSC osteogenic differentiation (**Figure 1**).

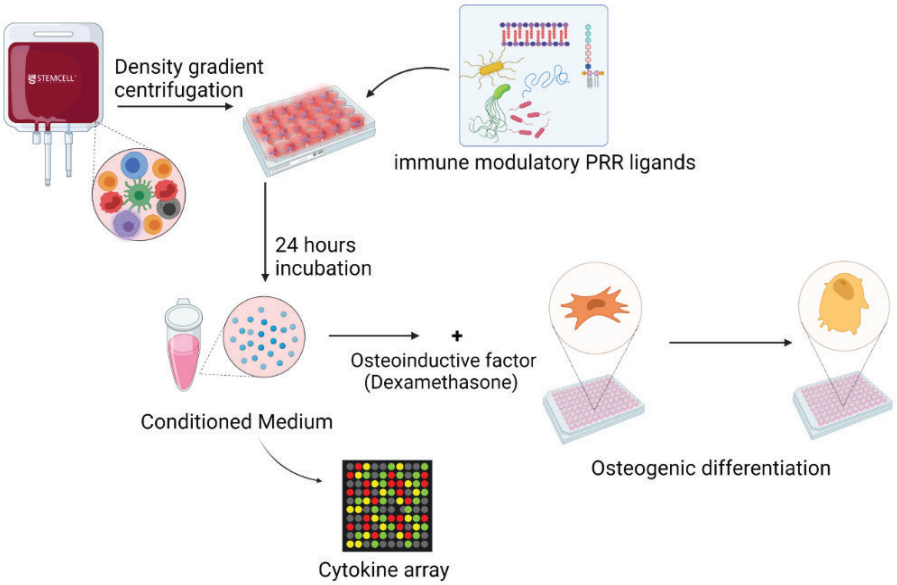


Figure 1. *In-vitro* model set up for investigating the response of immune-modulatory pathogen recognition receptor (PRR) ligands on osteogenesis *in-vitro.* Peripheral blood mononuclear cells were isolated from human blood and stimulated with immunomodulatory PRR ligands for 24 hours. Following incubation, the conditioned medium was used to investigate its effects on MSCs. The soluble immune cell products present in the conditioned medium were analysed using a cytokine array. Image has been created using biorender.com.

Reagents

C-class CpG Oligodeoxynucleotide (CpG ODN C) (M362) and high molecular weight Polyinosinic acid-polycytidylic acid (Poly(I:C)) were purchased from InvivoGen, San Diego, CA, USA. Lipopolysaccharide (LPS) (O55:B5, produced from *e. coli*) was purchased from Sigma Aldrich, St. Louis, Missouri, USA. The concentration range was based on the manufacturer's data sheet and literature[14].

PRR ligand	Receptor	Concentration range
CpG ODN C (M362)	TLR9	0.001 μg/mL to 10 μg/mL
High molecular weight Poly(I:C)	TLR3	0.001 μg/mL to 10 μg/mL
Lipopolysaccharide (LPS) O55:B5	TLR4	0.001 μg/mL to 10 μg/mL

Table 1. Overview of the tested PRR ligands and the Toll-like receptors (TLR) that they target.

Isolation of mononuclear cells from human peripheral blood and generation of conditioned medium

20 mL of Blood from healthy donors (n = 5) was obtained with informed consent from the blood bank (Sanquin, Utrecht, the Netherlands) since ~15 million cells were required for the experiments. The heparinized blood from individual donors was diluted 1:1 with phosphate-buffered saline solution (PBS) and layered on top of a 15mL density gradient medium (Ficoll- plaque plus, Cytiva, MA, USA) in SepMate PBMC tubes (Stem cell technologies, Cologne, Germany). These tubes were then centrifuged at 1200 g for 10 minutes with the brake on at room temperature. Following centrifugation, the supernatant containing serum and cells was quickly poured off into a new centrifuge tube and centrifuged at 300 g for 8 minutes to obtain a cell pellet. PBMCs were then suspended in a culture medium consisting of RPMI 1640 glutamax (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) heat inactivated FBS and 100 units/mL penicillin and 100 μg/mL streptomycin. To produce 1 mL of the conditioned medium, PBMCs obtained from individual donors were seeded in 24 well tissue culture plate at a density of 500,000 cells/cm^2 and cultured for 24 hours at 37 °C in a humidified atmosphere containing 5% CO_2. Subsequently, PBMCs were stimulated with different PRR ligands (**Table 1**). Control experiments were

performed in the absence of PRR ligands. After 24 hours of culture, the conditioned medium was collected, centrifuged at 1500 g for 5 minutes, and stored at -80ºC until use.

Isolation and culturing of MSCs

Human mesenchymal stromal cells (MSC) were isolated from bone marrow aspirates obtained from 4 subjects 50-80 years old that underwent orthopaedic surgery for knee or hip reconstruction and provided written consent for this procedure, for which approval was provided by the UMC Utrecht medical ethical committee under the protocols METC 08-001/K and METC 07-125/C. MSCs were isolated and cryopreserved after passage 2 as previously described in detail[28]. This standardized method of isolation yields multipotent cells as confirmed with assays into osteogenic, adipogenic, and chondrogenic lineages.

For the experiment, MSCs were thawed and expanded in an expansion medium (EM) consisting of minimum essential medium (α-MEM (Gibco)) supplemented with 10% (v/v) heat-inactivated FBS and 100 units/mL penicillin and 100 μg/mL streptomycin and 0.2 mM L-ascorbic-acid-2-phosphate (Sigma, St Louis, MO, USA). The addition of L-ascorbic-acid-2-phosphate is proven to help MSC proliferate and maintain their differentiation potency [29].The cells were plated at approximately 70% confluency during subsequent passages up to a maximum of 8 passages, as passaging more than 8 times has shown to affect MSC proliferative capacity and result in ageing and senescence [30,31]. All cell cultures were performed at 37 °C in a humidified atmosphere containing 5% CO_2.

Metabolic activity assay of MSCs

The metabolic activity of the MSCs was determined using the Alamar blue assay, a non-destructive assay that allows quantification of metabolism products that reflect expansion and/or activity. Alamar blue solution was prepared by dissolving 5.54 mg of Resazurin sodium salt (R7017, Sigma Aldrich, St. Louis, Missouri, USA) in 50mL PBS (10X) and sterile filtered. On days 1, 3, and 7 of MSC culture with conditioned medium and controls, the media of all the wells was replaced by the Alamar blue solution that was diluted in the MSC expansion medium. After 2 hours of incubation at 37 °C in a humidified atmosphere containing 5% CO2, the colour change in the media was measured using an absorbance plate reader (Clariostar plus, BMG labtech, Germany) at 570 nm and corrected at 600 nm.

Cell count and osteogenic differentiation assay of MSCs

Following 7 days of culture, cells were lysed with 0.2% (v/v) Triton-X 100/PBS for 30 mins at room temperature. Alkaline phosphatase activity was determined by using the hydrolysis reaction of p-nitrophenyl phosphate (pH =9.6) (SigmaFast Pnpp tablets, Sigma). The absorbance was measured at 405 nm using an absorbance plate reader and corrected at 655 nm. ALP was normalized to the DNA content in the wells. DNA quantification was performed using Quanti-kit (Invitrogen, MA, USA) according to the manufacturer's instructions.

Experiments

Dose determination of conditioned medium for MSC culture

To determine an appropriate dose of the conditioned medium that should be added to the MSCs, the conditioned medium from unstimulated PBMCs (n = 3) was added in different ratios to the MSC culture. The metabolic activity, cell count, and osteogenic differentiation assays were used as readout parameters.

To determine the effect of the conditioned medium on metabolic activity, MSCs (n = 3) were seeded and cultured in an expansion medium at a density of 15000 cells/ cm^2 in 96 wells tissue culture-treated plates. Upon confluency, the conditioned medium obtained from unstimulated PBMCs was added to the wells of the expansion medium (CM: EM) in the ratios 1:10, 1:4, and 1:1 for 7 days. Wells with expansion medium were used as control. The medium was refreshed every 3 days.

To test the effect of the conditioned medium on osteogenic differentiation, MSCs (n = 3) were similarly seeded and cultured. Upon confluency, the medium was replaced with an osteogenic differentiation medium (expansion medium supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone) alone or combined with the conditioned medium obtained from unstimulated PBMCs. The conditioned medium to osteogenic differentiation medium (CM: ODM) in ratios 1:10, 1:4, and 1:1 was added every 3 days.

MSC osteogenic differentiation following indirect stimulation of PRR ligands

MSCs (n = 4) were stimulated continuously with the conditioned medium obtained by stimulating the PBMCs with the PRR ligands (**Table 1**) for 24 hours. Since the effect of the conditioned medium obtained after the stimulation with

PRR ligands from 5 PBMC donors was similar in terms of osteogenic differentiation of MSCs (**Supplementary Figure S3**), conditioned medium from 1 PBMC donor was used. The MSCs cultured in standard osteogenic medium alone or combined with conditioned medium from unstimulated PBMCs were used as controls. The PRR ligand concentration that yielded high ALP activity in MSCs for osteogenic differentiation, upon indirect stimulation was chosen for further experiments (**Supplementary figure S1**). The final concentrations used were CpG ODN C (1μg/mL), Poly(I:C) (10μg/mL) and LPS (1μg/mL). The conditioned medium obtained from PRR stimulation was not supplemented with any additional amounts of PRR ligands for further analysis. All MSC experiments were performed with the osteogenic differentiation medium described above for 7 days, with the medium refreshed every 3 days. After 7 days, the ALP activity and DNA content were measured as described above.

Direct stimulation of PRR ligands on MSC osteogenic differentiation

Since the PRR ligands showed to enhance osteogenic differentiation of MSCs upon direct stimulation[14], this experiment was performed as a control for indirect stimulation experiments with effects of PRR ligands via the conditioned medium from PBMCs. The MSCs (n = 4) were also stimulated with CpG ODN C (1μg/mL), Poly(I:C) (10μg/mL), and LPS (1μg/mL) directly and cultured in osteogenic differentiation medium for 7 days. After 7 days, the ALP activity and DNA content were measured as described above.

Conditioned medium characterisation using cytokine array

To identify cytokines, chemokines, and growth factors present in the conditioned medium after stimulation with PRR ligands in one PBMC donor, a cytokine array assay (GSH-BMA-1000, RayBiotech, GA, USA) was performed to determine a panel of inflammatory mediators. The array included 41 proteins. Pro-inflammatory cytokines and chemokines TNF-α, Interleukins (IL-1α, IL-1β, IL-6, IL-8, IL-11, IL-17A), Monocyte chemoattractant protein-1 (MCP-1), Macrophages inflammatory protein-1a (MIP-1α) along with bone-related proteins like bone morphogenetic proteins (BMP-2, BMP-4, BMP-6, BMP-7, BMP-9), Osteoactivin, Osteopontin, Osteoprotegerin DKK-1, Activin A, FGF-1, amphiregulin, bFGF, E-selectin, CD54, IGF-1, MMP-2, MMP-9, MMP-13, P-Cadherin, RANK, SDF-1alpha, sonic hedgehog N-terminal, TGF-β 1, TGF-β 2, CD106, CDH5, DKK-1, MMP-3, TRANCE, PDGF-BB, and TGF-β3 were analysed according to manufacturer instructions. Briefly, the glass slide was first blocked with the sample diluent for 30 mins to prevent unspecific binding. Later, 100 μL of the conditioned medium

obtained from unstimulated PBMCs and PRR ligands stimulated PBMCs was added to different wells in the array slide and incubated for 2 hours at room temperature. Following incubation, the array was washed 3 times for 5 minutes each. Subsequently, a biotinylated antibody cocktail was added to the wells of the array and incubated for 2 hours at room temperature. Following washing, the array was incubated with Cy3 equivalent conjugated streptavidin for one hour. The signals were then visualized using a fluorescence laser scanner at a PMT gain of 50%. Data analysis was performed using R-data software.

To show the results, a Ratio Average (RA) plot was used. Essentially, the RA plot is a scatter plot that shows the differential expression of a cytokine in group "a" versus group "b" according to their Ratio (R=log2(a/b)), and Average (A=1/2log2(a*b)). In particular, "a" is the positive measure in the group of interest (here the medium of the PRR stimulated PBMCs), whereas "b" is the positive measure in the control group (here the medium of unstimulated PBMCs). Thus, overexpressed cytokines have R>0, whereas downregulated cytokines have R<0. A threshold of a 2-fold increase was applied to identify differences.

Statistical analysis

Statistical analysis was performed the analysis was done using R studio software (version 1.3.1093) and Prism GraphPad (San Diego, California, USA). The normality of the data was checked using the Pearson normality test and Q-Q plots. If the data were normally distributed, ordinary one-way ANOVA was performed and if data were not normally distributed, a Wilcoxon signed-rank test was used to determine significant differences between the groups.

Results

Dose determination of the conditioned medium from unstimulated PBMCs for MSC culture

The addition of the conditioned medium from unstimulated PBMCs in different dose ratios (conditioned medium (CM): expansion medium (EM)) did not affect the metabolism of the MSCs on day 1 and day 7 for all three donors (**Figure 2. a**). On day 3, a 50% increase in metabolic activity was observed for one of the three donors in the groups treated with the conditioned medium. This increase may be due to an increase in cell amount or due to high activity of cells, or a combination of both.

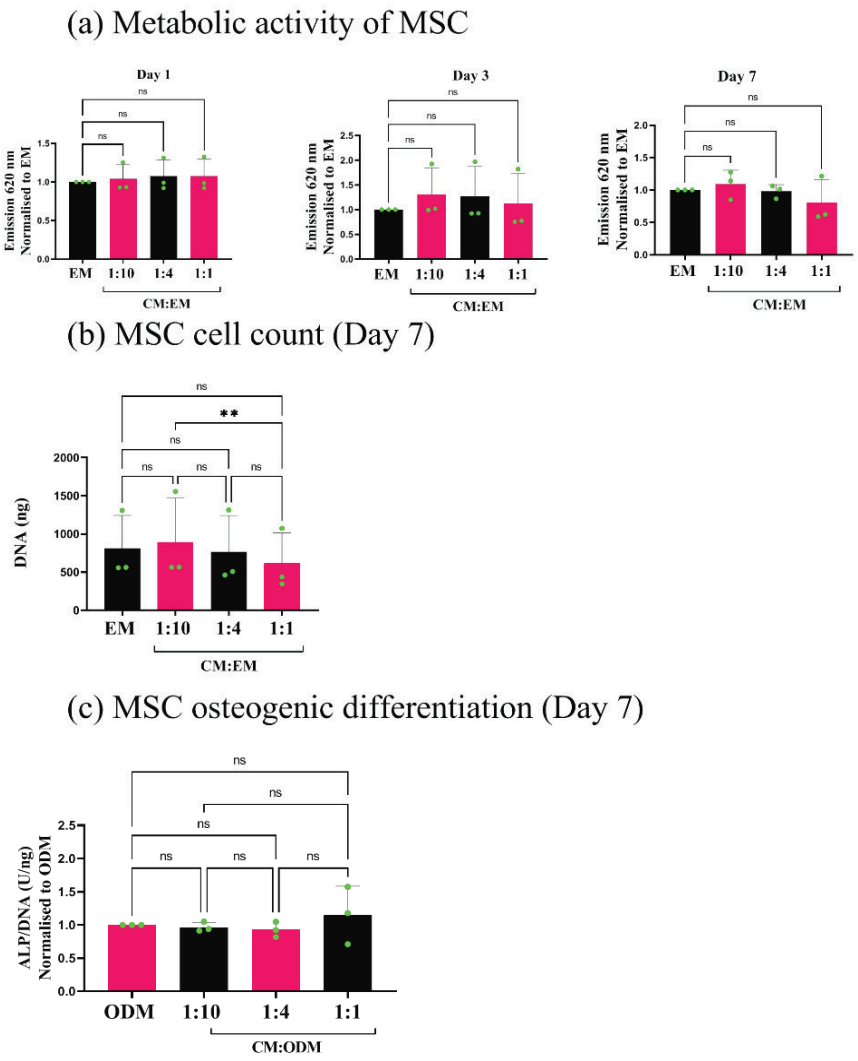


Figure 2. Effect of conditioned medium of unstimulated PBMCs (n = 3) on the metabolic activity, cell count, and osteogenic differentiation of MSCs (n = 3). (a) MSCs were cultured for 7 days either in expansion medium or a combination of expansion medium and conditioned medium obtained from unstimulated PBMCs in different ratios (1:10, 1:4, and 1:1). The metabolic activity of the MSCs was assessed using the Alamar blue assay at 24 hours, 3 days, and 7 days. The value of 1.0 is given to the expansion medium of MSCs that did not receive any conditioned medium. (b) DNA quantification was done on day 7. (c) Osteogenic differentiation was determined by alkaline phosphatase activity (ALP) after 7 days. MSCs were cultured for 7 days either in an osteogenic differentiation

medium (ODM) or a combination of and conditioned medium obtained from unstimulated PBMCs in different ratios (1:10, 1:4, and 1:1). ALP activity was corrected for the DNA content and normalized to the osteogenic differentiation control without the conditioned medium. The graph represents the mean ± standard deviation of the triplicates performed per donor for 2 individual MSC donors. Significance was tested using ordinary one-way ANOVA. ** indicates p-value <0.005.

The conditioned medium from unstimulated PBMCs significantly affected the cell count, except in the case of the highest dose in which the conditioned medium equalled the osteogenic medium (1:1) (**Figure 2. b**). Furthermore, the conditioned medium did not interfere with the MSC's osteogenic differentiation capacity (**Figure 2. c**), according to the alkaline phosphatase assay. Based on the latter results (ALP/DNA), we choose to conduct all further experiments with the ratio of 1:4 for conditioned medium to osteogenic differentiation medium. Also, the conditioned medium from unstimulated PBMCs did not promote the osteogenic differentiation of MSCs in the absence of dexamethasone (**Supplementary Figure S2**).

Effect of direct versus indirect stimulation of PRR ligands on osteogenic differentiation

Upon direct stimulation (PRR ligands directly with the osteogenic medium of MSCs), both CpG ODN C and LPS enhanced the ALP activity in MSCs by approximately 2-fold as compared to conditioned medium from unstimulated PBMCs (control CM) (**Figure 3**). Interestingly, the conditioned medium obtained from CpG ODN C stimulated PBMCs (indirect stimulation) induced even higher ALP activity (increase in donors ranging from 10% to 145%, p-value = 0.06) as compared to direct stimulation with CpG ODN C.

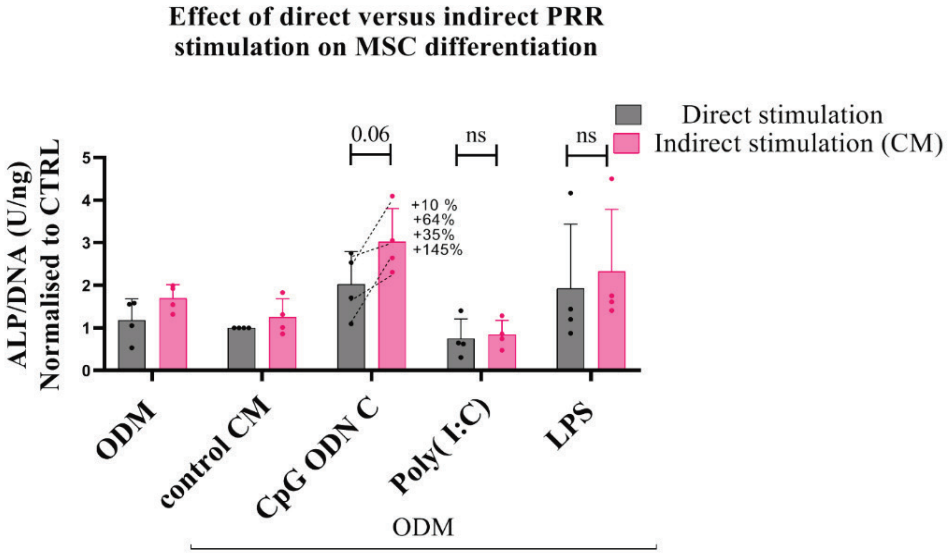


Figure 3. Alkaline Phosphatase levels in MSC cultures, corrected for DNA, after addition of PRR ligands to osteogenic differentiation medium (ODM). MSC stimulation with ligands was either direct (grey) or indirect (pink) via PBMC (n = 1) stimulation where unstimulated PBMC conditioned media (control CM) served as control situation for normalization. Immunomodulatory PRR ligands CpG ODN C (1µg/mL), Poly(I:C) (10µg/mL), and LPS (1µg/mL) were given either directly to the MSCs (with ODM) or used to stimulate the PBMCs, from which the conditioned medium was subsequently provided to the MSCs. The conditioned medium was added in the ratio of 1: 4 (CM: ODM). The graph represents the mean ± standard deviation of technical triplicates performed per donor for 4 individual MSC donors. Significance was tested using Wilcoxon signed-rank test, one-tailed.

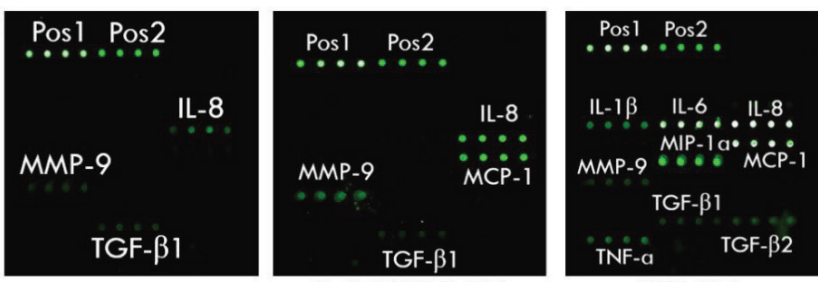
Conditioned medium characterisation using cytokine array

The cytokine array analysis was only performed for conditioned medium obtained from CpG ODN C as high ALP activity was seen due to this form of indirect stimulation. Conditioned medium from LPS stimulated PBMCs was used as a positive control since LPS is known to activate the NF-κβ signalling pathway leading to the release of pro-inflammatory cytokines. Stimulation with CpG ODN C resulted in the release of pro-inflammatory cytokines IL-8 (3-fold) and chemokine MCP-1 (4-fold), when compared to unstimulated PBMCs (**Figure 4b**). A 2-fold increase in Matrix metalloproteinase 9 (MMP-9) and Matrix metalloproteinase 13 (MMP-13) was observed in the CpG ODN C stimulated conditioned medium as compared to control conditioned medium. Upon LPS

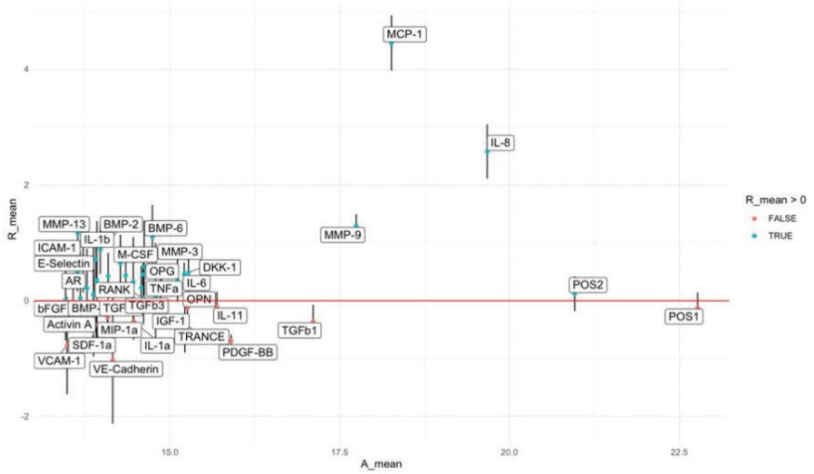
stimulation, the release of pro-inflammatory cytokines IL-6, IL-8, TNF-α, and IL-1β were seen along with chemokines MCP-1, and MIP-1α (**Figure 4c**).

Figure 4. Cytokine production by PBMCs upon stimulation with PRR ligands CpG ODN C and LPS after 24 hours. (a) Image obtained after using the fluorescence laser scanner. The green dots represent the detected cytokines/chemokines in the conditioned medium obtained from unstimulated PBMCs (left), CpG ODN C-stimulated PBMCs (centre), and LPS-stimulated PBMCs (right). (b) Data from the results are log-normalized to control (CM) from unstimulated PBMCs and shown as Ratio-Average (RA) plot. The x axis shows the intensity of the signal detected for a particular cytokine and y- axis shows the fold increase of the cytokine level as compared to the control CM. Ratio- average (RA) plot of CpG ODN C stimulated conditioned medium to unstimulated PBMC conditioned medium. (c) Ratio- average (RA) plot of LPS stimulated conditioned medium to unstimulated PBMC conditioned medium. The cytokines found above the line are upregulated upon CpG ODN C stimulation, whereas those below are downregulated. Data are shown as mean ± standard deviation of the quadruplicates as visible in panel (a) (experimental variation). N=1 donor. The red line separates up-regulated (R>0) and downregulated (R<0) cytokines versus the control.

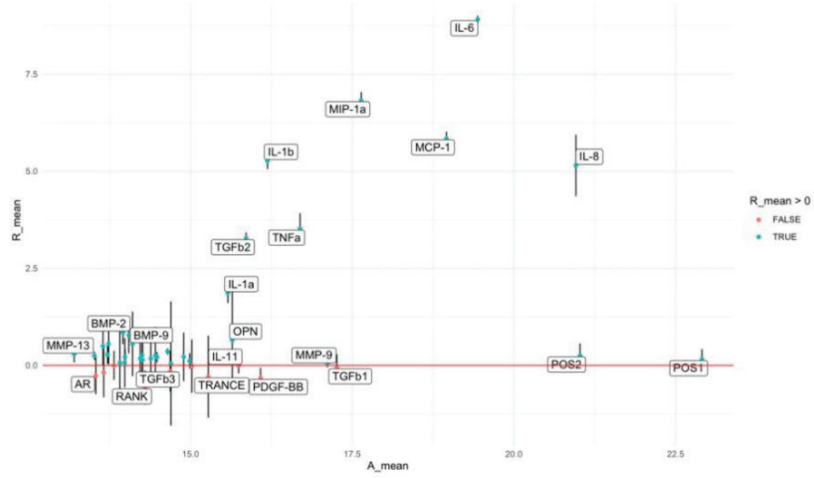
(a)



(b) Ratio Average plot of CpG ODN C - CM v/s control - CM



(c) Ratio Average plot of LPS - CM v/s control - CM



Discussion

Although the modulation of immune responses may be a way to enhance bone repair, an understanding of the optimum inflammatory environment to achieve this *in vivo* remains elusive. With the present work, we provide an *in-vitro* model that employs PBMCs isolated from peripheral blood, which includes a variety of immune cells from both the innate and adaptive immune systems, to study their effects on MSC osteogenesis. Herewith, we show that using the conditioned medium obtained from unstimulated PBMCs does not affect MSCs in terms of metabolic activity or osteogenic differentiation. We further applied this model to screen two immunomodulatory PRR ligands that may possess pro-osteogenic effects. Upon stimulation with CpG ODN C and LPS, directly or via a conditioned medium obtained by stimulating PBMCs, MSCs showed an increase in ALP activity as compared to controls with only osteogenic medium, whereas stimulation with Poly(I:C) directly or via the conditioned medium did not show an increase in ALP activity of MSCs. Moreover, the ALP activity was higher in the CpG ODN C stimulated conditioned medium group (indirect stimulation), indicating that the soluble factors secreted from PBMCs contributed to the pro-osteogenic effects of MSCs rather than the CpG ODN C itself (direct stimulation).

Guided by these observations, we investigated which cytokines and growth factors were present in the PBMC-derived conditioned medium upon stimulation with CpG ODN C. To do so, a cytokine array was performed on the conditioned media to quantify the release of multiple (most inflammatory) soluble factors simultaneously. Interestingly, we found that inflammatory mediators, mainly chemokines IL-8 and MCP-1 were upregulated upon stimulation with CpG ODN C. As previously described by others, MCP-1, also referred to as CCL-2, is an important chemokine involved in bone regeneration, since knock-out models for MCP-1 showed reduced bone formation [32,33]. This may be attributed to MCP-1 attracting monocytes and dendritic cells to locations of injury, the bone defect zone. These monocytes can further develop into macrophages and produce more MCP-1, with subsequent more monocyte attraction. As such the monocyte-macrophage feedback loop can be considered the key player in bone regeneration. Along with the chemokines, an upregulation in MMP-9 and MMP-13 was detected in CpG ODN C stimulated conditioned medium. MMPs are known to degrade the extracellular matrix and play a vital role in bone remodelling and skeletal repair [34]. It was shown that MMP-9 plays a crucial role in regulating the chondrogenic and osteogenic differentiation in early phases of fracture healing[35]. It was also shown that fractured tibiae of MMP-9 knockout mice developed non-unions[36]. Growth factors BMP-2 and BMP-6 were also secreted by PBMCs upon stimulation with CpG ODN C. Hence, although this cytokine array experiment was performed for one donor only, we found similarities with previous *in vivo* observations,

indicating the suitability of our model to screen for pro-osteogenic factors. Moreover, to confirm the reliability of our model, we screened for inflammatory mediators present in the conditioned media upon stimulation with LPS; a known stimulator of inflammatory cytokine production and for bone regeneration *in-vitro*[19]. As expected, the analysis revealed high expression of pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α[37] next to inducing TGF-β1, TGF-β2 and BMP-9, respectively. Further, studies recreating the composition of the conditioned medium in terms of cytokines/chemokines *in-vitro* can help in determining the exact role of the secretory cytokines and chemokines in maintaining the balanced inflammatory microenvironment created *in vivo* during bone healing process.

In the past decade, the effects of individual immune cell subpopulations during the initial phase of fracture healing have been studied extensively. At the fracture site, macrophages secrete cytokines and chemokines that recruit not only monocytes (via MCP-1) but also direct MSCs to the site of injury from their local niches to start the rebuilding of new bone. Macrophages support osteoblast formation by release of growth factors like TGF-β1, BMP-2 and BMP-4 and thus stimulate MSCs towards the osteogenic lineage[18,25]. Lymphocytes consisting of T- and B- cells, affect the differentiation and maturation of bone cells, other than their immune functionality and are present in fracture hematoma formed at the site of injury. Activated T-cells and their subsets can stimulate osteogenic differentiation of MSCs *in-vitro*[26]. In contrast to that , fracture healing was enhanced in the mice which lacked functional B- and T- lymphocytes, implying that adaptive immune cells somehow have a negative impact[38,39]. Further, it was reported that CD8+ T- cells, a subset of T lymphocytes impair the fracture healing process in humans and mice[40], while CD4+ cells have been shown to enhance osteogenic differentiation[41]. Recently, it was shown that activated T cells promote B cells to produce osteoprotegerin, which has a promoting effect on bone tissue regeneration via the CD40/CD40L signalling pathway[42]. Though roles of individual immune cell subpopulations during fracture healing are characterized to a certain extent, there is a need for combined models of bone (progenitor) cells and several types of immune cells *in-vitro* that recapitulate the cross talk, microenvironment, and key features of the bone healing process of the *in vivo* situation.

Our study identifies CpG ODN C as a potential osteo-immunomodulator. CpG ODN C is a synthetic bacterial DNA and currently investigated in clinical trials as an adjuvant for asthma and tumour therapies[43,44]. Recently, we showed that CpG ODN C enhances MSCs osteogenic differentiation in presence of dexamethasone[14]. CpG ODN C is recognized by intracellular receptor TLR9, which upon activation, initiates the NF-κβ and TIR domain-containing adapter-inducing interferon β (TRIF) signalling pathway, which results in the release of

pro-inflammatory cytokines like interferons, IL-8, IL-6[45,46]. In agreement, we found upregulation in IL-6 and IL-8 secretion in the CpG ODN C-stimulated PBMC group suggesting the activation of the NF-κβ signalling cascade. In this study, antibodies targeting interferons were not included in the cytokine array. It has been shown that CpG ODN C stimulation in macrophages leads to the release of MCP-1 via the c-Jun N-terminal kinase (JNK) signalling pathway, mainly due to the activation of TLR9[47]. Since CpG ODN C is reported to be a potent stimulator of B-cells, NK cells, and dendritic cells that induce secretion of immunoglobulins and interferon type 1 cytokines [48,49], it will be interesting to examine the presence of interferons in CpG ODN C stimulated conditioned medium. Thus, evaluating the contribution of the immune cells upon stimulation with CpG ODN C on MSC osteogenic differentiation represents an important step towards the use of CpG ODN C in enhancing bone repair. In the future, studies investigating the contribution of immune cell-secreted IL-8 and MCP-1 to bone formation should be carried out to provide a deeper insight into the mechanism benefitting osteogenesis.

As a limitation of this study, the evaluation of bone marker expression was limited to the investigation of ALP activity in MSCs, an early marker of osteogenesis. ALP activity is considered to be a superior predictor of bone formation *in vivo* [50] as compared to later osteogenic markers like osteocalcin, and osteonectin. The effect of the conditioned medium, stimulated with PRR ligands, should also be investigated on matrix mineralization, which is considered a functional endpoint assay for osteogenesis. Furthermore, characterization of the conditioned media obtained from stimuli like LPS, which are already investigated in detail[37], in terms of its cytokine content by using a larger cytokine array or enzyme-linked immunosorbent assays (ELISA) could provide invaluable insights into the microenvironment that might be beneficial for developing future therapies. Use of limited number of donors proved to be another limitation of this study. In our study the four donors used in the indirect stimulation experiments did show major differences (**Figure 3**). However, donor variation may have an important effect on our outcomes and further investigation with multiple donors should be performed before a next step to *in-vivo* experiments can start. Another limitation of this study lies in the fact that we use conditioned medium from the immune cells to stimulate MSCs rather than co-culturing of PBMCs and MSCs. The current model does not account for the effects of cell-to-cell contact on cytokine secretion. For instance, MSCs may have immunomodulatory effects on PBMCs that may suppress or stimulate the production of soluble mediators[51].

Nevertheless, the established *in-vitro* model resembles the physiological inflammatory state created *in vivo* and proves to be simplified practical tool that can be applied for various applications including the identification of new osteo-immunomodulators. Since it has been elucidated that both adaptive and innate

immune cells are key players in the immune-mediated bone deposition, developing new platforms that allow screening of potential bone enhancing immune modulators is of key importance to accelerate their employment in the clinic. Herein, we described a model that allows such tests and may as well help to identify immunomodulatory biomaterials and thereby supports *in vivo* experiments that are still required to clinically test bone regenerative compounds and materials.

Conclusion

In conclusion, in this study, we provide evidence of the suitability of a new *in-vitro* model based on PBMC-derived conditioned media produced upon stimulation with PRR ligands to study osteogenesis in MSCs. By culturing PBMCs in the presence of PRR ligands for 24 hours, the production of cytokines and growth factors was induced as shown by the cytokine array. In particular, stimulating PBMCs with CpG ODN C, a well-known adjuvant in clinical trials, and administrating the conditioned medium from the stimulated PBMCs to MSCs led to increased alkaline phosphatase levels as an indicator of osteogenic differentiation. No effects were observed when MSCs were cultured in the presence of conditioned media from unstimulated PBMCs, confirming the viability of this *in-vitro* model. Hence, the model described allows the screening of PRR ligands for osteoimmunomodulation and represents a promising strategy to better mimic the *in vivo* situation where multiple immune cell types interact with osteo-immunomodulators.

Funding

This article is supported by PPS allowance from the Health~Holland LSH-TKI (grant number: LSHM18011) and the EU's H2020 research and innovation program under Marie S. Curie Cofund RESCUE (grant agreement No 801540).

Acknowledgment

We would like to thank Dr. Margreet Wolfert, Department of Chemical biology and drug discovery, University Utrecht for her valuable help in reading the cytokine array a fluorescent laser scanner.

References

1. Claes, L.; Recknagel, S.; Ignatius, A. *Fracture Healing under Healthy and Inflammatory Conditions*; 2012; Vol. 8;.
2. Lu, H.; Kraut, D.; Gerstenfeld, L.C.; Graves, D.T. Diabetes Interferes with the Bone Formation by Affecting the Expression of Transcription Factors That Regulate Osteoblast Differentiation. *Endocrinology* **2003**, *144*, 346–352, doi:10.1210/en.2002-220072.
3. Kulemann, B.; Hoeppner, J.; Wittel, U.; Glatz, T.; Keck, T.; Wellner, U.F.; Bronsert, P.; Sick, O.; Hopt, U.T.; Makowiec, F.; et al. Perioperative and Long-Term Outcome after Standard Pancreaticoduodenectomy, Additional Portal Vein and Multivisceral Resection for Pancreatic Head Cancer. *J. Gastrointest. Surg. Off. J. Soc. Surg. Aliment. Tract* **2015**, *19*, 438–444, doi:10.1007/s11605-014-2725-8.
4. Carragee, E.J.; Comer, G.C.; Smith, M.W. Local Bone Graft Harvesting and Volumes in Posterolateral Lumbar Fusion: A Technical Report. *Spine J. Off. J. North Am. Spine Soc.* **2011**, *11*, 540–544, doi:10.1016/j.spinee.2011.02.014.
5. Gupta, A.; Kukkar, N.; Sharif, K.; Main, B.J.; Albers, C.E.; El-Amin III, S.F. Bone Graft Substitutes for Spine Fusion: A Brief Review. *World J. Orthop.* **2015**, *6*, 449–456, doi:10.5312/wjo.v6.i6.449.
6. Lehr, A.M.; Oner, F.C.; Delawi, D.; Stellato, R.K.; Hoebink, E.A.; Kempen, D.H.R.; van Susante, J.L.C.; Castelein, R.M.; Kruyt, M.C. Efficacy of a Standalone Microporous Ceramic Versus Autograft in Instrumented Posterolateral Spinal Fusion. *Spine* **2020**, *45*, 944–951, doi:10.1097/BRS.0000000000003440.
7. Lehr, A.M.; Oner, F.C.; Delawi, D.; Stellato, R.K.; Hoebink, E.A.; Kempen, D.H.R.; van Susante, J.L.C.; Castelein, R.M.; Kruyt, M.C.; Dutch Clinical Spine Research Group Increasing Fusion Rate Between 1 and 2 Years After Instrumented Posterolateral Spinal Fusion and the Role of Bone Grafting. *Spine* **2020**, *45*, 1403–1410, doi:10.1097/BRS.0000000000003558.
8. Safari, B.; Davaran, S.; Aghanejad, A. Osteogenic Potential of the Growth Factors and Bioactive Molecules in Bone Regeneration. *Int. J. Biol. Macromol.* **2021**, *175*, 544–557, doi: 10.1016/j.ijbiomac.2021.02.052.
9. Yun, Y.-R.; Jang, J.H.; Jeon, E.; Kang, W.; Lee, S.; Won, J.-E.; Kim, H.W.; Wall, I. Administration of Growth Factors for Bone Regeneration. *Regen. Med.* **2012**, *7*, 369–385, doi:10.2217/rme.12.1.
10. Chen, Z.; Klein, T.; Murray, R.Z.; Crawford, R.; Chang, J.; Wu, C.; Xiao, Y. Osteoimmunomodulation for the Development of Advanced Bone Biomaterials. *Mater. Today* **2016**, *19*, 304–321, doi: 10.1016/j.mattod.2015.11.004.
11. Walsh, M.C.; Kim, N.; Kadono, Y.; Rho, J.; Lee, S.Y.; Lorenzo, J.; Choi, Y. OSTEOIMMUNOLOGY: Interplay Between the Immune System and Bone

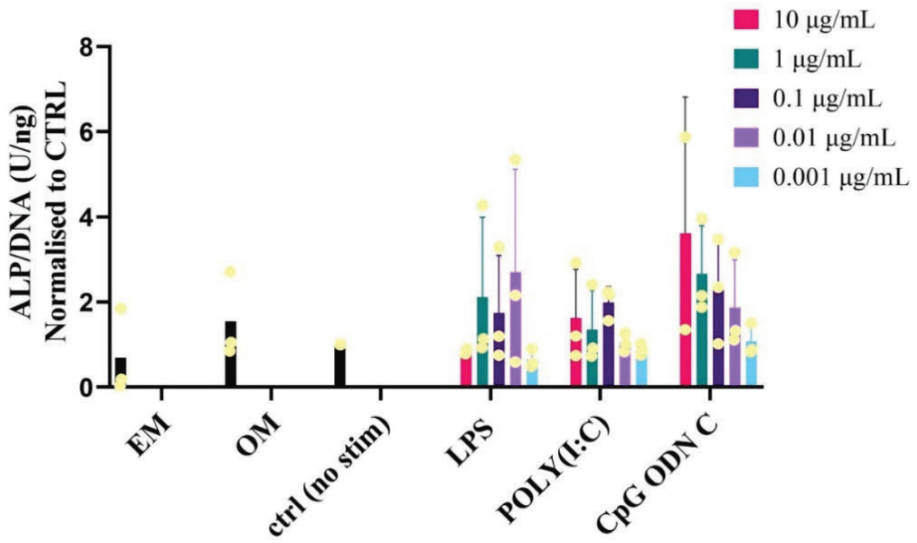
Metabolism. *Annu. Rev. Immunol.* **2006**, *24*, 33–63, doi: 10.1146/annurev.immunol.24.021605.090646.
12. Lopez, E.M.; Leclerc, K.; Ramsukh, M.; Parente, P.E.; Patel, K.; Aranda, C.J.; Josephson, A.M.; Remark, L.H.; Kirby, D.J.; Buchalter, D.B.; et al. Modulating the Systemic and Local Adaptive Immune Response after Fracture Improves Bone Regeneration during Aging. *Bone* **2022**, *157*, 116324, doi: 10.1016/j.bone.2021.116324.
13. Croes, M.; Wal, B.C.H.; Vogely, H.Ch. The Impact of Bacterial Infections on Osteogenesis: Evidence From *In vivo* Studies. *J. Orthop. Res.* **2019**, jor.24422, doi:10.1002/jor.24422.
14. Khokhani, P.; Rahmani, N.R.; Kok, A.; Öner, F.C.; Alblas, J.; Weinans, H.; Kruyt, M.C.; Croes, M. Use of Therapeutic Pathogen Recognition Receptor Ligands for Osteo-Immunomodulation. *Materials* **2021**, *14*, 1119, doi:10.3390/ma14051119.
15. O'Neill, L.A.J.; Golenbock, D.; Bowie, A.G. The History of Toll-like Receptors — Redefining Innate Immunity. *Nat. Rev. Immunol.* **2013**, *13*, 453–460, doi:10.1038/nri3446.
16. Baht, G.S.; Vi, L.; Alman, B.A. The Role of the Immune Cells in Fracture Healing. *Curr. Osteoporos. Rep.* **2018**, *16*, 138–145, doi:10.1007/s11914-018-0423-2.
17. Bucher, C.H.; Schlundt, C.; Wulsten, D.; Sass, F.A.; Wendler, S.; Ellinghaus, A.; Thiele, T.; Seemann, R.; Willie, B.M.; Volk, H.-D.; et al. Experience in the Adaptive Immunity Impacts Bone Homeostasis, Remodeling, and Healing. *Front. Immunol.* **2019**, *10*.
18. Michalski, M.N.; McCauley, L.K. Macrophages and Skeletal Health. *Pharmacol. Ther.* **2017**, *174*, 43–54, doi: 10.1016/j.pharmthera.2017.02.017.
19. Croes, M.; Oner, F.C.; Kruyt, M.C.; Blokhuis, T.J.; Bastian, O.; Dhert, W.J.A.; Alblas, J. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. *PLoS ONE* **2015**, *10*, 1–14, doi: 10.1371/journal.pone.0132781.
20. Bastidas-Coral, A.P.; Bakker, A.D.; Zandieh-Doulabi, B.; Kleverlaan, C.J.; Bravenboer, N.; Forouzanfar, T.; Klein-Nulend, J. Cytokines TNF-α, IL-6, IL-17F, and IL-4 Differentially Affect Osteogenic Differentiation of Human Adipose Stem Cells. *Stem Cells Int.* **2016**, *2016*, doi:10.1155/2016/1318256.
21. Sandberg, O.H.; Tätting, L.; Bernhardsson, M.E.; Aspenberg, P. Temporal Role of Macrophages in Cancellous Bone Healing. *Bone* **2017**, *101*, 129–133, doi: 10.1016/j.bone.2017.04.004.
22. Könnecke, I.; Serra, A.; El Khassawna, T.; Schlundt, C.; Schell, H.; Hauser, A.; Ellinghaus, A.; Volk, H.-D.; Radbruch, A.; Duda, G.N.; et al. T and B Cells Participate in Bone Repair by Infiltrating the Fracture Callus in a Two-Wave Fashion. *Bone* **2014**, *64*, 155–165, doi: 10.1016/j.bone.2014.03.052.

23. Alnaeeli, M.; Teng, Y.-T.A. Dendritic Cells: A New Player in Osteoimmunology. *Curr. Mol. Med.* **2009**, *9*, 893–910, doi:10.2174/156652409789105507.
24. Guihard, P.; Danger, Y.; Brounais, B.; David, E.; Brion, R.; Delecrin, J.; Richards, C.D.; Chevalier, S.; Rédini, F.; Heymann, D.; et al. Induction of Osteogenesis in Mesenchymal Stem Cells by Activated Monocytes/Macrophages Depends on Oncostatin M Signaling. *Stem Cells* **2012**, *30*, 762–772, doi:10.1002/stem.1040.
25. Nicolaidou, V.; Wong, M.M.; Redpath, A.N.; Ersek, A.; Baban, D.F.; Williams, L.M.; Cope, A.P.; Horwood, N.J. Monocytes Induce STAT3 Activation in Human Mesenchymal Stem Cells to Promote Osteoblast Formation. *PLOS ONE* **2012**, *7*, e39871, doi: 10.1371/journal.pone.0039871.
26. Croes, M.; Öner, F.C.; van Neerven, D.; Sabir, E.; Kruyt, M.C.; Blokhuis, T.J.; Dhert, W.J.A.; Alblas, J. Proinflammatory T Cells and IL-17 Stimulate Osteoblast Differentiation. *Bone* **2016**, *84*, 262–270, doi: 10.1016/j.bone.2016.01.010.
27. Acosta Davila, J.A.; Hernandez De Los Rios, A. An Overview of Peripheral Blood Mononuclear Cells as a Model for Immunological Research of Toxoplasma Gondii and Other Apicomplexan Parasites. *Front. Cell. Infect. Microbiol.* **2019**, *9*.
28. Pennings, I.; van Dijk, L.A.; van Huuksloot, J.; Fledderus, J.O.; Schepers, K.; Braat, A.K.; Hsiao, E.C.; Barruet, E.; Morales, B.M.; Verhaar, M.C.; et al. Effect of Donor Variation on Osteogenesis and Vasculogenesis in Hydrogel Cocultures. *J. Tissue Eng. Regen. Med.* **2019**, *13*, 433–445, doi:10.1002/term.2807.
29. Choi, K.-M.; Seo, Y.-K.; Yoon, H.-H.; Song, K.-Y.; Kwon, S.-Y.; Lee, H.-S.; Park, J.-K. Effect of Ascorbic Acid on Bone Marrow-Derived Mesenchymal Stem Cell Proliferation and Differentiation. *J. Biosci. Bioeng.* **2008**, *105*, 586–594, doi:10.1263/jbb.105.586.
30. Baxter, M.A.; Wynn, R.F.; Jowitt, S.N.; Wraith, J.E.; Fairbairn, L.J.; Bellantuono, I. Study of Telomere Length Reveals Rapid Aging of Human Marrow Stromal Cells Following *in vitro* Expansion. *Stem Cells Dayt. Ohio* **2004**, *22*, 675–682, doi:10.1634/stemcells.22-5-675.
31. Bonab, M.M.; Alimoghaddam, K.; Talebian, F.; Ghaffari, S.H.; Ghavamzadeh, A.; Nikbin, B. Aging of Mesenchymal Stem Cell *in vitro*. *BMC Cell Biol.* **2006**, *7*, 14, doi:10.1186/1471-2121-7-14.
32. Mulholland, B.S.; Forwood, M.R.; Morrison, N.A. Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) Drives Activation of Bone Remodelling and Skeletal Metastasis. *Curr. Osteoporos. Rep.* **2019**, *17*, 538–547, doi:10.1007/s11914-019-00545-7.
33. Ishikawa, M.; Ito, H.; Kitaori, T.; Murata, K.; Shibuya, H.; Furu, M.; Yoshitomi, H.; Fujii, T.; Yamamoto, K.; Matsuda, S. MCP/CCR2 Signaling Is Essential for Recruitment of Mesenchymal Progenitor Cells during the Early

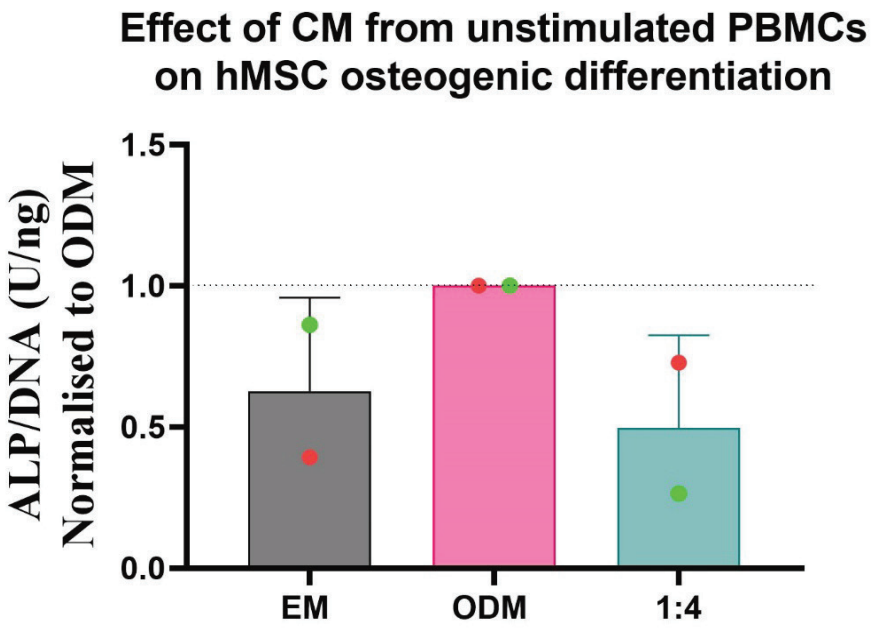
Phase of Fracture Healing. *PLOS ONE* **2014**, *9*, e104954, doi: 10.1371/journal.pone.0104954.
34. Behonick, D.J.; Xing, Z.; Lieu, S.; Buckley, J.M.; Lotz, J.C.; Marcucio, R.S.; Werb, Z.; Miclau, T.; Colnot, C. Role of Matrix Metalloproteinase 13 in Both Endochondral and Intramembranous Ossification during Skeletal Regeneration. *PLOS ONE* **2007**, *2*, e1150, doi: 10.1371/journal.pone.0001150.
35. Engsig, M.T.; Chen, Q.J.; Vu, T.H.; Pedersen, A.C.; Therkidsen, B.; Lund, L.R.; Henriksen, K.; Lenhard, T.; Foged, N.T.; Werb, Z.; et al. Matrix Metalloproteinase 9 and Vascular Endothelial Growth Factor Are Essential for Osteoclast Recruitment into Developing Long Bones. *J. Cell Biol.* **2000**, *151*, 879–889, doi:10.1083/jcb.151.4.879.
36. Colnot, C.; Thompson, Z.; Miclau, T.; Werb, Z.; Helms, J.A. Altered Fracture Repair in the Absence of MMP9. *Development* **2003**, *130*, 4123–4133, doi:10.1242/dev.00559.
37. Ngkelo, A.; Meja, K.; Yeadon, M.; Adcock, I.; Kirkham, P.A. LPS Induced Inflammatory Responses in Human Peripheral Blood Mononuclear Cells Is Mediated through NOX4 and Giα Dependent PI-3kinase Signalling. *J. Inflamm.* **2012**, *9*, 1, doi:10.1186/1476-9255-9-1.
38. Mombaerts, P.; Iacomini, J.; Johnson, R.S.; Herrup, K.; Tonegawa, S.; Papaioannou, V.E. RAG-1-Deficient Mice Have No Mature B and T Lymphocytes. *Cell* **1992**, *68*, 869–877, doi:10.1016/0092-8674(92)90030-g.
39. Toben, D.; Schroeder, I.; El Khassawna, T.; Mehta, M.; Hoffmann, J.-E.; Frisch, J.-T.; Schell, H.; Lienau, J.; Serra, A.; Radbruch, A.; et al. Fracture Healing Is Accelerated in the Absence of the Adaptive Immune System. *J. Bone Miner. Res. Off. J. Am. Soc. Bone Miner. Res.* **2011**, *26*, 113–124, doi:10.1002/jbmr.185.
40. Reinke, S.; Geissler, S.; Taylor, W.R.; Schmidt-Bleek, K.; Juelke, K.; Schwachmeyer, V.; Dahne, M.; Hartwig, T.; Akyüz, L.; Meisel, C.; et al. Terminally Differentiated $CD8^+$ T Cells Negatively Affect Bone Regeneration in Humans. *Sci. Transl. Med.* **2013**, *5*, 177ra36, doi:10.1126/scitranslmed.3004754.
41. Ye, F.; Li, J.; Xu, P.; Xie, Z.; Zheng, G.; Liu, W.; Ye, G.; Yu, W.; Lin, J.; Su, Z.; et al. Osteogenic Differentiation of Mesenchymal Stem Cells Promotes C-Jun-Dependent Secretion of Interleukin 8 and Mediates the Migration and Differentiation of CD4+ T Cells. *Stem Cell Res. Ther.* **2022**, *13*, 58, doi:10.1186/s13287-022-02735-0.
42. Roser-Page, S.; Vikulina, T.; Yu, K.; McGee-Lawrence, M.E.; Weitzmann, M.N. Neutralization of CD40 Ligand Costimulation Promotes Bone Formation and Accretion of Vertebral Bone Mass in Mice. *Rheumatology* **2018**, *57*, 1105–1114, doi:10.1093/rheumatology/kex525.
43. Adamus, T.; Kortylewski, M. The Revival of CpG Oligonucleotide-Based Cancer Immunotherapies. *Wspolczesna Onkol.* **2017**, *2*, 56–60, doi:10.5114/wo.2018.73887.

44. Kinjo, T.; Miyagi, K.; Nakamura, K.; Higa, F.; Gang, X.; Miyazato, A.; Kaku, M.; Fujita, J.; Kawakami, K. Adjuvant Effect of CpG-Oligodeoxynucleotide in Anti-Fungal Chemotherapy against Fatal Infection with Cryptococcus Neoformans in Mice. *Microbiol. Immunol.* **2007**, *51*, 741–746, doi:10.1111/j.1348-0421.2007.tb03963.x.
45. Krug, A.; Rothenfusser, S.; Hornung, V.; Jahrsdörfer, B.; Blackwell, S.; Ballas, Z.K.; Endres, S.; Krieg, A.M.; Hartmann, G. Identification of CpG Oligonucleotide Sequences with High Induction of IFN-Alpha/Beta in Plasmacytoid Dendritic Cells. *Eur. J. Immunol.* **2001**, *31*, 2154–2163, doi:10.1002/1521-4141(200107)31:7<2154::aid-immu2154>3.0.co;2-u.
46. Romieu-Mourez, R.; François, M.; Boivin, M.-N.; Bouchentouf, M.; Spaner, D.E.; Galipeau, J. Cytokine Modulation of TLR Expression and Activation in Mesenchymal Stromal Cells Leads to a Proinflammatory Phenotype. *J. Immunol.* **2009**, *182*, 7963–7973, doi:10.4049/jimmunol.0803864.
47. Lee, J.-G.; Lee, S.-H.; Park, D.-W.; Lee, S.-H.; Yoon, H.-S.; Chin, B.-R.; Kim, J.-H.; Kim, J.-R.; Baek, S.-H. Toll-like Receptor 9-Stimulated Monocyte Chemoattractant Protein-1 Is Mediated via JNK-Cytosolic Phospholipase A2-ROS Signaling. *Cell. Signal.* **2008**, *20*, 105–111, doi: 10.1016/j.cellsig.2007.09.003.
48. Blackwell, S.E.; Krieg, A.M. CpG-A-Induced Monocyte IFN-γ-Inducible Protein-10 Production Is Regulated by Plasmacytoid Dendritic Cell-Derived IFN-α. *J. Immunol.* **2003**, *170*, 4061–4068, doi:10.4049/jimmunol.170.8.4061.
49. Nierkens, S.; den Brok, M.H.; Garcia, Z.; Togher, S.; Wagenaars, J.; Wassink, M.; Boon, L.; Ruers, T.J.; Figdor, C.G.; Schoenberger, S.P.; et al. Immune Adjuvant Efficacy of CpG Oligonucleotide in Cancer Treatment Is Founded Specifically upon TLR9 Function in Plasmacytoid Dendritic Cells. *Cancer Res.* **2011**, *71*, 6428–6437, doi: 10.1158/0008-5472.CAN-11-2154.
50. Prins, H.-J.; Braat, A.K.; Gawlitta, D.; Dhert, W.J.A.; Egan, D.A.; Tijssen-Slump, E.; Yuan, H.; Coffer, P.J.; Rozemuller, H.; Martens, A.C. *In vitro* Induction of Alkaline Phosphatase Levels Predicts *in vivo* Bone Forming Capacity of Human Bone Marrow Stromal Cells. *Stem Cell Res.* **2014**, *12*, 428–440, doi: 10.1016/j.scr.2013.12.001.
51. Melief, S.M.; Schrama, C.L.M.; Roelofs, H. PBMC-MSC Co-Cultures for Induction of Treg Generation. *Bio-Protoc.* **2015**, *5*, e1383–e1383.

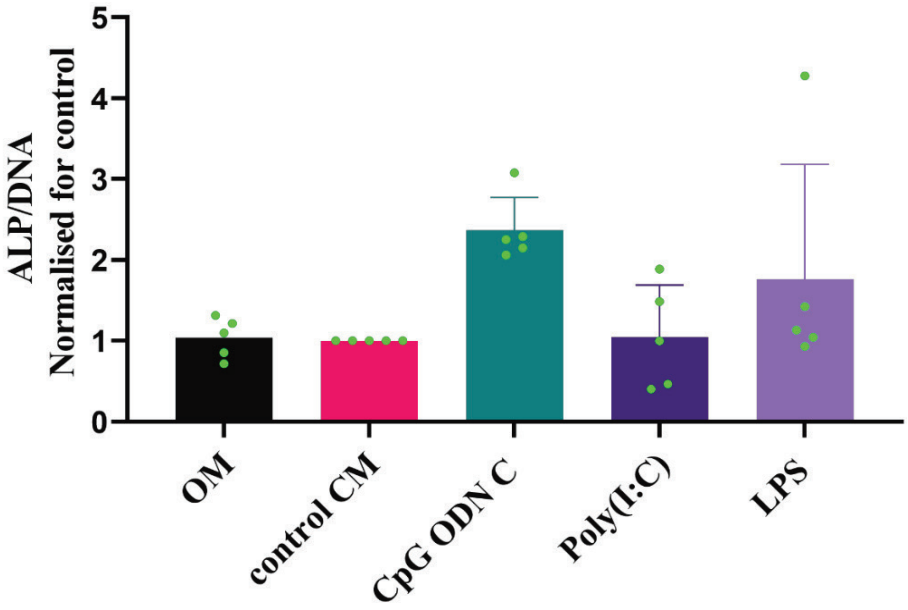
Supplementary File



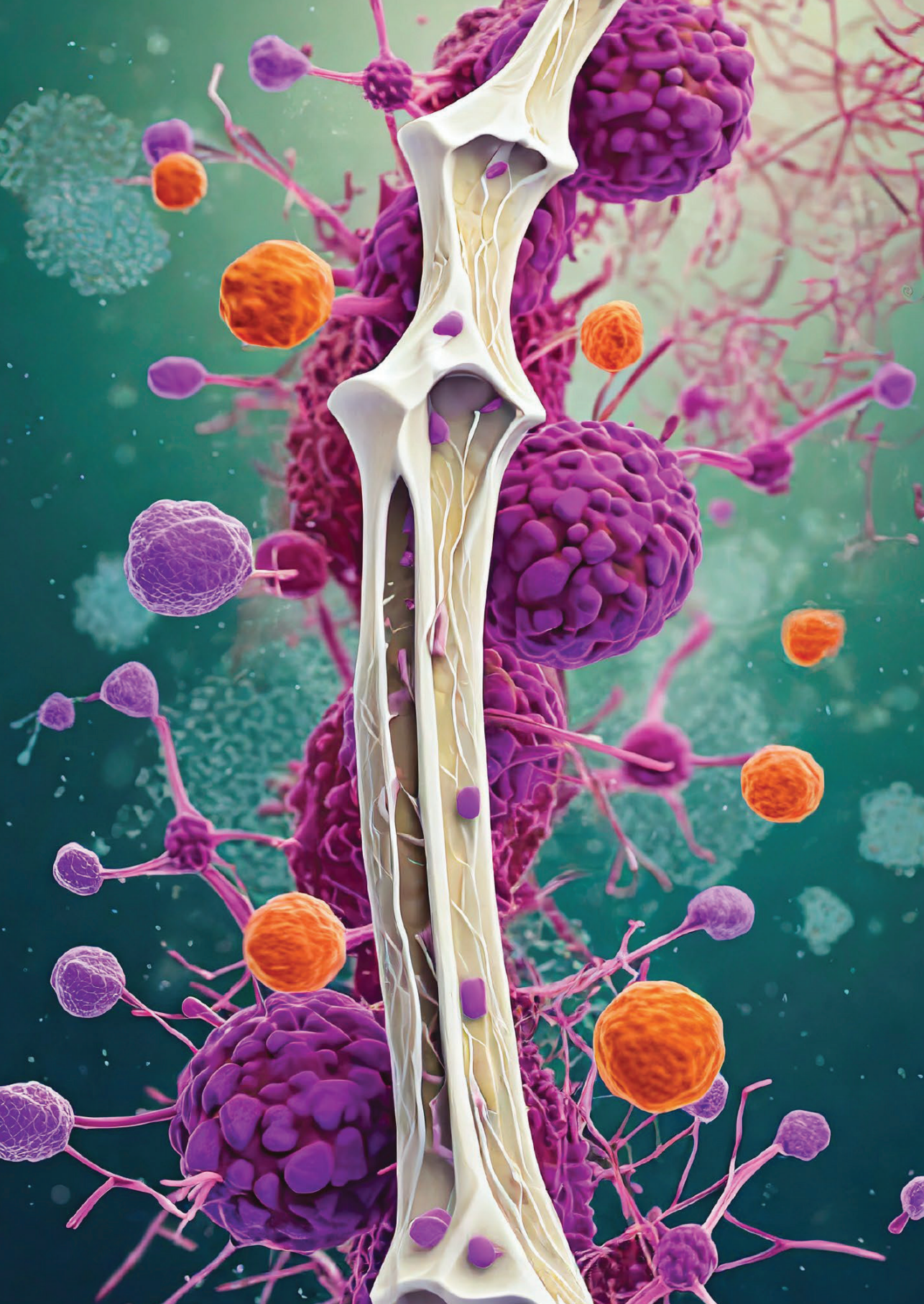
Supplementary Figure S1. Effect of the indirect stimulation of PRR ligands on osteogenic differentiation. ALP activity was corrected for DNA after MSC stimulation with PRR ligands indirectly with conditioned medium from stimulated PBMCs (indirect stimulation CM) measured after 7 days. Data were normalized to control CM consisting of osteogenic differentiation medium and conditioned medium from unstimulated PBMCs. The PBMCs were exposed to PRR ligands in the concentration range mentioned in Table 1. The conditioned medium was added in the ratio of 1:4 (CM: ODM). The graph represents the mean ± standard deviation of technical triplicates performed per donor for 3 MSC donors represented as yellow dots in the graph.



Supplementary Figure S2. Effect of the unstimulated PBMCs on osteogenic differentiation without dexamethasone. ALP activity was corrected for DNA after MSCs were cultured in expansion medium or expansion medium with conditioned medium from unstimulated PBMCs in the ratio of 1:4 after 7 days. Data were normalized to osteogenic differentiation medium. The graph represents the mean ± standard deviation of technical triplicates performed per donor for 2 MSC donors shown as red and green dots.



Supplementary Figure S3. Effect of the indirect stimulation of PRR ligands on osteogenic differentiation. ALP activity was corrected for DNA after MSC stimulation with PRR ligands indirectly with conditioned medium from stimulated PBMCs (indirect stimulation CM) measured after 7 days. Data were normalized to control CM consisting of osteogenic differentiation medium and conditioned medium from unstimulated PBMCs. The PBMCs (n = 5) were exposed to PRR ligands in the concentration range mentioned in Table 1. The conditioned medium was added in the ratio of 1:4 (CM: ODM). The graph represents the mean ± standard deviation of technical triplicates performed per donor for 5 MSC donors represented as green dots in the graph.



Chapter 4

Mixtures of PRR ligands partly mimic the immunomodulatory response of γi *Staphylococcus Aureus*, enhancing osteogenic differentiation of human mesenchymal stromal cells

Paree Khokhani[1], Kelly Warmink[1], Moyo Kruyt[1,4], Harrie Weinans[1,3], Debby Gawlitta[1,2]

1. Department of Orthopedics, University Medical Centre Utrecht, Utrecht, The Netherlands
2. Department of Oral and maxillofacial reconstruction surgery and Special Dental Care, University Medical Centre Utrecht, Utrecht, The Netherlands
3. Department of Biomechanical Engineering, 3ME, TU Delft, the Netherlands
4. Department of Developmental BioEngineering, University of Twente, the Netherlands

Manuscript Submitted

Abstract

Recent evidence indicates the potential of gamma-irradiated (γi) *S. aureus* to be used as an osteo-immunomodulator for bone regeneration. This study aims at characterizing the inflammatory milieu caused by the stimulation of γi *S. aureus* in immune cells and investigates its effects on MSC osteogenic differentiation. Furthermore, we aimed to recreate the immune-modulatory response exhibited by γi *S. aureus* by using a mixture of various synthetic PRR ligands consisting of TLR2, TLR8, TLR9, and NOD2 agonists. Human peripheral blood mononuclear cells (hPBMCs), isolated from healthy human donors, were exposed to γi *S. aureus* or seven different ligand mixtures. After 24 hours, the conditioned medium (CM) from the hPBMCs was collected, and its effects on hMSC osteogenic differentiation were investigated by assessing alkaline phosphatase activity (ALP) and matrix mineralization. The hPBMCs and their CM were also analyzed by bulk RNA sequencing and for cytokine secretion. CM from the γi *S. aureus* and the mixture consisting of Pam3CSK4, CpG ODN C, and Murabutide targeting TLR2, TLR9, and NOD2 showed a 5-fold increase in ALP and matrix mineralization in a donor-dependent manner. These effects were due to the upregulation of inflammatory signaling pathways, which led to an increase in cytokines and chemokines TNF, IL-6, IFN-γ, IL-1α, CXCL10, CCL18, CCL17, CXCL1, and CCL5. Upregulation of genes like BMP2R, BMP6R, BGLAP, and others contributed to the upregulation of osteogenic pathways in the hPBMCs stimulated with γi *S. aureus* and aforementioned mix. Thus, formulations with mixtures of PRR ligands may serve as immune-modulatory osteogenesis-enhancing agents.

Keywords

immune modulation, inflammation, PRR ligands, pathogen recognition molecular patterns, Staphylococcus aureus, bone regeneration

Introduction

New strategies to enhance off-the-shelf bone substitutes, especially biphasic calcium phosphates (BCPs), are of great interest, considering their non-inferiority to the existing autologous bone grafting to treat critical size defects, spinal fusion, and fracture non-unions [1,2]. BCPs are an attractive alternative to the current gold standard autografts as they are biocompatible, demonstrate osteo-conductivity, have an unlimited supply, and are easy to sterilize. However, autografts and BCPs only achieve a 58%-93% spinal fusion rate [2–5]. Adding cells or growth factors to these bone substitutes to boost their performance is explored extensively[6,7]. With increasing evidence of the immune system's role in bone healing and repair, the use of immune-modulatory factors to enhance bone substitutes holds excellent potential [8–10].

Generally, a balanced early inflammatory phase provides the vital microenvironment that coordinates osteogenic and angiogenic processes that are needed for optimal bone healing [11,12]. Pathological conditions such as osteomyelitis and heterotopic ossification, which involve the activation of inflammatory pathways leading to bone formation, can provide cues in identifying the underlying pro-osteogenic and angiogenic factors [13,14]. For instance, local responses to bacterial infections have been associated with new bone formation in heterotopic ossifications due to increased inflammation, indicating that bacterial antigens may act as osteo-immunomodulators [15,16]. Recently, we showed that gamma-irradiated (γi) *S. aureus* or its cell wall fragments induced bone formation in several rabbit models [17,18]. The mechanism behind this phenomenon is assumed to be the sterile inflammatory microenvironment created by γi *S. aureus* or the cell wall components that might be beneficial for bone formation. Hence, elucidating the mechanism through which γi *S. aureus*, and the immune cell secretome in response to γi *S. aureus* stimulation, affect bone formation is of great interest to define the inflammatory milieu that promotes bone regeneration.

The host immune system recognizes *S. aureus* by identifying the pathogen-associated molecular patterns (PAMPs) via the pathogen recognition receptors (PRRs) and inflammasome signaling, which lead to the release of various proinflammatory cytokines and antimicrobial peptides [19,20]. The primary PRR in *S. aureus* detection is the plasma membrane-bound TLR2, which is activated upon sensing bacterial Lipoteichoic acid (LTA), or synthetic ligand Pam3CysSerLys4 (Pam3CSK4), to form heterodimers TLR1/TLR2 and TLR2/TLR6 [21,22]. In addition, small peptidoglycan fragments of *S. aureus*, also synthetically known as Murabutide, induce cytosolic NOD-2 activation, resulting in the induction of various cytokines like interleukin IL-1β, IL-6, and antimicrobial peptides [23–25]. Upon degradation of *S. aureus* in the phagosome, the classical

induction of inflammatory cytokines and interferons is triggered due to the detection of CpG motifs by TLR9 [26] [27], via nuclear factor kappa beta (NF-κB), mitogen activated protein kinase (MAPK), type I interferons, and inflammasome signaling pathways [28]. To sum up, a synergistic crosstalk between the PRRs may be the reason for a controlled and yet specific immune response observed in *S. aureus* exposure.

Mixing different PRR ligands involved in the recognition of *S. aureus* via the immune system may prove to be an effective alternative for enhanced bone healing. PRR ligands are explored extensively for their ability to stimulate and enhance the host immune response. For example, they are currently clinically tested as vaccine adjuvants for cancer therapies and infectious diseases [29,30]. Recently, we also examined the effects of individual PRR ligands on osteogenic differentiation of human mesenchymal stromal cells (hMSCs) *in vitro*. Intracellular nucleic acid-based PRR ligands enhanced osteogenic differentiation when added directly or through conditioned medium from stimulated immune cells [31,32]. Considering this, PRR ligands offer a better defined, and more stable alternative to overcome the drawbacks like off-target side effects and batch-to- batch inconsistencies of γi *S. aureus* for orthopedic applications.

In this study, we evaluated the immunomodulatory effects and the underlying mechanism of γi *S. aureus* on osteogenic differentiation of hMSCs. Further, we aimed to mimic the effect of γi *S. aureus* on osteogenic differentiation by stimulating hMSCs with several mixtures of PRR ligands, as these have potential to provide a better defined and safer alternative compared to γi *S. aureus* for orthopedic applications. Additionally, hMSCs were indirectly stimulated via conditioned media from hPBMCs exposed to γi *S. aureus* or ligands, to mimic the *in vivo* situation where peripheral blood is exposed before hMSC arrive to the scene. To achieve this, we characterized hMSCs and hPBMCs stimulated with either γi *S. aureus* or mixtures to gain insight into the signaling pathways, secreted cytokines, and growth factors with the ultimate goals to improve bone (re)generation and to use a specific set of PRR ligands instead of γi *S. aureus.*

Materials and Methods

Study design

The osteogenic potential of gamma irradiated (γi) *S. aureus* and mixtures containing synthetic PRR ligands mimicking *S. aureus* was investigated by adding them either directly (direct stimulation) or via the conditioned medium obtained from stimulation of immune cells (indirect stimulation) to the human bone

marrow-derived mesenchymal stromal cells (hMSCs). The mixtures were based on the PRRs involved in the initial recognition of *S. aureus* mentioned in the literature [19] (**Table 1**).

Mixtures	Recognizing PRRs			
	NOD-2	TLR2	TLR8	TLR9
MIX 1	Murabutide	Pam3CSK4	--	CpG ODN C
MIX 2	Murabutide Peptidoglycan	--	--	CpG ODN C
MIX 3	Murabutide	--	--	CpG ODN C
MIX 4	Murabutide	CL429	Resiquimod	CpG ODN C
MIX 5	Murabutide	LTA	Resiquimod	CpG ODN C
MIX 6	Murabutide Peptidoglycan	--	--	CpG ODN C
MIX 7	Murabutide	CL429	--	CpG ODN C

Table 1. Overview of the *S. aureus* mimicking mixtures (MIX 1-7) that were prepared using the synthetic pathogen recognition receptor (PRR) ligands. Also, their corresponding recognition receptors are shown in the top row. LTA=Lipoteichoic acid, TLR=Toll-like receptors.

For indirect stimulation (immune-mediated stimulation), human peripheral blood mononuclear cells (hPBMCs) were isolated from peripheral blood obtained from healthy donors (n = 9). The pooled hPBMCs were seeded in a 24 wells plate and cultured in the presence/ absence of γi *S. aureus* or mixtures of PRR ligands (**Table 1**). The unstimulated hPBMCs served as the control. After 24 hours, the hPBMCs were prepared for bulk RNA sequencing experiments. The pooled supernatant from the hPBMCs donors was centrifuged at 1500 g for 5 mins to obtain cell-deprived conditioned medium that was stored at – 80°C. The CM was used to characterize its composition using a custom Luminex assay. Furthermore, the CM was added to the hMSC culture in the ratio of 1:4 (CM: osteogenic differentiation medium (ODM)). The osteogenic differentiation of hMSCs was evaluated by assessing the secretion of alkaline phosphatase on days 3, 7, 10, and 14 along with matrix mineralization on day 21.

Reagents

S. aureus (Wood 46) were cultured to mid-log phase in LB liquid medium. Freshly cultured bacteria were then subjected to 25 G kγ radiation. The absence of any viable bacteria was confirmed by plate cultures. The killed bacteria suspensions were stored at -80 ºC in phosphate-buffered saline solution (PBS, Gibco, Thermo Fisher Scientific, United States) with 20% (v/v) glycerol. The bacteria were washed thoroughly with PBS (300 g for 3 mins) before use. γ*i S. aureus* was used in the concentrations of 10^7 units/mL (high), 10^6 units/mL (medium) and 10^5 units/mL (low) for the experiments.

C-class CpG oligodeoxynucleotide (CpG ODN C) (M362) (1 μg/mL), Resiquimod (R848) (10 μg/mL), Pam3CSK4(Pam3CysSerLys4) (10 μg/mL), CL429 (Pam2C-conjugated Murabutide) (10 μg/mL), insoluble Peptidoglycan (from *S. aureus*) (10 μg/mL), lipoteichoic acid (LTA) (from *S. aureus*) (10 μg/mL) and Murabutide (10 μg/mL) were purchased from InvivoGen, USA. These PRR ligands were mixed to form mixtures 1-7 , of which the composition was based on PRRs involved in the recognition of *S. aureus* as mentioned in the literature [19]. The concentration of each PRR ligand was chosen based on the manufacturer's datasheet.

Isolation and culturing of human peripheral blood mononuclear cells (hPBMCs)

Human blood from healthy donors with ages ranging from 27 years to 62 years (n = 9) was obtained with the approval of the local medical ethical committee (University Medical Center Utrecht, Utrecht, The Netherlands) under the protocol METC 07-125/C, and written consent of the participants.

Peripheral mononuclear cells (hPBMCs) were isolated using the density gradient centrifugation method. Briefly, the heparinized blood was diluted with PBS in a ratio of 1:1 and layered on top of the 15 mL density gradient medium (Ficoll Paque plus, GE Healthcare, United States) into SepMate™ isolation tubes (Stem cell technologies, Germany). These tubes were then centrifuged at 1200 g for 10 mins with brakes on at room temperature. The supernatant containing the serum and cells was quickly poured off in a new 50 mL falcon tube and centrifuged at 300 g for 8 mins at room temperature. After centrifugation, hPBMCs were then suspended in a culture medium consisting of RPMI-1640 glutamax (Thermo Fisher Scientific, United States) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Biowest, United States) and 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco, Thermo Fisher Scientific, United States).

To produce 1 mL conditioned medium, hPBMCs from individual donors were seeded in a 24 wells plate at 500,000 cells / cm^2 and cultured for 24 hours at 37 °C

in a humidified atmosphere containing 5% CO_2. Subsequently, the hPBMCs were stimulated with either γ*i S. aureus* or one of the mixtures (Table 1). Unstimulated hPBMCs served as a control. After 24 h of culture, hPBMCs were stored in TRIzol® reagent (Thermo Fisher Scientific, United States) for extraction of RNA while the conditioned medium was pooled from all donors per condition, centrifuged at 1500 g for 5 min, and stored at -80°C until further use.

Isolation and culturing of human mesenchymal stromal cells (hMSCs)

Bone marrow was harvested from the vertebrae, or the iliac crest of female patients aged 15–62 years (n = 6) with the approval of the local medical ethical committee (University Medical Center Utrecht, Utrecht, The Netherlands) under biobank protocol 08/001K with broad consent. Human mesenchymal stromal cells (hMSCs) were isolated and cryopreserved at passage 2 according to a standardized method that yields multipotent cells [33]. hMSCs were thawed and expanded in a standard expansion medium consisting of minimum essential medium (α- MEM, Gibco, Thermo Fisher Scientific, United States) supplemented with 10 % (v/v) heat-inactivated FBS (Biowest, France) and 100 U/mL penicillin and 100 mg/ mL streptomycin and 0.2 mM l-ascorbic acid-2-phosphate (Sigma Aldrich, United States). The cells were plated at 70% confluency during subsequent passages up to a maximum of five passages. All cell cultures were performed at 37 °C in a humidified atmosphere containing 5% CO_2.

To test the effect of the γi *S. aureus* and the mixtures on early and late osteogenic differentiation, hMSCs were seeded at a density of 15,000 cells/cm^2 in a 96 wells plate or 24 wells plate in technical duplicates and cultured in the expansion medium. Upon 100% confluency, culture continued with either expansion medium or osteogenic medium (supplemented with 10 mM β-glycerophosphate and 10 nM dexamethasone, both from Sigma Aldrich, United States) and in the presence/ absence of the conditioned medium or by adding the γi *S. aureus* and the mixtures directly. hMSCs cultured in the osteogenic medium alone and in osteogenic medium containing 25% CM obtained from unstimulated hPBMCs were controls. The medium was refreshed every 3 days until day 14 for early analyses and day 21 for late osteogenic differentiation analyses.

hMSC osteogenic differentiation assays

Alkaline phosphatase secretion quantification

For quantification of alkaline phosphatase (ALP), cells were cultured in the osteogenic differentiation medium containing 10% FCS (Biowest, France). On

days 3, 7, 10, and 14, cells were lysed in 0.2% (*v*/*v*) Triton X-100/TBS for 30 min. ALP activity was measured by the conversion of the p-nitrophenyl phosphate liquid substrate system (pH = 9.6) (SigmaFast p-nitrophenyl phosphate tablets, Sigma-Aldrich). The cell lysate was also used to determine the DNA content with the Quant-It PicoGreen kit (Invitrogen), according to the manufacturer's instructions. The ALP/DNA ratio was normalized to the ratio of the controls that were treated with CM from unstimulated hPBMCs or osteogenic medium alone. The concentration of γi *S. aureus* and mixtures that yielded highest ALP activity upon indirect stimulation were chosen for further experiments (**Supplementary Figure S1**, the ones highlighted in red boxes).

Matrix mineralization assay

hMSCs were cultured in an osteogenic medium containing 10% FCS (Gibco, Thermo Fisher Scientific, United States) to assess and quantify the matrix mineralization after 21 days. Samples were incubated with 0.2% Alizarin Red S (ARS) for 60 min (pH = 4.2, Sigma) and examined using light microscopy. In addition, Alizarin Red was extracted from the monolayer of the cells by incubating in 10% (*w*/*v*) cetylpyridinium dissolved in 10 mM sodium di-phosphate buffer solution (pH = 7.2) (Sigma Aldrich) for 60 min. Absorbance was measured at 595 nm and corrected at 655 nm. The amount of calcium deposited in each well (experiments done in duplicates) was quantified using the standard curve obtained by dissolving a known concentration of ARS and considering 2 mol of Ca^{2+}/mol of dye in the solution.

Bulk RNA Sequencing and Analysis

Extraction of total RNA from hPBMCs stimulated with either γi *S. aureus* or the mixtures for 24 hours was carried out using the RNAeasy micro kit (Qiagen, Germany) according to the manufacturer's instructions. RNA concentration and quality were assessed using automated electrophoresis (4200 TapeStation system, Agilent). Bulk RNA sequencing was performed by Single Cell Discoveries (Utrecht, The Netherlands) using the CEL-seq 2 protocol with a sequencing depth of 10 million reads per sample. R data programming version 3.7 was used to analyze the data. The count normalization and differential gene expression were performed using the DESeq2 package v3.15 [34]. The log2-fold change of different genes between the experimental groups (i.e., unstimulated control versus γi *S. aureus*, control versus Mix 1, control versus Mix 4, control versus Mix 5) were determined. Log-fold change shrinkage was applied using the lfcShrink function with the adaptive Student's t prior shrinkage estimator, to reduce the number of

false positives [35]. This fold-change was used in all downstream analysis. A Wald test statistic was used to estimate fold-change significance, and p-values were adjusted for multiple testing using the Benjamini-Hochberg method. Genes with a shrunken log2-fold change above 1, and an adjusted p-value below 0.05 were considered differentially expressed. The top 100 differentially expressed genes between control and γi *S. aureus* were visualized across all samples in a heatmap, expressing normalized transformed counts, scaled per gene to represent the deviation from the average. In addition, predetermined genes of interest were visualized similarly in heatmaps and/or gene expression plots, by plotting the normalized transformed counts per condition.

The R package clusterProfiler (v.4.4.4) was used to perform gene set enrichment analysis (GSEA). The analysis was performed using gseGO to assess enrichment of gene ontology terms, with org.Hs.eg.db as organism database. A p-value below 0.05 was considered a significant enrichment, all p-values were corrected for multiple testing using the Benjamini-Hochberg method. Dot plot visualizations were generated to display the most significant gene sets, categorized by activation or suppression, as well as specific GO terms of interest related to bone development and inflammation.

Characterization of CM composition using Luminex assay

The conditioned medium was characterized for its protein composition using a human 36-target multiplex array (IL1RA, IL-1α, IL-1β, IL-6, IL-10, IL-17, IL-17F, TNFα, IFNα, IFNβ, IFNγ, TGFα MCP1, MIP1α, MIP1β, RANTES, MCP3, MCP2, CCL17, CCL18, GRO1a, CXCL5, IL8, MIG, IP10, SDF1α, OPG, OPN, SerpinF2, SerpinG1, FGFbasic, VEGF, MMP9, TREM1, IL1R1, THBS1). This experiment was performed at the Multiplex Core Facility (MPCF), University Medical Center, Utrecht, The Netherlands. The data were normalized to the unstimulated hPBMCs (control) and presented as Log2-change in a heatmap. The values that were out of range were shown in the heatmap as out of range (OOR); OOR > (above the highest concentration of the standard curve); or OOR < (below the lowest concentration of the standard curve).

Statistical Analysis

The normal Gaussian distribution of the data was tested using the Shapiro-Wilk normality test. Repeated measures ANOVA with Dunnett's post hoc correction for multiple comparisons was performed using SPSS (V24, IBM, USA). p-value < 0.05 was used as a threshold for significance. All data are presented as mean ± standard deviation with sample sizes mentioned in the figure legends. The

statistical analysis for the bulk RNA seq data is detailed under the respective paragraph.

Results

γi S. aureus and mixtures show different patterns of PRR activation

The gene expression in hPBMCs stimulated with γi *S. aureus* showed a significant upregulation in TLR1, TLR2, TLR6, TLR8, TLR9, and NOD2 expression along with a downregulation in TLR5 expression, as compared to the non-stimulated hPBMC control (**Figure 1**)[19]. Mix 1 shows upregulation in PRR expression similar to γi *S. aureus* except for NLRP3 and TLR6 expressions. Mix 4 and Mix 5, on the other hand, show a downregulation in NOD2, TLR1, TLR2, TLR4, and TLR7 as compared to γi *S. aureus*. Upregulation of TLR3 was observed in all mixtures and not in γi *S. aureus* (**Figure 1c**). Based on these, Mix 1 mimics γi *S. aureus* most closely in terms of PRR expression (**Figure 1b**).

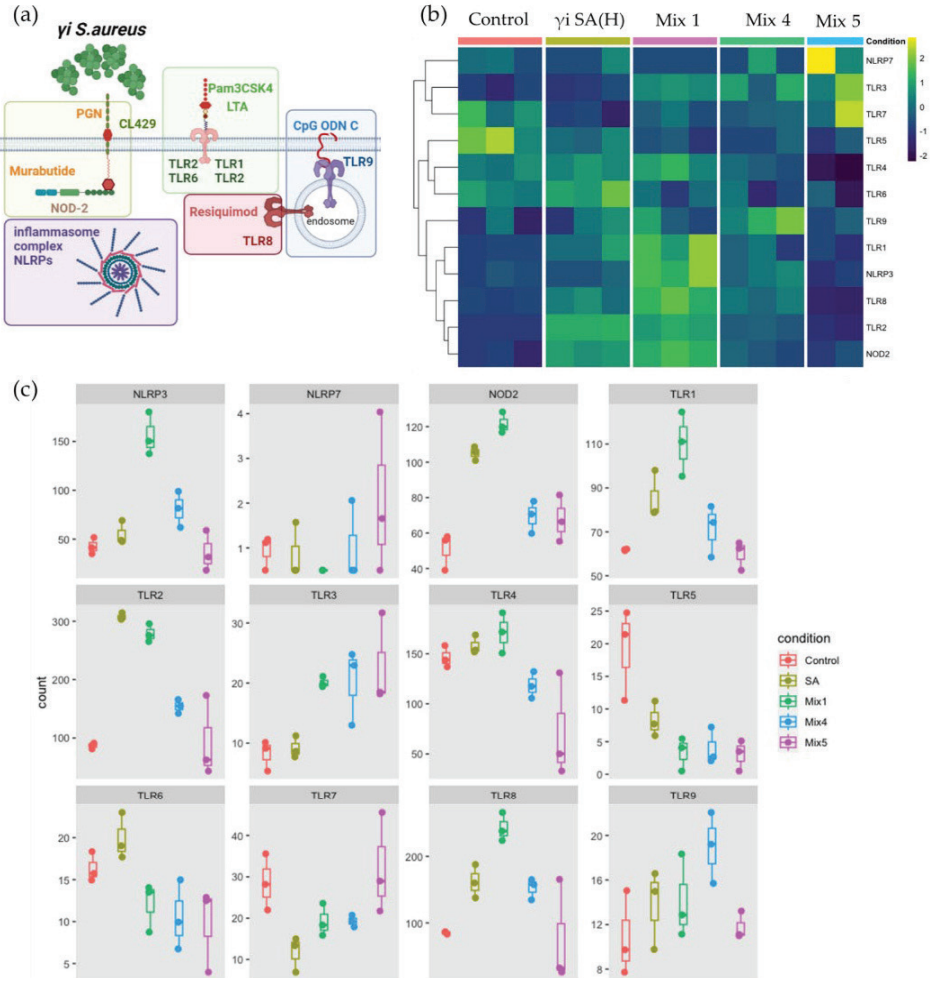


Figure 1. Effect of γi *S. aureus* and mixtures on pathogen recognition receptor expression at the gene level in pooled hPBMCs (n = 9). (a) Illustration showing the PRRs involved in *S. aureus* recognition based on literature[19]. Colored boxes correspond to colors in Table 1. (b) Heatmap showing the log2 normalized and scaled of the absolute gene count between groups. Each box depicts the technical replicates of the conditions. (c) Absolute gene count of the PRRs for all groups. Data are shown as box and whisker plots. The colored dots indicate the technical replicates used per condition.

Conditioned medium from γi S. aureus and mixtures of PRR ligands enhance osteogenic differentiation of hMSCs, while direct stimulation does not

hMSCs stimulated with a high concentration of γi *S. aureus* CM showed increased ALP activity at all time points as compared to the control group in 3/6 hMSC donors (**Figure 2c**). A similar trend was observed with conditioned media obtained from hPBMCs stimulated with mixtures, mainly Mix 1, Mix 4, and Mix 5 in 5/6 hMSC donors (**Figure 2 d, e, f**), while no effect was observed when individual PRR ligands were used (**Supplementary Figure S2**). The timing of the effect in all the groups was donor-dependent (**Figure 2g**). The direct stimulation with γi *S. aureus* and mixtures did not show an increase in the ALP activity (**Supplementary Figure S3**). In addition, no effect on ALP activity was observed when hMSCs were cultured in normal expansion medium, indicating a synergistic response of γi *S. aureus* and mixtures Mix 1, Mix 4, and Mix 5 with osteogenic medium (**Supplementary Figure S4**). In agreement with the early osteogenic marker ALP activity, hMSCs stimulated indirectly with a high concentration of γi *S. aureus* CM or with Mix 1 CM showed a five-fold increase compared to the control for matrix mineralization, a late marker for osteogenic differentiation (**Figure 2h**).

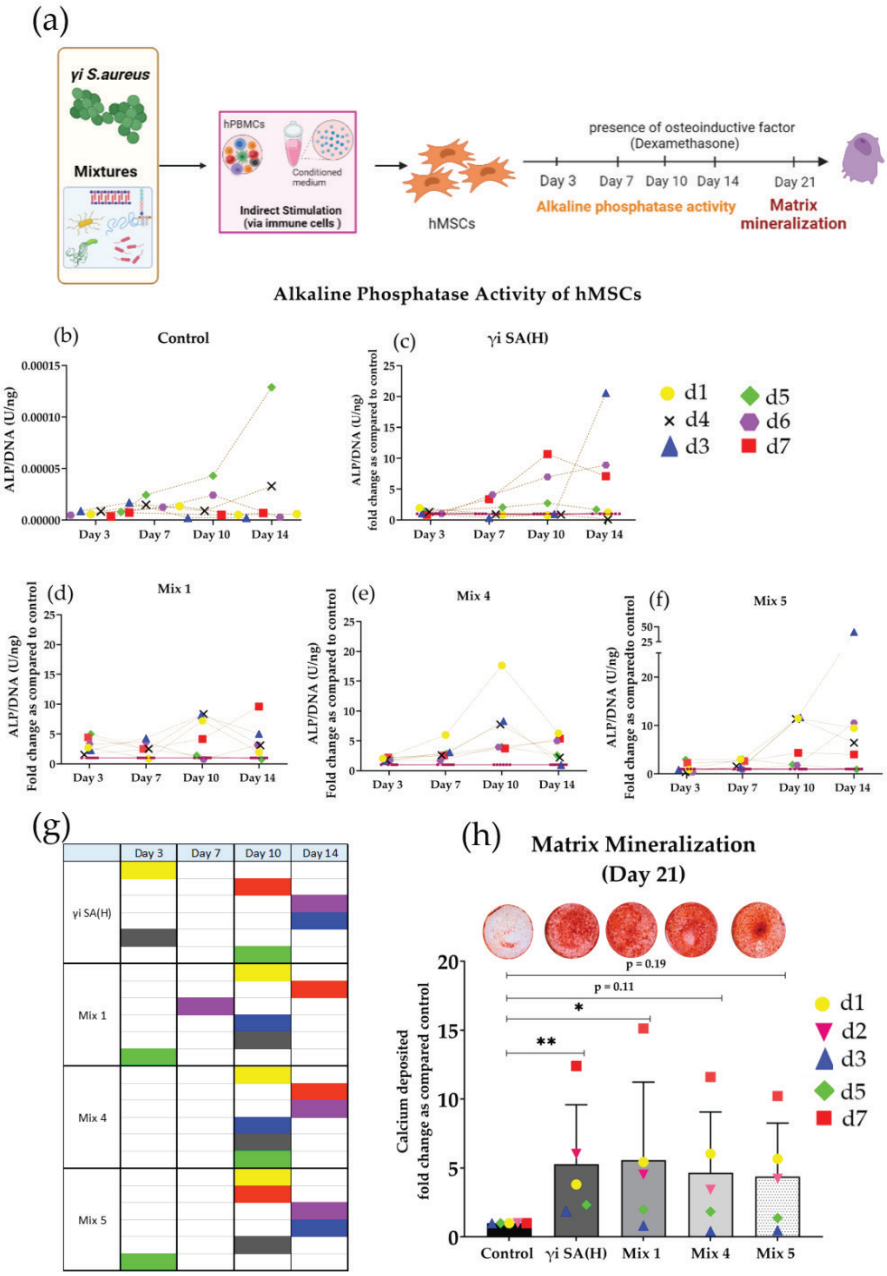


Figure 2. Indirect effect of γi *S. aureus* and mixtures on the hMSC osteogenic differentiation. (a) Study design set up. γi *S. aureus* and mixtures were added to hPBMCs (n =9) for 24 hours. The conditioned medium obtained from the stimulation was pooled and used to stimulate hMSCs (n=6) into osteogenic

differentiation. The CM was added in the ratio of 1:4 (CM: ODM). (b) Alkaline phosphatase activity was measured at days 3, 7, 10 and 14 and normalized to DNA content for the controls containing CM obtained from unstimulated hPBMCs. (c) The fold change of the normalized ALP activity to the controls is shown for γi *S. aureus*, (d) Mix 1, (e) Mix 4, and (f) Mix 5. The data are presented as mean of technical duplicates performed per donor for six individual donors. Significance was tested using repeated-measures ANOVA with Sidak's post hoc test for multiple comparisons. (g) Tabular format showing the upregulation of six hMSC donors (in colored cells) for ALP activity between groups. (h) Alizarin Red S staining was performed after hMSCs (n = 5) were stimulated with CM obtained from γi *S. aureus* and mixtures Mix 1, Mix 4, and Mix 5 stimulated hPBMCs (n = 9). The stained images (circular wells) show the calcium deposition in red for all groups (representative for 5 MSC donors). The total amount of calcium deposited per well was quantified and normalized to the control. Results show the mean + standard deviation for 5 MSC donors. Significance was tested using one-way ANOVA with Sidak's post hoc test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Differential gene expression and gene set enrichment analysis for γi S. aureus and mixtures

We identified the top 100 differentially expressed genes (**Figure 3**) between γi *S. aureus* and the control group and compared their gene expression across all experimental groups (**Figure 3b**). Of these 100 genes, 38 genes were upregulated in the γi *S. aureus* group as compared to control, while showing low expression for all the mixtures (**Figure 3b: highlighted in red**). Amongst these genes are transcription factors for various inflammatory markers like CXCL5, CCL22, IL-1R1, and growth factors like VEGF, TGFβ1, and degrading enzyme MMP9, and encoder of bone matrix protein SPP1 which are known to play a role in the fracture healing process. Fourteen genes showed similar patterns between γi *S. aureus* and Mix 1 (**Figure 3b: highlighted in pink**). These were mostly chemokine and pro-inflammatory cytokines, like IL1A and several CXCLs. Further, 12 genes showed a similar pattern between the three mixtures, all showing a high expression (**Figure 3b: highlighted in brown**) whereas the control and γi *S. aureus* group showed low expression levels. Lastly, 9 genes were found to show a similar pattern between γi *S. aureus*, and all mixtures as compared to the control (**Figure 3b: highlighted in blue**). Most notably, HIF-1α is upregulated, which is an important factor in vascular recruitment and subsequent bone formation [36]. All together indicating that Mix 1 shows the most overlap in the response to γi *S. aureus*.

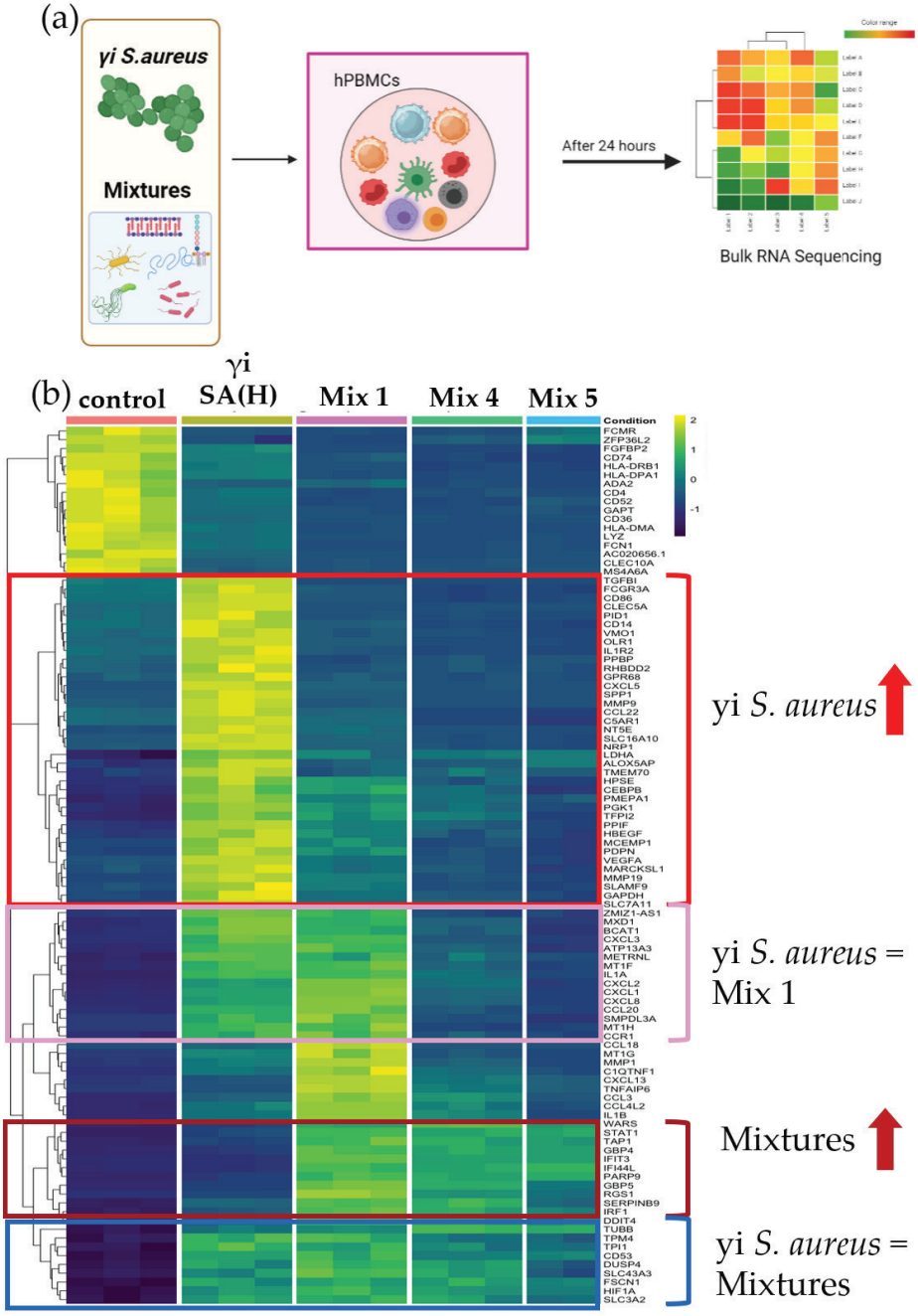


Figure 3. Differential gene expression analysis. (a) Illustration of the assay setup. hPBMCs (n=9) were stimulated with either γi *S. aureus* or mixtures for 24 hours. RNA was isolated and processed for bulk RNA sequencing. RNA pooled from

unstimulated hPBMCs (n = 9) served as the control. (b) A heatmap is showing gene expression of all groups of the top 100 most differentially regulated genes between control and γi *S. aureus*. Color scale indicates normalized transformed gene expression, scaled per gene to represent deviation from the average. The colored boxes highlight the patterns observed in the genes among all the groups.

To understand the biological processes and potential mechanisms involved in the immunomodulatory responses of the γi *S. aureus* and the mixtures, we performed gene set enrichment analyses for γi *S. aureus*, Mix 1, Mix 4, and Mix 5, compared to control on an *a priori* determined set of genes relating to bone and inflammatory pathways (**Figure 4**). The top 10 GO terms upregulated for the γi *S. aureus*, Mix 1, Mix 4, and Mix 5 also showed a similar pattern (**Supplementary Figure S5**). Especially γi *S. aureus*, Mix 1, and Mix 4 stimulation in hPBMCs resulted in the significant upregulation of mainly pro-inflammatory pathways of the NF-κB, ERK, and MAPK cascades, which are the known pathways involved in the secretion of various pro-inflammatory cytokines and chemokines as compared to the control. Mix 5 on the other hand, showed an upregulation of the NF-κB pathway with a suppression of MAP kinase. The interferon signaling pathway resulting in the secretion of IFN-α, -β, and -γ was upregulated in all the conditions (**Figure 4a**). Interestingly, γi *S. aureus* is the only group that showed an upregulation in the bone development and remodeling pathways. With one exception for Mix 1, which showed an upregulation in the bone mineralization pathway only (**Figure 4b**). Mix 4 and Mix 5 showed no enrichment of any bone-related pathway compared to control indicating that Mix 1 most closely mimics γi *S. aureus* response.

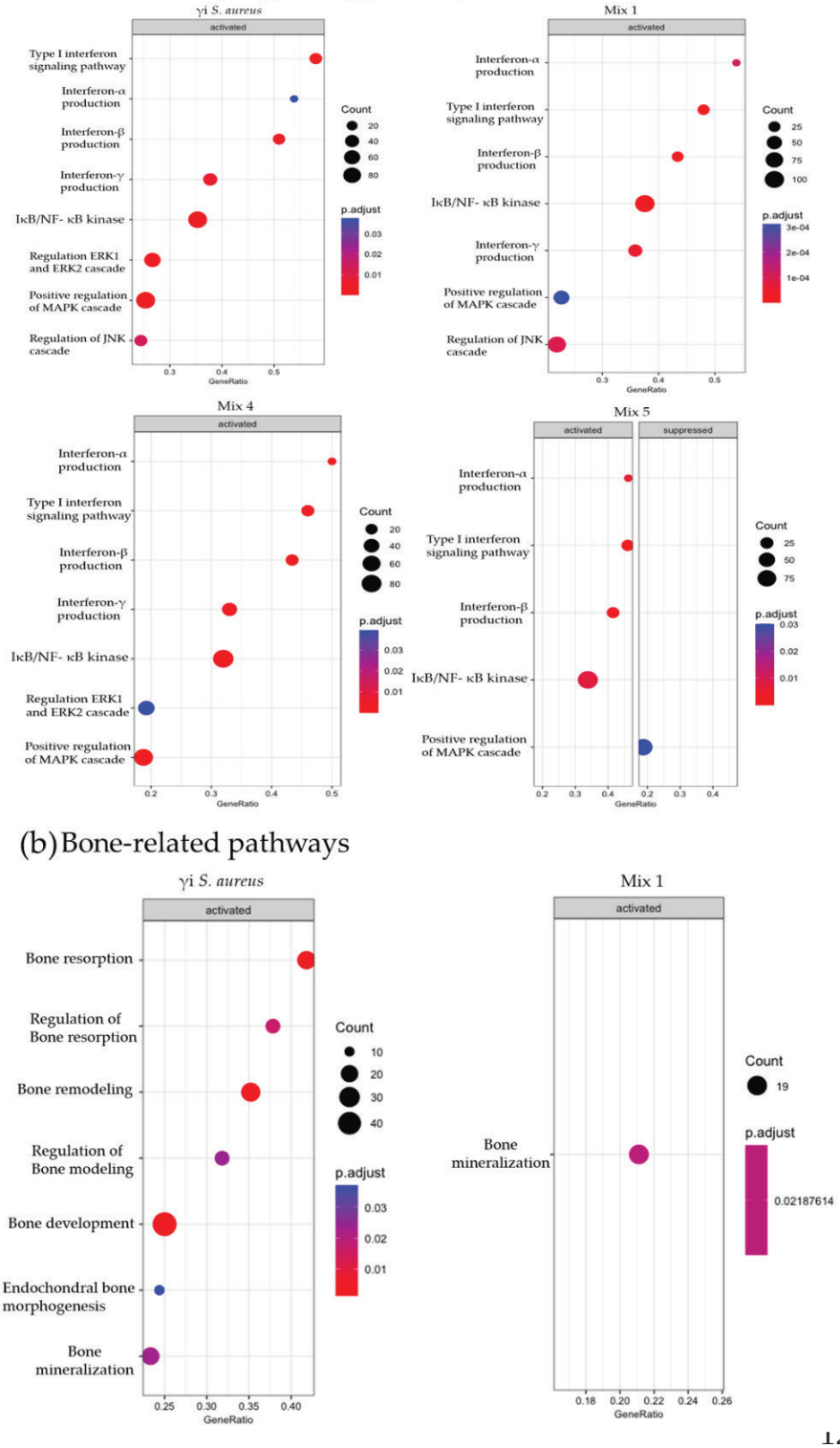

(a) Inflammatory signaling pathways
γi S. aureus
activated
Type I interferon signaling pathway
Interferon-α production
Interferon-β production
Interferon-γ production
IκB/NF- κB kinase
Regulation ERK1 and ERK2 cascade
Positive regulation of MAPK cascade
Regulation of JNK cascade
GeneRatio
Count
p.adjust
Mix 1
Mix 4
Mix 5
suppressed
(b) Bone-related pathways
Bone resorption
Regulation of Bone resorption
Bone remodeling
Regulation of Bone modeling
Bone development
Endochondral bone morphogenesis
Bone mineralization
0.02187614

Figure 4. The gene set enrichment analysis (GSEA), comparing Gene Ontology term enrichment for γi *S. aureus* and mixtures (Mix 1, Mix 4, and Mix 5) stimulated hPBMCs compared to unstimulated control. (a) Bubble plot showing the activation of inflammatory signaling pathways in all groups as compared to the control. (b)Bubble plot showing the activation of bone development and bone remodeling pathways in all conditions as compared to the control. Mix 1 was the only mixture to show an upregulation of bone related pathways. The number of the genes differentially expressed in a pathway is displayed using the size of the circle, GeneRatio displays the differentially expressed genes as a proportion of all genes in the pathway. The significance is shown as a color gradient (scale bar).

Protein expression in γi S. aureus and mixtures CM

On a protein level, γi *S. aureus* CM showed an immunomodulatory pattern when compared to the CM of mixtures, since the concentrations of the proinflammatory cytokines was found to be higher than that in γi *S. aureus* CM (**Figure 5b**). Significant upregulation was observed in proinflammatory cytokines and chemokines CCL8, IL-6, IL-1β, CXCL9, TNF-α, IFN-γ, CCL3, CXCL1, IL-1RN, CCL18, CCL4, IL-1α in the Mix 1 CM as compared to γi *S. aureus* CM (**Supplementary Figure S6**). Mix 4 CM and Mix 5 CM also show a similar pattern to Mix 1, except for downregulation in CXCL1, and CCL18. Interestingly, γi *S. aureus* CM showed an upregulation of cytokines IL-17, IL-17F, IL-1R1, chemokine CCL17, and growth factors TGF-α, VEGF, along with other factors like OPG, SERPING1 as compared to CM of the mixtures. Further, IFN-α was present in the higher concentrations in the mixtures CM as compared to that of γi *S. aureus* CM.

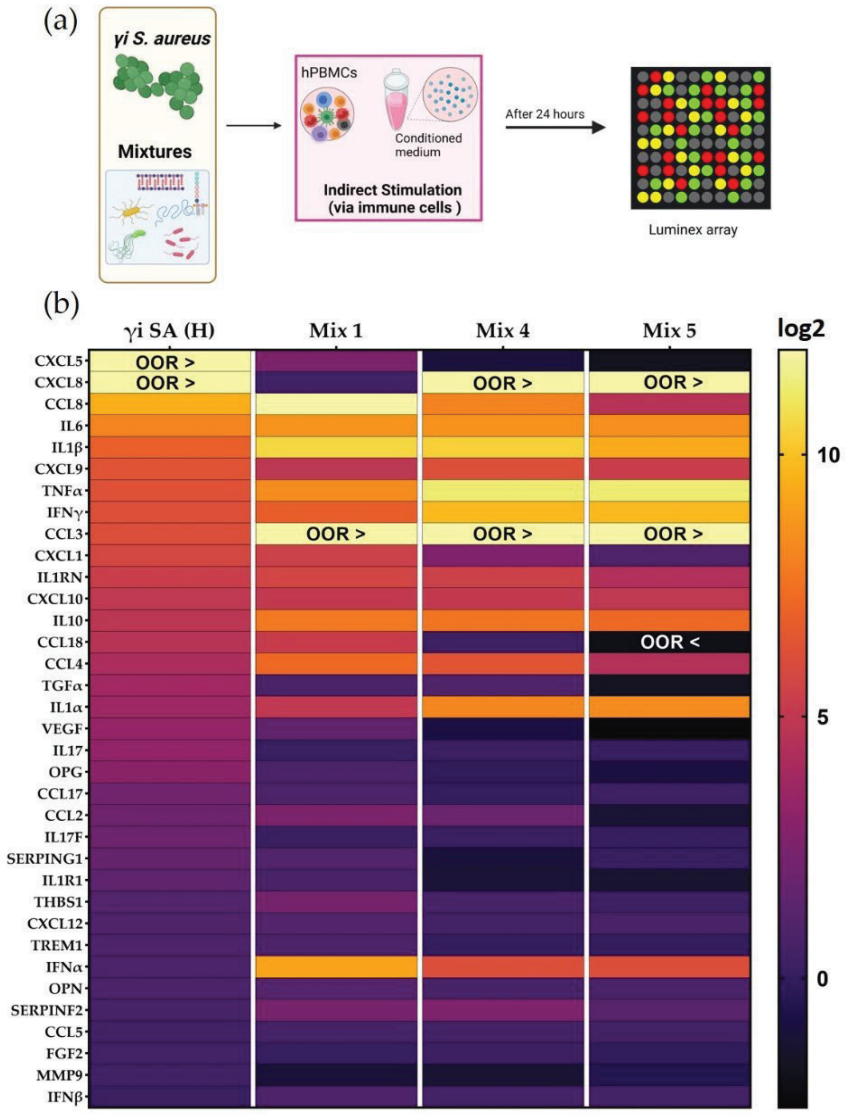


Figure 5. Characterization of proteins in CM. (a) hPBMCs (n=9) were stimulated with either γi *S. aureus* or mixtures for 24 hours. The conditioned medium obtained from the stimulation was pooled and used to characterize its composition using a Luminex assay. (b) Heatmap showing the log2-fold change from control of different proteins. All the groups were normalized to the control and transformed using log2. Samples that were above or below the detection limit were mentioned as out of range (OOR (above range OOR> and below range OOR<)).

Discussion

Harnessing bacterial antigens, especially those from *S. aureus* and their synthetic bacterial components known as PRR ligands, has gained attention to facilitate bone regeneration. Thus, understanding the mechanism and the inflammatory milieu instigated by *S. aureus* leading to bone formation is paramount to developing safe alternatives for patient treatment. To our knowledge, this is the first study to characterize the inflammatory milieu created by γi *S. aureus* and SA-mimicking mixtures in immune cells and its positive effect on hMSC osteogenic differentiation *in vitro*.

In summary, we found that conditioned medium from cultured PBMCs that were stimulated with γi *S. aureus* and mixtures of PRR ligands enhance osteogenic differentiation of hMSCs, while direct stimulation of hMSCs does not. The PRR mixture (Mix 1) mimicked the immunomodulatory response of γi *S. aureus* in hPBMCs most closely based on the differential gene expression patterns in hPBMCs seen from bulk RNA sequencing as well as in the inflammatory cytokine pattern derived from the Luminex assay. The same Mix 1 also showed the highest osteogenic stimulation of hMSCs in terms of both the alkaline phosphatase activity and the matrix mineralization assay. Most strikingly, the gene set enrichment analysis revealed that γi *S. aureus* stimulation upregulated genes like BMP6, BMP2, COL27A1, and BGLAP, that contribute to the upregulation of bone development, bone remodeling, bone mineralization and bone resorption pathways upon comparing to the pre-defined Gene ontology (GO) terms whereas Mix 1 was the only mixture that showed an upregulation in bone mineralization pathway. These findings might explain previous observations of increased bone formation by bacterial antigens in various rabbit models [10,17,18].

We observed that the osteogenic effects of γi *S. aureus* and PRR ligands are not directed to the MSCs (the precursors of osteoblasts) itself but have an indirect role via the stimulated PBMC secretome. On further characterization of the inflammatory milieu in hPBMCs conditioned medium, we found various cytokines like TNF-α, IL-6, IFN-γ, IL-1α, IL-1RN, IL-1R1 chemokines CXCL10, CCL18, CCL17, CXCL1, CCL5 and factors osteoprotegerin, SERPING1. These were present in response to γi *S. aureus* and the PRR ligands (in particular from Mix 1), which corresponds with activation of inflammatory signaling pathways NF-κB, MAPK, ERK and IFN type I upon comparing to the pre-defined Gene ontology terms as seen in the gene set enrichment analysis (**Figure 4** and **Figure 5**). The role of these proinflammatory cytokines in bone regeneration has been explored extensively. For example, TNF, IL-17, IL-1β, IL-6 were shown to enhance MSC osteogenic differentiation [37–39]. Interestingly, only hPBMCs stimulated with γi *S. aureus* showed an upregulation in genes that contribute to the

upregulation of bone development, bone mineralization, bone resorption, and bone remodeling pathways as compared to the predefined gene sets, indicating that γi *S. aureus* stimulation elicits a more comprehensive response in hPBMCs compared to PRR ligand mixtures (**Figure 4**). Nevertheless, the observed effect on matrix mineralization for conditioned medium of γi *S. aureus* and the three mixtures of PRR ligands, especially Mix 1, in hMSCs was quite similar. This suggests that the effect on osteogenic differentiation is primarily linked to the inflammatory pathways activated in hPBMCs upon stimulation with the PRR ligands.

This study aimed at mimicking γi *S. aureus's* immunomodulatory response by using various mixtures of PRR ligands. In agreement to numerous studies investigating the initial recognition mechanism of *S. aureus* in immune cells, we found that γi *S. aureus* upregulates the gene expression of TLR1, TLR2, TLR6, NOD2, TLR8, and TLR9 in human PBMCs as compared to control [19,22,23,40]. We expected the chosen mixtures to show a similar upregulation pattern in recognizing receptors, but only Mixture 1 closely mimicked the stimulation with γi *S. aureus*. Even though PRR ligands targeting TLR2 and NOD2 i.e., LTA, CL429, and Murabutide were included in Mix 4 and Mix 5, an upregulation in TLR2 and NOD2 receptor expression was not observed (Figure 1). Further, the differential gene expression in hPBMCs revealed that 14 genes including CXCL1, CXCL2, CXCL8, CXCL3, CCL20 showed similar expression when stimulated with γi *S. aureus* and Mix 1, indicating that Mix 1 most closely mimics γi *S. aureus* (**Figure 3**). The differences in the response of the mixtures might be explained from the differences in the chemical structure and origin of different PRR ligands resulting in non-specific binding and triggering of the target receptors [41,42]. Mixture 1 contained Pam3CSK4 to activate TLR2, compared to CL429 in Mix 4, and LTA in Mix 5. CL429 is a Pam2CSK4 conjugated with Murabutide designed to activate TLR2 and NOD2. However, CL429 does not form heterodimers with TLR1 and TLR6 receptors [43]. LTA requires a co-stimulatory molecule CD36 to activate TLR2 as compared to Pam3CSK4 [44]. These differences in the activation pattern of the receptors was shown to result in a delayed activation of NF-κB and MAPK pathways in murine macrophages [45]. Since all three mixtures (1, 4, 5) contained the same components for NOD2 and TLR9 activation, however, the components present in TLR2 activation was different among the mixtures. This implies that the choice of the PRR ligand will affect all inflammatory and osteogenic aspects of the bone healing milieu.

Despite the differences in the gene expression pattern of the PRR receptors involved in recognition of *S. aureus* and mixtures along with the differences in differential gene expression, the conditioned medium from all three mixtures of PRR ligands enhanced MSC osteogenic differentiation relative to the non-stimulated control. This was evident from the matrix mineralization at day 21

(**Figure 2**). This is likely the result of secretion of proinflammatory cytokines and chemokines in hPBMCs due to a synergistic activation of PRRs, leading to an upregulation of the signaling pathways NF-κB, MAPK and of interferons. This activation of these inflammatory pathways plays a role in bone formation and homeostasis. For example, the p38 MAPK signaling pathway is shown to regulate functions of osteoblasts and chondrocytes leading to its contribution in bone development phases both in *in vitro* studies as well as in genetically modified rat models [46,47]. Since all 3 mixtures show increased levels for some of the proinflammatory cytokines as well as the activation of the inflammatory signaling pathways as compared to γi *S. aureus*, it can be hypothesized that the mixtures possess immunomodulatory properties that can be harnessed for bone regeneration.

From analyzing the cytokine pattern (**Figure 5**), the composition of the conditioned medium of Mixture 1 most closely resembles the cytokine profile of γi *S. aureus*. With these findings, we showed that mixtures of PRR ligands can indeed at least partially recapitulate the immunomodulatory and osteoimmunological actions of γi *S. aureus* for bone regeneration. In addition to their safety advantages, the mixtures of PRR ligands offer a highly customizable approach. The combination of ligands and their concentrations could be further tailored to suit specific bone regeneration requirements, potentially offering the option to fine-tune the treatment for various bone-related conditions and injuries such as e.g., bone defect or non-unions. Due to their size and small molecular structure, they can be easily integrated with tissue engineering constructs, in prosthetic coatings and drug delivery platforms. For example, PRR ligands can be encapsulated in nanoparticles such as liposomes to facilitate controlled and sustained release [48,49]. In addition, their stability makes them ideal for use as bone-enhancing agents. Furthermore, they may offer a cheaper alternative to recombinant protein-based treatment options such as BMP-2. They can be easily immobilized on the surface of scaffolds, such as bioactive glass, titanium, or ceramics, using additive manufacturing techniques [50–53]. This allows for a smooth integration of PRR ligands into bone regenerative practices.

While these findings hold great promise, further research is essential to optimize mixture composition and to validate the safety and efficacy of mixtures of PRR ligands in clinically relevant animal models before considering human clinical trials. Continued investigation will help optimize the mixtures dosage and delivery for the most favorable bone regeneration outcomes, ensuring the successful translation of this novel approach into treatments for patients with bone fractures, bone defects, and degenerative bone diseases. Ultimately, mixtures of PRR ligands could represent a groundbreaking advancement in bone healing, ushering in a new era of safe and effective bone regeneration therapies.

Conclusion

In conclusion, mixtures of PRR ligands can partly mimic the immunomodulatory response of γi *S. aureus* and enhance osteogenic differentiation of hMSCs via stimulation of PBMCs. The PRR mixture (Mix 1) consisting of Pam3CSK4, CpG ODN C and Murabutide most closely mimicked the immunomodulatory γi *S. aureus* response in hPBMCs indicating that the complete response of *S. aureus* is still not fully understood and needs further investigation. Although all formulations of mixtures with PRR ligands possess potential to improve bone regeneration, considerable additional research is required for optimization and validation of these compounds to clinically advance bone healing.

Funding

This work is funded by PPS allowance from the Health~Holland LSH-TKI (grant number: LSHM18011) and the EU's H2020 research and innovation program under Marie S. Curie Cofund RESCUE (grant agreement No 801540).

Institutional Review Board Statement

Human material was obtained in accordance with the Declaration of Helsinki, with the approval of the local medical ethical committee (University Medical Center Utrecht, Utrecht, The Netherlands) under the protocols METC 08-001/K and METC 07-125/C.

References

1. Lehr, A.M.; Oner, F.C.; Delawi, D.; Stellato, R.K.; Hoebink, E.A.; Kempen, D.H.R.; van Susante, J.L.C.; Castelein, R.M.; Kruyt, M.C.; Dutch Clinical Spine Research Group Increasing Fusion Rate Between 1 and 2 Years After Instrumented Posterolateral Spinal Fusion and the Role of Bone Grafting. *Spine (Phila Pa 1976)* **2020**, *45*, 1403–1410, doi:10.1097/BRS.0000000000003558.
2. Lehr, A.M.; Oner, F.C.; Delawi, D.; Stellato, R.K.; Hoebink, E.A.; Kempen, D.H.R.; van Susante, J.L.C.; Castelein, R.M.; Kruyt, M.C. Efficacy of a Standalone Microporous Ceramic Versus Autograft in Instrumented Posterolateral Spinal Fusion. *Spine* **2020**, *45*, 944–951, doi:10.1097/BRS.0000000000003440.
3. Betz, R.R. Limitations of Autograft and Allograft: New Synthetic Solutions. *Orthopedics* **2002**, *25*, S561–S570, doi:10.3928/0147-7447-20020502-04.
4. Carragee, E.J.; Comer, G.C.; Smith, M.W. Local Bone Graft Harvesting and Volumes in Posterolateral Lumbar Fusion: A Technical Report. *Spine J* **2011**, *11*, 540–544, doi: 10.1016/j.spinee.2011.02.014.
5. Lee, S.-C.; Chen, J.-F.; Wu, C.-T.; Lee, S.-T. In Situ Local Autograft for Instrumented Lower Lumbar or Lumbosacral Posterolateral Fusion. *J Clin Neurosci* **2009**, *16*, 37–43, doi: 10.1016/j.jocn.2008.02.009.
6. Oliveira, É.R.; Nie, L.; Podstawczyk, D.; Allahbakhsh, A.; Ratnayake, J.; Brasil, D.L.; Shavandi, A. Advances in Growth Factor Delivery for Bone Tissue Engineering. *International Journal of Molecular Sciences* **2021**, *22*, 903, doi:10.3390/ijms22020903.
7. Ma, J.; Both, S.K.; Yang, F.; Cui, F.-Z.; Pan, J.; Meijer, G.J.; Jansen, J.A.; van den Beucken, J.J.J.P. Concise Review: Cell-Based Strategies in Bone Tissue Engineering and Regenerative Medicine. *STEM CELLS Translational Medicine* **2014**, *3*, 98–107, doi:10.5966/sctm.2013-0126.
8. Maruyama, M.; Rhee, C.; Utsunomiya, T.; Zhang, N.; Ueno, M.; Yao, Z.; Goodman, S.B. Modulation of the Inflammatory Response and Bone Healing. *Front. Endocrinol.* **2020**, *11*, doi:10.3389/fendo.2020.00386.
9. Mountziaris, P.M.; Spicer, P.P.; Kasper, F.K.; Mikos, A.G. Harnessing and Modulating Inflammation in Strategies for Bone Regeneration. *Tissue Engineering Part B: Reviews* **2011**, *17*, 393–402, doi: 10.1089/ten.teb.2011.0182.
10. Croes, M.; Kruyt, M.C.; Loozen, L.; Kragten, A.H.; Yuan, H.; Dhert, W.J.; Öner, F.C.; Alblas, J. Local Induction of Inflammation Affects Bone Formation. *Eur Cell Mater* **2017**, *33*, 211–226, doi:10.22203/eCM.v033a16.
11. Claes, L.; Recknagel, S.; Ignatius, A. Fracture Healing under Healthy and Inflammatory Conditions. *Nat Rev Rheumatol* **2012**, *8*, 133–143, doi:10.1038/nrrheum.2012.1.

12. Loi, F.; Córdova, L.A.; Pajarinen, J.; Lin, T.; Yao, Z.; Goodman, S.B. Inflammation, Fracture and Bone Repair. *Bone* **2016**, *86*, 119–130, doi: 10.1016/j.bone.2016.02.020.
13. Ramirez, D.M.; Ramirez, M.R.; Reginato, A.M.; Medici, D. Molecular and Cellular Mechanisms of Heterotopic Ossification. *Histol Histopathol* **2014**, *29*, 1281–1285.
14. Felix-Ilemhenbhio, F.; Pickering, G.A.E.; Kiss-Toth, E.; Wilkinson, J.M. Pathophysiology and Emerging Molecular Therapeutic Targets in Heterotopic Ossification. *International Journal of Molecular Sciences* **2022**, *23*, 6983, doi:10.3390/ijms23136983.
15. Urish, K.L.; Cassat, J.E. Staphylococcus Aureus Osteomyelitis: Bone, Bugs, and Surgery. *Infect Immun* **2020**, *88*, doi:10.1128/IAI.00932-19.
16. Pavey, G.J.; Qureshi, A.T.; Hope, D.N.; Pavlicek, R.L.; Potter, B.K.; Forsberg, J.A.; Davis, T.A. Bioburden Increases Heterotopic Ossification Formation in an Established Rat Model. *Clin Orthop Relat Res* **2015**, *473*, 2840–2847, doi:10.1007/s11999-015-4272-3.
17. Croes, M.; Kruyt, M.C.; Boot, W.; Pouran, B.; Braham, M.V.; Pakpahan, S.A.; Weinans, H.; Vogely, H.C.; Fluit, A.C.; Dhert, W.J.; et al. The Role of Bacterial Stimuli in Inflammation-Driven Bone Formation. *European cells & materials* **2019**, *37*, 402–419, doi:10.22203/eCM.v037a24.
18. Croes, M.; Wal, B.C.H.; Vogely, H.Ch. The Impact of Bacterial Infections on Osteogenesis: Evidence From *In vivo* Studies. *Journal of Orthopaedic Research* **2019**, jor.24422, doi:10.1002/jor.24422.
19. Askarian, F.; Wagner, T.; Johannessen, M.; Nizet, V. Staphylococcus Aureus Modulation of Innate Immune Responses through Toll-like (TLR), (NOD)-like (NLR) and C-Type Lectin (CLR) Receptors. *FEMS Microbiol Rev* **2018**, *42*, 656–671, doi:10.1093/femsre/fuy025.
20. Brandt, S.L.; Putnam, N.E.; Cassat, J.E.; Serezani, C.H. Innate Immunity to *Staphylococcus Aureus*: Evolving Paradigms in Soft Tissue and Invasive Infections. *J.I.* **2018**, *200*, 3871–3880, doi:10.4049/jimmunol.1701574.
21. Kim, J.; Yang, J.; Park, O.-J.; Kang, S.-S.; Kim, W.-S.; Kurokawa, K.; Yun, C.-H.; Kim, H.-H.; Lee, B.L.; Han, S.H. Lipoproteins Are an Important Bacterial Component Responsible for Bone Destruction through the Induction of Osteoclast Differentiation and Activation. *Journal of Bone and Mineral Research* **2013**, *28*, 2381–2391, doi:10.1002/jbmr.1973.
22. Gong, Z.; Zhang, J.; Zhang, S.; Cao, J.; Fu, Y.; Hu, X.; Zhao, J.; Gu, B.; Li, Q.; Zhang, K.; et al. TLR2, TLR4, and NLRP3 Mediated the Balance between Host Immune-Driven Resistance and Tolerance in Staphylococcus Aureus-Infected Mice. *Microbial Pathogenesis* **2022**, *169*, 105671, doi: 10.1016/j.micpath.2022.105671.
23. Deshmukh, H.S.; Hamburger, J.B.; Ahn, S.H.; McCafferty, D.G.; Yang, S.R.; Fowler, V.G. Critical Role of NOD2 in Regulating the Immune Response to

Staphylococcus Aureus. *Infection and Immunity* **2009**, *77*, 1376–1382, doi:10.1128/IAI.00940-08.

24. Chamaillard, M.; Hashimoto, M.; Horie, Y.; Masumoto, J.; Qiu, S.; Saab, L.; Ogura, Y.; Kawasaki, A.; Fukase, K.; Kusumoto, S.; et al. An Essential Role for NOD1 in Host Recognition of Bacterial Peptidoglycan Containing Diaminopimelic Acid. *Nat Immunol* **2003**, *4*, 702–707, doi:10.1038/ni945.

25. Hruz, P.; Zinkernagel, A.S.; Jenikova, G.; Botwin, G.J.; Hugot, J.-P.; Karin, M.; Nizet, V.; Eckmann, L. NOD2 Contributes to Cutaneous Defense against Staphylococcus Aureus through Alpha-Toxin-Dependent Innate Immune Activation. *Proc Natl Acad Sci U S A* **2009**, *106*, 12873–12878, doi:10.1073/pnas.0904958106.

26. Wolf, A.J.; Arruda, A.; Reyes, C.N.; Kaplan, A.T.; Shimada, T.; Shimada, K.; Arditi, M.; Liu, G.; Underhill, D.M. Phagosomal Degradation Increases TLR Access to Bacterial Ligands and Enhances Macrophage Sensitivity to Bacteria. *The Journal of Immunology* **2011**, *187*, 6002–6010, doi:10.4049/jimmunol.1100232.

27. Parker, D.; Prince, A. Staphylococcus Aureus Induces Type I IFN Signaling in Dendritic Cells Via TLR9. *The Journal of Immunology* **2012**, *189*, 4040–4046, doi:10.4049/jimmunol.1201055.

28. Brubaker, S.W.; Bonham, K.S.; Zanoni, I.; Kagan, J.C. Innate Immune Pattern Recognition: A Cell Biological Perspective. *Annu Rev Immunol* **2015**, *33*, 257–290, doi:10.1146/annurev-immunol-032414-112240.

29. Aleynick, M.; Svensson-Arvelund, J.; Flowers, C.R.; Marabelle, A.; Brody, J.D. Pathogen Molecular Pattern Receptor Agonists: Treating Cancer by Mimicking Infection. *Clin Cancer Res* **2019**, *25*, 6283–6294, doi: 10.1158/1078-0432.CCR-18-1800.

30. Li, D.; Wu, M. Pattern Recognition Receptors in Health and Diseases. *Sig Transduct Target Ther* **2021**, *6*, 1–24, doi:10.1038/s41392-021-00687-0.

31. Khokhani, P.; Belluomo, R.; Croes, M.; Gawlitta, D.; Kruyt, M.C.; Weinans, H. An In-Vitro Model to Test the Influence of Immune Cell Secretome on MSC Osteogenic Differentiation. *Tissue Eng Part C Methods* **2022**, doi: 10.1089/ten.TEC.2022.0086.

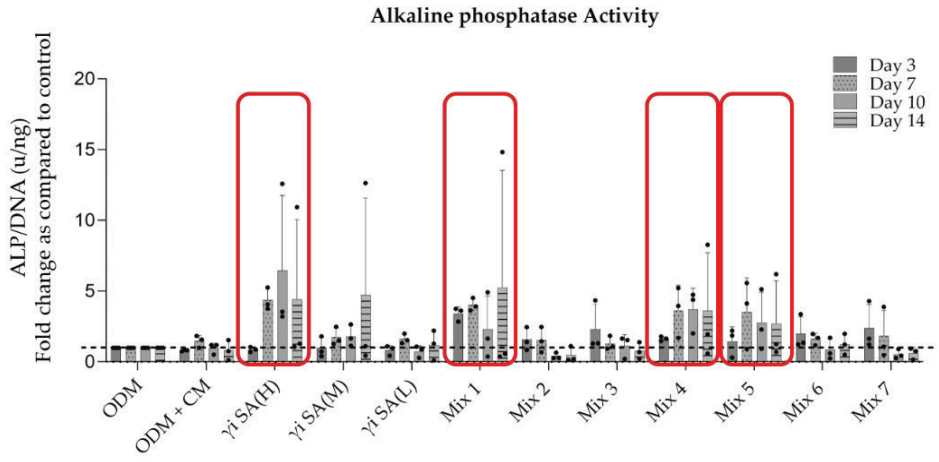
32. Khokhani, P.; Rahmani, N.R.; Kok, A.; Öner, F.C.; Alblas, J.; Weinans, H.; Kruyt, M.C.; Croes, M. Use of Therapeutic Pathogen Recognition Receptor Ligands for Osteo-Immunomodulation. *Materials* **2021**, *14*, 1119, doi:10.3390/ma14051119.

33. Pennings, I.; van Dijk, L.A.; van Huuksloot, J.; Fledderus, J.O.; Schepers, K.; Braat, A.K.; Hsiao, E.C.; Barruet, E.; Morales, B.M.; Verhaar, M.C.; et al. Effect of Donor Variation on Osteogenesis and Vasculogenesis in Hydrogel Cocultures. *Journal of Tissue Engineering and Regenerative Medicine* **2019**, *13*, 433–445, doi:10.1002/term.2807.

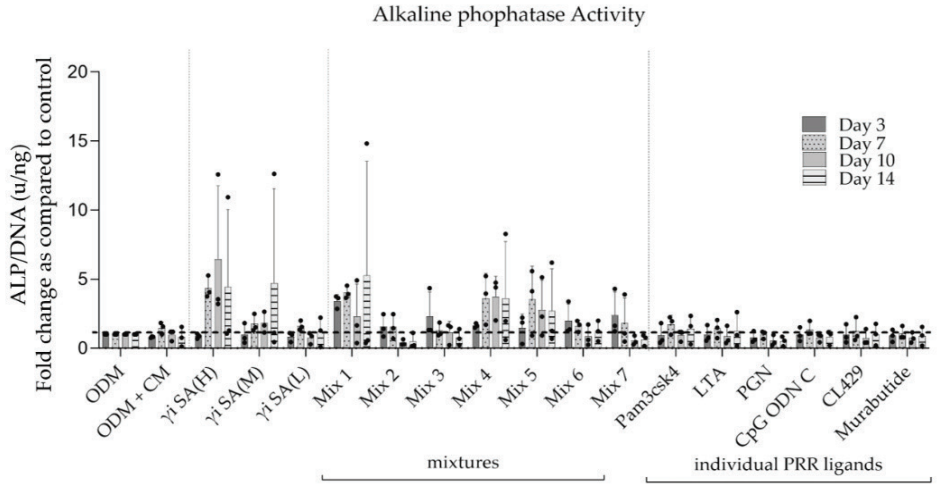
34. Love, M.I.; Huber, W.; Anders, S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biology* **2014**, *15*, 550, doi:10.1186/s13059-014-0550-8.
35. Zhu, A.; Ibrahim, J.G.; Love, M.I. Heavy-Tailed Prior Distributions for Sequence Count Data: Removing the Noise and Preserving Large Differences. *Bioinformatics* **2019**, *35*, 2084–2092, doi:10.1093/bioinformatics/bty895.
36. Riddle, R.C.; Khatri, R.; Schipani, E.; Clemens, T.L. Role of Hypoxia-Inducible Factor-1α in Angiogenic–Osteogenic Coupling. *J Mol Med* **2009**, *87*, 583–590, doi:10.1007/s00109-009-0477-9.
37. Croes, M.; Oner, F.C.; Kruyt, M.C.; Blokhuis, T.J.; Bastian, O.; Dhert, W.J.A.; Alblas, J. Proinflammatory Mediators Enhance the Osteogenesis of Human Mesenchymal Stem Cells after Lineage Commitment. *PLoS ONE* **2015**, *10*, 1–14, doi: 10.1371/journal.pone.0132781.
38. Souza, P.P.C.; Lerner, U.H. The Role of Cytokines in Inflammatory Bone Loss. *Immunological Investigations* **2013**, *42*, 555–622, doi:10.3109/08820139.2013.822766.
39. Bastidas-Coral, A.P.; Bakker, A.D.; Zandieh-Doulabi, B.; Kleverlaan, C.J.; Bravenboer, N.; Forouzanfar, T.; Klein-Nulend, J. Cytokines TNF-α, IL-6, IL-17F, and IL-4 Differentially Affect Osteogenic Differentiation of Human Adipose Stem Cells. *Stem Cells International* **2016**, *2016*, doi:10.1155/2016/1318256.
40. Pietrocola, G.; Arciola, C.R.; Rindi, S.; Di Poto, A.; Missineo, A.; Montanaro, L.; Speziale, P. Toll-Like Receptors (TLRs) in Innate Immune Defense Against Staphylococcus Aureus. *Int J Artif Organs* **2011**, *34*, 799–810, doi:10.5301/ijao.5000030.
41. Zähringer, U.; Lindner, B.; Inamura, S.; Heine, H.; Alexander, C. TLR2 – Promiscuous or Specific? A Critical Re-Evaluation of a Receptor Expressing Apparent Broad Specificity. *Immunobiology* **2008**, *213*, 205–224, doi: 10.1016/j.imbio.2008.02.005.
42. Kaur, A.; Kaushik, D.; Piplani, S.; Mehta, S.K.; Petrovsky, N.; Salunke, D.B. TLR2 Agonistic Small Molecules: Detailed Structure–Activity Relationship, Applications, and Future Prospects. *J. Med. Chem.* **2021**, *64*, 233–278, doi: 10.1021/acs.jmedchem.0c01627.
43. Buwitt-Beckmann, U.; Heine, H.; Wiesmüller, K.-H.; Jung, G.; Brock, R.; Akira, S.; Ulmer, A.J. Toll-like Receptor 6-Independent Signaling by Diacylated Lipopeptides. *Eur J Immunol* **2005**, *35*, 282–289, doi:10.1002/eji.200424955.
44. Hoebe, K.; Georgel, P.; Rutschmann, S.; Du, X.; Mudd, S.; Crozat, K.; Sovath, S.; Shamel, L.; Hartung, T.; Zähringer, U.; et al. CD36 Is a Sensor of Diacylglycerides. *Nature* **2005**, *433*, 523–527, doi:10.1038/nature03253.
45. Long, E.M.; Millen, B.; Kubes, P.; Robbins, S.M. Lipoteichoic Acid Induces Unique Inflammatory Responses When Compared to Other Toll-Like Receptor 2 Ligands. *PLOS ONE* **2009**, *4*, e5601, doi: 10.1371/journal.pone.0005601.

46. Greenblatt, M.B.; Shim, J.-H.; Zou, W.; Sitara, D.; Schweitzer, M.; Hu, D.; Lotinun, S.; Sano, Y.; Baron, R.; Park, J.M.; et al. The P38 MAPK Pathway Is Essential for Skeletogenesis and Bone Homeostasis in Mice. *J Clin Invest* **2010**, *120*, 2457–2473, doi:10.1172/JCI42285.
47. Thouverey, C.; Caverzasio, J. Focus on the P38 MAPK Signaling Pathway in Bone Development and Maintenance. *Bonekey Rep* **2015**, *4*, 711, doi:10.1038/bonekey.2015.80.
48. Jahanmard, F.; Khodaei, A.; Flapper, J.; Dogan, O.; Roohi, K.; Taheri, P.; Weinans, H.; Storm, G.; Croes, M.; Mastrobattista, E.; et al. Osteoimmunomodulatory GelMA/Liposome Coatings to Promote Bone Regeneration of Orthopedic Implants. *Journal of Controlled Release* **2023**, *358*, 667–680, doi: 10.1016/j.jconrel.2023.05.022.
49. Gale, E.C.; Roth, G.A.; Smith, A.A.A.; Alcántara-Hernández, M.; Idoyaga, J.; Appel, E.A. A Nanoparticle Platform for Improved Potency, Stability, and Adjuvanticity of Poly(I:C). *Advanced Therapeutics* **2020**, *3*, 1900174, doi:10.1002/adtp.201900174.
50. Cai, K.; Rechtenbach, A.; Hao, J.; Bossert, J.; Jandt, K.D. Polysaccharide-Protein Surface Modification of Titanium via a Layer-by-Layer Technique: Characterization and Cell Behaviour Aspects. *Biomaterials* **2005**, *26*, 5960–5971, doi: 10.1016/j.biomaterials.2005.03.020.
51. Chua, P.-H.; Neoh, K.-G.; Kang, E.-T.; Wang, W. Surface Functionalization of Titanium with Hyaluronic Acid/Chitosan Polyelectrolyte Multilayers and RGD for Promoting Osteoblast Functions and Inhibiting Bacterial Adhesion. *Biomaterials* **2008**, *29*, 1412–1421, doi: 10.1016/j.biomaterials.2007.12.019.
52. Guillot, R.; Gilde, F.; Becquart, P.; Sailhan, F.; Lapeyrere, A.; Logeart-Avramoglou, D.; Picart, C. The Stability of BMP Loaded Polyelectrolyte Multilayer Coatings on Titanium. *Biomaterials* **2013**, *34*, 5737–5746, doi: 10.1016/j.biomaterials.2013.03.067.
53. Amin Yavari, S.; Croes, M.; Akhavan, B.; Jahanmard, F.; Eigenhuis, C.C.; Dadbakhsh, S.; Vogely, H.C.; Bilek, M.M.; Fluit, A.C.; Boel, C.H.E.; et al. Layer by Layer Coating for Bio-Functionalization of Additively Manufactured Meta-Biomaterials. *Additive Manufacturing* **2020**, *32*, 100991, doi: 10.1016/j.addma.2019.100991.

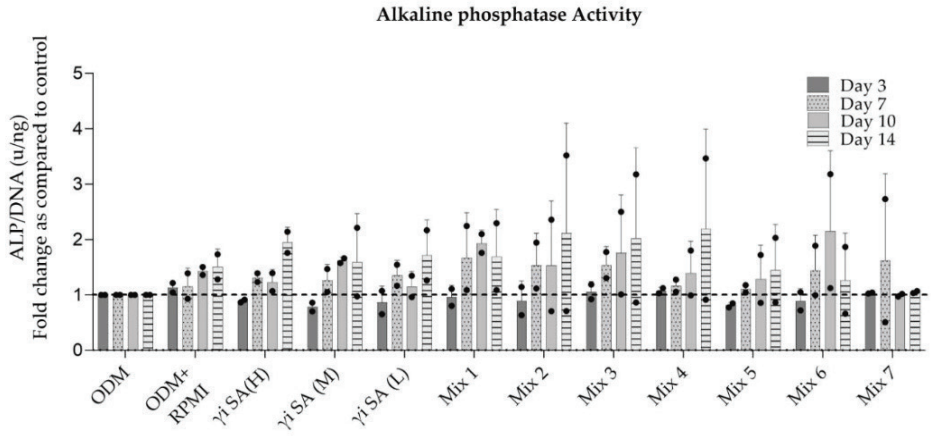
Supplementary Material



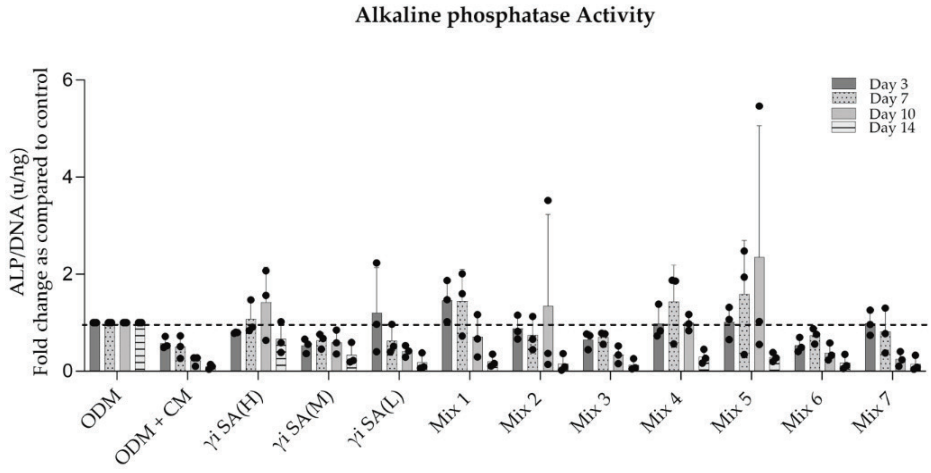
Supplementary Figure S1. Effect of indirect stimulation of γi *S. aureus* and mixtures on alkaline phosphatase levels in hMSC cultures, corrected for DNA on days 3,7,10, and 14. ALP/DNA ratios were normalized to the ratio of ODM cultured controls (dashed line). hMSC were cultured with conditioned medium obtained from hPBMCs (n = 6) stimulated with γi *S. aureus* in different concentrations and mixtures in the presence of osteogenic factor dexamethasone. The CM was added in the ratio of 1: 4 (CM: ODM). The graph represents the mean ± SD of technical triplicates performed per donor for three individual MSC donors. The groups γi *S. aureus* (H), Mix 1, Mix 4, and Mix 5 in (highlighted in the red boxes) were selected for further experiments.



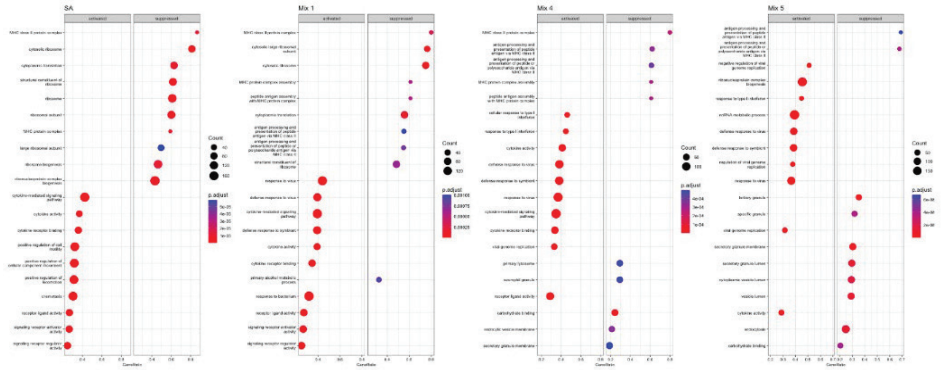
Supplementary Figure S2. Effect of indirect stimulation of γi *S. aureus* and mixtures on alkaline phosphatase levels in hMSC cultures, corrected for DNA on Days 3,7,10, and 14. hMSC were cultured with conditioned medium obtained from hPBMCs (n = 6) stimulated with γi *S. aureus* in 3 concentrations (H=high, M=middle, L=low), mixtures (1-7), and the individual PRR ligands in the presence of osteogenic factor dexamethasone. All data were normalized to the osteogenic medium control wells. The CM was added in the ratio of 1: 4 (CM: ODM). The graph represents the mean ± SD of technical triplicates performed per donor for three individual MSC donors (represented by dots).



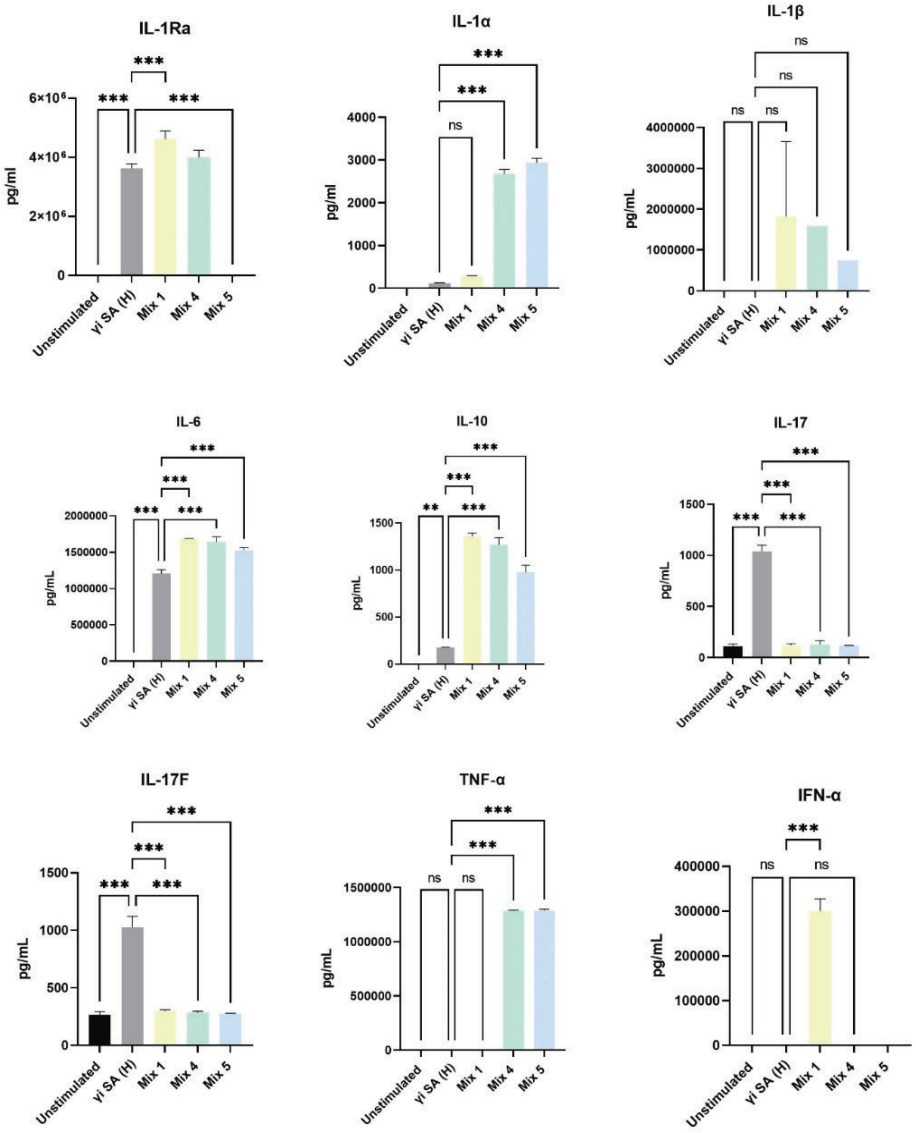
Supplementary Figure S3. Effect of direct stimulation of γi *S. aureus* and mixtures on alkaline phosphatase levels in hMSC cultures, corrected for DNA on Days 3,7,10, and 14. hMSC were cultured with conditioned medium obtained from hPBMCs (n = 6) stimulated with γi *S. aureus* in different concentrations and mixtures in the presence of osteogenic factor dexamethasone. All data were normalized to the osteogenic medium (ODM) control wells. The graph represents the mean ± SD of technical triplicates performed per donor for two individual MSC donors.

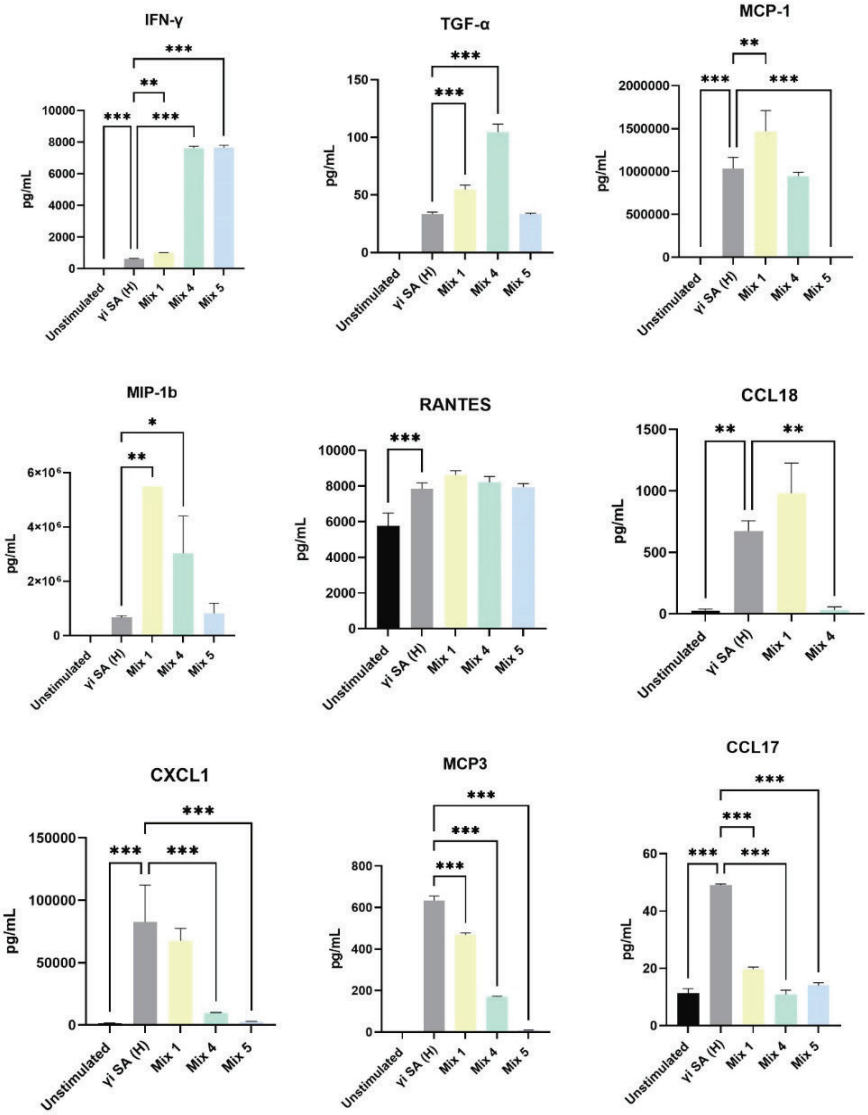


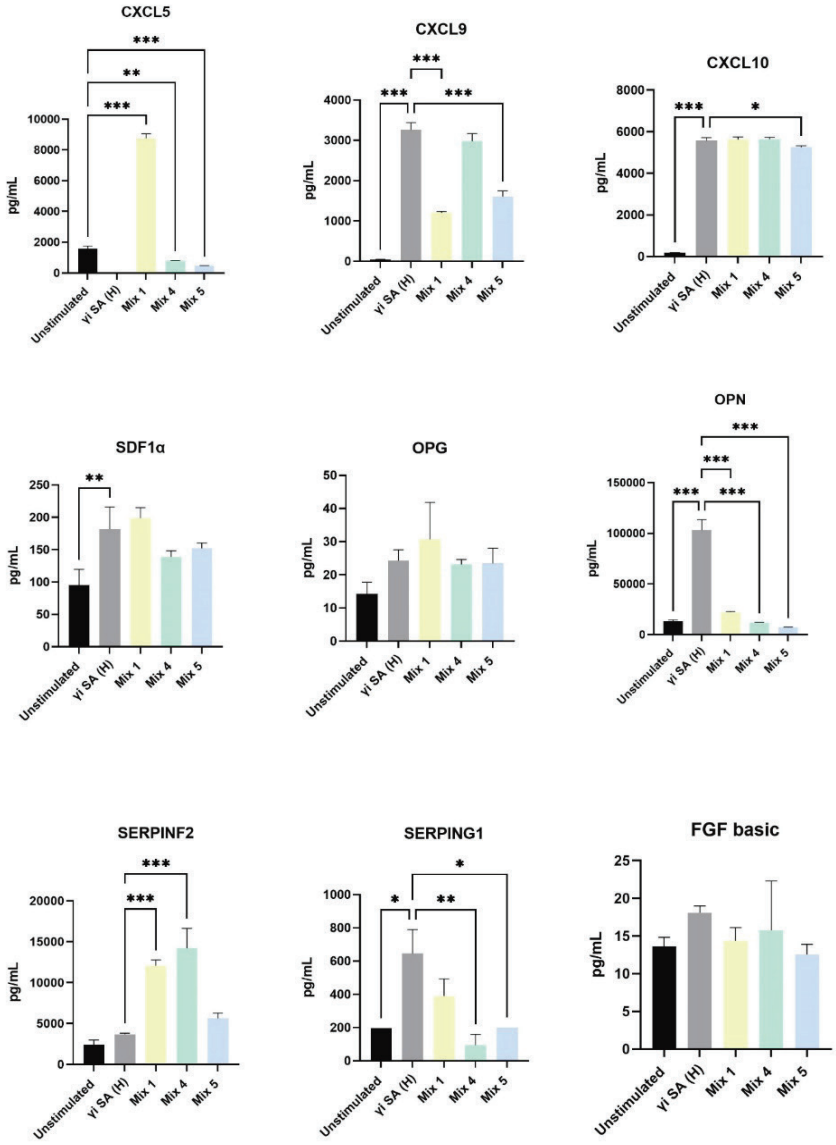
Supplementary Figure S4. Effect of indirect stimulation of γi *S. aureus* and mixtures on alkaline phosphatase levels in hMSC cultures, corrected for DNA on days 3, 7, 10, and 14. hMSC were cultured with conditioned medium obtained from hPBMCs (n = 6) stimulated with γi *S. aureus* in different concentrations and mixtures without osteogenic factor dexamethasone. All data were normalized to the osteogenic medium (ODM) control wells. The CM was added in the ratio of 1: 4 (CM: ODM). The graph represents the mean ± SD of technical triplicates performed per donor for three individual MSC donors.

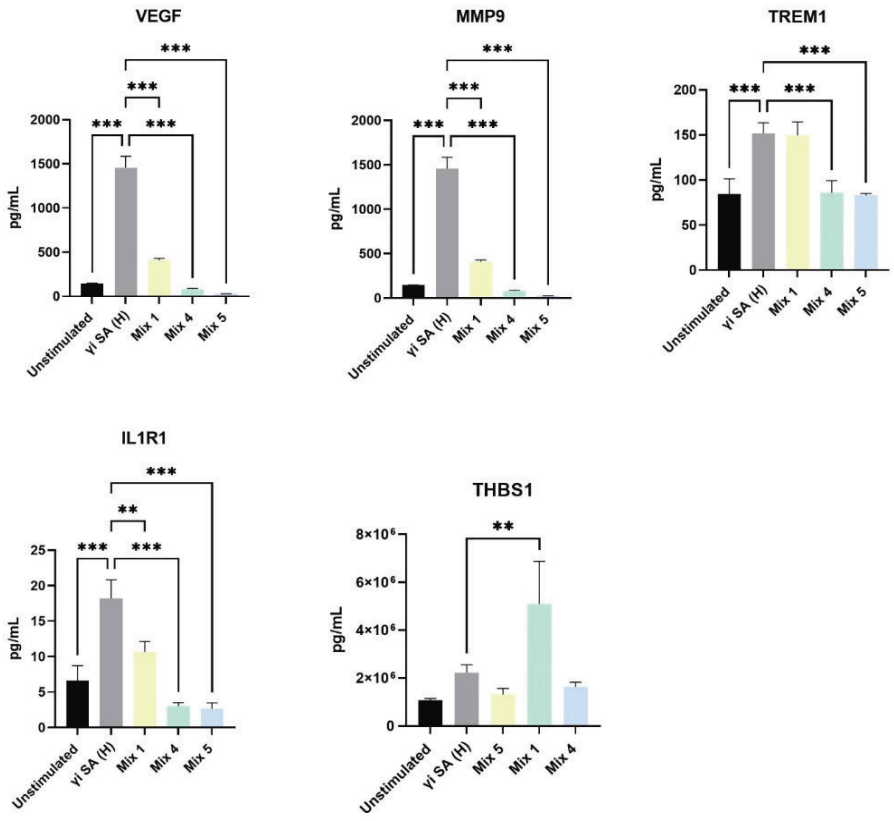


Supplementary Figure S5. The bubble plot displaying the top 10 GO terms upregulated in gene set enrichment analysis compared pre-defined GO resource for γi *S. aureus* and mixtures (Mix1, Mix 4, and Mix5) stimulated hPBMCs as compared to the unstimulated control.

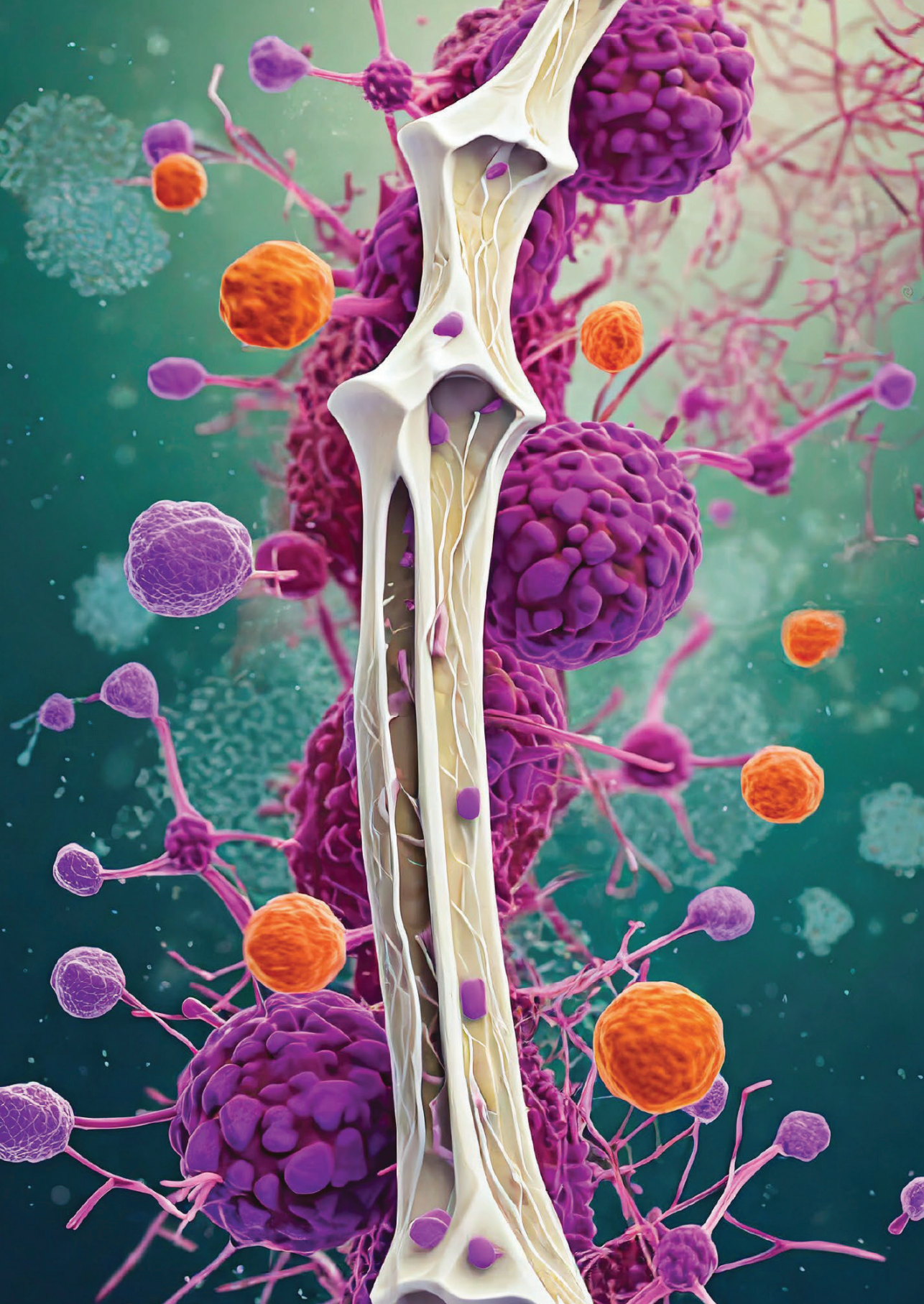

IL-1Ra
pg/ml
6×10⁶
4×10⁶
2×10⁶
0
***
***
***
IL-1α
pg/ml
4000
3000
2000
1000
0
***
***
ns
IL-1β
pg/mL
4000000
3000000
2000000
1000000
0
ns
ns
ns
ns
IL-6
pg/mL
2000000
1500000
1000000
500000
0
***
***
***
***
IL-10
pg/mL
1500
1000
500
0
**
***
***
***
IL-17
pg/mL
1500
1000
500
0
***
***
***
***
IL-17F
pg/mL
1500
1000
500
0
***
***
***
***
TNF-α
pg/mL
1500000
1000000
500000
0
***
***
ns
ns
IFN-α
pg/mL
400000
300000
200000
100000
0
***
ns
ns
Unstimulated
yi SA (H)
Mix 1
Mix 4
Mix 5


IFN-γ
pg/mL
10000
8000
6000
4000
2000
0
***
**
***
***
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5
TGF-α
pg/mL
150
100
50
0
***
***
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5
MCP-1
pg/mL
2000000
1500000
1000000
500000
0
**
***
***
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5
MIP-1b
pg/mL
6×10⁶
4×10⁶
2×10⁶
0
*
**
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5
RANTES
pg/mL
10000
8000
6000
4000
2000
0
***
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5
CCL18
pg/mL
1500
1000
500
0
**
**
Unstimulated
Yi SA (H)
Mix 1
Mix 4
CXCL1
pg/mL
150000
100000
50000
0
***
***
***
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5
MCP3
pg/mL
800
600
400
200
0
***
***
***
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5
CCL17
pg/mL
60
40
20
0
***
***
***
***
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5


CXCL5
CXCL9
CXCL10
SDF1α
OPG
OPN
SERPINF2
SERPING1
FGF basic
pg/mL
Unstimulated
Yi SA (H)
Mix 1
Mix 4
Mix 5



Supplementary Figure S6. Concentrations in the CM for selected proteins from figure 5. (a) hPBMCs (n=9) were stimulated with either γi *S. aureus* or mixtures for 24 hours. The conditioned medium obtained from the stimulation was pooled and used to characterize its composition using Luminex assay. All the groups were normalized to the unstimulated hPBMCs as the control. Data are presented in mean + standard deviation. Significance was tested using one-way ANOVA with post hoc Sidak's test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Chapter 5

In vivo screening of immune stimulants in an ovariectomy induced osteoporotic femoral fracture in rats

Paree Khokhani[1], Carissa Hing-Wai Hong[2], Michiel Croes[1], Simon Chow[2], Wing Hoi Cheung[2], Saber Amin Yavari[1], Debby Gawlitta[3], Moyo C. Kruyt[1,4], Harrie Weinans[1]

1. Department of Orthopedics, University Medical Center Utrecht, Utrecht, the Netherlands
2. Musculoskeletal Research Laboratory, Department of Orthopaedics and Traumatology, The Chinese University of Hong Kong, Hong Kong SAR, PR China
3. Department of Oral and Maxillofacial Surgery, Prosthodontics & Special Dental Care, University Medical Center Utrecht, Utrecht, the Netherlands
4. Department of Developmental BioEngineering, University of Twente, the Netherlands

**Manuscript Submitted*

Abstract

A competent inflammatory response that follows a precise pattern in time is required for optimal fracture healing. Gamma irradiated (γi) *C. albicans* or pathogen recognition receptor (PRR) ligands that are synthetically available such as CpG ODN C (bacterial DNA) or Poly(I:C) (bacterial dsRNA), target toll-like receptors expressed by immune cells. Local immune modulation by these factors could alter the timing of inflammation in a fractured bone and thereby provide a strategy to improve fracture healing. To test this hypothesis, a closed femoral fracture was performed in osteoporotic rats, which display delayed healing, and immune stimulants γi *C. albicans*, CpG ODN C, and Poly(I:C) were locally administered at the site of the fracture. The pro-inflammatory cytokine rhTNF-α served as the positive control, while a group with saline injections was used as the negative control. Cytokine expression was measured in the fracture hematoma (day 3) as an indication of the early inflammatory response. Weekly radiographs and micro-CT analysis at the endpoint (week 4) were performed to assess fracture callus morphometry as indicators of bone repair. No changes were observed in cytokine expression on day 3 in all groups compared to the saline control. No significant changes were observed in callus morphometry after week 4 assessed by both radiographic scores and micro-CT analysis. These findings question the current strategy of using local immune stimulation as a medication to improve bone fracture healing and indicate that further studies are needed to identify the role of immune modulation in enhancing fracture healing.

Keywords

Regenerative medicine, Osteoimmunology, Osteogenesis, Pathogen-recognition receptors, Cytokine, Inflammation, Pathogen-associated molecular patterns, fracture healing

Introduction

Fractures are one of the most common injuries in the musculoskeletal system. Generally, immobilization with a cast or surgical intervention is sufficient for healing, but about 30% of cases result in delayed healing [1]. Non-unions may occur in about 10% of cases with polytrauma, severe soft tissue damage, and/or comorbidities [2],[3]. Although biological substances like parathyroid hormone (PTH) [4–6] and bone morphogenetic protein-2 (BMP-2) [6],[7] have been used to stimulate fracture healing, they may cause side effects and may not outperform autologous bone grafts [9–11]. Physical stimulation therapies such as shock waves have also been used [11],[12] but are not universally accepted. Currently, there are no definitive therapeutic solutions for fast and effective bone healing.

Postmenopausal osteoporotic women more often suffer from delayed fracture healing than subjects with healthy bone stock, which may be attributed to low estrogen levels in osteoporosis patients [14]. This patient population often exhibits increased levels of circulating pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), along with a higher number of activated T-cells and B-cells [14],[15]. Impaired callus formation and angiogenesis have been observed in estrogen-deficient ovariectomized (OVX) rats, indicating that estrogen deficiency affects not only bone density but also the initial fracture healing process [16],[17]. Some studies have indeed reported increased local levels of pro-inflammatory cytokines, such as IL-6 and Midkine (a protein involved in regulating inflammation and wound healing), along with an increase in neutrophils on day 3 in the hematomas after a fracture in OVX animals compared to healthy rats [18],[19]. However, other studies have reported a downregulation of pro-inflammatory cytokines TNF-α and IL-6 in fracture hematomas of OVX rats compared to normal animals [21,22], indicating that estrogen deficiency alters the inflammatory response associated with fracture healing. Although the precise effects of estrogen deficiency on the local inflammatory response are not yet fully understood, it appears to disturb the optimal timing and tuning of the inflammation that is necessary for proper fracture healing.

Modulation of the immune response has garnered a lot of interest as a strategy to enhance fracture healing. This is because of increasing awareness of the interplay between immune cells and bone-forming cells [23]. Although excessive inflammation is deleterious for fracture healing, an absence of TNF-α and IL-6 signaling led to impaired healing [24],[25]. Also, injection of low dose rhTNF-α at the fracture site in a murine tibial fracture model led to improved callus formation and earlier remodeling [26],[27] indicating that a critical balance of the immune system was required. Recently, we showed that by using gamma-irradiated bacterial antigens, bone formation in various rabbit models could be enhanced

[28],[29]. This enhancement is postulated to be caused by the recognition of bacterial cell wall components by the immune system that result in the secretion of various cytokines, chemokines, and growth factors beneficial for bone healing. Furthermore, we demonstrated that synthetic bacterial cell wall components, known as pathogen recognition receptor (PRR) ligands, namely CpG ODN C and Poly(I:C), enhanced alkaline phosphatase activity in human bone marrow-derived mesenchymal stromal cells (hMSC) upon direct stimulation *in vitro* [30]. Immune cells recognize CpG ODN C, bacteria's DNA containing CpG motifs, and Poly(I:C), a synthetic analogue of double-stranded RNA, via Toll-like receptor 9 (TLR9) and Toll-like receptor 3 (TLR3), respectively. This recognition leads to the release of various inflammatory cytokines and interferons for host defense. In agreement, we showed that human peripheral blood mononuclear cells (PBMCs) secreted chemokines like MCP-1 and IL-6 in the presence of CpG ODN C that led to enhanced osteogenic differentiation in hMSCs [31]. These results indicate that the above classes of immune stimulants can modulate the immune system and thereby favor bone regeneration.

In this study, we further investigate the effect of immune stimulants as osteo-immunomodulators *in vivo*. We applied gamma-irradiated (γi) *C. albicans*, CpG ODN C and Poly(I:C) locally at the fracture site in a closed femur fracture model in ovariectomized rats. We hypothesized that immune stimulants modulate the early inflammatory phase in OVX rats, thereby leading to enhanced fracture healing. To test this hypothesis, we analyzed the cytokines present in the fractured hematomas obtained on day 3 and performed radiographic analysis and micro-CT assessments on the fractured bone.

Materials and Methods

Study design

Female Sprague Dawley (SD) rats (n = 60) aged 6 months received bilateral ovariectomy to induce osteoporosis. After 3 months (Day 0), all rats underwent a closed femoral fracture creation using a 3-point bending device followed by the insertion of an intramedullary fixation using a 1.2 mm k-wire [32]. After the fracture, rats were randomized into 3 immune stimulants (**Table 1**), or rhTNF- α (positive control), or saline solution (negative control) groups. Specified treatments were injected into the fracture site on the 2 following days (Day 1 and Day 2). Fracture hematomas were harvested from 5 rats per group on Day 3 for characterization of the inflammatory status. For bone healing, 7 rats per group were monitored by weekly radiography until 28 days when they were euthanized, and the femora were explanted for micro-CT (**Figure 1**).

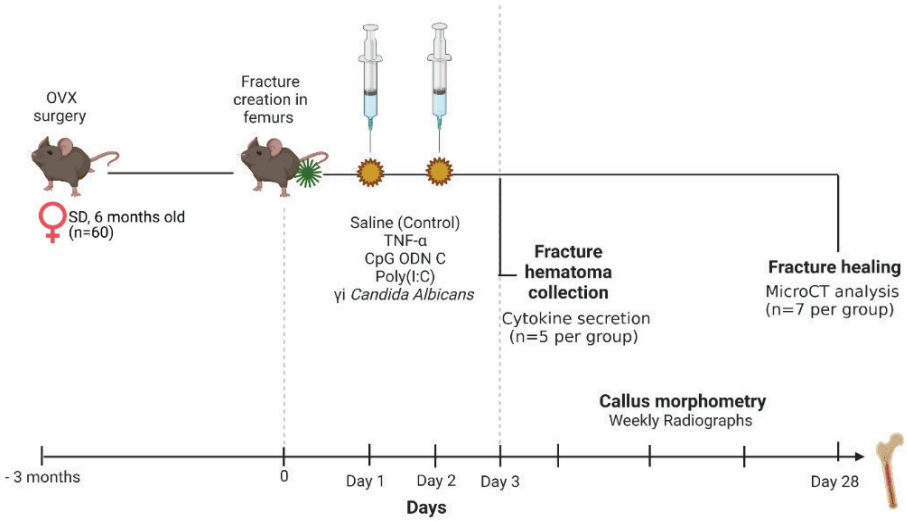


Figure 1. Experiment design. Six months old female SD rats (n = 60) underwent ovariectomy. After 3 months, these rats were considered osteoporotic and underwent unilateral femur fracture creation (Day 0). On days 1 and 2, local injections of different immune stimulants, rhTNF-α, or saline were given. On day 3 animals were euthanized and fracture hematomas were harvested to determine cytokine levels. Fracture healing was monitored with weekly radiographs, and on day 28, animals were euthanized, and the femurs were explanted for micro-CT analysis.

Animal model and interventions

Female Sprague Dawley rats aged 6 months were obtained from the laboratory animal services center of the Chinese University of Hong Kong and the experimental protocols were approved by the University animal experimentation ethics committee (Ref no: (19-989) in DH/HT& A/8/2/1 Pt.2) [32]. All rats were housed under a 12 h light-dark cycle. Free cage movement was allowed with ad libitum access to standard rat chow and tap water.

Rats received bilateral OVX operations at 6 months of age under intraperitoneal sedation with 50 mg/kg ketamine and 10 g/kg xylazine (Alfasan International B.V., the Netherlands). Further, OVX surgery was performed by bilateral incisions at the dorsal lower abdomen. Subsequently, osteoporosis developed during the following 3 months, as previously described [12]. For the creation of a closed fracture on the right femoral midshaft, rats were similarly anaesthetized and treated according to an established protocol [33,34]. A 15 mm long sterilized titanium k-wire with a diameter of 1.2 mm was inserted before fracture creation

at the diaphysis, using a customized 3-point bending apparatus [33]. At day 1 and 2, 100 µl of immune stimulants or control were injected into the fracture that was located by palpation (**Table 1**). At Day 3 (n = 5 per group) and Day 28 (n= 7 per group), animals were euthanized using an overdose of intraperitoneal injection of 200 mg/kg sodium pentobarbital (Alfasan International B.V.). At day 3, the fracture hematomas of 5 rats per condition were collected to determine the initial immune response. For the fracture healing assessment (day 28), only simple transverse fractures (fracture created perpendicular to femur's long axis) were included to minimize variability. This resulted in a reduction of the number of animals that could be studied per group, especially in the saline control group where only 2/7 animals were included.

Immune stimulants	Dose	No. of animals		
		Inflammatory cytokine analysis (Day 3)	Micro-CT analysis (Day 28)	
			Initial number	Included animals
Saline	-	5	7	2
Rh-TNF-α	50 ng	5	7	5
CpG ODN C	50 µg	5	7	4
Poly(I:C)	50 µg	5	7	6
γi C.albicans	10^7 units	5	7	6

Table 1. Overview of the immune stimulants that were injected into the fracture site with their doses and the numbers of animals included in the analyses.

Immune stimulants

Recombinant human (rh)TNF- α (source: *E. coli*) was purchased from Peprotech, USA. C-class CpG oligodeoxynucleotide (CpG ODN C) (M362) and high molecular weight Polyinosinic: polycytidylic acid (Poly(I:C)) were purchased from InvivoGen, USA. To obtain gamma-irradiated (γi) *C. albicans*, *C. albicans* was cultured into a mid-log phase in an LB liquid medium. Freshly cultured yeast was then subjected to 25 G kγ radiation. The absence of any viable microorganism was confirmed by plate cultures. The γi suspensions were stored at -80 ºC and washed with PBS before use in *in vitro* assays to confirm that there was no microorganism growth. The dosage for the study was determined by their ability to induce rat CXCL-1 and TNF- α production in whole rat blood in an ex vivo assay (**Supplementary Figure S1**).

Effect of immune stimulants on initial immune response post-fracture

The fracture hematoma was harvested and lysed in lysis buffer containing protease inhibitors (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, 0.02% NaN_3, 0.2 mM phenylmethylsulfonyl fluoride; and halt protease and phosphate inhibitor single-use cocktail (Thermo Fisher Scientific, USA)). Total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). The concentrations of cytokines TNF-α, IL-6, and IL-10 were assessed using an enzyme-linked immunosorbent assay (ELISA) (Duoset, R&D systems, USA), according to the manufacturer's protocol.

Effect of immune stimulants on fracture healing

Radiographic analysis

Weekly radiographs of the rat femora were taken with a cabinet X-ray system (UltraFocus DXA, USA). Radiographic healing is defined as complete mineralized callus bridging on both anteroposterior and lateral radiographs. Callus area and callus width are measured on digitized lateral view images using the built-in straight line and polygon selection tools in the image analysis software ImageJ (NIH, USA). Callus width is defined as the maximal outer diameter of the mineralized callus (d_2) minus the outer diameter of the femur (d_1); Callus area is calculated as the sum of the areas of the external mineralized callus in the lateral view according to previously established protocols [33,34].

Micro-computed tomography (micro-CT) analysis

The harvested fractured femora (Day 28) were scanned with a micro-CT system (VivaCT 40, Scanco Medical, Switzerland) at a 3D resolution of 19 microns, according to the established protocol [34]. The scan range covered a total of 8.02 mm (422 slides) proximal and distal to the fracture line, which is defined as the region of interest. The newly formed bone (low-density bone, threshold 165-300) and highly mineralized bone (high-density bone, threshold 350-1000) were reconstructed separately. The total tissue volume (TV) was defined as the volume of the entire region of interest including the spaces. The ratios of low-density (not fully mineralized) bone volume over total tissue volume (BV_l/TV), high-density (mineralized) bone volume over total tissue volume (BV_h/TV), total bone volume fraction (BV_t/TV) was defined as the ratio of the sum of low and high mineralized bone volume including the cortical bone over the total tissue volume, bone mineral density (BMD) defined as the density of mineral content including the

surrounding soft tissues in the total tissue volume and tissue mineral density (TMD) defined as the mineral density of the bone volume were calculated.

Statistical analysis

All data were tested for normal distribution using Shapiro-Wilk normality test. Repeated measures analysis of variance (ANOVA) using mixed models with Sidak's post hoc correction or one-way ANOVA with post hoc Bonferroni correction were used to compare the groups (SPSS V26, IBM, USA and GraphPad Prism, USA). Statistical significance was set at $p < 0.05$. Quantitative data are presented as mean ± standard deviation with the sample size indicated in the figure legends.

Results

Effect of immune stimulants on early immune response in fracture healing

We measured the protein concentration (**Supplementary Figure S2**) and cytokines TNF-α, IL-6, and IL-10 in the fracture hematoma on day 3 (**Figure 2**). No differences in cytokine expression were identified between the groups for all measured cytokines. Noteworthy, for two animals, an increased TNF-α and IL-10 concentration was observed (at least two-fold higher than all other values as shown in **Figure 2**) for γi *C. albicans* stimulated hematomas.

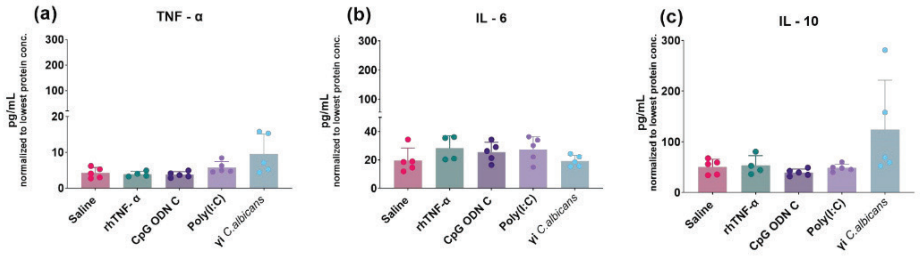


Figure 2. Cytokine concentrations in fracture hematomas (n = 5 per group) collected on day 3 post-fracture. The cytokine concentrations were normalized to the lowest protein concentration of the entire data set. Data are represented as mean ± standard deviation. Significance was tested using one way ANOVA.

Effect of the local injection of immune stimulants on fracture healing

Radiographic analysis

Weekly radiographs were taken to measure the callus bridging and temporal changes over 4 weeks. As indicated in **Table 1**, many of the fractures were not eligible for further analysis, as they did not meet the criteria of being a simple transverse type. A calcified bridge was found for 4/6 rats treated with Poly(I:C), 3/6 treated with γi *C. albicans*, 1/2 treated with saline, 2/5 rats treated with rhTNF-α, and 1/4 treated CpG ODN C (**Figure 3(a)**). Quantitatively, both callus width and callus area did not differ between groups receiving immune stimulants versus control treatment (**Supplementary table ST1**). Noteworthy, a trend towards faster remodeling for the Poly(I:C) treated groups could be discerned by week 4 for callus width (p value = 0.2295) seemed to increase faster in the stimulated conditions.

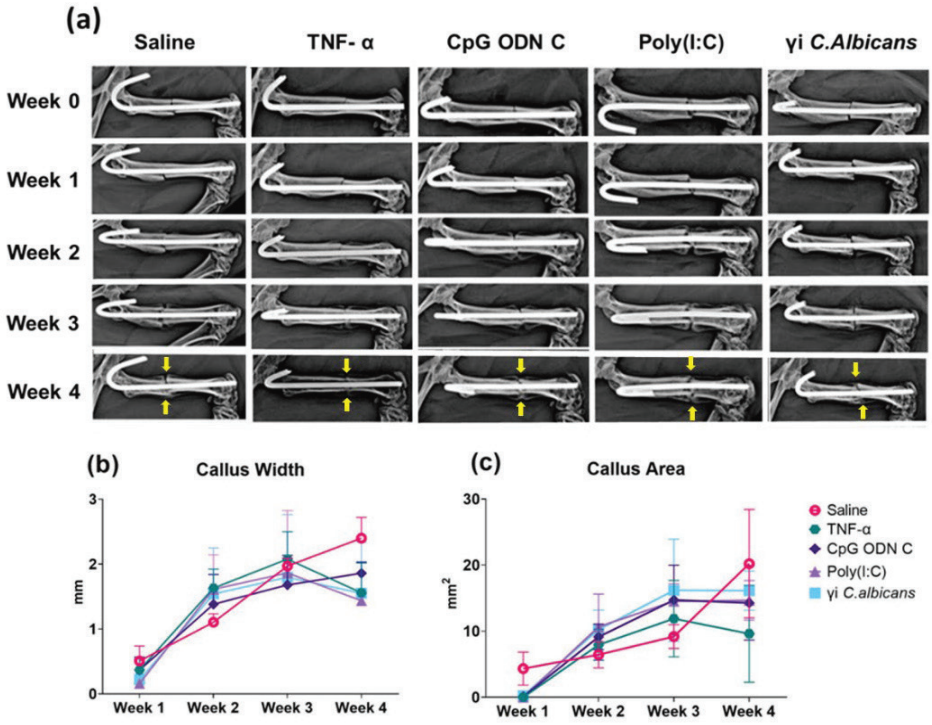


Figure 3. Weekly radiographic findings of the fractures. (a) A series of representative lateral radiographs taken at time points 0, 1-, 2-, 3-, and 4-weeks post-fracture. The arrows (yellow) indicate calcified bridges. The callus width (b) and (c) callus area over time is shown. Data are shown as the mean ± standard deviation. Significance was tested using repeated measures mixed model ANOVA with Sidak's post hoc correction.

Micro CT analysis

At day 28, no differences were observed in the total tissue volume (**Figure 4(a)**), low density bone fraction (**Figure 4(b)**), high-density bone fraction (**Figure 4(c)**), and bone and tissue mineral density (**Figure 4(d)** and **Figure 4(e)**) in the treatment groups as compared to the saline control. Variation within the groups was observed in all measured parameters.

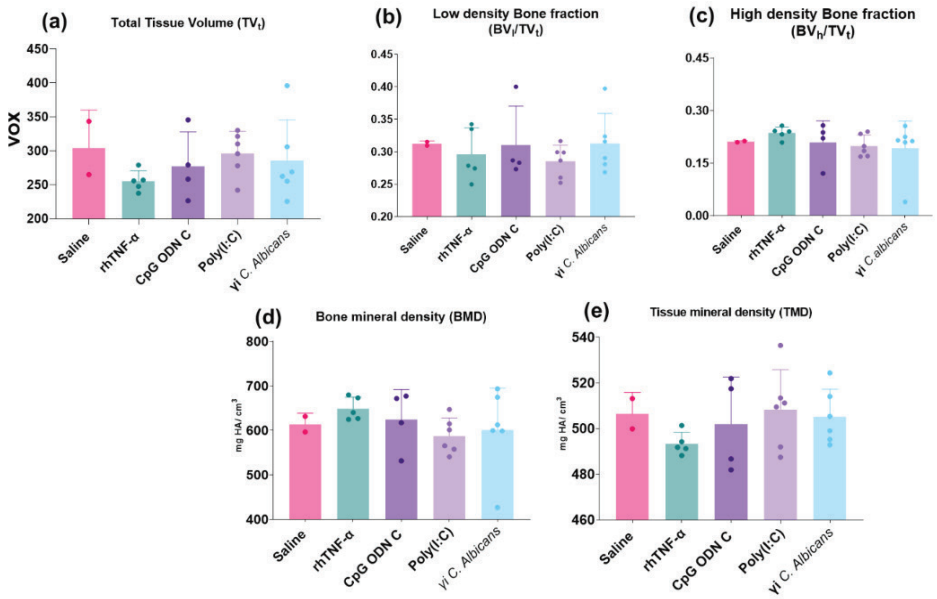


Figure 4. Micro-CT evaluation of the femora harvested after 4 weeks. (a) Total tissue volume (TV_t) (b) mineralized callus fraction (BV_l/TV), (c) high-density bone fraction (BV_h/TVt), (d) bone mineral density (BMD) and (e) tissue mineral density (TMD) was measured using 3D reconstruction. Data are shown as the mean ± standard deviation. Statistics was done using a one-way ANOVA.

Discussion

We investigated the effect of various immune stimulants including a whole microbe gamma-irradiated γi *C. albicans* and synthetic pathogen recognition receptor ligands CpG ODN C and Poly(I:C) on the early immune response and fracture healing using a closed femur fracture model of ovariectomized rats. The doses were determined based on a whole blood assay where an increase in cytokine production was elicited (**Supplementary Figure S1**). In contrast to the literature [19,20,22,35], we did not observe an obvious elevated inflammatory response in terms of increase of cytokines TNF-α, IL-6, and IL-10 in the fracture hematomas collected at day 3. Only γi *C. albicans* did show increased levels for two individual rats. This absence of an elevated inflammatory response may be the reason why we did not observe a noticeable effect on fracture healing. However, the present experimental design may have been limited in establishing such an effect.

We showed previously that bacterial agents enhance osteogenesis *in vivo* in several rabbit models and hypothesized that it was sterile inflammation that resulted in this phenomenon [29]. CpG ODN C and Poly(I:C) were shown to enhance osteogenic differentiation in human MSCs without altering osteoclast differentiation [27],[28].The delivery and timing of immune stimulants were likely suboptimal. Dosage was determined by cytokine production in a whole blood assay and literature on immune stimulants used in other applications [36–40]. Since a well-balanced inflammatory response facilitates efficient fracture repair, we injected the immune stimulants on day 1 and day 2 at relatively low doses to obtain mild immune activation. It has been observed by Glass and colleagues that administering immune stimulants within the first 24 hours, while the immune system is already primed, may result in a more balanced immune response[26,27]. On the other hand, if immune stimulants are administered after 24 hours, the immune response may shift towards a resolution phase, which is characterized by a decrease in inflammation and tissue remodeling. Furthermore, since immune stimulants exhibit a half-life ranging from minutes to hours *in vivo* [41], a controlled-sustained release with nanoparticles, liposomes, or other delivery methods may have been better [42–44]. Furthermore, the small number of samples, especially in the saline control group, which could be used for analysis limited the study outcomes.

We used rhTNF-α as a positive control group. Glass et al. also showed that injecting a low dose of rhTNF-α locally in the fracture site, immediately or after day 1 post-fracture, resulted in increased callus mineralization in OVX mice compared to healthy mice after 14 days, suggesting that fracture healing can be enhanced by modulating the early inflammatory stage [26]. Our study utilized a

higher dose of rhTNF-α compared to Glass et al to overcome the species related differences. It may be speculated that the used dosage of rhTNF- α in the positive control group of our study was too low since it did not show any changes from the control condition. It was likely not too high, as high doses of rhTNF-α may lead to impaired bone formation and increased osteoclast activity [45–47], which was not found here. Glass et al. and Chan et al. have shown that rhTNF- α has a positive effect on fracture healing in OVX mice [26,27]. However, it is important to note that their observations were made in a murine model of osteotomy, which differs from the closed femur fracture model. The closed femur fracture model is less impactful on surrounding tissues and blood flow compared to the osteotomy model, which causes severe damage to the periosteum, bone marrow cells, and surrounding tissues[48,49]. More inflammatory cells were also observed in the osteotomized rats as compared to the rats that received closed femur fractures, indicating that the early inflammatory environment created upon fractures differs between the models [50]. These differences could be the reason of varying outcomes despite receiving the same treatment.

One significant limitation is that we were unable to verify any changes in the inflammatory response of the fracture hematoma. This is in contrast to the alterations observed in the cytokine expression of the fracture hematoma, which has been analyzed in several studies[19–22,35].This may be due to simply missing the site of fracture where the immune stimulants were injected since the hematoma was identified by palpation only or a dose that was too low, but it could also be due to a test that was not sensitive enough. Furthermore, in the control group only 2 of 7 fractures were eligible making comparisons difficult. Although the two remaining animals in the control saline group with eligible fractures showed a slower callus area development in the first 3 weeks than in all the immune stimulated groups, at 4 weeks no differences were detected in the x-ray and micro-CT analyses.

Another limitation is that the final timepoint of the experiment was after 4 weeks. Rat femoral fracture healing including the remodeling phase, takes about 12–14 weeks to restore normal strength [51]. We chose week 4 for our analysis, since we expected to see an effect in the early phases of callus formation and bone regeneration based on previous studies [12,17] . Finally, we used callus morphometry assessment via radiographs and micro-CT analysis as the main outcome parameter for fracture repair instead of biomechanical testing. While biomechanical testing remains crucial for evaluating the functional strength and stability of the healed bone, early callus morphometry assessment offers the advantage of recognizing early effects of immune stimulants. Moreover, micro-CT analysis of the callus has been shown to be a more sensitive method of evaluating fracture healing than biomechanical testing as it allows for repeated measurements over time [52,53]. Nevertheless, early callus formation is not a

predictor of long term bone healing and eventual functional restoration of mechanical strength [53–57].

Conclusion

The present study determines the effect of immune stimulants on the early inflammatory phase and fracture healing in an ovariectomized rat model of closed femur fracture. In contrast to the observation that the immune stimulants did evoke an inflammatory response based on the whole blood assay performed ex vivo, we show that the immune stimulants did not have an effect on the cytokine levels present in the fracture hematoma collected on day 3 after the fracture. In agreement, no changes were observed in callus morphometry after week 4, assessed by both radiographic scores and micro-CT analysis. The unexpected absence of an effect of the immune stimulants compared to controls might be multi-factorial.

Funding

This work is funded by osteosynthesis and trauma care foundation OTC (reference number 2019 MCRC) and the EU's H2020 research and innovation program under Marie S. Curie Cofund RESCUE (grant agreement No 801540).

References

1. Stewart, S. Fracture Non-Union: A Review of Clinical Challenges and Future Research Needs. *Malays. Orthop. J.* **2019**, *13*, 1–10, doi:10.5704/MOJ.1907.001.
2. Shapiro, J.A.; Stillwagon, M.R.; Tornetta, P.I.; Seaver, T.M.; Gage, M.; O'Donnell, J.; Whitlock, K.; Yarboro, S.R.; Jeray, K.J.; Obremskey, W.T.; et al. Serology and Comorbidities in Patients With Fracture Nonunion: A Multicenter Evaluation of 640 Patients. *JAAOS - J. Am. Acad. Orthop. Surg.* **2022**, 10.5435/JAAOS, doi:10.5435/JAAOS-D-21-00366.
3. Nicholson, J.; Makaram, N.; Simpson, A.; Keating, J. Fracture Nonunion in Long Bones: A Literature Review of Risk Factors and Surgical Management. *Injury* **2021**, *52*, S3–S11, doi: 10.1016/j.injury.2020.11.029.
4. Peichl, P.; Holzer, L.A.; Maier, R.; Holzer, G. Parathyroid Hormone 1-84 Accelerates Fracture-Healing in Pubic Bones of Elderly Osteoporotic Women. *JBJS* **2011**, *93*, 1583, doi:10.2106/JBJS.J.01379.
5. Ellegaard, M.; Jørgensen, N.R.; Schwarz, P. Parathyroid Hormone and Bone Healing. *Calcif. Tissue Int.* **2010**, *87*, 1–13, doi:10.1007/s00223-010-9360-5.
6. Wojda, S.J.; Donahue, S.W. Parathyroid Hormone for Bone Regeneration. *J. Orthop. Res.* **2018**, *36*, 2586–2594, doi:10.1002/jor.24075.
7. Kanakaris, N.K.; Petsatodis, G.; Tagil, M.; Giannoudis, P.V. Is There a Role for Bone Morphogenetic Proteins in Osteoporotic Fractures?11Work Attributed to: Academic Unit, Trauma and Orthopaedic Surgery, Leeds General Infirmary, UK. *Injury* **2009**, *40*, S21–S26, doi:10.1016/S0020-1383(09)70007-5.
8. Blokhuis, T.J.; Buma, P.; Verdonschot, N.; Gotthardt, M.; Hendriks, T. BMP-7 Stimulates Early Diaphyseal Fracture Healing in Estrogen Deficient Rats. *J. Orthop. Res.* **2012**, *30*, 720–725, doi: 10.1002/jor.22013.
9. Lyon, T.; Scheele, W.; Bhandari, M.; Koval, K.J.; Sanchez, E.G.; Christensen, J.; Valentin, A.; Huard, F. Efficacy and Safety of Recombinant Human Bone Morphogenetic Protein-2/Calcium Phosphate Matrix for Closed Tibial Diaphyseal Fracture: A Double-Blind, Randomized, Controlled Phase-II/III Trial. *JBJS* **2013**, *95*, 2088, doi:10.2106/JBJS.L.01545.
10. Simmonds, M.C.; Brown, J.V.E.; Heirs, M.K.; Higgins, J.P.T.; Mannion, R.J.; Rodgers, M.A.; Stewart, L.A. Safety and Effectiveness of Recombinant Human Bone Morphogenetic Protein-2 for Spinal Fusion. *Ann. Intern. Med.* **2013**, *158*, 877–889, doi:10.7326/0003-4819-158-12-201306180-00005.
11. Fernandez, L.; Petrizzo, A. The Use of Bone Morphogenetic Protein 2 (BMP-2) in Spine Surgery: Is It Valuable? *Bull. NYU Hosp. Jt. Dis.* **2023**, *81*, 40–46.
12. Cheung, W.-H.; Chin, W.-C.; Qin, L.; Leung, K.-S. Low Intensity Pulsed Ultrasound Enhances Fracture Healing in Both Ovariectomy-Induced

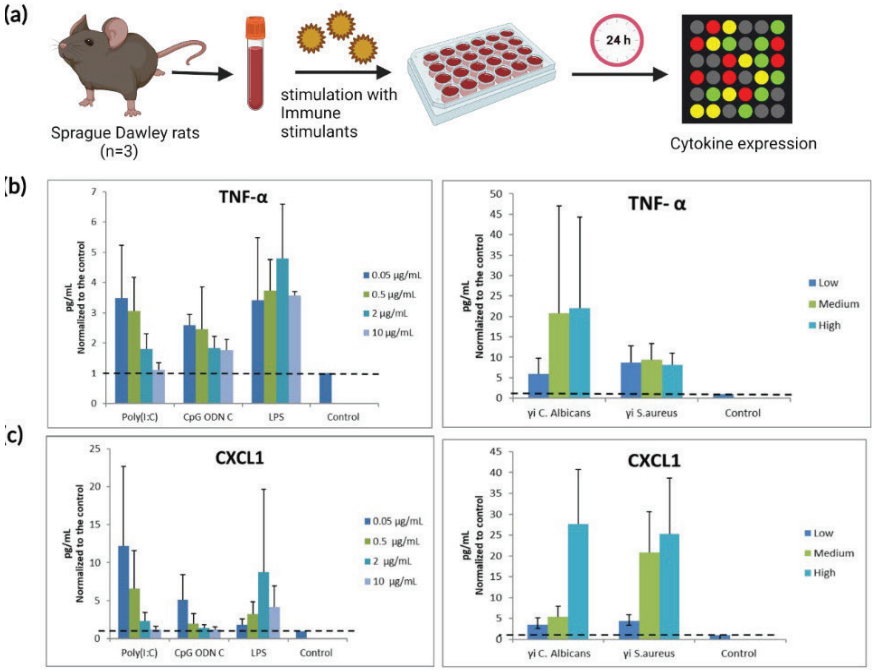
Osteoporotic and Age-Matched Normal Bones. *J. Orthop. Res.* **2012**, *30*, 129–136, doi: 10.1002/jor.21487.
13. Chung, S.-L.; Leung, K.-S.; Cheung, W.-H. Low-Magnitude High-Frequency Vibration Enhances Gene Expression Related to Callus Formation, Mineralization and Remodeling during Osteoporotic Fracture Healing in Rats. *J. Orthop. Res.* **2014**, *32*, 1572–1579, doi:10.1002/jor.22715.
14. Nikolaou, V.S.; Efstathopoulos, N.; Kontakis, G.; Kanakaris, N.K.; Giannoudis, P.V. The Influence of Osteoporosis in Femoral Fracture Healing Time. *Injury* **2009**, *40*, 663–668, doi: 10.1016/j.injury.2008.10.035.
15. Weitzmann, M.N.; Pacifici, R. Estrogen Regulation of Immune Cell Bone Interactions. *Ann. N. Y. Acad. Sci.* **2006**, *1068*, 256–274, doi:10.1196/annals.1346.030.
16. Pacifici, R. Estrogen Deficiency, T Cells and Bone Loss. *Cell. Immunol.* **2008**, *252*, 68–80, doi: 10.1016/j.cellimm.2007.06.008.
17. Namkung-Matthai, H.; Appleyard, R.; Jansen, J.; Hao Lin, J.; Maastricht, S.; Swain, M.; Mason, R.S.; Murrell, G.A.; Diwan, A.D.; Diamond, T. Osteoporosis Influences the Early Period of Fracture Healing in a Rat Osteoporotic Model. *Bone* **2001**, *28*, 80–86, doi:10.1016/s8756-3282(00)00414-2.
18. Kubo, T.; Shiga, T.; Hashimoto, J.; Yoshioka, M.; Honjo, H.; Urabe, M.; Kitajima, I.; Semba, I.; Hirasawa, Y. Osteoporosis Influences the Late Period of Fracture Healing in a Rat Model Prepared by Ovariectomy and Low Calcium Diet. *J. Steroid Biochem. Mol. Biol.* **1999**, *68*, 197–202, doi:10.1016/S0960-0760(99)00032-1.
19. Kolar, P.; Schmidt-Bleek, K.; Schell, H.; Gaber, T.; Toben, D.; Schmidmaier, G.; Perka, C.; Buttgereit, F.; Duda, G.N. The Early Fracture Hematoma and Its Potential Role in Fracture Healing. *Tissue Eng. Part B Rev.* **2010**, *16*, 427–434, doi: 10.1089/ten.teb.2009.0687.
20. Hoff, P.; Gaber, T.; Strehl, C.; Schmidt-Bleek, K.; Lang, A.; Huscher, D.; Burmester, G.R.; Schmidmaier, G.; Perka, C.; Duda, G.N.; et al. Immunological Characterization of the Early Human Fracture Hematoma. *Immunol. Res.* **2016**, *64*, 1195–1206, doi:10.1007/s12026-016-8868-9.
21. Chow, S.K.H.; Cui, C.; Cheng, K.Y.K.; Chim, Y.N.; Wang, J.; Wong, C.H.W.; Ng, K.W.; Wong, R.M.Y.; Cheung, W.H. Acute Inflammatory Response in Osteoporotic Fracture Healing Augmented with Mechanical Stimulation Is Regulated *In vivo* through the P38-MAPK Pathway. *Int. J. Mol. Sci.* **2021**, *22*, 8720, doi:10.3390/ijms22168720.
22. Chow, S.K.-H.; Chim, Y.-N.; Wang, J.-Y.; Wong, R.M.-Y.; Choy, V.M.-H.; Cheung, W.-H. Inflammatory Response in Postmenopausal Osteoporotic Fracture Healing. *Bone Jt. Res.* **2020**, *9*, 368–385, doi: 10.1302/2046-3758.97.BJR-2019-0300.R2.
23. Jahanmard, F.; Khodaei, A.; Flapper, J.; Dogan, O.; Roohi, K.; Taheri, P.; Weinans, H.; Storm, G.; Croes, M.; Mastrobattista, E.; et al. Osteoimmunomodulatory GelMA/Liposome Coatings to Promote Bone Regeneration of Orthopedic Implants. *J. Controlled Release* **2023**, *358*, 667–680, doi: 10.1016/j.jconrel.2023.05.022.

24. Gerstenfeld, L.; Cho, T.-J.; Kon, T.; Aizawa, T.; Tsay, A.; Fitch, J.; Barnes, G.; Graves, D.; Einhorn, T. Impaired Fracture Healing in the Absence of TNF-α Signaling: The Role of TNF-α in Endochondral Cartilage Resorption. *J. Bone Miner. Res.* **2003**, *18*, 1584–1592, doi:10.1359/jbmr.2003.18.9.1584.
25. Wallace, A.; Cooney, T.E.; Englund, R.; Lubahn, J.D. Effects of Interleukin-6 Ablation on Fracture Healing in Mice. *J. Orthop. Res.* **2011**, *29*, 1437–1442, doi:10.1002/jor.21367.
26. Glass, G.E.; Chan, J.K.; Freidin, A.; Feldmann, M.; Horwood, N.J. TNF- α Promotes Fracture Repair by Augmenting the Recruitment and Differentiation of Muscle-Derived Stromal Cells. **2011**, *108*, 1585–1590, doi:10.1073/pnas.1018501108.
27. Chan, J.K.; Glass, G.E.; Ersek, A.; Freidin, A.; Williams, G.A.; Gowers, K.; Espirito Santo, A.I.; Jeffery, R.; Otto, W.R.; Poulsom, R.; et al. Low-Dose TNF Augments Fracture Healing in Normal and Osteoporotic Bone by up-Regulating the Innate Immune Response. *EMBO Mol. Med.* **2015**, *7*, 547–561, doi:10.15252/emmm.201404487.
28. Croes, M.; Wal, B.C.H.; Vogely, H.Ch. The Impact of Bacterial Infections on Osteogenesis: Evidence From *In vivo* Studies. *J. Orthop. Res.* **2019**, jor.24422, doi:10.1002/jor.24422.
29. Croes, M.; Kruyt, M.C.; Boot, W.; Pouran, B.; Braham, M.V.; Pakpahan, S.A.; Weinans, H.; Vogely, H.C.; Fluit, A.C.; Dhert, W.J.; et al. The Role of Bacterial Stimuli in Inflammation-Driven Bone Formation. *Eur. Cell. Mater.* **2019**, *37*, 402–419, doi:10.22203/eCM.v037a24.
30. Khokhani, P.; Rahmani, N.R.; Kok, A.; Öner, F.C.; Alblas, J.; Weinans, H.; Kruyt, M.C.; Croes, M. Use of Therapeutic Pathogen Recognition Receptor Ligands for Osteo-Immunomodulation. *Materials* **2021**, *14*, 1119, doi:10.3390/ma14051119.
31. Khokhani, P.; Belluomo, R.; Croes, M.; Gawlitta, D.; Kruyt, M.C.; Weinans, H. An In-Vitro Model to Test the Influence of Immune Cell Secretome on MSC Osteogenic Differentiation. *Tissue Eng. Part C Methods* **2022**, doi: 10.1089/ten.TEC.2022.0086.
32. Chow, S.K.-H.; Chim, Y.-N.; Wang, J.; Zhang, N.; Wong, R.M.-Y.; Tang, N.; Leung, K.-S.; Cheung, W.-H. Vibration Treatment Modulates Macrophage Polarisation and Enhances Early Inflammatory Response in Oestrogen-Deficient Osteoporotic-Fracture Healing. *Eur. Cell. Mater.* **2019**, *38*, 228–245, doi:10.22203/eCM.v038a16.
33. Chow, S.K.; Leung, K.; Qin, L.; Wei, F.; Cheung, W. Callus Formation Is Related to the Expression Ratios of Estrogen Receptors-Alpha and -Beta in Ovariectomy-Induced Osteoporotic Fracture Healing. *Arch. Orthop. Trauma Surg.* **2014**, *134*, 1405–1416, doi:10.1007/s00402-014-2070-0.
34. Wei, F.-Y.; Leung, K.-S.; Li, G.; Qin, J.; Chow, S.K.-H.; Huang, S.; Sun, M.-H.; Qin, L.; Cheung, W.-H. Low Intensity Pulsed Ultrasound Enhanced Mesenchymal Stem Cell Recruitment through Stromal Derived Factor-1 Signaling

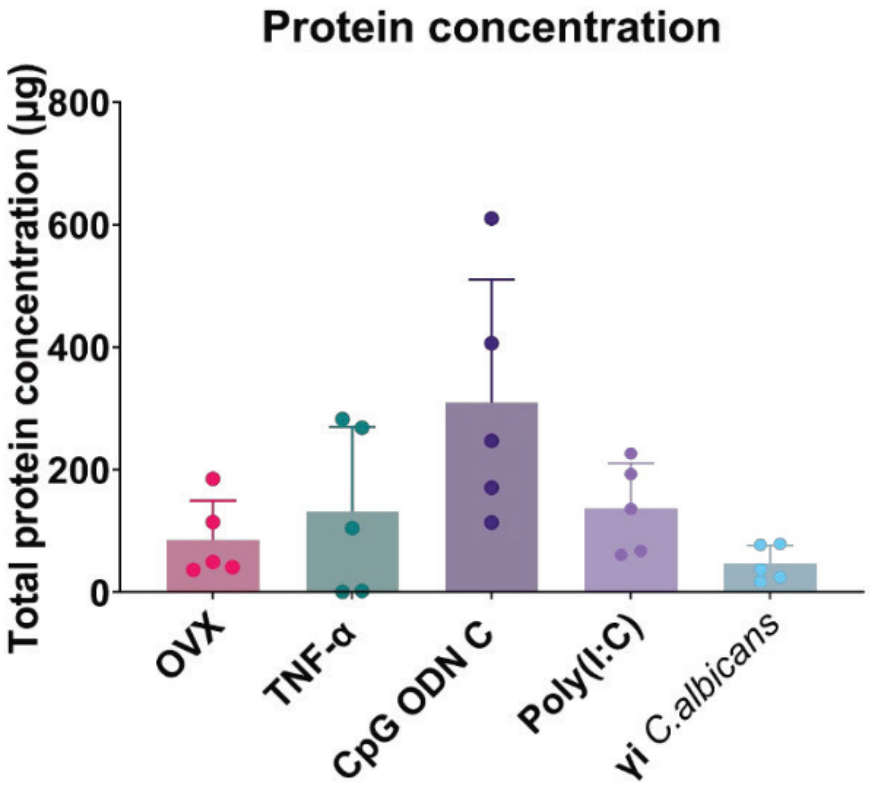
in Fracture Healing. *PLOS ONE* **2014**, *9*, e106722, doi: 10.1371/journal.pone.0106722.
35. Fischer, V.; Kalbitz, M.; Müller-Graf, F.; Gebhard, F.; Ignatius, A.; Liedert, A.; Haffner-Luntzer, M. Influence of Menopause on Inflammatory Cytokines during Murine and Human Bone Fracture Healing. *Int. J. Mol. Sci.* **2018**, *19*, 2070, doi:10.3390/ijms19072070.
36. Liu, L.; Shen, L.; Liu, X.; Yu, Y.; Li, Y.; Wang, L.; He, C.; Sun, J.; Li, B. A Safety Study of a B-Class CpG ODN in Sprague-Dawley Rats. *J. Appl. Toxicol. JAT* **2012**, *32*, 60–71, doi:10.1002/jat.1683.
37. de Oliveira, A.C.P.; Yousif, N.M.; Bhatia, H.S.; Hermanek, J.; Huell, M.; Fiebich, B.L. Poly(I:C) Increases the Expression of MPGES-1 and COX-2 in Rat Primary Microglia. *J. Neuroinflammation* **2016**, *13*, 11, doi:10.1186/s12974-015-0473-7.
38. Ewel, C.H.; Sobel, D.O.; Zeligs, B.J.; Bellanti, J.A. Poly I:C Accelerates Development of Diabetes Mellitus in Diabetes-Prone BB Rat. *Diabetes* **1992**, *41*, 1016–1021, doi:10.2337/diab.41.8.1016.
39. Gilmore, J.H.; Jarskog, L.F.; Vadlamudi, S. Maternal Poly I:C Exposure during Pregnancy Regulates TNFα, BDNF, and NGF Expression in Neonatal Brain and the Maternal–Fetal Unit of the Rat. *J. Neuroimmunol.* **2005**, *159*, 106–112, doi: 10.1016/j.jneuroim.2004.10.008.
40. Oligodeoxynucleotides Containing CpG Motifs Can Induce T Cell–Dependent Arthritis in Rats., doi:10.1002/art.11488.
41. Hafner, A.M.; Corthésy, B.; Merkle, H.P. Particulate Formulations for the Delivery of Poly(I:C) as Vaccine Adjuvant. *Adv. Drug Deliv. Rev.* **2013**, *65*, 1386–1399, doi: 10.1016/j.addr.2013.05.013.
42. Speth, M.T.; Repnik, U.; Müller, E.; Spanier, J.; Kalinke, U.; Corthay, A.; Griffiths, G. Poly(I:C)-Encapsulating Nanoparticles Enhance Innate Immune Responses to the Tuberculosis Vaccine Bacille Calmette–Guérin (BCG) via Synergistic Activation of Innate Immune Receptors. *Mol. Pharm.* **2017**, *14*, 4098–4112, doi: 10.1021/acs.molpharmaceut.7b00795.
43. Lin, S.-F.; Jiang, P.-L.; Tsai, J.-S.; Huang, Y.-Y.; Lin, S.-Y.; Lin, J.-H.; Liu, D.-Z. Surface Assembly of Poly(I:C) on Polyethyleneimine-Modified Gelatin Nanoparticles as Immunostimulatory Carriers for Mucosal Antigen Delivery. *J. Biomed. Mater. Res. B Appl. Biomater.* **2019**, *107*, 1228–1237, doi: 10.1002/jbm.b.34215.
44. Yu, C.-H.; Luo, Z.-C.; Li, M.; Lu, L.; Li, Z.; Wu, X.-Z.; Fan, Y.-Z.; Zhang, H.-L.; Zhou, B.-L.; Wan, Y.; et al. Synthetic Innate Defense Regulator Peptide Combination Using CpG ODN as a Novel Adjuvant Induces Long-lasting and Balanced Immune Responses. *Mol. Med. Rep.* **2016**, *13*, 915–924, doi:10.3892/mmr.2015.4581.
45. Yoshikawa, H.; Hashimoto, J.; Masuhara, K.; Takaoka, K.; Ono, K. Inhibition by Tumor Necrosis Factor of Induction of Ectopic Bone Formation by

Osteosarcoma-Derived Bone-Inducing Substance. *Bone* **1988**, *9*, 391–396, doi:10.1016/8756-3282(88)90121-4.
46. Hashimoto, J.; Yoshikawa, H.; Takaoka, K.; Shimizu, N.; Masuhara, K.; Tsuda, T.; Miyamoto, S.; Ono, K. Inhibitory Effects of Tumor Necrosis Factor Alpha on Fracture Healing in Rats. *Bone* **1989**, *10*, 453–457, doi:10.1016/8756-3282(89)90078-1.
47. Balga, R.; Wetterwald, A.; Portenier, J.; Dolder, S.; Mueller, C.; Hofstetter, W. Tumor Necrosis Factor-Alpha: Alternative Role as an Inhibitor of Osteoclast Formation *in vitro*. *Bone* **2006**, *39*, 325–335, doi: 10.1016/j.bone.2006.02.056.
48. Klein, M.; Stieger, A.; Stenger, D.; Scheuer, C.; Holstein, J.H.; Pohlemann, T.; Menger, M.D.; Histing, T. Comparison of Healing Process in Open Osteotomy Model and Open Fracture Model: Delayed Healing of Osteotomies after Intramedullary Screw Fixation. *J. Orthop. Res.* **2015**, *33*, 971–978, doi:10.1002/jor.22861.
49. Gao, H.; Huang, J.; Wei, Q.; He, C. Advances in Animal Models for Studying Bone Fracture Healing. *Bioengineering* **2023**, *10*, 201, doi:10.3390/bioengineering10020201.
50. Kratzel, C.; Bergmann, C.; Duda, G.; Greiner, S.; Schmidmaier, G.; Wildemann, B. Characterization of a Rat Osteotomy Model with Impaired Healing. *BMC Musculoskelet. Disord.* **2008**, *9*, 135, doi:10.1186/1471-2474-9-135.
51. Ekeland, A.; Engesæter, L.B.; Langeland, N. Mechanical Properties of Fractured and Intact Rat Femora Evaluated by Bending, Torsional and Tensile Tests. *Acta Orthop. Scand.* **1981**, *52*, 605–613, doi:10.3109/17453678108992155.
52. Morgan, E.F.; Mason, Z.D.; Chien, K.B.; Pfeiffer, A.J.; Barnes, G.L.; Einhorn, T.A.; Gerstenfeld, L.C. Micro-Computed Tomography Assessment of Fracture Healing: Relationships among Callus Structure, Composition, and Mechanical Function. *Bone* **2009**, *44*, 335–344, doi: 10.1016/j.bone.2008.10.039.
53. Eastaugh-Waring, S.J.; Joslin, C.C.; Hardy, J.R.W.; Cunningham, J.L. Quantification of Fracture Healing from Radiographs Using the Maximum Callus Index. *Clin. Orthop.* **2009**, *467*, 1986–1991, doi:10.1007/s11999-009-0775-0.
54. Powell, E.S.; Lawford, P.V.; Duckworth, T.; Black, M.M. Is Callus Calcium Content an Indicator of the Mechanical Strength of Healing Fractures? An Experimental Study in Rat Metatarsals. *J. Biomed. Eng.* **1989**, *11*, 277–281, doi:10.1016/0141-5425(89)90059-9.
55. Nyman, J.S.; Munoz, S.; Jadhav, S.; Mansour, A.; Yoshii, T.; Mundy, G.R.; Gutierrez, G.E. Quantitative Measures of Femoral Fracture Repair in Rats Derived by Micro-Computed Tomography. *J. Biomech.* **2009**, *42*, 891–897, doi: 10.1016/j.jbiomech.2009.01.016.
56. Mick, P.; Fischer, C. Delayed Fracture Healing. *Semin. Musculoskelet. Radiol.* **2022**, *26*, 329–337, doi:10.1055/s-0041-1740380.
57. Fisher, J.S.; Kazam, J.J.; Fufa, D.; Bartolotta, R.J. Radiologic Evaluation of Fracture Healing. *Skeletal Radiol.* **2019**, *48*, 349–361, doi:10.1007/s00256-018-3051-0.

Supplementary material



Supplementary Figure S1. (a) Peripheral blood was collected from 3 rats and stimulated with immune stimulants in different concentrations for 24 hours. After 24 hours, cytokine concentration in the supernatant was determined using enzyme-linked immunosorbent assay. (b) TNF-α and (c) CXCL-1 concentrations were used to determine the dosage of the immune stimulants.



Supplementary Figure S2. Protein concentrations in fracture hematomas (n = 5 per group) collected on day 3 post-fracture. Data are represented as mean ± standard deviation. Significance was tested using one way ANOVA.

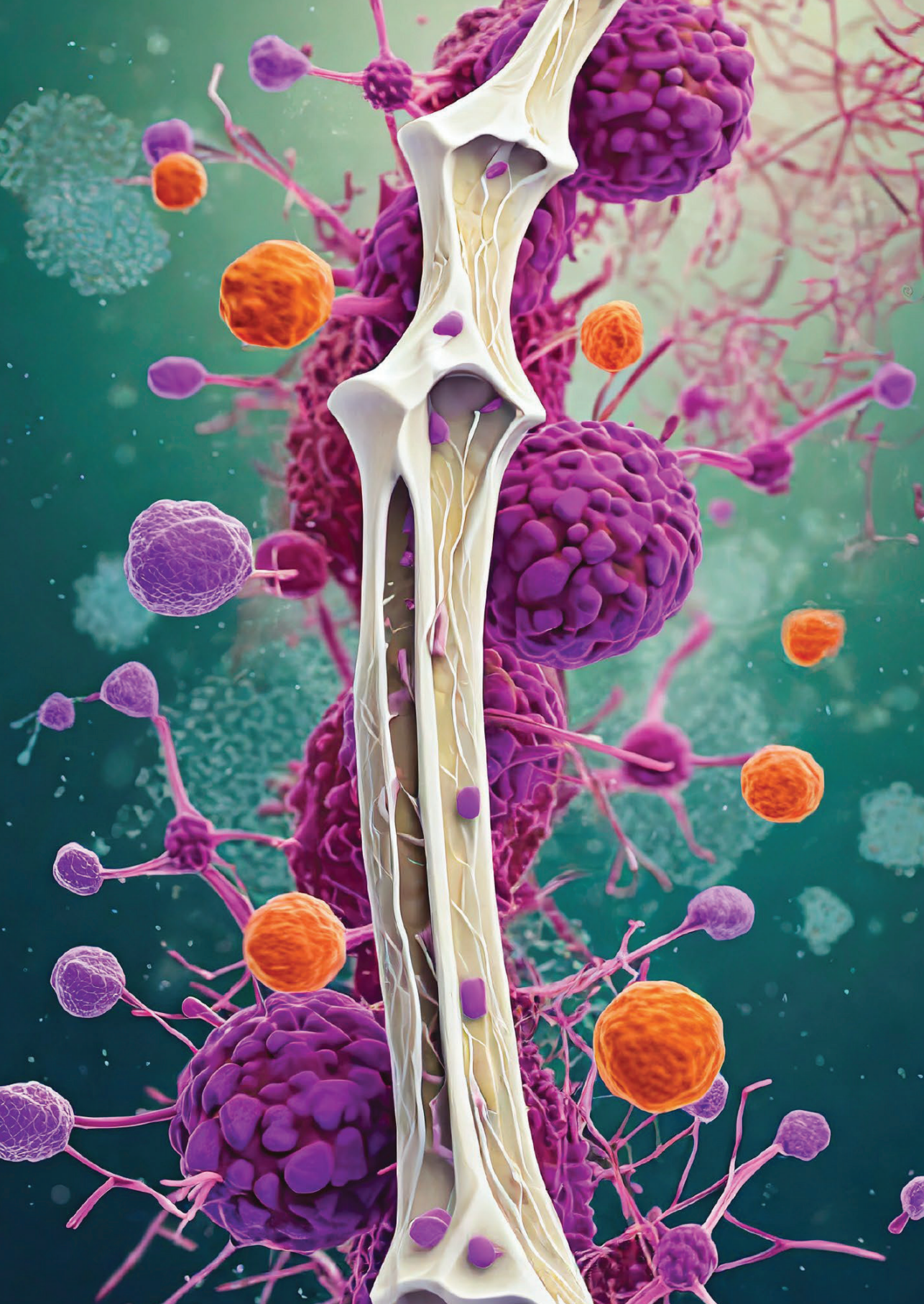
(a)

Parameter : Callus Width					
Šídák's multiple comparisons test					
Test details	Adjusted p-value				Summary
	Week 1	Week 2	Week 3	Week 4	
Saline vs. TNF-α	0.9585	0.0971	0.9812	0.2706	ns
Saline vs. CpG ODN C	0.9579	0.7958	0.7635	0.6312	ns
Saline vs. Poly(I:C)	0.7187	0.265	0.9985	0.2295	ns
Saline vs. γi C.albicans	0.7781	0.5976	0.9902	0.2955	ns

(b)

Parameter : Callus Area					
Šídák's multiple comparisons test					
Test details	Adjusted p-value				Summary
	Week 1	Week 2	Week 3	Week 4	
Saline vs. TNF-α	0.6766	0.9246	0.861	0.7214	ns
Saline vs. CpG ODN C	0.6847	0.6953	0.4336	0.9271	ns
Saline vs. Poly(I:C)	0.6766	0.4759	0.2086	0.9423	ns
Saline vs. γi C.albicans	0.7001	0.4637	0.2989	0.9764	ns

Supplementary Table ST1. p-value of Sidak's multiple comparison (control versus the different treatment groups) for (a) callus width and (b) callus area at different time points.



Chapter 6

Discussion

From infections to innovations

The journey from infections to innovations in osteoimmunology began with clinical observations of pathological conditions such as osteomyelitis, trauma-induced heterotopic ossifications, and cochlear ossifications where new bone formation was observed around the site of infections [1–3]. These observations revealed a fascinating connection: bacterial infections could trigger the formation of pathological new bone. This revelation ignited a quest to explore bacterial antigens as potential osteo-immunomodulatory agents to harness their power to enhance bone formation. Initial experiments conducted in various rabbit models provided promising results, demonstrating an increase in net bone volume upon the local application of killed bacteria in the form of injections or a simple loading of killed bacteria on calcium phosphate scaffolds [4–6]. However, the path to clinical translation was fraught with challenges. Issues such as off-target side effects and batch-to-batch inconsistencies overshadow the potential use of bacterial antigens as therapeutic agents.

To overcome these formidable hurdles, the synthetic PRR ligands were investigated as an alternative to bacterial antigens. These synthetic compounds offer a superior alternative, as they effectively mimic the actions of bacterial cell wall components and skew the immune response without the associated toxicity and variability. Furthermore, several synthetic PRR ligands are under investigation in clinical trials for various applications, such as tumors and asthma therapies. These characteristics make them a promising candidate that offers a safer and more consistent avenue for enhancing bone regeneration, as highlighted in **Chapter 1**.

Potential use of individual PRR ligands for bone regeneration

Using simple 2D *in vitro* models, we showed that direct stimulation with PRR ligands did not inhibit the osteogenic differentiation of MSCs and the differentiation of monocytes to osteoclasts. Although nucleic acid based PRR ligands, namely **CpG ODN C** and **Poly(I:C)**, particularly enhanced osteogenic differentiation of MSCs, the effect was highly dose and donor dependent. The immunomodulatory effects of PRR ligands were confirmed by measuring levels of cytokines TNF, IL-6, IL-10, and IL-8 secreted by stimulated monocytes and MSCs in **Chapter 2**.

Although **CpG ODN C** and **Poly(I:C)** stimulation enhanced osteogenic differentiation, the donor variable response poses a considerable challenge in clinical translation. The variability in the response towards these PRR ligands

could be due to the difference in PRR expression profile on MSCs of different donors [7]. Bone marrow-derived MSCs express TLR1,2,3,4,5,6,7,8,9, and 10, along with NOD1 and 2 [8–10]. Multiple studies have presented different expression outcomes of the PRRs on MSCs depending on the micro-environmental factors. For example, when exposed to inflammatory conditions, MSCs show an upregulation of TLR2, TLR4, and TLR7, while a downregulation of TLR6 was observed [11]. Other microenvironment conditions like hypoxia and viral infections encouraged an augmented expression of TLR 1,2,3,5,9 and 10 [12] .

Nevertheless, to facilitate the translation of PRR ligands to the clinics, one of the logical approaches would be to administer the PRR ligands at the fracture site when MSCs are present. However, there needs to be concrete proof of MSC presence at the fracture site in the initial stages for humans. We only know that MSCs are present in the callus of after Day 5 in mice and later on differentiate into chondrocytes or osteoblasts [13]. Moreover, even if they were found at the fracture site, we have yet to determine when they will be present or for how long. This situation would be like "shooting in the dark and hoping for the best," which is not ideal. Another way is to treat MSCs with the PRR ligands and then implant them at the fracture site [14,15]. However, this solution will still have to overcome the challenges of yielding enough cell amounts or increasing the survival rate of the MSCs in the scaffold/implant, as mentioned in **Chapter 1**.

Next, we showed that soluble factors secreted by **CpG ODN C** stimulated human peripheral mononuclear cells (PBMCs) enhanced the osteogenic differentiation capacity of MSCs compared to direct stimulation of MSCs, using our newly established *in vitro* model in **Chapter 3**. Characterizing these factors revealed that MCP-1, among others, could be responsible for this phenomenon [16,17]. The current outcomes prove that the PRR ligands, especially **CpG ODN C**, can activate the immune system, producing cytokines, chemokines, and various other factors that have a pro-osteogenic effect on MSCs.

While the PRR ligands have shown the aptitude to elicit the production of a variety of pro- as well as anti-inflammatory factors and influence differentiation capacity in **Chapters 2 and 3**, several studies have shown contrasting outcomes. For example, **Pam3CSK4** (targeting TLR2) and **LPS** (targeting TLR4) ligands increase osteogenic differentiation in bone marrow-derived MSCs [8,18]. At the same time, **CpG ODN C** (targeting TLR9) repressed it when these ligands were added to MSC cultures for five days [19]. In **Chapter 2,** however, we observed an opposite effect of these ligands on osteogenic differentiation of MSCs because these PRR ligands were added to the MSC cultures for 14-21 days. These discrepancies indicate the importance of PRR ligand stimulation's kinetics and the duration for which the ligands must be present in culture to interact with the cells needs to be considered before applying these ligands for bone regeneration.

Irrespective of the donor variation, these findings showed that **CpG ODN C**, a bacterial unmethylated CpG motif DNA, holds the potential to promote bone regeneration.

Next, to demonstrate the effect of PRR ligands on fracture healing and the early inflammatory stage of fracture healing, we injected the "most promising" PRR ligands, **CpG ODN C** and **Poly(I:C)**, at the site of the fracture in a closed fracture model of ovariectomized rats in **Chapter 5**. With the insights obtained from the *in vitro* models, we applied the PRR ligands locally for two days after the injury to stimulate the immune system to facilitate the secretion of cytokines and growth factors beneficial for fracture healing. Unfortunately, these PRR ligands showed no significant differences in the fracture healing outcomes or the early inflammatory stage. Although the reasons for these were most likely multifactorial, including the delivery of the PRR ligands, the lack of understanding of the ideal timing and kinetics of these agents *in vivo*, and identifying the correct assays and time points for outcome parameters, the most likely explanation for this distinct outcome is that the findings obtained during *in vitro* testing with human cells fail to translate to a rat model. This species-dependent response to the PRR ligands is mainly due to the difference in the expression of PRRs and their distribution in humans versus that in rats [20].

To summarize, exploring individual PRR ligands for bone regeneration shows promise through *in vitro* models. **CpG ODN C** and **Poly(I:C)** enhance osteogenic differentiation without inhibiting osteoclast differentiation and demonstrate immunomodulatory properties. However, the challenges include dose and donor-dependent effects, variability in PRR expression profiles, and discrepancies in the timing and kinetics of PRR ligand stimulation. Although soluble factors from **CpG ODN C**-stimulated cells enhance osteogenic differentiation, it's *in vivo* translation faces complexities, including species-dependent responses, delivery issues, and the need for a better understanding of treatment timing and ligand kinetics. Overall, while individual PRR ligands like **CpG ODN C** offer the potential for bone regeneration, addressing these challenges is crucial for practical clinical application.

Is it better to employ mixtures as opposed to single ligands?

With the demonstrated *in vivo* potential of gamma irradiated (γi) *S. Aureus* to promote bone formation in various rabbit models, we used γi *S. Aureus* to validate our newly developed *in vitro* model in **Chapter 3**. This model allowed us to gain insights into the underlying mechanism of γi *S. Aureus's* ability to enhance bone formation. We found that γi *S. Aureus* induces upregulation of NF-κB, MAPK, and

interferon signaling pathways in immune cells, leading to the secretion of various proinflammatory cytokines, chemokines, and other factors like osteoprotegerin, and VEGF. These factors, when added to the MSCs, enhanced their osteogenic differentiation. By studying γi *S. Aureus*, it was evident that a specific set of PRRs was involved in initial recognition by the immune system; thus, we aimed at mimicking this γi *S. Aureus*-specific composition using mixtures of PRR ligands in **Chapter 4**.

Within this chapter, we identified three potent mixtures, namely **Mix 1** consisting of **Pam3CSK4**, **CpG ODN C**, and **Murabutide**, **Mix 4** consisting of **CL429**, **Murabutide**, and **CpG ODN C** and finally **Mix 5** consisting of **LTA**, **CpG ODN C**, **Resiquimod**, and **Murabutide** that mirrored γi *S. Aureus's* effects on signaling pathways and the subsequent release of cytokines and chemokines. These mixtures demonstrated their prowess in enhancing the osteogenic differentiation of MSCs, surpassing the individual PRR ligands' efficacy. This ground-breaking approach not only offers a tailored solution for bone therapies but also has the potential to overcome resistance to a specific ligand that host might have due to pre-exposure and trigger a synergistic activation of PRRs, resulting in a tunable and robust immune response.

However, amidst these promising developments, it is imperative to acknowledge the challenges that lie ahead. The substantial donor variability observed in response to the mixtures and γi *S. Aureus* underscores the difficulty of achieving a "one-size-fits-all" therapeutic solution. Moreover, the complexities surrounding the formulation, pharmacokinetics, and *in vivo* administration of these mixtures for translation present a Herculean task. Nonetheless, armed with these remarkable discoveries, our future endeavors should involve rigorous testing of these mixtures in advanced animal models, such as the rabbit spinal fusion or segmental defect model, to assess their potential in bone formation for optimal outcomes, determination of the species specific PRR expression and responsiveness of *S. Aureus* towards the animal are a pre-requisite for *in vivo* testing. It is worth noting that administering these mixtures, in conjunction with osteo-inductive factors like BMP-2, will be crucial for bridging the gap between promising *in vitro* outcomes and real-world clinical applications.

Need for advanced models to test osteo-immunomodulatory strategies

This thesis employed simpler, straightforward in vitro models for the initial screening of the PRR ligands to investigate their effects on MSC differentiation into osteogenic and chondrogenic lineages. This approach enabled us to identify

the direct effects of the PRR ligands on MSCs as they undergo immunophenotypic changes and differentiate into osteoblasts and hypertrophic chondrocytes throughout the fracture healing process. A significant drawback of this model is that it completely ignores the influence of the crosstalk between the immune cells and bone cells that occurs *in vivo*. Thus, in addition to studying the effects of PRR ligands on MSC monocultures, a complex in vitro model is necessary. MSC–monocytes/macrophage coculture models are frequently used to study osteo-immunomodulatory strategies [21–23]. However, these models do not consider the role of other innate and adaptive immune cells contributing to the complex fracture healing cascade.

In **Chapter 4**, we designed a new in vitro model to study the effects of the immune cells and their secreted factors on bone-forming cells. We tested its feasibility by evaluating the immunomodulatory effects of PRR ligand's potential on MSC osteogenic differentiation. This model is advantageous as it includes all blood-resident immune cells, namely PBMCs, making it possible to discover the inflammatory milieu needed to study bone regeneration. Furthermore, this model is easy to use and contributes to studying the immunological and molecular dynamics among different cell types on a broader spectrum. In the current model, however, we do not consider the importance of cell-to-cell contact. Moreover, only the impact of the immune cell secretome on MSCs is examined, and not vice versa. MSCs regulate the immune cells' functions and responses, dampening the inflammatory response, which might indirectly affect bone formation as described in **Chapter 1** [24,25].

Various efforts are underway to address the shortcomings of using basic in vitro models, and advancements are being pursued to make them closely resemble the in vivo environment of fracture healing. For example, a model of the fracture hematoma has been created in vitro, which closely mimics the hematoma traits taken from human patients [26,27]. However, this model is still in its initial stages and is not yet dynamic enough to imitate the natural flow of blood and cells. Moreover, long-term experiments with these models are still not possible. De Wildt and colleagues have created a new 3D *in vitro* model that uses MSCs to differentiate osteoblasts and osteoclasts. This model allows us to simultaneously monitor the avascular bone formation and resorption process, mimicking the bone remodeling process in living organisms [28–30]. These models show much potential in improving the initial drug screening and testing phases, but they still need to be validated. An ideal *in vitro* model for studying osteo-immunomodulatory strategies would be a 3D coculture model of the immune cells and bone cells like MSCs and osteoclasts in a 3D setup and hypoxic environment to most closely mimic the initial phase of fracture healing. Further research should look into optimizing, validating, and refining these in vitro

models that can be used not only for predictive outcomes but also for fundamental research and development of drugs and personalized medicine.

In **Chapter 5**, the effects of PRR ligands on fracture healing and inflammation were tested in an ovariectomized rat model of closed femur fracture in this thesis. We aimed to define the initial inflammatory stage in the rats and then correlate its influence on fracture healing. However, the first and foremost challenge with this model is the more significant difference between rodent and human immunology, which makes testing any immunomodulatory strategy in this model critical [31]. Moreover, we also induced estrogen-deficient osteoporosis in this model without fully considering the effect of estrogen deficiency on the early inflammatory stage and how that would affect the PRR ligands activity. Further, limited PRR ligands can be tested as only one site of the injury is available, which does not go well with the principles of the 3Rs (replacement, reduction, and refinement) in animal testing. Besides this, the fracture defect created in this model is relatively small. It will eventually heal without interventions, making it impossible to investigate the difference between the PRR ligands at later time points. With these points, whether the OVX model is optimal for testing PRR ligands is questionable.

Typically, ectopic implantations in an animal are performed to test several immunomodulatory compounds and their dosage for bone formation[5,32,33]. However, since several implants are tested in the same animal, there is a possibility of systemic interference and local crossover effects of the compounds being tested. A standardized bio membrane pocket model, an improved version of the ectopic implantations, was developed by Croes et al. to study bone scaffolds together with a confined inflammatory response induced by the immunomodulatory compounds[34]. This model provided advantages over the classic ectopic implantations regarding better use of the animals. However, it is still uncertain whether this model minimizes the local crossover effects between adjacent implants and whether it can be applied to investigate the cellular and molecular content of the immune response towards the immunomodulatory compounds.

Further research is needed to develop and validate this model as a preclinical tool for testing various osteo-immunomodulatory strategies. Since the PRR ligands were investigated for bone regeneration purposes, especially for cases of fracture non-unions and spinal fusion, it is imperative to test their efficacy in functional models like Boden's spinal fusion model or segmental defect models, in which the osteoconductive and osteo-inductive properties of the PRR ligands incorporated in a bone scaffold could be investigated. However, these models only allow testing of a limited number of conditions and outcome parameters.

Lastly, it must be noted that the choice of animal species and its corresponding models influence the effectiveness of osteo-immunomodulatory strategies.

Moreover, all animal experiments are conducted in a controlled environment where their age and sex are carefully considered, neglecting the diverse effects the individuals' immune system might have on bone regeneration strategies. These standardized models can mask these variations, costing time and resources. Careful considerations must be made while testing new immunomodulatory strategies for clinical translation.

Concluding remarks and future perspective

This thesis investigates the PRR ligands' role in bone regeneration therapies. PRR ligands possess immunomodulatory properties that could modulate the initial inflammatory phase in the fracture healing process, leading to a successful healing outcome. One of the major obstacles with this approach is the modulation of the complex initial inflammatory phase, as described in **Chapter 1**. The transition from infections to innovations is a testament to the complex challenges and uncertainties inherent to osteo-immunomodulation research. While it marks a shift in the field, it also underscores the formidable obstacles that must be surmounted to achieve meaningful progress in enhancing bone growth and therapy.

Even with advancements due to the evolution of the osteoimmunology field, interventional therapies for fracture healing targeting the early phase are yet to be developed. There is an emerging need for these therapies as they can prevent the need for later stage secondary surgeries, avoiding patient suffering.

Modulating the inflammatory phase of fracture healing, especially using immunomodulatory agents like proinflammatory cytokines, bacterial antigens, or PRR ligands, which are known to induce inflammation at the injury site, is a "high-risk, high gain" situation. If these agents are not administrated in a controlled way, the probability of prolonged inflammation or chronic inflammation increases, which is definitely detrimental to bone formation. It all depends on being able to control these agents' delivery, dosage, activity duration and window, and pharmacokinetics. In order to fulfil all these requirements, a deeper understanding of the inflammation affecting the fracture healing cascade, including the defined roles of cellular and molecular components, is needed. The foremost question that needs to be answered is: What is the correct time frame for using proinflammatory agents? Complex in vitro and in vivo models are also needed to answer these questions and translate them into therapies.

Bacterial antigens and PRR ligands, whether single ligands or mixtures are particularly difficult to control in vivo, as they target multiple receptors in the immune system and the bone cells. The predictability of successful healing using

these agents is, as of now, seems too far away as their mode of action and secretion profile depend on numerous factors that still need to be discovered. These PRR ligands or bacterial antigens for bone formation are only tested in the presence of osteo inductive factors like BMP-2 or dexamethasone, making them a bone-adjuvant rather than a standalone agent. This is an important aspect that needs to be considered since BMP-2 has shown side effects in clinical use.

Nonetheless, if one wants to continue on the path of using bacterial antigens/ PRR ligands for bone therapies, it is advisable to start from the basics. For this, a combination of top-down and bottom-up approach should be considered. In bottom-up approach, characterizing the components of killed bacterial antigens that led to the increased bone volume in rabbit models would be a good starting point. This approach is beneficial especially when a formulation is developed to target a specific mechanism, in our case, we use the PRR ligands/ bacterial antigens to target the early immune stage of the bone regeneration process. This approach further equips and answers the question for successful application of these immunomodulatory agents for animal testing. That said, bottom-up approach can only help in optimizing the agents, but eventually their effects must be tested in a large animal model. A top-down approach can be utilized to screen for the most optimal bacterial antigen. As these bacterial antigens were only tested in the tibial model and ectopic implantations; their effect should be more rigorously investigated in functional models like spinal fusion and segmental defect models while also testing their influence on the immune response *in vivo* using the bio-membrane pocket model as a part of top-down approach. A large animal study is also advisable to screen for the effects of the bacterial antigens on bone formation. While using these models, the delivery methods to control the release of bacterial antigens/ PRR ligands should be considered. As for the PRR ligands, since the mixtures showed potential to enhance MSC osteogenic differentiation, efforts in testing their effects as well as developing their delivery system for *in vivo* testing should be taken into consideration.

In conclusion, PRR ligands, especially **CpG ODN C**, did show positive enhancement of MSC osteogenic differentiation *in vitro*; however, this positive effect failed to translate into an effect in the *in vivo* rat model. Mixtures of PRR ligands also promote MSC osteogenic differentiation *in vitro*; however, their efficacy for potential use needs to be investigated *in vivo*. Pending the *in vivo* outcomes, the road to clinical use of the mixtures of PRR ligands will be long and complicated, especially since the new formulations must undergo stringent regulatory assessments.

References

1. Edwards, D.S.; Kuhn, K.M.; Potter, B.K.; Forsberg, J.A. Heterotopic Ossification: A Review of Current Understanding, Treatment, and Future. *J. Orthop. Trauma* **2016**, *30*, S27, doi:10.1097/BOT.0000000000000666.
2. Felix-Ilemhenbhio, F.; Pickering, G.A.E.; Kiss-Toth, E.; Wilkinson, J.M. Pathophysiology and Emerging Molecular Therapeutic Targets in Heterotopic Ossification. *Int. J. Mol. Sci.* **2022**, *23*, 6983, doi:10.3390/ijms23136983.
3. Urish, K.L.; Cassat, J.E. Staphylococcus Aureus Osteomyelitis: Bone, Bugs, and Surgery. *Infect. Immun.* **2020**, *88*, doi:10.1128/IAI.00932-19.
4. Croes, M.; Wal, B.C.H.; Vogely, H.Ch. The Impact of Bacterial Infections on Osteogenesis: Evidence From *In vivo* Studies. *J. Orthop. Res.* **2019**, jor.24422, doi:10.1002/jor.24422.
5. Croes, M.; Kruyt, M.C.; Boot, W.; Pouran, B.; Braham, M.V.; Pakpahan, S.A.; Weinans, H.; Vogely, H.C.; Fluit, A.C.; Dhert, W.J.; et al. The Role of Bacterial Stimuli in Inflammation-Driven Bone Formation. *Eur. Cell. Mater.* **2019**, *37*, 402–419, doi:10.22203/eCM.v037a24.
6. Fiedler, T.; Salamon, A.; Adam, S.; Herzmann, N.; Taubenheim, J.; Peters, K. Impact of Bacteria and Bacterial Components on Osteogenic and Adipogenic Differentiation of Adipose-Derived Mesenchymal Stem Cells. *Exp. Cell Res.* **2013**, *319*, 2883–2892, doi: 10.1016/j.yexcr.2013.08.020.
7. Muzio, M.; Bosisio, D.; Polentarutti, N.; D'amico, G.; Stoppacciaro, A.; Mancinelli, R.; van't Veer, C.; Penton-Rol, G.; Ruco, L.P.; Allavena, P.; et al. Differential Expression and Regulation of Toll-Like Receptors (TLR) in Human Leukocytes: Selective Expression of TLR3 in Dendritic Cells. *J. Immunol.* **2000**, *164*, 5998 LP – 6004, doi:10.4049/jimmunol.164.11.5998.
8. Pevsner-Fischer, M.; Morad, V.; Cohen-Sfady, M.; Rousso-Noori, L.; Zanin-Zhorov, A.; Cohen, S.; Cohen, I.R.; Zipori, D. Toll-like Receptors and Their Ligands Control Mesenchymal Stem Cell Functions. *Blood* **2006**, *109*, 1422–1432, doi:10.1182/blood-2006-06-028704.
9. He, X.-X.; Bai, H.; Yang, G.-R.; Xue, Y.-J.; Su, Y.-N. [Expression of Toll-like receptors in human bone marrow mesenchymal stem cells]. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* **2009**, *17*, 695–699.
10. Kim, H.-S.; Shin, T.-H.; Yang, S.-R.; Seo, M.-S.; Kim, D.-J.; Kang, S.-K.; Park, J.-H.; Kang, K.-S. Implication of NOD1 and NOD2 for the Differentiation of Multipotent Mesenchymal Stem Cells Derived from Human Umbilical Cord Blood. *PLOS ONE* **2010**, *5*, e15369, doi: 10.1371/journal.pone.0015369.
11. Raicevic, G.; Rouas, R.; Najar, M.; Stordeur, P.; Id Boufker, H.; Bron, D.; Martiat, P.; Goldman, M.; Nevessignsky, M.T.; Lagneaux, L. Inflammation Modifies the Pattern and the Function of Toll-like Receptors Expressed by Human

Mesenchymal Stromal Cells. *Hum. Immunol.* **2010**, *71*, 235–244, doi: 10.1016/j.humimm.2009.12.005.
12. Dumitru, C.A.; Hemeda, H.; Jakob, M.; Lang, S.; Brandau, S. Stimulation of Mesenchymal Stromal Cells (MSCs) via TLR3 Reveals a Novel Mechanism of Autocrine Priming. *FASEB J.* **2014**, *28*, 3856–3866, doi:10.1096/fj.14-250159.
13. Knight, M.N.; Hankenson, K.D. Mesenchymal Stem Cells in Bone Regeneration. *Adv. Wound Care* **2013**, *2*, 306–316, doi:10.1089/wound.2012.0420.
14. Lin, T.; Pajarinen, J.; Nabeshima, A.; Lu, L.; Nathan, K.; Jämsen, E.; Yao, Z.; Goodman, S.B. Preconditioning of Murine Mesenchymal Stem Cells Synergistically Enhanced Immunomodulation and Osteogenesis. *Stem Cell Res. Ther.* **2017**, *8*, 277, doi:10.1186/s13287-017-0730-z.
15. Najar, M.; Krayem, M.; Meuleman, N.; Bron, D.; Lagneaux, L. Mesenchymal Stromal Cells and Toll-Like Receptor Priming: A Critical Review. *Immune Netw* **2017**, *17*, 89–102, doi:10.4110/in.2017.17.2.89.
16. Mulholland, B.S.; Forwood, M.R.; Morrison, N.A. Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) Drives Activation of Bone Remodelling and Skeletal Metastasis. *Curr. Osteoporos. Rep.* **2019**, *17*, 538–547, doi:10.1007/s11914-019-00545-7.
17. Shireman, P.K.; Contreras-Shannon, V.; Ochoa, O.; Karia, B.P.; Michalek, J.E.; McManus, L.M. MCP-1 Deficiency Causes Altered Inflammation with Impaired Skeletal Muscle Regeneration. *J. Leukoc. Biol.* **2007**, *81*, 775–785, doi: 10.1189/jlb.0506356.
18. Liotta, F.; Angeli, R.; Cosmi, L.; Filì, L.; Manuelli, C.; Frosali, F.; Mazzinghi, B.; Maggi, L.; Pasini, A.; Lisi, V.; et al. Toll-Like Receptors 3 and 4 Are Expressed by Human Bone Marrow-Derived Mesenchymal Stem Cells and Can Inhibit Their T-Cell Modulatory Activity by Impairing Notch Signaling. *Stem Cells* **2008**, *26*, 279–289, doi:10.1634/stemcells.2007-0454.
19. Nørgaard, N.N.; Holien, T.; Jönsson, S.; Hella, H.; Espevik, T.; Sundan, A.; Standal, T. CpG-Oligodeoxynucleotide Inhibits Smad-Dependent Bone Morphogenetic Protein Signaling: Effects on Myeloma Cell Apoptosis and *In vitro* Osteoblastogenesis. *J. Immunol.* **2010**, *185*, 3131–3139, doi:10.4049/jimmunol.0903605.
20. Shirjang, S.; Mansoori, B.; Solali, S.; Hagh, M.F.; Shamsasenjan, K. Toll-like Receptors as a Key Regulator of Mesenchymal Stem Cell Function: An up-to-Date Review. *Cell. Immunol.* **2017**, *315*, 1–10, doi: 10.1016/j.cellimm.2016.12.005.
21. Pirraco, R.P.; Reis, R.L.; Marques, A.P. Effect of Monocytes/Macrophages on the Early Osteogenic Differentiation of hBMSCs. *J. Tissue Eng. Regen. Med.* **2013**, *7*, 392–400, doi: 10.1002/term.535.
22. Guihard, P.; Danger, Y.; Brounais, B.; David, E.; Brion, R.; Delecrin, J.; Richards, C.D.; Chevalier, S.; Rédini, F.; Heymann, D.; et al. Induction of Osteogenesis in Mesenchymal Stem Cells by Activated Monocytes/Macrophages

Depends on Oncostatin M Signaling. *Stem Cells* **2012**, *30*, 762–772, doi:10.1002/stem.1040.

23. Jämsen, E.; Kouri, V.P.; Ainola, M.; Goodman, S.B.; Nordström, D.C.; Eklund, K.K.; Pajarinen, J. Correlations between Macrophage Polarizing Cytokines, Inflammatory Mediators, Osteoclast Activity, and Toll-like Receptors in Tissues around Aseptically Loosened Hip Implants. *J. Biomed. Mater. Res. - Part A* **2017**, *105*, 454–463, doi:10.1002/jbm.a.35913.

24. Kovach, T.K.; Dighe, A.S.; Lobo, P.I.; Cui, Q. Interactions between MSCs and Immune Cells: Implications for Bone Healing. *J. Immunol. Res.* **2015**, *2015*, doi:10.1155/2015/752510.

25. Kwon, M.-S.; Noh, M.-Y.; Oh, K.-W.; Cho, K.-A.; Kang, B.-Y.; Kim, K.-S.; Kim, Y.-S.; Kim, S.H. The Immunomodulatory Effects of Human Mesenchymal Stem Cells on Peripheral Blood Mononuclear Cells in ALS Patients. *J. Neurochem.* **2014**, *131*, 206–218, doi:10.1111/jnc.12814.

26. Pfeiffenberger, M.; Hoff, P.; Thöne-Reineke, C.; Buttgereit, F.; Lang, A.; Gaber, T. The *in vitro* Human Fracture Hematoma Model - a Tool for Preclinical Drug Testing. *ALTEX* **2020**, *37*, 561–578, doi:10.14573/altex.1910211.

27. Pfeiffenberger, M.; Damerau, A.; Ponomarev, I.; Bucher, C.H.; Chen, Y.; Barnewitz, D.; Thöne-Reineke, C.; Hoff, P.; Buttgereit, F.; Gaber, T.; et al. *An in vitro Human-Based Fracture Gap Model – Mimicking the Crosstalk between Bone and Immune Cells*; 2020; p. 2020.06.26.165456;

28. de Wildt, B.W.M.; Ansari, S.; Sommerdijk, N.A.J.M.; Ito, K.; Akiva, A.; Hofmann, S. From Bone Regeneration to Three-Dimensional *in vitro* Models: Tissue Engineering of Organized Bone Extracellular Matrix. *Curr. Opin. Biomed. Eng.* **2019**, *10*, 107–115, doi: 10.1016/j.cobme.2019.05.005.

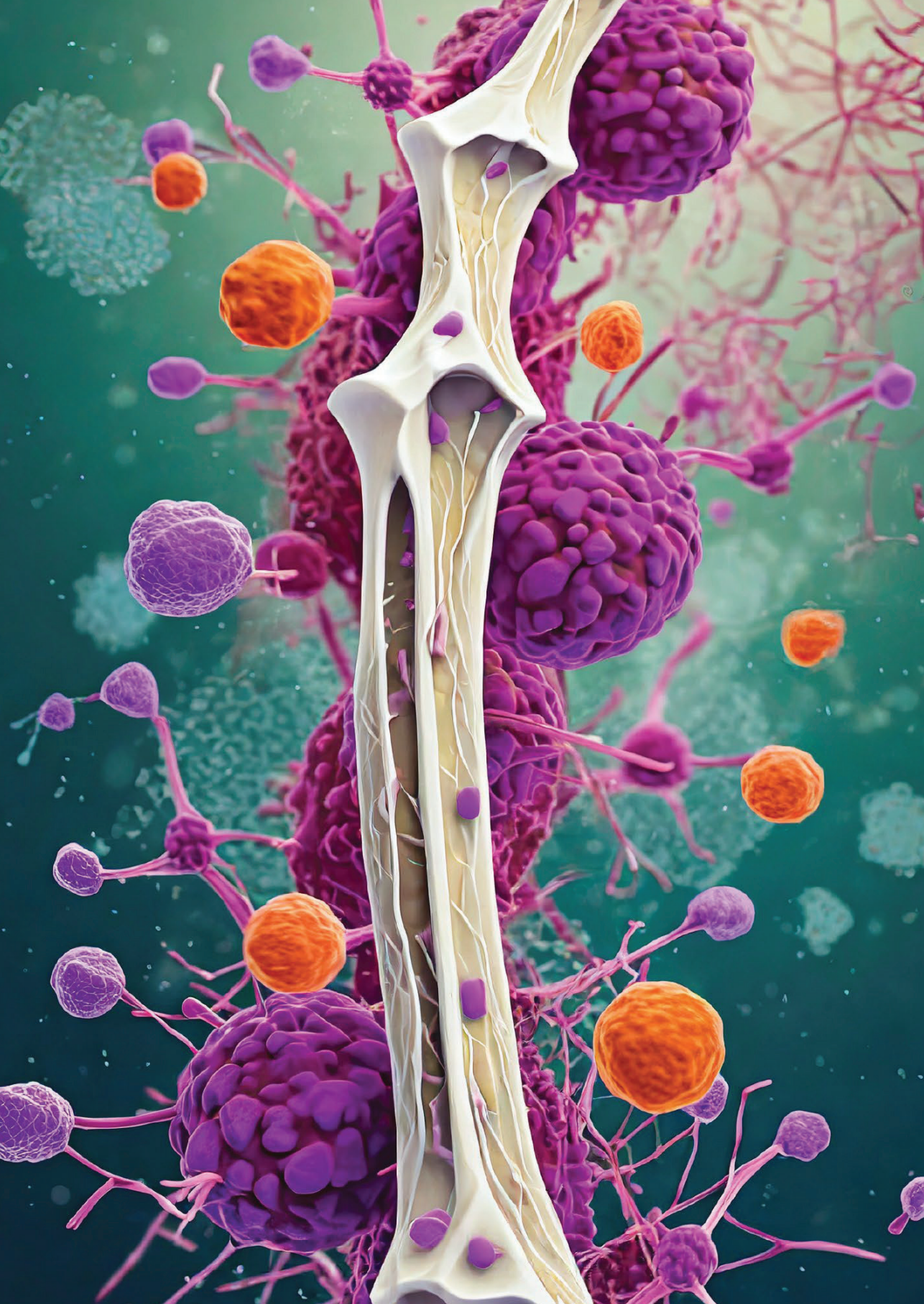
29. de Wildt, B.W.M.; Zhao, F.; Lauwers, I.; van Rietbergen, B.; Ito, K.; Hofmann, S. Characterization of Three-Dimensional Bone-like Tissue Growth and Organization under Influence of Directional Fluid Flow. *Biotechnol. Bioeng.* **2023**, *120*, 2013–2026, doi:10.1002/bit.28418.

30. de Wildt, B.W.M.; Cuypers, L.A.B.; Cramer, E.E.A.; Wentzel, A.S.; Ito, K.; Hofmann, S. The Impact of Culture Variables on a 3D Human *In vitro* Bone Remodeling Model: A Design of Experiments Approach. *Adv. Healthc. Mater. n/a*, 2301205, doi:10.1002/adhm.202301205.

31. Dinges, M.M.; Schlievert, P.M. Comparative Analysis of Lipopolysaccharide-Induced Tumor Necrosis Factor Alpha Activity in Serum and Lethality in Mice and Rabbits Pretreated with the Staphylococcal Superantigen Toxic Shock Syndrome Toxin 1. *Infect. Immun.* **2001**, *69*, 7169–7172, doi:10.1128/iai.69.11.7169-7172.2001.

32. Ruhé, P.Q.; Kroese-Deutman, H.C.; Wolke, J.G.C.; Spauwen, P.H.M.; Jansen, J.A. Bone Inductive Properties of rhBMP-2 Loaded Porous Calcium Phosphate Cement Implants in Cranial Defects in Rabbits. *Biomaterials* **2004**, *25*, 2123–2132, doi: 10.1016/j.biomaterials.2003.09.007.

33. Davison, N.L.; Gamblin, A.L.; Layrolle, P.; Yuan, H.; de Bruijn, J.D.; Barrère-de Groot, F. Liposomal Clodronate Inhibition of Osteoclastogenesis and Osteoinduction by Submicrostructured Beta-Tricalcium Phosphate. *Biomaterials* **2014**, *35*, 5088–5097, doi: 10.1016/j.biomaterials.2014.03.013.
34. Croes, M.; Kruyt, M.C.; Loozen, L.; Kragten, A.H.; Yuan, H.; Dhert, W.J.; Öner, F.C.; Alblas, J. Local Induction of Inflammation Affects Bone Formation. *Eur. Cell. Mater.* **2017**, *33*, 211–226, doi:10.22203/eCM.v033a16.



Closing Chapters

Summary in English

Fractures are the most common injury to the skeletal system. Typically, upon fracture, surgeons often use a cast to stabilize the damage, and the bone's regenerative capacity does the job of healing the fracture. However, 5-10% of fractures undergo delayed or impaired healing leading to a non-union. Significant bone defects and extensive bone loss after tumor resection or revision surgeries also pose a significant challenge when it comes to bone regeneration. The current practice to treat these is to transplant the patient's own bone, harvested from another site to the fracture site. This transplantation is known as autografting. Autografts possess similar regenerative properties as bone. However, they have drawbacks like limited availability, increased operation time, and a success rate of about 58%-93% in applications like spinal fusions. Synthetic bone substitutes, comprising metals, polymers, ceramics, and composites, aim to address these challenges but lack the regenerative properties of the bone. Calcium phosphates (BCPs) have recently gained attention due to their non-inferior performance to autografts in spinal fusion applications. However, a 100% fusion rate is yet to be achieved with these BCPs.

Researchers are working towards achieving a 100% fusion rate by fortifying bone substitutes with powerful bone-promoting agents. One of the strategies to enhance bone substitutes is to engage and manipulate the host's immune system. Upon injury, the immune system is the first responder. In the fracture healing process, the early inflammation stage plays a crucial role in deciding the fate of the healed fracture. The early inflammatory stage starts with recruiting various immune cells at the fracture site, leading to fracture hematoma formation. This hematoma then serves as a temporary scaffold that supports interaction between immune cells and various progenitor cells. These progenitor cells, namely mesenchymal stem cells, differentiate and carry out the functions of bridging the fracture gap. This initial immune response can be modulated using biomaterials with different topography or inflammatory soluble factors like pro-inflammatory cytokines TNF, IL-6, or a combination of both strategies.

Taking inspiration from pathological conditions like osteomyelitis, heterotopic ossification, and infections, in which bone growth can be observed in an inflammatory response to bacterial agents, the use of bacterial antigens to promote bone formation was explored. Previously, we injected gamma-irradiated (killed) bacteria or bacterial cell wall fragments into rabbit tibiae. Interestingly, an increase in net bone volume was observed in the tibiae. These results led to the hypothesis that the inflammatory response induced by the bacterial antigens resulted in this phenomenon. Although bacterial antigens prove to be a way to enhance bone

formation, they pose a challenge for clinical translation purposes, as their production can result in batch-to-batch inconsistencies and cause off-target side effects. As an alternative, synthetic bacterial fragments, also known as pathogen recognition receptor ligands (PRR ligands), are a safer and tunable option as an immunomodulatory agent to promote bone formation.

Bacterial cell wall components are Pathogen recognition receptor ligands. PRR ligands can comprise lipoproteins and lipopolysaccharides (LPS), unmethylated bacterial DNA, single-stranded RNA, viral RNA, peptidoglycans, etc. PRR ligands can elicit a specific immune response via selective PRR activation and are known for their stability in the host system, as described in **Chapter 1**. These PRR ligands are currently evaluated as potential therapies in applications like asthma and immunotherapies for treating tumors. Given the advantages of PRR ligands over killed bacteria, we explored using them as immunomodulating agents that could enhance bone formation.

Our investigation began with exploring the impact of therapeutic pathogen recognition receptor (PRR) ligands on bone cell differentiation and inflammation using simple *in vitro* models as described in **Chapter 2.** We tested the effects of several ligands, ranging from lipopolysaccharides to bacterial DNA, on human mesenchymal stem cells or multipotent stromal cells (hMSCs), precursors of bone-forming cells, and monocytes, a subset of the immune cell population. Notably, nucleic acid-based ligands, specifically Polyinosinic acid-polycytidylic acid (Poly(I:C)) targeting Toll-like receptor 3 (TLR3) and C-class CpG oligodeoxynucleotide (CpG ODN C) targeting TLR9, demonstrated a fourfold increase in alkaline phosphatase (ALP) activity on day 10, indicating early osteogenic differentiation in hMSCs. Significantly, these ligands did not influence osteoclast formation or the expression of late osteogenic markers such as osteocalcin and osteonectin. On the other hand, MPLA, Curdlan, and Pam3CSK4 targeting NOD2, TLR5, and TLR2, respectively, did not affect osteogenic differentiation but effectively inhibited tartrate-resistant acid phosphate (TRAP) activity in monocytes. This inhibition was accompanied by an upregulation of pro-inflammatory cytokines, including TNF-α, IL-6, IL-8, and IL-10. Our findings position nucleic acid-based ligands as promising osteo-immunomodulators, facilitating early osteogenic differentiation without provoking an exaggerated immune response or interfering with osteoclastogenesis.

In the quest to enhance bone formation, the pivotal role of immune cells and their soluble factors in the bone healing process cannot be overstated. Recognizing the potential of immune modulation as a strategy for influencing osteogenic differentiation, we developed and implemented an *in vitro* model that integrates innate and adaptive immune cells in **Chapter 3**. Human peripheral blood mononuclear cells (hPBMCs) were isolated and cultured for 24 hours. The

conditioned medium (CM) obtained from hPBMCs after stimulation was then employed to assess its impact on the metabolic activity and osteogenic differentiation capacity of human bone marrow-derived mesenchymal stromal cells (MSCs). This model was subsequently used to test the immune-modulatory agents—specifically, the nucleic-acid-based PRR ligands, CpG ODN C and Poly(I:C), and lipopolysaccharide (LPS) targeting TLRs 9, 3, and 4, respectively. Interestingly, the CM from CpG ODN C and LPS-stimulated hPBMCs significantly increased alkaline phosphatase activity (ALP) in MSCs by threefold as compared to the unstimulated control, while Poly(I:C) CM did not enhance ALP activity. Noteworthy, direct stimulation of MSCs with the PRR ligands tested in this model did not increase ALP. These findings underscore the significance of soluble factors present in CM from PBMCs stimulated with immune-modulatory agents in enhancing the osteogenic differentiation of MSCs. The developed *in vitro* model holds promise as a valuable tool for screening immune-modulatory stimulants across various immune cell types, shedding light on their (indirect) effects on osteogenic differentiation and identifying specific soluble factors that may play a role in modulating bone healing.

Since gamma-irradiated *S. aureus* was one of the first immunomodulatory agents that showed promising results in a rabbit's tibia, we tested gamma-irradiated *S. aureus* in our newly developed model to characterize the inflammatory milieu caused by *S. aureus* stimulation in immune cells, identify soluble factors and subsequently their effects on MSC osteogenic differentiation in **Chapter 4**. Furthermore, we also aimed at mimicking the immune response of *S. aureus* by using mixtures of PRR ligands consisting of TLR2, TLR8, TLR9, and NOD2 agonists. Human peripheral blood mononuclear cells (hPBMCs) are exposed to γi *S. aureus* or different ligand mixtures, and the conditioned medium (CM) from these cells is collected for further analysis. The results demonstrate a significant increase in alkaline phosphatase activity (ALP) and matrix mineralization in hMSCs treated with CM from γi S. aureus or a ligand mixture targeting TLR2, TLR9, and NOD2. This osteogenic enhancement is associated with the upregulation of inflammatory signaling pathways and the expression of key osteogenic genes. The findings suggest that formulations containing PRR ligands can serve as osteogenic immune-modulatory agents, pending their testing in *in vivo* models.

In **Chapter 5**, we tested the previously identified nucleic acid based PRR ligands, CpG ODN C and Poly(I:C) in an ovariectomized rat model of closed femur fracture. Along with these PRR ligands, we tested gamma-irradiated *C. albicans* and a proinflammatory cytokine TNF-α. The immune stimulants were injected at the fracture site in the femur on Days 1 and 2 after the fracture. One set of animals was euthanized on day 3 to collect fracture hematoma to test the effect of these immune stimulants on the early inflammatory stage of fracture healing. After 28

days (about four weeks), animals were euthanized, and rat femurs were scanned using microCT. Weekly radiographs and microCT were used to assess the fracture healing outcomes. No significant effects of the immune stimulants were observed on the early inflammatory stage and fracture healing outcomes. Even though the reasons were likely multifactorial, these findings raised a concern about the use of PRR ligands and immune stimulants as bone-enhancing substitutes.

In conclusion, the use of PRR ligands for bone regeneration for clinical applications is still at a considerable distance, as discussed in **Chapter 6**. Even though single or mixtures of PRR ligands showed promising results *in vitro*, their dosing, species-dependent, and donor-dependent effects pose an enormous challenge for clinical translation. Advanced models that consider the interactions of immune cells and bone cells should be developed to ensure effective preclinical testing of immunomodulatory strategies.

Samenvatting in Nederlands

Fracturen (botbreuken) zijn het meest voorkomend letsel van het skelet. Gewoonlijk gebruiken chirurgen gips om de fractuur te stabiliseren, waarna het bot-eigen regeneratievermogen de fractuur geneest. Bij 5-10% van de fracturen treedt echter vertraagde of geen genezing op, resulterend in een non-union. Aanzienlijke botdefecten en uitgebreid botverlies na tumorresectie of revisieoperaties vormen ook een grote uitdaging als het gaat om botherstel. De huidige standaard behandeling is het transplanteren van bot van de patiënt zelf, afkomstig van een andere locatie, naar de plaats van de breuk. Deze transplantatie staat bekend als 'autografting'. Autografts hebben vergelijkbare regeneratieve eigenschappen als bot. Er kleven echter nadelen aan deze methode, zoals een beperkte beschikbaarheid, een langere operatietijd en een succespercentage van ongeveer 58%-93% bij bijvoorbeeld spinale fusies (wervelfusie). Synthetische botsubstituten, bestaande uit metalen, polymeren, keramiek of composieten, proberen aan uitdagingen tegemoet te komen, maar missen de regeneratieve eigenschappen van het bot. Calciumfosfaten (BCPs) hebben onlangs de aandacht getrokken vanwege hun niet-inferieure prestaties ten opzichte van autografts. Met deze BCPs is echter nog geen 100% fusie bereikt.

Onderzoekers werken aan het bereiken van een 100% fusiegraad door botsubstituten te versterken met krachtige botbevorderende stoffen. Eén van de strategieën om botsubstituten te verbeteren is het inschakelen en manipuleren van het immuunsysteem van de gastheer. Bij letsel reageert het immuunsysteem als eerste. In het genezingsproces van fracturen speelt de vroege ontstekingsfase een cruciale rol bij het bepalen van het lot van de te genezen fractuur. De vroege ontstekingsfase begint met het rekruteren van verschillende immuuncellen op de plaats van de breuk, hetgeen leidt tot de vorming van een breukhematoom. Dit hematoom dient vervolgens als een tijdelijke steiger die de interactie tussen immuuncellen en verschillende voorlopercellen ondersteunt. Deze voorlopercellen, de mesenchymale stamcellen of multipotente stromale cellen (MSCs), differentiëren en zorgen voor het overbruggen van de breuk. Deze initiële immuunrespons kan worden gemoduleerd met behulp van biomaterialen met verschillende topografieën of inflammatoire factoren zoals TNF, IL-6, of een combinatie van beide strategieën.

Geïnspireerd door pathologische aandoeningen zoals osteomyelitis, heterotope ossificatie en infecties, waarbij botgroei werd waargenomen als reactie op ontsteking door bacteriële agentia, werd het gebruik van bacteriële antigenen om botvorming te bevorderen onderzocht. Eerder injecteerden we gamma-bestraalde (gedode) bacteriën of fragmenten van de bacteriële celwand in de tibiae van konijnen. Interessant genoeg werd een toename van het netto botvolume in de

tibiae waargenomen. Deze resultaten leidden tot de hypothese dat de ontstekingsreactie geïnduceerd door het bacteriële antigeen resulteerde in dit fenomeen. Hoewel bacteriële antigenen een manier blijken te zijn om botvorming te bevorderen, vormen ze een uitdaging met betrekking tot de vertaling naar de kliniek, omdat hun productie kan leiden tot inconsistenties tussen batches en ze off-target bijwerkingen kunnen veroorzaken. Als alternatief zijn synthetische bacteriële fragmenten, ook bekend als pathogeenherkenningsreceptorliganden (PRR-liganden), een veiligere en preciezere optie als immuunmodulerend agens om botvorming te bevorderen.

Bacteriële celwandcomponenten zijn ook pathogene herkenningsreceptorliganden. PRR-liganden bestaan bijvoorbeeld uit lipoproteïnen en lipopolysacchariden (LPS), niet-gemethyleerd bacterieel DNA, enkelstrengs RNA, viraal RNA, peptidoglycanen, enz. . PRR-liganden kunnen een specifieke immuunrespons uitlokken via selectieve PRR-activatie en staan bekend om hun stabiliteit in het gastheersysteem, zoals beschreven in hoofdstuk 1. Deze PRR-liganden worden momenteel geëvalueerd als mogelijke therapieën in toepassingen zoals astma en immuuntherapieën voor de behandeling van tumoren. Gezien de voordelen van PRR-liganden, onderzochten we het gebruik ervan als immunomodulerende middelen die de botvorming zouden kunnen verbeteren.

Ons onderzoek begon met het onderzoeken van de invloed van therapeutische pathogene herkenningsreceptoren (PRR-liganden) op botceldifferentiatie en ontsteking met behulp van eenvoudige in-vitromodellen zoals beschreven in **hoofdstuk 2**. We testten de effecten van verschillende liganden, variërend van lipopolysacchariden tot bacterieel DNA, op menselijke mesenchymale stamcellen (hMSCs), voorlopers van botvormende cellen, en monocyten, een subset van de immuuncelpopulatie. Met name de op nucleïnezuur gebaseerde liganden, meer specifiek polyinosinezuur-polycytidylzuur (Poly(I:C)) gericht tegen TLR3 en C-klasse CpG oligodeoxynucleotide (CpG ODN C) gericht tegen TLR9, toonden een viervoudige toename van de activiteit van alkalische fosfatase (ALP) op dag 10, wat duidt op vroege osteogene differentiatie in hMSCs. Het is veelzeggend dat deze liganden geen invloed hadden op de vorming van osteoclasten of de expressie van late osteogene markers zoals osteocalcine en osteonectine. Aan de andere kant hadden MPLA, Curdlan en Pam3CSK4, gericht tegen respectievelijk NOD2, TLR5 en TLR2, geen invloed op de osteogene differentiatie, maar remden ze effectief de activiteit van TRAP (tartraatresistent zuurfosfaat) in monocyten. Deze remming ging gepaard met een verhoging van pro-inflammatoire cytokines, waaronder TNF, IL-6, IL-8 en IL-10. Onze bevindingen positioneren op nucleïnezuur gebaseerde liganden als veelbelovende osteo-immuunmodulatoren, die vroege osteogene differentiatie bevorderen zonder een overdreven immuunrespons uit te lokken of de osteoclastogenese te verstoren.

In de zoektocht naar het verbeteren van botvorming, kan de centrale rol van immuuncellen en immuunfactoren in het botgenezingsproces niet onderschat worden. We erkenden het potentieel van immuunmodulatie als een strategie voor het beïnvloeden van osteogene differentiatie en ontwikkelden en implementeerden een in vitro model dat aangeboren en adaptieve immuuncellen in **hoofdstuk 3** integreert. Menselijke mononucleaire bloedcellen (hPBMCs) werden geïsoleerd uit perifeer bloed en gedurende 24 uur gekweekt. Het geconditioneerde medium (CM) dat na stimulatie uit hPBMCs werd verkregen, werd vervolgens gebruikt om de invloed ervan op de metabole activiteit en osteogene differentiatiecapaciteit van MSCs te beoordelen. Dit model werd vervolgens gebruikt om de immuunmodulerende stoffen te testen, met name de PRR-liganden op basis van nucleïnezuren, CpG ODN C en Poly(I:C), en lipopolysaccharide (LPS), gericht tegen respectievelijk TLRs 9, 3 en 4. Het was interessant om te zien dat het kweekmateriaal van CpG ODN C en Poly(I:C) een effect had op de metabole activiteit en de osteogene differentiatie van MSCs. Opmerkelijk was dat het supernatant van hPBMCs die gestimuleerd waren met CpG ODN C en LPS de alkalische fosfatase activiteit (ALP) in MSCs verdrievoudigde in vergelijking met de ongestimuleerde controle, terwijl supernatant van hPBMCs gekweekt met Poly(I:C) de ALP-activiteit niet verhoogde. Directe stimulatie van MSCs met de PRR-liganden die in dit model werden getest, verhoogde de ALP-activiteit niet. Deze bevindingen onderstrepen het belang van de geproduceerde immuunfactoren die aanwezig zijn in CM van PBMCs gestimuleerd met immuunmodulerende middelen bij het verbeteren van de osteogene differentiatie van MSCs. Het ontwikkelde in vitro model is veelbelovend als instrument voor het screenen van immuunmodulerende stimuli voor verschillende typen immuuncellen. Het kan licht te werpen op hun (indirecte) effecten op osteogene differentiatie en een rol spelen bij de identificatie van specifieke oplosbare immuunfactoren voor het moduleren van botgenezing.

Aangezien gammabestraalde S. aureus één van de eerste immunomodulerende middelen was die veelbelovende resultaten liet zien in de tibiae van konijnen, onderzochten we het effect van gammabestraalde S. aureus in ons nieuw ontwikkelde model op de productie van immuunfactoren door immuuncellen en vervolgens het effect op osteogene differentiatie van MSCs in **Hoofdstuk 4**. Verder probeerden we ook de immuunrespons van dode S. aureus na te bootsen met mengsels van PRR-liganden bestaande uit TLR2-, TLR8-, TLR9- en NOD2-agonisten. Menselijke mononucleaire bloedcellen (hPBMCs) werden blootgesteld aan bestraalde S. aureus of verschillende ligandmengsels, en het geconditioneerde medium (CM) van deze cellen werd verzameld voor verdere analyse. De resultaten tonen een significante toename in alkalische fosfatase activiteit (ALP) en matrixmineralisatie in hMSCs behandeld met CM van bestraalde S. aureus of een ligandmengsel gericht op TLR2, TLR9 en NOD2. Deze osteogene effecten

kunnen geassocieerd worden met de verhoging van inflammatoire signaaltransductieroutes en de daarop volgende expressie van belangrijke osteogene genen. De bevindingen suggereren dat formuleringen die PRR-liganden bevatten kunnen dienen als osteogene immuunmodulerende middelen, die hun werking in een in vivo model nog moeten laten zien.

In **hoofdstuk 5**, testten we de eerder geïdentificeerde PRR-liganden op basis van nucleïnezuren, CpG ODN C en Poly(I:C) in een rattenmodel (ovariëctomie) van een gesloten dijbeenfractuur. Samen met deze PRR-liganden testten we gammastraal bestraalde C. albicans en een pro-inflammatoire cytokine TNF. De immuunstimulerende middelen werden op dag 1 en dag 2 na de fractuur geïnjecteerd op de plaats van de fractuur in het dijbeen. Eén set dieren werd geëuthanaseerd op dag 3 om het fractuurhematoom te onderzoeken tijdens de vroege ontstekingsfase van de fractuurgenezing. Na 28 dagen werden de femora van de ratten gescand met microCT. Wekelijkse röntgenfoto's en microCT werden gebruikt om de resultaten van de fractuurgenezing te beoordelen. Er werden geen significante effecten van de immuunstimulerende middelen waargenomen op de vroege ontstekingsfase of op de fractuurgenezing. Hoewel werd aangenomen dat de redenen hiervoor multifactorieel zijn, gaven deze bevindingen aanleiding tot vraagtekens bij het gebruik van PRR-liganden en immuunstimuli als botversterkende substituten.

Concluderend kan gesteld worden dat het gebruik van PRR-liganden voor botregeneratie in klinische toepassingen nog ver weg is, zoals besproken in **hoofdstuk 6**. Hoewel enkelvoudige of mengsels van PRR-liganden veelbelovende resultaten lieten zien in vitro, vormen hun dosering, soort- en donorafhankelijke uitkomsten een enorme uitdaging voor vertaling naar de kliniek. Er moeten geavanceerde modellen worden ontwikkeld die rekening houden met de interacties tussen immuuncellen en botcellen om effectieve preklinische evaluatie van immunomodulerende strategieën te kunnen waarborgen.

Acknowledgment

Well, so far... everything you have read in this book was either way too technical or mind-blowingly interesting…but it wouldn't have been possible without my battalion providing me constant support, motivation, guidance, knowledge with occasional offers of shoulders to cry on or vent over a glass of wine. I want to take this opportunity to thank all of you who have been part of this incredibly intense journey of over 4 years!

Let me begin by acknowledging the efforts my promoters, Moyo, Harrie, and Debby, have put in throughout these years.

Moyo, you have always been there, providing critical and yet audacious remarks on everything. At first, I must say, I was quite intimidated by you, as you were always so on the point with your remarks. But with time, I looked forward to our discussions. I have always admired your passion for spines and hope that I find the courage to follow my passion too.

Harrie, I would have never made it to the finish line without you. I am immensely grateful for you to be on my side through my lows and highs. Thank you for believing in me and listening to me even when I was complaining about petty things. Your enthusiasm toward science has been a great inspiration. You found something good despite the not-so-good results and motivated me to move forward. You have been an amazing supervisor and mentor.

Debby, we met due to circumstances, but I am grateful you became my supervisor. You came to the project with a fresh perspective and many logical questions, which made me think outside the box. It was amazing to see how quickly we had gelled up. You always had my back, especially when I could not speak up for myself. Your love for cocktails was evident if you know what I mean 😊. I appreciate your efforts to try and bring the group together, whether through retreats, walks in the forest, or dinner parties.

Michiel and Saber, thank you so much for your guidance and help in the lab. Thank you for passing on all the skills and knowledge you gained from the lab. I am sure I have used many of the tricks and techniques that you taught throughout these years.

The main magicians behind all the experiments conducted in the lab are **Mattie** and **Anneloes**. You guys are just amazing. **Mattie**, you have been so patient with me, and somehow, you always find things in the lab that I could never find. From your restaurant suggestions to histology expertise, your diverse knowledge has been a compass guiding me through both the culinary and academic landscapes.

Anneloes, You have been so kind in helping me test all the new batches of FBS and taking over the coordination whenever I was on vacation.

Finally, **Anna**, **Lotte**, **Camilla**, and **Ruggero**, you guys were incredible contributors in shaping this thesis. I hope I managed to ignite the passion for science in all four of you. I certainly learned a lot from you all. May all your future endeavors be successful and fulfilling.

A big thank you to my meeting groups. Starting with the formerly known as iBONE… 'BONE' meeting group: **Flurina**, **Leanne**, **Kenny**, **Anneli**, **Nada**, **Zhiming**, **Alexandra**, and all the students, for your constructive and always stimulating discussions accompanied by occasional cakes and cookies. Thanks to **Flurina** for being supportive and patiently answering my questions and requests. I had a great time with you guys on retreats and dinner parties.

I am immensely grateful to be a part of the Friday meeting group known as the Harrie group with **Azin**, **Jaqueline**, **Kelly**, **Pardis**, **Zhiming**, **Zhengquing**, **Nada**, **Anneli**, **Lotte**, **Fatemeh**, **Ruben**, **Zijan** and all the students. Even though we all worked on different topics, the feedback, and ideas you gave were always helpful. **Nada**, you are the only one who understands the struggle of working in such a new and upcoming field. It was nice to talk to you since we were in the same boat. The conference in Greece with you was the most fun trip! **Azin** and **Jaqueline**, you guys are very approachable, whether talking about science or life; you always motivated and helped me with my questions. **Kelly**, thank you for collaborating with me on my projects; it was nice discussing immune cells and signaling pathways with someone equally enthusiastic. **Pardis**, I miss our coffee sessions so much! **Zhiming**, it was a pleasure working with you, showing you around in the lab and brainstorming discussions about new projects and PhD tips!

Anneli, you are amazing and a sweetheart! You have been a strong pillar of support throughout… you have been my doctor, guide, mentor, bestest friend, my reality check… in all, You are my Cristina to Meredith. From talking on the phone nonstop for 3 hours (latest record) discussing my millennial problems to our research discussions, from my first and last biking trip to Dreibergen to going in doubles on your bike, we did it all. You have always been my go-to person, from planning strategies to drafting email replies, calling you crying over silly issues, trying new hobbies, and going to new restaurants together. I am so grateful to the Netherlands because I met you. Cheers to our friendship, and I hope it stays forever and ever. You made the Netherlands homely for me, so thank you very very much for everything…

Matthijs and **Nicole**, you both have been very warm, welcoming, and perfect chaperones for my introduction to the Dutch culture. The one person who is loyal to my cooking is **Matthijs**. Thank you for that unique orange hat on King's Day.

You do make amazing pancakes! Still waiting for that stamppot that you promised! **Nicole**, thank you for bringing in those donuts on my bad day. Just having a drink and talking to you about it made it better. I will never forget that.

Thank you so much, **Akanksha** and **Ranjeet**. You both have been amazing roommates. Thank you for all the help in organizing crazy parties and listening to me crib over work after a very long day. I do miss our long meetings before every meal about what to cook and going for long walks after… **Varsha** and **Avinash**, you both rock! I wasn't sure what would kill me, sky diving or your jokes! we all should catch up soon! **Phani** and **Tanu,** thank you for being amazing friends throughout the years!

Now, I am moving on to my first family in the Netherlands, the **RESCUE** family. I am thankful to all of you for being there and helping each other get through the first few months. From parties to dinners, it has been a pleasure to know each one of you.

My very first friend in Holland**, Leonardo,** you are a great human with a unique personality, and I am grateful that I have the privilege to be your friend. You have always been available to listen to me and provide good suggestions, and most importantly, you taught me how to drink away all my worries, I hope you are proud of me now. It was big of you to make pizza and quiche for me that ne time. I will always cherish those long evening we spent chatting over dinner.

Lorenzo darling, you are a delight! Thank you for feeding me such amazing Italian food and teaching me only curse words. I enjoyed every conversation over coffee, especially the ones on the 4th floor of the UMC corridor; they were the best! You are the best girl gang**, TJ, Suji, and Vanessa**! Thank you for the beautiful memories we made together. You guys have been so motivating all these times. Thank you so much for everything! **Leanne**, thank you so much for being there and planning the best bachelorette party I could have ever dreamt of! **Ali, Madi, Stefan, Flurina, Alessandro**, and **Laura**, thank you for the amazing memories we made together, whether a simple outing or being drunk on Pride parade day. I will cherish them forever.

Thank you, **Bindi**, **Birwa, Harsh**, **Priyal**, **Milan**, **Mayur** and **Ranjani,** for being my long-distance cheer leaders!

Even though getting a PhD was my dream, it was way more important for my **Mummy** and **Papa**. Thank you, Mummy and Papa, for pushing me when I was lazy, taking my side when I was sad, even if I was wrong, and providing daily encouragement and motivation. You have been my pillar of strength through thick and thin. This one is for you, **Mummy Papa**!

Mansi, I don't even know how to thank you. No words describe how much you mean to me and how grateful I am for everything you have done for me. Thank you for sticking with me no matter what is in front. Thank you, **Jinay Jiju** and **Viransh**, for being supportive and encouraging.

Thank you, **Smita Mummy**, **Mahesh Papa, Dhara Didi, Ankit Kumar, Dhruva, and Vihaan;** I have reached this stage with your blessings and support.

Lastly, I want to take this opportunity to appreciate my soulmate and life partner, **Chirayu**. This journey has been difficult for both of us, but your unwavering faith in me got me this far. I admire the patience and positivity you have developed over the years to deal with me 😊. You never let me fall. It was hard for me to move to the Netherlands with you being in Germany, but you made it work for both of us and never complained. I am just glad that you came into my life and never left. I could have never imagined a better life partner than you, thank you so much, I love you!

List of Publication

Khokhani, Paree, Nada R. Rahmani, Anne Kok, F. Cumhur Öner, Jacqueline Alblas, Harrie Weinans, Moyo C. Kruyt, and Michiel Croes. "Use of therapeutic pathogen recognition receptor ligands for osteo-immunomodulation." Materials 14, no. 5 (2021): 1119.

Khokhani, Paree, Ruggero Belluomo, Michiel Croes, Debby Gawlitta, Moyo C. Kruyt, and Harrie Weinans. "An In Vitro Model to Test the Influence of Immune Cell Secretome on Mesenchymal Stromal Cell Osteogenic Differentiation." *Tissue Engineering Part C: Methods* 28, no. 8 (2022): 420-430.

Khokhani, Paree, Kelly Warmink, Moyo C. Kruyt, Harrie Weinans, and Debby Gawlitta. Mixtures of PRR ligands partly mimic the immunomodulatory response of γi *Staphylococcus Aureus*, enhancing osteogenic differentiation of human mesenchymal stromal cells. *Submitted.*

Khokhani, Paree, Carissa Hing-Wai Hong, Michiel Croes, Simon Chow, Wing Hoi Cheung, Saber Amin Yavari, Debby Gawlitta, Moyo C. Kruyt, and Harrie Weinans. *In vivo* screening of immune stimulants in an ovariectomy induced osteoporotic femoral fracture in rats. *Submitted.*

Nada R. Rahmani, Anneli Duits, **Khokhani, Paree**, Michiel Croes, Vela Kaludjerovic, Jacqueline Alblas, Debby Gawlitta, Harrie Weinans, and Moyo C. Kruyt. Testing the application of microbe-derived agents to promote osteogenesis. *Under preparation.*

About the Author

Paree Khokhani was born on 16 September 1993 in Mumbai, India. She graduated from Mumbai University in 2011. She started her bachelor's degree program in Biomedical Engineering at Thadomal Shahani Engineering College, under Mumbai University, India, to pursue her interests in Biology and technology. After graduation, she soon moved to Germany to pursue a master's in biomedical technologies from Eberhard Karl University of Tübingen. During her master's, she specialized in bioimaging and implantology. During this period, Paree carried out her master's thesis research project at NMI Institute, Reutlingen, under the supervision of Dr. Christopher Shipp. Here, she acquired skills in testing biomaterials and developed in vitro models to test immune compatibility towards biomaterials with different topographies and roughness. After graduating in December 2018, Paree continued to work on the same project for three months. In March 2019, she moved to the Netherlands to start a PhD at the Department of Orthopedics at the University Medical Center, Utrecht, under the regenerative medicine program. As a part of her PhD, Paree started working on strategies to promote bone regeneration, focusing on immune modulation. The results of her work are described in this thesis and published in peer-reviewed scientific journals. During her PhD, she attended several national and international conferences and supervised students during their internships. Post PhD, Paree is transitioning from academia to life sciences industry and currently in training for EU MDR regulation courses.

PhD training Certificate – Graduate School of Life Sciences

Discipline-specific educational activities	**#ECTS**
Introduction to Flow Cytometry	0.25
Introduction to Regenerative Medicine	1.5
RM PhD retreat	1.5
Enabling Technologies	1.5
Adobe Illustrator	0.4
Bench to Bedside	1.5
Supervising MSc students: Starter Kit	0.3
Introduction to R data programming in life sciences	3.3
Writing Scientific paper	1.5
Early clinical Evaluation	0.3
Statistics in Laboratory	1.5
Laboratory animals in Science	3
General educational activities	**#ECTS**
Responsible Conduct of Research (*compulsory*)	0.15
Dutch Language course for Fellows	1
Personal Development	0.1
Analytical Story telling	0.7
Giving effective presentations	0.6
Adobe inDesign essentials	0.6
Research planning and time management	0.4
Science communication clear and relevant	2
Ethics in RM	0.3
Symposia/conferences (oral or poster presentation) or other activities	**#ECTS**
RMU 'Meet your neighbours'	1
RM Retreats	3
Oral presentation at NBTE 2020	1
Oral presentation at TERMIS 2021	1
Oral presentation at osteoimmunology conference	1
Oral presentation at NBTE 2022	1
TOTAL	**29.4**