Osteomyelitis is an infectious disease affecting bone and bone marrow,1 with *Staphylococcus aureus* being the causative pathogen in 30% to 60% of cases in humans2 and staphylococci collectively causing approximately 75% of cases.3 Osteomyelitis generally develops from a hematogenous or traumatic source.1,4 Hematogenous osteomyelitis most often occurs in children aged <16 years as the result of hematogenous spread of bacteria (eg, due to bacteremia).1 The second route, traumatic, may occur secondary to fractures or surgery.4 Herein, the protective barrier of the skin is disrupted, allowing contaminating bacteria to enter the wound and invade bone tissue.4 In fact, all orthopedic surgeries are at risk of developing osteomyelitis via the traumatic route because of the risk of bacterial translocation from the skin to the surgical site intraoperatively.4

Osteomyelitis patients may display clinical symptoms, such as pain, swelling, purulent drainage, fistula and/or sinus presence, wound breakdown, erythema, and increased local temperature.5,6 In addition, the following parameters are considered: i) signs of bone destruction and the presence of sequestra and purulent collection in soft tissue assessed with imaging, ii) occurrence of organisms in more than one deep specimen verified with microbiological cultures, and iii) leukocyte counts, erythrocyte sedimentation rate, and C-reactive protein levels.5,6 Infection can be confirmed by histopathologic examination of deep tissue samples; the presence of microorganisms in deep tissue is examined using specific staining techniques for bacteria (eg, Gram stain or Ziehl-Neelsen stain for tuberculosis) or fungi (eg, Grocott methenamine silver stain).7 For culture-negative patients, the diagnosis of osteomyelitis can be confirmed histologically if there are signs of active bone resorption and

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remodeling and the presence of acute and chronic inflammatory cells.\textsuperscript{5} Acute inflammatory cell infiltrate is demonstrated by identifying more than five polymorphonuclear cells (PMNs) per high-power (×400 magnification) field.\textsuperscript{8} This criterion is also used for other orthopedic infections, such as periprosthetic joint infections\textsuperscript{9} and chronic/late-onset fracture-related infections.\textsuperscript{6} The complete absence of PMNs strongly correlates with aseptic nonunion (specificity, 98%; positive predictive value, 98%).\textsuperscript{6}

Treatment regimens in acute, uncomplicated cases of osteomyelitis may consist of antibiotic therapy alone if preconditions are met,\textsuperscript{7} which is typically administered for 4 to 6 weeks and is associated with a success rate of approximately 80%.\textsuperscript{4} In contrast, for chronic and for implant-associated osteomyelitis, the success of antibiotic therapy alone is relatively low and requires debridement (ie, surgical removal of infected bone and implant components) to achieve satisfactory success rates.\textsuperscript{3} These debridement or revision surgeries are often challenging, given that the extent of bone debridement can be difficult to judge, and the management of the resultant dead space can also require complex interventions and prolonged healing time.\textsuperscript{4} Despite best practice in medical and surgical therapy, there remains a 20% chance of treatment failure in such complicated cases.\textsuperscript{10}

Our understanding of the pathophysiology of osteomyelitis has evolved over recent years, and we now have a better insight into why chronicity and the presence of an implant require more vigorous treatment. For instance, we know that bacterial biofilm formation and bacterial invasion within the osteocyte lacuna-canalicular system are involved in chronic osteomyelitis.\textsuperscript{11} Furthermore, the host response to the infection and subsequent changes in bone morphology (eg, sequestrum or involucrum formation) are also better understood at the present time. Much of this new understanding of the underlying pathophysiology of osteomyelitis (eg, bone turnover, osteolysis, and bacteriological changes over time) has been determined from histologic analyses of individual human specimens\textsuperscript{12–14} and laboratory animal studies.\textsuperscript{15,16}

In other fields, advanced \textit{in vitro} cell culture models have been developed to reduce our reliance on laboratory animals. In particular, three-dimensional (3D) cell culture has become a standard in the fields of tissue engineering and cancer biology, where the organotypic 3D constructs have been shown to i) have their own microenvironment, ii) resemble \textit{in vivo} tissue organizations, and iii) have cellular behavior that more faithfully reflects the \textit{in vivo} setting. When using human cells, these systems may be more reflective of the human situation than currently used preclinical \textit{in vitro} two-dimensional culture systems.\textsuperscript{17} These models could also serve to improve our understanding of osteomyelitis, although until now, only one study has used an advanced cell culture system to study osteomyelitis,\textsuperscript{18} to the best of our knowledge.

In this review, we describe the pathophysiology of osteomyelitis and the currently used \textit{in vitro} systems for osteomyelitis research, with the focus on the most recent discoveries and improved mechanistic understandings of the pathophysiology of osteomyelitis. Furthermore, we review published advanced multicellular \textit{in vitro} models and their potential to further our understanding of human osteomyelitis, without requiring the use of experimental animals.

Pathophysiology of Osteomyelitis

Pathogen Factors

\textit{Staphylococcus aureus} arriving at the bone surface via iatrogenic or hematogenous routes can readily adhere to soft tissue, bone,\textsuperscript{1} or metal implants.\textsuperscript{19} The bacterium may achieve this through binding to extracellular matrix (ECM) proteins via microbial surface components recognizing adhesive matrix molecules, such as collagen-binding protein and bone sialoprotein binding protein.\textsuperscript{20} Herein, \textit{S. aureus} employs multiple survival strategies to not be affected by immune cells and therapies (Figure 1A). Adherent \textit{S. aureus} may multiply, aggregate, and form microcolonies,\textsuperscript{15,21} which are also known as staphylococcal abscess communities (SACs) (Figure 1D).\textsuperscript{22,24}

SACs are not exclusively found in bone tissue; they have been observed in skin, kidney, renal, and brain tissues.\textsuperscript{25–27} In most cases, the SACs form the center of an abscess structure with surrounding fibrin deposits.\textsuperscript{25,27,28} Specifically, \textit{S. aureus} can form fibrin by promoting polymerization of fibrinogen and secreting enzymes, such as coagulase and von Willebrand factor—binding protein, which activate endogenous prothrombin and contribute to fibrin formation. This fibrin network surrounding the bacterial SACs protects the bacteria from invasion and clearance by immune cells, such as PMNs,\textsuperscript{25} causing the immune cells to gather around the bacterial nidus (Figure 1D).\textsuperscript{25,29–27}

\textit{Staphylococcus aureus} adhering to implanted devices or sequestra may form an even more complex structure known as a biofilm (Figure 1D).\textsuperscript{19,23} Bacteria in biofilms are less susceptible to antibiotics, because of several factors, including reduced oxygen levels and metabolism.\textsuperscript{29} Furthermore, \textit{S. aureus} resident in biofilm may secrete the so-called extracellular polymeric substance matrix consisting of self-produced polysaccharides and proteins and possibly extracellular DNA from dead bacterial cells, forming a matrix that functions as a physical barrier to immune cell infiltration.\textsuperscript{29}

Besides clustering in SACs or into biofilm, \textit{S. aureus} can also invade the osteocyte-lacuna canaliculi networks within bone (Figure 1D).\textsuperscript{11,50} Recently, invasion of \textit{S. aureus} in the submicron channels buried deep within the dense mineral matrix of cortical bone has been discovered.\textsuperscript{11,50} Invasion of \textit{S. aureus} within the canalicular network could be a mechanism to promote persistence and chronic infection, with the potential to limit access to immune cells. This novel persistence mechanism was originally identified in a mouse model of implant-associated osteomyelitis, and was
subsequently confirmed in a human *S. aureus* diabetic foot infection.\(^1\,\^3\) This discovery is particularly concerning in the context of *S. aureus* osteomyelitis, as these canalicular networks may be impenetrable by immune cells, and bacteria can possibly survive in this space for a long period of time, using bone matrix as a nutrient source.\(^3\) However, this has not been proved yet.

As another defense mechanism, *S. aureus* has a wide range of toxins that target host cells. These toxins include exfoliative toxins, pore-forming toxins, and superantigens.\(^3\)

The superantigen toxic shock syndrome toxin 1 has been associated with bone infection.\(^3\) It has been shown that toxic shock syndrome toxin 1 can promote osteoclastogenesis and bone resorption activity of osteoclasts *in vitro*.\(^3\)

**Figure 1** A mechanistic illustration of the pathophysiology of osteomyelitis. **A:** Bacteria: survival strategies of *Staphylococcus aureus* in bone to circumvent immune cell responses and therapies are i) forming biofilm that contains an extracellular polymeric substance matrix, ii) growing as staphylococcal abscess communities (SACs) as part of an encapsulated abscess, or iii) performing intracellular colonization of host cells. **B:** Host: bacterial presence in bone initiates an influx of immune cells. During acute inflammation, polymorphonuclear cells (PMNs) predominate, and during chronic inflammation, macrophage numbers increase. PMNs secrete proinflammatory cytokines, including IL-1β and tumor necrosis factor (TNF)-α, and release neutrophil extracellular traps (NETs) to facilitate bacterial killing. Macrophages are skewed toward a wound healing and profibrotic phenotype. Adaptive immune responses include T- and B-cell responses. However, T-cell responses are skewed by *S. aureus* toward type 1 and type 17 helper T cell biased immune responses. *Staphylococcus aureus* protein A (SpA) binds to antibodies secreted by B cells and consequently blocks antibody-mediated phagocytosis. **C:** Bone: bacterial presence promotes i) host cells to secrete probone resorptive cytokines, causing, together with SpA binding, bone resorption by osteoclasts, ii) osteoblasts to form new bone because of internalization of the bacterium and SpA binding, and iii) secretion of the chemokine CXCL12 binding chemokines CXCL9, CXCL10, and CXCL11 for T-lymphocyte recruitment by (invaded) osteocytes. **D:** An overview of the different osteomyelitis components accompanied by *in vivo* images of the following: 1, a staphylococcal abscess commu (SAC) (asterisk) surrounded by immune cells (arrowheads), as observed in a hematoxylin and eosin stained paraffin-embedded section containing an *S. aureus* infected murine femur\(^2\); 2, biofilm on a polyether ether ketone fixation plate imaged with scanning electron microscopy\(^2\); 3, *S. aureus* within an osteocyte canalculus\(^1\); and 4, *S. aureus* bacterium (arrow) within a PMN\(^3\), both observed with transmission electron microscopy. Green cells, *S. aureus*; black strands, fibrous tissue; gray cells, dead cells; pink cells, PMNs; purple cells, macrophages; orange cells, generic cells; pink cell with purple-gray strands, PMNs undergoing NETosis; light green cells, T cells; blue cells, B cells; multinuclear yellow cells, osteoclasts; yellow cells, osteoblasts; elongated yellow cells, osteocytes. **D:** Image 1 reprinted from Brandt et al\(^2\) with permission (Copyright 2018. The American Association of Immunologists, Inc.); image 2 reprinted from Inzana et al\(^2\) with permission; image 3 reprinted from de Mesy Bentley et al\(^1\) with permission; and image 4 reprinted from Horst et al\(^2\) with permission. Images in A–C and the schematic in **D** were generated with BioRender (Toronto, ON, Canada). Scale bars: 100 μm (**D**, image 1); 2 μm (**D**, images 2 and 4); 1 μm (**D**, image 3).
Also, pore-forming toxins have been linked to bone infection. Staphylococcus aureus pore-forming toxins can be subdivided into leukotoxins, hemolysin-α, hemolysin-β, and phenol-soluble modulins; and these proteins affect host cell membrane integrity. For phenol-soluble modulins, it has been demonstrated in vitro to have a cytotoxic effect on osteoblasts, whereas the hemolysin-α caused both osteoblast and osteoclast cell death in vitro. Hemolysin-β may be involved in phagosomal escape, as shown in vitro in PMNs, and in vivo it stimulated biofilm formation. Furthermore, human osteomyelitis patients infected with an S. aureus strain that can secrete the leukotoxin Panton-Valentine leukocidin had a more aggressive and more difficult to treat infection.

In addition, S. aureus can invade and survive intracellularly in professional phagocytes, as well as nonprofessional phagocytes (Figure 1D). Staphylococcus aureus triggers phagocytic internalization by expressing fibronectin-binding proteins (A and B), adhering to fibronectin, and connecting to ε2β1 integrins on macrophages or neutrophils. After internalization, S. aureus evades cell death in these cells by persisting within vacuoles or by inhibiting phagosomal fusion. It can also infect and survive within non-professional phagocytes, such as primary human osteoblasts in vitro, mouse osteoclasts in vitro and in vivo, and human osteocytes in vitro and ex vivo. This intracellular persistence provides the pathogen with crucial protection needed against the onslaught of the immune system and antibiotic treatments. Staphylococcus aureus—infected human osteoblasts may also mature into osteocytes and remain infected. To survive intracellularly, S. aureus frequently adopts a dormant small colony variant phenotype, characterized by slow growth and reduced metabolic activity. The numerous mechanisms by which S. aureus is able to survive intracellularly within the bone niche for long periods is a primary cause of chronic and recurrent osteomyelitis.

Host Factors

The presence of bacteria within the bone tissue triggers a host response, which encompasses an innate immune response primarily driven by PMNs, macrophages, and adaptive responses mediated by T cells, B cells, and pathogen-specific antibodies (Figure 1B).

First, on bacterial recognition, resident macrophages in bone, osteocytes, and osteoblasts all appear able to secrete chemoattractants to initiate an influx of immune cells to the site of infection. An influx of PMNs during acute osteomyelitis occurs in both humans and in rodent osteomyelitis models. Inflammatory macrophages [myeloid related protein 8 (MRP8)/MRP14 positive] and CD4 T cells, potentially activating PMNs, have also been observed in humans; and many necrotic immune cells are present in rodent osteomyelitis models. PMNs can efficiently kill planktonic S. aureus via phagocytosis, oxidative bursts, and production of antimicrobial peptides, whereas secretion of proinflammatory cytokines and chemokines, such as tumor necrosis factor (TNF)-α, IL-1β, CXCL2, CXCL3, and others, activates and recruits PMNs, which ultimately leads to pathogen clearance. PMNs and macrophages also elicit direct host defense responses by forming neutrophil extracellular traps, to trap bacteria, which are eventually cleared by immune cells.

As the infection persists and becomes chronic, bacteria tend to form a biofilm phenotype, and the influx of viable PMNs decreases drastically, as demonstrated in mice and observed in humans with chronic osteomyelitis. In humans, most cells present at the site of chronic infection are wound-healing M2 macrophages (CD163 positive), accompanied by a small number of CD8 T cells and plasma cells.

Adaptive immune responses against bone infections include both T- and B-cell responses. Unfortunately, pathogens, such as S. aureus, have evolved numerous evasion mechanisms toward these responses, resulting in chronic osteomyelitis. For instance, in a porcine osteomyelitis infection model, it was observed that the antibody responses against intracellular S. aureus in biofilms are skewed to a predominantly type 1 and type 17 helper T cell biased immune response, which cannot effectively clear intracellular pathogens. Staphylococcus aureus can also efficiently manipulate B cells, affecting their survival and function via the secretion of staphylococcal protein A (SpA), which associates with the Fcγ and Fab domains of certain antibodies, blocking antibody-mediated phagocytosis and simultaneously causing proliferative B-cell apoptosis.

In addition, pathogen-specific antibodies produced by circulating plasmablasts and plasma cells are often not protective against chronic bone infections. Although further studies are needed to fully understand this, it may be that due to SpA, interference antibodies secreted against S. aureus do not confer protection against reinfection or chronic musculoskeletal infections or that these antibodies are nonneutralizing antibodies. In fact, anti-S. aureus IgG responses against certain antigens can lead to mortal outcomes. Nonetheless, these antibody responses can be useful diagnostic and prognostic biomarkers for identifying orthopedic infections.

Bacterial Interactions with Skeletal Cells

Bone is a mineralized organic matrix containing osteocytes, bone-forming osteoblasts, and bone-resorbing osteoclasts. All three bone cells are impacted directly and indirectly by S. aureus (Figure 1C).

Directly, SpA binding to TNF receptor-1 on osteoblasts results in an increase in apoptosis and a decrease in...
Osteomyelitis Pathology and Methods

Differentiation and calcium deposition of the osteoblasts. Moreover, internalization of S. aureus through fibronectin-binding protein A/B–α5β1 integrin bridging affects osteoblast viability and functioning. Both S. aureus internalization and SpA binding cause decreased bone formation and inhibition of matrix mineralization. Conversely, S. aureus infection increases periosteal bone formation by osteoblasts (as shown in rabbits) (Figure 2A) compared with noninfected controls (Figure 2B).

Osteoclasts up-regulate their bone resorption capacity because of TNF and epidermal growth factor receptor activation through SpA secreted by S. aureus. This leads to resorption lacunae formation and necrotic bone pieces, as observed in biopsies of human osteomyelitis patients (Figure 2C) and in vivo osteomyelitis models (Figure 2D). Indirectly, osteoclasts are activated and increase osteolysis activity by osteoblasts, osteocytes, and PMNs. These cell types secrete receptor activator of NF-κB ligand (RANK-L), which drives osteoclastogenesis and activates osteoclasts to resorb bone. Osteoclasts do so in response to a neighboring osteocyte that underwent apoptosis (eg, due to invasion of its lacunae by S. aureus). Moreover, osteoblasts up-regulate RANK-L expression when SpA is bound to TNF receptor-1 and by bacterial internalization, whereas PMNs up-regulate RANK-L secretion by toll-like receptor 4 activation. PMNs also drive osteoclastogenesis and bone resorption via osteoclast-mediated secretion of IL-8. Another contributor to osteoclastogenesis and osteoclast activity is the persistent inflammatory environment itself. This occurs initially because of the secretion of the proresorptive cytokines IL-6, TNF-α, and IL-1β by immune cells and osteoblasts, and subsequently because of hypoxia resulting from the persistent inflammation.

A crucial role of osteocytes is to mature and maintain the mineralized matrix, which is accomplished by their expression of enzymes capable of reversibly removing mineral and remodeling the organic phase of bone matrix, a process described as osteocytic osteolysis or perilacunar remodeling. The involvement of this process during osteomyelitis is currently poorly described, although matrix metalloproteinase expression was observed to be induced in S. aureus infected human osteocytes, suggesting that osteocytic osteolysis is affected by S. aureus. Another interesting function of osteocytes is their potential role in the recruitment of immune cells. A recent study demonstrated that human osteocyte-like cultures exposed to S. aureus resulted in the differential expression of >1500 genes, including the robust induction of a large number of chemokines and cytokines. Although classic PMN chemoattractants, such as CXCL1 and chemokine (C-C motif) ligand 5, were detected, CXC chemokine receptor 3 (CXCR3)-binding chemokines CXCL9, CXCL10, and CXCL11 were also expressed in abundance, suggesting the potential participation of osteocytes in the adaptive immune response to bacterial infection by recruiting cytotoxic and/or suppressive T-lymphocyte subsets to the infected sites. Further studies of the influence of the osteocyte in this regard will be of interest.

Taken together, S. aureus infection enhances osteoclastic bone resorption, possibly osteocytic osteolysis of bone, and inhibits bone formation, leading to an overall loss in bone tissue.

Conventional in Vitro Methods to Model Individual Aspects of Osteomyelitis

Although human biopsies and animal osteomyelitis models have contributed significantly to our understanding of osteomyelitis, conventional in vitro methods remain of value. Some of the mainstays of this approach include bacterial cultures and cocultures with host cells, which are described below.

Bacterial and Biofilm Cultures

Biofilm growth in vivo can be mimicked with conventional models, such as microtiter plate-based models or flow displacement biofilm models, as reviewed recently. These models can be used for antimicrobial compound testing and measuring bacterial colonization/biofilm formation on...
various substrates. One of the most common methods currently used to assess anti-biofilm efficacy is the minimum biofilm eradication concentration assay, which is a 96-well biofilm system using polystyrene pegs. Bacterial biofilms grown on the pegs can be simultaneously challenged with multiple antibiotic combinations at different concentrations for assessing the bactericidal and/or bacteriostatic efficacies of these antimicrobials.43

Conventional bacterial models can also be used to examine bacterial colonization on materials such as poly-methyl methacrylate and the efficacy of antibiotic coatings. Examples of orthopedic implant-related materials and coatings that have been tested for bacterial colonization have recently been reviewed.73 An interesting antibacterial coating that has been tested is a tissue plasminogen activator-containing coating to activate plasminogen and increase fibrin degradation.74 Fibrin can form a layer on biomaterials and promote adherence of pathogens to the biomaterial. Fibrin is also a component of the S. aureus biofilm matrix that facilitates antibiotic resistance due to poor penetration of the antibiotic into the biofilm. It was shown that the tissue plasminogen activator-containing coating reduced bacterial adherence to the biomaterial.74

In addition, adherent bacteria were more susceptible to antibiotics because the bacteria were not protected by a fibrin matrix.74 In a mouse model where S. aureus—infected implants were placed subcutaneously, the coating prevented biofilm-related infection.74

Bacterial Coculture with Host Cells

In an effort to increase the complexity and relevance of in vitro studies, bacterial cocultures with host immune or bone cells identified as key players in osteomyelitis have also been performed. For this review, a coculture is defined as a culture that combines bacteria with at least one host cell type.

Multiple groups have examined the effects of bacteria, usually S. aureus, on osteoblasts using two-dimensional cell culture models.43 To prevent bacterial overgrowth in static cultures, several techniques are routinely employed to remove extracellular bacteria. These include the use of antibiotics or the S. aureus—lysing enzyme lysostaphin, as well as rinsing to remove unbound bacteria. A variety of osteoblast coculture models have been developed using rodent and human cell lines, as well as human primary osteoblastic cells.43 Studies using S. aureus—infected human osteoblast cultures reported that the host cells underwent rapid and dramatic cell death after infection.75 One study examined the host cell response to several S. aureus strains at a fixed multiplicity of infection and showed that human primary osteoblasts exposed acutely or for short periods of time did not undergo cell death.76 Although relatively few intracellular bacteria were recovered, the primary cells secreted detectable levels of innate immune cell—relevant chemokines and cytokines, indicating the potential of osteoblasts to participate in innate immune responses and the utility of this model for studying this phenomenon.46 A study using bone explant-derived cells from the femoral heads of patients undergoing hip replacement surgery found that infections for up to 48 hours generated only low-level chemokine and cytokine responses, which the authors interpreted as indicating that osteoblasts may serve to internalize bacteria but not contribute significantly to the innate immune response.43 It is possible that matching the source of human primary cells and the pathology under investigation (osteomyelitis) may influence experimental outcomes. More specifically, periprosthetic joint infection most often occurs in patients treated for primary osteoarthritis, and osteoblastic cells derived from these donors display different phenotypic and behavioral qualities, such as aberrant in vitro mineralization, to those derived from nonosteoarthritis patients treated for fragility fractures of the hip.76 Furthermore, in hip osteoarthritis patients, the femoral head is usually diseased and the cells derived from this site may, therefore, be aberrant in their responses ex vivo. Thus, a site more distal from the joint (eg, the intertrochanteric region of the proximal femur) may be a more suitable source of disease-naïve cells. In a study using human osteoarthritis proximal femur-derived osteoblasts differentiated to an osteocyte-like stage, no cell death effect was observed in response to S. aureus infection for up to 30 days.36 Staphylococcus aureus formed small colony variant associated with the up-regulation of sigma B activity, consistent with establishment of a persistent intracellular infection. This correlated with observations of S. aureus bacteria inside viable osteocytes in clinical periprosthetic joint infection bone specimens.36 This model allows the study of both the host response and adaptation of the bacteria to intracellular infection.

Other studies have incorporated foreign biomaterials into the infection model. A typical application is the coculture of S. aureus and osteoblasts on a biomaterial surface to model the so-called race for the surface.77 Herein, the idea is that if host cells colonize the biomaterial first, bacterial adhesion is prevented. One way to study the race for the surface is by seeding a flow chamber with both staphylococci and osteoblasts.77 By using this method, different coatings that prevent bacterial adhesion and subsequently promote more host cell attachment can be studied, such as a coating containing the antibiotic levofloxacin.78 Biomaterials coated with levofloxacin had fewer adherent S. aureus compared with a non—levofloxacin-coated equivalent, and this enabled colonization by preosteoblasts.78

The effect of S. aureus on osteoblast-induced osteoclasisogenesis has also been studied in cocultures. These studies revealed that bacterial surface proteins could drive osteoclast formation because formaldehyde-fixed S. aureus induced RANK-L expression61 and IL-660 secretion by osteoblasts. Subsequently, this imbalances bone remodeling in favor of bone resorption.60 Furthermore, cocultures...
of *S. aureus* with osteoclasts have been performed; osteoclasts were seeded onto an inorganic crystalline calcium phosphate matrix mimicking bone, in presence of *S. aureus.* The infection promoted multinuclear osteoclast formation, which had a cellular area fourfold higher than noninfected osteoclasts and an increased bone resorption capacity, resulting from activation of the NF-κB pathway by *S. aureus.* Therefore, targeting osteoclast activity using antiresorptive drugs, such as bisphosphonates or denosumab (a monoclonal antibody targeting RANK-L), may be a means to prevent infection-induced osteolysis. Bisphosphonates or denosumab is effective in osteonecrosis of the jaw, indicating that further studies into antiresorptive drugs as a treatment option for osteomyelitis are required.

Bacterial biofilms can also be cocultured with immune cells. Immature and mature biofilms have been cocultured with PMNs to assess phagocytosis of biofilm-resident bacteria and the migration of PMNs to the biofilm. PMNs migrated toward the biofilm and engaged in phagocytosis of the biofilm, especially when the biofilm was in an immature state (<6 days old). Mature biofilm was less sensitive to PMN attack than immature biofilm because 15-day–old biofilm was subjected to significantly less phagocytosis by PMNs than 2- and 6-day–old biofilms. A possible reason for this may be that the ECM covering biofilm matures over time, thus preventing PMNs from reaching the bacteria in resident mature biofilm.

To our knowledge, only one multicellular model involving bacteria cocultured with both bone and host immune cells has been reported. To investigate competition for the surface of a polymethyl methacrylate plate, bacteria 

![Table 1 Aspects of Osteomyelitis That Are Achievable in Conventional or Theoretically Achievable in 3D Models](https://example.com/table1)

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<th>Aspects of osteomyelitis</th>
<th>Conventional models</th>
<th>3D models</th>
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<td>Generation of a fibrous encapsulation around a 3D structure</td>
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<td>Complex interactions between bacteria and multiple cell types</td>
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<td>A 3D structure with a necrotic core</td>
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3D, three dimensional.
**H. pylori** infection causes up-regulation of the NF-κB pathway in infected gastric organoids and, subsequently, an increase in IL-8, a neutrophil chemoattractant that promotes inflammation. In a more complex model, intestinal organoids from a human embryonic stem cell line were used to simulate *Escherichia coli* intestinal infection. The *E. coli* infected organoid was subsequently challenged with PMNs to thoroughly examine innate immune responses, such as reactive oxygen species production. Interestingly, the *E. coli* infection resulted in reactive oxygen species production by PMNs and migration of PMNs, but bacterial numbers did not decrease.

Another method to obtain a 3D organ structure is by using the RWV bioreactor. Cells are first grown in a monolayer and left to either aggregate onto a scaffold, such as ECM-coated microcarrier beads, then transferred into the RWV bioreactor, or self-aggregate by directly transferring the cells into the RWV bioreactor. In the RWV bioreactor, cells are subjected to a low shear force and fall gently in a restricted orbit, which first promotes 3D cell aggregation and then differentiation. To study host-pathogen interactions with the RWV bioreactor, 3D aggregates have been formed for tissues, such as lung, bladder, and intestinal tissue. 3D intestinal aggregates were used to study *Salmonella enterica* serovar typhimurium infection (Figure 3B). In this study, either RWV bioreactor-generated 3D intestinal aggregates or a monolayer culture of small intestinal epithelial cells (standardly used) was infected with *S. enterica* serovar typhimurium. *Salmonella* was less able to adhere to and invade the intestinal 3D aggregates compared with the monolayer of cells. It was concluded that the intestinal 3D aggregates more accurately replicate the *in vivo* environment, where most *S. enterica* serovar typhimurium remain extracellular.

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**Figure 3** A summary of three-dimensional (3D) infection models and the pros and cons of each model are listed. These 3D in vitro infection models include an intestinal organoid culture (A), rotating wall vessel (RWV) bioreactor-cultured intestinal aggregates (B), *Staphylococcus aureus* microcolonies in collagen gels (C), 3D printing with bacteria-containing inks (D), human skin equivalents (E), *ex vivo* bone models (F), microfluidic 3D models (G), and a 3D trabecular bone model (H). Images in A–H were partially generated with BioRender (Toronto, ON, Canada).
were observed for RWV bioreactor-formed lung aggregates infected with *P. aeruginosa*. In sharp contrast, monolayer cells were easily penetrable by *P. aeruginosa*, demonstrating that the RWV bioreactor-formed aggregates allowed for more *in vivo*—like infection by the bacterium given that the aggregates had more *in vivo*—like tight-junction complexes.96

*Staphylococcus aureus* microcolonies in a collagen gel supplemented with human fibrinogen have been developed to examine phagocyte-microbe interactions97 (Figure 3C). Supplementation of the collagen gel with fibrinogen was performed to facilitate fibrin-dependent formation of an inner pseudocapsule around the staphylococcal microcolony and an outer dense microcolony-associated mesh surrounding the pseudocapsule.97 The inner pseudocapsule formation was shown to be partially coagulase dependent, and the formation of the outer microcolony-associated mesh depended on von Willebrand factor—binding protein.97 When challenged with PMNs, the staphylococcal microcolonies were protected by the inner pseudocapsule and the outer dense microcolony-associated mesh against PMN infiltration.97

Also, 3D printing bacteria-containing ink into specific shapes is possible.98 *Acetobacter xylinum*, which produces bacterial cellulose, has been incorporated into hydrogel ink with hyaluronic acid, k-carrageenan, and fumed silica (Figure 3D). This bacteria-laden hydrogel ink was not toxic for the bacteria and has successfully been 3D printed into various shapes. In addition, because the bacteria present within the hydrogel retained their metabolic capacity, this technology resulted in functional materials that may be used for biomedical applications.98

Infection models examining the interactions between skin commensals, such as staphylococci and the epidermis, have been performed with human skin equivalents (HSEs). HSE cultures are developed by layering fibroblasts and keratinocytes, and then promoting their differentiation via air exposure.99 This generates HSEs consisting of a dermis and multilayered epidermis, with a functional epithelial barrier that blocks the entrance of the bacteria into the dermis.99 To infect the HSE, a bacterial suspension is applied on top of the model and the bacteria, in this case *S. aureus*, are allowed to colonize the HSE99 (Figure 3E). A similar approach is used to study airway infection; a bronchial epithelial model was used to clarify changes occurring to the bronchial epithelium in response to nontypeable *Haemophilus influenzae* infection, which was applied apically.100 Interestingly, nontypeable *H. influenzae* appeared to specifically migrate toward the stromal compartment of the bronchial epithelial model where the bacterium secreted a lipid-based structure.100

*Ex vivo* models have been established to investigate inflammatory bone destruction. For this model, 1-mm—thick murine mandibular slices were cultured in an air-liquid interface. Cells continued to proliferate, and protein synthesis was unaltered. Tissue was not infected with bacteria, but inflammation was achieved by supplementing media with lipopolysaccharide from *Porphyromonas gingivalis*, resulting in an increased number of osteoclasts in the *ex vivo* culture.101 Another study used an *ex vivo* human bone infection model for the investigation of osteocyte-bacterium interactions36 (Figure 3F). Fresh bone fragments without bone marrow, obtained from patients with femoral fracture (1 mm² in size) were cultured with *S. aureus* for 12 hours to achieve infection. Interestingly, *S. aureus* invaded osteocytes and lacunae of the *ex vivo* bone fragment, and the host cells responded in a manner similar to that of an *in vitro* differentiated two-dimensional culture of human primary osteocyte-like cells exposed to *S. aureus*.96 It was proposed that osteocytes may be an ideal host cell for long-term survival of the bacterium, where it adopts a small colony variant phenotype.36

A microfluidic 3D model has also been generated that promotes cells to form a 3D structure given the confined space and the microcirculation of nutrients and waste products in this system. For its development, a layer of human fibroenectin, *S. epidermidis*, and osteoblasts were applied into the microfluidic device.102 This resulted in an infected bone tissue model consisting of osteoblasts in a self-produced ECM of collagen fibers and calcium phosphate crystals, together with *S. epidermidis* forming biofilm102 (Figure 3G). This model allows the testing of treatments, such as antibiotics or wound-healing accelerators, by placing the microfluidic 3D model on inkjet-printed micropatterns containing the treatment.102 This model was used to test rifampicin-eluting biphasic calcium phosphate—containing beads, and it was demonstrated that these beads promoted osteoblast proliferation and ECM production, while simultaneously preventing biofilm formation.102

For the evaluation of the effect of biofilms on hematopoiesis during bone marrow infection, a 3D osteomyelitis model was developed18 (Figure 3H). More specifically, this model is a bone marrow analog that consists of a cationized bovine serum albumin scaffold resembling trabecular bone seeded with hematopoietic stem cells and mesenchymal stromal cells to mimic bone marrow. To infect this bone marrow analog, it was cocultured with a biofilm of methicillin-resistant *S. aureus* or *P. aeruginosa* grown on a titanium plate as a clinically relevant implant material. *Pseudomonas aeruginosa* caused cell death of both hematopoietic stem cells and mesenchymal stromal cells, whereas methicillin-resistant *S. aureus* stimulated IL-6 secretion by mesenchymal stromal cells and impaired differentiation of hematopoietic stem cells.18 To the best of our knowledge, this is the only reported 3D *in vitro* model realistically mimicking osteomyelitis pathophysiology. This model serves as an excellent starting point for further 3D osteomyelitis *in vitro* model development.
Outlook for in Vitro 3D Osteomyelitis Model Development

The previously described 3D models in other areas of infectious diseases offer great opportunities to translate the technological possibilities of 3D models to more faithfully model osteomyelitis.\(^{19,37,93,95–103}\) The previously described cationized bovine serum albumin scaffold developed by Raic et al\(^{18}\) offers an excellent starting point because it resembles bone marrow. Other studies have shown that such scaffolds could additionally be seeded with osteoblasts and osteoclasts.\(^{105}\) In which osteoblasts would form bone, and osteoclasts would resorb bone. Furthermore, it would be interesting to adapt a long-term ex vivo mechanically loading culture, such as the Zetos system, for the study of osteomyelitis.\(^{106}\) With the Zetos system, 3D cancellous bone tissue can be maintained ex vivo under physiological conditions, and loading and/or treatment with a variety of biochemical interventions can be applied.\(^{106}\) Using the Zetos system in combination with micro–computed tomography (or equivalent imaging technique), bone remodeling in response to infection could be monitored that would enable longitudinal observations of bone changes over time and response to therapy. Another interesting option would be to use the RWV bioreactor to culture sequestra from bone or bone mimics, and coculture with host cells to 3D model osteomyelitis. Once a source of the infection is present, the model may be exposed to different immune cells at multiple time points. Poor diffusion of nutrients, waste, and oxygen are traditionally considered complications for 3D models,\(^{108}\) but osteomyelitis-induced bone abscesses frequently contain such areas, which could thus be readily accommodated in these model systems.

Conclusions

The key features of osteomyelitis from the perspective of the pathogen include biofilm formation; SAC formation;\(^{22,23}\) intracellular infection; small colony variant phenotypes;\(^{35,36,39}\) and the invasion of the submicron channels of the canaliculi network.\(^{11,30}\) Immune responses and antibiotic therapy are often ineffective against bacteria in these locations, leading to chronic recurrent osteomyelitis and a skewing of bone remodeling in favor of osteolysis.\(^{60,61,63,67,68}\) Osteocytes themselves may also contribute to bone degradation in infection through the secretion of matrix metalloproteinases.\(^{96}\) Multicellular, 3D in vitro models of osteomyelitis have now also emerged as an exciting option to study the pathology of osteomyelitis using human cells, which offers promise in the advancement of our understanding of this disease, while also reducing animal use.

References

29. Edwards AM, Potts JR, Josefsen E, Massey RC: Staphylococcus aureus host cell invasion and virulence in sepsis is facilitated by the multiple repeats within FnBPA. PLoS Pathog 2010, 6:e1000964

Osteomyelitis Pathology and Methods


73. Hofstee et al


