RESEARCH ARTICLE

Toll-Like Receptor 2 Stimulation of Osteoblasts Mediates Staphylococcus Aureus Induced Bone Resorption and Osteoclastogenesis through Enhanced RANKL

Ali Kassem¹, Catharina Lindholm²,³, Ulf H Lerner¹,²*¹

¹ Department of Molecular Periodontology, Umeå University, Umeå, Sweden, ² Centre for Bone and Arthritis Research, Department of Internal Medicine and Clinical Nutrition at Institute for Medicine, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden, ³ Department of Rheumatology and Inflammation Research, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

*ulf.lerner@umu.se

Abstract

Severe Staphylococcus aureus (S. aureus) infections pose an immense threat to population health and constitute a great burden for the health care worldwide. Inter alia, S. aureus septic arthritis is a disease with high mortality and morbidity caused by destruction of the infected joints and systemic bone loss, osteoporosis. Toll-Like receptors (TLRs) are innate immune cell receptors recognizing a variety of microbial molecules and structures. S. aureus recognition via TLR2 initiates a signaling cascade resulting in production of various cytokines, but the mechanisms by which S. aureus causes rapid and excessive bone loss are still unclear. We, therefore, investigated how S. aureus regulates periosteal/endosteal osteoclast formation and bone resorption. S. aureus stimulation of neonatal mouse parietal bone induced ex vivo bone resorption and osteoclastic gene expression. This effect was associated with increased mRNA and protein expression of receptor activator of NF-kB ligand (RANKL) without significant change in osteoprotegerin (OPG) expression. Bone resorption induced by S. aureus was abolished by OPG. S. aureus increased the expression of osteoclastogenic cytokines and prostaglandins in the parietal bones but the stimulatory effect of S. aureus on bone resorption and Tnfsf11 mRNA expression was independent of these cytokines and prostaglandins. Stimulation of isolated periosteal osteoblasts with S. aureus also resulted in increased expression of Tnfsf11 mRNA, an effect lost in osteoblasts from Tlr2 knockout mice. S. aureus stimulated osteoclastogenesis in isolated periosteal cells without affecting RANKL-stimulated resorption. In contrast, S. aureus inhibited RANKL-induced osteoclast formation in bone marrow macrophages. These data show that S. aureus enhances bone resorption and periosteal osteoclast formation by increasing osteoblast RANKL production through TLR2. Our study indicates the importance of using different in vitro approaches for studies of how S. aureus regulates osteoclastogenesis to obtain better understanding of the complex mechanisms of S. aureus induced bone destruction in vivo.
Introduction

Severe *Staphylococcus aureus* (*S. aureus*) infections are a huge burden to healthcare systems worldwide. *S. aureus* causes a wide range of infectious diseases, from minor skin infections to life-threatening infections like endocarditis, toxic shock syndrome or sepsis. It can also cause post-operative wound- and implant-infections [1]. The emergence of multi-resistant *S. aureus* strains, Methicillin-resistant *S. aureus* (MRSA), causing infections that are difficult to treat makes the healthcare burden even more complicated [2]. *S. aureus* is a commensal bacterium, colonizing approximately 30% of the adult population [3] that can be highly opportunistic and invasive due to several virulence factors such as cell surface proteins and toxins. These virulence factors give *S. aureus* the ability to evade and destroy the host immune system and many of the clinical symptoms seen in patients are correlated with these virulence factors [4].

Osteomyelitis is a global, serious and morbid condition, especially in children, affecting bone tissue due to *S. aureus* infection in bone marrow. Acute osteomyelitis is characterized by rapid necrosis and destruction in bone and suppuration, while chronic osteomyelitis is often associated with sclerosing periosteal bone formation [3–8].

Arthritis can be a consequence of certain bacterial infections of which *S. aureus* is the most common pathogen in adults and children [9–11]. Infectious or septic arthritis is a rapid and progressive condition with high morbidity, characterized by joint swelling and early destruction of joint cartilage and bone, but also by systemic bone loss [12,13]. Septic arthritis has a prevalence of up to 0.01% in the general population and is seven times higher in patients with rheumatoid arthritis and prosthetic joints [5,14]. Bone loss in experimentally induced *S. aureus* septic arthritis in mice can be inhibited by treatment with bisphosphonate, OPG-Fc or RANK-Fc, demonstrating the importance of excessive osteoclast formation as a cause of bone loss [15,16]. Systemic bone loss is partly mediated by *S. aureus* lipoprotein since a lipoprotein-deficient mutant strain causes less bone loss [17].

Increased orthopedic implant failure facilitated by *S. aureus* infections constitutes a vast and costly issue for the health care system and the society [18]. *S. aureus* is also present abundantly in clinical sites of periodontitis and peri-implantitis [19]. Locally applied *S. aureus* in the gingiva causes osteoclast formation in alveolar bone and periodontal bone loss and, therefore, suggested being able to induce and synergistically enhance periodontal destruction [20,21].

Osteoclasts are multinucleated giant cells generated by fusion of hematopoietic mononuclear osteoprogenitor cells from the myeloid origin [22]. The differentiation of these progenitor cells requires activation of the receptor c-Fms (colony stimulating factor 1 receptor, CSF1R) by its ligand macrophage colony-stimulating factor (M-CSF or colony stimulating factor 1/CSF1), which stimulates proliferation and survival of the progenitors. Subsequent activation of the receptor activator of NF-κB (RANK) with RANK-Ligand, expressed by osteoblasts/osteocytes, induces differentiation along the osteoclastic lineage [23,24]. Interaction between RANKL and RANK can be inhibited by the decoy receptor osteoprotegerin (OPG), which binds and neutralizes RANKL. Osteoclasts resorb bone by initially dissolving the hydroxyapatite crystals in bone matrix through release of protons. Subsequently, degradation of organic matrix (mainly type I collagen) by various proteolytic enzymes will follow. One important bone matrix degrading enzyme is cathepsin K [25]. Osteoblasts, from mesenchymal origin, are the cells responsible for bone formation by producing bone matrix proteins and then depositing mineral crystals in the matrix. Osteoblasts/osteocytes also are key cells for the control of bone resorption by expressing and secreting RANKL [26,27].

Several studies have shown that *S. aureus* can be recognized by osteoblasts affecting their bone forming activities as well as their effects on osteoclastogenesis. It has been shown that *S. aureus* can inhibit bone formation and expression of bone formation genes *in vitro* in human
primary osteoblasts and osteoblastic cell line MG63 [28,29]. In the mouse osteoblastic cell line MC3T3-E1, *S. aureus* similarly decreases osteogenic differentiation and induces apoptosis [30]. *S. aureus* also upregulates RANKL mRNA and protein in primary mouse and human osteoblasts and in the mouse osteoblastic cell line MC3T3-E1 [17,28,31,32]. Interestingly, *S. aureus* protein A binds to tumor necrosis factor receptor-1 on osteoblasts causing decreased expression of bone formation genes and increased expression of inflammatory cytokines such as interleukin-6 (IL-6) [32,33]. It has also been reported that *S. aureus* can upregulate the expression of death inducing receptors (DR4 and DR5) leading to osteoblast apoptosis and increased OPG release [34]. These *in vitro* observations suggest that increased osteoclast formation caused by *S. aureus* may be due to *S. aureus* primarily targeting osteoblasts, which respond with increased RANKL expression.

In addition to the studies showing that *S. aureus* can interact with osteoblasts, it has been shown that this bacterium can be recognized by osteoclast progenitors. Using either live *S. aureus*, or *S. aureus* cell wall peptidoglycan or lipoteichoic acid, it has been found that all these preparations inhibit RANKL induced osteoclast formation in mouse bone marrow macrophage cultures [21,35,36], while stimulating differentiation along the macrophage lineage [36]. When using RANKL primed bone marrow macrophages, however, *S. aureus* cell wall peptidoglycan stimulated osteoclast formation [21]. In crude bone marrow cell cultures, containing both stromal cells/osteoblasts and hematopoietic cells, addition of surface-associated material from *S. aureus* enhanced osteoclastogenesis [37].

Due to the severity of *S. aureus* septic arthritis and to the increased use of prosthetic joint replacement with a risk of *S. aureus* infections, it is important to understand the bone destructive mechanisms exerted by *S. aureus* in order to develop new treatment strategies. Studies on effects by *S. aureus* on osteoclast formation have been performed using osteoclast progenitors from bone marrow showing either inhibition or stimulation of osteoclast formation. However, mature osteoclasts are formed exclusively on periosteal and endosteal surfaces and we, therefore, have studied how *S. aureus* can regulate osteoclastogenesis in the periostium/endosteum. For this purpose we have used either *ex vivo* organ cultures of mouse parietal bones, or cell cultures containing periosteal/endosteal osteoblasts and osteoclast progenitors. We found that bone resorption and osteoclast formation caused by stimulation of organ cultured parietal bones or periosteal/endosteal cell cultures with RANKL was not affected by *S. aureus*. In contrast, *S. aureus* abolished RANKL induced osteoclastogenesis in bone marrow macrophage cultures. These observations demonstrate that regulation of osteoclastogenesis is different using osteoclast progenitor cells from different tissues. Most importantly, *S. aureus* stimulated bone resorption and osteoclast formation in both organ cultured bone and periosteal/endosteal cell cultures, similar to *in vivo* observations in humans and rodents with *S. aureus* infections, and this response was dependent on TLR2-mediated increase of RANKL.

**Material and Methods**

**Bacteria**

The two *S. aureus* isolates, one Toxic shock syndrome toxin 1 (TSST-1) and Staphylococcal Enterotoxin A (SEA) producing, and one non-toxin producing strain, used in this study were originally isolated from healthy Swedish infants as previously described [38]. After 24 h growth on horse blood agar plates, harvested bacteria were washed in phosphate buffered saline (PBS), inactivated by exposure to UV-light (280–315 nm), and suspended in sterile PBS before use. Complete UV inactivation was confirmed by control cultures. Bacterial preparations were stored at—70°C until use.
Mice

CsA mice from our inbred colony, CB57BL/6J and B6.129 Tlr2^{tm1Kir}/J mice were from Jackson Laboratories. The mice were maintained (≤10 in each cage) under standard conditions of temperature and light, and were fed with standard laboratory chow and water ad libitum. Adult mice were killed by cervical dislocation and newborn mice by decapitation. 298 adult and newborn mice were used for this study. The Ethical committee of Umeå University, Umeå, Sweden has approved the animal care and experiments.

Reagents

Essentially fatty acid-free bovine serum albumin (BSA), tartrate-resistant acid phosphatase (TRAP) staining-kit (Sigma-Aldrich); alpha minimum essential medium (α-MEM), zoledronic acid, and indomethacin (Invitrogen); [45Ca]CaCl2 (Amersham Biosciences); oligonucleotide primers and probes, L-glutamine (Invitrogen or Applied Biosystems); TLR2 agonist (Palmitoyl-2-Cys-Ser-(Lys)4) Pam2, lipoprotein-containing lipopolysaccharide from Porphyromonas gingivalis (LPS \textit{P. gingivalis}), heat killed \textit{Listeria Monocytogenes} (HKLM) (InvivoGen); antibiotics (AstraZeneca); culture dishes, multiwell plates (Nunc Inc.); mouse recombinant OPG, RANKL, M-CSF, IL-1β, IL-6, IL-6SR, IL-11, oncostatin (OSM), leukemia inhibitory factor (LIF), tumor necrosis factor-α (TNF-α), anti-IL-1β (MAB401), anti-IL-6 (MAP406), anti-IL-11 (AF 418 NA), anti-OSM (AF 495 NA), anti-LIF (AF 449), anti-TNF-α (MAB 4101) (R&D Systems); RatLaps™ CTX ELISA kit (Immonodiagnosticsystems); Prostaglandin E₂ \textsuperscript{[125I]}-RIA \textsuperscript{K} Kit (Perkin-Elmer); RNAqueous–4 PCR \textsuperscript{K} kit (Ambion); High-Capacity cDNA Reverse Transcription \textsuperscript{K} Kit (Applied Biosystems); Kapa2G™ Robust HotStart PCR kit, Kapa™ Probe Fast qPCR kit (KapaBiosystems). Bacteria, antibodies and all other test substances, with the exception of indomethacin, were dissolved in culture media. Indomethacin was dissolved in ethanol; the final concentration of ethanol did not exceed 0.1%, a concentration which we have previously found not to affect bone resorption in the parietal bone cultures.

Organ culture of mouse parietal bones

Parietal bones from 5–7 days-old mice were dissected and cut either into halves for most of the experiments, or into quarters for mineral release analyses. Subsequently, the bones were incubated for 24 h in serum free α-MEM containing BSA (0.1%) and indomethacin (1 μM) to prevent the initial effect of released prostaglandins due to the dissection trauma \cite{39,40}. The bones were then washed extensively with sterile PBS and cultured in indomethacin free media with or without \textit{S. aureus} or other test substances.

Bone resorption assays

Bone resorption was analyzed by assessing either release of mineral (45Ca) or of matrix degradation fragment (CTX) from the bones to the culture media. 2–3 days-old mice were injected with 1.5 μCi 45Ca 4–5 days prior to dissection, and the amounts of radioactivity in bone and culture medium were analyzed by liquid scintillation at the end of the culture period. For the time-course experiments, the mice were injected with 12.5 μCi 45Ca, and the radioactivity was analyzed at different time points by withdrawal of small amounts of the culture media. Isotope release was expressed as the percent release of the initial amount of isotope (calculated as the sum of radioactivity in medium and bone after culture).

The release of collagen fragments (CTX) from the bone matrix into the media was analyzed by RatLaps™ ELISA kit.
Isolation and culture of parietal cells

Periosteal and endosteal cells were isolated from 2–3 days-old mouse parietal bones by sequential collagenase digestion [41]. Pooled cells from populations 1–10, containing both osteoblast and osteoclast progenitors capable of forming bone resorbing osteoclasts [41], were used for osteoclastogenesis experiments. These cells were initially cultured in 25 cm² flasks with α-MEM containing 10% FBS for 48 h to expand the number of cells. Cells were then washed and detached and subsequently seeded in 12-multiwells (10⁴ cells/cm²) and cultured with or without S. aureus or other test substances for 9 days. Cells were fixed and stained for TRAP.

Cells from populations 6–10 are enriched for osteoblastic cells and widely used for osteoblastogenesis experiments. These cells were expanded as described above, and seeded in 24-multiwells (10⁴ cells/cm²) and incubated with or without test substances for 48 h at which time point RNA was isolated and used for gene expression analyses.

Bone marrow macrophage isolation and cultures

Mouse bone marrow cells were isolated from tibia and femur as described [42]. The bone marrow macrophages were purified by incubating the cells on Corning dishes in the presence of M-CSF (30 ng/ml) for 48 h. The adherent bone marrow macrophages (BMM) were used as osteoclast progenitor cells. These cells do not contain T- or B-cells and all cells express the macrophage marker CD11b/Mac-1 [43]. After washing and detaching, cells were spot-seeded (5x10³ cells in 10 μl) at the center of 96-multiwells and left to adhere for 10 min. Then, the wells were added 200 μl medium containing M-CSF (30 ng/ml; controls) or M-CSF (30 ng/ml) +RANKL (4 ng/ml) with or without S. aureus or other test substances and incubated for 96 h. In experiments with committed osteoclast progenitors, cells were primed with RANKL (4 ng/ml) in presence of M-CSF for 24 h. Cells were then washed and medium containing M-CSF with or without test substances was added. At the end of the cultures, cells were fixed and stained for TRAP.

TRAP staining

Cells were fixed, washed and stained for TRAP using the Naphtol AS-BI phosphate kit from Sigma Aldrich. TRAP⁺ cells with at least three nuclei were counted as TRAP⁺ multinucleated osteoclasts (TRAP⁺MuOCL).

Gene expression analyses

RNA was isolated from bone tissue or cells using RNAqueous–4 PCR™ kit, according to manufacturer’s instructions. The RNA was quantified spectrophotometrically and single-stranded cDNA was synthesized from 0.1–0.5 μg of total RNA using High High-Capacity cDNA Reverse Transcription™ Kit. To ensure absence of genomic DNA in the samples, negative controls with no MultiScribe™ reverse transcriptase were included. The following predesigned real-time PCR assays from Applied Biosystems were used for gene expression assays: Acp5 (Mm00475698_m1), Calcr (Mm00432282_m1), c-Fos (Mm00487425_m1), Csf1 (Mm00432686_m1), Csf1r (Mm01266652_m1), Ctsk (Mm00484036_m1), Il1b (Mm00434228_m1), Il11 (Mm00434162_m1), Il6 (Mm00446190_m1), Lif (Mm00434761_m1), Nfatc1 (Mm00479445_m1), Oscar (Mm00558665_m1), Osm (Mm01193966_m1), Pigs2 (Mm00478374_m1), Tnfsf2 (Mm00443258_m1), Tnfsf11 (Mm00441908_m1), Tnfsf11a (Mm00437135_m1), Tnfrsf11b (Mm00435452_m1). β-actin (4352341E) was used as a reference gene to normalize for variability in amplification due to possible differences in starting mRNA concentrations. ABI PRISM 7900 HT Sequence Detection System and Software were used for the amplifications.
RANKL and OPG protein analyses
Assessment of RANKL and OPG protein was made using ELISA kits after lysing the bones in 1 ml 0.2% Triton X-100. The sensitivities of the immunoassays are 5 pg/ml.

Prostaglandin E\textsubscript{2} analysis
The amount of released PGE\textsubscript{2} in the culture media was measured by a radioimmunoassay kit.

Neutralizing antibody experiments
Neutralizing antibodies against mouse interleukin-1\textbeta{} (IL-1\textbeta{}), IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM) and tumor necrosis factor-\alpha{} (TNF-\alpha{}) were used to elucidate the role of these cytokines in \textit{S. aureus} induced bone resorption. We first validated the efficacy of the antibodies by verifying that the antibodies neutralized the effect of IL-1\textbeta{}, IL-6+sIL-6R, IL-11, LIF, OSM or TNF-\alpha{} (all used at maximally effective concentrations) on mRNA expression of \textit{Tnfsf11} in parietal bones. The parietal bones, after indomethacin pretreatment, were pre-incubated with the antibodies for 8 h prior to the stimulation with \textit{S. aureus}. To eliminate the possibility that several cytokines were responsible for the effects, we added the antibodies all together to the cell and organ cultures in a mixture at final concentrations of 1 \mu{}g/ml for anti-IL-6, anti-IL-11, anti-LIF and anti-TNF-\alpha{}, 3 \mu{}g/ml for anti-OSM and 5 \mu{}g/ml for anti-IL-1\textbeta{} and analyzed the response on the expression of \textit{Tnfsf11} mRNA or CTX release, respectively.

Statistics
Statistical analyses were performed using Paired t-test (S3A–S3D Fig) or one-way ANOVA (all other experiments) with Shapiro-Wilk’s normality test and Holm-Sidak’s \textit{post hoc} test using SigmaPlot software, (Systat Software Inc). The means and SEM shown in each figure are based upon 5–6 calvarial bones or cell culture wells in separate experiments, as specified in the legends to figures. All experiments were repeated with comparable results. Data were considered statistically significant when \(P<0.05\) (*), \(P<0.01\) (**), and \(P<0.001\) (***) or $\).

Results
\textit{S. aureus} stimulates bone resorption in parietal bones
Both the non-toxin producing \textit{S. aureus} and toxin producing \textit{S. aureus} (\textit{S. aureus Tox}) enhanced the release of mineral (\textsuperscript{45}Ca) from cultured neonatal parietal bones in a time-dependent manner (Fig 1A). The effect was statistically significant (\(P<0.05\)) already at 24 h. The stimulatory effect of \textit{S. aureus} on \textsuperscript{45}Ca release was concentration-dependent (Fig 1B). \textit{S. aureus} and \textit{S. aureus Tox} also significantly enhanced the release of bone matrix degradation fragments (CTX) from these bones (Fig 1C).

The \textit{S. aureus} and \textit{S. aureus Tox} induced CTX release from neonatal parietal bones was abolished by the bisphosphonate zoledronic acid (Fig 1D).

Mineral release and matrix degradation induced by \textit{S. aureus} and \textit{S. aureus Tox} were associated with time-dependent increased mRNA expression of \textit{Ctsk} (encoding cathepsin K; Fig 1E) and \textit{Acp5} (encoding TRAP; Fig 1F). The increased mRNA expression of \textit{Ctsk} and \textit{Acp5} was dependent on the concentration of \textit{S. aureus} (Fig 1G and 1H).

\textit{S. aureus}-induced osteoclast formation and bone resorption in parietal bones is mediated by enhanced RANKL
Osteoclastogenesis requires activation of c-Fms by its ligand M-CSF and activation of RANK by its ligand RANKL, with OPG being a decoy receptor for RANKL [22]. In addition,
activation of the receptor OSCAR (Osteoclast-associated receptor) is important for osteoclast differentiation [44,45]. Downstream signaling includes activation of NFATC1 (Nuclear factor of activated T-cells c1) which is regarded as the master transcription factor of osteoclastogenesis [46]. We assessed the effect by S. aureus on these cytokines, receptors and transcription factor in the parietal bones. 

*S. aureus* and *S. aureus* Tox time-dependently stimulated the mRNA expression of *Oscar*, *Nfatc1* and *Tnfsf11* (encoding RANKL) (Fig 2A–2C). The stimulatory effect on these transcripts was dependent on the concentration of *S. aureus* (Fig 2D–2F). The bacterium also
increased the expression of Csf1r (encoding the M-CSF receptor c-Fms) and Csfl (encoding M-CSF) (S1A–S1D Fig), whereas Tnfsf11a (encoding RANK) and Tnfsf11b (encoding OPG) mRNA were unaffected (S1E–S1H Fig).

In agreement with the mRNA analyses, S. aureus and S. aureus Tox significantly enhanced RANKL protein levels in the parietal bones (Fig 2G), without significantly affecting OPG protein (Fig 2H).

The importance of increased RANKL/OPG ratio for the stimulatory effect on bone resorption in neonatal parietal bones was demonstrated by the observation that bone matrix degradation (CTX release) in parietal bones challenged by S. aureus and S. aureus Tox was abolished when recombinant OPG (300 ng/ml) was added (Fig 2I). OPG also inhibited S. aureus induced mRNA expression of Ctsk (Fig 2J) and Nfatc1 (Fig 2K), indicating that OPG inhibited bone resorption.

**Fig 2.** The stimulatory effect on bone resorption in mouse parietal bones by *S. aureus* is dependent on increased RANKL. (A–C) *S. aureus* (3x10⁶ CFU/ml) time-dependently increased the mRNA expression of Oscar, Nfatc1 and Tnfsf11 in the parietal bones. (D–F) Concentration-dependent effect by *S. aureus* on Oscar, Nfatc1 and Tnfsf11 mRNA. (G) *S. aureus* (3x10⁶ CFU/ml) increased the cellular level of RANKL protein without affecting OPG protein. (I–L) The stimulatory effect by *S. aureus* (3x10⁶ CFU/ml) on CTX release and mRNA expression of Ctsk and Nfatc1 in the parietal bones was inhibited by OPG (300 ng/ml), without any effect of Tnfsf11 mRNA expression. Data are means of 5 (A–F, J–L) or 6 (G–I) observations and SEM is given as vertical bars when larger than the radius of the symbol. In Fig 2A, effects were statistically significant at 4 h (*P*<0.05) and at 12–48 h (*P*<0.001). In Fig 2B, effects at 4–48 h were statistically significant (*P*<0.001). In Fig 2C, effects were statistically significant at 4 h (*P*<0.05) and at 12–48 h (*P*<0.001). In Fig 2D, effects were statistically significant at 3 x 10⁶, 10⁷ and 3 x 10⁷ (*P*<0.001) and at 10⁶ and 3 x 10⁶ (*P*<0.01) CFU/ml. In Fig 2E and F effects at 3 x 10⁵–3 x 10⁷ CFU/ml were statistically significant (*P*<0.001). ***P*<0.001 compared to unstimulated control (G, I–L) or to *S. aureus* stimulated bones (I–K).

doi:10.1371/journal.pone.0156708.g002
resorption through inhibition of osteoclast differentiation. The fact that OPG did not affect Tnfsf11 mRNA (Fig 2L) shows that OPG acted downstream RANKL formation to inhibit osteoclastogenesis.

The role of osteoclastogenic cytokines and prostaglandins in *S. aureus* induced RANKL and bone resorption

*S. aureus* enhanced the mRNA expression of Il1b, Il6, Il11, Lif, Osm, Tnfsf2 (encoding TNF-α) and Ptgs2 (encoding cyclooxygenase-2) in the parietal bones in a dose- and time-dependent manner (Fig 3A–3H), as expected since TLR2 activation often results in enhanced expression of these proinflammatory molecules [47]. *S. aureus* as well as *S. aureus* Tox enhanced the release of PGE2 from the parietal bones (Fig 3I).

![Fig 3. S. aureus stimulates bone resorption in mouse parietal bones independently on cytokine and prostaglandin production. (A-D) Concentration-dependent effect by S. aureus on Il1b, Il11, Il6, Osm, Tnfsf2 and Ptgs2 mRNA expression in parietal bones. (E-H) Time-dependent effect by S. aureus (3x10^5 CFU/ml) on Il1b, Il11, Il6, Osm, Tnfsf2 and Ptgs2 mRNA expression in parietal bones. (I) Stimulation of PGE2 release from the bones by S. aureus (3x10^5 CFU/ml). (J, K) The stimulatory effect by S. aureus (10^5–10^7 CFU/ml) on CTX- release and by S. aureus (3x10^5 CFU/ml) on Tnfsf11 mRNA expression in parietal bones was unaffected by adding a mixture of antibodies neutralizing IL-1β, IL-6, IL-11, LIF, OSM and TNF-α. (L, M) Indomethacin (1 μmol/l) partially reduced Tnfsf11 mRNA induced by S. aureus (3x10^6 CFU/ml) but did not affect CTX-release. Data are means of 5 (A-H, K, L) or 6 (I, J, M) observations and SEM is given as vertical bars when larger than the radius of the symbol. In Fig 3A, effects on Il11 mRNA (P < 0.001) and Il1b mRNA (P < 0.01) were statistically significant at 3 x 10^5–3 x 10^7 CFU/ml. In Fig 3B, effects on Osm mRNA (P < 0.01) and Ptgs2 mRNA (P < 0.01) were statistically significant at 3 x 10^5–3 x 10^7 CFU/ml. In Fig 3C, effects on Tnfsf2 mRNA were statistically significant (P < 0.001) at 3 x 10^5–3 x 10^7 CFU/ml and on Tnfsf11 mRNA at 3 x 10^5 and 3 x 10^7 (P < 0.001) and at 10^5–10^7 (P < 0.01) CFU/ml. In Fig 3D, effects on Lif mRNA (P < 0.01) and at 10^5–10^7 (P < 0.01) CFU/ml were statistically significant. In Fig 3E, effects on Ptgs2 mRNA were statistically significant (P < 0.001) at 3 x 10^5–3 x 10^7 CFU/ml and on Tnfsf2 mRNA at 3 x 10^5 and 3 x 10^7 (P < 0.001). In Fig 3F, effects on Lif mRNA were statistically significant (P < 0.001) at 1–48 h and on Osm mRNA at 1, 4, 24 and 48 h (P < 0.001) and at 12 h (P < 0.01). In Fig 3G, effects were statistically significant on Ptgs2 mRNA at 4–48 h (P < 0.001) and on Tnfsf2 mRNA at 4, 24 and 48 h (P < 0.001) and at 12 h (P < 0.01). In Fig 3H, effects on Lif mRNA were statistically significant (P < 0.001) at 1–48 h. ***P<0.001 compared to unstimulated control (I-M) or *P<0.05 to S. aureus stimulated bones (L).
Since these cytokines and prostaglandins are osteoclastogenic and can promote bone resorption, we examined their possible role in the *S. aureus* induced bone resorption and enhanced Tnfsf11 mRNA expression by using specific antibodies neutralizing the cytokines and indomethacin to inhibit prostaglandin biosynthesis. We first confirmed the efficacy of the antibodies in the organ culture assay of parietal bones (S2A–S2C Fig), and then added a mixture of antibodies neutralizing IL-1β, IL-6, IL-11, LIF, OSM and TNF-α to *S. aureus* stimulated bones. Addition of antibodies did not affect bone resorption induced by the bacteria at optimal or suboptimal concentrations, as assessed by bone matrix degradation (CTX release; Fig 3J). Nor did the antibodies affect the increased Tnfsf11 mRNA expression induced by *S. aureus* (Fig 3K). Despite the partial decrease of *S. aureus* induced Tnfsf11 mRNA expression (Fig 3L) in parietal bones by indomethacin, bone resorption was not affected (Fig 3M).

**S. aureus** stimulates Tnfsf11 in mouse parietal osteoblasts independent of cytokine induction but dependent on TLR2

Osteoblasts are resident cells that communicate and activate osteoclastogenesis in bone tissue by producing RANKL in response to a variety of bone resorbing hormones and cytokines [48,49]. We, therefore, investigated if *S. aureus* could induce production of RANKL in mouse parietal osteoblasts. Stimulation of these cells by *S. aureus* caused a time- and concentration-dependent increase of Tnfsf11 mRNA expression but had no effect on Tnfrsf11b mRNA expression (Fig 4A and 4B).

*S. aureus* and *S. aureus* Tox increased the mRNA expression of Il1b, Il6, Il11, Lif, Osm and Tnfsf2 in the parietal osteoblasts (Fig 4C). Neutralization of these cytokines by a mixture of antibodies neutralizing IL-1β, IL-6, IL-11, LIF, OSM and TNF-α, did not affect the *S. aureus* induced Tnfsf11 mRNA expression (Fig 4D). *S. aureus* and *S. aureus* Tox also enhanced the expression of Ptgs2 mRNA (Fig 4E). Inhibition of prostaglandin biosynthesis by indomethacin abolished *S. aureus* and *S. aureus* Tox induced mRNA expression of Tnfsf11 (Fig 4F).

Others and we [50–52] have previously shown that osteoblasts express TLR2 and we, therefore, assessed if *S. aureus* induced Tnfsf11 expression was due to stimulation of TLR2. Using osteoblasts isolated from Tlr2 deficient mice, we found that Tnfsf11 mRNA induced by *S. aureus* was entirely dependent on Tlr2 expression (Fig 4G). Similarly, *S. aureus* did not upregulate Ptgs2 mRNA in osteoblasts in cells isolated from Tlr2 deficient mice (Fig 4H).

**S. aureus** differentially regulates osteoclast formation in bone marrow and periosteal cell cultures

Activation of TLR2 in RANKL stimulated BMM results in inhibition of osteoclast differentiation and formation [53,54]. We, therefore, asked ourselves why not *S. aureus* inhibited osteoclast formation in the ex vivo bone organ cultures. For this purpose, we compared the effects by *S. aureus* in three different osteoclastogenic systems, all stimulated by RANKL with or without *S. aureus*. *S. aureus*, similar to other TLR2 agonists (LPS *P. gingivalis* and Pam2), abolished osteoclastogenesis in RANKL stimulated BMM (Fig 5A). In contrast, *S. aureus* did not inhibit osteoclastogenesis induced by RANKL in periosteal/endosteal cell cultures (Fig 5B), or RANKL stimulated bone matrix degradation in parietal bone organ cultures (Fig 5C). These observations indicate that osteoclast progenitors in the periosteum/endosteum are different from those in bone marrow.

Interestingly, *S. aureus* stimulated osteoclast formation in the periosteal cells in the absence of exogenous RANKL (Fig 5D and 5E). The effect was associated with enhanced mRNA expression of Tnfsf11, Ctsk and Acp5 as markers of osteoclast differentiation (Fig 5F–5H).
Stimulation of TLR2 by its synthetic ligand (Pam2) in committed osteoclast progenitors (RANKL-primed for 24h) has been reported to promote osteoclastogenesis [17,54]. This finding suggests the possibility that one reason for the different response to *S. aureus* in osteoclast progenitors from periosteum and bone marrow co-treated with RANKL might be that the differentiation stage of osteoclast progenitors determines the response to *S. aureus*. We, therefore, assessed the effect by *S. aureus* in RANKL-primed BMM. In agreement with the previous observations [17, 54], we found that Pam2 and Pam3 enhanced osteoclast formation in RANKL-primed BMM to the same degree as treatment with RANKL (Fig 6A and 6B). Unlike the synthetic ligands Pam2 and Pam3, but similar to other bacterial TLR2 ligands such as HKLM and
LPS *P. gingivalis*, stimulation of RANKL-primed BMM with *S. aureus* resulted in formation of mainly mononuclear TRAP⁺ cells with only some few osteoclast-like cells (Fig 5A and 5B). This observation indicated that *S. aureus* can stimulate differentiation of RANKL-primed osteoclast progenitors but not to the same degree as Pam2 and Pam3 and not to the level where the progenitors fuse to typical multinucleated mature osteoclasts. Further evidence for the view that *S. aureus* can induce osteoclast progenitor cell differentiation were the observations that the mRNA expression of *Ctsk*, *Acp5* and *Calcr* was significantly induced by *S. aureus* (Fig 6C–6E), which was also true for the mRNA expression of the two osteoclastogenic transcription factors c-Fos and Nfatc1 (Fig 6F and 6G). However, the degree of upregulation of these genes induced by *S. aureus* was clearly less than that caused by Pam2, Pam3 and RANKL. Similarly, LPS *P. gingivalis* stimulation of RANKL-primed BMM with *S. aureus* resulted in formation of mainly mononuclear TRAP⁺ cells with only some few osteoclast-like cells (Fig 5A and 5B). This observation indicated that *S. aureus* can stimulate differentiation of RANKL-primed osteoclast progenitors but not to the same degree as Pam2 and Pam3 and not to the level where the progenitors fuse to typical multinucleated mature osteoclasts. Further evidence for the view that *S. aureus* can induce osteoclast progenitor cell differentiation were the observations that the mRNA expression of *Ctsk*, *Acp5* and *Calcr* was significantly induced by *S. aureus* (Fig 6C–6E), which was also true for the mRNA expression of the two osteoclastogenic transcription factors c-Fos and Nfatc1 (Fig 6F and 6G). However, the degree of upregulation of these genes induced by *S. aureus* was clearly less than that caused by Pam2, Pam3 and RANKL. Similarly, LPS *P. gingivalis* stimulation of RANKL-primed BMM with *S. aureus* resulted in formation of mainly mononuclear TRAP⁺ cells with only some few osteoclast-like cells (Fig 5A and 5B). This observation indicated that *S. aureus* can stimulate differentiation of RANKL-primed osteoclast progenitors but not to the same degree as Pam2 and Pam3 and not to the level where the progenitors fuse to typical multinucleated mature osteoclasts. Further evidence for the view that *S. aureus* can induce osteoclast progenitor cell differentiation were the observations that the mRNA expression of *Ctsk*, *Acp5* and *Calcr* was significantly induced by *S. aureus* (Fig 6C–6E), which was also true for the mRNA expression of the two osteoclastogenic transcription factors c-Fos and Nfatc1 (Fig 6F and 6G). However, the degree of upregulation of these genes induced by *S. aureus* was clearly less than that caused by Pam2, Pam3 and RANKL. Similarly, LPS *P.
S. aureus induced all these osteoclast genes but the degree of stimulation was less than that induced by the compounds stimulating mature osteoclast formation (Fig 6C–6G).

S. aureus inhibits the expression of osteoblast anabolic genes

Since S. aureus induced bone loss may not entirely depend on increased bone resorption we also assessed if the bacteria affected bone formation in the parietal bones. The mRNA expression of the bone matrix proteins osteocalcin (encoded by Bglap) and procollagen type I (encoded by procoll1a1), as well as of the enzyme alkaline phosphatase (encoded by Akp1) was substantially
decreased by *S. aureus* in organ cultured parietal bones (S3A–S3C Fig). This might be due to the decreased mRNA expression of the transcription factor Runx2 also observed (S3D Fig). Since Nfatc1 is expressed not only in osteoclasts but also in osteoblasts [55–58], and since *S. aureus* increased Nfatc1 mRNA in the parietal bones (Fig 2K), we assessed if increased Nfatc1 was involved in the decreased expression of osteoblast anabolic genes. We found, however, that inhibition of these genes induced by *S. aureus* was independent on Nfatc1 since the inhibition of Nfatc1 mRNA expression caused by OPG (Fig 2K) did not affect *S. aureus* induced down regulation of Bglap, Procolla1, Akp1 or Runx2 mRNA expression (S3E–S3H Fig).

**Discussion**

It is well recognized that *S. aureus* infections can cause local and systemic bone destruction [15–18] but the mechanisms by which *S. aureus* induces bone resorption are still not fully understood. Although several reports have shown that *S. aureus* can target osteoblasts *in vitro* causing apoptosis, decreased bone formation and decreased expression of osteoblastic genes, as well as enhanced RANKL expression [17,28,31,32], the data regarding effects on osteoclasts are more diverse. *S. aureus* has been shown both to inhibit [21,35,36] and stimulate [21] osteoclastogenesis in mouse bone marrow macrophage cultures depending on if the bacterium is exposed to the cells simultaneously with RANKL or after RANKL pretreatment, respectively. Since mature osteoclasts are formed only at bone surfaces we have studied the effect of *S. aureus* on osteoclast formation and bone resorption using osteoclast progenitors present at periosteal/endosteal surfaces.

To mimic the microenvironment of bone tissue where osteoclast formation and bone resorption take place *in vivo* we used *ex vivo* organ cultures of mouse parietal bones, exhibiting a periosteum and a thin endosteum. We show that *S. aureus* enhances bone resorption in the parietal bones through a process inhibited by bisphosphonate, demonstrating the importance of osteoclasts. The finding that *S. aureus* increased osteoclastic genes such as those encoding TRAP and cathepsin K, and the osteoclastogenic transcription factor NFATc1, showed that *S. aureus* induced bone resorption is due to enhanced differentiation and activation of osteoclasts.

Since the RANKL/OPG ratio is crucial for osteoclastogenesis and bone homeostasis [59,60], we next investigated the effect of *S. aureus* on RANKL/OPG ratio. *S. aureus* enhanced this ratio by increasing the expression at both mRNA and protein levels of RANKL, without affecting those of OPG in the parietal bones. The inhibition of bone resorption and osteoclastic gene expression, caused by exogenous OPG added to *S. aureus* stimulated bone organ cultures, further supports the essential role of RANKL in bone resorption due to *S. aureus* infection. Using osteoblasts from *wild type* and *Thr2* deficient mice, we show that osteoblastic TLR2 is the receptor utilized by *S. aureus* in the bones to enhance RANKL. These data show that *S. aureus* stimulates periosteal/endosteal osteoclast formation and bone resorption in organ-cultured bones by enhancing RANKL/OPG in osteoblasts. Our observations further indicate that *S. aureus* does not inhibit osteoclast progenitors in these bones, in contrast to observations in bone marrow cell cultures. We cannot exclude, however, that increased osteoclast differentiation by *S. aureus* targeting osteoclast progenitors stimulated by endogenous RANKL produced in the perios-teum/endosteum also may contribute to the enhanced bone resorption.

It has been reported that activation of TLR2 inhibits RANKL-induced osteoclast formation in BMM cultures [21,35,36]. We, therefore, wondered how *S. aureus* could increase osteoclastogenesis and bone resorption in the *ex vivo* parietal bone organ cultures. To investigate if osteoclast progenitors in parietal bones were different from those in bone marrow we next used cell cultures of periosteal/endosteal cells from parietal bones and mouse bone marrow cultures and
compared the effect of *S. aureus* on non-stimulated and RANKL-stimulated cells, respectively. When *S. aureus* was added together with RANKL to mouse bone marrow macrophage cultures we could confirm observations made by others [21,35,36] showing that the bacterium can abolish osteoclast differentiation. In contrast, when *S. aureus* was added together with RANKL to periosteal/endosteal cell cultures, no inhibition of osteoclastogenesis was observed. Similar to this finding, *S. aureus* did not affect bone resorption in the parietal bones stimulated by exogenous RANKL. These findings show that osteoclast progenitors in bone marrow and at bone surfaces are fundamentally different in their response to *S. aureus* and explain why *S. aureus* can stimulate osteoclast formation in intact bones despite its inhibitory effect on RANKL-stimulated bone marrow macrophages. Previously, we have similarly shown that also vitamin A and LPS *P. gingivalis* stimulate bone resorption in parietal bones and increase formation of bone resorbing osteoclasts in periosteal/endosteal cell cultures, while also inhibiting RANKL-stimulated osteoclast formation in bone marrow macrophage cultures [52,61,62]. All together, these observations indicate that studies on osteoclast formation should not only be based upon osteoclastogenesis in bone marrow macrophages but also include experiments using osteoclast progenitors present at the surfaces of bone.

When *S. aureus* was added to periosteal/endosteal cell cultures not stimulated with RANKL, we observed increased formation of osteoclasts, similar to the observations in the calvarial bones. This response was associated with increased mRNA expression of the osteoclastic genes *Acp5* and *Ctsk* as well as with *Tnsf11* mRNA, indicating that increased number of mature osteoclasts was due to increased differentiation of osteoclast progenitor cells due to increased RANKL in osteoblasts which are abundant in these cultures.

The reason for the different responsiveness of osteoclast progenitors in bone marrow and periosteum/endosteum is not known but could be due to differences in differentiation stage and/or to the microenvironment. If bone marrow macrophages are primed with RANKL before subsequent stimulation by LPS *E. coli* or *S. aureus* cell wall peptidoglycan, in the absence of RANKL, formation of mature osteoclasts is induced [21]. Similarly, the synthetic TLR2 agonists Pam2 and Pam3 [17,21], and the periodontal pathogen *P. gingivalis* acting through TLR2 [54], stimulate osteoclast formation in RANKL-primed bone marrow macrophages. These findings indicate that the differences between bone marrow macrophages and periosteal/endosteal osteoclast progenitor responses to stimulatory ligands may depend on the differentiation level of osteoclast progenitors. When we added *S. aureus* to RANKL-primed bone marrow macrophages, the cells started to differentiate and became TRAP⁺ but the mononuclear cells did not fuse to mature osteoclasts. In contrast, addition of the two synthetic TLR2 agonists Pam2 and Pam3 stimulated formation of mature osteoclasts to the same degree as RANKL. Similar to *S. aureus*, LPS *P. gingivalis* and HKLM, two other TLR2 agonists induced differentiation of TRAP⁺ mononuclear cells but not formation of mature osteoclasts. Pam2 and Pam3 robustly upregulated osteoclastic and osteoclastogenic genes such as *Ctsk, Acp5*, *Calcr*, *c-Fos* and *Nfatc1*, a response also observed after treatment with *S. aureus* and LPS *P. gingivalis* but to a much lesser degree.

We do not know if the reason why *S. aureus* was unable to induce mature osteoclast formation was due to the quantitative differences in gene expression, or if the TLR2 in the RANKL-primed osteoclast progenitors are not fully activated by the bacterial agonist, in contrast to the synthetic ligands. Interestingly, we have found that LPS *P. gingivalis*, HKLM, Pam2 and Pam3 stimulate bone resorption in *ex vivo* parietal bones and *Tnsf11* mRNA expression in osteoblasts through TLR2 to the same degree [52], indicating that TLR2 in osteoblasts and RANKL-primed bone marrow macrophages are not entirely similar. The fact that multinucleated osteoclast formation was observed in RANKL-primed bone marrow macrophages treated with *P. gingivalis* [54], in contrast to our findings showing differentiation of mononuclear osteoclasts, may be due to that whole bacteria was used instead of the LPS preparation used by us.
In agreement with the well-known consequence of TLR activation, *S. aureus* stimulated the expression of several cytokines such as IL-1β, IL-11, IL-6, LIF, OSM and TNF-α. Since these cytokines are potent stimulators of RANKL expression, osteoclast formation and bone resorption [22, 63–65], we assessed if RANKL and osteoclastogenesis induced by *S. aureus* was secondary to induction of these cytokines. Using neutralizing antibodies, we show, however, that the effect of *S. aureus* on bone resorption and RANKL formation in parietal bones and isolated osteoblasts is not mediated by these cytokines. *S. aureus* also enhanced the mRNA expression in parietal bones and isolated osteoblasts of *Ptgs2*, a key enzyme in prostaglandin biosynthesis. Although inhibition of prostaglandin biosynthesis in the parietal bones and osteoblasts decreased *S. aureus* induced *Tnfsf11* mRNA expression, no effect on bone resorption was observed; most likely due to the robust stimulation of *Tnfsf11* still observed in *S. aureus* stimulated bones co-treated with the prostaglandin inhibitor. The reason why inhibition of prostaglandin biosynthesis abolished *S. aureus* induced *Tnfsf11* mRNA expression in calvarial osteoblasts, while only partially decreased this response in calvarial bones, might be due to that *S. aureus* can induce RANKL in cells present in the calvarial bones, but not in the osteoblasts cultures, and that the calvarial cells are insensitive to prostaglandins. One such possibility is osteocytes which have been shown to be more important producers of RANKL than osteoblasts [26, 27].

*S. aureus* may not cause decreased bone mass only by increasing bone resorption but also by decreasing bone formation. Several studies using human and mouse osteoblasts have shown that *S. aureus* can inhibit expression of genes associated with osteoblast differentiation and bone formation [28–32]. We observed a similar effect using bone organ cultures in which *S. aureus* decreased the mRNA expression genes encoding osteocalcin, procollagen type I, alkaline phosphate and Runx2. *Nfatc1* is most well known as a master regulator of osteoclast differentiation [46], but is also expressed in osteoblasts. The role of *Nfatc1* in bone formation is controversial with both stimulatory [55, 56] and inhibitory [57, 58] effects observed. We found, however, that stimulation of *Nfatc1* by *S. aureus* in the calvarial bones was not involved in the inhibition of the osteoblast anabolic genes.

Since the array of symptoms displayed by patients with *S. aureus* infection is correlated to the arsenal of virulence factors exhibited by *S. aureus*, we used two different strains of *S. aureus* and evaluated the role of toxins in *S. aureus* induced bone resorption. Our findings demonstrate that the ability of toxin production has no significant effect on bone resorption stimulated by *S. aureus* in isolated *in vitro* assays. Most likely, the toxin production characteristics of certain *S. aureus* strains have favorable effects on invasiveness, escape and damage of the immune system and exacerbating the infection and inflammation *in vivo*.

In summary, *S. aureus* targets osteoblasts (or maybe osteocytes) through TLR2 causing increased RANKL and periosteal/endosteal osteoclast formation and bone resorption with no signs of *S. aureus* targeting the subpopulation of osteoclast progenitors present at the surfaces of bone. The finding that activation of TLR2 in a subpopulation of osteoclast progenitors present in bone marrow which have been primed by RANKL results in osteoclast differentiation indicate the possibility that *S. aureus* might increase bone resorption also through activation of osteoclast progenitors at a certain differentiation level. The relative importance of osteoblasts/osteocytes and osteoclast progenitors for the bone resorptive effect by *S. aureus* has to be assessed in mice (and/or bone organ cultures) with cell specific deletion of TLR2.

**Supporting Information**

S1 Fig. *S. aureus* and *S. aureus Tox* time- and concentration-dependently increased the mRNA expression in parietal bones of *Csf1r* (A, B), *Csf1* (C, D) without affecting the mRNA expression of *Tnfsf11a* (E, F) and *Tnfsf11b* (G, H). In A, effects were statistically
significant at 12 and 48 h \((P<0.01)\) and at 24 h \((P<0.001)\). In B, effects were statistically significant by \(10^6\) and \(3\times10^7\) \((P<0.01)\) and by \(3\times10^6\) and \(10^7\) \((P<0.001)\) CFU/ml. In C, effects at 1–48 h were statistically significant \((P<0.001)\). In D, effects were statistically significant by \(3\times10^6\)–\(10^7\) \((P<0.01)\) and by \(3\times10^7\) \((P<0.001)\) CFU/ml. No statistically effects were obtained in experiments shown in E-H.

(TIF)

S2 Fig. Anti-IL-1β and anti-TNF-α effectively inhibit \textit{Tnfsf11} mRNA in parietal bones induced by IL-1β and TNF-α, respectively (A), anti-IL-11, anti-LIF and anti-OSM effectively inhibit \textit{Tnfsf11} mRNA induced by IL-11, LIF and OSM, respectively (B) and anti-IL-6 inhibit \textit{Tnfsf11} mRNA induced by co-treatment with IL-6 and IL-6 soluble receptor (C).

\(^*P<0.05, ^**P<0.01\) and \(^***P<0.001\) compared to unstimulated control or to cytokine stimulated bones.

(TIF)

S3 Fig. A-D show that \textit{S. aureus} inhibits bone formation in organ cultured mouse parietal bones as assessed by decreased mRNA expressions of \textit{Bglap} (A), \textit{Akpl} (B), \textit{Procol1a1} (C) and \textit{Runx2} (D). In E-H is demonstrated that the osteoclast inhibitor OPG does not affect the inhibition of \textit{Bglap} (E), \textit{Akpl} (F), \textit{Procol1a1} (G) and \textit{Runx2} (H) induced by \textit{S. aureus} in the parietal bones. \(^*P<0.05, ^**P<0.01\) and \(^***P<0.001\) compared to unstimulated control bones.

(TIF)

Acknowledgments

Special thanks to Mrs. Ingrid Boström, Mrs. Inger Lundgren and Dr. Maria Bergquist for their technical assistance in the laboratory.

Author Contributions

Conceived and designed the experiments: UHL CL. Performed the experiments: AK. Analyzed the data: AK CL UHL. Wrote the paper: AK CL UHL.

References

1. Lowy FD (1998) Staphylococcus aureus infections. N Engl J Med 339: 520–532. PMID: 9709046
2. Calfee DP (2012) Methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci, and other Gram-positives in healthcare. Curr Opin Infect Dis 25: 385–394. PMID: 22614523
3. Tong SY, Chen LF, Fowler VG Jr. (2012) Colonization, pathogenicity, host susceptibility, and therapeutics for Staphylococcus aureus: what is the clinical relevance? Semin Immunopathol 34: 185–200. doi: 10.1007/s00281-011-0300-x PMID: 22160374
4. Foster TJ, Geoghegan JA, Ganesh VK, Hook M (2014) Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. Nat Rev Microbiol 12: 49–62. doi: 10.1038/nrmicro3161 PMID: 24336184
5. Wright JA, Nair SP (2010) Interaction of staphylococci with bone. Int J Med Microbiol 300: 193–204. doi: 10.1016/j.ijmm.2009.10.003 PMID: 19889575
6. Peltola H, Paakkonen M (2014) Acute osteomyelitis in children. N Engl J Med 370: 352–360. doi: 10.1056/NEJMra1213956 PMID: 24450893
7. Lew DP, Waldvogel FA (2004) Osteomyelitis. Lancet 364: 369–379. PMID: 15276398
8. Belli E, Matteini C, Andreano T (2002) Sclerosing osteomyelitis of Garre periositis ossificans. J Craniofac Surg 13: 765–768. PMID: 12457091
9. Tarkowski A (2006) Infection and musculoskeletal conditions: Infectious arthritis. Best Pract Res Clin Rheumatol 20: 1029–1044. PMID: 17127195
10. Dodwell ER (2013) Osteomyelitis and septic arthritis in children: current concepts. Curr Opin Pediatr 25: 58–63. PMID: 23283291
11. Garcia-Arias M, Balsa A, Mola EM (2011) Septic arthritis. Best Pract Res Clin Rheumatol 25: 407–421. doi: 10.1016/j.berh.2011.02.001 PMID: 22100289

12. Bremell T, Lange S, Yacoub A, Ryden C, Tarkowski A (1991) Experimental Staphylococcus aureus arthritis in mice. Infect Immun 59: 2615–2623. PMID: 1855981

13. Bremell T, Abdelnour A, Tarkowski A (1992) Histopathological and serological progression of experimental Staphylococcus aureus arthritis. Infect Immun 60: 2976–2985. PMID: 1612762

14. Dhaliwal S, LeBel ME (2012) Rapidly progressing polyarticular septic arthritis in a patient with rheumatoid arthritis. Am J Orthop (Belle Mead NJ) 41: E100–101.

15. Verdrengh M, Bokarewa M, Ohlsson C, Stolina M, Tarkowski A (2010) RANKL-targeted therapy inhibits bone resorption in experimental Staphylococcus aureus-induced arthritis. Bone 46: 752–758. doi: 10.1016/j.bone.2009.10.028 PMID: 19879986

16. Verdrengh M, Carlsten H, Ohlsson C, Tarkowski A (2007) Addition of bisphosphonate to antibiotic and anti-inflammatory treatment reduces bone resorption in experimental Staphylococcus aureus-induced arthritis. J Orthop Res 25: 304–310. PMID: 17089391

17. Kim J, Yang J, Park OJ, Kang SS, Kim WS, Kurokawa K, et al. (2013) Lipoproteins are an important bacterial component responsible for bone destruction through the induction of osteoclast differentiation and activation. J Bone Miner Res 28: 2381–2391. doi: 10.1002/jbmr.1973 PMID: 23633269

18. Montanaro L, Speziale P, Campoccia D, Ravaioi S, Cangini I, Pietrocola G, et al. (2011) Scenery of Staphylococcus implant infections in orthopedics. Future Microbiol 6: 1329–1349. doi: 10.2217/fmb.11.117 PMID: 22082292

19. Zhuang LF, Watt RM, Mattheos N, Si MS, Lai HC, Lang NP. (2014) Periodontal and peri-implant microbiota in patients with healthy and inflamed periodontal and peri-implant tissues. Clin Oral Implants Res.

20. Nagano F, Kaneko T, Yoshinaga Y, Ukai T, Kuramoto A, Nakatsu S, et al. (2013) Gram-positive bacteria as an antigen topically applied into gingival sulcus of immunized rat accelerates periodontal destruction. J Periodontal Res 48: 420–427. doi: 10.1111/jre.12021 PMID: 23137272

21. Kishimoto T, Kaneko T, Ukai T, Yokoyama M, Ayon Haro R, Yoshinaga Y, et al. (2012) Peptidoglycan and lipopolysaccharide synergistically enhance bone resorption and osteoclastogenesis. J Periodontal Res 47: 446–454. doi: 10.1111/j.1600-0765.2011.01452.x PMID: 22823724

22. Lorenzo J, Horowitz M, Choi Y (2008) Osteoimmunology: interactions of the bone and immune system. Endocr Rev 29: 403–440. doi: 10.1210/er.2007-0039 PMID: 18451259

23. Teitelbaum SL, Ross FP (2003) Genetic regulation of osteoclast development and function. Nat Rev Genet 4: 638–649. PMID: 12897775

24. Edwards JR, Mundy GR (2011) Advances in osteoclast biology: old findings and new insights from mouse models. Nat Rev Rheumatol 7: 235–243. doi: 10.1038/nrrheum.2011.23 PMID: 21386794

25. Costa AG, Cusano NE, Silva BC, Cremers S, Bilezikian JP (2011) Cathepsin K: its skeletal actions and role as a therapeutic target in osteoporosis. Nat Rev Rheumatol 7: 447–456. doi: 10.1038/nrrheum.2011.77 PMID: 21670768

26. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, et al. (2011) Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med 17: 1231–1234. doi: 10.1038/nm.2452 PMID: 21909105

27. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O’Brien CA (2011) Matrix-embedded cells control osteoclast formation. Nat Med 17: 1235–1241. doi: 10.1038/nm.2448 PMID: 21909103

28. Sanchez CJ Jr., Ward CL, Romano DR, Hurtgen BJ, Hardy SK, Woodbury RL, et al. (2013) Staphylococcus aureus biofilms decrease osteoblast viability, inhibits osteogenic differentiation, and increases bone resorption in vitro. BMC Musculoskelet Disord 14: 187. doi: 10.1186/1471-2474-14-187 PMID: 23767824

29. Lerner UH, Sundqvist G, Ohlin A, Rosenquist JB (1998) Bacteria inhibit biosynthesis of bone matrix proteins in human osteoblasts. Clin Orthop Relat Res: 244–254. PMID: 9577433

30. Chen Q, Hou T, Luo F, Wu X, Xie Z, Xu J (2014) Involvement of toll-like receptor 2 and pro-apoptotic signaling pathways in bone remodeling in osteomyelitis. Cell Physiol Biochem 34: 1890–1900. doi: 10.1159/000366387 PMID: 25503704

31. Somayaji SN, Ritchie S, Sahraei M, Marriott I, Hudson MC (2008) Staphylococcus aureus induces expression of receptor activator of NF-kappaB ligand and prostaglandin E2 in infected murine osteoblasts. Infect Immun 76: 5120–5126. doi: 10.1128/IAI.00228-08 PMID: 18765718

32. Widaa A, Claro T, Foster TJ, O’Brien FJ, Kerrigan SW (2012) Staphylococcus aureus protein A plays a critical role in mediating bone destruction and bone loss in osteomyelitis. PLoS One 7: e40586. doi: 10.1371/journal.pone.0040586 PMID: 22792377
33. Claro T, Widaa A, McDonnell C, Foster TJ, O’Brien FJ, Kerrigan SW (2013) Staphylococcus aureus protein A binding to osteoblast tumour necrosis factor receptor 1 results in activation of nuclear factor kappa B and release of interleukin-6 in bone infection. Microbiology 159: 147–154. doi: 10.1099/mic.0.063016-0 PMID: 23154968

34. Young AB, Cooley ID, Chauhan VS, Marriott I (2011) Causative agents of osteomyelitis induce death domain-containing TNF-related apoptosis-inducing ligand receptor expression on osteoblasts. Bone 48: 857–863. doi: 10.1016/j.bone.2010.11.015 PMID: 21130908

35. Yang J, Ryu YH, Yun CH, Han SH (2009) Impaired osteoclastogenesis by staphylococcal lipoteichoic acid through Toll-like receptor 2 with partial involvement of MyD88. J Leukoc Biol 86: 823–831. doi: 10.1189/jlb.0309206 PMID: 19602669

36. Trouilliet-Assant S, Gallet M, Nauroy P, Rasigade JP, Flammier S, Parroche P, et al. (2015) Dual impact of live Staphylococcus aureus on the osteoclast lineage, leading to increased bone resorption. J Infect Dis 211: 571–581. doi: 10.1093/infdis/jiu386 PMID: 25006047

37. Meghji S, Crean SJ, Hill PA, Sheikh M, Strannegård IL, et al. (2006) Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: an effect of hygienic lifestyle? Pediatr Res 59: 96–101. PMID: 16380405

38. Ljunggren O, Ransjo M, Lerner UH (1991) In vitro studies on bone resorption in neonatal mouse calvariae using a modified dissection technique giving four samples of bone from each calvaria. J Bone Miner Res 6: 543–550. PMID: 1887817

39. Granholm S, Henning P, Lindholm C, Lerner UH (2013) Osteoclast progenitor cells present in significant amounts in mouse calvarial osteoblast isolations and osteoclastogenesis increased by BMP-2. Bone 52: 83–92. doi: 10.1016/j.bone.2012.09.019 PMID: 23017661

40. Takeshita S, Kaji K, Kudo A (2000) Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res 15: 1477–1488. PMID: 10934646

41. Adlerberth I, Lindberg E, Aberg N, Hesselmar B, Saalman R, Strannegård IL, et al. (2006) Reduced enterobacterial and increased staphylococcal colonization of the infantile bowel: an effect of hygienic lifestyle? Pediatr Res 59: 96–101. PMID: 16380405

42. Kelly LA, Lerner UH, Ransjo M, Ransjo EJ, Garnovska F, et al. (2007) Estrogen inhibits osteoclast formation in mouse haematopoietic cells independently of transcriptional regulation by receptor activator of NF-(kappa)B and c-Fms. J Endocrinol 195: 415–427. PMID: 18000304

43. Kim N, Takami M, Rho J, Josien R, Choi Y (2002) A novel member of the leukocyte receptor complex regulates osteoclast differentiation. J Exp Med 195: 201–209. PMID: 11805147

44. Ljunggren O, Ransjo M, Lerner UH (1991) In vitro studies on bone resorption in neonatal mouse calvariae using a modified dissection technique giving four samples of bone from each calvaria. J Bone Miner Res 6: 543–550. PMID: 1887817

45. Matsumoto C, Oda T, Yokoyama S, Tominari T, Hirata M, Miyaura C, et al. (2012) Toll-like receptor 2 heterodimers, TLR2/6 and TLR2/1 induce prostaglandin E production by osteoblasts, osteoclast formation and inflammatory periodontitis. Biochem Biophys Res Commun 428: 110–115. doi: 10.1016/j.bbrc.2012.06.016 PMID: 23063683

46. Takeshita S, Kaji K, Kudo A (2000) Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts. J Bone Miner Res 15: 1477–1488. PMID: 10934646

47. Kassem A, Henning P, Lindholm C, Tominari T, Hirata M, Miyaura C, et al. (2012) Toll-like receptor 2 heterodimers, TLR2/6 and TLR2/1 induce prostaglandin E production by osteoblasts, osteoclast formation and inflammatory periodontitis. Biochem Biophys Res Commun 428: 110–115. doi: 10.1016/j.bbrc.2012.06.016 PMID: 23063683

48. Kassem A, Henning P, Lindholm C, Souza PP, Lindholm C, Lerner UH (2015) Porphyromonas gingivalis Stimulates Bone Resorption by Enhancing RANKL (Receptor Activator of NF-kappaB Ligand) through Activation of Toll-like Receptor 2 in Osteoblasts. J Biol Chem 290: 20147–20158. doi: 10.1074/jbc.M115.655787 PMID: 26085099

49. Kassem A, Henning P, Lindholm C, Tominari T, Hirata M, Miyaura C, et al. (2012) Toll-like receptor 2 heterodimers, TLR2/6 and TLR2/1 induce prostaglandin E production by osteoblasts, osteoclast formation and inflammatory periodontitis. Biochem Biophys Res Commun 428: 110–115. doi: 10.1016/j.bbrc.2012.06.016 PMID: 23063683

50. Kassem A, Henning P, Lindholm C, Souza PP, Lindholm C, Lerner UH (2015) Porphyromonas gingivalis Stimulates Bone Resorption by Enhancing RANKL (Receptor Activator of NF-kappaB Ligand) through Activation of Toll-like Receptor 2 in Osteoblasts. J Biol Chem 290: 20147–20158. doi: 10.1074/jbc.M115.655787 PMID: 26085099

51. Ji JD, Park-Min KH, Shin Z, Fajardo RJ, Goldring SR, McHugh KP, et al. (2009) Inhibition of RANK expression and osteoclastogenesis by TLRs and IFN-gamma in human osteoclast precursors. J Immunol 183: 7223–7233. doi: 10.4049/jimmunol.0900072 PMID: 19890054
54. Zhang P, Liu J, Xu Q, Harber G, Feng X, Michalek SM, et al. (2011) TLR2-dependent modulation of osteoclastogenesis by Porphyromonas gingivalis through differential induction of NFATc1 and NF-kappaB. J Biol Chem 286: 24159–24169. doi: 10.1074/jbc.M110.198085 PMID: 21566133

55. Koga K, Matsui Y, Asagiri M, Kodama T, Nakashima K, et al. (2002) NFAT and osterix cooperatively regulate bone formation. Nat Med 11:880–885.

56. Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G, et al. (2006) Calcineurin/NFAT signaling in osteoblasts regulates bone mass. Develop Cell 10:771–782.

57. Choo MK, Yeo H, Zayzafoon M (2009) NFATc1 mediates HDAC-dependent transcriptional repression of osteocalcin expression during osteoblast differentiation. Bone 45:579–589. doi: 10.1016/j.bone.2009.05.009 PMID: 19463978

58. Zanotti S, Smerdel-Ramoya A, Canalis E (2011) Reciprocal regulation of Notch and nuclear factor of activated T-cells (NFAT) c1 transactivation in osteoblasts. J Biol Chem 286:4576–4588. doi: 10.1074/jbc.M110.161893 PMID: 21131365

59. Boyce BF, Xing L (2007) Biology of RANK, RANKL, and osteoprotegerin. Arthritis Res Ther 9 Suppl 1: S1. PMID: 17634140

60. Walsh MC, Choi Y (2014) Biology of the RANKL-RANK-OPG System in Immunity, Bone, and Beyond. Front Immunol 5: 511. doi: 10.3389/fimmu.2014.00511 PMID: 25368616

61. Conaway HH, Pirhayati A, Persson E, Pettersson U, Svensson O, Lindholm C, et al. (2011) Retinoids stimulate periosteal bone resorption by enhancing the protein RANKL, a response inhibited by monomeric glucocorticoid receptor. J Biol Chem 286: 31425–31436. doi: 10.1074/jbc.M111.247734 PMID: 21715325

62. Conaway HH, Persson E, Halen M, Granholm S, Svensson O, Pettersson U, et al. (2009) Retinoids inhibit differentiation of hematopoietic osteoclast progenitors. FASEB J 23: 3526–3538. doi: 10.1096/fj. 09-132548 PMID: 19546303

63. Sims N, Walsh NC (2010) GP130 cytokines and bone remodelling in health and disease. BMB Reports 43:513–523. PMID: 20797312

64. Schett G, Gravallese E (2012) Bone erosion in rheumatoid arthritis: mechanisms, diagnosis and treatment. Nat Rev Endocrinol 8:656–664.

65. Souza PP, Lerner UH (2013) The role of cytokines in inflammatory bone loss. Immunol Invest 42:555–622. doi: 10.3109/088220139.2013.822766 PMID: 24004059