# Immune modulation in *Staphylococcus aureus*-induced arthritis by a combination of antibiotics and inhibition of Interleukin-15

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Petra Henning

**Thesis for the degree of Bachelor of Science**
**University of Iceland**
**Faculty of Medicine**
**School of Health Science**
**August 2012**

HÁSKÓLI ÍSLANDS

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## ABSTRACT

**Background:** *Staphylococcus aureus* arthritis is characterized by a severe and rapid joint destruction where many of the affected patients develop permanent joint damage despite adequate antibiotic treatment. The permanent nature of the damage and the inadequacy of antibiotic treatment spur a need for new therapeutic procedures. Interleukin-15 (IL-15) is a pluripotent, antiapoptotic, and pro-inflammatory cytokine, which has been previously implicated in osteoclastogenesis. We have recently shown that inhibition of IL-15 alone leads to a less destructive S. aureus induced arthritis without affecting morbidity and the host's ability to clear the bacteria. In this study we investigate the potential benefits of a combination of antibiotic and inhibition of IL-15, using anti-IL-15 (aIL-15), as a treatment in S. aureus-induced arthritis.

**Method:** Toxic shock syndrome toxin-1 producing *S.* aureus was intravenously inoculated in C57BL/6 wildtype mice. Treatment was started three days later with antibiotics and aIL-15ab or isotype control antibodies.

**Results:** Treatment with aIL-15ab combined with antibiotics reduced synovitis without significantly affecting bone and cartilage erosivity. Moreover, the treatment did not have a negative impact on mortality, morbidity nor bacterial clearance. Assessment of bone mineral density revealed that the treatment did not have an impact on general bone loss. We did not observe that expression of OPG, RANK or RANKL was influenced by the treatment, except for significantly lower serum levels of OPG in the treated mice at day 12 after bacterial inoculation. Preliminary *in vitro* data showed a trend towards decreased osteoclastogenesis in cultures with the aIL-15ab compared with cultures with the isotype control antibody.

**Discussion:** The combination of antibiotics and aIL-15 antibodies decreases the severity of synovitis in *S.* aureus-induced arthritis, but does not affect cartilage/bone destruction or general bone loss. Further, it does not have a negative impact on general infection. We conclude that, firstly, adding aIL-15ab to antibiotic treatment has limited additional benefits with respect to joint damage; and secondly, the effect of IL-15 on osteoclastogenesis is probably mediated via an indirect pathway.

**Key words:** IL-15; *S. aureus*; arthritis; osteoclasts; mice.

# ABBREVIATIONS

| | |
|---|---|
| aa | Amino acids |
| aIL-15ab | anti-IL-15 antibody |
| APC | Antigen-presenting cells |
| Bcl | B-cell lymphoma |
| BMC | Bone marrow cells |
| BMD | Bone mineral density |
| BMM | Bone marrow macrophages |
| CD | Cluster of differentiation |
| CFU | Colony forming units |
| CIA | Collagen-induced arthritis |
| CLP | Cecal ligation and puncture |
| CP(5) | Polysaccharide capsule (5) |
| DCs | Dendritic cells |
| ECM | Extracellular matrix |
| HIV | Human immunodeficiency virus |
| ELISA | Enzyme-linked immunosorbent assay |
| G&S | Van Gieson-Safranin O |
| HSC | Haematopoietic stem cell |
| H&E | Hematoxylin-eosin |
| IFN | Interferon |
| IL-(15) | Interleukin |
| IL-(15)R | Interleukin receptor |
| Ig | Immunoglobulin |
| LS | Lena Svensson |
| M-CSF | Macrophage-colony stimulating factor |
| MCP | Monocyte chemotactic protein |
| MHC II | Major histocompatibility complex class II |
| MSCRAMMs | Microbial surface components recognizing adhesive matrix molecules |
| NF-κB | Nuclear factor kappa B |
| NK | Natural killer |
| OPG | Osteoprotegerin |
| PBS | Phosphate buffered saline |
| PMN | Polymorphonuclear |
| PCR | Polymerase chain reaction |
| pQCT | Peripheral quantitative computed tomography |
| PTH | Parathyroid hormone |
| RA | Rheumatoid arthritis |
| RANK(L) | Receptor activator of nuclear factor kappa-B (ligand) |
| TCR | T-cell receptor |
| TNF | Tumor necrosis factor |
| TRAP | Tartrate resistant acid phosphatase |
| TSST-1 | Toxic shock syndrome toxin-1 |

# 1 INTRODUCTION

## 1.1 Septic arthritis

Septic arthritis is a disease where an infectious agent, most commonly a bacterium, causes an inflammatory response in the joint [1]. The main route by which the pathogen reaches the joint is through the blood [2] and therefore the arthritis is often accompanied by septic symptoms. Septic symptoms include hyperthermia/hypothermia, tachypnea and tachycardia. Sepsis can trigger a septic shock, a life-threatening condition, potentially resulting in multiple organ dysfunction. Examples of other routes by which pathogens reach the joints are direct inoculation due to trauma or an iatrogenic event, infected contiguous foci or a neighboring soft-tissue sepsis [3].

Bacterial arthritis is the most rapidly progressing and destructive joint disease [4, 5], and the estimated incidence is 0.002-0.01% in the general population and 0.03-0.07% in patients with rheumatoid arthritis (RA) and in patients with joint prostheses [2, 4]. The mortality rate in septic arthritis cases is high; approximately 5-20% of affected adults die due to the detrimental inflammatory response, and irreversible joint dysfunction follows infection in 25-70% (mean 40%) of patients despite the use of adequate antibiotic therapy [1]. *Staphylococcus aureus* is the most common etiological agent of human septic arthritis [2], isolated in 37–56% of cases [3], and the one which causes the most severe joint disease. Other common causative agents are *S. epidermidis* and the streptococci, and these aforementioned gram-positive cocci account for half of all cases of septic arthritis [6]. *Neisseria gonorrhoeae* and *N. meningitidis* are of most importance amongst gram-negative cocci, accounting for at least 20% of all cases of septic arthritis.

## 1.2 The characteristics of *S. aureus*

*S. aureus* is an ominous pathogen causing a broad spectrum of human diseases such as septic arthritis, endocarditis, toxic shock syndrome, scalded skin syndrome, pneumonia, meningitis, wound infections, skin infections, and food poisoning [1, 7]. It is also a common cause of hospital acquired infections, e.g. infected sutures in surgical wounds and infections accompanying foreign devices introduced in the body, such as catheters and prostheses. *S. aureus* is a facultative anaerobic gram-positive coccus with the capacity to produce a wide

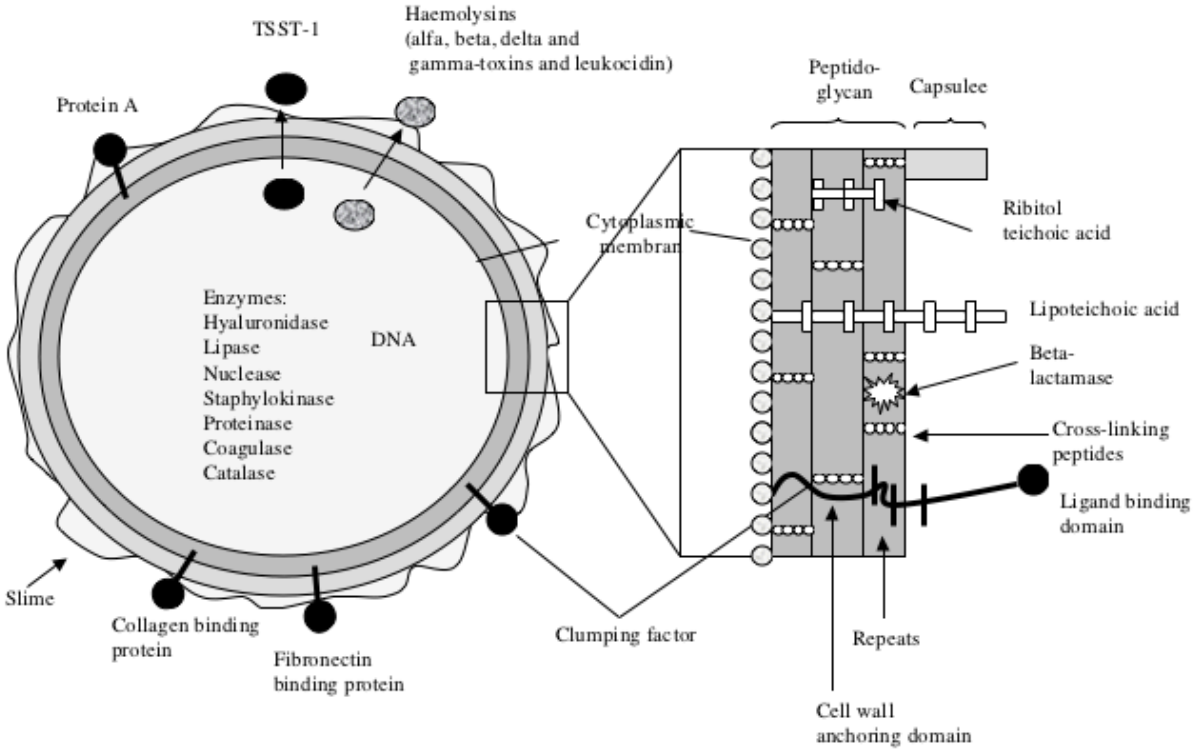


**Figure 1. Schematic overview over the structure of *Staphylococcus aureus* (adapted from Lowy, FD. NEJM 1998).**

range of virulence factors. The bacterium (Figure 1) is constituted of a slime layer, a capsule, a cell wall and a number of surface proteins, and can produce a vast array of enzymes and toxins [1, 7]. Many strains express a capsule, surrounding the cell wall, which is composed of polysaccharides. Many recognized subtypes of capsules exist where types 5 and 8 are the most common in diseases [7]. The polysaccharide capsule (CP) is an important virulence factor since it renders the bacterium more resistant to phagocytosis as opsonisation by antibodies and complement is obstructed [1]. CP5 encapsulated strains in *S. aureus*-induced arthritis are more virulent than non-encapsulated strains and CP5 expression leads to a higher frequency of arthritis and a more severe course of the disease as a result of decreased phagocytosis of the bacteria [8]. The cell wall is composed of peptidoglycans, teichoic acid and lipoteichoic acid. Peptidoglycans and lipoteichoic acid can function as recognition sites for the immune system and upon recognition, can lead to activation of the immune system [9]. Some of the staphylococcal surface proteins are able to function as adhesins which mediate adherence to host tissue and determine the ability of *S. aureus* strains to colonize the host tissues, enabling the bacteria to initiate infection [10, 11]. Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are a class of cell surface adhesins that mediate adherence to extracellular matrix (ECM) components such as fibronectin,

laminin, elastin, collagen and hyaluronic acid [10, 11]. Notable examples of MSCRAMMs in *S. aureus* infections include fibronectin-binding protein, clumping factor and collagen-binding protein [1, 5, 12]. **Fibronectin-binding protein A and B** bind fibronectin and are crucial virulence factors in the initiation of foreign body infections [5, 7, 12]; **clumping factor A** is an important virulence determinant for *S. aureus* septic arthritis [13]; **collagen-binding protein** gives the bacterium the ability to bind to collagen in various structures, for example cartilage; and **protein A** is either a cell wall anchored protein or a secreted soluble product and binds to the Fc region of the immunoglobulin (Ig) G molecule, making it unavailable for recognition by the Fc receptor on phagocytes, and obstructs phagocytosis [10]. *S. aureus* also secretes several **enzymes**, including, but not limited to, coagulase, beta-lactamase, staphylokinase, proteases, lipase, hyaluronidase, catalase and nuclease and these products provide protection from host phagocytes and penicillin, and facilitate the spread of infection to adjoining tissues. **Toxins** produced by *S. aureus* are numerous and include hemolysins and leukocidin, which are cytolytic, and superantigens. **Superantigens** (Figure 2) are pyrogenic toxins that can activate a large proportion of the host's T lymphocytes [14-17]. They bind to the variable region of the T-cell receptor (TCR) β chain (Vβ region) and to the outside of the major histocompatibility complex class II (MHC II) protein. The superantigen cross-links the TCR and MHC II molecule and induces extensive T-cell proliferation. The T-cell activation is not dependent on prior degradation by antigen-presenting cells (APC) and is therefore not antigen specific. The fraction of superantigen-

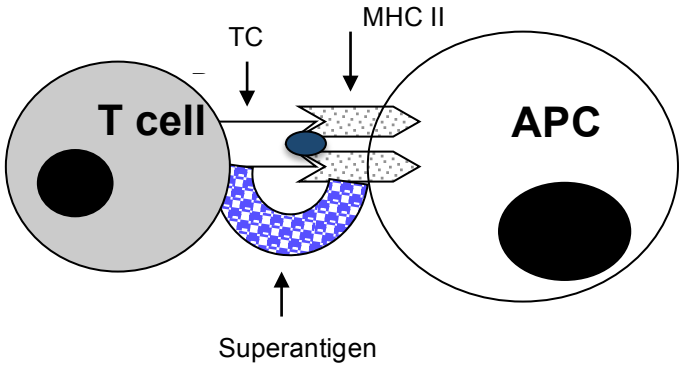


**Figure 2. A superantigen is activating an antigen presenting cell (APC) via MHC class II and a T cell via a specific Vβ-region on TCR.** This interaction activates around 5-20% of the T cells, and pro-inflammatory cytokines are extensively produced.

activated T-cells ranges from 5 to 20% of all T-cells. These toxins include the staphylococcal enterotoxins and toxic shock syndrome toxin-1 (TSST-1). The highly activated T-cells eventually die an apoptotic death and leave a "hole" in T cell repertoire. This T-cell activation and the subsequent production of high levels of pro-inflammatory cytokines can be very harmful, possibly causing fatal shock characterized by a rapid onset of high fever, hypotension and other acute toxic shock-like symptoms [1, 7, 18].

## 1.3 *S. aureus* infections

*S. aureus* can be found commensally on the skin or in the mucosal bacterial flora in the anterior nares [1, 7, 19, 20] of and 10-20% of healthy adults are persistently colonized, termed carriers. These carriers are at increased risk of getting *S. aureus* infections, but more importantly, carriers can serve as a mean of transmission of *S. aureus* and this is particularly relevant in hospitals as health care workers display higher carrier frequency than in the general population.

As mentioned earlier, the arthritis caused by *S. aureus* is extremely damaging, causing severe destruction of cartilage and subchondral bone early on in the infection [1, 5], subsequently giving rise to severe *S. aureus*-induced arthritis as well as inducing systemic bone loss [21]. The characteristic clinical feature is a monoarticular manifestation of a large joint, most often a knee [3, 6]. The joint is red, swollen, warm and acutely painful with decreased range of motion. Other symptoms are e.g. malaise and high fever. Particularly immunosuppressed patients do not all always respond with systemic symptoms or joint symptoms, which can lead to a delay in both diagnosis and treatment. During bacteremia the staphylococci are dispersed throughout the body but they often reside in the synovium and the kidneys, probably due to adherence characteristics and toxin production [5]. As mentioned, many of the bacterial products described have been shown to contribute to the joint damage, either directly or indirectly by activation of the immune system. It is not, however, the bacterium itself that is responsible for the severe joint damage observed, but rather, an overactivated immune system [1]. Prompt diagnosis and rapid initiation of therapy are essential to avoid a permanent reduction in joint function. Hitherto, it has proven difficult to effectively treat this type of infection. This stems from two conflicting factors: The importance of a strong immune response in killing the evading staphylococcus, and the necessity to down-regulate the exaggerated immune response that contributes to the tissue

destruction. Prognosis at the onset of septic arthritis is dependent on a few factors [1]. First, it is dependent on existing predisposing factors such as pre-existing joint disease, notably RA and prosthetic joint surgery, and states of compromised immunity e.g. malignancy, HIV infection, diabetes mellitus or immunosuppressive therapies. Second, a more negative outcome is expected if the joint manifestations are polyarticular, the patient is of older age and if there is a delay by patient, doctor or both.

Even with the eradication of the staphylococci by use of an efficient antibiotic therapy, the tissue destruction continues. This is evident in both human [7] and murine [22] septic arthritis and is caused by the aforementioned host immune response evoked by the bacteria, rather than by the presence of the bacterium itself. Thus, the permanent nature of the damage and the inadequacy of antibiotic treatment spur a need for new therapeutic procedures. Treatment with antibiotics combined with an unspecific inhibition of the immune system with corticosteroids has been shown to have a beneficial effect on the outcome of septic arthritis in both animal models and humans [22-24]. However, corticosteroids are *per se* strongly immunosuppressive and care must be taken to balance the immune response in such a way that the host defense is maintained.

Laboratory models of bacterial arthritis have proven beneficial in the research of the pathogenesis of this disease. The murine model is well suited for these studies for several reasons [1, 25, 26]: First, the immune system in mice is well-characterized, bearing great resemblance to the human system; second, there exists many inbred and genetically well-characterized strains; third, the existence of knock-out and transgenic mice; fourth, the much easier manipulation of mice in comparison to other animal models or humans; fifth, the ability to control the exact time of onset of the infection; sixth, the ability to test the efficacy of experimental prophylaxis and treatment; and lastly, the fact that the mouse develops spontaneous septic arthritis [27]. In these experiments, we used our well-established mouse model of systemic *S. aureus*-induced arthritis, established over twenty years ago [27].

Over twenty years ago, the technician Lena Svensson (LS) discovered that several mice spontaneously developed arthritis. This was found to be a TSST-1 producing *S. aureus* strain, which was named LS-1 [25-27]. After intravenous inoculation of a defined number of bacteria into one of the tail veins, the mouse develops a haematogenously spread septic arthritis. The bacterial dose in a particular mouse strain is pivotal with regard to the outcome of the septic

arthritis [26]. Too large of a dose can cause sepsis without causing arthritis, whereas a lower dose leads to the development of arthritis. In our mouse model the optimal dose is large enough to induce septic arthritis in the majority of the mice, without high mortality rates, and both clinical and histological signs of arthritis are present within 24 hours after inoculation [25]. Within a week, the infection has been cleared from all organs except for the kidneys and the joints, and therefore the bacterial clearance is measured by evaluating the bacterial dose in the kidneys after termination of the experiment.

## 1.4 Interleukin-15

Interleukin-15 (IL-15) is a pleiotropic, pro-inflammatory, antiapoptotic cytokine that has many diverse biological functions and signals to cells of both the innate and adaptive immune system [28-33]. IL-15 is a 15-17 kDa member of the 4-α-helix bundle cytokine family and shares many biological properties with another cytokine of the same family, IL-2 [33-36]. Unlike IL-2, which is secreted mainly by T-cells, IL-15 is produced by a variety of cells including epithelial and endothelial cells, fibroblast, muscle cells, monocytes/macrophages, dendritic cells and mast cells [33, 36, 37]. IL-15 interacts with a particular cell surface receptor, composed of three subunits [28, 34, 36, 38, 39]. This trimeric IL-15 receptor (IL-15R) shares two subunits with the IL-2 receptor, the IL-2 receptor beta (IL-2/15Rβ) and IL-2 receptor gamma/gamma common (IL-2Rγ/γc) chains. In addition the IL-15R has its own unique high affinity α chain, IL-15Rα. In both mice and humans, IL-15 exists in two mRNA isoforms as a consequence of alternative splicing. One form is shorter, stored intracellularly and has a signal peptide of 21 amino acids (aa). The alternative IL-15 isoform has a 48-aa signal peptide and binds the IL-15Rα [40, 41]. Recently, it has been shown that, in principle, the short IL-15 isoform can also participate in the cytokine-receptor complex formation with the high affinity α chain [42]. The most potent form of IL-15 is in this complex with IL-15Rα and this complex formation leads to a significant increase in production, stability, and tissue availability of bioactive IL-15 *in vivo* [43]. The IL-15/IL-15Rα complex is bound to the surface of its producing cells or a cleaved, secreted, soluble complex which binds to the β and γ subunits with considerably higher affinity compared to IL-15 alone [43, 44]. The formation of IL-15/IL-15Rα complexes on cell surfaces can induce presentation in *trans* to neighboring target cells expressing the IL-2R/15β and γ chains [45]. The intermediate affinity IL-15βγ complex is responsible for signal transduction, and the binding and signaling events can occur even in the absence of IL-15Rα. It was suggested that IL-15Rα was not able to signal [39] but

there is increasing evidence that IL-15Rα can, in fact, mediate signaling [46-50], and may function and signal independently of the other chains of the receptor complex.

The functions of IL-15 are very diverse and the cells found responsive to IL-15 are numerous. IL-15 is a prosurvival factor and upregulates antiapoptotic factors, such as Bcl-xL and Bcl-2, and hinders proapoptotic members of the Bcl-2 family, e.g. Bim and PUMA to act [31, 51]. It can therefore inhibit apoptosis in many cell types such as neutrophils, mast cells, CD8+ T-cells and dendritic cells (DCs). In addition, IL-15 is very important for lymphoid development and homeostasis, particularly for natural killer (NK) and CD8+ T cells [30, 52-54]. IL-15 has also been identified as a potent chemoattractant for T-lymphocytes [55]. IL-15 promotes the functional maturation of macrophages, neutrophils and DCs, enhances their phagocytic activity and stimulates monocytes to produce pro-inflammatory factors such as monocyte chemotactic protein-1 (MCP-1) and IL-8 which attract monocytes and neutrophils, respectively, to the site of inflammation [29, 56-58]. A novel role for intracellular IL-15 in mast cells was reported in the cecal ligation and puncture (CLP) mouse model of sepsis [37], where intracellular IL-15 was shown to suppress the activity of mouse mast cell chymase by acting as a specific negative transcriptional regulator. This IL-15-mediated repression of mast cell chymase activity has a profound negative effect on bacterial clearance and processing of neutrophil-recruiting chemokines. Subsequently, significantly higher survival rates were seen in IL-15 knockout mice after CLP because of the absence of intracellular IL-15 in mast cells [37]. IL-15 also affects non-immune cells such as epithelial cells, fibroblasts and endothelial cells, and protects these cells from apoptosis and induces angiogenesis [28].

## 1.5 IL-15 in arthritis

Excessive expression of IL-15 has been linked with a number of autoimmune and chronic inflammatory disorders, including RA, inflammatory bowel diseases, and diabetes mellitus [30]. RA is a chronic inflammatory disease characterized by a chronic inflammatory infiltration of the synovial membrane consisting of T-cells, monocytes/macrophages, mast cells, neutrophils and plasma cells, as well as an expanded population of activated synovial fibroblasts [30, 59]. This can be associated with a progressive destruction of articular cartilage and underlying bone of affected joints. High levels of IL-15 are detectable in the inflamed synovial fluid and tissues of RA patients and

IL-15 is known to play a part in the pathogenesis of RA [59, 60]. Fibroblast-like synoviocytes are an important source of IL-15. Together with endothelial cells, these synoviocytes can produce IL-15 and subsequently recruit and activate both CD4+ and CD8+ T-cells [59-61]. Via these effects on T-cells, IL-15 promotes cytokine and chemokine release directly or indirectly through maintenance of cytokine-activated T-cell cognate interactions with synovial macrophages [62]. IL-15 also promotes synovial neutrophil activation and survival, NK cell activation, osteoclast progenitor cell maturation and acts in synergy with other cytokines e.g. tumor necrosis factor (TNF) α, IL-18, IL-12 and IL-6 [30, 63]. All of the above factors contribute to the chronic synovial inflammation. Further, the importance of IL-15 for the development of inflammatory arthritis was demonstrated when administration of a soluble fragment of IL-15Rα profoundly suppressed the development of collagen-induced arthritis (CIA) in mice [64] and parallel ex vivo studies demonstrated the suppression of antigen-specific T-cell proliferation and cytokine production, as well as Ig synthesis. Taken together, IL-15 mediates pleiotropic effector functions in RA and monoclonal IL-15-blocking antibodies are being tested in clinical trials albeit with disappointing results [62, 63].

## 1.6 IL-15 and osteoclasts

Further, IL-15 has been implicated in osteoclastogenesis [65-67], the generation of osteoclasts, which is considered to play an important role in the loss of juxta-articular bone in arthritis [68-70]. A rapid and easily measurable systemic bone resorption, as a result of increased osteoclastic activity, has also been demonstrated in *S. aureus*-induced arthritis [21].

Bone turnover is mediated by osteoclasts and osteoblasts [71]. Osteoclasts are multinucleated cells, formed by the fusion of mononuclear progenitors of the monocyte/macrophage lineage. Osteoclasts are the main bone-resorbing cells, while osteoblasts are mononucleate cells that synthesize organic matrix for bone formation [72]. Osteoclast differentiation from haematopoietic stem cells (HSC) is regulated by and dependent on macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) (RANK) ligand (RANKL) [72-74]. M-CSF binds to its receptor, c-Fms, on preosteoclastic cells, and if the expression of M-CSF is increased, the

pool of preosteoclastic cell increases [75]. If there are mutations in the *M-CSF* gene, both osteoclasts and macrophages are influenced, which reflects the close relations between these two cell types [73, 76]. Also, mature monocytes and macrophages can differentiate into osteoclasts in a suitable microenvironment [74, 77].

RANKL is both produced as a soluble protein and expressed on the surface of stromal cells/osteoblasts (Figure 3) and its expression is regulated by osteoclastogenic factors, such as glucocorticoids, parathyroid hormone (PTH) and pro-inflammatory cytokines like IL-1 and IL-6 [71, 72]. Also, RANKL is produced during inflammation by a range of inflammatory cells, for example activated T-cells and synovial fibroblasts [71, 72]. Osteoclastogenesis is promoted by the binding of RANKL to its cognate receptor RANK, which is expressed on osteoclasts and their precursors. Osteoprotegerin (OPG) is a decoy receptor for RANKL and prevents its interaction with RANK, leading to a decrease in formation, activity, survival and proliferation of osteoclasts and reduced bone resorption [71, 72]. OPG is secreted by e.g. stromal cells/osteoblasts, lymphoid cells and fibroblast. Mice lacking RANK or RANKL, or overexpressing OPG [78-80], display profound osteopetrosis, a marked increase in bone density, as a result of a lack of active osteoclasts. OPG knockout mice develop early onset osteoporosis, characterized by a reduction in bone mass, due to both an increased number and activity by osteoclasts [81]. Any imbalance between the molecular triad of OPG, RANK and RANKL can lead to pathologic conditions such as bone tumor-associated osteolysis and osteoarticular pathology [71].

The OPG/RANK/RANKL triad can be modulated by a number of factors, such as hormones and cytokines [71, 75]. Among them, TNF-α and IL-1 stimulate M-CSF production and directly increase RANKL expression by stromal cells/osteoblasts. Glucocorticoids and PTH exert their effects on osteoclastogenesis by a negative regulation of OPG expression and an increased production of RANKL by stromal cells/osteoblasts. Many mediators, produced both during the inflammatory process in RA and *S. aureus*-induced arthritis, are implicated in osteoclastogenesis and function either directly, by acting on the cells of the osteoclast-lineage, or indirectly, by modulating the OPG/RANK/RANKL triad [72]. In RA, overexpression of RANKL can induce synovial macrophages to differentiate into active osteoclasts leading to periarticular bone loss [82]. Human trials of anti-RANKL and anti-osteoclast treatments have shown that these

treatments are beneficial in RA as they reduce the bone loss associated with inflammation [73], without affecting the underlying inflammatory processes such as joint swelling and pain [65, 73], which indicates that bone resorption and inflammation are closely related but distinct processes. Furthermore, RANKL-targeting treatment in combination with antimicrobial agents significantly reduces bone resorption in experimental *S. aureus*-induced arthritis [83].

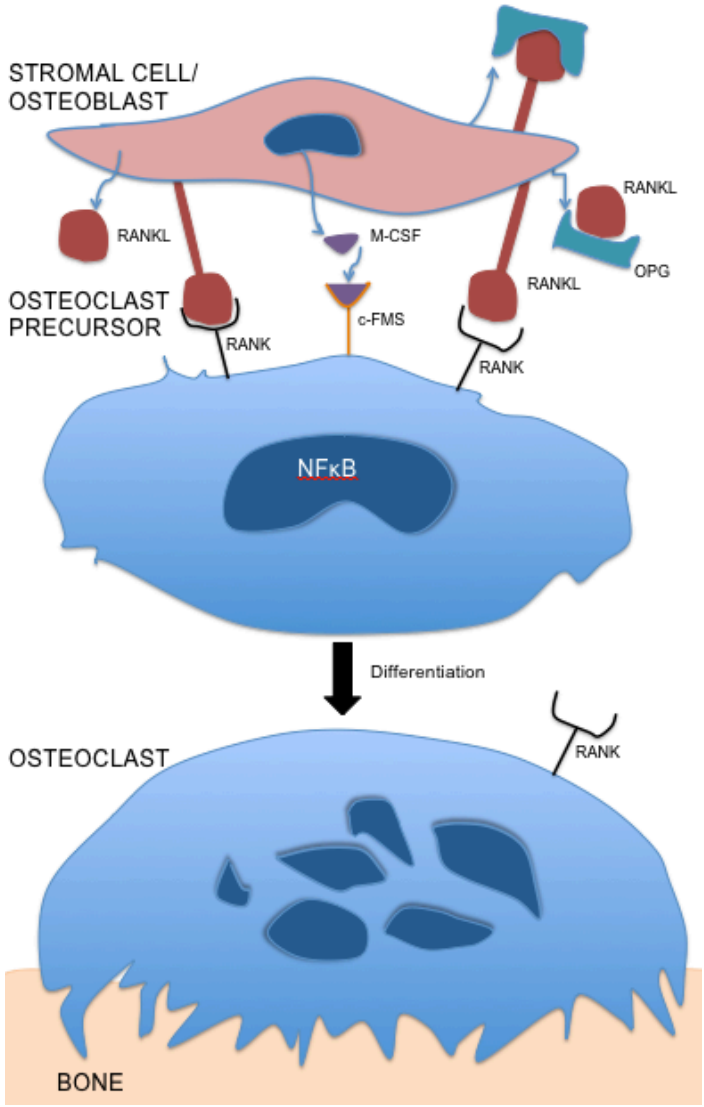


**Figure 3. Schematic overview over the mechanisms of osteoclastogenesis (adapted from Robbins and Cotran Pathologic Basis of Disease, 8. Edition, 2009** [84]**).** Stromal cells and osteoblasts express receptor activator of nuclear factor kappa B (NF-κB) (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which can interact with their receptors, RANK and c-FMS respectively, on osteoclast precursors. Osteoprotegerin (OPG), secreted by e.g. stromal cells and osteoblasts, inhibits osteolysis and blocks the interaction of RANKL/RANK.

## 1.7 *S. aureus*-induced arthritis and bone turnover

During the first day of *S. aureus* infection, polymorphonuclear (PMN) granulocytes and macrophages infiltrate the synovium, and a prominent T-cell infiltration, primarily of the CD4+ phenotype, soon follows [85]. Activated T-cells express RANKL so these T-cells might possess the ability to directly act on osteoclast-precursor cells to induce osteoclast differentiation [71, 72]. However, T-cells produce cytokines such as interferon (IFN) γ, IL-4 and IL-10, which are all inhibitory to *in vitro* osteoclastogenesis thus, the *in vivo* mechanisms behind T-cell-induced bone resorption are complex [72]. Macrophages, PMN granulocytes in the synovium and synovial fibroblasts are also an important source of pro-inflammatory cytokines. An example of such a cytokine is TNF-α, which induces RANKL expression on synovial fibroblasts and is the most potent osteoclastogenic cytokine produced during inflammation [71, 75]. Our cytokine of interest, IL-15, has been suggested to play an important role in osteoclast differentiation by inducing RANKL expression from stromal cells/osteoblasts, as well as from NK cells and synovial fibroblasts [65-67]. However, the exact role of IL-15 in osteoclastogenesis, osteoclast activation and bone remodeling *in vivo* remains unclear. IL-15 has been shown to directly stimulate the differentiation of osteoclast progenitors into preosteoclasts *in vitro* [66] and provide a costimulatory signal for RANKL-induced osteoclastogensis *in vivo* [65]. In mice with collagen-induced arthritis, treatment with an IL-15 antagonist reduced synovitis as well as bone erosion and cartilage destruction [86].

## 1.8 Conclusion

It was recently shown, using both IL-15 knockout mice or anti-IL-15 antibody (aIL-15ab)-treated mice, that inhibition of IL-15 leads to a less destructive *S. aureus*-induced arthritis compared with wildtype or control antibody treated mice [87]**,** while morbidity, mortality or bacterial clearance were not negatively affected. The aim of the present study was to investigate the potential benefits on the outcome of *S. aureus*-induced arthritis using a combination of aIL-15ab and antibiotic treatment. In addition, we wanted to assess the role of IL-15 in osteoclastogenesis in primary bone-marrow-derived macrophages and bone marrow cultures. We show that inhibition of IL-15 in combination with antibiotics significantly decreases the severity of synovitis in *S. aureus*-induced arthritis, while the joint destruction and general bone loss are not affected. Further, the combination treatment does not have a negative impact on general infection. We conclude that addition of aIL-15ab to antibiotic

treatment has limited effect with respect to joint damage; and secondly, the effect of IL-15 on osteoclastogenesis is probably mediated via an indirect pathway.

# 2 MATERIALS AND METHODS

## 2.1 Mice

Female wildtype C57BL/6 mice were obtained from Scanbur (Sollentuna, Sweden). 10 mice in each cage were maintained under standard conditions of temperature and light in the animal facility at the Department of Rheumatology and Inflammation Research, University of Gothenburg, Sweden. Permission from the local Animal Research Ethics Committee, in accordance with national animal welfare legislation, was obtained for the experiments.

## 2.2 Mouse model of systemic *S. aureus*–induced arthritis

The TSST-1-producing, LS-1 strain of *S. aureus* was used for infection [27]. Female wildtype C57BL/6 mice (n=20) were inoculated intravenously (Figure 4) in the tail with $0.8x10^8$ *S. aureus* LS-1/mouse in a total volume of 200 µl phosphate-buffered saline (PBS). To determine the number of bacteria injected, viable counts were performed. During the course of infection the mice were graded blindly for clinical arthritis severity and frequency. Arthritis was defined as visible erythema and/or swelling of finger/toe, and ankle/wrist joints. Arthritic index, a clinical scoring, was conducted every other day to evaluate the intensity of

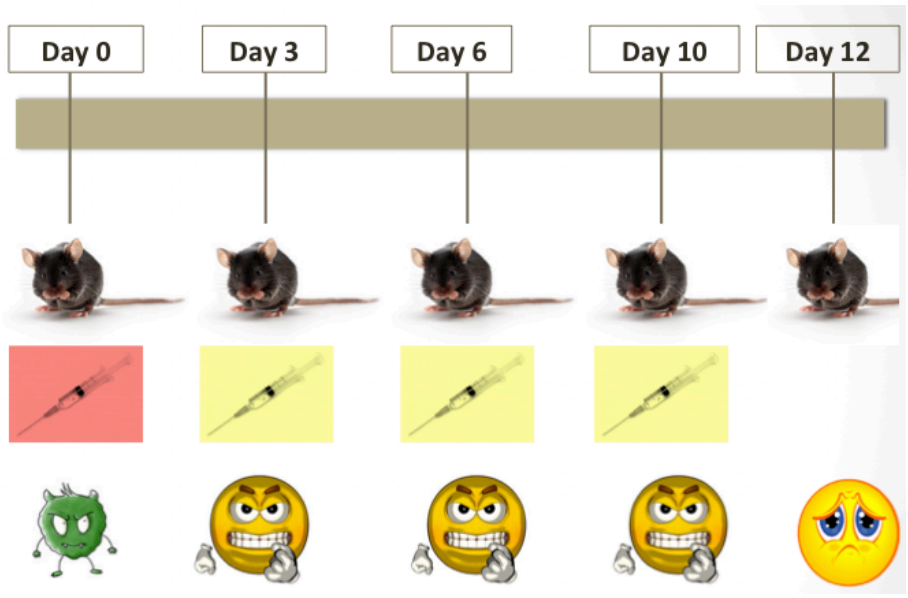


**Figure 4. Timeline of the experiments.** Female wildtype C57BL/6 mice (n=20) were inoculated intravenously with *S. aureus* at day 0. Treatment with anti IL-15 antibody or isotype control antibody, and cloxacillin was started at day 3 after bacterial inoculation. The antibodies were injected intraperitoneally at days 3, 6 and 10 after bacterial inoculation. At day 7, flucloxacillin was added to the drinking water. Termination of the experiment was at day 12 and blood, bone marrow, synovial tissue from knee joints, kidneys and limbs were obtained for further examination.

arthritis in which macroscopic inspection yielded a score of 0-3 points for each limb (0, neither swelling nor erythema; 1, mild swelling and/or erythema; 2, moderate swelling and erythema; 3, marked swelling and erythema). The total score was calculated by adding up all the scores within each animal tested. Each mouse was weighed and examined daily for assessment of the overall condition of the mouse and for signs of systemic infection, i.e. reduced alertness and ruffled coat. In cases of severe systemic infection, when a mouse was judged too ill to survive another 24 hours, it was culled and defined as dead due to sepsis.

## 2.3 Treatment of systemic *S. aureus*-induced arthritis with anti-IL-15 antibodies

Treatment with aIL-15ab (Amgen Inc, Seattle WA, USA) or isotype control antibody (IgG2a; Amgen Inc, Seattle WA, USA) and cloxacillin was started at day 3 after bacterial inoculation. The antibodies (25 $\mu$g/mouse) were injected intraperitoneally at days 3, 6 and 10 after bacterial inoculation. Injections of cloxacillin (150 $\mu$l/mouse) were given twice daily for 3 days and subsequently followed by flucloxacillin (0,5 mg/ml), which was added to the drinking water. Blood, bone marrow, synovial tissue from knee joints, kidneys and limbs were obtained at day 12 for further examination.

## 2.4 Bacterial load in kidneys

Kidneys were aseptically dissected, kept on ice, homogenized, serially diluted in PBS and spread on blood agar plates. After 24 hours of incubation at 37°C, the number of colony forming units (CFU) per kidney pair was determined.

## 2.5 Histology of inflamed joints

To evaluate synovial hypertrophy the joints were fixated, decalcificated and paraffin embedded. Tissue sections from fore- and hind paws were cut, deparaffinized and stained with hematoxylin-eosin (H&E; Histolab Products AB, Gothenburg, Sweden). All slides were coded and evaluated by two blinded observers. The degree of synovitis, and cartilage/bone destruction of finger/toes, wrists/ankles, elbows and knee yielded a score from 0 to 3. Occasionally, one paw was embedded in a way that made it impossible to evaluate the degree of bone destruction, and therefore the total score/mouse is divided by the number of evaluated joints.

## 2.6 A novel staining method using Safranin O and Weigert's Hematoxylin Van Gieson combined

To evaluate joint destruction the joints were fixated, decalcificated and paraffin embedded. Tissue sections from fore- and hind paws were cut, deparaffinized and stained with a newly developed staining method, in which Weigert's Iron hematoxylin Van Gieson (Histolab Products AB, Gothenburg, Sweden) staining procedure of differential staining of collagen and other connective tissue [88] is combined with Safranin O (G&S; Sigma-Aldrich AB, Stockholm, Sweden) staining with Fast Green counterstaining [88]. The degree of cartilage destruction was assessed using Safranin O, which staining intensity is directly proportional to the proteoglycan content in the cartilage [89]. This staining method made it possible to assess the cartilage and bone destruction, as well as the synovial hypertrophy, in a single slide. (Details of the staining method are available upon request from the author). All slides were coded and evaluated by two blinded observers, as described in the section above.

## 2.7 Enzyme-linked immunosorbent assay

Blood was collected before the *S. aureus* inoculation at day 0, at day 3 and at day 12, and centrifuged at 6000*g* for 10 minutes. The serum was collected and stored at -20°C for further analysis. The serum was analyzed for RANKL and OPG by DuoSet® enzyme-linked immunosorbent assay (ELISA) Development kit (mouse TRANCE/TNFSF11/RANKL (cat no DY462) and mouse OPG/TNFRSF11B (cat no DY459) respectively; R&D systems, Europe Ltd, Abingdon, UK) according to the manufacturer's recommendations. The assay was run on Spectra Max 340PC (Molecular Devices, Sunnyvale, CA) and analysis performed using SoftMax Pro 5.2 software (Molecular Devices, Sunnyvale, CA).

## 2.8 Assessment of bone mineral density

Femur and tibia from each mouse was fixed in 79% ethanol and further subjected to peripheral quantitative computed tomography (pQCT) scan with Stratec pQCT XCT Research M, software version 5.4 B (Norland, Fort Atkinson, WI) at a resolution of 70 μm. Trabercular bone mineral density (BMD) was determined with a metaphyseal scan, which was performed at a distance from the growth plate corresponding to 3% of the length of the femur. The inner 45% of the area was defined as the trabecular bone compartment. Cortical BMD was

determined with a mid-diaphyseal scan located 36% of the length of the femur from the growth plate.

## 2.9 *In vitro* generation of murine osteoclasts from bone marrow macrophages

Bone marrow cells from the tibia and femur of two C67BL/6 wildtype mice, were obtained. Bone marrow macrophages (BMM) were isolated and expanded as described by Takeshita et al [90]. Briefly, bone marrow cells were cultured in M-CSF (30 ng/ml) in suspension culture dishes (cat no 430591; Corning) where only macrophages adhere. Two days later the macrophages were used in RANKL stimulated osteoclastogenesis experiments: BMM were seeded in 96-well plates at a density of $1x10^6$ cells/$cm^2$. Cells were cultured in α-minimum essential medium (α-MEM; Gibco) supplemented with 10% fetal calf serum (FCS; Sigma), 50 μg/ml gentamicin (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (PEST; Gibco), 2 mM glutamax (Gibco) and with 30 ng/ml recombinant murine M-CSF (rmM-CSF; R&D Systems) and 2 ng/ml or 1 ng/ml recombinant mouse RANKL (rmRANKL; R&D Systems) with or without 50 ng/ml or 0,5 ng/ml IL-15 (Amgen, USA). Osteoclastic differentiation was maintained for 4 to 5 days prior to tartrate resistant acid phosphatase (TRAP; cat no 387A; Sigma) staining. Osteoclasts were identified as TRAP-positive cells with more than three nuclei.

## 2.10 *In vitro* generation of murine osteoclasts from unselected bone marrow cell culture

Bone marrow cells (BMC) from the tibia and femur of two C67BL/6 wildtype mice, were obtained. BMC were flushed from femur and tibiae and washed once in α-MEM culture medium (Gibco). BMC were seeded at $1x10^6$ cells/$cm^2$ in 96 well plates in α-MEM medium supplemented with 10% FCS (Sigma), 50 μg/ml gentamicin (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin (PEST; Gibco), 2 mM glutamax (Gibco) and with 30 ng/ml rmM-CSF (R&D Systems) plus 4 ng/ml or 2 ng/ml rmRANKL (R&D Systems) with or without anti-IL-15 antibody/isotype antibody (10, 100 or 1000 ng/ml; Amgen). After 4 and 5 days, with a medium change at day 3, cells were stained for TRAP (cat no 387A; Sigma). Finally TRAP-positive cells containing three or more nuclei, were counted as osteoclasts.

## 2.11 Real-time polymerase chain reaction (PCR) for the detection of mRNA levels of RANKL, RANK and OPG

Synovial tissues were obtained from the mice and RNA isolated using RNeasy Mini Kit

(QIAGEN, cat.no. 74104) according to the manufacturer's instructions. cDNA synthesis was performed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems, cat. no. 4368814). GAPDH was used as an internal control and the relative RANK, RANKL and OPG expression values were obtained by dividing each value by that calculated for GAPDH. All reactions were amplified using TaqMan Universal PCR Master Mix (Applied Biosystems, cat. no. 43044337) and analysed on a Viia7 system (Applied Biosystems).

## 2.12 Statistical analysis

Statistical analyses were performed using GraphPad Prism (La Jolla, CA). Statistical differences between independent groups were calculated using non-parametric Mann-Whitney U test. Kaplan-Meier survival plots were prepared and the log-rank test was used for comparison between the two survival curves. $P < 0.05$ was considered statistically significant.

# 3 RESULTS

## 3.1 Combination treatment ameliorates systemic *S. aureus*–induced arthritis and does not influence morbidity, mortality or bacterial clearance

Weight change is used as a measurement of general illness for this mouse model. The mice in the treatment group had less pronounced weight loss compared with those in the control group although the difference was only significant ($p < 0.05$ compared with control group) at days 9 and 10 (Figure 5A). There was no significant difference in mortality rate between the two treatment groups (Figure 5B). Clinical severity of arthritis was ameliorated in the treatment group compared to the control group and was significantly decreased in the treatment group at day 7 after intravenous inoculation of *S. aureus* (Figure 5C). Further, there was no significant difference in the frequency of arthritis between the groups (Figure 5D). The host's ability to clear the bacteria was measured as the CFU/kidney pair at day 12 after bacterial inoculation. Treatment with aIL-15ab combined with antibiotics does not influence the bacterial clearance (Figure 5E).

## 3.2 Combination treatment reduces the histopathological severity of synovitis but not bone and cartilage destruction in *S. aureus*-induced arthritis

Hematoxylin-eosin stained histological sections from day 12 revealed that mice in the treatment group developed a significantly less severe synovitis compared with control group ($p < 0.05$) (Figure 6A), whereas no significant differences could be observed in the degree of bone/cartilage destruction. Neither could the staining of bone and cartilage with van Gieson staining combined with Safranin O (G&S) staining at day 12 after *S. aureus* inoculation show any significant differences between the groups with respect to cartilage and bone destruction (Figure 6B). Figure 6C shows H&E staining of a hindpaw from a mouse belonging to the control group. The hypertrophy and proliferation of the synovial tissue is mild and no bone/cartilage destruction is observed. Figure 6D shows H&E stained section from another mouse of the control group with high-grade synovitis and extensive cartilage/bone destruction. Figures 6E-F show G&S stained sections from the same mice as in Figures 6C-D, respectively. The cartilage in Figure 6F is more clearly seen, stained brightly red, in the G&S stained sections and thus the cartilage destruction is more clearly observed in Figure 6F in comparison to the H&E stained specimen in Figure 6D. Likewise, the inflammatory infiltrate, stained green with G&S, is more prominent and so is the contrast between bone, stained purple with G&S, and the inflammatory cells as seen in Figure 6F compared to Figure 6D.

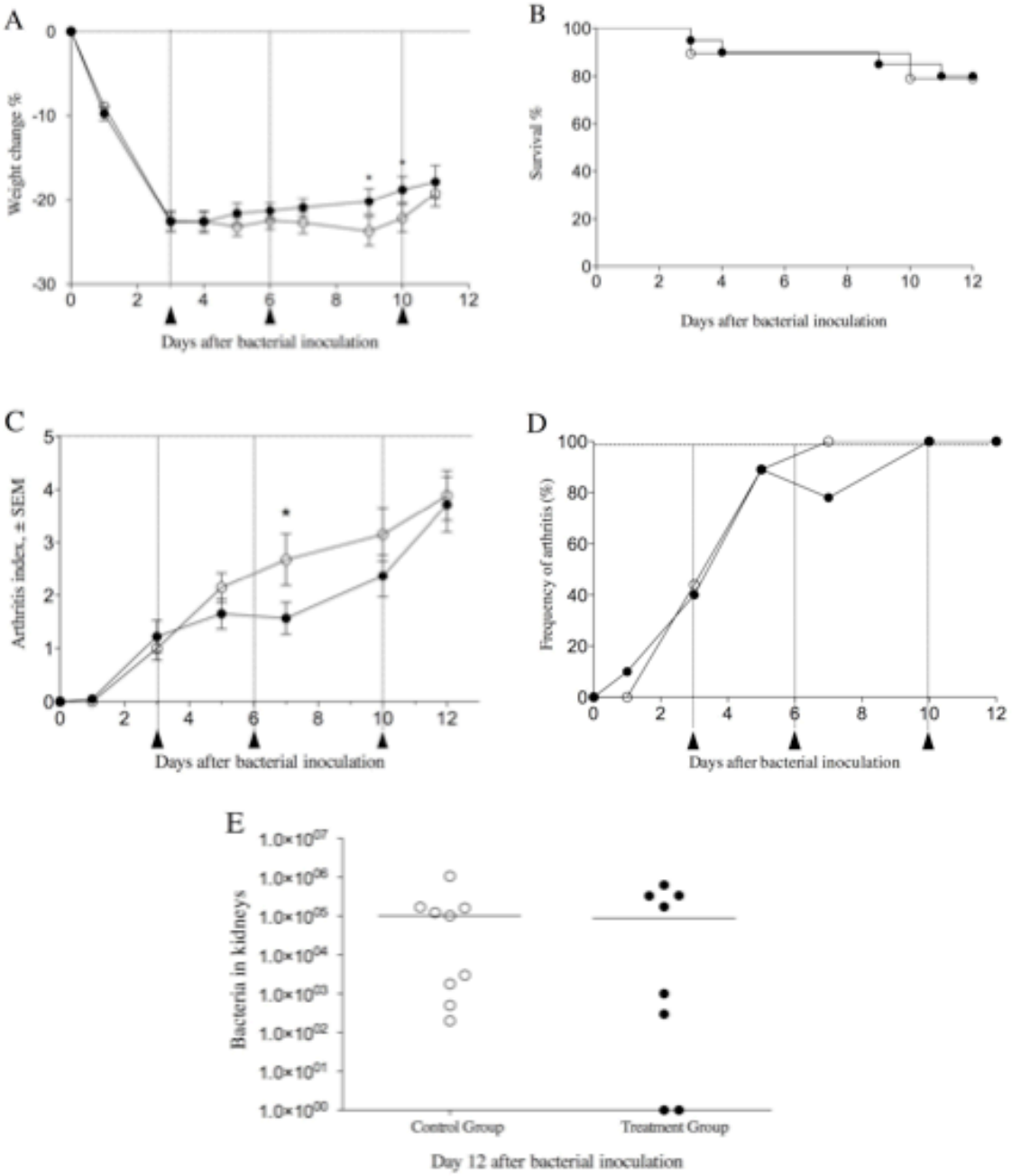


**Figure 5. Weight change, mortality, arthritis and bacterial persistence after induction of systemic *Staphylococcus aureus*–induced arthritis.** *A,* Weight change, as a percentage of initial weight and *(B)* mortality rate. *C,* Clinical severity of arthritis, indicated as arthritis index, and *(D)* frequency of arthritis. *E,* Bacterial persistence in kidneys at day 12. The arrows indicate intraperitoneal injections of anti-IL-15 antibody (aIL-15ab) or isotype control antibody. Antibiotics were added from day 3 and onwards. In panel *A* and *C*, bars show the mean ± standard error of the mean (SEM). In panel *E*, horizontal bars show median values. Statistical differences were calculated using Mann-Whitney U test. Kaplan-Meier survival plots were prepared and the log-rank test was used for comparison between the two survival curves. * = $p < 0.05$ compared with control group. *Open circles*: control group; *closed circles*: Treatment group.

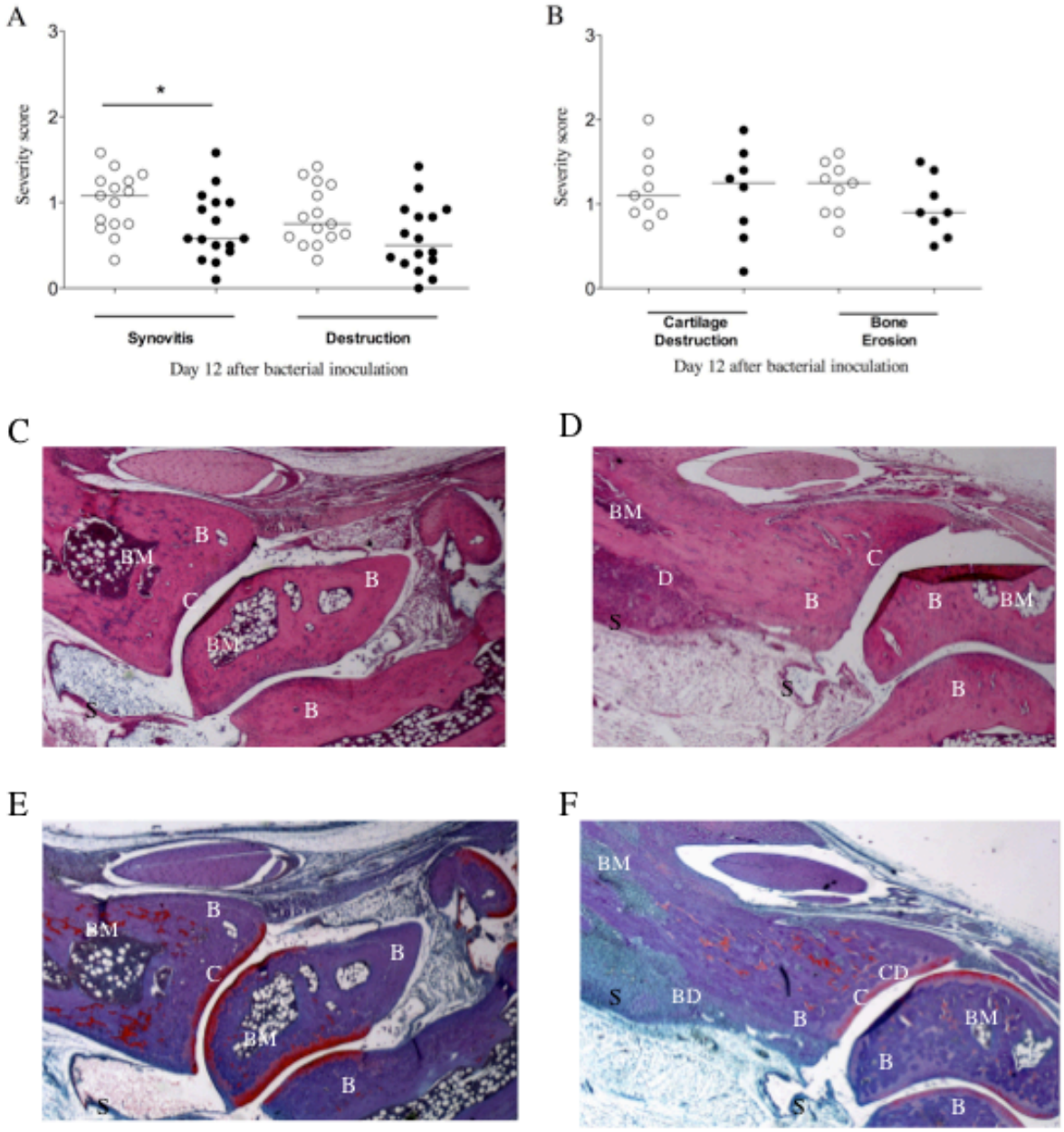


**Figure 6. Histopathological staining of joints after induction of systemic *Staphylococcus aureus*–induced arthritis.** *A,* Histology with hematoxylin and eosin (H&E) staining of joints from day 12 shows the degree of synovitis and bone/cartilage destruction. *B,* Bone van Gieson staining combined with cartilage Safranin O (G&S) staining of joints from day 12 shows the degree of cartilage and bone destruction. *C-D,* Sections from a H&E stained hind paw from an antibiotic treated mouse at day 12 with *(C)* mild synovitis and little destruction and *(D)* severe synovitis and destruction. *E-F,* Histopathological sections from a G&S stained hindpaw joint at day 12 of an antibiotic treated mouse with *(E)* mild synovitis and no cartilage or bone destruction and *(F)* severe synovitis, moderate cartilage destruction and severe bone erosion. Horizontal bars indicate median values. Abbreviations: B, bone; BM, bone marrow; BD, bone destruction; C, cartilage; CD, cartilage destruction; D, bone/cartilage destruction; S, Synovitis. Red staining indicates remaining cartilage, purple staining indicates bone and seagreen staining indicates inflammatory cells. Statistical differences were calculated using Mann-Whitney U test. * = $p < 0.05$ compared with control group. *Open circles*: control group; *closed circles*: Treatment group.

### 3.3 Combination treatment does not influence general bone loss in *S. aureus*-induced arthritis

*S. aureus*-induced arthritis results in a prominent general bone loss [21]. In order to investigate if the combination treatment would influence this feature, pQCT was performed on femur and tibia from mice of both groups. As shown in figure 7A, B and C, inhibition of IL-15 in addition to antibiotic treatment did not have a significant impact on cortical thickness, cortical density or trabecular BMD and thus, this treatment had not affected general bone loss at termination of the experiment, i.e. 12 days after bacterial inoculation.

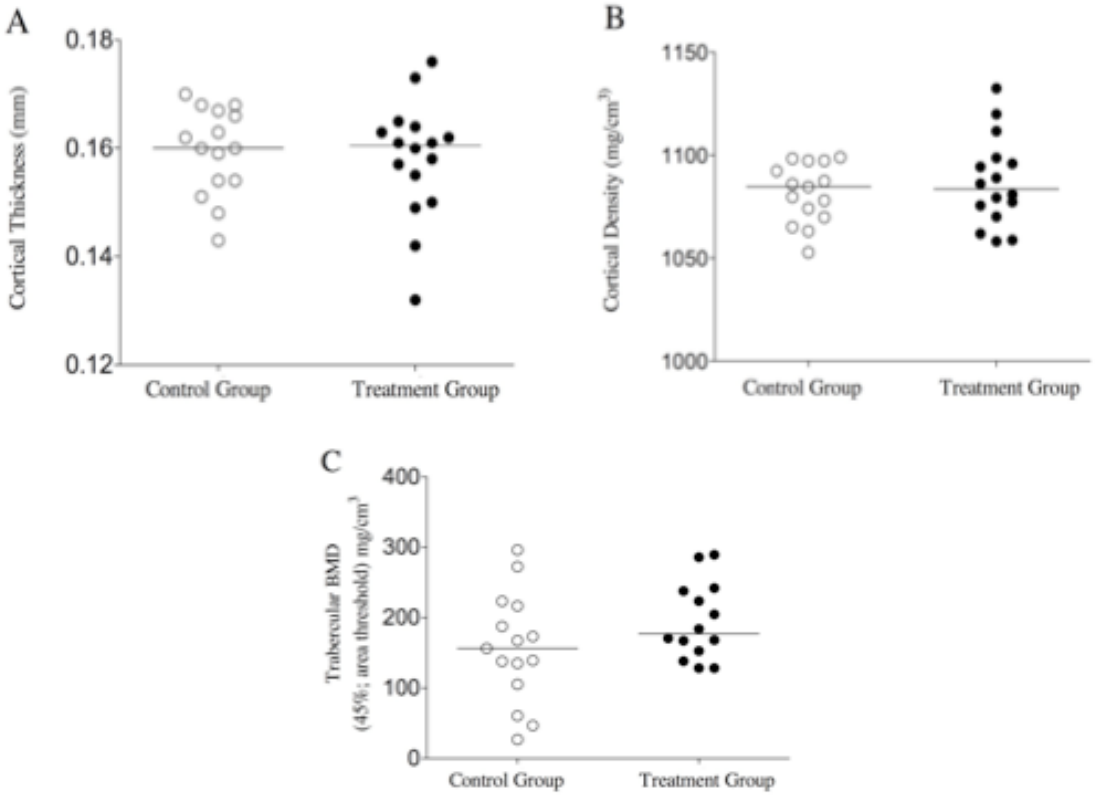


**Figure 7. Determination of general bone loss in femur and tibia at day 12 after induction of systemic *Staphylococcus aureus*–induced arthritis.** *A,* Cortical thickness, *(B)* cortical density (also named cortical bone mineral density, BMD) and *(C)* trabecular BMD. Horizontal bars indicate median values. Statistical differences were calculated using Mann-Whitney U test. *Open circles*: control group; *closed circles*: Treatment group.

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### 3.4 Combination treatment does not influence the synovial mRNA levels of RANKL, RANK or OPG

Using real-time PCR, the relative mRNA expression levels of RANKL, RANK and OPG in synovial tissues from the knee joints at day 12, were obtained. No significant differences between the groups were observed (Figure 8A-C).

### 3.5 Combination treatment decreases the serum protein levels of OPG at day 12 after *S. aureus* inoculation

Serum protein levels of OPG did not differ between the two groups at days 0 and 3 (Figure 9A), but were decreased at day 12 after *S. aureus* inoculation compared with the control group mice (Figure 9A). The serum protein levels of RANKL at day 12 did not differ between the treatment groups (Figure 9B).

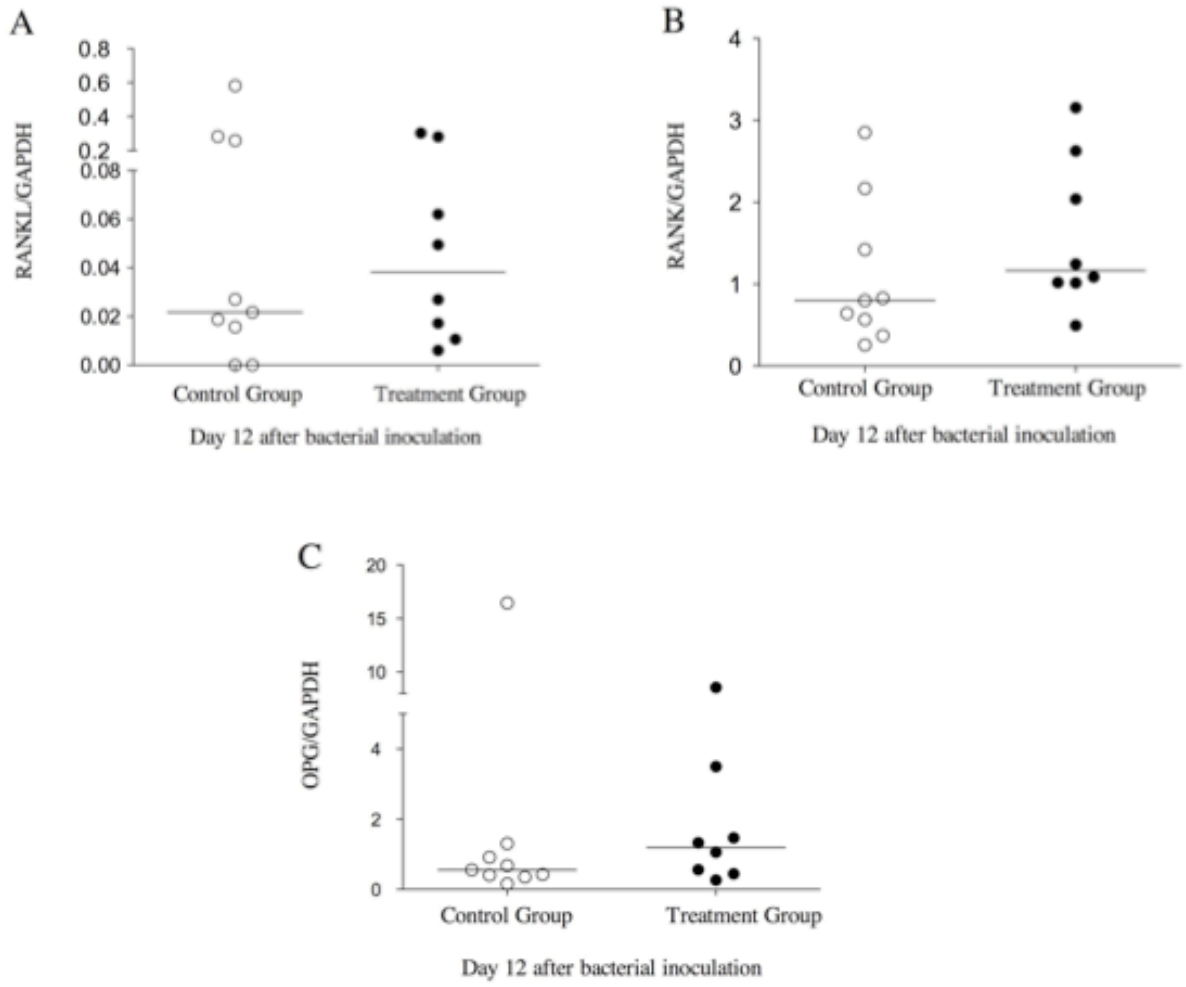


**Figure 8. Real-time Polymerase Chain Reaction (PCR) on synovial tissues obtained from knee joints at day 12 after induction of systemic *Staphylococcus aureus*-induced arthritis.** *A,* Synovial mRNA levels of RANKL, *(B)* RANK and *(C)* OPG with GAPCH as an internal control. Horizontal bars indicate median values. Statistical differences were calculated using Mann-Whitney U test. *Open circles*: control group; *closed circles*: Treatment group.

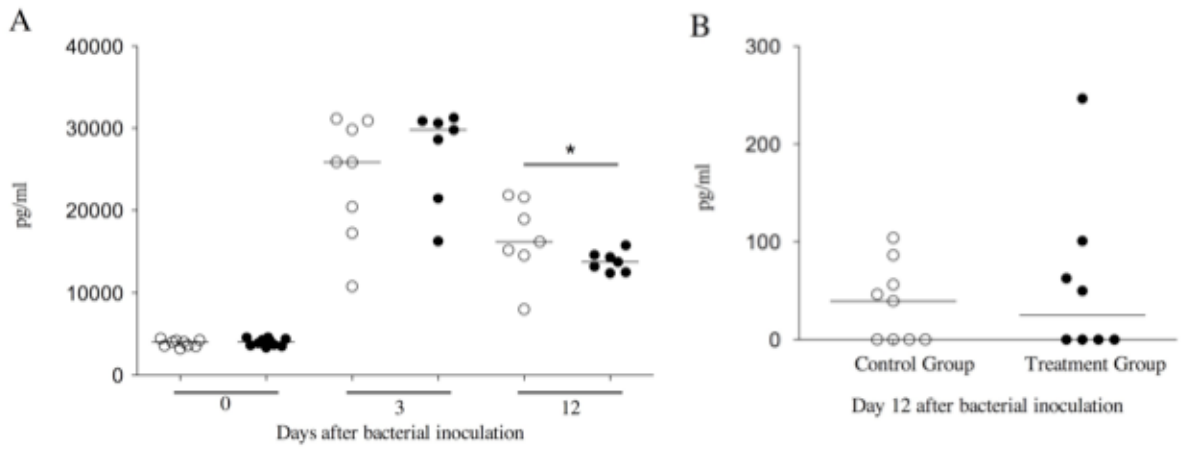


**Figure 9. Serum protein levels in mice after induction of systemic *Staphylococcus aureus*-induced arthritis.** *A*, Serum levels of OPG at baseline, and days 3 and 12 after inoculation of *S. aureus*. *B*, Serum levels of RANKL at day 12 after *S. aureus* infection. Horizontal bars indicate median values. Statistical differences were calculated using Mann-Whitney U test. * = $p < 0.05$ compared with control group. *Open circles*: control group; *closed circles*: Treatment group.

## 3.6 *In vitro* generation of murine osteoclasts from bone marrow macrophages

As mature monocytes and macrophages can differentiate into osteoclasts in the presence of RANKL and M-CSF [74, 77], bone marrow macrophages were isolated and co- cultured with these cytokines in different concentrations [90]. Recombinant IL-15 was added to the cultures in various concentrations (0,5 ng/ml and 50 ng/ml: Figure 10D-F). Other cultures were also performed: one with only added M-CSF (30 ng/ml; Figure 10A) and cultures with M-CSF (30 ng/ml), and various concentrations of RANKL (1 ng/ml and 2 ng/ml; Figure 10B and C). Osteoclasts were identified as TRAP-positive cells after 4-5 days in culture, and for some unknown reason very few osteoclasts had formed. Therefore, osteoclasts could not be counted and this experiment needs to be repeated.

## 3.7 *In vitro* generation of murine osteoclasts from unselected bone marrow cell culture

Bone marrow cells were obtained from wildtype mice and cultured with M-CSF (30 ng/ml) alone (Figure 11A) or together with various concentrations of RANKL (2 ng/ml and 4 ng/ml; Figure 11B and C) and with or without aIL-15ab/isotype control antibody (10 ng/ml, 100 ng/ml and 1 μg/ml; Figure 11D-G). After TRAP staining 4 and 5 days later and subsequent microscope analysis there seemed to be a trend towards decreased osteoclastogenesis in the cultures where aIL-15ab was present compared to cultures with isotype control antibody or RANKL alone. However, these cultures need to be repeated to verify this as osteoclasts were

identified as TRAP-positive cells with more than three nuclei and the cultivated osteoclasts were difficult to count using these parameters.

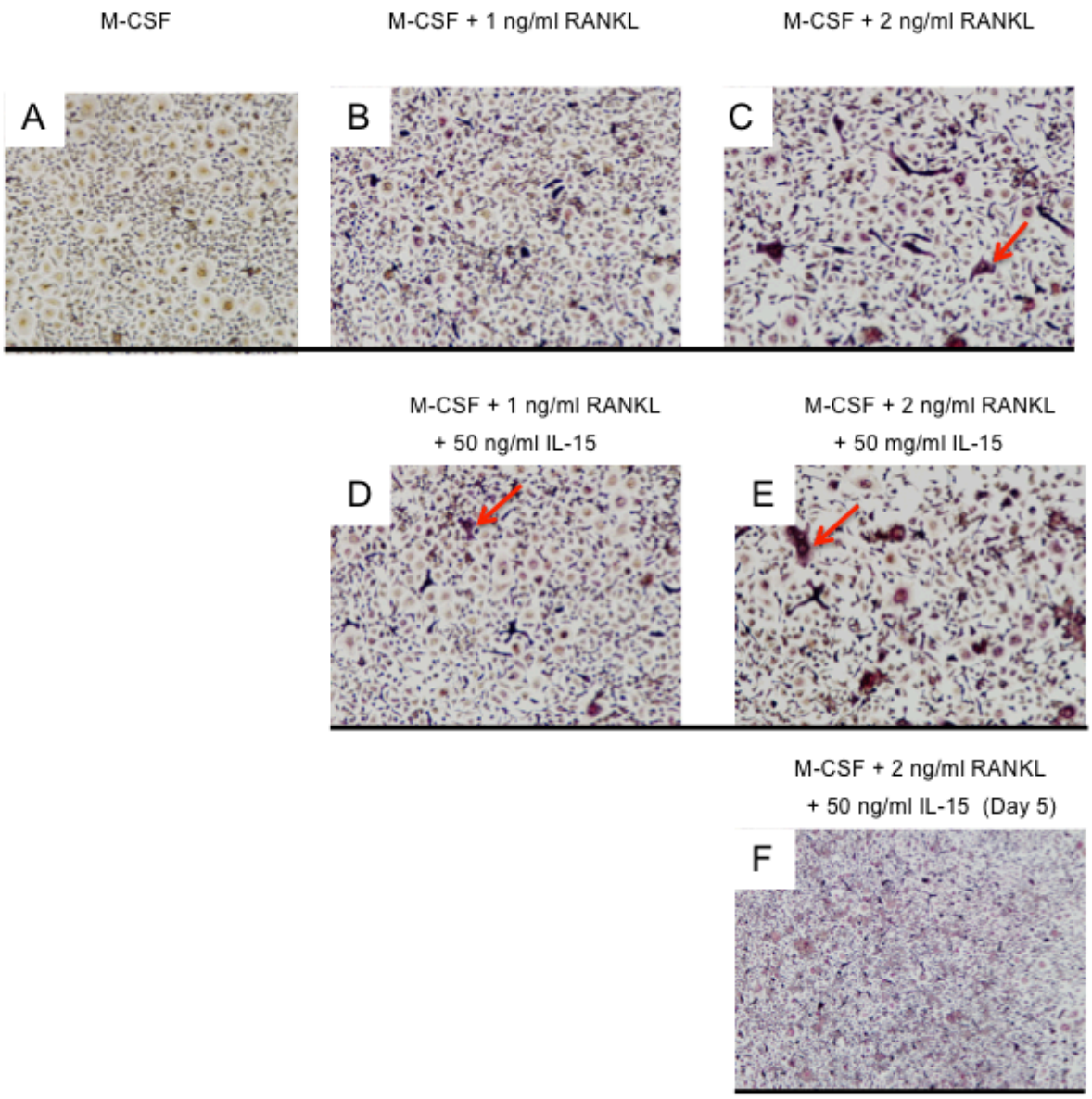


**Figure 10.** ***In vitro*** **generation of murine osteoclasts from bone marrow macrophages (BMM).** *(A-C)* Morphology of TRAP stained BMM cells is shown, in the presence of *(A)* M-CSF (30 ng/ml), *(B)* M-CSF (30 ng/ml) and RANKL (1 ng/ml) or *(C)* M-CSF (30 ng/ml) and RANKL (2 ng/ml) after 4 days culture. *(D-E)* TRAP staining of BMM cells in the presence of M-CSF (30 ng/ml), IL-15 (50 ng/ml) and *(D)* RANKL (1 ng/ml) or *(E)* RANKL (2 ng/ml) after 4 days culture. *(F)* TRAP staining of BMM cells in the presence of M-CSF (30 ng/ml), IL-15 (50 ng/ml) and RANKL (2 ng/ml) after 5 days culture. Red arrows point at osteoclasts.

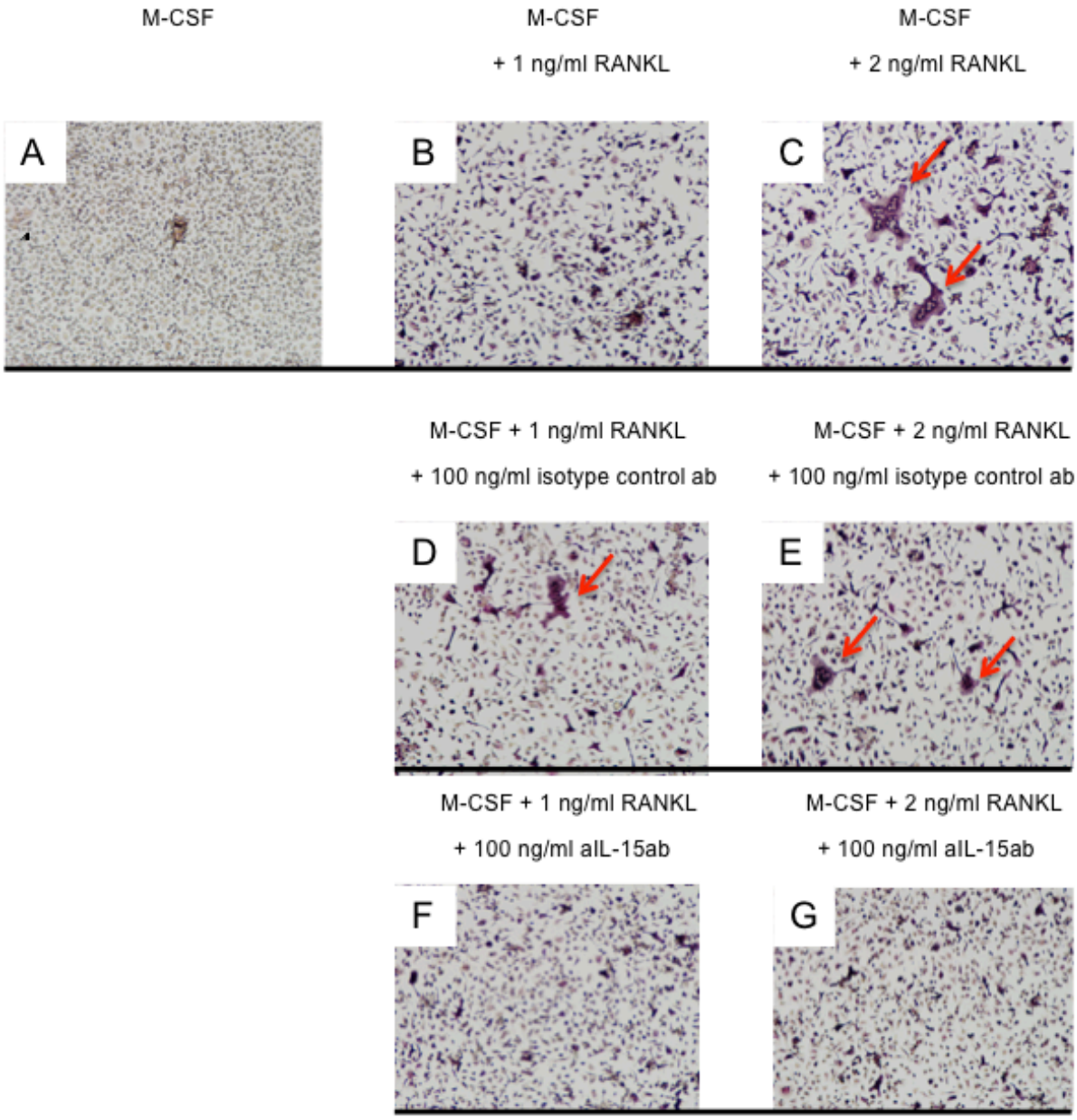


**Figure 11.** ***In vitro*** **generation of murine osteoclasts from bone marrow cell cultures (BMC).** *(A-C)* Morphology of TRAP stained BMC cells is shown, in the presence of *(A)* M-CSF (30 ng/ml), *(B)* M-CSF (30 ng/ml) and RANKL (2 ng/ml) or *(C)* M-CSF (30 ng/ml) and RANKL (4 ng/ml) after 4 days culture. *(D-E)* TRAP staining of BMC cells in the presence of M-CSF (30 ng/ml), isotype control antibody (100 ng/ml) and *(D)* RANKL (2 ng/ml) or *(E)* RANKL (4 ng/ml) after 4 days culture. *(F-G)* TRAP staining of BMC cells in the presence of M-CSF (30 ng/ml), anti-IL-15 antibody (100 ng/ml) and *(F)* RANKL (2 ng/ml) or *(G)* RANKL (4 ng/ml) after 4 days culture. Red arrows point at osteoclasts.

## DISCUSSION

We investigated the potential benefits of adding aIL-15ab to antibiotic treatment in systemic *S. aureus*-induced in mice. In addition, we also aimed to investigate further the role of IL-15 in osteoclastogenesis. We found that the clinical severity of arthritis and histopathological development of arthritis were ameliorated in mice treated with aIL-15ab in combination to antibiotics, compared to mice in the control group. However, no effects on cartilage destruction, bone destruction or systemic bone loss were observed. Importantly, the treatment did not have a negative impact on mortality, morbidity nor bacterial clearance. Inhibition of IL-15 did not seem to have major effects on the molecular triad of OPG/RANK/RANKL, as the mRNA levels in synovial tissues from knee joints at termination (i.e. 12 days after bacterial inoculation) were similar in both groups, as well as serum protein levels of RANKL at termination. However, serum protein levels of OPG were significantly decreased at termination in mice treated with aIL-15ab compared to control mice. Lastly, preliminary data from osteoclast cultures showed a trend towards decreased osteoclastogenesis in cultures where IL-15 was inhibited compared to cultures with isotype control antibody.

IL-15 can activate both the innate and adaptive immune systems [28-33] and has immunomodulatory effects on cells in both these systems. It is an important player in the protection against invading pathogens, particularly virus but also to some extent bacteria [87], and one would suspect that inhibition of IL-15 during an infection might prove harmful. Indeed, Inoue et al reported that mice receiving therapeutic administration of IL-15 had increased survival in the CLP model of sepsis and in a *Pseudomonas aeruginosa* pneumonia model [31]. They also reported that mice treated with aIL-15ab had significantly *worsened* mortality compared to septic mice that did not receive the antibodies. However, reports of IL-15 in sepsis are contradictory. Orinska et al [37] reported a novel role of intracellular IL-15 in a CLP mast cell-dependent sepsis model, as they observed *improved* survival in IL-15 deficient mice after CLP. Henningsson et al [87] have investigated inhibition of IL-15 in the model of *S. aureus*-induced arthritis, using IL-15 knockout mice and aIL-15ab treated mice, and they showed that, in fact, inhibition of IL-15 leads to a less destructive arthritis compared with wildtype or control antibody treated mice, *without* affecting mortality. Henningsson et al also investigated IL-15 in *S. aureus*-induced sepsis when IL-15 knockout mice were

inoculated with 10-fold higher bacterial dose and observed *no aggravation* in mortality, bacterial clearance or arthritis.

Henningsson et al also showed that the IL-15 knockout mice displayed significantly reduced severity and frequency of arthritis late during the infection, and lost significantly less weight compared to wildtype mice, but the host's ability to clear the bacteria was not affected. Further, histological evaluation yielded a significant reduction in cartilage destruction in the knockout mice compared with wildtype mice, which correlated to a significantly reduced number of osteoclasts in the joints of the IL-15 knockout mice. Thus, it was postulated that the lack of IL-15 ameliorated the joint destruction via a reduction in the number of osteoclasts in the joints. Furthermore, treatment with aIL-15ab alleviated *S. aureus*-induced arthritis as the aIL-15ab treated mice lost significantly less weight, had significantly reduced synovitis and joint destruction, and showed a significantly *increased* ability to clear the bacteria from the kidneys, compared with isotype control antibody treated mice. In the aforementioned study made by Orinska et al [37], an *enhanced* bacterial clearance was observed in the IL-15 knockout mice due to the absence of IL-15-dependent reduction of neutrophil recruitment to site of infection. As the bacterial clearance in the IL-15 knockout mice in our mouse model of *S. aureus*-induced arthritis was similar to the wildtype mice [87], the reduced clinical and histological severity of arthritis, as well as the decrease in weight loss, is *not* due to increased bacterial clearance. The aIL-15ab treatment alone, lead to a significant enhancement in bacterial clearance, which could be the reason for the amelioration of arthritis in the aIL-15ab treated mice. The differences in the findings of Orinska et al versus the findings of Henningsson et al, with respect to bacterial clearance in the knockout mice, could be due to the use of different infectious models and different pathogens, systemic *S. aureus* infection versus local CLP infection (which consists mainly of Gram-negative microbes). The explanation for the increased bacterial clearance measured in aIL-15ab treated mice versus the unaffected bacterial clearance in IL-15 knockout mice is uncertain, one being that knockout mice are known to compensate for the absence of various key components, resulting in an altered immunological situation. Thus, deletion of IL-15 may have activated compensatory mechanisms that were probably not established in the mice receiving aIL-15ab, in which the cytokine is not completely blocked.

Our results show that when antibiotics are added to aIL-15ab treatment the differences between the treatment groups, observed when they were only treated with aIL-15ab [87], decreased. In line with Henningsson et al [87] there are no differences in mortality between the treatment groups, however, we could not observe significant differences in weight loss, frequency or clinical severity of arthritis between the groups, although a trend towards milder weight loss was observed in the aIL-15ab treated mice. Thus, the effects of antibiotics completely overrule the benefits of aIL-15ab during *S. aureus*-induced infections.

As IL-15 knockout mice have a reduced numbers of osteoclasts in their joints as well as a significant reduction in joint destruction [87], and an important role of IL-15 in osteoclastogenesis has been reported [65-67], we hypothesized that adding aIL-15ab to antibiotic treatment would lead to a reduction of joint destruction, possibly via a decrease in osteoclastogenesis. However, we could not detect any significant differences between the groups with respect to cartilage and bone destruction in the joints and thus, our hypothesis appears to be false. Interestingly, synovitis in the aIL-15ab treated mice was significantly reduced compared to control mice, which probably is mediated by inhibition of IL-15's pro-inflammatory and antiapoptotic properties [31, 51], e.g. lack of stimulatory effects on neutrophils and T cells.

It has been shown that systemic bone loss is present in *S. aureus*-induced arthritis in mice [21], where the bacteria directly or indirectly trigger an increase in bone degradation as well as a decrease in *de novo* bone formation. Verdrengh et al [21] reported that 3 days after systemic inoculation with *S.aureus*, both total and trabercular BMD are already significantly reduced, compared with that in uninfected mice, and BMD continues to reduce during the infection. This was demonstrated to result from increased osteoclastic activity. IL-15 has been recognized as an important stimulator of osteoclastogenesis, providing a costimulatory signal for RANKL-induced osteoclastogensis [65] and directly stimulating early osteoclast differentiation *in vitro* [66]. It would therefore be expected that inhibition of IL-15 would have a negative effect on osteoclastogensis, thus decreasing the osteoclastic activity induced by the infection. We investigated if treatment with aIL-15 combined with antibiotics affected the general bone loss in *S. aureus*-induced arthritis by pQCT. To our surprise, inhibition of IL-15 in addition to antibiotic treatment did not have a significant impact on neither trabercular BMD, cortical density nor or cortical thickness. Instead, our results from both

treatment groups at day 12 are completely in line with the trabercular BMD measured in mice in the previous study conducted by Verdrengh et al [21].

Osteoclastogenesis is promoted by the interaction of RANKL and RANK and inhibited by OPG, a decoy receptor for RANKL. We determined the protein levels of these cytokines in serum before and during the infection as well as at termination, and as mRNA in synovial tissue at termination. Only the serum protein levels of OPG at termination were significantly reduced in mice treated with aIL-15ab combined with antibiotics, compared to isotype control treated mice. This could be due to the fact that these mice had significantly reduced synovitis, compared to control mice, but a substantial explanation is difficult to find.

Ogata et al [66] showed that IL-15 enhances osteoclastogensis by stimulating the formation of preosteoclasts, which occurs at an early stage of osteoclastogenesis, and Djaafar et al [65] demonstrated that IL-15 provides a costimulatory signal for RANKL-induced osteoclastogenesis, as well as stimulating the proliferation of T cells and their production of osteoclastogenic factors, such as RANKL. We wanted to investigate further the effect of IL-15 on osteoclastogensis and therefore a number of osteoclast cultures were made *in vitro*. *In vitro* generation of murine osteoclasts from bone marrow cell cultures was attempted and aIL-15ab or isotype control antibody was added. A trend towards a decrease in osteoclastogenesis in the cultures were IL-15 was inhibited, compared to control cultures, was observed by two blinded observers. The osteoclasts that formed were of such nature that they were difficult to count, as osteoclasts are defined as TRAP-positive cells with more than three nuclei. To assess whether the observed trend represents a significant decrease in osteoclastogensis in bone marrow cell cultures when IL-15 is inhibited, additional experiments need to be conducted. In addition, *in vitro* generation of murine osteoclasts from bone marrow macrophages, with or without the addition of various concentrations of IL-15, was conducted to see if IL-15 could drive the differentiation of bone marrow macrophages to osteoclasts. These experiments yielded a poor formation of osteoclasts and therefore, osteoclasts could not be counted. Thus, similarly to the other cultures, these experiments need to be repeated.

Taken together, neither the *in vivo* nor the *in vitro* studies support a direct role for IL-15 in bone and cartilage destruction and osteoclastogenesis.

The bacterial load in the kidneys from mice in both treatment groups at termination was substantial, considering that all of the mice had been treated with antibiotics [22]. Even though the mice were inoculated with an arthritogenic strain, the general illness (measured as weight loss) of mice in both groups was prominent, and indeed two mice out of twenty, died from sepsis, despite the antibiotic treatment. This further demonstrates the importance of finding an additional treatment to *S. aureus*-induced infections. Importantly, on the one hand aIL-15ab did not hamper the bacterial clearance, but on the other it did not either enhance it in the presence of antibiotics.

In conclusion, this study shows that the additional benefits of inhibiting IL-15 whilst treating with antibiotics in *S. aureus*-induced arthritis, are limited with respect to joint damage and prevention of systemic bone loss. In addition, the effect of IL-15 on osteoclastogenesis is probably mediated via an indirect pathway.

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