Running title: Antibiotic-killed *S. aureus* induces arthritis

Antibiotic-killed *Staphylococcus aureus* induces destructive arthritis in mice

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Abstract

Objective: Permanent reduction in joint function is a severe post-infectious complication in patients with *Staphylococcus aureus* septic arthritis. This reduction in joint function might be caused by persistent joint inflammation after the adequate eradication of bacteria by antibiotics.

Methods: We studied whether antibiotic-killed *S. aureus* induced joint inflammation in mice and elucidated the molecular and cellular mechanism of this type of arthritis.

Results: The intraarticular injection of antibiotic-killed *S. aureus* induced mild to moderate synovitis and bone erosions that lasted for a minimum of 14 days. The frequency and severity of synovitis were significantly reduced in tumor necrosis factor receptor 1 (TNFR1), receptor for Advanced Glycation End Products (RAGE), and toll like receptor 2 (TLR2) knockout mice compared with wild-type animals. The combined depletion of monocytes and neutrophils resulted in a significantly lower frequency of synovitis. Among bacterial factors, insoluble cell debris played a more important role than bacterial DNA or soluble components in inducing joint inflammation. Importantly, anti-TNF therapy abrogated the joint inflammation induced by antibiotic-killed *S. aureus*.

Conclusion: Antibiotic-killed *S. aureus* induced and maintained the joint inflammation that is mediated through TLR2, TNFR1, and RAGE receptor. The cross-talk between neutrophils and monocytes is responsible for this type

of arthritis. Anti-TNF therapy might be used as a novel therapeutic strategy, in combination with antibiotics, to treat staphylococcal septic arthritis.

Key words: *Staphylococcus aureus*, antibiotics, arthritis, TLR2, TNF-α, RAGE

Considered to be one of the most dangerous joint diseases, septic arthritis is usually caused by *Staphylococcus aureus*(1). The mortality in septic arthritis still remains high, varying from 10-25%, and 25-50% of all patients never regain full joint function(2). During the last decades, no new major therapies have been developed to reduce the joint deformation and deleterious contractures or to prevent joint dysfunction in septic arthritis(3).

The host's immune response protects the host against bacteria, but it can potentiate inflammation severity when staphylococcal danger signals trigger an exaggerated response(4). Permanent reductions in joint function are hypothesized to be caused by long-lasting joint inflammation after the adequate eradication of bacteria by antibiotics. Persistently high pro-inflammatory cytokine levels predict the severity of the later destruction of affected joints in septic arthritis patients(5). The addition of corticosteroids to antibiotic treatments ameliorated the course of *S. aureus* septic arthritis in mice(6). Recent studies demonstrated that the addition of glucocorticoid at the start of antibiotic treatment in children with septic arthritis led to a significantly more rapid clinical improvement(7, 8). However, the driving force behind the long-lasting joint inflammation after the elimination of *S. aureus* is unknown. Inflammatory bacterial components have recently been shown to persist near cartilaginous tissue after antibiotic treatment in Lyme disease; these components might contribute to the pathogenesis of antibiotic-refractory Lyme arthritis(9). Intriguingly, antibiotics were shown to induce the release of proinflammatory and chemotactic fragments e.g., staphylococcal

peptidoglycan and lipoteichoic acid(10, 11), capable of activating immune cells via toll-like receptors to release proinflammatory cytokines e.g., TNF-α and IL-6(12). It is therefore reasonable that components of antibiotic-killed *S. aureus* are capable of inducing and maintaining the chronic inflammation that later cause joint contracture and dysfunction.

In the present study, we demonstrated that antibiotic-killed *S. aureus* induces chronic inflammation and bone destruction. The arthritogenic properties of antibiotic-killed *S. aureus* are mediated through TLR2, TNFR1, and RAGE receptor. Therapy using a TNF-α inhibitor abrogated the joint inflammation induced by antibiotic-killed *S. aureus*.

MATERIALS AND METHODS

Mice

Female NMRI mice were purchased from Harlan Laboratories (Venray, the Netherlands). BALB/c and C57Bl/6 mice of both sexes were purchased from Charles River Laboratories (Sulzfeld, Germany). TNFR1-/-(13) (kindly provided by Dr. Wick, Gothenburg University), RAGE-/-(14, 15) (kindly provided by Prof. Arnold, Deutsches Krebsforschungszentum and Prof. Nawroth, University Clinical Centre of Heidelberg, Germany), B6.129-Tlr2^{tm1Kir}/J (TLR2-/-) mice(16), and IL-17A-/- mice(17), all on a C57BL/6 background, were bred and housed in the animal facility of the Department of

Rheumatology and Inflammation Research, University of Gothenburg. All mice were used for experiments at the age of 6-12 weeks. They were kept under standard conditions of temperature and light and were fed laboratory chow and water *ad libitum*. The study was approved by the animal research ethical committee of Gothenburg.

Reagents

Etoposide was purchased from Bristol Myers Squibb AB (Bromma, Sweden). The monoclonal antibody RB6-8C5 was produced and purified, as previously described in detail(18). As a control, monoclonal Ig-class-matched anti-ovalbumin antibodies were used(18). Cloxacillin (Cloxacillinnatr, Stragen Nordic, Sweden) was used to kill *S. aureus in vitro*. Lysostaphin was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Preparations of antibiotic-killed *S. aureus*

The *S. aureus* strain LS-1 was prepared as previously described(19), incubated with 33 mg/ml cloxacillin for 6-24 hours at 37ºC and thereafter for 60 minutes at 60ºC to eradicate any surviving bacteria. The cloxacillin (33 mg/ml) was treated similarly to be used as a control. The cloxacillin-killed *S. aureus* was stored at –70°C until used. To ensure sterility of the preparation, antibiotic-treated bacterial suspension was washed with PBS to remove the cloxacillin residues and cultured for 24 h. No bacterial growth was detected.

Supernatants of cloxacillin-killed *S. aureus* ($2.6x10^9$ bacteria/ml) were collected after a 5-minute centrifugation, and the pellets were suspended in equal volume of PBS as the supernatants. The supernatants and dead bacteria

were then stored at –70°C until used. To extract genomic DNA, cloxacillin-killed *S. aureus* ($1.3x10^{10}$ bacteria/ml) mixed with 200µg glass beads were lysed in Fastprep machine (BIO101). The extract was treated with RNase A and proteinase K, respectively, at 37^{o} and 55^{o} C for 30 minutes. Genomic DNA was purified by phenol/chloroform extraction and ethanol precipitation. The cell wall debris was isolated according to the method described previously(20). Cell-wall debris and bacterial DNA (0.35mg/ml) were suspended in PBS buffer.

Lysostaphin treatment of antibiotic-killed *S. aureus*

Antibiotic-killed *S. aureus* ($2.6x10^{9}$ bacteria/ml) was incubated overnight with lysostaphin (100 µg/ml) at 37°C. Previous study has shown that this procedure completely digest the peptidoglycan polymer(21). The absorbance of dead bacteria suspension at 620 nm was reduced 50% by the lysostaphin treatment.

***In vitro* spleen cell stimulation**

The preparation and stimulation of splenocytes from C57BL/6 and TNFR1-/- mice were performed as previously described(22). Splenocytes (1×10^{6}/ml) were incubated with various preparations of cloxacillin-killed *S. aureus* (concentrations between $4x10^{6}$-$1x10^{8}$ bacteria/ml) for 72 hours or with lipopolysaccharide (1 µg/ml) as a positive control.

Induction of arthritis with antibiotic-killed *S. aureus*

In all experiments, the mice were i.a. injected in the knee joint with a total volume of 20 µl of solution containing antibiotic-killed *S. aureus* in PBS

or with 20 µl of PBS with matching concentration (33 mg/ml) of cloxacillin. To study the kinetics of arthritis development, the NMRI mice were i.a. injected with antibiotic-killed *S. aureus* ($2.6x10^9$ bacteria/ml), and the contralateral knee joints with cloxacillin. The animals were sacrificed on following days: 1, 3, 7, 14, or 30. The knee joints were collected for histopathological evaluation. As cloxacillin did not induce any joint inflammation, further controls in the following experiments were deemed unnecessary.

To determine whether there was a dose-dependent pattern, various concentrations of antibiotic-killed *S. aureus* ($5.0x10^8$ to $1.3x10^{10}$ bacteria/ml; n=4-5 mice per group) were i.a. injected to NMRI mice.

In the second set of experiments, NMRI mice received an i.a. injection of supernatants (n=5) or bacterial debris (n=5) from antibiotic-killed *S. aureus* in 20µl of PBS ($2.6x10^9$ bacteria/ml) or the original suspension of antibiotic-killed *S. aureus*.

In the third experiment, NMRI mice were injected with purified bacterial components from antibiotic-killed *S. aureus* in 20µl of PBS ($1.3x10^{10}$ bacteria/ml) including staphylococcal DNA (n=10), bacterial debris (n=10), or a mixture of the fractions (n=20).

Injection protocols and cell depletion protocols

To examine the molecular mechanism of joint inflammation induced by antibiotic-killed *S. aureus*, four different gene knockout mice, including TLR2-/- mice (n=13), TNFR1-/- mice (n=20), RAGE-/- mice (n=17), and IL-

17A-/- mice (n=8), received i.a. antibiotic-killed *S. aureus* ($2.6x10^9$ bacteria/ml). C57Bl/6 wild-type mice (n=14, n=21, n=15, and n=8, respectively) served as controls. Knee joints were collected for analysis on day 3. For the TLR2-/- mice, TNFR1-/- mice, and RAGE-/- mice, two independent experiments were performed, and the results were pooled. One experiment was performed for the IL-17A-/- mice.

To further assess whether the synovitis differences observed on day 3 are constant even in a longer disease duration, TLR2-/- mice (n=9) and TNFR1-/- mice (n=18) received i.a. injection of high dose of antibiotic-killed *S. aureus* ($4.2x10^{10}$–$1.2x10^{11}$ bacteria/ml). C57Bl/6 wild-type mice (n=22) were used as controls. Two independent experiments were performed for TNFR1-/- and one was performed for TLR2-/-. Knee joints were analyzed on day 14.

To study different immune cells responsible for arthritis induction by antibiotic-killed *S. aureus,* 20 µl of dead bacteria ($2.6x10^9$ bacteria/ml) was i.a. injected into BALB/c mice depleted of blood monocytes using etoposide, depleted of neutrophils using RB6-8C5 mAb, or depleted of both cell types as previously described(23). Knee joints were analyzed on day 3.

To selectively modulate the CD80/CD86-mediated CD28 costimulatory signal required for full T-cell activation(24), abatacept (Orencia®, Bristol-Myers Squibb) was subcutaneously injected into NMRI mice (5 µg/g of body weight in 0.1 ml of PBS) daily starting on day 0 after i.a. injection of dead *S.*

aureus ($2.6x10^9$ bacteria/ml) and continuing until day 3 when the animals were sacrificed.

Treatment with anti-TNF compounds

Etanercept (Enbrel®; Wyeth Europa) is known to efficiently inhibit the activity of murine TNF-α(19, 25, 26). Two experimental settings were used to evaluate both the short- and long-term anti-TNF treatment effects on NMRI mice. Etanercept (5 μg/g of body weight in 0.1 ml of PBS) was given subcutaneously 1) every 24 hours starting on day 0 after the i.a. injection of antibiotic-killed *S. aureus* ($2.6x10^9$ bacteria/ml) and continuing until day 3 when the animals were sacrificed; 2) every 48 hours starting on day 0 after the i.a. injection of dead *S. aureus* ($4.2x10^{10}$ bacteria/ml) and continuing until days 7 or 14 when the animals were sacrificed and the knee joints were collected for histological examination and micro-CT scanning.

Histopathological examination of the joints

The knee joints were removed, fixed in 4% paraformaldehyde, decalcified, embedded in paraffin, sectioned and stained with hematoxylin-eosin. The joints receiving high dose of dead bacteria were chosen for tartrate−resistant acid phosphatase (TRAP) staining(27). All the slides were coded and assessed microscopically in a blinded manner by 2 observers (T.J. and A.A.) with regard to synovitis and cartilage/bone destruction. The extent of synovitis/ bone destruction was judged on a scale from grade 0 to grade 3 (0=no signs of inflammation or no bone erosion, 1=mild synovial hypertrophy and mild bone erosions, 2=moderate inflammation characterized by

hyperplasia of synovial membrane and influx of inflammatory cells throughout the synovial tissue or moderate bone erosions, and 3=marked synovial hypertrophy and inflammatory infiltration in synovial tissue or severe bone erosions).

Micro-CT imaging

The long-term effect (day 14) of anti-TNF therapy on bone destruction was also studied in the knee joints of mice receiving an i.a. injection of antibiotic-killed *S. aureus* by micro-CT scanning. A Skyscan1176 micro-CT (Bruker, Antwerp, Belgium) with a voxel size of 18 μm was used. The scanning was conducted at 45 kV/555 μA with a 0.2-mm aluminum filter. The exposure time was 199 ms. The X-ray projections were obtained at 0.5° intervals with a scanning angular rotation of 180°. The projection images were reconstructed into 3D images using NRECON software (version 1.5.1; Bruker) and analyzed using CT-vox (version 2.4; Bruker). The extent of cartilage and bone destruction was judged in a blinded manner by one observer (T.J.) on a scale from grade 0 to 3.

Measurement of cytokine levels

The levels of TNF-α, IL-6, Receptor activator of nuclear factor kappa-B ligand (RANKL) in the sample supernatants were determined using a DuoSet ELISA Development Kit (R&D Systems Europe, Ltd).

Statistical analysis

The statistical significance was assessed using the Mann–Whitney *U* test and the χ2 test. GraphPad Prism v. 6 (Graphpad Software) was used for the

calculations. The results are reported as the mean ± the standard error of the mean (SEM).

RESULTS

Antibiotic-killed *S. aureus* induces arthritis

All of the mice i.a. injected with antibiotic-killed *S. aureus* developed synovitis by day 1. After 2 weeks, both the synovitis severity and frequency had diminished significantly; however, after reaching a certain threshold, these symptoms remained stable until the experiment was ended (day 30). After the i.a. injection of cloxacillin, one of the four mice developed slight transient inflammation, which disappeared after a few days (Fig 1A). Bone destruction was not observed on day 3 after the i.a. injection of dead bacteria.

Different concentrations of antibiotic-killed *S. aureus* were i.a. injected, and the results showed a dose-dependent pattern regarding both the synovitis severity and frequency (Fig 1B).

Antibiotic-killed *S. aureus* induces release of proinflammatory cytokines

To study whether antibiotic-killed *S. aureus* has the potential to induce the production of pro-inflammatory cytokines, splenocytes from wild-type mice were stimulated with different concentrations of antibiotic-killed bacteria, and the cytokines were measured in cell culture supernatants after 24 and 48 hours (Fig 1C and D). Increased TNF-α and IL-6 levels were found after 24 hours of stimulation, and their concentrations remained high during 48 hours of culturing. Indeed, the stimulation of splenocytes with a low concentration of dead *S. aureus* ($4x10^6$ dead bacteria/ml) resulted in a several-fold increase in TNF-α production in the cell culture supernatants compared

with non-stimulated cells ($p< 0.001$, fig 2*A*); this result was even higher than the increase in TNF-α production in the LPS -stimulated cell culture.

In addition, the stimulation with antibiotic-killed *S. aureus* induced cell proliferation in a dose-dependent pattern (Fig 1*E*).

Receptor activator of nuclear factor kappa-B ligand (RANKL) was not detectable in those cell culture supernatants (data not shown).

The stimulation of spleen cells from TNFR1-/- mice (n=6) with dead *S. aureus* demonstrated similar increases in cytokine levels and cell proliferation to those detected in the wild-type supernatants (data not shown).

Arthritis induced by antibiotic-killed *S. aureus* is mediated through TLR2, TNFR1, and RAGE

Different knockout mice were used to study the signaling pathway of joint inflammation induced by dead *S. aureus.*

Mice lacking TNF receptor 1 had a significantly lower synovitis severity compared with their wild-type counterparts ($p<0.0001$, Fig 2A and 2D). The synovitis frequency was also significantly lower in the TNFR1-/- mice, with 35% of the mice developing synovitis compared with 80% of the wild-type controls ($p<0.0001$).

The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor and its activation has been shown to play a role in diverse experimental inflammatory and infectious diseases(28). The RAGE-/- mice

also had significant reductions in synovitis severity and frequency compared with their wild-type counterparts ($p<0.001$ and $p<0.05$, respectively, Fig 2B).

TLR2 is known to mediate the inflammation induced by the cell walls from gram-positive bacteria. TLR2-/- mice displayed a significantly lower synovitis severity compared with the wild-type controls ($p<0.01$, Fig 2C) and tended to have a lower synovitis frequency.

No significant difference could be observed in the synovitis severity and frequency between the mice lacking IL-17A and the wild-type mice (data not shown).

To verify whether observed differences are constant, TNFR1-/- and TLR2-/- mice were i.a. injected with high dose of antibiotic-killed *S. aureus* ($4.2x10^{10}$/ml) and observed 14 days (Fig 2D). TNFR1-/- mice had less severe synovitis (mean index: 1.15 versus 1.92, $p<0.05$) and bone destruction (mean index: 0.72 versus 1.4, $p<0.05$) compared with wild-type animals. Similarly, TLR2-/- mice tended to have less severe arthritis (mean index: 1.50 versus 1.92, $p=0.13$) and bone erosions (mean index: 1.01 versus 1.40, $p=0.08$) compared with wild-type mice. However, no differences were observed when extremely high dose of dead bacteria ($1.2x10^{11}$/ml) was injected into knee joints of TNFR1-/- and wild-type mice (data not shown), suggesting TNFR1 deficiency is not able to conquer the overwhelming effect caused by extremely high dose of dead bacteria.

Monocytes and neutrophils are essential for the inflammation induced by antibiotic-killed *S. aureus*

Antibiotic-killed *S. aureus* was i.a. injected into BALB/c mice depleted of monocytes and/or neutrophils to evaluate the roles of these cells in the development of joint inflammation (Table 1). No significant differences in the synovitis incidence and severity were observed in mice devoid of either monocytes or neutrophils alone compared with the control group. In contrast, the simultaneous depletion of both peripheral monocytes and neutrophils resulted in remarkable reductions in the frequency and severity of synovial inflammation, with only 14% of mice developing synovitis ($p<0.001$).

To study whether T cell activation is responsible for the induction of synovitis, NMRI mice treated with abatacept were i.a. injected with antibiotic-killed *S. aureus*. The abatacept treatment did not have a significant impact on the synovitis frequency and severity compared with the control group.

Anti-TNF therapy abrogates arthritis and bone destruction induced by antibiotic-killed *S. aureus*

We investigated first the short-term effects of anti-TNF therapy on synovitis induction by antibiotic-killed *S. aureus* (Fig 3A). On day 3 after the i.a. injection of dead bacteria, the majority of the animals developed synovitis. Importantly, mice receiving anti-TNF treatment exhibited a significant reduction in synovitis severity ($p<0.001$) and had a lower synovitis frequency (66% vs. 92%, $p<0.05$) compared with control animals.

We further studied the long-term effects of anti-TNF therapy in this type of arthritis. The i.a. injection of a high dose of antibiotic-killed *S. aureus* produced a drastic inflammatory infiltration in all injected joints and even

bone erosion in 55-60% of the joints on days 7 and 14 (Fig 3D-H). The joint inflammation persisted, and fibrotic synovial tissues started to appear after 14 days (Fig 3F). Radiological signs of bone erosion were also observed in the joints on day 14 after the i.a. injection of dead bacteria (Fig 3I). Anti-TNF therapy significantly reduced the severity of synovitis and tended to prevent the bone destruction on both days 7 and 14 (Fig 3D and 3G), indicating the crucial role of TNF-α in the maintenance of chronic inflammation and induction of bone erosions.

Remarkably, significant TRAP-positive staining was found between the bone surface and the erosive pannus of the synovia on day 7 and 14 (Fig 3J) after i.a. injection of dead bacteria, suggesting that osteoclast activation occurs during inflammatory process induced by antibiotic-killed *S. aureus*.

Bacterial debris is the main culprit for arthritis induced by antibiotic-killed *S. aureus*

To study whether soluble components released from *S. aureus* after antibiotic treatment are arthritogenic, supernatants from antibiotic-killed *S. aureus* were injected into the knee joints of NMRI mice. The supernatants induced less-severe synovitis compared with the suspension of whole killed bacteria (Fig 4A).

To further investigate which bacterial components are responsible for arthritis induction, purified staphylococcal DNA and cell wall debris extracted from antibiotic-killed *S. aureus* were i.a. injected (Fig 4B). The synovitis frequency was only 20% ($p<0.01$) after the i.a. injection of bacterial DNA

compared with 80% and 79% for the bacterial debris and whole killed bacteria, respectively.

Lysostaphin is a metalloendopeptidase capable of cleaving the crosslinking pentaglycine bridges in the staphylococcal cell wall. The enzymatic digestion of antibiotic-killed *S. aureus* by lysostaphin had no effect on the severity of arthritis. (n.s., Fig 4C).

DISCUSSION

Despite advances in medical practice, the permanent reduction of joint function is observed in approximately 20% of patients with *S. aureus* arthritis. This might be caused by an exaggerated immune response in infected joints even after the adequate elimination of live microbes by antibiotics. Indeed, our data demonstrate that cloxacillin-killed *S. aureus* induced joint inflammation in a dose-dependent pattern. Importantly, the i.a. injection of dead bacteria led to a long-lasting synovitis followed by cartilage and bone destruction. In line with *in vivo* findings, splenocytes stimulated with antibiotic-killed *S. aureus* released substantial amounts of pro-inflammatory cytokines.

Several potential components in antibiotic-killed *S. aureus* might be responsible for its arthritogenic properties. Beta-lactam antibiotics can induce the release of inflammatory and chemotactic fragments, such as soluble lipoteichoic acid and peptidoglycan from *S. aureus*(10, 11). However, we show that the arthritogenic property of soluble components released after the addition of antibiotics is negligible compared with bacterial debris. The i.a. injection of soluble peptidoglycan is known to induce arthritis, and the enzymatic digestion by lysostaphin was shown to abrogate this inflammation(29). Here, antibiotic-killed *S. aureus* digested by lysostaphin induced local inflammation to the same extent as its undigested counterpart, suggesting that components other than soluble peptidoglycan induced arthritis. A previous study(30) showed that bacterial DNA containing CpG motifs induced arthritis through the TNF-α pathway. In contrast, our results suggest

that bacterial DNA from antibiotic-killed *S. aureus* had very little effect on mediating arthritis, which is in agreement with the data that splenocytes do not produce inflammatory cytokines upon stimulation with bacterial DNA (data not shown). Bacterial debris appears to be the most crucial component in our setting. The components of a gram-positive bacterial cell wall are known to activate the innate immune system via the TLR2 (for peptidoglycan) or TLR4 (for lipoteichoic acid)(12, 31). TLR2 deficiency partially reduced the arthritis severity, indicating the potent role of staphylococcal cell walls in this type of joint inflammation.

Synovitis induced by antibiotic-killed *S. aureus* was most apparent in a relatively short time, suggesting that innate immunity was likely involved. T-cell activation does not appear to be involved in the early phase of joint inflammation induced by antibiotic-killed *S. aureus*, as abatacept therapy was not able to reduce the arthritis severity. The single depletion of neutrophils or monocytes did not affect the severity of arthritis, whereas the depletion of both cells markedly abolished the joint inflammation, indicating that the cross-talk between neutrophils and monocytes is crucial for the induction of this type of arthritis. The double depletion of neutrophils and monocytes was also shown to attenuate arthritis induced by the i.a. injection of HMGB1(23). Intriguingly, RAGE, a prototypic DAMP receptor that recognizes a variety of pro-inflammatory ligands generated during inflammation and infection, was also involved in the induction of arthritis caused by antibiotic-killed *S. aureus*. In addition to the S100/calgranulin proteins, that have potent proinflammatory

properties and are produced by neutrophils and macrophages(32), RAGE can also interact with beta2-integrin Mac-1 that has a key role in antimicrobial defense and promotes inflammatory cell recruitment(33). This suggests a subtle connection in the cross-talk among neutrophils and monocytes and RAGE receptors in this type of joint inflammation.

Bacterial components activate immune cells via TLRs to release inflammatory cytokines, e.g., TNF-α and IL-6(12, 34). Through TNF receptor 1, TNF-α exerts biological actions and up-regulates another potent cytokine, HMGB1(35). HMGB1 released from inflammatory or from necrotic cells in the inflamed joint has cytokine inducing and chemotactic activity, and can further exacerbate inflammatory responses through several receptors, such as RAGE, TLR2 and TLR4(23, 36). Indeed, both the synovitis index and frequency were significantly reduced in TNFR1-/- and RAGE-deficient animals. In addition, the arthritis severity was lower in TLR2-/- mice than in their wild-type controls. IL-17F is known to compensate for IL-17A in *S. aureus*-induced arthritis(37), especially in the short term, which might explain why no effect of IL-17A was observed on the antibiotic-killed *S. aureus* arthritis. Together, our data suggest that the signaling pathway in inflammation caused by antibiotic-killed *S. aureus* is most likely mediated through the TLR2, TNF, and RAGE axis. However, there was no such 'yes or no' effect in any of the tested gene-knockout settings, indicating that inflammatory signaling pathways are redundant and complicated.

The limitation of present study is that different strains of mice were used to identify the role of various immune cells in this arthritis model. However, our results from anti-TNF treatment on NMRI mice are consistent with the data obtained from TNFR1-/- mice on C57BL/6 background, suggesting that the involvment of TNF-α pathway in our model applies to different mice strains. An observandum is that cellular proliferation and levels of TNF-α and IL-6 did not differ in splenocytes from TNFR1-/- and wild-type mice, which is inconsistent with the result of *in vivo* experiments using TNFR1-/- mice. Further investigation is needed to elucidate the underlying mechanism.

The standard treatment for septic arthritis is antibiotics in combination with joint lavages. The therapeutic goal of joint lavages is to eliminate the bacterial components that trigger exaggerated host responses as well as the pro-inflammatory cytokines and infiltrated immune cells that may cause joint damage. Therapy with a corticosteroid against exaggerated host responses leads to a better clinical outcome in patients with bacterial infections, including bacterial meningitis(38) and septic arthritis in children(7, 8). However, glucocorticoids have several side effects, e.g., the induction of secondary osteoporosis. Of note, significantly increased osteoclastic activity already exists in *S. aureus* septic arthritis, which leads to systemic and rapid bone loss(39). The addition of glucocorticoids might aggravate the development of osteoporosis. We previously studied whether modulation of the inflammatory response by TNF inhibitors reduces the immune reaction and joint damage in septic arthritis. Indeed, combination therapy of antibiotics and

a TNF inhibitor resulted in a more rapid relief of clinical arthritis in mice with septic arthritis compared with the antibiotic monotherapy(19).

Here, we showed that antibiotic-killed *S. aureus* might be a cause of long-lasting joint inflammation that may lead to post-infectious complications of *S. aureus* septic arthritis. In septic arthritis, positive Gram staining and culture findings in the synovial samples become negative after short time (2-3 days) of antibiotic treatment(40). To prevent the chronic inflammation caused by dead bacteria in septic arthritis, we speculate that exaggerated immune responses can be modulated, e.g., by blocking TNF in combination with an efficient antibiotic treatment.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article and revising it critically for important intellectual content, and all authors approved the final version to be published. Ali and Dr. Jin had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ali and Jin.

Acquisition of data. Ali, Zhu, Kwiecinski, and Jin

Analysis and interpretation of data. Ali, Pullerits, and Jin.

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Figure 1. Antibiotic-killed *S. aureus* LS-1 induces *in vivo* joint inflammation following i.a. injection and *in vitro* the release of pro-inflammatory cytokines by splenocytes. (A) The severity and frequency of synovitis in NMRI mice (n =4/time point) 1, 3, 7, 14, and 30 days after the i.a. injection with cloxacillin-killed S. aureus (2.6×10^9 dead bacteria/ml). The same concentration of cloxacillin was i.a. injected as a control. (B) The severity and frequency of synovitis in NMRI mice (n=4-5) 3 days after the i.a. injection with different doses of cloxacillin-killed *S. aureus* in 20 μl of PBS (5.2×10^8, 2.6×10^9, and $1.3\text{x}10^{10}$ dead bacteria/ml). The data are presented as the mean±SEM of the synovitis index or arthritis frequency (%). (C) TNF-α and (D) IL-6 levels in the culture supernatants after the stimulation of spleen cells from wild-type C57Bl/6 mice with cloxacillin-killed *S. aureus* ($4\text{x}10^6$ to $1\text{x}10^8$ dead bacteria/ml) for 24 and 48 hours. The levels of cytokines are presented as the mean±SEM (pg/ml). (E) Proliferative responses of mixed spleen cells to cloxacillin-killed *S. aureus* ($4\text{x}10^6$ to $5\text{x}10^8$ dead bacteria/ml). LPS and PBS were used as positive and negative controls, respectively. The data are presented as the mean±SEM of counts per minute (cpm).

Figure 2. Antibiotic-killed *S. aureus* induces synovitis through TNFR1, RAGE receptor, and TLR2. The synovitis severity scores (mean±SEM) and arthritis frequency (%) are shown in (A) TNFR1-/- mice (n=20), (B) RAGE -/- mice (n=17), and (C) TLR2 -/- mice (n=13) 3 days after a single i.a. injection of cloxacillin-killed *S. aureus* LS-1 (2.6×10^9 dead bacteria/ml in 20 μl of PBS). Wild-type C57BL/6 mice (n=9-21) served as controls. Two independent experiments were performed, and their results were pooled. (D) To study long-term effect of TNFR1 deficiency, TNFR1-/- (n=8) and WT (n=12) mice were i.a. injected with a high dose of cloxacillin-killed *S. aureus* LS-1 ($4.2\text{x}10^{10}$ dead bacteria/ml in 20 μl of PBS) and the severity of synovitis and bone erosions evaluated on day 14.

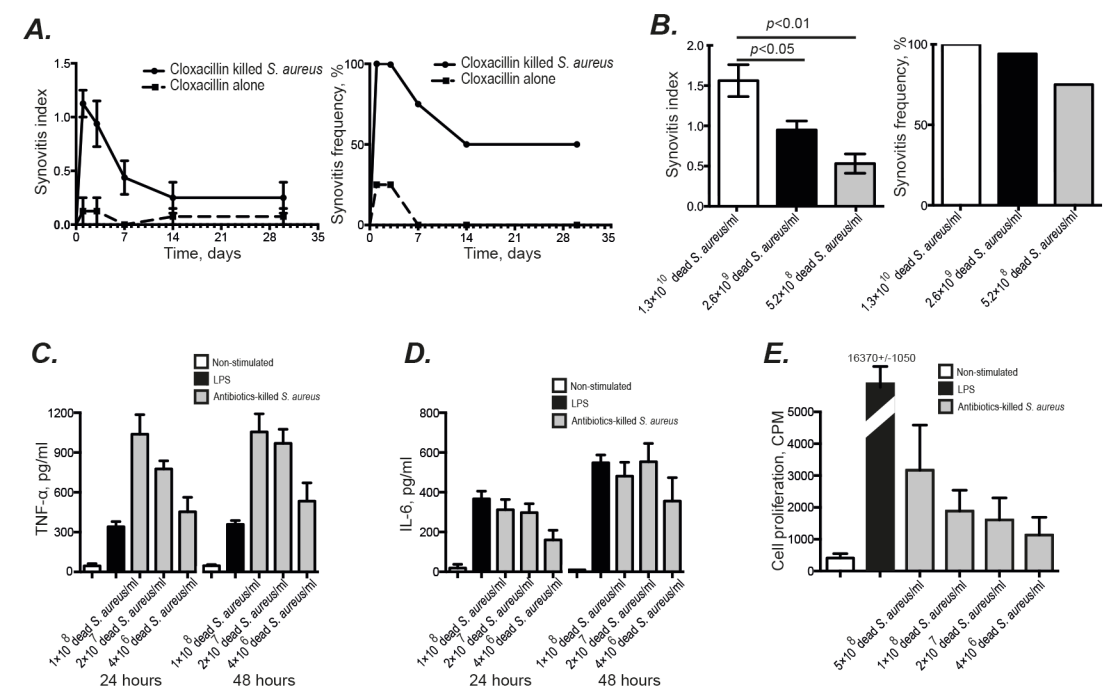
The data are presented as the mean±SEM of the synovitis and bone erosion index or synovitis frequency(%). Statistical evaluations were performed using the Mann–Whitney U-test, Fisher's exact test, or Chi-square test. n.s. = not significant.

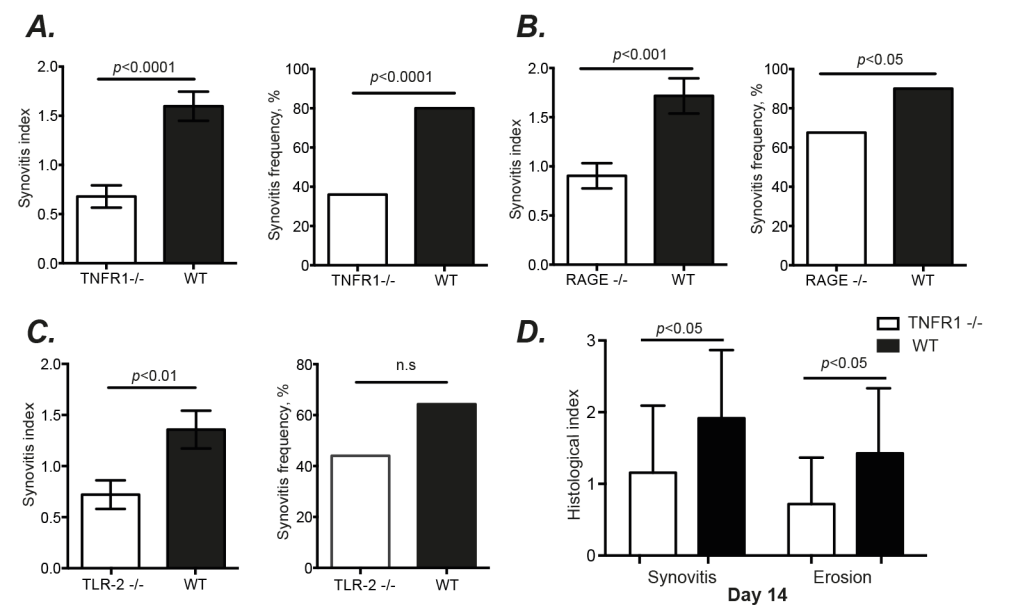
Figure 3. Anti-TNF treatment attenuates joint inflammation and bone destruction. (A) NMRI mice were i.a. injected with 20 μl of cloxacillin-killed *S. aureus* LS-1 (2.6×10^9 dead bacteria/ml) following s.c. injection of etanercept (5 μg/g of body weight) daily until day 3. Representative figures of joints from mice receiving PBS (B) or anti-TNF treatment (C) are shown. To study long-term effect of anti-TNF therapy, NMRI mice were i.a. injected with a high dose of cloxacillin-killed *S. aureus* LS-1 ($4.2\text{x}10^{10}$ dead bacteria/ml) following s.c. injection of Etanercept (5 μg/g of body weight) every 48 hours until day 14. The severity of synovitis and bone erosions were evaluated on day 7 (D) and 14 (G). The influx of inflammatory cells into the synovial tissue is evident on day 7 (E, arrow) and persistent on day 14 (F, arrow); pannus formation and cartilage erosions (E and H, arrowheads), were observed as early as day 7. On day 14 fibrotic tissues (F, arrow) appeared in the synovial tissues, and bone destructions were observed microscopically (G, arrowhead) and radiologically (I, arrowhead). TRAP-positive staining (J, arrowhead) was located between bone surface and erosive pannus.

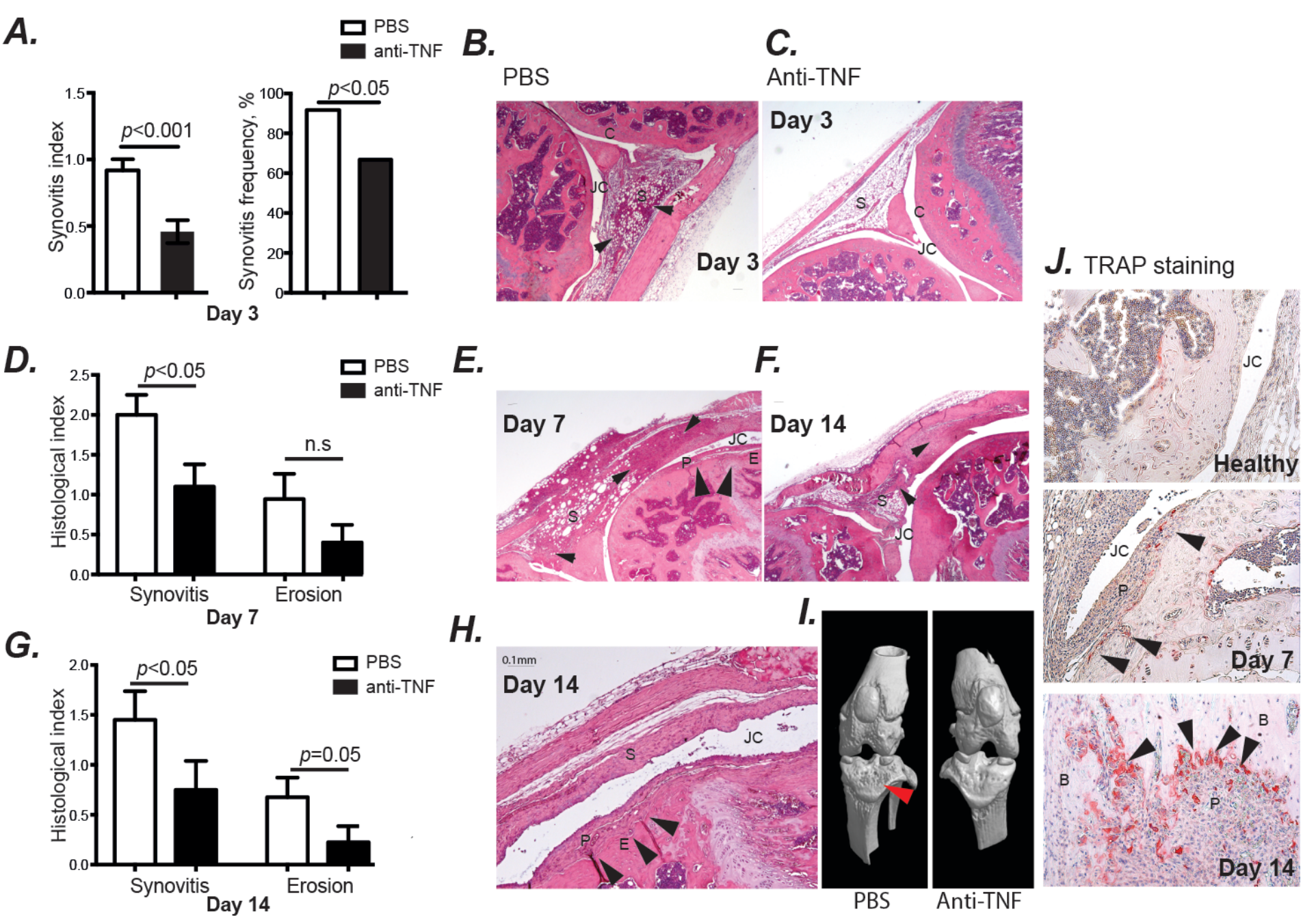
B, Bone; C, cartilage; E, bone erosions; JC, joint cavity; S, synovial tissue.

Figure 4. Cell debris from dead bacteria is responsible for joint inflammation induced by antibiotic-killed *S. aureus*. Synovitis severity scores (mean±SEM) and synovitis frequency (%) are shown in NMRI mice 3 days after a single i.a. injection of (A) supernatants or bacterial debris of cloxacillin-killed *S. aureus* LS-1 ($2.6\text{x}10^9$ dead bacteria/ml), (B) bacterial DNA or cell debris from cloxacillin-killed *S. aureus* LS-1 ($1.3\text{x}10^{10}$ dead bacteria/ml), and (C) antibiotic-killed *S. aureus* ($2.6\text{x}10^9$ dead

bacteria/ml) digested with lysostaphin (100 μg/ml) or undigested dead *S. aureus*. Statistical evaluations were performed using the Mann–Whitney U-test or Fisher's exact test.


A.
Cloxacillin killed S. aureus
Cloxacillin alone
Synovitis index
Synovitis frequency, %
Time, days
B.
p<0.01
p<0.05
Synovitis index
Synovitis frequency, %
1,3×10 10 dead S. aureus/ml
2,6×10 9 dead S. aureus/ml
5,2×10 8 dead S. aureus/ml
C.
Non-stimulated
LPS
Antibiotics-killed S. aureus
TNF-α, pg/ml
1×10 8 dead S. aureus/ml
2×10 7 dead S. aureus/ml
4×10 6 dead S. aureus/ml
24 hours
48 hours
D.
IL-6, pg/ml
E.
16370+/-1050
Cell proliferation, CPM
5×10 8 dead S. aureus/ml
1×10 8 dead S. aureus/ml
2×10 7 dead S. aureus/ml
4×10 6 dead S. aureus/ml


A.
Synovitis index
p<0.0001
TNFR1-/-
WT
Synovitis frequency, %
p<0.0001
TNFR1-/-
WT
B.
Synovitis index
p<0.001
RAGE -/-
WT
Synovitis frequency, %
p<0.05
RAGE -/-
WT
C.
Synovitis index
p<0.01
TLR-2 -/-
WT
Synovitis frequency, %
n.s
TLR-2 -/-
WT
D.
TNFR1 -/-
WT
Histological index
p<0.05
p<0.05
Synovitis
Erosion
Day 14


A.
PBS
anti-TNF
Synovitis index
p<0.001
Synovitis frequency, %
p<0.05
Day 3
B.
PBS
C.
Anti-TNF
Day 3
C
JC
S
Day 3
S
C
JC
J. TRAP staining
JC
Healthy
JC
P
Day 7
D.
PBS
anti-TNF
Histological index
p<0.05
n.s
Synovitis
Erosion
Day 7
E.
F.
Day 7
JC
P
E
S
Day 14
S
JC
H.
I.
0.1mm
Day 14
S
JC
P
E
PBS
Anti-TNF
B
B
P
Day 14
G.
PBS
anti-TNF
Histological index
p<0.05
p=0.05
Synovitis
Erosion
Day 14

A.
Synovitis index
2.5
2.0
1.5
1.0
0.5
p<0.01
n.s
p=0.06
Mixture
Dead bacteria
Supernatant
Synovitis frequency, %
100
80
60
40
20
p<0.05
n.s
Mixture
Dead bacteria
Supernatant
B.
Synovitis index
2.0
1.5
1.0
0.5
0.0
p<0.05
n.s
Killed bacteria
Debris
DNA
Synovitis frequency, %
100
80
60
40
20
0
p<0.01
n.s
Killed bacteria
Debris
DNA
C.
Synovitis index
1.5
1.0
0.5
0.0
p<0.05
p<0.01
killed bacteria
killed bacteria + lysostaphin
lysostaphin
Synovitis frequency, %
100
80
60
40
20
0
p<0.05
p<0.01
killed bacteria
killed bacteria + lysostaphin
lysostaphin