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Antibiotic-Killed *Staphylococcus aureus* Induces Destructive Arthritis in Mice

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Objective. **Permanent reduction in joint function is a severe postinfectious complication in patients with *Staphylococcus aureus* septic arthritis. We undertook this study to determine whether this reduction in joint function might be caused by persistent joint inflammation after the adequate eradication of bacteria by antibiotics.**

Methods. **After intraarticular injection of cloxacillin-killed *S aureus* into mouse knee joints, we investigated whether antibiotic-killed *S aureus* induced joint inflammation and elucidated the molecular and cellular mechanisms of this type of arthritis.**

Results. **Intraarticular injection of antibiotic-killed *S aureus* induced mild-to-moderate synovitis and bone erosions that lasted for a minimum of 14 days. Compared with wild-type animals, mice deficient in tumor necrosis factor receptor type I (TNFRI), receptor for advanced glycation end products (RAGE), or Toll-like receptor 2 (TLR-2) had a significantly reduced frequency and severity of synovitis. Combined depletion of monocytes and neutrophils also 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 joint inflammation induced by antibiotic-killed *S aureus*.**

Conclusion. **Antibiotic-killed *S aureus* induced and maintained joint inflammation mediated through TLR-2, TNFRI, and RAGE. The cross-talk between neutrophils and monocytes is responsible for this type of arthritis. Anti-TNF therapy might be used as a novel strategy, in combination with antibiotics, to treat staphylococcal septic arthritis.**

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% to 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 proinflammatory cytokine levels predict the severity of the later destruction of affected joints in patients with septic arthritis (5). The addition of corticosteroids to antibiotic treatments has been found to ameliorate the course of *S aureus* septic arthritis in mice (6), and more recent studies demonstrated that the addition of gluco-

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corticoids at the start of antibiotic treatment in children with septic arthritis led to 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 (TLRs) to release proinflammatory cytokines (e.g., tumor necrosis factor α [TNFα] and interleukin-6 [IL-6]) (12). It is therefore reasonable that components of antibiotic-killed *S aureus* would be 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 TLR-2, TNF receptor type I (TNFRI), and receptor for advanced glycation end products (RAGE). Therapy using a TNFα inhibitor abrogated the joint inflammation induced by antibiotic-killed *S aureus*.

MATERIALS AND METHODS

Mice. Female NMRI mice were purchased from Harlan Laboratories. BALB/c and C57BL/6 mice of both sexes were purchased from Charles River Deutschland. TNFRI$^{-/-}$ mice (13) (kindly provided by Dr. Mary J. Wick, University of Gothenburg), RAGE$^{-/-}$ mice (14,15) (kindly provided by Prof. Bernd Arnold, Deutsches Krebsforschungszentum, Heidelberg, Germany and Prof. Peter Nawroth, University Clinical Centre of Heidelberg, Heidelberg, Germany), B6.129-Tlr2^{tm1Kir}/J (TLR-2$^{-/-}$) 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 ages 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 Ethics Committee of Gothenburg.

Reagents. Etoposide was purchased from Bristol-Myers Squibb. The monoclonal antibody (mAb) RB6-8C5 was produced and purified as previously described in detail (18). Monoclonal Ig class–matched anti-ovalbumin antibodies were used as a control (18). Cloxacillin (Cloxacillinnatr; Stragen Nordic) was used to kill *S aureus* in vitro. Lysostaphin was obtained from Sigma.

Preparations of antibiotic-killed *S aureus*. *S aureus* strain LS-1 was prepared as previously described (19), then incubated with 33 mg/ml cloxacillin for 6–24 hours at 37°C and thereafter for 60 minutes at 60°C to eradicate any surviving bacteria. 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 phosphate buffered saline (PBS) to remove the cloxacillin residues and cultured for 24 hours. No bacterial growth was detected.

Supernatants of cloxacillin-killed *S aureus* (2.6×10^9 bacteria/ml) were collected after 5-minute centrifugation, and the pellets were suspended in a volume of PBS equal to the volume of the supernatants. The supernatants and dead bacteria were then stored at −70°C until used. To extract genomic DNA, cloxacillin-killed *S aureus* (1.3×10^{10} bacteria/ml) mixed with 200 μg glass beads was lysed in a FastPrep machine (BIO101; MP Biomedicals). The extract was treated with RNase A and proteinase K at 37°C and 55°C, respectively, for 30 minutes. Genomic DNA was purified by phenol–chloroform extraction and ethanol precipitation. The cell wall debris was isolated according to a previously described method (20). Cell wall debris and bacterial DNA (0.35 mg/ml) were suspended in PBS buffer.

Lysostaphin treatment of antibiotic-killed *S aureus*. Antibiotic-killed *S aureus* (2.6×10^9 bacteria/ml) was incubated overnight with lysostaphin (100 μg/ml) at 37°C. It has previously been shown that the peptidoglycan polymer is completely digested via this procedure (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 TNFRI$^{-/-}$ mice were performed as previously described (22). Splenocytes (1×10^6/ml) were incubated with various preparations of cloxacillin-killed *S aureus* (concentrations of 4×10^6–1×10^8 bacteria/ml) for 72 hours or with lipopolysaccharide (LPS; 1 μg/ml) as a positive control.

Induction of arthritis with antibiotic-killed *S aureus*. In all experiments, mice were injected intraarticularly (IA) in the knee with a total volume of 20 μl of solution containing antibiotic-killed *S aureus* in PBS or with 20 μl of PBS with a matching concentration (33 mg/ml) of cloxacillin. To study the kinetics of arthritis development, NMRI mice were injected IA with antibiotic-killed *S aureus* (2.6×10^9 bacteria/ml), and the contralateral knee joints were injected with cloxacillin. The animals were killed on days 1, 3, 7, 14, or 30. The knee joints were collected for histopathologic 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* (5.0×10^8–1.3×10^{10} bacteria/ml; n = 4–5 mice per group) were injected IA into NMRI mice. In the second set of experiments, NMRI mice received an IA injection of supernatants (n = 5) or bacterial debris (n = 5) from antibiotic-killed *S aureus* in 20 μl of PBS (2.6×10^9 bacteria/ml) or the original suspension of antibiotic-killed *S aureus* (n = 5). In the third experiment, NMRI mice were injected with purified bacterial components from antibiotic-killed *S aureus* in 20 μl of PBS (1.3×10^{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*, gene-knockout mice received IA injections of antibiotic-killed *S aureus* (2.6×10^9 bacteria/ml). These included TLR-$2^{-/-}$ mice (n = 13), TNFRI$^{-/-}$ mice (n = 20), RAGE$^{-/-}$ mice (n = 17), and IL-17A$^{-/-}$ mice (n = 8). C57BL/6 wild-type (WT) 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 TLR-$2^{-/-}$ mice, TNFRI$^{-/-}$ mice, and RAGE$^{-/-}$ mice, 2 independent experiments were performed and the results were pooled. One experiment was performed using IL-17A$^{-/-}$ mice.

To further assess whether the synovitis differences observed on day 3 are constant even with a longer disease duration, TLR-$2^{-/-}$ mice (n = 9) and TNFRI$^{-/-}$ mice (n = 18) received IA injections of a high dose of antibiotic-killed *S aureus* (4.2×10^{10}–1.2×10^{11} bacteria/ml). C57BL/6 WT mice (n = 22) were used as controls. Two independent experiments were performed using TNFRI$^{-/-}$ mice, and 1 was performed using TLR-$2^{-/-}$ mice. 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 (2.6×10^9 bacteria/ml) was injected IA 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 injected subcutaneously (SC) into NMRI mice (5 μg/gm body weight in 0.1 ml PBS) daily starting on day 0 after IA injection of dead *S aureus* (2.6×10^9 bacteria/ml) and continuing until day 3 when the animals were killed.

Treatment with anti-TNF compounds. Etanercept (Enbrel; Wyeth Europa) is known to efficiently inhibit the activity of murine TNFα (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/gm body weight in 0.1 ml PBS) was given SC either 1) every 24 hours starting on day 0 after IA injection of antibiotic-killed *S aureus* (2.6×10^9 bacteria/ml) and continuing until day 3 when the animals were killed, or 2) every 48 hours starting on day 0 after IA injection of dead *S aureus* (4.2×10^{10} bacteria/ml) and continuing until day 7 or 14 when the animals were killed and knee joints were collected for histologic examination and micro–computed tomography (micro-CT) scanning.

Histopathologic examination of the joints. Knee joints were removed, fixed in 4% paraformaldehyde, decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Joints receiving a high dose of dead bacteria were chosen for tartrate-resistant acid phosphatase (TRAP) staining (27). All slides were coded, and synovitis and cartilage/bone destruction were assessed microscopically in a blinded manner by 2 observers (AA, TJ). The extent of synovitis/bone destruction was judged on a scale of 0–3 (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. We also used micro-CT scanning to study the long-term effect (day 14) of anti-TNF therapy on bone destruction in knee joints of mice receiving an IA injection of antibiotic-killed *S aureus*. We used a SkyScan 1176 micro-CT apparatus (Bruker) with a voxel size of 18 μm. The scanning was conducted at 45 kV/555 μA with a 0.2-mm aluminum filter. The exposure time was 199 msec. The x-ray projections were obtained at 0.5° intervals with a scanning angular rotation of 180°. The projection images were reconstructed into 3-dimensional images using NRecon software (version 1.5.1; Bruker) and analyzed using CTVox software (version 2.4; Bruker). The extent of cartilage and bone destruction was scored on a scale 0–3 in a blinded manner by 1 observer (TJ).

Measurement of cytokine levels. Levels of TNFα, IL-6, and RANKL in sample supernatants were determined using a DuoSet ELISA Development Kit (R&D Systems Europe).

Statistical analysis. Statistical significance was assessed using the Mann-Whitney U test and the chi-square test. GraphPad Prism software, version 6 was used for the calculations. Results are reported as the mean ± SEM.

RESULTS

Arthritis induction by antibiotic-killed *S aureus*. All of the mice injected IA with antibiotic-killed *S aureus* developed synovitis by day 1. After 2 weeks, synovitis severity and frequency had both diminished significantly; however, after reaching a certain threshold, these symptoms remained stable until the experiment was ended (day 30). After IA injection of cloxacillin alone, 1 of the 4 mice developed slight transient inflammation, which disappeared after a few days (Figure 1A). Bone destruction was not observed on day 3 after IA injection of dead bacteria. Different concentrations of antibiotic-killed *S aureus* were injected IA, and results showed a dose-dependent pattern regarding both the severity and the frequency of synovitis (Figure 1B).

Induction of release of proinflammatory cytokines by antibiotic-killed *S aureus*. To study whether antibiotic-killed *S aureus* has the potential to induce the production of proinflammatory cytokines, splenocytes from WT mice were stimulated with different concentrations of antibiotic-killed bacteria, and cytokine levels were measured in cell culture supernatants after 24 and 48 hours (Figures 1C and D). Increased levels of TNFα and IL-6 were found after 24 hours of stimulation, and their concentrations remained high during 48 hours of culturing. Indeed, stimulation of splenocytes with a low concentration of dead *S aureus* (4×10^6 bacteria/ml) resulted in a severalfold increase in TNFα production in cell culture supernatants compared with unstimulated

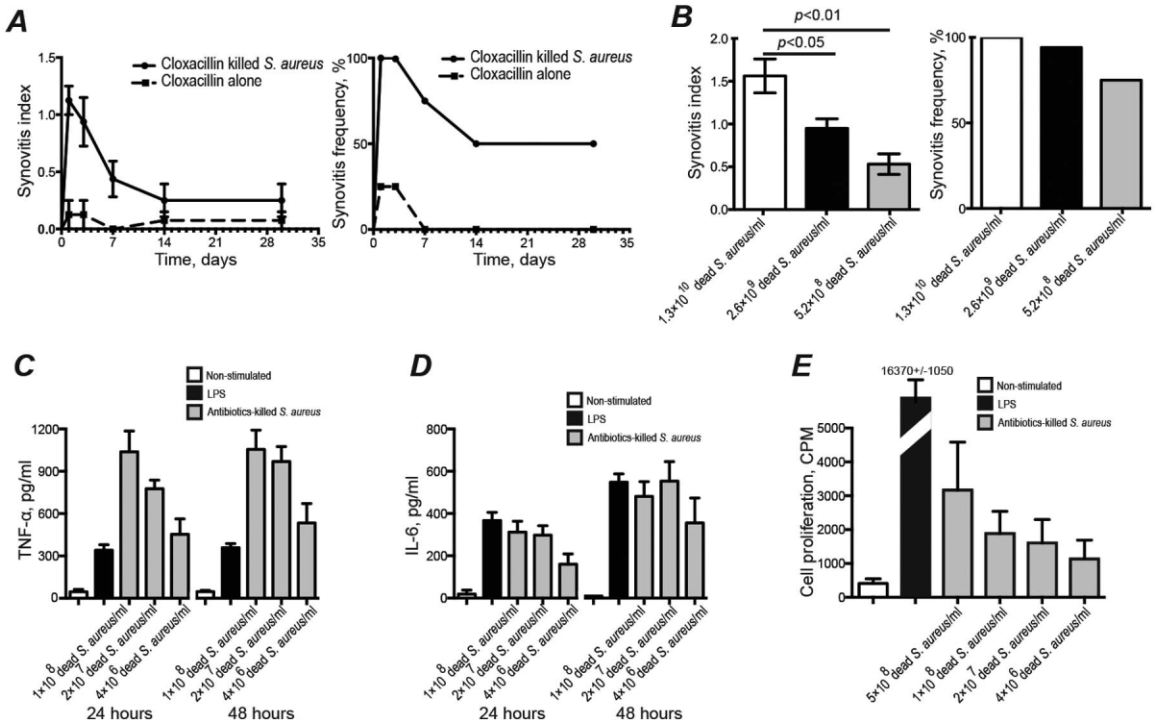

Figure 1. Antibiotic-killed *Staphylococcus aureus* LS-1 induces in vivo joint inflammation following intraarticular (IA) injection and also induces in vitro release of proinflammatory cytokines by splenocytes. **A,** Severity and frequency of synovitis in NMRI mice (n = 4 per time point) 1, 3, 7, 14, and 30 days after IA injection of cloxacillin-killed *S aureus* (2.6×10^9 dead bacteria/ml). The same concentration of cloxacillin was injected IA as a control. **B,** Severity and frequency of synovitis in NMRI mice (n = 4–5 per group) 3 days after IA injection of different doses of cloxacillin-killed *S aureus* (5.2×10^8, 2.6×10^9, and 1.3×10^{10} dead bacteria/ml) in phosphate buffered saline (PBS). In **A** and **B**, values for the synovitis index are the mean ± SEM. **C** and **D,** Levels of tumor necrosis factor α (TNFα) (**C**) and interleukin-6 (IL-6) (**D**) in culture supernatants after stimulation of spleen cells from wild-type C57BL/6 mice with cloxacillin-killed *S aureus* for 24 and 48 hours. Values are the mean ± SEM. **E,** Proliferative responses of mixed spleen cells to cloxacillin-killed *S aureus*. Lipopolysaccharide (LPS) and PBS were used as positive and negative controls, respectively. Values are the mean ± SEM.

cells ($P < 0.001$) (Figure 1C); this increase was even higher than the increase in TNFα production in LPS-stimulated cell culture. In addition, stimulation with antibiotic-killed *S aureus* induced cell proliferation in a dose-dependent pattern (Figure 1E).

RANKL was not detectable in these cell culture supernatants (data not shown). Stimulation of spleen cells from TNFRI$^{-/-}$ mice (n = 6) with dead *S aureus* demonstrated increases in cytokine levels and cell proliferation similar to those detected in culture supernatants of splenocytes from WT mice (data not shown).

Arthritis induced by antibiotic-killed *S aureus* is mediated through TLR-2, TNFRI, and RAGE. Different knockout mice were used to study the signaling pathway of joint inflammation induced by dead *S aureus.* Mice lacking TNFRI had significantly lower synovitis severity compared with their WT counterparts ($P < 0.0001$) (Figure 2A). The synovitis frequency was also significantly lower in TNFRI$^{-/-}$ mice, with 35% of the mice developing synovitis compared with 80% of the WT controls ($P < 0.0001$).

RAGE is a multiligand receptor, and its activation has been shown to play a role in diverse experimental inflammatory and infectious diseases (28). RAGE$^{-/-}$ mice also had significant reductions in synovitis severity and frequency compared with their WT counterparts ($P < 0.001$ and $P < 0.05$, respectively) (Figure 2B).

TLR-2 is known to mediate inflammation induced by cell walls from gram-positive bacteria. TLR-2$^{-/-}$ mice displayed significantly lower synovitis severity compared with WT controls ($P < 0.01$) (Figure 2C) and tended to have a lower synovitis frequency. No significant difference could be observed in synovitis severity and frequency between mice lacking IL-17A and WT mice (data not shown).

To verify whether observed differences were con-

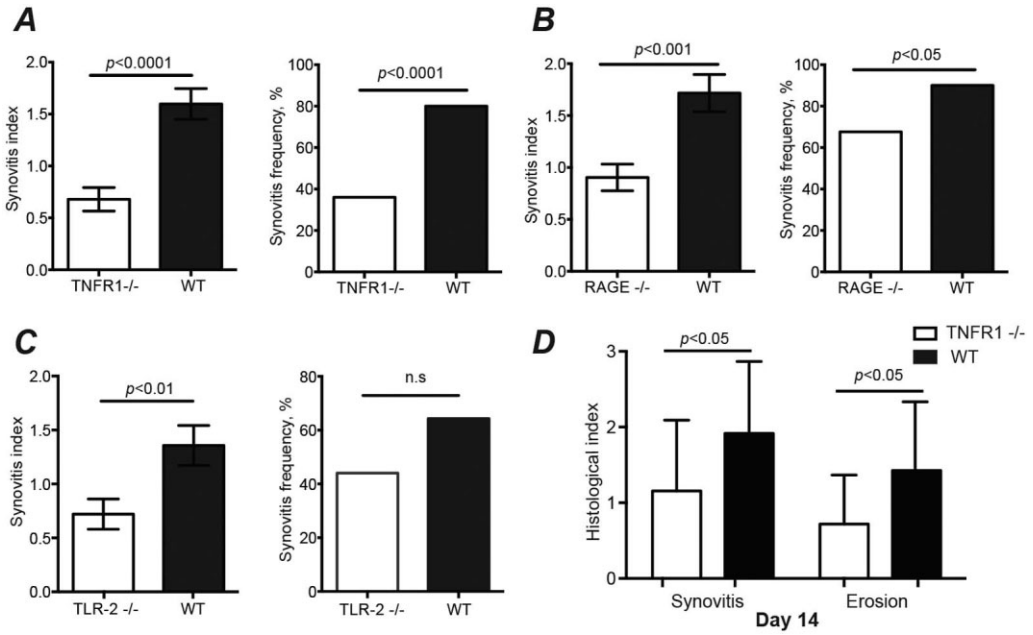

Figure 2. Antibiotic-killed *Staphylococcus aureus* induces synovitis through tumor necrosis factor receptor type I (TNFRI), receptor for advanced glycation end products (RAGE), and Toll-like receptor 2 (TLR-2). **A–C,** Synovitis severity scores and arthritis frequency in TNFRI$^{-/-}$ mice (n = 20) (**A**), RAGE$^{-/-}$ mice (n = 17) (**B**), and TLR-2$^{-/-}$ mice (n = 13) (**C**) were determined 3 days after a single intraarticular (IA) injection of cloxacillin-killed *S aureus* LS-1 (2.6×10^9 dead bacteria/ml in 20 μl of phosphate buffered saline [PBS]). Wild-type (WT) C57BL/6 mice (n = 9–21 per group) served as controls. Two independent experiments were performed, and their results were pooled. **D,** To study the long-term effect of TNFRI deficiency, TNFRI$^{-/-}$ mice (n = 8) and WT mice (n = 12) were injected IA with a high dose of cloxacillin-killed *S aureus* LS-1 (4.2×10^{10} dead bacteria/ml in 20 μl of PBS), and the severity of synovitis and bone erosions was evaluated on day 14. Values for the synovitis index and histologic indices are the mean ± SEM. *P* values were determined using the Mann-Whitney U test, Fisher's exact test, or chi-square test. NS = not significant.

stant, TNFRI$^{-/-}$ mice (Figure 2D) and TLR-2$^{-/-}$ mice (not shown) were injected IA with a high dose of antibiotic-killed *S aureus* (4.2×10^{10}/ml) and observed on day 14. TNFRI$^{-/-}$ 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 WT animals. Similarly, TLR-2$^{-/-}$ 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 WT mice.

Table 1. Incidence and severity of synovitis after intraarticular injection of cloxacillin-killed *Staphylococcus aureus* in mice depleted of neutrophils (using RB6-8C5) and/or monocytes (using etoposide) or in mice lacking efficient T cell activation (using abatacept to selectively modulate the CD80/CD86-mediated CD28 costimulatory signal required for full T cell activation)*

Group, treatment	Total number of joints	Synovitis frequency, %	Synovitis index, mean ± SEM
BALB/c mice, anti-ovalbumin	10	100	1.01 ± 0.18
BALB/c mice, RB6-8C5	10	70	1.35 ± 0.28
BALB/c mice, etoposide	6	67	0.67 ± 0.25
BALB/c mice, etoposide + RB6-8C5	7	14†	0.11 ± 0.07†
NMRI mice, PBS	10	100	0.95 ± 0.11
NMRI mice, abatacept	10	80	0.65 ± 0.13

* Monoclonal Ig class–matched anti-ovalbumin antibodies were used as a control. PBS = phosphate buffered saline.
† $P < 0.001$ versus BALB/c mice treated with anti-ovalbumin, by Fisher's exact test (for synovitis frequency) or Mann-Whitney U test (for synovitis index).

However, no differences were observed when an extremely high dose of dead bacteria (1.2×10^{11}/ml) was injected into knee joints of TNFRI$^{-/-}$ and WT mice (data not shown), suggesting that TNFRI deficiency is not able to counter the overwhelming effect caused by an extremely high dose of dead bacteria.

Necessity of monocytes and neutrophils for inflammation induction by antibiotic-killed *S aureus*. Antibiotic-killed *S aureus* was injected IA 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 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 injected IA with antibiotic-killed *S au-*

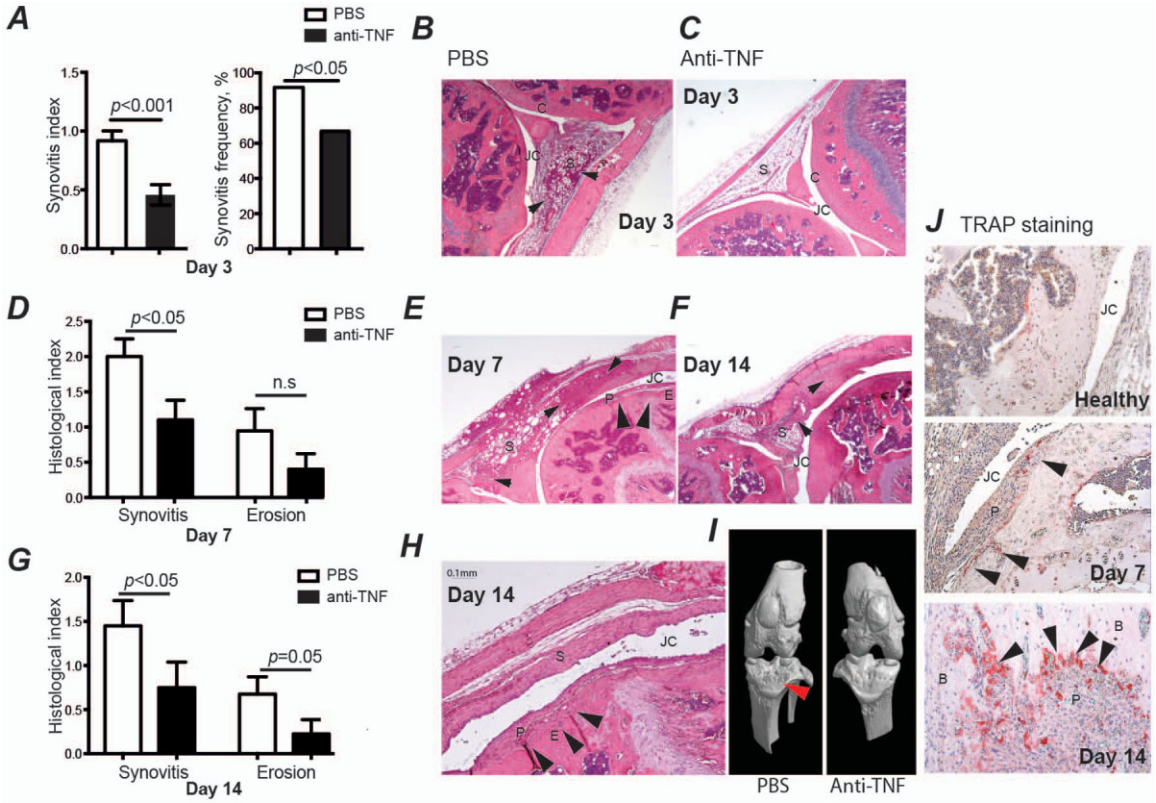

Figure 3. Anti–tumor necrosis factor (anti-TNF) treatment attenuates joint inflammation and bone destruction. **A,** The severity and frequency of synovitis in NMRI mice injected intraarticularly (IA) with 20 μl of cloxacillin-killed *Staphylococcus aureus* LS-1 (2.6×10^9 dead bacteria/ml) following subcutaneous (SC) injection of etanercept (5 μg/gm body weight) daily until day 3 were determined. **B** and **C,** Shown are representative photomicrographs of joints from mice receiving phosphate buffered saline (PBS) (**arrows** indicate severe inflammatory infiltration in synovium) (**B**) or anti-TNF treatment (**C**). **D** and **G,** To study the long-term effect of anti-TNF therapy, NMRI mice were injected IA with a high dose of cloxacillin-killed *S aureus* LS-1 (4.2×10^{10} dead bacteria/ml) following SC injection of etanercept (5 μg/gm body weight) every 48 hours until day 14. The severity of synovitis and bone erosions was evaluated on day 7 (**D**) and day 14 (**G**). In **A, D,** and **G,** values for the synovitis index and histologic indices are the mean ± SEM. NS = not significant. **E, F, H–J,** The influx of inflammatory cells into the synovial tissue was evident on day 7 (**arrows** in **E**) and persistent on day 14 (**arrows** in **F**). Pannus formation and cartilage erosions were observed as early as day 7. On day 14 fibrosis appeared in the synovial tissues (**arrows** in **F**), and bone destruction was observed microscopically (**arrowheads** in **E** and **H**) and radiologically (**arrowhead** in **I**). Tartrate-resistant acid phosphatase (TRAP)–positive staining (**arrowheads** in **J**) was located between bone surface and erosive pannus. **C** = cartilage; **JC** = joint cavity; **S** = synovial tissue; **P** = pannus; **E** = bone erosions; **B** = bone. Original magnification × 10 in **B, C, E,** and **F**; × 20 in **H** and **J**.

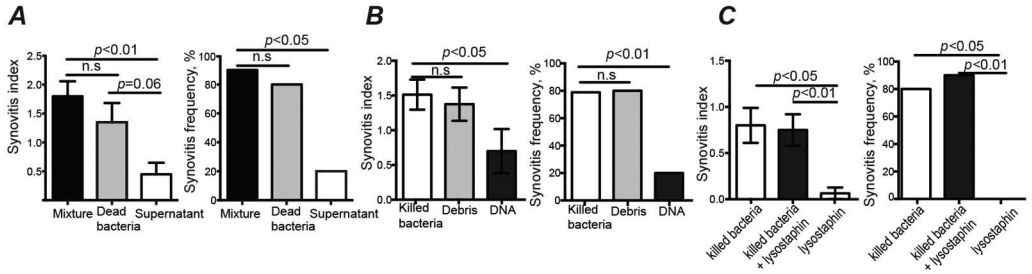

Figure 4. Cell debris from dead bacteria is responsible for joint inflammation induced by antibiotic-killed *Staphylococcus aureus*. Shown are synovitis severity and frequency in NMRI mice 3 days after a single intraarticular injection of supernatants, dead cloxacillin-killed *S aureus* LS-1 bacteria (2.6×10^9/ml), or the original suspension ("mixture") (**A**); bacterial DNA, cell debris from cloxacillin-killed *S aureus* LS-1 (1.3×10^{10} dead bacteria/ml), or a mixture of the fractions ("killed bacteria") (**B**); and cloxacillin-killed *S aureus* (2.6×10^9 dead bacteria/ml) digested with lysostaphin (100 μg/ml), undigested dead *S aureus,* or lysostaphin alone (**C**). Values for the synovitis index are the mean ± SEM. *P* values were determined using the Mann-Whitney U test or Fisher's exact test. NS = not significant.

reus. Abatacept treatment did not have a significant impact on synovitis frequency and severity compared with no treatment.

Anti-TNF therapy abrogates arthritis and bone destruction induced by antibiotic-killed *S aureus*. We first investigated the short-term effects of anti-TNF therapy on synovitis induction by antibiotic-killed *S aureus* (Figure 3A). On day 3 after the IA injection of dead bacteria, the majority of the animals had 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% versus 92%; $P < 0.05$) compared with control animals.

We further studied the long-term effects of anti-TNF therapy in this type of arthritis. IA 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 (Figures 3D–H). The joint inflammation persisted, and fibrotic synovial tissues started to appear after 14 days (Figure 3F). Radiologic signs of bone erosion were also observed in the joints on day 14 after IA injection of dead bacteria (Figure 3I). Anti-TNF therapy significantly reduced the severity of synovitis and tended to prevent bone destruction on both day 7 and day 14 (Figures 3D and G), indicating the crucial role of TNFα 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 days 7 and 14 after IA injection of dead bacteria (Figure 3J), suggesting that osteoclast activation occurs during the inflammatory process induced by antibiotic-killed *S aureus*.

Bacterial debris is the main cause of 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 (Figure 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 injected IA (Figure 4B). The synovitis frequency was only 20% after IA injection of bacterial DNA, compared with 80% and 79% for the bacterial debris and whole killed bacteria, respectively ($P < 0.01$).

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 (Figure 4C).

DISCUSSION

Despite advances in medical practice, permanent reduction of joint function is observed in ~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 demonstrated that cloxacillin-killed *S aureus* induced joint inflammation in a dose-dependent pattern. Importantly, the IA injection of dead bacteria led to long-lasting synovitis followed by

cartilage and bone destruction. Consistent with in vivo findings, splenocytes stimulated with antibiotic-killed *S aureus* released substantial amounts of proinflammatory cytokines.

Several 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 that of bacterial debris. IA injection of soluble peptidoglycan is known to induce arthritis, and enzymatic digestion by lysostaphin was shown to abrogate this inflammation (29). In the present study, antibiotic-killed *S aureus* digested with 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 TNFα pathway. In contrast, our results suggest that bacterial DNA from antibiotic-killed *S aureus* had very little effect on mediating arthritis, which is consistent with the finding that splenocytes do not produce inflammatory cytokines upon stimulation with bacterial DNA (data not shown). Bacterial debris appeared to be the most crucial component in our experimental setting. The components of a gram-positive bacterial cell wall are known to activate the innate immune system via TLR-2 (for peptidoglycan) or TLR-4 (for lipoteichoic acid) (12,31). TLR-2 deficiency partially reduced 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 after a relatively short period of 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 arthritis severity. The single depletion of neutrophils or monocytes did not affect severity of arthritis, whereas the depletion of both cells markedly abolished joint inflammation, indicating that cross-talk between neutrophils and monocytes is crucial for induction of this type of arthritis. The double depletion of neutrophils and monocytes was also shown to attenuate arthritis induced by IA injection of high mobility group box chromosomal protein 1 (HMGB-1) (23). Intriguingly, RAGE, a prototypic damage-associated molecular pattern molecule receptor that recognizes a variety of proinflammatory ligands generated during inflammation and infection, was also involved in induction of arthritis caused by antibiotic-killed *S aureus*. In addition to interacting with S100/calgranulin proteins, which have potent proinflammatory properties and are produced by neutrophils and macrophages (32), RAGE can interact with the β2 integrin Mac-1, which 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., TNFα and IL-6) (12,34). Through TNFRI, TNFα exerts biologic actions and up-regulates another potent cytokine, HMGB-1 (35). HMGB-1 released from inflammatory or necrotic cells in the inflamed joint has cytokine-inducing and chemotactic activity and can further exacerbate inflammatory responses through several receptors, such as RAGE, TLR-2, and TLR-4 (23,36). Indeed, we found that both the severity and the frequency of synovitis were significantly reduced in TNFRI$^{-/-}$ and RAGE$^{-/-}$ animals. In addition, arthritis severity was lower in TLR-2$^{-/-}$ mice than in their WT 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 there was no effect of IL-17A on arthritis induced by antibiotic-killed *S aureus*. Together, our data suggest that the signaling pathway in inflammation caused by antibiotic-killed *S aureus* is most likely mediated through the TLR-2–TNF–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.

A limitation of the 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 in NMRI mice are consistent with data obtained in TNFRI$^{-/-}$ mice on a C57BL/6 background, which suggests that involvement of the TNFα pathway in our model applies to different mouse strains. One observation is that cellular proliferation and levels of TNFα and IL-6 did not differ in splenocytes from TNFRI$^{-/-}$ and WT mice, which is inconsistent with the results of in vivo experiments using TNFRI$^{-/-}$ 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 lavage is to eliminate the bacterial components that trigger exaggerated host responses as

well as the proinflammatory cytokines and infiltrated immune cells that may cause joint damage. Use of glucocorticoids to target 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, compared with antibiotic monotherapy, the combination of antibiotics and a TNF inhibitor resulted in more rapid relief of clinical arthritis in mice with septic arthritis (19).

In the present study, we showed that antibiotic-killed *S aureus* might be a cause of long-lasting joint inflammation that may lead to postinfectious complications of *S aureus* septic arthritis. In septic arthritis, positive Gram staining and culture findings in the synovial samples become negative after a short period (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 TNF blockade in combination with efficient antibiotic treatment).

AUTHOR CONTRIBUTIONS

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

Study conception and design. Ali, Jin.

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