A murine *Staphylococcus aureus* fracture-related infection model characterized by fracture non-union, staphylococcal abscess communities, and myeloid-derived suppressor cells.

**Abstract:** Fracture-related infection (FRI) is a serious complication that can occur after surgical fixation of bone fractures. Affected patients may encounter delayed healing and functional limitations. Although it is well established that *Staphylococcus aureus* is the main causative pathogen of FRI, the pathophysiology of *S. aureus* FRI is not well characterized over time. Therefore, an experimental study in mice comparing *S. aureus*-inoculated and non-inoculated groups was performed that particularly focused on staphylococcal abscess communities (SACs) and host cellular response.

C57Bl/6N female mice received a double osteotomy of the femur, which was stabilized with a titanium 6-hole MouseFix locking plate and four screws. Animals were either *S. aureus*-inoculated or non-inoculated and euthanized between 1 and 28 d post-surgery. Histopathological evaluation showed normal bone healing for non-inoculated mice, whereas inoculated mice had no fracture consolidation and severe osteolysis. Within the bone marrow of inoculated mice, SACs were observed from 7 d, which increased in size and number over time. A fibrin pseudocapsule enclosed the SACs and they were surrounded by many Ly6G⁺ neutrophils with some Ly6C⁺ monocytes and F4/80⁺ macrophages, the majority of which were viable as indicated by immunohistochemistry. The abscesses were encapsulated by fibrin(ogen), collagen and myofibroblasts, with regulatory T cells and M2 macrophages at the periphery. Only bone marrow monocytes and...
neutrophils of inoculated mice displayed functional suppression of T cells, indicative of myeloid-derived suppressor cells (MDSCs). This study revealed that FRI in mice is persistent over time and associated with osteolysis, SAC formation, and an immunosuppressive environment.
Staphylococcus aureus fracture-related infection in mice is characterized by an immunosuppressive environment with staphylococcal abscess communities and myeloid-derived suppressor cells.

A murine Staphylococcus aureus fracture-related infection model characterized by fracture non-union, staphylococcal abscess communities, and myeloid-derived suppressor cells.


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Running title: SACs and MDSCs in mouse FRI
Abstract

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**Seventy-four** C57Bl/6N female mice received a double osteotomy of the femur, which was stabilized with a titanium 6-hole MouseFix locking plate and four screws. Animals were either *S. aureus*-inoculated or non-inoculated and euthanized between 1 and 28 d post-surgery. Histopathological evaluation showed normal bone healing for non-inoculated mice, whereas inoculated mice had no fracture consolidation and severe osteolysis. Within the bone marrow of inoculated mice, SACs were observed from 7 d, which increased in size and number over time. A fibrin pseudocapsule enclosed the SACs and they were surrounded by many Ly6G+ neutrophils with some Ly6C+ monocytes and F4/80+ macrophages, the majority of which were viable as indicated by immunohistochemistry. The abscesses were encapsulated by fibrin(ogen), collagen and myofibroblasts, with regulatory T cells and M2 macrophages at the periphery. Only bone marrow monocytes and neutrophils of inoculated mice displayed functional suppression of T cells, indicative of myeloid-derived suppressor cells (MDSCs).

This study revealed that FRI in mice is persistent over time and associated with osteolysis, SAC formation, and an immunosuppressive environment.

**Keywords:** *Staphylococcus aureus*, fracture-related infection, staphylococcal abscess community, myeloid-derived suppressor cell, immunosuppression.
**Introduction**

Fracture-related infection (FRI) is one of the most serious complications associated with surgical fixation of bone fractures. The rate of FRI in closed fractures ranges between 1-2%, whereas it can reach up to 30% for open fractures (Trampuz and Zimmerli, 2006). *Staphylococcus aureus* is the predominant causative pathogen in FRI, causing approximately 30% of cases (Trampuz and Zimmerli, 2006). Unfortunately, treatment efforts fail in 4 to 11% of cases, leading to further surgical revisions or lifelong suppression therapy or even amputation of the affected limb (Bezstarosti et al., 2019; Bose et al., 2015; Huh et al., 2011; Kanakaris et al., 2014). When specifically examining failure rates for FRI patients treated with implant retention, recurrence of infection varied from 0 to 14% for patients that had a revision surgery within 3 weeks of infection, 11 to 18% for patients that had a revision surgery within 3-10 weeks of infection, and 33% for patients that had a revision surgery after 10 weeks of infection (Morgenstern et al., 2021). Although early revision surgery does reduce the risk of recurrent infection, current treatment efforts are not ideal yet and may need to be combined with different strategies.

Traditionally, FRI has been classified as acute or chronic based upon time elapsed from initial surgery and symptom onset (Metsemakers et al., 2016). The distinction between acute and chronic FRI is also linked to the pathology pathophysiology and treatment (Metsemakers et al., 2016), however, this transition is poorly described in the literature and a clear time-related cut-off has never been scientifically defined (Morgenstern et al., 2021). In the early stages of acute FRI, the infiltrating immune cells are predominantly polymorphonuclear cells (PMNs), whilst in chronic FRI, the local inflammatory cells are predominantly macrophages (Klosterhalfen et al., 1996). Chronic FRI is also associated with osteolysis, sequestrum formation, and non-union (Alt et al., 2011; Metsemakers et al., 2016; Ochsner and Hailemariam, 2006).

One relatively recently described feature of the pathophysiology of *S. aureus* infection is the staphylococcal abscess community (SAC), which has been identified in renal and peritoneal tissue (Cheng et al., 2009; Cheng et al., 2010; Rauch et al., 2012), and also in bone marrow (Brandt et al., 2018; Farnsworth et al., 2017). SACs form the centre of abscesses and are surrounded by an amorphous pseudocapsule described to be comprised of fibrin deposits (Cheng et al., 2011; Cheng et al., 2010; Thomer et al., 2016b) formed by the action of the *S. aureus*-produced enzymes coagulase (Coa) and von Willebrand factor-binding protein (vWbp) (Bjerketorp et al., 2004; Friedrich et al., 2003; Thomer et al., 2016b). This pseudocapsule appears to serve as a barrier to invasion of immune cells, causing them to accumulate in high numbers at the periphery, leading to persistence of *S. aureus* (Brandt et al., 2018; Cheng et al., 2010; Hofstee et al., 2020a; Kobayashi et al., 2015; Thomer et al., 2016b).

Persistence of *S. aureus* has also been linked to myeloid-derived suppressor cells (MDSCs), which can inhibit proinflammatory responses of monocytes/macrophages, and has been mainly studied in mice (Heim et al., 2015a; Heim et al., 2018; Heim et al., 2015b; Heim et al., 2014; Peng et al., 2017). In mice, monocytic MDSCs are CD11b+ Ly6C<sup>high</sup> Ly6G<sup>+</sup>, whereas granulocytic MDSCs are CD11b<sup>-</sup>Ly6C<sup>low</sup>Ly6G<sup>+</sup>. Regular murine monocytes and neutrophils have the same phenotypical markers as monocytic and granulocytic MDSCs, which makes identifying murine MDSCs with immunohistochemical stains challenging. What sets monocytic and granulocytic MDSCs aside from regular monocytes and neutrophils is their...
immunosuppressive abilities, of these cells which are facilitated by the enzymes inducible nitric oxide synthase (iNOS) and/or arginase-1 (Arg-1) (Ostrand-Rosenberg and Fenselau, 2018; Veglia et al., 2018) and usually proven in functional T cell suppression assays (Bronte et al., 2016). For *S. aureus* infections, whether the MDSCs are distributed amongst the bone marrow or arranged in an organised manner is unknown at the present time. The aim of this study was to examine SAC formation in a murine FRI model, and investigate whether MDSCs are present within bone marrow of these mice and possibly are associated with SACs and/or abscess structures. Furthermore, the aim was to identify the host macromolecules associated with SAC formation including fibrinogen and collagen. A femoral double osteotomy mouse model was used where a femoral bone segment was created that was either not inoculated or inoculated with *S. aureus*, and the femur was stabilized with a titanium 6-hole MouseFix locking plate and animals were either inoculated with *S. aureus* or non-inoculated. Immuno(fluorescent) stainings, the histopathological osteomyelitis evaluation score (HOES) of Tiemann and colleagues (Tiemann et al., 2014), and ex vivo analyses were applied to investigate differences between FRI inoculated and non-inoculated mice, with a particular focus on the maturation of SACs over time and possible immunosuppressive properties of the involved host cells.

**Methods**

**Animals**

All animal experiments were approved by the ethical committee of the canton of Graubünden in Switzerland (approval number 2017_28 and 2019_10) and were carried out in an Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC) International accredited facility. In this study, 74 specific pathogen-free (SPF) C57Bl/6N female mice (Charles River, Sulzland, Germany) of 20 to 28 weeks old at the time of surgery were used. Animals were acclimatized for at least 2 weeks prior to the surgical intervention. Mice were divided randomly into *S. aureus* inoculated and non-inoculated groups. Timepoints for sacrifice were 1, 3, 7, 14, 21, and 28 d post-operative (*n* = 6 per subgroup) for inoculated mice and 3, 14 and 28 d (*n* = 6 per subgroup) for non-inoculated animals used for histological outcome parameters. Mice for colony forming unit (CFU) quantification and ex vivo cultures were sacrificed 21 d post-operative for both the inoculated group (*n* = 6) and non-inoculated group (*n* = 6). Cages were randomly assigned to the different subgroups. Individual ventilated cages (IVC; Techniplast, Schwerzenbach, Switzerland and Allentown, Schlieren, Switzerland) housed 2 to 6 mice per cage and were under 12 h light/dark cycle and enriched with a plastic house, paper, and wood for gnawing. Both water and food (3436, Provimi Kliba AG, Kaiseraugst, Switzerland) were provided *ad libitum*.

**Bacteria and inoculum preparation**

The bacterium *S. aureus* JAR 06.01.31 (the culture collection of Switzerland (CCOS) number 890, Wädenswill, Switzerland), obtained from a patient with an orthopaedic device-related infection (Moriarty et al., 2010), was used for inoculum preparation. An overnight culture was prepared the day before surgery by incubating one colony in 50 mL tryptic soy broth (TSB; Oxoid, Basel, Switzerland) at 37 °C while shaking. From the overnight culture, a fresh logarithmic phase culture was prepared in TSB. Bacteria were washed twice with phosphate buffered saline (PBS; Gibco, Basel, Switzerland) 1.5 h before surgery and diluted in PBS to
obtain a 1 µL inoculum containing approximately $1 \times 10^4$ CFU. One microliter sterile PBS was used as control.

**Surgery and animal welfare**

Prior to surgery, animals were anesthetized by induction with sevoflurane (ca. 5 % in O$_2$, flow rate 1 L/min; Sevoflurane Baxter, Baxter AG, Opfikon Switzerland). During surgery the anaesthesia was maintained with sevoflurane (ca. 2-3 % in O$_2$, flow rate 0.6-1 L/min).

Intraoperative analgesia consisted of buprenorphine (0.1 mg/kg s.c.; Bupaq, Streuli Pharma AG, Uznach, Switzerland) and carprofen (5 mg/kg s.c.; Norocarp, ufamed AG, Sursee, Switzerland) injected immediately after anaesthetic induction. After clipping and aseptically preparing the surgical field, a skin incision was made from tail base to the left stifle. The subcutaneous fascia lata was cut and the level between the quadriceps and biceps femoris muscle was bluntly dissected. The left femur was stabilized with a titanium 6-hole MouseFix locking plate (RISystems AG, Davos Platz, Switzerland) and 4-outermost screws were inserted. The 2-inner screw-holes were left empty and used to generate 2 osteotomies with the MouseFix Drill-&Saw guide (RISystems) and a Gigly hand saw (RISystems, 0.22 mm diameter). This created a 2 mm femoral segment. This segment was taken out and 1 µL of either the bacterial inoculum or sterile PBS was pipetted on top of the bone marrow of the segment. The inoculum or saline could absorb into the bone for 3 min before the segment was placed back into its original place without fixation. The fascia lata and the skin were closed with continuous sutures (5-0 Vicryl rapide, Ethicon, Courcelles, Belgium). Post-operative analgesia consisted of buprenorphine (0.1 mg/kg s.c.) every 4-10 h for 24 h as well as paracetamol (1.9 mg/mL) added to the drinking water for 7 d (Dafalgan Sirup for Children, Bristol-Myers Squibb SA, Steinhausen, Switzerland). Post-operative radiographs were taken to confirm that the osteotomy and plate position were correct and to check for fractures. The wellbeing of mice was checked daily by an animal caretaker and were scored twice a day until 5 d post-operative, followed by daily scoring up to 7 d and twice per week thereafter. The animals were monitored for their general and eating behaviour, and the load they placed on the operated leg. The external appearance and wound status were also monitored. The weight was monitored 3 and 7 d post-operative and weekly thereafter. Mice were sacrificed by first inducing general anaesthesia with sevoflurane followed by cervical dislocation.

**Fixation, sectioning, and stains**

Some of the left femoral bones ($n = 24$) were fixed in 70 % methanol immediately after euthanasia. The fixed samples were embedded in methyl methacrylate (MMA; Sigma-Aldrich, Buchs, Switzerland) with the implant left in place. Sections (200 µm) were made using a Leica 1600 rotating saw microtome (Leica Microsystems, Heerbrugg, Switzerland), rehydrated with 1-Acetoxy-2-methoxyethane and ethanol gradient after which the sections were stained with 15% (v/v) Giemsa and 1 % (v/v) eosin (G&E; both Sigma-Aldrich). Images were taken with an Olympus BX63 brightfield microscope (Olympus, Tokio, Japan).

For paraffin embedded samples ($n = 26$), left femora were fixed with formalin and subsequently decalcified with 12.5 % (w/v) ethylenediaminetetraacetic acid (EDTA; Roth AG, Arlesheim, Switzerland) and 1.25 % (w/v) sodium hydroxide (Sigma-Aldrich,) decalcification solution. Implants were then removed, and the remaining bone and soft tissue was embedded in paraffin (Leica Microsystems). Paraffin sections (5 µm) were cut with a paraffin HM 355 S microtome (Microm, ThermoFisher Scientific, Waltham, MA, USA), deparaffinized and rehydrated through a descending ethanol gradient. Sections were either stained with Mayer’s
haematoxylin and 1 % (v/v) eosin (H&E; both Sigma-Aldrich), Brown and Brenn (BB) (Brown and Brenn, 1931), a TUNEL assay to detect apoptotic cells (Abcam, Cambridge, United Kingdom; #ab206386, manufacturer’s protocol was used) Picro-Mallory, Picro-Sirius red, or immune(fluorescent) stains. For more details on the last three stains, see below.

**Picro-Mallory trichrome stain**

To allow the acid dyes to stain more intensely, sections were post-fixed with Bouin’s fluid (9 % (v/v) formaldehyde and 5 % (v/v) acetic acid in water saturated picric acid (0.9 % (v/v))) overnight at room temperature (RT). Sections were then rinsed in distilled water to remove the Bouin’s fluid. Weigert’s iron haematoxylin (Sigma-Aldrich) prepared following manufacturer’s instructions was applied for 20 min to stain nuclei, and samples were stained with a Picro-Mallory trichrome stain following a previously described protocol (Lendrum et al., 1962).

**Picro-Sirius red stain**

Sections were stained with Weigert’s iron haematoxylin for 8 min and differentiated in lukewarm running tap water. Then sections were placed into the Picro-Sirius Red solution (0.1 % (w/v) Sirius red F3B (Sigma-Aldrich) in water saturated picric acid) for 1 h. Samples were washed two times with 1 % (v/v) aqueous acidified acid and most of the water was removed by shaking the sections.

**Immuno(fluorescent) stains**

An antigen retrieval step was performed by boiling the sections in a sodium citrate buffer (0.1 M, pH 6.0; Sigma-Aldrich) for 15 min. Endogenous peroxidase was blocked by incubation for 30 min in methanol with hydrogen peroxide (0.3 % (v/v) H₂O₂ in absolute methanol) for samples subsequently stained with a peroxidase linked antibody. Samples stained with fluorescently labelled secondary antibodies were incubated for 5 min in absolute methanol. Sections were washed twice in PBS with 0.1 % (v/v) Tween 20 (PBS-T; both Sigma-Aldrich) and blocked by incubation with 5 % (v/v) animal-free blocker (Vector Laboratories, Burlingame, CA, USA) in PBS-T for 1 h at RT in a moist chamber. Blocking buffer was removed and sections were incubated overnight at 4 °C with primary antibodies in PBS-T with 0.5 % (v/v) animal-free blocker. Primary antibodies were omitted for negative control sections. See Table 1 for the different primary antibody combinations, concentrations, targets, and their matching secondary antibodies. Secondary antibodies were applied after overnight incubation with the primary antibodies. Samples were washed three times with PBS-T before each incubation step. All secondary antibodies were diluted 1:200 in PBS-T with 0.5 % (v/v) animal-free blocker and were incubated for 30 min at RT in the dark. Lastly, sections were incubated with Trueview and coverslipped with Vectashield vibration antifading mounting medium with DAPI (both Vector Laboratories) according to manufacturer’s instructions (Table 1; staining 1-6) or samples were incubated with ABC complex and ImmPACT DAB solution (both Vector Laboratories) following the manufacturer’s instructions and counterstained with Mayer’s haematoxylin (Table 1; staining 7-8).

Non-fluorescently stained paraffin samples were mounted with Eukitt mounting medium (Sigma-Aldrich) and imaged with an Olympus BX63 microscope (Olympus). Immunofluorescent stained samples were examined using a Zeiss LSM 800 inverse
fluorescence microscope and image processing was performed with the ZEN (blue edition) software (both Zeiss, Oberkochen, Germany).

**Histopathological osteomyelitis evaluation score**

G&E stained MMA embedded femurs samples \((n = 2 \text{ or } 3)\) with an of inoculated or non-inoculated bone segment mice were evaluated by one pathologist using the histopathological osteomyelitis evaluation score (HOES) (Tiemann et al., 2014). This score evaluates three acute osteomyelitis features (A1-3) and two chronic osteomyelitis features (C1-2). Osseonecrosis (A1), soft tissue necrosis (A2) and granulocyte infiltration (A3) were scored as indicators for acute osteomyelitis, whereas bone neogenesis/fibrosis (C1) and lymphocyte/macrophage infiltration (C2) were evaluated as features of chronic osteomyelitis. In this study the 5 above mentioned parameters were scored from 0 to 5 (Table 2). The HOES score was not applied for non-inoculated animals since this score focusses on osteomyelitis features. However, HOES score markers that were observed in non-inoculated animals are granulocyte infiltration in early samples and bone neogenesis in late samples.

**Staphylococcal abscess community size**

Images of SACs in paraffin or MMA embedded samples were taken with the Zeiss Axio Vert.A1 microscope (Zeiss). SAC number was determined for one section per mice and SAC size was established by measuring horizontal and vertical length of SACs with the measuring tool from the AxioVision SE64 software (Zeiss) and calculating the surface area (in mm\(^2\)) of the SACs.

**Bone marrow cell sorting**

Soft tissue and implants were removed from the operated femurs of inoculated \((n = 5)\) and non-inoculated mice \((n = 5)\) and used for CFU quantification. The outer ends of the femoral bones were cut off and bone marrow cells were flushed out with a 24G × 1 needle attached to a 2 mL syringe containing hank’s buffered salt solution (HBSS; Gibco). The collected cells were passed through a 70 µm cell strainer and centrifuged at 300 × g for 6 min at 4 °C. Bone marrow supernatant was collected for CFU quantification, and the cell pellet was incubated with 5 mL red blood cell lysis buffer (15 mM NH\(_4\)Cl, 1 µM KHCO\(_3\) (both Sigma-Aldrich) and 10 µM EDTA) for 5 min at RT after which 25 mL Roswell Park Memorial Institute (RPMI; Gibco) medium supplemented with 3 % (v/v) fetal bovine serum (FBS; Sigma-Aldrich) and cells were centrifuged at 300 × g for 6 min at 4 °C. In preparation of the fluorescence-activated cell sorting (FACS) staining, cells were washed with 10 mL of sorting buffer (HBSS with 0.5 % (w/v) bovine serum albumin (Sigma-Aldrich) and 2 mM EDTA). After centrifugating the suspension at 300 × g for 6 min at 4 °C and discarding supernatant, cells were incubated with FC-receptor blocking reagent (BD Bioscience, Allschwil, Switzerland) for 10 min at 4 °C. Subsequently, antibody mixtures containing anti-CD11b-APC, anti Ly6G-FITC, and anti Ly6C-PE (all Biolegend, Fell, Germany) were added to the samples and let to incubate for 30 min at 4 °C. Samples were washed twice with sorting buffer, resuspended in sorting buffer, passed through a 70 µm cell strainer and DAPI (Sigma-Aldrich) was added to stain dead cells. Sorting was performed with a BD FACSDiva (BD Bioscience) using a 100 µm nozzle and gating for CD11b\(^+\) Ly6C\(^-\) Ly6G\(^-\) and CD11b\(^+\) Ly6C\(^-\) Ly6G\(^-\) cell populations. Cells were sorted with an efficiency above 86 % and purified cells were kept on ice until further use.

**T cell suppression assay**
Splenocytes were isolated from spleens of non-inoculated mice by pushing the spleens through a 70 µm cell strainer placed on a 50 mL tube with the plunger of a 5 mL syringe. The cell strainer was washed with 5 mL RPMI with 3 % FBS, the cell suspension was centrifuged at 300 × g for 6 min at 4 °C, supernatant was discarded and red blood cells within the cell pellet were lysed with 5 mL lysis buffer for 4 min at RT. After adding 25 mL RPMI with 10 % FBS, cells were spun down at 300 × g for 6 min at 4 °C, washed with 10 mL RPMI with 10 % (v/v) FBS, centrifuged at 300 × g for 6 min at 4 °C and resuspended in 20 mL RPMI with 10 % FBS. The splenocyte suspension was filtered with a cell strainer and stained with the membrane dye PKH26 (1.2 µL per 1 × 10⁷ splenocytes; Sigma-Aldrich) using manufacturer’s instructions. Suppression of T cell proliferation was assessed by co-culturing 0.5 × 10⁵ PKH26-stained splenocytes/96-well plate well with previously sorted CD11b⁺ Ly6C⁺ Ly6G⁻ or CD11b⁺Ly6C⁺ Ly6G⁺ bone marrow cells from inoculated or non-inoculated mice in RPMI supplemented with 10 % FBS, 1 % (v/v) penicillin/streptomycin solution (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), and 50 µM β-mercaptoethanol (Roth). Splenocytes were stimulated with murine CD3/CD28 Dynabeads (1:1 ratio with splenocytes; ThermoFisher Scientific) and 30 U/mL murine rIL-2 (Peprotech, London, United Kingdom). Cells from non-inoculated mice were co-cultured 1:1, whereas cells from inoculated mice were co-cultured 1:1 and 0.5:1 with PKH26-stained splenocytes. The co-cultures were incubated for 4 d at 37 °C with 5 % CO₂ after which T cell proliferation was assessed by flow cytometry with CD3-FITC, CD4-Alexa Fluor 700, and CD8α-APC antibodies (all Biolegend) and DAPI to indicate dead cells. Proliferation was normalized to T cells stimulated with CD3/CD28 Dynabeads and rIL-2, which were set to 100 %. Flow cytometric data was analysed with Kaluza Analysis Software (Beckman Coulter Life Sciences, Indianapolis, IN, USA).

CFU quantification

CFU were quantified for soft tissue, bone, bone marrow supernatant and implant. Soft tissue or femoral bones were placed into 10 mL PBS and homogenized mechanically using Omni Tissue Homogenizer and Hard Tissue Homogenizing tips, respectively (both Omni International, Kennesaw, GA, USA). Implants were sonicated with 40 kHz for 3 min in 5 mL of PBS in an ultrasonic bath (Bandelin Sonorex, Berlin, Germany). Serial dilutions were made from all 4 type of samples per mouse and 10 µL of these dilutions were plated in triplicate on 5 % horse blood agar plates (Oxoid). Plates were incubated at 37 °C and CFUs were determined after 24 h and 48 h.

Data analysis

Data was analysed and visualized with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Normality of data was checked with a Shapiro-Wilk test and by visually assessing QQ plots from the data. Parametric data was assessed with a Sidak multiple comparison test or a two-way ANOVA, whereas non-parametric data was examined with a Dunn’s multiple comparison test. p-values of <0.05 were considered statistically significant.

Results

Clinical evaluation and animal welfare

Of the seventy-four operated mice, sixty mice were included in the final bacteriological and histological evaluation. Fourteen additional mice were operated but sacrificed before study
Radiographic and histopathological evaluation

The double femoral osteotomy model generated a 2 mm bone segment. At 3 d, non-inoculated mice did not show any signs of bone healing between the bone segment and the adjacent femoral bone (Figure 1A-B). By 14 d, some early signs of healing were observed (Figure 1C) with newly formed woven bone or soft callus, characterized by an irregular, not condensed appearance of the bone (Figure 1D; red arrow). Non-inoculated animals had complete healing of the femur revealed by complete bridging between the segment and adjacent bone after 28 d, although the newly formed bone was not yet compact (Figure 1E-F). At this time, some osteoclasts were present and remodelling of the callus had commenced (Figure 1F; black arrow). Bone marrow was observed within the newly formed bone (Figure 1F; yellow arrow). Inoculated animals displayed markedly different appearance, although at the earliest timepoint, 3 d, radiographic images were not significantly different from those of non-inoculated mice (Figure 1G). At this time, the bone marrow contained small bacterial aggregates, without any marked infiltration of immune cells (Figure 1H; arrowhead). By 14 d, there were no signs of bone healing (Figure 1I) and the bacterial colonies were still present (Figure 1J; arrowhead). Early signs of osteolysis were revealed by the irregularly edged bone (Figure 1J; black arrow). Some newly formed bone, which was not yet compact, was also present (Figure 1J; red arrow). In contrast to non-inoculated mice, no fracture consolidation was visible in the inoculated animals after 28 d and their osteotomy ends were irregularly shaped suggestive of osteolysis (Figure 1K). Bacterial aggregates and remnants of osteoclast activity were detected (Figure 1L; arrowhead, and black arrow, respectively). Newly formed bone that was loosely arranged could also be observed in inoculated mice 28 d post-operatively (Figure 1L; red arrow) but this was minor compared to non-inoculated mice and severe bone osteolysis prevailed.

Additionally, samples of non-inoculated and inoculated mice were evaluated by scoring for bone infection-related features using HOES (Tiemann et al., 2014) (Figure 2A) and immunostains were performed to depict the presence of neutrophils, fibrinogen, and macrophages in the distal part of the bone segment (Figure 2B-C). Non-inoculated mice had a low degree of osseonecrosis at 3 d, 14 d, and 28 d. Soft tissue necrosis was only observed at 3 d, whereas granulocytes were present in bone and surrounding tissue at 3 d and 14 d. A fibrin clot within the osteotomy gap was observed at 3 d, which evolved in fibrous tissue. Both the presence of fibrous tissue and bone neogenesis was noted from 14 d on, while lymphocytes and macrophages were present at all time points. Non-inoculated mice did not have acute osteomyelitis according to the HOES scoring system (i.e. the sum of osseonecrosis, soft tissue necrosis, and granulocyte infiltrate scores was lower than 6).
In inoculated mice, from 1 to 28 d, osseonecrosis was observed in the bone segment and adjacent femoral bone (Figure 2; white bars). The soft tissue above the implant became necrotic at 3 d and this increased at later time points. High numbers of granulocytic cells were located within muscle and bone tissue at all time points together with many fibrin(ogen) fibres. Regeneration started from 7 d and continued until 28 d, indicated by bone neogenesis and fibrous tissue formation. Aside from granulocytes, Furthermore, from 7 d on, lymphocytes and macrophages also began infiltrating into bone and soft tissue from 7 d onwards. From 14 d, animals no longer had an acute infection, but had transitioned to what is considered a chronically florid/active infection according to the HOES score (sum of all features was higher than 9). At later time points, the mice had still not transitioned to Animals did not have a chronic infection but a still active infection according to the HOES scoring system (i.e., since the sum score of bone neogenesis/fibrosis, and lymphocyte and macrophage infiltration was lower than 6); due to the consistent presence of granulocytes even after 28 d of infection; and because signs of only minor signs of bone regeneration were observed.

Staphylococcal abscess communities

In inoculated animals, abscess structures were observed within bone marrow from 3 d onwards (Figure 3A). In addition to the numerous abscesses observed, some biofilm on screws and implant was also present (data not shown). At the centre of the abscess, SACs were observed that appeared as dense blue-staining structures in G&E stained sections (Figure 3A, white arrows). From 14 d onwards, all inoculated animals had SACs, with significantly more SACs at 21 and 28 d compared to 1 d (Figure 3B). SAC size significantly increased from 1 d to 14 and 21 d (Figure 3C). Examination of a SAC and abscess in more detail showed that the centre of a SAC contained Gram-positive bacteria (Figure 3D; blue). These bacteria were covered with a pseudocapsule consisting of fibrin fibres (Figure 3E; magenta). Around the SAC (Figure 3F, asterisk) many leukocytes were present (Figure 3F, arrowheads), which, together with the SAC, were mostly alive (Figure 3G; green) but also a rim of apoptotic cells was observed that did not border the SAC (Figure 3G; brown). The SAC and its surrounding cells were encapsulated by layers of oriented but not consolidated collagen fibres (Figure 3H; red) and fibrin(ogen) strands (Figure 3I; red), and by alpha-smooth muscle actin (αSMA) positive myofibroblasts (Figure 3I; turquoise). Together, the SAC, adjacent immune cells, and encapsulation formed the complete structure of what we name an abscess in the text below.

Immune cell infiltration towards staphylococcal abscess communities

To investigate which immune cells are associated with early and late S. aureus aggregates / SACs present during acute or chronically florid infection, immunostainings with markers for different immune cells were performed at 7 d and 21 d, respectively. Bone marrow after 7 d of infection (overview in Figure 4A) contained a small number of S. aureus aggregates with few bacteria per aggregate together with some Ly6G positive neutrophils and F4/80 positive macrophages (Figure 4B). Successive sections showed that some Ly6C positive monocytes and CD11b positive myeloid cells were also dispersed around the same area of the S. aureus aggregates (Figure 4C). At this time, there were no iNOS, or Arg-1 expressing cells (Figure 4D) nor FoxP3 positive regulatory T cells (Tregs) were observed (Figure 4E), and no organised structures were seen.
By 21 d, clearly defined abscess structures were observed in the bone marrow (Figure 4F) with a central SAC staining positive for *S. aureus* (Figure 4G; edge of the SAC is depicted). The SAC was surrounded by highly Ly6G positive neutrophils and a small number of F4/80 positive macrophages at the border (Figure 4G; white arrowheads). Some cells within the abscess were positive for the monocyte marker Ly6C and a leukocyte marker CD11b (Figure 4H). iNOS and Arg-1 positive cells at the border of the abscess formed into somewhat organised arrangements within the abscess (Figure 4I; white arrows). FoxP3 positive Tregs cells were present outside the abscess (Figure 4J).

To further probe the cell populations within the abscess after 21 d of infection, and particularly to investigate neutrophil, monocyte and macrophage cell surface markers as well as the enzymes iNOS and Arg-1 used by macrophages but also MDSCs, further high magnification images were taken (Figure 5). Figure 5A-B shows the Ly6G neutrophil marker in combination with iNOS and Arg-1. The neutrophils (Ly6G) in close proximity to the SAC at the centre of the abscess expressed Arg-1 but not iNOS (Figure 5A and B). H&E staining of the same section confirmed these locations contained neutrophils with either banded or partially segmented nuclei (Figure 5C; white arrows). These neutrophils appeared intact and alive.

Co-localisation of iNOS and Arg-1 with Ly6C, a monocyte marker, is shown in Figure 5D-E. Highly Ly6C positive cells were observed at the border of the abscess where the stain co-localized with both iNOS and Arg-1 stain (Figure 5D and E). Such iNOS and Arg-1 positive monocytic cells were not present at the centre of the abscess, near the SAC. H&E staining showed cells with morphological characteristics of monocytes, in the same areas at the edge of the abscess where the Ly6C, iNOS and Arg-1 positive cells were located (Figure 5F; black arrows).

Co-localisation of iNOS and Arg-1 with F4/80, a macrophage marker, is shown in Figure 5G-H. Few F4/80 positive macrophages were observed, but those present were generally seen at the periphery of the abscess and were strongly Arg-1 positive and expressed iNOS to a lesser extent as well (Figure 5G-H), indicative of an anti-inflammatory, M2 phenotype. The H&E stain also confirmed the presence of macrophages at this location (Figure 5I; white arrowhead). F4/80 and Arg-1 expressing M2 macrophages were observed outside at the periphery of the abscess as well.

**T cell suppression by CD11b+Ly6C+Ly6G− or CD11b+Ly6C−Ly6G+ bone marrow cells**

To assess whether monocytes and/or neutrophils from non-inoculated or inoculated mice had immune suppressive capabilities, CD11b+Ly6C+Ly6G− monocytes or CD11b+Ly6C−Ly6G+ neutrophils were purified from non-inoculated bone marrow or bone marrow with many abscesses and SACs. CFU quantifications showed that non-inoculated mice did not have any detectable viable bacteria within soft tissue, bone, and bone marrow samples or on implants after 21 d (Figure 6A). In contrast, the samples from inoculated mice contained high numbers of bacteria varying from 5 to 6.5 log10 total CFU, demonstrating that the inoculated animals were indeed infected after 21 d.

There was a significant difference in percentage of monocytic CD11b+Ly6C+Ly6G− cells and neutrophilic CD11b+Ly6C−Ly6G− cells from the total bone marrow cell population between inoculated mice and non-inoculated mice (Figure 6B).

A T cell suppression assay was performed with the purified monocytic CD11b+Ly6C+Ly6G− cells or neutrophilic CD11b+Ly6C−Ly6G− cells to detect possible suppressive capabilities. When examining the effects on the total CD3+ CD4+ T cell fraction, it was observed that
monocytic CD11b+ Ly6C+ Ly6G+ cells from inoculated mice strongly suppressed T cell proliferation in a ratio-dependent manner, whereas the same cell type from non-inoculated mice did not suppress T cell proliferation (Figure 6C). Neutrophilic CD11b+ Ly6C+ Ly6G+ cells from non-inoculated mice also did not have suppressive activity towards CD3+ CD4+ T cells. However, neutrophilic CD11b+ Ly6C+ Ly6G+ cells from inoculated mice significantly suppressed CD3+ CD4+ T cell proliferation (Figure 6C). Similar results were observed for CD3+ CD8α+ T cell co-cultured with monocyte or neutrophil fractions from non-inoculated or inoculated mice, although neutrophilic cells from inoculated mice suppressed CD3+ CD8α+ T cells less than monocytic cells (Figure 6D).

Discussion

To get a better understanding of the pathophysiology of FRI, the histopathological changes occurring in a during FRI mouse model were examined over time was examined in a mouse model and compared to those changes with the normal fracture healing processes in non-inoculated mice. The infection in this model progressed into an active, a so-called chronically florid infection, with constant granulocytes presence, severe osteolysis of the operated femur, and with many abscesses containing SACs within the bone marrow of the osteotomy segment and adjacent femoral bone. As shown here, the SACs were covered by a fibrin pseudocapsule surrounded by neutrophils and by an early encapsulation comprised of fibrinogen and collagen. Furthermore, the S. aureus infection led to functionally suppressive monocytes and neutrophils, and immunohistochemistry revealed the presence of many neutrophils and a few monocytes, anti-inflammatory macrophages (M2), and Tregs.

The most obvious feature of the S. aureus infection in the histological sections were the S. aureus aggregates, or immature SACs, which were present in bone marrow of inoculated mice after 3 d. By inoculating the bacteria directly into the bone marrow of the double osteotomy-induced bone segment, we preferentially seeded a deep osteomyelitis with high chances of SAC formation, with relatively less chance for an implant-related biofilm infection. The SACs and abscesses surrounding them evolved into more distinct structures over time. Once matured, the abscesses appeared to be encapsulated by myofibroblasts with collagen and fibrin(ogen) fibres possibly as an attempt by the host to contain the infection (Kobayashi et al., 2011; Thomer et al., 2016b) and shield it off from neighbouring, healthy tissue. The SACs themselves were surrounded by a fibrin pseudocapsule, which is largely formed by the action of S. aureus within the SAC. S. aureus is known to bind fibrin(ogen) using proteins such as clumping factor A (ClfA), extracellular matrix protein (Emp), and extracellular adherence protein (Eap) (Chavakis et al., 2005; Foster et al., 2014). Furthermore, it has the ability to activate host prothrombin with Coa and vWbp to convert fibrinogen into fibrin (Bjerketorp et al., 2004; Friedrich et al., 2003; Thomer et al., 2016b). Others have suggested that the pseudocapsule around SACs contains fibrin (Cheng et al., 2011; Thomer et al., 2016b), which we confirmed in this study using a histochemical stain for fibrin. Immunostainings with an anti-fibrin(ogen) antibody did not stain the pseudocapsule directly around SACs in this study and previous studies (Cheng et al., 2010; Farnsworth et al., 2017). However, the fibrin pseudocapsule did stain positive for fibrin when using the histochemical Picro-Mallory trichrome stain (Figure 3E). The Picro-Mallory stain does not depend on antigen binding sites, but rather, by combining three different dyes and two staining differentiators, the acid fuchsin stain is retained specifically in fibrin (Lendrum et al., 1962). The fibrin pseudocapsule of an in vitro SAC also stained magenta, indicative for fibrin, when the Picro-Mallory stain was applied.
Although others have described most of the neutrophils within an S. aureus-related abscess to be dead (Cheng et al., 2011; Thomer et al., 2016b) and, normally, neutrophils are considered to be short-lived (Nicolás-Ávila et al., 2017), we believe the neutrophils surrounding the SAC in our mouse model to be alive, since they did not stain positive with the TUNEL assay and showed morphological features of live cells. Recent literature supports our observation of neutrophils potentially being long lived under certain conditions, such as — it has been stated that neutrophils may have a prolonged lifespan under certain inflammatory conditions. Moreover, they and are now believed to be more diverse in their capabilities, e.g. hyper proinflammatory or immunsuppressive, than previously thought (Nicolás-Ávila et al., 2017). Furthermore, microbiota-derived endotoxins and other molecules such as peptidoglycans can influence neutrophil aging and can prime neutrophil functions to be effective against the infection (Nicolás-Ávila et al., 2017). The bacteria within a SAC might secrete similar factors, which prolong the lifespan of the neutrophils close to the SAC and modulate them to have specific immunomodulatory capabilities, however, this remains unknown at the present time. The TUNEL assay in general identifies apoptotic cells and possibly also necroptotic cells and gave a few positive stained cells within the abscess but not bordering the SAC. It would be interesting to examine whether other types of cell death e.g. pyroptosis, autophagy or NETosis
(Dąbrowska et al., 2019) occur within an abscess to further elucidate the vitality and death of neutrophils associated with abscesses.

Fourteen mice in total were excluded from further analysis in this study, 12 of which were due to a fracture. The mouse model described in this study is complex and correct placement of the plate and screws is crucial. Consequently, when done inappropriately, there may be mechanical failures in the early post-operative period, which are not related to infection. At later time point when the infection progressed, there is a risk of screw and implant instability, and thus fractures, due to infection-induced osteolysis.

MDSCs were found in tissue surrounding the implant (Heim et al., 2015). The presence of these cells is in line with the development of persistent infection, since suppressive immune responses will interfere with clearance of infection (Heim et al., 2015a).

MDSCs suppress other immune cells by upregulating iNOS and/or Arg-1, and releasing molecules including reactive oxygen species (ROS), IL-10, TGF-β, and prostaglandin E2 (PGE2) to promote e.g. Treg and regulatory B cell (Breg) induction (Ostrand-Rosenberg and Fenselau, 2018; Veglia et al., 2018). At the border of the abscesses we identified Ly6C-monocytes simultaneously expressing iNOS and Arg-1, a feature observed only for monocytic MDSCs (Damuzzo et al., 2015). Interestingly, to the best of our knowledge, monocytes being part of an abscess has not been described to date. Furthermore, the Ly6G-neutrophils around the SAC expressed Arg-1, not a characteristic of neutrophils per se, but certainly of granulocytic MDSCs (Veglia et al., 2018). Cells positive for the Arg-1 protein have recently been reported the be present close to SACs as determined with spatially-targeted proteomics (Guiberson et al., 2020). Possibly, the neutrophils around the SAC and within the abscess and the monocytes at the outer rim of the abscess are MDSCs and have suppressive capabilities through Arg-1 or both Arg-1 and iNOS, respectively. This would explain the presence of FoxP3 positive Tregs and M2 macrophages around the abscess. More ex vivo/ in vitro investigations are required to assess the immunosuppressive capabilities of these cells specifically.

Additionally, it may be that the neutrophils and monocytes together with the M2 macrophages within the abscess initiate encapsulation by secreting high levels of TGF-β, a pro-fibrotic factor, which upregulates genes involved in extracellular matrix production by fibroblasts and myofibroblasts (Leask and Abraham, 2004). This would also be an interesting future direction of research.

To lower bacterial burden and prevent persistent infection in FRI, an option would be to target the MDSCs by using an antibody therapy against Ly6G, as performed in a murine femoral S. aureus orthopaedic biofilm infection model (Heim et al., 2014). This therapy decreased granulocytic MDSC levels and subsequently decreased bacterial loads in tissue surrounding the implant and the knee joint (Heim et al., 2014). Local application of anti-MDSC therapy may be key for this treatment approach (Tebartz et al., 2015).
Normally, and indeed observed in the non-inoculated mice (data not shown), a shift from proinflammatory, M1, towards anti-inflammatory, M2 macrophages occurs during fracture healing; the first 3 d after fracture M1 macrophages predominate, whereas by 7 d M2 macrophages prevail (Schlundt et al., 2018; Wasnik et al., 2018). However, in our study inoculated animals had minimal numbers of M1 macrophages and mostly M2 macrophages in tissue after 7 d. It has been shown that S. aureus biofilm can skew macrophages to an M2 phenotype in vivo (Thurlow et al., 2011). It has been proposed that this happens because toll-like receptor (TLR) ligands on S. aureus are not accessible for macrophages when S. aureus is within a biofilm (Thurlow et al., 2011). A similar process may occur for SACs, which are effectively sealed off from host cells by the fibrin pseudocapsule. When preventing S. aureus from growing in a fibrin pseudocapsule by one of the treatment strategies mentioned above, TLR ligands might be more exposed and macrophages might stay in a proinflammatory state instead of being skewed towards an anti-inflammatory state. Another interesting treatment option for FRI is metabolically reprogramming monocytes/macrophages to become proinflammatory with oligomycin nanoparticles as was shown to be effective in a mouse model of S. aureus PJI (Yamada et al., 2020). In addition to changing the metabolic state of monocytes, this therapy also indirectly changed the metabolic state of MDSCs (Yamada et al., 2020).

Taken together, in S. aureus FRI, abscesses contain a central S. aureus SAC within a fibrin pseudocapsule. The SAC is surrounded by live neutrophils with only few apoptotic cells, with a mixture of monocytes and M2 macrophages at the border of an abscess. The abscess encapsulation consists of αSMA-positive myofibroblast, collagen and fibrinogen. About the abscess neutrophils, M2 macrophages and Tregs were observed (Figure 7). The resulting condition in the tissue likely is anti-inflammatory and causally related to the persistent infection of S. aureus.

**Conclusion**

Overall, this study provided an in-depth description of the histopathology of S. aureus FRI as it progresses over time. It was shown here that fibrin-covered SACs are present within bone marrow abscesses and these abscesses are themselves encapsulated by collagen, fibrinogen, and myofibroblasts. Outside the SAC, and inside the abscess margins, the dense accumulation of cells, primarily neutrophils and a few monocytes, were largely vital and showed immunohistochemical markers consistent with granulocytic or monocytic MDSCs, respectively. The bone marrow also contained functionally immunosuppressive MDSCs, anti-inflammatory, M2 macrophages and Tregs, further indicating the local environment to be anti-inflammatory. By providing a better understanding of the local inflammatory conditions that lead to persistence of infection, and better understanding the physical features of SACs, we hope to enable tailored treatment regimens for FRI.

**Acknowledgement**

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617 Priority Program on Bone Infection.

Figure Legends

619 Fig. 1. Radiographic and histological appearance of non-inoculated and inoculated mice at 3, 14 and 28 d post-surgery. Representative radiographs and Giemsa and eosin (G&E) stained methyl methacrylate (MMA) embedded sections of non-inoculated (A-F) or S. aureus-inoculated (G-L) mouse femoral bone at 3 (upper row), 14 (middle row), and 28 d (lower row) post-operative. Mice received a double osteotomy within the femur, which created a bone segment, and a titanium 6-hole MouseFix locking plate for bone stabilization. Non-inoculated animals showed signs of fracture consolidation, whereas inoculated mice still had a non-union 28 d post-operatively. G&E stained bone pink and connective and soft tissue purple, whereas nuclei are dark blue. Red arrows indicate bone regeneration, black arrows show bone resorption pits created by osteoclasts, white arrowheads point out bacterial aggregates, and the yellow arrow demonstrates bone marrow within newly formed bone. Note, the samples of the non-inoculated mice stained more brown within the bone marrow. This is an artifact due to incomplete dehydration and defatting. Black debris were remains of the gigly wire used to make the double osteotomy. Scale bars: 1 mm.

620 Fig. 2. The histopathological osteomyelitis evaluation scores (HOES) of non-inoculated mice at 3, 14, and 28 d post-surgery and mice with a S. aureus-inoculated femoral bone segment assessed at 1, 3, 7, 14, 21, and 28 d post-infection (A). From bottom to top, the acute osteomyelitis features investigated were granulocyte infiltration (white), osseonecrosis (light grey) and soft tissue necrosis (medium grey), and the chronic osteomyelitis features assessed were bone neogenesis/fibrosis (dark grey), and lymphocyte and macrophage infiltrate (black). Data shown are medians of HOES scores with 95% confidence intervals from \( n = 2 \) for inoculated mice at 7 and 28 d post-operatively and \( n = 3 \) for the rest of the data 1, 3, 14, and 21 d post-operatively. Representative images of host responses in paraffin embedded murine femoral bone of non-inoculated (B) and S. aureus-inoculated mice (C) at 3, 14, and 28 d post-operative. For consistency, images were taken from the distal part of the bone segment of non-inoculated mice and inoculated mice. Immunofluorescent triple stains were performed for Ly6G (neutrophils, green), fibrin(ogen) (yellow), and F4/80 (macrophages, violet). DAPI (dark blue) was used as nuclear counterstain. Scale bar: 50 µm.

621 Fig. 3. Giemsa and eosin (G&E) stained methyl methacrylate (MMA) embedded femoral bone marrow of S. aureus-inoculated mice from 1, 3, 7, 14, 21, and 28 d post-operatively (A). Images are representative and focused on abscess structures with staphylococcal abscess communities (SACs), indicated with white arrows, present in the bone marrow. Scale bar: 200 µm. SAC number (B) and size (C) measured in paraffin and MMA embedded femoral bone marrow of S. aureus-inoculated mice 1, 3, 7, 14, 21, and 28 d post-operatively. Statistical test used: Dunn’s multiple comparison test. Data are medians ± max and min values of average SAC number and size per animal. \( N = 6 \) for 1, 3, and 14 d, \( n = 5 \) for 7 and 21 d and \( n = 4 \) for 28 d post-operatively. \(^* p < 0.05, \text{ } ^{**} p < 0.01. \) Representative images of SACs in paraffin embedded murine femoral bone inoculated with S. aureus from 21 or 28 d stained with a Brown and Brenn (BB) staining for Gram-positive bacteria (D; blue), a Picro-Mallory trichrome staining for fibrin (E;
magenta), haematoxylin & eosin (H&E) staining with a SAC indicated by an asterisk and host cells within an abscess by arrowheads (F), a TUNEL assay for apoptotic host cells with methyl green as counterstain (G; brown), a Picro-Sirius staining for collagen fibres (H; red), and immunofluorescent antibodies for fibrin(ogen) and alpha-smooth muscle actin positive myofibroblasts with DAPI as nuclear counterstain and the SAC indicated by a dashed white line (I; red, turquoise and dark blue, respectively).

**Fig. 4.** Host cells associated with *S. aureus* aggregates in paraffin sections of inoculated mice 7 and 21 d post-operative. Haematoxylin and eosin (H&E; A), immunofluorescent (B-D), and immunostaining (E) of bone marrow within the *S. aureus* infected segment after 7 d. H&E (F), immunofluorescent (G-I), and immunostaining (J) of bone marrow with an abscess containing SACs after 21 d. Successive sections were used for *S. aureus* (red), Ly6G (neutrophils, green) and F4/80 (macrophages, violet, white arrowheads), Ly6C (monocytic cells, turquoise), CD11b (pan-myeloid cells, red), iNOS (enzyme NO production, red), Arg-1 (enzyme urea cycle, violet), DAPI (nuclei, dark blue), and for FoxP3 (brown) to indicate regulatory T cells (Tregs).

Rim of cells positive for iNOS and Arg-1 are indicated by white arrows and co-localization of Ly6G, F4/80, and *S. aureus* signals shows as a light pink color. Scale bar: (B-D and G-I) 50 µm.

**Fig. 5.** Innate immune cells within a bone marrow abscess of an *S. aureus*-inoculated murine FRI model 21 d post-operatively. Immunofluorescent stainings for either Ly6G (A and B; neutrophils, green), Ly6C (D and E; monocytic cells, green) or F4/80 (H and G; macrophages, green) co-stained with iNOS (enzyme NO production, red) and Arg-1 (enzyme urea cycle, violet) and subsequently stained with haematoxylin and eosin (H&E; C, F and I). DAPI (nuclei, dark blue) was used as counter stain for the fluorescent lower magnification images. In the H&E images, white arrows indicate neutrophils, black arrows show monocytes, and a white arrowhead points out a macrophage. Scale bars: (A, D and G) 50 µm and (B, E and H) 10 µm.

**Fig. 6.** Mice received a double osteotomy within the femur, which created a bone segment that was inoculated with *S. aureus*, placed back and stabilized within the femur with a titanium 6-hole MouseFix locking plate. CFU quantification of soft tissues, bones, bone marrow supernatants and implants from non-inoculated (circles) or *S. aureus*-inoculated (triangles) mice used for purification of CD11b+ Ly6C+ Ly6G+, monocytic, or CD11b+ Ly6C- Ly6G+ neutrophilic bone marrow cells after 21 d post-operative (A). Data are means ± SD (n = 5). The percentage of monocytic CD11b+ Ly6C+ Ly6G+ or neutrophilic CD11b+ Ly6C- Ly6G+ cells from all alive bone marrow cells of non-inoculated (dark grey) or *S. aureus*-inoculated mice (B; light grey). Statistical test used: two-way ANOVA comparing cell percentages of inoculated and non-inoculated mice. Data presented are means ± SD (n = 5). Percentage proliferating CD3+ CD4+ T cells (C) or CD3+ CD8α+ T cells (D) cultured alone without or with CD3/CD28 Dynabeads and rIL2 stimulation (black – or +, respectively) or co-cultures with monocytic CD11b+ Ly6C+ Ly6G+ or neutrophilic CD11b+ Ly6C- Ly6G+ bone marrow cells from non-inoculated (1:1 ratio; dark grey) or *S. aureus*-inoculated mice (1:1 ratio; light grey or 0.5:1 ratio; striped light grey). Statistical test used: Sidak multiple comparison test. Data are means ± SD (n = 5), stimulated T cells cultured alone were set as 100 % and used as comparison for the statistical tests. N.S.= non-significant, *p < 0.05, **p < 0.01, and ***p < 0.001.
Fig. 7. A schematic overview of the different components of an abscess as observed in a murine FRI model which was partially generated with BioRender (Toronto, ON, Canada).
References


Table 1. The different primary antibody combinations with the dilutions used per antibody, and their corresponding secondary antibody mixtures.

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Table 1. The different primary antibody combinations with the dilutions used per antibody, and their corresponding secondary antibody mixtures.
Table 2. Severity scores used to score the five different acute and chronic osteomyelitis markers of the histopathological osteomyelitis evaluation score (HOES).

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Fig. 1. Radiographic and histological appearance of non-inoculated and inoculated mice at 3, 14 and 28 d post-surgery. Representative radiographs and Giemsa and eosin (G&E) stained methyl methacrylate (MMA) embedded sections of non-inoculated (A-F) or S. aureus-inoculated (G-L) mouse femoral bone at 3 (upper row), 14 (middle row), and 28 d (lower row) post-operative. Mice received a double osteotomy within the femur, which created a bone segment, and a titanium 6-hole MouseFix locking plate for bone stabilization. Non-inoculated animals showed signs of fracture consolidation, whereas inoculated mice still had a non-union 28 d post-operatively. G&E stained bone pink and connective and soft tissue purple, whereas nuclei are dark blue. Red arrows indicate bone regeneration, black arrows show bone resorption pits created by osteoclasts, white arrowheads point out bacterial aggregates, and the yellow arrow demonstrates bone marrow within newly formed bone. Note, the samples of the non-inoculated mice stained more brown within the bone marrow. This is an artifact due to incomplete dehydration and defatting. Black debris were remains of the gigly wire used to make the double osteotomy. Scale bars: 1 mm.
Fig. 2. The histopathological osteomyelitis evaluation scores (HOES) of non-inoculated mice at 3, 14, and 28 d post-surgery and mice with a S. aureus-inoculated femoral bone segment assessed at 1, 3, 7, 14, 21, and 28 d post-infection (A). From bottom to top, the acute osteomyelitis features investigated were granulocyte infiltration (white), osseonecrosis (light grey) and soft tissue necrosis (medium grey), and the chronic osteomyelitis features assessed were bone neogenesis/fibrosis (dark grey), and lymphocyte and macrophage infiltrate (black). Data shown are medians of HOES scores with 95 % confidence intervals from n = 2 for inoculated mice at 7 and 28 d post-operatively and n = 3 for the rest of the data. Representative images of host responses in paraffin embedded murine femoral bone of non-inoculated (B) and S. aureus-inoculated mice (C) at 3, 14, and 28 d post-operative. For consistency, images were taken from the distal part of the bone segment of non-inoculated mice and inoculated mice. Immunofluorescent triple stains were performed for Ly6G (neutrophils, green), fibrin(ogen) (yellow), and F4/80 (macrophages, violet). DAPI (dark blue) was used as nuclear counterstain. Scale bar: 50 µm.
Fig. 3. Giemsa and eosin (G&E) stained methyl methacrylate (MMA) embedded femoral bone marrow of S. aureus-inoculated mice from 1, 3, 7, 14, 21, and 28 d post-operatively (A). Images are representative and focused on abscess structures with staphylococcal abscess communities (SACs), indicated with white arrows, present in the bone marrow. Scale bar: 200 µm. SAC number (B) and size (C) measured in paraffin and MMA embedded femoral bone marrow of S. aureus-inoculated mice 1, 3, 7, 14, 21, and 28 d post-operatively. Statistical test used: Dunn’s multiple comparison test. Data are medians ± max and min values of average SAC number and size per animal. N = 6 for 1, 3, and 14 d, n = 5 for 7 and 21 d and n = 4 for 28 d post-operatively. *p < 0.05, **p < 0.01. Representative images of SACs in paraffin embedded murine femoral bone inoculated with S. aureus from 21 or 28 d stained with a Brown and Brenn (BB) staining for Gram-positive bacteria (D; blue), a Picro-Mallory trichrome staining for fibrin (E; magenta), haematoxylin & eosin (H&E) staining with a SAC indicated by an asterisk and host cells within an abscess by arrowheads (F), a TUNEL assay for apoptotic host cells with methyl green as counterstain (G; brown), a Picro-Sirius staining for collagen fibres (H; red), and immunofluorescent antibodies for fibrin(ogen) and alpha-smooth muscle actin positive myofibroblasts with DAPI as nuclear counterstain and the SAC indicated by a dashed white line.
(I; red, turquoise and dark blue, respectively).

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Fig. 4. Host cells associated with S. aureus aggregates in paraffin sections of inoculated mice 7 and 21 d post-operative. Haematoxylin and eosin (H&E; A), immunofluorescent (B-D), and immunostaining (E) of bone marrow within the S. aureus infected segment after 7 d. H&E (F), immunofluorescent (G-I), and immunostaining (J) of bone marrow with an abscess containing SACs after 21 d. Successive sections were used for S. aureus (red), Ly6G (neutrophils, green) and F4/80 (macrophages, violet, white arrowheads), Ly6C (monocytic cells, turquoise), CD11b (pan-myeloid cells, red), iNOS (enzyme NO production, red), Arg-1 (enzyme urea cycle, violet), DAPI (nuclei, dark blue), and for FoxP3 (brown) to indicate regulatory T cells (Tregs). Rim of cells positive for iNOS and Arg-1 are indicated by white arrows and co-localization of Ly6G, F4/80, and S. aureus signals shows as a light pink color. Scale bar: (B-D and G-I) 50 µm.

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Fig. 5. Innate immune cells within a bone marrow abscess of an S. aureus-inoculated murine FRI model 21 d post-operatively. Immunofluorescent stainings for either Ly6G (A and B; neutrophils, green), Ly6C (D and E; monocytic cells, green) or F4/80 (H and G; macrophages, green) co-stained with iNOS (enzyme NO production, red) and Arg-1 (enzyme urea cycle, violet) and subsequently stained with haematoxylin and eosin (H&E; C, F and I). DAPI (nuclei, dark blue) was used as counter stain for the fluorescent lower magnification images. In the H&E images, white arrows indicate neutrophils, black arrows show monocytes, and a white arrowhead points out a macrophage. Scale bars: (A, D and G) 50 µm and (B, E and H) 10 µm.
Fig. 6. Mice received a double osteotomy within the femur, which created a bone segment that was inoculated with S. aureus, placed back and stabilized within the femur with a titanium 6-hole MouseFix locking plate. CFU quantification of soft tissues, bones, bone marrow supernatants and implants from non-inoculated (circles) or S. aureus-inoculated (triangles) mice used for purification of CD11b+ Ly6C+ Ly6G- monocytic, or CD11b+ Ly6C+ Ly6G+ neutrophilic bone marrow cells after 21 d post-operative (A). Data are means ± SD (n = 5). The percentage of monocytic CD11b+ Ly6C+ Ly6G- or neutrophilic CD11b+ Ly6C+ Ly6G+ cells from all alive bone marrow cells of non-inoculated (dark grey) or S. aureus-inoculated mice (B; light grey). Statistical test used: two-way ANOVA comparing cell percentages of inoculated and non-inoculated mice. Data presented are means ± SD (n = 5). Percentage proliferating CD3+ CD4+ T cells (C) or CD3+ CD8α+ T cells (D) cultured alone without or with CD3/CD28 Dynabeads and rIL2 stimulation (black – or +, respectively) or co-cultures with monocytic CD11b+ Ly6C+ Ly6G- or neutrophilic CD11b+ Ly6C+ Ly6G+ bone marrow cells from non-inoculated (1:1 ratio; dark grey) or S. aureus-inoculated mice (1:1 ratio; light grey or 0.5:1 ratio; striped light grey). Statistical test used: Sidak multiple comparison test. Data are means ± SD (n = 5), stimulated T cells cultured alone were set as 100 % and used as comparison for the statistical tests. N.S. = non-significant, *p < 0.05, **p < 0.01, and ***p < 0.001.
Fig. 7. A schematic overview of the different components of an abscess as observed in a murine FRI model which was partially generated with BioRender (Toronto, ON, Canada).

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Response to reviewer Fig. 1. A haematoxylin-stained SAC (left; area within the dashed lines) and a SAC stained with an anti-S. aureus antibody (right; red). Due to the irregular shape of this specific SAC, 1) the SAC appears to be improperly stained while, in fact, S. aureus is only present in the red-stained shape seen in the image on the right-hand side and 2) the cells may appear at the center of this SAC, however, the cells are in fact outside the SAC.
Response to reviewer Fig. 2. An abscess that was cut through an entire cross section of a SAC stained for S. aureus, Ly6G (green; neutrophils), and F4/80 (violet; macrophages). The image shows in such case, there is no loss of signal at the center of a SAC using this staining protocol.