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A Quantitative Mouse Model of Implant-Associated Osteomyelitis and the Kinetics of Microbial Growth, Osteolysis and Humoral Immunity

Dan Li^{1,*}, Kirill Gromov^{1,2,*}, Kjeld Søballe², J. Edward Puzas¹, Regis J. O’Keefe¹, Hani Awad¹, Hicham Drissi¹, and Edward M. Schwarz^{1,3}

¹The Center for Musculoskeletal Research, University of Rochester, Rochester, New York

²The Department of Orthopedics, Aarhus University Hospital, Aarhus, Denmark

Summary

Although osteomyelitis (OM) remains a serious problem in orthopaedics, progress has been limited by the absence of an *in vivo* model that can quantify the bacterial load, metabolic activity of the bacteria over time, immunity and osteolysis. To overcome these obstacles, we developed a murine model of implant-associated OM in which a stainless steel pin is coated with *Staphylococcus aureus* and implanted transcortically through the tibial metaphysis. X-ray and micro-CT demonstrated concomitant osteolysis and reactive bone formation, which was evident by day 7. Histology confirmed all the hallmarks of implant-associated OM, namely: osteolysis, sequestrum formation and involucrum of Gram-positive bacteria inside a biofilm within necrotic bone. Serology revealed that mice mount a protective humoral response that commences with an IgM response after one week, and converts to a specific IgG2b response against specific *S. aureus* proteins by day 11 post-infection. Real-time quantitative PCR (RTQ-PCR) for the *S. aureus* specific *nuc* gene determined that the peak bacterial load occurs 11 days post-infection. This coincidence of decreasing bacterial load with the generation of specific antibodies is suggestive of protective humoral immunity. Longitudinal *in vivo* bioluminescent imaging (BLI) of *luxA-E* transformed *S. aureus* (Xen29) combined with *nuc* RTQ-PCR demonstrated the exponential growth phase of the bacteria immediately following infection that peaks on day 4, and is followed by the biofilm growth phase at a significantly lower metabolic rate ($p < 0.05$). Collectively, these studies demonstrate the first quantitative model of implant-associated OM that defines the kinetics of microbial growth, osteolysis and humoral immunity following infection.

Keywords

Osteomyelitis; *Staphylococcus aureus*; Osteolysis; Humoral Immunity; Bioluminescent Imaging

Introduction

Osteomyelitis (OM) is a common infectious disease characterized by progressive inflammation and bone destruction^{1–3}. This condition may be caused by a variety of microorganism, however, *Staphylococcus aureus* is responsible for >80% of these infections¹. Although the

³To whom correspondence should be addressed: Dr. Edward M. Schwarz, The Center for Musculoskeletal Research, University of Rochester Medical Center, 601 Elmwood Avenue, Box 665, Rochester, NY 14642, Phone 585-275-3063, FAX 585-756-4727, E-mail: Edward_Schwarz@URMC.Rochester.edu.

*These authors contributed equally to this work.

total number of osteomyelitis cases is high in that approximately 112,000 orthopaedic device-related infections occur per year in the US at an approximate hospital cost of \$15,000–70,000 per incident, the infection rates for joint prosthesis and fracture-fixation devices have been only 0.3–11% and 5–15% of cases respectively over the last decade ^{2,3}, which resulted in a low interest in rigorous prospective clinical studies. Another challenging issue hampering the progress in the OM research field is the absence of a quantitative animal model. It is well known that *in vitro* cultures rapidly select for growth of organisms that do not elaborate an extracellular capsule. As such, biofilm biology, which plays a critical role in resistance of chronic OM to antibiotic therapy by serving as a dominant protective barrier from the action of antibiotics, can only be studied with *in vivo* models ⁴. To date, much of our knowledge of the pathogenesis of OM comes from animal models ⁵, which have existed in chicken ⁶, rat ^{7,8}, guinea pig ⁹, rabbit ¹⁰, dog ¹¹, sheep ¹², goat ¹³ and most recently mouse ¹⁴. While these models have been used to confirm the importance of bacterial adhesins identified from *in vitro* assays ^{15–17}, none of them contain quantitative endpoints that can determine bacterial load or growth *in vivo*. Thus, they are of limited value for assessing drug effects, bacterial mutants and the role of host factors during the establishment of chronic OM.

In this study, we developed a novel murine model of implant-associated osteomyelitis in which a stainless steel pin is coated with *S. aureus* and implanted transcortically through the tibial metaphysis. The resulting infection closely resembles clinical OM. This generates a reproducible abscess without hematogenous spreading or death in >90% of mice. By adapting real-time quantitative PCR (RTQ-PCR) to detect the *S. aureus* specific *nuc* gene that is used to assess contaminated food ¹⁸, and a bioluminescent strain (Xen29) that can be quantified using longitudinal *in vivo* bioluminescent imaging (BLI) ¹⁹, we have elucidated the pattern of pathogenic growth during the establishment of implant-associated OM. We also performed serology and micro-CT analyses to assess the host response to infection. Our findings demonstrate the establishment of a quantitative small animal surrogate that can be used to evaluate the efficacy of novel interventions for OM.

Methods

S. aureus strains and pathogenic challenge

The UAMS-1 strain of *S. aureus* (ATCC 49230) was obtained from the American Type Culture Collection (Manassas, VA). The bioluminescent strain of *S. aureus*, Xen29 (derived from ATCC 12600), was obtained from Xenogen Inc. (Cranbury, NJ). Pathogenic challenge was initiated via a contaminated 0.25 mm diameter insect pin (Fine Science Tools, Foster City, CA) that was generated as follows. The pins were autoclaved and stored in 70% ethanol. After air-drying, the pins were incubated in 1.5 ml of an overnight luria broth culture of *S. aureus* for 20 min. Then, the pins were air dried for 5 minutes before trans-tibial implantation. The inoculating dose of bacteria was determined to be $9.5 \pm 3.7 \times 10^5$ CFU of UAMS-1 and $4.2 \pm 0.5 \times 10^5$ CFU of Xen29 per pin, by vigorously vortexing the pin in PBS to resuspend the bacteria and growing dilutions on agar plates.

Animal surgery

All animal studies were performed under University Committee for Animal Resources approved protocols. C57BL/6 female 6–8 week-old mice were anesthetized with Ketamine (100 mg/kg) and Xylazine (10 mg/kg), their left tibiae were shaved and the skin was cleansed with 70% ethanol. Infection was initiated by placing the pin transcortically through the tibia via medial to lateral implantation. The pin was bent at both ends for stability and cut adjacent to the skin on both ends, which allowed it to be covered by the skin and to eliminate additional environmental exposure. Once the mice recovered from the anesthesia, they were returned to

standard isolator cages without additional treatment until sacrifice, except for the gentamycin treatment group that received 0.1 mg/kg/day i.p.

***In vivo* radiology and bioluminescent imaging**

Longitudinal osteolysis was assessed radiographically using a Faxitron Cabinet x-ray system (Faxitron, Wheeling, IL, USA) as we have previously described²⁰. Bioluminescent imaging (BLI) of mice infected with Xen29 was performed on day 0, 4, 7, 11, 14, 18 post-infection using a Xenogen IVIS camera system (Xenogen Corporation, Alameda, Calif.). Five minute high sensitivity ventral images were taken at each time point. The BLI was quantified with the LivingImage software package 2.50.1 (Xenogen Corporation, Alameda, Calif.) by analyzing a fixed 1.5 cm diameter region of interest (ROI) centered on the pin. The photon signal was calculated as p/sec/cm²/sr.

Micro-CT analyses

After sacrifice, the pin was removed and the disarticulated tibiae were analyzed by high-resolution (10.5 μ m) micro-computed tomography (μ CT) (VivaCT 40; Scanco Medical AG, Basserdorf, Switzerland) to render 3D images of the diaphysis as we have previously described²¹. Images from each tibia were binarized individually at identical thresholds to allow for unbiased identification of the cortical pinholes and the extent of the osteolytic lesion in the cortices. The three-dimensional binary image was then rendered from the threshold slices, and sagittal sections through the tibia were digitally obtained so that the rendered images could be rotated to visualize the pinholes and osteolytic lesions on the lateral and medial cortical surfaces of the specimens. Since the infected pin was introduced through the medial side, and we observed more consistent and reproducible osteolysis on the medial side, we chose to only quantify the area of osteolysis on the medial side. The maximal osteolytic area was computed for each specimen using a semi-automated filter in Adobe Photoshop® CS (Adobe Systems, Inc., San Jose, CA) which thresholds the pinhole area, sums the number of pixels in the thresholded region, and then computes the area in mm² after calibrating against 1 mm scale bar (9216 pixels/mm²). This area measurement was then used to quantitatively describe the maximum size of the osteolytic cortical lesions for the group as mean $\pm$ SEM (Figure 1).

Histologic evaluation of OM

After μ CT, the tibial samples were processed for decalcified histology and stained with Orange G/alcian blue (H&E), Gram-stained, or tested for tartrate-resistant acid phosphatase (TRAP) activity as we have described previously²².

DNA purification and *nuc*/ β -actin real time quantitative PCR (RTQ-PCR)

Infected tibiae were disarticulated and separated from the surrounding soft tissues. Then the tibia was cut into small pieces with scissors, and demineralized individually with 0.5 M EDTA (pH 7.5) in 1.5 ml polypropylene tubes. The tubes were agitated on a shaker at 4°C for 24 h, centrifuged at 5500 rpm for 20 minutes and the precipitate was resuspended in fresh EDTA. This process was repeated 3 to 5 times until the bone fragments were completely decalcified. The samples were then washed three times with ddH₂O and the pellet was resuspended in 360 ml buffer ATL (QIAGEN, Valencia, CA) with 40 μ l proteinase K (QIAGEN, Valencia, CA), and incubated at 55°C until the bone tissues were fully digested and the solution was clear. Then 400 μ l buffer AL (QIAGEN, Valencia, CA) with 5 μ l proteinase K was added, and the samples were placed on ice and sonicated five times, at level 15, for 10 seconds with 1-minute resting intervals between the sonications (Microson Ultrasonic Cell Disruptor, Misonix Inc., Farmingdale, NY). After sonication, DNA was purified using DNeasy Tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. The final sample of DNA was eluted in 100 μ l of ddH₂O, and stored at -20°C.

RTQ-PCR for the *S. aureus*-specific *nuc* gene was performed to quantify the bacterial load with primers 5'-GCGATTGATGGTGATACGGTT -3' and 5'-AGCCAAGCCTTGACGAAGTAA -3' that amplify a 269-bp product, as previously described¹⁸. The reactions were carried out in a final volume of 20 μ l consisting of 0.3 μ M primers, 1 $\times$ Sybr Green PCR Super Mix (BioRad, Hercules, CA), and 2 μ l of the purified tibia DNA template. The samples were assayed in triplicate in a Rotor-Gene RG 3000 (Corbett Research, Sydney, AU). In order to control for the integrity of the DNA template between samples we performed RTQ-PCR for the mouse β -actin gene that detects a 124-bp product using primers 5'-AGA TGT GAA TCA GCAAGC AG-3' and 5'-GCG CAA GTT AGG TTT TGT CA-3'. In order to calculate the *nuc* gene copies in a tibia sample, we first generated a standard curve with *S. aureus* genomic DNA purified directly from an overnight culture. The mean of the 3 Ct values from each tibia sample were then plotted against this curve to extrapolate the number of *nuc* genes. This number was then normalized to β -actin and the data are presented as normalized *nuc* gene copies per sample.

Serology

In order to determine total immunoglobulin isotype (Ig) levels, blood samples were collected from the animals on days 0, 4, 7, 11 and 14 using retro-orbital bleeding, and an ELISA on the sera was performed using the Mouse Typer Sub-Isotyping Kit (BioRad, Hercules, CA) as we have previously described²³. Specific antibodies against *S. aureus* proteins were detected by western blotting. The protein was obtained from a 100 ml culture of bacteria in log phase, in which the protein extract was prepared using the Complete Bacterial Proteome Extraction Kit (Calbiochem, San Diego, CA) according to the manufacture's instructions. 20 μ g of total *S. aureus* protein per well was boiled in Laemmli loading buffer and separated in NuPAGETM 10% Bis-Tris SDS Gels (Invitrogen, Carlsbad, CA) by electrophoresis. The proteins were transferred to a PVDF membrane (Millipore, Billerica, MA) and stained with Ponceau Red (Sigma, St. Louis, MO) to control for protein loading and transfer efficiency. The membrane was then cut into single lanes and blocked with PBS, 0.1% Tween 20 (PBST) and 5% non-fat dry milk for 1 hr at room temperature. Afterwards, each lane was incubated with a unique serum (10 μ l serum in 5 ml of PBST) as the primary antibody for 1 hr at room temperature. The strips were then washed 3 times in 0.1% PBST, 15 minutes each at room temperature. The strips were then pooled and incubated with 1.5 μ l HRP-conjugated goat anti-mouse IgG antibody (BioRad, Hercules, CA) in 30 ml blocking buffer for 1 hr at RT. The strips were then washed 3 times in PBST, 15 minutes each at room temperature. Finally, the strips were reassembled with the molecular weight marker strip and imaged with ECL+ (Amersham) chemiluminescence autoradiograph.

Results

A murine transtibial model of implant-associated osteomyelitis

In our initial attempt to develop a quantitative model of implant-associated OM we chose an intramedullary implant approach, since this represents the serious infections in patients with total joint replacements. However, repeated attempts failed to provide evidence that a reproducible abscess could be generated (data not shown). This led us to conclude that it is challenging to consistently achieve a quantitative model of intramedullary implant associated-OM, although others have recently succeeded²⁴.

Next, we investigated a transtibial implant approach that mimics OM of external fixation pins. In our initial experiments, an insect pin coated with Xen29 was surgically implanted into the left tibia of mice. Longitudinal x-rays demonstrated osteolysis adjacent to the pin within 7 days (Figure 1A). Moreover, this mode of infection led to a highly reproducible localized abscess in >90% of the mice, and never resulted in detectable hematogenous spreading, sepsis or death.

In order to quantify the osteolysis, we performed a time-course study in which the infected tibiae were analyzed by μ CT (Figure 1B and C). These results are consistent with sequestrum formation in which osteoclastic bone resorption of cortical bone around the infected implant occurs with concomitant reactive periosteal bone formation.

The presence of OM in the mice was confirmed by histological analyses on tibiae that received infected or sterile pins. Figure 2 demonstrates that the tibial transcortical pin model contains all of the salient features of chronic OM including: sequestrum and involucrum formation, osteoclastic resorption of the cortical bone and Gram stained extracellular bacteria and biofilm that reside in the necrotic bone surrounding the implant. None of the negative controls, including heat killed *S. aureus* and non-pathogenic *E. coli* (data not shown), demonstrated these features.

Kinetics of infection and the host immunity during the establishment of OM

Since it is impossible to effectively extract live bacteria from infected bone to quantify the *in vivo* bacterial load due to the calcified matrix and biofilm, we adapted an RTQ-PCR method to determine the number of *nuc* gene per tibia as a surrogate outcome measure. Using PCR primers specific for murine β -actin and *S. aureus nuc*, we first validated the sensitivity and specificity of the assay (detection of <10 copies per sample with a single peak; Supplemental Figure A). Then, we used this assay to complete a time-course study on infected tibiae samples to assess the *in vivo* bacterial load during the establishment of OM. Figure 3A and B shows that the bacterial load peaked on day 11 post-surgery and dropped sharply thereafter. The sharp drop suggests that the host has generated an effective immune response that clears the bacteria.

Since effective clearance of *S. aureus* is partially dependent on humoral immunity, we evaluated antisera from infected mice over the course of infections. In order to determine when mice produce specific high affinity antibodies against *S. aureus* proteins after infections, we performed western blots on whole-cell bacterial extracts using sera from the challenged animals as the primary antibody. The results demonstrated the generation of specific anti-*S. aureus* IgG antibodies by day 11, which increase thereafter (Figure 3C). An assessment of total Ig levels in the sera of these challenged mice demonstrated an initial increase in IgM levels after one week, which was converted to IgG2b at two weeks (Figure 3D). Taken together, these results indicate that establishment of implant-associated OM is consistent with classical microbial pathogenesis and immunity in which the bacteria enjoy an initial growth period in the naïve host that is terminated by an acquired humoral response. The finding that mice are unable to completely eradicate *S. aureus* infection after generating a functional humoral response is consistent with the fact that antibodies cannot penetrate biofilm.

Bioluminescent imaging (BLI) quantification of bacterial metabolism during the establishment of OM

While the results of our RTQ-PCR studies demonstrate its ability to quantify bacterial load *in vivo*, the assay has two fundamental shortcomings that limit its utility. The first is the sacrificial endpoint, which prohibits longitudinal studies. The second is that RTQ-PCR cannot distinguish between metabolically active bacteria and dormant microbes within biofilm. Since this resolution is critical for the investigation of pathogenic mechanisms and evaluation of novel interventions, we examined the utility of BLI in our transtibial implant model. In our time-course studies with Xen29, only background signal was detected in mice that receive a sterile pin (Figure 4) or infected mice treated with parenteral gentamycin (data not shown). In contrast, the BLI of infected, untreated tibiae demonstrated a sharp 4-fold increase from baseline on day 4, which subsequently dropped to background levels by day 11.

Based on the finding that BLI peaks on day 4 and *nuc* levels peak on day 11 along with the facts that bioluminescent of *S. aureus* Xen 29 results from the reaction of enzymes and protein substrates synthesized by the *lux* operon and that *nuc* is a single copy gene in the *S. aureus* chromosome, we hypothesized that BLI is a measure of *S. aureus* metabolic activity (protein synthesis), while *nuc* levels are indicative of bacteria number. This theory also predicts that the BLI:*nuc* ratio is greater early on in infections compared to dormancy in latent infection. To test this, we performed linear regression analyses of BLI versus *nuc* gene copy number before the presence of biofilm on day 2 and on day 18 when humoral immunity limits the bacteria to biofilm growth. These analyses demonstrated that there is no relationship between BLI and *nuc* gene copy number on day 2 (Figure 5A), while there is a highly significant correlation between these outcome measures on day 18 (Fig 5B). Moreover, the finding that the BLI/*nuc* ratio is 15 times greater on day 2 vs. day 18 (Figure 5C) further substantiates BLI as an outcome measure of metabolic activity and *nuc* levels as a measure of bacterial load.

Discussion

Although infection rates following orthopaedic surgery are considered to be low^{2,3}, implant-associated OM remains a catastrophic outcome that often requires revision surgery and can lead to sepsis and death¹. This problem is compounded by the emergence of multi-drug resistant strains and the absence of effective treatments for patients with methicillin-resistant *S. aureus* (MRSA)^{1,3}. Progress in this area has been limited due to the absence of a quantitative animal model that can be utilized to elucidate molecular targets and evaluate novel interventions. From our assessment of the literature, we surmise that a quantitative OM model has not been developed for two reasons. First, the field has been overly concerned with intramedullary implant models, since this represents the more serious clinical condition^{8–13}. Unfortunately, we found that this model gave rise to highly variable (temporal and spatial) lesions, making a reproducible, quantitative model very challenging, although others have recently succeeded²⁴. In contrast, we have found that implantation of an infected transcortical pin always produces lesions adjacent to the pin, and never results in distal OM, hematogenous infection or death (Figure 1). Thus, while it could be argued that the mechanisms of infection and treatment modalities differ between intramedullary and external fixation implants, we find the transcortical pin model to be the best approach to study OM *in vivo*.

The second roadblock towards the development of a quantitative OM model is the difficulty in extracting individual live bacteria, classically known as colony forming units (CFU), from infected bone. Here we demonstrate two independent methods to overcome this obstacle. The first, *nuc* RTQ-PCR, is a highly specific and sensitive method that has been successfully used to quantify *S. aureus* levels in contaminated cheese¹⁸. It should be noted that although we control for DNA integrity via β -actin RTQ-PCR, we have no way of knowing the total *nuc* yield following our rigorous extraction procedures. Thus, it is likely that the *nuc* genes per tibia that we present represent a fraction of the total number of bacteria originally present in the sample. Furthermore, it is likely that our ability to extract *nuc* genes from infected tibiae becomes less efficient when the bacteria are residing in dense biofilm. Thus, the rather low *nuc* gene levels observed in latent infections (day 18), may be an under representation of the actual bacterial load. This may also explain the remarkable effects of the anti-*S. aureus* antibodies on *nuc* gene levels (Figure 3), as it is known that biofilm provide bacteria with an immune-privileged environment. Nevertheless, our RTQ-PCR results provide the first demonstration that: i) *in vivo* bacterial load levels can be quantified during the establishment of OM, and ii) that the peak bacterial load is coincident with the generation of humoral immunity against the bacteria.

As mentioned above, two critical aspects of OM research that cannot be addressed by our RTQ-PCR approach are longitudinal studies and assessment of microbial metabolic activity. As

research on the regulators of *in vivo* biofilm formation has become a central focus in this field, developing a surrogate outcome measure of pre and post-biofilm growth is of great value. To this end we have investigated the utility of BLI, which has emerged as a research technique with enormous potential²⁵. Contag and colleagues were the first to utilize bioluminescent strains of pathogens to study infection longitudinally in mice²⁶. Subsequent studies have proven the value of BLI over conventional methods in studying disease and therapeutic interventions in animals²⁵. More recently, this group generated bioluminescent *S. aureus* Xen29 for this purpose¹⁹. In our hands, Xen29 behaves essentially the same at UAMS-1, which is the most widely used strain of *S. aureus* used in OM research^{9–13}. Thus, we adopted this approach to be compatible with μ CT quantification of osteolysis and RTQ-PCR in our model of OM.

The first interesting result we obtained with BLI was that it peaked on day 4 (Figure 4), well before the peak of bacterial load and the appearance of adaptive immunity (Figure 3). Since BLI is a function of metabolic activity (protein synthesis), not bacterial load, our interpretation of these results is that there is a brief exponential bacterial growth phase that peaks on day 4, and is followed by the initiation of the biofilm growth phase that occurs at a low metabolic rate. Once this biofilm growth phase is initiated, perhaps due to the exhaustion of nutrients in the necrotic tissue, the bacterial load continues to increase. Microbes are shed from the biofilm until protective immunity is acquired on day 11. Afterwards, bacterial persistence is restricted to the biofilm in necrotic tissue indefinitely. To test this theory we postulated that at a given time during infection, *nuc* copy number (1 *nuc* gene per bacterium) only correlates with BLI (mean rate of *luxA-E* protein synthesis for all of the bacteria) during periods of homogenous growth. In support of this theory we found that no correlation exist during concomitant planktonic (high metabolic rate) and colonized (lower metabolic rate) bacterial growth, which is known to occur 48hr after infection, and when the inter-animal variability between the proportion of planktonic to colonized bacteria is expected to be great (Figure 5A). In contrast, when the bacteria are restricted to biofilm growth (low metabolic rate), due to acquired immunity at day 18, and there is no inter-animal variability between the proportions of planktonic to colonized bacteria, a significant correlation exists between *nuc* and BLI (Figure 5B). Furthermore, we found that the BLI:*nuc* ratio early on in infection (day 2) is 15-fold greater vs. chronic infection (day 18). While further studies are needed to formally demonstrate the kinetics of biofilm formation *in vivo*, our findings clearly distinguish these two phases of bacterial growth.

In summary, the development of a quantitative model of implant-associated OM opens the field to research that was previously unapproachable. New avenues include: the identification of biofilm genes through the characterization of mutant phenotypes, identification of diagnostic and/or protective antigens, and facile evaluation of infection-resistant implants such as the Selfprotective 'smart' devices²⁷, and novel drug efficacy. Although there are some fundamental limitations of this model with regard to its ability to accurately represent the true clinical problems associated with infected intramedullary implants and prostheses, we find that this transtibial mouse model, which displays all of the salient features of infected external fixation pins, to be a quantitative model suitable for most of these needs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Radiographic progression of trans-tibial implant-associated osteomyelitis

(A) Longitudinal x-rays of a representative mouse that received a trans-tibial pin coated with Xen29 *S. aureus*. Gentamycin and sterile pin controls at day 18 are also shown. X-rays were taken on the indicated day after surgery. Arrows indicate the osteolysis around the pin, which was evident at 7 days and thereafter. Also of note is the absence of any lesions distal to the pin. (B) Medial views of reconstructed μ CT images of representative tibiae from mice (N=5) that received a trans-tibial pin coated with Xen29 *S. aureus* and were sacrificed on the indicated day. Also shown are control mice that received a trans-tibial pin coated with Xen29 *S. aureus* and was treated with parenteral gentamycin (Gent), or received a sterile pin. (C) The osteolytic area around the pin was quantified as described in Material and Methods, and the

data are presented as the mean $\pm$ SD (* $p < 0.05$ vs Day 4; ** $p < 0.05$ vs. Gent Day 18). There was no difference in the osteolysis area between the gentamycin and sterile pin controls (data not shown).

Figure 2. Histological evaluation of the trans-tibial implant-associated model of osteomyelitis H&E (A–C), TRAP (D–F) and Gram stained (G, H) sections of histology sections at the pin site (*) adjacent to the tibial cortex (#), 9 days after implantation of a sterile pin (A,D,G), or a pin coated with UAMS-1 *S. aureus* (B,C,E,F,H). Of note is the new bone (h) that forms around the sterile pin (A,D,G) vs. the necrotic sequestrum (s) and involucrum (i) adjacent to the infected pin. While very few TRAP+ osteoclasts (yellow arrow heads) were present in the uninfected samples (D), numerous osteoclasts appear to be actively resorbing the cortex adjacent to the infected pin, and remodeling the new woven bone that is encasing the involucrum (E, F). Gram staining confirmed the absence of bacteria in the specimens with the

sterile pin (G) and their presence (black arrow heads) within the necrotic bone around the infected pins (H).

Figure 3. Kinetics of infection and host immunity during the establishment of OM

A time-course experiment was performed in which mice (N=5) received a trans-tibial pin coated with UAMS-1 *S. aureus* and were sacrificed at the indicated time after surgery. Genomic DNA was purified from the infected tibiae and used as the template for *nuc* and β -actin RTQ-PCR as described in Materials and Methods. (A) Data for each individual mouse, or (B) the mean for each group are presented with the standard deviation. (C) Sera were collected from mice (N=5) before (day 0) and on days 4, 7, 11 and 14 days after receiving a trans-tibial pin coated with *S. aureus* UAMS-1, and used as the primary antibody in Western blots of total *S. aureus* UAMS-1 protein extract. In the data shown from a representative animal, the black arrowhead indicates a pre-immune reactive band, and red arrowheads indicate the *S. aureus*

specific reactive bands. (D) Total Ig levels in sera of mice (N=5) obtained on the indicated day following surgery were determined by ELISA as described in Methods. The data from a representative mouse are presented as the mean $\pm$ SD of quadruplicates. Of note is the dominance of IgM on day 7 and IgG2b on day 14.

B.

Figure 4. Bioluminescent Imaging (BLI) quantification of bacterial growth during the establishment of chronic osteomyelitis

(A) BLI levels at the site of infection were assessed longitudinally in mice that received a sterile trans-tibial pin (Uninfected), or a pin coated with Xen 29 *S. aureus* (Infected), that were imaged on the indicated day. The circle in the top left image highlights the 1.5 cm diameter region of interest (ROI) that was assessed for BLI in each mouse at each time point. (B) The data from mice (N=5) that were Uninfected, Infected or infected and treated with parenteral antibiotics (Gentamycin) were assessed for BLI longitudinally at the indicated time following surgery. The data are presented as the mean $\pm$ SD (* Significantly greater vs. Day 0; $p < 0.05$).

Figure 5. BLI is a function of bacterial metabolism

Mice received a trans-tibial pin coated with Xen29, and the BLI and *nuc* levels were determined on Day 2 (A), or Day 18 (B). A linear regression analysis was performed and the line of best fit is displayed with its equation, correlation coefficient (R^2), and significance (P). (C) These data were also used to determine the mean BLI/*nuc* ratio $\pm$ SD (* $p < 0.05$ vs. Day 2).