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Single-Dose, Preoperative Vitamin-D Supplementation Decreases Infection in a Mouse Model of Periprosthetic Joint Infection

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Background: Despite recent advances, infection remains the most common etiology of arthroplasty failure. Recent work suggests that 25-hydroxyvitamin D (25D) deficiency correlates with the frequency of periprosthetic joint infection (PJI). We endeavored to examine whether 25D₃ deficiency leads to increased bacterial burden in vivo in an established mouse model of PJI and, if so, whether this effect can be reversed by preoperative 25D₃ supplementation.

Methods: Mice (lys-EGFP) possessing fluorescent neutrophils were fed a vitamin D₃-sufficient (n = 20) or deficient (n = 40) diet for 6 weeks. A group of 25D₃-deficient mice (n = 20) were “rescued” with 1 intraperitoneal dose of 25D₃ at 3 days before surgery. A stainless steel implant was inserted into the knee joint and the joint space was inoculated with bioluminescent *Staphylococcus aureus* (1×10^3 colony forming units [CFUs]). In vivo imaging was used to monitor bacterial burden and neutrophil infiltration. Blood was drawn to confirm 25D₃ levels 3 days before surgery and on postoperative days (PODs) 0 and 14. Mice were killed at POD 21, and CFUs were quantified after culture. Myeloperoxidase (MPO) and β -N-acetylglucosaminidase (NAG) were assayed to look at neutrophil infiltration and activated tissue macrophage recruitment, respectively.

Results: Serum values confirmed 25D₃ deficiency and repletion of the 25D₃-rescued group. Bacterial bioluminescence and neutrophil fluorescence were significantly greater ($p < 0.05$) in the 25D₃-deficient group. CFU counts from the joint tissue and implant were also significantly greater in this group ($p < 0.05$). Rescue treatment significantly decreased bacterial burden and neutrophil infiltration ($p < 0.05$). Compared with the 25D₃-sufficient and 25D₃-rescued groups, MPO activity was higher ($p < 0.02$) and NAG activity was lower ($p < 0.03$) in the 25D₃-deficient group.

Conclusions: This study demonstrated in vivo in a mouse model of PJI that (1) 25D₃ deficiency results in increased bacterial burden and neutrophil infiltration, and (2) this effect can be reversed with preoperative repletion of 25D₃.

Clinical Relevance: Considering that >65% of patients undergoing arthroplasty have insufficient or low levels of total 25D and that 25D levels can be replenished with ease using a U.S. Food and Drug Administration (FDA)-approved, oral 25D₃ product, 25D deficiency may be an important modifiable risk factor in humans undergoing joint replacement.

The number of arthroplasties performed in the U.S. is expected to exceed 3.8 million by 2030, and the annual number of periprosthetic joint infection (PJI) cases is estimated to increase from 17,000 in 2005 to >266,000¹. Despite advances in antiseptic protocols, surgical technique, and operating-room sterility, PJI remains the most common etiology

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of arthroplasty failure². PJIs are devastating, resulting in re-operations, prolonged antibiotic therapy, and extended disability and rehabilitation, with up to 7% of patients dying between the first and second stages of exchange arthroplasty^{3,4}. Medical costs are enormous, averaging \$145,000 per patient, resulting in a proposed projected annual U.S. health-care burden of \$8.6 billion by 2030^{5,6}.

While effort has been focused on perioperative antimicrobial therapies and modification of health-care systems protocols, host factors governing susceptibility to PJI have been understudied. Although smoking, obesity, and diabetes mellitus (among others) are known risk factors, mechanistic links have been difficult to ascribe, and efforts at modifying these factors have been challenging⁷⁻¹¹.

25-hydroxyvitamin D₃ (25D₃) holds promise as a risk modifier for 3 important reasons. First, epidemiologic data demonstrate that >65% of patients undergoing arthroplasty have an insufficient or low level of total 25-hydroxyvitamin D (25D; accounts for both 25D₂ and 25D₃ in the serum)¹². Second, recent epidemiologic work has suggested that vitamin-D deficiency is directly correlated with the frequency of PJI¹³. This coincides with literature highlighting the importance of the prohormone 25D as a locally active immune modulator for antigen-activated inflammatory cells. This is mediated through the intracellular enzymatic conversion of available 25D to 1,25-dihydroxyvitamin D (1,25D) via the CYP27B1-hydroxylase, which is coupled to the expression of the vitamin-D receptor. The vitamin-D receptor regulates the expression of genes critical to both the innate and adaptive immune responses in humans¹⁴⁻²⁰. Finally, 25D levels can be returned to normal with ease, rapidity, efficiency, and low cost using an available, U.S. Food and Drug Administration (FDA)-approved, orally administered 25D₃ product²¹. Taken together, 25D deficiency is prevalent in the population receiving total joint arthroplasty, appears to be correlated with PJI clinically and linked to the function of the innate immune response mechanistically, and can be easily improved, highlighting its promise as a modifiable risk factor.

The objective of the current study was to examine whether the immune system is suppressed in the 25D-deficient state, increasing the severity of a PJI. We aimed to test 2 primary hypotheses. First, does 25D₃ deficiency lead to an increased bacterial burden and decreased inflammatory response in vivo in an established mouse model of PJI? Second, if that is the case, does “rescue” with preoperative 25D₃ supplementation reverse this effect? If successful, such findings could pave the way for establishing a low serum 25D level as an easily modifiable host factor in humans to reduce the risk of PJI.

Materials and Methods

All animals were handled according to good animal practice as defined in the federal regulations set forth in the Animal Welfare Act, the 1996 Guide for the Care and Use of Laboratory Animals, and the Public Health Service Policy for the Humane Care and Use of Laboratory Animals as well as the policies and procedures of the University of California, Los Angeles (UCLA).

All animal experiments were approved by the UCLA chancellor’s Animal Research Committee.

Staphylococcus aureus Bioluminescent Strain

Staphylococcus aureus Xen36 (PerkinElmer) is a bioluminescent derivative of the *S. aureus* ATCC 49525 (Wright) strain, derived from a patient with *S. aureus* bacteremia. Xen36 has a gram-positive optimized luxABCDE operon stably integrated into a large native plasmid²². This strain emits a blue-green light with a maximal emission wavelength of approximately 490 nm from live, actively metabolizing bacteria and was previously shown to be optimal for use because of the strength and consistency of its signal⁶.

Preparation of *S. aureus* for Inoculation

Xen36 possesses a kanamycin-resistance selection marker, enabling isolation from contaminating background strains during culture. Thus, 200 µg/mL of kanamycin (Sigma-Aldrich) was added to all culture samples. Xen 36 was streaked onto tryptic soy agar plates (tryptic soy broth [TSB] plus 1.5% Bacto agar; BD Biosciences) and grown at 37°C overnight. Single colonies were cultured in TSB and grown overnight at 37°C in a shaking incubator (240 rpm) (MaxQ 4450; ThermoFisher Scientific). Midlogarithmic-phase bacteria were obtained after a 2-hour subculture of a 1:50 dilution of the overnight culture. Cells were pelleted, resuspended, and washed 3 times in phosphate buffered saline (PBS) solution. Bacterial concentrations were estimated by measuring the absorbance at 600 nm (BioMate 3; ThermoFisher Scientific). Colony forming units (CFUs) were verified after overnight culture of plates.

Mice

We used 4-week-old male lys-EGFP mice, a genetically engineered mouse line on a C57BL/6 background possessing green-fluorescent myeloid cells (mostly neutrophils) as a consequence of “knock-in” of enhanced green fluorescent protein (EGFP) into the lysozyme M gene^{23,24}. Animals were kept 3 mice per cage and fed either a standard diet (4,200 IU vitamin D₃ per kg of feed) or a vitamin D₃-deficient diet (0 IU vitamin D₃ added; Research Diets) with access to bottled water. Veterinary staff carried out daily assessments.

Vitamin-D Protocol

The mice were randomized to receive either a vitamin D₃-sufficient (n = 20) or vitamin D₃-deficient (n = 40) diet. On the basis of prior experiments, mice were fed the chosen diet for 6 weeks before surgery to ensure 25D₃ sufficiency or deficiency²⁵. Three days before surgery, a “rescued” group (n = 20) was created from the mice being fed a deficient diet by injecting 80 ng of 25D₃ (Enzo Life Sciences) intraperitoneally, as previously described²⁶; these mice were also switched over to a vitamin D₃-sufficient diet (Fig. 1). To confirm 25D₃ levels prior to rescue, prior to surgery, and in the postoperative period, retro-orbital blood draws were performed 3 days prior to surgery and on postoperative days (PODs) 0 and 14. Serum was separated via microcentrifuge. Individual samples (n = 10) from each group

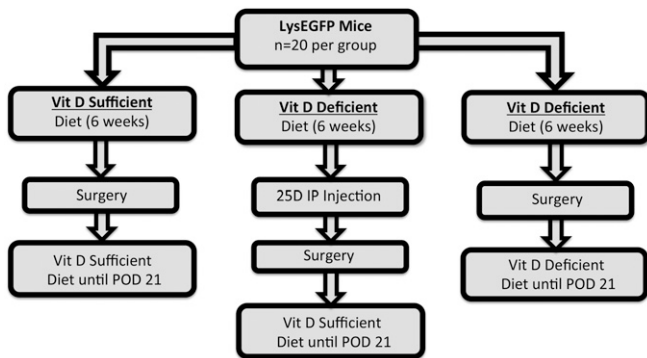


Fig. 1
Experimental protocol for 25-hydroxyvitamin D (25D₃) supplementation. Four-week old male lys-EGFP mice were randomly fed either a vitamin D₃-sufficient (n = 20) or D₃-deficient (n = 40) diet. A group of 25D₃-deficient mice (n = 20) were given 25D₃ by intraperitoneal (IP) injection 3 days prior to surgery and switched over to a vitamin D₃-sufficient diet for the remainder of the study to act as a “rescued” group. The remainder of the mice in the 25D₃-sufficient and deficient groups were fed their respective diets for the remainder of the study. POD = postoperative day.

were pooled on PODs -3, 0, and 14 to ensure adequate content of 1,25D₃ for radioimmunoassay and 25D₃ for liquid chromatography-mass spectrometry (Heartland Assays).

Surgical Procedure

Mice were anesthetized via inhalation of isoflurane (2%). The surgical procedure was previously described^{6,27}. A skin incision was made over the right knee. The distal part of the femur was accessed through a medial parapatellar arthrotomy. The femoral medullary canal was manually reamed with a 25-gauge needle. An orthopaedic-grade stainless steel Kirschner wire (0.6 mm in diameter; DePuy Synthes) was placed in a retrograde fashion and cut with 1 mm protruding into the joint space. An inoculum of 1×10^3 CFUs of Xen36 in 2 μ L of normal saline solution was pipetted into the joint space. The surgical site was closed with polyglycolic acid 5-0 sutures. Buprenorphine (0.1 mg/kg) was administered subcutaneously every 12 hours as an analgesic for the duration of the experiment. No antibiotics were given throughout the study period.

Quantification of Bacterial Burden Using Bioluminescence Imaging in Vivo and CFUs

Mice were anesthetized via inhalation of isoflurane (2%), and in vivo bioluminescence imaging was performed by using an in vivo imaging system (IVIS Lumina II; PerkinElmer)²⁸. Images were obtained on PODs 0, 1, 3, 5, 7, 10, 14, 18, and 21. Data were presented on a color scale overlaid on a grayscale photograph of the mouse and quantified as maximum flux (photons per second per cm² per steradian [photons/s/cm²/sr]) within a circular region of interest (16,103 pixels) by using Living Image software (PerkinElmer).

To confirm that the bioluminescence signals corresponded to the bacterial burden in vivo, bacteria were quantified. On POD 21, bacteria were detached from the implant by sonication in 1 mL of 0.3% polysorbate 80 in TSB for 10 minutes followed by vortexing for 5 minutes, as previously described⁶. In addition, bacteria in the surrounding joint tissue were measured by homogenizing bone and joint tissue (Pro200H Series homogenizer; PRO Scientific). The number of bacterial CFUs that were adherent to the implant and in the joint tissue was determined by counting CFUs after overnight culture of plates and was expressed as total CFUs harvested from the implant and joint tissue.

Quantification of Neutrophil Infiltration with Fluorescence Imaging in Vivo

Fluorescence imaging in vivo was performed using the IVIS Lumina system. EGFP-expressing neutrophils within the postoperative site were visualized by using the GFP filter for excitation (445 to 490 nm) and emission (515 to 575 nm) at an exposure time of 0.5 seconds^{6,28}. The observer was not blinded to the treatment group. Data were presented on a color scale overlaid on a grayscale photograph of the mouse and quantified as maximum radiant efficiency ([photons/s]/[μ W/cm²]) within a circular region of interest (16,103 pixels) using Living Image software.

Quantification of Neutrophil Infiltration with Myeloperoxidase Activity and Macrophage Recruitment with β -N-Acetylglucosaminidase Activity

At the conclusion of the experiment, 3 mice per group were killed and joint-tissue specimens were homogenized. The

TABLE I Pooled Serum 25-Hydroxyvitamin D₃ (25D₃) and 1,25-Dihydroxyvitamin D₃ (1,25D₃) Values from 25D₃-Deficient, Sufficient, and Rescued Mice*

	25D ₃ (ng/mL)			1,25D ₃ (pg/mL)		
	POD -3	POD 0	POD 14	POD -3	POD 0	POD 14
Sufficient	15.6	17.6	20.8	64.3	69.5	52.3
Deficient	2.1	1.8	1.5	25.7	34	21.5
Rescued	2.1	28.8	26.2	26.3	62.6	52.7

*N = 10 at each time point. POD = postoperative day.

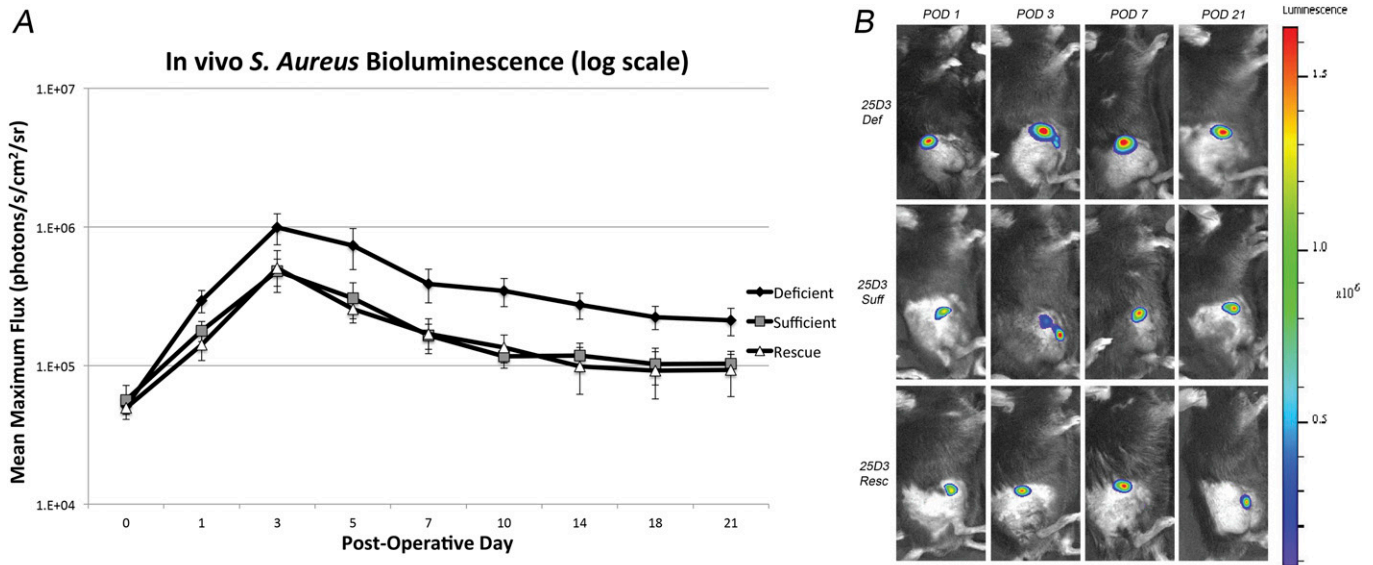


Fig. 2

Figs. 2-A and 2-B Measurement of bacterial burden in vivo using live-animal bioluminescence in 25D₃-deficient (def), sufficient (suff), and rescued (resc) mice. A stainless steel implant was inserted into the right knee joint of the mice (n = 20 per group), and the joint space was inoculated with Xen36 *Staphylococcus aureus* (1×10^3 colony-forming units) possessing the bioluminescent construct in a stable plasmid. **Fig. 2-A** Bacterial counts as measured by *S. aureus* bioluminescence in vivo (mean maximum flux and standard error of the mean [logarithmic scale]). **Fig. 2-B** Representative in vivo *S. aureus* bioluminescence on a color scale overlaid on a grayscale image of the mouse. POD = postoperative day.

homogenate was assayed for myeloperoxidase (MPO) activity levels, a surrogate for neutrophil infiltration, using the Myeloperoxidase Colorimetric Activity Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions and expressed as milliunits/mL²⁹. The tissue homogenate was also assayed for β -N-acetylglucosaminidase (NAG) activity levels, a surrogate for recruited tissue macrophage activity, using the β -N-Acetylglucosaminidase Assay Kit (Sigma-Aldrich) ac-

cording to the manufacturer's instructions and expressed as milliunits/mL³⁰.

Statistical Analysis

Each experimental group had 20 mice; in previous studies, our group determined that 20 animals per group were necessary to demonstrate significance at the $p < 0.05$ level^{6,29}. Data were compared using a Student t test (1 or 2-tailed where indicated).

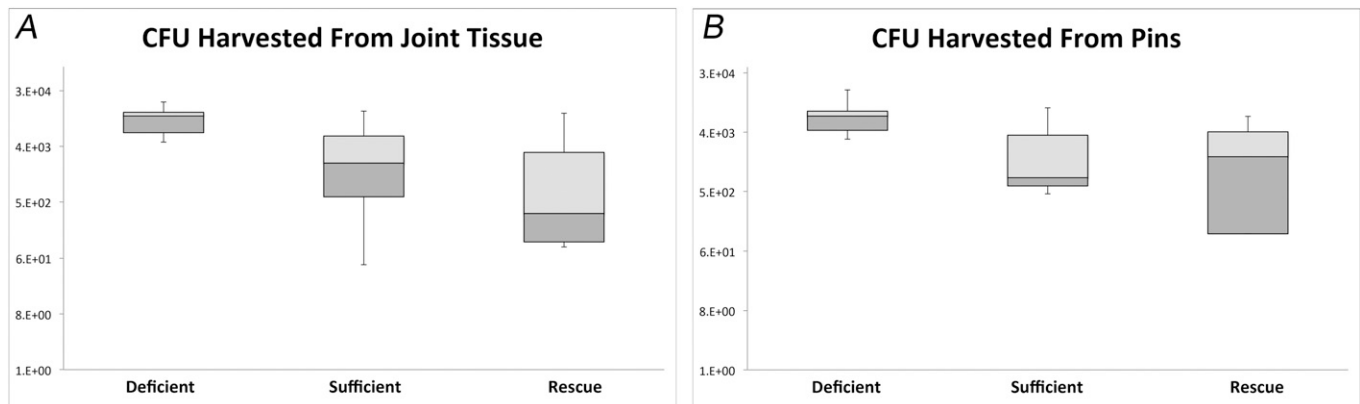


Fig. 3

Figs. 3-A and 3-B Confirmation of bacterial burden using colony-forming unit (CFU) counts. At postoperative day 21, mice were killed and bacteria from the implant and surrounding joint tissue were processed for culture. **Fig. 3-A** Box-and-whisker plots (logarithmic scale) of bacterial counts as measured by CFUs in the surrounding joint tissue of the right knee ($p < 0.05$ for the 25D₃-deficient mice compared with both 25D₃-sufficient and rescued mice). **Fig. 3-B** Box-and-whisker plots (logarithmic scale) of bacterial counts as measured by CFUs adherent to the implant ($p < 0.04$ for the 25D₃-deficient mice compared with both 25D₃-sufficient and rescued mice). The horizontal line within the boxes indicates the median, the outside borders indicate the medians of the upper half and lower half of the data, and the whiskers indicate the minimum and maximum values of the data set.

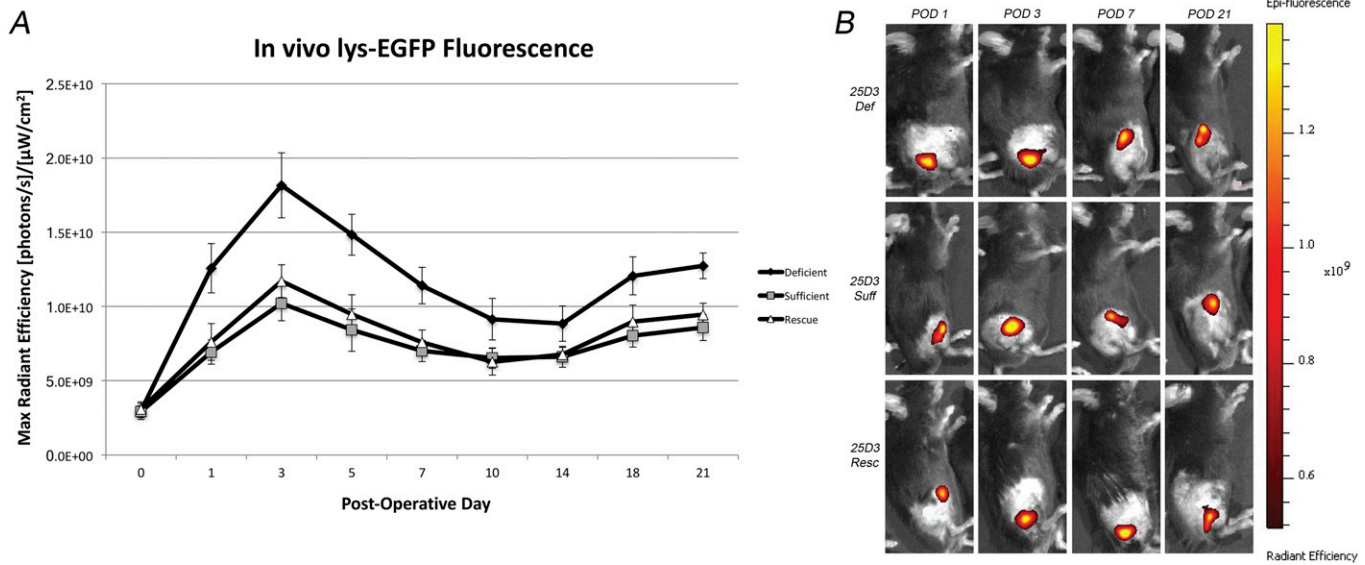


Fig. 4

Figs. 4-A and 4-B In vivo neutrophil EGFP fluorescence induced by the Xen36 *Staphylococcus aureus* strain in 25D₃-deficient (def), sufficient (suff), and rescued (resc) mice. A stainless steel implant was inserted into the right knee joint of the mice (n = 20 per group), and the joint space was inoculated with Xen36 *S. aureus* (1×10^3 colony-forming units) possessing the bioluminescent construct in a stable plasmid. **Fig. 4-A** Neutrophil infiltration (neutrophil EGFP fluorescence) as measured by fluorescence in vivo (mean maximum radiant efficiency and standard error of the mean). **Fig. 4-B** Representative in vivo neutrophil EGFP fluorescence on a color scale overlaid on a grayscale image of the mouse. POD = postoperative day.

All data were expressed as the mean and the standard error of the mean (SEM). Values of $p < 0.05$ were considered significant.

Results

Serum Vitamin-D Metabolite Levels

The 1,25D₃ and 25D₃ levels were higher in the mice that were fed a vitamin D₃-sufficient diet compared with those on a vitamin D₃-deficient diet for all 3 time points (PODs -3, 0, and 14) (Table I). Serum 25D₃ and 1,25D₃ levels were comparable between the 25D₃-deficient and rescued mice at POD -3;

the serum 25D₃ and 1,25D₃ in the rescued group increased to levels that were comparable with those in the 25D₃-sufficient group at PODs 0 and 14, confirming successful repletion.

Bacterial Burden Using Bioluminescence Imaging in Vivo and CFUs

The 25D₃-sufficient, deficient, and rescued groups had similar bioluminescent signals at POD 0 (5.64×10^4 , 5.08×10^4 , and 4.94×10^4 photons/s/cm²/sr, respectively) (Fig. 2). The bioluminescent signal for all 3 groups peaked at POD 3. From PODs

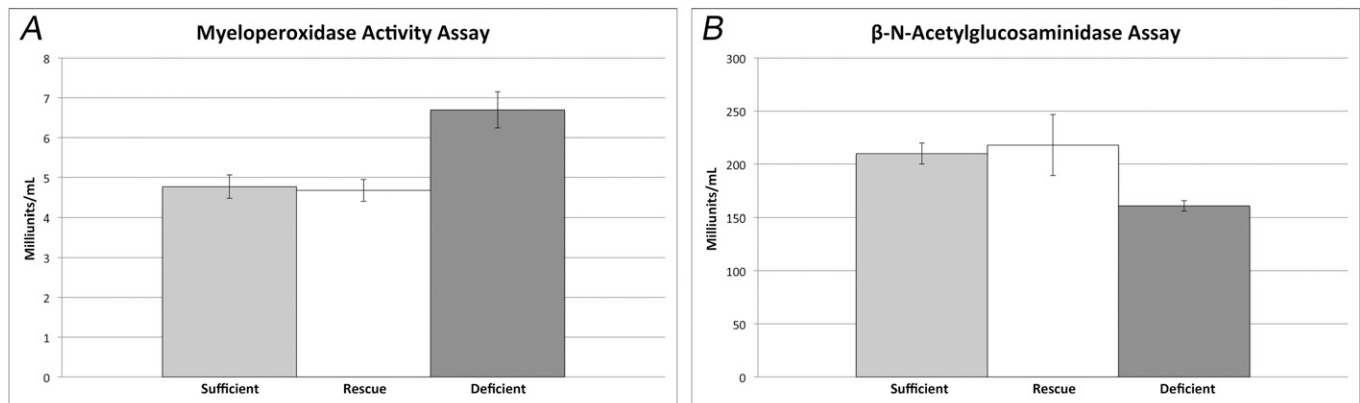


Fig. 5

Figs. 5-A and 5-B Myeloperoxidase (MPO) and β-N-acetylglucosaminidase (NAG) activity induced by the Xen36 *Staphylococcus aureus* strain in 25D₃-deficient, sufficient, and rescued mice. **Fig. 5-A** Mean MPO activity of the infected joint-tissue specimens (and standard error of the mean [SEM]) (n = 3 mice per group; $p < 0.02$ for the 25D₃-deficient mice compared with both 25D₃-sufficient and rescued mice). **Fig. 5-B** Mean NAG activity of the infected joint tissue specimens (and SEM) (n = 3 mice per group; $p < 0.03$ for the 25D₃-deficient mice compared with both 25D₃-sufficient and rescued mice).

1 through 21, the 25D₃-deficient group had a significantly higher bioluminescent signal than did the 25D₃-sufficient group ($p < 0.05$ for all time points). In addition, from PODs 1 through 21, the rescued group had a bioluminescent signal that was comparable to that of the 25D₃-sufficient group ($p > 0.05$ for all time points) and a significantly lower bioluminescent signal compared with the 25D₃-deficient group ($p < 0.05$ for all time points).

This finding was confirmed by CFU counts from the implant and surrounding joint tissue (Fig. 3). The average CFU count from the implant was 8.3×10^3 for the 25D₃-deficient group compared with 2.4×10^3 for the 25D₃-sufficient group ($p < 0.05$). The implant CFU count was 2.5×10^3 for the 25D₃-rescued group, which was equivalent to that of the 25D₃-sufficient group ($p > 0.05$) and significantly lower than that of the 25D₃-deficient group ($p < 0.04$). The average CFU count from the surrounding joint tissue was 1.2×10^4 for the 25D₃-deficient group compared with 5.4×10^3 for the sufficient group ($p < 0.05$). The CFU count from the surrounding joint tissue for the rescued group was 4.7×10^3 , which was also similar to that of the 25D₃-sufficient group ($p > 0.05$) and significantly lower than that of the 25D₃-deficient group ($p < 0.05$).

Neutrophil EGFP Fluorescence in Vivo

All 3 groups started with similar fluorescent signal on POD 0 (2.9×10^9 , 2.9×10^9 , and 3.1×10^9 [photons/s]/[$\mu\text{W}/\text{cm}^2$] in the 25D₃-deficient, sufficient, and rescued groups, respectively), and the signal peaked at POD 3 for all groups (Fig. 4). Interestingly, from PODs 1 through 21, the 25D₃-deficient group had significantly greater fluorescent signal compared with the sufficient group ($p < 0.05$ for all time points). The 25D₃-rescued group had fluorescent signal that was similar to that of the sufficient group ($p > 0.05$ for all time points) and significantly less fluorescent signal compared with the deficient group ($p < 0.05$ for all time points).

Quantification of Neutrophil Infiltration (MPO Activity) and Activated Tissue Macrophage Recruitment (NAG Activity)

Mice in the 25D₃-deficient group had significantly higher MPO activity, at 6.7 milliunits/mL ($p < 0.02$), than did mice in the 25D₃-sufficient and rescued groups, which were comparable at 4.8 and 4.7 milliunits/mL, respectively ($p > 0.05$) (Fig. 5). Mice in the 25D₃-deficient group had significantly less NAG activity, at 160 milliunits/mL ($p < 0.03$), than did mice in the 25D₃-sufficient and rescued groups, which were similar at 210 and 218 milliunits/mL, respectively ($p > 0.05$) (Fig. 5).

Discussion

Despite substantial research into its prevention, PJI remains the most common cause of failed total joint arthroplasty². Although advances have been made in antimicrobial therapies and modification of health-care systems protocols, host factors have been often neglected because of the perceived difficulty in modifying them^{7-11,31-34}. Recent work on circulating levels of 25D, the metabolite used to distinguish vitamin-D sufficiency of the host, has highlighted its importance as a host factor related to the

risk of infection^{13,15}. When considering the ease with which it can be replenished, a deficient serum 25D level has the potential to be an easily modifiable risk factor in the prevention of PJI²¹. With this in mind, we sought to use our previously established in vivo mouse model of PJI to (1) evaluate the differences in bacterial burden and host immune response between 25D₃-sufficient and deficient mice, and (2), test if this difference can be reversed with a single, preoperative dose of 25D₃.

In recent years, the physiological and pathophysiological roles ascribed to normal versus deficient vitamin-D balance have been broadened³⁵. Recent work has demonstrated a causal link between the prohormone 25D and the normal human innate immune response¹⁵⁻²⁰. In pathogen-associated molecular pattern (PAMP)-activated macrophages, characterized by co-upregulation of the expression of the 25D-activating enzyme gene (CYP27B1) and the vitamin-D receptor, 25D is converted to the active metabolite 1,25D intracellularly, which then binds to the vitamin-D receptor and turns on (1) antimicrobial peptide (AMP)-generating, (2) autophagy-stimulating, and (3) inflammasome-activating genes to combat infection, especially in the presence of *S. aureus*^{15,36}. This pathway is inoperable if extracellular levels of substrate 25D are inadequate. Clinical work by Maier et al. also demonstrated that there was a significant difference ($p < 0.001$) in serum 25D levels between patients who underwent primary total arthroplasty without PJI and those who developed a PJI¹³. In addition, 64% of the patients who underwent primary arthroplasty had low levels of 25D. Taken together, these recent findings highlight the potentially important role for 25D repletion in the prevention of PJI.

When comparing the 25D₃-sufficient and deficient mice, it was clear that the bioluminescent signal was greater in the deficient mice, indicating increased bacterial burden on the implant and surrounding tissues. This was also confirmed by CFU counts on POD 21. When examining the host immune response, the 25D₃-deficient mice had increased fluorescent signal, indicating greater neutrophil infiltration. This was confirmed by measuring MPO activity at the conclusion of the study. This is in contrast to decreased NAG activity, indicating diminished activated tissue macrophage recruitment. It is possible that inadequate host macrophage immune response leads to increased bacterial burden and this, consequently, leads to increased neutrophil infiltration in an attempt to more adequately combat the ongoing infection. Nonetheless, the data demonstrate that 25D₃ deficiency increases the severity of PJI. Moreover, the 25D₃-deficient mice that were replenished preoperatively with 25D₃ were “rescued” from this state and had a host response to induced infection that was similar to that of the mice that were 25D₃-sufficient. This finding opens the door to considerable further study of a low total 25D level as a risk factor that can be modified to help “prime” the immune system prior to arthroplasty.

There were several limitations to this study. One limitation was that these results were of a mouse model and thus may not be directly applicable to humans. Although this model is advantageous for the study of PJI, the size of the mice limited the blood available for testing of biomarkers and necessitated the

pooling of serum for analysis. Ideally, serum values of vitamin 25D₃ and 1,25D₃ would have been measured for each mouse at a variety of time points and correlated with the level of infection for each mouse. While this would have provided a more robust analysis of the role of 25D₃ and 1,25D₃ deficiency in PJI, the aggregate data presented here establish a foundation on which more detailed work on larger animals can be conducted. In addition, an extreme state of 25D₃ deficiency was tested, which may not be as clinically pertinent a scenario in humans, who would likely present with a lesser degree of deficiency. Furthermore, observers were not blinded to the treatment group when imaging the mice, leading to the potential for detection bias. To combat this, a predetermined region of interest in size and shape was used for both bioluminescence and fluorescence imaging.

A final limitation was the use of lys-EGFP mice containing fluorescent neutrophils instead of MacGreen mice, which contain fluorescent macrophages³⁷. As the macrophage is the primary cell responsible for initiating the “foreign-body reaction” to implanted hardware, it plays an important role in protecting the implant from contaminant bacteria³⁸. When the 25D level in the serum of the host is too low, this results in the failure of the macrophage to mount effective autophagy (killing) of bacteria, thus blunting the innate immune response³⁹. Longitudinal visualization of the difference in macrophage response might have yielded more direct information about the effect that 25D deficiency has on the innate immune response. Nevertheless, the lys-EGFP species was previously validated in our model²⁸. As such, it was thought to be more likely to produce a consistent, predictable result than a strain with a potentially different immunology. In addition, activated tissue macrophage recruitment was examined at the end of the study and demonstrated decreased activity in the 25D₃-deficient mice. Future study using MacGreen mice may be able to more fully evaluate the difference in macrophage recruitment over the course of a PJI.

Despite these weaknesses, we believe that this study successfully provides *in vivo* evidence that the 25D-deficient state of the host increases bacterial burden and neutrophil infiltration in the setting of PJI. Moreover, this effect can be reversed by preoperative administration of 25D₃. With 25D deficiency established as an important and easily modifiable risk factor *in vivo*, additional studies should (1) examine whether this single-dose “rescue” effect with 25D₃ administration also exists for humans, and (2) elucidate the exact mechanism behind the blunted immune response in the 25D-deficient population. In the future, a “personalized” approach to 25D₃ supplementation may be used for 25D-deficient patients prior to arthroplasty to help “prime” the immune system and prevent PJI. ■

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