Therapeutic assessment of \textit{N}-formyl-methionyl-leucyl-phenylalanine (fMLP) in reducing periprosthetic joint infection

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Abstract: & \textbf{INTRODUCTION:} Despite many preventive measures, including prophylactic antibiotics, periprosthetic joint infection (PJI) remains a devastating complication after arthroplasty, leading to pain, suffering, morbidity and a substantial economic burden. We have a powerful innate immune system which can effectively control infection, if alerted quickly. Unfortunately, pathogens use many mechanisms to dampen innate immune responses. We hypothesized that immunomodulators that can jumpstart and direct innate immune responses (particularly neutrophils) at the surgical site of implant placement would boost immune responses and reduce PJI, even in the absence of antibiotics. \\
& \textbf{METHODS:} To test our hypothesis, we used fMLP, (a potent chemoattractant for phagocytic leukocytes including neutrophils), in a mouse model of PJI with \textit{Staphylococcus aureus}. Mice receiving intramedullary femoral implants were divided into three groups: i) implant alone; ii) implant + \textit{S. aureus}, iii) implant + fMLP + \textit{S. aureus}. \\
& \textbf{RESULTS:} fMLP treatment reduced \textit{S. aureus} infection levels by ~2 log orders at day 3. Moreover, fMLP therapy reduced infection-induced peri-implant periosteal reaction, reduced focal cortical loss, and reduced areas of inflammatory infiltrate in the distal femurs of mice at day 10. Finally, fMLP treatment reduced pain behavior and increased weight-bearing at the implant leg in infected mice at day 10. \\
& \textbf{DISCUSSION:} Our data indicate that fMLP therapy is a promising novel approach in reducing PJI, if administered locally at surgical sites. Future work will be toward further enhancement and optimization of fMLP-based therapeutic approach through combination with antibiotics and/or
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implant coating with fMLP.

Note: The following files were submitted by the author for peer review, but cannot be converted to PDF. You must view these files (e.g. movies) online.

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Therapeutic assessment of an immunomodulator based on N-formyl-methionyl-leucyl-phenylalanine (fMLP)
in an approach to controlling periprosthetic joint infection

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ABSTRACT (no more than 250 words/currently 244 words)

INTRODUCTION: Despite many preventive measures, including prophylactic antibiotics, periprosthetic joint infection (PJI) remains a devastating complication after arthroplasty, leading to pain, suffering, morbidity and a substantial economic burden—a devastating complication that can lead to amputation, arthrodesis, antibiotic-induced organ cytotoxicity, or even death. We have a powerful innate immune system which can effectively control bacterial, fungal, and viral infections, if alerted quickly. Unfortunately, pathogens have evolved stealth mechanisms to avoid recognition by dampen innate immune system responses. We hypothesized that immunomodulators that can mobilize jumpstart and direct innate immune leukocytes responses (particularly neutrophils) toward the surgical site of surgical implant placement would enhance our ability to fight off invading pathogens and boost immune responses and be effective in controlling reduce PJI, even in the absence of prophylactic-antibiotics-control.

METHODS: To test our hypothesis, we first performed intra-articular injection of N-formyl-methionyl-leucyl-phenylalanine (fMLP) immunomodulator at the knee joint in mice to evaluate the neutrophil response to fMLP. We next evaluated our hypothesis using fMLP, a potent chemoattractant for phagocytic leukocytes including neutrophils, in a mouse model of PJI with Staphylococcus aureus (S. aureus). Mice received a right intramedullary femoral implant. Mice receiving intramedullary femoral implants were divided into three groups: i) implant alone; ii) implant + S. aureus; iii) implant + fMLP + S. aureus. Groups were assessed for bacterial burden, pathologic changes at the bone and joint tissue, and behavioral symptoms of PJI at specific time points over 10 days.

RESULTS: Consistent with our hypothesis, treatment with intra-articular administration of fMLP significantly increased neutrophil activity at the knee joint. In line with our hypothesis, fMLP treatment therapy significantly substantially reduced S. aureus infection levels substantially by over >~2 log—orders at day 3 at day 3. In addition, fMLP therapy reduced infection-induced peri-implant periosteal reaction, reduced focal cortical loss, and reduced areas of inflammatory infiltrate in the distal femurs of mice in the infected group that received fMLP at day 10 at day 10. Finally, fMLP treatment reduced pain behavior at the implant leg in infected mice and increased weight-bearing at the implant leg and in infected mice at day 10.

DISCUSSION: We propose Our data indicate that fMLP therapy is a promising novel approach in boosting reducing PJI, if administered locally at surgical sites—control, based on its ability to elicit a powerful neutrophil response stimulated a powerful neutrophil response at the knee joint, and we demonstrated that it could substantially reduce peak bacterial burden by over one log order at day 3, and reduce alleviate characteristic features of PJI (reduced weight-bearing and pain) at day 10 in a relevant PJI mouse model. Future work will be toward further enhancement and optimization of fMLP-based therapeutic approach enhancing the efficacy of fMLP through combination with antibiotics and/or implant coating with fMLP.
INTRODUCTION

Approximately 1-2% of patients with primary knee and hip replacement and about 3-6% of patients with revision knee and hip replacement acquire periprosthetic joint infection (PJI) (Izakovicova et al., 2019; Parvizi et al., 2017). Estimated risk of PJI can be over 20% in patients with a combination of demographic, surgical (e.g., prior procedure), and/or comorbidity risk factors (e.g., diabetes) (Tan et al., 2018). Moreover, PJI cases are projected to rise significantly within the next two decades due to increased number of joint replacements resulting from factors such as increased life expectancy and greater expectations of mobility in the older age population (Izakovicova et al., 2019; Kurtz et al., 2007). These alarming PJI statistics occur despite many prevention strategies which have been implemented to prevent surgical site infection (SSI), including surgical hand antisepsis, reduction of foot traffic in and out of the operating room, ventilation suits, use of intraoperative skin antiseptic agents, perioperative glycemic control, appropriate selection of surgical dressings, and prophylactic antibiotics (Allegranzi et al., 2016; Anesi et al., 2017; Bratzler et al., 2004; Campbell et al., 2014; Corona and Singer, 2010; Edmiston et al., 2013; Greif et al., 2000; Kamath et al., 2016; Khoshbin et al., 2015; Kroin et al., 2015; Kroin et al., 2016; Lindsay et al., 2011; Panahi et al., 2012; Parvizi et al., 2017; Sewick et al., 2012; Sidhwa and Itani, 2015; Stannard et al., 2012).

Although multiple bacterial pathogens have been associated with PJI, *Staphylococcus aureus* is often reported as the most common cause of PJI, accounting for greater than 50% of all PJIs (Aggarwal et al., 2014; Manning et al., 2020; Pulido et al., 2008; Tsai et al., 2019) (Teterycz et al., 2010). Treatments for PJI include long courses of antibiotics, debridement of infected tissue, and implant removal and replacement (Pelt et al., 2014; Segawa et al., 1999). However, antibiotic use is associated with emergence of antibiotic resistance, gut dysbiosis,
increased risk for *Clostridium difficile* infection, organ cytotoxicity, allergic reactions, and the success rates after these treatments are abysmal, often ranging between 50 to 70% (Becattini et al., 2016; Jakobsson et al., 2010; Korpela et al., 2016; Langdon et al., 2016; Pelt et al., 2014; Sampson et al.; Segawa et al., 1999; Yassour et al., 2016). There can be devastating consequences for patients that acquire PJI including amputation, arthrodesis, antibiotic-induced organ cytotoxicity, or even death (Pelt et al., 2014; Segawa et al., 1999). In addition, patients with PJI are also at increased risk for additional morbidities, such as deep vein thrombosis and pulmonary embolism (Lieberman and Hsu, 2005). These dismal outcomes underscore the need for new approaches to prevent/reduce and to treat PJI.

We have evolved a remarkable innate immune system that can recognize invading pathogens as “none-self” and mobilize its plethora of defenses to protect us against them (Brubaker et al., 2015; Guo et al., 2015; Janeway and Medzhitov, 2002; Kawai and Akira, 2011; Martinon et al., 2009; Schroder and Tschopp, 2010; Takeuchi and Akira, 2010). Recognition of microbial pathogens by pattern recognition receptors (PRRs) sets in motion multiple signaling cascades that culminate in the production of pro-inflammatory cytokines, which recruit effector innate immune leukocytes, particularly neutrophils, which in turn destroy invading pathogens by various direct or indirect mechanisms such as phagocytosis, bursts of reactive oxygen species (ROS), antimicrobial peptide (AMP) production, and neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Dovi et al., 2004). Unfortunately, pathogens have evolved various mechanisms, (e.g., reducing the production of pro-inflammatory cytokines), to dampen our innate immune responses, blocking production of pro-inflammatory cytokines, inducing cell death in target host cells, and blocking proliferation in target host cells (Faure et al., 2014; Hornef et al., 2002; Lai et al., 2009; Mohamed et al., 2021; Shafikhani and Engel, 2006; Shafikhani et al., 2008; Tolle et al., 2015; Wood et al., 2015a; Wood et al., 2015b). We hypothesized that local administration of immunomodulators that can accelerate and direct innate immune leukocyte responses (particularly neutrophils) toward the site of the surgically placed implant would enhance the immune responses toward infection and be effective in reducing PJI even in the absence of prophylactic antibiotics.

Although multiple bacterial pathogens have been associated with PJI, *Staphylococcus aureus* is the most common cause of PJI, accounting for greater than 50% of PJI (Aggarwal, 2014 #74417)(Kaplan, 2014) (Tetrezyez et al., 2010). Treatments for PJI include long courses of antibiotics, debridement of infected tissue, and implant removal and replacement (Pelt et al., 2014; Segawa et al., 1999). However, antibiotic use is associated with emergence of antibiotic resistance, gut dysbiosis, and increased risk for *Clostridium difficile* infection, organ cytotoxicity, and allergic reactions, and immunological and neurological diseases, and the success rates after these treatments are abysmal, often ranging between 50 to 70% (Becattini et al., 2016; Jakobsson et al., 2010; Korpela et al., 2016; Langdon et al., 2016; Pelt et al., 2014; Sampson et al.; Segawa et al., 1999; Yassour et al., 2016). In regard to antibiotic resistance, approximately 50% of bone and joint related infections is caused by methicillin resistant *S. aureus* (MRSA)(Kaplan, 2014), and a high percentage of implant associated infections are due to specifically to MRSA (Tetrezyez et al., 2010). There can be devastating consequences for patients that acquire PJI including amputation,
arthrodesis, antibiotic induced organ cytotoxicity, or even death (Pelt et al., 2014; Segawa et al., 1999). In addition, patients with PJI are also at increased risk for additional morbidities, such as deep vein thrombosis and pulmonary embolism (Lieberman and Hsu, 2003). These dismal outcomes underscore the need for new approaches to prevent and to treat PJI.

We have evolved a remarkable innate immune system that can recognize invading pathogens as “none-self” and mobilize its plethora of defenses to protect us against them (Brubaker et al., 2015; Guo et al., 2015; Janeway and Medzhitov, 2002; Kawai and Akira, 2011; Martinon et al., 2009; Schroder and Tschopp, 2010; Takeuchi and Akira, 2010). Recognition of microbial pathogens by pattern recognition receptors (PRRs) sets in motion multiple signaling cascades that culminate in the production of pro-inflammatory cytokines, which recruit effector innate immune leukocytes, particularly neutrophils (PMNs), which in turn destroy invading pathogens by various direct or indirect mechanisms such as phagocytosis, bursts of reactive oxygen species (ROS), antimicrobial peptide (AMP) production, and neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Dovi et al., 2004). Unfortunately, as powerful as our immune system is, there is a lag time (~18 – 72 hours) between the initial time of pathogen recognition and the time needed to mount a robust immune response that can effectively limit infection (Kim et al., 2008; Ng et al., 2011). Within this lag period, pathogens could potentially gain a foothold, making it more difficult to eradicate them. Moreover, pathogens have evolved various mechanisms to dampen our innate immune responses, such as reducing the production of pro-inflammatory cytokines, to dampen our innate immune responses (Faure et al., 2014; Hornet et al., 2002; Lai et al., 2009; Tolle et al., 2015). We hypothesized that local administration of immunomodulators that can accelerate and direct innate immune leukocyte responses (particularly neutrophils) toward the site of the surgically placed implant would enhance the immune responses toward infection and be effective in reducing PJI even in the absence of prophylaxis antibiotics. The evolution of pathogens that dampen antibiotic efficacy and the immune response, which can further lengthen this vulnerability phase and raise the possibility of increase vulnerability to surgical site infection (SSI).

To enhance the local immune responses at the time of surgery, we chose to locally administer N-formyl-methionyl-leucyl-phenylalanine (fMLP; a.k.a. fMLF) at the time of implant surgery, using an established mouse PJI mouse model (Bernthal et al., 2010; Bernthal et al., 2014; Hegde et al., 2017a; Thompson et al., 2018). We chose fMLP because it is a naturally occurring formyl peptide; product that is formyl peptides are released at injured tissues surgical sites from both injured tissues, such as surgical sites, although it can also be released and byand invading bacterial pathogens (Li et al., 2016; Raoof et al., 2010). We chose fMLP because it functions as a potent chemoattractant for neutrophils and other inflammatory leukocytes (Balazovich et al., 1996; Derian et al., 1995; Madianos et al., 2005; Panaro and Mitolo, 1999; Sabroe et al., 2003). In addition, fMLP interaction with FPR chemokine receptors also activates bactericidal functions in neutrophils, such as superoxide and reactive oxygen species (ROS) production, phagocytosis, and degranulation (Devosse et al., 2009; Dorward et al., 2015; Sengeløv et al., 1994). To enhance the local immune response at the time of surgery, we chose to locally administer N-formylmethionyl-leucyl-phenylalanine (fMLP; a.k.a fMLP) at the time of implant surgery. fMLP is a
formyl peptide, which is released at surgical sites from both injured tissues and invading bacterial pathogens (Li et al., 2016; Raoof et al., 2010) and functions as a potent chemoattractant for neutrophils and other inflammatory leukocytes (Balazovich et al., 1996; Derian et al., 1995; Madanos et al., 2005; Panaro and Mitolo, 1999; Sabroe et al., 2003). Phagocytic leukocytes, particularly neutrophils, play a major role defending wound from invading pathogens (Martin, 1997). The initial wave of neutrophil chemotaxis in wound and toward infection involves the activation of formyl peptide chemokine receptors (FPRs) by N-formyl peptides, (e.g., fMLP), released either by injured tissue or by invading bacteria (Afonso et al., 2012; de Oliveira et al., 2016; Futosi et al., 2013; Harvath et al., 1991; Liu et al., 2012). In addition to chemotaxis, fMLP interaction with FPR chemokine receptors also activates bactericidal functions in neutrophils (such as superoxide and reactive oxygen species (ROS) production, phagocytosis, and degranulation (Devesse et al., 2009; Dorward et al., 2015; Sengeløv et al., 1994). Given the potent immunodulatory effects of fMLP, and, to our knowledge, fMLP never being tested locally to control infection, we evaluated fMLP in the context of PJI. We next assessed whether local fMLP treatment would be able to reduce infection using an established mouse PJI model (Bernthal et al., 2010; Bernthal et al., 2014; Hegde et al., 2017a; Thompson et al., 2018). We hypothesized that local administration of immunomodulator fMLPs that can accelerate and direct innate immune leukocyte responses (particularly neutrophils) toward the site of the surgically placed implant would shorten the lag phase needed to mount effective immune responses toward infection in that environment and be effective in controlling PJI even in the absence of prophylaxis antibiotics.

MATERIALS AND METHODS

Animal experimentation: We have an approval from the Rush University Medical Center Institutional Animal Care and Use Committee (IACUC No: 19-623) to conduct research as indicated. All procedures complied strictly with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA). We obtained C57BL/6J mice from the Jackson Laboratories (Bar Harbor, ME). These mice were allowed to acclimate to the environment for 1 week prior to experimentation.

Intra-articular (IA) injection: To demonstrate that N-formyl-methionyl-leucyl-phenylalanine (fMLP - a.k.a fMLF) immunomodulator is capable of mobilizing and directing neutrophil response in the knee joint even in the absence of infection and/or injury, 12-week-old C57BL/6J mice (Jackson Laboratory) received local intra-articular injection of fMLP (Sigma Aldrich, F3506) or vehicle control at the right knee joint. Prior to injection, right hindlimbs were shaved and scrubbed with 70% ethanol followed by iodine. fMLP was prepared at a concentration of (1µg / µL) in a solution of 25% dimethylsulfoxide (DMSO) and 75% phosphate buffered saline (PBS). Vehicle control was a 5 µL solution of 25% DMSO and 75% PBS. Mice were anesthetized with 2% isoflurane. A total of 5 µL of fMLP solution or vehicle control was injected into the right hindlimb knee joint using a 5 µL Hamilton microliter syringe (Hamilton, 7634-01) and 27-gauge
needle as previously described (Nagao et al., 2017). The dosing of 5 µg of fMLP was chosen based on its maximum solubility in vehicle solution and maximum volume of solution that could be injected into the knee joint space. The syringe needle was applied to the intra-articular space directly through the patellar ligament with the knee flexed at a 90° angle with needle administration perpendicular to the apex of the flexed knee. A total of n = 5 mice received fMLP, and a total of n = 5 mice received vehicle control. Mice were evaluated for local neutrophil response at the knee joint using in vivo bioluminescence imaging (BLI) at 2 hours and 6 hours post injection. Mice were imaged using IVBIBLI under 2% isoflurane anesthesia.

Preparation of bacteria: We used bioluminescent S. aureus (Xen36 S. aureus; Perkin Elmer) that was derived from parental strain S. aureus ATCC 49525 (Wright). This strain has been used and validated in mouse PJI models (Bernthal et al., 2010; Bernthal et al., 2014; Carli et al., 2017; Hegde et al., 2017a). Xen36 S. aureus has been genetically modified to express the modified Photorhabdus luminescens luxABCDE operon on the native plasmid which encodes a luciferase enzyme, enabling this strain to produce a bioluminescent signal. Bacteria were incubated in tryptic soy broth (TSB) overnight on the day prior to surgery. On the day of surgery, Xen36 S. aureus was diluted in TSB to a spectrophotometer absorbance measurement of 600 nm at an optical density of 0.5 (against a TSB blank); this is equivalent to ~1.0 x 10^8 CFU/mL of S. aureus. This solution was further diluted to 5 x 10^5 CFU/mL in PBS. This solution was kept on ice for the day of surgery. At the time of surgery dropwise inoculation with 2 µL of 5 x 10^5 CFU/mL in PBS at the open knee joint surgical site with a 5 µL Hamilton syringe provided 1x10^3 CFU at the knee joint.

Mouse model of periprosthetic joint infection (PJI): To show that an fMLP immunomodulator is able to reduce PJI, we used an established PJI model with bioluminescent Staphylococcus aureus as described previously (Bernthal et al., 2010; Carli et al., 2017; Hegde et al., 2017a). Briefly, 2 days prior to surgery, the ventral and lateral surface of the right hindlimb of 12-week-old C57BL/6J mice were shaved. To ensure additional removal of hair, Nair hair removal spray was used. The day prior to surgery, mice underwent baseline testing for intravital fluorescence bioimaging (IVBI), pain behavior, and weight-bearing, and weight. The day prior to surgery, all non-sterilized instruments and implant material were autoclaved in self-sealing autoclave pouches. On the day of surgery, mice were first anesthetized with a mixture of ketamine (90mg/kg) and xylazine (4.5mg/kg). The right leg was scrubbed with 70% ethanol followed by iodine. Using aseptic technique and under guidance of a dissection microscope (Zeiss, Stemi 508), a skin incision over the right knee was performed followed by a medial parapatellar arthrotomy. Incisions were performed with a Micro Knives sterile scalpel (Fine Science Tools, 10315-12). To expose the femoral condyles, lateral displacement of the quadriceps patellar complex was performed. The intercondylar notch was located; a 25-gauge syringe needle attached to a 3-mL syringe was used to penetrate the intercondylar notch and ream the distal intramedullary canal at a distance of approximately 45–100 mm. An orthopedic-grade stainless steel Kirschner wire (K-wire) (diameter 0.6mm; Depuy Synthes) was surgically placed in a retrograde fashion into intramedullary canal with assistance of a pin holder (Fine Sciences Tools, 26018-17). The distal aspect of the K-wire was cut to a length of approximately 11mm with a wire.
cutter leaving approximately 1 mm protruding into the joint space. An orthopedic-grade stainless steel Kirschner wire (K-wire) (diameter 0.6 mm and length 15 mm; DePuy Synthes) was surgically placed in a retrograde fashion into the intramedullary canal with assistance of a pin holder (Fine Sciences Tools, 26018-17). The distal aspect of the K-wire was cut with a wire cutter leaving approximately 1 mm protruding into the joint space.

With the open knee joint surgical site and implant exposed, in the fMLP group, mice received a local intra-articular dose of 5 µg of fMLP in a 5 µL solution (25% DMSO and 75% PBS); this was the fMLP dose previously used for intra-articular injection at the healthy mouse knee joint to substantially increase local neutrophil activity (Discussed in the results). In the control groups, mice received 25% DMSO / 75% PBS solution as vehicle control. A 5 µL Hamilton Syringe was used to drop the solution into the exposed knee joint space. The exposed solution was left untouched at the knee joint space was exposed to a lamp for 5 minutes to enhance drying absorption of the solution at the knee joint. Following drying absorption, the quadriceps complex was reduced back to midline. The knee joint capsule was closed with 6-0 vicryl sutures (Ethicon). The skin was then closed with 6-0 prolene sutures (Ethicon). Mice were placed on a warming blanket for recovery following surgery. Mice received subcutaneous buprenorphine (0.1 mg/kg) every 12 hours for a duration of 48 hours following surgery for analgesia following surgery. Following surgery, living mice received longitudinal assessments with in vivo bioluminescence imaging (IVBI) up to (days 1, 3, 5, 7 and 10), and von Frey filament testing (day 10), weight-bearing assessment (day 10), and weight assessment (day 10). Mice were sacrificed at day 10 for gross morphologic assessment, microCT analysis, and histologic analysis. In total, 35 mice received femoral implant placement in the PJI model: n = 5 implant control, n = 15 implant + S. aureus, n = 15 implant + fMLP + S. aureus. A total of n = 6 mice in each of the two infected groups were harvested at day 3 for CFU analysis; these mice received bioluminescence imaging IVBI up to day 3. The remaining mice (n = 5 implant control, n = 9 implant + S. aureus, n = 9 implant + fMLP + S. aureus) in all groups were harvested at day 10 and received IVBI up to day 10, as well as von Frey filament testing (day 10), weight-bearing assessment (day 10), and weight assessment (day 10). These mice were then harvested at day 10 for gross morphologic assessment, microCT analysis, and histologic analysis. 

Preparation of bacteria: We used a bioluminescent Staphylococcus aureus strain (Xen36 S. aureus; Perkin Elmer) that was previously isolated from a patient suffering from bacteremia. This strain...
has been genetically modified to express the modified *Photorhabdus luminescens luxABCDE* operon which encodes a luciferase enzyme, enabling this strain to produce a bioluminescent signal. Bacteria was incubated in tryptic soy broth (TSB) overnight on the day prior to surgery. On the day of surgery, Xen36 *S. aureus* was diluted in TSB to a spectrophotometer absorbance measurement of 600 nm at an optical density of 0.5 (against a TSB blank); this is equivalent to ~1.0 x 10^8 CFU/mL of *S. aureus*. This solution was further diluted to 5 x 10^5 CFU/mL in PBS. This solution was kept on ice for the day of surgery. At the time of surgery dropwise inoculation with 2 µL of 5 x 10^5 CFU/mL in PBS at the open knee joint surgical site with a 5 µL Hamilton syringe provided 1x10^3 CFU at the knee joint.

**In Vivo Bioluminescence Imaging (IVBIBLI):** IVIS® Lumina II In Vivo Imaging System (PerkinElmer) was used to track neutrophil activity at the knee joint as well as for quantification of a bacterial abundance at the knee joint. For the assessment of neutrophil response in mice receiving knee injection of fMLP or vehicle control, IVBIBLI was performed at baseline prior to injection, as well as at 2 hours and 6 hours post injection. At 8 minutes prior to each imaging time-point, mice received intra-peritoneal (I.P.) injection of 100 mg/kg of luminol (Sigma-Aldrich, A4685) in PBS solution. Luminol is a chemiluminescent compound that produces a bioluminescent signal in the presence of reactive oxygen species (ROS) catalyzed by myeloperoxidase (MPO) in neutrophils and has been used previously as a measure of neutrophil activity in the joints and subcutaneous tissue of mice (Gross *et al.*, 2009; Tseng and Kung, 2013).

In the mouse PJI model, IVBIBLI to quantify bioluminescent signal from bioluminescent *S. aureus* Xen 36 was performed at baseline (prior to surgery), as well as at on days 1, 3, 5, 7, and 10 post-surgery. There is direct correlation between Xen36 *S. aureus* light intensity and quantified tissue and implant bacterial burdened measured by CFU count at the site of the implant and surrounding tissue (Bernthal *et al.*, 2010). In all IVBIBLI experiments, exposure time was performed for a total of 5 minutes. A standard circular region of interest (ROI) with a diameter spanning from the distal 1/4th of the femur and proximal 1/4th of the tibia/fibula was used. The ROI used for both assessment of neutrophil activity and bacterial bioluminescent signal using IVBIBLI are represented as red outlines in (Fig. 1a and Fig. 1B). Emission intensity at the ROI over time was quantified using mean maximum flux (photons/s/cm^2/sr). Mice were imaged using IVBIBLI under 2% isoflurane anesthesia. A total of *n* = 15 mice in each of the two infected groups were analyzed with IVBIBLI. For CFU analysis, *n* = 6 mice in each of the infected groups were harvested at day 3 and only received IVBIBLI up to day 3. The remaining mice (*n* = 5 implant, *n* = 9 implant + *S. aureus*, *n* = 9 implant + fMLP + *S. aureus*) received IVBIBLI up to day 10. Presentation of images of IVBIBLI for each group were selected for representation of mean values of mean maximum flux (photons/s/cm^2/sr) at the ROI.

**Infection burden assessment by Colony Forming Unit (CFU) analysis at knee joint and implant:** A subset of *n* = 6 mice in each of the infected groups (*n* = 6 implant + *S. aureus* and *n* = 6 implant + fMLP + *S. aureus*) underwent CFU analysis following bioluminescence imaging on day 3 post-surgery. Briefly, mice were sacrificed at on day 3 post-infection, and the distal 1/4th of the femur and the proximal 1/4th of the tibia/fibula were cut and harvested to isolate the knee joint...
and surrounding tissue. Skin was dissected removed from the harvested knee joint, and the remaining bone and soft tissue was used for CFU analysis. The implant was removed in anterograde fashion from the cut end of the femur. Bacterial loads from processed tissue and implants were determined using serial dilution and plating as previously described (Goldufsky et al., 2015; Kroin et al., 2015; Kroin et al., 2012; Kroin et al., 2016; Kroin et al., 2018). Samples were diluted in PBS to produce dilutions ranging from 10^{-1} to 10^{-5} with 96-well microplates. Aliquots of ten-five microliters were spot plated at (10^{0}-10^{-5}) on tryptic soy agar plates and incubated at 37°C for 24 hours. CFU counts were quantified the following day. Bacterial burden was assessed as CFU per gram tissue for knee joint and surrounding tissue. Bacterial burden for tissue implants were assessed as CFU per implant.

**Gross Morphologic analysis:** Following sacrifice, mice underwent further imaging with the aid of a Zeiss (Stemi 508) dissection stereomicroscope. Gross morphology was assessed on all mice that were harvested at day 10 post-surgery (n = 5 vehicle control implant, n = 9 implant + S. aureus, and n = 9 implant + fMLP + S. aureus mice group). Images were performed following skin incision, to image the knee joint capsule, and the knee joint capsule was opened to image the distal femur and implant. Knee capsule width measurements were performed using image J (NIH) using a known reference length from each image. Presentation of images of gross morphology for each group were selected as average representatives within each group. Images were performed following skin incision, to image the knee joint capsule, and full capsule dissection to image the distal femur and implant. Knee width measurements were performed using image J (NIH) using a known reference length from each image. Presentation of images of gross morphology for each group were selected as average representatives within each group.

**Histologic analysis:** The distal aspect of the femurs and surrounding tissues were fixed in 4% formaldehyde for 3 days at 4°C and were stored in 70% ethanol at 4°C for microCT analysis. Following microCT analysis, tissues were then decalcified in 0.5M EDTA (pH 8.0) for 14 days at 4°C. Following decalcification, tissues were embedded in paraffin. Serial 5 μm sagittal sections were performed at the distal femur tissues. Sections underwent hematoxylin and eosin (H&E) staining. Sections were imaged using an Olympus (BX43) light microscope. Image J was used to quantify cortical widths and inflammatory areas. We evaluated maximum cortical width at the distal ventral femur. Maximum cortical width was defined as maximum distance from cortical bone at the ventral surface to the beginning of contiguous marrow space. Furthermore, inflammatory infiltration areas were measured using image J as the largest contiguous areas of inflammatory infiltrates at the ventral 1/3rd of the femur epiphysis. Identification of inflammatory tissue eroding into the bone or marrow space in a mouse PJI model has been previously described (Thompson et al., 2018). Identification of inflammatory infiltrate was based on the following criteria: i) bone destruction, ii) fibrosis, and iii) inflammatory infiltrate consisting of leukocytes representing chronic inflammation/osteomyelitis, as previously described (Thompson et al., 2018; Tiemann et al., 2014). Black outline of the regions of interest of the ventral 1/3rd of the epiphysis used to measure inflammatory areas are represented in figure 4a. Representation of the inflammatory infiltrate areas, used in our calculations, are demarcated by the orange outlines in figure 4a. Histologic analyses were assessed on all mice that were harvested on day 10 post-
surgery ($n = 5$ implant, $n = 9$ implant + $S. aureus$, and $n = 9$ implant + fMLP + $S. aureus$). Presentation of images of histology for each group were selected for representation based on mean values for maximum cortical width at the distal femurs as well as inflammatory infiltration areas.

**Histologic analysis:** The distal aspect of the femurs and surrounding tissues were fixed in 4% formaldehyde for 3 days at 4°C and. This tissue was were stored in 70% ethanol at 4°C for microCT analysis. Following microCT analysis, tissues was were then decalcified in 0.5M EDTA (pH 8.0) for 14 days at 4°C. Following decalcification, tissues was were paraffin embedded in paraffin. Serial 5 µm sagittal sections were performed at the distal femur tissues. Sections underwent hematoxylin and eosin (H&E) staining. Sections were imaged using an Olympus (BX43) light microscope. Image J was used to quantify cortical widths and inflammatory areas. We evaluated maximum cortical width at the distal ventral femur, (defined as maximum distance from cortical bone at the ventral surface to the beginning of contiguous marrow space). Furthermore, inflammatory cell and tissue infiltration areas were measured using Image J as the largest contiguous areas of inflammatory infiltrates at the ventral 1/3 of the femur epiphysis.

Identification of inflammatory in the distal femur - from distal end of femur to the distal end of the growth plate - and the ventral 1/3 of the femur that represented inflammatory cell and fibrotic inflammatory tissue eroding tissue eroding into the bone or marrow space in a mouse PJI model as has been previously described (Thompson et al., 2018). Identification of inflammatory infiltrate consisting of leukocytes, representing chronic inflammation/osteomyelitis, as previously described (Thompson et al., 2018; Tiemann et al., 2014) and representing chronic inflammation/osteomyelitis. Black outline of the regions of interest of the distal femur ventral 1/3 of the epiphysis used to measure inflammatory areas was represented in figure 4a. Representation of the inflammatory infiltrate areas, used in our calculations, calculated is represented demarcated by the orange outlines in figure 4a. Histologic analyses were assessed on all mice that were harvested aton day 10 post-surgery ($n = 5$ vehicle control implant, $n = 9$ implant + $S. aureus$, and $n = 9$ implant + fMLP + $S. aureus$ mice groups). Presentation of images of histology for each group were selected for representation, based of mean values for maximum cortical width at the distal femurs as well as inflammatory cell and tissue infiltration areas.

**Micro computed tomography (MicroCT) assessment:** The distal 1/4th of fixed femur tissues at the implant leg day 10 post-surgery were assessed with microCT (Scanco, µCT50). MicroCT was assessed on all mice that were harvested at day 10 post-surgery ($n = 5$ vehicle control implant, $n = 9$ implant + $S. aureus$, and $n = 9$ implant + fMLP + $S. aureus$ mice groups). Three dimensional (3D) as well as mid-coronal microCT sections of the distal femur were evaluated. A maximum width of the distal femur was evaluated using a ventral view 3D microCT image and Image J. Increased maximum distal femur width has been previously used as a measure for infection in a mouse PJI model (Thompson et al., 2018) - 3D images and mid-coronal microCT sections were used to evaluate the following scoring criteria: i) periosteal reaction; ii) focal cortical loss; iii) trabecular loss; as well as total PJI scores. Total PJI score for each femur was the addition of three scores: i) periosteal reaction; ii) focal cortical loss; iii) trabecular loss. These scores were determined on a scale of 0-2, with 2 being the worst or most severe score. **Subjective scoring**
Criteria of bone were developed based on previous clinical radiographic evidence of PJI (Bauer et al., 2006) and mouse PJI model radiographic features and scoring (Carli et al., 2017). Criteria for the following periosteal reaction scores at the distal femur are as follows: 0) no or minimal periosteal reaction restricted to small regions; 1) moderate periosteal reaction with limited changes in cortical surface dimension and congruity; 2) severe and extensive spread of periosteal reaction with moderate to severe changes in cortical surface dimensions and congruity. Criteria for the focal cortical loss scores at the distal femur are as follows: 0) no or minimal areas of focal cortical loss; 1) definitive areas of focal cortical loss found in small regions of the distal femur containing near or full thickness cortical bone loss; 2) severe focal cortical loss causing full thickness cortical bone loss spread over large region(s) such as a femoral condyle. Criteria for trabecular loss score at the distal femur are as follows: 0) no or minimal loss of trabecular bone; 1) moderate loss of trabecular bone; 2) severe loss of trabecular bone. Scoring was performed with two blinded observers, and an interclass intraclass correlation (ICC) was calculated to estimate inter-rater reliability. ICC values were as follows: i) periosteal reaction (ICC 0.969; 95% CI 0.926-0.987); ii) focal cortical loss (ICC 0.986; 95% CI 0.966-0.994); iii) trabecular loss (ICC 0.929; 95% CI 0.832-0.970); iv) total PJI score (ICC 0.970; 95% CI 0.928-0.987).

Presentation of images of microCT for each group were selected for representation based on mean values for maximum distal femur width, as well as scored parameters: periosteal reaction, focal cortical loss, and trabecular loss.

**Weight-bearing assessment:** In addition to pain, impaired joint function is a common clinical symptomatic feature of PJI (Izakovicova et al., 2019). Weight-bearing at the implant leg was used as measure of pain and joint function. Mice were assessed for weight-bearing at the right hindlimb at baseline (pre-surgery) and at day 10 post-surgery. Weight-bearing was assessed on all mice that were harvested at day 10 post-surgery (n = 5 vehicle control implant, n = 9 implant + S. aureus, and n = 9 implant + fMLP + S. aureus mice group); baseline assessments for all of these mice were performed as well. Mouse weight-bearing was captured using slow motion video recording software on iPhone 10 as previously described (Carli et al., 2017). Grading at the right hindlimb was as follows: full weight-bearing (3 points); partial weight-bearing (2 points); toe-touch (1 point); non-weight-bearing (0 points). Detailed scoring criteria are illustrated in Video (online). Scoring was performed with two blinded observers, and ICC was 0.972; 95% CI 0.949-0.984.

**Pain behavior assessment by (von Frey filament testing):** One of the most common initial clinical findings or symptoms of PJI is pain (Izakovicova et al., 2019; Tande and Patel, 2014). As a measure of pain sensitization behavior in mice, mechanical allodynia was assessed using von Frey filament testing as previously described (Im et al., 2010; Miller et al., 2012). Mice can demonstrate allodynia, or pain behavior—(demonstrated for instance by leg withdrawal)—in response to normally innocuous stimulus, through application of various levels of mechanical stimulus (von Frey filaments applied at the plantar hindpaw) (Deuis et al., 2017). Mice were placed on top of a metal mesh stand (IITC mesh stand part# 408) within a small, weighted plastic enclosure. Calibrated von Frey filaments (Stoelting Touch Test Sensory Evaluator Kit) ranging from filament forces of 2.44 to 4.74 grams were used. Filaments were applied to the
plantar hindpaw with a force requiring the filament to bow. Filaments were held at the plantar surface for three seconds or until a pain withdrawal response was displayed. A modified up down method was used to calculate the force required to elicit withdrawal of the paw, which was quantified as paw withdrawal threshold (PWT) force in grams (g). Pain behavior was assessed on all mice that were harvested at day 10 post-surgery ($n = 5$ vehicle control implant, $n = 9$ implant + 	extit{S. aureus}, and $n = 9$ implant + fMLP + 	extit{S. aureus} mice group); baseline assessments for all of these mice were performed as well.

**Weight:** Mice were assessed for weight at baseline (pre-surgery) and at day 10 post-surgery. Weight measurements were taken to further evaluate a potential response of the mice to both surgery and infection as described previously (Carli et al., 2017), and to fMLP treatment. Weight was assessed in all mice that were harvested at day 10 post-surgery ($n = 5$ implant, $n = 9$ implant + 	extit{S. aureus}, and $n = 9$ fMLP + 	extit{S. aureus} mice). Weight was calculated in grams with an electronic balance (Ohaus, Scout SPX).

**Statistical analysis:** Statistical analysis for ICC was performed using SPSS statistics software version 27. The remainder of statistical analyses was performed using Prism software version 8. Comparison between two groups was evaluated with unpaired student’s $t$-test. Comparisons between more than two groups were evaluated with one-way ANOVA with post-hoc Tukey’s multiple comparison test. Comparisons of more than two groups over time were evaluated with mixed-effects analysis with post-hoc Tukey’s multiple comparison test. Data were represented as Mean ± SEM. Threshold for significance was set $p < 0.05$.

**RESULTS**

**fMLP treatment increases neutrophil activity at the knee joint.** To assess whether fMLP can initiate and direct inflammatory responses toward the implant surgical site, even in the absence of injury and infection, we hypothesized that administration of immunomodulators that can accelerate and direct innate immune leukocyte responses (particularly neutrophils) toward the site of the surgically placed implant would shorten the lag phase needed to mount effective immune responses toward infection in that environment and be effective in controlling PJI even in the absence of prophylaxis antibiotics. To evaluate our hypothesis, we chose N-formylmethionyl-leucyl-phenylalanine (fMLP) immunomodulator for these studies. fMLP is a formyl peptide, which is released at surgical sites from both injured tissues and invading bacterial pathogens (Li et al., 2016; Raoof et al., 2010), and functions as a potent chemoattractant for neutrophils and other inflammatory leukocytes (Balazovich et al., 1996; Derian et al., 1995; Madianos et al., 2005; Panaro and Mitolo, 1999; Sabroe et al., 2003).

We first assessed whether exogenous application of fMLP would be able to stimulate neutrophil recruitment in a healthy mouse knee joint in the absence of infection or surgery. Mice received either fMLP or vehicle control at the right knee joint by intra-articular injection, and
neutrophil response was assessed at baseline (0h) or at 2h or 6h post injection by in vivo bioluminescence imaging (IVBI), using luminol. Neutrophil response was assessed by in vivo In Vivo Bioluminescence Imaging (IVBIBLI), as described in Materials and Methods, using luminol, which produces a bioluminescent signal in the presence of reactive oxygen species (ROS) catalyzed by myeloperoxidase (MPO) in neutrophils (Gross et al., 2009; Tseng and Kung, 2013). IVBIBLI performed at baseline prior to injection demonstrated minimal to no neutrophil activity at the knee joint in either group. As compared to the mock group, mice receiving fMLP exhibited ~ 2-fold higher neutrophil response at 2h timepoint and 3-fold higher neutrophil response at 6h timepoint as assessed by IVBIBLI (Fig. 1a-b). These results demonstrated that fMLP is able to mobilize and direct a neutrophil response toward the knee environment even in the absence of infection or surgery.

fMLP treatment reduces S. aureus infection in a mouse model of periprosthetic joint infection (PJI). To show that an fMLP immunomodulator is able to reduce PJI, we used an established PJI model with bioluminescent Staphylococcus aureus as described previously (Bernthal et al., 2010; Carli et al., 2017; Hegde et al., 2017a) and in the Materials and Methods. We next assessed whether fMLP treatment would be able to reduce infection using an established PJI model (Bernthal et al., 2010; Carli et al., 2017; Hegde et al., 2017a). Mice underwent knee implant surgical procedure as described in the Materials and Methods. They were then divided into three groups: i) implant alone (n = 5); ii) implant + S. aureus (n = 15); iii) implant + fMLP + S. aureus (n = 15). In line with previous report (Bernthal et al., 2010), infection bacterial bioluminescent signal was significantly significantly higher in infected mice receiving vehicle as compared to non-infected mice at all timepoints, peaking at day 3 post-infection but declining overtime and, plateauing at on day 7-10 post-infection (Fig. 2a-b). Importantly, i

In contrast, infused mice receiving fMLP prior to infection trended toward lower infections at days 1 and 5 but had significantly reduced bacterial bioluminescent signal at on day 3 and trended toward reduced bacterial bioluminescent signals on days 1 and 5, as compared to mice receiving vehicle alone prior to infection and trended toward reduced bacterial bioluminescent signal at days 1 and 5. (Fig. 2a-b, p < 0.001). Given that the peak infection occurred at on day 3 in both mock and fMLP-treated mice, we harvested infected implants and tissue surrounding implants from mice as described in Materials and Methods, and assessed them for their bacterial infection burden by CFU analysis, as described (Goldufsky et al., 2015; Kroin et al., 2016; Kroin et al., 2018) determination using serial dilution and plating method as described previously (Goldufsky et al., 2015; Kroin et al., 2015; Kroin et al., 2012; Kroin et al., 2016; Kroin et al., 2018). CFU analysis analyses of the knee joint tissues at on day 3 post-surgery revealed an approximate 2-log order reduction of S. aureus bacterial numbers in the group that received fMLP as compared to the infected group without fMLP (Fig. 2c, p < 0.01). There was also a trend in reduction of CFUs on the implant itself in the group that received fMLP, but it did not reach statistical significance. Collectively, these data indicated that fMLP immunomodulator is able to reduce acute S. aureus peak infection in the PJI model, by mobilizing and directing immune leukocytes toward the surgical site in knee.
fMLP treatment reduces the pathologic effects of *S. aureus* infection at the knee joint tissue and bone in a mouse. Bone destruction and joint dysfunction are common clinical signs of PJI (Bozhkova et al., 2020; Izakovicova et al., 2019; Lima et al., 2013) (Carli et al., 2017; Izakovicova et al., 2019). Although, it was encouraging that fMLP was able to reduce infection in the PJI model, it remained a possibility that increased inflammatory environment due to the infection and the fMLP treatment could have dire long-term consequences causing damage and pathology to the knee joint and bone and the surrounding tissues. To address this concern, we next collected the knee joints and the bone tissues surrounding the implants at day 10 post-infection and evaluated them for the impact of infection and/or with or without the fMLP treatment.

A striking feature of knee joints in infected mice, particularly in the group without fMLP treatment, was their overall large sizes as compared to non-infected knee joints (Fig. 3a-b). This increased size was reflective of increased abscess formation within the knee-joint capsule. Infected mice without fMLP treatment had the greatest knee capsule width, which was significantly larger than non-infected mice (Figure 3a-b, p < 0.01). While the fMLP treatment group trended toward having a smaller knee capsule width as compared to mock-treated group, these differences did not reach statistical significance (Fig. 3a-b). Upon dissecting open the knee joint capsule and removing abscess debris, we next evaluated the distal femur surrounding the implant (Fig. 3a-c). In all groups, there appeared to be some level of adhesive tissue to the distal femur, which was likely a consequence of tissue reaction to implant surgery. In the implant group, characteristic features of the distal femur, such as the femoral condyles were abundantly clear. Infected mice receiving fMLP also appeared to retain somewhat natural morphology of the distal femur and femoral condyles; however, as compared to the non-infected group, there appeared to be greater adhesive tissue to the femoral condyles in this group. Mice infected without fMLP treatment had the highest level of adhesive tissue and abnormal morphology of the distal femur of any group. This was most clearly evidenced by loss of rounded contours of the femoral condyles in the infected group without fMLP, as indicated by black arrows (Figure 3a, black arrows).

To get a greater understanding of the effects of infection with or without fMLP treatment on the distal femur, we performed histologic analysis using hematoxylin and eosin (H&E) staining of the distal femur by hematoxylin and eosin (H&E) histological analyses on day 10 post-infection, as described previously (Citation) and in Materials and Methods. We evaluated maximum cortical width at the distal ventral femur, (defined as maximum distance from cortical bone at the ventral surface to the beginning of contiguous marrow space). The fMLP-treated group had significantly smaller cortical bone width as compared to the infected group without fMLP treatment (Fig. 4a-b, indicated by black dotted lines in magnified regions in orange boxes). Increased cortical width was likely a result of stimulation of bone production (periosteal reaction) from the overlying periosteum due to chronic inflammation, as has been reported in the context of infection and inflammation (Rana et al., 2009). The implant alone group without infection had the smallest cortical width.
As inflammatory infiltrate has been found eroding into the distal femur in a mouse PJI model (Thompson et al., 2018), prompting us to infection has been shown to cause severe damage to bone surrounding implants, due to increased inflammation (Citation), we assessed the pathological impact of infection with or without fMLP treatment on these effects. Inflammatory responses and on bone health. Data indicated that Furthermore, inflammatory cell and tissue infiltration area was measured using image J as the largest contiguous area in the distal femur—from distal end of femur to the distal end of the growth plate—and the ventral 1/3 of the femur that represented inflammatory cell and fibrotic inflammatory tissue eroding into the bone or marrow space. This inflammatory infiltrate was ubiquitously found and represented at the bone-implant interface found at the eroding into the distal femur, which has been previously demonstrated in a mouse PJI model (Thompson et al., 2018). Within this region, the standard defined region of interest (ROI) ROI in the ventral 1/3rd of the femur epiphysis, the mock infected group without fMLP had the greatest area of inflammatory cell and tissue infiltration, which was significantly higher than the fMLP-treated infected group (Figure 4a and 4b, p < 0.05, black arrow in magnified regions in blue boxes). In contrast, the uninfected implant group alone had the smallest regions of inflammatory cell and tissue infiltrate, which was likely a result of surgery and implant placement (Fig. 4a-b). At the area of interest in the distal femurs evaluated within the ventral 1/3rd of the femur epiphysis, the largest contiguous areas of inflammatory infiltrates were found at the bone-implant interface in all groups.

We next assessed the pathological impact of infection with or without fMLP treatment on bone by microCT, as described previously (Citation) and in Materials and Methods, as described in Materials and Methods. On ventral microCT 3D view, maximum width of the distal femur was measured. This was in particular used as a measure to quantify periosteal reaction, which would widen the femur width. Similar to non-infected group, the fMLP-treated group had significantly reduced femoral width as compared infected group without fMLP (Fig. 5a-b, p < 0.01, indicated by yellow dashed lines). The 3D images of the side view, dorsal view, and condyle view, as well as mid-coronal microCT sections were evaluated by two blinded observers and scored for i) periosteal reaction, ii) focal cortical loss, iii) trabecular loss, as well as combination of all three parameters were indexed as a combined microCT PJI score.

As compared to the infected group treated with vehicle, the fMLP-treated group exhibited significantly lowered peristeal reaction (Fig. 5a-b, indicated by green arrows, p < 0.01), and focal cortical loss (Fig. 5a-b, indicated by purple arrows, p < 0.05). Trabecular loss was also significantly higher in the infected group without fMLPs, as compared to non-infected group (p < 0.001), and trended higher as compared to the infected treated group treated with vehicle as compared to the fMLP group, but this effect did not reach statistical significance (Fig. 5a-b, indicated by red arrows). When all three parameters were combined into a net combined PJI microCT score, it was found the fMLP treatment significantly reduced the combined PJI microCT pathology score as compared to the infected group without fMLP treatment (Fig. 5b, p < 0.01). Collectively, these data indicated that fMLP treatment significantly
protected the periprosthetic joint tissue from the pathological impact of *S. aureus* PJI by lowering the infection burden.

fMLP improves behavioral symptoms in a PJI mouse model. Pain and joint dysfunction are a common clinical symptom associated with PJI in patients (Bozhkova et al., 2020; Izakovicova et al., 2019; Lima et al., 2013). While presence of bacteria, inflammation and immune cell responses, as well as tissue destruction are the hallmark pathologic features of PJI (Masters et al., 2019; Parvizi et al., 2011), one of the most common initial finding or symptom of PJI is pain (Izakovicova et al., 2019; Tande and Patel, 2014). Impaired joint function is also a common symptomatic feature of PJI (Izakovicova et al., 2019). Given our findings of reduced bacterial burden and subsequent reduced bone and tissue destruction in mice receiving fMLP, we wished to assess if fMLP treatment also positively impacted animal behavior with respect to pain and joint function. We evaluated pain behavior in these mice by two methods: weight-bearing on the right hindlimb (implant leg) and mechanical allodynia with von Frey filament testing, as previously described (Carli et al., 2017; Im et al., 2010; Miller et al., 2012) and in Materials and Methods. Weight-bearing was also used as an assessment for joint function (Carli et al., 2017). The weight-bearing animal behavior scoring methodology is discussed in the Methods and Materials and illustrated in the Video.

At baseline (Day 0), and prior to implant placement (Day 0), mice in all groups exhibited similar weight-bearing score and had the highest possible calculated withdrawal force threshold for evidence of mechanical allodynia (Fig. 6a-b). In contrast, at day 10 post-surgery and treatment, fMLP-treated infected mice exhibited significantly improved weight-bearing score on the implant leg as compared to infected mice without fMLP (Fig. 6c-d, *p* < 0.001). Furthermore, mice receiving fMLP, also exhibited significantly improved (increased) withdrawal force threshold as compared to infected mice without fMLP treatment (Fig. 5d-e, *p* < 0.05). Collectively, these data indicated that fMLP treatment significantly improves the behavioral symptoms that are associated with PJI (Bozhkova et al., 2020; Izakovicova et al., 2019; Lima et al., 2013).

fMLP treatment did not affect weight in a mouse PJI model. Prior to surgery, all groups had the same similar average weight (Fig. 6c). At day 10 post-surgery, all groups trended slightly toward having lower weight; however, this was not statistically significant. Furthermore, at day 10 post-surgery, infected mice without fMLP treatment trended toward slightly greater weight loss; however, this weight loss was not statistically significant between groups (Fig. 6f).

DISCUSSION

Despite many advances in infection control practices—including prophylaxis antibiotics—periprosthetic joint infection (PJI) remains a devastating complication after arthroplasty, leading to pain, suffering, morbidity and a substantial economic burden (Kapadia et al., 2016; Kurtz et al., 2012). There is an urgent need for alternative and/or adjunct measures to prophylactic antibiotics in addressing PJI. In this report, we sought to assess whether
immunomodulators that fMLP, which can harness and direct host innate immune responses (a potent immunomodulator that recruits and activates inflammatory leukocytes, particularly neutrophils) toward surgical implant placement, would be able to control PJI even in the absence of prophylactic antibiotics. We evaluated fMLP immunomodulator for this purpose. Our results data indicate that fMLP treatment significantly is highly effective in reducing S. aureus mediated infection in an established PJI model. Furthermore, fMLP can significantly protect against reduced infection-induced bone and tissue pathologies, and subsequently reduced and it can reduce infection-induced pain and improve weight-bearing behavior in infected animals.

Although, infection was significantly reduced in fMLP-treated mice, it was not completely abolished in this model. Whether this was due to low level of fMLP used in our studies, or the inability of fMLP to engage the adaptive immune responses which are also needed to fully control S. aureus infection (Lin et al., 2009) remains to be investigated. Intriguingly, administration of systemic vancomycin prophylaxis or intra-articular vancomycin powder treatment alone also failed to completely eradicate S. aureus infection when applied alone, but when combined together, they were highly effective in abolishing S. aureus PJI in rats, although they were more effective in reducing infection when combined (Edelstein et al., 2017). We posit that since antibiotics and immunomodulators (e.g., fMLP) combat infection through different mechanisms of action, combination treatment with both may also be more effective in eliminating PJI. Future studies are needed. It would be interesting to investigate whether fMLP in combination with prophylaxis or topical antibiotics would be more effective in controlling PJI. We posit that since antibiotics and immunomodulators (e.g., fMLP) combat infection through different mechanisms of action, combination treatment with both may be more effective in eliminating PJI.

It is encouraging that fMLP treatment reduced infection burden early after surgery and infection, given that bacteria have been shown to form biofilm on the implant surfaces early after infection (Carli et al., 2017; Lamret et al., 2020). Indeed, it has been demonstrated that higher neutrophil to S. aureus bacteria ratio at the implant surface is a determinative prognostic factor for reduced biofilm production at the implant surface (Ghimire et al., 2019). Furthermore, in regard to S. aureus biofilms, neutrophils are able to clear early immature biofilm formation; however, this sensitivity of biofilm to neutrophils is diminished with biofilm maturation and increased biofilm biomass (Gunther et al., 2009). Bacterial pathogens, including S. aureus, embedded in fully mature biofilms are protected from neutrophil killing when embedded in fully mature biofilms (de Vor et al., 2020; Gunther et al., 2009; Kristian et al., 2008). Due to the concern of early biofilm formation on the implant surface itself, we suggest propose that coating the implant with fMLP might better facilitate mobilize neutrophil recruitment directly to the implant interface, thus increasing neutrophil to S. aureus bacteria ratio at the implant surface, to prevent early biofilm formation. Future studies are needed to assess the impact of fMLP therapy (administered intra-articularly or via implant coating; alone or in combination with antibiotics) on biofilm production.

Inflammation and inflammatory cell activities responses are proportional to the bacterial burden and infection level and can culminate in severe tissue destruction (Bernthal et al., 2010;
Chronic inflammation in the setting of infection can suppress osteoblast activity and enhance osteoclast activity, and *S. aureus* infection can directly cause bone destruction as well as activate osteoclasts and inhibit osteoblasts leading to altered bone remodeling (Wright and Nair, 2010). We found substantial evidence of inflammation and bone loss and destruction particularly in the infected group without fMLP, as assessed by gross morphology, microCT, and histology. Furthermore, in the setting of osteomyelitis, periosteal reaction can occur through subperiosteal spread of inflammation which in turn elevates and stimulates the periosteum to lay down new layers of bone (Rana et al., 2009). Consistent with this report, we also found significant increases in inflammation and periosteal reaction in our mouse PJI model, as assessed by microCT, and histological analyses. Importantly, fMLP lowered these infection-induced bone pathologies.

Common clinical signs of PJI include joint pain and joint dysfunction (Bozhkova et al., 2020; Izakovicova et al., 2019; Lima et al., 2013)(Carli et al., 2017; Izakovicova et al., 2019). Intriguingly, infected mice treated with fMLP exhibited significant reduction in pain behavior and significant improvement in weight-bearing and reduced pain behavior, further highlighting the positive impact of fMLP therapy in reducing PJI peak bacterial load reduction at day 3 on CFU and IVBI analysis in the articular joint by fMLP immunomodulator. Severe weight loss can be a sign of systemic infection with bacteria such as *S. aureus* (Wu et al., 2017). We found a slight trend in weight loss among both infected and non-infected groups, although these differences did not reach statistical significance, suggesting that *S. aureus* infection in this model remains local. In line with our data, Carli et al., in a similar PJI mouse-*S. aureus* model infection study, reported using Xen36 *S. aureus*, infected and non-infected mice were weighed weekly for six weeks. Mice in both the infected and non-infected groups had lower weight at week one post-surgery but had similar weight at day 10 as pre-surgical weight at weeks 2-6; additionally, at all time points, there were no significant differences in weights between the infected and non-infected groups, although both groups exhibited slightly lower weight at week one post-surgery (Carli et al., 2017). Similarly, in our study, at day 10 as compared to pre-surgery, we found a slight trend in weight loss among all group that was not statistically significant. Furthermore, there were no statistically significant differences between all groups at day 10. Severe weight loss can be a sign of severe systemic infection with bacteria such as *S. aureus* (Wu et al., 2017). We likely did not find these weight differences due to the localization of the infection primarily to surgical knee joint over 10 days.

There is minimal literature to date on the therapeutic use of fMLP. Interestingly, Shin et al., evaluated the impact of local treatment with fMLP (delivered in a hyaluronic acid gel carrier) in a rabbit calvaria defect model and demonstrated that fMLP promoted osteogenesis and bone formation at the defect site as compared to vehicle control (Shin et al., 2011). Of note, no evidence of increased inflammation was found at the defect site in rabbits treated with fMLP at 4 weeks post treatment (Shin et al., 2011), suggesting that fMLP may have a positive effect on bone formation and healing after arthroplasty even in the absence of infection. Further studies should evaluate the positive or adverse impacts of local fMLP therapy on joint and surrounding tissue, as well as animal behavior, in an uninfected periprosthetic implant model cohort in the PJI model.
after arthroplasty, to further lay the groundwork for its therapeutic use to combat PJI. There is minimal literature to date reporting on the therapeutic use of fMLP. Interestingly, Shin et al., 2011 evaluated use of local application of fMLP in vitro in a rabbit calvaria defect. Use of the impact of local treatment with fMLP (local application of fMLP delivered in a hyaluronic acid gel carrier) in a rabbit calvaria defect model and demonstrated that it promoted osteogenesis and bone formation at the defect site as compared to vehicle control (Shin et al., 2011). Of note, no evidence of increased inflammation was found at the defect site in rabbits treated with fMLP were sacrificed at 4 weeks post treatment, and no evidence of severe inflammation was found at the defect site (Shin et al., 2011). While this report suggests that fMLP may have positive effect on promotes bone formation and healing after arthroplasty, further studies should evaluate the positive or adverse impacts of local fMLP treatment with fMLP on joint and surrounding tissue, as well as animal behavior, in uninfected periprosthetic implant model after arthroplasty, to further lay the groundwork for its therapeutic use including bone, given the robust ability of fMLP to stimulate the innate immune response. Additional studies are warranted to evaluate local fMLP on uninfected joints, and further studies are required to see if local fMLP treatment would have local or systemic adverse outcomes prior to consideration of a clinical application.

Immunomodulators have been previously shown to reduce infection in the setting of musculoskeletal infection. Use of motifs present in bacterial DNA, such as CpG (cytosine-phosphate-guanosine) oligodeoxynucleotide (CpG ODN) sequences, are known to stimulate innate and adaptive immune responses, including production of proinflammatory cytokines (Kanzler et al., 2007; Krieg, 2006). Sethi et al., have 2015 reported that in rats with a surgically placed tibial intramedullary implant with S. aureus infection, administration of CpG (cytosine-phosphate-guanosine) oligodeoxynucleotide (CpG ODN), which is found in bacterial DNA and shown to trigger inflammatory responses, CpG ODN led to ~67% an early reduction of infection burden at the tissue and implant early after infection, but did not significantly reduce prevent the development of chronic infection over time (Sethi et al., 2015). Our results are in line with these findings showing that fMLP significantly reduced early infection by nearly 2-log order on day 3 at the tissue implant perhaps setting up favorable conditions for traditional (i.e. antibiotic) treatments but did not completely abolish prevent persistent infection. Another avenue that has been explored to inhibit infection is using mechanisms primarily of the adaptive immune response through vaccination for instance against S. aureus or treatment with antibodies against S. aureus; the benefits of these studies have been limited due to S. aureus having a wide variety of virulence factors or antigens comparative to what virulence factors or antigens are targeted with vaccination or antibody treatment (Anderson et al., 2012; Muthukrishnan et al., 2019). Furthermore, not all antibodies against S. aureus antigens provide a protective benefit (Muthukrishnan et al., 2019).

CONCLUSION
Our proof-of-concept studies provide direct evidence in a mouse PJI model that fMLP immunomodulators that can mobilize and direct innate immune responses toward the surgical implant site will be effective in reducing acute infection and protecting against infection-induced bone and tissue damage and associated pain. Immunomodulators such as fMLP may provide an alternative or adjunct treatment therapeutic to prophylactic antibiotics for the prevention or treatment of PJI. Future studies should focus on optimization of immunomodulator-based approaches such as immunomodulator-fMLP dose assessment, implant coating with immunomodulator-fMLP, or combination of immunomodulator-fMLP with prophylactic antibiotics or other immunomodulators.
ACKNOWLEDGEMENTS: We would like to thank Ryan Ross and Rylan Martin for assistance with microCT; Yumei Lei for assistance with histology; and Lorenzo Girotto for assistance with surgery and tissue harvest. This work was supported by the National Institutes of Health (NIH) grant T32AR073157 to (to J.H. and M.W); the Grainger Chair of the Rush Arthritis and Orthopedics Institute Fund (to M.W), and NIH grants RO1DK107713 and RO1AI150668 and RO1DK107713 (to S.H.S).
**FIGURE LEGENDS**

**Fig. 1.** fMLP increases neutrophil activity at the articular joint. a) fMLP (5µg / 5µL) or vehicle (5 µL PBS + 25% DMSO) were injected into the right knee joint. Neutrophil recruitment was assessed by IVBI-BLI prior to knee injection (baseline), or at 2 hours and 6 hours post intra-articular knee injection and 8 minutes post I.P. luminol injection at each time point. Red dashed circle represents ROI used for IVBI-BLI quantification. b) Corresponding tabulated data are shown as the Mean ± SEM. (n = 5 mice/group, *p < 0.05, Student’s t-test).

**Fig. 2.** fMLP treatment reduces *S. aureus* infection in a mouse model of PJI. a) Mice received a surgically placed femoral implant and treated with vehicle alone, 10^3 Xen36 *S. aureus*, or fMLP + Xen36 *S. aureus* as described in Materials and Methods. Infection burden was assessed by IVBI BLI performed at baseline, as well as at days 1, 3, 5, 7, and 10 post-surgery and treatment. Red dashed circle represents ROI used for IVBI-BLI quantification. b) Corresponding tabulated data for mean maximum flux (photons/s/cm^2/sr) at the region of interest (ROI) are shown as the Mean ± SEM. (n = 5-15 mice/group, *p < 0.05, mixed-effects analysis with post-hoc Tukey’s multiple comparison test). c) Quantification of bacterial CFUs at the knee joint tissue and femoral implant at day 3. Data represented as the Mean ± SEM. (n = 6, *p < 0.05, Student’s t-test).

**Fig. 3.** fMLP reduces the pathological effects of infection at the knee joint tissue surrounding the implant. a) Gross morphologic assessment was performed at the knee joint capsule (knee capsule width in millimeters / measure of intra-capsular abscess) and distal femur for cartilage/bone erosion surrounding the implant at animal sacrifice on day 10 post-surgery and treatment. Yellow dashed line represents maximum knee capsule width. Black arrows represent region of the femoral condyles. b) Knee capsule width plotted as the Mean ± SEM. (n = 5-9, *p < 0.05, one-way ANOVA with post-hoc Tukey’s multiple comparison test).

**Fig. 4.** fMLP protects against infection and inflammatory induced bone damage and changes around the implant on histologic analysis. a) At day 10 post-surgery and treatment, mice were harvested for histologic assessment of the distal femur by H&E staining. Two parameters identified in the distal femur were dramatically higher in the infection group without fMLP, which were maximum cortical width (measure of traditional cortical bone in addition to new adjacent bone formation formed by periosteal reaction / yellow magnification / black dashed line) and inflammatory infiltrate (blue magnification / black arrow). Inflammatory infiltrate was measured as the largest contiguous area of inflammatory infiltrate in the ventral 1/3rd of the distal femur epiphysis from distal end of femur to the distal end of the growth plate (green star) - and the ventral 1/3 of the femur. Within blue magnification, ROI for inflammatory infiltrate outlined with black solid line and inflammatory area outlined by orange line. b) Quantification of maximum cortical width and inflammatory infiltrate. Data represented as mean ± SEM. (n = 5-9, *p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA with post-hoc Tukey’s multiple comparison test). Black error bar = 200 µm; Orange error bar = 70 µm; Blue error bar = 85 µm.
**Fig. 5.** fMLP protects against infection and inflammatory induced bone damage and changes around the implant on microCT analysis. Mice were sacrificed at day 10 post-surgery and treatment, and femurs at the implant sites their implant knee bones were evaluated for pathological features as follows. a) Distal femurs were assessed by microCT analysis on 3D imaging and mid-coronal sections. Green arrows point to periosteal reaction; purple arrows point to focal cortical loss; and red arrows point to trabecular loss. Yellow dashed line represents cortical width. b) The microCT quantification of maximum femur width in millimeters (based on ventral view 3D image), as well as peristomal reaction score, focal cortical loss score, and trabecular loss score, which were analyzed using scoring criteria based on assessment of 3D images and mid-coronal sections with two blinded observers. Scoring criteria were then quantified and combined as a single measure called combined microCT PJI score. Data represented as mean ± SEM. (n = 5-9, *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA with post-hoc Tukey’s multiple comparison test).

**Fig. 6.** fMLP treatment improves weight-bearing, decreases pain behavior, and has no effect on weight in a mouse model of PJI. Baseline assessment for weight-bearing (a) pain behavior (mechanical allodynia) on von Frey filament testing (b) and weight (c) performed prior to surgery or treatment (day 0). Assessment of weight-bearing (d) pain behavior (e) and weight (f) performed at day 10 post-surgery and treatment. Data represented as the Mean ± SEM. (n = 5-9, *p < 0.05, **p < 0.01, ***p<0.001, one-way ANOVA with post-hoc Tukey’s multiple comparison test).
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177x111mm (300 x 300 DPI)
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97x114mm (300 x 300 DPI)
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Therapeutic assessment of N-formyl-methionyl-leucyl-phenylalanine (fMLP) in reducing periprosthetic joint infection

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Running title: Immunomodulator for periprosthetic joint infection

Key words: Periprosthetic Joint Infection (PJI); Surgical site infection (SSI); Staphylococcus aureus (S. aureus); Neutrophil; Immunomodulator; N-formyl-methionyl-leucyl-phenylalanine (fMLP/fMLF)
ABSTRACT

INTRODUCTION: Despite many preventive measures, including prophylactic antibiotics, periprosthetic joint infection (PJI) remains a devastating complication after arthroplasty, leading to pain, suffering, morbidity and a substantial economic burden. We have a powerful innate immune system that can effectively control infection, if alerted quickly. Unfortunately, pathogens use many mechanisms to dampen innate immune responses. We hypothesized that immunomodulators that can jumpstart and direct innate immune responses (particularly neutrophils) at the surgical site of implant placement would boost immune responses and reduce PJI, even in the absence of antibiotics.

METHODS: To test our hypothesis, we used fMLP, (a potent chemoattractant for phagocytic leukocytes including neutrophils), in a mouse model of PJI with Staphylococcus aureus. Mice receiving intramedullary femoral implants were divided into three groups: i) implant alone; ii) implant + S. aureus, iii) implant + fMLP + S. aureus.

RESULTS: fMLP treatment reduced S. aureus infection levels by ~2 log orders at day 3. Moreover, fMLP therapy reduced infection-induced peri-implant periosteal reaction, reduced focal cortical loss, and reduced areas of inflammatory infiltrate in the distal femurs of mice at day 10. Finally, fMLP treatment reduced pain behavior and increased weight-bearing at the implant leg in infected mice at day 10.

DISCUSSION: Our data indicate that fMLP therapy is a promising novel approach in reducing PJI, if administered locally at surgical sites. Future work will be toward further enhancement and optimization of fMLP-based therapeutic approach through combination with antibiotics and/or implant coating with fMLP.
INTRODUCTION

Approximately 1-2% of patients with primary knee and hip replacement and about 3-6% of patients with revision knee and hip replacement acquire periprosthetic joint infection (PJI) (Izakovicova et al., 2019; Parvizi et al., 2017). Estimated risk of PJI can be over 20% in patients with a combination of demographic, surgical (e.g., prior procedure), and/or comorbidity risk factors (e.g., diabetes) (Tan et al., 2018). Moreover, PJI cases are projected to rise significantly within the next two decades due to increased number of joint replacements resulting from factors such as increased life expectancy and greater expectations of mobility in the older age population (Izakovicova et al., 2019; Kurtz et al., 2007). These alarming PJI statistics occur despite many prevention strategies which have been implemented to prevent surgical site infection (SSI), including surgical hand antisepsis, reduction of foot traffic in and out of the operating room, use of intraoperative skin antiseptic agents, perioperative glycemic control, appropriate selection of surgical dressings, and prophylactic antibiotics (Allegrenzi et al., 2016; Anesi et al., 2017; Bratzler et al., 2004; Campbell et al., 2014; Corona and Singer, 2010; Edmiston et al., 2013; Greif et al., 2000; Kamath et al., 2016; Khoshbin et al., 2015; Kroin et al., 2015; Kroin et al., 2016; Lindsay et al., 2011; Panahi et al., 2012; Parvizi et al., 2017; Sewick et al., 2012; Sidhwa and Itani, 2015; Stannard et al., 2012).

Although multiple bacterial pathogens have been associated with PJI, *Staphylococcus aureus* is often reported as the most common cause of PJI, accounting for approximately 13-44% of all PJIs (Aggarwal et al., 2014; Manning et al., 2020; Pulido et al., 2008; Tsai et al., 2019). Treatments for PJI include long courses of antibiotics, debridement of infected tissue, and implant removal and replacement (Pelt et al., 2014; Segawa et al., 1999). However, antibiotic use is associated with emergence of antibiotic resistance, gut dysbiosis, increased risk for *Clostridium difficile* infection, organ cytotoxicity, allergic reactions, and the success rates after these treatments are abysmal, often ranging between 50 to 70% (Becattini et al., 2016; Jakobsson et al., 2010; Korpela et al., 2016; Langdon et al., 2016; Pelt et al., 2014; Sampson et al.; Segawa et al., 1999; Yassour et al., 2016). There can be devastating consequences for patients that acquire PJI including amputation, arthrodesis, antibiotic-induced organ cytotoxicity, or even death (Pelt et al., 2014; Segawa et al., 1999). In addition, patients with PJI are also at increased risk for additional morbidities, such as deep vein thrombosis and pulmonary embolism (Lieberman and Hsu, 2005). These dismal outcomes underscore the need for new approaches to reduce and to treat PJI.

We have evolved a remarkable innate immune system that can recognize invading pathogens as “none-self” and mobilize its plethora of defenses to protect us against them (Brubaker et al., 2015; Guo et al., 2015; Janeway and Medzhitov, 2002; Kawai and Akira, 2011; Martinon et al., 2009; Schroder and Tschopp, 2010; Takeuchi and Akira, 2010). Recognition of microbial pathogens by pattern recognition receptors (PRRs) sets in motion multiple signaling cascades that culminate in the production of pro-inflammatory cytokines, which recruit effector innate immune leukocytes, particularly neutrophils, which in turn destroy invading pathogens by various direct or indirect mechanisms such as phagocytosis, bursts of reactive oxygen species (ROS), antimicrobial peptide (AMP) production, and neutrophil extracellular traps (NETs) (Brinkmann et al., 2004; Dovi et al., 2004). Unfortunately, pathogens have evolved various
mechanisms to dampen our innate immune responses, blocking production of pro-inflammatory cytokines, inducing cell death in target host cells, and blocking proliferation in target host cells (Faure et al., 2014; Hornef et al., 2002; Lai et al., 2009; Mohamed et al., 2021; Shafikhani and Engel, 2006; Shafikhani et al., 2008; Tolle et al., 2015; Wood et al., 2015a; Wood et al., 2015b). We hypothesized that local administration of immunomodulators that can accelerate and direct innate immune leukocyte responses (particularly neutrophils) toward the site of the surgically placed implant would enhance the immune responses toward infection and be effective in reducing PJI even in the absence of prophylactic antibiotics.

To enhance the local immune responses at the time of surgery, we chose to locally administer N-formyl-methionyl-leucyl-phenylalanine (fMLP; a.k.a. fMLF) at the time of implant surgery, using an established PJI mouse model (Bernthal et al., 2010; Bernthal et al., 2014; Hegde et al., 2017a; Thompson et al., 2018). fMLP is a naturally occurring formyl peptide; formyl peptides are released at injured tissues, such as surgical sites, and invading bacterial pathogens (Li et al., 2016; Raoof et al., 2010). We chose fMLP because it is a potent chemoattractant for neutrophils and other inflammatory leukocytes (Balazovich et al., 1996; Derian et al., 1995; Madianos et al., 2005; Panaro and Mitolo, 1999; Sabroe et al., 2003). In addition, fMLP interaction with FPR chemokine receptors also activates bactericidal functions in neutrophils, such as superoxide and reactive oxygen species (ROS) production, phagocytosis, and degranulation (Devosse et al., 2009; Dorward et al., 2015; Sengeløv et al., 1994).

MATERIALS AND METHODS

Animal experimentation: We have an approval from the Rush University Medical Center Institutional Animal Care and Use Committee (IACUC No: 19-623) to conduct research as indicated. All procedures complied strictly with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA). We obtained C57BL/6J mice from the Jackson Laboratories (Bar Harbor, ME). These mice were allowed to acclimate to the environment for 1 week prior to experimentation.

Intra-articular injection: To demonstrate that fMLP is capable of mobilizing and directing neutrophil response in the knee joint even in the absence of infection and/or injury, 12-week-old C57BL/6J mice (Jackson Laboratory) received local intra-articular injection of fMLP (Sigma Aldrich, F3506) or vehicle control at the right knee joint. Prior to injection, right hindlimbs were shaved and scrubbed with 70% ethanol followed by iodine. fMLP was prepared at a concentration of (1µg / µL) in a solution of 25% dimethylsulfoxide (DMSO) and 75% phosphate buffered saline (PBS). Vehicle control was a 5 µL solution of 25% DMSO and 75% PBS. Mice were anesthetized with 2% isoflurane. A total of 5 µL of fMLP solution or vehicle control was injected into the right hindlimb knee joint using a 5 µL Hamilton microliter syringe (Hamilton, 7634-01) and 27-gauge needle as previously described (Nagao et al., 2017). The dosing of 5 µg of fMLP was chosen based on its maximum solubility in vehicle solution and maximum volume of solution that could be
injected into the knee joint space. The syringe needle was applied to the intra-articular space directly through the patellar ligament with the knee flexed at a 90° angle with needle administration perpendicular to the apex of the flexed knee. A total of $n = 5$ mice received fMLP, and a total of $n = 5$ mice received vehicle control. Mice were evaluated for local neutrophil response at the knee joint using in vivo bioluminescence imaging (BLI) at 2 hours and 6 hours post injection. Mice were imaged using BLI under 2% isoflurane anesthesia.

**Preparation of bacteria:** We used bioluminescent *S. aureus* (Xen36 *S. aureus*; Perkin Elmer) that was derived from parental strain *S. aureus* ATCC 49525 (Wright). This strain has been used and validated in mouse PJI models (Bernthal *et al.*, 2010; Bernthal *et al.*, 2014; Carli *et al.*, 2017; Hegde *et al.*, 2017a). Xen36 *S. aureus* has been genetically modified to express the modified *Photorhabdus luminescens luxABCDE* operon on the native plasmid which encodes a luciferase enzyme, enabling this strain to produce a bioluminescent signal. Bacteria were incubated in tryptic soy broth (TSB) overnight on the day prior to surgery. On the day of surgery, Xen36 *S. aureus* was diluted in TSB to a spectrophotometer absorbance measurement of 600 nm at an optical density of 0.5 (against a TSB blank); this is equivalent to $\sim 1.0 \times 10^8$ CFU/mL of *S. aureus*. This solution was further diluted to $5 \times 10^5$ CFU/mL in PBS. This solution was kept on ice for the day of surgery. At the time of surgery dropwise inoculation with 2 µL of $5 \times 10^5$ CFU/mL in PBS at the open knee joint surgical site with a 5 µl Hamilton syringe provided $1 \times 10^3$ CFU at the knee joint.

**Mouse model of periprosthetic joint infection (PJI):** To show that an fMLP immunomodulator is able to reduce PJI, we used an established PJI model with bioluminescent *Staphylococcus aureus* as described previously (Bernthal *et al.*, 2010; Carli *et al.*, 2017; Hegde *et al.*, 2017a). Briefly, 2 days prior to surgery, the ventral and lateral surface of the right hindlimb of 12-week-old C57BL/6J mice were shaved. To ensure additional removal of hair, Nair hair removal spray was used. The day prior to surgery, mice underwent baseline testing for BLI, pain behavior, weight-bearing, and weight. The day prior to surgery, all non-sterilized instruments and implant material were autoclaved in self-sealing autoclave pouches. On the day of surgery, mice were first anesthetized with a mixture of ketamine (90mg/kg) and xylazine (4.5mg/kg). The right leg was scrubbed with 70% ethanol followed by iodine. Using aseptic technique and under guidance of a dissection microscope (Zeiss, Stemi 508), a skin incision over the right knee was performed followed by a medial parapatellar arthrotomy. Incisions were performed with a Micro Knives sterile scalpel (Fine Science Tools, 10315-12). To expose the femoral condyles, lateral displacement of the quadriceps patellar complex was performed. The intercondylar notch was located; a 25-gauge syringe needle attached to a 3-mL syringe was used to penetrate the intercondylar notch and ream the distal intramedullary canal at a distance of approximately 10 mm. An orthopedic-grade stainless steel Kirschner wire (K-wire) (diameter 0.6mm; Depuy Synthes) was surgically placed in a retrograde fashion into intramedullary canal with assistance of a pin holder (Fine Sciences Tools, 26018-17). The distal aspect of the K-wire was cut to a length of approximately 11mm with a wire cutter leaving approximately 1 mm protruding into the joint space.
With the open knee joint surgical site and implant exposed, in the fMLP group, mice received a local intra-articular dose of 5 µg of fMLP in a 5 µL solution (25% DMSO and 75% PBS); this was the fMLP dose previously used for intra-articular injection at the healthy mouse knee joint to substantially increase local neutrophil activity (Discussed in the results). In the control groups, mice received 25% DMSO / 75% PBS solution as vehicle control. A 5 µL Hamilton Syringe was used to drop the solution into the exposed knee joint space. The solution was left untouched at the knee joint for 5 minutes to enhance absorption of the solution at the knee joint. This was followed by administration of $1 \times 10^3$ CFU *S. aureus* in a 2 µL solution of PBS or PBS control with use of separate 5 µL Hamilton microliter syringes into the exposed knee joint space and on top of the implant. Administration of this and/or similar amounts of bacteria into the exposed knee joint space has been described previously in a mouse PJI model with *S. aureus* (Bernthal et al., 2010; Bernthal et al., 2014; Carli et al., 2017; Hegde et al., 2017b). The exposed knee joint space was then again left untouched for 5 minutes to enhance absorption of the solution at the knee joint. Following absorption, the quadriceps complex was reduced back to midline. The knee joint capsule was closed with 6-0 vicryl sutures (Ethicon). The skin was then closed with 6-0 prolene sutures (Ethicon). Mice were placed on a warming blanket for recovery following surgery. Mice received subcutaneous buprenorphine (0.1 mg/kg) every 12 hours for a duration of 48 hours following surgery for analgesia. Following surgery, living mice received assessments with BLI on days 1, 3, 5, 7 and 10; and von Frey filament testing, weight-bearing testing, and weight assessment on day 10. Mice were sacrificed on day 10 for assessment with microCT and histologic analysis. In a parallel study, mice were sacrificed on day 3 for infection assessment by bacteria count of the peri-implant knee joint tissue and implant, using colony forming unit (CFU) analysis. In total, 35 mice received femoral implant placement in the PJI model: $n = 5$ implant control, $n = 15$ implant + *S. aureus*, $n = 15$ implant + fMLP + *S. aureus*. A total of $n = 6$ mice in each of the two infected groups were harvested on day 3 for CFU analysis; these mice received BLI up to day 3. The remaining mice ($n = 5$ implant, $n = 9$ implant + *S. aureus*, $n = 9$ implant + fMLP + *S. aureus*) received BLI up to day 10, as well as von Frey filament testing (day 10), weight-bearing assessment (day 10), and weight assessment (day 10). These mice were then harvested at day 10 for gross morphologic assessment, microCT analysis, and histologic analysis.

**Bioluminescence Imaging (BLI):** IVIS® Lumina II In Vivo Imaging System (Perkin Elmer) was used to track neutrophil activity at the knee joint as well as for quantification a bacterial abundance at the knee joint. For the assessment of neutrophil response in mice receiving knee injection of fMLP or vehicle control, BLI was performed at baseline prior to injection, as well as at 2 hours and 6 hours post injection. At 8 minutes prior to each imaging time-point, mice received intra-peritoneal (I.P.) injection of 100 mg/kg of luminol (Sigma-Aldrich, A4685) in PBS solution. Luminol is a chemiluminescent compound that produces a bioluminescent signal in the presence of reactive oxygen species (ROS) catalyzed by myeloperoxidase (MPO) in neutrophils and has been used previously as a measure of neutrophil activity in the joints and subcutaneous tissue of mice (Gross et al., 2009; Tseng and Kung, 2013).

In the mouse PJI model, BLI to quantify bioluminescent signal from bioluminescent *S. aureus* Xen 36 was performed at baseline (prior to surgery), as well as on days 1, 3, 5, 7, and 10.
post-surgery. There is direct correlation between Xen36 *S. aureus* light intensity and quantified tissue and implant bacterial burdened measured by CFU count at the site of the implant and surrounding tissue (Bernthal *et al.*, 2010). In all BLI experiments, exposure time was performed for a total of 5 minutes. A standard circular region of interest (ROI) with a diameter spanning from the distal 1/4th of the femur and proximal 1/4th of the tibia/fibula was used. The ROI used for both assessment of neutrophil activity and bacterial bioluminescent signal using BLI are represented as red outlines in (Fig. 1a and Fig. 1B) respectively. Emission intensity at the ROI over time was quantified using mean maximum flux (photons/s/cm²/sr). Mice were imaged using BLI under 2% isoflurane anesthesia. A total of *n* = 15 mice in each of the two infected groups were analyzed with IVBI. For CFU analysis, *n* = 6 mice in each of the infected groups were harvested at day 3 and only received BLI up to day 3. The remaining mice (*n* = 5 implant, *n* = 9 implant + *S. aureus*, *n* = 9 implant + fMLP + *S. aureus*) received BLI up to day 10. Presentation of images of BLI for each group were selected for representation of mean values of mean maximum flux (photons/s/cm²/sr) at the ROI.

**Infection burden assessment by Colony Forming Unit (CFU) analysis at knee joint and implant:** A subset of *n* = 6 mice in each of the infected groups (*n* = 6 implant + *S. aureus* and *n* = 6 implant + fMLP + *S. aureus*) underwent CFU analysis following bioluminescence imaging on day 3 post-surgery. Briefly, mice were sacrificed on day 3 post-infection, and the distal 1/4th of the femur and the proximal 1/4th of the tibia/fibula were cut and harvested to isolate the knee joint. Skin was removed from the harvested knee joint, and the remaining bone and soft tissue was used for CFU analysis. The implant was removed in anterograde fashion from the cut end of the femur. Bacterial loads from processed tissue and implants were determined using serial dilution and plating as previously described (Goldufsky *et al.*, 2015; Kroin *et al.*, 2012; Kroin *et al.*, 2016; Kroin *et al.*, 2018). Samples were diluted in PBS to produce dilutions ranging from 10⁻¹ to 10⁻⁵ with 96-well microplates. Aliquots of five microliters were spot plated at (10⁻⁵–10⁻³) on tryptic soy agar plates and incubated at 37°C for 24 hours. CFU counts were quantified the following day. Bacterial burden was assessed as CFU per gram tissue for knee joint and surrounding tissue. Bacterial burden for tissue implants were assessed as CFU per implant.

**Gross Morphologic analysis:** Following sacrifice, mice underwent further imaging with the aid of a Zeiss (Stemi 508) dissection stereomicroscope. Gross morphology was assessed on all mice that were harvested on day 10 post-surgery (*n* = 5 implant, *n* = 9 implant + *S. aureus*, and *n* = 9 implant + fMLP + *S. aureus*). Images were performed following skin incision, to image the knee joint capsule, and the knee joint capsule was opened to image the distal femur and implant. Knee capsule width measurements were performed using Image J (NIH) using a known reference length from each image. Presentation of images of gross morphology for each group were selected as average representatives within each group.

**Histologic analysis:** The distal aspect of the femurs and surrounding tissues were fixed in 4% formaldehyde for 3 days at 4°C and were stored in 70% ethanol at 4°C for microCT analysis. Following microCT analysis, tissues were then decalcified in 0.5M EDTA (pH 8.0) for 14 days at 4°C. Following decalcification, tissues were embedded in paraffin. Serial 5 µm sagittal sections were cut from the distal 1/4 of the femur and the proximal 1/4 of the tibia/fibula were used for histologic analysis. Examination was performed using a Zeiss light microscope (Stemi 508 Dissection Stereomicroscope, Carl Zeiss, New Jersey). Images were captured using a Nikon digital camera (DS-L1, Nikon, Tokyo, Japan) and analyzed with Image J software (National Institute of Health, Bethesda, MD). Joint morphology and bone loss were measured and compared quantitatively with control values. Measurement of bone loss was performed by analyzing the region of the distal femur and proximal tibia/fibula that was adjacent to the implant. Histologic sections were analyzed for bone mineralization, bone resorption, and inflammatory cell infiltration. The presence of osteoclasts, osteoblasts, and fibroblasts was assessed using hematoxylin and eosin staining. The relative abundance of each cell type was determined using Image J software (National Institute of Health, Bethesda, MD). Results were expressed as the mean ± standard deviation of the mean (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Significance was set at *p* < 0.05.
were performed at the distal femur tissues. Sections underwent hematoxylin and eosin (H&E) staining. Sections were imaged using an Olympus (BX43) light microscope. Image J was used to quantify cortical widths and inflammatory areas. We evaluated maximum cortical width at the distal ventral femur. Maximum cortical width was defined as maximum distance from cortical bone at the ventral surface to the beginning of contiguous marrow space. Furthermore, inflammatory infiltration areas were measured using image J as the largest contiguous areas of inflammatory infiltrates at the ventral 1/3rd of the femur epiphysis. Identification of inflammatory tissue eroding into the bone or marrow space in a mouse PJI model has been previously described (Thompson et al., 2018). Identification of inflammatory infiltrate was based on the following criteria: i) bone destruction, ii) fibrosis, and iii) inflammatory infiltrate consisting of leukocytes representing chronic inflammation/osteomyelitis, as previously described (Thompson et al., 2018; Tiemann et al., 2014). Black outline of the regions of interest of the ventral 1/3rd of the epiphysis used to measure inflammatory areas are presented in figure 4a. Representation of the inflammatory infiltrate areas, used in our calculations, are demarcated by the orange outlines in figure 4a. Histologic analyses were assessed on all mice that were harvested on day 10 post-surgery (n = 5 implant, n = 9 implant + S. aureus, and n = 9 implant + fMLP + S. aureus). Presentation of images of histology for each group were selected for representation based on mean values for maximum cortical width at the distal femurs as well as inflammatory infiltration areas.

**Micro computed tomography (MicroCT) assessment:** The distal 1/4th of fixed femur tissues at the implant leg day 10 post-surgery were assessed with microCT (Scanco, µCT50). MicroCT was assessed on all mice that were harvested at day 10 post-surgery (n = 5 implant, n = 9 implant + S. aureus, and n = 9 implant + fMLP + S. aureus). Three dimensional (3D) as well as mid-coronal microCT sections of the distal femur were evaluated. A maximum width of the distal femur was evaluated using a ventral view 3D microCT image and Image J. Increased maximum distal femur width has been previously used as a measure for infection in a mouse PJI model (Thompson et al., 2018). 3D images and mid-coronal microCT sections were used to evaluate the following scoring criteria: i) periosteal reaction; ii) focal cortical loss; iii) trabecular loss; as well as total PJI scores. Total PJI score for each femur was the addition of three scores: i) periosteal reaction; ii) focal cortical loss; iii) trabecular loss. These scores were determined on a scale of 0-2, with 2 being the worst or most severe score. Subjective scoring criteria of bone were developed based on previous clinical radiographic evidence of PJI (Bauer et al., 2006) and mouse PJI model radiographic features and scoring (Carli et al., 2017). Criteria for the following periosteal reaction scores at the distal femur are as follows: 0) no or minimal periosteal reaction restricted to small regions; 1) moderate periosteal reaction with limited changes in cortical surface dimension and congruity; 2) severe and extensive spread of periosteal reaction with moderate to severe changes in cortical surface dimensions and congruity. Criteria for the focal cortical loss scores at the distal femur are as follows: 0) no or minimal areas of focal cortical loss; 1) definitive areas of focal cortical loss found in small regions of the distal femur containing near or full thickness cortical bone loss; 2) severe focal cortical loss causing full thickness cortical bone loss spread over large region(s) such as a femoral condyle. Criteria for trabecular loss score at the distal femur are as follows: 0) no or minimal loss of trabecular bone; 1) moderate loss of trabecular bone; 2) severe loss of trabecular bone. Scoring was performed with two blinded observers, and an intraclass
correlation (ICC) was calculated to estimate inter-rater reliability. ICC values were as follows: i) periosteal reaction (ICC 0.969; 95% CI 0.926-0.987); ii) focal cortical loss (ICC 0.986; 95% CI 0.966-0.994); iii) trabecular loss (ICC 0.929; 95% CI 0.832-0.970); iv) total PJI score (ICC 0.970; 95% CI 0.928-0.987). Presentation of images of microCT for each group were selected for representation based on mean values for maximum distal femur width, as well as scored parameters: periosteal reaction, focal cortical loss, and trabecular loss.

**Weight-bearing assessment:** In addition to pain, impaired joint function is a common clinical symptomatic feature of PJI (Izakovicova et al., 2019). Weight-bearing at the implant leg was used as measure of pain and joint function. Mice were assessed for weight-bearing at the right hindlimb at baseline (pre-surgery) and on day 10 post-surgery. Weight-bearing was assessed on all mice that were harvested at day 10 post-surgery (n = 5 implant, n = 9 implant + S. aureus, and n = 9 implant + fMLP + S. aureus). Mouse weight-bearing was captured using slow motion video recording software on iPhone 10 as previously described (Carli et al., 2017). Grading at the right hindlimb was as follows: full weight-bearing (3 points); partial weight-bearing (2 points); toe-touch (1 point); non-weight-bearing (0 points). Detailed scoring criteria are illustrated in Video (online). Scoring was performed with two blinded observers, and ICC was 0.972; 95% CI 0.949-0.984.

**Pain behavior assessment by von Frey filament testing:** One of the most common initial clinical findings or symptoms of PJI is pain (Izakovicova et al., 2019; Tande and Patel, 2014). To measure pain behavior in mice, mechanical allodynia was assessed using von Frey filament testing as previously described (Im et al., 2010; Miller et al., 2012). Mice can demonstrate allodynia, or pain behavior—(demonstrated for instance by leg withdrawal)—in response to normally innocuous stimulus, through application of various levels of mechanical stimulus (von Frey filaments applied to the plantar hindpaw (Deuis et al., 2017). Mice were placed on top of a metal mesh stand (IITC mesh stand part# 408) within a small, weighted plastic enclosure. Calibrated von Frey filaments (Stoelting Touch Test Sensory Evaluator Kit) ranging from filament forces of 2.44 to 4.74 grams were used. Filaments were applied to the plantar hindpaw with a force requiring the filament to bow. Filaments were held at the plantar surface for three seconds or until a pain withdrawal response was displayed. A modified up down method was used to calculate the force required to elicit withdrawal of the paw, which was quantified as paw withdrawal threshold (PWT) force in grams (g). Pain behavior was assessed on all mice that were harvested at day 10 post-surgery (n = 5 implant, n = 9 implant + S. aureus, and n = 9 implant + fMLP + S. aureus); baseline assessments for all of these mice were performed as well.

**Weight:** Mice were assessed for weight at baseline (pre-surgery) and on day 10 post-surgery. Weight measurements were taken to further evaluate a potential response of the mice to both surgery and infection as described previously (Carli et al., 2017), and to fMLP treatment. Weight was assessed in all mice that were harvested at day 10 post-surgery (n = 5 implant, n = 9 implant + S. aureus, and n = 9 implant + fMLP + S. aureus). Weight was calculated in grams with an electronic balance (Ohaus, Scout SPX).
Statistical analysis: Statistical analysis for ICC was performed using SPSS statistics software version 27. The remainder of statistical analyses were performed using Prism software version 8. Comparison between two groups was evaluated with unpaired student’s t-test. Comparisons between more than two groups were evaluated with one-way ANOVA with post-hoc Tukey’s multiple comparison test. Comparisons of more than two groups over time were evaluated with mixed-effects analysis with post-hoc Tukey’s multiple comparison test. Data were presented as Mean ± SEM. Threshold for significance was set \( p < 0.05 \).

RESULTS

fMLP treatment increases neutrophil activity at the knee joint. To assess whether fMLP can initiate and direct inflammatory responses toward the implant surgical site, even in the absence of injury and infection, mice received either fMLP or vehicle control at the right knee joint by intra-articular injection. Neutrophil response was assessed by in vivo bioluminescence imaging (BLI), as described in Materials and Methods, using luminol, which produces a bioluminescent signal in the presence of reactive oxygen species (ROS) catalyzed by myeloperoxidase (MPO) in neutrophils (Gross et al., 2009; Tseng and Kung, 2013). BLI performed at baseline prior to injection demonstrated minimal to no neutrophil activity at the knee joint in either group. As compared to the mock group, mice receiving fMLP exhibited ~2-fold higher neutrophil response at 2h timepoint and 3-fold higher neutrophil response at 6h timepoint as assessed by BLI (Fig. 1a-b). These results demonstrated that fMLP is able to mobilize and direct a neutrophil response toward the knee environment even in the absence of infection or surgery.

fMLP treatment reduces *S. aureus* infection in a mouse model of periprosthetic joint infection (PJI). To show that an fMLP immunomodulator is able to reduce PJI, we used an established PJI model with bioluminescent *Staphylococcus aureus* as described previously (Bernthal et al., 2010; Carli et al., 2017; Hegde et al., 2017a) and in the Materials and Methods. In line with previous report (Bernthal et al., 2010), bacterial bioluminescent signal was significantly higher in infected mice as compared to non-infected mice, peaking at day 3 post-infection but declining overtime and plateauing on day 7-10 post-infection (Fig. 2a-b). Importantly, infected mice receiving fMLP prior to infection had significantly reduced bacterial bioluminescent signal on day 3 and trended toward reduced bacterial bioluminescent signals on days 1 and 5, as compared to mice receiving vehicle alone (Fig. 2a-b, \( p < 0.001 \)). Given that the peak infection occurred on day 3 in both mock and fMLP-treated mice, we harvested infected implants and tissue surrounding implants from mice and assessed them for their bacterial infection burden by CFU analysis, as described (Goldufsky et al., 2015; Kroin et al., 2016; Kroin et al., 2018). CFU analyses of the knee joint tissues on day 3 post-surgery revealed an approximate 2-log order reduction in *S. aureus* bacterial numbers in the group that received fMLP as compared to the infected group without fMLP (Fig. 2c, \( p < 0.01 \)). There was also a trend in reduction of CFUs on the implant itself in the group that received fMLP, but it did not reach statistical significance. Collectively, these data indicated that fMLP immunomodulator is able to reduce acute *S. aureus* peak infection in the PJI model.
fMLP treatment reduces the pathologic effects of *S. aureus* infection at the knee joint tissue and bone. Bone destruction and joint dysfunction are common clinical signs of PJI (Bozhkova *et al.*, 2020; Izakovicova *et al.*, 2019; Lima *et al.*, 2013). We next collected the knee joints and the tissues surrounding the implants on day 10 post-infection and evaluated them for the impact of infection with or without fMLP treatment. A striking feature of knee joints in infected mice, particularly in the group without fMLP treatment, was their overall large sizes as compared to non-infected knee joints (Fig. 3a-b). This increased size was reflective of increased abscess formation within the knee-joint capsule. Infected mice without fMLP treatment had the greatest knee capsule width, which was significantly larger than non-infected mice (Figure 3a-b, *p* < 0.01). While the fMLP treatment group trended toward having a smaller knee capsule width as compared to mock-treated group, these differences did not reach statistical significance (Fig. 3a-b). Upon opening the knee joint capsule and removing abscess debris, we next evaluated the distal femur surrounding the implant (Fig. 3c). In all groups, there appeared to be some level of adhesive tissue to the distal femur, which was likely a consequence of tissue reaction to implant surgery. In the implant group, characteristic features of the distal femur, such as the femoral condyles were abundantly clear. Infected mice receiving fMLP also appeared to retain somewhat natural morphology of the distal femur and femoral condyles; however, as compared to the non-infected group, there appeared to be greater adhesive tissue to the femoral condyles in this group. Mice infected without fMLP treatment had the highest level of adhesive tissue and abnormal morphology of the distal femur of any group. This was most clearly evidenced by loss of rounded contours of the femoral condyles in the infected group without fMLP, as indicated by black arrows (Figure 3a, black arrows).

To get a greater understanding of the effects of infection with or without fMLP treatment on the distal femur, we performed histologic analysis of the distal femur by hematoxylin and eosin (H&E) histological analysis on day 10 post-infection, as described previously (Materials and Methods). The fMLP-treated group had significantly smaller cortical bone width as compared to the infected group without fMLP treatment (Fig. 4a-b, indicated by black dotted lines in magnified regions in orange boxes). Increased cortical width was likely a result of stimulation of bone production (periosteal reaction) from the overlying periosteum due to chronic inflammation, as has been reported in the context of infection and inflammation (Rana *et al.*, 2009). The implant group without infection had the smallest cortical width.

Inflammatory infiltrate has been found eroding into the distal femur in a mouse PJI model (Thompson *et al.*, 2018), prompting us to assess the pathological impact of infection with or without fMLP treatment on inflammatory responses and on bone health. Data indicated that within the defined region of interest (ROI) in the ventral 1/3rd of the femur epiphysis, the infected group without fMLP had the largest contiguous area of inflammatory cell and tissue infiltration, which was significantly higher than the fMLP-treated infected group (Figure 4a and 4b, *p* < 0.05, black arrow in magnified regions in blue boxes). In contrast, the uninfected implant group had the smallest regions of inflammatory cell and tissue infiltrate, which was likely a result of surgery and implant placement (Fig. 4a-b). Within the ventral 1/3rd of the femur epiphysis, the largest
contiguous areas of inflammatory infiltrates were found at the bone-implant interface in all groups.

We next assessed the pathological impact of infection with or without fMLP treatment on bone by microCT, as described in Materials and Methods. On ventral microCT 3D view, maximum width of the distal femur was measured. This was in particular used as a measure to quantify periosteal reaction, which would widen the femur width. Similar to non-infected group, the fMLP-treated group had significantly reduced femoral width as compared infected group without fMLP (Fig. 5a-b, \( p < 0.01 \), indicated by yellow dashed lines). The 3D images of the side view, dorsal view, and condyle view, as well as, mid-coronal microCT sections were evaluated by two blinded observers and scored for i) periosteal reaction, ii) focal cortical loss, iii) trabecular loss, as well as combination of all three parameters were indexed as a combined microCT PJI score.

As compared to the infected group treated with vehicle, the fMLP-treated group exhibited significantly reduced periosteal reaction (Fig. 5a-b, indicated by green arrows, \( p < 0.01 \)) and focal cortical loss (Fig. 5a-b, indicated by purple arrows, \( p < 0.05 \)). Trabecular loss was significantly higher in the infected group without fMLP, as compared to non-infected group (\( p < 0.001 \)), and trended higher as compared to the infected treated group treated with fMLP, but this effect did not reach statistical significance (Fig. 5a-b, indicated by red arrows). When all three parameters were combined into a combined PJI microCT score, it was found the fMLP treatment significantly reduced the combined PJI microCT pathology score as compared to the infected group without fMLP treatment (Fig. 5b, \( p < 0.01 \)).

fMLP improves behavioral symptoms in a PJI mouse model. Pain and joint dysfunction are common clinical symptom associated with PJI in patients (Bozhkova et al., 2020; Izakovicova et al., 2019; Lima et al., 2013). We evaluated pain behavior in these mice by two methods: weight-bearing on the right hindlimb (implant leg) and mechanical allodynia with von Frey filament testing, as previously described (Carli et al., 2017; Im et al., 2010; Miller et al., 2012) and in Materials and Methods. Weight-bearing was also used as an assessment for joint function (Carli et al., 2017). At baseline and prior to implant placement (Day 0), mice in all groups exhibited similar weight-bearing score and had the highest possible calculated withdrawal force threshold for evidence of mechanical allodynia (Fig. 6a-b). In contrast, at day 10 post-surgery, fMLP-treated infected mice exhibited significantly improved weight-bearing score on the implant leg as compared to infected mice without fMLP (Fig. 6d, \( p < 0.001 \)). Furthermore, mice receiving fMLP, also exhibited significantly improved (increased) withdrawal force threshold as compared to infected mice without fMLP treatment (Fig. 6e, \( p < 0.05 \)). Collectively, these data indicated that fMLP treatment significantly improves the behavioral symptoms that are associated with PJI (Bozhkova et al., 2020; Izakovicova et al., 2019; Lima et al., 2013).

fMLP treatment did not affect weight in a mouse PJI model. Prior to surgery, all groups had similar average weight (Fig. 6c). At day 10 post-surgery, all groups trended slightly toward having lower weight; however, this was not statistically significant. Furthermore, at day 10 post-
surgery, infected mice without fMLP treatment trended toward slightly greater weight loss; however, this weight loss was not statistically significant between groups (Fig. 6f).

**DISCUSSION**

PJI remains a devastating complication after arthroplasty, leading to pain, suffering, morbidity and a substantial economic burden (Kapadia *et al.*, 2016; Kurtz *et al.*, 2012). There is an urgent need for alternative and/or adjunct measures to antibiotic prophylaxis in addressing PJI. In this report, we sought to assess whether fMLP, (a potent immunomodulator that recruits and activates inflammatory leukocytes, particularly neutrophils), would be able to control PJI even in the absence of antibiotics. Our data indicate that fMLP treatment significantly reduced *S. aureus* infection in an established PJI model. Furthermore, fMLP reduced infection-induced bone and tissue pathologies, and subsequently reduced infection-induced pain and weight-bearing behavior in infected animals.

Although, infection was significantly reduced in fMLP-treated mice, it was not completely abolished in this model. Whether this was due to low level of fMLP used in our studies, or the inability of fMLP to engage the adaptive immune responses which are also needed to fully control *S. aureus* infection (Lin *et al.*, 2009) remains to be investigated. Intriguingly, administration of systemic vancomycin prophylaxis or intra-articular vancomycin powder treatment alone also failed to completely eradicate *S. aureus* PJI in rats, although they were more effective in reducing infection when combined (Edelstein *et al.*, 2017). We posit that since antibiotics and immunomodulators (e.g., fMLP) combat infection through different mechanisms of action, combination therapy with both may also be more effective in eliminating PJI. Future studies are needed to investigate whether fMLP in combination with prophylaxis or topical antibiotics would be more effective in controlling PJI.

It is encouraging that fMLP treatment reduced infection burden early after surgery and infection, given that bacteria have been shown to form biofilm on the implant surfaces early after infection (Carli *et al.*, 2017; Lamret *et al.*, 2020). Indeed, it has been demonstrated that higher neutrophil to *S. aureus* bacteria ratio at the implant surface is a prognostic factor for reduced biofilm production at the implant surface (Ghimire *et al.*, 2019). Bacterial pathogens, including *S. aureus*, are protected from neutrophil killing when embedded in fully mature biofilms (de Vor *et al.*, 2020; Gunther *et al.*, 2009; Kristian *et al.*, 2008). Due to the concern of biofilm formation on the implant surface itself, we propose that coating the implant with fMLP might better mobilize neutrophil recruitment directly to the implant interface, thus increasing neutrophil to *S. aureus* bacteria ratio at the implant surface to prevent early biofilm formation. Future studies are needed to assess the impact of fMLP therapy (administered intra-articularly or via implant coating; alone or in combination with antibiotics) on biofilm production.

Inflammation and inflammatory responses are proportional to the bacterial burden and infection level and can culminate in severe tissue destruction (Bernthal *et al.*, 2010; Carli *et al.*, 2017; Lamret *et al.*, 2020). Indeed, it has been demonstrated that higher neutrophil to *S. aureus* bacteria ratio at the implant surface is a prognostic factor for reduced biofilm production at the implant surface (Ghimire *et al.*, 2019). Bacterial pathogens, including *S. aureus*, are protected from neutrophil killing when embedded in fully mature biofilms (de Vor *et al.*, 2020; Gunther *et al.*, 2009; Kristian *et al.*, 2008). Due to the concern of biofilm formation on the implant surface itself, we propose that coating the implant with fMLP might better mobilize neutrophil recruitment directly to the implant interface, thus increasing neutrophil to *S. aureus* bacteria ratio at the implant surface to prevent early biofilm formation. Future studies are needed to assess the impact of fMLP therapy (administered intra-articularly or via implant coating; alone or in combination with antibiotics) on biofilm production.
Chronic inflammation in the setting of infection can suppress osteoblast activity and enhance osteoclast activity, and S. aureus infection can directly cause bone destruction as well as activate osteoclasts and inhibit osteoblasts leading to altered bone remodeling (Wright and Nair, 2010). We found substantial evidence of inflammation and bone loss and destruction particularly in the infected group without fMLP, as assessed by gross morphology, microCT, and histology. Furthermore, in the setting of osteomyelitis, periosteal reaction can occur through subperiosteal spread of inflammation which in turn elevates and stimulates the periosteum to lay down new layers of bone (Rana et al., 2009). Consistent with this report, we also found significant increases in inflammation and periosteal reaction in our mouse PJI model, as assessed by microCT, and histological analyses. Importantly, fMLP lowered these infection-induced bone pathologies.

Common clinical signs of PJI include joint pain and joint dysfunction (Bozhkova et al., 2020; Izakovicova et al., 2019; Lima et al., 2013). Intriguingly, infected mice treated with fMLP exhibited significant reduction in pain behavior and significant improvement in weight-bearing, further highlighting the positive impact of fMLP therapy in reducing PJI. Severe weight loss can be a sign of systemic infection with bacteria such as S. aureus (Wu et al., 2017). We found a slight trend in weight loss among both infected and non-infected groups, although these differences did not reach statistical significance, suggesting that S. aureus infection in this model remains local. In line with our data, Carli et al., in a similar PJI S. aureus infection study, reported no significant differences in weights between the infected and non-infected groups, although both groups exhibited slightly lower weight at week one post-surgery (Carli et al., 2017).

There is minimal literature to date on the therapeutic use of fMLP. Interestingly, Shin et al., evaluated the impact of local treatment with fMLP (delivered in a hyaluronic acid gel carrier) in a rabbit calvaria defect model and demonstrated that fMLP promoted osteogenesis and bone formation at the defect site as compared to vehicle control (Shin et al., 2011). Of note, no evidence of increased inflammation was found at the defect site in rabbits treated with fMLP at 4 weeks post treatment (Shin et al., 2011), suggesting that fMLP may have a positive effect on bone formation and healing after arthroplasty even in the absence of infection. Further studies should evaluate the positive or adverse impacts of local fMLP therapy on joint and surrounding tissue, as well as animal behavior, in an uninfected cohort in the PJI model, to further lay the groundwork for its therapeutic use to combat PJI.

Sethi et. al., have reported that in rats with a surgically placed tibial intramedullary implant with S. aureus infection, administration of CpG (cytosine-phosphate-guanosine) oligodeoxynucleotide (CpG ODN), which is found in bacterial DNA and shown to trigger inflammatory responses, led to ~67% reduction in infection burden early after infection, but did not prevent the development of chronic infection over time (Sethi et al., 2015). Our results are in line with these findings showing that fMLP reduced early infection by nearly 2-log order on day 3 but did not completely abolish infection.

CONCLUSION
Our proof-of-concept studies provide direct evidence in a mouse PJI model that fMLP immunomodulator was effective in reducing acute infection and protecting against infection-induced bone and tissue damage and associated pain. Immunomodulators such as fMLP may provide an alternative or adjunct therapeutic to antibiotics for reducing and or treating PJI. Future studies should focus on optimization of immunomodulator-based approaches such as fMLP dose assessment, implant coating with fMLP, or combination of fMLP with prophylactic antibiotics or other immunomodulators.
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FIGURE LEGENDS

Fig. 1. fMLP increases neutrophil activity at the articular joint. a) fMLP (5µg / 5µL) or vehicle (5 µL PBS + 25% DMSO) were injected into the right knee joint. Neutrophil recruitment was assessed by BLI prior to knee injection (baseline), or at 2 hours and 6 hours post intra-articular knee injection and 8 minutes post I.P. luminol injection at each time point. Red dashed circle represents ROI used for BLI quantification. b) Corresponding tabulated data are shown as the Mean ± SEM. (n = 5 mice/group, *p <0.05, Student’s t-test).

Fig. 2. fMLP treatment reduces S. aureus infection in a mouse model of PJI. a) Mice received a surgically placed femoral implant and treated with vehicle alone, 10^3 Xen36 S. aureus, or fMLP + Xen36 S. aureus. Infection burden was assessed by BLI performed at baseline, as well as at days 1, 3, 5, 7, and 10 post-surgery and treatment. Red dashed circle represents ROI used for BLI quantification. b) Corresponding tabulated data for mean maximum flux (photons/s/cm^2/sr) at the region of interest (ROI) are shown as the Mean ± SEM. (n = 5-15 mice/group, *p < 0.05, mixed-effects analysis with post-hoc Tukey’s multiple comparison test). c) Quantification of bacterial CFUs at the knee joint tissue and femoral implant at day 3. Data represented as the Mean ± SEM. (n = 6, *p <0.05, Student’s t-test).

Fig. 3. fMLP reduces the pathological effects of infection at the knee joint tissue surrounding the implant. a) Gross morphologic assessment was performed at the knee joint capsule (knee capsule width in millimeters / measure of intra-capsular abscess) and distal femur for cartilage/bone erosion surrounding the implant at animal sacrifice on day 10 post-surgery and treatment. Yellow dashed line represents maximum knee capsule width. Black arrows represent region of the femoral condyles. b) Knee capsule width plotted as the Mean ± SEM. (n = 5-9, *p <0.05, one-way ANOVA with post-hoc Tukey’s multiple comparison test).

Fig. 4. fMLP protects against infection and inflammatory induced bone damage and changes around the implant on histologic analysis. a) At day 10 post-surgery and treatment, mice were harvested for histologic assessment of the distal femur by H&E staining. Two parameters identified in the distal femur were dramatically higher in the infection group without fMLP, which were maximum cortical width (measure of traditional cortical bone in addition to new adjacent bone formation formed by periosteal reaction / yellow magnification / black dashed line) and inflammatory infiltrate (blue magnification / black arrow). Inflammatory infiltrate was measured as the largest contiguous area of inflammatory infiltrate in the ventral 1/3rd of the femur epiphysis. Within blue magnification, ROI for inflammatory infiltrate outlined with black solid line and inflammatory area outlined by orange line. b) Quantification of maximum cortical width and inflammatory infiltrate. Data represented as mean ± SEM. (n = 5-9, *p <0.05, **p <0.01, ***p <0.001, one-way ANOVA with post-hoc Tukey’s multiple comparison test). Black error bar = 200 µm; Orange error bar = 70 µm; Blue error bar = 85 µm.

Fig. 5. fMLP protects against infection and inflammatory induced bone damage and changes around the implant on microCT analysis. Mice were sacrificed at day 10 post-surgery and...
Distal femurs were assessed by microCT analysis on 3D imaging and mid-coronal sections. Green arrows point to periosteal reaction; purple arrows point to focal cortical loss; and red arrows point to trabecular loss. Yellow dashed line represents cortical width. The microCT quantification of maximum femur width in millimeters (based on ventral view 3D image), as well as periosteal reaction score, focal cortical loss score, and trabecular loss score, which were analyzed using scoring criteria based on assessment of 3D images and mid-coronal sections with two blinded observers. Scoring criteria were then quantified and combined as a single measure called combined microCT PJI score. Data represented as mean ± SEM. (*p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA with post-hoc Tukey’s multiple comparison test).

Fig. 6. fMLP treatment improves weight-bearing, decreases pain behavior, and has no effect on weight in a mouse model of PJI. Baseline assessment for weight-bearing (a) pain behavior (mechanical allodynia) on von Frey filament testing (b) and weight (c) performed prior to surgery or treatment (day 0). Assessment of weight-bearing (d) pain behavior (e) and weight (f) performed at day 10 post-surgery and treatment. Data represented as the Mean ± SEM. (*p <0.05, ** p <0.01, *** p<0.001, one-way ANOVA with post-hoc Tukey’s multiple comparison test).
REFERENCES


