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Mesenchymal Stem Cells**

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14. ABSTRACT

In this application, we propose the patient's own stem cells as a promising, autologous cell type for sustained local delivery of antimicrobial peptides, for use as an adjunct therapeutic for orthopaedic infections. **Rationale:** Adult mesenchymal stem cells (MSCs) can be easily isolated from various adult tissue sources such as bone marrow, adipose, bone and muscle, and retain their differentiation potential during in vitro culture expansion and manipulation. Recent studies have focused on adipose tissue-derived stromal/stem cells (ASCs) because they are easily isolated in numbers 100- to 2,500-fold greater than bone marrow-derived stem cells, for example, while possessing the ability to differentiate into many tissue and regulate immune responses. ASCs secrete many therapeutic cytokines that are anti-inflammatory and control immune responses. Of particular relevance to this proposal, MSCs have recently been shown to secrete antimicrobial peptides (AMPs) that act against a variety of bacteria. The benefits of AMPs over current treatments are that they do not induce antibiotic resistance and they selectively act against bacteria without negatively impacting the patient's tissues. The production of these AMPs is induced by exposure to bacterial products such as lipopolysaccharide (LPS), a component of bacterial cell walls. LL-37, a cleavage product of the protein Cathelicidin, is a major AMP produced by MSCs. LL-37 is antimicrobial and prevents the formation of biofilms that bacteria produce to protect themselves from a patient's immune system. We propose that the controlled stimulation of endogenous ASCs with LPS offers the potential to impart endogenous

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- 1. INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

The aim of this study is to develop a novel, autologous cell-based method to prevent or treat orthopaedic infections, thus minimizing morbidity from repeat surgeries, preventing antibiotic resistance, accelerating wound healing and enhancing the probability of tissue/limb salvage. We hypothesize that “Activated” adipose-derived stem cells (ASCs), as a result of simulated exposure to bacteria, will act as a bioreactor to produce anti-bacterial agents, such as LL-37, capable of retarding bacterial growth to treat, reduce, or prevent bacterial infection. This method may result in a commercializable product that utilizes immobilized agents, such as LPS, to activate a patient’s own ASCs, which are then injected back into a patient to prevent or treat infection.

- 2. KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Infection, Osteomyelitis, Adipose-derived stem cells, Antibacterial

- 3. ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

- Aim 1: Simulate/activate ASCs with LPS
 - o Milestone 1: Quantify the antimicrobial effects of adipose-derived stromal/stem cells (ASCs) potentiated by soluble or immobilized lipopolysaccharide (LPS) on *Staphylococcus aureus* and *Escherichia coli* cultures. (Timeline: 4/10/2015-4/9/2016, percentage of completion: 100 %)
 - o Milestone 2: Optimize the concentration of LPS to potentiate the antimicrobial effects of ASCs. (Timeline: 10/10/2015-4/9/2019, percentage of completion: 90 %)
- Aim 2: Design/test “one-step” ASC activation.

- o **Milestone 3:** Design and test a “point-of-care” product with solid-phase-bound LPS that readily provides biological activation of ASC antimicrobial activity. (Timeline: 10/10/2015-4/9/2017, percentage of completion: 100 %)
- **Aim 3:** Test activated ASCs in preclinical OM model
 - o **Milestone 4:** Characterize a model of osteomyelitis (OM) in a rabbit as a pre-clinical model. (Timeline: 10/10/2015-4/9/2019, percentage of completion: 100 %)
 - o **Milestone 5:** Test the efficacy of activated ASCs alone or as complement to standard OM treatment. (Timeline: 8/10/2016-12/31/20, percentage of completion: 100 %)

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major activities 1: Simulate and optimize infection-mediated activation of the bactericidal activities of MSCs by exposing human ASCs to activator (LPS or 1,25-dihydroxy vitamin D3 (VD₃))

Specific objectives: Human ASC isolation and culture. hASC isolation, culture, and characterization.

Significant results or key outcomes:

1. ASC isolation from Human Infrapatellar Fat Pad

Materials and Methods

The infrapatellar fat pad (IFP) was dissected from the knee and placed in a Petri dish with Hank's Balanced Salt Solution (HBSS, Hyclone). the IFP was minced into small pieces (~1 mm³), and any obvious fibrous tissue was removed. 10 mg /cc wet tissue Collagenase type I (Worthington) and 10 mg/cc Trypsin IIS (Sigma) were added to the conical tube containing the fat (up to 10 cc wet tissue). The fat was agitated continuously at 180 rpm, 37°C for 3 hours. Afterwards, the tissue digest was filtered through 250 µm cell strainer, centrifuged at 1,500 rpm (400g) for 5 min, resuspended in 25 mL growth medium (DMEM (Invitrogen) including 10% lot selected fetal bovine serum, 1x antibiotic-antimycotic (Invitrogen), 1 ng/mL fibroblast growth factor-2 (FGF-2; R&D System)). The adipose tissue digest was filtered again through 100 µm cell strainer, centrifuged at 1,500 rpm (400 g) for 5 min, with 3 mL red blood cell (RBC) lysis buffer added, and incubated for 10 min, after which 47 mL of growth medium was added and centrifuged at 1,500 rpm (400 g) for 5 min. Finally,

growth medium was added, and cells were counted and distributed to T-150 tissue culture flasks at -1 million cells/flask.

Patient	Age	Gender	CFU (per 100 cells)
1	65	Male	31
2	88	Female	40
3	61	Female	18
4	59	Female	17
5	71	Female	47
6	76	Female	46
7	68	Female	47
8	65	Female	24
9	68	Female	43
10	55	Male	38
11	59	Female	42
12	52	Female	45



Figure 1. Chondrogenic, osteogenic, and adipogenic differentiation in 2D culture

Table 1. MSC source and CFU.

2. Colony forming unit rate determination

Materials and Methods: hIFP-ASCs cultures were expanded to 70-80 % confluency, trypsinized, and counted. Cells were diluted in complete IFP-ASC growth medium, and plated at 100 cells per 100-mm tissue culture dish in ASC growth medium. Then, cells were incubated for 10-14 days at 37°C in 5% humidified CO₂, washed with phosphate-buffered saline (PBS) and stained with 0.5% Crystal Violet in methanol for 5-10 minutes at room temperature. Cells were washed with PBS twice. Stained colonies were counted.

3. Cell Differentiation assays

Material and methods: hIFP ASCs at passage 1 were trypsinized and seeded into a 6-well plate. After 2 days, when the cells reach to ~70% confluence, medium was changed to specific inductive medium for chondrogenesis, osteogenesis and adipogenesis, respectively. Each culture medium was changed twice a week for three weeks. After three weeks, cells were washed twice with PBS. Osteogenically and adipogenically differentiated cells were fixed with 10% formalin for 15 min, and then washed with tap water (for osteogenesis) and distilled water (for adipogenesis).

Chondrogenically differentiated cells were fixed with 70% ethanol and then rinsed with distilled water. **Alcian blue staining:** Fixed cells were rinsed with distilled water and incubated with 1 mL of 3% acetic acid (v/v) for 15 minutes. Cells were then incubated with Alcian Blue (EK industries) for 2 hours at room temperature, washed with acid alcohol until the supernatant ran

Patient	Differentiation		
	Chondrogenesis	Osteogenesis	Adipogenesis
1	2+	1+	3+
2	2~3+	Cells came off	3+
3	0	Cells came off	3
4	0	3	3
5	2+	3+	3+
6	2+	2+	3+
7	2~3+	2~3+	2+
8	2~3+	Cells came off	1+
9	0	1+	3+
10	1~2+	2~3+	1+
11	2+	0~1+	3+
12	2~3+	3+++	3+

Table 2. Differentiation assays.

clear, and then rinsed with distilled water for 3 times. Stained samples were scored upon examination under the microscope based on staining color intensity and abundance. Score 0: none, 1: weak, 2: mild, 3; strong. *Alizarin red staining*: Cells were fixed in 10% formalin for 15 minutes and washed with tap water. Then, cells were incubated with Alizarin Red (Rowely Biochemical Institute) at room temperature for 30 sec - 5 min with gentle shaking. And then, cells were then washed with tap water for 4 times for 5 minutes each. Stained samples were scored microscopically based on staining color intensity and abundance. Score 0: none, 1: weak, 2: mild, 3; strong. *Oil Red O staining*: Cells were fixed with 10% formalin for 15 min and washed with distilled water. Cells were then incubated with Oil Red O at room temperature for 2 hours, and washed with distilled water. Stained samples were scored upon examination under the microscope based on color intensity and abundance. Score 0: none, 1: weak, 2: mild, 3; strong.

Results:

IFP ASC were isolated from 12 patients and characterized by CFU (Table 1) and tri-lineage differentiation (Table 2; Figure 1). Using these results, IFP ASCs with similar CFU and differentiation capacities will be selected for all subsequent studies. Figure 2, compares antimicrobial activity versus tri-lineage differentiation. Four patients showed very strong antimicrobial activity in this model system (green arrow: Patient # 1 and #8; orange arrow: Patient # 2 and #7). It was observed that patient ASCs with strong tri-lineage differentiation (high total score) also had high antimicrobial activity. The correlation of antimicrobial activity and chondrogenesis was strongest. More studies are required; however, the current data suggests that differentiation capacity may predict the antimicrobial potential a patient ASC populations.

The task of “stimulating and optimizing infection-mediated activation of the bactericidal activities of human mesenchymal stem cells (hMSCs) was accomplished by exposing human ASCs to an activator (LPS or e.g. 1,25-dihydroxy vitamin D3).

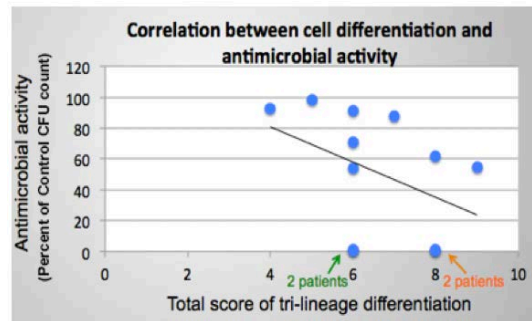


Figure 2. Correlation between cell differentiation and antimicrobial activity.

Other achievements: None

Major activity 2: Antibacterial effect of soluble and immobilized LPS (or e.g. 1,25-dihydroxy vitamin D3) on ASCs inoculated +/- E. coli/S. aureus (and or/P. aeruginosa and/or K. pneumonia).

Subtask 2-1: Optimize soluble LPS (or 1,25-dihydroxy vitamin D3 (VD₃)) concentration and treatment time. LPS or VD₃ will be used as activators.

Subtask 2-2: Optimize immobilized LPS or VD₃ concentration and treatment time. LPS or e.g. VD₃ will be used as activators.

Specific objectives: Determine dose and exposure duration needed to activate ASC antimicrobial activities using either LPS or

Significant results or key outcomes:

Material and Methods: Cells were treated with soluble LPS or VD₃, a known TLR4 activator, that should lead to LL-37 production, for various time periods. Collected cells and conditioned medium were then used for the following experiments as indicated in Figure 3: (1) qRT-PCR for LL-37, TLR4

using LPS- or VD₃-stimulated IFP ASCs/MSCs: or (2) analysis of antimicrobial activity as determined by CFU assay in which *S. aureus* were grown in conditioned.

(1) Bacterial culture

Staphylococcus aureus is the most common gram-positive organism present in osteomyelitis. *S. aureus* (ATCC 25923) cultures were grown in Tryptic Soy Broth (TSB) medium for 16 hrs at 37°C, and cultures were adjusted to 3.3×10^4 colony forming units CFU/mL on the basis of OD600 (OD600 = 1.0, which corresponds to 6.6×10^8 CFU/mL). Ampicillin (Amp) was used as the antibiotic against *S. aureus*. [Note: The bacterial concentration has been optimized in prior experiments to yield sufficient and countable colony numbers for better anti-microbial assessment.]

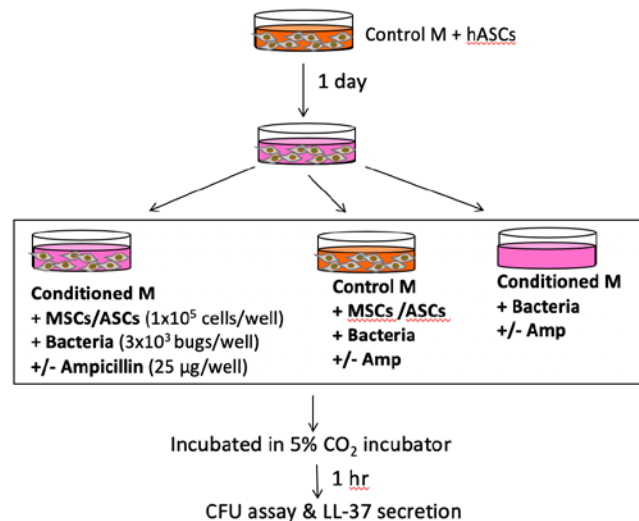


Figure 3. Experimental scheme.

(2) CFU assay 1 mL of conditioned medium was inoculated with 1.5×10^3 *S. aureus*, and incubated for 1 hour at 37°C in 5% CO₂ incubator. Unless otherwise specified, an antibiotic-free culture medium was used throughout the entire study. A cell-free control was processed under the same conditions. At the end of the incubation, a 50 µL aliquot of medium from each well (bacteria and medium) was plated on TSB agar for CFU counting. Plated dishes were incubated for 16-18 hrs at 37°C. Colonies were counted by using a colony counting pen (Fisher scientific, Waltham, MA).

(3) qRT-PCR for LL-37 and TLR4

Human ASCs were cultured in growth medium. Twenty-four hours before the experiment, culture medium was replaced with fresh antibiotic-free growth medium. On the day of experiment, cells were rinsed with PBS and trypsinized, and pelleted by centrifugation at 1,100 rpm for 5 minutes. The pelleted cells were resuspended in growth medium without antibiotics. Finally, the cells were replated in tissue culture treated 6-well plates (0.5×10^5 cells/2 mL/well). Cells were then allowed to sit for 2 hours. After 2 hours, cells were then exposed to varying concentrations of either LPS or VD₃ for various time periods (0-15 min-30 min -1 hr- 2 hrs - 4 hrs - 6 hrs). Unless otherwise specified, an antibiotic-free culture medium was used throughout the entire study. At the end of the desired exposure time, media was removed and saved for CFU assay (See below (3) CFU assay). The wells were washed twice with 2 mL of PBS. Then, 350 µL of RLT Plus buffer (Qiagen RNeasy Kit) was added to the well and allowed to interact with the cells for 10 minutes. Afterwards, the RNeasy sets were followed. Isolated RNA was converted cDNA by using First-Strand IV Synthesis Supermix (ThermoFisher) and analyzed by qRT-PCR for LL-37 and TLR4.

(4) Primers used in this study:

LL-37 (Located in N-terminal region) : Forward: 5'-GAA GGC TCC TGG TTG GG-3', Reverse: 5'-TCT GCC TCC CTC TAG CC-3', LL-37 (Located in the middle of the transcript): Forward: 5'-ATT GCC CAG GTC CTC AG-3', Reverse: 5'-GTC CCC ATA CAC CGC TTC AC-3', TLR-4:

Forward: 5'-TGG AAG TTG AAC GAA TGG AAT GTG-3', Reverse: 5'- ACC AGA ACT GCT ACA ACA GAT ACT-3', GAPDH: Forward, 5'-AGC CAC ATC GCT CAG ACA-3', Reverse, 5'- GCC CAA TAC GAC CAA ATC-3'.

Results: The antimicrobial activity of isolated ASCs was tested. The investigators found that maximal bacterial growth inhibition was observed in cells and their conditioned medium or conditioned medium alone (Figure 4). It was this decided that all future studies of this type would employ conditioned medium. The LPS and VD₃ concentrations and treatment times for IFP ASC activation were optimized (Figure 5A and 5B). IFP ASC conditioned medium alone showed antimicrobial activity in a time dependent manner. Longer incubation showed stronger antimicrobial activity. The data showed that VD₃ stimulation performs better than LPS. For IFP ASCs exposed to VD₃ for 1 - 6 hrs at 100 M, a reduction in colony forming units (CFU) was seen that increased with increasing VD₃ dose and length of exposure. In contrast, LPS treated IFP ASCs showed maximal antimicrobial activity at 1 hour (for all doses), with decreasing effectiveness at longer exposures at any LPS concentration. Based on these results, it was decided that only VD₃ would be used for further *in vitro/in vivo* study. Because VD₃ is a non-toxic, physiological metabolite it was anticipated that use of VD₃ for ASC activation would allow for a very simple, easy, quick treatment, minimizing cell toxicity and not requiring immobilization.

The investigators observed highly variable antibacterial activity in ASCs from different donors. ASCs from some donors almost completely blocked bacterial colony formation, but some others showed relatively low level of activities. As a consequence, results from different experiments show such large standard deviations throughout the study.

Attempts were made to use 16s RNA RT-PCR to quantify bacteria in inoculated medium. However, the data was not consistent and there was the possibility that some bacteria remained attached to cells, complicating the collection and all bacteria for quantitation by RT-

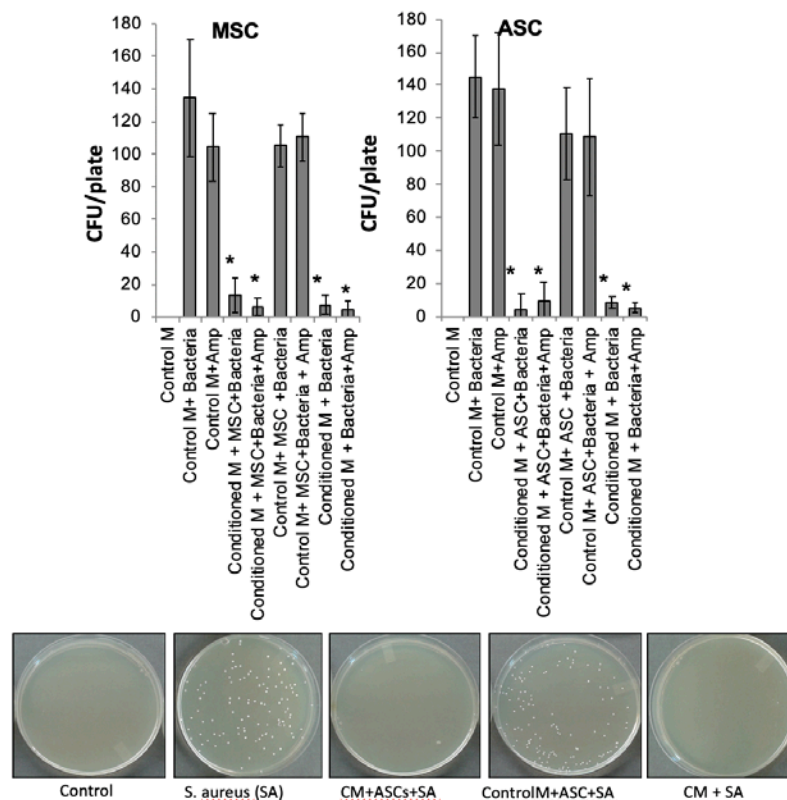


Figure 4. Antimicrobial activity of ASC/ASC.

PCR. Therefore, it was decided that CFU was the best means for quantifying bacterial number.

The task of assessing the “Antibacterial effect of soluble and immobilized LPS (or VD₃) on ASCs inoculated +/- *E. coli*/S. aureus (and or/ *P. aeruginosa* and/or *K. pneumonia*) was completed.

Other achievements: None

Major activity 3: Determine the mechanism of antimicrobial effects of LPS (or e.g. 1,25-dihydroxy vitamin D₃) -stimulated ASCs.

Subtask 3-1: Determine the expression of LL-37 and TLR4 in soluble LPS or VD₃-stimulated ASCs inoculated +/- *E. coli*/S. aureus (and/or *P. aeruginosa* and/or *K. pneumonia*). LPS or VD₃ will be used as activators.

Subtask 3-2: Determine the expression of LL-37 and TLR4 in immobilized LPS or VD₃-stimulated ASCs inoculated +/- *E. coli*/S. aureus (and/or *P. aeruginosa* and/or *K. pneumonia*). LPS or VD₃ will be used as activators.

Since we decided to use VD₃, these subtask are the same.

Significant objectives:

Based the results of Task 2 above, it was decided to use VD₃ as a stimulator of ASC anti-microbial activity. The sub-tasks were thus catered to analysis of VD₃ mechanisms of enhancing ASCs antimicrobial activities.

Significant results or key outcomes:

Rationale: LL-37: LL-37 expression by IFP ASCs was induced after exposure to 100 ng/mL LPS for 30 min – 60 minutes.(Figure 6A). In the case of VD₃ stimulation, statistically significant differences were observed after exposure to 12.5 nM VD₃ for 1 hr treatment (Figure 6B). These results suggest that a short time exposure has a positive effect on LL-37 expression.

Results: Based on CFU assay and LL-37 expression results, it was decided to use VD₃ as an activator. Exposure of IFP ASCs to 100 nM VD₃ for 1 hour induced the highest LL-37 gene expression and greatest antimicrobial activity (reduction in CFU). (b) **TLR-4:** Interestingly, contrary to our expectation, no significant effect on TLR-4 expression was observed in VD₃-stimulated hASCs (Figure 7). Utilizing the ELISA assay, the investigators were unable to detect any LL-37 production after stimulation of hMSCs with 25, 50, 200, 500 or 1000ng of immobilized LPS after 6-hour, 24-hour or 48-hour exposure, nor were they able to detect LL-37 production in IFP ASCs

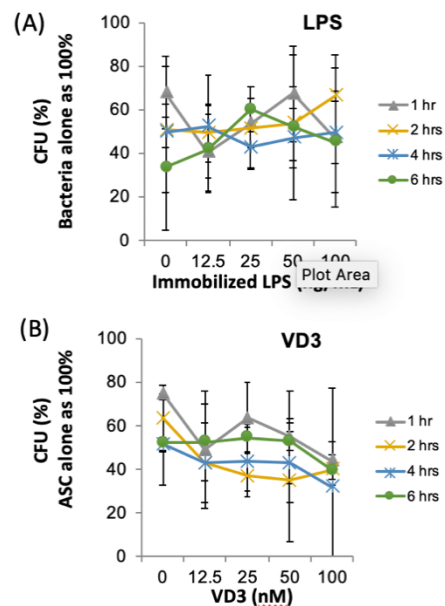


Figure 5. CFU Assays

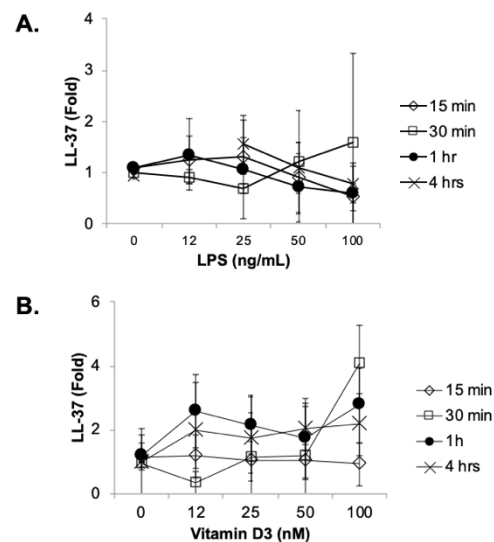


Figure 6. LL-37 expression in LPS- or Vitamin D₃-treated ASCs.

under the same conditions. These results are in contrast to earlier preliminary studies. Possible explanations include: (A) patient-to-patient variability in hMSCs; (B) the immobilized system did not work; or (C) LL-37 feedback inhibition. It is known that LL-37 decreases the effects of LPS inhibiting the binding of LPS to LBP, which delivers LPS to the TLR4 signaling complex (Shaykhiev et al, 2010). The investigators were able to complete the task of "Determining the expression of LL-37 and TLR4 in 1,25-dihydroxy vitamin D₃".

Other achievements: None

Subtask 3-3: TLR4 activation by measuring the level of NF-κB. LPS or e.g. 1,25-dihydroxy vitamin D₃ will be used as activators.

Significant objectives:

Based on the results indicated above, it was decided to use vitamin D₃ as an IFP ASC stimulator for further study. Studies were carried out to investigate the mechanisms mediating the antimicrobial effects of VD₃-activated IFO ASCs. LL-37 acts as its own feedback inhibitor at the TLR4 receptor, moderating the immune response. The majority of studies on VD₃ activation are on macrophages. Additional pathways of activation are currently being explored, but the consensus is that VD₃ induces expression of LL-37 through the Vitamin D receptor (VDR). Previous results showed that LPS-or VD₃-treated IFP ASCs did not show any changes in TLR4 expression. Subsequent studies assessed TLR4 involvement in our model system by treating IFP ASCs with TLR4 antagonists/agonists (Figure 8A) and applying conditioned medium from these cells to bacterial cultures and determining CFU rates.

Significant results or key outcomes:

Materials and methods:

(1) *Bacterial culture:* same as described in Specific Objective 1.

(2) *CFU Assay:* IFP ASCs were cultured in growth medium. Twenty-four hours before the experiment, culture medium was replaced with fresh antibiotic-free growth medium. On the day of experiment, cells were rinsed with PBS and

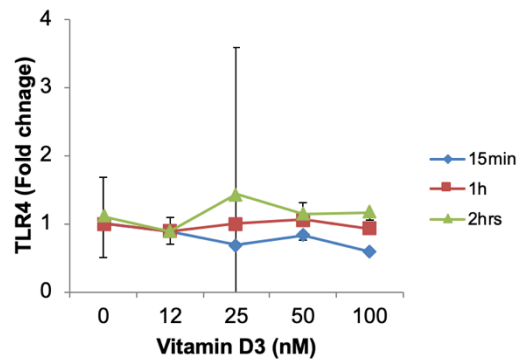


Figure 7. TLR4 expression with VD₃ treatment

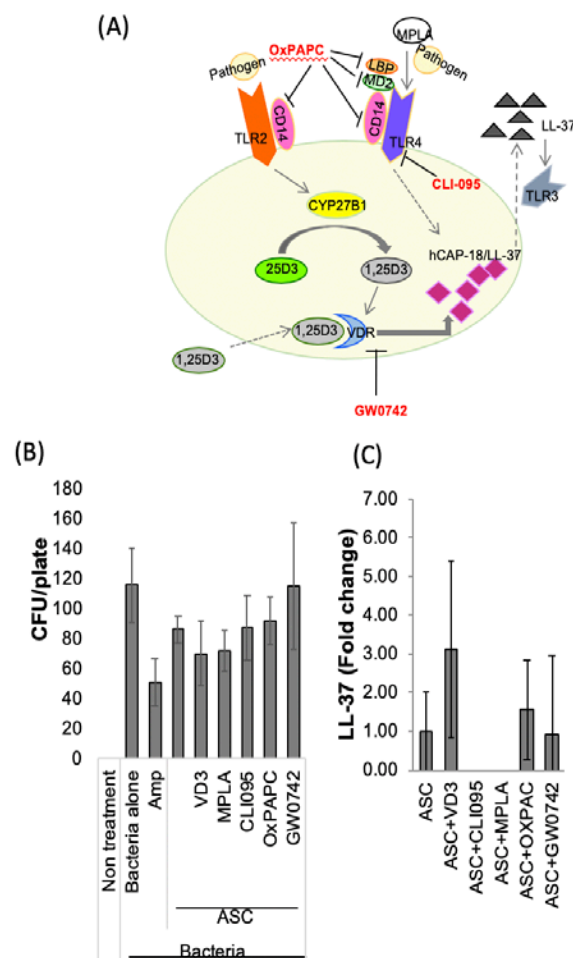


Figure 8. (A) LL-37 signaling pathway. MPLA (TLR4 agonist), CLI-095 (TLR4 antagonist), OXPAPC (TLR2 and TLR4 antagonist), GW0742 (Vitamin D signaling inhibitor). (B) CFU assays. (C) LL-37 expression

trypsinized, and pelleted by centrifugation at 1,100 rpm for 5 minutes. The pelleted cells were resuspended in growth medium without antibiotics. Finally, the cells were replated in non-tissue culture treated 6-well plates (1×10^5 cells/2 mL/well). Cells were then treated with various concentrations of TLR agonists (MPLA; monophosphoryl lipid A) or TLR 4 antagonist CLI-095, or TLR 2 /4 antagonist OXPAPC, and VD₃ for 1 day. 1 mL of conditioned medium was inoculated with 1.9×10^3 *S. aureus*, and incubated for 1 hour at 37°C in 5% CO₂ incubator. A cell-free control was processed under the same conditions. At the end of the incubation, a 50 µL aliquot of medium from each well (bacteria and medium) was plated on TSB agar for CFU counting. Plated dishes were incubated for 16-18 hrs at 37°C. Colonies were counted by using a colony counting pen (Fisher scientific, Waltham, MA). Unless otherwise specified, an antibiotic-free culture medium was used through the entire study.

Results:

Figure 8A shows the TLR4/LL-37 pathway already reported in other cell types in literature. Using ASCs, the investigators found that treatment with the TLR 4 antagonist CLI-095 and the TLR 2 /4 antagonist OxPAPC inhibited ASCs antimicrobial activity but not completely. TLR4 agonists MPLA did not affect the antimicrobial activity of IFP ASCs under the experimental conditions described here. These results suggest that both TLR4 and TLR2 signaling are be partially involved in the antimicrobial activity of IFP ASCs. Th einvestigators also observed that the antimicrobial activity of IFP ASCs was almost completely suppressed by the VD₃ signaling inhibitor GW0742 (Figure 8B). VD₃ enhanced LL-37 gene expression in ASCs (Figure 8C). OXPAC and GW0742 inhibited its expression. This result suggests that VDR plays a major role in the antimicrobial activity of IFP ASCs.

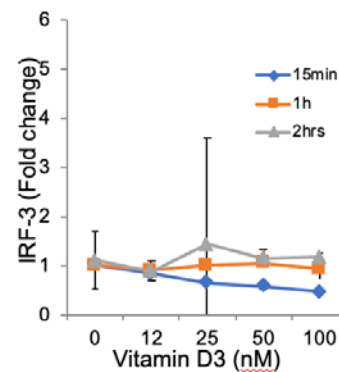


Figure 9. IRF-3 expression in VD₃ treated ASCs.

It has been reported that TLR4 signaling activates transcriptional factor NF- κ B and the unique intermediary IRF3 (Interferon Regulatory factor 3), Also, recent reports showed that once viral or bacterial infection triggers pattern recognition receptors (PRRs), TBK1 is activated and subsequently phosphorylates IRF3, which leads to the dimerization of IRF3 (Wang, 2016), 2). Activation of NF- κ B is also required for the dimerization of IRF3, suggesting crosstalk between NF- κ B and IRF3 (Goriely S, [Blood](#). 2006 Feb 1;107(3):1078-84). Inhibition of NF- κ B causes inhibition of IRF3. The investigators here did not observe changes in IFP ASC antimicrobial activity or TLR4 expression upon treatment with TLR4 agonists (see above). Therefore, the investigators assayed IRF-3 expression and activation as a member of both TLR4 and TBK1 signaling pathways. qRT-PCR for IRF3 and IRF3 ELISA was performed in VD₃ treated cells. However, the investigators did not observe significant IRF-3 gene expression (Figure 9). Also, there was no IRF-3 activation (by ELISA) observed under this model system. This suggests hat IFP ASC antimicobial activity may not be activated by the TLR4-IRF/- NF- κ B pathway. To confirm this results, the activation of NF- κ B in TLR4 agonist/antagonists-treated ASCs was investigated.

Major activity 4. Determine the mechanism of antimicrobial effects of LPS (or e.g. 1,25-dihydroxy vitamin D3) -stimulated ASCs.

TLR4 activation by measuring the level of NF- κ B. LPS or e.g. 1,25-dihydroxy vitamin D3 will be used as activators.

Specific objectives:

Based on our results previously reported, we decided to use vitamin D₃ as an IFP ASC stimulator for further study. We therefore carried out studies to investigate the mechanisms mediating the antimicrobial effects of VD₃-activated IFP ASCs.

Background: Vitamin D3 and antimicrobial protein expression: LL-37 regulates its own expression in a negative feedback loop via TLR4 receptor, moderating the immune response. The majority of studies on VD₃ activation are on macrophages. The field is testing additional pathways of activation, but the consensus is that VD₃ induces expression of LL-37 through the Vitamin D receptor (VDR). The investigators previously reported that both TLR4 and TLR2 signaling are partially involved in the antimicrobial activity of IFP ASCs and that the antimicrobial activity of IFP ASCs was almost completely suppressed by the VD₃ signaling inhibitor GW0742. They also reported that VD₃ enhances LL-37 gene expression in ASCs and that both OXPAC and GW0742 inhibited its expression. These results suggest that VDR plays a major role in the antimicrobial activity of IFP ASCs. **Mechanism of TLR4 regulation of LL-37 via NF- κ B:** It has been reported that TLR4 signaling activates the transcription factor NF- κ B via a unique intermediary IRF3 (Interferon Regulatory factor 3). Also, recent reports have shown that once viral or bacterial infection triggers pattern recognition receptors (PRRs), TBK1 is activated and subsequently phosphorylates IRF3, which leads to the dimerization and activation of IRF3 (Wang, 2016). Activation of NF- κ B is also required for the dimerization of IRF3, suggesting crosstalk between NF- κ B and IRF3 (Goriely S, [Blood](#). 2006 Feb 1;107(3):1078-84). Inhibition of NF- κ B causes inhibition of IRF3. Previously, the investigators did not observe changes in IFP ASC antimicrobial activity or TLR4 expression upon treatment with TLR4 agonists. Also, no IRF-3 activation was observed in this model system. This suggests that IFP ASC antimicrobial activity may not be activated by the TLR4-IRF/NF- κ B pathway. In this quarterly period, we investigated the activation of NF- κ B to confirm these results.

Significant results or key outcomes:

(1) Cell culture

Human ASCs (3 patients mixed, age 28-88) were cultured in growth medium. Twenty-four hours the day of experiment, cells were rinsed with PBS, trypsinized, and pelleted by centrifugation at 1,100 rpm for 5 minutes. The pelleted cells were resuspended in growth medium without antibiotics. Finally, the cells were re-plated in tissue culture treated 6-well plates (4×10^5 cells/2 mL/well). Cells were then exposed to 100 nM VD₃ for 1hr. After 1hr treatment, cells were collected using a cell scraper, rinsed with PBS, and then stored at -80°C until use.

(2) Isolation of Nuclear

extract Nuclear extract was isolated by using RayBio Nuclear Extraction Kit (RayBiotech). Protein estimation was determined by Pierce BCA protein assay kit (Thermo Fisher Scientific).

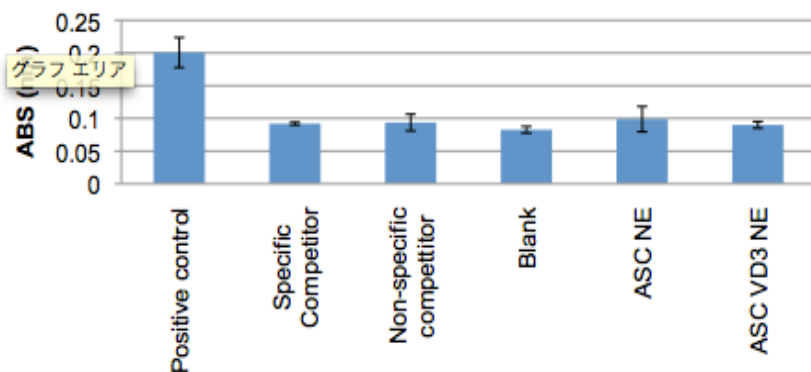


Figure 10. NF- κ B activation in ASCs with/without VD₃. ASC NE: Nuclear extract from non-treated ASC. ASC VD₃ NE: Nuclear extract from VD₃-treated ASC.

3) *NF-B activation by ELISA* NF-B activation was determined by using RayBio Human NF-B p65 transcription Factor Activity Assay Kit (RayBio). We used nuclear extract from 1×10^5 cells, which is the same cell number as we test antimicrobial activity.

These results show that the investigators did not observe activation of NF-B with/ without VD₃ treated ASCs (Figure 10). There are no significant difference between blank and ASC NE, blank and ASC VD₃ NE, or ASC NE and ASC VD₃ NE. These results suggest that ASC antimicrobial activity is not activated through the NF-B pathway.

Overall, these results show that VDR plays a major role in the antimicrobial activity of ASCs in the following way:

1. IFP-ASCs have greater bacterial (*S. aureus*) CFU reducing ability (antibiotic activity) when stimulated with vitamin D3 than with LPS.
2. The Vitamin D3-receptor inhibitor GW0742 blocks LPS and Vitamin D3-induced CFU reduction in IFP-ASCs almost 100%
3. Induction of LL-37 by vitamin D3 is independent of the TLR/IRF3/NF-kB signaling pathway dominant in macrophages.

At this point, the investigators have completed Specific Aim 1/Milestone in which they were to determine the mechanism of antimicrobial effects of LPS (or e.g. 1,25-dihydroxy vitamin D3) - stimulated ASCs.

Other achievements: None

Subtask 3-4: Stimu

late LL-37 expression using 1,25-dihydroxy vitamin D3 +/- *E. coli*/*S. aureus* (and/or *P. aeruginosa* and/or *K. pneumonia*).

This subtask is the same as 3-3 because it was decided that future studies should focus only on VD₃ activation of ASC antimicrobial activity (see above).

Other achievements: None

Major activities 5. Design a one-step, “point-of care” ASC activation device that employs timed exposure of autologous ASC to solid-phase-bound LPS (or e.g. 1,25-dihydroxy vitamin D₃).

Specific objectives:

- (1) Develop a one-step “point-of-care” product with solid-phase-bound LPS (now VD₃). (a) Immobilized LPS on polystyrene tube and optimize concentration of LPS. (b) Stimulate ASCs using LPS immobilized device. (c) Testing and trouble shooting of LPS immobilization.
- (2) Develop a one-step “point-of care” product with LPS immobilized-magnetic beads. If we use Vitamin D3, we do not need these steps.

Previous in vitro results showed that VD₃ is a more potent inducer of ASC anti-microbial activity than LPS. We will therefore use VD₃ instead of LPS for further study. We anticipate that VD₃ stimulation of IFP ASCs will be a relatively simple, easy, quick treatment because VD₃ is not toxic and does not require immobilization. VD₃ is a naturally occurring molecule, with no known toxicity at physiological levels. For this reason, the development of a special device to activate IFP ASC is not

necessary. Fresh VD₃ may be simply added to collected samples followed by incubation at 37°C under 5% CO₂. If a CO₂ incubator is not available under battlefield, there are many commercially available portable CO₂ tanks to supply CO₂ gas. However, it would be ideal if a system could be developed in which the battlefield medic or forward surgeon could culture/treat cells for few hours under an atmospheric CO₂ (i.e. NOT in an incubator) Medium with HEPES or MOPS will permit atmospheric treatment of IFP ASCs with VD₃ in the future.

Significant results or key outcomes Because VD₃ is the agent for ASC antimicrobial activity activation, the method of IFP ASC activation can be a very simple, easy, quick process, minimizing cell toxicity and not requiring immobilization.

Given the application parameters of VD₃ described above, the investigators have completed the Task to “Design a one-step, “point-of care” ASC activation device that employs timed exposure of autologous ASC to solid-phase-bound LPS (or e.g. 1,25-dihydroxy vitamin D₃)”, where VD₃ is the activator and solid phase exposure is not necessary.

Other achievements: None

Major activities 6. Apply an in vivo pre-clinical rabbit model of osteomyelitis (OM) to test the efficacy of LPS (or e.g. 1,25-dihydroxy vitamin D₃) -activated ASCs in the treatment of OM.

Specific objectives 1: Preparation of the animal protocol

Significant results or key outcomes: All protocols were approved by the local IACUC and ACURO. Given the potential suffering of the experimental animals, extensive meetings and collaboration occurred between the investigators, surgeons and the veterinary staff discussing the scope of study, work schedule and other requirements. (Subtask 1, preparation of animal protocol is completed)

Surgery group	Location	Survival rate at day 28 (%)	Signs of Illness	Osteomyelitis (Radiography)
Intra-articular bacterial injection with headed screw (intra-articular HS)	Femur	0	+	-
Intra-osseous bacteria injection + screw with head, no wax (intra-osseous HS)	Femur	0	+	-
Intra-osseous mGL-encapsulated bacteria implantation + screw with head, no wax (intra-osseous HS)	Femur	100	+/-	-
Intra-osseous bacteria injection + headless screw + wax (intra-osseous HLS)	Femur	100	+	+/-
Aseptic screw	Femur	100	-	-

Table 3. Survival, health and radiographic outcomes at 28 days after surgery

Specific objective 2 : Induction of Osteomyelitis in an in vivo rabbit model

Significant results or key outcomes: In establishing an OM model, two parameters were considered: (1) the best route of infection to produce OM and (2) the time course of OM. The objective was to develop OM associated with implant hardware.

For the demonstration and characterization of the OM model, 4 experimental groups were used: 1) Intra-articular injection, 2) Intra-articular injection + screw implantation, 3) mGL containing bacteria with screw implantation, 4) Intra-osseous bacteria injection into the screw hole, Groups 1-3 did not develop osteomyelitis (Table 3).

Material and Methods:

Rabbits: Rabbits (New Zealand White rabbits, 3 – 4 Kg, Female) were anesthetized and, in the case of screw implantation, their left femurs sterilely prepped, shaved and cleaned with alternating scrubs of Betadine and alcohol.

Bacteria preparation: *Staphylococcus aureus* is the most common gram-positive organism present in osteomyelitis. *S. aureus* (ATCC 25923) were grown in Tryptic Soy Broth (TSB) medium for 16 hrs at 37°C, and cultures were adjusted numbers on the basis of OD600 (OD600 = 1.0 corresponds to 6.6×10^8 CFU/mL).

Surgery groups: : 1) Intra-articular injection, 2) Intra-articular injection + screw implantation, 3) mGL containing bacteria with screw implantation, 4) Intra-osseous bacteria injection into the screw hole. 5 rabbits/group. The endpoint was 28 days or day of humane euthanasia.

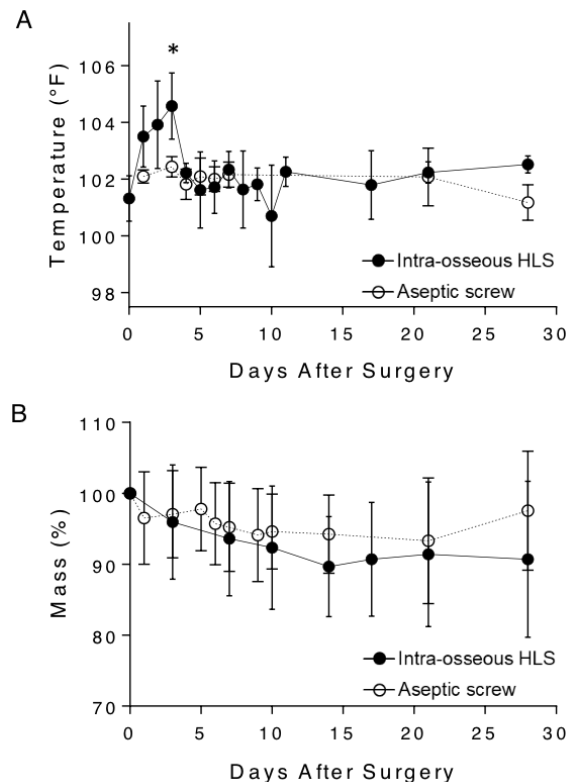


Figure 11. Physiologic markers of illness. (A) Temperature and (B) body mass of rabbits in the two groups of rabbits after surgery (n=5/group). * p < 0.05 vs aseptic group.

1) Intra-osseous bacteria injection into the screw hole in femur. Idea: Infections originate at the time of implant placement at the interface between the bone and implant. Free or encapsulated bacteria were used here exploring the idea that the periprosthetic area is immune-“privileged” enough to permit biofilm formation. Five Rabbits (New Zealand White rabbits, 3 – 4 Kg, Female) were used. A bone tunnel was created as described above. A 1µL bolus of a bacterial suspension of *S. aureus* (1×10^6 CFU/ µL) was introduced using a pipette after which a stainless screw was secured into bone as described above. The surgical site was closed.

Surgeries: Surgeries were performed and animals sacrificed as planned on day 5 and day 28. In the group testing intra-osseous bacteria delivery with headless screw implanted within the femoral condyle, all rabbits lost weight after surgery; however, they gained it back or maintained their weight (i.e. experiences less than 20% weight loss) over the following 28 days after surgery (Figure 11). Weight loss was

avoided through analgesia and antibiotic treatment (as described) as well as judicious food enrichment programs. Forced feeding, begun at 14% weight loss, was rarely required. All rabbits showed elevated ESR at day 3-7 that returned to normal by day 14.

Representative X-rays of a rabbit in the aseptic screw group 28 days after surgery (Figure 12A), and intra-osseous bacteria injection + screw group 28 days after surgery (Figure 12B). Neither the aseptic screw nor the intra-articular injection groups had any lysis around the implants. The intra-osseous group with metal implant into tibia did develop considerable peri-implant lysis by postoperative day 28 (data not shown). Figure 13 shows X-rays of rabbits in the intra-osseous bacteria injection + metal implant group (headless screw) in time-dependent manner.

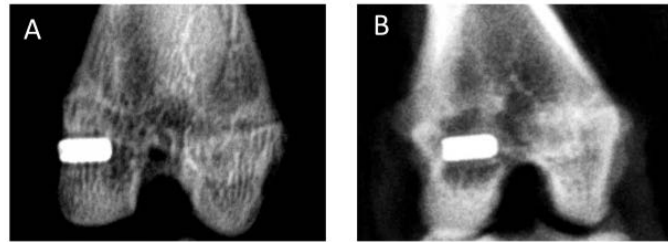


Figure 12. Representative X-rays of a rabbit. (A) Aseptic screw, (B) Intraosseous HLS

Reference: Odekerken JC, Arts JJ, Sutel DA et al. A rabbit osteomyelitis model for the longitudinal assessment of early post-operative implant. *Journal of Orthopaedic Surgery and Research*, 2013 (38).

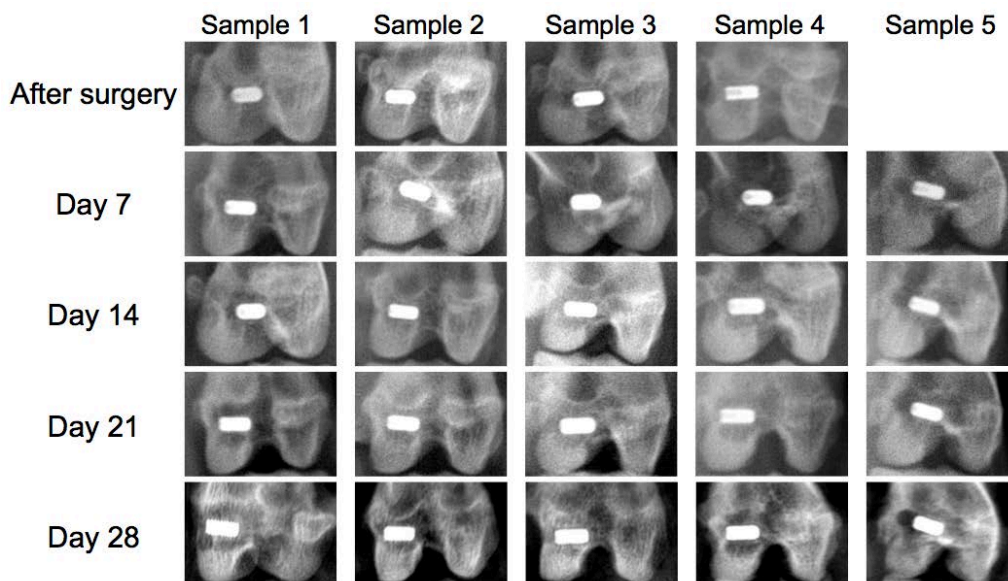


Figure 13. X-rays of rabbits in the intra-osseous bacteria injection + headless screw group

Blood Assays: Blood was collected at day 3, 5, 7, 14, 21 and 28 days from rabbits. The analysis of blood included quantification of erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and leukocyte count (WBC). These are common markers used to assess infection (and OM) in humans.

The data will help us characterize OM and compare the progression of the disease in rabbits and humans.

Whole blood was collected in commercially available anticoagulant treated tubes: The following assays were carried out:

- Erythrocyte sedimentation rate (ESR) : ESR level was determined by using the DISPETTE 2 kit (Fisher) within 24 hours blood collection.
- C-reactive protein (CRP): Collected whole blood into commercially available anticoagulant treated tubes. Cells are removed from plasma by centrifugation for 15 min at 2,000 x g. Centrifugation for 15 min at 2,000 x g depletes the plasma sample. Collected plasma was stored at -80 C° until use. CRP assay was performed by using C Reactive Protein (PTX1) Rabbit ELISA Kit (Abcam).
- Leukocyte count (WBC): A blood smear was prepared on a standard ColorFrost Plus™ slide and stained with polychromatic stains in the DipQuick stain kit (ThermoFisher). These stains will color acidic groups blue (DNA/RNA), basic groups orange (protein eosinophil granules), and metachromatic violet (mast cell and basophil granules). It is a valuable stain for blood cell differential counts and evaluations. Stained slides were dried out and kept at R.T. for analysis.

X-rays: X-rays were taken every week after bacteria inoculation. At the end of the experimental period, we collected both knees from each rabbits for X-ray and histological analysis.

Histology: Harvested knees were fixed in 10% Formalin for 5 days at 4C with shaking and then transferred into 70% ethanol until use. After gross analysis and digital photography, samples were stored in 70% ethanol at 4C until the next procedure. Before decalcification of samples, we carefully removed the screws and, using a fine toothed saw, first removed the condyles from the femoral shaft and second separated the condyles from each other. The lateral condyle was put into the 10% (v/v) neutral buffered paraformaldehyde (Electron Microscopy Sciences, PA, US) for 24hours. For decalcification, we submerged the tissue in Decal (StatLab, TX, US) for 1 day with gentle shaking and changing the solution every 8 hours. After ethanol dehydration, samples were dehydrated, equilibrated in xylenes, and finally embedded in paraffin. Samples were then sectioned to 6µm thickness from the lateral surface of the condyle toward to the notch. With this sectioning, we obtained anatomical sagittal plain sections perpendicular to screw direction. Sections were mounted on Superfrost Ultraplus O positively charged slides. We collected sections from 3 parts: superficial, middle and deep regions of the remaining screw hole. Each section was de-paraffinised with Histoclear™ and graded ethanol and then stained with Harris Hematoxylin (Sigme-Aldrich,

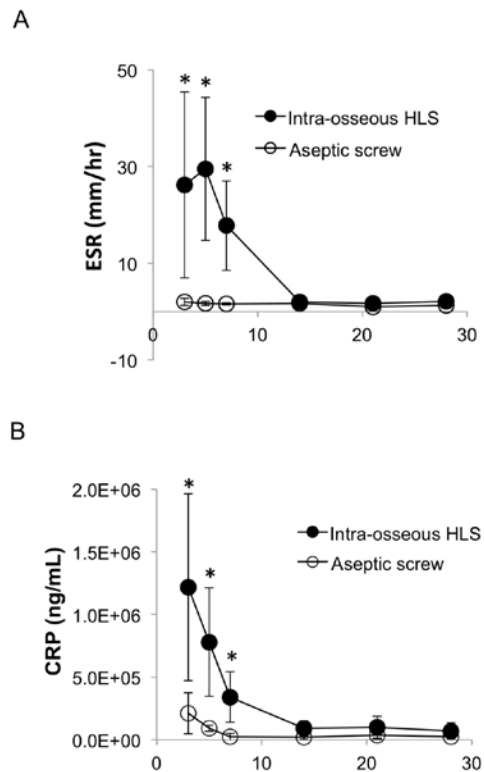


Figure 14. Hematologic evidence of inflammation. (A) ESR and (B) CRP values in the 2 groups of rabbits (n = 5/group, except at day 28, n = 4). * p < 0.05 vs aseptic screw group.

MO, US) and Eosin Y (Leica Biosystems, LO US). Stained sections were covered with mount medium and coverslip.

Results: Rabbits showed elevated CRP and ESR at day 3-7 that returned to normal by day 14 (Figure 14). Only rabbits from the aseptic screw group did not show elevated ESR. Laboratory values of white blood cell differentials revealed leukocytosis in the intra-osseous headless screw (HLS) model. Total WBC counts were elevated on both day 5 ($12,560 \pm 1128$ cells/ μ L) and day 28 ($13,060 \pm 737$ cells/ μ L) in the intra-osseous HLS group over aseptic screw controls ($6,580 \pm 826$ cells/ μ L and $6,780 \pm 750$ cells/ μ L, respectively) (Figure 15), while aseptic screw placement resulted in no increase in WBC over the duration of the experiment (approximately $6,680 \pm 751$ cells/ μ L). In comparison to the aseptic screw, intra-osseous HLS (Table 4) resulted in abnormal WBC differentials including increases in neutrophil count on day 5 ($51.8 \pm 5.6\%$) and day 28 ($51.6 \pm 0.2.7\%$) over control values ($32 \pm 5.2\%$ and $34.6 \pm 5.6\%$, respectively), and elevated monocytes on day 5 ($8.0 \pm 2.9\%$) and day 28 ($11.4 \pm 2.9\%$) over controls that remained unchanged ($2.9 \pm 1.5\%$) within normal ranges (Table 4). Lymphocytes remained in the normal range (Table 4) but were significantly lower on day 5 ($39.2 \pm 4.0\%$) and day 28 ($35.0 \pm 5.3\%$) than controls ($65.4 \pm 4.6\%$ and $62.0 \pm 5.3\%$, respectively) in this experiment. These laboratory values indicate a persistent infection and inflammation in the inoculated animals.

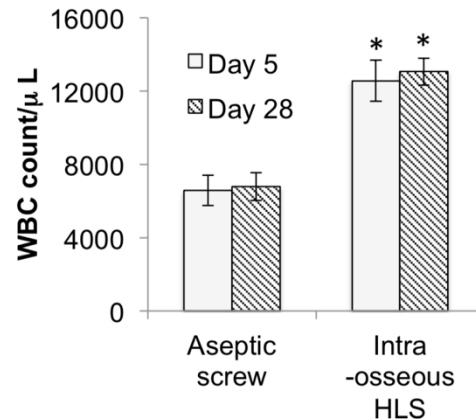


Figure 15. Hematologic evidence of infection. (WBC) in the 2 groups of rabbits. n=5/group). * p <0.05 vs aseptic screw group

After 28 days, no radiolucency or other radiographic parameters of osteomyelitis was observed surrounding the bone tunnel in the aseptic screw group and the samples were Gram stain negative (Figure 16 C, E). Hematoxylin and Eosin staining revealed fibrous tissue formation lining the bone tunnel in the intra-osseous injection group (Figure 16B). This fibrous tissue stained positively with the Gram stain (Figure 16D), and high magnification revealed Gram positive particles 1-2 microns in diameter characteristic of *S. aureus* cells (Figure 16F). This is our first histological indication of biofilm formation in this model.

	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Band Neutrophils (%)
Typical value	34-43	39-68	1.0-9.0	0.1-2.0	0.0
Day 5					
Aseptic screw	32.0 ± 5.2	65.4 ± 4.6	2.4 ± 1.1	0.2 ± 0.4	0.0
Intra-osseous HLS	51.8 ± 5.6 *	39.2 ± 4.0 *	8.0 ± 2.9 *	0.4 ± 0.9	0.4 ± 0.5
Day 28					
Aseptic screw	34.6 ± 5.6	62.0 ± 5.3	3.4 ± 1.8	0.0	0.0
Intra-osseous HLS	51.6 ± 2.7 *	35.0 ± 5.3 *	11.4 ± 2.9 *	1.0 ± 0.7 *	0.8 ± 0.8

* P < 0.05, compared to aseptic screw on the corresponding day

Table 4. Prevalance of selected white blood cells 5 and 28 days after surgery.

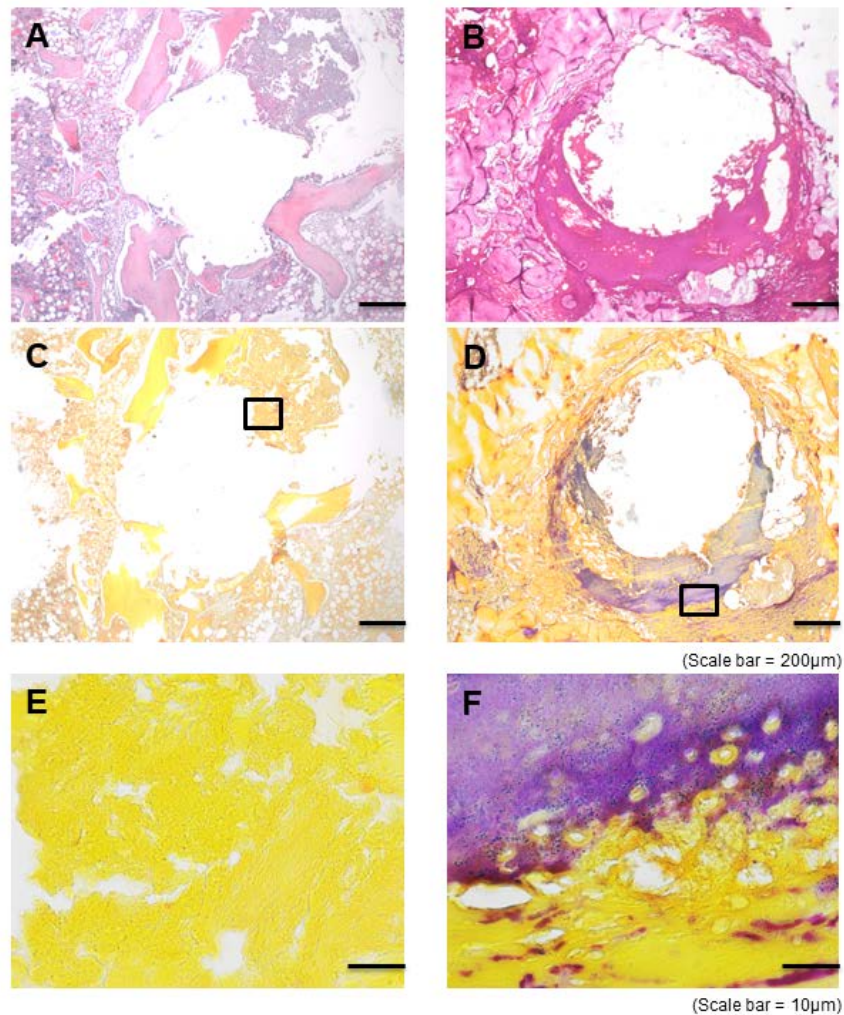


Figure 16. After 28 days, no radiolucency or other radiographic parameters of osteomyelitis was observed surrounding the bone tunnel in the aseptic screw group and the samples were Gram stain negative (Figure 16 C, E). Figure 16A, Hematoxylin and Eosin staining of aseptic screw group. H&E staining revealed fibrous tissue formation lining the bone tunnel in the intra-osseous injection group (Figure 16B). This fibrous tissue stained positively with the Gram stain (Figure 16D), and high magnification revealed Gram positive particles 1-2 microns in diameter characteristic of *S. aureus* cells (Figure 16F).

In summary, the investigators successfully created a rabbit model of peri-implant OM that can be used in future studies to test the safety and efficacy of various treatments. (Subtask 2, 2-1 completed)

Other achievements: None.

Specific objectives 3: Treatment of Osteomyelitis in an in vivo rabbit model

Significant results or key outcomes: For OM treatment, we will use allograft ASC. Therefore we need to isolate rabbit ASC from same rabbit species and test their antimicrobial activities before implantation.

ASC isolation from rabbit fat

Material and methods: 4 rabbits were obtained for harvest, culture and preparation of rabbit ASC and BM-MSCs.

For rabbit ASC collection, the rabbit fat pad was dissected from the intra-scapular region of the neck and placed in a Petri dish with Hank's Balanced Salt Solution (HBSS, Hyclone). The fat pad was minced into small pieces (~1 mm³), and any obvious fibrous tissue was removed. 4-20 mg /cc wet tissue Collagenase type I (Worthington) and 2-10 mg/cc Trypsin IIS (Sigma) were added to the conical tube containing the fat (up to 10 cc wet tissue). The fat was agitated continuously at 180 rpm, 37°C for 1-3 hours, and the tissue digest was filtered through 100 µm cell strainer, centrifuged at 1,500 rpm (400g) for 5min, resuspended in 25 mL growth medium (DMEM (Invitrogen) including 10% lot selected fetal bovine serum, 1x antibiotic-antimycotic (Invitrogen), 1 ng/mL fibroblast growth factor-2 (FGF-2; R&D System)). Cells were counted and plated in T-150 tissue culture flasks for expansion.

For rabbit BM-MSC collection, the bone marrow was debrided from rabbit femurs into 2% PSF DMEM rinsing medium. Repeated aspiration through a 2ml pipette was used to break the marrow into fine pieces. Marrow pieces were filtered through 40 µm cell strainers, centrifuged at 15000 rpm for 5 min, resuspended into growth medium to form a cell slurry/suspension. Cells were then distributed to T-150 tissue culture flasks for expansion.

Results: Rabbit ASCs and BM-MSCs were successfully harvested and expanded from rabbits (see

Antimicrobial activity of rabbit ASCs

Material and methods:

(1) Bacterial culture

Staphylococcus aureus is the most common gram-positive organism present in osteomyelitis. *S. aureus* (ATCC 25923) cultures were grown in Tryptic Soy Broth (TSB) medium for 16 hrs at 37°C, and cultures were adjusted to approx. 3×10^4 colony forming units CFU/mL on the basis of OD600 (OD600 = 1.0, which corresponds to 6.6×10^8 CFU/mL). Ampicillin (Amp) was used as the antibiotic against *S. aureus*. [Note: The bacterial concentration has been optimized in prior experiments to yield sufficient and countable colony numbers for better anti-microbial assessment.]

(2) CFU assay

Rabbit ASCs were cultured in growth medium. Twenty-four hours before the experiment, culture medium was replaced to fresh growth medium without antibiotics. On the day of experiment, conditioned medium was collected. Then, cells were rinsed with PBS and trypsinized, and pelleted by centrifugation at 1,100 rpm for 5 minutes. The pelleted cells were resuspended in growth medium without antibiotics. Finally, the cells were re-plated in non-tissue culture treated 6-well plates. Cells were treated with various concentration of VD₃ for 1 hr. And then, their conditioned medium were collected, inoculated with approx. 3×10^3 *S. aureus*, and incubated for 1 hour at 37°C in 5% CO₂ incubator. Unless otherwise specified, an antibiotic-free culture medium was used through the entire study. A cell-free control was processed under the same conditions. At the end of the 1-hour incubation, a 100 µL aliquot of medium from each well (bacteria and medium) was plated on TSB agar. Plated dishes were incubated for 16-18 hrs at 37°C. Colonies were counted by using a colony

counting pen (Fisher scientific, Waltham, MA).

Unless otherwise specified, an antibiotic-free culture medium was used through the entire study. A cell-free control was processed under the same conditions. At the end of the 1-hour incubation, a 100 μ L aliquot of medium from each well (bacteria and medium) was plated on TSB agar. Plated dishes were incubated for 16-18 hrs at 37°C. Colonies were counted by using a colony counting pen (Fisher scientific, Waltham, MA).

Results

In Figure 17, the antimicrobial activities of activated ASCs were tested and compared to that of naïve (unactivated) ASCs and BM-MSCs. Both rabbit ASC and BM-MSCs showed similar activities. It was decided that rabbit ASCs were to be used in the remainder of the study. for further study. Rabbit ASCs were expanded for a total of 40 million cells to each populations, frozen in 1 million cell stocks for the experiments described below.

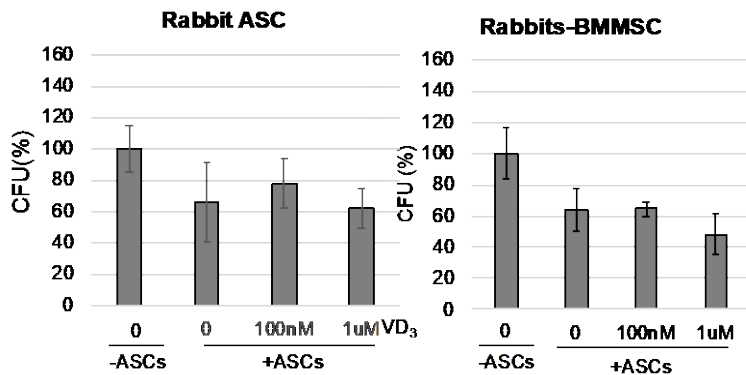


Figure 17. CFU assays. Treated with vitamin D3.

Significant results or key outcomes:

Treatment of Osteomyelitis in an in vivo rabbit model

As described above, the investigators established a rabbit OM model using an intra-osseous bacterial injection + headless screw (covered with bone wax). The investigators subsequently used this model to test the ability of activated ASC to improve the results of the standard clinical treatment (debridement, wash out and antibiotic treatment). The experimental groups for this study included induced osteomyelitis subsequently treated with rabbit ASC (20×10^6 cells/mL) encapsulated within a photocrosslinkable hydrogel (10% mGL) in the presence or absence of the activator (VD3) plus or minus systemic antibiotic treatment. After 28-day OM induction, the investigators performed the second surgery to remove metal implant and debride the unhealthy bone, followed by washout and filling of the defect with 1 of 3 materials (treatments): group 1: un-activated ASC treatment, group 2: activated ASC treatment, group 3: OM control [no cells]. All animals received systemic antibiotics to simulate the systemic antibiotic treatment utilized clinically. There were 5 rabbits/group with a 28-day endpoint: . Systemic antibiotics were given for 7 days after 1st and 2nd surgery and further analgesic treatment for 5-10 days after surgery and longer (at the discretion of

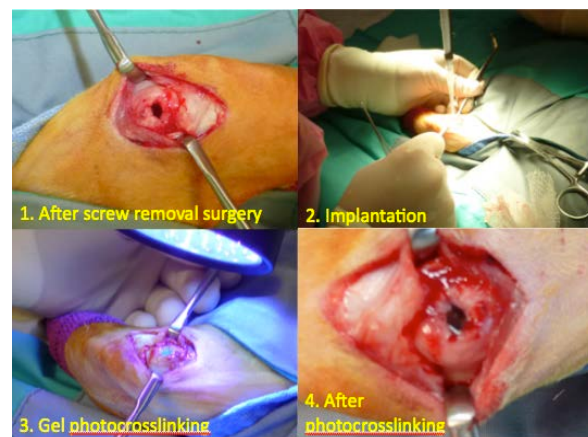


Figure 18. OM debridement and treatment procedure

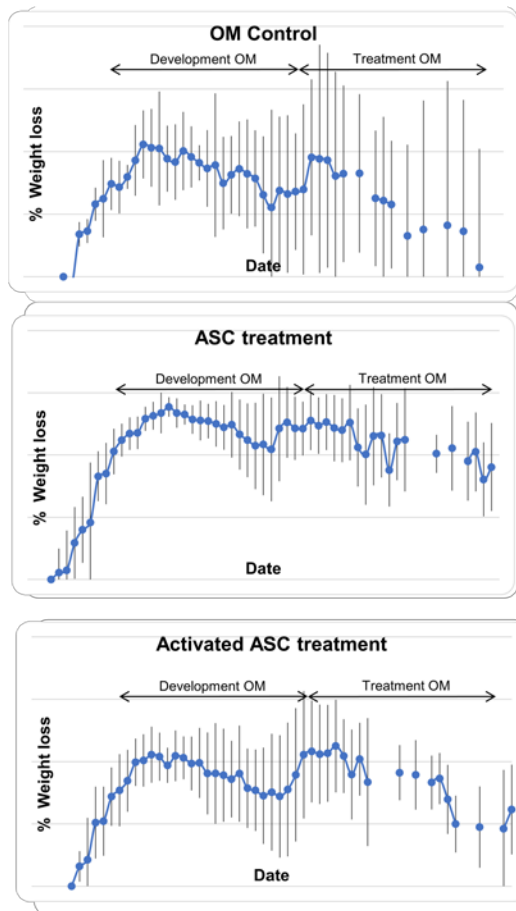


Figure 19. Weight. OM control (Upper, N=2), ASC treatment (Middle, N=4), activated ASC (Bottom, N=4).

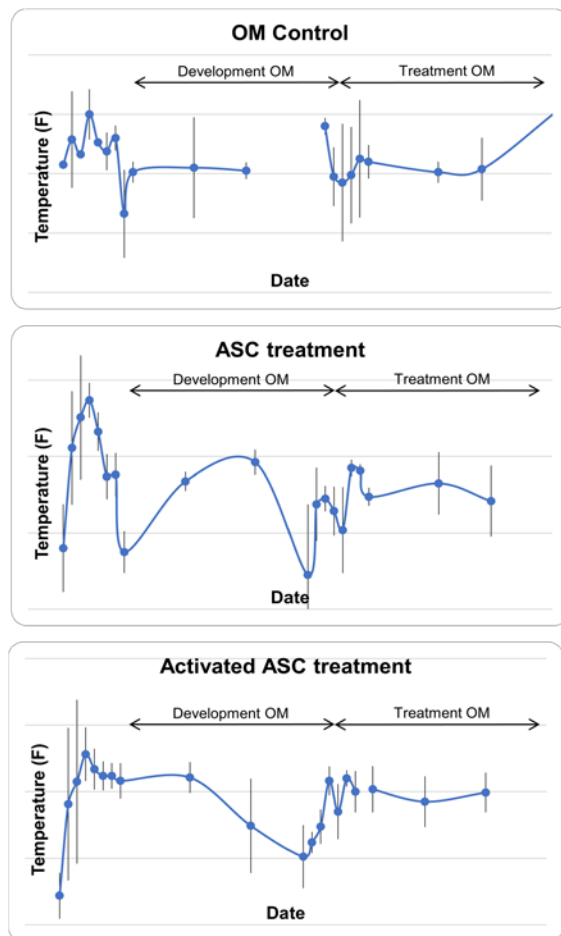


Figure 20. Body temperature. OM control (Upper, N=2), ASC treatment (Middle, N=4), activated ASC (Bottom, N=4).

the attending veterinarian) if necessary. Figure 18 depicts the major steps in the 2nd procedure involving removal of the screw, debridement and washout (Figure 18A), placement of the unpolymerized gel (containing cells or not) (Figure 18B), photo-polymerization (Figure 18C), and the final implant before closure (Figure 18D). Clinical parameters measured daily along with administration of care as required, 2x daily. In the course of the study, 5 rabbits were lost: 2 in groups 1 and 2 died of abdominal ulcers and 1 animal in group 3 (no cell treatment) died due to weight-loss. At 28 days all remaining samples were harvested, imaged, and processed for histology.

Assays and results:

1. No significant differences in body temperature (Figure 19) and mass (Figure 20) were observed between treatment groups over the course of the experiment. However, ASC treatment (whether naïve or activated) resulted in a plateau in temperature, whereas animals in the control, a-cellular treatment group seemed to suffer increased temperature near the end of the study (103.2F (N=2) at endpoint versus 102 F (N=5) at a week before endpoint). In comparison, animals in the

activated ASC group were more stable: (102F (N=4) at a week before endpoint, 101.8 F (N=4) at endpoint, $p=0.0057$ vs OM control).

2. Blood assays: blood was collected before surgery (baseline), and on days 3, 5, 7, 14, 21, and 28 before the second surgery (during development of OM), and after the debridement and treatment surgery on days 31, 33, and 35, 42, 49, and 56. Levels of CRP (Figure 21) and the ESR were assayed (Figure 22). No difference was seen between experimental groups in the CRP levels or ESR assays in the course of the experiment. Elevated CRP and ESR were observed in the first week, returning to normal by day 7 after treatment surgery. Interestingly, we did not observe an increase in ESR after debridement of the OM group (A-cellular implant). Both ESR and CRP are used to assess inflammation. In general, CRP is known to be a more sensitive marker of acute inflammation. WBC assays will be performed if necessary.
3. X-rays. Post-op x-rays were taken immediately after surgery and every week (7 days, 14, 21 and 28 pre-op and after debridement). Xray images were scored upon examination by 2 Orthopaedic

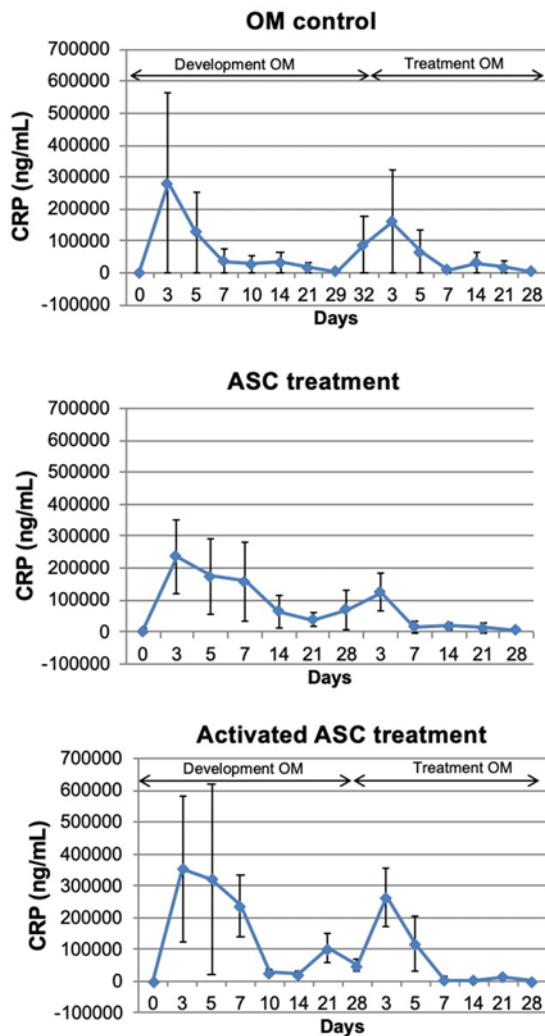


Figure 21. Level of CRP in plasma. OM control (N=3), ASC treated group (N=4), Activated ASC treated group (N=4).

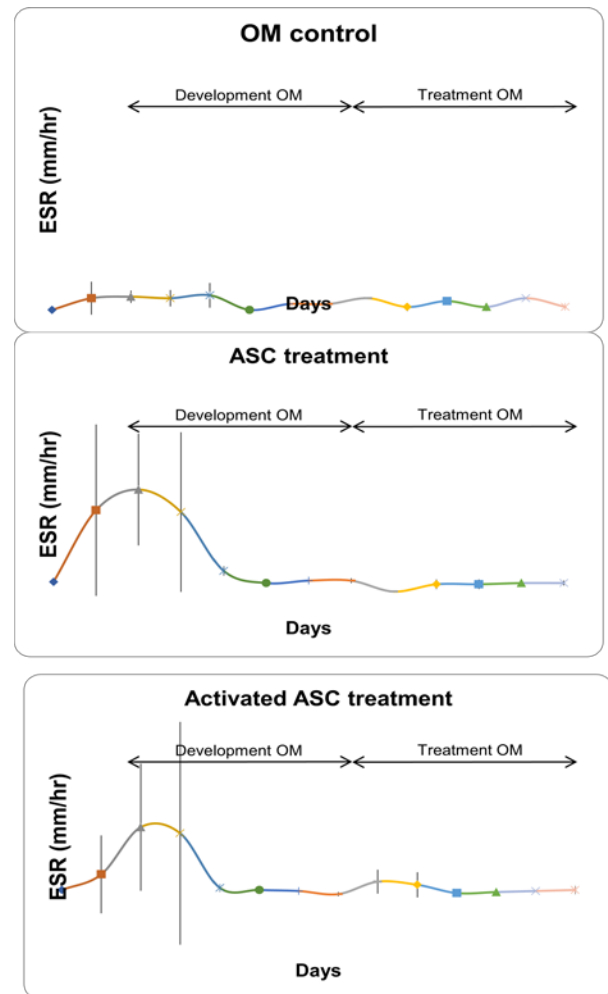


Figure 22. ESR. OM control (Upper, N=2), ASC treatment (Middle, N=4), activated ASC (Bottom, N=4).

surgeons based on imaging bone density. High scores indicate lower bone density. The scoring was as follows: Score 0: no bone, 1: weak bone signal, 2: mild bone, 3; strong bone signal (excellent healing) (Figure 23 and 24). Using this scoring system, no differences were observed between experimental groups, Complicating interpretation of the results, initial development of OM in the activated ASC group was not clear – the bone density at the time of second surgery was higher than the other groups and was unchanged after treatment surgery. In the activated ASC group there were 4 rabbits. 3 rabbits had a score of 1 or 0 (excellent bone/no OM) before treatment surgery. For the figures, data from only 1 rabbit was used, i.e. that rabbit with signs of OM, lower bone density (score = 2).

Subtask 3, “Test prepared ASCs in OM model” is completed.

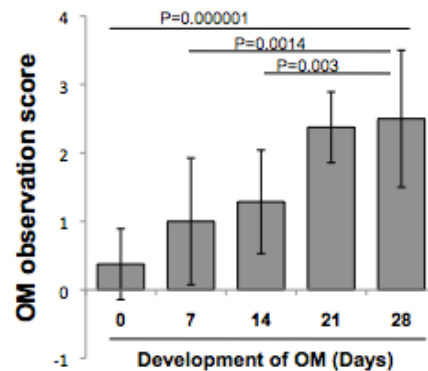


Figure 23. Osteomyelitis observation average score of development of OM. Xray images were scored upon examination by Orthopaedic surgeon based on imaging bone density. High score shows lower bone density. Score 0: none, 1: weak, 2: mild, 3; strong. (Total N=8, OM control, N=4; ASC treated group, N=4; Activated ASC group, N=1.)

Discussion: If systemic antibiotics are ineffective in treating OM, OM treatment involves (1) removal of hardware, (2) debridement of the infected bone, (3) extensive wash-out, (4) a temporary filler to restore bone that may contain antibiotics such as vancomycin, and (5) 6-12 weeks of systemic antibiotics. As adjunct therapy to the standard of care for OM (absent local vancomycin), naïve and activated ASCs had little detectable effect as compared to debridement and systemic antibiotics alone. The only indicator of ASC benefit was in the temperature of the animals at the close of the experiment, in which temperature on the a-cellular treatment group rose, indicative of continued OM, reflected in a slightly poorer bone quality score 28 days after treatment. WBS analysis and a shift in the neutrophil counts may provide insight into this possibility.

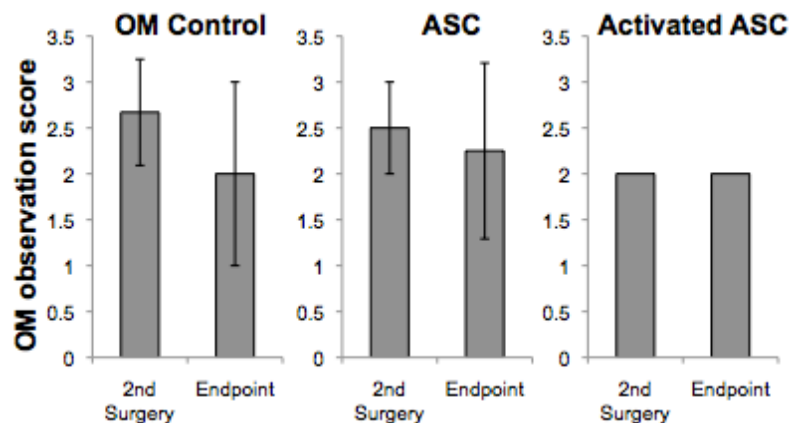


Figure 24. Osteomyelitis observation score of treatment of OM. Xray images were scored upon examination by Orthopaedic surgeon based on imaging bone density. High score shows lower bone density. Score 0: none, 1: weak, 2: mild, 3; strong. OM control, N=4; ASC treated group, N=4; Activated ASC group, N=1.

Shortcomings in the study:

1. Low animal numbers – effect of ASCs (activated or naïve) is small in the context of large individual variations in rabbit responses to surgery and therapy.
2. There should have been experimental groups not involving aggressive debridement and washing.
3. X-ray analysis is insufficient. X-rays of bone in vivo and after harvest and isolation affect interpretation of bone density. 3D CT or MRI should be used for analysis.
4. Despite excellent results in the study developing the OM model, some rabbits did not show clear development of OM – most notably in the control a-cellular group where animals showed no elevated ESR – though CRP levels were elevated. On the other hand, some rabbits suffered sepsis, causing loss of 2 animals in each ASC (activated and naïve) groups. Here again, a larger experimental group is required and standardization of OM before “treatment” needs to be developed.

In summary, the investigators established a peri-prosthetic OM model, demonstrated vitamin d3-activated ASC anti-microbial activity in vitro, but were unable to demonstrate this antimicrobial activity in vivo using the protocols used in this experiment.

Supplemental text and data:

Development of OM by using different methods:

Material and Methods:

Rabbits: Rabbits (New Zealand White rabbits, 3 – 4 Kg, Female) were anesthetized and, in the case of screw implantation, their left femurs sterilely prepped, shaved and cleaned with alternating scrubs of Betadine and alcohol.

Bacteria preparation: Staphylococcus aureus is the most common gram-positive organism present in osteomyelitis. S. aureus (ATCC 25923) were grown in Tryptic Soy Broth (TSB) medium for 16 hrs at 37°C, and cultures were adjusted numbers on the basis of OD600 (OD600 = 1.0 corresponds to 6.6×10^8 CFU/mL).

Surgery groups:

Intra-articular injection: Idea: infections may originate in the joint space and migrate along the margins of implanted hardware, defining the difference between acute planktonic (treatable) and chronic biofilm (“difficult/impossible” to treat). This group represents joint infection without an implant. After the left femurs were prepped, the bone was exposed by a small incision and immediately closed in layers and covered with a sterile dressing (a sham implant surgery). The patellar tendon was manually identified as an elastic structure on the anterior aspect of the knee. By visual control, a 25 G needle was then used to percutaneously deliver, at a medial parapatellar location, 0.1 mL of a bacterial (S. aureus) suspension of at a concentration of 2×10^5 CFU/0.1 mL to the in knee joint space .

Intra-articular injection + screw implantation: This group represents joint infection with implant. After the left femurs were prepped and the bone exposed, a 1.0 cm² area of periosteum was removed from the medial aspect of the medial femoral condyle. A 1.5 mm bit was used to drill a screw tunnel in the distal femoral metaphysis. A stainless screw (a 2.0 mm diameter by 10 mm length (#0 x 1/4” length, 90253A023, McMaster-Carr) was placed into screw tunnel and secured into bone. The surgical site was closed in layers and covered with a sterile dressing. As described above, 0.1 mL of a bacterial (S. aureus) suspension of at a concentration of 2×10^5 CFU/0.1 mL was then delivered to the in knee joint space.

mGL containing bacteria with screw implantation: Idea: Infections originate at the time of implant placement at the interface between the bone and implant. mGL was used to immobilize the bacteria to the periprosthetic area and permit development of a robust biofilm before the immune system can access the bacteria.

Rabbits (New Zealand White rabbits, 3 – 4 Kg, Female) were used. Following excision of a 1.0 cm² area of periosteum, a 1.5 mm bit was used to drill a screw tunnel in the distal femoral metaphysis. Then, a 1μL photopolymerized droplet of mGL containing bacteria was put into the screw hole. A stainless screw (a 2.0 mm diameter by 10 mm length (#0 x 1/4” length, 90253A023, McMaster-Carr) was placed into screw tunnel and secured into bone. The surgical site was closed in layers and covered with a sterile dressing

Direct bacteria injection into the screw hole Idea: Infections originate at the time of implant placement at the interface between the bone and implant. Free or encapsulated bacteria were used here exploring the idea that the periprosthetic area is immune-“privileged” enough to permit biofilm formation Five Rabbits (New Zealand White rabbits, 3 – 4 Kg, Female) were used. A bone tunnel was created as described above. A 1μL bolus of a bacterial suspension of S. aureus (1×10^6 CFU/ μL) was introduced using a pipette after which a stainless screw was secured into bone as described above. The surgical site was closed.

Direct bacteria injection into screw hole combined with head less screw as implant (Reported above). Throughout the previous studies, we remained concerned about unnecessary tissue injury caused by the screw head (as part of the implant) and bacteria leakage from edge of screw hole. To avoid these issues, we decided to use headless screws and bone wax to seal the bone-implant interface after implantation.

Intra-osseous bacteria injection into the screw hole in Tibia

For a symptomatic OM model, we will test (verify) a protocol described by Odekerken et al (2013), which reported a rabbit osteomyelitis model for the longitudinal assessment of early post-operative implant

infections. In this paper each animal received a 20-mm-long, 4-mm-wide metal implant in the proximal part of the tibia by transpatellar incision. Importantly, the investigators reported these rabbits survived for 6 weeks, initially losing weight but regaining it again by 6 weeks, post-operatively. In the tibial implant model, a 4-mm-wide defect was drilled into the tibia to open the tibial medullary canal. After reaming, the tibial medullary cavity was flushed with sterile saline to remove bone fragments and any forming haematoma. Each animal received a 20-mm-long, 4-mm-wide metal implant in the tibia. Immediately before insertion of the implant, the contamination group received an intramedullary contamination of a 1 μ L of a bacterial suspension of *S. aureus* (1×10^6 CFU/ μ L). The implant was press-fit into the tibial defect. After insertion, the defect was sealed with bone wax.

Rabbit #	ESR (mm/hr)					
	Day 3	Day 5	Day 7	Day 14	Day 21	Day 28
R241	33	91	75	2	2	2.5
R242	13	3	10	2	1	2
R243	27					
R244	29	11	30	5	2	2
R245	25	44	19	2	2	2.5
R247	49	60				
R248	31	68				
R249	2	53				
R250	17	107				
R148	2	38				

Supplemental Table 1. ESR assays. Direct bacteria injection into screw hole in Tibia model.

Results:

In the group testing intra-osseous bacteria delivery metallic implants within the medullary canal of the tibia, all rabbits lost weight after surgery; however, they gained back or maintained their weight over the following 28 days after surgery (less than 20% weight loss). Analgesia and antibiotic treatment (as described) as well as judicious food enrichment programs helped them to

keep their weight but not sufficient. Forced feeding was begun at 14% weight loss in all rabbits (n=5). 4 rabbits survived for 28 days while one was humanely euthanized due to weight-loss. Their ESR level increased at day 3 and got back to normal after 14 days (Supplemental Table 1). However, in our hand, their X-rays did not show any significant changes in their bones.

Summary, **We have successfully created a rabbit model of peri-implant OM that can be used in future studies to test the safety and efficacy of various treatments.** Our ongoing study is to develop a sub-acute, symptomatic model of OM with which to test anti-microbially activated ASC (with vitamin D3) as adjunct therapy to conventional treatment of osteomyelitis.

Reference:

Hang L, Cheng AW, Alexander PG, Beck, AM, Tuan RS., Cartilage Tissue Engineering Application of Injectable Gelatin Hydrogel with In Situ Visible-Light-Activated Gelation Capability in Both Air and Aqueous Solution. Tissue Eng Part A. 2014, p2402-2411.

Odekerken JC, Arts JJ, Sutel DA et al. A rabbit osteomyelitis model for the longitudinal assessment of early post-operative implant. Journal of Orthopaedic Surgery and Research, 2013 (38).

General outcomes are shown in Supplemental Table 2. Through out all these study, we finally established Rabbit OM model-**Intra-osseous bacteria injection into the screw hole in femur.**

Screw only, no bacteria	mGL with bacteria (2×10^5)	Intraarticular inoculation (2×10^5 or 2×10^6)	Injection of bacteria into screw hole (1×10^6)		
			Screw into femur	Headless screw into femur	Metal implant into tibia
Rapid return of weight bearing	Rapid return of weight bearing	Little to no weight bearing	Little to no weight bearing	Little to no weight bearing	On going study
Little to no weight loss	Little to no weight loss	Rapid weight loss (despite force feeding)	Rapid weight loss (despite force feeding)	Slow weight loss and gained back or maintain	
No increase in ESRs, slightly increase CRP, no fevers	No increase in ESRs, CRP, no fevers	Increase in ESRs, CRP, fevers	Increase in ESRs, CRP, fevers	Increase in ESRs, fevers	
		Purulence in joint	Purulence in joint, soft tissue abscesses	Soft tissue abscesses	
Sacrifice at planned time points	Sacrifice at planned time points	Early sacrifice	Early sacrifice	Sacrifice at planned time points	

Supplemental Table 2. General outcomes.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Residents and fellows in the Orthopaedic Surgery Department had a unique opportunity to work on OM in animal models. Those include: Antonia Chen, Adam Rothenberg, David Hirsch, Tomoya Iseki and Shinsuke Kihara.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Internal reports have been routinely submitted to the funding agency. Results have been presented at the MHSRS in Fort Lauderdale, FL (2017) and Orlando FL (2019), The Annual Orthopaedic Research Society Meeting in Austin TX (2019), the Annual Meeting of the Musculoskeletal Infection Society in Philadelphia, May 2019 and departmental and university Symposia.

2 papers have been submitted and are in review:

1. Development of a Large Animal Rabbit Model for Peri-Prosthetic Osteomyelitis, Bone and Joint Research
2. Antimicrobial activity of Mesenchymal Stem Cells Against Staphylococcus aureus, Stem Cell Research and Therapy

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

N/A (Final report)

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to Report.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*

- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

Development of a Large Animal Rabbit Model for Peri-Prosthetic Osteomyelitis, Haruyo Yagi PhD, Shinsuke Kihara MD, PhD, Peter N. Mittwede MD, PhD, Patrick A. Maher MD, Adam C. Rothenberg MD, Alyssa Falcione, Antonia F. Chen MD, MBA, Kenneth L. Urish MD, PhD, Rocky S. Tuan PhD, Peter G. Alexander PhD (Submitted to Bone and Joint Research, Current status is under review-revision)

Books or other non-periodical, one-time publications. Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).

- Yagi H, Chen AF, Rothenberg AC, Alexander PG, Tuan RS. (2015) Antimicrobial effects of adult mesenchymal stem cells. *The Pittsburgh Orthopaedic Journal*, Vol 26, p185.
- Yagi H, Alexander PG, Tuan RS. (2016) Antimicrobial effects of adult mesenchymal stem cells. *The Pittsburgh Orthopaedic Journal*, Vol 27, p178.
- Yagi H, Alexander PG, Tuan RS. (2017) Antimicrobial effects of adipose-derived stem cells. *The Pittsburgh Orthopaedic Journal*, Vol 28, p199.
- Kihara S, Yagi H, Mittwede P, Maher PA, Falcione A D, Sun A, Urish KE, Fu FH, Tuan RS, Alexander PG. Development of a Rabbit Model of Femoral Peri-Implant Osteomyelitis. *The Pittsburgh Orthopaedic Journal* (Submitted).

Other publications, conference papers and presentations. Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

- Treatment of Orthopedic Infections Using Activated Adult Mesenchymal Stem Cells. Tuan, R.S., US Army Medical Research and Materiel Command (USMRMC) Joint Program Committee (JPC-2) in –progress review, March 14, 2016, FT Detrick

- Treatment of Orthopedic Infections Using Activated Adult Mesenchymal Stem Cells. Tuan, R.S., US Army Medical Research and Material Command (USMRMC) Joint Program Committee (JPC-2) in –progress review, April, 2017
- Induced Expression of Cathelicidin by Bone Marrow and Adipose Derived Mesenchymal Stem Cells. Hirsch D, Residents' Research Day of Department of orthopaedic Surgery at University of Pittsburgh, June 1st, 2016.
- Antimicrobial effects of adipose-derived stem cells. Yagi H, Alexander PG, Tuan RS. (MHSRS, 2017)
- Development of a Rabbit Model of Femoral Peri-Implant Osteomyelitis, Yagi H, Mittwede PN, Kihara S, Maher PA, Falcione AD, Urish KE, Tuan RS, Alexander PG, , Department retreat, Department of Orthopaedic Surgery (University of Pittsburgh, June 29, 2018,)
- Development of a Rabbit Model of Femoral Peri-Implant Osteomyelitis, Yagi H, Mittwede PN, Kihara S, Maher PA, Falcione AD, Urish KE, Tuan RS, Alexander PG, (MSIS, Philadelphia, July 27-28, 2018)
- Development of a Rabbit Model of Femoral Peri-Implant Osteomyelitis, Shinsuke Kihara, Haruyo Yagi, Peter Mittwede, Patric A Maher, Alyssa D Falcione, Aaron Sun, Kenneth E Urish, Freddie H. Fu Rocky S. Tuan, Peter G Alexander. (ORS, Austin, TX, February 2-5, 2019).
- **Website(s) or other Internet site(s)**
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

ccme@pitt.edu
- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Development and characterization of a survivable (sub-lethal) model of OM in a rabbit
- **Inventions, patent applications, and/or licenses**
Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.
- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- data or databases;
- physical collections;
- audio or video products;
- software;
- models;
- educational aids or curricula;
- instruments or equipment;
- research material (e.g., Germplasm; cell lines, DNA probes, animal models);
- clinical interventions;
- new business creation; and
- other.

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith
 Project Role: Graduate Student
 Researcher Identifier (e.g. ORCID ID): 1234567
 Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

<i>Name:</i>	<i>Rocky S.Tuan</i>
<i>Project Role:</i>	<i>PI</i>
<i>Research Identifier:</i>	<i>University Employee ID# 124200</i>
<i>Nearest person month worked:</i>	<i>2.5 % effort (0.3 Person Months)</i>
<i>Contribution to Project:</i>	<i>Dr. Tuan provided oversight of data analysis, and supervised the day-to-day research activities of the project personnel.</i>
<i>Name:</i>	<i>Haruyo Yagi</i>
<i>Project Role:</i>	<i>Senior Research Specialist</i>
<i>Research Identifier:</i>	<i>University Employee ID# 129744</i>
<i>Nearest person month worked:</i>	<i>52% effort (6.24 Person Months)</i>
<i>Contribution to Project:</i>	<i>Haruyo is involved in all of the proposed Specific Aims.</i>
<i>Name:</i>	<i>Shinsuke Kihara</i>
<i>Project Role:</i>	<i>Research fellow</i>
<i>Research Identifier:</i>	<i>Badge ID# 175125</i>
<i>Nearest person month worked:</i>	<i>25% effort (Cost shared)</i>
<i>Contribution to Project:</i>	<i>Shinsuke is involved in Specific Aim 3.</i>
<i>Name:</i>	<i>Peter Alexander</i>
<i>Project Role:</i>	<i>Assistant Professor</i>
	<i>Badge ID# 124097</i>
	<i>10% effort (Cost shared)</i>
<i>Research Identifier:</i>	<i>Dr. Alexander is involved in Specific Aim 1-3, providing</i>
<i>Nearest person month worked:</i>	<i>oversight of data analysis, and supervised the day-to-</i>
<i>Contribution to Project:</i>	<i>day research activities of the project personnel.</i>
<i>Funding source:</i>	<i>Departmental</i>
<i>Name:</i>	<i>Masashi Taguchi</i>
<i>Project Role:</i>	<i>Research fellow</i>
<i>Research Identifier:</i>	<i>Badge ID# 175128</i>
<i>Nearest person month worked:</i>	<i>10% effort (Cost shared)</i>
<i>Contribution to Project:</i>	<i>Dr. Taguchi is involved in Specific Aim 3.</i>
<i>Funding source:</i>	<i>Departmental</i>
<i>Name:</i>	<i>Kenneth Urish</i>
<i>Project Role:</i>	<i>Assistant Professor</i>
<i>Research Identifier:</i>	<i>Badge ID# 71359</i>
<i>Nearest person month worked:</i>	<i>2% effort (Cost shared)</i>
<i>Contribution to Project:</i>	<i>Dr. Urish is involved in Specific Aim 3.</i>
<i>Funding source:</i>	<i>Departmental</i>
<i>Name:</i>	
<i>Project Role:</i>	<i>Do we have to add all the people who involved this</i>
<i>Research Identifier:</i>	<i>grant here?</i>

Nearest person month worked:

Contribution to Project

Funding source:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

- 9. APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*