

Infection burden and immunological responses are equivalent for polymeric and metallic implant materials *in vitro* and in a murine model of fracture-related infection

Edward T. J. Rochford,¹ Marina Sabaté Brescó,¹ Alexandra H. C. Poulsson,¹ Katharina Kluge,¹ Stephan Zeiter,¹ Mario Ziegler,² Liam O' Mahony,³ Robert Geoff Richards ,¹ Thomas Fintan Moriarty ¹

¹AO Research Institute Davos, Davos, Switzerland

²Swiss Institute of Allergy and Asthma Research (SIAF), University of Zurich, Davos, Switzerland

³Departments of Medicine and Microbiology, APC Microbiome Ireland, National University of Ireland, Cork, Ireland

Received 8 November 2017; revised 30 January 2018; accepted 18 February 2018

Published online 00 Month 2018 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.34202

Abstract: The development of an infection is a major complication for some patients with implanted biomaterials. Whether the material or surface composition of the used biomaterial influences infection has not been directly compared for key biomaterials currently in use in human patients. We conducted a thorough *in vitro* and *in vivo* investigation using titanium (Ti) and polyether-ether-ketone (PEEK) as both commercially available and as modified equivalents (surface polished Ti, and oxygen plasma treated PEEK). Complement activation and cytokine secretion of cell of the immune system was assessed *in vitro* for all materials in the absence and presence of bacterial stimulants. In a follow-up *in vivo* study, we monitored bacterial infection associated with clinically available and standard Ti and PEEK inoculated with *Staphylococcus aureus*. Complement activation was affected by material choice in the absence

of bacterial stimulation, although the material based differences were largely lost upon bacterial stimulation. In the *in vivo* study, the bacterial burden, histological response and cytokine secretion suggests that there is no significant difference between both PEEK and Ti.

In conclusion, the underlying material has a certain impact in the absence of bacterial stimulation, however, in the presence of bacterial stimulation, bacteria seem to dictate the responses in a manner that overshadows the influence of material surface properties. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res B Part B: 00B: 000–000, 2018.

Key Words: implant, material, surface, *S. aureus*, titanium, PEEK, osteosynthesis, implant-associated infection, electropolish, oxygen plasma treatment

How to cite this article: Rochford ETJ, Brescó MS, Poulsson AHC, Kluge K, Zeiter S, Ziegler M, Mahony LO, Geoff Richards R, Fintan Moriarty T. 2018. Infection burden and immunological responses are equivalent for polymeric and metallic implant materials *in vitro* and in a murine model of fracture-related infection. J Biomed Mater Res B Part B. 2018;9999:9999:1–12.

INTRODUCTION

Bacterial infection is a potential complication for all patients receiving implanted medical devices. The risk of infection arises from the potential for pathogens to access the implant site during the peri-operative period and adhere to and grow upon the biomaterial as a biofilm. Infection-associated implant failure rates for commonly implanted medical devices, such as prosthetic joints or dental implants, are between 1% and 5%^{1,2} and this number can increase for patients with soft tissue damage (e.g., open fractures)³ or with transcutaneous devices such as external fixators.⁴ Risk factors for developing an implant-related infection include age and health status of the patient⁵; the duration of the operative procedure; wound cleanliness; the implant site

location⁶; and the nature of the device itself (e.g., material, design, and insertion technique).^{3,7,8}

A wide range of biomaterials are used to manufacture medical devices, and the potential for these varied materials to differentially influence infection risk has been studied extensively, at least *in vitro*. Adhesion of bacteria to a biomaterial, considered to be the first step in biofilm infection development, can be influenced by material surface topography as has been shown *in vitro*.^{9–12} *In vivo* studies comparing the infection risk of different materials are comparatively scarce, however, the available literature indicates that material choice can also influence infection risk in laboratory animals in some,^{13,14} though not all cases.¹⁵ Studies have also shown that different materials can also differentially influence eukaryotic behavior (e.g., fibroblast, osteoblast,

Conflict of interest: None

Correspondence to: T. F. Moriarty; e-mail: fintan.moriarty@aofoundation.org

Contract grant sponsor: AOTrauma as part of the Clinical Priority Program on Bone Infection; contract grant number: AR2011_8

macrophage, and dendritic cell morphology and gene expression) *in vitro*,^{16–21} even to the extent that infection risk can also be influenced.²²

The most common materials used in the manufacture of orthopedic and trauma implants include the metals titanium (Ti) (and its alloys) and stainless steel, and polymers such as polyether-ether-ketone (PEEK). In applications involving direct bone contact, Ti typically is used with a microrough surface that supports osseointegration.²³ Standard machined PEEK implants are also manufactured with a microrough surface but the hydrophobic nature of the material is not as osseointegrative as Ti.^{16,24} However, the radiolucency of PEEK means that this material is advantageous in applications requiring close observations of bone healing. Both Ti and PEEK have been modified under experimental conditions with the aim of influencing tissue interactions for specific applications. For example, Ti has been polished to a smoother surface, which has been shown to reduce bony integration.²⁵ While modifications applied to PEEK to improve tissue integration include example oxygen-plasma treatment to increase hydrophilicity, or hydroxyapatite coatings.^{24,26} To date there is limited evidence as to how Ti and PEEK, either in their standard or modified forms, affect implant-associated infection risk. *In vitro* studies have shown bacterial adhesion is not significantly or consistently different between Ti and PEEK.^{9,10} In contrast, a rat model indicated Ti may facilitate greater bone formation adjacent to bacteria-colonized implants compared with equivalents composed of PEEK.²⁷ To date, no clinical study has compared infection rates between these two materials, although isolated case reports of infection associated with Ti and PEEK implants have been published.^{28,29}

Due to the lack of consensus regarding the comparative infection resistance of Ti and PEEK, we conducted a thorough *in vitro* and *in vivo* investigation using both commercially available and modified (surface polished titanium and oxygen plasma treated PEEK) forms of these implant materials. Specifically, we compared Ti, PEEK, and other implant materials in the presence and absence of bacteria and/or lipopolysaccharide (LPS) with regard to indicators of complement activation and immune response. In a follow-up *in vivo* study, we monitored bacterial infection associated with clinically available and standard Ti and PEEK using a detailed and clinically relevant fracture fixation model in two murine strains to determine if there is any significant difference in outcome as observed by quantitative bacteriology or histology. Two well-characterized and widely used inbred mouse strains with differing immune responses to infection were used in this study. C57BL/6 mice are T helper (Th)-1 skewed, and BALB/c mice are Th2-skewed and including both strains allowed us to determine if any significant findings were consistent across both mouse strains.

MATERIALS AND METHODS

Discs and implant materials

The *in vitro* studies used circular discs (13 mm diameter) manufactured from medical grade materials as listed in Table I. The metal discs were supplied by Synthes Holding

TABLE I. The Implant Materials Used in the *In Vitro* Study and Their Roughness Values as Measured by Atomic Force Microscopy

Disc Material	Abbreviation	Arithmetic Mean Roughness $\pm$ Standard Deviation (nm)
<i>Metallic materials</i>		
Commercially pure microrough titanium	TS	405.6 $\pm$ 113.3
Electropolished commercially pure titanium	TE	208.3 $\pm$ 23.5
Stainless steel	SS	139.4 $\pm$ 72.7
Microrough titanium–aluminum–niobium	NS	252.8 $\pm$ 10.8
Electropolished titanium–aluminum–niobium	NE	65.5 $\pm$ 16.3
<i>Polymeric materials</i>		
Injection molded PEEK	PO	84.96 $\pm$ 21.55
30-min Oxygen plasma treated PO	PO ₃₀	106.4 $\pm$ 13.95
Machined PEEK	PA	536.5 $\pm$ 335.76
30-min Oxygen plasma treated PA	PA ₃₀	385.0 $\pm$ 107.83

AG, Solothurn (CH), and the PEEK samples were supplied by Inivbio Biomaterial Solutions Ltd., Thornton-Cleveleys (UK). For the *in vivo* study, titanium and PEEK Mousefix implants were supplied by RIsystem AG (CH). The Ti implants were the standard 4-hole rigid Mousefix plates, while the PEEK implants were custom made from PEEK Optima[®] (raw material provided by Inivbio Biomaterial Solutions Ltd., Thornton-Cleveleys, UK) and were 0.2 mm thicker than the titanium plates to compensate for the reduced stiffness of the PEEK material. Both implant types were fixed in place with Titanium – 6% Aluminum 7% Niobium (Ti-6Al-7Nb, TAN) screws. All materials were cleaned and sterilized as previously described.³⁰

Surface characterization

The surface topography of the plates was measured by scanning electron microscopy (SEM) and atomic force microscopy (AFM) as previously described.⁹ For AFM measurements, a Multimode SPM with a Digital Instruments Nanoscope III controller (Veeco Instruments, UK) was used to measure the arithmetic mean roughness (R_a) of each material (from 60 μ m x 60 μ m areas in tapping mode with a silicon nitride tip) with Nanoscope 6.13R1[®] software (Digital instruments Veeco, 2002). For SEM analysis, PEEK discs were coated with 10 nm of gold and palladium using a BAL-TEC MED 020 sputter coater connected to a BALTEC MCS

010 and a BAL-TEC QSG 060 (BAL-TEC AG, FL). Images of all the material surfaces were acquired using a Hitachi S-4700 FESEM and Quartz PCI image management system (version 5.10; Quartz imaging corporation, CA).

Bacterial preparation

A clinical isolate of *Staphylococcus aureus* (JAR 06.01.31, available at the Culture collection of Switzerland, strain CCOS890) was used for both the *in vitro* and *in vivo* studies. For the *in vitro* studies, bacteria were pre-adhered to the discs (2×10^5 CFU/disc) using the bacterial adhesion chamber method previously described.⁹ After adhesion of bacteria to the discs, the samples were carefully removed to 24 well plates and washed by careful addition and removal of 1 mL of PBS to the wells. The samples were then maintained in PBS at 4°C until use (maximum of 18 h). The storage period did not influence bacterial viability (data not shown). For the *in vivo* study, the bacterial inoculum was introduced to the mice on pre-contaminated Mousefix plates prepared as previously described³⁰ at an inoculum known to induce consistent infection (approximately 9×10^5 CFU per implant). Both Ti and PEEK plates were found to yield equal bacterial burden in pilot adhesion tests (data not shown).

In vitro innate immune responses

Complement activation assay. Complement activation in the presence of materials with or without adherent bacteria was measured using C3a-desArg ELISA kits (TecoMedical AG, CH) and 1:15,000 diluted pooled human serum according to the manufacturer's instructions. The optical density of the sample and control wells was measured using a Mithras LB 940 ELISA plate reader (Berthold technologies, DE) at a wavelength of 450 nm. Calibration curves were generated using the four-parameter logistic analysis found on the readerfit.com. The materials were assessed in triplicate.

NF- κ B activation assay. THP-1 Blue™ cells (InvivoGen, US) were used to monitor NF- κ B activation. Duplicate discs were placed into 24-well plates and 1 mL of complete RPMI (cRPMI) containing 5×10^5 THP-1 cells was added. Duplicate negative control wells containing no discs and positive control wells containing no discs with 5 μ L of LPS (Sigma-Aldrich, CH) (100 μ g/mL) added to stimulate the cells were used in each plate. The plates were incubated for 24 h at 37°C, 5% CO₂. From each well, 20 μ L of the culture media were extracted in duplicate and added to 96 well plate wells containing 200 μ L of Quantiblue (InvivoGen, US). The plates were incubated in the dark for 24 h at 37°C. After incubation the optical density of each well was quantified at a wavelength of 620 nm using a Mithras LB 940 ELISA plate reader. The results were normalized to the negative controls, and cRPMI media controls were used to provide a blank measurement. All of the materials were assessed in triplicate.

PBMC isolation and culture. Whole blood from healthy human donors was diluted in sterile PBS (Life technologies, UK) and added into blood separation tubes (Bioswisstec AG, CH) which contained Bicoll (Biochrom AG, CH). The tubes

were then centrifuged at 800g for 20 min at room temperature to isolate mononuclear cell layer. Cells were passed through a 70 μ m pore size cell strainer (BD, CH) before being washed twice in PBS. An erythrocyte cell lysis step was performed for 30 s in ultrapure water. After repeating the washing step, the pellet was finally re-suspended in cRPMI. Subsequently, 5×10^5 cells/well were cultured in 24-well plates with the different material discs, with or without bacteria adhered. Cells cultured alone were used as negative controls and cells stimulated with LPS (100 μ g/mL) were used as positive controls. Each condition was assessed in duplicate. Plates were incubated 24 h at 37°C with 5% CO₂. Supernatants were then collected to measure cytokine production and cells were washed and kept in 350 μ L of RLT lysis buffer (from RNeasy Plus Mini Kit, Qiagen, CH) containing 10 μ L/mL of β -mercaptoethanol (ROTH, DE) at -20°C for real time PCR.

Real time PCR. RNA isolation was performed using Qiagen RNeasy plus mini kits (Qiagen, CH) according to the manufacturer's instructions. Following isolation, reverse transcription was performed with Reaction buffer (5x, Fermentas, CH), deoxynucleotide triphosphates (dNTPs, 1 mM, Fermentas, CH), random hexamer (0.2 μ g μ L⁻¹, Fermentas, CH), ribonuclease inhibitor (20 U μ L⁻¹, Fermentas, CH) and Revert Aid M-Multi V RT (200 U μ L⁻¹, Fermentas, CH). The program used for the reverse transcription was as follows: 10 min at 25°C, 60 min at 42°C, and 10 min at 70°C. RT-PCR of the samples was performed using the primers shown in Table 6.2. A 7900HT Fast Real-Time PCR system (Applied Biosciences, CH) was used to perform the absolute quantification. The data was processed using SDS (v2.2.2.; Applied Biosystems, CH), all of the data was normalized to the elongation factor and negative controls (PBMCs alone).

Cytokine detection. Cytokine measurements of *in vitro* stimulated PBMCs were conducted using BIO-RAD custom kits (BIO-RAD, CH) for the following analytes: interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF)- α . For each analysis, 50 μ L samples were used and the kits were used as described by the manufacturer. The concentration of the analytes was measured using a BIO-PLEX 200 systems connected to a BIO-PLEX HTF (BIO-RAD, CH).

In vivo assessment of the materials

Consent to perform this study was granted by the ethical committee of the Canton of Grisons in Switzerland and was carried out in an AAALAC accredited research institute (permission number 2012/15). Skeletally mature, female, specific pathogen free (SPF) C57BL/6 and BALB/c mice (used between 20 and 28 weeks old), were purchased from Charles River (Germany). Animal handling and surgery was performed as previously described.³⁰ Briefly, under general anesthesia with isoflurane (2–3% isoflurane in 100% O₂, 1 l/min) (Isofluran Baxter®, Baxter AG, Switzerland), the animal was placed in prone position and a skin incision was made from tail base to left stifle. The subcutaneous Fascia lata was cut and the tissue plane between the quadriceps and the biceps femoris muscle

was bluntly dissected. A Teflon foil was placed around the femur to protect the soft tissue from contamination. The implant was then fixed to the bone using four self-tapping, angular stable screws. Screw holes were predrilled with a 0.31 mm drill bit (RISystem AG, Switzerland). Once the implant was in place, a 0.44 mm defect osteotomy was performed using a jig and a Gigly wire. The foil was then removed and the Fascia lata and the skin were closed with continuous sutures (5-0 Vicryl rapide, Ethicon, Belgium). Following surgery, a lateral radiograph of the femur was taken to confirm proper positioning of the implant. Six mice were included per group, receiving either a Ti or PEEK plate. Animals were grouped into three experimental time-points (1-, 3-, and 7-day groups) with six animals in each group.

Post euthanasia sample processing

At the scheduled time-points, animals to be sampled were anesthetized in an induction box (5% isoflurane in O₂, 1 L min⁻¹) before cervical dislocation. The operated femur, the implant, and the soft tissue surrounding the implant were carefully dissected and placed in PBS in a sterile container. All tissue samples were immediately placed on ice. The tissue samples were subsequently homogenized (Omni Tissue Homogenizer, Omni International, USA). A quantitative assessment of bacterial load was performed from each tissue and from the implants as previously described.³⁰ Cytokine measurements of *in vivo* stimulated bone and soft tissue cells were performed as described above for the *in vitro* experiments.

Histology

Sixteen animals were operated to provide sections for histopathological evaluation of contaminated and control implants ($n = 4$ per group, per time-point). Animals were sacrificed at 3 and 7 days to evaluate the acute phase of the infection. The entire operated femur and surrounding musculature was removed and immediately fixed and embedded as previously described.³⁰ Sections were stained with Giemsa Eosin and qualitative histopathological analysis was performed using a light microscope (Olympus, Switzerland).

Statistical analyses

Data are presented as mean averages for each group with standard deviations (s.d.). A two-way ANOVA was used to compare differences between infected and non-infected groups versus un-operated controls for gene expression and cytokine secretion and Sidak's *post hoc* test was used to control for multiple comparisons. For flow cytometry data cells from non-infected animals were pooled to yield sufficient numbers. Therefore, the change in cell number over time was measured by a one-way ANOVA. In all cases, significance was set at: $*p < 0.05$; $***p < 0.01$; $****p < 0.001$, $*****p < 0.0001$. Prism software was used for all statistical tests (GraphPad Software Inc., La Jolla, CA).

RESULTS

Surface properties

The surfaces of the materials used in this study were characterized by AFM, the results of which can be seen in Table I

and Figure 1. Electropolished TAN displayed the smoothest surface. Machined PEEK and standard Ti were the roughest materials, caused by the respective manufacturing processes. Injection molded PEEK displayed a smoother surface than machined equivalents. Oxygen plasma treatment of the injection molded and machined PEEK led to an increase and decrease in surface roughness, respectively. The machined PEEK surfaces had the roughness of the peaks reduced, while injection molded PEEK experienced some pitting and general roughening of the surface as a result of the plasma treatment.

Complement and NF- κ B activation

Activation of complement by the materials alone and in the presence of *S. aureus* is shown in Figure 2(A). There were no observed differences between the standard materials (Titanium and PEEK), either alone or in the presence of bacteria. In general, the other metals (TE, SS, NS, and NE) all showed elevated complement activation relative to both the standard PEEK and Ti and the test polymers in the absence of bacteria. However, only TE was shown to differ from the standard or modified polymers ($p < 0.05$). Once bacteria were added to the discs however, differences were no longer observed between any of the materials.

NF- κ B activation in THP-1 monocytes is shown in Figure 2(B). In the presence of materials alone, PEEK induced higher levels of NF- κ B activation than Ti, although this did not reach statistical significance. In the presence of LPS, both materials induced a similar level of activation. All other test materials (metals and polymers), showed no significant differences either in the presence or absence of LPS.

In vitro gene expression

RT-PCR analysis of PBMCs exposed to the materials alone did not reveal any significant signal for any of the tested cytokines, indicating that the materials alone did not induce significant responses over and above exposure to tissue culture plastic (data not shown). Similarly, expression of the tested genes did not differ between the materials when LPS was used as a stimulus (data not shown). When PBMCs were stimulated by *S. aureus*, there was a slight increase in IL-10 and TNF- α , and a greater increase in IL-6 expression for all materials [Figure 3(A)]. There was no significant difference between the materials with regards to any of the tested cytokines other than an increase of IL-6 in the presence of PA30 in comparison with all others and between NS and NE [$p < 0.05$, Figure 3(A)].

In vitro cytokine secretion

Stimulation of PBMCs with each material and *S. aureus* did not result in any material-dependent differences for any of the tested cytokines (TNF- α , IL-10, GCSF, MIP 1 α , and IL-12) other than an increase in MIP-1 α for PO relative to NE [Figure 3(B)].

In vivo study

Implants used in the *in vivo* study were shown to display similar surface features as those tested in the *in vitro* study

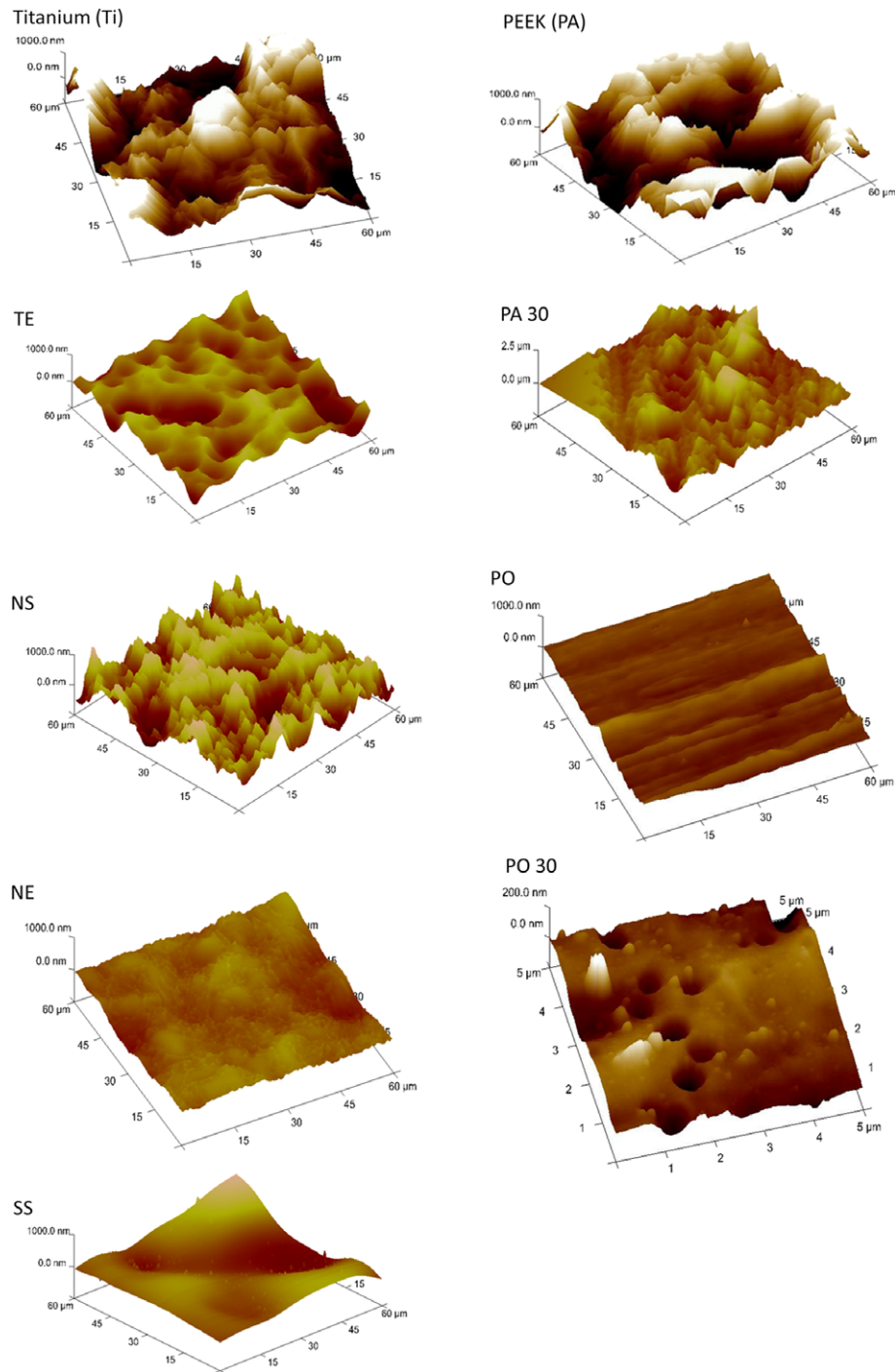


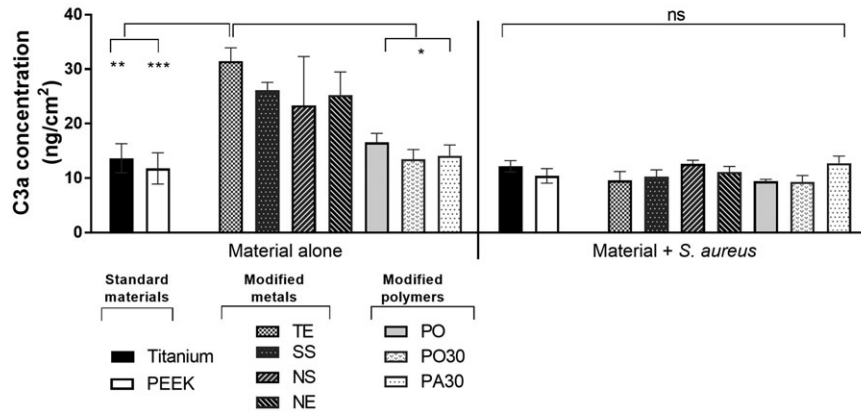
FIGURE 1. Representative 60 x 60 μm scan representing the metals tested in the study. Images represent surfaces generated by AFM in tapping mode. The Z-axis had a range of 1 μm and is disproportionate from the X and Y to illustrate the surface features of the material. Brightness is proportional to height. Abbreviations as per Table I.

when evaluated by SEM (Figure 4). Furthermore, bacterial adhesion to the implants prior to implantation was found to be statistically similar in both studies ($p = 0.543$).

All animals in the C57BL/6 and the BALB/c groups, were found to be culture positive for *S. aureus* at the scheduled euthanasia time-points in both the Ti and PEEK groups. The numbers of bacteria cultured from the implant, bone and

soft tissue for all groups is shown in Figure 5. Consistent with earlier findings using this model, bacteria rapidly increased from the pre-implantation number (9×10^5 CFU/Implant) to approximately 2×10^7 CFU/implant at Day 1 and Day 3 (Figure 5), and this was observed for both groups. Bacterial loads were highest in the soft tissue for C57BL/6 mice and on the implant for BALB/c mice. The

(A) Complement activation



(B) NF-κB activation

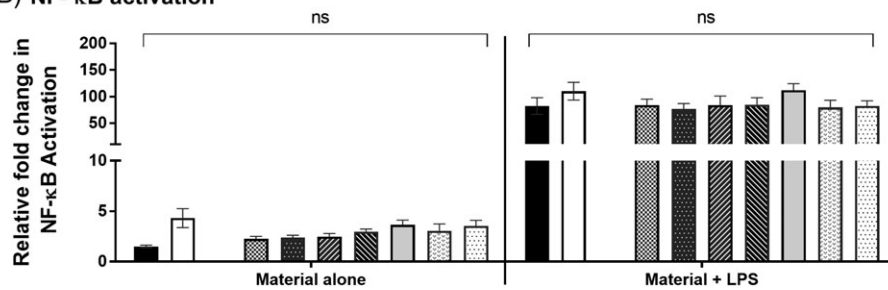
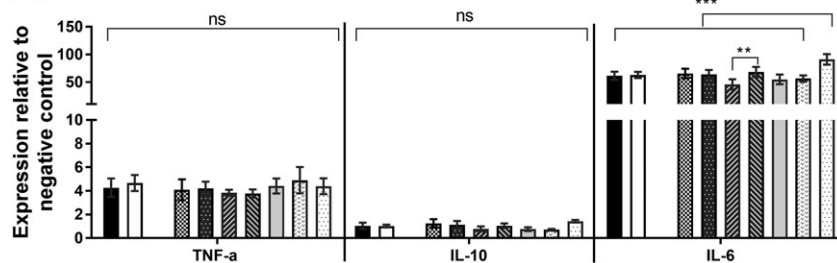


FIGURE 2. (A) Results of the C3a-desArg complement activation ELISAs (A) for the materials alone (left) and the materials with adherent *S. aureus* JAR (right) ($n = 3 \pm$ standard error). (B) Relative NF-κB activation in THP-1 cells inferred by secreted alkaline phosphatase in the absence (left) and presence (right) of LPS stimulation (normalized to the negative control, $n = 3 \pm$ standard error). LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; ns, not significant; NE, electropolished TAN; NS, standard TAN; PEEK, polyetheretherketone; PA30, 30-min oxygen plasma treated machined PEEK; PO, injection molded PEEK; PO30, 30-min oxygen plasma treated injection molded PEEK; TAN, titanium-aluminum-niobium; TE, electropolished titanium; SS, stainless steel. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(A) Gene expression



(B) Cytokine secretion

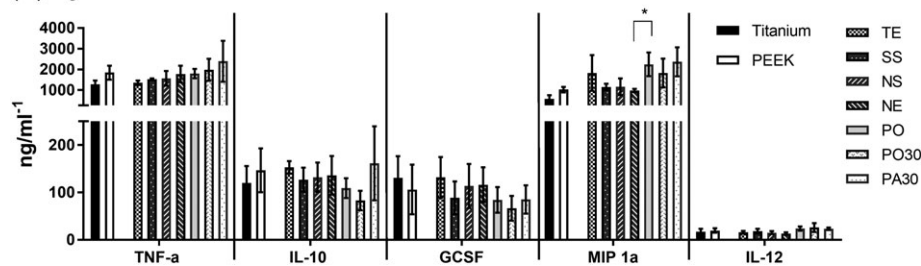


FIGURE 3. *In vitro* gene expression (A) and cytokine secretion (B) of PBMCs stimulated with *S. aureus* JAR. NE, electropolished TAN; ns, not significant; NS, standard TAN; PEEK, polyetheretherketone; PA30, 30-min oxygen plasma treated machined PEEK; PO, injection molded PEEK; PO30, 30-min oxygen plasma treated injection molded PEEK; TE, electropolished titanium; SS, stainless steel (* $p < 0.05$, *** $p < 0.001$).

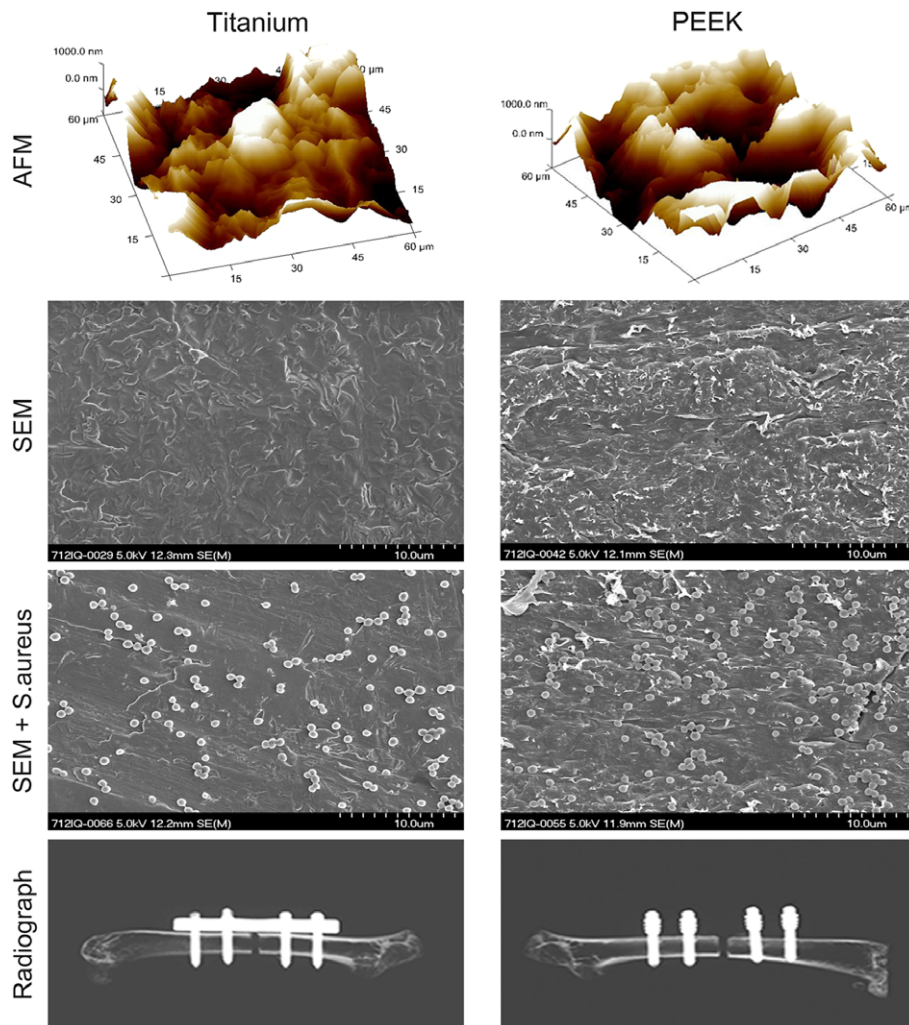


FIGURE 4. Surface visualization, bacterial adhesion and radiographic images of the Ti (left) and PEEK (Right) implants used in the *in vivo* study. Upper panel, representative 60 µm x 60 µm AFM image of the Ti and PEEK implant surfaces in tapping mode. Z-axis range: 2.5 µm. Second panel representative SE micrograph of the surface of the Ti and PEEK plates without bacteria, and with bacteria in the third panel. Bottom panel, contact radiograph taken at 3 days for Ti and PEEK implants, the PEEK plate is radiolucent and therefore not visible. AFM, atomic force microscopy; SEM, scanning electron microscopy.

quantity of bacteria on the implant, in the tissue, or in the bone did not differ significantly at any time-point between the Ti and PEEK groups, for either mouse strain, other than an increase in the Ti group at day 3 in the bone and soft tissue for C57BL/6 mice.

Histological evaluation of infection and healing

In non-inoculated animals at day 3, the tissue above the plate was infiltrated with a mixed, predominantly mononuclear cell population of inflammatory cells, with no significant difference between the materials [Figure 6(A,B)]. By Day 7, the soft tissue above the plate of non-inoculated animals appeared to have fewer inflammatory cells and a highly vascularized granulation tissue was formed alongside fibroblasts [Figure 6(C,D)]. The area below the plate of non-inoculated animals was infiltrated with few inflammatory cells at Day 3 [Figure 6(E,F)], and by Day 7 the incidence and mean grade of this inflammation decreased and the gap

below the plate was filled with low amounts of highly vascularized granulation tissue consisting of young fibroblasts and some inflammatory cells [Figure 6(G,H)]. There was no significant difference between the Ti or PEEK groups.

The infected animals displayed contrasting histological results, although there was once again no significant differences between the PEEK and Ti groups. The soft tissue above the plates of the infected animals revealed pronounced inflammatory cell invasion on day 3 [Figure 6(I,J)], consisting mostly of polymorphonuclear cells. By Day 7, the severity of the inflammation in the tissue overlying the plate in infected animals increased and changed to a non-encapsulated necrotizing type, characterized by large amounts of cellular debris and extracellular blue-staining material [Figure 6(K,L)]. The area below the plate, including the osteotomy area in infected animals also revealed pronounced inflammatory cell invasion by Day 3 [Figure 6(M, N)], and increased further by day 7 [Figure 6(O,P)]. There

C57BL/6

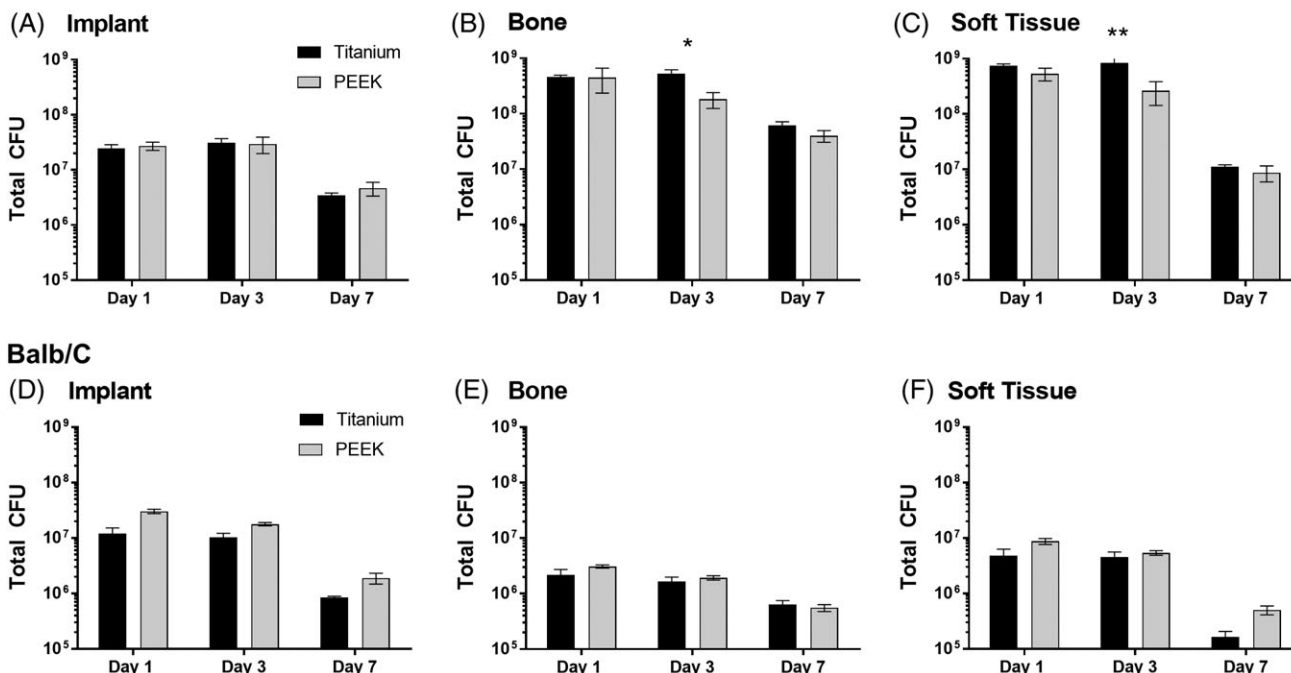


FIGURE 5. Quantitative bacteriological evaluation of tissue and hardware at euthanasia. At each scheduled euthanasia time-point, the soft tissues, implants (plate and screws) and the bone were separated and processed individually for quantitative bacteriology. Upper panel, C57BL/6, lower panel BALB/c ($n \geq 6$ at each time-point, mean $\pm$ s.d.). * $p < 0.05$, ** $p < 0.01$.

were no signs of a healing granulation tissue in infected animals from either the PEEK or the titanium group either above the plate or below the plate and in the osteotomy area. By Day 7, a minimal to moderate granulocytic to necrotizing myelitis, sometimes with beginning formation of micro-abscesses, was recorded in both groups. Bacteria were observed in the histological slides of the infected animals of both Ti and PEEK groups at day 3 [Figure 6(Q,R)] and day 7 [Figure 6(S,T)]. Bacteria were observed on fragments of bone [Figure 6(Q)] and adjacent to the plate of both groups [Figure 6(R-T)]. Overall, the histological analysis showed a significant progression in severity of symptoms in all groups over time. However, there was no marked difference between the materials in either the infected or non-infected groups.

Cytokine secretion

Cytokine secretion data from *ex vivo* stimulated single cell suspensions from bone and soft tissue homogenates is shown in Figure 5. Overall, very few significant differences were observed between PEEK and Ti groups. There was an increase in IL-17 expression in C57BL/6 mice receiving Ti implants compared to PEEK equivalents at day 7.

DISCUSSION

Biomaterials used for the manufacture of implantable medical devices are tested extensively prior to clinical implementation to determine biocompatibility, degradation and propensity to cause fibrous encapsulation. The risk of developing an

infection is a critical parameter in the performance of any medical device; however, preclinical investigation of this risk is generally not a prerequisite to commercial availability. In the present study, we assessed two commonly used implant materials – standard Ti and PEEK – both *in vitro* and *in vivo*, to determine if, and why, different materials can influence infection risk through diverse biological interactions.

In the *in vitro* study, complement activation was assessed as a measure of innate defense against both invading pathogens and foreign material. Although complement activation on different materials has been previously investigated,^{31–33} the effect of topography and chemistry on complement activation remains largely undescribed. In our study, there was a trend for the modified metals to induce greater complement activation than standard Ti and all PEEK materials in the absence of bacterial stimulation. The only material with statistically significantly higher complement activation was TE. The precise reason for this observation remains unclear as it is clearly not easily explained by topography or chemistry alone. When complement activation by the material surfaces with pre-adhered *S. aureus* was investigated, it was found that complement activation decreased, but there was very little difference between the different materials. This is an interesting result, as it seems that the presence of *S. aureus* contamination on the surfaces actually reduces complement activation and negates the effect of the underlying material under our experimental conditions. This may be due to the absence of bacterial exopolymers, removed during the wash step, or due to the binding of complement inhibiting proteins originating from the bacteria.³⁴ Further studies

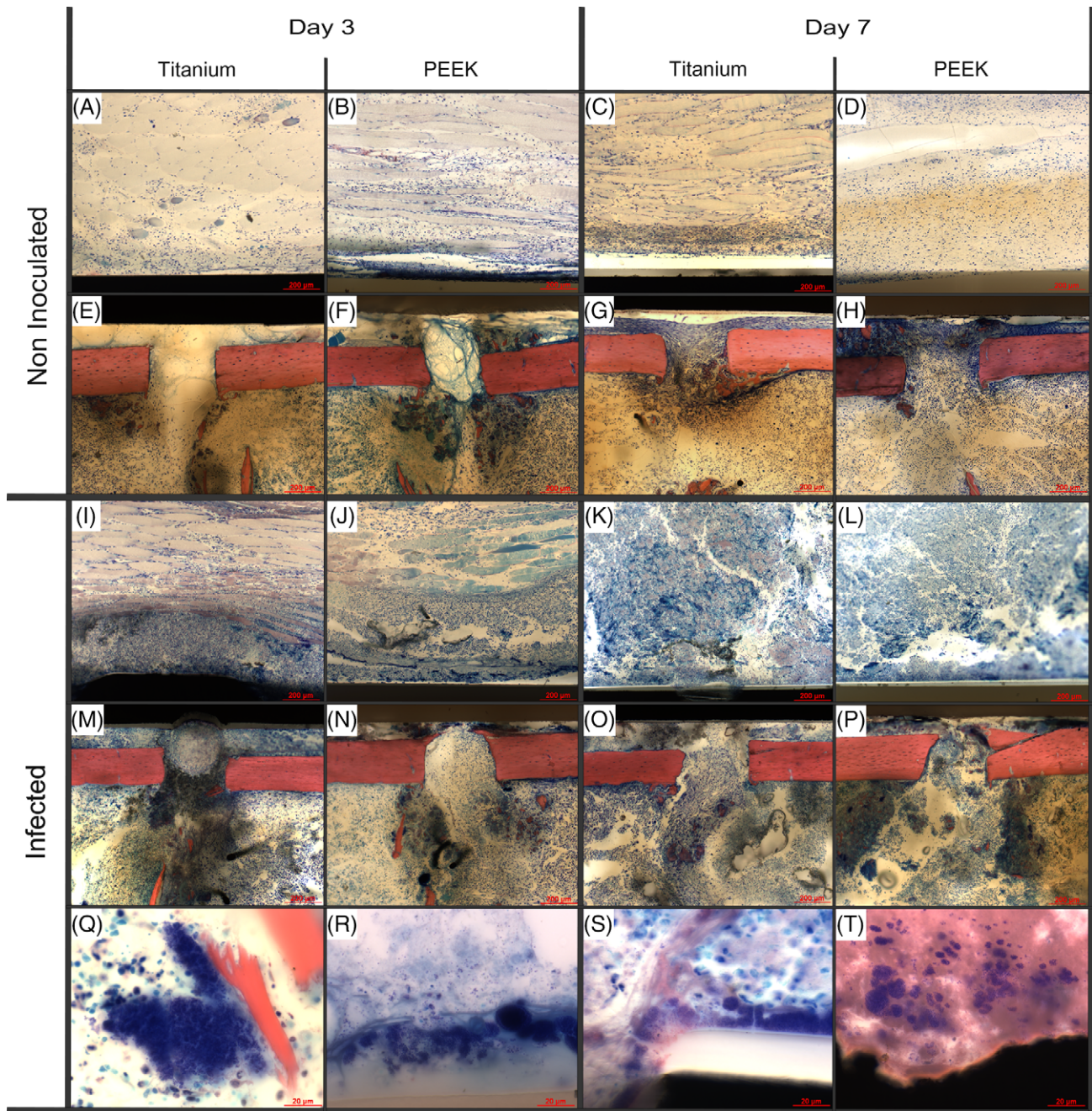


FIGURE 6. Histological sections showing critical features of tissue reactions 3 (left panel) and 7 days (Right panel) post-surgery. Upper panel, non-inoculated animals displayed signs of healing and a lack of inflammation in the tissues overlying the titanium (columns 1 and 3) and PEEK (columns 2 and 4) plates. Infected animals (bottom 3 panels of figure) displayed significantly different histological features, including elevated inflammatory cell infiltration. Infected animals displayed visible agglomerations of bacteria including adjacent to the plate for titanium and PEEK (bottom row). Similar features observed in both the PEEK and the titanium groups in all aspects. Giemsa Eosin stain, scale bar 20 μ m.

may look to investigate this phenomenon to develop more infection resistant biomaterial surfaces. As complement activation is among the earliest processes occurring around contaminated implants in a surgical site, mechanisms to increase antibacterial efficacy of complement may be an interesting avenue for future research.

In the current study, all the materials tested induced NF- κ B activity, although no material-dependent effect was observed. Similarly, PBMC gene expression and cytokine

secretion did not identify any pronounced differences between any of the materials. Rougher forms of titanium have previously been shown to increase TNF- α secretion by mouse macrophages *in vitro*,^{35,36} although we could not identify such a feature for microrough Ti in comparison with polished equivalents. This may be explained by the fact that this effect may be dependent on specific topographical features rather than any reduction in the degree of roughness alone. Previously it has been shown that the presence of LPS

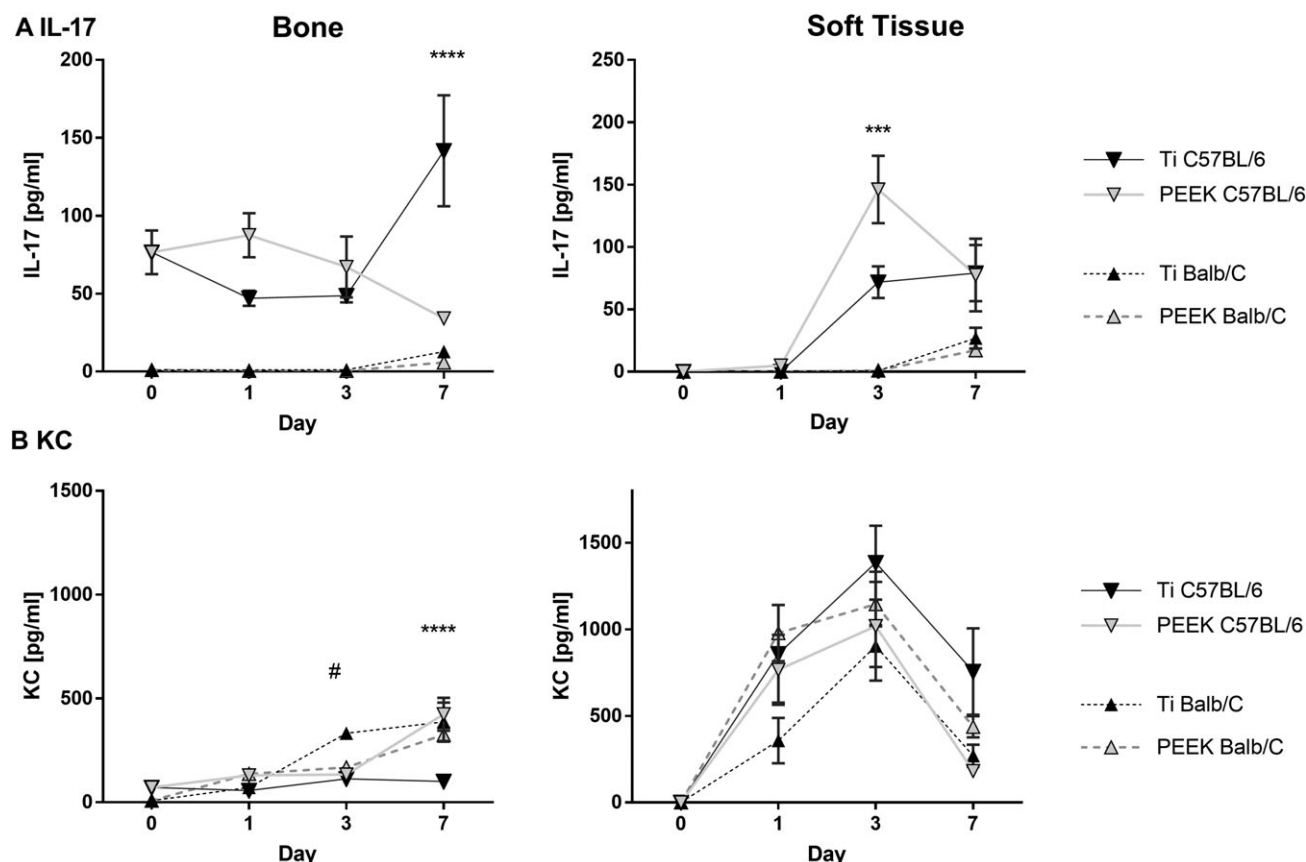


FIGURE 7. Expression of selected cytokine genes in the bone and soft tissue samples in C57BL/6 and Balb/C mice receiving titanium (Ti) or PEEK implants. All animals were inoculated with *S. aureus* and found to be culture positive at each time point. Bone and soft tissue single cell suspensions were stimulated with PMA/ionomycin for 24 h *in vitro*: $n = 6$ per group, *, # $p < 0.05$; **, ### $p < 0.01$; ***, #### $p < 0.001$; ****, ##### $p < 0.0001$ infected versus non-infected *C57BL/6, # Balb/C.

reduced the material induced differences in macrophage activation on titanium.³⁶ However, the effect of topographical differences has been shown to persist in the presence of LPS with other titanium surfaces.^{35,37}

The *in vivo* study was focused on the current clinical standards, Ti and PEEK. Under the controlled experimental conditions of our study, we could see few significant differences in the quantitative bacteriological outcome between the two groups in either mouse strain. In terms of the bacteria adherent to the implant, or biofilm, we could not detect any differences, which is interesting, as the biofilm represent the direct interface between the implant and the bacteria, and may be expected to show greatest differences due to differing substrate materials. Interestingly, there was an observable decrease in the number of bacteria in the bone and the soft tissue surrounding the PEEK implants in C57BL/6 mice on day 3 compared with the titanium. In contrast, the Balb/C mice data revealed a lower CFU count around the Ti implant (soft tissue and implant itself) than PEEK equivalents. The selection of a second mouse strain is, therefore, a valuable addition to this study to provide evidence that the differences observed are neither consistent nor significant overall.

Histologically, the infection around both implant materials also appeared equivalent, with mice receiving both

materials displaying features previously experienced in this model.³⁰ Interestingly, the fibrous encapsulation of PEEK, that has been previously described,²⁴ was not an apparent feature of the mice receiving PEEK implants in this study. Perhaps, the early time-points of 3 and 7 days do not allow sufficient time for encapsulation.

Of the limited studies that have previously studied clinically available biomaterials in human patients, both Ti and SS have been shown to produce similar degrees of inflammation in patients independently of the material used.³⁸ In terms of infection risk, the functional properties, or design, of the implant itself has been found to be more important than implant materials in most cases.^{13,15,39} In this study, the size of the implanted PEEK and Ti devices is approximately equal. A greater surface area represents a greater area for bacterial adhesion and is a larger foreign body. As such, the minor increase in surface area of the PEEK implants, due to the need for mechanical strength, could be considered a disadvantage for PEEK in this study. This effect is not expected to be significant due to the small increase in surface area, however, remains a difference between the study groups.

Microrough Ti is considered to be the most biocompatible of all the commonly used orthopedic implant materials

and integrates well with host tissue due to a combination of topography and chemistry.^{23,40} The increased roughness of titanium is believed to increase osseointegration,²³ yet yield greater bacterial adhesion, at least *in vitro*.¹¹ It may be that polished metals may have shown a reduced infection burden in this model, however, similar studies in rabbits could not identify a significant effect of surface topography on infection risk.^{13,15} PEEK on the other hand is biocompatible, inert,^{41–43} and has demonstrated limited osseointegration due to lower surface energy.^{27,44} One reason for this decreased osseointegration could be the low surface energy of the polymer,⁴⁵ though our previous *in vivo* study did not show a pronounced effect of the lower surface energy on osseointegration in a long term model in sheep.²⁴ Tantalum trabecular metal components are increasingly used in joint reconstruction prostheses and would be another interesting metal to compare in this model. Tantalum has been shown to be associated with reduced infection rate in clinical study,⁴⁶ however, this has not been supported by direct antibacterial activity *in vitro*.⁴⁷

This study has shown that regardless of different chemistry and topography of the PEEK and Ti surfaces, both materials displayed very similar profiles when infected, by all outcome measures used in this study. In conclusion, once infected, the choice of Ti or PEEK has little effect on the progression of the infection, as revealed both *in vitro*, *in vivo* and by either host immune response, bacterial burden, or histology.

ACKNOWLEDGMENTS

The authors would like to disclose and thank Invibio Biomaterial Solutions, UK, for PEEK material. Dr. N. Gadegaard, Centre of Cell Engineering, Glasgow University, UK, for AFM measurements. Dr. R. Heuberger, Robert Mathys Foundation, Switzerland for XPS measurements.

REFERENCES

- Ostman PO, Hellman M, Sennerby L. Ten years later. Results from a prospective single-centre clinical study on 121 oxidized (TiUnite) Branemark implants in 46 patients. *Clin Implant Dent Relat Res* 2012;14(6):852–860.
- Zimmerli W, Trampuz A, Ochsner PE. Prosthetic-joint infections. *N Engl J Med* 2004;351(16):1645–1654.
- Trampuz A, Zimmerli W. Diagnosis and treatment of infections associated with fracture-fixation devices. *Injury* 2006;37(Suppl 2):S59–S66.
- Kraemer P, Lee MB, Englehardt H, Chapman JR, Bransford RJ. Infectious pin complication rates in halo vest fixators using ceramic versus metallic pins. *J Spinal Disord Tech* 2010;23(8):e59–e62.
- Morgenstern M, Erichsen C, von Ruden C, Metsemakers WJ, Kates SL, Moriarty TF, Hungerer S. Staphylococcal orthopaedic device-related infections in older patients. *Injury* 2016;47(7):1427–1434.
- Yue C, Zhao B, Ren Y, Kuijter R, van der Mei HC, Busscher HJ, Rochford ET. The implant infection paradox: why do some succeed when others fail? Opinion and discussion paper. *Eur Cells Mater* 2015;29:303–310; discussion 10–3.
- Darouiche RO. Treatment of infections associated with surgical implants. *N Engl J Med* 2004;350(14):1422–1429.
- Patzakis MJ, Wilkins J. Factors influencing infection rate in open fracture wounds. *Clin Orthop Relat Res* 1989;243:36–40.
- Rochford ET, Poulsson AH, Salavarieta VJ, Lezuo P, Richards RG, Moriarty TF. Bacterial adhesion to orthopaedic implant materials and a novel oxygen plasma modified PEEK surface. *Colloids Surf B Biointerfaces* 2013;113C:213–222.
- Rochford ET, Subbiahdoss G, Moriarty TF, Poulsson AH, van der Mei HC, Busscher HJ, Richards RG. An *in vitro* investigation of bacteria-osteoblast competition on oxygen plasma-modified PEEK. *J Biomed Mater Res A* 2014;102(12):4427–4434.
- Harris LG, Meredith DO, Eschbach L, Richards RG. *Staphylococcus aureus* adhesion to standard micro-rough and electropolished implant materials. *J Mater Sci Mater Med* 2007;18(6):1151–1156.
- Harris LG, Richards RG. *Staphylococcus aureus* adhesion to different treated titanium surfaces. *J Mater Sci Mater Med* 2004;15(4):311–314.
- Moriarty TF, Debeve L, Boure L, Campoccia D, Schlegel U, Richards RG. Influence of material and microtopography on the development of local infection *in vivo*: experimental investigation in rabbits. *Int J Artif Organs*. 2009;32(9):663–670.
- Cordero J, Munuera L, Folgosa MD. Influence of metal implants on infection. An experimental study in rabbits. *J Bone Joint Surg Br* 1994;76(5):717–720.
- Metsemakers WJ, Schmid T, Zeiter S, Ernst M, Keller I, Cosmelli N, Arens D, Moriarty TF, Richards RG. Titanium and steel fracture fixation plates with different surface topographies: influence on infection rate in a rabbit fracture model. *Injury* 2016;47(3):633–639.
- Poulsson AH, Mitchell SA, Davidson MR, Johnstone AJ, Emmison N, Bradley RH. Attachment of human primary osteoblast cells to modified polyethylene surfaces. *Langmuir* 2009;25(6):3718–3727.
- Hayes JS, Khan IM, Archer CW, Richards RG. The role of surface microtopography in the modulation of osteoblast differentiation. *Eur Cells Mater* 2010;20:98–108.
- Hotchkiss KM, Reddy GB, Hyzy SL, Schwartz Z, Boyan BD, Olivares-Navarrete R. Titanium surface characteristics, including topography and wettability, alter macrophage activation. *Acta Biomater* 2016;31:425–434.
- Rostam HM, Singh S, Vrana NE, Alexander MR, Ghaemmaghami AM. Impact of surface chemistry and topography on the function of antigen presenting cells. *Biomater Sci* 2015;3(3):424–441.
- Kuhn S, Kroth J, Ritz U, Hofmann A, Brendel C, Muller LP, Förch R, Rommens PM. Reduced fibroblast adhesion and proliferation on plasma-modified titanium surfaces. *J Mater Sci Mater Med* 2014;25(11):2549–2560.
- Kou PM, Babensee JE. Validation of a high-throughput methodology to assess the effects of biomaterials on dendritic cell phenotype. *Acta Biomater* 2010;6(7):2621–2630.
- Muszanska AK, Rochford ET, Gruszka A, Bastian AA, Busscher HJ, Norde W, van der Mei HC, Herrmann A. Antiadhesive polymer brush coating functionalized with antimicrobial and RGD peptides to reduce biofilm formation and enhance tissue integration. *Biomacromolecules* 2014;15(6):2019–2026.
- Hayes JS, Richards RG. Surfaces to control tissue adhesion for osteosynthesis with metal implants: *in vitro* and *in vivo* studies to bring solutions to the patient. *Expert Rev Med Devices* 2010;7(1):131–142.
- Poulsson AH, Eglon D, Zeiter S, Camenisch K, Sprecher C, Agarwal Y, Nehrbass D, Wilson J, Richards RG. Osseointegration of machined, injection moulded and oxygen plasma modified PEEK implants in a sheep model. *Biomaterials* 2014;35(12):3717–3728.
- Hayes JS, Welton JL, Wieling R, Richards RG. *In vivo* evaluation of defined polished titanium surfaces to prevent soft tissue adhesion. *J Biomed Mater Res B Appl Biomater* 2012;100(3):611–617.
- Johansson P, Jimbo R, Naito Y, Kjellin P, Currie F, Wennerberg A. Polyether ether ketone implants achieve increased bone fusion when coated with nano-sized hydroxyapatite: a histomorphometric study in rabbit bone. *Int J Nanomed* 2016;11:1435–1442.
- Webster TJ, Patel AA, Rahaman MN, Sonny BB. Anti-infective and osteointegration properties of silicon nitride, poly(ether ether ketone), and titanium implants. *Acta Biomater* 2012;8(12):4447–4454.
- Khonsari RH, Berthier P, Rouillon T, Perrin J-P, Corre P. Severe infectious complications after PEEK-derived implant placement: report of three cases. *J Oral Maxillofac Surg Med Pathol* 2014;26(4):477–482.

29. Williams LR, Fan KF, Bentley RP. Custom-made titanium cranioplasty: early and late complications of 151 cranioplasties and review of the literature. *Int J Oral Maxillofac Surg* 2015;44(5):599–608.
30. Rochford ET, Sabate Bresco M, Zeiter S, Kluge K, Poulsson A, Ziegler M, Richards RG, O'Mahony L, Moriarty TF. Monitoring immune responses in a mouse model of fracture fixation with and without *Staphylococcus aureus* osteomyelitis. *Bone* 2016;83: 82–92.
31. Andersson M, Andersson J, Sellborn A, Berglin M, Nilsson B, Elwing H. Quartz crystal microbalance-with dissipation monitoring (QCM-D) for real time measurements of blood coagulation density and immune complement activation on artificial surfaces. *Biosens Bioelectron* 2005;21(1):79–86.
32. Janatova J. Activation and control of complement, inflammation, and infection associated with the use of biomedical polymers. *ASAIO J* 2000;46(6):S53–S62.
33. Tang L, Jennings TA, Eaton JW. Mast cells mediate acute inflammatory responses to implanted biomaterials. *Proc Natl Acad Sci USA* 1998;95(15):8841–8846.
34. van Wamel WJ, Rooijackers SH, Ruyken M, van Kessel KP, van Strijp JA. The innate immune modulators staphylococcal complement inhibitor and chemotaxis inhibitory protein of *Staphylococcus aureus* are located on beta-hemolysin-converting bacteriophages. *J Bacteriol* 2006;188(4):1310–1315.
35. Refai AK, Textor M, Brunette DM, Waterfield JD. Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines. *J Biomed Mater Res A* 2004;70(2):194–205.
36. Tan KS, Qian L, Rosado R, Flood PM, Cooper LF. The role of titanium surface topography on J774A.1 macrophage inflammatory cytokines and nitric oxide production. *Biomaterials* 2006;27(30): 5170–5177.
37. Waterfield JD, Ali TA, Nahid F, Kusano K, Brunette DM. The effect of surface topography on early NFkappaB signaling in macrophages. *J Biomed Mater Res A* 2010;95(3):837–847.
38. Voggenreiter G, Leiting S, Brauer H, Leiting P, Majetschak M, Bardenheuer M, Obertacke U. Immuno-inflammatory tissue reaction to stainless-steel and titanium plates used for internal fixation of long bones. *Biomaterials* 2003;24(2):247–254.
39. Arens S, Schlegel U, Printzen G, Ziegler WJ, Perren SM, Hansis M. Influence of materials for fixation implants on local infection. An experimental study of steel versus titanium DCP in rabbits. *J Bone Joint Surg Br* 1996;78(4):647–651.
40. Branemark R, Branemark PI, Rydevik B, Myers RR. Osseointegration in skeletal reconstruction and rehabilitation: a review. *J Rehabil Res Dev* 2001;38(2):175–181.
41. Hunter A, Archer CW, Walker PS, Blunn GW. Attachment and proliferation of osteoblasts and fibroblasts on biomaterials for orthopaedic use. *Biomaterials* 1995;16(4):287–295.
42. Morrison C, Macnair R, MacDonald C, Wykman A, Goldie I, Grant MH. In vitro biocompatibility testing of polymers for orthopaedic implants using cultured fibroblasts and osteoblasts. *Biomaterials* 1995;16(13):987–992.
43. Sagomonyants KB, Jarman-Smith ML, Devine JN, Aronow MS, Gronowicz GA. The in vitro response of human osteoblasts to polyetheretherketone (PEEK) substrates compared to commercially pure titanium. *Biomaterials* 2008;29(11):1563–1572.
44. Han CM, Lee EJ, Kim HE, Koh YH, Kim KN, Ha Y, Kuh SU. The electron beam deposition of titanium on polyetheretherketone (PEEK) and the resulting enhanced biological properties. *Biomaterials* 2010;31(13):3465–3470.
45. Kasemo B, Lausmaa J. Material-tissue interfaces: the role of surface properties and processes. *Environ Health Perspect* 1994;102(Suppl 5):41–45.
46. Tokarski AT, Novack TA, Parvizi J. Is tantalum protective against infection in revision total hip arthroplasty? *Bone Joint J* 2015; 97-B(1):45–49.
47. Harrison PL, Harrison T, Stockley I, Smith TJ. Does tantalum exhibit any intrinsic antimicrobial or antibiofilm properties? *Bone Joint J* 2017;99-B(9):1153–1156.