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The Gut Microbiota May be a Novel Pathogenic Mechanism in Loosening of Orthopedic Implants in Rats

Meghan M. Moran¹, Brittany M. Wilson¹, Jun Li², Phillip A. Engen³, Ankur Naqib^{1,3}, Stefan J. Green⁴, Amarjit S. Virdi¹, Anna Plaas², Christopher B. Forsyth³, Ali Keshavarzian³, D. R. Sumner¹

¹Department of Cell & Molecular Medicine, Rush University Medical Center, 600 S. Paulina St., Suite 507 AAC, Chicago, IL 60612, USA

²Department of Internal Medicine, Division of Rheumatology, Rush Medical College, Rush University Medical Center, 1750 W Harrison Street, Jelke 1302 Chicago, IL, USA

³Department of Internal Medicine, Division of Digestive Diseases and Nutrition, Rush Medical College, 1725 W. Harrison St., Suite 207, Chicago, IL 60612, USA

⁴Genome Research Core, Research Resources Center, University of Illinois-Chicago, 1819 West Polk Street, College of Medicine West, Room 329, Chicago, IL, USA.

Abstract

Particles released from implants cause inflammatory bone loss, which is a key factor in aseptic loosening, the most common reason for joint replacement failure. With the anticipated increased incidence of total joint replacement in the next decade, implant failure will continue to burden patients. The gut microbiome is increasingly recognized as an important factor in bone physiology, however, its role in implant loosening is currently unknown. We tested the hypothesis that implant loosening is associated with changes in the gut microbiota in a preclinical model. When the particle challenge caused local joint inflammation, decreased peri-implant bone volume and decreased implant fixation, the gut microbiota was affected. When the particle challenge did not cause this triad of local effects, the gut microbiota was not affected. Our results suggests that

Corresponding Author: Meghan M. Moran, 600 S. Paulina St., Suite 507 AAC, Chicago, IL 60612, USA, 312-563-1257, meghan_moran@rush.edu.

Author Contributions

Meghan M. Moran- experimental lead, active in surgery, daily experimental duties and sacrifice, drafted manuscript, edited manuscript, corresponding author, constructed figures, completed CD68 and MPO immunohistochemistry.

Brittany M. Wilson- active in surgery, daily experimental duties and sacrifice, completed select serum biomarker analysis and edited manuscript draft.

Jun Li- completed the hyaluronan immunohistochemistry and the Mason's Trichrome histology staining, edited manuscript draft.

Phillip A. Engen- completed bioinformatics statistical analysis and edited manuscript draft; Ankur Naqib- completed bioinformatics statistical analysis and edited manuscript draft.

Stefan J. Green- oversaw 16S rRNA gene amplicon sequencing and library building, consulted on bioinformatics experimental design and edited manuscript draft.

Amarjit S. Virdi- active in experimental design and surgery; edited manuscript draft.

Anna Plaas- coordinated hyaluronan immunohistochemistry, provided intellectual support in inflammation driven processes and edited manuscript draft.

Christopher Forsyth- consultant on experimental designs, provided training in microbiome sample collection and data analysis as well as intellectual support for gut endpoints.

Ali Keshavarzian- senior consultant on gut physiology and microbiome data analysis, edited manuscript draft

D. R. Sumner- senior consultant on bone phenotyping and implant fixation endpoints, edit manuscript drafts.

cross-talk between these compartments is a previously unrecognized mechanism of failure following total joint replacement.

Plain Language Summary

After joint replacement surgery, orthopedic implants shed small particles and these particles irritate the joint. This joint irritation induces bone loss around the implant and results in a loosened implant. This process is known as particle-induced implant loosening and it is the most common reason for joint replacement failure. The only therapy to fix a failed, loosened implant is revision surgery, which includes the surgical removal of the original, loosened implant and the replacement with a new implant. The gut microbiome is, in part, the bacteria found inside the intestine. This bacteria is now recognized as an important factor in bone health, but its role in implant loosening is currently unknown. In this study, we wanted to determine if implant loosening was associated with changes in the gut microbiome using a rat model that mimics the human condition of particle-induced implant loosening. We found that when the particles caused joint inflammation, loss of bone around the implant and loss of implant fixation (a measure of implant loosening), the gut bacteria was altered. When the particles did not cause this triad of effects in the knee joint, the gut bacteria was not altered. Our results suggest that there is a previously unrecognized communication between the implant and the gut that is likely involved in implant failure following total joint replacement. This is significant because our research may provide a new method for preventing or treating implant loosening and eventually reducing the need for revision surgery.

Keywords

Orthopedics; implant fixation; gut microbiome; bone volume; implant

Introduction

The gut microbiome is the collection of all microorganisms (i.e., microbiota: bacteria, archaea, fungi, viruses) and microbial genetic material (i.e., metagenome) in its environment (1–3). The microbiome has been shown to be altered by diet, host genetics, drug use, exercise, stress and disease (1, 2, 4, 5). The symbiotic relationship between gut microorganisms and the host is essential to overall health (6), as well as musculoskeletal system function (2, 7–15). Indeed, the gut microbiota-bone/joint axis has been implicated in osteoarthritis (16, 17), rheumatoid arthritis (8, 18, 19), fracture healing (20), and osteolysis (21). It is also known that the gut microbiota is affected by injuries and changes in systems remote from the gut, including burns to the skin (22–24), traumatic brain injury (25, 26), disrupted circadian rhythm (27, 28) and environmental and psychosocial stress (29–31). Thus, challenges remote from the gut can impact the gut microbiota composition and function, potentially leading to dysbiosis.

Although total joint replacement (TJR) is one of the most effective surgical interventions in modern medicine, aseptic loosening of implants remains a challenge (32, 33). The rate of TJR of the hip and knee is increasing world-wide (34–38) and there is a need for improved understanding of implant health and survivorship (39). Long-term resistance to loosening depends upon establishment and maintenance of the bone-implant interface, a process which

itself depends upon bone regeneration and remodeling (40). The primary mechanism of aseptic loosening involves particle-induced peri-implant osteolysis in which debris released from the surface of the implant triggers local inflammation, leading to the release of osteoclast-stimulating cytokines and eventually osteolysis (41–50). The wear debris is continuously worn from the surface and junctions of the implant and are isolated locally in the joint by inflammatory cells, but it is also known that particles translocate from the joint to other organ systems (51, 52). Particle-induced osteolysis is a key factor in failure of orthopedic implants (50, 53, 54).

In the current series of studies, we utilized a preclinical rat model of particle-induced implant loosening that mimics the human condition of aseptic particle-induced osteolysis (Fig. 1) (39, 54, 55) to test the hypothesis that implant loosening is associated with disruption of the gut microbiota composition.

Materials and Methods

Animal and Ethics Statement

We are reporting results from three separate experiments using an established rat model of osteolysis (39, 55). Sample sizes for all experiments were determined by *a priori* power calculations for the primary bone endpoint, implant fixation strength, which suggested $n = 9$ rats per study group ($\beta = 0.80$, $\alpha = 0.05$, PS Power and Sample Size Calculation, Nashville, TN). All rats were male and pair-housed in a facility maintained at 22 °C and 35–55% humidity with a 12-hour light/dark cycle (light 7am–7pm) at Rush University Medical Center (RUMC). Standard laboratory grade rodent diet (Teklab Global Rodent Diet 2018, 18% protein, Envigo) and water was provided *ad libitum*. Rat chow, water source and cage bedding were kept the same throughout each experiment to control for the three largest variables that are known to affect the gut microbiome (56). In addition cages were arranged to eliminate between-group cage placement effects on microbiome variability (56). All surgeries took place in the morning, in the animal unit at RUMC. All rats were sacrificed using carbon dioxide inhalation and secondary cardiac puncture blood draw. Tissues collected for analyses included blood from cardiac puncture, bilateral femurs, liver, cecum luminal content and distal colon. Culture swabs of the synovium were also taken for all experiments and they yielded no bacterial growth. All procedures were conducted in accordance with the institutional guidelines and approved by the Institutional Animal Care and Use Committee at Rush University (protocol #17-017) and NIH Guide for the Care and Use of Laboratory Animals.

Some data for Experiment 1 were recently reported (57). For experiment 1, 35 of 43 Sprague Dawley (SD) rats (380–400g, Envigo) underwent bilateral implant surgery to place titanium (Ti) rods (15mm $\times$ 1.5mm) in the femoral intramedullary canal (39) (Table 1). Beginning the day after surgery, 26 of the 35 rats were administered either clean (sterile) polyethylene (PE) particles (Ceridust® #3610, mean size = 1.75 μ m, size range of 0.06 – 11.06 μ m), lipopolysaccharide-doped PE (LPS-PE) particles or cobalt-chrome particles (CoCr, BioEngineering Solutions, Inc, mean size = 0.38 μ m, size range of 0.12–7.78 μ m) suspended in 6% rat serum vehicle. This size of CoCr particle has been shown to be clinically relevant to induce an inflammatory response (44, 58) The final PE concentration

was 2.6 mg/mL (4.7×10^7 particles/injection, cumulative 0.78 mg PE/knee after 6 weekly injections), as based on previously published studies (54, 55, 59). The final CoCr concentration was 3.4 mg/mL for a cumulative dose of 1mg per knee over 6 weeks. The total dose was one-half that typically given in a calvarial model where 2 mg are administered as a single injection (60). We used the 1 mg total dose to ensure that particles did not become packed too tightly in the joint and did not tear the joint capsule during repeated weekly intra-articular injections. Nine rats were administered vehicle without particles and 8 rats served as naïve controls (no surgery and no vehicle injections). The intra-articular injections of either particles or vehicle alone were administered weekly in 50µl volumes. All rats were sacrificed 6-weeks post-surgery. The surgeries took place in May and the experiment ended in June 2017.

In experiment 2, 42 of 61 SD rats (329– 374g, Envigo) underwent implant placement surgery (Table 1). Twenty-two of these rats were administered PE particles following the same administration schedule, dose and concentration of particles for 12 weeks. Twenty rats were administered vehicle without particles and 19 rats served as naïve controls. Twenty-nine rats were sacrificed 6-weeks post-surgery and 32 rats were sacrificed 12-weeks post-surgery. The surgeries took place in May and the experiment ended in June and August 2018. In experiment 3, 21 of 31 SD rats (355– 375g, Envigo) underwent implant placement surgery (Table 1). Of the 21 rats 11 were administered CoCr particles suspended in 6% rat serum vehicle. The final CoCr concentration was the same as Experiment 1, but administered over 12 weeks. Ten rats were administered vehicle without particles and 10 rats served as naïve controls. All rats were sacrificed at 12 weeks. The surgeries took place in February and the experiment ended in May 2019.

Micro-computed Tomography and Mechanical Testing

Methods for micro-computed tomography (microCT) and mechanical pull-out endpoints followed previously published techniques (57). The microCT results were reported in part (57), but in this manuscript, we included the naïve group, therefore the analysis is slightly different than reported previously for bone volume/total volume (BV/TV).

Microbial Community Analysis

Microbiota profiling was completed on n = 25 (Exp. 1), 61 (Exp. 2) and 31(Exp. 3) rats (Table S4). Total genomic DNA was extracted from the cecum luminal content utilizing the QIAamp DNA Microbiome Kit (QIAGEN, Germantown, MD, USA), and verified with fluorometric quantitation (Qubit, Life Technologies, Grand Island, NY). Genomic DNA was prepared for microbiome amplicon sequencing using a two-stage PCR protocol employing primers targeting the microbial small subunit ribosomal RNA (SSU or 16S rRNA) gene, as described previously(61). Primers 515F/926R containing linker sequences (*i.e.*, CS1_515F, 5' - ACACTGACGACATGGTTCTACAGTGYCAGCMGCCGCGGTAA -3' and CS2_926R, 5' - TACGGTAGCAGAGACTTGGTCTCCGYCAATTYMTTTRAGTTT-3'), targeting the V4-V5 variable regions of microbial 16S rRNA genes, were used for first stage polymerase chain reaction (PCR) (62). First stage PCR amplifications were performed in 10 microliter reactions in 96-well plates, using the MyTaq HS 2X mastermix. PCR conditions

were 95°C for 5 minutes, followed by 28 cycles of 95°C for 30", 50°C for 60" and 72°C for 90".

Subsequently, a second PCR amplification was performed in 10 microliter reactions in 96-well plates. A mastermix for the entire plate was made using the MyTaq HS 2X mastermix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; Item# 100-4876). These AccessArray primers contained the CS1 and CS2 linkers at the 3' ends of the oligonucleotides. Cycling conditions were as follows: 95°C for 5 minutes, followed by 8 cycles of 95°C for 30", 60°C for 30" and 72°C for 30". A final, 7-minute elongation step was performed at 72°C. Samples were pooled and purified using an AMPure XP cleanup protocol (0.6X, vol/vol; Agencourt, Beckmann-Coulter) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were sequenced using an Illumina MiSeq instrument, with a V3 kit and paired-end 300 base reads at the University of Illinois at Chicago Sequencing Core (UICSQC). Raw sequence data (FASTQ files) were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), under the Bioproject identifier PRJNA625979.

Raw FASTQ files for each sample were merged using the software package PEAR (Paired-end-read merger) (v0.9.8) (63, 64). Merged reads were quality trimmed and sequences shorter than 250 bases were removed (CLC Genomics Workbench, v10.0, CLC, Bio, Qiagen, Boston, MA). Sequences were screened for chimeras (usearch8.1 algorithm)(65), and putative chimeric sequences were removed from the dataset (QIIME v1.8)(66). Each fecal sample was rarefied (30,000 sequences/sample) and data were pooled, renamed, and clustered into operational taxonomic units (OTU) at a 97% similarity (usearch8.1 algorithm) threshold. Representative sequences from each OTU were extracted and classified using the uclust consensus taxonomy assigner (Greengenes 13_8 reference database). A biological observation matrix (BIOM)(67) was generated at each taxonomic level from phylum to species ("make OTU table" algorithm) and analyzed and visualized using the software packages Primer7(68) and the R programming environment (69).

Alpha (α) diversity (within sample) and beta (β) diversity indices (between samples) were used to examine changes in the cecum luminal content's microbial community structure between rat group samples, as described previously (70). Microbial community composition differences between samples, using the pairwise Bray-Curtis dissimilarity metric, was generated using the Primer7 software package, and used to perform analysis of similarity (ANOSIM) calculations. ANOSIM was performed at the taxonomic level of genus, using square-root transformed data. Also, Primer7 was used to conduct both metric multi-dimensional scaling (mMDS) and bootstrapping (average values and dispersion within each sample's group) plots to visualize each mice group's overall microbial differences, at the genus level.

The relative abundances of individual taxa (>1%) were assessed for significance using Kruskal-Wallis test, controlling for false-discovery rate (FDR), implemented within the software package QIIME (66). Furthermore, the rat cecum luminal content sample's community functional predictions were performed using PICRUSt (Phylogenetic

Investigation of Communities by Reconstruction of Unobserved States)(71), and inferred differences in Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog (KO) abundances between specified groups were identified using Kruskal-Wallis group significance testing with FDR-p corrections (72). Adjusted p-values were reported significant at FDR < 0.05 or at $p < 0.05$.

SCFA Gas Chromatography-Mass Spectrometry Analysis

Short-chain fatty acid analysis was completed on $n = 25$ (Exp. 1), 61 (Exp. 2) and 31 (Exp. 3) rats (Table S4). Absolute quantification of short chain fatty acids from rat cecum luminal content samples was completed by gas chromatography-mass spectrometry (GC-MS) (Proteomics & Metabolomics Facility, Colorado State University). Absolute quantities of acetate, propionate and butyrate were determined using spiked stable isotopes of acetate and butyrate. Briefly, 0.4 mL of cold 2 M HCl containing 75 $\mu\text{g/mL}$ of $^{13}\text{C}_2$ -acetic acid (Sigma-Aldrich) and 7.5 $\mu\text{g/mL}$ of $^{13}\text{C}_4$ -sodium butyrate (Santa Cruz Biotechnology) were added to a 20 mg sample of frozen cecum luminal content material. Samples were vigorously shaken and then sonicated in a cold water bath. Samples were vortexed and centrifuged at 4°C . Supernatants were recovered, and added to MTBE, which were stored at 4°C until analysis. The MTBE extract of SCFAs was injected into a Trace 1310 GC coupled to a Thermo ISQ-LT MS, at a 5:1 split ratio. SCFA separation was achieved on a 30m DB-WAXUI column (J&W, 0.25 mm ID, 0.25 μm film thickness). SIM mode was used to scan ions 45, 60, 62, 73, 74, 76, 87, 88, 92 at a rate of 10 scans/sec under electron impact mode.

Histology

Histology was completed on $n = 45$ knees, 19 livers and 43 colons (Exp. 1), 61 knees and colons (Exp. 2) and 13 knees and 31 colon (Exp. 3) samples. Right knee joints, distal colon samples and liver samples were collected for histology and immunohistochemistry (Table S4). Knee joints were fixed in 10% neutral buffered formalin (NBF) and decalcified in 10% ethylenediaminetetraacetic (EDTA) for 3 weeks. Implants were manually removed proximally to preserve the distal region of the implant. Liver samples were fixed in 10% NBF for 7 days, dissected to isolate the hepatic portal triad and paraffin embedded. H&E staining was completed by rehydrating sections in xylene and decreasing concentration of ethanol washes. Slides were then submerged in hematoxylin for 7 minutes, followed by a 3 minute water rinse and a 3 minute wash in Scott's bluing solution. Slides were then submerged in eosin for 1 minute, followed by dehydration in increasing ethanol solutions and xylene washes. Slides were then cover slipped using DPX and left to air-dry. H&E stained liver histology from selected rats per study group in Experiment 1 were read blindly by a veterinary pathologist (University of Pennsylvania Veterinary Comparative Pathology core lab). Decalcified femurs and livers were paraffin embedded and sectioned at $6\mu\text{m}$ on a Leica RM 2255 microtome. Distal colon samples were collected fresh and immediately embedded in OCT and flash frozen using 2' methylbutane on dry ice. OCT samples were sectioned at $10\mu\text{m}$ using a Leica CM 1850 cryostat.

Following previously published methods, H&E histology was completed on knees, liver and colon (57). DAB immunohistochemistry was completed using manufacturer's protocol for select inflammatory markers, specifically macrophages (CD68, AbCam, ab125212) on knee

joints. Antibody concentration for AbCam antibodies was 1:250. Briefly, sections underwent graded rehydration and antigen unmasking using Tris-EDTA (AbCam, ab93684) at 95°C. Sections were blocked with normal goat serum (AbCam, ab7481) at room temperature and secondary goat-anti-rabbit IgG HRP (ab205718) antibody was used in a 1: 2,000 concentration. Immunochemical localization for hyaluronan was carried as described (73) and Mason Trichrome staining carried out using the Trichrome Stain Kit (AbCam, ab150686). Liver histology and colon histology sections were scored by veterinary pathologists at the Department of Pathobiology at the University of Pennsylvania and Michigan State University Veterinary Diagnostic Laboratory, respectively. Liver sections were read in a blind fashion without disclosure of the experimental groups. Colon sections were first read with groups identified to identify a scoring pattern and then select slides were blinded and re-read.

Serum Analysis

Serum inflammatory markers were tested in n = 41 (Exp. 1), 61 (Exp. 2) and 31 (Exp. 3) rats and metal ion concentration was tested in n = 41 (Exp. 1), 0 (Exp. 2) and 31 (Exp. 3) rats (Table S4). Serum was processed from fasted whole blood collected from cardiac puncture at sacrifice. Enzyme-linked immunosorbent assays (ELISA) were completed following manufacturers protocol for C-reactive protein (CRP, 1:5 dilution, Cusabio, CSB-E07922r), lipopolysaccharide binding protein (LBP, 1:10 dilution, Cusabio, CSB-E11184r), interleukin- 6 (IL-6, 1:10 dilution, Cusabio, CSB-E04640r) and corticosterone (1:10 dilution, Immunodiagnostic Systems, AC-14F1). Systemic endotoxin levels were measured using an LAL kit (Pierce Limulus Amebocyte Lysate, 88282, Lonza, Walkerville Inc., Walkerville, MD) with a 1:10 dilution. All samples were measured in duplicate following the manufacturer's instructions on the same plate and all at the indicated dilution for each assay. In brief, 100µl of standard or samples were loaded per well and incubated at 37°C. Then biotin, HRP-avidin, TMB substrate and a stop solution were each incubated per well at 37°C. Standards and reagents were kit-specific.

Metal ions (chromium, manganese, molybdenum, titanium and cobalt) were tested for using a 20-fold dilution with a solution containing 0.5% EDTA and Triton X-100, 1% ammonium hydroxide, 2% propanol and 20 ppb of scandium, rhodium, indium and bismuth as internal standards (Michigan State University Veterinary Diagnostics Lab). Elemental analysis was performed using an Agilent 7900 Inductively Coupled Plasma – Mass Spectrometer (ICP/MS, Agilent Technologies Inc, Santa Clara CA). The ICP/MS was tuned to yield a minimum of 7500 cps sensitivity for 1ppb yttrium (mass 89), less than 1.0% oxide level as determined by the 156/140 mass ratio and less than 2.0% double charged ions as determined by the 70/140 mass ratio. Elemental concentrations were calibrated using a 5-point linear curve of the analyte-internal standard response ratio. Standards were from Inorganic Ventures (Inorganic Ventures, Christiansburg, VA). Lypochek (Bio-Rad, Hercules, CA) metal standards, Multi-Element QC-21 Specpure check standard solution (Alfa Aesar, Tewksbury, MA), and in-house serum pools were used as controls.

Statistics

Bioinformatics analyses were performed to identify implant + particle challenge effects. Differences between baseline and particle challenged samples for alpha diversity indices (Shannon, richness and evenness), Firmicutes-to-Bacteroides (F/B) ratio, micro-computed tomography (microCT), mechanical testing, serum biomarker, metal ion and SCFA metabolite concentrations were assessed for normality (Shapiro-wilks test). Significance using the one-way analyses of variance (ANOVA) for group effects with Bonferroni post-hoc test; two-way ANOVA for group and time effects with Bonferroni post-hoc test; or independent t-tests for inter-group differences were completed. All results are reported as means $\pm$ standard deviation with statistical testing performed with commercially available software (SPSS v.19 for Windows, Chicago, IL).

Results

Increased joint inflammation and fibrotic remodeling are associated with particle challenge.

Sections stained for hyaluronan (HA) identified regions of the joint capsule and synovium that displayed a soft tissue wound healing response (Fig. 2). Compared to naïve controls, a robust accumulation of HA-rich extracellular matrix (ECM) was present in the joint lining, the peri-meniscal synovium and the repair tissue between the distal aspect of the implant and the articular surface (Fig. 2A). There was extensive loss of subsynovial adipose tissue, which was accompanied by replacement with a dense connective tissue (Fig. 2B). To confirm that such HA-rich tissue remodeling consisted of fibrotic regions, adjacent sections were stained with Mason Trichrome as a marker of fibrotic collagenous ECM (Fig. 3A). The fibrotic remodeling was most widespread following CoCr particle administration (Fig. 3B).

The presence of immune cells was assayed with immunohistochemistry. Macrophages (CD68+ cells) were localized to the synovial lining (Fig. 4) and fibrotic regions of the peri-meniscal synovium (Fig. 5) after LPS-PE and CoCr injections, but were absent in joints injected with vehicle. Rats that were administered CoCr particles also demonstrated clustering of these cells around the particles. In addition, neutrophil accumulation was observed in the posterior synovium in rats treated with CoCr particles, as shown by myeloperoxidase (MPO) + staining (Fig. S1).

When particle challenge caused depressed implant fixation, the gut microbiota was also affected

The particle challenge led to decreased peri-implant bone volume and implant fixation only in Experiment 1 (Fig. 6). Specifically, in this experiment peri-implant bone volume (BV/TV) varied among the experimental groups (1-way ANOVA: $p = 0.001$) with significant decreases in both the CoCr (Bonferroni: $p < 0.001$) and LPS-PE (Bonferroni: $p < 0.001$) groups compared to naïve rats (57). CoCr (Bonferroni: $p < 0.001$) and LPS-PE (Bonferroni: $p = 0.001$) treated rats also had significantly lower BV/TV than rats treated with clean-PE (57). The loss of peri-implant BV/TV was accompanied by depressed implant fixation strength, a preclinical surrogate of implant loosening (1-way ANOVA: $p = 0.002$), with decreased fixation strength with CoCr treatment compared to vehicle treated (Bonferroni: p

= 0.007) and clean PE group (Bonferroni: $p = 0.002$) with a trend in the same direction compared to the LPS-PE group (Bonferroni: $p = 0.051$). In Experiments 2 and 3 there was an overall lack of response to the particle challenge assessed by peri-implant bone volume, implant fixation strength and gut microbiota (Fig. 6). The single exception was Experiment 3 where the CoCr particle challenge induced peri-implant bone loss (1-way ANOVA: $p < 0.001$), but did not cause depressed implant fixation (1-way ANOVA: $p = 0.743$) nor alterations in the gut microbial community (e.g., bacterial ratio of Firmicutes-to-Bacteroides, F/B ratio, was not significantly affected; 1-way ANOVA: $p = 0.716$).

We observed microbiota differences in rat cecum luminal content as assessed by cultivation-independent analyses of microbial community structure and metabolites (Experiment 1 only; Fig. 6). The cecum luminal content microbial community structure was significantly different between the five groups for the bacterial ratio of Firmicutes-to-Bacteroidetes (F/B) (1-way ANOVA: $p = 0.028$). Post-hoc analyses showed a significant increase of the F/B ratio in CoCr (Bonferroni: $p = 0.016$) and LPS-PE (Bonferroni: $p = 0.026$) treated rats, compared to naïve rats. Additionally, the F/B ratio was significantly increased in CoCr treated rats (Bonferroni: $p = 0.031$), and trending towards significance in LPS-PE treated rats (Bonferroni: $p = 0.059$), compared to vehicle treated rats. This phylum level microbial change was driven by a significant decrease in the relative abundance of Bacteroidetes in LPS-PE (FDR: $p = 0.03$) and CoCr (p-value: $p = 0.04$) compared to naïve, and a significant increase in relative abundance of Firmicutes in CoCr compared to vehicle (p-value: $p = 0.02$) and naïve (p-value: $p = 0.004$, FDR: $p = 0.09$). No significant differences in alpha (α)-diversity were observed at any taxonomic level among groups (data not shown). To assess effects of treatments on beta (β)-diversity, analysis of similarity (ANOSIM) was performed using pair-wise distance matrices derived from Bray-Curtis distance metrics (Table S1, Fig. S2). Overall microbial community compositions, at the taxonomic level of genus, were significantly different between vehicle and LPS-PE treated (ANOSIM: Global $R = 0.24$, $p = 0.02$); vehicle and CoCr treated (ANOSIM: Global $R = 0.35$, $p = 0.04$) and LPS-PE and CoCr treated (ANOSIM: Global $R = 0.30$, $p = 0.03$) rat cecum luminal content samples. The relative abundance of individual genus taxa (>1% abundance) were examined and depicted as stacked histograms between treatment groups (Fig. S3).

Taxon-by-taxon analyses were performed to identify individual taxonomic features significantly different between treatment groups. At the taxonomic level of family, the relative abundance of beneficial anti-inflammatory short-chain-fatty-acids (SCFA)-producing bacteria *Lachnospiraceae* was decreased in clean-PE (p-value: $p = 0.04$) and LPS-PE (p-value: $p = 0.04$) treated rats, compared to naïve rats. Additionally, *Lachnospiraceae* relative abundance increased in CoCr treated rats compared to vehicle (p-value: $p = 0.04$), clean-PE (p-value: $p = 0.01$) and LPS-PE (p-value: $p = 0.01$) treated rats. Similarly, the relative abundance of putative SCFA-producing bacteria family *Ruminococcaceae* was higher in vehicle (p-value: $p = 0.04$) and CoCr treated (p-value: $p = 0.01$) treated rats relative to naïve rats. CoCr treated rats had higher relative abundance of *Ruminococcaceae* than clean-PE (p-value: $p = 0.02$) and LPS-PE (p-value: $p = 0.01$) treated rats (Table S2).

Given the changes in the relative abundance of putative-SCFA producing bacteria from the rat treatment groups, we measured SCFA metabolite concentrations in the cecum luminal

content for all three experiments. In Experiment 1, acetate was significantly different (1-way ANOVA: $p = 0.043$) across all five groups. Post-hoc analysis showed that acetate was significantly decreased in LPS-PE treated compared to naïve (Bonferroni: $p = 0.042$) rats. With LPS-PE treatment, there was a significant decrease in acetate measurement, which may be associated with the decreased relative abundance of SCFA-producing bacteria *Lachnospiraceae*, compared to naïve rats. No significant effects of treatment on SCFA levels were observed in Experiments 2 and 3.

Using an *a priori* hypothesis for predictive assessment of the microbial community functional potential (PICRUSt metagenomics), we identified three inferred pathways involved in inflammation homeostasis with particle challenge: 1) pro-inflammatory pathways critical to LPS synthesis, 2) anti-inflammatory SCFA-related pathways, and 3) immune response and inflammatory pathways. Our analysis inferred the bacterial and immune response pathway abundance was significantly increased in vehicle (p -value: $p < 0.047$), LPS-PE (FDR: $p < 0.034$) and CoCr (p -value: $p < 0.028$) treated groups, compared to naïve rats. LPS-PE treated rats also had increased pathway abundance of bacterial and immune response, compared to the CoCr group (p -value: $p < 0.047$). The SCFA pathway abundance was only significantly increased in vehicle compared to naïve rats (p -value: $p = 0.028$, FDR: $p = 0.063$). The immune response pathway abundance was significantly increased in vehicle compared to naïve rats (p -value: $p = 0.028$, FDR: $p = 0.063$), and increased in CoCr treated rats compared to vehicle (p -value: $p < 0.047$), clean-PE (p -value: $p = 0.047$) and LPS-PE treated (p -value: $p = 0.016$) rats.

When particle challenge caused depressed implant fixation, there was also evidence of inflammation in the liver, but not in the colon

In the context of known particle migration and treatment effects on gut microbial community structure, we examined the liver and distal colon. Liver histology (H&E staining) showed mild inflammation characterized by sparse infiltrates of predominantly small lymphocytes in the portal areas. Approximately 30–60% of the portal areas contain inflammatory cell infiltrates in the effected livers. These results were classified into differential presence/absence of inflammation and vacuolation, depending upon treatment (chi-square: $p = 0.013$, Table 2). Specifically, 4 out of 4 CoCr treated rats and 3 out of 4 LPS-PE treated rats had inflammation present in the peri-portal region. Only one vehicle-treated rat had inflammation and none of the clean-PE treated rats had inflammation. No histological evidence of inflammation was observed in the distal colon. No differences in cell composition between groups were observed.

Serum metal ion levels, but not inflammatory cytokines, were altered with particle challenge

In Experiment 1, the serum levels of cobalt (1-way ANOVA: $p < 0.001$) and titanium (1-way ANOVA: $p < 0.001$) metal ions were significantly different between groups. Post-hoc analyses showed cobalt was significantly higher in the CoCr treated rats compared to all other study groups ($p < 0.001$) and titanium was significantly higher in CoCr treated rats than naïve ($p = 0.001$), clean-PE ($p < 0.001$) and LPS-PE ($p < 0.001$) groups. Titanium levels were not different in vehicle and CoCr treated rats ($p = 1.00$). There were no between-

group differences for manganese, molybdenum or chromium (Table S3). Serum endotoxin was not significantly different between groups (1-way ANOVA: $p = 0.673$) nor was LBP (1-way ANOVA: $p = 0.239$). Serum inflammatory markers, including cathepsin K, IL-6, CRP and corticosterone were also tested, but showed no between-group differences (Table S3).

Discussion

Our results support the hypothesis that a particle challenge leading to joint inflammation, peri-implant bone loss and diminished implant fixation (a proxy for aseptic loosening) are associated with changes in the gut microbiota composition. Specifically, in Experiment 1 we found that with particle administration 1) peri-implant trabecular bone volume was depressed, 2) implant fixation strength was depressed, 3) cecum luminal content's microbial community structure and composition (Firmicutes-to-Bacteroidetes ratio) was increased, 4) relative abundances of putative SCFA-producing bacteria were decreased and 5) acetate level concentration was significantly reduced. We also showed that when implant fixation was not depressed in the presence of a particle challenge, the gut was unaffected (Experiments 2 and 3). Our results suggest that if the peripheral particle challenge is sufficient to cause depressed implant fixation, the gut microbiome will also be affected, supporting the hypothesis that implant loosening alters the gut microbiota (bottom arrow, Fig. 7).

Particle-host response differed between our experiments. The lack of gut effects in Experiments 2 and 3 may be due to two factors, 1) LPS-PE and CoCr may signal through two different inflammatory pathways and 2) the timing of particle administration likely impacts the particle response. Our results are consistent with other studies that showed PE and CoCr particles elicit different inflammatory signaling pathways (50, 74–76). We showed LPS-PE and CoCr particles signal through different microbiota-to-implant pathways as shown by the difference in F/B expression. Specifically, the microbiota differences in Experiment 1 may be driven by the observed relative abundance of *Lachnospiraceae*, the putative beneficial anti-inflammatory SCFA-producing bacteria that is decreased in LPS-PE, compared to CoCr treated rats is consistent with the decrease in acetate concentration. We did not see a change in *Lachnospiraceae* abundance and there were no changes in SCFA-expression in Experiments 2 or 3. Further studies using shotgun metagenomics and metatranscriptomics analysis will be necessary to confirm these preliminary findings.

Particles were either administered over 6 weeks in Experiment 1, 6 or 12 weeks in Experiment 2 or 12 weeks in Experiment 3. While the particle administration over 6 weeks in Experiments 1 and 2 yielded comparable bone volume and fixation strength losses, administration of particles for an additional 6 week did not amplify these results, as we had expected. We now believe that the longer particle administration period may have resulted in an inoculation-like response to the particles and not a pro-inflammatory response, which may explain the lack of response in Experiments 2 and 3. This is supported by the loss of immune reactivity to particles in monocyte/macrophage cells as they differentiate into osteoclasts (77). While we did not look at osteoclast number in our samples, we do see an increase in CD68+ macrophages with 6 weeks of particle treatment.

Our data are consistent with other studies showing that peripheral injuries such as burns to the skin (22–24) and traumatic brain injury (25, 26) alter the gut microbiota. Other challenges that lead to alterations of the gut microbiota include disrupted circadian rhythm (27, 28) and environmental and psychosocial stress (29, 31). Proposed mechanisms for communication, between a peripheral injury and the gut microbiome, focus on inflammation leading to gut dysbiosis (changes in the gut microbiota composition and the gut barrier integrity). Studies have found that peripheral injuries induce increased gut permeability (22), decreased F/B ratio (23), decreased abundance of SCFA-producing bacteria (23, 24), increased pathogenic bacteria (26) and functional gene expression of the microbiome showed increased immunomodulatory pathway activity (24). While we do not currently understand the causal role of the microbiome in implant fixation, this is a common issue with microbiome-host interaction research (78), and we expect to expand our understanding of the observation we report here in the near future.

The innate immune response may be integral to promoting the inflammation-driven communication between a peripheral injury and the gut as evidenced by recruitment of neutrophils and CD68+ macrophages to particle-challenged joints. While we show infiltration of these inflammatory cells into the knee joint, we did not observe any increase in serum inflammatory markers. This is consistent with what has been observed in other diseases. Local inflammation occurs before systemic inflammation can be identified in COPD (79), stroke (80) and rheumatoid arthritis (81). It is also suggested that systemic inflammation may not be achieved until severe local inflammation is attained (79).

In our model the weekly administration of particles likely elicits a sinusoidal curve of inflammation that repeats with each intra-articular injection for the duration of the experiment. The local inflammatory effects of the last particle injection may have diminished by sacrifice, therefore not allowing for systemic reaction to be identified. The liver inflammation we identified in the LPS-PE and CoCr treated rats may be a mediator in the inflammatory pathway as it is known to synthesize CRP (82). We tested for and found no increase in CRP expression with particle challenge suggesting that local and liver inflammation likely occurs prior to systemic inflammation therefore suggesting that the inflammatory threshold to elicit change in the serum markers was not acquired in our model.

A downstream effect of this immune-driven inflammatory cascade is fibrosis. We observed fibrosis in the knee joint that appears similar to arthrofibrosis, the formation of scar tissue after a joint replacement surgery, which is well documented in patients (83, 84). Arthrofibrosis is known to be driven by an immune response after joint trauma and is mediated by T-cell activation and CD68+ macrophage recruitment to the synovium (85). We confirmed CD68+ macrophages were present with particle challenge in our model. In addition, the presence of neutrophils in the posterior synovium following CoCr particle challenge may point to prolonged joint inflammation in this group since these cells are normally cleared quickly (86). We did not observe neutrophils in PE particle-challenged groups in Experiment 1. While there is no established link between arthrofibrosis and the gut microbiome, other diseases resulting in fibrosis are associated with gut dysbiosis. Cystic fibrosis is associated with reduced gut microbial diversity and increased F/B ratio and

inflammatory markers (87, 88) and liver fibrosis and systemic scleroderma are associated with decreased diversity and abundance in the gut microbiome (89–92).

A second potential mechanism of communication between the implant microenvironment and the gut is the translocation of serum metal ions. We have shown in our model that elevated serum cobalt and titanium ions are associated with CoCr particle challenge. Metal ions released from the implant itself or from the oxidation of particles (93) can enter the blood stream and concentrate in red blood cells (44, 94). Excessive cobalt concentration can lead to multiple serious health concerns (44), promote the formation of free radicals and oxidative stress (95) and contribute directly to alterations in the gut microbiome (96, 97). Cobalt ions are known to be absorbed by the human small intestine when ingested (98). One recent rat study showed that oral administration of cobalt altered the β -diversity of the gut microbiome, leading to an increased abundance of bacteria from the phylum Verrucomicrobia (96), which may lead to changes in the mucosal layer and ultimately promote gut dysbiosis. Both the diversity and F/B ratio were decreased in Mongolian toads that were exposed to environmental heavy metal pollution (97). The presence of gut effects in experimental groups in Experiment 1 where metal ion levels were not raised (i.e., rats treated with LPS-PE), suggests that this potential mechanism may be contributory, but not necessary and reinforces the concept that LPS-PE and CoCr particles may signal through different implant-to-microbiota pathways.

A growing body of literature has established that targeting the gut with either antibiotics (7, 10, 99, 100), probiotics (2, 12–14, 101, 102) or prebiotics (103–106) can affect bone mechanical and structural properties as well as mineral metabolism (top arrow, Fig. 7). Some musculoskeletal diseases have also been targeted using these treatments. Administration of a probiotic has been shown to reduce arthritis-associated bone loss (19) and reduce particle-induced osteolysis in a calvarial model (21). Therefore, altering the gut microbiome can have an impact on the overall health of bone and the progression of musculoskeletal diseases. These observations along with our novel finding that pro-inflammatory alterations in the implant microenvironment are associated with alterations in the gut microbiome suggest that interventions to dampen implant loosening could target the gut-to-joint arrow or the joint-to-gut arrow in Fig. 7. With the anticipated increase in incidence of total joint replacement in the next few decades (37, 107), implant failures and revision surgery will continue to be a burden to patients. Our findings may provide a novel strategy for preventing or treating implant loosening and eventually reducing the need for revision surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-Standard Abbreviations List

ANOSIM	analysis of similarity
ANOVA	analyses of variance
BV/TV	bone volume/total volume
CoCr	cobalt-chrome particles
CRP	C-reactive protein
DAB	3,3'-Diaminobenzidine immunohistochemistry
DNA	deoxyribose nucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic
ELISA	Enzyme-linked immunosorbent assays
FDR	false-discovery rate
F/B	Firmicutes-to-Bacteroidetes
g	gram
GC-MS	gas chromatography-mass spectrometry
HA	hyaluronan
H&E	hematoxylin and eosin histology
HRP	horseradish peroxidase
IgG	immunoglobulin G
IL-6	interleukin-6
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Kyoto Encyclopedia of Genes and Genomes ortholog
LAL	Limulus Amebocyte Lysate
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
microCT	micro-computed tomography
µm	micrometer

μl	microliter
mg	milligram
mL	milliliter
mMDS	metric multi-dimensional scaling
MPO	myeloperoxidase
MTBE	methyl tert-butyl ether
NCBI	National Center for Biotechnology Information
NBF	neutral buffered formalin
OCT	optimal cutting temperature embedding medium
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PE	polyethylene particles
PICRUST	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
RNA	ribose nucleic acid
RUMC	Rush University Medical Center
SCFA	Short-chain fatty acid
SD	Sprague Dawley rats
SRA	Sequence Read Archive
TJR	total joint replacement

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Figure 1.
Radiograph of intramedullary implant in a rat. Scale bar= 10mm.

Figure 2.

Hyaluronan (HA) localization in soft tissues in naïve joints and joints after implant placement. A- 2x stitched images of sagittal sections. Implant track= *. Remodeling of the cartilage regions at the implant insertion site is marked by a black arrow. B- 4x images of the perimeniscal synovium and adjacent adipose tissue corresponding to the circled regions in Panel A. Note the loss of adipose tissue and replacement by dense fibrotic connective tissue (FIB) following implant placement, which strongly stained for HA. T= Tibia, F= Femur, M= Meniscus, PT= Patella Tendon, S= Synovial Lining, FIB= fibrotic tissue, AT= Subsynovial Adipose Tissue. Sections were taken from knee joint specimen prepared from rats in Experiment 1. Scale bar (A) = 500µm; Scale bar (B) = 200µm.

Figure 3. Mason's Trichrome Staining of Knee Joints. A- 2x stitched images of sagittal sections. Implant track= *. Remodeling of the cartilage regions at the implant insertion sites is marked by a black arrow. There is substantial thickening of the patellar tendon as shown by the increase in blue staining with CoCr particle challenge. B- 4x images of the perimeniscal synovium and adjacent adipose tissue corresponding to the circled regions in Panel A. The hyaluronan-stained dense connective tissue also stained strongly for collagenous extracellular matrix (blue stain), confirming that the synovial lining and the subsynovial adipose tissue were replaced by fibrotic tissue following implant placement. T= Tibia, F= Femur, M= Meniscus, PT= Patella Tendon, S= Synovial Lining, FIB= fibrotic tissue, AT= Subsynovial Adipose Tissue. Sections were taken from knee joint specimen prepared from rats in Experiment 1. Scale bar (A) = 500 μ m; Scale bar (B) = 200 μ m.

Figure 4. Immunohistochemical localization of CD68+ cells in the perimeniscal synovium. Implant placement + Vehicle had no detectable CD68+ cells whereas increased numbers of such cells were detected following intra-articular injection of LPS-PE into the joint following implant placement. FIB= fibrotic tissue, AT= Subsynovial Adipose Tissue; M= Meniscus; S= Synovial Lining. Sections were taken from knee joint specimen prepared from rats in Experiment 1. Scale bar A (4x) = 200 μ m; B (20x) = 50 μ m.

Figure 5.

Immunohistochemical localization of CD68+ cells in the perimeniscal synovium following intra-articular injection of CoCr particles after implant placement. CD68+ cells were predominantly clustered in fibrotic tissue regions containing abundant CoCr particles (encircled by dotted lines in Panel A. FIB= fibrotic tissue, M= meniscus. Sections were taken from knee joint specimen prepared from rats in Experiment 1. Scale bar A (4x) = 200µm; B (20x) = 50µm.

Figure 6.

Bone volume fraction (BV/TV), fixation strength and Firmicutes-to-Bacteroidetes Ratio (F/B Ratio) for Experiments 1, 2 and 3. For BV/TV and F/B Ratio, all study groups are normalized to the naïve group. For fixation strength, all particle-treated groups are normalized to vehicle. When implant fixation and peri-implant bone were both decreased the gut microbiota was also altered. In Experiment 2, LPS-PE particles administered for 6 or 12 weeks was not sufficient to induce depressed implant fixation nor peri-implant bone loss between groups. The gut microbiota was not altered. Means and SD. Significance bars labeled with p-values from post-hoc results. The BV/TV and Fixation Strength data for Experiment 1 were reported for some groups previously (57).

Figure 7.
The proposed model of bi-directional, cyclic cross-talk between the gut microbiome and implant microenvironment. Arthrofibrosis, as discussed in the text, may be a contributing factor.

Table 1.

Experimental Design. Study Groups and number of animals for Experiments 1, 2 and 3.

Group	Implant placement	Intra-articular particle challenge	End point (weeks)	Sample size
Experiment 1				
Naïve	No	No	6	8
Vehicle	Yes	No	6	9
PE	Yes	Yes (PE)	6	8
LPS-PE	Yes	Yes (LPS-doped PE)	6	9
CoCr	Yes	Yes (CoCr)	6	9
Total				43
Experiment 2				
Naïve	No	No	6	9
Vehicle	Yes	No	6	10
LPS-PE	Yes	Yes (LPS-doped PE)	6	10
Naïve	No	No	12	10
Vehicle	Yes	No	12	10
LPS-PE	Yes	Yes (LPS-doped PE)	12	12
Total				61
Experiment 3				
Naïve	No	No	12	10
Vehicle	Yes	No	12	10
CoCr	Yes	Yes (CoCr)	12	11
Total				31

LPS = lipopolysaccharide, PE = polyethylene, CoCr = cobalt chrome.

Table 2.

Chi-Square for Experiment 3 liver histology. Samples were coded for presence (yes) /absence (no) of liver inflammation as identified by peri-portal inflammation.

Group	No	Yes	TOTAL
Vehicle	4	1	5
clean-PE	4	0	4
LPS-PE	1	3	4
CoCr	0	4	4
TOTAL	9	8	17
	Value	df	Asymptotic Significance (2-sided)
Pearson Chi-Square	10.778	3	0.013
Likelihood Ratio	14.005	3	0.003
Linear-by-Linear Association	7.681	1	0.006
N of Valid Cases	17		