ORIGINAL RESEARCH

Bacterial Biofilm Components Induce an Enhanced Inflammatory Response Against Metal Wear Particles

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Purpose: Aseptic implant loosening is still a feared complication in the field of orthopaedics. Presumably, a chronic inflammatory response is induced by wear particles, which leads to osteoclast generation, bone degradation and hence loosening of the implant. Since it has been demonstrated in the literature that most implants are in fact colonized by bacteria, the question arises whether aseptic implant loosening is truly aseptic. The aim of this study was to investigate a possibly enhanced inflammatory response to metal wear particles in the context of subclinical infection.

Patients and Methods: Tissue samples were collected intra-operatively from patients undergoing implant-exchange surgery due to aseptic loosening. Histopathological analysis was performed, as well as gene expression analysis for the pro-inflammatory cytokine Interleukin-8. By a series of in vitro experiments, the effect of metal wear particles on human monocytes, polymorphonuclear neutrophiles and osteoblasts was investigated. Additionally, minor amounts of lipoteichoic acid (LTA) and the bacterial heat shock protein GroEL were added.

Results: Histopathology of tissue samples revealed an accumulation of metal wear particles, as well as a cellular infiltrate consisting predominantly of mononuclear cells. Furthermore, high expression of IL-8 could be detected in tissue surrounding the implant. Monocytes and osteoblasts in particular showed an increased release of IL-8 after stimulation with metal wear particles and in particular after stimulation with bacterial components and wear particles together.

Conclusion: We were able to show that minor amounts of bacterial components and metal wear particles together induce an enhanced inflammatory response in human monocytes and osteoblasts. This effect could significantly contribute to the generation of bone-resorbing osteoclasts and hence implant-loosening.

Keywords: aseptic loosening, metal wear particles, biofilm, implant-associated infection, Interleukin-8

Introduction

Metal implants are widely used in the field of orthopaedics and total joint replacement of the hip in particular has been termed one of the most successful surgeries in medicine. Despite great achievements in surgical technique and implant design, loosening of implants is still a severe complication, which causes patients' severe pain and eventually requires implant-exchange surgery.[1–4] Usually, two underlying pathomechanisms are distinguished: 1) Aseptic implant loosening, which is thought

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to be due to an inflammatory response elicited by implant wear particles.[5] Presumably, phagocytic cells are activated, because they readily take up particulate materials, and thereby produce inflammatory cytokines, which leads to a recruitment of more immuno-competent cells and also to a generation of bone-degrading osteoclasts.[6] 2) Infectious implant loosening: in these cases bacteria aggregate on an implant surface and surround themselves in a slimy matrix (extrapolymer polymeric substance). This process is referred to as biofilm formation.[7–9] Bacteria are much more difficult to eradicate once they have established a biofilm colony, which may result in a self-perpetuating inflammatory response in an effort to fight of infection.[10–12] In these cases, an infiltrate of mainly poly-morphonuclear neutrophils and mononuclear cells can be found in tissue surrounding the implant.[13] As the inflammatory response continues, a generation of osteoclasts is induced, which leads to bone degradation and hence implant-loosening.[14,15] Therefore, implant-loosening can be considered an unfortunate side effect of a chronic inflammatory process.

In our previous work, we were able to show that the immune response in aseptic and infectious implant loosening is similar, though the intensity of the reaction is usually higher in infectious cases, corresponding to the fact that patients with implant infections become symptomatic earlier on.[13] However, it is particularly difficult to distinguish between aseptic cases and so-called low-grade infections and it has been shown in the literature that up 40% of cases are falsely classified as aseptic.[16,17] Furthermore, bacterial DNA could be detected on the majority of routinely removed implants, even though patients were completely asymptomatic.[18,19] This raises the question whether aseptic cases are truly aseptic and whether it is reasonable to distinguish between these two entities rigorously.[20]

The aim of this study was to investigate the inflammatory response in patients suffering from aseptic implant loosening and a possibly enhanced response to metal wear particles in the context of subclinical infection. To simulate infection, minor amounts of lipoteichoic acid (LTA) and the bacterial heat shock protein GroEL were used. Lipoteichoic acid is a component of the cell wall of gram-positive bacteria, which are predominant in implant-associated infections.[21,22] GroEL is an evolutionary highly conserved protein that shares homologies with the human heat shock protein Hsp 60 and is essential for protein folding.[23–25] Furthermore, we were able to demonstrate

in our previous work that GroEL can be found within the extrapolymer polymeric substance of *Staphylococcus epidermidis* biofilms. Neutrophils and also local tissue cells – such as osteoblasts–recognize GroEL and respond by up-regulating defense-relevant function.[26,27]

Patients and Methods
Tissue Samples
Tissue samples were collected intra-operatively from 5 patients undergoing revision surgery due to aseptic loosening of a metal-on-metal hip replacement (ASR hip resurfacing system, DePuy, Warsaw, Indiana, USA). Diagnosis of loosening was based on patient's complaints, clinical examination and by conventional x-ray and/or CT-scan. Multiple tissue samples were taken from around the implant in a standardized fashion (from the joint capsule, from the area of the femoral shaft and the cup, as well as an intramedullary sample). The tissue samples were divided. One half was fixed in formalin for histological analysis and the other half placed in RNAlater (Ambion, Life technologies, Heidelberg, Germany) for quantitative PCR analysis.

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local ethic committee of Heidelberg University (S-424/2010). Informed consent was obtained from the patients.

Histology
The samples were fixed in formalin, embedded in paraffin and slices of 3–4 µm thickness were cut for eosin-haematoxylin staining.

Gene Expression Analysis
Gene expression analysis was carried out according to the previously reported protocol.[13,28] Cells were collected in 400 µL lysis buffer from the MagnaPure mRNA Isolation Kit I containing 1% DTT (v/w) (ROCHE Applied Sciences–RAS, Mannheim). mRNA was isolated with the MagnaPure-LC device using the mRNA-I standard protocol. Tissue samples were collected and stored in RNAlater (Ambion). After taking the samples out of the stabilizer, they were disrupted with the aid of a RiboLyser device (ThermoHYBAID, Heidelberg) in lysing matrix "D" tubes (Q-BIOgen, Heidelberg) containing 400 µL lysis buffer from the MagnaPure mRNA Isolation Kit I containing 1% DTT (v/w) (ROCHE Diagnostics, Mannheim). Ribolyser tubes were frozen at −80°C. After thawing 300 µL of the

lysate were collected and mixed with 120 µL lysis buffer containing DTT. After centrifugation at 13,000 rpm for 5 min, 400 µL of this mix was transferred into a MagnaPure sample cartridge and mRNA was isolated with the MagnaPure-LC device using the mRNA-standard protocol for cells. For both preparations, the elution volume was set to 50 µL. An aliquot of 8.2 µL mRNA was reversely transcribed using AMV-RT and oligo (dT) as primer (First Strand cDNA synthesis kit, Roche) according to the manufacturer's protocol in a thermocycler. After termination of the cDNA synthesis, the reaction mix was diluted to a final volume of 500 µL and stored at –20°C until PCR analysis.

Primer sets optimized for the LightCycler® (RAS, Mannheim Germany) were developed and purchased from SEARCH-LC GmbH (www.Search-LC.com). The PCR was performed with the LightCycler® FastStart DNA Sybr GreenI kit (RAS) according to the protocol provided in the parameter-specific kits. To control for specificity of the amplification products, a melting curve analysis was performed. The copy number was calculated from a standard curve, obtained by plotting known input concentrations of four different plasmids at log dilutions to the PCR-cycle number (CP) at which the detected fluorescence intensity reaches a fixed value.

To correct for differences in the content of mRNA, the calculated transcript numbers were normalized according to the expression of the housekeeping gene peptidylprolyl isomerase B (PPIB). Values were thus given as transcripts per 1000 transcripts of PPIB.

Isolation of Monocytes and Polymorphonuclear Neutrophils (PMN)

Monocytes and PMN were isolated from the peripheral blood of healthy donors according to the previously published protocol[29] (informed consent was obtained and the institutional guidelines were observed). The blood was drawn into heparin-coated tubes (Sarstedt, Nümbrecht, Germany) and centrifuged on PolymorphPrep (Axis-Shield, Oslo, Norway), which yields essentially two cell fractions separated according to their size and density. The neutrophil fraction was harvested, washed and resuspended in Hanks balanced salt solution, containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, Steinheim, Germany). This procedure yields more than 90% neutrophils as determined by flow cytometry determining expression of CD66. The mononuclear fraction was also harvested and monocytes were positively selected using anti-CD14 Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Generation of Osteoblasts

Bone marrow was collected intra-operatively from patients requiring an autologous bone graft due to fracture nonunion. Informed consent was obtained from all patients and the study was approved by the ethic committee of Heidelberg University. At the laboratory, the samples were then grinded with sterile scalpels and resuspended in osteoblast growth medium (PromoCell, Heidelberg, Germany) containing 0.5% penicillin/streptomycin (Gibco, Eggenstein, Germany). After cultivation for 4–8 days, an outgrowth of cells could be detected and the cells were then subcultivated after digestion with trypsin (0.05% Trypsin-EDTA, Life Technologies) for 5 min at 37°C. Once a homogenous cell layer could be seen (usually after 10–14 days), experiments were carried out as described below. Osteoblasts were used for a maximum of two passages.

Particles

To generate metallic abrasive wear particles, specimens of a Co29Cr6Mo alloy according to ISO 5832–12/ASTM F1537 were subjected to a wear test using a custom-made pin-on-plate simulator. As lubricant deionized water containing 35% v/v ethanol was used to prevent the formation of adherent endotoxins on the particles. In an earlier study, the current pin-on-plate setup including the lubricant has been found to produce particles which are comparable in size and morphology to clinically produced metal particles in joint revision.[30]

Stimulation of Neutrophils, Monocytes and Osteoblasts with Metal Particles, GroEL and LTA

In order to exclude a possible contamination of wear particles, a Limulus Amebocyte Lysate assay (Pierce LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher, Passau, Germany) was performed. No endotoxin could be detected. Metal wear particles were centrifuged twice for 5 min at 2000 rpm in a Vivaspin 20, 10.000 MWCO PES Ultrafiltration Unit (Sartorius, Göttingen, Germany), resuspended in RPMI medium (Gibco, Eggenstein, Germany) and counted microscopically. After isolation of monocytes (as described above), the cells were resuspended in RPMI containing foetal calf serum (10%), L-glutamine (1%) and

penicillin-streptomycin (1%) (all purchased from Gibco) and adjusted to a concentration of 3×10^6/mL/well using 24-well dishes (NuncTM, Wiesbaden, Germany). Neutrophils were also adjusted to a concentration of 3×10^6/mL/well in Aim 5 medium (Gibco, Eggenstein, Germany) containing inactivated, autologous serum. Osteoblasts were adjusted to a concentration of 3×10^4/mL/well in osteoblast growth medium (PromoCell, Heidelberg, Germany) containing 0.5% penicillin/streptomycin (Gibco Life, Eggenstein, Germany). The cells were stimulated with 1×10^6 metal wear particles, recombinant GroEL 0.1 µg/mL (Enzo Life Sciences, Loerrach, Germany), LTA 0.1 µg/mL (Sigma, Munich, Germany), and a combination of metal wear particles 1×10^6 with either GroEL 0.1 µg/mL or LTA 0.1 µg/mL together.

After 24 hours, cell supernatants were collected for ELISA analysis and cells were collected in lysis buffer for gene expression analysis.
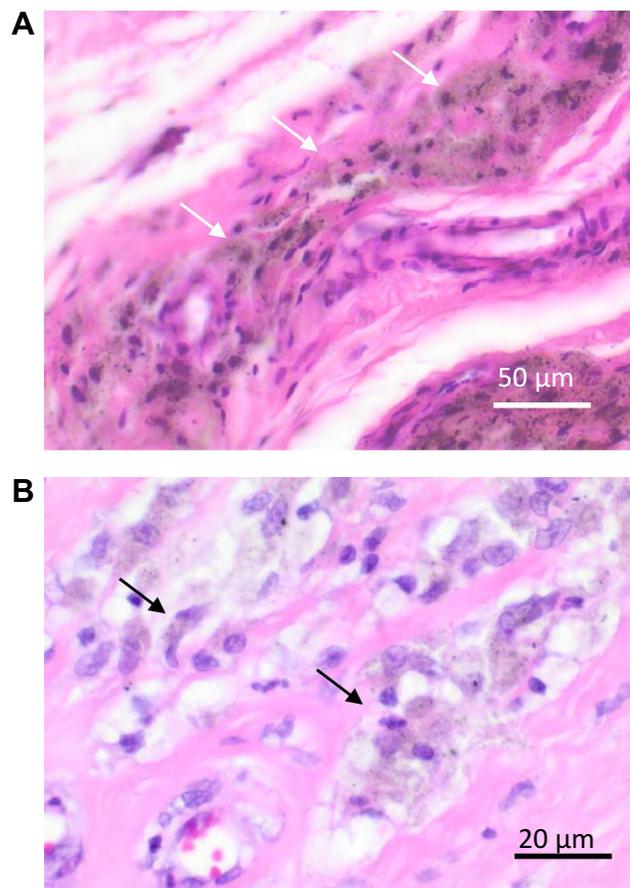
Figure 1 (**A** and **B**) Histopathology revealed high amounts of wear particles (white arrows) around the implant. Also, a high number of predominately mononuclear cells (black arrows) were detected.

ELISA

IL-8 in cell culture supernatants were determined using commercially available ELISA kits according to the protocol provided by the manufacturer. The human ELISA kits were purchased from R& D Systems (Minneapolis, USA).

Statistical Analysis

Differences between groups were calculated using Mann–Whitney test using GraphPad, Prism8 software. The significance level was determined as $p < 0.05$.

Results

Tissue samples were collected intra-operatively from 5 patients who underwent implant exchange surgery due to aseptic loosening and were evaluated by histopathology. High amounts of wear particles could be seen in direct vicinity to the implant, as well as a cellular infiltrate consisting mainly of mononuclear cells (Figure 1A and B).

Furthermore, gene expression analysis was performed from various tissue samples (intramedullary, cup, capsule, femoral) and compared to healthy muscle tissue. The pro-inflammatory cytokine IL-8 was evaluated (Figure 2). We were able to show that IL-8 was highly expressed in the surrounding tissue and gene expression of all samples (capsule, femoral, cup, intramedullary) differed significantly
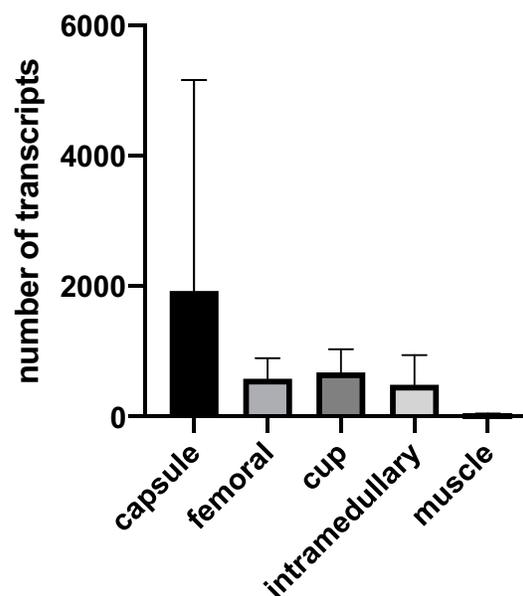
Figure 2 Gene expression of IL-8 in tissue samples. Tissue samples were collected intra-operatively in a standardized fashion (from the joint capsule; from the area of the femoral shaft and from the cup, as well as an intramedullary sample). Healthy muscle tissue served as control tissue. Gene expression analysis of IL-8 revealed significantly increased number of transcripts in all tissue samples when compared to muscle tissue (p<0.05; as calculated by Mann Whitney U-Test).

when compared to healthy control samples (muscle) (p<0.05, as calculated by Mann–Whitney *U*-test).

These results were followed up by in vitro analyses. Polymorphonuclear neutrophils (PMN) and monocytes were isolated from the peripheral blood of healthy donors (n=3). Osteoblasts were cultivated from human bone marrow, which was collected during surgery from patients (n=3) requiring an autologous bone graft. All three cell types were stimulated with metal wear particles. Additionally, minor amounts of lipoteichoic acid (LTA) and the bacterial heat shock protein GroEL were added to simulate a subclinical bacterial infection. The aim was to investigate a possibly enhanced reaction to wear particles in combination with bacterial products.

We were able to show that all three cell types released increased amounts of IL-8 after stimulation with wear particles, bacterial products and a combination thereof. The response of polymorphonuclear neutrophils was the least prominent (Figure 3A and B). Monocytes (Figure 4A and B) and osteoblasts (Figure 5A and B), however, showed a markedly increased release of the pro-inflammatory cytokine IL-8, particularly after stimulation with metal wear particles together with either GroEL or LTA. The experiments were repeated with cells of three different donors. Because the individual response of each donor was highly variable, unfortunately, no statistically significant differences could be detected (as calculated by Mann–Whitney *U*-Test). However, even though the individual response varied, each donor showed the same increased release of IL-8 after stimulation with wear particles or bacterial products (GroEL and LTA). The highest values for IL-8 were detected after stimulation with metal wear particles and bacterial products together. Each individual donor is additionally shown in Figures 4C and D and 5C and D.

Furthermore, the cells were collected for gene expression analysis. These experiments also demonstrated a high expression of IL-8 after stimulation with metal wear particles together with LTA or GroEL (Figures 6 and 7). The number of transcripts varied widely among donors, but the expression pattern was similar.

Discussion

In order to investigate the inflammatory response in patients suffering from aseptic implant loosening, we collected tissue samples during exchange surgery of metal-on-metal hip replacements. It has been described in the literature that some of these implants release a particular
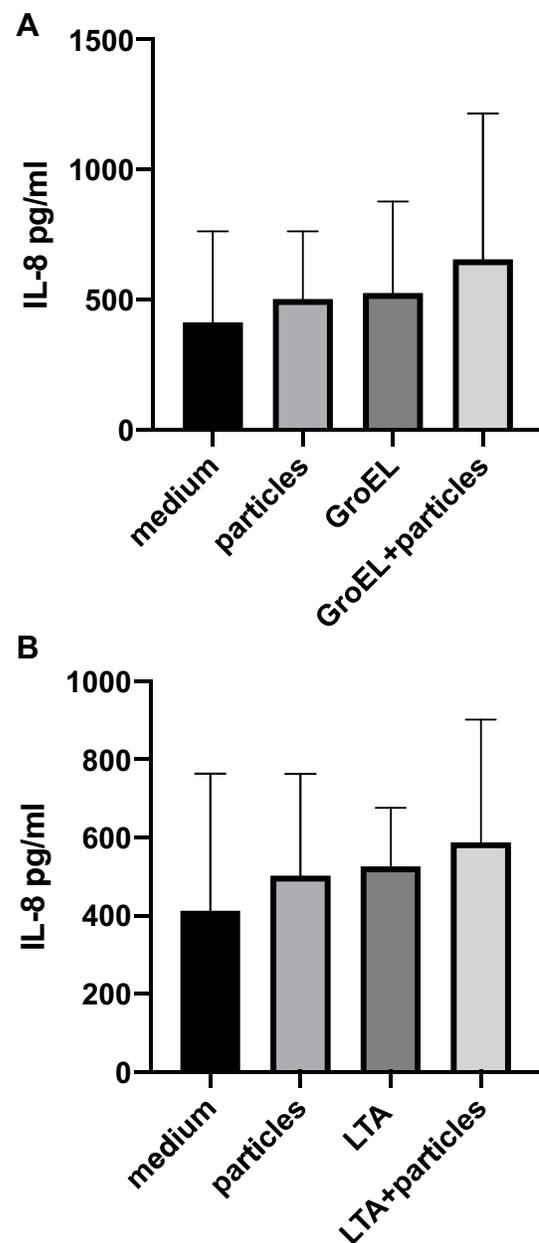

Figure 3 (**A** and **B**) polymorphonuclear neutrophils (PMN) were isolated from the peripheral blood of healthy donors. Release of the pro-inflammatory cytokine IL-8 was enhanced after stimulation with metal wear particles 1×10⁶, GroEL 0.1 μg/mL (**A**), LTA 0.1 μg/mL (**B**), and a combination of metal wear particles with either GroEL (**A**) or LTA (**B**). Mean values are shown.

high amount of wear particles, which causes adverse tissue reactions.[31–34]

Histopathology of tissue samples indeed revealed an impressive accumulation of wear particles surrounding the implant. Furthermore, predominately mononuclear cells could be detected in direct vicinity of the implant.

Monocytes are known to readily phagocytose particulate material, which leads to a release of various cytokines, whereby a pro-inflammatory peri-prosthetic environment
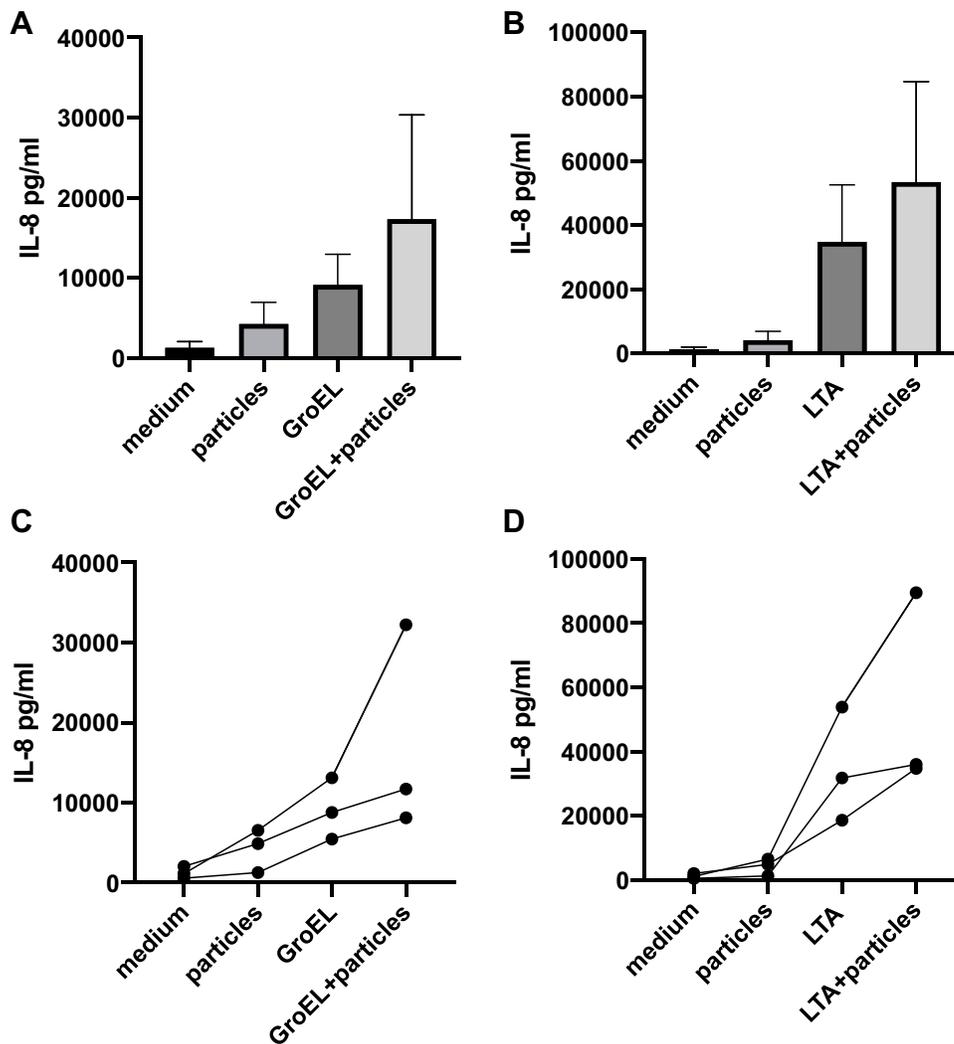
Figure 4 (**A–D**) Release of IL-8 by monocytes. Interleukin-8 release into the cell supernatant was increased after stimulation with metal wear particles 1×10⁶, GroEL 0.1 μg/mL (**A**), LTA 0.1 μg/mL (**B**) and in particular after stimulation with wear particles and bacterial products together (mean values are shown). The absolute numbers varied widely among the donors (p>0.05), but the stimulus-response pattern was similar. Therefore individual data of 3 donors are shown in (**C** and **D**).

is generated. This is followed by recruitment of more immune cells and as the inflammatory response persists, monocytes differentiate into osteoclasts, which causes bone degradation and further on implant loosening.[5,13]

Tissue samples were also collected for gene expression analysis and we were particularly interested in the multifunctional cytokine Interleukin-8, which promotes the inflammatory response by attracting and activating more immune cells. Furthermore, it has been shown in the literature that IL-8 is released by monocytes and directly induces osteoclast generation.[15,35] We were able to show that IL-8 was indeed highly expressed in various tissue samples from around the implant when compared to healthy muscle tissue.

These results were followed up by a series of in vitro experiments. Polymorphonuclear neutrophils, which are the first line of defense against bacterial infections[36,37] and monocytes were isolated from the blood of healthy donors. Monocytes in particular released increased amounts of IL-8 into the cell supernatant and showed enhanced expression of IL-8 as demonstrated by RT-PCR after stimulation with metal wear particles. The response was further enhanced after stimulation together with minor amounts of lipoteichoic acid (LTA) to simulate a subclinical infection. As expected, IL-8 release and the number of gene transcripts varied widely among each donor, which reflects inherent differences concerning responsiveness of leukocytes to irritants and their capacity
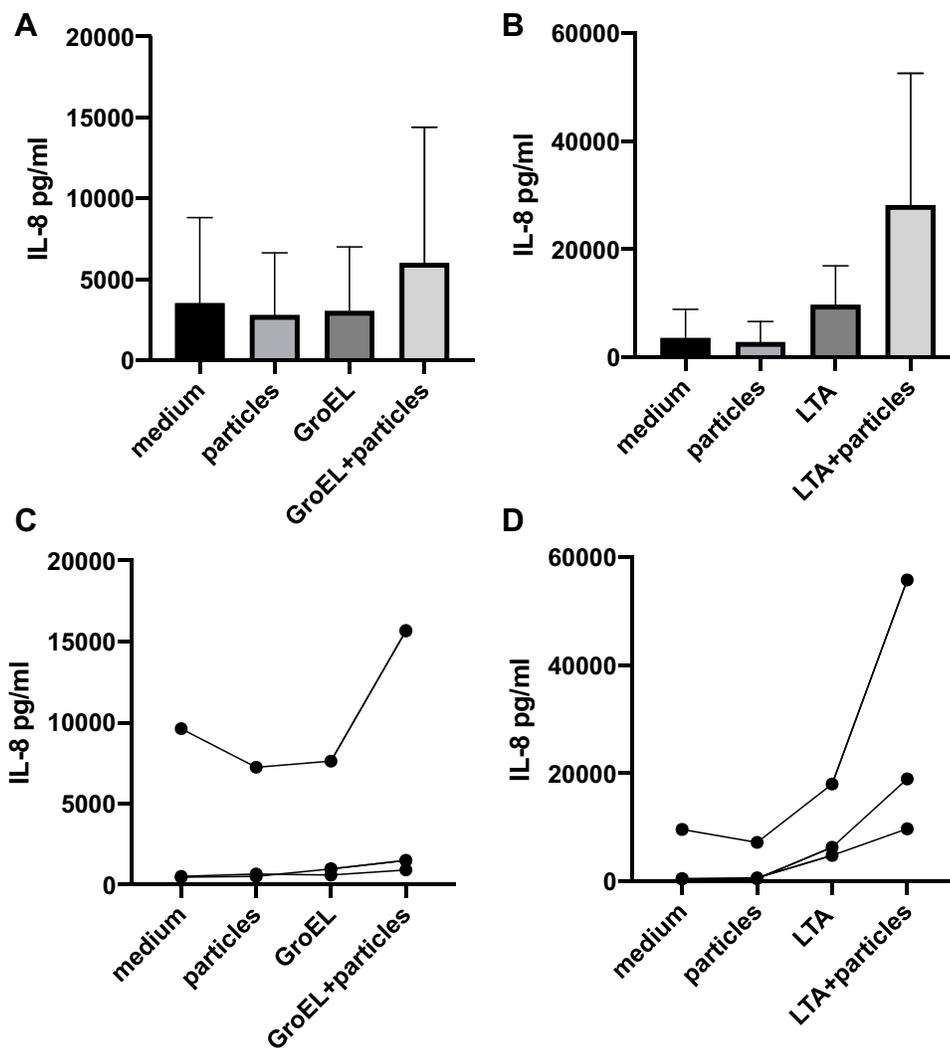
Figure 5 (**A–D**) Release of IL-8 by human osteoblasts. Osteoblasts were stimulated with metal wear particles 1×10⁶, GroEL 0.1 μg/mL (**A**), LTA 0.1 μg/mL (**B**), and a combination of wear particles and bacterial products together. An increased release of IL-8 could be detected (mean values are shown). The absolute numbers varied widely among the patients (p>0.05), but the stimulus-response pattern was similar. Therefore individual data of 3 patients are shown in (**C** and **D**).

to synthesize and release cytokines. However, each donor demonstrated an identical stimulus-response pattern, which was highest after stimulation with LTA and wear particles together. This is in line with data in the literature. Bi et al were able to show that adherent lipopolysaccharide (LPS)–which is a component of the cell membrane of gram-negative bacteria–on titanium particles induced osteoclast differentiation and an increased release of pro-inflammatory cytokines.[38]

In our previous work, we identified the bacterial heat shock protein GroEL as a component of the biofilm matrix, which is recognized by immune cells and induces an up-regulation of defence-relevant functions.[26] We were interested whether minor amounts of GroEL might also enhance the response to metal wear particles. Stimulation

with GroEL and wear particles together induced the highest response, similar to experiments with LTA. Since it has been reported in the literature, that most metal implants are colonized by bacterial biofilms,[18] the question arises whether a truly aseptic loosening even exists or whether the pro-inflammatory environment is triggered by both, wear particles and minor amounts of bacterial components.

Not merely immune cells, but also local tissue cells respond to an inflammatory stimulus. Osteoblasts, for example, are crucial in the context of bone turnover, but these cells also respond to a bacterial challenge by releasing pro-inflammatory cytokines such as Interleukin-8. Furthermore, osteoblasts produce defensin, a bactericidal molecule, which is typically associated with leukocytes and they can also acquire properties of antigen-presenting cells.[39–41]
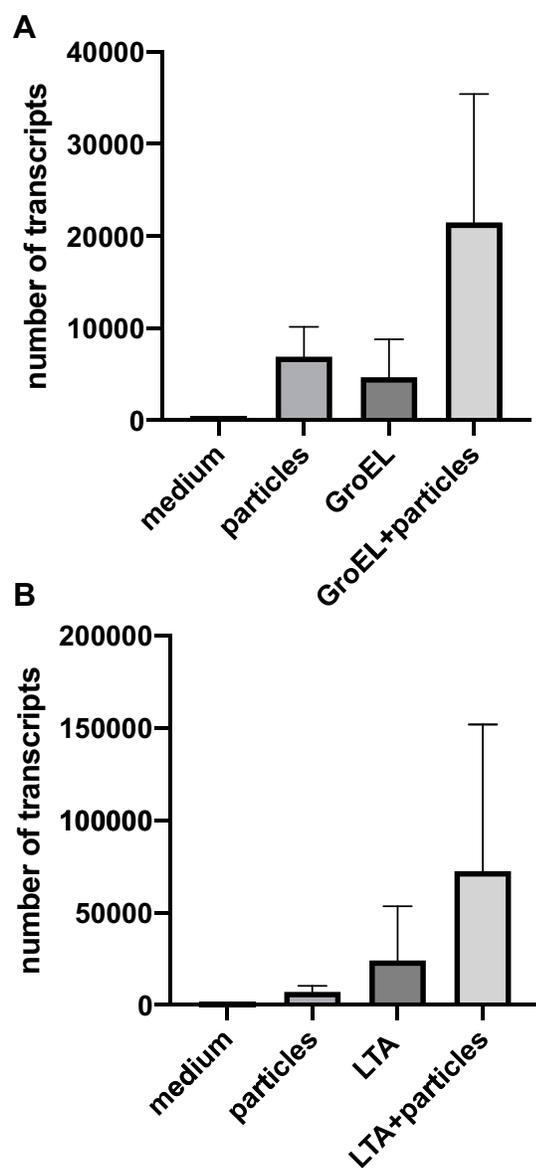
Figure 6 (**A** and **B**) Gene expression analysis of the pro-inflammatory cytokine IL-8 by monocytes by RT-PCR and quantified as number of transcripts. Mean values are shown. Expression of IL-8 was increased after stimulation with metal wear particles 1×10^6, GroEL 0.1 μg/mL (**A**), LTA 0.1 μg/mL (**B**) and in particular after stimulation with both, wear particles and bacterial products together. The number of transcripts varied widely among donors (p>0.05), but the expression pattern was similar.

Figure 7 (**A** and **B**) Gene expression analysis of the IL-8 by human osteoblasts by RT-PCR and quantified as number of transcripts. Mean values are shown. Expression of IL-8 was increased, particularly after stimulation with wear particles 1×10^6 and either GroEL 0.1 μg/mL (**A**) or LTA 0.1 μg/mL (**B**) together. The number of transcripts varied widely among donors (p>0.05), but the expression pattern was similar.

In the context of aseptic and/or infectious implant-loosening, we were able to demonstrate, that aside from mononuclear cells, also osteoblasts responded in a similar pattern to metal wear particles and components of a bacterial biofilm by increased expression and release of IL-8.

Since immune cells are not necessarily the first cells to encounter bacteria or wear particles, it seems plausible that local tissue cells also respond to these challenges and might therefore initiate or perpetuate an infl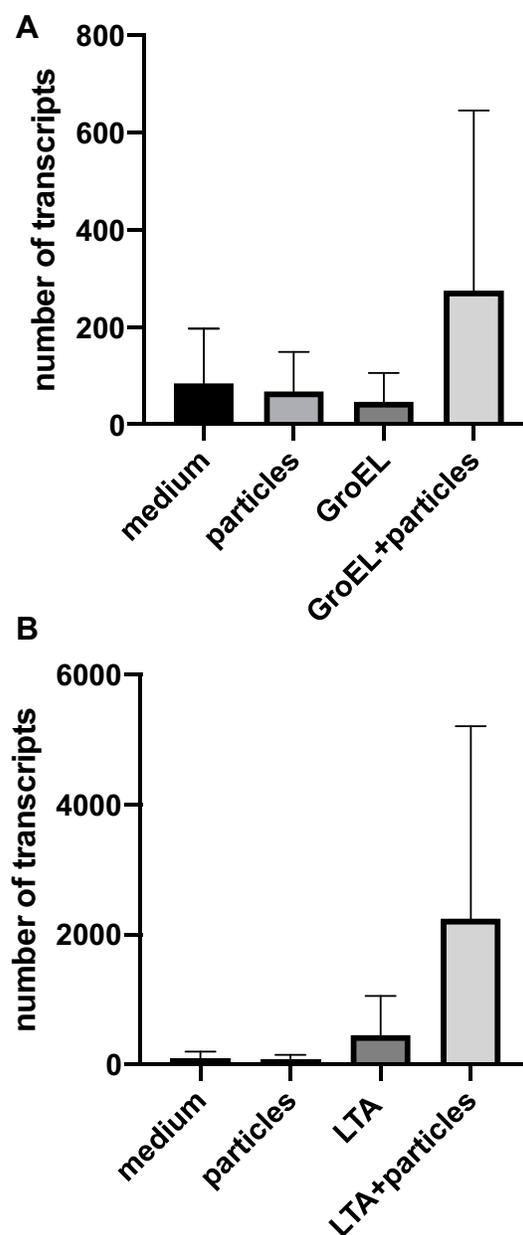ammatory response which leads to recruitment of immuno-competent cells, osteoclast generation and hence implant loosening.

Conclusion

Several excellent reports are available in medical literature, which demonstrate that aseptic loosening of implants is caused by an immune reaction directed towards wear particles.[42–44] Even though aseptic and infectious implant

loosening is considered to be two separate entities, we wondered whether subclinical infections might contribute to the inflammatory response directed against metal wear particles. Tissue samples of patients suffering from implant loosening (which represents the end result of a possibly longstanding inflammatory response) were collected intra-operatively and further on in vitro investigations were performed. We were able to demonstrate that metal wear particles induce an inflammatory response in human monocytes and osteoblasts, which indeed can be enhanced by minor amounts of bacterial biofilm components. Not only immune cells, but also local tissue cells could thereby contribute to an inflammatory response, which eventually results in bone degradation.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

Disclosure

Jan Philippe Kretzer reports grants from SpineServ, Endolab, IMA, Sintx, Aesculap AG, Peter Brehm Chirurgie-Mechanik e.K., Questmed, Permedica, Mathys, Falcon Medical, DOT, and Institut Straumann AG, and personal fees from Zentralstelle der Länder für Gesundheitsschutz bei Arzneimitteln und Medizinprodukten (ZLG), Arbeitsgemeinschaft Endoprothetik (AE), DePuy Synthes, AFOR Foundation, and Permedica, outside the submitted work. The authors report no other potential conflicts of interest in this work.

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