

Titanium induces proinflammatory and tissue-destructive responses in primary human macrophages

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Abstract

Implants and medical devices are efficient and practical therapeutic solutions for a multitude of pathologies. Titanium and titanium alloys are used in orthopedics, dentistry, and cardiology. Despite very good mechanical properties and corrosion resistance, titanium implants can fail due to inflammatory or tissue degradation-related complications. Macrophages are major immune cells that control acceptance of failure of the implant. In this study, for the first time, we have performed a systematic analysis of the response of differentially activated human macrophages, M(Control), M(IFN γ), and M(IL-4), to the polished and porous titanium surfaces in order to identify the detrimental effect of titanium leading to the tissue destruction and chronic inflammation. Transcriptome analysis revealed that the highest number of differences between titanium and control settings are found in M(IL-4) that model healing type of macrophages. Real-time quantitative polymerase chain reaction analysis confirmed that both polished and porous titanium affected expression of cytokines, chitinases/chitinase-like proteins, and matrix metalloproteinases (MMPs). Titanium-induced release and activation of MMP7 by macrophages was enhanced by fibroblasts in both juxtacrine and paracrine cell interaction models. Production of titanium-induced MMPs and cytokines associated with chronic inflammation was independent of the presence of *Staphylococcus aureus*. MMP7, one of the most pronounced tissue-destroying factors, and chitinase-like protein YKL-40 were expressed in CD68+ macrophages in peri-implant tissues of patients with orthopedic implants. In summary, we demonstrated that titanium induces proinflammatory and tissue-destructing responses mainly in healing macrophages, and the detrimental effects of titanium surfaces on implant-adjacent macrophages are independent on the bacterial contamination.

Keywords: chitinase-like protein, implant complications, macrophage activation, macrophages, MMP, titanium, transcriptomics

1. Introduction

Successful use of implants in medicine has been the reason for the increase in quality of life of millions of patients. Titanium is one of the main materials used in implantation. The main rationale for choosing titanium over other materials is its high corrosion resistance, excellent mechanical properties, low magnetic susceptibility, and biocompatibility.¹ Currently, titanium and its alloys are being used in a variety of medical applications such as dental implants, maxillofacial implants, femoral hip stems, artificial knee, spinal components and intramedullary nails, prosthetic heart valves, vascular stents, pacemakers, artificial larynx, and others.^{1–4}

Although titanium implants are generally well accepted by the host, they still carry a certain risk of implant failure. Common causes for implant failures are infection and aseptic loosening.^{5,6} While infection is often the reason for an early implant failure, aseptic loosening is the result of prolonged friction between articulating surfaces and high mechanical stress followed by a release of wear particles in the periprosthetic tissue.^{7,8} Recognition of the implant material or the released wear particles by the immune system as foreign bodies leads to the development of an inflammatory reaction. The main cells involved in initiation and coordination of the foreign body reaction are macrophages.⁹

Macrophages are multifunctional innate immune cells present in virtually all tissues. Due to their plasticity, they have a wide array of responses based on the microenvironmental signals. Thus,

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macrophages are able not only to recognize a foreign body and initiate an inflammatory reaction, but also to start a healing phase to re-instate homeostasis.¹⁰ The recognition of implants and initiation of the foreign body reaction by macrophages can occur through several mechanisms: (1) direct recognition of the material, (2) recognition of the protein layer adsorbed on the surface of the implant, and (3) phagocytosis.^{5,9,11} Several receptors, such as Fc gamma receptors (FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa), integrins (CD11B/CD18, αvβ3, αvβ5, α5β1), Toll-like receptors (TLRs) (TLR1, TLR2, TLR4), or scavenger receptors (SR-AI/II, MARCO) have been shown to be involved in foreign body recognition.¹² Activation of these receptors or phagocytosis leads to the activation of inflammatory signaling pathways such as nuclear factor κB (NF-κB), which trigger the release of proinflammatory cytokines (tumor necrosis factor α [TNFα], interleukin [IL]-1α, IL-1β, IL-6, IL-8, IL-11, IL-15), chemokines (CCL2, CCL3, CCL4, CCL8, CCL13), and growth factors (transforming growth factor α, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor [M-CSF], platelet-derived growth factor, epidermal growth factor).^{9,13–15} The released soluble factors attract other immune cells such as monocytes, lymphocytes, and neutrophils to the periprosthetic tissue, thus enhancing the inflammatory reaction.¹¹ Failure to resolve the inflammation can lead to the development of chronic inflammation, followed by formation of foreign body giant cells and, ultimately, to fibrous encapsulation of the implant.^{14,16}

The proinflammatory effect of titanium particles that mimic the implant debris was demonstrated in murine and human macrophage-like cell lines and primary murine bone marrow-derived macrophages. Vallés et al.¹⁷ demonstrated that titanium particles stimulate the secretion of TNFα, IL-1β, and IL-6 in THP-1 cells and in primary human macrophages. Similarly, Beidelschies et al.¹⁸ found that titanium particles enhance the expression of TNFα in RAW264.7 macrophages through the activation of ERK1/2/Egr-1 and NF-κB pathways. Yazdi et al.¹⁹ found that TiO₂ nanoparticles are able to activate the NLR pyrin domain containing 3 (Nlrp3) inflammasome and trigger secretion of IL-1β in mouse bone marrow-derived macrophages and THP-1 cells. Titanium nanoparticles have also been shown to promote secretion of CCL2 and CCL3 and increase production of reactive oxygen species in RAW264.7 macrophages.^{20,21} However, these studies do not shed light on the influence of titanium on macrophages in its bulk form, i.e., essential for the implant integration.

Macrophage reaction to titanium depends on number of factors. Response of primary human macrophages to titanium particles was shown to depend on their activation state.²² Human monocyte-like proliferating THP-1 cells cultured on titanium discs produced higher levels of TNFα, CCL2, CCL3, IL-1Ra when compared with titanium nanoparticles.²³ THP-1 cells respond to hydrophilic titanium surfaces by decreasing the expression and secretion of proinflammatory cytokines (TNFα, IL-1β, IL1R1, CCL1, CCL3, CXCL1).²⁴ Thus, the size of titanium particles as well as the contact with titanium surface can also influence macrophage foreign body reaction. However, to date, the reaction of primary human macrophage reaction on titanium surfaces was not systematically analyzed.

In the present study, for the first time we performed a systematic analysis of responses of different human primary macrophage subtypes, M(Control), M(IFNγ), and M(IL-4), to titanium surfaces. We found that M(IL-4), resembling the healing macrophage phenotype, has the strongest response to both polished and porous titanium surfaces. By using titanium microbeads and nanoparticles, we compared the reaction of primary human macrophages with titanium implant surfaces and to implant

debris. As bacterial contamination is a common problem with titanium-based implants, we examined whether bacterial infection can modulate macrophage responses to titanium implants and found that healing macrophages have a detrimental response to titanium in sterile conditions. Macrophage and peripheral blood mononuclear cell (PBMC) and macrophage and fibroblast coculture models were used to elucidate the effect of PBMCs and fibroblasts on macrophage reaction to titanium implants. We found that macrophages react to titanium independently on the cooperation with other cell types. Our study for the first time identifies the effect of titanium surface on the detrimental proinflammatory programming of primary macrophages that can explain the implant failure even on the later stages of the implantation.

2. Methods

2.1 Monocyte isolation and cultivation

Monocyte isolation and was performed as previously described.^{25,26} Briefly, buffy coats were diluted at a ratio of 1:1 with Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) (Biochrom). A total of 30 mL of diluted buffy coat was layered on top of the 15 mL of Biocoll solution (Biochrom) in a 50 mL falcon tube (Greiner). After a 30-min centrifugation in a swing out rotor (Hettich) at 420 *g* without breaks, the PBMCs were collected from the serum interphase. PBMCs were washed twice with PBS and layered on freshly prepared Percoll gradient. The Percoll gradient was prepared by mixing 13.5 mL Percoll (Pharmacia), 1.5 mL 10× Earle's Balanced Salt Solution (Sigma-Aldrich), and 15 mL of Minimal Essential Medium Eagle (Sigma-Aldrich). After a 30-min centrifugation at 420 *g*, the 2 upper layers were collected and 3 washing steps with PBS were performed. Collected cells were incubated with CD14 MicroBeads (Miltenyi Biotec) followed by a magnetic cell sorting step. Monocytes were cultured for 6 d at a concentration of 2 × 10⁶ cells/mL in macrophage serum-free medium SFM (Invitrogen) supplemented with 10⁻⁸ M dexamethasone (Sigma-Aldrich), 1 ng/mL M-CSF (PeproTech), and cytokines (as indicated): IL-4 at concentration of 10 ng/mL (PeproTech) and interferon γ (IFNγ) at concentration of 100 ng/mL (PeproTech).

2.2 Staining of tissue samples from patients

The Department of Orthopaedics and Trauma Surgery, University Medical Center Mannheim, University Heidelberg provided tissue samples of patients with orthopedic titanium implants. The study was approved by our Institutional Ethics Committee (file reference 2016-573N-MA). The tissue samples have been obtained during routine implant removal surgeries due to unclear/unspecific postoperative complications after osteosynthesis. Tissue samples have been embedded in Tissue-Tek (Sakura), and stored at -80 °C. Tissue samples were cut into sections of 5 μm thickness, fixed with acetone for 10 min at room temperature, and used for staining. Immunohistochemical staining was performed using primary antibodies: anti-human CD68 mouse monoclonal antibody (dilution 1:100; MSK055-05; Zytomed Systems); anti-YKL-40 guinea pig polyclonal antibody (dilution 1:500; PSL-010-2; generated in our lab); and anti-human matrix metalloproteinase 7 (MMP7) mouse monoclonal antibody (dilution 1:150; MAB9071; R&D Systems). Indirect immunofluorescent staining was performed first using primary antibodies: anti-CD68 rabbit monoclonal antibody (dilution 1:100; 76437; Cell Signaling Technology), a-YKL-40 guinea pig polyclonal antibody (dilution 1:500; PSL-010-2; generated in our lab), and anti-MMP7 rabbit polyclonal antibody (dilution 1:100; HPA051358; Sigma-Aldrich). Then, the following

secondary antibodies were used: Alexa Fluor 488–conjugated donkey anti-rabbit IgG (dilution 1:400; 711-545-152; Dianova), Alexa Fluor 488–conjugated donkey anti-guinea pig IgG (dilution 1:400; 706-545-148; Dianova); and Cy3-conjugated donkey anti-rabbit IgG (dilution 1:400; 711-165-152; Dianova). Nuclei were visualized with DRAQ5 (dilution 1:1000; 4084S; Cell Signaling Technology). Confocal microscopy analysis was performed using Leica Microsystems TCS SP8 laser scanning spectral confocal microscope in sequential scan modus.

2.3 Titanium samples

Medical-grade titanium discs of 13.7 × 2 mm size (both polished and porous) were autoclaved at 121 °C for 20 min at 18 psi. Medical-grade titanium microbeads (125 to 200 µm in size) were weighted in 1.5 mL Eppendorf tubes. A total of 45 mg of microbeads was sufficient to cover up to three-quarters of the area of a well (24-well plate). A total of 200 µL of 70% ethanol was added to each tube and incubated for 1 h. The beads were carefully washed twice with 400 µL sterile PBS. The beads were placed in the appropriate wells, and the plate was further sterilized under UV light for 1 h. Titanium discs and titanium microbeads (medical grade [grade 2] with 125 to 200 µm granulometry; Neyco) were provided by Protip Medical. TiO₂ nanoparticles (NanoAmor Europe) of 15 nm size were sterilized under UV light for 1 h. nTi particles were added in 0.0025 wt% per well.

2.4 Culture conditions for macrophage with PBMCs or fibroblasts

Isolated primary human monocytes and PBMCs were cultured at a concentration of 2×10^6 cells/mL in SFM medium (Invitrogen), supplemented with 10^{-8} M dexamethasone and 1 ng/mL M-CSF. Cells were seeded in 24-well plates. Three subtypes of monocytes were obtained by stimulation: M(Control), no additional stimulation; M(IFN γ), stimulated with 100 ng/mL; and M(IL-4), stimulated with 10 ng/mL IL-4. The same stimulations were used for PBMCs and PMBCs depleted of CD14⁺ monocytes (PBMC-CD14). Cells were cultured for 6 d.

To preserve the same conditions in all experiments, we used isolated primary human monocytes and BJ human foreskin fibroblasts (ATCC; kindly provided by Protip Medical). The coculture originally established, as described previously, demonstrated that BJ cells can survive in different cell culture media. In this study, BJ fibroblasts were cocultured in macrophage SFM medium supplemented with 10^{-8} M dexamethasone and 1 ng/mL M-CSF. The concentration of cells was 1×10^6 /mL for monocytes and 1×10^5 /mL for fibroblasts. Cells were seeded in 24-well plates, 1 mL/well. Monocytes were cocultured with BJ fibroblasts either directly in the same well (juxtacrine macrophage-fibroblast interaction [JMFI]) or in a Transwell system (0.4 µm pore size with PET track-etched membrane) (paracrine macrophage-fibroblast interaction [PMFI]). When cocultured in a Transwell system, 1 mL of monocyte suspension was added outside the Transwell insert (Sigma-Aldrich), while 10^5 fibroblasts were seeded inside the Transwell insert supplemented with 200 µL of culture medium. As controls, both monocytes and fibroblasts were cultured in monoculture. Additional stimulation with IFN γ and IL-4 was done as described previously. Cells were cultured for 6 d.

2.5 Activation of macrophages with heat-killed *Staphylococcus aureus*

Isolated primary human monocytes were cultured in SFM medium supplemented with 10^{-8} M dexamethasone and 1 ng/mL

M-CSF. Cells were seeded 24-well plates, 1.5 mL/well (2×10^6 cells/mL). Three subtypes of monocytes were obtained by stimulation: M(Control), no additional cytokines; M(IFN γ), stimulated with 100 ng/mL IFN γ ; and M(IL-4), stimulated with 10 ng/mL IL-4. 10^7 heat-killed *Staphylococcus aureus* (SA) cells per mL were used to additionally activate monocytes either immediately on day 0, or on the sixth day for next 24 h.

2.6 RNA isolation, complementary DNA synthesis, and real-time quantitative polymerase chain reaction

Total RNA was isolated using the E.Z.N.A. total RNA kit I (Omega Bio-tek) according to manufacturer's instructions. The RNA clean-up procedure was performed using an RNeasy Mini kit (Qiagen) according to manufacturer's instructions. For complementary DNA synthesis, the RevertAid H Minus First Strand Synthesis Kit (Thermo Fisher Scientific) was used according to manufacturer's instructions. The obtained complementary DNA was diluted 1:10 with double distilled water and 1 µL was used for real-time quantitative polymerase chain reaction (RT-qPCR).

Real-time qPCR analysis was performed using SensiMix II Probe No-ROX Kit (Bioline). The expression of CCL8 (Hs04187715_m1), CCL13 (Hs00234646_m1), CCL23 (Hs00270756_m1), CHI3L1 (Hs00609691_m1), CHIT1 (Hs00185753_m1), CSF1 (Hs00174164_m1), IL17RB (Hs00218889_m1), MMP8 (Hs01029060_m1), MMP9 (Hs00234579_m1), TNFSF14 (Hs00542477_m1), and TNFRSF21 (Hs01560899_m1) was analyzed using TaqMan Assays (Thermo Fisher Scientific). The following custom primers were used for the analysis of CCL18 expression: F2020 5'–ATA CCT CCT GGC AGA TTC CAC–3'; R2020 5'–GCT GAT GTA TTT CTG GAC CCA C–3'; and TaqMan probe Pr2020 FAM-CAA GCC AGG TGT CAT CCT CCT AAC CAA GAG AG-BHQ1. The following custom primers were used for the analysis of MMP7 expression: F2229 5'–CTT CCT GTA TGC TGC AAC TCA–3'; R2229 5'–GGG ATC TCC ATT TCC ATA GG–3'; and TaqMan probe Pr2229 FAM-TCC CAT ACC CAA AGA ATG GCC AA-BHQ1. For normalization the expression of GAPDH mRNA was measured using primers F848 5'–CAT CCA TGA CAA CTT TGG TAT CGT–3'; R848 5'–CAG TCT TCT GGG TGG CAG TGA–3'; and TaqMan probe Pr849 JOE-AAG GAC TCA TGA CCA CAG TCC ATG CC-BHQ1. Custom primers for qPCR were obtained from Eurofins. The experiments were performed using LightCycler 480 device (Roche). The following program was used: 95 °C for 10 min; followed by 50 cycles of 95 °C for 10 s and 60 °C for 60 s.

2.7 Affymetrix microarray analysis

RNA was tested by capillary electrophoresis on an Agilent 2100 bio-analyzer and high quality was confirmed. Hybridization of probes was done using arrays of human HuGene-1.0-st-type (Affymetrix). Biotinylated antisense complementary RNA was then prepared according to the Affymetrix standard labeling protocol with the GeneChip WT Plus Reagent Kit and the GeneChip Hybridization, Wash, and Stain Kit (both from Affymetrix). Afterward, the hybridization on the chip was performed on a GeneChip Hybridization oven 640, then dyed in the GeneChip Fluidics Station 450 and thereafter scanned with a GeneChip Scanner 3000. All of the equipment used was from Affymetrix. All the necessary procedures needed for hybridization and scanning of chips were performed in the Affymetrix Core Facility of Medical Research Center, Medical Faculty Mannheim. In pathway enrichment analysis, we applied the gene set of annotated pathways centered on myeloid innate immunity (open access on <https://nanosttring.com/resources/>

ncounter-hs-myeloid-innate-immunity-v2/), which underwent comprehensive enrichment analysis using gene set enrichment analysis (GSEA) software (v4.3.2). The GSEA results were visually represented with normalized enrichment scores (NES) displayed on a color scale that portrayed upregulation in red and downregulation in blue. Furthermore, pathway enrichment plots were generated to offer a holistic visualization of the biological implications.

2.8 Enzyme-linked immunosorbent assay

Concentration of human TNF α , IL-1 β , IL-1Ra, IL-6, CCL18, MMP7, and MMP9 in cell culture supernatants was measured using human DuoSet Elisa kits (R&D Systems) according to the manufacturer's recommendation. Samples were analyzed in duplicates and measured on a Tecan Infinite 200 microplate reader at 450 nm/570 nm wavelengths.

2.9 MMP activity assay

The concentrations of active MMP7 and MMP9 were determined using Human MMP7 (QZBmmp7H) and MMP9 (QZBMMP9H) activity assays (Quickzyme Biosciences) according to the manufacturer's recommendation. Samples were analyzed in duplicates and measured on a Tecan Infinite 200 microplate reader at 405 nm wavelength.

2.10 Statistical analysis

For statistical analysis of microarray data, a Custom CDF Version 19 with ENTREZ-based gene definitions was used to annotate the arrays. The raw fluorescence intensity values were normalized applying quantile normalization and RMA background correction. One-way analysis of variance was performed to identify differential expressed genes using a commercial software package SAS JMP10 Genomics version 6 (SAS Institute). A false positive rate of $\alpha = 0.05$ with false discovery rate correction was taken as the level of significance. Plots (3D Scatterplots and Venn diagrams) were made with JMP. Heat maps were generated in the Morpheus application from the Broad Institute. Gene families were selected from the Molecular Signatures Database from the Broad Institute. All statistical analysis for enzyme-linked immunosorbent assay (ELISA), RT-qPCR, and MMP activity assays was performed in GraphPad Prism 9 (GraphPad Software).

3. Results

3.1 Microarray analysis of macrophage responses to polished and porous titanium discs

Macrophages are key cells that induce immune reaction against foreign bodies. Previously, it has been shown that the surface of the implant can modulate macrophage immune response.²⁴ Macrophage reaction to titanium particles depends on their activation state.²² To analyze how specific subtypes of primary human macrophages react to titanium implants with different surfaces, macrophages were cultured on 2 types of medical-grade titanium: polished titanium discs as a model of bulk titanium implants (particularly dental implants) and porous titanium discs as a model of implants in which porosity is necessary for proper integration with the surrounding tissue (such as femoral or tracheal prostheses). As a control, macrophages were cultured on polystyrene cell culture plates. The transcriptome of macrophages differentiated for 6 d on the surfaces of polished and porous titanium discs was identified using Affymetrix microarrays.

Microarray analysis revealed that polished titanium discs altered the expression of 1,452 genes in M(IL-4), 214 genes in M(Control), and 150 genes in M(IFN γ) macrophages (Fig. 1A). Additionally, 14 genes were differentially regulated in both M(Control) and M(IFN γ), 98 genes in both M(Control) and M(IL-4), 21 genes in both M(IFN γ) and M(IL-4), and 7 genes across all 3 macrophage activation states (Fig. 1A). The differences in gene expression of macrophage subtypes cultured on polished titanium discs are visualized on a 3-dimensional (3D) scatterplot (Fig. 1B). Further, we focused on genes that are most likely to be involved in implant-related complications. A key role in these processes is attributed to mediators of inflammation and extracellular matrix remodeling.²⁷ Thus, genes from the cytokine-cytokine receptor, extracellular matrix disassembly, and Glyco_18 domain containing protein (chitinase-like proteins) families were analyzed in more detail. The expression of genes from these families with statistically significant change upon cultivation of M(Control), M(IFN γ), or M(IL-4) macrophages on polished titanium is illustrated as heatmaps (Fig. 1C–E). The full list of differentially expressed genes induced by polished titanium discs in M(Control), M(IFN γ), and M(IL-4) macrophages is provided as [Supplementary Table 1](#).

Similarly to the polished titanium, porous titanium induced differential expression of 3,560 genes in M(IL-4), 1,244 genes in M(Control), and 861 genes in M(IFN γ). A total of 37 genes were differentially regulated in both M(Control) and M(IFN γ), 713 genes in M(Control) and M(IL-4), 150 genes in M(IFN γ) and M(IL-4), and 59 genes across all 3 activation states of macrophages (Fig. 2A). The differences in gene expression of macrophage subtypes cultured on porous titanium discs are visualized on a 3D scatterplot (Fig. 2B). The expression of genes from the cytokine-cytokine receptor, extracellular matrix disassembly, and Glyco_18 domain containing protein (chitinase-like proteins) families with statistically significant change upon cultivation of M(Control), M(IFN γ), or M(IL-4) macrophages on porous titanium is illustrated as heatmaps (Fig. 2C–E). The full list of differentially expressed genes induced by porous titanium discs in M(Control), M(IFN γ), and M(IL-4) macrophages is provided as [Supplementary Table 2](#).

To further investigate the biological impact of titanium-induced differentially expressed genes, we performed GSEA, validating the gene screening results ([Supplementary Fig. 1](#)). Notably, the extracellular matrix remodeling pathway, with a central role played by the MMP family, showed significant upregulation in M(IL-4) in response to both polished and porous titanium discs ([Supplementary Fig. 2A, B](#)). We identified a consistent downregulation trend in the chemokine signaling pathway for macrophages culture on both polished and porous titanium discs ([Supplementary Fig. 2C, D](#)). Core genes within this pathway, such as CCL8 and CCL23, showed significantly decreased expression.

3.2 Confirmation of microarray results by RT-qPCR

To validate the results obtained from the microarray experiments, a selected list of genes from families of cytokine-cytokine receptors, extracellular matrix disassembly, and Glyco_18 domain containing protein (chitinase-like proteins) were further analyzed by RT-qPCR. The following genes were analyzed: CCL8, CCL13, CCL18, CCL23, CSF1, IL17RB, TNFRSF21, TNFSF14, MMP7, MMP8, MMP9, CHI3L1, and CHIT1. RT-qPCR analysis revealed that the expression of CCL8 was downregulated in M(Control) and M(IL-4) macrophages both on polished and on porous titanium discs (Figs. 3A and 4A). CCL13, CCL18, and CCL23 were downregulated only in M(IL-4) macrophages

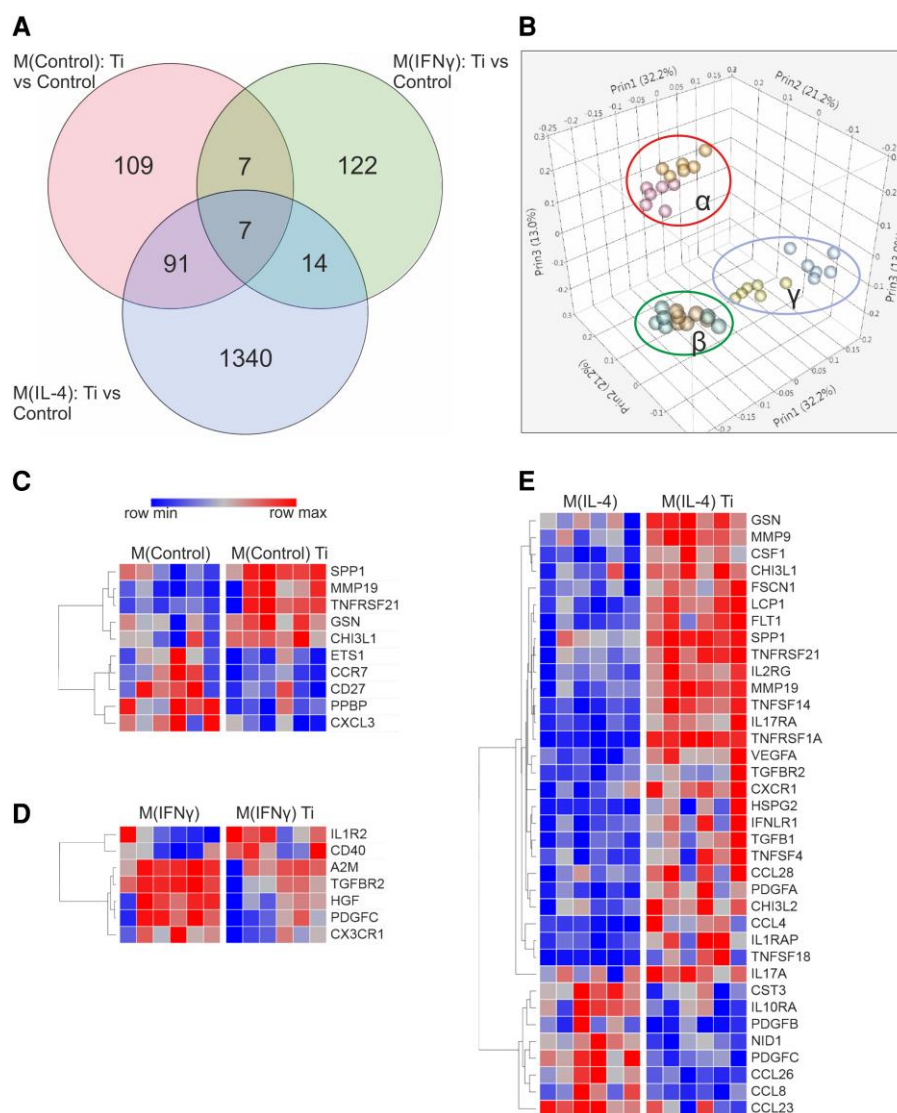


Fig. 1. Summary of microarray analysis of gene expression in macrophages cultured on polished titanium discs. (A) Venn diagram of differentially expressed genes in macrophages cultured with polished titanium vs control. Each number represents the number of genes differentially expressed in response to titanium. Each circle represents a population of macrophages with a different stimulation (red, control; green, IFN γ ; blue, IL-4). Intersecting points represent the number of genes differentially regulated by polished titanium in 2 or more stimulations. (B) Clustering of the microarray data in a 3D scatterplot. Each sphere represents genes from 1 donor. α indicates clustering of data from M(Control) (pink, control; orange, titanium); β indicates clustering of data from M(IFN γ) (green, control; brown, titanium); γ indicates clustering of data from M(IL-4) (yellow, control; cyan, titanium). (C–E) Heatmaps representing genes from cytokine-cytokine receptor, extracellular matrix disassembly, and Glyco_18 domain containing protein (chitinases and chitinase-like proteins) families with statistically significant difference in expression in macrophages in M(Control), M(IFN γ), and M(IL-4) cultured in the control wells and on polished titanium, respectively. Colors indicate relative expression of each gene between different stimulations in the presence or absence of polished titanium.

on both polished and porous titanium discs (Figs. 3B–D and 4B–D). Upregulation of CSF1 was observed in M(Control) and M(IL-4) macrophages cultured on polished titanium (Fig. 3E) and in M(Control) macrophages cultured on porous titanium (Fig. 4E). IL17RB was detectable only in M(IL-4) macrophages. Both polished and porous titanium induced downregulation of IL17RB (Figs. 3F and 4F). Polished titanium induced upregulation of TNFRSF21 in M(Control) macrophages, while TNFSF14 was upregulated in M(IL-4) macrophages (Fig. 3G, H). Similar tendencies in TNFRSF21 and TNFSF14 gene expression on porous titanium were observed (Fig. 4G, H).

RT-qPCR analysis also confirmed that titanium stimulates expression of genes involved in matrix disassembly. MMP7 was upregulated in M(Control) and M(IL-4) macrophages in porous and

in polished titanium discs (Figs. 3I and 4I). The expression of MMP8 was upregulated in M(IL-4) macrophages when cultured on porous titanium (Fig. 4J) but not on polished titanium (Fig. 3J). Both polished and porous titanium induced upregulation of MMP9 in M(IL-4) macrophages (Figs. 3K and 4K).

Expression of genes from the Glyco_18 domain containing protein family was also altered by titanium and confirmed by RT-qPCR. CHI3L1, a proinflammatory secreted protein induced by IFN γ in macrophages^{27–30} was upregulated in all 3 subtypes of macrophages cultured on polished titanium (Fig. 3L), while only in M(Control) and M(IL-4) macrophages when cultured on porous titanium (Fig. 4L). In contrast, expression of CHIT1 which hydrolytic activity has proinflammatory effect³¹ was upregulated in all 3 subtypes of macrophages cultured on porous

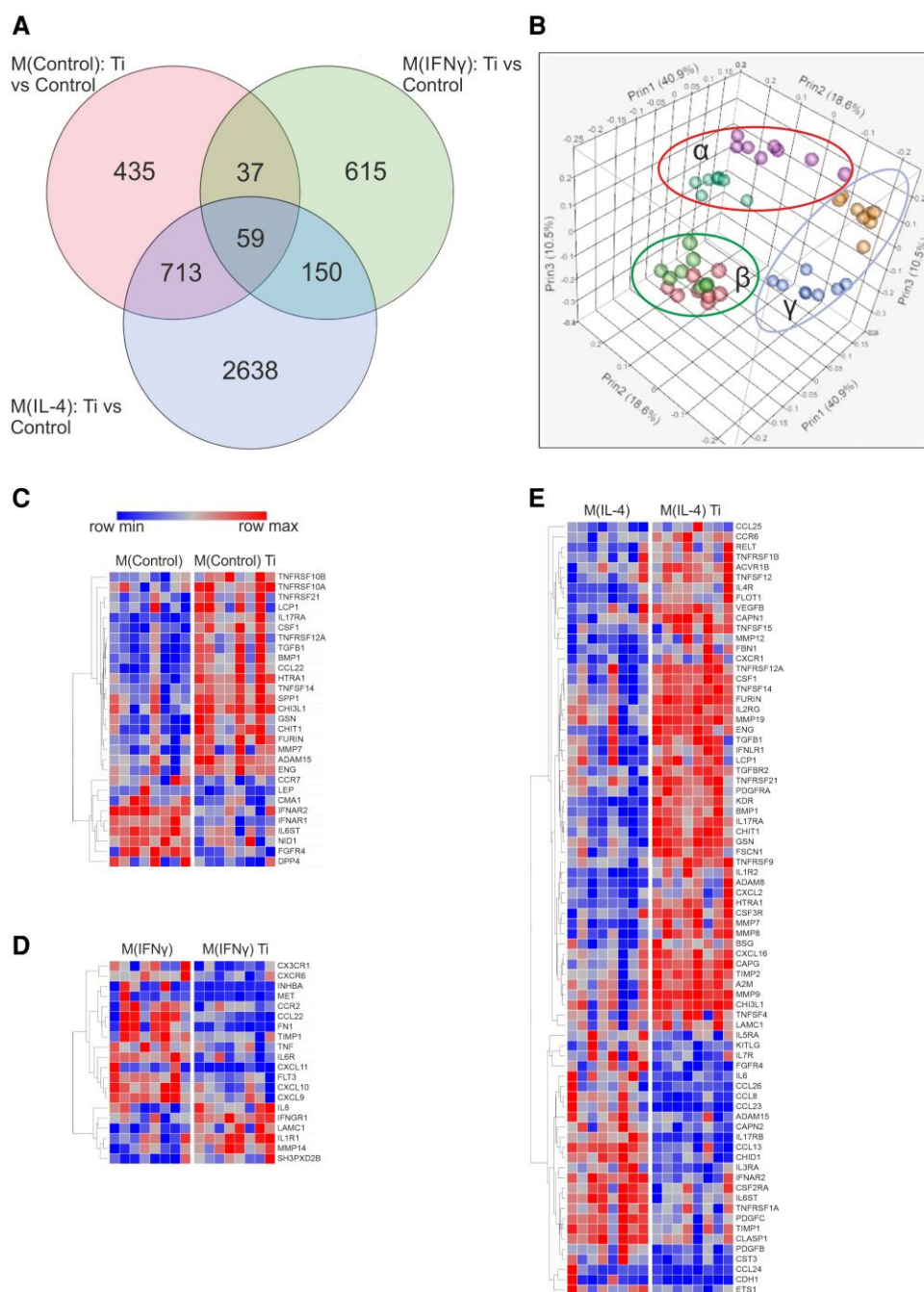


Fig. 2. Summary of microarray analysis of gene expression in macrophages cultured on porous titanium discs. (A) Venn diagram of differentially expressed genes in macrophages cultured with porous titanium vs control. Each number represents the number of genes differentially expressed in response to titanium. Each circle represents a population of macrophages with a different stimulation (red, control; green, IFN γ ; blue, IL-4). Intersecting points represent the number of genes differentially regulated by porous titanium in 2 or more stimulations. (B) Clustering of the microarray data in a 3D scatterplot. Each sphere represents genes from 1 donor. α indicates clustering of data from M(Control) (dark green, control; violet, titanium); β indicates clustering of data from M(IFN γ) (red, control; green, titanium); γ indicates clustering of data from M(IL-4) (blue, control; orange, titanium). (C–E) Heatmaps representing genes from cytokine-cytokine receptor, extracellular matrix disassembly, and Glyco_18 domain containing protein (chitinase-like proteins) families with statistically significant difference in expression between control and porous titanium conditions in the presence or absence of porous titanium.

titanium (Fig. 4M) but not on polished titanium (Fig. 3M). Because macrophage reactions to polished and porous titanium were similar, showing differences in the magnitude of gene expression levels and not in the direction of change (up- or downregulation), further experiments were conducted only using polished titanium discs.

3.3 Comparison of macrophage reaction to polished titanium discs, titanium microbeads, and nanoparticles

For THP-1 cells, reaction to the foreign body has been previously shown to depend not only on the surface physical and chemical properties of the material, but also on the size of the

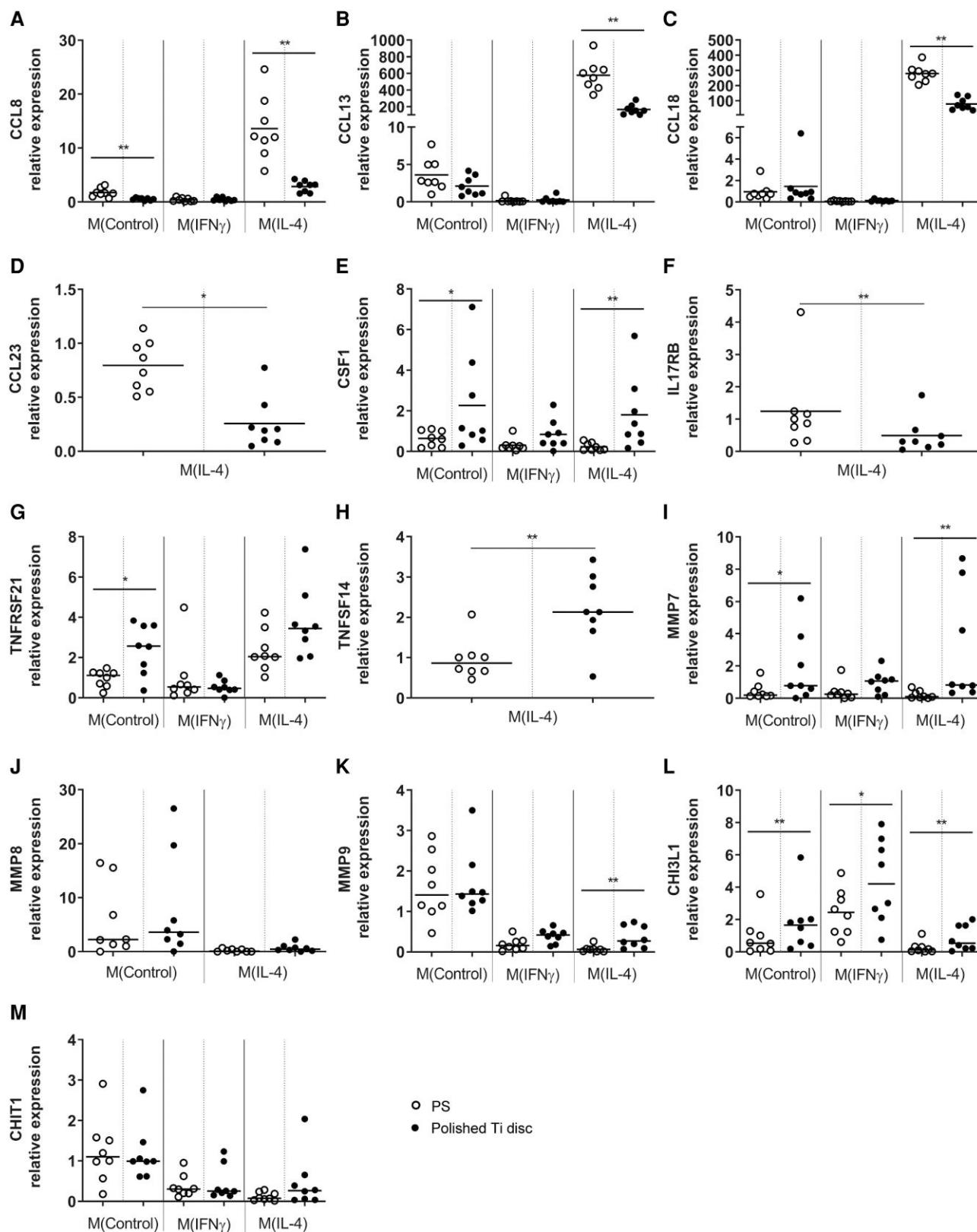


Fig. 3. RT-qPCR analysis of selected genes involved in inflammation and healing induced by polished titanium surfaces in the differentially activated macrophages. (A) CCL8 expression; (B) CCL13 expression; (C) CCL18 expression; (D) CCL23 expression; (E) CSF1 expression; (F) IL17RB expression; (G) TNFRSF21 expression; (H) TNFSF14 expression; (I) MMP7 expression; (J) MMP8 expression; (K) MMP9 expression; (L) CHI3L1 expression; (M) CHIT1 expression. All graphs represent the distribution of individual values from 8 donors, with the mean value represented by a horizontal line. (D, F, H, J) Only data for M(Control) and/or M(IL-4) are shown due to very low expression in other activation states (Ct > 40). For statistical analysis, the Wilcoxon matched-pairs rank test was used. *P < 0.05; **P < 0.01. PS = Plastic.

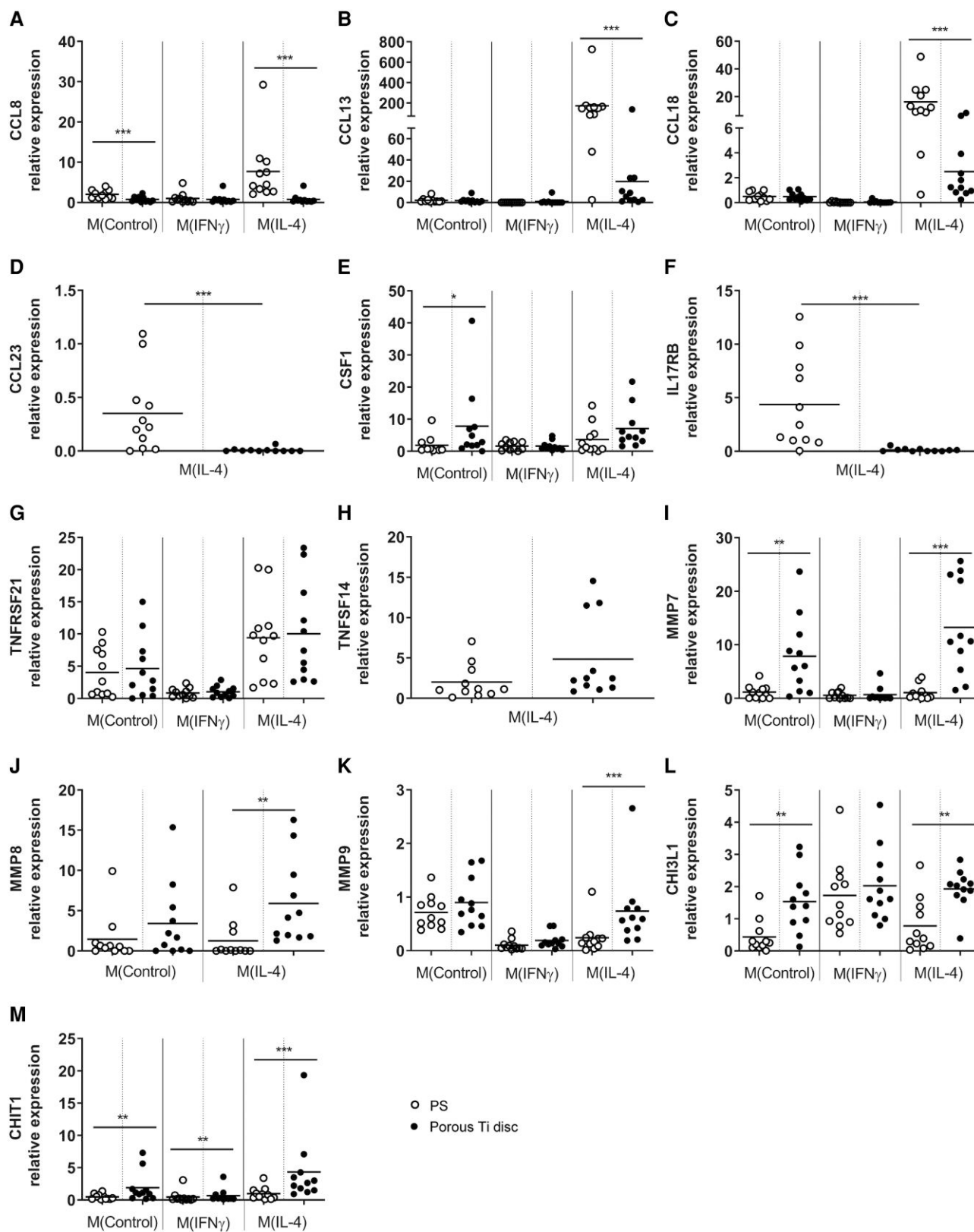


Fig. 4. RT-qPCR analysis of selected genes involved in inflammation and healing induced by porous titanium surfaces in the differentially activated macrophages. (A) CCL8 expression; (B) CCL13 expression; (C) CCL18 expression; (D) CCL23 expression; (E) CSF1 expression; (F) IL17RB expression; (G) TNFRSF21 expression; (H) TNFSF14 expression; (I) MMP7 expression; (J) MMP8 expression; (K) MMP9 expression; (L) CHI3L1 expression; (M) CHIT1 expression. All graphs represent the distribution of individual values from 11 donors with the mean values represented by horizontal lines. (D, F, H, J) Only data for M(Control) and/or M(IL-4) are shown due to very low expression in other activation states (Ct > 40). For statistical analysis, the Wilcoxon matched-pairs rank test was used. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. PS = Plastic.

material.^{23,24} To analyze whether primary human macrophages react differently on titanium surfaces and titanium particles (that mimics implant debris), macrophages were cultured on the surface of polished titanium discs (titanium discs), or in the presence of titanium microbeads (μ Ti) or titanium nanoparticles (nTi). Expression of genes that belong to families of cytokine-cytokine receptors, extracellular matrix components, and Glyco_18 domain containing proteins (chitinase and chitinase-like proteins) was analyzed by RT-qPCR. Major differences in the reaction of macrophages to nTi compared with titanium surfaces and to μ Ti have been found for the suppression of CCL8, CCL13, CCL23, and CCL18 expression in M(IL-4) (Fig. 5A–D), in which nTi had much more pronounced suppressive effect compared with titanium discs and microbeads. Titanium discs and μ Ti, but not nTi, suppressed expression of CSF1, a major macrophage growth and differentiation factor, in M(IFN γ) (Fig. 5E). In M(IL-4) titanium discs had the opposite effect and induced CSF1 expression (Fig. 5E). These data suggest that survival of healing M(IL-4) macrophages but not inflammatory macrophages is supported by titanium discs but not by nTi. Both titanium discs and nTi statistically significantly downregulated IL17RB (receptor for IL-17), while μ Ti also had a similar tendency; however, it did not reach statistical significance due to the high variability of individual reactions (Fig. 5F). No statistically significant effect in the expression of TNFSF14 was induced by any type of titanium materials compared with plastic control; however, nTi statistically significantly decreased the TNFSF14 expression in comparison with titanium discs (Fig. 5G).

To verify whether μ Ti or nTi can alter the expression of genes involved in matrix degradation found by us to be affected in M(IL-4) by titanium discs (Figs. 3 and 4), the expression of MMP7, MMP8, and MMP9 was analyzed by RT-qPCR. Due to notable individual variations, MMP7 and MMP8 did not show statistically significant induction by different titanium size variants (Fig. 5H, I). MMP9 was the main MMP in which expression was affected by the size of titanium. In M(IL-4), titanium discs upregulated MMP9 expression in comparison with plastic as well as with μ Ti and nTi (Fig. 5J). However, μ Ti also had stimulatory effect on MMP9 compared with plastic, while nTi still had a tendency for MMP9 expression stimulation but not statistically significantly so (Fig. 5J). Such stimulatory effect of Ti was profound only in healing macrophages M(IL-4), while in M(Control) and in M(IFN γ) nTi had even suppressive effect on the expression of MMP9 in comparison to cell culture plastic (Fig. 5J).

The size of titanium materials also influenced the expression of genes from the Glyco_18 domain containing protein family. In M(IFN γ), only nTi were able to suppress expression of CHI3L1 (YKL-40), a chitinase-like protein that can induce angiogenesis and cell proliferation (Fig. 5K). In M(IL-4), titanium discs and nTi, but not μ Ti, enhanced expression of CHI3L1 (Fig. 5K). Very high individual variability was found for the expression of enzymatically active chitinase CHIT1 involved in antipathogen defense. In case of M(Control) and M(IFN γ), no statistically significant effect of titanium discs or particles was detected (Fig. 5L). However, in case of M(IL-4), titanium disc and μ Ti had statistically significant stimulatory effect on CHIT1 expression (Fig. 5L).

In summary, both titanium surface and particles affected mostly M(IL-4) macrophages. Titanium discs had most pronounced effect on the tissue-distractive functions of macrophages, while nTi modulated the cytokine and chitinase profile involved in the macrophage defense functions. Such effects of implant surface and debris can explain loosening of implant several months after installation.

3.4 Effects of bacteria and titanium on macrophage cytokine secretion

Infection followed by biofilm formation on the surface of the implants is one of the major reasons of implant failure.⁶ SA is a common pathogen involved in metallic implant failures both in orthopedics and in dentistry.^{32,33} To determine whether bacteria contaminating orthopedic titanium implants can influence the secretion of inflammatory cytokines by macrophages in case of the contact with titanium surfaces, mature macrophages cultured on polished titanium discs with heat-killed SA. The secretion of proinflammatory cytokines (TNF α , IL-1 β , IL-6, and IL-8) and anti-inflammatory cytokine (CCL18) in response to SA stimulation was measured by ELISA. Secretion of TNF α was stimulated in M(IFN γ) macrophages both by titanium and SA. The effect was stronger when titanium and SA were used in combination, indicating that acute SA-induced inflammation is enhanced on the titanium implant surfaces (Fig. 6A). Secretion of IL-1 β was not significantly changed by SA regardless of macrophage activation state and stimulation with titanium (Fig. 6B). Release of IL-6 was strongly enhanced by SA in M(IFN γ), and this effect was suppressed by titanium. Nonetheless in M(Control) and M(IL-4), the combination of titanium and SA stimulated IL-6 production (Fig. 6C). Secretion of IL-8 was increased by SA in M(IFN γ) macrophages; however, no additional effect of titanium was found (Fig. 6D). To investigate whether SA exacerbates the inflammatory reaction by reducing M2-type cytokines, we analyzed the secretion of CCL18 under SA stimulation. As expected, the highest levels of CCL18 were detected in M(IL-4). SA stimulation alone did not decrease the secretion of CCL18, while a slight decrease was observed when SA was combined with titanium. No significant differences for CCL18 production were observed between titanium alone and in combination with SA (Fig. 6E).

Matrix degradation and tissue remodeling is a key mechanism of implant loosening.²⁸ Thus, we decided to analyze whether SA can support secretion of matrix remodeling molecules and measured the secretion of MMP7 and MMP9 by ELISA. SA stimulation alone did not induce higher levels of MMP7 or MMP9, regardless of the activation state of macrophages. In combination with titanium, higher levels of MMP7 and MMP9 were detected in M(Control) and M(IL-4) macrophages; however, no significant difference between titanium alone and SA in combination with titanium was found (Fig. 6F, G).

3.5 The effect of PBMCs and fibroblasts on macrophage MMP7 and MMP9 secretion and activity

Aseptic loosening is one of the main reasons for implant failure. A pivotal role in the development of aseptic loosening is attributed to matrix metalloproteinases, which lead to matrix degradation and tissue remodeling.²⁸ We have shown that both polished and porous titanium upregulate the expression of MMP7 and MMP9 in macrophages (Figs. 3I, 3K, 4I, and 4K). The next step was to examine whether the same patterns of expression can be observed on the protein level. Our results clearly show that polished titanium induces elevated secretion of both MMP7 and MMP9 in M(Control) and M(IL-4) macrophages (Fig. 7A, B).

MMPs require additional activation to induce their enzymatic activity.³⁴ Thus, we examined whether titanium can stimulate MMP7 and MMP9 activation. Our analysis revealed that the concentration of active MMP7, under titanium stimulation, was significantly elevated in M(Control), with similar effects in M(IL-4)

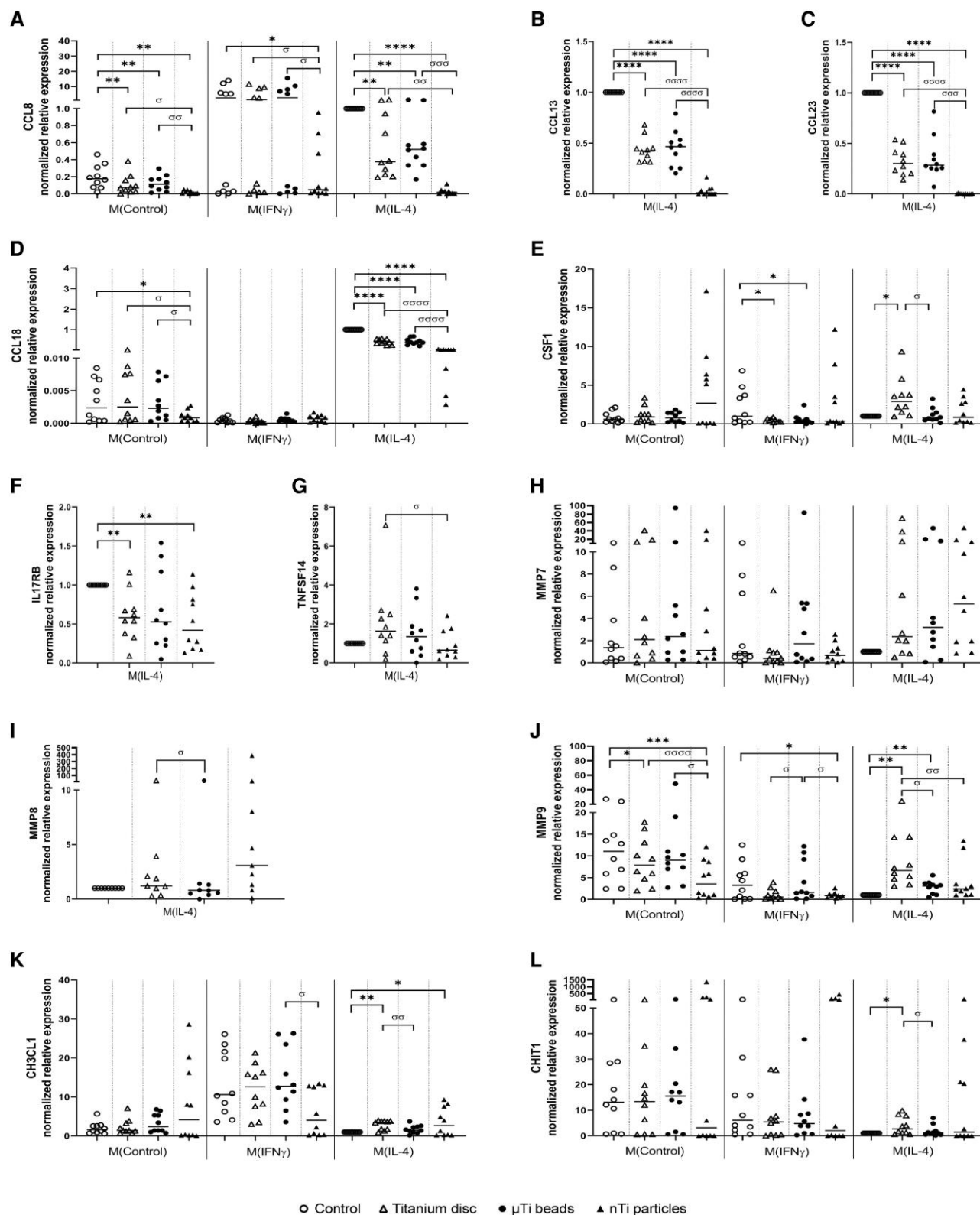


Fig. 5. RT-qPCR analysis of selected genes involved in inflammation and healing induced by ti discs, ti microbeads and ti nanoparticles in differentially activated macrophages. (A) CCL8 expression; (B) CCL13 expression; (C) CCL23 expression; (D) CCL18 expression; (E) CSF1 expression; (F) IL17RB expression; (G) TNFSF14 expression; (H) MMP7 expression; (I) MMP8 expression; (J) MMP9 expression; (K) CHI3L1 expression; (L) CHIT1 expression. All figures except for I ($n = 9$) represent the distribution of individual normalized values from 10 donors. (B, C, F, G, I) Only data for M(IL-4) are shown due to very low expression in other activation states ($Ct > 40$). The mean values were represented by horizontal lines. The data were normalized by setting the expression of M(IL-4) from each donor to 1. For statistical analysis, the 2-tailed paired t test was used. The asterisk indicates the statistical significance between different types of titanium materials and plastics of the respective activation state (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001), while σ denotes the statistical significance between different types of titanium materials (σP < 0.05; $\sigma\sigma P$ < 0.01; $\sigma\sigma\sigma P$ < 0.001; $\sigma\sigma\sigma\sigma P$ < 0.0001). PS = Plastic.

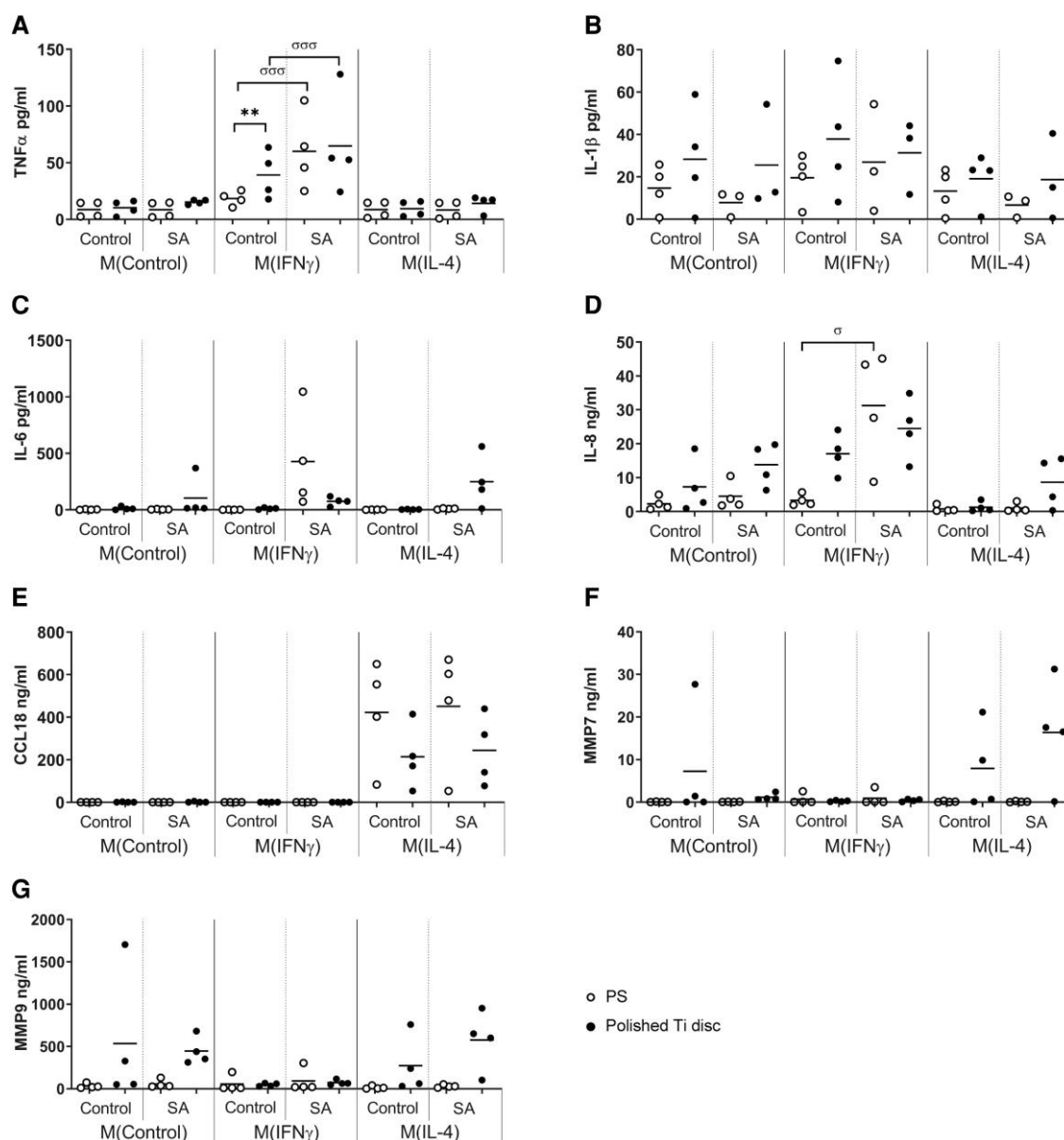


Fig. 6. Macrophage cytokine secretion upon stimulation with SA in the presence of titanium. SA was added to mature macrophages on day 6, and cells were cultured for additional 24 h. Protein concentrations were measured by ELISA on day 7 of culture. (A) TNF α secretion; (B) IL-1 β secretion; (C) IL-6 secretion; (D) IL-8 secretion; (E) CCL18 secretion; (F) MMP7 secretion; (G) MMP9 secretion. All graphs represent the distribution of individual values from 4 individual donors with the mean values represented by horizontal lines. For statistical analysis, the 2-way analysis of variance and Holm-Sidak post hoc test was used. The asterisk indicates the statistical significance between titanium and plastic of the respective activation state (** $P < 0.01$), while σ denotes the statistical significance between control and SA on same materials ($\sigma P < 0.05$; $\sigma\sigma P < 0.001$). PS = Plastic.

macrophages (Fig. 7C). Surprisingly, the concentration of active MMP9 was undetectable (data not shown).

In order to examine whether other cellular components of the periprosthetic tissue can induce MMP7 and MMP9 activation in the presence of titanium model culture systems that included macrophages + other PBMCs and macrophages + fibroblasts were used. We found that macrophages are the main fraction of PBMCs responsible for the secretion of both MMP7 and MMP9 in response to titanium. The fraction of PBMCs depleted of CD14 $^{+}$ cells (PBMC-CD14) secreted low amounts of MMP7 and MMP9 that did not vary between titanium and plastic conditions, while the full fraction of PBMCs secreted significantly higher amounts of MMP7 and MMP9 when cultured with titanium in both M(Control) and M(IL-4) stimulations (Fig. 7D, F). A statistically significant increase in the enzymatically active MMP7 was identified in the full fraction

of PBMCs in response to polished titanium (Fig. 7E). In contrast, no statistically significant changes were observed when analyzing concentration of active MMP9 (Fig. 7G). Collectively, these results show that PBMCs greatly enhance macrophage reaction to titanium. However, the concentration of active MMPs in comparison with the total amounts of MMPs secreted by PBMCs cultured on titanium surfaces is still below 10%, suggesting that other cells in the periprosthetic tissue can be responsible for their activation.

Because fibroblasts are pivotal in extracellular matrix deposition and remodeling, we analyzed whether fibroblasts alone or in coculture with macrophages are able to secrete and activate MMP7 and MMP9 upon stimulation with titanium. In order to distinguish between JMFI and PMFI, a Transwell system was used. In the JMFI model, titanium discs significantly upregulated MMP7 secretion in M(Control) compared with the plastic control (Fig. 7H). Titanium

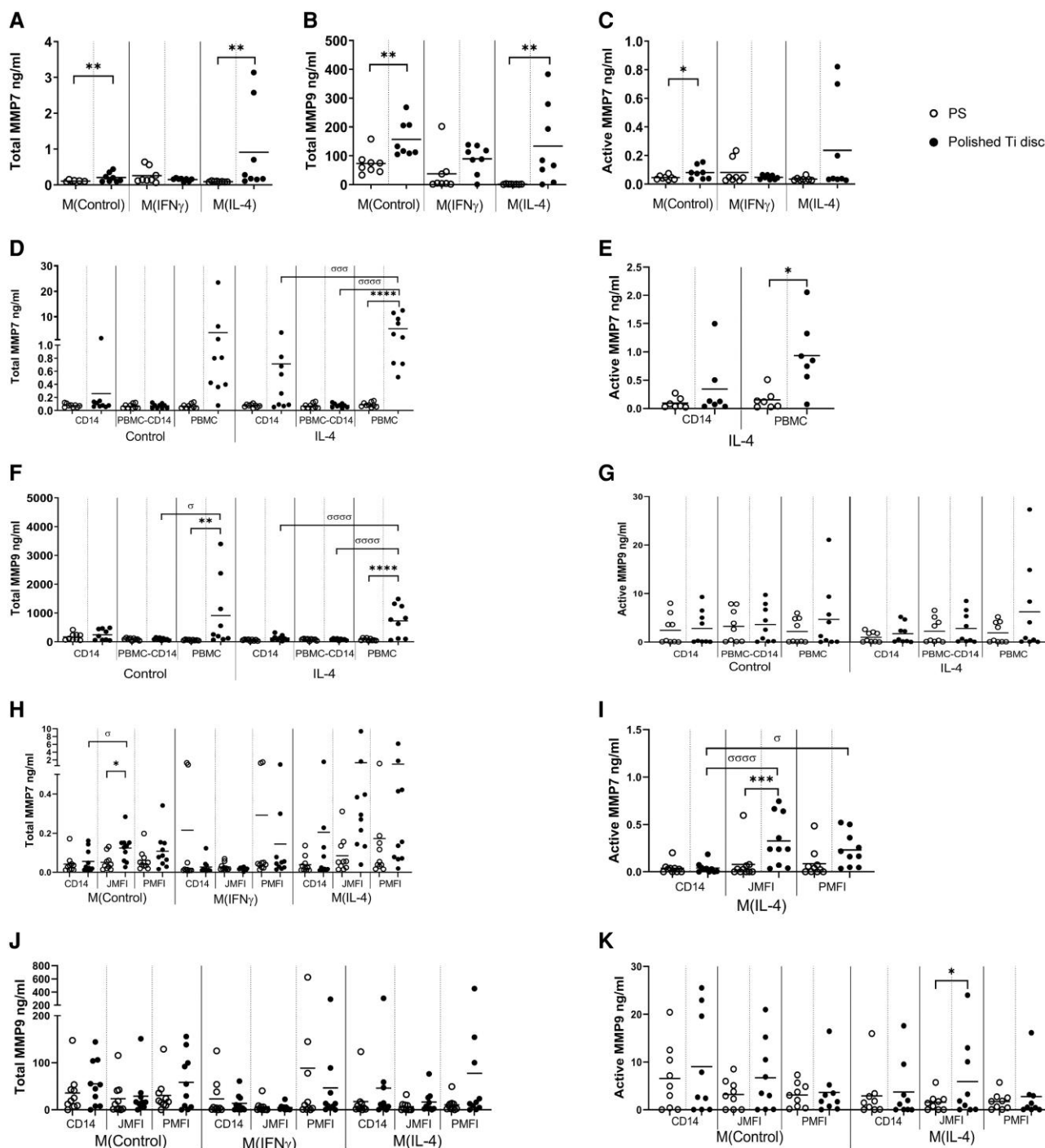


Fig. 7. The effect of titanium, PBMCs, and fibroblasts on macrophage MMP7 and MMP9 secretion and activity. (A, C) Titanium's effect on macrophage total and active MMP7 secretion; (B) Titanium's effect on macrophage total MMP9 secretion; (D–G) Effect of titanium and PBMCs on macrophage total and active MMP7 and MMP9 secretion; (H–K) Effect of titanium and fibroblasts on macrophage total and active MMP7 and MMP9 secretion. Graphs represent individual values from 10 (H–J), 9 (D, F, G, K), 8 (A–C), or 7 (E) donors with mean values represented by horizontal lines. For statistical analysis, a 1-way analysis of variance with post hoc Tukey test was used. The asterisk denotes statistical significance between titanium and control of the respective activation state (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$), while σ denotes statistical significance between different types of cell populations ($\sigma P < 0.05$; $\sigma\sigma P < 0.001$; $\sigma\sigma\sigma P < 0.0001$). PS = Plastic.

discs also induced significantly higher levels of MMP7 in M(Control) in the JMFI model compared with macrophages only (without fibroblasts) stimulated with titanium (Fig. 7H). MMP7 activity in M(IL-4) in the JMFI model was significantly enhanced by titanium compared with the plastic control (Fig. 7I). Furthermore, in M(IL-4) titanium induced significantly higher levels of active MMP7 in both the JMFI and

PMFI models compared with macrophages in monoculture (Fig. 7J). Due to the high variation between individual donors, no statistically differences in total secreted levels of MMP9 induced by titanium were found among monoculture and macrophage-fibroblast interaction models (Fig. 7J). However, in case of M(IL-4), in the JMFI model titanium triggered a significantly higher levels of enzymatically

Table 1. Expression of YKL-40 and MMP7 in CD68+ macrophages in tissues of patients with orthopedic implants.

Patient	Sex	Age (yr)	Localization of the implant	Time of postoperative implant removal	IF/confocal			
					YKL-40		MMP7	
					Adjacent	Distant	Adjacent	Distant
P1	Male	46	Clavicula/plate and screws	18 mo	+	++	++	+
P2	Male	50	Clavicula/plate and screws	4 mo	++	++	+	++
P3	Male	30	Proximal tibia/plate and screws	36 mo	—	—	—	+
P4	Male	49	Clavicula/plate and screws	14 mo	—	+	+	—
P5	Female	41	Proximal tibia/plate and screws	16 mo	—	—	+	++
P6	Male	28	Distal tibial shaftplate and screws	12 mo	++	++	++	++
P7	Male	40	Clavicula/plate and screws	3 mo	++	++	++	++
P8	Female	63	Distal fibula/ankle plate and screws	11 mo	+	—	+	+

+ indicates a single positive cell in the field; ++ indicates 2 to 5 positive cells in the field; +++ indicates more than 5 positive cells in the field.

active MMP9 compared with the plastic control (Fig. 7K). In summary, these results suggest that fibroblasts in periprosthetic tissue not only significantly enhance the secretion of MMP7 and MMP9 in healing macrophages, but also contribute to the activation of MMPs, particularly when macrophages and fibroblasts directly interact.

3.6 MMP7 and YKL-40 are expressed in peri-implant macrophages in patients with orthopedic titanium implants

Next, we analyzed whether inflammation-associated and tissue-distractive markers induced by titanium in human macrophages can be identified also in tissues of patients with titanium implants. We have selected YKL-40 as chronic inflammation-associated factor, and MMP7, as major tissue remodeling factor with broad spectrum of substrates in the extracellular microenvironment. In total, we analyzed tissue samples obtained from 8 patients with various orthopedic titanium implants (Table 1). CD68, YKL-40, and MMP7 were assessed by immunohistology for single markers (Fig. 8). CD68+ macrophages were abundant in the peri-implant tissues. Single YKL-40+ and MMP7+ cells we also identified in macrophage rich areas. Next, expression of YKL-40 and MMP7 in CD68+ cells was detected by immunofluorescent staining and confocal microscopy (Figs. 9 and 10). In 6 of 8 patients, YKL-40 was found in CD68+ macrophages (Table 1). In all 8 patients, MMP7 was expressed in CD68+ cells (Table 1). Thus, macrophages localized in close proximity to the titanium implant in patients express the markers of M2 macrophage response to titanium surfaces identified by Affymetrix chip analysis.

4. Discussion

Titanium is one of the most widely used implant materials and offers excellent mechanical properties and high biocompatibility.¹ However, even titanium implants carry a certain risk of implant failure, mainly due to infection or aseptic loosening.^{5,6} Release of wear particles in the periprosthetic tissue coupled with their recognition as foreign bodies by the immune system leads to the development of chronic inflammatory reactions.^{7–9} It is widely recognized that macrophages are essential cells involved in the recognition of foreign bodies and initiation of the foreign body reaction.^{12,35} A number of research groups have focused on studying macrophage reaction toward titanium, mainly in RAW264.7 cells,³⁶ THP-1 cells,¹⁷ and mouse bone marrow-derived macrophages.¹⁸ Because mouse bone marrow-derived macrophages, THP-1 cells, and human peripheral blood monocyte-derived

macrophages have a different gene expression and cytokine production profile,^{37,38} to better understand the macrophage-titanium interaction in human tissues, in this study human peripheral blood monocyte-derived macrophages were used. To simulate macrophage microenvironmental-dependent phenotypic plasticity,¹⁰ as well as distinguish between reactions of macrophages in different activation states, 3 distinct macrophage activation states were induced: M(Control) (no additional stimulation), M(IFN γ) (stimulation with IFN γ), and M(IL-4) (stimulation with IL-4). Both the surface and size of biomaterial can significantly alter macrophage reaction.^{39–41} We used several different titanium variants: polished titanium as a model of bulk titanium implants, commonly employed in dental applications⁴²; porous titanium as a model of porous implants used in femoral and tracheal prosthesis^{43,44}; μ Ti as a model for mechanical debris, which is difficult to phagocytose; and nTi, which also belong to the implant debris but are efficiently phagocytosed by macrophages.

Several models for foreign body response demonstrated the production of selected proinflammatory mediators upon the recognition of the foreign body by macrophages.^{9,11,25} Cytokines, chemokines, and growth factors are released in the tissue microenvironment, and attract other immune cells (monocytes, lymphocytes, and neutrophils) and exacerbate the inflammation.¹¹ Here, for the first time, we analyzed the whole transcriptome of macrophages in different activation states cultured on the surface of polished or porous titanium. We found that macrophage activation status plays a major role in determining the reaction to titanium. The highest number of genes affected by titanium was identified in M(IL-4) with 3,560 genes on porous and 1,452 genes on polished titanium, followed by M(Control) with 1,244 genes on porous and 214 genes on polished titanium, and last, by M(IFN γ) with 861 genes on porous and 150 genes on polished titanium. Plotting the results on a 3D scatterplot showed that in both polished and porous titanium M(Control) and M(IFN γ) activation states have more homogeneous populations, in comparison with M(IL-4). Thus, we have demonstrated that M(IFN γ) (model for acute inflammatory phase macrophages) do not react as strongly to titanium as M(IL-4) (model for healing macrophages). We hypothesize that the proinflammatory state induced by IFN γ overlaps with the one induced by titanium, thus masking its effects.

Our data clearly demonstrated the most pronounced effect titanium has on M(IL-4) that resemble the healing macrophage subtype. Our results come into contradiction with data presented by Pajarinen et al.,²² in which titanium affected more genes in M(IFN γ) (192 genes), followed by M(Control) (63 genes) and

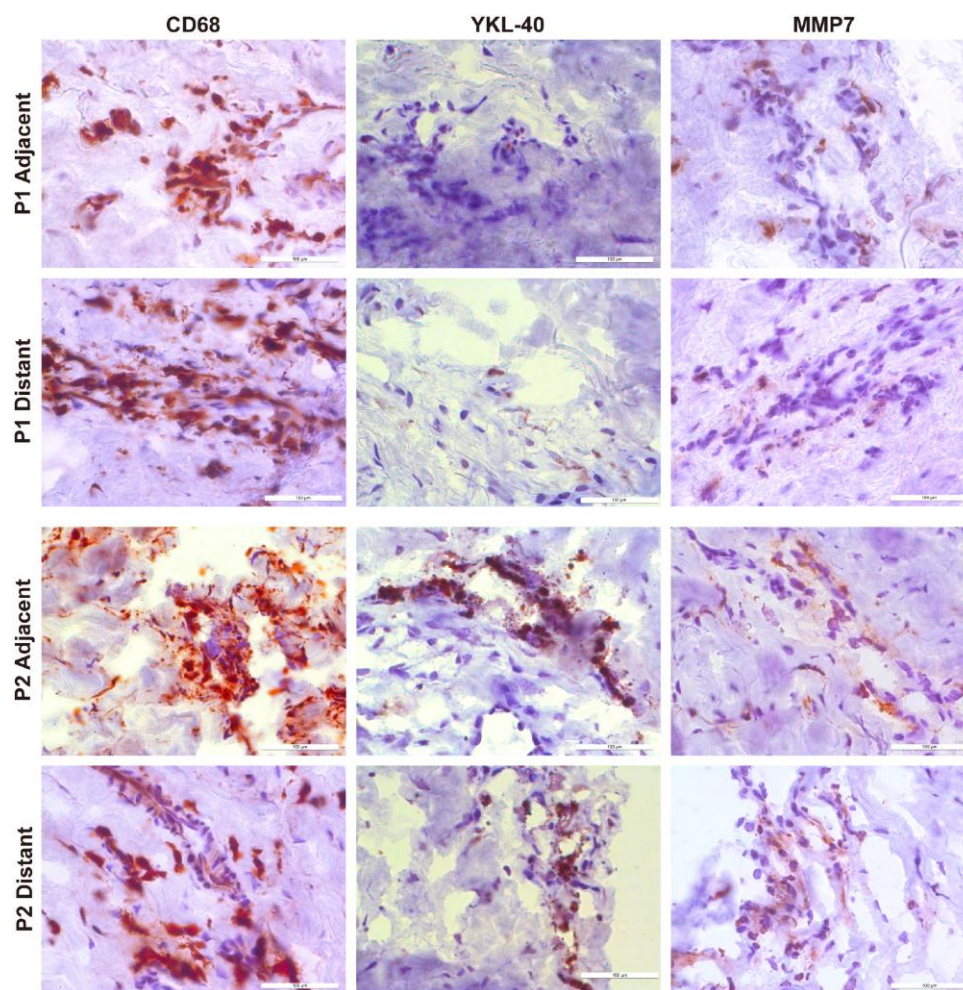


Fig. 8. Immunohistochemical (IHC) analysis of CD68, YKL-40, and MMP7 in macrophages in peri-implant tissue of the orthopedic patients. Tissues from the patients with titanium implants has been analyzed by IHC staining. Graphs represent the IHC staining of CD68, YKL-40, and MMP7 in macrophages in peri-implant tissue of 2 orthopedic patients (P1 and P2). Scale bars are 100 μ m.

M(IL-4) (59 genes). A deeper analysis of the data revealed that only 40 genes that were affected in both sets of data; however, the direction of change (up- or downregulation) did not always match. The discrepancy in results could be a consequence of different study conditions (titanium discs vs μ Ti; macrophage SFM medium + 1 ng/mL M-CSF + 10^{-8} M dexamethasone vs RPMI medium + 100 ng/mL M-CSF + 10% fetal bovine serum) and sample sizes (6 individual macrophages sets for polished titanium, 8 for porous titanium, and only 4 tested by Pajarinen et al.) as well as high variation among the tested samples. High concentrations of M-CSF, such as 100 ng/mL, is a potential stimuli known to program the M2 phenotype as strong as IL-4 does, which definitely interferes with the effects of IFN γ .⁴⁵

The use of polished and porous titanium discs in our experiments allowed us to compare the effects that titanium surface can have on macrophage transcriptome in 2D and quasi-3D conditions. Surface roughness has an effect on cell attachment and proliferation.⁴⁰ Macrophages cultured on materials with different topography or on materials designed to induce a specific cell shape acquire a particular activation state, which in turn alters the expression and secretion patterns of inflammatory factors.^{39,46} However, our data did not show a great difference in the expression pattern of genes from cytokine-cytokine receptor family between macrophages cultured on polished and porous

titanium discs, indicating that the nature of the material (titanium) is the dominant factor for macrophage programming. Although there were more cytokine-cytokine receptor family members affected by porous titanium (65 compared with 44), the direction of change (up- or downregulation) for the common genes was similar. For example, both types of titanium surfaces stimulated CSF1, IL1RN, and TNFSF14 and suppressed CCL8 and CCL23 expression. Some stimulation specific differences were also noted: (1) in M(IFN γ), a significant downregulation of INHBA, CXCL9, and CXCL10 was observed on porous titanium, with no effects on polished titanium; (2) in M(IL-4), CCL13 was strongly downregulated on porous titanium but not on polished titanium. Downregulation of CXCL9, CXCL10, and CCL13 by porous titanium is indicative for the suppression of specific inflammatory reactions, as all 3 genes are associated with adverse local tissue reaction.⁴⁷ Additionally, porous materials promote osseointegration and capillary formation⁴⁸; however, no significant differences in the expression of genes associated with wound healing and angiogenesis were noted between the surfaces.

Comparing the transcriptome of macrophages cultured on polished and on porous titanium discs, we have identified 1,102 genes that were common between these surfaces. A total of 899 genes were common in M(IL-4), 135 in M(Control), and 68 in M(IFN γ) activation states. Because mediators of inflammation and extracellular

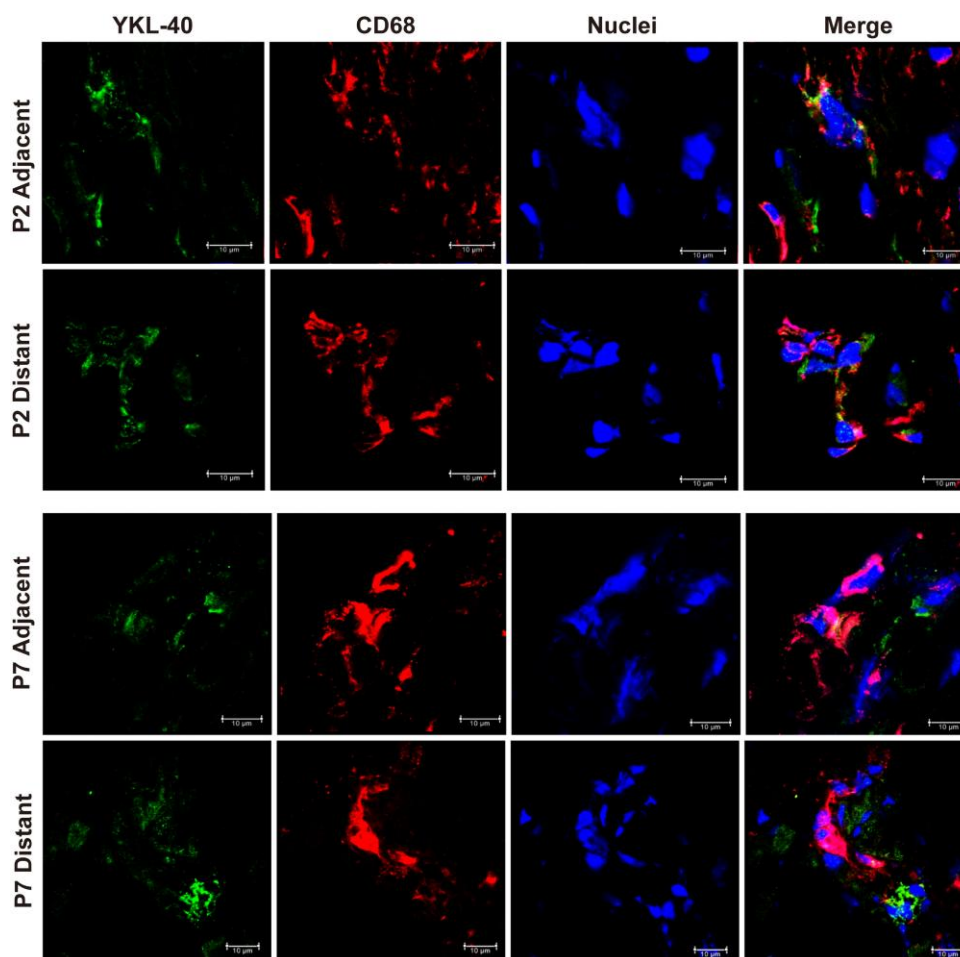


Fig. 9. YKL-40 is expressed in CD68+ macrophages in peri-implant tissue of the orthopedic patients. Tissue from a patient with a titanium implant has been analyzed by immunofluorescent staining. Graphs represent the immunofluorescent staining of CD68 and YKL-40 in macrophages in peri-implant tissue of 2 orthopedic patients (P2 and P7). YKL-40 is visualized in green and CD68 is visualized in red. Yellow indicates complete colocalization of green and red. Nuclei are visualized in blue. (Top) The tissue in very close proximity of the implant. (Bottom) The tissue distant from the implant. Scale bars are 10 µm.

matrix remodeling are mainly involved in implant complications, genes from cytokine-cytokine receptor, extracellular matrix disassembly, and Glyco_18 domain containing protein families were analyzed in more detail.²⁸ Thus, 14 genes with highest response to titanium were identified (Table 2). Interestingly, all of the selected genes followed the same direction of change regardless of the surface. Collectively, our comparison proves that titanium surface can affect gene expression in macrophages. However, the differences are mainly in magnitude of change and not in the direction of change (up- or downregulation) of gene expression. Thus, macrophages react stronger to titanium as a material than to its form.

Our results from microarray experiments were generally confirmed by RT-qPCR, in which several prominent genes were selected for analysis. Again, the highest reaction to titanium was observed in M(IL-4) activation state, both on porous and on polished titanium. Our analysis revealed that titanium upregulates CSF1, CHI3L1, CHIT1, MMP7, MMP8, MMP9, and TNFSF14. It is known that CSF1 and TNFSF14 are directly involved in osteoclastogenesis; CHI3L1 and CHIT1 promote chronic inflammation; and MMP7, MMP8, and MMP9 take part in extracellular matrix remodeling and promote local inflammatory reactions that may lead to implant failure.^{30,49–54} We suggest that upregulation of these genes by titanium in macrophages can lead to the implant-related complications.

Titanium also suppressed expression of several cytokines, including CCL8, CCL13, CCL18, and CCL23. In the periprosthetic tissue of patients with osteolysis and aseptic loosening, an upregulation of these genes was observed.^{47,55,56} CCL8, CCL13, CCL18, and CCL23 are involved in chemotaxis of lymphocytes, monocytes, basophils, and eosinophils.^{57,58} Moreover, we found that IL17RB, a gene expressed on M2 macrophages and involved in NF-κB activation,^{59,60} is also downregulated by titanium. A suppressive effect of titanium on the cytokine production can be considered as anti-inflammatory on the later stages of the implantation when the wound is healed; however, whether this effect is beneficial on the acute stage is questionable. Our data demonstrate that titanium can suppress a set of acute inflammatory chemokines; however, it also induces a set of factors related to chronic inflammation and tissue destruction that can act after the acute inflammation is resolved.

THP-1 cells react differently to titanium particles in comparison with titanium discs, with higher secretion of TNFα, CCL2, CCL3, and IL-1Ra when cultured on discs.²³ In our study, comparison of the reaction of human primary macrophages on titanium discs, µTi, and nTi revealed similarities in the induced gene expression. The strongest macrophage reactions were induced by nTi, with significant downregulation of CCL8, CCL13, CCL18, and CCL23 in comparison with titanium discs and µTi. In contrast to nTi, macrophage reactions to titanium discs and µTi were similar,

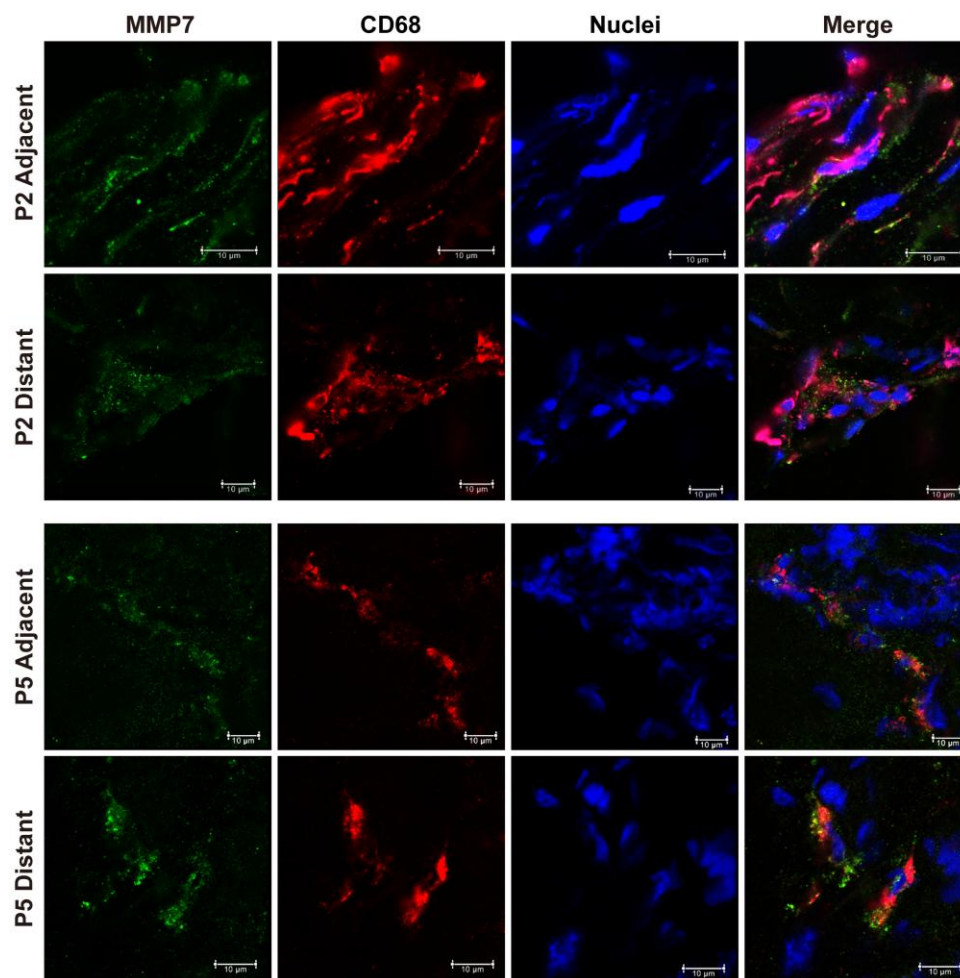


Fig. 10. MMP7 is expressed in CD68+ macrophages in peri-implant tissue of the orthopedic patients. Tissue from the patient with a titanium implant has been analyzed by immunofluorescent staining. Graphs represent the immunofluorescent staining of CD68 and MMP7 in macrophages in peri-implant tissue of 2 orthopedic patients (P2 and P5). MMP7 is visualized in green and CD68 is visualized in red. Yellow indicates complete colocalization of green and red. Nuclei are visualized in blue. (Top) The tissue in very close proximity of the implant. (Bottom) The tissue distant from the implant. Scale bars are 10 μm .

except CSF1 and CHI3L1 expression. The differences between (nTi) and non-(titanium discs and μTi) suggest that nanoparticle phagocytosis enhances macrophage reactions. Indeed, it was shown by others that particles that can be phagocytosed enhance the secretion of $\text{TNF}\alpha$, IL-1 β , IL-6, and PGE2 by macrophages, and increase their bone-resorbing activity.^{61,62} Our results confirm that macrophages respond differentially to titanium of different sizes. The difference in response can be explained by phagocytosis of the material, which takes place when the particles are smaller than 10 μm .¹⁴ However, these reactions differ only in their magnitude and are influenced by the initial activation state of macrophages. Therefore, in vivo, both the size of wear particles and micro-environmental signals will modulate the foreign body response.

One of the main reasons for implant failures is biofilm formation.⁶ Due to its resistance to antibiotics and host defense mechanism, biofilm formation on the surface of implants is a clinically significant problem.³³ SA is a common pathogen involved in orthopedic and dental implant failure.^{6,33,63} We have used inactivated SA to model a bacterial infection on titanium surface. Our results indicate that SA stimulation induces higher secretion of $\text{TNF}\alpha$ and IL-8 in M(IFN γ), while the secretion of IL-1 β , IL-6, and CCL18 as well as of MMP7 and MMP9 was

unchanged in all activation states. Although titanium induced higher levels of $\text{TNF}\alpha$ in comparison with control, in combination with SA, titanium did not enhance the effect of SA alone. Taken together, these results suggest that titanium and SA do not have synergistic effects on the proinflammatory reactions of macrophages. An important role in the recognition of SA by immune cells is attributed to TLR2 and its interaction with TLR1, TLR6, CD14, and CD36 (TLR2),⁶⁴ while titanium is recognized by a series of receptors.¹² Involvement of different receptors in SA and titanium recognition argue toward independent receptors and signaling pathways that do not amplify each other.

Another important reason for implant failures is aseptic loosening.^{5,28} We have shown that macrophages react to titanium by up-regulation of cytokines, chitinase-like proteins, and matrix metalloproteinases. Whole fractions of PBMCs induced significantly higher levels of MMP7 and MMP9, while no effect was detected in PBMCs depleted in monocytes. PBMCs mainly consist of 2 types of cells: lymphocytes (B cells, T cells, and natural killer cells) and monocytes. While lymphocytes display the ability to react to metals, it is also known that they enhance macrophage foreign body reaction in both a paracrine and juxtacrine manner.^{65,66} Still, the limited reaction of PBMCs depleted in monocytes on titanium reinforces the widely accepted concept that macrophages are

Table 2. Comparison of microarray data from macrophages cultured on polished and porous titanium.

Gene symbol	Polished titanium (6 donors)		Porous titanium (8 donors)	
	M(Control)	M(IL-4)	M(Control)	M(IL-4)
CCL8	▼*		Not changed	↓
CCL23	Not changed	↓	Not changed	↓
CHI3L1	↑	↑	↑	↑
CHIT1	Not changed	▲*	↑	↑
CSF1	▲*	↑	↑	↑
IL17RB	Not changed	▼*	Not changed	↓
IL1R2	▲*	↑*	▲*	↑
IL1RN	↑	↑*	↑	▲*
MMP7	▲*	↑*	↑	↑
MMP8	▲*	Not changed	↑*	↑
MMP9	Not changed	↑	Not changed	↑
SPP1	↑	↑	↑	↑*
TNFRSF21	↑	↑	▲*	↑
TNFSF14	↑*	↑	↑	↑

M(IFN γ) not shown due to absence of any significant changes in the regulation of these genes). ↑ indicates upregulation >2-fold; ↓ indicates downregulation >2-fold; ▲ indicates upregulation <2-fold; ▼ indicates downregulation <2-fold. *P > 0.05.

the main immune cells involved in foreign body recognition. Further, we tested whether fibroblasts can enhance macrophage reaction to titanium. Fibroblasts are known to secrete proinflammatory factors when stimulated with wear particles.⁶⁷ In our experiments, fibroblasts cocultured with macrophages stimulated secretion of MMP7 in both juxtacrine and paracrine fashion. MMP7, MMP8, and MMP9 are involved in osteoclastogenesis, osteolysis, and aseptic loosening.^{68–70} Because MMPs are secreted in a proenzyme form requiring additional activation, we examined whether other PBMCs and/or fibroblasts can increase the concentration of active MMP7 and MMP9.³⁴ The total concentration (pro-form and active form) of both MMP7 and MMP9 was significantly changed by PBMCs and fibroblasts when cocultured with macrophages on titanium, despite that the percentage of the concentration of their active form did not change substantially. Significantly enhanced titanium-induced production of active MMPs can be detrimental when healing phase has to be completed and can result in tissue destruction in close proximity to the implant. Despite that elevated levels of MMP7 and MMP9 in the periprosthetic tissue correlate with osseointegration at early stages of implantation,⁶⁷ long-term secretion of MMPs after the healing is completed can have detrimental effect on the tissue integrity. Interestingly, that elevated circulating levels of MMP7 and YKL-40 were identified as biomarkers indicative for shorter transplant-free survival of patients with idiopathic pulmonary fibrosis treated by antifibrotic therapy.⁷¹ The same biomarkers have been detected by us in the macrophages located in close proximity to the implant of patients with orthopedic implants. Moreover, for the first time we identified that MMP7, characteristic for M2 type macrophages, and YKL-40, expressed in M1 macrophages, are coexpressed in the same cell, indicating that titanium can induce mixed chronic-inflammatory macrophage phenotypes. The clinical effect of the titanium-induced metalloproteinases and proinflammatory mediators will on the types of tissue where implant is installed as well as time/phase of implant integration.

5. Conclusions

We identified that proinflammatory and tissue-destructive reactions of macrophages to titanium implants are defined by several

factors: (1) macrophage activation state, (2) titanium surface, (3) titanium size, (4) other cellular components of the periprosthetic tissue, and (5) donor-specific factors. However, macrophage reaction to titanium is rather independent on SA. We suggest that titanium-induced genes identified in our study can be used as biomarkers for the preimplantation diagnostic method to predict patient-specific innate immune reactions to titanium implants. Our model of healing macrophages can be applied for further examination of immune compatibility of newly developed implant materials, for making personalized clinical decision by selection optimal implant material for the individual patients and for validation of immunomodulatory approaches.^{25,26,72–74} Moreover, induction of MMP expression provides new sets of targets for potential immunomodulation around titanium implants, which can focus on decreasing active MMP amounts in order to prevent aseptic loosening, such as cytokine cocktail delivery systems.⁷³

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Author contribution

Conceptualization: J.K., H.K., N.E.V., U.O. Investigation: A.G., D.H., G.L., V.R., J.M., Q.L., C.S., Z.J., C.S., A.J. Data curation—formal analysis: J.K., A.G., V.R., C.S., G.L. Project Administration/oversight: J.K. Writing—original draft: J.K., S.G., N.E.V., H.K., V.R. Writing—review, editing, and revision: G.L., Q.L., J.K.

Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

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Conflict of interest statement. The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as potential conflicts of interest.

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