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# Pectin coating of titanium and polystyrene surfaces modulates the macrophage inflammatory response

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## ABSTRACT

Titanium has been used with success for bone anchoring of dental implants. However, when implant surfaces are exposed to the oral environment, the progression of peri-implantitis triggered by specific oral bacteria has been reported. Bacterial colonization of implants leads to prolonged immune cell activation and bone resorption. A new strategy to improve implant biocompatibility and prevent peri-implantitis is to develop pectin surface nanocoatings. These plant-derived polysaccharides are promising candidates for surface nanocoatings of titanium implants due to their osteogenic and anti-inflammatory properties. Therefore, the aim of the study was to evaluate the *in vitro* effect of nanocoating with plant-derived rhamnogalacturonan-I (RG-I) on pro- and anti-inflammatory responses of primary human monocyte-derived macrophages (HMDMs) induced by *Escherichia coli* LPS and *Porphyromonas gingivalis* bacteria. In the present study, two different types of surface materials, tissue culture polystyrene (TCPS) plates

and titanium (Ti) discs, coated with pectic polysaccharides, potato unmodified RG-I (PU) and potato dearabinanated RG-I (PA), have been examined. The inflammatory responses of HMDMs after *E. coli* LPS/*P. gingivalis* stimulation were investigated through gene expression measurements of pro- and anti-inflammatory cytokines. The results showed that PU and PA decreased expression of the proinflammatory genes tumour necrosis factor-alpha (*TNFA*), interleukin-1 beta (*IL1B*) and interleukin-8 (*IL8*) in activated HMDMs cultured on TCPS/Ti surfaces. In contrast, the effects on anti-inflammatory interleukin-10 (*IL10*) gene expression were not significant. The results indicate that RG-Is should be considered as a candidate for organic nanocoatings of titanium implant surfaces in order to limit host proinflammatory responses and improve bone healing.

**Keywords:** Rhamnogalacturonan-I; Titanium; Nanocoating; *Porphyromonas gingivalis*; LPS; Macrophage; Inflammation.

## 1. INTRODUCTION

The gold standard material for endosseous dental implants is titanium (Ti) due to its favorable physiochemical, mechanical and biological properties [1-3]. However, when dental implant surfaces are exposed to the oral environment, spontaneous progression of inflammation with bone and soft tissue destruction has been reported [4]. Therefore, titanium implant surface modifications are continuously developed to limit inflammation and enhance bone healing process following implant placement [5-7]. Nanocoating with organic molecules, such as proteins and polysaccharides, is one of the methods used to improve biocompatibility of dental implants. Plant-derived polysaccharides, mainly represented by rhamnogalacturonan-I (RG-I) from pectins, have been proposed as potential candidates for surface nanocoating of titanium implants due to their osteogenic and anti-inflammatory properties [8]. The structure of RG-I can easily be modified with various enzymes, which results in different physicochemical properties and their effect on the cellular response [8-12]. The results of *in vitro* studies showed increased proliferation and metabolic activity, as well as decreased proinflammatory response of different cells cultured on enzymatically modified RG-I with short arabinan side chains [12-15].

The success of biomaterial implantation depends on the outcome of the bone healing process following implant placement. Initially, implantation induces an acute inflammatory response to the implanted biomaterials, followed by repair processes resulting in bone healing [16, 17]. Macrophages, monocyte-derived cells, are one of the major cellular players in the host inflammatory response. Macrophages have recently come to the forefront in biomaterials research not just as mediators of tissue debris removal, capable of secreting proinflammatory cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and interleukin-8 (IL-8), but also potentially as key players in promoting new bone tissue formation at the implant surface through growth factor secretion [18, 19]. However, bacterial factors such as lipopolysaccharide (LPS, a cell wall component of Gram-negative bacteria) are potent macrophage activators, which inhibit bone for-

mation and stimulate production of proinflammatory mediators [20]. Prolonged macrophage exposure to bacterial LPS results in peri-implant inflammation and leads to tissue destruction around the dental implant.

The peri-implantitis is associated with biofilms comprising predominately of Gram-negative and anaerobic species of periodontal pathogens, such as *Porphyromonas gingivalis* [21]. This oral bacterium is capable of generating an arsenal of specialized virulence factors that contribute to its pathogenicity, including LPS, fimbriae, hemagglutinin and cysteine proteinases (gingipains) [22]. The *P. gingivalis* LPS is a stimulator of host proinflammatory response and bone resorption. It stimulates proinflammatory cytokine production of IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  in monocytes, as demonstrated *in vitro* [22, 23]. However, certain bacterial species, including *Escherichia coli*, that are less regularly detected in periodontitis could frequently be found at peri-implantitis site [24-26]. *E. coli* is an opportunistic pathogen in extensive variety of human infections and has impact on general host immune response, causing e.g. septic shock [27, 28]. It has been suggested that *E. coli* could migrate via the blood circulation from infections elsewhere in the human body and colonize peri-implant sites. These bacteria can favor the development of peri-implantitis, especially in immunocompromised host with a high risk of bacterial infection [25].

It has been reported that RG-Is inhibit inflammatory cell response in LPS-stimulated macrophages *in vitro* [8, 29]. However, knowledge of the effect of coating of titanium with RG-I molecules on pro- and anti-inflammatory responses of macrophages is still very sparse. Therefore, the aim of the present study was to evaluate the *in vitro* effect of nanocoating of titanium and polystyrene surfaces with potato unmodified RG-I (PU) and potato dearabinanated RG-I (PA) on pro- and anti-inflammatory responses of primary human monocyte-derived macrophages (HMDMs) stimulated by *E. coli* LPS and *P. gingivalis* bacteria. The expression levels of pro- and anti-inflammatory cytokines were investigated to determine inflammatory responses of activated HMDMs. The results of the present study could contribute to development of plant-derived pectin nanocoatings to prevent

inflammation and improve the bone healing process following implantation, especially in immunocompromised patients with poor tissue healing capacity and a high risk of bacterial infection.

## 2. MATERIALS AND METHODS

### 2.1. Isolation, modification and nanocoating of RG-I

RG-I was prepared as described previously by Gurzawska et al. [7] by treatment of potato pulp (P) with enzyme preparations. The arabinan side chains of potato RG-I were removed with  $\alpha$ -L-arabinofuranosidase and endo-arabinanase (Novozymes, Bagsvaerd, Denmark). The chemical properties, monosaccharide composition and linkage analysis of potato unmodified RG-I (PU) and potato dearabinanated RG-I (PA) have been presented in previous studies [7, 10]. Briefly, the results showed that the PU and PA is a homogeneous coating of 6-10 nm thick, defined as a nanocoating [10]. To evaluate the *in vitro* effects of PU and PA nanocoatings on cellular responses, two different types of material substrates were used: tissue culture polystyrene (TCPS) plates (Techno Plastic Product, Trasadingen, Switzerland) with a diameter of 60 mm and commercially pure (grade 2) machined titanium (Ti) discs (Dentsply, Mannheim, Germany) with a diameter of 60 mm. PU and PA (128  $\mu$ g/ml) were coated on the surface of 6-well TCPS plates and on the surface of the Ti discs placed in 6-well TCPS plates. The reaction was carried out at room temperature overnight in sterile conditions on a shaking platform (IKA-Werke GmbH & Co. KG, Staufen, Germany) at 100 rpm and then the plates and Ti discs were extensively rinsed in sterile water and dried in a laminar flow hood before *in vitro* experiments.

### 2.2. Detection of PU and PA nanocoatings

PU and PA RG-Is nanocoatings on Ti surface were visualized using immunofluorescence labeling and confocal microscopy. Uncoated and PU, PA-coated Ti discs were placed separately in polystyrene 6-well plate and blocked with 1 ml/well of 5% skimmed milk (5% solution of fat-free milk powder in phosphate-buffered saline (PBS), pH 7.2)

(Applichem, Darmstadt, Germany). After 15 minutes skimmed milk was removed. Anti-(1 $\rightarrow$ 4)- $\beta$ -galactan LM5 (Plant Probes, Leeds, UK) was diluted 1:10 in 5% skimmed milk and applied 1ml/well. The plate was placed on a shaker for 2 hours. All Ti discs were washed three times with 5% skimmed milk. 1 ml/well of secondary antibody, goat anti-rat IgG for LM5 linked to FITC (fluorescein isothiocyanate) (Sigma-Aldrich, Munich, Germany) diluted 1:200 in 5% of skimmed milk was added. The plate was covered with aluminum foil and placed on the shaker for 2 hours. Subsequently, Ti discs were washed three times with 1 M PBS. Confocal images were done with a Leica TCS-SP5 II confocal laser scanning microscope (Leica Microsystems, Exton, PA, U.S.A.) with PL Fluotar 10/ $\times$ 0.30 DRY objective. Excitation 488 and 633, zoom 1.8; beam splitter TD458/514/594, respectively emission bandwidth 500–600 nm; 633 nm laser; scan speed 100 Hz; emission bandwidth 644–714 nm.

### 2.3. *In vitro* studies

The TCPS and Ti discs with PU and PA nanocoatings were compared with control uncoated TCPS and Ti discs. Peripheral blood for preparation of mononuclear cells for all *in vitro* experiments was obtained from healthy volunteer donors. Written informed consent was obtained from all volunteer donors and the study was approved by the local research ethics committee of the Dental School, College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK (approval number 14/SW/1148).

#### 2.3.1. Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized (10 U/ml) blood by centrifugation on Ficoll-Paque<sup>TM</sup> Plus (Amersham Biosciences, Little Chalfont, UK) as previously described [18]. PBMC were resuspended at a density of  $1 \times 10^6$  cells/ml in Iscove's-modified Dulbecco's medium (Sigma-Aldrich, Poole, UK) supplemented with 2.5% human AB serum (BioSera, Ringmer UK), antibiotics (100  $\mu$ g/ml streptomycin, 100 U/ml penicillin) (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich). PBMC were

seeded at a cell density of  $1 \times 10^6$  per well in PU-/PA-coated and uncoated 6-well TCPS plates and on PU-/PA-coated and uncoated Ti discs placed in 6-well TCPS plates. The cells were incubated for 2 h at 37°C with 5% CO<sub>2</sub> to obtain adherent cells (monocytes). After 2 hours, the medium containing non-adherent cells was removed and replaced with fresh medium. The adherent monocytes were then incubated for 5 days at 37°C with 5% CO<sub>2</sub> to allow differentiation into macrophages. The cell morphology was observed before and after stimulation with LPS/*P. gingivalis* by inverted microscopy (Primovert, Zeiss, UK). Microscopic images were registered with a Zeiss AxioCam ERc 5s video camera.

### 2.3.2. *E. coli* LPS treatment and *P. gingivalis* invasion assay

After 5 days of incubation, adherent human monocyte-derived macrophages (HMDMs) cultured on tested and control TCPS and Ti surfaces were treated with *E. coli* serotype O26:B6 LPS (Sigma-Aldrich L5543; Sigma-Aldrich) at 100 ng/ml or heat-inactivated *P. gingivalis* bacteria (*Pg*; ATCC 33277) at a multiplicity of infection (MOI) of 100 ( $10^8$  bacteria/well) and incubated for 6 h at 37 °C with 5% CO<sub>2</sub> to RNA isolation.

In the present study *P. gingivalis* strain ATCC 33277 (Manassas, Virginia, USA) was cultivated under anaerobic conditions at 37°C as previously described [14]. After cultivation, the bacteria were harvested by centrifugation, washed in sterile PBS and heat-inactivated for 10 min at 100°C. The number of bacteria was determined by measuring the OD at 600 nm and appropriate dilutions were made to obtain the desired MOI.

### 2.3.3. RNA isolation, reverse transcription and real-time polymerase chain reaction (PCR)

The RNA extraction was carried out using TRI reagent (Sigma-Aldrich) and the RNeasy Mini Kit (Qiagen, Hilden, Germany). The protocol was followed according to the manufacturer's specification. The concentration of RNA was determined by UV spectrometry at 260 nm (Eppendorf, Hamburg, Germany). The RNA was reversed transcribed to cDNA using one-step high-capacity cDNA RT kit (Applied Biosystems, Warrington, UK). Real-time PCR reactions were performed using the Light Cycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany), utilizing Roche SYBR Green reagents according to the manufacturer's instructions. The primer sequences (Sigma-Aldrich) for the specific target genes including tumour necrosis factor-alpha (*TNFA*), interleukin-1 beta (*IL1B*), interleukin-8 (*IL8*) and interleukin-10 (*IL10*) and for beta-2-microglobulin (*B2M*) as the housekeeping gene are described in Table 1. Real-Time PCR reactions were carried out in 10 µl volumes in a 96-well plate (Roche Diagnostics GmbH) containing 1 µl of cDNA and 9 µl reaction mixture, according to the manufacturer's instructions. PCR conditions consisted of an initial denaturation step of 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s.

The comparative  $2^{-\Delta\Delta C_t}$  method was performed for analysis of relative gene expression data, as previously described by Livak et al. [30]. Relative expression levels were calculated for each sample after normalization against the housekeeping gene *B2M*. A pool of all the cDNA was used as calibrator in our study.

**Table 1.** Sequences of real-time PCR primers.

Target gene	Forward (5'-3')	Reverse (5'-3')
<i>B2M</i>	ACCCCACTGAAAAGATGA	ATCTTCAAACCTCCATGATG
<i>TNFA</i>	ATCCTGGGGGACCCAATGTA	AAAAGAAGGCACAGAGGCCA
<i>IL1B</i>	TTCGAGGCACAAGGCACAA	AAGTCATCCTCATTGCCACTGT
<i>IL8</i>	CTCCTTGGCAAACTGCACC	CAGAGACAGCAGAGCACACA
<i>IL10</i>	TGCCTTCAGCAGAGTG	GGGAAGAAATCGATGA

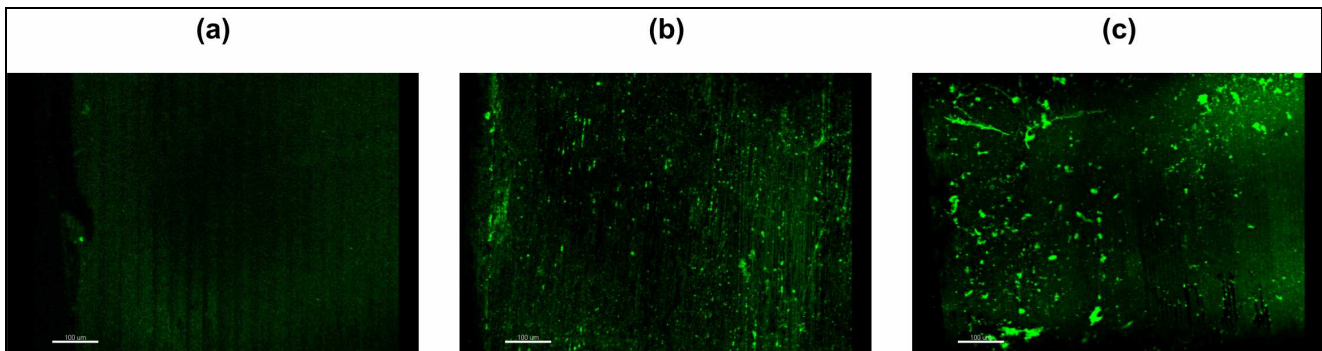
## 2.4. Statistical analyses

Descriptive statistics were used and mean values were calculated. Data are shown as mean  $\pm$  SEM and were analyzed using one-way ANOVA and post hoc Bonferroni test (IBM SPSS Statistic 22; IBM Corporation, Armonk, NY, USA). As significance level, a p-value of 5% was used throughout the study.

## 3. RESULTS

### 3.1. PU and PA nanocoatings of titanium surfaces visualized with confocal microscopy

The confocal images showed presence of PU and PA nanocoatings on the coated titanium disc surface compared to uncoated titanium disc surface (Figure 1).



**Figure 1.** Representative confocal images of PU and PA nanocoating visualized with immunofluorescence labeling on (a) titanium (Ti) surface without coating (C), on (b) Ti surface coated with unmodified RG-I (PU), and on (c) Ti surface coated with deacetylated RG-I (PA).

### 3.2. PU and PA nanocoatings of polystyrene surfaces decrease HMDM spreading

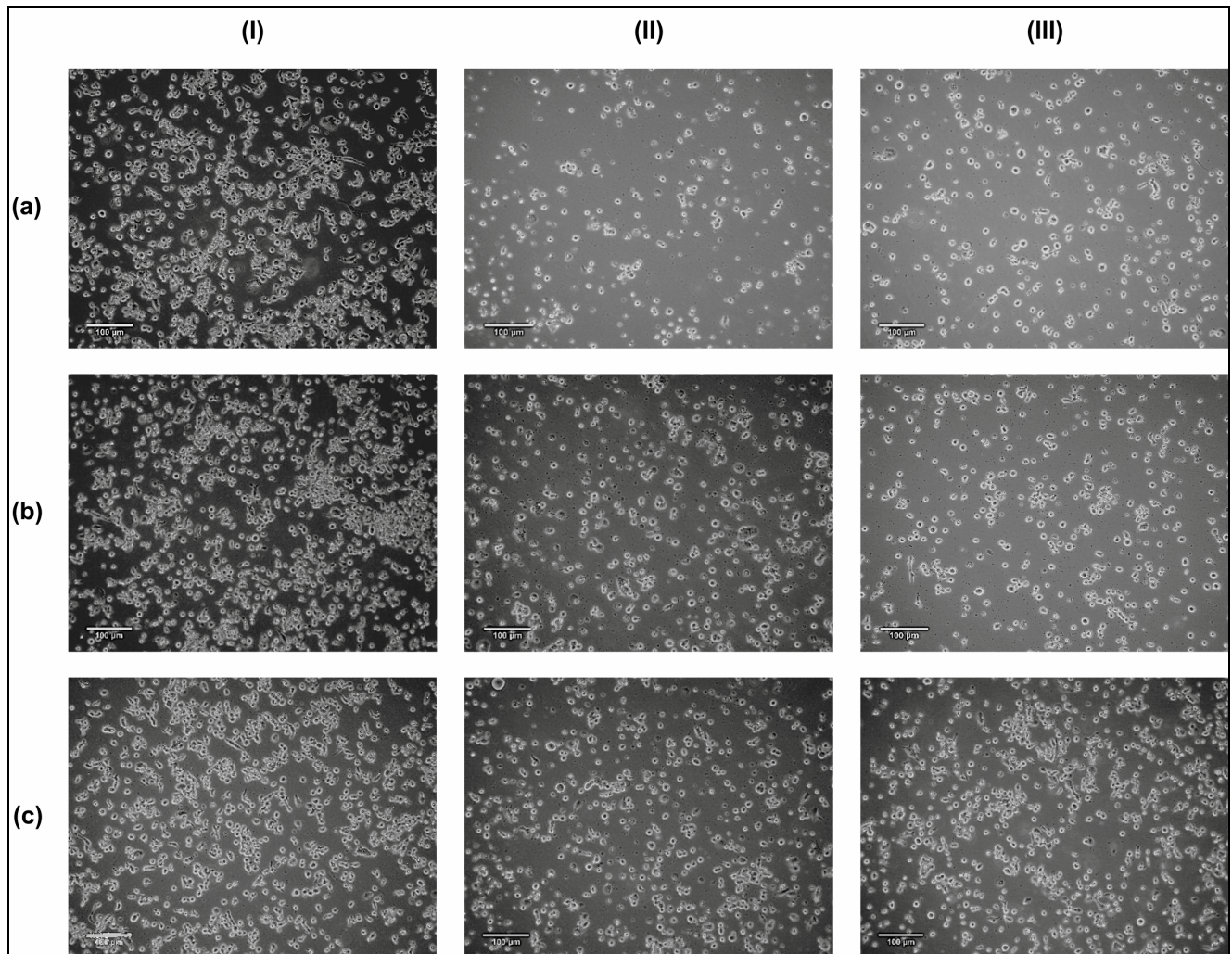
The morphology of HMDMs cultured on TCPS before and after stimulation with LPS/*P. gingivalis* is presented in Figure 2. The morphology of the unstimulated HMDM grown on the uncoated TCPS and PU, PA-coated TCPS surfaces looked quite similar, the cells were round and aggregated. LPS as well as *P. gingivalis* caused less cell spreading on surfaces coated with PU and PA.

### 3.3. PU and PA nanocoatings of polystyrene surfaces influence HMDM gene expression

We investigated the effect of RG-I (PU and PA) nanocoatings on pro- and anti-inflammatory gene expression in 5-day HMDMs cultured on TCPS surfaces. The relative expression of pro-inflammatory cytokine genes (*TNFA*, *IL1B*, *IL8*) and anti-inflammatory *IL10* gene was examined in HMDMs stimulated with *E. coli* LPS/*P. gingivalis* bacteria and in unstimulated HMDMs.

In general, proinflammatory *TNFA*, *IL1B* and *IL8* gene expression in activated HMDMs was highest on control TCPS surfaces and the lowest on PA-coated TCPS surfaces (Figure 3). PA significantly decreased *TNFA* ( $p < 0.001$ ) and *IL8* ( $p < 0.01$ ) expression in both *E. coli* LPS- and *P. gingivalis*-stimulated HMDMs compared with the TCPS control, while significant decrease of *IL1B* expression ( $p < 0.001$ ) in the presence of the PA nanocoating was observed only in HMDM cultures stimulated with *P. gingivalis* bacteria. PU significantly decreased *TNFA* ( $p < 0.01$ ) expression in HMDMs activated with *E. coli* LPS compared to the non-coated TCPS surfaces. No statistically significant differences were found between expression levels of proinflammatory genes in unstimulated HMDMs cultured on PU-/PA-coated and control surfaces.

As shown in Figure 3, neither nanocoating with PU nor PA significantly influenced the expression of *IL10* in unstimulated and *E. coli* LPS/*P. gingivalis*-stimulated HMDMs as compared to the corresponding TCPS control.



**Figure 2.** Representative images of HMDM morphology before (I) and after stimulation with LPS (II) and *P. gingivalis* (III) on (a) tissue culture polystyrene (TCPS) surfaces without coating (C), on (b) TCPS surfaces coated with unmodified RG-I (PU), and on (c) TCPS surfaces coated with dearabinanated RG-I (PA).

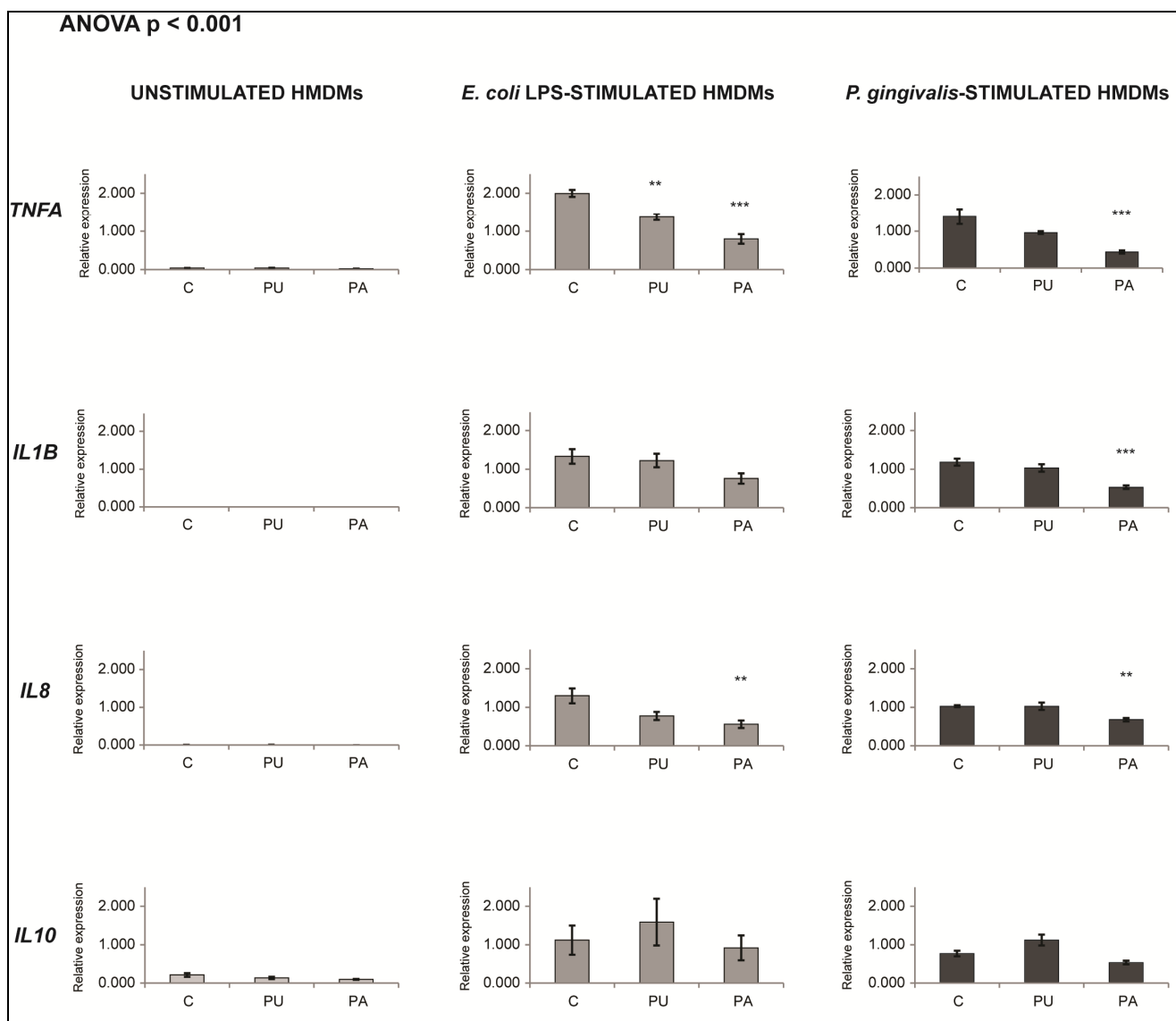
The level of *TNFA*, *IL1B*, *IL8*, and *IL10* expression, in general obtained higher values in HMDMs stimulated with *E. coli* LPS, rather than *P. gingivalis* bacteria. The lowest level of proinflammatory and anti-inflammatory gene expression was observed in unstimulated HMDM cultures.

### 3.4. PU and PA nanocoatings of titanium surfaces influence HMDM gene expression

The effect of RG-I (PU and PA) nanocoatings on Ti surfaces on pro- and anti-inflammatory gene expression was investigated in 5-day HMDMs. The relative expression levels of proinflammatory (*TNFA*, *IL1B*, *IL8*) and anti-inflammatory *IL10* genes were determined in unstimulated and *E. coli* LPS/*P. gingivalis*-stimulated HMDMs.

Generally, the expression of proinflammatory *TNFA*, *IL1B* and *IL8* genes in activated HMDMs was the highest on control Ti surfaces and the lowest on PA-coated Ti surfaces (Figure 4).

PA significantly reduced *IL8* expression in both *E. coli* LPS ( $p < 0.01$ ) and *P. gingivalis* ( $p < 0.05$ ) activated HMDMs compared to Ti control. The significant decrease of *TNFA* expression, in the presence of PA nanocoatings, was observed only in HMDM cultures stimulated with *P. gingivalis* bacteria ( $p < 0.01$ ) and in unstimulated HMDMs ( $p < 0.05$ ) compared to the non-coated Ti surfaces. No significant differences between PU-coated and control Ti surfaces were observed for proinflammatory gene expression in both stimulated and unstimulated HMDMs.



**Figure 3.** Relative expression of *TNFA*, *IL1B*, *IL8* and *IL10* in unstimulated and *E. coli* LPS/*P. gingivalis*-stimulated human monocyte-derived macrophages (HMDMs) cultured on tissue culture polystyrene (TCPS) surfaces without coating (C), on TCPS surfaces coated with unmodified RG-I (PU), and on TCPS surfaces coated with dearabinated RG-I (PA). Data are given as means  $\pm$  SEM ( $n = 9$ ) and were statistically analyzed using one-way ANOVA with Bonferroni's for multiple comparisons. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). RG-I: rhamnogalacturonan-I.

The expression of *IL10* in *E. coli* LPS/*P. gingivalis*-stimulated HMDMs was not significantly affected by different Ti surfaces analyzed, whereas in unstimulated HMDMs, PA nanocoatings significantly decreased *IL10* expression.

As shown in Figure 4, the relative expression level of *TNFA*, *IL1B*, *IL8* and *IL10*, in general resulted in higher values in HMDMs stimulated with *E. coli* LPS rather than *P. gingivalis* bacteria.

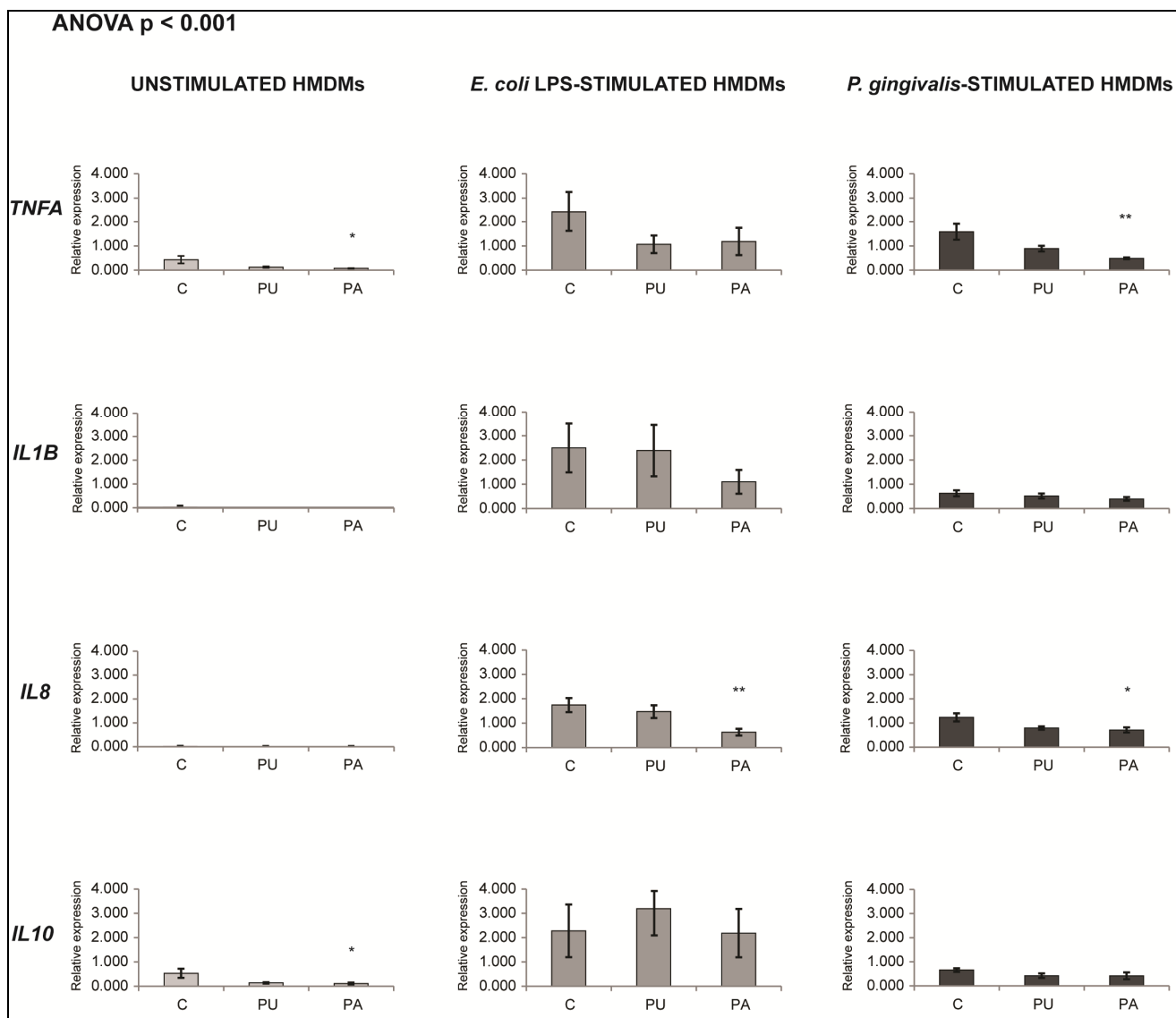
#### 4. DISCUSSION

Pectins from a variety of plants have been

shown to possess immunomodulatory activity, acting on immune cells such as macrophages [8, 29, 31, 32]. Macrophages play a key role in mediating the host inflammatory response to initial biomaterial implantation and in peri-implant infections [8, 33]. Thus, we examined the potential biomedical use of potato unmodified RG-I (PU) and potato modified RG-I (PA) as a nanocoating for Ti implant surfaces. For *in vitro* examination of organic nanocoatings, tissue culture polystyrene (TCPS) plates and Ti discs are frequently used for testing before *in vivo* studies [9]. Therefore, in the present study, adherent human monocyte-derived macro-

phages were cultured on coated/non-coated TCPS and Ti surfaces and activated in two different ways: (i) with *E. coli* LPS, a bacterial endotoxin which is a powerful macrophage activator [20], and (ii) by *P. gingivalis* invasion. Both *E. coli* and *P. gingivalis* bacteria have been frequently isolated from inflamed peri-implant tissues [2, 21, 24-26]. Moreover, it has

been reported that *P. gingivalis* and *E. coli* induce the host proinflammatory response through different Toll-like receptor (TLR)-independent mechanisms. While the LPS of *E. coli* is a strong activator of TLR4 responses, *P. gingivalis* LPS is predominately a TLR2 activator [34].



**Figure 4.** Relative expression of *TNFA*, *IL1B*, *IL8* and *IL10* in unstimulated and *E. coli* LPS/*P. gingivalis*-stimulated human monocyte-derived macrophages (HMDMs) cultured on titanium (Ti) surfaces without coating (C), on Ti surfaces coated with unmodified RG-I (PU), and on Ti surfaces coated with dearabinated RG-I (PA). Data are given as means  $\pm$  SEM ( $n = 9$ ) and were statistically analyzed using one-way ANOVA with Bonferroni's for multiple comparisons. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). RG-I: rhamnogalacturonan-I.

The results of morphology detection showed that PU and PA nanocoatings prevent HMDM spreading after LPS of *E. coli* as well as *P. gingivalis* stimulation. This findings is of great importance as

it has been demonstrated that the spreading of macrophages on different surfaces is a marker of activation [8].

We assessed the ability of PU and PA



nanocoatings to inhibit proinflammatory and stimulate anti-inflammatory response of activated HMDMs. To investigate inflammatory responses of HMDMs after *E. coli* LPS/*P. gingivalis* stimulation, gene expression levels were measured for pro- and anti-inflammatory cytokines. TCPS and Ti without PU and PA nanocoatings were used as control surfaces (positive controls). In parallel, non-activated HMDMs were cultured on tested and control TCPS/Ti surfaces (negative controls) to exclude the possibility that PU and PA molecules activate the inflammatory response of HMDMs.

Taken as a whole, our results clearly demonstrate that HMDMs are activated by *E. coli* LPS and *P. gingivalis* bacteria on TCPS/Ti surfaces with and without pectin nanocoatings. In general, nanocoatings with PU and PA decreased expression of genes coding for proinflammatory cytokines in *E. coli* LPS/*P. gingivalis*-stimulated HMDMs on TCPS/Ti surfaces, compared with uncoated controls.

In response to bacterial products such as LPS and other inflammatory stimuli, macrophages release large quantities of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IL-12 [35]. Increased level of these proinflammatory cytokines activates osteoclasts, bone-resorbing cells and leads to destruction of periodontal and peri-implant bone tissue [36, 37]. Therefore, the expression of proinflammatory genes: *TNFA*, *IL1B* and *IL8* in activated HMDMs was assessed on both PU and PA coatings. The expression of examined genes was downregulated in *E. coli* LPS/*P. gingivalis* activated HMDMs cultured on PU- and PA-coated TCPS and Ti. As our data showed, PU and PA nanocoatings decrease the expression of proinflammatory cytokines, it is possible that nanocoating with RG-I could inhibit macrophage activation, osteoclast recruitment and prevent bone destruction during bacterial infection. Recently, enzymatically modified RG-I from apple (MHR- $\alpha$ ) has been shown to decrease secretion of proinflammatory TNF- $\alpha$ , IL-6 cytokines and nitrites in J774.2 murine macrophages activated by bacterial LPS [8, 29]. The results of another study also demonstrate that Guar gum, a plant-derived polysaccharide, strongly inhibits nitric oxide generation and cytokine TNF- $\alpha$  secretion in LPS activated RAW 264.7 murine macrophages [31]. In contrast, some pectins have the capability to

induce proinflammatory responses in macrophages. Stimulatory effects of polysaccharide from *Solanum nigrum* on proinflammatory RAW 264.7 murine macrophage response have been reported [32]. The polysaccharide fraction isolated from *S. nigrum* promoted the secretion of TNF- $\alpha$  and IL-6 in RAW 264.7 cells. Such different results indicate that plant-derived polysaccharides regulate macrophage activation by various biochemical mechanisms, possibly caused by differences in RG-I structure [8].

To evaluate the anti-inflammatory effects of PU and PA nanocoatings, the expression of the anti-inflammatory cytokine gene *IL10* was also examined in HMDMs stimulated with *E. coli* LPS/*P. gingivalis* bacteria. IL-10 is an important immunoregulatory cytokine that inhibits the expression and production of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and IL-12 [35]. Generally, the expression of *IL10* in activated HMDMs was not affected by different surfaces analyzed. There were no significant differences in *IL10* expression between PU/PA-coated and control surfaces. Based on our *IL10* gene expression results, PU and PA appear to have no effect on the anti-inflammatory cytokine responses of activated HMDMs. The results may be due to different activation of molecular pathways. However, more *in vitro* studies need to be done to completely exclude anti-inflammatory activities associated with RG-I. A variety of cytokines with anti-inflammatory properties as well as several different signal transduction pathways are involved in suppression of inflammatory reaction [35].

In accordance with our previous studies, the results obtained from proinflammatory cytokine gene expression in activated HMDMs clearly indicate that PU and PA lead to different biological effects *in vitro*. PA, when compared to PU, seems to have a strong ability to reduce cellular proinflammatory responses. The sugar composition of PU and PA differs mainly in arabinose and galactose content. In this study, we also visualized PU and PA nanocoatings on Ti discs by immunofluorescence staining using the primary LM5 antibody, which specifically binds to galactan side chains. The results from confocal microscope detection showed higher amount of galactose on Ti surfaces coated with PA compared to PU. Galactose is known to be a specific high-affinity ligand for

galectin-3, a powerful proinflammatory mediator [38, 39]. Therefore, it can be speculated that the higher amount of galactose, a galectin-3 ligand, in PA's structure leads to more effective blocking of galectin-3 binding sites and attenuation of proinflammatory responses in activated HMDMs when compared to PU with lower amount of galactose residues. However, the biochemical mechanism underpinning inhibition of cellular proinflammatory response by PA nanocoatings requires further investigation.

Based on our examination of proinflammatory cytokine gene expression in non-activated HMDMs cultured on pectin coated TCPS/Ti surfaces, PU and PA molecules do not possess proinflammatory activity. The expression of *TNFA*, *IL1B* and *IL8* was downregulated in unstimulated HMDMs in the presence of PU and PA, when compared to negative controls, however the differences were not significant. The lack of proinflammatory properties of RG-Is is potentially of great importance for biomaterials applications.

## 5. CONCLUSIONS

The host immune response is a key factor influencing peri-implant bone regeneration. Our results indicate that it is possible to modulate cellular proinflammatory responses with plant-derived RG-I nanocoatings applied to titanium, a standard material used in dental implantation. Nanocoatings of TCPS and Ti surfaces with RG-Is (PU and PA) have the capacity to inhibit *in vitro* proinflammatory response of HMDMs stimulated with *E. coli* LPS and *P. gingivalis* bacteria, through downregulation of cytokine gene expression of *TNFA*, *IL1B* and *IL8*. Based on *IL10* gene expression results, PU and PA did not modulate anti-inflammatory response of activated HMDMs. However, due to RG-I's capacity to limit proinflammatory cellular responses, PU and PA nanocoatings are innovative candidates with considerable potential for improving the biocompatibility of implants and preventing immunopathological damage of peri-implant tissues. Better understanding of interactions between RG-Is and cells as well as *in vivo* investigation of RG-I's compatibility is needed to engineer novel immunomodulatory plant-

derived RG-I biomaterials able to suppress immune responses and promote regenerative processes.

## AUTHORS' CONTRIBUTION

AM: Conception and design, Development of methodology, Acquisition of data, Analysis and interpretation of data, Writing of the manuscript. JF: Development of methodology, Acquisition of data, Analysis and interpretation of data, Review of the manuscript. BB and OA: Study supervision, Administrative, technical and material support, Review of the manuscript. KG: Conception and design, Study supervision, Administrative, technical and material support, Review of the manuscript. All authors read and approved the final manuscript.

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## TRANSPARENCY DECLARATION

The authors declare that there is no conflict of interest.

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