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ORIGINAL ARTICLE

Assessment of lipopolysaccharide microleakage at conical implant-abutment connections

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Abstract
Objective The aim of this in vitro study was to assess lipopolysaccharide microleakage at conical implant-abutment connections of two-piece dental implants in terms of the expression levels of genes involved in lipopolysaccharide-mediated proinflammatory cytokine production.
Materials and methods Two implant systems with conical implant-abutment connections were inoculated with lipopolysaccharide and submerged in human whole blood. Positive-control blood samples (without implants) were stimulated with 4 μg/ml, 2 μg/ml, 200 ng/ml, and 20 ng/ml lipopolysaccharide. Sampling was performed after 1, 8, and 24 h of incubation. Changes of gene expression levels of Toll-like receptor 9, tumor necrosis factor-α, nuclear factor kappa light chain enhancer of activated B cells, interleukin-1β, and interferon-γ were assessed by real-time quantitative PCR. In addition, protein expression levels of interleukin-6, tumor necrosis factor-α, interleukin-1β, and interferon-γ were determined by immunoassay.
Results Changes in cytokine expression at the genomic and proteomic levels indicated lipopolysaccharide leakage at the interfaces of both tested implant systems, although some implants showed no sign of microleakage. Any tested concentration of lipopolysaccharide stimulated similar gene expression.
Conclusions Conical implant-abutment connections of two-piece dental implants do not prevent microleakage on a molecular level. Changes in lipopolysaccharide-induced proinflammatory cytokine gene expression facilitate the detection of lipopolysaccharide microleakage at implant-abutment interfaces.
Clinical relevance Small amounts of lipopolysaccharide released from intra-implant cavities can stimulate a detectable immunological response in human whole blood and may induce alveolar bone resorption via the osteoclast-activating pathway.

Keywords Proinflammatory cytokines · Immunological response · Implants · Lipopolysaccharides · Microleakage · Bone resorption

Introduction

Since the discovery of osseointegration of dental implants, intensive research has been conducted to assess the stability of the alveolar bone surrounding one- and two-piece implants. Using standardized longitudinal radiography and histometry, it could be shown that the intersection between the rough and the smooth part of implant surfaces as well as the location of microgaps between the implant and the abutment of two-piece dental implants might considerably affect marginal bone [1–5]. A chemotactic stimulus originating in intra-implant cavities and/or microgaps of two-piece dental implants possibly initiates and supports the recruitment of inflammatory cells such as neutrophils and mononuclear cells to the implant–alveolar bone interface, thus causing persistent inflammation and progress of alveolar bone loss [6]. This assumption is supported by in vivo and in vitro investigations verifying

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bacterial colonization of intra-implant compartments [7, 8] and leakage of bacteria and dyes along different implant-abutment interface geometries [9–12].

Lipopolysaccharides (LPS) are essential outer-membrane molecules and resemble the main surface antigens of virtually all Gram-negative bacteria [13]. LPS has been identified as a major bacterial bone-resorbing factor [14–16]. Because of the small molecular size of LPS (10^6 molecules per bacterium of *Escherichia coli*) [17, 18], tight implant-abutment connections seem to be very important to prevent the leakage of not only bacteria but also endotoxins. LPS leakage at the implant-abutment interface of LPS-inoculated two-stage dental implants was recently investigated using the *Limulus* amoebocyte lysate (LAL) assay [19]. The principle of this assay is the coagulation of hemolymph of the horseshoe crab upon contact with LPS [20]. Although the LAL assay detects the most common and potent LPS, it does not reflect the inflammatory potency of this molecule in humans. The present in vitro study was performed to elaborate such possible relationships.

Therefore, the hypotheses of the present study were that conical implant-abutment connections of two-piece dental implants do not prevent LPS microleakage and that the immunological response to leaking implants does not differ from that to LPS directly applied to human whole blood. To validate these hypotheses, LPS microleakage at the conical implant-abutment connections of two-piece dental implants was assessed in terms of the expression levels of genes involved in LPS-mediated proinflammatory cytokine production.

Materials and methods

Implants

Two titanium implant systems with long conical implant-abutment connections and antirotational elements below the conical part of the implant-abutment interface were used (Cone-log implants with Promote plus surface, Camlog Biotechnologies, Basel, Switzerland; Straumann BoneLevel implants with SLActive surface, Straumann, Basel, Switzerland) (Figs. 1–2). The dimensions (diameter × length) of the Conelog and BoneLevel implants were 4.3×11 and 4.1×10 mm, respectively. The standard abutments were used for both implant systems (Conelog Universal Abutment, Camlog Biotechnologies; RC Cementable Abutment, Straumann).

Blood collection

Whole blood (150 ml) was collected into EDTA tubes by venipuncture of the arm of a Caucasian healthy male donor. The blood was immediately transferred in 5-ml aliquots into 14-ml pyrogen-free Falcon tubes (Becton Dickinson, Meylan, France).

Inoculation

Seven implants of each system were inoculated with 1 μl LPS (from *Salmonella enterica* serotype Minnesota, Sigma-Aldrich, Munich Germany) in a final concentration of 20 μg/μl (test implants). Further, three implants of each

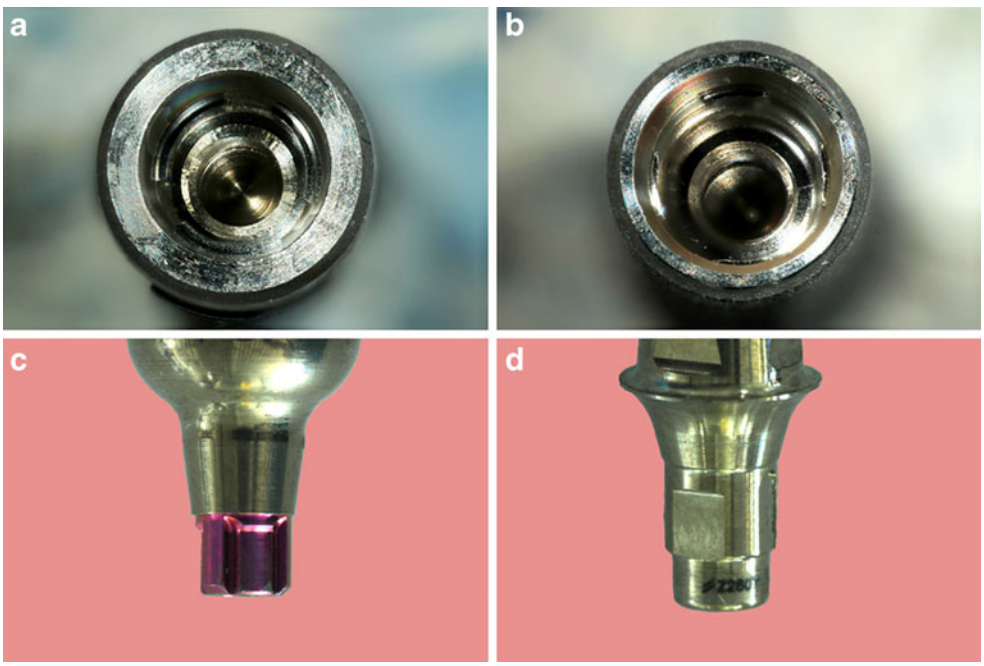

Fig. 1 **a** Conelog implant, **b** BoneLevel implant, **c** Conelog abutment, **d** BoneLevel abutment

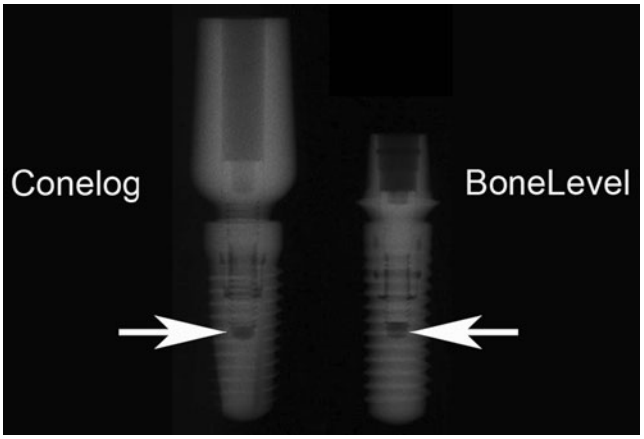

Fig. 2 X-ray images of the two tested implant systems with screwed abutments: in both the systems, intra-implant cavities are identifiable (*arrows*)

system were inoculated with 1 μl phosphate-buffered saline (PBS) (control implants). After inoculation, the implants and abutments were screwed together using the torque recommended by the implant manufacturers. Thereafter, the implants were immediately submerged in 5 ml blood each to ensure complete coverage of the microgaps between the implant and the abutment. Blood samples (2 ml) without implants were stimulated with 4 μg/ml, 2 μg/ml, 200 ng/ml, and 20 ng/ml LPS to serve as positive controls (PC); three blood samples at each concentration were prepared. Three blood samples (2 ml) without any stimulus served as negative controls (NC). The samples were incubated at 37°C in 5% CO_2 on a rotating platform (WT17, Biometra, Göttingen, Germany).

Sampling

Blood samples (500 μl) were collected after 1, 8, and 24 h of inoculation. Two aliquots of 200 μl blood from each group were mixed with 800 μl Prisure (Promolgene, Berlin, Germany) and stored at −80°C for RNA extraction, cDNA synthesis, and subsequent real-time qPCR. Blood serum for protein analysis was generated by centrifugation of the remaining (100 μl) blood. The resultant serum was transferred as 25-μl aliquots to new tubes and stored at −80°C until further analysis.

RNA extraction

After the blood samples were defrosted and incubated at room temperature for 5 min, RNA extraction was performed according to the method described by Chomczynski and Sacchi [21]. The resultant RNA pellet was dissolved in 20-μl diethylpyrocarbonate-treated water (Promolgene). RNA concentration was measured with a NanoDrop spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany), and RNA quality was determined by analyzing 2 μl (30 μg/μl) RNA with the Screentape Lab 901 System (Peqlab Biotechnologie). The remaining RNA was stored at −20°C until cDNA synthesis.

cDNA synthesis

The RNA was defrosted, and three aliquots of 200 ng total RNA from each blood sample were transcribed into cDNA according to the method described by Sambrook et al. [22] and using a cDNA synthesis kit (Promolgene) according to the manufacturer's protocol in combination with the provided oligo dT-V primer. cDNA synthesis was performed using a Thermocycler I (Biometra). The primer and RNA were incubated at 65°C for 5 min; the samples were immediately placed on ice, mixed with reverse transcriptase (RT) enzyme, RT buffer, and dNTPs, and incubated for 60 min at 37°C, followed by denaturation for 10 min at 72°C. The resultant cDNAs, corresponding to the three aliquots of RNA, were pooled. The remaining RT enzyme, RT buffer, and dNTPs were removed using spin columns and buffers provided with the cDNA synthesis kit. The cleaned cDNAs were each brought to a volume of 150 μl, divided into 50 μl aliquots, and stored at −20°C until further analysis.

Real-time qPCR

The gene expressions of TLR9, TNF-α, NF-κB, IL-1β, and IFN-γ were analyzed by real-time qPCR using a Rotor-Gene 3000 system (LTF Labortechnik, Wasserburg, Germany). To this end, 2.5 μl of the cleaned cDNAs (=10 ng RNA) was used per analysis, and the total reaction volume in each case was 25 μl. Each sample was run in duplicate. Gene-specific primers and SYBR Green-based qPCR mix were purchased from Promolgene. The threshold levels for Ct determination were chosen manually.

Immunoassay

Standardized immunoassay with Luminex technology (fixed-panel 8-plex assay, group I; single-plex assay for IL-1β) was performed in an external laboratory (Vaccine, Rostock, Germany) according to the manufacturers' protocol. The following protein expressions were analyzed: IL-6, TNF-α, IL-1β, and IFN-γ.

Data analysis and statistical evaluation

Data analysis was performed according to the ΔCt^{-} method [23] using the mean Ct value of three housekeeping genes: β-actin, 18S RNA, and B2M. Fold changes of the expression levels of the analyzed genes were calculated as described previously[23], and the obtained values were used

for statistical analysis with the GraphPad InStat 3 statistical pack. Statistical analysis was done by one-way analysis of variances followed by Tukey post hoc test. The p values ≤0.05 were accepted as statistically significant.

Results

Changes in the gene expression levels were found in five Conelog and three BoneLevel test implants, which were therefore classified as untight implants. Two Conelog and four BoneLevel test implants did not show any LPS-mediated responses and were therefore classified as tight implants. Differences between the tested implant systems were not statistically significant ($p>0.05$). Further, the different LPS concentrations did not induce dose-dependent effects in the PC group ($p>0.05$); therefore, the results obtained for one gene for all LPS concentrations used in the PC group were pooled, and mean and standard deviation were used for statistical and graphical analysis. No changes in the gene expression levels were observed in the NC group.

The expression levels of the three housekeeping genes (β-actin, 18S RNA, and B2M) were not affected by the LPS stimulation or the presence of the test or control implants. The mean qPCR efficiency, determined by analyzing 1:10 serial dilutions of cDNA in the presence of all primer pairs used, was 98.4±4.5%. The mRNA quantity and quality of all samples were sufficient to perform cDNA synthesis using an oligo dT-V primer, because the Screentape Disintegration Variable (SDV), calculated by the Screentape software, was below 10 for all the samples processed, indicating RNA of good, intact quality according to the manufacturer's protocol. No significant differences in SDVs were observed among the groups ($p>0.05$).

Real-time qPCR results

The TLR9, TNF-α, NF-kB, IL-1β, and IFN-γ genes were differentially regulated in the PC group and test-implant group, indicating LPS leakage in both tested implant systems (Fig. 3).

TLR9 After 1 h of incubation, the PC blood samples and untight implants of both tested systems showed increased TLR9 gene expression ($p\leq0.05$). After 8 and 24 h, the expression levels in the PC blood samples and untight test implants decreased but were still elevated when compared to the NC blood samples and tight test implants; data are not shown (Fig. 3a).

NF-kB The PC blood samples and untight test implants showed increased NF-kB gene expression levels after 1 h of incubation ($p\leq0.05$). These expression levels decreased after 8 and 24 h but were still higher than levels observed in the NC blood samples and tight test implants (data not shown) (Fig. 3b).

IL-1β The PC blood samples showed a significant ($p\leq0.05$) increase in IL-1β gene expression after 1 h, whereas the untight implants of both the tested systems showed increased gene expression after 8 h of incubation. After 24 h, the untight test implants and the PC blood samples still showed high IL-1β gene expression levels (Fig. 3c).

IFN-γ After 1 h of incubation, no differences in the expression levels were observed among the groups. However, IFN-γ gene expression levels increased ($p\leq0.05$) after 8 h of incubation in the PC blood samples and untight test implants. These samples showed only slight further increased gene expression levels after 24 h of incubation (Fig. 3d).

TNF-α An increase in gene expression levels ($p\leq0.05$) was observed after 1 h of incubation in the PC blood samples and untight test implants. Thereafter, the expression levels decreased to near baseline levels in these samples (Fig. 3e).

Immunoassay results

The proteomic analysis led to the identification of the same tight and untight test implants as the genomic analysis (Fig. 4). LPS-related changes in IL-1β and TNF-α protein expressions were detected ($p\leq0.05$), similar to the genomic data, but the IFN-γ protein levels did not change (data not shown). IL-6 protein was differentially ($p\leq0.05$) expressed in the PC group and untight test implants. Protein levels of the NC blood samples as well as the tight or PBS inoculated implants were below the detection limit of the assay for all parameters tested and therefore not shown in Fig. 4. Furthermore, similar to the genomic data, no clear LPS dose-related alterations were detected at the proteomic level. Therefore, all the data per sampling point were pooled, and their mean and standard deviation were used for statistical analysis.

IL-6 Although the protein levels were similar between the untight implants of both tested systems after 1 h of incubation, the Conelog implants showed increased IL-6 protein expression when compared to the BoneLevel implants after 8 and 24 h, without a significant difference between the tested systems (Fig. 4a).

TNF-α The protein levels were elevated to similar levels in the untight implants of both tested systems at all sampling points (Fig. 4b).

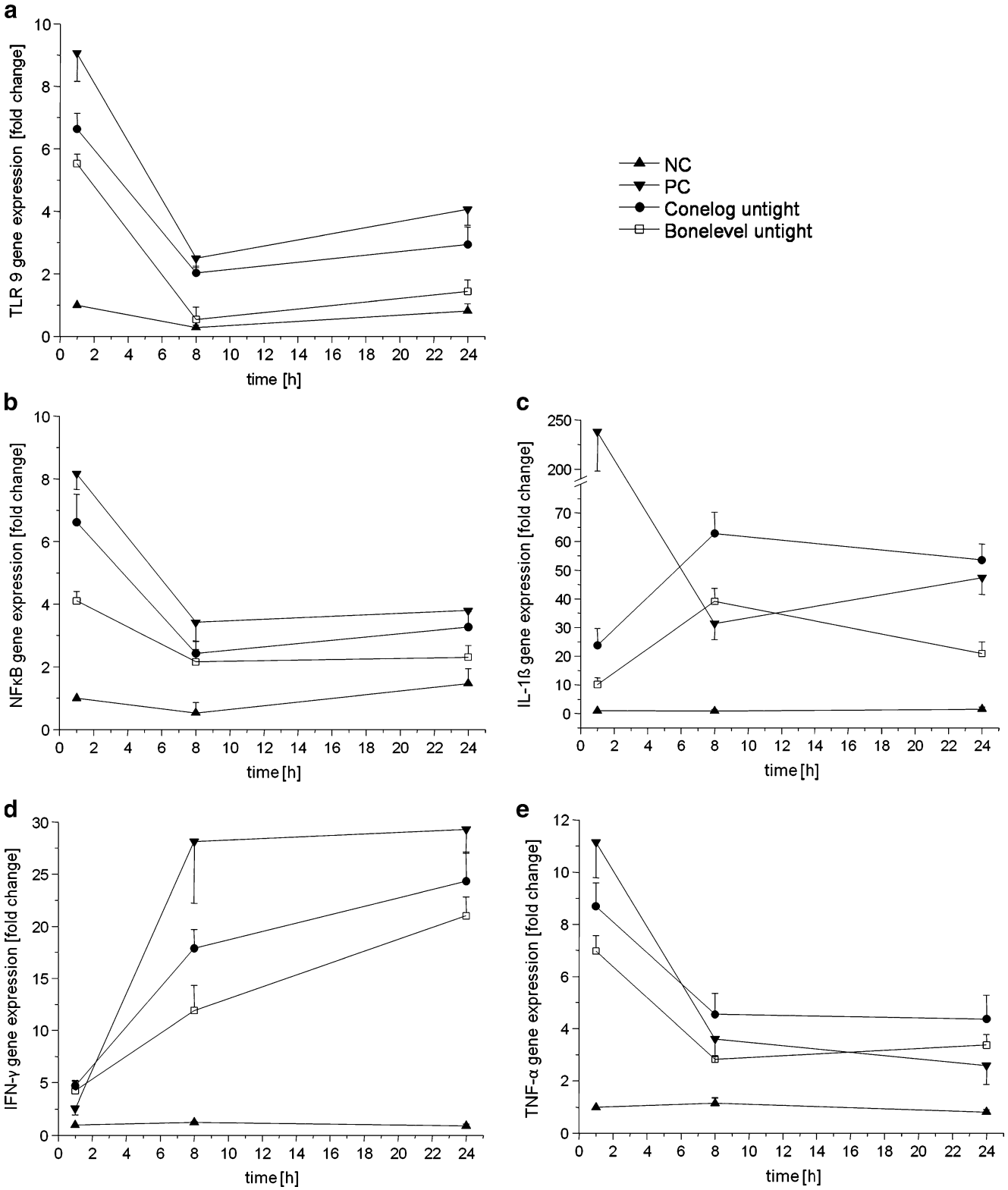

Fig. 3 Gene expression pattern at the three sampling points (1, 8, and 24 h) for negative controls (*NC*), positive controls (*PC*), and both LPS-inoculated implant systems (test implants) showing LPS release (Conelog, $N=5$; BoneLevel, $N=3$). **a** TLR9, **b** NF-kB, **c** IL-1β, **d** IFN-γ, **e** TNF-α

IL-1β The protein levels in the untight test implants increased after 8 h of incubation and remained high after 24 h of incubation, without a significant difference between the tested systems (4c).

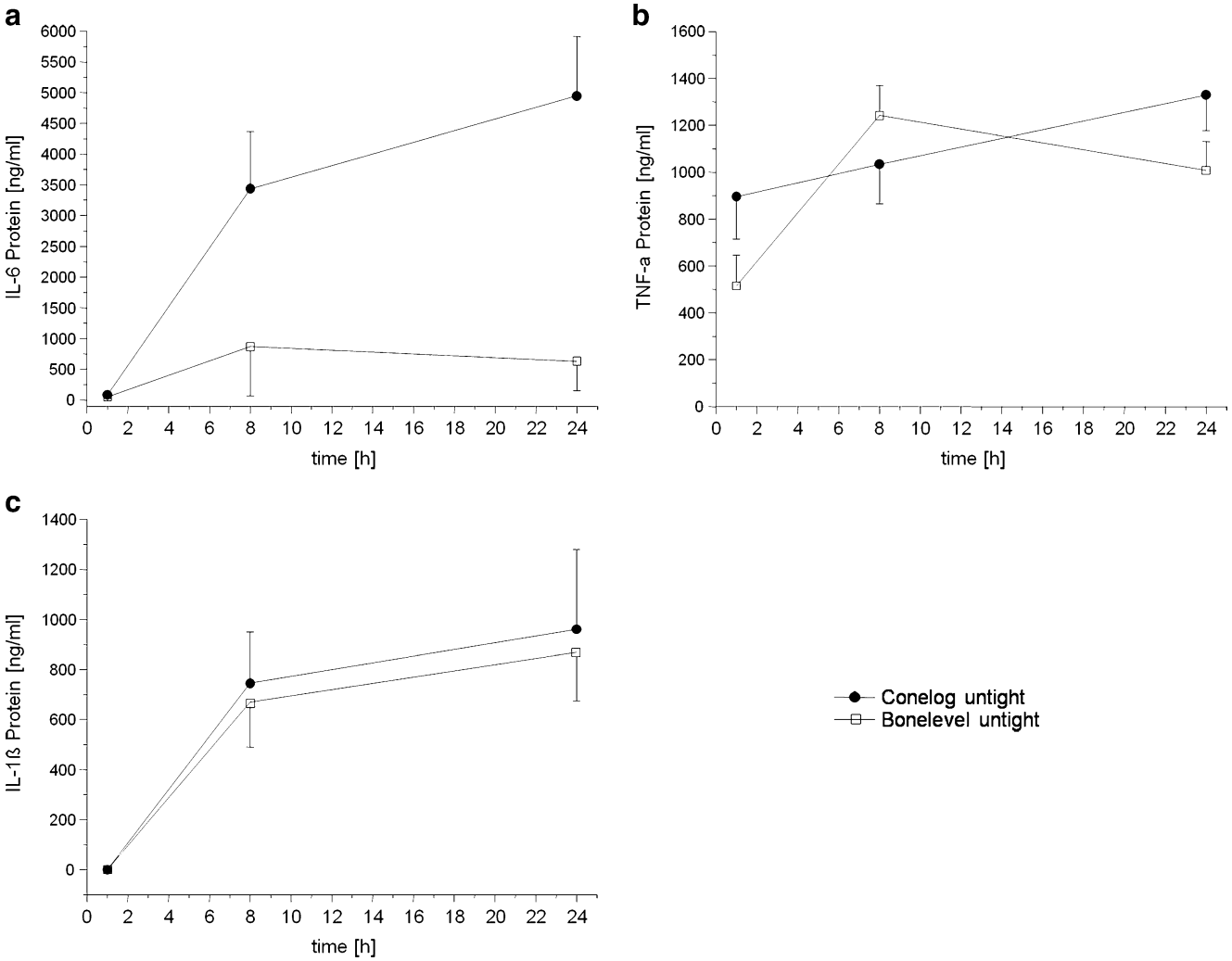

Fig. 4 Protein expression pattern at the three sampling points (1, 8, and 24 h). All data represent mean ± standard deviation: **a** IL-6, **b** TNF-α, **c** IL-1β

Discussion

In the present study, LPS microleakage at conical implant-abutment connections was assessed in terms of changes in the expression levels of genes and proteins involved in LPS-mediated cytokine production in human whole blood. Both tested implant systems showed significant changes in cytokine gene expression, indicating LPS microleakage from intra-implant cavities.

The purpose of the present in vitro study was to assess lipopolysaccharide microleakage at conical implant-abutment connections of two-piece dental implants in terms of the expression levels of genes involved in lipopolysaccharide-mediated proinflammatory cytokine production. Fresh human whole EDTA–blood was used to cover the test and control implants above the level of microgaps between the implant and the abutment. The use of human blood and assessment of cytokine release for LPS detection are well-described and established methods [24–28]. In contrast to other tests for assessing leakage at implant-abutment interfaces (e.g., dyes or bacteria), LPS detection offers the advantage of a test medium of biological origin and small molecular size, enabling penetration of even small gaps, if there are any. The high bioactivity of the LPS used in the present study allows safe and easy detection of LPS contamination of blood samples. However, LPS-induced changes in gene expression levels and the related release of cytokines in human blood show interindividual differences [26, 29]. Therefore, blood originating from a single donor was used to ensure comparable results. The results of the PC group clearly demonstrate that the blood samples showed a physiological response to LPS upon direct contact, which is in agreement with the current literature [30]. Furthermore, the immunological responses evoked in the untight test-implant groups were similar to the physiological responses in the PC group. LPS-related cytokine gene expression was absent in the NC

and tight test-implant groups, suggesting that titanium used for these implants does not evoke an inflammatory response on its own.

Clinical investigations have shown that the levels of proinflammatory cytokines such as IL-1β and TNF-α in the peri-implant crevicular fluid (PICF) depend on the microgap–bone distance, with increasing levels at smaller distances [31], and are significantly higher in the PICF of individuals susceptible to peri-implant bone loss. In a recent study, it could be demonstrated that higher levels of TNF-α at prospective implant sites seemed to be correlated to later peri-implant bone resorption [32]. Changes in levels of these factors are therefore valuable analytical tools for detecting and evaluating the prognosis of peri-implantitis. The extent to which the present in vitro findings reflect immunological events in vivo must be interpreted with caution because LPS was highly concentrated for inoculation in this study, and LPS from enteric bacteria shows different bioactivity to that from oral bacteria [33]. In various studies, the sealing capacity of two-piece dental implant systems with different connection types was evaluated. Studies revealed bacterial colonization of intra-implant cavities and abutment screws in vivo [7, 8] as well as bacterial leakage at implant-abutment interfaces in vitro [10, 11]. Previous in vitro radiological findings of the absence of micromovements at conical implant-abutment connections, indicating a good sealing capacity [34], must be reconsidered critically on the basis of the present findings. In addition, Rack et al. [35] visualized microgaps at conical implant-abutment connections under dynamic loading using monochromatic hard X-ray synchrotron radiation.

In the present study, all implant-abutment specimens were not exposed to static or dynamic load. The results of our study suggest that even under unloaded conditions, conical implant-abutment connections fabrication inherent fit tolerances of the components will often cause microleakage at a molecular level. These findings are in accordance with others studies indicating leakage of implants with screwed interference fit such as morse taper without static or cyclic loading [9, 12, 19, 36–38]. Therefore, it must be assumed that the number of leaking implants would be even higher if functional loading would be applied to the implant-abutment connection and that conical implant-abutment connections do not prevent microleakage at least on a molecular level.

Conclusions

Within the limitations of this study, the following conclusions can be drawn:

1. Conical implant-abutment connections of two-piece dental implants do not prevent LPS molecular microleakage, and leaking implants evoke immunological responses not different from those of LPS directly applied to human whole blood.
2. Small amounts of LPS released from intra-implant cavities might stimulate a detectable immunological response in human whole blood and may induce alveolar bone resorption via the osteoclast-activating pathway.
3. Further research is necessary to determine the bone resorption-stimulating effects of LPS microleakage originating from intra-implant cavities.

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Conflict of interests The authors declare that they have no conflicts of interest.

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