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ORIGINAL ARTICLE

Macrophage behavior and interplay with gingival fibroblasts cultured on six commercially available titanium, zirconium, and titanium-zirconium dental implants

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Abstract

Objectives The host-material interface has been a crucial relationship dictating the successful integration of biomaterials, including dental implants. The aim of the present study was to first investigate how macrophages behaved on various dental implant surfaces and thereafter to investigate their effect on soft tissue cells.

Materials and methods Macrophage adhesion, proliferation, and polarization towards either an M1 or M2 phenotype were investigated on six implant surfaces fabricated from pure titanium (Ti), pure zirconium (ZLA), and a titanium-zirconium (Ti-Zi) alloy of various surface topographies/chemistries. Thereafter, conditioned media (CM) collected from macrophages seeded on these various implant surfaces was cultured with murine gingival fibroblasts and investigated for their ability to promote collagen synthesis.

Results Macrophages attached and proliferated in similar levels on all implant surfaces; however, the modSLA hydrophilic surfaces tended to decrease the pro-inflammatory response by lowering the gene expression of TNF-alpha, IL-1, and IL-6 and promoting tissue resolution through the expression of an M2-macrophage cytokine IL-10. Thereafter, CM from macrophages were seeded with gingival fibroblasts on each implant surface. In general, CM from macrophages significantly promoted gingival fibroblast cell attachment on all implant surfaces at either 4 or 8 h and, most notably, significantly promoted fibronectin and TGF-beta gene expression on both Ti and Ti-Zi hydrophilic surfaces.

Conclusions and clinical relevance The present study found that implant surface topography and chemistry substantially impacted macrophage behavior. Most notably, modifications via hydrophilicity to both the pure Ti and Ti-Zi were shown to favor the secretion of macrophage pro-resolution markers and favored subsequent gingival fibroblast cell behavior when cultured with CM, whereas surface composition (Ti vs ZLA vs Ti-Zi) had little effect on macrophage polarization or gingival fibroblast behavior. This finding suggests that surface hydrophilicity would improve the soft tissue integration of dental implants, irrespective of material composition.

Keywords SLA · ZLA · Hydrophilic surfaces · Titanium · Zirconia · Dental implants

Introduction

The host-material interface between a biomaterial and a living organism is a complex interaction that dictates the fate of material integration [1]. At the center of these interactions are immune cells, a group of cell types responsible for dictating tissue adaptation [1]. Macrophages are key contributors towards the immune response to foreign materials with the ability to promote either a tissue-inflammatory (M1 macrophages) or tissue wound healing microenvironment (M2 macrophages). Despite their importance in tissue homeostasis to biomaterials, the study of macrophages has largely been omitted over the years [1].

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Dental implants are commonly fabricated utilizing pure titanium, a highly biocompatible material with long-term success rates routinely found in the 90–95% range [2–5]. More recently, implants have been strengthened with titanium-zirconium alloys or have been fabricated from pure zirconium to create ceramics [6–11]. Despite numerous implants being brought to market each year, a small percentage are lost for completely unknown reasons most likely due to a dysregulation in immune cells initially contacting the implant surfaces [1, 12, 13]. This is further complicated in the soft tissue healing zone, where both immune cells and regenerative cells are in potential contact with foreign body pathogens including bacteria and viruses. To achieve long-term stability of dental implants, peri-implant soft tissue healing is considered a crucial factor due to its function as a barrier from the oral cavity and their associated microbes [14, 15]. A quality soft tissue seal is further necessary to prevent gingival recession or in the worst case, biomaterial loss [14, 15].

Gingival fibroblasts play a prominent role in soft tissue integration around dental implants and act as the major cell-type responsible for creating a functional seal from the outside mucosa [14, 15]. Several studies have noted that the adhesion and function of gingival fibroblast on titanium are influenced by substratum surface topography [16–23]. Furthermore, hydrophilic implant surfaces have also been shown to promote better host-cell integration of implants into tissues in various in vitro and animal studies [24]. Nevertheless, while the majority of these studies have focused on gingival fibroblast behavior on various implant surfaces, their interplay with immune cells has not yet been fully characterized.

To date, studies on macrophages have demonstrated that additional surface roughness tends to promote an increase in M1 pro-inflammatory macrophages by secreting higher levels of pro-inflammatory "M1" cytokines including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and IL-1 [25–31]. While these studies have shown that surface topography may influence macrophage polarization [29], very little is known regarding how this microenvironment may affect soft tissue integration and fibroblast behavior on these biomaterial surfaces.

Therefore, the purpose of this study is twofold: First, the effect of six commercially available implant surface topography/chemistry/composition was investigated on macrophage polarization towards either an M1 or M2 macrophage phenotype including implants fabricated from pure titanium, pure zirconium, and a titanium-zirconium alloy with varying surface topographies. Thereafter, conditioned media (CM) collected from macrophages seeded on these various implant surfaces was harvested and cultured with gingival fibroblasts on their respective implant surfaces. Herein, we aim to investigate the influence of macrophage behavior on various implant surfaces and to determine which implant surface tends to more favorably generate an optimal micro-environment from host macrophages for soft tissue cell integration.

Materials and methods

Preparation of implant discs

All discs used in this experiment were provided by Straumann AG (Basel, Switzerland) with a 15-mm diameter. These discs fit directly into the bottom of 24-well culture plates. Briefly, smooth pickled (PT) titanium (Ti) surfaces were prepared using dilute nitric acid to clean the surfaces, followed by washing in reverse osmosis purified water. Roughened sand blasted and acid etched (SLA) topography surfaces were prepared by blasting the titanium with corundum particles, followed by etching with HCl/H2SO4. The modified SLA (modSLA) surface was produced with the same sandblasting and acid-etching procedure as for SLA but was rinsed under N_2 protection and continuously stored in a NaCl solution. For the titanium PT, Ti SLA, and Ti modSLA groups, commercial-grade 4 pure titanium was used for fabricating the discs. For the Ti-Zi SLA and Ti-Zi modSLA groups, a titanium-zirconium (TiZr) alloy consisting of Ti alloyed with 13–15% Zr was utilized for the discs. For the ZLA surfaces, zirconia oxide-fabricated yttria-stabilized tetragonal zirconia polycrystal was utilized and was sand blasted with corundum and acid-etched in HF at elevated temperatures, followed by rinsing in deionized water, air drying and packing in sterilized bags of sterilized H_2O_2 plasma.

Cell culture

The murine-derived macrophage cell line RAW 264.7 (China Center for Type Culture Collection) was used for macrophage experiments in the present study. RAW 264.7 cells were seeded on (1) Ti PT surfaces (TCP), (2) Ti SLA surfaces, (3) Ti modSLA surfaces, (4) Ti-Zi SLA surfaces, (5) Ti-Zi modSLA surfaces, or (6) ZLA surfaces in 24-well plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (HyClone, Thermo Fisher Scientific Inc.) at 37 °C in a humidified 5% CO_2 atmosphere.

Murine gingival fibroblasts were derived from 8-week-old healthy mice under approval of the Ethics Committee for Animal Research, Wuhan University, China. Briefly, the mandibles of the mice were collected after euthanasia. The collected mandibles were washed three times with phosphate-buffered saline (150 mM NaCl, 20 mM sodium phosphate pH 7.2) containing 1% penicillin/streptomycin, and then the gingival tissues were cut down with a sterilized surgical knife blade and then cut into small pieces with sterilized surgical scissors. The small pieces were then transferred into T25 tissue culture flasks containing minimal DMEM and allowed to adhere for 2 h. DMEM containing 20% fetal bovine serum and 1% penicillin/streptomycin was then added. After 1 week when the cells reached confluency, the gingival cells were

sub-cultured using trypsin and cultured in DMEM with 10% fetal bovine serum. All cells between the third and seventh passage were used for experiments.

Collection of conditioned media

The collection of conditioned media is intended to mimic the in vivo environment in which the macrophage on the implant surface secrets pro- and anti-inflammatory cytokines to influence the behavior of gingival fibroblasts as previously described [32]. Briefly, RAW 264.7 cells were cultured on Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA surfaces in the 24-well plates at a density of 10^4 cells per well. After 3 days, the culture medium was collected and centrifuged at 1500 rpm for 20 min at 4 °C to remove the cell debris, and then the supernatant was transferred to a new tube and frozen at − 80 °C until experimental seeding.

Adhesion and proliferation assay of RAW 264.7 cells cultured on different surfaces

For the adhesion and proliferation assays, RAW 264.7 cells were seeded on Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA surfaces in 24-well plates at a density of 10^4 cells per well. At time points of 4 and 8 h for cell adhesion and 1, 3 and 5 days for cell proliferation, the cell numbers of macrophages were determined using a Cell Counting Kit-8 (Dojindo, Japan) as previously described [32]. After incubation for 1 h, the culture medium with 10% CCK-8 was transferred to a 96-well plate and the absorbance was measured using a microplate reader scanning at 450 nm (PowerWave XS2, BioTek, Winooski, VT, USA). Samples were performed in triplicate with three independent experiments.

Inflammation-related gene expression of RAW 264.7 cells cultured on different surfaces

Real-time PCR was utilized to investigate the inflammatory gene expression of RAW cultured on different surfaces. RAW 264.7 was cultured on Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA surfaces in 24-well plates at a density of 10^4 cells per well for real-time PCR experiments. Total RNA was extracted from RAW 264.7 after 3 and 5 days using TRIzol (Invitrogen, USA) according to the manufacturer's instruction. The RNA concentration was determined by a NanoDrop 2000 UV-Vis Spectrophotometer as previously described [33]. Then, 1 μg of total RNA was used for cDNA synthesis using an Oligo(dT) and AMV reverse transcriptase (TaKaRa, Japan). RT-PCR was performed using a 20-μl final reaction volume of the QuantiFast SYBR Green PCR Kit (QIAGEN, Venlo, Holland), and the target gene expression was assayed on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, USA). The primer sequences for TNF-α, IL-1α, IL-6, IL-10, and GAPDH genes are listed in Table 1. Real-time PCR was performed under the following conditions: initial activation step at 95 °C for 10 min, followed by 40 cycles each consisting of a denaturation step at 95 °C for 30 s, annealing at 60 °C for 30 s, and an extension step at 72 °C for 1 min. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and normalized to control cells on the Ti PT group analyzed using the comparison Ct ($2^{-\Delta\Delta Ct}$) method. Each sample contained pooled mRNA collected from three titanium surfaces (one sample), and all samples were log-transformed. The experiments were performed in triplicate with three independent experiments.

Adhesion and proliferation assay of gingival fibroblasts cultured on different surfaces with or without conditioned media from macrophages

Gingival fibroblasts were seeded at a density of 10^4 cells per well on Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA surfaces in 24-well plates with or without CM and cultured for 4 and 8 h for the adhesion assay, as well as 1, 3, and 5 days for the proliferation assays. The cell numbers of macrophages were determined using a Cell Counting Kit-8 (Dojindo, Japan) as previously described [32]. After incubation for 1 h, culture medium with 10% CCK-8 was transferred to a 96-well plate, and the absorbance was measured using a microplate reader scanning at 450 nm (PowerWave XS2, BioTek, Winooski, VT, USA). Samples were performed in triplicate with three independent experiments.

Adhesion-related gene expression of gingival fibroblasts cultured on different surfaces with or without conditioned media from macrophages

Gingival fibroblasts were cultured on Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA surfaces in

Table 1 Primer pairs used in the qRT-PCR

Gene	Primer sequence
TNF-α F	TGTCTCAGCCTCTTCTCATT
TNF-α R	TGATCTGAGTGTGAGGGTCT
IL-1α F	CGAAGACTACAGTTCTGCCATT
IL-1α R	GACGTTTCAGAGGTTCTCAGAG
IL6 F	ATAGTCCTTCCTACCCCAATTTCC
IL6 R	GATGAATTGGATGGTCTTGGTCC
IL10 F	GGTTGCCAAGCCTTATCGGA
IL10 R	ACCTGCTCCACTGCCTTGCT
GAPDH F	GTGAAGGTCGGTGTGAACGG
GAPDH R	TCCTGGAAGATGGTGATGGG

24-well plates at a density of 10^4 cells per well either with or without CM for real-time PCR experiments. After 7 days of culture, total RNA was isolated from gingival fibroblasts to detect the expression of collagen 1 (Col1), fibronectin-1 (FN1), and transforming growth factor-β (TGF-β) genes using RT-PCR. GAPDH was used as a housekeeping gene, and the experiments were performed in triplicate with three independent experiments.

Statistical analysis

Statistical analysis was performed by one- and two-way ANOVA with a Bonferroni test using GraphPad Software v.6 (GraphPad Software, La Jolla, CA, USA), and statistical significance was set at $p < 0.05$. All data are expressed as the mean ± SE.

Results

Effect of surface topography, composition, and chemistry on macrophage behavior and polarization

In a first set of experiments, the effects of various implant topographies, compositions, and chemistries were investigated on macrophage adhesion, proliferation, and ability to produce either M1 tissue-inflammatory macrophages or M2 tissue-resolution macrophages. It was first found that macrophages attached on all implant surfaces in similar levels at 4 and 8 h; however, the most significant (highest) numbers were observed on the modSLA Ti surfaces (Fig. 1A, B). This trend was further apparent in macrophage proliferation experiments where modSLA promoted the highest cell number at both 3 and 5 days post-seeding compared to all other groups (Fig. 2). Thereafter, real-time PCR was utilized to investigate which surfaces led macrophages towards either an M1 or M2 phenotype (Fig. 3). First, the modified hydrophilic implant surfaces tended to decrease the pro-inflammatory response by modulating lower expressions of TNF-alpha (Fig. 3A), IL-1 (Fig. 3B), and IL-6 (Fig. 3C). Interestingly, IL-10, which was utilized as a tissue resolution M2 macrophage marker, was also significantly elevated on both modified titanium and Ti-Zi surfaces (Fig. 3D). Investigation of IL-10 concentrations performed by ELISA further revealed that both the Ti modSLA and Ti-Zi modSLA surfaces generated significantly higher IL-10 protein release from macrophages compared to their respective hydrophobic controls (data not shown).

Effect of macrophage conditioned media on fibroblast behavior cultured on various implant surfaces

In a second set of experiments, gingival fibroblasts were cultured on the various six implant surfaces with and without CM from macrophages to further investigate the effect of the macrophage micro-environment on soft tissue cell behavior. With respect to cell adhesion, CM from the majority of implant surfaces significantly promoted cell attachment of gingival fibroblasts irrespective of the implant topography/chemistry/composition at either 4 or 8 h post-seeding (Fig. 4A, B). Lastly, PCR was utilized to investigate the gene expression of fibronectin (FN), Col1a1, and TGF-beta1 (Fig. 5). While CM was able to significantly upregulate gene expression of FN on PT surfaces (Fig. 5A), it most notably significantly promoted Col1a1 on the hydrophilic Ti and Ti-Zi surfaces (Fig. 5B). Lastly, TGF-beta gene expression was also significantly upregulated, most notably on both these hydrophilic surfaces (Fig. 5C).

Discussion

The aim of the present study was to first investigate how macrophages behaved on various dental implant surfaces and thereafter to investigate their effect on soft tissue cells.

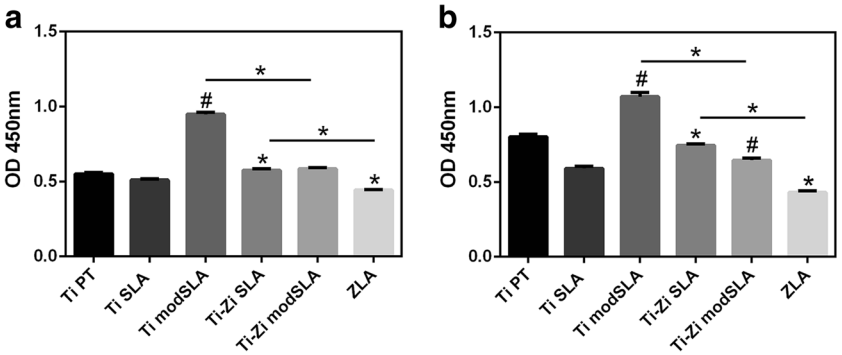

Fig. 1 Effect of surface topography, composition, and chemistry on RAW264.7 cell adhesion for Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA at 4 h (**A**) and 8 h (**B**). A significant (the highest) cell number was observed on modSLA Ti surfaces for cell adhesion at both time points. (*Significant difference between implant composition—Ti vs Ti-Zi vs ZLA; #significant difference between surface hydrophilicity—SLA and modSLA. $p < 0.05$. Data are means ± SE)

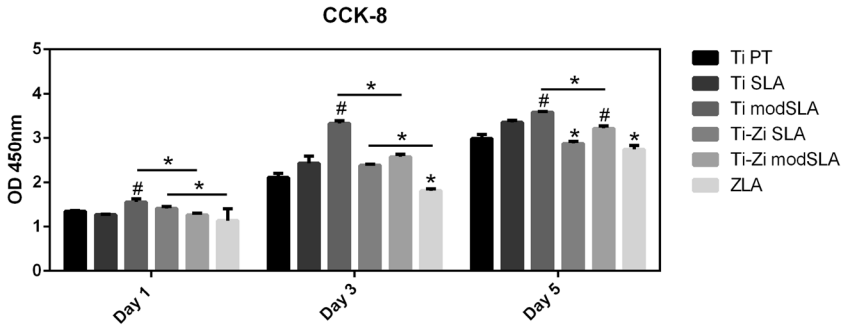

Fig. 2 Effect of surface topography, composition, and chemistry on RAW264.7 cell proliferation for Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA. The cell number on the modSLA surface was the highest at both 3 and 5 days post-seeding. (*Significant difference between implant composition—Ti vs Ti-Zi vs ZLA; #significant difference between surface hydrophilicity—SLA and modSLA. $p < 0.05$. Data are means ± SE)

While the majority of studies to date on dental implants have focused on hard and soft tissue integration either in vitro or in vivo, it remains important to note that the first cell type to come into contact with implanted biomaterials are immune cells derived from the monocyte lineage [1]. Notably, despite widespread study of immune cell interactions with various biomaterials in the medical field [34], their study in the field of implant dentistry has largely been omitted. A recent systematic review investigating the study of all cell studies on implant surfaces found that approximately 90% of all published literature focused primarily on mesenchymal cell behavior (either gingival fibroblasts, PDL cells, or osteoblasts) on implant surfaces with only 10% being dedicated to immune cell interactions, which comprise monocytes, macrophages, osteoclasts, leukocytes, and multinucleated giant cells [35]. This finding points to a lack of studies on immune modulation involving dental implants despite the fact that these cells are the first in contact with biomaterials [36].

In the present study, macrophages were first found to favor the attachment of modified SLA surfaces fabricated from titanium. It was also noteworthy that ZLA surfaces tended to decrease macrophage cell attachment (Fig. 1). This trend was further found at later time points following macrophage proliferation; after 3 days, macrophages seeded on Ti-SLA surfaces induced greater cell numbers. Thereafter, the results from macrophage polarization pointed to the fact that most

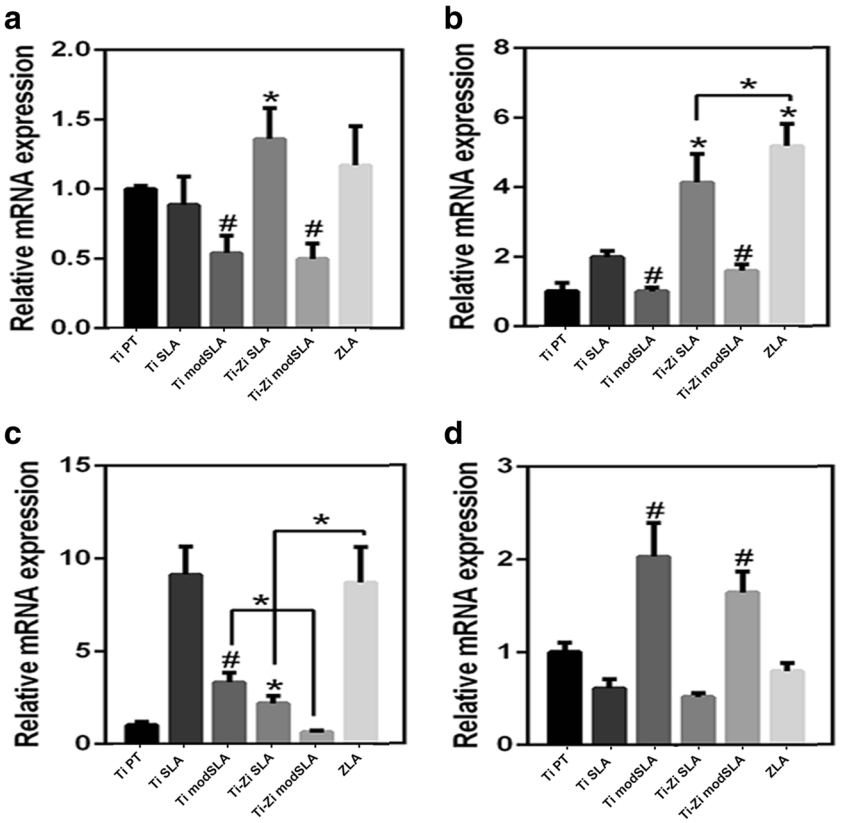

Fig. 3 Relative mRNA expression of inflammation-related genes. **A** TNF-α. **B** IL-1. **C** IL-6. **D** IL-10 for RAW264.7 seeded on Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA at 3 days post-seeding. GAPDH was used as housekeeping genes. (*Significant difference between implant composition—Ti vs Ti-Zi vs ZLA; #significant difference between surface hydrophilicity—SLA and modSLA. $p < 0.05$. Data are means ± SE)

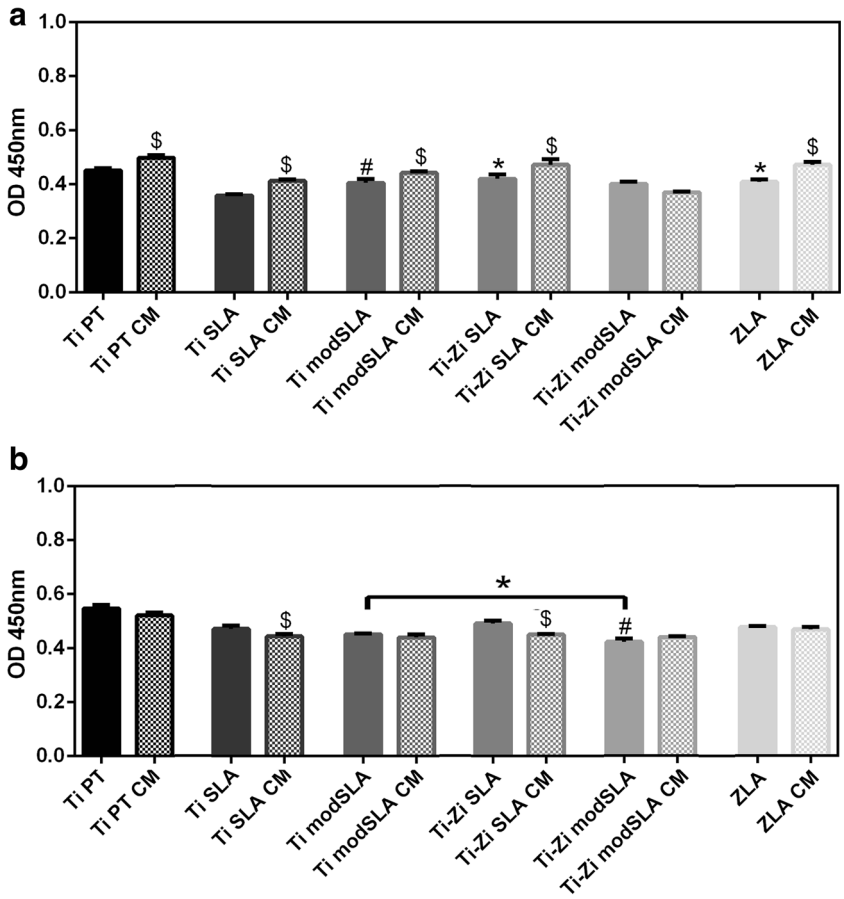

Fig. 4 Effects of surface topography, composition, chemistry, and conditioned media (CM) on gingival fibroblast cell adhesion at 4 h (**a**) and 8 h (**b**) post-seeding. CM from the majority of implant surfaces significantly promoted cell attachment of gingival fibroblasts irrespective of the implant topography/chemistry/composition. (*Significant difference between implant composition—Ti vs Ti-Zi vs ZLA; #significant difference between surface hydrophilicity—SLA and modSLA; $significant difference between normal and CM. $p < 0.05$. Data are means ± SE)

importantly, the modSLA surfaces (surfaces that are hydrophilic compared to their hydrophobic counterpart) were the most important feature promoting a M2 tissue resolution phenotype and lowered M1 pro-inflammatory gene expression (Fig. 3). This was further confirmed by ELISA, whereby the modSLA surfaces fabricated from either Ti or Ti-Zi both promoted significantly higher levels of IL-10 expression, an important transition marker for M2 macrophage polarization [1]. Therefore, the summary from the macrophage experiments demonstrated that micro-roughness of implant surfaces tended to promote a pro-inflammatory response compared to smooth PT surfaces and that a hydrophilic roughened surface was necessary to rescue this pro-inflammatory response and decrease the pro-inflammatory secretion of interleukins. Furthermore, while not directly investigated in the current study, implant chemistry is thought to play a pivotal role in macrophage behavior to implant surfaces [37, 38]. Hotchkiss et al. very recently showed that the combination of high-

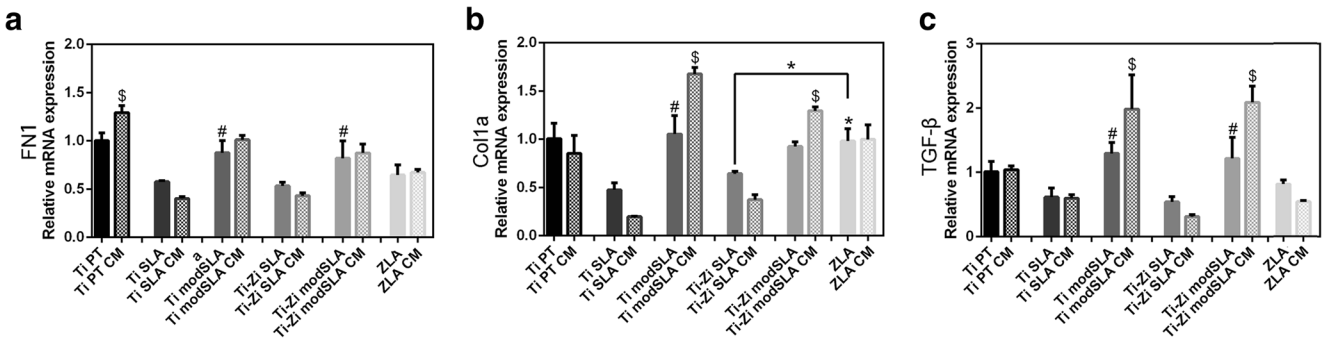

Fig. 5 Relative mRNA expression of **A** fibronectin (FN1), **B** collagen 1 (COL1), and **C** TGF-beta1 of gingival fibroblasts seeded on Ti PT, Ti SLA, Ti modSLA, Ti-Zi SLA, Ti-Zi modSLA, and ZLA at 3 days with and without conditioned media (CM). Most notably, CM significantly upregulated mRNA levels of COL1 on hydrophilic surfaces. (*Significant difference between implant composition—Ti vs Ti-Zi vs ZLA; #significant difference between surface hydrophilicity—SLA and modSLA; $significant difference between normal and CM. $p < 0.05$. Data are means ± SE)

energy and altered surface chemistry present on TiZr modSLA was able to influence macrophages to produce the greatest anti-inflammatory microenvironment and reduce extended pro-inflammatory factor release [39]. The macrophage response to hydrophilic implant surfaces has also been shown to regulate MSC recruitment and T-helper cell populations in vivo [40]. These data are in agreement with previous in vitro studies that have demonstrated that surface roughness tends to increase pro-inflammatory cytokine expression including IL-1beta, IL-6, TNF-alpha, or CCL2 [26–31].

Interestingly, while surface roughness tends to promote a pro-inflammatory response, it also encouraged gingival fibroblast cell attachment and the expression of growth factors. Therefore, surface roughness tends to play two roles: It promotes gingival fibroblast behavior while also inducing a pro-inflammatory response. We previously showed that the effect of macrophage polarization on pro-inflammatory M1 macrophages is more significant during tissue integration [32]. Therefore, the conclusion from these studies is that surface roughness tends to add both a positive response (towards gingival fibroblasts) but also a negative one (towards macrophages by increasing inflammation). Interestingly, the addition of surface hydrophilicity to implant surfaces both promoted soft tissue gingival fibroblast behavior and decreased pro-inflammatory mediators. Furthermore, when conditioned media from macrophages were cultured with gingival fibroblasts, an additional positive effect was noted (Figs. 4 and 5). Therefore, it must be pointed out that surface hydrophilicity was the primary factor in this study positively affecting cellular behavior. Interestingly, these findings were not affected by the material composition (pure Ti versus Ti alloy with Zi). Therefore, this study revealed that surface topography and, more importantly, surface hydrophilicity appear to be more prominent factors affecting cell behavior and the potential osseointegration of these implants compared to the material composition. Future in vivo study to confirm this finding is necessary.

The present study also revealed the marked impact of immune cells around implant surfaces. These cells are known to be capable of forming giant foreign body cells, and much research in recent years has focused on their importance and the implications of their long-term equilibrium [41–43]. This is further complicated by the fact that surface roughness at the trans-mucosal portion of dental implants has been linked to an increase in bacterial adherence and colonization [44, 45]. Therefore, it remains clinically relevant to note that numerous commercially available dental implants employ smooth trans-mucosal portions of the implant/abutment interface to prevent bacterial colonization and potential peri-implant disease [46, 47]. Despite these efforts, the rate of peri-implantitis remains high (often reported in the 11–47% range [48]), and further strategies to optimize these results are necessary.

Much research to date has focused on the effect of surface topography, both as it relates to hard tissue and soft tissue integration [17, 49]. Gingival fibroblasts have previously been demonstrated to favor micro-roughened surfaces or grooved surfaces capable of "guiding" fibroblast behavior [17, 49]. Based on these investigations, research on implant topography in the trans-mucosal region has attempted to optimize their design and orient aligned collagen fibers on the implant surfaces [18–20]. While implant topography certainly plays a predominant role, several recent studies have shown that surface wettability may also be an important factor [26–28]. These studies have shown that the modSLA surfaces are capable of decreasing TNF-alpha, IL1-beta, and IL-6 expression compared to control hydrophobic SLA surfaces and that they tended to promote M2 macrophage polarization both in vitro and in vivo [26–28]. Furthermore, various in vivo studies have suggested that the tissue-implant surface can be improved via macrophage polarization [26–28].

In summary, it remains of interest to further evaluate various implant surface features to better understand how to modulate macrophage behavior on their surfaces. The current study found that of the three investigated parameters, including material composition, surface topography, and surface hydrophilicity, the material surface hydrophilicity appeared to best modulate macrophage polarization, which subsequently facilitated gingival cell behavior. It remains of interest to better understand which factors and molecular mechanisms appear to drive multi-nucleated giant cell formation and foreign body reactions on these various implant surfaces and their interplay with material integration.

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Compliance with ethical standards

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

Ethical approval No ethical approval was required for this study, as human samples were not identified.

Informed consent For this type of study, informed consent to conduct the experiments outlined in this study was provided prior to blood draw.

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