Differential inflammatory landscape stimulus during titanium surfaces-obtained osteogenic phenotype

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ABSTRACT

Molecular mechanism governing inflammatory scenario in response to titanium (Ti)-nanotexturing surfaces needs to be better addressed. Thus, we subjected pre-osteoblast to different Ti-texturing surfaces, as follows: Machined (Mac), Double Acid-Etching (DAE) and nano-scaled hydroxyapatite-blasted titanium surface (nHA), considering the cells chronically responding either direct (when the cells were cultured onto surfaces) or indirectly (when the cells were challenged with the conditioned medium by the surfaces), up to 10 days. Our results showed there is a dynamic requirement of inflammatory-related genes activation in response to nHA by up-expressing *IL1ß*, *IL6*, *IL10* and *IL33* (direct condition) and *IL6*, *IL10*, *IL18* (indirect condition). Importantly, our data shows there is inflammasome involvement, once *NLRP3*, *ASC1* and *CASP1* genes were also required. As we found a strong signal of *IL10*, an anti-inflammatory cytokine, we further investigated Sonic Hedgehog (Shh) signaling cascade. Surprisingly, *Shh* ligand and Smoothened (*Smo*) genes were up-modulated in response to nHA, while Patched (*Ptc*) was down-modulated. Lastly, an interactome was built by using bioinformatics reinforcing Shh signaling cascade on modulating *IL10* transcripts by Src mediating this process and this prevalence of anti-inflammatory picture might explain the low profile of *RANKL* transcripts in response to nHA, compromising the osteoclastogenesis surrounding the implants. Taking our results into account, our data shows that the inflammatory landscape promoted by nHA is strictly modulated by Shh signaling-promoted anti-inflammatory pathways.

Key words: Implants; Bone; Titanium; Inflammation; Inflammasome; Sonic hedgehog; RANKL.

INTRODUCTION

Osseointegrative implants have been widely used in dentistry mainly to restore total, partial or complete edentulism[1], impacting the quality of life of a growing number of patients spread around the continents. Although the progress on the research covering macro-sized geometries, materials and techniques applied in dental implants, there is still much effort dedicated to improve the performance of those biomaterials[2,3], mainly in cases where there is a higher risk of treatment[4], such as patients with systemic alterations. To increase the success rate of dental implants, researchers has focused on better comprehension and control of implant's surface properties, such as topography, roughness and nano-activation and their impact on cell metabolism by considering gene expression and other molecular mechanism[5,6].

In this scenario, nanotechnology advances applied on the topography suggests an important tool for improving the performance of implants by triggering a guided molecular mechanisms requiring specific intracellular phosphorylation's cascade of specific proteins and consequently increasing bone neoformation [6,7]. In this way, Jimbo et al (2012) proposed that nanotexturized titanium surfaces do not only improve bone formation, but also ameliorate the strengthen of their biomechanical properties[8]. Although nanometric-scaled structures have important properties considering osseointegration, detailed interfacial interactions with osteogenic cells have not been fully addressed. On this matter, we have previously mapped the cellular phenotype of osteoblast when in response to nano hydroxyapatite-blasted surfaces (nHA), showing a decisive capacity of the nHA in stimulating osteoblast differentiation by requiring Src as pre-requisite[9,10]. However, in order to better comprehend the molecular mechanisms triggered by nHA, other aspects must be addressed such as inflammatory cytokines reprogramming.

Based on the surface's properties, peri-implant bone formation requires a sequence of partially-known biological events governing the success of the osseointegration of implants[11,12] and in this way the inflammation-related molecules have gained importance on this process[13]. Thus, we proposed evaluating a repertory of inflammatory-related genes in response to nHA, as well as Sonic Hedgehog (Shh) signaling cascade, a known signaling cascade involved with anti-inflammatory signals. Moreover, Shh

signaling cascade is an intricate signal transduction governing developmental processes guaranteeing osteoblast performance[14]. Summarizing, our results clearly shows the involvement of Shh signaling on modulating the dynamic repertory of the inflammatory-related genes activation proposing a new landscape for the molecular mechanism involved with the peri-implant cellular behavior in response to Ti-texturing surfaces, contributing with our better knowledge regard the success of the Ti-based implants osseointegration.

MATERIAL AND METHODS

Materials

Three different titanium surfaces (discs) were as follows: Machined (Mac; control), Dual Acid-Etched (DAE), and acid-etched nanoHA-blasted (nHA). Regarding the nHA, it was obtained by using the Promimic nano-HA-method, a detailed description can be found elsewhere [15,16]. Briefly, the samples were dipped into a stable particle suspension containing 10 nm in diameter HA particles followed by a heat treatment at 550°C for 5 min in nitrogen atmosphere. The surfactant-mediated process allows better control of the chemical composition of the coating[16]. The primers used in this study were purchased from Exxtend Soluções em OLIGOS (Campinas, São Paulo, Brazil). All of the titanium materials was sterilized by exposure to Gamma irradiation and donated by S.I.N. – Sistema Nacional de Implantes (São Paulo, SP, Brazil). Primers and experimental details of the qPCR are described in **Table 1**.

Cell culture

MC3T3-E1 lineage (subclone 4), mouse pre-osteoblastic cells, was used in this study. The cells were cultured in α-MEM supplemented with 10% of Fetal Bovine Serum (FBS) at 37°C and 5% CO_2. Sub-confluent passages were tripsinized and used in all experiments. Following the working flow, the cells were seeded either on the titanium surfaces (direct contact) or treated with the titanium-enriched medium (indirect contact, prepared in according with the ISO 10993: 2016) up to 10 days (for the enough time to obtaining osteogenic phenotype), as proposed by Bezerra et al (2017)[9]. During this timeline, the cells were maintained at 37°C in cell culture incubator and the culture medium changed every 3

days in order to maintain an adequate concentration of nutrients to the cells and/or titanium-enriched medium.

Quantitative PCR assay (qPCR)

Cells were directly cultured on different texturized titanium surfaces or challenged with titanium-enriched medium up to 10 days as detailed earlier, when the total mRNA was extracted with Ambion TRIzol Reagent (Life Sciences - Fisher Scientific Inc, Walthan, MA, USA) and treated with DNase I (Invitrogen, Carlsband, CA, USA). Thereafter, the cDNA synthesis was performed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. qPCR was carried out in a total of 10 µl, containing PowerUp™ SYBR™ Green Master Mix 2x (5µl) (Applied Biosystems, Foster City, CA), 0,4 µM of each primer, 50 ng of cDNA and nuclease free H_2O. Data were obtained and the results were expressed as relative amounts of the target gene using β-actin as housekeeping gene and using the cycle threshold (Ct) method.

<< Table 1. Expression primers sequences and PCR cycle conditions.>>

Bioinformatic analysis

String[17] was used to build a network using the input: Pycard, il13, il1b, il6, il18, nlrp3, casp1, spp1, ibsp, il33, il10, tnf, tnfsf11, sp7, tnfsf14, shh, ptch1, smo, mtor and src, for *homo sapiens* background. The String parameters was Text-mining, Experiments and Databases, with the minimum required interaction score 0.400 (medium confidence). Gene Ontology[18] and KEGG[19] were used for enrichment analysis of the network.

Statistical analysis

Mean values and standard deviation obtained for each test were calculated, and one-way ANOVA was performed (alpha error type set to 0.05) when adequate, with Bonferroni corrected post-test, or non-parametric analysis, using GraphPad Prysm 5 (GraphPad Software, USA).

RESULTS

We proposed evaluating the inflammatory landscape required in response to nano HA-blasted titanium surfaces using qPCR by exploring a repertory of related gene (**Fig.1a**).

Validation of the Ti-texturized surfaces on driving osteogenic phenotype

Firstly, we validated the capacity of nano HA-blasted titanium surface (nHA) in modulating osteoblast differentiation by considering classical osteogenic gene markers, as follows: Osterix (*Osx*) was up-expressed in response to DAE and nHA considering the direct contact (**Fig.1b**). Indirectly, other genes related with osteogenic phenotype were modulated - Osteoprotegerin (*Opg*) was up-expressed in response to DAE and down-regulated by nHA (**Fig.1c**), Bone sialoprotein (*Bsp*) was significantly down-modulated in response to DAE (**Fig.1d**), Osteopontin (*Opn*) was significantly up-expressed in response to both DAE and nHA, around 6- and 5-fold change, respectively, when compared with the control group (Mac) (**Fig.1e**). Lastly, receptor activator of nuclear factor kappa-B ligand (*Rankl*) was significantly down-regulated in response to both texturized surface DAE and nHA (**Fig.1f**), suggesting a low profile of osteoblast-modulated osteoclastogenesis.

Nano HA-blasted titanium surface requires a specific repertory of inflammatory-related genes

By considering the direct effect of the titanium-related surfaces on osteoblast behavior, we observed a dynamic reprogramming of inflammatory-related genes, as follows: *TNFα* was differentially modulated considering those Ti-surfaces, it being up-expressed in response to DAE, while nHA did not promote any change compared with the control (**Fig.2a**). In addition, nHA triggered significant up-modulation of *IL1ß* (**Fig.2b**), *IL6* (**Fig.2c**), *IL10* (**Fig.2d**), while *IL18* was significantly down-modulated (**Fig.2f**). The classical DAE surface promoted significant up-modulation of *IL6* (**Fig.2c**), *IL10* (**Fig.2d**), *IL33* (**Fig.2e**), *IL18* (**Fig.2f**) and *IL13* (**Fig.2g**). Moreover, *NFkB* was required in this scenario, but it was significantly up-expressed only in response to DAE (**Fig.2h**).

The indirect contact triggered particular cell responses, where we found that nHA promoted up-expression of *IL6* (**Fig.3c**), *IL10* (**Fig.3d**) and *IL18* (**Fig.3f**); while *IL1ß* (**Fig.3b**),

IL13 (**Fig.3g**) and *IL33* (**Fig.3e**) were down-modulated in this case. Although *TNFα* was significantly up-expressed in response to DAE (**Fig.3a**), *NFkB* was down-modulated (**Fig.3h**), suggesting specific modulation of the post-transcriptional mechanism on *TNFα* molecular processing.

Inflammasome complex-related genes landscape

We also considered evaluating whether inflammasome related genes were required in response to texturized titanium surfaces (**Fig.4a**). Direct contact promoted *NRLP3* significant up-modulation in response to DAE (**Fig.4b**), as well *CASP1* (**Fig.4d**), while *Asc1* showed only a slight up-modulation (**Fig.4c**). nHA-blasted titanium surfaces promoted up-modulation of *NLRP3* (**Fig.4b**), *Asc1* (**Fig.4c**) and *CASP1* (around 6-fold changes) (**Fig.4d**). Considering the indirect contact-related model, both DAE and nHA down-modulated *NLRP3* (**Fig.4e**) and *Asc1* (**Fig.4f**), while *CASP1* was up-modulated by nHA (3.5 fold-change) (**Fig.4g**).

Sonic hedgehog (Shh) signaling cascade-related genes were dynamically active

As Shh signaling modulates the inflammatory repertory, we decided evaluating whether they are involved in response to nHA. To address this issue, we explored Shh signaling members, such as Shh, Smo and Ptc (schematization in **Fig.5a**). Considering the direct contact model, *Shh* (**Fig.5d**) and *Smo* (**Fig.5d**) were up-modulated in response to DAE, while *Ptc* was down-modulated in response to both DAE and nHA (**Fig.5c**). In addition, nHA promoted up-modulation of *Smo* (around 70 fold-change, **Fig.5d**).

Indirect contact model promoted both *Shh* (**Fig.5e**) and *Ptc* (**Fig.5f**) down-modulation in response to the assayed surfaces, while *Smo* gene was significantly required in response to DAE (around 70 fold-change**, Fig.5g**).

Lastly, exploring bioinformatics, a new molecular landscape is proposed considering inflammatory panorama in response to nano hydroxyapatite-blasted titanium surface: string network shows that Shh members are able to process interleukins, mainly

through Src and mTor as linkers, providing molecular possibilities of Shh signaling in modulating IL10 processing (**Fig. 6**).

DISCUSSION

Although some progress has been reached over the last years[12], still little is known about the inflammatory scenario promoted by osteoblast responding to texturing titanium surfaces. In order to better address this issue, we explored direct and indirect contact-based models by using pre-osteoblast cells. To note, indirect contact model was also considered in this study once we have reported previously elsewhere that Ti-enriched medium affects profoundly osteoblast metabolism[20].

Our results showed nHA is able to promote osteoblast differentiation by driving the activation of a classical repertory of osteogenic gene markers and these results corroborate with Bezerra et al (2017)[9]. Important in this scenario is the capacity of both evaluated texturing-surfaces in promoting a down-activation of *RANKL* gene, a classical stimulating factor driving osteoclastogenesis[21]. At this first biological stage, our data shows that Ti-texturing surfaces controls preferentially osteoblast performance contributing consequently with the osseointegration of the implants, avoiding the generation of new osteoclasts.

In turn, osteoblast differentiation and further deposition of bone *de novo* surrounding the implants seems to be a complex mechanism[12,22] and now we suggest inflammatory-related landscape as a considerable factor to be considered on their understanding. Here, we focused on evaluating the inflammatory requirement by evaluating genetic machinery in response to the Ti-texturing surfaces. In fact, our data shows there is dynamic inflammatory-related gene activation - importantly, *IL1ß* and *IL6* were up-expressed by the challenged osteoblasts and they are related as signaling factors able to trigger osteoclastogenesis[23,24] by mediating RANKL expression. In conjunction, the increase on *IL1ß*, *IL6* and *TNFα* genes would stimulate osteoclast formation from mononuclear cells further inducing bone loss. However, in this study osteoblast adaptation to nHA surfaces seems requiring a molecular mechanism independent of *RANKL*, opening new questions about this molecular mechanism. In addition, as the tissue-based biological

response to Ti-based dental devices requires a multitude of other cells than osteoblasts[12,13] , we suppose a necessity to evaluate other cell's lines and functional approaches in this context, such as macrophages, and it is a technical limitation of this study.

Secondly, inflammasome was evaluated, as follows: *NLRP3*, *ASC1* and *CASP1* genes. Inflammasome are intracellular complexes able to convert pro-Interleukins (ILs) into biologically active ILs, such as *IL-1ß* and *IL18*[25]. On this scenario, it seems be a mechanism involved with the response to nHA, once our data shows *IL1ß* and *IL18* involvement on both direct and indirectly related models, while there was an significant up-modulation of *CASP1* (around 5-fold changes). Regarding caspase involvement, Mogi and Togari (2003) demonstrated that normal osteoblast differentiation requires a transient activation of several caspases, and they suggest a novel physiological function of caspases during osteoblast differentiation process[26]. Thus, we suggested this increase on profile of CASP1 transcripts be involved with the inflammasome-dependent ILs activation, reinforcing the importance of ILs on driving osteoblast differentiation, here in response to the Ti-texturing surfaces.

Lastly, as we and others have reported Sonic hedgehog (Shh) signaling in osteoblast biology[(14),(27)] and it has been also repeatedly linked with inflammatory picture[5,28], the present study also addressed Shh signaling members and we have shown the importance of this signaling pathway in responding to Ti-texturing surfaces. Although the exact role of Hh signaling in the regulation of inflammation is still poorly understood, this is proposed to serve as an anti-inflammatory factor at an autocrine manner[29]. In order to address this opening question, Zhou et al (2012) showed autocrine Shh as a ligand able to up-regulate IL-10[29], a well-known anti-inflammatory interleukin. This brief background is very helpful to understand the up-modulation of IL10 in response to both DAE and nHA, concomitant with the increase of Shh signaling members (*Ptc, Shh* and *Smo*). Thus, it is clear that even existing an increase of the pro-inflammatory gene machinery in a chronic response to the Ti-texturing surfaces (10 days) there is concomitantly a strong anti-inflammatory stimulus governed by Shh driving *IL10* expression. On this context, a draw of the molecular network of these findings was necessary, and bioinformatics hypothesizes a strong crosstalk between Shh signaling and *IL10* being mediated by Src and mTor proteins.

Unsurprisingly, we reported previously elsewhere the involvement of Src in the intracellular signaling in response to nano HA-blasted titanium surfaces[10] and this reinforces our previous hypothesis. This anti-inflammatory signal might explain the low profile of RANKL expression by osteoblasts, guaranteeing consequently a low profile of osteoclastogenesis at this stage, as already detailed.

By considering the experimental and technical limitations of this study, our results suggest that the inflammatory picture in response to nHA is regulated by anti-inflammatory mediated by Shh signaling cascade during osteoblast differentiation. Altogether, our results contribute with our better knowledge about the clinical success of the Ti-based dental implants.

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Competing financial interests

The authors declare no competing financial interests.

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FIGURE CAPTIONS

Fig. 1. Titanium-texturing surfaces promote osteoblastic gene markers modulation. a) Experimental design and timeline used in this study: the pre-osteoblasts were challenged by the Ti-texturing surfaces considering both direct and indirect contact related model, up to 10 days. **b)** *Osterix* gene was up-expressed by osteoblasts cultured directly on the surfaces, while Ti-enriched medium (indirect model) promoted a dynamic expression of *OPG* (**c**), *BSP* (**d**), *OPN* (**e**) and *RankL* (**f**). Letters were used when there were significance differences ($p<0.05$).

Fig. 2. Ti-texturing surfaces reprogram inflammatory gene landscape during osteogenic phenotype. There was a strict modulation of inflammatory related genes up to 10 days of pre-osteoblast cultured directly on Ti-texturing surfaces, as follows: *TNFα* (**a**), *IL1ß* (**b**), *IL6* (**c**), *IL10* (**d**), *IL33* (**e**), *IL18* (**f**), *IL13* (**g**), *NFkB* (**h**). Letters were used when there were significance differences ($p<0.05$).

Fig. 3. Ti-enriched medium reprograms inflammatory gene landscape during osteogenic phenotype. There was a dynamic modulation of inflammatory related genes up to 10 days of culturing pre-osteoblast in the presence of Ti-enriched medium (indirect model), as follows: *TNFα* (**a**), *IL1ß* (**b**), *IL6* (**c**), *IL10* (**d**), *IL33* (**e**), *IL18* (**f**), *IL13* (**g**), *NFkB* (**h**). Letters were used when there were significance differences ($p<0.05$).

Fig. 4. Inflammasome-related genes were dynamically modulated by the Ti-texturing surfaces. a) Draw of the inflammasome complex requiring $NLRP_3$, ASC and Caspase 1 is shown. Considering the osteoblast cultures, it was possible to observe a significant up-expression of the *NLRP3* (**b**), *ASC* (**c**) and *Caspase 1* (**d**). In addition, these genes were also modulated considering the indirect contact, when pre-osteoblasts were treated with Ti-enriched medium up to 10 days, as follows: *NLRP3* (**e**), *ASC* (**f**) and *Caspase 1* (**g**). Letters were used when there were significance differences ($p<0.05$).

Fig. 5: Sonic Hedgehog (Shh) signaling cascade-related genes was modulated considering both direct and indirect based models. Shh signaling cascade is an intricate signal transduction mechanism governing developmental processes as osteoblast differentiation. **a)** Schematization of the Shh signaling cascade: inactive signaling (left) occurs in the absence of Shh ligand wherein Patched (Ptc) inhibits a 7-transmembrane protein Smoothened (Smo). In the presence of Shh (right), Ptc suppression of Smo is abrogated resulting in the transcription of specific genes. Ti-texturing surfaces promoted a dynamic expression of Shh, Ptc and Smo by considering direct contact (**b-d**, respectively)

and indirect contact (**e-g,** respectively). ß-actin was considered a housekeeping gene. Letters were used when there were significance differences ($p<0.05$).

Fig. 6. Bioinformatics predicts an interactome and reveals dependency of Src within anti-inflammatory signaling. In conjunction, the signaling cascade requires a multitude of molecules highlighting a crosstalk between Shh signaling and interleukins processing (mainly IL10). Lastly, this anti-inflammatory picture might explain the low profile of RANKL by osteoblasts in response to Ti-texturing surfaces.

Table 1. Expression primers sequences and PCR cycle conditions.

Gene	Primer	5'-3' Sequence	Reactions Condition
Osterix	Forward	CCCTTCCCTCACTCATTTCC	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	CAACCGCCTTGGGCTTAT	
BSP	Forward	GTACCGGCCACGCTACTTTCT	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	GTTGACCGCCAGCTCGTTTT	
OPG	Forward	CAGAGACTAATAGATCAAAGGCAGG	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	ATGAAGTCTCACCTGAGAAGAACC	
Rank L	Forward	CGCTCTGTTCCTGTACTTTCGAGCG	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	TCGTGCTCCCTCCTTTCATCAGGTT	
OPN	Forward	TTTGCTTTTGCCTGTTTGGC	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	CAGTCACTTTCACCGGGAGG	
IL1β	Forward	GACCTTCCAGGATGAGGACA	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	AGCTCATATGGGTCCGACAG	
IL6	Forward	AGTTGCCTTCTTGGGACTGA	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	CAGAATTGCCATTGCACAAC	
IL10	Forward	CCAAGCCCTTATCGGAAATGA	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	TTTTCACAGGGGAGAAATCG	
IL13	Forward	CAGTCCTGGCTCTTGCTTG	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	CCAGGTCCACACTCCATACC	
IL18	Forward	ACTTTGGCCGACTTCACTGT	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	GGGTTCACTGGCACTTTGAT	
IL33	Forward	CCTTCTCGCTGATTTCCAAG	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	CCGTTACGGATATGGTGGTC	
TNF-α	Forward	CCACATCTCCCTCCAGAAAA	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	AGGGTCTGGGCCATAGAACT	
NFKβ	Forward	GGTTCAGGAGCTGCTGAAAC	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	GGTTCAGGAGCTGCTGAAAC	
NRLP3	Forward	ATTACCCGCCCGAGAAAGG	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	TCGCAGCAAAGATCCACACAG	
Caspase 1	Forward	TGAAAGAGGTGAAAGAATT	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	TCTCCAAGACACATTATCT	
ASC1	Forward	AGACATGGGCTTACAGGA	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	CTCCCTCATCTTGTCTTGG	
SHH	Forward	CCAACGTAGCCGAGAAGACC	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	TCCCGTGTTTTCCTCATCCT	
SMOOTH	Forward	GCTGGAGCTTTGCCTTATTGG	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	GGTCAAAAAGGTCTCAGTGAACT	

Ptch1	Forward	ACACTTCAGGGGCTACGACTATG	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	TGGGGCGACACTTTGATG	
β-Actin	Forward	TCTTGGGTATGGAATCCTGTG	95°C - 3s; 60°C - 8s; 72°C - 20s
	Reverse	AGGTCTTTACGGATGTCAACG