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**IN VITRO EFFECTS OF BETA-TITANIUM ALLOYS ON THE BIOLOGICAL BEHAVIOR OF MACROPHAGES AND THEIR CROSS-TALK WITH OSTEOBLASTS**

The inflammatory response is an important component of host response to biomaterials and therefore it represents a useful tool in assessing the biocompatibility both in the acute and chronic phases. Macrophages (MF) play a central role in triggering inflammatory and immune processes in the host organism. In addition, they contribute to the healing response of the host tissue, reaching before the arrival of undifferentiated mesenchymal cells at the site of implantation and, therefore, they may be responsible for directing the response of these progenitor cells, which will differentiate into osteoblasts. Consequently, the assessment of the MF behaviour on biomaterials is of major importance in understanding the host's response mediated by the implanted material.

Titanium (Ti) and Ti alloys are commonly used in medical applications due to their excellent biocompatibility and good mechanical properties [1]. However, the Ti-based devices, widely used as implants, generally have a Young's modulus higher than bone, which affects the long-term performance, due to the "stress shielding" phenomenon [2]. The effects associated with the stress at the bone-implant interface creates numerous unwanted reactions and ultimately, they can lead to the loss of tissue-implant contact, premature failure of the implant or infections induced by debris [3]. This explains why reducing the elastic stiffness must be regarded as a priority in the overall strategy of designing Ti-based alloys for biomedical applications. *In vivo* studies have demonstrated that Ti-based alloys with low Young's modulus are effective to inhibit the bone atrophy and to improve the formation of bone tissue [4-6]. Also, efforts are being made to replace aluminum (Al) and vanadium (V) from the commercial Ti alloys, which are cytotoxic, with other more safe alloying elements [7,8], which provide good mechanical properties, reduce the elastic modulus, while keeping the high resistance of the conventional alloys. Therefore, a new generation of β-Ti alloys without Al and V, with low modulus, began to be developed using elements that stabilizes β structure and, also, biocompatible elements such as Nb, Ta and Zr [9-19].

Initial studies elaborated within the project which aimed to evaluate the behavior of RAW 264.7 MF maintained in contact with the surface of β-Ti alloy, Ti23Nb0.7Ta2Zr0.5N, demonstrated the good biocompatibility of this material. Thus, it was revealed that this alloy does not exert cytotoxic effects ([Fig. 1](#)), having the ability to support the adhesion and proliferation of RAW 264.7 cells ([Fig. 2 and 3](#)), in the same way as the reference material, Ti6Al4V. Moreover, the results showed a high percentage of viable cells (positive for calcein) on the surface of the two metals, and a small number of cells in apoptosis (labeled in red with EthD-1) ([Fig. 4](#)).
Immunofluorescence studies investigating the cell morphology indicated that the Ti23Nb0.7Ta2Zr0.5N alloy surface supports the normal development of RAW 264.7 MF cytoskeleton (Fig. 5). Instead, MF grown in the presence of LPS (concentrations ranging from 10 to 1000 ng/ml) undergo major morphological changes, showing a considerably higher degree of spreading, elongated and stellate shape, lamelipodia and filopodia (Fig. 6).

On the two surfaces, punctate vinculin was distributed intracytoplasmic, on the base of the filopodia and lamelipodia which co-localized with actin, indicating the presence of focal contacts (Fig. 7). The pattern of vinculin expression on the two subtrates was similar, suggesting no differences in cell-support interactions.
Another objective of the present study was to highlight the podosomes by assessing the co-localization of vinculin and paxillin, and by staining the gelsolin. After 24 hours of culture, the fluorescent images revealed a punctiform pattern of gelsolin expression dispersed uniformly throughout the cell mass and its co-localization with actin. After 48 hours, regions with high concentrations of gelsolin were observed at the cell periphery (Fig. 8). Furthermore, the podosomes were labeled by immunofluorescent staining of the other two proteins, paxillin and vinculin, showing a similar distribution on the analyzed surfaces (Fig. 9).

Studies that aimed at determining the gene and protein expression profiles of the pro-inflammatory cytokines and chemokines produced by MF grown on Ti23Nb0.7Ta2Zr0.5N and Ti6Al4V surfaces, revealed their overexpression by MF treated with LPS and a strong inhibition of their expression at 48 h compared to 24 h post-seeding. A similar pattern of gene expression and protein secretion of cytokines and chemokines have been noticed by comparing the two substrates (Fig. 10 and 11).

In conclusion, the analyzed surfaces promote the transition from a pro-inflammatory profile of cytokines and chemokines at 24 hours post-seeding to a wound healing profile at 48 hours of cell culture, without affecting the adhesion, proliferation and viability of MF. Thus, the obtained data recommend Ti23Nb0.7Ta2Zr0.5N alloy as a suitable candidate for biomedical applications.
Investigation of some alloys from Ti-Mo-Nb system, which exhibit shape memory and superelastic properties, showed good compatibility with RAW 264.7 cells. The obtained data showed that β-Ti alloys from Ti-Mo-Nb system and the control alloy, Ti6Al4V, equally support cell adhesion and proliferation. Regarding the potential inflammatory responses, the results have shown little effect of these surfaces on the secretion of pro-inflammatory cytokines and chemokines, which is an important feature of the optimal tissue healing after the device implantation (data not shown).

A well-known and safe treatment to increase the materials performance for biomedical applications is the ion implantation, such as nitrogen. RAW 264.7 cells grown on the surface of the β-Ti alloy, Ti-25Nb-25Ta implanted with nitrogen ions, released into the culture medium equal quantities of inflammatory cytokines and chemokines to those produced by cells grown on pristine Ti-25Nb-25Ta alloy. After 48 h of culture, a marked decrease in the secretion of these factors was observed when cells were treated with LPS. Regarding the MF morphology, the results suggest that both TiTaNb-based substrates support the normal development of the actin cytoskeleton and of the microtubule network (data not shown). At 6 days post-seeding, the presence of actin filaments organized in podosomes was remarked in the cytoplasm of LPS-stimulated cells (Fig. 12). Moreover, it was noticed that LPS induces the MF fusion and the formation of multinucleated giant cells (FBGCs) (Fig. 13).

![Fig. 12. Fluorescent micrographs of RAW 264.7 cells labeled with phalloidin-FITC showing actin organization in podosome-like structures. MF were cultured for 6 days in the presence of LPS (100 ng/ml). Podosomes organization in: (A) clusters; (B) and (C) rosettes; (D) bands, is indicated by arrows.](image)

![Fig. 13. Fluorescent micrographs of RAW 264.7 cells cultured for 6 days in the presence of 1000 ng/ml LPS. (A) multinucleated giant cells formed by MF fusion after activation with LPS. (B) Microscopic fields with unfused MF; The fixed cells were labeled with Phalloidin-TRITC and DAPI to stain the actin and the nucleus, respectively.](image)

During the occurrence of FBR, a transient fibrin matrix is formed around the implanted material, primarily caused by the clotting process. To reach the inflammation site, MF must migrate through this extracellular matrix (ECM), which provides structural support to maintain tissue integrity and to promote cell adhesion. Matrix metalloproteinases (MMPs) are involved in ECM remodeling, having a crucial role in the migration of inflammatory cells and hence, in the progression of the whole inflammatory response. MMPs activation is an important control point that maintains the balance between matrix remodeling and destruction. Activated MF express high levels of MMPs to allow migration through ECM. The proteolytic activity of MMPs is controlled by their tissue inhibitors (TIMPs).

Therefore, another objective of our study was to analyze the MMPs and TIMPs expressed by RAW 264.7 MF grown on β-Ti alloy, Ti23Nb0.7Ta2Zr0.5N, as compared with Ti6Al4V. In the conditioned culture medium without serum, the gelatinolytic activities corresponding to MMP-9 and MMP-2 were similar in the case of MF grown in contact to Ti6Al4V and Ti23Nb0.7Ta2Zr0.5N (Fig. 14). This study was also performed in the serum containing culture media, the results being shown in Fig. 15. Similarly, a high expression of proMMP-2 and proMMP-9 was observed and the expression of proMMP-9 was stimulated by LPS. Western blot analysis for the detection of MMP-9 (Fig. 24) and MMP-2 (Fig. 25) in the
conditioned culture medium confirm the similar expression levels of the gelatinases in the culture medium of MF grown on Ti6Al4V and Ti23Nb0.7Ta2Zr0.5N, and also, the stimulatory effect of LPS on the expression of MMP-9.

Also, ELISA studies have shown no significant differences in the concentration of MMP-9 in the culture medium of MF grown on Ti6Al4V as compared to 8-Ti alloy, although lower levels of MMP-9 were detected on the latter, in pro-inflammatory culture conditions (data not shown).

Analysis of MMP and TIMPs expressed by RAW 264.7 MF grown on Ti23Nb0.7Ta2Zr0.5N as compared Ti6Al4V showed that 8-Ti alloy does not stimulate the synthesis and activation of MMPs in a higher extent than the control, which is desirable in the connective tissue surrounding a bone implant.

The body's defense mechanisms lead to the formation of multinucleated giant cells from monocytes-MF precursors as a part of the host response to foreign bodies, such as implanted materials. It has been shown that ADAM-9 (a transmembrane glycoprotein expressed at the interface of tissues with aseptically loosed implants) has an important role in the fusion of MF, event involved in implant failure. Thus, another objective of this study was to analyze the expression of ADAM-9 in the cultures of MF grown in contact with Ti23Nb0.7Ta2Zr0.5N and Ti6Al4V (Fig. 18). Results showed no differences in the pattern of ADAM-9 expression between samples.

![Fig. 14. Zymography reflecting gelatinolytic activities of MMPs secreted by RAW 264.7 MF in serum-free culture medium, after 5 days of maintaining the cells in contact with Ti6Al4V and Ti23Nb0.7Ta2Zr0.5N: 1 Ti6Al4V; 2- Ti23Nb0.7Ta2Zr0.5N; 3- Ti6Al4V + LPS; 4- Ti23Nb0.7Ta2Zr0.5N + LPS. MM - molecular weight marker.](image1)

![Fig. 15. Zymography reflecting specific gelatinolytic activities of MMPs expressed by RAW 264.7 MF in serum containing culture medium after 5 days of maintaining cells in contact with Ti6Al4V and Ti23Nb0.7Ta2Zr0.5N: 1- Ti6Al4V; 2- Ti23Nb0.7Ta2Zr0.5N; 3- Ti6Al4V + LPS; 4- Ti23Nb0.7Ta2Zr0.5N + LPS. MM - molecular weight marker.](image2)

![Fig. 16. Western blot analysis of MMP-9 secreted by RAW 264.7 MF in the culture medium, after 5 days of maintaining the cells in contact with Ti6Al4V and Ti23Nb0.7Ta2Zr0.5N: 1 Ti6Al4V; 2- Ti23Nb0.7Ta2Zr0.5N; 3- Ti6Al4V + LPS; 4- Ti23Nb0.7Ta2Zr0.5N + LPS. MM - molecular weight marker](image3)

![Fig. 17. Western blot analysis of MMP-2 secreted by RAW 264.7 MF in the culture medium, after 5 days of maintaining the cells in contact with Ti6Al4V and Ti23Nb0.7Ta2Zr0.5N: 1 Ti6Al4V; 2- Ti23Nb0.7Ta2Zr0.5N; 3- Ti6Al4V + LPS; 4- Ti23Nb0.7Ta2Zr0.5N + LPS. MM - molecular weight marker](image4)

![Fig. 18. Immunocytochemical study of ADAM-9 expression in multinucleated giant cells formed by the fusion of MF maintained in contact with Ti23Nb0.7Ta2Zr0.5N and Ti6Al4V under exposure to LPS and in unstimulated conditions, after 7 days of incubation.](image5)
Another study focused on the early inflammatory response of PPy films synthesized on the Ti6Al7Nb alloy by potentiostatic polymerization in the presence of surfactants: poly (sodium 4-stirenesulfonate) (PSS), polyetoxietanol t - octylphenoxy (Triton X-100) and N - dodecyl – β-D - maltoside (DM). RAW 264.7 MF response to PPy films synthesized on Ti6Al7Nb alloy was influenced by the substrate affinity for water. PPy/PSS film strongly inhibited the proliferation of these cells, while the other analyzed coatings had little effect on the proliferation of MF as compared to Ti6Al7Nb. The results of the cytokine and chemokine secretion showed a weak activation of MF grown on PPy/PSS, as compared to the other samples (data not shown).

Further experimental studies aimed to analyze the MF response to surface topography. Evaluation of the behavior of RAW 264.7 cells grown on Ti surfaces coated with TiO$_2$ nanotubes (Ti/TiO$_2$) has shown that the inflammatory response can be specifically modulated by nanoscale surface modification. The results obtained revealed that RAW 264.7 cells respond to a lower degree to Ti/TiO$_2$ surface as compared with the flat surface of Ti, both in terms of morphological characteristics and MF fusion in FBGCs (Fig. 19 and 20) and of the gene expression and extracellular protein secretion of pro-inflammatory cytokines/chemokines (Fig. 21 and 22).

![Fig. 19. Morphological characteristics of RAW 264.7 cells grown on Ti/TiO$_2$ and cpTi for 24 h and 48 h of culture, in standard or LPS-stimulated culture conditions; actin cytoskeleton was stained with phallolidin-TRITC](image)

![Fig. 20. Multinucleated giant cells formation by fusion of MF maintained in contact with Ti/TiO$_2$ and cpTi after 7 days of incubation: A) comparative fluorescent images of cells stimulated with LPS (1 μg/mL LPS); B) “Multinucleated index” determined based on the examination of 14-16 fields per sample.](image)

![Fig. 21. The effect of Ti/TiO$_2$ and cpTi on the pro-inflammatory cytokines and chemokines gene expression, at 24 h and 48 h after cell seeding, in the presence or absence of LPS.](image)
During the inflammatory processes, protein kinases from the family of MAPK (Mitogen Activated Protein Kinase) and NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) are activated and mediates signaling cascades that lead to gene expression for pro-inflammatory cytokines. Further, the studies developed in this project aimed to investigate whether the Ti alloys with different chemical compositions (Ti23Nb0.7Ta2Zr0.5N and Ti6Al4V) or different topographies (flat surface Ti and Ti coated with TiO2 nanotubes) can differentially modulate the activation of MAPK and NF-kB signaling pathways.

Investigation of the signaling pathways (MAPK and NF-kB) involved in inflammatory processes revealed a similar expression level of NF-kB and MAP kinases phosphorylation status in the MF maintained in contact with Ti23Nb0.7Ta2Zr0.5N and Ti6Al4V alloys (data not shown).

In case of the surface covered with TiO2 nanotubes it was shown that these nanostructures are able to induce the inhibition of the phosphorylation of some critical proteins involved in MAPK signaling pathway (ERK1/2, p38, JNK) (Fig. 23), as well as of some important components for the signal transduction in the NF-kB pathway (IKKβ, IkB-α, NF-kB-p65) (Fig. 24).

**Fig. 22.** Profiles of cytokines and chemokines secreted by MF grown on Ti/TiO2 and cpTi, normalized to cell number (pg/ml relative to 10⁵ cells), after 24 h and 48 h of maintaining the MF in the presence or absence of LPS.

**Fig. 23.** The effect of surface topography on MAPK activation in RAW 264.7 cells, unstimulated or stimulated with LPS; the level of phosphorylation of p38, ERK and JNK was detected by ELISA; * P <0.05 nanotubes vs Ti; * P <0.01 nanotubes vs Ti.

**Figure 24.** The effect of surface topography on NF-kB activation in RAW 264.7 cells, unstimulated or stimulated with LPS. (A) The level of phosphorylation of IKKβ (B) Representative micrographs of NF-kB-p65 (green fluorescent) localization.
These data support the results showing that the TiO$_2$ nanotubes significantly reduces the inflammatory activity of the MF in terms of cytokines/chemokines release, induction of multinucleated giant cells and the production of NO, thus alleviating the inflammatory response induced by LPS, as compared to Ti surface. In Fig. 25, a model for the possible mechanisms by which surface topography can modulate the functions of MF is proposed.

The osseointegration of a medical implant is characterized by a series of biological reactions involving bone tissue remodeling at the bone-implant interface, followed by a rapid repair of the host tissue. Osteoblasts and MF are key cells involved in the osseointegration process of a biomedical device. Coordinated interaction between these two cell types is critical for an optimal bone remodeling. Osteoblasts are responsible for generating bone ECM, and are also involved in the regulation of the activity and differentiation of osteoclasts, cells derived from MF [20]. Currently, there are relatively few studies in the field of tissue engineering that focuses on the interaction between MF and osteoblasts or on the inter-relationship between their secreted soluble factors. Thus, the MF-osteoblasts co-culture system is an attractive technique for the characterization of biomaterials in terms of their ability to maintain or to influence the interaction between the cells involved in bone remodeling. Furthermore, co-culture systems are considered to be much closer to the natural microenvironment conditions and might help to elucidate complex issues regarding the interaction between cells involved in bone formation and resorption.

In the last phase of the project, the studies aimed at evaluating the effect of Ti-based alloys surface characteristics on the interaction between RAW 264.7 MF and MC3T3-E1 pre-osteoblasts in mono- or co-culture systems. The obtained results showed similar capacities of β-Ti based alloy (Ti23Nb0.7Ta2Zr1.2O) and of the reference material, cpTi, to support the differentiation of MC3T3-E1 cells in terms of OPG and RANKL production, alkaline phosphatase activity and the mineralization of the extracellular matrix, both in mono- and co-culture. Furthermore, RAW 264.7 cells cultured on these surfaces exhibited similar secretion profiles of TNF-α, IL-6 and IL-1β. Regarding TiO$_2$ nanotubes, the obtained data indicated that their surface elicit an improved osteoblast function, being remarked an increase in alkaline phosphatase activity and in the rate of extracellular matrix mineralization. Moreover, the nanotubular Ti/TiO$_2$ surface lead to a decreased inflammatory response as compared with the flat Ti surface in both experimental conditions. Also, it has been observed that the early presence of MF in co-culture has a positive effect on the function of osteoblasts, while their chronic presence leads to a reduction in the expression of important osteogenic markers (data not shown).
In conclusion, the results of the studies carried out in this research project showed a different modulation of RAW 264.7 MF behavior in a manner dependent on the surface characteristics of Ti-based biomaterials. These studies have demonstrated that RAW 264.7 cells have the ability to adhere and proliferate on the surface of Ti-based biomaterials, irrespective of their type. However, the data show that MF have the ability to alter the secretion of cytokines/chemokines profiles depending on the surface characteristics of biomaterials. It is clear that the surface topography, especially at the nanoscale level, has a profound impact on the in vitro inflammatory response of the MF, promoting the transition from a pro-inflammatory profile of cytokines and chemokines to a profile prone to bone remodeling and tissue integration. Also, the results suggest that altering the biomaterials topography by creating nanostructures that are able to control the behavior of MF can be an effective strategy to improve the performance and functionality of biomedical implants. Therefore, this research project brings a significant contribution to elucidating the cellular and molecular mechanisms involved in the inflammatory response to implantable biomaterials. The accumulated information is of great importance for the development of next-generation medical devices with immunomodulating potential and, therefore, with a superior osteointegration ability.

References: