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Title: Effect of functionalized titanium particles with dexamethasone-loaded nanospheres on macrophage polarization and activity.

Running Head: Dexamethasone-doped nanoparticles effect on macrophages

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Abstract

Objective: The aim of this study was to determine the effect of titanium micro particles (TiP) previously functionalized with nanoparticles doped with dexamethasone (Dex) and doxycycline (Dox), on macrophage polarization and activity. Methods: Macrophages RAW264.7 were cultured in the presence TiP loaded with dexamethasone -NPs (Dex)- and doxycycline -NPs (Dox)-, and as control, TiP with or without doped NPs. Cells were tested with and without previous bacterial lipopolysaccharide endotoxin (LPS) stimulation. Their morphology, proliferation, cytotoxicity, phenotypic change, and cytokines release were assessed by LIVE/DEAD, DNA release, metabolic activity, brightfield and scanning electron microscopy. The test Kruskall-Wallis was used for comparisons, while the cytokine expression profiles were examined by hierarchical clustering ($p<0.05$). Results: Upon exposure with TiP macrophages were activated and polarized to M1, but without depicting cytotoxic effects. The particles were phagocytised, and vacuolized. When exposed to functionalised TiP with NPs(Dex) and NPs(Dox), the ratio M1/M2 was up to forty times lower compared to TiP alone. When exposed to LPS, TiP reduced cell viability in half. Functionalised TiP with NPs(Dex) inhibited the cytokine release exerted by TiP on macrophages. When macrophages were exposed to functionalised TiPs with NPs(Dex) with and without LPS, the effect of TiP on cytokine secretion was inhibited. Significance: Functionalised TiPs with NPs(Dex) and NPs(Dox) may potentially have beneficial effects on modulating titanium and LPS-related inflammatory reactions.

Key words:

Dexamethasone, doxycycline, macrophage, nanoparticles, polymers, titanium.

Highlights

The titanium particles were phagocytised by macrophages and vacuolized

Titanium particles induced M1 macrophages polarization without cytotoxic effects

Bacterial lipopolysaccharide plus titanium particles half-reduced macrophage viability

Titanium particles doped with dexamethasone reduced the ratio M1/M2 forty times

1. INTRODUCTION

Titanium dental implants are widely used for the replacement of the lost dentition; however, their use is not free of complications, being the most common, the presence of peri-implant diseases. These diseases, in its early forms (peri-implant mucositis) are characterised by peri-implant soft tissue inflammation initiated by the growth of bacterial biofilms adhered to implant and prosthetic surfaces. In some patients, this chronic inflammatory state may lead to dysbiosis within the mature biofilm and non-resolving inflammation, leading to bone resorption, which if untreated may result in implants loss [1,2]. In fact, peri-implantitis (PI) is defined as a pathological condition occurring in tissues around dental implants, characterized by inflammation in the peri-implant mucosa and progressive loss of supporting bone [3,4].

The main event in the pathobiology of PI is the interaction between the biofilm bacteria and the peri-implant soft tissues leading to non-resolving chronic inflammation [5]. Although these oral bacteria may also interact with the implant surface leading to material corrosion and thus facilitating the release of titanium particles and their accumulation within the peri-implant tissues [6,7]. Macrophages and multinucleated giant cells may phagocytize these particles and elicit an immune response which may also influence the pathological phenomena associated with peri-implantitis [8]. The specific influence of this titanium particle release on the pathogenesis of peri-implantitis is not fully understood and require further investigation [6]. In fact, recent investigations evaluating granulation tissue peri-implantitis lesions have evidenced the presence of titanium particles, but not associated with a foreign body reaction, but rather being inert within the tissues [7].

Macrophage polarization towards a M1 phenotype is characteristic of chronic inflammatory conditions, such as peri-implantitis [6,9], being activated by IFN-γ and by chemokines and bacterial endotoxins, such as the bacterial lipopolysaccharide endotoxin (LPS) [10]. Macrophages displaying M1 phenotype are characterized by secreting pro-inflammatory cytokines and producing reactive nitrogen and oxygen intermediates. Furthermore, macrophage exposure to certain signaling molecules, such as certain cytokines (IL-4, IL-10, IL-13), or glucocorticoid hormones, may change the polarization of macrophages towards a M2 phenotype, leading to tissue reconstruction and morphogenesis, characterized by angiogenesis and matrix remodeling [9,11].

This degree of macrophage polarization (M1/M2 ratio) thus becomes critical in wound healing processes, leading either to chronic inflammation and scar formation (M1) or to tissue reconstruction (M2) [11]. When the healing process occurs in presence of a contaminated environment (bacterial biofilm) macrophages will polarize towards M1 and to a chronic non-resolving inflammation [12]. What is still controversial is whether the presence of particle release when using titanium implants, may further contribute towards increased inflammation, since there is insufficient evidence to suggest a unidirectional role of titanium corrosion and particles release with peri-implantitis development [4,7]. However, there is a tendency to find more titanium particles in proximity to dental implants and to sites with peri-implantitis [13,14]. In addition, several studies using transcriptomic, proteomic and epigenomic tools have shown the biologic plausibility of titanium particles release as a mechanism in the pathogenesis of peri-implantitis [15].

When treating peri-implantitis, the regeneration of the lost bone is a challenging process, currently not highly predictable, since it requires the adequate interaction of cells, matrixes and signaling molecules, in absence of inflammation [16,17]. One of the possible key factors for success may be directing the immune response towards M2 polarization [18]. Therefore, the search for biomaterials with favorable osteo-immunomodulatory properties and the effective control of bacterial contamination has attracted recent research efforts [4,12,19].

A novel type of biocompatible polymeric nanoparticles (NPs) composed of 2-hydroxyethyl methacrylate, ethylene glycol dimethacrylate, and methacrylic acid connected covalently, has been synthesized by polymerization precipitation (surfactant-free) in a non-solvent medium [20–22]. These NPs may be functionalized with osteogenic, antimicrobial and immunomodulatory agents (as dexamethasone and doxycycline) [23,24], that can be chemically attached or adsorbed onto the polymers present on the surface of the NPs [23]. Doxycycline-doped NPs have shown to have antibacterial properties [24] and those loaded with both doxycycline or dexamethasone have also shown to promote human bone marrow mesenchymal stem cells differentiation [23].

It was the objective of this *in vitro* investigation to evaluate whether functionalized titanium with either dexamethasone or doxycycline NPs would elicit immunomodulatory responses in macrophage cell cultures, with or without the presence of LPS bacterial endotoxin.

2. MATERIALS AND METHODS

2.1. Specimens preparation

2.1.1. Nanoparticles production: Nanoparticles were obtained through a polymerization precipitation technique using a thermodynamic process according the Flory-Huggins model based on the Hansen solubility parameters. This model was based on the growth of polymeric chains through interactions with solvent molecules by hydrogen-bonding, dispersion, and polar forces [20]. The NPs were composed of 2-hydroxyethyl methacrylate as the backbone monomer, methacrylic acid as the functional monomer, and ethylene glycol dimethacrylate acting as a cross-linker. In brief, 14mL of a suspension of silicon dioxide nanoparticles at a concentration of 0.85mg mL^{-1} in acetonitrile was prepared. Then, 8.75mg of 2,2'-azodi(isobutyronitrile) (AIBN) were dissolved in a mixture of 0.170mL of ethylene dimethacrylate, 0.045mL of methacrylic acid and 0.137mL of 2-hydroxyethyl methacrylate (the monomers were previously purified). The monomer solution of AIBN was then mixed with the 14mL of suspension of NPs-SiO_2, shaken during 1 min, cooled at -8ºC and purged whit a soft flow of N_2 during 3min to remove the O_2. Contained in sealed bottles the polymerization took place at 75ºC without stirring. Once polymerized and centrifuged the resulting microspheres were washed three times with 30mL of methanol and dried at 80ºC in a vacuum until attaining a spherical shape and a mean particle size of about 250nm. These NPs did not agglomerate and had a zeta potential of -41eV and a polydispersity index of 0.05 [20,25].

The process of functionalization of the NPs with doxycycline and dexamethasone was carried out by immersion of 30mg of NPs in an aqueous solution (containing 40mgmL^{-1} of doxycycline hyclate (Sigma-Aldrich, Chemie Gmbh, Riedstr, Germany) or

dexamethasone (Sigma-Aldrich) for 4h, at room temperature and under constant shaking (rotator Orbit 300445, JP Selecta, Barcelona, Spain) at 12rpm [23]. The obtained suspensions were then centrifuged (Centrofriger BLT, JP Selecta, Barcelona, Spain) at 6,000rpm for 30min, the particles were detached from the supernatant and dried in an oven at 45°C (Selecta, JP Selecta, Barcelona, Spain) until achieving a constant weight. Using this process doped NPs have previously shown to effectively liberate drugs [26].

2.1.2. Loading of titanium particles with NPs: Commercial rutile TiO_2 microparticles (PCode 1002733097) were obtained from Sigma (Sigma Aldrich). They are presented as a white powder (<5μm in particle size; >99.9% trace metal basis). Adherent endotoxins on the particles were removed and tested for endotoxin-free using previously described methods [27,28]. The titanium particles and the doped or undoped NPs were then mixed (ratio 20:1%wt) in an ethanol suspension, for 4h, at room temperature and under constant shaking (rotator Orbit 300445) at 12rpm. Subsequently, the suspensions were centrifuged (Centrofriger BLT) at 6,000rpm for 30min, and the particles were detached from the supernatant and dried in an oven at 45 °C (Selecta, JP Selecta, Barcelona, Spain) until attaining a constant weight, being then sterilized by UV light. To demonstrate the loading of nanospheres on the titanium particles, they were suspended in ethanol ($1mgmL^{-1}$), placed in a sample holder, dried in a vacuum heater during 24h, carbon covered and analyzed by scanning electron microscopy, using a field emission scanning electron microscope (FESEM) (GEMINI, Carl Zeiss SMT, Oberkochen, Germany) at 5kV and 3.6mm working distance.

The obtained particles were then re-suspended in Dulbecco's modified Eagle's medium (DMEM) at a concentration of $100\mu gmL^{-1}$ and sonicated,[28] before the start of the cell experiments. Four different particles were tested: (a) Titanium microparticles (Ti), (b) Ti particles loaded with undoped NPs -TiNP()-; (c) Ti particles loaded with doxycycline doped NPs -TiNPs(dox)- and (d) Ti particles loaded with dexamethasone doped NPs -TiNPs(dexa)-.

2.2. Cells culture and treatment

We used the mouse macrophage cell line RAW264.7 obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). These cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin mixture ($50IUmL^{-1}$) (all from Sigma-Aldrich/Merck, Burlington, MA, USA) under standard culture conditions (37°C and 5% CO_2) [29].

RAW264.7 cells were plated at a cell density of $6x10^4$cells cm^{-2} and either a bacterial lipopolysaccharide endotoxin (LPS) at $0.01\mu g\ mL^{-1}$ (Sigma-Aldrich/Merck), or a diluent were added to the cell cultures and incubated for 24h. Macrophages were then exposed to the referred types of particles ($100\mu g\ mL^{-1}$), hence, establish the following study groups: *i*) Non-activated macrophages with particle treatment: Ti, TiNP(), TiNP(dox) TiNP(dexa) and *ii*) LPS-activated macrophages with particle treatment: aTi, aTINP(), aTiNP(dox), aTiNP(dexa) (Figure 1). In addition macrophages without particle treatment were used as positive control for non-activated -CTR(+)- or LPS-activated macrophages -aCTR(+)-.

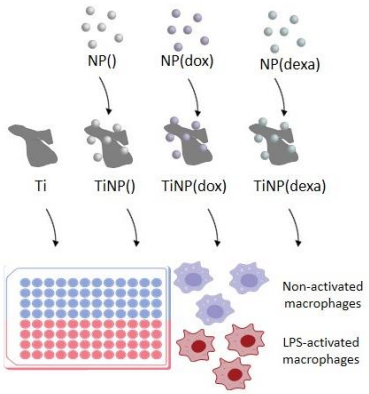


Figure 1. Schematic representation of planned experimental study groups.

2.3. Raw cells proliferation, cytotoxicity and morphology.

2.3.1. Cell Viability/Cytotoxicity Assays: The LIVE/DEAD Viability/Cytotoxicity kit (Invitrogen) was used to evaluate the cell viability within the different groups. In brief, cells were washed twice in phosphate-buffered saline (PBS) and cultured in the LIVE/DEAD reagent prepared as recommended by the manufacturer. Then, cells were incubated for 5min at room temperature in the dark. Finally, images were obtained using a ZOE Fluorescent Cell Imager (Biorad) [30].

Cell viability was also assessed by quantifying the DNA released from the cells to the culture medium. For this purpose, macrophages were seeded in 96-well cell culture plates (n=8). Aliquots were then taken from each well and the amount of DNA present in each sample was measured by determining the 260/280 nm absorbance using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Results were normalized using the negative CTR(-) cells in which cytotoxicity was induced adding 2% triton X-100 (considered as 100% DNA released) [30].

Finally, metabolic activity was evaluated by using the Cell Proliferation Reagent WST-1 (Roche, Basel, Switzerland). Cells were washed twice in PBS and the WST-1 reagent was added to each well diluted in culture medium, as recommended by the manufacturer. After 4 h of incubation at 37°C, the absorbance of the formazan dye - which correlates with the number of metabolically active cells-was measured with an Asys UVM-340 scanning microplate reader (Biochrom/Harvard Bioscience, Holliston, MA). Results were normalized with the results obtained in a positive CTR(+) (considered as 100% metabolic activity) and negative CTR(-) cells in which cytotoxicity was induced by adding 2% triton X-100 (considered as 0% metabolic activity) [30].

2.3.2. Evaluation of Apoptosis and Necrosis: For the evaluation of cell death mechanisms, macrophages were seeded in 24-well cell culture plates (n=6). After the

respective treatments, macrophages were studied using the Annexin V-FITC and propidium iodide (PI) apoptosis detection kit (BD Biosciences, Barcelona, Spain), with analysis of the results by flow cytometry to detect apoptosis and necrosis. Briefly, cells were carefully detached and washed with cold PBS. 100µl of the cell suspension were transfer to flow culture tube and 1µL of Annexin V-FITC and 1µL of PI were added. Then cells were gently vortexed and incubated for 15min at room temperature in the dark. After supernatant removal, 500µL of PBS was added to each sample and analysed by flow cytometry within 1h. Finally, cells were quantified with a NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA, USA). Here we included a negative CTR(-) in which cell death was induced by high temperatures (10min at 99ºC) [29].

2.3.3. Morphological Analysis: Macrophages were analyzed by brightfield microscopy and FESEM to evaluate the particle-induced morphological changes. For brightfield analysis, images were obtained using a ZOE Fluorescent Cell Imager (Bio-Rad Laboratories, CA, USA). For FESEM analysis, specimens were immersed in a 4% paraformaldehyde and 2.5% glutaraldehyde solution for 4h, at 4°C. Then, they were submitted to critical point drying technique. Specimens were fixed to the sample holder, dried in a vacuum heater during 24h, carbon covered and analyzed by FESEM (GEMINI, Carl Zeiss SMT, Oberkochen, Germany) at 3kV, and 3.7mm to 4.7mm working distances. Randomized images at 150x magnification were taken in all groups, and some cells were also observed at higher magnifications (600x to 6000x).

2.4. Phenotypic change of RAW cells.

The surface markers of stimulated macrophages were detected by flow cytometry. Briefly, RAW264.7 cells were collected from 24-well plates after stimulation for 24h (n=6). The cell suspension was washed twice with PBS and cells were incubated in 100µl of casein solution with the following antibodies: 0.25µl of FITC anti-mouse CD86 and 1ul of PE anti-mouse CD206 (both from Thermo Scientific) on ice for 30min in the dark. After washing twice, cells were resuspended in 200µl PBS and studied under a NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA, USA) [31].

2.5. Inflammatory response of RAW cells by interleukins and cytokines expression.

The Proteome Profiler™ Mouse Cytokine Array Kit (Panel A, ARY006) (R&D Systems, Inc. Minneapolis, MN 55413, USA) was used to measure the expression level of protein synthesized by the stimulated macrophages [32]. This array kit is a membrane-based sandwich immunoassay able to quantify 40 mouse cytokines, chemokines, and acute phase proteins simultaneously: CXCL13/BLC/BCA-1, C5a, G-CSF, GM-CSF, CCL1/I-309, CCL11/Eotaxin, ICAM-1, IFN-gamma, IL-1 alpha/IL-1F1, IL-1 beta/IL-1F2, IL-1ra/IL-1F3, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 p70, IL-13, IL-16, IL-17, IL-23, IL-27, CXCL10/IP-10, CXCL11/I-TAC, CXCL1/KCM-CSF, CCL2/JE/MCP-1, CCL12/MCP-5, CXCL9/MIG, CCL3/MIP-1 alpha, CCL4/MIP-1 beta, CXCL2/MIP-2, CCL5/RANTES, CXCL12/SDF-1, CCL17/TARC, TIMP-1, TNF-alpha and TREM-1. The macrophages-released cytokines were measured in cell culture medium after the different treatments. In brief, 50µl of cell culture medium were mixed with a cocktail of biotinylated detection antibodies and then incubated with a mouse chemokine or cytokine array membrane. After washing, streptavidin-conjugated horseradish peroxidase and chemiluminescent detection reagents were added and incubated for 1 min at room temperature prior to X-ray film exposure. The films were scanned, and signal intensity was quantified at each spot with

ImageJ software (version 1.53f51, Wayne Rasband, NIH, MD, USA) in two technical replicates included in each membrane. The results were normalized to positive and negative controls included in the array [33].

2.6. Statistical analysis

The results obtained in each experimental group for each analytical technique were compared with the positive CTR (+) and negative CTR(-) control groups and with its respective LPS-activated group. First, the normality of each distribution was tested by the Shapiro-Wilk test. Since none of the distributions fulfilled the criteria of normality, all comparisons were made using the non-parametric test of Kruskall-Wallis. The comparisons were done with Real Statistics Resource Pack software (Release 7.2) (Dr. Charles Zaiontz, Purdue University, West Lafayette, IN, USA), available at www.real-statistics.com. P value below 0.05 was considered statistically significant differences using two-tailed tests.

The differences in macrophage expression profile based on their cytokine release were examined using hierarchical clustering. With this purpose, average results obtained for the cytokine expression were analysed using ClustVis web tool for clustering of multivariate data [34]. Heatmaps and dendrograms displaying relative quantitative signals and representing higher or lower protein levels for each marker were generated by the software based on hierarchical clustering analysis using the Euclidean distance and the complete clustering method for both the rows and columns. The statistician was blinded to the group assignment when performing the statistical analysis.

3. RESULTS

3.1. Particles characterization.

Figure 2 displays SEM images of loaded titanium particles where the polymeric nanospheres can be observed in contact with the titanium microparticles.

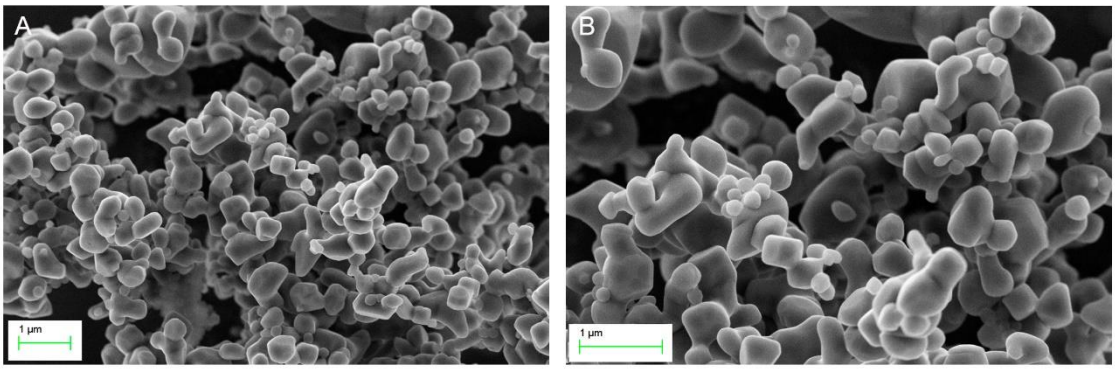


Figure 2. SEM images of titanium microparticles doped with polymeric spherical nanoparticles (20:1%wt) at 25,000x (A) and 40,000x (B) magnifications. Spherical nano-spheres may be observed on irregular titanium microparticles. Scales bars are 1 micron.

3.2. Raw cells proliferation, cytotoxicity and morphology.

3.2.1. Cell Viability/Cytotoxicity Assays DNA release testing, metabolic activity and dead cells assessment:

LIVE/DEAD microscopy images of LPS-activated and non-activated macrophages at the different experimental groups are presented in figure 3. Mean and standard deviations of the attained percentages of live and dead (apoptotic or necrotic) cells at the different tested groups are also displayed in figure 3. In all groups, there were significantly lower percentages of live cells compared with the negative control ($p<0.05$). Except for TiNP(Dox), all particles induced some cytotoxic effects (5 to 10%) on cultured cells (Figure 4) when compared with the positive control group ($p<0.05$). After LPS activation, those RAW cells cultured with titanium particles (in any mode) have reduced by half the percentage of live cells ($p<0.05$) and increased double-fold the percentage of dead cells ($p<0.05$), when compared to the positive control group. Moreover, live and dead cells percentages were significantly higher and lower, respectively in the non-activated cells groups if compared to their activated counterparts ($p<0.05$). Dead induction seems to be similarly due to apoptotic or necrotic processes. In general, proliferation rate decreased after macrophages activation when any kind of particle was added to the culture media (Figure 3).

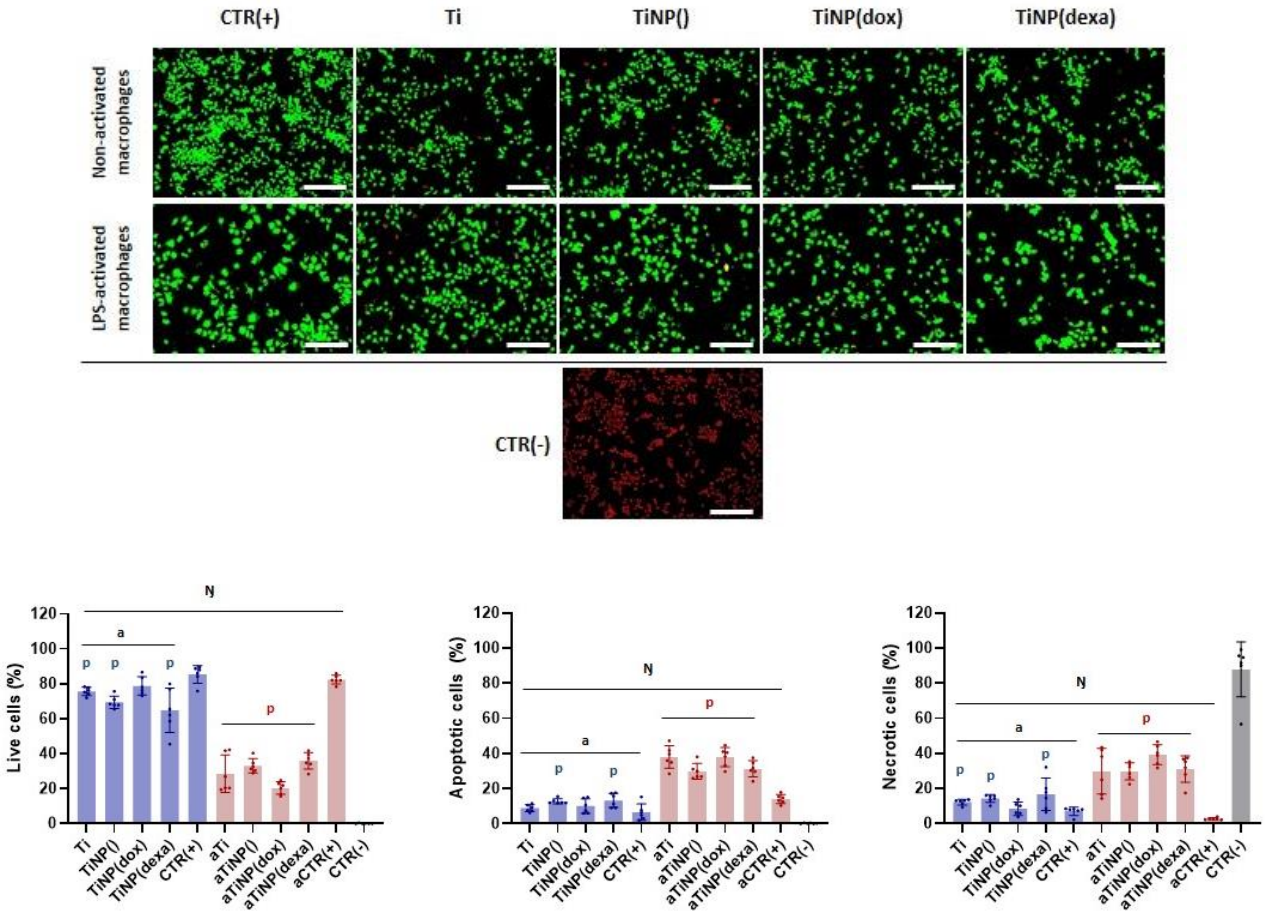


Figure 3. Cell viability assessment of macrophages after particle treatment. A) LIVE/DEAD microscopy images of LPS-activated and non-activated macrophages at the different experimental groups. Scales bars are 200μm. B) Mean and standard deviations of the percentages of live and dead (apoptotic or necrotic) RAW cells at the different tested groups. 'a' indicates differences of each group against its activated phenotype; 'Ɒ' point out differences of any group respect to the negative control and 'p' is used to mark differences between each experimental group and the positive control. Significant differences were considered at $p<0.05$.

Mean and standard deviations of DNA release (%) and metabolic activity (%) of RAW cells under the different tested conditions are displayed at figure 4. In general, a similar DNA release and metabolic activity was observed when RAW cells were cultured under the different conditions. None of the tested particles induced severe cytotoxic effects on RAW cells, either activated or not. All tested groups were different to the negative control ($p<0.05$). Significant differences with the positive control group were found in DNA release of activated macrophages for all the tested particle groups ($p<0.05$), except for those doped with dexamethasone.

Relative metabolic activity was different in all groups when compared to the negative control ($p<0.05$). Differences with the positive control were found for all non-activated raw cells groups ($p<0.05$). After LPS activation, cells in contact to unloaded Ti particles decreased metabolic activity respect to the positive control ($p<0.05$). Moreover, all activated cells increased in metabolic activity when compared to their non-activated phenotypes ($p<0.05$), except for the group in which unloaded Ti particles were added.

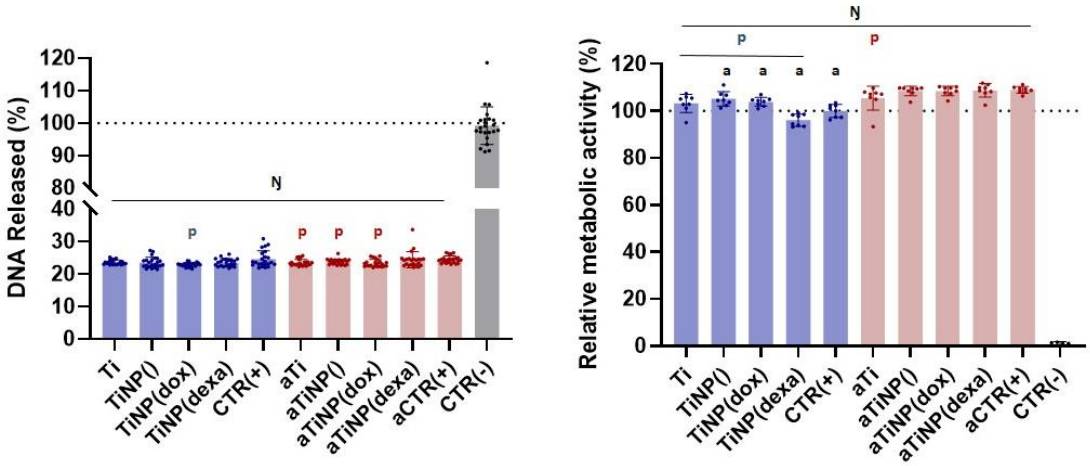


Figure 4. Mean and standard deviations of DNA release (%) and metabolic activity (%) of RAW cells under the different tested conditions. For DNA release and relative metabolic activity normalization was performed to CTR (-) 100%, and CTR(-) 0% plus CTR(+) 100%, respectively. 'a' indicates differences of each group against its activated phenotype; 'Ɲ' point out differences of any group respect to the negative control and 'p' is used to mark differences between each experimental group and the positive control. Significant differences were considered at $p<0.05$.

3.2.2. Morphological Analysis: Representative brightfield images of each group are displayed in figure 5. Evident morphological changes occurred in normal and LPS-activated macrophages after the addition of the different particles, which were phagocytised by the macrophages after 24h. A combination of round-shaped cells with elongated cells was observed in all conditions although the round morphology was predominant. In those groups where particles were present, vacuolization and particles were found in the cell cytoplasm. Cells appeared isolated and not organized into clusters. No clear changes of cell elongation were found when comparing the different experimental groups, except for the activated positive control group.

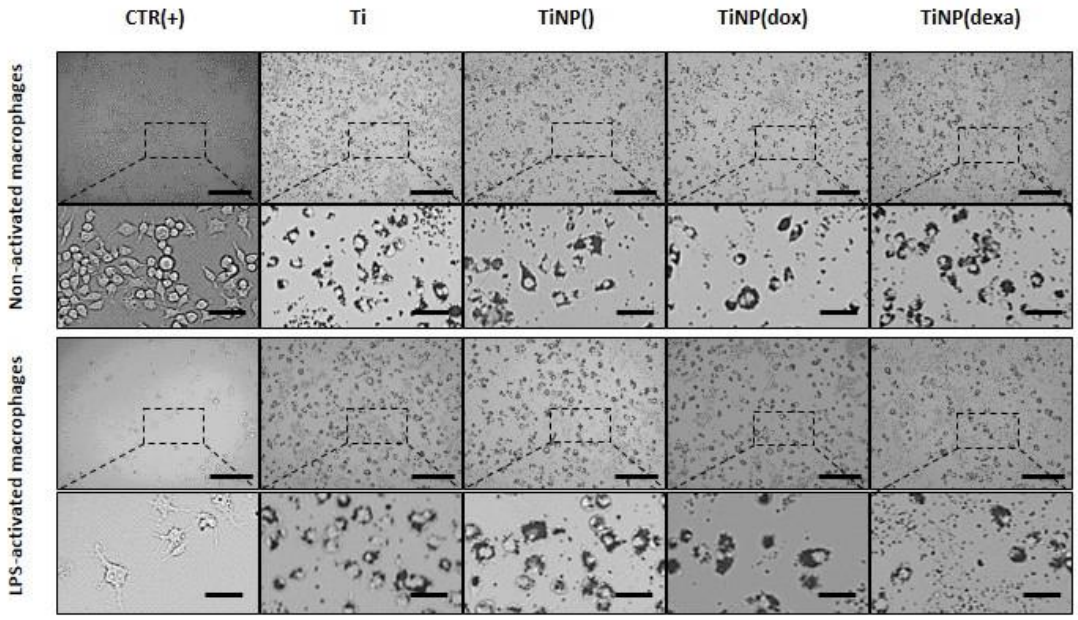


Figure 5. Brightfield microscopy images of LPS-activated and non-activated macrophages at the different experimental groups. Scales bars are 200μm and 50μm at low and high magnification images respectively.

FESEM images are depicted in figures 6 and 7. Both rounded and spindle-shaped cells were found in all groups, except for the non-activated control, where all cells were homogeneously rounded and forming clusters (Figure 6 A and F), and in the LPS-activated control, were most of the cells were elongated and star-shaped (Figure 7 A and F). After LPS stimulation macrophages became elongated and increased in size in all the tested groups (Figure 7). Once in contact with the titanium particles cells became irregular and developed evident vacuolization processes. Figure 8 depicts titanium particles (characterized by EDS) within the macrophage cytoplasm.

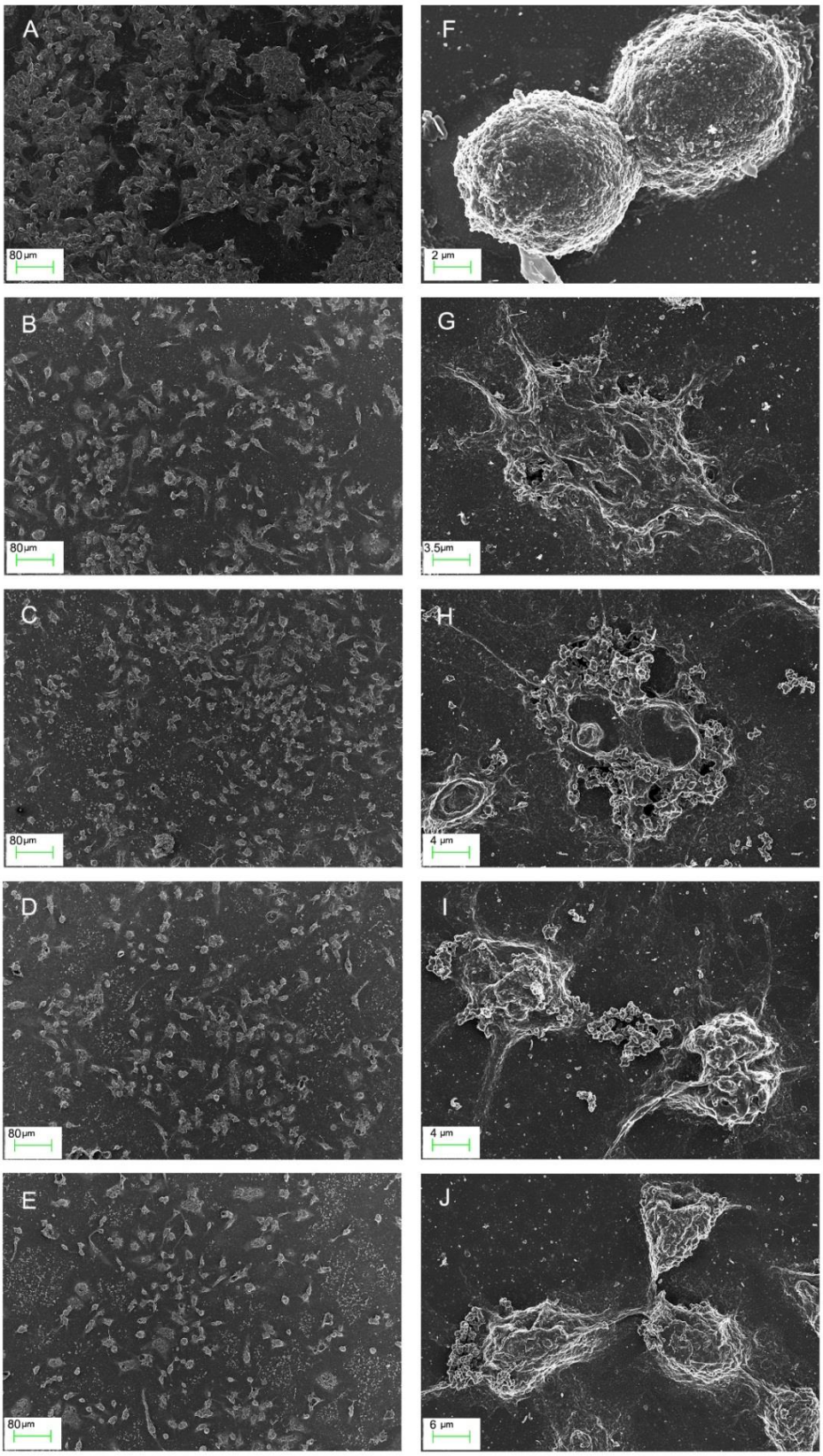


Figure 6. FESEM images of the cultured macrophages after the different particles addition, at 150x (A to E) and at higher magnifications (F to J). Control group without particles addition (A,F); titanium microparticles (Ti)(B,G), Ti particles loaded with undoped NPs -TiNP()- (C,H); Ti particles loaded with doxycycline doped NPs -TiNPs(dox)- (D,I) and Ti particles loaded with dexamethasone doped NPs -TiNPs(dexa)- (E,J).

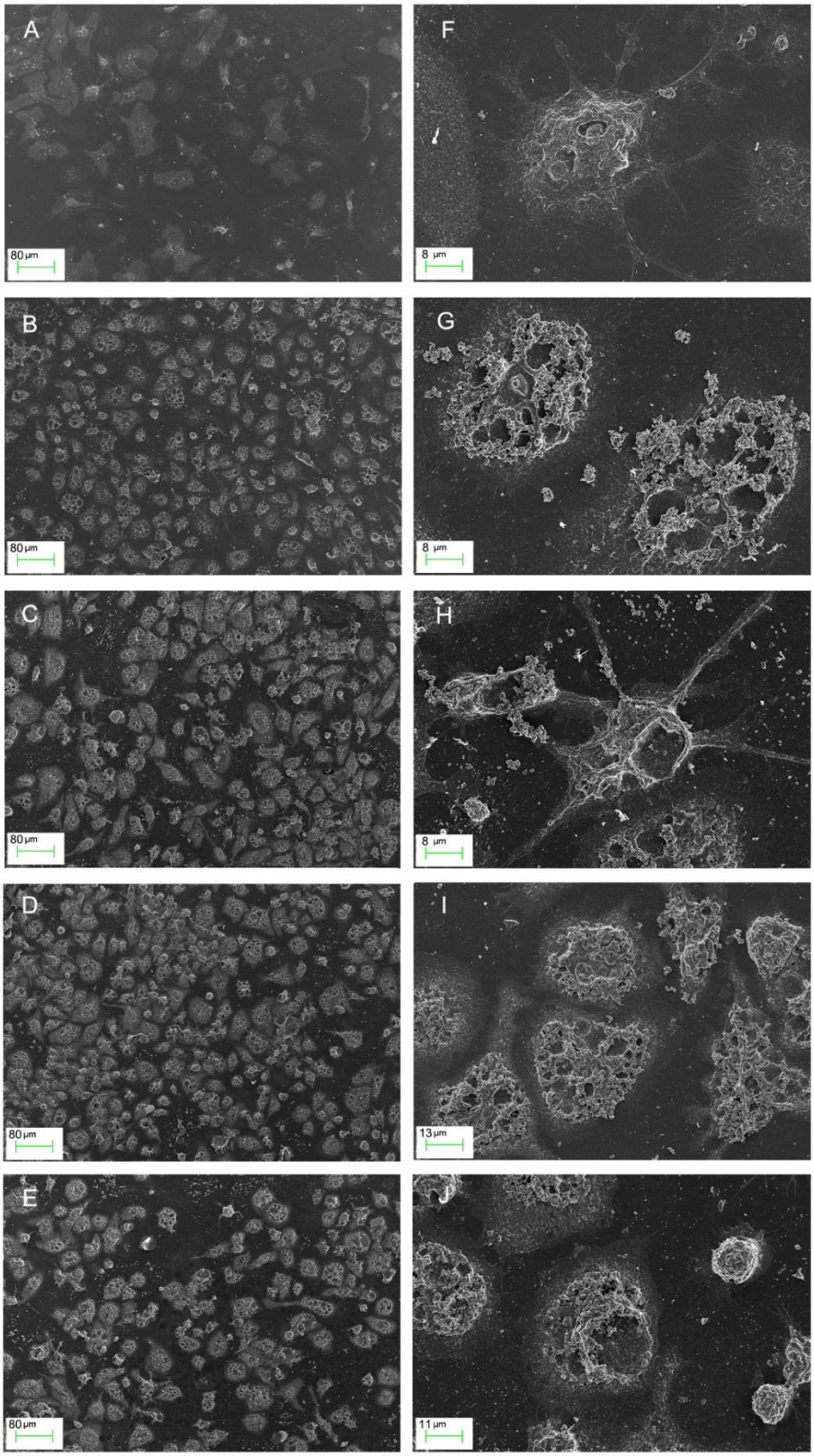


Figure 7. FESEM images of the cultured macrophages after LPS cell activation and further particles addition, at 150x (A to E) and at higher magnifications (F to J). Control group without particles addition (A,F); titanium microparticles (Ti)(B,G), Ti particles loaded with undoped NPs -TiNP()- (C,H); Ti particles loaded with doxycycline doped NPs -TiNPs(dox)- (D,I) and Ti particles loaded with dexamethasone doped NPs -TiNPs(dexa)- (E,J).

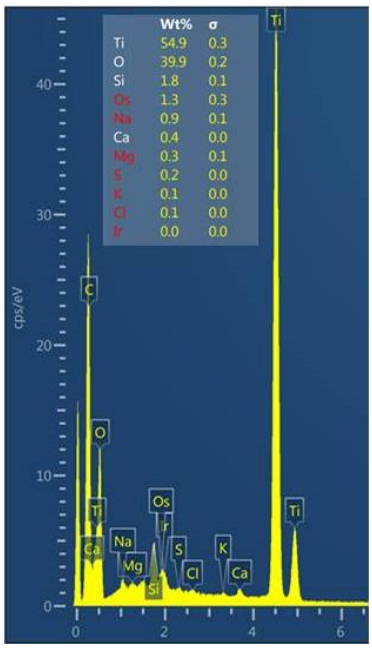


Figure 8. EDS Spectrum of titanium particles on cells cytoplasm.

3.3. Phenotypic changes of RAW cells.

Mean and standard deviations of RAW cells percentages exhibiting M1 (CD86) (pro-inflammatory) or M2 (CD206) (pro-regenerative) phenotype and M1/M2 ratios in the different experimental groups are presented in figure 9.

When titanium particles without doxycycline or dexamethasone were added, a higher percentage of M1 cells phenotype was present in comparison the positive control group ($p<0.05$). When RAW cells were activated with LPS, an even higher percentage of M1 macrophages were found in all experimental groups ($p<0.05$), if compared to the control group ($p<0.05$). M1 phenotype expression was always higher in the LPS-activated groups when compared to their non-activated counterparts ($p<0.05$), except in the group where un-doped titanium particles were used.

M2 macrophages were present in higher percentages in non-activated macrophages, compared with the control group with NPs. In NPs doped with dexamethasone the highest expression of M2 phenotype in the cultured RAW cells was reached (about 8-fold respect to the positive control) ($p<0.05$).

In the non-activated cells groups, the M1/M2 ratio was significantly higher than the positive control in the un-doped titanium particles group ($p<0.05$). With dexamethasone loaded NPs a significantly lower ratio than the positive control was found ($p<0.05$). When macrophages were activated with LPS, M1/M2 ratios at the different groups became significantly similar to the positive control group ($p>0.05$), just the positive control and macrophages cultured with dexamethasone loaded NPs were similar to the negative control ($p>0.05$).

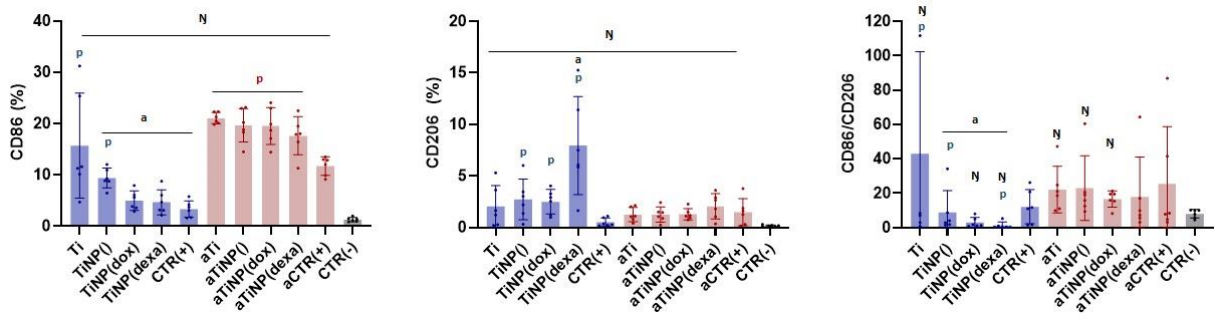


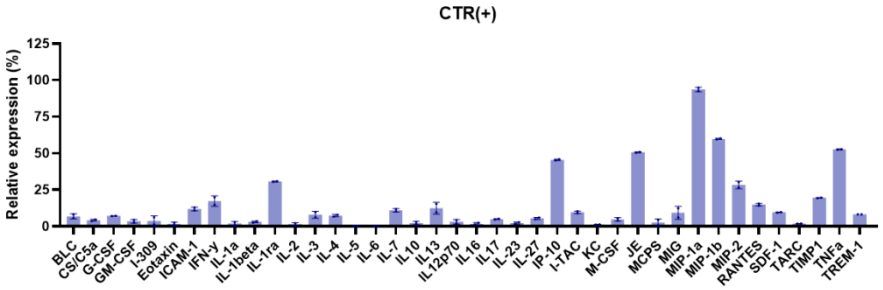
Figure 9. Percentage of RAW cells exhibiting M1 (CD86) or M2 (CD206) phenotype and M1/M2 percentage, at the different experimental groups is displayed. 'a' indicates differences of each group against its activated phenotype; 'Ɲ' point out differences of any group respect to the negative control and 'p' is used to mark differences between each experimental group and the positive control. Significant differences were considered at $p<0.05$.

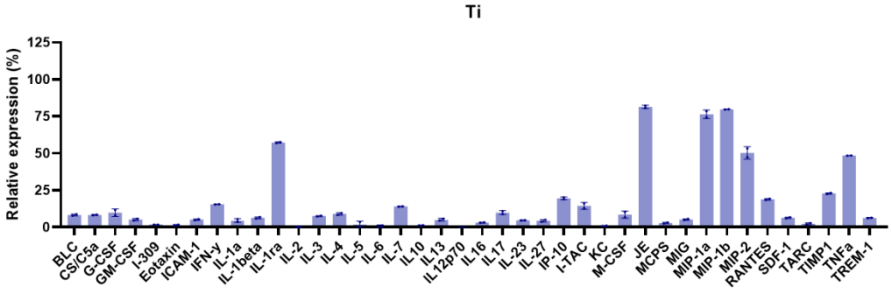
3.4. Inflammatory response of RAW cells: interleukins and cytokines expression.

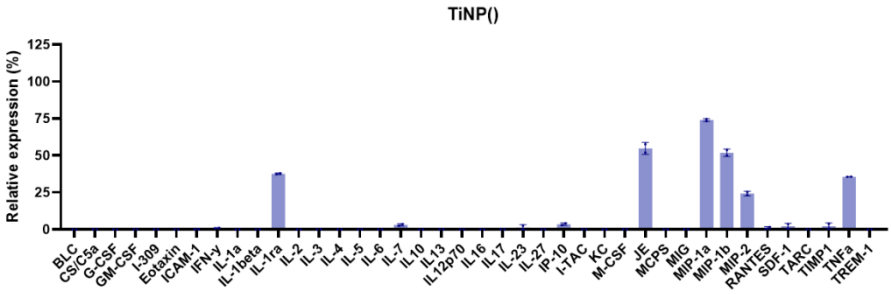
Mean percentage values of the relative expression of the different cytokines in the several experimental groups are displayed in Figure 10. Grouping after cluster analysis is shown in Figure 11. Differences were found between the inflammatory mediator landscapes after results from the proteome profiler kit were analysed.

When macrophages were activated by the LPS-endotoxin, the relative expression of most of the tested cytokines was increased if compared to control macrophages group. However, after LPS cells stimulation, a decrease in cytokines production in all groups was observed after Ti particles addition. In the presence of Ti particles or LPS-activation IL1-ra is highly increased.

After cluster analysis, it was found that when titanium particles were loaded with dexamethasone nanospheres, the macrophage cytokine secretion was similar to the non-activated cells (Figure 11). If LPS-stimulated macrophages were exposed to Ti particles, the cytokine release in the presence of doxycycline loaded NPs was similar to that of the LPS-stimulated cells, being the effect of Ti particles eliminated (Figure 11).


CTR(+)
Relative expression (%)
125
100
75
50
25
0
BLC
CS/C5a
G-CSF
GM-CSF
I-309
Eotaxin
ICAM-1
IFN-γ
IL-1a
IL-1beta
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-10
IL-13
IL12p70
IL-16
IL-17
IL-23
IL-27
IP-10
I-TAC
KC
M-CSF
JE
MCPS
MIG
MIP-1a
MIP-1b
MIP-2
RANTES
SDF-1
TARC
TIMP1
TNFa
TREM-1


Ti
Relative expression (%)
125
100
75
50
25
0
BLC
CS/C5a
G-CSF
GM-CSF
I-309
Eotaxin
ICAM-1
IFN-γ
IL-1a
IL-1beta
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-10
IL-13
IL12p70
IL-16
IL-17
IL-23
IL-27
IP-10
I-TAC
KC
M-CSF
JE
MCPS
MIG
MIP-1a
MIP-1b
MIP-2
RANTES
SDF-1
TARC
TIMP1
TNFa
TREM-1


TiNP()
Relative expression (%)
125
100
75
50
25
0
BLC
CS/C5a
G-CSF
GM-CSF
I-309
Eotaxin
ICAM-1
IFN-γ
IL-1a
IL-1beta
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-10
IL-13
IL12p70
IL-16
IL-17
IL-23
IL-27
IP-10
I-TAC
KC
M-CSF
JE
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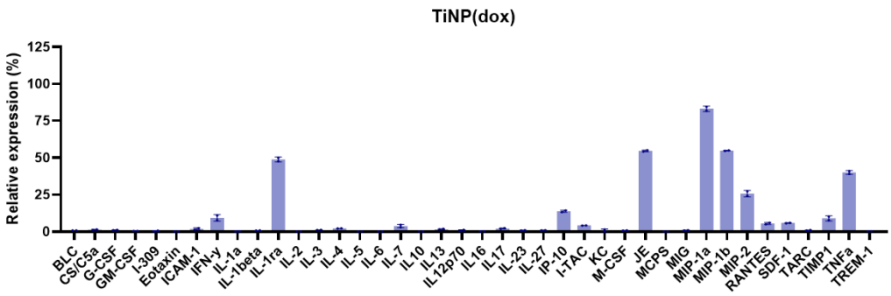
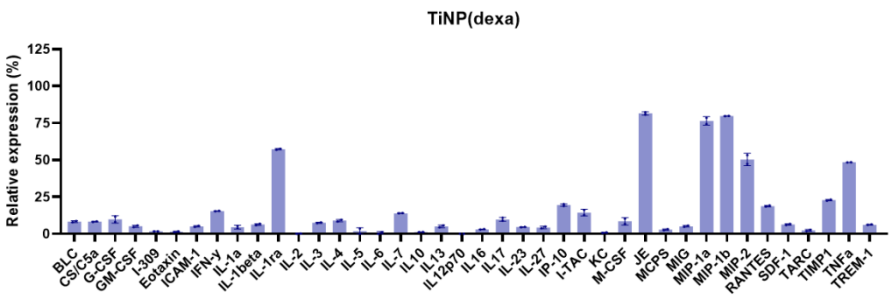
MIP-1b
MIP-2
RANTES
SDF-1
TARC
TIMP1
TNFa
TREM-1


TiNP(dox)
Relative expression (%)
125
100
75
50
25
0
BLC
CS/C5a
G-CSF
GM-CSF
I-309
Eotaxin
ICAM-1
IFN-γ
IL-1a
IL-1beta
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-10
IL-13
IL12p70
IL-16
IL-17
IL-23
IL-27
IP-10
I-TAC
KC
M-CSF
JE
MCPS
MIG
MIP-1a
MIP-1b
MIP-2
RANTES
SDF-1
TARC
TIMP1
TNFa
TREM-1


TiNP(dexa)
Relative expression (%)
125
100
75
50
25
0
BLC
CS/C5a
G-CSF
GM-CSF
I-309
Eotaxin
ICAM-1
IFN-γ
IL-1a
IL-1beta
IL-1ra
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-10
IL-13
IL12p70
IL-16
IL-17
IL-23
IL-27
IP-10
I-TAC
KC
M-CSF
JE
MCPS
MIG
MIP-1a
MIP-1b
MIP-2
RANTES
SDF-1
TARC
TIMP1
TNFa
TREM-1

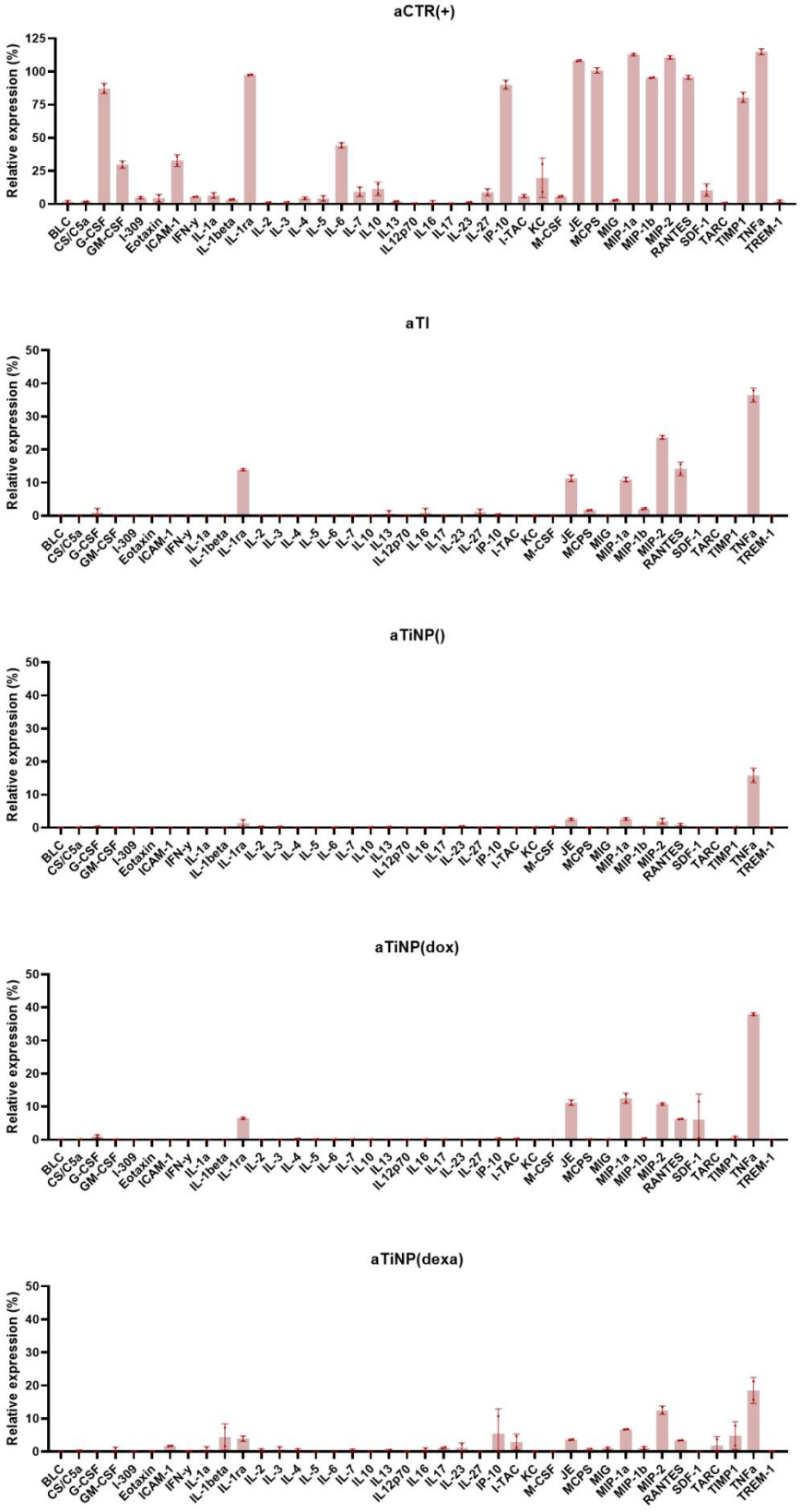


Figure 10. Relative expression (%) of the different interleukins and cytokines expression, at the experimental groups is presented. Without LPS stimulation (blue), and after LPS addition to cultured macrophages cells (pink). Relative expression (%) scales are reduced at: aTi, aTiNP(), aTiNP(Dox) and aTiNP(Dexa) groups.

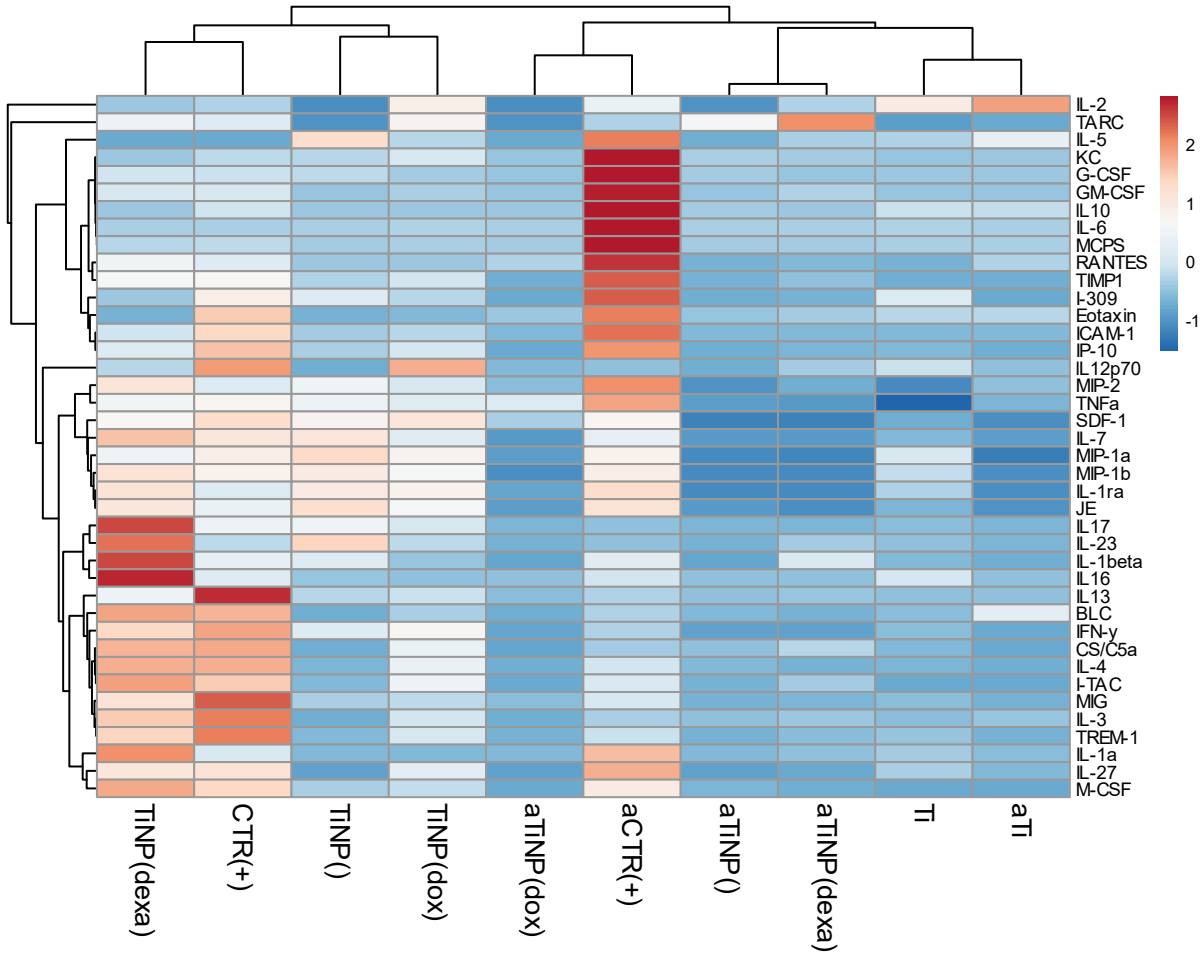


Figure 11. Heat map and dendogram of cluster analysis on the inflammatory response of RAW cells, tested trough cytokines and chemokines expression (Proteome Profiler™ cytokine array), at the different experimental groups.

4. DISCUSSION

This *in vitro* investigation was aimed to evaluate the differential response of macrophages to titanium microparticles, once functionalized with NPs containing doxycycline and dexamethasone and after being activated or not by bacterial LPS.

We selected the RAW264.7 murine monocytic/macrophagic cell line in this *in vitro* study since macrophages have been associated with the biologic events leading to osseointegration [35] and have been identified in high concentrations in lesions of peri-implantitis [7]. Although this is not a human cell line, these cells share most of the main characteristics of human macrophages [36,37] and elicit a robust, stable, and predictable cytokine response [9]. We used a TiO_2 microparticle size of <5μm, which is within the range for phagocytosis (5 ± 2 μm) and of a similar diameter to what has been described as titanium wear particles identified in the peri-implant tissues [28,37–40]. We functionalized the NPs with doxycycline and dexamethasone since they have proven cytocompatibility, antimicrobial effects (doxycycline loaded NPs) [24], and osteogenic potential (both doxycycline and dexamethasone loaded NPs) [23].

The tested Ti-particles exposed to the RAW cells demonstrated slight cytotoxic effects (Figure 3), what correlated with the resulting DNA release and metabolic activity shown in the different experimental groups (Figure 4). These results concord with previous investigations where macrophages when exposed to titanium particles of different sizes, compositions and concentrations have maintained their viability and stable proliferation rates [8,36,37,39]. In fact, titanium microparticles, at the present size range, have been shown to be less cytotoxic than nanoparticles [28]. The tested micro-particles were phagocytised by the macrophages, which appeared isolated and adopting a combination of round-shaped and elongated forms under the different experimental conditions, with presence of particle vacuolization within their cytoplasm (Figures 5,6,7 and 8) [39].

Ti particles when exposed to osteoblast cells in experimental studies have also demonstrated a decrease in their viability and being present within their cytoplasm [41]. Similar cytotoxicity elicited by Ti particles has also been described for fibroblasts from periodontal ligament [38]. These results have speculated that Ti particles may elicit a pro-inflammatory effect triggered by frustrated phagocytosis leading to different inflammasome activation [42]. However, this ascribed pathogenic role of released titanium particles associated with dental implants is still a matter of high controversy. While *in vitro* studies have shown reactivity of macrophages against Ti particles [8,36,37,39], a recent *ex vivo* study that identified Ti- particles in granulation tissue of peri-implantitis lesions did not evidence presence of phagocytes with intracytoplasmic Ti-particles or any foreign body reaction suggestive for a direct pathological effects these Ti-particles [7]. These differences may be explained by the short evaluation time periods of most of the *in vitro* studies, since prolonged exposures of titanium particles to macrophages (6 days) have shown to suppress scavenger-receptor-mediated endocytosis [43].

Once activated with LPS, the tested particles added to the cell culture increased the number of dead cells two-fold through both apoptotic and necrotic processes (Figures 3 and 4) in contrast with the group where LPS was added without Ti particles, where cell death mainly occurred by apoptotic processes (Figure 3). After LPS activation, macrophages increased their metabolic activity and changed their morphology

becoming enlarged and flattened, which is characteristic of M1 phenotype macrophages [43] (Figure 7).

Titanium particles also slightly increased the macrophage cytokine production, which is in line with previous *in vitro* experiments [4,27]. However, this cytokine production was increased when macrophages were activated with LPS (Figure 10) [36,40,44]. However, when cytokines were analyzed independently, macrophages activated with LPS resulted in an increased expression of pro-inflammatory cytokines (IL-10 and TNF-α and IL-6), and increased secretion of granulocyte macrophage-colony stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-SCF). This macrophage cytokine release associated to LPS activation had a clear pro-inflammatory pattern, as it has been described in previous reports [27,45], and interestingly it may stimulate early osteoclast activation, even without influencing the RANKL/OPG system [37]. In contrast, Ti particles stimulated in macrophages an increased secretion of interleukin-1 receptor-associated kinase-M (IL-1ra), which regulates toll-like receptors (TLRs) [27]. TLRs have also been associated with alterations in the local leading to peri-implantitis progression [44]. However, it is unknown whether this over-expression of IL-1ra stimulated by Ti-particles may negatively affect TLRs-mediated inflammation, resulting in a selective immunosuppression in peri-implant tissues [44,46]. This hypothesis should be tested in future investigations.

When LPS-activated macrophages were exposed to titanium particles, they secreted low levels of cytokines/chemokines, what is consistent with previous *in vitro* studies [27,40]. Although both LPS and Ti particles stimulated the macrophages, these cells may elicit 'endotoxin tolerance', which suppresses inflammatory reactions by altering signaling pathways when exposed to prolonged stimulation. While this 'endotoxin tolerance' may protect the host from excessive production of pro-inflammatory cytokines and an unresolved chronic inflammation, the resulting immunosuppression may also be detrimental [46], what has been reported in peri-implant pathology [47]. An *Ex vivo* observational study also reported absence of an inflammatory response associated with titanium particles in peri-implantitis sites [7]. Production of TNF-α (highly pro-inflammatory cytokine) was detected in the presence of LPS but not when just Ti particles were added to the culture media. IL-10 secretion was produced after LPS addition and not when Ti particles were added to the media.

The most relevant differences found when comparing the tested experimental groups were related to the macrophage's phenotypic changes. Non-functionalized Ti particles with either LPS activation or not, demonstrated higher M1/M2 ratio, what corroborates previous studies reporting that titanium particles may foster a pro-inflammatory macrophage polarization towards M1 phenotype [43,48]. These results are also consistent with findings from an *ex vivo* experimental study in which biopsies form peri-implantitis were examined and polarization towards M1 macrophages was also reported [7]. This event has also been recently stated in a systematic review of omics *in vitro* studies, in which it was showed that bacterial components such as LPS may have a synergistic effect with titanium in the development of inflammatory markers in oral cells [15]. In the included studies from this systematic review, titanium was hypothesized to increase the sensitivity of the oral epithelium to bacteria by increasing the expression of TLR4 or by acting as a second stimulus of LPS, thus enhancing the inflammation [15].

Interestingly, when Ti particles were functionalized with doxycycline or dexamethasone NPs, the percentage of M1 cells phenotype diminished (about three-fold). In fact, Ti particles functionalized with dexamethasone NPs demonstrated the highest expression of M2 phenotype (about 8-fold) compared to the control (Figure 9). The M1/M2 ratios for dexamethasone or doxycycline NPs groups were forty times lower compared with non-functionalised Ti particles (Figure 9). These results were consistent with the FESEM analysis, with higher number of cells exhibiting a round shape (Figures 6 and 7) in these groups. These results may be explained by the demonstrated activity of dexamethasone inducing the expression of cytokines and other signalling molecules with capacity to control the balance between inflammation and tissue remodelling [43,49]. Titanium particles have also been shown to increase the oxidative stress in exposed cells, thus increasing the ROS level in cells [15]. In this regard, dexamethasone has shown to inhibit reactive oxygen species (ROS) production and nitric oxide release in a dose dependent manner [50]. The use of sub-antimicrobial doses of doxycycline has also been shown in clinical studies, to reduce the production of ROS and nitro-oxidative stress in patients affected by periodontitis [51].

When titanium particles were loaded with dexamethasone nanospheres, the macrophage cytokine secretion was like the non-activated control cells (Figure 11), what suggests that dexamethasone modulated the effect of the Ti particles on the macrophage cytokine release. Similarly, when macrophages activated with LPS were exposed to Ti particles functionalised with doxycycline, the effect of Ti particles on the overall cytokine secretion was decreased, which may be explained by the previously mentioned anti-microbial, anti-inflammatory and antioxidant effects of dexamethasone and doxycycline [43,50,51].

A previous study did also state that dexamethasone and doxycycline nanoparticles were able to stimulate osteoblasts differentiation when applied on titanium surfaces, being considered potential inducers of osteogenic environment around titanium dental implants [52]. However, these results must be taken with caution, due to the inherent limitations of the *in vitro* investigations. It should also be taken into account that the assays were conducted at the early stages of the inflammatory response, when cytokine protein levels are low, and some cytokines could peak ahead of time [39]. It should be noted that in the present study, we used the macrophage cell line RAW264.7, what may possibly be a limitation of the study. The results should be further confirmed using primary cells.

In summary, this study evidenced the effect of Ti particles on the viability, polarization, and function of LPS-stimulated cultured macrophages. This demonstrated impact of the functionalized nanoparticles of dexamethasone and doxycycline on the macrophage response may be used as a strategy to limit macrophage-mediated inflammation in the progression of periimplantitis [27,53]. Furthermore, due to these demonstrated osteogenic, antibacterial and anti-inflammatory activities, functionalized nanoparticles may also be potentially used as an adjuvant to non-surgical treatment of periodontitis or in regenerative strategies. However, further research with adequately designed *in vivo* preclinical studies must be conducted before being clinically used.

Conflict of interest: The authors declare no conflict of interest.

Contribution statement: Concept and Design: MT-O, MA, MT, RO; Investigation: JC-A, MA, MT-O, RO; Data analysis and interpretation: JC-A, MT-O, MA, MT, MS, RO; Drafting article: JC-A, MT-O, MA, RO; Critical revision of article: MA, MT-O, MT, MS, RO; Statistics: JC-A; Funding acquisition: MT, RO. All authors have read and agreed to the published version of the manuscript.

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