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Titanium dioxide nanoparticles temporarily influence the sea urchin immunological state suppressing inflammatory-relate gene transcription and boosting antioxidant metabolic activity

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Titanium dioxide nanoparticles temporarily influence the sea urchin immunological state suppressing inflammatory-relate gene transcription and boosting antioxidant metabolic activity

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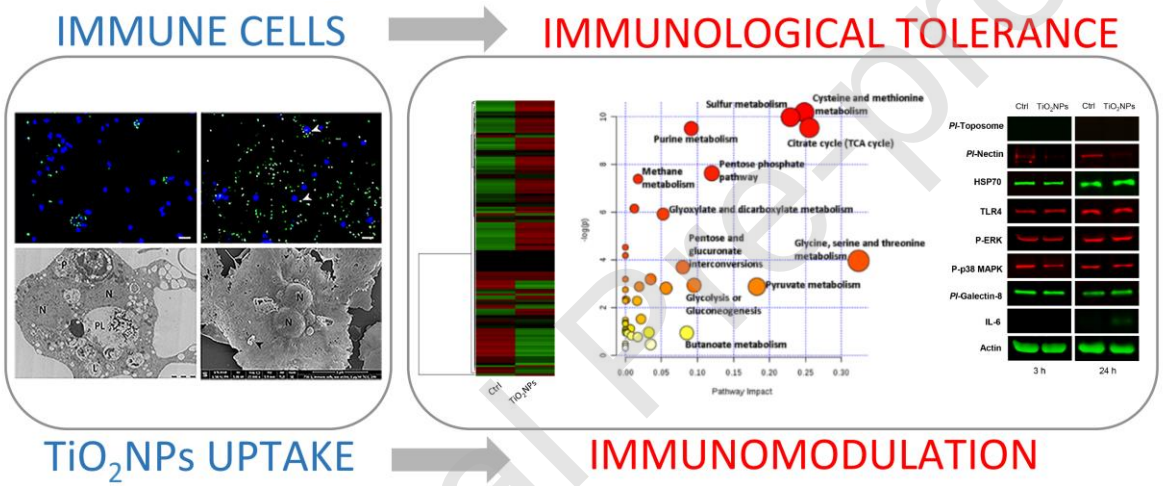
Graphical abstract



Highlights

- TiO_2NPs excite sea urchin immune cell adherence and phagocytic activity
- TiO_2NPs have a suppressive effects on transcripts involved in inflammation
- TiO_2NPs activate antioxidant defence metabolic pathways
- Immune system rewires metabolism promoting tolerance under TiO_2NP-exposure

Abstract

Titanium dioxide nanoparticles (TiO_2NPs) are revolutionizing biomedicine due to their potential application as diagnostic and therapeutic agents. However, the TiO_2NP immune-compatibility remains an open issue, even for ethical reasons. In this work, we investigated the immunomodulatory effects of TiO_2NPs in an

emergent *proxy to human non-mammalian* model for *in vitro* basic and translational immunology: the sea urchin *Paracentrotus lividus.* To highlight on the new insights into the evolutionarily conserved intracellular signaling and metabolism pathways involved in immune-TiO_2NP recognition/interaction we applied a wide-ranging approach, including electron microscopy, biochemistry, transcriptomics and metabolomics. Findings highlight that TiO_2NPs interact with immune cells suppressing the expression of genes encoding for proteins involved in immune response and apoptosis (e.g. *NF-κB, FGFR2, JUN, MAPK14, FAS, VEGFR, Casp8*), and boosting the immune cell antioxidant metabolic activity (e.g. pentose phosphate, cysteine-methionine, glycine-serine metabolism pathways). TiO_2NP uptake was circumscribed to phagosomes/phagolysosomes, depicting harmless vesicular internalization. Our findings underlined that under TiO_2NP-exposure sea urchin innate immune system is able to control inflammatory signaling, excite antioxidant metabolic activity and acquire immunological tolerance, providing a new level of understanding of the TiO2NP immune-compatibility that could be useful for the development in Nano medicines.

Keywords: Innate immunity; TiO_2NP-responsive genes; human gene networks; metabolic rewiring; homeostasis restoring

1. Introduction

1.1. Rationale for the choice of the NPs

Titanium dioxide nanoparticle (TiO_2NP) is a white, odourless, non-combustible and poorly soluble powder with two prevalent crystal structures, rutile and anatase. Among its polymorphs, anatase is of paramount importance since it exhibits higher activity, with a high refractive index and strong absorption of ultraviolet radiation (Fang et al., 2019). Due to their low dissolution rate, high surface area, anti-corrosive, antimicrobial and photocatalytic properties, TiO_2NPs are one of the most widespread and studied particles (Mou et al., 2014). Biosynthesis of TiO_2NPs have gained wide interest among researchers because of their cost effective, eco-friendly and reproducible approach (Zhu et al., 2015). Their exclusive chemical and physical properties combined with restricted noxiousness, inertness and biocompatibility make TiO_2NPs one of the most promising nanomaterials for applications in medicine (Yin et al., 2013). Compared to traditional molecular medicine, TiO_2NPs present distinctive bioactive properties (e.g. intermixing, ultrafast kinetics, diffusion, sensory response) promoting health or preventing disease (Yin et al 2013). On the other hand, concerns have been raised that these same properties may challenges their safety use. The potential toxicity greatly depends on the composition and concentration of the particle formulation. Although the definition identifies NPs as having dimensions below 0.1 μm, especially in the area of drug delivery relatively large NPs may be needed (>100 nm) and in large number for loading a sufficient amount of drug onto the particles (De Jong

and Borm, 2008). This strengthens the high levels of attention regarding the biosafety of TiO_2NPs on human and environment.

1.2. Rationale for the choice of the model

NP aggregates/agglomerates fall into the size, shape, geometry and surface chemistry of a wide range of bacteria, fungi and other pathogens of micro/nanoscale (Sweet et al., 2012). Therefore, the particulate nature of the NP aggregates/agglomerates dictates a preferential interaction with cells of the immune system deputed to recognition and elimination of foreign particulate matters. As an important protective system to defend organisms from foreign materials and hazard signals within the body, immune system plays a critical role in keeping tissue and cellular homeostasis. Nanomaterials can interact with the immune system in a number of ways either enhancing or suppressing immune system functions (Smith et al., 2014). A wide-ranging understanding of the immune-biosafety of TiO_2NPs has not been reached yet, due to huge gaps in knowledge on the biomedical potentiality, dealings with different biological factors (signaling molecules) and microenvironments (blood vessels, immune cells), risk management, particle disposal, and finally due to ethical issues.

A new frontier in immuno-nano research is the use of alternative approaches to promote the practical and vigilant application of the 3Rs principle (reduce, refine and replace) limiting living organism usage (Russell and Burch, 1959). In this light, primary cell culture straightforwardly represents the biological tissue microenvironment where cells reside within an organism, as cell–cell signaling pathways remain safeguarded (Bols et al., 2017). The sea urchin *Paracentrotus lividus* is an emergent *proxy to human non-mammalian* model for studying basic and translational immunology *in vitro-ex vivo* (Pinsino and Alijagic, 2019). Decoded sea urchin genome showed surprising relationship to humans (Sea Urchin Genome Sequencing Consortium, 2006; Cameron et al., 2008), highlighting the relevance of the sea urchin as a *proxy to human* model for biomedical research, mainly for immunological research. Sea urchin immune genes and related signaling pathways stand as an exceptional example of immune system complexity and sensing capability (Rast et al., 2006). Sea urchin immune cells sense environmental cues through an intricate repertoire of predicted extra- and intracellular immune receptors, regulators and effectors, including 703 innate pathogen recognition molecules, 423 chemical defensome genes and 67 cytokines and growth factors (Alijagic and Pinsino, 2017).

1.3. Rationale for the choice of the aim and the expected results at the nanoscale immune-machinery level

Immunity is the result of sophisticated signaling and behaviors, rising from dynamic and feedback-regulated interactions among many nano- cellular components (genes, transcripts, metabolites, proteins, cells). Specifically, environmental cues trigger signaling cascades consisting of dynamic events - including protein binding, phosphorylation, degradation, nuclear localization, gene expression changes, and production of effector molecules and proteins involved in keeping cellular homeostasis (Broz and Monack, 2013). Thus, immune functions are bio-energetically expensive, demanding accurate management of cellular metabolic pathways (e.g. oxidative metabolism, glycolysis, glutaminolysis) that fuel and regulate immune response

maturation (Pearce and Pearce, 2013; Ganeshan and Chawla, 2014). Diagnostic- and therapeutic release-aimed NPs require the highest degree of compatibility with the immune system to avoid inflammatory reactions or other noxious reactions. TiO_2NPs are considered inert and recent studies demonstrated that core/shell TiO_2NPs can be used as doxorubicin vector for cancer cells (Arora et al., 2012). On the other hand, the potential immune effects of TiO_2NPs are raising concern because other studies reported cell toxicity and genotoxicity (Zhang et al., 2012).

Although many aspects of the modulation of immune responses induced by nanoparticles have been investigated, a clear knowledge on the hierarchically organized set of molecular, cellular and organismal networks involved in the immune nanoscale interactions with TiO_2NPs and subsequent intracellular signal transduction, cellular trafficking and fate has not been achieved yet. Therefore, it is mandatory to address which type of interaction occur between nanomaterials and the nanoscale machineries of the immune system.

This study aims to characterize how TiO_2NPs interact with the sea urchin immune cells, focusing on the new insights into the intracellular signaling and metabolism pathways involved in immune-TiO_2NP recognition and interaction *in vitro.* For the first time, we demonstrated that TiO_2NPs influence the sea urchin immunological state: i) suppressing the expression of genes encoding for proteins involved in immune response-regulating cell surface receptor and apoptotic signaling (e.g. *NF-κB, FGFR2, JUN, MAPK14, FAS, VEGFR, Casp8*) and ii) boosting the immune cell antioxidant metabolic activity (e.g. pentose phosphate, cysteine –methionine, glycine-serine metabolism pathways). Our findings highlighted that under TiO_2NP-exposure sea urchin innate immune system is able to control inflammatory signaling (preventing NF-κB from inducing further inflammatory mediators) and proceeds to immune response resolution. Results establish that TiO_2NPs interact with sea urchin immune cells without eliciting harmful and irreversible effects on biological functions, and reinforce the recommendations about the use of sea urchin primary immune cell cultures as a *proxy to human* forceful model for nano- and translational- immunological studies.

2. Material and methods

2.1 Animal handling, cell cultures and titanium dioxide nanoparticle exposure

Adult sea urchins (*P. lividus*) were collected along the unpolluted coast of Sicily (Capo Gallo Marine Protected Area with restrictions for human activities, Italy). Sea urchins were acclimatized for a few weeks, and maintained under controlled conditions of temperature, pH, photoperiod, salinity and density in oxygenated artificial seawater (ASW) (Aqua Ocean Reef Plus Marine Salt, Aquarium Line, Italy) (Pinsino and Alijagic, 2019). Aeroxide TiO_2 P25 nanoparticles (declared particle size 21 nm, anatase and rutile 4:1) were acquired from Evonik Degussa (Essen, Germany). TiO_2NPs dispersed in salt water media were previously characterized by analytical techniques (transmission electron microscopy, Brunauer, Emmett and Teller method, dynamic light scattering) (Brunelli et al., 2013; Pinsino et al., 2015). TiO_2NP stock suspension (100-µg mL^{-1}) was prepared in

ultrapure water (18.2 MΩ cm^{-1}) (Purelab Option-Q System, UK), vortexed for 5 minutes and sterilized under UV light prior to usage.

Immune cell collection and primary cell culture were performed as recently described by Pinsino and Alijagic (2019). After harvesting, 1.5 x 10^6 cells were seeded into each well of a 25-well plate (Thermo Fisher Scientific, UK), and the TiO_2NP suspension was immediately added to CCM-based culture medium for 24-hours (hrs) (1 µg mL^{-1} nominal concentration). A dozen sea urchins were used for the primary cell cultures and they served for both control and exposed sample preparation. After immune cells harvesting, animals were mantained in dedicated tanks for over a 15–20 day period. At 3-hrs and 24-hrs time-points, cells were gently recovered from each well with a soft scraper, centrifuged (10 minutes, 9000 g, 4°C) and the pellets stored in aliquots at -80°C for following investigations. Seven controls and seven TiO_2NPs-exposed immune cells for each time (3 and 24 hours), obtained from the same donors, have been used for protein and mRNA investigations (28 wells in total). For metabolomic typing, immune cells were gently recovered from the wells, centrifuged (6 minutes, 4500 g, 4°C). Pellets were suspended in 0.5 mL ice-cold acetonitrile-water solution (70:30 v/v)(Sigma-Aldrich) and stored at -80°C until use. Five controls and five TiO_2NPs-exposed immune cells from the same donors have been used for metabolomic typing (10 wells in total).

2.2 Cellular imaging

2.2.1 Fluorescence-based *in vivo* phagocytosis assay and Leishman's eosin methylene blue labelling. Live immune cells (24 hrs- TiO_2NP exposure and control) were incubated into each well with nanobeads based on PD, Chromeon 470-marked, carboxylated (Sigma-Aldrich) for 1 hr in the dark at 16±2°C (0.9×10^9 nanobeads mL^{-1}). Cells were fixed in methanol at 20°C for 5 minutes and washed twice with ASW. Fixed cells were incubated with Hoechst 33342 trihydrochloride (Sigma-Aldrich) for 7 min (10 µg mL^{-1}) and washed three times with ASW before visualization. The fluorescent Hoechst dye was used to visualize cell nuclei.

Staining of the acidic and basic components of immune cells was performed by the use of 1.2 mg mL^{-1} ice-cold Leishman's eosin methylene blue solution (Merck) for 10 minutes. After labelling, solution was removed and cells were washed with ASW. Photomicrographs were acquired by Zeiss Axioskop 2 Plus microscope (Zeiss, Arese, Italy).

2.2.2. Scanning electron microscopy. Control and exposed immune cells (1 µg mL^{-1} nominal concentration, 24-hrs TiO_2NP exposure) adhering to 12 mm circular glass coverslips (Menzel Gläser, Germany) were fixed with a 1:1 mixture of 6% glutaraldehyde in 100 mM cacodylate buffer and ASW (pH 8 - 8.5) for 1-hr at room temperature . Coverslips were washed three times with cacodylate buffer, dehydrated through an alcohol series (25, 50, 75, 90, 96 and 100%) and dried in K850 Critical Point Dryer (Quorum Technologies Ltd, Ringmer, UK). The dried coverslips were sputter coated with 3 nm platinum using high-resolution Turbo-Pumped Sputter Coater Q150T (Quorum Technologies Ltd, Ringmer, UK). Cellular morphology was examined in FEI Nova NanoSEM scanning electron microscope (FEI, Czech Republic) at 5 kV using CBS and TLD detectors.

2.2.3. Transmission electron microscopy. Immune cells incubated in 15 mL tube (1 µg mL^{-1} nominal concentration, 24-hrs TiO_2NP exposure) under gentle agitation were fixed as described above. After exhaustive washing, cells were post-fixed with 1% osmium tetroxide in cacodylate buffer overnight at 4°C. Cells were washed three times in cacodylate buffer at 4°C and ddH_2O, then they were allowed to warm up to the room temperature and then embedded into 4% low-melting agarose. Solidified agarose was cut into small cubes 1 x 1 mm in size. The agarose cubes were dehydrated in alcohol series as already reported. Finally, agarose cubes were embedded into the epoxy resin (EMBed-812 Embedding kit; Electron Microscopy Sciences). Ultrathin sections were contrasted using uranyl acetate and lead citrate and were inspected through FEI Morgagni 268(D) electron microscope (FEI, Czech Republic).

2.3 Cell viability and toxicity assays

The assays were performed in white opaque-walled 96-well tissue culture plates (Thermo Fisher Scientific, UK) in the dark at 16 ± 2°C (1 x 10^5 cells/well-final density). TiO_2NPs were afterwards added, drop by drop, into the medium at the 1 µg mL^{-1} nominal concentration. Cell viability and cytotoxicity were measured using RealTime-Glo MT Cell Viability Assay (Promega, USA) and the non-lytic CellTox™ Green Cytotoxicity Assay (Promega, USA) as previously described (Pinsino and Alijagic, 2019). Luminescence and fluorescence were detected and quantified using GloMax Discover high-performance Microplate Reader (Promega). All assays involved at least five biological replicates from 5 donors.

2.4 RNA isolation and Real-time quantitative PCR (RT-qPCR)

Total RNA from control and exposed immune cells was isolated and reverse transcribed to cDNA as previously described (Pinsino et al., 2015). Exposure conditions (1 µg mL^{-1} TiO_2NPs nominal concentration, 24-hrs ending point) were chosen taking into account: i) the behavior of TiO_2NPs in synthetic and real salt waters (Brunelli et al., 2013); ii) the results obtained from our previous study performed *in vivo* (Pinsino et al., 2015); ii) the known early immune-activation of sea urchin immune cells upon insult (Pancer et al., 1999). To quantify *Pl-TLR*-4 like, *p38 MAPK, NF-kB, Jun* gene expression, cDNAs were amplified by SYBR Green technology, based on a Comparative Treshold Cycle Method (Livak and Schmittgen, 2001), according to the manufacturer's instructions (Applied Biosystems StepOnePlus instrument). *Pl*-z12-1 mRNA was used as an internal endogenous reference gene (Costa et al., 2012).

2.5 NanoString nCounter Gene Expression Assay

Total RNA from control and exposed immune cells was extracted using Qiagen RNeasy mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). A panel of *P. lividus* transcript sequences were selected and retrieved from the National Centre for Biotechnology Information (NCBI) database (Costa et al, in preparation). For each selected transcript, custom-made probes were developed and hybridized on total RNA (100ng/sample) through the nCounter NanoString technology (Diatech Labline) based on direct digital detection of mRNA molecules using target-specific color-coded probe pairs and capture probes carrying 35- to 50-base target-specific sequences (Kulkarni, 2011). The expression level of each gene was measured by

counting the number of times the color-coded barcode for that gene was detected, and barcode counts were then tabulated using S24 internal references genes. The resulting digital counts of each transcript were compared between control and TiO_2NP-exposed immune cells for each individual donor for two donors (Diatech Labline). Heat map was generated by a freely available web server (Babicki et al., 2016). Networks of human genes corresponding to the sea urchin genes analysed in this study was found by using Cytoscape software with automatically selected weighting method (Saito et al., 2012).

2.6 SDS-PAGE and immunoblotting

The goal of immunoblotting is to identify with a specific antibody a particular protein within a complex mixture of proteins that has been fractionated in a sodium dodecyl sulfatepolyacrylamide denaturing gel electrophoresis (SDS-PAGE) and immobilized onto a membrane. Protein contents of each sample were quantified by the Bradford method with the BioRad assay kit (Hercules, CA, USA). Fifteen micrograms of total protein per each cell extract were mixed with acetone (1:1), overnight, at -20°C. Acetone-precipitated proteins were suspended in SDS sample buffer supplemented by the β-mercaptoethanol, boiled for 5 minutes and run on 4-20% Mini-PROTEAN TGX precast polyacrylamide gels (BioRad, USA), and then transferred to a nitrocellulose membrane (Amersham, UK) according to standard procedures. Non-specific binding sites were blocked with Odyssey blocking buffer (LI-COR Biosciences, USA). Membranes were incubated with either one of the following primary antibodies which are known to recognize proteins mediating immune response: *Pl*-toposome (BEVIB12b8) 1:200; *Pl*-nectin 1:200; *Pl*-galectin-8 1:800; TLR4 (H-80) protein (Santa Cruz Biotechnology, sc-10741) 1:250; HSP70 (SIGMA, Cat N. H-5147) 1:1000; Phospho-p38 MAP Kinase (Tr180/Tyr182) (Cell Signaling, 9211) 1:250; Phospho-p42/44 MAP Kinase (ERK1/2) (Cell Signaling, 9101) 1:300; IL-6 (H-183) (Santa Cruz Biotechnology, sc-7920) 1:100; β-actin (SIGMA, A5441) 1:500. Membranes were washed three times with PBS-Tween20 prior to incubation with a fluorescein-labelled secondary anti-mouse and/or anti-rabbit antibody (LI-COR Biosciences) and visualized with Odyssey Infrared Imager (LI-COR Biosciences).

2.7 Mass spectrometry in untargeted liquid chromatography

Metabolites were extracted in 0.5 mL ice-cold 1% acetic acid water-acetonitrile solution (70:30 v/v). Supernatant containing both polar and non-polar metabolites were recovered in glass inserts for solvent evaporation. Samples were dried at 30°C for about 2.5 hrs (Concentrator plus / Vacufuge® plus, Eppendorf), re-suspended in 150 µL of H_2O LC-MS grade and injected in UHPLC–MS system for RPLC chromatography. Samples were analysed using a platform consisting of an UHPLC system (Agilent 1290 Infinity UHPLC system) coupled with a quadrupole-time of flight hybrid mass spectrometer (Agilent 6550 iFunnel Q-TOF) and equipped with an electrospray Dual JetStream source operated in negative mode. Mass spectra were recorded in centroid mode in a mass range from m/z 60 to 1050 m/z. Active reference mass correction was performed through a second nebulizer using the reference solution (m/z 112.9855 and 1033.9881) dissolved in the mobile phase 2-propanol-acetonitrile-water (70:20:10 v/v). Data analysis and isotopic natural

abundance correction were performed with MassHunter ProFinder and Mass Profile Professional software (Agilent).

2.8 Statistics

Statistical software GraphPad Prism Software 6.01 (USA) was used for data processing. All data from qPCR were analysed by the t-test and one-way analysis of variance (one-way ANOVA) compared with the respective control group. Statistical differences in cell viability and cell toxicity were estimated by one-way ANOVA followed by the multiple comparison test of Tukey's. Level of significance was set at $p \leq 0.05$ and data were presented as mean ± SD.

3. Results and Discussion

3.1 TiO_2NPs excite sea urchin immune cell adherence and phagocytic activity

P. lividus coelomic fluid (CF) carries signaling molecules and three different cell types of freely circulating blood like-cells (phagocytes, amoebocytes and vibratile cells) known to mediate immunity in adult sea urchins (Pinsino and Matranga, 2015). The innate immune system provides a first line of defense against pathogens through phagocytosis and activation/inhibition of several conserved signaling pathways (Schroder and Tschopp, 2010). Sea urchin CF contains host-associated intestinal microbiota and other "non-self" elements, making phagocytic activity essential in recognizing particle clearance (Smith et al., 2018). To focus on understanding the TiO_2NP-immune cells interaction *in vitro*, primary short-term cultures were performed as recently described by Pinsino and Alijagic (2019). Pristine TiO_2NP powder display a primary particle size ranging from 10 to 65 nm, and irregular and semi-spherical shape, categorised as mesoporous NPs, as summarized in our previous study for the same particle (Pinsino et al., 2015). The TiO_2NPs show a much larger hydrodynamic diameter after suspension in the water media highlighting that these particles consisted of hard aggregates ranging from 294 ± 37 to 375 ± 31 nm at 0.2 hrs of dispersion in synthetic and real waters (1 µg mL^{-1}) (Brunelli et al., 2013). TiO_2NPs suspended in the complex culture medium used in this study (10 µg mL^{-1}), appeared as polydispersed particle with a prevailing diameter ≥1000 nm (not shown). Sea urchin culture medium contains a modest average protein concentration that results in the adsorption of proteins onto these particle surface leading to a scenario of heteroaggregation (particle-protein complexes) as recently described by Alijagic et al (2019). Under exposure conditions, phagocytic cells - the most abundant adherent *P. lividus* immune cell type (>80%)- appeared much more strongly adherent, well spread and well

organized in bundles and fibres vs. control (compare Figure 1A-1B). Leishman's eosin methylene blue labelling highlighted an evident lodging in specific (very close to nuclei) sites in shades of a strong violet colour indicating a luminal acidification distinctive of vesicular structures in TiO_2NP-exposed cells (Figure 1B). An intense phagocytosis was confirmed by the increased encapsulation and internalisation of the fluorescent nanobeads upon 1 hr incubation (compare Figure 1C to 1D, green color). Consistently, transmission electron micrograph of a phagocytic cell depicted the ultrafine intracellular localization of TiO_2NPs within the endocytic/cytoplasmic vesicles close to the periphery of the cell membrane and adjacent to the nuclei (Figure 1E). Notably, TEM revealed a growing network of vesicle presenting different degree of maturation such as lysosomes, early phagosomes and growing phago-lysosomes (from 380 nm to 2.4 µm in diameter) containing aggregates/agglomerates (1.23 µm), thus confirming our previous study performed *in vivo* (Pinsino et al., 2015). Scanning electron micrograph of a phagocytic cell shows a particle aggregate/agglomerate partially engulfed (Figure 1F, black arrow).

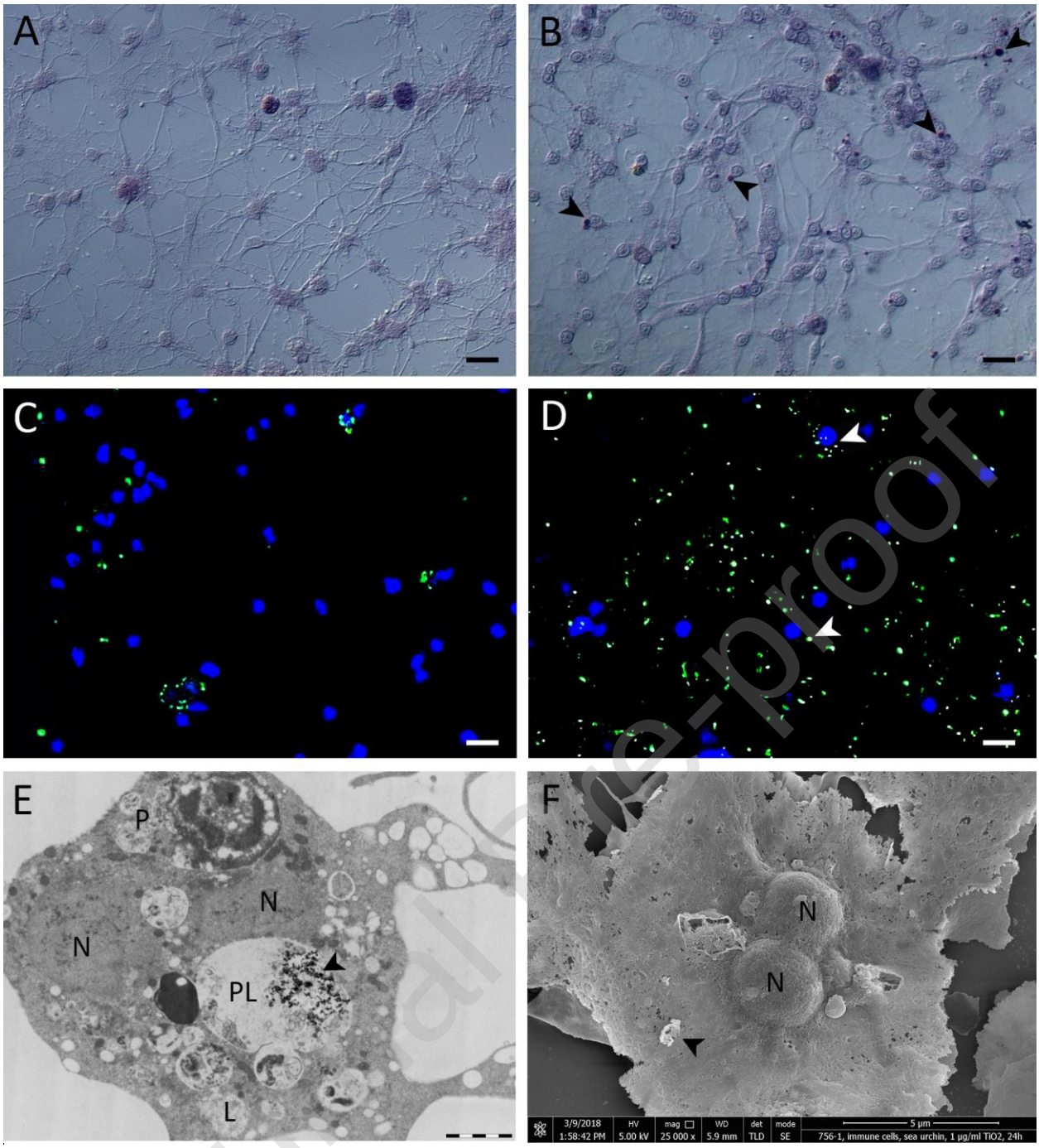


Figure 1. Sea urchin immune cells under the microscope. (A-B) Leishman's eosin methylene blue labelling in control (A) and TiO_2NP-exposed cells (B). Cells exposed to 1 µg/mL TiO_2NPs for 24-hrs showed specific sites in strong violet color that are indicative of a vesicular structure acidification (see black arrows). (C-D) Fluorescence-based *in vivo* phagocytosis assay after 1 h incubation. Immune cell nuclei labelled with Hoechst 33342 (blue color); green channel indicates internalized fluorescent nanobeads in control (C) and exposed cells (D) (see white arrows). Scale bar: 10 µm. E) Transmission electron micrographs depicting the ultrafine intracellular localization of TiO_2NPs (see black arrow) within vesicle presenting different degree of maturation Scale bar: 2 µm. F) Scanning electron micrograph of a phagocytic cell showing TiO_2NPs partially engulfment (see black arrow). N – Nucleus; L– lysosome; P – phagosome; PL – phago-lysosome. Scale bar: 5 µm.

3.2 Sea urchin immune cell transcriptomic profile *in vitro* deciphers a suppressive effect of the TiO_2NPs on transcripts involved in inflammation

A modulation of the intracellular and extracellular proteins ruling homeostatic changes can reflect a -positive or negative - transcriptional and/or post-transcriptional immune-regulation (Koppenol-Raab et al., 2017). Depending on the extent and severity of an insult, cells can either alter their signalling pathways and metabolism adopting a new state or die (Kucherenko and Shcherbata, 2018). Temporal fluctuations in the cellular microenvironment take place regularly, and such changes can alter cellular homeostasis. Over-all, alterations are reversible and of brief duration. In order to profile the sea urchin immunological state under TiO_2NP exposure *in vitro*, we investigated the gene expression profile and metabolism of primary sea urchin immune cell cultures by a high throughput approach. Currently, *P. lividus* whole-genome sequencing, assembly and annotation are still running (European *P. lividus* genome project consortium). Genome is expected to become publicly available very soon, along with ESTs, cDNA, BAC sequences and other transcriptome resources currently annotated in the GenBank and partially accessible through NCBI database. In this study, a digital multiplexed gene expression profiling of 127 selected *P. lividus* transcripts encoding for proteins involved in cellular growth, cell survival, homeostasis, development, stress, oncogenesis and immunity was drew up by nCounter NanoString technology (See Table 1S). Heat map combined with clustering methods (grouping genes based on the similarity of their gene expression pattern) showed that a good percentage of different genes appeared differentially expressed in 24-hr TiO_2NP-exposed and un-exposed sea urchin immune cells of two different donors (Figure 2). Clustering was done with the publically-available program in the Heatmapper web server using a Euclidean distance measurement and an average linkage (Babicki et al., 2016). Responsive genes found in only *n=1* donor, were not considered TiO_2NP responders, and transcripts presenting a digital counts lower than 50 in controls were excluded, according to the manufacturer's instructions. Comparison between immune gene basal expressions in both donors showed a high inter-individual variability (Figure 2, compare controls, column 1 and 3) but a small set of responsive genes (32 of 127) are reproducibly regulated by 24-hrs TiO_2NP exposure *in vitro* (≥1.5-fold higher or lower than controls) (Figure 2, column 2 and 4). Transcript levels of 29 target genes were found decreased in response to TiO_2NPs (24 hr exposure) (Figure 2, red names) while only three were found increased (Figure 2, green names).

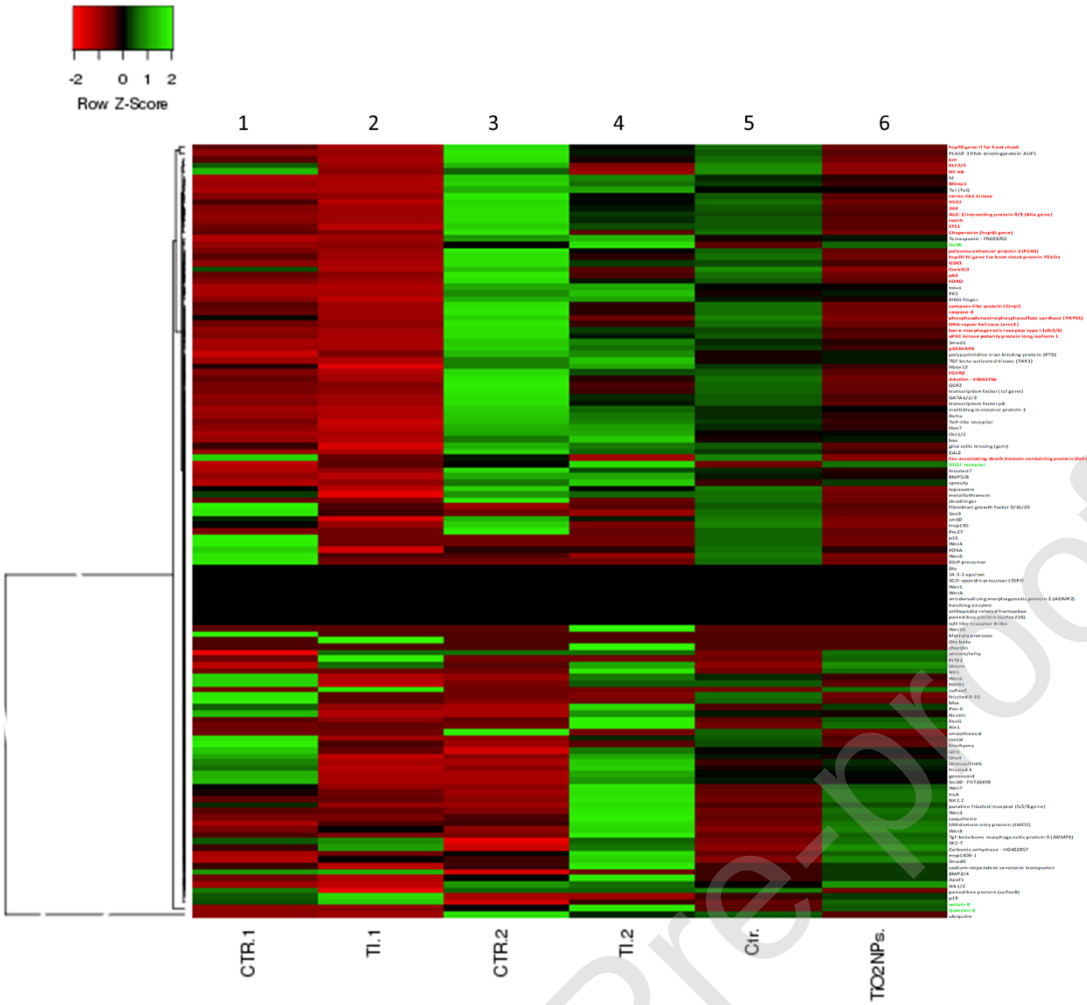


Figure 2. Heat map profiling of differentially expressed genes in TiO_2NP-exposed and un-exposed sea urchin immune cells, *in vitro*. Color and intensity of the boxes represent relative changes of gene expression. Data are displayed in a grid where each row represents a gene, each column from 1 to 4 represents a sample and each column from 5 to 6 represents mean raw data from control and exposed immune cells respectively. CTR1: control 1; Ti1= **TiO_2NP-exposed sample 1;** CTR2: control 2; Ti2= **TiO_2NP-exposed sample 2; Ctr: mean from controls; TiO_2NPs: mean from TiO_2NP-exposed samples.** A complete list of responder (red and green color) and non-responder (white color) genes following the sequence used for the heat map is reported in Table S1.

In order to focus on TiO_2NP-responsive gene interaction and to understand the related protein functions deduced by human gene ontology, we used Cytoscape, an open source network visualization and analysis tool, to draw networks by human gene ontology database corresponding to the sea urchin genes analysed in this study. An open software platform for visualizing complex networks and integrating these with any type of data does not exist yet for the sea urchin. Because of the evident homology between sea urchin and human genes, support for integrated models of biomolecular interaction networks was thus provided by Cytoscape. Sea urchin TiO_2NP-responsive genes (analogues to humans) were found predominantly involved in immune response-regulating cell surface receptor signaling (FGFR2, GSK3B, NF-κB, JUN), BMP signaling pathway (NOTH1), apoptotic and defense response signaling (HSP90B1, caspase 8, NF-κB, JUN, MAPK14, FAS, GSK3B), macrophage differentiation (caspase 8, VEGFA), and topologically incorrect protein response (HSP90B1, HSPA4, HSPA2 (Figure 3). Transcriptomic results decipher a suppressive effect of the TiO_2NPs on transcripts encoding for proteins mediating apoptotic signaling and inflammatory response (e.g. caspase 8, NF-κB, JUN,

MAPK14). In analogy, measurement of *Pl-p38 MAPK*, *Pl-NF-kB*, and *Pl-Jun* transcripts by comparative RT-qPCR confirmed decreasing levels in 24-hr TiO_2NP-exposed sea urchin immune cells (compared to controls), thus validating nCounter NanoString analysis, while after a few hour exposure, transcripts did not display any significant variations (Figure 4) in line with nCounter NanoString analysis at 3 hr exposure (not shown).

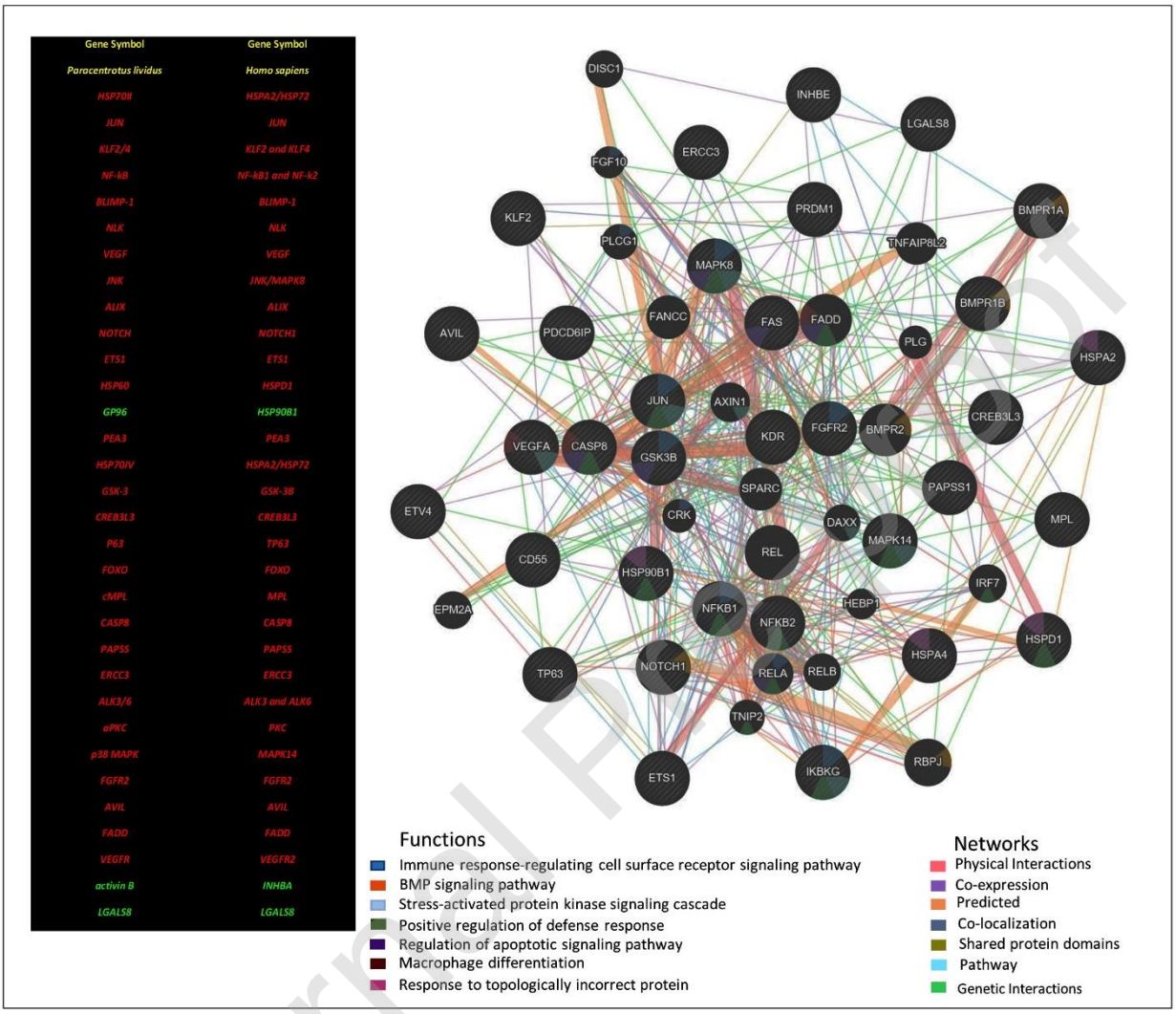


Figure 3. Interaction network model based on human gene ontology database. The network contains correlated genes obtained from the list shown on the left, with automatically selected weighting method generated by Cytoscape.

Notably, our findings highlight a down-regulation of few factors such as *VEGFR* and *FGFR* known to have the role of suppressing immune cell function. A selective VEGFR and FGFR signaling pathway inhibition is known to inhibit in turn tumour growing (Holmström et al., 2019).

Conversely, data highlight a stimulating effect on few transcripts such as *GP96* (also known as *HSP90B1*), *Activin B* and *Galectin 8*. The 96-kDa endoplasmic reticulum (ER)-resident glycoprotein (GP96) binds to cellular peptides and it is known to be induced by immune cytokines (e.g. interferon γ, interleukin 2) to elicit a tumor protective effect (Chen et al., 2003).

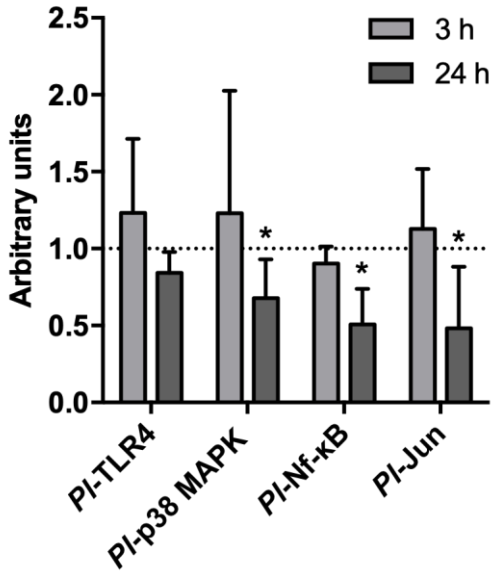


Figure 4. Expression levels of a few sea urchin inflammatory-related genes after 3 and 24-h TiO_2NPs exposure *in vitro*. Expression of *Pl-TLR4, Pl-p38 MAPK, Pl-Nf-κB* and *Pl-Jun* genes was analyzed by comparative qPCR. Levels are expressed in arbitrary units as fold increase compared to controls assumed as 1, using the endogenous gene *Pl-Z12-1* for normalization (N = 3 for 3-h samples and N= 4 for 24-h samples; data are presented as mean ± SD; *p ≤ 0.05).

Notably, while *GP96* was found up-regulated under TiO_2NP-exposure, 70-kDa-heat shock protein (*HSP70II* and *IV*), also known as *HSPA2/HSP72* in humans (normally compartmentalized on cytoskeleton, cytosol, plasma membrane, nucleus, and mitochondrion) was found down-regulated. The GP96 protein has important immunological functions due to its peptide-binding capacity. Reports highlight a putative interaction of GP96 with toll-like receptors to activate macrophages and dendritic cells and to mediate endocytosis (Vabulas et al., 2002; Huang et al., 2009). High quantities induce tolerance by incompletely understood mechanisms (Wolfram et al., 2013).

Although a potent immune response is crucial to reach the control and suppression of an insult, an excessive or inappropriate inflammation becomes harmful. Therefore, the innate immune system must control inflammatory signaling and down-regulate the inflammatory response once it is under resolution (Mogensen, 2009) through a down-regulation of NF-κB (Ruland, 2011). Notably, activin/inhibin and Galectin-8 signalling is considered crucial to maintain the balance between homeostatic and inflammatory signals, necessary for the optimal development and metabolic function of immune cells (Aleman-Muench and Soldevila, 2012; Karakostis et al., 2015; Brinchmann et al., 2018). Accordingly, activin and Galectin-8 seem to be required to mediate inflammation, stress, and immunity under TiO_2NP exposure.

3.3 Sea urchin immune system metabolic typing highlights antioxidant defense metabolic activity under TiO_2NP exposure *in vitro*

The activation and return to homeostasis of immune cells are intimately linked and dependent on dynamic changes in cellular metabolism (Pearce and Pearce, 2013; Buck et al., 2017). Metabolites control the duration and intensity of immune response and cell memory. In order to obtain integrated data on how the TiO_2NPs

influence the sea urchin immunological state, here we characterized the metabolic profile of exposed and un-exposed immune cells cultured for 24 hrs (Figure 5).

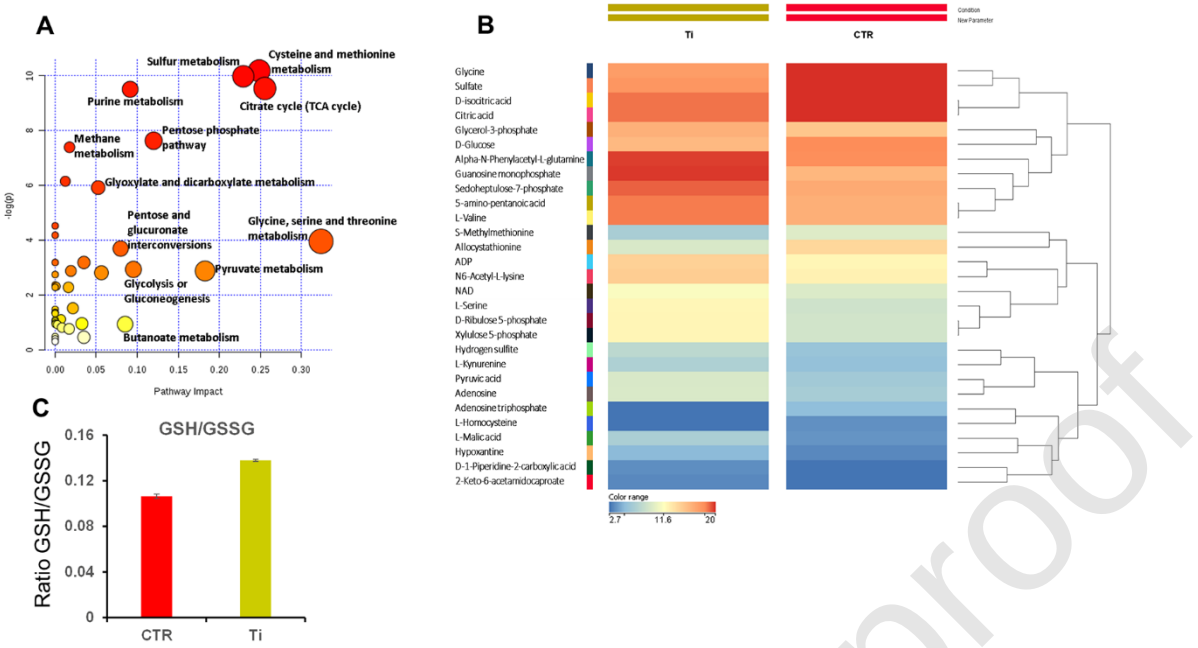


Figure 5. Innate immune-metabolic functional reprogramming in response to TiO_2NPs. A) Pathway enrichment analyses from control and TiO_2NPs-exposed immune cells. Enriched metabolic pathways were ranked according to their false discovery rate (FDR) values calculated by by Metabolomic Pathway Analysis (MetPa) method implemented in MetaboAnalyst 3.0 software. Pathway impact (a combination of the centrality and pathway enrichment results) is calculated summing up the importance measures of each of the matched metabolites and normalizing by the sum of the importance measures of all metabolites in each pathway. (B) Untargeted metabolic profiling *P. lividus* exposed and unexposed immune cells. Hierarchical clustering heatmaps show significantly ($p \leq 0.05$) different intracellular metabolites by LC-MS. C) GSH/GSSG ratio in control (CTR) and exposed immune cells (Ti) coming from relative abundance obtained by LC-MS analysis.

Our findings highlighted that TiO_2NPs interact with sea urchin immune cells *in vitro* increasing antioxidant metabolic pathways such as pentose phosphate pathway, cysteine-methionine and glycine-serine metabolism (Figure 5A). Specifically, it was worth noting the increased levels of: i) phenylacetyl glutamine, involved in metabolic degradation pathway as an alternative pathway for nitrogen secretion (urea cycle) (Brusilow, 1991; van Straten et al., 2017); ii) acetyl-L-lysine, involved in important mechanism of epigenetic control regulating chromatin accessibility and transcription (Kim et al., 2009); and iii) adenosine, involved in nucleotide metabolism (Figure 5B). Notably, nucleotide synthesis growing could indicate an increase in DNA replication and RNA production to support protein synthesis (Lane and Fan, 2015). Accordingly, increase in amino acids and peptides could highlight a faster protein turnover following a previous degradation. On the other hand, TiO_2NP exposure caused a decrease in ATP generation and decreasing levels of metabolites involved in TCA cycle (e.g. D-isocitric acid, citric acid) (Figure 5B) meaning that exposed immune cells are not in the inflammation genesis phase. As well known, activated mammalian dendritic cells (DCs) and

macrophages generate *extra* ATP to sustain the various cellular programs of activation (Williams and O'Neill, 2018).

Notably, the increased GSH/GSSG (reduced glutathione/oxidised glutathione) ratio observed under TiO_2NP exposure was a further confirmation of the increased defence antioxidant metabolic activity (Figure 5C). Our results are in agreement with a recent report highlighting the metabolic plasticity of the immune system in sea urchins adapted to life at sites under natural acidification (CO_2 vents), in which antioxidant capacity was found enhanced compared to control (Migliaccio et al., 2019). On the other hand, reactive oxygen (ROS) and nitrogen (RNS) species were found decreased.

Critically, our findings support the idea that *P. lividus* immune cells work under TiO_2NP exposure according to a simultaneously biphasic strategy: resistance and tolerance. Under perturbation (TiO_2NP exposure), immune system excites short-term and energetically expensive antioxidant metabolic activity (metabolic rewiring), preparing itself in the long-term activation of the tolerance-promoting survival (homeostasis restoring).

Our observation on the sea urchin immune system efficiency under immune-TiO_2NP recognition and interaction in vitro (TiO_2NP immune-compatibility) was confirmed by the results obtained from cell viability and toxicity assays (Figure 6). The viability and cytotoxicity of exposed immune cells did not change significantly compared to unexposed cells over 3-days.

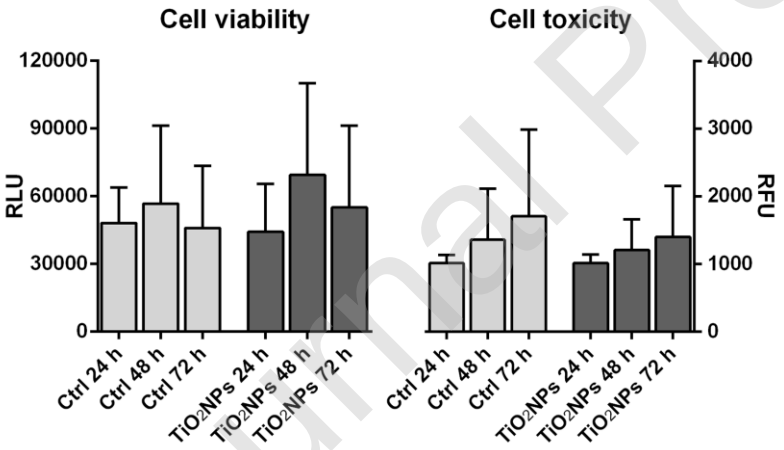


Figure 6. Real-time viability and toxicity assays highlight the TiO_2NP immune-compatibility. Results do not show significant changes in cell viability and toxicity upon TiO_2NP exposure (1 µg mL^{-1}) (24, 48, 72 hrs). Data are reported as the mean ± SD of at least five donors.

3.4 Highlighting the levels of a few key proteins involved in cellular adhesion and immune defence in the sea urchin

Finally, changes in activation/inactivation of a few mitogen-activated protein kinases (p-p38 MAPK, p-ERK), and variations of other key proteins involved in cellular adhesion (*Pl*-Toposome*, Pl*-Nectin, *Pl*-Galectin-8) and

immune/stress response (Toll-like receptor 4-like, Heat shock protein 70, Interleukin-6) were investigated at 3 hr and 24 hr exposure (Figure 7). An important issue for molecular biology is to establish whether an up- or down-regulation of a given gene corresponds to a modification in the levels of related proteins, these last executing an appropriate biological response. In general, TiO_2NP-exposed cells did not display significant variation in protein level compared to controls with some interesting exceptions: i) *Pl*-Nectin at both 3 hr and 24 hr exposure (decreased levels); ii) p-p38 MAPK at 3-hr exposure only (weak decreased levels); and iii) IL-6 at 24-hr exposure only (weak increased levels). Transcript and protein levels were not temporally correlated. Notably, as defined by NanoString nCounter Gene Expression Assay, none of 127 transcripts displayed significant variations after 3-hr TiO_2NPs exposure (not shown). In accordance, *Pl*-Toposome, Toll-like receptor 4-like and *Pl*-Nectin transcripts remained unvaried after 24 hr-exposure while *p38* MAPK transcript showed a weak decrease in immune cells exposed to TiO_2NPs (see Figure 2 and 4).

Pl-Nectin is a discoidin family member promoting cell adhesion and embryonic morphogenesis (Costa et al., 2010). The role of Nectins in regulating innate immunity has been more recently discovered establishing that they act as ligands for Natural Killer (NK) cell receptor promoting adhesion, cytotoxicity and cytokine release (Martinet and Smyth, 2015). Nectins induce the activation of intracellular signalling recruiting tyrosine kinase fibroblast endothelial kinase (Fyn) and serine threonine protein kinase C (PKC). Nectins can be either a co-stimulator or a co-inhibitor of immune cells. For example, Nectins are known to cooperate with E-cadherins and integrin αvβ3 to positively regulate growth factor receptors such as VEGF-mediated VEGFR2, acting as tumour regulators (Ogita and Takai, 2006; Mitola et al., 2006). In agreement, here we found both decreased level of *Pl*-Nectin protein and decreased expression of *αPKC*, *VEGF* and *VEGFR* genes at 24 hr-exposure (see figure 2 and 3). Critically, our findings support the idea that a mechanism inhibiting tumour growing (for promoting survival) is activated, as previously discussed (See Sea urchin immune cell transcriptomic profile *in vitro* deciphers a suppressive effect of the TiO_2NPs on transcripts involved in inflammation section). Finally, analyzing the intracellular protein levels of the interleukin-6 (IL-6) (a cytokine known to be abundantly produced during the inflammatory phase, mainly -but not only- a direct target of the NF-kB transcription factor) we found a weak increase at 24 hr-exposure, confirming that inflammatory response can be considered under resolution.

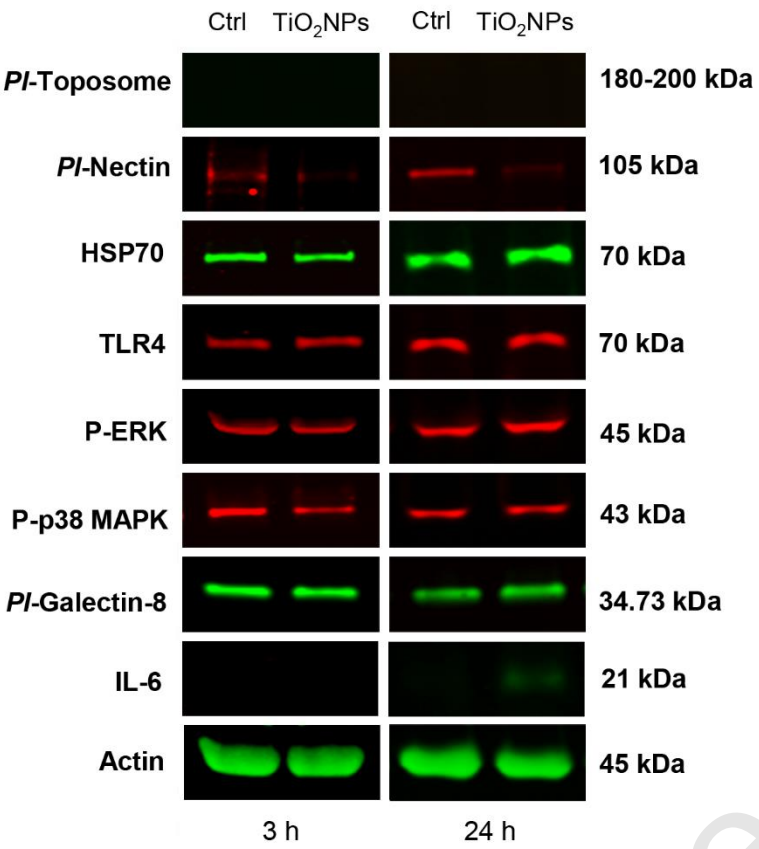


Figure 7. A cluster of selected proteins cross-linking cellular adhesion and innate immune response toward TiO_2NPs was assessed by immunoblotting. Immune cell exposure to TiO_2NPs elicited reduction in the level of Nectin after 3 and 24-hr exposure, a weak decrease of P-p38 MAPK at 3-hr and weak increase of IL-6 at 24-hr exposure.

4. CONCLUSIONS

Nanomaterials have the potential to offer promising strategies to optimize and improve the treatment of numerous disorders. The possibility to design nanomedicines able to transport biologically active molecules and target specifically the tissues makes them a promising tool for the implementation of personalized medicines (Halamoda-Kenzaoui and Bremer-Hoffmann 2018). When internalized, nanomaterials can interact immediately with the blood and the immune system triggering unexpected immunological challenges (Fadeel 2019). Here, we showed how TiO_2NPs influence the sea urchin immunological state, highlighting how metabolism regulates immune response maturation. Comparing sea urchin, human and other model organism (e.g. fruit fly, mouse, worm) genomes it was possible to highlight what happened during evolution (Sea Urchin Genome Sequencing Consortium, 2006). For example, among sequenced genomes, the genomes of fruit flies and mice are more distant from the human genome than that for the sea urchin genome. The

comparison of the sea urchin genes to the human genes shows which human genes are likely to be recent innovations in human evolution and which are ancient. Notably, between 4 and 5% of the sea urchin genes are directly involved in immune functions. The sea urchin presents a few genes known to have function in the adaptive immunity, but over 1000 immune genes falling into the innate immunity (as many genes in humans) (Rast et al., 2006). Under TiO_2NP-exposure, sea urchin innate immune system is able to trigger immune suppression, metabolic rewiring and homeostasis restoring. Our findings provide new understandings into the molecular mechanisms involved in the immune-TiO_2NP recognition and interaction *in vitro*; we obtained motivating results concerning the use of sea urchin primary immune cell cultures as *a proxy to human* forceful model (translational immunology) and highlight TiO_2NPs as effective immunosuppressant for drug delivery and regenerative medicine therapies.

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Competing interests

The authors declare no competing or financial interests.

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Author contributions

Conceptualization: A.P.; Methodology: A.P.; Validation: A.A., A.P.; Cellular/Biochemical Investigation: A.A., A.P.; Metabolomics Investigation: E.N., D.G.; Molecular Biology Investigation: R.R., C.C.; SEM and TEM Investigation: A.A., O.B., O.K.; Data curation: A.P.; Formal analysis: A.P., D.G., A.A.; Writing–original draft: A.P.; Supervision: A.P.; Project administration: A.P.

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