Silver nanoparticles’ impact on the gene expression of the cytosolic adaptor MyD-88 and the interferon regulatory factor IRF in the gills and digestive gland of *mytilus galloprovincialis*

**ABSTRACT**

Silver nanoparticles (AgNPs) have been reported as stressors for the bivalves’ immune system at different regulatory levels, impacting the detection step and receptors, and other mediators, as well as effector molecules. However, studies on how AgNPs impact the transmission of signals from receptors and whether they have an effect on mediators and transcription factors are still scarce. This study aims to investigate the effect of 12 hours of *in vivo* exposure to 100 μg/L of AgNPs on the gene expression of the cytosolic adaptor Myeloid, the differentiation protein 88 (MgMyD88-b), and the interferon regulatory factor (Me4-IRF) in the gills and digestive gland of *Mytilus galloprovincialis*. The results illustrate a tissue-specific gene expression of the MgMyD88-b and the Me4-IRF in the gills and digestive gland of *M. galloprovincialis*. In the gills, AgNPs did not significantly impact the expression of the two genes. However, blocking the caveolae-mediated endocytosis decreased the expression of Me4-IRF. Overall, the inhibition of the AgNPs’ uptake routes have highlighted their potential interference with the immune response through the studied mediators’ genes, which need to be studied further in future investigations.

1. Introduction

Bivalve’ mollusks, are organisms of a biological, ecological, and economic importance since they could be ideal candidates for critical, basic and applied research investigations in a large number of clades across the whole spectrum of biology and beyond like neuroendocrinology, host-pathogen interactions and symbiosis (Balbi et al. 2020, Destoumieux-Garzón et al. 2020), innate immunity, as well as in environmental biomonitoring (Fernández Robledo et al. 2019). This current study aims to investigate the impact of nanoparticles as environmental stressors on living organisms and their implications in environmental species like bivalves. Probing nanoparticles (NPs) across different environmental species has reported their potential impact affecting multiple molecular components and signaling pathways across living species, including invertebrates like the marine mussels *M. galloprovincialis* (Ale et al. 2019, Duroudier et al. 2019a,b). *M. galloprovincialis* are abundant in marine environments, and as filter-feeding organisms able to filter a large volumes of water, subsequently could retain a large amount of chemicals and particles, including nanoparticulate entities, have been proposed as target group in nano-ecotoxicology (Moore 2006, Canesi and Corsi 2016, Fernández Robledo et al. 2019).

Transcriptomic and proteomic studies have reported the influence of environmental contaminants on the molecular components of the immune system in species of environmental importance, including *M. galloprovincialis* and *Paracentrotus lividus* (Balbi et al. 2014, Pinsino et al. 2015, 2017, Châtel and Mouneyrac 2017, Granger et al. 2017, Duroudier et al. 2019a,b). However, environmental contaminants including polycyclic hydrocarbon, and nanoparticulate entities have been reported to induce pathogen-free chronic inflammation, also called “sterile” inflammation, and tissue damages (De Vico and Carelle 2012, Carella et al. 2015, Boraschi et al. 2017, Bouallegui et al. 2017b, 2018a, Alijagic et al. 2020). Such an inflammatory response was reported to be due to excessive production of reactive oxygen radicals and oxidative stress damages which have been illustrated...
through lysosomal membrane disruption, anti-oxidative stress enzymatic inhibition/induction, lipid peroxidation, and reduced glutathione depletion (Katsumiti et al. 2015, Châtel and Mouneyrac 2017, Bouallegui et al. 2017b, 2018a). They have also been shown to induce modifications and damages to the histology of tissue structures due to the excessive infiltration of hemocytes into the gills and digestive gland epithelia, as described in De Vico and Carelle (2012), Carella et al. (2015), and Bouallegui et al. (2017b, 2018a). In addition, modulation of the gene expression of immune response molecules like metalloproteins C1q containing domain proteins, and Toll-like receptors has been recorded (Katsumiti et al. 2015, Auguste et al. 2019, Jimeno-Romero et al. 2019). Toll-like receptors are a kind of pattern recognition receptors (PRRs) that could be triggered by pathogens aiming to activate the immune system (Auguste et al. 2019). However, not much information is available about the effects of NPs on mediator molecules that transmit signals from molecules at the external interfaces (i.e., PRRs) to cascading elements aimed to activate immune defenses (Lee and Kim 2007, Kawai and Akira 2010, Rauch et al. 2013, Toubiana et al. 2014, Châtel and Mouneyrac 2017, Ispanixtlahuatl-meraz et al. 2017). Previous studies have demonstrated that inflammatory lesions caused by silver nanoparticles AgNPs interfere with oxidative stress damages (Bhattacharya et al. 2017, Ispanixtlahuatl-meraz et al. 2017, Bouallegui et al. 2017b, 2018a). Aquatic environments commonly reported as final sinks of most chemicals waste made by humans. AgNPs have received special attention due to its exceptional broad spectrum bactericidal properties, relatively low cost of manufacturing AgNPs, unique properties and ability to form diverse nanostructures, and used in a diverse range of consumer products like food storage, coating materials, fabrics and clothing, toothbrushes, and antimicrobial coatings (Yu et al. 2013), aiming to predict the effect of their potential release through wastewater into aquatic environments and which end in living organisms. However, AgNPs toxicity is closely related to the release of Ag + ions, which are considered very toxic to aquatic species. Although, it is difficult to distinguish the effect of AgNPs from that of Ag + (Ale et al. 2019). Ag + ions have been commonly considered as the most toxic form of silver in water bodies prior to the focus on AgNPs (Lekamge et al. 2018). The reported estimated silver concentrations are of 0.03–0.1 ng/L in open seas, and of 0.14–1.29 ng/L in coastal regions, while calculated environmental concentrations of AgNPs are from 40 to 320 ng/L in surface waters, and of 2.3 µg/L in marine waters, as reported by Ale et al. (2019). Overall, the experimental design of this study is in line with previous experiments proposing new results that allow us to deepen further understanding and appraisal of AgNPs interactions in mussels’ immune system (Bouallegui et al. 2017a,b, 2018a,b,c). In so doing, our previous results of different exposure times to a single dose (100 µg/L as a commonly reported concentration) (Katsumiti et al. 2015) for different AgNPs sizes, before and after inhibition of endocytic uptake routes, showed a significant cytoxic effect on immune cells with changes in the percentages of different sub-populations of hemocytes involved, and significant variations in the histopathological indices of the inflammatory response. AgNPs showed an impact on the inflammation morphology and intensity, while redox proteomics showed redox-based changes in the proteome of the gills and the digestive gland. Overall, it was concluded that AgNPs’ impact was size- as well as exposure time-dependent with a greater effect caused by the smaller size. Endocytic routes were deeply involved in determining redox-based changes and immune response activation (Bouallegui et al. 2017a,b, 2018a,b,c). It is worth noting that clathrin- and caveolae-mediated endocytosis are described as receptor-dependent routes of NP’s entry into cells (Santos et al. 2011, Khan et al. 2015, Bouallegui et al. 2018a,b). In accordance with that, further studies demonstrated that the epithelium of tissues at the external interfaces play a role of oxygen exchange while the sub-epithelial tissues of the digestive gland that have a nutrient extraction role are among the most hemocyte-rich tissues and are enriched with patterns of the innate immune response (e.g., PRRs) (Allam and Pales Espinosa 2016, Lau et al. 2017). Immune defense factors associated with the surfaces of the epithelial tissues and the free-wandering hemocytes in bivalves’ open-circulatory system, make these contact surfaces a first checkpoint for any invading microorganisms or any source of danger like waterborne pollutants (Fernández Robledo et al. 2019). Such enriched epithelium are responsible for activating what is known as peripheral immunity (or also as local immunity), which thereafter has to activate and is involved in adjusting the systemic immune response (Allam and Pales Espinosa 2016, Wang et al. 2016).

In the current study we aim to highlight how AgNPs could impact the gene expression of the cytosolic adaptor Myeloid differentiation protein 88 (MgMyD88-b) and the interferon regulatory factor (Me4-IRF) as molecular mediators that deliver a signal from interaction sites through the surfaces’ receptors like TLRs to the downstream cascading reaction (Jeong and Lee 2011, Granger et al. 2017) which may hamper a proper evaluation of the immune response.

Mediator molecules like MgMyD88-b are universal mediators that deliver signals from different PRRs, including the entire TLR family, except TLR3, through the MyD88-dependent pathway (Philipp et al. 2012, Balbi et al. 2014, Toubiana et al. 2014, 2013, Granger et al. 2017, Perkins et al. 2018). The TLR3 is activated through the TRIF adpotor molecule containing the TIR domain (Toll-Interleukin 1-Domain), which is also described as the MyD88-independent pathway (Philipp et al. 2012, Tanguy et al. 2013, Toubiana et al. 2014, 2013, Zhao et al. 2015). However, TRIF has been reported to activate the Interferon Regulatory Factors (IRFs) to induce type 1 interferon (IFN) expression (Philipp et al. 2012, Tanguy et al. 2013, Toubiana et al. 2014, Gerdol and Venier 2015).

2. Materials and methods

2.1. Silver nanoparticles’ characteristics and preparation

Poly-vinyl- pyrrolidone (PVP)-coated AgNPs (<50 nm, 99.5% trace metal based, 99.1% purity) previously used in Bouallegui et al. (2017a) were used in the current study (See Supplemental material 1 for description of the synthesis
method and representative characterization measurements). Briefly, the TEM analysis (TECNAI G20, Ultra-Twin, FSB, Bizerte, Tunisia) showed that AgNPs <50 nm (Ag50) are homogeneously spherical with an approximate primary size of 50 nm and a size distribution with a median size of 41.6 ± 18.82 nm. The XRD pattern recorded on a D8 Advance diffractometer (Bruker, Bizerte) showed the crystalline nature of the AgNPs where the diffraction peaks matched the face centered cubic (fcc) phase of silver. The UV-Vis spectrum (T60; PG-instruments, Leicestershire, UK) of the colloidal AgNPs stock solution were prepared using artificial sea water (ASW [salinity = 35%, pH 8.0]) as previously described in Bouallegui et al. (2017a) and was performed prior to exposure. This clearly confirmed (λmax = 400 nm) that the AgNPs have a homogenous dispersion in aqueous solutions. The AgNP stock solution was mixed several times by inversion and an aliquot removed as a working solution that was sonicated for 15 min in alternating cycles (2 × 30 sec) in an ultrasonic bath (100 W; 40 KHz; VWR, Strasbourg, France).

### 2.2. Mussels and experimental design

Tissues used in the current study were aliquots previously collected, flash frozen and cryopreserved at −80 °C until their use in this molecular analysis (Bouallegui et al. 2017a). The experimental exposures are described as follows: *Mytilus galloprovincialis* with an average shell length of 75 ± 5 mm were collected from an aquaculture farm located in the Bizerte Lagoon (in northeast Tunisia) and immediately transported to the laboratory and maintained in oxygenated artificial sea water (ASW) (salinity 35%, pH 8) in static tanks under standard conditions (aeration, photoperiod: 12/12 h; T = 16 °C) where they were allowed to acclimatize (48 h) changing the water every 12 h before exposure. As previously reported in Bouallegui et al. (2017a), mussels were sorted into groups of ten individuals each (n = 10, exposure rate = 1 mussel/0.5 L ASW/tank). Each group was exposed for 12 h to 100 μg/L of AgNPs < 50 nm (Bouallegui et al. 2018a,b). The groups designed to probe the effect of uptake routes were exposed to pharmaceutical inhibitors prior to the exposure to AgNPs (Katsumiti et al. 2015). For inhibitor-treated groups, effective concentration ranges used were confirmed based on a previous study by Khan et al. (Khan et al. 2015). Groups aimed at assessing clathrin-mediated endocytosis blocking were incubated for 3 h with 100 μM of inhibitor amantadine (Sigma, Steinheim, Germany). They were then placed in AgNP exposure solutions for 12 h (without amantadine and containing 100 μg/L of AgNPs). To block caveolae-mediated endocytosis, selected groups were exposed to 50 μM nystatin/0.05% (v/v) Dimethyl-sulfoxide (DMSO) (Sigma, Steinheim, Germany) for 1 h *a priori*, with AgNPs being added for another 12 h (Khan et al. 2015). Control groups (n = 10 mussels each) constituted ASW, ASW with inhibitors (each inhibitor apart), DMSO (vehicle), and DMSO with AgNPs. Controls were maintained as comparative standards to normalize any undesirable effects. All exposures were done in triplicate. The mussels were dissected and then the gills and digestive gland were collected from the controls and the exposed groups, flash-frozen and kept at −80 °C until they were processed in the molecular analysis.

### 2.3. Expression of immune-related mRNA in mussels

30 mg of tissue samples (gills and digestive gland) from each experimental group (4 mussels from each group) were used to prepare tissue homogenate using a lysis buffer available from the Qiagen RNeasy mini-kit (Germany). The RNA was then isolated following the manufacturer’s instruction protocol (Qiagen RNeasy mini-kit, Germany). The concentration and quality of the RNA was determined by measuring the absorbance at 260/280 nm using a UV-Vis spectrophotometer (T60; PG-instruments, Leicestershire, UK). cDNA was transcribed from the RNA (2 μg) within the reverse transcription step which was followed by the PCR reaction, both carried out in the same tube according to the manufacturer’s protocol: QIAGEN One-step RT-PCR kit (Qiagen, Germany), containing a one-step RT-PCR kit enzyme mix (2 μl), a dNTP mix (10 mM), a 5X reaction buffer (containing 12.5 mM of MgCl₂), and the specific primer sets and conditions for each immune related mRNA (0.6 μM of each forward and reverse primer/reaction/tube) for a total volume of 50 μl of PCR reactions (each/PCR tube). The elongation factor-α (EF-1α) specific primer was also amplified and confirmed as an internal control that admitted minimal changes between different exposure samples. The hot start of the RT-PCR program used for the immune related mRNA was initialized with a reverse transcription step (same tube step) for the first strand cDNA. Synthesis was performed at 50 °C for 30 min followed by an initial PCR activation step at 95 °C for 15 min, followed by 30 amplification cycles at 94 °C for 30 s. The annealing temperature for each mRNA was as shown in Table 1, with an extension at 72 °C for 1 min followed by a final extension step at 72 °C for 10 min in the Applied Biosystems 2720 Thermal Cycler (Thermo-Fisher, USA).

The RT-PCR product was analyzed with 1.5% agarose gel electrophoresis, stained with 3% ethidium bromide, and visualized under ultraviolet light and documented using the Doc Print II system (VILBER LOURMAT, USA). Gene expression results were presented and semi-quantitatively determined from the ratio of band intensity to the internal control (EF-1α) of 4 biological replicates, using the ImageJ analysis program (from the NIH website by Scion Corporation, Frederick, MD). Each assay was carried out in triplicate for each reaction.

### 2.4. Statistical analysis

All assays were performed in triplicate. The gene expression rates were determined from three replicates of mRNA isolated from an average of four animals/treatments (extracts for each tissue). Results are presented as means ± SD of mRNA expression (relevant primer)/expression of EF-1α mRNA expression. The normal distribution and homogeneity of variance were tested using the Shapiro-Wilk and Bartlett tests prior to the statistical analysis. All samples showed a normal distribution. The statistical analysis was performed
endocytosis was inhibited (AgNPs (Figure 2). Changes when exposed to AgNPs (i.e., without inhibition)) increased the expression of IRF (Figure 2), and significantly increased the expression of Me4-IRF, while exposure to AgNPs when blocking the caveolae-mediated endocytosis increased the gene expression of Me4-IRF. However, the inhibition of clathrin-mediated endocytosis considerably decreased the expression of Me4-IRF, while the vehicle (DMSO) did not significantly affect the expression of both genes compared to the control (Figure 1). Moreover, in the digestive gland of mussels exposed for 1 and 21 days to 10 μg/L of AgNPs, the gene expression of MgMyD-88-b when caveolae-mediated endocytosis was inhibited (AgNPs + Nystatin) did not show any significant effect in all exposures. However, the expression of Me4-IRF showed a significant decrease (compared to DMSO) (Figure 2).

4. Discussion

Silver nanoparticles can exert toxicity through its particle form and through its Ag⁺ ions released during particles dissolution (Ale et al. 2019). The mechanisms of silver nanoparticles toxicity in mussels have been discussed in studies such as Katsumiti et al. (2015), and Ale et al. (2019). Although dissolution was not measured in this study, another study (Sikder et al. 2018) reports a dissolution rate constant (k) of 0.008 h⁻¹ for a 100 μg/L suspension of PVP-AgNPs in 20 ppt ASW measured over 120 hours (or 2.5 nm which is 13.5% of initial particle diameter; calculated according the graphs presenting data in Sikder et al. (2018)), therefore, the concentration and exposure time was chosen based on the estimated lower dissolution and it allows for comparison with other ecotoxicological studies. The focus on this study was on the NP entities’ effects on signaling molecular pathways. However, the effects of the release of ions which is one of the main toxic mechanisms that explain NPs’ toxicity, are beyond the objective of this study.

Table 1. Specific primers and conditions used for the determination of immune-related mRNA expression.

| Primers          | Primer sequences (5’ to 3’) | Annealing temp | Amplicon size (bp) | References | GenBank Acquisition n° |
|------------------|-----------------------------|----------------|-------------------|------------|------------------------|
| MgEF-1x-F        | GGAACCTGACCTGGTGTTG         | 57.8 °C for 30 sec | 223              | (Moreira et al. 2015) | AB162021 |
| MgEF-1x-R        | TGATGCCGTCTGATGAAA          |                |                   |            |                        |
| MgMyD88-b-F      | CTTGAGACATTTGAGCCAGT       | 57.8 °C for 30 sec | 147              | (Toubiana et al. 2013) | KC357761 |
| MgMyD88-b-R      | CTCACTCGGCCACACATAG         |                |                   |            |                        |
| Me4-IRF-F        | GATATGCGCCAGTCTTGGAT        | 59.85 °C for 30 sec | 333              | (Philipp et al. 2012) | HE609045 |
| Me4-IRF-R        | TCTATGCTCGGACACAGGA        |                |                   |            |                        |

Bold values indicate the size of the amplicon in base pair (bp), as illustrated in the figures of the gels.

using a one-way analysis of variance (ANOVA) with a Tukey’s HSD post-hoc test and significance determined at *p < 0.05 and **p < 0.01.

2.5. Ethical statements

Permits are not required for the field collection of Mytilus galloprovincialis nor for their use in laboratory testing. All experimental trials have been conducted following the principles of the Declaration of Helsinki.

The authors confirm that all mandatory laboratory health and safety procedures have been complied with during the course of this experimental work.

3. Results

The exposure to AgNPs has proven to insignificantly modulate the gene expression of MgMyD-88-b and Me4-IRF in the gills. However, the inhibition of clathrin-mediated endocytosis (AgNPs + Amantadine exposure) did not affect the modulation much. Although the inhibition of the caveolae-mediated endocytosis demonstrated an interference with MgMyD-88-b and significantly decreased the expression of Me4-IRF, while the vehicle (DMSO) did not significantly affect the expression of both genes compared to the control (Figure 1). Moreover, exposure to AgNPs when blocking the caveolae-mediated endocytosis resulted in a significant decrease in the expression of Me4-IRF, but not for MgMyD-88-b (Figure 1).

In the digestive gland, exposures to AgNPs significantly increased the gene expression of Me4-IRF. However, the inhibition of the clathrin-mediated endocytosis (AgNPs + Amantadine) decreased such expression (i.e., Me4-IRF) (Figure 2), and significantly increased the expression of the MgMyD-88-b expression (which did not show significant changes when exposed to AgNPs (i.e., without inhibition)) (Figure 2).

In the digestive gland of mussels exposed to AgNPs, the gene expression of MgMyD-88-b when caveolae-mediated endocytosis was inhibited (AgNPs + Nystatin) did not show any significant effect in all exposures. However, the expression of Me4-IRF showed a significant decrease (compared to DMSO) (Figure 2).

In general, bivalves are among the most studied invertebrate groups as an important target group for NP toxicity (Moore 2006, Canesi and Cors). Many investigations have focused on understanding the effects of AgNPs using conventional biomarkers (Jimeno-Romero et al. 2017, Duroudier et al. 2019a), and other methods like transcriptomics and proteomics (Rocha et al. 2016, Bouallegui et al. 2018b). However, complement information given by such tools still not enough. A major process of NPs to impact on immune response of different living organisms is their ability to mediate the modulation of signaling pathways at different checkpoints (Pinsino et al. 2015, Boraschi et al. 2017, Alijagic et al. 2020). Transcriptomic and proteomic studies on the digestive gland of mussels exposed for 1 and 21 days to 10 μg/L of AgNPs of 5 nm in different seasons, clearly indicating that exposure to AgNPs provoked significant alterations in the transcription levels of genes involved in the cytoskeleton reorganization (e.g., downregulations of the centrosome-associated 350-like isoform X2, calponin-1, and tropomyosin, alterations of paramyosin and actin proteins (Katsumiti et al. 2015, Duroudier et al. 2019a, Duroudier et al. 2019b). The same study documented alterations in cytoskeleton dynamics, which is a key process regulating vesicle and organelle transportation along the intracellular trafficking pathways. In addition, genes like Ras- and Rab-related, which are involved in endosomal trafficking were also found to be significantly affected...
altered (Duroudier et al. 2019a). It’s worth noting that in bivalves the endo-lysosomal trafficking system is also used as a nutrient digestion mechanism assured by the digestive gland. Subsequently, the alteration of such mechanisms would forcefully impact the hemostasis of exposed organisms. AgNPs have been documented to induce upregulations of the leucine-rich, repeat-containing, and low affinity immunoglobulin epsilon Fc receptor, and the serine threonine-kinase mitochondrial, simultaneously with downregulations of the calcium calmodulin-dependent kinase type IV. They have all been suggested to potentially experience interactions with bivalves’ immune system through Toll like receptors (TLR), which may directly interact with immunoglobulin E (IgE) synthesis, and together with the signal input, promote the immune response (Duroudier et al. 2019a).

Proteomic analyses showed that a putative C1q domain containing protein was also significantly altered after exposure of mussels to AgNPs, suggesting that the immune response of mussels was affected by the dietary exposure to AgNPs (Duroudier et al. 2019b). Previous studies (Jones et al. 2020), documented an important role allocated to such a category of molecules (C1q domain proteins) being members of mucosal surfaces covering epithelia of digestive gland and gill tissues, and involved a double-role as a nutrient selection process assured by bivalves as filter-feeder organisms to select suspended particles of nutrients (Jones et al. 2020).

Overall, Duroudier et al. (2019b), taking into account the role of the C1q domain containing proteins in the immune response, suggested that the under-expression of the C1q domain proteins after exposure to AgNPs is due to a disrupted cell-mediated immunity (Duroudier et al. 2019b).

**Figure 1.** (A) Photo of electrophoresis gels (1.5% agarose) of RT-PCR products of the analyzed mRNA expression from the gills after experimental exposures; A: control, B: AgNPs, C: Amantadine, D: Amantadine + AgNPs, E: DMSO, F: DMSO + AgNPs, G: Nystatin, H: Nystatin + AgNPs. (B) Expression levels of immune related mRNA in the gills from mussels relative to EF-1α in different experimental exposures. Data are presented as Means ± SD. Values significantly different from relevant compared groups at *p < 0.05 and **p < 0.01, n = 4.
Further studies documented differential regulations in the transcription levels of lysozyme, Mytilin B, Myticin B, the C1q-domain-containing protein (MgC1q), and the Toll-like receptor (TLR-I) as selected genes related to immune response, and were evaluated by RT-q-PCR in the digestive gland of CeO2NPs-exposed mussels, showing only significant variations in Mytilin B, while non-significant changes were recorded for lysozyme, Myticin B, and MgC1q, and almost no changes were seen in the TLR-I (Auguste et al. 2019). Similarly, Titanium dioxide nanoparticles (TiO2NPs) were reported to affect the protein expression of p38MAPK, extracellular receptor kinases (ERK), Toll-like receptor 4-like (TLR-4 like), HSP 70, and interleukin-6 (IL-6), in the urchin immune cells (Pinsino et al. 2015, Alijagic et al. 2020). Interestingly, receptor-ligand proteins like TLRs, act through adaptor molecules and activate various kinases and transcription factors mediating inflammatory response and apoptosis like caspase 8, Nuclear factor-RB (Nf-RB), JUN, and MAPK14 (Kawai and Akira 2010, Perkins et al. 2018). The current study shows the ability of AgNPs to modulate the expression of MgMyD88-b (significant downregulation in the digestive gland, and slight upregulation in the gills), which highlights the potential impact of AgNPs on mediators’ components linking the sensing phase to the action phase. Such findings are in line with previous studies that have demonstrated the ability of metal based pollutants to modulate the expression of TLRs through the MyD88 adaptor (Balbi et al. 2014, Granger et al. 2017). Other studies reported upregulations of the 96-kDa endoplasmic reticulum (ER)-resident glycoprotein (GP96) in sea urchin hemocytes exposed to TiO2NPs (Pinsino et al. 2015). It is worth noting that GP96 could interact with TLRs to activate macrophages and is known to be induced by cytokines like interferon-γ and interleukin-2 (Kawai and Akira 2010, Perkins et al. 2018). However, taking into account this possible activation of the interferon signaling pathway, the modulation of Me4-IRF by AgNPs recorded in our current results could be explained as such. Moreover, Granger et al. (2017) demonstrated the effect of exposure to cadmium on the expression of MyD88, TLR4-like, TLR2-like and TLR3 in

![Figure 2.](https://example.com/figure2.png)

(A) Photo of electrophoresis gels (1.5% agarose) of RT-PCR products of the analyzed mRNA expression from the digestive gland after experimental exposures; A: control, B: AgNPs, C: Amantadine, D: Amantadine + AgNPs, E: DMSO, F: DMSO + AgNPs, G: Nystatin, H: Nystatin + AgNPs. (B) Expression levels of immune related mRNA in the digestive gland from mussels relative to EF-1α in different experimental exposures. Data are presented as Means ± SD. Values significantly different from relevant compared groups at *p < 0.05 and **p < 0.01, n = 4.
the hemocytes of *Mytilus edulis*. Our results, have recorded the impact of AgNPs on the gene expression of MgMyD88-b and Me-4IRF, which might be linked to a putative activation of several TLR receptors (Jeong and Lee 2011, Rauch et al. 2013, Turabekova et al. 2014). Further, computational studies suggested as a mechanism of NP interferences with cell components the ability of nanoparticulate entities to associate with intracellular domains of receptors leading to block their dimerization, and/or mediating an unexpected hetero-dimerization and/or homo-dimerization (Turabekova et al. 2014). Blocking appropriate signals from being delivered to cascading mediators could hamper signal transmission (e.g., NPs ability to bind directly to extracellular domains of TLR4 and inhibit its activation) (Turabekova et al. 2014, Ispaniłatlahuat-meráz et al. 2017, Perkins et al. 2018). However, previous studies have demonstrated the ability of AgNPs to modulate the redox equilibrium of the proteome in *Mytilus galloprovincialis* tissues impacting its immune response (Bouallegui et al. 2018b,c). In this context, Jeong and Lee (2011) demonstrated the ability of sulfonamidophane, as a species resulting from a redox-modified-equilibrium, to bind directly to cystein residues of the TLR4 extracellular domain and inhibit its interaction with its relevant ligand, which might generate chronic inflammation (Jeong and Lee 2011).

Throughout this study we have shown that the inhibition of clathrin- and caveolae-mediated endocytosis, considered as NPs uptake pathways, has significantly decreased the expression of MgMyD88-b and Me-4IRF in the gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis* (in most cases). Interestingly, a potential involvement of the MyD88-independent pathway in recruiting specific receptors, such as for example the TLR3 which could be activated within intracellular vesicles formed by the clathrin- and caveolae-dependent endocytosis, both during the internalization of AgNPs, could interfere with the already activated immune response (Hromada-judycka 2014, Zhao et al. 2015, Perkins et al. 2018).

### 5. Conclusion

The current study has highlighted the possible interferences of AgNPs with the gene expression of mediator molecules which may alter the transmission signal from receptors at the cell surfaces (described as cellular sensors) aiming to activate the immune response. However, the results of the current study are preliminary and need to be further developed and integrated in other signaling pathways for a better understanding of the effect of NPs on the immune system of living organisms.

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### Disclosure statement

The authors report no conflict of interest.

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### References

Ale, A., et al., 2019. Exposure to a nanosilver-enabled consumer product results in similar accumulation and toxicity of silver nanoparticles in the marine mussel *Mytilus galloprovincialis*. *Aquatic Toxicology*, 211, 46–56.

Alijagic, A., et al., 2020. Titanium dioxide nanoparticles temporarily influence the sea urchin immunological state suppressing inflammatory-relate gene transcription and boosting antioxidant metabolic activity. *Journal of Hazardous Materials*, 384, 121389.

Allam, B., and Pales Espinosa, E., 2016. Bivalve immunity and response to infections: are we looking at the right place? *Fish Shellfish Immunology*, 53, 4–12.

Auguste, M., et al., 2018. Effects of nanosilver on *Mytilus galloprovincialis* hemocytes and early embryo Development. *Aquatic Toxicology*, 203, 107–116.

Auguste, M., et al., 2019. In vivo immunomodulatory and antioxidant properties of nanoceria (nCeO2) in the marine mussel *Mytilus galloprovincialis*. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, 219, 95–102.

Balbi, T., et al., 2014. Co-exposure to n-TiO2 and Cd2+ results in interactive effects on biomarker responses but not in increased toxicity in the marine bivalve *M. galloprovincialis*. *The Science of the Total Environment*, 493, 355–364.

Balbi, T., et al., 2020. Insight into the microbial communities associated with first larval stages of *Mytilus galloprovincialis*: possible interference by estrogenic compounds. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology*, 237, 108833.

Bhattacharya, K., et al., 2017. Cytotoxicity screening and cytokine profiling of nineteen nanomaterials enables hazard ranking and grouping based on inflammogenic potential. *Nanotoxicology*, 11 (6), 809–826.

Boraschi, D., et al., 2017. Seminars in immunity nanoparticles and innate immunity: new perspectives on host defence. *Seminars in Immunology*, 34, 33–31.

Bouallegui, Y., et al., 2017a. Impact of exposure time, particle size and uptake pathway on silver nanoparticle effects on circulating immune cells in *Mytilus galloprovincialis*. *Journal of Immunotoxicology*, 14 (1), 116–124.

Bouallegui, Y., et al., 2017b. Histopathology and analyses of inflammation intensity in the gills of mussels exposed to silver nanoparticles: role of nanoparticle size, exposure time, and uptake pathways. *Toxicology Methods and Mechanisms*, 27 (8), 582–591.

Bouallegui, Y., et al., 2018a. Histopathological indices and inflammatory response in the digestive gland of the mussel *Mytilus galloprovincialis* as biomarker of immunotoxicity to silver nanoparticles. *Biomarkers*, 23 (3), 277–287.

Bouallegui, Y., et al., 2018b. Role of endocytotic uptake routes in impacting theROS-related toxicity of silver nanoparticles to *mytilus galloprovincialis*: a redox proteomic investigation. *Aquatic Toxicology*, 200, 21–27.

Bouallegui, Y., et al., 2018c. Redox proteomic insights into involvement of Clathrin-mediated endocytosis in silver nanoparticles toxicity to *Mytilus galloprovincialis*. *PloS One*, 13 (10), e0205765.

Canesi, L., and Corsi, I., 2016. Effects of nanomaterials on marine invertebrates. *The Science of the Total Environment*, 565, 933–940.

Carello, F., et al., 2015. Quantitative histopathology of the Mediterranean mussel (*Mytilus galloprovincialis L*) exposed to the harmful dinoflagellate *Ostreopsis cf. ovata*. *Journal of Invertebrate Pathology*, 127, 130–140.

Châtel, A., and Mouneyrac, C., 2017. Signaling pathways involved in metal-based nanomaterial toxicity towards aquatic organisms.
Comparative Biochemistry and Physiology. Toxicology & Pharmacology, 196, 61–70.

De Vico, G., and Carelle, F., 2012. Morphological features of the inflammatory response in molluscs. Research in Veterinary Science, 93 (3), 1109–1115.

Destoumieux-Garzón, D., et al., 2020. Vibrio-bivalve interactions in health and disease. Environmental Microbiology, 22 (10), 4323–4341.

Duroudier, N., et al., 2019b. Changes in protein expression in mussels Mytilus galloprovincialis dietarily exposed to PVP/PEI coated silver nanoparticles at different seasons. Aquatic Toxicology, 210, 56–68.

Duroudier, N., et al., 2019a. Season influences the transcriptomic effects of dietary exposure to PVP/PEI coated Ag nanoparticles on mussels Mytilus galloprovincialis. Comparative Biochemistry and Physiology. Toxicology & Pharmacology, 222, 19–30.

Fernandez Robledo, J.A., et al., 2019. From the raw bar to the bench: bivalves as models for human health. Developmental and Comparative Immunology, 92, 260–282.

Gerdol, M., and Venier, P., 2015. Fish & shellfish immunology an updated molecular basis for mussel immunity carbohydrate recognition domain. Fish & Shellfish Immunology, 46 (1), 17–22.

Granger, P., et al., 2017. Fish & shellfish immunology functional and molecular responses of the blue mussel Mytilus edulis’ hemocytes exposed to cadmium - an in vitro model and transcriptomic approach. Fish & Shellfish Immunology, 67, 575–585.

Hromada-judycka, A., 2014. Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. Cellular and Molecular Life Sciences.

doi:10.1007/s00018-014-1762

Ispanixtlahuatl-mer, O., Schins, R.P.F., and Chirino, Y.J., 2017. Environmental science nano cell type specific cytoskeleton disruption induced by engineered nanoparticles. Environmental Science: Nano. doi:10.1039/C7EN00704C

Jeong, E., and Lee, J.Y., 2011. Intrinsic and extrinsic regulation of innate immune receptors. Yonsei Medical Journal, 52 (3), 379–392.

Jimeno-Romero, A., et al., 2017. Digestive cell lysosomes as main targets for Ag accumulation and toxicity in marine mussels, Mytilus galloprovincialis, exposed to maltose-stabilised Ag nanoparticles of different sizes. Nanotoxicology, 11 (2), 168–183.

Jimeno-Romero, A., et al., 2019. Bioaccumulation, tissue and cell distribution, biomarkers and toxicopatogenic effects of CdS quantum dots in mussels, Mytilus galloprovincialis. Ecotoxicology and Environmental Safety, 167, 288–300.

Jones, J., Allam, B., and Pales Espinosa, E., 2020. Particle selection in suspension-feeding bivalves: Does one model fit all? The Biological Bulletin, 238 (1), 41–53.

Katsumi, A., et al., 2015. Mechanisms of toxicity of Ag nanoparticles in comparison to bulk and ionic Ag on mussel hemocytes and gill cells. p. 1–30.

Kawai, T., and Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nature Immunology, 11 (5), 373–384.

Khan, F.R., et al., 2015. Inhibition of potential uptake pathways for silver nanoparticles in the estuarine snail Peringia Ulvae. Nanotoxicology, 9 (4), 493–499.

Lau, Y.Y.T., et al., 2017. Characterization of hemocytes from different body fluids of the eastern oyster Crassostrea virginica. Fish & Shellfish Immunology, 71, 372–379.

Lee, M. S., and Kim Y.-j., 2007. Signaling Pathways Downstream of Receptors and Their Cross Talk, no. II.

Lekamge, S., et al., 2018. The Toxicity of Silver Nanoparticles (AgNPs) to three freshwater invertebrates with different life strategies: Hydra vulgaris, Daphnia carinata, and Paratya australiensis. Frontiers in Environmental Science, 6, 152.

Moore, M.N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environment International, 32 (8), 967–976.

Moreira, R., et al., 2015. Evaluation of reference genes of Mytilus Galloprovincials and Ruditapes Philippinarum infected with three bacteria strains for gene expression analysis. Aquatic Living Resources, 152 (2014), 147–152.

Perkins, D.J., et al., 2018. Autocine-paracrine prostat glandin E2 signaling restricts TLR4 internalization and TRIF signaling. Nature Immunology, 19 (12), 1309–1318.

Philipp, E.E.R., et al., 2012. Massively Parallel RNA sequencing identifies a complex immune gene repertoire in the lophotrochozoan Mytilus edulis. PLoS One, 7 (3), e33091.

Pinsino, A., et al., 2017. Amino-Modified Polystyrene Nanoparticles Affect Signalling Pathways of the Sea Urchin (Paracentrotus Lividus) Embryos, 5390 (March).

Pinsino, A., et al., 2020. Probing the immune responses to nanoparticles across environmental species. A perspective of the EU Horizon 2020 project PANDORA. Environmental Science: Nano, 7 (11), 3216–3232.

Pinsino, A., et al., 2015. Titanium dioxide nanoparticles stimulate sea urchin immune cell phagocytic activity involving signalling pathway. Nature Publishing Group, no. May: 1–12. https://doi.org/10.1038/srep14492.

Rauh, J., et al., 2013. Big signals from small particles: regulation of cell signaling pathways by nanoparticles. Chemical Reviews, 113 (5), 3391–3406.

Rocha, T.L., Saboia-Morais, S.M.T., and Bebianno, M.J., 2016. Histopathological assessment and inflammatory response in the digestive gland of marine mussel Mytilus galloprovincialis exposed to cadmium-based quantum dots. Aquatic Toxicology, 177, 306–315.

Santos, T., et al., 2011. Effects of transport inhibitors on the cellular uptake of carboxylated polystyrene nanoparticles in different cell lines. PLoS One, 6 (9), e24438. .

Sikder, M., et al., 2018. A rapid approach for measuring silver nanoparticle concentration and dissolution in seawater by UV-Vis. The Science of the Total Environment, 618, 597–607.

Tanguy, M., et al., 2013. Sequence analysis of a normalized cDNA library of Mytilus edulis hemocytes exposed to Vibrio splendidus LGP32 strain. Results in Immunology, 3, 40–50.

Tobiana, M., et al., 2013. Toll-like receptors and MyD88 adaptors in Mytilus: complete cds and gene expression levels. Developmental and Comparative Immunology, 40 (2), 158–166.

Tobiana, M., et al., 2014. Toll signal transduction pathway in bivalves: complete Cds of intermediate elements and related gene transcription levels in hemocytes of immune stimulated Mytilus galloprovincialis. Developmental and Comparative Immunology, 45 (2), 300–312.

Turabekova, M., et al., 2014. Immunotoxicity of nanoparticles: a computational study suggests that CNTs and C 60 fullerenes might be recognized as pathogens by toll-like receptors β, 3488–95.

Wang, K., et al., 2016. Clam focal and systemic immune responses to QPX infection revealed by RNA-seq technology. BMC Genomics, 17, 146.

Yu, S.-j., Yin, Y.-g., and Liu, J.-f., 2013. Silver nanoparticles in the environment. Environmental Science: Processes & Impacts, 15 (1), 78–92.

Zhao, G.-n., Jiang, D.-s., and Li, H., 2015. Interferon regulatory factors: at the crossroads of immunity, metabolism, and disease. Biochimica et Biophysica Acta, 1852 (2), 365–378.