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# Gold nanoparticles coated with polyvinylpyrrolidone and sea urchin extracellular molecules induce transient immune activation

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1	Gold nanoparticles coated with polyvinylpyrrolidone and sea urchin extracellular molecules
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## 42 Abstract

43	We report that the immunogenicity of colloidal gold nanoparticles coated with polyvinylpyrrolidone
44	(PVP-AuNPs) in a model organism, the sea urchin Paracentrotus lividus, can function as a proxy for
45	humans for in vitro immunological studies. To profile the immune recognition and interaction from
46	exposure to PVP-AuNP (1 and 10 µg particles mL <sup>-1</sup> ), we applied an extensive nano-scale approach,
47	including particle physicochemical characterisation involving immunology, cellular biology, and
48	metabolomics. The interaction between PVP-AuNPs and soluble proteins of the sea urchin
49	physiological coelomic fluid (blood equivalent) results in the formation of a protein "corona"
50	surrounding the NP from three major proteins that influence the hydrodynamic size and colloidal
51	stability of the particle. At the lower concentration of PVP-AuNPs, the P. lividus phagocytes show a
52	broad metabolic plasticity based on the biosynthesis of metabolites mediating inflammation and
53	phagocytosis. At the higher concentration of PVP-AuNPs, phagocytes activate an immunological
54	response involving Toll-like receptor 4 (TLR4) signalling pathway at 24 hours of exposure. These
55	results emphasise that exposure to PVP-AuNP drives inflammatory signalling by the phagocytes and
56	the resolution at both the low and high concentrations of the PVP-AuNPs and provides more details
57	regarding the immunogenicity of these NPs.
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60 61	Sea urchin immune cells; Innate defence response; Nano-recognition; Immune metabolic rewiring; Immunoreactivity
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### 73 **1. INTRODUCTION**

74 Gold nanoparticles (AuNPs) are catching the attention of the scientific community due to the 75 promising progress made in synthesis technologies, in surface functionalization as well as in 76 biomedical, cosmetic and pharmaceutical applications<sup>1</sup>. A wide variety of specific properties (e.g., 77 small size and associated high surface to volume ratio, particle stability, low cost to produce, 78 electronic, magnetic and optical properties) makes AuNPs an ideal material for bioimaging, gene 79 diagnostics, cancer therapy, and drug delivery<sup>2</sup>. Accordingly, safety assessment becomes an 80 important issue for the beneficial usage of this new nanomaterial. However, increasing 81 nanobiotechnological applications result in the increased exposure of all living organisms and the 82 environment to NPs. This exposure, which is assumed to increase in the future, justifies the need to 83 identify, measure and manage the presumed risk. Chemical transformations of NPs in the 84 environment are poorly understood at present, largely due to the complexity arising from concurrent 85 transformations (e.g., oxidation, sulfidation, reduction reactions, surface adsorption of 86 macromolecular and ions) by the action of sunlight and microorganisms, as well as oxidants<sup>3</sup>. There 87 are a few analytical methods to determine the level of occurrence and the fate on AuNPs in water, 88 soil, air and biological matrices. In a realistic scenario, colloidal AuNPs may be trapped by the 89 sediment becoming unavailable for the majority of the organisms. However, filter feeders and other 90 species associated with the sediment may be the exceptions because they take up particles including 91 AuNPs that incorporate them into the food chain and undergoes biomagnification<sup>4</sup>. At present, there 92 are significant gaps in our knowledge of how gold particles impact human health because of their 93 chemical and physical properties but also because of complex interactions that may occur at various 94 biological levels including genes, transcripts, metabolites, proteins, enzymes, organelles, cells, 95 tissues, organs, organ systems, and whole organisms. Nano-structured materials display a privileged 96 interplay with the effector cells of the immune system, especially phagocytic cells, which, once 97 activated, recognize, interact, and eliminate foreign particles<sup>5</sup>.

98 The wide use of AuNPs strengthens concerns regarding their safety and compatibility with 99 the innate immune system, which is the primary defensive barrier that functions continuously, both 100 in healthy and diseased organisms. Although many aspects of the modulation of immune responses 101 induced by AuNPs have been investigated, results remain controversial<sup>6</sup>. AuNPs interact with the 102 immune system and induce downstream immune and inflammatory signalling that may both support 103 and suppress inflammation<sup>6</sup>. For example, macrophages engulf 3 nm-AuNPs efficiently through the 104 process of pinocytosis, without eliciting a pro-inflammatory cytokine release (e.g., tumour necrosis 105 factor- $\alpha$ , interleukin 1)<sup>7</sup>. In contrast, a different immunological scenario that is marked by a pro-106 inflammatory reaction (e.g., up- or down-regulation of IL-1, IL-6, TNF- $\alpha$ ) is evident when cells 107 interact with AuNPs of different size and/or functionalized surface<sup>8-10</sup>. In assessing the 108 immunogenicity of the AuNPs, it is important to give consideration not only to the particle size but 109 also to other biologically relevant properties (e.g., stability, dispersibility, reactivity) on which 110 depends their ability to recognize or to be recognized by cells. Because AuNPs tend to 111 aggregate/agglomerate in a biological medium<sup>11</sup>, the surface functionalization of the AuNPs may be 112 considered necessary to enhance both their stabilization and dispersibility in that medium.

113 Although nude particles can be toxic, coated particles (e.g., PVP, humic acid, serum) can be 114 harmless because the acquired chemical functions of the coat modify the properties of the NPs, which 115 influence bioavailability and biocompatibility. Biocompatibility is mandatory when the NPs are used 116 for applications that they be inert as in some medical, industrial and environmental applications. On 117 the contrary, targeting the immune system with NPs can be used to stimulate or inhibit the medical applications that can be anti-inflammatory, anti-cancer, anti-viral therapies<sup>12-13</sup>. This emphasizes the 118 119 basis for the great level of interest concerning the immunogenicity or immunocompatibility of 120 AuNPs.

The 3R principle of reducing, refining, and replacing animal experimentation has led to new 121 122 methodologies for in vivo and in vitro/ex vivo investigations, with increasing use of invertebrate 123 animal models, including the chance of "substituting" mammalian models with invertebrates<sup>14</sup>. Based 124 on this aim, the Mediterranean sea urchin, Paracentrotus lividus, a marine invertebrate, has become a swift and efficient popular model that is a proxy for humans for basic and translational nano-125 immunological studies in vivo<sup>15-16</sup> and, more recently, in vitro/ex vivo<sup>17-18</sup>. The genome sequence of 126 127 a different sea species emphasised some strong similarities between sea urchin and human immunity, 128 and also identified clues of the basis of alternative adaptive and anticipatory immune functions that 129 are shared with humans<sup>19</sup>.

130 To profile the sea urchin immune cell behaviour resulting from exposure to PVP-AuNPs at varying concentrations (1 and 10 µg mL<sup>-1</sup>), here we focus on the: i) characterisation of PVP–AuNPs 131 in the sea urchin coelomic fluid (CF), in which immune cells secrete functional biomolecules; ii) 132 133 behaviour of PVP-AuNPs (stability, dissolution, aggregation and particle transformation) in the CF 134 with a special focus on the main constituents shaping sea urchin PVP-AuNP protein corona; iii) sea urchin phagocytic cell topography and related PVP-AuNP surface distribution; iv) defensive 135 innate/inflammatory signalling leading the immune-PVP-AuNP recognition and interaction in vitro. 136 137 We demonstrate that protein-particle interaction triggers an extended network of immune-related 138 signalling, drawing a transient immune activation both at the low and the high concentrations of the 139 particles.

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### 141 2. MATERIALS AND METHODS

142 **2.1** Polyvinylpyrrolidone-functionalized gold nanoparticle synthesis and characterisation.

143 Chemical precursors. Tetrachloroauric (III) acid trihydrate (99.9% purity), sodium citrate
 144 tribasic dihydrate (≥99% purity), polyvinylpyrrolidone (PVP) (55 kDa) were purchased from Sigma 145 Aldrich.

146AuNP preparation. Citrate-stabilized AuNPs were synthesised according to the previously147developed seeded-growth methods<sup>20</sup>. Briefly, sodium citrate (Na<sub>3</sub>Ct; 2.2 mM) in milliQ (mQ) water148was boiled in a three necks flask under reflux, followed by the addition of chloroauric acid (HAuCl<sub>4</sub>;14925 mM). The citrate-stabilised AuNPs solution-phase synthesis was completed within a few minutes150and produced ~10 nm NP seeds. Following seeding, the desired size of the Na<sub>3</sub>Ct-coated AuNPs was151obtained by sequential growth steps.

152 *AuNP surface coating process by PVP and sample purification.* PVP (0.2 mM) in mQ water 153 plus Na<sub>3</sub>Ct-coated AuNPs (100  $\mu$ g mL<sup>-1</sup>) was stirred overnight. PVP-AuNPs were purified from the 154 excess of PVP by centrifugation (15 000 x *g*), the supernatant was aspirated, and particles were 155 resuspended in mQ water. Samples were washed twice in mQ water, and the final PVP-AuNP 156 concentration was 2  $\mu$ M PVP coating on 100  $\mu$ g mL<sup>-1</sup> AuNPs.

*PVP-AuNP characterisation*. Particle microstructural characterisation was carried out using a
 scanning electron microscope (SEM; FEI Magellan 400 XHR; eXtreme High Resolution) operated at
 20 kV. PVP-AuNPs were prepared by applying 4 μL onto a carbon-coated copper transmission
 electron microscopy (TEM) grid and dried at room temperature (RT). PVP-AuNPs (922 particles)
 from different regions of the grid were analysed.

162 UV-Vis Spectroscopy analyses. PVP-AuNPs (1 and 10 µg mL<sup>-1</sup>) were analysed for their 163 behaviour in a high salt solution (Coelomocyte Culture Medium; CCM<sup>21</sup>) and biological medium (sea 164 urchin cell-free CF in CCM; 1:1 diluted) <sup>22</sup>. Specifically, 1 mL of each sample was placed in a plastic 165 cuvette, and the spectral analysis was performed in an Agilent Cary 60 spectrophotometer (300–900 166 nm range, at RT). The concentration of particles in mQ water was used as reference baseline value to 167 normalize that particle concentration of each sample.

168 Size and Zeta Potential Measurements. The hydrodynamic diameter of the PVP-AuNPs before 169 and after incubation in CCM was determined by dynamic light scattering (DLS), and laser doppler 170 velocimetry respectively, using a Malvern Zetasizer Nano ZS instrument equipped with a light source 171 wavelength of 638.2 nm and a fixed scattering angle of 173°. Diameters of the NPs were reported as 172 intensity distribution calculated by non-negative least squares (NNLS) analysis; and as Z-average and 173 polydispersity index (PDI) calculated by cumulant analysis. The software was arranged with the 174 parameters of the refractive index and absorption coefficient of gold, and solvent viscosity of water 175 at 25°C.

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### 177 **2.2** Animal collection and maintenance, immune cell cultures and exposures.

Adult sea urchins (Paracentrotus lividus) were collected along the coasts of Sicily (Italy) that 178 179 are not human-impacted. Animals were acclimated for 1 week and kept under strictly controlled 180 conditions in tanks provided with flow-through oxygenated artificial seawater (Aqua Ocean Reef Plus 181 Marine Salt, Aquarium Line, Italy; salinity (38–39‰) and density (1.028–1.030 g/cm<sup>3</sup>)). Artificial 182 seawater ensures the absence of pollutants or organic matters putatively present in the natural 183 seawater, which could affect the sea urchin immune response. CF containing immune cells was 184 harvested from each donor in a medium containing ethylene glycol tetra-acetic acid (EGTA; 1 mM) 185 in CCM. Cells in the CF were withdrawn with a syringe preloaded with CCM and diluted 1:1. After 186 collection, half of the diluted CF was placed in culture and exposed to the NPs. The rest of the CF 187 was centrifuged, the cells were discarded, and the cell-free CF was exposed to NPs based on standard 188 procedures established for these experiments (see nanoparticles conditioning and protein corona 189 profiling section). Primary immune cell cultures were established following the procedures recently 190 described in Pinsino and Alijagic<sup>22</sup>. Briefly, harvested immune cells from eight healthy donors were 191 exposed to increasing concentrations of PVP-AuNPs (0, 0.1, 1.0, and 10  $\mu$ g mL<sup>-1</sup>), and maintained 192 in a custom-made cell culture incubator at 16±2°C, for 24 hours (h). Cells were collected by a soft 193 scraper, centrifuged at 9000 x g for 10 min at 4°C and stored at -80°C. Experimental conditions (e.g., 194 particle concentrations, exposure time) were set taking into account: i) the behaviour of AuNPs in salt water media<sup>23-24</sup>; ii) the detection limits of DLS for polydispersed colloids<sup>25</sup>; iii) the high ionic 195 strength of CCM<sup>22</sup>. 196

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### 198 **2.3 Real-time viability assay.**

199 Cell viability of immune cells obtained from five sea urchins was assessed in a real-time 200 measurement using the non-lytic and bioluminescent RealTime-Glo MT Cell Viability Assay 201 (Promega, USA) as previously reported<sup>22</sup>. Luminescence was calculated using GloMax Discover 202 high-performance Microplate Reader (Promega, USA). PVP-AuNPs were incubated alone with 203 reagents to identify any possible interference by the particles with the assay.

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## **205 2.4 Microscopy.**

Scanning electron microscopy coupled with energy-dispersive X-ray spectroscopy. Immune cells exposed to PVP-AuNPs (1 and 10  $\mu$ g mL<sup>-1</sup>, 24-h exposure) that adhered to glass coverslips (Menzel Gläser, Germany) were fixed and prepared for SEM as reported previously<sup>17</sup>. Cell morphology was observed with a FEI Nova NanoSEM scanning electron microscope (FEI, Brno, Czech Republic) and the EDS microanalysis was performed by Ametek® EDAX Octane Plus SDD detector and TEAM<sup>TM</sup> EDS Analysis Systems (Ametek, Inc.). Predictive theoretical model spectra were made using the NIST DTSA-II Kelvin software package<sup>26-27</sup>. 213 Immunofluorescence microscopy. Immune cells were distributed in wells of a 25-well plate 214 (Thermo Fisher Scientific) and incubated with 10 µg mL<sup>-1</sup> AuNPs for 24 h. Cells were fixed with 215 ice-cold methanol for 20 min and washed three times in CCM. Non-specific binding sites were 216 blocked with blocking buffer (3% bovine serum albumin (BSA), 5% inactivated normal goat serum 217 (NGS), 0.02% Triton X-100 in TBST) for 2 h, at RT. After blocking, cells were incubated overnight 218 at 16±2°C with anti-TLR4 (H-80) (Santa Cruz Biotechnology, sc-10741) and anti-α-tubulin (SIGMA, 219 T5168) both diluted 1:50 in blocking buffer. Cells were washed three times in TBST and incubated 220 for 1 h at 16±2°C with the secondary antibodies; i) goat anti-mouse-IgG (IRDye® 800CW, LI-COR) 221 and ii) goat anti-rabbit- F(ab')2 fragment (Alexa Fluor® 594, Life Technologies) both diluted 1:200 222 in blocking buffer. Cells were incubated with Hoechst dye (Hoechst 33342; 3 µM) for 7 min to 223 visualize nuclear DNA. Photomicrographs of immune cells were acquired with the Leica fluorescence 224 microscope using the 40x objective.

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### 226 **2.5 SDS-PAGE and immunoblotting.**

227 Cellular pellets were homogenised in a standard lysis buffer in preparation for SDS-PAGE 228 complemented with phosphatase and protease inhibitor cocktails<sup>28</sup>. Protein content of samples were 229 quantified by the BioRad assay kit (Hercules, CA, USA) followed by precipitation overnight at -20°C 230 in equal volume of acetone. Proteins pellet were resuspended immediately in SDS buffer containing 231 β-mercaptoethanol, denaturized at 100°C for 5 min plus cooling for 5 min, separated on 232 polyacrylamide gels (4-20% Mini-PROTEAN TGX precast BioRad, USA), and transferred to 233 nitrocellulose membranes (Amersham, UK), according to the manufacturer's instructions. Following 234 blocking, membranes were incubated with one of the following primary antibodies: anti-TLR4 (H-235 80) (Santa Cruz Biotechnology, sc-10741; diluted 1:250 in blocking buffer; anti-HSP70 (SIGMA, 236 Cat N. H-5147, 1:1000); anti-phospho-p38 MAP Kinase (Tr180/Tyr182) (Cell Signaling, 9211, 237 1:250); Phospho-p42/44 MAP Kinase (ERK1/2) (Cell Signaling, 9101, 1:300); IL-6 (H-183) (Santa 238 Cruz Biotechnology, sc-7920, 1:100); anti-MnSOD (Enzo Life Sciences, ADI-SOD-111, 1:200); 239 anti-NF-κB (H-286) (Santa Cruz Biotechnology, sc-715, 1:200); anti-β-actin (SIGMA, A5441, 1:500); anti-*Pl*-toposome (BEVIB12b8<sup>29</sup>, 1:200); anti-*Pl*-nectin<sup>30</sup>, 1:200); anti-*Pl*-galectin-8<sup>31</sup>, 240 241 1:800). Following washing, membranes were incubated with fluorescein-labelled secondary 242 antibodies (LI-COR Biosciences) and protein bands were visualised and captured by the Odyssey 243 Infrared Imaging System (LI-COR Biosciences).

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### 245 **2.6 Nanoparticles conditioning and protein corona profiling.**

246 PVP-AuNP (1 and 10  $\mu$ g mL<sup>-1</sup>) were added to 1 ml of cell-free CF diluted in equal volume of 247 CCM (cell-free CF in CCM) in TreffLab 1.5 mL microcentrifuge tubes and incubated with agitation 248 (shaking platform; bioSan, Latvia) for 24 h at 16±2°C in the dark. After incubation, the PVP-AuNPs 249 with newly-formed protein complexes were recovered by centrifugation at 21130 x g for 20 min at 250 4°C, followed by three energetic rinses in artificial seawater as already described<sup>17</sup>. PVP-AuNP-251 protein-complex pellets were resuspended in Laemmli buffer containing β-mercaptoethanol (Bio-252 Rad, USA), boiled for 5 min, and the PVP-AuNPs were pelleted at 21130 x g for 20 min at 4°C. 253 Eluted proteins were separated on polyacrylamide gels (4-20% Mini-PROTEAN TGX precast Bio-254 Rad, USA) that were stained with Coomassie Brilliant Blue R250 to identify the protein bands. 255 Protein binding to the PVP-AuNPs were performed four times with cell-free CF from different sea 256 urchins to ensure reproducibility. Gel scans were processed by the ImageJ software (NIH, USA).

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### 258 **2.7** Metabolomic typing by untargeted liquid chromatography and mass spectrometry.

259 Liquid chromatography-mass spectroscopy (LC-MS analysis) was performed according to 260 previously established protocols<sup>18</sup>. Cells exposed to PVP-AuNPs (1  $\mu$ g mL<sup>-1</sup>, 24-h exposure) were 261 gently recovered from the culture plate, centrifuged at 4500 x g for 5 min at 4°C, and pellets were 262 resuspended in 0.5 mL ice-cold 70% acetonitrile (Sigma-Aldrich). The supernatant fraction 263 containing both polar and non-polar metabolites was retrieved in glass vials followed by solvent 264 evaporation and drying at 30°C. Samples were resuspended in 150 µL of ultrapure water and injected 265 in the UHPLC–MS system to be analysed by reversed-phase chromatography<sup>18</sup>. Mass spectra were 266 recorded in a mass range of m/z 60 to 1050 m/z (centroid mode). A mass correction was performed 267 through a second nebuliser by a reference solution (m/z 112.9855 and 1033.9881) dissolved in the 268 mobile phase of 70% 2-propanol-acetonitrile-water. Analysis and isotopic natural abundance data 269 correction were performed by MassHunter ProFinder and Mass Profile Professional software 270 (Agilent).

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### 272 **2.8 Statistical Analysis.**

Statistical analyses were performed by GraphPad Prism Software 6.01 (USA). Statistical differences among selected groups were estimated by one-way or two-way ANOVA (followed by the multiple comparison tests). A *p*-value of less than 0.05 was deemed statistically significant. Data were reported as mean  $\pm$  standard deviation (SD) or mean  $\pm$  standard error (SE).

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### **3. Results and discussion**

# 3.1 Extracellular molecules in the cell-free CF affect stability and aggregation kinetics in a high salt medium.

281 Physicochemical properties of NPs determine their safety, stability, efficacy, as well as their 282 *in vitro* and *in vivo* behaviour. The leading mechanisms involved in NP transformation may depend 283 on the size and the concentration of the particles, the route of exposure to biological molecules, the 284 NP physicochemical features (e.g., surface chemistry, size, shape, geometry) and the nature of the environment in which the particles are found<sup>32</sup>. Metallic NPs such as AuNPs tend to form colloidal 285 286 aggregates and the stability of these particles is based on a balance between the electrostatic repulsion 287 and the universal attractive van der Waals force described by the DLVO theory. Salt concentrations 288 of the fluids tend to reduce electrostatic repulsions between head-to-head NPs and favours homo-289 aggregation, whereas the organic matters suspended in the fluids may aid in hetero-aggregation<sup>33</sup>. 290 Aggregation influences the fate of the NPs in the environment, particularly in aqueous environments, 291 which depends on ionic strength and composition, organic matter composition and pH. These 292 chemical and biochemical attributes may affect the particle surface charge stability, and in turn, 293 particle bioavailability and dispersion. Results from environmental monitoring of AuNPs in situ are 294 not available, and therefore *in silico* modelling of the particle behaviour is currently the only means 295 for estimating exposure to organisms<sup>34</sup>.

296 The appropriateness of NPs for a specific application in medicine, cosmetic and 297 pharmaceutical applications, depends on their average diameter, PDI and size, among other 298 parameters. Measurements of PVP- AuNPs showed a core diameter of  $25.0 \pm 3$  nm (Figure 1A, upper 299 panel). However, the physicochemical characterisation of PVP-AuNPs (10 µg mL<sup>-1</sup>) in CCM had a 300 hydrodynamic diameter of 63 nm with a polydispersity index of 0.122 (Figure 1A lower panel). The 301 zeta potential value in water was  $-15.3 \pm 0.6$  mV (conductivity 0.006 mS/cm), whereas the zeta 302 potential value in CCM was near neutral. This was because electrostatic charges were efficiently 303 screened when the ionic concentration of the solution was very high<sup>35</sup> as well as for the non-ionic 304 nature of the polymer<sup>36</sup>. The UV-vis spectrum of PVP-AuNPs (10 µg mL<sup>-1</sup>) dispersed in both CCM 305 (Figure 1B, red and blue lines) and mQ water (Figure 1B, green line) presented a typical profile of 306 monodisperse particles with a maximum size dispersion of 524 nm. UV-vis values did not show 307 significant changes in dynamic absorption at 0 h. Notably, at 24 h of exposure in CCM, a weak 308 decrease (<10%) in absorbance was noted but there were no changes in the UV-vis profile and DLS 309 (Figure 1B, compare red and blue line). This change simply suggested a reduction in the PVP-AuNP 310 concentration in the solution. It may be that even if PVP-AuNPs remain well dispersed in high salt 311 solutions, the increment of the polarity of the solvent may decrease their dispersibility. This would 312 lead to a partial adsorption of the PVP-AuNPs to some types of plastics or to glass surfaces, which 313 may modify their dissolution kinetic and related dispersibility depending on the material employed. 314 However, a partial loss of PVP coating AuNP cannot be excluded<sup>37</sup>. Overall, these data indicate good 315 monodispersity and stability of the particles also after suspension in CCM with high salt 316 concentration, including the chelating agent, EGTA that blocks cell aggregation<sup>21</sup>, NaCl, MgCl<sub>2</sub> and 317 HEPES. Remarkably, the immune cells are collected from P. lividus in CCM because this medium 318 maintains the sea urchin cell density and salinity very close that of the sea urchin CF, and it preserves 319 the morphological features of cells and blocks calcium-dependent cell clotting<sup>22</sup>.

The UV-vis spectrum of the PVP-AuNPs after 24 h of exposure to cell-free CF plus CCM 320 321 resulted in significant differences in both the shape and absorbance of the NPs, indicating that PVP-322 AuNPs interacted with sea urchin extracellular biomacromolecules and led to the formation of hetero-323 aggregates of particles of both concentrations (Figure 1C and D, blue lines). The decreased 324 absorbance values were related to both increased aggregation and decreased measurable particle 325 concentration under static conditions, as confirmed by the presence of noticeable precipitates. After 326 shaking to disperse the precipitates, samples showed similar spectra but increased absorbance and 327 baselines to that of static conditions (Figure 1C and D, dashed blue lines). This is due to the light 328 scattering effects that are common for larger NP aggregates. Our findings indicate that samples 329 underwent fast flocculation mediated by organic matter that created larger hetero-aggregates 330 (biomolecule-particle complexes) which are easier to dissociate than homo-aggregates (particle-331 particle aggregation). Notably, at 1 µg mL<sup>-1</sup>, the AuNPs showed slower aggregation, as expected. The 332 UV-vis spectrum of the PVP-AuNPs (1 and 10 µg mL<sup>-1</sup>) dispersed in cell-free CF *plus* CCM at 0 h 333 showed similar results to those obtained from CCM alone when evaluated at the same particle 334 concentration (compare Figure 1C and Figure 1B, red lines), as expected.

335 Biomacromolecules are able to change NPs properties, including their stability and related 336 interactions among particles<sup>38</sup>. The balance between homo- and hetero- aggregation scenarios is 337 driven by the relative collision frequencies of NPs together and with organic components, respectively<sup>39</sup>. Polymer coatings, including PVP, are stable under unperturbed conditions but other 338 ligands can well replace them<sup>40</sup>. Critically, our findings support the notion that CF 339 340 biomacromolecules may replace the PVP onto the AuNP surface, but this replacement is not able to 341 confer a satisfactory particle steric stabilisation. PVP-organic macromolecule exchanges onto the 342 AuNP surface are probably related to the stronger protein chemical moieties interaction than that of the cyclic amide groups of the PVP<sup>41</sup> (see below). We found that the interaction between sea urchin 343 344 extracellular organic matter and PVP-coated AuNPs formed a dynamic biomolecule coating causing 345 a lost in particle steric stabilisation, which induced a colour change of the biological medium from 346 red to purple (Figure 1E), indicative of particle aggregation<sup>42</sup>. AuNP aggregation correlated with the 347 extracellular protein adsorption onto the particle surface is a well-noted process in mammalian immune cell culture<sup>43</sup>. 348

349 Stabilising agents such as PVP play an important role in the corona formation as they minimise 350 the protein adsorption compared to that of an uncoated particle surface, in which an increase in the 351 conformational entropy and a decrease in the Gibbs free energy change, promote a strong 352 particle/protein interaction<sup>44</sup>. The capability to modulate the functional composition of the protein 353 corona is critical for the design of ideal and safe NPs because permit to modulate the bio-accessibility 354 and the nanotoxicity of the particles for guiding their design, synthesis, and effective applications in

- nanomedicine. In agreement, control over the protein corona of NPs by using PVP allows them to
   function better as drug delivery vehicles because decrease their cytotoxicity<sup>45</sup>.
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359 Figure 1. Physicochemical characterisation of PVP-coated AuNPs dispersed in CCM and cell-free CF plus CCM. 360 (A) SEM image of PVP-AuNPs in CCM (upper panel); DLS size measurement of PVP-AuNPs (10 µg mL<sup>-1</sup>) dispersed 361 in CCM at 0 and 24 h time period of exposure (lower panel, red and blue line). (B) UV-vis spectra of PVP-AuNPs (10 µg 362 mL-1) dispersed in mQ water, 0 and 24 h time period (green line) and in CCM at 0 h (red line), and in CCM at 24 h of 363 exposure (blue line). (C) PVP-AuNPs dispersed in cell-free CF plus CCM at 10 µg mL<sup>-1</sup> and 0 and 24 h of exposure (red line, and blue line respectively). (D) PVP-AuNPs dispersed in cell-free CF plus CCM at 1 µg mL<sup>-1</sup> and 0 and 24 h of 364 365 exposure (red line, and blue line respectively). (E) Macroscopic changes of PVP--coated AuNPs (10 µg mL<sup>-1</sup>) dispersed 366 in mQ water, CCM, and cell-free CF plus CCM, from 0 to 24 h of exposure. The biological medium (cell-free CF plus

367 CCM) changes colour from red to purple over the 1 day of monitoring. In C and D, dashed blue lines indicate spectra of
 368 shaking sample at 24 h of exposure. Physicochemical characterisation of PVP-coated AuNPs dispersed in the mQ water
 369 has been used as the reference point.

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### 372 **3.2** Sea urchin immune system interactions with PVP-AuNPs *in vitro*.

373 The immune cells (phagocytes, amoebocytes and vibratile cells) in the sea urchin, P. lividus, 374 are morphologically distinct with different functions and act as the defence system for protection 375 against exogenous and endogenous menaces to the host<sup>46</sup>. The phagocytes can be found in both 376 filopodial and lamellipodial morphology, are highly adherent, and are the most abundant (up to 80%) 377 cell type present in the sea urchin CF. They are associated with phagocytosis and encapsulation of 378 foreign invaders, allograft rejection, cytolytic/cytotoxic and inflammatory responses, antimicrobial 379 peptide secretion, etc<sup>47</sup>. To initiate our understanding the immune nanoscale interaction with PVP-380 AuNPs, we focused on sea urchin immune cell topography and the distribution of PVP-AuNPs (1 and 381 10 µg mL<sup>-1</sup>) on and within cells after 24 h of exposure *in vitro*. Observations by SEM coupled with 382 EDS showed that cells were spread and adherent to the culture plate (Figure 2A, 2D) as well as 383 organised in bundles and fibres at the lower concentration of PVP-AuNPs (Figure 2A, blue 384 arrowhead). The composition of the electron-dense gold particles was confirmed by EDS (see Figure 2S). Particle aggregates/agglomerates that were displayed on cell surfaces ranged in size from 0.195 385 386 to 1.6 µm in diameter (Figure 2A-F, yellow boxes). This association suggested a tight adhesion 387 between the PVP-AuNPs and the cell surfaces (Figure 2B, 2E; Figure 2C, 2F back-scattered electron images) that may have occurred by the interaction between cell surface receptors and sea urchin 388 389 extracellular protein ligands on the PVP-coated AuNP biocorona. Notably, back-scattered electron 390 approach permitted imaging that could discriminate both organic (dull shine) and inorganic (intense 391 brightness) materials inside of the cells (see Figure 2C, 2F), in agreement with Goldstein et al<sup>48</sup>. 392 Particle aggregates/agglomerates were located at the outer and the inner surface of the plasma 393 membrane (Figure 2B and 2E, yellow boxes; and Figure 1S). The SEM images suggests that sea 394 urchin phagocytes are able to take up PVP-AuNP aggregates/agglomerates. The composition of the 395 electron-dense gold particles was confirmed by EDS (see Figure 2S). The number of PVP-AuNP 396 aggregates/agglomerates that immune cells may takes up and accumulate, may be related to particle 397 concentration (see aggregates in Figure 2B, 2E). To the contrary, aggregate/agglomerate size may to 398 be independent of the concentration used (see Figure 2C, 2F). The general healthy state of sea urchin immune cells in culture and exposed to PVP-coated AuNPs was monitored over three days of the 399 400 experiment by the RealTime-Glo MT Cell Viability Assay for cells exposed at increasing 401 concentrations of the NPs (0.1, 1, 10,  $\mu$ g mL<sup>-1</sup>) (see Figure 3, three measurement points are shown). 402 The viability of the cells incubated with PVP-AuNPs (0.1, 1  $\mu$ g mL<sup>-1</sup>) over three days was not 403 different from the control cells that were cultured in the absence of NPs.





405 Figure 2. Scanning electron microscopy shows the interaction between PVP-AuNPs and sea urchin immune cells 406 *in vitro*. (A-C) Phagocytes exposed to 1  $\mu$ g mL<sup>-1</sup> PVP-AuNPs for 24 h. (D-F) Phagocytes exposed to 10  $\mu$ g mL<sup>-1</sup> PVP-407 AuNPs for 24 h. Back-scattered electron image (A, D) low-magnification (B-C, E-F) high-magnification. Yellow boxes 408 highlight aggregates/agglomerates at the outer and the inner surface of the plasma membrane.



Real-time cell viability

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411 Figure 3. Real-time viability assay reveals the impact of increasing concentrations of PVP-AuNP on sea urchin 412 immune cells in culture. Cell viability during three days of continuous monitoring shows three measurement points (24, 413 48, 72 h) for three difference concentrations of PVP-AuNPs (0.1, 1, 10  $\mu$ g mL<sup>-1</sup>). Only the highest dose of PVP-AuNPs 414 (10  $\mu$ g mL<sup>-1</sup>) results in a decrease in cell viability. Levels are expressed in arbitrary units as fold increase or decrease 415 compared to controls that were set to 1 (dotted line). Data are reported as the mean ± SD; asterisks (\*) indicate significant 416 differences among groups (\*\*p < 0.01; \*\*\*p < 0.001).

417 On the contrary, the viability was significantly decreased for cells incubated with the highest NP 418 concentration (10  $\mu$ g mL<sup>-1</sup>) compared to unexposed cells, which was in line with the reduced ability 419 of the cells to form dendritic protrusions as bundles and fibres across the surface of the plate. In 420 agreement with previous reports, AuNP exposure causes disruption in the cytoskeletal filament 421 content of human dermal fibroblast<sup>49</sup>. However, the level of viability in the cells exposed to PVP-422 AuNPs ( $10 \ \mu g \ mL^{-1}$ ) for 1 day was lower than that for three days, perhaps because these cells were 423 under recovery. We speculate that the sea urchin immune cells may have responded to a sub-toxic 424 exposure to PVP-AuNPs that activated a tolerogenic immunological response, possibly promoted by 425 excretion of the particle content. In vertebrates, a stimulus may induce a re-programming of the 426 immune system, resulting either in reduced reactivity (tolerance) or increased response (potentiation) 427 to a challenge<sup>50</sup>.

428 To elucidate whether extracellular proteins mediate the interactions between sea urchin phagocytes and PVP-AuNPs, particles (1 and 10 µg mL<sup>-1</sup> final concentration) were incubated with 429 430 cell-free CF collected from donor sea urchins and diluted in the CCM (1:1) for 24 h. Profiles of the 431 P. lividus extracellular proteins adsorbed onto and extracted from the PVP-AuNP surface showed that 432 PVP-AuNPs-protein complexes were composed of three major proteins of about 170, 100, and 40 433 kDa (Figure 4, black asterisks). The type of proteins adsorbed to the particles likely plays a key role in cellular recognition, uptake, and removal mechanisms of AuNPs<sup>51</sup>. Engineered metal nanoparticles 434 are able to acquire a biological identity in contact with the sea urchin CF based on both selective and 435 436 non-selective affinity<sup>17</sup>. Here, PVP-AuNPs presented the strongest selective affinity for the protein of about 40 kDa while, for example, TiO<sub>2</sub>NPs have the greatest affinity for the protein of about 100<sup>17</sup>, 437 438 confirming that each type of NPs acquires a selective biological identity which will define the 439 recognition of the NPs by the immune cells and their subsequent interactions. The effectiveness of 440 nanomaterials for biomedical purposes strongly depends on both the successful NP-uptake by cells 441 and their tolerogenic immunological response<sup>52</sup>.

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469 Figure 4. PVP-AuNP acquire a biological identity in contact with the sea urchin CF based on three major proteins.
 470 SDS-PAGE profiles the extracted protein corona acquired from cell-free CF *plus* CCM onto PVP-AuNPs (1 and 10 μg mL<sup>-1</sup>) after 24 h of incubation (left panel). Intensity analysis of the associated spectra profiles performed by the ImageJ software (NIH, USA) (middle and right panels).

# 474 **3.3** Sea urchin phagocytes undergo metabolic rewiring to control inflammatory responses upon

### 475 exposure to PVP-coated AuNP in vitro.

476 Mammalian phagocytes function to remove foreign invaders, and apoptotic inflammatory 477 cells, and to remodel the extracellular matrix. These innate immune cells also secrete cytokines, 478 chemokines, and growth factors leading their polarisation from a pro-inflammatory and the activation 479 of an immune response to anti-inflammatory and the resolution of a response<sup>53</sup>. The cellular metabolism represents the key to this functional plasticity by providing energy and chemical 480 481 constituents (metabolites) that are necessary to coordinate immune cell proliferation, quiescence, 482 differentiation, and the production of secretory molecules for managing intracellular and extracellular signals. To obtain integrated information on that state of phagocyte immune-metabolism and 483 484 associated tolerance, we employed LC-MS to characterise the metabolic profile of sea urchin immune 485 cells exposed to PVP-AuNPs (1 µg mL<sup>-1</sup>) for 24 h. We used the low concentration of NPs because 486 no harmful effects on the biological activities of the exposed cells were identified (see Figure 2 and 487 Figure 3). A hierarchical clustering heatmap was used to highlight the level of changes of 22 metabolites comparing un-exposed sea urchin immune cells (group 1) to immune cells exposed to 488 489 PVP-AuNPs (group 2). Results included analyses of amino acids (L-threo-3-methylaspartate, 490 glutamic acid, taurine, phenylalanine, glycine, glutamine), amino acid intermediates (O-acetylserine),

491 nucleotides (inosinic acid also known as inosine monophosphate, nicotinamide adenine dinucleotide 492 [NAD], guanosine monophosphate [GMP], uridine monophosphate [UMP], cytidine), organic acids 493 (aconitic acid, D-threo-isocitric acid, citric acid, N-acetylneuraminic acid, arachidonic acid, 494 juniperonic acid), enzymes (dehydroascorbate, 2,5-didehydro-D-gluconate), and sulfate metabolites 495 (sulfate) (Figure 5). Metabolites from phagocytes responding to PVP- AuNPs were predominantly 496 involved in mediating inflammatory response and phagocytosis. Specifically, inosinic acid and NAD 497 and GMP were found significantly increased in exposed cells compared to the controls; while sialic 498 acid and sulfate metabolites were significantly decreased. Inosinic acid exerts a broad range of anti-499 inflammatory effects (e.g., down regulation of TNF-α, IL-1, IL-6 and MIP-2) in animal models with 500 acute respiratory distress syndrome<sup>54</sup>. Induction of NAD synthesis regulates both macrophage 501 polarisation and phagocytosis, and refurbishes oxidative phosphorylation and cellular homeostasis<sup>55</sup>. 502 Phagocytosis efficiency is reduced when NAD salvage synthesis is inhibited<sup>56</sup>. Consequently, the 503 significantly increased NAD levels observed in phagocytes responding to PVP-AuNPs (1  $\mu$ g mL<sup>-1</sup>) 504 may be a signature of increased phagocytosis, thus reinforcing our results showing that the phagocytes 505 from *P. lividus* take up PVP-AuNP aggregates/agglomerates very efficiently (Figure 2). 506 Concordantly, guanosine monophosphate in the cyclic dimeric form (cGDP) activates and controls 507 the innate immune response of mammalian dendritic cells, macrophages, and monocytes<sup>57</sup>. Notably, 508 an increase in GMP as well as in UMP influence the immunological state of cells by increasing DNA 509 replication, gene transcription and corresponding protein synthesis<sup>58</sup>. N-Acetylneuraminic acid (sialic acid) is widely expressed on the surfaces of all cells in all animals of the deuterostome lineage<sup>59</sup>. It is 510 511 the major component of glycoconjugates as glycolipids, glycoproteins and proteoglycans. Reduced 512 sialic acid expression induces maturation of mammalian dendritic cells that is correlated with tolerance induction through cytokine production<sup>60</sup>. Heparin sulfate liberated from the extracellular 513 514 matrices by enzymes released during inflammation can promote immune activation by TLR4 515 signalling<sup>61</sup>. The trend of decreased amounts of sialic acid and sulfate metabolites in sea urchin cells exposed to PVP-AuNP (1 µg mL<sup>-1</sup>) suggest an ongoing metabolic shift in these cells to a resolution 516 517 of the inflammatory response. In general, these metabolic results highlight the broad metabolic 518 plasticity of phagocytes in *P. lividus* and their related adaptations to nanoparticles.



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**Figure 5. Sea urchin phagocytes show metabolic rewiring in response to PVP-AuNPs.** Untargeted metabolic profiling of exposed (1  $\mu$ g mL<sup>-1</sup>, 24 h) and un-exposed phagocytes (control cells). Hierarchical clustering heatmaps display significantly (p  $\leq$  0.05) different intracellular metabolites by LC-MS. Metabolic typing was performed on primary cultures of phagocytes from five individual *P. lividus* donors.

## 525 **3.4 PVP-AuNP induce an increase in the levels of a few proteins involved in defensive** 526 innate/inflammatory signalling at the highest concentration

527 The decision process for an immune system to respond to "non-self" in one of many possible 528 directions is determined by many elements acting synergistically through a hierarchically organised 529 set of molecular networks that are dependent on dynamic changes in cellular metabolism as implied 530 above (Figure 5). To profile the state of the sea urchin immune system, as implied from phagocyte exposure to PVP-AuNPs in vitro, we focused on the putative intracellular signalling that regulates 531 532 the effector molecules that elicit specific immunological functions. Specifically, changes in the levels 533 of a few proteins involved in canonical defensive innate/inflammatory signalling were evaluated. 534 These included phagocyte levels of Toll-like receptor 4, 38 mitogen-activated protein kinase (p38 535 MAPK), p42/44 MAP Kinase (ERK), nuclear factor-kB (NF-kB), interleukin-6 (IL-6), heat shock 536 protein 70 (HSP70), Manganese superoxide dismutase (MnSOD), and P. lividus cellular adhesion 537 proteins (*Pl*-toposome, *Pl*-nectin), and a lectin (*Pl*-galectin-8), upon exposure to PVP-AuNPs (1 and 10 µg mL<sup>-1</sup>) for 24 h *in vitro*. The biochemical analysis of sea urchin phagocytes exposed to PVP-538 539 AuNPs (1  $\mu$ g mL<sup>-1</sup>) was consistent with our previous observations (Figure 3) of the healthy state of 540 these cells; the protein levels in exposed cells were not different from the controls (see Figure 6A,

541 6B). This was in agreement with our finding that changes in the metabolism of phagocytes from *P*. 542 *lividus* regulates the metabolic plasticity and adaptation to PVP-AuNPs. The soluble substances from 543 the phagocytes, including cytokines, metabolites, and adhesion proteins function to detect and 544 respond to environmental changes, and if necessary, lead to inflammatory reactions. A successful 545 immunological defensive immune response acts decisively to control and dominate an insult, but 546 uncontrolled or excessive inflammation is detrimental.

547 Our findings emphasised that when sea urchin phagocytes were exposed to PVP-AuNPs at the highest concentration (10 µg mL<sup>-1</sup>), they activated an immunological response involving TLR4/ERK 548 549 signalling pathway based on increased protein levels (see Figure 6A, 6B). This result was in 550 agreement with a previous report that AuNPs induce cell proliferation through the ERK signalling 551 pathway in human primary osteoblasts<sup>62</sup>. In analogy, previous studies on sea urchin immune cells 552 exposed to TiO<sub>2</sub>NPs underlined the involvement of the TLR4/p38 MAPK signalling pathway in the sea urchin phagocyte immune response in vivo<sup>16</sup>. Specifically, cells exposed to PVP-AuNPs (10 µg 553 554 mL<sup>-1</sup>) showed a 3-fold increase in TLR4 protein levels compared to the controls. Immunofluorescent 555 images displayed that TLR4 was located on exposed-cells surfaces organized in clusters (Figure 6C, red spots). TLR4 is recruited to specific cell surface domains (lipid raft domains) to form nanoscale 556 557 clusters when activated by a ligand<sup>63</sup>. In agreement, our result further supports the key role for TLR4 558 in PVP-AuNP-induced sea urchin inflammatory response. Furthermore, exposed cells showed a weak 559 increase in the ERK phosphorylation level (1.5 fold), as well as a modest increase in the NF-κB and 560 in the IL-6 intracellular level (2-fold increase each; Figure 6B). On the contrary, p38 MAPK 561 phosphorylation (P-p38 MAPK) was not affected. NF-kB, the most sensitive transcription factor 562 associated with responses related to inflammation, is known to be the final protein that functions in 563 the TLR4/NF-kB signalling pathway. It controls the expression of an array of inflammatory cytokines<sup>64</sup> of which the IL-6 is a pleiotropic cytokine classified as pro-inflammatory, as well as the 564 565 expression of adhesion proteins and antioxidant enzymes<sup>65-66</sup>. Phagocyte exposure to PVP-AuNPs (10  $\mu$ g mL<sup>-1</sup>) stimulated an increase of the mitochondrial antioxidant enzyme MnSOD (1.8-fold 566 567 increase), whereas the NPs did not inhibit or enhance the level of HSP70 (Figure 6B). This finding 568 highlighted that exposed cells amplified their antioxidant activity perhaps for protection from the harmful effects of ROS overproduction<sup>67</sup>, but the cells did not activate an HSP70-dependent stress 569 570 response, likely because particles did not induce unfolding and aggregation of cell proteins. Finally, 571 immune cell adhesion proteins function in regulating several aspects of an immune response, including immune cell trafficking and activation of effector cells<sup>68</sup>. Two of the key cell adhesion 572 573 proteins involved in sea urchins (Pl-nectin and Pl-galectin-8) were found significantly increased (3.5 574 and 2-fold increase respectively) in response to PVP-AuNPs. In mammals, galectin-8 activates 575 dendritic cells and promotes cell cytoskeletal organisation, attachment, and spreading<sup>69-70</sup>, while 576 nectin is bound by immune receptors to mediate immune recognition<sup>71</sup>.



578 Figure 6. PVP-AuNP activate an immunological response involving TLR4/ERK signalling pathway at the highest 579 concentration. (A) Representative immunoblotting shows results for the selected set of proteins in cells exposed to PVP-580 AuNPs (1 and 10  $\mu$ g mL<sup>-1</sup>) for 24 h. (B) Histograms representing the means ±SE of five replicates after normalisation 581 with actin. Protein levels are expressed in arbitrary units as fold increase or decrease compared to controls that are set to 582 1. (C) Fluorescent imaging of phagocytes exposed to PVP-AuNPs (10  $\mu$ g mL<sup>-1</sup>) and control cells after 24 h. Cells are 583 evaluated with TLR4 and tubulin antibodies. Immune cell nuclei are labelled with Hoechst 33342 (blue colour). Scale bar 584 is 10  $\mu$ m.

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586 Overall, results show an astounding level of immunological plasticity, particularly at the 587 higher concentration of particles (Figure 6), in which an efficient ongoing inflammatory state may 588 progress a recovery-phase after three days of exposure (Figure 3). A summary view of the innate 589 immune signalling in the interaction between sea urchin phagocytes and PVP-AuNPs is schematized 590 in Figure 7, which underlines the putative role of each proteins found modulated at 10  $\mu$ g mL<sup>-1</sup> and 591 24 h of exposure. We speculate that the TLR4s activated by the PVP-AuNP-protein complexes, are 592 recruited to specific cell surface domains promoting phagocytosis and related immune signalling 593 cascade. Critically, we note that the complete categorized list of immune related genes in the sea 594 urchin genome sequence include gene sequences that encode all of the proteins analysed here, 595 excepting for IL-6. Failure to identify the IL-6 in the genome was because it was categorized with a 596 set of genes for which no evident homology with human genes or with encoded proteins of similar domain structure identified based on the search criteria detailed in the material and methods from Hibino et al<sup>19</sup>. Two explanations for this outcome may be i) there is a real absence of homologous genes encoding cytokines and chemokines in the echinoid lineage, or more likely ii) that the difficulties of detecting those genes is because they are small and evolve rapidly. Immunoblotting with IL-6 antibody shows a single immuno-reactive band of expected molecular weight only in stimulated cells, indicating that the antibody against human IL-6 is likely to cross-react with sea urchin IL-6. However, other plausible explanations cannot yet be excluded.

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607 Figure 7. Schematic representation of the sea urchin immune signalling putatively involved in the 608 response to PVP-AuNPs at the higher concentration of particles. Upon interaction with the CF, sea urchin 609 extracellular proteins form a complex protein corona on PVP-AuNP surfaces. The TLR4s are recruited to 610 specific cell surface domains to form dense nanoscale clusters because activated by a PVP-AuNP-protein 611 complex (see TLR4 clustering), and promote phagocytosis and related immune signalling cascade. TLR4s 612 initiate downstream signalling pathway by phosphorylation/activation of the p42/44 MAP Kinase (ERK) that 613 leads to the NF-kB activation. The TLR4/NF-kB signalling activation induces the production of the antioxidant 614 enzyme MnSOD, the cytokine IL-6, and the adhesion proteins *Pl*-nectin and *Pl*-galectin-8. The cell i) amplifies 615 their antioxidant activity for protection from the harmful effects of ROS overproduction induced by the 616 particle, ii) releases the *Pl*-galectin-8 for promote cell cytoskeletal re-organisation, attachment, and spreading, 617 and iii) releases the Pl-nectin to take part in corona modelling mediating particle immune recognition. Created 618 with BioRender.com. 619

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### 621 4. CONCLUSIONS

622 Since the recognition by Metchnikoff of the biological role of echinoderm phagocytic cells in 623 immunity<sup>72</sup> phagocytosis has been understood to function in all animals and is considered a key 624 cellular mechanism in the resolution of inflammation and in activating signals that suppress the release of proinflammatory cytokines<sup>73</sup>. By studying the interactions between sea urchin phagocytes 625 and PVP-AuNP in vitro we learned about the conserved mechanism of immune defence elicited by 626 627 the gold particles at the nanoscale level of immune-machinery. The relationship between PVP-AuNP and the sea urchin phagocytes is established by the binding of a few extracellular proteins existing in 628 629 the CF to the particle surface. Protein-particle interactions trigger an extended network of immune-630 related signalling in the phagocytes resulting in a transient immune activation both at the lower and 631 higher concentration of the particles.

632 Functionalised particle surface with the PVP capping agent shows considerable particle stability in pure water and in CCM, confirming a strong steric stabilisation nature of this particle. The 633 634 unexpected finding was that PVP does not favour hetero-aggregation in sea urchin CF in CCM, which 635 differ from the CCM only in the presence of the biomacromolecules only. Sea urchin extracellular 636 proteins promote a strong particle surface binding affinity but at the same time stimulate 637 aggregation/agglomeration after 24 h of exposure. However, while customised AuNPs do not show 638 expected stability during the exposure phase, our findings highlight the capability of particles to be 639 transiently immunogenic at both these concentrations. Thus, PVP-AuNPs may be considered 640 immunologically safe in organisms because they do not impair the physiological health of sea urchins, 641 preserving the defensive responses, and do not cause a pathologically chronic inflammatory response 642 with associated irreversible damage. Our results suggest that nano-immune studies in nonmammalian models that are proxy for humans, should be implemented and designed going over of 643 644 apparent problems to successfully translate results to human immunity<sup>74</sup>.

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### 646 **AUTHOR INFORMATION**

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# 648 Author Contributions649

Andi Alijagic: investigation, validation, formal analysis Francesco Barbero: validation, formal analysis
Daniela Gaglio: conceptualisation, methodology Elisabetta Napodano: conceptualisation, methodology.
Oldřich Benada: investigation Olga Kofroňová: investigation. Victor F. Puntes: methodology, funding
acquisition Neus G. Bastús: methodology Annalisa Pinsino: conceptualisation, data curation, supervision,
writing-original draft, funding acquisition, project administration.

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660 Notes

- 661 The authors declare no competing financial interest.
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### 671 **REFERENCES**

- 672 1. Zhang, J., Mou, L., & Jiang, X. (2020). Surface chemistry of gold nanoparticles for health-related 673 applications. *Chemical Science*.
- 674 2. Farooq, M. U., Novosad, V., Rozhkova, E. A., Wali, H., Ali, A., Fateh, A. A., Neogi, P. B., Neogi, A., &
- Wang, Z. (2018). Gold nanoparticles-enabled efficient dual delivery of anticancer therapeutics to HeLa cells.
   *Scientific reports*, 8(1), 1-12.
- 3. Nowack, B., Ranville, J. F., Diamond, S., Gallego-Urrea, J. A., Metcalfe, C., Rose, J., Horne, N., Koelmans,
  A. A., & Klaine, S. J. (2012). Potential scenarios for nanomaterial release and subsequent alteration in the
  environment. *Environmental toxicology and Chemistry*, 31(1), 50-59.
- 4. López-Lorente, Á. I., & Valcárcel, M. (2014). Determination of Gold Nanoparticles in Biological,
  Environmental, and Agrifood Samples. In *Comprehensive Analytical Chemistry* (Vol. 66, pp. 395-426).
  Elsevier.
- 5. Boraschi, D., Italiani, P., Palomba, R., Decuzzi, P., Duschl, A., Fadeel, B., & Moghimi, S. M. (2017,
  December). Nanoparticles and innate immunity: new perspectives on host defence. In *Seminars in immunology*(Vol. 34, pp. 33-51). Academic Press.
- 686 6. Dykman, L. A., & Khlebtsov, N. G. (2017). Immunological properties of gold nanoparticles. *Chemical* 687 *science*, 8(3), 1719-1735.
- 7. Shukla, R., Bansal, V., Chaudhary, M., Basu, A., Bhonde, R. R., & Sastry, M. (2005). Biocompatibility of
  gold nanoparticles and their endocytotic fate inside the cellular compartment: a microscopic overview. *Langmuir*, 21(23), 10644-10654.
- 691 8. Yen, H. J., Hsu, S. H., & Tsai, C. L. (2009). Cytotoxicity and immunological response of gold and silver 692 nanoparticles of different sizes. *Small*, 5(13), 1553-1561.
- 693 9. Sumbayev, V. V., Yasinska, I. M., Garcia, C. P., Gilliland, D., Lall, G. S., Gibbs, B. F., Bonsall, D. R.,
  694 Varani, L., Rossi, F., & Calzolai, L. (2013). Gold nanoparticles downregulate interleukin-1β-induced pro695 inflammatory responses. *Small*, 9(3), 472-477.
- 696 10. le Guével, X., Palomares, F., Torres, M. J., Blanca, M., Fernandez, T. D., & Mayorga, C. (2015).
  697 Nanoparticle size influences the proliferative responses of lymphocyte subpopulations. *RSC Advances*, 5(104),
  698 85305-85309.
- 11. Mahl, D., Greulich, C., Meyer-Zaika, W., Köller, M., & Epple, M. (2010). Gold nanoparticles:
  dispersibility in biological media and cell-biological effect. *Journal of Materials Chemistry*, 20(29), 61766181.
- Tran, T. H., & Amiji, M. M. (2015). Targeted delivery systems for biological therapies of inflammatory
   diseases. *Expert opinion on drug delivery*, 12(3), 393-414.

- 13. Riediker, M., Zink, D., Kreyling, W., Oberdörster, G., Elder, A., Graham, U., Lynch, I., Duschl, A., Ichihara, G., Ichihara, S., & Kobayashi, T. (2019). Particle toxicology and health-where are we?. *Particle and*
- 706 *fibre toxicology*, 16(1), 19.
- 14. Herrmann, K., Pistollato, F., & Stephens, M. L. (2019). Beyond the 3Rs: Expanding the use of humanrelevant replacement methods in biomedical research. *ALTEX* 36(3), 343-352.
- 709 15. Falugi, C., Aluigi, M. G., Chiantore, M. C., Privitera, D., Ramoino, P., Gatti, M. A., Fabrizi, A., Pinsino,
  710 A., & Matranga, V. (2012). Toxicity of metal oxide nanoparticles in immune cells of the sea urchin. *Marine*711 *environmental research*, 76, 114-121.
- 712 16. Pinsino, A., Russo, R., Bonaventura, R., Brunelli, A., Marcomini, A., & Matranga, V. (2015). Titanium
  713 dioxide nanoparticles stimulate sea urchin immune cell phagocytic activity involving TLR/p38 MAPK714 mediated signalling pathway. *Scientific reports*, 5, 14492.
- 715 17. Alijagic, A., Benada, O., Kofroňová, O., Cigna, D., & Pinsino, A. (2019). Sea urchin extracellular proteins
  716 design a complex protein corona on titanium dioxide nanoparticle surface influencing immune cell behaviour.
  717 *Frontiers in Immunology*, 10, 2261.
- 18. Alijagic, A., Gaglio, D., Napodano, E., Russo, R., Costa, C., Benada, O., Kofroňová, O., & Pinsino, A.
  (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.
- 19. Hibino, T., Loza-Coll, M., Messier, C., Majeske, A. J., Cohen, A. H., Terwilliger, D. P., Buckley, K. M.,
- Brockton, V., Nair, S. V., Berney, K., & Fugmann, S. D. (2006). The immune gene repertoire encoded in the
  purple sea urchin genome. *Developmental biology*, 300(1), 349-365.
- 20. Bastús, N. G., Comenge, J., & Puntes, V. (2011). Kinetically controlled seeded growth synthesis of citratestabilized gold nanoparticles of up to 200 nm: size focusing versus Ostwald ripening. *Langmuir*, 27(17),
  11098-11105.
- 21. Henson, J. H., Svitkina, T. M., Burns, A. R., Hughes, H. E., MacPartland, K. J., Nazarian, R., & Borisy,
  G. G. (1999). Two components of actin-based retrograde flow in sea urchin coelomocytes. *Molecular biology*of the cell, 10(12), 4075-4090.
- Pinsino, A., & Alijagic, A. (2019). Sea urchin *Paracentrotus lividus* immune cells in culture: formulation
  of the appropriate harvesting and culture media and maintenance conditions. *Biology open*, 8(3), bio039289.
- Pamies, R., Cifre, J. G. H., Espín, V. F., Collado-González, M., Baños, F. G. D., & de la Torre, J. G. (2014).
  Aggregation behaviour of gold nanoparticles in saline aqueous media. *Journal of nanoparticle research*, 16(4),
  2376.
- 24. Barreto, Â., Luis, L. G., Girão, A. V., Trindade, T., Soares, A. M., & Oliveira, M. (2015). Behavior of
  colloidal gold nanoparticles in different ionic strength media. *Journal of Nanoparticle Research*, 17(12), 493.
- 738 25. Tomaszewska, E., Soliwoda, K., Kadziola, K., Tkacz-Szczesna, B., Celichowski, G., Cichomski, M.,
  739 Szmaja, W., & Grobelny, J. (2013). Detection limits of DLS and UV-Vis spectroscopy in characterization of
  740 polydisperse nanoparticles colloids. *Journal of Nanomaterials*, 2013.
- 741 26. Ritchie, N. W. (2011). Getting Started with NIST\* DTSA-II. *Microscopy Today*, 19(1), 26-31.
- 742 27. Newbury, D. E., & Ritchie, N. W. (2013). Is scanning electron microscopy/energy dispersive X-ray
  743 spectrometry (SEM/EDS) quantitative?. *Scanning*, *35*(3), 141-168.
- Pinsino, A., Roccheri, M. C., & Matranga, V. (2014). Manganese overload affects p38 MAPK
  phosphorylation and metalloproteinase activity during sea urchin embryonic development. *Marine environmental research*, 93, 64-69.

- Pinsino, A., Thorndyke, M., & Matranga, V. (2007). Coelomocytes and post-traumatic response in the
  common sea star Asterias rubens. *Cell Stress & Chaperones*, *12*, 331-341.
- 30. Zito, F., Nakano, E., Sciarrino, S., & Matranga, V. (2000). Regulative specification of ectoderm in skeleton
   disrupted sea urchin embryos treated with monoclonal antibody to Pl-nectin. *Development, growth & differentiation*, 42(5), 499-506.
- 31. Karakostis, K., Costa, C., Zito, F., & Matranga, V. (2015). Heterologous expression of newly identified
  galectin-8 from sea urchin embryos produces recombinant protein with lactose binding specificity and antiadhesive activity. *Scientific reports*, *5*, 17665.
- 755 32. Jeevanandam, J., Barhoum, A., Chan, Y. S., Dufresne, A., & Danquah, M. K. (2018). Review on 756 nanoparticles and nanostructured materials: history, sources, toxicity and regulations. *Beilstein journal of* 757 *nanotechnology*, 9(1), 1050-1074.
- 33. Slomberg, D. L., Ollivier, P., Miche, H., Angeletti, B., Bruchet, A., Philibert, M., Brant, J., & Labille, J.
  (2019). Nanoparticle stability in lake water shaped by natural organic matter properties and presence of
  particulate matter. *Science of The Total Environment*, 656, 338-346.
- 34. Cohen, Y., Rallo, R., Liu, R., & Liu, H. H. (2013). In silico analysis of nanomaterials hazard and risk.
   *Accounts of chemical research*, 46(3), 802-812.
- 763 35. Cosgrove, T. (Ed.). (2010). Colloid science: principles, methods and applications. John Wiley & Sons.
- 36. Koczkur, K. M., Mourdikoudis, S., Polavarapu, L., & Skrabalak, S. E. (2015). Polyvinylpyrrolidone (PVP)
  in nanoparticle synthesis. *Dalton Transactions*, 44(41), 17883-17905.
- 37. Tejamaya, M., Römer, I., Merrifield, R. C., & Lead, J. R. (2012). Stability of citrate, PVP, and PEG coated
   silver nanoparticles in ecotoxicology media. *Environmental science & technology*, 46(13), 7011-7017.
- 38. Guerrini, L., Alvarez-Puebla, R. A., & Pazos-Perez, N. (2018). Surface modifications of nanoparticles for
   stability in biological fluids. *Materials*, 11(7), 1154.
- 770 39. Labille, J., Harns, C., Bottero, J. Y., & Brant, J. (2015). Heteroaggregation of titanium dioxide 771 nanoparticles with natural clay colloids. *Environmental science & technology*, *49*(11), 6608-6616.
- 40. Rostek, A., Mahl, D., & Epple, M. (2011). Chemical composition of surface-functionalized gold
  nanoparticles. *Journal of Nanoparticle Research*, 13(10), 4809-4814.
- 41. Barbero, F., Moriones, O. H., Bastús, N. G., & Puntes, V. (2019). Dynamic Equilibrium in the Cetyltrimethylammonium Bromide–Au Nanoparticle Bilayer, and the Consequent Impact on the Formation of the Nanoparticle Protein Corona. *Bioconjugate Chemistry*, *30*(11), 2917-2930.
- 42. Su, K. H., Wei, Q. H., Zhang, X., Mock, J. J., Smith, D. R., & Schultz, S. (2003). Interparticle coupling effects on plasmon resonances of nanogold particles. *Nano letters*, *3*(8), 1087-1090.
- 43. Park, J., Park, J. H., Ock, K. S., Ganbold, E. O., Song, N. W., Cho, K., Lee, S. Y., & Joo, S. W. (2011).
  Preferential adsorption of fetal bovine serum on bare and aromatic thiol-functionalized gold surfaces in cell culture media. *Journal of colloid and interface science*, *363*(1), 105-113.
- 44. Podila, R., Chen, R., Ke, P. C., Brown, J. M., & Rao, A. M. (2012). Effects of surface functional groups
  on the formation of nanoparticle-protein corona. *Applied physics letters*, 101(26), 263701.
- 45. Lu, X., Xu, P., Ding, H. M., Yu, Y. S., Huo, D., & Ma, Y. Q. (2019). Tailoring the component of protein
  corona via simple chemistry. *Nature communications*, 10(1), 1-14.
- 46. Pinsino, A., & Matranga, V. (2015). Sea urchin immune cells as sentinels of environmental stress. *Developmental & Comparative Immunology*, 49(1), 198-205.

- 788 47. Smith, L. C., Arizza, V., Hudgell, M. A. B., Barone, G., Bodnar, A. G., Buckley, K. M., Cunsolo, V.,
- Dheilly, N. M., Franchi, N., Fugmann, S. D., Furukawa, R., Garcia-Arraras, J., Henson, J. H., Hibino, T., Irons,
  Z. H., Li, C., Man Lun, C., Majeske, A. J., Oren, M., Pagliara, P., Pinsino, A., Raftos, D. A., Rast, J. P., Samasa,
- B., Schillaci, D., Schrankel, C. S., Stabili, L., Stensväg, K., & Sutton, E. (2018). Echinodermata: the complex
- immune system in echinoderms. In: Cooper, E.L. (Ed.), *Advances in Comparative Immunology*. Springer
- 793 International Publishing AG, pp. 409–501.
- 48. Goldstein, A., Soroka, Y., Frušić-Zlotkin, M., Popov, I., & Kohen, R. (2014). High resolution SEM imaging of gold nanoparticles in cells and tissues. *Journal of microscopy*, *256*(3), 237-247.
- 49. Mironava, T., Hadjiargyrou, M., Simon, M., Jurukovski, V., & Rafailovich, M. H. (2010). Gold
  nanoparticles cellular toxicity and recovery: effect of size, concentration and exposure
  time. *Nanotoxicology*, 4(1), 120-137.
- 50. Melillo, D., Marino, R., Italiani, P., & Boraschi, D. (2018). Innate immune memory in invertebrate metazoans: a critical appraisal. *Frontiers in immunology*, 9, 1915.
- 51. Ding, L., Yao, C., Yin, X., Li, C., Huang, Y., Wu, M., Wang, B., Guo, X., Wang, Y., & Wu, M. (2018).
  Size, shape, and protein corona determine cellular uptake and removal mechanisms of gold nanoparticles. *Small*, 14(42), 1801451.
- 52. Chen, F., Wang, G., Griffin, J. I., Brenneman, B., Banda, N. K., Holers, V. M., Backos, D. S., Wu, L.,
  Moghimi, S. M., & Simberg, D. (2017). Complement proteins bind to nanoparticle protein corona and undergo
  dynamic exchange in vivo. *Nature nanotechnology*, *12*(4), 387.
- 53. Zhang, S., Bories, G., Lantz, C., Emmons, R., Becker, A., Liu, E., Abecassis, M. M., Yvan-Charvet, L., &
  Thorp, E. B. (2019). Immunometabolism of phagocytes and relationships to cardiac repair. *Frontiers in cardiovascular medicine*, 6.
- 54. Liaudet, L., Mabley, J. G., Pacher, P., Virág, L., Soriano, F. G., Marton, A., Haskó, G., Deitch, E. A., &
  Szabó, C. (2002). Inosine exerts a broad range of antiinflammatory effects in a murine model of acute lung
  injury. *Annals of surgery*, 235(4), 568.
- 55. Minhas, P. S., Liu, L., Moon, P. K., Joshi, A. U., Dove, C., Mhatre, S., Contrepois, K., Wang, Q., Lee, B.
  A., Coronado, M., & Bernstein, D. (2019). Macrophage de novo NAD+ synthesis specifies immune function
- 815 in aging and inflammation. *Nature immunology*, 20(1), 50-63.
- 56. Venter, G., Oerlemans, F. T., Willemse, M., Wijers, M., Fransen, J. A., & Wieringa, B. (2014). NAMPTmediated salvage synthesis of NAD+ controls morphofunctional changes of macrophages. *PLoS One*, 9(5).
- 57. Cui, T., Cang, H., Yang, B., & He, Z. G. (2019). Cyclic dimeric guanosine monophosphate: activation and
  inhibition of innate immune response. *Journal of innate immunity*, *11*(3), 242-248.
- 820 58. Lane, A. N., & Fan, T. W. M. (2015). Regulation of mammalian nucleotide metabolism and 821 biosynthesis. *Nucleic acids research*, 43(4), 2466-2485.
- 59. Varki, A., & Gagneux, P. (2012). Multifarious roles of sialic acids in immunity. *Annals of the New York Academy of Sciences*, 1253(1), 16.
- 60. Lübbers, J., Rodríguez, E., & Van Kooyk, Y. (2018). Modulation of immune tolerance via Siglec-sialic
  acid interactions. *Frontiers in Immunology*, *9*, 2807.
- 826 61. Collins, L. E., & Troeberg, L. (2019). Heparan sulfate as a regulator of inflammation and 827 immunity. *Journal of leukocyte biology*, *105*(1), 81-92.
- 828 62. Zhang, D., Liu, D., Zhang, J., Fong, C., & Yang, M. (2014). Gold nanoparticles stimulate differentiation
- and mineralization of primary osteoblasts through the ERK/MAPK signaling pathway. *Materials Science and Engineering: C, 42, 70-77.*

- 63. Płóciennikowska, A., Hromada-Judycka, A., Borzęcka, K., & Kwiatkowska, K. (2015). Co-operation of
  TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. *Cellular and molecular life sciences*,
  72(3), 557-581.
- 64. Kawai, T., & Akira, S. (2007). Signaling to NF-κB by Toll-like receptors. *Trends in molecular medicine*, 13(11), 460-469.
- 65. Lockyer, J.M., Colladay, J.S., Alperin-Lea, W.L., Hammond, T., & Buda A.J. (1998). Inhibition of nuclear
  factor-κb-mediated adhesion molecule expression in human endothelial cells *Circulation Research*, *82*, *314 320*.
- 66. Yuan, S., Liu, X., Zhu, X., Qu, Z., Gong, Z., Li, J., Xiao, L., Yang, Y., Liu, H., Sun, L., & Liu F. (2018).
  The role of TLR4 on PGC-1α-mediated oxidative stress in tubular cell in diabetic kidney disease. *Oxidative*
- 841 *Medicine and Cellular Longevity*, 2018, 6296802.

67. Abdal Dayem, A., Hossain, M. K., Lee, S. B., Kim, K., Saha, S. K., Yang, G. M., Choi, H. Y., & Cho, S.
67. Abdal Dayem, A., Hossain, M. K., Lee, S. B., Kim, K., Saha, S. K., Yang, G. M., Choi, H. Y., & Cho, S.
67. Abdal Dayem, A., Hossain, M. K., Lee, S. B., Kim, K., Saha, S. K., Yang, G. M., Choi, H. Y., & Cho, S.
67. Abdal Dayem, A., Hossain, M. K., Lee, S. B., Kim, K., Saha, S. K., Yang, G. M., Choi, H. Y., & Cho, S.
67. Abdal Dayem, A., Hossain, M. K., Lee, S. B., Kim, K., Saha, S. K., Yang, G. M., Choi, H. Y., & Cho, S.
67. (2017). The role of reactive oxygen species (ROS) in the biological activities of metallic nanoparticles. *International journal of molecular sciences*, 18(1), 120.

- 845 68. Harjunpää, H., Llort Asens, M., Guenther, C., & Fagerholm, S. C. (2019). Cell adhesion molecules and 846 their roles and regulation in the immune and tumor microenvironment. *Frontiers in Immunology*, 10, 1078.
- 69. Levy, Y., Arbel-Goren, R., Hadari, Y. R., Eshhar, S., Ronen, D., Elhanany, E., Geiger, B., & Zick, Y.
  (2001). Galectin-8 functions as a matricellular modulator of cell adhesion. *Journal of Biological Chemistry*, 276(33), 31285-31295.
- 70. Carabelli, J., Quattrocchi, V., D'Antuono, A., Zamorano, P., Tribulatti, M. V., & Campetella, O. (2017).
  Galectin-8 activates dendritic cells and stimulates antigen-specific immune response elicitation. *Journal of leukocyte biology*, *102*(5), 1237-1247.
- Receptors (P1001).
   853 71. Gao, G. (2013). Nectin and nectin-like molecules: immune regulator, adhesion molecule and virus
   Receptors (P1001).
- 855 72. Gordon, S. (2016). Phagocytosis: the legacy of Metchnikoff. *Cell*, 166(5), 1065-1068.
- 73. Maderna, P., & Godson, C. (2003). Phagocytosis of apoptotic cells and the resolution of inflammation. *Biochimica et Biophysica Acta (BBA) Molecular Basis of Disease, 1639*(3), 141-151.
- 858 74. Boraschi, D., Alijagic, A., Auguste, M., Barbero, F., Ferrari, E., Hernadi, S., Mayall, M., Michelini, S.,
- 859 Navarro Pacheco, N. I., Prinelli, A., Swart, E., Swartzwelter, B. J., Bastús, N. G., Canesi, L., Drobne, D.,
- B60 Duschl, A., Ewart, M., Horejs-Hoeck, J., Italiani, P., Kemmerling, B., Kille, P., Prochazkova, P., Puntes, V.
- 861 F., Spurgeon, D. J., Svendsen, C., Wilde, C. J., & Pinsino, A. (2020). Addressing Nanomaterial Immunosafety
- by Evaluating Innate Immunity across Living Species. *Small*, 2020, 2000598.