Interaction between nanomaterials and the innate immune system across evolution

Diana Boraschi[1,2,3,4,*] ⓘ, Laura Canesi[5], Damjana Drobne[6], Birgit Kemmerling[7], Annalisa Pinsino[8] and Petra Prochazkova[9]

[1]*Shenzhen Institute of Advanced Technology (SIAT), Chinese Academy of Science (CAS), 1068 Xueyuan Blvd, 518071, Shenzhen, China*
[2]*Institute of Protein Biochemistry and Cell Biology (IBBC), CNR, Via Pietro Castellino 111, 80131, Naples, Italy*
[3]*Stazione Zoologica Anton Dohrn (SZN), Villa Comunale, 80132, Napoli, Italy*
[4]*China-Italy Joint Laboratory of Pharmacobiotechnology for Medical Immunomodulation (SIAT, CNR, SZN), Napoli, Italy*
[5]*Department of Earth, Environment and Life Sciences, University of Genova, Corso Europa 26, 16132, Genova, Italy*
[6]*Biotechnical Faculty, University of Ljubljana, Jamnikarjeva ulica 101, 1000, Ljubljana, Slovenia*
[7]*ZMBP - Center for Plant Molecular Biology, Plant Biochemistry, University of Tübingen, Auf der Morgenstelle 32, 72076, Tübingen, Germany*
[8]*Institute of Translational Pharmacology (IFT), National Council (CNR), Via Ugo La Malfa 153, 90146, Palermo, Italy*
[9]*Laboratory of Cellular and Molecular Immunology, Institute of Microbiology of the Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20, Prague, Czech Republic*

ABSTRACT

Interaction of engineered nanomaterials (ENMs) with the immune system mainly occurs with cells and molecules of innate immunity, which are present in interface tissues of living organisms. Immuno-nanotoxicological studies aim at understanding if and when such interaction is inconsequential or may cause irreparable damage. Since innate immunity is the first line of immune reactivity towards exogenous agents and is highly conserved throughout evolution, this review focuses on the major effector cells of innate immunity, the phagocytes, and their major sensing receptors, Toll-like receptors (TLRs), for assessing the modes of successful *versus* pathological interaction between ENMs and host defences. By comparing the phagocyte- and TLR-dependent responses to ENMs in plants, molluscs, annelids, crustaceans, echinoderms and mammals, we aim to highlight common recognition and elimination mechanisms and the general sufficiency of innate immunity for maintaining tissue integrity and homeostasis.

Key words: innate immunity, Toll-like receptors, phagocytes, plants, invertebrates, human, *in vivo*, *in vitro*.

CONTENTS

* Author for correspondence (Tel.: +86 133 02938464; E-mail: diana.boraschi@ibbc.cnr.it).

I. INTRODUCTION

The development of nanotechnologies and the consequent introduction of new man-made nano-entities into our environment has raised attention and concern about the possible detrimental effects of engineered nanomaterials (ENMs) on human and environmental health. Extensive evaluation has been implemented to understand the effects of the interaction of ENMs with biological systems (Oberdörster, Oberdörster & Oberdörster, 2005; Krug & Wick, 2011; Fadeel, 2012, 2019; Fadeel *et al.*, 2018; Zhu, 2020; Cronin *et al.*, 2020; Boraschi *et al.*, 2020).

Living organisms are endowed with powerful protective mechanisms, developed throughout evolution for counteracting invading pathogens (e.g. viruses, bacteria) and for eliminating threatening exogenous and endogenous entities (mechanical stress, dust, allergens, anomalous, senescent and dead cells). We can therefore expect that those mechanisms may be involved in the recognition and response to ENMs. Notably, ENMs may resemble some infectious agents in terms of size and shape, but their interaction with living organisms is passive, resembling that with dust. Thus, to understand the possible detrimental effects of ENMs, it is important to examine the features of their interaction with such protective mechanisms and assess whether and how ENMs may escape recognition or circumvent protective responses.

The protective mechanisms activated upon contact with invading agents are mainly those of immunity. We can define them as innate immune mechanisms, although this definition is only necessary for vertebrates, which also have another type of immunity (adaptive immunity). Innate immunity is the only type present in over 95% of living organisms and in vertebrates is estimated to account for about 80–90% of protective efficacy (R. Zinkernagel, personal communication; Murphy, Weaver & Berg, 2022; Chumakov *et al.*, 2021). Innate protection (physical, chemical and cellular barriers) prevents invasion by the majority of exogenous agents (about 97%), with the tissue-resident innate immune mechanisms subsequently tackling and eliminating (about 90%) those that successfully invade the body (Chumakov *et al.*, 2021). Innate immunity shows several evolutionarily conserved molecular and cellular features that are present with little variation from plants to mammals. We focus here

on two of these, the main defensive cells, i.e. phagocytes, and the main danger-sensing molecules, i.e. the pattern-recognition receptors (PRRs). Phagocytic cells are widespread across the tree of life, from unicellular organisms such as amoebae, with the capacity to engulf and digest exogenous material for nutrition, to specialised cells in multicellular animals that are involved in feeding and elimination of unwanted materials. Apart from plants, whose cells are not mobile, phagocytes are present in all multicellular eukaryotic organisms and can be found both as circulating cells and as tissue-resident sentinels, in particular in barrier tissues at the interface between the body and the external environment (Mowat, Scott & Bain, 2017). Thus, it can be assumed that interaction of ENMs with phagocytic cells is the first and primary interaction to occur. When a foreign agent comes into contact with phagocytes (or other cells in an organism), these cells have mechanisms for sensing and recognition, and for removal or destruction. PRRs are usually surface molecules, but can also be soluble, such as the complement components C1q and mannose-binding lectin (MBL) that perform this sensing function linked to an active downstream defensive/adaptation reaction. Notably, PRRs seem able to perceive different kinds of threats, including changes to environmental conditions (e.g. temperature, pH, salinity) that could hamper the organism's homeostasis, and can trigger appropriate adaptive responses. PRRs encompass a wide range of receptors that recognise sugars, nucleotides, glycoproteins, glycolipids and structural patterns typical of microorganisms (usually viruses and bacteria) although they are unable to recognise endogenous patterns unless damaged (Murphy *et al.*, 2022). Toll-like receptors (TLRs) are the best-known PRRs and are present in all multicellular organisms (homologous sequences have also been identified in bacteria).

In this review, we examine the interaction between ENMs and innate immunity by focusing on phagocytic cells and TLRs to understand whether and when such interaction results in successful removal of ENMs and under which circumstances it leads to detrimental consequences. We examine immune interactions in plants, invertebrates (molluscs, annelids, crustaceans, echinoderms) and vertebrates (with a focus on humans).

Notably, ENM physico-chemical characteristics are expected to play a major role in determining the mechanisms

of interaction with and uptake by phagocytes. However, after many years of research, no clear correlation between ENM characteristics and mechanisms of uptake has been identified that can allow us to predict the outcome of such interactions. The hope of 'safe by design' nanotechnological products is still unfulfilled. It is known that uptake depends on particle size, shape, stiffness and surface charge, among other characteristics, but the physiology of organisms remains the decisive parameter. The functional anatomy of the gut makes it a formidable barrier to ENMs. Some organisms, especially vertebrates, use mucus to keep adsorbed nanomaterial in the gut lumen and prevent it from interaction with epithelial cells. Invertebrates also have protective layers of cuticle or peritrophic membranes in the gut, which affect ENM uptake (Van der Zande *et al.*, 2020). Phagocytosis occurs only when ENMs are able to cross such barriers and reach the cells lining the gut. In organisms in which phagocytosis is mainly a defence mechanism and preserves tissue integrity, phagocytosed particles are directed towards destruction or, if indigestible, towards 'strenuous' isolation and seclusion from the rest of the body, as shown for example by tattoos (Baranska *et al.*, 2018). In addition, the health condition of the organism can affect the outcome of the interaction with ENMs, as well as the route of exposure and the type of affected cells.

II. IMMUNITY AND NANO-IMMUNE INTERACTIONS IN PLANTS

In plants, immunity does not rely on specialised tissues or organs but it is an autonomous process that can be activated in all cells. Innate immune receptors reside on the plasma membrane and intracellularly in the cytoplasm in all tissues and can recognise non-self structures on the surface (pattern-triggered immunity) and within the cell (effector-triggered immunity) (Zhou & Zhang, 2020; Ngou, Ding & Jones, 2022). Many cell surface receptors share similar features with TLRs, such as the extracellular leucine-rich domains. These receptors likely evolved independently in the plant and animal kingdoms and recognise pathogen-associated molecular patterns (PAMPs), but also other non-self and host-specific patterns that indicate hazards. The transmembrane domain transfers the signal into the plant cell and activates immune responses *via* intramolecular kinase domains in receptor kinases (complexes), which belong to the same phylogenetic family as the IL-1 receptor-associated kinases (IRAKs) in animals (Nürnberger *et al.*, 2004). Intracellular nucleotide oligomerisation domain (NOD) like receptors (NLRs) display a Toll-IL-1 receptor (TIR) domain similar to the TLR intracellular domain and can recognise intracellular effector molecules introduced by potential pathogens (Han, 2019). ENMs come into contact with plants mostly extracellularly, but some studies also report transport within the plant and into the cells (see Section II.1). Thus, in principle EMNs could contact both types of receptors.

Phytocytokines, endogenous peptides produced in response to stress, can amplify stress-adaptation reactions and alert neighbouring cells, similar to the role of animal cytokines (Gust, Pruitt & Nürnberger, 2017). Animal innate immune memory also has a plant counterpart in systemic acquired resistance (SAR). Primary local infections with a pathogen prime the plant not only at the infection site but also systemically, making the whole plant more resistant to a broad spectrum of subsequent infections, and suggesting that local ENM recognition could activate the defence repertoires within the entire plant (Vlot *et al.*, 2021). As in animals, immune responses in plants include the production of reactive oxygen species (ROS) and changes to the global transcriptome and proteome, enabling effective defence against the vast majority of microorganisms in the environment (Yu *et al.*, 2017).

(1) Uptake of ENMs

Plants do not have specialised phagocytic cells, instead particle uptake can result from interactions with all cells. The uptake of ENMs in plants is a controversial topic (Ali, Mehmood & Khan, 2021). In general, ENM uptake is prevented by physical barriers such as the cuticle, cell wall and the plasma membrane, which protect the plant from the entry of non-self-particles (Schwab *et al.*, 2016). Entry of ENMs into the plant cell can potentially occur *via* pores, channels or by endocytosis (Ali *et al.*, 2021). In aerial tissues, ENMs may be taken up through natural openings. In roots, they may enter the plant *via* cell wall pores and then travel apo- or symplastically. The cell wall pores are estimated to be 3–6 nm in diameter, thereby precluding the entry of larger molecules (Sabo-Attwood *et al.*, 2012). In the case of gold nanoparticles (AuNPs), 4 and 12 nm particles are blocked within the cell wall and cannot be taken up by *Arabidopsis thaliana* roots (Ferrari, 2022). Nevertheless, in several plant species uptake was reported for several ENMs larger than the cell wall pore size, suggesting that cell wall alterations, flexibility or engagement of active uptake processes can allow particle passage (Ali *et al.*, 2021). Small positively charged AuNPs were taken up more effectively than neutral or negatively charged larger particles (Spielman-Sun *et al.*, 2019; Ali *et al.*, 2021). Thus, depending on the species, ENM dose, size and surface charge, many types of nanomaterials have been shown to accumulate in roots (the majority) or shoots after soil or hydroponic exposure (Kranjc *et al.*, 2018). These include Ag particles, CuO, TiO_2, SiO_2, NiO, La_2O_3, ZnO and several others (Kranjc & Drobne, 2019). Some remain intact after uptake and are accumulated, while others undergo transformation either before adsorption through the roots or after uptake as nanoparticles (NPs) (Ali *et al.*, 2021). For example, in the soya bean *Glycine max*, CeO_2 NPs remain in this form after translocation from the soil, whereas ZnO particles are transformed into Zn-citrate within the plant tissues (Hernandez-Viezcas *et al.*, 2013). In maize *Zea mays*, they are taken up as Zn phosphate or Zn ions (Lv *et al.*, 2015). In general, uptake is often reported for

unstable NPs that can dissolve in the environment, suggesting that extracellular particle disintegration, uptake of ions and subsequent intracellular formation of new particles occurs rather than uptake of intact NPs (Taylor *et al.*, 2014). Thus, uptake mechanisms remain poorly understood and therefore unpredictable, and generalisations cannot be made on the nature of ENMs taken up, the plant species in which this occurs, or the conditions under which internalisation takes place.

(2) AuNP effects on immune responses

Caution is needed to interpret the toxic effects of ENMs on plants because, in some cases, the bulk material can be intrinsically toxic and, in other cases, the coatings can be toxic (Barrena *et al.*, 2009; Ferrari *et al.*, 2021). AuNPs are an example of a nanomaterial that is generally considered safe and inert, i.e. unable to cause direct toxic effects, and are therefore useful for identifying potential effects of the nanoparticulate form on plant immunity. Different and sometimes controversial effects of AuNPs on plants have been described, with plant responses to AuNPs depending on specific conditions and specific interactions (Siddiqi & Husen, 2016; Zia-ur-Rehman *et al.*, 2018; Sanzari, Leone & Ambrosone, 2019). Several studies show that there is no uptake of AuNPs (Taylor *et al.*, 2014; Zhang *et al.*, 2022; Ferrari, 2022). Any effects caused by AuNPs are therefore likely not caused by direct activation of receptors but are a consequence of other AuNP actions, e.g. clogging pores, or scavenging of environmental factors or PAMPs that induce defence responses (Barrena *et al.*, 2009; Asli & Neumann, 2009; Feichtmeier, Walther & Leopold, 2015; Ferrari *et al.*, 2021). In many studies no effects were detected using different assays and concentrations (Pacheco & Buzea, 2018). Negative results also may be underrepresented in the published literature because studies reporting no effect are often not published. At very high concentrations (≥100 mg/l) AuNPs have been reported to have detrimental effects on plants (Siegel *et al.*, 2018), with smaller particles (diameter < 5 nm) showing higher toxicity. This size is in the cell wall pore size range, implying that AuNPs may have direct access to plant cell surfaces. By contrast, lower concentrations of AuNPs were reported to enhance seed germination and chlorophyll content and improve growth and productivity in several crops and model plants (Arora *et al.*, 2012; Kumar *et al.*, 2013; Mahakham *et al.*, 2016; Ferrari *et al.*, 2021; Sabo-Attwood *et al.*, 2012).

A limited number of studies have investigated the effects of stable and well-characterised AuNPs on the plant immune system, with most focussed on oxidative burst phenomena. Kumar *et al.* (2013) reported that, in *Arabidopsis thaliana*, plant antioxidant potential under stressful conditions is enhanced by AuNP exposure through the activation of free radical scavenging activity. By contrast, other studies reported increased oxidative stress caused by AuNPs (Gunjan, Zaidi & Sandeep, 2014; Siddiqi & Husen, 2016). It was found that AuNPs at moderate concentrations can increase growth and

reduce PAMP-induced ROS bursts in *Arabidopsis thaliana* (Ferrari *et al.*, 2021), in agreement with similar results in *Brassica* plants (Arora *et al.*, 2012). AuNPs alone did not induce ROS bursts or other defence responses, suggesting that AuNPs do not activate self-destructive immune reactions. At the transcriptome and proteome level, growth and metal response genes overall were upregulated, while immune and oxidative stress-responsive genes/proteins were downregulated (Ferrari *et al.*, 2021). This indicates that the trade-off between growth and immunity could be shifted by AuNPs towards growth. Similar to the effects described for exposure to exogenous AuNPs, it was found that defence and oxidative stress-responsive genes are downregulated after Au ion treatment and intracellular formation of AuNPs (Tiwari *et al.*, 2016).

ENM–plant interactions are clearly very diverse and, based on current knowledge, cannot be predicted or extrapolated. AuNPs and several other ENMs do not appear to exert detrimental effects on *A. thaliana* plants at environmentally relevant concentrations, but rather display antioxidant and growth-promoting activity. A general scheme of the interaction of AuNPs with plants and plant immune mechanisms is provided in Fig. 1.

III. PHAGOCYTES AND TLRS IN MOLLUSC IMMUNITY AND NANOPARTICLE–IMMUNE INTERACTIONS

Phylum Mollusca includes aquatic and terrestrial bivalves, cephalopods, and gastropods. Haemocytes, molluscan immune cells, are generally classified on a morphological basis as hyaline, semigranular or granular. Different molluscan taxa show differences in abundance and function of these subtypes, reflecting environmental and endogenous factors including infection by different microorganisms (protozoans, bacteria, viruses) (Dyachuk, 2016).

Bivalves are sessile suspension-feeders abundant in freshwater and marine ecosystems, where they are constantly exposed to abiotic and biotic stressors. Due to their feeding habit involving gill-mediated water filtration, bivalves have highly developed processes for the uptake of nano- and micro-scale particles (endo- and phagocytosis), integral to physiological functions such as intracellular digestion and cellular immunity. Bivalves have become a target group for investigations of ENM toxicity (Canesi *et al.*, 2012) and to date represent the best studied group of invertebrates regarding NP–immune interactions (Canesi & Corsi, 2016).

(1) Immune effectors in bivalve molluscs

In bivalves, haemocytes are the main defence cells and act through a combination of phagocytosis and the production of humoral defence factors such as agglutinins (e.g. lectins), lysosomal enzymes (e.g. acid phosphatase, lysozyme), ROS and antimicrobial peptides (AMPs) (Canesi *et al.*, 2002). In the mussel *Mytilus galloprovincialis*, different haemocyte
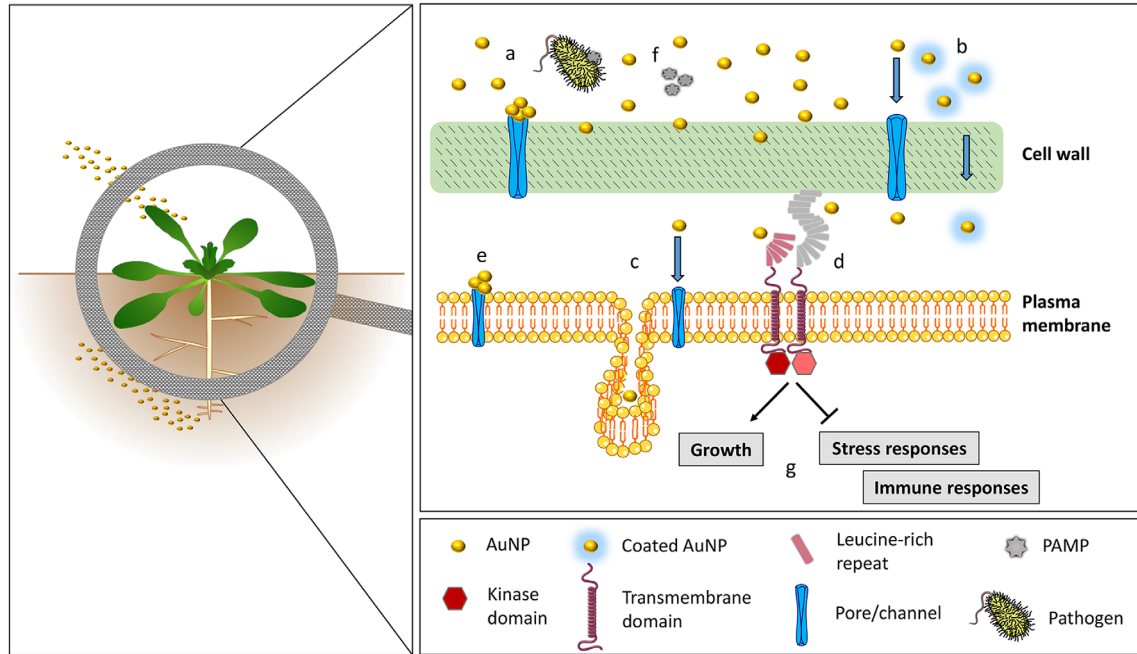
Fig. 1. Interaction of gold nanoparticles (AuNPs) with plants. AuNPs can access plants *via* natural openings in leaves or by uptake through the roots. Cell walls have a pore size of 3–6 nm that prevents access of pathogens and large engineered nanomaterials to the plasma membrane and the uptake machinery (a). AuNPs in nature are coated with a biocorona composed of environmentally adsorbed molecules, which may alter the cell wall barrier capacity and allow passage (b). Particles that cross the cell wall can pass across the plasma membrane through pores, channels or by endocytosis (c). Although uptake was not observed, Ferrari *et al.* (2021) found physiological changes in plants upon AuNP exposure. These might be caused by recognition of AuNPs or their effects by cell surface pattern recognition receptors (d), clogging of the cell wall and/or membrane pores (e), or interference with environmental factors (pathogen-associated molecular patterns, PAMPs) outside the plant (f). Stress responses in general or more specific immune responses might be affected directly or indirectly (g).

subpopulations were identified by cytofluorimetric analysis of size, granularity, phagocytic activity, and ROS and nitric oxide (NO) production capacity (García-García *et al.*, 2008). Representative images of *M. galloprovincialis* haemocytes are shown in Fig. 2.

Several cellular and soluble PRRs play a crucial role in activating the bivalve immune system. These include lectins, peptidoglycan recognition proteins, gram-negative binding proteins, TLRs, scavenger receptors, etc. (Canesi & Pruzzo, 2016; Gerdol *et al.*, 2018). The best studied PRRs
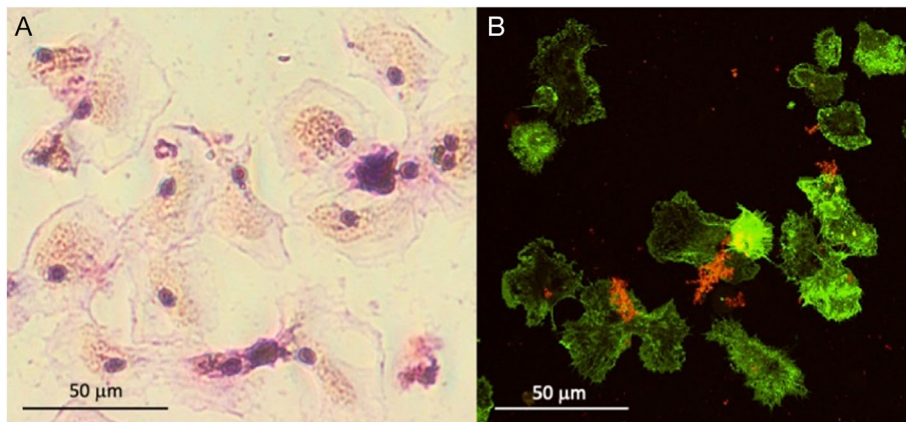
Fig. 2. *Mytilus galloprovincialis* haemocytes. (A) Giemsa staining with lysosomes in granular haemocytes in orange-yellow. (B) Confocal images of haemocytes stained with ActinGreen™ 488 (green) in the presence of 100 µg/ml fluorescent nanopolystyrene beads (100 nm nanoorange, Merck Sigma Aldrich®) (red). Image courtesy of Dr Caterina Ciacci, University of Urbino.

show high versatility and flexibility, although some degree of specificity could be identified (Canesi & Pruzzo, 2016). Compared with more basal phyla, many molluscan genomes exhibit a higher number of TLR-encoding genes; this expansion and divergence may contribute to molluscan immunity and their ability to adapt to stressors (Brennan & Gilmore, 2018). The recent application of single-cell RNA sequencing (scRNA-seq) techniques to bivalve haemocytes allowed identification, in the oyster *Crassostrea hongkongensis*, of 13 different subpopulations of immune cells, which have distinct expression patterns of genes encoding TLRs and other PRRs (Meng, Zhang & Wang, 2022).

Among the soluble factors, AMPs represent one of the main effectors in bivalve immunity. Several AMPs have been identified in different bivalve species. They are generally characterised by structural differences (peptide sequence, length, net charge, per cent hydrophobic residues), different activities and antimicrobial properties, differential tissue/cell expression, accessory functions, and often species-specific biological properties (reviewed in Zannella *et al.*, 2017; Gerdol *et al.*, 2018; Canesi *et al.*, 2022).

(2) Immune interaction with ENMs

A few studies have focused on the effects of ENMs on bivalve haemocyte function and expression of immune-related genes, including PRRs (Canesi, Auguste & Bebiano, 2019). Large variability was observed depending on the type of ENM used, bivalve species, and exposure conditions. In *M. galloprovincialis* exposed to TiO_2 NPs (4 days, 1–100 μg/l), a concentration-dependent decrease in lysosomal membrane stability and phagocytic activity was observed, together with an increase in extracellular ROS and NO production – indicating cellular stress and frustrated phagocytosis – and a general upregulation of messenger RNA (mRNA) levels for different AMPs and lysozyme (Barmo *et al.*, 2013). In *Tegillarca granosa*, prolonged exposure to higher concentrations of TiO_2 NPs (30 days, 10–100 mg/l) significantly reduced total haemocyte number and phagocytosis. Expression of genes encoding PRRs (*TLR1, TLR2, TLR4, TLR5, TLR6, RIG1*) and downstream immune-related molecules (*IRAK4, TRAF2, TRAF3, TRAF6, NFkb1, TRIM58*) was significantly downregulated (Shi *et al.*, 2017).

Most information on interactions between bivalve haemocytes and different types of ENMs has been obtained from *in vitro* studies with freshly isolated or primary cell cultures. In *M. galloprovincialis* haemocytes after short-term exposure to nano-carbon black (NCB) (Canesi *et al.*, 2008), concentration-dependent uptake of NCB was associated with a rapid increase in extracellular ROS production, lysozyme and NO release, and induction of pre-apoptotic processes. The effects were mediated by rapid activation of mitogen-activated protein kinases (MAPKs), which play a key role in immune and inflammatory responses. The results demonstrated that in mussel haemocytes, as in mammalian cells, exposure to ENMs can induce inflammatory processes, and indicate that bivalve immunocytes could represent a suitable model for investigating the effects and modes of action of ENMs in marine invertebrates (Canesi *et al.*, 2008). Several immunotoxic or immunomodulatory effects were observed upon exposure to different types of metal and carbon-based ENMs and nano-oxides in haemocytes from *M. galloprovincialis* and other bivalve species (clams, oysters); however, the effects were dependent on particle type and exposure conditions (reviewed in Canesi *et al.*, 2019). A summary of the effects of ENMs on bivalve haemocytes is reported in Table 1.

(3) Immune interaction with nanoplastics

In the last few years, increasing concern regarding the potential toxicity of nanoplastics (NPLs), mainly resulting from plastic degradation in oceans (Gigault *et al.*, 2018) has led to renewed interest in NP–immune interactions in bivalve haemocytes. In the absence of reliable 'natural' NPLs, studies have relied on the availability of different types of nanopolymers for use as proxies of NPLs, in particular polystyrene nanoparticles (PS-NPs) of different sizes and surface characteristics (e.g. plain or labelled with different fluorochromes, surface modified with cationic and anionic groups). *In vitro*, NPLs can be internalised in the lysosomal compartment of haemocytes and induce a wide range of responses, from lysosomal reactions to decreased phagocytosis and increased production of toxic radicals and apoptosis [see Auguste *et al.* (2021*a*,*b*) and references therein]. Again, the effects were dependent on particle characteristics (size, surface charge) and experimental conditions (exposure media, concentration, exposure time). Interestingly, distinct responses were observed in different haemocyte subpopulations, with large granular haemocytes (fully mature phagocytes), being most involved (Sendra *et al.*, 2020). None of these studies investigated the role of PRRs in mediating particle recognition and effects.

As in other invertebrate groups, in addition to cells, molluscan immune defences also rely on many soluble haemolymph factors, which can interact with non-self material often by targeting common (rather than specific) structural motifs (Gianazza *et al.*, 2021). Studies carried out with surface-modified NPLs reveal the role of individual haemolymph proteins in particle recognition. Results in mammals show that soluble serum components form a biomolecular corona on the ENM surface, which is responsible for the particle interaction with target cells. In molluscs, the influence of haemolymph serum (HS) on the effects of positively charged NPLs (50 nm amino-modified polystyrene, $PS-NH_2$) was investigated using *M. galloprovincialis* haemocytes. In the presence of HS, $PS-NH_2$ caused increased cellular damage and ROS production, mediated by dysregulation of p38 MAPK signalling (Canesi *et al.*, 2016). The putative C1q domain-containing protein 6 (MgC1q6) was identified as the only component of the 50 nm $PS-NH_2$ hard biocorona. MgC1q6 has a high affinity for cations, explaining why it can adhere to the positive surface charge of this type of NPL, and acts as an opsonin in immune recognition (Pezzati *et al.*, 2015).

Table 1. Effects of different engineered nanomaterials (ENMs) on phagocytic activity and Toll-like receptors (TLRs) in *in vitro* and *in vivo* studies in bivalve molluscs.

Species	ENM	Effect	References
In vitro			
Marine mussel *M. galloprovincialis*	50 nm PS-NH$_2$	• Decreased LMS • Decreased phagocytic activity	Auguste *et al.* ([2021a](#))
Marine mussel *M. galloprovincialis*	100 nm PS-NH$_2$	• Decreased LMS • Characterisation of a MgC1q44 corona in serum acting as a PRR	Auguste *et al.* ([2021a](#))
Marine mussel *M. galloprovincialis*	PS-NPs	• Increased release of toxic radicals • Increased apoptotic signals • Decreased phagocytic activity • Differential effect on haemocyte subpopulations (large granular cells most affected)	Sendra *et al.* ([2020](#))
Marine mussel *M. galloprovincialis*	n-CeO$_2$	• Decreased ROS production • Characterisation of a Cu,Zn-SOD corona in serum acting as a PRR	Canesi *et al.* ([2017](#))
Marine mussel *M. galloprovincialis*	50 nm PS-NH$_2$	• Increased haemocyte damage and ROS production • Dysregulation of p38 MAPK • Characterisation of a MgC1q6 corona in serum acting as a PRR	Canesi *et al.* ([2016](#), [2017](#))
Marine mussel *M. galloprovincialis*	NCB NPs	• NCB uptake • Increased extracellular ROS production • Increased lysozyme and NO release • Induction of pre-apoptotic processes	Canesi *et al.* ([2008](#))
Hong Kong oyster *Crassostrea hongkongensis*	37 nm Ag NPs	• Ag NP uptake • Increased granule formation • Induction of lysosomal dysfunction (due to Ag ions) • Induction of ROS production • Decreased phagocytosis	Luo & Wang ([2022](#))
In vivo			
Marine mussel *M. galloprovincialis*	50 nm PS-NH$_2$	• Decreased haemocyte LMS and ROS production **NH$_2$-NPS** • Increased serum lysozyme activity	Auguste *et al.* ([2021a](#))
Marine mussel *M. galloprovincialis*	100 nm PS-NH$_2$	• Increased ROS production • Increased serum lysozyme activity	Auguste *et al.* ([2021a](#))
Marine mussel *M. galloprovincialis*	50 nm PS-NH$_2$	**1st exposure** • Changes in haemocyte mitochondrial and lysosomal parameters, serum lysozyme activity, transcription of proliferation/apoptosis markers • Upregulation of MgC1q6, downregulation of lysozyme and mytilin B **2nd exposure** • Recovery of haemocyte functional parameters • Upregulation of immune-related genes • Increased bactericidal activity	Auguste *et al.* ([2020](#))
Marine blood clam *Tegillarca granosa*	TiO$_2$ NPs	• Decreased THC and phagocytosis • Downregulation of PRR genes (*TRR1-6, RIG*) • Downregulation PRR downstream genes (*IRAK4, TRAF2, TRAF3, TRAF6, NFKB1, TRIM58*)	Shi *et al.* ([2017](#))
Marine mussel *M. galloprovincialis*	TiO$_2$ NPs	• Decreased LMS, increased ROS and NO production • Upregulation of antimicrobial peptides and lysozyme	(Barmo *et al.*, [2013](#))

Abbreviations: LMS, lysosomal membrane stability; MAPK, mitogen-activated protein kinase; MgC1q6, *M. galloprovincialis* putative C1q domain containing protein 6; MgC1q44, *M. galloprovincialis* putative C1q domain containing protein 44; n-CeO$_2$, cerium nano-oxide; NCB, nano-carbon black; NO, nitric oxide; NP, nanoparticle; PRR, pattern recognition receptor; PS-NH$_2$, amino-modified polystyrene; PS-NP, polystyrene nanoparticle; ROS, reactive oxygen species; SOD, superoxide dismutase; THC, total haemocyte count.

The preferential association of PS-NH$_2$ with this opsonin thus is likely responsible for the observed effects. *In vivo*, repeated exposure to NPLs induced upregulation of *MgC1q6* (but not *TLR*) expression, associated with increased bactericidal activity (Auguste *et al.*, [2020](#)). By contrast, larger PS-NH$_2$ (100 nm, PS100-S) showed a peculiar behaviour in HS (absence of agglomeration and inversion of the zeta potential – the electrokinetic potential in a colloidal system) and weaker effects on lysosomal destabilisation and phagocytosis, suggesting that interactions with HS components can

reduce potential adverse effects. The main HS protein that forms a stable association with PS100-S is MgC1q44, an MgC1q complement component unrelated to MgC1q6 (Auguste *et al.*, 2021*a*). Similarly, decreased ROS production by haemocytes exposed to cerium nano-oxide (n-CeO$_2$) was associated with the formation of a biocorona mainly involving Cu,Zn-superoxide dismutase (SOD) (Canesi *et al.*, 2017). This evidence indicates that the formation of a biomolecular corona is ENM selective and involves distinct soluble serum factors, acting as PRRs for particle recognition, thereby mediating the interactions with immune cells and resulting in different functional outcomes (Canesi *et al.*, 2017). These data underline the importance of nano-bio interactions that confer a recognisable biological identity to ENMs in physiological exposure media. This is important when testing the potential environmental immunotoxicity of ENMs in model organisms (Canesi *et al.*, 2017), but also to reveal unexpected serum components than can act as PRRs for non-self particles.

The above results represent the most extensive data available on bivalve immune responses to NPL exposure. It should be noted, however, that an experimental approach using commercial spherical NPLs, necessitated by the absence of standardised environmental NPL samples for toxicity testing, has obvious limitations as underlined by Auguste *et al.* (2021*a*,*b*). Studies are underway for testing the effects of non-homogeneous NPLs, such as those obtained from weathering or fragmentation of larger plastic debris, fibres, and from different polymers. Recent data indicate that both *in vitro* and *in vivo* exposure to high concentrations of polyester microfibres (length 50–100 μm) arising from textile fragmentation/abrasion significantly affects haemocyte functions (adhesion, tissue infiltration) and immune responses (lysozyme release, NO and ROS production) in *M. galloprovincialis* (Auguste *et al.*, 2022).

A schematic depiction of the interaction of ENMs (including NPLs) with the bivalve immune system, and its functional consequences, is presented in Fig. 3.

IV. PHAGOCYTES AND TLRS IN ANNELID IMMUNITY AND NANOPARTICLE–IMMUNE INTERACTIONS

The annelids include marine worms (Polychaeta), earthworms (Oligochaeta), and leeches (Hirudinea). They rely on highly evolved mechanisms of innate immunity, especially in wound repair, clotting and coagulation, phagocytosis or cytotoxic killing, encapsulation reactions, and the action of antimicrobial factors. Since earthworms live in close contact with the soil, their coelomic cavity (which is in direct contact with the external environment through dorsal pores) is not aseptic and contains diverse microorganisms. Like earthworms, marine worms and freshwater leeches are also in direct contact with dispersed ENMs (Cuvillier-Hot, Boiddin-Wichlacz & Tasiemski, 2014; Bodó *et al.*, 2020*a*).

(1) Immunocytes and pattern-recognition receptors in annelids

The body cavity of an earthworm is filled with a protein-rich coelomic fluid containing several types of immune cells (coelomocytes) that differ in morphology and function. There are three main coelomocyte types: eleocytes have mainly nutritive functions and produce antimicrobial factors, and hyaline and granular amoebocytes represent effector immunocytes, responsible for phagocytosis and encapsulation (Engelmann *et al.*, 2016*a*) (Fig. 4). Amoebocytes engulf material including microbial components, foreign cells, inert particles, and ENMs. Similar to vertebrates, phagocytosis by coelomocytes can be modulated by humoral components, opsonins, which coat the particles and thus promote their phagocytosis (Bilej *et al.*, 2010). Similar cell types and immune defence principles have been described for leeches and marine worms, where three subtypes of granulocytes (G1, G2 and G3) were described (Lefebvre *et al.*, 2008; Cuvillier-Hot *et al.*, 2014).

In the phylum Annelida, only a few PRRs have been described, including TLRs, peptidoglycan recognition proteins (PGRPs), coelomic cytolytic factor (CCF), NLRs, and scavenger receptors (Bilej *et al.*, 2010; Engelmann *et al.*, 2016*b*). The first earthworm TLR with an immune function was described in *Eisenia andrei* (EaTLR) (Skanta *et al.*, 2013). Its significant intra-species variability suggests the possibility of a vast number of Toll-like gene paralogues in the *E. andrei* genome, for TLRs with tissue-specific expression profiles. Another *E. andrei* TLR-like protein (multiple cysteine cluster type of TLR, mccEaTLR), with a potential role in earthworm early development, was described by Prochazkova *et al.* (2019). In marine worms, 105 *TLR* homologues in *Capitella capitata* and 16 *TLR* homologues in *Helobdella robusta* genomes were found by *in silico* analysis of whole genomes (Davidson *et al.*, 2008). Of the four Hm-TLRs in the leech *Hirudo medicinalis*, Hm-TLR1 is associated with the leech central nervous system (Tasiemski & Salzet, 2017).

(2) Effects of ENMs on annelid innate immunity

A limited number of studies have investigated the effects of ENMs on phagocytic cells or their recognition by TLRs in annelids. Besides phagocytic activity, the production of ROS during phagocytosis has been assessed following ENM exposure. The involvement of TLRs in EMN recognition has mainly been studied in terms of changes to their expression after exposure to the particles.

Most studies on the effects of ENMs on annelid immunity examine the role of silver nanoparticles (AgNPs), especially using *in vitro* studies. Exposure of *Eisenia* earthworms to AgNPs led to the accumulation of NPs in cells (mainly amoebocytes), oxidative and mitochondrial stress, apoptosis, and altered expression of *TLR* and other genes involved in stress and intracellular signalling (Hayashi *et al.*, 2012; Bodó *et al.*, 2020*b*). Similarly, transcriptional profiling of cells exposed to nanosilver NM-300K revealed upregulation of
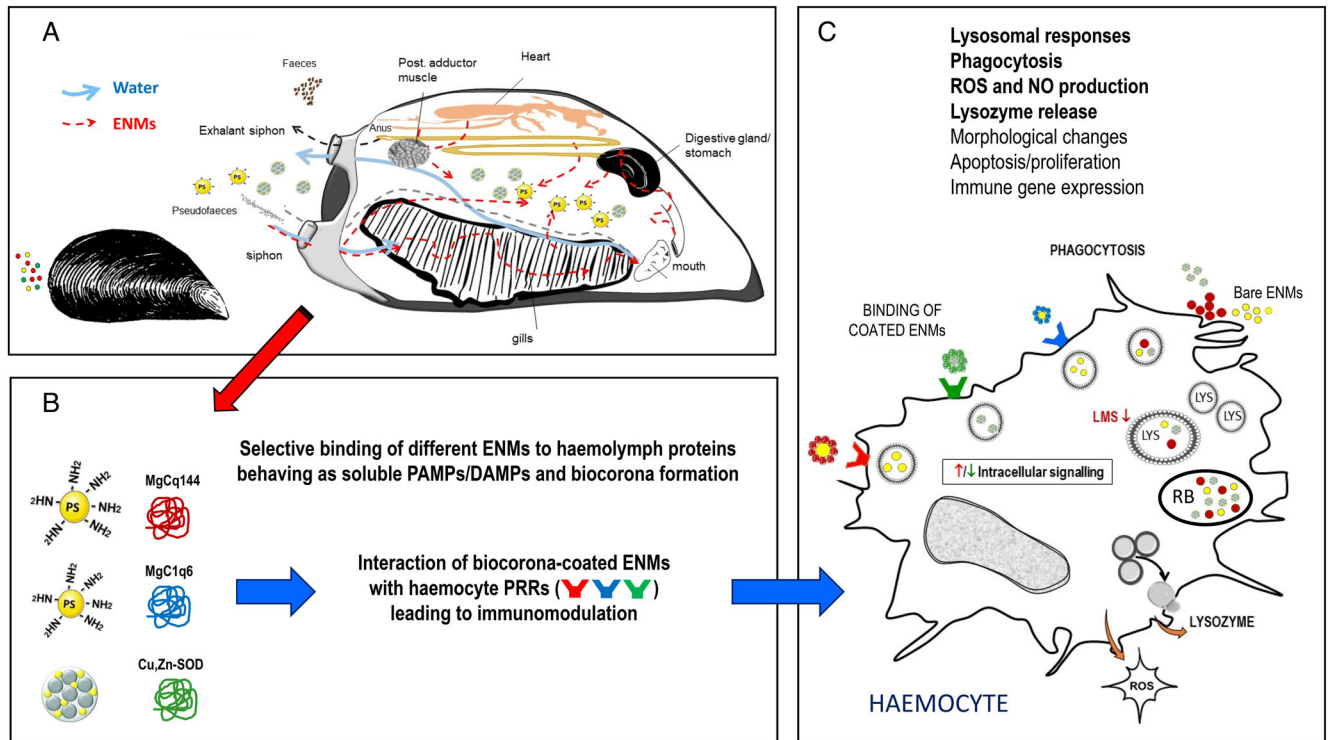
Fig. 3. Schematic representation of the interaction of engineered nanomaterials (ENMs) with the bivalve mollusc immune system and its functional consequences. (A) Simplified diagram of ENM uptake and routes of distribution to different mussel tissues and circulating fluid (haemolymph) (red dotted lines). ENMs taken up by the gills are partly ejected as pseudofaeces, and partly directed to the digestive system, where they can be accumulated or eliminated *via* faeces. Note that, due to the open circulatory system, ENMs can be transferred from any tissue to the haemolymph and *vice versa*. (B) Selective interactions of different ENMs with soluble haemolymph proteins lead to the formation of distinct biocoronas on the ENM surface in *Mytilus galloprovincialis*. Examples are reported of biocoronas with *M. galloprovincialis* putative C1q domain containing protein 44 (MgC1q44) on 100 nm amino-modified polystyrene (PS-NH$_2$), with *M. galloprovincialis* putative C1q domain containing protein 6 [MgC1q6, also known as extrapallial protein (EP) precursor] on 50 nm PS-NH$_2$, and with extracellular Cu,Zn superoxide dismutase (SOD) on CeO$_2$ (Canesi *et al.*, 2017; Auguste *et al.*, 2021a). Proteins on the ENM surface can act as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), which can be recognised by pattern recognition receptors (PRRs) on the haemocyte surface. (C) Interaction of biocorona-coated and bare ENMs with haemocytes can lead to activation or modulation of immune functions. Functional parameters that can be rapidly modulated by ENMs are indicated in bold. Biocorona-coated ENMs can interact with cellular PRRs (currently unidentified). Both coated and bare ENMs can be internalised by phagocytosis. Phagosomes can fuse with lysosomes (LYS) leading to decreased lysosomal membrane stability (LMS), the first marker of cellular stress. ENMs can accumulate in late lysosomes and in residual bodies (RB). ENMs can induce the rapid activation of functional immune parameters [reactive oxygen species (ROS), nitric oxide (NO) production, and lysozyme release] and also induce immunotoxic effects (lysosomal damage, inhibition of immune responses), depending on multiple factors (particle type and concentration, exposure conditions, biocorona coating). Later responses include changes in the expression of immune-related genes [encoding Toll-like receptors (TLRs), antimicrobial peptides, lysozyme] and apoptosis/proliferation-related genes (e.g. *p53*, *PCNA*).

TLR and signalling-related genes (Hayashi *et al.*, 2016). Increased phagocytic activity of earthworm coelomocytes was detected after exposure to CuO NPs and nanoscale zero-valent iron (nZVI), concomitant with higher levels of lipid peroxidation (Semerad *et al.*, 2020; Pacheco *et al.*, 2021a). Coelomocytes of *Lumbricus rubellus* treated with fullerene C60 NPs showed decreased gene expression of pattern recognition factor *CCF*, suggesting immunosuppression (van der Ploeg *et al.*, 2014). By contrast, except for altered *TLR* expression when cells were exposed to AuNPs, neither AuNPs nor TiO$_2$ NPs induced cytotoxicity or altered immune properties (Bodó *et al.*, 2020b; Pacheco *et al.*, 2021b). *In vitro* treatment of leech cells with multiwalled carbon nanotubes (MWCNTs) induced a strong inflammatory response, activation of macrophages, encapsulation of the foreign bodies, and increased apoptotic rate together with elevated ROS production (Girardello *et al.*, 2017, 2015a) (Table 2).

The majority of *in vivo* studies assessing the effects of ENMs on annelids focus on the toxic effect of AgNPs. Two transcriptomic studies revealed the upregulation of genes involved in phagocytosis and autophagy pathways as well as
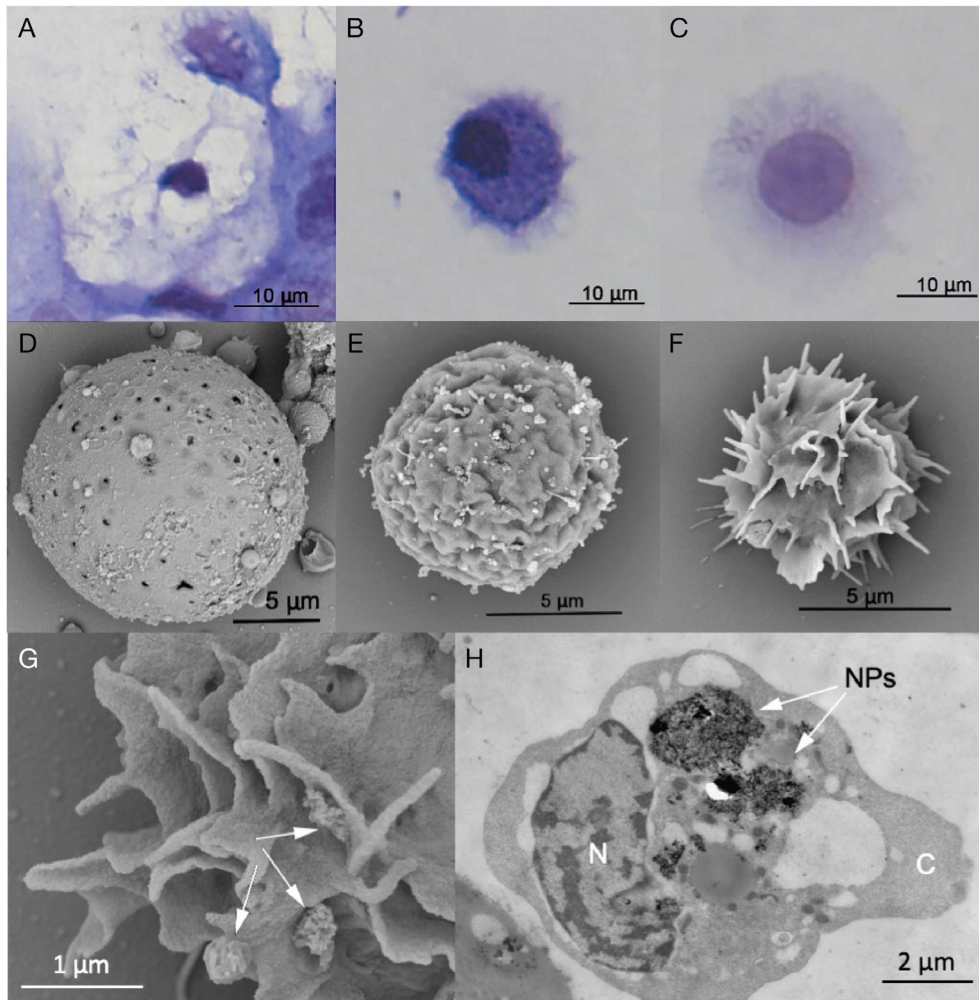
Fig. 4. Earthworm coelomocytes. (A) Eleocyte, (B) granular amoebocyte, and (C) hyaline amoebocyte, visualised by methylene blue staining. (D) Eleocyte, (E) granular amoebocyte and (F) hyaline amoebocyte, visualised by scanning electron microscopy (SEM). (G) Hyaline amoebocyte in contact with CuO NPs, visualised by SEM. (H) Coelomocytes with engulfed CuO NPs visualised by transmission electron microscopy. N, cell nucleus; NPs, nanoparticles; C, cytoplasm. The white arrows point to the CuO NP clusters.

in endocytosis (Novo *et al.*, 2015; Roelofs *et al.*, 2020). This is consistent with endocytosis being one of the possible routes by which ENMs can enter the tissues. Accumulation of AgNPs in tissues was detected in three earthworm species (Diez-Ortiz *et al.*, 2015; Garcia-Velasco *et al.*, 2016; Saleeb *et al.*, 2020). The greatest concentrations of AgNPs were found in the gut wall, liver-like chloragogenous tissue, and the excretory organ nephridia, suggesting potential uptake, detoxification, and excretion *via* this pathway. Furthermore, AgNPs induced oxidative stress, lipid peroxidation, apoptosis, and mass loss in earthworms (Lapied *et al.*, 2010; Gomes *et al.*, 2015; Saleeb *et al.*, 2020; Pacheco *et al.*, 2022). While CuO NPs did not induce changes in immune gene expression in the earthworm *E. fetida* (Swart *et al.*, 2020), a decrease in phagocytic activity and NO generation was observed in the earthworm *Metaphira posthuma* (Gautam *et al.*, 2018). Analysis of leech tissues after exposure to MWCNTs revealed internalised nanomaterial, a strong inflammatory response with

inflammatory cytokine expression, particle uptake by phagocytes, and encapsulation (Girardello *et al.*, 2015*b*). Few papers describe the effect of ENMs on marine worms. One study reported that exposure to CuO NPs provokes a substantial accumulation of Cu in tissues (Thit, Banta & Selck, 2015). Other work reported increased antioxidant enzyme activity and elevated lipid peroxidation as a consequence of MWCNT exposure (De Marchi *et al.*, 2018) (Table 2).

(3) Role of biomolecules adsorbed onto the ENM surface

Annelid coelomic fluid contains a large number of proteins, which form a biomolecular corona around NPs. The secretory protein lysenin was found to be an important component of the biocorona around nanosized silver particles in earthworms (Hayashi *et al.*, 2013), thus its opsonisation properties participate in ENM interaction with cells.

Table 2. Effects of different engineered nanomaterials (ENMs) on phagocytic activity and Toll-like receptors (TLRs) in *in vitro* and *in vivo* studies in annelids.

Species	ENM	Effect	References
In vitro			
Earthworm *Eisenia andrei*	TiO$_2$ NPs	• No change in ROS production, lipid peroxidation and phagocytic activity	Pacheco *et al.* (2021*b*)
Earthworm *E. andrei*	CuO NPs	• Attenuation of the phagocytic activity of hyaline amoebocytes • Elevated lipid peroxidation • Particle uptake by coelomocytes	Pacheco *et al.* (2021*a*)
Earthworm *E. andrei*	nZVI	• Increased ROS production • Elevated lipid peroxidation • Decreased phagocytic activity	Semerad *et al.* (2020)
Earthworm *Lumbricus rubellus*	Fullerene C60 NPs	• Decreased expression of CCF – immunosuppression	van der Ploeg *et al.* (2014)
Earthworm *E. fetida*	AgNPs	• Accumulation of particles in cells • Increased ROS production • Downregulation of stress-related genes • Upregulation of signalling-related genes	Hayashi *et al.* (2012)
Earthworm *E. andrei/fetida*	AgNPs	• Oxidative and mitochondrial stress • Apoptotic and genotoxic response • Altered *TLR* expression	Bodó *et al.* (2020*b*)
Earthworm *E. fetida*	nanosilver NM-300 K	• Accumulation of particles in cells • No ROS production • Upregulation of *TLR, MEKK1*	Hayashi *et al.* (2016)
Earthworm *E. andrei/fetida*	AuNPs	• No cytotoxicity • Altered *TLR* expression	Bodó *et al.* (2020*b*)
Earthworm *E. fetida*	ZnO NPs	• No DNA damage in coelomocytes below 3 mg/l	Gupta *et al.* (2014)
Leech *Hirudo medicinalis*	MWCNTs	• Inflammatory response • Activation of macrophages • Encapsulation	Girardello *et al.* (2015*a*)
Leech *H. verbana*	MWCNTs	• Decreased cell proliferation rate • Increased apoptotic rate • Increased ROS production	Girardello *et al.* (2017)
In vivo			
Earthworm *L. rubellus*	AgNPs	• Upregulation of phagocytosis and autophagy pathways	Roelofs *et al.* (2020)
Earthworm *L. terrestris*	AgNPs	• Increased apoptotic activity in tissues	Lapied *et al.* (2010)
Earthworm *L. rubellus*	AgNPs	• Distribution of NPs in tissues	Diez-Ortiz *et al.* (2015)
Earthworm *E. fetida*	AgNPs	• Upregulation of genes involved in endocytosis and cilia structures	Novo *et al.* (2015)
Earthworm *E. fetida*	AgNPs	• Accumulation of Ag in tissues	Garcia-Velasco *et al.* (2016)
Earthworm *Aporrectodea caliginosa*	AgNPs	• Accumulation of Ag in tissues • Increased antioxidant enzyme activities and lipid peroxidation	Saleeb *et al.* (2020)
Earthworm *E. fetida*	AgNPs	• Oxidative stress	Gomes *et al.* (2015)
Earthworm *E. fetida*	AgNPs	• Accumulation of Ag in coelomocytes, chloragogen tissue, and nephridial epithelium	Courtois *et al.* (2020)
Earthworm *E. andrei*	AgNPs	• Mass loss • NP uptake by coelomocytes • No impact on PO activity and lipid peroxidation • Imbalance in NO metabolism	Pacheco *et al.* (2022)
Earthworm *Metaphire posthuma*	CuO NPs	• Decreased phagocytic activity • Decrease in NO generation	Gautam *et al.* (2018)

(Continues on next page)

Table 2.   *(Cont.)*

Species	ENM	Effect	References
Earthworm *E. fetida*	CuO NPs	• No impact on the expression of immune markers	Swart *et al.* (2020)
Earthworm *L. rubellus*	Fullerene C60 NPs	• Downregulation of some stress- and immune-related genes • Tissue injury	van der Ploeg *et al.* (2013)
Leech *H. medicinalis*	MWCNTs	• Internalisation of particles • Inflammatory response, encapsulation • Accumulation of particles in phagocytic macrophages	Girardello *et al.* (2015a,b)
Marine worm *Nereis diversicolor*	MWCNTs	• Increased antioxidant enzyme activities and lipid peroxidation	De Marchi *et al.* (2018)
Marine worm *N. diversicolor*	CuO NPs	• Accumulation of particles in tissues	Thit *et al.* (2015)

Abbreviations: CCF, coelomic cytolytic factor; MWCNTs, multi-walled carbon nanotubes; NO, nitric oxide; NP, nanoparticle; nZVI, nanoscale zero-valent iron; PO, phenoloxidase; ROS, reactive oxygen species.

Particles covered by a biocorona may be eliminated by encapsulation and subsequently expelled *via* dorsal pores in the epidermis. Earthworms treated with nanosilver NM-300K showed suppressed lysenin expression and thus a reduction in its potential incorporation into the biocorona, thereby reducing lysenin-assisted ENM uptake (Hayashi *et al.*, 2016). It remains unclear whether the coating of ENMs with endogenous biomolecules is a way to facilitate ENM clearance or whether it results in concealment or in a do not-eat-me signal. Although some studies point to ENMs affecting TLRs, the exact mechanism is unknown. Since many ENMs are known to absorb bacterial lipopolysaccharide (LPS) readily onto their surface, the observed changes in TLR expression or activation may be due to interaction with LPS or other PRRs adsorbed on the particle surface. Particularly in annelids, whose body cavities are not aseptic, activation of TLR signalling is potentially more likely due to microbial molecules adsorbed on the ENM surface than to the ENMs themselves. Moreover, in earthworms, *TLR* expression changes during regeneration, which can be part of a response to ENM-caused tissue damage (Bodó *et al.*, 2021). A schematic depiction of the interaction of ENMs with annelid phagocytes and TLRs, and its functional consequences, is presented in Fig. 5.

V. PHAGOCYTES AND TLRS IN CRUSTACEAN IMMUNITY AND NANOPARTICLE–IMMUNE INTERACTIONS

Crustaceans are a large arthropod group comprising marine, freshwater and terrestrial species. As in other invertebrates, the crustacean innate immune response encompasses diverse humoral and cellular activities dependent on the activation of specific signal transduction pathways and is generally initiated by the recognition of PAMPs or endogenous damage-associated molecular patterns (DAMPs) by PRRs. Immune responses are also triggered by internal/endogenous and/or external/environmental stress signals (Lorenzon, 2005). For example, hyperglycaemia is a typical response of many aquatic animals, including crustaceans, to physical and chemical environmental changes. Exposure to environmental stresses (e.g. hypoxia, salinity changes, thermal stress, emersion, heavy metal pollutants, ENMs) results in increases in hyperglycaemic hormone levels, as is also the case for viral diseases or parasitic dinoflagellate infections. In infections, homeostasis is maintained by a complex neuroendocrine–immune regulatory network (Lorenzon, 2005).

(1) Crustacean haemocytes and pattern-recognition receptors

The central players in the immune response of crustaceans are haemocytes (Fig. 6), which are responsible for phagocytosis, encapsulation, and lysis of foreign particles and cells, and the production and release of different humoral components, such as coagulation factors, protease inhibitors, antimicrobial substances, lectins and others (Iwanaga & Lee, 2005; Griffith, Sokol & Luster, 2014). Phagocytosis by haemocytes is a crucial defence mechanism against infectious agents such as bacteria and viruses, and a scavenging mechanism to remove necrotic debris (reviewed in Liu *et al.*, 2020; Westman, Grinstein & Marques, 2020). Different receptors, such as lectins, scavenger receptors, immunoglobulin-related proteins, and fibrinogen-related proteins, have been reported to be involved in phagocytosis (Liu *et al.*, 2020). Soluble factors (opsonins) bind to pathogens or damaged cells, marking them for phagocytosis. A phagocytic receptor present only in arthropods is the Down Syndrome cell adhesion molecule (Dscam), which in its soluble isoforms binds with bacteria in an opsonisation-like manner, while membrane-bound Dscam promotes phagocytosis in haemocytes (Li *et al.*, 2018b). Dscams are highly variable immunoglobulin-related PRRs, they can be either constitutive or inducible during acute-phase reactions (Jin *et al.*, 2013; Tran *et al.*, 2020), and also have a role in promoting phagocytosis (Liu *et al.*, 2020). It has been suggested that
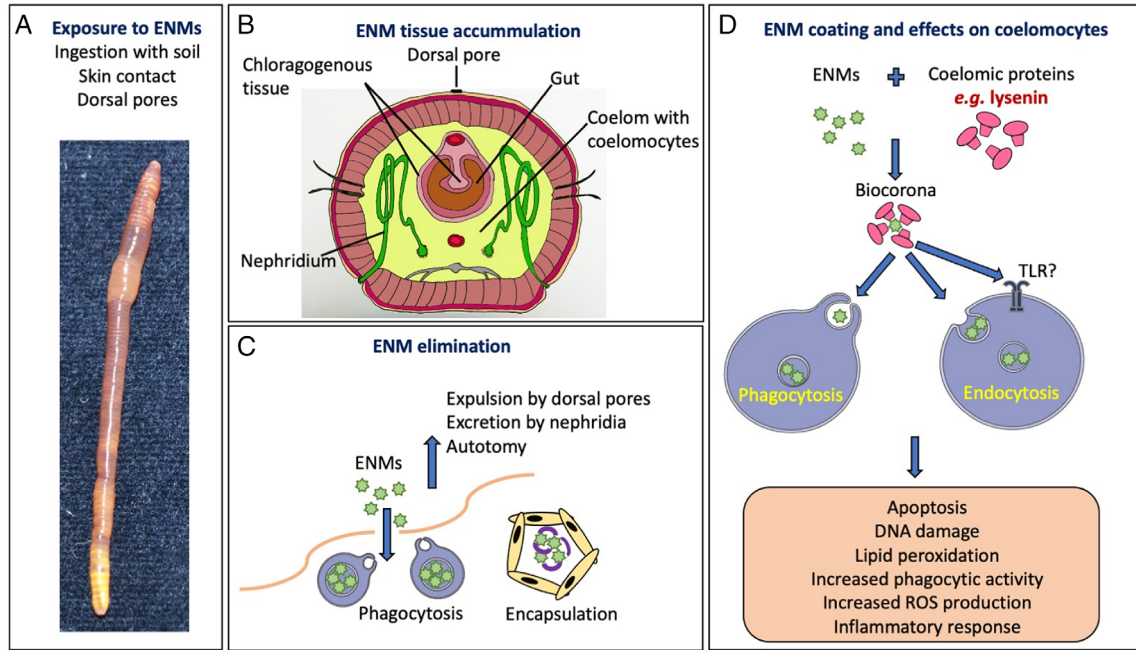
Fig. 5. Schematic representation of the interaction of engineered nanomaterials (ENMs) with the earthworm immune system and its functional consequences. (A) Earthworms are in direct contact with ENMs by skin contact. ENM uptake takes place primarily by the ingestion of soil (95% of total uptake) and by dorsal pores or nephridia. (B) The earthworm body contains a coelomic cavity, segmented by transversal septa, and filled with coelomic fluid. The coelomic fluid is in contact with all the organs and is in open contact with the external environment through a pair of dorsal pores and nephridia in each segment. Coelomocytes present in the coelomic fluid act as immune cells that can engulf ENMs. ENMs can also be bio-distributed to tissues such as the gut wall or chloragogenous tissue. (C) Exhausted phagocytes containing ENMs can be eliminated through the dorsal pores or excreted by nephridia. Aggregated ENMs can be eliminated by the processes of encapsulation and autotomy. (D) Proteins from coelomic fluid (e.g. lysenin) can form a biocorona around ENMs and facilitate their uptake by coelomocytes. A role of Toll-like receptors (TLRs) in sensing biocorona-coated ENMs is hypothesised. ENMs may induce several types of defensive cellular responses aiming at reducing cell/tissue damage. ROS, reactive oxygen species.
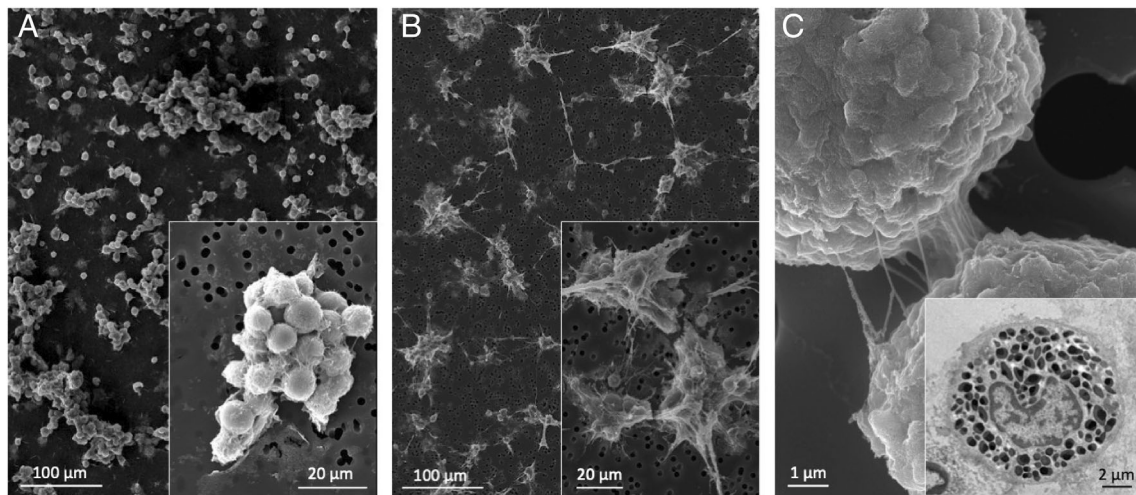
Fig. 6. Electron microscopy images of haemocytes of the terrestrial isopod crustacean *Porcellio scaber*. (A) Scanning electron microscopy (SEM) images of haemocytes isolated from naïve animals. (B) SEM images of haemocytes exposed *in vitro* to PBS for 15 min. Note the protrusions characteristic of activated haemocytes. (C) SEM and transmission electron microscopy images of haemocytes isolated from healthy animals.

membrane-bound and soluble Dscams can directly bind to pathogens to promote their elimination. Exposure to tyre particles for 4 days, but not 2 weeks, caused downregulation of the *Dscam* gene in the hepatopancreas of *Porcellio scaber* (Dolar *et al.*, 2022). This finding suggests that Dscams may have an important role in enabling arthropods to cope with external challenges and preserve homeostasis. Among factors enhancing phagocytosis, AMPs can bind to bacteria and promote their phagocytosis, in addition to their known direct antimicrobial functions (Liu *et al.*, 2015; Matos & Rosa, 2021). The expression of some *AMP* gene families is induced in response to tissue injury, suggesting a possible role in tissue repair.

In crustaceans, the main uptake mechanism for particles is direct uptake from the water through the gills by pinocytosis or phagocytosis (Ribeiro *et al.*, 2017). Traditionally, phagocytosis is assessed directly as a phagocytic index, i.e. the percentage of haemocytes containing endocytosed bacteria or latex beads in *in vitro* tests, or indirectly by measuring the production of ROS. Injected particles accumulate in the gills and can be eliminated together with haemocyte aggregates (Ikerd, Burnett & Burnett, 2015). When circulating haemocytes detect the presence of pathogens, foreign particles, or endogenous anomalous entities, they rapidly aggregate, entrapping the pathogens/particles. These aggregates are then trapped in the gill microvasculature where they are melanised and eliminated during the next moult (Martin *et al.*, 2000). While most studies pertain to bacteria and infectious events, there is little information on phagocytosis/activation as a consequence of exposure to pollutants, and the available evidence is mostly from terrestrial isopods in ecotoxicological studies. Mayall *et al.* (2021) reported an increase in NO concentration as an indirect indication of phagocytic activity resulting from CeO_2 NP exposure in the terrestrial crustacean *P. scaber*, explaining this as a (micro) damage-related response. The observed response of *P. scaber* to CeO_2 NPs was significantly different from that in AuNP-fed animals, both in terms of NO production and increased total haemocyte counts, suggesting that AuNPs are biologically inert compared to CeO_2 NPs (Mayall *et al.*, 2021). A summary of the available information on the interaction between ENMs and crustacean haemocytes is reported in Table 3.

Sensing of possible dangers by crustacean haemocytes mainly occurs through PRRs, among which the best described are TLRs (Wang & Wang, 2013). Eighteen *TLR* genes have been identified and studied extensively in several crustacean species. These are present on haemocytes and many other cells (Lai & Aboobaker, 2017; Habib & Zhang, 2020). As in insects, crustacean TLRs bind to Spätzle, an endogenous ligand generated by proteolysis of a pro-protein that is usually activated in response to an infectious agent. TLR engagement by Spätzle leads to the activation of an intracellular signalling cascade that initiates the expression of several immune genes, including those encoding anti-LPS factors (*ALF*s), crustins, penaeidins, histins, arasins, and hyastatin (Habib & Zhang, 2020; Sánchez-Paz &

Muhlia-Almazán, 2020). Components of the Toll pathway in malacostracan crustaceans (crabs, lobsters and shrimps) include a chain of interacting proteins: in addition to the TLRs themselves, these include the endogenous ligand Spätzle, the intracellular signalling molecules myeloid differentiation factor 88 (MyD88), Tube, Pelle, Dorsal, Cactus and the Toll-interacting protein (TOLLIP) (Arancibia *et al.*, 2007; Lai & Aboobaker, 2017).

(2) Crustacean immune reaction to environmental stressors

While the role of TLRs in pathogen recognition and reaction is established, much less is known about their role in responses to other types of stress. A low salinity shock can trigger an innate immune response by activating the TLR and MAPK signalling pathways (Zhao *et al.*, 2015). In the Indian spiny lobster, *Panulirus homarus*, environmental changes (salinity, dissolved oxygen levels, and ammonia–N concentration) affect immune responses, measured as changes in total haemocyte counts, phenoloxidase (PO) activity, and ROS production (Verghese, Radhakrishnan & Padhi, 2007). In the crab *Carcinus aestuarii*, extreme temperatures (both high and low) influence immune parameters (mainly haemocyte proliferation, haemolymph protein concentration, and PO activity), most likely as mechanisms to cope with temperature changes (Matozzo, Gallo & Marin, 2011). Such observations are not restricted to crustaceans: similar findings have been reported for other invertebrate and vertebrate species and underline the broader role of TLRs in sensing environmental changes and coordinating subsequent adaptive/protective responses.

Crustaceans can exhibit immune responses to other environmental stressors, including ENMs or micro- and nano-plastics (Table 3). In the terrestrial isopod *P. scaber*, immune stimulation in response to ingested or injected ENMs was assessed as increased or decreased expression of several immune-related genes (Table 3) (Dolar *et al.*, 2022). Whether and how particles are perceived as a potential threat by the crustacean immune system, and in particular by interaction with innate receptors such as TLRs according to Matzinger's danger model (Matzinger, 2002), may depend on the type of biomolecular corona on the particle surface. If the corona is composed of conformationally altered self-molecules, it can be recognised by the immune system as potentially dangerous altered/damaged self. Some proteins/molecules can undergo conformational changes upon adsorption onto particles (Li *et al.*, 2013). Thus, crustacean TLRs seem to be involved in recognition and defensive response to various stimuli and conditions other than infective agents, suggesting a wider function in safeguarding the body's integrity. In this context, evaluating immune reactions to particles may enhance our understanding of the fundamental mechanisms of crustacean immune defences. A schematic depiction of the interaction of ENMs (including NPLs) with the crustacean immune system, and its functional consequences, is presented in Fig. 7.

Table 3. Effects of different engineered nanomaterials (ENMs) on phagocytic activity and Toll-like receptors (TLRs) in *in vivo* studies in crustaceans.

Species *In vivo*	ENM	Effect	References
American lobster *Homarus americanus* Spiny lobster *Panulirus interruptus* Crayfish *Procambarus clarkia* Ridgeback prawn *Sicyonia ingentis*	India ink (Higgins) particles injected	• Particles found within nodules composed of multiple layers of flattened and melanised haemocytes; complete melanisation after 1 week	Martin *et al.* (2000)
Crayfish *Cherax quadricarinatus*	Fluorescent polystyrene beads 5 μm	• Granulocytes take up beads by endocytosis	Duan *et al.* (2014)
Crayfish *C. quadricarinatus*	Fluorescent polystyrene beads	• 0.2 μm particles phagocytosed by SGCs • 2 μm particles taken up by both GCs and SGCs	Li *et al.* (2018a)
Amphipod *Gammarus fossarum*	AgNPs and AuNPs 20–80 nm	• AgNPs detected in the cuticle and the gills • AuNPs detected in the gut area • CIT-NPs show higher bio-accumulation *versus* PEG-NPs • AgNPs upregulate gene encoding Cu,Zn SOD • AuNPs downregulate gene encoding Cu,Zn SOD • No phagocytic activity by blood cells detected	Mehennaoui *et al.* (2018)
Terrestrial isopod *Porcellio scaber*	AuNPs in diet (14 days)	• No increase in NO (no damage) • No evidence of phagocytic activity of damaged material	Mayall *et al.* (2021)
Terrestrial isopod *P. scaber*	CeO$_2$ NPs in diet (14 days)	• Increased PO-like activity • Reduced haemocyte viability • Increase in NO activity suggesting phagocytosis of damaged material	Mayall *et al.* (2021)
Terrestrial isopod *P. scaber*	AuNPs and CeO$_2$ NPs, injected	• Decreased haemocyte viability (CeO$_2$ NPs > Au NPs) • Increased NO production (CeO$_2$ NPs > Au NPs) suggesting phagocytosis	Mayall *et al.* (2021)
Terrestrial isopod *P. scaber*	AuNPs and CeO$_2$ NPs, injected with LPS	• Modulation of immune-related genes (*SOD2, NOS2, CLTC, MYD88, ATG5, CAT, FER, PRDX, MAS1L, CYPG, PXT*)	(D. Drobne *et al.*, unpublished data)
Terrestrial isopod *P. scaber*	Microplastics, ingestion	• Modulation of immune-related genes (*EEF2, CAT, SOD2, NOS2, CYPG, DSCAM, TOLL4, MAS1L, MYD88, PPAE2A*)	(D. Drobne *et al.*, unpublished data)
White-leg shrimp *Litopenaeus vannamei*	Exposure to microplastics	• Expression of immune-related genes (*proPO, TLR, IMD, ALF*)	Wang *et al.* (2021)
Chinese mitten crab *Eriocheir sinensis* (juvenile)	Exposure to microplastics	• Modulation of activity of immune-related factors and gene expression (*HC, AKP, PO, LSZ, ACP, MYD88, CASP*) • Decreased levels/activities of several immune-related factors (Hc, AKP, PO, LSZ, ACP)	Liu *et al.* (2019)

Abbreviations: ACP, acid phosphatase; AKP, alkaline phosphatase; CIT, citrate; GC, granular cell; HC, haemocyanin; *IMD*, immunodeficiency gene; *NOS2*, inducible nitric oxide synthase gene; GC, granular cell; LPS, lipopolysaccharide; LSZ, lysozyme; NO, nitric oxide; NP, nanoparticle; PEG, polyethylene glycol; PO, phenoloxidase; SGC, semigranular cell; SOD, superoxide dismutase.

VI. PHAGOCYTES AND TLR IN ECHINODERM IMMUNITY AND NANOPARTICLE–IMMUNE INTERACTIONS

Several immune-related traits are functionally and evolutionarily conserved among deuterostomes, of which echinoderms and chordates are the major extant members. These include danger-sensitive innate immune cells (e.g. phagocytic cells), stimulation of *TLR* orthologues, and activation of the inflammasome upon immune challenge.

Echinoderms include five extant clades: Crinoidea (sea lilies and feather stars), Asteroidea (sea stars), Ophiuroidea (brittle stars and basket stars), Echinoidea (sea urchins and sand dollars), and Holothuroidea (sea cucumbers). The Echinoidea and Holothuroidea are the most highly evolved classes.
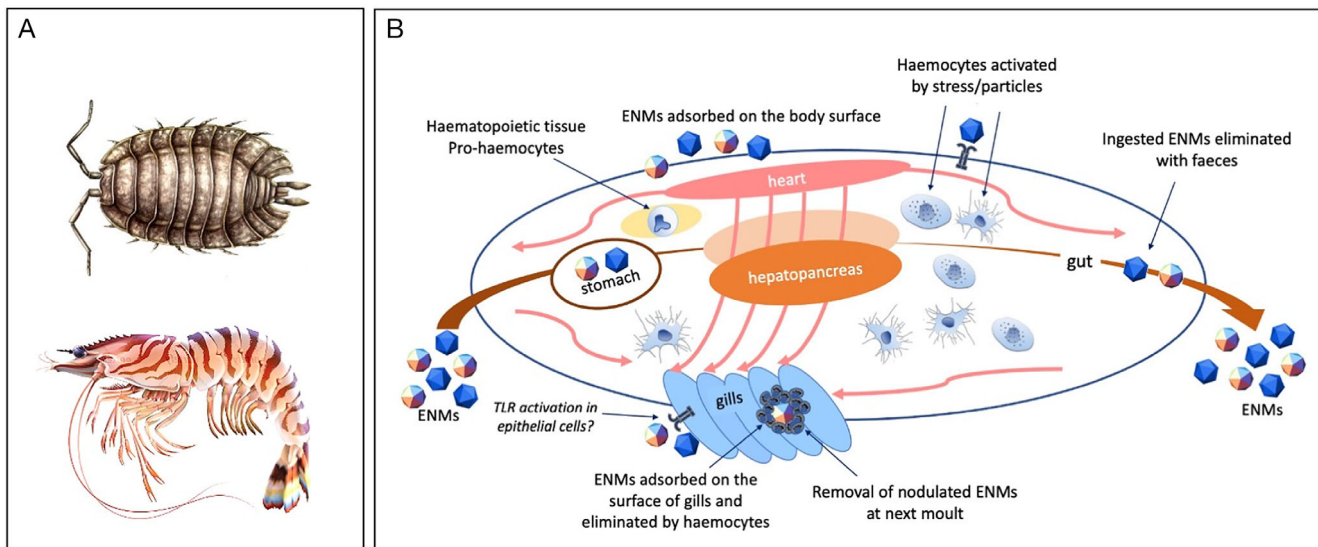
Fig. 7. Schematic representation of the interaction of engineered nanomaterials (ENMs) with the crustacean immune system and its functional consequences. (A) Both terrestrial (e.g. the isopod *Porcellio scaber*, top) and marine crustaceans (e.g. the tiger prawn *Penaeus monodon*, bottom) can be exposed to ENMs present in their environment. (B) ENM uptake and interaction with haemocytes in crustaceans. The contact points between ENMs and the crustacean body are the digestive system, the body surface and the gills. Ingested particles can be directly eliminated with faeces. A large proportion of contact occurs through the gills (Ribeiro *et al.*, 2017; Van der Zande *et al.*, 2020). Epithelial cells, through Toll-like receptor (TLR) activation, can release factors that activate haematopoiesis and the functions of mature haemocytes. Particles in the blood are taken up by haemocytes, which form aggregates that are subsequently cleared from the gills (Martin *et al.*, 2000). Haemocytes react to contact with ENMs with a stress-related activation profile that includes upregulation of immune-related genes and production of inflammatory/defensive molecules (see text for details).

(1) Phagocytes in echinoderms

Three major types of freely circulating immune cells [phagocytes (Fig. 8), amoebocytes, and vibratile cells] are relatively conserved among sea urchin and sea star species, but less so among species of sea cucumbers. They are present in all coelomic spaces, including the perivisceral coelomic cavities, the open water-vascular system, and in organs and tissues.

Although some cell types are better defined, less is known about the functions of the rarer types, including amoebocytes and vibratile cells. These cells are highly mobile and increase in number following an insult. Amoebocytes (red and white) are large motile cells with cytoplasmic granules rich in muco-polysaccharides and proteins. These cells are associated with the first phase of pathogen immobilisation (e.g. antibacterial and cytotoxic activity, encapsulation). Red amoebocytes in
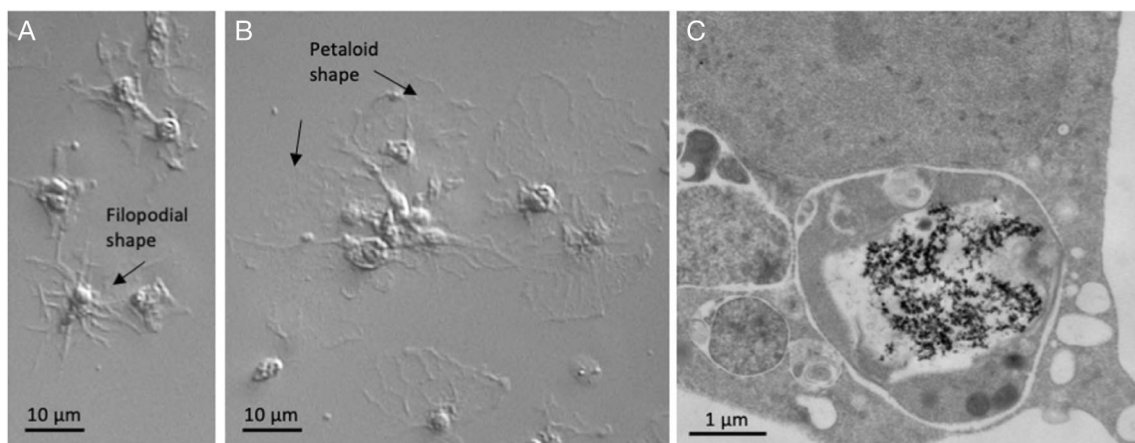
Fig. 8. Phagocytes in the sea urchin *Paracentrotus lividus*. (A) Phagocytes with filopodial shape. (B) Phagocytes with a petaloid shape. (C) Transmission electron microscopy image of a nanoparticle (NP)-containing vesicle within a phagocyte exposed in culture to TiO_2 NPs for 24 h. N, cell nucleus.

echinoderms produce the anti-bacterial agent echinochrome. White amoebocytes display cytotoxic activity against mammalian cells *in vitro*, which increases in the presence of phagocytes. Vibratile cells are fast-moving flagellated cells involved in clot formation, with cytoplasmic granules that contain clotting proteins.

In all echinoderms, phagocytic cells are the main actors in defensive reactions and in sea urchins and sea stars are often the most abundant free-circulating cells present in the coelomic fluid (Smith *et al.*, 2018; Andrade *et al.*, 2021; Barela Hudgell, Grayer & Smith, 2022). Phagocytes include a few subsets of cells based on their size and cytoskeletal structure, with additional distinctions based on function, including the production of complement components (e.g. SpC3), opsonisation, phagocytosis, encapsulation, bacterial growth inhibition, cytotoxicity, and stimulus-dependent expression of immune-related genes (e.g. genes encoding SpTransformer proteins) (Smith *et al.*, 2018; Clow *et al.*, 2000; Chou, Liu & Smith, 2018). Depending on the conditions, these cells can change from a petaloid/bladder shape (mainly involved in phagocytosis) to a filopodial shape (mainly involved in clotting formation) *via* reorganisation of cytoskeletal microfilaments (Fig. 8). Phagocytes produce several types of AMPs, including strongylocins (cysteine-rich), centrocins, β-thymosin, and paracentrin. Other humoral defensive factors are lectins, lysozymes, clotting proteins, lysins, and the prophenoloxidase (proPO) system (Smith *et al.*, 2018).

(2) TLRs in echinoderms

In Echinoidea, closely related but diversified variants of TLRs may respond to evolving pathogens with a higher dynamism than the PAMP-based systems of vertebrates and insects (Buckley & Rast, 2012). The sequence diversity and complexity of the Echinoidea *TLR* multigene families appears to be the result of an expansion specific to the echinoderm lineage; while a small number of TLRs may function similarly to those of vertebrates, the large number of TLRs likely implies novel immune-recognition functions. Depending on the species, the estimated size of the *TLR* gene family ranges from 68 in *Lytechinus variegatus* to 276 in *Mesocentrotus franciscanus* (syn. *Strongylocentrotus franciscanus*) (Smith *et al.*, 2018), with the level of complexity related to the average lifespan. For example, *M. franciscanus* (Pacific Ocean) can live for over 100 years (Ebert, 2008), while *L. variegatus* (Atlantic) has an estimated maximal lifespan of only 3–10 years (Russell *et al.*, 2012), which is a relatively short life expectancy among sea urchin species. Evidence for *TLR* expansion has not been detected in the genomes of other echinoderms, including the sea cucumber *Apostichopus japonicas* for which only two *TLR* genes were identified (*AjTLR3* and *AjToll*) (Sun *et al.*, 2013).

Genes involved in pathogen-specialised TLR signalling pathways are highly conserved and include the presence of TIR domain-containing adaptors (e.g. *MyD88*, *SARM1*), signalling mediators (e.g. *IRAK1/4*, *TRAF6*, *M3K7*, *TAB1*,

TAB2), and cytokines and transcription factors activated by infection (e.g. *NF-κB*, *TNF*s, *IL-17*, *IRF*s) (Tassia, Whelen & Halanych, 2017). The evolution of gene family expansion to generate TLR diversity and other immune receptors is not limited to sea urchins, but is also found in other invertebrate deuterostomes, including the lancelet *Branchiostoma floridae* (cephalochordate) (Tassia *et al.*, 2017), although it remains to be elucidated how *TLR* expansion is involved in pathogen recognition. Molecular phylogenetic analysis shows that most sea urchin or lancelet *TLR* candidates are paralogues, suggesting that these organisms independently underwent *TLR*-related gene family expansion (Satake & Sekiguchi, 2012).

(3) Interaction between ENMs and the echinoderm immune system

Echinoderms, particularly sea urchins, represent a suitable model in developmental biology for evaluating environmental risks posed by ENMs (Fairbairn *et al.*, 2011; Genevière *et al.*, 2020; Pikula *et al.*, 2020; Kukla *et al.*, 2022). Only one species, the sea urchin *Paracentrotus lividus*, has been extensively used for environmental nano-immunotoxicological studies, addressing the issue of 'nanosafety' (Pinsino & Di Bernardo, 2022). *P. lividus* is a common sea urchin on European coasts (Mediterranean Sea and Eastern Atlantic Ocean), with an estimated lifespan of 15 years (Tomšič *et al.*, 2010).

The open water-vascular system allows water-dispersed ENMs to come into contact with the coelomic fluid and interact with various extracellular proteins to form a complex biocorona on the particle surface. Biocorona-coated ENMs then come into contact with cells – mainly phagocytes which represent more than 80% of the total freely circulating cell population (Alijagic *et al.*, 2019, 2021*a*,*b*). *In vivo* studies on *P. lividus* immune cells show selective changes in specific pathways when exposed to a range of ENMs, a similar situation to that in humans (Falugi *et al.*, 2012; Pinsino *et al.*, 2015). For example, *in vivo* exposure of *P. lividus* to TiO₂ NPs for 24 h activates receptor-mediated phagocytosis involving the TLR4 and p38 MAPK signalling pathways coupled to an anti-inflammatory response (Pinsino *et al.*, 2015). Phagocytes exposed *in vitro* to the same NPs downregulate the expression of genes encoding TLR signalling-related proteins such as nuclear factor kappa B (NF-κB), c-Jun N-terminal kinase (JNK), p38 MAPK, caspase 8, and FAS-associated death domain protein (FADD), and increase their antioxidant metabolic activity (pentose phosphate pathway, cysteine–methionine and glycine–serine metabolism) that promotes immunological tolerance (Alijagic *et al.*, 2020). By contrast, AuNPs coated with polyvinylpyrrolidone (PVP-AuNPs) and sea urchin extracellular molecules can elicit an immune response through transient involvement of the TLR4/p42/44 MAPK (ERK) signalling pathway, accompanied by metabolic rebalancing, to control/downregulate the response (Alijagic *et al.*, 2021*a*). Upon exposure to PVP-AuNPs, phagocytes take up particles, and a rare cell subset shows increased expression of CD45 (which in mammals is

a pan-leukocyte antigen transmembrane tyrosine phosphatase) and CD14 (which in mammals is an LPS-binding protein expressed on monocytes and macrophages) (Alijagic *et al.*, 2021*b*). These cells may reflect an intermediate state of maturity and may be involved in several aspects of TLR-dependent immune activation. The particle-uptake mechanism is unknown, but may involve scavenger receptors and other PRRs and, as known for mammalian macrophages, a non-specific passive uptake mechanism, depending on the particle size (Geiser *et al.*, 2008). The biocorona on the ENM surface represents the interface that is perceived by cells, and particles coated with *P. lividus* extracellular proteins are readily phagocytosed by both petaloid and filopodial phagocytic cells (Pinsino *et al.*, 2015; Alijagic *et al.*, 2020). A summary of studies describing the interaction between ENMs and the echinoderm immune system is reported in Table 4, and a schematic depiction is presented in Fig. 9.

VII. PHAGOCYTES AND TLRS IN MAMMALIAN IMMUNITY AND NANOPARTICLE–IMMUNE INTERACTIONS

In vertebrates, the defensive role of phagocytes is similar to that in invertebrates, i.e. recognition and elimination of exogenous particles (scavenging), elimination of senescent or dead cells (surveillance), and initiation of an active immune/inflammatory response against exogenous agents such as pathogenic microorganisms. In mammals, including humans, there are two main types of phagocytic cells, mononuclear phagocytes (mainly blood monocytes and tissue-resident macrophages) (Fig. 10) and polymorphonuclear (PMN) phagocytes (mainly blood neutrophils). Monocytes and neutrophils exit the bloodstream to enter tissues where defensive reactions are ongoing, and are the main innate immune effector cells. PMN phagocytes represent the first defensive wave, with a very short lifespan and high phagocytic and degranulation capacity, followed by monocytes which may survive the immune reaction and remain in the tissue post infection. Other human leukocytes involved in inflammatory/innate immune reactions also resemble invertebrate defensive cells; these include circulating eosinophils and basophils and tissue mast cells, which are poorly phagocytic compared to macrophages and PMN phagocytes and have defensive roles mainly by releasing granules loaded with bioactive mediators.

(1) Pattern-recognition receptors in mammalian innate immunity

Phagocytes and other innate immune cells in vertebrates can sense, recognise and react to exogenous agents using a range of PRRs that selectively bind structural molecular patterns shared by different microbes and other particles. Vertebrate PRRs have much in common with those of invertebrates and include a large number of lectins, scavenger receptors, cytoplasmic NLRs and RIG-like receptors (RLRs), and TLRs (Murphy *et al.*, 2022; Brennan & Gilmore, 2018). In mammals, 13 TLR paralogue proteins have been described (of which 10 functional proteins are expressed in humans) that selectively recognise groups of molecular patterns characteristic of different microbes and/or anomalous

Table 4. Effects of different engineered nanomaterials (ENMs) on phagocytic activity and Toll-like receptors (TLRs) in *in vitro* and *in vivo* studies in the echinoderm *Paracentrotus lividus*.

Species	ENM	Effect	References
In vitro			
Sea urchin *Paracentrotus lividus*	TiO_2 NPs	• Anti-inflammatory • Phagocytosis (TLR/p38 MAPK-mediated) • Antioxidant activity	Alijagic *et al.* (2019, 2020)
Sea urchin *P. lividus*	PVP-coated AuNPs	• Inflammatory • Phagocytosis (TLR4/p42/44 MAPK-mediated) • Metabolic shift	Alijagic *et al.* (2021*a*)
Sea urchin *P. lividus*	PVP-coated AuNPs	• Particle uptake • Cell maturation (CD45 and CD14 expression)	Alijagic *et al.* (2021*b*)
Sea urchin *P. lividus*	Fe_3O_4 NPs	• NF-κB downregulation	(A. Pinsino, unpublished data)
In vivo			
Sea urchin *P. lividus*	TiO_2 NPs	• Anti-inflammatory • Phagocytosis (TLR/p38 MAPK-mediated)	Pinsino *et al.* (2015)
Sea urchin *P. lividus*	Fe_3O_4 NPs SnO_2 NPs CeO_2 NPs	• Particle uptake • ER and lysosome morphological alteration • Reduction in cholinesterase activity • Decreased levels of stress proteins	Falugi *et al.* (2012)

Abbreviations: CD14, cluster of differentiation 14; CD45, cluster of differentiation 45; ER, endoplasmic reticulum; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NP, nanoparticle; PVP, polyvinylpyrrolidone; TLR, Toll-like receptor.
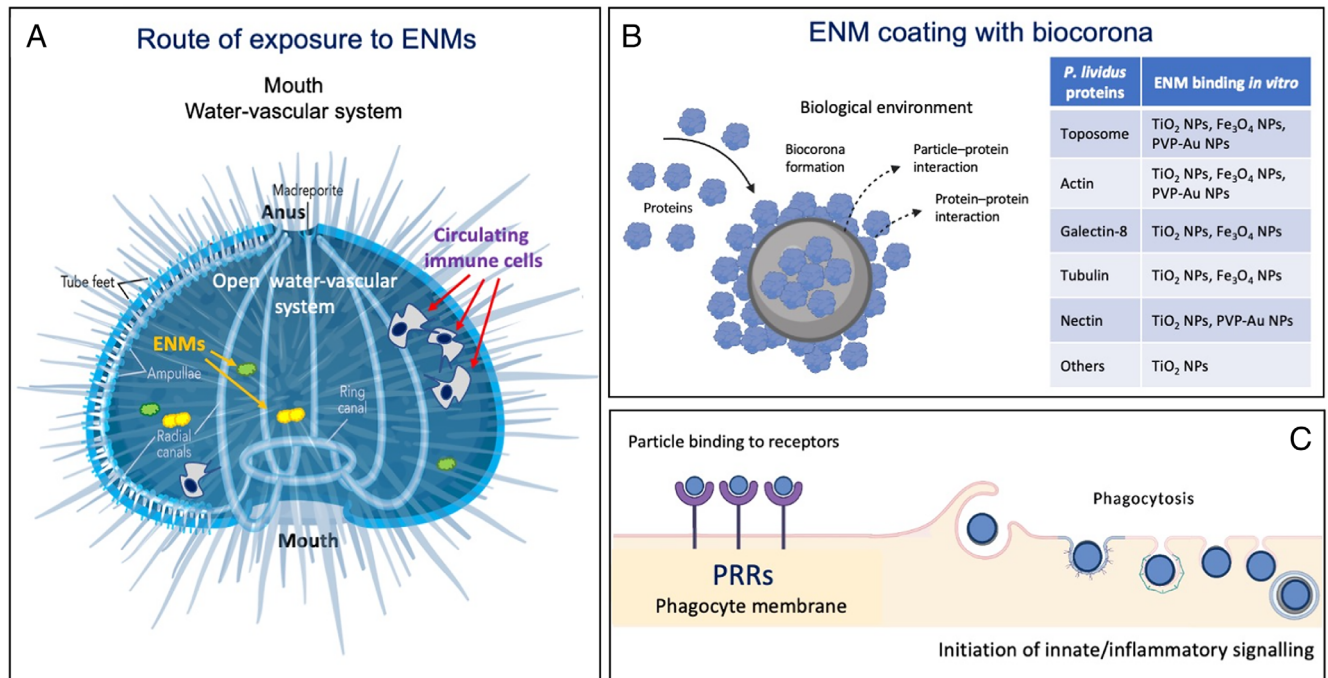
Fig. 9. Schematic representation of the interaction of engineered nanomaterials (ENMs) with the echinoderm immune system and its functional consequences. (A) Anatomical map of sea urchin showing routes of exposure to ENMs *via* the mouth and the open water-vascular system, where particles come into contact with soluble proteins and circulating phagocytes. (B) Schematic model representing the formation of a biocorona on the surface of particles within the *Paracentrotus lividus* water-vascular system. The table on the right lists the main *P. lividus* proteins known to interact with different types of ENMs (Alijagic *et al.*, 2019, 2021a). These include adhesion proteins (toposome, galectin-8, nectin) and other proteins that facilitate phagocytosis (actin, tubulin). (C) Biocorona-coated ENMs may be sensed/bound by Toll-like receptors (TLRs) or other pattern-recognition receptors (PRRs), initiating phagocytosis and a receptor- or phagocytosis-dependent innate/inflammatory signalling cascade. NP, nanoparticle.

endogenous molecules. Different types of professional phagocytes display different arrays of functional TLRs; for instance, monocytes/macrophages are able to express all of them at levels that depend on the activation conditions, whereas PMN leukocytes do not express TLR3 (Hayashi, Means & Luster, 2003). TLR expression levels also vary between mononuclear phagocytes from different anatomical sites (e.g. monocytes and alveolar macrophages), with TLR2 expressed more abundantly in monocytes and TLR9 in alveolar macrophages (Juarez *et al.*, 2010). In addition, there is compartmentalisation of TLR proteins within mononuclear phagocytes, with some TLRs present in the plasma membrane (TLR1, 2, 4, 5, 6, 10), others in endosomal membranes (TLR3, 4, 7, 8, 9), Golgi (TLR4), and endoplasmic reticulum (TLR3, 7, 8, 9) (Barton & Kagan, 2009). The presence of TLRs is not limited to professional phagocytes. The 'amateur' phagocytes, such as epithelial cells, endothelial cells and astrocytes, also express some TLRs and other PRRs, and use them for cell/particle recognition and subsequent elimination; their role mostly being the elimination of dead cells (efferocytosis) in synergy with professional phagocytes (Sihombing *et al.*, 2021). Mammary epithelial cells can phagocytose apoptotic cells using PRRs (Monks *et al.*, 2005). Intestinal enterocytes can use TLRs for the uptake of bacteria and their transcytosis (Neal *et al.*, 2006), a process that contributes to the maintenance of tolerance for commensal bacteria (Rakoff-Nahoum *et al.*, 2004), but that could also be the basis of systemic sepsis (Strober, Fuss & Blumberg, 2002). It is notable that colonocytes express TLR2, 4 and 5 only on the basolateral membrane, so that recognition and activation can only occur if bacteria pass the epithelial cell layer (i.e. in an invasive infection), whereas there is no recognition/activation upon contact of commensal bacteria with the apical cell membrane in the gut lumen. Conversely, enterocytes in the ileum also express TLR2 and TLR4 apically, while TLR5 is only detected basolaterally (reviewed in Yu & Gao, 2015). Different gut epithelial cells (crypt epithelial cells, Paneth cells, follicle-associated epithelial cells) differentially compartmentalise TLR proteins, with altered expression and compartmentalisation occurring in disease conditions (e.g. cancer, ulcerative colitis, Crohn's disease) (Yu & Gao, 2015). Thus, TLR expression and compartmentalisation depend both on cell types and microenvironmental conditions and allow modulation of the activation of cellular functions, adapting their responses to microenvironmental cues.

The modes of TLR recognition and signalling are almost identical to those described for *Drosophila* Toll and all
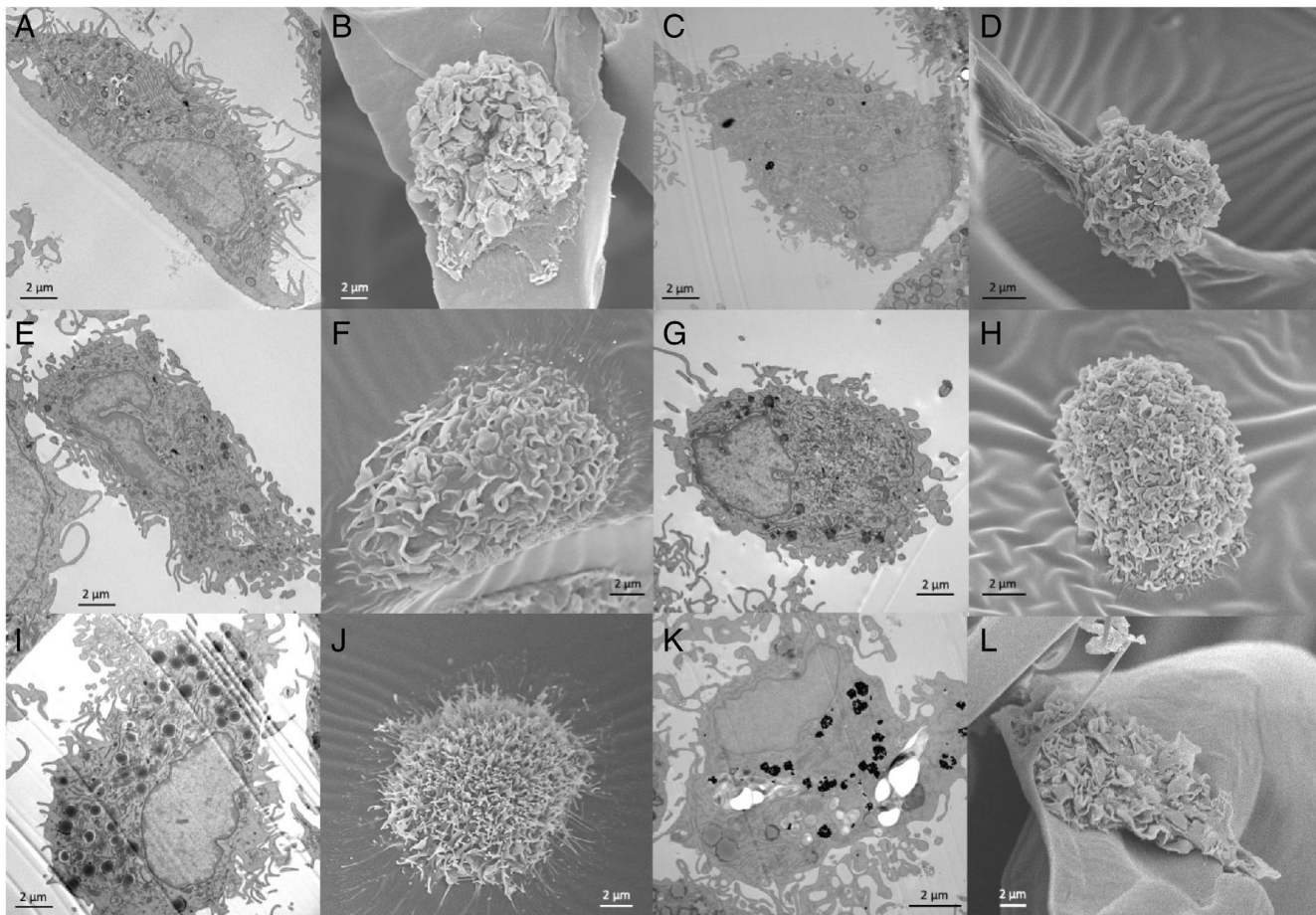
Fig. 10. Human mononuclear phagocytes. (A, B) Human monocytes in culture; (C, D) human monocytes exposed to gold nanoparticles (AuNPs); (E, F) human macrophages in culture; (G, H) human macrophages exposed to lipopolysaccharide (LPS); (I, J) human macrophages exposed to Bacillus Calmette-Guérin; (K, L) human macrophages exposed to AuNPs. Note the abundant particle uptake by macrophages compared to monocytes (compare K with C), the uptake of bacteria by macrophages (I), the morphological changes in activated macrophages, with the formation of surface vesicles and projections (H and J) compared to non-activated cells (characterised by broad flat protrusions; B, D, F and L). A, C, E, G, I, K are transmission electron microscopy (TEM) images; B, D, F, H, J, L are scanning electron microscopy (SEM) images. Images kindly provided by Dr B. J. Swartzwelter, with the collaboration of the microscopy facilities of IBBC-CNR (TEM) and SZN (SEM).

invertebrate TLRs. Signalling involves the presence of the highly conserved intracellular domain TIR that, in vertebrates, is present in another class of innate receptors, the interleukin-1 receptor (IL-1R) family. Molecules of the IL-1R family show binding specificity for endogenous cytokines of the IL-1 superfamily, which then activate an innate inflammatory response in target cells (including monocytes and macrophages) (Boraschi et al., 2018). Thus, the development of adaptive immunity in vertebrates is accompanied by the development of a new class of innate TIR-bearing receptors, which are likely involved in the crosstalk between innate and adaptive immunity (Boraschi, 2022).

(2) Innate immune interaction with ENMs

Similar to invertebrates, the interaction of ENMs with immune cells in vertebrates and, in particular, in mammals

occurs primarily with phagocytes (Fig. 10) and other innate immune cells, which are abundantly present in barrier tissues (Boraschi et al., 2017). Blood phagocytes are recruited to tissues following entry of a foreign object. These blood cells and tissue-resident phagocytes are the main cells that encounter ENMs and, when they recognise them as exogenous agents, engage in their elimination. ENMs in contact with biological systems readily become coated with biomolecules (e.g. serum proteins including complement and fibronectin, mucus components) that may function as opsonins and facilitate uptake by phagocytes through dedicated receptors (Barbero et al., 2017; Moghimi & Simberg, 2017). While the majority of ENMs contacting phagocytes are taken up and degraded intracellularly or secluded into vesicles (Krug & Wick, 2011; Baranska et al., 2018), high aspect ratio ENMs that cannot be phagocytosed because of their size can undergo extracellular degradation. This is the case for carbon-based ENMs such

as single-walled carbon nanotubes (SWCNTs) and graphene oxide (GO), which can be trapped by neutrophil extracellular traps (NETs) and degraded by neutrophil and eosinophil myeloperoxidases and NADPH oxidase (Kagan *et al.*, 2010; Andón *et al.*, 2013; Farrera *et al.*, 2014; Mukherjee *et al.*, 2015). The interaction between ENMs and mammalian macrophages has been studied mostly *in vitro*, mainly employing transformed cell lines of human or mouse origin (Boraschi & Italiani, 2016; Boraschi *et al.*, 2021; Andón & Bondarenko, 2021). In many cases, depending on the type and surface characteristics of ENMs, and on their size, shape, dose, and time of exposure, cytotoxic effects can be detected (inhibition of proliferation, cell death) or inflammatory activation, oxidative stress, and gene regulation can be observed (production of ROS and NO, inflammatory cytokines and chemokines, and enzymes) (Kodali *et al.*, 2013; Fadeel, 2012, 2019). Several caveats, however, limit the relevance of this large body of results, which should therefore be considered with caution. First is the extensive use of transformed cell lines instead of primary monocytes/macrophages. Although some studies have been performed on primary cells (mouse bone marrow-derived macrophages and human blood monocytes or monocyte-derived macrophages), the intrinsic donor-to-donor variability of responses with primary cells has led most studies to use more reproducible models such as those offered by continuous cell lines. The gain in reproducibility is, however, hampered by the loss in representativeness, since transformed, mostly tumoral cell lines do not fully reflect the functional and metabolic features of primary cells and may provide misleading information on the effects of interaction with ENMs (Boraschi *et al.*, 2021). Secondly, the exquisite sensitivity of human monocytes/macrophages to activation by bacterial agents such as LPS calls for caution in attributing to ENMs inflammatory effects that may be due to undetected contamination with LPS (Li & Boraschi, 2016; Li *et al.*, 2017). Likewise, the presence of a biomolecular corona on the ENM surface should always be considered since naked ENMs are unlikely to come into contact with immune cells. Eventually, and most importantly, the overall features of innate/inflammatory responses should be considered. ENMs that can kill macrophages *in vitro* or activate an inflammatory response are not necessarily immunotoxic or able to cause damage *in vivo*. Activation of an inflammatory response underlies the fact that macrophages can recognise ENMs as potentially dangerous and mount a defensive response. That macrophages die during an inflammatory reaction is the rule (for most macrophages and all PMN leukocytes), and, in most cases, the reaction is successful, and the dangerous agent eliminated without significant damage to the body (Ghezzi *et al.*, 2017). Thus, the overall outcome of the ENM–immune interaction cannot be judged reliably by examining the effects of a single ENM type on a single cell type in culture and cannot be assessed by examining a single time point after exposure since a successful innate immune reaction evolves over time and proceeds from activation to resolution and an eventual return to baseline. Anomalous immune reactions may only be captured by examining the entire course of the defensive response and by examining the balance between inflammatory and anti-inflammatory factors and mechanisms activated in response to ENMs (Li *et al.*, 2016, 2017; Italiani *et al.*, 2020; Swartzwelter *et al.*, 2020). Table 5 summarises the effects that should be considered for a realistic assessment of the functional outcomes of ENM–immune interactions in the mammalian/human context.

Some studies have proposed that ENMs may interact directly with TLRs or other PRRs, thereby activating innate/inflammatory responses of macrophages (Bastús *et al.*, 2009; Wolf-Grosse *et al.*, 2018; Mukherjee *et al.*, 2018), although conclusive proof is still not available. The fact that ENMs are often 'contaminated' with TLR ligands such as LPS suggests caution in the interpretation of results. On the other hand, the regularly patterned structure of engineered ENMs, similar to microbial patterns, implies that PRRs may indeed be able to recognise them. While the involvement of TLR activation is still questionable, it is known that ENMs can activate IL-1R family receptors indirectly by promoting the release of IL-1 superfamily ligands. The available information mostly concerns IL-1β, the prototypical inflammatory cytokine of this superfamily. Several types of ENMs can induce

Table 5. Comparison of cellular and organismal effects of engineered nanomaterials (ENMs) on mammalian innate immunity.

| ENM effect on cells	Type/extent of effect	Effect at the organism level
No effect	• Ignorance or silent uptake/elimination	No overall effect, ENMs are eliminated silently (respiration, urine, faeces)
Inflammatory activation	• Limited in time, as a normal response • Followed by resolution and anti-inflammation	No overall effect, limited damage repaired without consequences
Inflammatory activation	• Persistent and/or excessive (e.g. lack of ENM elimination) • Anti-inflammatory mechanisms become persistent	Long-term tissue damage, which may include both tissue destruction and neo-tissue formation (fibrosis, granulomas)
Death	• Limited to exposed cells • Exposed cells degrade ENMs • Dead cells replaced by new cells	No overall effect, limited damage repaired without consequences
Death	• Cells cannot eliminate ENMs (e.g. long fibres) • ENMs are intrinsically toxic • Death of new cells occurring	Long-term tissue damage possible
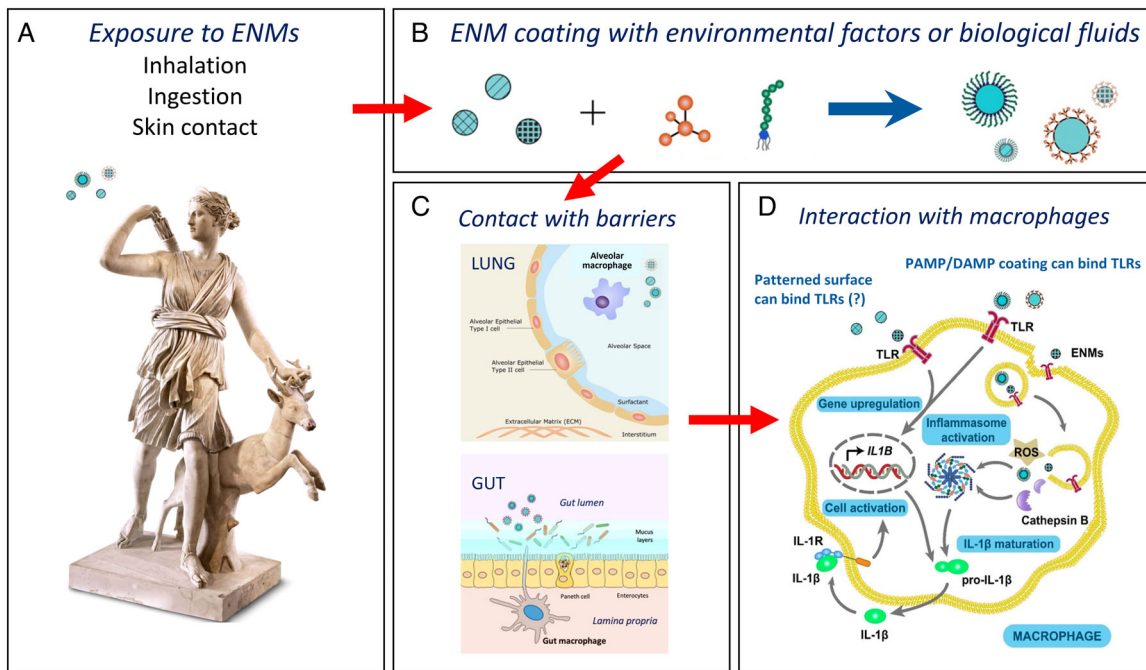
Fig. 11. Schematic representation of the interaction of engineered nanomaterials (ENMs) with the human immune system and its functional consequences. (A) Humans are exposed into ENMs mainly by skin contact, inhalation, or ingestion. (B) Most ENMs that come into contact with human tissues are coated with molecules/agents from the environment [e.g. bacterial lipopolysaccharide (LPS) and other pathogen-associated molecular patterns/damage-associated molecular patterns (PAMPs/DAMPs)] or adsorbed from biological fluids of the barrier tissues (e.g. mucus, surfactant). (C) Interaction between ENMs and immune cells at the barrier tissues occurs principally with macrophages (e.g. alveolar macrophages in the lung, gut macrophages in the intestine). (D) Toll-like receptors (TLRs) on the macrophage surface may interact with ENMs coated with microbial or altered endogenous molecules or, hypothetically, could recognise the orderly patterned ENM surface that resembles a microbial structure. TLR activation can lead to the upregulation of several inflammation-related genes, including the gene encoding interleukin-1β (*IL1B*), and the subsequent production of inflammatory factors, including the pro-IL-1β inactive precursor protein. ENMs can be internalised into endophagolysosomes. Several ENM types can cause vesicle membrane destabilisation, leading to the release into the cytoplasm of both particles and lysosomal enzymes (including cathepsin B). The cytoplasmic inflammasome complex can be activated by cathepsin B and by particulate matter [either directly or through induction of reactive oxygen species (ROS)], leading to the caspase-1-dependent maturation of pro-IL-1β into active IL-1β and its extracellular export. Mature IL-1β can further activate interleukin-1β receptor (IL-1R)-bearing cells and amplify their innate/inflammatory activation. Artwork by W.J. Yang, SIAT, Shenzhen, China.

IL-1β production, in particular by providing the intracellular signals necessary for the cleavage/maturation of the inactive pro-form of this cytokine, based on inflammasome activation (Martinon *et al.*, 2006; Dostert *et al.*, 2008; Hornung *et al.*, 2008; Yazdi *et al.*, 2010; Morishige *et al.*, 2010; Baron *et al.*, 2015), while in other cases they can interfere with the same mechanisms, resulting in decreased IL-1β production (Wu *et al.*, 2013). Whether ENMs may also provide the first signal required for IL-1β production, i.e. gene upregulation, is not clearly established, again because the undetected presence of bioactive agents such as LPS (which is an excellent inducer of *IL1B* gene expression) may lead to misinterpretation of results. In fact, LPS-free ENMs do not seem able to induce *IL1B* gene expression and consequent IL-1β production and are also unable to induce the production of the major IL-1β inhibitor, the IL-1 receptor antagonist IL-1Ra (Li *et al.*, 2016, 2017; Della Camera *et al.*, 2021; Verde *et al.*, 2021). Conversely, they seem able to modulate the generation of innate memory in

monocytes/macrophages, with cells primed with ENMs (alone or together with other agents) producing different amounts of IL-1β and IL-1Ra compared to unprimed cells (Italiani & Boraschi, 2017; Lebre *et al.*, 2020; Swartzwelter *et al.*, 2020; Della Camera *et al.*, 2021). A schematic representation of the interaction between human mononuclear phagocytes and ENMs is provided in Fig. 11.

VIII. CONCLUSIONS

(1) Nanoparticles interact with the host immune defensive mechanisms in a conceptually similar way to interactions that occur with microorganisms or other non-self/damaged-self materials. Since ENMs and other particles are not alive, the task for the innate immune system may be easier. Interactions, depending on the size, shape and surface properties

of the particles, are not different from those with inert microbial/organic particulate agents, which will depend on the same properties. A biocorona that determines the interface between particles and the immune system also can form on the surface of microorganisms (opsonisation) and likewise determines their interaction with the immune system.

(2) Immunity has developed and adapted to recognise and react to microorganisms and other biotic and abiotic materials (ashes, dust, etc.), we thus can expect it to deal successfully with ENMs as well.

(3) In the animal kingdom, phagocytic cells seem to have a major role in interactions with ENMs and can efficiently recognise and remove them without causing irreparable damage. The role of TLRs in ENM recognition is unclear, although the patterned structure of ENMs may facilitate their interaction with PRRs. Unlike plants, which do not have specialised immune cells, most animals appear to activate phagocyte-related defensive mechanisms, with some common to all (e.g. phagocytosis, production of ROS) and others restricted to particular clades (e.g. production of IL-1 in vertebrates).

(4) Observations on the capacity of innate immune mechanisms to recognise and eliminate ENMs across metazoans suggest that innate immunity can successfully deal with ENMs, without causing permanent damage to the organism (Boraschi *et al.*, 2020; Pinsino *et al.*, 2020; Swartzwelter *et al.*, 2021). In plants, interaction with ENMs appears to shift the growth/immunity balance towards enhanced growth. In animals, exposure to ENMs can trigger an inflammatory reaction in phagocytes, which may cause transient cell death and tissue damage responses. Since inflammation is a physiological defensive response with a limited duration and is concluded by resolution, tissue repair, and a return to homeostasis, this cannot be considered a toxic effect.

(5) Pathological consequences of ENM–immune interactions seem to be rare and due to either persistence of the triggering stimulus or anomalous development of the immune reaction, similar to many other infectious and immune-related diseases, such as autoimmunity or degenerative diseases. The effects of ENMs, similar to those of pathogens and other challenges, depend strongly on the type of host organism and its conditions. Thus, ENMs that are safe for humans may be toxic for marine crustaceans; and particles that are safe in young healthy individuals may cause chronic inflammation and damage in old or diseased individuals. The integrity and efficiency of body barriers and the performance of the immune system are key determinants in the 'toxicity' of ENMs (Boraschi, 2014), besides the material intrinsic characteristics. Thus, 'safe-by-design' ENMs are unlikely to exist, and the outcome of the ENM–organism interaction needs to be assessed case by case, in a strategy of 'personalised nanosafety'.

IX. ACKNOWLEDGEMENTS

X. REFERENCES

ALI, S., MEHMOOD, A. & KHAN, N. (2021). Uptake, translocation, and consequences of nanomaterials on plant growth and stress adaptation. *Journal of Nanomaterials* **2021**, 6677616.

ALIJAGIC, A., BARBERO, F., GAGLIO, D., NAPODANO, E., BENADA, O., KOFROŇOVÁ, O., PUNTES, V. F., BASTÚS, N. G. & PINSINO, A. (2021a). Gold nanoparticles coated with polyvinylpyrrolidone and sea urchin extracellular molecules induce transient immune activation. *Journal of Hazardous Materials* **402**, 123793.

ALIJAGIC, A., BENADA, O., KOFROŇOVÁ, O., CIGNA, D. & PINSINO, A. (2019). Sea urchin extracellular proteins design a complex protein corona on titanium dioxide nanoparticle surface influencing immune cell behavior. *Frontiers in Immunology* **10**, 2261.

ALIJAGIC, A., BONURA, A., BARBERO, F., PUNTES, V. F., GERVASI, F. & PINSINO, A. (2021b). Immunomodulatory function of polyvinylpyrrolidone (PVP)-functionalized gold nanoparticles in *Vibrio*-stimulated sea urchin immune cells. *Nanomaterials* **11**, 2646.

ALIJAGIC, A., GAGLIO, D., NAPODANO, E., RUSSO, R., COSTA, C., BENADA, O., KOFROŇOVÁ, O. & PINSINO, A. (2020). Titanium oxide nanoparticles temporarily influence the sea urchin immunological state suppressing inflammatory-related gene transcription and boosting anti-oxidant metabolic activity. *Journal of Hazardous Materials* **384**, 121389.

ANDÓN, F. T. & BONDARENKO, O. (2021). Recent discoveries in nanoparticle–macrophage interactions: in vitro models for nanosafety testing and novel nanomedical approaches for immunotherapy. *Nanomaterials* **11**, 2971.

ANDÓN, F. T., KAPRALOV, A. A., YANAMALA, N., FENG, W., BAYGAN, A., CHAMBERS, B. J., HULTENBY, K., YE, F., TOPRAK, M. S., BRANDNER, B. D., FORNARA, A., KLEIN-SEETHARAMAN, J., KOTCHEY, G. P., STAR, A., SHVEDOVA, A. A., ET AL. (2013). Biodegradation of single-walled carbon nanotubes by eosinophil peroxidase. *Small* **9**, 2721–2729.

ANDRADE, C., OLIVEIRA, B., GUATELLI, S., MARTINEZ, P., SIMÕES, B., BISPO, C., FERRARIO, C., BONASORO, F., RINO, J., SUGNI, M., GARDNER, R., ZILHAO, R. & VARELA COELHO, A. (2021). Characterization of coelomic fluid cell types in the starfish *Marthasterias glacialis* using a flow cytometry/imaging combined approach. *Frontiers in Immunology* **12**, 641664.

ARANCIBIA, S. A., BELTRÁN, C. J., AGUIRRE, I. M., SILVA, P., PERALTA, A. L., MALINARICH, F. & HERMOSO, M. A. (2007). Toll-like receptors are key participants in innate immune responses. *Biological Research* **40**, 97–112.

ARORA, S., SHARMA, P., KUMAR, S., NAYAN, R., KHANNA, P. K. & ZAIDI, M. G. H. (2012). Gold-nanoparticle induced enhancement in growth and seed yield of *Brassica juncea*. *Plant Growth Regulation* **66**, 303–310.

ASLI, S. & NEUMANN, P. M. (2009). Colloidal suspensions of clay or titanium dioxide nanoparticles can inhibit leaf growth and transpiration via physical effects on root water transport. *Plant, Cell & Environment* **32**, 577–584.

AUGUSTE, M., BALBI, T., CIACCI, C., CANONICO, B., PAPA, S., BORELLO, A., VEZZULLI, L. & CANESI, L. (2020). Shift in immune parameters after repeated exposure to nanoplastics in the marine bivalve *Mytilus*. *Frontiers in Immunology* **1**, 426.

AUGUSTE, M., BALBI, T., MIGLIOLI, A., ALBERTI, S., PRANDI, S., NARIZZANO, R., SALIS, A., DAMONTE, G. & CANESI, L. (2021a). Comparison of different

commercial nanopolystyrenes: behavior in exposure media, effects on immune function and early larval development in the model bivalve *Mytilus galloprovincialis*. *Nanomaterials* **11**, 3291.

AUGUSTE, M., JEMEC KOKALJ, A., LEONESSI, M., BALBI, T., BOZZO, M., RISSO, B., CIACCI, C. & CANESI, L. (2022). Effects of textile polyester microfibers on *Mytilus galloprovincialis*. 32nd ESCPB congress, Naples 28-31st August, 2022.

AUGUSTE, M., MAYALL, C., BARBERO, F., HOCEVAR, M., ALBERTI, S., GRASSI, G., PUNTES, V. F., DROBNE, D. & CANESI, L. (2021b). Functional and morphological changes induced in hemocytes by selected nanoparticles. *Nanomaterials* **11**, 470.

BARANSKA, A., SHAWKET, A., JOUVE, M., BARATIN, M., MALOSSE, C., VOLUZAN, O., VU MANH, T.-P., FIORE, F., BAJÉNOFF, M., BENAROCH, P., DALOD, M., MALISSEN, M., HENRI, S. & MALISSEN, B. (2018). Unveiling skin macrophage dynamics explains both tattoo persistence and strenuous removal. *Journal of Experimental Medicine* **215**, 1115–1133.

BARBERO, F., RUSSO, L., VITALI, M., PIELLA, J., SALVO, I., BORRAJO, M. L., BUSQUETS-FITÉ, M., GRANDORI, R., BASTÚS, N. G., CASALS, E. & PUNTES, V. (2017). Formation of the protein corona: the interface between nanoparticles and the immune system. *Seminars in Immunology* **34**, 52–60.

BARELA HUDGELL, M. A., GRAYFER, L. & SMITH, L. C. (2022). A flow cytometry-based approach to identify distinct coelomocyte subsets of the purple sea urchin. *Strongylocentrotus purpuratus*. *Developmental & Comparative Immunology* **130**, 104352.

BARMO, C., CIACCI, C., CANONICO, B., FABBRI, R., CORTESE, K., BALBI, T., MARCOMINI, A., POJANA, G., GALLO, G. & CANESI, L. (2013). In vivo effects of n-TiO₂ on digestive gland and immune function of the marine bivalve *Mytilus galloprovincialis*. *Aquatic Toxicology* **132–133**, 9–18.

BARON, L., GOMBAULT, A., FANNY, M., VILLERET, B., SAVIGNY, F., GUILLOU, N., PANEK, C., LE BERT, M., LAGENTE, V., RASSENDREN, F., RITEAU, N. & COUILLIN, I. (2015). The NLRP3 inflammasome is activated by nanoparticles through ATP, ADP and adenosine. *Cell Death & Disease* **6**, e1629.

BARRENA, R., CASALS, E., COLÓN, J., FONT, X., SÁNCHEZ, A. & PUNTES, V. (2009). Evaluation of the ecotoxicity of model nanoparticles. *Chemosphere* **75**, 850–857.

BARTON, G. M. & KAGAN, J. C. (2009). A cell biological view of Toll-like receptor function: regulation through compartmentalization. *Nature Reviews in Immunology* **9**, 535–542.

BASTÚS, N. G., SÁNCHEZ-TILLÓ, E., PUJALS, S., FARRERA, C., KOGAN, M. J., GIRALT, E., CELADA, A., LLOBERAS, J. & PUNTES, V. (2009). Peptides conjugated to gold nanoparticles induce macrophage activation. *Molecular Immunology* **46**, 743–748.

BILEJ, M., PROCHAZKOVA, P., SILEROVA, M. & JOSKOVA, R. (2010). Earthworm immunity. *Advances in Experimental Medicine and Biology* **708**, 66–79.

BODÓ, K., BARANZINI, N., GIRARDELLO, R., KOKHANYUK, B., NEMETH, P., HAYASHI, Y., GRIMALDI, A. & ENGELMANN, P. (2020a). Nanomaterials and annelid immunity: a comparative survey to reveal the common stress and defense responses of two sentinel species to nanomaterials in the environment. *Biology* **9**, 307.

BODÓ, K., HAYASHI, Y., GERENCSER, G., LASZLO, Z., KERI, A., GALBACS, G., TELEK, E., MÉSZÁROS, M., DELI, M. A., KOKHANYUK, B., NÉMETHA, P. & ENGELMANN, P. (2020b). Species-specific sensitivity of *Eisenia* earthworms towards noble metal nanoparticles: a multiparametric *in vitro* study. *Environmental Science: Nano* **7**, 3509–3525.

BODÓ, K., KELLERMAYER, Z., LASZLO, Z., BOROS, A., KOKHANYUK, B., NEMETH, P. & ENGELMANN, P. (2021). Injury-induced innate immune response during segment regeneration of the earthworm, *Eisenia andrei*. *International Journal of Molecular Sciences* **22**, 2363.

BORASCHI, D. (2014). Nanoparticles and immunological frailty. In *Nanoparticles and the Immune System: Safety and Effects* (eds D. BORASCHI and A. DUSCHL). Elsevier-Academic Press, Oxford, UK.

BORASCHI, D. (2022). What is IL-1 for? The functions of interleukin-1 across evolution. *Frontiers in Immunology* **13**, 872155.

BORASCHI, D., ALIJAGIC, A., AUGUSTE, M., BARBERO, F., FERRARI, E., HERNADI, S., MAYALL, C., MICHELINI, S., PACHECO, N. I. N., PRINELLI, A., SWART, E., SWARTZWELTER, B. J., BASTÚS, N. G., CANESI, L., DROBNE, D., *ET AL.* (2020). Addressing nanomaterial immunosafety by evaluating innate immunity across living species. *Small* **16**, 2000598.

BORASCHI, D. & ITALIANI, P. (2016). Model validity in nanoimmunosafety: advantages and disadvantages of *in vivo vs. in vitro* models, and human *vs.* animal models. *Current Bionanotechnology* **2**, 71–76.

BORASCHI, D., ITALIANI, P., PALOMBA, R., DECUZZI, P., DUSCHL, A., FADEEL, B. & MOGHIMI, S. M. (2017). Nanoparticles and innate immunity: new perspectives on host defence. *Seminars in Immunology* **34**, 33–51.

BORASCHI, D., ITALIANI, P., WEIL, S. & MARTIN, M. U. (2018). The family of the interleukin-1 receptors. *Immunological Reviews* **281**, 197–232.

BORASCHI, D., LI, D., LI, Y. & ITALIANI, P. (2021). *In vitro* and *in vivo* models to assess the immune-related effects of nanomaterials. *International Journal of Environmental Research and Public Health* **18**, 11769.

BRENNAN, J. J. & GILMORE, T. D. (2018). Evolutionary origins of toll-like receptor signaling. *Molecular Biology and Evolution* **35**, 1576–1587.

BUCKLEY, K. M. & RAST, J. P. (2012). Dynamic evolution of Toll-like receptor multigene families in echinoderms. *Frontiers in Immunology* **3**, 136.

CANESI, L., AUGUSTE, M., BALBI, T. & PROCHAZKOVA, P. (2022). Soluble mediators of innate immunity in annelids and bivalve mollusks: a mini-review. *Frontiers in Immunology* **13**, 1051155.

CANESI, L., AUGUSTE, M. & BEBIANNO, M. J. (2019). Sublethal effects of nanoparticles on aquatic invertebrates, from molecular to organism level. In *Ecotoxicology of Nanoparticles in Aquatic Systems* (eds J. BLASCO and I. CORSI), pp. 38–61. CRC Press, Boca Raton, FL.

CANESI, L., BALBI, T., FABBRI, R., SALIS, A., DAMONTE, G., VOLLAND, M. & BLASCO, J. (2017). Biomolecular coronas in invertebrate species: implications in the environmental impact of nanoparticles. *NanoImpact* **8**, 89–98.

CANESI, L., CIACCI, C., BETTI, M., FABBRI, R., CANONICO, B., FANTINATI, A., MARCOMINI, A. & POJANA, G. (2008). Immunotoxicity of carbon black nanoparticles to blue mussel hemocytes. *Environment International* **34**, 1114–1119.

CANESI, L., CIACCI, C., FABBRI, R., BALBI, T., SALIS, A., DAMONTE, G., CORTESE, K., CARATTO, V., MONOPOLI, M. P., DAWSON, K., BERGAMI, E. & CORSI, I. (2016). Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: role of soluble hemolymph proteins. *Environmental Research* **150**, 73–81.

CANESI, L., CIACCI, C., FABBRI, R., MARCOMINI, A., POJANA, G. & GALLO, G. (2012). Bivalve molluscs as a unique target group for nanoparticle toxicity. *Marine Environmental Research* **76**, 16–21.

CANESI, L. & CORSI, I. (2016). Effects of nanomaterials on marine invertebrates. *Science of the Total Environment* **565**, 933–940.

CANESI, L., GAVIOLI, M., PRUZZO, C. & GALLO, G. (2002). Bacteria-hemocyte interactions and phagocytosis in marine bivalves. *Microscopy Research & Technique* **57**, 469–476.

CANESI, L. & PRUZZO, C. (2016). Specificity of innate immunity in bivalves: A lesson from bacteria. In *Lessons in Immunity: From Single-Cell Organisms to Mammals* (eds L. BALLARIN and M. CAMMARATA), pp. 79–91. Academic Press, Oxford, UK.

CHOU, H.-Y., LUN, C. M. & SMITH, L. C. (2018). SpTransformer proteins from the purple sea urchin opsonize bacteria, augment phagocytosis, and retard bacterial growth. *PLoS One* **13**, e0196890.

CHUMAKOV, K., AVIDAN, M. S., BENN, C. S., BERTOZZI, S. M., BLATT, L., CHANG, A. Y., JAMISON, D. T., KHADER, S. A., KOTTILI, S., NETEA, M. G., SPARROW, A. & GALLO, R. C. (2021). Old vaccines for new infections: exploiting innate immunity to control COVID-19 and prevent future pandemics. *Proceedings of the National Academy of Sciences USA* **118**, e2101718118.

CLOW, L. A., GROSS, P. S., SHIH, C.-S. & SMITH, L. C. (2000). Expression of SpC3, the sea urchin complement component, in response to lipopolysaccharide. *Immunogenetics* **51**, 1021–1033.

COURTOIS, P., RORAT, A., LEMIERE, S., LEVARD, C., CHAURAND, P., GROBELAK, A., LORS, C. & VANDENBULCKE, F. (2020). Accumulation, speciation and localization of silver nanoparticles in the earthworm *Eisenia fetida*. *Environmental Science & Pollution Research International* **28**, 3756–3765.

CRONIN, J. G., JONES, N., THORNTON, C. A., JENKINS, G. J. S., DOAK, S. H. & CLIFT, M. D. J. (2020). Nanomaterials and innate immunity: a perspective of the current status in nanosafety. *Chemical Research in Toxicology* **33**, 1061–1073.

CUVILLIER-HOT, V., BOIDDIN-WICHLACZ, C. & TASIEMSKI, A. (2014). Polychaetes as anneli moels to study ecoimmunology of marine organisms. *Journal of Marine Science and Technology* **22**, 9–14.

DAVIDSON, C. R., BEST, N. M., FRANCIS, J. W., COOPER, E. L. & WOO, T. C. (2008). Toll-like receptor genes (TLRs) from *Capitella capitata* and *Helobdella robusta* (Annelida). *Developmental & Comparative Immunology* **32**, 608–612.

DE MARCHI, L., NETO, V., PRETTI, C., FIGUEIRA, E., CHIELLINI, F., MORELLI, A., SOARES, A. M. V. M. & FREITAS, R. (2018). The influence of salinity on the effects of multi-walled carbon nanotubes on polychaetes. *Scientific Reports* **8**, 8571.

DELLA CAMERA, G., MADEJ, M., FERRETTI, A. M., LA SPINA, R., LI, Y., CORTEGGIO, A., HEINZL, T., SWARTZWELTER, B. J., SIPOS, G., GIORIA, S., PONTI, A., BORASCHI, D. & ITALIANI, P. (2021). Personalised profiling of innate immune memory induced by nano-imaging particles in human monocytes. *Frontiers in Immunology* **12**, 692165.

DIEZ-ORTIZ, M., LAHIVE, E., KILLE, P., POWELL, K., MORGAN, A. J., JURKSCHAT, K., VAN GESTEL, C. A. M., MOSSELMANS, J. F. W., SVENDSEN, C. & SPURGEON, D. J. (2015). Uptake routes and toxicokinetics of silver nanoparticles and silver ions in the earthworm *Lumbricus rubellus*. *Environmental Toxicology & Chemistry* **34**, 2263–2270.

DOLAR, A., DROBNE, D., NARAT, M. & JEMEC KOKALJ, A. (2022). Tire microplastics exposure in soil induces changes in expression profile of immune-related genes in terrestrial crustacean *Porcellio scaber*. *Environmetal Pollution* **314**, 120233.

DOSTERT, C., PÉTRILLI, V., VAN BRUGGEN, R., STEELE, C., MOSSMAN, B. T. & TSCHOPP, J. (2008). Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. *Science* **320**, 674–677.

DUAN, H., JIN, S., ZHANG, Y., LI, F. & XIANG, J. (2014). Granulocytes of the red claw crayfish *Cherax quadricarinatus* can endocytose beads, *E. coli* and WSSV, but in different ways. *Developmental & Comparative Immunology* **46**, 186–193.

DYACHUK, V. A. (2016). Hematopoiesis in *Bivalvia* larvae: cellular origin, differentiation of hemocytes, and neoplasia. *Developmental & Comparative Immunology* **65**, 253–257.

EBERT, T. A. (2008). Longevity and lack of senescence in the red sea urchin *Strongylocentrotus franciscanus*. *Experimental Gerontology* **43**, 734–738.

ENGELMANN, P., HAYASHI, Y., BODÓ, K., ERNSZT, D., SOMOGYI, I., STEIB, A., ORBÁN, J., POLLÁK, E., NYITRAI, M., NÉMETH, P. & MOLNÁR, L. (2016a). Phenotypic and functional characterization of earthworm coelomocyte subsets: linking light scatter-based cell typing and imaging of the sorted populations. *Developmental & Comparative Immunology* **65**, 41–52.

ENGELMANN, P., HAYASHI, Y., BODÓ, K. & MOLNÁR, L. (2016b). New aspects of earthworm innate immunity: novel molecules and old proteins with unexpected functions. In *Lessons in Immunity* (eds L. BALLARIN and M. CAMMARATA), pp. 53–66. Academic Press, Oxford, UK.

FADEEL, B. (2012). Clear and present danger? Engineered nanoparticles and the immune system. *Swiss Medical Weekly* **142**, w13609.

FADEEL, B. (2019). Hide and seek: nanomaterial interactions with the immune system. *Frontiers in Immunology* **10**, 133.

FADEEL, B., FARCAL, L., HARDY, B., VÁSQUEZ-CAMPOS, S., HRISTOZOV, D., MARCOMINI, A., LYNCH, I., VALSAMI-JONES, E., ALENIUS, H. & SAVOLAINEN, K. (2018). Advanced tools for the safety assessment of nanomaterials. *Nature Nanotechnology* **13**, 537–543.

FAIRBAIRN, E. A., KELLER, A. A., MADLER, L., ZHOU, D., POKHREL, S. & CHERR, G. N. (2011). Metal oxide nanomaterials in seawater: linking physicochemical characteristics with biological response in sea urchin development. *Journal of Hazardous Materials* **192**, 1565–1571.

FALUGI, C., ALUIGI, M. G., CHIANTORE, M. C., PRIVITERA, D., RAMOINO, P., GATTI, M. A., FABRIZI, A., PINSINO, A. & MATRANGA, V. (2012). Toxicity of metal oxide nanoparticles in immune cells of the sea urchin. *Marine Environmental Research* **76**, 114–121.

FARRERA, C., BHATTACHARYA, K., LAZZARETTO, B., ANDÓN, F. T., HULTENBY, K., KOTCHEY, G. P., STARC, A. & FADEEL, B. (2014). Extracellular entrapment and degradation of single-walled carbon nanotubes. *Nanoscale* **6**, 6974–6983.

FEICHTMEIER, N. S., WALTHER, P. & LEOPOLD, K. (2015). Uptake, effects, and regeneration of barley plants exposed to gold nanoparticles. *Environmental Science & Pollution Research* **22**, 549–8558.

FERRARI, E. (2022). Risk assessment of gold nanoparticle in a stable *Arabidopsis* exposition system. PhD Thesis: University of Tübingen, Tübingen, Germany.

FERRARI, E., BARBERO, F., BUSQUETS-FITÉ, M., FRANZ-WACHTEL, M., KÖHLER, H.-R., PUNTES, V. & KEMMERLING, B. (2021). Growth-promoting gold nanoparticles decrease stress responses in *Arabidopsis* seedlings. *Nanomaterials* **11**, 3161.

GARCÍA-GARCÍA, E., PRADO-ALVAREZ, M., NOVOA, B., FIGUERAS, A. & ROSALES, C. (2008). Immune responses of mussel hemocyte subpopulations are differentially regulated by enzymes of the PI 3-K, PKC, and ERK kinase families. *Developmental & Comparative Immunology* **32**, 637–653.

GARCIA-VELASCO, N., GANDARIASBEITIA, M., IRIZAR, A. & SOTO, M. (2016). Uptake route and resulting toxicity of silver nanoparticles in *Eisenia fetida* earthworm exposed through standard OECD tests. *Ecotoxicology* **25**, 1543–1555.

GAUTAM, A., RAY, A., MUKHERJEE, S., DAS, S., PAL, K., DAS, S., KARMAKAR, P., RAY, M. & RAY, S. (2018). Immunotoxicity of copper nanoparticle and copper sulfate in a common Indian earthworm. *Ecotoxicology & Environmental Safety* **148**, 620–631.

GEISER, M., CASAULTA, M., KUPFERSCHMID, B., SCHULZ, H., SEMMLER-BEHNKE, M. & KREYLING, W. (2008). The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles. *American Journal of Respiratory Cell & Molecular Biology* **38**, 371–376.

GENEVIÈRE, A.-M., DERELLE, E., ESCANDE, M. L., GRIMSLEY, N., KLOPP, C., MÉNAGER, C., MICHEL, A. & MOREAU, H. (2020). Responses to iron oxide and zinc oxide nanoparticles in echinoderm embryos and microalgae: uptake, growth, morphology, and transcriptomic analysis. *Nanotoxicology* **14**, 1342–1361.

GERDOL, M., GOMEZ-CHIARRI, M., CASTILLO, M. G., FIGUERAS, A., FIORITO, G., MOREIRA, R., NOVOA, B., PALLAVICINI, A., PONTE, G., ROUMBEDAKIS, K., VENIER, P. & VASTA, G. R. (2018). Immunity in molluscs: recognition and effector mechanisms, with a focus on *Bivalvia*. In *Advances in Comparative Immunology* (ed. E. L. COOPER), pp. 225–341. Springer, Berlin, Heidelberg, Germany.

GHEZZI, P., FLORIDI, L., BORASCHI, D., CUADRADO, A., MANDA, G., LEVIC, S., D'ACQUISTO, F., HAMILTON, A., ATHERSUCH, T. J. & SELLEY, L. (2017). Oxidative stress and inflammation induced by environmental and psychological stressors: a biomarker perspective. *Antioxidants & Redox Signaling* **28**, 852–872.

GIANAZZA, E., EBERINI, I., PALAZZOLO, L. & MILLER, I. (2021). Hemolymph proteins: an overview across marine arthropods and molluscs. *Journal of Proteomics* **245**, 104294.

GIGAULT, J., TER HALLE, A., BAUDRIMONT, M., PASCAL, P.-Y., GAUFFRE, F., PHI, T.-L., EL HADRI, H., GRASSI, B. & REYNAUD, S. (2018). Current opinion: what is a nanoplastic? *Environmental Pollution* **235**, 1030–1034.

GIRARDELLO, R., BARANZINI, N., TETTAMANTI, G., DE EGUILEOR, M. & GRIMALDI, A. (2017). Cellular responses induced by multi-walled carbon nanotubes: in vivo and in vitro studies on the medicinal leech macrophages. *Scientific Reports* **7**, 8871.

GIRARDELLO, R., DRAGO, F., DE EGUILEOR, M., VALVASSORI, R., VIZIOLI, J., TETTAMANTI, G. & GRIMALDI, A. (2015a). Cytokine impregnated biomatrix: A new tool to study multi-wall carbon nanotubes effects on invertebrate immune cells. *Journal of Nanomedicine & Nanotechnology* **6**, 1000323.

GIRARDELLO, R., TASSELLI, S., BARANZINI, N., VALVASSORI, R., DE EGUILEOR, M. & GRIMALDI, A. (2015b). Effects of carbon nanotube environmental dispersion on an aquatic invertebrate *Hirudo medicinalis*. *PLoS One* **10**, e0144361.

GOMES, S. I., HANSEN, D., SCOTT-FORDSMAND, J. J. & AMORIM, M. J. (2015). Effects of silver nanoparticles to soil invertebrates: oxidative stress biomarkers in *Eisenia fetida*. *Environmental Pollution* **199**, 49–55.

GRIFFITH, J. W., SOKOL, C. L. & LUSTER, A. D. (2014). Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annual Review of Immunology* **32**, 659–702.

GUNJAN, B., ZAIDI, M. G. H. & SANDEEP, A. (2014). Impact of gold nanoparticles on physiological and biochemical characteristics of *Brassica juncea*. *Journal of Plant Biochemistry & Physiology* **2**, 133.

GUPTA, S., KUSHWAH, T. & YADAV, S. (2014). Earthworm coelomocytes as nanoscavenger of ZnO NPs. *Nanoscale Research Letters* **9**, 259.

GUST, A. A., PRUITT, R. & NÜRNBERGER, T. (2017). Sensing danger: key to activating plant immunity. *Trends in Plant Science* **22**, 779–791.

HABIB, Y. J. & ZHANG, Z. (2020). The involvement of crustacean toll-like receptors in pathogen recognition. *Fish & Shellfish Immunology* **102**, 169–176.

HAN, G.-Z. (2019). Origin and evolution of the plant immune system. *New Phytologist* **222**, 70–83.

HAYASHI, F., MEANS, T. K. & LUSTER, A. D. (2003). Toll-like receptors stimulate human neutrophil function. *Blood* **102**, 2660–2669.

HAYASHI, Y., ENGELMANN, P., FOLDBJERG, R., SZABÓ, M., SOMOGYI, I., POLLÁK, E., MOLNÁR, L., AUTRUP, H., SUTHERLAND, D. S., SCOTT-FORDSMAND, J. & HECKMANN, L.-H. (2012). Earthworms and humans *in vitro*: characterizing evolutionarily conserved stress and immune responses to silver nanoparticles. *Environmental Science & Technology* **46**, 4166–4173.

HAYASHI, Y., MICLAUS, T., ENGELMANN, P., AUTRUP, H., SUTHERLAND, D. S. & SCOTT-FORDSMAND, J. J. (2016). Nanosilver pathophysiology in earthworms: transcriptional profiling of secretory proteins and the implication for the protein corona. *Nanotoxicology* **10**, 303–311.

HAYASHI, Y., MICLAUS, T., SCAVENIUS, C., KWIATKOWSKA, K., SOBOTA, A., ENGELMANN, P., SCOTT-FORDSMAND, J. J., ENGHILD, J. J. & SUTHERLAND, D. S. (2013). Species differences take shape at nanoparticles: protein corona made of the native repertoire assists cellular interaction. *Environmental Science & Technology* **47**, 14367–14375.

HERNANDEZ-VIEZCAS, J. A., CASTILLO-MICHEL, H., ANDREWS, J. C., COTTE, M., RICO, C., PERALTA-VIDEA, J. R., GE, Y., PRIESTER, J. H., HOLDEN, P. A. & GARDEA-TORRESDEY, J. L. (2013). *In situ* synchrotron X-ray fluorescence mapping and speciation of CeO$_2$ and ZnO nanoparticles in soil cultivated soybean (*Glycine max*). *ACS Nano* **7**, 1415–1423.

HORNUNG, V., BAUERNFEIND, F., HALLE, A., SAMSTAD, E. O., KONO, H., ROCK, K. L., FITZGERALD, K. A. & LATZ, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nature Immunology* **9**, 847–856.

IKERD, J. L., BURNETT, K. G. & BURNETT, L. E. (2015). Effects of salinity on the accumulation of hemocyte aggregates and bacteria in the gills of *Callinectes sapidus*, the Atlantic blue crab, injected with *Vibrio campbellii*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **183**, 97–106.

ITALIANI, P. & BORASCHI, D. (2017). Induction of innate immune memory by engineered nanoparticles, an hypothesis that may become true. *Frontiers in Immunology* **8**, 734.

ITALIANI, P., MOSCA, E., DELLA CAMERA, G., MELILLO, D., MIGLIORINI, P., MILANESI, L. & BORASCHI, D. (2020). Profiling the course of resolving *vs.* persistent inflammation in human monocytes: the role of IL-1 family molecules. *Frontiers in Immunology* **11**, 1426.

IWANAGA, S. & LEE, B. L. (2005). Recent advances in the innate immunity of invertebrate animals. *Journal of Biochemistry & Molecular Biology* **38**, 128–150.

JIN, X. K., LI, W. W., WU, M. H., GUO, X. N., LI, S., YU, A. Q., ZHU, T. Y., HE, L. & WANG, Q. (2013). Immunoglobulin superfamily protein Dscam exhibited molecular diversity by alternative splicing in hemocytes of crustacean, *Eriocheir sinensis*. *Fish and Shellfish Immunology* **35**, 900–909.

JUAREZ, E., NUÑEZ, C., SADA, E., ELLNER, J. J., SCHWANDER, S. K. & TORRES, M. (2010). Differential expression of toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. *Respiratory Research* **11**, 2.

KAGAN, V. E., KONDURU, N. V., FENG, W., ALLEN, B. L., CONROY, J., VOLKOV, Y., VLASOVA, I. I., BELIKOVA, N. A., YANAMALA, N., KAPRALOV, A., TYURINA, Y. Y., SHI, J., KISIN, E. R., MURRAY, A. R., FRANKS, J., ET AL. (2010). Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. *Nature Nanotechnology* **5**, 354–359.

KODALI, V., LITTKE, M. H., TILTON, S. C., TEEGUARDEN, J. G., SHI, L., FREVERT, C. W., WANG, W., POUNDS, J. G. & THRALL, B. D. (2013). Dysregulation of macrophage activation profiles by engineered nanoparticles. *ACS Nano* **8**, 6007–7010.

KRANJC, E. & DROBNE, D. (2019). Nanomaterials in plants: a review of hazard and applications in the Agri-food sector. *Nanomaterials* **9**, 1094.

KRANJC, E., MAZEJ, D., REGVAR, M., DROBNE, D. & REMSKAR, M. (2018). Foliar surface free energy affects platinum nanoparticle adhesion, uptake, and translocation from leaves to roots in arugula and escarole. *Environmental Science Nano* **5**, 520–532.

KRUG, H. F. & WICK, P. (2011). Nanotoxicology: an interdisciplinary challenge. *Angewandte Chemie* **50**, 1260–1278.

KUKLA, S. P., SLOBODSKOVA, V. V., ZHURAVEL, A. V., MAZUR, A. A. & CHELOMIN, V. P. (2022). Exposure of adult sand dollars (*Scaphechinus mirabilis*) (Agassiz, 1864) to copper oxide nanoparticles induces gamete DNA damage. *Environmental Science & Pollution Research* **29**, 39451–39460.

KUMAR, V., GULERIA, P., KUMAR, V. & YADAV, S. K. (2013). Gold nanoparticle exposure induces growth and yield enhancement in *Arabidopsis thaliana*. *Science of the Total Environment* **461–462**, 462–468.

LAI, A. G. & ABOOBAKER, A. A. (2017). Comparative genomic analysis of innate immunity reveals novel and conserved components in crustacean food crop species. *BMC Genomics* **18**, 389.

LAPIED, E., MOUDILOU, E., EXBRAYAT, J. M., OUGHTON, D. H. & JONER, E. J. (2010). Silver nanoparticle exposure causes apoptotic response in the earthworm *Lumbricus terrestris* (Oligochaeta). *Nanomedicine* **5**, 975–984.

LEBRE, F., BOLAND, J. B., GOUVEIA, P., GORMAN, A. L., LUNDAHL, M. L. E., LYNCH, R. I., O'BRIEN, F. J., COLEMAN, J. & LAVELLE, E. C. (2020). Pristine graphene induces innate immune training. *Nanoscale* **12**, 11192–11200.

LEFEBVRE, C., VANDENBULCKE, F., BOCQUET, B., TASIEMSKI, A., DESMONS, A., VERSTRAETE, M., SALZET, M. & COCQUERELLE, C. (2008). Cathepsin L and cystatin B gene expression discriminates immune coelomic cells in the leech *Theromyzon tessulatum*. *Developmental & Comparative Immunology* **32**, 795–807.

LI, F., CHANG, X., XU, L. & YANG, F. (2018a). Different roles of crayfish hemocytes in the uptake of foreign particles. *Fish & Shellfish Immunology* **77**, 112–119.

LI, R., CHEN, R., CHEN, P., WEN, Y., KE, P. C. & CHO, S. S. (2013). Computational and experimental characterizations of silver nanoparticle-apolipoprotein bio-corona. *The Journal of Physical Chemistry B* **117**, 13451–13456.

LI, X. J., YANG, L., LI, D., ZHU, Y. T., WANG, Q. & LI, W. W. (2018b). Pathogen-specific binding soluble Down syndrome cell adhesion molecule (Dscam) regulates phagocytosis via membrane-bound Dscam in crab. *Frontiers in Immunology* **9**, 801.

LI, Y. & BORASCHI, D. (2016). Endotoxin contamination: a key element in the interpretation of nanosafety studies. *Nanomedicine* **11**, 269–287.

LI, Y., ITALIANI, P., CASALS, E., VALKENBORG, D., MERTENS, I., BAGGERMAN, G., NELISSEN, I., PUNTES, V. F. & BORASCHI, D. (2016). Assessing the immunosafety of engineered nanoparticles with a novel *in vitro* model based on human primary monocytes. *ACS Applied Materials & Interfaces* **8**, 28437–28447.

LI, Y., SHI, Z., RADAUER-PREIML, I., ANDOSCH, A., CASALS, E., LUETZ-MEIDL, U., COBALEDA, M., LIN, Z., JABERI-DOURAKI, M., ITALIANI, P., HOREJS-HOECK, J., HIMLY, M., MONTEIRO-RIVIERE, N. A., DUSCHL, A., PUNTES, V. F., ET AL. (2017). Bacterial endotoxin (LPS) binds to the surface of gold nanoparticles, interferes with biocorona formation and induces human monocyte inflammatory activation. *Nanotoxicology* **11**, 1157–1175.

LIU, N., LAN, J. F., SUN, J. J., JIA, W. M., ZHAO, X. F. & WANG, J. X. (2015). A novel crustin from *Marsupenaeus japonicus* promotes hemocyte phagocytosis. *Developmental and Comparative Immunology* **49**, 313–322.

LIU, S., ZHENG, S. C., LI, Y. L., LI, J. & LIU, H. P. (2020). Hemocyte-mediated phagocytosis in crustaceans. *Frontiers in Immunology* **11**, 268.

LIU, Z., YU, P., CAI, M., WU, D., ZHANG, M., CHEN, M. & ZHAO, Y. (2019). Effects of microplastics on the innate immunity and intestinal microflora of juvenile *Eriocheir sinensis*. *Science of the Total Environment* **685**, 836–846.

LORENZON, S. (2005). Hyperglycemic stress response in Crustacea. *Invertebrate Survival Journal* **2**, 132–141.

LUO, Y. & WANG, W.-X. (2022). Roles of hemocyte subpopulations in silver nanoparticle transformation and toxicity in the oysters *Crassostrea hongkongensis*. *Environmental Pollution* **305**, 119281.

LV, J., ZHANG, S., LUO, L., ZHANG, J., YANG, K. & CHRISTIED, P. (2015). Accumulation, speciation and uptake pathway of ZnO nanoparticles in maize. *Environmental Science Nano* **2**, 68–77.

MAHAKHAM, W., THEERAKULPISUT, P., MAENSIRI, S., PHUMYING, S. & SARMAH, A. (2016). Environmentally benign synthesis of phytochemicals-capped gold nanoparticles as nanopriming agent for promoting maize seed germination. *Science of the Total Environment* **573**, 1089–1102.

MARTIN, G. G., QUINTERO, M., QUIGLEY, M. & KHOSROVIAN, H. (2000). Elimination of sequestered material from the gills of decapod crustaceans. *Journal of Crustacean Biology* **20**, 209–217.

MARTINON, F., PÉTRILLI, V., MAYOR, A., TARDIVEL, A. & TSCHOPP, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237–241.

MATOS, G. M. & ROSA, F. D. (2021). On the silver jubilee of crustacean antimicrobial peptides. *Reviews in Aquaculture* **14**, 594–612.

MATOZZO, V., GALLO, C. & MARIN, M. G. (2011). Effects of temperature on cellular and biochemical parameters in the crab *Carcinus aestuarii* (Crustacea, Decapoda). *Marine Environmental Research* **71**, 351–356.

MATZINGER, P. (2002). The danger model: a renewed sense of self. *Science* **296**, 301–305.

MAYALL, C., DOLAR, A., JEMEC KOKALJ, A., NOVAK, S., RAZINGER, J., BARBERO, F. & DROBNE, D. (2021). Stressor-dependent changes in immune parameters in the terrestrial isopod crustacean, *Porcellio scaber*: a focus on nanomaterials. *Nanomaterials* **11**, 934.

MEHENNAOUI, K., CAMBIER, S., SERCHI, T., ZIEBEL, J., LENTZEN, E., VALLE, N., GUÉROLD, F., THOMANN, J.-S., GIAMBERINI, L. & GUTLEB, A. C. (2018). Do the pristine physico-chemical properties of silver and gold nanoparticles influence uptake and molecular effects on *Gammarus fossarum* (Crustacea Amphipoda)? *Science of the Total Environment* **643**, 1200–1215.

MENG, J., ZHANG, G. & WANG, W. X. (2022). Functional heterogeneity of immune defenses in molluscan oysters *Crassostrea hongkongensis* revealed by high-throughput single-cell transcriptome. *Fish and Shellfish Immunology* **120**, 202–213.

MOGHIMI, S. M. & SIMBERG, D. (2017). Complement activation turnover on surface of nanoparticles. *Nano Today* **15**, 8–10.

MONKS, J., ROSNER, D., JON GESKE, F., LEHMAN, L., HANSON, L., NEVILLE, M. C. & FADOK, V. A. (2005). Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. *Cell Death and Differentiation* **12**, 107–114.

MORISHIGE, T., YOSHIOKA, Y., INAKURA, H., TANABE, A., YAO, X., NARIMATSU, S., MONOBE, Y., IMAZAWA, T., TSUNODA, S.-I., TSUTSUMI, Y., MUKAI, Y., OKADA, N. & NAKAGAWA, S. (2010). The effect of surface modification of amorphous silica particles on NLRP3 inflammasome mediated IL-1β production, ROS production and endosomal rupture. *Biomaterials* **31**, 6833–6842.

MOWAT, A., SCOTT, C. & BAIN, C. (2017). Barrier-tissue macrophages: functional adaptation to environmental challenges. *Nature Medicine* **23**, 1258–1270.

MUKHERJEE, S. P., BONDARENKO, O., KOHONEN, P., ANDÓN, F. T., BRZICOVÁ, T., GESSNER, I., MATHUR, S., BOTTINI, M., CALLIGARI, P., STELLA, L., KISIN, E., SHVEDOVA, A., AUTIO, R., SALMINEN-MANKONEN, H., LAHESMAA, R. & FADEEL, B. (2018). Macrophage sensing of single-walled carbon nanotubes via Toll-like receptors. *Scientific Reports* **8**, 1115.

MUKHERJEE, S. P., GLIGA, A. R., LAZZARETTO, B., BRANDNER, B., FIELDEN, M., VOGT, C., NEWMAN, L., RODRIGUES, A. F., SHAO, W., FOURNIER, P. M., TOPRAK, M. S., STAR, A., KOSTARELOS, K., BHATTACHARYA, K. & FADEEL, B. (2015). Graphene oxide is degraded by neutrophils and the degradation products are non-genotoxic. *Nanoscale* **10**, 1180–1188.

MURPHY, K. M., WEAVER, C. & BERG, L. (2022). *Janeway's Immunobiology*, Tenth Edition. W.W. Norton & Company, New York.

NEAL, M. D., LEAPHART, C., LEVY, R., PRINCE, J., BILLIAR, T. R., WATKINS, S., LI, J., CETIN, S., FORD, H., SCHREIBER, A. & HACKMAN, D. J. (2006). Enterocyte TLR4 mediates phagocytosis and translocation of bacteria across the intestinal barrier. *The Journal of Immunology* **176**, 3070–3079.

NGOU, B. P. M., DING, P. & JONES, J. D. G. (2022). Thirty years of resistance: zig-zag through the plant immune system. *The Plant Cell* **34**, 1447–1478.

NOVO, M., LAHIVE, E., DIEZ-ORTIZ, M., MATZKE, M., MORGAN, A. J., SPURGEON, D. J., SVENDSEN, C. & KILLE, P. (2015). Different routes, same pathways: molecular mechanisms under silver ion and nanoparticle exposures in the soil sentinel *Eisenia fetida*. *Environmental Pollution* **205**, 385–393.

NÜRNBERGER, T., BRUNNER, F., KEMMERLING, B. & PIATER, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. *Immunologic Research* **198**, 249–266.

OBERDÖRSTER, G., OBERDÖRSTER, E. & OBERDÖRSTER, J. (2005). Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environmental Health Perspectives* **113**, 823–839.

PACHECO, I. & BUZEA, C. (2018). Nanoparticle uptake by plants: beneficial or detrimental? In *Phytotoxicity of Nanoparticles* (eds M. FAISAL, Q. SAQUIB, A. ALATAR and A. AL-KHEDHAIRY), pp. 1–61. Springer, Cham, Switzerland.

PACHECO, N. I. N., ROUBALOVA, R., DVORAK, J., BENADA, O., PINKAS, D., KOFRONOVA, O., SEMERAD, J., PIVOKONSKY, M., CAJTHAML, T., BILEJ, M. & PROCHAZKOVA, P. (2021a). Understanding the toxicity mechanism of CuO nanoparticles: the intracellular view of exposed earthworm cells. *Environmental Science: Nano* **8**, 2464–2477.

Pacheco, N. I. N., Roubalova, R., Semerad, J., Grasserova, A., Benada, O., Kofronova, O., Cajthaml, T., Dvorak, J., Bilej, M. & Prochazkova, P. (2021*b*). *In vitro* interactions of TiO$_2$ nanoparticles with earthworm coelomocytes: immunotoxicity assessment. *Nanomaterials* 11, 250.

Pacheco, N. I. N., Semerad, J., Pivokonsky, M., Cajthaml, T., Filip, J., Busquets-Fité, M., Dvorak, J., Rico, A. & Prochazkova, P. (2022). Effects of silver sulfide nanoparticles on the earthworm *Eisenia andrei*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 257, 109355.

Pezzati, E., Canesi, L., Damonte, G., Salis, A., Marsano, F., Grande, C., Vezzulli, L. & Pruzzo, C. (2015). Susceptibility of *Vibrio aestuarianus* 01/032 to the antibacterial activity of *Mytilus* haemolymph: identification of a serum opsonin involved in mannose-sensitive interactions. *Environmental Microbiology* 17, 4271–4279.

Pikula, K., Zakharenko, A., Chaika, V., Em, I., Nikitina, A., Avtomonov, E., Tregubenko, A., Agoshkov, A., Mishakov, I., Kuznetsov, V., Gusev, A., Park, S. & Golokhvast, K. (2020). Toxicity of carbon, silicon, and metal-based nanoparticles to sea urchin *Strogylocentrotes intermedius*. *Nanomaterials* 10, 1825.

Pinsino, A., Bastús, N. G., Busquets-Fité, M., Canesi, L., Cesaroni, P., Drobne, D., Duschl, A., Ewart, M.-A., Gispert, I., Horejs-Hoeck, J., Italiani, P., Kemmerling, B., Kille, P., Procházková, P., Puntes, V. F., et al. (2020). Probing the immunological responses to nanoparticles across environmental species: a perspective of the EU-funded PANDORA project. *Environmental Science: Nano* 7, 3216–3232.

Pinsino, A. & di Bernardo, M. (2022). Immunosafe(r)-by-design nanoparticles: molecular targets and cell signaling pathways in a next-generation model proxy for humans. *Advances in Protein Chemistry & Structural Biology* 130, 325–350.

Pinsino, A., Russo, R., Bonaventura, R., Brunelli, A., Marcomini, A. & Matranga, V. (2015). Titanium dioxide nanoparticles stimulate sea urchin immune cell phagocytic activity involving TLR/p38 MAPK-mediated signalling pathway. *Scientific Reports* 5, 14492.

Prochazkova, P., Roubalova, R., Skanta, F., Dvorak, J., Pacheco, N. I. N., Kolarik, M. & Bilej, M. (2019). Developmental and immune role of a novel multiple cysteine cluster TLR from *Eisenia andrei* earthworms. *Frontiers in Immunology* 10, 1277.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S. & Medzhitov, R. (2004). Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 118, 229–241.

Ribeiro, F., van Gestel, C. A. M., Pavlaki, M. D., Azevedo, S., Soares, A. M. V. M. & Loureiro, S. (2017). Bioaccumulation of silver in *Daphnia magna*: waterborne and dietary exposure to nanoparticles and dissolved silver. *Science of the Total Environment* 574, 1633–1639.

Roelofs, D., Makama, S., de Boer, T. E., Vooijs, R., van Gestel, C. A. M. & van den Brink, N. W. (2020). Surface coating and particle size are main factors explaining the transcriptome-wide responses of the earthworm *Lumbricus rubellus* to silver nanoparticles. *Environmental Science: Nano* 7, 1179–1193.

Russell, M. P., Ebert, T. A., Garcia, V. & Bodnar, A. (2012). Field and laboratory growth estimates of the sea urchin *Lytechinus variegatus* in Bermuda. In *Echinoderms in a Changing World* (ed. C. Johnson), pp. 133–139. CRC Press, Boca Raton, FL.

Sabo-Attwood, T., Unrine, J. M., Stone, J. W., Murphy, C. J., Ghoshroy, S., Blom, D., Bertsch, P. M. & Newman, L. A. (2012). Uptake, distribution and toxicity of gold nanoparticles in tobacco (*Nicotiana xanthi*) seedlings. *Nanotoxicology* 6, 353–360.

Saleeb, N., Robinson, B., Cavanagh, J., Ross, J., Munir, K. & Gooneratne, R. (2020). Antioxidant enzyme activity and lipid peroxidation in *Aporrectodea caliginosa* earthworms exposed to silver nanoparticles and silver nitrate in spiked soil. *Environmental Toxicology & Chemistry* 39, 1257–1266.

Sánchez-Paz, A. & Muhlia-Almazán, A. (2020). Uncovering and defragmenting the role of the Toll pathway in the innate immune responses of cultured crustaceans against viral pathogens. *Reviews in Aquaculture* 12, 1818–1835.

Sanzari, I., Leone, A. & Ambrosone, A. (2019). Nanotechnology in plant science: to make a long story short. *Frontiers in Bioengineering & Biotechnology* 7, 120.

Satake, H. & Sekiguchi, T. (2012). Toll-like receptors of deuterostome invertebrates. *Frontiers in Immunology* 3, 34.

Schwab, F., Zhai, G., Kern, M., Turner, A., Schnoor, J. L. & Wiesner, M. R. (2016). Barriers, pathways and processes for uptake, translocation and accumulation of nanomaterials in plants – critical review. *Nanotoxicology* 10, 257–278.

Semerad, J., Pacheco, N. I. N., Grasserova, A., Prochazkova, P., Pivokonsky, M., Pivokonska, L. & Cajthaml, T. (2020). *In vitro* study of the toxicity mechanisms of nanoscale zero-valent iron (nZVI) and released iron ions using earthworm cells. *Nanomaterials* 10, 2189.

Sendra, M., Carrasco-Braganza, M. I., Yeste, P. M., Vila, M. & Blasco, J. (2020). Immunotoxicity of polystyrene nanoplastics in different hemocyte subpopulations of *Mytilus galloprovincialis*. *Scientific Reports* 10, 8637.

Shi, W., Han, Y., Guo, C., Zhao, X., Liu, S., Su, W., Zha, S., Wang, Y. & Liu, G. (2017). Immunotoxicity of nanoparticle nTiO(2) to a commercial marine bivalve species, *Tegillarca granosa*. *Fish & Shellfish Immunology* 6, 300–306.

Siddiqi, K. S. & Husen, A. (2016). Engineered gold nanoparticles and plant adaptation potential. *Nanoscale Research Letters* 11, 400.

Siegel, J., Záruba, K., Svorcík, V., Kroumanová, K., Burketová, L. & Martinec, J. (2018). Round-shape gold nanoparticles: effect of particle size and concentration on *Arabidopsis thaliana* root growth. *Nanoscale Research Letters* 13, 95.

Sihombing, M. A. E. M., Safitri, M., Zhou, T., Wang, L., McGinty, S., Zhang, H.-J., Yin, Y., Peng, Q., Qiu, J. & Wang, G. (2021). Unexpected role of nonimmune cells: amateur phagocytes. *DNA and Cell Biology* 40, 157–171.

Skanta, F., Roubalova, R., Dvorak, J., Prochazkova, P. & Bilej, M. (2013). Molecular cloning and expression of TLR in the *Eisenia andrei* earthworm. *Developmental & Comparative Immunology* 41, 694–702.

Smith, L. C., Arizza, V., Barela Hudgell, M. A., 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., et al. (2018). Echinodermata: the complex immune system in echinoderms. In *Advances in Comparative Immunology* (ed. E. L. Cooper), pp. 409–501. Springer, Cham, Switzerland.

Spielman-Sun, E., Avellan, A., Bland, G. D., Tappero, R. V., Acerbo, A. S., Unrine, J. M., Giraldo, J. P. & Lowry, G. V. (2019). Nanoparticle surface charge influences translocation and leaf distribution in vascular plants with contrasting anatomy. *Environmental Science: Nano* 6, 2508–2519.

Strober, W., Fuss, I. J. & Blumberg, R. S. (2002). The immunology of mucosal models of inflammation. *Annual Reviews in Immunology* 20, 495–549.

Sun, H., Zhou, Z., Dong, Y., Yang, A., Jiang, B., Gao, S., Chen, Z., Guan, X., Wang, B. & Wang, X. (2013). Identification and expression analysis of two toll-like receptor genes from sea cucumber (*Apostichopus japonicus*). *Fish & Shellfish Immunology* 34, 147–158.

Swart, E., Dvorak, J., Hernadi, S., Goodall, T., Kille, P., Spurgeon, D., Svendsen, C. & Prochazkova, P. (2020). The effects of *in vivo* exposure to copper oxide nanoparticles on the gut microbiome, host immunity, and susceptibility to a bacterial infection in earthworms. *Nanomaterials* 10, 1337.

Swartzwelter, B. J., Barbero, F., Verde, A., Mangini, M., Pirozzi, M., de Luca, A. C., Puntes, V. F., Leite, L. C. C., Italiani, P. & Boraschi, D. (2020). Gold nanoparticles modulate BCG-induced innate immune memory in human monocytes by shifting the memory response towards tolerance. *Cell* 9, 284.

Swartzwelter, B. J., Mayall, C., Alijagic, A., Barbero, F., Ferrari, E., Hernadi, S., Michelini, S., Pacheco, N. I. N., Prinelli, A., Swart, E. & Auguste, M. (2021). Cross-species comparisons of nanoparticles interactions with innate immune system: a methodological review. *Nanomaterials* 11, 1528.

Tasiemski, A. & Salzet, M. (2017). Neuro-immune lessons from an annelid: the medicinal leech. *Developmental & Comparative Immunology* 66, 33–42.

Tassia, M. G., Whelen, N. V. & Halanych, K. M. (2017). Toll-like receptor pathway evolution in deuterostomes. *Proceedings of the National Academy of Sciences USA* 114, 7055–7060.

Taylor, A. F., Rylott, E. L., Anderson, C. W. N. & Bruce, N. C. (2014). Investigating the toxicity, uptake, nanoparticle formation and genetic response of plants to gold. *PLoS One* 9, e93793.

Thit, A., Banta, G. T. & Selck, H. (2015). Bioaccumulation, subcellular distribution and toxicity of sediment-associated copper in the ragworm *Nereis diversicolor*: the relative importance of aqueous copper, copper oxide nanoparticles and microparticles. *Environmental Pollution* 202, 50–57.

Tiwari, M., Krishnamurthy, S., Shukla, D., Kiiskila, J., Jain, A., Datta, R., Sharma, N. & Sahi, S. V. (2016). Comparative transcriptome and proteome analysis to reveal the biosynthesis of gold nanoparticles in *Arabidopsis*. *Scientific Reports* 6, 21733.

Tomsić, S., Conides, A., Radić, I. D. & Glamuzina, B. (2010). Growth, size class frequency and reproduction of purple sea urchin, *Paracentrotus lividus* (Lamarck, 1816) in Bistrina Bay (Adriatic Sea, Croatia). *Acta Adriatica* 51, 67.

Tran, N. T., Kong, T., Zhang, M. & Li, S. (2020). Pattern recognition receptors and their roles on the innate immune system of mud crab (*Scylla paramamosain*). *Developmental and Comparative Immunology* 102, 103469.

van den Ploeg, M. J., van den Berg, J. H., Bhattacharjee, S., de Haan, L. H., Ershov, D. S., Fokkink, R. G., Zuilhof, H., Rietjens, I. M. C. M. & van den Brink, N. W. (2014). *In vitro* nanoparticle toxicity to rat alveolar cells and coelomocytes from the earthworm *Lumbricus rubellus*. *Nanotoxicology* 8, 28–37.

van der Ploeg, M. J. C., Handy, R. D., Heckmann, L. H., van der Hout, A. & van den Brink, N. W. (2013). C-60 exposure induced tissue damage and gene expression alterations in the earthworm *Lumbricus rubellus*. *Nanotoxicology* 7, 432–440.

van der Zande, M., Jemec Kokalj, M., Spurgeon, D. J., Louriero, S., Silva, P. V., Khodaparast, Z., Drobne, D., Clark, N. J., van den Brink, N. W., Baccaro, M., van Gestel, C. A. M., Bouwmeester, H. & Handy, R. D. (2020). The gut barrier and the fate of engineered nanomaterials: a view from comparative physiology. *Environmental Science: Nano* 7, 1874–1898.

Verde, A., Mangini, M., Manago, S., Tramontano, C., Rea, I., Boraschi, D., Italiani, P. & de Luca, A. C. (2021). SERS sensing of bacterial endotoxin on gold nanoparticles. *Frontiers in Immunology* 12, 758410.

VERGHESE, B., RADHAKRISHNAN, E. V. & PADHI, A. (2007). Effect of environmental parameters on immune response of the Indian spiny lobster, *Panulirus homarus* (Linnaeus, 1758). *Fish & Shellfish Immunology* **23**, 928–936.

VLOT, A. C., SALES, J. H., LENK, M., BAUER, K., BRAMBILLA, A., SOMMER, A., CHEN, Y., WENIG, M. & NAYEM, S. (2021). Systemic propagation of immunity in plants. *New Phytologist* **229**, 1234–1250.

WANG, X. W. & WANG, J. X. (2013). Pattern recognition receptors acting in innate immune system of shrimp against pathogen infections. *Fish & Shellfish Immunology* **34**, 981–989.

WANG, Z., FAN, L., WANG, J., XIE, S., ZHANG, C., ZHOU, J., ZHANG, L., XU, G. & ZOU, J. (2021). Insight into the immune and microbial response of the white-leg shrimp *Litopenaeus vannamei* to microplastics. *Marine Environmental Research* **169**, 105377.

WESTMAN, J., GRINSTEIN, S. & MARQUES, P. E. (2020). Phagocytosis of necrotic debris at sites of injury and inflammation. *Frontiers in Immunology* **10**, 3030.

WOLF-GROSSE, S., MOLLNES, T. E., ALI, S., STENVIK, J. & NILSEN, A. M. (2018). Iron oxide nanoparticles enhance toll-like receptor-induced cytokines in a particle size- and Actin-dependent manner in human blood. *Nanomedicine* **13**, 1773–1785.

WU, H.-Y., CHUNG, M.-C., WANG, C.-C., HUANG, C.-H., LIANG, H.-J. & JAN, T.-R. (2013). Iron oxide nanoparticles suppress the production of IL-1 β via the secretory lysosomal pathway in murine microglial cells. *Particle & Fibre Toxicology* **10**, 46.

YAZDI, A. S., GUARDA, G., RITEAU, N., DREXLER, S. K., TARDIVEL, A., COUILLIN, I. & TSCHOPP, J. (2010). Nanoparticles activate the NLR pyrin domain containing 3 (NLRP3) inflammasome and cause pulmonary inflammation through release of IL-1α and IL-1β. *Proceedings of the National Academy of Sciences USA* **107**, 19449–19454.

YU, S. & GAO, N. (2015). Compartmentalizing intestinal epithelial cell Toll-like receptors for immune surveillance. *Cellular and Molecular Life Sciences* **72**, 3343–3353.

YU, X., FENG, B., HE, P. & SHAN, L. (2017). From chaos to harmony: responses and signaling upon microbial pattern recognition. *Annual Review of Phytopathology* **55**, 109–137.

ZANNELLA, C., MOSCA, F., MARIANI, F., FRANCI, G., FOLLIERO, V., GALDIERO, M., TISCAR, P. G. & GALDIERO, M. (2017). Microbial diseases of bivalve mollusks: infections, immunology and antimicrobial defense. *Marine Drugs* **15**, 182.

ZHANG, H., GOH, N. S., WANG, J. W., PINALS, R. L., GONZÁLEZ-GRANDÍO, E., DEMIRER, G. S., BUTRUS, S., FAKRA, S. C., DEL RIO FLORES, A., ZHAI, R., ZHAO, B., PARK, S.-J. & LANDRY, M. P. (2022). Nanoparticle cellular internalization is not required for RNA delivery to mature plant leaves. *Nature Nanotechnology* **17**, 197–205.

ZHAO, Q., PAN, L., REN, Q. & HU, D. (2015). Digital gene expression analysis in hemocytes of the white shrimp *Litopenaeus vannamei* in response to low salinity stress. *Fish & Shellfish Immunology* **42**, 400–407.

ZHOU, J.-M. & ZHANG, Y. (2020). Plant immunity: danger perception and signaling. *Cell* **181**, 978–989.

ZHU, S. (2020). 15 years of small: research trends in nanosafety. *Small* **16**, e2000980.

ZIA-UR-REHMAN, M., QAYYUM, M. F., AKMAL, F., MAQSOOD, M. A., RIZWAN, M., WAQAR, M. & AZHAR, M. (2018). Recent Progress of Nanotoxicology in plants. In *Nanomaterials in Plants, Algae, and Microorganisms* (eds D. K. TRIPATHI, P. AHMAD, S. SHARMA, D. K. CHAUHAN and N. K. DUBEY), pp. 143–174. Academic Press, Cambridge, MA.