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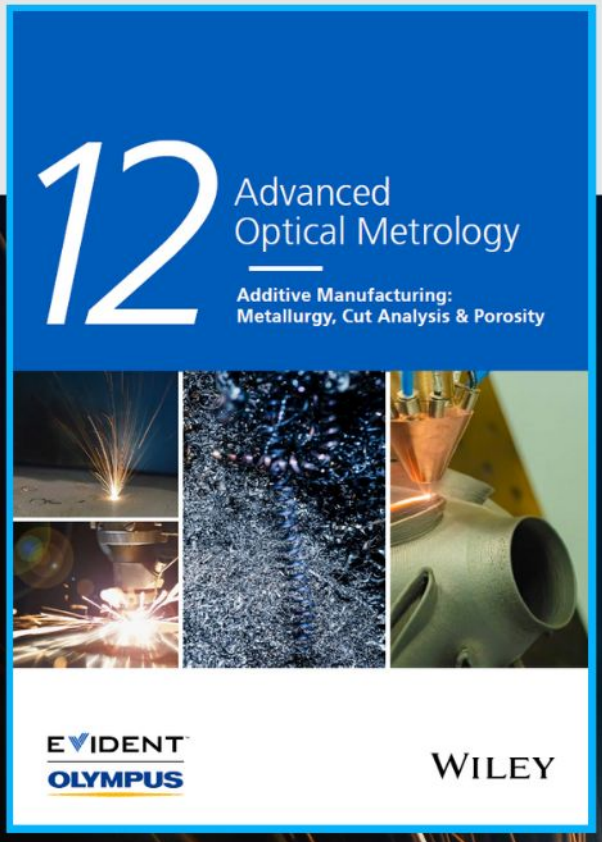

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Addressing Nanomaterial Immunosafety by Evaluating Innate Immunity across Living Species

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The interaction of a living organism with external foreign agents is a central issue for its survival and adaptation to the environment. Nanosafety should be considered within this perspective, and it should be examined that how different organisms interact with engineered nanomaterials (NM) by either mounting a defensive response or by physiologically adapting to them. Herein, the interaction of NM with one of the major biological systems deputed to recognition of and response to foreign challenges, i.e., the immune system, is specifically addressed. The main focus is innate immunity, the only type of immunity in plants, invertebrates, and lower vertebrates, and that coexists with adaptive immunity in higher vertebrates. Because of their presence in the majority of eukaryotic living organisms, innate immune responses can be viewed in a comparative context. In the majority of cases, the interaction of NM with living organisms results in innate immune reactions that eliminate the possible danger with mechanisms that do not lead to damage. While in some cases such interaction may lead to pathological consequences, in some other cases beneficial effects can be identified.

1. Introduction

Interaction of living organisms with their surrounding environment seeks to maintain a dynamic equilibrium that allows the organism's survival and development. When confronted with unknown environmental agents that may be potential threats (e.g., a pathogenic or damaging agent), organisms may behave in two ways. Either they react to the external challenge with a defensive response, or they change their physiological status as an adaptive response to their new conditions. This concept of dynamic equilibrium between defensive reaction and adaptive behavior is also relevant to the interpretation of nanotoxicological data. In this context, exposure of organisms to nanomaterials (NM) and the resulting response can be

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interpreted as a response to a foreign dangerous agent. On this basis, rethinking nano-immunosafety would imply a more comprehensive, holistic organism-based evaluation of the interaction of engineered NM with the immune system, as one of the major biological systems that living organisms use for probing the external environment, sensing potential threats and mounting defensive or adaptive responses.

What does immunosafety mean in a nanoecotoxicological context? If an organism meets a NM, recognizes it as an alien substance (potential threat) and mounts a successful immune reaction (e.g., that eliminates it), does this represents in itself an adverse response? In our opinion it does not, because a successful immune reaction is a physiological health-preserving defensive response to foreign agents and not alone a sign of toxicity. Thus, not only are the agents to which the organisms adapt (e.g., by actively preventing immune reaction) handled in a controlled way, but also those that are recognized as foreign may be directed to the appropriate cellular elimination system by an immune defensive response. A very important issue that we have to consider is that the real efficacy of a defensive immune reaction cannot be evaluated at the level of single cell types, as is the case in many in vitro toxicity assays. In fact, during a successful defensive immune reaction, which implies inflammation, many immune cells involved in the reaction may actually die, and some damage to the tissue may occur. However, the overall final outcome is the elimination of the threat, repair of the tissue damage and re-establishment of tissue integrity and function. Thus, the death of some cell types (such as polymorphonuclear phagocytes or monocytes) may not be alone a sign of immunotoxicity but of a normal homeostatic immune reaction. In this view, we should consider with critical attention the results obtained with single cell types in in vitro toxicological assays, as these may lead to incorrect assumption of systemic impacts when in an in vivo exposure, while such responses may actually be part of the normal immunological response to NM.

Although immune reactions may be part of a benign response to NM exposure, there are ways by which the presence of NM may affect immune responses in a fashion that could cause problems to the organism health. For example, could NM alter the normal defensive immune reaction to a threat, could the NM-induced metabolic or epigenetic changes in immune cells affect their capacity to mount an effective defensive response to a real danger, for instance an infection? Or could foreign or endogenous agents, when associated with NM, undergo structural changes thereby triggering an anomalous immune reaction, for instance become allergenic or triggering an autoimmune reaction? Also, could NM, alone or associated with other agents, trigger a potentially pathological immune reaction, such as a chronic inflammatory response with associated tissue damage? Based on such considerations, we can therefore consider NM as immunologically safe when they do not impair immune responses, and do not cause pathological immune reactions. Suitable assays, designed in a way that allows us to capture the relevant details of the nano-immune interaction, are required for adequately addressing these questions.

Nano-immunosafety, as in all toxicological approaches for any type of particulate and non-particulate agents, also depends on the nature of the exposure. NM concentration makes a difference, and the same NM can fail to induce an adverse immune response at a low dose, while triggering a strong reaction at higher concentrations. Although this concept may sound obvious in toxicological studies, it is nonetheless important to bear this in mind when assessing the putative toxicity of NM. Also, the immune system of each organism can react/adapt differently to the same NM at the same concentration. Thus, NM that elicit no adverse response at a specific concentration for immune cells (such as macrophages or dendritic cells (DC)) or for a given organism, may result in an adverse effect for other cells (such as epithelial cells) or other organisms at the same exposure level. This also is an obvious concept but it is worth

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reiterating in the context of a potential adverse effect. Finally, we should consider that the ability of the immune system to cope with NM strongly depends on the health conditions of the organism, and that immature, aging or damaged/diseased organisms usually have impaired immune reactivity and may be less able to adequately cope with potential threats or, in general, with foreign agents. Thus, we should be aware that it is impossible to define the immune response to a NM in a simple universal context, as its immunosafety will depend on the type of material, the exposure dose, route, and duration, the type of organism and the organism's health conditions. The concept of "safe-by-design" NM is therefore a chimera, and what we can and should aim at is a nanotechnology that is "safer-by-design" in these different contexts.

Why do we think that nano-immunosafety studies should focus on innate immunity, although adaptive immunity is such an excellent and highly specific defense mechanism in man? The reason is that innate immunity is the earliest and most potent type of immune reaction, thus the first to be engaged in the encounter with foreign materials. Most importantly, innate immunity is the common type of immunity shared by the majority of living organisms, from plants to mammals, and is the only type of immunity of over 95% of living eukariotic species (except higher vertebrates from bony fish to mammals that also display adaptive immune mechanisms). The high evolutionary conservation of innate immunity has produced a number of defensive mechanisms that are maintained with little variations across evolution. This may imply that, in the recognition and response to NM, different organisms may engage similar mechanisms. Eventually, it must be recognized that in the development of innate immune mechanisms a key role is played by the host interaction with bacteria. The immune system does not develop in germ-free animals, and this strongly suggests the interdependence and mutual cooperation of different living entities within an organism.

When assessing nano-immunosafety, we should consider the organism in its entirety (including its symbiotic bacteria) and also the environment in which it lives. In this review, we will try to address the interaction between NM and innate immunity across evolution by taking in consideration all the issues stated above.

2. Nanomaterials and Innate Immune Effector Cells

2.1. Innate Immune Cells across Living Organisms

In different living organisms, there are cells and mechanisms that are able to recognize potential threats and react to them for defending and preserving the organism's physical and functional integrity.

Most interestingly, in plants there are no specialized immune cells. However, plants, as animals, can sense "danger" and react to unfavorable conditions.[1] Danger signals are mainly external factors such as pathogens, abiotic factors, and potentially also NM. As mentioned, plant defense does not depend on specific immune cells, as in principle all cells can sense threat. The response to challenge includes the release of preformed factors after cell wall damage, activation of danger receptors and the secretion of endogenous danger signals, the so-called phytocytokines, that are able to alert adjacent cells.[2] The transport route for water and nutrients, through xylem and phloem, is used by agents such as small molecules, RNA, or peptides to achieve systemic activation of plant responses. NM can be also transported within the plant,[3] and therefore all plant tissues could potentially sense them. The extent by which NM can interfere with or be actively recognized by plants depends on the interaction interface, which is therefore a key element in determining uptake and subsequent reaction. Uptake of NM in plants is hindered by the cuticula in leaf tissue and the Casparian strip, as well as the suberized endodermis in roots. Cell walls are also a barrier, which only some NM can pass. Thus, NM uptake by plants depends on many parameters, including plant species, tissue, NM size, properties, and application route. Uptake is reported for a number of nanoparticles (NP) in above-ground and underground tissues of specific plant species, but it remains unpredictable, and no generalization can be made on the nature of NM that are taken up, the plant species and the conditions under which internalization occurs.[4]

Cells and tissues that form the interaction interface with NM in invertebrates represent the major animal recognition and defensive system in the many invertebrate phyla. They are worldwide distributed, in each environment, and are subjected to a wide range of threats even if their welfare is overlooked compared to the attention devoted to vertebrates. Invertebrate species are just as capable as vertebrates of experiencing stress and damage, and possess an evolutionarily conserved immune system acting as a first line of defense against exogenous and endogenous threats to the host, such as pathogenic infection, tissue damage, or cancer. The invertebrate immune system encompasses a number of innate defensive mechanisms, such as cellular responses involved in non-self recognition, phagocytosis, autophagy, cellular encapsulation, and nodulation.[5] The most frequently used invertebrate model systems are those from the clade Protostomia (e.g., cnidarians). Some other new model systems, such as the sea urchin (Echinodermata), are members of the Deuterostomia, the same superphylum that includes vertebrates, and thus have mechanisms that are likely to be more closely related to those occurring in humans.

Unlike vertebrates, invertebrates lack classical antibody-based adaptive immunity, and key molecular and cellular actors such as recombination-activating genes, B and T lymphocytes, except for some exceptions, are totally absent.[6] Notably, some indications of alternative adaptive or anticipatory immune functions and memory-like responses characterized by challenge-specific long-term protection were found in a few insect and echinoderm species.[7,8] Recent findings blur traditional distinctions between adaptive and innate immunity and emphasize that, throughout evolution, the immune system has used an unusually extensive selection of solutions to meet essentially comparable requirements for protection.[6] If the adaptive immune response is characterized by selective clonal gene rearrangements from a broad repertoire of antigen-specific receptors on T and B lymphocytes, the innate immune response is based mainly on different cell types recognizing invading pathogens and activating antimicrobial immune responses such as phagocytic cells, granulocytes, macrophages, and mast cells.[9]

The use of invertebrate models for immunosafety investigation requires a knowledge on the behavior of cells mediating immunity in each selected species, the degree of specificity (specific, quasi-specific, or nonspecific) and memory in their immune strategies, together with information on particle behavior in the environmental medium in which animals live and the route of cell and organism exposure to NM. As a rule of thumb, the cells essentially involved in the invertebrate cellular and humoral defenses are the circulating and sessile blood cells (currently termed hemocytes or celomocytes) as well as other cell types, including those residing in the fat body of insects, or in the hepatopancreas and gills in crustaceans.[10] Regardless of the adopted cell names of celomocytes and hemocytes, their defensive mechanisms are similar, and foreign material uptake and elimination is based on phagocytosis, enzyme activation (e.g., lysozyme), reactive oxygen species (ROS) formation, metabolic activation, and antimicrobial protein (AMP) production (e.g., defensins).[11]

In bivalve mollusks, hemocytes show both morphological and functional features resembling those of the mammalian monocyte/macrophage lineage.[12] As suspension feeders, bivalves have highly developed processes for the cellular internalization of nano- and microscale particles in the key biological functions of intracellular digestion and cell-mediated immunity. In this light, the hemocytes of different species (mussels, oysters, and clams) have been widely investigated as a possible target for the effects of NM.[13,14]

The coelomic fluid of the sea urchin *Paracentrotus lividus* carries three different freely circulating cells, phagocytes, amebocytes, and vibratile cells, which are morphologically and likely functionally distinct.[15] In the human body, macrophages recognize size and shape of their targets, facilitating internalization via phagocytosis. The phagocytes of the sea urchin interact with NM (e.g., TiO_2 NP) by internalizing them both in vivo and in vitro.[16–18] These cells have a dendritic-like morphology and are the most abundant cell type present in the sea urchin body cavity fluid (>80%). Upon interaction with NM, sea urchin phagocytic cells potently respond with active phagocytosis, becoming strongly adherent and metabolically active.[17,18]

Earthworm coelomocytes encompass two main subpopulations, the amebocytes and the eleocytes, that differ for morphology, cytochemistry, and gene expression profile.[19] Eleocytes are large cells filled with a substantial amount of chloragosomes (large granules containing fluorophores). Although their main function is nutritional (e.g., storage of glycogen and lipids), they also produce a number of bioactive molecules.[11] Hyaline and granular amebocytes represent the main effector immunocytes and are specifically involved in a broad range of defense functions including phagocytosis, encapsulation, ROS production, and cytotoxicity,[11] corresponding to the expression of many immune-related molecules found solely in amebocytes.[20] Cell viability is a meaningful immune marker in the earthworm, as it provides a general overview of the organism´s immune capacity. Immunocytes are reactive to a wide range of pollutants and may respond to NM in a distinct manner according to their immune repertoire. In pollutant-spiked soils the amebocytes/eleocytes ratio increases due to the higher mortality of eleocytes, which are more susceptible to pollutants than amebocytes. The most visible response difference is observed with metals, measured by neutral red uptake: after exposure, eleocytes started to die, while amebocytes showed enhanced phagocytosis.[21]

Arthropods, including insects and crustaceans, use a range of cellular and humoral immune defensive strategies to protect themselves from damage. The main cellular defense mechanisms are phagocytosis, nodule formation (nodulation), and encapsulation. Both nodule formation and encapsulation are responsible for the isolation and walling off of invading microbes and macroparasites.[22] In insects, there are circulating blood cells, sessile hemocytes, and various other cell types like cells in fat body. Several types of hemocytes were described in insects with the majority of species possessing only a few of them.[23] For example, *Drosophila* hemocytes include three cell types:[24] plasmatocytes, which are involved in phagocytosis and release of immune effector and signaling molecules; crystal cells, which contain pro-phenoloxidase (PO) and other proteases of the pro-PO activating system;[25] and lamellocytes, which are rare in naïve animals but differentiate during infection and participate to encapsulation. Within the hemocoel of terrestrial isopods (such as *Porcellio scaber*), nongranular or hyaline hemocytes are mainly responsible for phagocytosis.[26] Semigranular cells also show some phagocytic ability but are apparently more involved in encapsulation and nodulation. Granular cells are predominately connected with the PO system[27] and, along with semigranular cells, are thought to produce AMP and also contribute to antioxidant defense.[28] In crustaceans, there are reports that shrimp immune parameters (i.e., total hemocyte count, respiratory bursts, PO, and superoxide dismutase (SOD) activity) were significantly affected by exposure or ingestion of ZnO NP.[29] In a recent study on larvae, pupae, and adults of *Tenebrio molitor*, transmigration of nanodiamonds through the insect cuticle was followed by inhibition of cellular and humoral immune responses, presence of phagocytosed nanodiamond aggregates mainly in hemocytes, and also in fat body cells, but not in Malpighian tubule cells.[30]

Mammalian exposure to NM occurs at epithelial barriers such as the epidermis, at mucosal sites if inhaled or ingested, or directly in circulation, as in the case of nanomedical applications. Generally, NM do not cross the intact epidermal barrier,[31] and therefore the contact with immune cells can more easily occur upon mucosal or intravenous exposure. Cells of the innate immune system can be abundantly found in the mucosal tissues and in circulation, have the task of recognizing foreign objects such as NM, and initiate an appropriate response. As already mentioned, the response can be adaptation/lack of reaction (meaning that the foreign objects are considered harmless) or a defensive response. A successful response of these cells will lead to sequestration and elimination or destruction of potential threats, including NM, while insufficient or overactive responses may lead to infection and tissue damage.

In mammals, cells of the innate immune system are present in the blood and in all body tissues. While many functions, such as pattern recognition, are conserved across innate cells, each type contains specializations that benefit host defense. The most abundant of innate cells are the short-lived phagocytic and inflammatory cells called neutrophilic polymorphonuclear leukocytes or neutrophils, which comprise between 50% and 70% of the white blood cells found in circulation. Their primary function is phagocytosis and destruction of foreign objects,

and they enter challenged tissues in large numbers following a set of events known as the neutrophil recruitment cascade.[32] Once in the tissue, they are able to engulf objects for phagocytic destruction, release factors and enzymes aimed at degradation of invaders, and release the so-called neutrophil extracellular traps (NET) that can capture and degrade objects in the surrounding environment.[33] Eosinophils are short-lived cells mainly residing in tissues, involved in bacterial and parasite clearance. They function through release of granules containing different defensive molecules such as RNAses, peroxidases and soluble mediators (cytokines, chemokines, and growth factors). Eosinophils are additionally known to be involved in the pathogenesis of allergies and asthma.[34] Mast cells are large and probably long-lived tissue-resident cells with several functions. Among them, they are innate effector cells involved in allergic reactions and inflammation but also in anti-inflammatory activities, upon activation and subsequent release of chemical mediators by degranulation.[35] Within their defensive functions, mast cells are involved in pathogen clearance in many bacterial, viral and parasitic infections, upon release of proteases, AMP, and ROS production. Indeed, their primary evolutionarily conserved role seems to be inflammation and pathogen killing, with a specific capacity of toxin and venom detoxification. Innate lymphoid cells (ILC) are a heterogeneous group of innate cells of lymphoid origin classified into three subtypes according to their phenotypes, functions and cytokine expression profiles. ILC1 includes the classical natural killer cells, mainly secrete IFN-γ and can induce T helper 1 cell activation. ILC2, also known as natural helper cells or nuocytes, secrete a number of interleukins (IL-4, IL-5, IL-9, and IL-13) in response to parasitic infections, and are involved in T helper 2 responses, including allergic reactions. ILC3 produces IL-22 and or IL-17 and are involved in the activation of T helper 17 cells. As all innate cells, ILC are mainly activated by stress signals, the cytokine milieu of the surrounding tissue and microbial compounds.[36]

Eventually, the mammalian innate immune system is highly dependent on the multifunctional action of monocytes, macrophages and DC. Monocytes are phagocytic cells found in the blood, comprising ≈10% of the circulating white blood cell population. Upon tissue damage, monocytes are recruited to the tissue site and act as effective killer cells and initiators of a more general defensive inflammatory reaction. Most of the inflammatory monocytes that enter a tissue probably die during the local inflammatory reaction, but it is possible that some of them survive and differentiate into macrophages or DC within the healing tissue. Macrophages are the tissue-resident mononuclear phagocytes, mostly self-renewing tissue cells to which differentiated monocytes may contribute in different proportions (depending on the tissue).[37] Macrophages are mostly scavenging cells that keep the tissue clean by eliminating senescent, dead or anomalous cells and denatured proteins. Macrophages are also sentinel cells that perceive foreign agents (mostly invading microorganisms but also NM), can phagocytose and engulf them for destruction within a phagolysosome, or sequester them for eventual elimination. Notably, macrophages are poor inflammatory effector cells but, if required, they can initiate a defensive response by producing factors that recruit and activate circulating effectors (such as neutrophils and monocytes). Eventually, at the end of an inflammatory reaction macrophages participate to resolution of inflammation and tissue remodeling, thereby re-establishing the healthy physical and functional status of the tissue.[38] DC are similar to monocytes and macrophages in their phenotype and functions, although they are poorly phagocytic, and are specialized in antigen presentation (a feature shared by other cell types although less efficiently). DC are present in the circulation and in tissues (immature DC), and are activated upon interaction with foreign objects (mature DC), becoming able to efficiently present antigens.[39]

In conclusion, while in plants no specialized immune cells exist but all cells can sense and react to NM, in animals (from invertebrates to mammals) phagocytic cells are the major effector of the innate immune defensive responses (**Figure 1**). These cells may adopt different morphologies in different microenvironmental conditions and can encompass subpopulations with different names and more specialized roles in each organism, but they all have in common the capacity of rapidly adapting to the presence of foreign materials by uptaking and degrading/destroying them either directly or through the release of effector molecules. The modes of sensing and recognition of foreign agents determines the type of reaction by immune cells. A depiction of the interaction and uptake of NM with innate immune cells is presented in **Figure 2**.

2.2. Interaction of Nanomaterials with Innate Cells Depends on the Microenvironment

The ability of NM to induce any type of reaction in immune cells strongly depends upon the previous interaction of microenvironmental factors with the highly reactive NM surfaces. NM entry in a body implies a rapid interaction with body fluids. Biomolecules adsorb to the surface and create a "biomolecular corona." Thus, NM sensing is based on the biomolecular corona composition, and its particular spacing and structure, which depends both on the NM chemistry and size and on the biological tissue/fluid in which NM are present.[40] The corona can be a complex structure, sometimes 20–30 nm thick, consisting of soft and hard layers (soft and hard corona), based on the affinity of the NM–biomolecule interactions. Since the corona is composed by self-proteins and other self-molecules, these can mask the NM surface and prevent immune recognition. However, the interaction with the NM surface can alter the 3D structure and folding of proteins, thereby making them signals for non-inflammatory elimination by phagocytes or triggers for a defensive innate/inflammatory response.[41] Opsonization with complement components, one of the most efficient innate defensive mechanisms against bacteria, can occur upon adsorption of complement proteins in the NM biomolecular corona. Complement components can promote clearance by phagocytes through complement receptors, or trigger inflammation by activating the complement cascade.

Evidence for the implications of NM biocorona in immune recognition and response is also emerging in non-mammalian vertebrate and invertebrate models. In zebrafish blood plasma, sex-specific coronas were identified around SiO_2 NP,[42] with the prevailing contribution of vitellogenins conferring a "female"

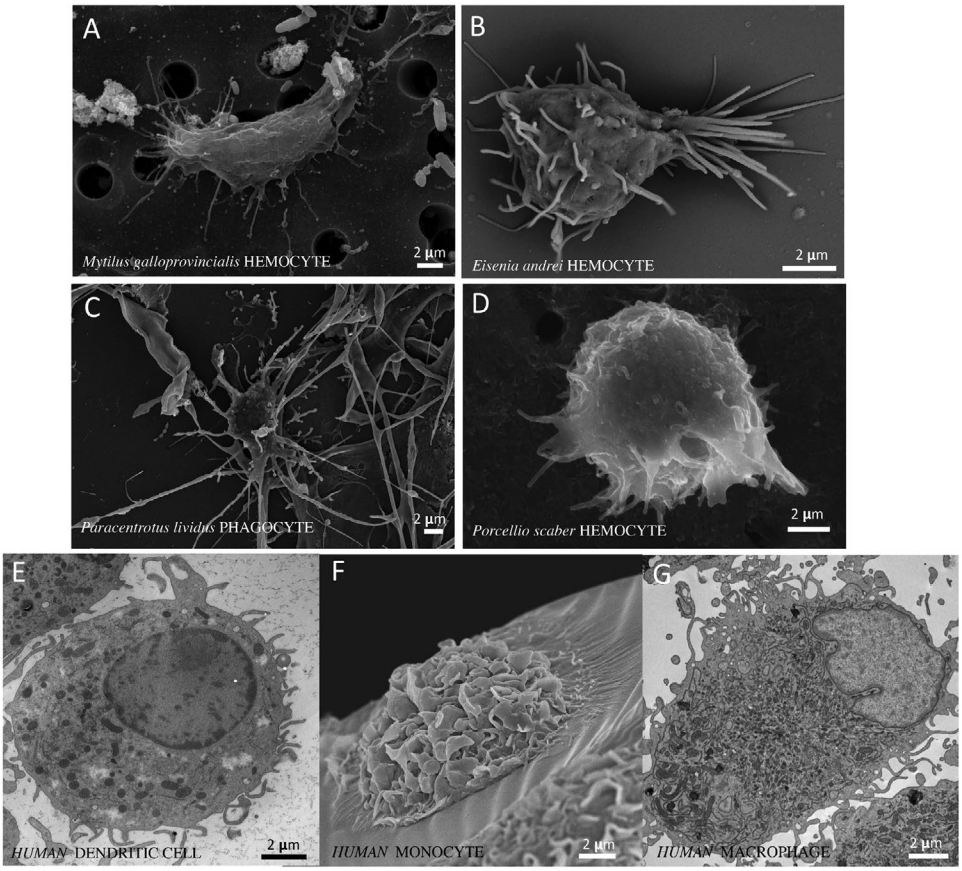

Figure 1. Innate immune cells across living species. SEM images of A) a hemocyte of the mussel *Mytilus galloprovincialis*, B) a hemocyte of the earthworm *Eisenia andrei*, C) a phagocyte of the sea urchin *Paracentrotus lividus*, and D) a hemocyte of the woodlouse *Porcellio scaber*. E) TEM image of a human monocyte-derived dendritic cell (after in vitro differentiation for 6 days with GM-CSF and IL-4). F) SEM image of a human blood monocyte after 7 days of culture on a collagen matrix. G) TEM image of a human monocyte-derived macrophage, differentiated for 7 days in culture with M-CSF.

biological identity to the NM. Moreover, zebrafish lymphoid and myeloid blood cells preferentially accumulated those NP with a female corona profile. The capacity of leukocytes to remove such NP–protein complexes from the bloodstream would prevent specific targeting of vitellogenin-coated NP to developing oocytes, and possible downstream consequences on reproduction.[42]

NM can be coated with proteins and other molecules also in biological fluids of invertebrates. In earthworms, the biocorona formed around Ag NP contains a predominant protein, lysenin, that ultimately triggers higher uptake by immune cells.[43] By analyzing gene transcription kinetics upon in vivo exposure to Ag NP, the transcription of lysenin was, after transient induction, gradually suppressed over time, with induction of a new secretome, suggesting a reshuffling of the NP biocorona that will change the interaction with immune cells.[44] In the marine mussel *Mytilus*, the complement component C1q and Cu,Zn-SOD represent the single protein types present in the biocorona that forms, upon in vitro interaction with mussel biological fluid, on different types of NM with positive (PS-NH_2 NP) or negative (CeO and TiO_2 NP) charge, respectively.[45] The formation of a distinct corona increased or decreased the immunotoxicity of either type of NM for hemocytes.

In the celomic fluid of the sea urchin *P. lividus*, the biocorona formed around PS-NH_2 was dominated by the toposome precursor protein. Such biocorona promoted Ca^{2+}-dependent recognition by phagocytes, leading to uptake followed by toxicity.[46] In contrast, the main constituents of the biocorona on the surface of TiO_2 NP exposed to the supernatant of cultured sea urchin immune cells were identified as a subset of adhesion and cytoskeletal proteins. Upon coating with these proteins, TiO_2 NP aggregated on the outer cell surface of phagocytes and were then internalized within well-organized vesicles without eliciting harmful effects.[16]

Overall, data so far obtained in invertebrates indicate that, in each species, NM can be coated by different and peculiar proteins, which makes the biological implications less straightforward.[45] In addition, the results obtained in marine invertebrates suggest that the net surface charge retained by different NM in biological fluids is an important factor in the formation of a stable surface coating and for the consequent interaction with immune cells.

It should be noted that a corona can form on NM surface even before entry into an organism, by surface adsorption of environmental agents (such as bacterial components or allergens). As in the case of the biomolecular corona mentioned above, the composition and steric/architectural features of the corona determines the subsequent interaction with living organisms. We should underline the fact that no naked NM can

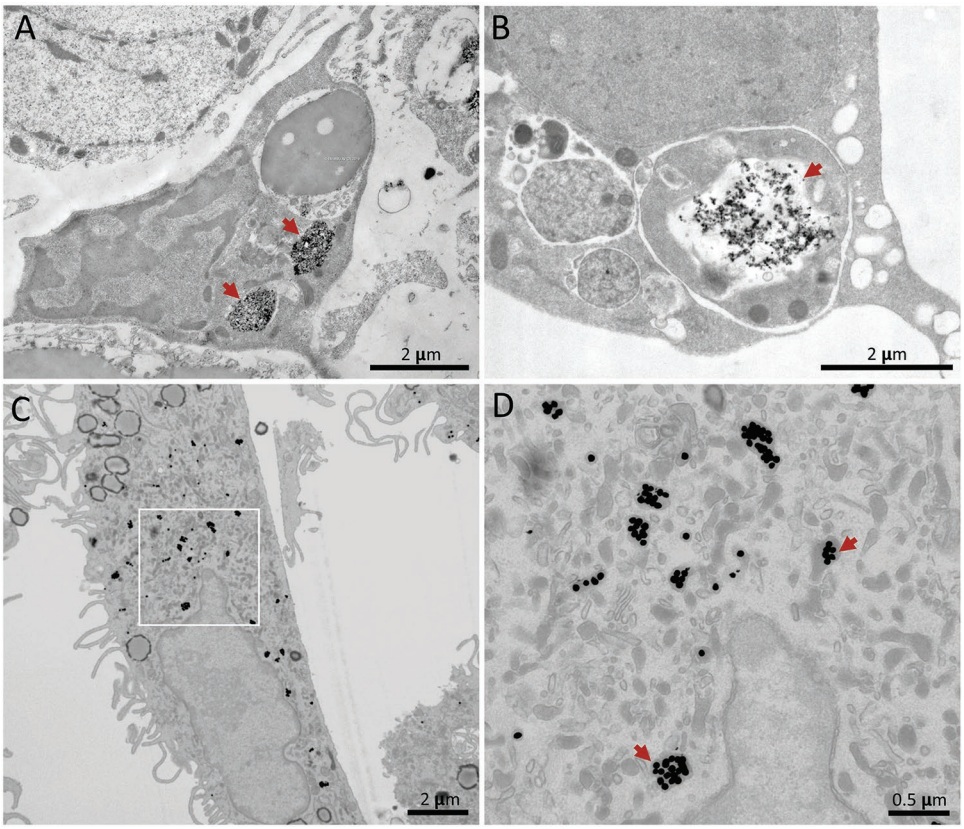

Figure 2. NM uptake by innate immune cells across living species. A) Representative TEM image of a hyaline hemocyte of the earthworm *Eisenia andrei* showing internalized CuO NP (red arrows) after in vitro exposure (100 µg mL^{-1} CuO NP, diameter 5–15 nm) for 2 h. B) TEM image of a phagocyte of the sea urchin *Paracentrotus lividus* showing internalized TiO_2 NP (red arrow) following in vitro exposure (1 µg mL^{-1} TiO_2 NP, diameter 10–65 nm) for 24 h. C) TEM image of a human monocyte-derived macrophage exposed to Au NP (20 µg mL^{-1}, 50 nm diameter) in vitro for 24 h. The white-framed area is magnified and displayed in (D), where red arrows indicate some of the internalized Au NP.

be found in our environment, and therefore the biological systems interact with "hybrid" NM that include environmentally borne non-characterized molecules on their surface.

2.3. Innate Sensing Mechanisms in the Recognition and Uptake of Nanomaterials

It is hypothesized that innate cells may sense NM upon interaction with surface receptors, mainly through typical innate immune pattern recognition receptors (PRR).

Plant PRR are similar to animal toll-like receptors (TLR), and can recognize microbe- or pathogen-associated molecular patterns (M/PAMP), thereby triggering defense responses that include ROS production, MAP kinase (MAPK) activation, and induction of defense genes, as well as immediate responses such as phytohormone release and callose deposition to strengthen the cell walls. The sum of these responses leads to enhanced resistance of the plant to invading pathogens, a phenomenon known as pattern triggered immunity (PTI). Some pathogens can suppress PTI by injecting virulence factors into the plant cells that are negatively affecting PTI signaling components, leading to enhance susceptibility of the plant. Plants can recognize some of these virulence factors by NOD-like intracellular receptors (NLR) that can trigger a fast defense response that includes ROS production and MAPK activation, but that can also lead to a hypersensitive response resulting in cell death of the infected plant parts and restricting nutrition to the invading pathogen.[47] If and how plants use these mechanisms to sense and react to NM is poorly known at present.

In invertebrates, the innate defense mechanisms also depend on sensing foreign material by several classes of immune receptors, which bind PAMP and damage-associated molecular patterns (DAMP) and activate rapid innate defensive reactions. As in plants, both cell surface receptors and intracellular sensing mechanisms can identify PAMP and initiate the innate immune response.[48] Echinoderms,[49] amphioxus[50] and, independently, also mussels[51] and sponges[52] have an expanded repertoire of PRR that include the intracellular NLR and the transmembrane TLR, while arthropods, nematodes, earthworms,[53,54] and tunicates have a more limited repertoire, all resulting from independent but combinatorially constrained evolution of domain architectures.[55] The invertebrate immune cells also express a wide range of inducible genes coding for extracellular recognition proteins, including lectins, peptidoglycan-recognition proteins, lipopolysaccharide (LPS) and β1,3-glucan-binding proteins, and fibrinogen-related proteins (FREP).[56–59]

Despite the presence of PRR and their clear role in pathogen recognition and immune reaction to threats, recognition of NM

Table 1. Cells and tissues interacting with NM across species.

Species	Cell/tissue	Cell function	NM type	Refs.
Zea mays L. (plant)	Xylem	Transport tissue in vascular plants	CuO	[3]
Mytilus galloprovincialis (mollusc)	Hemocytes	Phagocytosis, opsonization, cytotoxicity, inflammation, humoral response, ROS and NO production	TiO_2 PS-NH_2 CeO NP	[14, 45]
Eisenia fetida (earthworm)	Amoebocytes eleocytes	Phagocytosis, opsonization, cytotoxicity, inflammation, humoral response, ROS and NO production, graft rejection	Ag	[14, 44]
Paracentrotus lividus (sea urchin)	Phagocytes	Phagocytosis, opsonization, clotting, cytotoxicity, inflammation, humoral response, graft rejection, ROS and NO production, clearance	TiO_2	[16–18]
Penaeus semisulcatus (shrimp)	Hemocytes	Encapsulation, cytotoxicity, PO activity	ZnO	[29]
Homo sapiens	Macrophages	Phagocytosis, clearance, inflammation	TiO_2	[41]

by celomocytes through PRR sensing has been reported only in the case of recognition and uptake of TiO_2 NP by sea urchin phagocytes through TLR4.[18] Otherwise, these is no evidence of a direct receptor activation by NM with subsequent inflammatory activation.

In vertebrates, both TLR and NLR are present, generally in a smaller number than in invertebrates (possibly because of the presence in higher vertebrates of adaptive immunity).[55] On mammalian innate cells additional PRR include scavenger receptors (SR) and C-type lectin receptors, which can sense different types of microbe-associated molecular pattern (MAMP), PAMP, and DAMP, allowing cells to react appropriately to changes in their tissue conditions.[60,61] Upon activation, PRR initiate a series of intracellular signals that lead to an immune response intended to eliminate the threat and restore tissue condition. Several NM have been reported as able to activate the NLRP3 inflammasome,[62–66] thereby inducing caspase-1 activation and the production of inflammatory IL-1β. Mechanisms of inflammasome activation by NM include induction of K^+ efflux and ROS generation and lysosomal membrane destabilization. Caspase-1 activation and pulmonary inflammation by silica particles has been shown depending on the presence of the scavenger receptor SR-B1.[67]

Generally, NM seem readily taken up by phagocytic cells. In some cases it has been shown that PRR such as SR and TLR participate to the uptake,[68,69] and it is important to note that the presence and characteristics of the biomolecular corona can dictate the type of interaction of NM with SR or other innate receptors (e.g., complement receptors for complement-opsonized NM). After interaction with the cell membrane, NM uptake also depends upon factors such as shape, size, surface charge and surface chemistry. It was shown that particles with a diameter ≤0.5 μm as well as rod/fiber-shaped NM were preferentially taken up by DC compared to larger particles or those with cubic or spherical shape.[70–73] Smaller NM (≤500 nm) are mainly internalized via pino- or micro-pinocytosis and via clathrin- or caveolin-mediated endocytosis, while NM of >500 nm are usually taken up by phagocytosis.[74–76] Thus, it appears that mechanical forces, as well as opsonizing proteins interacting with the various receptors, are responsible for the majority of NM endocytic uptake, with a major role for NM size and shape in determining the type of uptake mechanism.

These NM internalized via endocytosis are encapsulated into early endosomes that then fuse with lysosomes, to form endolysosomes. Elimination of endocytosed particles may be achieved with lysosomal enzymes (if the NM are sensitive to them) or by autophagic processes.[77–79] However, intracellular degradation of several NM, for instance gold or tattoo pigment particles, is not readily achieved. Consequently, many particles that are engulfed by cells may remain as such for prolonged periods without inducing toxic effects, or can be passed from macrophage to macrophage during time or upon cell death[74,80] Innate immune cells can also capture and degrade NM without engulfing them. Indeed, neutrophils, eosinophils, and mast cells can react to the interaction with NM with degranulation and release of proteases and other enzymes. Several studies have shown that neutrophil and eosinophil peroxidases (EPO) can degrade both multi-wall and single-wall carbon nanotubes.[81,82]

Another mechanism of particle capture and clearance that does not imply their internalization is the formation of extracellular traps (ET).[83] Upon challenge resulting in cell death, cells release a net of chromatin fibers, which are used to immobilize extracellular particles, such as pathogens or non-self objects. The innate cells that can form ET include eosinophils, monocytes, macrophages, neutrophils, and mast cells.[34,84] This process is evolutionarily conserved, and ET have been described also in plants and invertebrates.[85,86] ET are often coupled with local release of ROS and enzyme-containing intracellular granules, which contribute to trapped particle degradation. Indeed, this mechanism acts efficiently also for NM, as shown in a study describing the peroxidase-dependent extracellular degradation of single-walled carbon nanotubes trapped in NET.[87] NM can trigger ET formation in vivo possibly through lysosomal leakage and ROS production.[88,89]

Based on these observations, we can generally conclude that NM are sensed by innate cells through different receptors/mechanisms and either internalized and tagged for elimination, or can induce cell activation (mainly associated to cell death) that leads to ET formation and eventual particle handling. **Table 1** summarizes the reported examples of NM–innate cell interaction across species and their functional consequences.

3. Nanomaterials and Activation of Innate Immune Genes/Factors

In addition to, and as part of, cell-based mechanisms of sensing and reaction to external challenges, a wide array of soluble

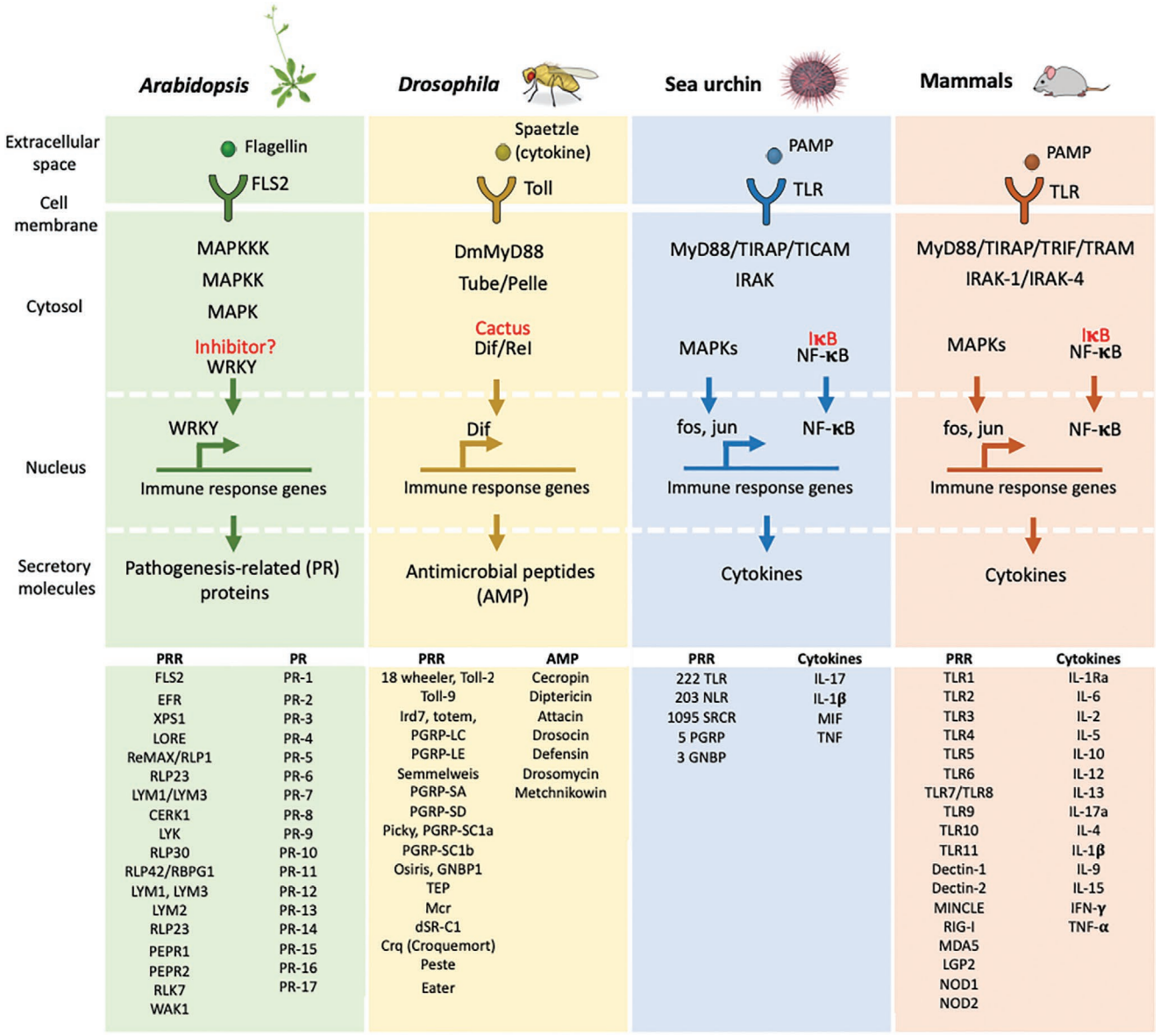

Figure 3. Innate immune molecules and signaling across living species. Upper panels: comparative examples of evolutionary conserved innate signaling in plants (*Arabidopsis*), insects (*Drosophila*), sea urchins, and mammals. Signaling cascades initiate when pattern recognition receptors (PRR) on the cell membrane recognize specific pathogen-associated pattern (PAMP). Signal is transduced within the cytosol by a set of signaling proteins (mainly protein kinases), which in turn activate evolutionary conserved transcription factors that will orchestrate transcription of inflammatory related genes. Consequently, each organism produces a panel of soluble molecules (pathogenesis-related proteins, antimicrobial peptides, cytokines, and others) that contribute to coping with different foreign agents. Lower panels: comparative list of PRR and soluble molecules (pathogenesis-related proteins (PR), antimicrobial peptides (AMP), and cytokines) known in plants (*Arabidopsis*), insects (*Drosophila*), sea urchins, and mammals. Artwork by Andi Alijagic.

molecules are produced that either directly exert defensive functions or communicate with surrounding tissues for mounting a defensive response. **Figure 3** depicts the main molecules and pathways involved in the immune defensive reactions across species.

Upon cell damage or PRR activation, plants can release preformed factors and phytocytokines, which function as endogenous danger signals that alert adjacent cells.[2] Most of the defensive factors produced by plants are soluble molecules that reach all plant tissues using the same transport route as water and nutrients. Depending on the type of pathogen, plants react by producing ROS and phytohormones such as salicylic acid, jasmonic acid, and ethylene.[90] NM are reported to activate some of these molecular defense responses, mainly ROS production and gene activation, suggesting that responses to NM may share similarities with immune responses.[91] Interestingly, recent transcriptomic analysis in *Arabidopsis thaliana* revealed that exposure to different types of NP (e.g., ZnO, fullerene soot, or TiO_2) repressed a significant number of genes involved in phosphate starvation, pathogen and stress responses, with possible negative effects on plant root development and defense mechanisms. A recent systems biology approach, including

omics data from tobacco, rice, rocket salad, wheat, and kidney beans, confirmed that metal NM provoke a generalized stress response, with the prevalence of oxidative stress components.[92,93] However, a general view on NM-induced immune responses is difficult, as contradictory results have been obtained by different groups, possibly due to different experimental conditions.

Invertebrate immune cells (hemocytes and celomocytes) are responsible for immune reactions through phagocytosis and the production of a number of cytotoxic factors, such as hydrolytic enzymes, AMP, ROS, and nitric oxide (NO).[11,12,14,94,95] Upon exposure to TiO_2 NP, earthworm engulf NP by phagocytosis, and simultaneously modify mRNA expression of immune proteins and metallothioneins, thereby affecting the molecular response of immune and detoxification systems.[96] Immune cells also express many inducible immune-related genes that are able to bind microbial patterns. Some of these are membrane proteins involved in recognition, e.g., lectins and lectin-like molecules (see previous section), while others are released as soluble proteins, such as FREP and AMP.[11,97]

In the marine mussel *Mytilus*, hemocyte functional parameters and transcription of different immune-related genes are affected by exposure to different NM types with a high degree of specificity.[13,98] Echinoderm gene sequencing reports over 1000 immune genes falling into the innate immunity area,[55] and exposure of *P. lividus* to NM induces selective changes in immunological pathways and molecules.[99] As an example, exposing *P. lividus* to TiO_2 NP activates suppressive mechanisms by down-regulating the expression of genes encoding immune-related and apoptotic proteins (e.g., *NFκB*, *FGFR2*, *JUN*, *FAS*, *VEGFR*, and *Casp8*); elicits metabolic rewiring by boosting the immune cell antioxidant activity (e.g., pentose phosphate, cysteine-methionine, glycine-serine metabolism pathways); and restores homeostasis by keeping at physiological levels some key immune-related proteins (e.g., TLR4, IL-6, MAPK, heat shock protein 70 (HSP70)).[17]

The earthworm celomic fluid also contains various antimicrobial factors or peptides, such as lysozyme and lumbricin/lumbricin-related protein, and hemolytic molecules such as fetidin and lysenins. In *Eisenia* earthworms, lysenin turned out as a key protein in the earthworm secretome upon interaction with Ag NP, and it is possibly involved in opsonization-induced cellular interaction.[43] The supplementation of culture medium with celomic fluid supports celomocyte survival during in vitro culture. Such protein-rich environment, present both in the earthworm body and in the supplemented culture medium, leads to the formation of a biomolecular corona on the NM surface during exposure. The biomolecular corona can function as opsonin that promotes phagocytosis. Indeed, earthworms can efficiently opsonize microbial particles by means of the soluble form of the receptor protein celomic cytolytic factor (CCF).[11,100] As membrane protein CCF participates in the activation of the pro-PO cascade, while its soluble form has opsonizing capacity.[101] Upon incubation of synthetic NM (HEMA particles) with celomic fluid, CCF was adsorbed onto the particle surface in a manner that was recognizable by CCF-specific monoclonal antibodies (i.e., surface-adsorbed CCF retained its native conformation). This adhesion capability of CCF is an early example of pattern recognition-independent opsonization of non-biological particles.[102] One of the mechanisms by which NM may provoke cell toxicity is ROS generation. Oxidative stress is usually monitored by assessing lipid peroxidation and measuring antioxidant enzymes.[103] Although exposing celomocytes to metallic NP can lead to ROS production,[104] it is still controversial whether such effect can be ascribed to NP or to released metal ions. Celomocytes exposed to metal-based NP (Ag and carbon) show modulated expression of oxidative stress genes and induction of ROS production.[105–107] The antioxidant enzyme catalase is more effective in ROS neutralization than superoxide dismutase in earthworms.[108] Upon catalase down-regulation, the accumulation of H_2O_2 was shown to accelerate NP dissolution.[109]

In arthropods (including crustaceans), lectins from the hemolymph are important in immune recognition and phagocytosis. A major defense molecule in the immune response of crustaceans is the conserved Cu-containing enzyme PO. A number of pattern-recognition proteins (such as PGBP, LGBP, and BGBP) participate in immune defense by recognizing pathogen-associated carbohydrate-containing molecules, i.e., peptidoglycans, lipopolysaccharides, and β-glucans.[110] In insects, the tracheae, epidermis, gonads, and gut epithelium are involved in release of PO and AMP and in the production of ROS.[111] As in many other invertebrates, melanin synthesis is a central mechanism of innate immunity and a major response to a variety of immune challenges.[112–116] Part of the melanin synthesis pathway is catalyzed by the enzyme PO. The PO cascade produces melanin, and also induces multiple potent bioactive agents, such as peroxinectin and ROS, that aid in phagocytosis and cell adhesion. Proper PO modulation is crucial to ensure survival of the organism. The majority of invertebrates activate the PO cascade from the pro-PO enzyme upon recognition of foreign matter.[117] However, in a limited number of *Crustacea*, including *P. scaber*, the production of melanin is not initiated by pro-PO, and it has been suggested that hemocyanin, the oxygen transporter in isopod hemolymph, is responsible for initiating the cascade by undergoing a conformational change upon interaction with non-self material.[118]

Reactive nitrogen species are chemically reactive species that include NO, and are produced through normal metabolism, after an injury, during infections and disease and in response to environmental pollutants and radiation. NO is known to modulate many biological processes including inflammation and cytotoxicity.[119] In crayfish, the hemocyte-derived NO was able to promote hemocyte-bacterial adhesion and increase the hemocyte bactericidal activity.[120]

AMP are another major component of the invertebrate defensive system. They are small cationic, amphipathic molecules effective against Gram-positive and Gram-negative bacteria, yeasts, fungi, and some protozoa and enveloped viruses. AMP are found in the isopod hemolymph, function by disrupting the target cell membrane integrity via destabilization or pore formation, and there is some evidence that they may be translocated into the microorganisms' cytoplasm where upon they can interact with specific intracellular targets to cause cell death.[121]

In vertebrates, and more specifically in mammals, it is known that several types of NM (e.g., carbon, silica, polystyrene, and latex NM) can interfere with the immune system, inducing oxidative stress, complement activation and release of soluble mediators such as cytokines, chemokines and growth

factors. Although an immunomodulatory effect has been shown for several types of NM, the extent of activation of the immune response is specific for every type of object, from minimal (Au NM) to substantial (carbon nanotubes). When NM are used as biomedical products, they are often actively injected in the bloodstream where they encounter a complex environment of plasma proteins and immune cells. NM can be sensed and come in contact with monocytes, platelets, polymorphonuclear leukocytes, and DC and subsequently with tissue resident phagocytes. The encounter between NM and immune cells/factors at the site of injection can lead to several reactions, which could ultimately result in the complete clearance of the NM. Hemolysis, thrombogenicity, and complement activation are the main processes considered relevant, from a toxicological point of view, when NM are injected in the bloodstream.[122] For example, it has been reported that Ag NP at high concentrations can induce hemolysis and affect lymphocyte viability in human and rat blood in vitro, while unable to activate complement and induce platelet aggregation, and also increase sensitivity to thrombogenic factors in vivo in the rat.[123,124] The effects obtained at very high doses used in these studies do not appear at lower concentrations. Depending on their physico-chemical properties, NM may trigger the activation of the complement system, which is crucial for the rapid detection and elimination of circulating particles and pathogens by phagocytic cells. The complement system acts with three main mechanisms. The first is the coating of pathogens/foreign objects with proteins acting as opsonins (e.g., the complement components C1q or C3b) that can be recognized by specific receptors on the surface of phagocytic cells thereby promoting clearance via phagocytosis. The second is the production of chemotactic peptides (e.g., the complement component C5a) for recruiting monocytes and neutrophils to the site of reaction. Finally, the third mechanism is the formation of the membrane attach complex on the microorganism surface that leads to lysis of the pathogen.[125,126] Several studies reported the capacity of NM to activate the complement system, with NM surface charge playing a most prominent role. Charged NM (e.g., lipid and polycation-based NP) could easily bind human serum complement, compared to their neutral PEGylated counterparts.[127] Surface characteristics also are predictive of NM–complement interactions. It has been shown that dextran coating in loop configuration increases cleavage of C3 into C3a and C3b upon incubation in human serum compared to polymers in brush formation,[128] and that the switch of polymer configuration is associated with a shift from the classical to the lectin complement pathway.[129] Although complement activation has been claimed as an important safety issue in relation to medicinal NM, it seems that a major role is instead played by activation of innate cells.[130] This stresses the need of examine in depth the complex interrelationships among innate immune mechanisms activated by exposure to NM, in particular in the design and production of nano-medicaments.

As in invertebrates, NM can also induce ROS production and oxidative stress in mammalian cells. Although ROS are natural byproducts of cellular metabolism with an important role in cell survival, death, differentiation, signaling, and inflammation, an imbalance in their generation and neutralization can result in oxidative damage, leading to cell death. Several NM, such as TiO_2, ZnO, CeO_2, Mn_3O_4, and Ag NP, have been reported able to induce oxidative stress signaling cascades in mammalian cells.[131,132] During an inflammatory reaction, innate immune cells produce matrix metalloproteinases (MMP), which are factors involved in the degradation and remodeling of the extracellular matrix during wound healing. Recently it has been reported that different NM (Ag, Au, Zn, and carbon-based NP) can regulate the synthesis of some MMP,[133,134] although the variability of study models and of NM used does not yet allow full understanding. Overall, the possibility of using NM to modulate/control the immune cell activation and their ROS and MMP production opens promising perspectives for new therapeutic approaches.

Also in the case of mammals, AMP are produced for controlling the interaction with bacteria. AMP, which include defensins and cathelicidins, are involved in maintaining healthy commensal gastro-intestinal flora and in bacterial clearance, with functions ranging from direct bactericidal activity to initiation of innate immune responses.[135] Experimental evidence shows that different types of NM can promote and amplify the antibacterial capacity of AMP, as in the case of the synergistic effect of Ag NP with the bacterial AMP polymyxin B, likely caused by the polymyxin B-driven permeabilization of the bacterial outer membrane that enhances the intracellular antimicrobial activity of Ag NP.[136] Conversely, as in the case of carbon NP and cathelicidin LL-37, NM may alter the AMP structure, thereby hampering their bactericidal effect.[137]

A number of innate immune cells produce intracellular granules that store a range of soluble factors (e.g., proteolytic enzymes, RNAses, peroxidases, histamine, and other mediators). Upon activation, these cells (eosinophils, neutrophils, basophils, mast cells, monocytes, and macrophages) can degranulate and release the active mediators in the surrounding tissues. Proteins such as myeloperoxidase (MPO), EPO, MBP (major basic protein) and ECP (eosinophil cationic protein) are very efficient in pathogen killing, although they can also induce toxicity in several tissues. MPO and EPO, mainly involved in bacterial and parasite clearance, were also found able to degrade single-walled carbon nanotubes in vitro and in vivo, a fact that underlines the role of leukocytes in NM clearance.[81,138] The fact that NM can promote NET formation, as bacteria do, further supports the notion that neutrophilic leukocytes are among the central innate effectors in NM clearance.[88,89]

Very important immune soluble factors are the cytokines, chemokines, and growth factors, which regulate crucial immune and cellular processes such as proliferation, activation, differentiation and migration. The ability of NM to induce an immune response is, therefore, often characterized by their capacity to provoke the production or secretion of inflammatory cytokines and chemokines. A comprehensive study on the capacity of engineered NM to activate in vitro the human monocytic cell line THP-1 to produce cytokines and chemokines showed that, in response to 19 different NM, there is little correlation between activation of innate immunity (i.e., production of innate cytokines and chemokines) and cytotoxic effects.[139] Despite the significant limitation due to the use of a highly proliferating tumor cell line instead of primary monocytes/macrophages, an interesting result is that NM can be roughly grouped into those that can promote inflammation

Table 2. Molecular defense responses activated by NM across species.

Organism	Exposure type	Innate immune gene/factor activation	NM type	Refs.
Plants	In vitro (Tobacco BY-2 cells), in vivo (*Arabidopsis thaliana*, rice, corn)	Oxyradical production, gene activation (e.g., MAPK signaling, salicylic acid signaling, and stress response), secondary metabolite release	Ag, ZnO, Al_2O_3, TiO_2	[91]
Earthworms	In vitro (primary immune cell culture) and in vivo	Gene modulation (fetidin, metallothionein, coelomic cytolytic factor, and superoxide dismutase), lysenin release, apoptotic signaling, oxyradical production	TiO_2 Ag	[43, 96, 104, 106]
Mussels	In vitro (short-term primary immune cell culture) and in vivo	Oxyradical production, stress-activated MAPK signaling, apoptotic signaling, lysozyme release, AMP production	TiO_2 SiO_2 ZnO CeO_2	[14]
Sea urchins	In vitro (primary immune cell culture) and in vivo	Gene and protein modulation (e.g., MAPK, NFκB, FGFR2, JUN, FAS, VEGFR, and Casp8), metabolites rewiring (e.g., pentose phosphate, cysteine-methionine, glycine-serine metabolism pathways), cytokine production	TiO_2	[17, 18]
Mammals	In vitro (primary immune cell culture) and in vivo	Hemolysis, thrombogenicity, complement activation, NFκB and oxidative stress signaling, production of oxyradicals, opsonins, chemotactic peptides, MMP, AMP, proteolytic enzymes, RNAses, peroxidases, histamine, cytokines, chemokines, growth factors	Ag, Fe_3O_4, TiO_2, ZnO, CeO_2, Mn_3O_4, Au, carbon	[81, 122–124, 127, 131–136, 138–140]

(innate immune activation) and those that can apparently downregulate inflammation/innate immune activation by blocking the expression and production of inflammatory mediators. The induction of inflammatory mediators by NM correlates with the block of peroxisome proliferator-activated receptor (PPAR)/LXR pathways. PPAR can antagonize the action of inflammatory transcription factors, such as nuclear factor κB (NFκB) and activator protein-1 (AP-1), thereby inhibiting the production of many inflammatory mediators and cytokines.[140] PPAR activation in monocytes/macrophages, obtained by targeting a PPAR agonist to phagocytes with a nanocarrier, achieved promising results in a preclinical model of myocardial infarction.[141] Modulation of PPAR activity via NM may therefore be developed as a novel therapeutic strategy for anti-inflammatory and pro-healing effects.

The maturation and secretion of IL-1β and IL-18, two key mediators of inflammation, is triggered by a cytoplasmic multiprotein complex termed the inflammasome, of which the NLRP3 inflammasome is the most common in monocytes and macrophages. Since IL-1β is a potent pyrogen that can induce an inflammatory form of programmed cell death (pyroptosis), but is also a key immune-amplifying factor in the development of defensive reactions, NLRP3 inflammasome activation and consequent production of mature IL-1β are factors that can either predict NM immunotoxicity or NM-mediated promotion of immune defensive capacity. Abundant evidence shows the ability of NM to activate the NLRP3 inflammasome and promote the maturation of IL-1β by mechanisms that include ROS production, K^+ efflux, lysosomal membrane destabilization and release of cathepsin G.[62,63,65,66] The stimulation of monocytes or macrophages with by SiO_2 and TiO_2 NP could increase local production of adenosine, leading to an accumulation of intracellular ATP that promotes/prolongs the activation of the NLRP3 inflammasome resulting in an enhanced release of IL-1β and IL-18.[142] It should be underlined that the production of IL-1β is by no means a marker of detrimental/pathological inflammation, but only a sign of an active defensive reaction. Most defensive reactions rapidly resolve, and only in rare cases these degenerate into chronic destructive anomalous inflammation (such as those observed in rheumatoid arthritis or multiple sclerosis). Thus, when assessing the possible NM immunotoxicity, the induction of an innate/inflammatory response must be considered as a normal effect, while only an anomalous reaction (too prolonged in time, of excessive or insufficient extent) might be considered as potentially harmful.

Overall, the interaction of NM with the mammalian innate immune system can provoke a reaction, in terms of production of defensive factors, which mainly results in the re-establishment of tissue homeostasis. Only in rare cases were NM found able to provoke significantly harmful effects at the organism level. Conversely, the increasing body of data accumulated so far shows great promise for NM as agents for modulating and directing innate immune responses for future therapeutic applications. **Table 2** summarizes the main immune pathways activated and defensive molecules produced in response to NM across species.

4. Nanomaterials and Global Defense to Infection/Damage

In all living organisms, NM represents foreign elements with their own physico-chemical properties, which therefore may interfere with the normal physiological defensive mechanisms of the embryos, growing organisms, and adults against infections or other challenges. From the available information, it appears that, at environmentally relevant concentrations, NM are unlikely to have a widespread detrimental effect on living organisms except under certain local release scenarios. However, subtle effects of NM to immune pathways resulting from exposures need to be understood.

In plants, several reports have addressed the activity of NM on plant immune defense. Field and lab studies have described the protective effects of different NM against a number of microbial plant pathogens. Protection of plant from fungal and bacterial diseases has been reported for several inorganic NP, such as ZnO, Cu, SiO_2, TiO_2, CaO, MgO, MnO, and Ag NP.[143,144] For example, ZnO NP can reduce infection by *Fusarium graminearum*, *Penicillium expansum*, *Alternaria alternata*, *Fusarium oxysporum*, *Rhizopus stolonifer*, *Mucor plumbeus*, and *Aspergillus flavus*, as well as by phytopathogenic bacteria such as *Pseudomonas aeruginosa*.[144–147] Cu NP were more effective against *Phytophthora infestans* infection compared to non-nano Cu formulations in tomato.[148,149] The best studied NM for protection against infections are Ag NP. Their protective activity is likely due to the antimicrobial effect of Ag ions. The same applies to Cu NP, as Cu ions are active against fungal phytopathogens and are widely used in bioorganic farming as fungicides. Cu in low doses is also essential for many plant processes as a micronutrient that supports the plant defensive mechanisms against infection. Other NM such as Zn, Ce, SiO_2, and TiO_2 NP also show suppressive effects on plant infections, and might also function as micronutrients. Nano formulation might make the elements more accessible to the plant and allow for lower-dose applications and potentially better uptake rates.[148]

In crustaceans, exposure to dietary NM enhances immune functions. In the freshwater prawn *Macrobrachium rosenbergii* postlarvae, improved survival, growth, total hemocyte counts and digestive enzyme activity were reported after exposure to dietary ZnO, MgO, or Cu NP.[150–152] Treatment of Pacific white shrimp *Litopenaeus vannameinano* with capsules containing the antioxidant lipoic acid induced positive response on immune parameters,[153] and Au NP exerted immunostimulatory and protective effects against *Vibrio parahaemolyticus*.[154] In the crab *Eriocheir sinensis*, dietary CeO_2 NP promote growth, relieve ammonia nitrogen stress, and improve immune protection against infection with the pathogen *Aeromonas hydrophila*.[155]

In marine invertebrates, including bivalves, a number of in vivo studies have shown that, although NM at predicted environmental concentrations are generally not toxic, they may induce sub-lethal responses both at the molecular level and at the overall organism level. In addition to the observed immunomodulatory effects on circulating hemocytes, different NM have been shown to modulate inflammatory and stress responses systemically.[13,98] The overall impact on the physiological defense responses can be positive, as shown in *Mytilus* for certain types of CeO_2 NP, which upregulate the expression of several immune-related genes in hemocytes, and exert antioxidant properties at the tissue level as in mammalian models.[156] Moreover, exposure of animals to TiO_2 NP increases lysozyme and ROS production and upregulates transcription of AMP in hemocytes, thus resulting in increased bactericidal capacity of whole hemolymph.[157] On the whole, the results so far available on the effects of NM on innate immunity in bivalves indicate that, in realistic environmental exposure conditions, NM are not immunotoxic but can even have a beneficial effect on immune functions.

Changes in immune cell reactivity can be observed in the sea urchin *P. lividus* exposed in vivo to a range of NP (e.g., Fe_3O_4, CeO_2, and SnO_2), which include reduction in the production of the HSP70 protein, inhibition of the activity of cholinesterases and decrease of the basal protein levels of glucose-regulated protein 78.[158] These changes correlate with global toxicity, with animals exposed to 10^{-2} g L^{-1} of NP surviving for only 1–2 days, while those exposed to a lower NP dose (10^{-4} g L^{-1}) showed pathological signs (lack of spines, slow mobility) after 5 days. On the other hand, immune cells exposed to TiO_2 NP in vivo do not show altered HSC70 levels, and take up particles through a mechanism that involves the TLR4/p38 MAPK signaling pathway without activating an inflammatory response.[18] Notably, animals exposed to TiO_2 NP do not display any pathological state, showing a normal locomotion capacity, regular movement of the spines and tube feet, characteristic adhesion ability, no loss of spines, no excess of excretion, no loss of ectoderm, and no signs of bald sea urchin disease (a bacterial infection that affects several species of sea urchins).[95]

In mammals, a recent study in mice showed that SiO_2 NP increased mice susceptibility to *P. aeruginosa*. However, NP-loaded alveolar macrophages were fully efficient in bacterial clearing, highlighting that particle effects are related to alterations of the alveolar-capillary barrier while not affecting the normal course of the inflammatory responses.[159] Metal and metal oxide NM, including Ag and ZnO NP, are highly effective in bactericidal activity, and CuO and TiO_2 NP can also show microbicidal capacity against various microbes including *Klebsiella pneumoniae*, *Shigella dysenteriae*, *Vibrio cholerae*, *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecium*, *P. aeruginosa*, and *Staphylococcus aureus*.[160] More recently, it was reported that vitamin C lipid NP, used to deliver antimicrobial peptide to macrophage lysosomes, show remarkable antimicrobial activities in fighting multidrug-resistant bacteria, including *S. aureus* and *E. coli*, even in immunocompromised septic mice.[161]

It should be noted that the vast majority of the results showing little toxic or immunomodulatory effects of NM was obtained in healthy animals or under standard cell culture conditions. The possibility that NM may impact the innate immune defensive mechanisms of weaker, immunocompromised organisms, or under chronic inflammatory conditions is, therefore, still possible and remains under-investigated. In a murine model of ovalbumin (OVA)-induced lung inflammation, SiO_2 NP act as adjuvant of allergic airway inflammation, suggesting that they might worsen the disease in allergic individuals.[162] Additional studies confirmed the allergy-promoting effects of SiO_2 NP and report similar effects for metal oxide NP. Indeed, the particular individual conditions play a crucial role in determining the immune reaction to NP; while in non-pregnant mice the allergy-promoting effect of TiO_2 NP is only moderate, OVA-induced lung inflammation is clearly exacerbated in pregnant mice. This indicates that, possibly due to a pro-allergy reactive hormone/cytokine milieu in pregnant mice, inflammatory responses to NM may be enhanced during pregnancy.[163] Because NM have the ability to penetrate physiological barriers including the placental barrier,[164] potential effects of accumulating NP in embryonic tissues require attention. Many types of NM, including Si, Ag, and TiO_2 NP, were reported having effects on embryonic development in fish, amphibian, bird, and mammalian embryos, with reduced weight, deformities and an increase in toxicity.[165]

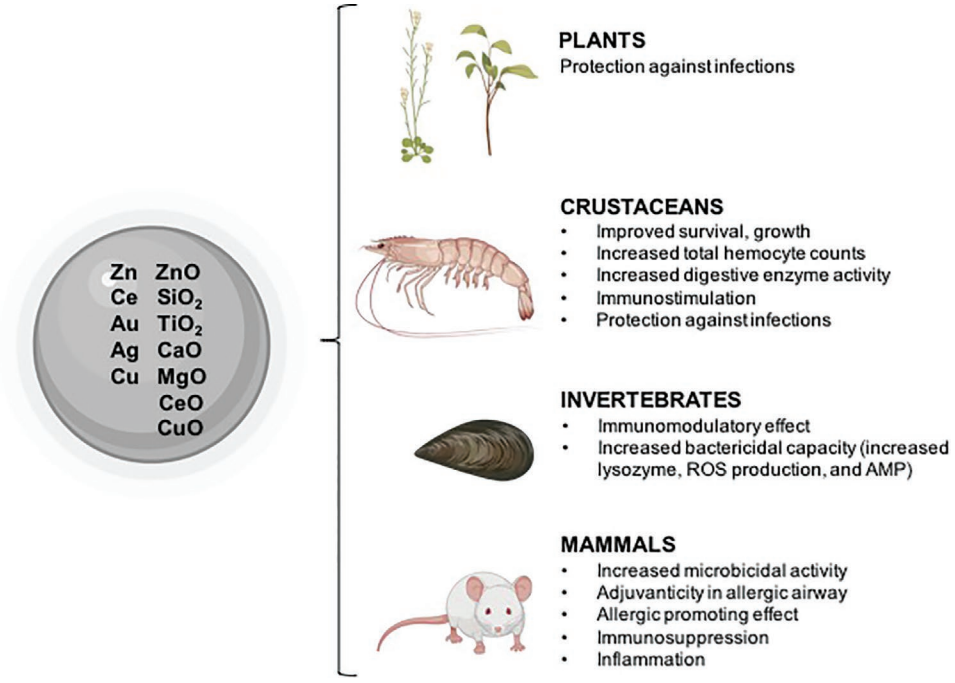

Figure 4. Effect of NM on innate immune defensive functions across living species. The figure depicts the effects of different NM, listed on the left, on innate immune defensive functions of different living species, i.e., plants (upper right), crustaceans and other invertebrates (center right), and mammals (lower right). Details are given in the text. Artwork by Paola Italiani.

While most studies explain potential pathologic effects of NM by their ability to induce toxicity and inflammation, a growing body of evidence suggests that some NM, especially metal, carbon and CeO_2 NP, can display immunosuppressive effects. This is through direct interference with inflammatory signaling pathways, preventing inflammasome assembly or acting as radical scavengers, thereby suppressing oxidative stress and inflammatory responses. While potentially useful in suppressing excessive inflammation (e.g., in chronic inflammatory diseases), a potential immunosuppressive effect due to the NM should be considered in nanomedicine, in particular in nanoparticulate anticancer therapies, because the NM could reduce efficient antitumor responses and increase susceptibility to infections.[166] The multiple effects of NM and their dual role in inflammation and immunosuppression thus further increases the complexity of understanding the contribution of NM to immunity. Developing embryos, young immunologically immature animals, damaged organisms or disease-affected individuals (e.g., chronic inflammatory diseases, cancer, and autoimmunity) may all react to NM in an inadequate manner, failing to adapt or to respond by elimination, thereby risking pathological consequences.

The effects of different NM on innate immune defenses of living species, from plants to mammals, are summarized in **Figure 4**.

5. Nanomaterials and Interaction with Bacteria

The interaction between NM and microorganisms is significant, and it is at the basis of an emerging discipline dubbed nanomicrobiology. It is notable that microorganisms naturally synthesize NP as a mechanism to detoxify their environment from toxic elements, normally heavy metals,[167] or use them as electrodes to assist direct interspecies electron transfer[168] or as reservoirs to release with molecular precision some essential elements (upon NM dissolution) at the site of action, for instance bactericidal ions (Ag^+)[169] or pro-proliferating ones (Fe^{2+}).[170] The NM size and water solubility allow them to finely integrate into the biological machinery. The interaction of prokariota with inorganic natural elements is clearly more intense and effective when compared to eukariota.

Regarding the interaction of NM with the immune system through microbiota, one has to bear in mind that the immune system develops upon interaction with microbiota. The system reaches an equilibrium in which commensal microbiota populate different organs and fluids of the organism without triggering an immune reaction, while only invading microorganisms induce a defensive immune response. Thus, NM can trigger immune responses only when they localize in some usually secluded tissues and present particular surface patterns that can be recognized by PRR or other innate receptors.[171] Triggering immunity may be undesirable if the activation is excessive, prolonged, or inducing severe tissue damage, but may result into an increase of host defenses against potential infection. Effects can be even more basal because of the possible similarities between microorganisms and NM that mimic biological structures, since the immune reactivity and competence develops as a consequence of the interaction with microorganisms.[172]

NM may admix with microbial communities and interfere with their biology, and induce population shifts and

modifications in the different consortia by empowering or restraining different species. The possibility of shaping the microbiota communities with the use of NM can be used at our advantage, as increasing evidence indicates that our health status[173] and therapy response[174] are strongly dependent on the gut microbiota. NM can provide microbiota with different essential elements, from iron to magnesium and zinc, to ultratrace ones such as selenium, cobalt or nickel,[175] or they can provide a hostile environment to pathogens or undesirable species colonizing our gut.[176]

5.1. Host–Bacteria Interactions under Nanomaterial Exposure

A major application of NM is as an antimicrobial agent. NM efficacy as antimicrobial agents is thought to be linked to the physical interaction with the bacterial walls, followed by formation of lesions in the cell membrane and subsequent intracellular accumulation of toxic ions.[177,178] Over the last decade, the increased availability and accessibility of high-throughput sequencing methods has greatly enhanced our understanding of the diversity and functionality of the microbial world and its role in animal health. The roles microbes can play in animal health can be diverse and include both beneficial as well as pathogenic interactions. Well described beneficial roles of microbes include the provision of nutrients,[179] aid in digestion[180] and degradation of complex organic molecules.[181,182] Notably, commensal microbiota play an important beneficial role in immune defense by allowing for the adequate development of local and systemic immunity and by preventing the establishment of microbial invaders.[172] The interaction between microbes and host is highly dynamic, and it can switch from a beneficial or commensal equilibrium to a pathogenic/invasive disequilibrium, for example when microbes move from their original niche to another.[183] The immune system of animals regulates the host–microbe interaction at the interaction/barrier surfaces (e.g., in the gut), maintaining microbes that are beneficial to the host and preventing invasion and barrier breaches. Immune recognition of microbes through PRR does not really distinguish between pathogenic and non-pathogenic bacteria, and threat is associated to the capacity of invasion (breaches of the barrier). This is the reason why most of the innate immune cells that can sense microbial patterns are located right below the barrier. Thus, in the case of damage of the barrier (as in some diseases, or mechanical or toxic) the underlying innate cells recognize and react also to commensal bacteria, because of their entry into the body. Chemical exposure can lead to disturbance of the microbiota (dysbiosis),[184,185] which can negatively affect host immunity, thereby promoting susceptibility to infections. As an example, in the tsetse fly antibiotic-induced loss of symbiotic bacteria severely compromises host immune functions making the host unable to control bacteria that are non-pathogenic under normal conditions.[186]

Since microbiota and immune functions are integrally linked, assessment of the immunosafety of NM and chemicals should include studies on impact on the interaction between bacteria and host immunity especially for those agents that are designed to target microbes (biocides). Studies on the impact of biocidal NM on host-associated microbiota are still rather limited in number and have been reviewed in detail elsewhere.[185,187,188] Many of these studies have used rodents, and only a few have used nonrodent vertebrates or invertebrates as model species. The outcomes are somewhat contrasting with regards to the capacity of biocidal NM to cause disturbance to the host-associated microbiota. Some studies, for example, indicate a clear negative effect of Ag NP on the relative abundance of *Firmicutes* and *Lactobacillus*,[189,190] whereas other studies indicate a positive or no impact of Ag NP on the relative abundance of these taxa.[191–193]

The absence of clear effects of biocidal NM on the bacterial community structure, as reported by some,[193,194] is surprising given the high activity of biocidal NM in in vitro exposure studies. This lack of toxicity on gut microbial communities has been linked to physical–chemical conditions in the gastric environment. At low pH, as in some gastrointestinal tracts, Ag NP can quickly dissolve but can subsequently precipitate to form the much less toxic AgCl NP.[195] Intestinal anoxia is another possible driver of toxicity of biocidal NM. In anoxic conditions, both Cu NP and Ag NP can quickly sulfidize,[196–198] a reaction that strongly reduces their toxicity.[199–201] In addition, gut microbiota may be physically protected from exposure to biocidal NM due to their embedment into intestinal folds. Physical and chemical conditions in the intestine are thus likely modulating the toxicity of NM, as well as other toxicants,[184] which could explain the discrepancy in outcomes between in vitro and in vivo NP toxicity studies.[193]

Studies that have looked at disruption of mucosal integrity induced by NM have yielded contrasting results, with some studies showing no evidence for any histological change under biocidal NM exposure[192,194] and others showing strong signs of epithelium and crypt damage.[189] It is thus not fully understood under which conditions NM induce histological damage to intestinal tissue. Recent studies showed that in the bivalve *Mytilus galloprovincialis* the immunomodulatory effects of exposure to TiO_2 NP is associated with a shift in hemolymph microbial composition, indicating the interplay between the microbiota and the immune system.[157] Similarly, in rodents, NM-induced alterations of the gut microbiota is linked to altered expression of host cytokines.[189,190] The co-occurring modulation by NM of the host-associated microbiota and the host immune system, as reported in both invertebrates and vertebrates, thus reflects the integrated link between microbes and immunity, and indicates the need to include microbiota analysis in nano-immunosafety assessment.

5.2. Interaction of Bacterial Molecules with Nanomaterials

The effect of NM in the host–microbiota interaction is of course not limited to direct effects on bacterial communities or on the immune system. The interaction of microbial factors with NM can modulate immune reactions in a way that is different from those achieved by microbial molecules or NM separately. In a recent study, human DC were exposed in culture to SiO_2 NP together with supernatants from either commensal or pathogenic bacterial cultures.[202] In this study, NP and bacterial molecules together induced the production of inflammation-related cytokines, with differences between bacterial molecules (those from pathogenic bacteria could induce cytokines only in the presence of toxic NP concentrations) and depending on the

NP concentration (non-pathogenic bacterial molecules could induce the production of different cytokines if in the presence of non-toxic vs toxic NP concentrations). The effect was truly synergistic and not due to adsorption of bacterial molecules on the NP surface.

It is an important issue in immunotoxicity studies the notion that bacterial molecules can be adsorbed on the NM surface giving rise to hybrid entities with new functional characteristics. Of particular importance is the interaction between NM and bacterial endotoxins, highly inflammatory and pyrogenic lipopolysaccharidic molecules (i.e., LPS). Given the high abundance of endotoxin in the outer membrane of Gram-negative bacteria, efficient recognition of endotoxin by the immune system is a key mechanism in host defense. Humans are particularly susceptible to LPS, which induces potent innate and inflammatory reactions by activating cells mainly through TLR receptors. It has been shown that LPS can adsorb on the surface of citrate Au NP, stabilize them and reduce their ability to form a biocorona in human plasma.[203] LPS on the NP surface is still active and can induce an inflammatory response in human monocytes comparable to that of free endotoxin.[203] Similarly, the inflammatory effect of TiO_2 NP on murine macrophages is significantly increased upon LPS adsorption.[204] Compared to free LPS, LPS absorbed on TiO_2 NP induces similar levels of NOS, but higher expression of NOS2 proteins, whereas the effect of NP in combination with unbound LPS is low.[204] Whether adsorption of LPS on NM increases, decreases or leaves unchanged the inflammatory effectiveness of LPS appears to strongly depend on the physical properties of NM, the dose and timing of administration and the target cell type.[205–209] We can in any case assume that LPS-coated NM behave differently from free LPS and uncoated NM, suggesting that LPS-coated NM represent new nano-objects with different characteristics, which will have to be considered as such in nanotoxicological studies.

LPS is a widespread and thermostable bacterial molecule that represents a ubiquitous potential source of contamination even in the absence of viable bacteria. Humans are highly sensitive to LPS and respond to 1000× lower LPS concentrations compared to mice.[210,211] Careful and reliable detection of potential endotoxin contamination of NM is therefore crucial, especially in the fields of immunology, drug development, and drug delivery.[212,213] This also applies to NM synthesis.[214] It has been shown that specific components, including citrate, Triton X-100, and bovine serum albumin hinder reliable LPS recognition by commercially available LPS detection assays.[215] From these data, we can conclude that effects of NM on immune cells must be analyzed very carefully to distinguish potential contaminating LPS effects from real NM-mediated effects. However, due to their ability to interact with endotoxin and due to their optical and physical properties, novel LPS detection platforms involving NM may be developed in the future.[216]

6. Nanomaterials and Innate Memory

Immune memory is a key defensive mechanism that allows living organism to adapt to their environmental conditions by mounting efficient defense against the threatening agents that are present in the same environment. In plants and invertebrates, which do not have adaptive immunity, the term "immune memory" is sufficient for defining the phenomenon, as they display only one type of immunity, i.e., the innate defensive mechanisms. On the other hand, in higher vertebrates in which adaptive and innate immunity coexist, the term "innate immune memory" specifically defines the evolutionarily conserved memory mechanisms of innate immunity, distinct from the better-known adaptive immune memory. Innate memory enables organisms to cope in a more efficient way with external challenges, after a first encounter, thereby ensuring increased survival and fitness. How NM may induce innate memory or modulate the establishment of memory following the interaction of organisms with foreign agents is an open question that needs attention, as it can have a significant long-term impact on the living organisms' capacity to cope with/adapt to their environment.

Innate memory in plants corresponds to systemic acquired resistance (SAR), a phenomenon that is induced in plants by local pre-infection with pathogens.[217] This local infection leads to the activation of defense responses in the local tissue but also in distant uninfected tissue and is effective not only against the pre-infecting pathogen but to a broad spectrum of diverse pathogens. Such response is effective after a few hours and lasts for several weeks, and it primes the plant for defense to subsequent infection and thereby renders the locally pre-infected plant resistant in all parts and against multiple pathogens.[218] The activation of SAR is characterized by an accumulation of the phytohormone SA, which acts as a signaling molecule in the induction of pathogenesis-related (PR) proteins. Azelaic acid is supposed to be a mobile signal that transports the priming information to distant tissues.[217]

Only very few studies concerning the role of NM on the induction of SAR have been conducted, and so far the mechanisms of these effects on the induced resistance are mostly unknown. Changes in the expression of *A. thaliana* genes involved in SAR after treatment with Ag NP coated with polyvinylpyrrolidone and silver ions (Ag^+) were analyzed by Kaveh et al.[219] The study shows that the flagellin pattern recognition receptor FLS2 and the azaleic acid induced gene 1 (AZI1) are downregulated after NM treatment, suggesting that Ag NP have a negative impact on innate memory in plants.[219] The expression of the PR genes *PR1, PR2,* and *PR5* was examined in *A. thaliana* after application of nanosized Ag–silica hybrid complexes and Ag NP. Genes implicated in SAR were significantly upregulated after treatment with NM, but the hybrid complexes caused a faster activation of *PR* genes.[220] Another study examined the capacity of Ag NP to induce SAR against tomato mosaic virus and potato virus, and their direct ability to suppress viral infections on tomato plants. Plants treated with Ag NP and later inoculated with the virus showed a lower disease rate. Indeed, Ag NP were able to bind virus particles and/or block viral replication in the case of many different viruses in the absence of toxicity for the host cells.[221] In contrast with the study described above, the expression of the SAR marker gene *PR1* was repressed in *Solanum lycopersicum* plants treated with chitosan-polyvinyl alcohol (PVA) hydrogels plus copper NP (Cs-PVA + Cu NP) and Cs-PVA alone. In both cases the expression of *PR1* was repressed in normal condition

but also under saline stress.[222] Imada et al.[223] reported that nano-MgO treated roots rapidly generated ROS along with upregulation of *PR1*, jasmonic acid, ethylene, and systemic resistance-related genes.[148]

The development of nanotechnology has opened up new frontiers in order to solve technical problems in efficiently delivering plant immune inducers. In particular, mesoporous silica NP, already used as anticancer drug delivery systems, are expected to be a promising carrier of resistance inducers.[224] To date engineered NP have been successfully used as phytohormone delivery systems[225] or like pesticide carriers in order to target their activity and reduce the indiscriminate leaking of toxic compounds, alleviating the potential adverse effects on the environment.[226] Functionalized mesoporous silica NP with a redox-responsive gatekeeper system, able to specifically release the resistance inducer and avoiding premature leakage in the delivery process, demonstrated their potential application in agriculture. In fact, this system has proven to be an efficient technique to introduce resistance inducers and reduce *Phytophthora* infections in pineapple.[224] NM application in plants is still in its infancy but promising strategies are emerging. Therefore, some NM might in the future offer applications to stimulate innate immune responses in plants and help agronomists to reduce yield losses caused by biotic factors without extensive use of conventional pesticides.

Invertebrates can adapt and survive solely relying on innate immunity. This implies the presence of complex and sophisticated mechanisms of immune specificity, and the capacity to mount a faster/more effective response upon re-exposure to threatening agents, which is the function of innate memory or "immune priming." The innate memory of invertebrates is a well-known phenomenon, observed and exhaustively described in practically all phyla as consequence of infection immunity, natural transplantation immunity, individual, and transgenerational immune priming.[227] However, molecular and functional immune parameters responsible of the induction of innate memory sensu stricto (as response to a challenge of a quiescent immune system that has been previously primed) remains largely unknown. Memory is usually induced by priming with infective microorganisms, and the effect is examined as survival to reinfection.[228–230] Notably, memory, which is based on epigenetic changes, can be inherited by the progeny of primed animals, thereby enhancing the fitness of the progeny in the same environmental challenges encountered by the parents.[228,231,232]

Despite the widespread studies on invertebrate immune memory, information on memory responses to NM is virtually absent. The only available data come from an unpublished study in bivalves. The mussel *Mytilus spp*. has extremely powerful immune defenses to cope with different potential pathogens and contaminant stressors, including NM. The possibility that NM may participate in immune memory has been recently investigated in *M. galloprovincialis* subjected to repeated exposure to nanoplastics (amino-modified polystyrene PS-NH_2), by examining the modulation of several functional and molecular parameters in hemocytes and hemolymph. First exposure indicates the occurrence of stress conditions in the hemocytes, which, however, did not result in changes in the overall bactericidal activity (suggesting a reaction that resolves without consequences). After second exposure, it was possible to observe a shift in granular hemocyte subpopulations, together with re-establishment of basal functional parameters and of proliferation/apoptotic markers. Moreover, the hemolymph bactericidal activity was increased, as well as transcription of immune related genes coding for secreted proteins. The results indicate both tolerance and potentiation as compensatory mechanisms to maintain immune homeostasis after a second encounter with PS-NH_2, representing a form of immune memory.[233]

The same forms of innate memory, "tolerance" and "trained immunity/potentiation," are documented in mammals/vertebrates. Although the innate memory can be induced by any type of exposure, most of our knowledge on the tolerance phenomenon comes from study on LPS exposure, while that on trained immunity/potentiation is mainly related to exposure to Bacillus Calmette–Guérin (BCG) or to fungal cell wall β-glucans. The concept of innate memory in higher vertebrates, known for decades, has recently experienced renewed interest, with a wealth of studies describing the effect of "priming," either in vivo or in vitro, on the subsequent reactivity of innate immune cells (especially macrophages and monocytes) to an unrelated challenge. A typical experiment on the induction of human innate memory in vitro showed that human monocytes primed with β-glucan from *Candida albicans* developed an enhanced inflammatory cytokine production after restimulation with TLR1/2 agonists.[234] The molecular mechanisms underlying the development of innate memory in mammals include metabolic changes (i.e., shift from oxidative phosphorylation to aerobic glycolysis—"Warburg Effect)" and epigenetic reprogramming (e.g., DNA hypermethylation and histone modifications). Human monocytes and macrophages primed with LPS or β-glucan present specific histone modifications,[234] and β-glucans from *C. albicans* induce a metabolic reprogramming of human macrophages toward aerobic glycolysis that is possibly involved in the development of innate memory.[235] The interplay between cellular metabolism and chromatin remodeling associated with the development of innate memory has been reviewed elsewhere.[236,237]

Older evidence of the existence of the innate memory in humans in vivo comes from epidemiological studies showing that vaccination with *Mycobacterium bovis* BCG (a live attenuated tuberculosis vaccine) provides protection against non-related infections.[238,239] A recent study highlighted the effect of BCG vaccination on the development of the innate memory both in vivo and in vitro. The authors observed that BCG vaccination protects humans from viral challenges (simulated with the administration of Yellow Fever Vaccine in volunteers) through the involvement of IL-1β production, and confirm in vitro the role of IL-1β in inducing epigenetic reprogramming of monocytes.[240] The BCG-induced memory was shown to persists one year after vaccination.[241] This prolonged persistence of BCG-induced memory is hard to explain because of the shorter lifespan of the innate immune cells that develop memory following priming (mostly monocytes and macrophages). To explain the persistence of innate memory, recent evidence shows that BCG can access the bone marrow and change the transcriptional landscape (i.e., the areas of chromatin accessibility) of hematopoietic stem cells (HSCs) and multipotent progenitors, leading to local cell expansion and enhanced myelopoiesis.[242,243] The

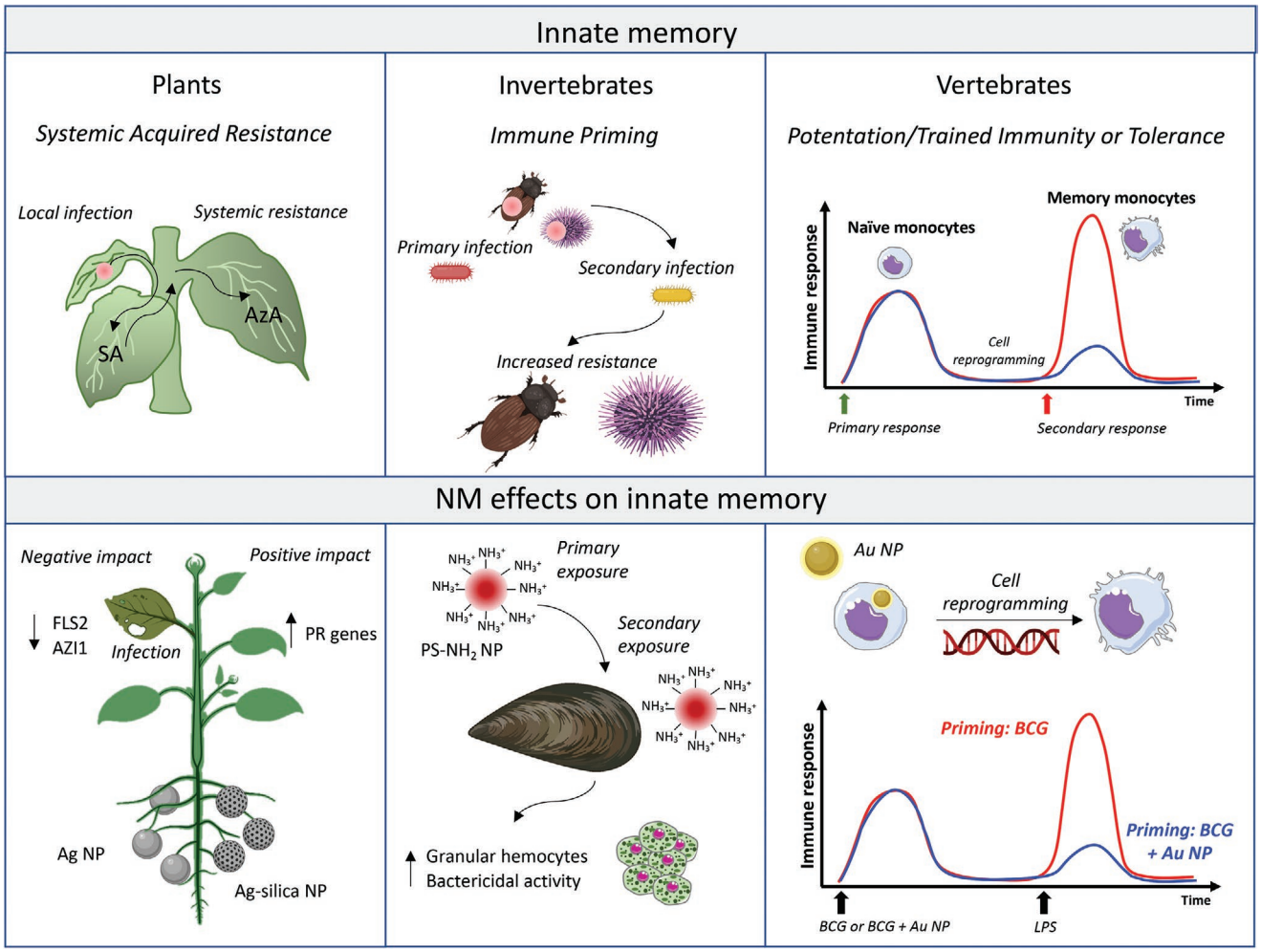

Figure 5. Innate immune memory across living species. Upper panels: main mechanisms and effects of innate immune memory in plants, invertebrates, and vertebrates. Lower panels: effects of NM on the innate immune memory mechanisms in plants, invertebrates, and vertebrates. SA, salycilic acid; AzA, azaleic acid; FLS2, flagellin pattern recognition receptor; AZI1, azaleic acid induced gene 1; PR, pathogenesis-related. See text for detailed description. Artwork by Paola Italiani.

authors demonstrate that BCG-educated HSC generate epigenetically modified macrophages able to protect mice against virulent *Mycobacterium tuberculosis* infection, and that BCG-induced HSC reprogramming occurs in vivo.

Despite the growing number of studies on innate immune memory in mammals/humans, and the large body of literature on the interaction of NM with the mammalian innate immune system,[62] NM effects on the development or modulation of the innate memory to date remains practically unstudied. The hypothesis that NM may have an effect on innate immune memory is possible and supported by a series of considerations arising from the fact that NM can have an effect on the molecular mechanisms underlying the development of innate memory,[244] which are mainly based on epigenetic and metabolic reprogramming. Indeed, NM are able to induce both epigenetic[245] and metabolic changes.[246] Thus, it is reasonable to presume that NM may be able to induce or modulate innate memory and, therefore, affect the capacity of innate cells to react to a second exposure. Indeed, preliminary studies on human primary monocytes in vitro showed that exposure to Au NP could change the subsequent response to LPS and that changes could differ in different individuals.[244] Another study has proven the significant effect of Au NP in reducing the BCG-induced memory in human primary monocytes.[247] Overall, the putative capacity of NM to interact with innate immune processes at the level of innate memory has very interesting implications, opening the way to the medical use of NM for controlling subsequent innate/inflammatory responses for a significant period of time, with beneficial effects in autoimmunity, infections and immune-related diseases.

A schematic depiction of the mechanisms of innate memory and of the effects of NM on its establishment and effects is provided in **Figure 5**.

7. Conclusions and Future Challenges

When assessing the immunosafety of NM, we mainly need to examine the interaction of exogenous NM with the innate

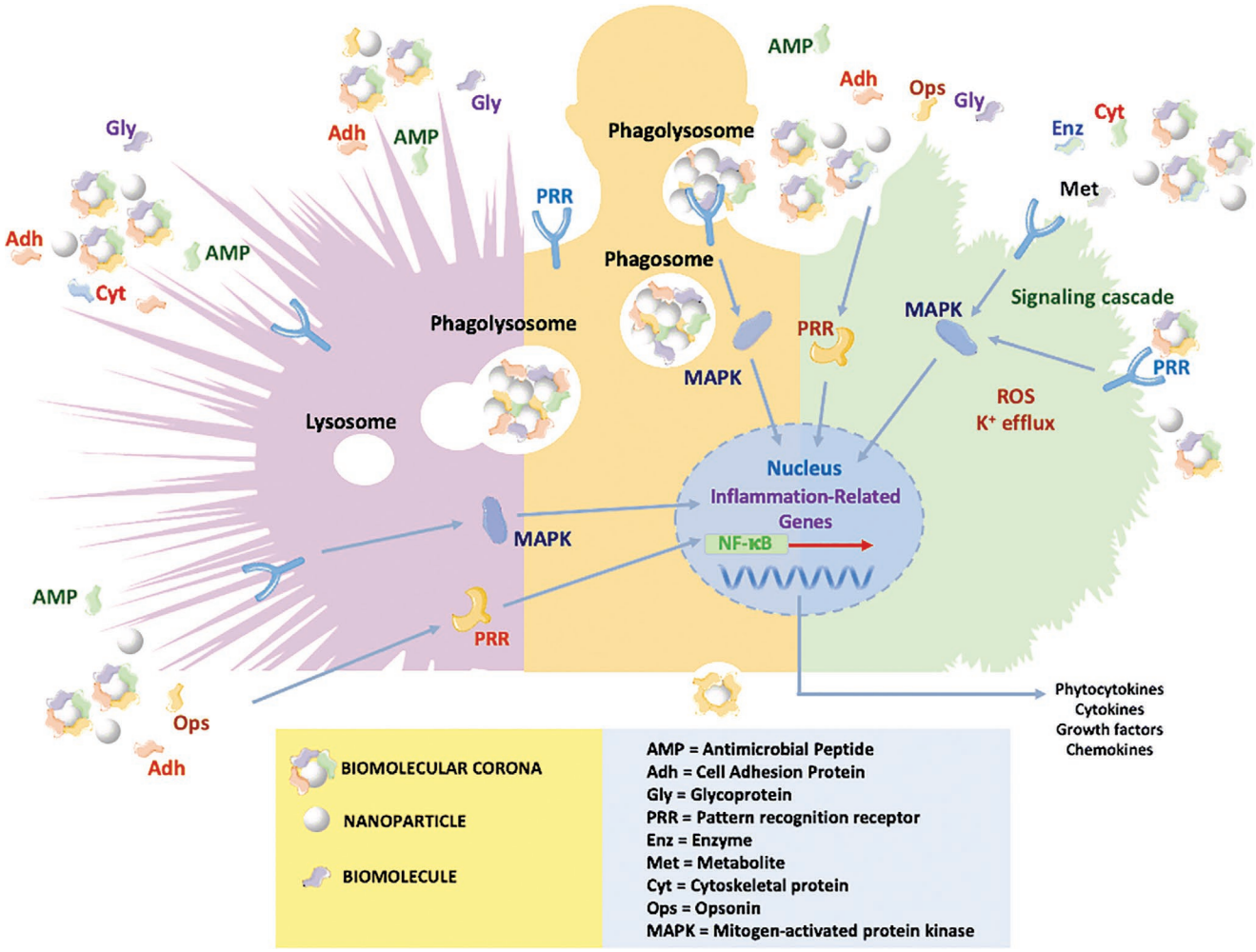

Figure 6. Common immune mechanisms induced by NM across living species. Schematic depiction of the common mechanisms of immune reaction of living species (plants, green silhouette; invertebrates, purple silhouette; vertebrates, yellow silhouette). Upon interaction with the microenvironment, biomolecules adsorb to the NM surface and create a "biomolecular corona." NM–biomolecule interactions signal for non-inflammatory clearance (opsonization and endocytosis) or trigger a defensive innate/inflammatory response through an expanded repertoire of PRR initiating an appropriate signaling cascade. Immune responses include ROS, MAPK activation, defense gene induction, and production of cytokines, chemokines, and growth factors. The combination of a multitude of cellular effects have, as result, the preservation of cell/tissue integrity and function. Artwork by Andi Alijagic.

immune system of living organisms. Innate immunity is, for over 95% of living eukaryotic organisms, from plants to mammals, the only type of immunity displayed. This fact makes adaptive immunity (coexisting with innate immunity in higher vertebrates) a less relevant actor in the nano-immune interaction. Indeed, innate immunity is the first, most powerful, and fastest line of immune defense. The mechanisms of innate immunity are fundamentally similar across species and evolution, despite the fact that they are to some extent the result of parallel and challenge-specific evolution and that there are species- and environment-specific differences. Natural and anthropogenic NM are one of the many foreign challenges present in the environment that come in contact with living organisms and their immune system. For NM as for all foreign agents, based on sensing and sorting molecules and mechanisms, the immune system decides the best way to cope with them with the ultimate goal of preserving the organism's health and fitness. There are essentially three ways to cope with NM. Ignoring them (e.g., in marine filter feeders, they are largely expelled with water), adapting to them (e.g., taking and eliminating/storing them with non-inflammatory non self-damaging mechanisms), or reacting to them with an active innate/inflammatory reaction. We should consider as immunosafe those NM that fall in each of the three cases, including those that trigger an innate/inflammatory reaction. Only when such reactions turn out to be anomalous (too prolonged in time or too short/inefficient, excessive or too weak), then they could cause pathological consequences. A summary view of the innate immune mechanisms engaged in the interaction with NM is presented in **Figure 6**, which underlines the similarities across species.

In light of the above considerations, we should re-think immuno-nanosafety by critically examining the validity and predictivity of the data produced by the scientific community, and by identifying the future challenges and most promising

avenues. One of the drawbacks for understanding relevant effects is the use of high concentrations of NM in experimental settings that do not represent real life situations. Cell death in vitro upon exposure to high concentrations of NM may not reflect toxicity for the same cells in vivo within a complex organism and at realistic concentrations. Also, experimental studies still suffer of many limitations that hamper their reliability and that may not provide useful information. For instance, studies on human innate reactivity to NM are usually restricted to in vitro systems that, however complex they may be, still cannot reproduce the in vivo situation. The use of tumor cell lines instead of primary cells further decreases the possibility that the results obtained may be valid for normal cells. In addition, studies of chronic exposure are not feasible with in vitro models. In vivo experimentation in rodents is also not ideal because of a number of significant differences, including the geometry of exposure (for instance how inhaled NM will deposit in the lungs of animals based on gait) and some critical innate immune mechanisms (e.g., complement). Therefore, one of the future challenges for human nano-immunosafety is that of identifying reliable experimental models that could realistically represent the in vivo immune reactions to NM and the true risk for health. Stepping from short-term to long-term exposure models will be a particular challenge. An interesting approach is to focus on invertebrates and plants, in which the global response to NM exposure can be assessed much more easily and over a long time, although even among invertebrates we should identify representative and predictive models.

Another challenge that we will have to face in assessing nano-immunosafety is the fact that different cells can have different sensitivity to NM challenge. Indeed, as mentioned before, some highly proliferating cells can be more susceptible to chemicals or other compounds, while quiescent cells can be more resistant. In the context of a particular organ (e.g., the lung) some cells can die while others are untouched, but what counts is the final outcome, i.e., how the organ succeeds in eliminating the foreign potentially dangerous agents and re-establishing the organ physical and functional integrity. This means that the susceptibility or resistance to toxicity of one single cell type may not reflect the events in real life conditions. Notably, the reaction in one organ may be limited spatially to that organ (or part of the organ, or some cells within the organ), without affecting survival or fitness.

The immunosafety of NM should also consider the pre-existing or ongoing health conditions of the organisms. Plants or animals that are infected or damaged or senescent can be less able to adequately react to new challenges. Thus, in human nano-immunosafety, the effects of NM that are harmless in healthy conditions can still be potentially harmful in immunologically frail conditions. As health in invertebrates and vertebrates also depends on the composition of the associated microbiota, an additional very important area of investigation will be the NM–microbiota interaction, and the health-related consequences of such interaction for the whole organism.

Finally, a very interesting new area of investigation in nano-immunosafety is the possible effect of NM in shaping innate memory, i.e., in determining changes in the protective immune response to subsequent challenges. Immune memory is the mechanism by which organisms "remember" previous exposure to foreign agents, so that they respond better (with a more effective immune response) to recurring challenges. While the phenomenon of innate memory is well known in plants, invertebrates, and also in vertebrates, very little is known on the possibility that NM may alter the establishment of protective memory or even induce an anomalous memory. This is an area that needs addressing, as NM that have no direct effect and are therefore considered immunosafe could nevertheless modify immune memory with unpredictable consequences.

As we have summarized in this review, examining the interaction of NM with the innate immune defensive mechanisms in plants and animals leads to the overall feeling that NM in general do not pose significant threats to the organisms' survival and fitness, as they rarely induce direct effects or interfere with ongoing innate reactions under realistic conditions. Thus, it seems that the major immune responses to NM is silent elimination (adaptation) or successful immune elimination (innate/inflammatory reaction with full resolution). Of course, it is not possible to generalize, and caution should always be taken, and the new challenges opened by the advancements in our knowledge and technology should be confronted. Some of them, already discussed above, are listed hereafter:

1) Realistic and predictive experimental models.
2) Organ/tissue restriction of NM immune effects.
3) NM effects in immunologically frail organisms.
4) NM effects on organism-associated microbiota.
5) NM effects on innate immune memory.

Overall, we should aim at defining NM immunosafety not in absolute terms, but relative to specific conditions of use/exposure (organism, tissue/organ, exposure features, health conditions). While the "safe-by-design" concept seems unfeasible, we can successfully target a "safer-by-design" nanotechnology.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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