European Journal of Pharmaceutics and Biopharmaceutics 95 (2015) 3–12

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research Paper

It takes two to tango: Understanding the interactions between engineered nanomaterials and the immune system

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ARTICLE INFO

Article history:
Received 9 November 2014
Revised 13 February 2015
Accepted in revised form 3 March 2015
Available online 11 March 2015

Keywords:
Nanomaterials
Nanotoxicology
Macrophages
Bio-corona
Inflammasome
Danger signals

ABSTRACT

The immune system represents our primary defense system against foreign intrusion, including pathogens as well as particles. In order to understand the potential toxicity of engineered nanomaterials of ever increasing sophistication, it is necessary to understand the sophistication of the immune system with its multiple, specialized cell types and soluble mediators. Moreover, it is important to consider not only material-intrinsic properties of the pristine nanomaterial, but also the acquired, context-dependent 'identity' of a nanomaterial in a living system resulting from the adsorption of biomolecules on its surface. The immune system has evolved to recognize a vast array of microbes through so-called pattern recognition; we discuss in the present review whether engineered nanomaterials with or without a corona of biomolecules could also be sensed as 'pathogens' by immune-competent cells.

1. Introduction

The ability to manipulate matter at the nano-scale enables many new properties that are both desirable and exploitable, but the same properties could also give rise to unexpected (if not entirely novel) toxicities that may adversely affect human health [1]. Delineating the physico-chemical properties that are driving the toxicity of nanomaterials remains a challenge [2]. However, being able to link material properties to toxicity would enable the prediction of nanomaterial hazards and facilitate the design of nanomaterials that retain their useful properties, but display reduced toxicity (i.e., safety-by-design). Automated, high-throughput screening of well-defined libraries of nanomaterials is likely to aid in this endeavor and data generated through this approach can be used for structure–activity relationship (SAR) modeling using *in silico* methods [3]. If one can delineate the nanomaterial 'properties of concern' then assays for screening of nanomaterials could be refined and patterns would begin to emerge that allow for grouping of nanomaterials. In addition, systems biology approaches whereby quantitative measurements of molecular and functional changes are determined using gene expression profiling or other omics-based methodologies combined with computational modeling of the molecular interactions may also aid in defining the interactions of nanomaterials and other chemicals with biological systems [4].

In this context, it may be pertinent to recall that the immune system has evolved to protect us from foreign intrusion, including bacteria, viruses, parasites as well as particles [5]. Indeed, viruses, may be viewed essentially as self-replicating, biological nanoparticles that 'hijack' the biological machineries of the host for their own purposes. Notably, immune cells belonging to the innate (or, 'primitive') arm of the immune system use so-called pattern recognition receptors, including Toll-like receptors (TLRs), to recognize conserved molecular motifs on the surface of microbes and this allows for the recognition of a multitude of different microorganisms through the recognition of a limited number of molecular 'signatures' [6]. Thus, there are important lessons to learn from the field of immunology in terms of understanding how nano- or micro-scale objects are perceived by the immune system. Conversely, as highlighted by Hubbell et al. [7] material sciences have a great deal to offer immunology and medicine; the purposeful design of vaccine platforms is one example, as we will discuss below. However, the aim of the present essay is not to provide a

Abbreviations: DAMPs, danger-associated molecular patterns; DCs, dendritic cells; EPO, eosinophil peroxidase; GO, graphene oxide; HMGB1, high-mobility group box 1 protein; LPS, lipopolysaccharide; MPO, myeloperoxidase; PAMPs, pathogen-associated molecular patterns; NAMPs, nanomaterial-associated molecular patterns; NASPs, nucleic-acid scavenging polymers; NETs, neutrophil extracellular traps; NLRP3, nucleotide-binding domain, leucine-rich family (NLR), pyrin-containing 3; PRR, pattern recognition receptor; ROS, reactive oxygen species; TLRs, Toll-like receptors; SPIONs, superparamagnetic iron oxide nanoparticles; SWCNTs, single-walled carbon nanotubes; TNF, tumor necrosis factor.

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http://dx.doi.org/10.1016/j.ejpb.2015.03.007
0939-6411/

litany of all relevant nanomaterials studied, but rather to extract illustrative examples from the literature.

Nanotoxicology may be viewed as the study of the undesirable interference between man-made nanomaterials and biological nano-scale structures [5]. Here, we will focus, in particular, on nanomaterial interactions with the innate and adaptive arms of the immune system. We shall also consider the notion that engineered nanomaterials may, in fact, be recognized as 'pathogens' by cells of the immune system, including macrophages and other phagocytes, and the finding that carbon-based nanomaterials are enzymatically degraded by different innate immune cells much like bacteria and fungi.

2. Nanotoxicology: understanding the identities of nanomaterials

Most nanomaterials that have been studied to date are relatively simple materials; however, future developments may incorporate several different nano-sized components into complex assemblies of nanomaterials and suitable methodologies with which to assess adverse effects of such composite structures are therefore needed. In addition, as pointed out recently [8], increasingly sophisticated materials, including active materials responding to environmental cues or self-assembling materials present new, dynamic risks that are currently not well understood. Nanomaterial-enabled products also need to be assessed for any potential hazard throughout the product life cycle.

Numerous studies in recent years have shown that engineered nanomaterials may display toxicities *in vitro* and *in vivo*, even though it has been argued that many of these studies are fraught with shortcomings related to the use of unrealistically high doses, or the lack of reference materials with which to benchmark the results, and so on [9,10]. Moreover, it is important to determine whether toxicities of nanomaterials are 'novel' or merely scalable (and therefore, in principal, predictable, based on the study of larger particles or fibers). Indeed, it has been argued that "the final common pathways for pathological effects, that is, oxidative stress, inflammation, and genotoxicity are entirely shared by both nanoparticles and conventional particles and no novel pathogenic pathways are anticipated" [11]. Yet, it remains possible that the proximal events leading to the initiation of a common downstream program of cellular demise and subsequent organ dysfunction may nonetheless be related to the size of the offending particle. For instance, nanoparticles of a certain size have been shown to interject themselves into the inter-endothelial cell adherens junction thereby eliciting a size-dependent toxicity, manifested in an *in vivo* model as vascular leakiness [12]. Moreover, as noted recently, future materials of ever-increasing sophistication are more likely to resemble the complexity of natural nano-scale machineries rather than the apparent simplicity of chemicals [8]. This means that the assays used to assess for toxicity need to be sophisticated too, and that care should be taken to exclude assay interferences related to the nanoparticles themselves. Furthermore, nanoparticles that come into contact with biological fluids are thought to be rapidly covered by biomolecules forming a 'corona' that, in turn, interfaces with biological systems [13]. Indeed, it has been suggested that the interactions of engineered nanomaterials with cells and tissues are determined by the combination of material physico-chemical properties (the 'synthetic identity') and the context-dependent properties arising from the bio-corona the composition of which depends on the biological compartment in question [2]. This may be of particular relevance when considering interactions with the immune system as macrophages and other professional phagocytic cells of the immune system are equipped with receptors that recognize opsonized microorganisms and particles (opsonization is the process whereby a pathogen is marked for engulfment through coating with a substance, such as a protein).

2.1. The intrinsic, synthetic identity of nanomaterials

The importance of material-intrinsic properties, such as size, shape, surface charge and colloidal stability, for toxicological effects of nanomaterials cannot be overstated [2]. Together, these properties constitute the 'synthetic identity' of a material. Size is important as size can affect, for example, cellular uptake or the ability of particles to traverse biological barriers. In a seminal study, Choi et al. [14] followed the fate of intratracheally instilled near-infrared (NIR) fluorescent nanoparticles that were varied systematically in size, surface modification and core composition and determined that nanoparticles with hydrodynamic diameter less than 34 nm with non-cationic surface charge translocate rapidly from the lungs to regional lymph nodes in rats following intratracheal instillation. Furthermore, nanoparticles with a hydrodynamic diameter less than 6 nm were found to traffic from the lungs to lymph nodes and the bloodstream, ultimately being cleared from the body through the kidneys [14]. Moreover, nanoparticle behavior was found to depend strongly on surface coating which affects protein adsorption in body fluids; hence, for charged nanoparticles, nonspecific adsorption of endogenous proteins, mostly albumins, resulted in a large increase in hydrodynamic size, and this affected the biodistribution of the nanoparticles [14]. It is noteworthy that particles of the same chemical composition can elicit different responses depending on their shape. Hence, using highly stable, polymer micelle assemblies known as filomicelles, Geng et al. [15] compared the transport and trafficking of filamentous particles (filomicelles) with spherical particles of similar chemistry in an animal model. The filomicelles persisted in the circulation up to one week after intravenous injection which is about ten times longer than their spherical counterparts. Using a flow chamber with immobilized phagocytic cells, long filomicelles were found to flow past the cells, while smaller micelles and spheres were captured [15]. Surface charge also impacts on the interaction of nanoparticles with cells; higher toxicity of positively charged nanoparticles is generally correlated to their enhanced cellular uptake [16]. It has been suggested that the adsorption of a bio-corona of proteins may effectively equalize the surface charge of different nanoparticles [17]. However, it remains possible that the bio-corona is stripped off inside the cell, for instance in lysosomes [18], thereby revealing the intrinsic surface charge of the nanoparticle itself. Dissolution (of metal or metal oxide nanoparticles) and other forms of biotransformation may also occur in a living system and this has been shown to drive the toxicity of various nanoparticles (see [19] for a recent review). Finally, surface coating or functionalization impacts on the interaction of nanoparticles with cells. For instance, functionalization of carbon nanotubes (CNTs) and so-called carbon nano-onions, i.e., spherical carbon nanoparticles, with benzoic acid has been shown to diminish their inflammogenic properties, as assessed by decreased secretion of IL-1β and reduced recruitment of neutrophils and macrophages after intraperitoneal injection into mice [20]. Similarly, Li et al. [21] showed that functionalization determines pulmonary toxicity of multi-walled CNTs insofar as strongly cationic, polyetherimide (PEI)-modified CNTs induced significant lung fibrosis while anionic functionalization (carboxylation) decreased the extent of fibrosis in mice. These differences could be attributed to differences in cellular uptake and lysosomal damage leading to inflammasome activation (see below for a further discussion). Gao et al. [22] showed that the recognition of CNTs by macrophage-differentiated THP.1 cells can be regulated through surface chemistry modifications leading to a 'switch' from mannose receptor-mediated to scavenger receptor-mediated

recognition of the CNTs, with attendant reduction in toxicity, and production of pro-inflammatory TNF-α. In sum, physico-chemical properties of nanomaterials are important determinants of toxicity, and the examples provided here serve to illustrate that material properties such as size, shape and surface charge can greatly influence the interaction of engineered nanomaterials with immune cells, not least with macrophages, professional phagocytes of the immune system.

2.2. The acquired, biological identity of nanomaterials

Upon entry into a biological system, nanomaterials adsorb biomolecules forming a bio-corona on the surface [13]. It is generally believed that the bio-corona has two components, a 'hard' corona and a 'soft' corona; the hard corona is stable and crucial in providing the nanomaterials with their 'biological identity'. Whether or not the hard corona covers the surface of the nanomaterial completely remains unresolved. Notwithstanding, the binding of proteins to nanoparticle surfaces may not only afford a new 'identity' to the nanoparticle, but this interaction may also affect the protein itself. In an illustrative example, Deng et al. [23] showed that poly(acrylic acid)-coated gold nanoparticles bind fibrinogen, a protein involved in blood clot formation, in a charge-dependent manner inducing unfolding of the protein and that binding to integrin receptors on the surface of the monocytic cell line, THP-1 led to activation of the NF-κB pathway and secretion of the pro-inflammatory cytokine, TNF-α. The notion that the unfolding of proteins on nanoparticles may impact on cellular interactions is further underscored by the recent work of Yan et al. [24] who noted that albumin was found to undergo conformational changes upon adsorption onto nanoporous polymer particles leading to a significant decrease in internalization in undifferentiated THP.1 cells, in comparison with the bare particles, while scavenger receptor-mediated uptake in differentiated, macrophage-like THP.1 cells was enhanced by the presence of the unfolded albumin. The latter work thus emphasizes that adsorbed proteins, such as albumin, may act as opsonins to promote uptake, or, on the contrary, as 'dysopsonins'. In a recent, comprehensive study using silica and polystyrene nanoparticles of various size and surface functionalization, Tenzer et al. [25] could show that plasma protein adsorption occurs very rapidly and that it affects hemolysis (i.e. lysis of red blood cells), thrombocyte activation, cellular uptake and endothelial cell death. However, it remains to be firmly demonstrated if and how the specific identity of the corona proteins is linked to toxicity. Dobrovolskaia et al. [26] reported that the composition of the protein corona did not correlate with compatibility of colloidal gold nanoparticles with cells of the blood. Ge et al. [27] reported that the toxicity of SWCNTs was mitigated after the binding of serum proteins, but this does not necessarily prove that specific proteins are involved; the serum proteins may have saturated the binding sites of the CNTs thereby preventing further interactions with cellular proteins. Indeed, the presence or absence of a protein corona affects toxicity of silica nanoparticles, possibly through passivation of the particle surface [28–30]. Interestingly, we found that the presence of a 'hard' corona of plasma proteins affected macrophage uptake as well as the magnetic properties of silica-coated superparamagnetic iron oxide nanoparticles or SPIONs [31]. Taken together, while there is increasing evidence for the importance of the protein corona in regulating the cellular interactions of nanoparticles, not least cellular uptake of nanoparticles, it appears difficult at this stage to pinpoint a role for individual protein(s) in the corona. In a recent study, so-called serum protein corona 'fingerprinting' of a library of 105 surface-modified gold nanoparticles implicated a set of hyaluronan-binding proteins as mediators of nanoparticle-cell interactions [32], although the significance of this observation was not validated in functional studies. Nevertheless, the study showed that the core material was shown to exert a greater influence on protein corona composition than core size or surface functional group. This may seem surprising; however, as pointed out by the authors, even if the ligand-protected core does not make direct contact with proteins in the biological environment, it determines the density, arrangement, and orientation of the associated ligands [32]. Thus, the synthetic and biological

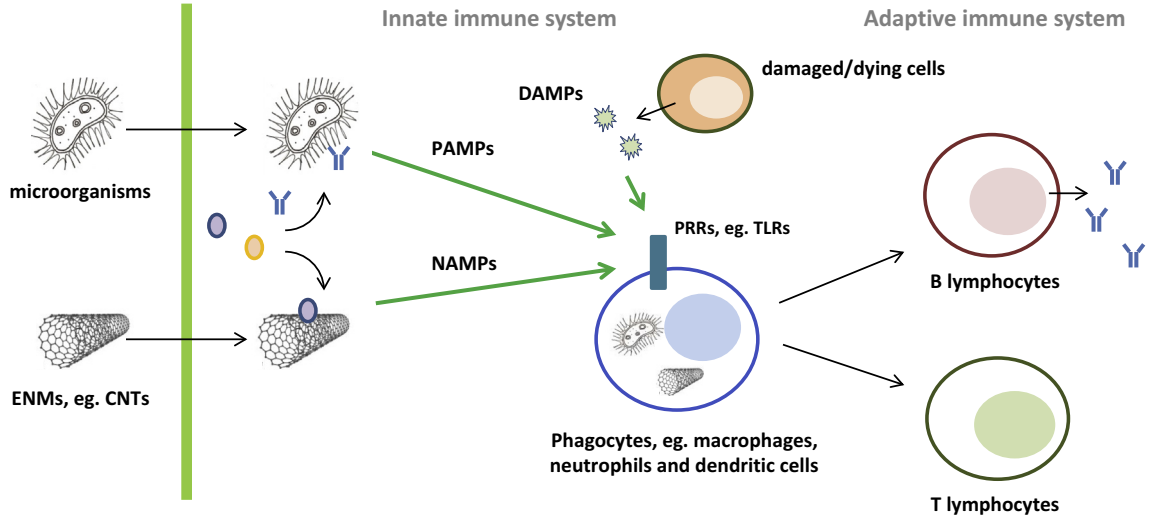

Fig. 1. The innate or primitive immune system handles microorganisms and engineered nanomaterials in an analogous fashion. This cartoon depicts how microorganisms and nanomaterials (here: carbon nanotubes (CNTs)) undergo opsonization and coronation, respectively; this marks the invading microbes or particles for phagocytosis by cells of the innate immune system, such as macrophages. Corona formation endows nanomaterials with a "biological identity" (consult text for a further discussion). Phagocytes are equipped with so-called pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns or PAMPs as well as damage-associated molecular patterns or DAMPs emanating from dying or damaged cells and tissues. We have speculated that proteins or other biomolecules adsorbed onto the surface of nanomaterials could be recognized or "sensed" in a similar manner as nanomaterial-associated molecular patterns or NAMPs. Alternatively, nanomaterials *per se* could be recognized as pathogens by virtue of their specific physico-chemical properties (i.e., the "synthetic identity"). In addition, microbes (bacteria, fungi) and oxidized CNTs have been shown to undergo myeloperoxidase (MPO)-dependent degradation in neutrophils as well as in neutrophil extracellular traps or NETs (not shown). Finally, phagocyte "sensing" of microbes or nanomaterials leads to cytokine and/or chemokine-mediated signaling between the innate and adaptive immune system.

'identities' of nanoparticles are interrelated (Fig. 1). In sum, when considering the toxicities of engineered nanomaterials one cannot disregard the biological context, i.e., the specific compartment in the body and the composition of bio-fluids in that compartment as this may influence subsequent interactions with cells. The natural extension of the bio-corona concept is that this phenomenon could be exploited through the purposeful design of material surfaces in order to control protein binding and targeting of nanoparticles to specific cell types [33].

3. Nanotoxicology: interactions with the two arms of the immune system

The immune system consists of an army of dedicated cell types that work in concert using complex detection, communication and execution systems to defend us from any external or internal harm. The immune system is typically divided into the 'primitive' or innate immune system which is quick to react to foreign intrusion and the more delayed but highly specific adaptive immune system which is endowed with immunological 'memory' after an initial response to a specific pathogen, leading to an enhanced response to subsequent encounters with that same pathogen; this is the basis for vaccination, one of the triumphs of modern medicine [34]. Importantly, there is cross-talk between the innate and the adaptive arms of the immune system, and some cell types that were previously considered as being 'primitive' innate immune cells, such as the neutrophil, are also involved in the orchestration of adaptive responses (see [35] for a review). Based on the current literature, we propose that most if not all of the adverse effects of engineered nanomaterials are exerted via direct effects on cells of the innate immune system, including macrophages, or via dendritic cells (DCs), phagocytosis-competent, antigen-presenting cells that act as a bridge between the two arms of the immune system, while the effects on the adaptive immune system (B cells and T cells) appear to be indirect. In the following sections, we shall explore some of the relevant evidence. This is not intended as an exhaustive account of nanomaterial interactions with the immune system as there are other reviews covering various aspects of this topic [36,37]. We shall also discuss the possibility that immune cells 'sense' nanomaterials as pathogens.

3.1. Nanomaterials and the innate immune system

Phagocytosis – the process whereby macrophages, DCs and other myeloid cells such as neutrophils internalize various targets including microbes or cell debris – is a key mechanism of innate immunity [38]. Phagocytosis is not merely a process of waste disposal but also has an important role in 'interrogating' the target, in order to determine the most appropriate response to the threat posed to the organism. When the target is physically too large for the macrophage to engulf this may trigger 'frustrated phagocytosis' leading to the release of pro-inflammatory mediators; this is seen, for instance, when asbestos fibers or long, rigid multi-walled CNTs are injected into mice [39].

Understanding how phagocytes – key sentinels of the immune system – interact with nanoparticles is of key importance. In addition, it may be important to consider differences between the various populations of macrophages, depending on tissue of origin and activation status. Activated macrophages can be divided into M1, so-called classically activated macrophages, or M2, alternatively activated macrophages [40]. Hence, while M1 macrophages are thought to be more pro-inflammatory and oriented toward pathogen killing, M2 macrophages have been linked to anti-inflammatory, wound healing and tissue repair functions. The presence of Th1 cytokines has a tendency to polarize macrophages toward the M1 phenotype while Th2 immune responses can induce macrophage polarization toward the M2 phenotype. In a recent study, Jones et al. [41] showed that mouse strains that are prone to Th1 immune responses clear nanoparticles at a slower rate than Th2-prone mice. Interestingly, the authors could show that both granulocytes – mainly neutrophils – and macrophages participated in the clearance observed in Th2-prone mice. The implication of this study for the human situation is that the global immune status of an individual may impact on nanoparticle clearance.

An undesired effect of nanoparticles is the impairment of phagocytic activities. For instance, SWCNTs were described to impair macrophage engulfment of apoptotic target cells [42]. Similar observations have been made for ultrafine carbon particles, which impair ingestion of microorganisms by human alveolar macrophages [43]. Thus, inhalation of nanoparticles could lead to increased susceptibility to pulmonary pathogens as their clearance might be impaired. Indeed, delayed bacterial clearance is seen in mice exposed to SWCNTs via pharyngeal aspiration [44]. Furthermore, Kodali et al. [45] have shown that pre-treatment of macrophages with iron oxide nanoparticles at non-cytotoxic doses caused extensive transcriptional re-programming in response to subsequent challenge with bacterial LPS. Macrophages exposed to nanoparticles displayed a phenotype suggesting an impaired ability to transition from an M1 to M2-like activation state, associated with a diminished phagocytic activity toward certain bacteria. The authors concluded that biological effects of nanoparticles may be indirectly manifested only after challenging normal cell function, such as clearance of bacteria [45].

Several studies in recent years have shown that CNTs are susceptible to degradation by naturally occurring plant and human enzymes (peroxidases) [46]. Myeloperoxidase (MPO) is a component of the microbicidal system of phagocytes, especially neutrophils, and thus an important part of the innate immune response. Importantly, MPO expressed in primary human neutrophils is capable of mediating biodegradation of oxidized SWCNTs [47]. Furthermore, our studies have shown that neutrophil extracellular traps (NETs) produced by activated neutrophils can 'capture' and digest SWCNT in an MPO-dependent manner [48]. We have also shown that eosinophil peroxidase (EPO), the major oxidant-producing enzyme in eosinophils, degrades SWCNTs [49]. Moreover, Kagan et al. [50] provided evidence that SWCNTs can undergo biodegradation in macrophages, through a superoxide/peroxynitrite-driven oxidative pathway, while Girish et al. [51] documented macrophage-mediated degradation of carboxyl-functionalized graphene following intravenous administration in mice, although the specific mechanism of degradation was not identified in the latter study. Thus, innate immune cells in mice and humans are capable of degrading not only microbes, but also carbon-based nanomaterials.

3.2. Engineered nanomaterials as danger signals: focus on inflammasomes

Pattern recognition receptors (PRRs) localized at the cell surface, in intracellular vesicles or in the cytosol, are a primitive part of the immune system and these receptors enable cells at the front line of host defense, such as macrophages and epithelial cells to detect and respond to the presence of so-called danger- and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively). PAMPs include bacterial cell wall components (eg., lipopolysaccharide, LPS, and peptidoglycan, PGN) or viral DNA/RNA, as well as fungal glucans. DAMPs, on the other hand, are endogenous stress signals (sometimes referred to as 'alarmins') and several such factors have been identified including high-mobility group box 1 protein (HMGB1), uric acid, nucleotides, and heat shock proteins (HSPs).

DAMPs are released from activated or dying cells and their recognition by DCs and macrophages via different PRRs results in immune cell maturation and the production of pro-inflammatory cytokines [52].

Several members of the NOD-like receptor (NLR) family, a subset of cytoplasmic PRRs, are able to sense PAMPs and DAMPs and subsequently induce the assembly of a multi-protein signaling platform called the inflammasome [53]. The inflammasome serves as an activation platform for the mammalian cysteine protease caspase-1, a central mediator of innate immunity (Fig. 2). Active caspase-1, in turn, promotes the maturation and release of interleukin-1β (IL-1β) and IL-18. It appears that DAMPs and PAMPs synergize to permit secretion of IL-1β: PAMPs (such as LPS) stimulate synthesis of pro-IL-1β, but not its secretion, while DAMPs can stimulate assembly of an inflammasome and activation of caspase-1, which cleaves pro-IL-1β into IL-1β. It is noted that inflammasome activation is not the same in all cell types, and caspase-1 activation is not the only mechanism leading to the processing of pro-IL-1β into IL-1β. For instance, neutrophil-derived serine proteases and pathogen-released enzymes can also process and activate IL-1β (see [54] for further discussion). Dostert et al. [55] initially demonstrated that asbestos fibers and crystalline silica trigger activation of the NLRP3 [nucleotide-binding domain, leucine-rich family (NLR), pyrin-containing 3] inflammasome, and several other studies have subsequently shown that the NLRP3 inflammasome also responds to a range of different engineered nanomaterials including polystyrene nanoparticles, metal oxide nanoparticles, and carbon-based nanomaterials, including both 'needle-like' CNTs and spherical carbon nanoparticles [20,56–58]. There are several inflammasomes [53]. However, while all inflammasomes recognize certain PAMPs or DAMPs, it is a distinctive feature of the NLRP3 inflammasome that it is activated by many, diverse stimuli making NLRP3 the most versatile of the inflammasomes. However, as pointed out recently, it does not seem plausible that NLRP3 is able to detect all of its triggering agents through direct interactions; instead, it is more likely that NLRP3 is activated by generic cellular stress signals [59]. Overall, this implies that innate immune cells may respond in a conserved manner to pathogens (PAMPs) as well as to nanomaterials and other exogenous substances, such as asbestos fibers, crystalline silica, and aluminum salt (alum) that is frequently used as vaccine adjuvants to boost immune responses [60].

The mechanism of activation of the NLRP3 inflammasome has been the subject of much research, and reactive oxygen species (ROS) production, either through the activation of the NADPH oxidase or through perturbation of mitochondrial function, as well as lysosomal damage or a drop in intracellular potassium concentrations have all been implicated in this process. Specifically, the release of the cysteine protease, cathepsin B from lysosomes has been repeatedly implicated in inflammasome activation. Indeed, in a recent study, Li et al. [61] reported that rare earth oxide nanoparticles triggered inflammasome activation through a biotransformation process within lysosomes resulting in lipid membrane dephosphorylation and organelle damage, with release of

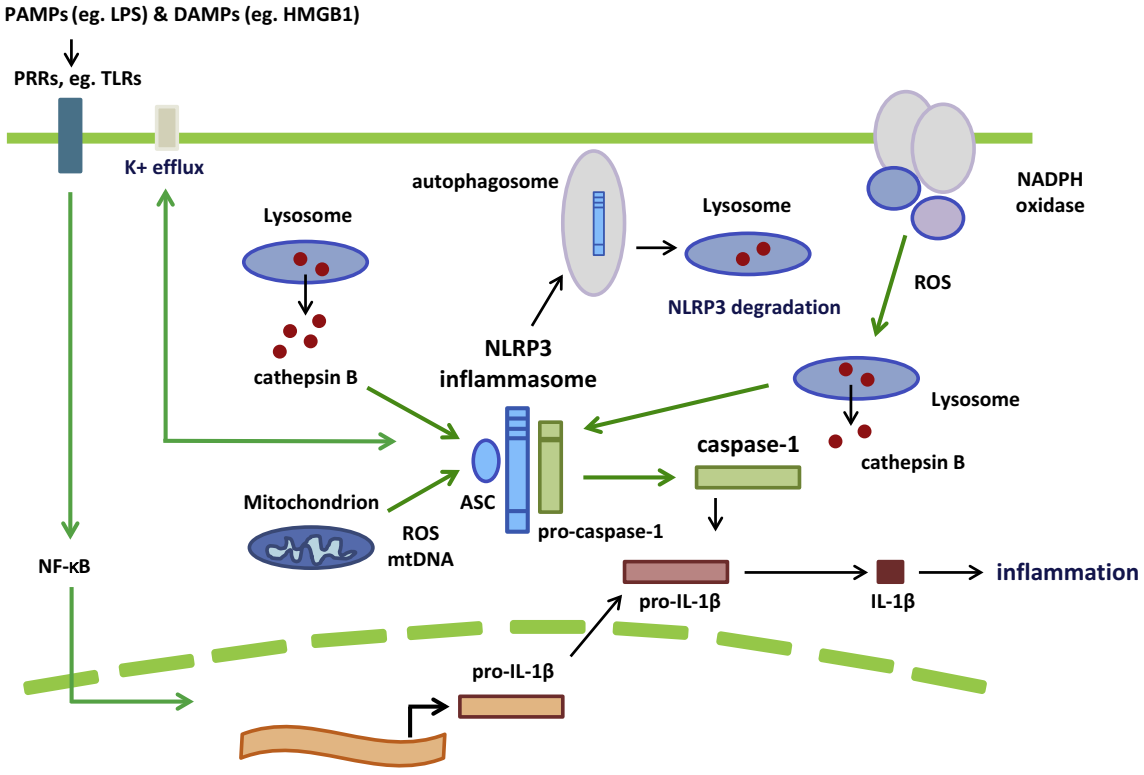

Fig. 2. Schematic illustration of NLRP3 inflammasome activation. The NLR family, pyrin domain-containing 3 (NLRP3) inflammasome is a multi-protein complex that activates caspase-1, leading to the processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18. The NLRP3 inflammasome is activated by a diverse range of endogenous and microbial 'danger' signals, as well as by xenobiotic compounds, including asbestos fibers, crystalline silica, alum (adjuvants) and nanomaterials. The molecular mechanism of NLRP3 activation is the subject of much research and considerable debate, and evidence has been put forward for a role of reactive oxygen species (ROS), lysosomal rupture with release of lysosomal proteases including cathepsin B, and a lowering of K^+ concentrations through cellular efflux of K^+. The release of other mitochondrial factors such as mtDNA has also been shown to lead to activation of the inflammasome, and blockade of mitochondrial autophagy/mitophagy has been found to lead to the accumulation of damaged mitochondria which in turn activates the inflammasome (not shown). The inflammasome is kept in check through ubiquitination and autophagic degradation of inflammasomes. As discussed in the present essay, nanomaterials may activate the NLRP3 inflammasome, leading to IL-1β production and inflammation *in vitro* and *in vivo*, and they may do so in several different ways, for instance through activation of the NADPH oxidase and/or by damaging lysosomal membranes with release of cathepsin B. Nanomaterials may also interfere with the process of autophagy.

cathepsin B. Lysosomes are involved in the degradation of intracellular pathogens, damaged organelles and proteins through a process called autophagy [62]. Moreover, it has been suggested that autophagy accompanies inflammasome activation to 'temper' inflammation by eliminating active inflammasomes, thereby limiting IL-1β production [63]. Notably, in a subsequent study, Li et al. [64] found that rare earth oxide nanoparticles interfered with autophagosome fusion with lysosomes, thereby disrupting the homeostatic regulation of activated NLRP3 complexes, leading to enhanced IL-1β production. Together, these findings suggest a novel pathway for nanoparticle-induced lysosomal interference leading to inflammasome activation, at least for certain nanoparticles [61,64]. In a very recent study, Sun et al. [65] provided evidence, using genetic and pharmacological approaches, for NADPH oxidase-generated ROS in macrophages that had encountered high aspect-ratio nanomaterials such as MWCNTs, and showed that NADPH oxidase activation was directly involved in lysosomal damage and IL-1β production.

Overall, the NLRP3 inflammasome appears to function as a sensor and integrator for pro-inflammatory stimuli [66]. It should be noted that the *in vitro* assessment of inflammasome activation is usually performed using LPS-primed cells, implying that nanoparticles exposure alone might not be sufficient to induce inflammasome activation and that other concomitant signals would be required in a living organism. Interestingly, MWCNT exposure was recently shown to increase secretion of extracellular HMGB1 (an endogenous stress signal or DAMP) in alveolar macrophages, and neutralization of extracellular HMGB1 reduced MWCNT-induced IL-1β secretion *in vivo* [67]. This provides a basis for the sterile inflammation triggered by MWCNTs.

As discussed in a previous section, nanomaterials adsorb proteins and other biomolecules onto their surface, and these proteins may undergo conformational changes leading to the exposition of cryptic epitopes that could be recognized by immune cells. In fact, on the basis of these findings, we suggested previously that engineered nanomaterials might be 'sensed' as NAMPs or nanomaterial-associated molecular patterns [68]. This could, in principle, be accomplished via surface-adsorbed biomolecules (notably, not only proteins, but also sugars, lipids, and nucleic acids could bind nanoparticles), or, this could result from the recognition of nanomaterials *per se*. Graphene oxide (GO) was shown to trigger necrosis of macrophages via TLR4 signaling [69], while Tsai et al. [70] found that gold nanoparticles attenuated TLR signaling in macrophages in a size-dependent manner. In the latter study, the nanoparticles were found to bind to HMGB1 (which is involved in the regulation of TLR9 signaling) inside lysosomes. Additionally, previous studies showed the specific sensing of peptides conjugated to gold nanoparticles by murine macrophages occurred through a TLR4-dependent pathway; this suggests novel ways of modulating immune responses using nanoparticles [71]. Importantly, endotoxin-mediated effects were excluded in the latter study. Moreover, ZnO nanoparticles were suggested to exert adjuvant properties to a known allergen, ovalbumin (OVA) in mice via upregulation of TLRs and downstream adaptor molecules such as Myd88 [72]. However, in these examples, the findings may be explained through indirect effects on TLR signaling. In contrast, recent computational studies have shown that the internal hydrophobic pockets of some TLRs might be capable of direct binding of carbon nanomaterials (i.e., 5,5 armchair SWCNTs containing 11 carbon atom layers and C_{60} fullerenes) [73]. Further studies exploring the potential for direct 'recognition' of nanomaterials by TLRs or other PRRs are warranted and could support the view that engineered nanomaterials may be sensed by innate immune cells as pathogens. For comparison, recent studies using zebra fish embryos have shown that the transcriptional response to poly(amidoamine) dendrimers is consistent with the activation of the innate immune response [74]. Moreover, certain dendrimers were shown to act as nucleic acid 'scavengers' (so-called nucleic-acid scavenging polymers, or NASPs) and were shown to inhibit activation of nucleic acid-sensing TLRs; importantly, systemic administration of NASPs prevented fatal liver injury caused by pro-inflammatory nucleic acids in an acute toxic shock model in mice [75]. This may thus be considered as an example of beneficial bio-corona formation that prevents immune activation. Further studies on the bio-corona of host- and pathogen-derived proteins, lipids, nucleic acids and sugars on nanomaterials and its impact on the recognition of PAMPs or DAMPs, and studies designed to address whether nanomaterials themselves may act as NAMPs, are warranted.

3.3. Nanomaterials and the adaptive immune system

The adaptive immune system is composed of B cells and T cells. B cells are responsible for humoral (antibody-mediated) immunity while T cells are involved in cell-mediated responses. T helper cells (CD4+) are needed to support the production of antibodies by B cells; T helper cells, in turn, are subdivided into Th1, Th2, and Th17, depending on their specific role and cytokine profiles. Cytotoxic T cells (CD8+) are required for killing of virus-infected and malignant cells, while regulatory T cells are required for maintenance of immune tolerance, which is important for the discrimination between 'self' and 'non-self' (i.e., pathogens). DCs, in turn, constitute the bridge between the innate and the adaptive arms of the immune system. These cells are effective phagocytic cells that also exhibit a capacity for processing and presentation of antigens (i.e., a substance that provokes an adaptive immune response) [76]. Importantly, DCs migrate from peripheral tissues to lymph nodes, where they can stimulate T cells and B cells. Targeting DCs may be advantageous, for instance, in vaccination strategies (see below). However, undesired targeting of DCs may be linked to nanomaterial toxicity. In fact, several studies have reported that nanoparticles may disturb the functions of DCs, which may affect B cells and/or T cells. For instance, MWCNTs were found to alter the capacity of human monocytes to differentiate into DCs [77], and pulmonary exposure to SWCNTs has been shown to induce diminished proliferation of splenic T cells in mice through direct effects on DCs [78]. Furthermore, graphene oxide (GO), but not fullerenes, was shown to suppress antigen presentation by DCs [79]. As previously discussed, surface coating may serve to mitigate nanomaterial effects on immune cells. Zhi et al. [80] studied the effects of GO with and without polyvinylpyrrolidone (PVP) coating on human DCs, T cells and macrophages, and found that PVP-coated GO exhibited lower immunogenicity compared with uncoated GO in terms of inducing DC differentiation and maturation. It seems unlikely that nanomaterials *per se* are immunogenic, i.e., have the capacity to induce a specific, adaptive immune response. However, it is a well-known phenomenon in immunology that certain small molecules, referred to as haptens, can elicit an immune response when coupled to a protein carrier, but are otherwise non-immunogenic [36]. Indeed, immunization of mice with a C_{60} fullerene derivative conjugated to a protein (bovine thyroglobulin) yielded a population of fullerene-specific IgG antibodies [81]. On the other hand, it remains possible – even plausible – that the bio-corona on the surface of nanoparticles may induce specific immune responses, for instance if proteins bound to nanoparticles undergo conformational changes, thereby revealing cryptic epitopes. Further studies are certainly needed to address this issue.

Nanomaterials can elicit either immunostimulatory or immunosuppressive effects [82]. Mitchell et al. [83] found that inhalation of MWCNTs produced a systemic immunosuppression in mice with decreased T cell proliferation in the spleen due to a

signal from the lung, likely TGF-β secreted by alveolar macrophages. As already mentioned, nanoparticles have been shown to influence T cell proliferation, typically through an effect on antigen-presenting cells, leading to an enhanced T cell stimulatory capacity [84–86]. Using a fully human autologous immunological construct as a non-animal alternative to monitor nanoparticle responses, Schanen et al. [87] showed that exposure to TiO_2 nanoparticles led to elevated levels of pro-inflammatory cytokines and increased maturation of DCs. Additionally, the nanoparticles effectively primed activation and proliferation of naive CD4+ T cells in comparison with micrometer-sized TiO_2. Myeloid-derived suppressor cells (MDSC), a heterogenous population of myeloid progenitors, are increasingly being recognized as playing a key role in the immunosuppression induced by tumors. This is accomplished either through direct or indirect mechanisms, i.e., by directly influencing effector T cells, or through the generation and/or expansion of other regulatory cell populations, such as regulatory T cells [88]. Shvedova et al. [89] reported that metastatic establishment and growth of Lewis lung carcinoma (LLC), a commonly used model of pulmonary metastatic disease, is promoted by SWCNTs and that this effect is likely mediated by increased accumulation of MDSC, as their depletion using anti-Gr-1 antibodies abrogated the tumorigenic activity of the SWCNTs.

Exosomes are cell-derived, nano-sized (30–100 nm) vesicles that may play a role in cell-to-cell communication. Zhu et al. [90] reported that exposure to magnetic iron oxide nanoparticles results in significant exosome generation in the alveolar region of Balb/c mice. Through exosome-initiated signals, immature DCs were found to undergo maturation and differentiation, while macrophages were activated and differentiated to the M1 subtype. Simultaneously, these cells released various Th1 cytokines driving T cell activation and differentiation. Th1-polarized immune activation is associated with delayed-type hypersensitivity, and the authors hypothesized that nanoparticle-induced exosomes might underlie the long-term inflammatory effects associated with nanoparticle exposure [90]. The study underlines the importance of not only assessing immune cells in isolation, but also the *communication* between different immune cells; nanoparticles may certainly be cytotoxic for immune cells, depending on the dose, which would obviously affect their function, but could also affect the function of immune cells at lower doses (as discussed above) and/or impact on cell-to-cell signaling.

4. Immune modulation using nanoparticles: the other side of the coin

Moon et al. [91] have provided an excellent overview of recent progress in the design of synthetic micro- and nanoparticles that can be used for 'tuning or taming' of the immune system. Considerable attention is thus devoted to the use of engineered particles as artificial antigen-presenting cells, and the use of particles for delivery of antigen plus immunostimulatory molecules for vaccination purposes. Synthetic particles are of interest because they can mimic microorganisms that are themselves nanoparticles (viruses) without the complications of anti-vector immune responses that are often elicited by recombinant viral vaccines [91]. The fact that synthetic vaccines (i.e., cross-linked liposomes that act as a controlled release reservoir of antigen) can elicit immune responses comparable to live vector vaccines is certainly very encouraging [92]. In a previous section, we discussed how the innate immune system decodes endogenous and exogenous danger signals (i.e., DAMPs and PAMPs, respectively); however, it is important to consider whether 'danger' necessarily is undesirable. It should be noted that vaccine adjuvants (such as alum) are substances that enhance the immunogenicity of a co-administered antigen, thereby boosting protective immune responses [93]. Nanomaterials could perhaps be used to deliver adjuvants or act as adjuvants themselves [7]. Reddy et al. [94] demonstrated size-dependent targeting of pluronic-stabilized polypropylene sulfide (PPS) nanoparticles to lymph node-residing DCs in mice. In addition, the surface chemistry of the nanoparticles activated the complement cascade, generating a 'danger' signal leading to activation of DCs. Using nanoparticles conjugated to the model antigen OVA, the authors demonstrated generation of humoral and cellular immunity in a size- and complement-dependent manner [94]. In another key study, Kasturi et al. [95] reported that immunization of mice with synthetic nanoparticles based on the biodegradable synthetic polymer, poly(D,L-lactic-co-glycolic acid) (PLGA) containing antigen plus ligands that signal through TLR4 and TLR7 induces synergistic increases in antigen-specific, neutralizing antibodies compared to immunization with nanoparticles containing antigen plus a single TLR ligand. Immunization afforded significant protection against lethal avian and swine influenza virus strains. The vaccination strategy was also tested in non-human primates, which – like humans – show a different TLR7 expression pattern on DCs compared to mice [95]. The nanoparticle-based vaccine resembled a virus both in size and in composition and recapitulated the immunogenicity of live viral vaccines. In a different twist on the same theme, Hu et al. [96] reported a new nanoparticle-based toxin-detainment approach that could be used to deliver pore-forming toxins for immune processing. As compared to vaccination with heat-inactivated toxin, mice vaccinated with the nanoparticle-detained toxin (or 'nanotoxoid') displayed superior protective immunity.

Several groups have explored the applicability of CNTs as carriers of immunologically active molecules (see [97] for a review). Villa et al. [98] used SWCNTs as carriers for delivery of peptides into antigen-presenting cells to induce humoral (IgG) immune responses against weak tumor-associated antigens. No antibody responses to SWCNTs themselves were detected. Using PEG-modified SWCNTs armed with the glucocorticoid-induced TNFR-related receptor (GITR), which has shown higher expression on intratumor versus peripheral regulatory T cells, Sacchetti et al. [99] demonstrated *in vivo* targeting of regulatory T cells residing in a B16 melanoma (skin cancer) in mice. In this context, it is worth noting that innate immune cells can also enzymatically 'digest' PEGylated CNTs [100]. In other words, such CNTs could potentially be handled by the immune system and are therefore promising as vectors for drug or gene delivery in nanomedicine. In sum, different classes of nanomaterials are emerging as a promising tool for immunomodulation. The long-term safety of these nanomaterial carriers is of course of paramount importance if they are to reach the clinic.

5. Conclusions

As the title of this essay suggests, it takes two to tango, that is to say: nanomaterials possess two 'identities' (intrinsic and acquired), while the immune system consists of two branches (innate and adaptive); the title could also imply that the interactions between nanomaterials and the immune system are reciprocal in the sense that nanomaterial exposure may trigger inflammation with involvement of innate immune cells, while innate immune cells may, in some cases, 'digest' nanomaterials, thereby mitigating their toxicity [101]. Moreover, as we have attempted to highlight here, the 'coronation' of nanomaterials with proteins and other biomolecules can be likened to the opsonization of pathogens, a process that facilitates their uptake by phagocytes. Indeed, we have discussed the notion that engineered nanomaterials – with or without a corona of biomolecules – may be recognized by phagocytes via

pattern recognition receptors. Further studies are certainly warranted in order to support or refute this possibility. Overall, a detailed understanding of the synthetic and biological identities of engineered nanomaterials and their interactions with living systems is an important goal [102].

Finally, it is important to note not only that nanomaterials can be exploited for the 'tuning and taming' of the immune system for therapeutic purposes [91], but also that precisely tuned libraries of nanomaterials could be employed to investigate biological processes, for instance, the role of size and shape for the induction of immune responses [103–105].

Acknowledgments

The authors are supported, in part, by the Swedish Research Council, the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), and the European Commission (FP7-MARINA, Grant Agreement No. 263215; FP7-NANOREG, Grant Agreement No. 310584; FP7-NANOSOLUTIONS, Grant Agreement No. 309329; FP7-SUN, Grant Agreement No. 604305; GRAPHENE Flagship Project, Grant Agreement No. 604391). This review is based on an Invited Lecture at the 10th International Conference on Biological Barriers, in Saarbrücken, Germany.

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