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Mechanisms of immune response to inorganic nanoparticles and their degradation products

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Abstract

Careful assessment of the biological fate and immune response of inorganic nanoparticles is crucial for use of such carriers in drug delivery and other biomedical applications. Many studies have elucidated the cellular and molecular mechanisms of the interaction of inorganic nanoparticles with the components of the immune system. The biodegradation and dissolution of inorganic nanoparticles can influence their ensuing immune response. While the immunological properties of inorganic nanoparticles as a function of their physicochemical properties have been investigated in detail, little attention has been paid to the immune adverse effects towards the degradation products of these nanoparticles. To fill this gap, we herein summarize the cellular mechanisms of immune response to inorganic nanoparticles and their degradation products with specific focus on immune cells. We also accentuate the importance of designing new methods and instruments for the *in situ* characterization of inorganic nanoparticles in order to assess their safety as a result of degradation. This review further sheds light on factors that need to be considered in the design of safe and effective inorganic nanoparticles for use in delivery of bioactive and imaging agents.

Keywords

Drug delivery; Nanotoxicology; Immunotoxicity; Oxidative stress; Inflammation; Metal homeostasis; Inflammasome; Mechanism of degradation; Genotoxicity; Epigenetic toxicity

1. Introduction

The immune response is the reaction of the host to foreign substances which is regulated by the cells and molecules of the immune system. The act of the immune system is to eliminate unwanted foreign substances from the body and to maintain homeostasis [1]. >400 million

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years of evolution has resulted in the immune system to develop into the highly complex and adaptable defense mechanism that it is today [2]. There continues to be a need to modulate the immune system for better diagnostic or therapeutic outcomes. This modulation can be on activation of the immune system against new infections, or suppression of it to prepare the body to receive therapeutic interventions. Nanoparticles are one class of carriers for therapeutic agents. They can also act intrinsically as therapeutics or imaging systems. Understanding the *in vitro* and *in vivo* fate of nanoparticles and their related immunotoxicity is crucial to inform their choice for therapeutic and diagnostic applications.

The potential of inorganic nanoparticles in biomedical applications in general, and drug delivery in specific, is widely acknowledged [3–6]. Inorganic nanoparticles ranging in size from 1 to 1000 nm can contain metals, metal oxides, metal alloys, and semiconductors [7,8]. Some examples of inorganic nanoparticles include gold, iron oxide, silver, zinc oxide, silica and silicon dioxide, and titanium dioxide nanoparticles, as well as quantum dots [9]. Due to their unique physical, electronical, and optical properties, in many cases well-established synthetic methods, the ability to tune physicochemical properties, ease of scale-up, often low cost, and superparamagnetic and exhibition of quantum confinement effects, inorganic nanoparticles show promise in many biomedical applications. These include, but are not limited to, drug and gene delivery, as antibacterial agents, in cell and tissue imaging and labeling, and as diagnostics and/or theranostics [6,10,11]. Despite the fact that these particles have been investigated for decades, few of them have advanced to clinical applications in drug delivery. This is in contrast to some of the organic nanoparticles such as polymeric- or lipid-based systems where systematic evaluation of their biological fate has informed their translation to clinical use.

The relation between nanoparticles and immune cells is highly dynamic. The detailed life cycle of nanoparticles inside the immune cells and the cell reaction to them are still poorly understood. Administration of inorganic nanoparticles into the body, as a foreign substance, activates the host immune response which may lead to desirable (*e.g.*, activation of the immune response as a vaccine adjuvant) or undesirable (*e.g.*, autoimmunity or allergic reaction) immune reactions [12]. The interaction of inorganic nanoparticles with the immune system and the alteration of normal immune function raises concerns about the safety of these materials. Such interaction can result from the intact nanoparticles, as well as their degradation and dissolution products. Once administered, the physicochemical properties of nanoparticles start to change. They may interact with proteins and macromolecules, aggregate or agglomerate, and potentially biodegrade and dissolve. The biological milieu will then encounter different particulate products than the original nanoparticle formulation, with different physical and chemical properties than the parent particles. This change will pose simple and very important questions: What would happen when the immune system encounters these modified products? What would be the fate and function of the immune cells and the ensuing molecular events in response to these modified inorganic nanoparticles and their degradation or dissolution products? Finding the answers to these questions is instrumental for the design of safe and effective inorganic nanoparticles with minimal immunotoxicity.

Extensive research has been conducted to study the interaction of inorganic nanoparticles with the immune system. In the present review, we aim to provide a summary of the key cellular mechanisms of immune responses observed to various inorganic nanoparticles with focus on delivery applications. We describe the degradation profile of selected inorganic nanoparticles and the current knowledge regarding their fate *in vitro* and *in vivo*. The importance of the understanding of immunological properties of these nanoparticles and their degradation products, and the different factors that influence their immune response will be discussed. We then discuss the challenges, critical gaps, and future directions for better understanding of the immunological properties of inorganic nanoparticles and their degradation products.

2. Immunogenicity of inorganic nanoparticles and cellular mechanisms of their immune response

The ability of nanoparticles serving as immunogen to stimulate the immune response in particular species is called immunogenicity [12]. The immunogenicity of nanoparticles depends on their physicochemical properties and the genetic capacity of the host defense [13,14]. Numerous reports have reviewed the immunogenicity of gold [15], silica [16], silver [17–19], titanium dioxide [20], zinc oxide [21], and iron oxide nanoparticles [22]. Altogether, these reports have demonstrated that inorganic nanoparticles interact with different immune cells including monocytes and macrophages, dendritic cells, B and T lymphocytes, and natural killer cells, as well as other immune function cells including mast cells and endothelial cells. Upon interaction, the nanoparticles will be taken up by and processed in these cells and can in turn influence the fate and function of the immune cells. The cellular response to inorganic nanoparticles may be desirable or undesirable. Production of inflammatory or anti-inflammatory cytokines may occur due to response to a toxic dose of inorganic nanoparticles or it might be the response of the cells to heal the damaged tissue. Cellular oxidants or antioxidants would also have a dual effect. The antioxidant mechanisms such as catalase, glutathione peroxidase, or superoxide dismutase help the cells to reduce the oxidative stress response and maintain the overall oxidative balance [23]. Therefore, to understand the mechanisms of immunotoxicity to inorganic nanoparticles, it is critical to check the anti-inflammatory or antioxidant response. The result of the activation of these mechanisms will qualitatively indicate the nanoparticle effect and final cell response to them. The balance between pro-and anti-inflammatory cytokines will determine the final cellular inflammation response.

Various cellular and molecular mechanisms have been reported as a result of the interaction of inorganic nanoparticles with the immune system. Fig. 1 represents some of the cellular mechanisms of inorganic nanoparticle-mediated immune response. Below we summarize the main mechanisms of immune response to inorganic nanoparticles reported so far with specific focus on the immune cells and organs.

2.1. Inflammation and disruption of cell signaling pathways

One of the extensively studied immunological properties of inorganic nanoparticles is their ability to induce inflammation. Inorganic nanoparticles can alter pro- or anti-inflammatory

pathways *in vitro* and *in vivo*. For example, titanium oxide nanoparticles (anatase crystals) influence human polymorphonuclear neutrophils morphology and function in a concentration- (20, 500, and 100 μg/mL) and time-dependent manner [24]. These particles induce rapid phosphorylation of extracellular signal-regulated kinases-1/2 (Erk-1/2) and p38 mitogen-activated protein kinase, which are involved in apoptosis [24]. Titanium oxide nanoparticles also induced pro-inflammatory mediators, including the chemokines macrophage inflammatory protein-1 α/β, Interleukin (IL)-6, IL-8, and Gro-α in human neutrophils. These cytokines could attract and activate other immune cells, macrophages, natural killer cells, lymphocytes, eosinophils, and dendritic cells, and induce inflammation [24]. There are also reports that the imbalance of Th_1/Th_2 cytokines might be one of the mechanisms of immunotoxicity of lung injury induced by 21 nm TiO_2 nanoparticles in male Sprague Dawley rats (administrated intratracheally at 0.5, 4, and 32 mg/kg dose, twice a week for four weeks) (Fig. 2A) [25]. Alteration in the expression level of interferon (IFN)-γ, IL-4, and T-bet and GATA-3, two transcription factors determining Th cell differentiation, was observed after higher dose injection of these nanoparticles [25]. However, *in vivo* mechanistic study of subchronic accumulation in the spleen and thymus and immunotoxicity of TiO_2 particles (90 days with intragastric administration) in the spleen have shown overexpression of macrophage inflammatory protein (MIP)-1α, MIP-2, protein tyrosine phosphatase and kinase 1, vascular cell adhesion molecule-1, monocyte chemotactic protein-1, IL-13, INF-γ, some fibroblast growth factors, but significantly decreased levels of NKG2D, NKp46, and 2B4 expression involved in immune response and apoptosis (Fig. 2B and 2C) [26]. Splenocyte proliferative response and cytokine secretion were also observed after oral administration of commercial gold nano colloid in a dose-dependent manner over 7–28 days [27]. 0.25 ppm nanogold enhanced the synthesis of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and showed immunostimulating effect. 25 PPM was considered immunotoxic by a drastic decrease in the proliferative activity of lymphocytes. However, 2.5 PPM dose inhibited the synthesis of pro-inflammatory cytokines of macrophages, while stimulated the proliferation of lymphocytes. All the doses overexpressed IL-2, which may implicate their effect on the immunoregulatory mechanisms of the spleen [27]. Interleukin-2dependent anti-proliferation effect of silver nanoparticles (<100 nm) has also been observed on $CD4^+$ T lymphoblastoid WE17/10 cell line by mechanism involving CD25 overexpression without significant alteration of the level or phosphorylation of ERK1/2, Stat5, and JNK (signaling pathways activated by IL-2 receptor) [28]. Anti-cell proliferation effect of silver and gold nanoparticles on leukemic cell lines (T-lymphocytic Jurkat and monocytic U937 cells) have been shown by distinct signaling pathway response to inhibit or stimulate cytokine production [29]. For example, gold nanoparticles inhibit IL-2 and IL-6 production in Jurkat and U937 cells, respectively, while inducing TNF-α through c-Jun N-terminal kinase in U937 cells. Silver nanoparticles inhibit TNF-α in Jurkat cells while involving extracellular-signal regulated protein kinase but not the c-Jun N-terminal kinase pathway [29].

Inorganic nanoparticles cause *in vivo* immunotoxicity such as blood immunotoxicity, tissue inflammation, and damage to the immune organs. Oral administration (750 mg/kg/day for 14 days) of zinc oxide nanoparticles (20 nm and 100 nm) to C57BL/6 mice suppressed the activity of natural killer cells, serum levels of IL-1β, TNF-α, and IL-10, T helper-1

cytokines (interferon-c and IL12p70), and decreased nitric oxide in splenocytes compared to control animals in a size and charge-dependent manner [30]. The alteration of T cell and innate immune cell homeostasis such as reduction of lymphocyte subsets including $CD3^+$, $CD4^+$, $CD8^+$, B cell, and natural killer cells, were observed in the TiO_2 nanoparticle treated female ICR mice thymus after daily intragastric feeding for nine months. These nanoparticles exerted toxic effects via activation of the NF-κB-mediated mitogen-activated protein kinases pathway [31]. The ability of zinc oxide nanoparticles (26.6 nm, 350 mg/kg by oral gavage) to induce oxidative stress, inflammation, and DNA strand break in immune organs, thymus, and spleen of male Wistar albino rats was also evaluated by Abass M. A. *et al.* [32]. The thymus and spleen immunohistochemical analysis revealed an increase in the number of cells expressing the positive reaction of anti-p53 and a decrease in the number of cells expressing positive reactions of anti-PCNA. These results also noted significant upregulation of the immunomodulatory (CD3, CD11b, heme oxygenase (HO^{-1})) and the inflammatory genes (toll-like receptor 4 and 6 (TLR4 and TLR6)), thymic and splenic malondialdehyde and DNA shearing, as well as proinflammatory cytokines IL-4, IL-10, IL-1β, TNF-α, and INF-γ. ZnO nanoparticles enhanced cell maturation marker (CD11b), an important marker required for macrophage activation [32]. Six-week pulmonary administration of cadmium oxide nanoparticles (9.82 nm) to female ICR mice (0.195 μg CdO/g body weight) resulted in increased percentage of $CD3e^+CD8a^+$ cells in the thymus, enhanced splenocyte proliferation and production of inflammatory cytokines and chemokines [33]. Altogether, these examples illustrated that various inorganic nanoparticles can disrupt different cellular pathways in immune cells and tissues and trigger inflammation.

2.2. Oxidative stress

Oxidative stress is a condition of exceeding generation of reactive oxygen species with decreased activity of antioxidants which can trigger the inflammatory response [34]. Inorganic nanoparticles cause oxidative stress through various mechanisms including generation of toxic radicals, such as reactive oxygen species (ROS) and nitric oxide (NO), catalysis of Fenton reactions, participation in redox reactions, increasing membrane lipid peroxidation, and decreasing intracellular glutathione (GSH) levels [23]. The free radicals can be generated from the surface of nanoparticles or upon interaction with other free radicals in the aqueous suspension [35]. When particles start to dissolve, reduction of their size and change of the electronic properties on their surface can result in the creation of reactive groups for electron donors or accepters [36]. These active radicals can produce ROS through Fenton-type reaction [37]. Surface stabilizing of nanoparticles can decrease their reactivity and reduce the generation of these types of radicals. On the other hand, some of the inorganic nanoparticles have radical quenching properties which cause scavenging of intracellular ROS [38]. For example, it has been reported that Cerium nanoparticles reduce oxidative stress in PC12 cells up to 50% [39].

Oxidative stress might have proinflammatory responses or immunosuppressive capacity in immune cells [23]. For example, the exposure of 30 nm ZnO nanoparticles to human macrophage THP-1 cells led to the release of pro-inflammatory cytokines (TNF-α and IL-1β) via activation of redox-sensitive NF-κB and MAPK signaling pathways with the increase in dose-dependent oxidative and nitrative stress and decrease in glutathione (GSH)

levels [40]. The ability of nickel oxide nanoparticles (17 nm) to induce oxidative stress by generating ROS and peroxidation of the lipids in the human peripheral blood lymphocytes was evaluated [41]. Fe_2O_3 nanoparticles (30–35 nm) also induced concentration-dependent oxidative stress with an increase in ROS and lipid peroxidation levels, and depletion of antioxidant enzymes and glutathione in lymphocytes of healthy male Wistar rats that led to morphologic changes in these cells [42]. In this study, lipid peroxidation and antioxidant imbalance were observed in all vital organs of female Wistar rats 24 h post intravenous administration of 5 mg $Fe.kg^{-1}$. Dextran-coated and poly(ethylene glycol)-coated superparamagnetic iron oxide nanoparticles, 45 ± 9. 8 nm, 89 ± 0.4 nm, and 67 ± 4.6 nm, respectively, influenced antioxidant and tissue nitrate levels, which resulted in mast cell infiltration in some organs such as liver, lung, and heart of female Wistar rat model [43]. Silica nanoparticles also triggered oxidative stress *in vitro* and *in vivo* [44]. *In vitro* generation of ROS and the GSH level of Raw 264.7 macrophages, and *in vivo* activation of peritoneal macrophages along with the release of nitric oxide, overexpression of pro-inflammatory genes (IL-1, IL-6, Cox-2, TNF-α), and abnormal distribution of the immune cells (NK cells, T cells, and B cells) were reported for intraperitoneally administered silica nanoparticles into ICR mice [44]. Therefore, oxidative stress induction is a common immune cell response upon interaction with various types of inorganic nanoparticles.

2.3. Metal homeostasis disruption

Animal cells naturally contain different metal ions including zinc, copper, iron, nickel, cobalt, manganese, and magnesium [45,46]. These metals play important roles in different cell functions including cell proliferation and growth, oxidative reactions, enzymatic reaction, gene transcription, as well as immune function [45,46]. Concentration, localization, and homeostasis of these metals and their related free ions are critical to keep the cells alive and functional [46]. Most of these metals have been used for inorganic nanoparticle synthesis. It is clear that these particles can dissolve or degrade to their parent element in the physiological solutions. The biological outcome of inorganic nanoparticles exposure is an influence of the intact particles and their degradation products, including metal elements and ions. The fundamental question regarding metal contained inorganic nanoparticles is whether their accumulation inside immune cells influences the molecular pathways and cellular trafficking as a result of the cells' effort to keep the metal homeostasis. There are few studies on the metal homeostasis disruption of the immune cells upon dissolution of inorganic nanoparticles. It has been reported that zinc, copper, iron oxide, and silver nanoparticles are capable to disrupt metal homeostasis *in vitro* and *in vivo* [47]. For example, Feraheme@ influences intracellular iron homeostasis of the primary human T cells after intracellular iron accumulation [48]. ZnO dissociation also can disrupt cellular zinc homeostasis in Raw macrophages [49]. Copper oxide nanoparticles have been reported to interfere with Cu and Zn homeostasis in hepatocytes at their sub-toxic dose [50].

Numerous studies have described the overexpression of metallothionein as a marker of exposure to metal ions. The metallothionein protein family have the capacity to bind both physiological and xenobiotic metals (such as zinc, copper, selenium, silver, arsenic) and localize at the membrane of the Golgi apparatus. These proteins contain several Zn(II) ion exchangeable metal-binding sites which cause translocation of Zn(II) from the cytosol to the

nucleus in the presence of excess metal, and activate the transcription factors involved in the control of metal homeostasis [51]. Higher expression of Met-RNA has been reported for the silver, zinc, and copper oxide nanoparticles [47]. However, in some instances significant gene expression in the proteins involved in metal homeostasis pathways after treatment of hepatocytes with zinc, copper, and silver oxide nanoparticles was not observed [47]. There are gaps in the mechanistic understanding of the effect of free ions released from metal nanoparticles to the cells including in ROS generation which need further clarification.

2.4. Inflammasome activation

The inflammasomes are large intracellular signaling platforms present in the cytosol of stimulated immune cells that mediate the activation of inflammatory caspases consisting of the protease Caspase-1, Apoptosis-associated Speck-like protein containing a C-terminal caspase recruitment domain (ASC), and a pattern-recognition receptor of the NOD-like family of receptors [52,53]. Nucleotide-binding oligomerization domain-like receptor (NLR) inflammasome complexes are the most characterized to date. Among these, activation of the pattern recognition receptor NLRP3 is a well-known and crucial signaling node that controls the maturation of proinflammatory interleukin (IL)-1 family of cytokines [54]. Inflammasome activation requires signaling of both the Toll-like receptor (TLR) and NLRP3 in the antigen-presenting cells [55]. Activation of inflammation and production of NLRP3 inflammasome-5 for immunotoxicity of inorganic nanoparticles [56]. Several studies have investigated the mechanisms of inflammasome activation in the immune cells recognized by amorphous and crystalline silica nanoparticles. Activation of NLRP3 inflammasomes after the generation of ROS potentially due to leakage of cathepsin B after lysosomal rupture, induction of superoxide and deterioration of the mitochondrial membrane, Caspase-1-mediated inflammatory responses after activation of scavenger receptor B1 in mouse macrophages and human peripheral blood monocytes are among these mechanisms [57,58]. Gómez D. M. *et al.* demonstrated the effect of 12 nm and 200 nm silica nanoparticles on the expression of pro-inflammatory cytokines (IL-1β, IL-6, IL-18) in a dose-dependent manner, and NLRP3 inflammasome components in human primary neutrophils and peripheral blood mononuclear cells (PBMCs) [59]. Canonical inflammasomes convert procaspase-1 into the catalytically active enzyme, whereas noncanonical inflammasome promotes activation of procaspase11 [60]. Kusaka T. *et al.*, primed bone marrow-derived macrophages (BMDMs) with lipopolysaccharide (LPS) followed by silica nanoparticles of various diameters (at concentration of 0.3 μg/mL for 2 h) [61]. They observed over-secretion of IL-1β induced by 30 nm and 300 nm silica nanoparticles compared to 3 mm in diameter silica nanoparticles without any significant difference in Caspase-1 activation or IL-1β maturation between bone marrowdrived macrophages (BMDMs) treated with all sizes of silica nanoparticles [61]. These results indicated that silica nanoparticles induce activation of caspase 1, and that size-dependent induction of IL-1β secretion does not correlate with the level of Caspase-1 activity and IL-1β maturation [61]. Pulmonary inflammation induced by intratracheal instillation of spherical 16.75 nm silica nanoparticles in C57BL/6 mice through ROS/PARP/TRPM2 signaling pathways was also reported by Wang M. and coworkers [62]. Tao X. *et al.* reported treatment of J774A.1 macrophages with 50 nm copper oxide nanoparticles causing lysosomal damage, release of cathepsin B, and induction of IL-1β- mediated inflammasome through myeloid differentiation factor 88 -dependent TLR4 and activation

of NF-κB signaling pathways (Fig. 3 A-C) [63]. Previous results highlighted the potential of silver nanoparticles to activate inflammation in THP-1 cells and primary blood monocytes by overexpression of IL-1, IL-6, and cleavage and release of pro-IL-1β [64]. *In vivo* studies also reported evidence of inflammasome activation in rodent models. A study on the impact of titanium dioxide nanoparticles on inflammasome in an allergic asthma mouse model showed IL-1β, IL-18, NLRP3, and Caspase-1 were increased and led to the production of active Caspase-1 in the lung (Fig. 3D). This suggests that targeting inflammasomes may assist in controlling TiO_2 induced airway inflammation (Fig. 3E) [65].

The inflammasome activation in immune cells is dependent on the physicochemical and structural characteristics of inorganic nanoparticles. For example, mouse bone marrow-derived dendritic cells (BMDCs) treated with ultrasmall gold nanoparticles (<10 nm) activated the NLRP3 inflammasome for Caspase-1 maturation and led to interleukin-1b production, while the larger size particles (>10 nm) triggered the NF-κB signaling pathway [66]. Among the three different sizes of silica nanoparticles, (30, 79, and 10 nm in diameter), small-sized particles stimulated LPS-primed mouse liver KUP5 macrophages to induce ROS through cell-membrane NADPS oxidase. The released ROS caused activation of inflammasome via activation of P2X7 receptor and ATP-binding receptor which play important roles in intracellular signaling [67]. It should be noted that a very common mistake in nanoparticle toxicity assays is when cytotoxic materials result in cell death via mechanisms unrelated to inflammasomes where the IL-1 detected in the culture medium as a marker of inflammasome activation is in fact immature IL-1 precursor that simply leaks from the cell. Therefore, the activation of inflammasome upon receiving inorganic nanoparticles by immune cells should be checked by measuring the level of mature IL-1 along with other signals.

2.5. Autophagy

Autophagy is a formation of double-membrane vacuolar autophagosomes which would fuse with lysosomes and stimulate self-digesting of long-lived proteins and damaged organelles. Autophagy might cause cell death which can be stimulated by various stress situations, or it can be a protective mechanism to improve cell survival [68]. There are many evidences that autophagy is one of the mechanisms to respond to or sequester inorganic nanoparticles and products inside cells [69,70]. The function of autophagy upon receiving nanoparticles could be a result of oxidative stress, a natural process to maintain cell homeostasis in stress conditions, and also separating degradation fragments or leakage of inorganic nanoparticles from endolysosomes in the cytoplasm [71]. This pathway can be activated directly upon nanoparticle entry into the immune cells, or indirectly following impairment of the function of organelles such as lysosomes, mitochondria, endoplasmic reticulum, and Golgi to clean the damaged organelles. On the other hand, it could be cell death or cell survival promoted mechanisms. For example, autophagy protects Raw 264.7 macrophages from silica nanoparticles at sub-toxic doses (10 or 50 μg/mL) of SiO_2Aerosil200 (12 nm) [72], and cylindrical and wormlike particles at 50 μg/mL [73]. Silica nanoparticles with the same size (100 nm) but different interior structures and porosities, trigger autophagy in mesenchymal stem cells extracted from the bone marrow of femur and tibia of Sprague Dawley rats via upregulation of LC3-II through ERK1/2 and

AKT/mTOR signaling pathways. Autophagy is also associated with increased differentiation potential of mesenchymal stem cells [74]. Upregulation of Beclin-1 and LC3II dots have been observed in Raw 264.7 cells upon receiving mesoporous silica nanoparticles. The enhanced autophagy attenuates the inflammation mediated by the NF-κB pathway, whereas autophagy inhibition contributes to inflammation [75].

Different formulations of iron oxide nanoparticles for example have been reported to induce autophagy in various immune cells including B lymphocytes, dendritic cells, monocytes, and macrophages *in vitro* and *in vivo* [76–79]. Feraheme (Ferumoxytol) and Resovist (Ferucarbotran), two clinically approved dextran-coated superparamagnetic iron oxide nanoparticles (SPIONs) induced autophagy in Raw 264.7 macrophages through activation of TLR4-p38-Nrf2-p62 signal which triggers inflammatory responses (overexpression of cytokines, IL-1β, IL-2, IL-12p40/70, TNF-α, and IL-10 and chemokines MCP-1 and SDF-1a) (Fig. 4) [76]. Autolysosomes were filled with SPIONs following degradation and metabolism in the autolysosomes. These particles induced endogenous LC3-II transformation in bone marrow-derived macrophages (BMDMs) obtained from both tibia and femur of male Balb/c mice upon intravenous injection and triggered time-dependent LC3B accumulation in mouse Kupffer cells when intravenously administered (Fig. 4) [76]. Lactosylated N-Alkyl polyethylenimine coated superparamagnetic iron oxide nanoparticles can induce protective autophagy by LC3 conversion (from LC3-I to LC3-II) in RAW 264.7 cells [80], and dendritic cells extracted from bone marrow precursors of Balb/c mice [77]. These nanoparticles which have been used for dendritic cell labeling, promote cell maturation which is an essential process for migration and antigen presentation, and can enhance the vaccine functions of these cells. In addition, inhibition of the autophagy flux using 3-Methyladenine could lead to apoptotic cell death [77]. Autophagosome formation was also observed with increased expression of LC3-II protein in human peripheral blood monocytes which were incubated with 5–10 nm dextran-coated SPIONs. The authors noticed that autophagy is a protective mechanism in these cells against cytotoxicity of nanoparticles while inhibition of autophagy attenuated cell survival and accelerated inflammation [78]. Fe_3O_4 nanoparticles (15 to 20 nm) would result in significant accumulation of autophagosomes (detect the distribution of endogenous LC3 proteins) in the kidney and spleen of female mice which were injected via peritoneal cavity at a single dose of 10 mg iron oxide/kg for 30 days. However, 300 nm core–shell nanostructures comprising a PLGA shell and magnetic Fe_3O_4 did not show the same effect [81].

Various studies reveal autophagy regulation in immune cells by other inorganic nanoparticles and their dissolution products. Johnson B. *et al.*, reported release of free ion (Zn^{+2}) from zinc oxide nanoparticles can be taken up by primary and immortalized immune cells, where increased levels of intracellular reactive oxygen species and level of LC3A resulted in autophagic cell death [82]. Silver nanoparticles (<30 nm) downregulated surface marker CD11b and response to lipopolysaccharide stimulation and therefore prevented THP-1 differentiation from monocytes to macrophages by blockade of autophagic flux through blockage of P62 degradation, an autophagy substrate [83]. Autophagy also has been found to have an anti-inflammatory effect to promote M2 Raw 264.7 macrophage differentiation after exposure to silver nanoparticle-loaded TiO_2 nanotubes (130–140 nm) via inhibiting PI3K/Akt pathways [84]. Whole-body chamber inhalation of TiO_2

nanoparticles (19.3 ± 5.4 nm) to A/J Jms Slc mice (males and females) for 28 days induced ER stress and mitochondria in the lung leading to abnormal dose-dependent accumulation of autophagy (defined by overexpression of LC3, p62, and Beclin 1 proteins levels) [84]. Activation of autophagy pathways may occur following several mechanisms such as inflammation, ER stress, organelle dysfunctions upon exposure of immune cells to inorganic nanoparticles. Such activation is considered one of the main mechanisms of immunotoxicity or immunosuppression of nanoparticles.

2.6. Organelle dysfunction

Another effect of inorganic nanoparticles on immune cells is the dysfunction of the cellular organelles such as mitochondria, lysosomes, endoplasmic reticulum, and Golgi apparatus. Mitochondria, an organelle with major metabolic pathways, plays an important role in cell physiology, function, death, and survival [85]. Endoplasmic reticulum serves multiple function in cells including synthesis, modification, folding, and transport of proteins and involves various cell signaling pathways [86]. Mitochondrial damage and endoplasmic reticulum stress are two of the mechanisms that induce apoptosis in macrophages and lymphocytes upon receiving inorganic nanoparticles. Raw macrophages exposed to magnetic iron oxide nanoparticles (50 $\mu g.mL^{1}$) overexpressed the mitochondrial superoxide dismutase (SOD), but not cytosolic SOD, while increasing with the number of cells that generated ROS [87].However mitochondrial calcium levels and apoptosis did not increase at 24 h after exposure, but chromatin condensation and mitochondrial swelling were observed [87]. Feraheme, an injectable form of iron oxide nanoparticles, also induced mitochondrial oxidative stress which altered mitochondrial dynamics, architecture, and membrane potential and resulted in a decrease in cytokine production and proliferation of primary human T cells (Fig. 5C-E). In this study the authors did not observe mitochondrial damage in ironcontaining complex drug formulations [48]. PEG-COOH-coated iron oxide nanoparticles (PEG-Fe_3O_4, 50 nm) induced mitochondrial instability in bone marrow-derived dendritic cells from male C57BL/6J mice [88]. The mechanism was evaluated to be the activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) pathway resulting in promotion of mitochondrial biogenesis and therefore impairing mitochondrial dynamics. PEG-Fe_3O_4 particles decreased autophagy and inhibited mitochondrial degradation and facilitated mitochondrial fragmentation by increasing dynamin-related GTPases level, dynamin-related protein 1 and mitofusin-2, which are involved in mitochondrial fission and fusion, causing impairment in the functionally immature state of dendritic cells [88].

Lysosomes are the membrane-bounded organelles containing digestive enzymes which receive and digest cargo from the inside and outside of the cells [89]. There is evidence that nanoparticles cause lysosomal dysfunction directly by accumulation inside them, or indirectly through other cellular pathways which lead to cell death. Porosity dependent lysosomal activity alteration for inorganic silica nanoparticles has been reported [90,91]. Various sizes of silica nanoparticles (30–3000 nm) caused lysosome destabilization in BMDM cells (Fig. 5 A, B) [61]. RNA sequencing analysis of Raw 264.7 macrophages receiving a sub-toxic dose of mesoporous and non-porous silica nanoparticles of approximately 500 nm in diameter revealed that mesoporous particles are capable of

changing gene expression related to inflammatory response and higher lysosomal activity, while gene transcription was minimally affected. The mechanism behind this phenomenon was suggested to be early lysosome alkalization by influencing Vo and V1 protein complexes of vacuolar H+(V)-ATPase expression levels [91]. Alkalization and decrease of lysosomal membrane stability have also been observed in the THP-1 cells treated with silver nanoparticles [83]. Changing the geometry of anatase TiO_2 nanomaterial into a fiber structure (>15 μm) initiates an inflammatory response in alveolar macrophages extracted from C57BL/6. Results showed that these macrophages were not able to sequester TiO_2 nanofibers into lysosomes which led to lysosomal instability and disruption, secretion of cathepsin B, and formation of the NALP3 inflammasome [92]. Cho W. *et al.*, also reported that zinc oxide nanoparticles (10.7 ± 0.7 nm) release Zn^{+2} ions in the acidic condition of the lysosomes of human macrophage THP-1 cells that cause destabilization and loss of integrity of lysosomes, however, the same results were not observed for titanium oxide nanoparticles (30.5 ± 1.8 nm) [93].

The endoplasmic reticulum (ER) is another membrane-bound organelle which plays important roles in the synthesis, folding, and maturation of proteins, lipid metabolism, and calcium storage [94]. ER dysfunction and stress lead to loss of ER homeostasis and accumulation of unfolded proteins which was found to be associated with toxicity of nanoparticles [95–97]. 24 h treatment of human monocyte THP-1 cells with non-toxic dose (25 μg/mL) of slightly negatively charged silver nanoparticles (15 nm) induced rapid processing of activating transcription factor 6 (ATF-6), an indicator of ER stress, in parallel with activation of the NLRP-3 inflammasome [98]. Huo L. and coworkers studied the downstream proteins in the ER stress signaling pathway including Caspase-12, eIF2a, and CHOP proteins, and expression of ER marker genes including *chop, xbp-1s*, and *bip*. ER stress responses were observed in the lung, liver, and kidney by intratracheal instillation of 20 nm AgNPs (NM-300 K) into male ICR mice in two doses of 0.1 and 0.5 μg/g of body weight. These toxic doses caused significant apoptosis only in the lung and kidney [99]. Upregulation of ER stress-related genes promoting oxidative stress, inflammation, autophagy, and apoptotic cell death were also reported for copper oxide [100], silica [101], and titanium oxide [102,103], ultra-small superparamagnetic [104], and magnetic iron oxide nanoparticles [87].

Exosomes are small extracellularly secreted membrane vesicles that originate from multivesicular endosomes and contain protein, lipids, nucleic acids, and glycoconjugates. Upon release from the cells, exosomes activate different signal transduction pathways including immune signaling [105]. Previous studies have revealed exosome generation upon nanoparticle exposure. Zhu M. *et al.*, were able to see a significant number of exosomes in the alveolar region of Balb/c mice upon respiratory-system-exposed magnetic iron oxide nanoparticles (43 nm). These exosomes would activate splenic T lymphocytes, and induce dendritic cell maturation [106]. Kasper J. Y. and coworkers have suggested silica nanoparticle treatment of the inflamed endothelium during chronic inflammatory bowel disease can inhibit exosome systemic communication via decreased secretion of ICAM/E-selectin proteins [107], On the other hand, Andersson-Willman B. *et al.*, have shown titanium oxide (21 nm) and zinc oxide (10 nm) nanoparticles have no effect on exosome production in the primary human lymphocyte and dendritic cells (PBMC and MDDC) [108].

There are reports that inorganic nanoparticles can induce other cell organelle dysfunctions such as Golgi apparatus fragmentation in cancerous cells. For example, oxidative stress induced by Ag@ZnO nanoparticles (mean thickness 16 nm for the Ag core and 3 nm for the ZnO shell) has been reported to lead to Golgi fragmentation [109]. However, it still remains to be seen whether dysfunction of other organelles occur in the immune cells, and the molecular mechanisms behind such exposure to inorganic nanoparticles need further elucidation.

2.7. DNA damage and genotoxicity

Another mechanism reported for the toxicity of inorganic nanoparticles or their free ions is to damage cellular genetic information via direct interaction with DNA sequence or structure and influencing gene expression. Genotoxicity of silica [110,111], silver [112], titanium oxide [113], zinc oxide [111], and iron oxide nanoparticles [114] have been extensively reviewed, including in the immune cells. Significant genotoxic and DNA damage potential of zinc (30 nm) and nickel (17 nm) oxide nanoparticles on human monocyte and peripheral blood lymphocytes have been reported, respectively [40,41]. Comet assay data showed an extensive increase in DNA damage of THP-1 cells treated with ZnO nanoparticles (20 μg/mL) and an increase in the number of micronucleated cells in comparison with control [40]. Comet and cytokinesis-block micronucleus assays also revealed a significant time- and dose-dependent genotoxic potential of nickel oxide nanoparticles at higher doses of 25 and 50 μg/mL in human peripheral blood lymphocytes [41]. Shahbazi M. *et al.*, demonstrated genotoxicity of five different types of porous silicon nanoparticles on various immune cells, i.e., Raji (B-cell), Jurkat (T-cell), U937 (monocyte), and RAW 264.7 (macrophage), as a function of nanoparticle surface chemistry and charge in a concentration- and time-dependent manner [115]. Their results showed that the immunotoxicity of the particles is predominantly surface chemistry- and charge-dependent whereby the surface charge played the predominant role compared to hydrophobicity. In the same study cells with lower metabolic activity and longer doubling time (T-cells and monocytes) were more sensitive to the concentration- and time-dependent toxicity of particles (B-cells and macrophages) [115]. DNA strand breaks in female Wistar rat PBMCs were observed after one day exposure to titanium oxide nanoparticles (about 21 nm) [116]. These particles induced DNA breaks, not DNA oxidation, in human PBMCs in a time- and dose-dependent manner (75 $\mu g.cm^{-2}$ after 4 h exposure, 15 $\mu g.cm^{-2}$ and 75 $\mu g.cm^{-2}$ after 24 h exposure) [116]. Size-dependent DNA damage and micronuclei formation in white blood cells, Jurkat Clone E6–1, and THP1, was also reported for various sizes of silver nanoparticles (10–100 nm). Bulter K. S. *et al.*, reported smaller size of silver nanoparticles induced more genotoxic response [117]. Genotoxicity of iron oxide nanoparticles may also depend on their surface coating. For example, Oleatecoated iron oxide nanoparticles induced DNA damage in human lymphoblastoid TK6 cells [118]. Other studies demonstrated that polyacrylic acid-coated and non-coated iron oxide nanoparticles (4, 20, and 100 μg/mL for 2 days) were not directly genotoxic in human T lymphocytes extracted from blood [119]. Global gene analysis of murine macrophages response to mesoporous and non-porous silica nanoparticles also showed porosity-dependent genotoxicity [91]. The mesoporous SiO_2 nanoparticles altered gene expression at sub-toxic doses, however comparable non-porous particles, independent

of their size, did not change gene expression despite having higher cytotoxicity at the same dose [91].

Few studies have been done to evaluate if the genotoxicity of inorganic nanoparticles is from the intact nanoparticles or their released ions. For example, Li Y. *et al.*, have explored the different mechanisms of genotoxicity of silver nanoparticles and their related ions (Ag+, released from silver nitrate) in human spleen lymphoblast, TK6 cells by micronucleus assay [120]. Their results showed that genotoxic effects are primarily due to intact nanoparticles rather than the released ions [120]. Different sizes of silica nanoparticles (6–55 nm) also influenced DNA damage in human peripheral blood lymphocytes, where smaller sizes were the most potent [121]. The same study revealed soluble products released from SiO_2 nanoparticles did not increase DNA damage [121]. Higher DNA damage was caused by cobalt nanoparticles compared to cobalt ions in human T lymphocytes [122]. Drawing a general conclusion regarding the genotoxicity of inorganic nanoparticles and their degradation products needs detailed elucidation of experimental conditions. More comprehensive studies are needed to evaluate the long-term genotoxicity and mutagenicity of inorganic nanoparticles and the role of the immune response in different animal models and sexes as well as upon various routes of administration.

2.8. Epigenetic toxicity

Epigenetic modification is alteration in the genome without changing DNA sequences. These include DNA methylation, histone modification, chromatin remodeling, and alteration in expression of non-coding RNA [123,124]. Concerns have been raised regarding the epigenetic toxicity of nanomaterials including inorganic nanoparticles on immune cells [125–128]. Increasing evidence suggests the potential of inorganic nanoparticles to induce epigenetic toxicity. For example, alteration of different miRNAs has been reported upon 6 h and 24 h exposure to metal-based inorganic nanoparticles (zinc oxide, silver oxide, and titanium dioxide) at their sub-toxic doses in human THP-1 macrophages [129]. Pulmonary administration of surface-coated (polyalcohol) nanoTiO_2 to female C57BL/6 BomTac mice (42.4 ± 2.9 mg nanoTiO_2.m^3, 1 h/day) resulted in changes in the expression of genes associated with inflammation and immune response (upregulation of miR449a, miR-1, and miR-135b) five days post-exposure [130]. Ag nanoparticles (<100 nm) and their degradation products (Ag ions) where shown to induce miRNA alteration in human Jurkat lymphocyte cells after one-day treatment at 0.2 mg/L via different epigenetic mechanisms. Ag ions induced ENDOGL1 expression regulated by miR 654–3p. However, Ag nanoparticles induced MT1F and TRIB3 regulated by miR-219–5p [131]. MicroRNAs regulate gene expression post-transcriptionally by binding to the 30 untranslated region (30-UTR) of target mRNAs. These also participate in the expression of the epigenetic regulators such as histone deacetylases, polycomb group genes, and DNA methyltransferases [132]. Ag nanoparticles 25 nm in diameter coated with poly (vinylpyrrolidone) (PVP) altered histone 3 methylation status significantly in the mouse erythroleukemia cells, derived from a B-cell lymphoma sub-lethal dose (8 μg/mL), while Ag ion-treated cells showed no alterations [133]. Copper oxide nanoparticles (58.7 nm, 0.5 and 30 μg/mL) caused DNA methylation in LINE-1 and Alu/SINE, two most abundant transposable elements (TEs)-associated DNA in mammalian genomes, in human and murine macrophages (THP-1 and RAW 264.7) and in Balb/c

mouse lung after intratracheal administration (at dose of 2.5 mg/kg) [134,135]. Intratracheal administration of gold nanoparticles to male Balb/c mice also showed CpG methylation changes in some genes (increase in *Gsr, Cdk, Atm* and decrease in *Gpx, Gsr,* and *Trp53*) in the lung for which the *Trp53* methylation was nanoparticle size dependent [136]. DNA methylation alteration within CpG sequences influences their interaction with methyl-CpG binding proteins, which may induce chromatin conformational modifications. The result of this chromatin remodeling is inhibition of the access of the transcriptional machinery to gene promoter, and therefore altering gene expression levels [137]. However, there are still significant gaps in the understanding of the mechanism of long-term epigenetic and also developmental toxicity of inorganic nanoparticles in the immune system.

2.9. Immunosuppressive response

The reduction or suppression of the activity of the immune system is called immunosuppression [138]. Anti-inflammatory response is one example of an immunosuppressive reaction. Along with the immunotoxicity reports, several studies have shown that metal and metal oxide nanoparticles can be immunosuppressive based on their structure [138]. Dobrovolskaia M. and coworkers have produced comprehensive summaries of immunosuppressive and anti-inflammatory properties and methods of analysis for nanomaterials [139,140]. Ngobili T. and Daniele M. also highlighted the immunosuppression of metal nanoparticles such as gold and silver, and metal oxide nanoparticles such as iron oxide, titanium oxide, and cerium oxide nanoparticles in various immune cells [138]. For example, single intravenous exposure of OVA-sensitized mice to Resovist, commercial medicine containing iron oxide nanoparticles (28 mg iron/mL), suppressed IL-17, IL-6, and ROR-γt and attenuated Th17 immune responses [141]. Investigation on J774A.1 murine macrophage cells showed cerium oxide nanoparticles play anti-oxidant and anti-inflammatory roles *in vitro* [142]. Silver nanoparticles also have shown to suppress the immune response. For example, Yilma A. N. *et al.* observed different sizes of silver-poly (vinyl pyrrolidone) nanoparticles (10–80 nm) downregulated pro-inflammatory cytokines such as IL-6 and TNF in mouse macrophages infected with the sexually transmissible infection *Chlamydia Trachomatis* [143]. Such downregulation was more pronounced for smaller PVP-coated silver nanoparticles.

One fundamental question is whether inorganic nanoparticles show antigenicity properties or not. Antigenicity is the ability of nanoparticles to be recognized specifically by antibodies or with receptors of T cells stimulated and presented to MCHC during an immune response [13]. There is significant debate in the current literature regarding the source of antibodies against inorganic nanoparticles and their contribution to immunotoxicity. These antibodies could be formed in response to inorganic nanoparticle fragments or surface modifications (such as PEGylation). The antigen-specific immunity towards iron oxide nanoparticles has been reported [144,145]. Shen C. *et al.* have demonstrated serum production of T cell-dependent antigen ovalbumin (OVA)-specific antibodies (IgG1 and IgG2a) in male Balb/c, 7 days after bolus intravenous administration of iron oxide nanoparticles [144]. It has been shown that anti-PEG antibodies form upon exposure to organic nanoparticles (such as liposomes), although PEGylated gold nanoparticles did not generate anti-PEG antibodies [145]. The immunomodulation of inorganic nanoparticles is complex and various

parameters including size, composition, surface chemistry, protein binding, dose, and route of administration define their immunostimulation or immunosuppression behavior [146].

3. Clinical application of inorganic nanoparticles and theirimmunotoxicity

Inorganic nanoparticles have been used for different applications in the clinic. Gold, iron oxide, and silica nanoparticles received the U.S. Food and Drug Administration (FDA) approval for thermal ablation of tumors, chronic kidney disease, cancer imaging, imaging probe for magnetic resonance imaging (MRI) and computed tomography (CT), and for treatment of anemia [10,147,148]. For example, Ferumoxytol has been approved by the FDA as a contrast agent for gastrointestinal imaging upon oral administration and treatment of anemia in patients with chronic kidney disease [149]. Another application of inorganic nanoparticles is in *ex vivo* cell labeling to enhance contrast of cellular target such as in MRI or long-term tracking of stem cells [150]. For example, Fridex and Ferucarbotran received FAD approval as MRI contrast agents [151]. It has been demonstrated that glucosamine-modified iron oxide nanoparticles can be used for long-term stem cell labeling due to their biocompatibility, high and sensitive *in vivo* and *in vitro* detection in MRI and high cellular Fe dose [152]. There are additional inorganic nanoparticles in various phases of clinical trials which seek FDA and/or the European Medicines Agency (EMA) approval [153,154]. Beside these opportunities, there are still challenges for loading efficacy, *in vivo* targeting, and clearance of these nanoparticles which need to be investigated further.

Safety of nanoparticles is crucial and should be assessed to draw out their potential in the clinic. Many *in vitro* studies have been done on immunotoxicity of inorganic nanoparticles, however detailed and long term *in vivo* studies are limited. The distribution, clearance, and long-term toxic effect on various immune cells need to be investigated *in vivo* as a function of nanoparticles' physicochemical properties and potential degradation patterns. There are scattered reports on immunogenicity and immunotoxicity of various inorganic nanoparticles. The physical or chemical stability of inorganic nanomaterials under certain conditions may be desirable or undesirable for the ultimate end-use. For example, the physicochemical stability of drug-loaded nanocarriers until they reach the target site may be desirable. However, such stability is unfavorable for clearance of the carriers from the body. Therefore, more mechanistic studies are needed to evaluate the immunostimulation or immunotoxicity of these particles and their degradation products. Immune response may be desirable for using inorganic nanoparticles as vaccine adjuvants (*e.g.*, modulating specific hormonal or cellular immunogenicity for the designed antigen), or undesirable when using these particles for imaging or delivery of other bioactive agents [14]. Immunosuppression, may also be desirable or undesirable as well based on the application of nanoparticles. Therefore, evaluation of immune response to inorganic nanoproducts need to be discussed in the context of their specific clinical application. This means induced immune response does not necessarily preclude the application of a specific nanomaterial in the clinic, and the biological outcome depends on the type of immune response, duration, and reversibility for that specific application.

Current FDA guidance for evaluating the safety of new nanoproducts applies based on their application and all new pharmaceuticals should be investigated for their immune

related response using standard toxicity assays [155]. FDA safety guidelines for engineered nanomaterials including inorganic nanoparticles is the same as other therapeutic frameworks with the focus on understanding the risk to benefit ratio in a case-by-case basis for each product [156]. Dobrovolskia M. has reported the challenges and strategies for preclinical immunotoxicity evaluation of nanoformulated-drugs [157]. For example, lower immunotoxicity due to TNF-α secretion has been observed upon reformulation in PEG-coated colloidal gold nanoparticles [145]. General safety evaluation with respect to the route of administration, dose, and duration of exposure should be demonstrated and correlated with careful physicochemical characterization, pharmacokinetics, and pharmacodynamic evaluation of the new materials. In case of using inorganic nanoparticles as vaccine adjuvants, FDA and EMA require preclinical immunogenicity studies for the full products and the adjuvant by itself, including their potency, tolerability, short and long term toxicity during treatment, and also following the recovery time (*e.g.*, two weeks or more after last exposure) at the highest dose (which is going to be used in clinical trials) in relevant animal species and strains [158]. Preclinical *in vitro* and *in vivo* studies are needed to screen immunological properties of inorganic nanoparticles formulated as drugs or vaccines [157]. The evaluation of the serological response, hormonal and cellular immune response, and lack of immune interference with all antigen components in the vaccine should be reported to the FDA and EMA. The regulatory agencies will then determine the risk assessment of the product based on a weight-of-evidence approach [155]. Assessment approaches may differ based on type, severity, duration, and desirable or undesirable response of immunity. FDA and EMA will then decide if the immune response can be tolerated or is reversible and safe enough to enable approval of the nanoproduct.

4. Degradation mechanisms of inorganic nanoparticles

Much research has been done about the degradation and dissolution of various inorganic nanoparticles. Different terminologies are used to describe the inorganic nanoparticles' susceptibility to degradation, disintegration, and dissolution including biopersistent, durable, stable, labile, nondegradable or degradable. Inorganic nanoparticles can degrade into smaller fragments, or to their precursors, *e.g.*, metal ions or metal oxides in air, in solution, *in vitro*, or *in vivo*. Here, we first define different terminologies for stability and degradability of inorganic nanoparticles. Based on one classification, there are three levels of inorganic nanoparticle stability or integrity: *i) Colloidal stability* of the nanoparticles refers to the ability of nanoparticle dispersion to resist aggregation or agglomeration into large entities that could be segregated from the solution [159]; *ii) Chemical stability* refers to the preservation of the chemical properties of the nanoparticles [160]; and *iii) Biofunctional stability* of the nanoparticles refers to the ability of empty nanoparticles, functionalized nanoparticles, or active ingredient-carrying nanoparticles to stay functional in the environment. Such stability of nanoparticles may be lost during storage, or in media upon *in vitro* treatment, or under physiological conditions *in vivo* in blood, tissues, or cells [161,162]. The process of instability might happen via two phenomena: *i) Clumping or gathering:* which is the assembly of nanoparticles together or to other available molecules or species that results in bigger particles or particle assemblies, for example, aggregation of inorganic nanoparticles (strong cluster of nanoparticles), agglomeration (loose gathering

of nanoparticles), and macromolecular adsorption on the surface of nanoparticles (such as protein corona); *ii) Disintegration or corrosion:* Which is the process of dissolution of nanomaterials in the respective environment such as metal release from metal or metal oxide nanoparticles [163,164]. These phenomena are highly dynamic and can be reversible or irreversible.

Various terms are used to describe the process of the gradual loss of integrity of inorganic nanoparticles, such as degradation, erosion, and dissolution. The prefix “bio” has been used for the same process in the living organism [165]. To reduce this confusion regarding terminology, here we will use the following definitions: *i) Degradation* refers to any chemical process that cleaves a covalent bond. This can be hydrolysis, oxidative, or enzymatic in nature; *ii) Dissolution* refers to any physical changes in the structure of nanoparticles in the environment such as the capability of forming non-covalent interactions of precursors with the solvent or crystal structure that needs to be broken up and release ions; *iii) Erosion* includes both physical (such as dissolution) and chemical processes (such as backbone cleavage) (Fig. 6) [165]. When any biological agent or physiological condition causes these processes, they are called biodegradation, biodissolution, or bioerosion. This biological agent can be any macromolecules such as enzymes, or simply mimic of biological environments such as lysosomal pH [165]. Therefore, acid etching of nanoparticles inside the endolysosomes is a biodissolution process, and the process of disulfide bond cleavage of nanoparticles in the presence of glutathione (GSH) is a biodegradation process, while these phenomena occurring intracellularly is called bioerosion. Degradation, dissolution, and erosion of inorganic nanomaterials can start from the inside or core of the particles (core erosion), or from the surface of the particles (surface erosion), or in the entire structure of the particles (balk erosion) (Fig. 6). It is important to note that the degradation of inorganic nanomaterials can occur in the absence of erosion due to dissolution and conversely dissolution of particles may be observed without biodegradation [165]. Further, the degradation or dissolution of nanoparticles is likely an uneven and nonlinear process between the populations of nanoparticles with the same composition and even throughout the structure of one nanoparticle [166].

The degradation and dissolution kinetics of inorganic nanoparticles in the blood, target tissue, cells, and subcellular organelles may define the extent and type of immune responses. The intracellular degradation of inorganic nanoparticles is often due to etching in the acidic condition of the endosomal and lysosomal compartments or due to enzymatic activity [162,167]. For example, lysosomal degradation of iron oxide nanoparticles in the macrophages has been reported after *in vivo* administration and splenic and hepatic accumulation [168]. The magnetic properties of these nanoparticles were reduced over time upon degradation [168]. The higher pH of cytosol may have accelerated the degradation process of silica nanoparticles if they escape the lysosomes or endosomes [169]. Variations in the pH of the environment may change the degradation and dissolution of inorganic nanoparticles as a function of their physicochemical properties [167]. For example, Hadipour S. P. *et al.*, investigated the 28-day degradation profiles of the synthesized SiO_2 nanoparticles in different simulated biological fluids, including simulated gastric fluid (pH 1.2), lysosomal fluid (pH 4.5), intestinal fluid (pH 6.5), body fluid (pH 7.4), and in deionized water (pH 6.5) [170]. The results showed very slow degradation in the gastric

fluid over 28 days, while the degradation rate was much higher in the intestinal fluid. Mesoporous silica nanoparticles (100 nm, and 500 nm) degraded faster at higher pH (Fig. 7) [170]. *In vivo* degradation of inorganic nanoparticles is also critical for their metabolism in the body. For example, iron metabolism occurs mostly in the liver [171]. Biodistribution studies demonstrate that iron oxide nanoparticles tend to distribute in the liver and spleen for about two weeks and their degradation and clearance is dependent on their properties including size and surface coating [172]. Liver endothelial and Kupffer cells exhibit equal distribution and degradation (ferritin and hemosiderin) of superparamagnetic iron oxide nanoparticles following bolus IV administration (5 mg Fe/weight of rat) [173].

The effects of inorganic nanoparticles (such as Fe_3O_4, Au, Ag, SiO_2, Cds, Pt) on the structure and activity of several enzymes, such as glucose oxidase and dehydrogenase, protein disulfide isomerase, peroxidase, nitrate reductase, lysozyme, lactase, microbial esterase, peroxidase, DNA methyltransferase, among other enzymes have been investigated [174]. There are few reports on the enzymatic degradation of nanoparticles [175]. The degradation of inorganic nanoparticles with disulfide bonds by GSH has been established. Disulfide-based biodegradable mesoporous silica nanoparticles for example underwent both hydrolysis and disulfide reduction in the presence of glutathione and disintegrated into smaller fragments over a period of 28 days [170]. Due to the hydrophobic nature of the precursor, these particles did not exhibit higher degradation rates compared to mesoporous silica nanoparticles without disulfide bonds [170]. This demonstrates the importance of additional factors such as porosity, surface area, and hydrophobicity on degradation, besides the nature of the degradable bond.

Stability of inorganic nanoparticles depends on their physicochemical properties [176]. The kinetics of loss of the integrity and dissolution of inorganic nanomaterials is highly dependent on their physicochemical properties as well as the chemical properties of the environment (Fig. 8). Multiple factors influence the biodegradation or bio-persistence of inorganic nanoparticles. These include: *i)* surface reactivity of nanoparticles such as their tendency to agglomerate in the biological environment, adsorption of macromolecules on the surface of nanoparticles (such as protein corona), oxidation or reduction of metal from the surface or core of metal nanoparticles, availability and influence of enzymes (such as hydrolytic enzymes) and chelators, and the storage of metals in the environment; *ii)* intracellular distribution of nanoparticles; *iii)* and their exposure modes such as route of administration and *in vivo* biodistribution in various organs [177]. The fate of iron oxide nanotubes (with amphiphilic polymer shell and poly(ethylene glycol) coating) was monitored in mice over 14 days upon intravenous administration [177]. The results showed that the degradation rate of these nanoparticles depend on polymer surface coating and the accessibility of chelating agent to the core of nanoparticles [177]. Degradation of silica nanoparticles has also been shown to depend on several factors such as size, porosity, composition, pH of the environment, and surface coating. The nonporous dense Stöber silica nanoparticles (100 nm in diameter, negatively charged) underwent surface (external) degradation, however the same size mesoporous particles degraded both on the surface and in bulk due to the porous scaffold [170]. Size-dependent-dissolution rate of amorphous silica nanoparticles reveals that the kinetics of dissolution depends on the surface area or mass-normalized data. Farfrom-equilibrium surface area normalized data showed smaller size

particles have a slower rate of dissolution in aqueous solutions at neutral and basic pH. But, mass normalized dissolution rates were shown to be independent of SiO_2 nanoparticle size [178]. 50 nm core–shell structured magnetic mesoporous silica nanoparticles contained a single Fe_3O_4 nanoparticle core center located inside the mesoporous silica matrix, dissolved from the inside of the particles around the magnetic core, while poly(ethyleneimine) coating retarded the silica degradation due to the slightly basic environment (Pk_a value > 10) [179]. Yang S. *et al.* have reported that the amine groups in media or blood components can play an important role in the erosion of silica nanoparticle surface layers [169]. It has been shown that low pH environment of the lysosome and endosomes plays a significant role in metabolism of Clariscan™ ferromagnetic particles after IV injection for liver imaging [180]. Their *in vivo* dissolution data have revealed pH-dependent dissolution of these particles in a period of one week with complete dissolution in 10 mM citrate, pH 4.5, in 4–7 days, likely due to intracellular ferritin dissolution [181]. There is evidence that PEGylated sliver-iron nanoparticles tend to be cleared from the liver making them suitable for potentially clinically applicable biodegradable MRI contrast agent [182]. Amendola V. *et al.*, have shown Ag-Fe nanoparticles are renally cleared over 30 days in Balb/c mice [182]. Therefore, a combination of the environmental factors and nanoparticle characteristics define the outcome of the degradation of inorganic nanoparticles. There is a significant gap in the understanding of the fate of degradation products of inorganic nanoparticles in dendritic cells, lymphocytes, natural killer cells, and also immune organs which need to be studied in the future.

The physicochemical properties of nanoparticles, and the activities which arise from these properties such as superparamagnetism, quantum confinement, and extreme catalytic activity, may be partially or progressively changed once the particles are in the physiological environment [163]. For example, partial loss of surface coating can lead to reduced colloidal stability of nanoparticles following by their agglomeration. Balforier A. *et al.* observed size-dependent degradation of gold nanoparticles (4 to 22 nm) in primary human fibroblasts. The released gold ions underwent a biomineralization process and recrystallized into a new nanostructure inside the cells [183]. Subsequently, as nanoparticles come into contact with tissues or cells, they are exposed to many challenges that change their properties compared to the well-defined pristine systems. For example, the cells may encounter a biodegradation product which can cause a different set of molecular alterations compared to the parent compound. When nanoparticles enter the cells, the protein corona around them may be digested enzymatically by proteases in the lysosomes or phagosomes. Nanoparticle fragments, after digestion inside the cells or tissue, might be recognized as a different antigen for the host and trigger different immune responses. Degradation products of inorganic nanoparticles might start new changes in the physicochemical properties of existing particle fragments and therefore the downstream pathways. Aggregated particles lead to new surface functionality, mobility, and concentration [163]. This alters their blood circulation, biodistribution into different organs of the body and their immunogenicity. Detailed characterization, accurate and appropriate dosimetry, and investigation of changes in physicochemical properties in relevant biological assays are crucial for proper investigation of immune reaction to nanoparticles in general, and inorganic nanoparticles

in specific. These aspects need further investigation in nanotoxicology studies, including in immunotoxicity.

5. The influence of degradation products on the immune system

The degradation and dissolution of silver, iron, zinc oxide nanoparticles, and quantum dots, as well as their biological fate have been reviewed [162,184]. The released metal ions from the etched nanoparticles may be toxic even at low concentrations (*e.g.* Ag^{+}, Au^{+}, and Cd^{+}), or may participate in different cellular pathways (*e.g.* Zn^{+2}, Fe^{+2}), or induce ROS and changes in metal homeostasis of the cells [162]. For example, the released ions from metal nanoparticles, trapped inside endosomes or lysosomes, may cause different outcomes for both the nanoparticles and the cells: *i)* cause re-growing of the nanoparticle core which involves reshaping of the existing nanoparticles [167]; *ii)* depending on their solubility, particles may gradually dissolve in the cells; *iii)* might be excreted from the cells through exocytosis pathways; and *iv)* might relocate to different organelles causing downstream effects such as altering the equilibrium concentration of metals inside the cells and subsequent long-term cytotoxicity or genotoxicity. A clear understanding of the fate of these dissolution and degradation products inside the cells and the body, including their immune properties, is crucial for safe and effective use of inorganic nanoparticles.

Metal ion release has been reported for zinc oxide, silver, cadmium, selenium (CdSe), and iron oxide nanoparticles [162]. Although gold nanoparticles have been considered “inert” or stable, there is concern regarding the release of Au from the surface of the particles by intracellular thiol reductase resulting in the shape transition of nanoparticles [166]. The toxicity of metal ions released from these nanoparticles depends on their rate of dissolution, and also the properties of the corresponding dissolved metal ions [47]. There are few studies which compare the toxicity of intact inorganic nanoparticles with their aqueous extract which contain the degradation and dissolution products. Cho W.-S. *et al.* investigated the relative role of water-soluble metal ions released from metal oxide nanoparticles, nickel (10–20 nm), zinc (<10 nm), and copper oxide (<50 nm) nanoparticles, and their pro-inflammatory effects *in vitro* and *in vivo* [185]. Unlike NiO nanoparticles, the aqueous extract from them showed no significant cytotoxicity in the adenocarcinoma human alveolar basal epithelial cells, A549, nor inflammatory response in the rat lungs. The aqueous extract from ZnO and CuO nanoparticles showed toxicity and proinflammatory response *in vitro* and *in vivo* by a mechanism of early recruitment of the polymorphonuclear leukocytes when instilled into the rat lungs [185]. Four weeks toxicity studies revealed that metal ion solutions of these particles induced sustained inflammatory effects. However, the intact particles induced inflammation in the lymphocytes and polymorphonuclear leukocytes along with eosinophilic infiltrate into the bronchoalveolar tissue upon intratracheal instillation [185]. In another study, no cytotoxicity was observed upon 24 h exposure of RAW 264.7 macrophages to free silicic acid [$Si(OH)_4$], released from silica nanoparticles, in the range of 0.0625 to 400 μg/mL [170]. A comparative study of copper nanoparticles (23.5 nm), microparticles (17 μm), and ions (commercially available $CuCl_2 2H_2O$) revealed that the median lethal doses were 413, 5000, and 110 mg/kg, respectively, after oral ingestion in ICR mice showing slightly higher toxicity for ions. However, sex-dependent toxicity was observed only for copper nanoparticles where male mice were shown to be

more sensitive than female mice [186]. 28-day oral exposure to poly(vinylpyrrolidone) (PVP)-coated silver nanoparticles (<15 nm, [Ag] = 90 mg/kg) to Sprague Dawley rats appeared to be very similar to exposure to silver salts, $AgNO_3$ ([Ag] = 9 mg/kg] with no significant effect on the immune function [187]. On the other hand, zinc ion (such as zinc sulfate) showed more acute toxicity compared to the same mass (250 mg/kg) of oral administration of zinc nanoparticles via increasing glutamicoxalacetic transaminase activity in the serum, alteration of intestinal microbiota, and decreasing body weight of CD-ICR male mice [188]. The comparison of the cytotoxic effect of fully dissolved $ZnSO_4$ with an equivalent amount of Zn in the form of zinc oxide nanoparticles, showed significantly higher cell death for zinc oxide nanoparticles [49]. Dissolution plays an important role in zinc oxide nanoparticle induced cytotoxicity. ZnO dissolution and release of toxic Zn^{2+} in the cell culture medium of Raw 264.7 macrophages disrupts cellular zinc homeostasis, produces proinflammatory cytokines, leading to oxidative cell injury, intracellular Ca^{2+} release, mitochondrial depolarization, lysosomal damage, and cell death [49]. There are contradictory reports regarding the toxicity of intact nanoparticles and their aggregation or degradation products, which highly depend on the environment of nanoparticles after administration, concentration, and time of the study. More mechanistic studies are needed to evaluate the immune response to the nanoparticles' downstream products *in vitro* and *in vivo*.

6. Fate of inorganic nanoparticles inside the immune cells

Low degradation rate of many inorganic nanoparticles, along with their rapid clearance by the reticuloendothelial system (RES) continues to pose a major problem for use of these systems in delivery applications. For example it has been reported that a very low percentage of intravenously injected nanoparticles reach the target solid tumors [189,190]. This limitation coupled with a low loading capacity of many of inorganic nanoparticles may result in a high dose or frequency of administration in order for the therapeutic cargo to reach a clinically relevant dose. This raises the concern for the potential of inorganic nanoparticles to "saturate" or overwhelm the mononuclear phagocytic system (MPS) [191]. Further, the therapeutic doses of nanoparticles are not given to healthy individuals. The immune response and metabolism of particles in diseased conditions would likely be different and therefore the risk of saturated MPS may be higher in patients. Currently, most toxicology studies utilize healthy animals and cell models which have normal rates of particle clearance. There are important and clinically relevant questions that need to be addressed when considering nanoparticle clearance: what would happen to the patients upon receiving inorganic nanoparticles whose rate of metabolism and clearance are not as high as in a healthy host? What happens when MPS is saturated? How long does it take for the nanoparticles to degrade and be cleared from MPS? How does this influence the function of the immune system? Are saturated macrophages able to do their normal functions such as clearance of foreign or abnormal agents, for example bacteria or cancer cells? More comprehensive studies are needed to address these important gaps.

Although there are significant investigations on the entry and uptake of inorganic nanoparticles via different endocytic pathways into immune cells, less is known about their fate beyond the endosomal compartment, the journey and fate of nanoparticles

(and their fragments thereof) inside the cells, and their exocytosis. It has been reported that Raw 264.7 murine macrophages can uptake silica nanoparticles up to a “threshold” of concentration [73]. The cells tend to compartmentalize particles (and their resulted fragments) inside vacuoles for a long period of time [73]. Therefore, there is a point of saturation or overloading of the nanoparticles inside these macrophages. Cytoplasmic vacuolization upon receiving spherical gold and iron oxide nanoparticles, 4 and 14 nm in diameter respectively, into Raw 264.7 macrophages have also been observed which results in cell pyroptosis, promotion of the cells toward M1 polarization through NF-κB signaling pathway, and therefore enhanced immune response [192]. Alternatively, direct depletion of RES macrophages and overwhelming liver macrophages with pre-or co-injection of inorganic materials (RES blockade techniques), such as gold [193] and silica [194] nanoparticles, has shown to be effective to various degrees in increasing tumor accumulation and in enhancement of therapeutic efficacy [195,196]. Therefore, the saturation of RES macrophages (intentionally or unintentionally) with inorganic nanoparticles is a significant challenge for the fate of the injected bare nanoparticles and their degradation products as well as subsequent long-term immunotoxicity. A study in Balb/c mice reported that repeated administration of sub-toxic doses of silica nanoparticles did not saturate the MPS, however, changes in the blood chemistry and histopathology were noticed in the animals exposed to the nanoparticles [197]. The amount of nanoparticle uptake relative to the capacity of phagocytes can reprogram the intracellular pathways of macrophages and affect their ability for the normal function of phagocytosis of foreign entities, and induction of cytokines which need further investigation.

Inorganic nanoparticles may have two main fates *in vivo*: i) excretion from the body (through urine, feces, or hepatobiliary elimination), and ii) metabolism to materials or elements which can be used by cells. Excretion of inorganic nanoparticles is highly dependent on their size. Nanoparticles or their downstream products with generally <5.5 nm can be quickly cleared by the urinary system, and >6 nm through hepatobiliary clearance [198]. Other physicochemical properties of nanoparticles can influence their *in vivo* biodistribution, and biodegradation profile, and therefore indirectly change their size and thus clearance. There are many reports regarding the long-term retention of inorganic nanoparticles (or their degradation products) in the body [199]. For example Miller M. R. *et al.*, have detected gold in the blood and urine of the individuals up to three months after 2 h inhalation (116 ± 12 μg. m^3; $5.8 \pm 0.3 \times 10^6$ particles.cm^3) while the levels were greater for smaller particles (5 nm) [200]. Kreyling and colleagues also demonstrated the persistence of radiolabeled iridium nanoparticles in the lung of rats six months after a 1 h inhalation [201,202]. Mohammadpour R. *et al.* detected silicon in the liver, spleen, and rarely lung of female and male Balb/c mice two months after bolus intravenous administration of silica nanoparticles with variations in size and porosities at their 10-days maximum tolerated dose (100–300 mg/weight of mice) [203]. In this study although the particles and their degradation products were cleared over two months, immunotoxicity was observed six months to one year after administration [204]. Kolonsjaj-Tabi J. and coworkers observed that the gold part of intravenously administrated gold/iron oxide nanoheterostructures persist in the liver and spleen of female C57/Bl6 mice for up to one year [205]. These studies again emphasize that inorganic nanoparticles can accumulate in the body, have the potential

to saturate the immune cells, degrade over time, and influence the immune system during this period. More effective strategies are needed to track the nanoparticles and degradation fragments *in vitro* and *in vivo* to elucidate their long-term immunotoxicity as a function of time and as a result of the changes in their physicochemical characteristics.

7. Conclusion, challenges, and future directions

In summary, various cellular mechanisms are involved in immune responses to inorganic nanoparticles. These responses may be to the intact nanoparticles, and/or to their degradation fragments and dissolution products. The degradation products of inorganic nanoparticles may be a biologically relevant compound which cells already have (such as iron), or it might be a nonrelevant compound (such as degradation fragments). The biologically relevant compound might be involved in the existing cellular metabolic pathways; however, it may change the cell homeostasis based on concentration and reactivity. When nanoparticles are administered, the immune system encounters structures that are different from the pristine nanoparticles. Detailed understanding of the influence of such changes and the degradation process on the mechanisms of immunotoxicity is limited.

Several unresolved issues remain in the understanding of the influence of inorganic nanoparticle physicochemical properties and their degradation products on immunotoxicity. The efforts to synthesize nanoparticles with low polydispersity are not yet efficient. Nanoparticle batches vary in size and size distribution without careful characterization in a relevant biological environment, over time, and in storage conditions. Our current knowledge at best elucidates a correlation between nanoparticles' physicochemical properties and immune response. However, we do not completely know the mechanisms. We do not clearly understand how we can change a specific nanoparticle to reduce the risk to benefit ratio of immunotoxicity before clinical applications. The ability to track the physicochemical stability of nanoparticles until they reach the target cell or tissue is limited. The nanoparticle feature in various tissues, cells, and subcellular compartments is dynamic, and this makes understanding the mechanisms of immune response difficult. Any changes in nanoparticle properties may result in the ensuing variation in the immunological properties of the host. These changes and their effects need to be examined more carefully.

Nano-immunotoxicology is a cross-disciplinary science. Improvement in our characterization methods, especially understanding the physicochemical properties and behavior of nanoparticles in the biological environment will help in the better understanding of the correlation between nanoparticle physicochemical properties and immune response. Appropriate animal or cell culture models, nanoparticle dosimetry, relevant dose, time, and frequency of administration, the appropriate endpoints of assays, and correlation of agglomeration, aggregation, dissolution, and sedimentation with immunotoxicity are important issues for better understanding of the immune response to inorganic nanoparticles [206]. High-resolution imaging methods can assist in the characterization of particles *in situ*, such as their aggregation, agglomeration, and release of ions. A better understanding of nanoparticle immune effects is possible with the appropriate choice of assays where the nanoparticle does not interfere with the test assay. Dosimetry and dose extrapolation and discrepancies between *in vivo* and *in vitro* exposure are significant challenges. It

still remains to be determined whether nanoparticles' mass, number, or surface area play the predominant roles on their biological fate in general and immunotoxicity in specific. Whereas numerous studies have been conducted to correlate physicochemical properties such as size, geometry, and charge of inorganic nanoparticles with their immunological properties, less attention is devoted to the route and frequency of administration, and in different immunologically-biased animal models, as well as variation in animal sex. Specific surface groups including hydrophilic, hydrophobic, and lipophobic materials, density and type of such groups and their spatiotemporal presentation could influence the stimulation of immune pathways which need to be elucidated in more detail.

Studies of the interactions of inorganic nanoparticles with the immune system have mostly focused on macrophages. Little work has been done on the fate of nanoparticles in other types of immune cells such as natural killer cells, lymphocytes, and dendritic cells. It is important to understand the fate and function of immune cells that carry inorganic nanoparticles or their degradation fragments. There is a clear need for more quantitative studies on subchronic and chronic immunotoxicity of inorganic nanoparticles *in vivo*. Finally, one should also take into account that it is crucial to study the changes in the normal function of immune cells, such as clearance of bacteria or cancerous cells from macrophages, while the cells are carrying nanoparticles or their degradation products over a period of time. Addressing these and other knowledge gaps are needed in order to design nanoparticles with improved safety profile and reduced adverse effects.

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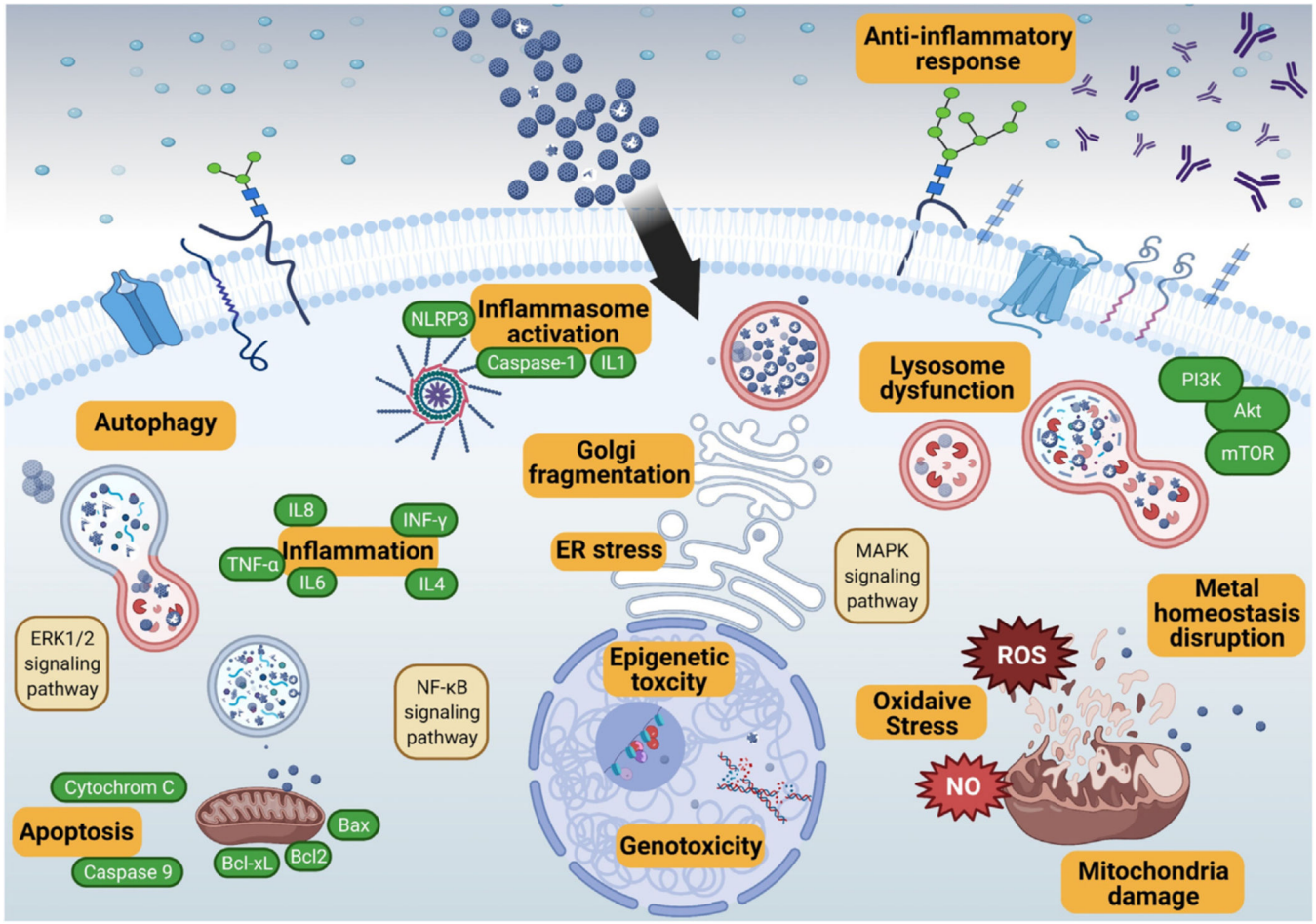

Fig. 1.

Schematic representation of various intracellular mechanisms for inorganic nanoparticle-mediated immune response. Inorganic nanoparticles may cause pro- or anti-inflammatory responses and oxidative stress. They can influence different organelles inside cells causing mitochondria and lysosome damage, ER stress, and Golgi apparatus fragmentation. Genotoxicity and epigenetic toxicity also may occur following the direct and indirect effects of inorganic nanoparticles inside the immune cell. Various molecular mechanisms including MAPK, NF-κB, ERK1/2, and apoptotic signaling pathways might alter in response to inorganic nanoparticles. All these processes determine the fate and function of immune cells.

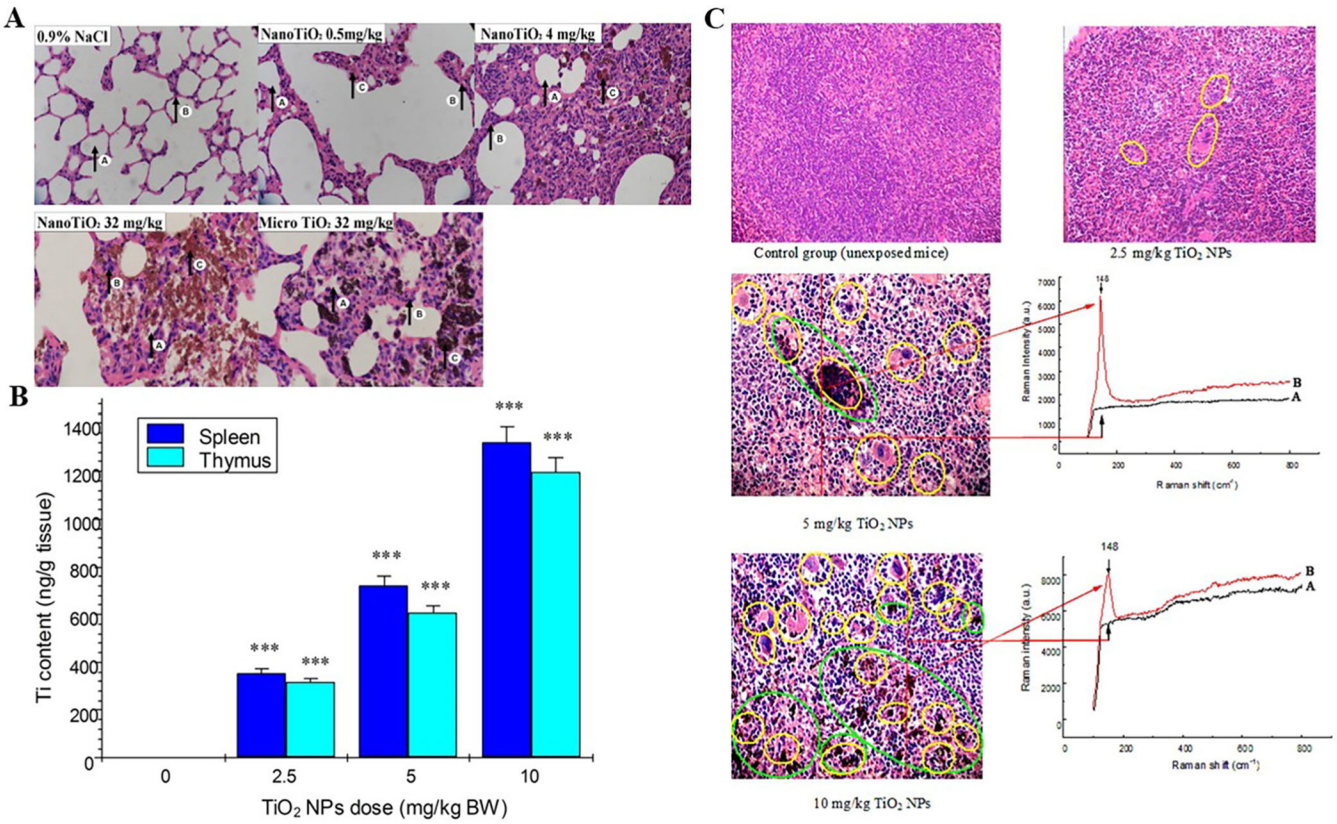

Fig. 2.

Immunotoxicity of titanium oxide nanoparticles. A) Aggregation of lymphocytes and macrophages, pulmonary emphysema, collapse of terminal bronchioles, and massive nanoparticle deposition in the lung tissue following intra-tracheal installation of different doses of TiO_2 (21 nm) into male Sprague Dawley rats [25]. B) 90 days consecutive intragastric administration of TiO_2 nanoparticles (mean hydrodynamic diameter about 294 nm) caused significant accumulation of the particles in immune organs, spleen and thymus [26]. C) macrophage infiltration and nanoparticle aggregation with corresponding Raman spectra shows spleen histopathology of female mice following subchronic (90 days) intragastric administration of TiO_2 nanoparticles [26]. Figure is included with permission [25,26].

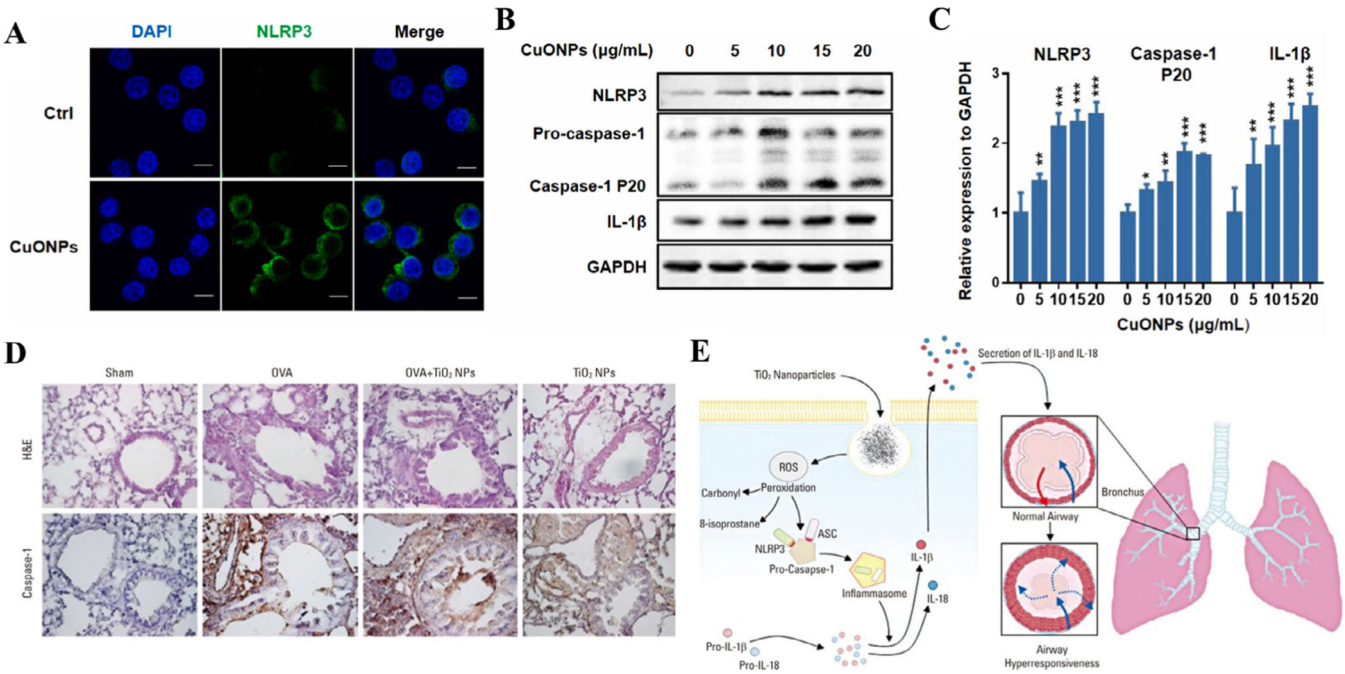

Fig. 3.

Inorganic nanoparticles activate inflammasome *in vitro* and *in vivo*. Copper oxide nanoparticles (50 nm) significantly increased the expression of NLRP3 protein in J774A.1 as immunofluorescence staining showed (A) [63]. The western blot assay also confirmed increases in the level of IL-1β, caspase-1 p20, and NLRP3 in the same cells (B and C) [63]. Caspase-1 expression was also significantly increased in the lung tissue of female Balb/c mice that received titanium dioxide nanoparticles and ovalbumin plus TiO_2 nanoparticles compared with saline-treated mice. Schematic shows the possible pathway of inflammasome activation upon intraperitoneal injection of titanium dioxide nanoparticles [65]. Figure is reproduced with permission [63,65].

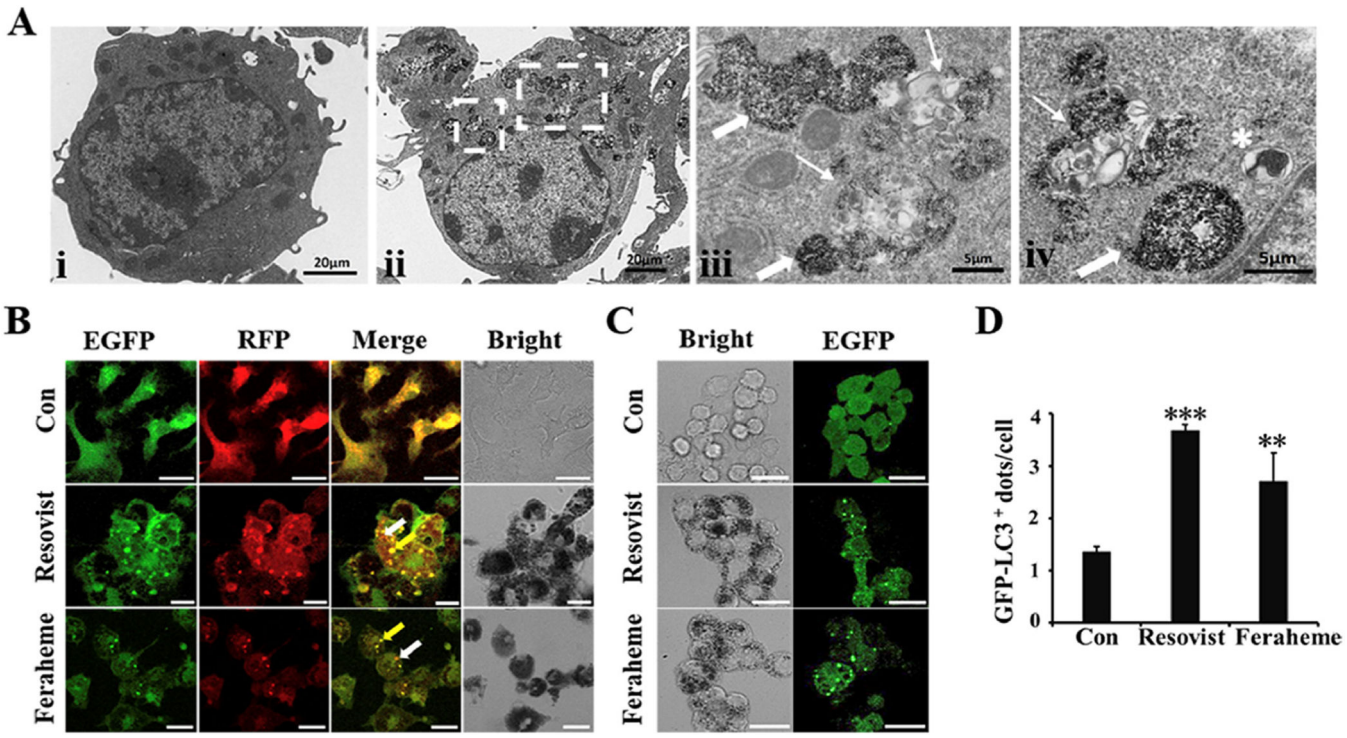

Fig. 4.

Autophagy induced in Raw 264.7 and BMDM macrophages after treatment with analysis of SPIONs [76]. A) TEM represents endosomes (thick arrows), early autophagic vacuoles(asterisk), and autolysosomes (thin arrows) containing SPIONs in Raw 264.7 macrophages. B, C) Confocal laser scanning microscopy represents colocalization of SPIONs in BMDMs infected with mRFP-GFP-LC3 adenovirus. A significant increase was shown in the number of GFP-LC3^{+} puncta in Raw264.7 cells treated with Feraheme and Resovist (D) [76]. Figure is reproduced with permission [76].

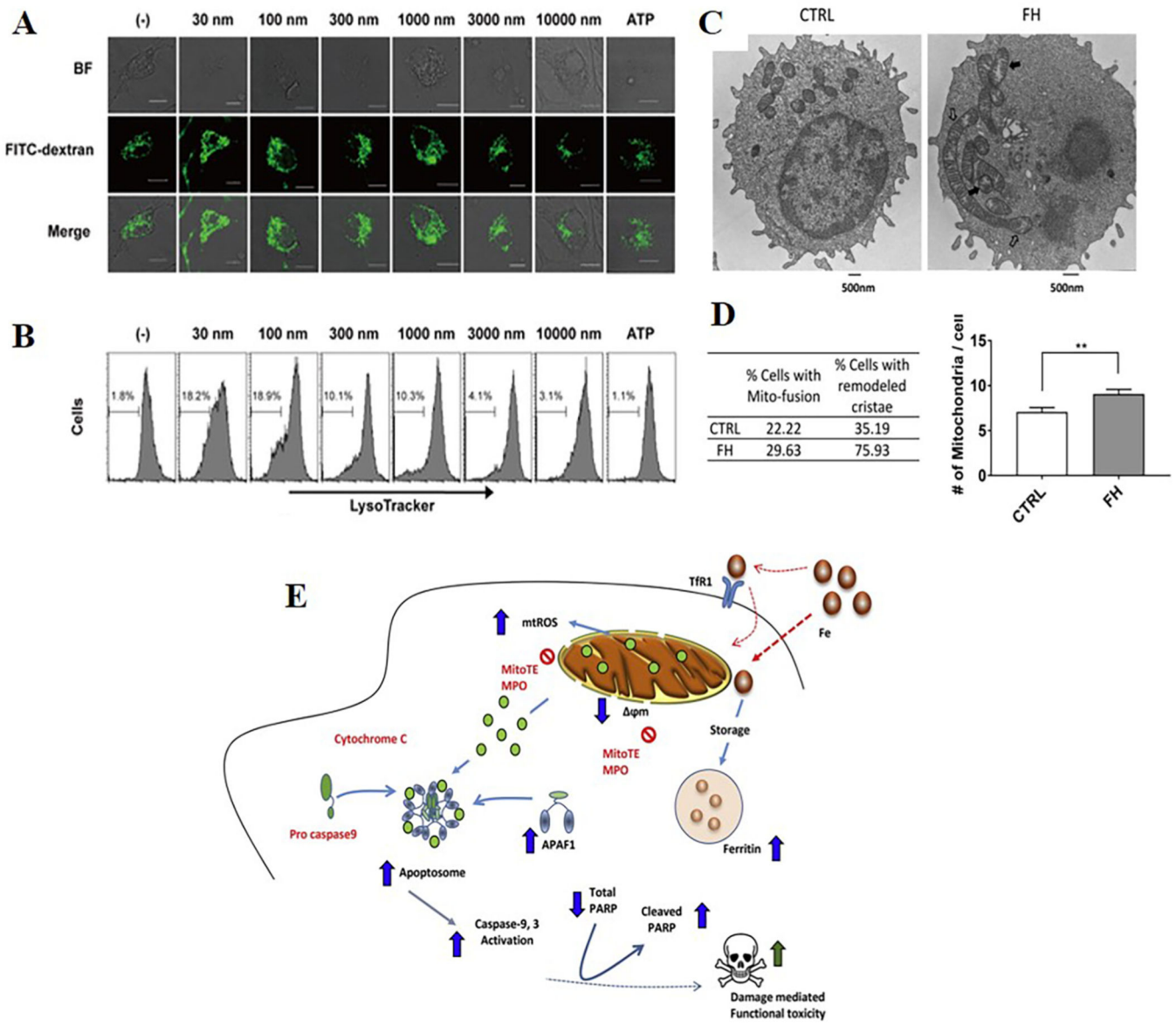

Fig. 5.

Organelle dysfunction by silica nanoparticles and iron oxide-based nanoparticles. A, B) BMDMs exposed to 30 nm–3000 nm silica particles, caused swollen lysosomes and damage as shown by confocal microscopy analysis and LysoTracker navigation [61]. T lymphocytes treated with Feraheme showed mitochondria with remodeled cristae (black arrows) fused mitochondria (open arrows) (C), and increased the average number of mitochondria per cell (D) [48]. E) Schematic represents the immunosuppression mechanism of Feraheme via increase in the iron-storage protein ferritin and subsequently ROS which caused mitochondria damage. This led to apoptosome formation and activation of caspase-mediated cascades of signals, caused toxicity along with suppression of cytokine and proliferative responses [48]. Figure is reproduced with permission [48,61].

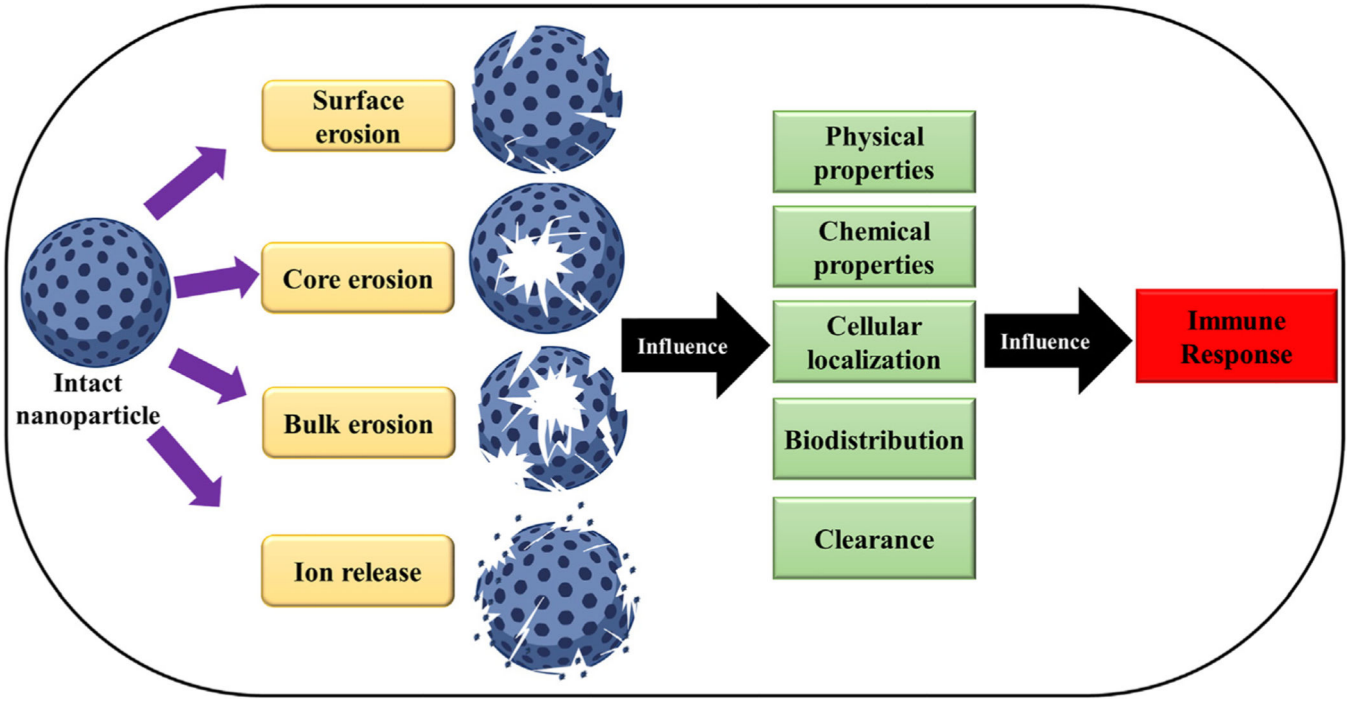

Fig. 6.

Schematic of degradation and dissolution patterns of inorganic nanoparticles. Degradation of inorganic nanoparticles can happen through surface erosion, core erosion, bulk erosion, and ion release. The kinetics of nanoparticle degradation can influence their physicochemical properties, the *in vitro* and *in vivo* localization, and clearance. These parameters can determine the immune response to inorganic nanoparticles.

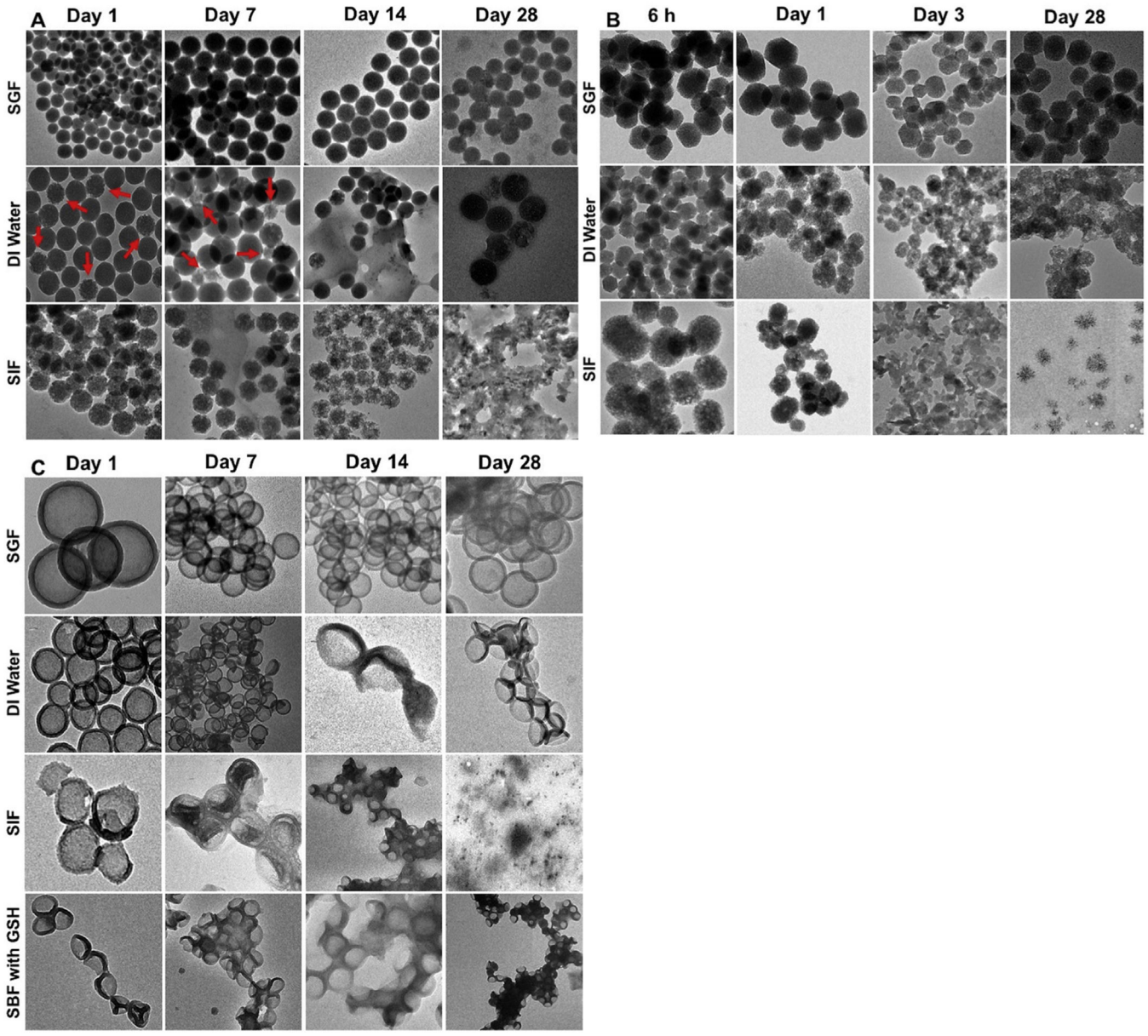

Fig. 7.

Degradation and dissolution pattern of silica nanoparticles (SNPs) with various physicochemical properties (dense Stöber (A), mesoporous (B), and hollow (C) SNPs; and similar size of approximately 100 nm in dimeter) in simulated gastric, intestinal, and body fluid compared to DI water, over 28 days [170]. TEM images represent surface erosion of Stöber SNPs and surface and bulk erosion of mesoporous SNPs over time. Hollow SNPs ruptured into small fragments. The fabricated SNPs degraded faster in simulated intestinal fluid irrespective of their physicochemical characteristic. Reprinted with permission from reference [170].

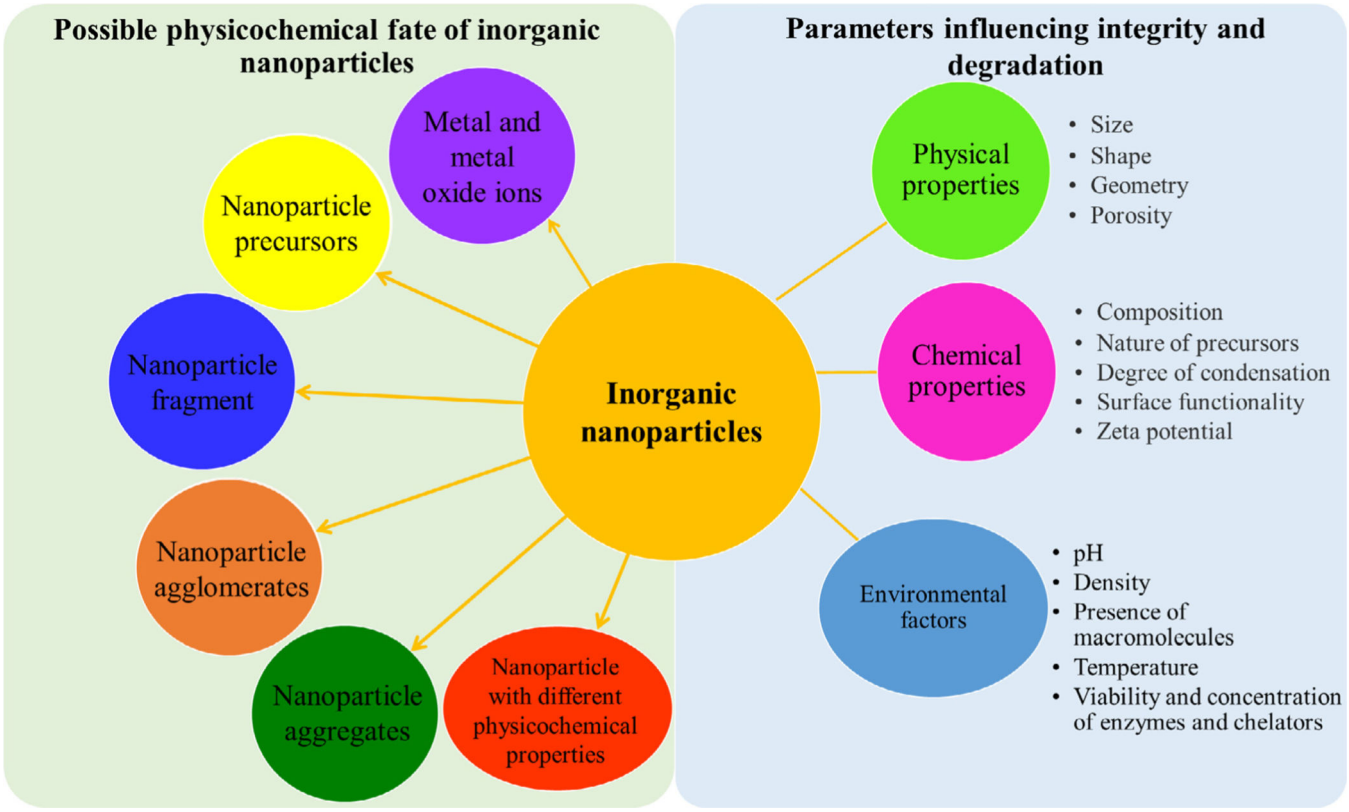

Fig. 8.

The possible physicochemical fate of inorganic nanoparticles and parameters influencing their integrity and degradation. Inorganic nanoparticles can degrade into fragments, precursors, or metal and metal ions. They might aggregate or agglomerate in the environment. These changes can change their physicochemical properties. Various physical and chemical properties as well as environmental factors influence their integrity and degradation outcome.