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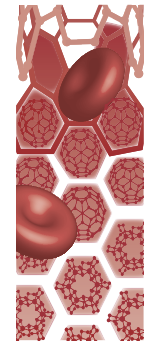

The toxicity of silica nanoparticles to the immune system

Liangjiao Chen[1], Jia Liu[2], Yanli Zhang[2], Guilan Zhang[2], Yiyuan Kang[2], Aijie Chen[2], Xiaoli Feng[2] & Longquan Shao*[,2]

[1]Key Laboratory of Oral Medicine, Guangzhou Institute of Oral Disease, Stomatology Hospital of Guangzhou Medical University, Guangzhou 510140, PR China

[2]Nanfang Hospital, Southern Medical University, Guangzhou 510515, PR China

*Author for correspondence: shaolongquan@smu.edu.cn

Silicon-based materials and their oxides are widely used in drug delivery, dietary supplements, implants and dental fillers. Silica nanoparticles (SiNPs) interact with immunocompetent cells and induce immunotoxicity. However, the toxic effects of SiNPs on the immune system have been inadequately reviewed. The toxicity of SiNPs to the immune system depends on their physicochemical properties and the cell type. Assessments of immunotoxicity include determining cell dysfunctions, cytotoxicity and genotoxicity. This review focuses on the immunotoxicity of SiNPs and investigates the underlying mechanisms. The main mechanisms were proinflammatory responses, oxidative stress and autophagy. Considering the toxicity of SiNPs, surface and shape modifications may mitigate the toxic effects of SiNPs, providing a new way to produce these nanomaterials with less toxic impaction.

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The immune system, and especially the innate immune system, are where innate effector cells and humoral factors provide the first line of defense against foreign microbes and particulate materials. Nanomaterials (NMs) can interact with immunocompetent cells and induce immunotoxicity. Interactions of nanoparticles (NPs) with the immune system have different outcomes that mostly depend on the characteristics of the NPs [1,2]. The effector cells that are mainly involved in this reaction are monocytes/macrophages, peripheral blood monocytes and polymorphonuclear leukocytes. NPs also interact with and become internalized by dendritic cells (DCs; the key antigen-presenting cells (APCs) of the immune system), lymphocytes, mastocytes, and so forth. The toxicity of NMs to immune cells includes their ability to cause direct cell damage such as apoptosis and necrosis. The function of immune cells changes after interactions with NMs, and the immune-specific signaling pathways are influenced. These features are measured by evaluating cell functions, proinflammatory responses, reactive oxygen species (ROS) generation and so on [3,4]. Evaluating the interaction of NMs and cells is critical for the safety consideration. Silica NPs (SiNPs) are widely utilized in biomedical applications due to their unique chemical and physical properties [5,6]. The safety of SiNPs is becoming a concern of an increasing number of people [7]. The influence of SiNPs on basic biology, medicine and agro-nanoproducts was recently reviewed [8]. Epidemiological studies have drawn inconsistent conclusions regarding amorphous silica toxicity [9]. SiNPs interact with the immune system in many ways. The physicochemical characteristics, such as particle size, shape, composition and crystallinity, affect toxicity towards immune cells and organs. However, the toxic effects of SiNPs on the immune system have rarely been reviewed [10]. Assessments of immunotoxicity include the evaluation of cell dysfunction, cytotoxicity, genotoxicity and the underlying mechanisms. This review focuses on these points.

Silica nanoparticles

Silicon, or Si, is one of the chemical elements found on Earth's crust. Its oxide forms are silicate (SiO_4) and silica (silicon dioxide, SiO_2). Si is often used in industry, while its oxide forms are often utilized for biomedical

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applications. SiNPs possess a variety of rare properties such as being easily synthesized, having a modifiable surface, having robust mechanical properties and possessing a relatively inert chemical composition. They have been used as biomaterials for decades. There are two basic forms of silica: crystalline and amorphous. Both forms have the same molecular formula [11], but their structural arrangements are different [12]. Crystalline silica lattices are arranged regularly, while amorphous silica lattices are arranged irregularly. Crystalline silica has multiple forms. A well-known form is α-quartz, which can be transformed into β-quartz, tridymite and cristobalite by heating. A porous crystalline silica called porosil also exists. All porosils are synthetic products. The terms nanoporous, mesoporous and microporous are used based on the diameters of the pores. 'Nanoporous' refers to materials with pore diameters smaller than 2 nm, 'microporous' refers to materials with pore diameters greater than 100 nm and 'mesoporous' refers to materials with pore diameters between 2 and 100 nm [13]. Mesoporous silicon and silica particles are ideal candidates for the controlled release of drugs, because of their rare properties such as high surface areas, large pore volumes, tunable pore sizes and good chemical and thermal stability [14]. Amorphous silica can be classified into three groups: a natural form, a by-product of power stations and metallurgical processing and a synthetically created form. Amorphous silica is considered a very promising candidate for gene carriers and molecular imaging, mostly because of its highly tunable biocompatibility and stability [15,16]. In addition, it has also been used in dietary supplements [17], catheters, implants [18] and dental fillers [19]. By 2013, millions of tons of SiNPs had already been placed on the worldwide market, and SiNPs became one of the three most produced NMs [20]. Approximately a fifth of nano-based products listed in a consumer product inventory claim to contain SiNPs [21]. The risk of human exposure to SiNPs at workplaces was increased [22]. Such growing potential for exposure raises concern regarding the toxicity and adverse health effects of SiNPs [23]. The health effects associated with silica exposure, especially crystalline silica, have been widely studied. Studies have shown that occupational crystalline silica exposure induces silicosis (a fibrotic lung disease) in workers and that this exposure is associated with other lung diseases, such as lung cancer, emphysema and pulmonary tuberculosis [24]. Amorphous silica was previously considered less harmful than crystalline silica. However, recent studies have showed that amorphous SiNPs have a similar potential toxicity as crystalline particles [25]. Physicochemical properties of SiNPs other than crystallinity cause different toxic effects in *in vitro* and *in vivo* studies. This subject was comprehensively reviewed by Napierska *et al.* [26]. Safety and potential adverse effects, especially those affecting the immune system, should be considered.

Toxicity to immune cells

NP characteristics influence NP uptake by a plethora of immune cells and the subsequent immune responses. Immune cells play an important role in host exposure to NMs. Phagocytes, particularly macrophages, are the 'first responder' cells, and other cell lines such as DCs and T-lymphocytes engage particles immediately. They uptake and process NPs, but cell functions change after exposure. Host biological responses are mediated by activated inflammatory signaling pathways, reactive oxygen species (ROS), and so forth.

Monocytes/macrophages

Changes to cell function

The activity and abilities of macrophages are influenced by SiNP treatment. Phagocytic monocytes/macrophages effectively capture SiNPs [27] and are mostly localized in vesicles and/or in phagolysosomes [28]. Macrophages are first metabolically reprogrammed after exposure: their glycolytic activity is increased, tricarboxylic acid cycle is altered and ATP generation is reduced. These changes are consistent with a proinflammatory response. Amino acids (possibly arising from autophagy), the creatine kinase/phosphocreatine system and a few osmolytes and antioxidants emerge as important players in the metabolic reprogramming of macrophages exposed to silica [29,30]. Second, the activity of macrophages is influenced by SiNPs. SiNPs influence activity-related gene expression. The phagocytosis ability of RAW 264.7 was also reduced, decreasing as much as 50% after exposure to 10-nm SiNPs, while larger SiNPs caused this ability to be reduced by a lesser degree. However, SiNPs influenced macrophage phagocytosis without altering surface markers and cytokine production *in vitro* [31]. Amorphous SiNP exposure causes sensitivity to DNA-alkylating agents, such as styrene oxide, even at doses that do not result in appreciable cell death [32]. They also promote monocyte adhesion to human endothelial cells in a size-dependent manner [33].

The phenotypes of macrophages were also changed by SiNP exposure. The state of macrophage polarization influences the steady state of the immune system and plays an important role in the processes of many diseases. Resting macrophages (M0) polarize into different phenotypes (proinflammatory [M1] or anti-inflammatory [M2]) under different physiological or pathological conditions. Different phenotypes perform different roles in local

microenvironments. In addition, the polarized macrophages can also reverse their phenotype after exposure to a changed milieu. Macrophages are targeted by NPs after exposure. NPs can polarize and reprogram macrophages, but their immunological function has already been affected, furtherly affecting the pathological process of disease [34]. NPs can differentially modulate macrophage polarization and reprogramming based on various physicochemical features such as chemical composition, size and surface modifications. SiNPs stimulate *IL-1* and *TNF-α* (inflammatory cytokines) expression after macrophage polarization toward the M1 phenotype [35]. The same NPs can drive macrophage polarization in different directions depending on exposure times or doses used for stimulation. For instance, α-quartz delays the occurrence of inhalation toxicity. α-Quartz activates M0 macrophages toward the M2 phenotype at an early stage. With continued exposure, particles interact with the M2 phenotype when M2 macrophages cannot accommodate more particles. M1 macrophages express various inflammatory cytokines, such as *IL-1* and *TNF-α*, resulting in granuloma formation [36]. The results depend on different exposure/incubation times [37]. Furthermore, different macrophage activation statuses also determine the internalization of particles [38]. This effect on macrophage polarization is not always toxic to cells; this effect means that SiNPs can be used as immune-regulating materials. In bone regeneration, the proper immune environment regulated by macrophage statues is needed. SiNPs may be used for drug delivery or as a carrier for tissue regeneration to adjust the proper immune environment.

Cell damage to monocytes/macrophages

The toxicity of SiNPs to monocytes/macrophages is dependent on the physicochemical properties of particles [39]. Numerous recent studies have shown that size impacts toxicity [40]. The uptake of SiNPs into RAW 264.7 macrophages is highly dependent on size rather than porosity or amount of adsorbed proteins [41]. Amorphous SiNPs have a higher cytotoxicity to macrophages than submicron-sized particles [42]. The toxicity of nano- and micron-sized silica particles (14 nm and 1–5 μm, respectively) was compared. Apoptosis was observed only upon exposure to NPs. The decrease in cell viability was based on particle size and occurred in a dose-dependent manner [43].

The cytotoxicity of SiNPs against RAW 264.7 cells is dose dependent, in other words, SiNPs are nontoxic at low dosages and cell viability decreases at high dosages [44]. An outstanding dose-dependent cytotoxicity was observed after exposure to SiNPs. Concentrations greater than 200 μg/ml are large enough to induce cytotoxic and genotoxic effects in RAW 264.7 cells [45]. The biological reactions to silicon NPs (3 nm in diameter) and silicon microparticles (100–3000 nm in diameter) in RAW 264.7 cells are also assayed in terms of cytotoxicity/cell viability and inflammatory responses. SiNPs at concentrations less than or equal to 20 mg/ml exhibit no cytotoxicity or inflammatory responses; however, silicon NPs and silicon microparticles at concentrations of more than 20 and 200 mg/ml, respectively, have greater cytotoxicity than controls [46]. The presence of positively charged, large mesoporous SiNPs (MSNs, ≥100 nm diameters) at high treatment doses (≥500 μg/ml) results in an accumulation of internalized MSNs in cells that is sufficient to induce a significant release of ROS and oxidative stress, causing inflammatory gene upregulation. The cytotoxicity of MSNs is thus correlated with the number of MSNs taken up by cells, which is positively dependent on the particle size and dosage [47].

Furthermore, other physicochemical properties play different roles in toxicity [48]. The cytotoxicity of SiNPs with different surface topologies was investigated. The use of different synthetic routes results in different biological effects on cells. Colloidal and Stöber silica, which are amorphous SiNPs, exert toxicity via mechanisms like those of crystalline silica. Certain physicochemical properties such as surface area and microporosity promote cytotoxicity [49]. For example, cytotoxic activity against J774 macrophages increased with external surface area and decreased with increasing micropore volume [50]. Aggregation of particles also plays important roles in the cytotoxicity of amorphous SiNPs [51].

Except for the physicochemical properties mentioned above, the different zeta potentials (cationic, anionic or neutral) may have different toxic effects on immune cells. However, there is no detailed information on this point. More researches should focus on this area.

Many cell lines in the immune system take up silica particles, and macrophages might show extreme sensitivity [52]. Differential cytotoxic and inflammatory potency of SiNPs of similar sizes was observed in different cell lines [53]. Published results on the toxicity of amorphous silica against different cells and tissues conflict [48,54]. Macrophages are sensitive to amorphous and crystalline silica particles after phagocytosis, and a small number of internalized particles kill cells [52]. Different macrophage phenotypes produce significant differences in toxicity. M1 macrophages

take up many particles, while M2 cells take up fewer particles. SiNPs can be highly toxic to M1 cells in both *in vitro* and *in vivo* studies [55].

Genotoxic effects on monocytes/macrophages

The genotoxic effects of SiNPs are controversial. Several studies have investigated the genotoxic effects of SiNPs [56]. The cytotoxicity and genotoxicity of SiNPs are well correlated [45]. When the cell membrane is damaged, DNA damage and apoptosis occurs [57]. SiNPs induce more DNA damage than microscale SiO_2 particles in RAW 264.7 cells [58], which is manifested as broken DNA strands and changes in chromosomes [59]. Dose uptake by cells affects the genotoxicity. SiNPs (15 nm) slightly increase DNA damage after rats are intravenously exposed to their maximum tolerated dose. The DNA damage was measured by *in vivo* comet and micronucleus assays. Both colloidal and amorphous SiNPs induce genotoxic effects in lung cells *in vitro* at comparatively high doses [60]. A small amount of DNA damage is caused by SiNPs and secondary to the inflammation/immune response [61]. However, SiNPs also demonstrate no genotoxicity in both *in vitro* and *in vivo* systems. No large increase in DNA damage was observed in synthetic amorphous silica NM-(SAS-NM) exposed animals, although SAS NMs cause hepatotoxicity, thrombocytopenia and even death to animals [62]. The controversial results are due to variations in evaluation methods and the different size and physicochemical properties of the particles used in studies. In general, food-graded SiNPs may be safer than common-graded SiNPs.

Changes to tissue-resident macrophages

Kupffer cells

Kupffer cells are phagocytic cells located on the surface of the liver sinus. Kupffer cells play a vital role in the liver inflammation induced by SiNPs [63]. SiNPs might be distributed in Kupffer cells after intravenous exposure [64]. Kupffer cells are activated by SiNPs, which increases the expression of *TNF-α* and the release of nitric oxide (NO) and ROS [65]. The P2X7 receptor plays an important role in the exposure of Kupffer cells to SiNPs. The P2X7 receptor releases ATP, which induces the formation of ROS via NADPH oxidase. The ROS activate inflammasomes, leading to caspase-1-dependent processing of *IL-1β* [66].

Microglia

Microglia are a type of glial cell that act as a macrophage in the brain and spinal cord, and are the first and most important line of defense in the CNS. SiNPs are taken up by microglial cells in a time- and particle-dependent manners. The maximal uptake is reached after 4 h, and NPs are found in the endoplasmic reticulum (ER) and in lysosomes [67]. Very low levels of SiNPs can alter microglial function [68]. Microglial cells release proinflammatory mediators after being activated by SiNPs, which results in cell dysfunction and cytotoxicity [69].

Dendritic cells

Changes to cell functions

The antigen presentation of DCs is a central orchestrator of the adaptive immune response. SiNPs enhance antigen-specific cellular immune responses and change cell functions. Langerhans cells are a type of immature DC. Langerhans cells mature into a professional APC after capturing and processing exogenous toxins. DCs sense SiNPs after exposure. The effect on DC function is size and concentration dependent. 70 and 100 nm-sized SiNPs enhance exogenous antigen entry into the cytosol from endosomes and induce cross-presentation, whereas submicron-sized silica particles (>100 nm) do not [70]. The cytotoxicity against Langerhans cells is size-dependent. SiNPs are taken up by Langerhans cells after they penetrate the stratum corneum of mouse skin. SiNPs become more cytotoxic against Langerhans cells with decreasing particle size [71]. Furthermore, SiNPs (70 nm) induce a higher level of ROS generation than micron-sized particles [72]. SiNPs and crystalline silica upregulate MHC-II, CD80 and CD86 levels on DCs. Furthermore, these particles stimulate the inflammasome, clearly resulting in *IL-1β* secretion in wild-type (WT) but not caspase-1- or NLRP3-deficient mice. In addition, activation of *p38 and NF-κB* may be critical for the proinflammatory effect of SiNPs on DCs [73]. Size- and concentration-dependent effects were seen in viability, uptake and immune regulatory markers. Small particles and low concentrations affected human monocyte-derived DCs (MDDCs) to a lesser degree than large particles and high concentrations [74].

Cell damage in & genotoxic effects on DCs

SiNPs have size- and concentration-dependent cytotoxic effects on DCs. SiNPs have different effects on DCs with different statuses. The exposure of DCs to ultrafine SiNPs decreased cell viability and induced cell death in size- and concentration-dependent manners [75]. For example, small particles and low concentrations affected the viability, uptake and immune regulatory markers of MDDCs to a smaller degree than large particles and high concentrations [74]. Immature and unprimed DCs that interact with food-grade SAS particles fail to experience cytotoxicity and do not release *IL-1α* or *TNF-α*. However, SAS particles activate completely immature DCs by causing maturation markers to display on the cell surface. In steady-state DCs, SAS particles directly activate endosomal MyD88-dependent pathogen patterns and the signaling pathway [76]. Although SiNPs are widely used, data on their potential genotoxicity to DCs are limited. No studies have focused on the genotoxicity of SiNPs against Langerhans cells.

Lymphocytes: T & B lymphocytes & natural killer cells

Changes to cell functions

Lymphocytes are the smallest kind of white blood cell, are produced by the lymphoid organs and are an important cellular component of the immune response function of the body. Lymphocytes exhibit the identification function of the immune cell lines and can be divided into T cells, B lymphocytes (B cells) and natural killer (NK) cells. The viability and proliferative activity of lymphocytes are dependent on particle size, concentration, charge and other physicochemical characteristics. The presence of 100 μg/ml SiNPs produces an outstanding cytotoxic effect. SiNPs cause the death of all phytohemagglutinin-activated lymphocytes, while doses of 1 and 10 μg/ml have no effect on cell proliferation [77]. Apoptosis and postapoptotic necrosis are the main results of SiNP exposure, which directly decreases cellular viability and results in cell membrane damage [78].

Furthermore, alterations in the levels of antioxidants cause DNA injury and chromosomal aberration in human lymphocytes [79]. Specifically, SiNPs with negative charge reduce the levels of antioxidants more significantly than other SiNPs. Negatively charged SiNPs show the most potent *in vivo* immunotoxicity. They inhibit lymphocyte proliferation, suppress the killing activity of NK cells and decrease proinflammatory cytokine and NO production [80].

Genotoxic effects on lymphocytes

The genotoxic effect on lymphocytes is like macrophages. Genotoxic effects are controversial, mostly due to the use of NPs with different particle sizes and physicochemical properties. SiNPs were claimed to be genotoxic to the DNA of lymphocytes. SiNPs cause DNA fragmentation in lymphocytes in a dose- and size-dependent manners [78,81]. The genotoxicity of SAS in human lymphocytes was evaluated. SAS was unable to induce micronuclei and has no obvious genotoxicity [82].

Other immune function cells: mast cells & endothelial cells

Mast cells

Mastocytes are involved in adjusting the immune response against exogenous toxins and cause proinflammatory effects. The impact of SiNPs on mast cells and their function has been studied. NPs are located primarily on secretory granules after their internalization by mast cells. Nonporous and porous SiNPs decrease the number of molecules released per granule, with nonporous SiO_2 also inducing a decrease in amperometric spike frequency and therefore having a large impact on cell function [83]. SiNPs activate mouse bone marrow-derived mast cells to produce ROS and inflammatory mediators [84]. No studies have focused on the genotoxicity of SiNPs to mast cells. Deeper research on the toxicity of mast cells is needed.

Endothelial cells

Changes to cell function

Endothelial cells form a class of nonprofessional APCs. Endothelial dysfunction precedes cardiovascular disease and is often accompanied by mitochondrial impairment and dysfunction. Endothelial cells take up SiNPs in a dose-dependent manner. SiNPs increase the intracellular level of mitochondrial ROS, eventually resulting in the collapse of the mitochondrial membrane potential; impairments in ATP synthesis, cellular respiration and the activities of three ATP-dependent enzymes (Na^+/K^+-ATPases, Ca^{2+}-ATPases and Ca^{2+}/Mg^{2+}-ATPases); and an

elevated intracellular calcium level. Furthermore, mitochondria in SiNPs-treated human umbilical vein endothelial cells (HUVECs) display a fission phenotype. Accordingly, dysregulation of the expression of key genes (*FIS1, DRP1, OPA1, Mfn1 and Mfn2*) involved in fission/fusion events further certifies the SiNP-induced perturbation of mitochondrial dynamics [85].

SiNPs, therefore, trigger endothelial toxicity by targeting mitochondria, inducing mitochondrial dysfunction and perturbing their dynamics and biogenesis [86]. Moreover, SiNPs disturb the balance of the NO/nitric oxide synthase (NOS) system, inducing proinflammatory effects and eventually leading to endothelial dysfunction via the PI3K/protein kinase B (Akt)/mTOR (PI3K/Akt/mTOR) pathway [87]. In another study, autophagic activity was induced in endothelial cells. SiNPs disturbing endothelial cell homeostasis, subsequently impairing angiogenesis. *'"he VEGFR2-mediated autophagy pathway may take a significant role in maintaining endothelial and vascular homeostasis"* [88]. SiNPs cause systemic inflammation *in vivo*, impair vascular homeostasis and alter vascular reactivity [89].

Cell damage to & genotoxic effects on endothelial cells

Particle size, surface area and microporosity influence the cytotoxic activity of SiNPs [51]. SiNPs cause cytotoxic damage to and decrease the cell survival of endothelial cells in a dose-related manner. The surface area of the tested particles is also an important parameter in determining the toxicity of SiNPs [90]. SiNPs significantly elicit apoptosis in HUVECs, and the apoptotic rates were both dose- and size-dependent. The p53-caspase pathway is the main mechanism of SiNPs-mediated apoptosis in HUVECs [91]. Many studies have focused on the mechanism of the cytotoxicity of SiNPs. In primary human endothelial cells, SiNPs work through exocytosis of the von Willebrand factor and necrotic cell death [92]. A DNA damage response via the Chk1-dependent G2/M checkpoint signaling pathway may be the underlying mechanism of the toxic effect on endothelial cells [93]. Although the cytotoxicity of SiNPs to endothelial cells has been the subject of several studies, data on their cytotoxicity and genotoxicity are limited. The toxicity of SiNPs on immune cells was listed in Table 1.

Immunotoxicity to tissues & organs

In vivo data may be not consistent with *in vitro* data. The *in vivo* immunotoxicity of SiNPs has been investigated in many studies. ROS generation, cytokine expression, hematology profiles and other traits are evaluated as *in vivo* immunotoxicity. The size-dependent toxicity of silica has been observed, and other physicochemical properties and the administration route influenced the toxicokinetics [94]. After being absorbed, SiNPs translocate to tissues and organs through several routes, including intravenous injection, pulmonary inhalation, skin contact and gastrointestinal routes. Liver, lung, spleen and heart are the target organs of SiNPs. However, direct research on toxic effects on immune organs and influence on immune functions are lacking. The direct research may provide more useful information for immunotoxicity evaluation.

Liver

SiNPs cross different biological barriers into the liver by intramuscular and hypodermic injection, but the absorption rate is very low. When orally administered, SiNPs are absorbed into the intestinal tract and are then located in the liver. SiNPs are administered by intravenous injection, mainly persisting in the liver [95]. SiNPs are mainly distributed in the resident macrophages (Kupffer cells) of the liver after being intravenously administered, and induce macrophage proliferation in the liver. Lymphocytic infiltration, granuloma formation and hydropic degeneration in liver hepatocytes were observed by histological examination [96]. After intravenous exposure, mast cell recruitment in the liver occurs before fibrosis [97]. Furthermore, SiNPs increase the release of lipid peroxides and reduce antioxidant enzyme activities in the liver after repeated intravenous exposure [98]. SiNPs are absorbed into liver macrophages and persist there for 4 weeks after a single injection [99]. However, repeated intravenous dosing of SiNPs at subtoxic levels (nontoxic at single acute or repeated dosing levels) does not saturate bioaccumulation in liver or spleen macrophages [100]. No toxicity is found in brain, liver, lung, spleen, heart, kidneys, intestine, eyeballs, ovaries and testes after oral and topical ocular applications of nonporous SiNPs in Sprague–Dawley rats [101].

Spleen

SiNPs are mainly distributed in spleen and induce macrophage proliferation, which results in megakaryocyte hyperplasia in the spleen after intravenous administration [96]. SiNPs that are trapped by macrophages in the spleen remain there for 4 weeks after a single injection [99]. Colloidal SiNPs of different sizes and charges were administered

Table 1. Summarizing the studies of SiNP toxicity.

Cell type	Cell line	Types of SiNP	Particle primary size	End point	Assay methods	Results	Study (year), Ref.
Monocytes/ macrophages	RAW 264.7	SiNPs	850, 500, 250 and 150 nm	Cell damage, inflammation oxidative stress	LDH release TNF-α ROS production	Size-dependent increase	Leclerc *et al.* (2012), [40]
	RAW 264.7	SiNPs	12 nm	Cytotoxicity, genotoxicity	WST-8 cell viability, Hoechst/PI apoptosis assay Micronucleus analysis, Comet assay	Dose-dependent increase	Hashimoto and Imazato (2015), [45]
	RAW 264.7	SiNPs	3 nm	Cytotoxicity, NO production, Inflammatory responses	MTT assays ELISA	Size- and dose-dependent increase	Choi *et al.* (2009), [46]
	Human monocyte-derived macrophages	MSNs	50, 100 and 250 nm with a positive surface charge and 100 nm with a negative surface charge	Cytotoxicity oxidative stress, inflammatory gene	Cellular-/animal-level end point tests Gene expression analysis	Charge- and size-dependent	Chou *et al.* (2017), [47]
	THP-1	SiNPs	Twelve different types of SNPs with varying size, surface topology (porous vs nonporous)	Cytotoxicity oxidative stress	MTT assays DEFH-DA	Porous/negative decrease cell viability, no oxidative stress	Kettiger *et al.* (2015), [49]
	J774 macrophages	SiNPs	2–335 nm surface area BET, 16–422 m^2/g, micropore volume, 0–71 μl/g	Cytotoxicity	WST1 assay	Increased with external surface area, decreased with micropore volume	Rabolli *et al.* (2010), [50]
	RAW 264.7	SiNPs	31.25, 125 and 500 mg/ml SiNPs Positive control: 500 mg/ml microscale SiO_2	Cytotoxicity DNA injury	MTT, SCGE and flow cytometry	Dose-related decline, DNA injury	Yang *et al.* (2016), [58]
	THP-1	S-SiNPs	2, 16, 60 and 104 nm	Cytotoxicity	LDH assay	Size-dependent increase	Napierska *et al.* (2009), [90]
	THP-1 and A549 coculture			Proinflammatory response	Cytometric bead array	Increase of cytokines in presence of 2 and 60 nm (except TNF-α)	
	U-937 cells	SiNPs	15 nm	Proinflammatory response	ELISA	M1 polarization IL-1β and TNF-α	Lucarelli *et al.* (2015), [35]
	PBMC	SiNPs	10 and 100 nm	Proinflammatory response	Multiplex bead array	Size and dose-dependent increase in cytokines	Mendoza *et al.* (2014), [115]
				Oxidative stress	GSH depletion	Size- and dose-dependent increase	
				Oxidative stress	WB	Dose-dependent increase in proteins with free radicals only with 10 nm	

AU: Comet assay; BET: Specific surface area; BMDC: Bone marrow-derived dendritic cell; C-SiNP: Crystalline silica nanoparticle; DC: Dendritic cell; DCFH-DA: 2′,7′-dichlorodihydrofluoresceindiacetate; DEFH-DA: 2-chloro-2-fluoresceinacetoacetate; FAC: Flow cytometry; GSH: Glutathione; HUVEC: Human umbilical vein endothelial cell; KC: Kupffer cells; LDH: Lactate dehydrogenase; LPO: Lipid peroxidation; LSCM: Laser scanning confocal microscope; MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; MDc: Dansylcadaverine; M-SiNP: Mesoporous silica nanosphere; MSN: Mesoporous silica nanoparticle; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO: Nitric oxide; NOS: Nitric oxide synthase; NP: Nanoparticle; PBMC: Peripheral blood mononuclear cell; PI: Propidium iodide; Pr-SiNP (NM 200 & 201): Precipitated silica; Py-SiNP (NM 202 & 203): Pyrogenic silica; ROS: Reactive oxygen species; RT: Reverse transcription; SCE: Sister chromatid exchange; SCGE: Single cell gel electrophoresis assay; SEM: Scanning electron microscopy; SiNP: Silica nanoparticle; SOD: Superoxide dismutase; S-SiNP: Amorphous silica nanoparticle; TEM: Transmission electron microscopy; WB: Western blot.

Table 1. Summarizing the studies of SiNP toxicity (cont.).

Cell type	Cell line	Types of SiNP	Particle primary size	End point	Assay methods	Results	Study (year), Ref.
				Proinflammatory response	ELISA	Dose-dependent increase of TNF-α and decrease of IL-6 (only for bare SiNPs. IL-6 decrease for microparticles	
	RAW 264.7	S-SiNPs M-SiNPs	115 nm (with or without amine modification)	Cell viability	WST-8 assay	Dose-dependent reduction only in RAW 264.7. Amine-modified SiNPs were less toxic	Yu *et al.* (2011), [39]
	RAW 264.7	C-SiNPs	20 and 100 nm (uncoated and L-arginine coated)	Cell viability	WST-8 assay	Size- and charge-dependent reduction	Kim *et al.* (2014), [80]
	J774.1	C-SiNPs M-SiNPs	100 nm	Cell viability	MTT assay	Reduction only for C-SiNPs	Lee *et al.* (2011), [113]
				Apoptosis	Annexin V/PI staining	Caspase-3 activation	
				Proinflammatory response	RT-PCR and WB	TNF-α, IL-6 and IL-1β increase	
				Pathway analysis	RT-PCR and WB	Activation of MAPKs and NF-κB	
	RAW 264.7	SiNPs	12 nm	Cell viability	WST-8 assay	Dose-dependent reduction	Hashimoto and Imazato (2015), [45]
				Genotoxicity	Hoechst/PI staining	Deformation of nuclei at both concentrations	
				Genotoxicity	Comet assay	Dose-dependent increase	
				Genotoxicity	Micronuclei induction	Increase	
			Cell uptake	SEM and TEM	NPs detected in vesicles and nucleus		
DCs	DCs	S-SiNPs	70 and 100 nm	Cross-presentation	Cross-presentation assay	70 and 100 nm enhance cross-presentation >100 nm do not	Hirai *et al.* (2012), [70]
	Intestinal DCs	S-SiNPs C-SiNPs	-	Cell disfunction	FAC	Upregulation of MHC-II, CD80 and CD86	Winter *et al.* (2011), [73]
	Human monocyte-derived DCs	S-SiNPs	270 nm, 2.5 μm	Viability Immune regulatory markers	MTT TME FAC	Size- and concentration-dependent	Vallhov *et al.* (2007), [74]
	BMDC and murine DC line	Ultrafine SiNPs	<100 nm	Viability phenotypic changes Inflammatory response	Annexin V and 7-AAD staining Flow cytometry RT-PCR	Size- and concentration-dependent CD11c, CD54, CD80, CD86 and MHC class II change p38 and NF-κB activation	Kang and Lim (2012), [75]

AU: Comet assay; BET: Specific surface area; BMDC: Bone marrow-derived dendritic cell; C-SiNP: Crystalline silica nanoparticle; DC: Dendritic cell; DCFH-DA: 2′,7′-dichlorodihydrofluoresceindiacetate; DEFH-DA: 2-chloro-2-fluoresceinacetoacetate; FAC: Flow cytometry; GSH: Glutathione; HUVEC: Human umbilical vein endothelial cell; KC: Kupffer cells; LDH: Lactate dehydrogenase; LPO: Lipid peroxidation; LSCM: Laser scanning confocal microscope; MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; MDc: Dansylcadaverine; M-SiNP: Mesoporous silica nanosphere; MSN: Mesoporous silica nanoparticle; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO: Nitric oxide; NOS: Nitric oxide synthase; NP: Nanoparticle; PBMC: Peripheral blood mononuclear cell; PI: Propidium iodide; Pr-SiNP (NM 200 & 201): Precipitated silica; Py-SiNP (NM 202 & 203): Pyrogenic silica; ROS: Reactive oxygen species; RT: Reverse transcription; SCE: Sister chromatid exchange; SCGE: Single cell gel electrophoresis assay; SEM: Scanning electron microscopy; SiNP: Silica nanoparticle; SOD: Superoxide dismutase; S-SiNP: Amorphous silica nanoparticle; TEM: Transmission electron microscopy; WB: Western blot.

Table 1. Summarizing the studies of SiNP toxicity (cont.).

Cell type	Cell line	Types of SiNP	Particle primary size	End point	Assay methods	Results	Study (year), Ref.
Lymphocytes	Human peripheral lymphocytes	Pr-SiNPs Py-SiNPs	14–16 nm	Genotoxicity	Cytokinesis block micronuclei induction	No effect	Tavares *et al.* (2014), [82]
	Human peripheral lymphocytes	SiNPs	10–20 nm	Cell viability	MTT assay	Dose-dependent reduction	Rajiv *et al.* (2016), [79]
				Cytotoxicity	LDH assay	Dose-dependent increase	
				Oxidative stress	DCFH-DA assay	Dose-dependent increase	
				Oxidative stress	LPO assay	Dose-dependent increase of MDA formation	
				Oxidative stress	GSH depletion	Dose-dependent increase	
				Oxidative stress	SOD assay	Dose-dependent increase	
				Oxidative stress	Catalase assay	Dose-dependent increase	
				Genotoxicity	Comet assay	Increase	
				Chromosomal aberrations	Giesma staining and microscopy	No effect	
				NO production	Griess reagent	Dose-dependent increase of NO in KCs	
	Peripheral blood lymphocytes	SiNPs	6, 20, 50 nm	Genotoxicity	AU values	Increased the SCE frequency and DNA damage	Battal *et al.* (2015), [81]
Mast cell	Mast cell	Nonporous, mesoporous SiNPs	-	Cell uptake Cell function	TEM carbon-fiber microelectrode Amperometry measurements	Less uptake on nonporous SiO_2 Cell function decrease	Maurer-Jones *et al.* (2010), [83]
Langerhans cells	XS52 cells	S-SiNPs	70, 300 and 1000 nm	Cellular uptake cytotoxicity	TEM CCK-8	Size-dependent cytotoxic effects	Nabeshi *et al.* (2010), [71]
	XS52 cells	S-SiNPs	70 nm vs microsize	Oxidative stress	FAC ELISA	ROS generation in nm than microsize	Yoshida et al. (2012), [72]
Kupffer cells	Kupffer cells	S-SiNPs		Oxidative stress	ELISA colorimetric assay DCFH-DA	ROS generation TNF-α and NO expression	
Microglial cells	Rat brain macrophage-like cells	SiNPs	20 nm	Cell viability	MTT assay	No reduction	Xue *et al.* (2012), [69]
				Proinflammtory response	ELISA	Mild increase of cytokines such as IL-6, TNF-α and IL-1β	
				NO production	Griess reagent assay	No effect	
				Nuclear-binding activity	RT-PCR	No effect	
				Inflammatory factors	WB	No effect	

AU: Comet assay; BET: Specific surface area; BMDC: Bone marrow-derived dendritic cell; C-SiNP: Crystalline silica nanoparticle; DC: Dendritic cell; DCFH-DA: 2′,7′-dichlorodihydrofluoresceindiacetate; DEFH-DA: 2-chloro-2-fluoresceinacetoacetate; FAC: Flow cytometry; GSH: Glutathione; HUVEC: Human umbilical vein endothelial cell; KC: Kupffer cells; LDH: Lactate dehydrogenase; LPO: Lipid peroxidation; LSCM: Laser scanning confocal microscope; MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; MDc: Dansylcadaverine; M-SiNP: Mesoporous silica nanosphere; MSN: Mesoporous silica nanoparticle; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO: Nitric oxide; NOS: Nitric oxide synthase; NP: Nanoparticle; PBMC: Peripheral blood mononuclear cell; PI: Propidium iodide; Pr-SiNP (NM 200 & 201): Precipitated silica; Py-SiNP (NM 202 & 203): Pyrogenic silica; ROS: Reactive oxygen species; RT: Reverse transcription; SCE: Sister chromatid exchange; SCGE: Single cell gel electrophoresis assay; SEM: Scanning electron microscopy; SiNP: Silica nanoparticle; SOD: Superoxide dismutase; S-SiNP: Amorphous silica nanoparticle; TEM: Transmission electron microscopy; WB: Western blot.

Table 1. Summarizing the studies of SiNP toxicity (cont.).

Cell type	Cell line	Types of SiNP	Particle primary size	End point	Assay methods	Results	Study (year), Ref.
Endothelial cell	HUVECs line	SiNPs	–	Cellular uptake Autophagy Oxidative stress Proinflammation	LSCM TEM MDc staining ELISA WB	Dose-dependent in cell uptake Autophagic process NO/NOS system imbalance TNF-α, IL-1, IL-6 expression PI3K/Akt/mTOR pathway inhibited	Duan *et al.* (2014), [87]

AU: Comet assay; BET: Specific surface area; BMDC: Bone marrow-derived dendritic cell; C-SiNP: Crystalline silica nanoparticle; DC: Dendritic cell; DCFH-DA: 2′,7′-dichlorodihydrofluoresceindiacetate; DEFH-DA: 2-chloro-2-fluoresceinacetoacetate; FAC: Flow cytometry; GSH: Glutathione; HUVEC: Human umbilical vein endothelial cell; KC: Kupffer cells; LDH: Lactate dehydrogenase; LPO: Lipid peroxidation; LSCM: Laser scanning confocal microscope; MAPK: Mitogen-activated protein kinase; MDA: Malondialdehyde; MDc: Dansylcadaverine; M-SiNP: Mesoporous silica nanosphere; MSN: Mesoporous silica nanoparticle; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO: Nitric oxide; NOS: Nitric oxide synthase; NP: Nanoparticle; PBMC: Peripheral blood mononuclear cell; PI: Propidium iodide; Pr-SiNP (NM 200 & 201): Precipitated silica; Py-SiNP (NM 202 & 203): Pyrogenic silica; ROS: Reactive oxygen species; RT: Reverse transcription; SCE: Sister chromatid exchange; SCGE: Single cell gel electrophoresis assay; SEM: Scanning electron microscopy; SiNP: Silica nanoparticle; SOD: Superoxide dismutase; S-SiNP: Amorphous silica nanoparticle; TEM: Transmission electron microscopy; WB: Western blot.

(750 mg/kg per day) orally in female C57BL/6 mice for 2 weeks. In this case, the proliferation of immune cells such as B and T cells in the spleen is lower in all groups fed SiNPs than the control group. Specifically, the SiNPs with negative charges decrease cell proliferation most significantly. In addition to that of T and B cells, NK cell activity in SiNP-fed mice is significantly suppressed. NO production and the release of inflammatory cytokines in serum are also decreased. Lymphocyte populations are altered in the spleen, and IgG and IgM levels in serum increase and cause histological changes after treatment with mesoporous silica (MPS) [102]. The relative populations of NK and T cells are higher in the spleen of animals receiving a single intraperitoneal dose of 50 mg/kg nanosilica than in the spleen of control animals [103]. However, no toxicity is found in spleen after topical ocular applications of nonporous SiNPs to Sprague–Dawley rat [101]. In addition, a subtoxic (nontoxic at a single acute or repeated dose) level of 10-nm SiNPs does not cause toxicity after intravenous administration to mice for up to 8 weeks. SiNPs do not saturate bioaccumulation in spleen macrophages, and no major changes in the splenocyte population are observed [100].

Lung

Size-dependent injury is found in the lung after exposure to SiNPs. Nano-sized silica causes greater injury to lung tissue than micron-sized silica. Neutrophilic infiltration and pulmonary injury are greater in lung tissue treated with nano-sized silica than silica at the micron scale [104]. Increased mast cell abundance is found in the lung post intravenous treatment [96].

Heart

The toxic effects of low-dose exposures of SiNPs on cardiac function in zebrafish embryos via intravenous microinjection have been investigated. SiNPs induce whole-embryo oxidative stress and neutrophil-mediated cardiac inflammation in zebrafish. Inflammatory cells are observed in the atria of SiNPs-treated zebrafish hearts by histopathological examination [105]. Increased mast cell abundance is found in heart postintravenous treatment [97].

Hemocytes & blood circulation

The effects of SiNPs on hemocytes were investigated by administering via vascular injection into silkworms. SiNPs are rapidly accumulated in granulocytes, oenocytoids and spherulocytes, which have immune functions in the circulating hemolymph. SiNPs initiate autophagy and apoptosis via the lysosomal/mitochondrial pathway when they enter hemocytes. However, the damage caused by high doses of SiNPs to hematopoiesis is self-healing. Limited damage is caused to the hematopoietic tissues by SiNPs that enter the hematopoietic stem cells in the circulating hemolymph [106]. The number of neutrophils in arterial blood is significantly reduced after respiratory exposure. The levels of blood alanine aminotransferase and lactate dehydrogenase are significantly increased after exposure [107].

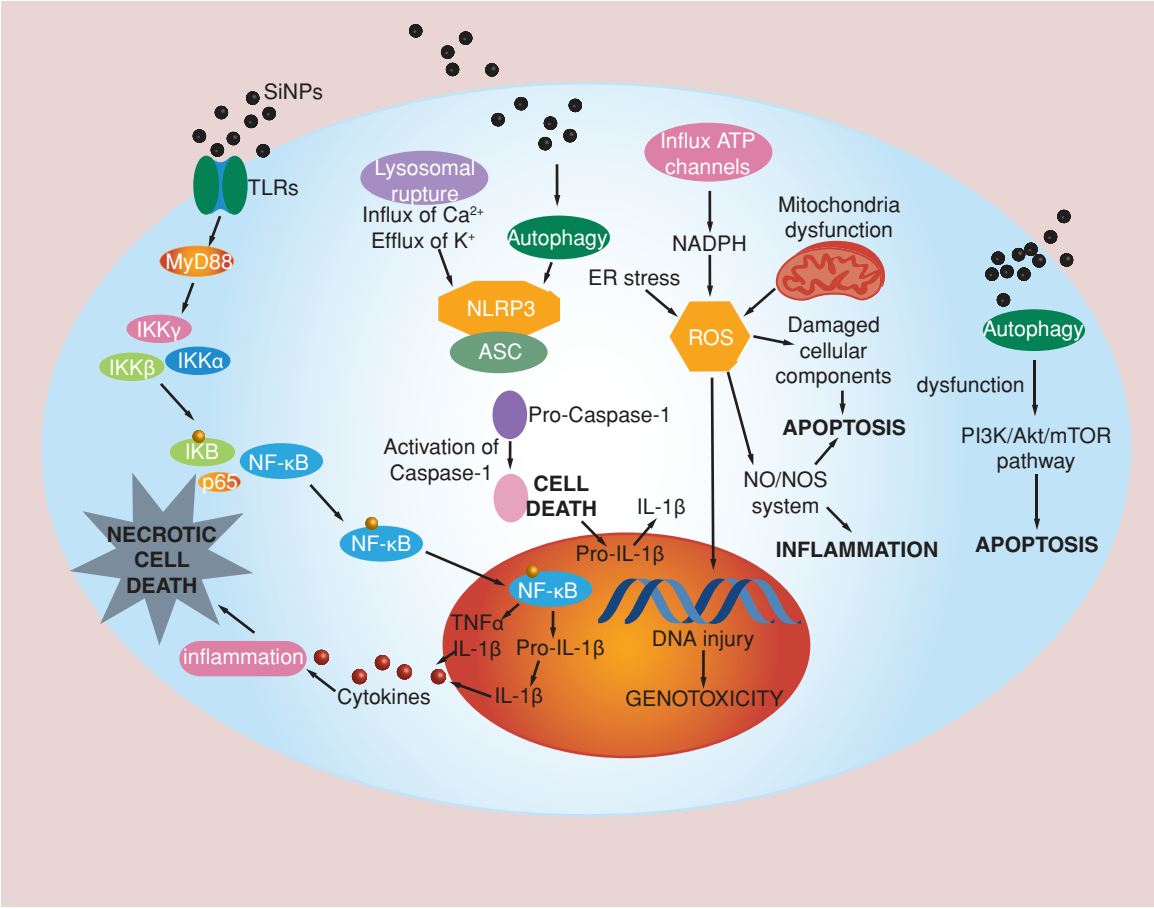

Figure 1. Mechanisms of the toxicity of silica nanoparticles.

Mechanism of the toxicity

SiNPs are toxic to the immune system, but the exact mechanisms leading to these effects are still obscure. Proinflammatory responses, ROS and autophagy are accepted as the main mechanisms of the immunotoxicity. The mechanism of toxicity was described in Figure 1.

Proinflammatory response

Proinflammatory responses are an important element in the evaluation of NM-induced immunotoxicity [108]. Cytokines, chemokines and interferons, which are released by immune cells, are widely used as biomarkers of inflammation in response to cellular stress, damage and/or pathogens. Due to their small size, NMs may escape particle-clearing defensive mechanisms (e.g., phagocytosis), and some NMs trigger a direct or indirect inflammatory response [109]. Professional phagocytes recognize and uptake NPs when a body is exposed to NPs intentionally or accidentally. Immune cells such as macrophages and polymorphonuclear granulocytes play a key role in NM-induced inflammation [110].

Different physicochemical properties such as size and crystallinity influence proinflammatory responses to exposure. Assessing the differences between NPs and microparticles is helpful in evaluating their safe use in biological applications [47]. Micro-sized SiO_2 rarely causes inflammation, unlike its NPs [111]. The expression of proinflammatory cytokines, such as *IL-1β* and *TNF-α*, has a bell-shaped distribution versus time curve after cells are exposed to silica particles. SiNPs with a diameterof 50 nm cause maximal secretion, while particles with smaller or larger diameters have progressively smaller effects [112]. Differences in the effects of MSNs and colloidal silica on inflammation were also evaluated. Exposure to MPS NPs decreases the expression of proinflammatory cytokines such as *TNF-α, IL-1β* and *IL-6* in macrophages. The reduced inflammatory response and apoptosis elicited by MPS NPs results from a decrease in the concentrations of mitogen-activated protein kinases, NF-κB and caspase-3.

In contrast to MPS NPs, colloidal SiNPs act as immunogenic sensitizers and induce contact hypersensitivity [113]. In addition, the effects of two types of amorphous SiNPs, the pyrogenic NM-203 and the precipitated NM-200, on two murine macrophage cell lines (MH-S and RAW 264.7 cells) were investigated. Pyrogenic NM-203 NPs interact with macrophages more strongly than the precipitated NM-200 NPs and trigger a more evident inflammatory response, which is evaluated using the secretion of *TNF-α*, *IL-6* and *IL-1β*, and NOS_2 induction and NO production [114].

In contrast to macrophages, lymphocytes, peripheral blood mononuclear cells (PBMCs), Kupffer cells and microglia are activated by SiNPs, leading to the release of *TNF-α,* NO and ROS from these cells [65]. Mendoza *et al.* reported the effects of SiNPs with different particle sizes on human PBMCs. Cytokines (TNF-*α, IL-1ra*, *IL-6, IL-8, IL-1β* and *IFN-γ*) are expressed after cells are exposed to 10- and 100-nm NPs. *IL-1β* production is enhanced by the presence of 10- and 100-nm NPs, and the cytotoxicity of the NPs is associated with increases in the *IL-1β*/*IL-6* ratio. The use of 100-nm NPs at concentrations that did not induce the loss of cell viability enhanced *IL-1β* and *IL-6* levels to an extent like phytohemagglutinin, a T-cell mitogen [115].

The synergistic toxicity of combinations of NMs was also reported. SiO_2 and TiO_2 NPs synergistically trigger macrophage inflammatory responses [116].

Quartz, nanosilica and rutile particles induce significant chemotaxis in NR8383 rat alveolar macrophages but do not cause cytokine release. Chemotaxis caused by quartz and nanosilica is accompanied by elevated transcription of *CCL3, CCL4, CXCL1, CXCL3* and *TNF-α* [117]. Nanosilica-stimulated macrophages express genes that enrich cytokine–cytokine receptor interactions [118]. Quartz and silica particles cause chemotactic attraction of neutrophils [119].

NLRP3 inflammasome

Upon their internalization by macrophages, SiNPs activate signals [120,121] for NLRP3 inflammasome activation and the translesion synthesis pathway. The physicochemical characteristics of the SiNPs such as size and surface charge play an important role in the activation of signal pathways. For example, positively charged NPs elicit greater *IL-1β* secretion than negatively charged or neutral NPs [122]. Similarly, small SiNPs induce higher levels of *IL-1β* secretion in murine bone marrow-derived macrophages than silica particles larger than 1 μm [123]. Furthermore, inflammation was ameliorated by genetic deletion of IL-1β *in vivo* [124]. *IL-1β* is a strong proinflammatory factor, and its secretion requires the activation of specific signaling pathways [125,126].

The inflammasomes are a family of multiprotein complexes that are major mediators of the inflammatory responses of the innate immune system. Inflammasomes are intracellular molecular platforms expressed in macrophages and other innate immune cells and are required for the oligomerization and activation of procaspase-1 to caspase-1, which then converts *pro-IL-1β to IL-1β*, leading to *IL-1β* secretion [127]. NLRP3 is the most studied inflammasome and responds to a wide range of different stimuli, including a variety of NPs [128,129]. NLRP3 activation is one of the initial steps in an inflammatory cascade against NPs, which is required for inflammatory-induced programmed cell death (pyroptosis).

SiNPs strongly activate macrophages to induce *IL-1* secretion by activating NLRP3 [120,123]. NLRP3 inflammasome activation is crucial for toxicity, while NLRP3-deficient macrophages and mice do not secrete IL-1β in response to NPs [130,131]. The activation of inflammasomes occurs via several mechanisms [108]. The NLRP3 inflammasome is activated by lysosomal rupture and the consequent release of cathepsin enzymes [130], the stimulation of channels (efflux of K^+ ions or influx of Ca^{2+} ions through ion channels or the influx of ATP via ATP channels) [132,133] and cellular stress induced by ROS [134]. The central pathway for activating the NLRP3 inflammasome is lysosome membrane permeabilization. Lysosome acidification is a prerequisite for particle-induced lysosome membrane permeabilization, and the resultant leak of lysosome cathepsins is a primary regulator of ongoing NLRP3 inflammasome activity and the release of high-mobility group box-1 [135]. Furthermore, the inhibition of phagocytosis, the suppression of endosomal acidification and suppresion of cathepsin B activity decrease the *IL-1β* responses to different particles to a similar extent [136]. K^+ efflux is a common feature of inflammasome activation by bacterial toxins and particulate matter (i.e., silica, aluminum or calcium pyrophosphate crystals). A reduction in the intracellular K^+ concentration activates NLRP3, whereas intracellular Na^+ ion level increases modulated activation but were not strictly required for inflammasome activation. Thus, a drop in cytosolic K^+ levels is a common step to activate the NLRP3 inflammasome, which is necessary and sufficient for caspase-1 activation [137]. In addition, the release of ATP and subsequent ATP, ADP and adenosine receptor signaling is required for inflammasome activation. The NLRP3 inflammasome is activated by extracellular adenosine in two ways: by interacting with adenosine receptors

and through cellular uptake by equilibrate nucleoside transporters. SiNPs significantly increase *P2Y1, P2Y2, A2A* and/or *A2B* receptor expression, whereas the P2X7 receptor is downregulated. IL-1β secretion in response to NPs is increased by enhanced ATP and ADP hydrolysis and decreased by adenosine degradation or selective A2A or A2B receptor inhibition [138]. P2R-mediated pathways play important roles in SiNPs-mediated *IL-1β* and *IL-18* production in mouse bone marrow DCs. SiNPs activate purinergic signaling in matured mouse bone marrow DCs by inducing ATP release via the P2X7 receptor. ATP induces ROS production through NADPH oxidase, and the ROS subsequently activate inflammasomes, leading to caspase-1-dependent signaling and the release of *IL-1β* and *IL-18* [139].

The mechanism that activates *IL-1β* after inflammasomes are activated has been investigated in many studies. Scavenger receptors SR-A1 and SR-B1 are silica receptors that are associated with caspase-1-mediated inflammatory responses in mouse macrophages and human peripheral blood monocytes [112,140]. The caspase-1 inhibitor zYVAD and RNA silencing of the NALP3 receptor decrease particle-induced *IL-1β* expression in RAW 264.7 macrophages. *IL-1α* also affects the production of *pro-IL-1β*. Recombinant IL-1α significantly induces *pro-IL-1β* production, which is not induced by IL-33 and high-mobility group box-1. Neutralization or deletion of *IL-1α* reduces *IL-1β* production and neutrophil accumulation after silica exposure *in vivo*. The amount of *IL-1α* released after *in vitro* exposure to a range of micro- to nano-particles of silica correlates with the degree of lung inflammation induced by these particles *in vivo* [141].

Toll-like receptors (TLRs)

Proinflammatory responses are mediated by pathogen-associated molecular patterns such as lipopolysaccharides and lipoproteins. These pathogen-associated molecular patterns stimulate Toll-like receptors (TLRs) to activate NF-κB, leading to the production of pro-IL-1β and NLRP3. The NF-κB signaling pathway plays important roles in SiNPs-induced inflammation [120,121]. NF-κB is a pivotal transcription factor activated by silica in macrophages and other types of cells. TLR activation and MyD88 (a downstream adapter of TLR pathways) play important roles in the NF-κB signaling pathway [76]. MSNs induce inflammatory gene upregulation through NF-κB and eventually lead to proinflammatory responses and autophagy-mediated necrotic cell death [48]. Immature and unprimed DCs internalize food-grade SAS particles by endocytic uptake, which fails to release *IL-1α* or *TNF-α*. However, the steady-state DC uptake of SAS particles leads to induction of the precursor pro-IL-1β, which is subsequently cleaved by the inflammasome for the secretion of mature IL-1β. The pro-IL-1β induction is suppressed by pharmacologic inhibitors of endosomal TLR activation or by genetic ablation of MyD88. Large doses of MSNs induce severe and selective nephrotoxicity, which is closely related to the inflammation mediated by the NF-κB pathway. Moreover, enhanced autophagy attenuates the inflammation mediated by the NF-κB pathway, whereas the inhibition of autophagy contributes to inflammation [142]. TLRs can be stimulated by endotoxin contamination, which adhere to particles [143]. However, most original articles do not mention whether SiNP samples were free of endotoxin contamination. The endotoxin contamination should be considered when evaluating the proinflammatory response.

Oxidative stress

Oxidative stress is an underlying mechanism that causes immunotoxicity. NPs cause oxidative stress by increasing membrane lipid peroxidation and the levels of ROS and by decreasing intracellular glutathione (GSH) levels. Oxidative stress damages the cellular components and leads to cell death via apoptosis. It can affect cell proliferation and induce DNA damage, resulting in genotoxicity [144]. It also plays an important role in cell signaling and inflammatory responses. Oxidative stress disturbs the NO/NOS system, which is tightly correlated with inflammatory responses. NO is implicated in phagocytosis as well as the pathogenesis of inflammation [145,146]. NP characteristics such as size, shape and deformability also influence NP uptake by immune cells and the subsequent oxidative stress responses [147]. Experimental evidence proves that SiNPs have caused oxidative stress in various cell lines such as those of endothelial cells, colon carcinoma cells and keratinocytes [148,149]. Silica crystals produce ROS in immune cells [150]. The cytotoxicity induced by SiNPs is closely correlated to increased oxidative stress. The generation of ROS induces cell membrane damage by lipid peroxidation that may subsequently increase cellular permeability [151], disturb intracellular calcium homeostasis and alter signaling pathways [152,153]. SiNPs disturb the NO/NOS system, which stimulates the release of cytoprotective NO and upregulates inducible NOS mRNA while downregulating endothelial NOS and ET-1 mRNA. Oxidative stress responses to amorphous SiNPs (average primary size 12 nm) have been investigated. RAW 264.7 cells were exposed to SiNPs (5–40 ppm) *in vitro*, resulting in ROS generation and decreased intracellular GSH levels as well as increased levels of NO release [102]. SiNPs elicit ROS generation in

a size-dependent manner [154]. NPs (10 nm) are more cytotoxic against and induce higher oxidative stress in human PBMCs than 100-nm NPs [115]. Amorphous SiNPs with a particle size of 70 nm induce more ROS generation in XS52 cells than micron-sized amorphous silica particles [72]. XS52 cells are a Langerhans cell-like line. The crystallinity of silica affects oxidative stress. Quartz and vitreous silica display stable surface radicals and the sustained release of OH radicals. Vitreous silica and pure quartz show remarkably high cytotoxicity, nitrite release and TNF-α production, suggesting a common behavior in inducing oxidative stress [155]. The particle size and presence of iron were determined under low-dose, noncytotoxic conditions that are likely to approximate actual exposure levels, in contrast with higher dose conditions under which cytotoxicity occurs. Smaller particle sizes and the presence of iron increase superoxide production, lipid peroxidation and the induction of proinflammatory cytokine mRNA expression [156]. Human lymphocytes play a major role in the immune system, and their antioxidant levels can change when exposed to NPs. Exposure to SiNPs decreases the cellular viability and increases the cell membrane damage of human lymphocytes. SiNPs induce the generation of ROS, lipid peroxidation, the depletion of catalase and decreases in the levels of GSH and superoxide dismutase in human lymphocytes. Alterations in the levels of antioxidants causes DNA damage and chromosomal aberration in human lymphocytes [79] (Table 1).

NADPH oxidase pathway

The mechanisms underlying the production of ROS by SiNPs were investigated. In addition to the intrinsic ROS production by the particles themselves, the NADPH oxidase pathway, damage to mitochondria that leads to the expression of death receptors and ER stress play important roles [157]. NADPH activation is an important pathway for the induction of ROS production. The transient receptor potential melastatin-2 channel acts as an oxidative stress sensor that plays a dual role in SiNPs-induced cytotoxicity by differentially regulating NADPH oxidase activity. This channel is a cellular redox potential sensor that provides an important pathway for increasing the Ca^{2+} ion concentration under oxidative stress [158]. Silica particles cause mitochondrial dysfunction by increasing the expression of death receptors and/or their ligands, which initiate apoptosis [159]. In addition, ER stress is involved in silica-induced apoptosis of macrophages. Silica exposure induces nuclear condensation and caspase-3 expression in RAW 264.7 cells, and the expression of binding protein and CCAAT-enhancer-binding protein homologous protein was also increased in silica-stimulated cells [160].

MAPK pathway

Signaling pathways that respond to the generation of oxidants by silica particles include those that involve the MAPK/ERK kinase, extracellular signal-regulated kinase (ERK) phosphorylation and the activation of specific transcription factors such as NF-κB and AP-1 [161]. SiNPs induce oxidative stress, proinflammatory effects and endothelial dysfunction *in vitro* via activation of the MAPK/Nrf2 pathway and NF-κB signaling (Figure 1) [162]. Nuclear factor Nrf2 signaling and its regulated antioxidant genes play critical roles in maintaining redox homeostasis [163]. SiNPs inhibit macrophage activity and angiogenesis via the downregulation of the MAPK signaling pathway [31].

Autophagy

Autophagy dysfunction is considered a potential toxic mechanism of NMs. Autophagy deficiency in macrophages enhances NLRP3 inflammasome activity and chronic lung disease following silica exposure [164]. SiNPs induce autophagy even at the subtoxic level and block autophagic flux at the high doses. Low concentrations of SiNPs trigger autophagy, which is evidenced by morphological and biochemical hallmarks such as autophagolysosomes and increased levels of LC3-II, which serve to protect cells from cytotoxicity [165,166]. However, SiNPs inhibited autophagosome degradation via lysosomal impairment, resulting in autophagy dysfunction at large doses or long exposure times [167]. SiNPs trigger autophagy dysfunction via the PI3K/Akt/mTOR pathway [88]. SiNPs also induce ER autophagy in human colon cancer cells [168].

Other mechanisms

In vitro studies have shown that exposure of macrophages and macrophage cell lines to crystalline silica results in cell death by activation of the intrinsic apoptotic pathway [169,170]. Silica-induced lysosomal rupture is a very early apoptotic event, preceding the activation of caspases, the disruption of transmembrane mitochondrial potential and DNA fragmentation; these later apoptotic events are directly correlated to the magnitude of lysosomal leakage [171,172].

IL-1β-dependent NO-mediated apoptosis may play a role in silica-induced apoptosis. NO release and apoptosis might be inhibited by a neutralizing anti-IL-1β antibody or the NOS inhibitor N(G)-nitro-L-arginine-methyl ester *in vitro*. An *in vivo* study exposed IL-1β knockout (IL-1β$^{-/-}$) mice, inducible NOS knockout (iNOS$^{-/-}$) mice and WT mice to 250 mg/m^3 silica for 5 h per day for 10 days using an inhalation chamber. IL-1β$^{-/-}$ mice and iNOS$^{-/-}$ mice had significantly less apoptosis and inflammation than WT mice [124].

Quartz and amorphous silica are considered toxic because they cause strong apoptotic effects in RAW 264.7 macrophages [173].

The modification of immunotoxicity

Some modifications to NPs were investigated to attenuate the toxicity of SiNPs. Physicochemical properties of particles are often considered important factors in the development of safer forms of NMs. The toxic effects of SiNPs are dramatically attenuated by NPs surface-functionalized with amino and phosphate groups [174]. The surfaces of unmodified SiNPs (nSP70) or SiNPs modified with amine (nSP70-N) or carboxyl groups (nSP70-C) were investigated. Compared with the unmodified nSP70 SiNPs, nSP70-N and nSP70-C have a smaller effect on DNA synthesis activity. Analysis of the intracellular localization of the SiNPs revealed that nSP70 penetrated into the nucleus, whereas nSP70-N and nSP70-C showed no nuclear localization. These results suggest that intracellular localization is a critical factor underlying the cytotoxicity of these SiNPs [175]. Similarly, surface-modified silica particles were internalized by lymphocytes with varying efficiency and expressed no cytotoxic or genotoxic effects as determined by various methods (cell viability, apoptosis/necrosis, oxidative DNA damage and chromosome aberration assays). However, these particles affected the proliferation of the lymphocytes because their mitotic index value and cell cycle progression decreased [176]. In addition, surface functionalization of SiNPs can mitigate toxicity by reducing free radical production [177]. Thus, the surface properties of SiNPs play an important role in determining their safe use. Asymmetric MSNs with controllable head–tail structures have been investigated for biocompatibility. The head particle type is tunable (solid or porous), and the tails have dendritic large pores. The tail length and tail coverage on head particles are adjustable. Compared with spherical SiNPs with a solid structure (Stöber spheres) or large-pore symmetrical MSNs with fully covered tails, asymmetrical head–tail MSNs show superior hemocompatibility because they cause less membrane deformation of red blood cells and lower levels of ROS. Moreover, compared with Stöber spheres, asymmetrical head–tail MSNs exhibit a higher level of uptake and *in vitro* maturation of immune cells, including DCs and macrophages [178]. Surface and shape modifications mitigate the toxic effects of SiNPs, providing a new way to produce safer NMs. In addition, MSNs can be used for drug delivery to attenuate the toxic effects. MSNs with extra-large pores (XL-MSNs; 30 nm) have been used *in vivo* for the delivery of macrophage-polarizing cytokines. XL-MSNs were used to deliver IL-4, which is an M2-polarizing cytokine and very quickly degraded *in vivo*, to macrophages and polarize them into anti-inflammatory M2 macrophages *in vivo*. IL-4-loaded XL-MSNs induce a low level of ROS production and no inflammatory cytokines in RAW 264.7 cells *in vitro* and in peritoneal macrophages extracted from mice after intravenous administration [30]. Therefore, M2 polarization of macrophages promotes NP internalization, and the phenotypical differences between macrophage subsets should be considered in future investigations of nanosafety [179]. Furthermore, we showed that surface modification of SiNPs suppresses cross-presentation. Although further studies are required to determine whether surface-modified SiNPs suppress immune-modulating effects *in vivo*, the current results indicate that appropriate regulation of the characteristics of SiNPs, such as size and surface properties, will be critical for the design of safer SiNPs [70].

Limitations of current research & future perspectives

The toxicity of SiNPs to the immune system is important in safety considerations. The toxicity is size, dose, crystallinity and cell-type dependent. The biological matrices and dispersion of NMs may determine the results of toxicity assessment. However, there is a lack of research focusing on these points. The purity of SiNPs should be evaluated in details. The presence of impurities, such as the presence of surfactants (e.g., cetyltrimethyl ammonium bromide or hexadecyl trimethylammonium chloride used to make mesoporous silica) may influence the results of toxicity evaluation.The immunotoxicity of SiNPs includes cell dysfunction, cytotoxicity, genotoxicity and *in vivo* toxicity. Although many studies have focused on the cytotoxicity and genotoxicity to monocytes/macrophages, data on their genotoxicity to other immune cells such as lymphocytes and mast cells are limited. Although many studies have paid attention to the *in vivo* toxicity to organs, data on the direct toxicity of SiNPs to immune organs such as bone marrow, thymus and lymph node are very limited. Furthermore, no study has focused on the effects of

SiNPs regulating the immune response; they may activate or suppress immunity. Therefore, we should more deeply investigate the toxicity of SiNPs to immune systems, especially their direct influence on immune organs. Different routes of administration of SiNPs *in vivo* have different results. The comparison of different administration route is lacking. Proinflammatory responses, oxidative stress and autophagy are the main toxicity mechanisms of SiNPs. The endotoxin contamination and the sterility of SiNPs affect the proinflammatory effect and ROS. The detailed information of original articles should be clear and deeply research should consider this point. This is important because such contamination may confound the results of the toxicity. The interrelationships and interactions of these three mechanisms were unclear. For example, some studies have demonstrated that the MAPK signaling pathway plays important roles in the ROS generated by SiNPs. However, the exact regulatory effects were unclear. Therefore, we need to understand the mechanism more deeply. Although surface modification provides strategies for the production of NMs with less health impact for humans, mitigating the toxic effects of SiNPs still require much work.

Executive summary

Toxicity to immune cells

- Monocytes/macrophages/Kupffer cells and microglia. Silica nanoparticles (SiNPs) change the activity and abilities of macrophages/monocytes. The cytotoxicity of SiNPs is dependent on the physicochemical properties of particles.
- Dendritic cells (DCs) and Langerhans cells. SiNPs enhance antigen-specific cellular immune responses and change cell functions, and have size- and concentration-dependent cytotoxic effects on DCs.
- Lymphocytes. The viability and proliferative activity of lymphocytes are dependent on particle size, concentration, charge and other physicochemical characteristics.
- Mast cells. These cell functions were changed after exposure. No studies have focused on the genotoxicity against these cells.
- Endothelial cells. Cell function was changed.

Immunotoxicity to tissues & organs

- Liver. Lymphocytic infiltration, granuloma formation and hydropic degeneration in liver hepatocytes were observed.
- Spleen. The proliferation of immune cells such as B and T cells in spleen is decreased. Lymphocyte populations are altered in the spleen, and IgG and IgM levels in serum increase and cause histological changes.
- Lung. Neutrophilic infiltration, increased mast cell abundance and pulmonary injury are greater in the lung.
- Heart. SiNPs induce neutrophil-mediated cardiac inflammation *in vivo*.
- Hemocytes and blood circulation. SiNPs initiate autophagy and apoptosis when they enter hemocytes.

Mechanism of the toxicity

- Proinflammatory responses are an important element in SiNPs-induced immunotoxicity. NLRP3 inflammasome and Toll-like receptors are the main signal in inducing proinflammatory responses.
- Oxidative stress is an underlying mechanism that causes immunotoxicity. NADPH oxidase pathway and MAPK pathway are the main signal in cause oxidative stress.
- Autophagy. Autophagy dysfunction is considered a potential toxic mechanism of SiNPs.
- Other mechanisms: Activation of the intrinsic apoptotic pathway plays important role in cytotoxicity.

The modification of immunotoxicity

- The toxic effects of SiNPs are dramatically attenuated by nanoparticles surface-functionalized with amino and phosphate groups.
- Shape modifications mitigate the toxic effects of SiNPs, providing a new way to produce safer nanomaterials.

Limitations of current research & prospects

- The biological matrices and dispersion of nanomaterials may determine the results of toxicity assessment. However, there is lack of research focusing on these points.
- The purity of SiNPs should be evaluated in details. The presence of impurities, such as the presence of surfactants (e.g., CTAB or CTAC used to make mesoporous silica) may influence the results of toxicity evaluation.
- Data on the direct toxicity of SiNPs to immune organs such as bone marrow, thymus and lymph node are very limited. Furthermore, no study has focused on the effects of SiNPs regulating the immune response; they may activate or suppress immunity.
- Different routes of administration of SiNPs *in vivo* have different results. The comparison of different administration route is lacked.
- The endotoxin contamination and the sterility of SiNPs affect the proinflammatory effect and reactive oxygen species. The detailed information of original articles should be clear and deeply research should consider this point.
- The interrelationships and interactions of these three mechanisms were unclear.

Conclusion

SiNPs cause toxicity to immune cells and tissues. The main mechanisms were pro-inflammatory responses, oxidative stress, autophagy and so on. Surface and shape modifications may mitigate the toxicity effects of SiNPs, providing a new way to produce these NMs with less toxic impact.

Financial & competing interests disclosure

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