Research Article

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Therapeutic Delivery

Evidence of size-dependent effect of silica micro- and nano-particles on basal and specialized monocyte functions

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Aim: To analyze the effect of silica particles on monocyte/macrophage functions. **Materials & methods:** Silica micro- and nanoparticles were obtained by the Stöber method. Their effect on monocyte/macrophage proliferation, activation, membrane integrity and metabolic activity were determined. **Results:** Silica particles inhibit cell proliferation while 10 nm nanoparticles (NPs) did not affect it. Similarly, silica particles induced strong cell activation. However, 10 nm NPs do not alter IL-12 or nitrite levels. Furthermore, bigger NPs and microparticles increase cell membrane damage and reduce the number of living cells but smallest NPs (10 and 240 nm) did not. **Conclusion:** Cell activation properties of silica particles could be useful tools for immune stimulation therapy, while 10 nm NPs would be suitable for molecule transportation.

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Keywords: activation markers • cell activation • cytokine secretion • monocytes/macrophages • silica nanoparticles

The employment of nanoparticles (NPs) in the biomedical field has gained great attraction in recent years owing to the potential therapeutic use of these materials, for example, drug delivery and tissue engineering [1–3]. Especially, the possibility of designing multifunctional nanosystems with different approaches enhances the efficacy of therapeutic treatments [4–6]. There is also great interest on nanocomposites concerning biopolymeric matrices and bioactive nanosized fillers. Biodegradability, high mechanical strength and controlled drug delivery are properties required for those materials [7,8]. Several materials are employed to build NPs. Among this, magnetic and silica NPs are of great interest because of the possibility to introduce chemical functional groups on their surfaces to tune NP–biological molecule interaction.

Silica vehicles offer a number of advantages over organic polymers. These include its stability, hydrophilic properties that make it easy to disperse in aqueous media and suitability to transport many therapeutic agents [9,10]. Moreover, silica as the main and natural constituent of sand and glass, is an adaptable material due to the large number of chemical and physical modifications that can be applied to it, and have recently been widely used for drug transport [11,12]. The great motivation for their employment is the versatility of Si chemistry that permits control of the size, porosity and to easily introduce various functional groups such as carboxylic, amino or thiol onto the particle's surface and to incorporate several drugs through compartmentalization approaches [13–15]. The chemical modification of the inorganic and/or bio-organic component allows the adjustment of the interactions between the silica particles and the biomolecules (proteins, polysaccharides, lipids and nucleic acids) [16]. Thus, it is possible to tune the characteristics of the NPs to meet the requirements of each specific cell and desired application [17]. Moreover, once the silica surface has been modified, biomolecules such as antibodies can be linked

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to the NPs for drug-targeting purposes [18,19]. These characteristics make silica NPs true candidates to develop more effective drug delivery and targeted safe methods [20–23].

Despite the many works that exist on the synthesis of silica NPs and their potential as target drug delivery systems [24,25], there is still little information on their *in vivo* behavior and fate. Particularly, the interaction of silica NPs with different biological systems is an important aspect to analyze for future application of NPs in medical treatments and is essential for the safe use of nanotechnology.

The great advances in the application of silica NPs also require a careful characterization of the biocompatibility, immune response and toxicological effects that they may cause [26–28]. The immune response against NPs is actually being studied [29–31] for multiple reasons such as the elucidation of new targets for drug delivery systems [32,33], as vaccination adjuvants [34,35] or for toxicological studies [36–39]. It is worth mentioning that the immune system interacts with NPs in different ways, having the possibility to trigger both the innate and adaptive immune response [40]. Innate and adaptive immune responses function in a coordinated manner to maintain effective immune response that prevents or protects not only against foreign antigens, mostly microorganisms, but also particles that enter affecting the organism.

In this work, we analyze the effect of silica particles of various sizes (from 10 to 1430 nm) on monocyte/macrophage cells which are among the first line of defense against foreign aggression. Basal function processes common to all types of cells (i.e., viability and proliferation) and specialized cell functions (i.e., cytokine secretion, nitrites production and activation markers) were carefully analyzed, providing a clear evidence of the size and time dependent differential effect of silica microparticles and NPs on basal and specialized monocyte functions.

Materials & methods

Materials

RPMI 1640 medium, glutamine, pyruvate, penicillin and streptomycin were from Life Technologies (Paisley, UK). Inactivated fetal bovine serum (FBS) was from Natocor (Córdoba, Argentina). Anti-human CD14-PE-Cy7, CD11-PE-Cy5, CD40-FITC, CD86-FITC and CD80-FITC conjugate monoclonal antibodies (mAbs) were obtained from BD Pharmingen (CA, USA). Calcitriol (1,25-dihydroxy vitamin D3) was obtained from Calbiochem (Darmstadt, Germany). Tetraethyl orthosilicate (98%) was purchased from Sigma-Aldrich (MO, USA). Ammonium hydroxide (30%) was obtained from Carlo Erba Reagents (Barcelona, Spain). All other reagents used were of analytical grade and were commercially available.

Synthesis of silica particles

Bare silica micro- and NPs were obtained by the Stöber method [41,42]. In this method, when the concentration of the silica precursor is higher than solubility, nucleation phenomenon takes places and generates homogeneous particles. Briefly, tetraethyl orthosilicate was added dropwise to a stirred solution of ultrapure water, absolute ethanol and ammonium hydroxide. The concentration of reagents used depends on the size of the synthesized particle. The solution was stirred overnight at room temperature. Particles were washed twice with absolute ethanol, once with deionized water and then centrifuged (10,000 × g). The obtained pellet was dried under vacuum and stored in a closed flask. At last, silica particles were suspended to achieve a concentration of 5 mg ml^{-1} in an aqueous solution of 10 mM KCl and then analyzed by dynamic light scattering and by zeta potential measurements.

Cells & cultures

We employed THP-1 cell line (a human monocytic leukemia cell line) because it is a widely used model for the study of the immune system, mainly for phagocytic cells [43]. THP-1 cells were obtained from ATCC (VA, USA) and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 1 mM pyruvate, 100 U of penicillin per ml and 100 µg of streptomycin per ml (complete medium) at 37°C with 5% CO_2 and humidified environment. Prior to each assay, 1×10^5 cells ml^{-1} were treated with either 1,25-dihydroxyvitamin D3 (VD3) (0.05 µM) for 72 h, or left untreated. Differentiation of THP-1 cells with 1,25-dihydroxyvitamin D3 is known to result in a noncomplete macrophage phenotype. For this reason, increased cytoplasmic volume and CD14 expression in the cell membrane, a marker of mature monocytes, were analyzed before each assay (Supplementary Figure 1). Viable cells were counted in Newbauer chamber after staining with Trypan Blue.

Effect of silica particles on cellular proliferation

THP-1 cells (1×10^5 cells ml^{-1}) were cultivated in complete medium at 37°C with 5% CO_2 for 24–168 h in the presence of the different silica particles (between 0.3 and 2.4 mg ml^{-1} of silica concentration) obtained as previously described. All culture assays were done in 24-well plates with 1 ml as final volume. Biological activity as indicator of cellular proliferation was determined with the tetrazolium assay (MTT) [44]. Briefly, cells were centrifuged, resuspended in a 5 mg ml^{-1} MTT solution in phosphate-buffered saline and incubated at 37°C with 5% CO_2 and humidified environment for 4 h. After that, the cells were washed three-times with phosphate-buffered saline and dimethyl sulphoxide was added. The absorbance of the solution was read at 570 nm with a spectrophotometer Multiskan Ex (Thermo Scientific, MA, USA). Cells not exposed to particles and particles alone were used as basal control. Results were expressed as mean ± standard deviation (SD) from triplicate experiments.

Effect of silica particles on cell activation

THP-1 cells (1×10^5 cells ml^{-1}) were cultured in complete medium in the absence or presence of silica particles for different times (24–96 h) in 24-well plates in order to verify the effect of silica particles on cell activation. After that, cells were washed and incubated with the following mAbs: anti-human CD11-PE-Cy5, CD14-PE-Cy7, CD40-FITC, CD80-FITC and CD86-FITC (according to manufacturer's instructions) followed by flow cytometry analysis. Isotype controls were done. From each sample, 20,000 events were collected on a Partec PAS III flow cytometer (Görlitz, Germany) and analyzed with the Winmdi Software (The Scripps Institute, Flow Cytometry Core Facility, CA, USA). Cells present in M1 region were considered positive. For double fluorescence experiments, the flow cytometer was properly compensated with FloMAX software.

Subsequently, culture supernatants were tested for IL-8 and IL-12 by ELISA (R&D Systems, Oxon, UK) as indicated by the manufacturer. Nitrite determination was performed by Griess reactive. Briefly, 50 µl of supernatants or standard controls (different concentration of sodium nitrite) were mixed with 50 µl of commercial reagents (sulfanilic acid and α-naphthylamine, Laboratorios Britania, Argentina), incubated for 15 min and reaction was read at 540 nm. Cells cultivated without particles and particles alone were used as controls. Experiments were repeated at least three times.

Membrane integrity & metabolic activity determination

THP-1 cells were cultured (1×10^5 cells ml^{-1}) in the presence of different silica particles for 24–96 h, as described above, and membrane integrity and metabolic activity were analyzed. Analyses were performed by flow cytometry using the LIVE/DEAD® viability/cytotoxicity kit (Invitrogen, CA, USA) following manufacturer's instructions. This two-color assay identifies live versus dead cells on the basis of esterase activity and membrane integrity, respectively. Cells cultivated without particles and with particles, but without fluorescence reactivity were used as controls. Experiments were repeated at least three times.

Statistical analysis

All data were expressed as the mean ± SD of triplicate experiments. Results were analyzed using one-way ANOVA, followed by the Dunnett's multiple comparison test; $p < 0.05$ was considered significant. Two-way ANOVA, followed by the Bonferroni post-test were used for MTT assays when different NP concentration was evaluated.

Results

Characterization of silica particles

Monodisperse spherical bare silica particles (SiOH) were obtained by the Stöber method. The mean diameters for silica particles were 10 ± 2 nm (Si10), 240 ± 4 nm (Si240), 380 ± 8 nm (Si380) and 1430 ± 37 nm (Si1430). The zeta potential (ζ) of all these particles ranged between circa -28 mV and -40 mV (-28 ± 5 mV for Si10, -35 ± 8 mV for Si240, -40 ± 8 mV for Si380 and -38 ± 7 mV for Si1430), suggesting that there is sufficient electrostatic repulsion to ensure colloidal stability. These particles were employed for the following experiments.

Effect of bare silica particles on cell proliferation

With the aim to evaluate the effect of SiOH particles over cell cultures, THP-1 cells were cultivated in 24-well plates in the presence of the different SiOH particles for a period of 24–168 h and cellular proliferation was determined with the MTT test. When different NP concentrations were assayed, between 0.3 and 2.4 mg ml^{-1} of silica, similar cell behavior was observed with the most remarkable differentiation with Si10 NP. THP-1 proliferation was not

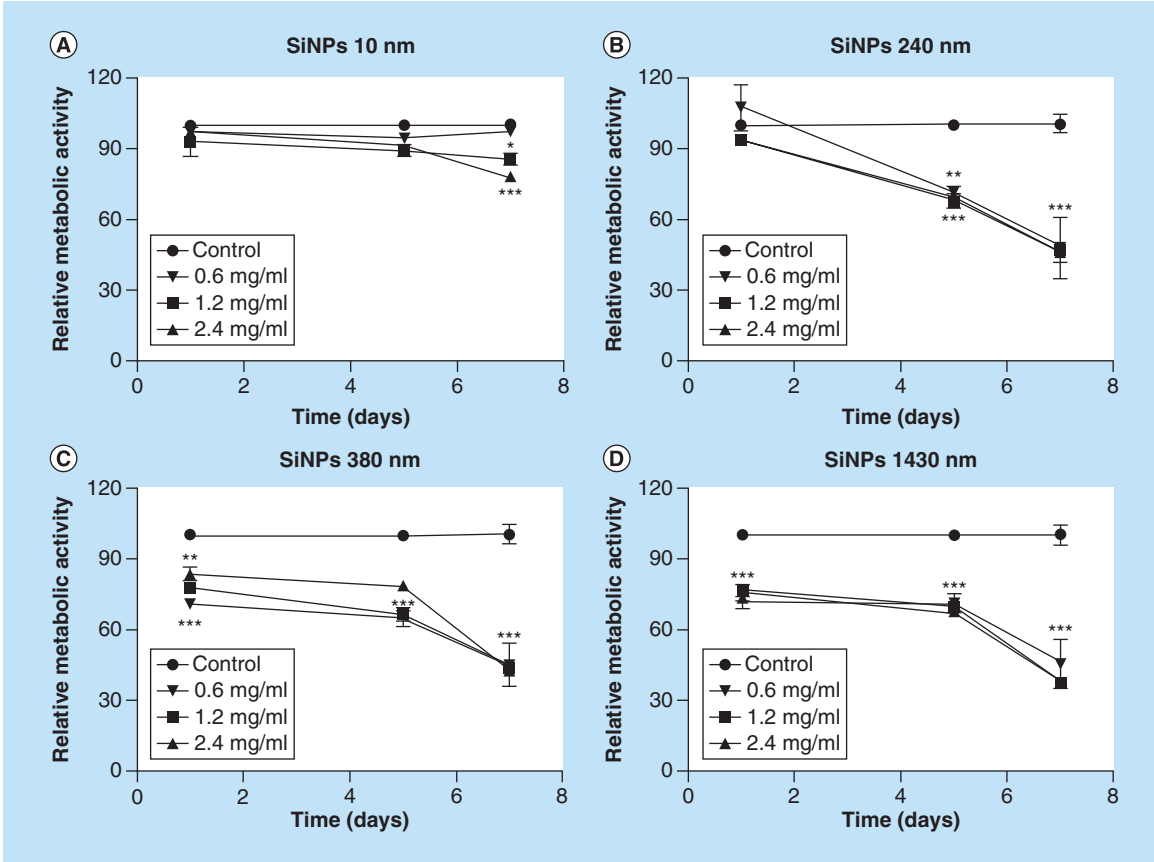

Figure 1. THP-1 metabolic activity in the presence of silica particles.
THP-1 (1×10^5 cells ml^{-1}) cells were cultured alone (control) or in the presence of different particles (0.6, 1.2 or 2.4 mg ml^{-1} of total silica concentration) during a 24–168 h period and their metabolic activity was determined by the MTT assay. Control cells were considered as 100% proliferation at each time tested. **(A)** Si10; **(B)** Si240; **(C)** Si380; **(D)** Si1430. Results represent the mean ± SD of triplicate experiments.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
SD: Standard deviation.

affected by the presence of 0.3 and 0.6 mg ml^{-1} of Si10 by all times analyzed, but when 1.2 and 2.4 mg ml^{-1} of Si10 were added, cell proliferation was significantly inhibited after 5 days of culture (Figure 1). All results described later refer to cells cultivated in the presence of 0.6 mg ml^{-1} SiOH particles because it is the highest concentration used that showed a differential performance between Si10 and other NP sizes.

It was observed that during the analyzed period, THP-1 cells exposed to particles were able to proliferate but at a different rate depending on the size of the particle employed. Indeed after 24 h exposure to the bigger particles (Si380 and Si1430 nm), the proliferation was significantly lower (~70% with respect to control). There were no statistically significant differences in cells exposed to the Si10 and Si240 particles (Figure 2A). After 48 h of culture, Si240 particles also inhibit THP-1 cells' proliferation (~80% with respect to control), but to a lower extent than Si380 and Si1430 nm (*ca*. 60–70% with respect to control). At this time, cells exposed to the smallest NPs of 10 nm do not present proliferation differences with the control cells (Figure 2B). This behavior is maintained during the following culture times assayed (Figure 2C–F). Figure 2G shows the proliferation of the cells not exposed and exposed to the different particles as a function of time where it can be observed a clear inhibition in the percentage of proliferation of the cells exposed to bigger particles.

Evaluation of cytokine secretion

Supernatants of THP-1 cells cultured in the presence and absence of SiOH particles were analyzed to evaluate cytokine secretion. It was found that all silica particles induced the production of IL-8, IL-12 and nitrites. The production of IL-8 by cells exposed for 24 h to Si380 particles was three-times higher (~150 pg ml^{-1}) than by

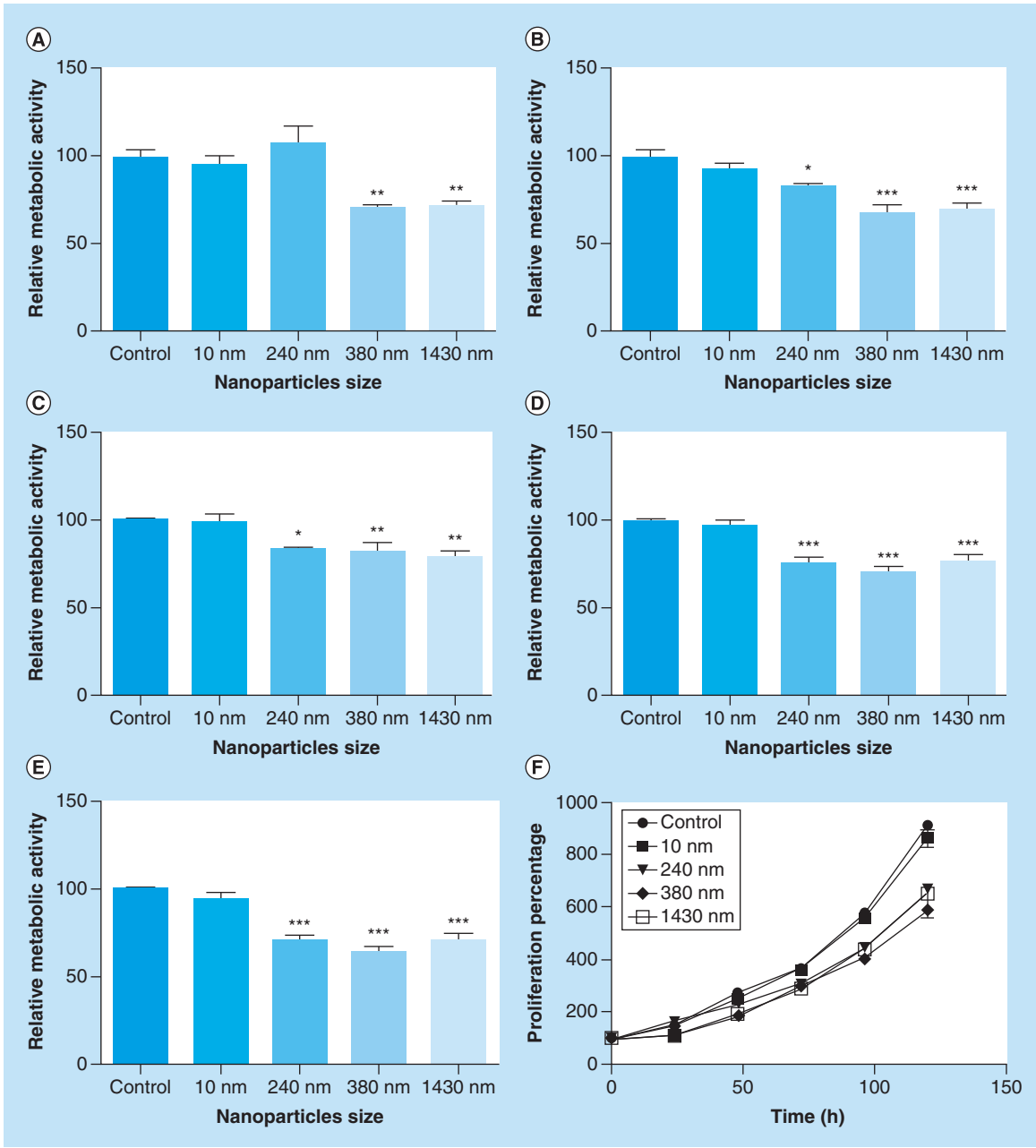

Figure 2. THP-1 metabolic activity in the presence of silica particles.
THP-1 (1×10^5 cells ml^{-1}) cells were cultured alone (control) or in the presence of different particles during a 24–120 h period and their metabolic activity was determined by the MTT assay. Control cells were considered as 100% proliferation at each time tested. **(A)** 24 h; **(B)** 48 h; **(C)** 72 h; **(D)** 96 h; and **(E)** 120 h. Results represent the mean ± SD of triplicate experiments. **(F)** Proliferation of the cells exposed to particles of different sizes assayed at different times of culture. In this case, 100% proliferation was assigned to culture cells at time 0 h.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
SD: Standard deviation.

the cells exposed to the other particles (~50 pg ml^{-1}) (Figure 3A). The levels of cytokine secretion continue to increase and after 48 h, the highest production of IL-8 was obtained with the cells exposed to Si380 particles but the levels were only 1.5-times higher than what were obtained with the other particles (Figure 3B). During the rest of the times evaluated (72–96 h), the levels of IL-8 reached 2400 pg ml^{-1} with Si380, 1500 pg ml^{-1} with Si240 and Si1480 and the lowest level was obtained with Si10 NPs (Figure 3C–D). Similar results were obtained when

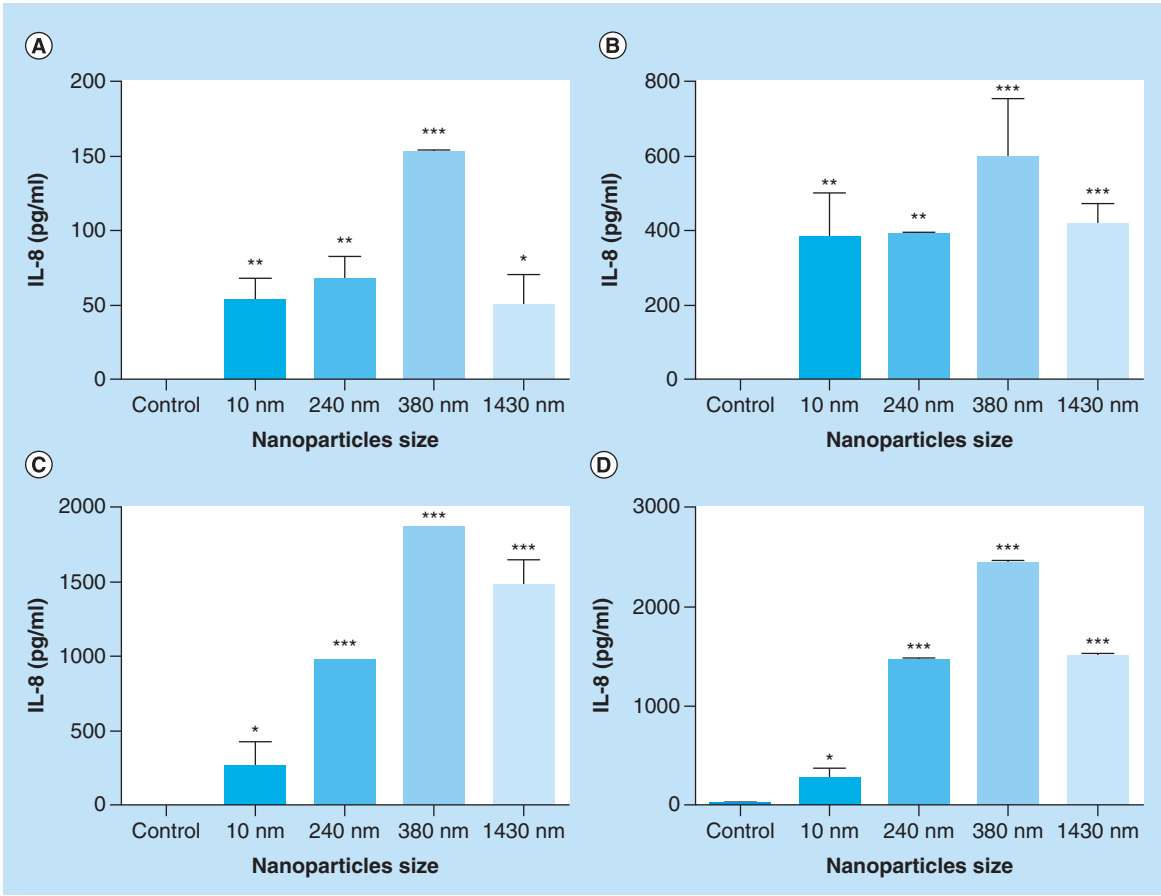

Figure 3. IL-8 secretion.
Supernatants of monocytes/macrophages cultured with different size nano- and microparticles were analyzed by ELISA for IL-8 production at **(A)** 24 h; **(B)** 48 h; **(C)** 72 h; and **(D)** 96 h. Results represent the mean ± SD of triplicate experiments.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
SD: Standard deviation.

IL-12 was analyzed. An enhanced production of IL-12 was observed in cells exposed to Si240 particles after 24 h of culture (~22 pg ml^{-1}) (Figure 4A). After 48 h, the highest production was obtained with Si1430 particles (~28 pg ml^{-1}), followed by Si380 and Si240 (ca. 11 pg ml^{-1}) (Figure 4B). Interestingly, cells exposed to Si1430 particles begin to markedly increase secretion after 72 h (Figure 4C) and after 96 h, the level of IL-12 secretion by cells exposed to these particles reached 1200 pg ml^{-1} (Figure 4D). The levels of cytokine secretion increase during the times evaluated. Once again, 10 nm silica NPs do not induce IL-12 secretion all over the different times evaluated. In parallel, an enhanced production of nitrite by cells exposed to Si240, Si380 and Si1430 particles at early hours of culture (i.e., 24 h) (Figure 5A) was observed. SiOH NPs of 10 nm did not induce the production of this important molecule implied in microbial destruction by monocyte/macrophage cells. Only cells exposed to Si380 particles produced significantly higher levels of nitrites after 48 h of culture (Figure 5).

Effect of silica particles on cell activation

In order to further analyze the effect of silica particles over cell activation, THP-1 cells were cultured in complete medium in the absence or presence of particles in 24-well plates. Later, we analyzed the expression of five membrane molecular markers CD11, CD14, CD40, CD80 and CD86 by FACS and using WinMDI software. It was observed that after 48 h of culture with Si240 particles, the CD14 expression slightly increased (~100%), while in the same period with the other particles, the CD14 expression increased by a factor of 6 (~500%) (Figure 6A). All differences have statistical significance except for Si240 particles. When CD40 was analyzed, an increment of the mentioned membrane marker expression when cells were cultivated with SiOH particles was observed, but this behavior was

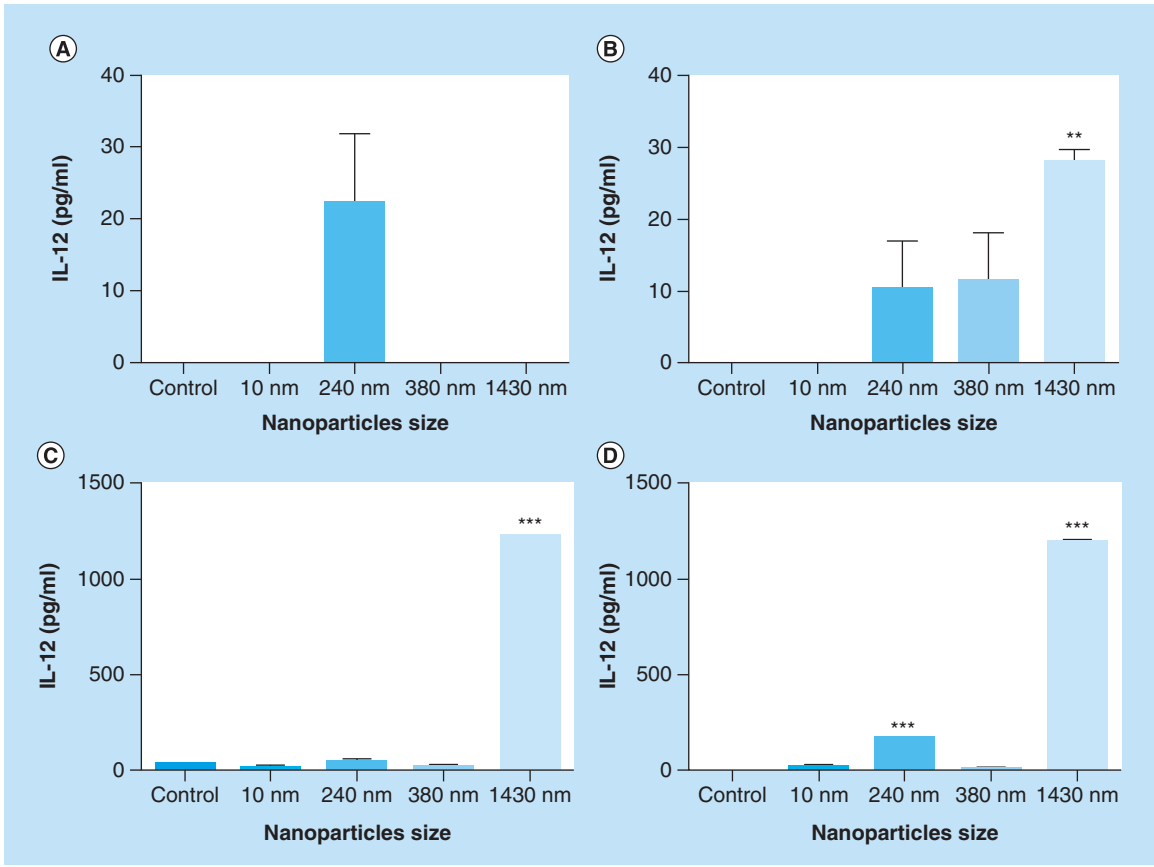

Figure 4. IL-12 secretion.
Supernatants of monocytes/macrophages cultured with different size nano- and microparticles were analyzed by ELISA for IL-12 production at **(A)** 24 h; **(B)** 48 h; **(C)** 72 h; and **(D)** 96 h. Results represent the mean ± SD of triplicate experiments.
**p < 0.01; ***p < 0.001.
SD: Standard deviation.

more significant for Si10 and Si240 particles (~300%) followed by Si380 (~200%) and at last Si1340 (~150%) (Figure 6B). In parallel, results obtained for CD80 and CD86 determinations showed that the exposure to SiOH particles increased the expression of co-stimulatory markers in THP-1 cell membranes. In both cases, Si240 particles cause the higher increase in the expression of these markers (Figure 6C & D). On the contrary, all SiOH particles, except Si10 NPs, decrease THP-1 cells CD11 expression and most of them do this in a size-dependent significant way (~ 50–70%) (Figure 6E).

Monodisperse spherical bare silica particle-induced cell death & membrane damage

With the aim to study the membrane integrity and cell viability, cells were incubated with different particles during 24–96 h and analyzed with the LIVE/DEAD® viability/cytotoxicity kit (L-3224, Molecular Probes, CA, USA) in accordance with the manufacturer's instructions. This two-color assay allows the discrimination of living versus dead cells based on esterase activity (resazurin) and membrane integrity (Sytox green incorporation), respectively. Upper right and lower right quadrants are positive for Sytox green incorporation; upper right and upper left quadrants are positive for resazurin determinations. We observed, at 24 h of culture, that Si380 and Si1430 particles increased the incorporation (~100%) of Sytox green by THP-1 cells in comparison to nontreated cells (Figure 7A–E).

Moreover, Si1430 particles decreased the metabolic activity (~65%) of THP-1 cells with respect to controls, as determined by resazurin incorporation (Figure 7A–E). These results suggest that most of these particles affect the viability of cells, especially bigger ones such as Si1430, where a significant reduced number of living cells were detected. Unexpected results were observed with Si240 particles, since this behavior is similar to Si10 without altering cell metabolic activity and membrane integrity. When cells were analyzed after 48 h of culture, similar

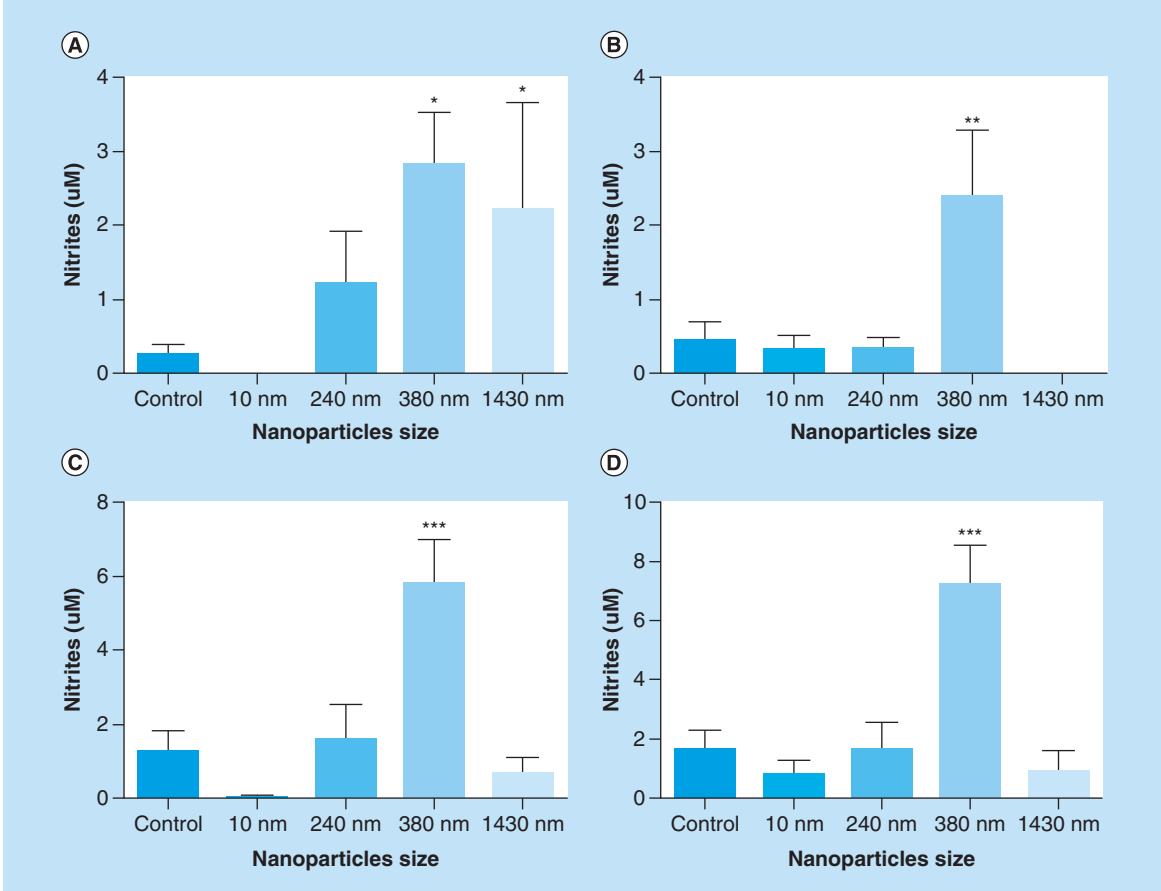

Figure 5. Nitrites secretion.
Supernatants of monocytes/macrophages cultured with different size nano- and microparticles were tested using Griess reactive for nitrites production at **(A)** 24 h; **(B)** 48 h; **(C)** 72 h; and **(D)** 96 h. Results represent the mean ± SD of triplicate experiments.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
SD: Standard deviation.

results were obtained. SiOH particles increased the incorporation of Sytox green by THP-1 cells in comparison to nontreated cells, principally Si380 and Si1430 ones (more than 100%) (Figure 7F–J). Moreover, the same particles after 48 h of culture, significantly decreased the metabolic activity of THP-1 cells with respect to controls, as determined by resazurin incorporation (Figure 7F–J). Comparable results were observed at 72 h of cultures where SiOH particles of 380 and 1430 nm increased the incorporation of Sytox green by THP-1 cells (Figure 7K–O). Furthermore, Si1430 particles decreased THP-1 cell metabolic activity with respect to control as observed for 24 and 48 h of culture (Figure 7K–O).

Discussion

We obtained SiOH particles by the Stöber method, with diameters ranging from 10 to 1430 nm. All particles presented negative charge between -28 and -40 mV, suggesting that there is sufficient electrostatic repulsion to ensure colloidal stability.

The effect of SiOH particles over cell cultures was evaluated for a period of 24–168 h. Cells exposed to different particles were able to proliferate but to a lower extent when compared with control cells not exposed to particles. A clear inhibition in the percentage of proliferation of the cells exposed to bigger particles for all concentrations assayed (Figure 1) was observed. The strange kinetics of cells in the presence of particles of 380 and 1430 nm could be due to the fact that at 7 days of culture, the cells began to die due to the high density of the cells. At this time, overproliferation induces cell death in all conditions, but is greater with the larger particles, so that their relative metabolic activity is lower. As mentioned before, most results described refer to cells cultivated in the presence of

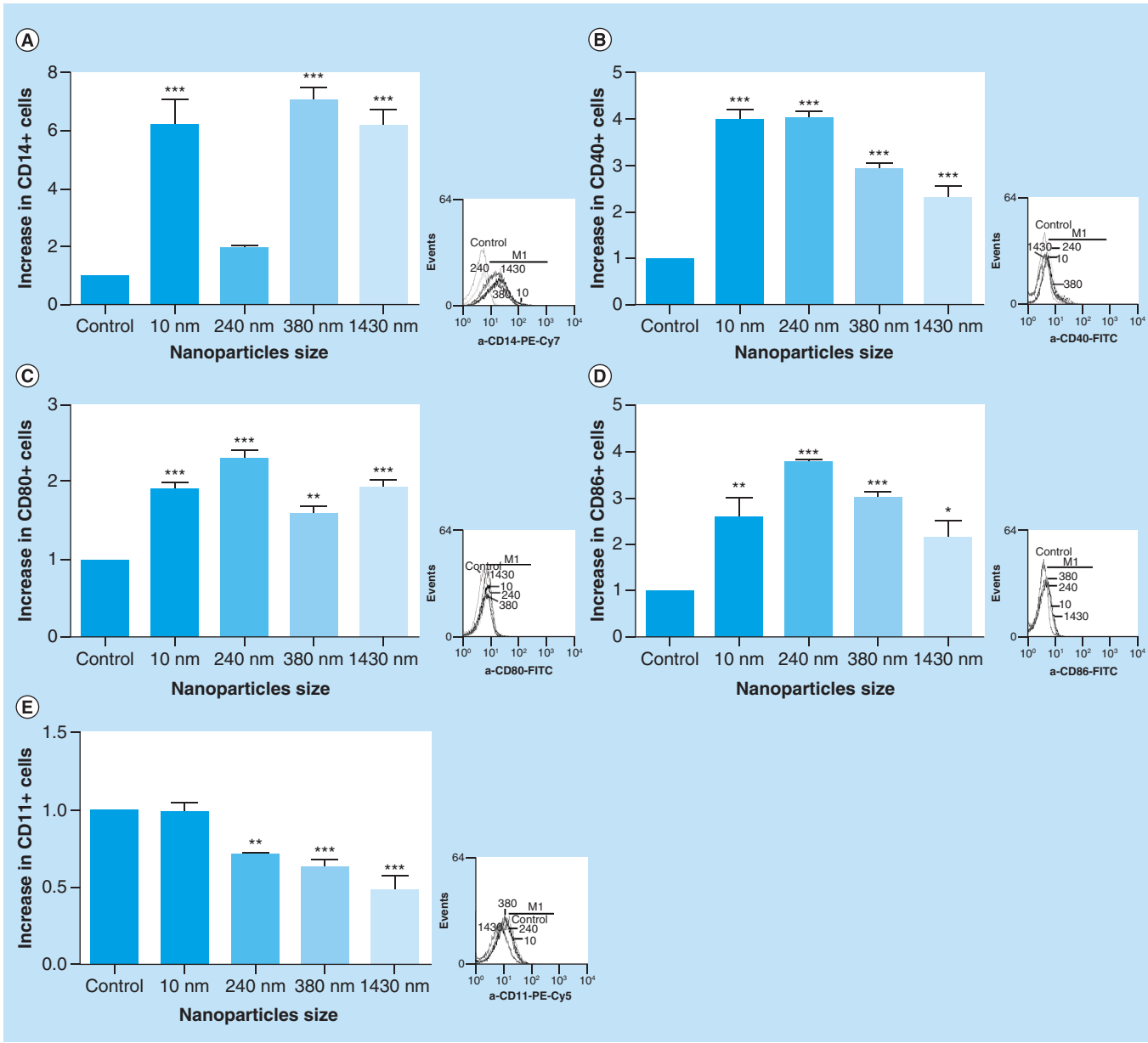

Figure 6. Effect of silica particles over THP-1 cell membrane markers.
THP-1 cells were treated with negative particles and then incubated with anti-human CD14, CD40, CD80, CD86 and CD11 antibodies. Flow cytometry results were expressed as the percentage of positive cell population presented in the M1 region of cells treated with particles/control cells (untreated). **(A)** CD14; **(B)** CD40; **(C)** CD80; **(D)** CD86; and **(E)** CD11 expression on THP-1 cell membrane treated with particles. Insets: cytometry histogram. Results represent the mean ± SD of triplicate experiments.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
SD: Standard deviation.

0.6 mg ml^{-1} SiOH particles, the high concentration used with differential performance between Si10 and others. The observed cell proliferation is between 40 and 50% lower in the presence of SiOH particles (Si240, Si380 and Si1430) with respect to control (Figure 2). This behavior confirms the existence of a size-dependent effect on cell proliferation when exposed to silica particles. Indeed, the proliferation of the cells was not affected only by NPs of 10 nm (Figure 2). As can be observed in Figure 2, until 120 h exposure to 10 nm SiOH NPs, the viability and proliferation was similar to control cells. In contrast, Fedeli *et al.*, [45] reported that 24 nm SiO_2-NPs have an IC_{50} around 30 ug ml^{-1}, 1 day after using human blood primary monocytes. There are several plausible explanations to this differential concentration-dependent effect. In one hand, the difference between primary cultures and

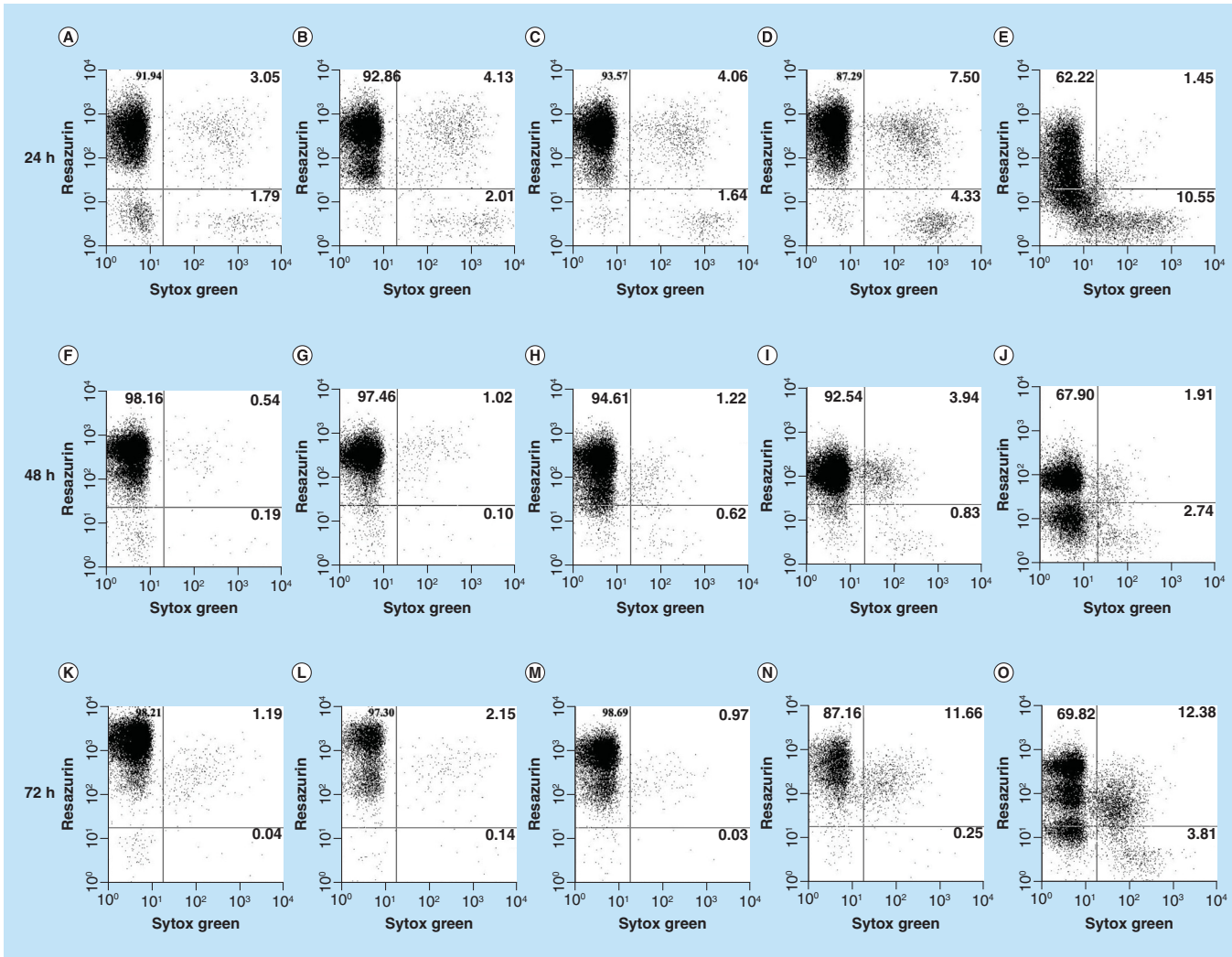

Figure 7. Cell membrane damage by 24–72 h of culture.
Monocytes were incubated with silica particles, stained with Sytox green/resazurin and finally analyzed by flow cytometry. Live/dead analysis of THP-1 cell cultures without **(A, F and K)** or with NPs of 10 nm **(B, G and L)**, 240 nm **(C, H and M)**, 380 nm **(D, I and N)** and 1430 nm **(E, J and O)**.
NP: Nanoparticle.

cultures of cell lines is well-documented, thus it is possible that the nanotoxicity is also different. In line with this observation, the mentioned authors also analyzed the HeLa cell line and observed a significantly higher IC_{50} either because it was not a phagocytic cell or because it was a cell line. In addition, Liu *et al.* [46] observed a toxic effect of SiO_2 NPs (25 nm) over human umbilical vein endothelial cells (HUVECs), but not over THP-1 cells. It is worth mentioning that THP-1 is a cell line established as a model for phagocytosis. Moreover, 1,25-dihydroxyvitamin D3-differentiated THP-1 cells do not develop a complete macrophage phenotype. Thus, they retain an important proliferation capacity whereas most primary culture monocytes do not. In conclusion, the difference lies in the fact that the NPs were incorporated into a culture with cells that retain important proliferative capability. Another difference is that THP-1 culture cells are homogenous cells whereas primary cultures usually have a high percentage of the desired cells but usually have greater heterogeneity. On the other hand, it is true that NPs of 10 nm are smaller than those of 24 nm, so it cannot be ruled out that they have different behaviors. In addition, the adsorption of proteins would be higher in smaller NPs due to their higher surface to weight ratio. Thus, the effect of FBS would be responsible for an increase in the IC_{50} [47,48], in agreement with the results obtained in this work.

When we analyzed cytokine secretion by THP-1 cells, we found that silica particles induced the production of IL-8 (Figure 3), IL-12 (Figure 4) and nitrites (Figure 5), and that the levels of these molecules in culture supernatants

augment during the time. On the contrary, we observed that 10 nm silica NPs do not induce IL-12 or nitrite secretion all over the different times evaluated; they only slightly stimulated IL-8 secretion. The strong increase in IL-12 levels between 48 and 72 h of culture was noteworthy. This could be due to a strong proinflammatory reaction and a related response to the toxic shock induced by said particles. In summary, it is possible that the cells have unconventional reactions as they are inquiring and trying to phagocyte particles that are approximately the size of a bacterium but many of the pathogen-associated molecular pattern (PAMP) receptors that would normally be activated are not doing so.

IL-12 is an important link between the innate and adaptive immune response and induces the secretion of IFN-γ by natural killer cells and T cells, which will increase the activation of macrophages. IL-8 is a chemokine involved in attracting neutrophils while nitrite levels are indicator of macrophages activation. The levels of proinflammatory cytokines produced by cells exposed to SiOH particles are in agreement with the increased levels described previously with different particles. Indeed, similar effects were previously reported with NPs produced with poly(γ-glutamic acid). It was reported that poly(γ-glutamic acid)-NPs induced the production of TNF-α, IL-12 and IL-6 by macrophages [49]. But, not all NPs could induce nitrite secretion and even less maintain their secretion over time. Nitrites are part of microbicidal mechanism used by phagocyte cells to destroy invading agents. Particles and bigger NPs are able to markedly induce this mechanism but smaller ones are not. Indeed, Si1430 are microparticles and their behavior may be different. Altogether, this evidence put silica particles as potential immunomodulatory agents. Our results show that SiOH particles induce innate immune cell activation *in vitro* and could have vast potential both as an adjuvant and in delivery of antigens in a vaccine development [49]. Other authors showed that the NALP3 inflammasome is activated by alum, uric acid, silica as well as PLGA NPs [50]. In the same way, Liu *et al.* reported that SiO_2 particles upregulated the production of IL-6, IL-8, MCP-1, TNF-α or IL-1β in a monocyte/HUVEC co-culture [46]. But they did not find that THP-1 cells alone cultured with SiO_2 particles secreted detectable IL-6, IL-8, MCP-1, TNF-α or IL-1β. They proposed that SiO_2 particles can indirectly activate monocytes, contrary to our findings. Other authors suggested a proinflammatory role for same SiOH NPs [51]. Results obtained with Si10 NPs suggest that these NPs are more biocompatible and less proinflammatory, with the potential to be used in delivery of molecules for several purposes unlike immune stimulation.

All particles affect the expression of the different membrane markers assayed (CD11, CD14, CD40, CD80 and CD86). Cell activation by silica particles are evidenced by an increased expression of CD14, CD40, CD80 and CD86 molecules on monocyte/macrophage membrane (Figure 6). These evidences propose a proinflammatory role of bare silica particles. Previously, Uto *et al.* described that poly(γ-glutamic acid)-NPs enhance the expression of co-stimulatory molecules, such as CD40, CD80 and CD86 on macrophage membrane [49]. On the contrary, Liu *et al.* did not find CD40 expression in THP-1 cells exposed to SiO_2 particles [46]. No relationship was found between CD increased expressions and SiOH particle sizes. The increased expression of CD14, a molecule involved in PAMP recognition, is vastly significant in the context of a potential infection. In addition, enhanced CD80 and CD86 molecules would amplify co-stimulatory signal for antigen presentation and CD40 would increase lymphocyte activation. CD40 and CD40L act as co-stimulatory molecules that are indispensable for antigen-presenting cells and activated $CD4^+$ T cells. This is an important aspect, since these molecules, employed in correct concentrations, could be used as an adjuvant for immune response against same microbial antigens [43]. In contrast, results with CD11 molecules, which decrease expression when cells are exposed to SiOH particles, are contradictory. Low expression of CD11 could affect the adhesion of monocytes to the endothelium and among its ligands is the fragment of the complement factor iC3b (key in the innate immune defense) and PAMPs, among others. It was reported that in dendritic cells, CD11 expression is important in the regulation of the quantity and quality of immune responses [52].

The fact that some SiOH particles inhibit THP-1 cell proliferation suggest that those particles could alter immune response affecting one of the antigen presenting cells, monocytes, proliferation and consequently, altering the number of cells able to present antigens. But activation observed as increment of CDs in cells treated with bigger particles, could be associated to a monocyte maturation phenomenon. A different behavior is observed with the smallest NPs (i.e., 10 nm), where cells could be activated without affecting cell proliferation.

When we studied the effect of SiOH particles on cell viability and membrane integrity, we observed that Si380 and Si1430 particles increased membrane damage and decreased cell metabolic activity (Figure 7A–E). These results suggest that bigger NPs and microparticles affect the viability of cells, where a significant reduced number of living cells were detected. Smallest NPs (Si10 and Si240) did not alter metabolic cell activity and membrane integrity. Similar results were obtained during different times evaluated (Figure 7F–O). These results are partially

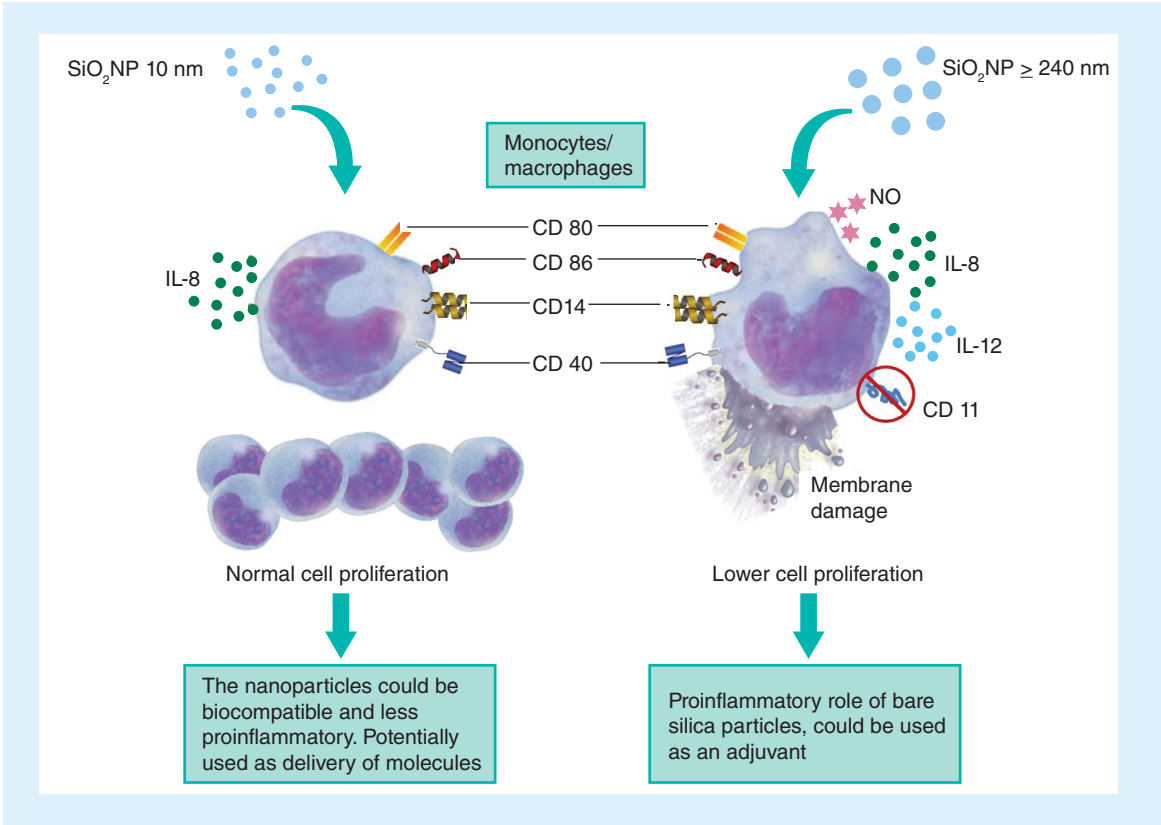

Figure 8. Schematic representation of the effect of silica micro- and nano-particles on basal and specialized monocyte functions.

in agreement with those reported by Liu *et al.* [46], where they observed a toxic effect of SiO_2 particles (25 nm) over HUVECs, but not over THP-1 cells.

Conclusion

We observed the existence of a particle size-dependent effect on cell proliferation. When THP-1 culture cells were exposed to SiOH particles, only particles (≥200 nm) induced significantly lower cell proliferation. On the other way, we found that most SiOH particles increased the production of IL-8, IL-12 and nitrite with respect to control cells. The levels of cytokine secretion increase during the times evaluated. SiOH NPs of 10 nm did not induce the production of IL-12 or nitrites during all times assayed. Similarly, we observed that all SiOH particles induce cell activation, evidenced by an increased expression of CD14, CD40, CD80 and CD86 molecules on the THP-1 cell membrane but all SiOH particles except 10 nm ones, decrease CD11 expression. At last, we observed that the biggest SiOH particles affect membrane integrity and viability of cells (Figure 8). All these evidences suggest a proinflammatory role of bare silica particles since these molecules, employed in the proper concentrations, could be used as an adjuvant for immune response against microbial antigens. On the contrary, results with NPs (10 nm) suggest that these NPs could be more biocompatible and less proinflammatory with the potential to be used in delivery of small molecules, as chemical drugs, for several purposes unlike immune stimulation. Other molecules could be transported on 10 nm NP surface. This conclusion could not only be applied for the size of 10 nm, since we still have to analyze what happens with particles in the range of 10–200 nm. Our results contribute to the development of safely therapeutic applications of nanotechnology.

Future perspective

Several diseases of different origin are currently affecting our planet and hopefully others already controlled may resurface as well as new or unknown will appear in the coming years. Therefore, the development of appropriate immunotherapy strategies will be necessary for the control of diseases. NPs offer a very important alternative for

immunotherapeutic approaches. These may, in the future, not only be used for the transport of molecules but may also transport antigens and immunomodulatory molecules. NPs with intrinsic immunostimulation capacity could be the basis of new vaccine strategies that slowly release the antigens they carry or use in immunostimulatory formulations. Alternatively, NPs with less proinflammatory activity may be used for the transport of drugs used in the treatment of endocrine diseases, cancer, along other important diseases.

Summary points

- Monodisperse spherical bare silica (SiOH) particles ≥200 nm induced significantly lower THP-1 cell proliferation.
- SiOH particles ≥200 nm increased the production of IL-8, IL-12 and nitrites by THP-1 cells but 10 nm ones did not induce the production of IL-12 or nitrites.
- All SiOH particles induce cell activation (by an increased expression of CD14, CD40, CD80 and CD86 molecules on the THP-1 cell membrane) but decrease CD11 expression.
- SiOH particles ≥380 nm affect membrane integrity and viability of cells.
- SiOH particles ≥200 nm are proinflammatory and could be used as an adjuvant for immune response against microbial antigens.
- Nanoparticles (10 nm) are more biocompatible and less proinflammatory with the potential to be used in delivery of molecules for several purposes unlike immune stimulation.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

1 Wang X, Ahmed NB, Alvarez GS *et al.* Sol-gel encapsulation of biomolecules and cells for medicinal applications. *Curr. Topics med. Chem.* 15(3), 223–244 (2015).

2 Johnson NR, Wang Y. Drug delivery systems for wound healing. *Curr. Pharm. Biotechnol.* 16(7), 621–629 (2015).

3 D'este M, Eglin D, Alini M, Kyllönen L. Bone regeneration with biomaterials and active molecules delivery. *Curr. Pharm. Biotechnol.* 16(7), 582–605 (2015).

4 Baeza A, Manzano M, Colilla M, Vallet-Regí M. Recent advances in mesoporous silica nanoparticles for antitumor therapy: our contribution. *Biomater. Sci.* 4(5), 803–813 (2016).

5 Martínez-Carmona M, Colilla M, Vallet-Regí M. Smart mesoporous nanomaterials for antitumor therapy. *Nanomaterials* 5(4), 1906–1937 (2015).

6 Vallet-Regí M, Ruiz-Hernández E, González B, Baeza A. Design of smart nanomaterials for drug and gene delivery. *J. Biomater. Tissue Eng.* 1(1), 6–29 (2011).

7 Pina S, Oliveira JM, Reis RL. Natural-based nanocomposites for bone tissue engineering and regenerative medicine: a review. *Adv. Mater.* 27(7), 1143–1169 (2015).

8 Hélary C, Desimone MF. Recent advances in biomaterials for tissue engineering and controlled drug delivery. *Curr. Pharm. Biotechnol.* 16(7), 635–645 (2015).

9 Chen Y, Chen H, Ma M *et al.* Double mesoporous silica shelled spherical/ellipsoidal nanostructures: synthesis and hydrophilic/hydrophobic anticancer drug delivery. *J. Mater. Chem.* 21(14), 5290–5298 (2011).

10 Song M, Song J, Ning A *et al.* Feasibility study of silica sol as the carrier of a hydrophobic drug in aqueous solution using enrofloxacin as the model. *Mater. Sci. Eng. C* 30(1), 58–61 (2010).

11 Du L, Liao S, Khatib HA, Stoddart JF, Zink JI. Controlled-access hollow mechanized silica nanocontainers. *J. Am. Chem. Soc.* 131(42), 15136–15142 (2009).

12 Liu Y, Lou C, Yang H, Shi M, Miyoshi H. Silica nanoparticles as promising drug/gene delivery carriers and fluorescent nanoprobes: recent advances. *Curr. Cancer Drug Targ.* 11(2), 156–163 (2011).

13 Alvarez GS, Alvarez Echazú MI, Olivetti CE, Desimone MF. Synthesis and characterization of ibandronate-loaded silica nanoparticles and collagen nanocomposites. *Curr. Pharm. Biotechnol.* 16(7), 661–667 (2015).

14 Alvarez GS, Hélary C, Mebert AM, Wang X, Coradin T, Desimone MF. Antibiotic-loaded silica nanoparticle-collagen composite hydrogels with prolonged antimicrobial activity for wound infection prevention. *J. Mater. Chem. B* 2(29), 4660–4670 (2014).

15 Mebert AM, Aimé C, Alvarez GS *et al.* Silica core-shell particles for the dual delivery of gentamicin and rifamycin antibiotics. *J. Mater. Chem. B* 4(18), 3135–3144 (2016).

16 Voisin H, Aimé C, Coradin T. Understanding and tuning bioinorganic interfaces for the design of bionanocomposites. *Eur. J. Inorg. Chem.* 2015(27), 4463–4480 (2015).

17 Du X, He J. Spherical silica micro/nanomaterials with hierarchical structures: synthesis and applications. *Nanoscale* 3(10), 3984–4002 (2011).

18 Coradin T, Boissière M, Livage J. Sol-gel chemistry in medicinal science. *Curr. Med. Chem.* 13(1), 99–108 (2006).

19 Hudson SP, Padera RF, Langer R, Kohane DS. The biocompatibility of mesoporous silicates. *Biomaterials* 29(30), 4045–4055 (2008).

20 Niu Y, Yu M, Zhang J *et al.* Synthesis of silica nanoparticles with controllable surface roughness for therapeutic protein delivery. *J. Mater. Chem. B* 3(43), 8477–8485 (2015).

21 Foglia ML, Alvarez GS, Catalano PN *et al.* Recent patents on the synthesis and application of silica nanoparticles for drug delivery. *Recent Pat. Biotechnol.* 5(1), 54–61 (2011).

22 Heinemann S, Coradin T, Desimone MF. Bio-inspired silica-collagen materials: applications and perspectives in the medical field. *Biomater. Sci.* 1(7), 688–702 (2013).

23 Qu Q, Ma X, Zhao Y. Targeted delivery of doxorubicin to mitochondria using mesoporous silica nanoparticle nanocarriers. *Nanoscale* 7(40), 16677–16686 (2015).

24 Dai L, Zhang Q, Shen X *et al.* A pH-responsive nanocontainer based on hydrazone-bearing hollow silica nanoparticles for targeted tumor therapy. *J. Mater. Chem. B* 4(26), 4594–4604 (2016).

25 Hu JJ, Liu LH, Li ZY, Zhuo RX, Zhang XZ. MMP-responsive theranostic nanoplatform based on mesoporous silica nanoparticles for tumor imaging and targeted drug delivery. *J. Mater. Chem. B* 4(11), 1932–1940 (2016).

26 Mebert AM, Camporotondi DE, Foglia ML *et al.* Controlling the interaction between cells and silica nanoparticles. *J. Biomater. Tissue Eng.* 3(1), 108–121 (2013).

27 Popat A, Hartono SB, Stahr F, Liu J, Qiao SZ, Qing Lu G. Mesoporous silica nanoparticles for bioadsorption, enzyme immobilisation and delivery carriers. *Nanoscale* 3(7), 2801–2818 (2011).

28 Mihalache R, Verbeek J, Graczyk H, Murashov V, Broekhuizen PV. Occupational exposure limits for manufactured nanomaterials: a systematic review. *Nanotoxicol.* doi:10.1080/17435390.2016.1262920 1–35 (2016) (Epub ahead of print).

29 Mody KT, Popat A, Mahony D, Cavallaro AS, Yu C, Mitter N. Mesoporous silica nanoparticles as antigen carriers and adjuvants for vaccine delivery. *Nanoscale* 5(12), 5167–5179 (2013).

30 Deville S, Baré B, Piella J *et al.* Interaction of gold nanoparticles and nickel(II) sulfate affects dendritic cell maturation. *Nanotoxicol.* 10(10), 1395–1403 (2016).

31 O'keefe SJ, Feltis BN, Piva TJ, Turney TW, Wright PFA. ZnO nanoparticles and organic chemical UV-filters are equally well-tolerated by human immune cells. *Nanotoxicol.* 10(9), 1287–1296 (2016).

32 Demuth P, Hurley M, Wu C, Galanie S, Zachariah MR, Deshong P. Mesoscale porous silica as drug delivery vehicles: synthesis, characterization and pH-sensitive release profiles. *Micropor. Mesopor. Mater.* 141(1–3), 128–134 (2011).

33 Heidegger S, Go, Schmidt A *et al.* Immune response to functionalized mesoporous silica nanoparticles for targeted drug delivery. *Nanoscale* 8(2), 938–948 (2016).

34 Carvalho LV, Ruiz RDC, Scaramuzzi K *et al.* Immunological parameters related to the adjuvant effect of the ordered mesoporous silica SBA-15. *Vaccine* 28(50), 7829–7836 (2010).

35 Mercuri LP, Carvalho LV, Lima FA *et al.* Ordered mesoporous silica SBA-15: a new effective adjuvant to induce antibody response. *Small* 2(2), 254–256 (2006).

36 Berntsen P, Park CY, Rothen-Rutishauser B *et al.* Biomechanical effects of environmental and engineered particles on human airway smooth muscle cells. *J. Royal Soc. Interf.* 7(Suppl. 3), S331–S340 (2010).

37 Fadeel B, Garcia-Bennett AE. Better safe than sorry: understanding the toxicological properties of inorganic nanoparticles manufactured for biomedical applications. *Adv. Drug Deliv. Rev.* 62(3), 362–374 (2010).

38 Nel A, Xia T, Mädler L, Li N. Toxic potential of materials at the nanolevel. *Science* 311(5761), 622–627 (2006).

39 Wittmaack K. In search of the most relevant parameter for quantifying lung inflammatory response to nanoparticle exposure: particle number, surface area, or what? *Environ. Health Perspect.* 115(2), 187–194 (2007).

40 Mody KT, Mahony D, Zhang J *et al.* Silica vesicles as nanocarriers and adjuvants for generating both antibody and T-cell mediated immune resposes to Bovine Viral Diarrhoea Virus E2 protein. *Biomaterials* 35(37), 9972–9983 (2014).

41 Stöber W, Fink A, Bohn E. Controlled growth of monodisperse silica spheres in the micron size range. *J. Colloid Interf. Sci.* 26(1), 62–69 (1968).

42 Thomassen LCJ, Aerts A, Rabolli V *et al.* Synthesis and characterization of stable monodisperse silica nanoparticle sols for *in vitro* cytotoxicity testing. *Langmuir* 26(1), 328–335 (2010).

43 De Marzi MC, Todone M, Ganem MB *et al.* Peptidoglycan recognition protein–peptidoglycan complexes increase monocyte/macrophage activation and enhance the inflammatory response. *Immunology* 145(3), 429–442 (2015).

44 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* 65(1), 55–63 (1983).

45 Fedeli C, Selvestrel F, Tavano R, Segat D, Mancin F, Papini E. Catastrophic inflammatory death of monocytes and macrophages by overtaking of a critical dose of endocytosed synthetic amorphous silica nanoparticles/serum protein complexes. *Nanomedicine* 8(7), 1101–1126 (2012).

46 Liu X, Xue Y, Ding T, Sun J. Enhancement of proinflammatory and procoagulant responses to silica particles by monocyte-endothelial cell interactions. *Particle Fibre Toxicol.* 18(9), 36 (2012).

47 Fedeli C, Segat D, Tavano R *et al.* Variations of the corona HDL: albumin ratio determine distinct effects of amorphous SiO2 nanoparticles on monocytes and macrophages in serum. *Nanomedicine* 9(16), 2481–2497 (2014).

48 Fedeli C, Segat D, Tavano R *et al.* The functional dissection of the plasma corona of SiO2-NPs spots histidine rich glycoprotein as a major player able to hamper nanoparticle capture by macrophages. *Nanoscale* 7(42), 17710–17728 (2015).

49 Uto T, Akagi T, Yoshinaga K, Toyama M, Akashi M, Baba M. The induction of innate and adaptive immunity by biodegradable poly(γ-glutamic acid) nanoparticles via a TLR4 and MyD88 signaling pathway. *Biomaterials* 32(22), 5206–5212 (2011).

50 Look M, Bandyopadhyay A, Blum JS, Fahmy TM. Application of nanotechnologies for improved immune response against infectious diseases in the developing world. *Adv. Drug Deliv. Rev.* 62(4–5), 378–393 (2010).

51 Winter M, Beer H-D, Hornung V, Krämer U, Schins RPF, Förster I. Activation of the inflammasome by amorphous silica and TiO2 nanoparticles in murine dendritic cells. *Nanotoxicology* 5(3), 326–340 (2011).

52 Kohrgruber N, Halanek N, Gröger M *et al.* Survival, maturation and function of CD11c- and CD11c+ peripheral blood dendritic cells are differentially regulated by cytokines. *J. Immunol.* 163(6), 3250–3259 (1999).