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### Mesoporous silica nano-adjuvant triggers pro-inflammatory responses in Caco-2/ peripheral blood mononuclear cell (PBMC) co-cultures

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#### Abstract

The aim of this study was to evaluate the cytotoxicity and immune-stimulatory effect of Mesoporous silica nanoparticle (MSN) Nano-adjuvant on pro-inflammatory cytokines and pattern recognition receptors (PRR) genes expression in Caco-2/PBMC co-culture model. MSNs were synthesized and characterized by scanning electron microscope (SEM), Brunauer Emmett Teller (BET) and Barrett Joyner Halenda (BJH) techniques. The BET specific surface area of MSNs was around 947 m<sup>2</sup>/g and the total pore volume and average pore diameter were 1.5 cm<sup>3</sup>/g and 8.01 nm, respectively. At the concentration of 10 µg/mL, MSN showed a low and time-dependent cytotoxicity on Caco-2 cells, while no cytotoxic effect was observed for 0.1 and 1 µg/mL concentrations after 24, 48 and 72 h. The expression of pro-inflammatory cytokines genes (IL-1, IL-8 and TNF- $\alpha$ ) in co-cultures treated with different concentrations of MSN showed a dose-dependent significant increase up to 17.44, 2.722 and 4.34 folds, respectively, while the expression augmentation of IL-1 gene was significantly higher than the others. This indicates slight stimulation of intestinal inflammation. Different concentrations of MSN significantly increased TLR4 and NOD2 expression to 4.14 and 2.14 folds, respectively. NOD1 was not affected significantly. It can be concluded that MSN might increase protective immune responses against antigens as a vaccine adjuvant candidate. It seems that stimulation of TNF- $\alpha$ , IL-1, and IL-8 expression in enterocytes probably transpires through the agonistic activity of MSN for TLRs including TLR4, while NOD2-associated signaling pathways are also involved. This study provides an overall picture of MSN as a novel and potent oral adjuvant for mucosal immunity.

#### **Keywords**

Caco-2, IL-1, IL-8, MSN, TLR, TNF- $\alpha$ 

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#### Introduction

Nanotechnology is the science of matter manipulation at the nanometer scale. This technology aids the development of medical devices.<sup>1</sup> A nanoparticle is categorized into different types based on dimensionality, morphology, composition, etc.<sup>2</sup> Several nanoparticles have hollow structure with adsorptive capacity and selective permeation.<sup>3</sup> Mesoporous silica nanoparticle (MSN) is composed of silica and possesses the nanopore sizes ranging from 2–50 nanometer (nm). MSN is suggested as a suitable carrier of drugs, enzymes, genes and protein delivery system. Some

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Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access page (https://us.sagepub.com/en-us/nam/open-access-at-sage). characteristics of MSN, such as porous structure, suitable surface area and biocompatibility makes it an appropriate oral vaccine adjuvant candidate.<sup>4,5</sup>

One of the main entry routes of the nanoparticle is through the gastrointestinal tract via oral digestion.<sup>6</sup> Nanoparticles are internalized by phagocytic cells (i.e. macrophage), thereby the interplay between them and the immune system can elicit an unfavorable immune response, such as inflammation.<sup>7</sup> They can enter into nonprofessional phagocytic cells, such as enterocytes via endocytosis and cause DNA damage.<sup>8</sup> Enterocytes play a critical role as intestinal barrier by inducing innate immunity and inflammatory responses as well as mucin and antimicrobial peptide production.<sup>9</sup> It is a fact that intestinal epithelial cells (IECs) are an important source of pro-inflammatory cytokines like IL-1, IL-8, and TNF- $\alpha$  which participate in an inflammatory process.<sup>10</sup> Following nanoparticles uptake by IEC, they translocate and accumulate in lymphoid rich organs, which might induce inflammation.<sup>8</sup> IL-1, IL-8 and TNF- $\alpha$  genes expression enhance the exposure of cell culture to more nanoparticles.<sup>11</sup> Furthermore, IEC affects immune cells beneath the epithelium by cytokine release and stimulate local inflammation, which elicits a stronger immune response to nano-vaccine adjuvant.12

recognition receptors (PRR) Pattern including nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) and toll-like receptors (TLRs) play a critical role in the recognition of pathogenassociated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs) and nanoparticle-associated molecular patterns or NAMPs which lead to activation of nuclear factor-jB (NF-jB) and interferon regulatory factors (IRFs).<sup>13,14</sup> TLRs and NODs stimulation is associated with induction of pro-inflammatory cytokines thereby evoking immune response by antigen-presenting cells.<sup>15</sup> Therefore, providing a concise overview of the role of a mucosal adjuvant in provoking immunity is of great significance from clinical perspectives.

Efficacy and toxicity of most mucosal adjuvants seem to be intrinsically linked, leading to the concept that a perfect adjuvant would hardly be achieved. This has moved the community toward designing effective mucosal adjuvant with reduced toxicity. This study intended to evaluate the immunostimulatory effect of different concentrations of MSN nano-adjuvant on IL-1, IL-8 and TNF $\alpha$  gene expressions in a co-culture model of human epithelial colorectal adenocarcinoma *cells* (Caco-2) and Peripheral Blood Mononuclear Cells (PBMCs). Furthermore, the study aimed to assess the probable contribution of TLRs in immune stimulation of MSN.

#### Materials and methods

The local Ethics Committee of Tarbiat Modares University reviewed and approved this study (IR.TMU.REC.1395. 339).

#### Cell culture

In this experimental study, human colon carcinoma cell line (Caco-2 cells) were obtained from Cell Bank of Iran (Pasteur Institute of Iran, Tehran) and cultivated in Dulbecco's Modified Eagle (DMEM) F12 (Gibco, Thermo scientific, USA) containing L-glutamine and high glucose, substituted with 10% fetal bovine serum (Gibco, Thermo scientific, USA) and 1% penicillin-streptomycin. The cell culture flasks were incubated at 37°C in an ambient humidity of 5% CO2.<sup>16</sup> Upon confluency, polarized Caco-2 cells express markers of colonocyte and macromolecules are sorted and retained between apical and basolateral surfaces.

#### Mesoporous silica nanoparticle synthesis (MSN)

MSN was synthesized according to the method by Wang and Kwon.<sup>17</sup> Briefly, 800 mg of CTAB was dissolved in a double-distilled water (D.D.W). Subsequently, 800  $\mu$ L of aqueous ammonia, 20 mL ethyl ether, and 20 mL ethanol was added and mixed in a round bottom flask and vigorously stirred. About 2.5 mL of tetraethyl ortho-silicate (TEOS) was added and vigorously stirred. After 4 h, the product was collected by centrifugation, washed and air dried. Calcination was done in 550°C for 5 h.

#### Characterization of the nanoparticles

SEM microscopy was performed to determine the morphology and size of the nanoparticles, using the KYKY-EM3200 device (Ontario, CA, USA). The MSNs surface area was examined via the nitrogen gas adsorption experiment using the Brunauer Emmett Teller (BET) technique in the range of a relative pressure of 0–1.0 mmHg. The sample was degassed for 2 h at 200°C before analysis. The pore size distribution (PSD) was calculated by the Barrett Joyner Halenda (BJH) method from the desorption branch of the isotherm curve.<sup>18</sup>

#### Assessment of MSN cytotoxicity for Caco-2 cells

MTT (Dimethyl thiazolyl diphenyl-tetrazolium bromide) assay was performed to evaluate the cytotoxic effect of MSN on Caco-2 cells.<sup>19</sup> Upon 90% confluency, the Caco-2 cells were trypsinized and placed into a 96 well plate (SPL life science, South Korea) at the density of  $2 \times 10^4$  cells/mL. Caco-2 cells were incubated for 24 h at 37° C in an atmosphere containing 5%CO2, treated with different concentrations of MSN (0.1, 1 and 10 µg/mL) for 24, 48 and 72 h. Afterward, 50 µL of the supernatant medium was replaced with MTT reagent and incubated for 4 h at 37°C. Subsequently, 100 µL of DMSO was added, mixed and related absorbance was measured at 570 nm. The following formula was used to calculate cell viability:

Cell viability (%) = OD of sample/OD of control  $\times$  100.<sup>19</sup>

#### Caco-2/(PBMCs) co-culture model

Caco-2/PBMC co-culture was used to mimic the enterocyte and mucosal immune system in the lumen. PBMCs were purified from buffy coat of a healthy adult volunteer whole blood by Ficoll-Hypaque centrifugation method (400×g, 20 min).<sup>20,21</sup> Purified PBMCs were washed twice with PBS and cultivated in DMEM and substituted with 10% fetal bovine serum and 1% penicillin-streptomycin and maintained at 37°C with 5% CO2. The Caco-2 cells were seeded in a 25 cm<sup>2</sup> cell culture flask at a density of  $2.5 \times 10^5$  cell/mL. Subsequently, 5 mL of DMEM F12 medium was enriched with 10% FBS and 1% penicillinstreptomycin, and was subsequently added to cultured PBMC at a density of  $2 \times 10^6$  cell/mL.<sup>22</sup>

#### RNA extraction and cDNA synthesis

Caco-2/PBMC co-culture was exposed to different concentration of MSN (1, 5, 10 and 50 µg/mL). Untreated co-culture was used as the control in all experiments. After treatment of the co-cultured model with different concentrations of MSN (0.1, 1, 10 and 50 µg/mL) for 24 h at 37°C in the CO2 atmosphere, Caco-2 cells were collected, lysed, and homogenized in trizol reagent (Sigma Aldrich, Germany) for RNA extraction. Chloroform was added to cell lysate and centrifuged (for 15 min at 4°C with speed of  $1200 \times g$ ) and the aqueous phase was collected. The 2-propanol was added to the liquid phase and re-centrifuged. Total RNA was extracted, washed, dried and dissolved in diethyl pyrocarbonate (DEPC) treated water. The concentration of RNA was measured by spectrophotometer (OD = 260 nm) and OD260/280 was used to estimate RNA purity. Purified samples with a ratio of 1.8  $\pm 2.0$  were used for cDNA synthesis by cDNA Synthesis Kit (Yekta TajhizAzma, IRAN). Briefly, 100 ng of total RNA, 50 µM Random hexamer, 50 µM Oligo-dT and DEPC treated water were blended and maintained at 70°C 5 min; thereafter, M-MLV (Moloney for Murine Leukemia Virus reverse transcriptase) (10,000 U), dNTP 10 mM, RNasin (40 u/µL), 5× first-strand buffer were added and the blend was treated for 60 min at 37°C. The reaction was completed by heating it at 70°C for 5 min.

#### Assessment of pro-inflammatory cytokines expression by real-time PCR

Real-time PCR was carried out, using Applied Biosystems 7500 (Thermo Fisher Scientific, Inc). The qPCR was accomplished, using Takara SYBR-Green assay kit under the following conditions: Pre-incubation at 95°C for 5 min, 45 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s Amplification of *IL-1*, *IL-8*, and *TNF-α* genes was performed, using the forward and reverse primers, shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as an internal control in all the experiments. The  $2^{-\Delta\Delta CT}$  value was calculated to achieve the fold changes.<sup>23</sup>

## Evaluation of stimulatory effect of MSN on of TLR4, NOD1 and NOD2 expression

TLR4, NOD1 and NOD2 are the first line of defense against pathogens in the gut which act as environmental sensors promoting innate immunity stimulation. The stimulatory effect of MSN on of TLR4, NOD1 and NOD2 expression was assessed by Real-time PCR using primers described in Table 1. Real-time PCR instrument and procedure was the as process described above. The expression of GAPDH gene was considered as the internal control for each sample and the  $\Delta$ CT (CT target – CT reference) of each sample and expression fold change were calculated. Each real-time PCR reaction was carried out in triplicate and results were presented as the Mean of triplicates.

#### Statistical analysis

All experiments were performed in triplicates and the data presented as Mean  $\pm$  SD. The Kruskal-Wallis nonparametric test was used to analyzes statistical data (using SPSS software, ver. 16) to compare the gene expression between treated and non-treated Caco-2/PBMC co-culture cell groups. *P* value <0.05 was considered to be statistically significant. The correlation between the MSN concentrations and different levels of gene expression was assessed by the Spearman test.

#### Results

#### Preparation and characterization of MSN

Analysis of MSNs by SEM microscopy exhibited the spherical shape and sized around 350-500 nm in diameter (Figure 1). The gas adsorption revealed many useful data about the surface and pore diameter of the nanoparticle. As it is depicted in Figure 2, nanoparticle showed Type IV adsorption-desorption isotherm which is the main characteristic of the mesoporous materials. The Brunauer-Emmett-Teller (BET) specific surface area of MSNs was detected around 947 m<sup>2</sup>/g and the total pore volume and average pore diameter were 1.5 cm<sup>3</sup>/g and 8.01 nm, respectively.

#### Cytotoxic effect of MSN on Caco-2 cell

MTT assay was performed to assess the MSN cytotoxic effect on Caco-2 cells. At the concentration of  $10 \,\mu\text{g/mL}$ , MSN showed 7, 8 and 10% inhibitory effect on Caco-2

| Gene  | Forward primer                | Reverse primer                  | Reference |
|-------|-------------------------------|---------------------------------|-----------|
| IL-1  | 5'-CCTGTCCTGCGTGTTGAAAGA-3'   | 5'-GGGAACTGGGCAGACTCAAA-3'      |           |
| IL-8  | 5-CTGGCCGTGGCTCTCTTG-3        | 5'-TTAGCACTCCTTGGCAAAACTG-3'    | 25        |
| ΤΝFα  | 5'-CCCTGGTATGAGCCCATCTATC-3'  | 5'-AAAGTAGACCTGCCCAGACTCG-3'    | 26        |
| TLR4  | 5'-GGAAGTTGAACGAATGGAATGTG-3' | 5'-ACCAGAACTGCTACAACAGATACT-3'  | 27        |
| NODI  | 5'-TCCCAGTTTAAGATGCGTGAG-3'   | 5'-AAGCGAAGAGCTGACCAAATAC-3'    | 28        |
| NOD2  | 5'-GGAAGCGAGACTGAGCAGACA-3'   | 5'-GCCACGGTGAAAGCGAAT-3'        | 29        |
| GAPDH | 5'-GGAAGGTGAAGGTCGGAGTCA-3'   | 5'-GTCATTGATGGCAACAATATCCACT-3' | 30        |

Table 1. The oligonucleotide primer sequences used for real-time PCR.



Figure 1. SEM photography of MSNs. The morphology and size are indicated.

cells in 24, 48 and 72 h, respectively, compared to untreated Caco-2 cell. In fact, MTT results showed time-dependent cytotoxicity of MSN on Caco-2 cells at the concentration of 10  $\mu$ g/mL, while no cytotoxic effect was observed for 0.1 and 1  $\mu$ g/mL concentrations of MSN on Caco-2 cells in 24, 48 and 72 h

## Stimulatory effect of MSN on TLR4, NOD1 and NOD2 expression in Caco-2/PBMC co-culture

The expression of TLR-4, NOD1 and NOD2 was assessed in response to different concentrations (1, 5, 10 and 50 µg/ mL) of MSN nano-adjuvant (Figure 3). The expression of TLR4 was increased to 2.83, 3.87, 3.91 and 4.14-fold, respectively (P < 0.05). The NOD2 expression was increased to 1.30, 1.51, 1.74 and 2.14 folds, respectively (P < 0.05), while NOD1 was not affected significantly (P > 0.05).

#### MSN impact on pro-inflammatory genes expression in Caco-2/PBMC co-culture

Real time-PCR was carried out to evaluate the gene expression levels of *IL-1*, *IL-8*, and *TNF-\alpha* in the co-culture model

which was exposed to different concentration of MSN (Figure 3). The expression rate of *IL-1* in co-cultures treated with different concentrations of MSN (1, 5, 10 and 50  $\mu$ g/mL) was significantly increased (P<0.05) by 2.12, 12.99, 15.34 and 17.44 folds, respectively in comparison with the untreated co-culture control. Moreover, in Caco-2/ PBMC co-culture after treatment with the same concentrations of MSN, IL-8 expression was significantly increased (P<0.05) (1.1, 1.739, 1.892 and 2.722 folds, respectively), in comparison with the controls. TNF- $\alpha$ gene expression level also revealed significant increase to 1.71, 3.02, 3.16 and 4.34 folds, respectively (P < 0.05), after exposure of co-culture cells to different concentrations of MSN. Expression augmentation of IL-1 gene was significantly higher than IL-8 and TNF- $\alpha$  genes (P<0.05) (Table 2).

#### Discussion

Mucosal adjuvants can trigger protective local and systemic immune responses, which is necessary for effective vaccinations against some infectious agents. Some mucosal adjuvants benefit from both immunostimulatory effect and delivery, among which mesoporous nanoparticles are of the major concern. Due to MSN unique characteristics, it is a potential adjuvant candidate for the oral vaccines. These properties are as follows: (1) a large amount of antigen can be loaded into MSN (2) release rate of antigen from this nanoparticle is slow (3) incorporation of antigen in MSN leads to retain antigen activity.<sup>6</sup> Considering that some nanoparticles are immunotoxic, the measurement of pro-inflammatory cytokine levels is a useful test for the assessment of nanoparticle immunotoxicity.31

Previous study by our laboratory has shown high efficacy of MSN nanoparticles in antigen drug stability and delivery, as well as adjuvant activity.<sup>32</sup> Moreover, immunostimulatory effect of MSN nanoparticles was also reported in many other studies.<sup>33</sup> This arouse the need for better understanding of probable mechanisms involved in immunostimulatory properties of MSN as a potent adjuvant.

TLR agonists are used as novel vaccine adjuvants which are able to provide long-lasting protection.<sup>34</sup> Our results



Figure 2. (A) The BET adsorption/desorption isotherm of MSN nanoparticles, (B) BJH graph of MSNs nanoparticles.

showed that MSN increases TLR4 expression levels in an intestinal co-culture model. It seems that MSN could play an important role as TLR4 agonist. TLR4-induced autophagy stimulates digestion of pathogens in autophagosomes.35 TLR4 agonists, such as aminoalkvl glucosaminide phosphates (AGPs), monophosphoryl lipid A (MPLA), and the MPLA-modified formulation (AS02 and AS04), has been demonstrated as immune adjuvants in preclinical experiments and clinical applications.<sup>36</sup> In a study by Tamavo and colleagues, it was revealed that Poly (Anhydride) nanoparticles has TLR2, 4 and 5 agonistic activity.<sup>37</sup> TLR4 is characterizes as the first line of defense against pathogens in the gut and is an environmental sensor promoting autophagy in innate immunity. Overall, therapeutic intervention that modulates autophagy through TLR4 may serve as an effective strategy for vaccine or adjuvant development. Hence, it can be concluded that MSN can be used as a promising nano-adjuvant due to TLR4 agonistic properties.

The NLRs are cytoplasmic receptors that play a crucial role in the innate immune response by recognizing various ligands from microbial pathogens (peptidoglycan, flagellin, viral RNA, fungal hyphae, etc.). They activate inflammatory responses and promote inflammasome formation, signaling transduction, transcription activation, and autophagy to remove pathogens at the site of bacterial entry. Inflammasome formation activates caspase-1.<sup>14</sup> which leads to the processing and maturation of proinflammatory cytokines as well as an inflammatory cell death termed pyroptosis.<sup>14</sup> Among NLRs, NOD1 and NOD2 were selected as they play a major contribution in pathogen recognition. Following treatment of Caco-2/PBMC co-cultures with MSN, NOD2 expression was significantly increased, while NOD1 showed no significant change. NOD1 recognizes D-glutamyl-meso-diaminopimelic acid (iE-DAP) of Gram-negative bacteria.<sup>14</sup> while NOD2 recognizes MDP from both Gram-positive and Gram-negative **Table 2.** The expression level of *IL-1, IL-8*, TNF- $\alpha$ , TLR4, NOD1 and NOD2 genes when exposed to different concentration of MSN ( $\mu$ g/mL) compared to non-treated co culture cells.

| Gene          | Expression rate (Fold changes) in different concentrations of MSN |               |              |               |               | b value |
|---------------|---|---------------|--------------|---------------|---------------|---------|
| Gene          | None  | l (μg/<br>mL) | 5(µg/<br>mL) | 10(µg/<br>mL) | 50(µg/<br>mL) | p-value |
| IL-I          | 1.00  | 2.12          | 12.99        | 15.34         | 17.44         | <0.05   |
| IL-8          | 1.00  | 1.1           | 1.739        | 1.892         | 2.722         | <0.05   |
| TNF- $\alpha$ | 1.00  | 1.71          | 3.02         | 3.16          | 4.34          | <0.05   |
| TLR4          | 1.00  | 2.83          | 3.87         | 3.91          | 4.14          | <0.05   |
| NODI          | 1.00  | 0.22          | 0.22         | 0.23          | 0.25          | >0.05   |
| NOD2          | 1.00  | 1.30          | 1.51         | 1.747         | 2.14          | <0.05   |

bacteria and viral ssRNA. Both NOD1 and NOD2 activate the nuclear factor kappa B (NF-kB) and MAPK signaling pathways, which play important role in regulating the host immune response, and enhance transcription of pro-inflammatory cytokines.<sup>14</sup> This suggests that MSN by induction of NOD2 expression contributes in general defense against Gram-positive and Gram-negative bacteria and viral pathogens.

In this study, Real time-PCR was carried out to evaluate the gene expression levels of *IL-1*, *IL-8*, and *TNF-\alpha* in the co-culture model which was exposed to different concentration of MSN. The expression rate of *IL-1*, *IL-8* and *TNF-\alpha* in co-cultures treated with different concentrations of MSN nanoparticle was significantly increased (*P* < 0.05) in comparison with the untreated co-culture control. MSN stimulation of cytokines production occurred in a dose-dependent manner which is important in governing the extent of stimulation of innate immunity and preventing inflammatory responses by adjusting the adjuvant dose in experimental challenges.

The reactogenicity of MSN nanoparticle was assessed by IL-8 measurement.<sup>38</sup> It was indicated that the stimulation of



Figure 3. (A) Non stimulated enterocyte, (B) MSN-stimulated enterocyte.

*IL-8* expression was significantly lower than *IL-1* and *TNF-\alpha* which confirms the low reactogenicity of nano-adjuvant (*P* < 0.05).

Pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-1, and IL-8 contribute to the inflammatory responses. TNF- $\alpha$  and IL-1 are associated with apoptosis, cell proliferation, cytokine production and induction of inflammation.<sup>39</sup> TNF $\alpha$  is an initiator of the innate immune response which is secreted by circulating macrophages in response to activation by either bacterial products or foreign particles including adjuvants. TNF $\alpha$  induces surrounding endothelial cells to produce interleukin-8 and E-selectin which are involved in phagocyte recruitment and phagocytic cell adhesion to endothelium, respectively.40 the surrounding Subsequently, IL-1 and additional TNF $\alpha$  are produced by macrophages.<sup>41</sup> IL-8 is produced by macrophage, monocyte and IECs while its expression is controlled by TNF- $\alpha$  and IL-1.<sup>42</sup> IL-8 is a potent inducer of neutrophil recruitment and the main cause of reactogenicity.<sup>38</sup> Our data revealed that MSN enhances TNF- $\alpha$ , IL-1 and IL-8 expression level (*P*<0.05) in the co-culture of Caco-2 cells and PBMC as a co-culture model of the intestine. Hence, it can be assumed that MSN increases TNF- $\alpha$  and IL-1 production in Caco-2 cells, which presumably cause inflammation in the small intestine.

A previous study by Nogueira et al. showed that titanium oxide nanoparticle enhances TNF- $\alpha$  production and promotes inflammation in the small intestine of mice model.<sup>43</sup> According to the study by Murugadoss and Fritsch-Decker, silica nanoparticles increase the production of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-1 through the human vein endothelial cells (HUVEC) and human colon cancer cells (HCT116).<sup>44,45</sup> Another study revealed that gold and silver nanoparticles increase the expression level of *TNF-* $\alpha$  and *IL-1* genes in macrophage cells.<sup>13</sup> Likewise, double-walled carbon nanotubes induce IL-1 production in human monocytes.<sup>46</sup> Kruger et al. showed that titanium dioxide nanoparticles stimulate IL-8 expression in Caco-2 cells. Indeed, an increase in IL-8 secretion in Caco-2 cells treated with silver nanoparticles was shown.<sup>47</sup> Brzicova et al. found that silver and silica nanoparticles enhance IL-8 expression levels in THP-1 macrophage-like cells.<sup>48</sup>

In contrast, in the site of vaccine delivery, a transient inflammation leads to immune cells attraction and an effective antigen uptake; thereby evoking better adaptive immunity responses.<sup>17</sup> It seems that MSN as adjuvant candidate increases the production of TNF- $\alpha$  and IL-1 through its TLR1 and TLR4 agonist activity, and promote local inflammation which induces an effective immune response. It is difficult to prove that a mucosal adjuvant is indeed safe. It seems that some adjuvants act as a two-wing flying object which trigger innate immunity and inflammation in each side. The balance between the two wings determines the efficacy of an adjuvant molecule.

#### Conclusion

In conclusion, MSNs (i) significantly stimulated the expression of TNF- $\alpha$ , IL-1 in intestinal co-culture model which is supposed to promote intestinal inflammation and eliciting a better protective immune response as a vaccine adjuvant candidate, (ii) MSN stimulation of cytokines production occurs in a dose-dependent manner which is important in controlling innate immunity vs. inflammatory responses, (iii) the reactogenicity of nano-adjuvant was significantly lower than its stimulatory effect and (IV) stimulation of TNF- $\alpha$ , IL-1, and IL-8 expression in enterocytes probably transpires through agonistic activity of MSN for TLRs including TLR4. This study provides an overall picture of MSN as a novel and potent oral vaccine adjuvant for mucosal immunity although these findings would greatly benefit from further studies in animal model.

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#### Author contributions

M.G. performed all the laboratory tests, also has major contribution in drafting the manuscript, B.B. supervised the study entirely and has participated in writing the results and conclusions, R. K. and S.S. had contribution in analysis of the data as well as drafting the manuscript.

#### **Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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#### **Ethics** approval

The study was reviewed and approved by the Medical Ethics Committee of Tarbiat Modares University (IR.TMU.REC.1395.339) before it began.

#### **Consent for publication**

Not applicable.

#### Availability of data and materials

The datasets of the current study are available within article file or can be obtained from corresponding upon request.

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