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Biomimetic nanoassembly for targeted antigen delivery and enhanced Th1-type immune response†

Zhenhua Li,[ab] Kai Dong,[ab] Yan Zhang,[c] Enguo Ju,[ab] Zhaowei Chen,[ab] Jinsong Ren*[a] and Xiaogang Qu*[a]

A new type of biomimetic nanoassembly for targeted antigen delivery and enhanced Th1-type response is reported for the first time, to combat the major challenges in the treatment of infected cells.

The development of vaccines constitutes one of the major medical breakthroughs in the treatment of numerous infectious diseases.[1] Many of the vaccines that are currently administered are derived from live-attenuated viruses or inactivated pathogens.[2,3] Although great efforts have been made for a large number of diseases, safety concerns have led to shifting the focus from traditional vaccines to protein-based subunit vaccines.[4,5] However, without an optimized delivery strategy or adjuvants, these protein-based vaccines are often unable to cross intestinal mucosal tissues due to degradation by blood proteases. In this regard, adjuvants are often added to the formulation for improving the immunogenicity of subunit vaccines.[6] Aluminium-based compounds (aluminium phosphate or hydroxide) have been widely used as licensed adjuvants for human vaccines, including diphtheria-tetanus-pertussis and hepatitis vaccines.[7] Nevertheless, aluminum salts induce mainly T helper (Th) 2 responses, which generally induce a humoral response in the defense against extracellular pathogens.[8] However, for major killers such as HIV, Hepatitis B and C, Th1-type response is necessary to elicit cellular immune responses, especially CD8 cytotoxic T cells (CTLs).[9,10] In particular, to achieve a potent anti-tumor CTL response, cell-mediated immunity is highly desired.[11] Thus, the development of less toxic and more effective vaccine adjuvants that induce Th1-type response would be highly important.

In the past few years, application of nanotechnology has provided researchers with a robust platform for the assembly of subunit vaccines.[12] Several particle-based vaccine delivery systems such as polymeric, lipid-based and mesoporous silica nanoparticles (MSNs) have been explored for protein-based immunotherapies.[13,14] These strategies have been shown to increase the uptake of antigens by antigen presenting cells (APCs), thereby enhancing the immunogenicity of the antigen. Although nanoparticles themselves may function as adjuvants through improving the uptake of antigens by APCs, they generally lack intrinsic immune-stimulatory effects. Recently, our group and others have shown that co-delivering antigens and immuno-stimulatory unmethylated cytosine-phosphate-guanine oligo-nucleotide (CpG ODN) to the same APC improves antigen-specific immune responses.[15,16] CpG ODN is a type of therapeutic nucleic acid with strong immunostimulatory activities through binding to Toll like receptor 9 (TLR9).[17,18] Since TLR9 is not a plasma membrane receptor, nanoparticles that present CpG ODN cannot be efficiently recognized and internalized by APCs.[19] Thus, these CpG ODN-based co-delivery systems are still confronted with the problem that particles cannot be efficiently recognized by targeted APCs.

Recently, lipopolysaccharide (LPS) derived from the cell wall of Gram-negative bacteria has been provided as an alternative to CpG ODN. LPS is a highly potent stimulus of the human immune system *via* the activation of TLR4 signaling.[20] LPS activates T cells to produce IFN-γ for enhancing cellular immune response.[21,22] Furthermore, unlike TLR9, TLR4 localizes at the plasma membrane of monocytes, and the binding of LPS to TLR4 induces the translocation of TLR4 from the cell surface to the endosome. Although LPS activates a Th1 immune response, it is responsible for a large variety of side effects including high fever, hypoperfusion, and metabolic dysfunction.[23] To overcome these limitations, large amounts of detoxified LPS have been prepared, including OM-174 and sodium phthalate salt of parent LPS (SP-LPS).[24,25] However, the application of detoxified LPS as both a targeted ligand and TLR4 agonist has not been realized.

In this work, we develop a biomimetic nanoassembly for targeted antigen delivery and enhanced Th1-type response based on detoxified LPS conjugated large pore MSNs (Scheme 1). We have previously demonstrated that large pore materials possessed

[a] *Laboratory of Chemical Biology and State Key Laboratory of Rare Earth Resources Utilization, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China. E-mail: jren@ciac.ac.cn, xqu@ciac.ac.cn; Fax: +86 431 85262625*

[b] *University of Chinese Academy of Sciences, Beijing 100039, China*

[c] *College of Life Science, Jilin University, Changchun, Jilin 130012, P. R. China*

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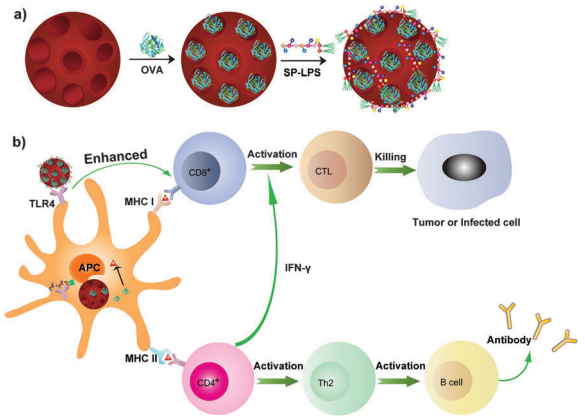

Scheme 1 (a) General scheme for the construction of protein antigens and the detoxified LPS co-delivery system. (b) A scheme depicting the strategy of how to restore the OVA-specific immunity by employing large pore MSN-based vaccines.

important advantages for functional and efficient biomacromolecule delivery.[26,27] In our system, MSNs with large pores were chosen as the hosts for antigen encapsulation. SP-LPS, a detoxified LPS, was conjugated to the surface of MSNs to assemble bacteria-like nanostructures. In this way, the SP-LPS modified MSNs (MSN–SP-LPS) would have the ability to mimic the behavior of bacteria with the capability of continued stimulation of APCs and skew the immune response to the Th1-type. In addition, owing to the specific interactions between SP-LPS and TLR4, surface modification of MSNs with SP-LPS would further enhance the recognition and internalization of antigen-loaded nanoparticles by targeted APCs. We envision that these antigen-loaded biomimetic nanoassemblies would provide a unique targeted co-delivery system for promoting a Th1-type immune response and CTL activity.

In our experiments, the used MSNs with large pore channels were synthesized according to the method reported previously.[28] Characterization of large pore materials is described in detail in Fig. S1 (ESI†). It is known that LPS has the ability to induce generation of high levels of H_2O_2 in APCs.[29] Thus arylboronic esters were chosen as the linkers because of their sensitivity to H_2O_2 in a physiological environment.[30] An advantage of this linkage strategy was that LPS could be released after internalization by APCs in the case of the possible influences on immunomodulatory and the subsequent release of antigens. Phenylboronic acid groups were introduced onto the outlet of amino-modified MSNs (MSN–CBA) using a carbodiimide reaction. The functional MSN was monitored by FTIR spectroscopy (Fig. S2, ESI†). Then, LPS was made nontoxic by reacting with phthalic anhydride. Subsequently, the arylboronic ester containing linker was grafted onto the surface of MSNs by the reaction of polysaccharide with CBA. The increase of the SP-LPS group was verified by FTIR. Thermogravimetric analysis (TGA) was performed to determine the density of SP-LPS grafted onto the MSN host (Fig. S3, ESI†). The cellular toxicities of MSN–SP-LPS were then evaluated by a MTT assay (Fig. S4, ESI†). MSN–SP-LPS was nontoxic up to the highest testing concentration of 1.6 mg mL^{-1}, indicating that nanocarriers were favorably biocompatible.

Ovalbumin (OVA) was chosen as a model vaccine in this study because it was one of the best characterized and understood antigens. The loading efficiency of OVA was determined at varied pH and the results showed that more OVA loaded into MSN at pH 5.0 (Fig. S5, ESI†). The high loading efficiency at low pH was likely attributed to the fact that low pH reduced the electronegativity of OVA (isoelectric point 5.0), resulting in decreasing electrostatic repulsion between the negatively charged nanoparticles (Fig. S6, ESI†) and OVA. To determine the saturation level of OVA loading on MSNs, we mixed MSN solutions with different concentrations of OVA at pH 5.0. The maximal OVA loading efficiency was 50% (w/w) under this loading condition (Fig. S7, ESI†). To investigate the OVA release behavior *in vitro*, FITC-modified OVA was prepared and loaded into RITC-labeled MSNs. As shown in Fig. S8a (ESI†), overlaying the images taken from RITC (red) and FITC (green) channels yielded many yellow spots, suggesting that most of the antigen molecules were trapped inside the pores after 1 h of incubation. However, many green spots were observed upon prolonging the incubation time, indicating that OVA was released from a nanocomplex (Fig. S8b, ESI†).

We then investigated the influence of SP-LPS surface modification on the internalization of nanoparticles by APCs. MSNs with and without SP-LPS were incubated with RAW264.7 cells and internalization was assessed by flow cytometry and confocal fluorescence microscopy (Fig. 1). Detoxified LPS-modified nanoparticles were internalized more effectively than MSNs alone after 2 h of incubation. Corresponding confocal fluorescence microscopy images were consistent with the flow cytometry results. The enhanced macrophage phagocytosis upon TLR stimulation was attributed to the special interaction between SP-LPS and TLR4. Previous reports have indicated that binding of LPS to TLR4 leads to the activation of NF-κB through the induction of H_2O_2 in APCs.[31] Then, NF-κB would regulate the expression of multiple immune molecules. We next studied the involvement of intracellular reactive oxygen species (ROS) generation upon TLR4 activation by SP-LPS-modified MSNs. DCFH, a commercially available fluorescent probe, was utilized in our experiment to prove the generation of ROS in stimulated macrophages. As shown in Fig. S9b (ESI†), strong intracellular fluorescence signals were observed after MSN@RITC-SP-LPS stimulation. In contrast, no distinct red fluorescence was seen when RAW264.7 cells were stimulated by MSN@RITC (Fig. S9d, ESI†). NF-κB comprises a family of transcription factor subunits that control expression of multiple immune-related genes in both innate cells and lymphocytes. In resting cells, NF-κB subunit p65 resides in the cytoplasm in an inactive form bound to inhibitor molecule IκB protein. TLR4-activation results in the nuclear translocation of p65 for initiating immune-related gene transcription, which enhances antigen presentation in APCs. We then tested the activation and translocation of NF-κB in RAW264.7 cells by immunostaining the p65 subunit of NF-κB parallel to DAPI. Confocal fluorescence microscopy images showed that nuclear translocation of NF-κB was observed in cells treated with MSN@FITC-SP-LPS (Fig. S9f, ESI†). These findings supported the idea that detoxified LPS modified larger pore MSNs could be efficiently internalized by APCs.

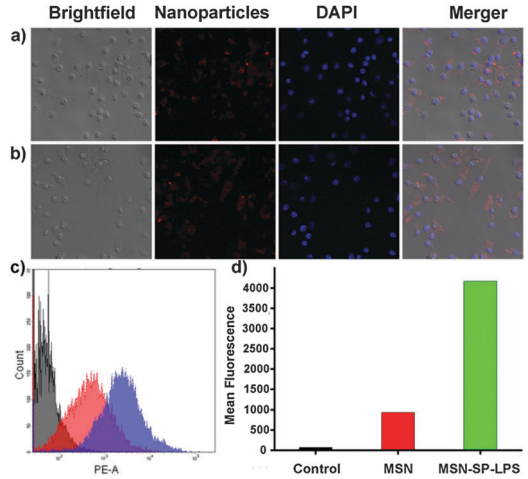

Fig. 1 Evaluation of uptake efficiency. RAW264.7 cells were incubated with MSN@RITC (a) and MSN@RITC-SP-LPS (b) and analyzed by confocal microscopy. (c) Flow cytometry analysis of RAW264.7 cells pulsed with MSN@RITC (red) and MSN@RITC-SP-LPS (blue). (d) Mean fluorescence is shown.

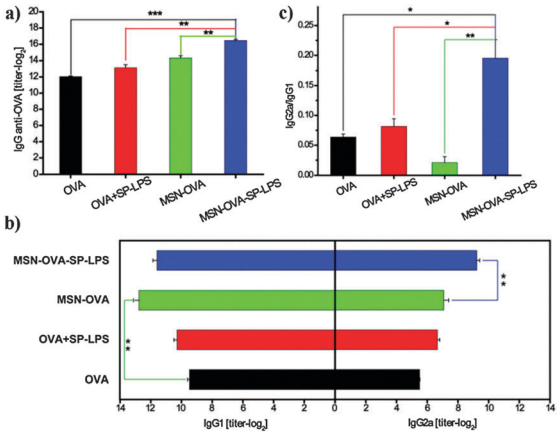

Fig. 2 ELISA results of anti-OVA IgG (a), IgG 1 and IgG 2a (b) titers elicited by different stimulus. (c) Each bar represents the average of the IgG2a : IgG1 ratio ± SD for each group. *$p < 0.01$, **$p < 0.005$ and ***$p < 0.001$.

The activation of innate immune systems is associated with the secretion of cytokines such as TNF-α. Activation of the RAW264.7 cells was then quantified by TNF-α secretion. Fig. S10 (ESI†) clearly illustrates that RAW264.7 cells treated with MSN–SP-LPS produced at least 15 times more TNF-α than that of MSN–CBA groups. This result established that MSN–SP-LPS could effectively stimulate the immune cells to an activated state. To study if this improved APC activation ability *in vitro* indeed correlated with the immunogenicity, mice were vaccinated subcutaneously with samples and the OVA-specific total serum IgG titres were measured (Fig. 2a). Since detoxified LPS-modified nanoparticles may cause a harmful effect or disease, we first investigated the long-term toxicity through histological assessment. No tissue damage or any other adverse effect was observed after the administration of MSN–SP-LPS (Fig. S11, ESI†). In addition, we did not find the infiltration of inflammatory cells in these organs, indicating that nanoparticles were nontoxic and would not induce systemic inflammation. Then we investigated the immune response arising from biomimetic nanoassemblies. OVA-loaded large pore MSNs positively affected the IgG response compared to OVA. Nanoparticles could protect antigens from degradation before being internalized by APCs and enhance the uptake of antigens. Importantly, we found that mice immunized with MSN–OVA–SP-LPS developed a much higher level of anti-OVA IgGs than the mixture of OVA and SP-LPS. We ascribed this effect to the effective internalization of OVA and SP-LPS into the same APCs. These results indicated that co-encapsulation of antigens and the TLR4 agonist in one nanoparticle could intensely enhance antigen-specific immune response.

Th1 type immune responses are necessary to elicit cellular immune responses, which is crucial in the treatment of chronic viral infections and cancer. To further investigate the type of immune response stimulated by the vaccination, the antibody subclasses IgG2a (Th1-type) and IgG1 (Th2-type) were analyzed (Fig. 2b). The main IgG subtype immunized with plain OVA was mainly IgG1 and the addition of the TLR4 agonist induced equally elevated IgG1 and IgG2a levels. Mice immunized with OVA-loaded MSNs produced more IgG1 antibodies than other formulations. However, the introduction of SP-LPS, MSN–OVA–SP-LPS formulation, resulted in the reduction in IgG1 antibodies and a significant increase in IgG2a antibodies. Furthermore, co-delivery of OVA with SP-LPS significantly shifted the IgG2a/IgG1 balance towards IgG2a, indicating that MSN–OVA–SP-LPS has the capabilities of skewing the immune response to the Th1-type (Fig. 2c). This alteration was mainly attributed to the fact that SP-LPS activated TLR4 and then induced the production of cytokine in relation to Th1-type response. The Th1-inducing effect of administered MSN–OVA–SP-LPS was further confirmed by the T-cell activation study. Splenocytes from immunized mice were restimulated with OVA and then OVA-specific IFN-γ was analyzed in the culture supernatants (Fig. 3). Splenocytes from mice vaccinated with OVA and MSN–OVA did not produce more IFN-γ, demonstrating a weak Th1 response. In contrast, the introduction of SP-LPS into OVA-loaded nanoparticles resulted in significantly more IFN-γ secretion from those splenocytes than that from others. IFN-γ secretion results showed that the addition of SP-LPS strongly shifted the T-cell polarization

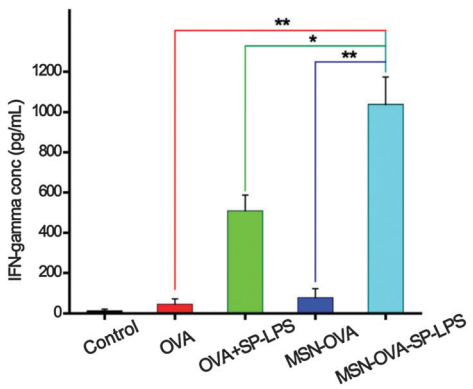

Fig. 3 IFN-γ production by splenocytes after restimulation with OVA. *$p < 0.01$, **$p < 0.005$.

towards the Th1 direction which was in agreement with antibody subclass results. Overall, SP-LPS modified MSN has the capabilities of eliciting strong Th1 type response, making it a promising vaccine carrier for antigens.

In summary, a new paradigm for promoting a Th1-type immune response and CTL activity has been demonstrated. In our system, detoxified LPS was conjugated to the antigen loaded large pore MSNs to assemble bacteria-like nanostructures, which could efficiently modulate the Th1 immune response through activating TLR4 signaling. Specifically, the TLR4 surface decoration effectively enhances the cellular uptake of nanoparticulate carriers and improves the selectivity between APCs and normal cells. As a result, our biomimetic nanoassembly can efficiently enhance Th1 immune response, which is essential to fully eliminate the infecting virus and cancer. Additionally, the nanoassembly reveals no significant cytotoxicity and adverse effects towards mammalian cells. With an excellent immune effect and high biocompatibility, our system provides a promising avenue to developing vaccines against a range of infectious diseases and cancer.

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