

Mesoporous silica particles potentiate antigen-specific T-cell responses

Aim: To study the adjuvant effect of mesoporous silica particles and their capability of modifying an already existing allergic Th2-like immune response. **Materials & methods:** The adjuvant effect of Santa Barbara Amorphous-15 (SBA-15) mesoporous silica particles was studied in an antigen-specific ovalbumin (OVA) system *in vitro* and *in vivo*. The capacity of the OVA-loaded SBA-15 particles (SBA-15-OVA) to modify an existing immune response was assessed in a murine allergy model. **Results:** SBA-15-OVA induced significantly stronger OVA-specific splenocyte proliferation compared with OVA alone. Significantly higher IFN- γ production was observed in *ex vivo* OVA-stimulated splenocytes from SBA-15-OVA-immunized mice compared with mice injected with only SBA-15 or OVA. Treatment of OVA-sensitized mice with SBA-15-OVA modified the immune response with significantly lower serum levels of OVA-specific IgE and higher IgG levels compared with the alum-OVA-treated group. **Conclusion:** The results are promising for the continued development of mesoporous silica materials for therapeutic applications.

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Keywords: adjuvant • allergy model • cytokine • mesoporous silica particle • ovalbumin • splenocyte • T cell

New efficient vaccines against different diseases are in high demand. Some important factors impeding vaccine development are poor immunogenicity of candidate antigens and lack of appropriate delivery systems that could elicit the desired immune responses. The discovery of novel vaccine adjuvants, capable of enhancing immunogenicity of the antigens and providing T-cell help can circumvent these problems. The most frequently used adjuvants until now are aluminium salts, generally called 'alum' [1]. Alum has a good safety record, but comparative studies show that it is a weak adjuvant for antibody induction to subunit vaccines and a poor adjuvant for cell-mediated immunity [2]. Furthermore, alum induces primarily Th2-like immune responses and could promote and enhance nontarget IgE synthesis in a genetically vulnerable subpopulation [3].

Alum is therefore less effective as an adjuvant in vaccines against diseases that require a more Th1-like response such as allergy [4], or problematic intracellular pathogens such as HIV and malaria parasites [5]. For these reasons, new adjuvants able to induce more Th1-like immune responses are desired.

Materials can be engineered in order to modulate immune responses. They can act by targeting specific intracellular pathways [6]. This can be exploited to control how antigens are presented to T cells by antigen-presenting cells (APCs) [6]. Mesoporous silica materials offer the possibility of delivering and releasing antigens in a controlled manner, which can lead to increased efficiency of the triggered immune response [7]. Mesoporous silica particles are thermally and chemically stable porous materials that can be synthesized to achieve different shapes and

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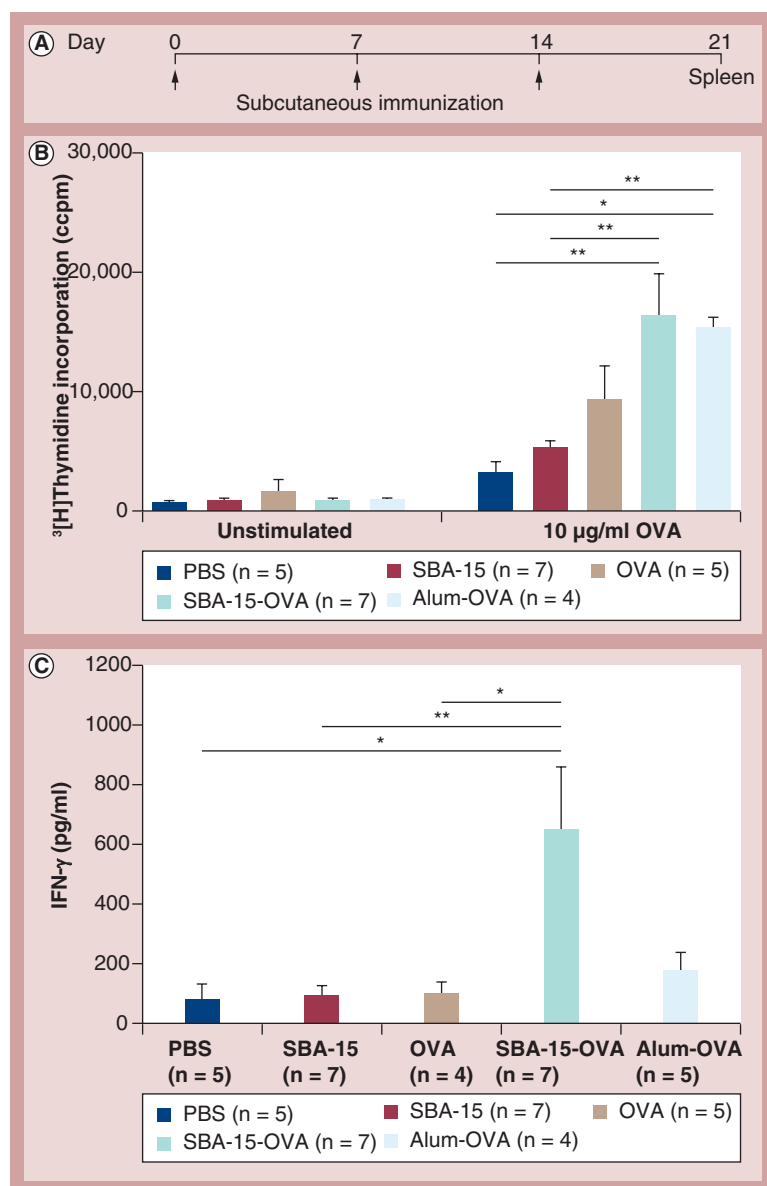


Figure 1. *In vitro* stimulation of splenocytes with ovalbumin from SBA-15-OVA-immunized mice induce increased cell proliferation and elevated levels of IFN-γ production. (A) Immunization protocol. BALB/c mice were immunized subcutaneously with SBA-15-OVA (containing 10 µg OVA), SBA-15 alone (same amount of SBA-15 as in the SBA-15-OVA stimuli) or 10 µg OVA three times in weekly intervals. Positive and negative control groups received alum-OVA (10 µg OVA at 1:1 ratio) and PBS, respectively. Mice were sacrificed on day 21 and spleens were collected for *ex vivo* stimulation. (B) Splenocytes from individual mice from all five groups were cultured *ex vivo* in triplicate in the presence or absence of OVA. [3H] thymidine, 1 µCi/ml, was added to the cultures after 96 h of incubation and the proliferation was measured after an additional 18 h. Results are expressed as mean ± standard error of the mean cell count per minute for each group. (C) The supernatants from the 96 h splenocyte cultures were analyzed for the presence of IFN-γ by ELISA. Results represent the mean ± standard error of the mean. Significant differences using a Mann–Whitney U test are indicated with asterisks (**p* < 0.05; ***p* < 0.01).

Alum-OVA: Alum-formulated ovalbumin; OVA: Ovalbumin; PBS: Phosphate-buffered saline; SBA-15: Santa Barbara Amorphous-15; SBA-15-OVA: Ovalbumin-loaded Santa Barbara Amorphous-15.

particle sizes [8]. They possess ordered porosity, high surface areas (~1000 m²/g), large pore volumes and can be loaded with substantial amounts of functional molecules within the pores. By using mesoporous particles as drug delivery systems, the solubility and stability of biomolecules and active pharmaceutical ingredients can be enhanced, and their release rate can be controlled. Those properties, amongst others such as stability and biocompatibility, make them promising as drug delivery systems, and as adjuvants in biomedical applications [8].

Recently, we have shown that mesoporous silica particles are able to tune naive T-cell responses via dendritic cell (DC) signalling *in vitro*. When human pure T cells were cocultured with monocyte-derived DCs pre-exposed to Santa Barbara Amorphous-15 (SBA-15) mesoporous silica particles, they could elicit both Th1 and Th2 cytokines [9]. SBA-15 possesses a 2D hexagonal mesoporous structure with pore sizes that can be increased up to 130 Å by addition of swelling agents during the synthesis [10]. Hence, SBA-15 is capable of entrapping large molecules and is especially attractive for biomedical applications such as delivery systems for immobilization or controlled release of enzymes [11], antigens [12] and drugs [13]. SBA-15 has been shown to enhance antibody production both in mice [12] and sheep [14] without producing local toxic effects or granuloma formation on the injection site, common for oily adjuvants. SBA-15 demonstrated adjuvant properties when used as a delivery vehicle for a single *Escherichia coli* bacterial protein (Int 1β) and venom proteins in mice resulting in increased serum antibody levels [12]. Furthermore, Carvalho *et al.* showed that while alum was unable to enhance the humoral response (IgG) against bovine serum albumin (BSA), SBA-15 loaded with BSA improved and modulated the anti-BSA antibody response of low-responder mice to the same level as high-responder mice, both through the intramuscular and oral routes of administration [15]. Considering the fact that SBA-15 has a good safety record and can modulate the antigen-specific immune response, in this study we aimed to investigate the adjuvant efficacy and immune modulatory properties of SBA-15 particles. Particles were loaded with hen egg ovalbumin (OVA; SBA-15-OVA) and tested both *in vitro* and *in vivo* in a murine OVA sensitization model with the aim to develop new treatments for allergy.

Materials & methods

Particle preparation

All chemicals were purchased from Sigma-Aldrich (Stockholm, Sweden) and used as received. The synthesis of SBA-15 has been described previously [16].

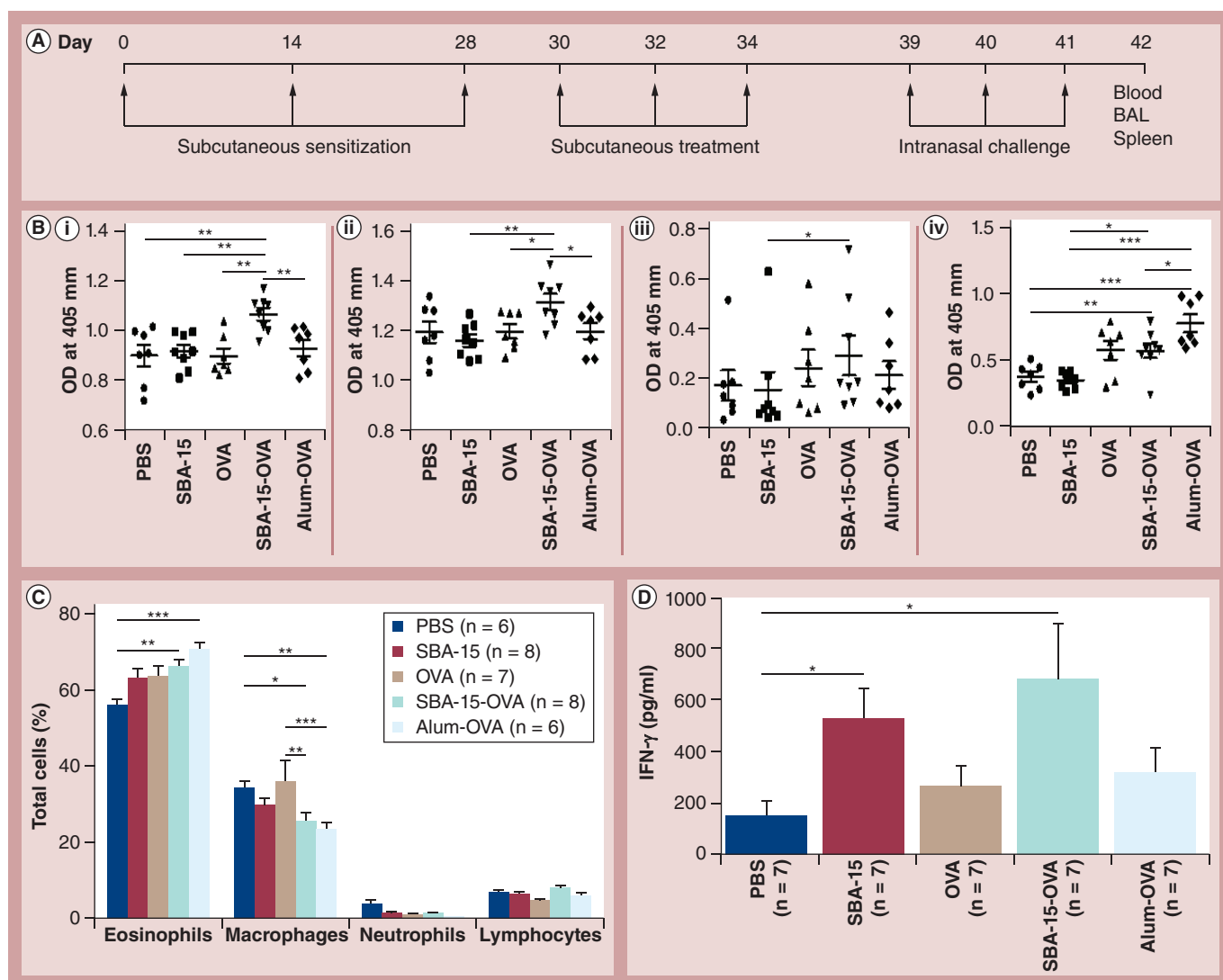


Figure 2. Treatment with ovalbumin-loaded Santa Barbara Amorphous-15 can modify an ongoing immune response to ovalbumin. (A) Treatment protocol. Balb/c mice were sensitized subcutaneously with 10 µg OVA three times every second week. Mice were then treated subcutaneously three times every second day with either SBA-15-OVA (containing 10 µg OVA), SBA-15 (same amount of SBA-15 as in the SBA-15-OVA stimuli) or 10 µg OVA. Alum-OVA and PBS were included as positive and negative controls, respectively. Daily intranasal challenge with 10 µg OVA followed during 3 days. Blood, BAL and spleen samples were collected at day 42. (B) Humoral response was assessed by OVA-specific ELISA. Horizontal lines indicate means. (B,i) IgG, (B,ii) IgG₁, (B,iii) IgG_{2a} and (B,iv) IgE. (C) Differential BAL cell count by light microscopy after cytopspin and staining with May-Grünwald/Giemsa stain. (D) Splenocytes were cultured and stimulated as described in the legend to Figure 1B. After 96 h of incubation the supernatants were analyzed for the presence of IFN-γ by ELISA. Results represent the mean ± standard error of the mean. The Mann-Whitney U test was used for statistical analysis in (B & D). Two-way analysis of variance with Bonferroni post-test was used for statistical analysis of the BAL cell count in (C). Significant differences are indicated with asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).

Alum-OVA: Alum-formulated ovalbumin; BAL: Bronchoalveolar lavage; OD: Optical density; OVA: Ovalbumin; PBS: Phosphate-buffered saline; SBA-15: Santa Barbara Amorphous-15; SBA-15-OVA: Ovalbumin-loaded Santa Barbara Amorphous-15.

Briefly, P123 triblock copolymer was dissolved together with 1,3,5-trimethylbenzene in acidic conditions in a Teflon® (Thermo Fisher Scientific Inc./VWR International AB, Spånga, Sweden) bottle and stirred until complete dissolution at 40°C. Tetraethyl orthosilicate was then added under stirring conditions and the solution was stirred for 8 min at

40°C followed by 24 h at static conditions at 40°C. The final molar composition was: 0.016 P123:0.31 1,3,5-trimethylbenzene:176 H₂O:4.5 HCl:1 tetraethyl orthosilicate. Hydrothermal treatment of the precipitated solution was performed in an oven at 100°C for 24 h followed by filtration and washing with 20 ml distilled water three times. The material was left

to dry at room temperature for 24 h prior to calcination at 550°C for 6 h. SBA-15 particles (300 mg) were stirred at 4°C in 50 ml phosphate-buffered saline (PBS) 1× containing 0.1 g of OVA (grade V, Sigma-Aldrich) for 3 days.

Characterization of SBA-15 particles

Nitrogen adsorption/desorption isotherms

Nitrogen adsorption/desorption isotherms were measured at liquid nitrogen temperature (-196°C) using a TriStar II volumetric adsorption analyzer (Micromeritics Instrument Corp., GA, USA). Before being measured, the samples were outgassed for 3 h at 40°C followed by 2 h at 60°C. The Brunauer–Emmett–Teller equation [17] was used to calculate the surface area from the adsorption data obtained in the relative pressure range of 0.05 and 0.3. The total pore volume was calculated from the amount of gas adsorbed at a relative pressure of 0.91. Pore size distribution curves were derived using the density functional theory method assuming a cylindrical pore model [17].

Thermogravimetric analysis

Thermogravimetric analysis of the SBA-15-OVA samples was performed on a Mettler Toledo thermogravimetric analysis instrument (Mettler Toledo AB, Stockholm, Sweden). The temperature was increased from 25 to 900°C at a heating rate of 5°C/min on an alumina holder under the flow of air at 20 ml/min.

Scanning electron microscopy

Scanning electron microscopy images were obtained using a Gemini LEO 1550 (Zeiss AG, Oberkochen, Germany) operating at 2–3 kV with no gold coating.

Transmission electron microscopy

Transmission electron microscopy was conducted with a JEOL-3010 microscope (JEOL Ltd, Tokyo, Japan), operating at 300 kV (Cs 0.6 mm, resolution 1.7 Å). Images were recorded using a charged-coupled camera model Keen View, SIS Analysis Specialized Imaging (Olympus Soft Imaging Solutions, Münster, Germany; size 1024 × 1024, pixel size 23.5 × 23.5 µm) at 30,000–100,000× magnification using low-dose conditions on as-synthesized and calcined samples.

Measurements of particle size & zeta-potential

The size and zeta-potential of the particles were measured in Milli-Q water (Millipore Corp., MA, USA), PBS 1× pH 7.4 and in complete cell culture media in a particle-size laser diffraction analyzer (Nano ZS ZEN3600, Malvern Instruments Ltd, Worcestershire,

UK). The refraction indexes used were of 1.33 for the dispersant and 1.650 for the particles. Prior to being measured, the particle solutions were sonicated over a period of approximately 1 min.

OVA release

The analysis of the release of OVA from the SBA-15-OVA particles was carried out at 37°C at a stirring rate of 100 rpm in the PTWS 800D water bath (Pharma Test Apparatebau AG, Hamburg, Germany) connected to a CECIL 3021 spectrophotometer (Cecil Instruments Ltd, Cambridge, UK) in 250 ml PBS, pH 7.4. The absorbance at 280 nm was automatically measured every 20 min for 5 h. The OVA concentration of the solution was obtained from a standard curve of absorbance versus known OVA concentration. The release kinetic curve was fitted to power law and Higuchi equations [18].

Lipopolysaccharide analysis

The amount of lipopolysaccharide contamination in the OVA used and on the particles was measured by the end point chromogenic limulus amoebocyte lysate test method using the Endochrome-K™ limulus amoebocyte lysate kit (Charles River Endosafe, MA, USA) according to the manufacturer's instructions. Lipopolysaccharide concentrations were 119.6 ng endotoxin/mg OVA, 10.9 ng endotoxin/mg SBA-15-OVA and 0.87 ng endotoxin/mg SBA-15, which in the *in vivo* experiments correlates to 1.2, 1.09 and 0.07 ng endotoxin per subcutaneously (sc.) administrated dose for OVA, SBA-15-OVA and SBA-15, respectively.

Particle formulation

Prior to each addition to the cell culture or sc. administration, the particles were suspended in the required volume of cell culture media or PBS at the desired concentrations (see the section titled '*In vitro* proliferation of OVA transgenic splenocytes') and sonicated over a period of approximately 1 min to obtain homogenous particle suspensions.

In vitro proliferation of OVA transgenic splenocytes

Spleens from OVA MHC-II T-cell receptor transgenic female mice (8–20 weeks old, DO11.10, BALB/c background) were a kind gift from B Heyman (Uppsala University, Sweden). Single cell suspensions of the spleens were prepared by mechanical disruption followed by lysis of red blood cells with ammonium chloride-potassium lysis buffer (1 EDTA:100 KHCO₃:1509 NH₄Cl molar composition). Cells were washed three times with PBS, counted by the trypan blue exclusion method and seeded into 96-well

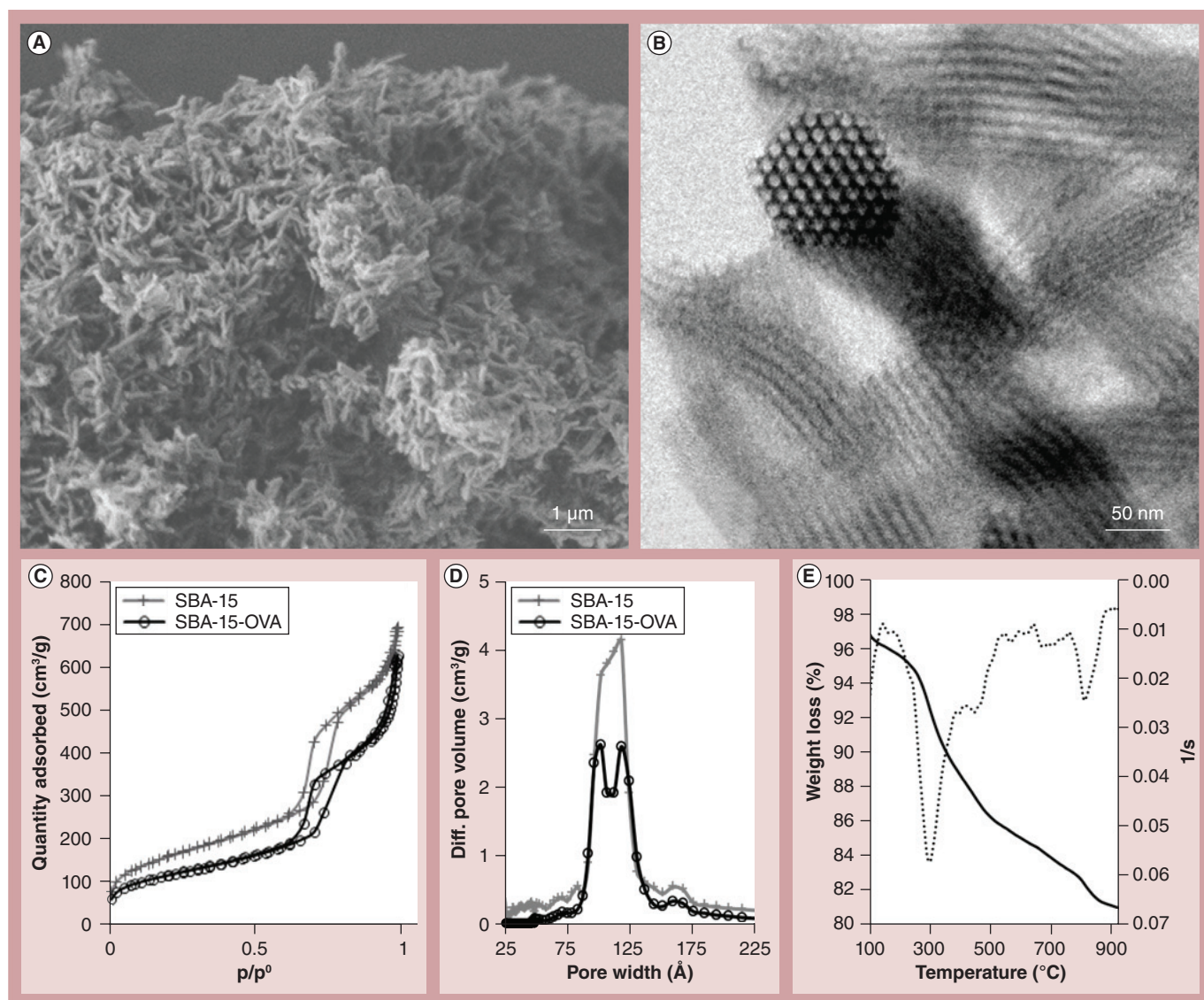


Figure 3. Particle characterization. (A) A scanning electron microscopy image of SBA-15 showing elongated rod-like particles. (B) A transmission electron microscopy image of SBA-15 particles showing hexagonal arrangement of pores with pores parallel and perpendicular to the beam direction. (C) Nitrogen adsorption–desorption isotherms of SBA-15 (gray) and SBA-15-OVA (black) samples and (D) their corresponding pore size distributions derived using the density functional theory method [17] assuming a cylindrical pore geometry. (E) Thermogravimetric analysis of SBA-15-OVA showing that the ovalbumin content in the material is 10% w/w. The solid line corresponds to the weight loss versus temperature graph, while the dotted line is the first derivative showing decomposition peaks.

Diff.: Differential; SBA-15: Santa Barbara Amorphous-15; SBA-15-OVA: Ovalbumin-loaded Santa Barbara Amorphous-15.

cell culture plates (Corning® Costar®, Corning Incorporated, NY, USA) in complete cell culture media; Roswell Park Memorial Institute 1640 medium supplemented with 10% heat inactivated fetal calf serum (Gibco, Invitrogen, Stockholm, Sweden), 1 mM sodium pyruvate, 100 IU/ml penicillin/streptomycin, 200 mM L-glutamine, 50 μM β-mercaptoethanol (Sigma Aldrich) at a cell concentration of 2×10^5 cells/well. The cells were cocultured in triplicates either with OVA or SBA-15-OVA (at 1, 10 and 25 μg/ml concentrations based on the OVA amount) or SBA-

15 (at concentrations corresponding to the amount of SBA-15 in the SBA-15-OVA stimulus) and incubated at 37°C in an atmosphere of 6% CO₂. Concanavalin A (5 μg/ml, Sigma-Aldrich) stimulated or unstimulated cells were included as positive and negative controls, respectively. After 96 h, 10 μl [³H]-thymidine (1 μCi/well, GE HealthCare AB, Stockholm, Sweden) was added for another 18 h. Cell proliferation was measured as cell count per minute in a scintillation counter (Wallac 1205 Betaplate, WALLAC Oy, Turku, Finland).

Table 1. Particle sizes by dynamic light scattering and zeta-potential values in Milli-Q (Millipore Corp., MA, USA) water, phosphate-buffered saline (pH 7.4) and in complete cell culture media.			
Sample	Media	Size (nm [PDI])	Zeta-potential (mV)
SBA-15	Water	382.9 (0.445)	-17.6, -18.0
	PBS	339.6 (0.359)	-18.9 [†]
	CCM	385.0 (0.492)	-10.0, -9.96
SBA-15-OVA	Water	272.9 (0.245)	-33.8, -34.0
	PBS	757.4 (0.477)	-17.0, -17.0
	CCM	1336 (0.455)	-16.9, -16.9
[†] Only one measurement. Size results and respective PDIs are presented as means of three independent measurements. Zeta-potential values were performed in duplicates. CCM: Cell culture media; PBS: Phosphate-buffered saline; PDI: Polydispersity index; SBA-15: Santa Barbara Amorphous-15; SBA-15-OVA: Ovalbumin-loaded Santa Barbara Amorphous-15.			

Mice

Female BALB/c mice (6–8 weeks old) were purchased from Harlan Laboratories (Zeist, The Netherlands) and housed under 12-/12-h light/dark cycle with food and water *ad libitum* at the animal facility at the Department of Microbiology, Tumor and Cell Biology (Karolinska Institutet, Stockholm, Sweden). Animal care and treatment were approved by the local ethics committee (Stockholm, Sweden).

Immunization protocol

The immunization protocol is illustrated in Figure 1A. Briefly, five groups of mice, with four to seven mice per group, were immunized three times in 7-day

intervals with either SBA-15-OVA (containing 10 µg OVA) or SBA-15 alone (same mass of SBA-15 particles as in the SBA-15-OVA stimuli), sc. over the neck in a total volume of 200 µl sterile PBS. Alum (Sigma-Aldrich)-formulated OVA (alum-OVA) (1:1 ratio) and PBS groups were included as positive and negative controls. After 1 week from the last injection, mice were sacrificed and spleens were collected for *ex vivo* stimulation as described above.

Treatment protocol

The treatment protocol is illustrated in Figure 2A. Briefly, seven mice per group were sensitized in 2-week intervals three times sc. with 10 µg OVA per mouse formulated in alum in a 1:1 ratio. At 2 days after the last sensitization dose, mice were injected sc. three times in 2-day intervals with either SBA-15 (same mass of SBA-15 particles as in the SBA-15-OVA stimuli), 10 µg OVA, SBA-15-OVA (containing 10 µg OVA) or alum-OVA (1:1 ratio, 10 µg OVA) in total volumes of 200 µl PBS (pH 7.4). PBS alone was used as a negative control. At 5 days after the treatment, all mice were exposed to intranasal challenge with 10 µg OVA for 3 successive days. The following day, mice were sacrificed and blood, bronchoalveolar lavage (BAL) fluid and the spleen were collected to evaluate the treatment efficacy. Blood samples were centrifuged and serum was stored at -20°C until analysis. Spleens were used directly for cell cultures (see above).

Determination of OVA-specific antibodies in serum

Indirect ELISA was performed to determine serum levels of OVA-specific antibodies. Briefly, 96-well microtiter plates (Corning Costar) were coated with 10 µg/ml of OVA protein (grade V, Sigma-Aldrich) and incubated overnight. The plates were washed, followed by incubation overnight with serial dilutions of

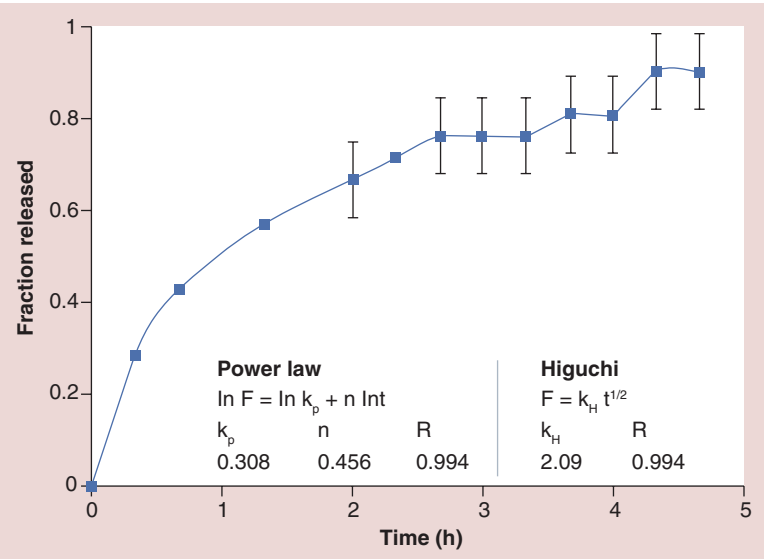


Figure 4. Release of ovalbumin from ovalbumin-loaded Santa Barbara Amorphous-15 particles in phosphate-buffered saline, pH 7.4, at 37°C. Parameters of power law and Higuchi equations [18] for the release curves of ovalbumin from SBA-15. Results are expressed as means ± standard deviation of three samples.
F: Fraction released; k_H : Kinetic constant for Higuchi equations; k_p : Kinetic constant for power law; $\ln$: Natural logarithm; n : Release exponent; R : Correlation coefficient; t : Time.

sera (in duplicates). Isotypes of the reactive antibodies were determined by incubating the plate with alkaline phosphatase-conjugated goat anti-mouse IgG, IgG₁ or IgG_{2a} (Southern Biotechnology Inc., AL, USA) antibodies. The enzyme–substrate reaction was developed using p-nitrophenyl phosphate disodium (Sigma Diagnostics, MO, USA).

Sandwich ELISA was performed to determine OVA-specific IgE antibodies. Briefly, microtiterplates were coated with 2 µg/ml of rat anti-mouse IgE (Mabtech AB, Nacka Strand, Sweden) overnight, followed by incubation with serially diluted sera. Biotinylated OVA (AbD Serotec, Oxford, UK) was used as the detection antibody and the plates were developed with streptavidin–alkaline phosphatase (Mabtech AB).

Optical density was measured at 405 nm at different time points by an ELISA reader (EnSpire® Multilabel Reader, PerkinElmer Sverige AB, Upplands Väsby, Sweden).

Cytokine analysis

Supernatants from the splenocyte cultures were analyzed for the presence of IFN-γ by ELISA (Mabtech AB; detection limit 4 pg/ml) in duplicates according to the manufacturer's instructions.

Bronchoalveolar lavage

After sacrifice, 1 ml PBS was injected through the trachea to lavage the lungs three times. The total number of live cells in the lavage was then counted and calculated back to cells per milliliter BAL. BAL cells were resuspended in PBS, cytospun and stained with May-Grünwald/Giemsa (Sigma-Aldrich). A minimum of 300 cells were counted per BAL sample for the cell differential count.

Statistical analysis

The Mann–Whitney U test was used for the statistical analysis of *in vitro* and *ex vivo* experiments, since it is appropriate for both normally and not normally distributed data, and the test samples were considered too small for an appropriate normal distribution analysis. A two-way analysis of variance with Bonferroni post-test was used for statistical analysis of the BAL cell count. GraphPad Prism software version 4.03 (GraphPad Software, CA, USA) was used for all statistical analysis. A p-value <0.05 was considered statistically significant.

Results & discussion

Particle characterization

The physical and structural properties of SBA-15 were determined by scanning and transmission electron microscopy, respectively. The scanning

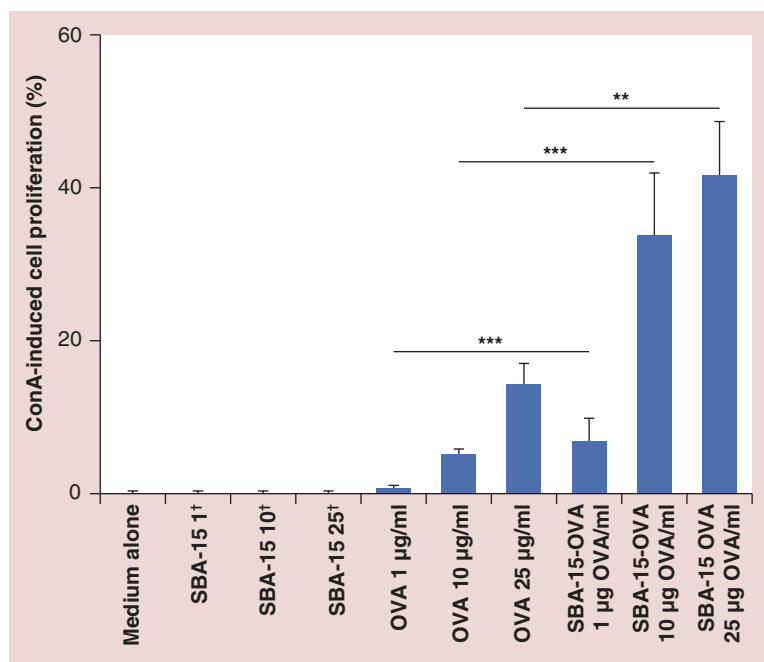


Figure 5. Ovalbumin-loaded mesoporous silica particles induce ovalbumin-specific splenocyte proliferation in a dose-dependent manner compared with ovalbumin alone. Splenocytes from OVA T-cell receptor transgenic DO11.10 mice were cultured in triplicates in the presence of either OVA or SBA-15-OVA (at different concentrations based on the OVA amount in the particles: 1, 10 and 25 µg/ml) or SBA-15 alone at particle concentrations corresponding to the amounts in respective SBA-15-OVA stimuli. Medium alone and ConA (5 µg/ml) were included as negative and positive controls, respectively. [³H]thymidine was added to the cultures after 96 h of incubation and the proliferation was measured after additional 18 h. Results represent the mean ± standard error of the mean from three independent experiments (n = 3 spleens) as a percentage of ConA-induced cell proliferation. Significant differences using Mann–Whitney U test are indicated with asterisks (**p < 0.01, ***p < 0.001).

*Particle concentrations corresponding to the amount of particle in the corresponding SBA-15-OVA stimuli containing 1, 10 and 25 µg/ml OVA, respectively.

ConA: Concanavalin A; OVA: Ovalbumin; SBA-15: Santa Barbara Amorphous-15; SBA-15-OVA: Ovalbumin-loaded Santa Barbara Amorphous-15.

electron microscopy images showed agglomerated particles (in powder form) with thin, elongated, rod-like morphology (Figure 3A). Transmission electron microscopy images of the same samples showed an ordered hexagonal pore structure within the elongated particles (Figure 3B).

The hydrodynamic particle sizes were measured by dynamic light scattering in water, PBS or complete cell culture medium in order to measure size and surface chemistry in relevant experimental conditions (Table 1). SBA-15 showed similar hydrodynamic particle sizes in all three media. A higher ionic environment as expected in the buffer solutions does not appear to affect the particle size. A media-dependent size variation causing agglomeration has been observed for smaller mesoporous particles [19].

The highest hydrodynamic particle sizes for SBA-15-OVA were measured in cell culture media, followed by PBS and then water (Table 1). The larger hydrodynamic particle sizes of SBA-15-OVA in PBS and cell culture media, in comparison to unloaded SBA-15, are due to the presence of OVA on the particle surface as well as to increased electrostatic interactions between particles, and in cell culture media due to the adsorption of serum proteins on the particle surface [20]. In cell culture medium, particles are likely to show the most similar conditions after injection *in vivo*, where particles are rapidly covered with serum proteins [21]. We have previously shown that DCs in culture pre-exposed to mesoporous silica particles (AMS-6 and SBA-15) are capable of tuning autologous naive T cells into different effector cells. The T cells showed different cytokine patterns depending on the size and functionalization of the particles [9]. This implicates that not only the presence of OVA, but also the increased hydrodynamic particle size of SBA-15-OVA, compared with SBA-15 (when measured in cell culture media), could have a role in determining the adjuvant effect of the particles *in vitro* and *in vivo*.

The zeta-potential of SBA-15-OVA in water resulted in a more negative value in comparison to SBA-15, probably due to the presence of negatively charged OVA. This difference was absent when comparing the size of the particles in PBS. The slight difference in zeta-potential measured in complete cell culture media could be explained by the formation of negatively charged SBA-15-OVA–serum protein agglomerates, consistent with an increased dynamic light scattering particle size (Table 1).

Nitrogen adsorption–desorption isotherms of SBA-15 and SBA-15-OVA are typical for mesoporous materials [17] and can be classified as type IV according to the International Union of Pure and Applied Chemistry nomenclature (Figure 3C). The isotherm gives two indications of the presence of OVA inside the pores. First, the pore volume was lower for SBA-15-OVA compared with SBA-15 (Figure 3D). Second, although the pore size distributions curves showed no shift of the pore width, there was a dip in the amount of pores in the 100–130 Å width range, where OVA most probably fits, in SBA-15-OVA compared with SBA-15 (Figure 3D). Thermogravimetric analysis indicated the presence of OVA in the pores, since the decomposition peak of OVA in the SBA-15-OVA sample was slightly shifted to higher temperatures compared with OVA alone. The OVA content in the SBA-15-OVA particles was determined to be 10% w/w by this method (Figure 3E).

OVA release kinetics

Microparticles are often employed as delivery systems for vaccine formulation to improve targeting

and minimize antigen loss and breakdown [7]. The antigen release kinetics or depot effect is an important phenomenon for vaccine development since the slow release of antigens at the site of injection results in a persistent stimulation of the immune system, which in turn leads to increased recruitment and activation of APCs [22]. Particulate adjuvants such as alum and (potentially) mesoporous silica particles have the capability to bind antigens to form multimolecular aggregates, which improve uptake by APCs [22]. For these reasons, the capability of the SBA-15 particles to exert a depot effect was studied by performing OVA release experiments from the SBA-15-OVA particles. We observed that after 5 h of incubation at 37°C in PBS, pH 7.4, the total fraction of OVA released was between 0.8 and 1 (Figure 4). Up to 0.7 of the released fraction, the kinetic release curve for OVA can be fitted with power law and Higuchi equations (Figure 4) [18], which indicate that the release of OVA from SBA-15 is controlled by diffusion forces. Using a power law, an *n*-value of 0.456 is obtained (Figure 4). An *n*-value of 0.45 indicates Fickian diffusion from cylindrical pores [18]. Although $0.45 < n < 0.89$ indicate anomalous transport (diffusion controlled release in combination with swelling-controlled release), the results suggest that OVA is kept inside the pores and thereby exerting a depot effect. The release rate is likely to be different *in vivo* compared with in PBS, as measured here, but our data still suggest a delayed release and a possible depot effect, supporting the suitability of the particles as adjuvants.

In vitro proliferation of OVA transgenic splenocytes in response to SBA-15-OVA

Since the characterization of the SBA-15-OVA particles showed promising results indicating that they might be useful for vaccine delivery, we first aimed to evaluate the ability of SBA-15-OVA to elicit OVA-specific T-cell responses *in vitro* in a T-cell receptor transgenic mouse model (DO11.10). This is advantageous over wild-type mice since a clear antigen-specific immune response can be monitored within a shorter time period [23]. Exposure to SBA-15-OVA induced significantly stronger proliferation of DO11.10 splenocytes in a dose-dependent manner compared with the proliferation elicited with OVA alone, whereas SBA-15 alone induced no proliferation of DO11.10 splenocytes (Figure 5). OVA is a weak immunogen by itself and needs adjuvant help to elicit a strong immune response *in vivo* [24]. These results suggest that the SBA-15 particles support efficient antigen presentation to T cells, which indeed enhanced the expansion of the specific T cells *in vitro*. Furthermore, the adjuvant

effect of SBA-15, when administrated together with the antigen, could probably be regulated by controlling the adjuvant/particle ratio and amount.

Mesoporous silica particles can easily be taken up by APCs such as DCs [25] and macrophages [19]. As adjuvants, the particles act as delivery systems concentrating the antigen to the particles, and this may enhance the grade of antigen uptake by the APC. The progressive release of antigen from the particles, within the cell or at the site of application, may also delay clearance, improving antigen exposure to the immune system. Although the mechanism(s) through which silica particles exert an effect on immune competent cells is still unclear, it has been discussed that it is probably mediated through scavenger receptors such as MARCO as demonstrated for transfected Chinese hamster ovary cells [26] and murine alveolar macrophages [27] giving rise to cell signalling. The particles most probably interact with APCs such as DCs in the splenocyte cultures. Particles can affect/activate APCs leading to changes on the costimulatory molecules on their surface and cytokine expression, which in turn results in the activation of other cell types such as T cells, as has been shown previously [9]. An analysis on the phenotype and cytokine production pattern of APCs after *in vivo* immunization would help us to understand the mechanisms underlying our observed immune effect. There are, however, many subpopulations of APCs, mainly located in tissues, and they might migrate to different sites upon activation. Further studies are needed to address the specific *in vivo* APC-mediated mechanisms.

In vivo immunogenicity of SBA-15-OVA

Following the above described *in vitro* studies where we saw an efficient expansion of the antigen-specific T cells when exposed to SBA-15-OVA, immunization studies were performed to evaluate the capacity of SBA-15-OVA to stimulate antigen-specific T cells *in vivo* (see immunization schedule in Figure 1A). The ability of SBA-15-OVA to deliver OVA antigen to APC was indirectly measured in an *ex vivo* splenocyte assay in response to OVA. Immunizations with SBA-15-OVA and alum-OVA induced significantly higher splenocyte proliferation levels compared with SBA-15 or PBS (Figure 1B), and higher, although not significant, proliferation compared with OVA alone. SBA-15-OVA immunization induced significantly higher IFN- γ production, compared with only SBA-15 or OVA (Figure 1C). Secretion of IFN- γ is characteristic of a Th1 type of response and indicative of cellular immunity [28]. Hence, the IFN- γ results suggest a potent cellular response to SBA-15-OVA.

Differences in formulation can have quite dramatic effects on both the magnitude and type of immune response induced by different antigens. The uptake of antigen by APCs is favored in particulate form rather than soluble form [29]. Hence, the adjuvant effect exerted by the particles could be, at least in part, explained through enhanced antigen uptake. Particles have been shown to improve the immune responses towards an antigen through other mechanisms as well. These include the depot effect and kinetics of the antigen delivery, as well as the route of antigen trafficking within the APC, which will be determined by the particle size and composition. Reports regarding particle size correlation with humoral or cell-mediated immunity are, however, conflicting [29]. Concerning adequacy of the adjuvant, it is noteworthy that alum- or oil-based adjuvants promote lump formation under the skin after intramuscular immunization, while the silica solution does not [15]. Thus, it is necessary to determine the route, uptake mechanisms, depot effects, frequency and different doses of antigen formulations in different experimental settings to achieve a desired immune response.

Effect of SBA-15-OVA in an OVA allergy model

From our previous data regarding the adjuvanticity of SBA-15 [25] and from the *in vitro* experiments presented here, it is apparent that SBA-15-OVA has the potency to modulate the immune system to induce IFN- γ production. Since IFN- γ production is characteristic of a Th1-like immune response [30], and hence might play a beneficial role against allergy, we wanted to assess whether SBA-15-OVA could modify an already existing immune response to OVA in an OVA allergy model [31]. OVA-sensitized mice were treated with SBA-15-OVA, OVA, SBA-15, alum-OVA, or PBS on three occasions with 2-day intervals before challenge with OVA (see protocol Figure 2A). The results show that SBA-15-OVA treatment led to significantly higher levels of IgG compared with SBA-15, OVA, alum-OVA and the PBS control. The IgG₁ levels were higher in the SBA-15-OVA group compared with all other groups, with the exception of the PBS-treated group (Figure 2B). A slight increase of IgG_{2a} was seen in response to SBA-15-OVA compared with SBA-15 alone (Figure 2B). Increased levels of IgG_{2a} are indicative of a Th1 skewed immune response in mice [32]. The OVA-specific IgE levels between the OVA and the SBA-15-OVA groups were similar. Interestingly, the OVA-specific IgE levels in the SBA-15-OVA-treated group were, however, significantly lower than in the alum-OVA treated group (Figure 2B). This, in combination with the IgG analysis, which showed an increased IgG level compared with both the alum-OVA- and OVA-

treated groups (Figure 2B), suggest that SBA-15-OVA is directing the immune response away from an allergic phenotype compared with both alum-OVA and OVA. Low IgE production is desired in allergy treatment strategies since IgE triggers mast cells and eosinophil effector functions, which play important roles in allergic diseases [33]. However, both the SBA-15-OVA and the alum-OVA groups showed an increased number of eosinophils in BAL fluid compared with the groups receiving only PBS (Figure 2C). *In vitro* stimulation with OVA of the *ex vivo* splenocytes showed enhanced production of IFN- γ in the SBA-15-OVA- and SBA-15-treated groups (Figure 2D). Those results are in accordance with previous work where SBA-15 was shown to have an adjuvant effect in itself [9]. Thus, both the humoral *in vivo* response and the high IFN- γ production by *ex vivo* stimulated splenocytes indicated a modification of the immunological response with SBA-15-OVA. The lower OVA-specific IgE and higher IgG serum levels, and the higher IFN- γ secretion by the OVA-stimulated splenocytes induced by SBA-15-OVA treatment suggest that SBA-15 particles are promising adjuvants for the treatment of diseases that require a Th1-like immune response. However, there were no differences in the BAL fluid cellular count between the SBA-15-OVA- and alum-OVA-treated groups (Figure 2C). This might be due to the relatively short duration of the experiment; in humans allergen-specific immunotherapy can last for several years [34]. It should also be noticed that the cell counts in the BAL fluid does not change in parallel to other measurements of airway inflammation such as the airway hyper responsiveness [35]. A protocol as short as the one used in the present study is mainly aiming at detecting acute effects on the T- and B-cell level, and does not induce remodeling of the airways characteristic to asthmatic disease, such as an increase in the smooth muscle cell layer. Therefore, a more chronic model of airway inflammation that better mimics the inflammation observed in humans is needed in order to find an effective protocol for treatment of allergic inflammation using SBA-15 particles as adjuvants.

Conclusion

Silica mesoporous particles can be engineered to encapsulate antigens of different sizes and compositions by modulating their pore size and/or surface functionalization to obtain a desired strength in the antigen-particle system as well as a desired release kinetic profile. This study shows the feasibility of loading SBA-15 particles with antigen (OVA), which is readily released to immune competent cells.

The release of OVA from SBA-15 in PBS, pH 7.4 and at 37°C is controlled by diffusion forces and

reached the total release of OVA after 5 h under sink conditions. It is speculated that the time it takes for OVA to be released from SBA-15 will be longer at the injection site, creating a depot effect. This is possible since the dissolution volume is smaller and the release media is not as dynamic (stirring conditions) as in the *in vitro* release experiment.

Splenocytes from DO11.10 mice showed that OVA loaded into SBA-15 induced a much higher cell proliferation compared with the same amount of free OVA. The *in vitro* system was found to be a quick assay to detect adjuvant potential of particles. The adjuvant effect exerted by the particles may be regulated by controlling the adjuvant/particle ratio in order to potentially tune the immunological response against specific antigens. In the immunization protocol in Balb/c mice, the SBA-15-OVA particles boosted an antigen-specific immune response with high IFN- γ production. Treatment of allergic inflammation with SBA-15-OVA induced lower OVA-specific IgE and higher IgG production than alum-OVA. These results suggest that mesoporous silica particles might modulate existing antigen-specific immune responses. Thus, mesoporous silica particles are interesting adjuvant candidates for vaccine/immunotherapy applications.

Future perspective

Although the mechanism underlying the immune-stimulating effect of mesoporous silica particles when codelivered with antigens requires further investigation, our results indicate that mesoporous silica are very promising materials and may be used as adjuvants in the future. Further development of the materials within life sciences including their role as adjuvants may lead to effective vaccine delivery and adjuvant formulations that are able to control the type and strength of an appropriate immune response depending on the specific vaccine.

Novel approaches in vaccine development require the development of new adjuvants that not only selectively induce the desired immunological response depending on the antigen; they should also confer stability, have reasonable production cost, be suitable to a broad range of vaccines and have minimal adverse effects. Mesoporous silica particles can potentially solve these issues. They may be loaded with a wide range of vaccine components, improve their stability by allowing their storage in dry conditions until their formulation prior to injection or oral/mucosal administration. They can enhance the immunological response against an antigen and hence may lead to a decrease of the antigen amount needed in the vaccine formulation. This may be due to the depot effect,

which results from the slow release of the antigen as well as the effective uptake of the mesoporous particles by APCs, which contribute to the targeted delivery of antigens to those cells. The availability of vaccines could be improved since vaccine production has become less dependent on antigen availability. Future development of mesoporous silica particles as vaccine components/adjuvants should include efforts to improve the required issues for the production of effective, stable and accessible vaccines as well their suitability for different routes of administration depending on the antigen. The strong induction of IFN- γ is also promising for future use of SBA-15 in therapeutic settings for cancer.

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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.

Executive summary

***In vitro* proliferation of ovalbumin transgenic splenocytes in response to ovalbumin-loaded Santa Barbara Amorphous-15**

- Ovalbumin-loaded Santa Barbara Amorphous-15 (SBA-15-OVA) induced significantly stronger ovalbumin (OVA)-specific splenocyte proliferation compared with OVA alone in a T-cell receptor transgenic mouse model. This antigen specific *in vitro* system was found to be an effective assay to detect adjuvant potential of the particles.

***In vivo* immunogenicity of SBA-15-OVA**

- *Ex vivo* stimulation of splenocytes from SBA-15-OVA immunized mice induced IFN- γ production.

Effect of SBA-15-OVA in an OVA allergy model

- Treatment with SBA-15-OVA in a murine OVA allergy model induced a stronger Th1-like response compared with the alum-OVA-treated group.

Conclusion

- The presented results are promising for the further development of mesoporous silica particles as adjuvants in vaccine systems or as therapeutic tools.

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