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Uptake of Chitosan-Based Viscoelastic Hydrogel Particles by Antigen Presenting Cells and Activation of Innate Immune Responses

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Abstract

The chitin-derived biopolymer chitosan has been shown to possess immunostimulatory properties in several systems and has rendered interest as a candidate adjuvant in vaccine formulations. The mechanisms underlying this effect are not completely understood at the cellular and molecular level, but activation of inflammasome and caspase-1 in antigen presenting cells (APC) was recently suggested to be vital for immune activation. In this study, chitosan-based viscoelastic hydrogel particles of two sizes, 10 µm and 200 µm, were evaluated in regard to cellular uptake and activation of APCs. Macrophages derived from the human cell line THP-1 were shown by flow cytometry and confocal laser scanning microscopy to take up FITC-labelled chitosan particles of both sizes via an active process that could be inhibited by cytochalasin D. To investigate if the viscoelastic chitosan particles cause inflammasome activation, NFκB and IL-1β was measured in THP-1 derived macrophages after 24 h incubation with the chitosan particles with or without priming of the cells with LPS. We found that chitosan particles of both sizes stimulated upregulation of NFκB and IL-1β in the absence of LPS. Finally the dependence of this effect on inflammasome-mediated activation of caspase-1 was assessed. Active caspase-1 was not detected in THP-1-derived macrophages stimulated with chitosan-based viscoelastic hydrogel particles, neither alone nor in combination with LPS. In conclusion, we show that viscoelastic chitosan particles in the size range of 10-200 µm, are taken up by human APCs. Moreover, our study suggests that the chitosan particles stimulate NFκB upregulation and IL-1β secretion through inflammasome activation via a caspase-1 independent pathway.

Keywords: Adjuvant; Caspase-1; Chitosan; Hydrogel; IL-1β; NFκB; THP-1

Abbreviations:

APC: Antigen Presenting Cell; Alum: Aluminium Salt; Cyt D: Cytochalasin D; DD: Degree of Deacetylation; DC: Dendritic Cell; IL-1β: Interleukin-1β; LPS: Lipopolysaccharide; MDDC: Monocyte-Derived DC; NLRP3: Nucleotide-Binding Oligomerization Domain-Like Receptor Family Pyrin Domain Containing Protein 3; NFκB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells; TLR: Toll-Like Receptor

Introduction

The development of vaccines constitutes one of the most important achievements of modern medicine. Vaccinations against lethal or harmful diseases protect vast populations, using simple and cost-effective methods with little side effects. Nevertheless, there is still a demand for new approaches to boost the immune system to react against poorly immunogenic antigens [1]. This could be achieved with the use of adjuvants; substances that enhance antigen-specific immune responses. Today there are only a few adjuvants approved for clinical use in humans, of which aluminium salts (alum) are the most widely used [2]. Much effort has been put into unravelling the immunostimulatory properties of alum, and various mechanisms have recently been proposed [3-6]. However, the mechanisms underlying the immune activation of most adjuvants, in particular novel adjuvant candidates, remain elusive.

Chitin-building blocks of exoskeletons in insects and crustaceans can be chemically modified into chitosan, a polymer composed of N-acetyl-glucosamine and glucosamine. Chitosan has generated interest for its immunostimulatory properties and as an adjuvant candidate [7-10]. It is vital that chitosan applied in immunological studies is well-defined, since the polysaccharide exists in many forms and various degrees of purity. The chemical and physical properties of a given chitosan preparation, as well as possible contaminants, are decisive for its effect on the immune system [11]. Methods have recently been developed to process chitosan into grades suitable for medical use and further to viscoelastic hydrogel particles of defined sizes. These well-characterized particles have previously been shown to potentiate the immune system in a mouse model using a glycoconjugate vaccine to Haemophius influenzae type B, Act-HIB, as model antigen [12]. Furthermore, the chitosan particles were well tolerated in humans when administrated with an Act-HIB vaccine in a phase I/IIa clinical trial [13]. So far it is not known by which mechanism the viscoelastic chitosan particles affect the immune system *in vitro* or *in vivo*.

It has been established that several adjuvants stimulate uptake and activation of antigen presenting cells (APC). Moreover, it has been demonstrated that the Nucleotide-binding oligomerization domain-like receptor family pyrin domain containing protein 3 (NLRP3) inflammasome is activated in cells taking up various types of chitosan *in vitro* [6,7,14]. Inflammasome activation by chitosan has been shown to be dependent on cell priming by Toll-like receptor (TLR) activation,

and the NLRP3 inflammasome pathway in APCs to be stimulated by chitosan only in combination with TLR ligands, lipopolysaccharide (LPS) [3,7] or CpG [6]. Engagement of TLR receptors triggers nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) stimulated upregulation of pro-interleukin (IL)-1β transcription [15,16]. The subsequent conversion of pro-IL-1β to active IL-1β by caspase-1, a subunit of activated NLRP3, is considered a key event in the immunoactivation by the NLRP3 inflammasome.

The aim of the present study was to provide mechanistic data on how the hydroelastic chitosan particles previously investigated by us [12,13] activate the immune system. As APCs serve as a link between innate and adaptive immunity and play a central role in determining the magnitude and quality of the immune response to vaccination, the activation of dendritic cells (DC) and macrophages are highly interesting targets for adjuvant development. Therefore a suitable model for this study was to investigate if APCs take up viscoelastic chitosan particles, and to examine their effect on these cells.

Material and Methods

Reagents

Viscoelastic chitosan particles were produced as previously described [12,17]. In brief, chitosan (Viscosan®, Viscogel AB, Solna, Sweden) with a 50% degree of deacetylation (DD) was cross-linked with 3,4-diethoxy-3-cyclobutene-1,2-dione (Acros Organics, Thermo Fisher Scientic, Waltham, MA, USA) for 3 days at 40°C, until a viscoelastic hydrogel (ViscoGel®) was obtained. Gel particles with an average diameter of either 10 or 200 μm were obtained by mechanical processing. The LPS content of the particles was <7.5 EU/g. To acquire FITC labeled viscoelastic chitosan particles, a solution of 23.2 mg FITC (Sigma Aldrich, Stockholm, Sweden) in 50 ml methanol was added to a stirred solution of 0.5% (w/w) chitosan (Viscosan®, DD 50%) in 100 g water (pH 6.6) [18]. The reaction was left overnight with stirring at room temperature and protected from light. Purification was done by dialysis (Spectrum Laboratories, Rancho Domingues, CA, US), molecular weight cut-off 12-14 kDa, first against ethanol/water (1:3) and then extensively against distilled water. The FITC-labeled Viscosan was filtered and lyophilized, and could then be subjected to preparation of viscoelastic chitosan particles.

THP-1 derived macrophages

THP-1-blue NFκB cells (InvivoGen, Carlsbad, CA, USA) were cultivated according to the manufacturer's instructions. In brief, cells were cultured in complete RPMI, i.e. RPMI medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin 100 μg/ml streptomycin, 25 μg/ml Gentamicin and 10% fetal bovine serum (all from Sigma Aldrich), at a density between 7×10^5 and 2×10^6 cells per ml at 37°C and 5% CO_2. For macrophage differentiation 2×10^5 cells in 200 μl were cultivated per well in a 96 well flat-bottomed well plate for 72 hours in complete RPMI medium supplemented with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) [19]. Cells were then washed twice to remove non-adherent cells.

Monocyte derived dendritic cells

Buffy coats were obtained from healthy volunteers, provided by Karolinska University Hospital. Peripheral blood mononuclear cells were prepared from the buffy coats and monocytes were differentiated into dendritic cells as described in [20].

Uptake experiments

To the PMA differentiated THP-1 cells, 30 μl FITC labeled viscoelastic hydrogel with particle size of 10 or 200 μm were added using 28G insulin syringes (BD Biosciences, San José, CA, US). After 24 hours at 37°C, the cells were washed and detached by incubation in PBS supplemented with 2mM EDTA for 15 minutes at 37°C. FITC positive cells were detected by flow cytometry (using a FACS Verse instrument, BD Biosciences), and internalization of FITC was confirmed by quenching external fluorescence with trypan blue and re-analyzing the cells. To inhibit active uptake, 10 ng/ml of cytochalasin D (Cyt D) (Sigma Aldrich) was added per well 30 minutes prior to addition of FITC labelled viscoelastic chitosan particles. Data were analyzed using FlowJo X software (Tree Star, Ashland, OR, US). Uptake by MDDCs from two individual blood donors was tested using the protocol described above. Cell viability was >90% after 24 hrs incubation with the viscoelastic chitosan particles, as shown by staining with Zombie Aqua™ Fixable Viability Stain (BioLegend, San Diego, CA, USA) followed by flow cytometry analysis.

Confocal laser scanning microscopy

THP-1-derived macrophages were cultured as described above in 8-well Nunc™ Lab-Tek™ Chambered Coverglass with a 1.0 borosilicate bottom (Thermo Scientific, Stockholm, Sweden) and maintained for 72 hours, followed by stimulation with FITC labelled viscoelastic chitosan particles for 2, 6 or 24 hours. Cell membranes were stained with Cell Mask Orange (Invitrogen, Carlsbad, CA, US) according to the manufacturer's instructions and the cells were imaged using confocal laser scanning microscopy LSM 510 ConfoCor 3 system with Zeiss LSM Image Brovser V 4.2.0 (Carl Zeiss, Jena, Germany). FITC fluorescence was excited using the 488 nm laser line of the ArKr laser, and Cell Mask Orange fluorescence using the HeNe 543 laser. Fluorescence signals were separated using a secondary dichroic beam splitter (NFT545) and further spectrally narrowed before detection using a band pass filter (BP 505-530) for FITC and a long pass filter (LP580) for Cell Mask Orange.

Activation and analysis of NFκB

THP-1 derived macrophages were incubated with 30 μl of viscoelastic chitosan particles or 15 μl alum (Aluminium hydroxide gel 2%, Brenntag, Fredrikssund, Denmark), with or without 1 μg/ml LPS (from E. coli serotype O26:B6, Cat No L8274, Sigma Aldrich). After 24 hours, 5 μl of the supernatant was added to 190 μl of SEAP blue detection medium (InvivoGen), incubated for 15 minutes at 37°C and then analyzed at 620 nm. All experiments were performed in triplicates and repeated three times.

Cytokine analysis by ELISA

Supernatants from stimulated THP-1 derived macrophages were analyzed for IL-1β by ELISA according to manufacturer's instructions (Mabtech, Stockholm, Sweden). Murine IL-1β and IL-18 were analyzed using ELISA (Abnova, Taiwan, and R&D Systems, UK, respectively) according to the manufacturers' protocols.

In vivo activation by viscoelastic chitosan particles

C57BL/6 mice (6-8 weeks of age, from Charles River, Sulzfeld, Germany) were injected subcutaneously with 100 μl viscoelastic chitosan particles (200 μm) in one flank and PBS as control in the

opposite flank. Mice (two mice per time point) were sacrificed 4 or 12 hours post injection. Exudates from chitosan/PBS exposed tissue were obtained by mashing the tissue from the injection site with 500 µl PBS through a 100 µm cell strainer. The supernatant was filtered through a 40 µm cell strainer, and analyzed for IL-1β and IL-18. Mouse experiments were approved by the local ethics committee for animal welfare.

Caspase-1 activity

Caspase-1 was detected by FLICA 660 (Nordic Biosite, Stockholm, Sweden) using flow cytometry, according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad prism 6.01 software (GraphPad Software Inc, San Diego, CA, US). Levels of uptake, NFκB, IL-1β and IL-18 were analyzed using the parametric one-way ANOVA and Tukey´s multiple comparison test, after testing for normal distribution using D`Agostino and Pearson omnibus normality test. P values <0.05 were considered significant. All experiments were performed in triplicates and repeated three times unless otherwise stated.

Results

Both 10 µm and 200 µm viscoelastic chitosan particles are taken up by APCs

To determine if the viscoelastic chitosan particles were internalized by APCs, THP-1 derived macrophages were inspected with confocal laser scanning microscopy (Figure 1). Internalized FITC from labelled 10 µm particles was not detected after 2 hours (Figure 1A), but could be detected after 6 hours of incubation (Figures 1B). FITC positive stain from both 10 and 200 µm particles was seen within the cells after 24 hours (Figures 1C-1D). Transmission images show that the cell culture is viable after 24h incubation (Figure 1E) and the intracellular location of the detected FITC was verified by performing Z-stack scanning (Figures 1F-1H), which showed that the FITC was internalized, as opposed to surface bound. The size of the particles after internalization appears to be 2-5 µm. To further examine the uptake of 10 and 200 µm viscoelastic chitosan particles by THP-1-derived macrophages, the cells were incubated with FITC labeled viscoelastic chitosan particles of both sizes and analyzed by flow cytometry after 24 hours. FITC-positive cells were detected after incubation with particles of size, 10 and 200 µm, however, cells treated with 10 µm viscoelastic chitosan particles were FITC positive to a higher degree than cells treated with 200 µm particles (27% versus 19%) (Figure 2A). Fluorescence quenching of extracellular FITC by Trypan blue led to a reduction of the FITC-positive proportion (significant for the 10 µm particles), revealing that approximately the same percentage of cells had internalized 10 and 200 µm sized particles, 10% and 12%, respectively. Next we examined if the viscoelastic chitosan particles were taken up by an active process. Cells were treated with Cyt D, a broad inhibitor of actin dependent phagocytosis, prior to the incubation with FITC-labelled particles. Cyt D treatment resulted in a significant reduction of FITC-positive cells, indicating that an active process was responsible for the uptake of chitosan particles (Figure 2B). Finally we investigated the ability of MDDCs to take up viscoelastic chitosan particles, and could show that these cells readily internalized the particles (Supplementary Figure S1A). The MDDC-mediated internalization of chitosan particles was solely actin-dependent (Supplementary Figure S1B).

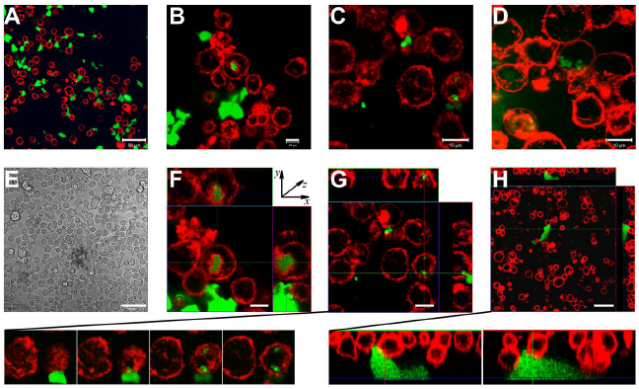

Figure 1: Uptake of viscoelastic chitosan particles by THP-1 derived macrophages. A-H Analysis by confocal laser scanning microscopy of THP-1 derived macrophages incubated with 10 µm FITC labeled viscoelastic chitosan particels (green) for 2 hours (A), 6 hours (B) or 24 hours (C) and 200 µm particles for 24 hours (D). Cell membranes were stained using Cell Mask orange (red). Transmission image of cells in culture after 24 h incubation (E). Visualization of confocal image z-stacks in three orthogonal sections (F-H). Inset: Corresponding consecutive z-stack sections showing uptake of a 10 µm viscoelastic chitosan particle (left). Images of a 200 µm viscoelastic chitosan particle in the xz and yz planes (right). 40x magnification, scale bar indicates 50 µm (A and E) or 10 µm (B-D and F-H).

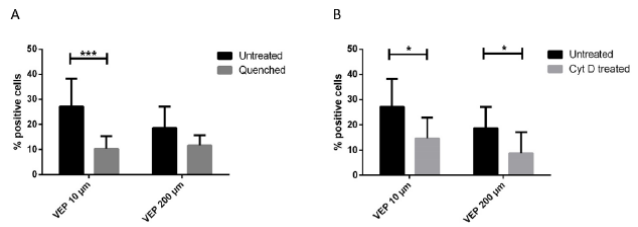

Figure 2: Flow cytometry analysis of THP-1 derived macrophages exposed to FITC labelled viscoelastic chitosan particles (10 and 200 µm) for 24 hours. Percentage of FITC-positive cells found in total in unquenched sample (untreated) and intracellular stained particles in cells quenched with Trypan blue (quenched) (A). Percentage of FITC positive cells after incubation with viscoelastic chitosan particles in the absence (untreated) or presence of cytochalasin D (Cyt D treated) (B). Data presented as mean plus SD. Statistical analysis was performed using one way ANOVA, Tukey´s multiple compairsion; *p<0.05, ***p<0.001. VEP: Viscoelastic Chitosan Particles; Cyt D: Cytochalasin D.

Induction of NFκB and IL-1β by viscoelastic chitosan particle *in vitro* and *in vivo*

The ability of viscoelastic chitosan particles to induce NFκB was assessed in THP-1 derived macrophages by measuring SEAP blue

activity. Compared to unstimulated cells, both 10 and 200 µm particles significantly induced NFκB production, while alum did not (Figure 3). However, when LPS was present in the cell culture, neither the viscoelastic particles, nor alum, were able to further enhance NFκB compared to LPS alone (Figure 3). Transcription of pro-IL-1β is triggered by NFκB, therefore we investigated production of IL-1β. Chitosan particles of both sizes induced significantly higher levels of IL-1β than did alum alone, and compared to unstimulated cells (Figure 4). When assessing LPS stimulated cells, alum triggered the highest level of IL-1β production, significantly higher than both 10 µm and 200 µm chitosan particles in combination with LPS. The levels of IL-1β produced in response to stimulation with the 10 µm and 200 µm chitosan particles in combination with LPS were not significantly higher than those stimulated by LPS alone.

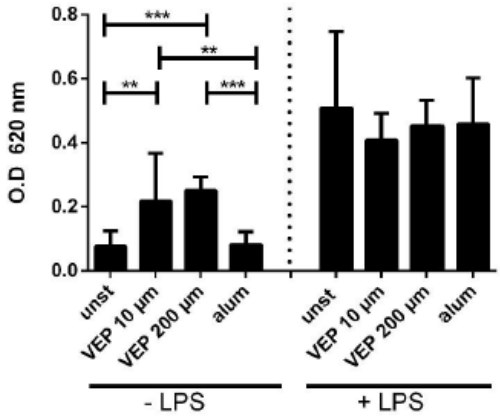

Figure 3: Viscoelastic chitosan particles activate NFκB. NFκB activity detected in THP-1 derived macrophages after 24 hours stimulation with viscoelastic chitosan particles of 10 and 200 µm size, and with alum, alone (−LPS) or with the addition of LPS (+LPS). Data presented as mean plus SD. Data presented as mean plus SD. Statistical analysis was performed using one way ANOVA, Tukey´s multiple compairsion; **p<0.01, ***p<0.001. VEP: Viscoelastic Chitosan Particles; unst: cells incubated in medium alone.

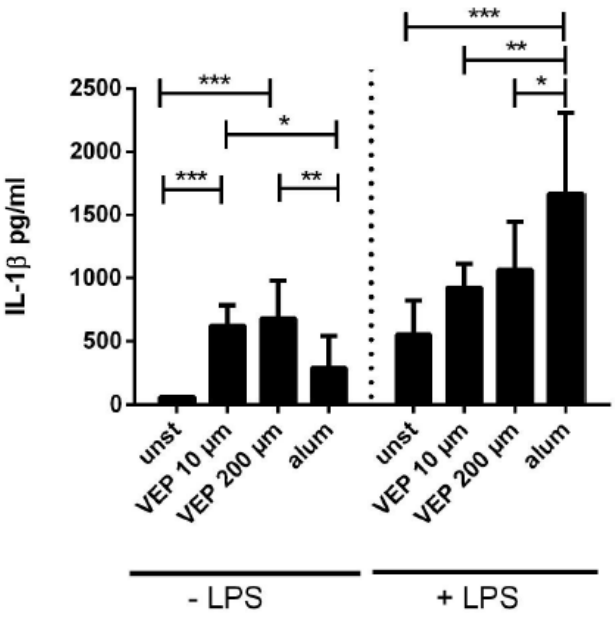

Figure 4: Viscoelastic chitosan particles stimulate the production of IL-1β. IL-1β measured in supernatants of THP-1 derived macrophages after 24 hours stimulation with viscoelastic chitosan particles of 10 and 200 µm size, and with alum, alone (−LPS) or with the addition of LPS (+LPS). Data presented as mean plus SD. Statistical analysis was performed using one way ANOVA Tukey´s multiple comparison. The concentration of IL-1β in cultures of unstimulated cells was set to 62.5 pg/ml, i.e., the detection limit of the assay, since all data points were ≤62.5 pg/ml. *p<0.05, **p<0.01, ***p<0.001. VEP: Viscoelastic Chitosan Particles; unst: cells incubated in medium alone.

In order to confirm the viscoelastic chitosan particles' ability to stimulate IL-1β production *in vivo*, mice were injected subcutaneously with 200 µm chitosan particles in one flank and PBS as a negative control in the opposite flank. An enhanced production of IL-1β could be detected in the exudate from the chitosan-injected flank, compared to the PBS control injection site both 4 hours and 12 hours after injection (Supplementary Figure S2A). In addition, since IL-18 production is also stimulated by inflammasome activation, IL-18 was measured in the exudates. Increased levels of IL-18 could be detected in exudates from the chitosan injection sites, compared to the PBS injection sites (Supplementary Figure S2B).

A key event in NLRP3 inflammasome formation is the conversion of pro-capsase-1 to active caspase-1. Caspase-1 was measured in THP-1 derived macrophages after labeling the cells with activated caspase-1 specific FLICA 660. No upregulation of caspase-1 was detected in cells stimulated with viscoelastic chitosan particles of either size, alone or in combination with LPS (data not shown). In contrast, alum potently induced caspase-1, both alone and to a significantly higher level in combination with LPS (data not shown).

Discussion

Chitosan has gained much interest as an adjuvant candidate [11,21]. We have previously shown that viscoelastic chitosan particles are able to activate cellular and humoral immune responses *in vivo* in mice [12]. In this study we investigated mechanisms for how chitosan particles of two sizes, 10 and 200 μm, activate the innate immune system. First the ability of THP-1 derived macrophages to take up particles of both sizes was confirmed by confocal laser scanning microscopy and by flow cytometry. Notably, particles of both sizes were taken up to a similar degree by an actin polymerization dependent process. Similar results were obtained with MDDCs, showing that 10 and 200 μm particles were readily taken up by dendritic cells via an active process. Confocal laser scanning microscopy revealed that the particles taken up by THP-1-derived macrophages were 3-5 μm in size, and the plasma membrane surrounding the particle was intact. It should be noted that the hydrogel particles consist of 1% FITC-labelled chitosan and 99% water and that they are not homogenous in shape. They can be viewed as gel blocks with an average diameter of 10 or 200 μm rather than spherical particles with a fixed diameter. Our findings imply that either the chitosan polymers are degraded into smaller pieces or the hydrogel is processed into smaller sized particles in the cell culture.

Previously it has been reported that positively charged micro- and nanoparticles stimulate the NLRP3 inflammasome and promote the conversion of pro-IL-1β into IL-1β via caspase 1 activity [4,14,22]. These compounds include both alum and chitosan particles, but in both cases a primary signal to induce NFκB activity is needed, such as LPS signaling via TLR4 [6,8]. Therefore expression of the NFκB reporter gene in THP-1 derived macrophages was used to detect activation by our essentially LPS-free chitosan viscoelastic particles alone or by particles in combination with LPS. Our results show that, in contrast to alum, the chitosan particles stimulate an increase in NFκB signaling even in the absence of LPS. However, LPS alone stimulated higher levels of NFκB and neither the chitosan particles, nor alum, were able to enhance the LPS-stimulated NFκB signaling in our experimental system. We cannot exclude that the NFκB levels detected in LPS treated cells represent a maximal stimulation, and thus an inability to further enhance the LPS-induced NFκB production. Also secretion of IL-1β was induced by chitosan particles of both sizes even in the absence of LPS, which is in contrast to previously reported results where chitosan was able to induce IL-1β only in TLR-triggered cells [7,8,14]. This discrepancy could possibly be explained by unique properties of the viscoelastic hydrogel chitosan particles [12,17]. They contain a chitosan with lower degree of deacetylation (50% randomly distributed acetyl groups) and are presented to the APCs in a different physical form, i.e. viscoelastic hydrogel microparticles, compared to previously investigated chitosan preparations [6-8]. Despite detecting products of NLRP3 inflammasome activation in response to stimulation with the viscoelastic chitosan particles both *in vitro* and *in vivo*, we could not detect any active caspase-1 in THP-1 derived macrophages stimulated with the particles, neither alone nor in combination with LPS. Possibly the activation is mediated by alternative inflammasome activation mechanisms. In caspase-1 knockout mice, IL-1β release independent of caspase-1 via a non-canonical pathway has been reported [23]. It was also recently shown that alum stimulates release of IL-1β and IL-1β at the site of injection by an NLRP3 independent mechanism, which was instead dependent on cathepsin S [24].

It is vital to understand the mechanisms behind the immunostimulatory properties of both already licensed adjuvants and adjuvants under development [1]. Such knowledge would allow potential fine-tuning and directing of the immune response after vaccination. The viscoelastic chitosan particles that were investigated in the present study have several desired properties of a potential adjuvant, such as the possibility to produce the particles in different forms designed for various antigens and suitable administration routes, manufacture under GMP conditions and to meet regulatory bodies' demands for medical and pharmaceutical products. Here we have shown that chitosan from particles in the size range from 10 to 200 μm, are taken up via an active process by human APCs. Moreover, our data show that the chitosan particles stimulate NFκB production and IL-1β secretion by APCs *in vitro*, indicative of NLRP3 inflammasome activation. However, the activation is independent of LPS and does not seem to occur through a conventional caspase-1 dependent pathway.

Conflicts of Interest

JB, TNA, HG and GG have previously been employed by Viscogel AB.

Acknowledgement

Unlabeled and FITC-labeled viscoelastic hydrogel (ViscoGel®) chitosan particles were kindly provided by Viscogel AB, Solna, Sweden. We gratefully acknowledge Johan Öckinger, Department of Medicine, Karolinska Institute, for his expert advice on THP-1 activation *in vitro*.

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