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Adjuvant activity of CpG-ODN formulated as a liquid crystal

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ABSTRACT

The adjuvants approved in human vaccine with recombinant/purified antigens induce weak cellular immune response and so the development of new adjuvant strategies is critical. CpG-ODN has successfully been used as an adjuvant (phase I–III clinical trials) but its bioavailability needs to be improved. We investigated the adjuvant ability of CpG-ODN formulated with a liquid crystal nanostructure of 6-O-ascorbyl palmitate (Coa-ASC16). Mice immunized with OVA/CpG-ODN/Coa-ASC16 elicited a potent specific IgG1, IgG2a, Th1 and Th17 cellular response without systemic adverse effects. These responses were superior to those induced by OVA/CpG-ODN (solution of OVA with CpG-ODN) and to those induced by the formulation OVA/CpG-ODN/Al$(OH)_3$. Immunization with OVA/CpG-ODN/Coa-ASC16 resulted in a long-lasting cell-mediated immune response (at least 6.5 months). Furthermore, Coa-ASC16 alone allows a controlled release of CpG-ODN in vitro and induces local inflammatory response, independent of TLR4 signaling, characterized by an influx of neutrophils and Ly6C^{high} monocytes and pro-inflammatory cytokines. Remarkably, the adjuvant capacity of CpG-ODN co-injected with Coa-ASC16 (OVA/CpG-ODN plus Coa-ASC16) was similar to the adjuvant activity of OVA/CpG-ODN, supporting the requirement for whole formulation to help CpG-ODN adjuvanticity. These results show the potential of this formulation, opening a new avenue for the development of better vaccines.

1. Introduction

Vaccine formulations are steering away from live attenuated microorganisms toward subunits of pathogens (highly purified or recombinant molecules). Although the latter antigens are intrinsically safer, they are often poorly immunogenic due to the lack of an inherent immunostimulatory property and so they need a vaccine adjuvant to trigger an effective immune response.

Approved adjuvants for human vaccines are still limited and include aluminum salts, oil-in-water emulsion (MF59 and ASO3) and 3-O-desacyl-4′-monophosphoryl lipid A (MPL) adsorbed onto aluminum hydroxide (ASO4) [1]. When these licensed adjuvants are used with pure proteins, they induce a robust antibody response but they are poor adjuvants for building up potent and durable T cell-mediated immunity, which is crucial for vaccines against intracellular pathogens and cancer [2,3].

Many substances have been assayed as adjuvants in experimental models or in clinical trials, including synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN), ligands of Toll-like receptor 9 (TLR9) [1,4]. Over the last decade many human clinical trials have been carried out with CpG-ODN, some of which are in phase III trials in the vaccine area [5]. The key features of CpG-ODN used as a vaccine adjuvant, in contrast to currently licensed adjuvants, include the ability to elicit antibody, Th1 cell and, but only under certain conditions, CD8+ cytotoxic T cell responses [6,7]. An additional benefit of CpG-ODN is its ability to divert the preexisting Th2 response in neonates and elderly mice toward a Th1 phenotype [8–11]. However, CpG-ODN still presents some limitations such as a short half-life, unfavorable pharmacokinetics and biodistribution, high binding to plasma protein, a lack of specificity for target cells, poor cellular uptake, and CpG motif-independent side effects that subsequently restrict its clinical application [7,12–14]. The development of an efficient drug carrier system could overcome these drawbacks and improve the adjuvant properties of CpG-ODN. To this end, a great number of formulations have been explored, such as liposomes, nano/microparticles constructed in a

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variety of ways using different materials and modifications in its structure [13–17]. Although some of these formulations appeared promising, they also had some problems mainly related to manufacturing issues, such as the scaling-up of production, and toxicity associated with cationic materials [14,18,19].

In the present study, we formulated CpG-ODN with coagel (Coa-ASC16) formed by self-assembly of 6-O-ascorbyl palmitate (ASC16) as previously described by us [20]. Alkyl ascorbic acid derivatives (ASCn) are obtained through the esterification of the hydroxyl group in position 6 of ascorbic acid with fatty acids of variable chain length (Fig. 1) [20]. Since a hydrophobic portion (alkyl chain) and a polar group (ascorbic acid) are present in this structure, this ester behaves as an amphiphilic molecule. Being amphiphilic allows these compounds to form supramolecular aggregates, mainly lamellar mesophases. The solubility in water of ASCn increases with temperature, and form transparent dispersions above the critical micelle temperature (CMT), at which the solubility reaches the critical micelle concentration (CMC). On cooling, water dispersions of ASCn form coagels, regardless of the length of the aliphatic chain [20]. Coagels are liquid crystalline phases and their lamellar structure produces at least one highly ordered dimension, so they exhibit sharp X-ray diffraction patterns and optical birefringence [21]. We have reported the phase diagram of ASC16 in water, one interesting feature of this system is the way the interlamellar water interacts with assembled ASC16 molecules [22]. CpG-ODN is a water-soluble compound, so it is to be expected that dissolved CpG-ODN may be trapped in the interlamellar water and its release from the system can be modulated by the three-dimensional structure of the lamellar liquid crystal.

In previous studies, we have shown that these systems are able to substantially increase the solubility [23] and the stability of certain drugs [24]. In this way, ASCn coagel increased the permeation of ibuprofen through hairless mouse skin in comparison to the commercial formulation Arfen® [25]. In addition, anthralin solubilized in Coa-ASC16 was more stable than in ethanolic/aqueous solutions [20] and the permeation of anthralin from ASCn coagels applied on rat skin was increased compared to other pharmaceutical systems [26].

Based on all these considerations, we hypothesized that Coa-ASC16 could improve the adjuvant activity of CpG-ODN. To test this, we formulated CpG-ODN with Coa-ASC16, and examined its adjuvant activity in mice with the ovalbumin (OVA) antigen.

2. Materials and methods

2.1. Reagents

We used OVA from Worthington Biochemical Corp (Lakewood, NJ) as an antigen [27–29]. OVA stock solution and CpG-ODN stock solution were prepared in sterile apyrogenic 0.9% NaCl saline solution (B. Braun Medical S.A, Mar del Plata, Buenos Aires, Argentina). ASC16 was purchased from Fluka Analytical (Milan, Italy). Sterile apyrogenic 5% dextrose solution was purchased from Laboratorios Roux-Ocefa (Buenos Aires, Argentina). Alu-Gel-S (2% aluminum hydroxide) was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany). LPS (*Escherichia coli* strain 0111:B4) was obtained from Sigma–Aldrich (Buenos Aires, Argentina).

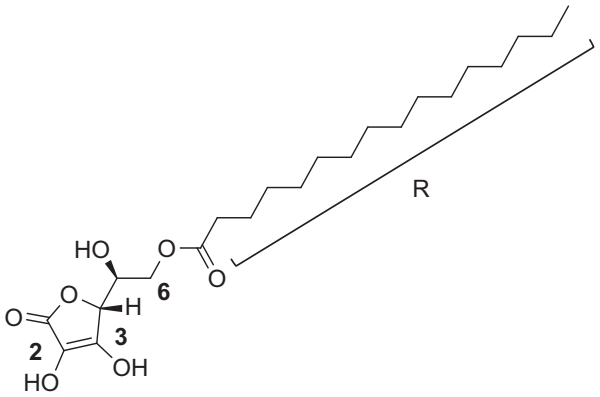


Fig. 1. Schematic chemical compositions of 6-O-alkyl ascorbic acid derivatives (ASC_n). $R = (CH_2)x\text{-}CH_3$.

2.2. Synthetic oligodeoxynucleotides

The CpG-ODN (sequence 5′-TCCATGACGTTCCTGACGTT-3′) was synthesized with a nuclease-resistant phosphorothioate backbone (CpG-ODN (PS)) (1826, B-class oligodeoxynucleotide) or with a natural phosphodiester backbone (CpG-ODN (PO)). In all tests shown in this work we used CpG-ODN (PS) and in the nuclease digestion assay we also used CpG-ODN (PO) (Operon Technologies, Alameda, CA, USA). The endotoxin content in oligodeoxynucleotide after reconstitution, determined by a standard Limulus amebocyte lysate assay (BioWhittaker Inc., Walkersville, MD, USA), was <1 endotoxin unit/ml.

2.3. Preparation of Coa-ASC16

The samples were prepared by mixing the components (ASC16 and 5% dextrose solution) in the appropriate proportions in closed glass tubes. The dispersions were heated up to 72 °C (CMT) and then homogenized in an ultrasonic bath for 15 min and left to reach room temperature in small, hermetically closed plastic tubes.

CpG-ODN and/or OVA were incorporated into ASC16/dextrose mixture and then the Coa-ASC16 was prepared as described above. In all cases the ASC16 concentration was 2% (W/V). Coa-ASC16 has a semisolid consistency.

2.4. In vitro release of CpG-ODN and OVA from Coa-ASC16

The in vitro release kinetics of CpG-ODN and OVA from Coa-ASC16 was performed in a modified Franz diffusion cell assembly at 37 ± 1 °C. Plain sintered disc (17 mm diameter and 5 mm thickness) was placed between the donor and receptor compartment. 1 ml of Coa-ASC16 loaded with 300 μl of CpG-ODN solution (1 mg/ml) and/or 24 μl of OVA solution (10 mg/ml) were placed in the upper compartment. The receptor compartment was filled with 4.3 ml of Tris–HCl buffer pH 7.2 and stirred at 200 rpm with a teflon-coated magnetic stirring bar. Periodically, 0.4 ml aliquots were withdrawn and replaced by the same volume of receptor medium. Data were corrected for dilution. CpG-ODN concentration was determined by HPLC and the Bradford method was used for OVA determinations. The assays were performed in triplicate.

2.5. Nuclease digestion assay

To evaluate the effect induced by Coa-ASC16 on CpG-ODN stability, we performed a nuclease digestion assay, exposing solutions of CpG-ODN (PS) or CpG-ODN (PO) or both formulated in Coa-ASC16, to a 3′-exonuclease enzyme solution. We chose this enzyme because it was reported that degradation of phosphorothioate oligodeoxynucleotides administered subcutaneously occurred predominantly by 3′-exonucleases [30]. Samples were mixed with the reaction buffer (670 mM glycine–KOH pH 9.5 at 25 °C, 67 mM $MgCl_2$, 10 mM DTT) and were incubated at 37 °C with 0.5 μl of 3′-exonuclease I (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). The reaction was inactivated by heating at 80 °C for 15 min at different time points. The amount of CpG-ODN remaining at each time point was quantified by HPLC.

2.6. Mice

Wild-type 8 to 10 week-old female BALB/c and C57BL/6 mice were provided by Fundación Facultad de Ciencias Veterinarias (Universidad Nacional de la Plata, La Plata, Argentina). Toll-like receptor 4 $(TLR4)^{-/-}$ mice, which have a defective response to lipopolysaccharide stimulation, were provided by The Jackson Laboratory. Mice were maintained in our animal facility according to the standards of the *Guide to the Care and Use of Experimental Animals*, published by the Canadian Council on Animal Care, with the assurance number A5802-01 delivered by the Office of Laboratory Animal Welfare (National Institutes of Health).

2.7. Immunizations

Mice were subcutaneously immunized with a solution of OVA with CpG-ODN (OVA/CpG-ODN), OVA formulated in Coa-ASC16 (OVA/Coa-ASC16), OVA and CpG-ODN co-formulated in Coa-ASC16 (OVA/CpG-ODN/Coa-ASC16), OVA/CpG-ODN + Coa-ASC16 (OVA/CpG-ODN solution and Coa-ASC16 administered separately at the same injection site, co-injection), OVA/$Al(OH)_3$ or OVA/CpG-ODN/$Al(OH)_3$. We used two different immunization schedules: in the first, immunizations were performed at days 0, 7 and 14; in the second, they were applied only twice, at days 0 and 7. Each mouse was immunized with an entire dose (250 μl) equally distributed at five sites: tail, back, the neck region and both hind limbs (50 μl/site). CpG-ODN was administered at 75 μg/mouse/dose or 30 μg/mouse/dose. The OVA dose injected was the same for all experimental groups (60 μg/mouse/dose). $Al(OH)_3$ was administered at 500 μg/mouse/dose in all cases.

2.8. Antibody detection assay

Specific antibodies against OVA were determined by ELISA. Briefly, 96-well flat-bottom plates (Greiner Bio One, Frickenhausen, Germany) were coated with OVA (1 μg/well) in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C and blocked with 0.5% gelatin PBS. Then, after washing, they were incubated with serial dilutions of plasma in 0.05% Tween® 20 0.5% gelatin PBS for 1 h at 37 °C. Next, plates were incubated with HRP-conjugated anti-mouse IgM (polyclonal), IgG (polyclonal) (both from Sigma–Aldrich), IgG1 (clone X56), IgG2a/c (clone R19-15) (both from Becton Dickinson Argentina SRL, Buenos Aires, Argentina) detection antibodies. Anti-mouse IgG2a/c recognizes an epitope in the CH3 domain of mouse IgG2a, with strong reactivity to the Igh-I[a] (IgG2a, BALB/c) allotype and weaker reactivity to Igh-I[b] (IgG2c, C57BL/6). It does not react with other isotypes, therefore we employed this antibody to detect IgG2a in BALB/c and IgG2c in C57BL/6. Finally, plates were examined on a microplate at 490 nm after incubation with H_2O_2 and *o*-phenylenediamine. Titers were calculated as the reciprocal of the last plasma dilution that yielded an absorbance at 490 nm above that of twice the mean value of blank. The plasmas from non-immunized mice were not reactive to OVA.

2.9. Spleen cells culture

We used GIBCO® RPMI 1640 medium (Life Technologies, Buenos Aires, Argentina) supplemented with 10% heat-inactivated fetal bovine serum (PAA Laboratories GmbH, Linz, Austria), 2 mM GIBCO® Glutamax, 100 U/ml Penicillin and 100 μg/ml Streptomycin (all from Life Technologies) and 50 μM 2-mercaptoethanol (Sigma–Aldrich). Splenocytes (1×10^6 cell/well) were incubated in 96-well U-bottom plate at 37 °C and 5% CO_2 with medium or OVA (100 μg/ml). Cytokine production was measured by ELISA in the supernatant samples collected after 72 h. For the detection of intracellular cytokines, cell suspensions were cultured for 48 h and GolgiStop (Monensin 4 μl/6 ml of cell culture) (Becton Dickinson Argentina SRL) was added for the last 5 h of cell culture.

2.10. Cytokine detection assay

Concentrations of different cytokines were measured by standard sandwich ELISA following instructions from the manufacturer. All assays were standardized with recombinant murine cytokines. The antibody pairs used were as follows (listed by capture/biotinylated detection): IL-6, MP5-20F3/MP5-32C11; IL-12p40, C15.6/C17.8; IFN-γ, R4-6A2/XMG1.2; IL-17A, 17CK15A5/17B7; IL-5, TRFK5/TRFK4; IL-4, 11B11/BVD6-24G2; TNF-α 1F3F3D4/XT3/XT22; IL-1β, B122/polyclonal; IL-10, JES5-2A5/JES5-16E3; GM-CSF, MP1-22E9/MP1-31G6. All antibodies were obtained from Becton Dickinson Argentina SRL or eBioscience (San Diego, CA).

2.11. Intraperitoneal injection assay

Mice were injected intraperitoneally with 5% dextrose solution (control) or Coa-ASC16 (50 μL in both cases). At different time points postinjection, peritoneal lavage was obtained. The peritoneal cavity was washed 3 times with ice-cold HBSS (1 ml per turn) and the resulting lavage was centrifuged at 2000 rpm for 5 min in order to separate the supernatant from the cells.

2.12. Flow cytometry

Cells were pre-incubated with anti-CD16/32 (clone 2.4G2) for 15 min at 4 °C and then stained with fluorochrome or biotin-labeled antibodies for 30 min at 4 °C. The antibodies used were against mouse CD11b (clone M1/70), Ly-6G (clone 1A8), Ly-6C (clone AL-21), CD19 (clone 1D3), NK1.1 (clone DX5), CD3 (clone 145-2C11), CD4 (H129.9), CD8 (clone 53-6.7) and F4/80 (clone BM8). All antibodies were purchased from Becton Dickinson Argentina SRL or eBioscience, except for F4/80 (clone BM8) which was from Life Technologies. Biotin-labeled antibodies were revealed with fluorochrome-conjugated streptavidin.

To measure the frequency of IFN-γ producing cells, splenocytes were stained for surface markers, fixed and permeabilized using BD Cytofix/Cytoperm™ Plus Kit (Becton Dickinson Argentina SRL), and stained for intracellular IFN-γ using monoclonal antibody against mouse IFN-γ (XMG1.2) (Becton Dickinson Argentina SRL) or isotype-matched control antibody.

Cells were acquired on a FACSCanto II flow cytometer. Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

2.13. ALT and AST measurement

The plasma concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using an enzymatic standard biochemical assay purchased from Wiener Lab (Rosario, Argentina), under the established manufacturer protocols.

2.14. Histology

Tissue samples were fixed in 10% neutral-buffered formalin, cleared in xylol, embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological assessment. All histological observations were performed by an expert pathologist unfamiliar with the experiment.

2.15. Statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA). Data analysis included one-way ANOVA followed by a Bonferroni post-test for multiple comparisons and the unpaired Student *t* test. All data were considered statistically significant for p values <0.05.

3. Results

3.1. Release and protection properties of formulation

Because our adjuvant strategy involved the formulation of OVA and CpG-ODN in Coa-ASC16, we examined how this platform might affect the release profile of both molecules. These are shown in Fig. 2A–C. CpG-ODN alone was quickly released (more than 90% in 15 min) from the aqueous solution (Fig. 2A). The inclusion of OVA in the solution did not affect this release pattern (Fig. 2B). However, the co-formulation of both molecules (CpG-ODN and OVA) in Coa-ASC16 significantly affected their release rate (Fig. 2B). When CpG-ODN was formulated with Coa-ASC16, the amount released was just about 45% at 60 min (Fig. 2A), and the inclusion of OVA in the formulation scarcely affected CpG-ODN release, which was about 30% in this case in the same period of time (Fig. 2B). On the other hand, the OVA release rate was also affected when this molecule was formulated in Coa-ASC16 (Fig. 2C).

In order to evaluate if Coa-ASC16 could exert a protective effect on CpG-ODN, we performed a nuclease digestion assay. First, we used the native form CpG-ODN (PO), which is extremely susceptible to degradation by nucleases, as a positive control. As shown in Fig. 2D, the percentage of oligodeoxynucleotide remaining after 0.8 h treatment with the enzyme was higher for CpG-ODN (PO)/Coa-ASC16 than for CpG-ODN (PO) in solution, showing that Coa-ASC16 is exerting a protective effect of 3′-exonuclease. We did not observe the same for CpG-ODN (PS) because this is already much more resistant to nucleases, remaining completely intact even after 24 h digestion. However, after 48 h it seems that there is a difference between both study groups in the percentage of the adjuvant that is intact after treatment (Fig. 2E).

Given that Coa-ASC16 generates a controlled liberation of CpG-ODN (PS) and that current clinical trials are using CpG-ODN synthesized with phosphorothioate-modified backbones [7], we chose to use CpG-ODN (PS) in all in vivo tests.

3.2. Assessment of adjuvant efficacy

3.2.1. Comparison of CpG-ODN adjuvant activity under different formulations

In order to evaluate the adjuvant activity of CpG-ODN formulated in Coa-ASC16, mice were subcutaneously immunized at days 0, 7 and 15 with OVA/CpG-ODN, OVA/Coa-ASC16 or OVA/CpG-ODN/Coa-ASC16.

CpG-ODN/Coa-ASC16 induced higher amounts of anti-OVA IgG, IgG1 (associated with Th2-biased response) and IgG2a (associated with Th1-biased response) compared with CpG-ODN (Fig. 3A). In addition, CpG-ODN/Coa-ASC16 elicited higher amounts of IgG2a than Coa-ASC16 alone. Splenocytes from vaccinated mice were re-stimulated ex vivo with OVA to measure cytokine production, a parameter indicative of the development of antigen-specific cellular immune response. Splenocytes from mice immunized with OVA/CpG-ODN/Coa-ASC16 showed higher antigen-specific IFN-γ and IL-17 secretion compared to those immunized with OVA/CpG-ODN and OVA/Coa-ASC16 (Fig. 3B). IL-4 concentrations in all groups were close to the detection limit (data not shown). In order to identify the source of IFN-γ in the spleen cells and to evaluate the quality of the T cell response, we performed an

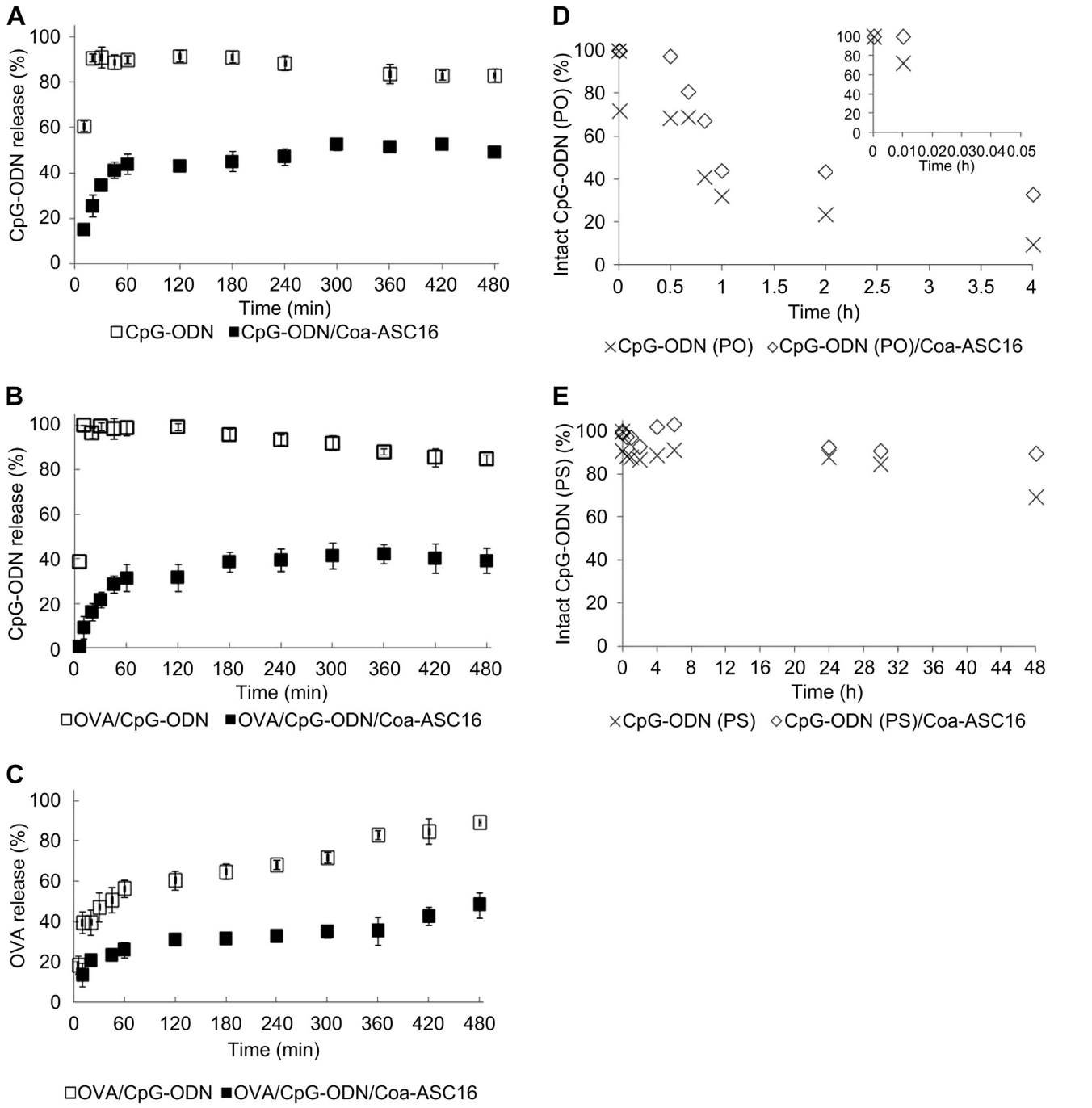


Fig. 2. Release of CpG-ODN and OVA from Coa-ASC16 formulations and nuclease digestion assay. (A–C) In vitro CpG-ODN and OVA release profiles in buffer Tris–HCl. The oligodeoxynucleotide used was CpG-ODN (PS). In A, CpG-ODN release compared between CpG-ODN in solution and formulated in Coa-ASC16. In B, CpG-ODN release compared between OVA/CpG-ODN solution and OVA/CpG-ODN formulated in Coa-ASC16. In C, OVA release compared between OVA/CpG-ODN solution and OVA/CpG-ODN formulated in Coa-ASC16. (D–E) Nuclease digestion assay. The oligodeoxynucleotides used were CpG-ODN (PS) and CpG-ODN (PO). Percentage of the intact oligodeoxynucleotide after treatment with 3′-exonuclease enzyme. The percentage is based on measurement of the total amount of oligodeoxynucleotide in the reaction medium by HPLC. Data are representative of two independent experiments performed.

intracellular IFN-γ staining of splenocytes. As shown in Fig. 3C, spleen from mice immunized with OVA/CpG-ODN/Coa-ASC16 contained a higher frequency of OVA-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells than spleen from mice immunized with OVA/CpG-ODN, clearly showing that Coa-ASC16 helps to induce a T cell response.

Next, we compared the efficiency of CpG-ODN/Coa-ASC16 as an adjuvant with CpG-ODN formulated in aluminum salts, a well-known adjuvant licensed for human use [1]. As shown in Fig. 3D, immunization with OVA/CpG-ODN/Coa-ASC16 induced the highest titers of OVA-specific IgG1 and IgG2a. Similarly, IFN-γ and IL-17 secretions in spleen from mice immunized with OVA/CpG-ODN/Coa-ASC16 were much higher than in mice immunized with OVA/CpG-ODN/$Al(OH)_3$. In contrast, IL-4 secretion was almost only observed in spleen from OVA/$Al(OH)_3$ vaccinated mice (Fig. 3E). Thus, the CpG-ODN/Coa-ASC16 based vaccine was significantly more efficient than CpG-ODN/$Al(OH)_3$ to induce specific humoral, Th1 and Th17 cellular immune responses.

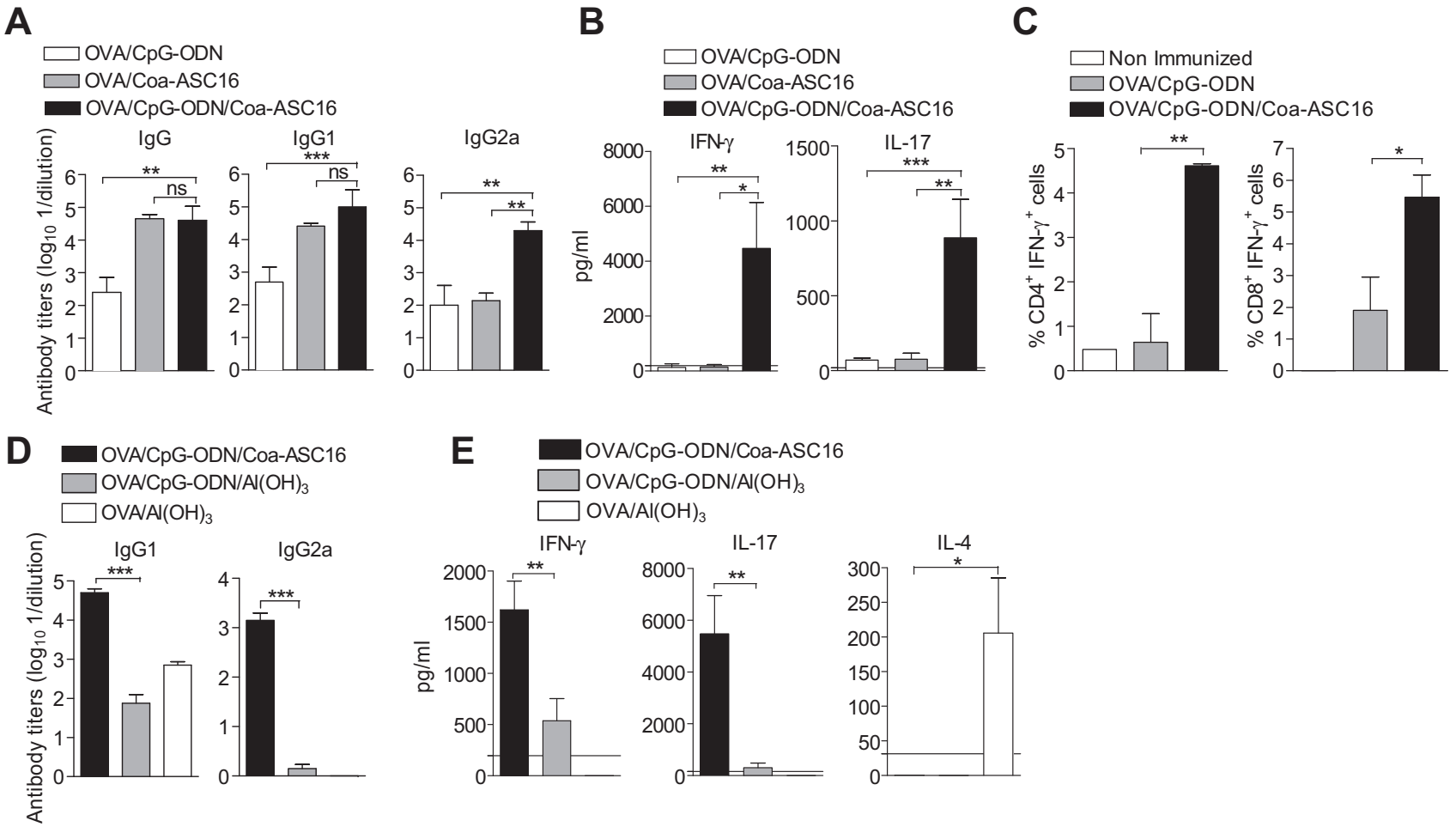


Fig. 3. Coa-ASC16 improved CpG-ODN adjuvant activity. BALB/c mice were subcutaneously immunized with OVA/CpG-ODN, OVA/Coa-ASC16, OVA/CpG-ODN/Coa-ASC16, OVA/CpG-ODN/Al(OH)$_3$ or OVA/Al(OH)$_3$ at days 0, 7 and 14 (CpG-ODN (PS) at a dose of 75 μg/mouse). At day 21 after the first immunization, plasma and spleen were obtained. (A and D) OVA-specific IgG, IgG1 and IgG2a titers. (B and E) OVA-specific cytokine production of spleen cell suspensions cultured with medium or OVA (100 μg/ml, 1×10^6/well) for 72 h measured by ELISA. The concentrations of cytokine production in response to OVA were calculated by subtracting concentrations measured in cultures without OVA. (C) Frequency of IFN-γ producing CD4$^+$ and CD8$^+$ cells in spleen determined by intracellular staining and flow cytometry. Data was calculated based on the isotype control and subtracting the percentage of IFN-γ$^+$ cells from cultures without OVA. The data show the mean ± SEM of individual values (3–4 mice/treatment group in each experiment) and are representative of three/four independent experiments performed. The full line represents the detection limit of the assay. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Similar results were obtained with TLR4$^{-/-}$ mice, showing that no putative endotoxin contaminants were responsible for the adjuvant activity of CpG-ODN/Coa-ASC16 (Supplementary Fig. 1A–C).

To summarize, these results indicate that this new strategy of vaccine adjuvant is strong enough to generate a specific antibody and T-cells immune response and works independently of TLR4 signaling. Moreover, the formulation of CpG-ODN in Coa-ASC16 increased the magnitude but did not change the Th1 response profile, which indicated that CpG-ODN maintains its known Th1 polarizing effect.

3.2.2. Evaluation of memory immune response

To study the persistence of the induced immune response, mice received three subcutaneous immunizations (at days 0, 7 and 14) with OVA/CpG-ODN or OVA/CpG-ODN/Coa-ASC16 and were in vivo re-stimulated with OVA at day 190. Both groups were able to sustain the OVA-specific humoral response at least 6.5 months. Remarkably, without restimulation, mice immunized with OVA/CpG-ODN/Coa-ASC16 always showed significantly higher amounts of anti-OVA IgG, IgG1 and IgG2a than those observed in plasma of OVA/CpG-ODN immunized mice (Fig. 4A). Next, at day 190, mice were challenged with an intraperitoneal injection of OVA to simulate protective efficacy of immunization. One week later (day 197), the amounts of anti-OVA IgG, IgG1 and IgG2a were similar in both groups (Fig. 4A) and there were no differences in IgG2a/IgG1 ratio at any of the study days (Fig. 4B). Moreover, splenocytes from OVA/CpG-ODN/Coa-ASC16 immunized mice produced greater quantities of antigen-specific IFN-γ and IL-17 than OVA/CpG-ODN immunized mice. There were no differences between the two experimental groups in the production of IL-4 (Fig. 4C). These data indicate that the CpG-ODN/Coa-ASC16 formulation facilitates the generation of a more robust memory immune response than the soluble CpG-ODN form (principally cellular immunity).

3.2.3. Comparison of alternative immunization schemes

In order to evaluate the potency of CpG-ODN/Coa-ASC16 as an adjuvant using shorter immunization schedules, we subcutaneously immunized mice at days 0 and 7 with OVA/CpG-ODN or OVA/CpG-ODN/Coa-ASC16. At day 13 post first immunization, OVA/CpG-ODN/Coa-ASC16-immunized mice showed stronger amounts of anti-OVA IgM, IgG, IgG1 and IgG2a than those immunized with OVA/CpG-ODN (Fig. 5A). These results clearly show that Coa-ASC16 makes it possible to reach titers of immunoglobulin more rapidly than CpG-ODN alone.

Then, we evaluated the efficiency of two vs three immunizations. For this purpose, mice received two (days 0 and 7) or three (days 0, 7 and 15) subcutaneous immunizations with OVA/CpG-ODN or OVA/CpG-ODN/Coa-ASC16 and the antigen-specific response was evaluated at day 21 post first immunization. As shown in Fig. 5B, the amounts of IgG1 and IgG2a anti-OVA were always higher in mice immunized with OVA/CpG-ODN/Coa-ASC16 than in OVA/CpG-ODN-immunized mice. However, the IgG2a titer was lower when OVA/CpG-ODN/Coa-ASC16 mice received two instead of three immunizations. In addition, three immunizations induced higher amounts of antigen-specific IFN-γ and similar amounts of IL-17 (Fig. 5C).

Finally, since Coa-ASC16 increases the adjuvant activity of CpG-ODN, the use of lower quantities of CpG-ODN for inducing the antigen-specific immune response was evaluated. Mice received three subcutaneous immunizations (at days 0, 7 and 14) with OVA/CpG-ODN(75 μg)/Coa-ASC16 or OVA/CpG-ODN(30 μg)/Coa-ASC16. The OVA-specific humoral and cellular immune responses were evaluated at day 21 post first immunization. Both doses of CpG-ODN (75 and 30 μg) formulated in Coa-ASC16 induced equivalent amounts of anti-OVA IgG1, IgG2a and IFN-γ, IL-17 (Fig. 5D and E). Furthermore, the IgG2a/IgG1 ratio showed no significant differences between the two doses of CpG-ODN (Fig. 5F).

All these results provide evidence that Coa-ASC16 notably increases the adjuvanticity of CpG-ODN.

3.3. Study of systemic toxicity

We have previously observed that mice immunized with OVA/CpG-ODN showed no sign of toxicity [11]. ASC16, a Generally Recognized As Safe (GRAS) substance, is a non-toxic component when used as an antioxidant in oral pharmaceutical formulations and food products [31]. However, we cannot assume that this is also true for its derivate, Coa-ASC16. Therefore, we evaluated the effect of vaccination with CpG-ODN formulated with Coa-ASC16 through biochemical and histological assays.

All mice survived the duration of the vaccine trial (21 days or 6.5 months) and no macroscopic evidence of organ damage was observed. At days 21 and 197 after first immunization, plasma ALT and AST concentrations in OVA/CpG-ODN/Coa-ASC16 and OVA/CpG-ODN immunized mice were not significantly different from those observed in non-immunized mice (Supplementary Fig. 2A and B). Histological analyses in liver, spleen, kidney and lung performed at days 21 and 197 after first immunization showed no signs of toxicity in any of these groups (data not shown).

All these results together indicate that the formulation CpG-ODN/Coa-ASC16 was able to stimulate a vigorous specific immune response without inducing adverse biological effects.

3.4. Injection site studies

We found that the formulation CpG-ODN/Coa-ASC16 is a potent adjuvant for Th1 and Th17 responses. To begin to elucidate the mechanisms involved in the improvement of the specific immune response, we tested whether the immune system could sense Coa-ASC16 at the injection site.

We injected mice subcutaneously with OVA, Coa-ASC16 or OVA/Coa-ASC16. Forty-three hours later, plasma and tissue samples from the injection site were obtained. Histological examination of the injection site in mice injected with OVA showed scarce cellular infiltrate (Fig. 6A and B). In contrast, the tissue samples obtained from mice injected with Coa-ASC16 or OVA/Coa-ASC16 presented an infiltration mainly composed of neutrophils in dermis and subcutaneous tissue (Fig. 6A and B). In addition, Fig. 6C shows a macroscopic view of the injection site of Coa-ASC16-injected mice. To dissect local and systemic effects of Coa-ASC16, pro-inflammatory plasma cytokines concentrations were assessed 43 h after subcutaneous injection. IL-6 and TNF-α were not detected in any group studied (data not shown), indicating that at this time any effects of Coa-ASC16 were distant from the injection site. In addition, plasma amounts of hepatic enzymes (ALT and AST) were similar in all groups (data not shown), which is also evidence that Coa-ASC16 has no systemic effects.

Mice subcutaneously injected with OVA/CpG-ODN had a slight cellular infiltration at the injection site, principally composed of mononuclear cells (Fig. 6A). This finding matches others previously reported [32,33]. Mice subcutaneously injected with OVA/CpG-ODN/Coa-ASC16 presented higher cellular recruitment than the OVA/CpG-ODN group (Fig. 6A). Additionally, Coa-ASC16 altered the cellular composition of the cell influx, inducing an increased percentage of neutrophils (Fig. 6A, OVA/CpG-ODN/Coa-ASC16 vs OVA/CpG-ODN). This clearly shows that the injection site response is mainly driven by Coa-ASC16 rather than CpG-ODN.

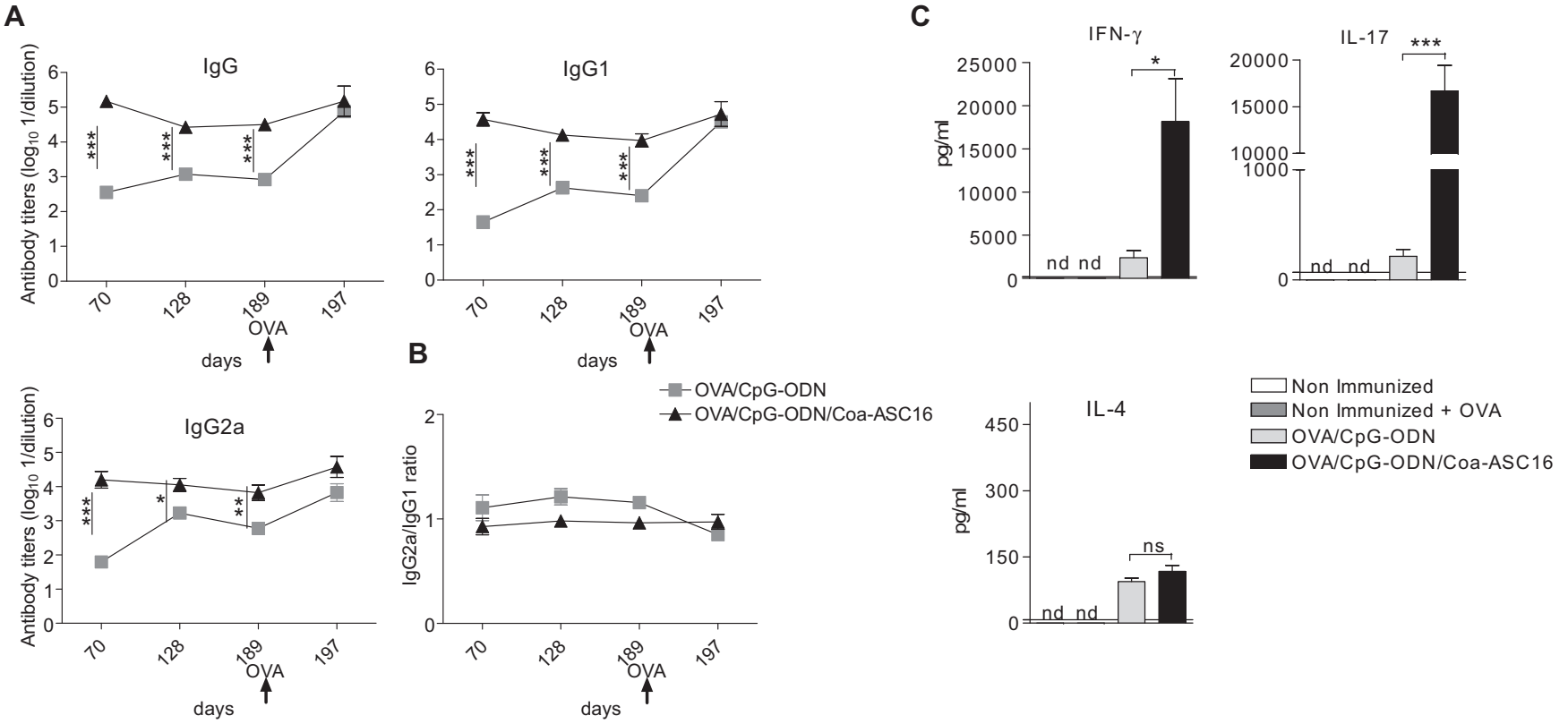


Fig. 4. Enhancement of the specific immune response by Coa-ASC16 is long-lasting. BALB/c mice were subcutaneously immunized with OVA/CpG-ODN or OVA/CpG-ODN/Coa-ASC16 at days 0, 7 and 14 (CpG-ODN (PS) at the dose of 75 μg/mouse). At day 190 after the first immunization, mice were challenged intraperitoneally with OVA. Plasma samples were collected at different time points and spleens were obtained at day 197. (A) OVA-specific antibody production as a function of time. The titers of IgG, IgG1, IgG2a were measured at days 70, 128, 189 and 197 after the first immunization. (B) IgG2a/IgG1 ratio. (C) OVA-specific cytokine production of spleen cell suspensions cultured with medium or OVA (100 μg/ml, 1×10^6/well) for 72 h measured by ELISA. Cytokine production levels in response to OVA were calculated by subtracting concentrations measured in cultures without OVA. Data show the mean ± SEM of individual values (3–4 mice/treatment group in each experiment) and are representative of two/three independent experiments performed with the same results. The full line represents the detection limit of the assay. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, nd: not detected, ns: not significant.

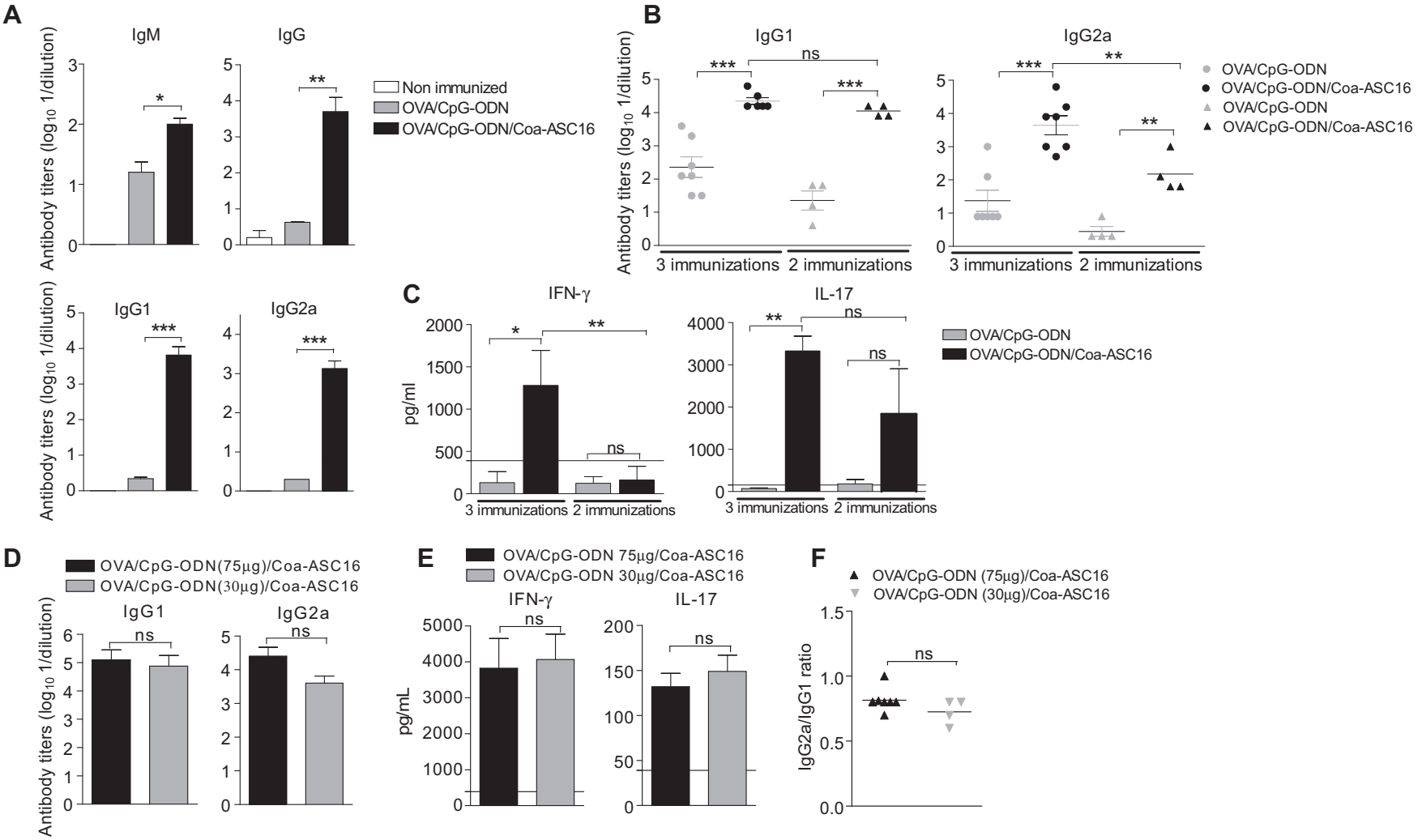


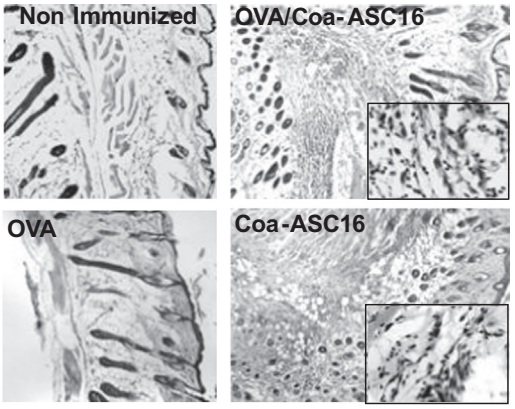
Fig. 5. Evaluation of adjuvant activity of CpG-ODN formulated in Coa-ASC16 using alternative immunization schemes. BALB/c mice were subcutaneously immunized with OVA/CpG-ODN or OVA/CpG-ODN/Coa-ASC16. (A–C) Mice were immunized two (days 0 and 7) or three times (days 0, 7 and 14) (CpG-ODN (PS) at a dose of 75 μg/mouse). (D–F) Mice were immunized at days 0, 7 and 14 using 75 or 30 μg/mouse of CpG-ODN (PS). (A, B and D) Titers of OVA-specific IgM, IgG, IgG1 and IgG2a measured at day 13 (A) or at day 21 post first immunization (B and D). (C and E) Spleens were removed at day 21 post first immunization and OVA-specific cytokine production of spleen cell suspensions cultured with medium or OVA (100 μg/ml, 1×10^6/well) for 72 h measured by ELISA. Cytokine production levels in response to OVA were calculated by subtracting concentrations measured in cultures without OVA. (F) IgG2a/IgG1 ratio. In B and F, each data point represents an individual animal and mean values are indicated by a horizontal line. In A, C, D and E, the data show the mean ± SEM of individual values (3–4 mice/treatment group in each experiment) and are representative of two/three independent experiments performed. The full line represents the detection limit of the assay. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant.

A

Experimental group	Cell recruitment	Celular composition
Non immunized	absent	
OVA	+	
Coa-ASC16	++++	
OVA/Coa-ASC16	++++	
OVA/CpG-ODN	+	
OVA/CpG-ODN/Coa-ASC16 (co-formulation)	+++/++++	
OVA/CpG-ODN + Coa-ASC16 (co-injection)	++/+++	

M N E

B



C

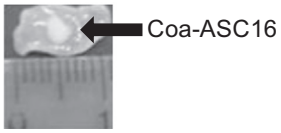


Fig. 6. Coa-ASC16 induces inflammatory response at the injection site. BALB/c mice were shaved 7 days before the beginning of the experiment. At day 7, each mouse was subcutaneously injected with OVA, Coa-ASC16, OVA/Coa-ASC16, OVA/CpG-ODN, OVA/CpG-ODN/Coa-ASC16 or OVA/CpG-ODN + Coa-ASC16 in five sites as indicated in Materials and methods (CpG-ODN (PS) at the dose of 75 μg/mouse). After 43 h, full-thickness skin samples were taken from the injection site and processed for histological assessment. Slides were inspected under a Star Zeiss microscope, and representative fields were photographed with a digital camera (Moticam 2000). (A) Semiquantitative evaluation and differential cell counts of the cellular infiltrate at the injection site in sections stained with hematoxylin and eosin. We defined cell recruitment as "absent" in the case of the cellularity observed in non-immunized mice, corresponding to the resident cell population in skin, (+) "scarce", (++) "moderate", (+++) "abundant" and (++++) "very abundant". M: mononuclear cells, N: neutrophils, E: eosinophils. (B) Representative microphotographs of the injection site. Low power (×40 original magnification) images and higher magnification images (inset, ×400 original magnification). (C) Macroscopic view of the injection site of mice injected with Coa-ASC16. Arrowhead indicates the presence of Coa-ASC16. Results shown are from two independent experiments (3–4 mice/treatment group in each experiment).

To go more deeply into the characterization of the inflammatory response induced by Coa-ASC16, we employed the peritoneal injection route as being more accessible and easier to study. Mice were injected with Coa-ASC16 or dextrose (control group) and, 2 and 6 h later, we obtained peritoneal lavages. Coa-ASC16 induced a recruitment of neutrophils at 2 and 6 h, and also of Ly6C^{high} monocytes at 6 h after injection into the peritoneal cavity (Fig. 7A and B). At the same time, 2 and 6 h after injection, there was a dramatic reduction in the number of resident macrophages and B cells in mice receiving Coa-ASC16, with no significant differences in the number of NK, NKT and T cells (data not shown). We also determined the amount of pro-inflammatory as well as regulatory cytokines in peritoneal lavage supernatants. The secretion of IL-6 (principally), IL-12 and IL-1β was promoted by Coa-ASC16. Concentrations of TNF-α, IFN-γ, IL-17, GM-CSF and IL-10 were similar in mice injected with Coa-ASC16 and in those injected with dextrose (Fig. 7C and data not shown).

Similar experiments were also carried out in TLR4$^{-/-}$ mice with similar results (Supplemental Fig. 1D and E). Collectively, all these findings indicate that Coa-ASC16 is sensed by the immune system, initiating a sterile inflammatory response independent of TLR4 signaling.

3.5. Comparison of co-formulation vs co-injection regimen of immunization

Based on all these results, we hypothesized that the enhancer action of Coa-ASC16 on CpG-ODN adjuvant activity may either reflect the activation of complementary biological processes, since both molecules have demonstrated stand-alone inflammatory activity, or it may depend on the whole formulation. Therefore, we performed a comparative analysis of the specific immune responses induced by immunization with OVA/CpG-ODN/Coa-ASC16 (co-formulation) vs immunization with OVA/CpG-ODN plus Coa-ASC16 (co-injection). In mice subcutaneously immunized with the co-injection regimen, Coa-ASC16 was administered at the same injection site of OVA/CpG-ODN solution using the same needle, without being removed between the injections. The administration of OVA/CpG-ODN plus Coa-ASC16 (co-injection regimen) resulted in a very similar antigen-specific humoral and cellular response to that elicited by OVA/CpG-ODN, and thus under these conditions Coa-ASC16 did not increase the adjuvant activity of CpG-ODN (Fig. 8A and B). Moreover, the injection site of mice immunized by the co-injection regimen showed quantitative and qualitative differences in local cell infiltration to those that received the co-

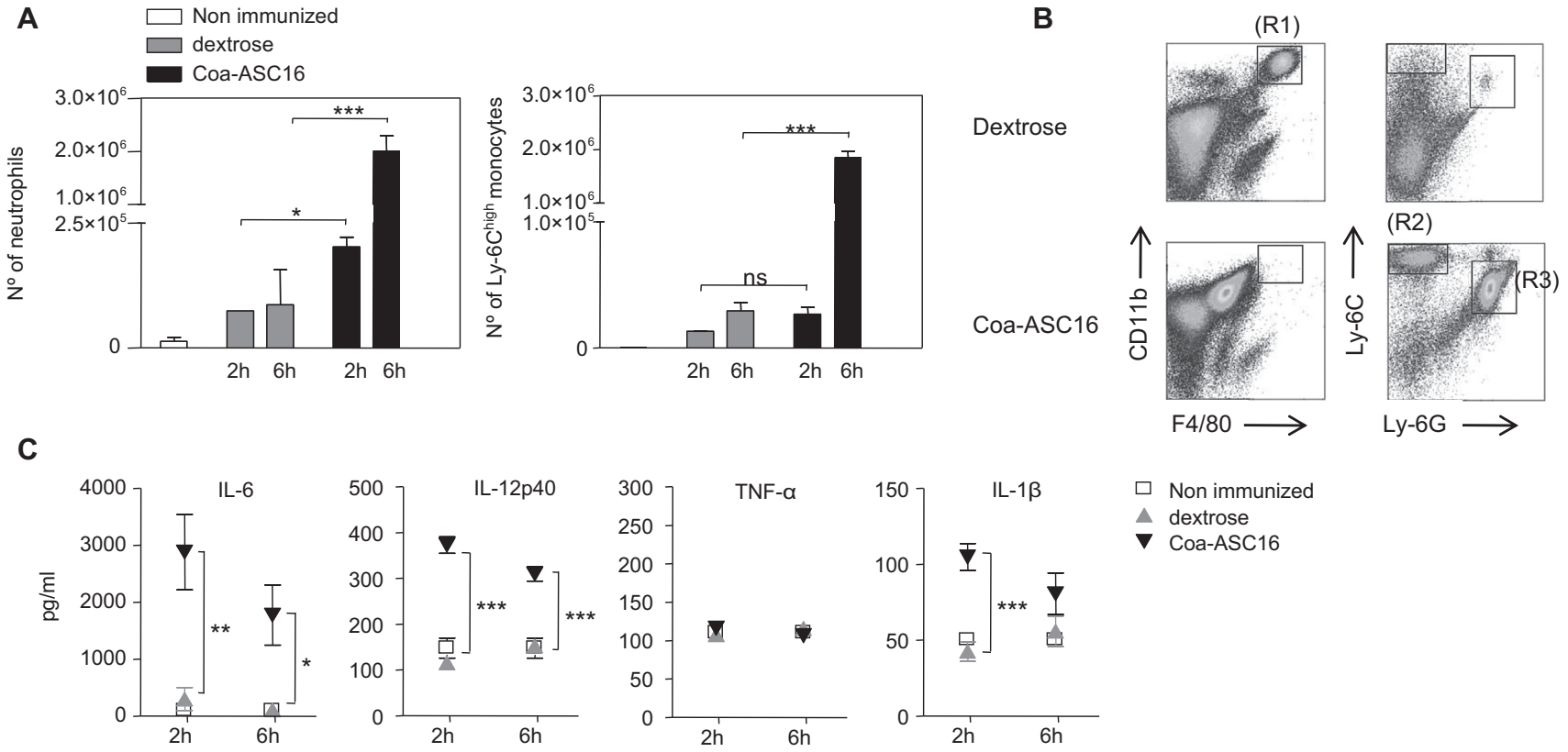


Fig. 7. Coa-ASC16 recruits neutrophils and Ly6C^{high} monocytes into peritoneal cavity. BALB/c mice were intraperitoneally injected with Coa-ASC16 or dextrose (control). After injection, the peritoneal cavity was washed at 2 and 6 h with HBSS at 4 °C. (A) Number of neutrophils (Ly-6G^{high} CD11b^{+} F4/80^{neg} Ly-6C^{+}) and monocytes (CD11b^{+} Ly6G^{neg} F4/80^{neg} Ly-6C^{high}) in peritoneal cavity. (B) Density plot graphic representative of mice injected with Coa-ASC16 or dextrose 6 h after injection. (R1) macrophages, (R2) Ly-6C^{high} monocytes, (R3) neutrophils. (C) Concentration of IL-6, IL-12p40, TNF-α, IL-1β measured by ELISA in peritoneal lavage supernatants. Data show the mean ± SEM of individual values (3–4 mice/treatment group in each experiment) and are representative of four/five independent experiments performed. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant.

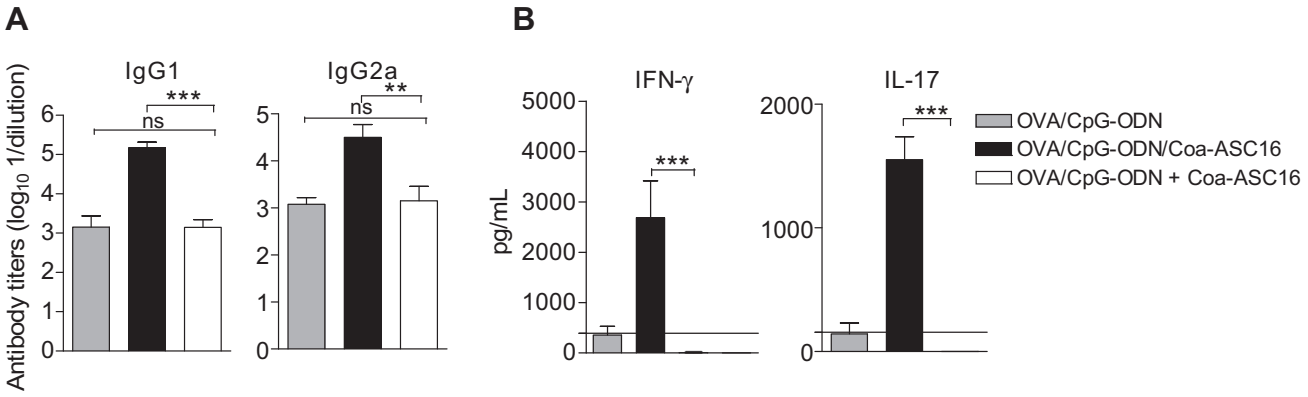


Fig. 8. Immunization with co-formulation vs co-injection regimen. BALB/c mice were subcutaneously immunized on days 0, 7 and 14 with OVA/CpG-ODN, OVA/CpG-ODN/Coa-ASC16 (co-formulation) or OVA/CpG-ODN + Coa-ASC16 (co-injection) (CpG-ODN (PS) at a dose of 75 μg/mouse). At day 21 post first immunization, plasma and spleen were obtained. (A) OVA-specific IgG1 and IgG2a titers. (B) OVA-specific cytokine production of spleen cell suspensions cultured with medium or OVA (100 μg/ml, 1×10^6/well) for 72 h measured by ELISA. The concentrations of cytokine production in response to OVA were calculated by subtracting concentrations measured in cultures without OVA. Data show the mean ± SEM of individual values (3–4 mice/treatment group in each experiment) and are representative of two independent experiments performed. The full line represents the detection limit of the assay. $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant.

formulation regimen. Mice immunized by co-injection showed a mixed pattern between CpG-ODN alone and Coa-ASC16 alone (Fig. 6A).

These results together indicate that the whole formulation is needed in order to enhance CpG-ODN adjuvant activity and that Coa-ASC16 seems not to help CpG-ODN adjuvanticity when administered separately.

4. Discussion

Vaccines can be classified into three broad groups: live attenuated, inactivated and subunit vaccines. The latter group is less immunogenic and therefore the adjuvant has a central role in generating an effective vaccine response [34]. In addition, it was recently shown that genetic and environmental exposure differences between populations around the world determine differences in the innate response to adjuvants, leading to heterogeneity in vaccine efficacy [35]. Other factors such as aging, nutritional status, chronic diseases and emerging infections can also modify adjuvant efficacy. There is thus an urgent need to develop vaccine strategies that target and protect specific vulnerable groups [36].

In this context, we analyzed a new adjuvant strategy consisting in formulating CpG-ODN with Coa-ASC16 using OVA as a model antigen. We showed that CpG-ODN formulated with Coa-ASC16 dramatically enhances the magnitude of both OVA-specific humoral (IgG1, IgG2a) and cellular (IFN-γ, IL-17) immune responses (even when we halved the dose of CpG-ODN) compared to CpG-ODN in solution (Fig. 3A–C and Fig. 5D–F). We also observed that Coa-ASC16 accelerates seroconversion (Fig. 5A). The enhancement of CpG-ODN activity could be most relevant in domestic animals and humans where CpG-ODN is not as potent as in rodents, presumably due to differences in TLR9 expression between the species [7,37]. Although the cost of CpG-ODN may not be the main limiting factor for human vaccines, it would not be economic in large animal vaccines. So, the possibility of reducing the dose of CpG-ODN is another benefit of this new adjuvant strategy. In addition, the use of smaller doses of adjuvant and the increased speed of the initial vaccine-specific response may be critical in pandemic situations.

Our findings are consistent with several previous reports demonstrating enhanced CpG-ODN adjuvant activity achieved by formulation with a wide range of components [13–17,38]. However, it is difficult to compare different formulations of CpG-ODN reported in the literature because the amount and the type of CpG-ODN and antigen as well as mouse strain are divergent. For example, two different reports showed a profound increase of anti-OVA immune response after OVA and CpG-ODN formulation with anionic nanoliposomes [39] or ISCOMATRIX [40]. Erikci et al. reported that Balb/c mice immunized with OVA and CpG-ODN (A or D-type) co-encapsulated in anionic nanoliposomes showed increased IgG1 and IgG2a titers at two weeks post-boost, similar to the measured by us one week post-boost in mice immunized with OVA/CpG-ODN/Coa-ASC16 [39]. Similarly, McCluskie et al. showed in C57Bl/6 mice immunized with the combination OVA, CpG-ODN and ISCOMATRIX, IgG2c titers similar to those obtained in mice immunized with OVA/CpG-ODN/Coa-ASC16 [40].

The goal of vaccination is to generate long-lasting immunological memory that mediates host protection from infection or cancer [41]. One distinguishing feature of the CpG-ODN/Coa-ASC16 formulation is its enhancing effect on Th1 and Th17 cellular responses and the fact that this remains over a long period of time (Fig. 4).

Many clinical trials have examined the vaccine adjuvant activity of TLR9 ligands, focusing principally on “B” (also called “K”) class CpG-ODN. Among those trials, CpG-ODN was evaluated combined with licensed vaccines designed to prevent infections. One example is the administration of the commercial Engerix-B® vaccine (recombinant hepatitis B surface antigen alum-adsorbed) plus CpG-ODN, which induced protective antibody titers in HIV-infected adults who were hyporesponsive to vaccination with Engerix-B® alone (completed phase 1b) [5,42]. In the present study, we found that the CpG-ODN/Coa-ASC16 formulation is much better than CpG-ODN/$Al(OH)_3$ at inducing Th1 and Th17 cell responses (Fig. 3D–E). Recently, several groups have shown that Th1 and Th17 responses are crucial for the induction of protective immunity against a wide range of pathogens [43]. However, licensed human vaccine adjuvants are poorly effective promoters of Th1 and Th17 responses. Freund’s complete adjuvant, which is experimentally used, is among the most potent adjuvants leading immune responses toward Th1 and Th17 [44], but it is associated with significant adverse events and so its use is not approved in humans [45]. It would be premature to suggest that CpG-ODN/Coa-ASC16 would not induce adverse side effects in future clinical studies. However, taking into account the preclinical systemic toxicology studies of blood and organ histopathology performed in this work (Supplementary Fig. 2 and data not shown), CpG-ODN/Coa-ASC16 seems not to have these limitations. This formulation is thus an

interesting approach to developing a new vaccine against many pathogens, cancer and for specific patient groups such as elderly people. Further studies will examine these specific topics.

CpG-ODN is not optimally effective when used in soluble form and, in consequence, several groups have developed different approaches to enhancing the adjuvant efficacy of CpG-ODN in vivo [13–17,38]. Our new adjuvant strategy (CpG-ODN nanoformulated with Coa-ASC16) fits some of the characteristics defining an "ideal vaccine" (elicits long-term immunity, few doses, easy and low cost production, contains a minimal number of biological molecules, easy logistics). Coa-ASC16 has many advantages that make it a very attractive platform for biomedical use: (i) it is a compound formed by ascorbic acid and palmitic acid, both biodegradable components, (ii) ascorbic acid preserves its antioxidant property [31], (iii) ASC16 is listed as a GRAS substance, (iv) it is easy to prepare and inexpensive.

Another important point to note is that mice immunized with OVA/CpG-ODN solution in this study were found to develop a weak specific immune response. This finding is similar to those reported by other authors using highly purified antigens [40,46] but differs from other published studies. We and other authors have previously shown that mice immunized with OVA plus CpG-ODN solution developed a good specific immune response [10]. A possible explanation for this discrepancy may be the type of OVA used, because some commercially available OVA preparations usually contain contaminations. In addition, in this work we showed that in the absence of TLR4, the enhancement of a specific immune response elicited by OVA/CpG-ODN/Coa-ASC16 was similar to that obtained in wild type counterpart mice (Supplementary Fig. 1A–C). This excludes the possibility that residual traces of endotoxins in OVA, CpG-ODN or Coa-ASC16 might be responsible for this enhancer effect. Therefore, these results indicate that OVA/CpG-ODN/Coa-ASC16 is a very good adjuvant strategy to induce specific immune response when the antigen has weak intrinsic immunogenicity (subunit and recombinant antigens).

In immunology, the term "vaccine adjuvant" comprises all molecules/compounds or formulations that are able to modify the specific immune response when used in combination with vaccine antigens. The adjuvants change the adaptive immune response by one or more pathways, including formation of an antigen depot (slow sustained vaccine components release), local inflammatory response, chemotaxis of appropriate immune cells to the antigen administration site, prolonging bioavailability and in consequence increasing the duration of action of immunomodulatory compounds, specific delivery of antigen and/or adjuvant, etc. [2,4,47–49]. Many adjuvants were found empirically, and progress in understanding their mechanism of action has been slow, which partly explains why the number of adjuvants approved for human use is still low [50]. This work showed that the formulation CpG-ODN/Coa-ASC16 has significant adjuvant properties. The mechanism of action of CpG-ODN has been well characterized. CpG motifs, characteristic of bacterial DNA, are a "danger signal" for the innate immune system [7] and TLR9 was identified as the mediator of its immune stimulatory effects [51]. In relation to Coa-ASC16, we were surprised when our studies showed that Coa-ASC16 injected alone (without antigen) was sensed by the innate immune system, triggering per se an early inflammatory response at the injection site (Fig. 6A–B). This response was characterized by rapid cell recruitment dominated by neutrophils and Ly6C^{high} monocytes, associated with the production of IL-6, IL-12 and IL-1β, and TLR4 signaling was not required (Fig. 7 and Supplementary Fig. 1D–E). In addition, when Coa-ASC16 was used to formulate OVA/CpG-ODN, the inflammatory response induced by OVA/CpG-ODN was modified quantitatively and qualitatively (Fig. 6A). Since Coa-ASC16 was administered without antigen (some experimental groups of Figs. 6 and 7), the observed activation of innate immune cells is antigen independent and may be acting upstream of dendritic cells. Considering these results, by analogy with other recognized adjuvants [28,52–54], we hypothesized that the particular local immunocompetent environment created by Coa-ASC16 may significantly impact the final OVA-specific immune response. This could thus be one of the mechanisms through which Coa-ASC16 increases the immunogenicity of OVA/CpG-ODN. However, our studies are insufficient to build a connection between inflammation and the enhancer adjuvant effect induced by Coa-ASC16 on the downstream adaptive response. In addition, our data do not consider the nature of the molecular mechanism/s that initiates interactions between innate immune cells and Coa-ASC16, which requires further studies. This is a challenge for the basic immunobiology that has emerged in recent years from the engineering of numerous new biomaterials. Several reviews related to how these emerging biomaterials can modulate immune-cell function may be found in the literature [55,56]. It is interesting that the injection of Coa-ASC16 alone stimulated an inflammatory response at the injection site without systemic immune activation at 43 h. This is a useful quality of Coa-ASC16, because establishing a local immunocompetent environment for vaccine adjuvanticity is generally associated with the development of minimum vaccine risks [57–59].

Finally, we investigated the dependence of adjuvant enhancer activity of the whole formulation through comparative analysis of immunization with the co-formulation regimen vs the co-injection regimen. Under the co-injection regimen, no increase of the adjuvant ability of CpG-ODN was observed (Fig. 8). Therefore, the formulation of CpG-ODN in Coa-ASC16 is strictly required, as the inflammatory response induced by Coa-ASC16 seems to be insufficient to help CpG-ODN adjuvanticity when they are separately administered. The injection with whole formulation may provide other benefits in vivo, not addressed in this work, such as forming a depot to keep the vaccine components at the injection site. It is noteworthy that structures such as Coa-ASC16 have a certain rigidity which can modulate the release of molecule/s into the biological medium or provide stability to loaded molecules [20]. Here, when OVA/CpG-ODN was formulated with Coa-ASC16, we detected in vitro a sustained release of both adjuvant and antigen (Fig. 2A–C). This kind of release kinetics may work in vivo as a depot effect, which often makes it possible to reduce the dose and/or the number of immunizations required for optimal response. Related to this, using CpG-ODN formulated with Coa-ASC16, it is possible to use a half-dose of the adjuvant (Fig. 5D–F). Moreover, specific humoral response following two immunizations (days 0 and 7) with OVA/CpG-ODN/Coa-ASC16 was higher than that observed in mice receiving three immunizations (days 0, 7 and 14) with OVA/CpG-ODN (Fig. 5B). Evidently, some kind of depot effect is produced after injection, making this strategy a reservoir of the vaccine components at the injection site. We cannot affirm that the enhancer effect of Coa-ASC16 on CpG-ODN adjuvant activity may be given, in part, by the effect of protection from degradation by nucleases, since we worked with CpG-ODN (PS), which is relatively nuclease resistant (Fig. 2E). However, we do not underestimate the protective effect that Coa-ASC16 exerts on CpG-ODN (PO), because this strategy would make it possible to replace CpG-ODN (PS) with CpG-ODN (PO) in future vaccination trials.

In summary, Coa-ASC16 improves CpG-ODN adjuvant activity, using more than one mechanism and the whole formulation is critical in order to achieve optimal results. CpG-ODN/Coa-ASC16 is therefore a potent adjuvant formulation that could be considered as a promising candidate to promote humoral and, more remarkably, cellular immune response.

5. Conclusions

In this study, we demonstrate that the nanostructure formed by self-aggregation from ASC16 is not immunologically inert since it creates a local inflammatory response. The formulation of the immune modulator CpG-ODN with this nanostructure leads to the enhancement of the specific humoral and cellular immune response, which is long-lasting. However, we still do not know the exact mechanism of action of Coa-ASC16. We believe that more than one mechanism may be involved, the eliciting of proper cytokine milieus and the formation of a depot probably are key elements. This last one is supported by the fact that only the formulation (OVA/CpG-ODN/Coa-ASC16) was able to improve CpG-ODN adjuvant activity. We showed the potential of combining a nanostructure and a TLR9 agonist as a Th1 and Th17 response-promoting adjuvant. Thus, the system described here is ideal for investigating the wide variety of poorly immunogenic emerging antigens and immune modulators, using this formulation to design more effective vaccines.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.12.002.

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