Journal of Controlled Release 287 (2018) 67–77

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Cationic lipids as one-component vaccine adjuvants: A promising alternative to alum

Malvina Pizzuto[a,*,1], Pascal Bigey[b,c,d,e], Anne-Marie Lachagès[b,c,d,e], Céline Hoffmann[b,c,d,e], Jean-Marie Ruysschaert[a], Virginie Escriou[b,c,d,e,2], Caroline Lonez[a,f,2]

[a] Structure and Fonction of Biological Membranes, Université Libre de Bruxelles, Boulevard du Triomphe, 1050 Brussels, Belgium
[b] CNRS, Unité de Technologies Chimiques et Biologiques pour la Santé (UTCBS), UMR 8258, F-75006 Paris, France
[c] INSERM, UTCBS U 1022, F-75006 Paris, France
[d] Université Paris Descartes, Sorbonne-Paris-Cité University, UTCBS, F-75006 Paris, France
[e] Chimie ParisTech, PSL Research University, UTCBS, F-75005 Paris, France
[f] Department of Veterinary Medicine, University of Cambridge, Madingley Rd, Cambridge CB3 0ES, United Kingdom

ARTICLE INFO

Keywords:
One-component vaccine adjuvants
Toll-like receptors
Inflammasome
Cationic lipids
Nanocarriers
Immunostimulant
Delivery system
Lipopolyamines
Adjuvant
Vaccine

ABSTRACT

Effective vaccine formulations consist of several components: an antigen carrier, the antigen, a stimulator of cellular immunity such as a Toll-like Receptors (TLRs) ligand, and a stimulator of humoral response such as an inflammasome activator. Here, we investigated the immunostimulatory and adjuvant properties of lipopolyamines, cationic lipids used as gene carriers. We identified new lipopolyamines able to activate both TLR2 and TLR4 and showed that lipopolyamines interact with TLRs via a mechanism different from the one used by bacterial ligands, activating a strong type-I IFN response, pro-inflammatory cytokines and IL-1β secretion. The TLR and inflammasome stimulations, together with the antigen carrier properties of lipopolyamines, resulted in both humoral and cellular immunity in mice vaccinated against OVA and make lipopolyamines promising one-component vaccine adjuvants.

1. Introduction

Vaccine made of proteins or genes that mimic or produce the active unit of pathogens (antigen) are commonly used and are well tolerated but usually require additional components called adjuvants to fully activate the immune system. The adjuvant formulations include several components in order to both promote antigen transport and uptake from antigen presenting cells (APCs) and activate the innate immune response. The quality and quantity of adaptive immune responses induced by adjuvants can be controlled by the type of activated pathways [1, 2].

Depending on the cytokines secreted by APCs, and on the co-stimulatory molecules and the type of Major Histocompatibility Complex (MHC) recruited, naïve T cells differentiate into type 1 (Th1), type 2 (Th2) Helper or cytotoxic (CTL) T cells [3, 4].

Th1 differentiation is induced by Type-I Interferon (IFN) cytokines [5, 6], while Th2 one by NOD-like receptor Pyrin domain containing 3 (NLRP3) inflammasome activation among others [7, 8]. In turn, the generated Th cells support the immune response by secretion of specific cytokines that activate neighboring cells to perform specific functions or chemokines that recruit new immune cell subsets to sites of pathogen encounter. Th2 differentiation induces a humoral immune response (B-cell activation and antibodies production) through secretion of IL-4 and IL-5 among others, while Th1 activate memory cells and macrophages contributing together with CTL to the cell-mediated immunity through secretion of IFN-γ, tumor necrosis factor (TNF)-α and Interleukin (IL)-2 [9–11].

Aluminum hydroxide, also called Alum, is the most common adjuvant used in human vaccines. It promotes antigen transport and uptake and induces the production of IL-1β generating a T helper 2 (Th2) humoral response [2, 12].

In order to induce also the Th1 cell-mediated immunity, which is more effective to induce an antiviral and antibacterial immunization, Alum is combined with weak Toll-like receptor-4 (TLR4) agonists, such

* Corresponding author.
E-mail address: malvina.pizzuto@gmail.com (M. Pizzuto).
[1] Present address: Molecular Inflammation Group, Biomedical Research Institute of Murcia (IMIB-Arrixaca), 30,120 Murcia, Spain.
[2] Equal contribution.

https://doi.org/10.1016/j.jconrel.2018.08.020
Received 27 February 2018; Received in revised form 23 July 2018; Accepted 10 August 2018
Available online 12 August 2018

as monophosphoryl lipid A (MPLA) as an immunostimulant [2, 13].

TLR4 is one of the 11 TLRs, transmembrane proteins that recognize pathogen-associated molecular patterns (PAMPs). The recognition induces TLR dimerization and, in the intracellular domain, recruitment of molecular adaptors, TIR-domain-containing adapter-inducing interferon-β (TRIF) or myeloid differentiation primary response 88 (MyD88) [14].

The secretion of pro-inflammatory cytokines such as TNF-α and IL-6 depends mainly on MyD88-driven nuclear factor kappa B (NF-κB) activation, is common to all TLRs and may originate from endosome or plasma membrane. By contrast, the type-I IFN response originates exclusively from the endosome and depends on interferon regulatory factor (IRF) activation. IRF activation can be TRIF or MyD88-driven depending on the TLR [15] and leads to cytokine secretion such as IFN-γ and IP-10, among others, more typical of viruses but also observed for some TLR4 and TLR2 bacterial ligands. [14, 16–19]. Finally, the TRIF-endosomal pathway may also induce NF-κB activation and pro-inflammatory cytokine secretion but with a slower mechanism [20]. It is generally considered that the TRIF-dependent Type-I IFN response rather than the MyD88-dependent pathway is needed for TLR4-induced adjuvant effect [21, 22].

Some vaccine formulations use cationic lipids to enter the endosomal pathway and increase the adjuvanticity of both antigen and immunostimulants through their presentation to endosomal TLRs in APCs [1, 23]. The use of multiple components in vaccine formulation increases cost and time of vaccine development because, according to the European and American guidelines, each adjuvant must be tested for stability, safety and mechanism alone, in combination with antigen and with other adjuvants and also the efficiency and kinetics of adsorption of all components must be described [24, 25]. Therefore, combining all the adjuvant effects into a single molecule implies minor cost and less systemic adverse effects.

The recently highlighted TLR and inflammasome-dependent pro-inflammatory properties of the cationic lipid diC14-amidine [26, 27], have suggested that cationic lipids may simultaneously transport the antigen and activate both humoral and cellular immunity and therefore, be used as one-component vaccine adjuvants [23, 28]. Importantly, cationic lipids are safer because less immunostimulatory than MPLA and, because of their synthetic origin, lack contamination risk. However, diC14-amidine alone has shown promising adjuvanticity properties in a vaccine against dust mite allergy (IgE antibodies) but failed in inducing IgG antibodies production [29] and was an effective adjuvant only when mixed with Alum [30] or coated with Mannan [31].

We have previously shown that cationic lipids lipopolyamines have TLR2 and NLRP3-dependent pro-inflammatory properties [32, 33]. While such inflammatory properties are deleterious in gene therapy, they can be exploited to develop new effective one-component vaccine adjuvants.

In order to investigate this alternative use, here we studied the immunostimulatory properties and ability to induce cellular and humoral responses of several lipopolyamines used previously as gene carriers [34–36] (Table 1).

2. Materials and methods

2.1. Reagents and cell lines

Human embryogenic kidney cells were purchased from the American Type Culture Collection ([HEK293] (ATCC® CRL1573™ RRID:CVCL_0045); RAW 264.7 (ATCC® TIB-71™ RRID:CVCL_0493) and human acute monocytic leukemia cell line (THP1 ECACC 88081201 RRID:CVCL_0006) were obtained from European Collection of Authenticated Cell Cultures. RPMI 1640 (Roswell Park Memorial Institute) and DMEM (Dulbecco's Modified Eagle's Medium) media, L-glutamine, sodium pyruvate, penicillin and streptomycin were from Lonza. Phorbol 12-myristate 13-acetate (PMA), FBS from North America, ovalbumin and albumin-FITC were from Sigma Aldrich. Fetal bovine serum (FBS) from South America was from Lonza. Aluminum salts were from Serva Electrophoresis (Alu-Gel-S suspension, ref. 12,261). Ultrapure standard lipopolysaccharide (LPS) from E. coli 0111:B4, Pam_3CSK_4, and Pam_2CSK_4, human TLR2 and TLR4 neutralizing antibodies (Cat. Code pab-hstlr2 RRID:AB_11124921, pab-hstlr1 RRID:AB_11124904, pab-hstlr6 RRID:AB_11124897 and pab-hstlr4 RRID:AB_11125132), RAW-Blue™ cells (RRID:CVCL_X594), Zeocin™, ultrapure standard lipopolysaccharide (LPS) from E. coli 0111:B4, RS-LPS and Quanti-Blue™ were from InvivoGen.

2.2. Lipid nanoparticle preparation

Lipopolyamines were synthesized as described earlier [34] and stored as powder at −20 °C. Lipid films were formed by dissolving powder in chloroform, followed by solvent evaporation under nitrogen stream and vacuum drying overnight, and kept at −20 °C. Liposomes were freshly formed by suspending lipid films into filtered Hepes 10 mM heated at 56 °C and sonicated for 5 min (BioRuptor, Diagenode) before each experiment.

2.3. Cell culture

HEK293 and RAW cells were maintained in DMEM supplemented with 10% heat-inactivated FBS from North America, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin. THP-1 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS from South America, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin. Raw-Blue cells were cultured in DMEM supplemented with 10% heat-inactivated FBS from South America, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin and 200 μg/ml Zeocin. All cells were incubated at 37 °C in a 5% CO_2 atmosphere.

All cell lines were tested for mycoplasma contamination on a regular basis. To avoid divergence from the parent line, cell cultures were passaged up to 10 times.

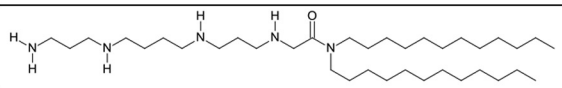
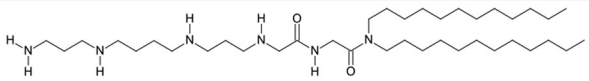
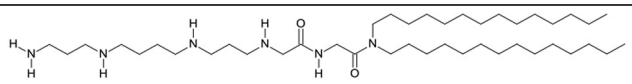
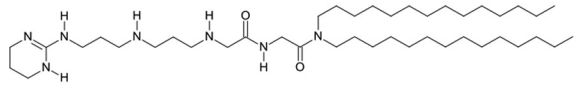
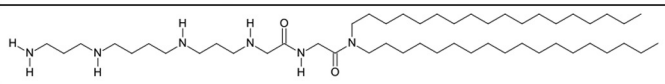
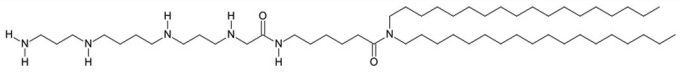
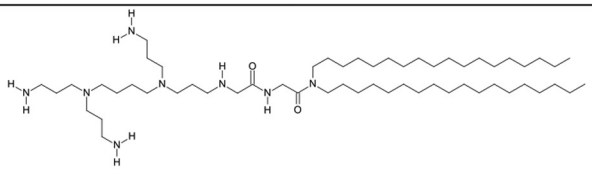
2.4. HEK293 cell transfection and stimulation

Cells were seeded at 4×10^4 cells/ml in 96-well plates (200 μl/well) and transiently transfected 4 days later. Expression vectors containing an NF-κB transcription reporter vector encoding firefly luciferase (10 ng/well pNF-κB-luc from Clontech), and a constitutively active reporter vector encoding Renilla luciferase (5 ng/well phRG-TK; Promega), together with empty vector (pcDNA3.1 from Invitrogen Cat. N. V79020) and cDNA encoding human membrane CD14 (3 ng/well) and human TLR2 (0.5 ng/well) or human TLR4/MD2 (3 ng/well) (kindly provided by Prof. Clare Bryant, University of Cambridge) were mixed with jetPEI (Polyplus transfection Cat.N. 101-10 N) and incubated with cells according to manufacturer's instructions. After 48 h, the medium was replaced with serum-free medium and cells were incubated for 1 h, additionally washed with serum-free medium, then directly incubated for 6 h with lipopolyamines (in serum-free medium) or PAMPs (in complete medium). Cells were washed with PBS and then lysed with passive lysis buffer (Promega). Luciferase and Renilla activity on cell lysates were quantified on a BioTek Synergy HT microplate reader using home-made luciferase reagent with luciferin (Biosynth) or coelenterazine (Biosynth) as previously described [33]. Firefly and Renilla luciferase activity on cell lysates were normalized and data were expressed as fold induction as compared to unstimulated conditions.

2.5. RAW- Blue™ cell experiments

RAW-Blue cells were seeded the day before the experiment at 5×10^5 cells/ml in 48-well plates (500 μl/ well). The day of

Table 1
List of lipids used in this study and summary of their TLR activity. These are lipopolyamines varying in the length of their double hydrophobic chain and the type and size of their polar head. The ability of each lipid to induce NF-κB via TLR2 and/or TLR4 was measured on HEK293 cells transfected with plasmids encoding [a] human TLR2 and CD14 or [b] human TLR4, MD2 and CD14 together with luciferase reporter plasmid dependent on NF-κB activation and compared to control cells. As shown in the table, the data come either from previous work already published or from Fig. 1B. − indicates no detectable or significant induction of that specific TLR, + corresponds to a fold induction between 2 and 10 as compared to non-stimulated control.

					NF-κB fold induction		
Name	**Formula**	**Polar Head**	**Structure**	**Chain length**	**TLR2[a]**	**TLR4[b]**	**Ref**
209204	$C_{36}H_{77}N_5O_1$	small linear		C12	+	+	This work Fig. 1B
122767	$C_{38}H_{80}N_6O_2$	medium linear		C12	+	+	
122766	$C_{42}H_{88}N_6O_2$	medium linear		C14	+	+	
206252	$C_{42}H_{86}N_7O_2$	small + cycle linear		C14	+	-	[32]
120525	$C_{50}H_{104}N_6O_2$	medium linear		C18	+	-	[33]
128506	$C_{54}H_{112}N_6O_2$	large linear		C18	+	-	
120535	$C_{56}H_{118}N_8O_2$	large branched		C18	+	-	

stimulation, cells were washed twice in serum-free medium and incubated 1 h in serum-free medium, then with lipopolyamines (in serum-free medium) or PAMPs (in complete medium) for 22 h. Cell culture supernatants were collected and assayed for murine TNF-α, IL-6 and IL-1β using DuoSet ELISA kits from R&D Systems, according to manufacturer's instructions. NF-κB activation was therefore evaluated by measuring the NF-κB-dependent alkaline phosphatase (SEAP) secreted by RAW-Blue cells in the collected supernatants by using the Quanti Blue™ reagent (InvivoGen), according to manufacturer's instructions and a BioTek Synergy HT Microplate Readers.

2.6. THP-1 cell stimulation

THP-1 cells were primed by suspending them in fresh medium containing 50 nM PMA and seeding them at 3.5×10^6 cells/ml in 96-well plates (200 μl/well) two days prior to stimulation. After 24 h of incubation with PMA, the medium was replaced with fresh one, and cells were incubated overnight to further allow cell differentiation.

The day of stimulation, cells were washed twice in serum-free medium and incubated 1 h in serum-free medium. Where specified cells were incubated for 1 h in the presence of antibodies blocking human TLR2 or TLR4, at final concentrations of 30 μg/ml in serum-free medium, as appropriate, prior to 5 h stimulation with lipopolyamines (in serum-free medium) or PAMPs (in complete medium).

After stimulation, cell culture supernatants were collected and assayed for human TNF-α, using DuoSet ELISA kits from R&D Systems, according to manufacturer's instructions and a BioTek Synergy HT Microplate Readers.

2.7. Antigen uptake

RAW cells were seeded in 24-well plates at 4×10^5 cells/well the day prior to uptake assay. The cells were then incubated in complete medium with albumin-FITC (1 μg) alone or in complex with 209,204 or 128,506 (4 nmol) or Alu-Gel-S (26 μg) in 10 μl saline buffer. After 24 h of incubation, the cells were washed three times in PBS and fixed with PFA (4% in PBS) for 20 min. After additional washing, nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole, 300 nM in PBS) for 30 min. Cells were then analyzed with a Zeiss AxioVert microscope.

2.8. Analysis of antigen-lipopolyamine complexes formation by gel retardation

Ovalbumin-lipopolyamines complexes were prepared with 20 nmol of lipids 209,204 or 128,506 mixed with ovalbumin (5 μg) in saline solution. For non-denaturing conditions, samples were diluted two times with 2× native loading buffer (glycerol 20%) before loading on 4–20% acrylamide gel and electrophoresed in Tris 25 mM, Glycine 192 mM at 125 V for 2 h at 4 °C. For denaturing conditions, the same samples were diluted two times with 2× denaturing loading buffer (glycerol 20%, SDS 10%), heated at 95 °C for 5 min and loaded on 4–20% acrylamide gel and electrophoresed in Tris 25 mM, Glycine 192 mM, SDS 0.1% at 125 V for 2 h. Gels were then placed in ethanol 50%, acetic acid 10% for 30 min (fixation), then in Coomassie Blue 0.05%, and acetic acid 10% for 1 h (coloration) and finally three times in acetic acid 10% for 15 min followed by overnight incubation (discoloration).

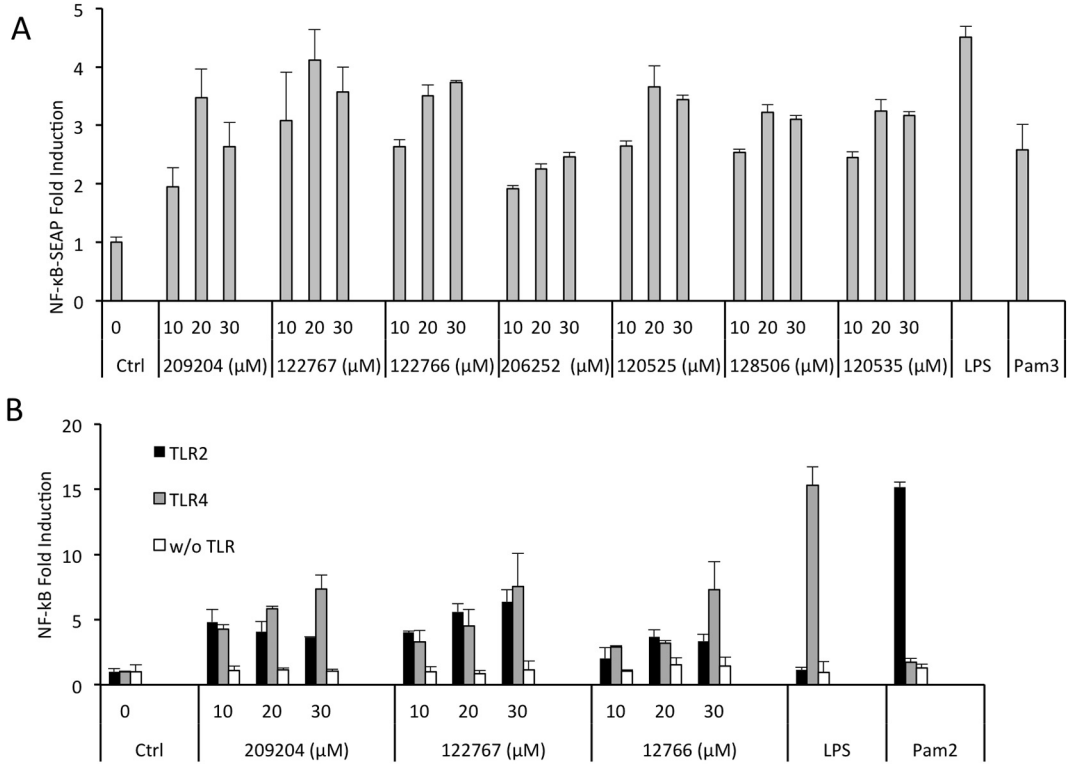


Fig. 1. Lipids 209,204, 122,766 and 122,767 activate both TLR2 and TLR4 and induce NF-κB activation comparable to TLR2-activating lipopolyamines 206,252, 120,525, 120,535, 128,506 and PAMPs.

A) Raw-blue cells were incubated for 22 h with increasing concentrations of lipopolyamines, LPS (100 ng/ml) or Pam_3CSK_4 (Pam3, 30 ng/ml) then supernatants were collected. NF-κB activation was measured by quantifying SEAP activity in collected supernatants (Quanti-blue test). B) HEK293 cells were transfected with human TLR2 and CD14 (TLR2), human TLR4, MD2 and CD14 (TLR4), or empty pcDNA3.1 vector (w/o TLR) together with luciferase reporter plasmid dependent on NF-κB activation and a constitutively active reporter vector encoding renilla luciferase. Two days after transfection, cells were incubated for 6 h with the indicated concentrations of lipids 209,204, 122,766 or 22,767, or Pam_2CSK_4 (Pam2, 30 ng/ml) or LPS (100 ng/ml). Luciferase and renilla were quantified in the cell lysate, normalized and reported here as fold induction as compared to control. Each bar represents the mean + standard deviation of three replicated values ($n = 3$). The experiment is representative of at least 3 independent replicates.

2.9. Vaccination titration of antibodies against OVA in serum

Swiss female mice (5 week-old) were obtained from Janvier (Le Genest-Saint-Isle, France) and maintained according to the recommendations prescribed under European Parliament and Council Directive 2010/63/EU. They were housed in standard conditions of temperature (22 °C) and lightning (12 h/12 h light/dark cycle) with free access to water and food and entered experiments after a 7-day adaptation period. This immunization protocol was approved by the local ethics committee (registration number: APAFIS N° 2,016,060,916,594,433-V3).

Female 6 week-old Swiss mice were injected intraperitoneally at day 0 and day 14 with 25 μg of ovalbumin in saline solution, alone or mixed with 0.65 mg of Alum, or 10 nmol or 100 nmol of lipopolyamines 209,204 or 128,506. Blood was collected after further 10 days (Day 24) by retro-orbital puncture and stored at −20 °C before being assayed as previously described [37].

Microtiter plates were coated with ovalbumin (10 μg/ml in PBS) overnight at room temperature and were blocked with PBS-0.1% Tween 20–2% bovine serum albumin (BSA) for 30 min at room temperature. The plates were washed three times with PBS-0.1% Tween 20–0.2% BSA, and serial twofold dilutions of mouse serum samples in PBS-0.1% Tween 20–1% BSA (starting at 1:100) were then added (100 μl/well). The plates were then incubated for 2 h at 37 °C and washed three times. For total IgG titer determination, peroxidase-conjugated anti-mouse IgG (1/2000) antibodies were used. For IgG1/IgG2a ratio determination, biotin-conjugated anti-mouse IgG1 and IgG2a (LO-MG1–2 biotin and LO-MG2a-9 biotin, respectively; Abcys) were used at 1:4000 and 1:2000 dilutions, respectively. The plates were washed three times, and peroxidase-conjugated streptavidin (1/4000) was added and incubated 1 h at room temperature. The revelation was done with 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA (Sigma) and the reaction was stopped with 3 M HCl (50 μl/well). The plates were read with a microplate reader (490 nm).

Absorbance readings were plotted against the reciprocal of the dilution. The antibody titer of a serum was determined graphically and calculated as the reciprocal of the dilution where the absorbance of the serum was 0.3 units above that of the control serum.

2.10. Peritoneal cells: preparation, culture and cytokine secretion assay

Mice were injected intraperitoneally as described above either once (1B1) or three times (3B1) at days 0, 14 and 30. The day after the last injection, mice were euthanized and peritoneal cells were recovered as described [38]. After washing with RPMI, cells were seeded in 24-well plates at 1×10^6 cells/well. After 2 h, medium was carefully removed and replaced with fresh one. Cells were cultured overnight at 37 °C in 5% CO_2 atmosphere. When indicated, cells were incubated for two days with activators. Cell culture supernatants were collected and assayed for mouse TNF-α and IL-5 using ELISA MAX Standard Set (BioLegend), and mouse IP-10 using Mouse CXCL10/IP-10/CRG-2 DuoSet ELISA (R&D) according to manufacturer's instructions.

2.11. Statistical analysis

Experimentation results were analyzed with the unpaired non-

parametric Mann–Whitney test using two-tailed *p*-values. Results with $p < .05$ were considered significant.

3. Results

3.1. The studied lipopolyamines have pro-inflammatory properties

We first incubated immortalized murine macrophages (Raw-blue cells) with increasing amounts of lipid 209,204, 122,767, 122,766, 206,252, 120,525, 128,506, 120,535 or LPS or Pam_3CSK_4 as positive controls. All lipopolyamines activated NF-κB (Fig. 1A) in a dose-dependent manner with an induction factor ranging from 2 to 4 compared to untreated cells. We previously showed that lipids 128,506, 120,535, 120,525 and 206,252 are activators of TLR2 ([32, 33] and Table 1). In order to identify the receptor involved in the immunostimulatory properties of lipids 122,766, 122,767 and 209,204, we investigated their ability to activate NF-κB in HEK293 cells transfected with an empty plasmid (w/o TLR) or encoding TLR2 or TLR4. The tested lipopolyamines activated NF-κB in a dose-dependent manner in cells transfected with TLR2 or TLR4, whereas no activity was measured in non-transfected cells revealing the peculiar ability of 122766, 122767 and 209204 to activate both receptors (Fig. 1B). Taken together, past and present data show that all lipids interact with TLR2 but only lipids 209204, 122767 and 122766 also interact with TLR4.

Since we highlighted that some lipopolyamines activate TLR4 and others do not, we wondered if this was reflected in a different spectrum of secreted cytokines. We measured cytokine secretion characteristic of different pathways in supernatants of murine macrophages stimulated for 22 h with lipopolyamines (Fig. 2). All lipopolyamines induced a significant secretion of TNF-α (Fig. 2A) and IP-10 (Fig. 2B) cytokines. Lipopolyamines induced much more IP-10 secretion than PAMPs, especially 122,766 but also 122,767 that showed a dose-dependent decrease probably due to toxicity.

In addition to TNF-α and IP-10 measured for all the compounds, we chose one lipid as representative of lipids activating both TLR2 and TLR4 (lipid 209,204) and one as representative of lipids activating only TLR2 (lipid 128,506) to measure IL-6, IL-10, IL-12 and IL1-β. No significant differences were found: both lipopolyamines induced IL-6 and IL-1β secretion in a dose-dependent manner (Fig. 2 C and D); neither IL-12 nor IL-10 were detected (data not shown).

3.2. Lipopolyamines interact with TLR differently from bacterial ligands

In order to compare the activity of non-microbial TLR activators like lipopolyamines, with the classical bacterial lipopolysaccharide (LPS) and lipopeptides (Pam_3CSK_4), we investigated the role of co-receptors in the pro-inflammatory activity of lipopolyamines and tested different timing of NF-κB activation. Moreover, using two lipopolyamines, we determined the ability of TLR2 and TLR4-activating lipopolyamines to induce an inflammatory response in human cells and the relative role of TLR2 and TLR4. We measured TNF-α secretion in primed THP-1 cells incubated for 5 h with lipids 209,204 or 122,766 and Pam_3CSK_4 or LPS as controls, after pre-incubation with or without antibodies blocking TLR2 or TLR4 (Fig. 3). Lipids 209,204 and 122,766 induced a significant TNF-α secretion that was strongly depleted by antibodies blocking TLR2, but not by antibodies blocking TLR4. Pam_3CSK_4 and LPS-induced TNF-α secretion was depleted by antibodies blocking TLR2 and TLR4 respectively, demonstrating that the antibodies were effective and specific at the used concentrations. The TNF-α secretion induced by 209,204 and 122,766 was decreased by about 70% with antibodies

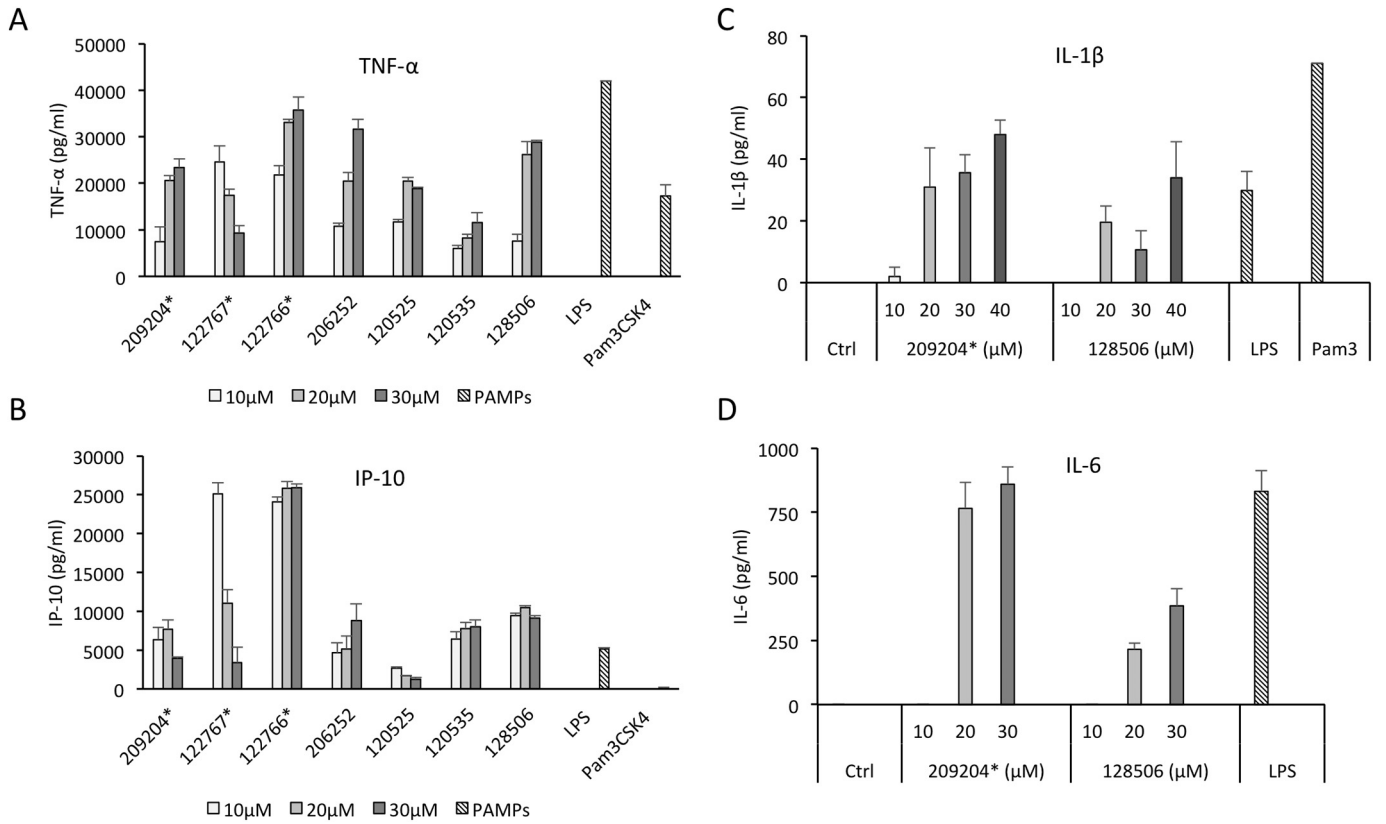


Fig. 2. Lipopolyamines induce cytokine secretion in murine macrophages.
Raw-blue cells were incubated for 22 h with the indicated concentrations of lipopolyamines or PAMPs (LPS, 100 ng/ml; Pam_3CSK_4, 30 ng/ml) then supernatants were collected. TNF-α (A), IP-10 (B), IL-1β (C) and IL-6 (D) were quantified by ELISA. (A and B data reported after subtraction of Ctrl value). * indicates lipids that activate both TLR2 and TLR4. Each bar represents the mean + standard deviation of three replicated values ($n = 3$). The experiment is representative of at least 3 independent replicates.

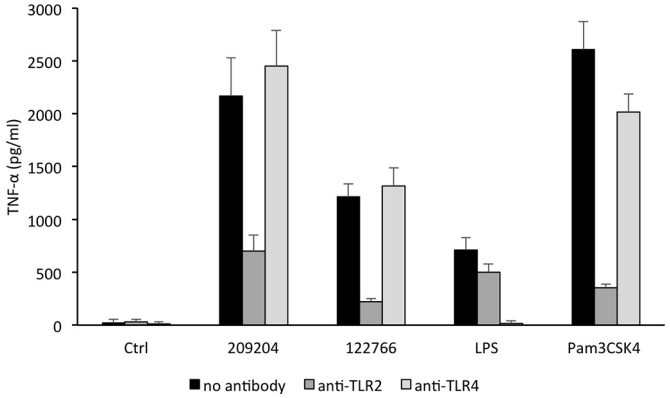


Fig. 3. TNF-α secretion in human macrophages is mainly dependent on TLR2. Primed THP1 cells were incubated for 1 h in serum-free medium, then 1 h with or without (no antibody) 60 μg/ml (30 μg/ml final concentration after addition of tested compounds) antibodies blocking TLR2 (anti-TLR2) or TLR4 (anti-TLR4). Then cells were incubated for 5 h with serum-free medium alone (Ctrl), lipids 209,204 or 122,766 (20 μM) or LPS (50 ng/ml) or Pam_3CSK_4 (30 ng/ml). TNF-α was quantified by ELISA. Each bar represents the mean + standard deviation of three replicated values (n = 3). The experiment is representative of at least 3 independent replicates.

blocking TLR2, suggesting that the secretion was mainly due to human TLR2.

Fig. 4A and B shows HEK293 cells transfected with human TLR4 (A) or TLR2 (B) with or without CD14, then stimulated with lipopolyamines or bacterial TLR2 or TLR4 ligands in serum-free or complete medium. A serum-free condition was chosen to ensure the absence of CD14. We chose two lipids as representative of the lipopolyamines activating only TLR2 (128,506 and 120,535) and two as representative of the ones activating both TLR2 and TLR4 (209,204 and 122,766). As expected LPS-dependent TLR4 activation was very poor in the absence of serum and totally abrogated in the absence of CD14 (Fig. 4A), whereas Pam_3CSK_4 was able to activate TLR2 in both conditions, although impaired in the absence of serum (Fig. 4B). In contrast to LPS, lipopolyamines activated TLR4 without CD14 and serum, although the TLR4 activation was enhanced in the presence of CD14 (Fig. 4A). This effect was more important for the C14-lipopolyamine (122766) than for the C12 one (209204) (Fig. 4A). Additionally, differently from Pam_3CSK_4, TLR2 activation by lipopolyamines was enhanced by CD14 (Fig. 4B). The enhancement was more important for the C18– (128,506, 120,535) over the C12– and C14-lipopolyamines (122,766, 209,204) (Fig. 4B).

PAMPs recognition by the innate system induces a strong immune response and NF-κB activation is normally observed after 5 h. However, in Fig. 1B, we tested 22 h of stimulation to ascertain to observe even weaker or slower responses such as the ones that involve endocytosis. In order to compare the timing of activation of lipopolyamines to the PAMPs one, we also measured the activation of NF-κB in murine macrophages (Raw-Blue cells) after 5 h of stimulation. Interestingly only the two C12 lipopolyamines (Lipids 209,204 and 122,767) and one C14 (lipid 206,252) activated significantly after 5 h (Fig. 5), like natural PAMPs (Pam_3CSK_4 and LPS) do, whereas most cationic lipids activated only after 22 h of stimulation.

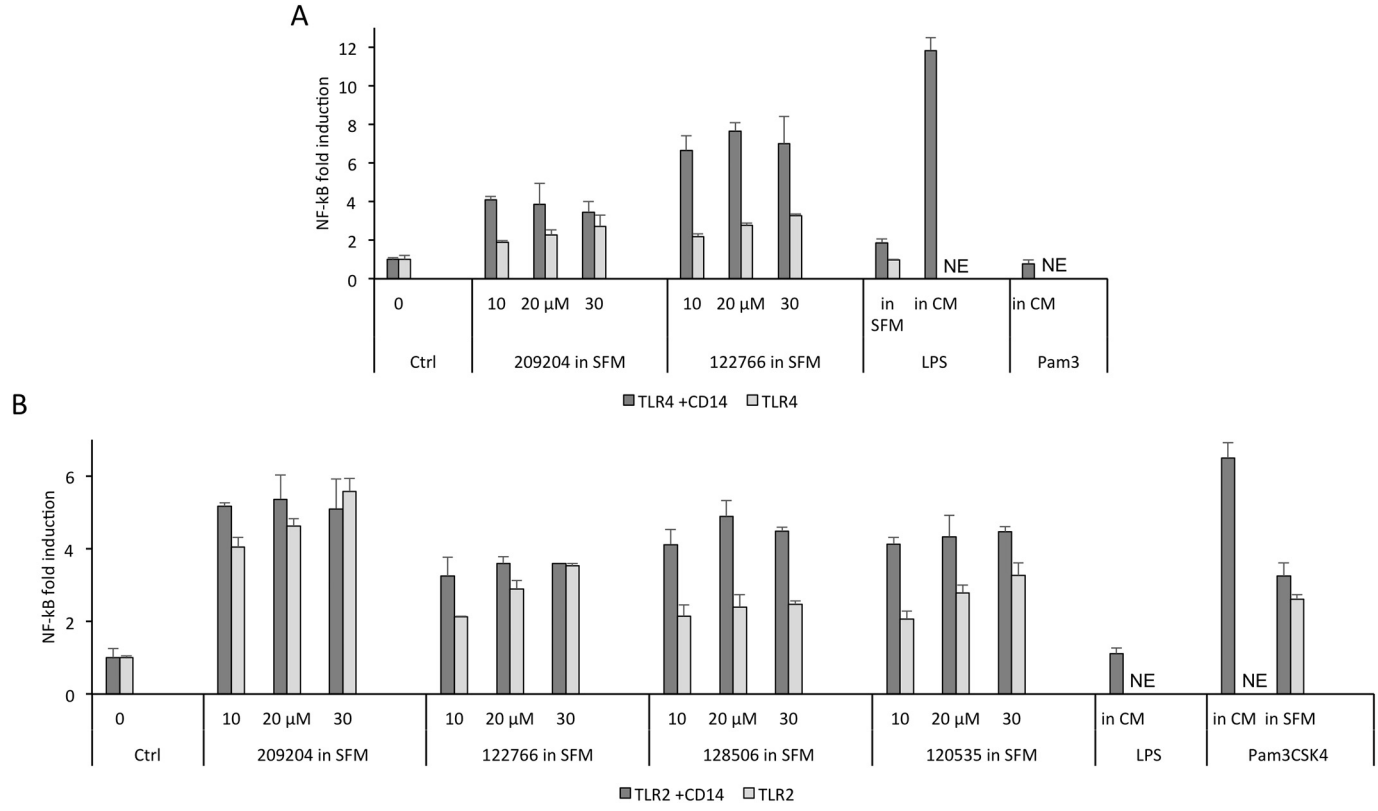


Fig. 4. Lipopolyamines do not require CD14 or serum to activate TLRs.
HEK293 cells were transfected with TLR4/MD2 (A) or TLR2 (B) plus CD14 (dark grey bars) or not (light grey bars), together with luciferase reporter plasmid dependent on NF-κB activation and a constitutively active reporter vector encoding renilla luciferase. Two days after transfection, cells were incubated for 6 h with the indicated concentrations of lipids 209,204, 122,766, 128,506, 120,535 in serum-free medium, LPS (100 ng/ml) or Pam_3CSK_4 (30 ng/ml) in serum-free (SFM) or complete medium (CM). Luciferase and renilla were quantified in the cell lysate, normalized and reported here as fold induction as compared to control. NE: not evaluated. Each bar represents the mean + standard deviation of three replicated values (n = 3). The experiment is representative of at least 3 independent replicates.

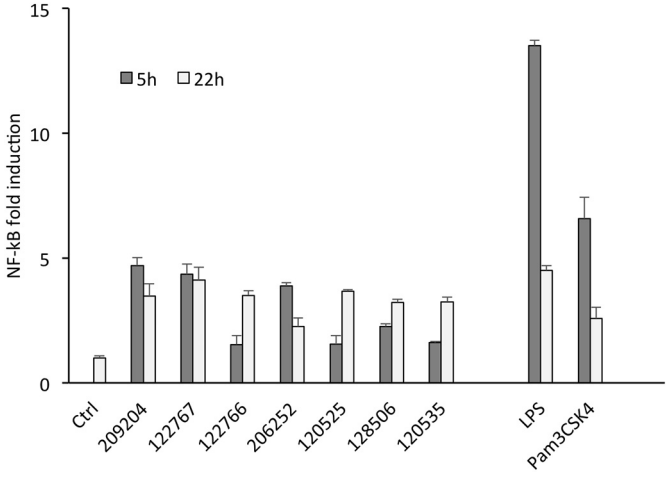


Fig. 5. Only 209,204, 122,767 and 206,252 activate a significant NF-κB in murine macrophages after 5 h of stimulation.
Raw-blue cells were incubated for 5 or 22 h with lipopolyamines (20 μM), LPS (100 ng/ml) or Pam_3CSK_4 (30 ng/ml), supernatants were collected and NF-κB activation was measured by quantifying SEAP activity (Quanti-blue test). Each bar represents the mean + standard deviation of three replicated values ($n = 3$). The experiment is representative of at least 3 independent replicates.

3.3. Lipopolyamines 209,204 and 128,506 have properties of immunological adjuvant

In the first part, we have shown that all the studied lipopolyamines were able to activate NF-κB, induce the production of pro-inflammatory cytokines by murine or human macrophages and interact with TLRs (TLR2 alone or TLR2 and TLR4 according to lipids) with behaviors that differ from the natural ligands of these receptors. These properties are compatible with an immunological adjuvant activity. To test this hypothesis, we chose two lipids inducing a strong induction of NF-κB, one activating both TLR2 and TLR4 (209204) and one activating only TLR2 (128506), and first studied their ability to form a complex with a protein antigen and to be internalized by cells in the form of this complex.

We associated ovalbumin, an antigen widely used in vaccination experiments, with lipopolyamines 209,204 or 128,506 as described above. To provide evidence of the formation of a complex between the lipid and the antigen, we loaded the mixture on an acrylamide gel under non-denaturing conditions (Fig. 6A, left part). Compared to ovalbumin alone, when associated with lipid 209,204 or 128,506, ovalbumin did not migrate through the gel. As a control, we assayed the same samples in denaturing conditions (Fig. 6A, right part) that demonstrate the dissociation of the complex in lipid (lower band) and ovalbumin (upper band). Lipid-antigen complexes were efficiently taken up by macrophages as shown in Fig. 6B. The 128,506-antigen and 209,204-antigen complexes were internalized in larger amounts than antigen alone, for which only a few fluorescence spots are detected, but less efficiently than Alu-gel-antigen complexes.

We then tested the adjuvanticity of these lipopolyamines using the model antigen ovalbumin (OVA). We injected mice intraperitoneally, twice (day 0 and day 14), with 25 μg of OVA in saline buffer alone or mixed with lipopolyamines (10 or 100 nmol) or aluminum salts (Alu-Gel-S, 0.65 mg). After 10 days (day 24), we assayed OVA-specific serum total IgG titers by ELISA. At higher doses (100 nmol) both lipids induced total IgG titers comparable to aluminum salts and significantly higher than OVA alone ($p < .01$, Mann-Whitney statistical analysis) (Fig. 7A).

To evaluate the Th2 humoral and Th1 cellular response induced by the adjuvants, we evaluated the IgG1/IgG2a ratio in the same sera using anti-IgG1 or IgG2a biotinylated antibodies. Differently from OVA alone or Alum, which induced exclusively IgG1, lipopolyamines induced both IgG1 and IgG2a antibodies production (Fig. 7B). In order to evaluate the possible toxicity of the lipopolyamine-OVA complexes, we measured the liver enzymes ALT (Alanine Transaminase) and AST (Aspartate Transaminase) as well as the inflammatory cytokine TNF-α in the serum of the injected mice one post injections (D1 and D15) and ten days later (D24) and examined the liver of injected mice at the end of the vaccination protocol (D25) by performing hematoxylin and eosin staining on slices. As shown in Fig. S3, no toxicity was detected neither in serum nor on liver slices.

3.4. Analysis of in vivo pro-inflammatory properties of lipopolyamines 209,204 and 128,506

Mice were injected intraperitoneally either once (1B1) or three times (3B1, at days 0, 14, 30) with ovalbumin/lipid (209,204 or 128,506) or ovalbumin/Alu-Gel-S complexes and, 24 h after the last injection, cells were recovered from the peritoneal cavity and counted. Fig. S1 shows that from the first injection, the number of cells was multiplied by 2 to 2.5 compared to the number of cells recovered in untreated mice. The number of cells recovered did not increase significantly with the number of injection and did not change according to the type of complex injected, lipid or alum.

The recovered cells were then cultured for 48 h in order to determine the inflammatory cytokines present in the culture supernatant (Fig. 7C, D and E). We quantified IP-10 and TNF-α, cytokines produced by activated lymphocytes and which are the signature of the Th1 activation pathway (cellular) (Fig. 7C and D), and IL-5 (Fig. 7E, left panel), a cytokine produced by activated lymphocytes that signal the Th2 activation pathway (humoral). Peritoneal cells recovered from mice injected three times with ovalbumin/209204 or ovalbumin/128506 produced, without the addition of any activator, levels of TNF-α and IP-10 significantly higher than peritoneal cells recovered from mice injected only once (Fig. 7C and D). Interestingly, three injections of ovalbumin/Alu-gel-S did not lead to significant secretion of IP-10 nor TNF-α in peritoneal cells (Fig. 7C and D). IL-5 was detectable in the supernatant of cells from mice injected three times with ovalbumin/209204, ovalbumin/128506 or Alu-gel-S complexes (Fig. 7E, left panel). Nevertheless, due to data dispersion, only the cells resulting from the treatment with ovalbumin/209204 had a level significantly different from the cells from untreated mice. However, upon activation by ovalbumin, IL-5 secretion level in the cells from vaccinated mice increased significantly compared to untreated mice, regardless of the adjuvant (Fig. 7E, right panel). This antigen-specific production of IL-5 is consistent with the antibody titers.

4. Discussion

4.1. The mechanism of interaction between lipopolyamines and TLRs

The cationic lipids 209,204, 122,766 and 122,767 have similar or identical polar head group but shorter alkyl chains (12 or 14 carbon atoms) compared to the C18-lipopolyamine 120,525, 128,506 and 120,535 (Table 1). Differently from these C18-lipopolyamines, which activates only TLR2 [33], 209,204, 122,766 and 122,767 are able to activate both TLR2 and TLR4 HEK-transfected cells (Fig. 1B).

However, the TNF-α secretion in THP-1 cells was mainly dependent on TLR2 as demonstrated by the strong inhibition (70%) induced by antibodies blocking TLR2 (Fig. 3). Although we did not observe any inhibition induced by antibodies blocking TLR4, we do not exclude a contribution of TLR4 in the residual 30% activity. Indeed, we have previously characterized a C14-cationic lipid (diC14-amidine) that activates TLR4 through a mechanism that involves residues located at the dimerization interface between the two TLR4 monomers far from the LPS binding site [26]. The observed inefficiency of antibodies blocking TLR4 in inhibiting TNF-α secretion may be explained by the fact that

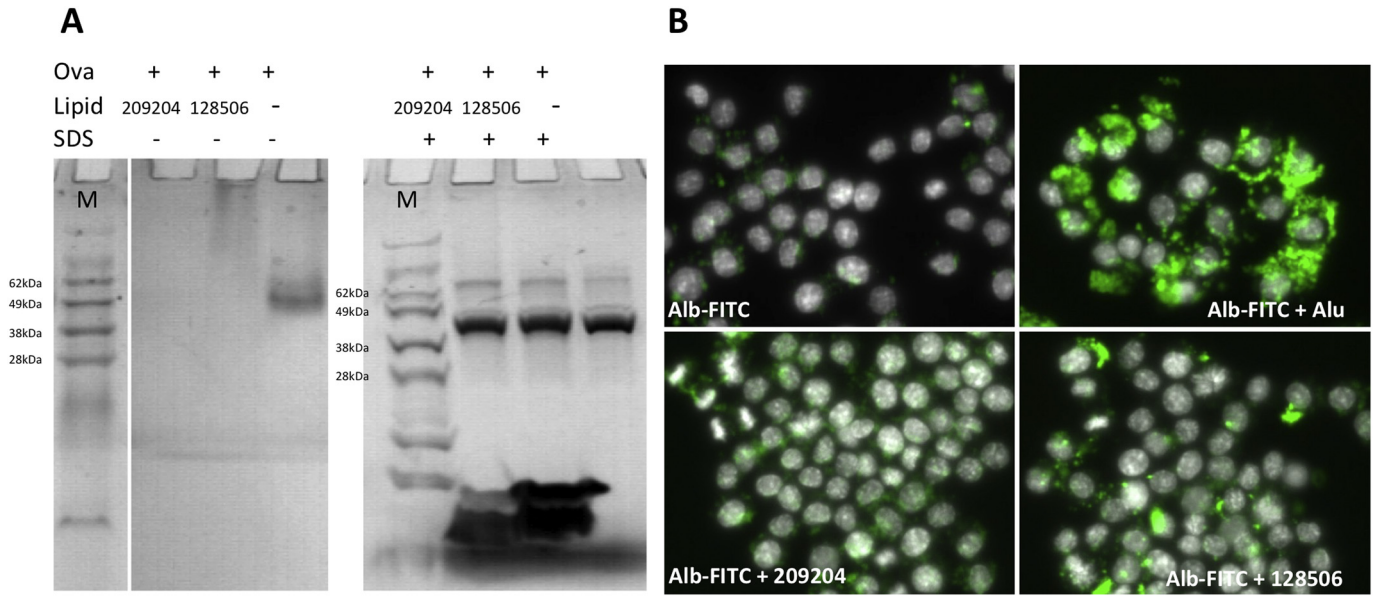


Fig. 6. Lipids 209,204 and 128,506 form complexes with albumin and help its cellular uptake.
A) 209,204 or 128,506 (20 nmol) were mixed with ovalbumin (5 μg) in saline solution before loading on acrylamide gel in non-denaturing (SDS -, left part) or denaturing (SDS +, right part) conditions. M: molecular weight markers. B) Raw cells were incubated for 24 h with mixtures of 209,204 or 128,506 or Alu-Gel and FITC-Albumin or FITC-albumin alone. Fluorescence microscopy of washed and fixed cells (green: FITC-Albumin; white: DAPI labeling of nuclei).

the cationic lipids characterized in this study follow the same mechanism as diC14-amidine and therefore standard TLR4 antibodies are not able to inhibit the interaction within this region.

Further differences were observed with respect to the mechanism of recognition of bacterial ligands by TLRs. LPS needs LPS Binding Protein (LBP), present in serum, to activate TLR4 (Fig. 4A) and Pam_2CSK_4 TLR2 activation is increased in the presence of serum (Fig. 4B). By contrast, lipopolyamines do not require serum (all experiments) and their activity is impaired when it is present (data not shown). Additionally, TLR4 activation by lipopolyamines does not require CD14, which is instead an essential co-receptor for TLR4 activation by LPS (Fig. 4A). Finally, lipopolyamines have an enhanced ability to activate TLR2 in the presence of CD14, which is not the case of the natural ligand Pam_2CSK_4 (Fig. 4B).

The type and amount of cytokines secreted after stimulation with lipopolyamines were comparable to bacterial TLR4 or TLR2 ligands, with the exception of IP-10 that was lower for LPS and absent for Pam_2CSK_4 (Fig. 2). IP-10 is typical of endosomal TLR/IRF signaling whereas TNF-α and IL-6 are cytokines representative of the TLR/MyD88/NF-κB pro-inflammatory pathway that originates from the plasma membrane [15]. However, the endosomal pathway may also induce a late NF-κB activation [20], which is compatible with the timing of NF-κB activation and cytokine secretion induced by lipopolyamines (Figs. 1 and 2). TLR/MyD88/NF-κB pathway that originates from the plasma membrane is faster and activated after few hours as can be observed in cells incubated with PAMPs (Fig. 5). Such differences in IP-10 secretion and timing suggest that lipopolyamines interaction with TLRs occur mainly in the endosomes compared to bacterial ligands.

However, the two C12 (209,204 and 122,767) and the C14-one with a pyrimidinium group (206252) activate NF-κB after 5 h as PAMPs (Fig. 5). It is likely that those lipids are the most fusogenic of the group. Indeed, according to the literature, shorter lipid chain and aromatic group in the polar head confer more fusogenicity [39, 40]. The observed different timing of NF-κB activation depending on fusogenicity suggests that fusogenic lipids fuse with the plasma membrane and activate MyD88-pathway (short timing), whereas longer chains would insert into the membrane only after endocytosis and would activate exclusively the TRIF pathway.

Even for LPS chemotype that does not require CD14 for MyD88 signaling because hydrophobic enough to insert into the membrane by itself, CD14 is necessary to induce TLR4 endocytosis and activate the endosomal pathway [41]. The known ability of cationic liposomes to interact with plasma membrane and induce endocytosis [42] may induce a CD14-independent endosome signalization previously proposed for diC14-amidine [43].

CD14 has been proposed to help LPS insertion to the cell membrane instead of directly transfer LPS to TLR4 [44]. This model may explain why CD14 is not required for lipopolyamines but merely enhances NF-κB activation and why such enhancement is more significant for longer (hence less fusogenic) lipids (Fig. 4A and B). Membrane insertion is probably required for cationic lipids to reach the binding site near the TLR4 transmembrane region, which has been shown crucial for diC14-amidine activity [26]. This binding site is big enough to host C18-lipopolyamines, but their inability to activate TLR4 may be due to their low fusogenicity. C18-lipopolyamines may be not sufficiently fusogenic to insert into the membrane and activate TLR4 even with the help of CD14.

4.2. *Adjuvanticity of TLR and NLRP3 activating lipopolyamines*

We also demonstrated here that lipopolyamines induce IP-10, IL-6 and IL-1β secretion in murine macrophages and TNF-α in murine and human macrophages. TNF-α and IL-6 are pro-inflammatory cytokines typical of the NF-κB induction. IP-10 is instead the signature of Type I IFN antiviral and T-cell stimulating response and is typical of the IRF induction. Finally, IL-1β secretion demonstrates the concomitant activation of the NF-κB pathway, which expresses pro-IL-1β, and of the inflammasome one that cleaves it. NLRP3 Inflammasome activation has been previously shown for the lipid 206,252 [32]. Activation of the inflammasome signaling is an important property of adjuvants such as Alum [45]. However, Alum has to be combined with TLR ligands to achieve antiviral or antibacterial immunization (Th1) [2]. The activation of both TLR and inflammasome pathways highlighted in this study, combined with the carrier properties makes cationic lipid lipopolyamines excellent candidates as one-component vaccine adjuvants.

Adjuvant properties have been demonstrated in this study by the presence of specific IgG antibodies in mice vaccinated with ovalbumin

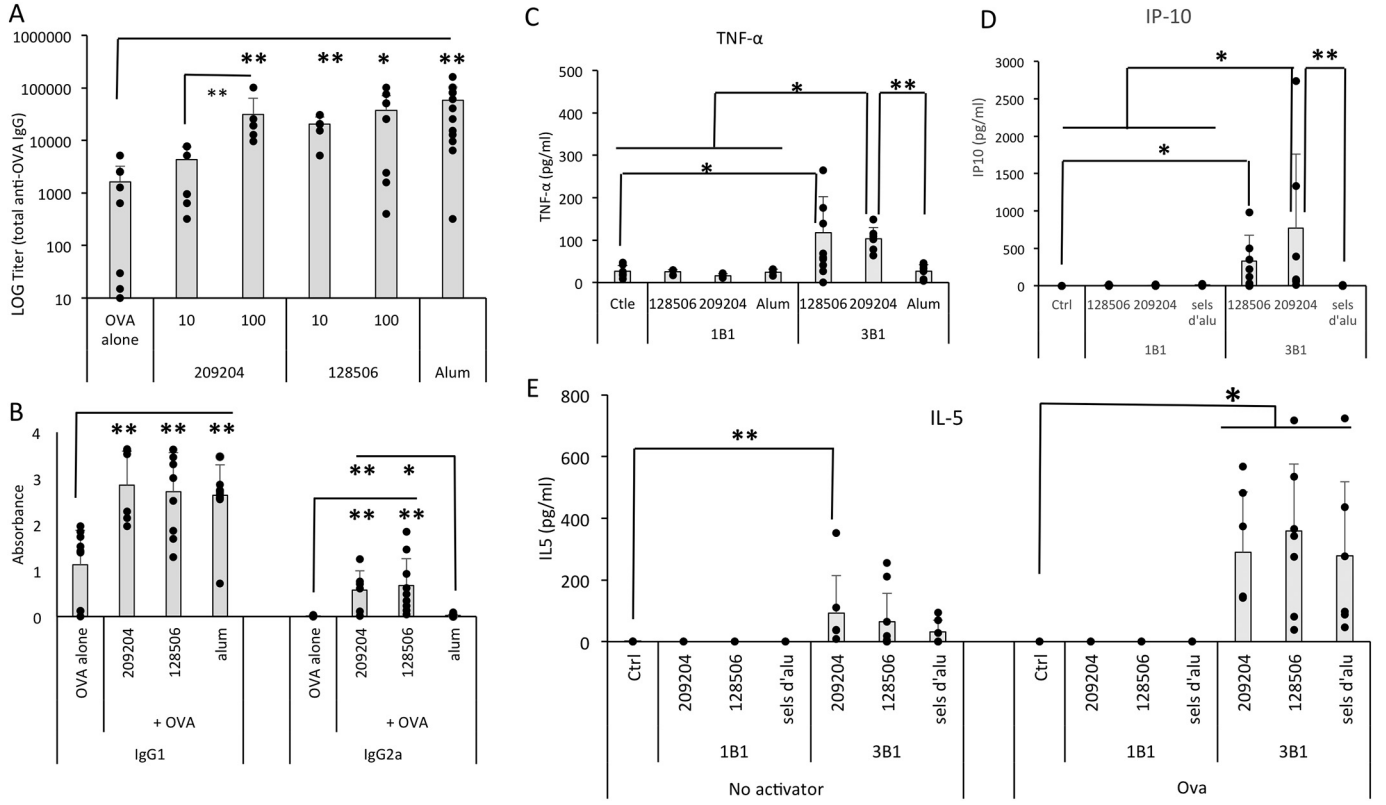


Fig. 7. Lipids 209,204 and 128,506 have an adjuvant effect similar to aluminum salts but induce a higher cellular Th1 response.
A, B) Swiss mice were injected at D0 and D14 with 25 μg of OVA alone or mixed with lipopolyamines 209,204 or 128,506 (10 or 100 nmol in A, 100 nmol in B) or 0.65 mg Alu-Gel-S, then sera were collected ten day later (D24). A) IgG titers determined by ELISA assay in collected sera. Statistic Mann-Whitney compared to OVA alone or to the other dose of the same lipid * $0.010 < p < .05$; ** $p < .01$; $n = 6$ (209,204_100 + OVA), 15 (alum + OVA) or 9 (the other conditions). B) Collected sera were diluted to one hundredth and tested in parallel by ELISA with anti IgG1 or IgG2a antibodies. Each bar represents the mean ± standard deviation of replicated values (n = 6 (209,204 + OVA), 12 (alum + OVA) or 9 (the other conditions)). Statistic Mann-Whitney compared to OVA alone or OVA + Alum * $0.010 < p < .05$; ** $p < .01$. C, D, E) Swiss mice were injected once at D0 (1B1) or three times at D0, D14 and D30 (3B1) with 25 μg of OVA mixed with lipopolyamines 209,204 or 128,506 (100 nmol) or 0.65 mg Alu-Gel-S. 24 h after the last injection, peritoneal cells were recovered, counted and seeded (10^6 cells/well). After 48 h supernatant was collected and TNF-α (A), IP-10 (B) and IL-5 (C) were assayed using ELISA and compared with cells recovered from untreated mice (Ctrl). Cells were cultured without added activator (C, D and E left panel) or in the presence of OVA 100 μg/ml (E right panel). Each bar represents the mean + standard deviation of replicated values (n = 6 (209,204 + OVA), 12 (alum + OVA) or 9 (the other conditions)). Statistic Mann-Whitney compared to OVA alone or OVA + Alum * $0.010 < p < .05$; ** $p < .01$.

simply mixed to 206,252 (data not shown), 209,204 or 128,506 (Fig. 7A). The titers of IgG antibodies were comparable to aluminum salts, the most used adjuvant in human vaccine [1]. The vaccination resulted in an immune response against OVA, which was not present in non-vaccinated mice (Fig. 7E and Fig. S2).

No significant differences were observed between only-TLR2 or TLR2 and TLR4-activating lipopolyamines, suggesting an equal role of both receptors or a main role of TLR2. However, more experiments are needed to investigate the type of immune response and the role of TLR and inflammasome pathway on immunization.

Following the simplified classical Th1/Th2 model, IgG1 antibodies against protein antigens are produced after T helper cells differentiation to Th2 during a humoral immune response against extracellular bacteria [46]. By contrast, antibodies against polysaccharide antigens, IgG2a, are typical of a Th1 cellular response against intracellular pathogens. Differently from aluminum salts, lipopolyamines induced both IgG1 and IgG2a antibodies production (Fig. 7B) strongly suggesting that both Th1 and Th2 differentiation are triggered by lipopolyamines. The differences in antibodies production are consistent with the secretory profile of the peritoneal cells recovered in the vaccinated mice (Fig. 7C, D and E): cells from mice vaccinated with lipopolyamines secreted cytokines typical of both Th1 (IP-10 and TNF-α) and Th2 pathway (IL-5), whereas vaccination with Alum induced only IL-5 secretion. This means that lipopolyamines may overcome the main restriction of Alum use: the lack of protection against intracellular pathogens for which a cell-mediated immunity (Th1) is required [2]. On the other hand, cationic lipids have already been used in human vaccine formulation but mainly for their ability to transport the antigen to APCs. Cationic lipids alone were not sufficiently immunostimulatory and had to be combined with stronger Th1 and Th2 immunostimulants. [1, 23]. By contrast, our work demonstrates that, without the addition of any other stimulants, immunogenic lipopolyamines are able to fulfill three functions in one (carrier, Th1 and Th2 immunostimulations) therefore achieving mice immunization against OVA.

Furthermore, like Alum, lipopolyamines can interact with the antigen and facilitate the transport (Fig. 6). But if in Alum complexes the antigen is simply absorbed [47] the lipopolyamines investigated in this study also form stable complex with DNA used for transfection [34, 48] and hence could be used for gene-based and anticancer vaccine as well [49]. Finally, their synthetic nature allows to explore structure modifications in order to enhance and direct immunostimulations.

5. Conclusions

The mechanism used by lipopolyamines to activate TLR2 and/or TLR4 endosomal pathways is different from bacterial PAMPs and probably linked to their fusogenic properties. The activation of both TLR and inflammasome pathways induce both humoral and cellular

immunization; which, combined with the ability of lipopolyamines to transport negatively charged molecules such as antigens, make lipopolyamines promising one-component vaccine adjuvant for vaccine formulations of minor cost and less adverse effects.

Author contribution

Conceptualization VE and MP; Validation PB, MP and CL; Formal Analysis VE and MP; Investigation AML, PB, CH, VE and MP; Resources AML, PB, JMR and CL; Writing-Original Draft and Visualization VE and MP; Writing-Review & Editing AML, PB, VE, JMR, MP and CL; Supervision JMR, VE and CL.

Acknowledgments

We are grateful to the Animal Platform, CRP2 -UMS 3612 CNRS -US25 Inserm-IRD –Faculté de Pharmacie de Paris, Université Paris Descartes, Paris, France. MP would like to acknowledge the FRIA-FNRS (F3/5/5-MCF/XH/FC-17514) and the Wiener-Anspach Foundation for their financial support. CL is grateful to the foundation Wiener-Anspach and the Marie Curie Actions (TLR4-CAT PIEF-GA-2012-326481) for financial support. We are also grateful to Nicolas SORHAINDO, Plateforme de Biochimie, CRI, Faculté de Médecine Site Bichat, Paris.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.08.020.

References

[1] C.R. Alving, Z. Beck, G.R. Matyas, M. Rao, Liposomal adjuvants for human vaccines, Expert Opin. Drug Deliv. 13 (2016) 807–816, https://doi.org/10.1517/17425247.2016.1151871.
[2] S. Awate, L.A. Babiuk, G. Mutwiri, Mechanisms of action of adjuvants, Front. Immunol. (2013) 4.
[3] D.A. Hume, Macrophages as APC and the dendritic cell myth, J. Immunol. Baltim. 1950 (181) (2008) 5829–5835.
[4] T. Kambayashi, T.M. Laufer, et al., Nat. Rev. Immunol. 14 (2014) 719–730.
[5] L.M. Bradley, D.K. Dalton, M. Croft, A direct role for IFN-gamma in regulation of Th1 cell development, J. Immunol. Baltim. 1950 (157) (1996) 1350–1358.
[6] R.B. Smeltz, J. Chen, R. Ehrhardt, E.M. Shevach, Role of IFN-gamma in Th1 differentiation: IFN-gamma regulates IL-18R alpha expression by preventing the negative effects of IL-4 and by inducing/maintaining IL-12 receptor beta 2 expression, J. Immunol. Baltim. 1950 (168) (2002) 6165–6172.
[7] M. Bruchard, C. Rebé, V. Derangère, D. Togbé, B. Ryffel, R. Boidot, E. Humblin, A. Hamman, F. Chalmin, H. Berger, A. Chevriaux, E. Limagne, L. Apetoh, F. Végran, F. Ghiringhelli, The receptor NLRP3 is a transcriptional regulator of TH2 differentiation, Nat. Immunol. 16 (2015) 859–870, https://doi.org/10.1038/ni.3202.
[8] I.S. Okoye, M.S. Wilson, CD4+ T helper 2 cells – microbial triggers, differentiation requirements and effector functions, Immunology 134 (2011) 368–377, https://doi.org/10.1111/j.1365-2567.2011.03497.x.
[9] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter, Helper T Cells and Lymphocyte Activation, https://www.ncbi.nlm.nih.gov/books/NBK26827/, (2002) , Accessed date: 28 January 2018.
[10] G.E. Kaiko, J.C. Horvat, K.W. Beagley, P.M. Hansbro, Immunological decision-making: how does the immune system decide to mount a helper T-cell response? Immunology 123 (2008) 326–338, https://doi.org/10.1111/j.1365-2567.2007.02719.x.
[11] S.F. Ziegler, Division of labour by CD4(+) T helper cells, Nat. Rev. Immunol. 16 (2016) 403, https://doi.org/10.1038/nri.2016.53.
[12] N. Petrovsky, J.C. Aguilar, Vaccine adjuvants: current state and future trends, Immunol. Cell Biol. 82 (2004) 488–496.
[13] A.M. Didierlaurent, S. Morel, L. Lockman, S.L. Giannini, M. Bisteau, H. Carlsen, A. Kielland, O. Vosters, N. Vanderheyde, F. Schiavetti, AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity, J. Immunol. Balt. Md. 1950 (2009) 6186–6197.
[14] N.J. Gay, M. Gangloff, Structure and function of Toll receptors and their ligands, Annu. Rev. Biochem. 76 (2007) 141–165, https://doi.org/10.1146/annurev.biochem.76.060305.151318.
[15] N.J. Gay, M.F. Symmons, M. Gangloff, C.E. Bryant, Assembly and localization of Toll-like receptor signalling complexes, Nat. Rev. Immunol. 14 (2014) 546–558, https://doi.org/10.1038/nri3713.
[16] G.M. Barton, J.C. Kagan, et al., Nat Rev Immunol. 9 (2009) 535–542.
[17] G.M. Barton, R. Medzhitov, et al., Science 300 (2003) 1524–1525.
[18] N. Dietrich, S. Lienenklaus, S.G. Weiss, N.O. Murine, et al., PLoS One 5 (2010) 10250.
[19] J. Stack, S.L. Doyle, D.J. Connolly, L.S. Reinert, K.M. O'Keeffe, R.M. McLoughlin, S.R. Paludan, A.G. Bowie, TRAM is required for TLR2 endosomal signaling to type I IFN induction, J. Immunol. Balt. Md. (2014) 6090–6102.
[20] H. Häcker, P.-H. Tseng, M. Karin, Expanding TRAF function: TRAF3 as a tri-faced immune regulator, Nat. Rev. Immunol. 11 (2011) 457–468.
[21] V. Mata-Haro, C. Cekic, M. Martin, P.M. Chilton, C.R. Casella, T.C. Mitchell, The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4, Science 316 (2007) 1628–1632.
[22] S. Schülke, A. Flaczyk, L. Vogel, N. Gaudenzio, I. Angers, B. Löschner, S. Wolfheimer, I. Spreitzer, S. Qureshi, M. Tsai, MPLA shows attenuated pro-inflammatory properties and diminished capacity to activate mast cells in comparison with LPS, Allergy 70 (2015) 1259–1268.
[23] D. Christensen, K.S. Korsholm, P. Andersen, E.M. Agger, Cationic liposomes as vaccine adjuvants, Exp. Rev. Vaccines 10 (2011) 513–521.
[24] Guidance For Industry For the Evaluation of Combination Vaccines for Preventable Diseases; Production, Testing and Clinical Studies, (2018), p. 23 n.d..
[25] Guideline on Adjuvants In Vaccines, 5 (2004), p. 18.
[26] C. Lonez, K.L. Irvine, M. Pizzuto, B.I. Schmidt, N.J. Gay, J.-M. Ruysschaert, M. Gangloff, C.E. Bryant, Critical residues involved in Toll-like receptor 4 activation by cationic lipid nanocarriers are not located at the lipopolysaccharide-binding interface, Cell. Mol. Life Sci. (2015), https://doi.org/10.1007/s00018-015-1915-1.
[27] T. Tanaka, A. Legat, E. Adam, J. Steuve, J.-S. Gatot, M. Vandenbranden, L. Ulianov, C. Lonez, J.-M. Ruysschaert, E. Muraille, M. Tuynder, M. Goldman, A. Jacquet, DiC14-amidine cationic liposomes stimulate myeloid dendritic cells through toll-like receptor 4, Eur. J. Immunol. 38 (2008) 1351–1357, https://doi.org/10.1002/eji.200737998.
[28] C. Lonez, M. Vandenbranden, J.-M. Ruysschaert, Cationic liposomal lipids: from gene carriers to cell signaling, Prog. Lipid Res. 47 (2008) 340–347, https://doi.org/10.1016/j.plipres.2008.03.002.
[29] A. Jacquet, J.-F. Vanderschrick, M. Vandenbranden, A. Elouahabi, M. Magi, L. Garcia, J.M. Ruysschaert, Vaccination with the recombinant allergen ProDer p 1 complexed with the cationic lipid DiC14-amidine prevents allergic responses to house dust mite, Mol. Ther. J. Am. Soc. Gene Ther. 11 (2005) 960–968.
[30] A. Wilmar, C. Lonez, M. Vermeersch, M. Andrianne, D. Pérez-Morga, J.-M. Ruysschaert, M. Vandenbranden, O. Leo, S.T. Temmerman, The cationic lipid, diC14 amidine, extends the adjuvant properties of aluminum salts through a TLR-4- and caspase-1-independent mechanism, Vaccine 30 (2012) 414–424.
[31] S. Sasaki, J. Fukushima, H. Arai, K.-I. Kusakabe, K. Hamajima, N. Ishii, F. Hirahara, K. Okuda, S. Kawamoto, J.-M. Ruysschaert, Human immunodeficiency virus type-1-specific immune responses induced by DNA vaccination are greatly enhanced by mannan-coated diC14-amidine, Eur. J. Immunol. 27 (1997) 3121–3129.
[32] C. Lonez, M. Bessodes, D. Scherman, M. Vandenbranden, V. Escriou, J.-M. Ruysschaert, Cationic lipid nanocarriers activate toll-like receptor 2 and NLRP3 inflammasome pathways, Nanomed. Nanotechnol. Biol. Med. 10 (2014) 775–782, https://doi.org/10.1016/j.nano.2013.12.003.
[33] M. Pizzuto, M. Gangloff, D. Scherman, N.J. Gay, V. Escriou, J.-M. Ruysschaert, C. Lonez, Toll-like receptor 2 promiscuity is responsible for the immunostimulatory activity of nucleic acid nanocarriers, J. Control. Release Off. (2016), https://doi.org/10.1016/j.jconrel.2016.12.029.
[34] G. Byk, C. Dubertret, V. Escriou, M. Frederic, G. Jaslin, R. Rangara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz, D. Scherman, Synthesis, activity, and structure-activity relationship studies of novel cationic lipids for DNA transfer, J. Med. Chem. 41 (1998) 229–235.
[35] V. Escriou, C. Ciolina, F. Lacroix, G. Byk, D. Scherman, P. Wils, Cationic lipid-mediated gene transfer: effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes, Biochim. Biophys. Acta 1368 (1998) 276–288.
[36] B. Frisch, M. Carrière, C. Largeau, F. Mathey, C. Masson, F. Schuber, D. Scherman, V. Escriou, A new triantennary galactose-targeted PEGylated gene carrier, characterization of its complex with DNA, and transfection of hepatoma cells, Bioconjug. Chem. 15 (2004) 754–764, https://doi.org/10.1021/bc049971k.
[37] C. Trollet, Y. Pereira, A. Burgain, E. Litzler, M. Mezrahi, J. Seguin, M. Manich, M.R. Popoff, D. Scherman, P. Bigey, Generation of high-titer neutralizing antibodies against botulinum toxins A, B, and E by DNA electrotransfer, Infect. Immun. 77 (2009) 2221–2229, https://doi.org/10.1128/IAI.01269-08.
[38] X. Zhang, R. Goncalves, D.M. Mosser, The isolation and characterization of murine macrophages, Curr. Protoc. Immunol. (2008), https://doi.org/10.1002/0471142735.im1401s83 Unit 14.1.
[39] P. Bhargava, M. Singh, V. Sreekanth, A. Bajaj, Nature of the charged headgroup determines the fusogenic potential and membrane properties of lithocholic acid phospholipids, J. Phys. Chem. B 118 (2014) 9341–9348, https://doi.org/10.1021/jp504104a.
[40] A. Mori, A. Chonn, L.S. Choi, A. Israels, M.A. Monck, P.R. Cullis, Stabilization and regulated fusion of liposomes containing a cationic lipid using amphipathic poly-ethyleneglycol derivatives, J. Liposome Res. 8 (1998) 195–211.
[41] Z. Jiang, P. Georgel, X. Du, L. Shamel, S. Sovath, S. Mudd, M. Huber, C. Kalis, S. Keck, C. Galanos, M. Freudenberg, B. Beutler, CD14 is required for MyD88-independent LPS signaling, Nat. Immunol. 6 (2005) 565–570.
[42] A. Elouahabi, J.M. Ruysschaert, Formation and intracellular trafficking of lipoplexes and polyplexes, Mol. Ther. J. Am. Soc. Gene Ther. 11 (2005) 336–347.
[43] B. Schmidt, J.M. Ruysschaert, M. Vandenbranden, Insight Into The Activation Mechanism Of Toll-Like Receptor, (2014) (Schmidt 2014-Thesis manuscript).
[44] T. Vasselon, E. Hailman, R. Thieringer, D.P. A, Internalization of monomeric lipopolysaccharide occurs after transfer out of cell surface CD14, J. Exp. Med. 4 (1999) 509–521.

[45] S.C. Eisenbarth, O.R. Colegio, W. O'Connor, F.S. Sutterwala, R.A. Flavell, Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants, Nature 453 (2008) 1122–1126.
[46] C. Snapper, W. Paul, Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production, Science 236 (1987) 944–947.
[47] L.S. Burrell, J.L. White, S.L. Hem, Stability of aluminium-containing adjuvants during aging at room temperature, Vaccine 18 (2000) 2188–2192.
[48] H. Rhinn, C. Largeau, P. Bigey, R.L. Kuen, M. Richard, D. Scherman, V. Escriou, How to make siRNA lipoplexes efficient? Add a DNA cargo, Biochim. Biophys. Acta 1790 (2009) 219–230, https://doi.org/10.1016/j.bbagen.2009.01.005.
[49] J.A. Berzofsky, J.D. Ahlers, I.M. Belyakov, Strategies for designing and optimizing new generation vaccines, Nat. Rev. Immunol. 1 (2001) 209–219.