International Journal of Pharmaceutics: X 2 (2020) 100054

Contents lists available at ScienceDirect

International Journal of Pharmaceutics: X

journal homepage: www.journals.elsevier.com/international-journal-of-pharmaceutics-x

A potent novel vaccine adjuvant based on straight polyacrylate

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ARTICLE INFO

Keywords:
Polyacrylate
SPA09
Adjuvant
Vaccine
Cytomegalovirus (CMV)

ABSTRACT

A structure-activity study was conducted to identify the structural characteristics underlying the adjuvant activity of straight (*i.e.* non-crosslinked) polyacrylate polymers (PAAs) in order to select a new PAA adjuvant candidate for future clinical development. The study revealed that the adjuvant effect of PAA was mainly influenced by polymer size (Mw) and dose. Maximal effects were obtained with large PAAs above 350 kDa and doses above 100 µg in mice. Small PAAs below 10 kDa had virtually no adjuvant effect. HPSEC analysis revealed that PAA polydispersity index and ramification had less impact on adjuvanticity. Heat stability studies indicated that residual persulfate could be detrimental to PAA stability. Hence, this impurity was systematically eliminated by diafiltration along with small Mw PAAs and residual acrylic acid that could potentially affect product safety, potency and stability. The selected PAA, termed SPA09, displayed an adjuvant effect that was superior to that of a standard emulsion adjuvant when tested with CMV-gB in mice, even in the absence of binding to the antigen. The induced immune response was dominated by strong IFNγ, IgG2c and virus neutralizing titers. The activity of SPA09 was then confirmed on human cells *via* the innate immune module of the human MIMIC® system.

1. Introduction

Although several new adjuvants have been approved in human vaccines over the last decade (Bonam et al., 2017; Del Giudice et al., 2018), yet the panel of adjuvants in licensed human vaccines remains fairly small. There is definitely a need for additional adjuvants that could be safely used in humans, would be easy to source and to formulate with a wide range of antigens and would be broadly applicable to a wide range of vaccines. In this regard, different studies indicated that polyacrylates (PAAs) in different forms and formats were safe and biocompatible and adjuvant active within a range of polymer sizes, doses, formulations and routes of administration compatible with vaccination (Coucke et al., 2009; Cranage et al., 2011; De Clercq, 2010; Diamantstein et al., 1971; Gartlan et al., 2016; Hicks et al., 1989; Krashias et al., 2010; Kreuter and Haenzel, 1978; Mustafaev, 1996; Parker et al., 2009; Powell et al., 2015; Regelson, 1979; Wegmann et al., 2015; Zondlo Fiume, 2002). Among the adjuvant-active PAAs, one can find plain PAAs (Diamantstein et al., 1971; Hilgers et al., 1998a), PAA dendrimers (Zaman et al., 2010, 2011), PAA alkyl esters (Hilgers et al., 2000, 1998b; Tifrea et al., 2011) and the crosslinked PAAs (*i.e.* Carbomers™ and Carbopols™) that are present in various experimental and commercial veterinary vaccines (Angelos et al., 2016; Deville et al., 2011, 2008; Gelfi et al., 2010; Gualandi et al., 1988; Hoogland et al., 2006; Kim et al., 2012; Liu et al., 2005; Mair et al., 2015; Minke et al., 2006; Mumford et al., 1994; Tollersrud et al., 2002; Zhang et al., 2018). Potent adjuvant systems were also obtained by combining Carbopol™ with oil-in-water emulsions (Dey et al., 2012; Lai et al., 2012; Sastry et al., 2017) or by co-formulating Carbomer™ with lecithin in an adjuvant system termed Adjuplex™ (Advanced BioAdjuvants LLC) (Chakrabarti et al., 2013; Gasper et al., 2016; Sastry et al., 2017; Wegmann et al., 2015). The latter recently entered human phase 1 clinical testing as an adjuvant in the adenovirus-based cocaine addiction vaccine candidate dAd5GNE (Havlicek et al., 2016; Hicks et al., 2014; Maoz et al., 2013).

In the aim of selecting a new PAA adjuvant candidate for future clinical development, straight PAAs available from commercial sources were screened for adjuvant activity in mice. Simple, non-crosslinked PAAs were preferred over Carbomers™ or Carbopols™, which are large

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https://doi.org/10.1016/j.ijpx.2020.100054
Received 21 May 2020; Received in revised form 6 July 2020; Accepted 18 July 2020
Available online 23 July 2020

PAAs randomly crosslinked with polyalkenyl ethers or divinylglycol forming quite viscous suspensions. In order to correlate PAA structural characteristics with adjuvant effects, the commercial PAAs were characterized for polymer size and branching by using a triple detection high performance size exclusion chromatography (HPSEC) system. This technique was useful for the structural analysis and quantitation of PAAs as crude raw materials and as adjuvants in final vaccine formulations. The adjuvant activity of the PAAs was then tested with the recombinant glycoprotein gB from human cytomegalovirus (CMV-gB) in mice, since CMV-gB, which is felt to be an important antigen for a human CMV vaccine, requires a potent adjuvant to become an effective immunogen, especially when used as a highly purified recombinant glycoprotein (Krause et al., 2013; Pass, 2009; Pass et al., 1999; Wang and Fu, 2014). Moreover, CMV-gB was already used in previous work with emulsion adjuvants, which could serve to benchmark the PAA adjuvant activity (Haensler et al., 2015). Finally, the selected PAA termed SPA09 was confirmed for its capacity to stimulate human immune cells *in vitro via* the innate immune module of the human MIMIC® system (Ma et al., 2010). This report describes the selection process, and the main properties and specifications of SPA09, the PAA adjuvant that was selected for future human clinical trials.

2. Materials and methods

2.1. CMV-gB antigen

The CMV-gB subunit antigen consists of the entire extracellular, glycosylated domain and the entire intracellular domain of the envelope glycoprotein B (gB) of CMV Towne strain. The recombinant gene deleted from the transmembrane domain was expressed in Chinese hamster ovary (CHO) cells (Sanofi Pasteur CHO cell line) and secreted as a protein of 807 amino acids with 19 putative N-linked glycosylation sites. The CMV-gB antigen was purified from the CHO cell line supernatant to ≥99% purity as assessed by SDS PAGE.

The antigen was stored frozen (−70 °C) at 0.8 mg/ml in 10 mM phosphate, 270 mM NaCl, 10 mM histidine, 3 mM EDTA, 0.005%Tween-80, pH 7.0 (CMV buffer).

2.2. Adjuvants

2.2.1. Emulsion adjuvants used as benchmarks

MF59-like squalene emulsions were used as benchmark adjuvants in *in vivo* and *in vitro* studies. For the *in vivo* study, MF59 was manufactured as a twofold concentrated squalene emulsion by using a M110-S Microfluidizer (Microfluidics, Newton, MA) according to the process and composition published for MF59 (O'Hagan and Singh, 2007) and contained 5% v/v squalene, 0.5% w/v Tween80, and 0.5% w/v Span85 in 10 mM citrate buffer pH 6.5. Alternatively, a commercial MF59-like emulsion termed AddaVax™, with the same composition and characteristics, was obtained from InVivogen (San Diego, CA) and was used in *in vitro* studies.

2.2.2. PAA polymers

Polyacrylic acid sodium salts of different sizes ranging from 2 to > 1000 kDa were obtained from Polysciences (Eppelheim, Germany) and from Polymer Source (Montreal, Canada) in the form of dry powders or concentrated solutions in water. In order to standardize the materials, they were resuspended with sterile water, adjusted to pH 7.0 with NaOH or HCl, further diluted to a concentration of about 20 mg/ml and dialyzed against 150 mM NaCl or PBS (3 consecutive baths) by using 2 kDa cutoff dialysis cassettes (Thermo Fischer Scientific, Courtaboeuf, France) at research stage. At pilot scale, the commercial materials were received as 10% w/w solutions in water adjusted to pH 7.0 with NaOH (Polysciences). The commercial solutions were then diafiltered upon receipt into PBS pH 7.4 on a 10 kDa or a 50 kDa Pellicon XL Biomax PES diafiltration cassette (Merck-Millipore, Molsheim, France). At this stage, the concentration of PAA was determined either by total organic carbon analyzis using a TOC VCPH apparatus (Shimadzu, Duisburg, Germany) or by high performance size exclusion chromatography (see section 2.4). Total organic carbon determinations were performed on 10-fold diluted samples and PAA concentration calculation was based on 3 carbon atoms per acrylate monomer, representing 38.3% of the total sodium acrylate monomer mass. The dialyzed PAA solutions were then diluted to 8 mg/ml into phosphate buffered saline (PBS pH 7.4) (or Tris buffered saline (TBS pH 7.4) for a specific study), sterilized by filtration through 0.2 µm PVDF membranes (research scale) or 0.2 µm Millipore Express SHC (PES) membranes (pilot scale) and kept stored at 4 °C. The > 1000 kDa PAA solution was too viscous to pass the 0.2 µm filtration step and was dropped at this stage.

PAA solutions for *in vivo* studies were always controlled for low endotoxin content (< 5 IU/ml) by using Endosafe® cartridges and an Endosafe®-PTSTM spectrophotometer (Charles River Laboratories International, Inc., Wilmington, MA).

2.3. Preparation of adjuvanted formulations used for immunization

CMV-gB antigen stock solution was first diluted to 80 µg/ml (2× solution) in CMV buffer. The dialyzed PAA polymer was diluted in PBS pH 7.4 to a twofold concentrated adjuvant solution (1–16 mg/ml of PAA). Adjuvanted formulations for preclinical testing were prepared by mixing extemporaneously one volume of a twofold concentrated adjuvant solution with one volume of 2× CMV-gB solution as to yield experimental vaccines containing 40 µg/ml of CMV-gB and the desired amount of PAA (from 0.5–8 mg/ml) or 2.5% v/v squalene in case of MF59. By this way, one mouse dose of PAA-adjuvanted vaccine always contained 2 µg of CMV-gB and 25–400 µg of PAA under a final volume of 50 µl.

2.4. High performance size exclusion chromatography (HPSEC) with triple detection for the characterization of PAAs

PAA average molecular weight (Mw), polydispersity index (PI), branching (Mark-Houvink coefficient) and concentration were determined by HPSEC as previously described (Cotte et al., 2017). In brief, separation was achieved through two A6000M (300 × 8 mm) columns (Malvern Instruments) connected in series. PBS at a flow rate of 0.6 ml/min was used as eluent. PAA samples of 100 µl were injected at approximatively 0.4 mg/ml for the determination of precise concentration by refractive index (RI) detection, Mw and PI by right angle light scattering (RALS) detection, and branching by viscosity (VIS) detection and using the Mark Houwink equation, which correlates polymer intrinsic viscosity and Mw through the following formula: $[h] = KMw^a$, where "*K*" and "*a*" are constants for the given polymer-solvent system. The Mark-Houwink coefficient "*a*" in this equation represents the slope in plots where the log of intrinsic viscosity is represented as a function of the log of Mw. When "*a*" is greater than 0.7, the polymer can be considered as linear (Masuelli, 2014). A 400 kDa pullulan standard (Agilent, santa-Clara, CA) was used as a control for all analyses. The column void volume (V0) and total permeation volume (Vt) were determined by injection of high-molecular-weight DNA and sucrose, respectively.

2.5. High performance anion exchange chromatography (HPAEC) with conductivity detection for the quantification of residual acrylate and persulfate in PAA

Acrylate and persulfate impurities in raw PAA materials were analyzed simultaneously by HPAEC with conductivity detection. A Dionex ICS-3000 ion chromatography system was used. It was equipped with a SP-1 single gradient pump, an autosampler, an IonPack™ AS11-HC analytical column (250 × 4 mm) and IonPack™ AG11-HC guard

column (50 × 4 mm) and a conductivity detector (Thermo Fisher Scientific, Pittsburgh, PA). An IonPack™ ATC-3 carbonate trap column (9 × 24 mm) was installed between the gradient pump and the injection valve. A Dionex Anion Electrolytically Regenerated Suppressor 500 (AERS 500; 4 mm) was installed between the column and the detector to improve the quality of the signal. The elution was performed with a NaOH gradient from 25 mM NaOH (eluent A) to 200 mM NaOH (eluent B). The gradient conditions were: 0% B (12 min), 0–40% B (5 min), 40–100% B (8 min), 100% B (25 min), 100–0% B (1 min), 0% B (9 min). The flow rate was 1 ml/min and the injection volume was 50 µl. In these conditions, retention times for sodium acrylate, sodium oxalate and sodium persulfate were around 4, 11, and 45 min, respectively. Before chromatography, the PAA samples were diluted with water to 10 mg/ml and sodium oxalate (Sigma Aldrich) was added at 50 µg/ml as an internal standard to correct for deviations in sample preparation. Then, 0.5 ml of sample was successively centrifuged (14,000 *g* for 30 min) on Amicon Ultracel 0.5 ml-100 K and 0.5 ml-3 K units (Merck Millipore, Darmstadt, Germany) to remove the high molecular weight species. The concentration of acrylate and persulfate in the samples was derived from calibration curves obtained by injecting increasing concentrations (1–100 µg/ml) of sodium acrylate (Sigma Aldrich) and sodium persulfate (Fisher Scientific) into the system.

The testing of residual persulfate and acrylate impurities in PAA samples after the diafiltration step was performed as a limit test. The persulfate and acrylate concentration limits not to exceed were established at 40 µg/ml and 1 µg/ml, respectively. For the persulfate impurity limit test the same chromatographic conditions were used. For the acrylate impurity limit test, the same HPAEC system was used but the analytical separation was achieved on a CarboPac™ SA10 analytical column (250 × 4 mm) and CarboPac™ SA10G guard column (50 × 4 mm) eluted with a NaOH gradient from 30 mM NaOH (eluent A) to 200 mM NaOH (eluent B). The gradient conditions were: 0% B (14 min), 0–100% B (6 min), 100% B (15 min), 100–0% B (1 min), 0% B (9 min). In these conditions, the retention time of sodium acrylate was around 8 min.

2.6. Accelerated heat stability studies

Samples of selected commercial lots of PAA raw material containing 10% w/w of PAA in water at pH 7.0 (Polysciences) were autoclaved for 15 min at 121 °C or placed for 15 min at 120 °C on a heated sand bath. The lots were characterized for residual acrylate, persulfate and sulfate by HPAEC (section 2.5). The polymer size, viscosity and branching were determined prior and after autoclaving or heating by HPSEC as described in section 2.4 to assess the integrity of the polymer. To confirm the detrimental role of residual sodium persulfate on PAA heat stability, a sample of heat stable PAA was spiked with 150 ppm of sodium persulfate and placed for 15 min at 120 °C on a heated sand bath.

2.7. Stability of a diafiltered PAA in comparison with PAA raw material

PAA lot#668942 containing 10% w/w of PAA and 401 ppm of residual sodium persulfate in water at pH 7.0 (Polysciences) was filled into type-I glass vials and placed at +5 °C (cold room), +25 °C or + 37 °C (thermostatic oven). At different time points (0, 1, 3 and 6 month) samples were analyzed for PAA integrity by HPSEC (see section 2.4). The stability of this raw material lot was compared to the stability of lot#658002 diafiltered into PBS and diluted to 8 mg/ml prior filling into type-I glass vials.

2.8. Mice immunization

C57BL/6 J female mice (10 per group), aged 6–8 weeks (body weight 18–20 g) at the time of first immunization were obtained from Charles River Laboratories. Mice were immunized twice, at 3–4 weeks interval, with the recombinant CMV-gB antigen (2 µg/injection) combined or not with adjuvant under a final volume of 50 µl by slow intramuscular (IM) injection into the left quadriceps. All mice of a given group received the same formulation at first and second immunization, but formulations were prepared freshly for each immunization. The test always comprised as controls, a group (n = 10) immunized twice with CMV-gB alone, a group (n = 10) immunized twice with CMV-gB combined with MF59 and a group (n = 5) injected with PBS for the determination of background levels in the cytokine assays.

All animal studies were performed in accordance with French national regulations and European Directives (Agreement #04991.02 from the French Ministry of Research). Studies were conducted in animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and by the French Ministry of Research and Education (Accreditation Number: C 69127 0302). Mouse studies were conducted under the accreditation number 04991.02 from the French Ministry of Research and Education. The protocols were approved by the Sanofi Pasteur Animal Ethics Committee under the internal reference number RE12087.

2.9. Immune readouts

Serum antibody and spleen cytokine responses were determined two weeks following the second immunization. CMV-gB specific IgG1 and IgG2c antibody titers were measured by an automated version of the previously described ELISAs (Haensler et al., 2015). The secretion of IFN-γ and IL-5 in cultures of splenocytes isolated from the immunized mice was quantified after 5 days of *in vitro* stimulation with 5 µg/well of CMV-gB by using the Mouse Th1/Th2 CBA-Flex Set assay (BD Biosciences, San Jose, CA) as described previously (Haensler et al., 2015). Neutralizing and binding antibody levels were measured individually on the 10 mice per group. Cytokine levels were determined in only 5 mice per group (arbitrarily chosen) and plotted after subtraction of background levels.

CMV-neutralizing antibody titers were determined on both MRC-5 fibroblasts and ARPE-19 epithelial cells. The previously described SN50 virus neutralization assay was used on MRC5 fibroblasts (Haensler et al., 2015) while an improved virus neutralization assay (µPRNT50) was used on epithelial cells. In brief, ARPE-19 epithelial cells were seeded in 96-well dark-walled, clear- and flat-bottomed microtiter plates and kept in a 5% CO_2 cell culture incubator at 37 °C. The next day, two-fold serial dilutions of heat-inactivated sera from immunized animals in DMEM/F12 containing 1% fetal bovine serum (FBS) were mixed with an equal volume of BADrUL131-Y4 CMV virus (Fluorescent restored AD169 strain provided by Dr. Thomas Shenk (Wang and Shenk, 2005)) containing approximately 800 fluorescent focus forming units (FFU) and 5% of baby rabbit complement. After 1 h at 37 °C in an atmosphere of 5% CO_2, the mixture was transferred onto the ARPE-19 cell monolayers. After four days of incubation, culture supernatants were removed. Cells were fixed with 100 µl of 1% formaldehyde in PBS for 1 h at room temperature (RT). The plates were washed three times with PBS and air dried at RT before analysis on a fluorescent plate reader (Microvision Instruments, Evry, France) to count the number of infected cells in each well. Negative controls contained no virus, and positive controls contained 400 FFU per ml. The mean number of infected cells in the positive control wells, representing 50% of the specific signal value, defined the threshold of seroneutralization. Cell counts were fit by nonlinear regression, and the seroneutralization titer was obtained by four-parameter curve fitting and defined as the interpolated reciprocal serum dilution that induced a 50% reduction in infected cell count. If fitting failed due to low neutralizing activity, a titer of 5 was assigned (half of the first dilution (1:10) tested for each individual serum sample). Geometric mean (GM) neutralizing antibody titers were calculated for each group.

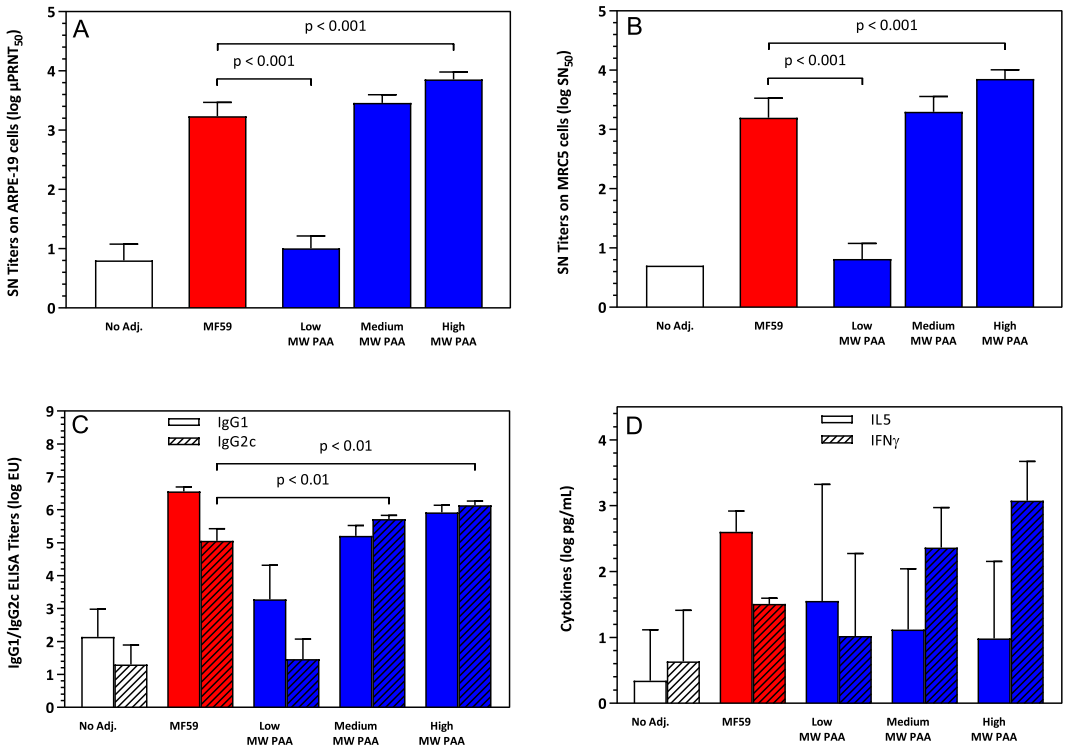

Fig. 1. Adjuvant activity of PAA is strongly dependent on polymer size. C57BL/6J mice (n = 10/group) were immunized 2 times, 4 weeks apart, by IM injection of 2 µg of recombinant CMV-gB without adjuvant (white bars) or with MF59 (red bars) or PAA (blue bars) under a final volume of 50 µl. Low Mw PAA (lot#617193; Mw = 9 kDa and PI = 3.1), medium Mw PAA (lot#601318; Mw = 134 kDa and PI = 2.4) and high molecular weight PAA (lot#566253; Mw = 489 kDa and PI = 3.8) were all tested at 200 µg/dose. Two weeks after the second immunization (D42), specific serum antibody responses and spleen cell cytokine responses were determined as described in the Materials and Methods section. Panel A: CMV neutralizing antibody titers measured on ARPE-19 epithelial cells. Panel B: CMV neutralizing antibody titers measured on MRC-5 fibroblasts. Panel C: CMV-gB-specific IgG1 and IgG2c titers. Panel D: IL-5 and IFN-γ productions from restimulated splenocytes cultures. Titers were plotted as geometric mean titers with 95% Confidence Interval (CI) on a log scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.10. In vitro Peripheral Tissue Equivalent (PTE) module of the MIMIC® system

2.10.1. Human blood processing

Donors included in this study were healthy and free of conventional blood-borne pathogens. Donor blood samples were obtained from OneBlood (Orlando, FL) using IRB protocols that were approved by Chesapeake Research Review Inc. (Columbia, MD). PBMCs were derived by density gradient separation and cryopreserved in dimethylsulphoxide-containing freezing media for liquid nitrogen vapor phase storage.

2.10.2. In vitro PTE module preparation

The innate module of the MIMIC® system, named Peripheral Tissue Equivalent (PTE) was prepared as described previously (Schanen and Drake 3rd, 2008). Briefly, EA.hy926 cells (ATCC, MD) were cultured on a transwell membrane (EMD MilliPore) in M199 media (Lonza Bioscience, Wlakersville, MD) supplemented with 20% fetal bovine serum and 1% Penicillin, Streptomycin and Glutamax until confluent. Once the cells reached confluency, the serum-supplemented media was replaced with serum-free XVIVO15 media (Lonza Bioscience). Frozen PBMCs were thawed and resuspended in XVIVO15 and applied onto the endothelial monolayer on the transwell insert. Then, the plates were incubated for 6 h at 37 ˚C to allow for migration of PBMCs across the endothelial monolayer and into the bottom compartment. After this period, the transwell inserts containing PBMCs were replaced with EA-cultured transwell inserts without PBMCs. Twenty four hours after initial PBMC application, different doses of PAA (SPA09), commercial MF59-like adjuvant, AddaVax (Invivogen, San Diego, CA) or an assay control consisting in a mixture of 100 ng/ml of *Pseudomonas aeruginosa* lipopolysaccharide (LPS) (Millipore Sigma, Burlington, MA), and 10 µg/ml of Resiquimod (R848) (InVivoGen) were added to the migrated cells in the bottom compartment for 48 h. Twenty four hours after the treatments were added, the transwell inserts were discarded. After 48 h of treatment, migrated dendritic cells were harvested for flow cytometry analysis (see section 2.10.3), and culture supernatants were collected for the dosage of cytokine/chemokine secretion (see section 2.10.4).

2.10.3. Flow cytometry (FACS)

Dendritic cell (DC) phenotype was determined using flow cytometry. The migrated cells from the MIMIC® PTE module were first incubated with Live Dead Aqua (Thermo Fisher Scientific), a cell viability marker to stain dead cells for 15 min. Thereafter, the cells were rinsed with PBS and then blocked with murine immunoglobulin G1 (IgG1; Sigma) to block Fcγ receptors. Next, the cells were labeled for 20 min with a fluorescent antibody cocktail. Finally, the cells were washed twice in buffer and then evaluated on a flow cytometer. Viable cellular events were acquired on a Fortessa cytometer (BD Biosciences) equipped with FACSDIVA software (BD Biosciences). FLOWJO software (Tree Star Inc., Ashland, OR) was used to analyze the data.

2.10.4. Cytokine and chemokine analysis

Culture supernatants from the MIMIC® PTE module were evaluated for cytokine/chemokine production using a Beadlyte human 22-plex multi-cytokine detection system (Millipore, Billerica, MA). For this purpose, assay samples or cytokine standards were incubated at RT for 2 h with multi-cytokine beads in a filter plate. After a wash step, the beads were incubated with multi-cytokine biotin for 90 min and then Beadlyte strepavidin-PE. After 30 min, supernatants were removed, and

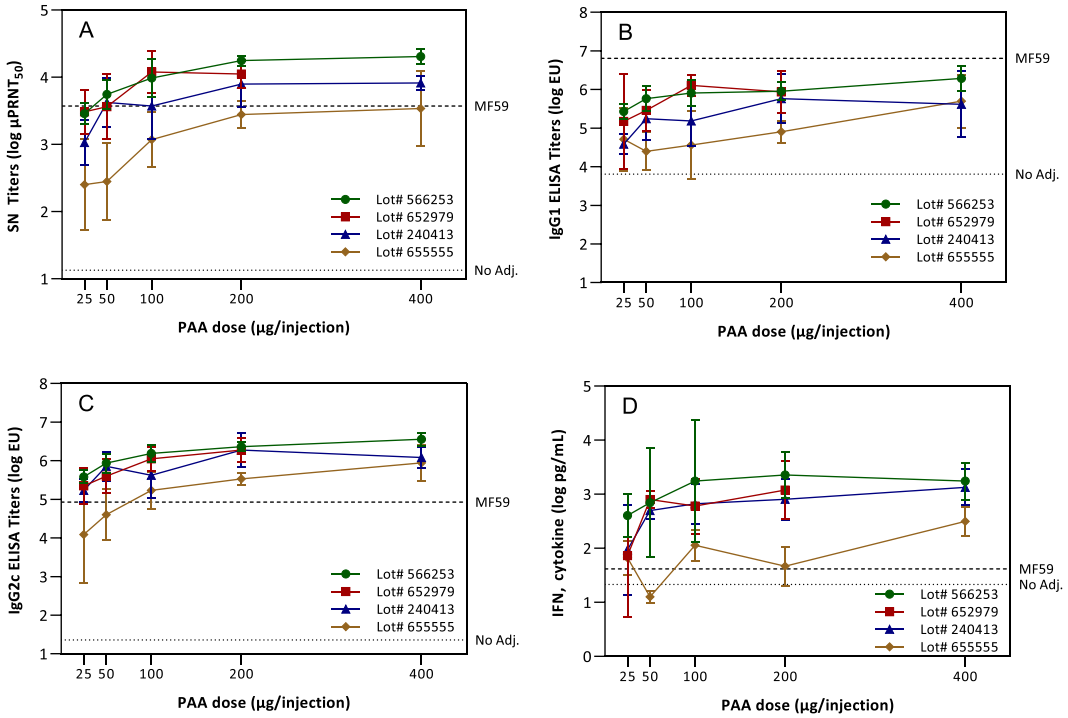

Fig. 2. Adjuvant activity of different technical lots of PAA is dependent on polymer size and dose and not on polymer branching. C57BL/6J mice (n = 10/group) were immunized 2 times, 25 days apart, by IM injection of 2 μg of recombinant CMV-gB without adjuvant or with MF59 or different PAAs at increasing concentrations (25-50-100-200-400 μg/dose) under a final volume of 50 μl. The PAAs used in this experiment were lots # 566253 (○), #652979 (□), #240413 (Δ) and #655555 (◊) characterized in Table 1. Two weeks following the second administration (D39), CMV neutralizing titers determined on ARPE-19 cells (Panel A), CMV-gB-specific IgG1 (panel B) and IgG2c (panel C) titers and spleen cell IFN-γ responses (panel D) were determined as in Fig. 1. Antibody and IFN-γ levels obtained in mice injected with CMV-gB alone or combined with MF59, are shown as dotted and dash lines, respectively. No IL-5 was detected in the PAA groups in this study.

the beads were then brought up in 125 ul of assay buffer. Analysis was performed using a Bio-Plex 200 system (Bio-Rad, Hercules, CA). Analyte concentrations were calculated based on relevant standard curves using the Bio-rad BIO-PLEX software manager.

2.11. Statistical analyses

For mouse study depicted in Fig. 1, an Analysis of Variance (ANOVA) with Dunnett adjustment for comparison to the MF59 reference was used. Concerning study depicted in Fig. 2, an Analysis of CoVariance (ANCOVA) was used. Two factors were included in the model, the factor "adjuvant" considered in a categorical way and the factor "dose" considered in a continuous way. Analyses were performed with SAS v9.1® and v9.2® software (SAS Institute, Cary, NC). For *in vitro* studies in the MIMIC® system depicted in Figs. 3 and 4, statistical analyses and graphs were prepared using GraphPad InStat version 5.00 (GraphPad Software, San Diego, CA, USA). Analysis of variance and Tukey's honestly significant difference (HSD) were employed to determine statistical significance. In all studies, P values $< .05$ were considered statistically significant.

3. Results

3.1. Formulation and characteristics of the commercial PAAs and interaction with CMV-gB

The aim of the present study was to select an adjuvant active PAA polymer from straight sodium polyacrylates, *i.e.* with no crosslinking, available from commercial sources and to identify the critical structural and quality attributes responsible for polymer stability and adjuvant activity. Before *in vitro or in vivo* applications, formulations were prepared and characterized carefully. Particularly, since the ability of PAA to interact with antigens through electrostatic interactions would directly depend on its degree of ionization, all PAA stock solutions were brought to pH 7 before engaging further process steps. This is important as well from a polymer conformational standpoint, since linear PAAs form random coils at low pH where most acid groups are protonated, and progressively expand upon pH increase as a result of electrostatic repulsions between the deprotonated carboxylate groups (Nagasawa et al., 1965; Vink, 1986). Then, to purify the samples from potential small molecular weight impurities (*e.g.* residual traces of polymerization initiators, solvents, unreacted monomer or very low molecular weight polymers) that could potentially affect PAA adjuvanticity, stability and safety, all PAA samples were extensively dialyzed against normal saline solution (150 mM NaCl; pH 7.0) or PBS pH 7.4. After this step, product identity and purity were confirmed by NMR. In some PAA batches, traces of poly-3-hydroxypropionate (poly-3HP), with no impact on adjuvanticity, were detected by ^{1}H NMR analysis (from 0 to 10% w/w; data not shown). Poly-3HP is a known process impurity in the synthesis of PAA, especially when the starting acrylic acid is obtained from 3-hydroxypropionic acid (Li et al., 2018).

To test for residual sodium sulfate and persulfate used as polymerization initiators in PAA synthesis and for residual acrylic acid monomers, an ion exchange chromatography method with conductivity detection (Khan and Adewuyi, 2011) was applied. It turned out that most of the batches of commercial PAA contained such impurities (Table 1), and that sodium persulfate could be detrimental to the heat stability of PAA, as already reported by others (Shukla et al., 2009). This is of importance in the case of sterilization by autoclaving. As shown on Table 2, a persulfate containing PAA (lot#658002) did not resist to autoclaving or to a 15 min heating step at 120 °C, as indicated by a decrease in average Mw, while a persulfate-free PAA (lot#566253) resisted the heating step. To demonstrate this was due to persulfate, PAA lot#566253 was spiked with 150 ppm of sodium persulfate and

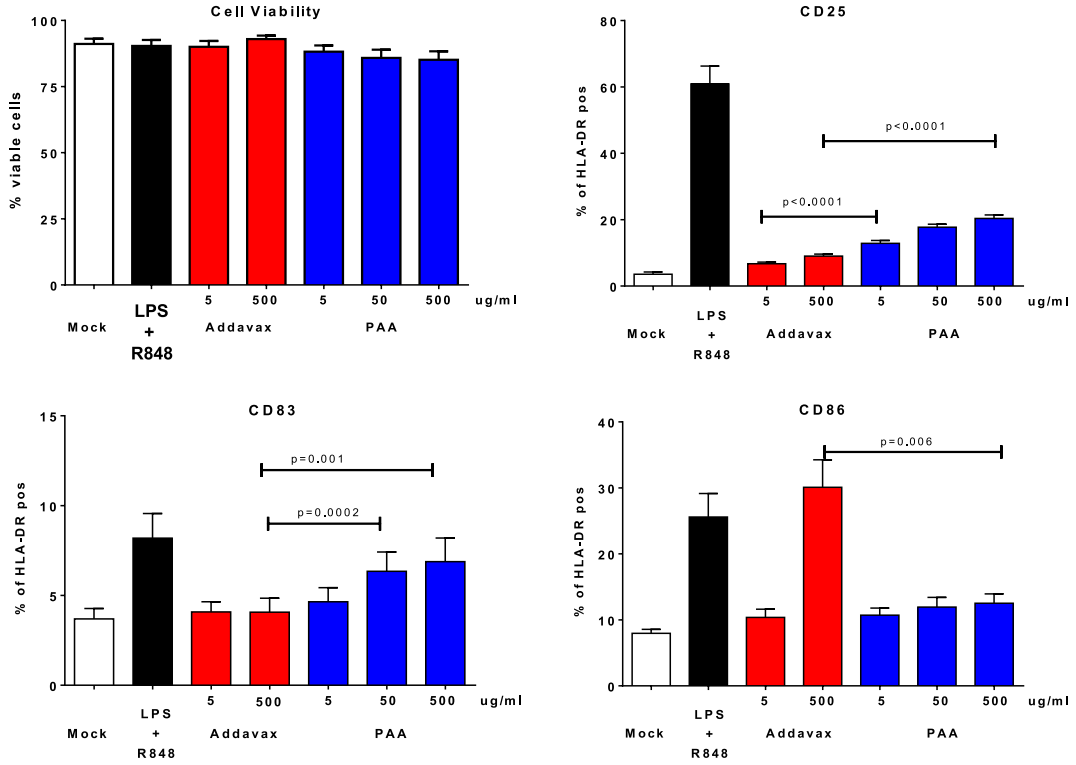

Fig. 3. PAA stimulation increased expression of certain antigen-presenting cells costimulatory markers. PBMCs were applied to the MIMIC® PTE module and treated with high Mw PAA (SPA09, blue bars), AddaVax™ (MF59-like emulsion, red bars) or LPS + R848 (positive control ■) as described in Material and Methods. After 48 h, the cells were harvested, labeled with Live Dead Aqua, anti-HLA-DR, anti-CD14, anti-CD25, anti-CD83 and anti-CD86, visualized by flow cytometry, analyzed using FlowJo, and plotted in % of cells (mean ± SEM). Data from 12 donors tested in two separate experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

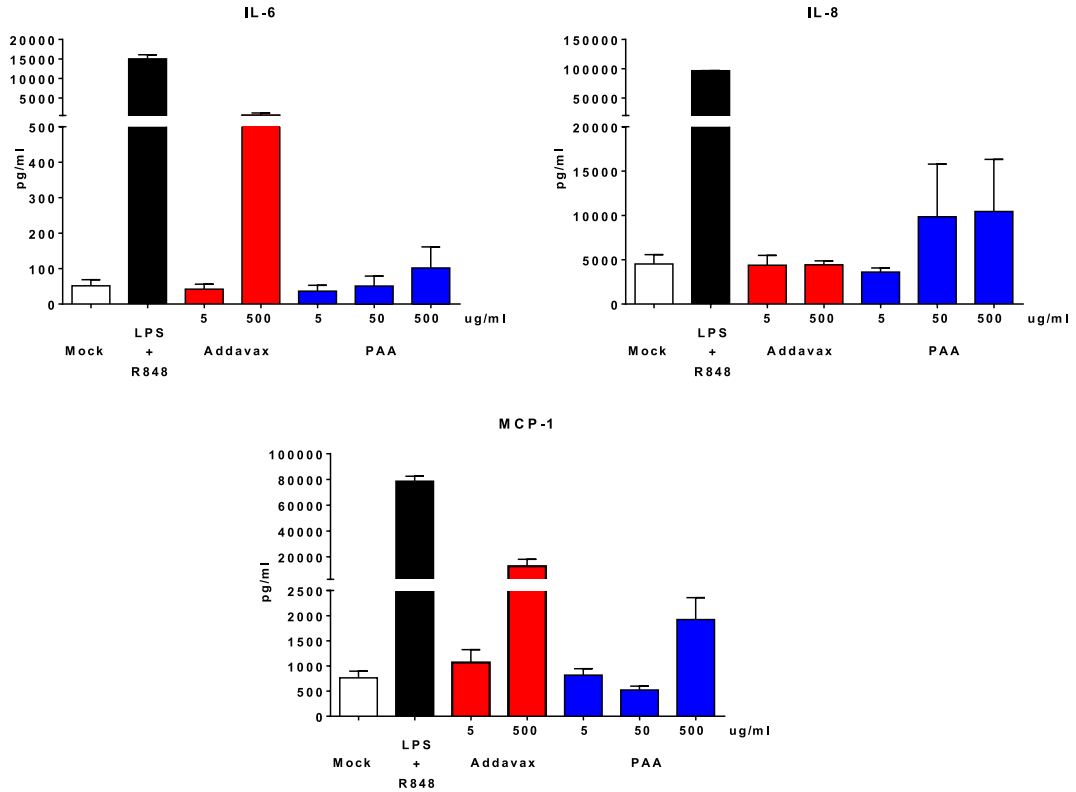

Fig. 4. PAA stimulation induced low cytokine and chemokine production from antigen-presenting cells. Culture supernatants from the study depicted in Fig. 3 were collected after 48 h and the secretion of cytokines and chemokines was assayed using multiplex array, analyzed and plotted in mean = SEM (pg/ml). Data from 12 donors tested in two separate experiments.

Table 1
Characteristics of PAA lots used in batch consistency studies.

PAA Lot# supplier	Stage	Mw (kDa)	Rh (nm)	PI	IV (dl/g)	Mark-Houwink coef.	Residual acrylate (ppm)	Residual persulfate (ppm)	Residual sulfate (ppm)
566253	Raw material	429	20.0	3.2	1.5	0.55	186	< DL (< 1 ppm)	1075
PolySciences	After diafiltration (on a 50 kDa unit)	564	24.6	1.9	2.0	0.55	< DL	< DL	< DL
652979	Raw material	404	26.8	4.1	3.6	0.93	257	169	497
PolySciences	After diafiltration (on a 50 kDa unit)	522	32.2	1.6	4.5	0.88	< DL	< DL	< DL
240413	Raw material	430	31.1	1.1	4.5	0.90	ND	NA	NA
Polymer Source	After PBS dilution (not diafiltered)	430	31.1	1.1	4.5	0.90	ND	NA	NA
655555	Raw material	90	10.9	3.0	1.1	0.95	ND	ND	ND
PolySciences	After diafiltration (on a 10 kDa unit)	120	13.5	1.4	1.4	0.95	ND	ND	ND
656483	Raw material	200	17.3	3.1	2.0	0.86	ND	2469	4057
PolySciences	After diafiltration (on a 50 kDa unit)	268	20.4	1.6	2.3	0.77	< DL	< DL	< DL
659589	Raw material	526	31.5	4.5	4.5	0.87	133	208	371
PolySciences	After diafiltration (on a 50 kDa unit)	583	34.2	2.1	4.9	0.89	< DL	< DL	< DL
658002	Raw material	405	26.6	4.3	3.5	0.86	201	138	506
PolySciences	After diafiltration (on a 50 kDa unit)	452	28.8	2.3	3.9	0.86	< DL	< DL	< DL

PAA characteristics were obtained by using a predefined value of 0.165 ml/g for the dn/dc of PAA.
Mw = average molecular weight; Rh = Hydrodynamic Radius (nm); PI = polydispersity index; IV = intrinsic viscosity; ppm = parts per million (mg/L); DL = detection limit; ND = not done; NA = not applicable.

heated for 15 minutes at 120 °C. As shown on Table 2, sodium persulfate spiking abolished the resistance of the polymer to heat. Hence, commercial PAAs were systematically neutralized and dialyzed (or diafiltered) upon receipt. The dialyzed or diafiltered PAA solutions were then diluted to 8 mg/ml into phosphate or TRIS buffered saline pH 7.4 (PBS or TBS), sterilized by filtration through a 0.2 μm PVDF membrane and kept stored at +4 °C until use. An accelerated heat stability study (Table 3) revealed that a PAA solution prepared by this method was stable for at least 6 months at +25 °C and +37 °C while a solution of raw (unprocessed) PAA material degraded after one month storage at these temperatures, as indicated by a decrease in Mw.

Since synthetic PAAs can vary in size (or molecular weight) and branches are easily formed during PAA synthesis by radical chain polymerization when the reaction is not well controlled (Yu-Su et al., 2011), it was important to use a reliable characterization method to enable correlating PAA structural characteristics with adjuvant activity. HPSEC with a triple detection system (RI, RALLS and viscosity) was applied to precisely determine the polymer average molecular weight (Mw), polydispersity index (PI) and branching through the Mark-Houwink equation correlating polymer size and branching with intrinsic viscosity (Masuelli, 2014). This method was applied to characterize the PAAs prior and after dialysis or diafiltration and to ultimately define the attributes that were important for adjuvant activity (Cotte et al., 2017). As shown in Table 1, the polydispersity indices of PAAs obtained from Polysciences were comprised between 3.1 and 4.5 and were characteristic of fairly heterogeneous polymer products. The processing of PAA samples by dialysis or diafiltration increased the Mw and decreased the PIs as determined by HPSEC, indicating that some low molecular weight polymer chains could be removed during this purification step. Interestingly, the PAA obtained from PolymerSource (lot#240413) had a Mw of 430 kDa and a small PI of 1.1, even without diafiltration, due to a specific manufacturing or purification process that was not disclosed by the vendor.

As described earlier, the HPSEC method was also used for PAA quantification in the absence and presence of CMV-gB. Interestingly, the retention time and elution profile of PAA was not affected by CMV-gB, indicating that HPSEC quantification of PAA would remain possible in the final CMV-gB vaccine formulation (Cotte et al., 2017). This also suggested that there was no strong association between PAA and CMV-gB, as a strong association would have created larger polymer-protein complexes with different elution profiles (Topuzoğulları et al., 2007).

3.2. Assessment of the adjuvant activity in mice and correlation with PAA structure

The adjuvant activity of various PAAs was assessed by using the CMV-gB as model antigen and a MF59-like squalene emulsion as benchmark adjuvant in C57BL/6J mice. The characteristics of a typical

Table 2
Impact of residual sodium persulfate on the heat stability of PAA.

PAA Lot# supplier	Stage	Mw (kDa)	PI	IV (dl/g)	Mark-Houwink coeff.	Residual acrylate (ppm)	Residual persulfate (ppm)	Residual sulfate (ppm)
658002	Prior heat exposure*	405	4.3	3.5	0.86	201	138	506
PolySciences	Autoclaved*	189	3.0	1.9	0.86	104	< DL	859
	After heat exposure	206		2.1	0.84			
566253	Prior heat exposure	435	3.2	1.6	0.55	186	< DL	1075
PolySciences	After heat exposure	429		1.6	0.55			
	After sodium persulfate spiking (150 ppm) and heat exposure	323		1.4	0.55			

Mw = average molecular weight; PI = polydispersity index; IV = intrinsic viscosity; ppm = parts per million (mg/L); DL = detection limit; ND = not done; NA = not applicable.
* Autoclaving is 15 min at 121 °C; heat exposure is 15 min at 120 °C on a heated sand bath.

Table 3
Stability of PAA lots prior and after diafiltration.

	PAA raw material Lot#668942					PAA Lot#658002 after diafiltration*	
Time/T^{ure}	Mw (kDa)	PI	Acrylate (ppm)	Persulfate** (ppm)	Sulfate** (ppm)	Mw (kDa)	PI
T0	470	4.3	201	401	367	444	2.2
3 M; 5 °C	467	4.8	219	366	414	451	2.6
6 M; 5 °C	486	5.2	ND	ND	ND	456	2.4
1 M; 25 °C	399	4.0	141	306	502	442	2.3
3 M; 25 °C	346	4.1	84	209	648	421	2.5
6 M; 25 °C	336	5.8	ND	ND	ND	455	2.2
1 M; 37 °C	310	4.0	33	54	862	443	2.1
3 M; 37 °C	302	3.9	41	< 13	1026	419	2.3
6 M; 37 °C	321	4.2	ND	ND	ND	436	2.3

Mw = average molecular weight; PI = polydispersity index; ppm = parts per million (mg/L).
* Persulfate is totally eliminated during the diafiltration step (see Table 1).
** Persulfate degrades into sulfate over time.

Table 4
Characterization of an adjuvant-active PAA formulation.

	[PAA] mg/ml	[CMV-gB] µg/ml	pH	Osmolality (mOsm/kg)	Viscosity (mPa.s)
CMV-gB in CMV buffer	–	80	7.0	280	–
PAA lot#566253 in PBS	8.0		7.5	278	3.7
CMV-gB + PAA (vol/vol)	4.0	40	7.4	277	2.7
CMV buffer + PAA (vol/vol)	4.0	–	7.4	275	2.6

CMV Buffer = 5 mM NaPO4, 135 mM NaCl, 5 mM Histidine, 1.5 mM EDTA, 0.0025% Tween 80, pH 7.0.
PBS = 9.6 mM K/NaPO4, 2.7 mM KCl, 137 mM NaCl, pH 7.4.

CMV-gB/PAA formulation used for immunization in terms of pH, osmolality and viscosity are shown in Table 4. All formulations characteristics were compatible with IM administration.

The first study consisted in evaluating the effect of polymer size, *i.e.* Mw, on adjuvant activity. Three PAAs obtained from Polysciences were tested after dialysis and sterile filtration: a low Mw PAA (Mw = 9 kDa), a medium Mw PAA (Mw = 134 kDa) and a higher Mw PAA (Mw = 489 kDa), all at a 200 µg dose. A very high Mw PAA (> 1000 kDa), which failed to pass 0.2 µm filtration due to high viscosity was dropped at this stage.

CMV neutralizing titers elicited by CMV-gB combined with PAAs of different molecular weights are shown on Fig. 1**, panel A and B**. Similar virus neutralization profiles were obtained on MRC5 (fibroblasts) and ARPE (epithelial) cells leading to the same conclusions for group comparison. No or low neutralizing antibody titers were detected in mice immunized with non-adjuvanted CMV-gB (GMT = 6). Marked adjuvant effects were observed with the MF59-like squalene emulsion and with the medium and high Mw PAAs but not with the low Mw PAA. When compared to the titers induced by CMV-gB/MF59, the titers obtained with low Mw PAA were significantly lower (*p*-value < .001) whereas those obtained with high Mw PAA were significantly higher (p-value < .001). There was no statistical difference between the titers measured in the MF59 and medium Mw PAA groups (p-value > .05). Corresponding CMV gB-specific IgG1 and IgG2c antibody responses are reported in Fig. 1 **panel C**. Low IgG1 and IgG2c antibody titers were detected in mice immunized with non-adjuvanted CMV-gB (mean titers in log were 2.15 and 1.30, respectively). The medium and high Mw PAAs, but not the low Mw PAA, induced IgG2c titers that were superior to those obtained with MF59 (all *p*-values < .01), whereas MF59 remained the strongest IgG1 inducer. This result confirmed the correlation between seroneutralization activity and high IgG2c titers.

Concerning spleen cytokine responses, means of IL-5 and IFN-γ concentrations in splenocyte cultures are shown on Fig. 1**, panel D**. Due to the broad distribution of data points within individual groups, no statistical analysis was performed but overall, the PAA adjuvants were less effective than MF59 at inducing IL-5 responses and more effective at inducing IFN-γ responses, especially the medium and high Mw PAAs. This was in accordance with the capacity of PAA to promote IgG2c titers in mice and confirmed that the adjuvant profile of high Mw PAA was Th-1 biased. Note also that when formulated in TRIS buffered saline instead of PBS the adjuvant activity of PAA remained unchanged (data not shown). This study indicated that the adjuvant activity of PAAs was strongly dependent on polymer size, with the larger PAAs (Mw > 350 kDa) displaying and adjuvant activity superior to that of MF59.

In the aim of studying the consistency of the PAA raw material and the robustness of the adjuvant activity, different PAA batches from Polysciences, and batches from an alternative supplier, Polymer Source, were compared in mice. These studies provided also an opportunity to better understand PAA structure-activity relationships. One such study is depicted in Fig. 2. In this study, three PAA lots from Polysciences (lots#566253, 652979 and 655555) and one from Polymer Source (lot#240413) were compared for adjuvant activity (Table 1). Concerning the PAAs obtained from Polysciences, lots# 566253, 652979 and 655555 had respective Mw of 567 kDa, 522 kDa and 120 kDa after diafiltration into PBS (Table 1). Among the high Mw PAAs from Polysciences, lot#652979 was essentially linear with a Mark-Houwing coefficient of 0.9 and lot#566253 was branched with a Mark-Houwing coefficient of 0.55 (Table 1). These results suggested that robust PAA synthesis was not trivial and that radical polymerization needed to be well-controlled to guaranty lot-to-lot consistency. To test the adjuvant activity of the different PAAs, increasing amounts of the diafiltered PAAs, from 25 to 400 µg, were added to 2 µg of CMV-gB and used to immunize C57BL/6J mice. The levels of CMV-neutralizing and CMV-gB binding antibody titers from this study are shown as a function of PAA batch and dose on Fig. 2**, Panels A-C**, and the mean IFN-γ levels are shown on **Fig. 2, Panel D**. No IL-5 was detected in the PAA groups in this study (data not shown). The results indicated that whatever the nature of the PAA, the adjuvant effects measured on antibody and IFN-γ production increased with the PAA dose up to the 100 µg dose. Statistical analysis, taking into account the antibody and cytokine titers induced at all concentrations, indicated an absence of significant differences in terms of antibody and IFN-γ induction between PAA lot#652979 (Polysciences; Mw = 522 kDa; linear) and PAA

lot#566253 (Polysciences; Mw = 567 kDa; branched) (p = .63 for neutralizing titers, p = .54 for IgG1 titers, p = .050 for IgG2c titers and p = .55 for IFN-γ titers, t-tests). In contrast, PAA lot#240413 (Polymer Source; Mw = 430 kDa; linear) and lot#655555 (Polysciences; Mw = 120 kDa; linear) induced significantly lower antibody and IFN-γ titers than lot#566253 (all p-values < .001 for neutralizing antibodies, IgG1, IgG2c and IFN-γ). PAA lot#566253 was found to display superior adjuvant effects compared to MF59 on neutralizing, IgG2c and IFN-γ titers (all p-values < .001) with plateau effects observed between 100 and 200 μg per dose. Note also that all PAAs tested in this study tended to induce a Th-1 type immune response based on antibody isotypes and cytokine profiles. Taken together, these results confirmed that the adjuvant effect of PAA was mainly influenced by polymer size and dose, with maximum effects obtained with Mw above 430 kDa and doses of 100 μg and above in mice. Polymer ramification, as defined by the Mark-Houwing coefficient, and homogeneity as defined by the polydispersity index, had less impact on the PAA adjuvant activity. This was observed as well in another such study, comparing PAA lots#658002 (Mw = 452 kDa; linear), 659589 (Mw = 583; linear), 656483 (Mw = 268 kDa; linear) and 652979 (Mw = 522 kDa; linear) described in Table 1, and showing no significant difference between lots#658002, 569589 and 652979, whereas lot#656483 displayed a reduced adjuvant effect (data not shown). Based on antibody isotypes and cytokine profiles, strong Th-1 responses were obtained in these studies with all PAAs of Mw 405kD (lot#658002) and above. From a general safety standpoint, even at the highest injected doses (400 μg of PAA) there were no macroscopic injection site reactions and the immunization regimen with PAA-adjuvanted CMV-gB had no effect on mouse normal behavior and body weight gain. Subsequently, the Mw specifications for an ideal PAA adjuvant were set to be comprised between 350 and 650 kDa, as determined by HPSEC after diafiltration. Although polymer polydispersity index (PI) and branching (Mark-Houwink coefficient) did not seem to have a significant impact on the adjuvant activity of PAA, these characteristics were controlled as well, essentially for the sake of product batch-to-batch consistency. Thus, an upper limit of 2.5 was set for polydispersity index and a lower limit of 0.7 was set for Mark-Houwink coefficient, to favor non-branched polymers with a controlled size distribution. In addition, residual small molecular weight impurities were also controlled to make sure these were all eliminated during the diafiltration step. A high Mw linear PAA that would fulfill these different criteria was termed SPA09.

3.3. Stimulation of human immune cells by high Mw linear PAA (SPA09) in vitro

To further support entry into clinical development, the high Mw linear PAA (SPA09) was studied in the MIMIC® peripheral tissue equivalent module (Schanen and Drake 3rd, 2008). In this study none of the tested adjuvants induced a reduction in cell viability at the tested dose range (Fig. 3). As also shown on Fig. 3, the stimulation of the MIMIC PTE module with SPA09 generated a dose-dependent increase in the expression of the costimulatory markers, CD25 and CD83, at a level significantly higher (p < .05) than induced by the MF59-like adjuvant, AddaVax™. In contrast, SPA09 only minimally induced the upregulation of CD86 on the PTE-derived antigen presenting cells (APCs). While there were differences in the activation profiles induced by AddaVax™ and SPA09, both induced the activation of the APCs, which can be considered an important step for the induction of downstream adaptive responses (Hoebe et al., 2004).

In addition to evaluating alterations in APC activation marker profiles, the analysis of chemokine and cytokine expression patterns was used as a second measure of adjuvant activity in the MIMIC PTE module. As depicted in Fig. 4, stimulation with SPA09 induced the secretion of MCP-1, IL-8, and IL-6, although not to the levels achieved with AddaVax™. This difference in the level of cytokine/chemokine secretion can be explained by the difference in composition and pathways activated by the MF59-like emulsion compared to a synthetic polyanionic polymer as reported for the adjuvanted influenza vaccines (Tregoning et al., 2018). These chemokines and cytokines play key roles in multiple immune pathways, including cell recruitment and migration and stimulation of phagocytosis, and have been reported as important innate immune factors induced by MF59 and other adjuvants (Tregoning et al., 2018). Overall, these data suggest that SPA09 has the potential to induce human APC activation and cytokine/chemokine secretion in a manner consistent with other vaccine adjuvants (Seubert et al., 2008).

4. Discussion

The present study revealed that the adjuvant activity of straight PAAs was essentially controlled by polymer size (Mw) and concentration, and not so much by branching and polydispersity. The strong correlation between adjuvant activity and polymer Mw had already been reported for several polyelectrolyte adjuvants, including polyacrylates and polymethacrylates (Kreuter and Haenzel, 1978). However, since radical polymerization processes used for PAA synthesis are hard to keep under control, reproducibility and batch-to-batch consistency can be challenging. Hence, a reliable triple detection HPSEC method was used control PAA size (Mw), structure (branching and polydispersity) and concentration (Table 1). The polymer sizes determined by this method were generally different from the polymer sizes obtained from the supplier. This could be explained by the use of a different method for Mw determination, as already observed and discussed by others (Yohannes et al., 2005).

Triple detection HPSEC could also be used for the determination of polymer ramification based on the Mark-Houwing equation linking the degree of ramification of a given polymer to its viscosity at a given concentration in solution (Masuelli, 2014). The preference for linear PAAs was motivated by the fact that linear PAAs may be more susceptible to elimination from the body by excretion (with urine or bile for example) than branched, circular or crosslinked PAAs (Chen et al., 2009; Fox et al., 2009; Nasongkla et al., 2009), which is an important consideration given the minimally-biodegradable nature of PAAs (Zondlo Fiume, 2002). HPSEC was also used for accurate determination of polymer concentration. Indeed, while total organic carbon determination was useful for the dosage of PAA stock solutions in water and PBS, the HPSEC method was needed for PAA quantification in the presence of CMV-gB antigen (or in the presence of other sources of carbon). Moreover, HPSEC could be applied to study adjuvant-antigen association, a potentially important aspect for polymer adjuvant activity (Andrianov et al., 2005; Flanary et al., 2009; Mustafaev et al., 1996), but for CMV-gB no association was detected, suggesting that high molecular weight PAAs can exert adjuvant effects even in the absence of interaction with the antigen (Cotte et al., 2017). In the case of CMV-gB with a theoretical isoelectric point at 6.4 (based on amino-acid composition), a high degree of glycosylation and a negative charge at neutral pH, no strong electrostatic interaction with the polyanionic PAA polymer was expected. This was confirmed not only by HPSEC but by a panel of other techniques including DLS, gel and capillary electrophoresis, and thermal analyses (DSC and ITC) used to study antigen-adjuvant interactions (P. Dinadayala et al., Sanofi Pasteur, Manuscript in preparation).

Despite the lack of interaction with CMV-gB, SPA09 induced stronger neutralizing antibody and IFN-γ titers than MF59 in mice immunized with CMV-gB plus SPA09. SPA09 was also able to increase the level of CMV-specific memory B cells over that achieved with a squalene emulsion, in accordance with the induction of a more durable virus neutralizing immune response that lasted for at least 180 days (WO2019/052975). Since one of the weaknesses of the CMV-gB/MF9 vaccine candidate in the field was the short duration of the induced immune response (Pass, 2009; Pass et al., 2009), SPA09 could represent an interesting adjuvant alternative to MF59 for this vaccine candidate.

Overall, the adjuvant profile of SPA09 was Th-1 biased and this was observed with several different antigens in different models including CMV-gB in mice, a *Staphylococcus aureus* polysaccharide conjugate in mice, (WO2017218819A1) and a recombinant RSV F antigen in mice and monkeys (V. Pavot et al., Sanofi Pasteur, Manuscript in preparation). Similar observations were recently reported for Carbopol-based adjuvants as well (Gartlan et al., 2016).

The adjuvant activity of SPA09 was then further characterized on mouse and human cells *in vitro*. In preliminary *in vitro* studies using either isolated mouse splenocytes or human PBMCs, it had been very difficult to evidence any sign of immunostimulation in terms of cell activation/proliferation or chemokine/cytokine secretion upon direct incubation of these cells with up to 1 mg/ml of SPA09 (not shown). Similarly, Wegmann and colleagues did not observe any cell activation when isolated mouse bone marrow dendritic cells were directly incubated with the Carbopol-lecithin adjuvant, Adjuplex™ (Wegmann et al., 2015). However, in the Peripheral Tissue Equivalent (PTE) module of the MIMIC® system, which includes PBMCs applied to transwell membranes containing a cultured monolayer of EA cells that induce the differentiation of the PBMCs to a dendritic cell (DC) phenotype expressing HLA-DR (Schanen and Drake 3rd, 2008), an upregulation of co-stimulatory molecules and the secretion of pro-inflammatory chemokines such as, IL-8 and MCP-1, could be observed. Although the innate immunity profile of SPA09 in the MIMIC® PTE module has some differences when compared to AddaVax™, a commercial MF59-like benchmark adjuvant, this profile was in accordance with *in vivo* observations in animal models. Taken together, these data confirm the value of SPA09 as a potential human vaccine adjuvant candidate based on a low inflammatory profile with innate immune activation, which could lead to T cell responses and antibody secretion as reported for other adjuvants with similar composition (Zhang et al., 2018).

5. Conclusion

This work provides a new PAA adjuvant, SPA09, that is composed of straight (*i.e.* non-crosslinked) high molecular weight, linear sodium polyacrylate. This adjuvant is advantageous in terms of simplicity, cost of goods and easiness of quality control by using NMR and HPSEC techniques. It has the potential for broad application in human vaccines requiring the induction of high antibody titers and Th-1 biased immune responses, provided safety is confirmed in pivotal non-clinical safety studies and in first-in-human trials. SPA09 can exert strong adjuvant effects even in the absence of antigen binding, as observed with CMV-gB, through mechanisms that remain to be fully elucidated. Such mechanistic studies, looking at immune responses induced by SPA09 in the MIMIC® system, in selected knockout (KO) *versus* wild type mice, and in different animal species with conventional and multi-OMIC readouts are underway.

Credit authorship contribution statement

Marie Garinot: Conceptualization, experimental design and data analysis, Analytical characterization and Formulation and stability. **Fabienne Piras-Douce:** Conceptualization, experimental design and data analysis, Writing original draft and Animal studies. **Patricia Probeck:** Conceptualization, experimental design and data analysis, Analytical characterization, and Formulation and stability. **Véronique Chambon:** Conceptualization, experimental design and data analysis, Writing original draft and Analytical characterization. **Kucku Varghese:** Conceptualization, experimental design and data analysis and MIMIC studies. **Yuanqing Liu:** Conceptualization, experimental design and data analysis and Animal studies. **Ernesto Luna:** Conceptualization, experimental design and data analysis, Writing original draft and Animal studies. **Donald Drake:** Conceptualization, experimental design and data analysis, Writing original draft and Animal studies. **Jean Haensler:** Conceptualization, experimental design and data analysis, Writing original draft, Analytical characterization and Formulation and stability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are thankful to Dr. Thomas Shenk and Princeton University for providing the GFP-labeled CMV virus.

The authors wish to thank Stéphanie Guinamand, Laurent Revet, Séverine Nury, Olivier Engel, Bénédicte Bourdeau, Franck Vallery, Isabelle Zamora, Isabelle Jaudinaud, Sonia Cognet, Antonin Asmus, Sandrine Painchaud, Julie Barrier, John Escobar and Maria E Boone for expert technical assistance, Christophe Charnay for animal care and Sylviane Gautheron for expert statistical analyses. This work was funded by Sanofi Pasteur.

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