Research paper

Targeting the deep lungs, Poloxamer 407 and a CpG oligonucleotide optimize immune responses to *Mycobacterium tuberculosis* antigen 85A following pulmonary delivery

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**Abstract**

The current Bacille Calmette–Guérin vaccine provides variable protection against tuberculosis and new vaccination approaches are urgently needed. Pulmonary vaccination could be the best way to induce a protective immunity against *Mycobacterium tuberculosis* as it targets its natural site of infection. The aim of this study was to investigate the potential of Poloxamer 407 (P407) combined with a CpG oligonucleotide (CpG) to enhance immune responses to *M. tuberculosis* antigen 85A (Ag85A) following pulmonary delivery in BALB/c mice. An additional goal of this study was to localize the optimal delivery site of Ag85A within the lungs for generating the most intense immunity. We also investigated the capacity of P407 to prolong the residence time of the antigen within the lungs and we studied the safety of the adjuvants following pulmonary delivery. Targeting the antigen to the deep lungs produced more intense specific immune responses than targeting it to the upper airways. P407 and CpG further increased humoral immune responses and splenocyte proliferation in vitro. CpG strongly increased the Th-1 immune response with high IgG2a/IgG1 ratio, high IFN-γ and TNF-α productions by spleen mononuclear cells in vitro. P407 tended to induce a Th-2 response, as indicated by the slight decrease in IgG2a/IgG1 ratio and the slight increase in IL-5 levels. The combination of P407 and CpG produced the highest Th-1 and Th-17 responses by generating IFN-γ, TNF-α, IL-2, and IL-17A cytokines. Targeting the antigen to the deep lungs and the presence of P407 increased the residence time of the antigen within the lungs. This might explain the enhancement of immune responses induced by these factors. CpG did not induce inflammation in the lungs while P407 produced a reversible alteration of the alveolo-capillary barrier. Adding CpG to P407 did not further increase this alteration of the alveolo-capillary barrier. In conclusion, delivery of Ag85A formulated in a combination of P407 and CpG to the deep lungs induced strong immune responses, with a polyfunctional T cells phenotype.

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**1. Introduction**

Tuberculosis (TB) is a global health problem, with one-third of the world’s population currently infected with the TB bacillus *Mycobacterium tuberculosis*. In 2010, 1.4 million people died of tuberculosis (including 450,000 patients infected with human immunodeficiency virus, HIV) and there were an estimated 8.8 million new TB cases [1]. Human immunodeficiency virus infection dramatically increases the risk of TB and TB disease is the leading killer in HIV-infected patients. Although the Bacille Calmette–Guérin vaccine (BCG) is widely administered, its protective efficacy against TB is highly variable. BCG protects against systemic tuberculosis in children but fails to prevent pulmonary tuberculosis in adults. In addition, BCG is a full contraindication in HIV-infected infants due to the risk of BCG dissemination [2]. Therefore, an adequate and truly effective vaccine is lacking and new vaccination approaches are being developed, some of which are currently being tested in phase 1 and phase 2 clinical trials [3,4].

Because *M. tuberculosis* uses the respiratory tract as portal of entry into the body, pulmonary vaccination could be the best way to induce a specific immunity in the lungs and to protect against the disease [5]. Pulmonary vaccination has the advantage to be needle-free and to generate a mucosal but also a systemic immunity [6].
However, there remains a crucial need to optimize the efficacy of pulmonary vaccination. Live attenuated vaccines present the potential disadvantage of being reactogenic. For instance, BCG produces an ulcerative lesion at the cutaneous vaccination site that drains for several weeks [7]. Therefore, an alternative would be to develop vaccines made of highly purified antigens formulated in appropriate adjuvants. So far, administration of adjuvanted vaccines to the lungs has been little studied and particularly requires demonstration of safety due to the vital character of the organ [8].

P407 is a non-ionic hydrophilic triblock copolymer of ethylene oxide (EO) and propylene oxide (PO) conforming to the formula polyEO–polyPO–polyEO, in which the polyPO portion has an average molecular weight of 4000 Da (first two digits in 407 multiplied by 100) and the polyEO portion has a percentage by weight of 70% (third digit in 407 multiplied by 10). This gives a molecular weight of about 12,600 Da for P407 [9]. P407 is a pharmaceutical excipient approved by the Food and Drug Administration and used in a variety of oral, ophthalmic, topical and injectable formulations for its emulsifying, solubilizing, and stabilizing properties. Above the critical micellar concentration and temperature, copolymer molecules self-aggregate into micelles. Additionally, P407 presents a reverse ical micellar concentration and temperature, copolymer molecules self-aggregate into micelles. Additionally, P407 presents a reverse thermogelation in which aqueous solutions are liquid at or below ambient temperature and form gels at body temperature. Therefore, P407 can be administered in liquid form and acts as sustained release depot at body temperature [10].

The goal of this study was to investigate the potential of P407 combined with synthetic CpG C274 oligonucleotide to enhance immune responses to antigen 85A (Rv3804c) of M. tuberculosis following pulmonary delivery as well as to orientate immunity toward a polyfunctional T cells phenotype [17,18]. While the CpG oligonucleotide was used as Th1-immunostimulant, P407 was used as a vaccine vehicle that could prolong the duration of contact of the antigen and immunostimulant with the respiratory mucosa through its gelation properties. The mycolyl-transferase Ag85A is a member of the well characterized antigen 85 complex of M. tuberculosis. Ag85A is a promising vaccine candidate that induces strong protective responses in experimental animal models [11] and which is actually used (as Modified Vaccinia Ankara 85A) in phase 2b clinical trials as a boosting vaccine of BCG vaccinees and PPD-positive persons [12]. An additional goal of this study was to locate the optimal delivery site of Ag85A within the lungs for generating the most intense immunity. We also investigated the capacity of P407 to prolong the residence time of the antigen within the lungs and we studied the safety of the adjuvants following pulmonary delivery.

2. Materials and methods

2.1. Mice

Specific-pathogen-free female BALB/c mice, aged between 8 and 10 weeks, were used for the experiments (Janvier elevage; Le Genest-St-Isle, France). All experimental protocols in mice were approved by the Institutional Animal Care and Use Committee of the Université catholique de Louvain.

2.2. Materials

Hexa-histidine tagged Ag85A protein from M. tuberculosis was purified from recombinant Escherichia coli, as described before [13]. Cloning in expression vector pQE-80L (Qiagen) and purification were performed, as described before [11]. The endotoxin level, measured with the Limulus Amebocyte Lysate kinetic chromogenic assay (Lonza Verviers Sprl, Belgium), was less than 25 EU/ml (endotoxin units per milliliter) or 0.065 EU/μg of purified protein.

The I-Eα restricted, immunodominant peptide 11, spanning amino acids 101–120 sequence of the mature Ag85A sequence [14], was synthesized by Prommune Ltd., Oxford, UK. The peptide was initially dissolved in dimethyl sulfoxide. Stock solutions were subsequently prepared in RPMI 1640 culture medium at 1 mg/ml. Aliquots were stored frozen at −20 °C.

P407 solutions of 10% to 20% w/v were prepared by dissolving P407 in PBS buffer under stirring in ice. The solution was kept in the fridge overnight to improve the dissolution. The viscosity of the solutions was measured by using a Rheometer RM180 Rheomat (Lamy Rheology, Champagne Au Mont d’Or, France). For the 13% w/v solution, particle size distribution was determined using dynamic light scattering (NanoSizer ZS; Malvern Instruments, Malvern, UK). Several concentrations of P407 (Sigma, USA) were administered by intratracheal instillation (IT) in BALB/c mice namely, 10%, 13%, 15%, and 20% (w/v). The survival of mice was observed immediately after delivery. Deposition in the deep lungs was verified by adding the patent blue violet (Sigma, USA) to the solutions and by visually assessing the blue coloration of resected lungs.

2.3. Vaccination protocol

BALB/c mice were anesthetized with intraperitoneal injection of ketamine/xylazine at 75 mg/kg and 8.4 mg/kg, respectively. Next, mice received the vaccine by IT either in the upper airways (UA) or in the deep lung (DL). A 10 μl sterile solution was instilled in the trachea of the mouse lying horizontally on its back in order to reach upper respiratory airways. In order to reach the deep lungs, a 20 μl sterile solution was instilled in the trachea of the mouse positioned with a 45° angle of tilt followed by insufflation of a 500 μl air bolus. Mice were instilled three times at 3 weeks intervals (on days 1, 21, 42), with 5 μg of purified recombinant Ag85A alone or combined with 13% (w/v) P407 and/or 5 μg CpG C274 (sequence 5‘-TCG-TCG-AAC-GTT-CCA-GAT-GAT-3‘, Eurogentec S.A, Seraing, Belgium). Control mice were injected subcutaneously (SC) in the back three times, at 3 week intervals (on days 1, 21, 42), with 5 μg of purified recombinant Ag85A alone or emulsified in Gerbu adjuvant in a total volume of 100 μl. Gerbu adjuvant is a colloidal suspension composed of biodegradable cationic lipid nanoparticles (octa-/hexa-decane). The suspension is completed with a cell wall subunit of Lactobacillus bulgaricus (GMDP), which is a powerful immunomodulator inducing T cell response and lasting cellular immunity. Additional immunomodulators, cimetidine and saponin, are also included as general enhancers of the immune response (GERBU Biochemicals, Germany). Additional control mice were instilled in the deep lungs with saline (PBS), P407, and/or CpG without Ag85A.

2.4. Samples collection and antibody ELISA assays

Sera were collected from the retro-orbital plexus before the first vaccination (day 0) and 3 weeks after the third vaccination (day 63) and stored at −20 °C until assayed. On day 70, mice were killed with an overdose of pentobarbital, spleens were removed aseptically and broncho-alveolar lavages (BALs) were performed with 1 ml Hanks’ Balanced Salt solution (Sigma, USA) by positioning a cannula in the trachea toward the lungs, as described before [15]. Ag85A-specific IgG, IgG1, and IgG2a were measured by ELISA in sera. Ag85A-specific IgA and Ag85A-specific IgG were measured in BAL fluid. Specific anti-Ag85A antibodies were determined, on 2-fold serial dilutions of sera and BALs, using recombinant Ag85A for coating (8 μg/ml), appropriate detection antibodies (rat antimouse total IgG, IgG1, IgG2a, IgA labeled with peroxidase from IMEX, UCL, Brussels, Belgium) and orthophenylendiamine for revelation (Sigma, USA). Optical densities were read at 492 nm. Antibody titers were
defined as the dilution corresponding to an optical density of 0.2 at 492 nm. Individual IgG2a/IgG1 ratios were calculated by dividing individual IgG2a titers by individual IgG1 titers. Antigen-specific antibody levels were undetectable in the PBS group and in the control group vaccinated in the deep lung with the combination of the adjuvants alone (data not shown).

2.5. Proliferative responses and cytokines production

Splenocytes were adjusted to a concentration of $4 \times 10^6$ white blood cells/ml and cultured 180 μl per well in 96-well round-bottom microtiter plates (Escolab, Kruibeke, Belgium) in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 0.05 mM 2-mercaptoethanol, 1% (v/v) sodium pyruvate, antibiotics, and 1% (v/v) non-essential amino acid (MEM) (Gibco, Merelbeke, Belgium). Cells were incubated at 37 °C in a humidified CO2 incubator and stimulated with purified recombinant Ag85A (5 μg/ml), synthetic Ag85A101–120 peptide (10 μg/ml) or Concanavalin A (5 μg/ml). Negative control cultures were left unstimulated. All cultures were incubated for 48 h and then pulsed overnight with 0.05 M Ci [3H] thymidine per well. Cells were harvested onto microplate unifilters and the activity was counted using a TopCount scintillation counter (PerkinElmer, Zaventem, Belgium). Results are expressed in counts/min (c.p.m). Supernatants were collected either after 24 h for interleukin-2 (IL-2) or after 72 h for interleukin-5 (IL-5), interleukin-17A (IL-17A), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) assay. Cytokine concentrations were measured in supernatants using mouse IL-2, IL-5, TNF-α, and IFN-γ Duoset ELISA development kit (R&D Systems Europe Ltd., Abingdon, UK), according to the manufacturer’s protocols. IL-17A levels were quantified by sandwich ELISA using coating antibody MM173C9, biotinylated antibody MM17F3 (Experimental Medicine Unit, UCL, Belgium) and the standard murine recombinant IL-17A (R&D Systems Europe Ltd., Abingdon, UK). The detection limits were 4, 7.8, 15, 10.4, and 21 pg/ml for IL-17A, IL-2, IL-5, TNF-α, and IFN-γ, respectively.

2.6. Residence time

BALB/c mice were anesthetized by intraperitoneal injection of ketamine–xylazine before IT of 5 μg of ovalbumin (Grade V, Sigma, USA) with or without 13% (w/v) P407 in the upper airways or in the deep lungs. The endotoxin level, measured with the Limulus Amebocyte Lysate kinetic chromogenic assay (Lonza Verviers Sprl, Belgium), was 1.78 UE/μg of protein. We verified that no pulmonary inflammation resulted from the administration of 5 μg of OVA to the deep lungs (data not shown). Mice were instilled once. BAL fluid was collected at 0, 24, and 48 h after instillation. The lungs were lavaged four times with 1 ml Hanks’ Balanced Salt solution. The supernatants of the lavages were stored frozen at −20 °C until assay for ovalbumin content. Ovalbumin concentration was measured by ELISA using an affinity purified rabbit anti-ovalbumin antibody for coating and a peroxidase conjugated rabbit anti-ovalbumin for detection (MyBioSource, San Diego, USA). The detection limit was 0.6 ng/ml. Ovalbumin, instead of Ag85A, was used because sensitivity of Ag85A-specific ELISA was insufficient using available custom-made monoclonal antibodies against Ag85A.

2.7. Toxicity study protocol

BALB/c mice were anesthetized by intraperitoneal injection of ketamine–xylazine before receiving adjuvants alone by instillation in the deep lungs, as described above. Mice were instilled once with 13% (w/v) P407 and/or 5 μg CpG C274. Negative and positive control mice were instilled with PBS and 5 μg of LPS (E. coli O111:B4, Sigma, USA), respectively. BAL fluid was collected 4 h, 24 h, 72 h, 7 days, and 14 days after intratracheal administration. The lungs were lavaged twice with 1 ml Hanks’ Balanced Salt solution, as described above. The lavages were then centrifuged (281g at 4 °C for 10 min). The supernatants of the first lavages were stored frozen at −20 °C for TNF-α and serum albumin assays.

2.8. BAL biochemistry parameters and cellular components

BAL total protein content and lactate dehydrogenase (LDH) activity were spectrophotometrically assayed in the supernatants of the first lavages using the Synchron LX Systems (Beckman Coulter Inc.; Brea, USA). Total protein content was assayed by monitoring the formation of a purple color complex of pyrogallol red and molybdate at 600 nm using human serum albumin as standard while LDH activity was assayed by monitoring the reduction of NAD+ to NADH in the presence of lactate at 340 nm.

Serum albumin and TNF-α levels were measured by ELISA according to the manufacturer’s protocols. Mouse albumin assay was performed using a commercial kit (Imtec Diagnostics N.V., Antwerpen, Belgium) with a detection limit of 3 ng/ml and mouse serum albumin as standard. TNF-α assay was performed using a BD Biosciences kit (BD Biosciences, San Jose, USA) with a detection limit of 33 pg/ml.

The cells of both BAL aliquots were pooled and the total number of live cells in BAL fluid was determined by Türk’s solution method (VWR International, Leuven, Belgium). Differential cell counts were obtained by cytocentrifugation and coloration with Diff quick (Dade NV/SA).

2.9. Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). One-way ANOVA and Tukey’s post-test were performed on normalized data to demonstrate statistical differences using the software GraphPad Prism 5 for Windows. Groups of 6 to 8 mice were used for the immunogenicity, toxicity, and residence time studies. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. The choice of P407 concentration

In a first step, we selected the maximal concentration of P407 that could be readily delivered deeply in the lungs of mice. From a concentration of 20% w/v and higher, the mice did not survive the intratracheal instillation of P407 because the extremely viscous solution obstructed respiratory airways and prevented mice to properly breathe (Table 1). For a concentration of 15% w/v and higher, the viscosity of the solution prevented it to go deep into the lungs. A P407 concentration of 13% w/v allowed delivery of the solution to the lungs periphery (Table 1). Therefore, a P407 concentration of 13% was chosen for further experiments. The average viscosity of the 13% solution was 17 mPa s at 37 °C, which means a 17-times increased viscosity as compared to pure water (Table 1). The average size of P407 micelles at this concentration was 27 nm.

3.2. The choice of the delivery site of Ag85A within the lungs

Both the delivery site of Ag85A within the lungs and P407 had an impact on serum anti-Ag85A IgG titers (Fig. 1). In order to reach the deep lungs, a 20 μl solution of Ag85A was instilled in the trachea of the mouse tilted at an angle of 45° and an air bolus
was immediately insufflated in the trachea following administration. In order to deposit the Ag85A solution in upper airways, a 10-μl solution was instilled in the trachea of the mouse lying horizontally on its back and no air bolus was insufflated. The targeting of the different lung regions obtained using these techniques was previously demonstrated using ovalbumin as a tracer compound [15]. The administration of Ag85A to the deep lungs generated 2log10 higher anti-Ag85A IgG titers in sera than its delivery to upper airways. Both in the deep lungs and upper airways, P407 strongly enhanced antigen-specific serum IgG titers (Fig. 1A). The administration of Ag85A to the deep lungs tended to induce increased splenocyte proliferation and IFN-γ production as compared to its administration to upper airways (Fig. 1B and C). These results confirm the importance of the antigen delivery site within the lungs for an optimal immune response [15]. They also highlight the potential of P407 as a carrier for pulmonary vaccination. Because targeting the antigen to the deep lungs enhanced immune responses, this lung region was chosen as antigen delivery site for the subsequent experiments.

3.3. Humoral immune responses to Ag85A following its pulmonary delivery in combination with P407 and CpG

P407 and CpG C274 both strongly increased humoral immune responses to Ag85A following delivery to the deep lungs (Fig. 2A). Serum anti-Ag85A IgG titers increased by 2log10

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### Table 1

Dynamic viscosity of P407 at increasing concentrations and two temperatures.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Mouse survival</th>
<th>Deep lungs deposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 °C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>15 °C</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>20 °C</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

*At these concentration and temperature, P407 did not follow a Newtonian behavior and viscosity is given at 6.5 cm shear rate.*

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**Fig. 1.** Impact of Ag85A delivery site on humoral and cellular immune responses. (A) Serum anti-Ag85A IgG titers. (B) Proliferation of spleen mononuclear cells following in vitro Ag85A or peptide 11 stimulation or without stimulation. (C) The supernatants from the culture of spleen mononuclear cells were assayed for IFN-γ. Limit of detection = 21 pg/ml.

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**Fig. 2.** Impact of P407 and CpG on antigen-specific humoral immune responses. (A) Serum anti-Ag85A IgG titers. (B) BAL anti-Ag85A IgG titers. (C) Serum anti-Ag85A IgG subclass titers. # indicates significant difference with Ag85A-Gerbu SC group. + indicates significant difference with the Ag85A delivered alone in the deep lung. Two symbols indicate p < 0.01, and three symbols indicate p < 0.001.
when P407 or CpG was added to the antigen. The pulmonary administration of Ag85A together with either P407 or CpG induced similar serum anti-Ag85A IgG titers as the subcutaneous administration of a same dose of Ag85A without adjuvant. Yet, when combining P407 and CpG with the antigen, serum anti-Ag85A IgG levels were comparable to those obtained following subcutaneous administration of Ag85A formulated in Gerbu adjuvant (Fig. 2A). The BAL anti-Ag85A IgG levels paralleled serum IgG levels, in support of IgG passive diffusion from serum into the lungs (Fig. 2B). Antigen-specific IgA levels in broncho-alveolar lavages could not be detected in any of the groups, probably due to their high dilution in BAL samples.

Serum IgG2a and IgG1 subclasses were analyzed as an indirect assessment of the polarization of T-helper cell populations. As compared to subcutaneous administration, pulmonary vaccination of Ag85A alone tended to generate a higher IgG2a to IgG1 ratio (Fig. 2C), indicating a slight shift toward a T-helper type-1 phenotype. Adding the CpG oligonucleotide to the antigen increased IgG2a to IgG1 ratio following pulmonary vaccination as compared to the pulmonary vaccination with Ag85A alone, with a ratio reaching over 1. However, P407 presented the reverse tendency to CpG and decreased the IgG2a to IgG1 ratio as compared to the pulmonary vaccination with Ag85A alone. The formulation of Ag85A in a combination of P407 and CpG induced a IgG2a to IgG1 ratio...
intermediate between the Ag85A–CpG and Ag85A–P407 groups (Fig. 2C).

3.4. Cellular immune responses to Ag85A following its pulmonary delivery in combination with P407 and CpG

Both P407 and CpG enhanced cellular immune responses to Ag85A following pulmonary administration (Fig. 3). Positive splenocyte proliferation was seen in the groups vaccinated in the deep lungs against M. tuberculosis with P407, CpG, or the combination of both molecules (Fig. 3A). Positive splenocyte proliferation was also seen in the groups vaccinated subcutaneously without or with Gerbu. Yet, the antigen delivered alone to the deep lungs did not induce proliferative responses. The combination of P407 and CpG delivered to the lungs induced the highest splenocyte proliferation and a proliferation even higher than that generated by the subcutaneous injection of Ag85A with Gerbu (Fig. 3A). When stimulated with Ag85A101–120 peptide, splenocytes proliferated in the same proportions as following stimulation with the whole antigen.

Similarly to the IgG2a to IgG1 ratios, the in vitro splenocyte cytokine production indicated that formulation in CpG oligonucleotide induced a shift of the Ag85A-specific immune response toward a Th-1 phenotype. The IFN-γ production by Ag85A-stimulated splenocytes in vitro was 7-fold and 4-fold increased in the groups of mice vaccinated with Ag85A in CpG and in the combination of P407 and CpG, respectively, in comparison with the group vaccinated with Ag85A alone (Fig. 3B). CpG as well as the combination of P407 and CpG significantly increased TNF-α production as compared to the group vaccinated with Ag85A alone (Fig. 3C).

P407 alone did not enhance splenocyte production of IFN-γ, TNF-α, and IL-2 levels. These cytokines were only increased by the combined use of P407 and CpG (Fig. 3D). Significant IL-5 levels were only detected in the group vaccinated subcutaneously with
Ag85A alone or vaccinated in the deep lungs with Ag85A formulated in P407 (Fig. 3E). IL-17A levels were increased both by the combined use of P407 and CpG and by the Gerbu adjuvant (Fig. 3F). BAL samples were concentrated to assay local IFN-γ. Nevertheless, IFN-γ levels were undetectable in the BALs of all the groups.

3.5. Residence time

In order to assess whether antigen delivery to the deep lungs and P407 led to increased residence time of the antigen within the lungs, we measured ovalbumin content in broncho-alveolar fluid in terms of time after instillation of ovalbumin in the upper airways or deep lungs, without and with P407 (Fig. 4A). Twenty-four hours post-instillation, the lungs retained 20% and 11% of the time zero-recovered ovalbumin dose following delivery to the deep lungs and upper airways, respectively. When P407 was added, 24 h lung retention increased to, respectively, 36% and 27% (Fig. 4B). Forty-eight hours post-instillation, 20% of the ovalbumin dose was still present in the lungs following deep lung delivery in P407 while only 3% to 8% of ovalbumin remained for the other groups (Fig. 4B).

3.6. BAL biochemical parameters

We analyzed several biochemical markers in broncho-alveolar lavages in order to check whether P407 and CpG induced inflammation in the lungs. Total proteins and albumin were measured to detect permeability alteration to the alveolo-capillary barrier. The extracellular release of lactate dehydrogenase (LDH), an intracellular enzyme, allows detecting injury to pulmonary cells. TNF-α was measured in BAL as an early inflammation marker. Total proteins were significantly increased in the P407 group from day 1 until day 14 after pulmonary administration but tended to return to baseline values at day 14. The combination of P407 and CpG significantly increased total proteins at day 3 and day 14 as compared with the P407 group (Fig. 5A). Albumin followed the same rise as total proteins following P407 delivery and tended to return to baseline values at day 14 as well. Albumin was similarly increased in the P407–CpG group but decreased faster than in the P407 alone group. Therefore, albumin was significantly lower in the combination group versus the P407 alone group at day 7 (Fig. 5B). Total proteins distributed on a lower scale than albumin and this might be explained by the use of a non-specific standard for the quantification of total proteins (Fig. 5B). Intratracheal instillation of P407 induced a slight and apparently reversible increase in LDH content. P407–CpG tended to induce a higher increase in LDH than P407 alone. However, there were no significant differences between both groups (Fig. 5C). P407 and P407–CpG did not induce modification to TNF-α levels (Fig. 5D). Pulmonary delivery of CpG as pulmonary delivery of saline, the negative control, did not modify the levels of any of these biochemical markers (Fig. 5A–D). Intratracheal instillation of LPS, the positive control, induced a sharp and reversible increase in total proteins, LDH and TNF-α but no alteration of albumin (Fig. 5A–D).

3.7. BAL cellular components

Counting of total cells and analysis of cell distribution in BAL fluid can give an indication of pulmonary inflammation as well. Total cells were not increased following pulmonary administration of P407 and/or CpG (Fig. 4A). However, the number of macrophages increased in the P407 and P407–CpG groups and was significantly different from the saline group at day 14 (Fig. 6A). The number of lymphocytes tended to increase in the group P407–CpG at day 7 (Fig. 6B). In contrast, intratracheal instillation of LPS (the positive control) induced a large increase in the number of total cells recovered, and this increase was due to an influx of neutrophils from day 1 to day 3, an influx of lymphocytes from day 3 to day 7 and an influx of macrophages at day 7 (Fig. 6A–D).
4. Discussion

The goal of this study was to assess the potential of a formulation composed of P407 and a CpG to optimize immune responses to Ag85A following pulmonary delivery. Our objective was to generate a TB-specific immunity that could protect the host against infection by airborne *M. tuberculosis* through non-invasive pulmonary vaccination. In a first step, we showed that targeting Ag85A to the deep lungs, as compared to the upper airways, induced higher antigen-specific immune responses (Fig. 1). In a second step, we demonstrated that formulation of Ag85A in P407 and CpG further increased humoral and cellular responses and generated a polyfunctional T cells phenotype (Figs. 2 and 3). In a third step, we showed that deep lung targeting and P407 enhanced the residence time of the antigen within the airway lumen, in support of our starting hypothesis (Fig. 4). Finally, we showed that intratracheal instillation of CpG did not cause inflammation in the lungs but that intratracheal instillation of P407 induced an alteration of the alveolo-capillary barrier (Figs. 5 and 6).

Pulmonary delivery of Ag85A combined with P407 and CpG C274 generated a strong immune response needed for protection against infection with *M. tuberculosis* [7]. IgG diffusing from the bloodstream can block initial infection at the mucosal surface by preventing the binding of the mycobacterium to host receptors (Fig. 2B). T lymphocytes migrate back and forth across the mucosal epithelium and production of interferon-γ, tumor necrosis factor-α, and interleukin 2 by CD4+ Th-1 cells can activate alveolar macrophages infected with aerosolized *M. tuberculosis* to produce intracellular microbialic activities (Fig. 3). CD4+ T cells expressing IL-17 may also contribute to the adaptive immune responses to mycobacteria by triggering expression of chemokines in the lung, which in turn may mediate recruitment of Th1 cells to the airways (Fig. 3F) [16]. The generation of multifunctional T cells has been shown to correlate with protection against tuberculosis (Figs. 3B–D and F) [17,18].

Poloxamer 407 alone or combined with CpG increased immune responses to Ag85A following pulmonary delivery. Our starting hypothesis was that P407 would enhance the residence time of the antigen and adjuvant within the lungs due to the increased viscosity of the solution made of P407. This enhancement was in fact verified by measuring ovalbumin content in BAL fluid in terms of time after delivery of an ovalbumin solution formulated or not in P407 (Fig. 4). P407 did not induce inflammation in the lungs (Figs. 5 and 6), but P407 modulated the immune responses and acted as an adjuvant probably by forming an antigen depot and extending the delivery of the antigen (Fig. 4). Although P407 altered the permeability of the alveolo-capillary barrier (Fig. 5), this did not translate in increased antigen transport from the alveolar space into the lung interstitium since the antigen amount recovered from the alveolar space was higher when P407 was present in the instillate (Fig. 4). No synergy between P407 and CpG was observed as to the toxicity to the alveolo-capillary barrier since adding CpG to P407 did not further increase albumin content in BAL (Fig. 5B). Sustaining the release of the antigen and adjuvant within the lungs in order to increase immune responses is a strategy that has not been tested yet by the pulmonary route, in contrast to particulate carriers well explored to target vaccines to antigen presenting cells in the lungs [19,20]. Pulmonary administration of poly(propylene sulfide) nanoparticles, stabilized with P407 as emulsifier surfactant, targeted lymphoid tissue and led to enhanced Ag85B–specific mucosal and systemic Th-1 and Th-17 immune responses [19]. P407 has also been used as adjuvant for vaccines delivered by the intranasal route or by injection and has already been shown to increase humoral immune responses. After intranasal delivery, P407 combined with chitosan increased IgA antibodies to *Bordetella bronchiseptica* antigens [21] and IgG and IgA antibodies specific to tetanus toxoid [22]. P407 has previously been shown to be synergistic with CpG following injection. Their combination formulated with tetanus toxoid, diphtheria toxoid, and anthrax recombinant protective antigen resulted in enhancement of IgG antibody responses as compared to the antigens mixed with CpG alone [23]. Yet, the mechanism behind the adjuvant action and the sustained antigen release P407 provides has not been verified in these studies. Cellular immune responses have also been little analyzed [24,25].

Targeting Ag85A to the deep lungs induced more intense antigen-specific immunity than targeting it to upper airways (Fig. 1). This confirms our previous results on a split influenza virus vaccine, which generated stronger specific immunity following delivery to the lung periphery than following delivery to the nasal cavity or to the upper or central airways [15]. At that time, we hypothesized that the increased efficacy of deep lung vaccination might originate from the ensuing longer residence time of the antigen within the lungs. Here, we confirm this hypothesis by directly measuring ovalbumin content in BAL fluid in terms of time after delivery to the deep lungs or upper airways (Fig. 4). Ronan et al. have compared intranasal instillations targeting a recombinant adenovirus expressing Ag85A to the nasal cavities or to the whole respiratory tract in mice. They showed that only deep lung immunization induced strong immune responses in the lungs and that these correlated with protection against an aerosol *M. tuberculosis* challenge [26].

The analysis of IgG subclasses and cytokines secreted by splenocytes in culture indicated that the CpG induced a Th-1 cellular response (Figs. 2 and 3). This effect can be related to the binding of CpG to Toll-like receptor-9 in the intracellular compartment of immune cells [27]. The particular CpG used in this study (CpG C274) belongs to class C CpG oligonucleotides, which provide both the effect of class A (activation of plasmacytoid dendritic cells) and class B (activation of B cells). In addition, CpG C274 is a particularly potent inducer of IFN-α, IFN-γ, TNF-α, and IL-6, as compared to other class C oligonucleotides [28]. Formulation of Ag85A in P407 induced a Th-2 polarization of the immune response, but the mechanism behind this orientation remains to be determined (Figs. 2 and 3). Newman et al. have shown that poloxamers with 10% polyOE increased Th-2 responses to ovalbumin, whereas more lipophilic copolymers augmented both Th-1 and Th-2 responses [29]. Lipophilic copolymers rapidly adhere to cell membranes, incorporate into them, and increase membrane fluidization, thereby facilitating the non-specific transfer of protein antigens into the cytoplasm. On the contrary, hydrophilic copolymers as P407 adhere to cell membranes but do not incorporate into them [30]. P407 could increase the efficiency of antigen delivery to dendritic cells through endocytosis of micelles and this could augment the T cell response. Formulation in CpG adjuvant and P407 carrier produced the highest splenocyte proliferation (Fig. 3A) and induced a mixed Th-1 and Th-17–oriented response with high levels of IFN-γ, TNF-α, IL-2, and IL-17A (Fig. 3B–F).

The CpG and P407 did not induce any inflammation in the lungs but P407 increased the permeability of the alveolo-capillary barrier (Fig. 5). Adding CpG to P407 did not further increase the alteration of the alveolo-capillary barrier. By adhering to the surface of cell plasma membranes, P407 limits the lateral mobility of membrane lipids and thereby causes membrane solidification [30]. There might be a link between this adherence to cell surfaces and the increase in epithelial permeability. However, the alteration of the alveolo-capillary barrier by P407 is reversible over time and its reversible nature could come from the clearance of the polymer from the lungs. P407 has a molecular weight of 12,600 Da, and macromolecules of this size are known to be mostly cleared from the lungs within 1 day. Yet, P407 micelles might present a slower clearance than P407 unimers since nanoparticles can remain in the lungs for weeks [31]. After absorption in the systemic
circulation, P407 unimers are rapidly excreted in the urine [32]. The alteration of the alveolo-capillary barrier induced by P407 could possibly be reduced by decreasing P407 concentration in the solution delivered. Because sodium chloride and phosphates have been shown to increase the strength of P407 gels, these ions could be added to the P407 solution in order to keep its viscosity constant using lower polymer concentrations [33].

5. Conclusion

This study showed that targeting the deep lungs, P407 and CpG all increased immune responses to Ag85A. CpG led to a T-helper type-1 phenotype and the combination with the carrier P407 generated a more polyfunctional T cells phenotype. This work also showed that these effects could be explained, at least in part, by the enhanced residence time of the antigen within the alveoli. However, in order to consider further development of this formulation, the key experiment will involve the assessment of the protection it confers against A. tuberculosi s challenge as compared to BCG.

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