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PLGA-encapsulation of the *Pseudomonas aeruginosa* PopB vaccine antigen improves Th17 responses and confers protection against experimental acute pneumonia

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Abstract

The *Pseudomonas aeruginosa* type III secretion system protein PopB and its chaperon protein PcrH, when co-administered with the adjuvant curdlan, elicit Th17 responses after intranasal immunization of mice. These PopB/PcrH-curdlan vaccines protect mice against acute lethal pneumonia in an IL-17-dependent fashion involving CD4 helper T cells secreting IL-17 (Th17 cells). In this study, we tested whether encapsulation of PopB/PcrH in poly-lactic-co-glycolic acid (PLGA) nanoparticles could elicit Th17 responses to PopB. Recombinant PopB/PcrH or PcrH alone was encapsulated into PLGA nanoparticles. Mice (FVB/N) were intranasally immunized with the PLGA-PopB/PcrH nanoparticles, PLGA-PcrH nanoparticles, PLGA alone, or PopB/PcrH alone. The protective efficacy was assessed in an acute lung infection model with a lethal dose of an ExoU-producing version of *P. aeruginosa* strain PAO1. Th17 responses were assayed by intracellular flow cytometry and by ELISA for IL-17 in supernatants of splenocytes co-cultured with purified PopB/PcrH. PLGA-PopB/PcrH-immunized mice showed 3–4-fold higher Th17 responses both in the lung and in the spleen compared to mice immunized with empty PLGA or PopB/PcrH alone. After challenge with *P. aeruginosa*, PLGA-PopB/PcrH-immunized mice showed significantly lower bacterial counts in the lungs and improved survival. In conclusion, encapsulation of PopB/PcrH in PLGA nanoparticles can elicit Th17 responses to intranasal vaccination and protect mice against acute lethal *P. aeruginosa* pneumonia.

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Keywords

vaccine; *Pseudomonas aeruginosa*; PLGA; nanoparticle; Th17; IL-17

Introduction

Pseudomonas aeruginosa causes a wide variety of serious infections in humans, particularly in immunocompromised patients, burn victims, and those with cystic fibrosis [1–4]. *P. aeruginosa* is also the leading Gram-negative pathogen in hospital-acquired pneumonia [5]. Despite extensive efforts, there is currently no licensed vaccine for *P. aeruginosa* infections. The failures of multiple clinical trials of *P. aeruginosa* vaccines in humans, including the passive therapy trial in ICU patients more than 30 years ago [6] and the more recent failure of the OprF/I hybrid fusion protein vaccine, strongly suggest a missing link in prior vaccine strategies. Our early studies using live-attenuated *P. aeruginosa* vaccine strains found that helper T cells secreting the cytokine IL-17 (Th17 cells) can provide cross-serogroup protection against infection in an antibody-independent fashion [7].

The importance of IL-17 in controlling bacterial infections centers on neutrophil recruitment via induction of CXC chemokine secretion (KC and MIP-2 in rodents, GRO and IL-8 in humans) along with granulopoietic factors (such as G-CSF) that lead to increased bone marrow production and/or prolonged survival of neutrophils [8]. IL-17 can also activate macrophage-mediated bacterial killing [9]. Kolls and co-workers showed that vaccination of mice with *K. pneumoniae* outer membrane proteins protected mice from pneumonia in a Th17-dependent manner [10]. By enhancing phagocyte recruitment to the site of infection, which is an indirect mechanism of protection, Th17-based vaccines have low selective pressure for bacterial escape mutants [11].

Previously, using a reverse vaccinology approach, we found that the *P. aeruginosa* type III secretion system protein PopB is a Th17 antigen and, when mixed with the Th17 adjuvant curdlan, elicits protective Th17 responses in mice after intranasal vaccination [12]. IL-17 receptor-deficient mice were not protected by PopB vaccination, demonstrating this protection was dependent on IL-17 [12].

In an attempt to enhance the protective efficacy of the PopB vaccine, which is co-expressed and co-purified recombinantly with the chaperone protein PcrH, we encapsulated recombinant PopB/PcrH, into poly-lactic-co-glycolic acid (PLGA) nanoparticles. PLGA is biocompatible and biodegradable copolymer of poly lactic acid and poly glycolic acid. PLGA has been used in the clinic as a vehicle for controlled delivery of small molecules and/or proteins. Pulendran *et al.* found that encapsulation of toll-like receptor (TLR) agonists (specifically, the TLR4 and TLR7 agonists monophosphoryl lipid A and imiquimod, respectively) in PLGA nanoparticles led to marked improvement in the magnitude and duration of antibody responses in mice and non-human primates to yellow fever and influenza vaccines [13]. More recent studies suggest that encapsulation of proteins in PLGA can induce Th17 responses [14]. We hypothesized that vaccination of PopB/PcrH encapsulated with PLGA will elicit a Th17 response and be protective against *P. aeruginosa* pneumonia. Indeed, we found that PopB/PcrH encapsulated in PLGA stimulated a Th17

response in the absence of additional adjuvants. This response was able to protect mice against acute lethal *P. aeruginosa* pneumonia. The level of Th17 response and protection were comparable to those after immunization with PopB/PcrH mixed with curdlan. These data suggest that PLGA-encapsulated PopB/PcrH vaccines, without standard adjuvants, are a promising new approach for vaccination against *P. aeruginosa*.

Methods

Ethics statement

All animal protocols and procedures were approved by the Brigham and Women's Hospital Institutional Animal Care and Use Committee (assurance number A3431-01). The specific protocol number is 404. All animal protocols are complement with NIH Office of Laboratory Animal Welfare, Guide for the Care and Use of Laboratory Animals, The US Animal Welfare Act, and PHS Policy on Humane Care and Use of Laboratory Animals.

Construction of PLGA nanoparticles

Recombinant PopB with its chaperone PcrH was expressed and purified from *E. coli* as previously described [12]. Poly-lactic-co-glycolic acid (PLGA) nanoparticles were generated using a published water-in-oil-in-water emulsion technique [13, 15]. Briefly, protein solutions in polyvinyl alcohol (PVA) as excipient were homogenized with 10% w/v PLGA in dichloromethane. The water-in-oil emulsion was then added to a 5% w/v solution of PVA for the second emulsion step, and the water-in-oil-in-water double emulsion was allowed to evaporate at room temperature. Sizing of nanoparticles was done using dynamic light scattering. Protein encapsulation levels were measured using Bradford assay [16]. LPS was quantified using a Pierce LAL Chromogenic Endotoxin Quantitation Kit according to manufacturer's instructions.

Immunization of mice and murine pneumonia model

Wild type FVB/N mice (6–8 weeks, female) were obtained from the Jackson Laboratories and maintained at the animal facility of Brigham and Women's Hospital. Mice anesthetized with ketamine/xylazine given intraperitoneally were intranasally immunized with the PLGA-PopB/PcrH nanoparticles, PLGA alone, PopB/PcrH alone, or PopB/PcrH plus curdlan (Th17 adjuvant) suspended in phosphate-buffered saline (PBS). Each vaccine dose (20 μ L) contained an equivalent dose of PopB/PcrH (20 μ g/mouse). The PLGA dose was 1 mg per mouse and the curdlan dose was 200 μ g per mouse. Immunization was conducted once per week for 3 weeks. For the murine pneumonia model, mice anesthetized with ketamine/xylazine were intranasally challenged with 1×10^6 CFU of *P. aeruginosa* strain ExoU⁺ PAO1 three weeks after last immunization. A subset of immunized, infected mice were euthanized at 18 hours after infection by CO₂ overdose, lungs were excised and homogenized, and dilutions were plated to determine bacterial load. Other mice were monitored for 6 days, and moribund mice were euthanized.

Collection of white blood cells from lung tissue and intracellular flow cytometry analysis

White blood cells from lungs were isolated and cultured from collagenase/DNase-digested lungs following published protocols [17] and then stimulated *ex vivo* with PMA and

ionomycin for 6 hours. Cell samples were washed and adjusted to a concentration of 5×10^5 /mL with flow cytometry buffer (2% FCS in PBS). Samples were then treated with Fc block (anti-CD16/32, clone 2.4G2) in flow cytometry buffer for 20 min on ice. Samples were first stained for surface markers, followed by intracellular cytokine staining. For surface-marker staining, cells were incubated with fluorochrome-conjugated monoclonal antibodies against mouse CD45 (30-F11) and CD4 (GK1.5), all from BioLegend. For intracellular cytokine staining, cells were processed with Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) according to manufacturer's instruction. Samples were then incubated with fluorochrome-conjugated antibodies against IL-17A (TC11–18H10.1, BioLegend) and analyzed using a BD FACScalibur flow cytometer.

Splenocyte isolation and co-culture assays for IL-17 secretion

Splenocytes were isolated and stimulated with vaccine antigens as previously described. Briefly, spleens were aseptically removed and suspended in PBS containing 2% heat-inactivated FCS. Spleens were disaggregated by passing through 100-micron nylon screens into a petri dish. Erythrocytes were lysed using a Mouse Erythrocyte Lysing Kit (R&D Systems) per the manufacturer's protocol. Cells were centrifuged and resuspended in 5 mL cR10 (RPMI with 2 mM glutamine, 1 mM sodium pyruvate, 1X non-essential amino acids solution, 1x penicillin/streptomycin, 55 μ M 2-mercaptoethanol, and 10% heat-inactivated FCS, all from Invitrogen), adjusted to 1×10^6 cells/mL, and seeded into 96-well round-bottom polystyrene plates. Cells were stimulated with 1 μ g/mL protein for 5 days at 37°C in 5% CO₂. Supernatants were collected and assayed for IL-17 by ELISA (R&D Systems).

Isolation of bone marrow derived dendritic cells and stimulation with vaccine constructs.

Bone marrow cells from naïve male 6–8 week-old FVB/N mice were collected and erythrocytes lysed using a Mouse Erythrocyte Lysing Kit (R&D Systems). Bone marrow cells were then suspended in cR10. Cells were adjusted to 2×10^5 cells/mL and cultured for up to 9 days in the presence of 10 ng/ml recombinant mouse GM-CSF and IL-4 (both from BioLegend). Media were changed every 3 days. BMDCs were collected and stimulated with the vaccine constructs for 24 hours, after which supernatants were collected for determination of IL-1 β and IL-6 by ELISA (R&D Systems).

Opsonophagocytic Assays

Opsonophagocytic assays were performed as previously described [7]. For assessment of PopB antisera, *P. aeruginosa* strain PAO1 was grown in LB containing 5 mM EGTA to induce the type III secretion system [18]. Killing less than 50% in these assays is not considered biologically significant.

ELISAs

ELISAs to measure PopB-specific IgG titers were performed with plates coated with proteins at 1 μ g/mL, as previously reported [7]. Sera from mice immunized with curdlan alone were previously described [12].

Statistical analyses

All analyses were performed using Prism (Graphpad Software). Survival data were analyzed with the Kaplan-Meier method and the log-rank test. Parametric data were analyzed by t test or by ANOVA with Dunnett's multiple comparison test.

Nonparametric data were analyzed by Mann Whitney U test or Kruskal Wallis test with Dunn's multiple comparison test.

Data availability

The data that support the findings of this study are available from the corresponding author upon request.

Results

Characteristics of PLGA-PopB/PcrH nanoparticles.

His-tagged recombinant versions of the *P. aeruginosa* type III secretion system protein PopB along with its chaperon protein PcrH were expressed from *E. coli* and encapsulated in PLGA nanoparticles. PopB alone could not be expressed in *E. coli* so it was necessary to co-express and purify PopB with its chaperon PcrH [12]. The average diameter of PLGA-nanoparticle constructs was 600–700 nm, with approximately 2% protein loading (Table 1). We measured the amount of PopB/PcrH release from PLGA nanoparticles in PBS solution and found that the PLGA nanoparticles showed relatively quick release, with loss of half the protein content within 12 hours, and almost 100% in the first 24 hours (Figure 1).

PLGA-PopB/PcrH nanoparticles elicit IgG and Th17 responses.

Mice were intranasally immunized with PLGA-PopB/PcrH nanoparticles, PLGA-PcrH nanoparticles, PLGA alone, PopB/PcrH alone, PopB/PcrH plus curdlan, or curdlan alone once a week for 3 weeks. Intranasal immunization of mice with all vaccine constructs was well-tolerated. Three weeks after the last immunization, sera were collected and pooled within groups. Antibody titers against PopB/PcrH were determined using an ELISA. Mice that were immunized with PLGA-PopB/PcrH nanoparticles, PopB/PcrH alone, or PopB/PcrH plus curdlan all generated a statistically significant IgG response to PopB/PcrH, although encapsulation in PLGA led to somewhat diminished IgG levels (Figure 2A). As expected, mice immunized with PLGA or curdlan alone did not elicit a PopB/PcrH-specific antibody response. We also measured the ability of sera from immunized mice to mediate killing of *P. aeruginosa* strain PAO1 by human neutrophils in the presence of complement using an opsonophagocytic assay. As previously seen [12], sera from mice immunized with PopB/PcrH had no significant opsonophagocytic killing activity (Figure 2B) despite sera containing antibodies against PopB/PcrH. Likewise, sera from mice immunized with PLGA-PopB/PcrH nanoparticles also had no opsonophagocytic killing activity (Figure 2B). As a control, antiserum from mice immunized intranasally with a live-attenuated vaccine derived from strain PAO1 called PAO1 *aroA* [19] mediated high opsonophagocytic killing activity against *P. aeruginosa* strain PAO1 (Figure 2B).

We next measured the Th17 response elicited by the different vaccines. Three weeks after the third immunization, we quantified the proportion of T cells within collagenase/DNase-digested lungs that produced IL-17 by flow cytometry using intracellular staining. We found that immunization with PLGA-PopB/PcrH nanoparticles or PopB-PcrH-curdan resulted in an increase in the proportion of IL-17-producing T cells in the lungs compared to mice immunized with empty PLGA particles, PLGA-PcrH nanoparticles, or unencapsulated PopB/PcrH (Figure 3A). This increased production of IL-17 occurred in the absence of typical adjuvants. We further measured the Th17 response elicited by immunization by stimulating splenocytes with purified PcrH, PopB/PcrH, or FpvA/SecB (negative control antigen [12]) for 5 days. We found that splenocytes from mice immunized with PLGA-PopB/PcrH particles showed increased IL-17 secretion when stimulated with purified PopB/PcrH compared to splenocytes collected from mice immunized with PLGA-PcrH nanoparticles, PLGA alone, or PopB/PcrH alone, although the IL-17 production was not as high as from mice immunized with PopB/PcrH mixed with curdian (Figure 3B). Co-culture of PcrH with the PLGA-PopB/PcrH vaccine led to minimal IL-17 production, far less than that induced by the PopB/PcrH vaccine mixed with curdian as adjuvant.

PLGA-PopB/PcrH nanoparticle vaccines protect against a lethal *P. aeruginosa* challenge

To determine if immunization with PLGA-PopB/PcrH nanoparticles is able to protect against *P. aeruginosa* challenge, we immunized mice with PLGA-PopB/PcrH nanoparticles, PopB/PcrH alone, PopB/PcrH plus curdian, or PLGA alone once a week for three weeks and then three weeks later intranasally challenged with a highly virulent cytotoxic *P. aeruginosa* strain called ExoU⁺ PAO1 (1×10^6 CFU/mouse), which expresses ExoU from a plasmid [20]. Infected mice were monitored for survival for 6 days after infection. As shown in Figure 4A, mice vaccinated with PLGA-PopB/PcrH nanoparticles had significantly better survival than mice vaccinated with PopB/PcrH or PLGA alone (* $P < 0.01$ by logrank test). In separate experiments, mice that were immunized with PLGA-PopB/PcrH or PopB/PcrH plus curdian had a 5-log₁₀ reduction in the median number of bacteria within the lungs compared to mice immunized with PopB/PcrH or empty PLGA particles (Figure 4B). Mice immunized with PLGA-PopB/PcrH or PopB/PcrH plus curdian had no detectable bacteria in spleen (a marker for dissemination), while 43% of mice in the PopB/PcrH alone group and 25% of mice in the PLGA alone group had detectable bacteria in the spleen (median 0 CFU/g; IQR 0, 1.6×10^4 CFU/g for PopB/PcrH, and median 0 CFU/g, IQR 0, 1.1×10^3 CFU/g for PLGA alone; $P = 0.05$ for PopB/PcrH in comparison to either PopB/PcrH plus curdian or PLGA-PopB/PcrH by Kruskal-Wallis test with Dunn's multiple comparisons test).

PLGA-PopB/PcrH nanoparticles elicit an IL-6 and IL-1 β response in dendritic cells

We measured the amount IL-6 and IL-1 β produced from bone-marrow-derived dendritic cells (BMDC) isolated from naïve mice in order to determine a potential mechanism for induction of the Th17 response. An inflammatory milieu (particularly the proinflammatory cytokine IL-6, with amplification by IL-1 β) has been shown to be important for the development of a Th17 responses [21]. BMDC were stimulated PcrH alone, PopB/PcrH, PLGA-PopB/PcrH, PopB/PcrH plus curdian, PLGA alone, LPS isolated from *P. aeruginosa*, or curdian alone for 24 hours, and then IL-6 and IL-1 β was measured. The PLGA-PopB/PcrH nanoparticles and PopB/PcrH plus curdian both elicited strong IL-6 and IL-1 β

responses (Figure 5), suggesting a potential mechanism of induction of the priming of the Th17 response to PLGA-encapsulated protein antigens. To address the possibility of LPS contamination of recombinant proteins contributing to the responses observed, we measured LPS levels in the vaccines (Supplementary Table 1). LPS at concentrations of 30,000 EU/mL, which was 5,000-fold higher than that found in any vaccine preparation, induced similar levels of IL-6 as PLGA-PopB/PcrH nanoparticles and PopB/PcrH plus curdlan (Figure 5A).

Discussion

In the current study, we sought to improve vaccines based on the *P. aeruginosa* type III secretion system protein PopB by encapsulation in PLGA nanoparticles. Intranasal vaccination of mice with PLGA-encapsulated PopB (which also included PopB's chaperone PcrH) was well tolerated and induced a PopB-specific Th17 response that was associated with protection against a lethal challenge. This Th17 response and protection was acquired without an additional adjuvant and was dependent on encapsulation in PLGA-particles. PLGA encapsulation led to diminished IgG responses to PopB, but we previously showed that IgG to PopB was not protective [12]. Similar to vaccination with PopB/PcrH plus curdlan, vaccination with PLGA-encapsulated PopB/PcrH led to significant protection from a lethal *P. aeruginosa* challenge and a significant reduction in the number of bacteria within the lungs (Figure 4). This protection requires PopB as our previous work found that vaccination with the chaperone protein, PcrH, or curdlan alone did not elicit protection against the same challenge strain [12]. We also observed no viable bacteria in the spleens of mice immunized with PLGA-PopB/PcrH, indicating protection against bacterial dissemination from the lung. It should be pointed out, however, that animal models of *P. aeruginosa* pneumonia with cytotoxic strains, as used in these studies, suggest that bacteremia is just a marker of lung damage and that leakage of cytokines from the damaged lungs is what produces septic shock [22].

Although the experiments described here were limited to challenge with a single *P. aeruginosa* strain, the high conservation of PopB among clinical isolates [23] and the antibody independence of the protection induced by PopB-based vaccines make it highly likely that protection will span multiple LPS serotypes. Since cystic fibrosis (CF) isolates are typically LPS-rough, they are not virulent in this acute pneumonia model, so other models will need to be tested to evaluate protection in the setting of CF. Furthermore, it is possible that vaccine-induced Th17 responses will be harmful in the setting of CF, where Th17-characterized lung inflammation has been observed [24] (albeit in the context of chronic *P. aeruginosa* infection).

Despite an abundance of vaccine candidates and trials, there is no FDA-approved *P. aeruginosa* vaccine. Early vaccines targeted LPS O antigen and were able to generate immunity, but this protection was LPS serotype-specific, and, with more than 30 subtypes of LPS O antigen, this approach has met significant challenges [25, 26]. Outer membrane proteins (OMPs) F and I [27, 28], antibodies to the PcrV antigen component of the type III secretion system [29], and antibodies to the biofilm polysaccharide Psl [30] have all been evaluated as potential vaccine strategies. While protection against heterologous serogroups

has sometimes been seen, the protection of these non-LPS-based vaccines has, as a rule, been of comparatively low potency [31]. In addition, monoclonal antibodies to bacterial antigens run the risk of escape mutants from small alterations in the antigen. Our recent studies have found that the ability of a vaccine to stimulate a Th17 response may be the missing piece necessary for an effective *P. aeruginosa* vaccine [12]. Growing evidence suggests that Th17 cells [32, 33] play a critical role in innate and adaptive antibacterial host defense [8, 10, 34, 35] as well as in autoimmunity [36]. By enhancing phagocyte recruitment to the site of infection, which is an indirect mechanism of protection, Th17-based vaccines have low selective pressure for bacterial escape mutants [11]. They also have the theoretical benefit that they might protect against mixed infections of *P. aeruginosa* with other bacterial species. Notably, a vaccine based on a *Candida* protein called Als3p has been shown to confer IL-17-dependent protection in mice against both *Candida* and *S. aureus* bloodstream infections [13].

An inflammatory milieu, especially the proinflammatory cytokines IL-6 and IL-1 β , help to prime Th17 responses [21]. We found that exposing PLGA-PopB/PcrH nanoparticles or PopB/PcrH with curdlan to naïve BMDC were able to induce both IL-6 and IL-1 β , suggesting a likely mechanism of the induction of the Th17 response. Interestingly, the amount of IL-1 β induced by PLGA-PopB/PcrH nanoparticles was significantly greater than the amount induced with PopB/PcrH or PLGA alone, suggesting the sustained exposure and release of the PopB/PcrH or the targeting of PopB/PcrH to antigen-presenting cells by PLGA facilitates the Th17 response. Future studies will help elucidate this mechanism. We believe LPS contamination of the vaccine preparations only minimally contributed to the observed IL-6 and IL-1 β responses. LPS was unable to induce an IL-1 β response from naïve BMDC (Figure 5B), whereas LPS at concentrations of 30,000 EU/mL could induce an IL-6 response at levels similar to BMDC exposed to PopB/PcrH with curdlan, which contained approximately 5,000-fold less EU.

PLGA is FDA-approved for a number of slow-release drug formulations and biomaterials, although no PLGA-encapsulated vaccines are currently in clinical use. Several other studies have evaluated vaccine antigens encapsulated in PLGA particles as vaccination strategies [13, 14, 37–39]. These studies have assessed protection against a diverse set of pathogens, including both bacteria and viruses. In one of these studies, Singh *et al.* was able to elicit protection and a Th17 response to *Brucella abortus* using PLGA nanoparticles encapsulating the rL7/L12 protein, an immunodominant antigen of *Brucella*, without the addition of an adjuvant [38]. The authors hypothesized that the PLGA-rL7/L12 nanoparticles were internalized by antigen-presenting cells, potentially leading to high local concentrations of antigen that helped prime T cell responses without additional adjuvant. Two recent studies demonstrated that PLGA nanoparticles encapsulating vaccine proteins or adjuvants are taken up by macrophages and dendritic cells, with subsequent antigen processing and presentation [40, 41] [42]. We suspect that the PLGA-PopB/PcrH nanoparticles studied here are working via a similar mechanism.

Conclusion

In summary, we demonstrate a novel approach to develop a *P. aeruginosa* vaccine by encapsulating PopB/PcrH in PLGA nanoparticles for inducing Th17-mediated protective responses. Future vaccine candidates incorporating other *P. aeruginosa* antigens, particularly those that induce opsonophagocytic antibodies, will likely lead to even more potent protection by adding protective antibody responses to phagocyte-stimulating Th17 responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Pseudomonas aeruginosa* PopB was encapsulated into PLGA nanoparticles.
- Vaccination with PopB-PLGA protected against lethal *P. aeruginosa* challenge.
- PopB-PLGA vaccination induced a Th17 response.
- Th17 response and protection occurred without additional adjuvant.

Figure 1. Composition of PLGA molecule and release kinetics of PLGA nanoparticles. Release of encapsulated PopB/PcrH protein over time. Protein concentration measured by Bradford assay in PBS at 37 °C. Error bars are SD.

Figure 2. Vaccination with PopB/PcrH encapsulated in PLGA nanoparticles induces an anti-PopB/PcrH IgG response without opsonophagocytic activity.

Mice (n=4/group) were immunized intranasally with PLGA alone, PLGA-PopB/PcrH particles, PLGA-PcrH particles, PopB/PcrH, PopB/PcrH plus curdlan, or curdlan alone once per week for 3 weeks, and sera were obtained and pooled 3 weeks later. (A) Anti-PopB/PcrH IgG in sera was measured by ELISA. The EC₅₀ for mice immunized with PLGA-PopB/PcrH was significantly different compared to mice immunized with PopB/PcrH or PopB/PcrH-Curdlan as was mice immunized with PLGA-PcrH compared to PLGA-PopB-PcrH by comparing 95% CI of non-linear regression line fits. Sera from mice immunized with curdlan alone were previously described [12]. (B) *P. aeruginosa* strain PAO1-targeted opsonophagocytic killing activity of pooled sera from mice immunized with indicated vaccines. As a positive control, sera from mice immunized with live-attenuated PAO1 *aroA* [19] was diluted 1:100 before use, while the other sera were diluted 1:5 before use (all sera were diluted again 1:4 in the final mixture). **p* < 0.05 by ANOVA with Dunnett's multiple comparisons test compared to no sera.

Figure 3. Vaccination with PopB/PcrH encapsulated in PLGA nanoparticles induces a Th17 response.

Mice were immunized intranasally with PLGA alone, PLGA-PopB/PcrH particles, PLGA-PcrH particles, PopB/PcrH, PopB/PcrH plus curdian, or curdian alone once per week for 3 weeks, and then lungs and spleens were harvested 3 weeks after last immunization. (A) Lung Th17 cells were quantified by intracellular staining of collagenase/DNase-digested lungs stimulated *ex vivo* with PMA and ionomycin for 6 hours. * $p < 0.05$ compared to empty PLGA group by Kruskal Wallis test with Dunn's multiple comparison test. (B) Splenocytes were stimulated with PcrH, PopB/PcrH, or SecB/FpvA (negative control) for 5 days and then IL-17 in supernatant was measured by ELISA. * $p < 0.05$; ** $p < 0.01$ by ANOVA with Dunnett's multiple comparison test using mice immunized with PLGA empty and stimulated with PcrH as control comparator.

Figure 4. Vaccination with PopB/PcrH encapsulated in PLGA nanoparticles protects against lethal *P. aeruginosa* pneumonia.

Mice were immunized intranasally with PLGA alone, PLGA-PopB/PcrH particles, PopB/PcrH, or PopB/PcrH plus curdlan, once per week for 3 weeks, and then challenged 3 weeks later with *P. aeruginosa* strain ExoU⁺ PAO1 (1×10^6 CFU). (A) Disease progression after challenge was monitored over 6 days. $p < 0.01$ by logrank test in comparison to empty PLGA or PopB/PcrH group. Each group consisted of 10 mice, and data shown are representative of two separate experiments. (B) Counts of viable bacteria in the lungs 18 hours after challenge. * $p < 0.05$ by Kruskal-Wallis test using log₁₀-transformed data, with Dunn's multiple comparison test. Dotted line denotes limit of detection.

Figure 5. PopB/PcrH encapsulated in PLGA nanoparticles stimulates IL-6 and IL-1 β from naïve bone marrow-derived dendritic cells (BMDC).

BMDC were obtained from naïve FVB/N mice after cultured for 7 days with GM-CSF and IL-4. BMDC were then stimulated with PopB/PcrH, PopB/PcrH plus curdian, PLGA-PopB/PcrH, PLGA only, LPS (30,000 EU/mL), or left untreated for 24 hours when IL-6 (A) and IL-1 β (B) in the supernatant was measured by ELISA. * $p < 0.05$ compared to unstimulated by ANOVA with Tukey's multiple comparison test.

Table 1.

Characteristics of PLGA-nanoparticle constructs.

Vaccine	Size (nm) mean $\pm$ SD	Protein Loading (% weight)
PLGA-PopB/PcrH	628 $\pm$ 111.2	2.1
PLGA-PcrH	703 $\pm$ 95.6	2.3
PLGA (empty)	628 $\pm$ 326 nm	N/A