Th17-stimulating Protein Vaccines Confer Protection against *Pseudomonas aeruginosa* Pneumonia

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Rationale: New vaccine approaches are needed for *Pseudomonas aeruginosa*, which continues to be a major cause of serious pulmonary infections. Although Th17 cells can protect against gram-negative pathogens at mucosal surfaces, including the lung, the bacterial proteins recognized by Th17 cells are largely unknown and could be potential new vaccine candidates.

Objectives: We describe a strategy to identify Th17-stimulating protein antigens of *Pseudomonas aeruginosa* to assess their efficacy as vaccines against pneumonia.

Methods: Using a library of *in vitro* transcribed and translated *P. aeruginosa* proteins, we screened for Th17-stimulating antigens by coculturing the library proteins with splenocytes from mice immunized with a live-attenuated *P. aeruginosa* vaccine that is protective via Th17-based immunity. We measured antibody and Th17 responses after intranasal immunization of mice with the purified proteins mixed with the Th17 adjuvant curdlan, and we tested the protective efficacy of vaccination in a murine model of acute pneumonia.

Measurements and Main Results: The proteins PopB, FpvA, FptA, OprL, and PilQ elicited strong IL-17 secretion in the screen, and purified versions of PopB, FpvA, and OprL stimulated high IL-17 production from immune splenocytes. Immunization with PopB, which is a highly conserved component of the type III secretion system and a known virulence factor, elicited Th17 responses and also enhanced clearance of *P. aeruginosa* from the lung and spleen after challenge. PopB-immunized mice were protected from lethal pneumonia in an antibody-independent, IL-17-dependent manner.

Conclusions: Screening for Th17-stimulating protein antigens identified PopB as a novel and promising vaccine candidate for *P. aeruginosa*.

Keywords: vaccine; pneumonia; Th17; IL-17; *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a major cause of serious, often antibiotic-resistant, lung infections in humans, particularly in

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Despite more than 40 years of vaccine research and development, there is no clinically available vaccine for the bacterial pathogen *Pseudomonas aeruginosa*, which continues to be a major cause of serious pulmonary infections in humans. Th17 cells are a recently described lineage of helper T cells that can enhance antibacterial mucosal defenses and can potentially mediate protective vaccine-induced responses.

What This Study Adds to the Field

Here we designed a screening method to discover Th17-stimulating protein antigens expressed by a plasmid library encoding *P. aeruginosa* proteins. Our screen uncovered the type III secretion system protein PopB as an effective stimulator of Th17 immunity, and immunization with purified PopB enhanced survival from pneumonia in a murine model in an antibody- and serogroup-independent fashion. Thus PopB, which harnesses a novel mechanism of immunity, is a promising vaccine candidate for *Pseudomonas aeruginosa*.

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patients receiving mechanical ventilation and those with cystic fibrosis (1, 2). Most *P. aeruginosa* vaccines developed to date, including those based on the LPS O antigen (3), the outer membrane proteins F and I (4, 5), or the type III secretion system component PcrV (6), have relied on conventional protective mechanisms—namely, antibody-mediated opsonophagocytic killing and/or antibody-mediated toxin inhibition. Although LPS O antigen-based vaccines can mediate high levels of immunity to *P. aeruginosa*, the protection is limited to strains having the same LPS serogroup (3). The failure of the Federal Hyperimmune Immunoglobulin Trial (7), which found no benefit in critically ill adults passively immunized with *P. aeruginosa* LPS O antigen-specific IgG, perhaps best illustrates that antibody-mediated protective mechanisms are not sufficient.

Th17 cells have recently been shown to mediate antibody-independent host defense against *Klebsiella pneumoniae* (8), although the bacterial proteins recognized by the Th17 cells in those studies were not fully characterized. In our own evaluations of live-attenuated *P. aeruginosa* vaccines, we found that IL-17 was essential for LPS serogroup-independent protection against pneumonia in the absence of opsonophagocytic antibody and was associated with rapid recruitment of neutrophils to the airways (9). We thus turned to a reverse vaccinology (10) approach to capitalize on the Th17-based mechanism of protection elicited by live-attenuated *P. aeruginosa* vaccines. In reverse

vaccinology, the annotated bacterial genome sequence is subjected to bioinformatics analysis to identify possible surface proteins. The genes encoding these proteins are then cloned, overexpressed in *E. coli*, and purified for use as vaccines in mice. Typically, proteins that can induce bactericidal or opsonophagocytic antibodies are selected and tested as protective antigens (11). With this strategy, novel protective antigens have been identified for serogroup B *Neisseria meningitidis* (12), Group B *Streptococcus* (13) and extraintestinal pathogenic *Escherichia coli* (14). As noted above, this approach has generally been based on humoral immune responses rather than T-cell responses.

In this study, we identified the protective protein components of a live-attenuated *P. aeruginosa* vaccine using a Th17-based reverse vaccinology strategy. We used a library of *P. aeruginosa* outer membrane and secreted proteins identified by bioinformatics, with their respective genes cloned into expression vectors (15). The proteins were produced with an *in vitro* transcription and translation system and used to stimulate splenocytes from mice immunized with a live-attenuated *P. aeruginosa* vaccine strain. His-tagged purified version of three proteins from the library (OprL, PopB, and FpvA) stimulated IL-17 production in immune splenocytes, indicating their potential Th17-stimulating properties. We hypothesized that Th cell epitopes identified by such a screen would also elicit Th17 responses if combined with Th17-inducing factors during priming of the immune response. Indeed, it is not the nature of the protein antigen that determines the lineage decisions of immature Th cells but rather the context of the initial interaction of the naive T cell with antigen-presenting cells (16). Thus, we tested whether a known Th17 adjuvant, curdlan (17, 18), would improve the Th17 responses after immunization with the purified proteins. We found that immunization of mice with PopB-curdlan elicited strong Th17 responses against *P. aeruginosa* and conferred IL-17-dependent protection from lethal *P. aeruginosa* lung infection in the absence of opsonophagocytic antibody. A portion of the results of these studies has been previously reported in abstract form (19).

METHODS

Detailed methods are available in the online supplement.

Protein Library

The construction of the *P. aeruginosa* outer membrane and secreted protein library followed previously described methods (15).

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in these experiments are listed in Table E1 in the online supplement. Primers used in this study are listed in Table E2.

Expression and Purification of Proteins from *E. coli*

The His-tagged proteins were purified using a previously described method (20) with slight modifications detailed in the online supplement.

Immunization of Mice and Murine Pneumonia Model

All animal experiments complied with institutional and federal guidelines regarding the use of animals in research. Immunizations with bacteria were performed as previously described (9). For immunization with proteins, mice were immunized three times with proteins or proteins plus curdlan (10 mg/ml) (Sigma-Aldrich, St. Louis, MO) suspended in phosphate-buffered saline (PBS), also at weekly intervals. The dose of each protein used in immunization was 35 µg per mouse. For the murine pneumonia model, mice were anesthetized and inoculated intranasally with *P. aeruginosa* strain ExoU⁺ PAO1 (a cytotoxic version of strain PAO1 [21]) at doses indicated in the figure legends.

Cells and supernatants obtained from bronchoalveolar lavage fluid (BALF) were analyzed for cytokines and intracellular IL-17 staining using methods detailed in the online supplement.

Opsonophagocytic Assays

Opsonophagocytic assays were performed by standard methods, as previously described (9). For assessment of PopB antisera or BALF, bacteria were grown in LB (lysogeny broth) containing 5 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid to induce the type III secretion system (TTSS) (22). Killing less than 50% in these assays is not considered biologically significant.

ELISAs

ELISAs to measure protein- or *P. aeruginosa*-specific IgG titers were performed with plates coated with proteins at 1 µg/ml or whole live bacteria (grown in TTSS-inducing conditions [22] for PopB antisera), as previously described (9).

Cytotoxicity Assay

Cytotoxicity assays were performed as previously reported (23) with modifications described in detail in the online supplement.

Statistical Analyses

All analyses were performed using Prism software (GraphPad Software, La Jolla, CA). Survival data were analyzed with the Kaplan-Meier method and the log-rank test. Normally distributed data were analyzed by *t* test or by analysis of variance with Dunnett multiple comparison test using PBS-immunized cells or antisera as the control comparator group. Nonparametric data were analyzed by Mann-Whitney *U* test or Kruskal-Wallis test with Dunn's multiple comparison test.

RESULTS

Screen for Antigens Recognized by Th17 Cells Isolated from PA14ΔaroA-immunized Mice

To identify Th17-stimulating antigens, we screened a *P. aeruginosa* outer membrane and secreted protein library with splenocytes isolated from mice immunized with the live-attenuated *P. aeruginosa* vaccine strain PA14ΔaroA (9). A total of 258 recombinant proteins were produced with an *in vitro* transcription/translation system, and the whole reaction mixtures were directly used to stimulate the splenocytes. After 3 days of coculture, IL-17 production in the supernatant was measured by ELISA. As summarized in Figure 1A, wells containing the transcription/translation system without plasmid did not stimulate IL-17 production, whereas the positive-control wells containing gentamicin-killed *P. aeruginosa* strain PAO1 stimulated high IL-17 production, which is approximately 1.5-fold higher than the mean level of all the tested proteins. Proteins that stimulated similar levels of IL-17 production as the positive control were further analyzed and reconfirmed by polymerase chain reaction of the vectors to be OprL, PopB, FpvA, FptA, and PilQ. The functions of these proteins are summarized in Table E4, as described on the *Pseudomonas* Genome Database (www.pseudomonas.com).

Overexpression and Purification of Identified Proteins

To confirm the Th17-stimulating properties of the proteins identified in the screen, we first overexpressed and purified OprL, PopB, FpvA, and FptA from *E. coli*. The purified N-terminal His-tagged OprL (Figure E1A) had very low solubility in PBS, precluding large-scale *in vivo* studies. C-terminal His-tagged PopB (PopB-His) was not detected in *E. coli*, and after attempted

Figure 1. Screening an *in vitro* transcribed and translated *Pseudomonas aeruginosa* protein library can identify Th17-stimulating antigens. (A) IL-17 production induced by positive “hits” of *P. aeruginosa* library proteins. Splenocytes from PA14ΔaroA-immunized mice (FVB/N) were stimulated with 258 *in vitro* transcribed and translated library proteins individually. IL-17 production was determined 3 days later by ELISA. Stimulation with gentamicin-killed *P. aeruginosa* strain PAO1 (multiplicity of infection [MOI] = 10) was used as positive control. (B) Stimulation of IL-17 production by purified His-tagged proteins. Splenocytes from PA14ΔaroA-immunized mice were stimulated with gentamicin-killed PAO1 (MOI = 10) or purified proteins at 0.2 or 1 μg/mL. Bars represent averages, and error bars are SD. Cells were pooled from three to four mice each group. **P* < 0.01 compared with *Escherichia coli* group by analysis of variance with Tukey multiple comparison test. Gm = gentamicin.

induction of its expression with IPTG (isopropyl β-D-1-thiogalactopyranoside), no increase in PopB-His (about 40 kD) was observed in the whole bacteria lysate (Figure E1B, lanes 3 and 4). However, coexpression of His-tagged PcrH (His-PcrH), the intracellular chaperone of PopB (24), apparently stabilized PopB, and a strong band of the predicted size was observed when lysates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure E1B, lanes 5 and 6). When copurified with His-PcrH, PopB-His was soluble in PBS (25) at a concentration at 3.5 μg/μl. Similar to PopB, C-terminal His-tagged FptA (FptA-His) was not stable in *E. coli* (Figure E1C, lanes 5 and 6). Coexpression of SecB (N-terminal His-tagged, designated His-SecB), a chaperone of the general secretion (Sec) pathway (26), stabilized FptA-His (Figure E1C, lanes 9 and 10) and kept the purified FptA-His soluble in PBS at a concentration of 3.5 μg/μl (Figure E1D). The purified C-terminal His-tagged FpvA (FpvA-His) was not soluble in PBS, but coexpression and purification with His-SecB kept FpvA-His soluble at a concentration of 1 μg/μl (Figure E1D). Purified PilQ was not soluble in PBS so was not further evaluated.

Th17-stimulating Properties of Identified Proteins

On coculture of the purified proteins with splenocytes from PA14ΔaroA-immunized mice, His-OprL as well as PopB-His and FpvA-His, together with their copurified chaperones, induced IL-17 production (Figure 1B). Notably, the chaperones, His-PcrH and His-SecB, did not elicit IL-17 production, which

suggests that PopB and FpvA, rather than their chaperones, are the antigens that stimulate IL-17 production. FptA-His with His-SecB did not induce IL-17 production, indicating a false-positive result from the screen (Figure 1B).

Protective Efficacy of PopB Vaccination

Because PopB is a component of the type III secretion system and is a known virulence factor of *P. aeruginosa* (27–30), we focused on PopB for *in vivo* immunization experiments. Moreover, PopB has been reported to elicit a humoral immune response in patients with cystic fibrosis (CF) with *P. aeruginosa* lung infection (15, 31), indicating its immunogenicity during infection. We did not assess the vaccine potential of FpvA due to the recently described high genetic variation of the *fpvA* gene, resulting in extensive sequence polymorphism of this protein when expressed by different *P. aeruginosa* strains (32, 33). We used an intranasal immunization route and combined the proteins with the Th17 adjuvant curdlan (a β-(1, 3)-linked glucose polymer that composes the β-D-glucan of the fungal cell wall), which has been shown to promote Th17 responses (17, 18). Importantly, despite mucosal administration of a Th17 adjuvant and a protein with Th epitopes (PopB) during vaccination, there was no evidence of lung inflammation on histology (Figure E2A), and BALF neutrophil numbers were not elevated when assessed 3 weeks after the third intranasal immunization (Figure E2B). The number of CD4 cells in BALF, however, was significantly higher in the PopB-immunized mice (Figure E2C), indicating probable vaccination-induced expansion and tissue persistence.

Vaccination with PopB combined with its chaperone PcrH (PopB/PcrH) conferred significant protection, with 62.5% survival after lung challenge with *P. aeruginosa* strain ExoU⁺ PAO1, whereas immunization with PcrH alone did not provide protection (Figure 2A). Curdlan did not further increase the protective efficacy of PopB (Figure 2B). Mice vaccinated with PopB had significantly lower levels of viable bacteria in the lung and spleen 18 hours after challenge (Figure 2C).

Antigen-Specific Serum Antibodies from Immunized Mice

To test the humoral immune responses elicited by immunization with the protein antigens, sera from immunized mice were collected 3 weeks after the third immunization and the antigen-specific IgG titers were determined by ELISA. Both PcrH and PopB/PcrH immunization elicited antigen-specific IgG (Figures 3A and 3B). Interestingly, higher antigen-specific IgG titers were detected in sera from PopB/PcrH-immunized mice compared with sera from PcrH-immunized mice, indicating stronger immunogenicity of PopB compared with PcrH. Notably, addition of curdlan to the proteins increased the serum IgG titers (Figures 3A and 3B). Curdlan alone did not elicit IgG specific to the protein antigens. BALF contained PopB-specific IgG (Figure E3A) but no significant PopB-specific IgA (Figure E3B).

Opsonic Killing Activities and Recognition of *P. aeruginosa* by Serum Antibodies from Immunized Mice

We next used opsonophagocytic assays to test whether the antigen-specific Abs in the sera from protein-immunized mice could mediate killing of *P. aeruginosa* strain PAO1 by polymorphonuclear leukocytes in the presence of complement. Unlike the antisera from mice immunized intranasally with a live-attenuated vaccine derived from strain PAO1 called PAO1ΔaroA (34), the sera from mice immunized with the protein antigens (Figure 4A) showed very low opsonophagocytic killing activity against strain

Figure 2. Mice immunized with the Th-stimulating *Pseudomonas aeruginosa* protein PopB and its chaperone PcrH mixed with the Th17 adjuvant curdlan are partially protected against *P. aeruginosa* pneumonia. Mice were challenged with *P. aeruginosa* strain ExoU⁺ PAO1 (7×10^5 cfu per mouse) after intranasal immunization with PcrH or PopB/PcrH either alone (A) or mixed with curdlan (B). *P* values are by log-rank test. (C) Viable counts of *P. aeruginosa* in the lungs and spleens of PopB-vaccinated mice 18 hours after challenge. Mice were challenged with *P. aeruginosa* strain ExoU⁺ PAO1 (1×10^6 cfu per mouse) after intranasal immunization with PopB/PcrH mixed with curdlan or curdlan alone. Each point represents one mouse. Bars represent medians. Dashed line in right panel is the lower limit of detection. *P* values are by Mann-Whitney *U* tests.

PAO1 despite growth conditions that induce type III secretion (22). Similarly, there was no killing activity of BALF from PopB-immunized mice (Figure E3C). We also determined the IgG titers in the sera of the immunized mice against whole cells of *P. aeruginosa* strain PAO1 by ELISA using plates coated with live PAO1 grown in type III secretion-inducing conditions. As shown in Figure 4B, no reactivity was observed in the antisera from mice immunized with the protein antigens compared with antisera to PAO1ΔaroA (34). This suggests that the epitopes recognized by the antigen-specific antibodies are not accessible on the bacterial surface, which likely contributes to the lack of opsonic killing activity.

Figure 3. Intra-nasal immunization with PopB/PcrH plus curdlan elicits high serum IgG titers. Sera from mice immunized with His-tagged PcrH or PopB/PcrH, with or without curdlan, were tested for antigen-specific IgG levels by ELISA with plates coated with PcrH (A) or PopB/PcrH (B). Sera from PBS- or curdlan-immunized mice were used as controls. Each point is the average of duplicates using pooled sera from 7 to 10 mice, and error bars are SDs. PBS = phosphate-buffered saline.

Anti-PopB Antibody Effects on *P. aeruginosa*-induced Cytotoxicity

As noted above, PopB is a component of the TTSS of *P. aeruginosa*, which directly translocates cytotoxic effector proteins into host cells (35) and plays an important role in *P. aeruginosa* pathogenesis (27, 30). PopB and another TTSS component, PopD, form a ring-like structure in the host cell membrane to facilitate the translocation of effector proteins (25, 36). The assembly of this PopB/PopD translocation pore is also dependent on PcrV (37). Because anti-PcrV IgG has been demonstrated to protect cells from TTSS-mediated cytotoxicity (6), we tested whether antisera from PopB-immunized mice can inhibit cytotoxicity using a cell detachment assay. As shown in Figure 4C, the PopB antisera did not protect cells from *P. aeruginosa*-induced cytotoxicity. This result suggests that the protective effect conferred by PopB immunization is not due to the inhibition of TTSS-mediated cytotoxicity.

Th17 Responses Elicited by PopB-Curdlan Vaccination

We next investigated the levels of Th17 responses elicited by intra-nasal immunization with PopB mixed with the Th17 adjuvant curdlan. Three weeks after immunization, splenocytes were isolated and cocultured for 5 days with purified proteins or gentamicin-killed PAO1. Secreted IL-17 was then measured by ELISA. When stimulated with PopB/PcrH, splenocytes from mice immunized with PopB/PcrH or PcrH plus curdlan, but not with proteins alone, produced significant levels of IL-17 (Figure 5A). Although PcrH alone did not stimulate IL-17 secretion from PA14ΔaroA-immune splenocytes (Figure 1B), immunization with PcrH mixed with curdlan (but not with PcrH alone) did

Figure 5. Immunization with PopB/PcrH plus curdlan engenders Th17 responses. Splenocytes isolated from mice immunized with His-tagged PcrH or PopB/PcrH, with or without curdlan, were stimulated with increasing concentrations of purified His-tagged PopB/PcrH (A) or with gentamicin-killed PAO1 (multiplicity of infection = 10) (B). Cells were pooled from three to four mice. After 5 days' incubation, IL-17 levels in the supernatants were measured by ELISA. Bars represent averages, and error bars are SDs. *At 1 μg/ml PopB/PcrH, $P < 0.01$ in comparison with curdlan, PcrH+curdlan or PopB/PcrH group. #At 1 μg/ml PopB/PcrH, $P < 0.05$ in comparison with curdlan group. These comparisons were analyzed by analysis of variance (ANOVA) with Bonferroni multiple comparison test. † $P < 0.05$ in comparison with phosphate-buffered saline (PBS) control group by ANOVA with Dunnett's multiple comparison test.

the PopB vaccine to elicit protection in an inbred mouse background having a different MHC haplotype from that used in the screen.

To investigate further the mucosal immune mechanisms of protection, we assessed the BALF cytokine and T-cell composition 6 hours after challenge of immunized mice. BALF IL-17 levels were significantly higher in PopB-immunized mice (Figure 7A), whereas BALF levels of the Th1 cytokine IFN- γ were all below 20 pg/ml (not shown). Consistent with the well-established role of IL-17 in neutrophil recruitment to the airways (9, 39, 40), there was a trend toward higher neutrophil numbers in BALF from PopB-immunized mice (Figure 7B). Intracellular cytokine staining showed a significantly higher proportion of IL-17-positive CD4 cells in the PopB-immunized mice (Figure 7C) as well as higher staining for the Th17 transcription factor ROR γ t (Figure 7D). Taken together, these results show that the protection elicited by vaccination with PopB/PcrH mixed with the Th17 adjuvant curdlan is independent of antibody, dependent on IL-17, and associated with enhanced mucosal IL-17 and Th17 responses.

DISCUSSION

In this study, we screened a *P. aeruginosa* protein library (15) and identified OprL, PopB, and FpvA as Th17-stimulating

Figure 6. The protective efficacy of vaccination with PopB/PcrH plus curdlan is antibody independent but IL-17 dependent. (A) Survival of mice after passive immunization with antisera to PopB/PcrH plus curdlan, curdlan alone, or live-attenuated *Pseudomonas aeruginosa* strain PAO1ΔaroA. Mice were challenged with *P. aeruginosa* ExoU⁺ PAO1 (9×10^5 cfu per mouse). (B) Survival of PopB/PcrH+curdlan-immunized mice after antibody-mediated neutralization of IL-17 and xOU⁺ PAO1 (1×10^6 cfu per mouse). (C) Survival of PopB/PcrH+curdlan-immunized wild-type or IL-17 receptor knock-out (IL-17R KO) mice after challenge with ExoU⁺ PAO1 (9×10^6 cfu per mouse). P values were calculated by log-rank test.

protein antigens. We further evaluated PopB, as it is a highly conserved, known virulence factor, and found that PopB possesses strong Th epitope(s) as well as one or more nonprotective B-cell epitopes. Intranasal immunization of mice with PopB mixed with the Th17 adjuvant curdlan induced a systemic and mucosal Th17 response to the T-cell epitope(s) and was partially protective on challenge in a murine model of acute *P. aeruginosa* pneumonia. The ability of the PopB vaccine to improve survival after pulmonary challenge with a lethal dose of *P. aeruginosa* is notable in comparison with Th17-based protein vaccines for pneumococcus (41), which protected mice against nasal colonization but not lethal pneumonia. Our results add to a growing number of reports that Th17 responses are important for host defense against an expanding list of pathogens, including *K. pneumoniae* (8, 40), *Salmonella enterica* (42), *Streptococcus pneumoniae* (43), *Mycobacterium tuberculosis* (44), and *Candida albicans* (45).

The proteins for our screen were generated using an *in vitro* transcription/translation system composed of eukaryotic cell

Figure 7. Mucosal IL-17 and Th17 cells correlate with vaccine-induced protection. (A, B) IL-17 levels (A) and neutrophil numbers (B) in bronchoalveolar lavage fluid (BALF) 6 hours after challenge with *Pseudomonas aeruginosa* ExoU⁺ PAO1 (1×10^6 cfu per mouse). Eight mice/group. Bars in (A) depict means and error bars the SD. *P* value by unpaired *t* test. Each point in (B) depicts one mouse; bars show medians. *P* value by Mann-Whitney *U* test. (C, D) Intracellular staining for IL-17 (C) and RORγt (D) in leukocytes isolated from BALF 6 hours after challenge with *P. aeruginosa* strain ExoU⁺ PAO1 (1×10^6 cfu per mouse). Eight mice/group. Bars show means, error bars show SDs. *P* value by unpaired *t* test.

protein expression components, which can efficiently produce proteins directly from plasmid DNA and, importantly, do not activate splenocytes. Thus, this method is suitable for high-throughput screening for T cell-stimulating antigens. It should be noted that eukaryotic cell-based translation systems might process or modify proteins differently from live bacteria, which could result in different immunogenicity. Indeed, in bacteria, the N-terminal signal peptide of proteins secreted by the *sec* pathway (e.g., OprL and FpvA) is cleaved off during secretion (46).

Despite eliciting high titers of anti-PopB IgG after vaccination with the purified protein (Figure 3A), these antibodies could neither mediate opsonophagocytic killing (Figure 4A) nor recognize whole cells of PAO1 (Figure 4B). These findings may relate to the fact that PopB is likely not exposed on the surface of live bacteria in the conditions we used *in vitro*. During infection, PopB and PopD, which are components of the *P. aeruginosa* type III secretion system (TTSS), form a ring-like structure on the host cell membrane, which depends on another TTSS protein, PcrV. However, the location of PopB before contact with the host cell is not known. In the closely related *Yersinia* TTSS, the PopB homolog YopB is not located on the tip or inside the channel of the TTSS needle, whereas the PcrV homolog LcrV is on the top of the TTSS needle (47). Anti-PcrV IgG was able to protect the cell from TTSS-mediated cytotoxicity (6), whereas our

PopB antiserum could not block TTSS-mediated cytotoxicity. These results suggest that PopB is not exposed on the surface of *P. aeruginosa*.

Although our findings that the antisera to PopB had neither opsonophagocytic nor anticytotoxic activity support the validity of our screen and the dependence of *in vivo* protection on Th17 responses, the presence of opsonophagocytic activity is overall a desirable feature of bacterial vaccines. Opsonophagocytic antibodies would be expected to work synergistically with Th17 responses in that IL-17 can recruit neutrophils rapidly to the site of infection, where they can mediate opsonophagocytic bacterial killing. Our current work is focusing on the development of conjugate vaccines composed of PopB conjugated to either a variety of LPS O antigens or with alginate congeners.

Caution must be exercised if applying our results with a model of acute *P. aeruginosa* pneumonia to the chronic *P. aeruginosa* infection in the setting of CF. Indeed, humans with CF have high sputum and bronchoalveolar lavage levels of IL-17 as well as infiltration of the airway submucosa with Th17 lymphocytes (48, 49). Although those findings do not rule out the potential of Th17 responses to be effective before infection is established, it is possible that vaccine-induced Th17 responses will be ineffective in the CF lung and may even exacerbate the neutrophilic airway inflammation of CF.

Although a large body of research is currently focusing on the role of IL-17 and Th17 responses in autoimmune inflammatory diseases, such as multiple sclerosis and rheumatoid arthritis, our findings here underscore the evolutionary importance of Th17 responses in protection of the host from infection. Our screening approach could be generalized to other pathogens that are also controlled by Th17 responses and will serve as the basis for the further development of novel *P. aeruginosa* vaccines.

Author disclosures are available with the text of this article at www.atsjournals.org.

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