



ORIGINAL ARTICLE

Immunization with outer membrane proteins (OprF and OprI) and flagellin B protects mice from pulmonary infection with mucoid and nonmucoid *Pseudomonas aeruginosa*



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Abstract *Background:* *Pseudomonas aeruginosa* is a Gram-negative opportunistic bacterium, which considered as a common cause of nosocomial infection and life-threatening complications in immunocompromized and cystic fibrosis patients. Here, we evaluate the protective effect of recombinant vaccines composed of outer membrane proteins OprF and OprI alone or in combination with flagellin B against mucoid and nonmucoid pseudomonas infection. *Methods:* BALB/C mice were immunized subcutaneous using OprF and OprI with or without flagellin B and antibody titers were determined. Serum bactericidal and opsonophagocytosis activities of immunized and control sera were estimated against mucoid and nonmucoid pseudomonas strains. Lung tissue sections from immunized and nonimmunized mice were analyzed and the levels of peripheral neutrophils infiltration into the lung and tissue inflammation were scored. *Results:* Subcutaneous immunization using OprF and OprI with or without flagellin B elicited higher antibody titers against OprF, OprI, and flagellin B. The produced antibodies successfully opsonized both mucoid and nonmucoid strains with subsequent activation of the terminal pathway of complement that enhances killing of nonmucoid strains via complement-mediated lysis. Furthermore, opsonized mucoid and nonmucoid strains showed enhanced opsonophagocytosis via human peripheral neutrophils, a mechanism that kills *P. aeruginosa* when complement mediated lysis is not effective especially with mucoid strains. Immunized mice also showed a significant prolonged survival time, lower bacteremia, and reduced lung damage when compared with control nonimmunized mice.

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Conclusion: Our data showed that mice immunized with OprF/OprI or OprF/OprI and flagellin B are significantly protected from infection caused by mucoid and nonmucoid strains of *P. aeruginosa*. Copyright © 2017, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is considered to be one of the most important bacterial pathogens responsible for serious opportunistic infections among cystic fibrosis (CF) and immunocompromized patients.^{1,2} Multidrug resistance among *P. aeruginosa* has emerged causing serious complications and representing a major health problem.³ The development of new vaccines that can limit the spread of antibiotic-resistant pseudomonas is now a major challenge. Many antigens of *P. aeruginosa* have been tested as vaccine candidates but unfortunately; none of them were effective as universal immunogens. These include cell wall lipopolysaccharide, high molecular mucoexopolysaccharide, alginate and ribosomes.^{4–8} By contrast, outer membrane proteins (OprF and OprI) have shown to be antigenically cross-reactive within different serogroups and clinical isolates of *P. aeruginosa*.^{9–12} Furthermore, flagella of *P. aeruginosa* are very immunogenic,^{13–15} showing promising results when used alone or in combination with outer membrane proteins in protection against nonmucoid pseudomonas strains.^{9,11} However, it has been reported that the produced antibodies failed to enhance complement mediate lysis of mucoid strains of *P. aeruginosa* *in vitro*. Mucoid pseudomonas strains are well characterized by its overproduction of exopolysaccharide (alginate) that protects pseudomonas from phagocytosis.^{7,8}

Here, we study the protective role of cocktails of antigens formed of recombinant OprF, OprI, and flagellin B in protection of BALB/C mice against acute infection caused by mucoid and nonmucoid *P. aeruginosa* strains.

Materials and methods

Ethics statement

All animal procedures were conducted in accordance with the ethical guidelines of the World Medical Association, Declaration of Helsinki: ethical principles for animal care.

P. aeruginosa strains

Mucoid *P. aeruginosa* clinical isolates DM125 and DM126 (with type B flagellin) were kindly provided by Department of Microbiology and Immunology, Faculty of Pharmacy, Mansoura University, Egypt. The clinical isolates were oxidase and pyocyanin positive and further confirmed as *P. aeruginosa* using API 20NE system. The production of mucoid layer was confirmed using Muir test and secretion of thick slimy layer on agar after 48 hours of incubation at

37°C.¹⁶ Nonmucoid *P. aeruginosa* strains PAO1 and PAK were kindly provided by Dr Kumar Rajakumar, Department of Infection, Immunity and Inflammation, University of Leicester, UK.

Cloning, expression, and purification of recombinant Opr I, Opr F, and flagellin B

The open reading frame of each targeted protein was amplified from the genomic DNA of *P. aeruginosa* PAO1 using the primers listed in Table 1. The stop codon of each open reading frame (OprF, OprI, and flagellin B) was mutated to be in frame with the 6-histidine tag provided by the bacterial expression vector pRSET-B (Promega, San Luis Obispo, CA, USA). The recombinant proteins were expressed in *Escherichia coli* BL-21 (DE3) PlyS and purified as previously described.¹⁷ The expression of recombinant proteins was induced by adding 1 mM of isopropyl-β-D-thiogalactopyranoside. Cells were subsequently harvested and the bacterial pellet was lysed by sonication on ice and then centrifuged at 10,000 ×g for 10 minutes. The supernatant containing the recombinant protein was purified on Ni²⁺ Sepharose 6 fast flow column (GE Health Care, Uppsala, Sweden) according to the manufacturer's instructions. Purified recombinant protein was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by Coomassie brilliant blue G-250 staining and Western blot analysis (Figures S1–3).

Immunization of mice with the recombinant proteins

Eight-week-old female BALB/c mice (20–25 g) were immunized subcutaneously with 50 µg of OprF, OprI, Flagellin B, mixture of OprI/OprF or combination of OprI/OprF/Flagellin B in complete Freund's adjuvant. Control groups were immunized with complete Freund's adjuvant alone. Mice were boosted every week for 3 weeks with 25 µg of the same antigens combinations in incomplete

Table 1 Primers used for amplification of open reading frame sequences of OprF, OprI and flagellin B.

Primer name	Sequence (5' to 3' direction)
OprF-F-BamHI	GGATCCTATGAACTGAAGAACACCTTAGGC
OprF-R-EcoRI	GAATTCTTACTTGGCTTCGGCTTCTAC
OprI-F-BamHI	GGATCCTATGAACAACGTTCTGAAATTCTCTG
OprI-R-EcoRI	GAATTCTTACTTGGCTTCGGCTTCTAC
Fla-B-F-XhoI	CTCGAGCATGGCCCTTACAGTCAACACG
Fla-B-R-HindIII	AAGCTTTTAGCGCAGCAGGCTCAGG

Freund's adjuvant. Blood samples were collected 2 weeks after the immunization course to determine the antibody titer against each individual recombinant protein. For infection study, mice were infected 2 weeks after the last immunization.

Determination of antibody titer in sera from immunized mice

Nunc Maxisorp enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 μ L of either 10 μ g/mL of OprI, OprF or flagellin B in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6). ELISA plates were blocked at room temperature with 250 μ L of 1% (w/v) bovine serum albumin in TBS buffer (10 mM Tris-HCl, 140 mM NaCl, pH 7.4). After 2 hours, ELISA plates were washed three times with 250 μ L of TBS with 0.05% Tween 20 and 5 mM CaCl₂ (wash buffer). Serial dilutions of serum in 100 μ L of 4 mM barbital, 145 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, pH 7.4 were incubated with the precoated ELISA plates for 90 minutes at room temperature. ELISA plates were washed again and bound mouse immunoglobulin (IgG) was detected using alkaline phosphatase conjugated goat anti-mouse antibody and the chromogenic substrate *para*-nitrophenyl phosphate (Sigma-Aldrich, St Louis, MO, USA).

Serum bactericidal assay

Killing of *P. aeruginosa* by serum was estimated by measuring the decrease in the viable count over time. A liquid culture at mid-logarithmic phase of *P. aeruginosa* (PAO1, PAK, DM125, and DM126) was washed using Hank's balanced salt solution (HBSS) and resuspended to a final concentration of 10⁶ colony-forming units (CFU)/mL. *Pseudomonas* (10⁵ CFU) was opsonized with 20% mouse serum (immunized or control mouse serum) for 1 hour at 37°C in a final volume of 250 μ L. Samples were taken at 0 minutes, 30 minutes, 60 minutes, and 120 minutes. To determine the viable bacteria, samples were serially diluted in HBSS and plated onto Luria agar followed by overnight incubation at 37°C.

Opsonophagocytosis assay

Human peripheral blood neutrophils and polymorphonuclear leukocytes (PMNs) were isolated according to Sigma-Aldrich protocol using histopaque-1119 and histopaque-1077 as previously described.^{18,19} The isolated PMNs were re-suspended in HBSS to a final concentration of 1 $\times$ 10⁷ cells/mL. *P. aeruginosa* was opsonized by incubation with 20% control serum or serum from immunized mice at 37°C for 30 minutes. PMNs (1 $\times$ 10⁶ cells) were mixed with 1 $\times$ 10⁵ CFU preopsonized or nonopsonized bacteria in a final volume of 250 μ L in HBSS and incubated at 37°C on a rotary mixer. Samples were taken at 0 minutes, 30 minutes, 60 minutes, and 120 minutes. Killing of *P. aeruginosa* by PMNs was estimated by measuring the decrease in viable bacteria over time. To determine viable bacteria, samples were serially diluted in HBSS and plated on Luria agar followed by incubation at 37°C.

Intranasal infection of mice with *P. aeruginosa*

Preimmunized and control mice ($n = 12$) were infected with each of the *P. aeruginosa* strains PAO1, PAK, DM125, and DM126 in four independent experiments. Mice were lightly anaesthetized with 2.5% (v/v) fluothane (AstraZeneca, UK) over oxygen (1.5–2 L/min), and 50 μ L phosphate-buffered saline containing 10⁷ CFU/50 μ L *P. aeruginosa* was then administered into the nostrils of the mice.¹⁹ Mice were monitored for clinical signs and disease progression. Blood samples were taken and bacterial viable count was calculated after serial dilution in phosphate-buffered saline and plating out.

Histology

Two days postinfection, mice were culled and whole lungs were collected and fixed in 10% formalin for 12 hours. Lung tissues were embedded in paraffin and 7 μ m lung sections were cut and stained with hematoxylin and eosin. The levels of PMNs infiltration into the lung and tissue inflammation were scored blindly and the results were calculated as means ($\pm$ standard error of the mean) of triplicates.

Statistical analysis

Graph Pad Prism software package (version 5.01) was used to calculate mean, standard deviation and standard error. Data were analyzed according to ANOVA and two-sample *t* test and assigned significant when $p < 0.05$ or $p < 0.01$. The statistical analysis of survival was performed using Mantel–Cox log-rank test; with $n = 12$ /group. All results were calculated from the mean of three separate experiments.

Results

Immunization of BALB/C mice with OprF or flagellin B but not OprI induced a strong immune response

To assess the immune response in mice following immunization, serum samples were collected from the immunized and control mice. Antibody titer (IgG) was assessed using ELISA plates precoated with the purified recombinant proteins. Mice immunized with flagellin B or OprF showed a robust immune response after immunization, while mice immunized with OprI alone showed a lower immune response compared to the previous groups. Interestingly, the antibody titer of OprI did not increase when injected with OprF or in combination with OprF and flagellin B (Figure S4). Control mice immunized with Freund's adjuvant alone showed no response against the tested antigens.

Immunization of mice with the divalent (OprF and OprI) or the trivalent (OprF, OprI and flagellin B) vaccines enhanced serum bactericidal activity against nonmucoid pseudomonas strains

The bactericidal activity of 20% mouse immune sera was assessed against mucoid and nonmucoid pseudomonas

strains. The mucoid *Pseudomonas* strains were resistant to lysis by the immune sera (Figure 1A and B). This observation was also confirmed when using a higher serum concentration (1:1) of immunized or control mouse sera (data not shown). Interestingly, nonmucoid *Pseudomonas* strains PAO1 and PAK were susceptible to serum bactericidal activity of the divalent or the trivalent immune sera, and, to a lesser extent, to OprF immune sera (Figure 1C and D).

Opsonisation of *Pseudomonas* strains with the divalent or the trivalent immune serum enhanced phagocytosis of both mucoid and nonmucoid strains by PMNs

In opsonophagocytosis assay, *Pseudomonas* strains were opsonized with immune or control mouse sera and then incubated with freshly isolated PMNs. Mucoid and nonmucoid *Pseudomonas* opsonized with serum from mice immunized with the divalent or trivalent vaccine showed a significant increase in phagocytosis by PMNs when compared with bacteria opsonized with control mouse serum (Figure 2). Only, the nonmucoid strains (PAO1 and PAK) showed enhanced phagocytosis when opsonized with immune serum from mice immunized with OprF or flagellin B alone (Figure 2C and D).

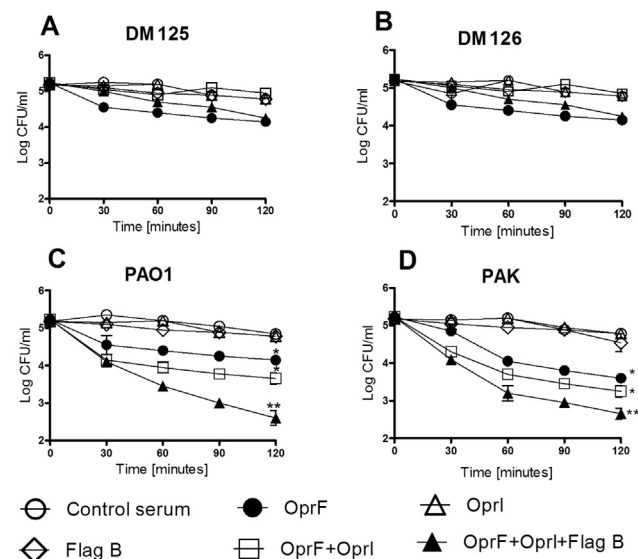


Figure 1. Antibodies against the divalent vaccine (OprF and OprI) or the trivalent vaccine (OprF, OprI, and flagellin B) enhance bactericidal activity of mouse serum on nonmucoid strains of *Pseudomonas aeruginosa*. *P. aeruginosa* (1×10^5 colony-forming units) was incubated with 20% immunized mouse serum or nonimmune control serum. Samples were taken at the time points indicated and the viable count was determined. Antibodies against the divalent or the trivalent vaccines showed no bactericidal activity against mucoid *Pseudomonas* strains (A) DM125 and (B) DM126, whereas a significant killing effect was observed against nonmucoid strains (C) PAO1 and (D) PAK. Results are means ($\pm$ standard error of the mean) of triplicates. * $p < 0.05$; ** $p < 0.01$.

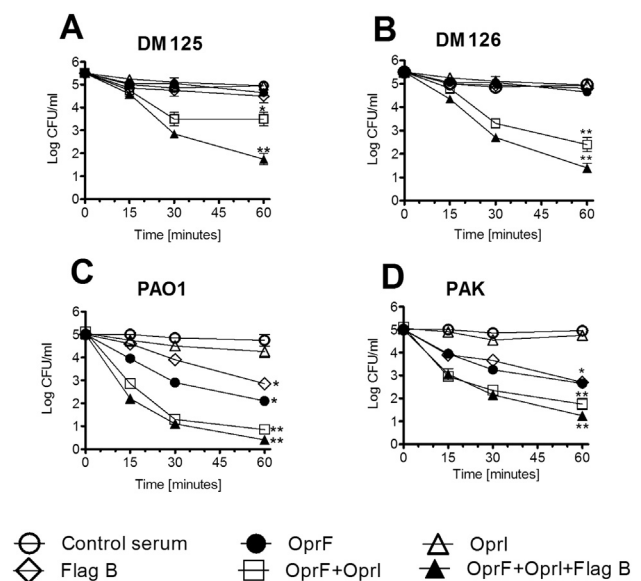


Figure 2. Opsonization with antibodies against OprF, OprI and flagellin B enhance phagocytosis of mucoid and nonmucoid strains of *Pseudomonas aeruginosa* by polymorphonuclear leukocytes (PMNs). Samples were taken from the *Pseudomonas aeruginosa*/PMN mixture at the time points indicated and viable bacteria were determined. Serum from mice immunized with the divalent or the trivalent vaccine significantly enhance opsonophagocytosis of mucoid *Pseudomonas* strains (A) DM125 and (B) DM126 by PMNs, whereas immune serum from mice immunized with OprF, flagellin B, the divalent or the trivalent vaccine efficiently opsonized the bacteria, facilitating killing of nonmucoid *Pseudomonas* strains (C) PAO1 and (D) PAK by PMNs. Controls were run in parallel containing bacteria opsonized with nonimmunized wild serum. Results are means ($\pm$ standard error of the mean) of triplicates. * $p < 0.05$; ** $p < 0.01$.

Immunization with trivalent vaccine enhanced survival of mice after infection with either mucoid or nonmucoid *P. aeruginosa* strains

The survival analysis showed that mice vaccinated with recombinant OprF were protected after infection with PAO1 ($p < 0.01$) or PAK ($p = 0.0372$; Figure 3A). The development of bacteremia after infection with PAO1 or PAK was significantly lower than in control nonimmunized infected mice (Figure 3B). Immunization of mice with OprI alone failed to protect mice from infection either by PAO1 or PAK strains (Figure 3C and D).

Mice immunized with recombinant flagellin B were significantly protected after infection with PAO1 strain ($p = 0.0134$) or PAK strain ($p = 0.041$). Bacteremia was significantly higher in control mice and increased consistently during the first 48 hours, at which point the experiment was stopped (Figure 3E and F). Although immunization of mice with a single recombinant vaccine (OprF or flagellin B) results in a significant protection against infection caused by nonmucoid *Pseudomonas* strains PAO1, this single vaccine strategy was not encouraging as the level of protection from infection caused by

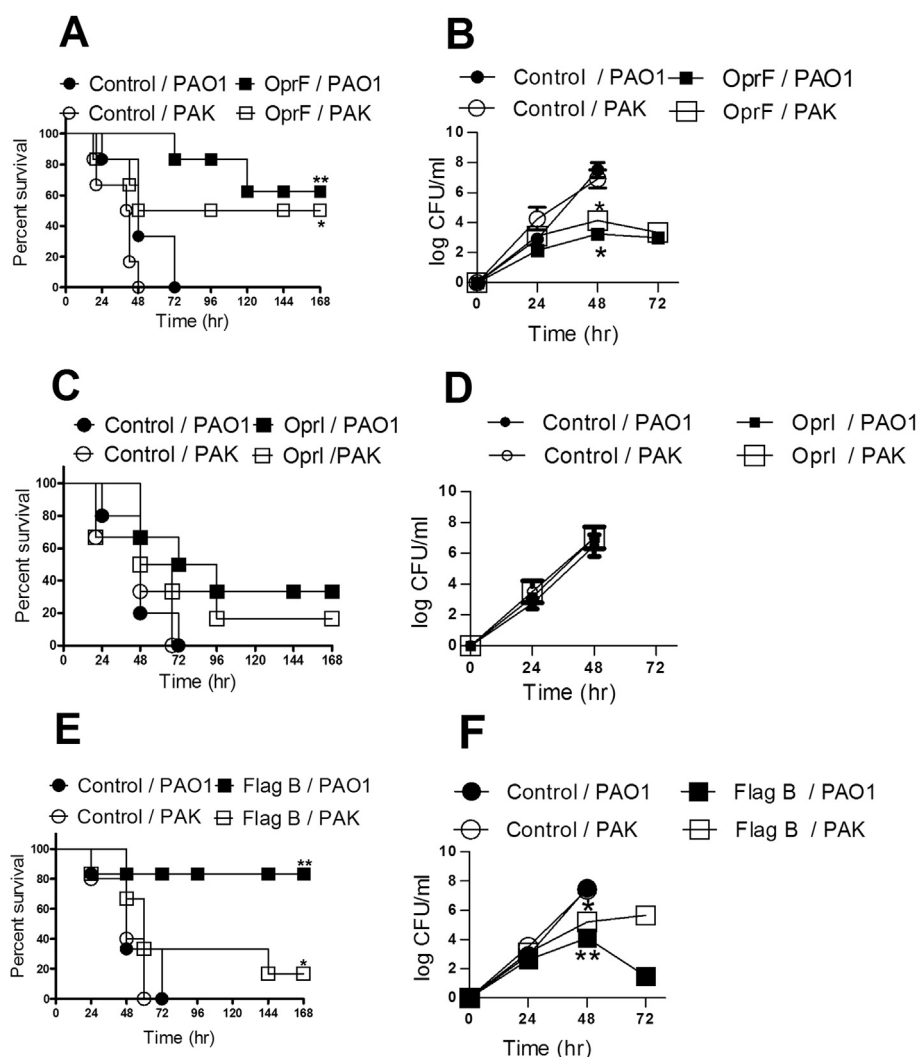


Figure 3. Immunization of mice with OprF or flagellin B, but not OprI significantly protects mice after infection with nonmucoid pseudomonas strains PAO1 or PAK. Immunization with OprF enhances survival time and protects mice from bacteremia caused by PAO1 and PAK strain (A, B). Immunization with OprI did not protect mice against infection caused by PAO1 or PAK (C, D). Immunization with flagellin B protects mice from infection caused by PAO1 and, to a lesser extent, from PAK infection (E, F). Flagellin B prolongs the survival of mice after infection with PAK. * $p < 0.05$; ** $p < 0.01$ (Mantel–Cox log-rank test; $n = 12/\text{group}$).

nonmucoid PAK was not satisfactory and hence single vaccine protocol was not tested against mucoid pseudomonas strains.

Immunization of mice with the divalent or the trivalent vaccines showed a significantly lower bacterial load in blood 24 hours and 48 hours postinfection compared to nonimmunized mice. In addition, the recombinant vaccines significantly increased the survival of mice following infection with mucoid pseudomonas strains (Figure 4A–D). As expected, the divalent and trivalent vaccines showed a significantly higher degree of protection against nonmucoid pseudomonas strains PAO1 ($p < 0.001$) and PAK ($p < 0.01$) with significantly less bacterial load in blood at 24 hours and 48 hours postinfection when compared with the control nonimmunized groups (Figure 4E–H).

Immunization with the divalent or the trivalent vaccines reduced lung tissues damage in mice after infection with mucoid and nonmucoid strains of *P. aeruginosa*

Histological analysis of lung tissue sections from immunized and nonimmunized mice showed pathological and histological differences after infection with mucoid and nonmucoid *P. aeruginosa*. Two days postinfection, lungs from control mice showed heavy leukocytes, infiltration compared to the lungs from immunized mice. Bronchiole wall thickening was increased, and solid fibrous tissue and exudates filling the bronchioles and alveolar spaces had appeared. Additionally, cellular infiltration at this time point was increased, with infiltration of inflammatory cells

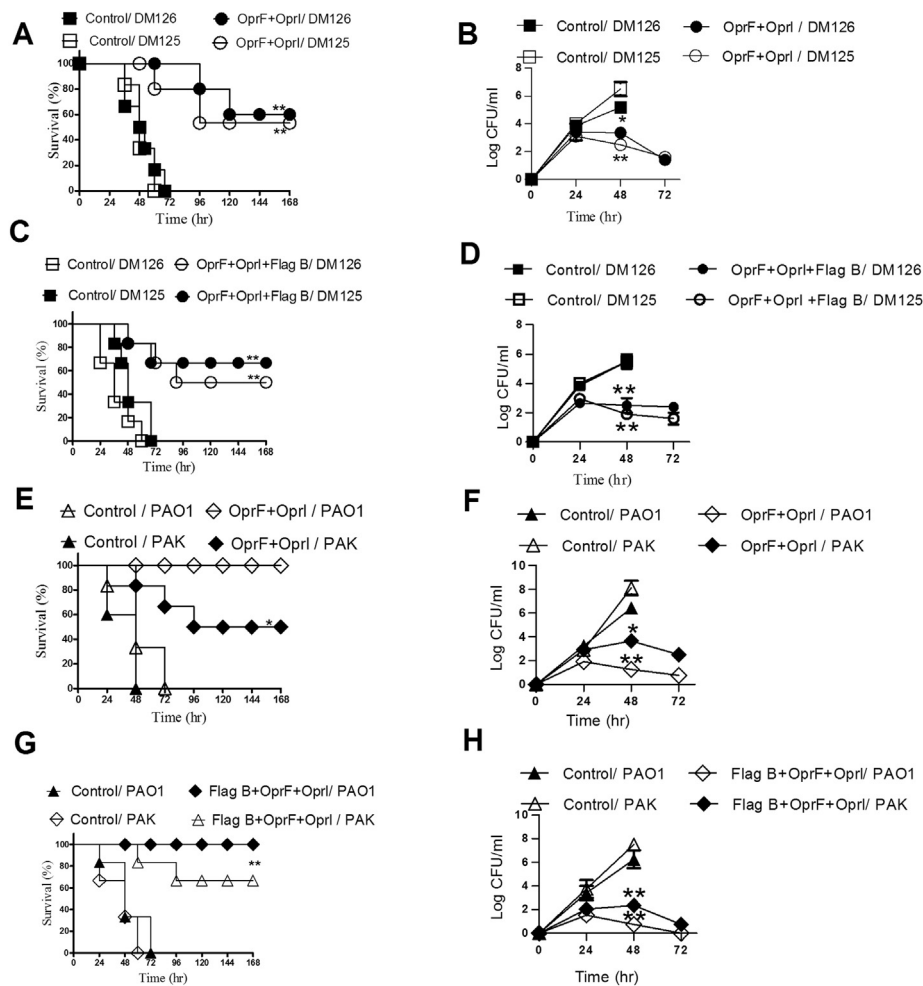


Figure 4. Immunization with divalent or trivalent vaccine increases the survival time and protects mice from bacteremia caused by intranasal infection with mucoid and nonmucoid *Pseudomonas aeruginosa* strains. Mice were infected intranasally with 1×10^7 colony-forming units of *Pseudomonas aeruginosa* (mucoid or nonmucoid). Immunized mice showed a significantly improved survival time after infection with (A, C) mucoid or (E, G) nonmucoid *Pseudomonas aeruginosa* when compared to control nonimmunized mice (Mantel–Cox log-rank test; $n = 12$ /group). (B, D, F, H) Viable *P. aeruginosa* count in peripheral blood at the indicated time points after infection (means $\pm$ standard error of the mean; $n = 12$). * $p < 0.05$; ** $p < 0.01$.

from bronchioles and perivascular areas into the surrounding lung parenchyma with several focal areas of consolidation becoming larger and more diffuse. In contrast, lungs from immunized mice (either with the divalent or the trivalent vaccine) exhibited a limited infiltration of the inflammatory cells into the lung bronchioles and lung parenchyma. This limitation was associated with a lower level of pathological tissue damage. The reduced pathological changes in the lungs of immunized mice were observed in the lungs from mice infected either with mucoid or nonmucoid strains of *P. aeruginosa* (Figure 5).

Discussion

P. aeruginosa is a leading cause of life-threatening infections in immunocompromised, CF, and burn patients. It is an etiological agent of bacteremia, urinary tract infections, and pneumonia.^{20,21} The present study aims to test the effect of divalent (OprF and OprI) or trivalent

vaccines (OprF, OprI, and flagellin B) against lung infection caused by mucoid and nonmucoid *Pseudomonas aeruginosa* strains.

Complement mediated lysis has been reported to play a major role in clearance of Gram-negative bacteria including *P. aeruginosa*.²² Some *Pseudomonas* strains have been reported to resist complement lysis and identified as serum-resistant.²³ Our results showed that mucoid strains were highly resistant to serum lysis compared to nonmucoid strains after opsonization with antibodies against OprF, OprI, and flagellin B (Figure 1). This could be explained by the presence of long lipopolysaccharide O side chain on the surface of mucoid strains, which interferes with the deposition of C3b and C9 on the bacterial surface and subsequent insertion of C9 into the outer membrane of mucoid *Pseudomonas* strains. Absence of that lipopolysaccharide O chain in nonmucoid *Pseudomonas* facilitates complement deposition and increases the susceptibility of *Pseudomonas* to complement-mediated lysis.²³

Opsonization of *P. aeruginosa* with antigen specific antisera against OprF/OprI or against OprF/OprI/flagellin B

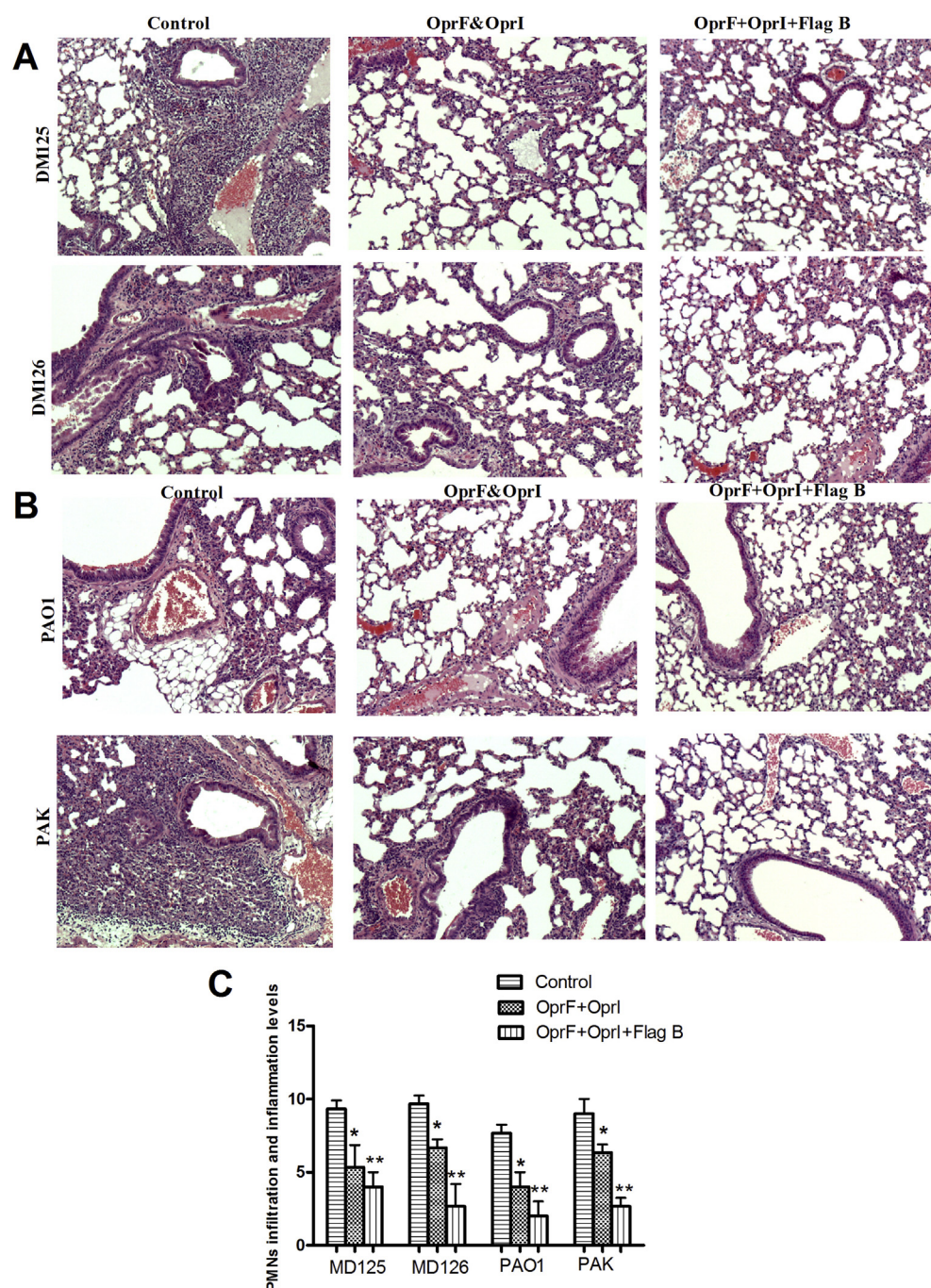


Figure 5. Lung sections from immunized mice with the divalent or the trivalent vaccine showed a significant decrease in leukocytes infiltration and less tissue damage 48 hours postinfection with mucoid or nonmucoid *Pseudomonas aeruginosa* strains. Lung sections (7 μ m) from infected mice (immunized and nonimmunized) were stained by hematoxylin and eosin stain. (A) Lung sections from mice infected with mucoid strains DM125 and DM126. (B) Lung sections from mice infected with nonmucoid strains PAO1 and PAK. Heavy leukocyte infiltration and sever pulmonary edema was observed in lungs from control nonimmunized mice, whereas immunized mice showed a better outcome and significant improvement in lung pathology with less pronounced tissue exudates and significantly less leukocyte infiltration into the lung tissues after infection with mucoid or nonmucoid *P. aeruginosa* strains. (C) The levels of PMN infiltration and the lung inflammation were scored blindly and the results were calculated as means ($\pm$ standard error of the mean) of triplicates. * $p < 0.05$; ** $p < 0.01$.

showed a significant enhanced phagocytosis of mucoid and nonmucoid *P. aeruginosa* strains (Figure 2). This finding was in agreement with a previous observation that opsonization of mucoid and nonmucoid pseudomonas by serum from CF patients has a significant high opsonophagocytosis levels by

PMNs when compared to serum from normal volunteers.²⁴ This high level of opsonic activity with subsequent phagocytosis was attributed to the higher antibody titer against pseudomonas in serum from CF patients compared to normal individuals.²⁴

The previous findings showed that both opsonophagocytosis and, to some extent, complement mediate lysis of pseudomonas strains are two effective mechanisms that work synergistically to clear mucoid and nonmucoid strains after infection. To confirm this, an *in vivo* mouse model of pseudomonas infection was established and the effect of our recombinant divalent and trivalent vaccines was tested against mucoid and nonmucoid *P. aeruginosa*.

The protective efficacy of outer membrane proteins against *P. aeruginosa* infection has been shown in various animal models and in clinical trials.^{25–28} Our results showed that mice immunized with OprF were significantly protected from death from acute pneumonia caused by nonmucoid strains with a significant decrease in bacterial blood count (Figure 3A and B). Vaccination with OprF alone was not protective against infection caused by mucoid strains. The produced anti-OprF antibodies effectively opsonized nonmucoid pseudomonas strains with subsequent activation of serum mediated lysis and opsonophagocytosis where mucoid strains were resistant to lysis. Although anti-OprI antibodies were detected in the sera of mice immunized with OprI alone, the protection afforded by this antigen was minimal where no significant difference was observed between immunized and control groups, indicating that immunization with OprI has no protective effect against infection caused by mucoid or nonmucoid pseudomonas strains (Figure 3C and D). The degree of protection achieved by vaccination with OprI alone is limited since the total copy number of OprI exposed at the bacterial surface is considerably low and hence promoting the binding of fewer antibody molecules to the bacterial surface.²⁹ Vaccination with OprF/OprI was protective against mucoid DM125, 126 infection. The combined vaccine showed a significant protective effect (Figure 4A and B). This protection could be explained by synergistic effect of both antibodies that binds to different epitopes on the surface of the bacteria.³⁰

Vaccines consisting of a mixture of different outer membrane proteins or fusion components of these proteins could increase the degree of protection against pseudomonas infection.³¹ Addition of flagellin B as an adjuvant to vaccine cocktail elicits activation of the innate immune response via TLR5 that enhances protective inflammatory response and enhance recruitment of PMNs and facilitate pseudomonas clearance post infection.³² Immunization of mice with flagellin B alone showed a significant protection against infection caused by nonmucoid strain (PAO1 and PAK) but not against infection caused by mucoid strains when compared to control groups (Figure 3E and F). These results were consistent with several other studies that reported the protective effect of flagellin B vaccine against infection caused by nonmucoid pseudomonas strains.^{9,33} Although PAK strain has type A flagellin, subcutaneous immunization of mice with flagellin B significantly enhanced the survival time of these mice after intranasal infection with PAK. Type A and B flagellin amino acid sequences showed 63–65% homology and so the raised antibodies against type B flagellin are predicted to cross react with flagellin A.¹⁵ Campodónico et al³³ have shown a similar phenotype when mice were immunized with type A flagellin, where a significant degree of protection was observed after infection with *P. aeruginosa* PAO1 strain that has type B flagellin.

In 2009, Weimer et al⁹ assessed a fusion protein consists of OprF_{190–342}, OprI, type A and type B flagellins in mouse model of pseudomonas infection. In line with our results, Weimer et al⁹ found that this vaccine enhanced clearance of *P. aeruginosa* from lung of immunized mice. These results were consistent with several other studies showing the protective effect of the combined vaccine against only nonmucoid pseudomonas strains and failed to enhance complement mediate lysis of mucoid strains of *P. aeruginosa* *in vitro*.^{29,31} Weimer et al⁹ also reported that immunization with their vaccine elicited high affinity IgG antibodies specific to flagellin, OprI and OprF. They added that this combination exhibited a high degree of functional activity that trigger the highest level of C₃ deposition and subsequent complement-mediated lysis of nonmucoid *P. aeruginosa*. In addition, they reported that the produced antibodies failed to enhance complement mediate lysis of mucoid strains of *P. aeruginosa* *in vitro* and hence they concluded that their fusion vaccine would have no effect in clearance of mucoid pseudomonas strains *in vivo*. Interestingly, the produced antibodies against our cocktail vaccine (OprF/OprI) or (OprF/OprI/flagellin B) successfully opsonized mucoid pseudomonas strains and enhanced complement-mediated phagocytosis via PMNs *in vitro* (an observation that was not detected by Weimer et al).⁹ In addition, mice immunized with the cocktail vaccines, showed a significant longer survival time and a significant lower bacterial load in blood when compared to nonimmunized mice (Figures 4 and 5). Mucoid pseudomonas strains are distinguished by accumulation of exopolysaccharide alginates to form the mucoid layer around the bacterium.³⁴ Once infection is established the alginate biosynthesis gene increases alginate production that protects pseudomonas from phagocytosis.⁸ Previous study reported that pseudomonas starts to produce the mucoid layer shortly post infection and the thickness of the mucoid layer increases by time and this achieve an effective mechanism against opsonisation by antibodies against surface exposed antigens.³⁵ Induction of high antibodies titers via immunization against pseudomonas surface antigens (OprF, OprI, and flagellins) increases the level of opsonization and enhances bacteria killing via phagocytosis. Opsonization at early stages of infection with mucoid pseudomonas strains before the formation of well-established mucoid layer enhances opsonophagocytosis and clearance of mucoid pseudomonas strains.

In conclusion, immunization with outer membrane proteins (OprF and OprI) in presence of flagellins enhances killing of nonmucoid pseudomonas strains via serum mediated killing and facilitates clearance of mucoid strains of pseudomonas via opsonophagocytosis in a mouse model of acute lung infection caused by mucoid or nonmucoid *P. aeruginosa* strains.

Conflicts of interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jmii.2016.08.014>.