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Intradermal vaccination with a *Pseudomonas aeruginosa* vaccine adjuvanted with a mutant bacterial ADP-ribosylating enterotoxin protects against acute pneumonia

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ABSTRACT

Respiratory infections are a leading cause of morbidity and mortality globally. This is partially due to a lack of effective vaccines and a clear understanding of how vaccination route and formulation influence protective immunity in mucosal tissues such as the lung. *Pseudomonas aeruginosa* is an opportunistic pathogen capable of causing acute pulmonary infections and is a leading cause of hospital-acquired and ventilator-associated pneumonia. With multidrug-resistant *P. aeruginosa* infections on the rise, the need for a vaccine against this pathogen is critical. Growing evidence suggests that a successful *P. aeruginosa* vaccine may require mucosal antibody and Th1- and Th17-type $CD4^+$ T cells to prevent pulmonary infection. Intradermal immunization with adjuvants, such as the bacterial ADP-Ribosylating Enterotoxin Adjuvant (BARE) double mutant of *E. coli* heat-labile toxin (dmLT), can direct protective immune responses to mucosal tissues, including the lungs. We reasoned that intradermal immunization with *P. aeruginosa* outer membrane proteins (OMPs) adjuvanted with dmLT could drive neutralizing antibodies and migration of $CD4^+$ T cells to the lungs and protect against *P. aeruginosa* pneumonia in a murine model. Here we show that mice immunized with OMPs and dmLT had significantly more antigen-specific IgG and Th1- and Th17-type $CD4^+$ memory T cells in the pulmonary environment compared to control groups of mice. Furthermore, OMPs and dmLT immunized mice were significantly protected against an otherwise lethal lung infection. Protection was associated with early IFN-γ and IL-17 production in the lungs of immunized mice. These results indicate that intradermal immunization with dmLT can drive protective immunity to the lung mucosa and may be a viable vaccination strategy for a multitude of respiratory pathogens.

1. Introduction

The mucosal surfaces of the gastrointestinal, respiratory, and genital tracts serve as the main portal of entry for most pathogens. The lack of success in the development of vaccines against some mucosal pathogens such as human immunodeficiency virus, herpes viruses, and various respiratory bacterial infections, may be partly due to the inability of current vaccine strategies to adequately stimulate multiple arms of the innate and adaptive immune systems and to target essential immune responses to infected mucosal tissues. The paucity of vaccines against pulmonary bacterial pathogens and the emerging multidrug resistance among many of these bacteria highlights the need for rational vaccine design and alternative delivery strategies for eliciting both local and mucosal immunity against this collective group of pathogens.

Pseudomonas aeruginosa is a Gram-negative bacterium and a significant human pathogen, capable of causing infections of the respiratory tract, urinary tract, skin and soft tissues, eyes, and ears, with infections occurring primarily in those patients with physical, phagocytic, or immunologic defects in host defense mechanisms. As a nosocomial pathogen, *P. aeruginosa* poses an enormous burden on the health care system and is responsible for 17% of ventilator associated pneumonias [1] and 9% of other healthcare associated pneumonias [2]. Importantly, infection with *P. aeruginosa* is emerging as a healthcare crisis, with 6700 multidrug resistant infections occurring in the United States annually [3]. Multidrug resistance severely limits the choice and efficacy of antibiotic treatment regimens against *P. aeruginosa*. A prophylactic vaccine that could

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prevent infection with drug-resistant *P. aeruginosa*, particularly in the lung, would have a profound impact on reducing human morbidity and mortality worldwide.

Growing clinical and experimental evidence suggests that a successful *P. aeruginosa* vaccine must elicit antibodies combined with both Th1- and Th17-type $CD4^+$ T cell responses to provide protection against pulmonary infection [4–9]. Intradermal immunization is an attractive option for directing vaccine-induced immune responses to the mucosa because (1) the dense network of lymphatic vessels located within the dermis facilitates vaccine antigen migration to the nearby draining lymph nodes [10] and (2) T cells activated in the relevant draining lymph nodes can exhibit a migratory preference for mucosal tissues, as effector and memory T cells exhibit distinct tissue tropism depending on adjuvant selection [10–12]. To this effect, recent work by our group suggests that vaccine-induced mucosal immune responses can be achieved through dermal immunization with a vaccine containing the bacterial ADP-ribosylating enterotoxin (BARE) double mutant of *E. coli* heat-labile toxin (dmLT) adjuvant

[13–17]. Adjuvants are an important component of vaccine formulation, as they initiate the innate immune response, promote the activation and maturation of antigen presenting cells, and promote downstream adaptive immune responses to co-delivered antigens. Depending on their mechanism of action, adjuvants can skew the $CD4^+$ T cell response towards a Th1-, Th2, or Th17-like response. dmLT adjuvant has been shown to induce both antibodies and Th1 and Th17 CD4 T cell responses in the pulmonary milieu and other mucosal tissues and appears to require the engagement of particular dendritic cell subsets, including $CD103^+$ dendritic cells [14,18].

Despite the emerging immunological benefits to intradermal immunization, the majority of previous *P. aeruginosa* vaccine studies utilized the standard intramuscular route of immunization. Here we investigate the systemic and mucosal immune responses elicited by intradermal immunization with a *P. aeruginosa* vaccine incorporating dmLT adjuvant. We show that inclusion of dmLT helps promote *Pseudomonas*-specific antibodies and Th1/Th17 $CD4^+$ T cells in the lungs of immunized mice. We further show that intradermal immunization with the dmLT-adjuvanted vaccine provides significant protection against an otherwise rapidly lethal lung infection with *P. aeruginosa*. Finally, we demonstrate that protected mice display an early Th1- and Th17-type immune response in the lung following infection.

2. Methods

2.1. Ethics statement

This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publications No. 8023, revised 1978). The protocols were approved by the Tulane University Institutional Animal Care and Use Committee. For survival studies, death was not used an as endpoint. Mice were humanely euthanized once they displayed >20% weight loss, paralysis, or were unresponsive to handling. Mice were observed at least three times daily, including weekends. Euthanasia was performed in mice by CO_2 overdose and confirmed by cervical dislocation.

2.2. Bacterial strain

The PAO1 strain of *P. aeruginosa* was used for all studies. PAO1 is a derivative of the PAO clinical isolate and is a commonly used strain for *P. aeruginosa* research. To prepare *P. aeruginosa* for mouse challenge experiments, a single colony of PAO1 was inoculated into 5 ml Tryptic Soy Broth (TSB, Fluka Analytical) and incubated overnight shaking at 233 rpm at 37 °C. The optical density (OD) was then measured and an OD_{600} of 2.0 was added to 20 ml TSB. The culture was incubated for 4 h shaking at 233 rpm at 37 °C until an OD_{600} of 3.5–4.0 was reached. The bacteria were pelleted and resuspended in PBS. Bacterial concentration was adjusted to obtain a concentration of 2.8×10^8 CFU/ml.

To prepare heat-inactivated bacteria, two primary overnight cultures of *P. aeruginosa* in 5 ml LB each were grown to confluency and two secondary overnight cultures were made, diluting the primary culture 1:10 in 50 ml of LB. After overnight incubation at 37 °C with shaking at 233 rpm, the optical density of these two 50 ml was assessed and the bacterial concentration was confirmed by plating for colony forming units (CFU) on *Pseudomonas* Isolation Agar (PIA). The bacterial cultures were combined and centrifuged at $9000 \times g$ for 10 min. The bacterial pellet was resuspended in 5 ml dH2O and heat-inactivated for four hours at 60 C. After inactivation, 10% of the bacterial suspension was plated on PIA to confirm killing. A Bradford assay was used to determine protein concentration after inactivation, and heat-inactivated bacteria were stored at −20 °C until use.

2.3. Outer membrane protein extraction

Outer membrane proteins (OMPs) were isolated from PAO1 using the method described by Hancock [19] with the following modifications. *P. aeruginosa* was grown in LB and bacteria were pelleted by centrifugation for 10 min at $8000 \times g$ at 4 °C in a RC 5C Plus centrifuge (Sorvall) with an SLA-1500 rotor and resuspended in cold 20% sucrose in 10 mM Tris (pH 8.0), then frozen in −20 °C until processing. The bacteria were passed through a Microfluidizer ® (Microfluidics) with a H102 100 μm attachment once to break the cells and then centrifuged at $1800 \times g$ for 10 min at 4 °C to remove cell debris. A two-step sucrose gradient with 70% and 50% sucrose in 10 mM Tris was used to produce a crude separation of the inner and outer membranes. To do so, the sucrose gradient was centrifuged overnight at $41{,}474 \times g$ with a SW28 rotor (Beckman) in an Optima XL-100K Ultracentrifuge (Beckman Coulter). The outer membrane protein fraction was carefully removed, and this fraction was pelleted by centrifugation for 1 h at $221{,}824 \times g$ in a 50.2 Ti rotor (Beckman) in Optima XL-100K Ultracentrifuge (Beckman Coulter). The pellet was resuspended in HyClone water. Protein concentration was determined by Bradford assay. OMPs were separated by SDS-PAGE and stained with Coomassie blue to visualize protein content and to assess continuity across preparations (Fig. 1a).

2.4. Mouse immunization experiments

Six to twelve-week-old C57BL/6 mice were purchased from Charles River (Wilmington, MA). Mice were housed in microisolators with specific-pathogen free conditions. Food and water were available *ad libitum*. Groups of mice were immunized intradermally with 1 μg *P. aeruginosa* OMPs resuspended in HyClone™ water (GE Life Sciences) with 1 μg dmLT diluted in HyClone™ water, 1 μg *P. aeruginosa* OMPs alone, 1 μg dmLT alone, or saline (0.9% NaCl, Braun Medical) in a total volume of 50 μl. Vaccines were administered three times, two weeks apart.

2.5. Western blot

Ten (10) μg *P. aeruginosa* OMPs were separated on a 4–20% SDS-PAGE gel (BioRad) alongside a protein molecular weight ladder (BioRad). After separation, the proteins were transferred from the gel to a nitrocellulose membrane (iBLOT) using the iBLOT system per manufacturer instructions. The membrane was then blocked

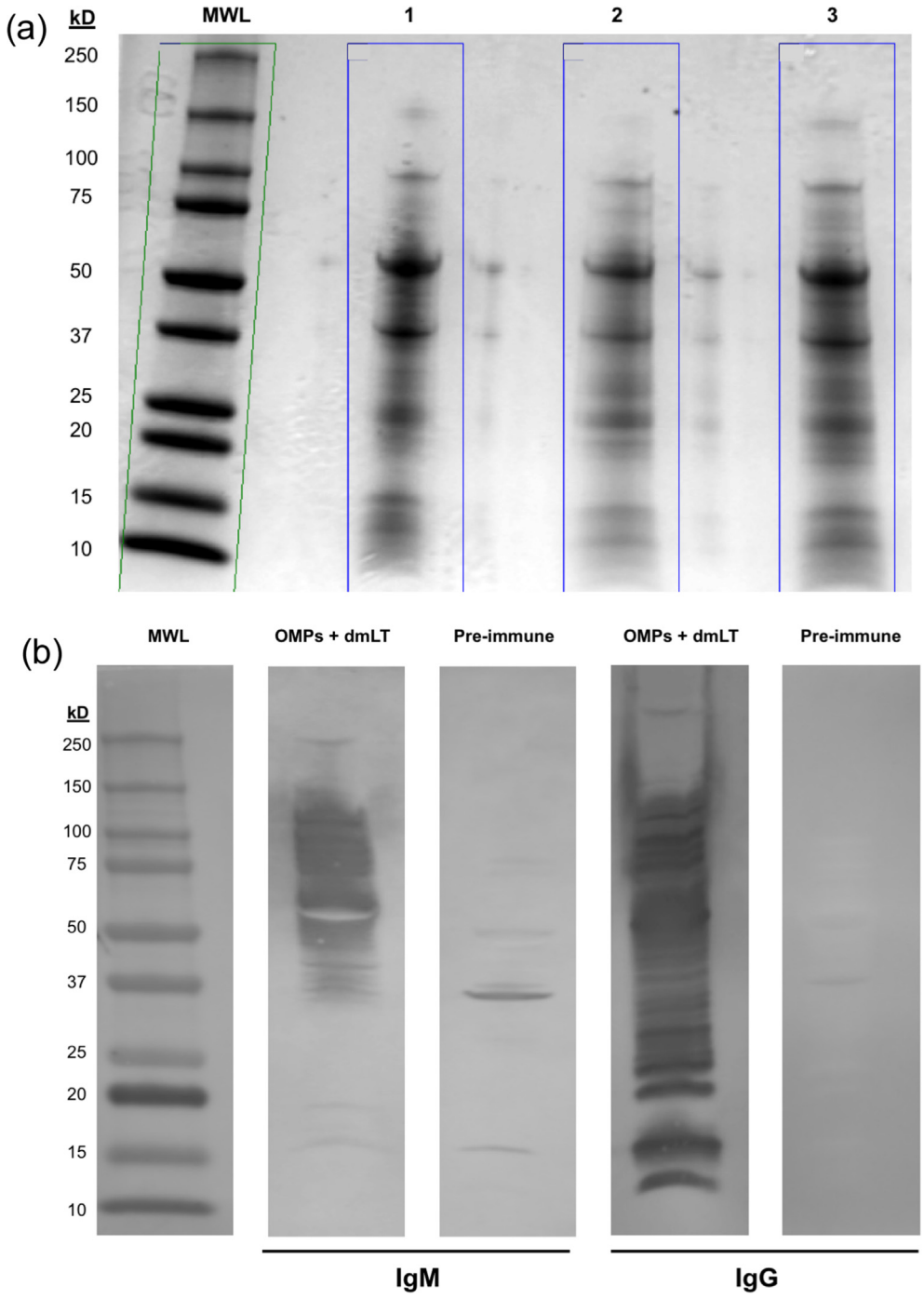

Fig. 1. *P. aeruginosa* outer membrane protein (OMPs) preparations contain numerous proteins that are highly immunogenic. (a) Three independent batches of OMPs were prepared from *P. aeruginosa* strain PAO1 to demonstrate reproducibility of the preparations. OMPs (1–3) were separated by SDS-PAGE and stained with Coomassie blue. (b) Ten micrograms of *P. aeruginosa* OMPs were probed using pre-immune sera or sera pooled from mice immunized intradermally with *P. aeruginosa* OMPs + dmLT. Lanes 2–3 were probed with anti-mouse IgM and lanes 4–5 were probed with anti-mouse IgG. MWL = molecular weight ladder. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in 1.5% bovine serum albumin (BSA) in tris-buffered saline with 1% Tween-20 (TBS-T) for 1 h, then washed and incubated overnight at 4 °C with pooled pre-immune sera or sera from mice immunized with *P. aeruginosa* OMPs and dmLT. Sera were diluted 1:200 for IgG Western Blots and 1:100 for IgM Western blots. The following day, membranes were washed and incubated for 1 h with goat anti-mouse IgM (AbCam) diluted 1:2000 in TBS-T or goat anti-mouse IgG (BioRad) diluted 1:3000 in TBS-T. Membranes were washed and developed with the Opti-4CN kit (BioRad) according to manufacturer instructions and imaged using a GE AI600 RBG Imager.

2.6. Analysis of antibody responses

Two weeks after the final immunization, mice were sacrificed by CO_2 asphyxiation. Blood was collected by cardiac puncture, centrifuged in serum separator tube (BD) at 7000 × *g* for 10 min, and serum was collected and kept at −80 °C until analysis. To collect

bronchoalveolar lavage fluid (BAL), the mouse trachea was exposed, and a tracheostomy was performed. A luer stub adaptor (BD) attached to a 1 ml syringe (Fisher) was filled with cold Complete Protease Inhibitor (PI) Cocktail (Roche) and inserted into the trachea that was then tied with thread. The lungs were washed three times with the Complete PI Cocktail and the collected BAL fluid was kept at −80 °C until analysis. Enzyme-linked Immunosorbent Assays (ELISA) were performed to determine antigen-specific IgG concentrations in immunized mice. To perform the ELISAs, flat bottom 96-well polystyrene plates (Costar) were coated overnight at 4 °C with heat-inactivated *P. aeruginosa* at a concentration of 1 μg/well in coating buffer or with purified mouse standards (IgG1, Sigma or IgA, Sigma). Plates were then washed three times with PBS + 0.5% Tween 20, hereafter referred to as PBS-T. Blocking buffer with 2% skim milk powder in PBS-T was added to the wells for 1 h. Wells were then washed three times with PBS-T and samples were added in a volume of 100 μl/ well. Samples were diluted serially in a dilution buffer of 0.2% skim milk powder with PBS-T. Serum and BAL samples were added to the coated plates, and samples were incubated for 1 h at room temperature. Plates were washed 3 times with PBS-T. Detection by IgG ELISAs was performed using AKP-conjugated rabbit anti- mouse IgG (Sigma) diluted 1:300 in dilution buffer. Detection by IgA ELISAs was performed using HRP-conjugated goat anti- mouse IgA (Abcam) diluted 1:500 in dilution buffer. Secondary antibodies were added at a volume of 100 μl/ well and incubated for 1 h at room temperature. Plates were then washed 5 times with PBS-T. For detection of IgG, p-nitro-phenyl-phosphate (Sigma) was dissolved in diethanolamine buffer at a concentration of 1 mg/ml and 100 μl of this solution was added to the wells. After development of the assay, the reaction was stopped using 50 μl/well 2 M NaOH. Plates were read immediately at 405 nm to determine optical density (OD). For the detection of IgA, TMB Substrate kit (KPL) was used according to the manufacturer's instructions and the reaction was allowed to occur for 2.5 min before it was stopped using 1M H3PO4. Plates were read immediately at 450 nm to determine OD. Results were expressed as ELISA units/ml (EU/ml) using an average of three sample dilutions closest to the midpoint of the standard curve. Samples in which the OD readings were less than 3 standard deviations above the reading of the blank were considered to be non-responders.

2.7. Analysis of T cell responses

Lungs were harvested from a subset of mice (n = 3 per group) two weeks after the final immunization. Mice were sacrificed by CO_2 asphyxiation and lungs were excised to prepare single cell suspensions as follows. The excised lungs were cut into pieces approximately 1–2 mm in size and placed in 5% RPMI with 0.2 Wünsch units/ml LiberaseTM (Roche) then incubated for 1 h at 37 °C in a shaking incubator at 233 rotations per minute. After incubation, lungs were placed on a 70 μm nylon cell strainer (Fisher) and homogenized with a rubber syringe plunger from a 5 ml syringe (Fisher). The screen was intermittently rinsed with RMPI (Gibco) containing 1% fetal bovine serum (FBS, Atlanta Biologicals). The cell suspension was centrifuged at 460 × *g* for 10 min at 4 °C. Supernatant was decanted and the cells were resuspended in 2 ml ACK red blood cell lysis buffer (Invitrogen) and incubated at room temperature for 3 min with occasional shaking. To stop the reaction, 20 ml of RMPI (Gibco) containing 10% fetal bovine serum (FBS, Atlanta Biologicals), hereafter referred to as 10% RPMI, was added to the cells. Cells were then centrifuged at 300 × *g* for 10 min, supernatant was decanted, and the cells were resuspended in 5 ml 10% RPMI. The viable cells were counted on a Cellometer (Nexcelom Bioscience) using Trypan Blue (Sigma) then centrifuged at 300 × *g* for 10 min and resuspended in a final volume of 1×10^6 cells/ml.

For the restimulation assay, the following were added to the wells containing 10% RPMI in a volume of 100 μl: 2 μg/ml anti-CD28 antibody, 2 μg/ml anti-CD28 antibody plus 1 μg/ml heat-inactivated *P. aeruginosa*, or 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 μg/ml Ionomycin (Sigma) as a positive control. Cells were incubated at 37 °C for 2 h, then treated with Golgi Plug (BD) according to manufacturer's instructions and incubated for an additional 6 h at 37 °C. Cells were then centrifuged for 5 min at 300 × *g*, washed with 200 μl phosphate buffered saline (PBS) and resuspended in 50 μl of sorter buffer with 5 μl/ml Fc Block (anti-CD16/CD32, BD) and incubated for 10 min. Cells were then stained for viability, and the expression of CD3, CD4, and CD44 on the cell surface for 20 min in the dark at room temperature (See Supplemental Table 1 for details on flow antibodies). Stained cells were then washed twice with sorter buffer, fixed and permeabilized using 100 μl/well of Cytofix/Cytoperm Solution (BD). After 20 min, cells were washed twice with Perm/Wash buffer (BD) and resuspended in antibodies to stain for intracellular IFN-γ and IL-17. After an overnight incubation in the dark at 4 °C, cells were washed twice with Perm/Wash buffer (BD), resuspended in sorter buffer, and stored at 4 °C. Prior to flow cytometry collection, all samples were filtered through a 100 μm nylon mesh filter. Samples were collected using a BD Biosciences Fortessa cytometer and data were analyzed in FlowJo (TreeStar). Cells were gated using granularity to include lymphocytes, then by size to include only single cells. Cells were then assessed for viability, then expression of CD3 on the surface and exclusion of B220, CD11b, CD11c, CD19, F4/80, and NK1.1 on the surface to confirm T cells. The CD3+ B220- CD11b-CD11c- CD19- F4/80- NK1.1- T cell population was then gated to include $CD4^+$ T cells. $CD4^+$ T cells were then gated to include those antigen-experienced ($CD44^+$) cells producing the cytokines IFN-γ, IL-4, or IL-17A. All gating was done using a fluorescence minus one (FMO) technique to confirm negative and positive populations. Representative cytokine flow plots are depicted in Supplemental Fig. 1.

2.8. Mouse challenge experiments

Two weeks after the final immunization, mice were administered *P. aeruginosa* directly to the lungs to induce an acute pneumonia infection. A method of oropharyngeal aspiration was used to administer bacterial inocula to mice [20]. Mice were anesthetized with 1–5% isoflurane gas in oxygen continuously. Once fully anesthetized, mice were suspended vertically by the upper incisors on a nylon filament. The nares were pinched with curved forceps and the tongue was gently extracted from the mouth and displaced using blunt forceps. The base of the tongue and pharynx was visualized. A 50 μl suspension of bacteria containing approximately 10^7 CFU was placed in the posterior pharynx with a micropipetter. The mouse was allowed to aspirate the bacteria for 15 breaths while respiration was monitored to ensure the suspension was fully aspirated. The tongue and nares were released, and mice were allowed to recover in hand, held in a vertical position until fully awake. Mice were closely monitored for 10 days and humanely sacrificed if they reached a moribund state (ruffled fur, shaking, and loss of mobility). At day 10 survivors were euthanized and tissues (blood, lung, spleen) were harvested and homogenized, then plated to determine bacterial cfu. Three independent challenge experiments were performed with an average challenge dose of 1.4×10^7 CFU (doses ranged from 7.0×10^6 cfu to 1.6×10^7 cfu).

For lung cytokine analyses, BAL fluid was collected from a subset of challenged mice (n = 5 per group) 24 h after infection and stored at −80 C until analysis. The BAL fluid was evaluated by Luminex assay using a murine 25-plex cytokine kit (Millipore) and read on a Bioplex 200 reader (BioRad). Cytokine concentra-

tions, determined as pg/ml, were standardized by BAL fluid protein content, and presented as pg/mg protein.

2.9. Statistics

All data were analyzed in GraphPad Prism version 6. Statistical significance was determined using a one way ANOVA with Tukey's multiple comparisons test, a Two-tailed *t* test, or log rank Mantel-Cox test as indicated in the figure legends. Statistical significance is indicated as ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001.

3. Results

3.1. Intradermal immunization with P. aeruginosa outer membrane proteins (OMPs) and dmLT promotes anti-pseudomonal IgG in the lung

Given that dmLT can promote mucosal immune responses to co-delivered antigens, we sought to determine if dmLT could elicit mucosal humoral immune responses against *P. aeruginosa*. Mice were immunized with dmLT adjuvant combined with *P. aeruginosa* OMPs (Fig. 1a and b) three times, 14 days apart. Control groups of mice received OMPs alone or saline (sham). Antigen-specific IgG was measured in the serum and BAL fluid by ELISA two weeks after the final immunization. Immunization with *P. aeruginosa* OMPs plus dmLT induced higher levels of anti-pseudomonal IgG in the serum compared to immunization with OMPs alone ($p = 0.0505$) or sham ($p < 0.01$) (Fig. 2a). Moreover, immunization with *P. aeruginosa* OMPs and dmLT induced the production of significantly more anti-pseudomonal IgG in the pulmonary environment compared to immunization with OMPs alone ($p < 0.05$) or sham ($p < 0.01$) (Fig. 2b). The antibody responses were directed against multiple protein antigens as indicated by Western blot (Fig. 1b). There was no difference in the levels of antigen-specific IgA in serum (Supplemental Fig. 2), and antigen-specific IgA in BAL was undetectable (data not shown). These results demonstrate that intradermal immunization with *P. aeruginosa* antigens plus dmLT adjuvant promotes antigen-specific IgG responses in the blood and lung mucosa.

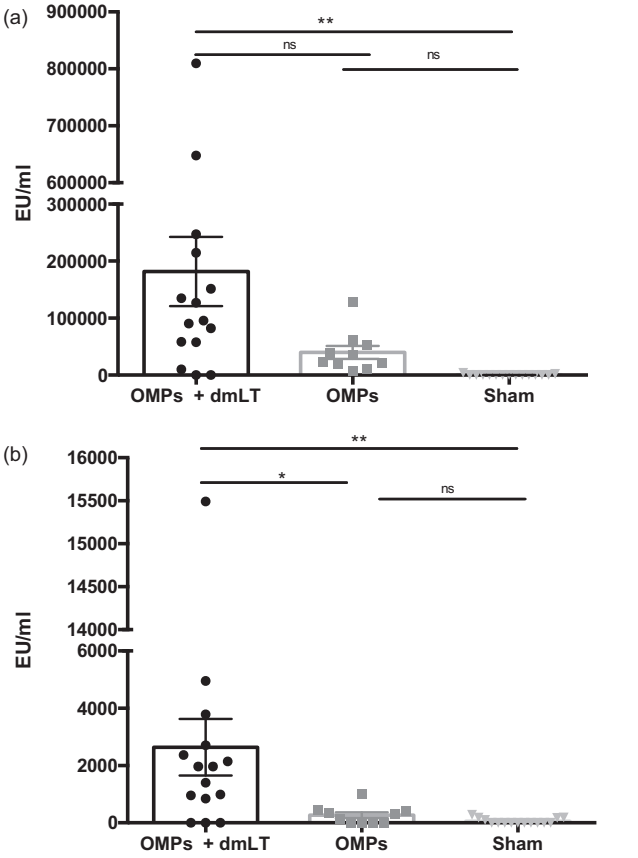

Fig. 2. Intradermal immunization with *P. aeruginosa* OMPs and dmLT elicits anti-pseudomonal IgG in the blood and lung. Mice were immunized with *P. aeruginosa* OMPs + dmLT (n = 15), OMPs alone (n = 10), or sham (n = 10) intradermally. Antigen-specific (a) serum and (b) bronchoalveolar lavage fluid (BAL) IgG were measured by ELISA using microtiter plates coated with heat-inactivated *P. aeruginosa*. Results are expressed as EU (ELISA units) per ml. Data shown is the cumulative result of three independent experiments. ns = not significant, * $p < 0.05$, ** $p < 0.01$ using a one way ANOVA with Tukey's multiple comparisons test.

3.2. Intradermal immunization with P. aeruginosa OMPs and dmLT elicits pulmonary IFN-γ^+ and IL-17$^+$ CD4$^+$ T cells

Given that dmLT increased *Pseudomonas*-specific antibody in the lung, we next examined cellular immune responses elicited by immunization with *P. aeruginosa* OMPs and dmLT to determine if the inclusion of dmLT adjuvant could increase T cell responses in the lungs of immunized mice. CD4$^+$ T cells were harvested from the lungs of mice two weeks after the final immunization, restimulated with heat-inactivated *P. aeruginosa*, and analyzed by intracellular cytokine staining and flow cytometry. Mice immunized with *P. aeruginosa* OMPs plus dmLT possessed a significantly greater percentage of antigen-experienced CD4$^+$T cells in the lung that produced IFN-γ after restimulation compared to mice immunized with OMPs alone ($p < 0.01$) or sham ($p < 0.01$) (Fig. 3a). Mice immunized with *P. aeruginosa* OMPs plus dmLT also had a significantly greater percentage of antigen-experienced CD4$^+$ T cells in the lung that produced IL-17A compared to mice immunized with OMPs alone ($p < 0.05$) or sham ($p < 0.05$) (Fig. 3b). There was no significant difference in the percentage of antigen-experienced IL-4 producing CD4$^+$T cells in immunized mice (Fig. 3c). There was no significant difference in the percentage of lung CD4$^+$ T cells that were CD44$^+$ among the groups (Supplemental Fig. 3). We did not observe any significant differences in the percentage of cytokine-producing antigen-specific CD4$^+$T cells in the spleens of immunized mice (data not shown). These results indicate that intradermal immunization with *P. aeruginosa* OMPs plus dmLT promoted a mixed Th1/Th17-type CD4$^+$ T cell response in the pulmonary tissue itself and that inclusion of dmLT adjuvant was required for achieving this mucosal response.

3.3. Intradermal immunization with P. aeruginosa OMPs and dmLT provides protective immunity against P. aeruginosa

To evaluate the protective efficacy of intradermal immunization with *P. aeruginosa* OMPs and dmLT against subsequent *P. aeruginosa* challenge, mice were immunized as above and then challenged with an average dose of 1.4×10^7 cfu, of *P. aeruginosa* by oropharyngeal aspiration two weeks after the final immunization. Mice injected with *P. aeruginosa* OMPs plus dmLT were significantly protected against an otherwise rapidly lethal lung infection ($p < 0.0001$, Fig. 4a). OMPs plus dmLT immunized mice displayed 53% survival over the 10-day study period, whereas mice immunized with OMPs alone, dmLT alone, or sham completely succumbed to infection within 1–3 days. Immunized mice that survived challenge began recovering after day 3 and steadily regained weight (Fig. 4b). All surviving animals were euthanized at the 10-day study endpoint and blood, lung, and spleen homogenates were plated to evaluate bacterial persistence. *P. aeruginosa* was undetectable in all tissues examined suggesting that surviving mice had cleared the infection (data not shown).

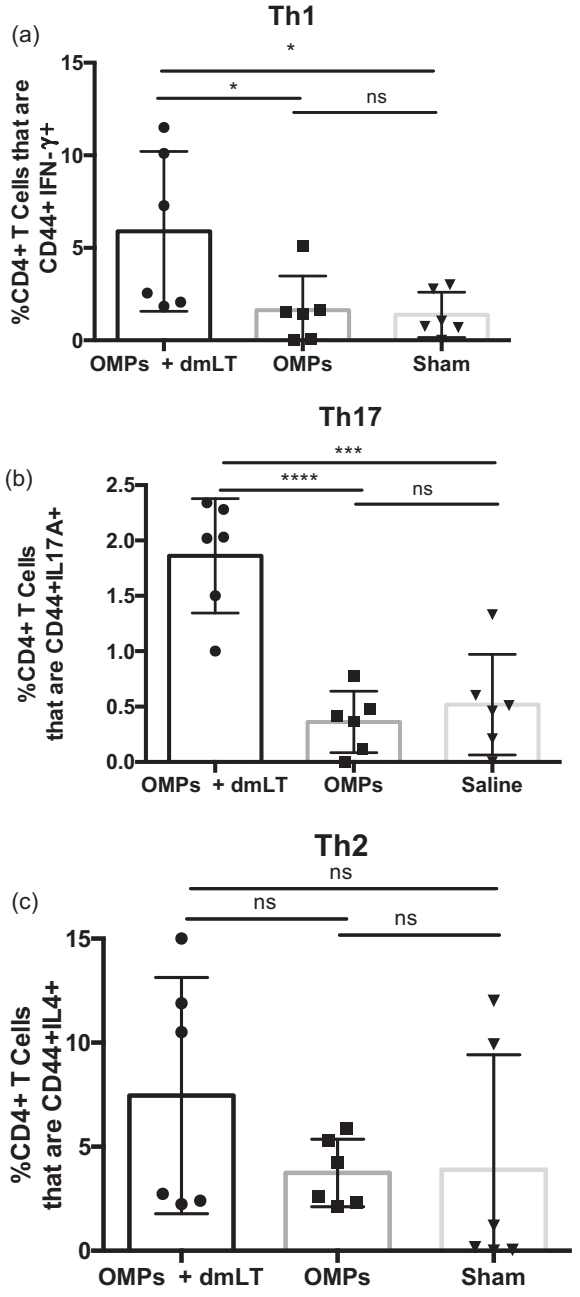

Fig. 3. Intradermal immunization with *P. aeruginosa* OMPs and dmLT elicits Th1- and Th17-type $CD4^+$ T cells in the lung. Mice (n = 6 per group) were immunized with *P. aeruginosa* OMPs + dmLT, *P. aeruginosa* OMPs alone, or saline (sham) intradermally. *P. aeruginosa*-specific CD4 + T cell response was assessed 14 days after final immunization. Lungs were collected, dissociated, and plated into tissue culture dishes. Tissue explants were restimulated with α-CD28 and heat-inactivated *P. aeruginosa* followed by intracellular cytokine staining for (a) IFN-γ (b) IL-17 or (c) IL-4. Percentage of $CD4^+$ T cells that are CD44 + and IFN-γ+, IL-17 + or IL-4 + were analyzed. Results shown are the cumulative data from two independent experiments. ns = not significant, * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA with Tukey's multiple comparisons test.

3.4. Protected mice mount a Th1/Th7-type immune response early during lung infection

Given that intradermal immunization with *P. aeruginosa* OMPs and dmLT elicits pulmonary IFN-γ^+ and IL-17^+ $CD4^+$ T cells, we sought to determine if the cytokine profiles in protected immunized mice differed from that of control-immunized mice that succumbed rapidly to *P. aeruginosa* infection. Mice immunized with

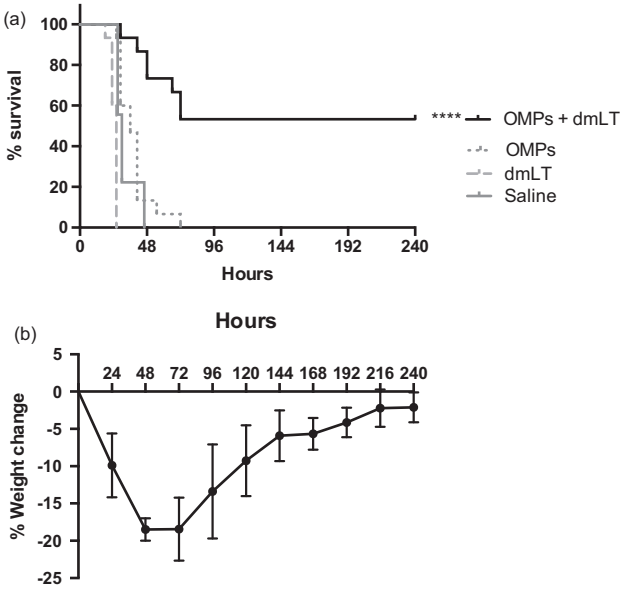

Fig. 4. Intradermal immunization with *P. aeruginosa* OMPs and dmLT protects mice against acute *P. aeruginosa* lung infection. (a) Mice were immunized with *P. aeruginosa* OMPs + dmLT (n = 15), *P. aeruginosa* OMPs (n = 15), dmLT (n = 15), or saline (n = 9). Animals were challenged with 1.4×10^7 CFU delivered by oropharyngeal aspiration 14 days after immunization. *P. aeruginosa* OMPs + dmLT immunized mice were significantly protected against an otherwise lethal *P. aeruginosa* lung infection (****$p < 0.0001$ by log rank Mantel-Cox test). (b) Immunized mice rapidly lost weight after challenge but survivors began recovering by day 3 post-infection. Data shown is the cumulative result of three independent experiments.

OMPs plus dmLT displayed a significant increase in the Th1 cytokine IFN-γ ($p < 0.01$) and the Th17 cytokine IL-17 ($p < 0.05$) in the BAL fluid (Fig. 5a and b) at 24 h post-infection compared to control mice (dmLT). This was accompanied by an increase in other Th1-associated cytokines, including interferon-γ inducible protein (IP10, Fig. 5c, $p < 0.0005$), IL-2 (Fig. 5d, $p < 0.01$), RANTES (Fig. 5e, $p < 0.05$), and IL-12p70 (Fig. 5f, $p < 0.05$) [21–23]. No significant differences were noted for the Th2-associated cytokines IL-4 and IL-5 [23] (data not shown) or for the anti-inflammatory cytokine IL-10 [24] (Fig. 5h). Additionally, OMPs plus dmLT immunized mice had significantly greater levels of IL-7, a lymphocyte survival cytokine [25], compared to control mice ($p < 0.005$, Fig. 5g). Taken together, these results demonstrate that immunization with OMPs plus dmLT helps promote a Th1- and Th17-type immune response within the first 24 h after *P. aeruginosa* lung infection.

4. Discussion

Pulmonary infections remain a significant cause of morbidity and mortality globally. As antibiotic resistance continues to rise among bacteria that cause severe respiratory infections, the development of vaccines that drive protective pulmonary immune responses is critical. In this work, we demonstrate that intradermal immunization with *Pseudomonas* OMPs and dmLT adjuvant elicits cellular and humoral immune responses in the lung and provides protection against an otherwise lethal *P. aeruginosa* pulmonary infection. This is the first study of its kind to include dmLT as an adjuvant in a vaccine against *P. aeruginosa*. We chose to evaluate dmLT in our vaccine formulation because it has been shown to promote the production of IFN-γ- and IL-17-producing $CD4^+$ T cells at mucosal surfaces [16]. Several studies have shown that Th1 and Th17 $CD4^+$ T cells are important for vaccine-mediated immunity to pulmonary pathogens, including *P. aeruginosa*, *Mycobacterium tuberculosis*, *Bordetella pertussis*, *Streptococcus pneumoniae*, and

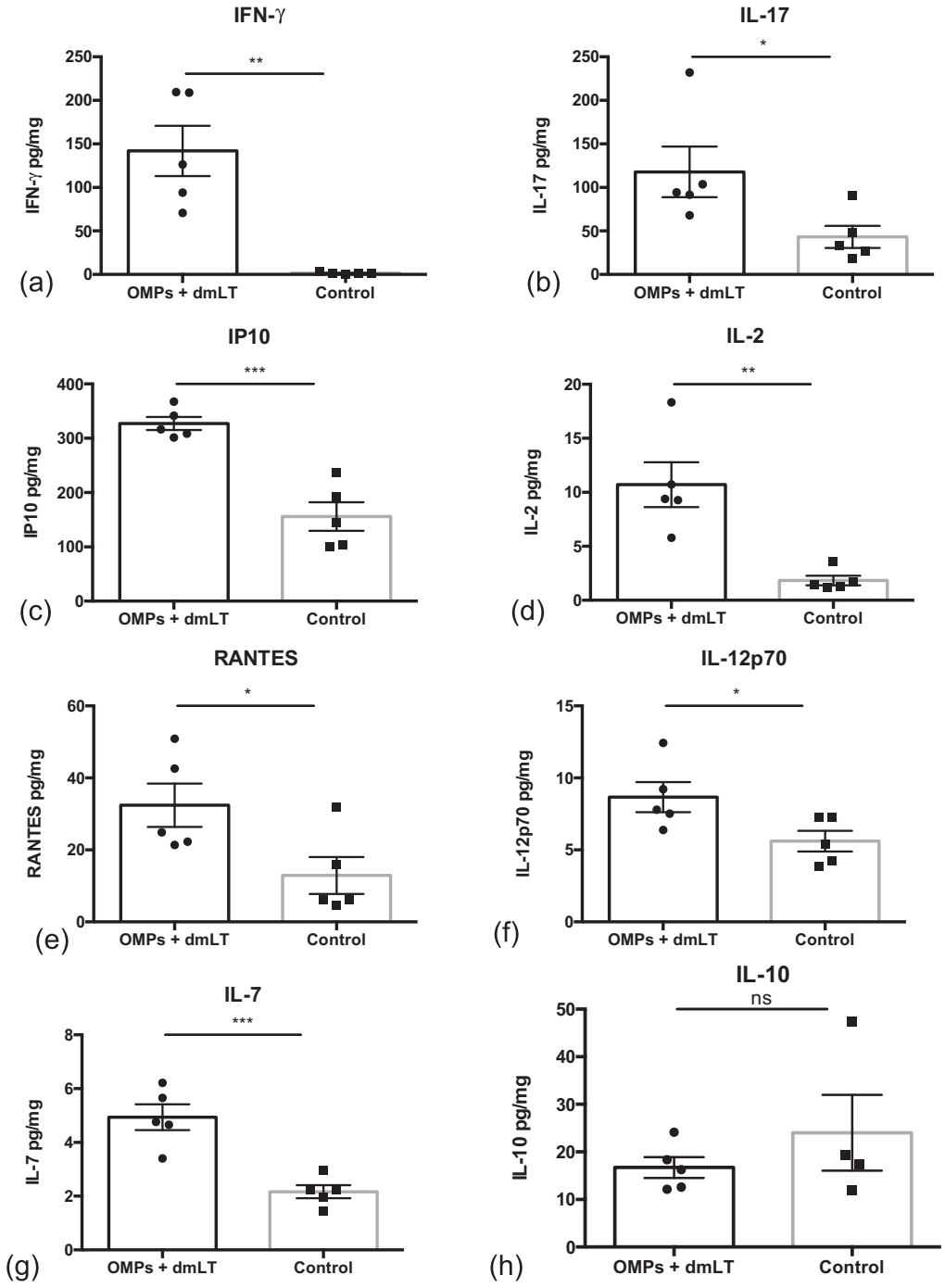

Fig. 5. Intradermal immunization with *P. aeruginosa* OMPs and dmLT promotes a Th1- and Th17-type cytokine profile during *P. aeruginosa* infection. Mice (n = 5 per group) were immunized with *P. aeruginosa* OMPs + dmLT or control (dmLT alone) intradermally. All animals were challenged with 1.4×10^7 CFU delivered by oropharyngeal aspiration 14 days after immunization. The concentration of cytokines present in the BAL fluid 24 h after infection was measured by luminex assay. Cytokine levels were standardized by BAL protein content. ns = not significant, * $p < 0.05$, ** $p < 0.01$, ***$p < 0.005$ by Two-tailed *t* test.

Klebsiella pneumoniae [26,27]. In our study, addition of dmLT to the *P. aeruginosa* OMP vaccine promoted antigen-specific IFN-γ^+ and IL17$^+$ CD4$^+$ T cells in the lungs of immunized mice. Following *P. aeruginosa* challenge, the inflammatory response in the lungs of immunized, protected mice was composed of cytokines known to be associated with the homing, migration, and proliferation of effector and memory T cells. Among those, IL-7, which is required for T cell development, has been shown to be protective against *P. aeruginosa* pneumosepsis and intra-abdominal peritonitis when administered as an immunotherapy [28]. In addition, the levels of IFN-γ and IL-17 were significantly elevated in protected animals, supporting the importance of a Th1/Th17-type cellular immune response during acute *P. aeruginosa* lung infection. This balanced Th1/Th17-type response is consistent with previous findings that *E. coli* LT mutants promote the production of IL17 and IFN-γ [14,15,18,29], resulting in protection against lung infection with the respiratory pathogen, *S. pneumoniae* [30].

Despite decades of research, few *P. aeruginosa* vaccine studies have focused on vaccine-induced cellular immunity and the role of T cells in protection. Live-attenuated vaccines (LAV) can be

effective in this regard, and a number of LAV platforms have shown protective efficacy against *P. aeruginosa* in murine models. For example, intranasal immunization with a LAV composed of multiple *Pseudomonas* strains, Habs16/IT3/PAO1Δ*aroA*, provided 75% protection against intranasal challenge with strain Habs16 [31]. The protection observed was mediated by $CD4^+$ T cells and dependent upon IL-17 production [7,31]. However, safety can be a concern for LAV, as there can be some risk of reversion to wild type virulence or infection from the attenuated strain. This is particularly a concern for use in immunocompromised patient populations, including those who are at the highest risk of developing *P. aeruginosa* pneumonia. A safer alternative to LAV are subunit vaccines, such as those composed of *P. aeruginosa* OMPs. OMPs have received considerable attention as they are immunogenic and highly conserved across bacterial strains [32–39]. A trivalent recombinant subunit vaccine, composed of PcrV-OprI-Hcp1, provided up to 70% survival in an otherwise lethal acute *P. aeruginosa* pneumonia model [40]. Immunization with a recombinant OprF-OprI vaccine adjuvanted with complete Freund's adjuvant (CFA) provided 100% and 50% protection against intranasal challenge with strains PAO1 and PAK, respectively, for up to one week post-infection; however, CFA is highly reactogenic and can cause local tissue necrosis, thereby excluding its utility in human vaccines [41]. In our study, OMPs administered intradermally with dmLT adjuvant provided 53% protection against a highly lethal pulmonary challenge. Notably, dmLT has already been shown to be safe in multiple clinical trials and thus has the potential to be included in a new *Pseudomonas* vaccine [42]. Taken together, these studies demonstrate the potential for subunit vaccines to promote protective immunity against *P. aeruginosa* in the lung. Moreover, our study demonstrates that protection in the lung can be achieved by parenteral vaccine administration. While many studies have investigated parenteral routes of immunization, they often utilize aluminum salts (i.e. alum) as the adjuvant. Alum is known to polarize immune responses toward Th2, resulting in high antibody titers at the expense of Th1 cellular immune responses. In our study, *P. aeruginosa* OMPs administered with dmLT induced significantly more *Pseudomonas*-specific IgG in the pulmonary airways compared to immunization with OMPs alone. LT and LT mutants have been shown to enhance antigen-specific serum and BAL fluid IgG for numerous pathogens [14,43–46]. The ability of dmLT to support antibody responses in addition to Th1/Th17 type cellular immunity is significant and offers a distinct advantage over alum, particularly for those pathogens that require both arms of the immune response. It is worth noting that in our studies, around 20% of mice failed to seroconvert in response to the vaccine. Other studies have found that this kind of variability, even during potent infections, likely depends on infection or vaccination dose or attributes of the infection or vaccine as well as genetic background, age and overall immunologic state of the animal being infected or vaccinated [47]

It is well-established that the adjuvant dmLT can enhance mucosal immune responses when administered via dermal immunization routes [13,17,44,48]. Efficient antigen delivery to draining lymph nodes and subsequent uptake of antigen by lymph node-resident dendritic cells occurs via a dense network of lymphatic vessels that are more broadly distributed in the dermis than in muscle or subcutaneous spaces. This allows for the rapid and direct migration of intradermally-administered antigen to nearby lymph nodes [10], subsequent activation of T cells, and T cell migration to the proximal mucosa, including respiratory tissue [11]. Intradermal immunization with *Francisella tularensis* Live Vaccine Strain resulted in higher numbers of lung IFN-γ^+CD4^+ T cells compared with intranasal immunization [49]. Similarly, intradermal immunization improved the efficacy of a recombinant protein vaccine against *M. tuberculosis* compared to subcutaneous administration [50]. These studies, together with our present findings, lend support to our hypothesis that the intradermal route may be a key pathway for promoting vaccine-induced immunity in the lung. It is worth noting that the majority of vaccines currently licensed in the United States are administered parenterally via the intramuscular route [51]. The ability of intradermal vaccination to drive both humoral and cellular immunity against *F. tularensis*, *M. tuberculosis*, and *P. aeruginosa* in the lung underscores the value of this route for vaccination against pulmonary pathogens.

In summary, our results demonstrate that an intradermal vaccine composed of *P. aeruginosa* OMPs plus dmLT is capable of inducing pathogen-specific antibody, IFN-γ^+ and $IL17^+$ $CD4^+$ T cells in the lungs of mice. This combination of vaccine-induced humoral and cellular immunity provided protection against an otherwise lethal acute *P. aeruginosa* pneumonia and is consistent with the leading argument that antibodies and CD4 T cells, but not CD8 T cells, are important for protection against this organism [52]. These results further support the premise that inclusion of dmLT adjuvant in intradermally-administered vaccines can drive protective immunity to sites of mucosal infection, such as the respiratory tract. This feature may be particularly helpful in designing vaccines to combat infections in special patient populations such as those with cystic fibrosis or chronic obstructive pulmonary disease although this remains to be tested. Nonetheless, inclusion of dmLT in intradermal vaccine platforms for *P. aeruginosa* and other respiratory pathogens deserves further investigation.

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Declaration of Interest

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.12.053.

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