Research Article

Intranasal Vaccination with rePcrV Protects against Pseudomonas aeruginosa and Generates Lung Tissue-Resident Memory T Cells

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1. Introduction

Pseudomonas aeruginosa (P. aeruginosa), a prevalent opportunistic pathogen and Gram-negative bacteria, tends to cause acute and chronic severe pulmonary infections [1–3]. P. aeruginosa infections are particularly problematic in mechanically ventilated patients, chronic obstructive pulmonary disease (COPD) patients, and cystic fibrosis (CF) patients [4–9]. Recently, the emergence of multidrug-resistant (MDR) P. aeruginosa has become a serious clinical challenge, posing a serious threat to effective infection control in clinical [9–11]. Over the past decades, enormous efforts have been focused on P. aeruginosa vaccines. Regrettably, no approved vaccines are available for treatment of P. aeruginosa infections [12], because of its high diversity and variability.

Tissue-resident memory (T_{RM}) cells are a new subpopulation of memory T cells recently identified, which embedded within peripheral tissues [13–15]. T_{RM} cells serve as immune sentinels at the respiratory tract and provide rapid and broad-spectrum protective effects against a variety of respiratory infection pathogens [15–17]. Induction of memory T and B cells has now been widely accepted as the principal disciplines for effective vaccine design which could provide robust protective immunity against pathogens caused by prior infection [18–20]. Both CD4 and CD8 T_{RM} cells in mucosal could be produced by natural infection [21]; however, natural infection could be lethal. Thus,
Figure 1: Continued.
Finding an effective way to induce highly protective T<sub>RM</sub> cells could be an ideal choice especially for the prevention of <i>P. aeruginosa</i>. Previous study showed the type of vaccines and adjuvants, and the route of vaccination could influence the efficacy of T<sub>RM</sub>. For pulmonary infectious diseases, mucosal immunization via the intranasal pathways is more effective than intramuscular route in inducing and stimulating immune protection of T<sub>RM</sub> [20, 22].

Th17 has been regarded as a major player in the anti-<i>P. aeruginosa</i> immunity; indeed, in our previous study, we identified a soluble <i>P. aeruginosa</i> antigen called rePcrV which could induce Th17 response and provide protection against <i>P. aeruginosa</i> by intranasal immunization [23]. Another substrate, 1,3-β-glucan, derived from <i>Alcaligenes faecalis</i>, has also been reported to prompt a Th1/Th17 response [24, 25]. Therefore, we combined rePcrV and 1,3-β-glucan supplemented with curdlan as an adjuvant. After immunization with the vaccine by intranasal administration, we observed that the ratio of CD44<sup>+</sup>CD62L<sup>-</sup>CD69<sup>+</sup>CD4<sup>+</sup> T<sub>RM</sub> cells induced by this vaccine was significantly increased, and IL-17A production of this subpopulation was notably enhanced after in vitro stimulation. Vaccinated mice infected with <i>P. aeruginosa</i> showed a sharp reduction in the bacterial burden. What is more, our results showed that CD4<sup>+</sup> T<sub>RM</sub> may involve the recruitment of neutrophils and provide partial protection against <i>P. aeruginosa</i>. Better understanding the underline mechanism could provide new strategies for the development of vaccines for <i>P. aeruginosa</i> and other respiratory-targeted vaccines.

2. Materials and Methods

2.1. Animals and Strains. Adult female C57BL/6 mice (6-8 weeks) were purchased from Beijing HFK Bioscience.
Figure 2: Continued.
20 adult female mice were vaccinated intranasally (i.n.) with P. aeruginosa by the intratracheal injection of XN-1. The sublethal dose of P. aeruginosa mouse. The lethal dose of P. aeruginosa XN-1 was 1.0 × 10⁷ CFU per mouse. The sublethal dose of P. aeruginosa XN-1 was 1.3 × 10⁶ CFU per mouse.

2.2. Immunization Procedure. For active immunization, adult female mice were vaccinated intranasally (i.n.) with 20 μL of curdlan (10 mg/mL, Sigma) or purified proteins (25 μg/mouse) plus curdlan (10 mg/mL, Sigma), on days 0, 14, and 21. Mice were challenged at day 35 and were anesthetized with isoflurane or pentobarbital sodium followed by the intratracheal injection of P. aeruginosa XN-1. The lethal dose of P. aeruginosa XN-1 was 1.0 × 10⁷ CFU per mouse. The sublethal dose of P. aeruginosa XN-1 was 1.3 × 10⁶ CFU per mouse.

2.3. FTY720 Treatment. FTY720 (Cayman Chemical) dissolved in saline was continuously administered i.p. (0.5 mg/kg) to mice for a period of 7 d before infection [26].

2.4. Isolation of Lung Lymphocyte. At day 36 after treatment, mice were sacrificed under overdose isoflurane. The lungs were dissociated with collagenase D (150 UmL⁻¹, Gibco) and DNase I (1 unit/μL, Sigma) at 37°C on a rocker at 260 rpm for 1 h. Then, lung tissues were transferred to a 70 μm cell strainer (Beyotime) to obtain cell suspensions. Monocytes were separated using by Percoll (Cytiva) [27].

2.5. Flow Cytometry. Mice were intravenously injected with 3 μg APC/Cy7 anti-mouse CD45 (BioLegend) diluted in 300 μL saline [28], 10 min before euthanasia. Then, lung mononuclear cells were stimulated with leukocyte activation cocktail, with BD GolgiPlug (BD Pharmingen) for 4-6 h. PerCP/Cyanine5.5 anti-mouse CD4 (BioLegend), PE/Cy7 anti-mouse CD44 (BioLegend), FITC anti-mouse CD69 (BioLegend), and PE anti-mouse CD62L (BioLegend) were used for cell surface marker staining. APC anti-mouse IL-17A (BioLegend) and Brilliant Violet™510 anti-mouse IFN-γ (BioLegend) were used for cell surface marker staining. APC anti-mouse IL-17A (BioLegend) and Brilliant Violet™510 anti-mouse IFN-γ (BioLegend) were used for intracellular staining. Zombie NIR™ Fixable Viability Kit (BioLegend) was used to distinguish between living and dead cells. For RNA-profiling, CD4⁺CD44⁺CD69⁺CD62L⁻ cells were sorted into DMEM (Gibco) with 20% fetal bovine serum (FBS, Gibco) on ice using BD FACS Aria II SORP before RNA extraction.

2.6. Real-Time PCR. RNA was extracted from sorted CD4⁺CD44⁺CD69⁺CD62L⁻ cells using MicroElute Total RNA Kit (OMEGA) according to the manual and stored at -80°C. Hobit, Blimp-1, RORγt, and T-bet were quantified using QuantiTect Probe RT-PCR Kit (200) (Qiagen) with SYBR-Green. The primers used were as follows: Hobit, forward: 5'-CTCAGCCACTTGAGACTCA-3', reverse: 5'-
Figure 3: Intranasal vaccination with rePcrV primes the CD4+ TRM cells response. (a) Schematic of the experimental protocol. Representative intracellular staining profiles and pooled data of IL-17A and IFN-γ in CD4+CD44+CD69+CD62L- T cells in the lungs of immunized mice (rePcrV + curdlan i.n.) or unimmunized mice (n = 4). (b) Hobit, Blimp-1, ROR-γt, and T-bet expressions of CD4+ T cells in the lung tissues of immunized mice (rePcrV + curdlan i.n.) or unimmunized mice (n = 4). (c) Representative immunofluorescence images of the lung tissues stained with DAPI (blue), anti-CD69 (green), and anti-IL-17A (red) from immunized mouse (rePcrV + curdlan i.n.) or unimmunized mouse (n = 4). Data are presented as mean ± SEM. P values were calculated by Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Figure 4: Continued.
CTGTCGGTGGAGGCTTTGTA-3'; Blimp-1, forward: 5'-TTCTCTTGGAAAAACGTGGG-3', reverse: 5'-GGAGCCGAGCTAGACTTG-3'; RORγt, forward: 5'-CAGAGGAATGTCAGAGGCT-3', reverse: 5'-TGCAAATGTGATCCGAGCGAC-3'; and T-bet, forward: 5'-TATGGCAGCAGCAG-3' and reverse: 5'-GGAGCCCCTTTGTTGACCG-3'.

2.7. Histology and Immunofluorescence. The lungs were collected and fixed in 4% paraformaldehyde (BioSharp) and embedded in paraffin. Pathological changes were evaluated by hematoxylin and eosin stain (H&E stain) [29]. Anti-CD69 and anti-IL-17A were used for immunofluorescence staining of lung samples.

2.8. IL-7, IL-17A, and IFN-γ Neutralization. Mice were administrated 50 μg/mouse of an IL-7-neutralizing antibody (BioXCell, clone M25) at days 27, 30, 32, and 34 of the first immunization (day 0) [30]. IL-17A was blocked using 300 μg anti-mouse IL-17A mAb [31] (BioLegend, Clone TC11-18H10.1) administered i.v. into mice 2 d before P. aeruginosa XN-1 infection (at days 33 and 34). For neutralization of IFN-γ, mice were given intravenous injection 2 days of 300 μg anti-mouse IFN-γ mAb [32] (BioLegend, Clone R4-6A2) before P. aeruginosa XN-1 infection (at days 33 and 34).

2.9. Neutrophil Depletion. Mice were daily injected intraperitoneally (i.p.) with anti-Ly6G antibody (BioXCell, clone 1A8, 50 μg/mouse) for a period of 7 d before challenge [33] (at days 28, 29, 30, 31, 32, 33, and 34).

2.10. Statistical Analysis. Data are presented as mean ± SEM. Student’s t-test and Mann–Whitney U test were conducted, according to the data distribution. The survival rate was analyzed by the Kaplan-Meier survival curves. GraphPad Prism 8.0 (GraphPad Software) was used for data analyses. P values less than 0.05 were considered significant.

3. Results

3.1. Intranasal Vaccination with rePcrV Enhanced Protection against P. aeruginosa Compared with Intramuscular Vaccination. PcrV has been proved to have immune protective effect by intramuscular or intraperitoneal immunization [34, 35]. In our study, we firstly compared the immune protective effects of these two different vaccination routes, intramuscular (i.m.) vaccination with the rePcrV protein formulated with aluminum adjuvant and intranasal (i.n.) immunization with curdlan. As expected, intranasal immunization route improved the efficacy of vaccine. The survival of the i.n. was higher (P < 0.0013) than the rate of i.m. at day 14 postinfection (Figure 1(a)). Then, mice were administrated a sublethal dose of P. aeruginosa. A histological analysis of lung tissues of rePcrV i.n. suggested a further increase in inflammatory cell infiltration. Meanwhile, the rePcrV i.n. showed significant reduction (P < 0.001) in lung pathology score (Figure 1(b)). Furthermore, the bacterial burdens of the rePcrV i.n. were significantly decreased (rePcrV i.n. vs. rePcrV i.m. P < 0.01, Figure 1(c)). The mRNA expression of IL-6 (rePcrV i.n. vs. rePcrV i.m. P < 0.001, Figure 1(d)) and TNF-α (rePcrV i.n. vs. rePcrV i.m. P < 0.05, Figure 1(e)) was also reduced in rePcrV i.n. Thus, intranasal vaccination with rePcrV enhanced protection
Immunized + FTY720

Unimmunized + FTY720

IL-17A-APC-Cy7

CD69-FITC IFN-γ-PE-Cy7

Cells in CD69+ CD62L− CD4+ T cells (%)

IL-17A+ cells (%)

IFN-γ+ cells (%)

(a)

DAPI CD69 IL-17A MERGE

Immunized + FTY720

Unimmunized + FTY720

(b)

Day: 0 7 21 33 34 35 49

Immunized + FTY720 + anti-IL-17A vs Immunized + FTY720 + anti-IFN-γ

P = 0.0003

(c)

Figure 5: Continued.
Figure 5: rePcrV vaccine efficacy depends on the IL-17A expression by CD4+ T_{RM} cells. (a) The representative dot plots showed CD4+CD44+CD69+CD62L- T cells in the lungs of rePcrV-immunized mice with FTY720 treatment or naive C57BL/6 mice with FTY720 treatment. The graph indicates the number of CD4+CD44+CD69+CD62L- T cells per mouse (n = 4). (b) Schematic of the experimental protocol. Survival in the FTY720 treatment-immunized mice with anti-IL-17A or IFN-γ Ab treatment (n = 10). ***P < 0.001 by log-rank test. (d) Representative H and E stains in the FTY720 treatment-immunized mice with anti-IL-17A or IFN-γ Ab treatment (n = 5). Significant differences are designated by using Student’s t -test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

against P. aeruginosa compared with intramuscular vaccination.

3.2. CD4 T Cells Were Essential for rePcrV-Mediated Protection in P. aeruginosa Pneumonia. To inquire the role of lymphocyte-mediated immune responses during rePcrV-induced protection, adult female CB-17 SCID mice were vaccinated with rePcrV plus curdlan or rePcrV plus aluminum. Mice were challenged with P. aeruginosa XN-1 and were observed to survive for 14 days. As shown in Figure 2(a), there was no statistical difference (P = 0.1316) in survival rate between rePcrV-immunized SCID mice and -unimmunized mice, indicating that a complete lymphocyte system was required for protection after rePcrV immunization in P. aeruginosa pneumonia. In order to determine the relative requirements for humoral immunity and cellular immunity, rePcrV vaccine tested the protection in μMT mice (which lack mature B cells), CD8 T cell KO mice, and CD4-depleted mice (by intraperitoneal injection of anti-CD4 antibody GK1.5). As shown in Figure 2(b), rePcrV-immunized μMT mice were significantly protected (P < 0.001) after P. aeruginosa XN-1 challenge, compared with unimmunized mice which were not protected. The result of CD8 T cell KO mice was the same (P < 0.001, Figure 2(c)). However, the rePcrV-immunized CD4-depleted mice (P = 0.4728, Figure 2(d)) were not protected after P. aeruginosa XN-1 challenge. These data suggested the key role for CD4 T cells in mediating protection after immunization with rePcrV.

3.3. Intranasal Vaccination with rePcrV Initiates the CD4+ T_{RM} Cell Response. The result above showed that CD4+T cells are essential for the anti-P. aeruginosa immunity. However, it is still unknown whether circulating or resident CD4+ T cell is the major player. To this end, the lungs were dissociated into a single cell suspension and detected by flow cytometry. A dramatic increase in CD4+CD44+CD62L-CD69+TRM cells was observed in vaccinated mice compared with unimmunized mice (P < 0.001, Figure 3(a)). Transcriptional analysis of T_{RM} cells showed that they expressed a unique transcription factor profile. Since Hobit together with Blimp-1 regulates the differentiation and maintenance of T_{RM} cells [36], we purified T_{RM} cells from immunized or unimmunized mice and determined the level of Hobit, Blimp-1, RORγt, and T-bet mRNA. As shown in Figure 3(b), the level of Hobit, Blimp-1, and RORγt was increased in mice immunized with rePcrV compared with unimmunized (P < 0.001, respectively). To examine the expression of IL-17A production in CD4 T_{RM} cells, we employed immunofluorescence staining. The result revealed that the IL-17A expression was enhanced in immunized mice (Figures 3(a) and 3(c)). Representative gating strategies were shown in figure S1.

3.4. CD4+ T_{RM} Cells Partially Protected against Pseudomonas aeruginosa Pulmonary Infection. To exclude the contribution of circulating memory cells to the recall responses, we administered FTY720 [37, 38] (a SIP inhibitor that blocks the egress of T cells from repositioning from secondary...
lymphoid organs to the tissue). We found that FTY720 treatment followed by a P. aeruginosa XN-1 challenge induced higher survival in immunized mice (P < 0.0001) but not in unimmunized mice (Figure 4(a)). Vaccine efficacy was maintained in vaccinated mice with FTY720 treatment, as measured indirectly by global disease score (Figure 4(b)) and weight loss (Figure 4(c)). Furthermore, the bacterial load of immunized mice treated with FTY720 decreased significantly (P < 0.01, Figure 4(d)). In contrast, immunized mice significantly alleviated pathological damage (P < 0.001, Figure 4(e)). It should be noted that, compared with immunized mice without FTY720 treatment, immunized mice with FTY720 treatment diminished partial protection, which suggested that circulating T cells also played a role in preventing P. aeruginosa infection.

3.5. rePcrV Vaccine Efficacy Depended on IL-17A Expression by CD4+ TRM Cells and Remained Independent of IL-7. Lung CD4+ TRM cells in vaccinated mice with FTY720 treatment showed higher level of IL-17A secretion compared with cells from FTY720-treated unimmunized mice (P < 0.01, Figures 5(a) and 5(b)). We treated mice with anti-IL-17A antibody and anti-IFN-γ antibody before and during vaccination to determine whether IL-17A or IFN-γ was required for rePcrV vaccine efficacy in the lungs. Anti-IL-17A-immunized mice were not protected against the challenge of P. aeruginosa XN-1 (Figure 5(c)). In line with this, the histological analysis of the lung tissues of anti-IL-17A-immunized mice revealed a further increase of peribronchial inflammatory cell infiltration (P < 0.001, Figure 5(d)). IL-7 signaling is regarded as a key mediator for homeostatic proliferation of CD4 T cells, which could explain the long-term and circulatory independent maintenance of TRM cells. To assess whether IL-7 mediated the population expansion of TRM cells and contributed to its survival, we applied a neutralizing antibody to IL-7 (anti-IL-7) at days 27, 30, 32, and 34 of the first immunization (day 0). The results showed that neutralization of IL-7 did not increase the bacterial load (P = 0.7836, Figure 6(a)), and there was no statistical difference in histopathological examination between groups (P = 0.1599, Figure 6(b)).

3.6. Depletion of Neutrophils Impaired the Clearance of Pseudomonas aeruginosa from the Lung. Neutrophils are main orchestrators of lung inflammation and play a unique role in the connection between innate and adaptive...
Immunity [39]. In order to investigate whether neutrophils play a role in CD4+ TRM cells mediated protection against P. aeruginosa, neutrophils were deleted before challenge. The results showed that in the neutrophil depletion mice, CFU counts were increased in the lungs of mice treated with anti-Ly6G (P < 0.01, Figure 7(a)), and lung damage was worse (P < 0.0001, Figure 7(b)). Flow cytometry showed that neutrophil depletion did not impact the CD4+ TRM cell population (P = 0.7296, Figure S2). These data indicated that CD4+ TRM cells may be involved in recruitment of neutrophils and provided partial protection against P. aeruginosa.

4. Discussion

According to the different types of cytokines secreted, CD4+ TRM cells are divided into Th1, Th2, or Th17 subtypes. Generally, CD4+ TRM cells in viral infection and tumors mainly secreted IFN-γ, while CD4+ TRM cells induced by bacterial or fungal infection mainly expressed IL-17A. The study indicated that dermal Candida albicans infection preferentially produces CD4+ IL-17A+ TRM cells. When reinfected with Candida albicans, TRM cells could rapidly clear infection challenges [40]. Previous work showed that lung TRM cells were elicited by heat-killed K. pneumoniae [41]. By using IL-17A tracking-fate mouse models [42], CD4+ TRM cells were found derived from effector Th17 cells [27]. Our previous study found that repCrv could induce Th17 response and enhanced protection [23]. The results of this study initially demonstrate that repCrv intranasal immunization could induce the generation of CD4+ TRM cells secreting IL-17A in lung tissues of mice, and these cells produced a protective immune response after P. aeruginosa infection. Therefore, the origin of CD4+ IL-17A+ TRM cells and their relationship with Th17 cells need to be further investigated in subsequent experiments.

FTY720 not only blocks the egress of T cells but also prevents migration of B cells from lymph nodes to the circulation [15, 43]. Indeed, FTY720 treatment appeared to affect
bacterial burdens and survival in the immunized group, suggesting that circulating T cells or antibody-producing cells were also required in preventing *P. aeruginosa* infection. However, treatment with FTY720 did not affect $T_{RM}$ cell expansion in the lungs. Our data showed that there was no statistical significance between mice with FTY720 treatment and mice without FTY720 treatment (Figure S3).

Long-term survival in peripheral tissues is another important characteristic of $T_{RM}$ cells [13, 14, 44]. Furthermore, researches showed that the survival and expansion of $T_{RM}$ cells in peripheral tissues were mainly regulated by the type of vaccines (inactivated vaccine and live vaccine). Comparing the intranasal or injected inactivated **pertussis** vaccine BPZE1 can resist the infection caused by *B. pertussis* [50]. On the contrary, nasal inoculation of attenuated pertussis vaccine BPZE1 can resist the infection caused by *B. pertussis* [50]. Comparing the intranasal or injected influenza vaccines, we found that the route of administration and the type of vaccines (inactivated vaccine and live vaccine) also affect the production of CD4+ $T_{RM}$ cells. Nasal vaccination with a live attenuated influenza vaccine (Flumist) induced antigen-specific CD4+ $T_{RM}$ cells in the lung, mediating long-term protection against heterologous influenza virus strains. However, inactivated influenza virus vaccine (Fluzone) did not elicit $T_{RM}$ cell production after nasal inoculation but induced strain-specific neutralizing antibody production [50]. Hence, choosing the appropriate vaccination route and vaccine type is an important means to induce respiratory $T_{RM}$ cell production.

**Data Availability**

All the data are included in this paper.

**Ethical Approval**

The animal study was reviewed and approved by the Animal Ethical and Experimental Committee of the Army Medical University.

**Conflicts of Interest**

The authors declare no competing interests.

**Authors’ Contributions**

Y.X.O., Y.W., and Q.F.Z. designed the experiment and are responsible for data integrity and accuracy. T.Y., X.C., and Z.Y.C. contributed to the data generation and analysis. W.J.Z. and J.G. supervised the experiments. Y.X.O., Q.F.Z., J.G., and Q.M.Z. contributed to manuscript writing and revision. Yangxue Ou and Ying Wang contributed equally to this work and shared first authorship.

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**Supplementary Materials**

Figure S1: representative gating strategies for CD44+ CD62L CD69+CD4+ $T_{RM}$ cells in the lungs. CD44+ CD62L CD69+CD4+ $T_{RM}$ cells were gated on live CD45 cells. Figure S2: the graph indicates the number of CD44+ CD64+CD69+ CD62L T cells per mouse ($n = 4$) found in the lungs of FTY720 treatment-immunized mice with or without anti-Ly6G Ab. Figure S3: CD4+ $T_{RM}$ cells protect against *Pseudomonas aeruginosa* pulmonary infection. (A) The representative dot plots showed CD4+CD44+CD69− CD62L T cells in the lungs of rePcrV-immunized mice with or without FTY720 treatment. (B) The graph indicates the number of CD4+CD44+CD69− CD62L T cells per mouse ($n = 4$) found in the lungs of immunized mice with or without FTY720 treatment. (Supplementary Materials)

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