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Source-specific priming of lung NK cells by *Pseudomonas* LPS enhances resistance to bacterial pneumonia

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Bacterial pneumonia is a major cause of morbidity and mortality in elderly individuals, yet how mucosal innate immunity changes with age remains unclear. We detected bacterial DNA, particularly signatures of *Pseudomonas*, in the circulation of healthy elderly individuals, suggesting that chronic exposure to low-dose microbial components occurs during aging. Using murine models, we found that intratracheal delivery of a low dose of *Pseudomonas aeruginosa* lipopolysaccharide (LPS) strongly protected against bacterial pneumonia through a microbiota-dependent mechanism, whereas *Escherichia coli* LPS had no detectable protective effect. This protective response occurred in the absence of adaptive immunity and was mediated by lung-resident natural killer (NK) cells. Transcriptomic profiling revealed that *P. aeruginosa* LPS selectively upregulated the complement receptor C3aR1 in pulmonary NK cells, increasing their IFN- γ production and LFA-1-dependent adhesion to target cells. Adoptive transfer of *P. aeruginosa* LPS-conditioned pulmonary NK cells conferred protection to naïve recipients, whereas silencing C3aR1 abrogated this effect. This localized innate training occurred without systemic inflammation and required the presence of a commensal microbiota. These findings reveal that structurally distinct LPS from *P. aeruginosa* programs lung NK cells toward a C3aR1-driven, high-affinity effector state that enhances resistance to bacterial pneumonia. Source-specific innate conditioning of mucosal NK cells may provide a tissue-targeted strategy to strengthen pulmonary host defense, particularly in aging populations vulnerable to respiratory infections.

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INTRODUCTION

The existence of a blood microbiome in healthy individuals remains a controversial and actively debated topic.¹ Traditionally, the human circulatory system has been regarded as a sterile compartment. While a few studies have reported the presence of viable microorganisms in the peripheral blood of healthy individuals, presumably through the intestinal route,^{2–6} a large-scale population analysis of 9770 subjects strongly refuted the presence of viable microorganisms under physiological conditions, reinforcing the view that live microbes are absent from the bloodstream.⁷ This distinction is important because the absence of culturable or viable bacteria does not necessarily exclude the presence of microbial products. Bacterial DNA fragments, outer membrane vesicles, metabolites, and cell wall components may enter the circulation transiently or at low abundance, especially under conditions of age-associated barrier dysfunction. Such signals may be rapidly cleared and therefore difficult to detect

consistently, but even brief or low-level exposure could be sufficient to shape systemic immune tone. In this context, aging is accompanied by progressive structural and functional deterioration of mucosal barriers, particularly in the gut and lung, characterized by epithelial barrier weakening, altered mucus production, impaired antimicrobial defense, and shifts in local microbial communities. These changes may permit commensal bacteria or their components to translocate across mucosal barriers and enter the circulation without causing overt infection, suggesting that circulating bacterial signals may represent subclinical microbial sensing associated with physiological aging rather than sustained bacteremia. In immunocompetent individuals, viable microbes that translocate across weakened barriers are unlikely to persist in the bloodstream or distal organs. In contrast, bacterial molecular components, such as nucleic acids and lipopolysaccharide (LPS), may readily enter the circulation and influence systemic immune responses.⁸

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To determine whether circulating bacterial signals are associated with healthy aging, we examined blood samples from a cohort of relatively healthy older adults and used these observations to guide mechanistic studies in mice. We reasoned that if microbial components are detectable during physiological aging, their biological significance may extend beyond serving as markers of barrier dysfunction. Instead, repeated low-level exposure to bacterial molecules could tune innate immune responsiveness and alter host defense at mucosal sites. These observations led us to investigate whether age-associated bacterial signals can influence mucosal innate immunity and alter susceptibility to infectious challenge.

Acute respiratory infections are the third leading cause of death worldwide,⁹ and pneumonia is frequently listed as a secondary cause of death in elderly individuals, even if it is not the primary underlying condition leading to hospitalization.¹⁰ The increased susceptibility to pulmonary infections in elderly individuals is attributed to both immunosenescence and age-related deterioration of respiratory tract immune function. Among common respiratory pathogens, *Pseudomonas aeruginosa* (*P. aeruginosa*) is of particular concern because of its virulence, multidrug resistance, and high mortality rates in cases of nosocomial pneumonia.¹¹ Innate and adaptive immune responses in the lower airways are tightly regulated to maintain a balance between microbial clearance, immune tolerance, and tissue repair. The commensal lung microbiota plays a critical role in maintaining pulmonary immune homeostasis.¹² Thus, age-associated changes in microbial exposure may influence pulmonary host defense by reshaping the baseline responsiveness of resident innate immune cells. Notably, microbial components such as LPS have been shown to epigenetically reprogram tissue-resident macrophages, increasing their transcriptional responsiveness and resistance to subsequent infections.¹³ However, a substantial body of work also indicates that canonical LPS-driven innate immune programming more often results in a tolerized (hyporesponsive) state, particularly in macrophages.¹⁴ While macrophage priming and training in innate immunity are well recognized, the contribution of natural killer (NK) cells—key innate effectors involved in immunosurveillance—has not been systematically investigated in this context. This gap is particularly relevant in the lung, where NK cells can rapidly produce IFN- γ , interact with myeloid cells, and participate in early antimicrobial responses before the development of adaptive immunity. Whether the low levels of circulating bacterial components observed in healthy elderly individuals contribute to NK cell-mediated innate conditioning and mucosal protection remains to be determined.

Building on these human observations, we employed murine models of bacterial pneumonia to determine whether low-level exposure to bacterial components can modulate pulmonary innate immunity. We found that intratracheal exposure to low-dose *P. aeruginosa*-derived LPS conferred robust protection against subsequent bacterial pneumonia through a microbiota-dependent mechanism. This protection was mediated by lung-resident NK cells, which acquired enhanced effector function and increased C3aR1-dependent, LFA-1-mediated adhesion to target cells. Importantly, this NK cell-centered protective program differed from the canonical macrophage endotoxin tolerance response induced by LPS and was not reproduced by *E. coli*-derived LPS, highlighting the source-specific, cell type-specific, and tissue-context-dependent outcomes of LPS exposure. Together, our findings support a localized NK cell conditioning pathway that links age-associated microbial signals to enhanced pulmonary host defense and provides new insight into how bacterial components may shape mucosal immunity during aging.

RESULTS

Pseudomonas-associated bacterial DNA and elevated lipopolysaccharide-binding protein (LBP) are detectable in the blood of healthy elderly individuals

We recruited a cohort of 103 healthy elderly individuals (aged > 65 years) in collaboration with the Beicai Community Health Service

Center in Pudong New District, Shanghai, China (Fig. 1a). Clinical laboratory test results at enrollment are summarized in Supplementary Table 1. The median age of the cohort was 70 years, with females comprising 60.2% of the participants (Fig. 1b). To quantify the bacterial burden in blood, digital PCR targeting the 16S rRNA gene was performed on blood samples. Correlation analysis between bacterial DNA levels and clinical variables revealed a significant positive association with age but not with other clinical parameters, including hematological indices (Fig. 1c and Supplementary Fig. 1). Importantly, the positive association between blood bacterial DNA burden and age was independently replicated in a separate cohort of 37 healthy elderly individuals (Supplementary Fig. 2), supporting the robustness of our observations. We found no evidence for culturable/viable bacteria in their blood under the conditions tested (Supplementary Table 2). Notably, among individuals with higher bacterial DNA burdens ($\log_{10} > 5.5$), the levels were positively correlated with circulating monocyte counts (Fig. 1d) but no longer associated with age (Supplementary Fig. 3), suggesting a potential state of immunological activation in those individuals. In contrast, in individuals with bacterial DNA levels below this threshold, the levels remained positively associated with age but not with inflammatory markers (Fig. 1c and Supplementary Fig. 1), indicating that a low bacterial DNA burden represents a feature of healthy aging.

To assess systemic exposure to gram-negative bacterial components, we compared serum levels of lipopolysaccharide-binding protein (LBP) between healthy elderly individuals ($n = 38$) and sex-matched healthy younger adults ($n = 31$). LBP levels were significantly higher in the elderly group (Fig. 1e). These results indicate that the levels of both bacterial DNA and LBP, a surrogate marker of systemic exposure to LPS, are elevated in the circulation of healthy elderly individuals. To explore the microbial composition of circulating bacterial DNA, we performed 16S rRNA gene sequencing of the V3–V4 region on blood samples. On the basis of the bacterial burden level (BBL; upper-quartile cutoff, $\log_{10} = 4.5$), participants were classified into two subgroups: a high-bacterial-burden group (BBL_{high}) and a low-burden group (BBL_{low}). Compared with the BBL_{low} group, the BBL_{high} group presented significantly reduced bacterial α diversity and richness (Fig. 1f–l), suggesting that the taxonomic diversity of translocated bacteria in the blood is relatively limited.

Moreover, principal coordinate analysis (PCoA) based on the Bray–Curtis distance of amplicon sequence variants (ASVs) revealed significant compositional differences between the two groups ($P = 0.001$, PERMANOVA with 999 permutations; Fig. 1m). To identify key taxa enriched in the BBL_{high} group, a sparse partial least squares discriminant analysis (sPLS-DA) was performed (Fig. 1n and Supplementary Fig. 4). Two representative ASVs (ASV2 and ASV39) enriched in the BBL_{high} group were classified as *Pseudomonas* (Fig. 1n). The relative abundance of *Pseudomonas* was consistently significantly greater in the BBL_{high} group than in the BBL_{low} group (Fig. 1o) and was positively correlated with total bacterial DNA levels in blood (Fig. 1p) but was not significantly associated with clinical parameters (Fig. 1q). Collectively, these findings suggest that bacterial components, particularly those derived from *Pseudomonas*, are detectable in the circulation of healthy elderly individuals and have no overt impact on systemic health.

Low-dose LPS derived from *Pseudomonas aeruginosa*

(*P. aeruginosa*) confers protection against bacterial pneumonia in both adult and aged mice

To investigate the immunological status of aged mice, we performed flow cytometric analysis of immune cells isolated from the lungs, mesenteric lymph nodes (MLNs), spleen, and Peyer's patches (PPs) of 18-month-old male C57BL/6 mice. Compared with young adult controls (2 months old), aged mice presented increased expression of CTLA-4 and PD-1 on T cells (Fig. 2a–c;

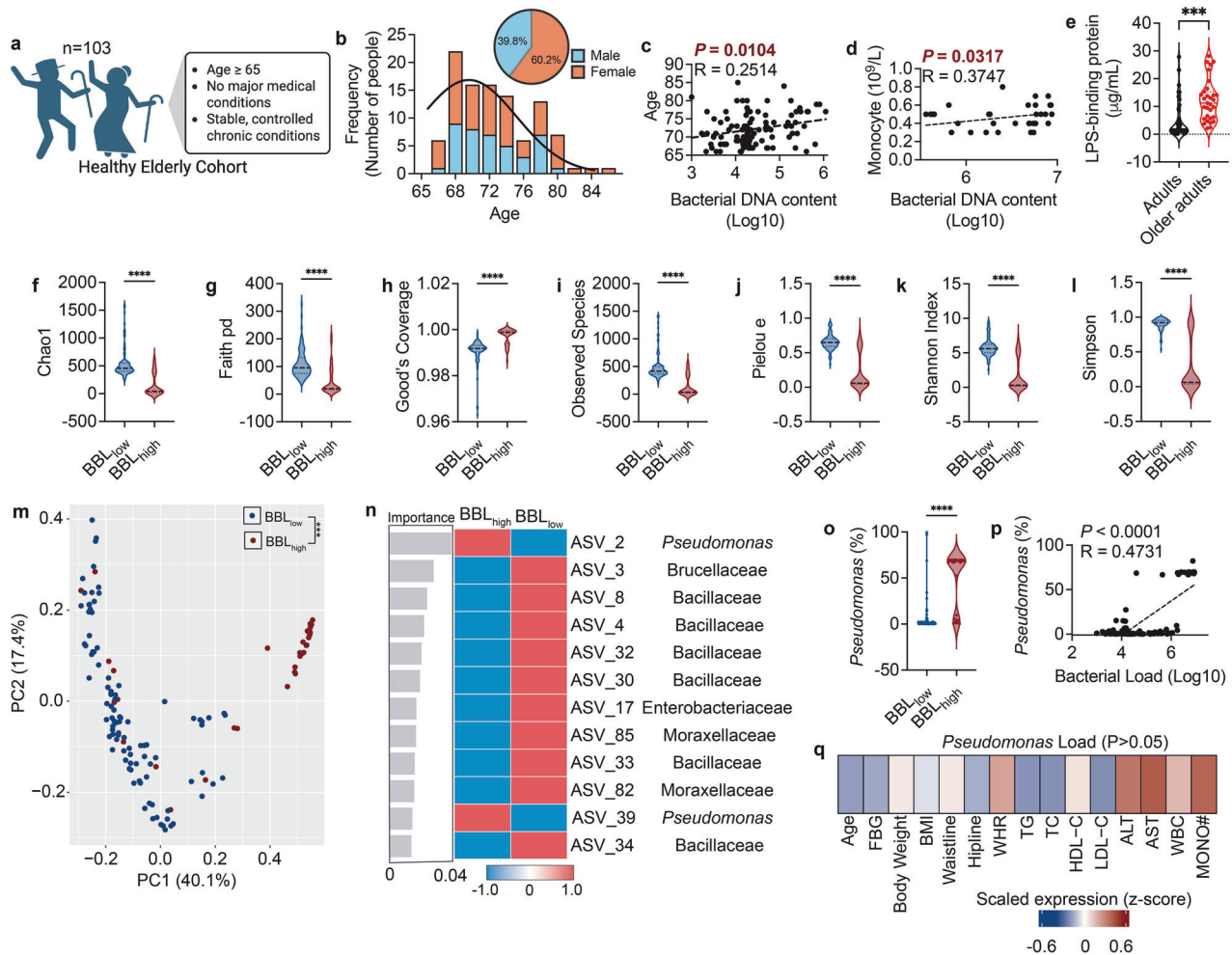


Fig. 1 Circulating *Pseudomonas* signatures are detectable in healthy elderly individuals. **a** A schematic diagram illustrates the simplified inclusion and exclusion criteria used to recruit 103 healthy elderly participants. **b** Histogram showing the age and sex distributions of the cohort. **c** Correlations between blood bacterial 16S rRNA gene load and age. **d** Correlations between elevated blood bacterial 16S rRNA gene load (log10-transformed >5.5) and peripheral blood monocyte frequency. **e** Plasma concentrations of LPS-binding protein (LBP) in adults versus elderly individuals (adults, $n = 38$; older adults, $n = 31$). α -diversity metrics based on 16S rRNA gene sequencing of blood samples from the BBL_{low} and BBL_{high} groups, including **f** the Chao index, **g** Faith's phylogenetic diversity, **h** Good's coverage, **i** observed species, **j** Pielou's evenness, **k** the Shannon index, and **l** the Simpson index. **m** Principal coordinate analysis (PCoA) based on Bray-Curtis distances between BBL_{low} and BBL_{high} samples. **n** Sparse partial least squares discriminant analysis (sPLS-DA) identifying the top 12 amplicon sequence variants (ASVs) contributing most to group separation. **o** Relative abundance of *Pseudomonas* in BBL_{low} versus BBL_{high} samples. **p** Correlations between total 16S rRNA gene load and *Pseudomonas* relative abundance. **q** Associations between *Pseudomonas* abundance and selected clinical parameters in the elderly cohort. The data in **c**, **d**, **p**, and **q** were analyzed using Spearman's correlation. Data in **e** and **o** were compared using two-tailed unpaired t tests. Data in **f**–**l** were evaluated using the Mann-Whitney U test (Wilcoxon rank-sum test). The difference in community composition in **m** was assessed using PERMANOVA with 999 permutations. Statistical analysis: Spearman's correlation was used for (**c**), (**d**), (**p**) and (**q**); two-tailed unpaired Student's t tests were used for (**e**) and (**o**); Mann-Whitney U tests were applied for (**f**–**l**); and community composition differences in **m** were assessed by PERMANOVA with 999 permutations. *** $P < 0.001$, **** $P < 0.0001$. BBL_{low} individuals with log10-transformed blood 16S rRNA gene load <4.5 , BBL_{high} individuals with log10-transformed blood 16S rRNA gene load >4.5 , FBG fasting blood glucose, BMI body mass index, TG triglycerides, TC total cholesterol, ALT alanine aminotransferase, AST aspartate aminotransferase, WBC white blood cells, MONO# monocyte count. **a** was generated using BioRender, and the appropriate publication license was obtained

absolute flow cytometry cell counts are provided in Supplementary Fig. 5), reflecting an immunosuppressive and hyporesponsive immune phenotype.

Given our finding that *Pseudomonas*-derived components were detectable in the circulation of healthy elderly individuals, we hypothesized that chronic exposure to low levels of bacterial products may modulate local mucosal immunity. Among *Pseudomonas* species, *P. aeruginosa* is a common opportunistic pathogen capable of invading the respiratory tract and is a major cause of fatal infections in hospitalized elderly patients.¹⁵ To test whether low-dose *P. aeruginosa*-derived LPS could enhance mucosal immune defense, we preconditioned mice by intratracheal

administration of LPS followed by respiratory challenge with live *P. aeruginosa*. To determine a physiologically relevant LPS dose, varying doses of LPS were administered intratracheally to adult mice, and acute inflammatory responses were assessed by monitoring core abdominal temperature 24 hours post-administration. High-dose LPS (1.6 mg/kg) caused hypothermia, whereas both 0.16 mg/kg and 0.8 mg/kg induced transient hyperthermia without lethality or overt sickness (Fig. 2d). On the basis of these results, both aged (18 months) and young adult (2 months) mice were treated with 0.16 mg/kg LPS, and core body temperature was monitored daily. Body temperature returned to baseline in all the mice by day 7 after treatment (Fig. 2e, f),

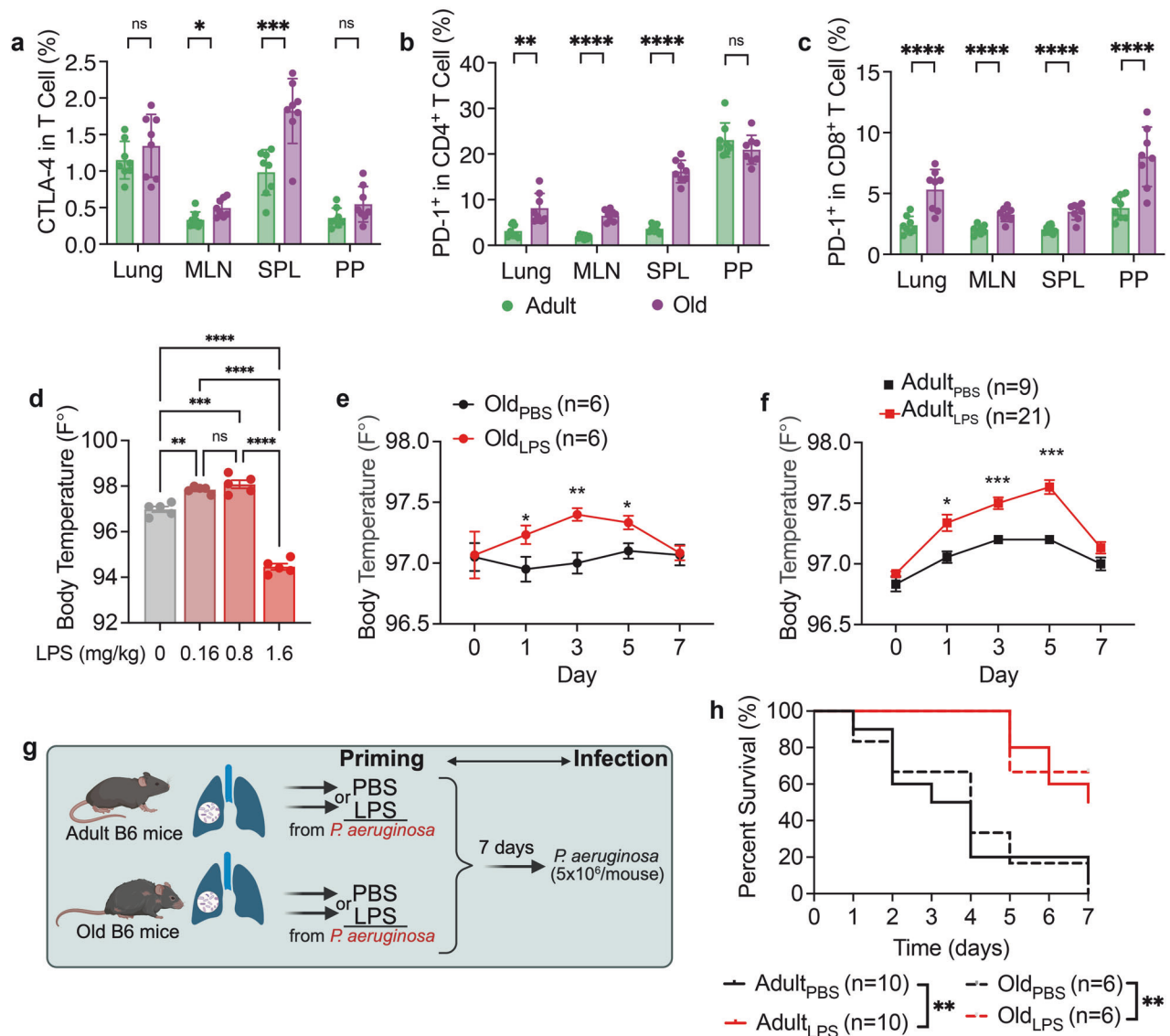


Fig. 2 Low-dose *Pseudomonas aeruginosa* (*P. aeruginosa*) LPS confers protection against bacterial pneumonia in both young adult and aged B6 mice. **a–c** T-cell phenotypes in the lung and secondary lymphoid tissues of young adult (2 months) and aged (18 months) B6 mice (each group, $n = 8$). **a** Frequencies of CTLA-4⁺ T cells, **b** PD-1⁺ CD4⁺ T cells, and **c** PD-1⁺ CD8⁺ T cells. **d** Body temperature was measured 12 hours after the intratracheal administration of different doses of *P. aeruginosa* LPS (each group, $n = 5$). Body temperature over time in aged mice (**e**) and young adult mice (**f**) treated with intratracheal *P. aeruginosa* LPS (0.16 mg/kg) versus PBS controls, measured on days 1, 3, 5, and 7 posttreatment (Old_{PBS}, $n = 6$; Old_{LPS}, $n = 6$; Adult_{PBS}, $n = 9$; Adult_{LPS}, $n = 21$). **g** Schematic of the experimental design: aged and young adult wild-type B6 mice were preconditioned with *P. aeruginosa* LPS, followed by pulmonary challenge with live *P. aeruginosa*. **h** Survival curves following *P. aeruginosa* infection in LPS-pretreated and control mice (Old_{PBS}, $n = 6$; Old_{LPS}, $n = 6$; Adult_{PBS}, $n = 10$; Adult_{LPS}, $n = 10$). Statistical analysis: Data in **a–f** are presented as the mean $\pm$ SEM. **a–c**, **e**, **f** were analyzed using unpaired two-tailed Student's *t* test; **d** was analyzed using one-way ANOVA; and **h** was analyzed using Kaplan–Meier analysis with the log-rank test. ns not statistically significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The data shown are derived from at least two independent experiments. Spl spleen, MLN mesenteric lymph nodes, PP Peyer's patch, Adult_{PBS} 8-week-old mice treated intratracheally with sterile PBS (control group), Adult_{LPS} 8–12-week-old mice treated with 0.16 mg/kg *P. aeruginosa* LPS, Old_{PBS} 18-month-old mice treated with sterile PBS, Old_{LPS} 18-month-old mice treated with 0.16 mg/kg *P. aeruginosa* LPS. **g** was generated using BioRender, and the appropriate publication license was obtained

suggesting that aged mice tolerated this LPS dose and fully recovered from LPS-induced acute inflammation.

Next, we evaluated the effect of *P. aeruginosa*-derived LPS pretreatment on *P. aeruginosa*-induced pneumonia. Both aged and young adult mice received intratracheal administration of LPS followed by intratracheal infection with 5×10^6 CFU of *P. aeruginosa* seven days later. Survival was then monitored (Fig. 2g). Compared with the PBS-treated control mice, all the LPS-preconditioned mice survived significantly better regardless of age (Fig. 2h). Moreover, we performed the same survival experiment in 3-, 6-, and 12-month-old mice and observed consistent protection, with LPS-preconditioned

mice exhibiting significantly improved survival compared with that of PBS-treated controls across these age groups (Supplementary Fig. 6).

Given the comparable degree of protection observed across the full age range tested (Fig. 2h and Supplementary Fig. 6) and in accordance with the 3 R principles of animal research, young adult mice were used for subsequent mechanistic studies.

LPS-induced innate protection against bacterial pneumonia is microbiota dependent and associated with NK cell activation. LPS is known to activate innate immune signaling through the TLR4 pathway.^{16–18} To distinguish the innate immune contribution

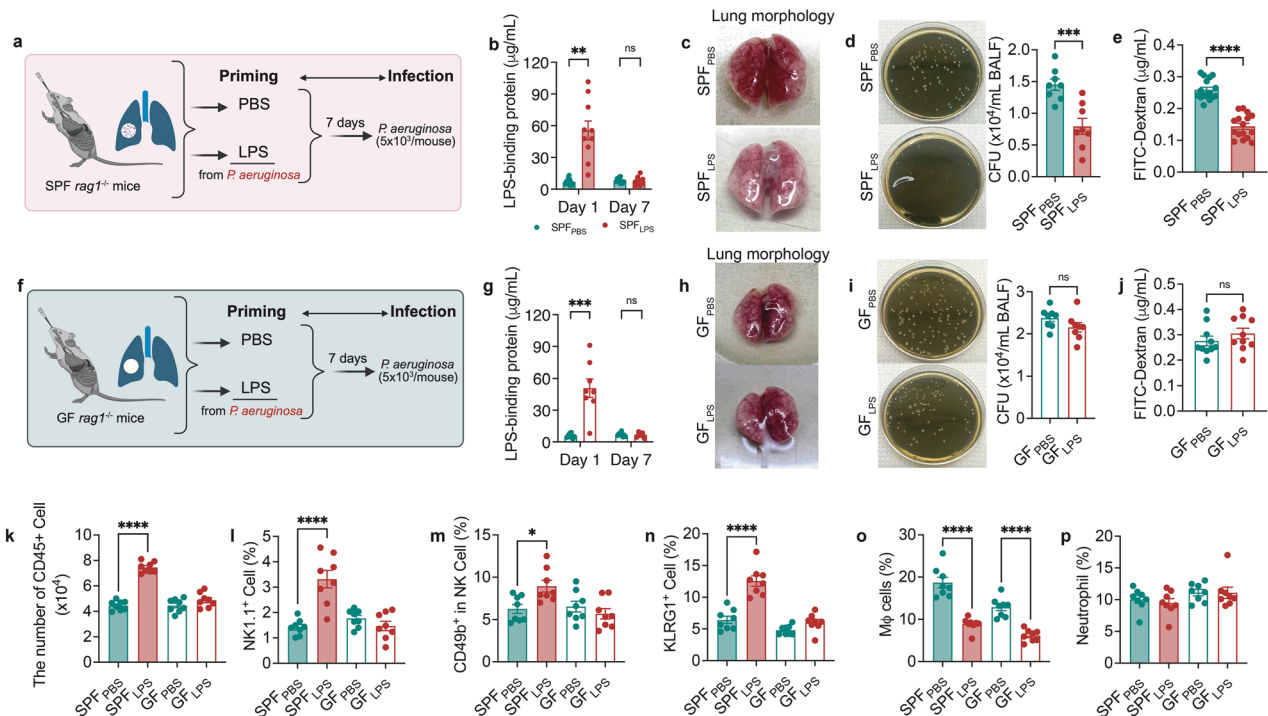


Fig. 3 LPS-induced innate protection against bacterial pneumonia is microbiota dependent. **a** Schematic of the experimental design: specific pathogen-free (SPF) *Rag1*^{-/-} mice were preconditioned with *P. aeruginosa* LPS, followed by pulmonary challenge with live *P. aeruginosa*. **b** Plasma concentrations of lipopolysaccharide-binding protein (LBP) in SPF mice (each group, *n* = 10) on day 1 and day 7. **c** Representative gross morphology of perfused lungs from SPF mice 7 days after *P. aeruginosa* infection. **d** Representative images and quantification of bacterial colonies from bronchoalveolar lavage fluid (BALF) cultured for 24 hours post-infection in SPF mice pretreated with PBS or LPS on day 7 (each group, *n* = 10). **e** Serum FITC-dextran concentrations in SPF mice 7 days after PBS or LPS preconditioning (each group, *n* = 16). **f** Schematic of the experimental design: germ-free (GF) *Rag1*^{-/-} mice were preconditioned with *P. aeruginosa* LPS, followed by pulmonary challenge with live *P. aeruginosa*. **g** Plasma LBP levels in GF mice (each group, *n* = 8). **h** Gross morphology of perfused lungs from GF mice 7 days post-infection. **i** Representative images and quantification of bacterial colonies from the BALF of infected GF mice cultured for 24 hours (each group, *n* = 5). **j** Serum FITC-dextran concentrations in GF mice after PBS or LPS preconditioning (each group, *n* = 10). Flow cytometric analysis of pulmonary immune cell populations in SPF and GF mice 7 days after PBS or LPS treatment: **k** total CD45⁺ leukocyte count, **l** frequency of NK1.1⁺ cells, **m** frequency of CD49b⁺ NK cells, **n** frequency of KLRG1⁺ NK cells, **o** frequency of macrophages, and **p** frequency of neutrophils (*n* = 8 in each group). Statistical analysis: All quantitative data are presented as the mean ± SEM. Comparisons were performed using an unpaired two-tailed Student's *t* test. ns not statistically significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. The data shown are derived from at least two independent experiments. SPFB_{PBS} 8-week-old SPF *Rag1*^{-/-} B6 mice pretreated intratracheally with sterile PBS, SPFL_{PBS} SPF *Rag1*^{-/-} B6 mice pretreated with *P. aeruginosa* LPS, GFB_{PBS} germ-free *Rag1*^{-/-} B6 mice pretreated with PBS, GFL_{PBS} germ-free *Rag1*^{-/-} B6 mice pretreated with *P. aeruginosa* LPS. **a**, **f** were generated using BioRender, and the appropriate publication licenses were obtained

from adaptive immune effects, we utilized *Rag1*-deficient C57BL/6 mice, which lack mature B and T cells. The mice were preconditioned with an intratracheal instillation of *P. aeruginosa*-derived LPS (0.16 mg/kg) and challenged with a sublethal dose (5×10^3 CFU) of *P. aeruginosa* (Fig. 3a) seven days later. As expected, serum levels of LBP were markedly elevated 24 hours after LPS administration but returned to baseline by day 7 (Fig. 3b). The pulmonary morphology in perfused lung tissues collected on day 7 post infection appeared poorer in PBS-treated control mice than in LPS-preconditioned mice (Fig. 3c). Quantification of pulmonary bacterial burden by serial dilution of bronchoalveolar lavage fluid (BALF) and colony formation on ampicillin-containing agar plates revealed significantly reduced bacterial loads in the LPS-preconditioned mice (Fig. 3d). Moreover, LPS treatment preserved pulmonary barrier integrity, as evidenced by reduced permeability, as measured by intratracheal administration of FITC-dextran followed by quantification of FITC-dextran levels in the blood (Fig. 3e). Collectively, these findings demonstrate that *P. aeruginosa*-derived LPS enhances resistance to bacterial pneumonia through innate immune mechanisms in the absence of adaptive immunity, resulting in decreased bacterial colonization and reduced lung damage.

Given the crucial role of the commensal microbiota in shaping mucosal immunity, we next investigated whether the protective effect of LPS was microbiota dependent. Germ-free (GF) *Rag1*^{-/-} C57BL/6 mice were treated with LPS following the same protocol (Fig. 3f). Serum LBP levels increased on day 1 post treatment but returned to baseline by day 7 (Fig. 3g), which is consistent with observations in SPF counterparts. However, LPS preconditioning failed to protect GF mice from *P. aeruginosa*-induced pneumonia, as evidenced by the absence of detectable differences in lung morphology, bacterial burden, or pulmonary permeability between the LPS-treated and control groups (Fig. 3h–j). These findings indicate that the innate immune-enhancing effects of LPS are dependent on the presence of the commensal microbiota.

To examine changes in pulmonary innate immune cell populations following LPS treatment, we isolated nonepithelial single-cell suspensions from the lungs of SPF and GF mice on day 7 post-LPS treatment. Flow cytometric analysis revealed a significant increase in total CD45⁺ immune cells in the lungs of LPS-treated SPF mice but not in those of GF mice (Fig. 3k). Notably, the proportion of NK cells nearly doubled in the LPS-treated SPF mice, whereas no significant changes were observed in their GF counterparts (Fig. 3l and Supplementary

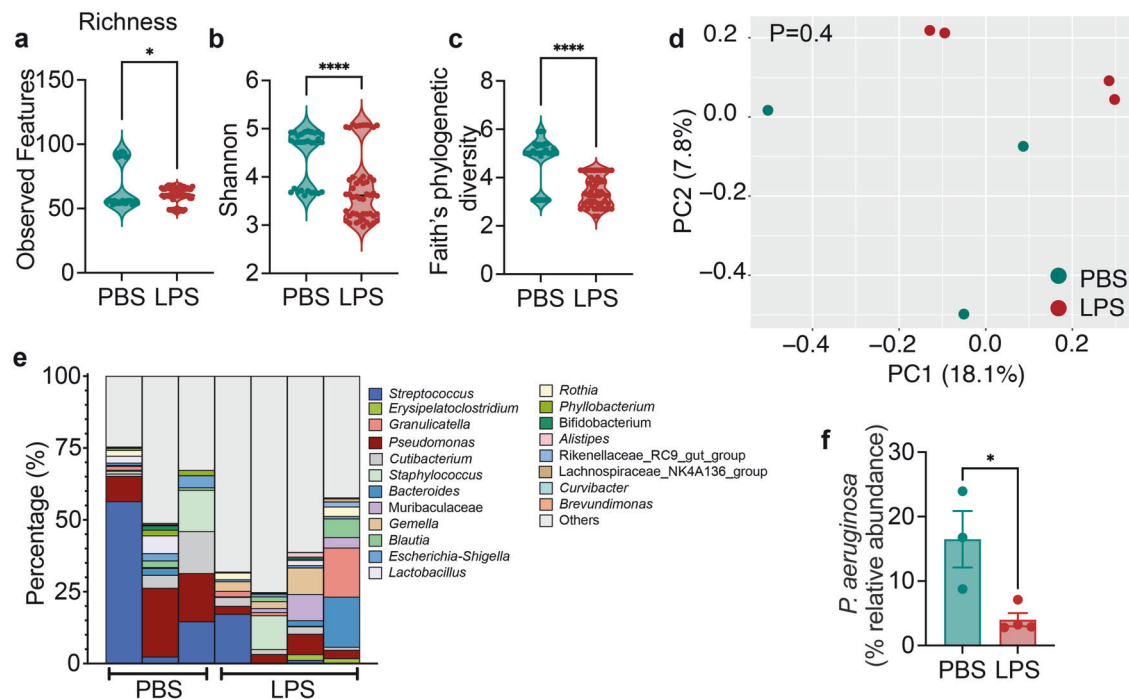


Fig. 4 Lung microbiota profiling in *Rag1*^{-/-} B6 mice following *P. aeruginosa* challenge with or without prior LPS conditioning. The lung microbiota composition was assessed 7 days after pulmonary *P. aeruginosa* infection in SPF *Rag1*^{-/-} B6 mice pretreated with either PBS or *P. aeruginosa* LPS. Alpha diversity was calculated on the basis of 16S rRNA gene sequencing: **a** observed features, **b** Shannon index, and **c** Faith's phylogenetic diversity. **d** PCoA based on Bray-Curtis distances comparing the PBS- and LPS-pretreated groups. **e** Relative abundance of the top 20 most abundant taxa in the lung microbiota of the PBS and LPS groups (PBS, *n* = 3; LPS, *n* = 4). **f** Relative abundance of *P. aeruginosa* in the lungs of PBS- and LPS-treated mice. Statistical analysis: Data in **a–c** were analyzed using the Mann-Whitney U test (Wilcoxon rank-sum test). The data in **f** were analyzed using an unpaired two-tailed Student's *t* test. **P* < 0.05, *****P* < 0.0001. PBS SPF *Rag1*^{-/-} B6 mice pretreated intratracheally with PBS, followed by pulmonary infection with 5×10^3 CFU/mouse *P. aeruginosa*; samples were collected 7 days post-infection. LPS, SPF *Rag1*^{-/-} B6 mice pretreated with *P. aeruginosa* LPS, followed by identical infection and sampling schedules

Fig. 7a). Moreover, both mature (CD49b⁺) and activated (KLRG1⁺) NK cell subsets were selectively expanded in LPS-treated SPF mice (Fig. 3m, n and Supplementary Fig. 7b, c). In contrast, macrophage frequencies were significantly reduced following LPS treatment in both SPF and GF mice (Fig. 3o and Supplementary Fig. 7d), whereas neutrophil populations remained unaffected across all groups (Fig. 3p and Supplementary Fig. 7e).

LPS preconditioning has a minimal effect on overall lung microbiota structure following infection

To evaluate how LPS preconditioning is related to lung microbial community features in the context of ongoing and/or resolving infection, we performed 16S rRNA gene (V3–V4 region) sequencing on lung tissue collected from *Rag1*^{-/-} mice on day 7 post infection. At this time point, the infection itself is expected to influence community composition; therefore, these analyses are intended to compare microbiota features under postinfection conditions rather than infer baseline effects of LPS in the absence of infection. Compared with control mice, LPS-preconditioned mice exhibited significantly reduced bacterial richness (Fig. 4a) and α -diversity (Fig. 4b, c). However, principal coordinate analysis (PCoA) based on the Bray-Curtis distance of amplicon sequence variants (ASVs) revealed no significant differences in overall community composition between the two groups (*P* = 0.4, PERMANOVA with 999 permutations; Fig. 4d). Analysis of the 20 most abundant genera revealed that the relative abundance of *Pseudomonas* was significantly lower in LPS-preconditioned mice (Fig. 4e), which is consistent with the reduced *P. aeruginosa* burden observed in BALF plating assays. Taxonomic assignment of *P. aeruginosa* further confirmed that compared with the control treatment, LPS preconditioning specifically reduced its relative abundance by approximately 80% (Fig. 4f). These findings suggest

that on day 7 post infection, LPS preconditioning is primarily associated with reduced *P. aeruginosa* abundance, without substantially altering the overall lung microbiota structure.

The protective efficacy of LPS against bacterial pneumonia depends on its bacterial origin

LPS is a conserved component of the outer membrane of gram-negative bacteria that activates innate immunity via the TLR4 pathway, largely independent of its microbial origin. However, recent studies have demonstrated that subtle differences in the acylation and phosphorylation patterns of bacterial glycolipids can profoundly influence immune responses, distinguishing proinflammatory from tolerogenic responses in the gut.¹⁹ To determine whether the bacterial source of LPS affects its protective capacity against bacterial pneumonia, after verifying that the two LPS preparations exhibited comparable in vitro biological activity in macrophage assays (Supplementary Fig. 8a–d), we compared the effects of *Escherichia coli* (*E. coli*)-derived LPS with those of *P. aeruginosa*-derived LPS in both SPF and GF *Rag1*^{-/-} C57BL/6 mice (Fig. 5a). Seven days after *P. aeruginosa* challenge, the bacterial colony counts in the BALF did not significantly differ between the *E. coli*-LPS-treated and control mice under either SPF or GF conditions (Fig. 5b). Although *E. coli*-derived LPS modestly preserved lung barrier integrity in SPF mice (Fig. 5c), this effect was markedly weaker than that observed with *P. aeruginosa*-derived LPS (Fig. 3e). Notably, *E. coli*-LPS provided no protection in GF mice (Fig. 5b, c), which is consistent with the dependence on the microbiota observed in response to *P. aeruginosa*-LPS treatment.

To determine whether the protective effect of LPS is pathogen specific and to elucidate the role of innate immunity, we preconditioned SPF *Rag1*^{-/-} mice with LPS derived from either *P. aeruginosa*- or *E. coli* and then challenged the mice with

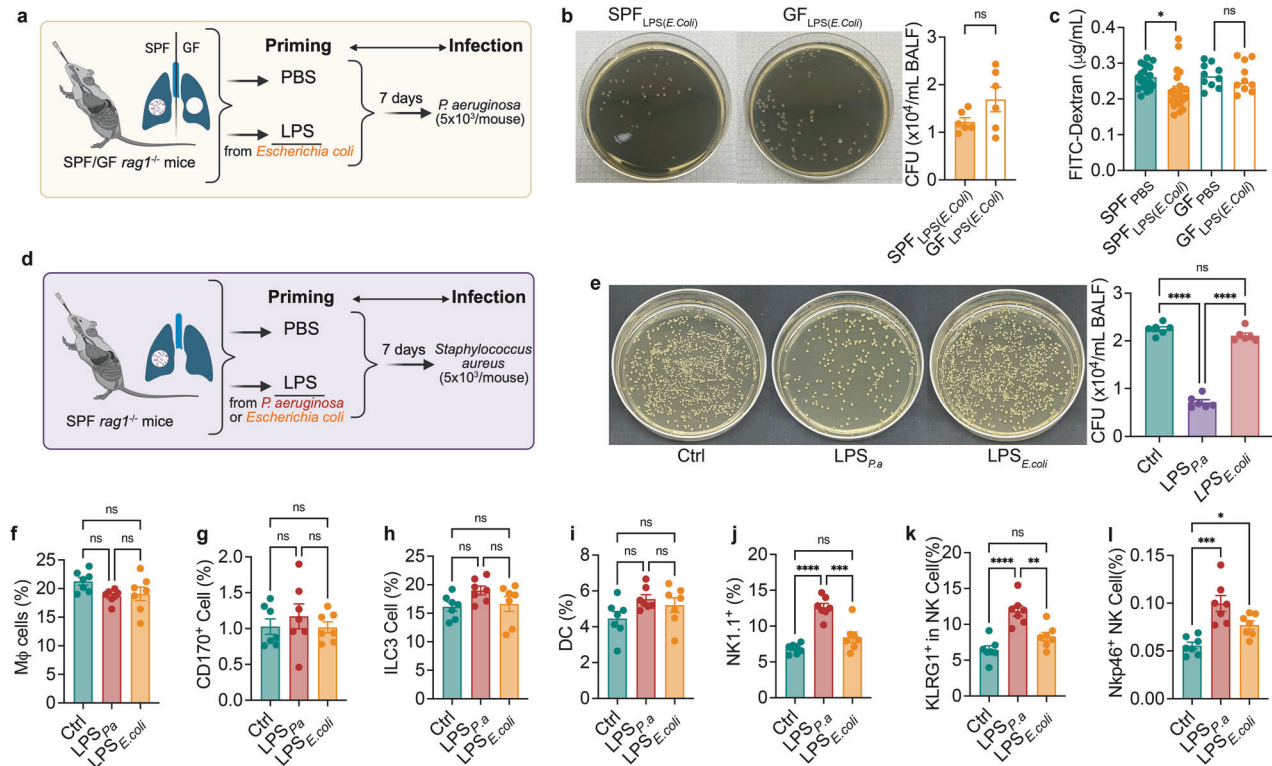


Fig. 5 The protective efficacy of LPS against bacterial pneumonia depends on its bacterial source. **a** Schematic illustration of the experimental design: SPF and GF *Rag1*^{-/-} mice were preconditioned intratracheally with *Escherichia coli* (*E. coli*)-derived LPS, followed by pulmonary challenge with live *P. aeruginosa*. **b** Representative images and quantification of bacterial colonies from the BALF of SPF and GF mice 7 days after *E. coli* LPS preconditioning and *P. aeruginosa* infection (each group, *n* = 6; the SPF control group is shown in Fig. 3d, and the GF control group is shown in Fig. 3i). **c** Serum FITC-dextran concentrations measured 7 days after PBS or *E. coli* LPS preconditioning in SPF and GF mice (SPF_{PBS}, *n* = 20; SPF_{LPS(E. coli)}, *n* = 22; GF_{PBS}, *n* = 10; and GF_{LPS(E. coli)}, *n* = 10). **d** Schematic of the experimental design: SPF *Rag1*^{-/-} mice preconditioned with either *E. coli* or *P. aeruginosa* LPS, followed by pulmonary infection with *Staphylococcus aureus* (*S. aureus*) for 7 days. **e** Representative images and quantification of bacterial colonies from the BALF of SPF mice 7 days after *E. coli* or *P. aeruginosa* (*P.a*) LPS preconditioning and *S. aureus* infection (each group, *n* = 6). Flow cytometry analysis of pulmonary immune cell subsets in SPF mice 7 days after PBS, *E. coli* LPS, or *P. aeruginosa* (*P.a*) LPS treatment revealed differences in the frequencies of **f** macrophages, **g** CD170⁺ cells, **h** ILC3 cells, **i** dendritic cells, **j** NK1.1⁺ cells, **k** KLRG1⁺ NK cells, and **l** Nkp46⁺ NK cells (each group, *n* = 7). Statistical analysis: The data in **b** were analyzed using an unpaired two-tailed Student's *t* test; the data in **c**, **e**, **f**–**l** were analyzed using one-way ANOVA. All values are presented as the mean ± SEM. ns not statistically significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. The data shown are derived from at least two independent experiments. Ctrl PBS-treated control, SPF_{LPS(E. coli)} SPF *Rag1*^{-/-} B6 mice preconditioned with *E. coli* LPS and infected with 5 × 10³ CFU/mouse *P. aeruginosa*, GF_{LPS(E. coli)} GF *Rag1*^{-/-} B6 mice preconditioned with *E. coli* LPS and infected with 5 × 10³ CFU/mouse *P. aeruginosa*, LPS_{P.a} SPF *Rag1*^{-/-} B6 mice preconditioned with *P. aeruginosa* LPS and infected with 5 × 10³ CFU/mouse *S. aureus*, LPS_{E.coli} SPF *Rag1*^{-/-} B6 mice preconditioned with *E. coli* LPS and infected with 5 × 10³ CFU/mouse *S. aureus*. Immune cell profiling in **f**–**l** was performed 7 days after different LPS treatments. **a**, **d** were generated using BioRender, and the appropriate publication licenses were obtained

Staphylococcus aureus USA300 (*S. aureus*), a common gram-positive pathogen that also causes bacterial pneumonia (Fig. 5d). Strikingly, only *P. aeruginosa*-derived LPS significantly reduced the pulmonary *S. aureus* burden, whereas *E. coli*-derived LPS conferred no protection (Fig. 5e). Our results suggest that the bacterial origin of LPS determines its ability to elicit protective innate immune responses against bacterial pneumonia. To identify cellular mediators underlying this differential protection, we analyzed pulmonary innate immune populations by flow cytometry, focusing on lung macrophages (CD45⁺ CD11c⁺ CD11b⁺ F4/80⁺), eosinophils (CD45⁺ CD11b⁺ CD170⁺), ILC3s (CD45⁺ Lin[−] RORγt⁺ CD117⁺), dendritic cells (CD45⁺ CD11c⁺ CD11b[−]), and NK cells (CD45⁺ NK1.1⁺). No significant differences in macrophages, eosinophils, ILC3s, or DCs were detected between the two LPS treatment groups (Fig. 5f–i and Supplementary Fig. 9a–d). In contrast, both total NK cells and activated NK cell subsets (KLRG1⁺ or Nkp46⁺) were significantly greater in *P. aeruginosa*-LPS-treated mice than in *E. coli*-LPS-treated mice (Fig. 5j–l and Supplementary Fig. 9e–g). Although total macrophage numbers remained unchanged (Fig. 5f and Supplementary Fig. 9a), subtype analysis revealed alterations in macrophage subsets following both LPS

treatments (Supplementary Fig. 10a–e), which is consistent with previous reports.^{20,21} However, the macrophage polarization changes did not correlate with the protection phenotype across LPS sources (i.e., similar polarization shifts were observed with *E. coli* LPS despite the absence of protection), suggesting that macrophage polarization alone is unlikely to explain the differential antibacterial protection observed in this study. Systemically, we found that most of the innate immune cell profiles in the spleen were in line with those in lung tissue, indicating that the expansion of activated NK cells (Supplementary Fig. 11a–l) and that localized pulmonary innate immune responses may promote systemic innate immunity, contributing to protection. It is conceivable that the protective effect of *P. aeruginosa*-derived LPS is likely mediated through the activation of natural killer (NK) cells, highlighting the source-specific modulation of innate immunity.

P. aeruginosa-derived LPS elicits lung-specific immune activation with prominent NK cell transcriptional responses. To elucidate the transcriptional programs underlying the differential immunomodulatory effects of LPS, we purified pulmonary

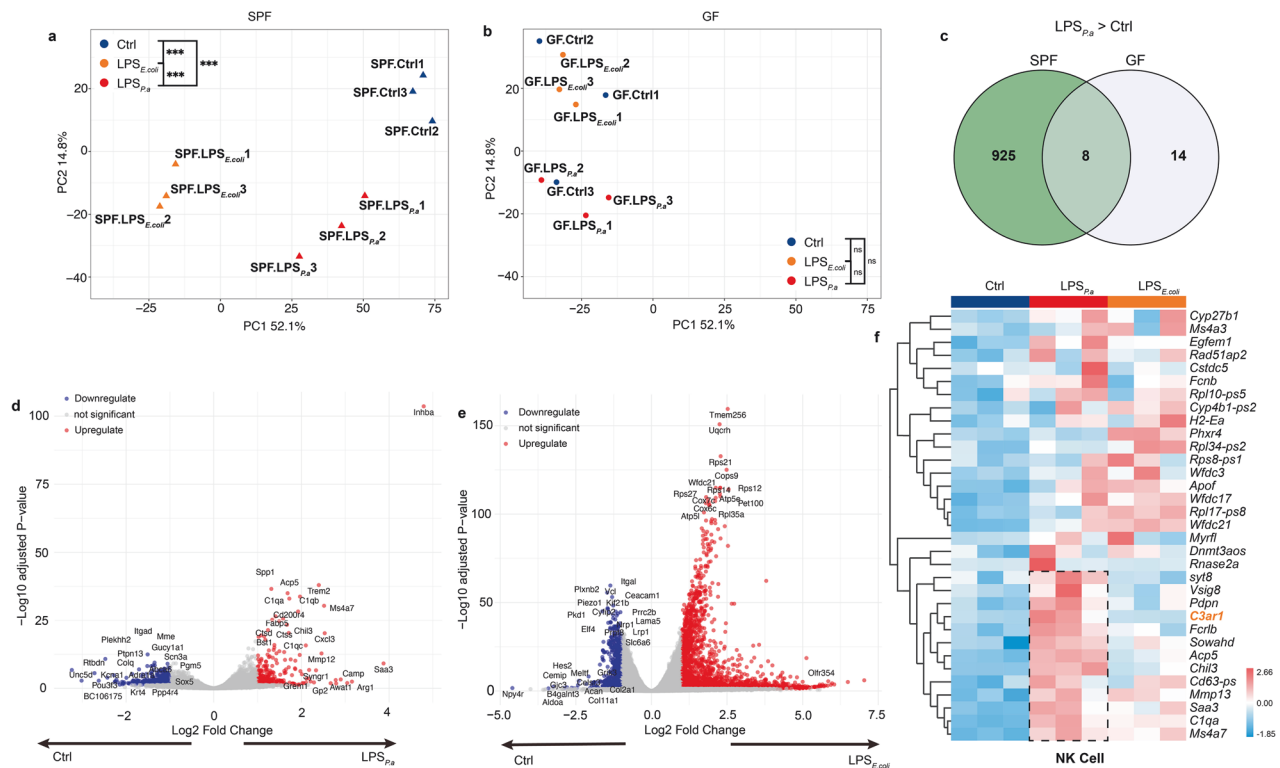


Fig. 6 Lung innate immune transcriptomic responses to LPS preconditioning differ by bacterial source. RNA-seq was performed using CD45⁺ cells purified from lung tissue. Principal component analysis (PCA) of global gene expression in CD45⁺ pulmonary immune cells from **a** SPF and **b** GF *Rag1*^{-/-} B6 mice reveals distinct clustering among the control (Ctrl), *E. coli* LPS (LPS_{E.coli}), and *P. aeruginosa* LPS (LPS_{P.a.}) treatment groups. **c** Venn diagram displaying the number of upregulated genes in LPS_{P.a.}-treated versus Ctrl mice, highlighting gene overlap between the SPF and GF cohorts. Volcano plots of differentially expressed genes (DEGs) between the LPS-treated and Ctrl groups in SPF *Rag1*^{-/-} B6 mice: **d** LPS_{P.a.} vs Ctrl and **e** LPS_{E.coli} vs Ctrl; the x-axis represents the log2-fold change, and the y-axis shows the -log10 adjusted P values. **f** Heatmap of the relative expression levels of key genes associated with NK cell identity in SPF *Rag1*^{-/-} B6 mice after LPS_{P.a.} and LPS_{E.coli} treatments were compared on the basis of ImmGen annotations. Genes significantly upregulated in the LPS_{P.a.} group are highlighted by black dashed boxes. Each group, *n* = 3. Statistical significance of clustering in **a** and **b** was assessed using PERMANOVA (999 permutations) based on Euclidean distances. ****P* < 0.001. Ctrl *Rag1*^{-/-} B6 mice intratracheally administered sterile PBS (vehicle control), LPS_{P.a.} *Rag1*^{-/-} B6 mice preconditioned with 0.16 mg/kg *P. aeruginosa* LPS via intratracheal instillation, LPS_{E.coli} *Rag1*^{-/-} B6 mice preconditioned with 0.16 mg/kg *E. coli* LPS via intratracheal instillation

CD45⁺ cells from SPF and GF *Rag1*^{-/-} mice treated with *P. aeruginosa*-derived LPS, *E. coli*-derived LPS, or PBS as a control and performed bulk RNA sequencing. Principal component analysis (PCA) of global gene expression profiles revealed clear separation among the three treatment groups in SPF mice, with significant differences between all pairs (*P* = 0.001, PERMANOVA with 999 permutations; Fig. 6a). In contrast, the same treatment groups of GF mice were not significantly different (*P* > 0.05, PERMANOVA; Fig. 6b). Differential expression analysis revealed that 933 genes were upregulated in SPF mice compared with only 22 genes in GF mice, with only 8 genes shared between the two conditions (Fig. 6c). Comparative analysis of LPS-induced gene signatures further revealed pronounced transcriptional differences between *P. aeruginosa*-LPS and *E. coli*-LPS treatments (Fig. 6d, e). The genes upregulated by *P. aeruginosa*-LPS included complement-related components and proinflammatory chemokines, whereas those upregulated by *E. coli*-LPS were associated mainly with genes associated with ribosomal (e.g., *Rps21*, *Rps14*, *Rps12*, *Rps27*, *Rpl35a*) and mitochondrial (e.g., *Cox6c*, *Cox7c*, *Pet100*) functions, with limited direct relevance to immune activation.

To gain insight into which immune compartments might contribute to the bulk CD45⁺ lung RNA-seq signals, we applied an ImmGen reference-based deconvolution framework to the transcriptomic data. We emphasize that this analysis infers cell-type contributions and signature enrichment from bulk RNA-seq, rather than providing definitive cell type-resolved transcriptional

changes as would be achieved with single-cell RNA-seq. Using this approach, we found that the transcriptional differences induced by *P. aeruginosa* LPS in SPF mice were most consistent with an NK-cell-associated signature, and we identified a subset of genes whose predicted NK-associated expression increased in response to *P. aeruginosa* LPS but not *E. coli* LPS (Fig. 6f). Among these genes, the complement-related genes *C1qa* and *C3ar1* were particularly notable. *C3ar1*, which encodes complement component 3a receptor 1, has been implicated in immunomodulation, inflammation, chemotaxis, and regulation of NK cell function.^{22,23} Applying the same deconvolution framework, we also detected signature patterns consistent with multiple immune lineages (including dendritic cell- and ILC-associated signatures) in SPF (Supplementary Fig. 12a, b) and GF samples (Supplementary Fig. 13a–c). Notably, in GF mice, we did not observe evidence of an increased NK-associated *C3ar1* expression signal following *P. aeruginosa* LPS exposure (Supplementary Fig. 13a). Together, these inferred transcriptomic patterns, together with our flow cytometric findings, motivated subsequent functional experiments to directly test the role of NK cells in LPS-associated protection against bacterial pneumonia.

P. aeruginosa LPS-primed pulmonary NK cells mediate protection against pneumonia

Given that the ImmGen platform infers cell type-specific gene expression profiles from bulk RNA-seq data, we sought to validate

whether the observed upregulation of *C3ar1* also occurred directly in purified lung-resident NK cells following *P. aeruginosa*-derived LPS treatment. To this end, we purified NK cells from the lungs and spleens of LPS- or PBS-treated *Rag1*^{-/-} mice, as well as lung CD45⁺ cells depleted of NK cells, and assessed *C3ar1* expression by qPCR (Fig. 7a). *C3ar1* expression remained unchanged in the splenic NK cells across the groups. In contrast, compared with control cells, pulmonary NK cells from LPS-treated mice presented approximately 50-fold higher *C3ar1* expression, whereas no difference in *C3ar1* expression was observed in the NK-depleted CD45⁺ population (Fig. 7a). These findings support our transcriptomic results and confirm that *P. aeruginosa*-LPS selectively upregulates *C3ar1* expression in lung NK cells but not in splenic or non-NK lung immune cells. Given that IFN- γ is the primary cytokine produced by NK cells, we next examined whether LPS treatment enhanced NK cell effector function. We cultured purified pulmonary NK cells from LPS- or PBS-treated mice in the presence of different doses of IL-2 and quantified IFN- γ secretion after 48 hours. Compared with control cells, NK cells from the LPS group secreted significantly higher levels of IFN- γ at all the concentrations of IL-2 tested (0.1, 0.5, and 1 KU/mL; Fig. 7b).

Next, we investigated whether LPS-primed pulmonary NK cells could confer protection against *P. aeruginosa* infection in vivo. NK cells were isolated from the lungs of *P. aeruginosa* LPS- or PBS-treated SPF *Rag1*^{-/-} mice and transferred (0.5×10^6 cells per mouse) via intratracheal instillation into naïve wild-type SPF C57BL/6 recipients (Fig. 7c). Twelve hours post-NK cell transfer, we measured IFN- γ levels in the BALF of the recipient mice. Strikingly, the IFN- γ levels were significantly elevated in the BALF from the recipients of LPS-primed NK cells (Fig. 7d). To assess their protective capacity, SPF C57BL/6 mice were challenged intratracheally with live *P. aeruginosa* (5×10^5 CFU/mouse) 7 days after NK cell transfer (Fig. 7c). By day 7 post *P. aeruginosa* infection, gross inspection suggested less apparent lung consolidation in mice that received LPS-primed NK cells (Fig. 7e). Consistent with the enhanced IFN- γ secretion observed in vitro (Fig. 7b), the bacterial burden in the BALF was significantly lower in the LPS-NK recipients, and the colonies appeared more homogeneous, whereas the PBS-NK recipients presented higher bacterial loads and greater heterogeneity in terms of colony appearance—potentially reflecting increased bacterial virulence (Fig. 7f). In addition, NK cells from aged wild-type (WT) mice could also be conditioned by *P. aeruginosa* LPS, resulting in increased *C3ar1* expression (Supplementary Fig. 14a, b), and the adoptive transfer of these LPS-conditioned aged WT NK cells into aged WT recipients reduced the pulmonary *P. aeruginosa* bacterial burden (Supplementary Fig. 14c). The proinflammatory cytokines TNF- α , IL-1 β , and IL-6 were also significantly reduced in the BALF from the LPS-NK recipients (Fig. 7g–i). Flow cytometric analysis confirmed increased total and activated (KLRG1⁺) NK cells in the lungs of the LPS-NK recipients compared with those in the lungs of the controls (Fig. 7j, k and Supplementary Fig. 15a, b). The proportion of alveolar macrophages (CD11c⁺, F4/80⁺, and CD11b^{mid/high}) was also significantly increased (Fig. 7l and Supplementary Fig. 15c), with a greater proportion of these cells displaying an M1 phenotype characterized by elevated CD86 expression (Fig. 7m and Supplementary Fig. 15d), which was likely driven by enhanced IFN- γ signaling.^{22,23} To evaluate adaptive immune responses in recipient mice, we analyzed T-cell and B-cell populations. Total T cells and CD4⁺ T cells remained unchanged (Fig. 7n, o and Supplementary Fig. 15e, f), whereas the number of CD8⁺ T cells did not significantly increase in the LPS-NK (NK_{LPS}) group ($P = 0.057$; Fig. 7p and Supplementary Fig. 15g), possibly due to IFN- γ -mediated bystander activation. No significant differences in total B cells or IgA⁺ B cells were detected between the groups (Fig. 7q, r and Supplementary Fig. 15h, i). Notably, LPS-conditioned NK cells isolated from aged WT donors also reduced the pulmonary

bacterial burden in recipients across multiple ages (3, 6, 12, and 18 months), indicating that this NK cell-mediated protective effect is conserved across the age range tested (Supplementary Figs. 14 and 16). Finally, to determine whether microbiota exposure is required during the LPS-conditioning (donor) phase versus the effector (recipient) phase, we transferred LPS-conditioned pulmonary NK cells (generated as described in Supplementary Fig. 14) into germ-free (GF) C57BL/6 mice. These conditioned NK cells still significantly reduced pulmonary *P. aeruginosa* CFUs in GF recipients following infection (Supplementary Fig. 17). These findings suggest that microbiota exposure is important for establishing an immune-competent context that supports NK-cell conditioning or licensing during the LPS pretreatment phase, whereas the effector phase of protection mediated by conditioned NK cells does not require recipient microbiota.

Taken together, these results demonstrate that pulmonary NK cells preconditioned with *P. aeruginosa*-derived LPS acquire enhanced antimicrobial properties and are sufficient to confer protection against *P. aeruginosa* pneumonia following adoptive transfer. This protective effect is associated with increased IFN- γ production and the activation of local innate effector responses, and it is evident even in germ-free recipients. These findings support a model in which microbiota exposure is most critical during the NK-cell conditioning phase rather than the effector phase of protection.

C3aR1 enhances NK cell cytotoxicity by promoting high-affinity LFA-1-mediated adhesion to target cells

To determine whether LPS-induced C3aR1 upregulation was responsible for the enhanced effector function of NK cells, we isolated pulmonary NK cells from *Rag1*^{-/-} mice pretreated with *P. aeruginosa*-derived LPS and transfected them ex vivo with a pooled siRNA cocktail targeting *C3ar1* (Fig. 8a). After 48 hours, *C3ar1* mRNA levels were reduced by approximately 80% (Fig. 8b), confirming efficient knockdown. To assess the functional consequences of *C3ar1*-silenced NK cells in vivo, we transferred *C3ar1*-deficient NK cells or control NK cells (transfected with nontargeting siRNA) intratracheally into the lungs of wild-type C57BL/6 recipient mice. Twelve hours after transfer, IFN- γ concentrations in the BALF were significantly reduced in mice that received *C3ar1*-deficient NK cells, indicating impaired natural killer (NK) cell activation (Fig. 8c).

To assess the function of C3aR1-silenced NK cells during infection, a parallel cohort of mice was intratracheally challenged with *P. aeruginosa* (5×10^5 CFU/mouse) seven days post-NK cell transfer (Fig. 8a). Compared with control mice, mice receiving C3aR1-silenced NK cells displayed more severe pulmonary congestion following cardiac perfusion (Fig. 8d) and significantly greater bacterial burdens in the BALF (Fig. 8e). Furthermore, cytokine analysis of the BALF revealed elevated levels of TNF- α , IL-1 β , and IL-6 (Fig. 8f–h) in the recipients that received C3aR1-silenced NK cells, which suggested that inflammation was dysregulated and impaired bacterial clearance. Flow cytometric analysis of perfused lung tissue revealed a marked reduction in both total NK cells and activated NK cell subsets in recipient mice that received C3aR1-deficient NK cells (Fig. 8i, j and Supplementary Fig. 18a, b). Alveolar macrophages (CD11c⁺, F4/80⁺ and CD11b^{mid/high}) were also decreased (Fig. 8k and Supplementary Fig. 18c), accompanied by a significant reduction in M1-like macrophages (CD86⁺) (Fig. 8l and Supplementary Fig. 18d). Minimal changes were observed in adaptive immune compartments (Fig. 8m–q and Supplementary Fig. 18e–i), underscoring the predominant role of the innate immune response in our model system. Collectively, these findings demonstrate that C3aR1 is essential for the *P. aeruginosa* LPS-induced NK cell conditioning phenotype, supporting both direct effector functions and downstream orchestration of innate immune responses during pulmonary infection. C3aR1 has been shown to facilitate

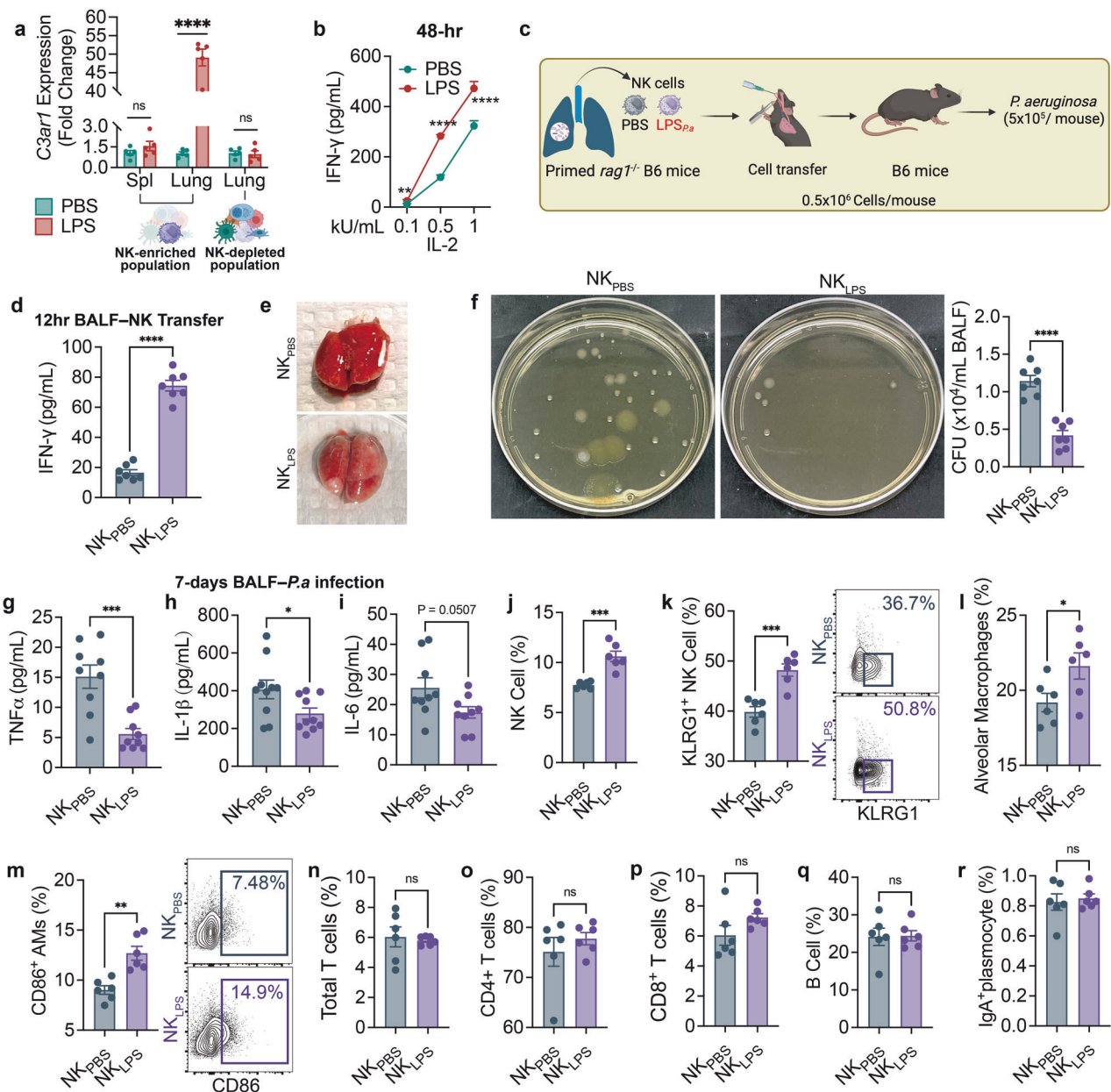


Fig. 7 *P. aeruginosa* LPS-primed pulmonary NK cells mediate protection against pneumonia. **a** Expression of *C3ar1* in NK cells from the spleen and lung and in NK-depleted lung cells isolated from PBS- or *P. aeruginosa* LPS-treated *Rag1*^{-/-} B6 donor mice after 7 days ($n = 7$ /group). **b** IFN- γ levels in the supernatants of pulmonary NK cells isolated from PBS- or LPS-treated *Rag1*^{-/-} mice after 48 h of in vitro culture with varying concentrations of IL-2 ($n = 3$ /group). **c** Schematic of the adoptive transfer experiment: pulmonary NK cells (0.5×10^6 cells/mouse) from PBS- or LPS-treated *Rag1*^{-/-} donor mice were intratracheally transferred into SPF wild-type B6 recipients. The mice were then challenged with intrapulmonary infection with 5×10^5 CFU *P. aeruginosa*, and samples were collected 7 days post-infection. **d** IFN- γ levels in the BALF of recipient mice 12 h after NK cell transfer (each group, $n = 7$). **e** Representative gross morphology of perfused lungs from recipient mice 7 days after infection. **f** Representative images and quantification of *P. aeruginosa* colonies cultured from BALF after 24 hours of culture (each group, $n = 7$). Levels of the inflammatory cytokines **g** TNF α , **h** IL-1 β , and **i** IL-6 in the BALF of the recipient mice after 7 days (each group, $n = 9$). Flow cytometric analysis of CD45⁺ lung immune cells in recipient mice, showing the frequencies of **j** total NK cells, **k** KLRG1⁺ NK cells, **l** alveolar macrophages, **m** CD86⁺ alveolar macrophages, **n** total T cells, **o** CD4⁺ T cells, **p** CD8⁺ T cells, **q** B cells, and **r** IgA⁺ plasma cells (each group, $n = 6$). Statistical analysis: The data in **a**, **b**, **d**, **f**, **g**–**r** are presented as the mean $\pm$ SEM and were analyzed using an unpaired two-tailed Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The data shown are derived from at least two independent experiments. Spl spleen, PBS control donor mice treated with PBS, LPS donor mice treated with *P. aeruginosa* LPS, NK_{PBS} recipient mice receiving pulmonary NK cells from PBS-treated *Rag1*^{-/-} donors, NK_{LPS} recipient mice receiving pulmonary NK cells from LPS-treated *Rag1*^{-/-} donors. **c** was generated using BioRender, and the appropriate publication license was obtained

conformational changes in lymphocyte function-associated antigen-1 (LFA-1, integrin α L β 2/CD11a–CD18) that increase its affinity for intercellular adhesion molecule-1 (ICAM-1), a key adhesion receptor that supports firm leukocyte adhesion and stable cell–cell interactions during immune responses.²⁴ To test

whether the adhesion of LPS-conditioned NK cells is improved via this pathway, we performed a target-cell binding assay using recombinant mouse ICAM-1-Fc-coated plates to assess the affinity of LFA-1 for ICAM-1 (Fig. 8r). Pulmonary NK cells from *P. aeruginosa* LPS-treated mice exhibited significantly increased

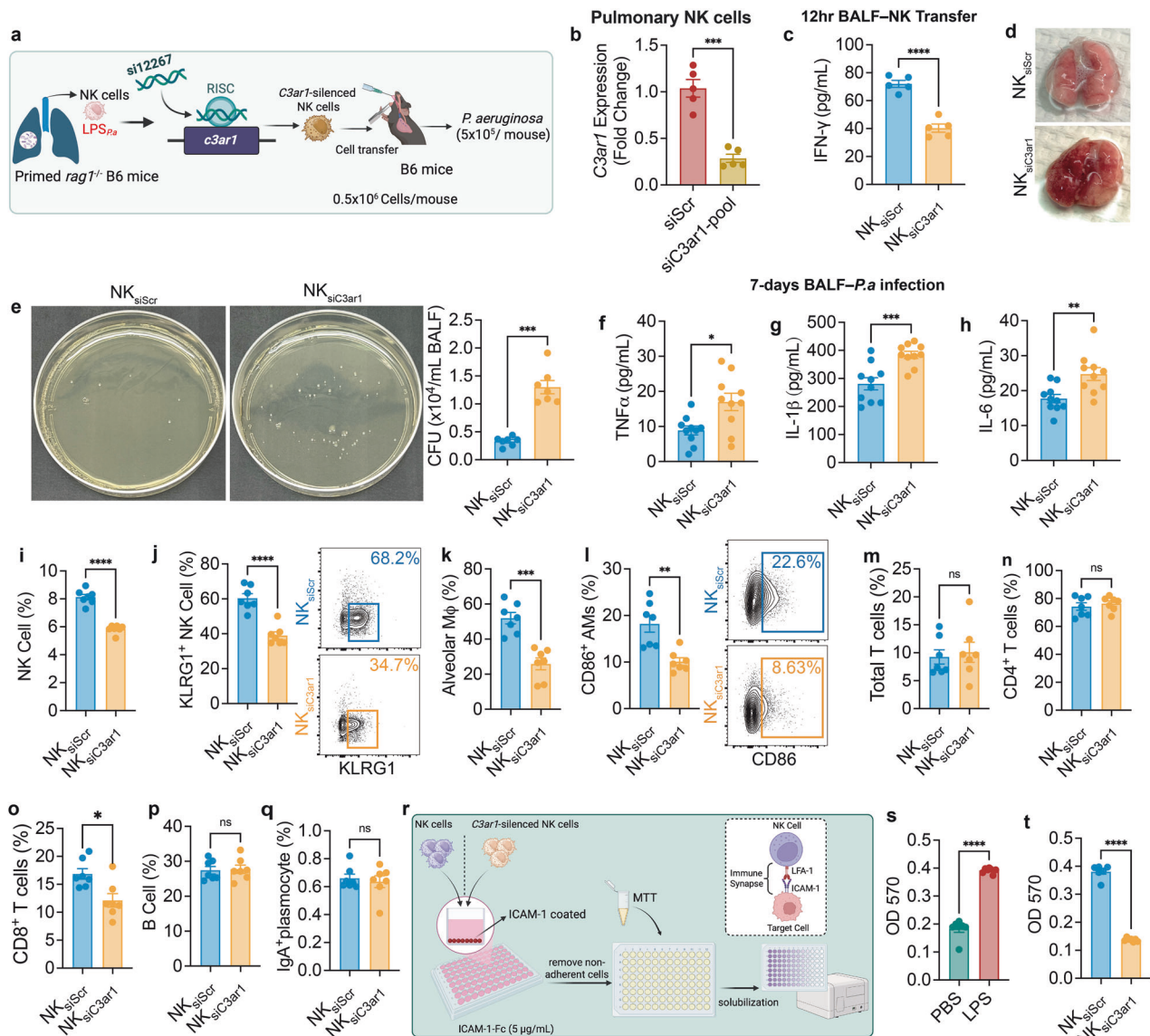


Fig. 8 C3ar1 enhances the target-cell adhesion of pulmonary NK cells following *P. aeruginosa* LPS treatment. **a** Schematic of the adoptive transfer experiment: pulmonary NK cells were isolated from *P. aeruginosa* LPS-treated *Rag1*^{-/-} mice, transfected in vitro with either control siRNA (siScr) or a pool of *C3ar1*-targeting siRNAs (siC3ar1), and intratracheally transferred into SPF wild-type B6 recipient mice. Recipients were then challenged with 5×10^5 CFU of *P. aeruginosa*, after which the lungs were harvested 7 days later. **b** Relative expression of *C3ar1* in pulmonary NK cells after siRNA transfection. **c** IFN- γ concentrations in the BALF of recipient mice 12 hours after NK cell transfer (each group, $n = 5$). **d** Representative gross morphology of perfused lungs from recipient mice 7 days post-infection. **e** Representative images and quantification of *P. aeruginosa* colonies cultured from BALF after 24 h (each group, $n = 7$). Quantification of the levels of **f** TNF α , **g** IL-1 β , and **h** IL-6 in the BALF ($n = 5$ /group). Flow cytometric analysis of lung CD45⁺ immune cells, including the frequencies of **i** total NK cells, **j** KLRG1⁺ NK cells, **k** alveolar macrophages, **l** CD86⁺ alveolar macrophages, **m** total T cells, **n** CD4⁺ T cells, **o** CD8⁺ T cells, **p** B cells, and **q** IgA⁺ plasma cells (each group, $n = 7$). **r** Schematic of the NK cell adhesion assay using recombinant mouse ICAM-1-Fc-coated plates to assess LFA-1-mediated binding. **s** Adhesion of pulmonary NK cells from PBS- and LPS-treated mice (each group, $n = 7$). **t** Adhesion capacity of siScr- and siC3ar1-transfected NK cells (NK_{siScr} and NK_{siC3ar1}) measured by MTT assay (OD₅₇₀ absorbance). Statistical analysis: The data in **b**, **c**, **e**–**q**, **s**, **t** are presented as the mean $\pm$ SEM and were analyzed using an unpaired two-tailed Student's *t* test. ns not statistically significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. The data shown are derived from at least two independent experiments. PBS control mice treated with PBS, LPS mice treated with *P. aeruginosa* LPS, siScr pulmonary NK cells from LPS-treated *Rag1*^{-/-} mice transfected with control siRNA, siC3ar1 pulmonary NK cells from LPS-treated *Rag1*^{-/-} mice transfected with *C3ar1*-targeting siRNA pool, NK_{siScr} recipient SPF wild-type B6 mice receiving siScr-transfected NK cells, followed by *P. aeruginosa* challenge, NK_{siC3ar1} recipient SPF wild-type B6 mice receiving siC3ar1-transfected NK cells, followed by *P. aeruginosa* challenge. **a**, **r** were generated using BioRender, and the appropriate publication licenses were obtained.

binding, as quantified by the MTT signal (Fig. 8s). Importantly, knockdown of C3ar1 abrogated this increase in adhesion, indicating that C3ar1 mediates high-affinity NK–target cell conjugation through LFA-1 (Fig. 8t). Together, these data suggest

that *P. aeruginosa* LPS augments NK cell function by upregulating C3ar1, which promotes high-affinity LFA-1-mediated immune synapse formation, supporting robust IFN- γ production and coordinated activation of local antibacterial effector programs.

DISCUSSION

Lower respiratory tract infections are among the most common causes of mortality in elderly individuals. In the United States, approximately 17.7% of deaths among older adults are attributed to respiratory diseases.²⁵ There is an urgent need to better define the immune mechanisms—particularly innate immune programs—that decline with age but remain critical for protection against common pulmonary pathogens. In this study, we identify a previously unrecognized mechanism by which the innate immune system combats bacterial pneumonia—one of the leading causes of mortality in elderly individuals—through localized conditioning of lung-resident natural killer (NK) cells.

Traditionally, innate immunity is viewed as a nonspecific defense system lacking memory. However, recent advances in the concept of “trained immunity” have shown that certain stimuli—such as β -glucan and BCG vaccines—can induce long-lasting epigenetic and metabolic reprogramming in innate immune cells, thereby enhancing their responsiveness to subsequent, unrelated pathogen challenges, even years later.^{26,27} In contrast, repeated exposure to LPS has historically been associated with endotoxin tolerance, a state in which innate immune cells become hyporesponsive to further stimuli.^{28,29} However, these paradigms sometimes overlook the structural diversity of LPS molecules derived from different bacterial species, which can profoundly modulate immune outcomes.

In contrast to classical trained-immunity paradigms that emphasize broad heterologous protection, our data show that the LPS source/chemotype shapes distinct pulmonary conditioning programs that protect against bacterial pneumonia and can extend to heterologous challenges. This protection is dependent on the host microbiota and is mediated by pulmonary NK cells through the complement receptor C3aR 1 (C3aR1). Adoptive transfer of LPS-conditioned pulmonary NK cells protected naïve mice from pneumonia, whereas C3aR1 knockdown in these NK cells abolished this protection. Unlike systemic immune imprinting, this form of lung NK conditioning represents a localized persistent conditioning phenotype of the innate immune system. Rather than claiming classical trained immunity, we report that distinct LPS sources elicit nonequivalent pulmonary NK-cell conditioning programs. *P. aeruginosa*-derived LPS conditioned pulmonary NK cells and conferred protection against *P. aeruginosa* pneumonia while also providing protection against heterologous bacterial challenge.

C3aR1 signaling has been linked to LFA-1 activation and increased affinity for ICAM-1.²⁴ In solid tumors, excessive adhesion has been proposed to restrain NK cell motility and limit infiltration, whereas in acute airway infection, enhanced LFA-1/ICAM-1-dependent adhesion may instead promote stable immune synapse formation and retention near infected foci.²⁴ Importantly, we do not propose that adhesion alone directly mediates the killing of extracellular bacteria.^{30,31} Rather, we suggest that increased adhesion supports the spatial delivery of NK effector functions, including IFN- γ release and contact-dependent communication with local innate effector cells (e.g., alveolar macrophages), thereby facilitating more effective antibacterial responses in the lung.³²

In our study, the ability of *P. aeruginosa* LPS to enhance pulmonary NK cell function was observed in SPF mice but not in GF mice, suggesting that this conditioning phenotype requires a microbiota-exposed (i.e., immune-competent) host context. Previous studies have shown that microbial colonization during the mouse weaning period promotes immune maturation and enhances host resistance to infections.³³ Consistent with this, GF mice—having developed entirely in the absence of microbial exposure—exhibit broadly immature immune systems, including impaired innate immune cell functionality.³⁴ Under these conditions, *P. aeruginosa* LPS preconditioning failed to increase NK cell responses, suggesting that a baseline level of immune

competence is required for this training effect to occur. However, we wish to emphasize that our findings do not necessarily imply a direct requirement for the microbiota in LPS-mediated NK cell training. Notably, LPS conditioning induced only minimal changes in pulmonary microbial composition, with no significant changes in β -diversity. This suggests that the observed “microbiota dependency” may instead reflect the global immune immaturity characteristic of GF mice rather than a direct role for commensals in driving NK cell training. To directly test whether the pulmonary microbiota contributes to NK cell preconditioning by *P. aeruginosa* LPS, the selective depletion of lung bacteria using antibiotics is needed. However, the complex architecture and large surface area of the lung, with extensively branched airways, make complete and sustained microbial clearance by intratracheal antibiotic delivery technically impractical. These limitations underscore the challenges of dissecting the role of site-specific commensals in shaping local innate immune training. However, our data indicate that microbiota exposure is required primarily during the NK-cell LPS-conditioning phase rather than during the effector phase because the pulmonary bacterial burden is still significantly reduced in response to the transfer of LPS-conditioned NK cells into germ-free recipients following *P. aeruginosa* challenge.

Notably, LPS from *E. coli* failed to confer similar protection. Although both LPS preparations were gently purified using gel-filtration chromatography to preserve the native structure, RNA-seq analyses of pulmonary CD45⁺ cells revealed that only *P. aeruginosa* LPS robustly upregulated genes associated with complement activation and chemotaxis. In contrast, *E. coli* LPS primarily affected ribosomal and mitochondrial function, with minimal effects on immune-related pathways. These differences likely arise from distinct structural features in the lipid A moiety of LPS: *E. coli* LPS contains canonical hexa-acylated, highly phosphorylated lipid A, a potent TLR4 agonist, whereas *P. aeruginosa* LPS contains more heterogeneous, hypophosphorylated lipid A species that elicit weaker TLR4 activation.^{35–38} Such structural nuances may enable low-level *P. aeruginosa* LPS to persist in healthy elderly individuals without triggering overt inflammation while subtly tuning NK cell function toward a heightened state of vigilance.

Importantly, our study highlights the spatial specificity of immune training, reinforcing the concept that the anatomical site of immune activation critically affects functional outcomes. As early as 1969, Siskind and colleagues reported that the location of immune activation profoundly influences immune outcomes.³⁹ Lung-resident immune cells, such as alveolar macrophages and tissue-resident natural killer (NK) cells, have distinct developmental trajectories and functions that are not mirrored by those of their peripheral counterparts.⁴⁰ In support of this notion, previous studies have shown that lung-specific cytokine environments and memory cell formation differ markedly from those in the blood or spleen.^{41,42} Consistent with these findings, we found that C3aR1 expression was selectively upregulated in pulmonary, but not splenic, NK cells following LPS treatment. To ensure this spatial restriction, we delivered LPS intratracheally, avoiding nasopharynx-associated lymphoid tissue (NALT)-driven responses that can arise with intranasal administration.^{43–45} Our data underscore the importance of the local tissue context in shaping immune training and have implications for the design of targeted immunotherapies.

Our adoptive transfer experiments further validated the functional significance of trained NK cells. Compared with those from control mice, lung-derived NK cells from LPS-pretreated mice exhibited highly elevated IFN- γ secretion *in vitro* and enhanced protection when transferred into naïve recipients prior to *P. aeruginosa* challenge. These NK cells promoted M1 macrophage recruitment and reduced the inflammatory cytokine burden without affecting systemic adaptive immunity. Although 2–3-month-old adult mice were used in many experiments in this

study, we also demonstrated that LPS treatment similarly increased antibacterial defense in 18-month-old aged mice, indicating that the underlying mechanisms are conserved across age groups. These findings have potential translational implications. Age and immunosenescence are major risk factors for pneumonia, yet current prophylactic strategies, such as vaccines, often show reduced efficacy in elderly or immunocompromised individuals. Our results raise the possibility that localized, innate immune training, for example, through agents such as hypoinflammatory LPS analogs, could enhance mucosal immunity in a tissue-specific, antigen-independent manner. Importantly, the NK cell-centered mechanism demonstrated in our study may offer a rapid and broad-spectrum alternative to conventional adaptive immunity-based approaches.

To relate these mechanistic findings to human biology, we note that our elderly cohort comprised relatively healthy older adults without overt infection at enrollment. In this setting, the detection of low-level *Pseudomonas*-derived genetic material in the blood is more plausibly interpreted as a feature of aging-associated barrier changes and altered microbial translocation and clearance, rather than evidence of active bacteremia. We propose that such low-grade, chronic microbial exposure may help sustain baseline innate immune tone—particularly NK-cell readiness—in otherwise healthy older adults, thereby partially offsetting age-related immune hyporesponsiveness. One potential source of these signals is increased mucosal barrier permeability with aging, which may permit intermittent, low-level entry of microbial products into the circulation. We speculate that this may represent an adaptive trade-off (or compensatory mechanism): while barrier weakening may increase infection risk, low-grade microbial sensing may help preserve immune vigilance. Importantly, this concept does not contradict the increased susceptibility of elderly individuals to clinical pneumonia, which likely reflects the combined effects of immunosenescence, comorbidities, and exposure to high pathogen burden during acute infection.

We also considered that sex-based immunological differences may contribute to the disparate pneumonia outcomes observed in clinical populations. Compared with women, men exhibit higher pneumonia-related mortality, potentially because of lower estrogen-mediated immune activation and higher smoking prevalence.^{46–50} To minimize confounding biological variability and isolate mechanistic effects, we conducted all the experiments using male mice. However, future studies in female models will be essential to fully elucidate sex-specific responses. Collectively, our findings suggest that the lung-specific, NK-centered mechanism described here may represent a viable nonvaccine-based innate immune intervention tailored for elderly or immunocompromised individuals.

Despite these novel findings, our study has several limitations. First, while our use of germ-free mice demonstrated the microbiota-dependence of LPS-induced NK cell training, the specific microbial taxa, either from the lung or gut, and their relevant metabolites remain undefined. Future studies using targeted reconstitution of lung and gut microbiota could help clarify the role of compartmentalized commensals in shaping local innate immunity, especially given emerging evidence highlighting important crosstalk between the gut and lung (“gut–lung axis”). Second, although intratracheal delivery enables spatial specificity, this approach may not fully recapitulate natural routes of pathogen exposure. It is also not a practical method of therapeutic delivery in humans. The development of clinically feasible delivery platforms, such as aerosolized or nanoparticle-based formulations, will be crucial for the translation of these findings into therapeutic applications. Third, while our study focused on acute bacterial pneumonia, whether similar mechanisms operate in other respiratory diseases remains to be determined.

In conclusion, our study identifies a novel, source-specific mechanism of lung-localized NK cell training driven by the

structurally distinct LPS of *P. aeruginosa*. This paradigm suggests a potential strategy for enhancing pulmonary innate immunity against bacterial pneumonia, particularly in vulnerable populations. Low-grade, localized innate immune training may offer a safer and more targeted alternative to systemic immune activation—particularly in elderly individuals—who remain disproportionately susceptible to severe and often fatal respiratory infections.

MATERIALS AND METHODS

Human cohort and blood bacterial signal detection

Elderly participants were recruited from individuals undergoing routine physical examinations at the Beicai Community Health Service Center of Pudong New District, Shanghai, China. Eligible participants were >65 years old, had no active infectious disease, had not received antibiotics within one month before enrollment, and had no major uncontrolled medical conditions. The study was approved by the Medical Ethics Committee of Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (2017LCSY069) and registered in the Chinese Clinical Trial Registry (ChiCTR1800014364). Written informed consent was obtained from all participants.

Bacterial DNA in human blood was quantified by droplet digital PCR targeting the bacterial 16S rRNA gene. Each sample was analyzed in technical triplicates, with no-template controls, extraction blanks, and environmental exposure controls included to monitor contamination. Serum lipopolysaccharide-binding protein (LBP) levels were measured as a surrogate marker of systemic exposure to Gram-negative bacterial components. For taxonomic profiling, the V3–V4 region of the 16S rRNA gene was sequenced, and potential contaminants were identified and removed before downstream analyses.

Mice and animal experimental design

Male C57BL/6 and *Rag1*^{−/−} C57BL/6 mice were maintained under specific pathogen-free (SPF) or germ-free (GF) conditions at the Yale Animal Resource Center. Young adult mice were 8–12 weeks old, and aged mice were 18 months old unless otherwise indicated. Mice were randomly assigned to treatment groups. Intratracheal administration of PBS, LPS, bacteria, or NK cells was performed under light isoflurane anesthesia in a total volume of 50 μ L. All animal procedures were approved by the Yale University Institutional Animal Care and Use Committee (IACUC; protocol 2022-07911).

LPS conditioning and bacterial pneumonia models

For LPS conditioning, mice received intratracheal PBS or LPS derived from *Pseudomonas aeruginosa* or *Escherichia coli* at 0.16 mg/kg body weight. Bacterial challenge or tissue collection was performed 7 days after LPS or PBS administration. For *P. aeruginosa* pneumonia, mice were challenged intratracheally with live *P. aeruginosa* ATCC 27853. Survival experiments used 5×10^6 CFU/mouse in SPF C57BL/6 mice, whereas non-lethal infection and downstream immunological analyses used 5×10^3 CFU/mouse in SPF or GF *Rag1*^{−/−} mice. For heterologous challenge, SPF *Rag1*^{−/−} mice were challenged with live *Staphylococcus aureus* USA300 at 5×10^3 CFU/mouse 7 days after PBS, *E. coli* LPS, or *P. aeruginosa* LPS conditioning.

Outcome measurements

Bronchoalveolar lavage fluid (BALF) was collected by flushing the airways with cold sterile PBS. Bacterial burden was quantified by plating diluted BALF on LB agar plates and counting colony-forming units. BALF cytokines, including IFN- γ , TNF- α , IL-1 β , and IL-6, were measured by ELISA. Pulmonary permeability was assessed by intratracheal administration of 4 kD FITC-dextran followed by plasma fluorescence measurement. Lung immune cell populations were analyzed by flow cytometry using the gating strategies

shown in Supplementary Fig. 19 and the antibody panel is provided in Supplementary Table 3.

Pulmonary NK-cell isolation, adoptive transfer, and C3aR1 silencing

For adoptive transfer, donor *Rag1*^{-/-} mice were treated intratracheally with PBS or *P. aeruginosa* LPS. Seven days later, pulmonary NK cells were isolated from perfused lungs and transferred intratracheally into naïve SPF C57BL/6 recipients at 5×10^5 cells per mouse. Recipients were challenged intratracheally the following day with live *P. aeruginosa* at 5×10^5 CFU/mouse.

To test the requirement for C3aR1, pulmonary NK cells from *P. aeruginosa* LPS-treated donor mice were transfected ex vivo with C3aR1-targeting siRNA or non-targeting control siRNA for 48 h. C3aR1-silenced or control NK cells were transferred into naïve SPF C57BL/6 recipients, followed by *P. aeruginosa* challenge as described above.

NK-cell adhesion assay

LFA-1-mediated NK-cell adhesion was assessed using recombinant mouse ICAM-1-Fc-coated plates. Freshly isolated pulmonary NK cells were allowed to adhere to coated wells, and adherent cells were quantified by MTT assay at OD570. PMA stimulation, EDTA treatment, and anti-CD11a blocking antibody were used as controls to validate LFA-1-dependent adhesion.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10. Comparisons between two groups were performed using unpaired two-tailed Student's *t* tests, and comparisons among multiple groups were performed using one-way ANOVA unless otherwise indicated. Survival curves were analyzed using Kaplan–Meier analysis with the log-rank test. A *P* value < 0.05 was considered statistically significant.

Supplementary methods

Additional methodological details, including reagent information, primer sequences, antibody panels, sequencing parameters, animal welfare procedures, and extended experimental protocols, are provided in the Supplementary Materials and Methods.

DATA AVAILABILITY

The raw Illumina sequencing data for 16S rRNA gene amplicons derived from human samples have been deposited in the Genome Sequence Archive for Humans (GSA-Human), National Genomics Data Center (NGDC), and China National Center for Bioinformation (CNCB) under accession number HRA014258. The raw RNA-seq data generated in this study have been deposited in the Genome Sequence Archive (GSA), National Genomics Data Center (NGDC), and China National Center for Bioinformation (CNCB) under accession number CRA033687. The raw 16S rRNA gene amplicon sequencing data from lung bacterial samples have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) and are available under BioProject accession number PRJNA1356906.

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AUTHOR CONTRIBUTIONS

X.Y., L.W., R.W., and H.Q.L. were responsible for drafting the manuscript. X.Y., H.Q.L., and Q.H.X. conducted the experiments, including data collection and analysis. Q.H.X. participated in the experimental work and performed data verification. X.Y., R.W., and Q.H.X. also contributed to the analyses of the high-throughput sequencing data. G.J., J.P., H.X.S., D.H., X.M.R., X.C., L.Q.G., H.G., Y.Z., Y.L.H., and L.Z. reviewed and confirmed the experimental results and contributed to all phases of project planning and implementation. E.H. helped with the isolation of pulmonary immune cells and intratracheal administration. F.S.W. contributed to manuscript writing and editing. L.W., R.R.W., and G.J. supervised the overall progress of the study. X.Y., L.W., R.W., and J.G. conceived and directed the experimental design. All the authors have read and approved the article.

ADDITIONAL INFORMATION

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