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Early Decline of Conventional NK Cells During Acute
Pseudomonas aeruginosa Lung Infection

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Abstract

Pseudomonas aeruginosa (PA) is a key pathogen In the context of hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP), challenging clinical medicine. This study intends to clarify the features of the host's innate immune response in the wake of inhalational PA infection. We established a mouse model through aerosolized intratracheal (i.t.) inoculation with PA and performed a comprehensive analysis encompassing protein, cellular, and gene expression levels. Protein analysis revealed a substantial increase in inflammatory proteins in the bronchoalveolar lavage fluid and serum, indicating a robust inflammatory response in the lungs and systemic circulation. Cellular investigations showed an increase in neutrophils, monocytes, and alveolar macrophages (AM) during infection. To assess gene expression changes, RNA-sequencing technology was employed to map the temporal shifts in the transcriptional profile of the host lung post-infection. We analyzed the expression patterns and dynamic transcriptional characteristics of differentially expressed genes (DEGs), describing the inflammation progression. Our results highlighted the critical roles of TNF, IL-17, NF-κB, and MAPK signaling pathways in the immune pathogenesis of PA infection. Noteworthy, we identified genes such as Saa3, Serpina3n, Lcn2, and Orm1, which may interfere with inflammation development and present significant targets for future research. Importantly, Through the analysis of publicly available single-cell RNA sequencing (scRNA-seq) datasets, we observed the reduction in conventional natural killer (cNK) cells, rather than tissue-resident natural killer (trNK) cells in the early stages of PA infection. Our findings provide novel insights into the pathogenesis of PA pulmonary infections and offer valuable suggestions for inhalational infection protection and immunotherapy.

Keywords: Pseudomonas aeruginosa, aerosolized intratracheal inoculation, conventional NK cells, innate immunity, RNA-sequencing

**Introduction**

Pseudomonas aeruginosa (PA) is a parthenogenic anaerobic, Gram-negative bacterium from the Pseudomonas family, recognized as a conditionally pathogenic organism [1]. It interacts directly with host cells via pili, flagella, lipoproteins, lipopolysaccharides (LPS), and a type III secretion system situated in its outer membrane [2-4], It utilizes quorum sensing (QS) to modulate the release of virulence factors, which not only harm host cells but also engage in crosstalk with them via both direct and indirect mechanisms[5], and mediate cytokine production and immune cell recruitment, which is key to the tissue damage, invasion and spread of PA. PA is resistant to a variety of antibiotics and can form biofilms in vivo and on the surface of medical devices [6].Given the rising prevalence of drug resistance in Pseudomonas aeruginosa, the World Health Organization has designated it as a "Priority 1: Critical" pathogen, necessitating urgent research and the development of new effective treatments [7].

Prior research has demonstrated that PA infection triggers the release of proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-18, which contribute to cell death, lung injury, and associated pathology [8-10]. The induction of a potent inflammatory immune response and subsequent lung injury contributes to the mortality caused by PA. Inflammation, as a mechanism used by the body for self-protection, is the first line of defense against tissue damage, directs the immune system to ensure host protection, and is an effective response to injury and infection [11, 12]. However, excessive inflammatory responses can lead to life-threatening diseases such as a cytokine storm [13] and acute respiratory distress syndrome (ARDS) [14]. Excessive inflammation, especially acute pneumonia, is associated with a compromised respiratory system and must be controlled [15]. Therefore, The innate immune response serves as a double-edged sword. An appropriate inflammatory response protects the host, while an excessive response can harm host tissues. A more thorough understanding of the innate immune response related to the pathogenesis of acute PA pneumonia is essential. In recent years, with the changing epidemiology of pneumonia and an increase in opportunistic infections, the innate immune system of the lung has received increasing attention [16]. Future clinical and public health protection requires us to expand the conceptual framework of pneumonia pathogenesis and reduce ineffective treatment and overuse of antibiotics.

In this study, we focus on the characteristics of the host’s innate immune response after PA infection. We used the type culture strain PAO1 to establish an acute pneumonia model via aerosolized intratracheal inoculation, supported by high bacterial burdens and lung injury. We utilized high-throughput liquid-on-chip lab technology to assess the expression of inflammation-related factors by examining crucial cytokines in serum and bronchoalveolar lavage fluid (BALF) at time intervals of 0, 12, 24, 48, and 96 hours after infection with PA.Mouse lungs were sampled at 0, 12, 24, 48, and 96 h after infection with PA. The genome-wide transcriptome expression of these lungs was examined using RNA-seq, and the frequency of different immune cell populations were evaluated using flow cytometry. Through the analysis of single-cell sequencing public datasets, we investigated the dynamic changes in NK cell subsets during the acute infection process. Our study aims to identify targets for intervention in organismal inflammation, with potential applied value for PA infection prevention and immunotherapy.

**Materials and Methods**

Mice and Bacteria Strain

This study follows the ARRIVE guidelines for reporting animal research[17]. Wild-type female C57BL/6 mice, aged 8 weeks and weighing 18-20 grams, were acquired from Vital River Laboratory Animal Technology Co., Ltd., located in Beijing, China. Female C57BL/6 mice with knockouts Il-6-/-, Tnf-/-, Ccl2-/-, Il-1β-/- were purchased from Cyagen (Guangzhou, China). Mice were maintained on a 12:12 h light: dark cycle with free access to food and water under specific pathogen-free (SPF) conditions.All procedures involving animals adhered to the protocols outlined by the Institutional Animal Care and Use Committee (IACUC), and the Academy of Military Medical Science (AMMS) approved all animal experiments under the approval number IACUC-IME-2021-031. Pseudomonas aeruginosa strain PAO1 was kindly provided by the Institute of Pathogen Biology, Chinese Academy of Medical Sciences.

To cultivate PA bacteria, 20 μL of the PAO1 bacterial stock solution was added to 20 mL of brain heart infusion (BHI) broth and shaken at 200 rpm at 37 °C for 16 hours to yield a first-generation stationary phase culture. Next, 100 μL of this culture was transferred to 20 mL of BHI broth and incubated under the same conditions for 4 hours, resulting in a second generation culture at the mid-logarithmic growth phase. This process was repeated by inoculating the second-generation culture in 20 mL of BHI medium at a 1:200 dilution and incubating for another 4 hours to produce a third generation culture also at the mid-logarithmic phase. Finally, the optical density at 600 nm (OD600) of this culture was adjusted to 1.0, correlating to approximately 8×108 CFU/mL, for further experiments.

Mouse Pneumonia Model

Mice underwent aerosolized intratracheal inoculation challenge as previously described [18]. Prior to the experiment, mice underwent weight measurement and received anesthesia through an intraperitoneal injection of 1% sodium pentobarbital solution at a dosage of 70 mg/kg body weight. The trachea of the mouse was visualized with a laryngoscope (Huironghe Company, Beijing, China) and a micro sprayer (Hui Ronghe Company, Beijing, China) was inserted into the trachea for inoculation. Mice were inoculated with different doses (1 × 107 CFU, 5 × 106 CFU, and 1 × 106 CFU/mouse) in 50 µl PBS and control mice were inoculated with 50 µl PBS. The survival of mice was checked twice per day for 7 days post-infection.

Bacterial burdens detection and analysis

Mice were sacrificed at 0, 12, 24, 48, and 96 hpi; the lungs of the mice were dissected, weighed and immersed immediately in 1 ml sterile PBS buffer. The lungs were mechanically homogenized using a MagNA Lyser (Roche, Basel, Switzerland). Blood and lung homogenate were serially diluted and cultured on BHI plates at 37 °C overnight to detect bacterial burdens. Data are expressed as the mean ± SEM log10 CFU per gram of organ and mean ± SEM log10 CFU per ml.

Histopathology analysis

Mice were euthanized at 0, 12, 24, 48, and 96 hours post-infection; their lungs were then preserved in 4% paraformaldehyde and encased in paraffin. Following standard practices, tissue sections were treated with hematoxylin and eosin (H&E) stains. Light microscopy (BX60, Olympus, Japan) was used to identify pathological changes. A qualified pathologist, in a blinded evaluation, assessed the tissue samples using a standardized scoring system: 0 indicated normal; 1 indicated minimal changes; 2 indicated mild changes; 3 indicated moderate changes; and 4 indicated severe changes. The severity of tissue lesions was evaluated based on previously mentioned specific factors[19].

Cytokines/chemokines levels detection and analysis

Mice were sacrificed at 0, 12, 24, 48, and 96 hours post-infection (hpi). The trachea was rapidly exposed with the mice in a dorsal recumbent position. The bronchoalveolar lavage fluid (BALF) and serum were collected by centrifugation at 3000 g for 10 minutes at 4°C. All cytokine assays were performed using the Cytokine & Chemokine 36-Plex Mouse ProcartaPlex™ Panel 1A on the Bio-Plex Multiplex Immunoassay System (Bio-Plex 200, CA, USA).

Flow cytometry detection and analysis

Mice were euthanized at 0, 12, 24, 48, and 96 hours post-infection, and their lungs were immersed in 5 ml of tissue digestion solution for 30 minutes at 37 °C to obtain a single-cell suspension using the gentleMACS™ Octo dissociator (Miltenyi Biotec, Germany). Single-cell suspension treatment and detection method as described previously[20, 21].The following antibodies were used for flow staining: fixable viability stain 510 (BD Biosciences, 564406), BUV395 rat anti-mouse CD45 (BD Biosciences, 564279), BV421TM anti-mouse MerTK (BioLegend, 151510), PE-Cyanine7 anti-mouse Ly6C (BioLegend, 128018), BV650TM anti-mouse Ly6G (BioLegend, 127641), BV605TM anti-mouse CD11b (BioLegend, 101237), PE anti-mouse CD11c (BioLegend, 117308), APC anti-mouse CD64 (BioLegend, 139305), APC-Cyanine7 anti-mouse I-A/I-E (BioLegend, 107628), BV711TM anti-mouse CD19 (BioLegend, 115555), BV421 anti-mouse CD3e (BioLegend, 562600), APC anti-mouse NK1.1 (BioLegend, 108710), BV786 rat anti-mouse CD326 (BD Biosciences, 740958), FITC rat anti-mouse CD31 (BD Biosciences, 561813). Data were analyzed with Flowjo, version 10 (Ashland, OR, USA).

RNA extraction, library construction, and sequencing

Mice were sacrificed at 0, 12, 24, 48, and 96 hpi and lungs were submerged in RNAlater™ stabilization solution (Invitrogen). Total RNA was extracted from the lung tissue using the PureLink™ RNA mini kit (Thermo Fisher Scientific, Waltham, MA, USA). RNA concentration and A260/280 ratio were measured by Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). RNA quality was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to examine RNA integrity. Library construction and sequencing were conducted by the Novogene Company (Beijing, China). Libraries for transcriptome sequencing were constructed with NEBNext® Ultra™ RNA Library Prep Kit (NEB, Ipswich, MA, USA) and qualified libraries were pooled into flowcell and then sequenced on the Illumina sequencing platform (NovaSeq 6000, Illumina, San Diego, CA, USA). After sequencing, low-quality reads of raw data were filtered out to obtain clean data. Clean reads were mapped to the reference genome (mouse) using Hisat2 v2.0.5. Gene expression levels were estimated by calculating the fragments per kilobase of exon per million fragments mapped (FPKM) of each gene [22].

Processing of RNA-seq data

Using edgeR, we identified genes with significant differential expression [23]. The Benjamini & Hochberg method was used to adjust the P-value for multiple tests. Fold Change ≥2 or ≤0.5 and P-value <0.05 was taken as the criterion to identify when a gene was differentially expressed. Principal component analysis (PCA) was used to examine the distribution of samples and assess the quality of the data. Functional enrichment and pathway analysis were conducted using the Gene Ontology (GO) database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database..

Time Series Gene clustering

The temporal expression patterns of differentially expressed genes (DEGs) were analyzed using the Mfuzz R package, employing the fuzzy c-means algorithm for microarray data soft clustering. This method effectively represents the association strength between a gene and its cluster. The analysis included 16 clusters, with a coefficient m of 1.71.

Immune Cell infiltration analysis

ImmuCellAI-mouse (Immune Cell Abundance Identifier for mouse, http://bioinfo.life.hust.edu.cn/) is a tool to estimate the abundance of 36 immune cells or subtypes based on gene expression profiles from RNA-Seq or microarray data [27]. ImmuCellAI-mouse can be used to estimate the difference in immune cell infiltration among diverse groups.

It categorizes the 36 cellular phenotypes into three tiers, utilizing a stratification approach that simulates the methodology of flow cytometry analysis.

PMN and NK cell depletion experiments.

Mice were divided into four groups, with 10 mice per group: PMN depletion group, PMN isotype control group, NK cell depletion group, and NK cell isotype control group. One day prior to infection, mice in the PMN depletion group were intraperitoneally injected with 100 μl of anti-mouse Ly6G/Ly6C (Gr-1) antibody (1 mg/ml), while mice in the PMN isotype control group received 100 μl of mice IgG2b isotype control antibody (1 mg/ml). Similarly, mice in the NK cell depletion group were intraperitoneally injected with 100 μl of anti-mouse NK1.1 antibody (1 mg/ml), and mice in the NK cell isotype control group received 100 μl of mouse IgG2a isotype control antibody (1 mg/ml). The next day, all mice were subjected to aerosol lung infection via liquid aerosol delivery, with an infection dose of 1×106 CFU per mouse. After the infection procedure, mice were returned to individual ventilation cages (IVC) and maintained in a head-elevated position. Three hours post-infection, the mice were monitored for recovery; any unexpected deaths were recorded and those mice were removed from the experimental group. The remaining mice were observed every 12 hours for a total of 14 days, with survival status recorded to generate survival curves.

Single-cell RNA-Seq (scRNA-seq) analysis

The single-cell data were obtained from the NCBI Gene Expression Omnibus (GEO) database, specifically the dataset with the accession number GSE192890. The data were generated using the 10x Genomics platform. The processing steps, including single-cell data quality control, normalization, and cell type identification, were described in a previous study [1]. Detailed analysis was conducted using the R software package Seurat (version 4.4.0). After performing Canonical Correlation Analysis (CCA) for data integration, we proceeded with clustering at a resolution of 0.2. Within this context, we identified that cluster 2 exhibited high expression of the Gzma gene and lacked expression of the Cd3e gene. Based on these gene expression characteristics, we defined cluster 2 as NK cells. Subsequently, we extracted the NK cells for further analysis. UMAP analysis was conducted. Heatmap plots were generated using the dittoSeq (version 1.1.7). we scored every cell using Seurat's AddModuleScore function for the genes within the module. Acquisition of trNK and cNK gene sets from published literature[28]. We utilized the clusterProfiler R package to statistically analyze the enrichment of marker genes within KEGG pathways.

Real-Time PCR

RNA was isolated from lung tissue using RNAprep Pure Tissue Kit (TIANGEN, Beijing, China) according to the manufacturer’s instructions. RNA (1 μg) was used to synthesize cDNA using ReverTra Ace® qPCR RT Master Mix Kit (TOYOBO, ShangHai, China). Primer sequences were listed in Table 2. SYBR green qPCR Master Mix (Servicebio, Wuhan, China) was used following the manufacturer’s instructions with 7500Fast DX Real-time PCR (Life Technologies Holdings Pte Ltd, Singapore). All primers were verified to produce a single specific PCR product using a melting curve program. The relative expression of each gene was calculated using the 2-ΔΔCt method with β-actin as an internal reference.

Statistical analysis

Statistical analysis was done using GraphPad Prism8 software. Statistical analysis of survival curves was done using a log-rank test. P <0.05 was considered statistically significant. Bar graphs are expressed as mean ± SEM.

**Results**

Mouse pneumonia model induced by PA

Mice were intratracheally inoculated with different doses of aerosolized PA to induce pneumonia and then assessed at different time points post-infection (Figure 1A). The mortality rate of mice inoculated with higher doses (1 × 107 CFU and 5 × 106 CFU) of PA was 100% within 48 hpi, while all mice inoculated with the lowest dose (1 × 106 CFU) survived (Figure 1B). Mice inoculated with a dose of 1×106 CFU/mouse had significant body weight decreases at 24 and 48 hpi compared to the control group (Figure 1C). Lung bacterial burdens started to decrease after infection, and most bacteria were cleared from the lungs within 48 hpi (Figure 1D). Over time, blood became bacteria-colonized (Figure 1E). These data suggest that a low-dose PA infection induces transitional pneumonia in which inflammation might contribute to bacterial clearance. Mice infected with 1 × 106 CFU did not show acute mortality and stimulated the body to produce an immune response. Therefore, we selected the dose of 1 × 106 CFU to infect mice for subsequent experiments.

PA infection induces an inflammatory response

The lung tissue was structurally intact with normal tracheal/bronchial and vascular wall structures and no inflammatory changes in the control group mice (0 hpi). Compared with the control group, the lungs of infected mice showed alveolar septal thickening and inflammatory cell infiltration. In addition, the degree of edema and vascular leakage of the lung tissue increased from 12 to 48 hpi, with serious pathological changes of the lung observed at 48 hpi, before beginning to recover after 48 hpi. The histopathological changes in lung tissues indicated a pronounced and progressively escalating infiltration of inflammatory cells, predominantly consisting of neutrophils and monocytes, throughout the course of the infection. (Figure 2A, 12-48 hpi).

Histological scoring revealed that the severity of lung lesions increased from 12 to 48 hpi and the degree of tissue pathological lesions was minimal at 96 hpi. Histopathological analysis confirmed that PA induced acute inflammation and acute lung damage in mice. Accordingly, a mouse model for acute primary PA pneumonia was successfully established through aerosolized intratracheal inoculation, and all subsequent experiments were carried out using this animal model.

We detected inflammatory cytokine secretion during PA pneumonia by assessing the expression of various inflammation-related cytokines and chemokines in the BALF and serum in mice (Figure 2B, C). The results of cytokine assays in BALF and serum of PA-infected mice were visualized using a heat map with darker colors indicating higher levels of cytokine secretion. Large amounts of inflammatory proteins were detected in BALF and serum in infected mice, indicating a severe inflammatory response in the lung. After infection, large amounts of inflammatory proteins such as IL-1β, IL-6, and TNF were detected in BALF and serum of mice, indicating that the inflammatory response of the organism occurred rapidly, secretion levels started to decrease after 48 hpi and had returned to pre-infection levels by 96 hpi.

PA pneumonia alters immune cell infiltration and accumulation in the lung

To investigate the cellular changes in the lungs during acute Pseudomonas aeruginosa infection, we employed flow cytometry to assess the numbers and proportions of different cell populations (Figure 3A, B). After infection at a dose of 1 × 106 CFU, the proportion and number of alveolar macrophages (AM) in mice decreased until 48 hpi (Figure 3C). This may be due to apoptosis triggered by phagocytosis of PA, with AM numbers gradually returning to homeostasis as inflammation subsides. The proportion and number of neutrophils increased significantly until 48 hpi (Figure 3D). A large number of neutrophils were recruited from the peripheral blood to the lungs and triggered an intense inflammatory response, which started to return to homeostasis after 48 hpi. The proportion and number of inflammatory monocytes tended to increase after infection, and Ly6Chi inflammatory monocytes (iMonos) were recruited to the lungs in response to the inflammatory response (Figure 3E). The proportion and number of NK cells decreased after infection (Figure 3F).

Overview of the transcriptomic analysis

In order to assess the reliability of the transcriptomic sequencing data, we conducted principal component analysis (PCA) on the samples, utilizing the transcript expression data. (Figure 4A). The first principal component (PC1) accounted for 43.6% of the total expression variance for the top 1000 most variable genes. Control and PA-infected groups separated along the PC1 axis. The expression matrix of 12, 24, and 48 hpi groups were at a distance from the control group while the the 96 hpi group was closer to the control group. Thus, the PCA revealed clear differentiation among all groups and good duplication within groups and differences in gene expression profiles identify lung intrinsic alterations of transcriptional signatures after PA infection. DEGs were identified using the edgeR package in R software [23] and 6758 DEGs were identified in the lungs at four time points after infection (Figure 4B, C). Overall, the 24 hpi group had the highest number of modulated genes (n = 2452). The number of differentially expressed genes upregulated and downregulated at each time point showed a trend of increasing and then decreasing, suggesting a transitional process of inflammation generation and regression (Figure 4F). A total of 165 upregulated genes and 18 downregulated genes occurred in common for all time points (Figures 4D, 4E). To functionally characterize the transcriptional phenotypes, we performed GO and KEGG enrichment analysis to identify key molecular processes regulated in each group. GO enrichment analysis shows that early after PA infection induced an acute phase inflammation, biological processes associated with the inflammatory response are activated, such as leukocyte migration, response to bacterial molecules, regulation of immune effector processes, and myeloid leukocyte activation (Figure 4G). KEGG pathway analysis (Figure 4H) indicated that pro-inflammatory and M1-like signaling pathways were activated in 12-48 hpi groups. Pathways were mainly enriched in cytokine receptor interaction, TNF, NOD-like receptor and TOLL-like receptor signaling pathways, which play a key role in the intrinsic immune response and recognizing pathogen-associated molecular patterns (PAMPs) that rapidly generate immune responses upon pathogen invasion. Extracellular matrix (ECM) receptor interaction and cell cycle were activated at 96 hpi, these play an important role in tissue repair and regeneration. Overall, our data reveal time-specific gene expression in lung tissues after PA infection, highlighting a molecular profile of distinct pulmonary responses, suggesting that PA infection causes a severe inflammatory response in the lung that leads to acute lung injury, followed by a repair process in lung tissue post-injury that ameliorates inflammation to restore homeostasis.

**Analysis of expression patterns of DEGs**

To gain a comprehensive understanding, differentially expressed genes (DEGs) were grouped into sixteen distinct clusters based on their temporal expression profiles (Figure 5A). The functional processes linked to each temporal cluster were then assessed using Gene Ontology (GO) enrichment analysis (Figure 5B) and KEGG pathway analysis (Figure 5C). Only a few functional processes were commonly enriched, suggesting that the gene sets identified by Mfuzz possess distinct functions. Furthermore, the processes we identified were consistent with the molecular pathophysiology of disease progression.

Clusters 1 and 16 exhibited a pattern of initially decreasing gene expression levels, followed by subsequent increases that was mainly enriched in calcium ion transmembrane transport, the Wnt signaling pathway, associated with cytoskeleton and migration [29], and the Rap-1 pathway, associated with cell junctions and cell adhesion [30], suggesting structural damage in the lung after infection.

Cluster 3 and cluster 8 genes showed a trend of increasing gene expression levels and then decreasing, which were mainly related to immune response regulation, including leukocyte migration, positive regulation of cytokine production, cytokine signaling pathways, TNF, IL-17, NF-κB signaling pathway, and cytokine receptor interactions.

Cluster 2 and 5 exhibited a pattern of initially rising expression levels followed by a decline, primarily linked to processes related to tissue repair, encompassing DNA replication, DNA repair mechanisms, chromosome segregation, and cell cycle regulation.

**Functional modules identified by WGCNA**

WGCNA was performed on the detected DEGs and finally 10 different colored modules were obtained (Figure 6A, B). The gray module is the default module, including the genes that cannot be clustered, and the rest of the module colors were randomly assigned. Modules exhibiting a correlation coefficient exceeding 0.5 between the infection process (hpi) and module genes were selected for enrichment analysis.

TThe black module showed a strong positive correlation with 12 hpi. GO analysis indicated that this module was predominantly associated with the acute inflammatory response, response to lipopolysaccharide, regulation of cytokine production, and leukocyte migration. KEGG analysis indicated enrichment in TNF, IL-17, NF-κB, Jak-STAT, MAPK, Toll-like receptor, NOD-like receptor signaling pathway, and cytokine receptor interaction, which are all highly correlated with an inflammatory response (Figure 6C). The green module was highly positively correlated with 24 hpi. GO analysis was mainly enriched in biological processes such as inflammatory response, leukocyte migration, response to lipopolysaccharide, cytokine production, myeloid leukocyte activation, and response to IFN-γ. KEGG analysis indicated enrichment in signaling pathways such as IL-17, NOD-like receptor signaling pathway, and cytokine receptor interaction (Figure 6D). The turquoise module was highly positively correlated with 48 hpi. GO analysis was mainly enriched in cell division, DNA replication, and positive regulation of the cell cycle. KEGG analysis was mainly enriched in the cell cycle and the p53 signaling pathways, which are associated with maintaining genome integrity and tissue repair (Figure 6E).

To further explore key regulatory hubs, we performed a protein-protein interaction (PPI) analysis of the genes in these significantly enriched modules; the top 10 genes with the most nodes in the three modules are shown in Table 1. Black and green modules were mainly inflammation-related genes such as Tnf, Il6, and Il1b. Turquoise module genes were mainly associated with injury repair, mostly expressed by cycling basal cells and fibroblasts, such as Cdk1, Plk1, Ccnb1, Mad2l1, which are related to cell cycle protein-dependent serine/threonine kinase activity and regulation of the cell cycle, demonstrating that fibroblasts also play an important role in the repair process after acute lung injury.

**Immune cell abundance analysis**

To assess the differential infiltration of various immune cell types in lung tissue at multiple time intervals after infection, we used the ImmuCellAI-mouse tool. Figure 7A shows the overall immune infiltration score, with an overall trend of increasing immediately after infection and then decreasing, suggesting that large numbers of immune cells were recruited to the lung after infection, rapidly provoking an inflammatory response, with the most intense immune cell infiltration in the lung at 24 hpi, followed by the beginning of reduced inflammation. Neutrophils, macrophages, monocytes, and NK T cells all showed a trend of increasing and then decreasing; conversely, NK cells showed a trend of decreasing and then increasing (Figure 7B). () This suggests innate immunity plays a dominant role after infection.

Validation of seleted DEGs associated with inflammatory response by real-time PCR

To validate the reproducibility and repeatability of DEGs identified from transcriptome sequencing, 14 DEGs associated with inflammatory response were selected for qRT-PCR validation, namely, Tnf、Il-1b、Il-6、Cxcl1、Cxcl2、Cxcl5、Cxcl10、Ccl2、Csf2、Saa3、Lcn2、Lcp2、Ncf4、Itgam, using β-actin as an internal reference gene (Figure S1). RT-PCR results showed that the 14 genes showed a trend of increasing followed by decreasing, which was consistent with the trend of gene expression patterns in RNA-seq results,the RNA-seq outcomes reliably indicated gene expression trends, confirming the accuracy of the transcriptome sequencing data.

Survival curve of mice after knockout and depletion of key factors

Subsequently, we selected several factors and cells that play an important role in the anti-infection process for phenotype verification, including IL-1β, Ccl2, IL-6, Tnf, Csf2, neutrophils, AM, and NK cells. For this experiment, two doses were chosen to infect mice, a non-lethal dose of 1 × 106 CFU and a lethal dose of 1 × 107 CFU. The non-lethal dose of PA-induced dramatic and fast 100% mortality in mice depleted of neutrophils (Figure 8A). Using the same non-lethal dose, only 20% of mice survived after AM depletion (Figure 8B). Depletion of NK cells increased mice mortality during infection, with a 60% survival rate at 72 h compared to the control group (Figure 8C).

Similarly, knockout mouse strains experienced mortality with the non-lethal dose. Only 50% of Csf2-/- mice survived the 1 x 106 dose (Figure 8D). The Csf2 gene encodes GM-CSF, the deficiency of which increases the susceptibility of mice to Gram-negative bacteria and causes impaired AM function, resulting in impaired bacterial clearance and decreased survival of mice. Using the lethal (1 × 107 CFU) dose, all control mice died within 48 h, while Ccl2-/- (Figure 8E), Tnf-/- (Figure 8F), Il-1β-/- (Figure 8G), and Il-6-/- (Figure 8H) mice survived longer, suggesting rate of death was slowed, probably due to the reduced degree of inflammatory response and lung injury in mice after pro-inflammatory factor knockout.

A reduction in circulating NK cells has been observed during the early stage of PA pneumonia

By utilizing flow cytometry to detect dynamic changes in NK cell numbers (Figure 3F) during the infection process, combined with deconvolution analysis of transcriptomic data (Figure 7B), we confirmed a trend of decreased NK cell numbers in the early stages of infection. To elucidate the specific changes in NK cells during the infection process, we downloaded single-cell sequencing data of mice with acute infection from public datasets. We extracted NK cell subsets from the dataset for subsequent analysis. After performing Harmony integration and dimensionality reduction clustering (Figure 9A, B), we classified NK cells into trNK and cNK cells. To validate the accuracy of cell classification, we extracted gene signatures of trNK and cNK cells from previously published articles[28] and performed gene set scoring on our defined cells. The results indicated a high accuracy of cell definition (Figure 9C). The comparison of NK cell subsets between different groups revealed that the proportion of cNK cells in the acute group was lower compared to the chronic and control groups (Figure 9D). By extracting high-marker genes from different cell subsets, we found that the gene expression levels varied among different cell subsets (Figure 9E). cNK cells exhibited high expression of genes such as Zeb2, Spn, S1pr5, Cx3cr1, Cma1, Klrg1, Fgl2, Ly6c2, Kcnj8, and Klra9. In contrast, trNK cells showed high expression of genes including Nfkbia, Gimap5, Plscr1, Map3k8, Socs3, S100a8, S100a9, Xcl1, Ctla2a, and Emb. Differential gene enrichment analysis of the two cell subsets revealed that cNK and trNK cells tend to perform different functions, Through KEGG analysis (Figure 9F), it was found that trNK cells tend to be involved in Natural killer cell mediated cytotoxicity, whereas cNK cells are associated with the IL-17 signaling pathway and the TNF signaling pathway. In summary, our analysis of NK cell subsets revealed that during acute pulmonary infection with Pseudomonas aeruginosa in mice, there is a reduction in the number of cNK cells rather than trNK cells. Additionally, different subsets exhibit distinct functional states.

**Discussion**

Pneumonia is a heterogeneous and complex disease; despite advances in diagnosis, treatment, and prevention, it remains a major source of global morbidity and mortality [31]. With the changing epidemiology of pneumonia and the increase in opportunistic infections, the innate immune system of the lung has received increasing attention. Future clinical and public health protection requires us even more to expand the conceptual framework of pneumonia pathogenesis and to reduce ineffective treatment and overuse of antibiotics. In this study, a C57BL/6J mouse model infected with PA strain PAO1 was successfully constructed via aerosolized intratracheal (i.t.) inoculation and evaluated by the survival curve, bacterial burdens, and histopathology.

Subsequently, we investigated the characteristics of the host immune response utilizing this model at the protein, gene, and cellular levels within the lungs of infected mice across various time points. This approach aims to enhance our understanding of pulmonary infections caused by PA, systematically elucidate disease progression, identify potential new intervention targets for managing inflammation within the organism, and provide a foundational reference for protection against inhalational infections and immunotherapeutic strategies for respiratory pathogens.

At the protein level, large amounts of inflammatory proteins were detected in the alveolar lavage fluid and serum of infected mice, indicating an intense inflammatory response in the lungs and organism. IL-1β, IL-6, and TNF-α are typical inflammatory factors with elevated expression in inflammatory states[32-34]. IL-1β and IL-18 play a central role in the development of acute lung injury, both of which further amplify the inflammatory response and induce more pro-inflammatory cytokine secretion[35].

It has been observed that minimizing the inflammatory response, especially by lowering IL-1β levels, results in improved outcomes in acute pulmonary infections caused by Pseudomonas aeruginosa. This includes enhanced survival rates, diminished lung tissue injury, and more effective clearance of bacteria within the airways and lung parenchyma.[36, 37].

TNF-α is mainly secreted by activated macrophages and lymphocytes, and its increased secretion stimulates PMN adhesion to lung capillaries and triggers the secretion of a range of inflammatory molecules[38]. IL-6 promotes innate and adaptive immune responses and drives T cell differentiation[39]. During bacterial infection, T lymphocytes play an important role in the immune response, and Th17 cells mainly secrete IL-17A and IL-22[40]. IL-17A improves bacterial clearance and survival in mice, and IL-17A is important in fighting extracellular bacterial pathogens[41]. During PA infection, IL-22 upregulates the expression of IFN-λ, both of which are protective in mice[42]. IFN-γ promotes innate immunity by activating immune effector pathways, initiating response to LPS and leukocyte migration, and lack of IFN-γ impairs bacterial clearance in mice[43]. GM-CSF stimulates the proliferation and differentiation of neutrophils and monocytes/macrophages, which are critical for monocyte/macrophage lineage development and differentiation[44]. GM-CSF-/- mice have reduced survival and increased bacterial load in the blood and spleen of mice after PA infection, and GM-CSF deficiency increases the susceptibility of mice to Gram-negative bacteria[45]. MCP-1, or Monocyte Chemoattractant Protein-1, recognized as Chemokine (CC-motif) Ligand 2 (CCL2), plays a crucial role in inflammatory processes, where it attracts or enhances the expression of other inflammatory cells and factors such as IL-6 and TNF-α[46]. It has been reported that infection with PA can elevate inflammation by inducing over expression of TNF-α and MCP-1, which leads to the infiltration of inflammatory cells and tissue injury[8].

As lung tissue is a crucial target organ for PA infection and has been relatively understudied in terms of host transcript levels in previous research, it is essential to provide a comprehensive description of the host transcriptome following infection in order to elucidate the mechanisms underlying PA-host interaction.

RNA-Seq is an advanced transcriptome sequencing technology that allows for the examination of dynamic alterations in host gene expression upon pathogen infection, commonly utilized in disease and infection research [47]. Using this technology, we conducted time-resolved RNA sequencing on lung tissues from mice with initial PAO1 pneumonia. We utilized a mix of bioinformatics analysis methods, such as Mfuzz temporal clustering, WGCNA, and ImmuCellAI-mouse, to examine the alterations in lung transcriptional profiles post-PAO1 infection, and to identify crucial genes or other significant research targets. This assists in enhancing our knowledge of the host's innate immune characteristics and the pathogenesis.

Lung tissue, being a crucial target for PA infection and underexplored regarding host transcript levels, necessitates a thorough transcriptome analysis post-infection to elucidate PA-host interactions. Utilizing RNA-Seq, a high-throughput transcriptomic method, we analyzed host gene expression dynamics during PA infection. Among the top 20 genes significantly upregulated at 12-48 hpi in the differential gene volcano map, some are associated with the chemotaxis and activation of immune cells such as Cxcl2, Cxcl3, Ccl3, Ccl9, Cxcr2, Ccr2 and some are associated with inflammatory responses mediated by Gram-negative bacterial PAMPs such as CD14, Saa3. The Serpina3n gene regulates serine-type endopeptidase inhibitor activity and can play an active defense role in pathogenic microbial infestation and has an important role in a range of physiological processes such as immune response and inflammation, and is also associated with chronic obstructive pulmonary disease and cystic fibrosis[48]; Saa3 encodes the inflammatory response acute phase protein SAA3, which can act as an antimicrobial agent. The upstream regulatory sequence of Saa3 promoter has activator response elements such as IL-1 and IL-6, which play an important role in the protective response to infection, trauma and inflammatory stimuli, and the expression of SAA in the acute phase reduces LPS-induced tissue damage[49]; Orm1 encodes an acute phase response protein (orosomucoid-1) that regulates the response of immune cells to TNF-α and can induce monocytes to release the inflammatory cytokines such as IL-1 and IL-6, thereby amplifying the inflammatory response[50], Orm1 also plays a role in cell proliferation, migration and differentiation, apoptosis and tissue repair[51]; Lcn2 encodes a neutrophil gelatinase-associated lipid transport protein (LCN2), and increased expression of LCN2 facilitates inflammation production, binding and sequestering bacterial iron carriers, thereby depriving bacteria of the iron that provides them with nutrients and having an antibacterial effect[52]. Serpina3n and Orm1 have not been reported for PA infection and can be focused as targets for subsequent studies.

WGCNA analysis is suitable for complex data patterns and can be used to study the response at different time points after pathogenic bacteria infection[53]. Both the black and green modules of WGCNA analysis were highly correlated with the inflammatory response, suggesting that the inflammatory response was rapidly provoked after infection and a large number of inflammatory pathways were activated, including TNF, IL-17, NF-KB, NOD-like receptor and Toll-like receptor signaling pathways. PAMPs of PA are recognized by pattern recognition receptors (NLR, TLR), which activate host innate immune responses, induce different signaling pathways and lead to inflammatory responses[54]. During PA infection, the IL-17 family interacts with its receptors and activates downstream pathways to induce the secretion of various pro-inflammatory mediators, such as IL-6, TNF-α and IL-1β, activating innate immune signaling[55]. NF-κB is a classical signaling pathway mediating the inflammatory response in the lung, and LPS is a potent stimulus for triggering MAPK and NF-κB signaling pathways[56]. The turquoise module was highly associated with maintaining genome integrity, tissue repair, and most genes were expressed by circulating basal cells and fibroblasts, suggesting that 48 hpi may be a turning point in the inflammatory response, where the lung tissue parenchyma may switch from an pro-inflammatory phenotype to an anti-inflammatory phenotype, and the biological processes associated with tissue repair were highly activated, with fibroblasts also playing an important role in the injury repair process. We found that the extracellular matrix receptor interaction (ECM-receptor interaction) pathway was upregulated at 96 hpi. The ECM provides anchoring sites for cells and plays a key role in signal transduction and maintenance of homeostasis[57]. ECM not only regulates the local inflammatory response, but also recognizes damage-associated molecular patterns ((DAMPs) to promote interaction with immune cells and tissue remodeling[58], which has positive implications for the repair of tissue damage caused by PA infection. In addition, effective alveolar epithelial repair during lung injury repair may also attenuate fibrosis progression[59]. During lung injury, normally dormant AT2 cells express a large number of cell cycle-related factors and can also secrete and activate matrix metalloenzymes (MMPs), which play an important role in biological processes such as tissue remodeling, tissue defense and immune response[60]. Increased expression of matrix metallase genes such as MMP-3, MMP-8, and MMP-9 were also detected in our RNA-seq data. Tissue repair plays an important role in host survival and restoration of homeostasis, epithelial cells and fibroblasts play important functions in repair, tissue remodeling and regeneration after lung injury, with subsequent attention to the role of non-immune cells in the development of inflammation.

At the cellular level, flow cytometry results showed significant changes in AM, neutrophils, inflammatory monocytes, and NK cells. AM is the first line of host defense in the clearance of extracellular pathogens from the lung, and pulmonary macrophages coordinate the innate immune response during bacterial infection[61]. Pseudomonas aeruginosa exotoxin induces apoptosis in phagocytes[62]. Macrophages and lung epithelial cells undergoing apoptosis can help modulate the immune response, as apoptotic cells are known to possess the ability to suppress immune system functions. Consequently, this process could prevent an extreme immune response, potentially avoiding cytokine storm and life-threatening scenarios [63]. Thus, a controlled apoptotic response in lung cells is necessary for effective clearance of invading PA and prevention of lung infections. Our flow cytometry results showed a decreasing trend in AM numbers after infection, verifying the fact that PA infection leads to AM apoptosis. .Recent findings indicate that PA infection triggers a swift activation of acid sphingomyelinase (Asm), resulting in the creation of ceramide-enriched biofilm platformss. These platforms may facilitate JNK activation, a crucial component of the MAPK signaling pathway, consequently promoting the apoptosis of alveolar macrophages during Pseudomonas aeruginosa infection.[64]. Neutrophils are the most abundant polymorphonuclear and granulocytic leukocytes in the blood, which can be rapidly recruited to the site of inflammation through circulation and are an important component of the innate immune system[65]. A large number of neutrophils are recruited from the peripheral blood to the lungs and trigger an intense inflammatory response, which is important for the host to resist PA acute lung infection. Our data showed that after PA infection, a large number of cytokines related to neutrophil chemotaxis and activation were detected in the lungs, with an exponential increase in the number of neutrophils and massive neutrophil infiltration in the lungs, suggesting that neutrophils are important for host resistance to PA acute infection. Recent studies have revealed that neutrophils have multiple biological functions in innate and adaptive immunity, and can directly interact with other immune cells, thus regulating immune responses[66]. Neutrophils can switch phenotypes to different subpopulations in different microenvironments, and these phenotypes reflect the heterogeneous nature of neutrophils[67, 68]. The phenotypic heterogeneity and functional diversity of neutrophils make them important regulators of inflammatory and immune responses[69], which can be further explored as a research direction in bacterial infections, revealing the differentiation of neutrophils in steady state and during bacterial infections, the functions of different subpopulations.

Monocytes originate from progenitor cells in the bone marrow, circulate through the vasculature and patrol the vascular endothelium, infiltrating through the blood to peripheral tissues in the presence of infection and other inflammatory conditions[70]. The principal subsets of circulating monocytes have been classified as 'inflammatory' monocytes, denoted as Ly6Chi, and 'patrolling' monocytes, referred to as Ly6Clo cells.[71]. The ‘inﬂammatory’ monocytes can enter a developmental program to transform into macrophages, decrease the production of pro-inflammatory cytokines and simultaneously upregulating M2 markers, thus transform into an anti-inflammatory phenotype, which is associated with wound healing and tissue regeneration.[72]. Monocytes are not only required for the generation of macrophages but also contribute to the overall coordination of immunity[73]. Inflammatory monocytes were recruited to the lung in large numbers after PA infection and were involved in regulating neutrophil activation, and a portion may be converted to M2 macrophages during infection to suppress the development of inflammation in a dynamic equilibrium.

NK cells constitute the predominant fraction of the resident lymphocyte population in the pulmonary environment, and they serve a crucial function in mediating the innate immune response within the respiratory system.[74]. Activated NK cells produce INF-γ and exhibit strong cytotoxicity, which is a key factor in antimicrobial immune defense [72]. NK cell depletion led to increased clearance of Listeria monocytogenes[75], and in Streptococcus pneumonia mice, NK cell depletion led to a significant increase in mouse survival[76]. In contrast, depletion of NK cells increased the susceptibility of mice to Pseudomonas aeruginosa[77]. PA invades natural killer (NK) cells and induces phagocytosis-induced cell death (PICD) of lymphocytes. PA-mediated NK cell apoptosis was dependent on activation of MAPK signaling pathway activation and the generation of reactive oxygen species (ROS)[78]. The decrease in the number of NK cells after PA infection was consistent with our results, and the MAPK pathway was also found to be activated after infection in RNA-seq analysis, which was associated with NK cell apoptosis. Through the analysis of single-cell sequencing data, cNK cells and trNK cells are two distinct subsets of natural killer (NK) cells that differ significantly in their tissue localization, activation mechanisms, gene expression profiles, and responsiveness to specific cytokines[28]. we found that during the early stages of Pseudomonas aeruginosa infection in the lungs, the number of cNK cells rapidly decreased while the number of trNK cells remained unchanged. The effects of NK cells on the organism in defense against bacterial infection may be detrimental or beneficial, these contrasting outcomes may be related to different mechanisms of bacterial pathogenesis and bacterial-host interactions. Therefore, focusing on the role of NK cells in PA lung infections may provide new therapeutic ideas that deserve further investigation. Next, we will focus on the functional differences between trNK and cNK cells in lung infection, as well as the reasons behind the decrease in cNK cell numbers while the number of trNK cells remains largely unchanged.

In this study, we constructed a mouse model of PAO1 inhalation infection via aerosolized intratracheal inoculation and systematically revealed the characteristics of the host innate immune response after PA inhalation infection at the protein, cellular, and gene expression levels, demonstrated the progression of inflammation, and found that Serpina3n and Orm1 were not reported about PA inhalation infection, which may be target genes for subsequent studies. NK cells play a crucial role in the acute lung infection caused by Pseudomonas aeruginosa, with cNK cells rapidly decreasing during the early stages of infection.

In summary, our research not only corroborates the findings of earlier investigations into PA pneumoniae pulmonary infections but also offers novel perspectives on these infections, which may serve as valuable references for protective measures against Pseudomonas inhalational infections and for immunotherapeutic strategies.

**Author Contributions**

DZ, LH and HY conceived and designed the experiments. NX and YW performed the experiments. FZ and DS analyzed and interpreted the results. FZ and NX wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

**Date availability statement**

The data that support the findings of this study are openly available in Gene Expression Omnibus (GSE272417) and available on request from the corresponding author.

**Ethics statement**

The animal study was reviewed and approved by the Institute of Animal Care and Use Committee (IACUC) at the Academy of Military Medical Sciences.

**Declarations of Interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1 Top 10 hub nodes in progression associated modules, as identified by genes modules in weighted gene co-expression network analysis (WGCNA)

Table 2 Primers sequences used for RT-PCR in this study

Figure Legends

Figure 1. Pseudomonas aeruginosa induced pneumonia model. (A) Aerosolized intratracheal inoculation of Pseudomonas aeruginosa leads to inflammation responses. (B)Survival curves of C57BL/6 mice infected with 1×107, 5×106, 1×106 CFU PAO1 (n = 10 per group). (C)Time course evaluation of body weight change during infection with 1× 106 CFU/ mouse compared with control group (n = 10 per group). (D-E) Bacterial burdens in lungs and blood of mice at 0, 12, 24, 48, 96h infected with 1× 106 CFU/ mouse. Bar graphs are expressed as mean ± SEM. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and \*\*\*\* P < 0.0001, compared with 0h.

Figure 2. Pseudomonas aeruginosa induced inflammatory response. (A) Lung histological (200×) analysis and pathological scores of infected mice at 0, 12, 24, 48, 96 hpi. Scoring standard: 0, no pathological lesions; 1, minimal; 2, mild; 3, moderate; 4, severe. (B) Histogram of cytokine secretion in bronchoalveolar lavage fluid (BALF) and serum of mice infected with 1×106 CFU PAO1. Bar graphs are expressed as mean ± SEM. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and \*\*\*\* P < 0.0001, compared with 0 hpi. Hpi= hours post-infection.

Figure 3. Dynamic changes of important immune cells in lung tissues were analyzed by flow cytometry. (A) Flow cytometry gating strategy used to identify innate immune cells among myeloid cells in mice. Myeloid cells were gated on CD11c versus CD11b. (B) Flow cytometry gating strategy used to identify lymphocytes and tissue cells. (C-F) Detection and analysis of dynamic changes of immune cells and epithelial cells by flow cytometry. The proportion and number of alveolar macrophages (AMs), neutrophils, inflammatory monocytes (iMonos), natural killer (NK) cells. Bar graphs are expressed as mean ± SEM. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001 and \*\*\*\* P < 0.0001 compared with 0 hpi.

Figure 4. Differentially expressed genes (DEGs) in a mouse model of Pseudomonas aeruginosa-induced pneumonia derived by RNA-seq. (A) Principal components analysis (PCA) of the normalized RNA-seq data of lung tissues in response to Pseudomonas aeruginosa infection. The same color and symbol represent the same stages during infection. (B) Heatmap plot of DEGs displaying the pattern of gene expression. (C) Volcano plot of RNA-seq transcriptome data displaying the pattern of gene expression. Significantly differentially expressed genes (FDR, p ≤ 0.05) are highlighted in red (up-regulated) or blue (down-regulated). Curated genes with specialized biological functions are indicated. (D) Venn diagram comparing the upregulated DEGs. (E) Venn diagram comparing downregulated DEGs. (F) Histogram showing the number of DEGs in differential experimental conditions. (G) Gene ontology (GO) terms in the biological processes describing each condition's upregulated and downregulated genes. (H) Terms from the Kyoto Encyclopedia of genes and Genomes (KEGG) describe each condition's upregulated and downregulated genes. All transcriptome experiments were performed in biological triplicate. DEGs = differentially expressed genes, hpi = hours post-infection.

Figure 5. Cluster analysis of significantly regulated genes and related biological function and KEGG pathway. (A) Clustering by Mfuzz identified 16 distinct temporal patterns of gene expression. (B) Heatmap showing the significance of the GO terms in the biological processes describing each of the 16 clusters. (C) Heatmap showing the significance of the KEGG terms describing each of the 16 clusters.

Figure 6. Genes modules in weighted gene co-expression network analysis (WGCNA) and enrichment analysis of modules that were highly positively correlated with different infection stages. (A) Topological overlap matrix plot showing pairwise gene correlations within each module. Genes within different modules are labeled with different colors according to WGCNA's conventions. (B) Heatmap chart showing module–trait relationships. Red denotes a positive correlation (0 < r < 1), and blue indicates a negative correlation (−1 < r < 0) between the module and infection stages. (C-E) Enrichment analysis of GO BP (biological process) terms and KEGG terms within modules that are highly positively correlated with different infection stages, (C) black module, (D) green module, (E) turquoise module.

Figure 7. Immune cell infiltration analysis. (A) Infiltration score of all samples at different stages post-infection. (B) The abundance of the major innate immune cells at different stages. Bar graphs are expressed as mean ± SEM. \*P <0.05, \*\*P <0.01, \*\*\*P <0.001 and \*\*\*\*P <0.0001, compared to 0 hpi. hpi = hours post-infection.

Figure 8. Survival curve of mice after knockout and depletion of key factors. (A) Survival curve of mice infected with 1×106 CFU PAO1 after depletion of neutrophils by the anti-Gr-1 antibody (n=10). (B) Survival curve of mice infected with 1 × 106 CFU PAO1 after depletion of alveolar macrophages by clodronate liposomes (n=10). (C) Survival curve of mice infected with 1 × 106 CFU PAO1 after depletion of NK cells by the anti-NK1.1 antibody (n=10). (D) Survival curve of Csf2-/- mice infected with 1 × 106 CFU PAO1 (n=8). (E) Survival curve of Ccl2-/- mice infected with 1 × 107 CFU PAO1 (n=10). (F) Survival curve of Tnf-/- mice infected with 1 × 107 CFU PAO1 (n=10). (G) Survival curve of Il-1b-/- mice infected with 1 × 107 CFU PAO1 (n=8). (H) Survival curve of Il6-/- mice infected with 1 × 107 CFU PAO1 (n=10).

Figuer 9. Single-cell sequencing analysis shows a significant reduction in NK cell subsets during the early stages of acute infection. (A) Two-dimensional UMAP visualization of NK cells. (B) The distribution of NK cells in each of the three groups. (C) trNK and cNK score. (D) Boxplot of the proportion of NK subset. (E) Heat map showing the expression of top 10 genes in each NK cell subset. (F) KEGG enrichment analysis in NK cell subset.

Figure S1 . Validation of selected DEGs in response to Pseudomonas aeruginosa lung infection. C57BL/6 mice were infected with 1× 106 CFU of PAO1, and lungs mRNA expression of these DEGs at 0, 12, 24, 48, 96 hpi were detected by real-time PCR (n = 3). Bar graphs are expressed as mean ± SEM.\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, \*\*\*\*P < 0.0001 compared with 0h.