

# Role of immunodeficiency in *Acinetobacter baumannii* associated pneumonia in mice

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## Abstract

**Background:** *Acinetobacter baumannii* (*A. baumannii*) has become one of the most important opportunistic pathogens inducing nosocomial pneumonia and increasing mortality in critically ill patients recently. The interaction between *A. baumannii* infection and immune response can influence the prognosis of *A. baumannii* related pneumonia. The target of the present study was to investigate the role of immunodeficiency in *A. baumannii* induced pneumonia.

**Methods:** Male BALB/c mice were randomly divided into the normal immunity control (NIC) group, normal immunity infection (NIA) group, immune compromised control (CIC) group, and immune compromised infection (CIA) group ( $n = 15$  for each group). Intraperitoneal injection of cyclophosphamide and intranasal instillation of *A. baumannii* solution were used to induce compromised immunity and murine pneumonia, respectively. The mice were sacrificed at 6 and 24 h later and the specimens were collected for further tests. Seven-day mortality of mice was also assessed.

**Results:** After *A. baumannii* stimulation, the recruitment of neutrophils in mice with normal immunity increased sharply ( $P = 0.030$  at 6 h), while there was no significant raise of neutrophil counts in mice with compromised immune condition ( $P = 0.092$  at 6 h,  $P = 0.772$  at 24 h). The Th cell polarization presented with pulmonary interleukin (IL)-4 and interferon (IFN)- $\gamma$  level in response to the *A. baumannii* in CIA group were significantly depressed in comparison with in NIA group (IFN- $\gamma$ :  $P = 0.003$  at 6 h;  $P = 0.001$  at 24 h; IL-4:  $P < 0.001$  at 6 h;  $P < 0.001$  at 24 h). The pulmonary conventional dendritic cell accumulation was even found to be inhibited after *A. baumannii* infection in immunocompromised mice ( $P = 0.033$ ). Correspondingly, *A. baumannii* associated pneumonia in mice with compromised immunity caused more early stage death, more severe histopathological impairment in lung.

**Conclusion:** *A. baumannii* could frustrate the immune response in immunocompromised conditions, and this reduced immune response is related to more severe lung injury and worse outcome in *A. baumannii* induced pneumonia.

**Keywords:** *Acinetobacter baumannii*; Compromised immunity; Dendritic cells; Helper T cell; Neutrophilic granulocytes; Pneumonia

## Introduction

*Acinetobacter baumannii* (*A. baumannii*) which is an emerging nosocomial, opportunistic pathogen causing a wide range of clinical manifestations, has become a significant challenge in intensive care unit (ICU) patients. Although *A. baumannii* is considered to have limited virulence, the occurrence of multiple antimicrobial resistance and the biofilm formation enhance their spread and limit our ability to eliminate *Acinetobacter* species.<sup>[1]</sup> The prevalence of *A. baumannii* colonization/ infection in ICU was 1.23 to 4.35 cases/1000 patient-days causing a mortality of 27.8% to 36.5%.<sup>[2-4]</sup> Our own study showed that *A. baumannii* was the most common multi-drug resistant bacterium in ICU.<sup>[5]</sup> The most frequent clinical

manifestations of *A. baumannii* infection in critically ill patients is pneumonia. The rate of pneumonia in *A. baumannii* infection ranges from 46.2% to 91.9%.<sup>[2,4,6]</sup> Likewise, *A. baumannii* has become one of the most important opportunistic pathogens in nosocomial pneumonia.<sup>[7]</sup> In China, its infection accounted for 25.8% of the hospital-acquired pneumonia (HAP) and 28.4% of the Gram-negative bacteria induced HAP.<sup>[8]</sup>

The specific structures of *A. baumannii* facilitate the biofilm formation and bacteria colonization on skin, conjunctiva, oral, respiratory, rectal, and genitourinary tracts of patients by promoting bacterial adherence to various surfaces.<sup>[9,10]</sup> The colonization rate in critically ill patients detected with *A. baumannii* positive is as high as

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44.3%.<sup>[6]</sup> The colonization on the respiratory tract can advance to pneumonia preferentially in immunocompromised patients.<sup>[4,11,12]</sup> On the other side, *A. baumannii* was also found to be able to impair immune functions.<sup>[13,14]</sup> The interaction of host immunity and *A. baumannii* influences the severity of infection,<sup>[1,15]</sup> as well as the clinical outcome. Correspondingly, immunomodulatory therapeutics in patients with compromised immune status may be a beneficial strategy for treatment of *Acinetobacter* infections. However, it is dependent on a more complete understanding of their mutual effects.

As both innate and adaptive immune response are involved in the pathogenesis of *A. baumannii* induced pneumonia,<sup>[16-18]</sup> in the present study, we observed the response of the neutrophilic granulocytes, conventional dendritic cells (cDCs) and lymphocytes to *A. baumannii* in *A. baumannii* induced pneumonia mice model under different immune conditions to explore the relationship between the immune conditions and the lung injury induced by *A. baumannii* infection.

## Methods

### Animals

Eight- to 10-week-old BALB/c male mice were provided by the Laboratory Animal Center of Yangzhou University (Yangzhou, China). Mice were housed under specific pathogen-free conditions and were given free access to sterile water and certified mouse food. The experimental procedures involving animals in this study complied with the National Research Council's guidelines and were approved by the Care of Experimental Animals Committee of the Southeast University (No. 20140518002).

### Experimental protocol

Mice were randomly divided into four groups ( $n = 15$  for each group): the normal immunity control (NIC) group, the normal immunity with *A. baumannii* infection (NIA) group, the compromised immunity control (CIC) group and the compromised immunity with *A. baumannii* infection (CIA) group. Mice in CIC and CIA groups were intraperitoneally injected with 75 mg/kg cyclophosphamide (CTX, Jiangsu Hengrui Medicine, China) on the first day and 50 mg/kg CTX on the fourth day to induce immunocompromised model. Meanwhile, the mice in the NIC and NIA groups were injected intraperitoneally the same volume of saline. Subsequently, murine pneumonia in NIA and CIA group was induced on the fifth day by intranasal instillation of 50  $\mu$ L bacterial solution containing  $10^8$  colony forming units/mL of *A. baumannii* (ATCC-19606, American Type Culture Collection, ATCC, Manassas, VA, USA) following anesthesia with 10% chloral hydrate. For the NIC and CIC groups, mice were administered with the same volume of saline intranasally. Five mice were sacrificed at 6 or 24 h, respectively after bacteria administration. The left lung was collected to detect the wet weight to dry weight ratio, the right superior lobe was preserved in liquid nitrogen to detect enzyme-linked immunosorbent assay (ELISA) and Western blotting subsequently, the right inferior lobe was fixed

in formalin for subsequent hematoxylin and eosin staining, and the right middle lobe was separated to prepare for flow cytometry. The other five mice in each group were kept for evaluating the 7-day mortality.

### Hematoxylin and eosin staining and lung injury scoring

The right inferior lobe was embedded in paraffin. Then the embedded blocks were sliced sagittally at 5  $\mu$ m thickness. The sections were stained with hematoxylin and eosin. The severity of lung injury was semi-quantitatively evaluated according to the criteria of Smith *et al.*<sup>[19]</sup> Briefly, edema, alveolar, and interstitial inflammation and hemorrhage, atelectasis, necrosis, and hyaline membrane formation were each scored using a 0 to 4 point scale: 0, no injury; 1, injury in 25% of the field; 2, injury in 50%; 3, injury in 75%; and 4, injury throughout the field. The sum of scores reflected the extent of lung injury. Ten randomly selected high-power fields (400 $\times$ ) in each slide were analyzed by two investigators who were blinded to the mouse groups.

### Lung wet/dry weight ratio

Lung edema was determined using the lung wet/dry weight ratio. Briefly, the whole left lung was removed, blotted, and weighed. Subsequently, the lung was placed in an oven at 60°C for 48 h to dry and then was reweighed. Wet/dry weight ratios were then calculated.

### Neutrophilic granulocyte and lymphocyte counts

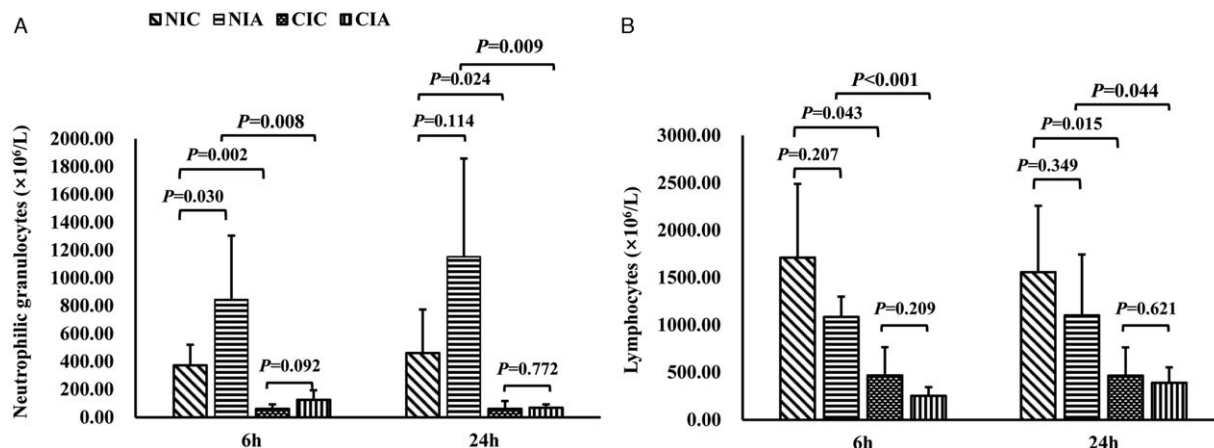
After sacrificing the mice, 0.5 mL blood was collected from the heart for neutrophilic granulocyte and lymphocyte counts using a hematology analyzer.

### Lung cell isolation

Single-cell suspensions were obtained according to our previous protocol to analyze the phenotypes of lung cDCs.<sup>[20]</sup> Briefly, the right middle lobe was finely dissected into small pieces with scissors. It then was digested using type V collagenase (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, digested lung debris were pressed through a 70- $\mu$ m sterile mesh and were cleared of red cells with red blood cell lysis buffer to generate single-cell suspensions.

### Flow cytometry

To analyze the accumulation and maturation of pulmonary cDCs with flow cytometry, the pulmonary single-cell suspensions were stained with monoclonal antibodies including phycoerythrin (PE)-labeled anti-CD11c monoclonal antibody (clone: REA754) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), Percpcy5.5-labeled anti-CD11b monoclonal antibody (clone: M1/70) (eBioscience, San Diego, CA, USA) and fluorescein isothiocyanate (FITC)-labeled anti-MHC II monoclonal antibody (clone: REA813) (MACS Phamingen) after blocking Fc binding. Phenotypic expression was acquired using the flow cytometer (FACS Calibur, BD) and analyzed by Flow Jo software (Treestar, San Carlos, USA). For each analysis, 100,000 events were collected. Cells in the single-cell suspensions isolated from the lung tissue with CD11c+ and



**Figure 1:** Neutrophilic granulocytes and lymphocytes in mice of *A. baumannii*-induced pneumonia with different immune conditions. Blood neutrophilic granulocytes (A) and lymphocytes (B) in mice with or without *A. baumannii* instillation in normal or compromised immune conditions at 6 and 24 h were analyzed. *n* = 5 for each group at each time point. CIA: Immune compromised infection; CIC: Immune compromised control; NIA: Normal immunity infection; NIC: Normal immunity control.

CD11b+ were defined as cDCs. MHC II+ in CD11c+ and CD11b+ lung cells reflect the maturation of cDCs. Each sample derived from one mouse was analyzed one time.

**ELISA**

Level of interferon (IFN)- $\gamma$  and interleukin (IL)-4 in lung homogenates were assessed by the commercialized sandwich ELISA kit (Cusabio Biotech Co., Ltd, China) strictly according to the instructions of the manufacturer. The concentrations of these cytokines were calculated from a standard curve and were expressed in picograms or nanograms/milligram lung tissue. Each sample was duplicated tested.

**Western blotting**

Total protein lysates were extracted from the right superior lobe using radio-immunoprecipitating assay lysis buffer (Beyotime Institute of Biotechnology, China). Following separation by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were electro-transferred to polyvinylidene fluoride membranes (Millipore, Bedford, USA). These were then blocked for 1 h at room temperature and were incubated at 4°C overnight with primary antibodies to GATA-3, T-bet, and  $\beta$ -actin (Santa Cruz Biotechnology, Santa Cruz, USA), respectively. On the following day, the immunoreactive bands were detected with a chemiluminescence imaging system (ChemiQ 4800mini, Ouxiang, China) after incubation with a horseradish peroxidase-conjugated secondary antibody (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at room temperature. Each protein was repeated three times with protein collected from different mice.

**Statistical analyses**

Continuous variables were presented as the mean  $\pm$  standard deviation and the median (interquartile range) based on the distribution of quantitative variables. Statistical analyses were performed using SPSS 12.0 (SPSS Inc.,

Chicago, IL, USA). Groups with normal distribution were compared using Student’s *t* test or one-way analysis. Non-parametric tests of comparison were used for variables evaluated as not normally distributed. The differences in categorical variables were assessed using the  $\chi^2$  test or the Fisher exact test.

**Results**

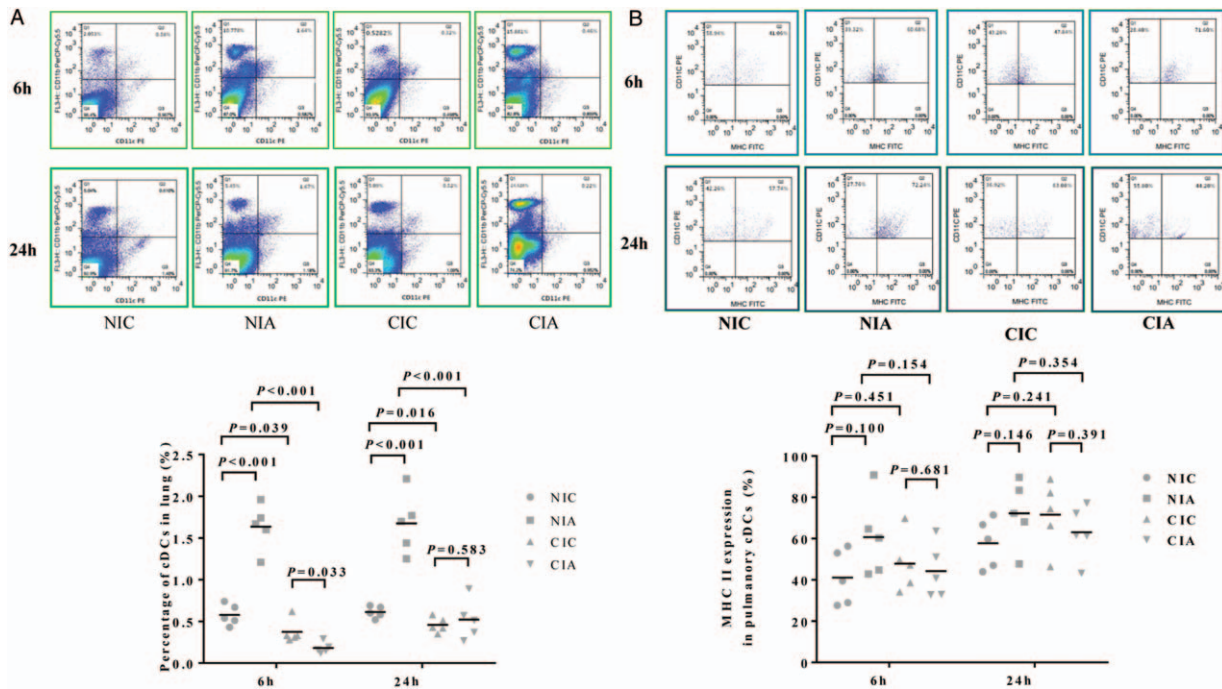
**Induction of immunocompromised condition with CTX**

The immunocompromised mice exhibited decreased appetite and activity, dullness and tarnished fur after CTX treatment. Compared with control mice in the NIC group, a significant reduction of neutrophilic granulocytes was observed in the CIC group (CIC vs. NIC:  $62.00 \pm 31.14 \times 10^6/L$  vs.  $374.00 \pm 147.24 \times 10^6/L$  at 6 h, *P* = 0.002;  $62.00 \pm 55.41 \times 10^6/L$  vs.  $462.50 \pm 311.17 \times 10^6/L$  at 24 h, *P* = 0.024) [Figure 1A]. There was also an inhibition of lymphocytes in CIC group compared with NIC group ( $470.00 \pm 296.31 \times 10^6/L$  vs.  $1712.50 \pm 776.80 \times 10^6/L$  at 6 h, *P* = 0.043;  $468.00 \pm 296.77 \times 10^6/L$  vs.  $1560.00 \pm 699.29 \times 10^6/L$  at 24 h, *P* = 0.015) [Figure 1B]. The pulmonary cDC counts defined as CD11c+ and CD11b+ with flow cytometry was less in CIC group than in NIC group ( $0.37\% \pm 0.14\%$  vs.  $0.58\% \pm 0.12\%$  at 6 h, *P* = 0.039;  $0.46\% \pm 0.09\%$  vs.  $0.61\% \pm 0.07\%$  at 24 h, *P* = 0.016) [Figure 2A], but there was no difference in the maturation percentage of cDCs (CD11c+, CD11b+, MHC II+) between these two groups ( $47.84\% \pm 13.78\%$  vs.  $41.06\% \pm 13.29\%$  at 6 h, *P* = 0.451;  $71.60\% \pm 16.41\%$  vs.  $57.74\% \pm 12.02\%$  at 24 h, *P* = 0.241) [Figure 2B].

**Effect of A. baumannii on neutrophilic granulocytes in mice with different immune conditions**

In mice with normal immunity, the neutrophilic granulocyte counts after *A. baumannii* stimulation (NIA) increased about 2.5 to 2.8 times compared with NIC mice ( $846.00 \pm 459.71 \times 10^6/L$  vs.  $374.00 \pm 147.24 \times 10^6/L$  at





**Figure 2:** The effect of *A. baumannii* instillation on the quantity and maturation of pulmonary cDCs in mice with different immune conditions. (A) The percentage of cDCs (CD11c+ and CD11b+) in the single-cell suspensions isolated from the lung tissue in each group was analyzed using flow cytometry. (B) The percentage of mature pulmonary cDCs marked with MHCII+ in CD11c+ and CD11b+ was examined using flow cytometric analysis. *n* = 5 for each group at each time point. cDCs: Conventional dendritic cells; CIA: Immune compromised infection; CIC: Immune compromised control; NIA: Normal immunity infection; NIC: Normal immunity control.

6 h,  $P=0.030$ ;  $1154.00 \pm 704.61 \times 10^6/L$  vs.  $462.50 \pm 311.17 \times 10^6/L$  at 24 h,  $P=0.114$ ). However, there was no significant raise of neutrophil counts in mice with compromised immune condition at 6 or 24 h of bacteria activation (CIA vs. CIC:  $126.00 \pm 68.04 \times 10^6/L$  vs.  $62.00 \pm 31.14 \times 10^6/L$  at 6 h,  $P=0.092$ ;  $70.00 \pm 22.36 \times 10^6/L$  vs.  $62.00 \pm 55.41 \times 10^6/L$  at 24 h,  $P=0.772$ ). Additionally, as for the neutrophilic granulocytes after bacteria stimulation in mice with different immune condition, it was found that neutrophilic granulocytes in mice with compromised immune condition (CIA) were significantly less than neutrophilic granulocytes in mice with normal immune condition (NIA) ( $126.00 \pm 68.04 \times 10^6/L$  vs.  $846.00 \pm 459.71 \times 10^6/L$  at 6 h,  $P=0.008$ ;  $70.00 \pm 22.36 \times 10^6/L$  vs.  $1154.00 \pm 704.61 \times 10^6/L$  at 24 h,  $P=0.009$ ) [Figure 1A].

**Effect of *A. baumannii* on lymphocytes in mice with different immune conditions**

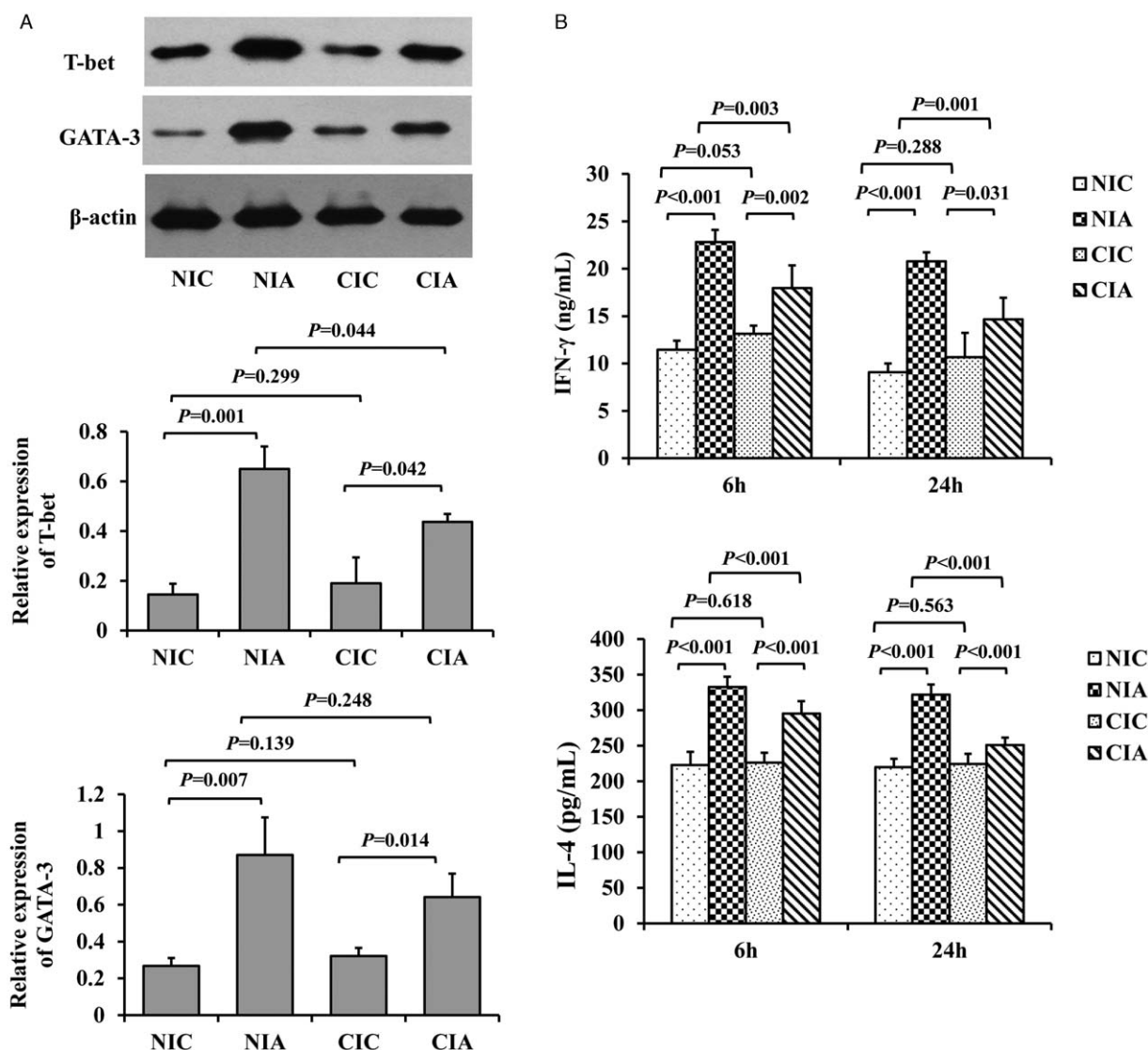
There was no significant difference in lymphocyte counts between mice in NIC group and NIA group ( $1712.50 \pm 776.80 \times 10^6/L$  vs.  $1087.50 \pm 212.35 \times 10^6/L$  at 6 h,  $P=0.207$ ;  $1560.00 \pm 699.29 \times 10^6/L$  vs.  $1102.00 \pm 644.26 \times 10^6/L$  at 24 h,  $P=0.349$ ) or between mice in CIC and CIA group ( $470.00 \pm 296.31 \times 10^6/L$  vs.  $255.00 \pm 90.37 \times 10^6/L$  at 6 h,  $P=0.209$ ;  $468.00 \pm 296.77 \times 10^6/L$  vs.  $390.0 \pm 163.71 \times 10^6/L$  at 24 h,  $P=0.621$ ) [Figure 1B]. When we compare lymphocytes after bacteria stimulation in mice with different immune conditions, much less lymphocytes were observed in mice with compromised immune condition (CIA) than in mice

with normal immune condition (NIA) ( $255.00 \pm 90.37 \times 10^6/L$  vs.  $1087.50 \pm 212.35 \times 10^6/L$  at 6 h,  $P<0.001$ ;  $390.00 \pm 163.71 \times 10^6/L$  vs.  $1102.00 \pm 644.26 \times 10^6/L$  at 24 h,  $P=0.044$ ).

**Accumulation and maturation of pulmonary cDCs in *A. baumannii* induced pneumonia mice with different immune conditions**

*A. baumannii* instillation increased the accumulation of pulmonary cDCs in mice with normal immunity (NIA group) compared with the control mice in NIC group at either 6 h ( $1.64\% \pm 0.27\%$  vs.  $0.58\% \pm 0.12\%$ ,  $P<0.001$ ) or 24 h ( $1.67\% \pm 0.36\%$  vs.  $0.61\% \pm 0.07\%$ ,  $P<0.001$ ). However, there was no significant difference in pulmonary cDCs rate in the mice with compromised immunity (CIA group) and the control mice of CIC group at 6 h ( $0.18\% \pm 0.07\%$  vs.  $0.37\% \pm 0.14\%$ ,  $P=0.033$ ) or 24 h ( $0.52\% \pm 0.24\%$  vs.  $0.46\% \pm 0.09\%$ ,  $P=0.583$ ) [Figure 2A].

As for the cDC maturation, *A. baumannii* instillation had the trend to increase the maturation of pulmonary cDCs in mice with normal immunity (NIA group) compared with the control mice in NIC group, but no statistically significant difference was found ( $60.68\% \pm 19.34\%$  vs.  $41.06\% \pm 13.29\%$  at 6 h,  $P=0.100$ ; or  $72.24\% \pm 16.15\%$  vs.  $57.74\% \pm 12.02\%$  at 24 h,  $P=0.146$ ). Similarly, there was no significant difference between CIA and CIC groups at neither 6 h ( $44.20\% \pm 13.16\%$  vs.  $47.84\% \pm 13.78\%$ ,  $P=0.681$ ) nor 24 h ( $63.08\% \pm 13.11\%$  vs.  $71.60\% \pm 16.41\%$ ,  $P=0.391$ ) [Figure 2B].



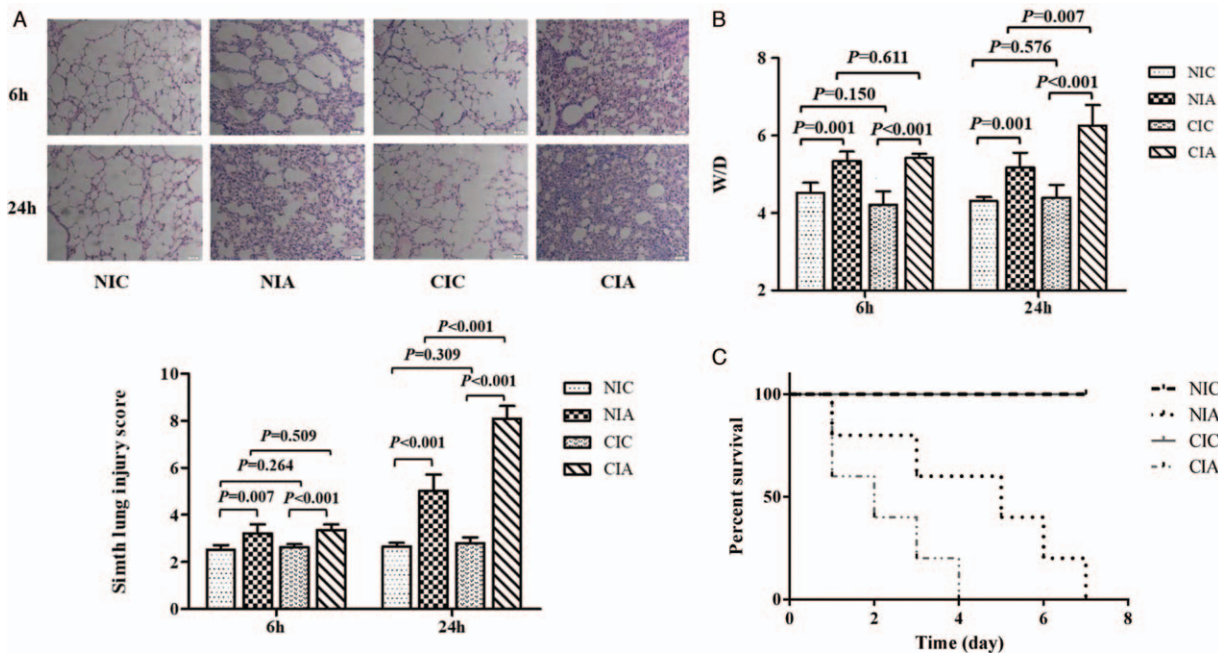
**Figure 3:** Th cell polarization in mice of *A. baumannii*-induced pneumonia with different immune conditions. (A) The expression of T-bet, a Th1-inducing transcription factor and GATA-3, a Th2 related transcription factor in mice receiving or not the instillation of *A. baumannii* with different immune conditions at 6 and 24 h were examined by Western blotting ( $n = 5$  for each group at each time point). (B) Pulmonary Th1- and Th2-associated cytokines, IFN- $\gamma$  and IL-4 respectively, with or without the induction of *A. baumannii*-associated pneumonia in mice with different immune conditions at 6 or 24 h were examined by ELISA tests.  $n = 5$  for each group at each time point. CIA: Immune compromised infection; CIC: Immune compromised control; ELISA: Enzyme-linked immunosorbent assay; IFN: Interferon; IL: Interleukin; NIA: Normal immunity infection; NIC: Normal immunity control.

### Polarization of Th cells after *A. baumannii* infection under different immune conditions

To assess Th cell polarization, the expression of T-bet protein, a Th1-inducing transcription factor and the expression of GATA-3, a Th2-related transcription factor at 24 h were examined by Western blotting. Also, the production of IFN- $\gamma$  and IL-4, the Th1 and Th2 related cytokine respectively, in mouse lung at 6 and 24 h were evaluated using ELISA. There was no significant difference in the expression of these proteins between NIC and CIC groups (the relative expression of T-bet:  $0.14 \pm 0.04$  vs.  $0.19 \pm 0.10$ ,  $P = 0.299$ ; the relative expression of GATA-3:  $0.26 \pm 0.04$  vs.  $0.32 \pm 0.04$ ,  $P = 0.139$ ). Although we found that T-bet and GATA-3 increased after *A. baumannii* infection with both normal (the relative expression of T-bet in NIA vs. NIA:  $0.14 \pm 0.04$  vs.

$0.65 \pm 0.09$ ,  $P = 0.001$ ; the relative expression of GATA-3 in NIC vs. NIA:  $0.27 \pm 0.04$  vs.  $0.87 \pm 0.20$ ,  $P = 0.007$ ) and compromised immunity (the relative expression of T-bet in CIC vs. CIA:  $0.19 \pm 0.10$  vs.  $0.43 \pm 0.03$ ,  $P = 0.042$ ; the relative expression of GATA-3 in CIC vs. CIA:  $0.32 \pm 0.04$  vs.  $0.64 \pm 0.12$ ,  $P = 0.014$ ), the increment among mice with normal immunity was more significant than mice with compromised immunity (the relative expression of T-bet in NIA vs. CIA:  $0.65 \pm 0.09$  vs.  $0.43 \pm 0.03$ ,  $P = 0.044$ ; the relative expression of GATA-3 in NIA vs. CIA:  $0.87 \pm 0.20$  vs.  $0.64 \pm 0.12$ ,  $P = 0.248$ ) [Figure 3A].

In consistent with the results of T-bet and GATA-3 proteins, no changes in IFN- $\gamma$  and IL-4 level between NIC and CIC group were observed (IFN- $\gamma$ :  $11.45 \pm 0.95$  vs.  $12.71 \pm 0.80$  ng/mL at 6 h,  $P = 0.053$ ;  $9.09 \pm 0.91$  vs.



**Figure 4:** The effect of *A. baumannii* instillation on the pulmonary histopathology, pulmonary permeability, and mortality among mice with different immune conditions. (A) Hematoxylin and eosin staining and Smith score analysis to evaluate the histopathological changes in lung tissues from mice in each experimental group were performed at 6 and 24 h after *A. baumannii* administration (original magnification  $\times 200$ ). (B) Lung edema was measured by the ratio of lung wet weight to dry weight (W/D) at 6 and 24 h of the induction of *A. baumannii*-associated pneumonia in mice with different immune conditions.  $n = 5$  for each group at each time point. (C) The mortalities of mice in different experimental groups were analyzed seven days after the administration with *A. baumannii* ( $n = 5$  for each group). Comparing the NIA and CIA group, the hazard ratio is 0.37 (95% confidence interval, 0.05–0.85;  $P = 0.066$ ). CIA: Immune compromised infection; CIC: Immune compromised control; NIA: Normal immunity infection; NIC: Normal immunity control.

10.67  $\pm$  2.56 ng/mL at 24 h,  $P = 0.228$ ; IL-4: 222.74  $\pm$  18.84 vs. 226.47  $\pm$  13.72 pg/mL at 6 h,  $P = 0.618$ ; 220.10  $\pm$  11.53 vs. 224.33  $\pm$  14.30 pg/mL at 24 h,  $P = 0.563$ ). Both the levels of IFN- $\gamma$  and IL-4 were significantly increased at 6 and 24 h after *A. baumannii* challenge in mice with normal (IFN- $\gamma$  in NIC vs. NIA: 11.45  $\pm$  0.95 vs. 22.83  $\pm$  1.28 ng/mL at 6 h,  $P < 0.001$ ; 9.09  $\pm$  0.91 vs. 20.79  $\pm$  0.94 ng/mL at 24 h,  $P < 0.001$ ; IL-4 in NIC vs. NIA: 222.74  $\pm$  18.84 vs. 332.68  $\pm$  14.63 pg/mL at 6 h,  $P < 0.001$ ; 220.10  $\pm$  11.53 vs. 271.56  $\pm$  10.85 pg/mL at 24 h,  $P < 0.001$ ) or compromised immunity and the IL-4 and IFN- $\gamma$  level were higher in the NIA group than those in the CIA group (IFN- $\gamma$  in CIC vs. CIA: 12.71  $\pm$  0.80 vs. 17.95  $\pm$  2.40 ng/mL at 6 h,  $P = 0.002$ ; 10.67  $\pm$  2.56 vs. 14.67  $\pm$  2.27 ng/mL at 24 h,  $P = 0.031$ ; IL-4 in CIC vs. CIA: 226.47  $\pm$  13.72 vs. 295.36  $\pm$  17.49 pg/mL at 6 h,  $P < 0.001$ ; 224.33  $\pm$  14.30 vs. 251.03  $\pm$  10.28 pg/mL at 24 h,  $P < 0.001$ ). However, the increase of IL-4 and IFN- $\gamma$  in mice with normal immunity were much higher than in the immunocompromised mice (IFN- $\gamma$  in NIA vs. CIA: 22.83  $\pm$  1.28 vs. 17.95  $\pm$  2.40 ng/mL at 6 h,  $P = 0.003$ ; 20.79  $\pm$  0.94 vs. 14.67  $\pm$  2.27 ng/mL at 24 h,  $P = 0.001$ ; IL-4 in NIA vs. CIA: 332.68  $\pm$  14.63 vs. 295.36  $\pm$  17.49 pg/mL at 6 h,  $P < 0.001$ ; 271.56  $\pm$  10.85 vs. 251.03  $\pm$  10.28 pg/mL at 24 h,  $P < 0.001$ ) [Figure 3B].

**Effect of *A. baumannii* on lung impairment and mortality among mice under different immune conditions**

The pulmonary histopathology of each group after 6 or 24 h of administration with bacterium or saline was

analyzed. We found that control mice in the NIC and CIC groups had relatively normal lung tissue, while the *A. baumannii* infection resulted in a thickened alveolar wall, alveolar and interstitial inflammatory cell infiltration, alveolar exudates and edema 6 h later. The pulmonary histopathology was exacerbated at 24 h with more significant inflammatory cell infiltration and hemorrhage. The Smith score for quantification of the lung injury was also raised in NIA or CIA group at 6 or 24 h compared with their control mice (NIC vs. NIA: 2.52  $\pm$  0.19 vs. 3.22  $\pm$  0.38 at 6 h,  $P = 0.007$ ; 2.66  $\pm$  0.15 vs. 5.02  $\pm$  0.65 at 24 h,  $P < 0.001$ ; CIC vs. CIA: 2.64  $\pm$  0.11 vs. 3.36  $\pm$  0.24 at 6 h,  $P < 0.001$ ; 2.80  $\pm$  0.24 vs. 8.08  $\pm$  0.55,  $P < 0.001$ ). Comparatively, the extent of lung injury was much more serious in CIA group at 24 h compared with lung injury in NIA group (3.22  $\pm$  0.38 vs. 3.36  $\pm$  0.24 at 6 h,  $P = 0.509$ ; 5.02  $\pm$  0.65 vs. 8.08  $\pm$  0.55 at 24 h,  $P < 0.001$ ) [Figure 4A].

Lung wet/dry weight ratio was calculated to evaluate permeability of pulmonary capillary and the injury of lung tissue. We found that the control mice with normal or compromised immunity had similar wet/dry ratio (NIC vs. CIC: 4.52  $\pm$  0.26 vs. 4.22  $\pm$  0.34 at 6 h,  $P = 0.150$ ; 4.31  $\pm$  0.10 vs. 4.40  $\pm$  0.32 at 24 h,  $P = 0.576$ ), but after infection with *A. baumannii*, the wet/dry weight ratio was significantly higher at either 6 or 24 h (CIA or NIA group) than their respective control (NIC vs. NIA: 4.52  $\pm$  0.26 vs. 5.35  $\pm$  0.24 at 6 h,  $P = 0.001$ ; 4.31  $\pm$  0.10 vs. 5.18  $\pm$  0.38 at 24 h,  $P = 0.001$ ; CIC vs. CIA: 4.22  $\pm$  0.34 vs. 5.42  $\pm$  0.11 at 6 h,  $P < 0.001$ ; 4.40  $\pm$  0.32 vs. 6.25  $\pm$  0.54 at 24 h,  $P < 0.001$ ). The increase seemed most



significant in CIA group than in NIA group especially at 24 h ( $5.35 \pm 0.24$  vs.  $5.42 \pm 0.11$  at 6 h,  $P = 0.611$ ;  $5.18 \pm 0.38$  vs.  $6.25 \pm 0.54$  at 24 h,  $P = 0.007$ ) [Figure 4B].

Additionally, we also assessed the seven-day mortality among these groups. Seven-day mortality of the mice in NIC and CIC group were 0% and 100% in NIA and CIA group. However, after *A. baumannii* infection, the immunocompromised mice (CIA group) all died within four days, while only 2/5 mice died within 4 days in the NIA group. It seemed that the infected mice with compromised immunity died earlier and faster than mice with normal immunity. Comparing the NIA and CIA group, the hazard ratio is 0.37 (95% confidence interval, 0.05–0.85;  $P = 0.066$ ) [Figure 4C].

## Discussion

Based on these results, a reduced immune response to *A. baumannii* instillation could be found in mice with compromised immune condition than mice with normal immunity. As expected, the compromised immunity was related to more severe lung injury and worse outcome in mice of *A. baumannii* pneumonia.

CTX was intraperitoneally administered in our study to induce immune deficiency in mice. CTX is a kind of alkylating agent which has been widely used as an immunosuppressive drug to inactivate the rapidly cycling immune cell population in some other investigations.<sup>[21,22]</sup> The relatively moderate dose of CTX used in our study was verified to be able to induce granulopenia, while also avoided increased mortality of mice receiving CTX administration within seven days. Besides neutrophilic granulocytes, CTX also took negative effect on lymphocytes and dendritic cells.

Neutrophils play a critical role in host resistance to respiratory *A. baumannii* infection. Early recruitment of neutrophils which take responsibilities in rapidly and continuously engulfing and killing bacteria is critical for initiating an efficient host defense against respiratory *A. baumannii* infection.<sup>[23,24]</sup> It was found that neutropenia was related to inhibited bacteria clearance, alteration of pro-inflammatory cytokine release thus leading to more severe microbial disease in *A. baumannii* infection.<sup>[25,26]</sup> In our study, at 6 h of *A. baumannii* administration, the recruitment of peripheral neutrophils sharply rose in mice with normal immunity. However, no changes in the neutrophil counts between the immunocompromised mice receiving or not receiving *A. baumannii*. The interactions between neutrophils and *A. baumannii* remain largely unknown. Some recent studies revealed that *A. baumannii* had negative effects on neutrophils. In the study of Kamoshida *et al*,<sup>[27]</sup> it was found that *A. baumannii* suppressed the adhesion ability of neutrophils through suppression of the surface expression of CD11a, thereby inhibiting PMA-induced NET formation. Another study found that *A. baumannii* could influence the neutrophil chemotaxis through the accumulation of phenylacetate.<sup>[28]</sup> The negative effects of *A. baumannii* on neutrophils might become more evident in compromised immune conditions. Still, it was hard to totally exclude the toxicity of CTX.

cDCs are specialized in the processing and presentation of antigens. They also activate the adaptive immune response.<sup>[29,30]</sup> Pulmonary cDCs mainly reside in the airway epithelium, alveolar septa or around pulmonary vessels with immature phenotypes.<sup>[31]</sup> In response to infection/inflammation, cDCs accumulate rapidly in lung, in some circumstances peaking as early as 2 h after challenge.<sup>[32,33]</sup> DCs play a positive role in the defensive immune response in infections resulting from multiple pathogenic bacteria.<sup>[34,35]</sup> For example, after fungal infection, DCs capture the fungal particles and present antigens to T cells, which induce a specific Th1 adaptive immune response to fungus.<sup>[36–39]</sup> Pene *et al*<sup>[40]</sup> found that intratracheal administration of DC in mice with compromised immunity infected with *Pseudomonas aeruginosa* could significantly improve the survival of these mice. The similar results found by Bohannon *et al*<sup>[41]</sup> indicated that DC could accelerate the clearance of *Pseudomonas aeruginosa* in burned mice. cDCs are also involved in the immune response to *A. baumannii*. Once mobilized to the lung, DCs sample incoming antigens and undergo the maturation process which is phenotypically characterized by the upregulation of cell-surface MHC II and costimulatory molecule expression (eg, CD80, CD86),<sup>[31]</sup> and elicited a specific Th1 response to enhance the immunity to *A. baumannii*.<sup>[18]</sup> In a recent study, it was found that the outer membrane vesicle from an *A. baumannii* clinical strain could activate bone marrow-derived DC to promote Th2 activity.<sup>[42]</sup> In our study, the finding that a rapid accumulation and maturation of cDCs in the lung of mice with normal immunity after *A. baumannii* stimulation suggested that cDCs were involved in the immune response to *A. baumannii*.

However, we noticed a significant inhibition of cDC counts presented after the induction of *A. baumannii* related pneumonia in immunocompromised mice. This may be explained by the virulence of *A. baumannii* to DCs. According to the study of Lee *et al*,<sup>[14]</sup> higher concentrations of *A. baumannii* could attack the mitochondria of DCs, thus causing apoptosis and necrosis of DCs as well as inhibition of both innate and adaptive immunity through the large quantities of reactive oxygen species produced.

Adaptive immunity is also involved in the immune response to *A. baumannii*. Upon receiving signals through the binding of antigen to the T-cell receptor in the presence of polarizing cytokines, naive Th precursor cells differentiate into Th1, Th2, and so on.<sup>[43]</sup> Interestingly, in our study, the lymphocyte counts had a decreased trend after *A. baumannii* instillation in mice with either normal or compromised immunity. It seems that the innate immunity may take a more critical role in the antimicrobial immune response to *A. baumannii*. The role of the adaptive immunity in *A. baumannii* infection as well as the effect of *A. baumannii* on adaptive immunity were rarely concerned. In our study, CTX administration did not influence significantly the Th cells polarization. However, the activation of either Th1 or Th2 polarization after *A. baumannii* stimulation was not as evident as in mice with normal immunity. The impairment of both Th1 and Th2 adaptive immunity in response to *A. baumannii* stimulation may be resulted from the compromised activity of cDCs.

There were some limitations to this paper. First, we used standard strains of *A. baumannii*, which may be different from the multi-drug resistant *A. baumannii* confronted in the clinic. Accordingly, the results may not fully match actual clinical situations. Second, we used CTX to induce the immunocompromised conditions, which had a wide inhibited effect on various immune cells. It was hard to clarify whether the reduced immune response to *A. baumannii* could be explained by the virulence of *A. baumannii* on immune cells under deficient immune condition or the negative effect of immunodeficiency induced by CTX. Further exploration about the specific effect and mechanisms were lack in this study which should be carried out in the upcoming investigation.

In summary, we may come to the conclusion that *A. baumannii* infection may induce an inhibited immune response in compromised immune conditions, and this reduced immune response is related to more severe lung injury and worse outcome in *A. baumannii* induced pneumonia. Further studies are needed to clarify the mutual effects of *A. baumannii* on certain immune cells and the underling mechanisms.

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### Conflicts of interest

None.

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