Cytokines help suggest aplastic anemia with pulmonary bacterial or co-fungal infection

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Although aplastic anemia (AA) does not come under the category of blood malignant diseases, the infection that frequently occurs in this bone marrow failure can make it worse. Pulmonary infection is the most prevalent but limiting clinical diagnosis. To find biomarkers predicting bacterial or bacterial-combined fungal infections in the lungs, we reviewed 287 AA medical records including 151 without any infection, 87 with pure pulmonary bacterial infection, and 49 with bacterial and fungal infection were reviewed. There were substantial changes in IL-17F, IL-17A, IFN-γ, IL-6, IL-8, and IL-10 levels between the non-infected and lung bacterial infection groups (P < 0.05). Further, a significant variation in IL-17A, TNF-β, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-22, and IL-12p70, between the uninfected group and the pulmonary bacterial and fungal infection group (P < 0.05) was observed. The results further revealed significant differences in TNF-β, IL-12p70, IL-6, IL-8, and IL-10 between the pulmonary bacterial infection group and the fungal infection group (P < 0.05). Moreover, by calculating ROC and cut-off values, we determined that IL-6 (AUC = 0.98, Cut-off = 14.28 pg/ml, P = 0.0000) had a significant advantage than other cytokines, body temperature (AUC = 0.61, P = 0.0050), PCT (AUC = 0.57, P = 0.0592), and CRP (AUC = 0.60, P = 0.0147) in the detection of lungs bacterial infections. In addition, IL-6 (AUC = 1.00, Cut-off = 51.50 pg/ml, P = 0.0000) and IL-8 (AUC = 0.87, Cut-off = 60.53 pg/ml, P = 0.0000) showed stronger advantages than other cytokines, body temperature (AUC = 0.60, P = 0.0324), PCT (AUC = 0.72, Cut-off = 0.63 ng/ml, P = 0.0000) and CRP (AUC = 0.79, Cut-off = 5.79 mg/l, P = 0.0000) in distinguishing bacteria from fungi. This may suggest that IL-8 may play a role in differentiating co-infected bacteria and fungi. Such advantages are repeated in severe aplastic anemia (SAA) and very severe aplastic anemia (VSAA). In conclusion, aberrant IL-6 elevations in AA patients may predict the likelihood of bacterial lung infection. The concurrent increase of IL-6 and IL-8, on the other hand, should signal bacterial and fungal infections in patients. These findings may help to suggest bacterial or fungal co-infection in patients with AA (Focus on VSAA and SAA).

“Aplastic” refers to the inability of bone marrow to form blood, the end-organ effect of diverse pathophysiologic mechanisms. Mostly, Aplastic anemia (AA) patients have pancytopenia, with decreased levels of platelets and white blood cells\textsuperscript{2,3}. The immune function of AA patients is always low, which makes AA patients susceptible to infection, and infection is also a significant cause of death in AA patients\textsuperscript{4,5}. Infection, especially early pulmonary infection, has always been a fundamental problem for hematologists, mainly because pulmonary infection, especially early infection, is not easy to diagnose. Body temperature, PCT, and CRP were less-sensitive in multiple infection studies on hematological malignancies\textsuperscript{6,7}. The main problem with commonly used sputum culture is the low positive rate\textsuperscript{7}. Computerized tomography (CT) is still the most important tool for lung infection clinical diagnosis. However, it may not well diagnose the disease at the early stages, and may be restricted by the health center.

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None of the laboratory tests for lung infections are perfect. In addition to the above examinations, lung lavage fluid culture and lung lavage fluid microbial next-generation sequencing (mNGS) have a wider and more accurate identification of pathogenic microorganisms in basic research and clinical experiments, and are also subject to many restrictions in clinical use (such as price, long sampling process and time to obtain results), especially for some blood system diseases. Like most developing countries or economically backward regions, the biggest problem we have is that because of economic reasons, the lung lavage fluid culture and lung lavage fluid microbial next-generation sequencing (mNGS), especially mNGS, can only be developed among a small number of people. Therefore, more auxiliary examinations are needed to strengthen the identification of pulmonary infection.

The limitations of these common examination items can easily lead to early lung infection not being detected in time, which further develops into serious infection, affecting the treatment of patients, and even affecting the survival of patients. In other words, the initial pulmonary infection is often found and treated after the infection is aggravated. Therefore, it is important to provide evidence of infection, which may help to accelerate the diagnosis of pulmonary infection. Cytokines are now gaining more attention, and previous studies have found cytokines to be more sensitive to lung infection than PCT, CRP, and body temperature in the absence of other diseases. Furthermore, cytokines have the advantages of requiring less quantity of samples for detection, eliminating the need to consider secondary pollution, and allowing for faster flow cytometry detection. Although cytokines have been shown to have a strong relationship with inflammation, they have also been used to predict inflammation.

Our team has also been working on studying cytokines indicating pulmonary infection in patients with hematological diseases. Previous research results suggest that cytokines predicting pulmonary infection also apply to hematological tumors. But we also found differences in cytokine expression between different types of blood diseases; considering the difference in disease, especially the difference between non-tumor disease and tumor disease. The primary focus of the current study is to investigate AA patients (Focus on VSAA and SAA) and explore the expression pattern of cytokines in the lungs of AA patients with bacterial infection and find the best potential biomarkers.

Material and methods

Declaration. All methods were performed in accordance with the relevant guidelines and regulations. The “Declaration of Helsinki” and the “International Code of Ethics for Human Biomedical Research” jointly formulated by the International Council of Medical Science Organizations and the World Health Organization were strictly followed throughout this study. At the same time, the project was approved by the Ethics Committee of the First People’s Hospital of Yunnan Province, number: KHLL2022-KY075. All study participants provided informed consent simultaneously.

Study participants and data collection. A total of 287 non-children AA medical records from patients who visited Yunnan First People’s Hospital’s Department of Hematology from June 2017 to May 2022 were evaluated for this study. It consists of 70 VSAA cases and 217 SAA cases. At the initial examination of the visit factor, we recorded the body temperature, Th1/Th2/Th17, CRP, and PCT cytokines. Refer to the following diagnostic criteria for bacterial lung infection; bacterial lung infection, suspected or confirmed, irrespective of the presence or absence of bacteria in respiratory specimens, and is classified based on clinical symptoms and diagnostic standards. Cough that is becoming worse or new, shortness of breath, hypoxia, or dyspnea are all clinical criteria. Chest X-rays to detect inflammatory changes and sputum culture to detect bacterial infection or other auxiliary tests. In certain patients with suspected pulmonary disease, chest CT scans were used to confirm bacterial infection. For patients with bacterial and fungal infection, we only included patients with positive bronchoalveolar lavage fluid (BAL) and β-D glucan test (G test) or galactomannan test (GM test) culture.

Statistical analysis. The data were initially examined for normality to see whether they have a normal distribution. Then, a t test or Mann–Whitney U test was conducted on independent samples. Continuous data are represented by the median and interquartile range (IQR), whereas percentages and frequencies describe categorical variables. We calculated the area under the curve (AUC) using the ROC curve to evaluate the diagnostic value of each parameter. Using the highest Youden index, the optimal Cut-off was determined. The GraphPad Prism 7 software was utilized to generate distribution plots, and the IBM SPSS 21.0 (Armonk, NY, USA) was used for statistical analysis. P-values of <0.05 on (two-sided) were considered statistically significant.

Determination of cytokines. The kit used to detect serum cytokines is Aimplex Cytokine (QuantoBio, Tianjin, China) with a detection range of 1–2500 pg/ml, and the detected cytokines include Th (1/2/17), namely: interferon (IFN) -γ, IL-1β, 2, 4, 5, 6, 8, 10, 12p70, 17A, 17F, 22) and tumor necrosis factor (TNF-α and β) using flow cytometry (NovoCyte D3000).

Results

Patients characteristics. Among the 287 medical records, there were 125 females and 162 males, the median age was 29 (22–49) years, 151 patients without any infection, the median body temperature was 36.8 (36.6–37.0) °C (the mean value is 36.86 ± 0.46 °C), and the median PCT was 0.06 (0.04–0.2) ng/ml, median CRP 3.00 (1.55–8.78). Eighty-seven patients had a simple pulmonary bacterial infection, the median body temperature was 36.90 (36.70–37.60) °C (the mean value is 37.07 ± 0.60 °C), the median PCT was 0.12 (0.04–0.32) ng/ml, and the median CRP was 5.00 (2.48–29.00). Forty-nine patients had bacterial and fungal infections, with a median body temperature of 36.8 (36.7–37.6) °C (the mean value is 37.08 ± 0.63 °C), median PCT of 0.26 (0.08–1.10) ng/ml, and median CRP of 19.10 (10.04–39.15) (Table 1 and Fig. 1A–C).
Table 1. Patient characteristics. The difference in the expression of Age, body temperature, PCT and CRP between the patient's lungs with bacterial infection and without infection was detected by the Mann–Whitney U test. P < 0.05 means there is a statistical difference.

|                | A: no infection (n = 151) | B: bacterial infection of the lungs (n = 87) | C: bacterial and fungal infection of the lungs (n = 49) | P (A VS B) | P (A VS C) | P (B VS C) |
|----------------|---------------------------|---------------------------------------------|------------------------------------------------------|------------|------------|------------|
| Gender (male/female) | 74/77                     | 53/34                                       | 35/14                                                | -          | -          | -          |
| Age (years old)     | 35.00 (23–52)             | 26.00 (20–52)                               | 25.00 (25–42)                                        | 0.1322     | 0.3976     | 0.0076     |
| Body temperature (°C) | 36.8 (36.6–37.0)          | 36.9 (36.7–37.6)                            | 36.8 (36.7–37.6)                                     | 0.0048     | 0.0324     | 0.7677     |
| PCT (ng/ml)         | 0.06 (0.04–0.2)           | 0.12 (0.04–0.32)                            | 0.26 (0.08–1.10)                                     | 0.0462     | 0.0000     | 0.0006     |
| CRP (mg/l)          | 3.00 (1.55–8.78)          | 5.00 (2.48–29.00)                           | 19.10 (10.04–39.15)                                  | 0.0147     | 0.0000     | 0.0003     |

Figure 1. The Mann–Whitney U test was used to test the differences among patients with respiratory tract bacterial infection, patients with respiratory tract bacterial complicated with fungal infection and patients with no bacterial infection. (A) Body temperature, (B) PCT, (C) CRP, (D) IL-6, (E) IL-8, (F) IL-10. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns no statistical difference.
The difference in the expression of cytokines between the patient’s lungs with bacterial infection and without infection was detected by the Mann–Whitney U test. P < 0.05 means there is a statistical difference.

**Cytokine expression.** In our study cohort IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-1β, IL-2, IFN-γ, TNF-α, TNF-β, IL-17A, IL-17F and IL-22 The median expression levels in non-infected patients were 2.72 (1.82–3.95) pg/ml, 2.15 (1.59–2.98) pg/ml, 8.19 (6.10–10.41) pg/ml, 14.13 (6.75–35.46) pg/ml, 4.38 (3.3–6.44) pg/ml, 3.94 (3.27–5.71) pg/ml, 1.64 (1.16–2.16) pg/ml, 3.08 (2.23–4.80) pg/ml, 2.87 (1.89–3.82) pg/ml, 2.84 (2.02–3.93) pg/ml, 3.16 (2.57–3.90) pg/ml, 2.29 (1.37–3.33) pg/ml, 3.36 (2.47–4.67) pg/ml, 1.01 (0.58–1.80) pg/ml (Table 2).

The median expression levels in patients with pulmonary bacterial infections were 2.84 (1.80–5.00) pg/ml, 2.29 (1.71–3.64) pg/ml, 26.43 (17.40–37.48) pg/ml, 26.62 (14.19–60.58) pg/ml, 4.96 (3.95–8.20) pg/ml, 4.29 (3.44–6.52) pg/ml, 1.75 (1.41–2.32) pg/ml, 3.49 (2.60–5.28) pg/ml, 3.94 (3.44–6.52) pg/ml, 3.08 (2.02–3.93) pg/ml, 2.29 (1.37–3.33) pg/ml, 2.29 (1.37–3.33) pg/ml, 1.64 (1.16–2.16) pg/ml, 3.08 (2.23–4.80) pg/ml, 2.87 (1.89–3.82) pg/ml, 2.84 (2.02–3.93) pg/ml, 3.16 (2.57–3.90) pg/ml, 2.29 (1.37–3.33) pg/ml, 3.36 (2.47–4.67) pg/ml, 1.01 (0.58–1.80) pg/ml (Table 2).

There were significant differences in IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-1β, IL-2, TNF-β, IL-17, and IL-22 between the uninfected group and the pulmonary bacterial and fungal infection group (P < 0.05) (Table 2).

### Table 2. Cytokine expression

| Parameter | A: no infection | B: bacterial infection | C: fungal and bacterial infection | P (A VS B) | P (A VS C) | P (B VS C) |
|-----------|----------------|------------------------|---------------------------------|------------|------------|------------|
| IL-4 (pg/ml) | 2.72 (1.82–3.95) | 2.84 (1.80–5.00) | 3.55 (2.08–6.67) | 0.3332 | 0.0170 | 0.1406 |
| IL-5 (pg/ml) | 2.15 (1.59–2.98) | 2.29 (1.71–3.64) | 2.19 (1.45–3.71) | 0.1389 | 0.8949 | 0.4078 |
| IL-6 (pg/ml) | 8.19 (6.10–10.41) | 26.43 (17.40–37.48) | 104.73 (75.80–260.86) | 0.0000 | 0.0000 | 0.0000 |
| IL-8 (pg/ml) | 14.13 (6.75–35.46) | 26.62 (14.19–60.58) | 103.15 (46.06–209.31) | 0.0001 | 0.0000 | 0.0000 |
| IL-10 (pg/ml) | 4.38 (3.3–6.44) | 4.96 (3.95–8.20) | 7.08 (4.18–15.61) | 0.0062 | 0.0000 | 0.0075 |
| IL-12p70 (pg/ml) | 3.94 (3.27–5.71) | 4.29 (3.44–6.52) | 5.80 (4.32–6.83) | 0.0741 | 0.0196 | 0.0167 |
| IL-1β (pg/ml) | 1.64 (1.16–2.16) | 1.75 (1.41–2.32) | 1.93 (1.38–2.45) | 0.1343 | 0.0001 | 0.1668 |
| IL-2 (pg/ml) | 3.08 (2.23–4.80) | 3.49 (2.60–5.28) | 4.44 (3.04–6.03) | 0.0910 | 0.0087 | 0.0949 |
| IFN-γ (pg/ml) | 2.87 (1.89–3.82) | 3.07 (2.43–4.10) | 3.56 (2.10–4.56) | 0.0244 | 0.0742 | 0.8305 |
| TNF-α (pg/ml) | 2.84 (2.02–3.93) | 3.07 (2.05–4.42) | 3.17 (2.42–4.36) | 0.4348 | 0.1165 | 0.2170 |
| TNF-β (pg/ml) | 3.16 (2.57–3.90) | 3.20 (2.66–3.75) | 3.73 (2.99–4.09) | 0.8191 | 0.0258 | 0.0324 |
| IL-17A (pg/ml) | 2.29 (1.37–3.33) | 2.69 (1.59–4.23) | 3.40 (1.93–4.69) | 0.0321 | 0.0232 | 0.1358 |
| IL-17F (pg/ml) | 3.36 (2.47–4.67) | 4.23 (13.06–6.50) | 3.99 (2.48–5.69) | 0.0005 | 0.0642 | 0.2383 |
| IL-22 (pg/ml) | 1.01 (0.58–1.80) | 1.24 (0.62–2.59) | 1.39 (0.92–1.98) | 0.1850 | 0.0392 | 0.1514 |

**Determination of specific indicators.** Cut-off values were calculated from ROC curves, retaining potentially valuable cytokines, and compared with body temperature, PCT, and CRP. Our results show that IL-6 (AUC = 0.98, Cut-off = 14.8 pg/ml, P = 0.0000) has better sensitivity than other cytokines, body temperature AUC = 0.61, P = 0.0050), PCT (AUC = 0.57, P = 0.0592) and CRP (AUC = 0.60, P = 0.0147) for predicting bacterial infection of the lung (Table 3 and Fig. 2A). Further, the AUC and Cut-off values between no infection and bacterial and fungal infection were calculated. Surprisingly, IL-6 (AUC = 1.00, Cut-off = 51.50 pg/ml, P = 0.0000) and CRP (AUC = 0.79, Cut-off = 5.79 mg/l, P = 0.0000) were significant. Among the three groups of patients, there were significant differences in IL-6, IL-8, IL-10, IFN-γ, IL-17A, and IL-17F between the non-infection group and the pulmonary bacterial infection group (P < 0.05) as given in Table 2.

There were significant differences in IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-1β, IL-2, TNF-β, IL-17, and IL-22 between the uninfected group and the pulmonary bacterial and fungal infection group (P < 0.05) (Table 2). There were significant differences in IL-6, IL-8, IL-10, IL-12p70, and TNF-β between the pulmonary bacterial infection group and the pulmonary bacterial, fungal infection group (P < 0.05) (Table 2).
Discussion

Infection is prevalent in AA with bone marrow failure because immunosuppression is prevalent during the treatment of hematological diseases Among them, SAA and VSAA are the most frequently infected in clinic. Due to the lack of corresponding clinical symptoms of early bacterial infection in the lungs, it is easy to be ignored or misdiagnosed at the time of diagnosis. This often leads to aggravation or even death of patients, so it is necessary to increase auxiliary diagnosis indicators. In this study, the abnormal expression of IL-6 was found suitable for the

| Parameter          | Cut-off values of cytokines (AUC ≥ 70), CRP, PCT and body temperature between no infection and bacterial lung infection. Calculate the Cut-off values of cytokines (AUC ≥ 70), CRP, PCT and body temperature by ROC curve. | Cut-off values of cytokines (AUC ≥ 70), CRP, PCT and body temperature between no infection and pulmonary bacterial and fungal infection. |
|--------------------|-------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| Body temperature (°C) | 0.61 0.0050 – 0.60 0.0324 | 0.61 0.0062 – 0.71 0.0000 |
| PCT (ng/ml)          | 0.57 0.0592 – 0.72 0.0000 0.63 | 0.57 0.0000 – 0.72 0.0000 0.63 |
| CRP (mg/l)           | 0.60 0.0147 – 0.79 0.0000 5.79 | 0.60 0.0147 – 0.79 0.0000 5.79 |
| IL-6 (pg/ml)         | 0.98 0.0000 14.18 1.00 0.0000 51.50 | 0.98 0.0000 14.18 1.00 0.0000 51.50 |
| IL-8 (pg/ml)         | 0.65 0.0001 – 0.87 0.0000 60.53 | 0.65 0.0001 – 0.87 0.0000 60.53 |
| IL-10 (pg/ml)        | 0.61 0.0062 – 0.71 0.0000 6.83 | 0.61 0.0062 – 0.71 0.0000 6.83 |

Table 3. Cut-off values of cytokines (AUC ≥ 70), CRP, PCT and body temperature between no infection and bacterial lung infection. Calculate the Cut-off values of cytokines (AUC ≥ 70), CRP, PCT and body temperature by ROC curve. \( P < 0.05 \) means there is a statistical difference.

Figure 2. (A) the ROC curves of cytokine, PCT, Body temperature and CRP in patients with respiratory tract bacterial infection were compared with those without respiratory tract infection. (B) the ROC curves of cytokine, PCT, Body temperature and CRP in patients with respiratory tract bacterial and fungal infection compared with those without infection.
pulmonary bacterial infection of AA. When the expression level is high, it may indicate a pulmonary bacterial and fungal infection in AA patients. In addition, the abnormal expression of IL-6 may help distinguish whether AA patients develop a bacterial co-fungal infection, which is different from the previous report that the increase of cytokines contributes to the bacterial lung infection in hematological cancers. Simultaneous expression of IL-10, IL-8, and IL-6 is a decisive advantage in newly diagnosed hematological malignancies and pediatric patients.

Consistent with lymphoma and other reports, IL-6 was the most dominant of all markers, our findings provide insight into the availability of IL-6 in AA for predicting bacterial lung infection. Our results revealed that IL-10, IL-8, IL-6, IFN-γ, IL-17A, and IL-17F levels in patients with bacterial lung illness were abnormally higher than those without infection (Table 2). This is different from our study on non-Hodgkin’s lymphoma, where only IL-6, IL-8, IL-10, and IFN-γ have intersection indicators, while non-Hodgkin’s lymphoma Abnormal TNF-β is also present. These differences may be primarily due to the disease itself, e.g., TNF-β can be related to tumor burden. In addition, TNF-β is also a non-Hodgkin’s poor prognostic factor. Therefore, it is also necessary to study the cytokines of each blood system disease separately, which is beneficial to increase the reliability of the application of cytokines in different conditions.

Previous research has found that invasive pulmonary aspergillosis causes an increase in Th1/17 cytokines in individuals with hematological diseases and that IL-6 and IL-8 are strongly related to invasive pulmonary aspergillosis. Aspergillus fumigatus protease induces IL-6 and IL-8 release from A549 lung epithelial cells and primary epithelial cells. In addition, fungal spores also promote the secretion of IL-8 from A549 lung epithelial cells. IL-6 expression plays a crucial role in protective immunity in mice infected with Aspergillus. Further, the Aspergillus flavus can also induce the expression of IL-8.

Furthermore, abnormally high expression of IL-6 and IL-8 may indicate poor mortality in individuals with hematological malignancies who have pneumococcal infection. These data suggest that IL-6 and IL-8 are over-expressed in fungal-infected patients, and numerous hematological malignancies have been documented. Our results complement that IL-6 and IL-8 are similarly aberrant in AA expression in hematological non-neoplastic diseases and may have a role in discriminating bacterial or co-fungal infections because only IL-6 had a solid sensitivity to bacterial lung infection in our results. But after bacterial infection with a fungus, the IL-6 has a strong sensitivity. IL-8 also possesses better sensitivity than PCT, CRP, and body temperature. In Table 3, Fig. 2A,B.

However, we analyzed SAA and VSAA separately, and the results were repeated, which may indicate that this model is applicable to both SAA and VSAA (Supplementary Table 2, Supplementary Fig. 2A–D and Table 3).

Our study is a retrospective study, and the biggest regret is that the number of patients with accurate identification of pathogenic bacteria is small and we did not include it in the analysis. For lung infection, the technology widely used to identify pathogenic bacteria in most countries and regions is still sputum culture, but the positive rate of sputum culture is only about 30%. The more popular studies are obtaining samples directly from affected lesions by bronchoalveolar lavage (BAL) or by safe brushing with bronchoscopy followed by culture or mNGS, which is an invasive procedure. For the invasive examination of bone marrow failure disease AA in affected lesions by bronchoalveolar lavage (BAL) or by safe brushing with bronchoscopy followed by culture or burden. In addition, TNF-β is also a non-Hodgkin’s poor prognostic factor. Therefore, it is also necessary to study the cytokines of each blood system disease separately, which is beneficial to increase the reliability of the application of cytokines in different conditions.

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non-specific cytokines, the colonized flora generally does not lead to cytokine disturbance. In addition, the higher rates of mNGS in AA patients with concurrent bacterial and fungal infections in the lungs of SAA and VSAA patients.

Data availability
All data generated or analysed during this study are included in this published article [and its supplementary information files].
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**Author contributions**

J.Z. wrote the manuscript and graphs, Z.Y. analyzed the data and prepared the tables. P.H., X.G., C.Z. and Y.Z. jointly counted the data. H.L. and T.Y. corrected the language and logic of the manuscript. Y.C. helped to check the data. R.Z. designed the research protocol and corrected the methodology. Z.L. participated in the design of the protocol, supporting the progress of the study and continuing the related research on cytokines in hematological diseases.

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**Competing interests**

The authors declare no competing interests.

**Additional information**

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