Characteristics and clustering analysis of peripheral blood lymphocyte subsets in patients with ANCA-associated vasculitis

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Abstract

Objective

This study aimed to investigate the clusters of lymphocyte subset in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) and their correlation with clinical characteristics.

Methods

A total of 247 active AAV patients, 70 AAV patients with induced remission, and 252 healthy controls (HCs) were enrolled. Based on lymphocyte subsets, results were visualised by principal component analysis, and subgroups of patients were identified by cluster analysis.

Results

The absolute number of total lymphocytes and lymphocyte subsets were lower in patients with AAV than in HCs. All lymphocyte subsets were negatively correlated with BVAS ($p<0.01$) in patients with AAV, except for B cells. A decrease in lymphocyte subset count was associated with renal damage in patients with AAV. T lymphocyte subset features recovered during remission compared with the active disease state. However, the percentage and absolute number of B cells markedly decreased in the induced remission group ($p<0.0001$). Cluster analysis classified patients into three distinctive subgroups: Patients in cluster 2 had the highest white blood cell count, serum albumin level, monocyte count, and eGFR, whereas they had the lowest serum creatinine level and ESR ($p<0.05$). Patients with decreased lymphocyte subsets showed poor disease outcome. The count of $CD3^+$ T lymphocytes had the best predictive power for identifying disease outcome in AAV patients.

Conclusion

The counts of lymphocyte are lower in patients with active AAV than HCs. Decreased lymphocyte subsets may serve as a biomarker for assessing disease severity and predict poor outcome in AAV patients.

Key words

cluster analysis, lymphocyte subsets, ANCA-associated vasculitis, predict outcome

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Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a complex and heterogeneous autoimmune disease characterised by autoantibody-mediated necrotising inflammation of small blood vessels and severe organ damage (1-3). The precise mechanism of AAV development remains unclear. Nevertheless, lymphocyte subsets comprising T, B lymphocyte, and natural killer (NK) cells play a pivotal role in the occurrence and development of AAV. Their level changes dynamically with different disease statuses (4, 5).

The pathogenic role of T and B cells in AAV pathogenesis has been implicated. In patients with AAV, B cells are central in disease development and ANCA production, thereby mediating the disease through various pro-inflammation mechanisms (6). Meanwhile, deregulated T cells interact with autoreactive B cells, subsequently activating neutrophils and inducing vascular inflammation and necrosis of small blood vessel walls (7, 8). The pathogenic role of NK cells in AAV has also been suggested (9). However, studies analysing the AAV-related alterations in lymphocyte subset distribution are limited.

AAV is a heterogeneous autoimmune disease, and immune dysfunction mediated by lymphocyte subsets is critical to the onset and progression of AAV (10, 11). Accordingly, we speculated that lymphocyte-subset clustering may help identify different clinical patterns of patient, assess disease severity, and predict disease prognosis (12). Recent studies have shown that the number of lymphocytes in the blood of AAV patients decreases (13). However, an investigation on lymphocyte subsets in a large cohort has not been conducted.

The present study aimed to investigate peripheral blood lymphocyte subsets in patients with AAV from a large cohort. The heterogeneity of lymphocyte subsets in patients with AAV was evaluated by clustering analysis and explored their correlation with clinical parameters. The clinical significance of lymphocyte subset alterations in patients with AAV was also explored.

Materials and methods

Study population

We enrolled 247 patients with active AAV, 70 AAV patients with induced remission, and 252 age- and sex-matched healthy controls (HCs) recruited from the First Affiliated Hospital of Zhengzhou University between September 2017 and June 2023. All patients with AAV included in our study were diagnosed according to the definition established at the Chapel Hill Conference (14). All patients were older than 18 years, and patients with insufficient data or other autoimmune diseases were excluded. The disease activity of all patients was assessed using the Birmingham Vasculitis Activity Score (BVAS) at the baseline.

Clinical and laboratory data

Clinical and laboratory data were collected from inpatient medical records, including demographic characteristics, clinical symptoms, routine blood test, erythrocyte sedimentation rate (ESR), serum levels of C-reactive protein (CRP), serum levels of creatinine (Cr), serum albumin, plasma concentration of immunoglobulin, and serum complement C3 and C4. Detection of lymphocyte subset was conducted by FCM and phenotypes were collected from the clinical data of study populations and HCs. This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University (2021-KY-0486), and written informed consent was obtained from all subjects. The clinical characteristics of active AAV patients are reported in Supplementary Table S1.

Measurement of lymphocyte subset by flow cytometry (FCM)

Detection of lymphocyte subset was conducted by FCM. In a typical procedure, 100 μL of blood sample was drawn into tubes for FCM and then incubated with FITC-conjugated anti-CD3, PE-conjugated anti-CD4, APC-conjugated anti-CD8, PerCP-conjugated anti-CD19, PE-conjugated anti-$CD16^{+}56$, and controls (BD Biosciences) for 30 min. After adding FACS lysing solution according to the manufacturer's protocol, the samples were

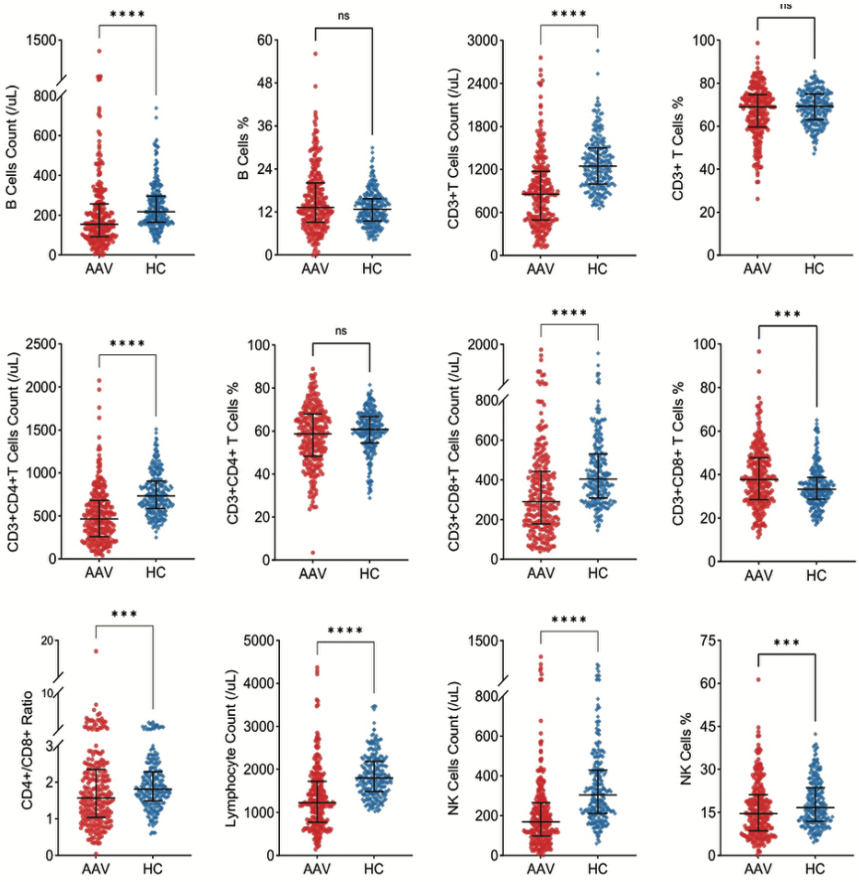

Fig. 1. Lymphocyte subsets of AAV patients and HCs.
ns: not significant *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

analysed on a Canto II flow cytometer (BD Biosciences). Total lymphocyte count, $CD3^+$ T cell, $CD3^+CD4^+$ T cell, $CD3^+CD8^+$ T cell, B cell, and NK cell were detected.

Cluster analysis

R software (v. 4.3.1) with the prcomp function in stats R packages and hclust function in cluster R packages was used to analyse the data of selected variables (total of lymphocyte, $CD3^+$ T cell, $CD3^+CD4^+$ T cell, $CD3^+CD8^+$ T cell, B cell, NK cell, and $CD4^+/CD8^+$ ratio). Principal component (PC) analysis (PCA) was conducted to visualise the clustering results. Hierarchical cluster analysis (HCA) was performed with the package *cluster* by Wards.D2 method.

Statistical analysis

Data were analysed using SPSS 26.0 software (SPSS, Chicago, IL, USA). Descriptive statistics are expressed as the mean ± standard deviation or median (interquartile range) for quantitative data, frequency, and percentage for qualitative data. The Mann-Whitney U or Kruskal-Wallis test was used to compare continuous data, and χ^2 test was used to compare categorical data. For correlation analysis, the Spearman rank correlation coefficient was used to examine the relationship of lymphocyte subsets and clinical variables. Receiver operating characteristic (ROC) curves were plotted, and the area under the curve (AUC) was calculated to evaluate the performance of lymphocyte subsets for patient outcome. We considered p<0.05 to be statistically significant.

Results

Characteristics of lymphocyte subsets in AAV patients

We compared the number of total lymphocyte and lymphocyte subsets between AAV patients and HCs. The number of total lymphocytes significantly decreased in AAV patients than in controls (p<0.0001) (Fig. 1). For lymphocyte subsets, the percentage and absolute number of $CD3^+CD8^+$ T cells in AAV patients was statistically lower than in controls (p<0.001 and p<0.0001, respectively). Moreover, the count of $CD3^+$ T and $CD3^+CD4^+$ T cell was lower in AAV patients than in controls (p<0.0001), but not the percentage of cells. However, the ratio of $CD4^+/CD8^+$ in AAV patients was obviously lower than that in HCs (p<0.001), indicating the aberrant immune status of AAV.

Interestingly, we also found that the absolute number of B cells and NK cells in AAV patients markedly decreased compared with in controls (p<0.0001). The percentage of NK cells was significantly lower in patients with AAV than in HCs (p<0.001), but not in B cells. Therefore, compared with HCs, the number of lymphocytes and all lymphocyte subsets in AAV patients significantly decreased (all p<0.0001).

Lymphocyte subsets in AAV patients with induced remission

According to the disease activity, the study populations were divided into 247 active and 70 remission groups. Several lymphocyte subset features recovered during remission compared with the active state. Lower counts of $CD3^+$ T, $CD3^+CD4^+$ T, and $CD3^+CD8^+$ T cell were observed in the peripheral blood of AAV patients during the active stage (all p<0.05) (Fig. 2). However, no significant differences in the total lymphocytes, NK cell, and $CD4^+/CD8^+$ ratio were found among the two groups. Interestingly, the percentage and absolute number of B cell markedly decreased in the induced remission group (p<0.0001). This finding indicated that the maintenance of remission in AAV patients based on B cell repopulation was crucial.

Relationship of lymphocyte subsets with clinical parameters

We analysed the relationship between these lymphocyte subsets and the AAV disease activity and clinical parameters. The total lymphocytes and all lymphocyte subsets, except B cells, were negatively associated with the BVAS (p<0.01) (Fig. 3). We found that the peripheral count of total lymphocyte, $CD3^+$ T, $CD3^+CD4^+$ T, $CD3^+CD8^+$ T,

and NK cells were positively correlated with the eGFR and serum albumin but negatively correlated with serum Cr levels. Furthermore, the count of total lymphocyte and subsets in the renal-damage group were significantly lower than those in the non-renal-damage group of patients with AAV (Suppl. Table S2). Meanwhile, the count of circulating total lymphocytes and all lymphocyte subsets were positively correlated with the number of monocytes (all $p<0.05$). These results implied that aberrant lymphocyte subsets may be potential markers to evaluate disease activity and predict organ involvement. Thus, lymphocyte subsets were involved in the occurrence of AAV and closely correlated with disease development.

Hierarchical cluster analysis divided AAV patients into three clusters

To explore the clinical significance of lymphocyte subsets, PCA was performed in patients with AAV based on the lymphocyte-subset count. We used PCA to extract 7 PCs and visualised the distribution of lymphocyte subsets in AAV patients. The cumulative percentage of PC1 and PC2 was 78.11%, as demonstrated in Figure 4A. The positive side contained B cell, $CD3^+CD4^+$ T cell, and $CD4^+/CD8^+$ ratio. By contrast, the negative side primarily included $CD3^+CD8^+$ T cell and NK cell (Fig. 4B). Three AAV patient clusters (clusters 1, 2, and 3) were identified through HCA based on the peripheral lymphocyte profiles of 247 active AAV patients. The results are shown in a combination of a dendrogram and a heatmap. Two-dimensional PCA further confirmed that the three clusters of AAV patients had distinct lymphocyte subset profiles (Fig. 4D, E, F).

Clinical and laboratory characteristics of three patient clusters

The three identified AAV patient clusters were further explored to determine whether different immune cell profiles led to different clinical characteristics. The results are presented in Figure 5 and Table I. Among the three clusters, cluster 2 (n=38) had the highest count of total lymphocytes, $CD3^+$ T, $CD3^+CD4^+$ T, $CD3^+CD8^+$ T, NK cells, and B cells (all

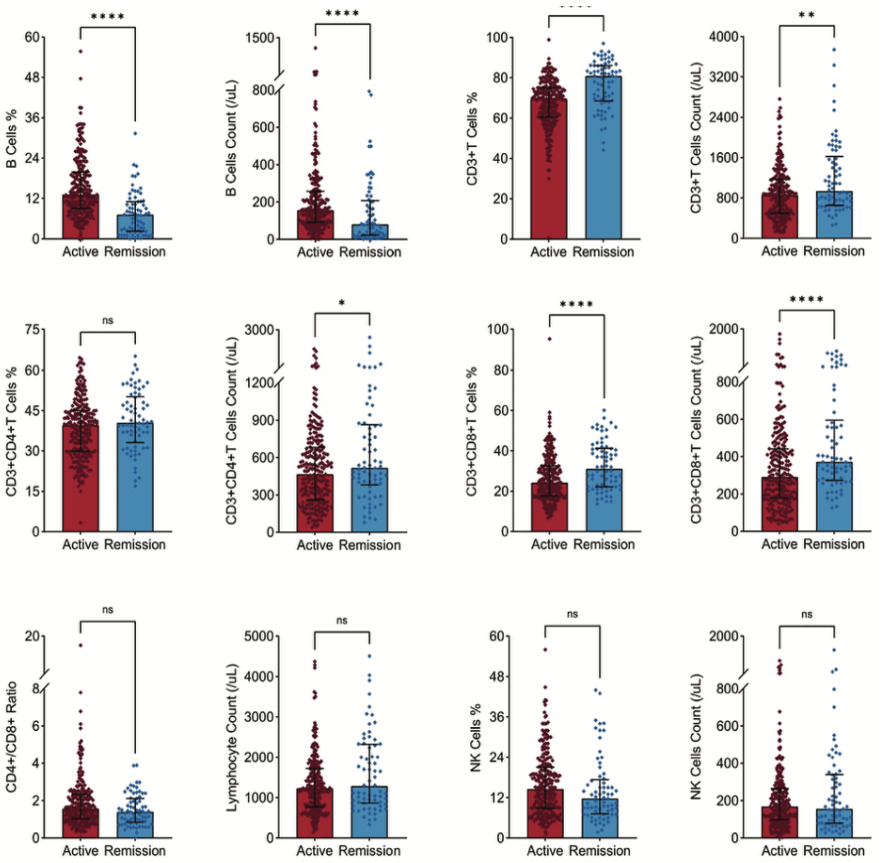

Fig. 2. Lymphocyte subsets in active and remission AAV patients.

$p<0.0001$). Conversely, cluster 1 (n=92) had the lowest count of total lymphocytes and lymphocyte subsets (all $p<0.0001$), whereas cluster 3 (n=117) had a moderate lymphocyte-subset count and the highest $CD4^+/CD8^+$ ratio. Cluster 2 had higher white blood cell count, monocyte count, serum albumin, total protein, and eGFR compared with clusters 3 and 1, whereas cluster 2 had lower serum Cr and BVAS than the other two clusters. The lowest ESR were found in cluster 2 ($p=0.049$), CRP levels had no difference among the three clusters. Decreased percentage of renal involved was found in cluster 2, and no difference in other organs damage were found among the three clusters. These results suggested the clinical heterogeneity among three AAV patient clusters.

Lymphocyte subsets predicted the outcome of AAV patients

To further investigate the association between lymphocyte subsets and disease prognosis in patients with AAV, we examined the ability of lymphocyte subsets to predict disease outcome by ROC analysis. In this study, poor outcome including serious infection, all cause of death, and end-stage renal disease (ESRD). ESRD was defined as dialysis dependence for greater than 3 months, which can be used to indicate renal survival.

The AUC of $CD3^+$ T lymphocyte in predicting outcome was 0.833, indicating that a lower number of $CD3^+$ T cells corresponded with poorer outcome (Fig. 6). The optimal cut-off value was 543.86 /μL, corresponding with a sensitivity of 92.3% and a specificity of 63.3%. Similarly, reduced peripheral counts of $CD3^+CD8^+$ T cell, $CD3^+CD4^+$ T cell, and NK cell were associated with poor outcomes. The AUCs of total lymphocyte, $CD3^+CD4^+$ T cell, $CD3^+CD8^+$ T cell, B cell, and NK cell to predict the outcome of patients were 0.822, 0.806, 0.812, 0.646, and 0.773, respectively. Hence, the $CD3^+$ T lymphocyte count had the best predictive power for identifying disease outcome in AAV patients.

Discussion

The involvement of immune cell subsets in the development of autoimmune

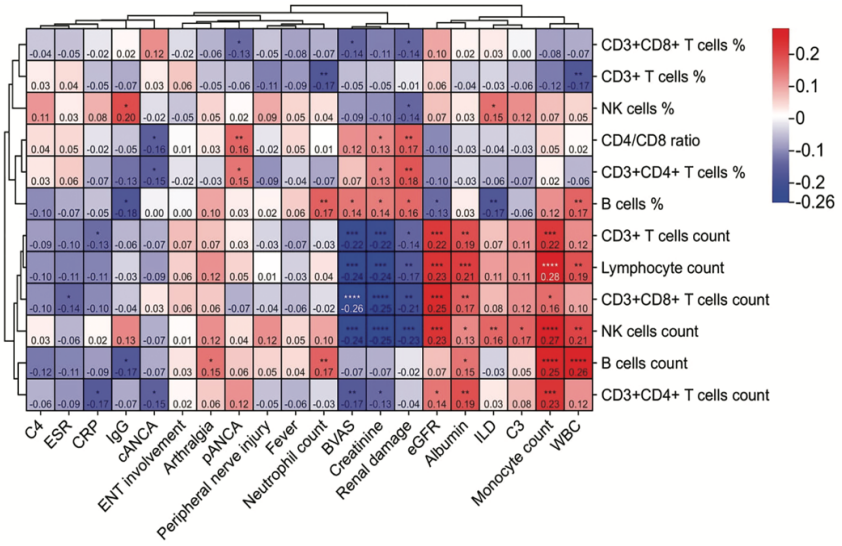

Fig. 3. Correlation heatmap between lymphocyte subsets and clinical parameters in active AAV patients. Red colour indicates positive correlation coefficient, blue colour indicates negative correlation coefficient.

diseases is receiving increased research interest. Extensive studies have revealed that various lymphocyte subsets play crucial roles in AAV development. Changes in blood lymphocytes in AAV patients may be due to the transfer of lymphocytes into the target organ to participate in the local immune response (15). The present study demonstrated that the lymphocyte-subset count decreased in patients with AAV. Lymphocyte subsets except B cells were negatively associated with the disease activity of AAV. The decreased number of $CD3^+$ T cell had the best power in predicting poor outcome for patients. In summary, our study systematically described the changes in lymphocyte subsets in AAV patients and found a significant correlation between lymphocyte subsets and the disease activity of AAV.

We found that the blood lymphocyte count in active AAV patients was significantly lower than that in the HC group, similar finding to previous clinical research (16). This finding indicated that lymphocytes played an important role in disease development. The count of T lymphocyte subsets in the peripheral blood decreased in the active group compared with the remission group. Several lymphocyte subset features recovered during remission compared with the active disease state, consistent with a previous study on AAV. Several of reasons contribute to peripheral lymphopenia, including infection, neoplasms, standard induction therapy, and intense recruitment of lymphocytes into inflammatory tissue (17, 18). To exclude interference by the induction therapy, we included newly diagnosed and relapse AAV patients.

An increase in tubulointerstitial infiltra-

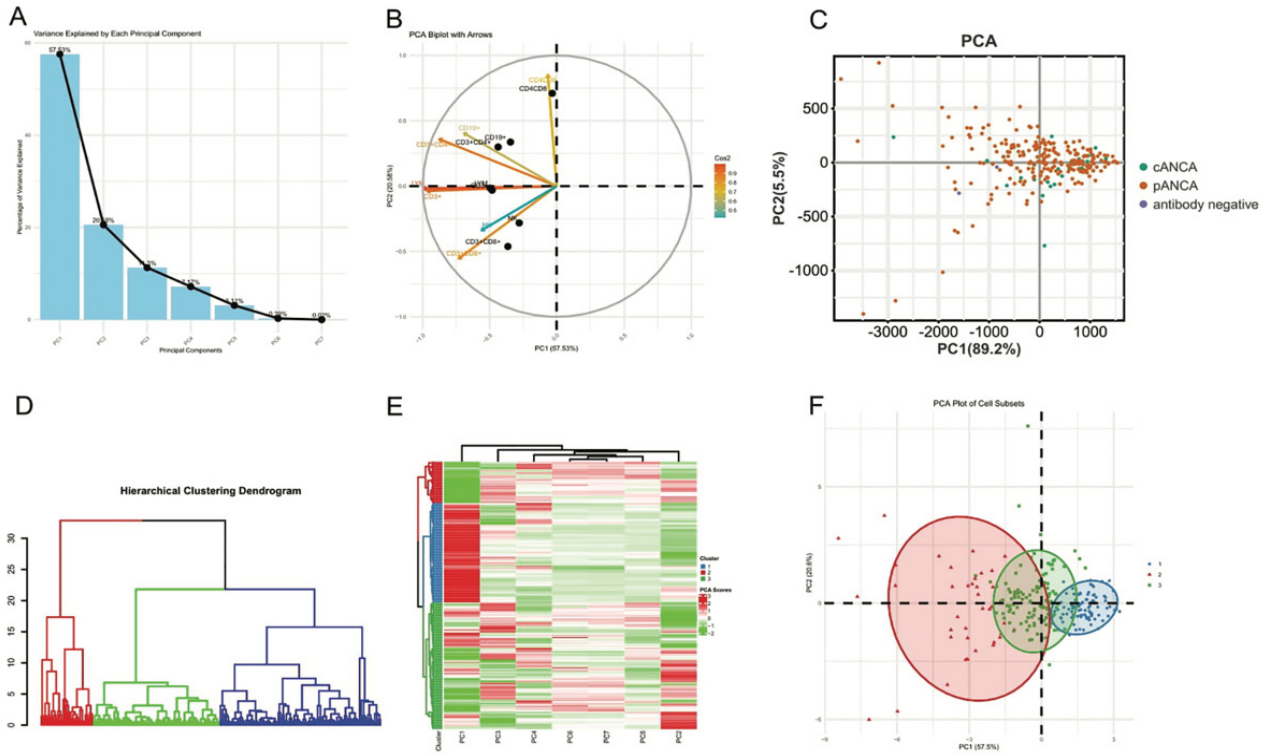

Fig. 4. Results of PCA and HCA based on lymphocyte subsets in AAV patients. (**A**) Scree plot for PCA of lymphocyte subsets in AAV patients. (**B**) Circle plot of lymphocyte subsets correlating with PCs in AAV patients. (**C**) Scatterplot showing the PC1 and PC2 values in AAV patients with different ANCA subtype. (**D-E**) Hierarchical cluster analysis divided AAV patients into three distinctive clusters. (**F**) The scatterplot performed by PCA to show the three clusters of AAV patients. PCA: principal component analysis; PCs: principal components; HCA: hierarchical cluster analysis; cos2: square cosine.

Table I. Clinical and laboratory characteristics of three patient clusters.

	Cluster 1	Cluster 2	Cluster 3	*p*-value
Age, years	64 (57, 71)	63.5 (49.5, 70)	66 (52.5, 72)	0.381
Female, n (%)	47 (51.1%)	21 (55.3%)	65 (55.6%)	0.798
WBC, $\times10^9$/L	8 (6.0, 10.0)	10.2 (7.8, 13.0)	8.1 (6.8, 11.4)	0.036
Neutrophil count, $\times10^9$/L	6.7 (4.6, 8.2)	7.4 (4.9, 8.2)	6.0 (4.6, 9.1)	0.556
Lymphocyte count, $\times10^9$/L	0.8 (0.6, 1.1)	2.1 (1.7, 2.7)	1.4 (1.0, 1.6)	<0.0001
Monocyte count, $\times10^9$/L	0.4 (0.3, 0.6)	0.6 (0.4, 0.9)	0.5 (0.4, 0.7)	0.001
Total protein, g/L	62.6 ± 8.7	66.5 ± 8.8	63.1 ± 8.1	0.053
ALB, g/L	30.9 (29.3, 33.8)	36.3 (31.4, 41.2)	33.3 (28.7, 36.6)	0.0004
CREA, µmol/L	134 (78, 316)	84 (63, 132.8)	112 (60.5, 370)	0.014
eGFR, ml/min×1.73 m^2	39.7 (13.8, 81.7)	75.1 (42.9, 90.5)	49.7 (12.8, 94.4)	0.033
ESR, mm/H	90 (49.5, 105.5)	58 (38.5, 83.0)	70 (30, 96)	0.049
CRP, mg/L	62.1 (14.9, 104.6)	20.9 (6.7, 88.0)	31.8 (5.0, 85.0)	0.167
C3, g/L	1.04 (0.86, 1.31)	1.11 (0.92, 1.35)	1.12 (0.97, 1.22)	0.212
C4, g/L	0.26 (0.21, 0.32)	0.24 (0.19, 0.32)	0.24 (0.18, 0.32)	0.55
IgG, g/L	13.1 (10.9, 16.4)	12.9 (10.5, 17.0)	12.3 (9.2, 15.7)	0.361
pANCA, n (%)	79 (85.9%)	35 (92.1%)	104 (88.9%)	0.579
cANCA, n (%)	13 (14.1%)	2 (5.3%)	12 (10.3%)	0.321
Fever, n (%)	29 (31.5%)	10 (26.3%)	37 (31.6%)	0.811
Arthritis/arthralgia, n (%)	5 (5.4%)	6 (15.8%)	8 (6.8%)	0.117
ENT involvement, n (%)	10 (10.9%)	8 (21.1%)	16 (13.7%)	0.309
ILD, n (%)	38 (41.3%)	19 (50.0%)	41 (35.0%)	0.241
Renal damage, n (%)	66 (71.7%)	21 (55.3%)	81 (69.2%)	0.173
Peripheral nerve injury, n (%)	10 (10.9%)	3 (7.9%)	9 (7.7%)	0.706
BVAS	16.2 ± 5.5	12.8 ± 6.6	15.5 ± 6.1	0.013

tion of $CD3^+CD4^+$ T and $CD3^+CD8^+$ T cell is reportedly correlated with lower eGFR (19). Similarly, we found a positive correlation between counts of lymphocyte subset and eGFR, and a negative correlation with serum Cr. The peripheral counts of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells were lower in active AAV patients with ESRD. Recent study suggested that urinary $CD3^+CD4^+$ T cell counts could identify patients with AAV at a substantial risk of renal relapse within 6 months (20). Clustering analysis based on peripheral lymphocyte profiles showed that cluster 2 had the highest count of total lymphocytes and lymphocyte subsets. Cluster 2 also had higher white blood cells count, monocytes count, serum albumin, and eGFR than clusters 3 and 1, whereas cluster 2 had lower serum Cr and BVAS than the other two clusters. Taken together, those observations suggested that the changes in lymphocyte levels were likely to play pathological roles in AAV patients, and that the number of lymphocytes was closely correlated with target-organ injury in AAV patients.

Activated $CD8^+$ T cells are known to differentiate into cytotoxic T cells, which kill the target cells and inflict organ damage (21). Nevertheless, how cytotoxic lymphocytes work in AAV patients requires further research. Consistent with previous studies, we found a significant decrease in T cell counts in AAV patients (16). Further analysis on T cell subpopulations revealed significantly reduced counts of $CD3^+CD8^+$ T cell in AAV patients and recovery from the remission state of disease, and the most relevant between counts of $CD3^+CD8^+$ T cell and BVAS were found ($r=-0.26$, $p<0.0001$). These findings indicated that $CD3^+CD8^+$ T cells were deeply involved in the pathogenesis and disease activity of AAV.

NK cells are cytotoxic lymphocytes that are important components of innate immune. The primary function of NK cells is to eliminate cells infected with viruses or cancerous cells (22, 23). The onset of AAV is a multifactor-mediated process that includes the innate and adaptive immune systems, and NK cells may be involved in the pathogenesis of AAV (9). A previous study has reported that NK cells are lower in the peripheral blood of patients with AAV than in HCs (24). Notably, our work also showed that the counts of NK cells in AAV patients significantly decreased, and NK cell counts significant negatively correlation with BVAS, suggesting that NK cell may be involved in AAV pathogenesis.

In AAV development, auto-reactive B cells are central to the development of the disease and the persistent production of ANCAs, which are involved in the disease through various pro-inflammatory mechanisms (25). Interestingly, we found that no relationship existed between B cell counts and BVAS. Unlike other lymphocyte subsets, the percentage and absolute number of B cell markedly decreased in the induced remission group, indicating that the maintenance of remission of AAV based on B cell depletion strategy was very important (26). However, therapeutic strategies targeted to other subsets of lymphocytes require further exploration.

Some correlations also existed between clinical parameters and peripheral lymphocyte subsets (12). For instance, the counts of total lymphocyte, T cells were positively correlated with the eGFR and negatively correlated with serum Cr levels. Furthermore, the immune system always works together; different lymphocyte subsets had wide-ranging interactions with each other (27, 28). Given that AAV is caused by an imbalance of the immune system, in which almost each subset of lymphocyte is involved, taking a full view of the changes in lymphocyte subsets for AAV patients is necessary. In our clustering analysis, a lower percentage of renal damage was found in cluster 2. Meanwhile, cluster 2 had higher white blood cell count, monocyte count, serum albumin, and eGFR. These results suggested a clinical heterogeneity among the three AAV patient clusters.

A recent study has revealed that lymphopenia is correlated with the severity of AAV glomerulonephritis and predicts poor renal outcome (29). To further investigate the association between lymphocyte subsets and outcome in patients with AAV, we examined the ability of lymphocyte subsets to predict outcome by ROC analysis. The $CD3^+$ T lymphocyte count had the best predictive power for identifying disease outcome in AAV patients. Moreover, the AUC of total lymphocytes, $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, and NK cells to predict the outcome of patients were 0.822, 0.806, 0.812, and 0.773, respectively. Accordingly, we hypothesised that a decrease in $CD3^+$ T cells related to the disease activity of AAV, participate in the pathogenesis of AAV,

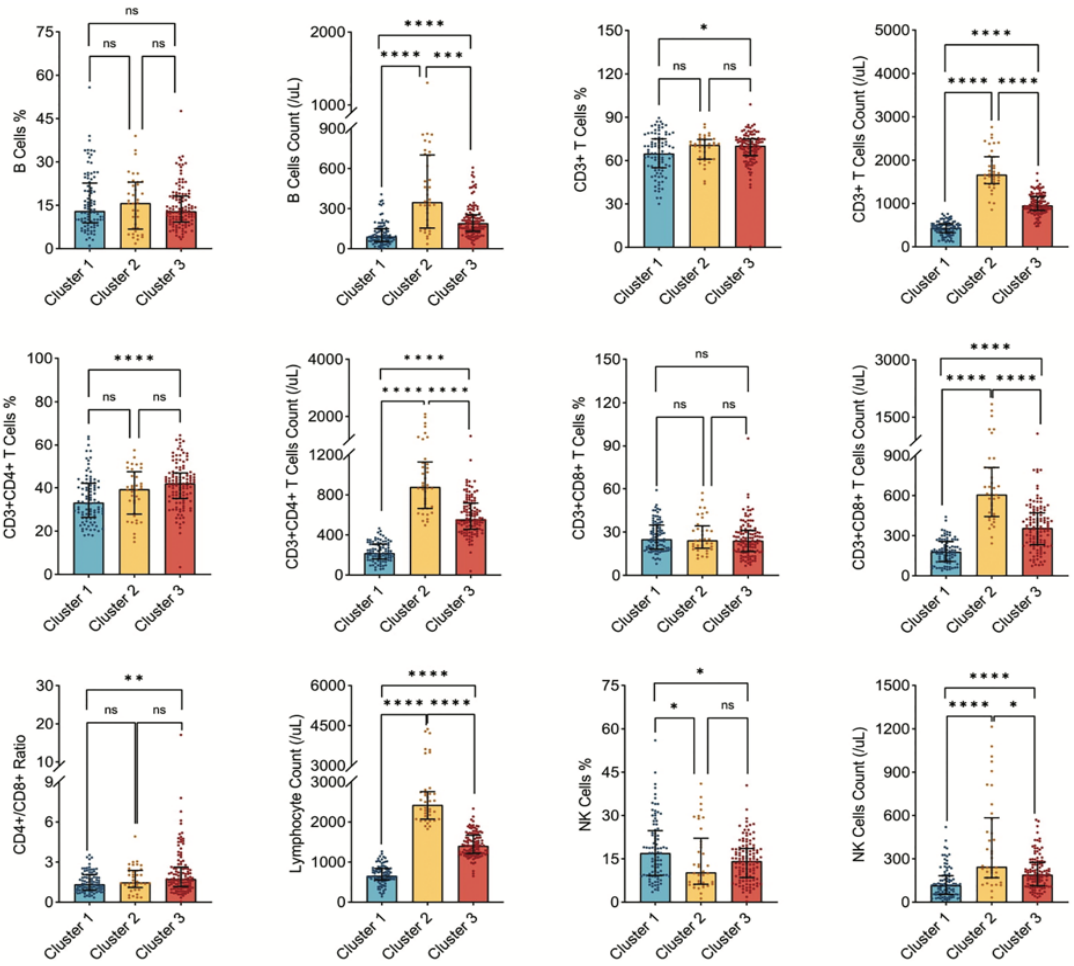

Fig. 5. Distribution of lymphocyte subsets in three clusters of AAV patients.

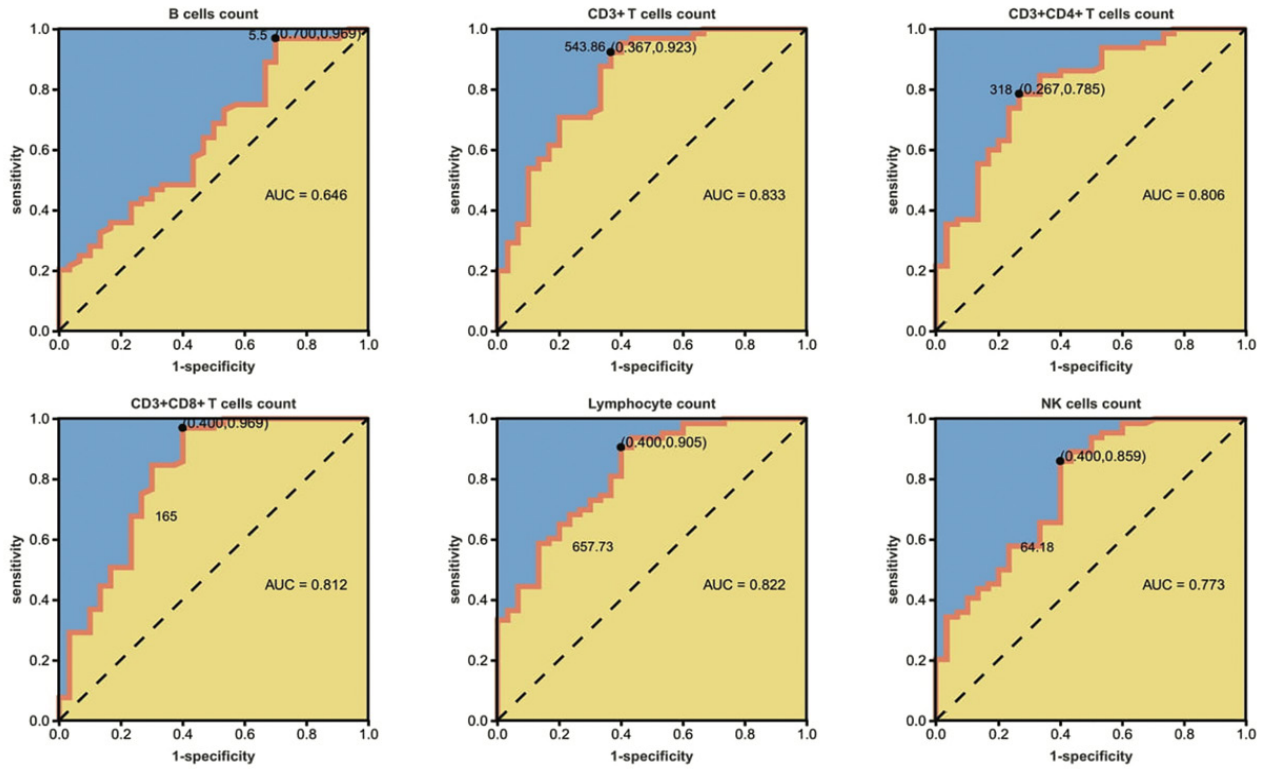

Fig. 6. ROC curve analysis of lymphocyte subsets to identify outcome in patients with AAV.

and may be a distinguishing biomarker for poor outcome.

Latest research suggests that a higher baseline total B cell number with a decreased risk of severe infections (30). However, the AUC of B cell counts was 0.646 for predicting poor outcome in our study. Additionally, no relationship existed between B cell counts and disease activity and most clinical parameters. The number of B cell also markedly decreased in the induced remission group. These results may be due to the strategy of targeting B cell for AAV standard treatment (31). Our results further indicated that AAV was associated with changes in lymphocyte subsets, so these alterations were closely linked to the active state of AAV. More investigations involving larger groups of patients are required to fully elucidate the pathogenesis of the disease.

Conclusion

A decrease in total lymphocytes and lymphocyte subset counts in the peripheral blood of active AAV patients was correlated with clinical disease activity. Low lymphocyte subsets especially low $CD3^+$ T cell count could predict a poor disease outcome in patients with AAV. These results can help us better understand the pathogenesis of AAV and develop novel treatment strategies for AAV.

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