Flow cytometry-based peripheral blood analysis as an easily friendly tool for prognostic monitoring of acute ischemic stroke: a multicenter study

Kang Lu
Zhejiang Provincial of Traditional Chinese Medicine, The First Affiliated Hospital of Zhejiang University of traditional Chinese Medicine

Juanqing Yue
Hangzhou First People's Hospital

Wanmao Ni
Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College

Jing Du
Zhejiang Provincial People's Hospital, Hangzhou Medical College

Yanchun Li
Hangzhou First People's Hospital

Xiangmin Tong
Hangzhou First People's Hospital

Guo-Bo Chen
Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College

Ying Wang (✉ nancywangying@163.com)
Hangzhou First People's Hospital

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Abstract

**Background and Objective** Acute ischemic stroke (AIS) is a leading cause of mortality, severe neurological and long-term disability world-wide. Blood-based indicators may provide valuable information on identified prognostic factors. However, currently, there is still a lack of peripheral blood indicators for the prognosis of AIS. We aimed to identify the most promising prognostic indicators and establish prognostic models for AIS.

**Methods** 484 patients enrolled from four centers were analyzed immunophenotypic indicators of peripheral blood by flow cytometry. Least absolute shrinkage and selection operator (LASSO) regression was applied to minimize the potential collinearity and over-fitting of variables measured from the same patient and over-fitting of variables. Univariate and multivariable Cox survival analysis of differences between and within cohorts was performed by log-rank test. The areas under the receiving operating characteristic (ROC) curves were used to evaluate the selection accuracy of immunophenotypic indicators in identifying AIS subjects with survival risk. The prognostic model was constructed using a multivariate Cox model, consisting of 402 subjects as a training queue and 82 subjects as a testing queue.

**Results** In the prospective study, 7 immunophenotypic indicators of distinct significance were screened out of 72 peripheral blood immunophenotypic indicators by LASSO. In multivariate cox regression, CTL (HR: 1.18, 95% CI: 1.03-1.33), monocytes/μl (HR: 1.13, 95% CI: 1.05-1.21), non-classical monocytes/μl (HR: 1.09, 95% CI: 1.02-1.16) and CD56$^{high}$ NK cells/μl (HR: 1.13, 95% CI: 1.05-1.21) were detected to decrease the survival probability of AIS, while Tregs/μl (HR: 0.97, 95% CI: 0.95-0.99, p=0.004), $B_M$/μl (HR: 0.90, 95% CI: 0.85-0.95, p=0.023) and CD16$^+$NK cells/μl (HR: 0.93, 95% CI: 0.88-0.98, p=0.034) may have the protective effect. As for indicators’ discriminative ability, the AUC for CD56$^{high}$NK cells/μl attained the highest of 0.912. In stratification analysis, the survival probability for AIS patients with a higher level of Tregs/μl, $B_M$/μl, CD16$^+$NK cells/μl, or lower levels of CD56$^{high}$NK cells/μl, CTL (HR, non-classical monocytes/μl, Monocytes/μl were more likely to survive after AIS. The multivariate Cox model showed an area under the curve (AUC) of 0.805, 0.781 and 0.819 and 0.961, 0.924 and 0.982 in the training and testing cohort, respectively.

**Conclusion** Our study identified 7 immunophenotypic indicators in peripheral blood may have great clinical significance in monitoring the prognosis of AIS and provide a convenient and valuable predictive model for AIS.

**INTRODUCTION**

Stroke has life-threatening characteristics that is one of the leading causes of death and long-term disability in the world\(^1\). Nearly 800,000 patients experience stroke each year in the US, nearly 700,000 of which are acute ischemic stroke (AIS). Acute ischemic stroke (AIS) is defined by the sudden loss of blood flow to an area of the brain\(^2–4\). Biomarkers have the potential value in predicting the prognosis of AIS and
improving the diagnosis and management of AIS patients, but have not yet shown sufficient sensitivity, specificity, rapidity, and accuracy. In short, there is an unmet need to accurately predict prognosis after acute ischemic stroke to guide early decision making. It's reported that Benjamin Dieplinger and his team have developed novel multi-markers model that combines NIHSS, IL-6, and NT-pro BNP for simple and accurate risk stratification. Nonetheless, their multi-marker prototype approach requires further validation in an independent cohort of patients with acute ischemic stroke, and should eventually also be implemented and tested in the decision making process as well\(^5\).

Owing to blood-based biomarkers may provide additional information for identified prognostic factors. As far as we know, in previous reports, the number of reports on blood biomarkers for ischemic stroke prognosis has increased, but methodological shortcomings still remains\(^6\), there are no reliable indicators to evaluate the prognosis of AIS patients\(^7,8\). In addition, because of the limited prognostic models available for AIS, predicting the prognosis of ischemic stroke patients remains challenging. Therefore, there is an urgent need to find new peripheral blood biomarkers or to develop prognostic models for early-stage prediction and accurate assessment of the prognosis of AIS.

In vitro diagnostics (IVD) has become one of the hot fields in the medical and health industry recently. It conducts in vitro testing and analysis of human samples (such as blood, body fluids, tissues, etc.) to obtain diagnostic information and judge the state of the body. IVD plays an important role in disease prevention, diagnosis, treatment monitoring, prognosis observation, health evaluation and prediction of hereditary diseases. Besides, in vitro diagnostics plays an indispensable role in chronic disease management\(^9\).

Professor Zhang Jing has innovated and developed a stable and rapid assay method for quantitative determination of nervous system-derived plasma extracellular vesicles (EVs) in plasma, through the innovative nano-flow cytometry detection technology, and innovated the discovery of a novel peripheral blood neurogenic EVs related marker NMDAR2A, to evaluate its diagnostic value for Alzheimer's Disease (AD)\(^10\). However, there is not widely accepted IVD strategy for prognostic prediction of AIS. The inflammatory response after cerebral ischemia has attracted much attention in the past few years. Stroke can induce acute immune responses that involve both local and peripheral immune compartments. Immune regulation after stroke includes the accumulation of microglia and the infiltration of macrophages, lymphocytes, dendritic cells (DCs), and neutrophil streams in the ischemic hemisphere. Numerous studies have demonstrated the critical role of the cellular and humoral immune systems in post-ischemic brain injury, and the degree of neuronal damage appears to correlate with the degree of innate immune activity\(^11,12\). In experimental animal models such as mice and rats, the crucial functions of invading immune cells and proinflammatory cytokines have been well investigated\(^13–16\), while the whole immune reaction pictures in human ischemic stroke is barely unknown. Even the poststroke immune regulation was mainly focused on the local lesion, such as resident immune cells and cytokines, whereas the activation in peripheral blood circulation was limited. Given the differences of systemic blood and immune responses in between animal models and human, it poses a challenge how to
transform discoveries in animal models into ‘druggable’ mechanisms of ischemic stroke. Thus, studies on systemic immune responses after ischemic stroke are scarce, and whether immune responses are beneficial or harmful remains controversial. Circulating immunoassays may be promising and valuable in predicting the prognosis of AIS, however, no such attempts have been made so far.

Analysis of immune subpopulations of lymphocytes in blood is one of the most important clinical applications of flow cytometry (FCM). FCM is sensitive, rapid, and multi-parametric in its analysis and detects immunophenotypes in blood more accurately than other methods. To examine the value of peripheral blood immune cells in predicting the prognostic outcome of AIS, we conducted a study with a large time span and sample size. FCM was used to obtain comprehensive information on the composition, phenotype, and function of peripheral blood immune cells. The aim of this study was to develop a prognostic model of AIS using FCM and to try to understand the logic behind this model for better use in clinical application.

**METHOD**

**Data acquisition**

This was a multicenter, prospective study that recruited a total of 484 participants from 4 centers across China from January 2016 to July 2019. The training cohort and testing cohort were from Zhejiang Provincial People's Hospital, Zhejiang Provincial Hospital of Traditional Chinese Medicine, the Second Affiliated Hospital of Zhejiang University of Traditional Chinese Medicine, and Hangzhou First People's Hospital. A disease stratified random sampling method was used to select 255 subjects from the AIS cohort and 147 subjects from the control cohort as the training cohort to construct the prognostic model. All personal privacy information was well protected and removed during the analysis and publication process. This study was approved by ethics committee of Zhejiang Provincial People's Hospital (No. 2020QT295) and was exempt from informed consent as shown by the IRB approval letter. The inclusion and exclusion criteria were detailed in the study-design workflow (Fig. 1). Specifically, subjects with AIS were defined as those who meet all the following criteria: (1) Age is ≥ 18 years old; (2) Diagnosed as AIS and confirmed by radiography; (3) AIS symptom onset ≤ 4.5 hours; (4) National Institutes of Health Stroke Scale (NIHSS) 5–25 (inclusive); (5) First stroke attack, or pre-stroke MRS ≤ 1 if with previous stroke. Those who with (1) intracranial hemorrhage; (2) other contraindications or complications; were excluded from the AIS cohort. Subjects with other mild neurological diseases but not vascular diseases were defined as controls who meet all of the following criteria during their hospital visits: (1) Age is ≥ 18 years old; (2) Diagnosed with myasthenia gravis, dizziness, peripheral neuropathy or Parkinson; (3) mild to moderate. Patients with vascular diseases such as ischemic, hemorrhage, vascular malformations were excluded from the control group. All subjects were evaluated and screened by a neurologist, 500 eligible subjects were selected, with 300 for AIS and 200 for control group. 45 and 53 subjects were excluded from the AIS and control group respectively, since their baseline or immunophenotypic data were missing. The remaining 255 subjects of the AIS group and 147 subjects remained in the control
group (73 myasthenia gravis, 40 dizziness, 17 peripheral neuropathy and 17 Parkinson) were used for model construction. In addition, we followed the survival status and time of 82 AIS patients after two years and collected their peripheral blood immunophenotypic indicators as a testing queue for model construction.

**Baseline characteristics and flow cytometry**

Baseline characteristics include age, sex, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), the history of smoking, alcohol consumption, as well as the use of antihypertension or lipid-lowering drugs. Height and weight were measured with the participants standing without shoes or heavy outer garments. BMI was calculated by dividing weight in kilograms by height in meters squared. Blood pressure was detected before other parameters, including smoking, alcohol and drug history etc. were obtained by questioning during their first outpatient visit.

**Sample collection and processing**

Within 24 hours of admission, the peripheral blood samples from subjects diagnosed with AIS were collected, anticoagulated with heparin and stained. Each sample was divided into five tubes and antibodies were added to each flow tube in turn according to the assay protocol, then 100 microliters of peripheral blood was taken and incubated for 15 minutes under light-free conditions, followed by 1× ammonium chloride lysate (0.15 M NH4CL, 10mM NaHCO3, 1 mM EDTA-Na2) mixed thoroughly for 10 minutes without exposure to light, and finally centrifuged at 500 rpm for 5 minutes, then the supernatant was removed and the sample was resuspended and mixed with 200 microliters PBS and assayed on a computer. A more detailed test procedure is described in the reference13. The assay protocol is listed in Supplementary Table 1.

**Model construction and statistical analysis**

The data set was divided into two parts: training queue and testing queue. Marker negative proportion and subgroup proportion were used as modelling features. The training queue which included 255 AIS subjects and 147 control subjects, and testing queue consisted of 82 AIS subjects with a 2-year follow-up. Modeling was processed in two phases: (1) developing the model using the training queue and generating prediction estimates; (2) then validating it in the testing queue. Due to the excessive number of features, there may be some collinearity and non-significant features, so feature screening is needed to filter out the important and useful features. At the first stage, among these 72 immunophenotypic indicators, statistically significant indicators between the male and female groups were excluded to eliminate gender bias. After that, 41 indicators were retained for comparison with the control group, and finally 22 indicators were statistically significant with a \( p \) threshold of 0.05. Least absolute shrinkage and selection operator (lasso) regression, 10-fold cross-validation and penalty was used to construct the AIS prognostic model through the “glmnet” R package. Lasso regression is a widely used machine learning algorithm; compared with traditional logistic regression, it uses a penalty term, which can actively select
impactful parameters from many potential multicollinearity variables in the regression, helping to reduce prediction errors.

Finally, univariate and multivariate Cox regression models were constructed by selecting 7 immunophenotypes, and the obtained data were used to draw a nomogram to predict the survival probability of AIS patients. The prediction line was drawn upward to confirm the points obtained from the nomogram. The sum of these points lies on the “total points” axis; Subsequently, a line was drawn down the bottom scale to determine the probability of survival. The area under the curve (AUC) value of ROC curve was used to evaluate the performance of univariate and multivariate cox regression models. In the second stage, testing set of 82 AIS subjects follow-up for 2 years was used to verify and evaluate the cox regression model, and the area under the ROC curve (AUC) value was used to evaluate the performance of univariate and multivariate cox regression models. At the same time, AIS subjects were divided into “high” group and “low” group according to the average of the 7 indicators, and Kaplan-Meier curve was applied to reflect the survival difference between two groups. The heatmap was showed seven immune indicators for model construction as well as following clinical features, containing gender, age (≤ 60 or > 60 years), the status of alcohol and tobacco use (yes, no and NA) and living status (alive, dead and NA).

Besides, quantitative variables were presented as the median and interquartile spacing by using the Mann–Whitney U test. Categorical variables were expressed as the frequency (percentage) and were compared using chis-square test. A p-value of < 0.05 was considered for statistical significance and all statistical analysis was performed with R software version 4.0. Moreover, based on R software (v4.1.2), we analyzed the correlation between clinical features and immune indicators for model construction in AIS patients with R package–Complex Heatmap [2.13.1].

RESULTS

Demographic characteristics of cohorts

A total of 402 patients were included in this study, including 255 with AIS, 147 with other mild neurological diseases and without any vascular diseases as controls. Baseline demographic characteristics were presented in Table 1. Patients in AIS group were predominantly males (173, 63.8%), with an older mean age (70.2±13.3) and higher systolic (152.4±20.7) and diastolic (81.7±13.2) blood pressures than controls. In addition, the proportion of alcohol consumption (244, 96.8%), use of antihypertensive drugs (157, 94.0%), and lipid-lowering drugs (156, 94.0%) were higher in the AIS group compared with the control group. The differences between the above indicator groups were statistically significant (p<0.05). BMI and smoking history were balanced and not statistically significant.

Table 1. Characteristics of study subjects
**Demographics**

<table>
<thead>
<tr>
<th></th>
<th>AIS group (n = 255)</th>
<th>Control group (n = 147)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex male, n (%)</td>
<td>173 (67.8)</td>
<td>69 (46.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age, years, (IQR)</td>
<td>71 (62,81)</td>
<td>58 (41.25,67)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body mass index, (IQR)</td>
<td>23.49 (21.21,25.78)</td>
<td>25.95 (22.25,52.50)</td>
<td>0.546</td>
</tr>
<tr>
<td>Systolic BP (IQR), mmHg</td>
<td>152 (143.25,166)</td>
<td>118.50 (73,138)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic BP (IQR), mmHg</td>
<td>80 (73,90)</td>
<td>82 (71,119.25)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Having smoking history, n (%)</td>
<td>80 (31.7)</td>
<td>20 (21.3)</td>
<td>0.056</td>
</tr>
<tr>
<td>Having alcohol history, n (%)</td>
<td>244 (96.8)</td>
<td>11 (11.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Using antihypertension drugs, n (%)</td>
<td>157 (94.0)</td>
<td>67 (84.8)</td>
<td>0.018</td>
</tr>
<tr>
<td>Using lipid-lowering drugs, n (%)</td>
<td>156 (94.0)</td>
<td>67 (84.8)</td>
<td>0.019</td>
</tr>
</tbody>
</table>

AIS = acute ischemic stroke; Systolic BP = systolic blood pressure; Diastolic BP = diastolic blood pressure

**Survival analyses**

To reflect the survival difference, the Kaplan-Meier curve was used to compare the survival probability of the AIS group with that of the control group, as shown in Figure 2A. It could be found that within 10 months, the survival probabilities of the two groups tended to be close to each other and remained around 1; then after 10 months, the survival probabilities of patients in the AIS group decreased significantly compared with the control group, and the difference was statistically significant (log-rank: \( p < 0.001 \)); while the survival probabilities of patients in two groups tended to be equal in the next 30 months. To avoid gender bias as indicated by the differences between the two groups, a subgroup analysis was done to explore the survival differences between the male and the female groups. The results showed that survival probabilities decreased in both male and female groups after 10 months and leveled off after 30 months, with no statistically significant difference between the two groups (log-rank: \( p = 0.70 \)).

**Feature selection and model construction**

To better understand whether systemic immune function reflects the progression and prognosis of AIS, we performed a group-wide study including 72 immunophenotypic indicators using flow cytometry on peripheral blood samples within 24 hours after the onset of AIS. We observed that among the 72 immunophenotypic indicators, we first excluded 31 indicators that were statistically different between the male and female groups to eliminate gender bias. Details were shown in Supplementary Table 2. Then, 22 of the 41 indicators were selected by univariate analysis of AIS versus controls (Supplementary Table...
and correlation analysis was performed on these 22 indicators, which showed that there was no strong correlation between them. Then, the least absolute shrinkage and selection operator (lasso) regression was applied to screen $CD56^{\text{high}}$NK cells/$\mu l$, Tregs/$\mu l$, $CD16^{+}$NK cells/$\mu l$, $B_M$/$\mu l$, CTL (%), non-classical monocytes/$\mu l$ and Monocytes/$\mu l$. These indicators were then applied to construct prognostic model (Figure 3).

Then, we performed univariate and multivariate Cox regression analysis to explore the immunophenotypic indicators associated with prognosis. And a forest plot was used to represent these indicators and their Hazard ratio (HR), 95% CI and $p$ values between AIS and the control group, as detailed in Figure 4. Univariate analysis revealed that CTL (%) [HR: 2.03, 95% CI: 2.00-2.06, $p$ < 0.001], Monocytes/$\mu l$ [HR: 2.00, 95% CI: 1.90-2.10, $p$<0.001], Non-classical monocytes/$\mu l$ [HR: 1.51, 95% CI: 1.20-1.82, $p$=0.015], $CD56^{\text{high}}$NK cells/$\mu l$ [HR:1.70, 95% CI:1.50-1.90, $p$=0.008] and $CD16^{+}$NK cells/$\mu l$ [HR:1.05, 95% CI:1.01-1.11, $p$=0.005] may contribute to decreasing the survival probability of AIS, while Tregs/$\mu l$ [HR:0.96, 95% CI:0.94-0.99, $p$=0.003] and $B_M$/$\mu l$ [HR: 0.97, 95% CI: 0.95-0.99, $p$<0.001] were prognostic protective factors of AIS. On the other hand, multivariate Cox regression analysis showed that CTL (%) [HR: 1.18, 95% CI: 1.03-1.33, $p$=0.034], Monocytes/$\mu l$ [HR: 1.13, 95% CI: 1.05-1.21, $p$=0.043], Non-classical monocytes/$\mu l$ [HR: 1.09, 95% CI: 1.02-1.16, $p$=0.041] and $CD56^{\text{high}}$NK cells/$\mu l$ [HR: 1.13, 95% CI: 1.05-1.21, $p$<0.001] decreased the survival probability in the AIS group, while Tregs/$\mu l$ [HR:0.97, 95% CI: 0.95-0.99, $p$=0.004], $B_M$/$\mu l$ [HR:0.90, 95% CI: 0.85-0.95, $p$=0.023] and $CD16^{+}$NK cells/$\mu l$ [HR:0.93, 95% CI: 0.88-0.98, $p$=0.034] may have a protective effect on the prognostic likelihood of survival in patients with AIS. The differences between the groups of the above indicators were statistically significant ($p$<0.05).

**Survival probability nomogram development and performance of the Cox model**

Based on the above seven indicators, a multivariate Cox regression model was constructed, and the 7 indicators in the cox regression model were integrated to the nomogram to predict the survival rate of patients in the next 1, 2, and 3 years, respectively. For each AIS patient, the higher the total points, indicated the lower survival probability. For example, if the patient had a CTL (%) of 40, Tregs of 5$\mu l$, $B_M$ (%) of 10, monocytes of 600$\mu l$, non-classical monocytes of 80, $CD56^{\text{high}}$NK cells of 25$\mu l$ and $CD16^{+}$NK cells of 30$\mu l$, then the corresponding points would be approximately 10, 10, 15, 10, 10, 40 and 50, respectively. The total score would be approximately 145, indicating an estimated survival probability of 48% for the next two years and 32% for the next three years for this case. More details can be found in Figure 5.

To assess the accuracy of predicting AIS adverse events, the AUC values of the univariate cox regression model ROC curves were calculated. The AUC of $CD56^{\text{high}}$NK cells/$\mu l$ was higher at 0.912 (0.884-0.954), while the AUC value of $CD16^{+}$NK cells/$\mu l$ was lower at less than 0.8 (0.72, 95% CI: 0.654-0.758). The accuracy of the other indicators in predicting AIS prognosis ranged from 0.820 to 0.879. Detailed information on the AUC of each indicator and its 95% confidence interval was shown in Supplementary Figure 2. Based on the performance of these indicators in the univariate analysis, we combined seven
indicators to construct a multivariate Cox model. The results showed that the AUC of the integrated Cox model was 0.805 (0.781–0.819), which could be used to predict the prognosis of AIS. To further validate the performance of the multivariate model in the prediction of AIS survival, we followed up 82 patients at 2 years and collected their peripheral blood immunophenotypic indicators as a test set for analysis. Notably, the multivariate Cox model achieved a high AUC of 0.961 (0.924-0.982) in the test set, indicating that the model is relatively stable and has a good predictive performance (Figure 6).

To further confirm the prognostic value of these immunophenotypic indicators, we employed stratified analysis and divide AIS patients into high and low subgroups based on the mean of each indicator. Notably, AIS patients with higher levels of Tregs/μl, B_{M}/μl or CD16+NK cells/μl had higher survival probability than AIS patients with lower levels. In contrast, for AIS patients with lower levels of CD56^{high}NK cells/μl, CTL (%), non-classical monocytes/μl or Monocytes/μl, survival probability may be higher than in the high-level group (Figure 7).

The heatmap showed the relationship between the seven immunophenotypic indicators used to construct the model and as the clinical characteristics, including gender, age (≤60 or >60 years), drinking and smoking status (yes, no and NA) and living status (alive, dead and NA) (Figure 8).

**DISCUSSION**

Immune microenvironment and inflammatory response are involved in the whole process of AIS development\(^{16,18}\). Although the local immune microenvironment of AIS has been well studied, the relationship between AIS and systemic immunity is rarely explored. Immune cells in the central nervous system (CNS) engage in crosstalk with infiltrated peripheral immune cells, forming a complicated inflammatory network that may indirectly influence BBB integrity. Therefore, circulating, and cerebral immune cells play a profound and dual role in BBB disruption following ischemic stroke. Cerebral immune cells (CICs) and peripheral immune cells (PICs) form a subtle and complex network, and the interference may indirectly affect the integrity of blood-brain barrier (BBB) during ischemic stroke. Peripheral immune cells (including macrophages) migrate through the injured BBB to the injured site and activate host immune cells, such as microglia. Infiltrating macrophages and activated microglia release more cytokines, chemokines, and other molecules, leading to further damage or protection of the ischemic brain\(^{11,19}\). To the best of our knowledge, this is the first time to use human peripheral blood immune cell profile to establish a prognostic prediction model for AIS patients, and the sample size involved in this study is large. Previous studies mainly focused on the local inflammatory response after stroke, and few studies on the peripheral immune status. This study fills this gap. In this study, we constructed an AIS prognostic model by considering peripheral blood immunophenotypic indicators to predict the prognosis of patients with acute ischemic stroke. In addition, to make our model more consistent with a natural population cohort and to avoid the failure of normal individuals to reflect the disease characteristics of AIS, we selected neurological patients from the same time, i.e., those diagnosed with non-vascular neurological disease, and age-matched cases to controls, which helped to better
assess the predictive power of peripheral blood immunophenotypic indicators for AIS prognosis. We determined the relationship between overall survival (OS) and peripheral blood immunophenotypic indicators by immunophenotyping using flow cytometry. Among the seven indicators selected by Lasso, the model showed that Monocytes/µl, non-classical monocytes/µl and CD56\textsubscript{high}NK cells/µl were risk factors for the prognosis of AIS, while Tregs/µl, B\textsubscript{M}/µl and CD16\textsuperscript{+}NK cells/µl were protective and validated by Test set. When comparing the discriminative ability, the AUC for CD56\textsubscript{high}NK cells/µl was the highest at 0.912; while other indicators of AIS prognosis had AUC values of 0.820 to 0.879. Besides, based on the multivariate Cox regression model, a nomogram for predicting survival was established and the multivariate model was validated using follow-up data (AUC: 0.961). Stratification analysis revealed that the survival rates for AIS patients with high levels of Tregs/µl, B\textsubscript{M}/µl or CD16\textsuperscript{+}NK cells/µl was higher than that of the low level group, and the survival rate of AIS patients with low levels of CD56\textsubscript{high}NK cells/µl, CTL (%), Non-classical monocytes/µl or Monocytes/µl was higher than that of the high level group.

The follow-up data of testing queue which contained 82 AIS patients confirmed the good accuracy and compliance of the model, and the visualized and personalized nomogram model may provide clinicians with a simple and intuitive practical prediction tool.

The exact role of monocytes in BBB disruption remains unclear, since clinical observations are controversial. Some studies suggested that their roles were harmful to BBB disruption. Previous studies indicated that monocyte counts did not change during AIS, the counts cannot predict long-term mortality\textsuperscript{20}. A recent investigation demonstrated that lower MHRs were independently associated with increased HT risk, especially sICH in AIS patients\textsuperscript{21}. Elevated levels of classical monocytes were associated with early clinical worsening and higher mortality in ischemic stroke, while non-classical monocytes were significantly decreased in stroke patients. Decreased levels of non-classical monocytes was inversely related to poor prognosis. Thus, distinct subpopulations may exert different roles in BBB integrity and stroke outcome. Ralf Stumm and his team demonstrated that Cxcr4 ablation reduces monocyte infiltration after tMCAO, which is associated with a deteriorated outcome and altered molecular responses of monocyte-derived macrophages (MDMs) and microglia\textsuperscript{22}. Intriguingly, by univariate analysis, it could be found that non-classical monocytes/µl and Monocytes/µl may be associated with reduced survival in patients with AIS. Overall, our findings are consistent with those previously reported in the literature. Circulating innate immune cells are quickly engaged at the onset of arterial occlusion, ultimately resulting in invasion of the ischemic brain by blood-borne immune cells and activation of brain-resident cells, which can be either beneficial or detrimental. Monocytes enter the CSF through the choroid plexus after ischemic stroke\textsuperscript{23}. NK cells also contribute to ischemic brain injury. Yan Feng and his team indicated that miR-1224 suppresses NK cell function through Sp1 after ischemic stroke, especially in the periphery. These results suggest that blocking miR-1224 biogenesis or administering a miR-1224 antagonist might be a viable therapeutic approach for poststroke immunosuppression and infection\textsuperscript{24}. They also confirmed that miR-1224 suppressed NK cell activation and cytotoxicity specifically in the periphery rather than in the brain. Our data established that it is possible to enhance the cytotoxicity of peripheral NK cells by targeting miR-1224 while preserving the immunosuppression of brain-infiltrating

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NK cells to avoid aggravated intracerebral inflammation. This result is interesting and seemingly paradoxical because studies by our team and others have all suggested that NK cells can migrate into the brain parenchyma after brain ischemia\(^{24}\). However, in our study, by stratification analysis, revealed that CD16\(^+\)NK cells/\(\mu l\) may play a protective role and CD56\(^{\text{high}}\)NK cells/\(\mu l\) may become a dangerous biomarker in prognosis of AIS. NK cells have been demonstrated to exacerbate brain infarction after ischemic stroke by promoting local inflammation and neuronal hyperactivity\(^{25}\).

Treg cells play important immunosuppressive functions in maintaining immune homeostasis and curbing inflammatory responses in different diseases. Some studies have shown that CNS-infiltrating Treg cells, brain-resident microglia, and oligodendrocytes interact to manage white matter (WM) repair and functional recovery in the chronic stage of stroke. We have discovered that Treg cells enhance post-stroke oligodendrogenesis, at least partially, in a microglia-dependent manner. Osteopenia has been identified as a mediator between Treg cells and microglia. Boosting Treg cell numbers might be a practical and druggable approach to improve WM repair and functional recovery\(^{26}\). It is apparent that the mechanisms underlying the effects of Treg cells in an ischemic brain are complicated, involving crosstalk between the CNS and peripheral immune system and interactions between Treg cells and many other types of cells. Besides, in an ischemic brain, Treg cells transmit inhibitory signals to neutrophils via PD-L1/PD-1 interactions, thereby inhibiting matrix metallopeptidase 9 (MMP-9) production and protecting the integrity of the blood-brain barrier\(^{27}\). Thus, Tregs may be a promising candidate for cell-based therapies targeting post-stroke inflammatory disorders and neurovascular dysfunction. This is consistent with the results of our analysis that Tregs are protective factors in the development of AIS\(^{28–30}\).

To date, neuroinflammation is a complex event regulated by multiple factors that play a crucial role not only in the pathogenesis of the ischemic injury but also in determining its evolution. The brain insult that follows ischemic stroke results in necrosis and apoptosis; all of this drives an inflammatory reaction controlled by the discharge of ROS, chemokines, and cytokines. This process springs up in the microcirculation and involves several cytotypes, such as innate immune cells (i.e., the microglia) and adaptive immune cells (i.e., lymphocytes) causing neuronal death\(^{31}\).

It has been well acknowledged that inflammation plays a key role and is involved in the whole process of acute ischemic stroke, and one of the important stages is the recruitment of leukocytes from the peripheral circulation into the ischemic tissue\(^{32}\). In general, changes in peripheral blood lymphocyte subsets may reflect local inflammation in the central nervous system\(^{33}\). The specific mechanism between lymphocytes and AIS may be explained by lymphocytes infiltrating ischemic tissue and mediating inflammatory responses, which increase the levels of anti-inflammatory cytokines and inhibit the production of pro-inflammatory cytokines\(^{32}\). During the ischemic stroke cascade, infiltration of immune cells and release of pro-inflammatory cytokines reduce blood-brain barrier damage, brain edema, and infarct volume.
Several limitations of this study need to be acknowledged. First of all, 402 samples were recruited in this study, so larger sample trial is required to verify the relationship between peripheral blood immune indicators and the prognosis of AIS. The study participants came from 4 centers in Zhejiang Province. They were samples from a single region, so to some extent, they were not internationally representative. However, we believe that given the nature of this immunophenotypic assay model, the current approach can be implemented across different ethnic groups and regions, although the parameters of the model will be modified to some extent. Secondly, we only examined the peripheral blood immunophenotype indicators within 24hrs after AIS onset but did not monitor the fluctuations of those indicators continuously to better show the correlation with the prognosis. Third, our study confirmed the validity of peripheral blood immunophenotypic indicators in the prognostic testing of AIS, and whether the strategy can be used for other related ischemic diseases is vague. The aim of our study was to develop a validated prognostic tool for acute ischemic stroke and to confirm the performance of our model through validation.

**CONCLUSION**

In conclusion, we demonstrated the utility of a deep immune profiling approach with flow cytometry to characterize the systemic immune response comprehensively and functionally within 24 hours after AIS in peripheral blood samples. Immunophenotypic indicators of early acute stroke outcome may contribute to AIS treatment. The AIS model constructed based on the peripheral blood immunophenotypic indicators provides a new approach to predict the survival of AIS patients and provide a basis for comprehensive human studies with important implications for clinical routine. Therefore, we may provide an easily and friendly approach for the prognostic of AIS. Besides, our attempt may also lay the foundation for future systemic immunological method for AIS.

**Abbreviations**

AIS = Acute ischemic stroke; AUC = area under the curve; CTL (%) = The percentage of Cytotoxic T cells; Tregs/ul = Absolute number of total Treg cells; $B_M/\mu l$ = Absolute number of Memory B cells; FCM = flow cytometry

**Declarations**

**Ethics approval and consent to participate**

Name of the ethic committee: Medical Ethics Committee of Zhejiang Provincial People's Hospital

Approved No. of ethic committee: 2020QT295

Registration number: ChiCTR2000040207

**Consent for publication**
Not applicable

**Availability of data and materials**

Please contact author for data requests.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

KL and YW drafted manuscript text. KL and J.Y. generated figures and tables. W.N. and JD and Y.L. contributed on the testing of immunophenotypic indicators in acute ischemic stroke. G.C. and X.T. contributed to manuscript design and final review. YW is responsible for the manuscript design and final writing. All authors read and approved the final manuscript.

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Not applicable

**Authors' information**

KL, J.Y. and W.N. contributed to the work.

KL: Department of Laboratory Medicine, Zhejiang Provincial of Traditional Chinese Medicine, The First Affiliated Hospital of Zhejiang University of traditional Chinese Medicine, Hangzhou, Zhejiang, 310006, China

J.Y.: Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, 310006, China

W.N.: Clinical Research Institute, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, Zhejiang, 310014, China

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**Figures**
Figure 1

Study design
Figure 2

Survival probability between (A) AIS and control group, (B) male and female group.
Figure 3

(A) Least absolute shrinkage and selection operator (LASSO) coefficient profiles of the fractions of 22 indicators. (B) Tenfold cross-validation for tuning parameter selection in the LASSO model. (C) Absolute value of coefficient of 7 immunophenotypic indicators.
Figure 4

(A) Univariate and (B) multivariate Cox regression analysis for 7 selected immunophenotypic indicators

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Hazard ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Univariate Analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTL (%)</td>
<td>2.03 (2.00–2.06)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tregs/ul</td>
<td>0.96 (0.94–0.99)</td>
<td>0.003</td>
</tr>
<tr>
<td>B_{δ}/ul</td>
<td>0.97 (0.95–0.99)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Monocytes/ul</td>
<td>2.00 (1.90–2.10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non-classical monocytes/ul</td>
<td>1.51 (1.20–1.82)</td>
<td>0.015</td>
</tr>
<tr>
<td>CD56^high NK cells/ul</td>
<td>1.70 (1.50–1.90)</td>
<td>0.008</td>
</tr>
<tr>
<td>CD16^+ NK cells/ul</td>
<td>1.05 (1.01–1.11)</td>
<td>0.005</td>
</tr>
<tr>
<td>(B) Multivariate Analysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTL (%)</td>
<td>1.18 (1.03–1.33)</td>
<td>0.034</td>
</tr>
<tr>
<td>Tregs/ul</td>
<td>0.97 (0.95–0.99)</td>
<td>0.004</td>
</tr>
<tr>
<td>B_{δ}/ul</td>
<td>0.90 (0.85–0.95)</td>
<td>0.023</td>
</tr>
<tr>
<td>Monocytes/ul</td>
<td>1.13 (1.05–1.21)</td>
<td>0.043</td>
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<tr>
<td>Non-classical monocytes/ul</td>
<td>1.09 (1.02–1.16)</td>
<td>0.041</td>
</tr>
<tr>
<td>CD56^high NK cells/ul</td>
<td>1.13 (1.05–1.21)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD16^+ NK cells/ul</td>
<td>0.93 (0.88–0.98)</td>
<td>0.034</td>
</tr>
</tbody>
</table>
Figure 5

Nomogram for the survival probability of multiple indicators of AIS patients

Figure 6

The ROC curve of the multivariate Cox model in training and testing set
Figure 7

Kaplan-Meier curves for high and low groups stratified by the mean of each immunophenotype indicator in AIS group.
Figure 8

Heatmap of the relationship between seven immunophenotypic indicators used for model construction and AIS clinical features.

AIS = acute ischemic stroke; CTL = Cytotoxic T lymphocytes; $B_M$ = absolute number of Memory B cells

NA, not available.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx
- SupplementaryTable2.docx
• SupplementaryTable3.docx