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Changes of T cell subsets across treatments associated with prognosis in newly diagnosed follicular lymphoma

Qiuhui Jiang[1,2,5], Feng Lin[1,2,5], Zhifeng Li[1,2,5], Hongpeng Duan[1,2], Chong Jiang[3], Xingxing Yu[1,2], Caiyan Wang[1,2], Li Zhang[1,2], Xiuhua Sun[4], Jie Zha[1,2], Long Liu[1,2]✉, Zhijuan Lin[1,2]✉ & Bing Xu[1,2]✉

Follicular lymphoma (FL) is an immune-responsive tumor with spontaneous remission. T cells play a pivotal role in the anti-lymphoma immune response. However, the dynamics of T cells during treatment, their impact on FL clinical outcomes, and the risk factors contributing to T-cell cytopenia remain largely unexplored. T-cell and their subsets in the peripheral blood of FL patients at diagnosis, during 2–4 cycles and after 6 cycles of treatment, as well as healthy individuals were detected by flow-cytometry. The predictive effects of T cells for early progression and risks for T-cell cytopenia were analyzed. FL patients exhibited a significant decrease in CD3+, CD4+, and CD8 + T cells compared to healthy individuals, with aging intensifying the decline of CD3+, and CD4 + T cells. Notably, a reduction in CD4 + T cells, predominantly contributing to treatment-related T-cell reduction, was only observed in patients undergoing Bendamustine-based regimens. Moreover, a significantly decreased CD4 + and CD8 + T-cell at diagnosis rather than after induction therapy was observed in patients with treatment failure. Furthermore, lower CD4 + T-cell (< 260/uL) at baseline was independently correlated to early progression within 24 months. Finally, disease stage and albumin were the independent predictive factors for the decline of CD4 + T cells in FL patients. Overall, FL patients demonstrated compromised T-cell immunity, with a lower CD4 + T cell count at diagnosis correlating with treatment response and early progression. Therefore, monitoring CD4 + T cells at diagnosis might reflect immune status and aid in stratifying FL patients.

Keywords Follicular lymphoma, T cell subsets, Lymphocyte, Outcomes

Follicular lymphoma (FL) is the most common indolent B-cell lymphoma mostly with balanced chromosomal translocation t (14;18) (q32; q21) and a highly heterogeneous clinical course[1,2]. Notably, FL is recognized as an immunocompetent tumor. Spontaneous remission is observed in 20–30% of patients, and chemo-free regimens utilizing immune modulatory drugs, such as lenalidomide/rituximab, have demonstrated comparable efficacy to standard immunochemotherapy. Therefore, the immune status of patients plays a critical role in the pathogenesis and progression of FL[3–5].

T cells serve as the primary effector immune cells in anti-lymphoma immunity. The amount of T cells infiltrating the tumor microenvironment is associated with anti-lymphoma response and could predict the progression of disease[6–10]. In addition to T cells within the lymphoma site, circulating T-cell counts in peripheral blood closely correlate with immune status, which in turn is associated with prognosis[11–13]as well as the risk of infection[14]. He et al[15]. found that low absolute CD4 + T cell counts in peripheral blood are associated with inferior survival in FL. Additionally, T-cell cytopenia is not only present at diagnosis but also tends to occur more frequently post-immunochemotherapy[16]. Currently, the first-line immunochemotherapy for FL alternates

[1]Department of Hematology, Institute of Hematology, School of Medicine, The First Affiliated Hospital of Xiamen University, Xiamen University, Xiamen, China. [2]Key Laboratory of Xiamen for Diagnosis and Treatment of Hematological Malignancy, Xiamen, Fujian Province, China. [3]Department of Nuclear Medicine, West China Hospital, Sichuan University, Chengdu, Sichuan Province, China. [4]Department of Hematology, The Second Affiliated Hospital of Dalian Medical University, Dalian, Liaoning Province, China. [5]Qiuhui Jiang, Feng Lin and Zhifeng Li contributed equally to this work share first authorship. ✉email: kucangyaoren@126.com; zjuan_lin@126.com; xubingzhangjian@126.com

between regimens combining anti-CD20 antibodies with CHOP/like treatments and those combining anti-CD20 antibodies with Bendamustine[1,17]. As reported, CD4 + T cell cytopenia was linked to a higher incidence of infection after Bendamustine and rituximab treatment in the indolent non-Hodgkin lymphoma[18]. However, the dynamics of T cells during treatment, their role in FL, and the risk factors for T-cell cytopenia incidence remain largely unexplored[8,19,20].

Consequently, our aim was to investigate the longitudinal changes in T-cell subsets during FL treatment, their predictive role in treatment responses and prognosis, and to identify risk factors contributing to T-cell cytopenia.

Methods

Patients and sample collection

As illustrated in Fig S1, we retrospectively included 96 newly diagnosed (ND) FL patients who underwent lymphocyte subset testing at baseline from March 2019 to January 2024. These patients were from the Department of Hematology of the First Affiliated Hospital of Xiamen University ($N=67$), the West China Hospital of Sichuan University ($N=19$), and the Second Affiliated Hospital of Dalian Medical University ($N=10$). Meanwhile, we selected 30 healthy controls (HC) who matched the FL patients in terms of gender and age. Patients with FL who had suffered histologic transformation to Diffuse large B-cell lymphoma (DLBCL) upon diagnosis were excluded. Initial treatment regimens after diagnosis and response to treatment, such as complete remission (CR) rate, were recorded and assessed using the Lugano criteria[21]. As for treatment dose, we followed standard guidelines, administering full dosages for patients under 65 and a 20% reduction for those aged 65 and older or deemed unfit. POD24 was defined as disease progression within 24 months after initial chemoimmunotherapy. This study was conducted in accordance with the Declaration of Helsinki. Approval was granted by the Ethics Committee of the First Affiliated Hospital of Xiamen University, the West China Hospital of Sichuan University, and the Second Affiliated Hospital of Dalian Medical University. All included patients consented to participate in this study and provided their signatures on the informed consent form.

The lymphocyte subsets analyzed in this study included T cell counts (CD3+, CD4+, CD8+) and CD4/CD8 ratios. Data for T cell subsets were gathered at three distinct time points: pre-treatment, mid-treatment (between 2 and 4 cycles), and post-treatment (following 6 cycles). Of the 96 patients with FL, 54 underwent monitoring of T cell subsets pre-, mid-, and post-treatment. Meanwhile, 70 patients were monitored for T cell subsets at both baseline and post-treatment stages.

Flow cytometry analyses

Fresh peripheral blood samples were collected into heparin anticoagulation tubes, then co-incubated with fluorescence-coupled monoclonal antibodies (such as CD4, CD8) in absolute counting tubes for 15 min at room temperature, followed by the addition of lysing solution (15 min, room temperature), centrifuge, discard the supernatant and add the appropriate amount of phosphate-buffered saline to wash it again, add the fixative to resuspend the cells, and then immediately put on the machine for detection. Samples that could not be analyzed immediately were stored at 2–8 °C until analysis, but no longer than 24 h. The monoclonal antibodies used were anti-human CD3 (FIFC), CD4 (PE-Cy^{TM}7), and CD8 (APC-Cy7) from BD Biosciences, USA. Flow cytometry was conducted using a BD FACSCanto system by local laboratories in each hospital, following institutional protocols.

Statistical analysis

Data for continuous variables were presented as mean ± standard deviation (SD). Paired samples were compared using the Wilcoxon signed-rank test. The t-test or Mann-Whitney U-test was used to assess the statistical significance of differences between two groups. Comparisons of variables across multiple groups were analyzed using either ANOVA or the Kruskal-Wallis test, with multiple comparisons made using the Bonferroni method. Categorical information was presented as ratios (%), and the chi-square test was used for comparison. Risk factors for progression of disease within 24 months (POD24) and T-cell cytopenia were identified using univariate and multivariate logistic regression analyses, with the associated risks quantified as odds ratios (OR) and 95% confidence intervals (95% CI). Receiver operating characteristic (ROC) analysis was performed to determine the optimal cut-off values of T cell subsets for predicting progression-free survival (PFS). All statistical analyses were performed using R version 4.3.0 and GraphPad Prism version 8.0. All tests were two-tailed, and a P value of <0.05 was considered statistically significant.

Results

Patients' characteristics

The baseline characteristics of the patients included in this study are listed in Table 1. Of the 96 FL patients included in the study, 76.0% were over 60 years old and 38.5% were male. At the time of admission, a large proportion of patients were at Ann Arbor Stage III or IV (81.3%), and bone marrow involvement was found in 38.5% of the participants. Elevated levels of Beta-2-microglobulin (β-MG) and lactate dehydrogenase (LDH) were observed in 33.3% and 20.8% of patients, respectively. The high-risk patients, as scored by FLIPI and FLIPI-2, accounted for 25.3% and 25.0%, respectively. Following diagnosis, 50.0% of patients were administered a rituximab (R)-based regimen, while 42.7% underwent an Obinutuzumab (G)-based regimen. A CHOP (Cyclophosphamide, doxorubicin, vincristine, and prednisone)-like regimen was used in 50.0% of FL patients, while a Bendamustine (B)-based regimen was administered to 28.1% of the patients.

Characteristics	Patients ($N=96$)
Age, n (%)	
≤ 60 years	23 (24.0)
> 60 years	73 (76.0)
Sex, n (%)	
Male	37 (38.5)
Female	59 (61.5)
Ann Arbor Stage, n (%)	
I/II	18 (18.8)
III/IV	78 (81.3)
Histological grade, n (%)	
I/II	57 (59.4)
IIIA	39 (40.6)
B symptoms, n (%)	
Yes	18 (18.8)
No	78 (81.3)
Bone marrow involvement, n (%)	
Yes	37 (38.5)
No	56 (58.3)
Serum β2-MG, n (%)	
Elevated	32 (33.3)
Normal	63 (65.6)
Serum LDH, n (%)	
Elevated	20 (20.8)
Normal	75 (78.1)
FLIPI risk category, n (%)	
Low-risk	35 (36.8)
Intermediate-risk	26 (27.4)
High-risk	24 (25.3)
FLIPI-2 risk category, n (%)	
Low-risk	48 (52.2)
Intermediate-risk	21 (22.8)
High-risk	23 (25.0)
Treatment, n (%)	
R-based regimen	48 (50.0)
G-based regimen	41 (42.7)
CHOP-like regimen	48 (50.0)
B-based regimen	27 (28.1)
Observation	4 (4.2)

Table 1. Baseline characteristics of enrolled patients. Abbreviations: β2-MG, β2-microglobulin; LDH, lactate dehydrogenase; R, Rituximab; G, Obinutuzumab; CHOP, Cyclophosphamide, doxorubicin, vincristine, and prednisone; B, Bendamustine.

CD4 + T cells decrease in FL patients and its association with clinical features

As depicted in Fig. 1, we analyzed T cell subset counts from the peripheral blood of 96 ND-FL patients and 30 healthy controls (HCs). The CD3 + T cells (Fig. 1A) were significantly reduced in FL patients at diagnosis compared to HCs; Further subset analysis showed that counts of both CD4 + T cells (Fig. 1B) and CD8 + T cells (Fig. 1C) were decreased in FL patients compared to HCs. However, there was no significant difference in the CD4/CD8 ratio between the two groups (Fig. 1D). In addition, we analyzed the association between T-cell subsets and the clinical characteristics of FL patients. The counts of CD3 + T and CD4 + T lymphocytes were significantly lower in FL patients aged over 60 years than in those aged 60 years or less, while the number of CD8 + T lymphocytes did not significantly differ between the two age groups (Fig. 1E). However, factors such as bone marrow involvement, Ann Arbor staging, and histological grading did not significantly affect the counts of CD3+, CD4+, and CD8 + T cells (Fig. 1F and G, and 1H). Notably, a significant negative correlation was observed between FLIPI scores and both CD3 + T cells (Fig. 1I) and CD4 + T cells (Fig. 1J), but not with CD8 + T cells (Fig. 1K) or the CD4/8 ratio (Fig. 1L).

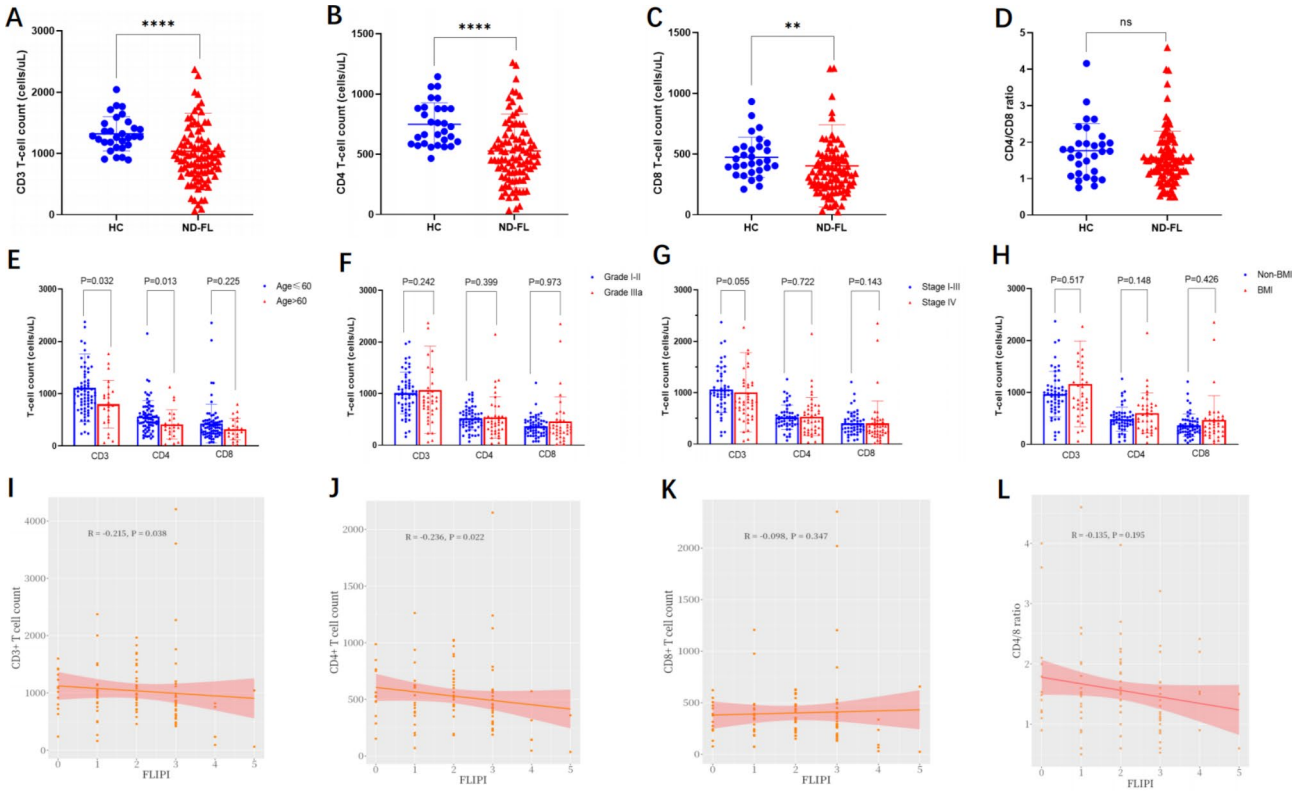

Fig. 1. Comparative analysis of T lymphocyte subsets and CD4/8 ratio in newly-diagnosed follicular lymphoma (ND-FL) patients and healthy controls (HCs): (A) CD3 + T cells, (B) CD4 + T cells, (C) CD8 + T cells, and (D) CD4/8 ratio. Statistically significant differences were indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. The association of baseline T cell subsets with patient characteristics was also shown, including (E) age, (F) histological grade, (G) Ann Arbor Stage, and (H) bone marrow involvement (BMI). Further analyses were conducted to explore the relationship between FLIPI score and CD3 + T cells (I), CD4 + T cells (J), CD8 + T cells (K), and CD4/8 ratio (L).

Decline of CD4 + T cells contribute predominantly to the treatment-related T-cell reduction

We analyzed the counts of T cells at baseline, after 2–4 cycles, and after 6 cycles of treatment in 54 patients. CD3 + T cells showed an overall decreasing trend after 2–4 treatment cycles and decreased significantly after 6 cycles of treatment (Fig. 2A). Further analysis of subpopulations revealed a dramatic decrease in CD4 + T cells (Fig. 2B) after 6 cycles of treatment, rather than CD8 + T cells (Fig. 2C). Fig.S2, which illustrated longitudinal changes of T cells before and after 6-cycle treatment in 70 patients, demonstrated a consistent trend. Notably, a decrease in CD4 + T cells emerged quickly after 2–4 treatment cycles, which was earlier than the decrease in CD3 + T cells. Consistently, the CD4/CD8 ratio also decreased significantly after 6 treatment cycles (Fig. 2D). Additionally, we analyzed the effects of different treatment regimens on the dynamics of T cells. Bendamustine-based (B-based) regimens contributed to the decline of CD3 + T (Fig. 2E) and CD4 + T cell (Fig. 2F) but not CD8 + T cells (Fig. 2G) after 6 cycles while Non-B-based regimens did not lead to a significant decline of T cells. However, a significant disruption of CD4/CD8 ratio were both observed in patients received B-based and Non-B-based regimens (Fig. 2H). Furthermore, the type of CD20 regimen (G vs. R) did not influence the dynamics of T cell subsets (Fig.S3).

Decreased frequency of T cells at diagnosis is associated with treatment failure

As shown in Table S1, 68 (70.8%) of the FL patients in our cohort achieved CR, and 84 (87.5%) achieved overall response after the initial treatment regimen. We analyzed the association between T-cell counts at diagnosis and treatment response. As a result, significantly lower frequency of CD4 + T cells ($P = 0.014$) and CD8 + T cells ($P = 0.047$) at diagnosis were observed in patients with disease progression (PD). Whereas, CD3 + T cells showed no significant difference between PD group and non-PD group (Fig. 3A). Additionally, we compared T-cell counts after 6 treatment cycles between the PD group and the non-PD group (Fig. 3B). However, the counts of CD3+, CD4+, and CD8 + T cells were similar in the PD and non-PD groups. Furthermore, no significant differences were observed in the changes in counts of CD3+, CD4+, and CD8 + T cells across the course of treatment between the PD and non-PD groups (Fig. 3C).

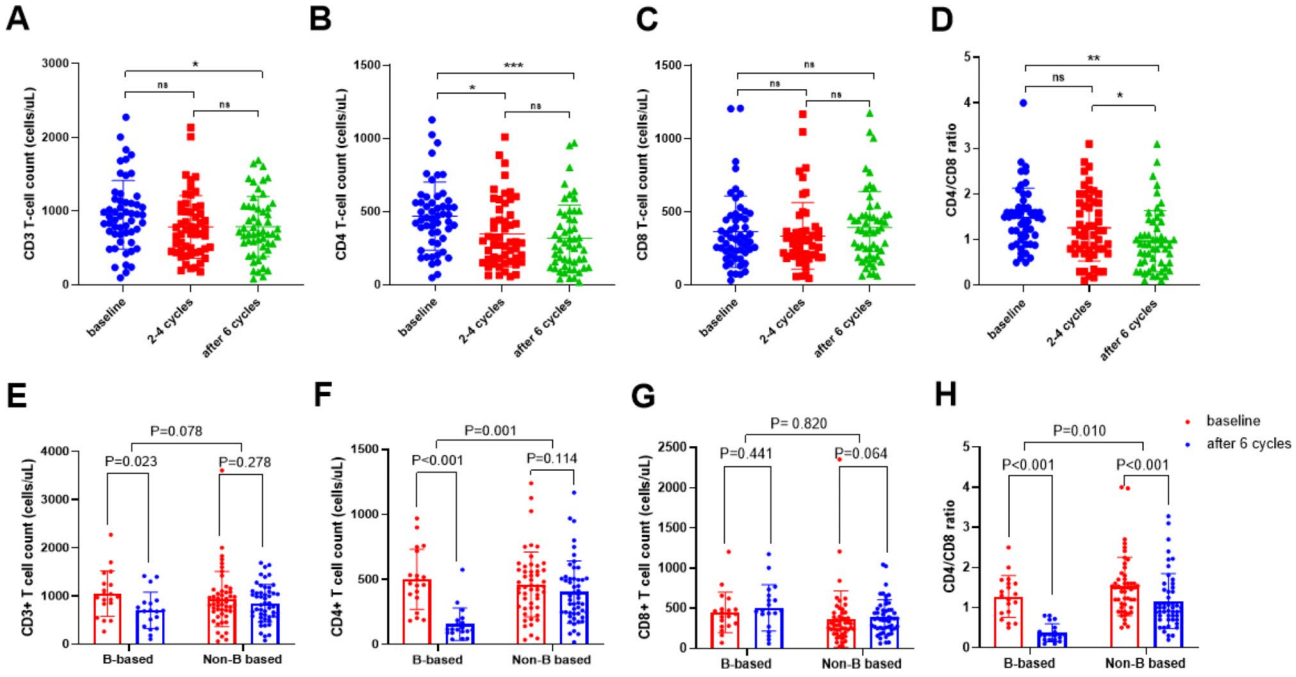

Fig. 2. Longitudinal changes in **(A)** CD3+, **(B)** CD4+, **(C)** CD8 + T cell subsets, and **(D)** CD4/8 ratio across treatment in FL patients. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Further, changes in **(E)** CD3+, **(F)** CD4+, **(G)** CD8 + T cell counts, and **(H)** CD4/8 ratio were compared between FL patients using the Bendamustine-based (B-based) regimen and the Non-B-based regimen.

Lower CD4 + T cells at diagnosis were linked to early progression of FL

Early progression of FL, generally defined as disease progression within 24 months (POD24) from the initiation of treatment, is associated with poor outcomes. We explored the role of T-cell counts at diagnosis in POD24 and identified potential risk factors using univariate and multivariate logistic regression analyses (Table 2). We used ROC curve analysis to calculate the optimal threshold for differentiating between the low and high CD4 + T cell groups. This analysis revealed that patients with CD4 + T cell counts lower than 260/uL had a significantly higher POD24 rate (HR = 10.43, $P = 0.001$, Fig. 4). After adjusting for confounding factors, our results indicated that a lower baseline CD4 + T cell count was an independent risk factor for POD24 in FL. Additionally, we further investigated the risk factors associated with a baseline CD4 + T cell count of < 260/uL (Table 3). The results of the multifactorial logistic regression analysis revealed that FL patients with lower plasma albumin levels (OR: 0.61, 95% CI: 0.46–0.79; $P < 0.001$) and a higher Ann Arbor stage (OR: 5.61, 95% CI: 1.14–27.61; $P = 0.034$) were at a significantly higher risk of having a low CD4 + T cell count (< 260/uL).

Discussion

In this study, we characterized the T-cell profiles of FL patients by comparing the levels of T-cell subsets with those of healthy subjects. We found that CD4 + T-cell counts at diagnosis could predict treatment response and were associated with progression of disease within 24 months (POD24). Moreover, we observed that a decline in CD4 + T cells contributed predominantly to treatment-related T-cell depletion. These results suggest that CD4 + T-cell counts might reflect the immune status of patients and are linked to the early progression of FL.

FL is recognized as an immune functional tumor, given its potential for spontaneous remission driven by anti-tumor immunity[2]. B. Milcent et al[22]. reported no significant difference in the percentages of CD4 + and CD8 + T cells between FL patients and healthy individuals. However, they did not explore the absolute number of T cells. In this study, we found that the number of CD3+, CD4+, and CD8 + T cells in FL patients was significantly reduced compared with those in healthy donors. Moreover, we controlled for the impact of age on T-cell counts, as evidenced by the similar median age between the FL and healthy groups. The result indicated that patients with FL display compromised T-cell immunity. Additionally, we identified clinical features linked to more compromised T-cell immunity. Specifically, older FL patients (> 60 years) had significantly lower CD3 + and CD4 + T-cell counts, but not CD8 + T-cell counts. Aging could lead to a reduction in naïve CD4 + T cells, which could subsequently contribute to the overall decrease in CD4 + T cells[23]. Moreover, the FLIPI score, which is widely used as an indicator to assess the prognosis of FL, reflects the severity of the disease[24]. Our results revealed a linear negative correlation between the counts of CD3 + and CD4 + T cells and the FLIPI score in FL patients. Since neither the disease stage nor bone marrow involvement were associated with the decline in CD4 + T cells, the significant association between the FLIPI score and T-cell counts might be attributed to age. Therefore, older FL patients may experience a more severe deficiency in T-cell immunity.

Treatment-related lymphocytopenia frequently occurs in patients with FL, especially among those receiving Bendamustine-based regimens[18]. In the BRIGHT and STiL trials, grade 3–4 lymphopenia was observed in 62%

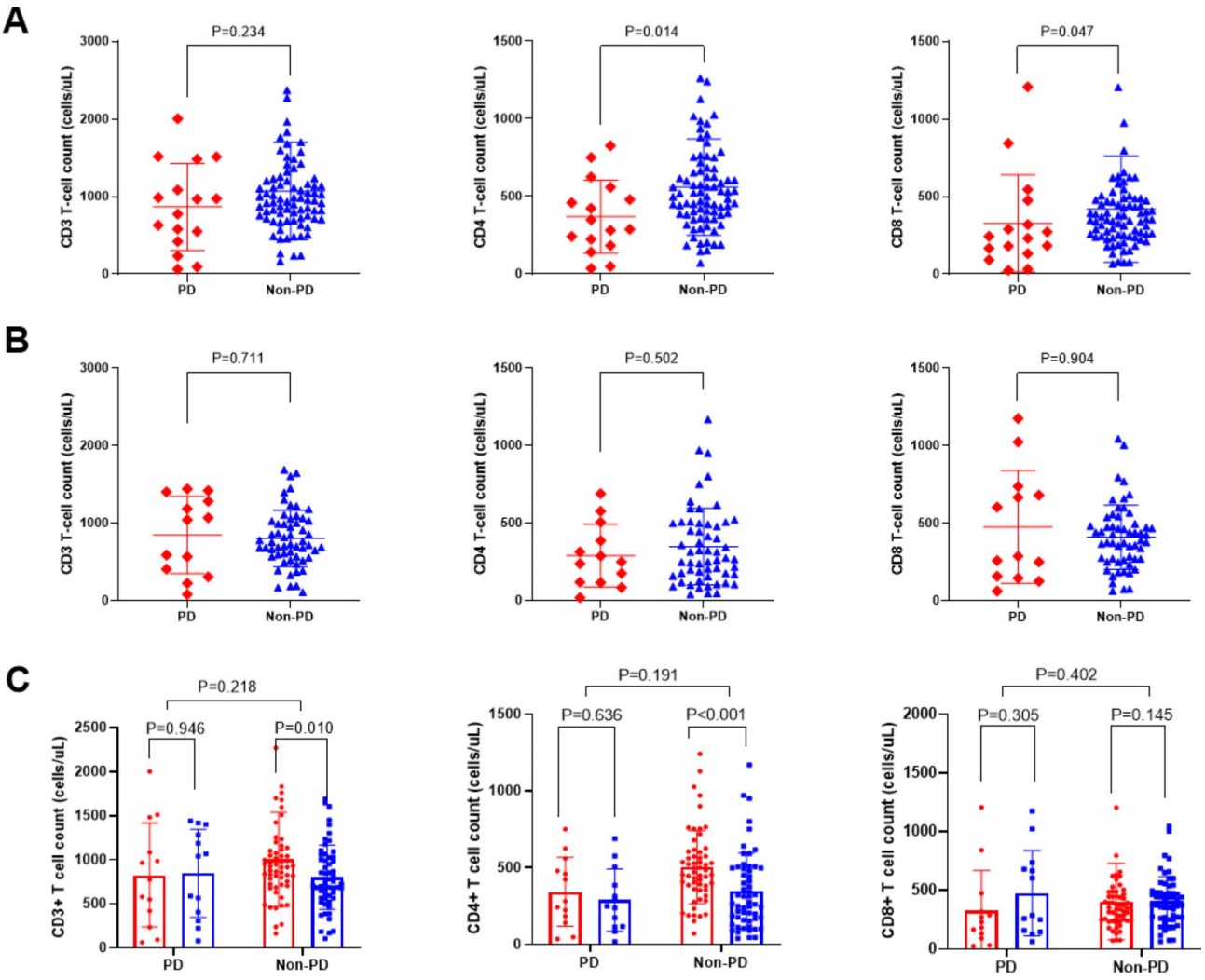

Fig. 3. Baseline immune signatures between FL patients with or without progression disease (PD) **(A)** at baseline and **(B)** after 6-cycle treatment; Within-group and between-group comparisons of the changes of T-cell subsets **(C)** across treatment in PD/Non-PD group were performed using the Wilcoxon paired-sign rank test and the Wilcoxon rank sum test.

	Univariate					Multivariate				
Variables	**β**	**S.E**	**Z**	***P***	**OR (95%CI)**	**β**	**S.E**	**Z**	***P***	**OR (95%CI)**
Histologic grade (IIIa vs. I to II)	2.40	1.14	2.11	0.035	11.00 (1.18 ~ 102.35)					
CD4 + T cell count < 260/uL	2.93	0.99	2.96	0.003	18.75 (2.68 ~ 130.94)	2.86	0.99	2.88	0.004	17.5 (2.50 ~ 122.50)
LDH elevated	1.22	0.89	1.37	0.170	3.37 (0.59 ~ 19.21)					
β2-MG elevated	1.27	0.86	1.48	0.139	3.56 (0.66 ~ 19.11)					
HGB	-0.06	0.03	-2.16	0.031	0.94 (0.89 ~ 0.99)					
Age > 60	1.06	0.87	1.22	0.224	2.89 (0.52 ~ 16.03)					
Gender (Male)	-0.56	0.91	-0.62	0.537	0.57 (0.10 ~ 3.38)					
FLIPI high risk	2.77	1.15	2.41	0.016	16.00 (1.68 ~ 152.01)					
Stage IV	0.52	0.84	0.63	0.532	1.69 (0.33 ~ 8.73)					
Abbreviations: POD24, disease progression within 2 years after receiving first-line therapy; OR: Odds Ratio, CI: Confidence Interval; LDH, lactate dehydrogenase; β2-MG, β2-microglobulin; HGB, Hemoglobin.										

Table 2. Prognostic risk factors for POD24 determined in univariate and multivariate logistic regression analysis.

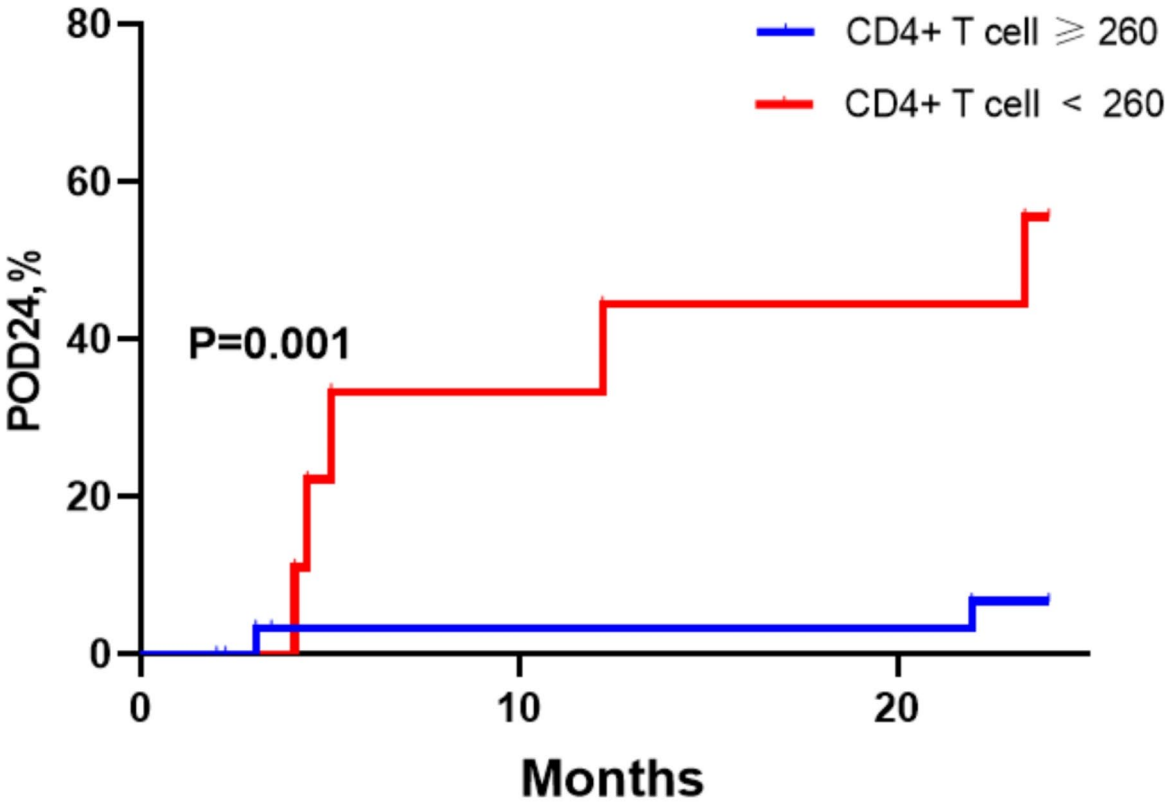

Fig. 4. Kaplan–Meier curves for the association of time to progression within 24 months (POD24) with CD4 + T cell counts at baseline in FL. Abbreviations: HR, hazard ratio; CI, confidence interval.

Variables	Univariate					Multivariate				
	β	S.E	Z	*P*	OR (95%CI)	β	S.E	Z	*P*	OR (95%CI)
Age > 60 years	0.92	0.59	1.55	0.121	2.51 (0.78 ~ 8.03)					
B-symptoms	1.34	0.61	2.19	0.028	3.83 (1.15 ~ 12.74)					
FLIPI high-risk	1.19	0.58	2.06	0.039	3.30 (1.06 ~ 10.28)					
Ann Arbor Stage (IV vs. I to III)	0.92	0.59	1.55	0.121	2.50 (0.78 ~ 7.97)	1.72	0.81	2.12	0.034	5.61 (1.14 ~ 27.61)
Ki67 > 40%	0.92	0.63	1.46	0.145	2.51 (0.73 ~ 8.69)					
ALB	-0.42	0.11	-3.93	< 0.001	0.66 (0.53 ~ 0.81)	-0.50	0.14	-3.69	< 0.001	0.61 (0.46 ~ 0.79)
Abbreviations: OR: Odds Ratio, CI: Confidence Interval; ALB, albumin;										

Table 3. Risk factors for CD4 + T cell < 260/uL at baseline identified by univariate and multivariate logistic regression analysis.

and 74% of patients receiving BR, respectively, compared to 30% and 43% of patients receiving R-CHOP[16,25]. In the GALLIUM trial[26], at least 75% of patients experienced CD4 + lymphopenia at the end of induction, and nearly 25% of patients in the Bendamustine-treated group continued to exhibit this condition at the three-year mark. The Bendamustine-based group showed marked and prolonged reductions in T-cell counts, which were significantly more pronounced than those in the CHOP and CVP groups. The severe CD4 + lymphopenia induced by the BR regimen was also demonstrated in the study by Ito and Yutaka et al[17,27].. Similarly, our results indicated that B-based regimens, rather than non-B-based regimens, led to a significant decrease in CD4 + T-cell counts. However, only the level of CD4 + T cells at diagnosis, not after induction, correlated with treatment response. These data suggest that the level of CD4 + T cells at diagnosis may reflect the immune status of FL patients and could be linked to treatment response. However, a decline in CD4 + T-cell counts after treatment may merely reflect a temporary, treatment-related immune deficiency.

Given the predictive role of T cells in treatment response, we also analyzed their role in predicting survival. Shafer et al[28]. demonstrated an association between low peripheral blood CD4 + T cell counts and shorter overall survival (OS), a finding similarly reported by Wei Liu et al[13].. Conversely, Junlén et al. found that neither absolute T cell counts nor T cell subset counts predicted clinical outcomes in FL[29]. Given that the POD24 is the surrogate endpoint for the FL studies[30], we performed a regression analysis of POD24 and found that the counts of CD4 + T cells less than 260/uL at diagnosis was an independent risk factor for POD24. Additionally, we analyzed factors associated with CD4 + T cell counts less than 260/uL and found that advanced disease stage and lower albumin (ALB) levels were independent risk factors for lower CD4 + T cell levels. Overall, the detection of baseline CD4 + T cell counts can help identify FL patients at the highest risk for early progression.

Despite our comprehensive collection and analysis of T cell subset test results from several centers and the clinical information of the patients, several limitations of the current study need to be noted. Firstly, the retrospective nature of the study prevented us from accurately setting the time point for determining T cell counts after treatment, which may have introduced some bias. A carefully designed prospective study is needed to obtain more accurate data in the future. Secondly, our study was observational, which means there could be many confounding influences on the associations between variables and prognosis. By adjusting for confounders, we did address this limitation to some extent, but some residual confounding may remain. Thirdly, the relatively small size of our study population and the diverse treatment regimens they underwent may limit the generalizability of our conclusions. Finally, due to the associated costs, detailed T lymphocyte subgroup testing was not routinely performed for most patients. Thus, further phenotypic characterization of the T cell populations (such as naïve or memory) was not analyzed.

Overall, our data suggest a compromised T-cell immunity among FL patients, with lower CD4 + T cell counts at diagnosis correlating with treatment response and early progression. Therefore, the CD4 + T cell count at diagnosis may reflect the immune status, and monitoring these levels could help stratify FL patients.

Data availability

Data will be made available upon request to Bing Xu.

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Author contributions

BX and ZL designed the study. QHJ, FL, CJ, and XHS collected the data. QHJ performed statistical analysis and wrote the draft of manuscript. LL, BX, and JZ edited the paper. All authors were involved in the design, conduction, and review of this study and approved the final manuscript.

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Declarations

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the First Affiliated Hospital of Xiamen University (2022-070), the West China Hospital of Sichuan University, and the Second Affiliated Hospital of Dalian Medical University (2024 – 1010). Written informed consent was waived by the Institutional Review Board, owing to the retrospective nature of the study. Animal studies: N/A.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to L.L., Z.L. or B.X.

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