Original Article

Increase of $CD3^+CD7^-$ T cells in bone marrow predicts invasion in patients with T-cell non-Hodgkin's lymphoma

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Background: The T-cell non-Hodgkin's lymphoma (T-NHL) patients with bone marrow (BM) invasion have a poor prognosis. Although BM biopsy is still a confirmed diagnosis method, the low sensitivity restricts its use to detect the minimal BM invasion. It is of great clinical significance to establish a rapid and highly sensitive method to evaluate BM invasion.

Methods: We conducted a retrospective study of 85 patients with new diagnosed T-NHL patients enrolled in our institute. The bone marrow mononuclear cells (BMMNCs) cells were isolated, stained with different combinations of antibody and subjected to flow cytometry analysis.

Results: We found that $CD3^+CD7^-$ T cells increased significantly in the BM in T-NHL patients with BM invasion. The patients were divided into the low and high groups according to the cutoff value of 1.035% obtained by analyzing the receiver operating characteristic (ROC) curve of the percentage of $CD3^+CD7^-$ T cells of nucleated cells at diagnosis. The ratio of invasion in high group was markedly higher than that in low group. Furthermore, $CD3^+CD7^-$ T cells presented significantly higher level of programmed cell death-1 (PD-1), lymphocyte-activation-gene-3 (LAG3) and CD4/CD8 ratio.

Conclusions: Our study revealed the percentage of $CD3^+CD7^-$ T cells of nucleated cells in BM was a potential diagnostic predictor of BM invasion with T-NHL.

Keywords: T-cell non-Hodgkin's lymphoma (T-NHL); $CD3^+CD7^-$ T cells; prognostic predictor

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Introduction

T-cell lymphoma (TCL) is a malignant clonal proliferative disease originating from T lymphocytes, accounting for 10–15% of non-Hodgkin's lymphoma (NHL) (1). It is a malignant cancer with rapid progress, poor prognosis and high recurrence rate. The 5-year survival rate is about 30–40% (2). Patients with bone marrow (BM) invasion are in stage IV according to the Ann Arbor staging. Although T-NHL has a lower BM invasion rate compared to B-NHL (3), T-NHL patients with BM invasion have a shorter survival time than B-NHL patients (4). So far, BM biopsy and BM pathology are still the gold standard for diagnosis of BM invasion in T-NHL (5). However, with the technology development, several methods such as positron

emission tomography-computed tomography (PET-CT) (6,7), T-cell receptor (TCR) gene rearrangement (3,8), flow cytometry (9-11) have been greatly improved, and gradually become required examinations when the BM invasion of T-NHL was assessed. Although a growing body of evidence manifests that BM biopsy is still an irreplaceable diagnostic method (11) and the current pathological diagnosis has certain limitations, a pathological evaluation is one of the most important methods for the diagnosis of malignant lymphoma with BM invasion (12). The method based on flow cytometry has a high sensitivity, making it easier to detect the minimal BM invasion (9).

CD7 is a cell surface costimulatory molecule expressed on T and natural killer (NK) cells and on cells in the early stages of T-, B-, and myeloid cell differentiation. Interestingly, under physiological and certain pathological conditions, the loss of CD7 molecule occurs in a subset of $CD4^+$ memory T cells. Specific absence of CD7 antigen expression on T cells has been observed in various pathologic conditions such as HIV infection (13), rheumatoid arthritis, and kidney transplantation with the consequence of chronically repeated T cell stimulation (14,15). While loss of *CD7* gene expression indicates malignancy in the peripheral blood T cells and has been observed in a great variety of pathologic conditions such as cutaneous T cell lymphoma, adult T-cell leukemia/lymphoma, primary hepatic peripheral T-cell lymphoma, chronic myeloid leukemia, and Sezary syndrome (SS) (16). However, the number and activity of $CD7^-$ T cells as well as the potential pathophysiological significance of this T cell subset in the BM have not been discussed. Additionally, the CD7 monoclonal antibodies (17) or CD7 chimeric antigen receptor (CAR)-T cells (18) have exhibited powerful cytotoxic killing effects on $CD7^+$ hematological malignant diseases.

Immune checkpoints include stimulatory and inhibitory checkpoint molecules. Over the past decade, inhibitory checkpoints, including cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), programmed cell death-1 (PD-1), lymphocyte-activation-gene-3 (LAG-3) and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), have been identified to suppress anti-tumor immune responses in solid tumors. Novel drugs targeting immune checkpoints, such as specific PD-1 blockades, have already achieved approval in several solid tumors such as melanoma, non-small cell lung cancer, and kidney cancer (19,20). As for hematological malignancies, clinical benefits of immune checkpoint blockades are observed in only limited tumor types such as Hodgkin's lymphoma that are particularly characterized by a high infiltration of immune cells. The main reason is primary and acquired resistance that remains a barrier in utilizing them against a broad range of hematological malignancies.

In our study, we found that $CD3^+CD7^-$ T cells increased significantly in the BM in T-NHL patients with BM invasion. The proportion of $CD3^+CD7^-$ T cells in nucleated cells in BM was a potential diagnostic predictor of BM invasion in T-NHL. This may be related to increased expression of immune inhibitory checkpoints in these cells. We present the following article in accordance with the STARD reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2666/rc).

Methods

Enrolled patients and BM sample

This study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (No. 2017-044), and all participants signed written informed consent. The study was carried out in accordance with the Declaration of Helsinki (as revised in 2013). A total of 85 patients who were newly diagnosed with T-NHL lymphoma consist of 59 males and 26 females with a median age of 60 years old (range, 17–83 years old). The diagnosis and classification depend on pathological examination after lymph node puncture or biopsy and PET-CT. The stages and other clinical characteristics of patients are summarized in *Table 1*. Bone marrow mononuclear cells (BMMNCs) were isolated using ficoll-hypaque density gradient centrifugation and stored.

Immunophenotyping

BMMNCs were stained with different combinations of anti-human CD3 (Beckman Coulter Cat# IM2467, RRID:AB_130788), CD4 (Beckman Coulter Cat# IM2468, RRID:AB_130781), CD7 (Beckman Coulter Cat# IM3613U, RRID:AB_10643230), CD8 (Beckman Coulter Cat# IM0451U, RRID:AB_10638218), CD28 (Beckman Coulter Cat# 6607111, RRID:AB_1575955), CD45 (Beckman Coulter Cat# A96416, RRID:AB_2888654), human leucocyte antigen DR (HLA-DR) (Beckman Coulter Cat# A74781, RRID:AB_2892134) for 20 min in dark and PD-1 (BD Biosciences Cat# 557946, RRID:AB_647199; San Jose, CA, USA), LAG3 (BioLegend Cat# 369310,

Table 1 clinical characteristics of patients with T-NHL

Characteristics	$CD3^+CD7^-$ ≥1.035% (n=24)	$CD3^+CD7^-$ <1.035% (n=61)	P value
Median age [range] (years)	63.5 [28–77]	57 [17–83]	0.430
Male/female	15/9	44/17	0.438
Ann Arbor stage, n (%)			
I	3 (12.5)	16 (26.2)	0.249
II	3 (12.5)	11 (18.0)	0.748
III	5 (20.8)	18 (29.5)	0.589
IV	13 (54.2)	15 (24.6)	0.012
Unclassified	0	1 (1.6)	1.000
Diagnostic criteria, n (%)			
Pathology	19 (79.2)	58 (95.1)	–
PET-CT	20 (83.3)	57 (93.4)	–
Marrow invasion, n (%)	12 (50.0)	12 (19.7)	0.008
Marrow invasion criteria, n (%)			
Pathology	3 (12.5)	3 (4.9)	–
PET-CT	1 (4.2)	2 (3.3)	–
Flow cytometry	12 (50.0)	8 (13.1)	–
Bone marrow smear	10 (41.7)	7 (11.5)	–
TCR gene rearrangement	11 (45.8)	17 (27.9)	–

PET-CT, positron emission tomography-computed tomography; T-NHL, T-cell non-Hodgkin's lymphoma; TCR, T-cell receptor.

RRID:AB_2629753; San Diego, CA, USA), C-C motif chemokine receptor 7 (CCR7) for 30 min in dark. Cells were then hemolyzed for 15 min and washed twice. The immunophenotyping was performed using Navios flow cytometer (Beckman Coulter, Brea, CA, USA). Live lymphocytes were gated according to CD45 and appropriate forward and side scatter. Gating of CD3 was set to determine the frequency of $CD3^+CD7^-$ T cells. The proportion of LAG3, PD-1, CCR7, HLA-DR or CD28 was determined in $CD3^+CD7^-$ T cells, $CD3^+CD7^+$ T cells, $CD3^+CD7^-CD4^+$ T cells, and $CD3^+CD7^+CD4^+$ T cells. The gating standards of CD28, HLA-DR, PD-1, CCR7 and LAG3 were set using isotype controls recommended by the manufacturer. A total of 500,000 cells per sample was acquired and analyzed.

Statistical analysis

The data were presented as mean ± standard error of the mean (SEM) and analyzed by *t*-test, one-way ANOVA or Chi-square test to determine the differences. In this study, the proportion of $CD3^+CD7^-$ T cells of nucleated cells was measured in all 85 patients, and the receiver operating characteristic (ROC) curve was subsequently used in clinical monitoring to calculate the appropriate values for sensitivity and specificity. Differences at $P<0.05$ were considered statistically significant, and all tests were two-tailed. All statistical analyses were performed using GraphPad Prism 5.0 software.

Results

Higher frequency of $CD3^+CD7^-$ T cells is shown in BM in patients with T-NHL BM invasion

All BM samples of 85 patients who were newly diagnosed with T-NHL were analyzed by flow cytometry. All cells in the $CD45^{high}/SSC^{low}$ population were predominantly T-cells with regular CD3 expression. It has been reported that in cutaneous TCLs, especially SS, a large number

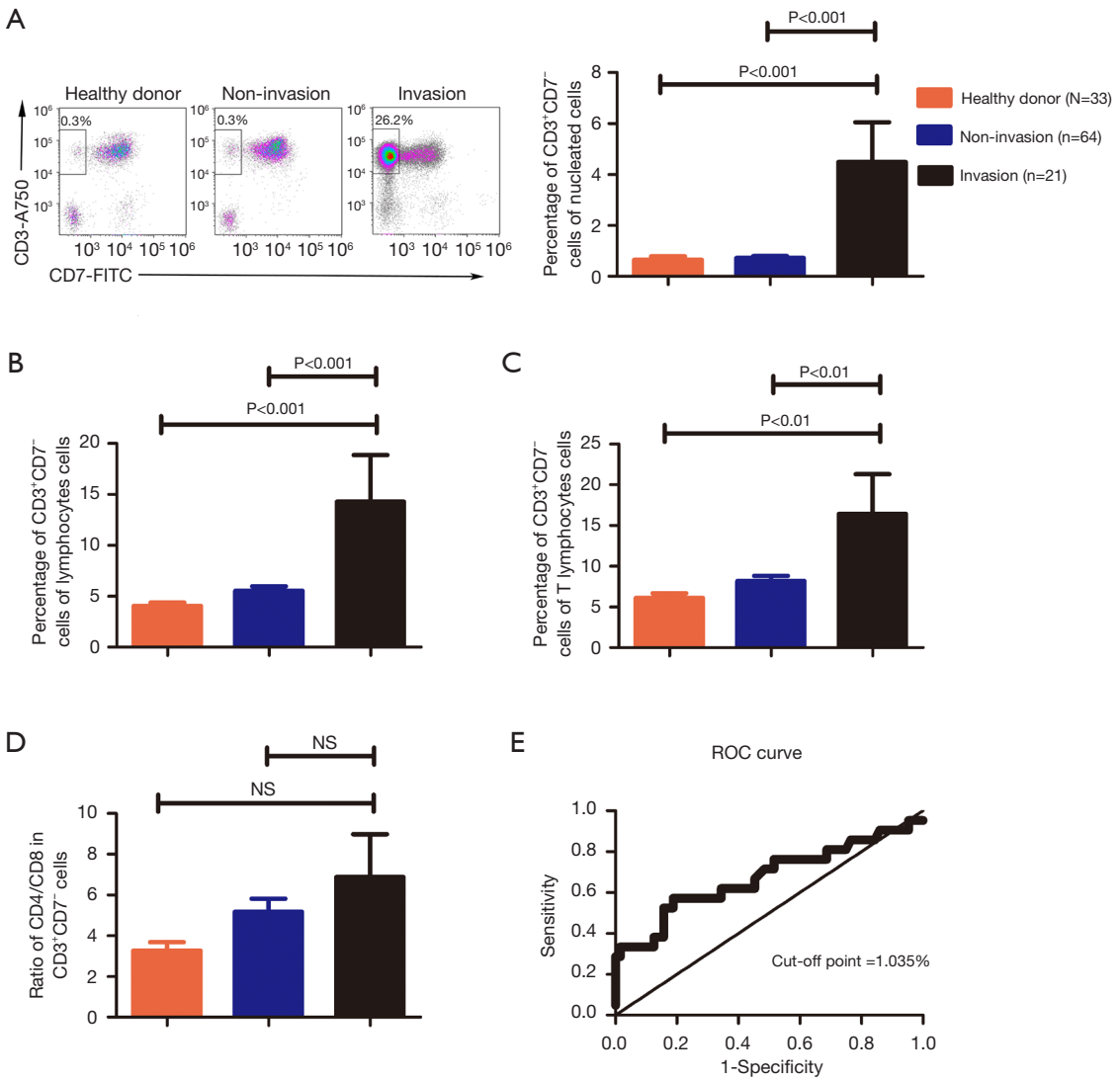

Figure 1 The frequency CD3⁺CD7⁻ T cells is increased in bone marrow in T-NHL patients with bone marrow invasion. (A) Proportion of CD3⁺CD7⁻ T cells of nucleated cells was shown. (B) Proportion of CD3⁺CD7⁻ T cells of lymphocytes was shown. (C) Proportion of CD3⁺CD7⁻ T cells of T lymphocytes was shown. (D) Ratio of CD4/CD8 in CD3⁺CD7⁻ T cells was shown. (E) ROC curve analysis of proportion of CD3⁺CD7⁻ T cells of nucleated cells in T-NHL patients was shown. NS, not significant; ROC, receiver operating characteristic; T-NHL, T-cell non-Hodgkin's lymphoma.

of circulating CD7⁻ T cells are derived from the clonal expansion of CD7⁻ T cells in tumor tissues (14,16). We found that the frequency of CD3⁺CD7⁻ T cells in BM was notably higher in T-NHL patients with BM invasion than that patients without BM invasion or healthy donors (*Figure 1A-1C*). The ratio of CD4/CD8, although high, was not statistically significant between the BM invasion patients and patients without BM invasion or healthy donors (*Figure 1D*). In this study, BM invasion was defined as positive results in two or more examination including BM biopsy, PET-CT, BM smear, flow cytometry and *TCR* gene rearrangements.

The ROC curve of the proportion of CD3⁺CD7⁻ T cells of nucleated cells was used to determine the values (*Figure 1E*). The area under the curve (AUC) of proportion of CD3⁺CD7⁻ T cells of nucleated cells was 0.6749 [95% confidence interval (CI): 0.5226 to 0.8271], and the optimal value was 1.035%, with 57.1% sensitivity and 81.2%

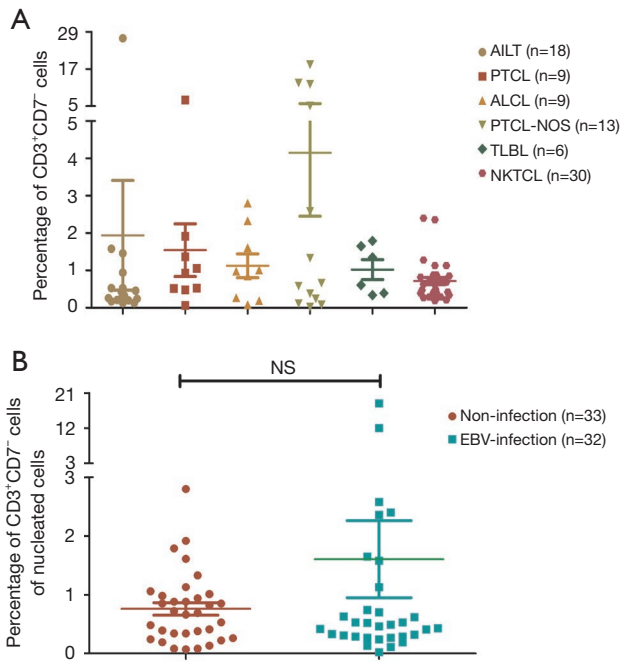

Figure 2 Percentage of $CD3^+CD7^-$ T cells has no difference in a number of subtypes and EBV infection. (A) There is no obvious difference in proportion of $CD3^+CD7^-$ T cells of nucleated cells in different subtypes of T-NHL. (B) There is no obvious difference in proportion of $CD3^+CD7^-$ T cells of nucleated cells in T-NHL patients with or without EBV infection. AITL, angioimmunoblastic T-cell lymphoma; ALCL, anaplastic large cell lymphoma; EBV, Epstein-Barr virus; NKTCL, natural killer/T-cell lymphoma; NS, not significant; PTCL-NOS, peripheral T-cell lymphoma not otherwise specified; TLBL, T cell lymphoblastic lymphoma; T-NHL, T cell non-Hodgkin's lymphoma.

specificity, P=0.017. There were 24 (28.3%) patients with high proportion of $CD3^+CD7^-$ T cells of nucleated cells (≥1.035%) and 61 patients with low proportion of $CD3^+CD7^-$ T cells of nucleated cells (<1.035%). The ratio of invasion in high group was markedly higher than that in low one (P=0.0015). The association between proportion of $CD3^+CD7^-$ T cells of nucleated cells and laboratory indicators was summarized in Table S1. However, there was no significant difference in the overall survival (OS) between those two groups (Figure S1).

Analysis for $CD3^+CD7^-$ T cells in different subtypes and viral Epstein-Barr virus (EBV)-DNA

T-NHL is a disease with obvious heterogeneity. There are considerable differences in clinical manifestations and immunological phenotypes between different subtypes. 85 patients enrolled, included 18 (21.2%) cases of angioimmunoblastic T-cell lymphoma (AITL), 9 (10.6%) cases of peripheral T-cell lymphoma (PTCL), 9 (10.6%) cases of anaplastic large cell lymphoma (ALCL), 13 (15.3%) cases of peripheral T-cell lymphoma not otherwise specified (PTCL-NOS), 6 (7.1%) cases of T cell lymphoblastic lymphoma (TLBL), and 30 (35.3%) cases of natural killer/T-cell lymphoma (NKTCL). There was no difference in the proportion of $CD3^+CD7^-$ T cells of nucleated cells among different types (*Figure 2A*).

EBV infection is closely related to the occurrence of T cell lymphoma. A total of 65 cases in our sample were tested for the level of EBV-DNA in peripheral blood, of which 32 cases were positive. No difference was observed in the proportion of $CD3^+CD7^-$ T cells in nucleated cells between two groups (*Figure 2B*).

$CD3^+CD7^-$ T cells express higher level of immune inhibitory checkpoints

In order to investigate the mechanism by which $CD3^+CD7^-$ T cells may cause BM invasion, the expression of immune markers on these cells were analyzed. Compared with $CD3^+CD7^+$ T cells, the expression of immune checkpoints like LAG3 and PD-1 was higher in $CD3^+CD7^-$ T cells (*Figures 3A,3B*), but there was no significant difference on the expression of CCR7, CD28 and HLA-DR (Figure S2). Furthermore, we found that these $CD3^+CD7^-$ T cells had a higher ratio of CD4/CD8 (*Figure 3C*). Additionally, we also found that the expression of PD-1 was higher in $CD3^+CD7^-CD4^+$ T cells than that in $CD3^+CD7^+CD4^+$ T cells (*Figure 3D*). These data suggested that $CD3^+CD7^-$ T cells may be more exhausted compared to $CD3^+CD7^+$ T cells.

Discussion

$CD7^-$ T cells are usually considered as late memory cells characterized by a high activation threshold, low effector capacities, and high sensitivity to activation-induced cell death (AICD) (14,21).The $CD7^-$ T cells can be found in peripheral blood, especially in patients with SS or infection. Viruses infection including EBV and cytomegalovirus (CMV), or the immunization with attenuated measles virus, may provoke a clonal but benign expansion of $CD3^+CD7^-$ T cells (22). In acute myeloid leukemia (AML), CD7 had already been confirmed as an independent prognostic factor for poor disease-free survival and post-remission

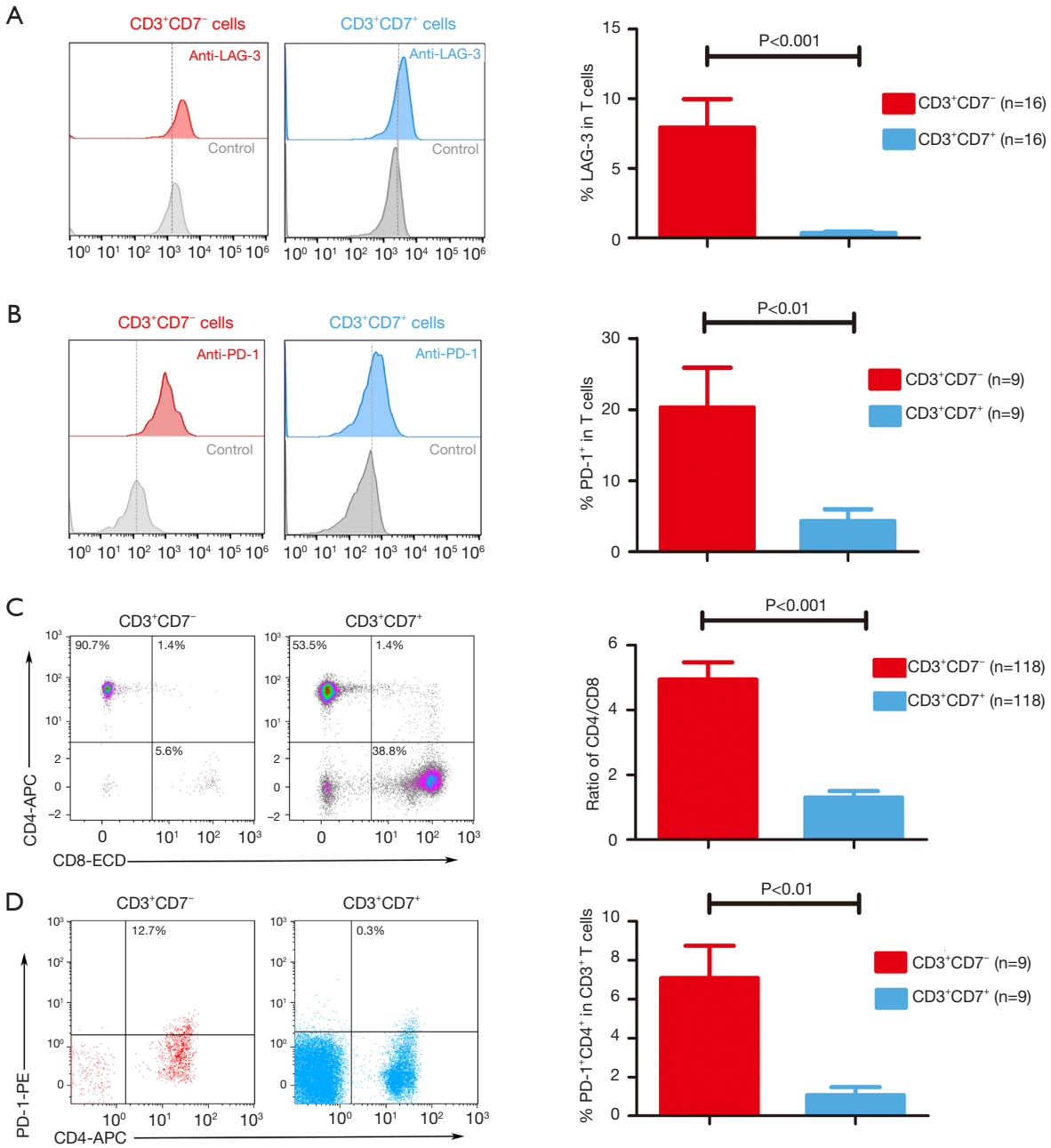

Figure 3 Increased expression of immune inhibitory checkpoints was shown in CD3+CD7− T cells in T-NHL patients with BM invasion. (A) CD3+CD7− T cells had a higher LAG-3 expression than CD3+CD7+ T cells. (B) CD3+CD7− T cells had a higher PD-1 expression than CD3+CD7+ T cells. (C) CD3+CD7− T cells had a higher ration of CD4/CD8 than CD3+CD7+ T cells. (D) CD3+CD7−CD4+ T cells had a higher PD-1 expression than CD3+CD7+CD4+ T cells. APC, allophycocyanin; BM, bone marrow; LAG-3, lymphocyte-activation-gene-3; PD-1, programmed cell death protein 1; PE, phycoerythrin; T-NHL, T-cell non-Hodgkin's lymphoma.

survival (23). In lymphoma, especially T-NHL, CD7 has become one of the therapeutic targets (18). However, the pathophysiological significance of $CD3^+CD7^-$ T cells in the BM remains elusive in T-NHL.

Here, we found that the proportion of $CD3^+CD7^-$ T cells was especially higher in T-NHL patients with BM invasion. We divided all patients into two subgroups according to ROC curve, and the results showed that patients with a high proportion of $CD3^+CD7^-$ T cells of nucleated cells had a higher BM invasion rate than those with a low level. However, in T-NHL patients, EBV infection did not cause a change in the proportion of $CD3^+CD7^-$ T cells, which may be due to its own T cell function impairment in T-NHL patients, especially in BM-invasion patients.

CD28, a cell surface glycoprotein receptor, which has sequence homology with CTLA-4 and is expressed on T cells after activation, regulates T cell proliferation and differentiation and plays an important role in the immune response pathway *in vivo* (24). HLA-DR exists on the surface of macrophages, B lymphocytes, and T lymphocytes. In addition to being involved in antigen presentation, HLA-DR is also recognized as a marker of T cell activation. PD-1, a well-recognized immune checkpoint, has been extensively studied in hematological malignancies (25-27). The application of PD-1 inhibitors has received approval in Hodgkin's lymphoma (28). LAG3 is a key immune checkpoint with relevance in autoimmunity, cancer and infectious diseases (29). In this study, we found that $CD3^+CD7^-$ T cells had higher expression of PD-1 and LAG3 compared with $CD3^+CD7^+$ T cells. Since LAG3 and PD-1 contribute to T-cell exhaustion during chronic virus infection (30), high expression of these two immune checkpoints on $CD3^+CD7^-$ T cells indicated that these cells were exhausted during the immune response against T-NHL. The CD4/CD8 ratio in normal BM generally fluctuates within a certain relatively fixed range. We found a significantly higher ratio of CD4/CD8 in $CD3^+CD7^-$ T cells, which may be also related to immune dysfunction.

Except for targeted therapy, the CAR-T cell therapy has been widely used in B-cell lymphoma and multiple myeloma. However, it is just beginning in T-cell hematological malignancies. CD7 CAR-T cells exhibit a powerful cytotoxic killing effect on $CD7^+$ hematological malignancies (18). In this study, increased $CD7^-$ T cells were observed in the BM of T-NHL patients with BM invasion. Therefore, we think that it is necessary to fully evaluate the proportion of $CD7^-$ T cells in the BM before selecting CAR-T treatment.

Of course, we must admit that our retrospective analysis certainly has limitations. As recruitment was performed in a single institution, election bias might be difficult to be well balanced. Secondly, because of the limited follow-up time, we drew OS curve under the grouping of ROC curves, and there was no significant difference between two groups, which may due to the limiting time and cases, we will finish it in our subsequent studies. Additionally, although we found that the expression of immune inhibitory checkpoints increased in $CD3^+CD7^-$ T cells, the specific mechanism need be further studied.

In conclusion, we believe that the frequency of $CD3^+CD7^-$ T cells is significantly increased in T-NHL with BM invasion, and the proportion of $CD3^+CD7^-$ T cells of nucleated cells in BM was a potential diagnostic predictor of BM invasion in T-NHL. In addition, increased expression of several immune inhibitory checkpoints was found on $CD3^+CD7^-$ T cells, which can guide clinical diagnosis and medication to a certain extent. This study supplies an auxiliary method which may indicate that some indolent T lymphomas are in a progressive state or may indicate a poor prognosis.

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2666/rc

Data Sharing Statement: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2666/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-21-2666/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (No. 2017-044), and all participants signed written informed consent. The study was carried out in accordance with the Declaration of Helsinki (as revised in 2013).

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Supplementary

Table S1 Baseline characteristics of patients with T cell lymphoma

Characteristics	$CD3^+CD7^-$ ≥1.035% (n=24)	$CD3^+CD7^-$ <1.035% (n=61)	P value
Mean WBC count (10^9/L)	6.87	7.33	0.679
Mean RBC count (10^{12}/L)	3.78	4.16	0.024
Mean hemoglobin (g/L)	116.0	125.4	0.059
Mean platelets count (10^9/L)	198.5	216.4	0.391
Mean prothrombin time (s)	13.92	13.68	0.393
Mean fibrinogen (g/L)	3.667	3.771	0.800
Mean APTT (s)	40.03	40.40	0.824
Mean thrombin time (s)	16.9	17.3	0.458
Mean D-dimer (mg/L)	1.16	1.98	0.143
Mean IgG (g/L)	13.9	14.2	0.886
Mean IgA (g/L)	2.78	3.10	0.438
Mean IgM (g/L)	1.63	1.48	0.737
Mean LDH (U/L)	439.5	501.0	0.676
Mean β2-microglobulin (μg/mL)	3.36	3.67	0.570
Mean ferritin (μg/L)	1,165.8	1,481.4	0.721

WBC, white blood Cell; RBC, red blood cell; APTT, activated partial thromboplastin time; LDH, lactate dehydrogenase.

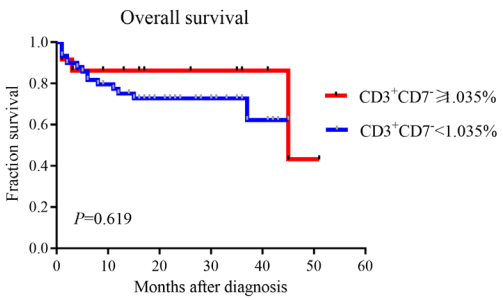

Figure S1 There was no difference in the overall survival in two groups between $CD3^+CD7^-$ ≥1.035% and $CD3^+CD7^-$ <1.035%. The log-rank method was used to test for differences in overall survival.

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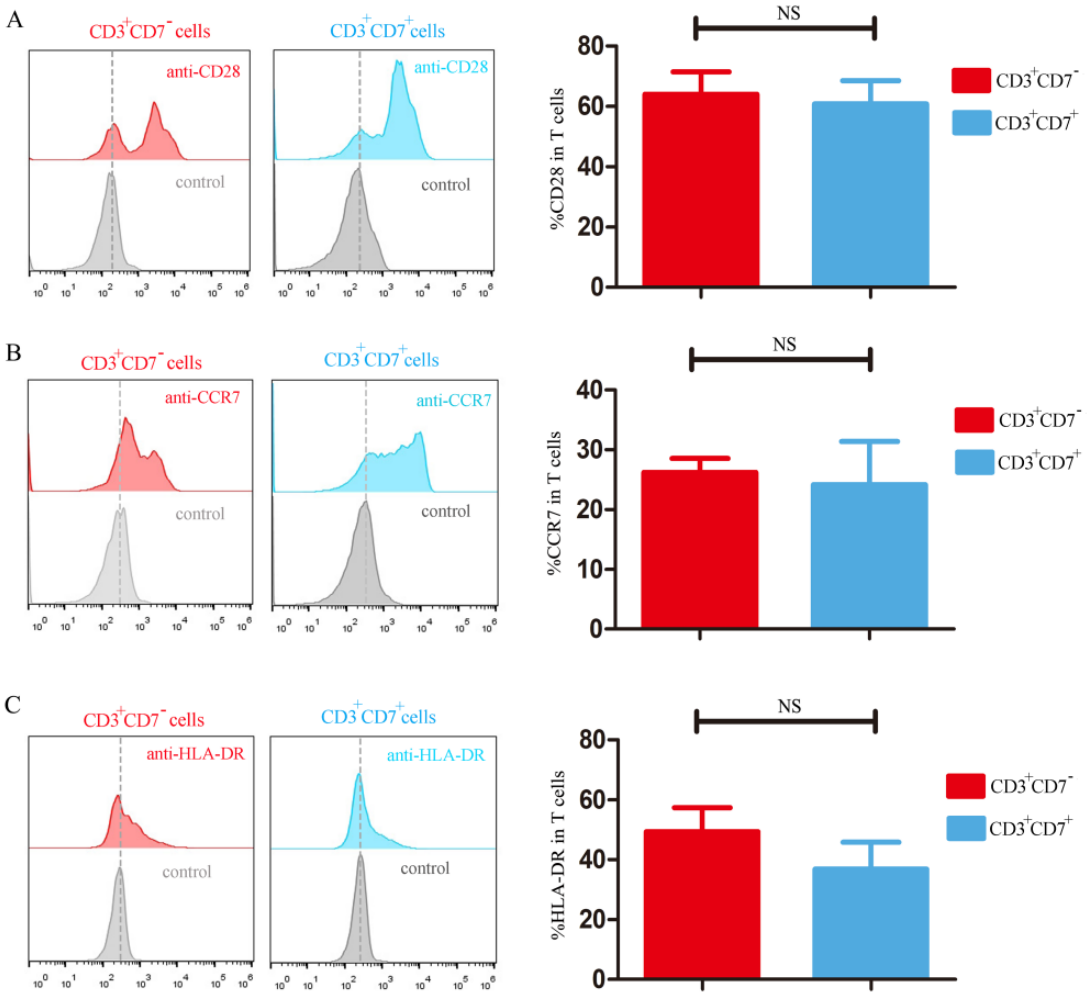

Figure S2 The expression of CD28 (A), CCR7 (B) and HLA-DR (C) in $CD3^+CD7^-$ T cells and $CD3^+CD7^+$ T cells in T-NHL patients with BM invasion. CCR7, C-C motif chemokine receptor 7; HLA-DR, human leucocyte antigen DR; T-NHL, T-cell non-Hodgkin's lymphoma. CCR7, C-C motif chemokine receptor 7; HLA-DR, human leucocyte antigen DR; NS, not significant.

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