INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a hematological tumor that occurs mostly in the middle-aged and elderly population. The disease is characterized by the clonal proliferation of mature B lymphocytes in peripheral blood, bone marrow, spleen, and lymph nodes. The number of newly diagnosed CLL patients in China has been increasing year by year. It is currently believed that the pathogenesis of CLL is closely related to immune dysfunction, especially the abnormal T cell subsets in CLL patients. The levels of Galectin-9 and IL-10 were significantly elevated in patients of CLL, especially in stages of Binet B, and C. However, IFN-γ decreased. Moreover, Galectin-9 in CLL patients was positively correlated with the number of Tim-3+ Treg cells and the level of IL-10. Interestingly, when the Tim-3/Galectin-9 pathway was blocked in vitro, the level of IL-10 in the culture supernatant of CD4+ T cells was significantly reduced, while the levels of IFN-γ and TNF-α were increased. After co-culture with activated Th1 cells, the apoptosis of CLL cells was significantly increased, and this effect was reversed after treatment with Tim-3+ Tregs. In summary, Galectin-9/Tim-3 are elevated in CLL and associated with disease progression. This pathway might become the potential target of immunotherapy in CLL patients.

KEYWORDS
chronic lymphocytic leukemia (CLL), CD4+ T cell subset, Tim-3/Galectin-9 signal pathway
lymphocyte immune response. At present, lenalidomide is used in the treatment of CLL patients, which can promote the infiltration of T cells in the tumor microenvironment and polarization of T cells to the Th1/IFN-γ direction.

CD4+ T cells are divided into Th1, Th2, Th17, and Treg subsets, which coordinate and antagonize each other to maintain a relative balance of immune responses. Th1 and Th2 cells mediate cellular and humoral immune responses by secreting IFN-γ, TNF-α, IL-4, etc. Treg cells secrete IL-10 and TGF-β to downregulate the immune response and play an important role in tumor immunity. Th17 cells mainly secrete IL-17A, IL-17F, IL-21, and participate in inflammatory reactions, autoimmune diseases, and tumors. It is shown that there is a change in Th1/Th2 homeostasis in patients with CLL and shift to Th2 is considered to be related to the progression of CLL. In addition, Beyer et al found that the increase in the proportion of Treg in CLL patients was related to disease progression. Although the protective effect of Th17 cells in different solid tumors has been confirmed, its role in CLL is controversial. Moreover, our previous study also found that the ratio of Treg/Th17 cells in peripheral blood of CLL patients was changed, Treg cells increased significantly in CLL patients, indicating that Treg/Th17 imbalance is involved in the development of CLL.

Abnormal expression of immune checkpoints in the tumor microenvironment leads to the change in CD4+ T cell subsets. Similar to PD-1/PD-L1, T cell immunoglobulin mucin-3 (Tim-3) is a newly identified negative checkpoint, which inhibits T cell-mediated immune response by binding to its ligand (Galectin-9), thus inducing immune tolerance. Currently, Tim-3 has been extensively studied in solid tumors such as liver cancer and gastric cancer. In liver cancer patients, Tim-3 inhibited Th1 cell function and promoted its apoptosis. In gastric cancer patients, Tim-3 on CD4+ T cells was closely related to tumor invasion, lymph node metastasis, TNM staging, and prognosis. However, in hematological malignancies, especially in CLL, the function of Tim-3/Gal-9 is still unclear. Allahmoradi et al have found that the overexpression of Tim-3 on CD4+ T cells in peripheral blood of CLL patients. However, the role of Tim-3 in the differentiation and function of CD4+ T subsets in CLL patients is still unclear.

Here, in this study, we used flow cytometry to detect the expression of Tim-3 on CD4+ T cell subsets (Th1, Th2, Treg, Th17) and in peripheral blood of CLL patients. Levels of IFN-γ, TNF-α, IL-4, IL-10, IL-17, and Galectin-9 were detected in the serum to analyze the relationship between changes in Gal-9/Tim-3 and the function of CD4+ T cells in CLL. Our results may provide a theoretical basis for Tim-3 to become a new potential therapeutic target of CLL.

2 MATERIALS AND METHODS

2.1 Healthy volunteers and patients

A total of 40 patients with untreated CLL admitted from January 2018 to September 2019 were enrolled in this study and were assigned as the CLL group, including 27 males (67.50%) and 13 females (32.50%), with an average age of 64.00 ± 9.46 years. The diagnosis and staging of these patients were based on the reported standards. Another 36 healthy individuals, including 23 males and 13 females, with an average age of 62.06 ± 10.16 years, were used as healthy control. There was no statistically significant difference in the ages between the two groups. Their clinical data are shown in Table 1. Peripheral blood was collected from each participant. The diseases, including diabetes, hypertension, cardiovascular disease, pregnancy, acute/chronic infectious disease, or metastatic tumor history, were excluded from all subjects. This study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (20180223-78), and all subjects signed the informed consent.

2.2 Flow cytometry analysis and cell sorting

CD4+ T subset biomarkers were chosen according to the immunophenotyping standardized by the Human Immunology Project Consortium. In detail, freshly isolated blood samples (100 μL) were incubated with the following

| TABLE 1 Clinical data of the study cohort (mean ± SD) |
|-----------------------------------------------|
| Groups | Age (year) | While blood cell number (×10^9/L) | Lymphocyte absolute number (×10^9/L) | Lymphocyte percentage (%) | Hemoglobin (g/L) | Platelets (×10^9/L) |
|--------|------------|---------------------------------|-------------------------------------|--------------------------|-----------------|-------------------|
| CLL (n = 40) | 64.00 ± 9.46 | 63.03 ± 82.82 | 53.48 ± 75.66 | 76.52 ± 14.01 | 117.28 ± 28.66 | 149.06 ± 69.07 |
| HC (n = 36) | 62.06 ± 10.16 | 6.37 ± 1.45 | 2.16 ± 0.72 | 32.36 ± 7.59 | 139.83 ± 17.29 | 227.47 ± 33.58 |
| t value | 0.99 | 4.10 | 4.07 | 16.81 | 4.10 | 6.18 |
| P value | >.05 | <.05 | <.05 | <.05 | <.05 | <.05 |

Abbreviations: CLL, chronic lymphocytic leukemia; HC, healthy control.
fluorescent-labeled antibodies: 1) CD4-PerCP, CD8-FITC, CCR6-PE-cy7 (CD196), CXCR3- APC (CD183), CD3-V450, and Tim-3-PE for Th17, Th1, Th2 cells; 2) CD4-PerCP, CD3-V450, CD25-APC, CD127-FITC and Tim-3-PE for Treg cells; 3) CD4-PerCP, CD8- FITC, CD3-V450, and IgG-PE for isotype control. The incubation was performed at room temperature in the dark for 20 minutes, and then erythrocytes were lysed by adding erythrocyte lysis solution. After washing with PBS twice, cells were suspended in 500 μL of PBS and detected by flow cytometry (Aria II, BD, USA). CD4+ T cells and Tim3+ Treg cells were separately sorted from PBMCs of CLL patients, and Th1 cells were further sorted from PBMCs of healthy controls by FACS Aria II (BD). The data were analyzed by FlowJo software (TreeStar, USA). All antibodies and reagents were purchased from BD Biosciences (San Jose, CA, USA).

2.3 | CD4+ T cell treatment

2.3.1 | Anti-Tim-3 antibody blocking experiment

The sorted CD4+ T cells were cultured in vitro with CD3 and CD28 (clone OKT3, CD28.2, 0.5 μg/mL, eBioscience, San Diego, CA, USA) for 48 hours to stimulate the production of IFN-γ, TNF-α, IL-4, IL-10, and IL-17. These cells were further incubated with anti-Tim-3 monoclonal antibody (BioLegend, 10 μg/mL) or/and Galectin-9 recombinant protein (ACRO Biosystems, 5 μg/mL), at 37°C for 24h. Then the culture supernatant was collected, and the changes in cytokines under different treatments were detected by ELISA.

To determine the effect of Tim-3 on Treg cells in the CLL microenvironment, Tim-3+ Treg and CLL strain (BeNa culture collection, Wuhan) were co-cultured with Th1 cells at a 1:20:5 ratio for 48 hours. At the same time, the anti-Tim-3 blocking antibody or/and Galectin-9 recombinant protein was added to the co-culture system. After that, the cells were collected and analyzed with flow cytometry for apoptosis. The culture supernatant was used for ELISA.

2.4 | ELISA

After centrifugation for 10 minutes (2500 r/min), serum was isolated from peripheral blood. IFN-γ, TNF-α, IL-4, IL-10, IL-17, and Galectin-9 levels in serum and in culture supernatant were detected by the corresponding ELISA kits (eBioscience, San Diego, CA, USA), and the experimental procedures were carried out in strict accordance with the instructions. Finally, the absorbance at 450 nm (OD450) was measured with a microplate reader (Thermo Fisher, USA). The cytokine level was calculated according to the standard curve.

2.5 | Statistical analysis

SPSS 17.0 software was used for statistical analysis, and all data were expressed as mean ± SD. The t test was used for comparison between the two groups. Analysis of variance was used for comparison between the three groups followed by the LSD method for comparison between groups. If there is no homogeneity of variances, the rank sum test was used for comparison between groups. Spearman correlation analysis was performed. A P value < .05 is considered statistically significant.

3 | RESULTS

3.1 | Increased Treg and Treg/Th17 imbalance in peripheral blood of CLL patients

Flow cytometry was used to detect CD4+ T cell subsets in peripheral blood of CLL and HC, including Th1 cells (CD183+ CD196+ CD4+), Th2 cells (CD183- CD196- CD4+), Th17 cells (CD183- CD196+ CD4+) and Treg cells (CD25hi CD127low CD4+). The representative flow cytometry results of HC and CLL groups are shown in Figure 1A, B, respectively. Statistically, the proportions of Th1 and Th2, and the Th1/Th2 ratio in CLL patients were not significantly different from those in the HC group (P > .05, Figure 1C). However, the proportion of Treg cells in CLL patients was significantly higher than that in the HC group (P < .01, Figure 1D). Th17 cells in the CLL group were only slightly higher than those in the HC group, without significant difference (P > .05, Figure 1D). In addition, the Treg/Th17 ratio in the CLL group was significantly higher than that in the HC group (P < .01, Figure 1D). The above results indicate that Treg cells in peripheral blood of CLL patients increase significantly, resulting in an imbalance of the Treg/Th17 ratio.

3.2 | Tim-3 overexpresses on the surface of Th1 and Treg cells in CLL patients

We also detected Tim3 expression on CD4+ T cell subsets. The representative flow cytometry results are shown in Figure 2A. Compared with the HC group, the percentages of Tim-3+ Th1 and Tim-3+ Treg cells of CLL patients were significantly increased (P < .05, Figure 2B, C). However, the difference in percentages of Tim-3+ Th2 and Tim-3- Th17 cells was not statistically significant between the two groups (P > .05). Furthermore, the ratio of Tim-3+ Th1/Tim-3+ Th2 and Tim-3+ Treg/Tim-3+ Th17 in CLL group was significantly higher than that in HC group (P < .01) (Figure 2B, C). This result suggests that the Tim-3 is highly expressed on the surface of Th1 and Treg cells in CLL patients.
**FIGURE 1** Percentage of Th1, Th2, Treg, and Th17 cells in CLL and HC. The percentages of Th cells in total CD4⁺ T-cell numbers were determined by flow cytometry. A-B. Cells were first gated on CD4⁺ T cells. Different cell subsets were distinguished according to different cell labels, i.e., the Treg cells was CD4⁺ CD25⁺ CD127⁻, Th1 was CD4⁺ CD183⁺ CD196⁻, Th2 was CD4⁺ CD183⁻ CD196⁻, Th17 was CD4⁺ CD183⁻ CD196⁺. C–D, Percentage of CD4⁺ T cell subsets, Th1/Th2 and Treg/Th17 ratios in the peripheral blood of CLL patients and HC. HC: healthy control, CLL: Chronic lymphocytic leukemia.

**FIGURE 2** The expression of Tim-3 on CD4⁺ T cell subsets. A, Cells were first gated on Th1, Th2, Th17, and Treg cells, and Tim-3 expression on the surface of CD4⁺ T cells in CLL patients and HC were analyzed. B-C, Comparisons of the percentages of Tim-3⁺ Th1, Tim-3⁺ Th2, Tim-3⁺ Th17, and Tim-3⁺ Tregs cells in the peripheral blood of the HC and CLL groups. HC: healthy control, CLL: Chronic lymphocytic leukemia.
3.3 | Changes in serum cytokines in patients with CLL

In order to explore the changes in cytokines in the serum of CLL patients, we performed ELISA. Compared with the HC group, the levels of Galectin-9 and IL-10 in the CLL group were significantly increased, while the levels of IFN-γ were significantly reduced ($P < .05$) (Figure 3A). However, the levels of TNF-α, IL-17, and IL-4 in CLL patients did not significantly change ($P > .05$). In the CLL group, there was a significant increase in the ratio of IL-10/IL-17, while an obvious decrease in the ratio of IFN-γ/IL-4 ($P < .05$) (Figure 3B). These results indicate that the change in IFN-γ, IL-10, and Galectin-9 is related to the imbalance of CD4+ T subsets in CLL patients.

3.4 | The relationship between cytokines and other CD4+ T cell subsets

Next, we conducted a correlation analysis of Galectin-9, T cell subsets, and cytokines. As shown in Figure 4, the level of Galectin-9 in CLL patients was positively correlated with the percentage of Tim-3+ Treg cells and the level of IL-10 ($r = 0.581$, $P < .01$; $r = 0.608$, $P < .01$). However, it did not relate to the number of Th1 cells, Tim-3+ Th1, Th2, Tim-3+ Th2, Treg, Th17, Tim-3+ Th17 cells or the levels of IFN-γ, IL-4, and IL-17 (all $P > .05$) (Figure 4). These results suggest that in CLL patients, high levels of Galectin-9 may promote the proliferation and differentiation of Treg cells.

3.5 | The levels of Galectin-9 and IL-10 are associated with the advanced Binet stage of CLL patients

We then analyzed the CD4+ T cell subset percentage and cytokine level in CLL patients with different Binet stages. As shown in Figure 5A, the percentage of Tim-3+ Th1 cells in Binet stage C patients was higher than that in Binet stage A and stage B ($P < .05$). Tim-3+ Treg cells in Binet stage A, stage B, and stage C patients were in increasing order, and Tim-3+ Treg cell percentage in each stage had significant difference with each other ($P < .05$) (Figure 5B). There was no statistical difference in Tim-3+ Th2 and Tim-3+ Th17 cells among the three Binet stages ($P > .05$). Among them, the level of Galectin-9 was highest and the level of IFN-γ was lowest in patients with Binet C ($P < .05$) (Figure 5C). The change trend of IL-10 in the Binet A phase, Binet B phase, and Binet C phase was similar to that Tim-3+ Treg cells. The later the stage, the higher the IL-10 level and IL-10/IL-17 ratio ($P < .05$) (Figure 5D). This result indicates that the high percentage of Tim-3+ Treg cells, especially Galectin-9 and IL-10 may be closely related to the disease progression.

3.6 | The changes in cytokines with and without blocking the Gal-9/Tim-3 pathway

We sorted out CD4+ T cells (with purity above 97%) from CLL patients with flow cytometry and incubated with CD3 and CD28 for 48 hours. The stimulated CD4+ T cells in vitro were then incubated with or without anti-Tim-3 for 24h. Cytokine levels (IFN-γ, TNF-α, IL-4, IL-10, and IL-17) in the culture supernatant were detected by ELISA. As shown in Figure 6A,B, IFN-γ and TNF-α levels were decreased in the Galectin-9 group, while significantly increased after blocking the pathway by anti-Tim-3 antibody ($P < .05$). The level of IL-10 was reversed (Figure 6C). IL-4 and IL-17 levels were not significantly different among groups (Figure 6D,E).

These data suggest that the function of CD4+ T subsets is affected by blocking the Tim-3/Galectin-9 pathway.

3.7 | Tim-3+ Treg inhibit the function of Th1 effector depends on activated Tim-3/Gal-9 pathway in vitro

To further confirm the identification of the Tim-3/Galectin-9 pathway, we co-cultured the sorted Tim3+ Treg, Th1, and CLL cells in vitro with or without anti-Tim-3/Gal-9 antibody.

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**FIGURE 3** Cytokines changes in CLL patients. A, Serum levels of Galectin-9, IFN-γ, TNF-α, IL-4, IL-10, and IL-17 in HC and CLL groups were detected with ELISA. Compared with the HC group, *$P < .05$, **$P < .01$. HC: healthy control, CLL: Chronic lymphocytic leukemia. B, Ratios of IL-10/IL-17 and IFN-γ/IL-4
Figure 7). Among all groups, as exposure to Tim-3+ Treg and Gal-9, not only apoptosis of CLL cells (Figure 7A,B) but also the levels of IFN-γ and TNF-α in the culture supernatant were decreased mostly in Tim-3+ Treg and Gal-9 group (Figure 7C,D). These results indicate that the Galectin-9/Tim3 signaling pathway promotes Tim3+ Treg to suppress Th1 effector function. In anti-Tim-3 antibody + Tim-3+ Treg+ Gal-9 group, the rate of CLL cell apoptosis increased, at the same time, IFN-γ and TNF-α secretion increased significantly compared with Tim-3+ Treg and Gal-9 group (Figure 7). These results show that after blocking Tim-3/Galectin-9, the inhibitory effect of Tim-3+ Treg cells on Th1 cells is reserved.

Figure 4  Correlation analysis of cytokines in CLL patients. Data were analyzed with Spearman correlation analysis. Galectin-9 were positively correlated with the concentration of IL-10 ($P < .01, r = 0.608$) and Tim3+ Tregs ($P < .01, r = 0.581$) but not others.
DISCUSSION

Various immune cells and negative signaling molecules are involved in the immunopathogenic mechanism of CLL. Tim-3 is a newly identified negative immunoregulatory factor, expressed on Th1, CD8+ T, Th17, and Treg cells. It can bind to its ligand (Gal-9) and play an important role in infectious diseases and tumors; its continuous expression leads to effector T cell exhaustion. Studies have shown that Tim-3 is expressed at high levels in tumors such as kidney cancer and lung cancer, and can promote tumor progression. However, the change and role of the Tim-3/Gal-9 pathway in CLL are still unclear. In this study, we found that the expression of Tim-3 on Th1 and Treg cells was upregulated, levels of Gal-9 and IL-10 were significantly increased, and IFN-γ was lower in newly diagnosed CLL patients. Tim-3/Gal-9 was closely associated with the progression of CLL. We further studied the change and function of CD4+ T cell subsets by blocking Tim-3/Gal-9 pathway in vitro. The results showed that the level of IL-10 in the culture supernatant of CD4+ T was significantly reduced, while the levels of IFN-γ and TNF-α were increased. The apoptosis of CLL cells was also increased after blocking Tim-3/Gal-9. These data indicate that elevated Gal-9/Tim-3 pathway regulate the balance and function of CD4+ T subsets, especially the role of Th1 and Treg cells in CLL.

In the previous study, Karmali et al. found that the expression levels of Th2 cytokines were dominant, while Th1 cells related cytokines were reduced in CLL patients. Subsequent study also found that Treg cells in newly diagnosed CLL patients increased significantly and that with the progressing of Binet staging, Treg cells gradually increased, which ultimately affected the prognosis. As the disease progresses in CLL patients, the number of Treg cells increases and Tregs play a role by suppressing the anti-tumor immune response. It is shown that the occurrence of CLL may be related to the downregulation of Th17 expression and the differentiation of Treg cells, leading to the proliferation of leukemia cells. In this study, Treg cell percentage was significantly increased, and there was a Treg/Th17 imbalance in CLL patients. However, the immunopathogenic mechanism of Treg cells in CLL patients is currently unknown.

The present study found that Tim-3 was mainly expressed on the surface of T1 and Th17 cells. After binding to its ligand Gal-9, Tim-3 suppresses effector Th1 and Th17 cells to downregulate T cell-mediated immune responses. In this study, we further analyzed the expression of Tim-3 on CD4+ T cell subsets and found that Tim-3 on Th1 and Treg cells were upregulated in CLL patients. At the same time, the levels of Gal-9 and IL-10 were significantly increased, while IFN-γ decreased in the serum of CLL patients, which suggests that the negative signaling pathway of Tim-3/Gal-9 is excessively activated in CLL patients. Previously, Taghiloo et al. found that Gal-9 was overexpressed in patients with CLL and...
**FIGURE 6** The changes in cytokines with and without blocking the Gal-9/Tim-3 pathway. CD4+ T cells were isolated from CLL patients. They were first stimulated for 48 hours and then treated with TIM-3 or Galectin-9. Culture supernatant of these lymphocytes was collected after 24 hours. Levels IFN-γ (A), TNF-α (B), IL-4 (C), IL-10 (D), and IL-17 (E) in culture supernatant were tested by ELISA assay. The comparisons were determined by One-way ANOVA NS: no significant

**FIGURE 7** Inhibition of the Tim-3 pathway on Tregs induces the apoptosis of CLL and increases the secretion of IFN-γ and TNF-α in vitro. Th1 cell, Tim-3+ Treg, and CLL strain were co-cultured with or without anti-Tim-3 antibody/Galectin-9 recombinant protein for 48 hours. The ratio of apoptotic cells was determined by flow cytometry. A, Representative flow cytometry results of different culture systems. B, Quantitative flow cytometry results of different culture systems. The levels of IFN-γ (C) and TNF-α (D) were determined by ELISA. Compared with PBS group, *: P < .05, **: P < .01. Compared with Tim-3+ Treg+ Gal-9 group in each group, △P < .05, △△P < .01
that Galectin-9 expression was higher in patients in the advanced stage than in patients in the early stage. They believed that the upregulation of Galectin-9 was related to the poor prognosis of CLL patients, and Galectin-9 may be used as a biomarker of disease prognosis. This is consistent with our findings. We further analyzed the expression of Galectin-9 and Tim-3 on T cell subsets (Th1, Th2, Th17, and Treg), and the correlation between Galectin-9 and cytokines. We found that Galectin-9 was positively correlated with the percentage of Tim-3+ Treg cells and IL-10 in CLL patients. Moreover, Galectin-9 and Tim-3+ Treg were higher especially in stages of Binet C. In addition, studies have reported that Galectin-9 can upregulate Foxp3 to promote the differentiation of naive T cells into Treg cells and that the number of Tregs in Galectin-9 knockout mice is reduced. Thus, Tim-3/Galectin-9 may be closely related to a poor outcome of CLL. Studies have shown that Galectin-9/Tim-3 enhances Treg cell differentiation and inhibits Th17 cell differentiation. Therefore, we believe that high expression of Tim-3 on Treg cells in CLL patients may promote the proliferation and function of Treg cells after binding to Galectin-9, and eventually lead to Treg/Th17 imbalance in CLL patients. This indicates that increased Tim-3+ Treg cells may secrete large amounts of IL-10 to induce immune escape of tumor.

In gastrointestinal tumors, the continuous expression of Tim-3 led to the exhaustion of T cell function, otherwise blocking Tim-3 signaling could restore the function of tumor-infiltrating lymphocytes both in vivo and in vitro. In order to further directly confirm the effect of Tim-3/Galectin-9 on the function of CD4+ T cell subsets, we used the anti-Tim-3 antibody to block the Tim-3/Galectin-9 signaling pathway in vitro and found that IFN-γ and TNF-α levels were significantly increased, while IL-10 level was decreased. Allahmoradi et al. found that CD4+ T cells isolated from CLL patients showed a lower production of IL-2, IFN-γ, and TNF-α, and higher production of IL-10. Therefore, we speculate that Tim-3/Galectin-9 signaling pathway may participate in the development of CLL by regulating the function of Th1 and Treg cells. Aue et al. found that lenalidomide induced T-cell secretion of IFNγ and IL-2, which promoted Th1 differentiation in the CLL microenvironment. In our study, to determine the role of the Tim-3/Galectin-9 pathway on CD4+ T subsets, the sorted Th1 cell, Tim-3+ Treg, and CLL cells were co-cultured with or without anti-Tim-3 antibody. Exposure to Tim-3+ Treg and Gal-9 decreased the apoptosis of CLL cells as well as reduced the levels of IFN-γ and TNF-α. Further analyses showed that blocking the Tim-3/galectin-9 pathway resulted in the suppression of Tregs in vitro, thereby significantly increasing IFN-γ and TNF-α production from T1 effector cells. Moreover, blockade of Tim-3 on Treg in vitro with an anti-Tim-3 antibody accelerated the apoptosis of CLL cell, which may possibly be achieved by increased IFN-γ and TNF-α production. These results indicate that Tim-3+ Treg could inhibit the function of the Th1 effector via activating the Tim-3/Gal-9 pathway.

**FIGURE 8** Schematic diagram illustrating the mechanisms of Tim-3/Gal-9 signal pathway in regulating the differentiation of Treg cells in CLL patients. In the CLL tumor microenvironment, Th1 and Treg cells express upregulated Tim-3, resulting in the depletion of Th1 cells by the binding of ligand Gal-9. At the same time, it causes excessive proliferation and activation of Treg cells, which ultimately promotes the progression of CLL. After blocking the Tim-3/Gal-9 pathway, IL-10 is suppressed, thereby enhancing the ability of Th1 cells to secrete IFN-γ and TNF-α, which is beneficial to the clearance of CLL cells.
In summary, Tim-3 and Galectin-9 significantly increases in CLL patients, which is closely related to disease progression. Elevated Gal-9/Tim-3 pathway regulates the balance and function of CD4+ T subsets. Activated Galectin-9/Tim3 signaling pathway promotes Tim3+ Treg to suppress Th1 effector function in control CLL cells (Figure 8). Blocking the Tim-3/Galectin-9 signaling pathway can attenuate Treg function. Therefore, the Tim-3/Galectin-9 signaling pathway may become a novel target for immunotherapy in patients with CLL.

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CONFLICT OF INTEREST
The authors declare no potential conflict of interest.

AUTHOR CONTRIBUTIONS
N. Pang prepared the manuscript. X. Alimu collected the data. R. Chen searched the literature. M. Muhashi, F. Zhao and L. Wang analyzed the data. J. Ma and G. Chen interpreted the data. J. Qu collected the funds. J. Ding designed the study.

ORCID
Jianbing Ding https://orcid.org/0000-0001-5506-7665

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