The role of follicular T helper cells in patients with malignant lymphoid disease

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ABSTRACT

Objectives: To investigate the dynamic change of follicular T helper cells (TFH) in patients with malignant lymphoid disease (MLD) and to explore its clinical significance.

Methods: The dynamic change of TFH cells, ICOS+ and PD-1+ TFH cells at pretreatment and different treatment periods was determined by flow cytometry in 85 MLD patients. Concentration of interleukin 21 (IL-21) was evaluated by ELISA, and the correlation between clinical prognosis and the ratio of TFH cells was analyzed.

Results: Significantly increased ICOS+ and PD-1+ TFH cells were found in MLD patients at pretreatment compared to healthy controls. Decreased or even close to normal levels of ICOS+ and PD-1+ TFH cells were found at the end of treatment. However, in the patients with progressive disease, high levels of ICOS+ and PD-1+ TFH cells were found. Moreover, a significantly increased plasma IL-21 level was found in MLD patients. Negative correlation was found between the level of ICOS+, PD-1+ TFH cells, as well as IL-21 and the prognosis of MLD.

Conclusions: Significantly increased TFH cell ratios were found in patients with MLD, and decreased TFH cells ratios could be expected in those treatment-effective patients, which could be used as the therapeutic efficacy index.

KEYWORDS
Acute lymphoblastic leukemia; multiple myeloma; non-Hodgkin’s lymphoma

Introduction

Malignant lymphoid diseases (MLDs), including acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma (NHL), and multiple myeloma (MM), are malignancies which arise from lymphocytes residing in lymphoid tissue outside of the marrow. Until now, the detailed pathogenesis of MLD has not been fully explored and abnormal immune system is considered to be the main cause of MLD. Moreover, multiple factors, including gene mutation, epigenetic alterations and change of hematopoietic microenvironment, could induce the development and progression of MLD. Understanding the detailed behaviors of immune system could be helpful in the effective treatment of MLD.

Follicular T helper cells (TFH), which are different from Th1, Th2, Th17, and Tregs, are a novel subset of CD4+ T cells located in germinal center (GC) of B cells. The TFH cells display the features of CXCR5, ICOS, PD-1 expression, and IL-21 secretion, which could be further involved in the B-cell activation, antibody production, and humoral immune response [1–3]. The abnormal change of TFH cells has been proved in multiple diseases, including immune disease, rheumatic diseases, immunodeficiency disease, and tumor [4–7].

In the present study, we aimed to investigate the dynamic change of TFH in MLD patients during the treatment process and to explore its clinical significance.

Materials and methods

Subjects

From June 2014 to January 2016, a total of 85 patients treated in the Department of Hematology of our hospital were included in this study. Among these patients, 13 cases were acute lymphoblastic leukemia (ALL), 52 cases were NHL, and 20 cases were multiple myeloma (MM). All the patients were diagnosed according to the diagnosis and treatment criteria of hematological disease. The detailed clinical characteristics of these patients are shown in Table 1. We also recruited age-matched health control subjects from the physical examination department and a total of 10 cases were included, including 5 males and 5 females, with a median age of 50 (44–65) years. The Institutional Review Board at the Second Affiliated Hospital of Soochow University approved all protocols, and informed consent was obtained from all adult donors.

Peripheral blood mononuclear cells and plasma sampling

A total of 4 mL heparin anticoagulant peripheral blood was collected at pretreatment and the end of second, fourth, and sixth treatment course. The plasma used for IL-21 concentration determination was obtained by 3000 rpm centrifugation for 10 min. The remaining blood was diluted with phosphate-buffered saline...
Table 1. Clinical characteristics of MLD.

| Clinical characteristics | ALL (n = 13) | NHL (n = 52) | MM (n = 20) |
|--------------------------|--------------|--------------|-------------|
| Sex                      |              |              |             |
| Male                     | 5            | 31           | 11          |
| Female                   | 8            | 21           | 9           |
| Age                      | 49.6 ± 15.9  | 55.8 ± 16.0  | 57.9 ± 11.9 |
| Age ≤60                  | 9            | 23           | 8           |
| Age >60                  | 4            | 29           | 12          |
| Gene mutation            |              |              |             |
| RBB2H                    | 2            | 1            | 2           |
| RBB2H and CEBPA          | –            | –            | 1           |
| FLT3-TKD                 | 1            | –            | 1           |
| FLT3-ITD                 | –            | 1            | –           |
| Karyotypic anomalies     | 2            | 1            | 5           |
| Deletion                 | 2            | 1            | 3           |
| Malposition              | –            | –            | 2           |
| Lab test                 |              |              |             |
| Hemoglobin (g/l)         | 88.94 ± 14.04| 105.30 ± 9.73| 92.96 ± 9.98|
| PLT (×10^9/l)            | 56.3 ± 40.6  | 82.1 ± 34.9  | 134.2 ± 49.7|
| LDH level (U/L)          | 568.65 ± 147.34| 458.66 ± 78.23| 382.57 ± 89.48|
| ≥244 U/L                 | 9            | 18           | 13          |
| β2-microglobulin (mg/L)  | 4.91 ± 2.15  | 3.88 ± 1.01  | 5.36 ± 2.72 |

Values are presented as mean ± standard deviation or n.

ALL: acute lymphoblastic leukemia; NHL: non-Hodgkin lymphoma; MM: multiple myeloma; PLT: platelet count; LDH: lactate dehydrogenase.

Flow cytometry analysis of TFH cells

PBMCs were thawed and adjusted to a density of 1 × 10^6 cells/mL with PBS. One hundred microliter of PBMCs solution was aliquoted and a volume of 10 µL PerCP-CD4 or Isotype IgG and FITC-CXCR5, PE-ICOS, and PE-Cy7-PD1 were added. After incubation for 30 min, the cells were processed in a flow cytometry and CD4⁺ cells were used for gating. Here, we defined CD4⁺CXCR5⁺ cells as TFH cells and further analyses were also performed by detecting the ICOS⁺ and PD1⁺ expression. The ratio of peripheral TFH cells was defined as the ratio of CXCR5⁺CD4⁺ cell to CD4⁺ cells, whereas the ICOS⁺ and PD1⁺ peripheral TFH cells stand for the ratio of ICOS⁺CXCR5⁺CD4⁺ cell to CXCR5⁺CD4⁺ cells and PD1⁺ ICOS⁺CXCR5⁺CD4⁺ cell to CXCR5⁺CD4⁺ cells, respectively. All the data were analyzed by the Summit 4.0 software. All the antibodies used here were purchased from eBioscience Co. Ltd.

ELISA

The plasma inflammatory factor IL-21 was measured by quantitative colorimetric sandwich ELISA’s kit (R&D, Minneapolis, MN).

Statistical analysis

All the data were processed with SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. Student’s t-test or one-way analysis of variance was used to examine differences between groups. Multiple linear regression was performed for the correlation analysis. A p-value of <0.05 was considered significant.

Results

Characteristics of the MLD patients

As shown in Table 1, there were 5, 31, and 11 males in the groups of ALL, NHL, and MM, respectively. The average ages were 49.6 ± 15.9, 55.8 ± 16.0, and 57.9 ± 11.9 in the three groups, respectively. The numbers of gene mutation including RBB2H, RBB2H and CEBPA, FLT3-TKD and FLT3-ITD, and Karyotypic anomalies which include deletion and malposition are also listed in Table 1. Lab tests, including concentration of hemoglobin, platelet, lactate dehydrogenase, and β2-microglobulin, are also recorded in Table 1.

Elevated peripheral TFH cells in MLD patients

We observed significantly increased ratio of peripheral TFH cells in MLD patients compared to healthy controls (32.06 ± 8.06% vs. 11.06 ± 0.88%, p < 0.001). Different ratios of peripheral TFH cells in different type of MLDs: NHL (36.04 ± 6.39%) > ALL (30.03 ± 6.07%) > MM (23.01 ± 4.69%), which showed a significant difference among these groups (p < 0.001). Significantly decreased ratio of peripheral TFH cells in those MLD patients who achieved an effective treatment at the end of second, fourth, and sixth treatment course compared to pretreatment (p < 0.05). A similar or even lower ratio of peripheral TFH cells could be observed in MLD patients, especially in those patients with complete remission, at the end of sixth treatment course compared to healthy controls (p > 0.05); however, a higher ratio of peripheral TFH cells was still found in those patients with partial remission (p < 0.05). In addition, a higher ratio of peripheral TFH cells was found in NHL and MM patients with disease progression compared to pretreatment, while slightly lower ratio of peripheral TFH cells was found in ALL patients with disease progression compared to pretreatment. These results suggested that the ratio of peripheral TFH cells of a patient who is treated and responds well reverts towards that in a normal subject.

Elevated ICOS⁺- and PD1⁺-peripheral TFH cells were found in MLD patients

Further analysis showed that the ratio of ICOS⁺- and PD1⁺-peripheral TFH cells in healthy controls was 16.08 ± 5.51% and 20.68 ± 2.50%, whereas significantly elevated ICOS⁺- and PD1⁺-peripheral TFH cells were found in MLD patients. The ratio of ICOS⁺- and
PD1⁻/⁺-peripheral T follicular helper (TFH) cells was 39.21 ± 5.04% and 47.52 ± 6.57% in ALL patients, 45.01 ± 9.14% and 53.52 ± 7.16% in NHL patients, and 31.58 ± 4.20% and 43.14 ± 5.01% in MM patients, which showed a statistical difference compared to healthy controls (*p < 0.001) (Figure 1).

Dynamic changes of ICOS⁻/⁺ and PD1⁻/⁺ peripheral TFH cells during the treatment process

Significantly decreased ICOS⁺ peripheral TFH cells were found in those patients with complete remission (CR) and partial remission (PR) (*p < 0.001). The ratio of ICOS⁺ peripheral TFH cells at the end of second, fourth, and sixth treatment course was, respectively, 29.59 ± 4.21%, 23.63 ± 4.17%, and 18.07 ± 6.14% in ALL patients, 38.77 ± 4.12%, 27.58 ± 4.22%, and 19.34 ± 5.08% in NHL patients, and 28.18 ± 4.46%, 21.23 ± 5.15%, and 17.55 ± 6.17% in MM patients (Figure 2(a)). In addition, in those patients with no response or progressive disease, no significant difference was found on the ratio of ICOS⁺ peripheral TFH cells at the specific treatment time point compared to pretreatment (Figure 3(a)).

PD1⁺ peripheral TFH cells showed a similar pattern as ICOS⁺ peripheral TFH cells, significantly decreased PD1⁺ peripheral TFH cells were found in those patients at the end of second, fourth, and sixth treatment course (*p < 0.05). Ratio of PD1⁺ peripheral TFH cells in different treatment courses was, respectively, 39.82 ± 5.00%, 33.24 ± 5.91%, and 24.77 ± 5.96% in ALL patients, 46.40 ± 4.53%, 34.66 ± 5.82%, and 23.07 ± 4.49% in NHL patients, and 37.33 ± 3.70%, 29.93 ± 5.56%, and 24.33 ± 6.29% in MM patients (Figure 2(b)). Similar ratio of PD1⁺ peripheral TFH cells was found in NHL and MM patients at the end of sixth treatment course, while significantly increased ratio of PD1⁺ peripheral TFH cells was found in ALL patients compared to healthy controls. In addition, no difference was found on PD1⁺ peripheral TFH cells in those patients with no response or progressive disease (Figure 3(b)).

Elevated plasma IL-21 was found in MLD patients

Interleukin (IL)-21, a cytokine, has a fundamental role in the development of T-cell-dependent B-cell responses and, therefore, we determined the plasma IL-21 in all these patients. The plasma IL-21 was 471.40 ± 33.95 pg/mL, 498.74 ± 70.77 pg/mL, and 444.18 ± 37.56 pg/mL in ALL, NHL, and MM patients, respectively, which showed a significant increase compared to that in healthy controls (336.57 ± 40.56 pg/mL, *p < 0.001) (Figure 1(c)). Moreover, significant difference of IL-21 level was found between NHL and MM patients (**p < 0.01). Concentrations of IL-21 at the end of second, fourth, and sixth treatment course were, respectively, 364.13 ± 38.27 pg/mL, 334.78 ± 36.22 pg/mL, and 313.38 ± 37.40 pg/mL in ALL patients, 366.27 ± 49.97 pg/mL, 337.20 ± 45.38 pg/mL, and 310.14 ± 46.44 pg/mL in NHL patients, 348.13 ± 36.05 pg/mL, 328.23 ± 43.28 pg/mL, and 319.18 ± 41.52 pg/mL in MM patients (Figure 2(c)), and significant differences could be found between second and fourth treatment course in ALL patients (*p < 0.05), between second and fourth treatment course (**p < 0.01) and between fourth and sixth treatment course (**p < 0.05) in NHL patients, and between pretreatment and second treatment course in ALL (**p < 0.001), NHL (***p < 0.001), and MM (**p < 0.001) patients. Similarly, there were no differences between the concentrations of IL-21 in those patients on pretreatment and with no response or progressive disease (Figure 3(c)).

Correlations between prognosis and the ratio of peripheral TFH cells

We performed the correlation analyses between the prognosis and the ratio of peripheral TFH cells, and the results are shown in Table 2. According to the results, negative correlation was found between the prognosis and ratio of PD1⁺ and ICOS⁺ peripheral TFH cells. Similarly, negative correlation was found between the prognosis and concentration of IL-21 (Table 2).
Discussion

Early since 1960s, Claman and Miller first proposed the role of T helper cells especially Th2 cells in B-cell activation and antibody production using irradiated or thymectomized mouse models [8,9]. The critical role of Th2 cells in B-cell function mature was emphasized for a long time until the discovery of follicular helper T cells (TFH). Studies have verified TFH cells as the truly functional cells in promoting B-cell proliferation, differentiation, antibody secretion, and involvement in humoral immune response [10–13].

As a novel subpopulation of CD4+ T cells, TFH cells play a critical role in maintaining the immune homeostasis. Previous studies have elucidated the functional role of TFH cells in immune system. The interactions between TFH cells and B cells could be realized via high expression of chemokine receptor CXCR5 on the surface of TFH and the expression of its ligand CXCL13 in the follicular GC of the B cells, which could result in the migration and location of TFH into the GC [14,15]. Moreover, the expression of costimulatory molecule ICOS on the surface of TFH could further induce the production and function maintenance of TFH cells in participating in the generation of GC and memory B cells via interaction with its ligand ICOS-L. Several studies have shown that downregulation expression of ICOS could result in decreasing number of TFH, thereby affecting the mature and class switching of B cells, suggesting the irreplaceable role of ICOS in TFH function [16–18]. Programmed death receptor 1 (PD), which could interact with its ligand PD-L1 to transmit inhibition signaling to affect the T-cell differentiation and Treg production, is another important marker on TFH cells. Blocking the PD-1/PD-L1 signaling could lead to the massive proliferation of TFH cells, thereby resulting in the B-cell proliferation and antibody production [19–21]. IL-21 is a key cytokine involved in the TFH cell production, and it can upregulate the expression of CXCR5 and ICOS through an autocrine manner, thereby promoting the TFH cell differentiation and function development. In addition, IL-21 was also considered to be involved in the GC

Figure 2. Changes of ratio of ICOS+ and PD1+ follicular help T cells (TFH) as well as concentration of IL-21 in patients with MLD after chemotherapy at second, fourth, and sixth treatment course. The ratio of TFH was determined by flow cytometry analysis. Plasma IL-21 level was determined by enzyme-linked immunosorbent assay. *p < 0.05, **p < 0.01, and ***p < 0.001 for between-group comparison.
formation, B-cell proliferation and differentiation, and IgG class switching [22,23].

In the present study, we found that TFH cells, ICOS, PD-1, and IL-21 were participated in the development and progression of MLD. Significantly elevated TFH and the expression of ICOS, PD-1, and IL-21 were found at pretreatment compared to healthy controls, whereas significantly decreased TFH cells, ICOS and PD-1 were found at the end of second, fourth, and sixth treatment course in those patients with CR or

Table 2. Relationship between changes of surface markers of the follicular help T cells (TFH) and prognosis of MLDs.

| Patients | Changes (means ± SD) | R²   | p-Value | Changes (means ± SD) | R²   | p-Value | Changes (means ± SD) | R²   | p-Value |
|----------|----------------------|------|---------|----------------------|------|---------|----------------------|------|---------|
| ALL      |                      |      |         |                      |      |         |                      |      |         |
| CR       | 29.01 ± 4.42         | 0.701| 0.000   | 26.65 ± 3.91         | 0.42 | 0.016   | 212.55 ± 26.88       | 0.56 | 0.003   |
| PR       | 25.93 ± 3.83         | 0.440| 0.000   | 23.60 ± 9.07         | 0.116| 0.014   | 151.78 ± 42.91       | 0.152| 0.004   |
| PD       | 15.21 ± 3.38         |      |         | 14.78 ± 5.58         |      |         | 119.39 ± 39.39       |      |         |
| NHL      |                      |      |         |                      |      |         |                      |      |         |
| CR       | 35.24 ± 4.67         | 0.440| 0.000   | 29.18 ± 10.75        | 0.116| 0.014   | 209.33 ± 79.62       | 0.152| 0.004   |
| PR       | 30.08 ± 7.60         |      |         | 22.79 ± 9.45         |      |         | 199.06 ± 51.02       |      |         |
| PD       | 18.30 ± 10.79        |      |         | 20.55 ± 9.88         |      |         | 116.90 ± 91.74       |      |         |
| MM       |                      |      |         |                      |      |         |                      |      |         |
| CR       | 36.39 ± 5.39         | 0.325| 0.009   | 33.50 ± 9.20         | 0.305| 0.012   | 153.34 ± 38.94       | 0.314| 0.010   |
| PR       | 34.81 ± 3.11         |      |         | 31.80 ± 12.45        |      |         | 127.46 ± 67.41       |      |         |
| PD       | 27.85 ± 6.61         |      |         | 19.51 ± 4.95         |      |         | 80.44 ± 45.30        |      |         |

Data were presented as mean ± standard deviation.
ALL: acute lymphoblastic leukemia; NHL: non-Hodgkin lymphoma; MM: multiple myeloma; CR: complete remission; PR: partial remission; PD: disease progression.

Figure 3. Ratio of ICOS+ and PD1+ follicular help T cells (TFH) as well as concentration of IL-21 among the patients with the prognosis of complete remission (CR), partial remission (PR), and disease progression (PD). (a,d,g) The differences in the ratios of ICOS+ TFH, PD1+ TFH, and concentration of IL-21 in ALL among CR, PR, and PD, respectively. (b,e,h) The differences in the ratios of ICOS+ TFH, PD1+ TFH, and concentration of IL-21 in NHL among CR, PR, and PD, respectively. (c,f,i) The differences in the ratios of ICOS+ TFH, PD1+ TFH, and concentration of IL-21 in multiple myeloma (MM) among CR, PR, and PD, respectively. *p < 0.05, **p < 0.01, and ***p < 0.001 for between-group comparison.
PR. However, the decreasing of $T_{FH}$ cells and its related molecules ICOS and PD-1 did not reach to the normal level although most of patients achieved a remission statue. At the end of sixth treatment course, a similar ratio of $T_{FH}$ cells in the MLD patients could be observed, indicating the treatment duration-dependent property of $T_{FH}$ in MLD, which might be served as a prognostic index in clinical practice. Further comparisons between the CR and PR patients revealed that significantly lower level of $T_{FH}$ cells could be expected in CR patients than in PR patients, suggesting a negative correlation between $T_{FH}$ number and therapeutic effect. We also found that significantly increased or similar amount of $T_{FH}$, ICOS and PD-1 in those patients with no response or progressive disease at the end of treatment course. Combining with the correlation analysis, we concluded that late staging of patient could result in poor prognosis, which is illustrated by no decrease in the $T_{FH}$ cell number. Moreover, owing to the similar increasing pattern of $T_{FH}$ cells as the disease progress, we proposed a close relationship between $T_{FH}$ cells and MLD, and $T_{FH}$ cells could be employed as a novel indicator in peripheral blood for disease progression. We compared the difference in the $T_{FH}$ cells, ICOS, PD-1, and IL-21 among all the three group of patients, and the results showed the highest level of $T_{FH}$ cells, ICOS, PD-1, and IL-21 in NHL patients and lowest in MM patients. A recent study also indicated the relationship between plasma cells inhibition and TFH. Pelletier et al. [24] demonstrate that isotype-switched plasma cells expressed major histocompatibility complex (MHC) class II, the costimulatory molecules CD80 and CD86, and the intracellular machinery required for antigen presentation. Antigen-specific plasma cells accessed, processed, and presented sufficient antigen in vivo to induce multiple helper T-cell functions. They found that antigen-primed plasma cells failed to induce IL-21 or the transcriptional repressor Bcl-6 in naive helper T cells and actively decreased these key molecules in antigen-activated $T_{FH}$ cells. Mice lacking plasma cells showed altered $T_{FH}$ cell activity, which provided evidence of this negative feedback loop. Hence, antigen presentation by plasma cells defines a previously unknown layer of cognate regulation that limits the antigen-specific $T_{FH}$ cell program that controls ongoing B-cell immunity.

Recent studies have also shown the clinical significance of $T_{FH}$ cells in patients with immune thrombocytopenia (ITP), and they concluded that higher proportion of TFH cells, which could be rectified by hormone therapy, might account for the decreased platelet counts to be further involved in the immunological pathogenesis of children ITP [25,26]. Moreover, Dogan et al. [27] reported that abnormal expression of CXCL13, a chemokine critical for GC formation and one of the most highly upregulated genes in the GC T helper cell subset, in the majority of angioimmunoblastic T-cell lymphoma cases, provided further support for the role of GC T helper cells as the cells of origin for angioimmunoblastic T-cell lymphoma. Battistella et al. [28] identified five cases of cutaneous T-cell lymphoma with a peculiar pathologic aspect and expression of $T_{FH}$ markers CD10 in four of five biopsy specimens further showed medium-sized to large-sized atypical T-cell skin infiltrate expressing $T_{FH}$ markers (CD10, Bcl-6, PD-1, CXCL13, and ICOS), and this is the first report of the presence of $T_{FH}$ in lymphoma. They also proposed to examine the expression of TFH cells to improve prognosis in those patients with no response to CD20 antibody treatment.

On the basis of the elucidated role of $T_{FH}$ cell in some of the diseases and PD-1 was confirmed as the early marker expressed on the $T_{FH}$ cells, we proposed PD-1 as the main marker in tumors and the use of PD-1 blocking therapy might serve as a promising treatment intervention in different types of tumors to improve the overall survival in these patients. Recently, the development of PD-1 monoclonal antibody, such as pembrolizumab, has been employed in the treatment of melanoma and non-small lung cancer and the mechanism includes blocking its interaction with PD-L1 and PD-L2, thereby affecting tumor escaping and activation of immune system [29].

In conclusion, we found that upregulation of $T_{FH}$ cells, ICOS, PD-1, and IL-21 was found in the peripheral blood of MLD patients, which could be decreased to normal level in those patients with response to the treatment. Moreover, a close level of $T_{FH}$ to normal could be expected in CR patients than PR patients, suggesting the involvement of $T_{FH}$ in the progression of MLD. We concluded that consistent level of $T_{FH}$ cells and disease activity could be found in MLD patients and treatment targeting $T_{FH}$ might serve as an efficacious way to improve the disease state of the patients.

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