Phenotypic characterization of circulating CD4/CD8 T-lymphocytes in β-thalassemia patients

Egarit Noulsri,[1] Surada Lerdwana,[1] Suthat Fucharoen[2] and Kovit Pattanapanyasat[1]

Summary

Background: **Infection is one of the most common causes of death in β-thalassemia patients. This may be due in part to an underlying immunological abnormality. During the past decade, a subset of CD3+ T cells that express both CD4+CD8+ (DP) T-cells were discovered and have been described in several pathological conditions. However, phenotypic characterization of this unique T-lymphocyte subset in patients with β -thalassemia has not yet been investigated.**

Methods: **Flow cytometry was used to determine the frequency of such CD4+CD8+(DP) cells in concert with frequencies of CD4+, CD8+, NKT cells and γδ-TCR T-lymphocytes in the peripheral blood of β-thalassemia/HbE patients. The frequencies of these lymphocyte subsets were compared with those in blood samples from healthy volunteers.**

Results: **The results showed that the frequency of lymphocytes was significantly increased in splenectomized β-thalassemia/HbE patients but the frequencies of CD3+, CD4+ and CD8+ T-lymphocytes were not significantly different among the studied groups. However, analysis of unconventional T-lymphocytes revealed a significant increase in the frequency of CD4-CD8- in splenectomized β-thalassemia/HbE patients. The frequencies of CD4-CD8dim and CD4+CD8+ in β-thalassemia/HbE patients were similar to the controls. Further classification of the CD4+CD8+ cells revealed that β-thalassemia/HbE patient expressed significantly high levels of CD4brightCD8dim, with a marked increase found in non-splenectomized patients. Furthermore, significant increases in the frequency of γδ-TCR and NKT cells were also demonstrated in these splenectomized β-thalassemia/HbE patients.**

Conclusion: **Our findings show the alteration of unconventional T-lymphocyte subsets in β-thalassemia/HbE patients, which may be responsible or may reflect the impaired immune response in β-thalssemia disease.** ***(Asian Pac J Allergy Immunol 2014;32:261-9)***

Keywords: *T-lymphocytes, CD4+CD8+, CD4-CD8-, γδ-TCR, NKT, and β-thalassemia*

Introduction

Infection is a common complication associated with significant morbidity and mortality in β-thalassemia disease.[1] Besides the association of blood-borne infections with multiple transfusions, the contribution of co-existent immune abnormalities have also been reasoned to confer an increased susceptibility of these patients to infection. Studies of the immune system in these patients have shown a wide spectrum of immune abnormalities which include increased immunoglobulin production, deficient activity of the complement system, decreased opzonization and granulocyte phagocytosis.[2-5] Evidence showing abnormalities in the cell-mediated immune response (CMI) in thalassemia patients has also been documented.[6] However, it is not quite clear why thalassemia patients are susceptible to infection.

During the past decade, several phenotypically unique T-lymphocyte subsets have been identified along with function(s) unique to such T lymphocyte subsets. Mature CD3+ T-lymphocytes are generally classified as either those that co-express CD4 or CD8 molecules. In addition to these conventional subsets, there is considerable evidence that a readily detectable number of CD4+CD8+ or double positive (DP) and CD4-CD8- or double negative (DN) T-lymphocytes exist in the peripheral blood. Although these DP and DN T-lymphocytes are present at a

From 1. Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

2. Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Nakornpathom, Thailand

Corresponding author: Kovit Pattanapanyasat

E-mail: grkpy@mahidol.ac.th

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low frequency in humans, the relative percentage of these otherwise minor cell populations can be increased spontaneously in healthy individuals and in patients suffering from certain disease conditions.[7] These disease conditions include infection with HIV, human T cell leukemia virus, Epstein–Barr virus, human herpes viruses and there is also an association with aging. In each of these conditions there is evidence for a significant increase in the percentage of DP T-lymphocytes in the peripheral blood.[8-13] Experimental inoculation of mice with either reovirus or recombinant adenovirus has also been shown to lead to an increased population of DP cells.[14] Other examples of these abnormalities include the occasional presence of low numbers of DN T-lymphocytes in otherwise healthy individuals and an increase of DN cells in association with lymphoproliferative disorders, graft-versus-host disease and selected autoimmune diseases.[15-17] An immunoregulatory role of DN T-lymphocytes has also been suggested. Thus, data from some recent studies have shown that these DN T-lymphocytes play a significant role in the initiation of acute cellular rejection or a possible regulatory role in the immunologic changes associated with transplant rejection.[16] While these DP, DN and the SP T cells that express either CD4 or CD8 molecules also express the α and β heterodimeric T cell receptor (TCR), there is another T cell subset that expresses the γ and δ heterodimeric TCR.[18] These T-lymphocytes subsets are classified as αβ and γδ T-lymphocytes, based on TCR expression. In healthy individuals, γδ T-lymphocytes represent an average between 1–5% of the peripheral blood T-lymphocytes. The functions ascribed to these γδ T-lymphocytes include cytotoxicity, lymphokine secretion, and a regulatory effect on the functions of B cells, αβ T-lymphocytes, natural killer cells, and macrophages.[19] These γδ lymphocytes have also been shown to have a protective role in infectious diseases by recognising phosphorylated low molecular weight molecules produced by a number of micro-organisms.[20] Another subset of unconventional T-lymphocytes are the natural killer T (NKT) cells. These cells have the functional capability of both T and NK cells and they co-express T cell receptors (TCRs), as well as markers associated with NK cells such as CD56 and/or CD16.[21] The role of NKT cells in humans is not completely understood. Results from a recent study suggest that the function of NKT cells is primarily associated with priming and regulating the immune responses in cancer, autoimmunity and infectious diseases.[22] Although changes in the frequency of these unconventional T-lymphocyte subsets have been demonstrated in several pathological conditions, to our knowledge no study to date has been conducted on defining the presence and frequencies of such unconventional T-lymphocyte subsets in patients with β-thalassemia.

The present study was therefore conducted in an effort to phenotypically characterize the frequencies of such unconventional T-lymphocyte subsets in the peripheral blood of splenectomized and non-splenectomized β-thalassemia/HbE patients and compare the values with those obtained from the peripheral blood of healthy volunteers using standard flow cytometry.

Methods

Patients and Blood Samples

Blood samples were collected from healthy volunteers and β-thalassemia patients. The group of β-thalassemia patients included splenectomized- and non-splenectomized β-thalassemia/HbE individuals. The diagnosis of thalassemia for all subjects was made by standard hematological techniques and hemoglobin (Hb) analysis. All subjects had normal G6PD levels and no evidence of concurrent infection and none had been hospitalized or received a blood transfusion for at least three months prior to the initiation of the study or during the sampling period. These studies were approved by the Institutional Review Board of Sriraj Hospital, Mahidol University School of Medicine, Bangkok, Thailand. After obtaining informed consent, 3 ml of venous blood was collected in K_3EDTA (Becton Dickinson Biosciences {BDB}; San Jose, CA). All samples were collected at room temperature and processed within 2 hours.

Monoclonal Antibody Reagents

The monoclonal antibodies used in this study were directly conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP) or allophycocyanin (APC). The monoclonal antibodies utilized included CD3-APC, CD4-PE, CD8-FITC, CD45-PerCP, CD16/56-PE and γδ-TCR-PE. Isotype controls utilized included IgG-PE and IgG-FITC. All monoclonal antibodies and isotype controls were purchased from BDB.

Phenotypic Staining of Whole Blood and Flow Cytometric Analysis

Fifty microliters whole blood samples were incubated with 5 µl each of the CD8-FITC, CD4-PE, CD45-PerCP and CD3-APC for 15 min at room temperature in the dark followed by the addition of 450 µl FACS Lysing Solution (BDB) for another 10 minutes. Stained samples were analyzed using the FACSCalibur™ flow cytometer (BDB). The flow cytometric gating strategy is illustrated in Figure 1. Lymphocytes were distinguished on the basis of linear amplification of the forward and side scatter (FSC/SSC) signals and CD45 staining. Lymphocytes were identified as cells that displayed high CD45 staining intensity and low SSC cells (Figure 1a). A narrow gate was used to select small lymphocytes to minimize artifactual DP staining due to the potential formation of doublets of CD4+ and CD8+ single population (SP) T-lymphocytes. The CD3 vs SSC dot plot was used to gate CD3+ T-lymphocytes (Figure 1b). Subsequently, the cells from the previous gate (R1 and R2) were logically selected to analyze CD4+, CD8+ and the other subsets of unconventional T-lymphocytes (Figure 2a). For characterization of γδ and NKT cells, lymphocytes were indentified as cells with high CD45-PerCP staining intensity and low SSC (Figure 3a and 4a). The CD3-APC vs. SSC dot plot was used to gate CD3+ T-lymphocytes (Figure 3b and 4b). Cells from the previous gates (R1 and R2) were analyzed to generate histogram plots of γδ-TCR-PE and CD16+56-PE expressing cells (Figure 3c and 4c). Typically, data obtained on 5,000-10,000 gated CD3+ events were collected using FL-4 (CD3-APC) as a fluorescent trigger. Data were acquired and analyzed using CellQuest™ software (BDB).

Statistical Analysis

Data were collected and graphed using Graphpad Prism™ software. The statistical significance of difference observed between groups was determined using an unpaired two-tailed t-test. The p-values of 0.05 or less were considered as statistically significant.

Results

Distribution of lymphocytes, CD3+, CD4+ and CD8+ SP T-lymphocytes

Hematological findings in healthy donors, non-splenectomized and splenectomized β-thalassemia patients are summarized in Table 1. We initially determined the frequency of total lymphoid cells and the frequency of CD3+ T cells that either expressed CD4 or CD8 (the major T-lymphocyte subsets) in β-thalassemia/HbE patients and healthy volunteers. As shown in Figure 1a, the frequency of lymphocytes as a percent of total white blood cells in the peripheral blood from splenectomized β-thalassemia/HbE patients was significantly higher than in that from normal volunteers and non-splenectomized β-thalassemia/HbE patients. However, the frequency of lymphocytes in the WBCs from non-splenectomized patients was similar to that in controls. In contrast, the frequency of CD3+T cells or the CD4+ or CD8+ T cells in the peripheral blood from both splenectomized and non-splenectomized β-thalassemia/HbE patients was not significantly different from the values obtained from the healthy volunteers (Figure 1b, 2b and 2c). Means sd of the data are also summarized in Table 2.

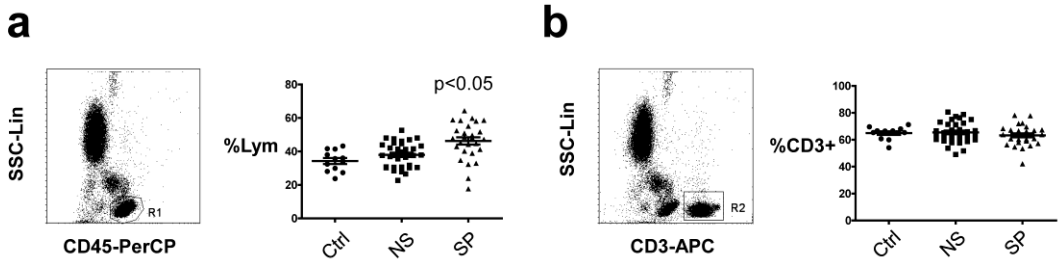

Figure 1. Frequency distribution of lymphocytes and CD3+ T-lymphocytes. (a) Representative dot plots showing total lymphocytes in R1 and their frequency respectively. (b) Representative dot plots showing CD3+ T-lymphocytes in R2 and their frequency respectively among splenectomized (closed triangle, n=27), non-splenectomized (closed square, n=33) β-thalassemia/HbE patients and in healthy controls (closed circle, n=12). The p-values between groups are indicated.

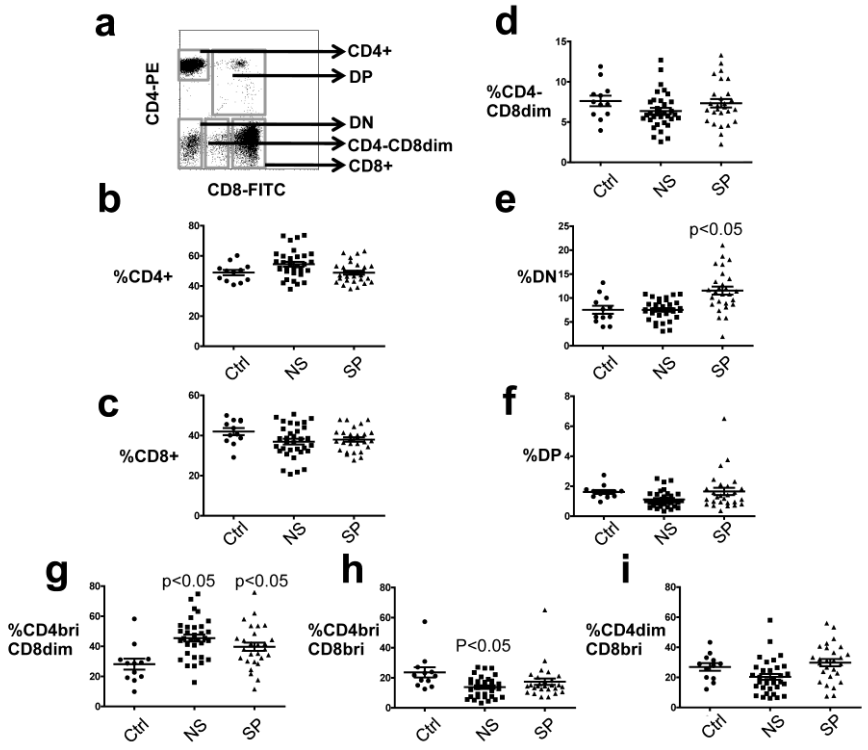

Figure 2. Frequency distribution of CD4/CD8 T-lymphocytes. (a) Representative dot plot showing T-lymphocyte sup-populations, based on the previous gate R1 and R2. Cumulative data showing frequency of CD4+ (b), CD8+ (c), CD4-CD8dim (d), DN (e), DP (f), CD4briCD8dim (g), CD4briCD8bri (h) and CD4dimCD8bri (i) among splenectomized (closed triangle, n=27), non-splenectomized (closed square, n=33) β-thalassemia/HbE patients and in healthy controls (closed circle, n=12). The p-values between groups are indicated.

Phenotypic characterization of unconventional T-lymphocytes and their frequency distribution

To characterize the frequency of the unconventional subset of T-lymphocytes, we first utilized the relative densities of CD4 and CD8 expression and identified 3 different cell populations in β-thalassemia/HbE patients and healthy volunteers.

As seen in Figure 2a, these three different populations are apparent. These include the CD4-CD8dim, DN and DP cell populations. In efforts to determine whether the DP cells reflected the existence of blast like immature cells, the FSC and SSC profiles of the DP cells were examined. These analyses revealed in fact that the DP cells had similar FSC and SSC characteristics when compared to the CD4 or CD8 single positive cells, suggesting that these cells were unlikely to be immature cells. It is important to note that the CD4 and CD8 staining on the DP T-lymphocytes was not a result of non-specific binding, since alternative fluorescent combinations of reagents specific for CD4 and CD8 utilized yielded similar results for DP T-lymphocyte expression. In addition, it is reasoned that the DP cells identified in the studies presented herein were not present due to a coincident artifact because the frequency of these DP CD4+CD8+ T-lymphocytes remained constant even though the flow rate had been changed. Moreover, the intensity of CD4 expression on the DP cells was similar to the CD4+ SP cells (MFI = 1024 and 1098 respectively) but the intensity of CD8+ expression on the DP cells was lower than the CD8+ SP cells (MFI = 54 and 103, respectively). Taken together, these data suggest the potential presence of unconventional T-lymphocytes as defined by the level of CD4 and CD8 expression in the peripheral blood.

Results obtained based on these criteria for defining these 3 cell populations (Figure 2a) showed that the frequencies of CD4-CD8dim in both splenectomized and non-splenectomized β-thalassemia/HbE patients were not significantly

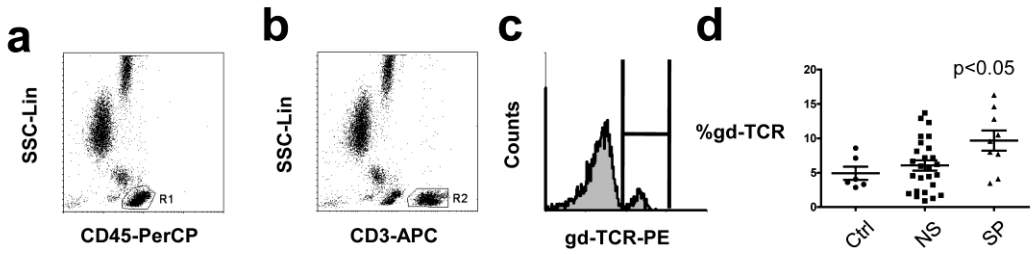

Figure 3. Frequency distribution of γδ-TCR. (a), (b) and (c) showing the flow cytometry gating strategy for γδ-TCR T-lymphocytes. (d) showing the frequency of γδ-TCR as a percentage of circulating CD3+ T-lymphocytes among splenectomized (closed square, n=9), non-splenectomized (closed triangle, n=26) β-thalassemia/HbE patients and in healthy control (closed circle, n=6). The p-values between groups are indicated.

different from those in the control group (Figure 2d). Interestingly, a significant increase in the frequency of DN CD4-CD8- T-lymphocytes was found in splenectomized β-thalassemia/HbE patients (Figure 2e). While initial analysis of these data showed no significant differences in the frequency of DP CD4+CD8+ T-lymphocytes among the 3 studied groups (Figure 2f), when the data were examined in more detail interesting differences emerged. When the expression of CD4 and CD8 molecules by cells within the DP T-lymphocyte population were utilized, the analysis revealed the existence of cells expressing CD4brightCD8dim, CD4dimCD8bright and CD4brightCD8bright. The use of these criteria revealed that there was a marked increase in the frequency of the CD4brightCD8dim T-lymphocyte subset in the peripheral blood of β-thalassemia/HbE patients, with the highest frequency noted for samples from the non-splenectomized β-thalassemia/HbE patients (Figure 2g). By contrast, the frequency of CD4brightCD8bright T-lymphocyte subset populations was significantly decreased in the peripheral blood of both non-splenectomized and splenectomized β-thalassemia/HbE patients, when compared with the healthy volunteers (Figure 2h). No statistically significant difference of the CD4dimCD8bright subset was found among the 3 studied groups (Figure 2i).

Frequency of γδ-TCR and NKT cells

Previous investigations have shown that the frequency of DN T-lymphocytes is directly proportional to the frequency of γδ-TCR T-lymphocytes,[23] suggesting that most γδ-TCR + T-lymphocytes are negative for CD4 and CD8. We thus examined the frequency of γδ-TCR T-lymphocytes. As shown in Figure 3d, while there was a significant increase in the frequency of γδ-TCR T-lymphocytes in the peripheral blood specimens from the splenectomized β-thalassemia/HbE patients, there was no significant difference in the frequency of γδ-TCR T-lymphocytes in non-splenectomized β-thalassemia/HbE patients.

Studies on the frequency of NKT-lymphocytes have shown that the peripheral blood from the splenectomized β-thalassemia/HbE patients contains a markedly higher frequency of NK-T cells (calculated as the percentage of total CD3+ T-lymphocytes), as compared to controls (Figure 4d). While a slight increase in the frequency of NKT-lymphocytes was observed in non-splenectomized patients as compared with controls, this increase was not statistically significant. Taken together, our data suggest that there are statistically significant alterations in the frequencies of both γδ-TCR and NKT-lymphocyte population, especially in the splenectomized β-thalassemia/HbE patients.

Discussion

Susceptibility to infectious diseases is a common complication in patients with β-thalassemia and this has been thought to be partly due to immunological abnormalites. However, conclusive data to document such abnormalities have been lacking. Recently, a number of studies have documented the appearance of high frequencies of "unconventional" lymphocytes in the peripheral blood of patients with a variety of pathological conditions.[13,16, 21, 24, 25] However, to date, studies of the presence of such subsets of unconventional lymphocytes in the peripheral blood from patients with β-thalasssemia have not been reported. The results of the studies reported herein for the first time document the presence of significant increases in the distribution of unconventional lymphocytes in

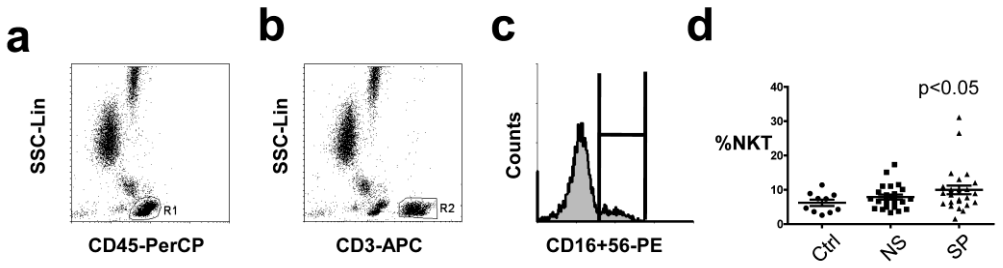

Figure 4. Frequency distribution of NKT-lymphocytes. (a), (b) and (c) showing the flow cytometry gating strategy for NKT cells. (d) showing the percentage of NKT cells as circulating CD3+ T-lymphocytes in splenectomized (closed square, n=26), non-splenectomized β-thalassemia/HbE patients (closed triangle, n=24) and controls (closed circle, n=10). The p-values between groups are indicated.

the peripheral blood from β-thalassemia patients. Additional differences noted included an increased frequency of the absolute number of lymphocytes in splenectomized β-thalassemia/HbE patients. The possible explanation for lymphocytosis could be that the spleen, as a secondary lymphoid organ, serves as a large reservoir of lymphoid cells and when the organ is removed, lymphocytes which would normally target to the spleen accumulate in the circulating pool of peripheral blood cells. This observation is in agreement with those previously reported in children and adults who have been shown to be at an increased risk of infection following splenectomy.[26] A similar finding has also been observed in experimental animals.[27] Moreover, we have confirmed the findings from previous studies that have reported no significant differences in the frequency of conventional CD3+, CD4+ and CD8+ T-lymphocytes in β-thalassemia/HbE patients, as compared with results from healthy individuals.[28] Taken together, these results suggest that, although the relative absolute number of lymphocytes is increased in splenectomized β-thalassemia/HbE patients when compared to healthy volunteers and non-splenectomized β-thalassemia/HbE patients, these differences did not result in increases in the number of conventional T-lymphocyte subpopulations.

A small population of DP T-lymphocytes has been reported in the peripheral blood of healthy donors and in patients with various viral infections.[13] The results of the studies reported herein show that, whereas the level of CD4+ in DP T-lymphocytes was comparable to CD4+ single positive T-lymphocytes, the level of CD8 expressed differently on DP T-lymphocytes. Varying levels of CD8 expression in the DP T-lymphocyte have been demonstrated in humans, swine and rats,[24] while on CD8+ single positive T cells it was homogenous suggesting that a peripheral blood DP T cells exists in vivo and is phenotypically distinct from that of CD4 and CD8 SP T-lymphocytes. Given the similar frequency of DP T-lymphocytes as in the controls, such observation could be due to the transient nature of these cells when responding to the site of infection during the course of infection.[29]

Table 1. Hematological parameters in healthy donors, and non-splenectomized and splenectomized β-thalassemia patients.

	Healthy donors	Non-splenectomize β-thalassemia	Splenectomized β-thalassemia
Hb (g/dl)	14±1.4	6.4±1.9	6.3±1.6
RBCs ($\times 10^6$/μl)	4.8±0.58	3.5±1.2	3.2±0.9
HCT(%)	42.5±4	20.5±5.4	22.4±5.3
MCV (fl)	87.3±4.8	59.7±8.2	72.5±8.9
MCH(pg)	29.1±1.5	18.6±2.6	20.1±2.4
MCHC(g/dl)	33.2±0.8	30.9±1.8	27.8±2.4
Platelet (/μl)	270,055.6±44,487.2	334,053.6±173,057	626,390±148,731.5
WBC (/μl)	6,055.6±1,067.3	15,858.3±39,696	58,266.4±42,277.1

Table 2. Frequency of lymphocyte sub-populations in healthy controls, and non-splenectomized and splenectomized β-thalassemia patients.

	Healthy donors	Non-splenectomize β-thalassemia	Splenectomized β-thalassemia
Lymphocyte	34.24±6.22	37.91±7.53	46.23 11.19
CD3+	64.88±4.67	65.42±7.28	63.22±7.77
CD4+	48.91±6.06	54.44±9.29	48.81±7.13
CD8+	41.96±6.13	36.93±8.13	37.98±5.80
CD4-CD8dim	7.62±2.16	6.36±2.25	7.37±2.71
CD4-CD8-	7.53±2.90	7.51±2.13	11.53±4.34
CD4+CD8+	1.62±0.45	1.12±0.55	1.65±1.26
γδ-TCR T-lymphocyte	4.88±2.48	6.06±3.79	9.68±4.40
NKT lymphocyte	4.47±0.27	9.26±2.86	13.9±8.99

When the subpopulation of the DP T-lymphocytes were further characterized, based on the relative density of CD4 and CD8 expression into CD4dimCD8bright, CD4brightCD8bright and CD4brightCD8dim, the results showed that the proportion of CD4brightCD8dim in β-thalassemia/HbE patients was significantly increased compared to that in healthy controls. These CD4brightCD8dim T-lymphocytes typically express CD8αα homodimers and are believed to be of extrathymic origin. The transient and persistent expansion of this subset has been observed in both healthy individuals and those with a wide range of unrelated diseases, including viral infections, various autoimmune diseases and T and B cell leukemias and lymphomas.[7] Further studies are needed to better understand the immunological role and physiological significance of such CD4brightCD8dim T-lymphocytes in β-thalassemia disease. Interestingly, a significant increase in the frequency of DN T-lymphocyte subset was also found in splenectomized β-thalassemia/HbE patients. Previous investigations of DN T-lymphocytes in patients with systemic lupus erythematosus (SLE) have found that the DN T-lymphocyte population is increased in association with the production of the IL-4 and IFN-γ.[15] The fact that the DN T-cells synthesize a significant amount of IL-4 and IL-10 upon in vitro activation and the finding that human DN NKT cells expressing invariant TCR α chains show increased production of IL-4 and IFN-γ[15] suggests that the DN T-lymphocyte are likely involved in the down-regulation of the immune response. Thus, it is possible that DN T-lymphocytes could be important in the suppression of immune responses in these β-thalassemia patients.

The increased frequency of γδ-TCR T-lymphocytes in splenectomized β-thalassemia/HbE patients reported herein is of interest. The requirement for γδ-TCR T-lymphocytes in the response to infection has been demonstrated in a number of investigations. A study using TCR-γδ-deficient mice showed both increased susceptibility and exaggerated inflammatory responses during infection with viruses, bacteria and parasites.[30-32] In addition, a recent investigation has suggested that γδ-TCR T-lymphocytes are required to down-modulate the inflammatory response and eliminate activated macrophages.[33] To our knowledge, the dual roles of these γδ-TCR T-lymphocytes have not been yet demonstrated in β-thalassemia patient. Several lines of experimental data have demonstrated that cellular subsets such as host macrophage, donor natural killer cells and donor NKT cells are involved in the pathogenesis of graft-versus-host disease (GVHD).[34] Recently, the results of a study using several mouse models suggested that the host γδ-TCR T cells exacerbate GVHD by enhancing the allo-stimulatory capacity of host antigen-presenting cells.[35] Hence, the elimination of host γδ-TCR T cells might be a novel therapeutic strategy to reduce GVHD in β-thalassemia patients, since allogeneic bone marrow transplantation is the therapeutic treatment of choice. In addition, the monitoring of the frequency of γδ-TCR T-lymphocyte subsets may be of interest in patients undergoing immunosuppressive therapy, which may have additional value for the diagnosis of graft rejection.

It has been known for some time that both endothelial injury and inflammation have an important role in the pathogenesis of thalassemia, as evidenced by the elevation of pro-inflammatory cytokines such as IL-6 and the inflammatory markers such as C reactive protein (CRP) in thalassemic patients.[36] Recently, a study of the role of NKT cell in ischemia-perfusion injury in sickle cell disease (SCD) has suggested that NKT cells play an important role in sustaining inflammation by the production of IFN-γ and the chemotactic CXCR3 chemokine.[37] The findings of a significant increase in the frequency of NKT cells in the peripheral blood of splenectomized β-thalassemia/HbE patients reported herein suggests that these NKT cells may be an extrinsic factor for thrombotic risk caused by vascular injury, particularly in high-risk patients such as β-thalassemia patients.

In conclusion, our study has shown for the first time the presence of increased frequencies of "unconventional" lymphocyte subsets. Studies of the potential mechanisms that lead to such an increase and the potential pathogenic role such cells play in patients with β-thalassemia/HbE may provide important clues to our understanding of the pathogenesis of this disease.

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