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The prevalence and function of $CD4^+CXCR5^+Foxp3^+$ follicular regulatory T cells in diffuse large B cell lymphoma

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ABSTRACT

$CD4^+CXCR5^+Foxp3^+$ follicular regulatory T (Tfr) cells possess critical roles in suppressing the germinal center reaction, B cell activation, and follicular helper T cell (Tfh) cytokine secretion. Since diffuse large B cell lymphoma (DLBCL) can arise from B cells undergoing germinal center reaction and/or differentiation, we hypothesized that Tfr cells might be involved in DLBCL. In the present study, we recruited thirty-five DLBCL patients and twenty-five healthy controls. Data showed that DLBCL patients presented an enrichment of circulating $CD4^+CXCR5^+Foxp3^+$ Tfr cells compared to controls. In the primary tumor isolated from enlarged lymph nodes, Tfr cells made up of roughly 3% to 16% of infiltrating T cells. Higher levels of tumor-infiltrating Tfr cells were observed in patients with less advanced DLBCL stages, and in patients that stayed in remission 24 months after the initial R-CHOP treatment. High BCL6 and high FOXP3 expression was observed in Tfr cells *ex vivo*. After anti-CD3/CD28 and IL-2 stimulation, the Tfr cells more closely resembled Treg cells and presented high IL10 and TGFB1 expression. $CD4^+CD25^+CXCR5^+$ Tfr cells and $CD4^+CD25^+CXCR5^-$ non-Tfr Treg cells could suppress $CD4^+CD25^-$ Tconv cell and $CD8^+$ T cell proliferation with similar capacity. However, Tfr cells were less capable of suppressing IFNG expression than Treg cells, and although both cell types supported $CD19^+$ tumor cell proliferation, Tfr cells were less supportive than the non-Tfr Treg cells. Overall, this study suggested that Tfr cells were involved in intratumoral immunity, were likely beneficial to DLBCL patients, and were functionally distinctive from non-Tfr Treg cells. The distribution pattern and the prognostic value of Tfr cells in DLBCL should be examined in further studies.

1. Introduction

Follicular helper T (Tfh) cells provide critical stimulatory cytokines and co-stimulatory molecules to assist B cell affinity maturation, antibody class switch, and plasmablast differentiation. In both the germinal center and the peripheral blood, $CD4^+CXCR5^+$ Tfh cells represent the main B-helping subset of $CD4^+$ T helper (Th) cells [1]. Recently, a subset of Tfh cells with $CD4^+CXCR5^+Foxp3^+$ expression is discovered in the germinal center [2,3]. Due to the shared expression of Foxp3 with the canonical Treg cells, this $CD4^+CXCR5^+Foxp3^+$ subset is termed follicular regulatory T (Tfr) cells. Like the canonical Tfh cells, Tfr cells highly express CXCR5, PD-1, and ICOS, and express Bcl6 at a level lower than canonical Tfh cells but higher than other Th subsets [4]. But unlike Tfh cells, Tfr cells suppress the germinal center reaction by suppressing both B cells and Tfh cells [4,5]. Tfr cells were shown to suppress B cell activation and antibody production, and possibly inhibited plasma cells migration from lymph nodes to bone marrow [2,6–8]. The human Tfr cells ($CD4^+CXCR5^+CD25^+CD127^-$) significantly suppressed antibody production *in vitro* [9]. In co-culture with Tfh cells together with B cells or dendritic cells (DCs), Tfr cells suppress the production of multiple cytokines by Tfh cells, including IFN-γ, IL-4, IL-10, IL-21, and TNF-α, and inhibit Tfh cell cycling [6]. CTLA-4, an inhibitory molecule expressed by both canonical Treg cells and Tfr cells and with high binding affinity to CD80 and CD86, is critical to Tfr cell-mediated inhibition of B cells and Tfh cells [10,11]. Alterations in the Tfh cell frequency and function may affect the course of many diseases. It was shown that Tfr cells were expanded in human chronic HIV, HBV, and HCV infections, and might present a role in suppressing the formation of broadly neutralizing antibodies in HIV infection [6,12–14]. The potential role of Tfr cells in other diseases should be further examined.

Diffuse large B cell lymphoma (DLBCL) is the most common type of

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Table 1
Demographic and clinical characteristics of study participants.

Characteristics	Healthy (N = 25)	DLBCL		
		Stage I (N = 10)	Stage II (N = 10)	Stage III (N = 15)
Age (years)	52 (40–63)	49 (39–55)	53 (35–61)	51 (43–62)
Sex (F/M)	9/16	4/6	3/7	5/10
B symptoms (N, %)		1, 10	2, 20	4, 27
Extranodal involvement (N, %)		0, 0	2, 20	13, 87

non-Hodgkin's lymphoma and an aggressive and rapidly progressing disease. With the recent implementation of anti-CD20 rituximab and combinatorial chemotherapies, most DLBCL patients can now be cured [15]. However, approximately one-third of the DLBCL patients demonstrate poor response to current therapies and have a recurrent or refractory disease [16]. Since further treatment options are limited, these patients eventually succumb to DLBCL and related complications. The oncogenic pathways of DLBCL are still not completely understood. Interestingly, the Tfh cell presents altered prevalence and cytokine expression in DLBCL patients [17]. Furthermore, IL-10-expressing Tfh cells are shown to promote the survival of $CD19^+$ tumor cells [18]. It remains unclear whether the Tfr cells are involved in the oncogenesis and the clinical progression of DLBCL.

With the goal of characterizing Tfr cells in DLBCL patients, we examined the frequency of $CD4^+CXCR5^+Foxp3^+$ T cells in adult DLBCL patients and healthy controls. In the peripheral blood, the frequency of $CD4^+CXCR5^+Foxp3^+$ T cells was significantly higher in patients. In the tumors, the frequency of $CD4^+CXCR5^+Foxp3^+$ T cells was associated with cancer stage. Moreover, patients that presented high intratumoral $CD4^+CXCR5^+Foxp3^+$ T cell frequencies had lower risk of poor clinical response to current therapy. Both the $CD4^+CXCR5^+CD25^+$ Tfr cells and $CD4^+CXCR5^-CD25^+$ Treg cells could suppress the proliferation and cytokine expression by $CD4^+CD25^-$ Tconv cells and $CD8^+$ T cells, but the Tfr cells presented lower capacity in suppressing IFNG expression. The Tfr cells were also less supportive of $CD19^+$ tumor cell proliferation than Treg cells.

2. Methods

2.1. Study participants

The collection and use of human samples were approved by Changhai Hospital Institutional Ethics Board. Diagnosis of primary DLBCL was confirmed by surgical removal of the bulking lymph node and examination of the tumor biopsy under a microscope. Staging was performed according to the Ann Arbor Classification System [19]. Ten stage I, ten stage II and fifteen stage III subjects were included in this study. All thirty-five DLBCL patients and twenty-five healthy controls were adults between 18 and 65 years of age. In addition to not presenting DLBCL, the healthy controls were also free from chronic virus infections, acute infections, autoimmune diseases and other forms of malignancy. All participants provided written informed consent. The baseline characteristics of the patients and controls are presented in the following Table 1.

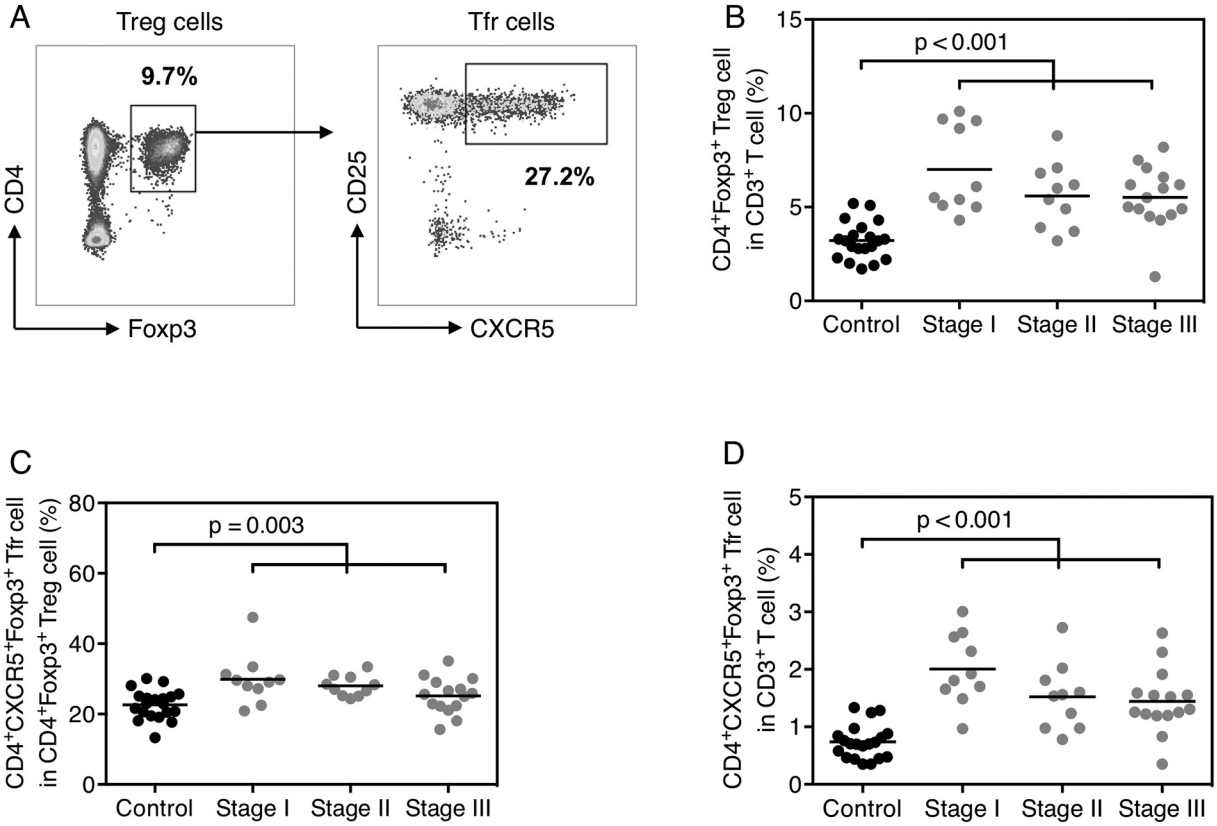

Fig. 1. Frequency of circulating Tfr cells in DLBCL patients and healthy controls. (A) The Tfr cells are identified as $CXCR5^+$ cells in $CD4^+Foxp3^+$ Treg cells. Plots were from one representative DLBCL patient. Cells shown were pre-gated on live $CD3^+$ T cells. (B) The frequency of $CD4^+Foxp3^+$ Treg cells in $CD3^+$ T cells. (C) The frequency of $CD4^+CXCR5^+Foxp3^+$ Tfr cells in $CD4^+Foxp3^+$ Treg cells. (B) The frequency of $CD4^+CXCR5^+Foxp3^+$ Tfr cells in $CD3^+$ T cells. Differences between healthy controls (black) and DLBCL patients (grey) were examined by Mann-Whitney test. Differences among various DLBCL stages were examined by Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test.