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Differences in frequency and regulation of T follicular helper cells between newly diagnosed and chronic pediatric immune thrombocytopenia

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ABSTRACT

Objective: This study aims to investigate the role of T follicular helper (TFH) cells in the immunopathogenesis of pediatric immune thrombocytopenia (ITP), as well as differences in TFH expansion and its regulation between newly diagnosed ITP (nITP) and chronic pediatric ITP (cITP).
Methods: Eighty-five children with ITP and 20 age-matched healthy controls were enrolled into this study. TFH cell frequencies and TFH cell-associated regulatory factors before and after treatment were analyzed by flow cytometry, RT-PCR and ELISA.
Results: The percentages of TFH cells were significantly elevated in both nITP and cITP compared with controls. RT-PCR revealed significant differences in Bcl-6, c-Maf, Blimp-1, ICOSL, TACI and BAFFR mRNA expression in $CD4^+$ T or $CD19^+$ B cells between patients and controls, and further between nITP and cITP, before and after treatment. Moreover, there were significant differences in serum IL-4, IL-21 and BAFF between patients and controls.
Conclusion: The overactivation of TFH cells may contribute to the immunopathogenesis of pediatric ITP. IL-21 and IL-4 serum levels may affect the differentiation of TFH cells in ITP patients. The aberrant balance between BAFFR-BAFF/TACI-BAFF may be a factor that caused the persistent high expression of ICOSL in pediatric cITP, which consequently lead to the over activation of TFH cells in pediatric cITP.

1. Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder associated with increased platelet destruction and impaired platelet production, and both of which are mediated by antiplatelet autoantibodies [1–3]. Autoantibodies produced by autoreactive B-cells against self-antigens, specifically immunoglobulin G (IgG) antibodies against glycoprotein IIb (GPIIb)/IIIa and/or GPIb/IX, have been considered to play a crucial role in ITP [4]. In addition to humoral immunity, multi-dysfunctional features of cellular immunity contributes to the pathogenesis of ITP including abnormalities in T-cells (T helper 1, Th1), the defective suppressive function of regulatory T-cells, and platelet destruction by cytotoxic T-cells (CTLs), B-cells, co-stimulating factors and altered cell communication [5–11].

There is a $CD4^+$ T cell subset in germinal centers (GC) called follicular T helper (TFH) cells, which are defined by the expression of chemokine (C-X-C motif) receptor 5 (CXCR5), inducible costimulator (ICOS) and programmed cell death 1 (PD-1), as well as the high expression level of B cell lymphoma 6 (Bcl-6). TFH cells are capable of supporting Ig class switching, and are involved in the initiation and maintenance of responses that generate memory B cells and long-lived plasma cells during immune responses [12,13]. Recent studies have shown that TFH cells have a role in the development of pathologies in autoimmunity such as systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [14–16]. Several studies have also shown that splenic (or bone marrow) TFH frequencies in adult patients with ITP are higher, and might participate in B cell differentiation and antiplatelet antibody production. However, this immune mechanism which leads to the aberrant expansion of TFH cells remains to be explored. In this study, we further demonstrated that the frequencies of TFH cells significantly increased in peripheral blood of pediatric patients with ITP. Through the analysis of the alteration of TFH cell ratio and TFH cell-associated regulatory factors before and after treatment in newly diagnosed ITP (nITP) and chronic ITP (cITP) children, our results suggest that the overexpression of ICOSL on B cell that resulted in excessively increased levels of BAFF may be a major driver for the persistent expansion of TFH cells in cITP.

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2. Materials and methods

2.1. Patients

A total of 85 patients diagnosed with ITP in Shenzhen Children's Hospital (China) between June 2012 and June 2013 were recruited. Patients were classified into two groups: newly diagnosed ITP (nITP, $n = 59$) group, defined as patients with ITP within three months from diagnosis; chronic ITP (cITP, $n = 26$) group, defined as patients with ITP that lasted for >12 months (Table 1B). Diagnosis and treatment (intravenous immunoglobulin, corticosteroids, or both) were carried out according to clinical guidelines [17,18] and the updated American Society of Hematology evidence-based practice guideline for immune thrombocytopenia [19] in 2011. A total of 20 healthy controls (control group) were enrolled (Table 1C). Informed consent was obtained from parents and guardians of the children. This study was approved by the Local Hospital Medical Ethics Committee. Blood samples were initially collected when patients were diagnosed with ITP. Blood samples were collected again after platelet counts recovered to normal levels or platelet counts did not rise to normal level, but bleeding symptoms disappeared after the primary treatment. There was no significant difference in the distribution of age and gender between the control and ITP groups, as shown in Table 1A.

2.2. Blood samples

Venous blood (5 mL) was obtained from patients with ITP and healthy children using ethylene diamine tetraacetic acid (EDTA) Na_2 as an anti-coagulant. Blood samples were immediately analyzed without the stimulation of mitogens or culture *in vitro* unless specifically indicated. Whole blood (2 mL) was prepared for flow cytometric analysis. $CD4^+$ T cells or $CD19^+$ B cells were immediately isolated from peripheral blood by microbead (Dynabeads® CD4 11145D, Invitrogen Dynal AS, Oslo, Norway; Dynabeads® CD19 pan B 11143D, Invitrogen Dynal AS, Oslo, Norway), according to the manufacturer's instructions. Plasma was obtained after centrifugation and stored at −80 °C for detection of enzyme-linked immunosorbent assay (ELISA). As assayed by 0.05% trypan blue staining, purified cells were identified to have >97% purity by flow cytometry, while viability was >95%.

2.3. Flow cytometry

Antibodies CD4-eFluor-450 and CD19-APC-eFluor-780 were obtained from Beckman Coulter, Inc. (Miami, FL, USA). ICOS-PE, CXCR5-Alexa Fluor-647, ICOSL-PE and mouse IgG1-PE were purchased from eBioscience (San Diego, CA, USA). Whole blood (100 μL) was incubated with relevant antibodies for 30 min at 4 °C. After incubation, red blood cells were lysed using red blood cells lysis buffer, and the remaining white blood cells were washed twice with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and 0.1% NaN_3. Immediately afterwards, the expression of cell surface markers was analyzed by flow cytometric analysis using an Epics-XL4 flow cytometer equipped with EXPO32 ADC software (Beckman Coulter, San Diego, CA, USA). Data are presented as proportions of cells expressing antigen (%).

Table 1A
Characteristics of patients with ITP($\bar{x} \pm$ SD).

	Controls	ITP	nITP	cITP
n	20	85	59	26
Gender (M/F)	11/9	48/37	34/25	14/12
Age (month)	31.3 ± 14.1	28.8 ± 19.0	26.53 ± 18.1	35.56 ± 30.2
Platelet count ($\times 10^9$/L)	217.7 ± 25.9	18.7 ± 13.5	17.4 ± 13.8	21.7 ± 12.6
GPIIb/IIIa (OD value)	0.27 ± 0.10	0.64 ± 0.69	0.68 ± 0.71	0.55 ± 0.65
GPIb/IX (OD value)	0.33 ± 0.12	0.57 ± 0.69	0.58 ± 0.75	0.55 ± 0.53
Bleeding symptoms	0/20	76/85	57/59	19/26

Table 1B
Characteristics of patients with cITP.

Number	Gender	Age (month)	Treatment	Platelet count before (after) treatment (10^9/L)	Relapse times
1#	Male	30	IVIg	2/104	2
2#	Female	64	Corticosteroids	48/145	2
3#	Male	24	Corticosteroids	36/100	1
4#	Male	36	Corticosteroids + IVIg	3/188	2
5#	Female	12	Corticosteroids + IVIg	15/190	1
6#	Female	48	Corticosteroids	9/110	2
7#	Male	54	Corticosteroids	5/151	2
8#	Female	22	IVIg	12/162	1
9#	Female	110	Corticosteroids	22/124	3
10#	Male	36	Corticosteroids	43/219	1
11#	Female	78	Corticosteroids	38/106	3
12#	Male	14	Corticosteroids + IVIg	23/295	1
13#	Male	17	Corticosteroids	6/225	1
14#	Male	24	IVIg	26/105	1
15#	Female	48	Corticosteroids + IVIg	12/137	2
16#	Female	102	Corticosteroids	23/98	4
17#	Male	19	IVIg	26/105	1
18#	Male	20	IVIg	37/100	1
19#	Male	15	IVIg	20/169	1
20#	Female	28	Corticosteroids + IVIg	28/266	2
21#	Male	12	IVIg	30/121	1
22#	Female	31	Corticosteroids	28/174	2
23#	Female	41	Corticosteroids	28/153	1
24#	Male	20	Corticosteroids + IVIg	19/138	1
25#	Male	24	Corticosteroids	7/146	2
26#	Female	18	Corticosteroids + IVIg	17/263	1

2.4. Total RNA extraction and cDNA synthesis

Total RNA from $CD4^+$ T cells or $CD19^+$ B cells was prepared using a Versagene RNA Kit (Gentra, 0050C; USA), according to manufacturer's instruction. DNase I (0050D, Gentra) was used to eliminate the trace DNA during extraction. Isolated total RNA integrity was verified by average optical density (OD) with OD_{260}/OD_{286} absorption to cDNA with an oligodeoxythymidylic acid (oligo-dT) primer using RevertAid™ H

Table 1C
Characteristics of healthy controls.

Number	Gender	Age (month)	Platelet count (10^9/L)
1#	Female	21	204
2#	Male	40	187
3#	Male	12	259
4#	Male	30	237
5#	Female	48	214
6#	Male	20	199
7#	Male	24	216
8#	Male	24	176
9#	Male	61	181
10#	Female	30	254
11#	Male	12	221
12#	Female	35	218
13#	Female	29	227
14#	Female	56	229
15#	Male	18	169
16#	Female	33	220
17#	Female	24	245
18#	Male	37	212
19#	Female	52	250
20#	Male	20	236