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CCR6 defines a subset of activated memory T cells of Th17 potential in immune thrombocytopenia

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Summary

Current researches have determined the significance of C-C chemokine receptor (CCR)6 expression as either a marker of T helper cells (Th) or an effector and regulator of T cell function. However, the roles of CCR6 in the pathogenesis of immune thrombocytopenia (ITP) are unclear. In this study, we aimed to investigate the phenotype and functional characteristics of circulating CCR6$^+$ T cells in blood from chronic ITP patients and healthy controls. We found that the frequency of CCR6$^+$CD4$^+$ cells was higher in ITP patients than in healthy controls. Anti-CD3/anti-CD28 stimulation induced rapid expansion of CCR6$^+$CD4$^+$ cells in ITP patients. CCR6$^+$CD4$^+$ cells had a phenotype of activated cells and predominantly expressed CD45RO. Forkhead box protein P3 (FoxP3) and CD25-positive cells were exclusively detected within the CCR6$^+$CD4$^+$ cells. In ITP patients, CCR6$^+$ regulatory T cells (T_{reg}) were decreased and positively correlated with platelet counts and transforming growth factor (TGF)-β plasma levels. In contrast to CCR6$^-$ counterparts, CCR6$^+$CD4$^+$ cells produced higher levels of interleukin (IL)-17A. The frequency of CCR6$^+$ Th17 was higher in ITP patients and positively correlated with IL-17A levels in supernatant. Most importantly, CCR6$^+$CD4$^+$ cell subpopulations, but not CCR6$^-$CD4$^+$, were closely correlated to treatment response of ITP patients. These findings suggest that circulating CCR6$^+$CD4$^+$ cells in ITP patients have characteristics of activated memory Th17 phenotype and could be used to monitor disease activity and treatment response.

Keywords: CCR6, immune thrombocytopenia, memory, regulatory T cell, T helper cell

Introduction

Immune thrombocytopenia (ITP) is currently defined as an acquired autoimmune disorder characterized by a low peripheral blood platelet count [1]. The underlying abnormalities involved in the pathogenesis of ITP appear to be complex. In addition to the classical mechanism of glycoprotein (GP)-specific autoantibody-mediated platelet destruction, cellular dysfunction has been implicated in ITP pathogenesis, such as antigen-presenting cell (APC) defects, T cell-mediated immunity disorders and B cell activation [1–3].

Abnormal T cells have been described in patients with ITP, including a higher T helper cell (Th) reactivity against platelets [4–6] and decreased number and function of regulatory T cells (T_{reg}) [7–9]. Th cells are central organizers in immune response [4] and can be distinguished by differential expression of chemokine receptors that facilitate inflammation response [10–13]. For instance, Th1 cells express C-X-C chemokine receptor (CXCR)3 and C-C chemokine receptor (CCR)5 [10,11], whereas Th17 cells express CCR4 and CCR6 [12,13]. Chemokine receptors are dynamically modulated during the process of naive CD4$^+$ T cell activation and differentiation [14]. Meanwhile, these chemokine receptors are thought to function in positive feedback loops to amplify Th1 and Th17-like inflammatory responses [15,16]. Human T_{regs} characterized as CD4$^+$CD25$^+$forkhead box protein 3 (Foxp3$^+$) cells play an important role in maintaining

immune tolerance by suppressing both cell-mediated and antibody-mediated immune responses [17]. T_{regs} are reported to express chemokine receptors such as chemokine receptor 6 (CCR6), CCR4, C-X-C motif chemokine receptor 3 (CXCR3) and CCR10 [18]. Heterogeneous subsets of FoxP3$^+$ T_{reg} cells identified by chemokine receptors are functionally suppressive and respond differently to Th1 and Th17 responses [18]. Constitutive expression of CCR4 and CCR8 on the majority of circulating T_{reg} cells may control the dynamics of other effector T cells for interacting with APCs [19]. Because the expression of chemokine receptors is closely linked to the function of the cells, identifying chemokine receptors by immune cells is important in determining favorable immune outcomes in both disease and therapeutic strategies.

CCR6 is a seven transmembrane G-protein-coupled receptor (GPCR) that is found predominantly on T cells, B cells and monocyte-derived dendritic cells (DCs) [20]. C-C motif chemokine ligand 20 (CCL20) is identified to be the sole ligand for CCR6 on peripheral blood T cells [20]. CCR6 is present on a substantial fraction of regulatory CD25$^+$CD4$^+$ T cells [21,22]. Furthermore, CCR6$^+$ T_{reg} cells exhibit the phenotype of activated effector-memory cells and suppress immune responsiveness [21,22]. CCR6$^+$ T_{reg} cells have been identified in some diseases, including recurrent miscarriage and RA, and might play a role in disease development [23,24]. The CCR6$^+$ Th cell population is heterogeneous, and several subpopulations can be distinguished, including interleukin (IL)-17, IL-22 and interferon gamma (IFN)-γ-secreting cells [25]. CCR6$^+$ Th cells and over-production of their signature cytokines are associated with persistent inflammation and autoimmune diseases [26,27]. Therefore, CCR6 has a role in balancing inflammation *versus* immune intolerance by regulating the balance between opposing Th cells and T_{reg} cells [15,18,25].

Pathogenic roles of such alterations in CCR6 activity and nature of expression by immune cells in ITP have been unexplored to date. In this study, we have characterized the phenotypical differences between CCR6$^+$ and CCR6$^-$ subsets to assess its prevalence and activity in peripheral CD4 cells and analyze the correlation with the response of treatment in patients with chronic ITP.

Materials and methods

Patient data and design

Peripheral blood samples from 36 patients with ITP who had not been treated with any therapy for at least 1 week before sampling were enrolled at our hospital between November 2015 and July 2017. All the cases met the diagnostic criteria of chronic ITP, as described previously [28]. Among them, 26 patients had evaluation of platelet antibodies. We followed-up on 31 patients (platelet count < 30×10^9/l) at baseline and at a later time-point (28 days) following treatment to analyze the dynamic change of CCR6$^+$CD4$^+$ T subsets. The response after each treatment was recorded. A complete response (CR) is defined as any platelet count of at least 100×10^9/l, and a response (R) is defined as a platelet count greater than 30×10^9/l and at least a doubling of the baseline level. No response (NR) is defined as any platelet count lower than 30×10^9/l or less than doubling of the baseline count [28]. Age- and sex-matched healthy controls (n = 43) were obtained from the medical examination center. Characteristics of ITP patients and healthy controls can be found in Table 1. The study was approved by the Institutional Review Board and written informed consent was obtained from each study subject.

Plasma and cell preparation

Plasma obtained from centrifuged peripheral blood samples anti-coagulated with ethylenediamine tetraacetic acid (EDTA) was stored at –80°C until assay. Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation over a Ficoll-Hypaque gradient (Amersham Pharmacia Biotech, Little Chalfont, UK) and resuspended in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) for future use.

Table 1. Main demographic and clinical characteristics of the patients with chronic immune thrombocytopenia and healthy controls

Characteristics	Patients (n = 36)	Controls (n = 43)
Female/male, *n*	18/18	25/18
Age, mean ± s.d.	46·5 ± 13·9	40·0 ± 13·7
Platelet count, × 10^9/l	17·9 ± 11·2	218·6 ± 40·6
Disease duration, months, mean ± s.d.	35·1 ± 21·3	
Anti-platelet autoantibody pattern, *n*/total number (%)		
GPIIb/IIIa	4/26 (15·4)	
GPIb/IX	3/26 (11·5)	
GPIIb/IIIa$^+$GPIa/IIa	4/26 (15·4)	
GP IIb/IIIa$^+$GPIa/IIa$^+$GPIb/IX	1/26 (3·8)	
None	14/26 (53·9)	
Therapy, *n*/total number (%)		
GC	11/36 (30·6)	
GC$^+$IVIg	5/36 (13·8)	
GC$^+$DNZ	4/36 (11·1)	
GC$^+$rhTPO	2/36 (5·6)	
GC$^+$IVIg$^+$rhTPO	8/36 (22·2)	
GC$^+$IVIg$^+$VCR	1/36 (2·8)	
None	5/36 (13·9)	

M = male; F = female; GC = glucocorticoid; IVIg = intravenous immunoglobulin; DNZ = danazol; VCR = vincristine; rhTPO = recombinant human thrombopoietin.

Cell culture and stimulation

PBMCs were adjusted to 1 × 10^6/ml in complete RPMI-1640 medium, and cultured in 24-well culture plates and incubated in humidified air in 5% CO_2 at 37°C. PBMCs were treated with coated 1 μg/ml anti-CD3 (OKT3; Biolegend, San Diego, CA, USA) and 0·5 μg/ml anti-CD28 (CD28.2; Biolegend) for up to 12, 24, 48 or 72 h. For T_{reg} cells, 20 U/ml IL-2 was added to the cultures and cells were cultured overnight.

Flow cytometric analysis

For surface marker detection, PBMCs were labeled by APC anti-human CD4, APC anti-human CD8, phycoerythrin-cyanin 7 (PE-Cy7) anti-human CCR6, fluorescein isothiocyanate (FITC) anti-human CD25, APC anti-human CD69 or their respective immunoglobulin (IgG) isotype controls (all from Biolegend). For intracellular detection, PBMCs were stimulated with 50 ng/ml phorbol–myristate–acetate (PMA) and 500 ng/ml ionomycin (Sigma-Aldrich, St Louis, MO, USA) for 5 h in the presence of GolgiStop (BD Biosciences, San Diego, CA, USA). After incubation, the cells were first stained for surface markers, fixed and permeabilized according to the manufacturer's instructions (BD Biosciences). Cells were then incubated with a selected combination of the following antibodies: FITC anti-human IFN-γ, PE anti-human IL-17A, PE anti-human IL-22, PE anti-human FoxP3 or their corresponding IgG isotype controls (all from Biolegend). Stained cells were tested on a fluorescence activated cell sorter (FACS) Canto II flow cytometer.

Cytokine assays

PBMCs were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin at 37°C before supernatants were collected. IFN-γ, IL-17A and IL-22 production in supernatant as well as transforming growth factor (TGF)-β and IL-10 plasma levels were measured by enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (Westang Biotech, Shanghai, China and NeoBioscience Technology, Shenzhen, China). The assay was performed in duplicate. The lower limits of detection were 15 pg/ml for TGF-β, 0·4 pg/ml for IL-10 and 8 pg/ml for IFN-γ, IL-17A and IL-22.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). The results were expressed as mean ± standard deviation (s.d.). Statistical significance was performed by Student's *t*-test, one-way analysis of variance (ANOVA) or paired *t*-test. Correlations were examined using Spearman's rank correlation analysis. A *P*-value < 0·05 was considered statistically significant.

Results

Circulating $CCR6^+CD4^+$ T cells are elevated in ITP patients

The frequency of $CD4^+$ T cells expressing CCR6 in the peripheral blood was significantly increased in patients with ITP compared with healthy controls (34·03 ± 7·15% *versus*·21·97 ± 5·76%, $P < 0·001$, Fig. 1a). No significant difference was found in the frequency of $CCR6^+CD8^+$ T cells between ITP patients and healthy controls (6·15 ± 3·12% *versus*·6·69 ± 4·24%, $P = 0·673$, Fig. 1b). In contrast, the frequency of $CCR6^+CD19^+$ B cells in ITP patients was lower than that in healthy controls (72·04 ± 16·17% *versus*· 86·47 ± 8·43%, $P = 0·002$, Fig. 1c). When ITP patients alone were analyzed, there was no significant association between the frequency of $CCR6^+CD4^+$ cells and platelet counts or the presence of autoantibodies (data not shown).

$CCR6^+CD4^+$ T cells in ITP patients exhibit greater expansion and activation

Based on the finding that $CCR6^+CD4^+$ cells were significantly increased in ITP, we further sought to determine whether T cell activation can lead to the expansion of

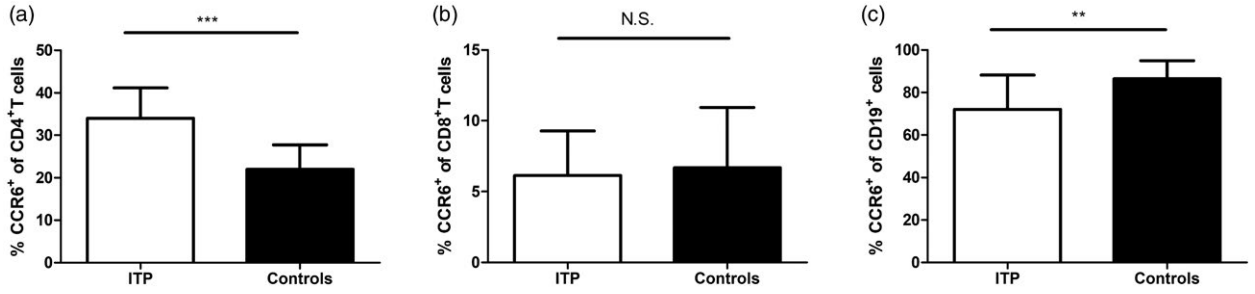

Fig. 1. C-C chemokine receptor (CCR)6 expression in immune thrombocytopenia (ITP) patients. The scatter-plots showed the frequencies of CCR6 on (a) $CD4^+$ T cells, (b) $CD8^+$ T cells and (c) $CD19^+$ B cells of peripheral blood in ITP patients ($n = 16$) and healthy controls ($n = 18$). Bars are shown as means ± standard deviation (s.d.). $^{**}P < 0·01$, $^{***}P < 0·001$; n.s. = not significant.

$CCR6^+CD4^+$ populations. PBMCs were stimulated *in vitro* with anti-CD3 and anti-CD28, then the subsequent frequency of CCR6 was measured (Fig. 2a). CCR6 was induced on $CD4^+$ T cells, starting at 24 h and persisting until at least 72 h in ITP patients (30·22 ± 3·30% at 12 h, 33·18 ± 3·89% at 24 h, 41·84 ± 4·59% at 48 h, 56·29 ± 7·27% at 72 h, Fig. 2b). However, the increase of CCR6 began at 48 h in heathy controls (19·35 ± 2·07% at 12 h, 20·51 ± 2·64% at 24 h, 27·49 ± 4·11% at 48 h, 37·23 ± 5·04% at 72 h, Fig. 2b). The frequency of $CCR6^+CD4^+$ T cells in ITP was higher than that in healthy controls after stimulation ($P < 0·001$, Fig. 2b). Thus, CD4 cells were capable of up-regulating CCR6 early in response to stimulation via CD3 and CD28 in ITP patients.

Additionally, we examined the expression of several activation markers on $CCR6^+CD4^+$ cells. Although more $CCR6^+$ cells and $CCR6^-$ cells expressed the activation markers CD25 and CD69 after stimulation ($P < 0·05$ in ITP patients and controls, Supporting information, Table S1), the fractions of $CD25^+/CD25^-$ and $CD69^+/CD69^-$ were higher for circulating $CCR6^+$ compared with $CCR6^-$ cells at every time-point ($P < 0·05$ in ITP patients and controls, Supporting information, Table S1). The frequencies of $CCR6^+CD25^+CD4^+$ and $CCR6^+CD69^+CD4^+$ cells were increased markedly following increased time of activation ($CCR6^+CD25^+CD4^+$ cells, $P = 0·002$ for ITP patients and $P = 0·007$ for controls; $CCR6^+CD69^+CD4^+$ cells, $P = 0·009$ for ITP patients and $P = 0·008$ for controls, Fig. 2c,d). In contrast, the frequencies of $CCR6^+CD25^-CD4^+$ and $CCR6^+CD69^-CD4^+$ cells were slightly decreased or remained unchanged, which did not reach significance ($P > 0·05$, Fig. 2c,d). Activation markers CD25 and CD69 on $CCR6^+CD4^+$ cells were also significantly increased in ITP patients compared with healthy controls ($P < 0·001$, Fig. 2c,d). Our data suggest that in response to stimuli, increased $CCR6^+CD4^+$ cells may represent activated $CD4^+$ T cells in ITP.

Peripheral $CCR6^+CD4^+$ T cells in ITP patients exhibit a memory phenotype

We next sought to characterize the phenotype of these $CCR6^+CD4^+$ T cells in the peripheral blood of patients with ITP. CD45RO is a well-established marker of memory T cells [18,21]. CCR6 was expressed on more than 50% of $CD45RO^+$ cells (Fig. 3a). The frequency of CCR6 was greatly increased on $CD45RO^+$ than on $CD45RO^-CD4^+$T cells (54·67 ± 7·88% *versus* 11·49 ± 7·07% for ITP patients, $P < 0·001$; 46·94 ± 8·62% *versus*· 6·63 ± 4·83% for controls, $P < 0·001$, Fig. 3b). The frequency of $CCR6^+CD4^+$ cells expressing $CD45RO^+$ was increased in ITP patients compared with healthy controls ($P < 0·01$, Fig. 3b).

$CCR6^+$ T_{reg} cells are decreased in ITP patients

As our results suggested a memory phenotype for $CCR6^+CD4^+$ T cells, we therefore evaluated classic T_{reg} cell markers in $CCR6^+CD4^+$ T cells (Fig. 4a). The proportion of $CD4^+CD25^+$ cells in ITP patients was similar to that observed in controls ($P = 0·9654$, Fig. 4b), while the $CD4^+CD25^+$ cells expressing FoxP3 were 2·35 times less frequent in ITP patients than in controls (2·79 ± 1·15% *versus* 6·56 ± 2·94%, $P = 0·0012$, Fig. 4b). $CD4^+$ cells that expressed both CD25 and FoxP3 had significant enrichment for CCR6 expression ($CCR6^+$ *versus* $CCR6^-$ 2·82 ± 1·15 *versus* 0·13 ± 0·08% for ITP patients, $P < 0·001$; 4·75 ± 2·09% *versus* 0·16 ± 0·12% for controls, $P = 0·001$, Fig. 4c) and 2·82 ± 1·15% of $CCR6^+CD25^+CD4^+$ cells in ITP patients were $FoxP3^+$, whereas 4·75 ± 2·09% of $CCR6^+CD25^+CD4^+$ cells expressed FoxP3 in controls ($P < 0·01$, Fig. 4c). The frequencies of $CD4^+CD25^+FoxP3^+$ and $CCR6^+CD25^+CD4^+FoxP3^+$ cells were significantly correlated with platelet counts in ITP patients ($r^2 = 0·1734$, $P = 0·0481$ and $r^2 = 0·2256$, $P = 0·0220$, respectively, Fig. 4d,e).

TGF-β and IL-10 are primarily considered anti-inflammatory cytokines [29]. We found lower circulating TGF-β levels in ITP patients compared with controls (1063·67 ± 454·11 pg/ml *versus* 156 122·57 ± 599·47 pg/ml, $P = 0·0053$, Fig. 4f). TGF-β levels were correlated with $CD4^+CD25^+FoxP3^+$ and $CCR6^+CD25^+CD4^+FoxP3^+$ cells in ITP patients ($r^2 = 0·1990$, $P = 0·0487$ and $r^2 = 0·2341$, $P = 0·0263$, respectively, Fig. 4g,h). Concurrently, we also tested levels of circulating IL-10 and found no difference in levels of IL-10 between ITP patients and healthy controls (25·96 ± 13·67 pg/ml *versus* 35·96 ± 26·35 pg/ml, $P = 0·1775$, Fig. 4f). No significant difference was found between platelet counts and TGF-β or IL-10 levels in ITP patients (data not shown).

$CCR6^+CD4^+$ T cells are a major source of IL-17A in ITP patients

Next, we detected the secretion of IL-17A, IL-22 and IFN-γ within $CCR6^+CD4^+$ T cells (Fig. 5a). As shown in Fig. 5b, increased secretion of IL-17A, IFN-γ and IL-22 by $CD4^+$ T cells was found in ITP patients compared with healthy controls (1·51 ± 1·03% *versus*·0·97 ± 0·31%, $P = 0·016$; 15·95 ± 6·03% *versus* 9·53 ± 3·84%, $P = 0·001$; 1·91 ± 1·20 *versus*·1·01 ± 0·72, $P = 0·004$, Fig. 5b). $CCR6^-CD4^+$ cells produced significantly higher levels of IFN-γ compared to $CCR6^+$ counterparts (11·58 ± 5·44% *versus*·4·37 ± 2·67% for ITP patients, $P = 0·001$; 6·74 ± 2·5% *versus*·2·78 ± 1·64% for controls, $P < 0·001$, Fig. 5c). In contrast, $CCR6^+CD4^+$ cells produced more IL-17A than their $CCR6^-$ counterpart (20·08 ± 0·99% *versus* 0·41 ± 0·45% for ITP patients,

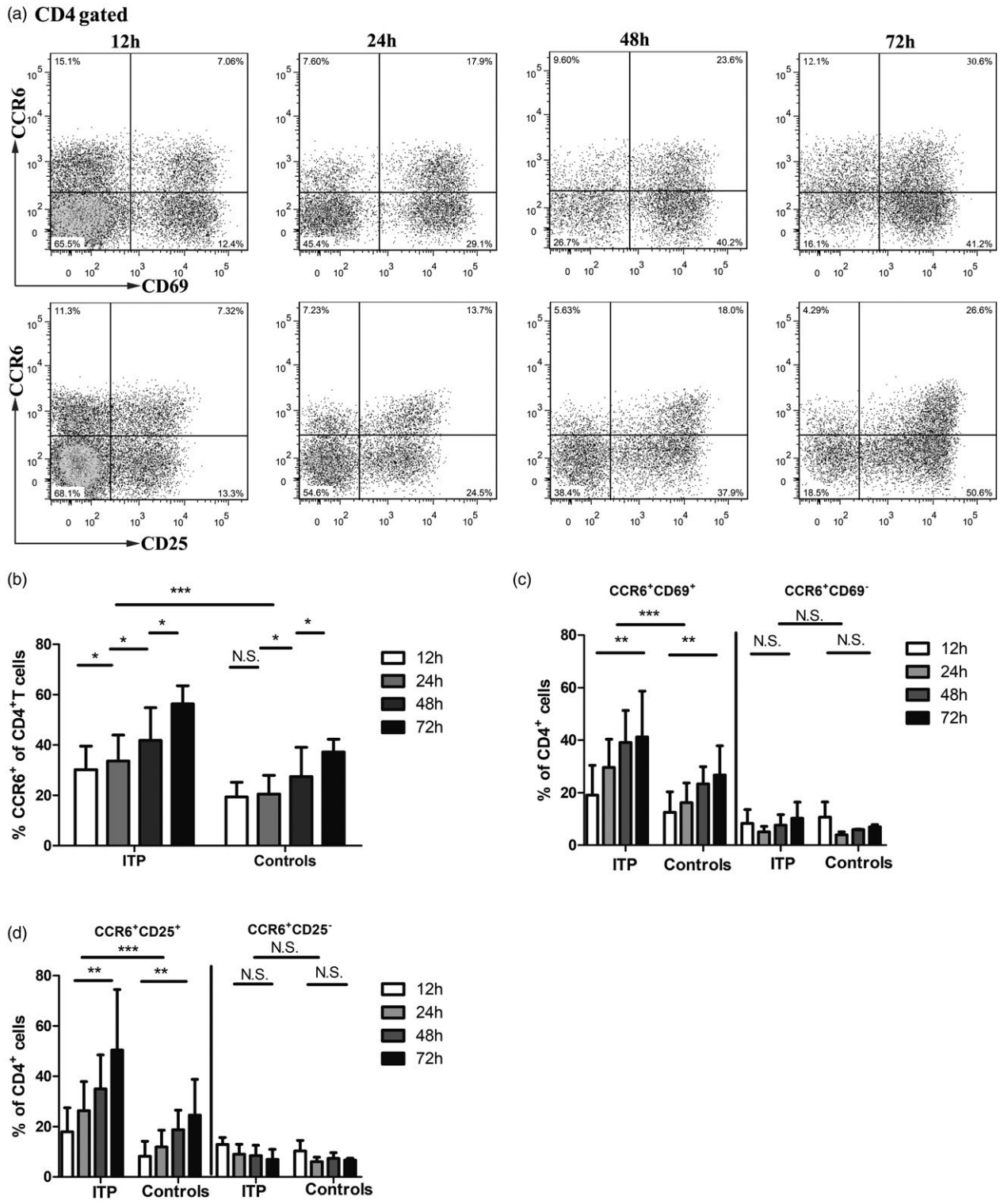

Fig. 2. C-C chemokine receptor (CCR)6 expression following stimulation *in vitro*. (a) Peripheral blood mononuclear cells (PBMCs) from a healthy control were stimulated using plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (0·5 μg/ml) antibodies. At various times, as shown, the frequency of CCR6 and CD69 or CD25. (b) The frequency of CCR6 among CD4 cells in culture with anti-CD3 and anti-CD28 at different times (12, 24, 48 and 72 h) in immune thrombocytopenia (ITP) patients (n = 8) and healthy controls (n = 8). (c) The frequency of CD25 in the $CCR6^+$ *versus* $CCR6^-$ subset in culture with anti-CD3 and anti-CD28 at different times (12, 24, 48 and 72 h) in ITP patients (n = 8) and healthy controls (n = 8). (d) The frequency of CD69 in the $CCR6^+$ *versus* $CCR6^-$ subset in culture with anti-CD3 and anti-CD28 at different times (12, 24, 48 and 72 h) in ITP patients (n = 8) and healthy controls (n = 8). Bars are shown as means ± standard deviation (s.d.). $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$; n.s. = not significant.

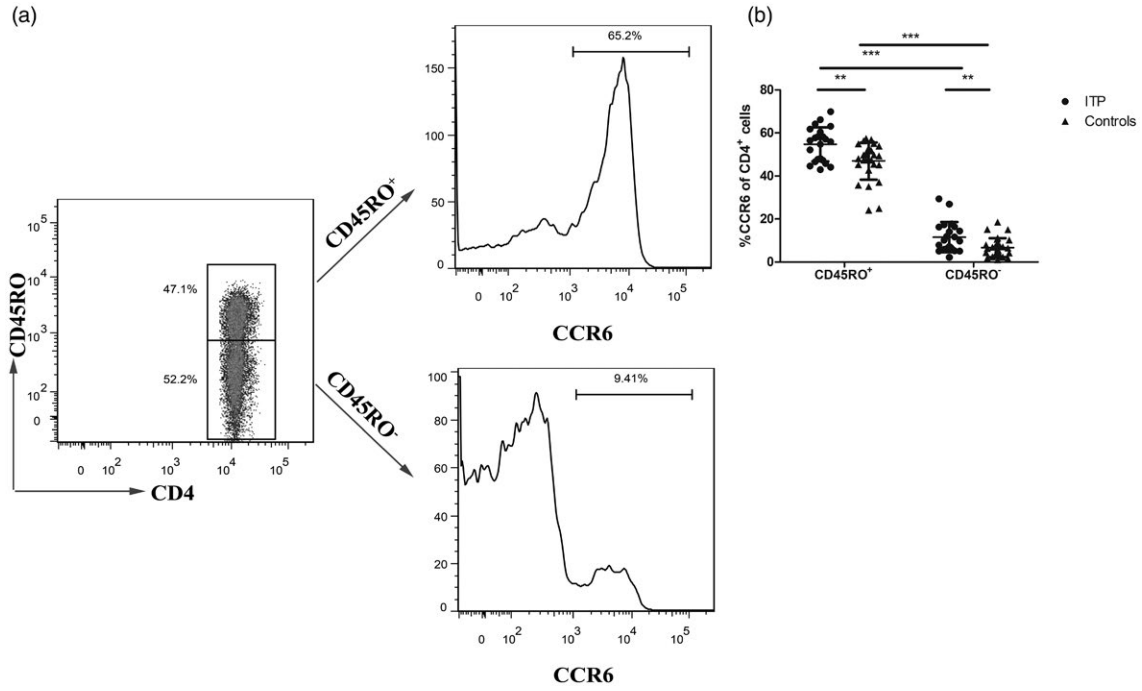

Fig. 3. Phenotype of C-C chemokine receptor (CCR)6$^+$ CD4$^+$ cells in immune thrombocytopenia (ITP) patients. (a) Flow cytometry study of CD45RO status in peripheral blood CD4$^+$ T cells from a healthy control. The frequency of CCR6$^+$ cells among CD45RO$^+$ cells (top) and CD45RO$^-$ cells (bottom) was also evaluated. (b) The frequencies of CCR6 on CD45RO$^+$ and CD45RO$^-$CD4$^+$ cells were analyzed in ITP patients (n = 23) and healthy controls (n = 21). Each symbol represents one individual; horizontal bars indicate means ± standard deviation (s.d.). $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

P = 0·001; 0·80 ± 0·35% *versus* 0·36 ± 0·21% for controls, P = 0·005, Fig. 5d). IL-22 production was equivalent between CCR6$^+$ and CCR6$^-$CD4$^+$ cells (0·81 ± 0·24% *versus* 0·91 ± 0·51% for ITP patients, P = 0·468; 0·57 ± 0·33% *versus* 0·46 ± 0·25% for controls, P = 0·658, Fig. 5e). Our study showed that the frequency of CCR6$^+$CD4$^+$ cells was increased by 1·54-fold in ITP patients compared with healthy controls (34·03 ± 7·15% *versus* 21·97 ± 5·76%, $P < 0{\cdot}001$).The IL-17A, IFN-γ and IL-22 subsets of CCR6$^+$CD4$^+$ population increased by 2·6-, 1·57- and 1·42-fold in ITP patients compared with controls (2·08 ± 0·99% *versus* 0·80 ± 0·35%; 4·37 ± 2·67% *versus* 2·78 ± 1·64%; 0·81 ± 0·24% *versus*· 0·57 ± 0·33%, all $P < 0{\cdot}05$, Fig. 5c–e). The frequencies of IFN-γ- and IL-22-secreting CD4$^+$ T cells in the CCR6$^-$ population were 1·78- and 1·97-fold in ITP patients compared with healthy controls (P = 0·025 and P = 0·003, respectively, Fig. 5c,e). Furthermore, the ratio of CCR6$^+$CD4$^+$IL-17A/CCR6$^+$ T_{reg} in ITP patients was increased significantly when compared with that in controls (1·03 ± 0·63 *versus* 0·16 ± 0·11, $P < 0{\cdot}001$, Fig. 5f).

Similarly in supernatant, the levels of IL-17A, IFN-γ and IL-22 in ITP patients were higher than those in controls (17·47 ± 2·71 pg/ml *versus* 15·08 ± 2·59 pg/ml, P = 0·0071; 59·16 ± 11·19 pg/ml *versus* 34·49 ± 7·18 pg/ml, $P < 0{\cdot}001$; 43·78 ± 16·69 pg/ml *versus* 25·59 ± 7·68 pg/ml, $P < 0{\cdot}001$, Fig. 5g). Moreover, the frequency of CCR6$^+$CD4$^+$IL-17A$^+$ cells was significantly correlated with IL-17A levels in the culture supernatant in ITP patients (r^2 = 0·2256, P = 0·0343, Fig. 5h). There was no significant difference between CCR6$^+$CD4$^+$IFN-γ$^+$ cell frequency and IFN-γ levels or between CCR6$^+$CD4$^+$IL-22$^+$ cell frequency and IL-22 levels (data not shown).

CCR6$^+$CD4$^+$ T cells are associated with treatment response in ITP patients

To further address whether CCR6$^+$CD4$^+$ T cells were associated with treatment response, 31 ITP patients receiving treatment were investigated. CR was observed in eight patients, R in 12 patients and NR in 11 patients. There was no significant difference in the percentages of CCR6$^+$CD4$^+$ T cell subsets between responding and non-responding patients before treatment ($P > 0{\cdot}05$). However, the frequencies of circulating CCR6$^+$CD4$^+$, CCR6$^+$CD4$^+$IFN-γ$^+$, CCR6$^+$CD4$^+$IL-17A$^+$ and CCR6$^+$CD4$^+$IL-22$^+$ cells as well as the ratio of CCR6$^+$CD4$^+$IL-17A$^+$/CCR6$^+$ T_{reg} were decreased in CR and R patients compared with pretreatment ($P < 0{\cdot}05$, Fig. 6a–d,f), while the frequency of circulating CCR6$^+$ T_{reg} cells was increased in CR and R patients ($P < 0{\cdot}05$, Fig. 6e). Furthermore, CCR6$^+$ T_{reg} cell frequency after treatment

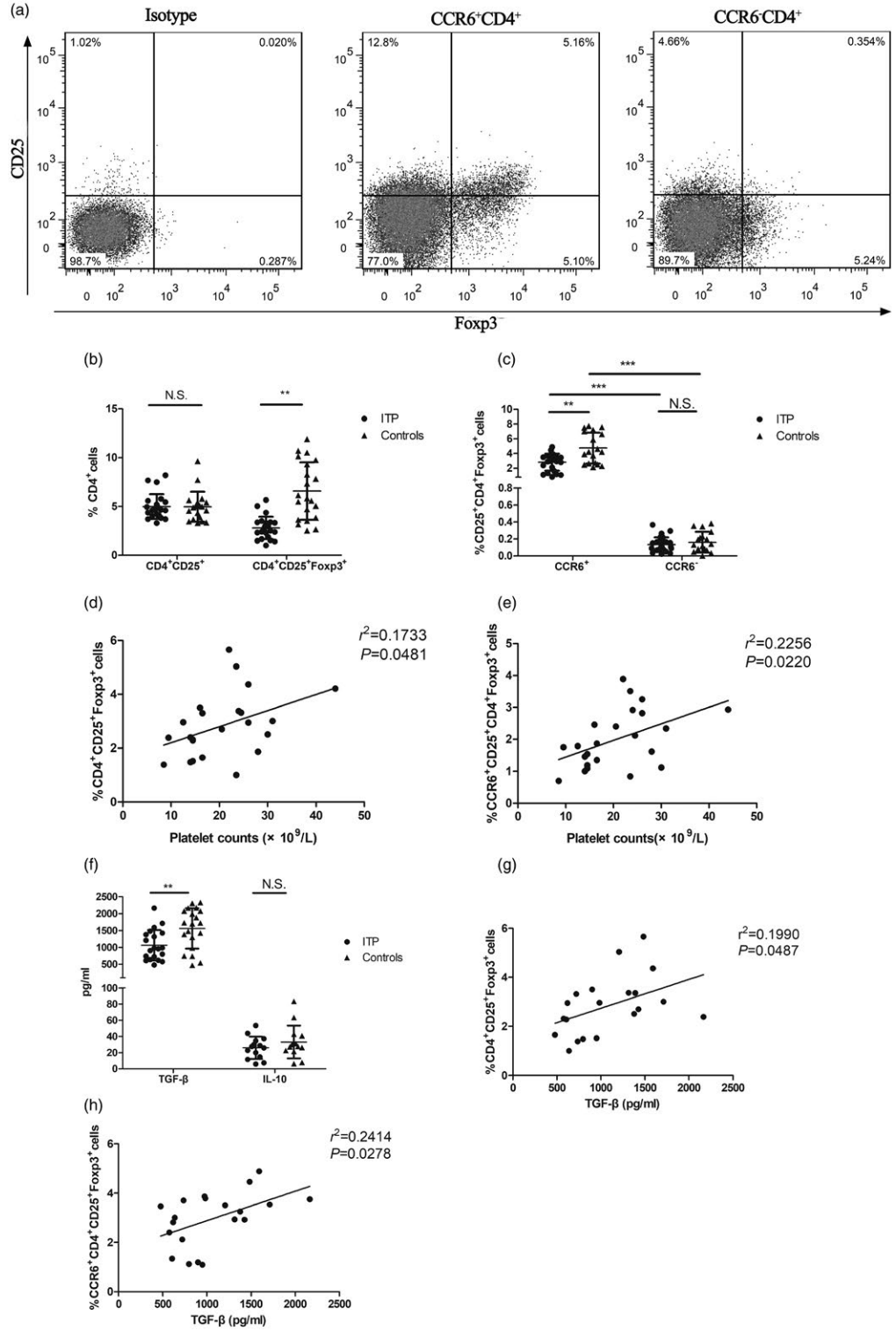
(a)
Isotype
CCR6+CD4+
CCR6-CD4+
CD25
Foxp3
1.02%
0.020%
98.7%
0.287%
12.8%
5.16%
77.0%
5.10%
4.66%
0.354%
89.7%
5.24%
(b)
N.S.
**
% CD4+ cells
CD4+CD25+
CD4+CD25+Foxp3+
ITP
Controls
(c)
***
***
**
N.S.
%CD25+CD4+Foxp3+ cells
CCR6+
CCR6-
ITP
Controls
(d)
r2=0.1733
P=0.0481
%CD4+CD25+Foxp3+ cells
Platelet counts (× 10^9/L)
(e)
r2=0.2256
P=0.0220
%CCR6+CD25+CD4+Foxp3+ cells
Platelet counts(× 10^9/L)
(f)
**
N.S.
pg/ml
TGF-β
IL-10
ITP
Controls
(g)
r2=0.1990
P=0.0487
%CD4+CD25+Foxp3+ cells
TGF-β (pg/ml)
(h)
r2=0.2414
P=0.0278
%CCR6+CD4+CD25+Foxp3+ cells
TGF-β (pg/ml)

Fig. 4. C-C chemokine receptor (CCR)6$^+$ regulatory T cells (T_{reg}) cells in immune thrombocytopenia (ITP) patients. (a) Representative example of a flow cytometry density plot of CD25 and forkhead box protein 3 (FoxP3) expression in the population of CCR6$^+$CD4$^+$T cells and CCR6$^-$CD4$^+$ T cells from a healthy control. (b) Summary of frequencies of CD4$^+$CD25$^+$ cells and CD4$^+$CD25$^+$FoxP3$^+$ cells in ITP patients (n = 23) and healthy controls (n = 21). (c) Frequencies of CD4$^+$CD25$^+$FoxP3$^+$ cells in the CCR6$^+$ *versus* CCR6$^-$ subset were analyzed in ITP patients (n = 23) and healthy controls (n = 20). Correlation between the frequency of (d) CD4$^+$CD25$^+$FoxP3$^+$ cells or (e) CCR6$^+$CD4$^+$CD25$^+$FoxP3$^+$ cells and platelet counts in ITP patients (n = 23). (f) Transforming growth factor (TGF)-β and interleukin (IL)-10 in plasma were measured in ITP patients (n = 20 and n = 16, respectively) and healthy controls (n = 20 and n = 16, respectively). Correlation between the frequency of (g) CD4$^+$CD25$^+$FoxP3$^+$ cells or (H) CCR6$^+$CD4$^+$CD25$^+$FoxP3$^+$ cells and TGF-β levels in ITP patients (n = 20). Each symbol represents one individual; horizontal bars indicate means ± standard deviation (s.d.). $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$; n.s. = not significant.

was significantly higher in CR and R patients compared with NR patients ($P < 0{\cdot}01$, Fig. 6e). In comparison with pretreatment, the frequencies of CCR6$^+$CD4$^+$, CCR6$^+$CD4$^+$IFN-γ$^+$ and CCR6$^+$CD4$^+$IL-17A$^+$ cells in NR patients remained unchanged ($P > 0{\cdot}05$, Fig. 6a–c), while the frequencies of CCR6$^+$CD4$^+$IL-22$^+$ and CCR6$^+$ T_{reg} cells were significantly changed ($P < 0{\cdot}05$, Fig. 6d,e). Although the frequencies of CCR6$^-$CD4$^+$IFN-γ$^+$ and CCR6$^-$CD4$^+$IL-22$^+$ cells were decreased in CR and R patients compared with pretreatment ($P < 0{\cdot}05$, Fig. 6f,h), the frequencies of CCR6$^-$CD4$^+$IL-17A$^+$ and CCR6$^-$ T_{reg} cells had no difference regarding reaction to treatment ($P > 0{\cdot}05$, Fig. 6g–i). No significant changes of CCR6$^-$CD4$^+$IFN-γ$^+$, CCR6$^-$CD4$^+$IL-17A$^+$, CCR6$^-$CD4$^+$IL-22$^+$ and CCR6$^-$ T_{reg} cells from NR patients were observed in our study ($P > 0{\cdot}05$, Fig. 6g–i). Additionally, CCR6$^+$CD4$^+$ subsets in CR and R patients changed in GC alone and combined therapy groups after treatment (Supporting information, Table S2). However, a significant difference was found in patients receiving combined therapy not in patients receiving GC alone, probably because of the small number observations in GC alone group (Supporting information, Table S2).

Discussion

In this study, we provide new information on CCR6 in ITP and report for the first time that the frequency of CCR6$^+$CD4$^+$ cells was increased in ITP patients. We also demonstrate that TCR triggering rapidly induced an increase of CCR6$^+$CD4$^+$ cells, which were enriched of activation markers in ITP patients. CCR6$^+$CD4$^+$ cells exhibiting memory phenotype were particularly susceptible to skew into Th17-like effector T cells in ITP patients. Finally, we show an association between CCR6$^+$CD4$^+$ cell subsets and the response of treatment in ITP patients.

Among T cell subsets, we found expression of CCR6 on both CD4 and CD8 cells, but a higher frequency of the former stained positive compared with the latter. Stimulation of T cells with anti-CD3/anti-CD28, a more physiological stimulus, induced the proportion of CCR6 in CD4 cells, which contained high fractions positive for activation markers (CD69 and CD25). Activated CD4 cells that expressed CCR6 increased following the time of T cell receptor (TCR) stimulation, similar to that reported recently for significant up-regulation of CCR6 expression on CD4$^+$ cells exposed to proteolipid protein 139-151 ($PLP_{139–151}$) [27]. This is in contrast to other studies, which have observed no increase in CCR6 surface expression and gene expression by treating cells with IL-2 for 5 days or with antibody against CD3 for 3 days [20]. The differences are probably related to the use of different stimuli, timing of analyses and heterogeneity in the population studied. Although CCR6$^+$ and CCR6$^-$CD4$^+$ subsets contained a greater proportion of activated phenotype cells after stimulation, activated CCR6$^+$CD4$^+$ occurred rapidly in ITP patients. In all eight patients, each of the activated markers tested was over-represented in the CCR6$^+$ subpopulation of CD4 cells at 24 h, whereas a small fraction of CCR6$^-$CD4$^+$ cells were activated phenotype cells. Thus, CCR6 might be an early marker of CD4$^+$ T cell activation in ITP. Combinatorial analysis of activation markers including CCR6 may improve the assessment of T cell activation in ITP.

CCR6$^+$CD4$^+$ T cells expressed higher levels of conventional memory marker CD45RO and classic T_{reg} cell markers (CD25 and FoxP3). Conversely, these markers were weakly or not expressed in CCR6$^-$ counterparts. Our findings might suggest that CCR6$^+$CD4$^+$ cells are characterized by memory-like phenotype. Furthermore, we observed that the frequency of CCR6$^+$ T_{reg} was lower in ITP patients and positively correlated with platelet counts. Circulating TGF-β levels were decreased in ITP patients compared with healthy controls, while IL-10 levels did not differ between two groups. Previous studies have reported that the inhibitory cytokine TGF-β and IL-10 produced by T_{reg} cells play a role in immune tolerance [29]. Activated CCR6$^+$ T_{reg} cells exhibit stronger suppressive activity compared with CCR6$^-$ T_{reg} cells due to enhanced IL-10 production [21,30]. However, we found that TGF-β, but not IL-10, levels were positively correlated with T_{reg} and CCR6$^+$ T_{reg} frequencies in ITP patients. These data indicate that a decrease of CCR6$^+$ T_{reg} and TGF-β might be involved in the pathogenesis of ITP, and CCR6$^+$ T_{reg} are more capable of exerting TGF-β-mediated suppression effects. TGF-β has also been shown to be released by platelet activation/degranulation and is essential for maintenance of T_{reg} suppressive functions [31]. It is

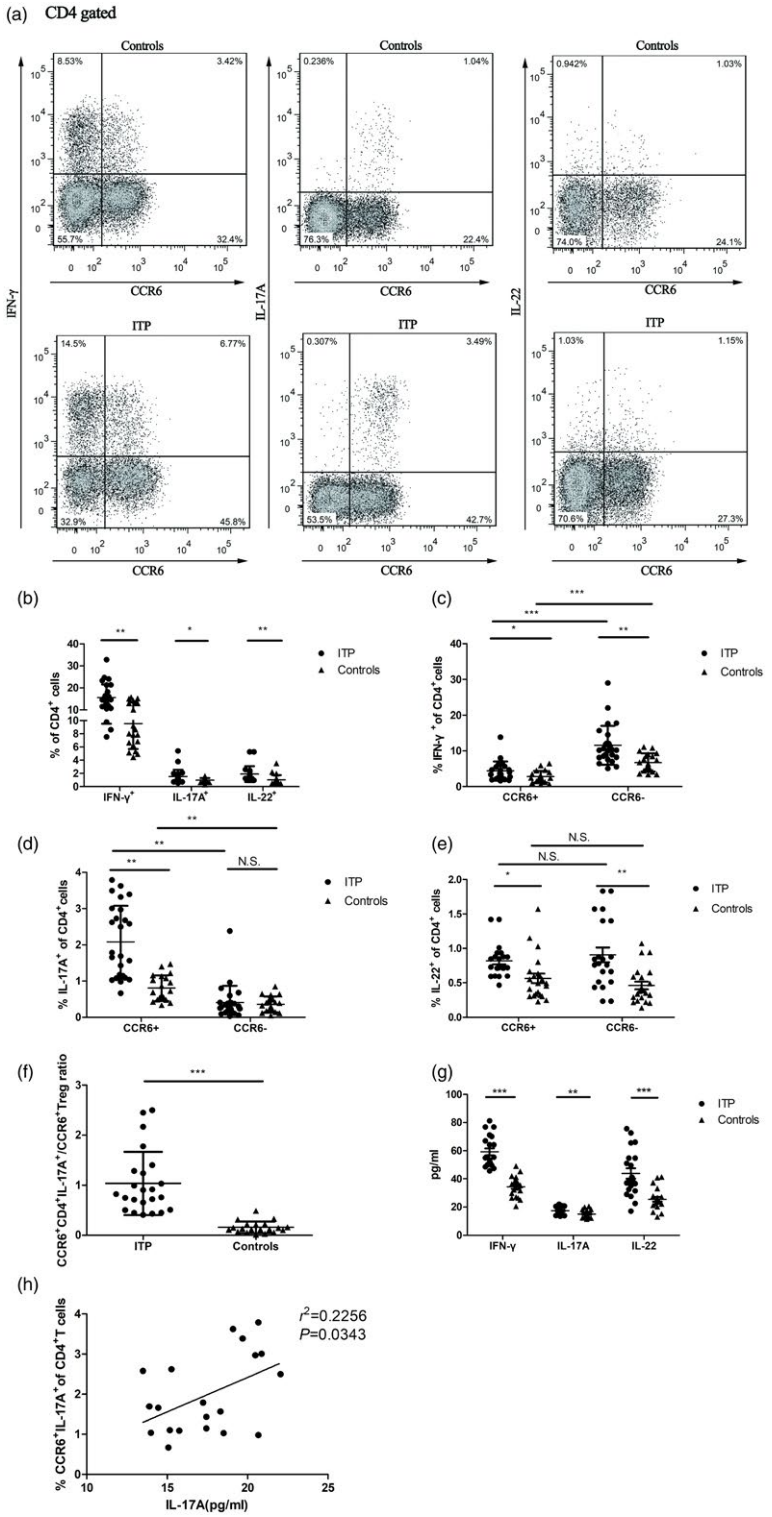
(a) CD4 gated
Controls
ITP
IFN-γ
IL-17A
IL-22
CCR6
8.53%
3.42%
55.7%
32.4%
0.236%
1.04%
76.3%
22.4%
0.942%
1.03%
74.0%
24.1%
14.5%
6.77%
32.9%
45.8%
0.307%
3.49%
53.5%
42.7%
1.03%
1.15%
70.6%
27.3%
(b)
% of CD4+ cells
IFN-γ+
IL-17A+
IL-22+
ITP
Controls
(c)
% IFN-γ+ of CD4+ cells
CCR6+
CCR6-
(d)
% IL-17A+ of CD4+ cells
N.S.
(e)
% IL-22+ of CD4+ cells
(f)
CCR6+CD4+IL-17A+/CCR6+Treg ratio
(g)
pg/ml
IFN-γ
IL-17A
IL-22
(h)
% CCR6+IL-17A+ of CD4+T cells
IL-17A(pg/ml)
r2=0.2256
P=0.0343

Fig. 5. The frequencies of interferon (IFN)-γ$^+$, interleukin (IL)-17A$^+$ and IL-22$^+$-secreting C-C chemokine receptor (CCR)6$^+$CD4$^+$ cells in immune thrombocytopenia (ITP) patients. (a) IFN-γ, IL-17A and IL-22 secretion in CD4$^+$ T cells were analyzed based on CCR6 expression. Representative dot-plots from an ITP patient and a healthy control. (b) Summary of frequencies of CD4$^+$ T cells producing IFN-γ, IL-17A and IL-22 in ITP patients (n = 25) and healthy controls (n = 25). Frequencies of CD4$^+$ T cells producing (c) IFN-γ, (d) IL-17A and (e) IL-22 in the CCR6$^+$ *versus* CCR6$^-$ subset were analyzed in ITP patients (n = 20) and healthy controls (n = 20). (f) The ratio of CCR6$^+$CD4$^+$IL-17A$^+$/CCR6$^+$ regulatory T cells (T_{reg}) was increased in ITP patients (n = 23) compared to healthy controls (n = 20). (g) IFN-γ, IL-17A and IL-22 in supernatant of ITP patients (n = 20) and healthy controls (n = 20). (h) Correlation between the frequency of CCR6$^+$CD4$^+$IL-17A$^+$ and IL-17A in supernatant in ITP patients (n = 20). Each symbol represents one individual; horizontal bars indicate means ± standard deviation (s.d.). $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$; n.s. = not significant.

possible that, with decreased platelet counts in ITP, there is attenuated release of TGF-β. Thus, the relatively lower level of TGF-β fails to effectively promote T_{reg} cell development. In this study, we were unable to find a correlation between platelet counts and TGF-β levels in ITP patients. Whether the decrease in the frequency of CCR6$^+$ T_{reg} cells is a consequence of TGF-β released by platelets in ITP requires further investigation.

Of the cytokines examined, circulating CCR6$^+$CD4$^+$ cells were a mixed IL-17A, IFN-γ and IL-22 secretion population. IL-17A-producing cells were more frequent in the CCR6$^+$ than in the CCR6$^-$ subsets. In contrast to IL-17A, there was a greater frequency of IFN-γ-producing cells among the CCR6$^-$ subsets. CCR6$^+$CD4$^+$ cells produced comparable IL-22 cytokines to CCR6$^-$ subsets. In ITP patients, CCR6$^+$CD4$^+$ cells produced more IL-17A compared with healthy controls. This is consistent with previous reports in which significant increases in CCR6$^+$ Th17 cells were observed in RA and psoriasis patients [25,32]. After short-term stimulation, IL-17A, IFN-γ and IL-22 levels in supernatant were higher in ITP patients than in controls. However, only up-regulated IL-17A was correlated with CCR6$^+$ Th17 cell frequency in ITP patients. These results may indicate that CCR6 expression defines the major source of IL-17A-producing T cells that have inflammatory potentials in ITP patients.

T_{reg} cells and Th17 cells have a reciprocal relationship. Conversion of T_{reg} to Th17 cells is regulated partly by epigenetic modifications of transcription factors and signature cytokines [15,33]. The T_{reg}/Th17 ratio may be a useful marker for assessing the severity of diseases in ITP [34]. Interestingly, we found that the CCR6$^+$Th17/CCR6$^+$ T_{reg} ratio was higher in ITP patients compared with healthy controls and correlated with the response of treatment, suggesting that the CCR6$^+$Th17/CCR6$^+$ T_{reg} imbalance might be involved in the pathogenesis of ITP. As reported from earlier studies [15,25], CCR6 is important for Th17 migration to inflammatory tissues and may modulate human Th17 differentiation to sustain inflammatory responses. Similarly, CCR6 on T_{reg} cells is also required for T_{reg} cell localization and migration [15]. CCR6$^+$ T_{reg} cells co-localize with different Th cell subsets and exhibit superior suppressive capacity *in vitro* [18]. These studies suggested that CCR6 might regulate the migration of both proinflammatory and T_{reg} cells in response to inflammatory cues from the immune environment [15,25]. Thus, we postulate that CCR6 may play important roles in dictating the balance between tolerance and immunity. Further studies are needed to delineate precisely how CCR6 control the balance of pro- and anti-inflammatory response in CD4 cells of ITP.

Different strategies have been considered for the treatment of chronic ITP, such as glucocorticoid, intravenous immunoglobulin, danazol, vincristine, thrombopoietin receptor agonists (TPO-RA) [35] and recombinant human thrombopoietin (rhTPO) [36,37]. As we know, effective treatment might increase bone marrow platelet production, correct the immune dysregulations and lead to a response in patients. In this study, we did not observe any statistical significance in the frequency of circulating CCR6$^+$CD4$^+$ cells between responders and non-responders before treatment. However, our data showed a significant relationship between CCR6$^+$CD4$^+$ cell subsets and the response to treatment. CCR6$^+$ T_{reg} and CCR6$^+$CD4$^+$IL-17A$^+$ cells differed significantly in patients with CR/R response between pre- and post-treatment, while CCR6$^-$ T_{reg} and CCR6$^-$CD4$^+$IL-17A$^+$ cells in ITP patients did not alter after treatment. In comparison with pretreatment, the ratio of CCR6$^+$Th17/CCR6$^+$ T_{reg} was decreased in patients with CR/R response. Irrespective of treatments, CCR6$^+$CD4$^+$ cell subsets and the CCR6$^+$Th17/CCR6$^+$ T_{reg} ratio in CR/R patients differed between pre- and post-treatment. On that basis, we postulate that CCR6$^+$CD4$^+$ cells might possibly be involved in the clinical efficacy of ITP treatment.

Our current work reveals that CD4$^+$T cell subsets expressing the chemokine receptor CCR6 are enriched in blood of ITP patients. CCR6$^+$CD4$^+$ cells exhibit characteristics of activated memory effector Th17, rather than regulatory phenotype in ITP patients. Finally, CCR6$^+$CD4$^+$ cell subsets are correlated with the response of treatment. Altogether, our results offer a potential biomarker for a subpopulation of T cells with effector properties, which might contribute to the pathogenesis of ITP.

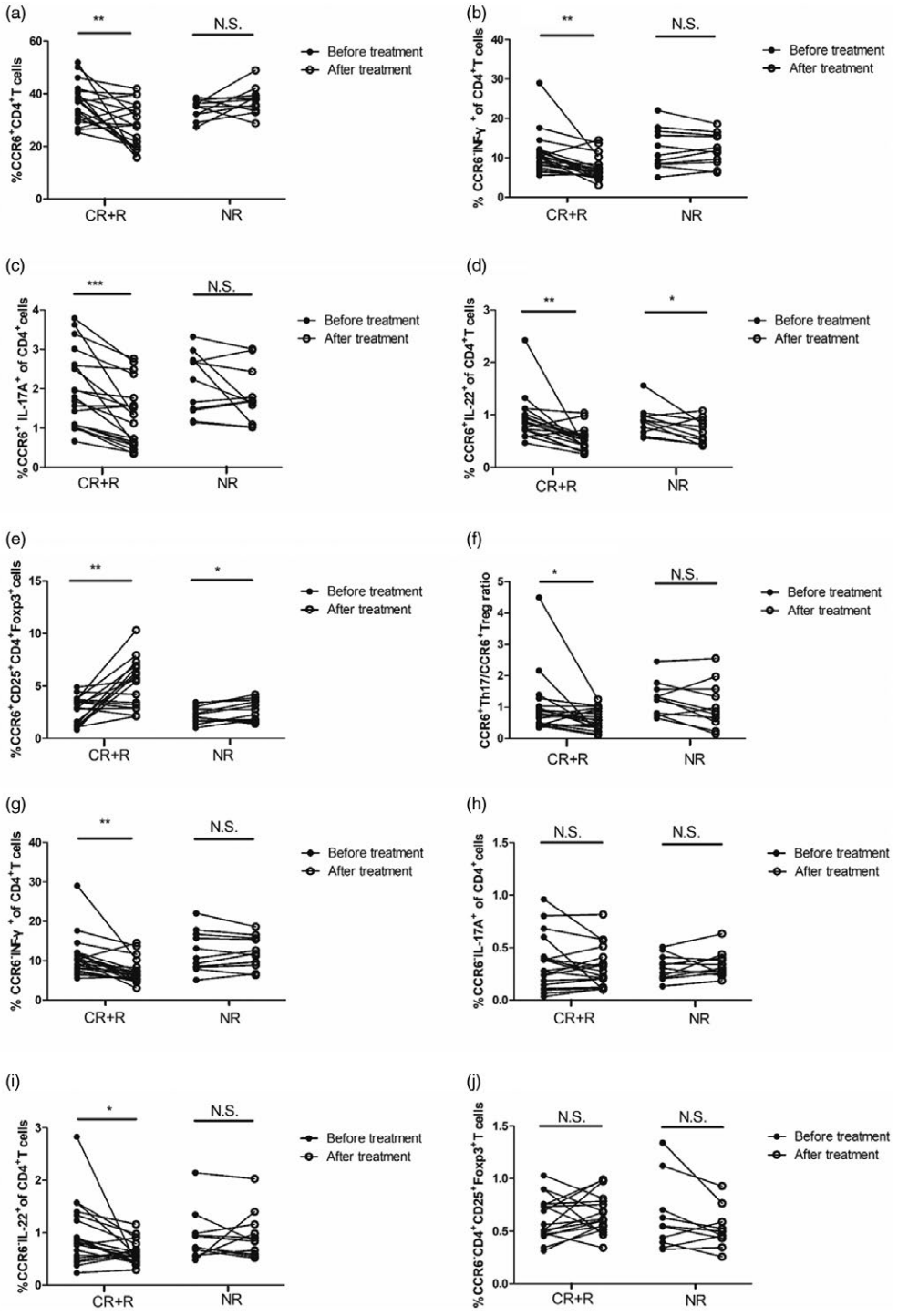

Fig. 6. Comparison of C-C chemokine receptor (CCR)6$^+$CD4$^+$ T cell subsets between responder (n = 20) and non-responder patients (n = 11). The frequencies of (a) CCR6$^+$CD4$^+$, (b) CCR6$^+$CD4$^+$ interferon (IFN)-γ$^+$, (c) CCR6$^+$CD4$^+$interleukin (IL)-17A$^+$, (d) CCR6$^+$CD4$^+$IL-22$^+$, (e) CCR6$^+$CD4$^+$CD25$^+$forkhead box protein 3 (FoxP3$^+$), (f) CCR6$^+$CD4$^+$IL-17A$^+$/CCR6$^+$CD4$^+$CD25$^+$FoxP3$^+$ratio, (g) CCR6$^-$CD4$^+$IFN-γ$^+$, (h) CCR6$^-$CD4$^+$IL-17A$^+$, (i) CCR6$^-$CD4$^+$IL-22$^+$ and (j) CCR6$^-$CD4$^+$CD25$^+$FoxP3$^+$ cells were evaluated in responders and non-responders before and after treatment. Each symbol represents one individual. *P < 0·05, **P < 0·01, ***P < 0·001; n.s. = not significant; CR = complete responders; R = responders; NR = non-responders.

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Disclosure

All authors have no conflicts of interest.

Author contributions

M. L. and R. Y. participated in designing the research, analyzing and interpreting the data and writing the manuscript. M. L., Y. Li and Y. H. contributed to performing the experiments. C. L., Y. H., B. S., H. L., F. X. and X. L. contributed to sample collection and essential reagents or tools.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. The ratio of positive vs. negative phenotypic markers on CCR6+ and CCR6-CD4+ T cells in peripheral blood.
Table S2. CCR6+CD4+ T cells within ITP patients stratified by different treatment groups.