

# Abnormal numbers of CD4<sup>+</sup> T lymphocytes and abnormal expression of CD4<sup>+</sup> T lymphocyte-secreted cytokines in patients with immune-related haemocytopenia

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Received September 22, 2018; Accepted June 12, 2019

DOI: 10.3892/mmr.2019.10663

**Abstract.** In the past decade, a group of cases with persisting haemocytopenia were separated from those with idiopathic cytopenia of undetermined significance due to the optimal response of these patients to immunosuppression therapy and due to the detection of autoantibodies in the bone marrow of haemopoietic cells. This condition was termed immune-related haemocytopenia (IRH). However, the quantity of T lymphocytes remained unknown. In the present study, the percentage of CD4<sup>+</sup> T-cell subsets and related cytokines was measured using flow cytometry and an enzyme-linked immunosorbent assay. An abnormal number of CD4<sup>+</sup> T cell subsets was found, including increased percentages of T helper (Th)2, Th9 and Th17 cells and a decreased number of regulatory T (Treg) cells. In addition, the results showed downregulation in the levels of interleukin (IL)-2, transforming growth factor- $\beta$  and IL-35, and upregulation in the levels of IL-4, IL-6, IL-17, IL-23 and interferon- $\gamma$  in patients who did not receive therapy (untreated patients). These levels were significantly associated with the number of peripheral blood cells and were recovered following treatment. In conclusion, an abnormal number of CD4<sup>+</sup> T cell subsets and corresponding abnormal levels of regulatory cytokines resulted in the stimulation of B1 lymphocytes to produce autoantibodies in IRH, which may be considered as markers to evaluate disease prognosis and treatment strategies.

## Introduction

Previous studies have shown that certain patients with idiopathic cytopenia of undetermined significance can respond well to treatment with adrenocortical hormone and/or intravenous immunoglobulin (IVIG) (1,2). Autoantibodies on the membrane of bone marrow (BM) haemopoietic cells have been detected (1-5). Additional studies have indicated that autoantibodies inhibit the function of BM or reduce the number of BM haemopoietic cells by macrophage phagocytosis or by activating complement and inhibiting functional antigen formation (6-8). These findings indicate that persisting haemocytopenia may be a type of autoimmune disease, termed immune-related haemocytopenia (IRH). The production of autoantibodies in IRH may occur due to the abnormal production of B lymphocytes, notably B1 lymphocytes (CD5<sup>+</sup>) (9,10).

The function of B lymphocytes is regulated by T lymphocytes, including T helper (Th)1, Th2, Th9 and Th17 cells and regulatory T (Treg) cells. Usually, naïve CD4<sup>+</sup> T cells mature into Th1, Th2, Th9, Th17 or Treg cell subsets in response to innate immune signals, costimulatory interactions with antigen-presenting cells (APCs), paracrine cytokine signals, and due to mTOR-mediated changes in energy metabolism (11,12). Th1 cells are the quintessential cell type involved in cell-mediated inflammation and delayed-type hypersensitivity reactions, which are considered important for immunity against intracellular pathogens (12). Th2 cells were initially described as anti-inflammatory cells based on their ability to suppress cell-mediated immunity in Th1 models of autoimmune disease (11). Th17 cells have been shown to serve an important role in promoting and enhancing inflammation, including autoimmune tissue injury (13,14). The mechanisms of Treg-mediated immune suppression include the secretion of anti-inflammatory cytokines, the expression of inhibitory receptors and cytokine deprivation (15).

Previous research has shown that the percentage of Th17 cells is increased in IRH (16). The quantity and function of Treg cells in IRH were significantly lower than those of normal controls (17), whereas the quantity and function of

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**Key words:** cytopenia, autoantibodies, T lymphocytes, cytokines, relative telomere length.

T follicular helper cells (Tfh cells) were significantly increased in patients with IRH, which were positively associated with the presence of BM mononuclear cell antibodies, disease activity and the response to treatment (18). These preliminary studies indicated that Th cells maybe involved in the pathogenesis of IRH. To date, the number of studies that have examined the function and quantity of T lymphocytes in IRH is limited. The present study focused on the evaluation of several T cell subsets in patients with IRH, including Th1/2, Th17, Th9 cells and Tregs, and their secreted regulatory cytokines in order to elucidate their pathogenic mechanism of action. The relative telomere length (RTL) was also detected in patients with untreated IRH and control subjects in order to assess the degree and number of abnormal CD4<sup>+</sup> T cells. The number of CD19<sup>+</sup> B cells was also measured to observe the potential association between these cells and the incidence of IRH.

## Materials and methods

**Patients.** A total of 44 (25 women and 19 men) patients with IRH, with a median age of 36 years (range 11-69 years), were enrolled in the present study, including 18 patients who did not receive therapy (untreated patients; 11 women and seven men, median age 44.5 years, range 16-68 years) and 26 patients in remission (14 women and 12 men, median age 31 years, range 11-69 years). All patients were inpatients of the Department of Haematology, Tianjin medical University General Hospital, who were admitted between October 2015 and October 2016 and were diagnosed with IRH according to He *et al* (2). The patients received corticosteroids (prednisone, 0.5 mg/kg/day), cyclosporine (CsA; 3 mg/kg/day), and high-dose IVIG (Chengdu Institute of Biological Products, Sichuan, China, 0.4 g/kg/day for 5 days) if they were dependent on blood transfusion. Complete blood count and BM examination were performed regularly. The response criteria were measured according to those used for aplastic anaemia (AA) (19). The median follow-up time was 16 months (range 3-60 months) for all patients and 32 months (19-60 months) for patients in remission. A total of 15/26 patients in remission received CsA immunosuppressive therapy for >1 year following remission, whereas therapy administered in the remaining 11 patients was terminated within 1 year.

A total of 20 healthy volunteers (10 women and 10 men) with a median age of 32 years (range 22-48 years) were enrolled in the study as control subjects. A total of 10 ml of peripheral blood (PB) was obtained from the patients and the control subjects. The present study was approved by the Ethics Committee of Tianjin Medical University. Written informed consent was obtained from the patients and/or their parents in case the participants <16 years old.

**Flow cytometry.** Several T cell subsets, including Th1 [CD4<sup>+</sup> interferon (IFN)- $\gamma$ <sup>+</sup>], Th2 [CD4<sup>+</sup>interleukin (IL)-4<sup>+</sup>], Treg [CD4<sup>+</sup>CD25<sup>+</sup> forkhead box P3 (FoxP3<sup>+</sup>)], regulatory B (Breg; CD19<sup>+</sup>IL-10<sup>+</sup>), Th9 (CD4<sup>+</sup>IL-9<sup>+</sup>) and Th17 (IL-17<sup>+</sup>CD4<sup>+</sup>) cells and the CD5<sup>+</sup>CD19<sup>+</sup> B cell subset were detected by flow cytometry. For the Th1, Th2, Th9, Th17 and Breg cells, peripheral blood mononuclear cells (PBMCs) were incubated with 50 ng/ml of phorbol ester (Beyotime Institute of Biotechnology, Jiangsu, China), 1  $\mu$ g/ml of Brefeldin A (Beyotime Institute of

Biotechnology) and 1  $\mu$ g/ml of ionomycin (Beyotime Institute of Biotechnology) at 37°C for 5 h.

Briefly, fresh PB (400  $\mu$ l) was collected and separated into four tubes with EDTA-anticoagulant. A total of 20  $\mu$ l of mouse IgG1-FITC (cat. no. 551954), mouse IgG1-PE (cat. no. 555749) and mouse IgG1-APC (cat. no. 555751) antibodies (all BD Pharmingen, San Diego, CA, USA) were added into the negative tube. A total of 20  $\mu$ l of antibody against CD4-FITC (cat. no. 561842; BD Biosciences, Franklin Lakes, NJ, USA), CD25-APC (cat. no. 560987; BD Pharmingen) and CD5-FITC (cat. no. 555352; BD Biosciences), CD19-APC (cat. no. 561742; BD Biosciences) were separately added into different test tubes. Following incubation in the dark at 4°C for 30 min, the red blood cells were lysed with 5 ml of erythrocytolysin solution (BD Biosciences) and subsequently centrifuged at 150 x g for 5 min at room temperature. Following washing with PBS, the cells were permeabilised using a Cytotfix/Cytoperm Buf kit (BD Pharmingen) in the dark for 10 min and further washed with PBS. A total of 20  $\mu$ l of antibody against FoxP3-PE (cat. no. 560852), IL-17-PE (cat. no. 560436), IL-11-APC (cat. no. 560228), IL-10-APC (cat. no. 558458), IL-9-PE (cat. no. 560807), IFN- $\gamma$ -APC (cat. no. 551385) and IL-4-PE (cat. no. 559333; all BD Biosciences) were added separately into three test tubes and incubated for 30 min in the dark. Subsequently, the cells were washed twice and resuspended with PBS. At least 300,000 counts were obtained using a BD FACSCalibur flow cytometer (BD Biosciences). The results were analysed using CellQuest software 5.2.1. (BD Biosciences).

The fresh heparinized BM samples (400  $\mu$ l) were washed with PBS three times, separated into four tubes and stained with either 20  $\mu$ l of mouse IgG1-FITC (cat. no. 551954), 20  $\mu$ l of mouse IgG1-PE (cat. no. 555749), or 20  $\mu$ l of mouse IgG1-APC (cat. no. 555751) as a negative control, or stained separately with 20  $\mu$ l of CD15-FITC (cat. no. 555401), 20  $\mu$ l of GlyCoA-FITC (cat. no. 565234) and 20  $\mu$ l of CD34-FITC (cat. no. 560942; all BD Pharmingen). A total of 20  $\mu$ l of anti-human IgG-PE (cat. no. 555787) and anti-human IgM-APC (cat. no. 551062; BD Pharmingen) were added to each tube. Following incubation in the dark for 30 min at 4°C, the cells were incubated with 2 ml erythrocyte lytic solution (BD Pharmingen) for 10 min at room temperature and washed three times with PBS. Finally, at least 30,000-100,000 cells were acquired and analysed on a FACSCalibur flow cytometer.

**Enzyme-linked immunosorbent assay (ELISA).** The serum levels of IL-2, IL-4, IL-6, IL-17, IL-23, transforming growth factor (TGF)- $\beta$  and IFN- $\gamma$  in the patients with untreated IRH, remission and control subjects were measured using ELISA reagent kits for IL-2 (cat. no. 171B5003M) and TGF- $\beta$  (cat. no. 171W4001M; Bio-Rad Laboratories, Inc., Hercules, CA, USA), IL-6 (cat. no. D6050) and IL-23 (cat. no. D2300B; R&D Systems, Inc., Minneapolis, MN, USA) and IL-4 (cat. no. SEA077Hu), IL-17 (cat. no. SEA063Hu), IL-35 (cat. no. SEC008Hu) and IFN- $\gamma$  (cat. no. SEA033Hu; USCNLIFE, Inc., Wuhan, China).

Briefly, diluted standards and patient serum (100  $\mu$ l) were added in duplicate and incubated at 37°C for 2 h. Following five washes with 1X wash buffer concentrate, 100  $\mu$ l of antibody was added to each well and incubated at room

temperature for 90 min. Subsequently, HRP was added to each well. Following incubation at 37°C for 30 min, the wells were washed five times. Subsequently, TMB solution was added to each well, and the samples were incubated in the dark at room temperature for 20 min. Finally, a stop solution was added, and the optical density (OD) was read at 450 nm within 15 min.

**Sorting of CD4<sup>+</sup> and CD19<sup>+</sup> lymphocytes by magnetic-activated cell sorting (MACS).** The PBMCs were isolated from the heparinized anticoagulant venous blood of the IRH and control subjects using Ficoll-Hypaque density gradient centrifugation at room temperature for 20 min at 600 x g. The CD4<sup>+</sup>T and CD19<sup>+</sup>B lymphocytes were purified using the respective anti-CD4 or anti-CD19 mAb-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. A total of 10,000,000 cells were resuspended in 90  $\mu$ l of buffer. Subsequently, 20  $\mu$ l of CD4 or CD19 microbeads (Miltenyi Biotec) were added and incubated at 4°C in the dark for 20 min. Following washing with 2 ml of buffer, the cells were centrifuged at 300 g for 5 min and resuspended in 500  $\mu$ l of buffer. The MS column was placed in the magnetic field of a suitable MACS separator (Miltenyi Biotec). Following preparation of the column by rinsing with 1.5 ml of buffer, the cells were added to the column. The column was washed with 1.5 ml of buffer and all flow-through unlabelled cells were collected. The magnetically-labelled cells were immediately flushed out by firmly pushing the plunger into the column. The purity of the enriched isolated CD4<sup>+</sup> and CD19<sup>+</sup> lymphocytes was evaluated by flow cytometry and was typically >90%.

**Non-adherent cell culture of MOLT-4.** MOLT-4 cells (National Infrastructure of Cell Line Resource, Beijing, China) were used as control cells and were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin (Gibco BRL; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were grown for 2-3 days in liquid.

**FLOW-fluorescence in situ hybridization (FISH).** The analysis was conducted according to the telomere PNA kit/FITC used for flow cytometry (Dako, Carpinteria, CA, USA). A total of 1 and/or 2x10<sup>6</sup> sorted cells and control cells were diluted in PBS and divided into two tubes, namely A and B. The DNA was denatured at 82°C for 10 min in an Eppendorf tube in the presence of hybridization solution with or without a fluorescein-conjugated PNA telomere probe. Subsequently, hybridization was performed in the dark at room temperature overnight. The samples were washed twice in washing solution at 40°C for 10 min each. Finally, the cells were resuspended in DNA-staining solution and stored in the dark at 2-8°C for 2-3 h prior to flow cytometric analysis. The specific fluorescence activity was proportional to the telomere staining and was detected in FL1, whereas the fluorescence derived from DNA staining was detected in FL3. Finally, at least 20,000 cells were acquired and analysed using the fluorescence-activated cell sorter (FACSCalibur) flow cytometer (BD Biosciences). The DNA index of the cells was determined as follows: RTL = (mean FL1 sample cells with probe-mean FL1 sample cells without probe) x DNA index of control cells x 100/(mean

FL1 control cells with probe-mean FL1 control cells without probe) x DNA index of sample cells.

**Statistical analysis.** SPSS 21.0 software (IBM Corp.) was used for statistical analysis. Data are presented as the mean  $\pm$  standard deviation. The significance of the differences was assessed by one-way ANOVA and the independent sample t-test. Relapse rates were assessed using the chi-square test (Fisher's exact test) in the two groups. Data that exhibited correlation were assessed using Spearman's rank correlation. P<0.05 was considered to indicate a statistically significant difference.

## Results

**CD4<sup>+</sup> T cells exhibit abnormal number in untreated patients with IRH, as demonstrated by increased percentages of Th2, Th9, Th17 and Breg cells and decreased percentages of Treg cells.** The percentages of CD4<sup>+</sup> T cells, Th1, Th2, Th9, Th17, Treg and Breg cells are shown in Table I. It was found that the percentages of the CD4<sup>+</sup> T cell lymphocyte populations were decreased in the untreated patients with IRH compared with those noted in the patients of the remission group (P=0.004) and control group (P<0.001, Fig. 1A). The percentages of Th1 and Th2 cells in the CD4<sup>+</sup> T lymphocyte population were increased significantly in the untreated patients with IRH compared with those noted in the control subjects (P=0.013 and P<0.001, respectively). In addition, the percentage of Th1 cells was higher in patients in the remission group than that in patients in the control group (P=0.042). The percentage of Th2 cells was reduced significantly following treatment (P<0.001), although it was higher than the percentage noted in the control subjects (P=0.008). The ratio of Th1/Th2 lymphocytes was further evaluated, and the results indicated that the ratio in the untreated IRH group was significantly lower than the ratios in the IRH remission group and control group (P=0.043 and P<0.001, respectively, Fig. 1B). These results indicated that Th2 cells may be important in the development of IRH. The percentage of Th9 cells in the untreated patient group was significantly increased compared with that noted in the remission group and control group (P<0.001, Fig. 1C).

The percentage of Th17 cells was also significantly increased in the untreated patients compared with that in the control subjects (P<0.001). Following treatment, the percentage of Th17 cells decreased significantly (P=0.007), but remained at a higher level than that in the control group (P=0.006, Fig. 2A). The percentage of Treg cells in the untreated patients was significantly decreased compared with that in the remission patients (P=0.048) and control subjects (P<0.001). In addition, the levels of Treg cells in the remission group was significantly lower than that of the control group (P=0.001, Fig. 2B). In contrast to the Treg cells, the percentages of Breg cells in the untreated and remission patients were significantly increased compared with that in the control subjects (P=0.018 and P=0.032, respectively, Fig. 2C). No significant differences were noted with regard to these parameters between the untreated and remission groups.

A value of autoantibodies >4.0% was defined as positive in IRH. From these findings, the IRH patients were divided into two groups: i) IRH patients with a value of  $\geq$ 10%; ii) IRH patients with a value of <10%. There was no statistical significance between the two groups in terms of an abnormal

Table I. Percentages of different CD4<sup>+</sup> T lymphocyte subsets.

	CD4 <sup>+</sup> T cell (CD4 <sup>+</sup> /Lym) %	Th1 (CD4 <sup>+</sup> IFN- $\gamma$ +/ CD4 <sup>+</sup> ) %	Th2 (CD4 <sup>+</sup> IL-4+/ CD4 <sup>+</sup> ) %	Th1/Th2	Th9 (CD4 <sup>+</sup> IL-9+/ CD4 <sup>+</sup> ) %	Th17 (CD4 <sup>+</sup> IL-17+/ CD4 <sup>+</sup> ) %	Treg (CD4 <sup>+</sup> CD25 <sup>+</sup> FoxP3 <sup>+</sup> /CD4 <sup>+</sup> ) %	Breg (CD19 <sup>+</sup> IL-10 <sup>+</sup> / CD4 <sup>+</sup> ) %
Untreated	29.8039 $\pm$ 3.8688 <sup>a,b</sup>	3.1367 $\pm$ 2.0143 <sup>b</sup>	4.9783 $\pm$ 1.8487 <sup>a,b</sup>	0.6630 $\pm$ 0.345 <sup>a,b</sup>	2.73 $\pm$ 1.96 <sup>a,b</sup>	4.8311 $\pm$ 2.5255 <sup>a,b</sup>	1.4333 $\pm$ 0.7255 <sup>a,b</sup>	26.5020 $\pm$ 6.9294 <sup>b</sup>
Remission	34.1265 $\pm$ 4.3872 <sup>c</sup>	2.0973 $\pm$ 0.9592 <sup>c</sup>	1.8927 $\pm$ 0.9811 <sup>c</sup>	1.2527 $\pm$ 0.496 <sup>c</sup>	1.17 $\pm$ 0.79	2.6134 $\pm$ 1.1236 <sup>c</sup>	1.9127 $\pm$ 0.8079 <sup>c</sup>	22.5186 $\pm$ 9.8210 <sup>c</sup>
Control	38.1215 $\pm$ 3.9399	1.2527 $\pm$ 0.4963	1.1345 $\pm$ 0.5741	1.8525 $\pm$ 1.488	0.67 $\pm$ 0.40	1.8035 $\pm$ 0.9225	2.7095 $\pm$ 0.7158	12.0657 $\pm$ 4.2323
Ab% ( $\geq$ 10)	29.7370 $\pm$ 3.4924	3.2300 $\pm$ 1.6574	4.4664 $\pm$ 1.5671	1.0366 $\pm$ 0.814	2.42 $\pm$ 1.50	4.6517 $\pm$ 2.1363	1.9238 $\pm$ 0.4042	28.0968 $\pm$ 4.6957
Ab% (<10)	30.5481 $\pm$ 4.1705	2.1257 $\pm$ 1.5139	4.4603 $\pm$ 1.5581	1.5122 $\pm$ 0.212	1.86 $\pm$ 1.44	3.6513 $\pm$ 1.5129	2.7241 $\pm$ 1.0677	25.9168 $\pm$ 7.8114

<sup>a</sup>P=0.004 (CD4<sup>+</sup> T cell); P<0.001 (Th2); P=0.043 (Th1/Th2); P<0.001 (Th9); P=0.007 (Th17); P=0.048 (Treg) vs. remission. <sup>b</sup>P=0.007 (CD4<sup>+</sup> T cell), P<0.001 (Th2), P<0.001 (Th1/Th2), P<0.001 (Th9), P<0.001 (Th17), P<0.001 (Breg) vs. control. <sup>c</sup>P<0.001 (CD4<sup>+</sup> T cell), P=0.042 (Th1), P=0.008 (Th2), P=0.034 (Th1/Th2), P=0.006 (T17), P=0.001 (Treg), P=0.032 (Breg) vs. control. Ab%, the percentage of autoantibodies; Lym, lymphocyte; Th, T helper cell; Treg, regulatory T cell; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; FoxP3, forkhead box P3.

percentage of CD4<sup>+</sup> T cell subsets or regulatory cytokines regulated B1 lymphocytes, but the trend in accordance with the present results (Table I).

The correlation analysis between clinical data and the percentages of the different T lymphocyte subsets is shown in Table II. A negative correlation was noted between the percentages of Th1 and Th2 lymphocytes and the levels of haemoglobin (Hb) in patients with IRH. A negative correlation was also noted between the percentages of Th1 and Th2 lymphocytes and the white blood cells (WBC) and platelet (Plt) numbers in the patients with IRH. Furthermore, the percentage of Th17 cells exhibited a negative correlation with the levels of Hb and Plt numbers. In contrast to Th17 cells, the percentage of Treg cells exhibited a positive correlation with the levels of Hb and the WBC and Plt numbers. However, the percentage of neutrophils and reticulocytes showed no significant correlation with the percentage of T lymphocyte subsets.

*CD5<sup>+</sup> B cell numbers are increased in patients with untreated IRH.* The percentage of CD5<sup>+</sup>CD19<sup>+</sup> B lymphocytes in the CD19<sup>+</sup> B lymphocyte population was significantly increased in the untreated IRH patient group (26.6006 $\pm$ 9.1446%) compared with that in the remission group (17.7075 $\pm$ 8.9295%, P=0.011) and that in the control subjects (12.2995 $\pm$ 3.5353%, P<0.001, Fig. 2D). The percentage of CD5<sup>+</sup>CD19<sup>+</sup> B lymphocytes in the IRH patients exhibited a positive correlation with the percentage of Th1 (r=0.262, P=0.046), Th2 (r=0.447, P=0.002) and T17 (r=0.318, P=0.035) cells, and a negative correlation with that of Treg lymphocytes (r=-0.341, P=0.024; Table II).

*Secreted levels of abnormal cytokines are associated with the percentage of CD4<sup>+</sup> T cells in patients with untreated IRH.* In order to examine the regulation of CD4<sup>+</sup> T cells in the present study, the levels of several cytokines were measured in the patients with IRH, including IL-2, IL-4, IL-6, IL-17, IL-23, IL-35, TGF- $\beta$  and IFN- $\gamma$ , which are shown in Table III. It was found that the levels of IL-4, IL-6, IL-17 and IL-23 in the untreated patients were increased compared with those in the remission patients and control subjects. The levels of these cytokines in the remission group were significantly higher than those in the control subjects (Fig. 3). The levels of IL-2, IL-35 and TGF- $\beta$  were decreased significantly in the untreated patients compared with those in the remission patients and control subjects, while the corresponding levels in the remission patients were significantly lower than those in the control subjects (Fig. 3). Furthermore, the levels of IFN- $\gamma$  were increased in the untreated patients compared with those in the remission patients and control subjects, whereas no significant difference was noted between the latter two groups.

*RTLs of CD4<sup>+</sup> T cells and CD19<sup>+</sup> B cells are shortened in patients with untreated IRH.* The RTLs of the CD19<sup>+</sup> B and CD4<sup>+</sup> T lymphocytes of the untreated IRH group and of the control group were measured using flow cytometry (Fig. 4A and B). The RTLs of CD19<sup>+</sup> B and CD4<sup>+</sup> T lymphocytes in the untreated IRH patient group were 22.1360 $\pm$ 15.3903 and 7.2725 $\pm$ 3.2566%, respectively, which were shorter than those in the control group (42.7313 $\pm$ 14.7974%, P=0.003, Fig. 4C-a,

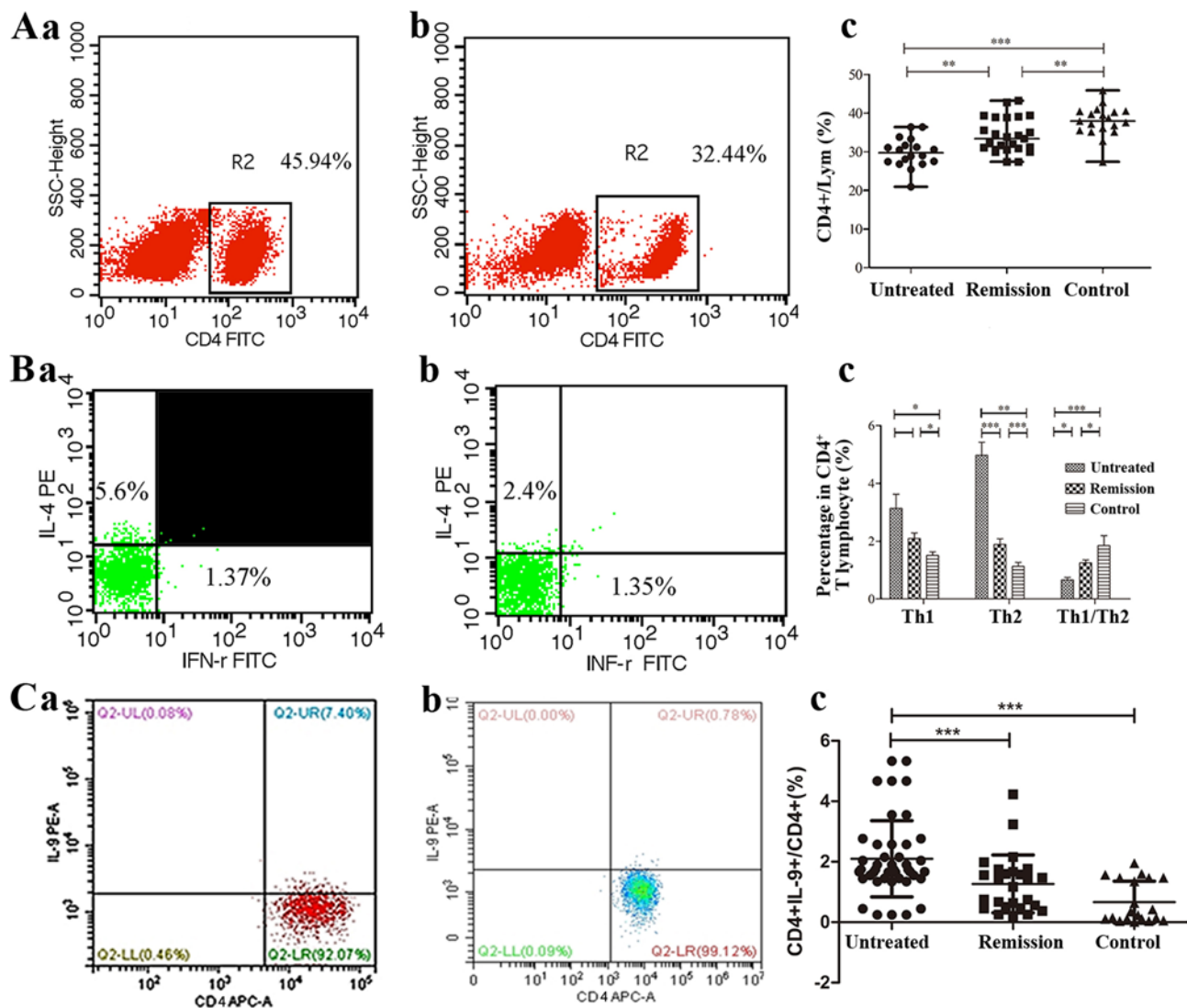


Figure 1. Percentages of different CD4<sup>+</sup> T lymphocyte subsets in patients with IRH and control subjects detected by flow cytometry. (A) CD4<sup>+</sup> T cells in (a) patients with untreated IRH and (b) control subjects; (c) significant differences were noted among the groups. (B) Th1 and Th2 lymphocytes in (a) patients with untreated IRH and (b) control subjects; (c) significant differences were noted among the groups. (C) Th9 lymphocytes in (a) patients with untreated IRH and (b) control subjects; (c) the percentage of Th9 cells was significantly higher in the IRH group than that in the untreated patients. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. IRH, immune-related haemocytopenia; IL, interleukin; IFN, interferon; Th, T helper; APC, antigen-presenting cell.

vs.  $12.0657 \pm 2.4007\%$ ,  $P < 0.001$ , Fig. 4C-b). The RTLs of the CD19<sup>+</sup> B and CD4<sup>+</sup> T lymphocytes of the IRH patient group were negatively correlated with the percentages of Th1 cells ( $r = -0.79$ ,  $P = 0.002$  and  $r = -0.602$ ,  $P = 0.013$ , respectively), Th2 cells ( $r = -0.676$ ,  $P = 0.016$  and  $r = -0.655$ ,  $P = 0.021$ , respectively) and Th17 cells ( $r = -0.657$ ,  $P = 0.02$  and  $r = -0.531$ ,  $P = 0.075$ , respectively). These percentages were also positively correlated with those of Treg lymphocytes ( $r = 0.748$ ,  $P = 0.005$  and  $r = 0.678$ ,  $P = 0.015$ , respectively; Table II).

**Relapse rates of patients in remission.** All patients in remission were followed up in order to observe their treatment and relapse rates. Of 26 patients in remission, 15 patients received CsA as immunosuppressive therapy for >1 year following remission, whereas the remaining 11 patients underwent therapy termination within 1 year. The median follow-up time for the patients in remission was 32 months (19-60 months). Subsequently, the relapse rates in the 2-year follow-up period

were compared between the two groups. The results showed that 45.5% (5/11) of the patients relapsed in the intermittent treatment group, which was higher than that noted in the continuous group (20%, 3/15,  $P = 0.218$ ).

## Discussion

IRH is one type of autoimmune BM failure disease, which is mediated by autoantibody secretion on the membrane of BM haemopoietic cells. Autoantibodies can inhibit haemopoiesis and thereby induce haemocytopenia (5).

In the present study, it was demonstrated that the percentages of Th1 and Th2 cells were increased in the patients with untreated IRH, whereas the Th1/Th2 ratio was decreased, indicating that Th2 cells serve a major role in the development of this disease. It is well known that Th2 cells are responsible for humoral-mediated immunity, while Th1 cells are responsible for cell-mediated immunity. In addition, Th2 cells



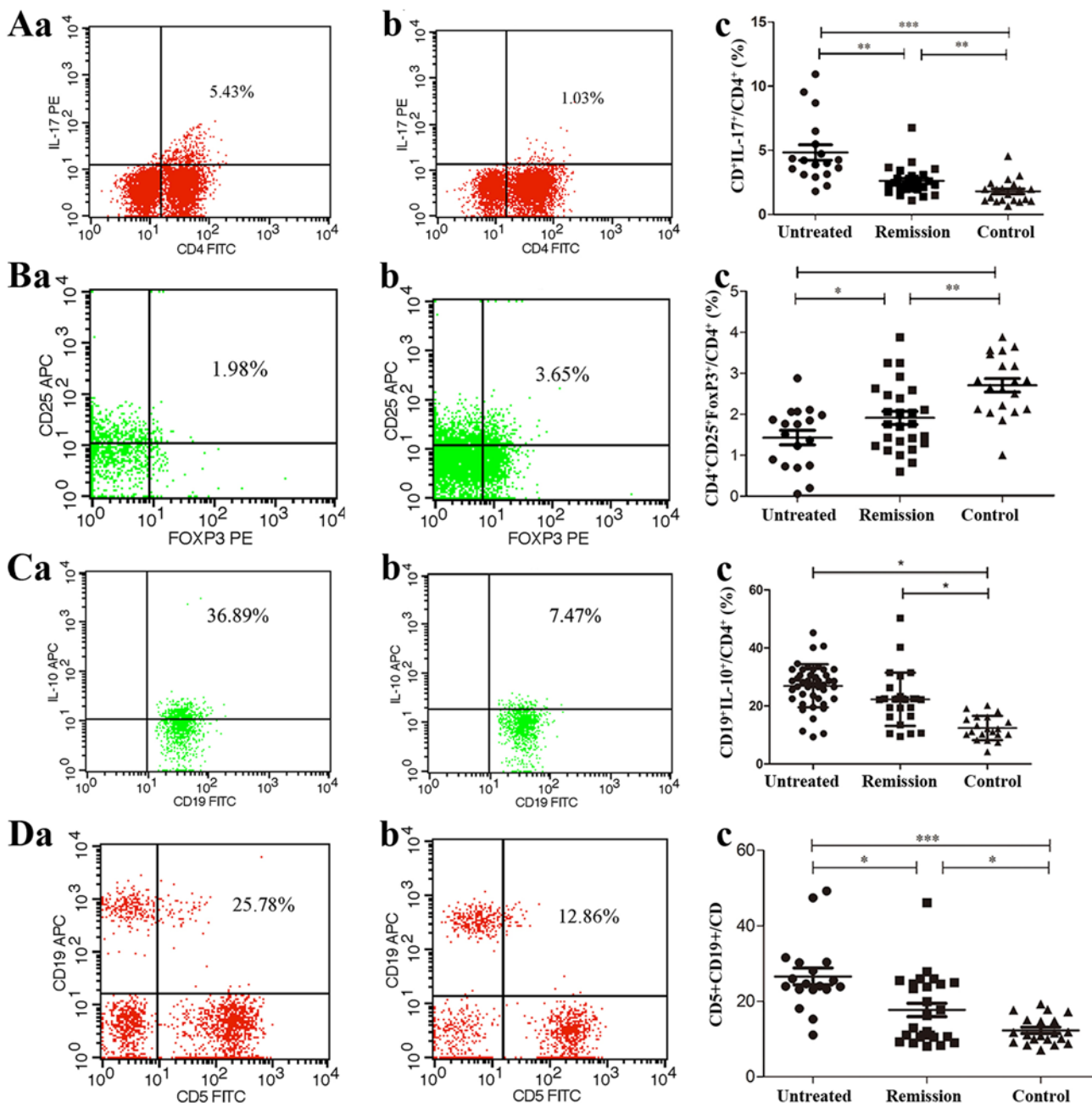


Figure 2. Percentages of different CD4<sup>+</sup> T lymphocyte subsets in patients with IRH and control subjects detected by flow cytometry. (A) Th17 lymphocytes in (a) patients with untreated IRH and (b) control subjects; (c) the percentage of Th17 cells was significantly higher in patients with IRH than that in untreated patients. (B) Percentages of Treg cells in patients with (a) untreated IRH and (b) control subjects; (c) the percentages of Treg cells in untreated and remission patients were significantly decreased compared with that in control subjects. (C) Percentages of Breg cells in patients with (a) untreated IRH and (b) control subjects; (c) the percentages of Breg cells in untreated and remission patients were significantly increased compared with that in control subjects. (D) Percentages of CD5<sup>+</sup>CD19<sup>+</sup> B lymphocytes in (a) patients with untreated IRH and (b) control subjects; (c) the percentage of CD5<sup>+</sup>CD19<sup>+</sup> B lymphocytes was increased significantly in patients with IRH. The data are expressed as the mean  $\pm$  SD. (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001). IRH, immune-related haemocytopenia; IL, interleukin; FoxP3, forkhead box P3; Th, T helper cell; Treg, regulatory T cell; APC, antigen-presenting cell.

secrete IL-4, which is closely associated with the production of IgG1 by B cells (20). In the present study, the levels of IL-4 were upregulated in patients with untreated IRH and were higher than those in control subjects following remission. Therefore, the present study indicated that IRH is a type of autoantibody-mediated autoimmune disease.

The data revealed a significant decrement in the levels of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells in patients with IRH compared with those in control subjects. Naturally occurring

CD4<sup>+</sup>CD25<sup>+</sup> Treg cells are key in immune tolerance. Foxp3 serves a central role in the differentiation and maintenance of Treg cells. It has been shown that Foxp3 gene transfer can convert naïve CD4<sup>+</sup>CD25<sup>-</sup> T cells into a functional regulatory population (21). In addition, Treg cells exert suppressive effects on the effector T cells by secreting IL-10, which can inhibit APC maturation and exert direct suppressive effects on effector T cells (22). Tregs can further inhibit the proliferation and cytokine production of Th1 and Th2 cells *in vitro*

Table II. Correlation analysis between PB cells, RTLs of lymphocytes and levels of T lymphocytes.

Factor	n	Mean $\pm$ SD	Cell	+/-	P-value	r
Hb (g/l)	44	94.2954 $\pm$ 27.6023	Th1	-	0.012 <sup>a</sup>	-0.377
			Th2	-	0.009 <sup>b</sup>	-0.387
			Th17	-	<0.001 <sup>c</sup>	-0.504
			Treg	+	0.001 <sup>b</sup>	0.501
WBC ( $\times 10^9$ )	44	4.4843 $\pm$ 2.3569	Th1	-	0.015 <sup>a</sup>	-0.364
			Th2	-	0.012 <sup>a</sup>	-0.374
			Th17	N	0.084	-0.263
			Treg	+	0.037 <sup>a</sup>	0.316
N (%)	44	50.1046 $\pm$ 19.6863	Th1	N	0.379	-0.136
			Th2	N	0.350	0.144
			Th17	N	0.261	0.173
			Treg	N	0.877	0.024
Plt ( $\times 10^9$ )	44	54.4545 $\pm$ 37.5143	Th1	-	0.001 <sup>b</sup>	-0.474
			Th2	-	0.034 <sup>a</sup>	-0.32
			Th17	-	0.001 <sup>b</sup>	-0.484
			Treg	+	0.001 <sup>b</sup>	0.481
Ret (%)	44	1.8959 $\pm$ 0.8766	Th1	N	0.375	-0.137
			Th2	N	0.211	-0.192
			Th17	N	0.387	-0.134
			Treg	N	0.098	0.253
CD5 <sup>+</sup> CD19 <sup>+</sup> /CD19 <sup>+</sup> (%)	44	21.3447 $\pm$ 10.1374	Th1	+	0.046	0.262
			Th2	+	0.002 <sup>b</sup>	0.447
			Th17	+	0.035 <sup>a</sup>	0.318
			Treg	-	0.024 <sup>a</sup>	-0.341
RTLs of CD19 <sup>+</sup> B cells	12	22.1360 $\pm$ 15.3903	Th1	-	0.002 <sup>b</sup>	-0.79
			Th2	-	0.016 <sup>a</sup>	-0.676
			Th17	-	0.020 <sup>a</sup>	-0.657
			Treg	+	0.005 <sup>b</sup>	0.748
RTLs of CD4 <sup>+</sup> T cells	12	7.2725 $\pm$ 3.2566	Th1	-	0.013 <sup>a</sup>	-0.692
			Th2	-	0.021 <sup>a</sup>	-0.655
			Th17	N	0.075	-0.531
			Treg	+	0.015 <sup>a</sup>	0.678

<sup>a</sup>P<0.05; <sup>b</sup>P<0.01; <sup>c</sup>P<0.001. +, positive correlation; -, negative correlation; Th, T helper cell; Treg, regulatory T cell; Hb, haemoglobin; WBC, white blood cells; N, neutrophils; Plt, platelets; Ret, reticulocytes; RTLs, relative telomere lengths; N, no correlation.

and *in vivo* (23) and possibly suppress B cell activation (24). IL-2 has been shown to regulate the expression of CD25 (25) and was critically required for the peripheral maintenance of natural CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, which consequently sustained immunologic self-tolerance (25,26). TGF- $\beta$  serves a key role in the differentiation and function of Treg cells in mice by inducing the expression of Foxp3 *in vivo* and *in vitro* (27). Furthermore, TGF- $\beta$  produced by Treg cells suppresses immune responses in different target cells (28). A newly discovered cytokine that is secreted by CD4<sup>+</sup>CD25<sup>+</sup> Tregs has been identified as IL-35. IL-35 can promote the suppressive function of Treg cells and restrict the differentiation and function of Th1/Th17 cells (29). In patients with untreated IRH, the levels of IL-2, IL-35 and TGF- $\beta$  were decreased compared with those in control subjects, indicating that these cells serve a protective role in the progression of IRH.

It has already been shown that the development of Th17 cells is prompted by a combination of IL-6 and TGF- $\beta$ , which require the expression of STAT3 and the retinoic acid-related orphan receptor  $\gamma$ t (30,31). Th17 cells are not terminally differentiated, as they are able to switch to Th1 cells and are therefore implicated in several inflammatory reactions (32-34). The main secretory cytokine of Th1 cells is IL-17. In the present study, the percentage of Th17 cells and the serum levels of IL-17 were significantly higher than those of control subjects. IL-17 acts as a pleiotropic cytokine and as a chemoattractant for neutrophils (34). It is also a suppressor of Th1 and Treg cells (34). IL-23 is known to be a survival and proliferative factor for Th17 cells (35). Emerging data suggest that not all Th17 cells are pathogenic, and that the pathogenic state is induced only following exposure to APCs that secrete IL-23. IL-23 is crucial for the ability of Th17 cells to induce

Table III. Serum levels of cytokines measured by enzyme-linked immunosorbent assay.

Cytokine (pg/ml)	Untreated	Remission	Control
IL-2	149.2868±45.8402	192.5027±55.0910 <sup>c</sup>	268.2111±62.8435
IL-4	9.7976±1.9017 <sup>a,b</sup>	4.9491±2.8450 <sup>c</sup>	2.5726±2.0220
IL-6	22.2948±13.0507 <sup>a</sup>	12.8473±6.5653 <sup>c</sup>	7.1145±2.4324
IL-17	273.4800±58.0620 <sup>a</sup>	206.1831±44.0075 <sup>c</sup>	170.5465±26.4704
IL-23	182.4770±82.8248 <sup>a</sup>	96.6372±53.6834 <sup>c</sup>	56.2377±32.1117
IL-35	20.5905±6.0470 <sup>a,b</sup>	30.7670±10.6000 <sup>c</sup>	98.4509±57.0162
IFN- $\gamma$	152.9567±32.8728	93.0603±30.7645	63.7026±40.2122
TGF- $\beta$	15.6375±5.2789 <sup>a,b</sup>	20.4485±5.8137 <sup>c</sup>	28.1787±8.9327

<sup>a</sup>P=0.022 (IL-2), P<0.001 (IL-4), P=0.033 (IL-6), P=0.001 (IL-17), P=0.002 (IL-23), P=0.001 (IL-35), P<0.001 (IFN- $\gamma$ ) and P=0.025 (TGF- $\beta$ ) compared with remission patients. <sup>b</sup>P<0.001 (IL-2), P<0.001 (IL-4), P=0.001 (IL-6), P<0.001 (IL-17), P<0.001 (IL-23), P<0.001 (IL-35), P<0.001 (IFN- $\gamma$ ) and P<0.001 (TGF- $\beta$ ) compared with normal controls. <sup>c</sup>P<0.001 (IL-2), P=0.007 (IL-4), P=0.001 (IL-6), P=0.005 (IL-17), P=0.01 (IL-23), P<0.001 (IL-35) and P=0.005 (TGF- $\beta$ ) compared with normal controls. IL, interleukin; INF, interferon; TGF, transforming growth factor.

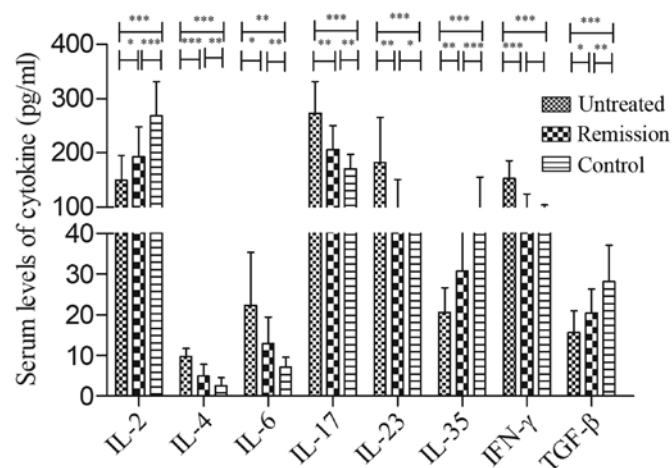


Figure 3. Expression of serum levels of different cytokines in patients with IRH and control subjects, as determined by enzyme-linked immunosorbent assay. Data are expressed as the mean  $\pm$  SD. (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001). IRH, immune-related haemocytopenia; IL, interleukin; IFN, interferon; TGF, transforming growth factor.

autoimmunity. It acts on newly generated IL-23R-expressing Th17 cells and causes a shift in function in order to produce IFN- $\gamma$  (36,37). In addition, the present study found that serum levels of IL-23 were increased in patients with IRH and that they activated Th17 cells to induce the autoimmune status of the disease. Th9 cells require the combination of TGF- $\beta$  and IL-4 for their development. Th9 cells are important in certain autoimmune diseases through the production of IL-9. IL-9 induces CD4<sup>+</sup> T cells to differentiate into Th17 cells. IL-9 enhances the suppressive function of Treg cells. TGF- $\beta$  promotes Treg maturation, whereas IL-4 induces Th2 cell activation (38). The results of the present study indicated that the levels of Th9 and Th17 were increased.

The balance between suppressive Treg cells and pathogenic Th17 cells is critical in orchestrating autoimmune responses (39). IL-6 serves a major role in determining this balance (39). In the present study, a significant increase in the plasma levels of IL-6 was noted in patients with IRH. This

pleiotropic inflammatory cytokine is produced by T cells, monocytes, macrophages and synovial fibroblasts, and mediates various functions by binding to its receptor IL-6R (40). As a potent activator of STAT3, IL-6 has the capacity to switch immune responses from the induction of suppressive Treg cells to the development of pathogenic Th17 cells (41). Furthermore, the IL-6-induced B cell help requires the expression of the signal transducer and activator STAT3, suggesting that the ability to provide help to B cells is not restricted to a well-defined Th1 or Th2 effector population (31).

The hyperfunction and increased levels of B1 lymphocytes may be caused by the imbalance of Th1/Th2 cells. It has been shown that the abnormal number and function of B1 lymphocytes may be one factor causing the production of autoantibodies in patients with IRH (42). In the present study, the percentage of abnormal T lymphocytes and CD5<sup>+</sup> B lymphocytes gradually returned to normal in patients with IRH who were treated with immunotherapy and presented with disease remission and/or curative effects. No significant difference was noted between the cured patients and control subjects. Therefore, the levels of abnormal T lymphocytes and CD5<sup>+</sup> B lymphocytes can be used to evaluate the treatment of patients with IRH and the prognosis of the disease. B1 cells, notably B1a cells, have been shown to contribute to autoimmune pathogenesis by the production of autoantibodies (43), antigen presentation, activation of CD4<sup>+</sup> T cells (44,45) and cytokine production (46). The results of the present study further revealed a significant increase in the levels of CD5<sup>+</sup>CD19<sup>+</sup> B1 lymphocytes in patients with IRH. Our previous studies showed a positive correlation between the levels of IgG1 and the proportion of CD5<sup>+</sup> B1 lymphocytes, and that haematopoietic cells may be targeted by IgG1 autoantibodies in certain patients with IRH (47). Furthermore, B1 cells can induce T cells to express IL-17 and IFN- $\gamma$  to a greater extent than that observed with either B2 cells or dendritic cells (44). Accumulating evidence has shown the multifaceted functions of B1 cells, which include several processes other than antibody production. These functions are implicated in the development of autoimmune diseases, including autoimmune arthritis and systemic lupus



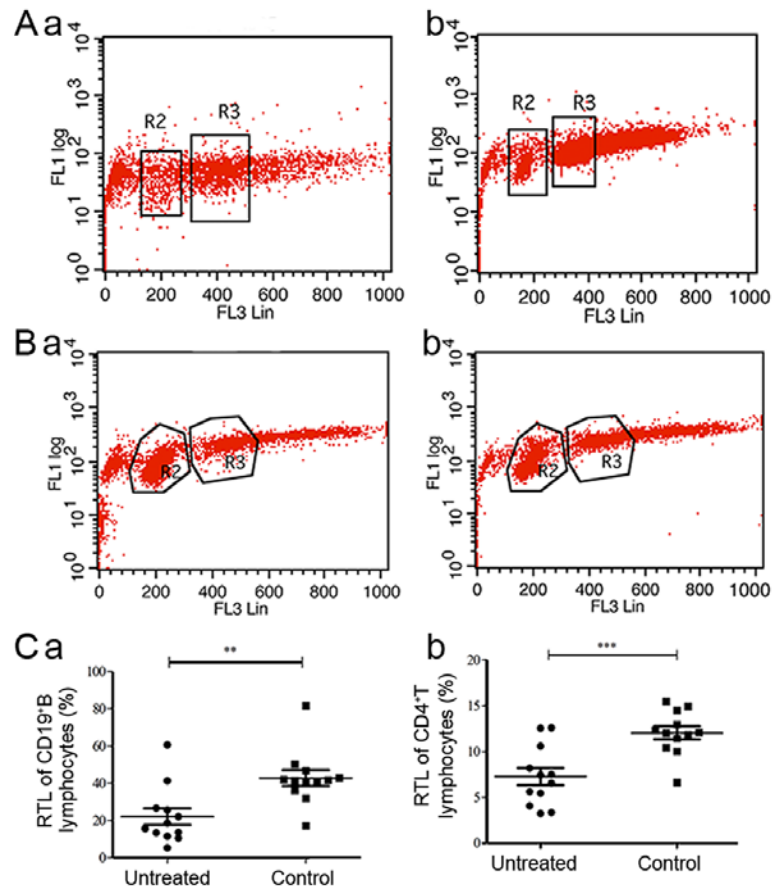


Figure 4. RTL (%) of different subtypes of lymphocytes in PB. R2 indicates the sorted cells from patients, and R3 indicates MOLT-4 cells. (A) Flow cytometry was used for the sorting of (a) CD19<sup>+</sup> B lymphocytes without a PNA fluorescent probe and of (b) CD4<sup>+</sup> T lymphocytes without a PNA fluorescent probe. (B) Flow cytometry was used for the sorting of (a) CD19<sup>+</sup> B lymphocytes with a PNA fluorescent probe and (b) CD4<sup>+</sup> T lymphocytes with a PNA fluorescent probe. (C) RTL of (a) CD19<sup>+</sup> B lymphocytes and (b) CD4<sup>+</sup> T lymphocytes in patients with untreated IRH and control subjects. Data are expressed as the mean  $\pm$  SD. (\*\*P<0.01, \*\*\*P<0.001). RTL, relative telomere length; IRH, immune-related haemocytopenia.

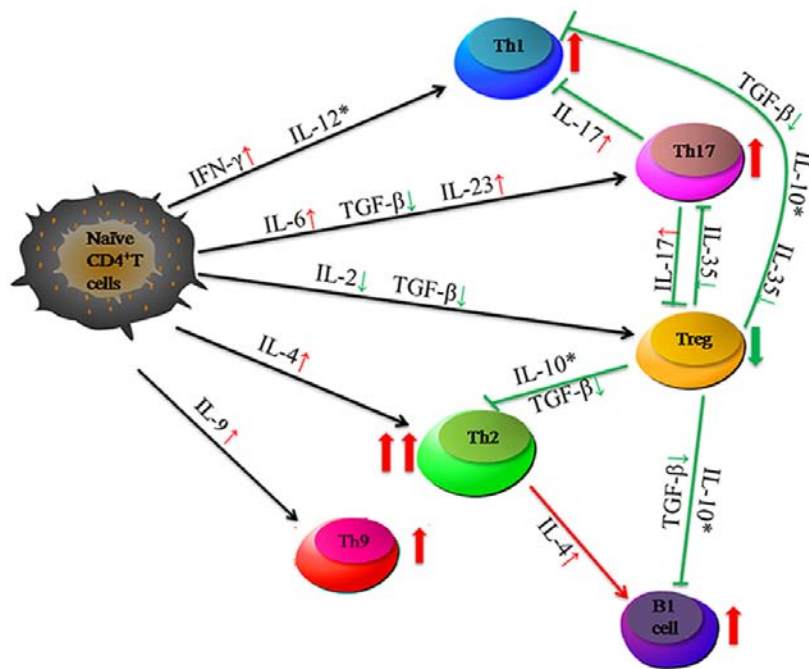


Figure 5. Abnormal percentage of CD4<sup>+</sup> T cell subsets and abnormal expression levels of regulatory cytokines in patients with untreated IRH. The percentages of Th1, Th2, Th9 and Th17 cells were increased, whereas the percentage of Treg cells was decreased in patients with IRH. Several cytokines were abnormal and were involved in the regulation of CD4<sup>+</sup> T lymphocytes. These CD4<sup>+</sup> T cells and related cytokines further regulated CD5<sup>+</sup> B lymphocytes to produce autoantibodies. Thin red arrows indicate a small increase, while large red arrows indicate a large increase. The green arrows indicate a decrease. \*, cytokines not detected in the present study. IRH, immune-related haemocytopenia; Th, T helper; Treg, regulatory T cell; IL, interleukin; IFN, interferon; TGF, transforming growth factor.

erythematosus (SLE) (48,49). The main cytokines secreted by CD4<sup>+</sup> T cells that are involved in IRH are presented in Fig. 5.

Breg cells induce IL-10, which suppresses the release of IFN- $\gamma$  and TNF- $\alpha$ . Breg cells can inhibit naïve T cell differentiation into Th1 and Th17 cells and the activation of CD4<sup>+</sup>CD25<sup>-</sup> T cells to Tregs. In rheumatoid arthritis, Breg cells are suppressed and do not produce IL-10, which in turn leads to reduced Th17 development (50). In SLE, the percentages of Breg cells have been shown to be increased, although these cells were refractory to CD40 stimulation and produced less IL-10, thus failing to suppress T cell proliferation (51). The present study indicated that the number of Breg cells was increased in IRH. Breg cells are increased in order to inhibit the activity of abnormal T lymphocytes. Concomitantly, the present study examined whether the function of Breg cells was defective.

Telomeres are heterochromatic structures with tandem DNA repeats of 5'-TTAGGG-3' at the chromosomal ends. With each cell division, telomeres shorten progressively due to the 'end-replication problem'. Various studies have documented that accelerated telomere loss in different lymphocyte subsets is a common feature of autoimmune disease, including SLE, RA and psoriasis (52-55). The present study demonstrated for the first time, to the best of our knowledge, that the telomere length of several lymphocyte subgroups (CD19<sup>+</sup> B and CD4<sup>+</sup> T lymphocytes) was significantly shortened in patients with IRH compared with that of control subjects. Furthermore, it was shown that the RTL significantly correlated with the percentage of T lymphocyte subgroups. Telomere shortening is an important suppressive mechanism that limits cellular proliferative capacity via regulating senescence checkpoint activation. The immune system is in constant self-renewal and is consequently dependent on efficient telomere maintenance. As telomeres protect chromosomes and genetic information from damage and erosion, their shortening mainly depends on antigen irritation and various stimulatory factors (56). Telomere shortening in IRH may be due to the chronic activation/proliferation occurring in autoimmune conditions and therefore could lead to premature immune senescence, involving exit from the mitotic cycle and/or cell death.

Furthermore, the present study examined the association between the abnormal CD4<sup>+</sup> T cell subtypes and the clinical data of the patients; the results showed that the increased percentages of Th17, Th1 and Th2 cells correlated negatively with Hb levels and Plt count, whereas the decreased percentages of Treg cells and Th1/Th2 cells correlated positively with Hb levels and Plt count (Table II). This indicated that these markers can be used for evaluation of the severity of the disease. In addition, abnormal percentages of CD4<sup>+</sup> T cell subtypes and abnormal expression of corresponding cytokine levels, including reduced levels of IL-2, TGF- $\beta$  and IL-35, were noted following successful application of immunotherapy (normal blood cell counts). Although the levels of IL-4, IL-6, IL-17, IL-23 and IFN- $\gamma$  were simultaneously increased in these patients, they did not reach the normal baseline levels, which illustrated that clinical immunosuppressive therapy should be prolonged. Finally, patient treatment and disease relapse rates were examined. It was found that 45.5% (5/11) of the patients relapsed in the intermittent treatment group, which was higher than the number

in the continuous treatment group (20%, 3/15) ( $P=0.218$ ). Therefore, it was concluded that it is necessary for patients in remission to undergo extension of their immunosuppressive therapy.

Additional T cell subsets are present that were not detected in the present study. For example, Tfh cells provide a helper function to B cells and represent one of the most numerous and important subsets of effector T cells (57). Tfh cells can express Bcl-6 and CXCR5, providing assistance to B-cells via the production of IL-21 (58). It has already been demonstrated in a previous study that the increased frequency and hyperfunction of Tfh cells in patients with IRH is associated with disease progression, which includes the presence of autoantibodies, disease activity and response to treatment (18). Th9 cells undergo a maturation program similar to that of Th2 cells, with IL-4 inducing the activation of STAT6 and producing IL-9 and IL-10. However, unlike Th2 cells, they require TGF- $\beta$  for maturation. This cytokine provides protection against intestinal helminth infections (59). Th22 cells are phenotypically and functionally related to Th17 cells, which are involved in wound repair and protection against bacterial, viral and fungal infections on epithelial surfaces, including the skin and gastrointestinal tract (60). Th25 cells can produce IL-25 and stimulate non-lymphoid cells in order to secrete effector cytokines in response to extracellular pathogens (61). However, the effects of Th9, Th22 and Th25 cells on IRH remain to be fully elucidated. The pathogenesis of IRH has been associated with the cytokine network, notably the B and the T lymphocyte subsets. Further investigations are required to elucidate the exact mechanism of IRH.

In conclusion, the present study demonstrated increased percentages of Th17 cells, decreased percentages of Treg cells and regulatory cytokines and a decreased ratio of Th1/Th2 in patients with IRH. These immune mediators regulated CD5<sup>+</sup> B lymphocyte function in order to produce autoantibodies in IRH. The abnormal percentage of CD4<sup>+</sup> T subtypes and the abnormal levels of their corresponding cytokines may be used as indicators for evaluating the severity of this disease and for extension of the immunosuppressive therapy used in the clinic.

## Acknowledgements

Not applicable.

## Funding

The present study was supported by the National Natural Science Foundation of China (grant nos. 81570106, 81600088, 81600093, 81400085 and 81770110), the Tianjin Municipal Natural Science Foundation (grant nos. 14JCYBJC25400, 15JCYBJC24300 and 16ZXMJSY00180) and the Science and Technology Foundation of Tianjin Municipal Health Bureau (grant nos. 2011kz115 and 2014KZ120).

## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

RF designed the research plan and revised the manuscript. JC, HL and LijL performed the experiments, analysed the data and wrote the manuscript. HW, YL, YW, KD, SH and YS contributed to the experimental work. LijL, JS, GW and ZS recorded the clinical characteristics of the patients with IRH. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Tianjin Medical University. Written informed consent was obtained for all patients included in the clinical trial.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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