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Aberrant frequency of IL-10-producing B cells and its association with the balance of Treg/Th17 in children with inflammatory bowel disease

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Regulatory B cells (Breg) are a distinct B cell subset, which contribute to the pathogenesis of autoimmune disorders. Interleukin-10 (IL-10) plays a pivotal function to Breg. It is well described in adults but little is known in a pediatric population. This study was to investigate the role of IL-10-producing B cell (B10) and its association with Treg and Th17 subsets in the children with inflammatory bowel diseases (IBD). Peripheral blood mononuclear cells from IBD children patients and controls were stimulated with PMA, ionomycin, and brefeldin A. The frequencies of $CD19^+IL\text{-}10^+$ B cells, $CD3^+CD4^+IL\text{-}17^+$Th17 cells, and $CD4 + CD25^{hi}Foxp3^+$ Treg cells were analyzed by flow cytometry. The mRNA expression of Foxp3, IL-17a and RORγt was detected by real-time quantitative PCR. The number of B10 cells was elevated in IBD children patients. There was a positive correlation between B10 cells and Tregs in IBD. The ratio of Treg/Th17 decreased in IBD, and it strongly correlated with B10 cells. The frequency of B10 cells is elevated in IBD and it correlates with both the Tregs counts and the Treg/Th17 ratio. B10 cells to regulate functional T cell subsets might be impaired in paediatric patients with IBD.

1. Introduction

Inflammatory bowel disease (IBD) classically includes two forms, ulcerative colitis (UC)and Crohn's disease (CD), and is thought to originate from an aberrant immune response against resident intestinal bacteria resulting in chronic inflammation (Lawrance et al. 2001). Like other autoimmune processes, the causes of these disorders remain unknown but likely involve some interplay between genetic vulnerability and environmental factors. In recent years, many experimental and clinical observations suggest that the restoration of tolerance and protective immunity play a role key for the IBD pathogenesis (Danese et al. 2004). Tolerance is mediated by regulatory T cells (Tregs), a sub-population of $CD4^+$ T cells that control immune responses in the gut by inhibiting the proliferation and effector functions of other Tcells (Fehérvari and Sakaguchi 2004). Although the mechanism through which Tregs suppress proliferation of other T cells isnot clear, there is evidence that they play an important role in preventing autoimmunity and controlling colitis and gastritis *in vivo* (Suri-Payer and Cantor 2001).

IL-10, which mediates suppression of inflammation by a number of mechanisms, has been recently shown to be produced by myeloid cells which is crucial for the maintenance of Foxp3 expression and regulatory T cell (Treg) function and ultimately controls the severity of colitis. IL-10 expressionin the mucosal environment is also critical for immunologic tolerance (Murai et al. 2009). In the absence of these regulatory effects, $IL\text{-}10^{-/-}$ mice spontaneously develop IBD(Rennick and Fort 2000). In addition, recent studies have shown that regulatory B cells (Bregs) are potent immune response regulators and play important roles in autoimmune diseases. A number of regulatory B cell subsets have been reported, among which the most widly characterized is the interleukin-10-producing B cell subset (B10 cells). There is growing evidence that IL-10 produced by B cells may be important for maintaining the balance between Tregs and Th17 cells (Carter et al. 2011).

Studies in a variety of mouse models of immune mediated disorders have demonstrated that B10 cells are of crucial importance in preventing disease development and ameliorating established symptoms (Carter et al. 2012). B10 cells appear during chronic inflammation and suppress the progression of intestinal inflammation by down-regulating inflammatory cascades. In mouse and human, it has also been shown that the frequencies of B10 cells are correlated with disease activity in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and chronic hepatitis B virus infection (Hua et al. 2014). However, little is known about the characteristics of B10 cells in IBD. Given the important immune regulatory functions of B10 cells and the broken immune homeostasis in IBD, to better dissect the features of B10 cells is essential both for the understanding of the pathogenesis of IBD and for the development of new treatment strategies. In this study, we investigated the profile of circulating IL-10-producing B cell subset in newly diagnosed IBD children patients, as well as its role in maintaining the balance of Treg/Th17.

2. Investigations and results

2.1. Subjects

The average age ± SEM of CD, UC children patients and health controls that donated peripheral blood were 12.5 ± 1.8, 13.8 ± 1.5 years and 13.1 ± 2.1 years, respectively. All the 56 IBD patients that donated blood samples were in a state of disease inactivity. Intestinal biopsy samples were collected from 27

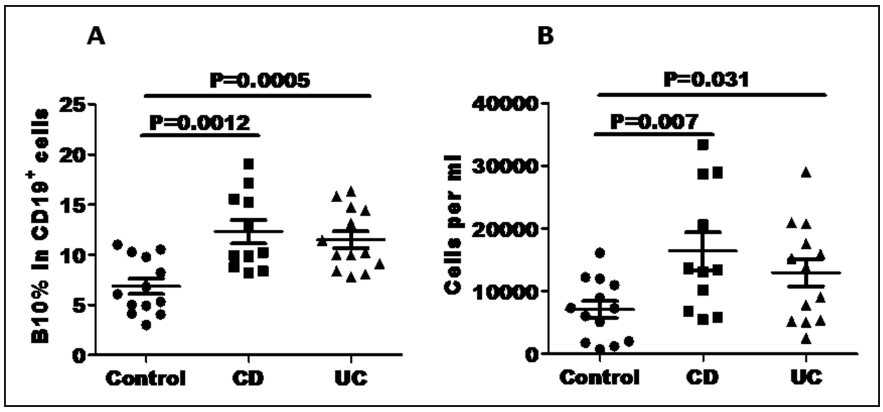

Fig. 1: Detection of human number of IL-10-producing B cells by flow cytometry. The percentage of IL-10-expressing B cells in total CD19 + B cells was higher than that in controls (A). At the diagnosis of UC and CD children patients, healthy controls showed relative lower number of IL-10 + CD19 + B cells when compared with IBD children patients (B).

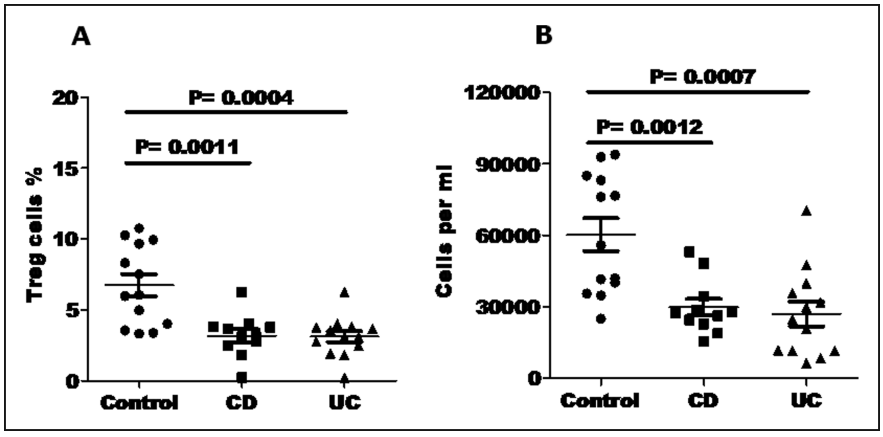

Fig. 2: Quantification of Treg in IBD children patients. Absolute numbers of Treg cells were calculated using lymphocyte counts and the frequency of CD4 + CD25bright Foxp3 + cells determined by flow cytometry. Decreased frequency of Tregs can be improved in UC and CD children patients (A). The defiiency in the number of Tregs was measured in IBD patients compared with the children controls (B).

IBD patients, and the average age ± SEM of CD, UC and control patients were 13.2 ± 2.2, 12.5 ± 1.3 and 14.2 ± 1.9 years, respectively. Out of the total 27 IBD biopsies collected, 8 were from patients with moderate disease activity, 9 had mild disease activity and 10 had inactive disease, based on global colonoscopic appearance and histologic reports (SA Pathology, Adelaide, South Australia).

2.2. Increased frequency of IL-10-producing B cells in children patients with IBD

After stimulation with PMA and ionomycin for 24 h, the percentage of IL-10$^+$CD19$^+$ B cells in total CD19$^+$ B lymphocytes in the PBMCs were found to be significantly higher in both CD patients (12.28 ± 3.85%) and UC patients (11.49 ± 3.03%) compared to the control group (6.85 ± 2.78%). Similarly, the absolute nmber of IL-10$^+$CD19$^+$ B cells were found to be significantly higher in both CD patients ($1.64 \pm 1.00 \times 10^4$/mL, P = 0.0012) and UC patients ($1.29 \pm 0.79 \times 10^4$/mL, P = 0.0005) compared to the healthy subjects ($0.71 \pm 0.49 \times 10^4$/mL) (Fig. 2).

2.3. Positive correlation between IL-10-producing B cells and Tregs in IBD patients

Having shown the effects of increased IL-10$^+$CD19$^+$ B cells on Tregs in IBD patients, B10 cells facilitate the differentiation and expansion of Tregs within the same IBD patient and control subjects. The percentage of CD4$^+$ CD25^{bright} Foxp3$^+$Tregs was significantly decreased in CD and UC patients when compared with that in normal controls (3.18 ± 1.50%), (3.12 ± 1.42%) *versus* (6.75 ± 2.81%). The absolute numbers of CD4$^+$CD25^{bright} Treg (mean ± SEM per ml of whole blood) in the peripheral blood were found to be significantly lower in both CD patients ($2.98 \pm 1.15 \times 10^4$/mL, P = 0.0012) and UC patients ($2.69 \pm 1.85 \times 10^4$/mL, P = 0.007) compared to the control group ($6.02 \pm 2.49 \times 10^4$/mL). Interestingly, in the CD and UC patients, both the percentage and the absolute number of B10 cells were positively correlated with those of Tregs. However, there is no correlation between the frequency of IL-10$^+$CD19$^+$ B cells and Tregs in the healthy controls.

2.4. IL-10-Producing B cells correlated with Treg/Th 17 ratio in IBD patients

As the Treg/Th17 imbalance plays an important role in the pathogenesis of IBD, an imbalance of Treg and Th17 cells occurs in IBD. Having shown a decrease in Treg and concomitant increase in Th17 in IBD, this relationship was further explored by investigating the balance of Treg and Th17 within the same IBD patient and control subjects. To do this the numbers of Treg and Th17 cells in the peripheral blood of IBD patients were directly compared by simultaneous measurement. The balance of Treg and Th17 cells was assessed in 13 control, 11 CD and 13 UC patients. While the numbers of Treg and Th17 cells were equivalent incontrols, the balance of Treg and Th17 cells in the peripheral blood was disrupted in IBD patients (Fig. 3). The imbalance oserved was characterised by a decrease in Treg and

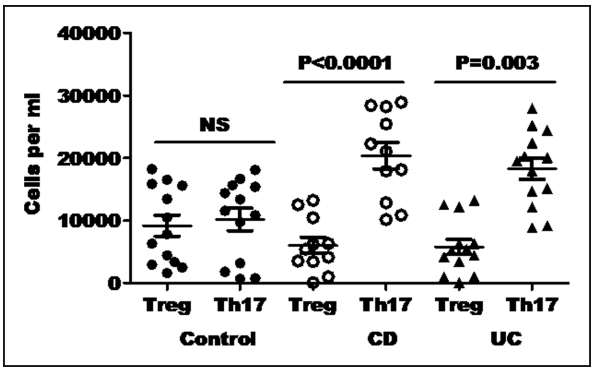

Fig. 3: The balance of Th17 and regulatory T cell numbers is disrupted in IBD. Absolute numbers of CD4 + CD25bright Foxp3 + Treg and Th17 cells were determined using the same patient lymphocyte samples. Each datum point represents an individual patient sample. The horizontal line represents median values for each group.

an increase in Th17 cells in the peripheral blood of both CD ($P < 0.0001$) and UC ($P = 0.003$).

2.5. Expression of Foxp3, IL-17a and RORγt are increased in the intestinal mucosa of IBD patients

In order to determine whether Treg and Th17 cells contribute to the maintenance of inflammation in the intestinal mucosa of IBD patients, the expression of Foxp3, IL-17a and RORγt in the intestinal mucosa was measured by real time RT-PCR. A 5-fold decrease in Foxp3 expression was observed in CD patients ($P < 0.0001$) compared to controls, and a4-fold increase in Foxp3 expression was observed in UC patients ($P = 0.003$). A a 3.8-fold increase in IL-17a expression was observed in CD patients ($P = 0.0087$) compared to controls, and a 3.0-fold increase in IL-17a expression was observed in UC patients ($P = 0.0093$). Meanwhile, RORγt was expressed at significantly higher levels in the intestinal mucosa of CD ($P = 0.0018$) and UC ($P = 0.0003$) patients compared with the controls.

3. Discussion

Previous work has shown that anti-immune cells/cytokines influence and constrain each other to keep a delicate balance plays a role key in immune system. The functional and numerical deficiencies in regulatory immune cells are to a great extent responsible for the disruption of immune homeostasis in autoimmune disorders (Roberts-Thomson et al. 2011). The present study shows that, in addition to regulatory T cells, the number of circulating IL-10-producing B cells is also queer in IBD patients, indicating that both regulatory B and T cells participate in the development and perpetuation of IBD.

Our data showed that the number of circulating B10 cells is significantly increased in IBD children patients at the diagnosis of the disease, which is in line with results from some other studies on autoimmune disorders such as systemic lupus erythematosus, autoimmune myasthenia gravis, rheumatoid arthritis, and Wiskott-Aldrich syndrome (Valencia and Lipsky 2007; Sleasman 1996). Recent research has indicated that B10 cells are ableto up-regulate the expression of Foxp3 and thus expand the population of Tregs. The number of IL-10-producing B cells might correlate with clinical outcome in IBD patients. However, no correlation was found between B10 cells and Tregs in healthy controls. Yanaba et al. (2009) also evaluated the correlation between these two cell subsets; they found that although B10 cells positively correlated with Tregs in patients with disease, but no association was found in healthy controls.

In the present study, the numerical deficiency of $CD4^+CD25^+Foxp3^+$ Treg subset was confirmed in IBD children patients. However, there is a positive correlation between the frequency of IL-10-producing B cells and $CD4^+CD25^+Foxp3^+$ Tregs in diagnosed IBD children patients. The opposite changes in B10 cells and Tregs, together with the positive mutuality between the two subsets, imply that the ability of B10 cells to diminish the population of Tregs might be insufficient in diagnosed IBD children patients. Next, we demonstrated that IBD is characterized by a decrease of Treg and an increase in Th17 cells in the peripheral blood of children patients in disease remission. Some studies suggest that human IL-10-producing B cells do not affect Th17 differentiation (Iwata et al. 2011). In contrast, Flores-Borja et al. (2013) have recently demonstrated that healthy $CD19^+CD24^{hi}CD38^{hi}$ B cells, a subset of B cells that produce a large amount of IL-10, do inhibit naive T cell differentiation into Th17 cells. In the current study, we could not find any correlation between $IL\text{-}10^+CD19^+$ B cells and Th17 cells, indicating that the ability of B10 cells to inhibit Th17, if any, is largely dampened in IBD. Interestingly enough, there is a strong correlation between B10 cells and Treg/Th17 ratio inIBD children patients. The Treg/Th17 ratio is believed to be relevant to the clinical diversity of IBD and it might have prognostic role in IBD patients, which is in accordance with the result that the number of B10 cells is relatively higher in UC patients than that in CD patients. That is, despite the impaired functions of B10 subset in orchestrating Treg andTh17 cells in IBD, it might provide useful information for clinical management and therapeutic options.

Taken together, our data demonstrate the increased frequency of $IL\text{-}10^+CD19^+$ B cells and its association with essential T cell subsets in paediatric patients with IBD. Considering immune regulatory functions of B10 cells, a conflict exists between the increased number of $IL\text{-}10^+CD19^+$ B cells and the disrupted immune homeostasis in IBD. Mizoguchi et al. (2000) first coined the term regulatory B cell to describe B cells that suppress disease in a mouse IBD model. There is definite evidence that the activation signals from T cells initiate regulatory properties in B cells that modulate T cell responses, forming a negative feedback loop involving T cell activation and regulatory B cells (Brandman and Meyer 2008). We have previously demonstrated that the secretion of IL-10 by a subset of B cells is impaired in IBD children patients, which might lead to functional impairment of B10cells. However, the underlying mechanism of impaired IL-10secretion in IBD is currently unknown. Further studies that exactly dissect the properties of regulatory B cells in IBD are obviously warranted.

In summary, in addition to Treg and Th17 cells, the frequency of IL-10-producing B cells is also aberrant in pediatric patients with IBD. CD and UC children patients show relative higher number of B10 cells, suggesting that B10 cells might associate with clinical outcome in IBD pediatric patients. Interestingly, B10 cells positively correlate with both Treg cells and Treg/Th17 ratio, indicating that this may be a characteristic feature of pathologic inflammatory disorders. However, the elevated number of B10 cells together with Treg/Th17 imbalance suggests that the ability of B10 cells to regulate functional T cell subsets might be insufficient in pediatric patients with IBD. The role of B10 cells for the development and perpetuation of IBD still needs to be further investigated.

4. Experimental

4.1. Patients and controls

IBD children patients were recruited from the Department of Pediatric Surgery at Wuxi People's Hospital of Nanjing Medical University. Informed consent was obtained from all patients before collection of samples. Twenty-

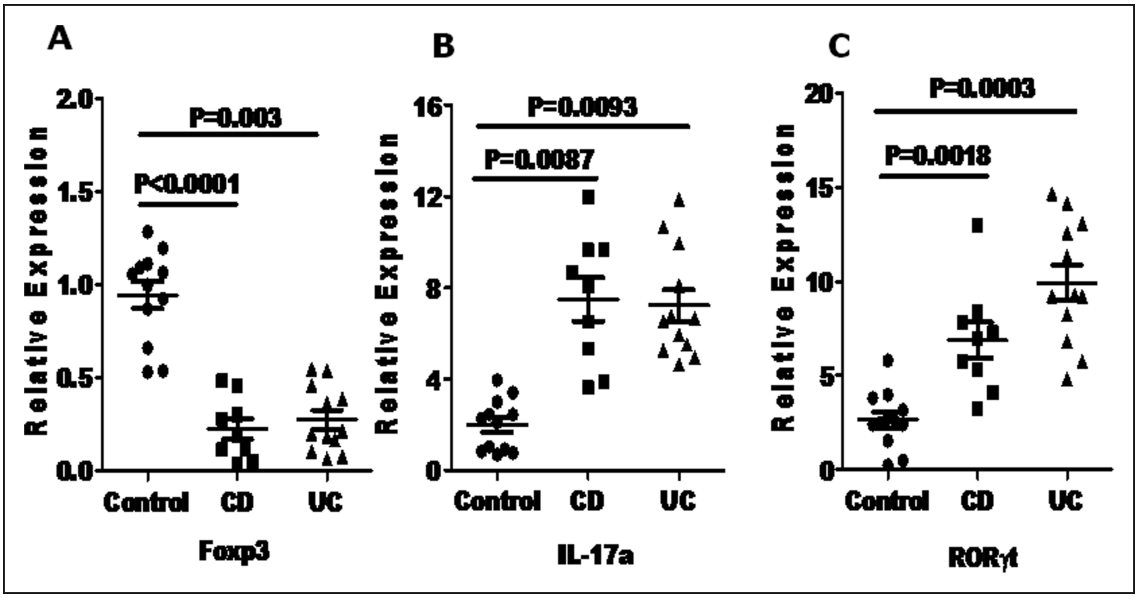

Fig. 4: RNA was extracted from intestinal biopsies with Foxp 3 (A), IL-17a (B) and RORγt (C) were determined by real-time RT-PCR and normalized to GAPDH expression. Horizontal lines indicate median values for each group. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with the control group; $^{*}p<0.05$, $^{**}p<0.01$ compared with the model group.

seven CD, 29 UC, and 18 control patients were recruited for blood collection. Control subjects had non-inflammatory disorders (non-ulcer dyspepsia, irritable bowel syndrome, reflux) or were healthy volunteers. Intestinal biopsies were obtained from an additional subset of IBD patients in various states of disease activity. Biopsies were collected from 10 CD, 12 UC and 12 control subjects at colonoscopy. Biopsy samples were frozen immediately and stored at-80 °C until further processing.

4.2. Isolation of peripheral blood mononuclear cells (PBMCs)

Venous blood samples were collected in 3-mL ethylenediamine tetraacetic acid-treated tubes and diluted 1:2 with Hanks balanced salt solution (HBSS) before Ficoll-Hypaque gradient centrifugation (2,200 rpm at room temperature for 15 min). Washed and resuspended, PBMCs were cryopreserved in fetal bovine serum containing 10% dimethyl sufloxide (DMSO), and stored in liquid nitrogen for future flow cytometric analysis (FCM).

4.3. Flow cytometric analysis

Cryopreserved PBMCs were thawed at 37°C, washed twice with HBSS, and stained with trypan blue to test cell viability. 1×10^6 PBMC were distributed for Treg flow cytometric analysis. PBMCs were stained with PE-conjugated anti-CD25 and PE-Cy5-conjugated anti-CD4 or isotypes (BD, Sydney, NSW, Australia) for 20 min at 4°C, washed twice, fixed, and permeabilized and then stained with Alexa Fluor 488-conjugated anti-Foxp3 for analysis of Treg subpopulation. For B10 and Th17 detection, cultured cells were stained with surface FITC-conjugated anti-CD19 and intracellular APC-conjugated anti-IL-10 (B10) or FITC-conjugated anti-CD3, PE-conjugated anti-CD4, and intracellular Alexa Fluor 647-conjugated anti-IL-17 (Th17). Stained cells were tested on a FACS Aria II flow cytometer (BD, USA) and then analyzed using Flowjo software version 7.6.1.

4.4. RNA isolation and RT-PCR

Total RNA was extracted from intestinal biopsies with Trizol reagent (Invitrogen, USA) and converted into cDNA using a Prime Script RT reagent kit (Takara, Japan) according to the manufacturer's instructions. The gene expression levels of Foxp3, RORγt and IL-17a were determined by real-time quantitative reverse-transcription polymerase chain reaction with the use of ABI 7700 and specific primers as reported previously (Hua et al. 2014; Bullens et al. 2006). Specific PCR primer pairs for the target genes were:

GAPDH forward: 5'-GGTGGTCTCCTCTGACTTCAACA-3'
GAPDH reverse: 5'-GTTGCTGTAGCCAAATTCGTTGT-3'
Foxp3 forward: 5'-GTGGCATCATCCGACAAGG-3'
Foxp3 reverse: 5'-TGTGGAGGAACTCTGGGAAT-3'
RORγt forward: 5'-GTGCTGGTTAGGATGTGCCG-3'
RORγt reverse: 5'-GTGGGAGAAGTCAAAGATGGA-3'
IL-17a forward: 5'-CAATCCCACGAAAT CCAGGATG-3'
IL-17a reverse: 5'-GGTGGAGATTCCAAGGTGAGG-3'

4.5. Statistical analysis

The statistical differences between IBD and the control group were evaluated using the two-tailed Mann Whitney ranked sum test. Comparison of paired samples was carried out utilizing a paired samples t-test. D Data are expressed as mean ± standard error of the mean. Statistical significance was achieved when $P<0.05$. All analyses were performed using the SPSS statistical software for Windows, version 10.1.4.

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