RESEARCH ARTICLE

Peripheral cytokine profile in Chilean patients with Crohn's disease and ulcerative colitis

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ABSTRACT. Crohn's disease (CD) and ulcerative colitis (UC) belong to the group of inflammatory bowel diseases (IBD), with complex ethiopathogenic factors that include an unbalanced immune and inflammatory response to commensal and food antigens. The differential diagnosis between CD and UC is performed using clinical, endoscopic, histopathological, serological and radiological methods; however between 10-15% of IBD patients are diagnosed as "unclassified colitis". Further research into IBD is necessary in order to develop additional diagnostic tools. The aim of this work was to see if the Th1, Th17 or Th2 immune pattern, represented by $CD4^+$ lymphocytes producing IFN-γ, IL-17 and IL-5 or IL-13, respectively (CD4/IFN-γ+, CD4/IL-17+,CD4/IL-5+ or, CD4/IL13+), are useful peripheral markers which can be used to differentiate between UC and CD. Peripheral blood samples were taken from IBD patients from the Clinic Hospital of the University of Chile. The percentage of IFN-γ-, IL-17-, IL-5- or IL-13-expressing CD4+ cells was determined by flow cytometry in phorbol ester- (PMA) and calcymycin-activated blood samples. The percentages of the CD4+ cell populations producing each cytokine were compared between UC and CD. IFN-γ production by CD4+ lymphocytes was significantly higher in CD compared to UC and the control. The percentage of IL-17-expressing cells was significantly higher in CD patients compared to to the control; however, there were no differences between UC and CD; or between UC and healthy individuals. No significant differences were observed between the different groups as regards the representative Th2 cytokines. This study suggests that, under pathogenic conditions, several immune profiles may be operating, in the development of IBD. Although peripheral IFN-γ and IL-17 expression, as indicators of the immune pattern, may help in the diagnosis of IBD, other cytokines and adaptive immune markers should be analyzed to allow better differentiation between the two entities.

Keywords: inflammatory bowel disease, cytokines, Crohn disease, ulcerative colitis, Th17, interferon gamma

Inflammatory bowel disease (IBD) occurs as heterogeneous phenotypes including: ileitis, ileocolitis, pancolitis left colitis, proctosigmoiditis and proctitis, and this determines the therapeutic approach [1, 2]. In spite of its phenotypic diversity, the diagnosis of IBD is based upon the clinical course, supported by endoscopic, histological, radiological and serological tests that, at the moment, allow the clinician to classify IBD as either ulcerative colitis (UC) or Crohn's disease (CD). When these features overlap, the disease is described as unclassified colitis, which makes up 10-15% of all IBD patients [3].
IBD susceptibility, phenotype and response-to-treatment may be determined by the complex interaction between genetic, immunity and environmental components of the intestine [4, 5]. The mechanisms involved in the development of the uncontrolled and chronic inflammatory response seen in IBD remain unknown. However, it has been suggested that a loss of peripheral and central tolerance to food antigens and commensal flora, as well as to host antigens, might be involved in the development of IBD [6], leading to a destructive immune response.
Antigen-presenting cells can lead to a cellular (Th1 or Th17) or humoral (Th2) immune response to intestinal lumen antigens [7, 8]. Th1 cells produce IFN-γ and TNF-α, and mostly induce pro-inflammatory responses due to activation of cellular immunity in response to intracellular microbes. Th2 cells produce IL-4, -5, -10 and -13 that can lead to non-inflammatory processes, and to B lymphocyte proliferation against gastrointestinal nematodes and associated allergic disorders [9-11].

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Recently, a novel subset of T-CD4+ cells, distinct from Th1 and Th2, named Th17 has been described [12]. Generally, the polarization of the immune response into Th1, Th17 or Th2 cellular types varies according to the physiological state. Furthermore, some pathological conditions may arise from an imbalance between the production of Th1, Th17 and Th2 cytokines. Common examples of these include arthritis, asthma, leukemia, cancer, as well as IBD [13-17]. Evidence indicates that the altered local immune response in IBD might be mediated by T-CD4+ lymphocytes, with either a Th1, Th17 or Th2 phenotype [18-20]. CD patients have a predominant, local, Th1 immune pattern, in contrast to UC, which shows a Th2 predominance [18]. However, this concept of peripheral CD4+-producing cytokines is unclear in IBD [21]. Moreover, a Th17 pattern has been described in the intestinal mucosa of CD patients that correlates with increased IL-17 in serum and peripheral T-CD4+ lymphocytes [20, 22].

Here we examined the T-CD4+ cell population in peripheral blood from CD and UC patients and from healthy individuals, and the *ex vivo* production of stimulated and unstimulated IFN-γ, IL-17, IL-5 and IL-13, with the purpose of establishing if a peripheral immune profile can be associated with CD or UC.

DONORS AND METHODS

The study group consisted of 21 IBD patients (13 UC and 8 CD, with three in each group being in remission), recruited from the Gastroenterology Department of the Clinic Hospital University of Chile, and with a follow-up of at least one year. Seven healthy individuals were also included in the study.

Clinical data for the IBD patients were obtained retrospectively from the patients' clinical charts. Patients were diagnosed, and UC and CD were each confirmed according to the Montreal classification system [23]. Those individuals with non-classifiable IBD or indeterminate colitis were excluded. The following information from the IBD patients were obtained: age, age-at-diagnosis, gender, familial or spontaneous diseases (familial disease was considered as one, first- or second-degree relative diagnosed with IBD), smoking habits (current smoking, history of smoking, or never smoked), disease localization, disease pattern, extraintestinal manifestations (articular, ophthalmological, dermatological and hepatic manifestations), occurrence of colorectal cancer, perianal disease and surgery.

The control group comprised healthy individuals who had no history of immune-mediated diseases.

The study was approved by the Ethics Committee, and written, informed consent was obtained from all participants involved in the study.

IMMUNE-STAINING OF CELL SURFACE ANTIGENS, AND INTRACELLULAR CYTOKINES

Intracellular cytokines were studied by flow cytometry as has been described previously [24]. Briefly, heparinized whole blood (WB) was diluted 1:1 v/v in culture medium consisting of RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin, and incubated in 12 x 75-mm polystyrene, round-bottomed tubes for six hours at 37°C, 5% CO_2 under agitation, and in the presence of 10 ng/mL of phorbol 12-myristate 13-acetate (PMA), 1 mM of calcymycin and 10 mg/mL brefeldin A (Sigma, St Louis, MO, USA). Cells were stained with CD4-FITC-conjugated monoclonal antibody at 4°C in the dark for one hour. Excess antibody was removed with phosphate-buffered saline (PBS), and erythrocytes were lysed using ACK lysis buffer. The cells were fixed with 1% paraformaldehyde. Thereafter, the remaining cells were washed and permeabilized with 0.05% saponin in PBS and stained with PE-conjugated IFN-γ, IL-17, IL-5 and IL-13. Isotype-matched controls were included for each antibody to evaluate non-specific binding. Finally, cells were washed with 0.05% saponin, resuspended in 1% paraformaldehyde and analysed by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson, San Diego, CA, USA). Gate analyses were set on lymphocytes according to forward and side scatter properties. CD4+ cells were analysed in accordance with the following criteria: (1) the lymphocyte population must be clearly depicted in the scatter plot, (2) the density plot must show a clear separation between the negative and positive CD4 population [24]. At least 10,000 CD4+ cells were collected and evaluated. Data were analysed using CellQuest (version 3.3) software (Beckton Dickinson, USA). We defined cell populations as follows: Th1: CD4+ IFN-γ+; Th2: CD4+ IL-5+ or IL-13+ and Th17: CD4+ Th17+. All data were corrected for autofluorescence as well as for non-specific binding using isotype-matched controls.

Reagents and antibodies

All reagents were from Sigma (St Louis, USA), and conjugated antibodies for flow cytometry were from Becton Dickinson (CA, USA) and eBioscience (CA, USA).

Statistical analysis

A comparison of IFN-γ-, IL-17-, IL-5- and IL-13-producing cells was made between the patients with CD, UC and healthy individuals. The differences were compared using Student's t test: P values of < 0.05 were considered significant.

RESULTS

In the CD group, mean age-at-diagnosis was 52 yr (range 25-68); four patients were female. Of these patients, based on the Montreal Classification (17), four had disease location L2 ± L4 (colonic with or without upper tract disease), three had L1 ± L4 (ileum with or without upper tract disease), and one patient had L3 ± L4 (ileocolonic with or without upper tract disease). The distribution of disease behavior at diagnosis was: four patients had B1 ± P (non-penetrating, non-perforating with or without perianal disease), and four had B2 ± P (structuring with or without perianal disease). Four patients had

arthralgia. Two patients needed surgery. In the UC group, the mean age-at-diagnosis was 37.8 yr (range 18-61), seven patients were female. Nine patients had pancolitis, two had left-sided colitis and two had proctitis. Ten patients had extra-intestinal manifestation. None of the patients needed surgery.

The evaluation of the cytokine pattern in peripheral whole blood from IBD patients and healthy individuals was made using flow cytometric analysis of PMA/calcymycin-stimulated and non-stimulated CD4+ lymphocytes *(figure 1)*. There were differences in the production of cytokines, such as IFN-γ, IL-17, IL-5 and IL-13, between CD, UC patients and healthy controls. *figure 2* shows that the stimulated, peripheral CD4+ lymphocyte population from CD patients produced significantly higher levels of IFN-γ (mean 29.79 ± 7.13) than UC patients (mean 15.74 ± 3.91; $p = 0.037$) or healthy control (mean 9.56 ± 2.69; $p = 0.013$). Moreover, intracellular expression of IL-17 by CD4+ cells was significantly higher in CD (mean 3.70 ± 0.53), in comparison to healthy controls (mean 1.68 ± 0.59; $p = 0.012$). However, there were no differences between the Th17 immune pattern detected in UC (mean 3.45 ± 1.15) and CD, or between UC and healthy individuals *(figure 2)*. For the Th2 immune profile, represented by CD4+/IL-5+ and CD4+/IL-13+, the percentages were lower than those obtained for IFN-γ and IL-17. Nevertheless, there were no statistically significant differences in the expression of Th2 cytokines between each patient group. The CD4 intracellular expression of IL-5 was lower in healthy individuals than in IBD patients. There was a tendency towards increased IL-13 expression in CD4+ cells from CD patients in comparison to UC patients and healthy individuals *(figure 2)*. Similar levels for cytokine production were observed in the blood of the healthy population as reported previously [25-27].

DISCUSSION

In health, there is a finely-tuned balance between the immune response to pathogenic bacteria and tolerance to commensal flora and diet antigens. When this balance is disturbed, unregulated cytokine production occurs and IBD develops. IBD patients show a disturbed intestinal immune response, with a Th1, Th17 or Th2 phenotype [18, 20, 28-30]. CD patients have a predominantly local, Th1 pattern, in contrast to UC which develops a Th2 profile as shown by an increased production of IL-5 by stimulated, lamina propria T cells [18, 28]. However, no difference in lamina propria immunoreactivity against IL-4/CD3+ cells was assessed in UC specimens [18, 28]. A Th17 immune pattern has been described in peripheral cells and in mucosa from IBD patients, particularly those with CD [20, 22].

In this study, an increased production of IFN-γ and IL-17 by peripheral CD4+ T lymphocytes from patients with IBD is shown in comparison to healthy subjects. CD patients present elevated CD4+/IFN-γ expression levels in comparison to UC patients and healthy controls, supporting previous evidence described for local immune patterns [18, 28]. These results are in agreement with

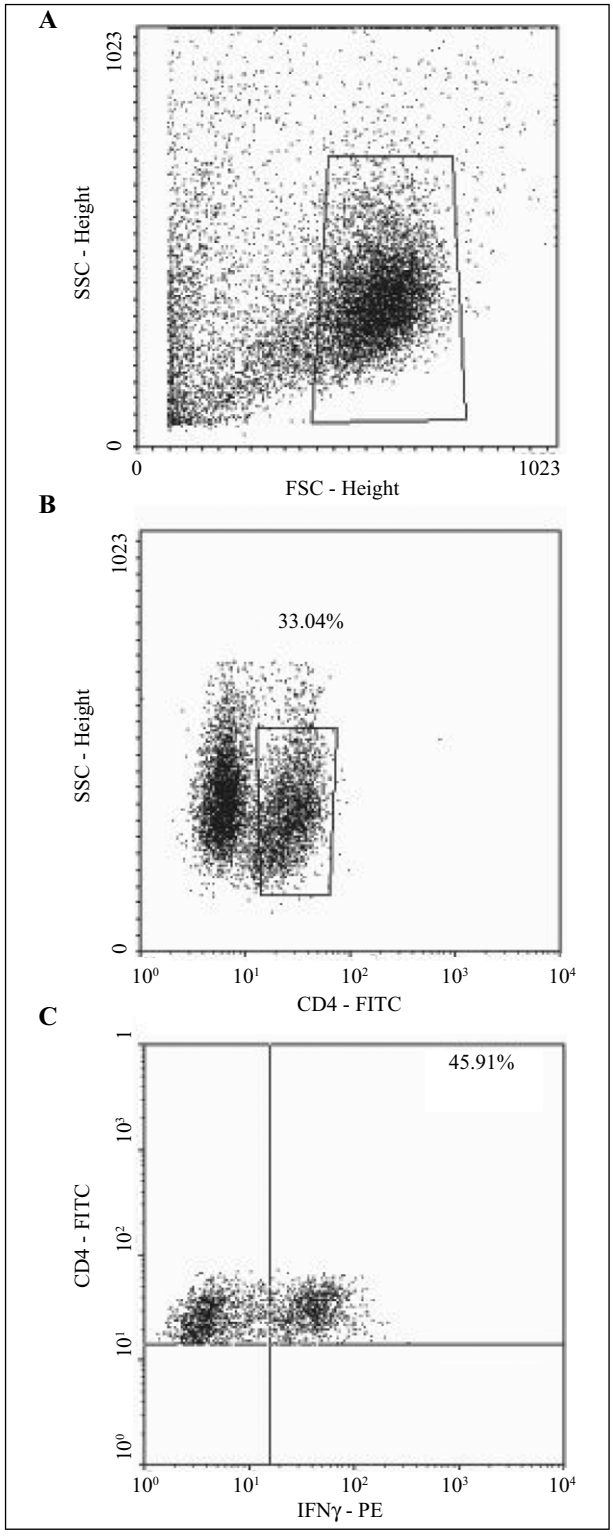

Figure 1

Flow cytometric detection of intracellular IFN-γ production by T-helper cells. Whole blood stimulated with PMA/calcymycin was stained with cytokine-specific antibodies (IFN-γ-PE for T-helper-1 cells, IL-5 and IL-13-PE for T-helper-2 cells and IL-17-PE for T-helper-17). (**A**) Scatter plot of all cells, a circular gate was placed around the live lymphocyte population, based on size (FS, forward scatter) and granularity (SS, side scatter). Of the total number of lymphocytes, only the CD4+ T-helper cells were selected (right peak, **B**). These T-helper cells were examined for Th1/Th2/Th17 cytokine production (**C**), with cells that stained positive for IFN-γ only (upper right quadrant) classified as Th1 cells; similar analyses were performed for the other cytokines.

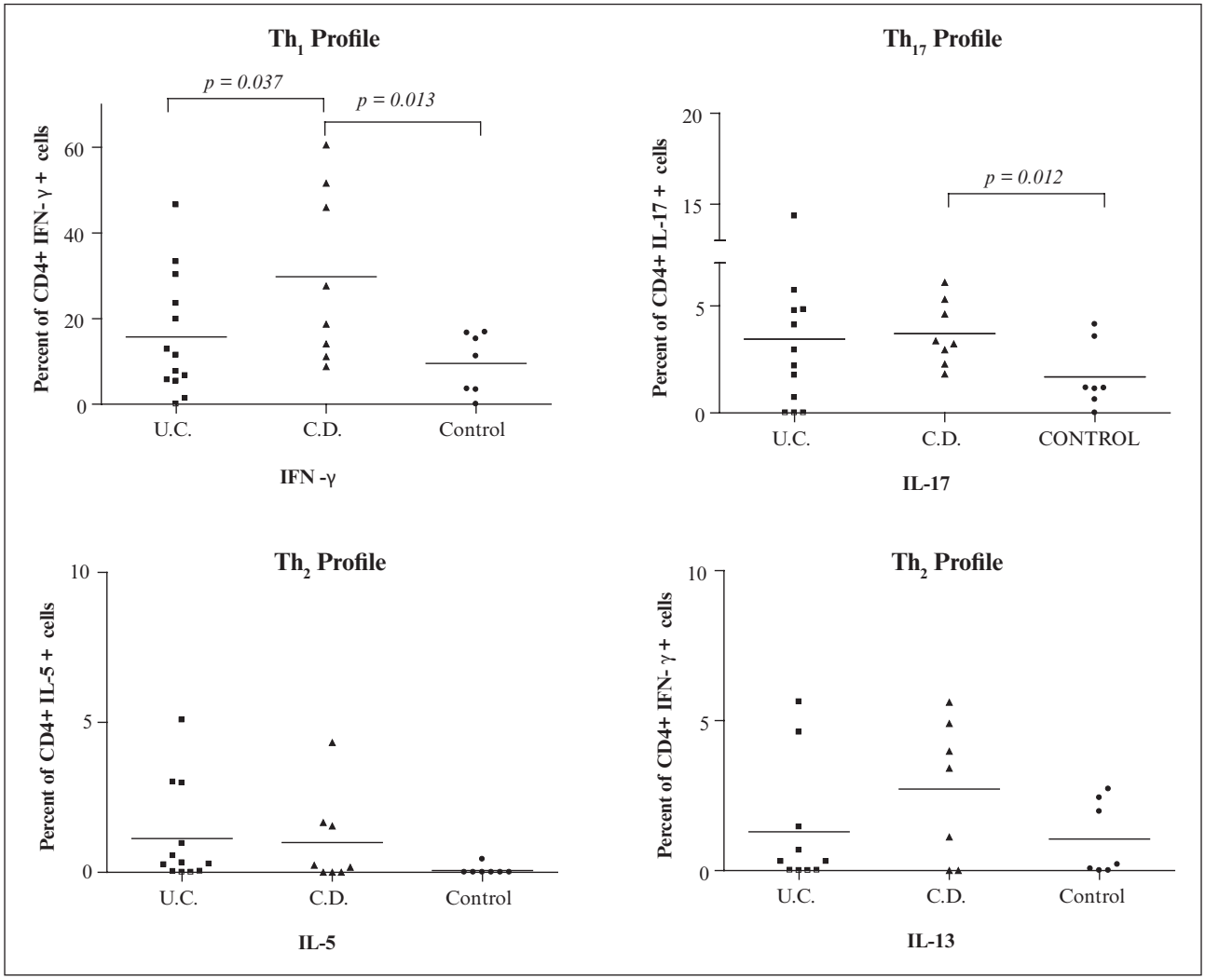

Figure 2

Cytokine production by CD4 lymphocytes: intracellular cytokine expression in peripheral whole blood CD4 lymphocytes. Whole blood was stimulated with PMA-calcymycin for eight h in the presence of brefeldin. The figure represents the cell populations of peripheral blood from patients with Crohn's disease (CD), ulcerative colitis (UC) or healthy controls. Each symbol represents a single patient or control; horizontal lines represent means.

other studies, which have described increased levels of IFN-γ mRNA and IFN-γ-producing T-cells in inflamed mucosa from adult patients with IBD as compared to controls [29-32]. Moreover, increased production of Th1 cytokines has been well-characterized in murine IBD models [18, 33]. Decreased IFN-γ production by peripheral CD4+ cells has been reported in children and adults with CD [16, 20]. We have previously observed a significantly different IFN-γ expression pattern in a cohort of active and inactive Chilean IBD patients with CD and UC, suggesting that peripheral pro-inflammatory cytokine levels correlate with the state of the disease (unpublished data, Falk symposia; [34]).

A Th17 immune pattern in IBD patients has been demonstrated as the predominant response in inflamed mucosa, and increased IL-17 serum levels in CD patients [22]. However, the specific T cell subtype producing IL-17 was not evaluated in that work. A Th17 phenotype, responsible for the development of IBD, has been described in a murine colitis model [35, 36]. Here we described an increased peripheral CD4+ T cell population, producing IL-17, in both forms of IBD, suggesting that a strong cellular response, together with a Th1 immune phenotype, are involved in this pathology. Investigation of double positive Th17/Th1 cytokine-expressing T CD4+ cells was not conducted in our study; however, no difference in local CD4+ cell population sharing features of both cytokines, in the gut of patients with CD, UC or healthy individual has been observed previously [37].

In relation to Th2 immune profiles in IBD, we detected low expression of IL-5 and IL-13 in CD4+ peripheral cells in IBD patients as well as controls. Studies have not defined any tendency in IL-13 levels between periphery and mucosa from IBD patients [21, 38]. These results are in agreement with the lower IL-4, IL-5 and IL-13 mRNA and secreted protein levels previously reported in intestinal mucosa of IBD patients and a healthy population [18, 20, 30, 39]. Lamina propria T cells from CD patients were shown to secrete IFN-γ, but no differences where observed for IL-4 or IL-5 production, contrary to what was observed in UC patients [40]. In UC pathogenesis, IL-13 expression was detected in lamina propria NK cells, bearing a cyto-

toxic phenotype [40]. Moreover, as reported in patients with different autoimmune pathologies [25, 26], we detected less than 10% of IL-5- and IL-13-producing T cell in peripheral blood from CD and UC patients.

In summary, our study has indicated that over-activation of the Th1, Th2 or Th17 immune profile may occur in the pathogenesis of IBD. The combination of immune responses might be due to a loss or defect in oral tolerance, which may suggest an inflammatory immune response, with high levels of pro-inflammatory cytokines. A larger number of patients with both IBD pathologies, as well as a wider range of cytokines should be included in future studies, to provide results that might be useful in the monitoring of disease and the development of treatment.

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