The levels of IL-17A and of the cytokines involved in Th17 cell commitment are increased in patients with chronic immune thrombocytopenia

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ABSTRACT

Th17 cells have been associated with immune-mediated diseases in humans but it has still not been determined whether they play a role in immune thrombocytopenia. We evaluated representative cytokines of the Th17, Th1, Th2 and Treg cell commitment in the serum of patients with chronic immune thrombocytopenia, as well as the cell source of IL-17A. Higher levels of IL-17A and Th17-related cytokines, and an increased percentage of IL-17A producing CD4+ and neutrophils were observed in patients. The levels of cytokines involved in Th1 cell commitment IFN-γ, IL-2, IL12-p70 and the percentages of Th1 cells were also increased, but IL-4 was not detected. Although the concentrations of IL-10 were higher, the levels of TGF-β were similar in both groups. In conclusion, our results point to a putative role for Th-17 cells/IL-17A cytokine in the pathogenesis of chronic immune thrombocytopenia.

Key words: immune thrombocytopenia, Th17 cell, Th1 cell, Th2 cell.

Introduction

Chronic immune thrombocytopenia (cITP) is an acquired immune-mediated disease characterized by auto-antibody dependent accelerated destruction and impaired production of platelets.12 The auto-antibodies, detected in approximately 70% of the patients with cITP, are usually directed to surface glycoproteins GPⅡb/Ⅲa and GPⅠⅡa/Ⅸ.2

The etiology of cITP remains a question for debate but genetic and environmental factors are thought to play a role in the development of the disease. Although events associated with autoreactive B lymphocytes and abnormal production of antibodies, such as molecular mimicry, cross-reaction and expression of cryptic epitopes, play a major role in the pathogenesis of the disease, several abnormalities of cellular immunity have also been described.3-5 As observed in immune-mediated diseases, such as systemic lupus erythematosus (SLE), a CD4+T lymphocyte (Th) type 1 polarized immune response with an increased expression of interleukin (IL)-2 and interferon (IFN)-γ and reduced expression of IL-4 has been demonstrated in patients with cITP.5,7 In addition, reduced transforming growth factor (TGF)-β and IL-10 serum levels,8 and deficiency or altered functions of T-regulatory (Treg) cells9 have also been described in cITP patients suggesting that a defective immune regulation might play a part in the pathogenesis of the disease.

Recently, a novel subset of Th cells characterized by the production of IL-17A has been described.10 Th17 cells play an important role in host protection against fungal and extracellular bacterial infections and are also involved in autoimmune diseases.11 Increased serum levels of IL-17A have been associated with immune-mediated diseases in humans, such as SLE.12 However, few studies to date have evaluated the Th17 cell associated cytokines in cITP, all of them included only Chinese populations and there is little agreement between results.13-16 Whereas Zhang et al.12 demonstrated increased intracellular levels of Th17 and Th1 cell cytokines in patients with cITP, no significant differences in the serum levels or in the expression of IL-17 by peripheral blood mononuclear cells were observed in patients with cITP in the studies of Ma et al.15 and Guo et al.,14 respectively.
Therefore, our aim was to determine the serum levels of cytokines associated with the Th17 cell commitment in cITP adult patients from a Western country. In order to identify the origin of IL-17A production, we evaluated the percentage of T cells and polymorphonuclear cells producing intracelluar IL-17A. Because the Th17 cell produces large amounts of tumor necrosis factor (TNF-α) that mediates tissue damage in autoimmune diseases, the serum TNF-α concentration was also assessed. We also evaluated the levels of representative cytokines of the Th1, Th2 and Treg cell lineages.

**Design and Methods**

This study was approved by the Ethics Committee of the Federal University of Minas Gerais and the Fundação Hemominas, Brazil. Informed consent was obtained from all subjects.

From 2004 to 2010, we prospectively studied 98 patients (37 male, mean age 46.3±16.1 years, range 18-83 years) with cITP selected from among those who attended the Hematology Service, University Hospital, Universidade Federal de Minas Gerais. cITP was diagnosed as isolated thrombocytopenia (platelets < 100x10^9/L) that persisted for more than 12 months in patients in whom no other causes of thrombocytopenia could be identified. Patients who had received previous treatment for cITP, including corticosteroid or immunosuppressive agents, or who had undergone splenectomy were also excluded. Patients' platelet count ranged between 19-99x10^9/L, with a mean (±SD) of 75 (±22)x10^9/L. The duration of the disease ranged from one to 28 years with a mean (±SD) of 4.3 (±6.2) years.

The control group consisted of 150 subjects randomly selected from among 541 voluntary blood donors from the Fundação Hemominas, Minas Gerais matched for ethnic origin.

Representative cytokines of the Th17 (IL-1β, IL-6, IL-17A, IL-23), Th1 (IL-2, IFN-γ and IL-12p70), Th2 (IL-4), and Treg (IL-2, IL-10 and TGF-β) cell commitment, as well as TNF-α were evaluated.

**Figure 1.** Box plots representing the serum levels (pg/mL) of IL-1β, IL-2, IL-6, IL-10, IL-12p70, IL-17, IL-23, IFN-γ, TGF-β and TNF-α in patients with chronic immune thrombocytopenia (n=98) and controls (n=150). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively; the horizontal bar across the box indicates the median and the ends of the vertical lines indicate the minimum and maximum data values. *P<0.001.
The serum cytokine concentrations (picogram per milliliter, pg/mL) were assayed in duplicate by ELISA (Biosource, Camarillo, CA, USA). Ultra-sensitive kits were used to quantify the levels of IL-1β, IL-4, IL-6, IL-10 and TNF-α. The minimum detectable levels are 0.06 pg/mL (IL-1β), 4.0 pg/mL (IL-2), 0.27 pg/mL (IL-4), 104 fentogram per milliliter (fg/mL) (IL-6), 0.2 pg/mL (IL-12p70), 2.0 pg/mL (IL-17A), 15.0 pg/mL (IL-23), 4.0 pg/mL (IFN-γ), 15.6 pg/mL (TGF-β) and 0.09 pg/mL (TNF-α). All values below the detection levels were considered undetectable and were valued at 0.

Intracellular cytokines were analyzed by flow cytometry in 10 patients and 10 randomly selected controls. Briefly, heparinized peripheral blood (750 µL) with an equal volume of RPMI 1640 medium (Gibco, Grand Island, NY, USA) was incubated for 4 h at 37°C in a 5% CO2 humidified atmosphere in the presence of 50 ng/mL phorbol 12-myristate 13-acetate-PMA, 1 µg/mL ionomycin, and 10 µg/mL of Brefeldin A-BFA (Sigma Chemical Company, St Louis, MO, USA). After incubation, the cells were stained with anti-human monoclonal antibodies (mAbs) to cell surface markers, including anti-CD3 labeled with fluorescein isothiocyanate (FITC), plus anti-CD4 or anti-CD8, labeled with phycoerythrin cyan dye Cy5 (PECy5) and peridinin chlorophyll protein complex (PerCP), respectively. Cells were then fixed, permeabilized, and stained with phycoerythrin (PE)-labeled anti-cytokine mAbs, including anti-IFN-γ and IL-17. All antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Flow cytometry acquisition and analysis were performed in a FACSscalibur™ flow cytometer equipped with a four color detection system (Becton Dickinson, San Jose, CA, USA) using CELLquest software (Franklin Lakes, NJ, USA). After acquiring 30,000 events per tube, specific gating strategies were used to analyze the different cytokine-expressing leukocyte subsets, including innate (neutrophils) and adaptive immunity cells (CD4+; CD8+ T-cell subsets). Selective analysis of neutrophils was performed by establishing a specific scatter gate using the dot plot distribution of anti-CD4PECy5 and laser side
scatter (SSC) to identify the neutrophils as SSChighCD4+. The selection of T-cell subsets was performed by initially gating the lymphocytes on forward scatter (FSC) versus SSC dot plot distribution, followed by analysis on CD8 T cells. The leukocyte subset anti-CD8FercP or anti-CD4PECy5 was then selected and the frequency of cytokine+ cells was determined using quadrant statistics over FL-3/anti-cell surface marker-PercP or PECy5 versus FL-2/anti-cytokine-PE dot plot distribution. The results were expressed as percentages of cytokine positive cells for the different gated leukocyte subpopulations analyzed.

Data were analyzed with SPSS statistical software package version 17.0 (SPSS Inc., Chicago, IL, USA). In addition, to visual examination of histogram and box plots, the Kolmogorov-Smirnov goodness-of-fit model was used to assess the normality of the data. Significant departures from normality were identified for almost all cytokines evaluated. Because in most of them the data did not become normal after log transformations, comparisons between the groups were made by the two-tailed Mann-Whitney U test. Correlations were evaluated by Spearman’s correlation test. The level of significance was set at \( P < 0.05 \).

**Results and Discussion**

A 5.1-fold increased serum level of IL-17A, the major pro-inflammatory cytokine produced by Th17 cells, was observed in the patients with cITP compared with controls (\( P < 0.001 \)). Also, the serum concentration of the cytokines involved in the Th17 cell activation and maintenance\(^{11,12} \) i.e. IL-1β, IL-6 and IL-23, were increased 1.8, 3.4 and 16.2-fold, respectively, in patients when compared with controls (\( P < 0.001 \) for all) (Figure 1).

To identify the cell source of IL-17A, we determined the percentages of T cells and neutrophils expressing IL-17A. Similarly to the results of Zhang et al.,\(^{13} \) the percentage of CD4+/IL-17+ cells was significantly higher (\( P = 0.05 \)) in patients with cITP (1.63±1.23) than in controls (0.62±0.21) (Figure 2A and B), whereas the percentage of CD8+/IL-17+ cells did not differ (\( P = 0.50 \)) between patients (0.78±0.29) and controls (0.59±0.41). Notably, we also demonstrated that the percentage of neutrophils expressing IL-17A was significantly higher (\( P = 0.001 \)) in patients with cITP than in controls (0.98±0.32 and 0.51±0.19, respectively) (Figure 2A and B).

One possible link between IL-17A and autoimmune diseases has been recently proposed by Doreau et al.\(^{12} \) The authors demonstrated that IL-17 alone or in combination with B-cell activating factor (BAFF) protects B cells from apoptosis, promotes B-cell proliferation and drives plasma cell differentiation, probably playing a role in the pathogenesis of SLE. Similar mechanisms might be involved in the pathogenesis of cITP, a disease also marked by a loss of B-cell tolerance, abnormal production of auto-antibody and high serum levels of BAFF that promotes the survival of CD19+ and CD8+ cells.\(^{14} \) In addition to IL-17A, Th17 cells also induce the expression of neutrophil stimulating factors (granulocyte-monocyte colony-stimulating factor and granulocyte colony-stimulating factor) as well as chemokines that attract polymorphonuclear cells.\(^{15} \) In addition, we demonstrated that neutrophils are also an important source of IL-17A in cITP patients.

The levels of TNF-α, also produced by Th17 cells in large amounts, were increased 8.4-fold in patients (\( P < 0.001 \)) compared with controls (Figure 1). TNF-α has been considered a cytokine that mediates tissue damage in autoimmune diseases. Although studies evaluating TNF-α in the context of cITP are scarce, the use of a recombinant TNF-α receptor to neutralize TNF-α ameliorates the disease.\(^{21} \)

In agreement with previous results,\(^{15,16} \) we observed that cITP patients have an upregulation of the Th1 lineage characterized by no detectable serum IL-4 (\( P = 0.001 \), Figure 1) and increased serum median levels of IL-2 (17.3-fold increase), IL-12p70 (54.8-fold increase) and IFN-γ (1.6-fold increase) (\( P < 0.001 \) for all, Figure 1), with a concomitant increased percentage of CD4+/IFN-γ+ cells (16.5±11.3 and 5.9±2.29 in patients and controls, respectively, \( P = 0.04 \)) (Figure 2A and B). We also observed that the serum level of IL-2 was correlated with IFN-γ (\( r = 0.42, P < 0.001 \)). Increased levels of IL-12p70, that have a pivotal role in Th1 cell differentiation, and of IL-2, that promotes expansion of Th1 cells leading to an IFN-γ production, favor an inflammatory response, a breakdown of tolerance, and promote the synthesis of IgG1 and IgG3 isotype antibodies with opsonic and complement-fixing effects in humans. Thus, conditions that are associated with increased Th1 cytokine production may play a part in the pathogenesis of the disease. In fact, in a previous study we demonstrated\(^{22} \) that cITP is associated with polymorphisms in genes linked to Th1 cell response. Increased levels of IL-1β and of IL-2/INF-γ were observed in the carriers of the polymorphic alleles of IL1RN VNTR and IL2-330, respectively. No association of these polymorphisms with the other cytokine levels was observed in the present study (data not show).

Because it has been suggested that a defective immune regulation might play a part in the pathogenesis of cITP, we evaluated representative cytokines of the Treg cell commitment. In contrast to the results of other studies,\(^{7,8} \) IL-10 concentration was higher in patients than in controls (\( P < 0.001 \), Figure 1). However, considering that we did not observe increased serum levels of TGF-β (\( P = 0.26 \), Figure 1), and that the levels of IL-2, that has a non-redundant role in the Treg cell commitment, were not correlated with the levels of IL-10 (\( P = 0.49 \)) and TGF-β (\( P = 0.35 \)), we might speculate that Treg cell is not the source of IL-10 in our patients. Reinforcing this hypothesis, a deficient number or an altered function of Treg cells has been described in cITP patients.\(^{23} \)

In conclusion, our study showed increased levels of IL-17A and of Th17-related cytokines, as well as IL-17A producing CD4+ and neutrophils, in cITP patients. This suggests that these cytokines contribute to the pathogenesis of the disease and that they are promising targets for therapeutic intervention.

**Authorship and Disclosures**

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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