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Altered fraction of regulatory B and T cells is correlated with autoimmune phenomena and splenomegaly in patients with CVID

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ABSTRACT

Common variable immunodeficiency (CVID) is a heterogeneous primary immunodeficiency disease, leading to recurrent bacterial airway infections and often also autoimmune complications. To shed light on the regulatory lymphocytes from these patients, we analyzed the levels of regulatory B (pro-B10) cell and regulatory T (Treg) cell subpopulations in PBMCs from twenty-six patients diagnosed with CVID using multi-color flowcytometry. Pro-B10 cells were induced by 48 h in vitro stimulation prior to analysis. Suppressor function was measured on a subset of patients with splenomegaly and autoimmune complications. The levels of regulatory B and T cells were correlated to clinical manifestations, including autoimmunity, splenomegaly and CVID EUROclass subgroups. We demonstrate a significant association between elevated levels of pro-B10 cells, decreased levels of Tregs and autoimmune phenomena in CVID patients. The finding of marked abnormalities in regulatory lymphocyte populations contribute to our understanding of the pathogenesis of CVID and potentially be valuable in the clinical management and treatment of patients.

1. Introduction

Common variable immunodeficiency (CVID) comprises a large heterogeneous group of patients with primary antibody deficiency of unknown ethiology, and is the most common clinically significant primary immunodeficiency disease with an estimated prevalence between 1:20,000 and 1:50,000 [1–4]. It is a severe and potentially fatal disease characterized by low IgG in combination with low-level IgM and/or low-level IgA concentrations [1]. Decreased antibody responses are believed to be due to alterations in B cell differentiation, which is altered in a heterogeneous manner in CVID patients [2,5]. The alterations in B cell subpopulation frequencies have lead to the generation of the EUROclass classification system, which divides CVID patients into eight subgroups based on B cell sub-population frequencies [2]. CVID patients are susceptible to recurrent infections of the upper and lower respiratory tracts, and have an increased risk of developing lymphoproliferative, granulomatous and autoimmune diseases [4]. The frequency of autoimmunity in CVID patients is estimated to be as high as 20%, and may even present before hypogammaglobulineamia [6–8].

It has been suggested that development of autoimmunity in CVID patients could be a breakdown of self-tolerance caused by alterations in immune regulatory mechanisms. Several reports have shown that CVID patients demonstrate a reduced frequency and effector function of natural regulatory T cells (Tregs), which is most pronounced for the autoimmune group [9–14]. Indeed, reduced levels and functionality of Tregs have been firmly associated with human systemic autoimmune disorders, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and others [15].

The classical definition of Tregs is a population of T cells expressing the surface markers CD4 and CD25 and the transcription factor Forkhead box protein 3 (FoxP3). Recently, Miyara et al. added a layer of complexity by demonstrating that Tregs can be divided into three

Abbreviations: AHA, autoimmune hemolytic anemia; AI, autoimmune; aTreg, activated regulatory T cell; CVID, common variable immunodeficiency; ESID, European Society for immunodeficiencies; Foxp3, Forkhead box protein 3; IL-10, interleukin-10; ITP, immune thrombocytopenia; NAI, non-autoimmune; PIB, PMA, Ionomycin and BFA; RA, rheumatoid arthritis; rTregs, resting regulatory T cell; SLE, systemic lupus erythematosus; TLR, Toll-like receptor; Treg, regulatory T cell.

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subpopulations comprised of resting Tregs (rTregs), activated Tregs (aTregs) and non-Tregs using CD45RA and FoxP3 [16]. The non-Treg population (hereafter referred to as fraction III cells) does not possess any regulatory potential. The existing knowledge about aTregs and rTregs in human autoimmune diseases is sparse, and it is currently not known whether the Treg-reduction observed in CVID patients is due to an overall reduction of the total pool of Tregs or rather due to a decrease in only one subset.

Regulatory B cells are another type of regulatory lymphocytes, which have received increasing attention in a number of immune-mediated disorders, such as RA and immune thrombocytopenia (ITP) [17–21]. Although the idea of regulatory B cells is not novel, it is only recently that this cell type has been described in humans [21–23]. Regulatory B cells are generally defined by the ability to produce the immune-regulatory cytokine interleukin (IL)-10 [24]. Importantly, this B cell subset has been suggested to play a role in human disease, but the lack of adequate immunological surface markers and availability has made these cells difficult to study in humans. However, a method for studying human regulatory B cells has recently been described by Iwata et al. [23]. They used prolonged treatment with the TLR9 agonist CpG-ODN to induce a population of IL-10-producing B cells termed pro-B10 cells. This method has been used to study the frequencies of regulatory B cells in a number of human diseases. Among others, it has recently been demonstrated that RA patients have reduced levels of pro-B10 cells, and that this decrease is inversely correlated with disease severity [17,18]. Studies on ITP patients have revealed decreased levels of Tregs in these patients, but surprisingly it has recently been reported that these patients also have increased frequencies of pro-B10 cells [20,25,26]. Moreover, increased fractions of pro-B10 cells have also been observed in chronic hepatitis B patients [19].

The status of the regulatory potential of the B cell pool in CVID patients is currently unknown. It is likewise not known how pro-B10 cell levels are correlated to the relative frequency of Tregs in CVID, a relationship that has only been studied sparsely in any human diseases. In the present work we report that CVID patients have altered frequencies of Tregs and B10-pro cells, and that this reduction is most pronounced in patients with autoimmunity and splenomegaly. Importantly, we demonstrate that the B10-pro cells retain suppressor function in these patients. These findings may have implications for the basic understanding of this B cell disorder and contribute to the knowledge on the pathogenesis underlying autoimmune phenomena and splenomegaly in CVID.

2. Methods

2.1. Patients and inclusion criteria

The entire cohort of CVID patients (thirty-four patients) attending the outpatient clinic at the international center for immunodeficiencies (ICID), Department of Infectious Diseases, Aarhus University Hospital Skejby, Denmark were contacted, and twenty-six of these were recruited into this study. All patients were diagnosed with CVID according to the ESID criteria: IgG and IgA levels at least 2 SD below the mean for age, onset of disease at greater than two years of age, absent isohemagglutinins and/or poor response to polysaccharide vaccine and no other defined cause of hypogammaglobulinemia [1]. The control group consists of eleven healthy controls recruited from the blood bank at Aarhus University Hospital Skejby, Denmark. The study was approved by the Danish Ethics Committee (ref. nr. 41,532) and the Danish Data Inspectorate (ref. nr. 1-16-02-551-13), and all material was handled in accordance with the guidelines from these institutions.

2.2. Isolation of PBMCs

Peripheral blood was collected in Heparin tubes and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density centrifugation, frozen at 1 °C/min and stored in liquid nitrogen.

2.3. Intracellular IL-10 expression analysis

Cells analyzed for intracellular IL-10 levels were thawed rapidly in pre-heated media, washed and seeded in 48-well plates at a concentration of 1×10^6 cells/well in RPMI media supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. The cells were either mock treated or treated with CpG-ODN type B (2006) (10 μg/ml) (Invivogen, Toulouse, France) or CpG + rhCD40L (1 μg/ml) (Invivogen) for 43 h. The CpG and CpG + rhCD40L treated cells were subsequently treated for an additional 5 h with phorbol,12-myristate,13-acetate (PMA) (50 ng/ml), Ionomycin (1 μg/ml) and Brefeldin A (BFA) (10 μg/ml) (all purchased from Sigma-Aldrich, St. Louis, USA). All three stimulants were added to cells as a mixed solution with media. The cells were washed in PBS and stained with LIVE/DEAD Near-IR Dead Cell Stain Kit according to the manufacturers instructions (Invitrogen, Life Technologies, USA). The cells were blocked in 10% FCS and surface stained with the following antibodies: mouse APC anti-human CD3 SK7 (BioLegend, San Diego, USA), mouse APC anti-human CD4 SK3 (BioLegend), mouse APC anti-human CD14 M5E2 (BioLegend), mouse BrilliantBlue515 anti-human CD19 HIB19 (BD Bioscience, New Jersey, USA) and mouse PerCP anti-human CD20 L27 (BD Bioscience). The cells were subsequently fixed and stained with rat BrilliantViolet421 anti-human IL10 JES3-9D7 (BioLegend) using the Cytofix/Cytoperm kit (BD Bioscience) following the manufacturers instructions. The cells were stained according to the fluorescence-minus-one (FMO) principle. The cells were analyzed using a FACSVerse flowcytometer (BD Bioscience) with a 405 nm violet laser, a 488 nm blue laser and a 633 nm red laser. The acquired data were analyzed using the FlowJo software (FLOWJO LLC, USA), in which a derived parameter was generated from the CD19 and the CD20 acquisitions. IL-10^+ gates were set at the top-border of the 98% population of the FMO control in order to avoid subjectivity in the gating.

2.4. Intracellular FoxP3 expression analysis

Cells used for Treg subset analysis were thawed rapidly in pre-heated media, washed and blocked in 10% FCS and surface-stained with the following antibodies: mouse FITC anti-human CD3 SK7 (BioLegend), mouse PerCP anti-human CD4 OKT4 (BioLegend), mouse PE-Cy7 anti-human CD25 (M-A251), mouse APC anti-human CD45RA (HI100) (BD Bioscience). The cells were subsequently stained for intra-nuclear FoxP3 levels with mouse PE anti-human FoxP3 (236a/E7) (BD Bioscience) using the Transcription Factor Buffer Set (BD Bioscience), following the manufacturers instructions. A subset of samples were also stained with mouse anti-human CTLA-4 (CD152) (BNI3) (BD Bioscience) in an eight-color stain. The cells were stained using the FMO principle. The cells were analyzed using a FACSVerse flowcytomtre (BD Bioscience) with a 405 nm violet laser, a 488 nm blue laser and a 633 nm red laser. The acquired data was analyzed using the FlowJo software (FLOWJO LLC, USA). Tregs were defined as $CD3^+CD4^+CD25^+FoxP3^+$, rTregs were defined $CD3^+CD4^+CD45RA^+FoxP3^+$, aTregs were defined as $CD3^+CD4^+CD45RA^-FoxP3^{hi}$, fraction III cells were defined as $CD3^+CD4^+CD45RA^-FoxP3^{low}$. rTregs and aTregs were further defined as $CD25^+FoxP3^+$ by back-gating the $CD25^+FoxP3^+$ gate onto the CD45RA:FoxP3 plot (Fig. 2A).

2.5. T cell suppression assay

PBMCs were thawed rapidly in pre-heated media, and B cells were isolated by negative isolation using the MACSxpress B Cell Isolation Kit (Miltenyi, Germany). Isolated B cells were washed and seeded in 48-well plates at a concentration of 1×10^6 cells/well in RPMI media supplemented with 10% FCS and penicillin/streptomycin. The cells were either mock treated or treated with CpG-ODN type B (2006) (10 μg/ml) (Invivogen) for 43 h. The CpG treated cells were subsequently treated for an additional 5 h with PMA (50 ng/ml) and Ionomycin (1 μg/ml)