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

Emil Kofod-Olsen [a,b,c,*], Sofie Eg Jørgensen [c,e,1], Sara Konstantin Nissen [c,e,1], Lena Westh [c], Bjarne K. Møller [a,d], Lars Østergaard [a,c], Carsten S. Larsen [a,c], Trine H. Mogensen [a,c,e]

[a] International Center for Immunodeficiency Diseases, Aarhus University Hospital Skejby, Aarhus, Denmark
[b] Department of Health Science and Technology, Aalborg University, Aalborg, Denmark
[c] Department of Infectious Diseases, Aarhus University Hospital, Skejby, Aarhus, Denmark
[d] Department of Clinical Immunology, Aarhus University Hospital, Skejby, Aarhus, Denmark
[e] Department of Biomedicine, Aarhus University, Aarhus, Denmark

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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.

* Corresponding author at: Department of Health Science and Technology, Aalborg University, Aalborg, Denmark.
E-mail address: ekol@hst.aau.dk (E. Kofod-Olsen).

[1] Authors contributed equally.

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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)

(Sigma-Aldrich). After stimulation, the B cells were moved to 96-well plates pre-coated with anti-CD3 (LEAF-purified anti-CD3 UCHT1) (BioLegend). $CD4^+$ T cells were isolated from freshly thawed PBMCs by negative isolation using the MACSxpress CD4 T cell Isolation Kit (Miltenyi), and stained with Cell Proliferation Dye eFluor 670 Cell Labeling Kit (eBioscience) following the manufacturers instructions. The eFluor labeled $CD4^+$ T cells were then added to the CD3-coated wells with B cells at a concentration of 10^5 cells/well (1:1). The cells were subsequently treated with anti-CD28 (LEAF-purified anti-CD28 CD28.2) (BioLegend) and incubated in the dark for 96 h. The cells were stained with mouse PerCP-Cy5.5 anti-human CD3 (BD Bioscience), and analyzed using a FACSVerse flowcytomtre (BD Bioscience). The acquired data was analyzed using the proliferation algorithm of the FlowJo legacy version 8.8.7 software (FLOWJO LLC).

2.6. Statistics

Comparison between patients and controls was carried out by non-parametric analysis using the unpaired Mann–Whitney signed-rank test. Comparison of ratios was conducted using the unpaired non-parametric Kolmogorow–Smirnow test. Correlation analysis was performed using the Spearmans non-parametric test. *P* values less than 0.05 (*) were considered significant; *P* values less than 0.01 (**) were considered highly significant.

3. Results

3.1. Clinical characteristics of the CVID patients

Demographic and clinical details of the patients are summarized in Tables 1 & 2. All patients were on immunoglobulin replacement therapy at the time of inclusion. Seven patients were reported to have splenomegaly and one patient was splenectomized. Three patients had granulomatous disease and fourteen patients were diagnosed with autoimmune disorders. There was a large degree of heterogeneity in the observed autoimmune diseases, and many patients had multiple diseases. The splenomegaly patients also presented with various autoimmune disorders. This group, thus represent a subset of patients with very severe disease.

3.2. Analysis of regulatory T cells

It has previously been reported that CVID patients have an overall reduction in regulatory T cells (Tregs) [9,11,13,14]. In order to verify these findings in our patient group, we analyzed the frequency of $CD4^+CD25^+FoxP3^+$ T cells in patient PBMCs compared to control PBMCs (Fig. 1A). We first assessed the $CD4^+$ T cell distribution, where we found that patients and controls displayed comparable median levels, although the patient group exhibited a wider distribution spread of the population (Fig. 1B). We next assessed the Treg distribution and found that patients had significantly reduced levels of Tregs compared to the control group (Fig. 1C). When the patient group was divided into autoimmune (AI) and non-autoimmune (NAI) patients, we observed a tendency to a further reduction in the AI patients (patients $p = 0.0431$ vs. AI $p = 0.0246$), and a slightly higher level in the NAI patients (Fig. 1C). The NAI patients were not significantly different from the control group, however this could be due to low sample size, as the median is close to that of the CVID patients. When we analyzed patients with splenomegaly, we found that this group displayed the lowest Treg portion of the CVID patients, and was highly significantly reduced compared to the controls ($p = 0.0003$), and significantly reduced compared to the NAI group (Fig. 1C). To estimate the FoxP3 protein levels in the Tregs, we analyzed the median fluorescence intensity (MFI) of the Treg gate, which did not reveal any difference in FoxP3 expression between patients and controls, as previously reported in another study [12] (Fig. 1D). Taken together, we find significantly reduced levels of Tregs in CVID patients, which is more pronounced within the group of patients with autoimmunity and particularly evident in the group of patients with splenomegaly.

To determine if the observed reduction in Tregs may be due to an overall reduction in the total pool of Tregs or rather due to a skewed reduction of any of the three Treg subsets, we assessed the distribution of the aTregs, rTregs and Fr.III cells within our patient group. To analyze these three Treg sub fractions, we gated the cells for CD45RA and FoxP3, followed by back-gating of the $CD25^+FoxP3^+$ gate (Fig. 2A). We then defined the three sub-gates as described by Miyara et al. [16]. We found that the patients had a significant reduction in the rTreg frequencies compared to controls, and both AI, NAI and splenomegaly subgroups showed a tendency towards a reduction, although this did not reach statistical significance (Fig. 2B). We did not observe any reduction in the aTreg frequencies for neither the total patients nor the AI and NAI groups (Fig. 2C). Importantly, the patients with splenomegaly showed a highly reduced aTreg frequency compared to the controls (Fig. 2C). We did not observe any drop in the Fr.III cells in either of the groups (Fig. 2D). To assess the amount of rTregs and aTregs out of the total Treg pool, we calculated the rTreg/aTreg ratio. This analysis revealed that the CVID patient group consisted of two separate populations (Fig. 2E). Importantly, the splenomegaly patients were predominantly found in the group expressing high rTreg/aTreg ratio (Fig. 2E).

In summary we found a significantly reduced level of rTregs in the patient group and a highly significant reduction of aTregs in the splenomegaly group, and an increased rTreg/aTreg ratio within the splenomegaly groups.

When we estimated the total fraction of $CD45RA^+CD4^+$ T cells in the patient group, we observed a significant reduction compared to the control group (Fig. 2F). This reduction was particularly pronounced within the AI and splenomegaly groups, whereas the NAI group only showed a modest non-significant reduction (Fig. 2F). To rule out that

Table 1
Summary of demographic and clinical data for patients and controls.

Age (controls)	48.55	±17.73
Number (controls)	11	
Gender M/F (controls)	5/6	
Age (patients)	49.27	±15.53
Number (patients)	26	
Gender M/F (patients)	12/14	
Age at diagnosis	41.81	±19.17
years of symptoms before diagnosis	8	4–22
Years of Ig treatment	11	5–15
IgG (at diagnosis) (g/l)	3.6	1.2–5.5
IgM (at diagnosis) (g/l)	0.08	0.012–0.23
IgA (at diagnosis) (g/l)	0.14	0.06–0.24

Age and age at diagnosis is shown as mean ± SD and the rest is shown as median with interquartile range.

Table 2
Clinical summary.

Disease	*n*
Splenomegaly	7
Granulomatous disease	3
Autoimmunity	14
–ITP	7
–Microscopic colitis	3
–IBD	2
–Sjögren's syndrome	2
–Grave's disease	1
–SLE	1
–Psoriasis	1
–Diabetes mellitus type I	1
–Atopic dermatitis	1
–Chronic urticaria	1
–Ankylosing spondylitis	1
–Hypothyroidosis	1

Number of patients with non-infectious complications.

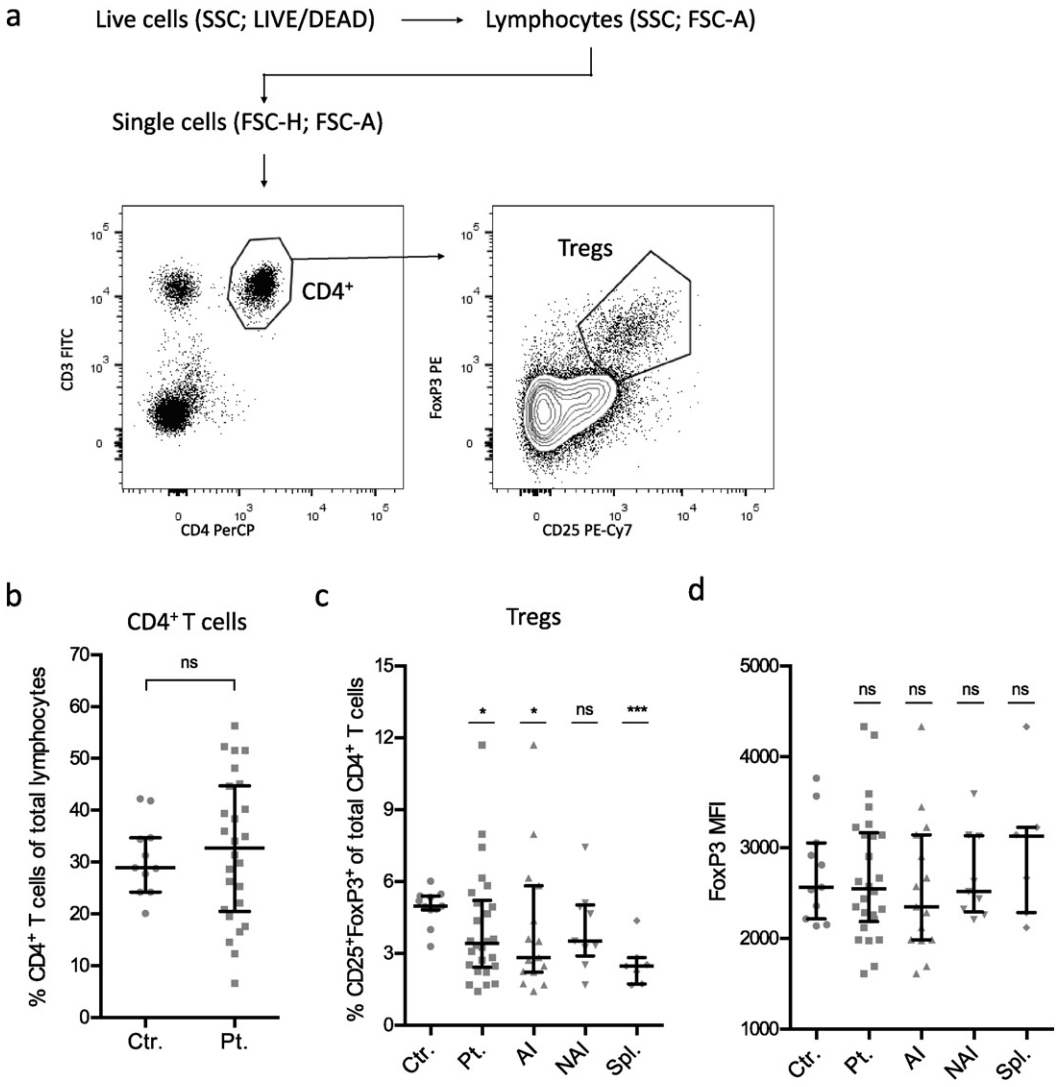

Fig. 1. Treg frequencies. A) Flowcytometric gating strategy. B) CD4+ T cell frequency as percentage of the total single cell lymphocytes in Ctr. (control) and Pt. (patient) groups. P value = 0.7250. C) Treg frequency as percentage of total CD4+ T cells in Ctr., Pt., AI (autoimmune), NAI (non-autoimmune) and Spl. (splenomegaly) groups. Statistical bars mark difference from controls. P values: Pt. = 0.0431, AI = 0.0442, NAI = 0.1711 and Spl. = 0.0003. D) FoxP3 median fluorescence intensity (MFI) of the Treg gate. P values: Pt. = 0.7503, AI = 0.4434, NAI = 0.9837 and Spl. = 0.4756. All plots show median with interquartile range. * < 0.05, ** <0.005, *** <0.0005, ns = non significant.

the observed reduction in rTregs were due to the reduction in CD45RA, we conducted correlation analysis, which revealed that the Treg, aTreg and rTreg frequencies where not correlated to CD45RA levels (Suppl. Fig. 1A–C).

It has previously been shown that Tregs from CVID patients had decreased expression of CTLA-4, which is highly expressed on Tregs, and plays a major role in the inhibition of effector T cells [12,27,28]. To assess the expression levels of CTLA-4 on the surface of Treg subsets in the most severe cases in our patient group (AI + Spl.), we stained PBMCs with Treg markers and CTLA-4. This analysis showed that the patients had a significant reduction in CTLA-4 expression in all subsets except the rTregs (Fig. 2G).

3.3. Analysis of regulatory B cells

A central question of the present study was to examine the fraction of pro-B10 cells in CVID patients. In order to measure pro-B10 cells, we treated patient and control PBMCs with the TLR9 agonist CpG or CpG + CD40L for 43 h, followed by a 5 h stimulation with PMA, Ionomycin and BFA (PIB), as previously described by Iwata et al. [23]. B cells were defined as $CD3^-CD4^-CD14^-CD19^+CD20^+$ lymphocytes, and pro-B10 cells were further identified as $IL\text{-}10^+$ B cells (Fig. 3A). When we examined the reproducibility of the method, we found a strikingly good reproducibility of pro-B10 cell frequencies (Fig. 3B). We did not observe any difference between CpG + PIB and CpG + CD40L + PIB stimulation.

When we analyzed the frequency of B cells in our patients, we found no difference between patients and controls, although the patient group demonstrated a very large variation (Fig. 3C).

We next analyzed the fraction of pro-B10 cells. Here we found that the patient group had a highly significantly increased pro-B10 cell fraction compared to the control group, after both CpG + PIB and CpG + CD40L + PIB stimulation (Fig. 3D). When we further divided the patients into AI and NAI groups, we found that the AI patients had an even more significant expression of pro-B10 cells in contrast to the total patients (Pt. $p = 0.005$ vs. AI $p = 0.0028$) whereas the NAI patients were not significantly different from the controls (Fig. 3E). Patients with splenomegaly had a significantly elevated level of pro-B10 cells compared to control, although this was less pronounced than the entire patient group.

Importantly we found no correlation between overall B cell frequencies and pro-B10 cell expansion, suggesting that the increase in pro-B10 cells is independent of B cell levels (Fig. 3F). We next wanted to analyze if there was correlation between the pro-B10 cell levels and the Treg

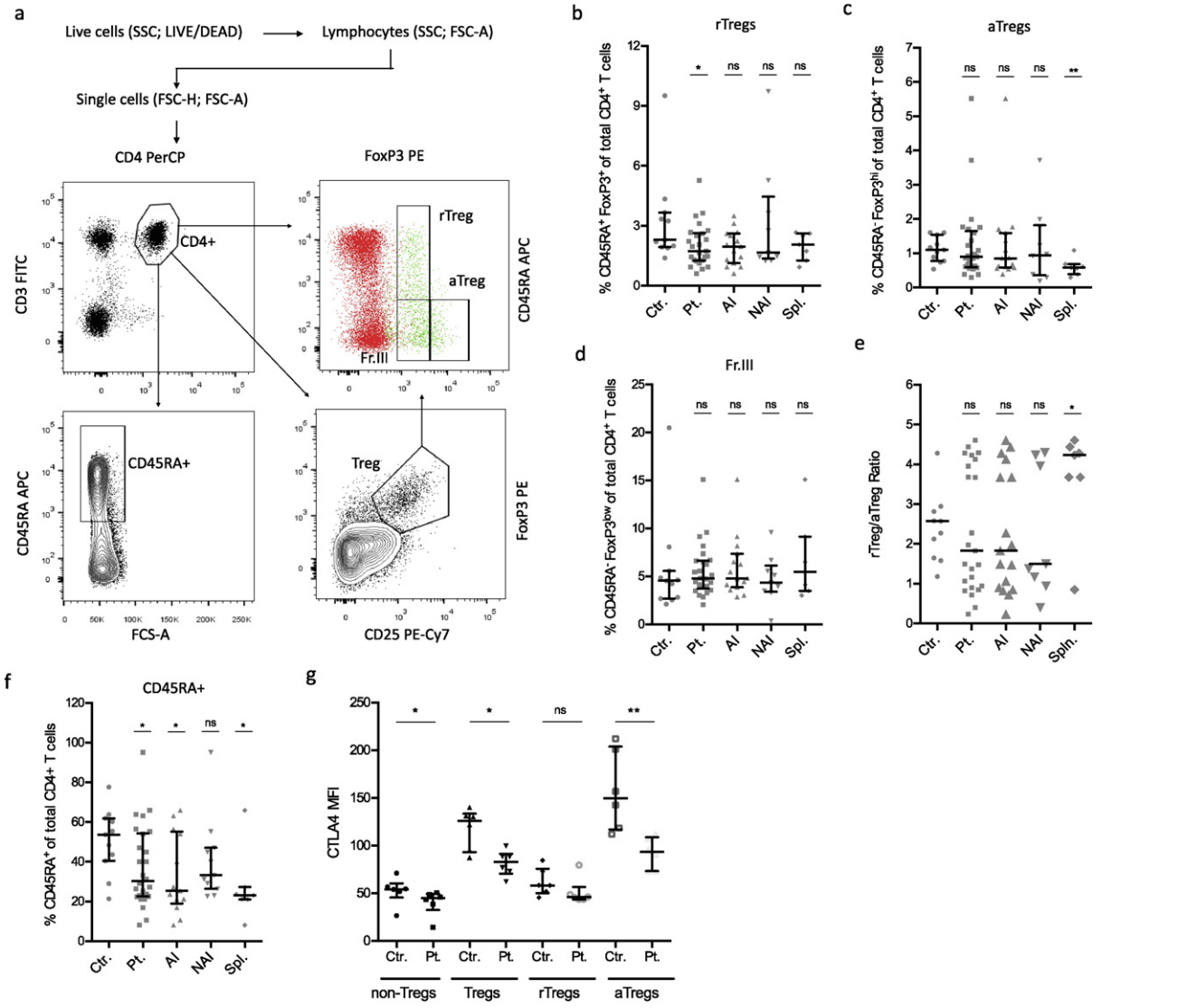

Fig. 2. Treg subgroup frequencies. A) Flowcytometric gating strategy. CD4+ T cells were analyzed for CD45RA expression, Tregs and Treg sub-fractions. The Treg gate was back-gated onto the CD45RA-FoxP3 scatterplot (green cells). B) Resting Treg (rTreg) frequency as percentage of total CD4+ T cells in Ctr. (control), Pt. (patient), AI (autoimmune), NAI (non-autoimmune) and Spl. (splenomegaly) groups. Statistical bars mark difference from controls. P values: Pt. = 0.0445, AI = 0.0381, NAI = 0.4023 and Spl. = 0.1741. C) Activated Treg (aTreg) frequency as percentage of total CD4+ T cells in Ctr. Pt. AI, NAI and Spl. groups. P values: Pt. = 0.5821, AI = 0.3609, NAI = 0.8444 and Spl. = 0.0059. D) Fraction III (Fr.III) cell frequency as percentage of total CD4+ T cells Ctr. Pt. AI, NAI and Spl. groups. P values: Pt. = 0.3508, AI = 0.4413, NAI = 0.8679 and Spl. = 0.3730. E) The rTreg/aTreg ratio in Ctr. Pt. AI, NAI and Spl. groups. P values: Pt. = 0.1977, AI = 0.2907, NAI = 0.2355, Spl. = 0.0241. F) CD45RA+ cell frequency as percentage of total CD4+ T cells in Ctr. Pt. AI, NAI and Spl. groups. P values: Pt. = 0.0478, AI = 0.0979, NAI = 0.1155 and Spl. = 0.0203. G) CTLA-4 median fluorescence intensity (MFI) of the different Treg gates from the patients in the Spl. group. P values: non-Tregs = 0.0411, Tregs = 0.0238, rTregs = 0.1016, aTregs = 0.0022. All plots show median with interquartile range. * < 0.05, ** <0.005, *** <0.0005, ns = non significant.

levels. We observed no correlation between the two parameters (Fig. 3G). We found similar observations when analyzing pro-B10 cell correlation to aTregs and rTregs (data not shown). Therefore, we do not find it likely that there are any correlations between the frequency of pro-B10 cells and any of the Treg subsets.

In order to determine if the pro-B10 cells from the patients were functionally deficient, we analyzed suppression of $CD4^+$ T cell proliferation in controls and the most severely ill patient group (AI + splenomegaly). B cells from controls and patients were isolated by negative selection and subsequently treated with or without CpG for 43 h, followed by 5 h and PMA/Ionomycin treatment to enrich pro-B10 cells. The B cells were then mixed with freshly isolated eFluor 670-labeled $CD4^+$ T cells from the same donor, after which the mixture of cells were treated with anti-CD3 and anti-CD28 to promote T cell proliferation. The cells were grown for 96 h and analyzed by flowcytometry for eFluor 670 and CD3 expression. We found that the $CD4^+$ T cells mixed with mock-treated B cells proliferated to a similar level as $CD4^+$ T cells without B cells for both controls and patients (Fig. 4A&B). When the $CD4^+$ T cells were mixed with pro-B10 enriched B cells and media from this culture, the proliferation was almost completely blocked for both controls and patients, although there was a significant minor increase in the patient group (Fig. 4A&B). When we analyzed the individual T cell generations, we found no difference between patient and control cells (Fig. 4C–E).

3.4. Regulatory cells and EUROclass categorization

We wanted to analyze the distributions of pro-B10 cells, Tregs, aTregs, rTregs and CD45RA CD4 T cells within the different EUROclass subgroups. The EUROclass sub-goupings are based on four different criteria: i) frequency of B cells out of the total number of lymphocytes (B), ii) frequency of switched memory B cells out of the total B cells (smB), iii) frequency of transitional B cells out of the total B cells (Tr), and iv) freqency of CD21-low B cells out of the total B cells ($CD21^{low}$).

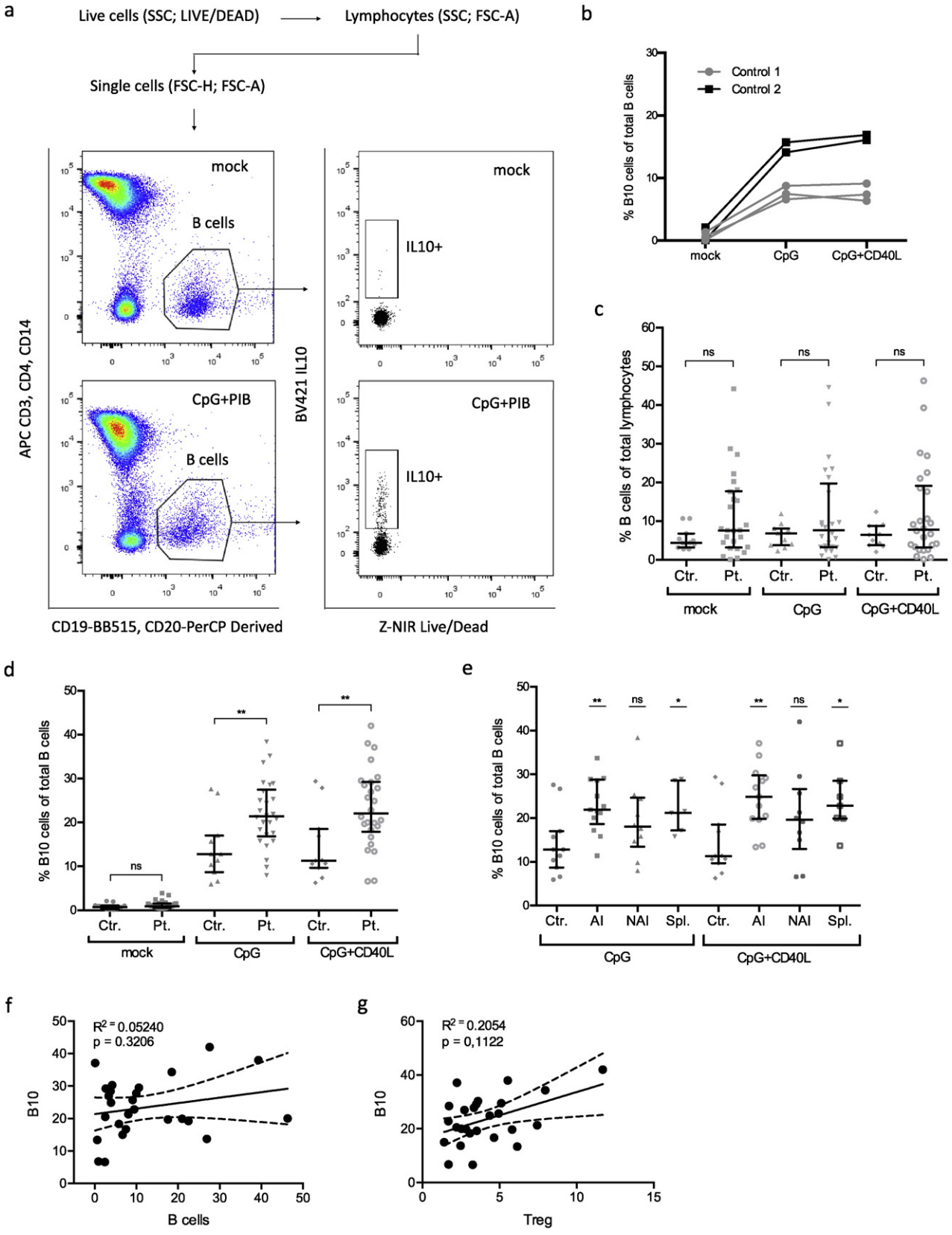

Fig. 3. B10 cell frequencies. PBMCs were mock stimulated or stimulated with CpG or CpG + CD40L for 43 hrs + PIB for 5 hrs. A) Flowcytometric gating strategy. B) Repeated measurements of two controls. C) B cell frequencies as percentage of total single cell lymphocytes in Ctr. (control), and Pt. (patient) groups. D) B10 cell frequencies as percentage of total B cells in Ctr. and Pt. groups. P values: mock = 0.2011, CpG = 0.005, CpG+CD40L = 0.0067 E) B10 cell frequencies in AI (autoimmune), NAI (non-autoimmune) and Spl. (splenomegaly) groups. Statistical bars mark difference from controls. F & G) Correlation analysis of B10 cell frequency (CpG+CD40L) and B cell and Treg frequencies. All plots show median with interquartile range. * < 0.05, ** <0.005, *** <0.0005, ns = non significant.

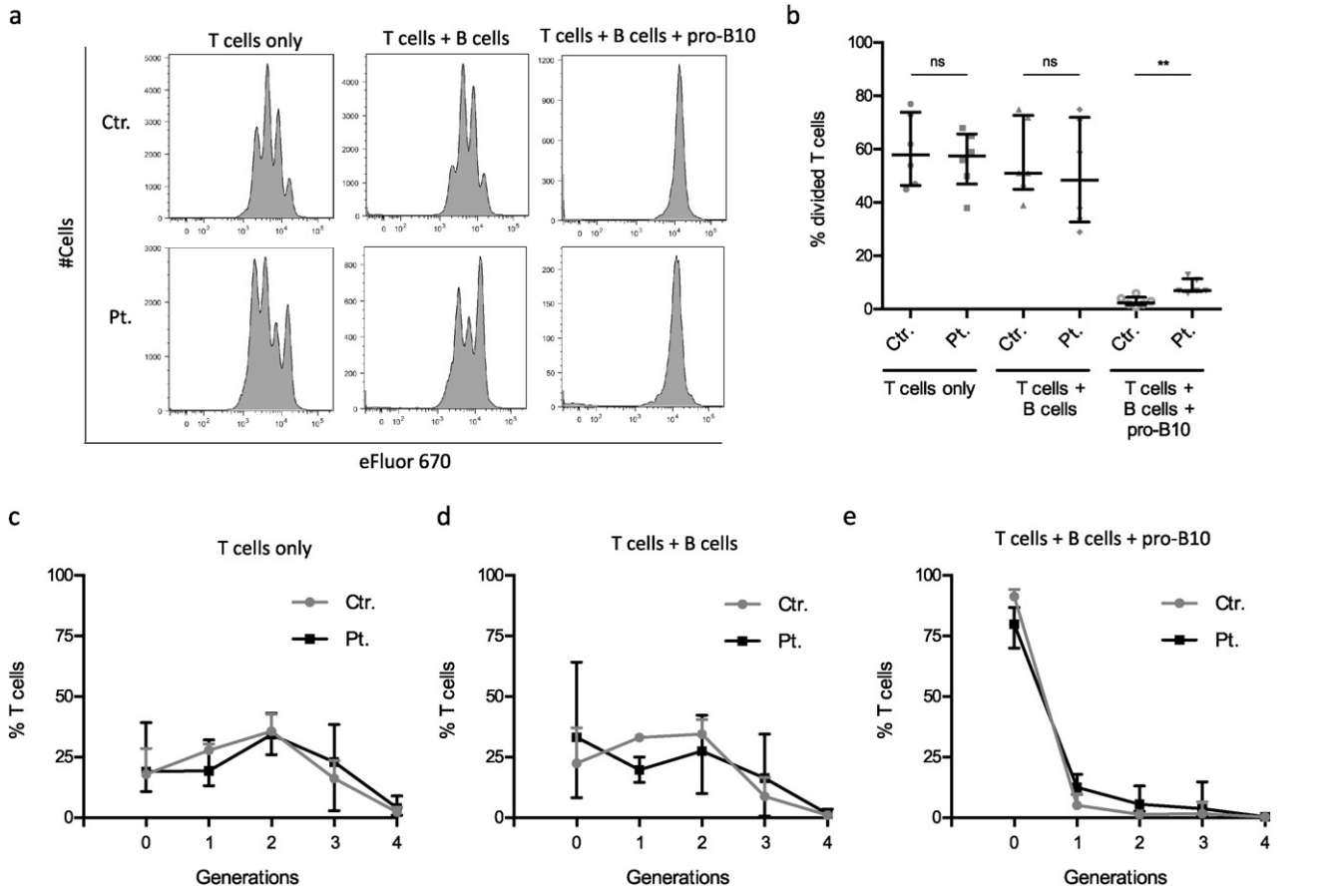

Fig. 4. pro-B10 dependent CD4+ T cell suppression. Labeled CD4+ T cells were incubated with media (T cells only), B cells (T cells + B cells) and B cells enriched for pro-B10 cells (T cells + B cells + pro-B10), and analyzed for proliferation. A) Representative histograms of eFluor staining after 96 hours. B) % divided cells from controls and patients from the Spl. group. P values: T cells only = 0.7879, T cells + B cells = 0.5043, T cells + B cells + pro-B10 = 0.0043. C – E) % divided cells in each generation from controls and patients from the Spl. group. All plots show median with interquartile range. * < 0.05, ** <0.005, *** <0.0005, ns = non significant.

The criteria required to fall into either of these groups are shown in Supplemental Table 1. The EUROclass sub-groupings are then generated on basis of all four criteria, generating the groupings shown in Supplemental Table 2. The distribution of patients within the EUROclass subgroups, and the number of patients with AI and splenomegaly within each group is shown in Supplemental Table 2. Due to the relatively small size of our data set, we chose to restrict our analysis to diminished groups, where we omitted the CD21 criteria (Table 3). This criteria was then analyzed independently of the other three criteria, thus defining patients as either $CD21^{low}$ or $CD21^{norm}$ (Table 3 bottom rows).

The most striking finding for all the regulatory T cell subsets was that the $CD21^{low}$ patients demonstrated very low median levels for Tregs, rTregs and aTregs (Table 3). Furthermore this group also had an increased occurrence of splenomegaly (Table 4). All other subgroups showed levels similar to those observed in the entire patient group. In contrast to this, $smB^{-}Tr^{hi}$ patient group and the $CD21^{norm}$ patients exhibited normal levels of Tregs, aTregs and rTregs (Table 3), and had reduced occurrence of splenomegaly (Table 4).

Analysis of the $CD45RA^{+}CD4^{+}$ T cells, revealed that all subgroups had reduced levels of this subpopulation, except for the patients in the $smB^{+}Tr^{norm}$ group, which showed normal levels of these cells (Table 3).

We found that the pro-B10 cell levels within CVID patients were significantly elevated compared to controls in all subgroups, with the exception of the $smB^{+}Tr^{norm}$ and $CD21^{norm}$ groups (Table 5).

Table 3
Frequencies of regulatory T cells within CVID subgroups.

Group	*n*	*Tregs*		*P*	*aTregs*		*P*	*rTregs*		*P*	*CD45RA*		*P*
Controls	11	4.9	4.6-5.2	–	1.1	0.8-1.5	–	2.3	1.9-3.7	–	53.6	40.5-61.9	–
Patients	26	3.4	2.4-5.2	0.0431*	0.9	0.6-1.1	0.5821	1.7	1.3-2.6	0.0445*	30.2	22.6-54-3	0.0478*
B-	2	1.9	1.7-2.2	0.0256*	0.7	0.4-1.1	0.2308	1.9	0.9-2.8	0.3333	19.6	8.0-31.2	0.0769
$smB^{+}Tr^{norm}$	5	3.1	2.5-3.3	0.0018**	0.9	0.7-0.9	0.1376	1.5	1.0-2.1	0.0371*	45.0	40-0-63.5	0.9338
$smB^{-}Tr^{norm}$	11	3.5	1.7-5.5	0.2668	0.9	0.6-1.8	0.8315	1.7	1.2-2.5	0.0715	24.3	22.1-35.7	0.0241*
$smB^{-}Tr^{hi}$	6	5.3	3.3-8.9	0.1276	1.2	0.6-2.6	0.9517	2.5	1.3-2.7	0.3167	27.3	21.3-47.1	0.0266*
$CD21^{norm}$	7	4.4	3.3-5.8	0.8260	0.9	0.6-1.7	0.8805	2.3	1.4-3.1	0.5723	25.4	21.2-47.1	0.0838
$CD21^{low}$	12	2.7	1.8-4.9	0.0437*	0.8	0.6-1.6	0.3429	1.7	1.3-2.4	0.0373*	26.9	22.7-38.3	0.0156*

Frequencies of Treg sub-populations CD45RA CD4 T cells within EUROclass subgroups without the CD21 criteria (rows 3–6) and within $CD21^{norm}$ and $CD21^{low}$ (elevated levels of $CD21^{low}$ cells) patients (rows 7–8). Values are given as median with interquartile range and *P* value.

* Marks significance.

** Marks highly significant.

Table 4
Autoimmunity and splenomegaly within CVID subgroups.

Group	*n*	*Autoimmunity*		*Splenomegaly*	
Controls	11	–		–	
Patients	26	14	(53.8%)	7	(26.9%)
B-	2	1	(50%)	1	(50%)
$smB^{+}Tr^{norm}$	5	3	(60%)	–	
$smB^{-}Tr^{norm}$	11	5	(45.6%)	4	(36.4%)
$smB^{-}Tr^{hi}$	6	4	(66.7%)	2	(33.3%)
$CD21^{norm}$	7	4	(57.1%)	1	(14.1%)
$CD21^{low}$	12	6	(50%)	5	(41.7%)

Number and frequency of patients with autoimmune manifestations and splenomegaly within the reduced EUROclass subgroups.

4. Discussion

In this study we examined the levels and functionality of pro-B10 cells, as well as the levels of Tregs, rTregs and aTregs within a cohort of CVID patients and correlated our findings to CVID EUROclass subgroups and clinical characteristics related to autoimmune phenomena and splenomegaly.

We report that our cohort of CVID patients have a reduced level of Tregs, which was most pronounced within the groups of patients with autoimmunity and splenomegaly. In agreement with these results, previous studies also found reduced levels of Tregs and association with autoimmunity and splenomegaly in CVID patients [9,11]. Previous studies have focused on Tregs, defined as $CD25^{+}FoxP3^{+}$ T cells. However, this population has been shown to include a subpopulation, which is non-regulatory [16]. Here we demonstrate that the rTreg population was reduced in CVID patients. Moreover, we found that the aTreg population was highly reduced only in the most severely ill patients with both autoimmunity and splenomegaly. It thus appears that the CVID patients fall into two groups, one that produce lower amounts of rTegs, but still sufficient to maintain a normal level of aTregs, and one that produce to few rTregs to maintain their aTreg pool, with the latter constituting the most severely ill patients. In addition, we found that the rTreg/aTreg ratio is high in a subgroup of AI patients, which includes the splenomegaly patients. We found no change in the non-regulatory Fr.III cells. Treg function has been associated with the inhibitory receptor CTLA-4 [27,28]. It has previously been reported that Tregs from CVID patients have a reduced expression of CTLA-4 mRNA [12]. We found that CVID patients with severe progression of disease have a reduced expression of CTLA-4 on Tregs, and in particular on aTregs, which indicates that these cells may be dysfunctional. The low expression of CTLA-4 on Tregs may thereby play a role in development of autoimmunity in these patients.

Interestingly, when we analyzed the Treg subpopulations, we noticed a general reduction in CD45RA expression on $CD4^{+}$ T cells in CVID patients. We found that the reduced levels of CD45RA, was associated with autoimmunity, and particularly with splenomegaly. The reason why we observe this association between CD45RA and severity of disease is not clear. CD45RA is primarily expressed on naïve and effector T cells, whereas memory T cells express the CD45RO variant [29]. It thus appears that the severity of disease is associated with a reduction in naïve and effector T cells. This is supported by the findings that CVID patients express a reduced level of naïve $CD4^{+}$ T cells [30].

It is evident that there is a strong association between reduced Treg activity and the presence of autoimmunity/splenomegaly in CVID patients. This is confirmed by the large amount of data linking impaired Treg levels and activity with autoimmunity in both humans and mice [15].

The main finding of the present study is that CVID patients have a highly increased level of pro-B10 cells after induction with either CpG or CpG + CD40L followed by a five hour PIB stimulation. We did not observe any difference between the two kinds of stimuli, and thus conclude that the induction of IL-10 in B cells in our setup is independent of T cell help. We found that the expansion of pro-B10 cell fractions was most pronounced within the CVID patients with autoimmunity and splenomegaly, whereas CVID patients without autoimmunity only showed a modest increase. The finding that high pro-B10 cell levels is associated with autoimmunity is surprising in relation to murine studies and the studies in rheumatoid arthritis, which have suggested an association between diminished levels of pro-B10 cells and these autoimmune conditions [17,18,31–33]. However, one study has previously demonstrated that patients with the autoimmune disease ITP have highly increased levels of pro-B10 cells, which was correlated with the levels of Tregs [20]. This study supports our findings that autoimmunity may be associated with elevated levels of pro-B10 cells in humans, although we did not observe any correlations between pro-B10 levels and Treg levels. A study by Blair et al. found that $CD19^{+}CD24^{+}CD38^{hi}$ peripheral blood B cells, a subset enriched for regulatory B cells, were able to inhibit T cell differentiation in healthy individuals, but failed to do so in SLE patients [21]. In light of our findings, it evident that there are fundamental differences in the underlying mechanism leading to autoimmunity in SLE and CVID patients.

The underlying mechanistic link between elevated pro-B10 cell levels and autoimmunity in CVID patients is, thus, not clear at the moment. Since we observed reduced levels of Tregs with association to autoimmunity, it could be speculated that pro-B10 cells may be increased as a secondary phenomenon to compensate for the impaired Treg maturation and function. This idea is supported by our findings that pro-B10 cells from the most severely ill CVID patients in our group were able to suppress $CD4^{+}$ T cell proliferation to a level close to that observed in healthy controls. It is thus likely that the immune-regulatory defect in CVID patients is due to a reduced and dysfunctional population of Tregs, and that pro-B10 cells are up-regulated as a compensatory mechanism to increase the amount of Tregs. An alternative explanation for the observed up-regulated levels of pro-B10 cells in CVID patients could be that CVID patients have a skewed distribution of B cell sub-population, in particular in the memory subsets. However, this is not likely, since recent findings by Lin et al., show that memory and naïve B cell populations exhibit comparable levels of IL-10 secretion after stimulation [34].

The massive suppression of T cell proliferation observed in our study, was a bit surprising since Bouaziz et al. previously reported a more moderate inhibition in co-culture assays [35]. However, the B cells in that study had been activated with CpG and anti-Ig, whereas we used CpG and PMA/Ionomycin. Anti-IgM has been reported to suppress regulatory B cell function, which may explain the observed differences [36].

Due to the size of our cohort, we were not able to analyze our data in relation to all EUROclass subgroups, but chose instead to analyze the data according to a slightly simplified system (Table 4).

We demonstrate that $CD21^{low}$ patients have particularly low levels of Tregs and particularly high levels of pro-B10 cells, and are overrepresented within the splenomegaly group, whereas $CD21^{norm}$ patients have normal levels of both pro-B10 cells and Tregs, and have a reduced proportion

Table 5
Frequencies of B10 cells within CVID subgroups.

Group	*n*	*B10*		*P*
Controls	11	11.3	9.7–18.5	–
Patients	26	22.1	17.9–29.3	0.0067**
B-	2	21.9	6.7–37.1	0.9231
$smB^{+}Tr^{norm}$	5	20.5	12.5–27.4	0.3773
$smB^{-}Tr^{norm}$	11	21.3	17.7–28.5	0.0104*
$smB^{-}Tr^{hi}$	6	22.1	13.6–36.2	0.0365*
$CD21^{norm}$	7	19.7	13.4–27.0	0.1507
$CD21^{low}$	12	20.9	17.5–27.8	0.0129*

Frequencies of B10 cells (CpG + CD40L treated) within EUROclass subgroups without the CD21 criteria (rows 3–6) and within $CD21^{norm}$ and $CD21^{low}$ (elevated levels of $CD21^{low}$ cells) patients (rows 7–8). Values are given as median with interquartile range and *P* value.
* Marks significance.
** Marks highly significant.

of splenomegaly. We thereby link the $CD21^{low}$ B cell subset with altered regulatory lymphocyte populations and severity of disease. In support of this, expanded $CD21^{low}$ B cell populations have also been observed in the autoimmune disease SLE [37]. Furthermore, it has been demonstrated in another study that there is a negative correlation between the $CD21^{low}$ population levels and FoxP3 frequency in CVID patients [11].

In addition to this, we find that the pro-B10 cell levels in $smB^{+}Tr^{norm}$ subgroup patients were not significantly different from the control group, and none of these patients had splenomegaly, indicating that abnormalities in pro-B10 levels may be linked to the presence of splenomegaly. Interestingly, this patient subgroup also had normal levels of $CD45RA^{+}CD4^{+}$ T cells. We further demonstrate that $smB^{-}Tr^{hi}$ patients have normal levels of Tregs, rTregs and aTregs; however we observed no reduction in autoimmunity and splenomegaly in this patient subgroup.

In conclusion, we here demonstrate that CVID patients have a reduced level of Tregs and an increased level of functional pro-B10 cells, and these alterations are strongly associated with both autoimmunity and splenomegaly. The reduction in Tregs is primarily found within the rTreg subpopulation, whereas the aTregs levels are particularly low in splenomegaly patients. Finally we demonstrate an association between decreased Tregs, increased pro-B10 cells, splenomegaly and $CD21^{low}$ subgroup patients. We hereby provide novel data, which associate the autoimmunity and splenomegaly observed in CVID patient with both Tregs and pro-B10 cells. It will be interesting in future studies to gain more knowledge on the mechanisms, whereby abnormalities in regulatory B and T cells mediate autoimmune manifestations in CVID patients. Such knowledge may provide a link between the B cell deficiency in CVID with the autoimmune phenotype and may predict disease severity with respect to autoimmunity and splenomegaly in the clinic.

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Appendix A. Supplementary data

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