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ORIGINAL ARTICLE

Modulation of the Interleukin-21 Pathway with Interleukin-4 Distinguishes Common Variable Immunodeficiency Patients with More Non-infectious Clinical Complications

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Abstract

Purpose Common variable immunodeficiency (CVID) is characterized by hypogammaglobulinemia and clinical manifestations such as infections, autoimmunity, and malignancy. We sought to determine if responsiveness to interleukin-21 (IL-21), a key cytokine for B cell differentiation, correlates with distinct clinical phenotypes in CVID.

Methods CVID subjects were recruited through the Canadian Primary Immunodeficiency Evaluative Survey registry. Peripheral blood mononuclear cells were cultured with anti-CD40 ± interferon-gamma, interleukin-4 (IL-4), IL-21, and/or IL-4+IL-21. B cell subpopulations and IgG production were determined at baseline and day 7 by flow cytometry and ELISA. Clinical complications were compared using contingency tables.

Results CVID subjects exhibited decreased $CD27^+$ B cells and IgG production after 7 days of stimulation with anti-CD40+IL-21 ($p < 0.05$). In a subset of subjects [CVID responders (R)], the addition of IL-4 led to significant increases in $CD27^+$ B cells and IgG ($p < 0.05$). In CVID non-responders (NR), $CD27^+$ B cells and IgG remained lower despite the addition of IL-4. CVID NR experienced significantly more non-infectious clinical complications of CVID than R [OR 8.8, 95% confidence interval (CI) 1.6 to 48.13]. Previous studies reported that CVID subjects with ≤ 2% class-switched memory B cells were more at risk of these complications, but no significant association was found among this cohort of subjects [OR 3.5, CI 0.9 to 13.3]. In fact, 34.6% of CVID NR had > 2% class-switched memory B cells at baseline.

Conclusions The IL-4 and IL-21 in vitro assays distinguish two groups of CVID subjects and can be used with baseline B cell subpopulation phenotyping to better identify patients experiencing more vs. fewer clinical non-infectious complications and potentially to modulate therapy.

Keywords Common Variable Immunodeficiency · interleukin-4 · interleukin-21 · B lymphocytes · humoral immune response · antibody production

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Introduction

Primary immune deficiencies (PIDs) are a group of diseases that are important causes of severe recurrent infections and autoimmunity and have exceedingly high use of medical resources. Common variable immune deficiency (CVID) is one

of the most frequently diagnosed humoral immune defects [1] and represents a syndrome rather than a single diagnostic entity [2]. It is characterized by decreased serum immunoglobulins (Ig), impaired specific antibody responses to vaccines, and increased risks of severe infections [3]. Despite proper diagnosis and treatment with Ig replacement therapy, some patients still have reduced survival rates of 64–67% at 20 years (compared to 92–94% for the general population) with morbidity and mortality secondary to infections, autoimmunity, or malignancies [4–7]. Specific and/or curative therapies for CVID are not available presently, and a genetic etiology is unknown in more than 70% of cases despite whole-exome sequencing screening for up to 269 PID-causing genes [6, 8].

One frequent finding in CVID is a decrease in mature, isotype class-switched (CS) memory B cells [9] which correlates with a more severe disease course [10, 11]. The development of normal memory B cell population is milieu-dependent, with the cytokine interleukin-21 (IL-21) being among the most important signals for inducing B cell memory [12, 13]. Abnormal signaling through the IL-21R/γ_c/JAK3/STAT3 pathway has been related to decreased specific antibody responses following vaccination and to increased susceptibility to encapsulated bacterial infections, as found in patients with the hyper-IgE syndrome and X-linked and JAK3-related forms of severe combined immunodeficiency and in individuals with loss-of-function mutations in the IL-21 receptor gene [14]. Moreover, patients with CVID can have primarily infectious manifestations or have their disease complicated by autoimmunity, lymphoproliferation, and/or malignancy. With these principles in mind, we hypothesized that impaired B cell development in CVID is related to improper immune responses to IL-21. Using the Canadian Primary Immunodeficiency Evaluative Survey (C-PRIMES) [15, 16], we recruited a cohort of subjects with CVID and healthy controls matched for age and sex to assess if the development of $CD27^+$ B cells and IgG production in response to IL-21 stimulation in vitro could be a tool to differentiate clinical phenotypes in CVID.

Methods

Recruitment CVID subjects were recruited through the C-PRIMES registry at the McGill University Health Centre (MUHC) and at the Centre Hospitalier Universitaire de Québec. Subjects receiving intravenous Ig (IVIG) or subcutaneous Ig (SCIG) replacement therapies from all sites were screened and recruited based on the following inclusion criteria: male or female (4 years of age and older) with clinical symptoms classified as probable and/or possible CVID according to the European Society of PID diagnostic criteria [17]. Subjects were excluded if they had defined causes of hypogammaglobulinemia such as known genetic defects (e.g., X-linked agammaglobulinemia, hyper-IgM syndrome, X-linked lymphoproliferative disease, chromosomal anomalies, CD19 deficiency, ICOS deficiency), evidence of significant T cell dysfunction (e.g., combined immune deficiencies, ataxia-telangiectasia, Wiskott-Aldrich syndrome), or having required immunosuppressive medication within 6 weeks of recruitment.

CVID subjects and healthy controls were matched for age and sex. Information regarding age at symptom onset, age at diagnosis, type of infections (e.g., ears, nose and throat, pneumonia, septicemia, warts), complications (e.g., autoimmune cytopenia, other autoimmunity, chronic diarrhea, bronchiectasis, biopsy-proven granuloma, splenomegaly, hematologic malignancies, or other neoplasia), and laboratory results and management (e.g., IVIG, SCIG, dosage, and antibiotic prophylaxis) was collected. The research project was approved by institutional research ethics boards (REB), and informed consents were obtained for subjects and controls.

B and T Cell Phenotyping Laboratory analyses were performed at the Meakins-Christie Laboratories of the Research Institute of the MUHC from a single blood procurement of 15 to 30 mL collected at recruitment. Five-color analysis was performed for specific B cell subpopulations: naive B cells ($CD19^+$ $CD27^-$ IgM^+), marginal-zone B cells ($CD19^+$ $CD27^+$ IgM^+), transitional B cells ($CD19^+$ $CD38^{++}$ IgM^{high}), isotype CS memory B cells ($CD19^+$ $CD27^+$ IgM^-), and CS plasmablasts ($CD19^+$ $CD27^+$ $CD38^+$ IgM^-). Day 7 analysis was performed to demonstrate $CD19^+$ $CD27^+$ cells gated on live peripheral blood mononuclear cells (PBMCs), and a complementary analysis of $CD27^{++}$ $CD38^{+++}$ (after $CD19^+$ characterization) gated on live PBMCs was performed. Additional flow cytometry phenotyping was performed to establish baseline proportions of $CD3^+$ T cells, $CD4^{+/-}$, CD45RA/CD45RO T cell subpopulations, and IL-21 receptor expression. Mean percentages of cells were compared between CVID subjects and controls. Flow cytometric analyses were performed on 100 μL of blood or 1×10^6 PBMCs/mL with an LSRII (Becton Dickinson, Missisaugua, ON, Canada) using FlowJo software. Antibodies included Zombie Aqua fixable viability dye (BioLegend), PeCy7-conjugated mouse anti-human CD19 (BioLegend), allophycocyanin (APC)-conjugated mouse anti-human CD27 (BioLegend), FITC-conjugated mouse anti-human CD38 (BioLegend), PE-conjugated mouse anti-human IgM (BD PharMingen), phycoerythrin (PE)-conjugated mouse anti-human CD3 (BD PharMingen), APC-conjugated mouse anti-human CD4 (BioLegend), FITC-conjugated mouse anti-human CD45RA (BioLegend), APC-Cy7-conjugated mouse anti-human CD45RO (BioLegend), and APC-conjugated mouse anti-human IL-21 receptor (BD PharMingen) with appropriate isotype controls: PE-Cy7 IgG_1 k (BioLegend), APC IgG_1 k (BD PharMingen), FITC IgG_1 k (BD PharMingen), FITC

IgG_{2b} k (BioLegend), PE IgG_1 k (BD PharMingen), and APC-Cy7 IgG_{2a} k (BioLegend). Proper antibody titrations were performed.

Cell Culture Blood was resuspended in wash medium (RPMI 1640 supplemented with 2 mM L-glutamine, 2% FBS, 100 U/mL penicillin and streptomycin, and 0.5μg/mL amphotericin B) and layered onto a Ficoll-Paque (Pharmacia, Upsalla, Sweden) gradient. Upon centrifugation, the Ficoll interface was collected and washed. Purified peripheral blood mononuclear cells (PBMCs) were resuspended at 1×10^6 PBMCs mL in complete medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 15 mM HEPES buffer (pH 7.0), and 100 U/mL penicillin/streptomycin). PBMCs were cultured with anti-CD40 (Clone G28.5, ATCC, 1 μg/mL) ± interferon-gamma (R&D Systems, 10 ng/mL), interleukin-4 (IL-4, R&D Systems, 200 U/mL), IL-21 (Peprotech Inc., 50 ng/mL), or IL-4+IL-21 at 37 °C and 5% CO_2 for 7 days. Culture medium was replenished at days 3 and 5 of cell culture without additional cytokines. Culture supernatants were frozen at − 80 °C.

Quantification of IgG by ELISA IgG production in culture supernatant was determined by ELISA on day 7 culture supernatants. Ninety-six-well plates (Costar, Corning Corp, Acton, Mass) were coated overnight at 4 °C with goat anti-human IgG (Bethyl, 1 mg mL, working dilution of 1:500) in 0.5 M carbonate-bicarbonate buffer, pH 9.6. After washing three times with PBS/0.1% Tween-20, the plates were blocked with blocking buffer (TRIS 30 mM, NaCl 0.14 M, and 1% BSA) for 30 min at room temperature, washed, and then incubated for 1 h with cell culture supernatants or serial dilutions of human IgG standards (4.4 mg mL). After washing, HRP-conjugated goat anti-human IgG (Bethyl, 1 mg mL, working dilution of 1:50,000) was added and incubated for 1 h. After incubation and washing, tetramethylbenzidine (BioLegend) was added, and the plate was incubated for 10 min. The reaction was stopped with a 1 N H_3PO_4, and the absorbance was measured with an ELISA plate reader (Tecan Infinite M1000) at 450 nm. The limit of IgG detection was 0.586 ng/mL.

Statistical Analyses One-way ANOVA with Bonferroni post-tests or Student's *t* test was performed to compare culture conditions between CVID subjects and controls using PRISM 5 software (Graphpad, Carlsbad, CA). Proportions of clinical complications between CVID responders (R) and non-responders (NR) or the percentage of CS memory B cells was compared using a contingency table and Fisher exact tests. A p value < 0.05 was considered as statistically significant.

Results

Demographic and Clinical Characteristics of CVID Subjects Fifty subjects were recruited (Table 1) and of these, 7 subsequently met exclusion criteria because of known genetic anomalies or other humoral defects (sIgA deficiency $n = 2$, X-linked agammaglobulinemia $n = 1$, hyper IgM $n = 1$, STAT1 mutation $n = 1$, Turner's syndrome $n = 2$) following medical chart review. Another subject was excluded because of a past medical history or chronic lymphocytic leukemia. Of the included CVID subjects, 65.9% were female and the mean age at time of recruitment was 41.6 years. The mean delay between onset of symptoms and diagnosis was 14 years. The most frequent clinical manifestations or complications were infections (Fig. 1) followed by splenomegaly (40.0%), bronchiectasis (27.5%), autoimmune cytopenias (17.5%), and chronic diarrhea (15.0%). Baseline B and T cell characteristics determined by flow cytometry are presented in Table 2. Fifty-one (51.0%) percent of CVID subjects were on regular SCIG (mean dose 0.6 g/kg) and 46.9% on regular IVIG (mean dose 0.7 g/kg). One subject was temporarily not receiving Ig replacement therapy at the time of chart revision because of adverse IVIG infusion reactions. The management plan was under revision by the medical team for this subject. Twenty-two (22.5%) percent received continuous antibiotic prophylaxis (thrimethoprim-sulfamethoxazole, amoxicillin, or azithromycin) in combination with SCIG/IVIG. No statistical difference was observed regarding the rate of clinical complications between CVID subjects receiving or not antibiotic prophylaxis.

IL-21 Pathway Differentiates Two Groups of CVID Subjects We stimulated PBMCs from subjects with CVID using anti-CD40+IL-21(±IL-4), to promote B cell development and IgG production. Data on IgG production in culture supernatant at day 7 was available for 39/42 CVID subjects. However, only the second half of the cohort (23 CVID subjects) had extensive B cell characterization at day 7. CVID subjects exhibited proportionally decreased $CD27^+$ B cells (Fig. 2a, b, $p < 0.05$), $CD27^{++}$ $CD38^{+++}$ plasmablasts ($p < 0.05$, data not shown), and IgG production in culture supernatants (Fig. 2c, $p < 0.05$) compared to controls. However, the in vitro response to anti-CD40+IL-4+IL-21 stimulation discriminated two groups of CVID subjects. A subset of CVID [designated responders (R)] exhibited an increase in $CD27^+$ B cells (Fig. 3a–c, from 1.8 to 6.0%, $n = 9$, $p<0.05$), $CD27^{++}$ $CD38^{+++}$ plasmablasts (Fig. 3d, from 0.6 to 4.8%, $n = 6$, $p < 0.05$), and IgG production (Fig. 3e, from 0.5 to 3.0 mg/L, $n = 13$, $p < 0.05$) approaching that of the controls upon anti-CD40+IL-4+IL-21 stimulation. CVID subjects were classified as R if their percentage of $CD27^+$ B cells or their IgG production in response to anti-CD40+IL-4+IL-21 stimulation (for the subjects that did not have day 7 B cell

Table 1 Demographic and laboratory characteristics of common variable immunodeficiency (CVID) subjects

Age [mean (SD), years]	41.6 (16.5)
Age at first symptoms [mean (SD), years][a]	18.1 (16.8)
Age at diagnosis [mean (SD), years][a]	32.1 (15.4)
Caucasian (% subjects)	93.2
Female (% subjects)	65.9
Serum IgG at diagnosis [mean (SD), g/L]	2.8 (1.9)
Serum IgA at diagnosis [mean (SD), g/L]	0.2 (0.5)
Serum IgM at diagnosis [mean (SD), g/L]	0.3 (0.3)
Serum diphtheria-specific IgG [mean (SD), IU/mL][b]	0.0 (0.2)
Serum tetanus-specific IgG [mean (SD), IU/mL][b]	0.7 (1.2)
Number of serum pneumococcal serotype-specific IgG ^{3} 1.3 [mean(SD)][c]	1.9 (3.2)

Of the initial 50 recruited subjects, 8 were excluded from the analysis based on genetic anomalies, other humoral defects, or previous history of chronic lymphocytic leukemia

SD standard deviation

[a] Mean age at first symptoms and at diagnosis was calculated based on 21 subjects with available information

[b] Specific antibody response was assessed post vaccination booster. Response was considered abnormal if less than fourfold increase compared to baseline or below laboratory threshold despite booster vaccination

[c] Fourteen pneumococcal serotypes were assessed post vaccination booster. Response was considered abnormal if less than 70% (50% in children) of serotypes were ≥ 1.3

characterization) was equal to or above one standard deviation of the mean compared to the control group. In CVID non-responders (NR), the percentage of $CD27^+$ B cells [from 1.7 to 1.9%, $n = 14$, no significant difference (NS)] and of $CD27^{++}$ $CD38^{+++}$ plasmablasts [from 0.5 to 0.5%, $n = 12$, NS] remained very low, as did IgG production (from 0.1 to 0.4 mg/L, $n = 26$, $p = 0.05$) despite stimulation with anti-CD40+IL-4+IL-21. In fact, the percentage of $CD27^+$ B cells ($p < 0.05$), $CD27^{++}$ $CD38^{+++}$ plasmablasts ($p < 0.05$), and IgG production ($p < 0.05$) remained significantly lower for CVID NR compared to control levels. Following 7 days of stimulation with anti-CD40+IL-4+IL-21, CVID R had more $CD27^+$ B cells and $CD27^{++}$ $CD38^{+++}$ plasmablasts and exhibited a 226-fold increase in IgG production compared to a 28-fold increase among NR. No significant differences between CVID subjects and controls were observed regarding B cell responses to interferon-gamma stimulation in vitro.

CVID R and NR Have Different Baseline B Cell Characteristics CVID NR had significantly more naive B cells (Fig. 4, $p < 0.05$) and transitional B cells ($p < 0.05$), with fewer CS memory B cells ($p < 0.05$) and CS plasmablasts ($p < 0.05$) compared to controls. CVID R had significantly more $CD27^+$ IgM^+ B cells ($p < 0.05$) and CS memory B cells ($p < 0.05$) compared to CVID NR with fewer CS plasmablasts ($p < 0.05$) compared to controls. Although CVID NR had fewer CS memory B cells compared to controls at baseline, their mean percentage was 2.9% and 9/26 CVID NR had > 2% CS memory B cells (8/13 CVID R, data not shown). The percentage of transitional B cells was higher among CVID NR, but a strong expansion (≥ 9%) was present in only four CVID NR and one CVID R. CVID NR and R as well as controls had similar proportion of overall $CD3^+$ T cells at baseline (Supplemental Fig. 2A). Further analysis of T cell subpopulations revealed that CVID R had slightly lower

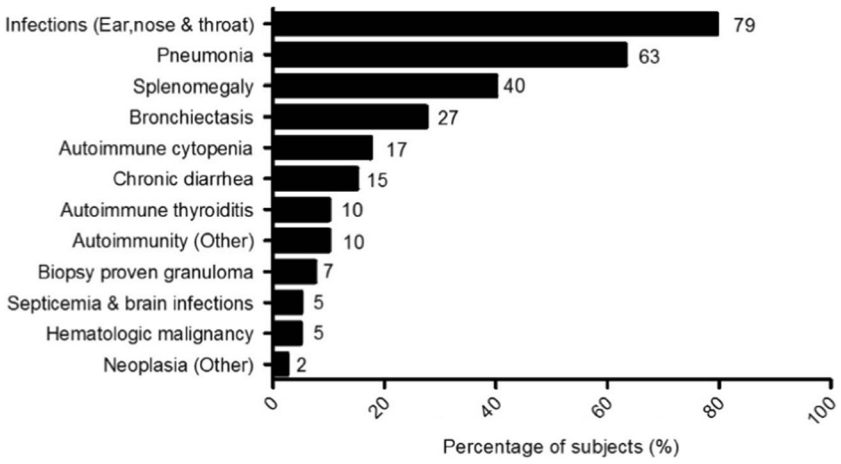

Fig. 1 Clinical manifestations and complications of common variable immunodeficiency (CVID) subjects. Clinical manifestations and complications reported at diagnosis or during follow-up after diagnosis. Information was available for 41/42 subjects

Table 2 B and T cell flow cytometry phenotyping of common variable immunodeficiency (CVID) subjects and controls at baseline

	CVID (n = 42)	Healthy controls (n = 32)	p value
Lymphocytes [mean (SEM), number of cells × 10^9/L]	1.5 (0.1)	1.9 (0.2)	0.08
Lymphocytes [mean (SEM), %]	27.5 (2.1)	29.4 (3.1)	0.61
$CD19^+$ B cells [mean (SEM), %]	8.3 (1.0)	8.7 (0.6)	0.92
$CD27^-$ IgM^+ naive B cells [mean (SEM), %]	38.4 (3.6)	16.8 (2.8)	< 0.05
$CD38^{++}$ IgM^{high} transitional B cells [mean (SEM), %]	2.5 (0.8)	1.4 (0.4)	0.21
$CD27^+$ IgM^+ marginal B cells [mean (SEM), %]	7.2 (1.2)	6.7 (1.3)	0.79
$CD27^+$ IgM^- CS memory B cells [mean (SEM), %]	5.4 (1.5)	16.6 (1.3)	< 0.05
$CD27^+$ $CD38^+$ IgM^- CS plasmablasts [mean (SEM), %]	1.2 (0.2)	3.8 (0.5)	< 0.05
$CD3^+$ T cells [mean (SEM), %]	58.0 (3.0)	58.9 (2.9)	0.31
$CD4^+$ T cells [mean (SEM), %]	58.9 (2.3)	69.1 (2.4)	0.10
$CD4^-$ T cells [mean (SEM), %]	39.8 (2.4)	27.3 (2.3)	< 0.05

Statistical analyses were performed by Student's t test between the two groups

SEM standard error of the mean, % percentage, *CS* class-switched

proportion of $CD3^+$ $CD4^+$ T cells ($p < 0.05$) and CVID NR had more $CD3^+$ $CD4^+$ CD45RO T cells (Supplemental Fig. 2B, $p < 0.05$) compared to controls. The proportion of $CD45^+$ $CD3^+$ $CD4^-$ $CD8^-$ double-negative T cells could be retrieved from the C-PRIMES registry for 26 CVID subjects: CVID R (n = 11) had significantly more double-negative T cells compared to CVID NR (Supplemental Fig. 2C, $p < 0.05$, n = 15). One CVID NR appeared as an outlier with a percentage of $CD45^+$ $CD3^+$ $CD4^-$ $CD8^-$ double-negative T cells of 14.4%.

CVID NR Had More Non-infectious Clinical Complications Compared to CVID R Retrospective analysis of the clinical characteristics of the CVID R and NR groups demonstrated a distinctive pattern that predominated in the NR group. Although both groups exhibited similar rates of infectious manifestations and bronchiectasis (Fig. 5), CVID NR had significantly more complications such as autoimmune cytopenia, splenomegaly, and biopsy-proven granuloma compared to CVID R (difference = 46.1%, $p < 0.05$) and only CVID NR had symptoms of chronic diarrhea/enteropathy.

Discussion

Since its initiation in 2012, the C-PRIMES registry had enrolled over 300 Canadian patients with PID [18, 19]. This study describes the first analysis of CVID subjects enrolled in C-PRIMES. Studies on large cohorts of CVID subjects showed two main clinical phenotypes, one presenting primarily with recurrent infections and a second with infections as well as other complications (autoimmunity, granulomatous disease, and/or malignancy). Strategies that allow early identification of these clinical phenotypes are needed and could lead to better monitoring and management of patients. We described for the first time the IL-4 and IL-21 in vitro assays that distinguish two groups of CVID subjects and can be used as an additional tool to better identify patients with non-infectious complications of CVID.

This cohort was composed primarily of adult CVID subjects with only six subjects diagnosed before 18 years of age. Similar to previously described adult cohorts [20, 21], there was a slight female predominance (Table 1). The mean delay in CVID diagnosis in our group (14 years) was significantly greater than recent data from European countries reporting a mean delay of 4 to 5 years [20] and may reflect a lack of recognition of CVID signs and symptoms among healthcare providers or a perception that PIDs are rare diseases in Canada leading to a low index of suspicion. This was reflected in a previous report from our dedicated PID clinic, where only 17% of referrals were from primary care physicians and only one third had pre-referral quantitative assessment of serum Ig [22]. One long-term consequence of diagnostic delay in humoral immune deficiencies such as CVID is the development of irreversible chronic lung disease. A higher proportion of our CVID subjects (27.5%) were reported with bronchiectasis compared with the US (11.2%, [21]) and European cohorts (23.0%, [20]).

Our laboratory studies regulatory events in B cells in relation to antibody production, immune defects, and severe allergic inflammation involving Th2 cytokines [23, 24]. IL-21 was first described in 2000 by Parrish-Novak et al. [25] and Ozaki et al. [26] as a key cytokine for secondary lymphoid organ B cell development. Abnormal signaling through the IL-21R/γ_c/JAK-3/STAT3 pathway was associated with impaired specific antibody responses and defective establishment of B cell memory in PID diseases [14]. Our study addresses the synergistic combination of IL-4 and IL-21 in driving in vitro B cell

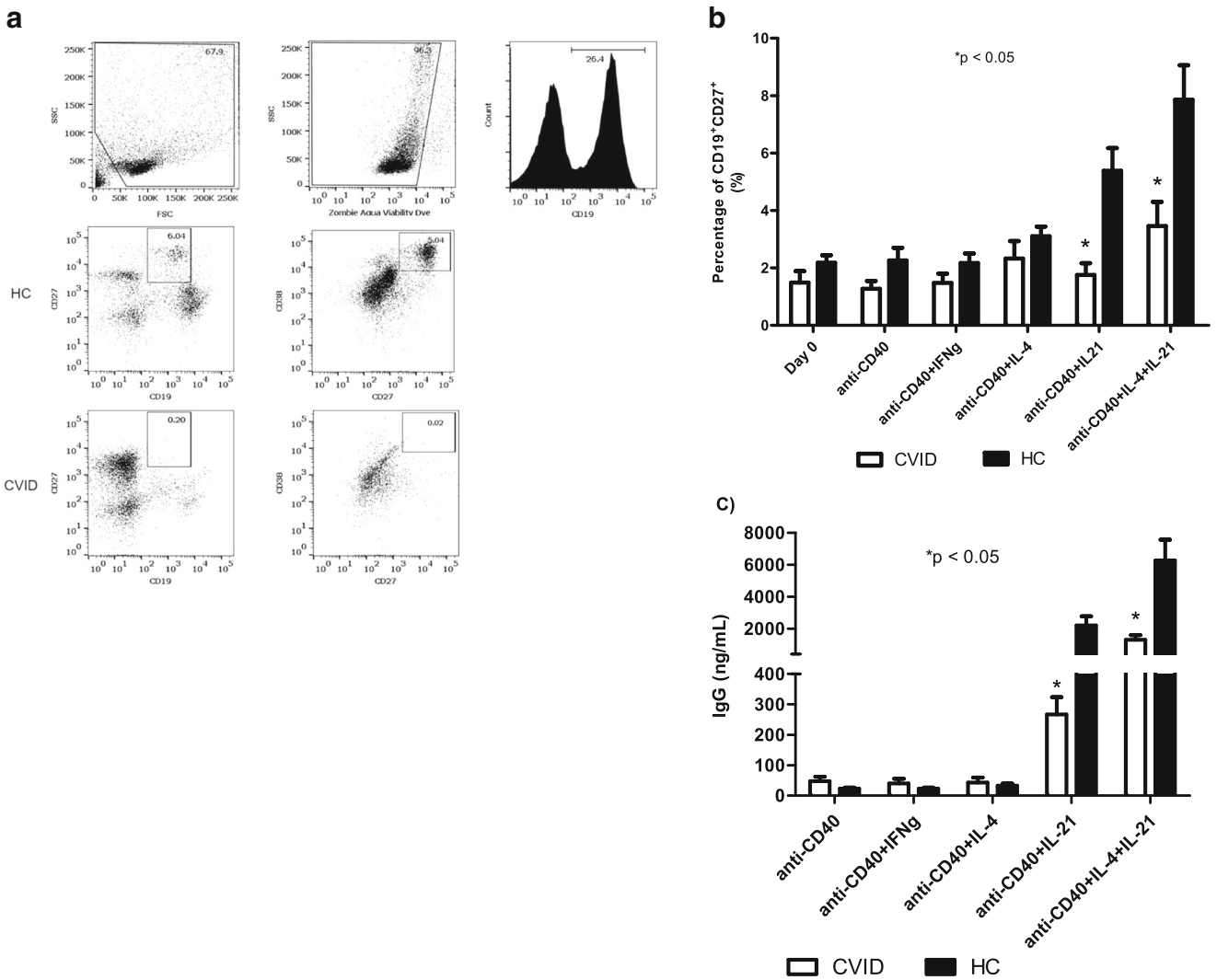

Fig. 2 Common variable immunodeficiency (CVID) subjects have impaired development of $CD27^+$ B cells and IgG production upon 7-day in vitro stimulation with interleukin-21 (IL-21). **a** Peripheral blood mononuclear cells (PBMCs) were analyzed by flow cytometry. After gating on non-debris cells by forward (FSC)/side scatter (SSC), live cells were selected based on the negative expression of Zombie Aqua viability dye. The proportion of $CD19^+$ $CD27^+$ B cells and $CD27^{++}$ $CD38^{+++}$ plasmablasts (after $CD19^+$ characterization) is presented for a representative healthy control (HC, second line) and CVID subject (third line) after anti-CD40+IL-21 stimulation. **b** CVID subjects ($n = 23$) had significantly fewer $CD27^+$ B cells after anti-CD40+IL-21 and anti-CD40+IL-4+IL-21 stimulation compared to HC ($n = 19$, $^*p < 0.05$). No significant differences were observed between CVID subjects and HC for other culture conditions. Results were presented as a percentage of live PBMCs. **c** PBMCs from CVID subjects ($n = 39$) produced significantly less IgG after anti-CD40+IL-21 and anti-CD40+IL-4+IL-21 stimulation compared to HC ($n = 19$, $^*p < 0.05$). No significant differences were observed between CVID subjects and HC for other culture conditions. Statistical analyses were performed by one-way ANOVA with Bonferroni post-tests. INFg interferon-gamma, IL-4 interleukin-4

differentiation in CVID. Using our in vitro culture system, we established that IL-21 promoted PBMC differentiation into $CD27^+$ B cells and $CD27^{++}$ $CD38^{+++}$ plasmablasts, as well as IgG production, in the presence of T-dependent co-stimulation (CD40L/CD40) in controls. PBMCs were cultured for 7 days with media replenishment to allow B cell proliferation and differentiation and limit in vitro cell death. CVID subjects had impaired responses to this stimulation. These results are in line with those of Clemente et al. [27] who demonstrated that purified B cells from CVID subjects had poorer differentiation into $CD38^+$ B cells and produced less IgG and IgA following anti-CD40+IL-21+anti-IgM stimulation but differ from another study [28] suggesting that Ig production could be restored by IL-21. This disparity may be explained by the use of different methodologies, such as ELISPOT counting Ig-secreting cells [28] vs. measurement of actual Ig protein by cytometric bead array [27] or ELISA.

Previous studies suggested that the effect of IL-21 on IgG switching could be enhanced by the addition of IL-4 to the cytokine milieu, independently of cell proliferation. The combination of CD40L+IL-4+IL-21 was found to induce IL-6 and IL-10 which provided an autocrine signal contributing to increased IgG switching [29] which, in some patients, may have therapeutic implications. Indeed, we found a subset of CVID

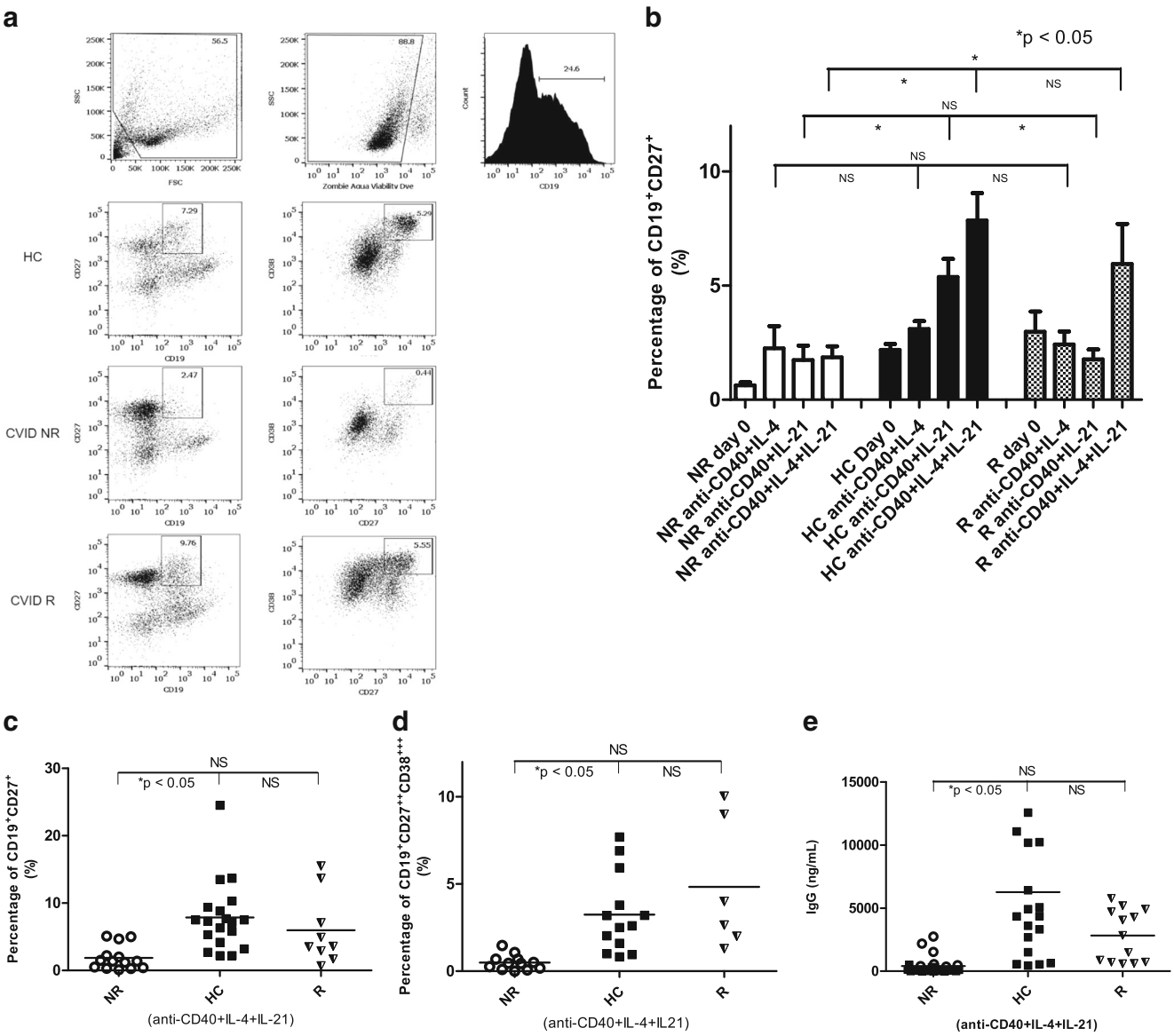

Fig. 3 Modulation of interleukin-21 (IL-21) pathway with interleukin-4 (IL-4) differentiates two groups of common variable immunodeficiency (CVID) subjects. **a** Peripheral blood mononuclear cells (PBMCs) were analyzed by flow cytometry. After gating on non-debris cells by forward (FSC)/side scatter (SSC), live cells were selected based on the negative expression of Zombie Aqua viability dye. The proportion of $CD19^+$ $CD27^+$ B cells and $CD27^{++}$ $CD38^{+++}$ plasmablasts (after $CD19^+$ characterization) is presented for a representative healthy control (HC, second line), CVID non-responders (NR, third line), and CVID responders (R, fourth line) after anti-CD40+IL-4+IL-21 stimulation. **b** The percentage of $CD27^+$ B cells was low after anti-CD40+IL-21 stimulation for CVID NR ($n = 14$) and R ($n = 9$) compared to HC ($n = 19$, $^*p < 0.05$), while it was similar between CVID NR and R [no significant difference (NS)]. The percentage of $CD27^+$ B cells was similar after anti-CD40+IL-4 stimulation between CVID NR, R, and HC (NS). The addition of IL-4 to the culture media significantly increased $CD27^+$ B cells toward control level for CVID R (NS). The percentage of $CD27^+$ B cells remained significantly lower for CVID NR compared to HC despite the addition of IL-4 ($^*p < 0.05$). Results were presented as a percentage of live PBMCs. **c** Dot plot representation of $CD27^+$ B cells among CVID NR, R, and HC after anti-CD40+IL-4+IL-21 stimulation. **d** The percentage of $CD27^{++}$ $CD38^{+++}$ plasmablasts was low after anti-CD40+IL-4+IL-21 stimulation for CVID NR ($n = 12$, $^*p < 0.05$), while CVID R ($n = 6$) were statistically similar to HC ($n = 13$, NS). Results were presented as a percentage of live PBMCs. **e** The IgG production was low after anti-CD40+IL-4+IL-21 for CVID NR ($n = 26$, $^*p < 0.05$), while CVID R ($n = 13$) were statistically similar to HC ($n = 19$, NS). Statistical analysis was performed by one-way ANOVA with Bonferroni post-test or Student's *t* test

subjects (R), in whom the percentage of $CD27^+$ B cells, $CD27^{++}$ $CD38^{+++}$ plasmablasts, and IgG production was increased toward control levels following anti-CD40+IL-4+IL-21 stimulation. Our results are consistent with data [30] showing that Toll-like receptor (TLR7 and TLR9) activation of B cells in the presence of interferon-alpha normalized isotype CS $CD27^+$ B cells and improved IgG production in a subset of CVID subjects who had higher baseline percentages of $CD27^+$ B cells compared to CVID subjects with lower baseline $CD27^+$ B cells. Together with our results, this suggests

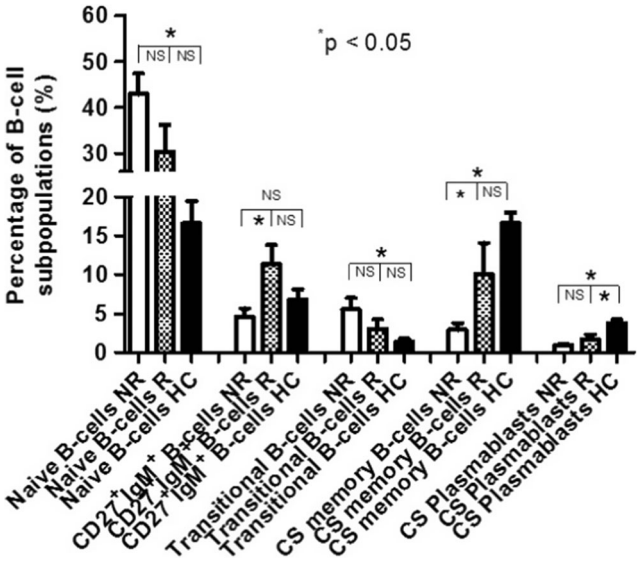

Fig. 4 Common variable immunodeficiency (CVID) groups have different baseline B cell characteristics. **a** CVID non-responders (NR, $n = 26$) had significantly more naive B cells ($*p < 0.05$) and transitional B cells ($*p < 0.05$) with fewer class-switched (CS) memory B cells ($*p < 0.05$) and CS plasmablasts ($*p < 0.05$) compared to controls (HC, $n = 32$). CVID responders (R, $n = 13$) had significantly more $CD27^+$ IgM^+ marginal and CS memory B cells compared to CVID NR ($*p < 0.05$) with fewer CS plasmablasts compared to HC ($*p<0.05$). Results were presented as a percentage of B cells. Statistical analyses were performed by one-way ANOVA with Bonferroni post-tests. NS, no significant difference

that the CVID R group has the functional and enzymatic B cell machinery to increase Ig production in vitro if cultured in the appropriate stimulatory milieu. On subsequent analysis, it was found that CVID R had lower proportion of $CD3^+$ $CD4^+$ "helper" T cells at baseline. CD40L, IL-4, and IL-21 are major molecules related to germinal center (GC) follicular helper T cells (Tfh) to induce B cell proliferation and differentiation. The role of Tfh is to provide transient help to GC B cells. A high level of PD-1 expression found on Tfh contributes to limit Tfh cell proliferation for proper B cell expansion [31]. This is a clinically relevant distinction as it is associated with significantly lower rate of non-infectious complications of CVID and better clinical and prognostic outcomes. In fact, the most important potential finding of our study was the observation that an in vitro assay assessing B cell development and IgG production in response to IL-4 and IL-21 was able to distinguish between two CVID phenotypes (Table 3, odds ratio 8.8, 95% confidence interval of 1.6 to 48.3). Pioneer studies [32, 33] already suggested the characterization of CVID subjects according to their in vitro B cell responses, although at the time, classifications were not related to patients' clinical phenotype. The EUROClass trial [9] also established that low levels of CS memory B cells (≤ 2%) were associated with a higher risk for splenomegaly and granulomatous disease in CVID. However, using the cutoff of ≤ 2% CS memory B cells described in the EUROClass trial, 34.6% of CVID NR who experienced granuloma, autoimmune cytopenia, and/or splenomegaly would not have been identified (Table 4, odds ratio 3.5, 95% confidence interval of 0.9 to 13.3). The mean percentage of isotype CS memory B cells in this cohort was 16.6% (standard deviation 7.3%, standard error of the mean 1.3%, range of 4.6 to 33.7%) for controls which was comparable to the normal range of 6.5 to 29.2% for IgM^- IgD^- $CD27^+$ memory B cells reported by the EUROClass trial. Also, 56.4% of CVID subjects in our study had less than or equal to 2% isotype CS memory B cells at baseline again similar to 58% described in the EUROClass trial [9]. No differences in terms of clinical characteristics between CVID groups such as age at first symptoms (Supplementary Fig. 1), at diagnosis, and at recruitment; serum IgG, IgA, or IgM levels; or serum diphtheria-, tetanus-, or pneumococcal-specific IgG were predicting their outcome (data not shown). One interesting observation was that CVID R who experienced less non-infectious complications showed significantly more $CD45^+$ $CD3^+$ $CD4^-$ $CD8^-$ double-

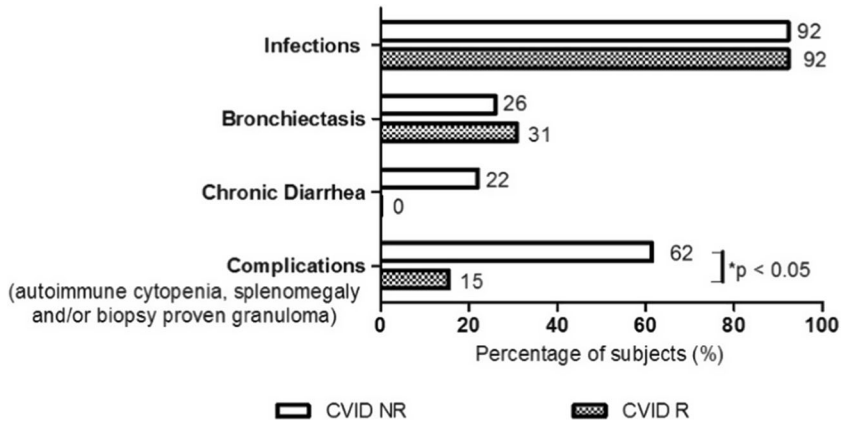

Fig. 5 Common variable immunodeficiency (CVID) non-responders (NR) to interleukin-4 (IL-4) and interleukin-21 (IL-21) in vitro assays have higher rates of non-infectious clinical complications. CVID NR ($n = 26$) have significantly more complications such as autoimmune cytopenia, splenomegaly, and/or biopsy-proven granuloma than CVID R ($n = 13$, $*p < 0.05$). Symptoms of chronic diarrhea occurred only among subjects of the NR group. No other statistical differences were observed. Statistical analyses were performed by Fisher exact test

Table 3 IL-4 and IL-21 in vitro assay

	Complications		Total
	Yes	No	
CVID NR	16	10	26
CVID R	2	11	13
Total	18	21	39

Odds ratio (OR) 8.8 [95% confidence interval (CI) 1.6 to 48.3, Fisher's exact test $p < 0.05$]. Complications included in the analysis were autoimmune cytopenia, splenomegaly, and/or biopsy-proven granuloma

NR CVID non-responders, *R* CVID responders

negative T cells, also described as helper-like immune regulatory cells (Supplementary Fig. 2C) [34].

Prospective validation of the IL-4 and IL-21 in vitro assays in a larger cohort of newly diagnosed CVID subjects is needed and could determine if the assays can identify CVID subjects at risk of less frequent complication, such as other forms of autoimmunity and malignancy. Also, as suggested by Rösel et al. [35], functional in vitro PBMC assay that identify CVID subjects with residual B cell function could potentially be a tool to modulate therapy. Future prospective trial could determine if these CVID subjects can be treated differently with lower Ig replacement doses, decreasing the cost and side effects of therapy as well as improving patients' quality of life (reduction of infusion time and/or frequency).

After the initiation of our study, familial cases of loss-of-function mutations in the IL-21R gene were described leading to mutant proteins retained in the endoplasmic reticulum that do not traffic or are expressed at the cell surface [36–38]. Affected patients suffered from recurrent upper respiratory tract infections and exhibited decreased CS memory B cells similar to CVID. However, they also suffered from cryptosporidium-associated chronic cholangitis and liver disease, opportunistic infections involving *Pneumocystis jirovecii*, or, in the case of IL-21 deficiency, early-onset inflammatory bowel disease presenting before 1 year of age [39]. These manifestations are unusual in CVID. No specific gene testing for IL-21/IL-21R defects was performed as part of our study protocol, but the distinct clinical features described above were not found among members of the CVID cohort. The IL-21R alpha-chain MFI expression was comparable between CVID groups and controls at baseline (Supplement Fig. 3).

Table 4 Baseline B cell subpopulation flow cytometry phenotyping

	Complications		Total
	Yes	No	
CS memory B cells ≤ 2%	13	9	22
CS memory B cells > 2%	5	12	17
Total	18	21	39

OR 3.5 (95% CI 0.9 to 13.3, Fisher's exact test $p = 0.11$). Complications included in the analysis were autoimmune cytopenia, splenomegaly, and/or biopsy-proven granuloma

CS class-switched

Another observation that may explain the different B cell responses among our CVID groups was that CVID R had increased IgM^+ $CD27^+$ marginal B cells at baseline. Although IgM^+ $CD27^+$ marginal B cells have a decreased tendency to undergo IgG switching compared to naïve B cells, Avery et al. reported that for this cell subpopulation, stimulation with CD40L+IL-4+IL-21 could support IgG_2 production [29]. Additionally, IL-21 induces intracellular signaling through the signal transduction and activator of transcription 3 (STAT3). Functional STAT3 was shown to be required for proper differentiation of naïve B cells into long-lived memory and plasma cells [12]. It was proposed that memory B cells had a lower threshold for STAT3 activation compared to naïve B cells [40]. With this in mind, it is possible that CVID R, through their increased proportion of IgM^+ $CD27^+$ marginal B cells at baseline, had increased responsiveness and STAT signaling following anti-CD40+IL-4+IL-21 stimulation compared to CVID NR. It was also proposed that impaired responses to IL-21 stimulation could be explained by decreased rescue from apoptosis of $CD27^+$ B cell in some CVID subjects [41]. We performed a pilot flow cytometry staining for annexin V/propidium iodide of eight CVID and seven controls and found no significant difference in live vs. apoptotic or necrotic cells upon IL-21(±IL-4) stimulation in vitro (data not shown). It is also unlikely that CVID NR would have become R if their PBMCs were cultured for longer than 7 days, because the cell viability in vitro, already between 35 and 55%, decreased after 7 days.

A possible limitation of this study was the use of PBMCs instead of purified B cells in the culture system, since some CVID subjects may have defects in other cell types, such as T cells [42, 43]. On subsequent analysis, it was found that although both CVID groups and controls had similar proportion of total $CD3^+$ T cells at baseline, CVID R had slightly decreased proportion of $CD3^+$ $CD4^+$ T cell subpopulation compared to controls (Supplementary Fig. 2A). PBMCs were chosen for practical reasons, since subjects with immunodeficiency often have lower proportions of B cells in the peripheral blood, and manipulation through isolation of specific B cell populations can result in further cell loss. The culture system provides co-stimulation with anti-CD40 and cytokines directly to B cells (decreasing the need for T cell help) and experiments, such as IgG quantification and flow cytometry phenotyping, which were performed specifically to target B cells. To date, this system has shown high reproducibility and findings

consistent with previous studies [21, 24] similar to assays performed on pure B cell cultures. We plan to expand our flow cytometry panel to include more B cell markers, like CD21, CD24, IgD, and cytokines. Defect in the development of regulatory B cells, for example, could be related to non-infectious complications in CVID. Overall, the use of PBMCs has the advantage of decreasing the cost of the assay, which would be an asset if performed on a larger number of patients in the future.

Conclusions

CVID subjects have impaired in vitro development of $CD27^{+}$ B cells, $CD27^{++}$ $CD38^{+++}$ plasmablasts, and IgG production in response to IL-21 stimulation. In a subset of CVID, we demonstrated that the percentage of $CD27^{+}$ B cells and $CD27^{++}$ $CD38^{+++}$ plasmablasts, as well as IgG production, can be increased through modulation of the IL-21 pathway with IL-4, suggesting that these CVIDs retain functional B cell responses. This phenotype appears to be associated with significantly fewer clinical complications such as autoimmune cytopenia, splenomegaly, and biopsy-proven granuloma. The IL-4/IL-21 assay can be used in addition to baseline B cell subpopulation phenotyping to better identify CVID patients experiencing less vs. more non-infectious complications as well as subjects with residual in vitro B cell responses. Prospective studies are required to determine if these patients could be treated with lower Ig replacement doses which could possibly decrease therapy cost and side effects and improve patients' quality of life.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

References

1. Gathmann B, Grimbacher B, Beauté J, Dudoit Y, Mahlaoui N, Fischer A, et al. The European internet-based patient and research database for primary immunodeficiencies: result 2006-2008. Clin Exp Immunol. 2009;157(Suppl 1):3–11.
2. Castigli E, Geha R. Molecular basis of common variable immunodeficiency. J Allergy Clin Immunol. 2006;117(4):740–6.
3. Kainulainen L, Vuorinen T, Rantakokko-Jalava K, Osterback R, Ruuskanen O. Reccurent and persistent respiratory tract viral infections in patients with primary hypogammaglobulinemia. J Allergy Clin Immunol. 2010;126:120–6.
4. Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. Clin Immunol. 1999;92(1):24–48.
5. Glocker E, Ehl S, Brimbacher B. Common variable immunodeficiency in children. Curr Opin Pediatr. 2007;19(6):685–92.
6. Park M, Li J, Hagan J, Maddox D, Abraham R. Common variable immunodeficiency: a new look to an old disease. Lancet. 2008;372(9637):489–502.
7. Ahn S, Cunningham-Rundles C. Role of B cells in common variable immune deficiency. Expert Rev Clin Immunol. 2009;5:557–64.
8. Maffucci P, Filion CA, Boisson B, Itan Y, Shang L, Casanova J-L, et al. Genetic diagnosis using whole exome sequencing in common variable immunodeficiency. Front Immunol. 2016;7:220.
9. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood. 2008;111(1):77–85.
10. Warnatz K, Denz A, Drager R, Braun M, Groth C, Wolff-Vorbeck G, et al. Severe deficiency of switched memory B cells (CD27(+)IgM(−)IgD(−)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood. 2002;99(5):1544–51.
11. Piqueras B, Lavenu-Bombled C, Galicier L, Bergeron-van der Cruyssen F, Mouthon L, Chevret S, et al. Common variable immunodeficiency patient classification based on impaired B cell memory differentiation correlates with clinical aspects. J Clin Immunol. 2003;23(5):385–400.
12. Avery DT, Deenick EK, Ma CS, Suryani S, Simpson N, Chew GY, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. J Exp Med. 2010;207(1):155–71.
13. Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, et al. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J Exp Med. 2010;207(2):365–78.
14. Desjardins M, Mazer B. B-cell memory and primary immune defiencies: interleukin-21 related defects. Curr Opin Allergy Clin Immunol. 2013;13(6):639–45.
15. O'Keefe A, Halbrich M. Primary immunodeficiency for the primary care provider. Paediatr Child Health. 2016;21(2):e10–e4.
16. McCusker C, Warrington R. Primary immunodeficiency. Allergy Asthma Clin Immunol. 2011;7(Suppl 1):S11.
17. Conley M, Notarangelo L, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (pan-American group for immunodeficiency) and ESID (European society for immunodeficiencies). Clin Immunol. 1999;93(3):190–7.
18. Al Rushood M, McCusker C, Mazer B, Alizadehfar R, Grimbacher B, Depner M, et al. Autosomal dominant cases of chronic mucocutaneous candidiasis segregates with mutations of signal transducer and activator of transcription 1, but not of Toll-like receptor 3. J Pediatr. 2013;163(1):277–9.
19. Halbrich M, Ben-Shoshan M, McCusker C. Autoimmune hemolytic anemia in a teenager: a wolf in sheep's clothing. Eur J Haematol. 2013;91(3):262–4.
20. Gathmann B, Mahlaoui N, Gérard L, Oksenhendler E, Warnatz K, Schulze I, et al. Clinical picture and treatment of 2212 patients with common variable immunodeficiency. J Allergy Clin Immunol. 2014;134:116–26.

21. Resnick E, Moshier E, Godbold J, Cunningham-Rundles C. Morbidity and mortality in common variable immune deficiency over 4 decades. Blood. 2012;119:1650–7.
22. Srinivasa B, Alizadehfar R, Desrosiers M, Shuster J, Pant Pai N, Tsoukas C. Adult primary immune deficiency: what are we missing? Am J Med. 2012;125(8):779–86.
23. Hajoui O, Zheng H, Guay J, Letuve S, Fawaz L, Mazer B. Regulation of IL-13 receptor alpha 1 expression and signaling on human tonsillar B-lymphocyte subsets. J Allergy Clin Immunol. 2007;120(6):1425–32.
24. Hajoui O, Janani R, Tulic M, Joubert P, Ronis T, Hamid Q, et al. Synthesis of IL-13 by human B lymphocytes: regulation and role in IgE production. J Allergy Clin Immunol. 2004;114(3):657–63.
25. Parrish-Novak J, Dillan S, Nelson A, Hammond A, Sprecher C, Gross J, et al. Interleukin-21 and its receptor are involved in NK cells expansion and regulation of lymphocyte function. Nature. 2000;408:57–63.
26. Ozaki K, Kikly K, Michalovich D, Young P, Leonard W. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. Proc Natl Acad Sci U S A. 2000;97(21):11439–44.
27. Clemente A, Pons J, Matamoros N, Iglesias J, Ferrer JB. Cells from common variable immunodeficiency patients fail to differentiate to antibody secreting cells in response to TLR9 ligand (CpG-ODN) or anti-CD40 + IL-21. Cell Immunol. 2011;268:9–15.
28. Borte S, Pan-Hammarstrom Q, Liu C, Sack U, Borte M, Wagner U, et al. Interleukin-21 restores immunoglobulin production ex vivo in patients with common variable immunodeficiency and selective IgA deficiency. Blood. 2009;114:4089–98.
29. Avery D, Bryant V, Ma C, Malefyt R, Tangye S. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. J Immunol. 2008;181:1767–79.
30. Yu J, Zhang L, Radigan L, Sanchez-Ramon S, Cunningham-Rundles C. TLR-mediated B cell defects and INF-alpha in common variable immunodeficiency. J Clin Immunol. 2012;32(1):50–60.
31. Crotty S. T follicular helper cell differentiation, function, and roles in disease. Immunity. 2014;41(4):529–42.
32. Saiki O, Ralph P, Cunningham-Rundles C, Good RA. Three distinct stages of B-cells defects in common varied immunodeficiency. Proc Natl Acad Sci U S A. 1982;79(19):6008–12.
33. Bryant A, Calver NC, Toubi E, Webster ADB, Farrant J. Classification of patients with common variable immunodeficiency by B cell secretion of IgM and IgG in response to anti-IgM and interleukin-2. Clin Immunol Immunopathol. 1990;56(2):239–48.
34. Lu X, Su B, Xia H, Zhang X, Liu Z, Ji Y, et al. Low double-negative CD3+CD4-CD8- T-cells are associated with incomplete restoration of CD4+ T-cells and higher immune activation in HIV-1 immunological non-responders. Front Immunol. 2016;7:579.
35. Rösel AL, Scheibenbogen C, Schliesser U, Sollwedel A, Hoffmeister B, Hanitsch L, et al. Classification of common variable immunodeficiencies using flow cytometry and a memory B-cell functionality assay. J Allergy Clin Immunol. 2015;135(1):198–208.
36. Kotlarz D, Zietara N, Uzel G, Weidemann T, Braun C, Diestelhorst J, et al. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. J Exp Med. 2013;210:433–43.
37. Stepensky P, Keller B, Abuzaitoun O, Shaag A, Yaacov B, Unger S, et al. Extending the clinical and immunological phenotype of human interleukin-21 receptor deficiency. Haematologica. 2015;100:e72–e6.
38. Erman B, Bilic I, Hirschmugl T, Salzer E, Çagdas D, Esenboga S, et al. Combined immunodeficiency with CD4 lymphopenia and sclerosing cholangitis caused by a novel loss-of-function mutation affecting IL21R. Haematologica. 2015;100:e216–e9.
39. Salzer E, Kansu A, Sic H, Májek P, Ikincioğullari A, Dogu F, et al. Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL-21 deficiency. J Allergy Clin Immunol. 2014;133:1651–9.
40. Deenick E, Avery D, Chan A, Berglund L, Ives M, Moens L, et al. Naive and memory human B cells have distinct requirements for STAT3 activation to differentiate into antibody-secreting plasma cells. J Exp Med. 2013;2(12):2739–53.
41. Clemente A, Pons J, Lanio N, Matamoros N, Ferrer J. CD27+ B cells from a subgroup of common variable immunodeficiency patients are less sensitive to apoptosis rescue regardless of interleukin-21 signalling. Clin Exp Immunol. 2013;174:97–108.
42. Berrón-Ruiz L, López-Herrera G, Vargas-Hernández A, Santos-Argumedo L, López-Macías C, Isibasi A, et al. Impaired selective cytokine production by CD4(+) T cells in common variable immunodeficiency asssociated with the absence of memory B-cells. Clin Immunol. 2016;166-167:19–26.
43. Azizi G, Rezaei N, Kiaee F, Tavakolinia N, Yazdani R, Mirshafiey A, et al. T cell abnormalities in common variable immunodeficiency. J Investig Allergol Clin Immunol. 2016;26(4):233–43.