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Skewed Invariant Natural Killer T (iNKT) Cells, Impaired iNKT:B Cell Help and Decreased SAP Expression in Blood Lymphocytes from Patients with Common Variable Immunodeficiency

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Abstract

Common variable immunodeficiency (CVID) is a syndrome with predominantly defective B cell function. However, abnormalities in the number and function of other lymphocyte subpopulations in peripheral blood (PB) have been described in most patients. We have analysed the distribution of iNKT cell subpopulations in the PB of CVID patients and the ability of these cells to provide *in vitro* cognate B cell help. The total of iNKT cells was reduced in the PB of CVID patients, especially CD4+, CD4-/CD8- and CCR5+/CXCR3+. These findings were associated with an enrichment of memory-like and a tendency towards a reduction in TNF-α-expressing effector iNKT cells in the peripheral blood mononuclear cells (PBMC) of CVID patients. Moreover, an accumulation of follicular helper iNKT cells in the PB of CVID patients was demonstrated. CVID αGalCer-pulsed iNKT cells are not able to induce autologous B cell proliferation although they do induce proliferation to healthy donor B cells. Interestingly, autologous and heterologous co-cultures did not differ in the amount of immunoglobulin secreted by B cells *in vitro*. Finally, reduced intracellular SAP expression in iNKT cells and other lymphocytes in the blood from CVID patients was observed. These results provide further insights into the immunological mechanisms underlying the iNKT cell defects and the potential targets to improve B cell help in CVID.

Introduction

Invariant natural killer T (iNKT) cells are a subset of T lymphocytes that express an invariant T cell receptor (iTCR), which recognize glycolipids presented in the context of CD1d on antigen-presenting cells [1, 2]. CD1d also plays a role in the positive selection of iNKT cells in the thymus, a process dependent on the SLAM-associated protein (SAP) [3, 4]. In addition, SAP modulates the cytotoxic iNKT cell function in the periphery, promoting the formation of immunological synapses [5]; it also elicits cognate iNKT:B cell help [6]. Unlike other T cell subpopulations, most iNKT cells in their steady state express homing receptors for extralymphoid tissues (CCR2, CCR5 and CXCR3), and they produce different immunoregulatory cytokines such as interleukin (IL)-4, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, a hallmark of effector T cells [7–10].

Cognate iNKT:B cell crosstalk is important for the proper development of humoral immune responses. This cooperation occurs in a conventional T cell-independent manner, resulting in an extrafollicular response with immunoglobulin class switching, but no B cell memory [11–13]. Conversely, non-cognate iNKT:B cell stimulation enhances T cell-dependent responses, inducing germinal centre maintenance, sustained B cell memory and immunoglobulin (Ig) class switching [6, 14]. The key role of iNKT cells in B cell function is also supported by the abnormalities in the iNKT cell compartment described in B cell-mediated autoimmune diseases, such as systemic lupus erythematosus (SLE) [15] and rheumatoid arthritis [16]. Moreover, iNKT cells are also decreased in the peripheral blood (PB) of patients with predominantly antibody immunodeficiencies such common variable immunodeficiency (CVID) [17–22].

CVID is a heterogeneous primary antibody syndrome characterized by hypogammaglobulinaemia, poor response

SCANDINAVIAN JOURNAL OF Immunology AN INTERNATIONAL MONTHLY JOURNAL

to vaccination and susceptibility to gastrointestinal and respiratory infections [21]. Mutations in several genes associated with B cell terminal differentiation have been described in these patients [23]. Studying a cohort of 47 CVID patients, Fulcher *et al.* reported a consistent decrease in the percentages of iNKT cells in PB, particularly in CVID patients with a low frequency of switched memory B cells (smB) as compared with healthy donors (HD) and individuals with X-linked agammaglobulinaemia [17], a finding that is not affected by the immunoglobulin replacement therapy [22]. Carvalho *et al.*, also demonstrated high frequencies of activated CCR5+/CD69+/CXCR6-, as well as skewed percentages of CD4+ and CD8+ iNKT cells, in cryopreserved peripheral blood mononuclear cells (PBMC) from CVID patients [18]. Finally, Gao *et al.* described the low production of cytokines such as IL-13 and IL-17 and they also noted low proliferative capacity in enriched iNKT cell cultures from PBMC of CVID patients [20]. Therefore, iNKT cells are impaired both in number and function in CVID.

Although these investigations provide an insight into iNKT cell abnormalities in CVID, questions remain about how this affects B cell development and function. Therefore, we have evaluated the number, phenotype and function of iNKT cells obtained from freshly isolated PBMC and we explored the *in vitro* capacity of these cells to provide cognate B cell help in patients with CVID.

Materials and methods

Study population. Twenty-two individuals diagnosed with CVID, according to the criteria of the European Society for Immunodeficiencies (http://esid.org/Resources/Diagnostic-Criteria-PID), were included in this study. The patients were further classified by taking into account the Euroclass B cell subpopulation profiles in PB [24]. In addition, 22 age- and sex-matched HD were voluntarily recruited. This study was reviewed and approved by the Institutional Review Board from the University of Antioquia. Written informed consent was obtained from the children's parents and adults, according to the standards of the Declaration of Helsinki.

Blood sampling and lymphocyte subpopulation staining. EDTA blood samples were collected from all the individuals, and the frequency and phenotype of the different lymphocyte subpopulations were determined by flow cytometry. Briefly, PB cells were incubated with the corresponding monoclonal antibodies (mAbs) for 20 min at room temperature in the dark. Erythrocytes were lysed by incubation with the fluorescence-activated cell sorting (FACS) lysing solution (Becton Dickinson, BD, San Jose, CA, USA) following the manufacturer's instructions. FACS was performed using the FACScanto II (BD) and analysed using the FlowJo V8-2 software (Tree Star, Inc., Ashland, OR, USA).

CD19+ B cell (anti-CD19 clone SJ25C1) subsets were defined as follows: anti-IgD (clone IA6-2) and anti-CD27 (clone M-T271) mAbs were used to classified naïve, switched memory and marginal zone-like B cells. Transitional B cells and plasmablasts were recognized as CD38++/IgD++ and CD38++/IgD-, respectively, using the corresponding mAbs (anti-CD38 clone HIT2, anti-IgD clone IA6-2). Also, the expression of CD1d (anti-CD1, clone CD1d42) was analysed in CD19+ B cells. iNKT cells were identified as CD3 (anti-CD3, clone SK7) and iTCR (clone 6B11) double-positive cells, and their subsets were studied using the mAbs against different surface molecules, as follows: CD4 (clone UCHL1), CD8α (clone OKT8) and β (clone 2ST8.5H7), CD45RO (clone UCHL1), CXCR5 (clone RF8B2), CD62L (clone DRE656), CCR7 (clone 150503), CXCR3 (clone 1C6/CXCR3) and CCR5 (clone 2D7/CCR5). All antibodies were purchased from BD.

In addition, PBMC were isolated from heparinized blood, and they were subsequently washed with phosphate-buffered saline and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Surface markers (CD40L and CD69) and intracellular cytokine production were determined in PBMC cultures (1 million cells/ml in six-well plates) using phorbol 12-myristate 13-acetate (PMA) (20 ng/ml, Sigma-Aldrich Co., St. Louis, MO, USA) and ionomycin (1 μg/ml; Sigma-Aldrich Co.) for 6 h. To assess cytokine production, Brefeldin A (10 μg/ml; Sigma-Aldrich Co.) was added 4 h after PMA/ionomycin stimulation and cells were incubated for the additional 2 h. The intracellular staining was performed using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit. The intracellular cytokines were evaluated using specific mAbs against IFN-γ (clone 4S.B3), TNF-α (clone 6401.1111) and IL-4 (clone 8D4-8). Intracellular SAP expression was measured by indirect staining with the anti-SH2D1A (clone 1C9) and secondary anti-IgG2a (clone M2A-15F8) antibodies. The SAP expression index was established as the Median Fluorescence Intensity (MFI) of SAP-positive cells minus the MFI of cells incubated with the secondary antibody, divided by the MFI of cells incubated with the secondary antibody.

iNKT cells enrichment, purification and cloning ex vivo. To expand iNKT polyclonal cells *in vitro*, 1×10^6 freshly isolated PBMC were stimulated with α-galactosylceramide (αGalCer) (50 ng/ml, KRN7000 Funakoshi, Tokyo, Japan); and 24 h later, recombinant human IL-2 (rhIL-2) (20 U/ml; BD) was added to the cell cultures. Cells were maintained under rhIL-2 (40 U/ml) supplementation every 48 h for the following 15–20 days. Thereafter, CD3+ iTCR+ CD4+ iNKT cells were sorted using a MoFlo™ XDP (Beckman-Coulter, Brea, CA, FL, USA). These polyclonal iNKT cells were used as helper cells for the first set of iNKT:B cell co-cultures. Considering that human iNKT cells have largely been characterized using

clones obtained following their expansion with α-GalCer and long-term cell culture with autologous-irradiated mononuclear cells and cytokines (e.g. IL-2, IL-7) [25], we also obtained iNKT clones cells as follows: CD3+/iTCR+/CD4+ iNKT cells were sorted at 1 cell/well in U-shaped 96-well plates containing 200 μl of RPMI complete medium, 0.5 μg/ml of phytohaemagglutinin (Sigma-Aldrich Co.), 50 U/ml of rhIL-2 and 1×10^6/ml of pooled PBMC obtained from three different donors and irradiated at 8.000 rad. iNKT cells clones were grown in 37 °C and 5% CO2 for 15 days and then used for the monoclonal set of iNKT:B cell co-cultures.

B cell stimulation and iNKT:B cell co-culture. B cells were isolated from PB by negative selection using the Human B Cell Enrichment Cocktail RosetteSep™ (StemCell, Vancouver, BC, Canada) and then seeded alone (as negative control) or stimulated with CD40L trimers plus IL-21 (generously provided by Hermann Eibel, Center for Chronic Immunodeficiency, Freiburg, Germany) (as positive control). B cells were also stimulated with polyclonal or monoclonal CD4+ iNKT cells (1:10 ratio) with or without αGalCer. After 5 days of incubation, B cell proliferation in either CD19+ or CD20+ cells was determined by staining with carboxyfluorescein diacetate succinimidyl ester (CFSE; ThermoFisher Scientific, Walthman, MA, USA). Cell culture supernatants were collected at day 10 and stored at −70 °C to measure IgM, IgG and IgA levels by enzyme-linked immunosorbent assay (ELISA), using the Ready-SET-Go kits following the manufacturer's instructions (Affymetrix, eBioscience, Santa Clara, CA, USA).

Statistical analysis. Results are presented as mean ± standard deviation (SD). Statistical comparison between two different groups was performed using either the Mann–Whitney U-test, with a confidence level of 95% or the Student's t-test. We also applied the Shapiro–Wilk test to evaluate normality (Statistical version 5) and Bartlett's test to evaluate equality of variances (GraphPad Prism 5.0 Software, Inc. La Jolla, CA, USA). A P-value <0.05 was considered significant.

Results

Description of study population

All our patients exhibited >1% of B cells from total lymphocytes in PB. Of these patients, fifteen showed ≤2% of smB cells and seven presented with >2% of smB cells (referred to as smB− and smB+, respectively) of the total B cells. All CVID patients and HD exhibited CD1d expression in more than 88% of PB B cells (data not shown) with the exception of three smB− patients, who exhibited CD1d expression in 72%, 70%, 31% of the PB B cells, respectively. Homogeneous demographic and clinical characteristics were observed in both CVID groups, as summarized in Tables 1 and 2. Early-onset CVID (age at first symptom <10 years) was observed in ten (66.6%) and two (28.6%) patients from the smB− and smB+ groups, respectively. Sinopulmonary infections were the most common clinical manifestations in both CVID groups, followed by infections of the skin and soft tissues. Bronchiectasis and autoimmunity were observed with similar frequencies in both groups. Only two patients from the smB− group manifested each either meningitis or a duodenal diffuse large B cell lymphoma. Most patients were asymptomatic at the time of the evaluation, with the exception of four that presented with mild respiratory symptoms as well as one with diarrhoea.

Skewed iNKT cell subpopulations in the PB of CVID patients

As reported by others [17–22], we found significantly reduced frequencies and absolute numbers of total PB iNKT cells in CVID patients, irrespective of the Euroclass B cell subpopulation profiles (Fig. 1A). No differences were observed in the PB iNKT cell percentages and absolute numbers of patients with early- or late-onset disease (data not shown).

To establish whether decreased iNKT cells were subset-specific in CVID, we evaluated the different iNKT cells subpopulations in the PB of these patients. CD4+ iNKT cells were reported to induce activation of conventional CD4+ T, B and natural killer (NK) cells, producing more IL-4 and IL-2 than other iNKT subpopulations [26]. On the other hand, CD8 α/β+ and DN (CD4−/CD8α−) iNKT cells showed a Th1 phenotype and cytotoxic activity; however, DN iNKT cells are also able to produce IL-17 [26]. A reduction in the CD4+ and DN iNKT cell numbers in CVID patients was observed in comparison with those in HD with no differences among smB+ and smB- groups (Fig. 1B). Although increased percentages in double-positive (CD4+/CD8α+, DP) and CD8 α/β+ iNKT

Table 1 Demographic description of the study population.

	CVID patients		
Demographic data	smB-	smB+	HD
n	15	7	22
Sex (% females)	53.3	42.8	45.5
Current age[a]	37 (13–63)	33 (17–58)	37 (12–62)
Age at diagnosis[a]	26 (6–49)	16 (7–45)	–
Age at first symptom[a]	5 (0.5–42)	11 (0.4–38)	–
Age at last follow-up[a]	39 (15–60)	32 (12–56)	–
Delay in the diagnosis[a]	10 (1–44)	5 (3–12)	–
IgG[b]	231.5 (0–823)	612 (0–741)	–
IgA[b]	18.7 (12–129)	39.5 (2–394)	–
IgM[b]	27.3 (12–81.1)	92.5 (6–351)	–

[a]Median (min-max) in years.
[b]Median (min-max) of the immunoglobulin levels at diagnosis (mg/dl).

Table 2 Clinical parameters of the CVID patients included in this study.

	No. of CVID Patients (%)	
Clinical features	smB−	smB+
Pneumonia	11 (73.3)	5 (71.4)
Sinusitis	9 (60)	2 (28.6)
Otitis	8 (53.3)	2 (28.6)
Tonsillopharyngitis	5 (33.3)	2 (28.6)
Skin and soft tissue infections	5 (33.3)	3 (42.9)
Urinary tract infections	0	2 (28.6)
Gastroenteritis	5 (33.3)	2 (28.6)
Bronchiectasis	3 (20)	2 (28.6)
Autoimmunity	4 (26.6)	2 (28.6)

cell subpopulations were also observed in CVID, the absolute numbers were similar to those in HD (data not shown).

To ascertain whether the skewed iNKT cell distribution in the PB of CVID patients was due to a differential migratory response, we evaluated the *ex vivo* expression of homing receptors on these cells. In humans, the double expression of CD62L and CCR7 was described as a hallmark of central memory iNKT cells [27], whereas CCR5+/CXCR3+ double-positive iNKT cells were identified as effector cells in a mouse model [28]. We observed similar CD62L+/CCR7+ iNKT cell counts in CVID as compared to those in HD, but profoundly reduced numbers of CCR5+/CXCR3+ iNKT cells (Fig. 1C). No differences were observed in these iNKT cell subpopulations among smB+ and smB- groups.

We postulated that these differences in the distribution of iNKT cells in CVID are due to an exaggerated recruitment of effector cells to inflammed tissues, as was demonstrated in other disease models [15, 16], with an enrichment of other cell subsets. Consistent with this hypothesis, we observed significant increased CD40L+ iNKT cell percentages in the PBMC of CVID patients, as compared with those of HD (Fig. 1D); these cells that have been shown to exhibit a memory-like phenotype and reduced inflammatory-homing capabilities [29]. This finding was not due to an increased steady-state activation of PBMC iNKT cells in CVID, as the percentage of CD69 expression in these cells was comparable to that observed in HD cells (Fig. 1D). Moreover, the PBMC of CVID patients were enriched in iNKT cells with a reduced capacity of intracellular IFN-γ and TNF-α production upon stimulation with PMA/Ionomycin, although this did not reach statistical significance (Fig. 1E). We also wanted to investigate the amount of follicular helper iNKT cells ($iNKT_{fh}$) in the PB of HD and CVID patients. Interestingly, we observed an increased frequency of $iNKT_{fh}$ cells in the PB of CVID patients (Fig. 1F). Again, all these differences could not be attributed to the smB CVID profile.

These data demonstrate that, disregarding the smB profile from the CVID patients, they still exhibit a differential distribution of iNKT cell subsets in PB compared to HD. These findings provide further evidence in support of an increased output of effector CD4+ and DN iNKT cells to inflamed tissues, as well as retention of those cells with a memory-like and a follicular helper phenotype in PB from these patients.

CVID iNKT cells are functionally able to provide B cell help in vitro

CD4+ iNKT cells primarily induce B cell proliferation and immunoglobulin secretion [11, 12, 30]. In addition to examine the phenotype of these cells, we investigated whether the remaining CD4+ iNKT cells in the PB of CVID patients might also induce proliferation and immunoglobulin secretion when co-cultured with B cells. These proliferative responses were compared with those in B cells exposed to medium alone (as negative control) or CD40L trimer+IL-21 (as positive control). In the B cells from HD, proliferation in response to CD40L trimer+IL-21, and αGalCer-pulsed autologous iNKT cells was observed (Fig. 2A and representative contour plots in Fig. 2C; HD1). Interestingly, as HD B cells were co-cultured with heterologous CVID iNKT cells, we detected B cell proliferation comparable to that obtained from autologous co-cultures (Fig. 2A, C). All the CVID patients' B cells exhibited a heterogeneous but considerable response to CD40L+IL-21 stimulation (Fig. 2A and representative contour plots in Fig. 2C; P1). However, CVID iNKT cells were unable to induce autologous B cell proliferation (Fig. 2A and representative contour plots in Fig. 2C; P1). This impaired response was also observed, as patients' B cells were co-cultured with heterologous CD4+ iNKT cells from HD (Fig. 2A, C). These findings were confirmed using clones from iNKT cells obtaining similar results (representative example in Fig. 2C, HD2 and P2). Again, no differences were observed in the ability of iNKT cells to provide B cell help *in vitro* among smB+ and smB- groups (data not shown).

Next, we investigated the B cell immunoglobulin production following exposure to iNKT cells by ELISA in the supernatants of these co-cultures (Fig. 2B). Stimulation with CD40L trimer+IL-21 induced the secretion of IgM and IgA in the B cells of CVID patients, which was comparable to that observed in HD. In contrast, IgG secretion was statistically reduced in the B cells from CVID patients when stimulated *in vitro* with CD40L trimer+IL-21, as compared with that from HD B cells. More interestingly, both autologous and heterologous αGalCer-pulsed iNKT cells were able to induce comparable IgM, IgA and IgG secretion in the B cells of CVID patients and HD. Thus, CVID CD4+ iNKT cells from PB are able to induce proliferation and immunoglobulin secretion in HD B cells, demonstrating their functional capabilities. However, these cells cannot rescue

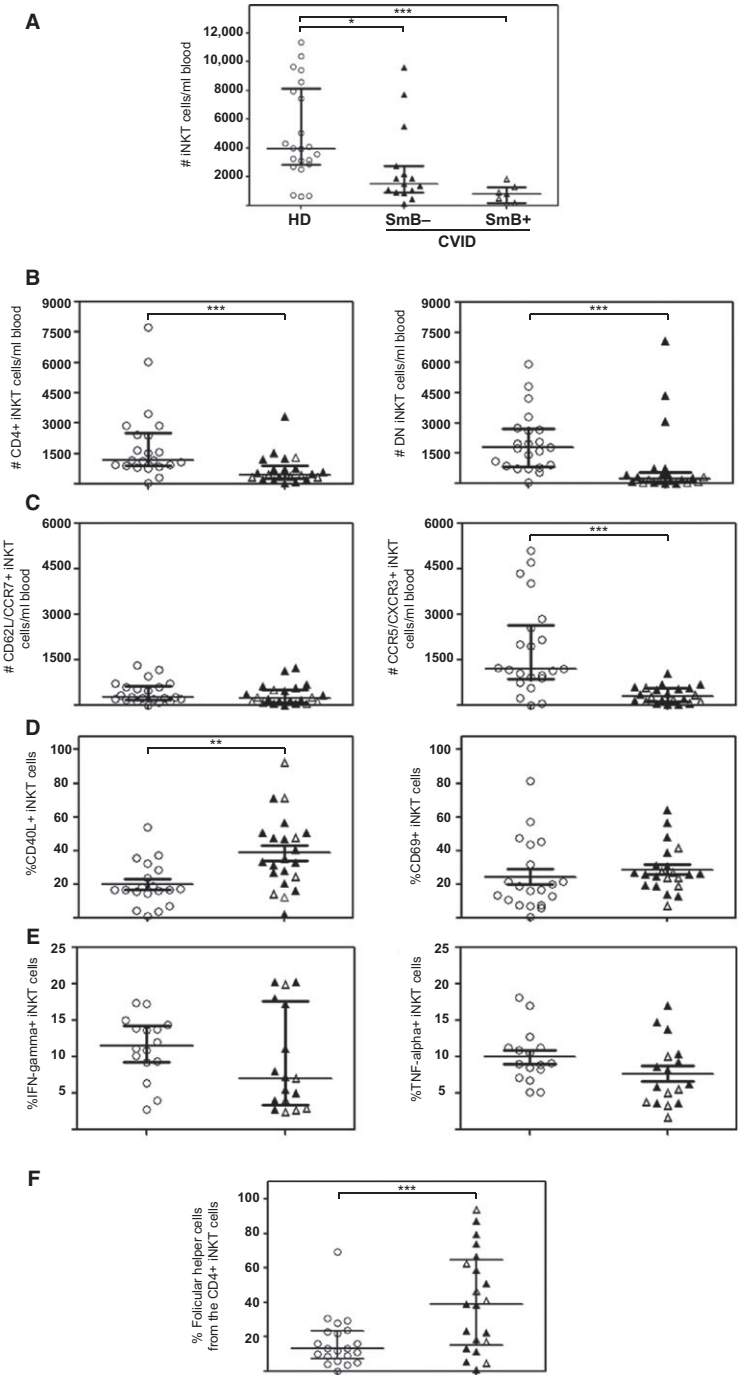

Figure 1 iNKT cells subpopulations in the PB and PBMC of CVID patients. PB was collected and stained with anti-CD3 and anti-iTCR and analysed by flow cytometry, as described in materials and methods. Absolute numbers and mean ± SD of total (A), CD4+ and DN (B), and CD62L+/CCR7+ or CCR5+/CXCR3+ (C) iNKT cells in PB are shown. Percentages of CD40L+ or CD69+ iNKT cells in steady-state PBMC (D) and IFNγ- and TNFα-producing iNKT cells in PMA/Ionomycin-stimulated PBMC (E) are also depicted. PB $iNKT_{fh}$ cells from HD and CVID defined as CD3+/iTCR+/CD4+/CD45RO+/CXCR5+ cells are also shown (F). Open circles represent data from HD, whereas open or filled triangles represent data from smB– and smB+ CVID patients, respectively. *p*-values were calculated using the Mann–Whitney test. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$.

proliferation in autologous B cells, even though they are able to induce immunoglobulin secretion, demonstrating a specific impaired iNKT:B cell signalling defect affecting the patients' B cell proliferation.

Altered mechanisms of cognate iNKT:B cell help in CVID patients

The data presented so far (Fig. 2) seem to demonstrate that it is the CVID patient B cells that have a lack of responsiveness and not the iNKT cells that are impaired in providing help to B cell *in vitro*. However, we evaluated the intracellular expression of SAP in iNKT cells obtained from the PBMC of CVID and HD, due to the importance of SAP in the induction of iNKT cell-mediated B cell proliferation [6]. These findings were compared to the SAP expression in PB NK and T cells. The SAP expression index was significantly reduced in the iNKT cells from PBMC, as well as in NK and T cells obtained from the PB of CVID patients when compared with those in HD (Fig. 3A, B).

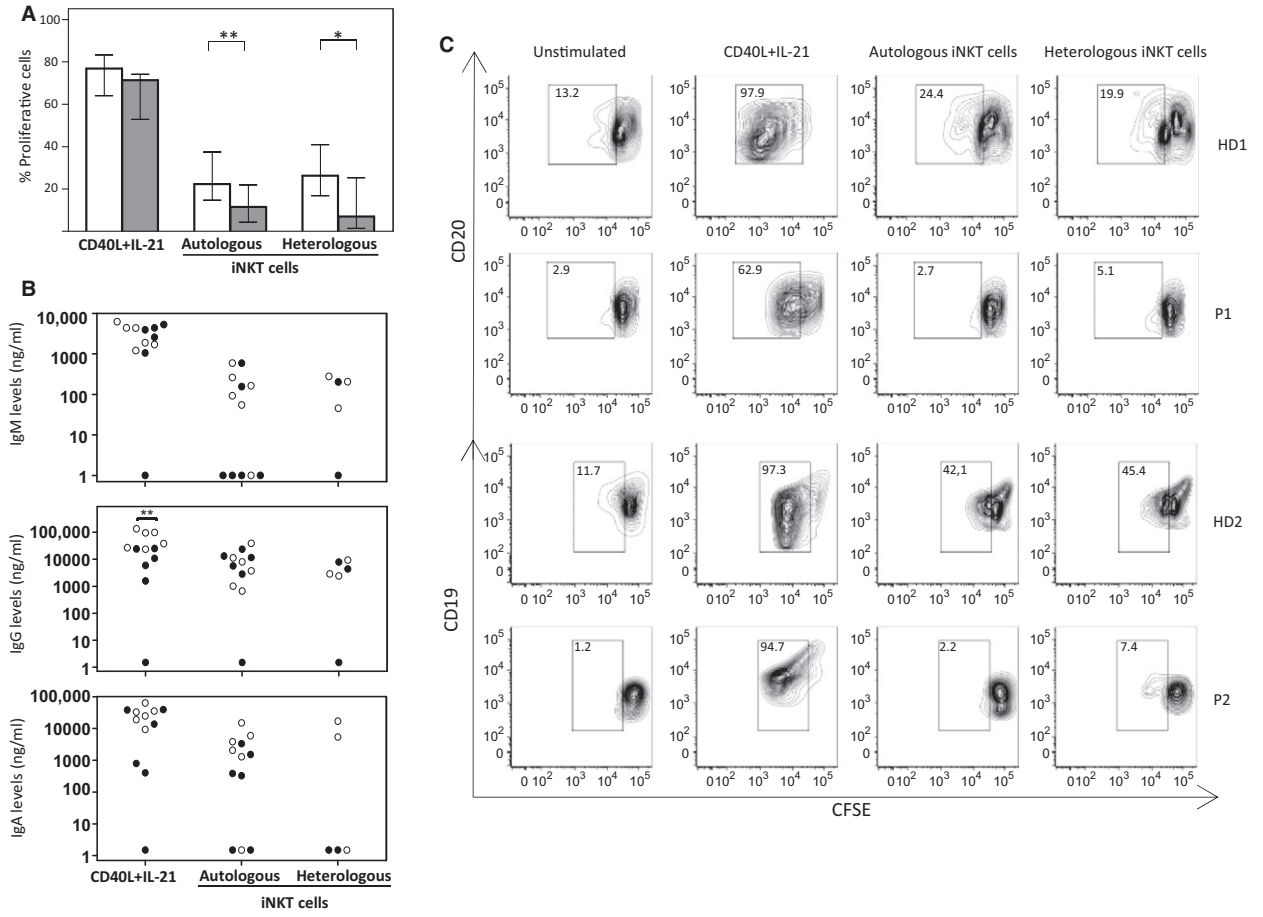

Figure 2 *In vitro* cognate iNKT:B cell help in CVID. Proliferation percentages of purified B cells from 11 HD and 8 CVID patients upon stimulation either w/o CD40L trimer+IL-21, αGalCer-pulsed autologous or heterologous polyclonal CD4+ iNKT cells is shown in (A). An example of these co-cultures is depicted in C (HD1 and P1). The results from co-cultures using clones of iNKT cells are also shown in (C) (HD2 and P2, representative data from three HD and four CVID patients, respectively). The percentages of proliferating B cells are enclosed and indicated below the squares. Immunoglobulin levels in the culture supernatants were measured using ELISA and showed in (B). Open and filled circles and squares represent data from HD and CVID patients, respectively. *P*-values were calculated using the Mann–Whitney *U*-test. $^{**}P < 0.001$.

Discussion

Previous reports have described numerical, migratory and functional impairment in PB iNKT cells from CVID patients [17–20]; however, the mechanisms underlying these defects were not well understood. In the present study, we investigated the phenotype of PB iNKT cell subsets and their capacity to support cognate B cell help in CVID patients. As previously reported, we observed a consistent reduction in the numbers of total iNKT cells in the PB of CVID patients, particularly in PB CD4+ and DN iNKT cells. These results partly differ from those published by Carvalho *et al.*, who reported low absolute numbers of total and CD4+ iNKT cells, as well as CD8α+ iNKT cells in CVID [18]. However, Carvalho *et al.* used cryopreserved iNKT cells and they did not measure DN and CD8 α+/β+ iNKT cell subpopulations, representing a methodological difference that may account for the variable findings. Also, a significant reduction in the absolute number of the effector CCR5+/CXCR3+ iNKT cells in PB was observed in our patients. Although more experiments are necessary to ascertain our hypothesis, taking into account the high frequency of infections and chronic inflammation observed in these patients, our data suggest that CD4+ and DN effector iNKT cells leave the PB to exert their function on extralymphoid tissues, a phenomenon that has been observed in other inflammatory human diseases. Specifically, a recall of CCR5+/CXCR3+ T cells in the synovial fluid of inflamed joints has been observed in patients with juvenile idiopathic arthritis, which is related to the persistence of destructive inflammation in the synovial joints [31]. Additionally, higher percentages of CCR5+/CXCR3+ T cells in the cerebrospinal fluid have also been reported in patients with multiple sclerosis [32], who also presented with a reduced frequency of total and DN iNKT cells in PB [33].

In addition, we detected greater CD40L surface expression in steady-state iNKT cells of CVID patients, and also a

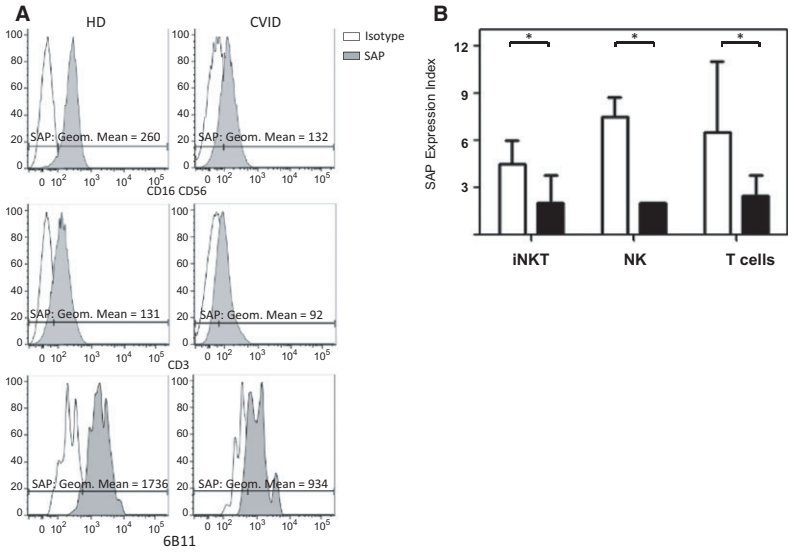

Figure 3 intracellular lymphocyte SAP expression in CVID. Mean ± SD of SAP index expression in PBMC CD3+/iTCR+ iNKT cells as well PB CD56+/CD16+ NK and CD3+ T cells is included. A representative example and data from 10 HD and 8 CVID patients are depicted in (A) and (B). Open and filled bars represent data from HD and CVID patients, respectively. *p*-values were calculated using the Student's t-test. $*p < 0.05$.

tendency towards a reduction in the frequency of TNF-α-producing iNKT cells upon stimulation. The increased expression of CD40L in human CD8+ T cells has been associated with a memory phenotype, and these cells also have a reduced inflammatory-homing capability [28]. These data are in agreement with our findings, suggesting that iNKT cells in the PB of CVID patients are depleted because they do migrate to inflamed tissues. Interestingly, steady-state $CD40L^{high}$-expressing T cells in PB have been associated with an unfavourable prognosis in rheumatoid arthritis patients [34], and they also favours the production of autoantibodies in SLE-derived B cells [35]. Whether this might be related to the chronic inflammation and autoimmune abnormalities observed upon CVID progression deserve more investigation. With respect to cytokine production in iNKT cells, our data differ from those published by Gao *et al.*, who reported a bias towards IFN-γ-producing cells following PBMC αGalCer stimulation in three CVID patients. However, we also showed that the IFN-γ production in stimulated PBMC from CVID patients is highly variable; thus, an analysis featuring a larger number of patients is necessary to yield consistent results.

Finally, we assessed the ability of CD4+ iNKT cells to provide cognate B cell help in CVID patients. CVID CD4+ iNKT cells are able to induce proliferation of HD B cells, as compared to those from HD. These results demonstrated that CD4+ iNKT cells from CVID patients are still able to provide cognate B cell help and pointed out towards a proliferative defect of CVID B cells following iNKT cell stimulation. Interestingly, despite the defective B cell proliferation in CVID after iNKT cell cognate help, these cells are still able to secrete comparable amounts of immunoglobulins in the presence of autologous and heterologous αGalCer-pulsed iNKT cells. A closer look at CVID iNKT cells also showed a reduction in the intracellular SAP protein expression, a finding also observed in CVID T and NK lymphocytes. We propose that the diminished expression of SAP in cells within the context of CVID's genetic background may reduce the contact time during the cognate iNKT:B cell interaction [36], leading to an impaired B cell proliferation; however, it does allow a preliminary cycle of antibody responses. We hypothesized that the low intracellular expression of SAP in CVID iNKT cells is also associated with the retention of $iNKT_{fh}$ cells in the PB of CVID patients, as SAP deficiency has been found to impair follicular helper T cells recruitment into nascent GC via the accumulation of these cells in the follicular mantle [36]. Questions remain about the ability of CVID iNKT cells to induce proliferation in HD B cells, despite the decreased SAP intracellular expression in our experiments. However, previous studies have demonstrated that the ability of SAP-deficient T cells to modulate B cell responses is highly dependent on the genetic background [37]. Thus, B cells from a SAP-sufficient environment (HD) may overcome the low expression of this molecule in CVID iNKT cells *in vitro*.

Taken together, our results demonstrate a specific reduction in effector CD4+ and DN iNKT cells in the PB of CVID patients. Moreover, iNKT cells expressing high levels of CD40L seemed to be enriched in the PBMC of these patients. Also, we show compelling evidence that the cognate iNKT:B cell help is impaired in CVID, a finding that may be related to the decreased expression of the intracellular SAP protein in the lymphocytes of these patients. These low SAP levels may be associated with the retention of high numbers of $iNKT_{fh}$ cells in the PB of CVID patients. Further studies are necessary to elucidate the mechanism responsible for the decrease in intracellular SAP in CVID-derived lymphocytes. This knowledge will provide new insights about alternative therapeutic approaches to restore the B cell dysfunction in CVID.

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