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# Defective Bcl-2 expression in memory B cells from common variable immunodeficiency patients

L. del Pino Molina ,*
J. M. Torres Canizales ,*
O. Pernía,† R. Rodríguez Pena ,*
I. Ibanez de Caceres†
and E. López Granados *
*Clinical Immunology Department, La Paz University Hospital, Lymphocyte Pathophysiology in Immunodeficiencies Group La Paz Institute for Health Research (IdiPAZ) and Center for Biomedical Network Research on Rare Diseases (CIBERER U767), and †Cancer Epigenetics Laboratory, INGEMM, Biomarkers and Experimental Therapeutics in Cancer Group, La Paz Institute for Health Research (IdiPAZ), Madrid, Spain

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Correspondence: L. del Pino Molina, Clinical Immunology Department, La Paz University Hospital and Lymphocyte Pathophysiology in Immunodeficiencies Group, La Paz Institute for Health Research (IdiPAZ) and Center for Biomedical Network Research on Rare Diseases (CIBERER U767), Paseo de la Castellana 261, Madrid 28046 Spain.
E-mail: lucia.delpino@idipaz.es

## Summary

**Common variable immunodeficiency (CVID) is a primary immunodeficiency characterized by hypogammaglobulinemia and different degrees of B cell compartment alteration. Memory B cell differentiation requires the orchestrated activation of several intracellular signaling pathways that lead to the activation of a number of factors, such as nuclear factor kappa B (NF-κB) which, in turn, promote transcriptional programs required for long-term survival. The aim of this study was to determine if disrupted B cell differentiation, survival and activation in B cells in CVID patients could be related to defects in intracellular signaling pathways. For this purpose, we selected intracellular readouts that reflected the strength of homeostatic signaling pathways in resting cells, as the protein expression levels of the Bcl-2 family which transcription is promoted by NF-κB. We found reduced Bcl-2 protein levels in memory B cells from CVID patients. We further explored the possible alteration of this crucial prosurvival signaling pathway in CVID patients by analysing the expression levels of mRNAs from anti-apoptotic proteins in naive B cells, mimicking T cell-dependent activation *in vitro* with CD40L and interleukin (IL)-21. *BCL-XL* mRNA levels were decreased, together with reduced levels of *AICDA*, after naive B-cell activation in CVID patients. The data suggested a molecular mechanism for this tendency towards apoptosis in B cells from CVID patients. Lower Bcl-2 protein levels in memory B cells could compromise their long-term survival, and a possible less activity of NF-κB in naive B cells, may condition an inabilityto increase *BCL-XL* mRNA levels, thus not promoting survival in the germinal centers.**

**Keywords:** anti-apoptotic proteins, B cells, common variable immunodeficiency

## Introduction

Common variable immunodeficiency (CVID) is the most frequent symptomatic primary immunodeficiency (PID), with an estimated prevalence of 1 : 100 000 to 1 : 10 000 of the population [1]. CVID is characterized by hypogammaglobulinemia and recurrent severe infections. Some patients, however, also present autoimmune complications, lymphoproliferative manifestations, granulomas, enteropathy and increased risk of malignancy [2–4]. A defining feature in all CVID patients is some degree of impairment in B cell differentiation into plasma cells (maturation). Different patterns of B cell alterations have been identified based on the stage in which B cell differentiation is halted [5]. Most CVID patients present normal numbers of peripheral B cells but with a poorly differentiated memory B cell compartment.

Despite significant efforts to identify the monogenic causes of CVID, fewer than 20% of CVID patients present a monogenic defect [6]. Several genes have been associated with CVID, including *CD19* [7], *CD81* [8], *CD20* [9], *CD21* [10], *ICOS* [11], *TACI* [12], *BAFFR* [13], *LRBA* [14], *CTLA4* [15], *PI3KCD* [16], *IKAROS* [17], *NFKB2* [18] and some others (*TWEAK*, *CD27*, *IL-21*, *IL-21R*, *NFKB1*, *PRKCD*, *PIK3R1*, *VAV1*, *RAC2*, *BLK* and *IRF2BP2* [19–21]. Loss-of-function variants of nuclear factor kappa B subunit 1 (NF-κB1) have recently been proposed as the most common monogenic cause of CVID in a European cohort [22]. The

recent general consensus is that the pathogenesis of CVID is complex and multi-factorial in most patients, rather than the consequence of a monogenic defect [6]. A plausible hypothesis for the pathogenesis of CVID is that of a polygenic or even epigenetic heterogeneous predisposing genetic background [6,23], inducing several cumulative defects and leading to the deregulation of B cell differentiation.

B cells recognize a multitude of pathogens through the diversity of the Bcell receptor (BCR) and generate specific antibodies with diverse functional characteristics. This variability relies upon the variability, diversity and joining [V(D)J] recombination mechanism in the first stages of development in bone marrow and on the somatic hypermutation (SHM) and class-switch recombination (CSR) processes in the germinal centers (GC) [24,25].

B cell survival depends upon various signals through the BCR, B cell activating factor receptor (BAFFR) and cytokine receptors that trigger internal signal transduction pathways and ultimately activate several transcription factors that modulate the transcription of target genes [25–30]. The nuclear factor of kappa B light chain (NF-κB) family consists of various subunits: NF-κB1 (p105/p50), NF-κB2 (p100/p52), Rel A (p65), Rel B and c-Rel, a key transcription factor that translocates to the nucleus and enhances the expression of survival proteins such as the Bcl-2 family, among others [31–34]. The Bcl-2 anti-apoptotic family consists of several integral membrane proteins located in the mitochondria, endoplasmic reticulum and nuclear membranes. Membrane B cell receptors, as well as BAFFR and CD40L bound by their ligands, induce intracellular signaling pathways that lead to NF-κB activation and anti-apoptotic gene expression [35,36]. NF-κB activity is, therefore, the result of the convergence of several signaling pathways.

The aim of this study was to determine whether disturbed B cell differentiation, survival and activation in CVID patients could be related to defects in intracellular signaling pathways. For this purpose, we selected intracellular readouts measurable by flow cytometry that reflected the strength of homeostatic signaling pathways in resting cells, as the protein expression levels of the Bcl-2 family which transcription is promoted by NFκB. We further explored the possible alteration of this crucial prosurvival signaling pathway in CVID patients by analysing the expression levels of mRNAs from anti-apoptotic proteins in naive B cells, mimicking an *in-vitro* T cell-dependent activation with CD40L and interleukin (IL)-21.

## Materials and methods

### Samples

Heparinized peripheral blood samples from CVID patients (according to the criteria of the European Society for Immunodeficiencies) and healthy donors (HD) were collected at La Paz University Hospital after obtaining their informed consent. The study was approved by the hospital's ethics committee (PI-2833 and 2009/3348/I), and adhered to the principles set out in the Declaration of Helsinki. The clinical data were obtained from the clinical records updated during routine medical visits for the diagnosis and follow-up at the outpatient clinic of the Clinical Immunology Department.

Routine enumeration of lymphocyte subpopulations, including the analysis of the B cell peripheral compartment, was performed on a fluorescence activated cell sorter (FACSCalibur) and FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA).

### Intracellular proteins analysed by flow cytometry

We performed an intracellular staining protocol to evaluate Bcl-2 expression in naive ($CD19^+IgD^+CD27^-$), unswitched memory B cells (USm) $CD19^+IgD^+CD27^+$ and switched memory B cells (Sm) ($CD19^+IgD^-CD27^+$). Briefly, 300 µl of heparinized whole blood was lysed with FACS lysing solution (BD Biosciences) and permeabilized with BD FACS permeabilizing solution 2, according to the manufacturer's recommendations. Samples were stained with intracellular Bcl-2 fluorescein isothiocyanate (FITC) (clone Bcl-2/100; BD Biosciences), CD19 peridinin chlorophyll-cyanin 5.5 (PerCP-Cy5.5) (clone SJ25C1; BD Biosciences), CD27 allophycocyanin (APC) (clone M-T271, Miltenyi Biotec, San Diego, CA, USA), IgD phycoerythrin (PE) (Southern Biotech, Birmingham, AL, USA) and an isotype control mouse IgG1 FITC κ (BD Biosciences). Samples were analysed by flow cytometry using FACSCalibur and CellQuest software (BD Biosciences).

Intracellular and surface staining for Bcl-xl expression was performed in 100 µl of whole blood, based on the manufacturer's instructions and the original from Chow *et al.* [37]. Whole blood was stained with CD27 BV421 (clone M-T271; BD Biosciences), then fixed with 10% formaldehyde and treated with 0·1% Triton X-100. Cells were permeabilized with 50% methanol on ice. The cells were then stained with intracellular marker and the other surface markers: CD3 APC (clone UCHT1; BD Biosciences), CD19 phycoerythrin (PE)-Cy7 (clone J3-119; Beckman Coulter, Pasadena, CA, USA), IgD PE (Southern Biotech) and Bcl-xl rabbit antibody (clone 54H6), conjugated with Alexa Fluor 488 (Cell Signaling, Danvers, MA, USA). The specific isotype control anti-rabbit IgG for Bcl-xl (Cell Signaling) was added at the same concentrations as the specific antibodies. The mean fluorescence intensity (MFI) values for each protein were obtained over the specific isotype controls. Samples were acquired in FACS Canto II (BD Biosciences). We performed the data analysis by employing FlowJo software LLC (Ashland, OR, USA).

### Naive B cell purification and cell culture

Freshly obtained peripheral mononuclear cells (PBMCs) were isolated after performing Ficoll density gradient centrifugation. Naive B cells were isolated from PBMCs using the MACS naive B cell isolation kit II in an Automacs (Miltenyi Biotech). Naive B cell enrichment was assessed using CD19 PerCP-Cy5.5, CD27 FITC, CD3 APC (BD Biosciences) and IgD PE (Southern Biotech). We resuspended $1 \times 10^5$ purified naive B cells in 200 µl RPMI-1640 supplemented medium (10% fetal calf serum, 100 U/ml penicillium, 100 µg/ml streptomycin and 2 mM glutamine). Cells were activated for 4 days with 2 µg/ml of MegaCD40L soluble human recombinant (Enzo Life Sciences, Farmingdale, NY, USA) and 100 ng/ml of human recombinant IL-21 (Gibco, Life Technologies, Carlsbad, CA, USA); unstimulated samples were performed in parallel. Cell cultures were maintained for 4 days at 37°C in a 5% $CO_2$ atmosphere. Cell activation was measured with the monoclonal antibody combinations CD19 PerCP-Cy5.5, CD27 FITC, CD69 PE (clone L78) and CD86 APC (clone 2331 Fun-1) (BD Biosciences). Responsiveness to stimulation was calculated as the fold increase of MFI in CD69 and CD86 in stimulated cells to the same markers in the unstimulated cells (fold change = MFI-stimulated/MFI-unstimulated). The analysis was performed by flow cytometry using FACS Canto II (BD Biosciences) and the FlowJo Software (LLC).

### Molecular biology

*BCL-2*, *BCL-XL* and *AICDA* gene expression levels were detected by quantitative real-time polymerase chain reaction (qPCR). Total RNA was extracted using the miRNeasy micro Kit (Qiagen, Valencia, CA, USA) and quantified in the Nanodrop 2000c (Thermo Scientific, Waltham, MA, USA). C-DNA was synthesized from 50 ng of total RNA using the high-capacity RNA-to-c DNA kit (Applied Biosystems, Foster City, CA, USA). We performed qPCR using the TaqMan gene expression master mix (Applied Biosystems) in an Applied Biosystems 7900HT fast real-time PCR system, with the primer/probe real-time PCR assays for *BCL-2* (Hs00608023_m1), *BCL-XL* (Hs00236329_m1), *AICDA* (Hs00757808_m1) and *18 S* (Hs 99999901_s1) (all from Applied Biosystems). PCR fragments were amplified for 2 min at 50°C, 10 min at 95°C followed by 50 cycles consisting of 15 sec at 95°C and 1 min at 60°C. Samples were amplified in triplicate, and target gene expression was normalized with 18S ribosomal RNA. We calculated the relative expression using the $2^{-\Delta\Delta Ct}$ method and employed SDS Software and RQ Manager (Applied Biosystems) for the data analyses. We calculated the relative mRNA levels as the ratio of the relative gene expression in stimulated cells at day 4 compared with the relative gene expression in the unstimulated cells at day 4 of the culture, expressing the results as the fold increase.

### Statistics

We performed the data analysis using GraphPad Prism version 6.0 software (San Diego, CA, USA). Statistical differences between CVID patients and controls were determined using the non-parametric Mann–Whitney test. Differences were considered statistically significant for *P*-values < 0·05, < 0·01 and < 0·001.

## Results

### Anti-apoptotic protein levels

To determine whether unbalanced expression of Bcl-2 family proteins could contribute to the disturbance in B cell compartment from CVID patients, we first analysed Bcl-2 anti-apoptotic protein levels in different B cell subsets, naive ($CD19^+IgD^+CD27^-$), USm B cells and Sm B cells, from 43 CVID patients and 41 healthy donors (HD). Naive B cells from CVID patients presented similar levels of intracellular Bcl-2 protein as healthy donors (expressed as mean MFI = 10·98 *versus* 10·92). However, USm B cells from CVID patients showed reduced Bcl-2 expression compared with healthy controls (mean MFI = 17·31 *versus* 18·72) and significant Bcl-2 protein levels in Sm B cells (mean MFI = 14·57 *versus* 18·11 in HD) ($P$ = 0·0021) (Fig. 1a,b). B cells from healthy donors presented an increase in Bcl-2 intracellular expression from naive to Sm B cells. In contrast, this increase was reduced in CVID patients ($P$ < 0·0001) (Fig. 1c). The classification of patients according to B cell phenotype showed impaired Bcl-2 expression in Sm B cells from CVID with a reduction in total memory B cells ($P$ = 0·0567) (mean MFI = 15·53 *versus* 18·11 in HD, Fig. 2a). In CVID patients with a severe reduction in Sm B cell counts (< 2%), Bcl-2 protein levels were lower in the unswitched (mean MFI = 16·59 *versus* 18·72 in HD; $P$ = 0·0431) and in Sm cells (mean MFI = 14·61 *versus* 18·11 in HD; $P$ = 0·0202) (Fig. 2b). CVID patients with expansion of $CD21^{low}$ B cells (> 10%) also presented lower Bcl-2 levels in Sm B cells compared with the Sm B cells in healthy donors (mean MFI = 12·92 *versus* 18·11; $P$ = 0·0100) (Fig. 2c).

To test whether the reduced Bcl-2 protein levels were accompanied by other anti-apoptotic proteins, we detected Bcl-xl protein levels in B cell subsets from 37 CVID patients and 27 healthy donors. We detected no differences in MFI expression in naive, unSm memory B cells and Sm B cells between CVID patients and healthy donors (Fig. 2d).

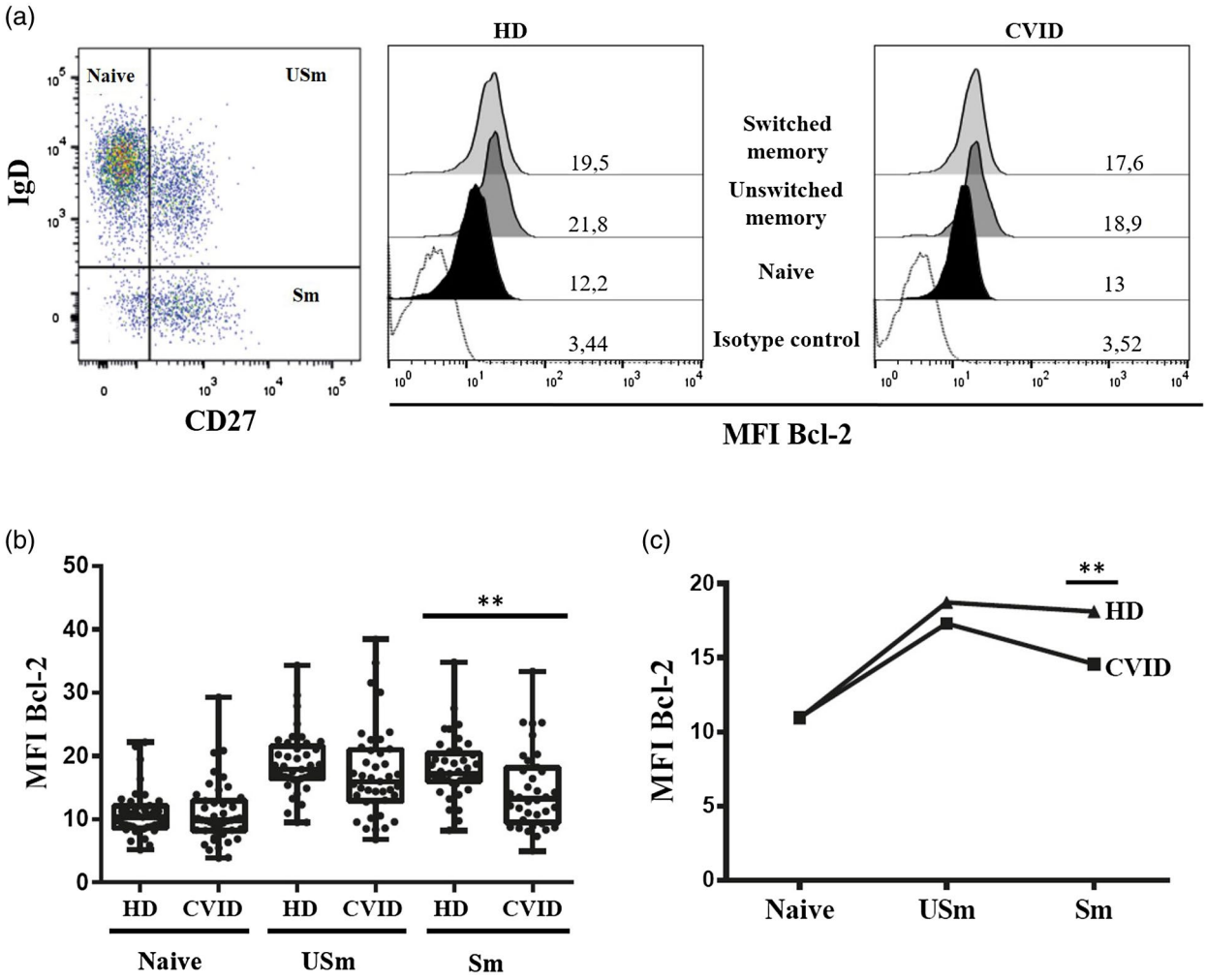


**Fig. 1.** (a) Dot-plots showing distribution of naive ($CD19^+IgD^+CD27^-$), unswitched memory (USm) ($CD19^+IgD^+CD27^+$) and switched memory (Sm) ($CD19^+IgD^-CD27^+$) B cells. Histograms of representative healthy donor (HD) and common variable immunodeficiency (CVID) patient showing mean fluorescence intensity (MFI) of isotype control and MFI of Bcl-2 in naive, USm and Sm B cells; (b) MFI of Bcl-2 in naive, USm and Sm B cells. Box and whiskers represent mean and range. (c) Up-regulation of Bcl-2 protein expression in the transition from naive to Sm B cells. The *P*-value is shown for the cases with a statistically significant difference using the Mann–Whitney test (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$).

### Naive B cells from CVID presented reduced up-regulation of anti-apoptotic mRNA levels after activation with CD40L and IL-21

To determine whether the reduced counts of Sm B cells together with the reduced Bcl-2 protein levels were related to impaired B cell activation, we analysed the mRNA levels of Bcl-2 and Bcl-xl, two target genes of NF-κB, upon naive B cell activation. Surface expression of the B cell activation proteins CD69 and CD86 were measured by flow cytometry as an internal control of proper naive B cell activation.

We examined the induction of target genes *BCL-2* and *BCL-XL* together with *AICDA*, as a positive control of activation, in naive B cells after 4 days of *in-vitro* stimulation with CD40L and IL-21, mimicking a GC reaction (Fig. 3). CVID patients presented decreased *BCL-2* induction expressed as a fold change (mean = 1·9 *versus* 2·8) compared with healthy donors (although the difference was not statistically significant) (Fig. 3a). The relative expression of *BCL-XL* induced after activation was diminished in CVID patients (mean = 4·72 *versus* 11·86 in HD; $P = 0{\cdot}0371$) (Fig. 3b). *AICDA* was included in this study because *AICDA* was not expressed in naive B cells, but was induced after naive B cell activation during the GC reaction. We found that CVID patients and healthy donors expressed *AICDA* after naive B cell activation, but this increase was lower in CVID patients in comparison with healthy donors ($P = 0{\cdot}019$) (Fig. 3c). The decreased up-regulation of this mRNA did not appear to be related to a defective activation response of CVID naive B cells,

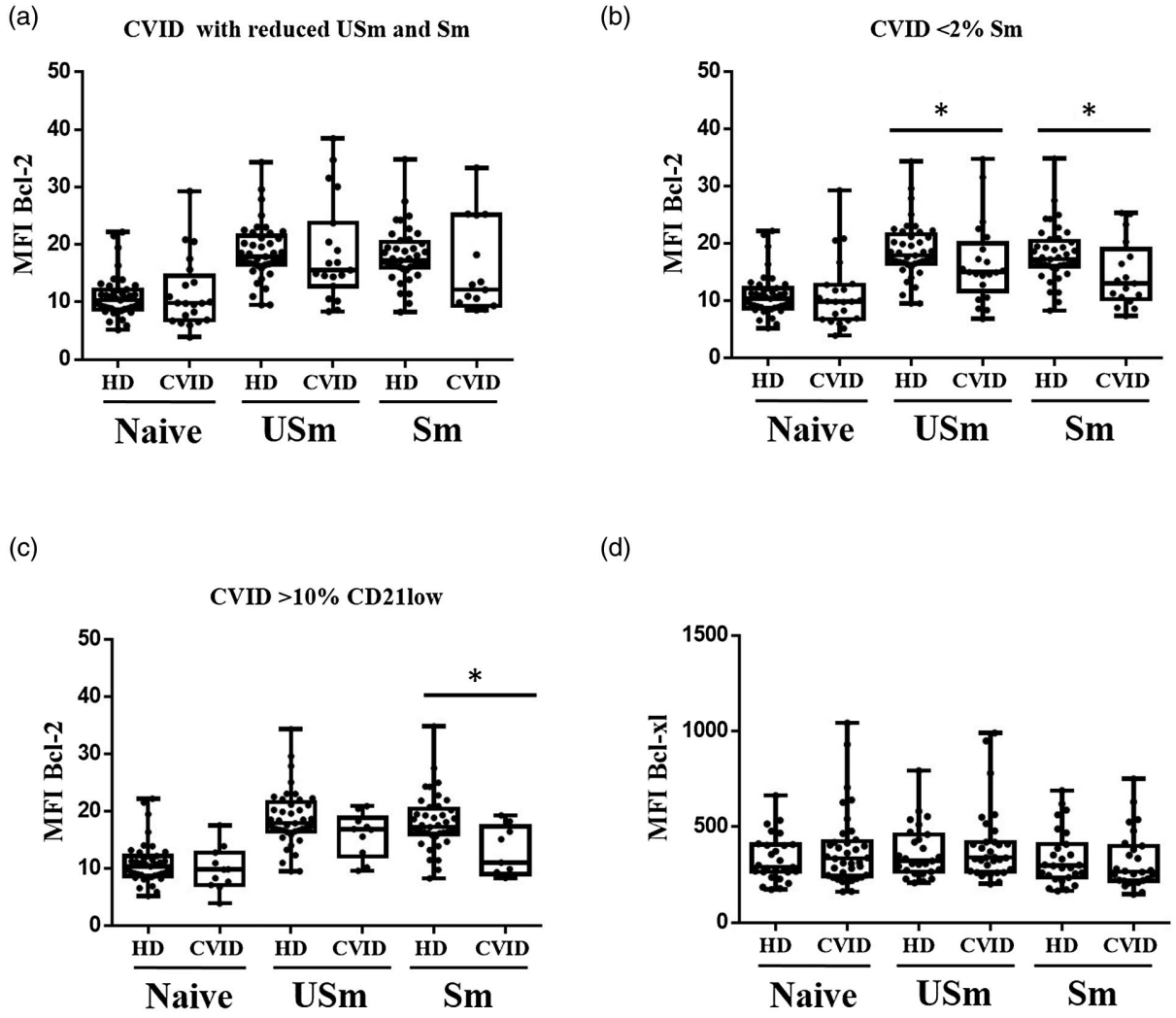


**Fig. 2.** Mean fluorescence intensity (MFI) Bcl-2 protein expression in naive, unswitched memory (USm) and switched memory (Sm) B cells according to B cell phenotype. (a) Common variable immunodeficiency (CVID) patients with reduced USm and Sm B cells. (b) CVID patients with severe reduction of Sm B cells (< 2%). (c) CVID patients with expansion of $CD21^{low}$ B cells (> 10%). (d) MFI Bcl-xl protein expression in naive, USm and Sm B cells from HD and CVID patients. Box and whiskers represent mean with range. Significant *P*-values using the Mann–Whitney test (**P* < 0·05; ***P* < 0·01; ****P* < 0·001).

due to similar induced levels of CD69 and CD86 after 4 days of stimulation with CD40L and IL-21 in CVID patients and healthy controls (Fig. 4a,b).

## Discussion

The pathogenesis of CVID is still poorly understood [1]. Adequate B cell activation allows the differentiation of naive B cells into memory B cells to enable a quick and efficient response during the immune reaction. The main differences between naive and memory B cells are the various gene expression profiles and the variety of stimuli they receive, including that from T cells [38]. The maintenance of circulating resting mature and memory B cells requires signaling via B cell antigen receptor (BCR), the tumor necrosis factor receptor (TNFR) family [via B cell activating factor receptor (BAFFR)] and cytokine receptors leading to the activation of key transcription factors, such as NF-κB. The NF-κB family consists of various subunits: NF-κB1 (p105/p50), NF-κB2 (p100/p52), Rel A (p65), Rel B and c-Rel [31,32]. Signaling through NF-κB effectors eventually up-regulates the expression of anti-apoptotic members of the Bcl-2 family, such as Bcl-xl and Mcl-1, with the reported increased of Bcl-2 expression in memory B cells of particular relevance [35,39].

Both anti-apoptotic proteins Bcl-2 and Bcl-xl share functional homology, and their transcription is tightly

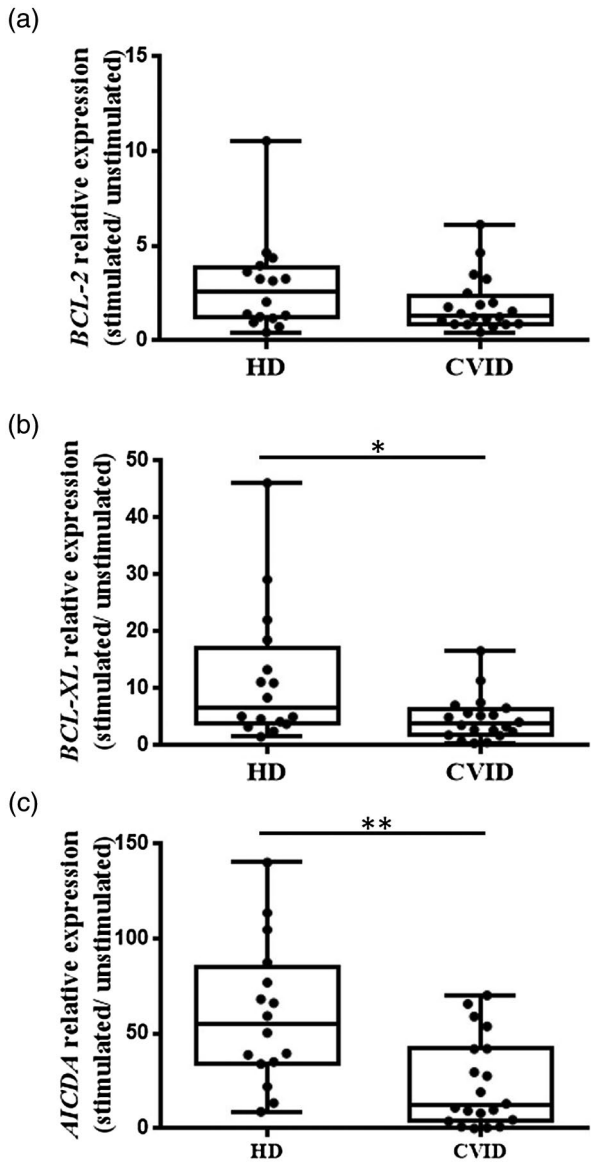


**Fig. 3.** Relative gene expression (a) *BCL-2*, (b) *BCL-XL* and (c) *AICDA* in naive B cells from healthy donor (HD) and common variable immunodeficiency (CVID) patients after 4 days' stimulation with CD40L and interleukin (IL)-21. Relative mRNA levels were normalized to the expression of 18S ribosomal RNA and were calculated as the ratio of relative gene expression in stimulated cells at day 4 to the relative gene expression in unstimulated cells at day 4 of culture. Box and whiskers represent mean and range. Significant *P*-values using the Mann–Whitney test (*$P < 0{\cdot}05$; ** $P < 0{\cdot}01$; ***$P < 0{\cdot}001$).

regulated by NF-κB activity but differs in the regulation of B cell survival in different subpopulations. Bcl-2 has a relevant role for naive and memory B cell survival. In this study, we showed reduced Bcl-2 protein levels in memory B cells from CVID patients, in particular in Sm B cells. In healthy donors, Bcl-2 expression showed an increase in the transition from naive to Sm B cells, which was less evident in the B cells from CVID patients.

Whereas Bcl-xl is weakly expressed in naive and memory B cells, Bcl-xl is highly expressed in GC B cells where its regulator c-Rel is constitutively expressed. Hence, the kinetics observed in our results barely showed changes in Bcl-xl expression between the naive and Sm B cells [33]. To determine the signaling defect that prevents B cells from increasing the Bcl-2 expression essential for progression to more differentiated stages, we activated naive B cells with CD40L and IL-21. This stimulation leads to an activation and differentiation of naive B cells, mimicking the stimuli received during the *in-vivo* GC reaction [40,41]. B and T cell co-operation activates both canonical and non-canonical NF-κB pathways for anti-apoptotic *BCL-XL* gene expression [34], given that c-Rel (NF-κB subunits) regulates the *BCL-XL* promoter [33].

Activated naive B cells from CVID patients presented impaired up-regulation of *BCL-2* and *BCL-XL* mRNA levels. The increase in *BCL-XL* levels appears to be crucial for GC B cells *in vivo* [33], precisely where *BCL-XL* is highly expressed. Stimulated naive B cells from CVID patients showed impaired induction of *BCL-XL* expression, which was even more diminished in the patients with reduced memory B cells. These results agree with those of Keller *et al.* [42], showing reduced induction of *BCL-XL* after anti-IgM stimulation in B cells from CVID patients. Recently, López-Gómez *et al.* [43] also showed the B cell defect of $CD27^-$ B cells in CVID patients, analysing by flow cytometry the induction of Bcl-2 and Bcl-xl proteins after 20 h activation with anti-BCR and anti-CD40.

For the first time, to our knowledge, our results show alterations in Bcl-2 protein expression in B cell subsets from CVID patients and reveal the complementary functions of the two anti-apoptotic members of the Bcl-2 family. Bcl-2 has a relevant role in naive and memory B cells and Bcl-xl in GC B cells. In the GC phase that we attempted to reproduce *in vitro*, the reduced *BCL-2* expression in this stage has been reported previously [33]. Bcl-2 reduction can act as a tolerance mechanism to mediate apoptosis to eliminate possible autoreactive B cells that might be generated during the CSR and SHM process [44]. *BCL-2* has also been reported to be less inducible than *BCL-XL* in memory B cells, even from healthy donors stimulated for 24 h with CD40L [45].

The discovery of NF-κB signaling defects suggests a possible role for increased apoptosis as a contributor to the B cell defect in CVID. The inability to increase *BCL-XL* mRNA levels would not favor survival in the GC maturation process, while expressing lower Bcl-2 protein levels could compromise

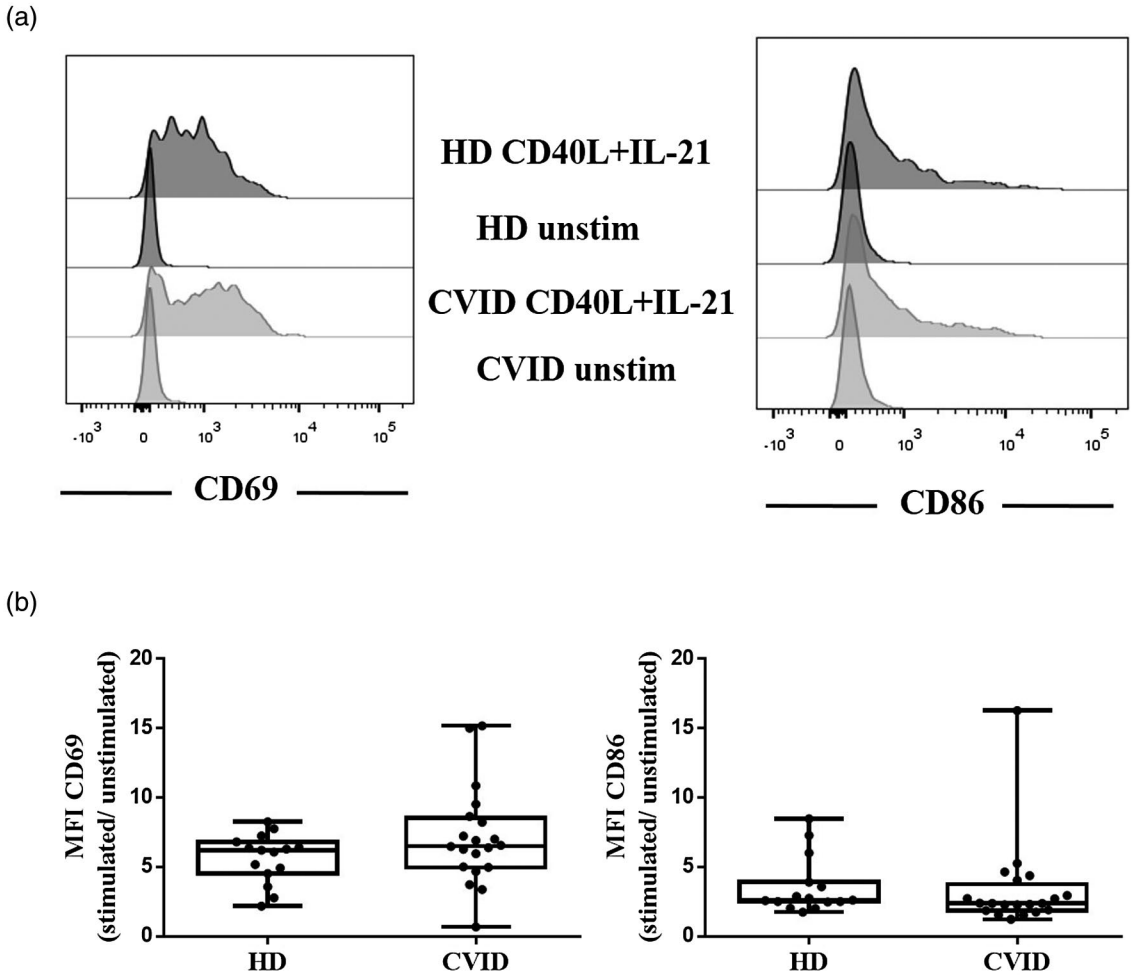


**Fig. 4.** (a) Representative mean fluorescence intensity (MFI) in CD69 and CD86 activation markers expression in unstimulated and stimulated naive B cells with CD40L and interleukin (IL)-21 from healthy donor (HD) and common variable immunodeficiency (CVID) patients. (b) No differences were detected in MFI expression in CD69 and CD86 in HD and CVID patients, represented as fold induction of naive B cells stimulated with CD40L and IL-21 compared with unstimulated naive B cells. Box and whiskers represent mean and range.

the long-term survival of memory B cells. Other studies have recently described more related alterations in NF-κB signaling in B cells from CVID patients. Keller *et al.* [42] identified impaired signaling in the canonical NF-κB pathway in B cells from CVID patients, with diminished IκBα degradation and reduced p65 translocation, particularly in CVID patients with expanded $CD21^{low}$ B cells.

Deregulation in apoptosis in the CVID context has been previously reported, such as spontaneous apoptosis in B cells from CVID patients [46,47], and the increased expression of cell death receptor FAS and TNF-related apoptosis-inducing ligand (TRAIL) in patients with low memory B cells [48]. T cells in CVID patients also showed higher levels of CD95, which was related to impaired survival and $CD4^+$ lymphopenia [46,49].

We also included *AICDA* in the study as a positive control, because *AICDA* is only expressed after naive B cell activation. The induction of *AICDA* expression could be mediated by both the canonical and alternative NF-κB pathways [50]. Activated naive B cells from CVID patients and controls could increase *AICDA* expression, but the expression was dampened in CVID patients, particularly in those with reduced numbers of Sm B cells. Diminished *AICDA* induction together with low Sm B cells reveal the blockage in CSR and SHM present in CVID patients, with the reduced SHM rate observed in our CVID cohort [23].

Despite impaired *BCL-XL* and *AICDA* induction, we found CD69 and CD86 expression levels comparable in the patients and controls after 4 days of activation of naive B cells with CD40L and IL-21. The defects observed *in vitro* did not appear to be due to a lower responsiveness of the general activation in patients' B cells. Previous studies have shown a defect in CD69 and CD86 induction; Lougaris *et al.* [51] observed reduced CD69 and CD86 expression in total PBMCs

stimulated with cytosine– phosphate–guanosine (CpG). Groth *et al.* [52] also observed decreased expression in naive B cells from CVID patients stimulated with anti-IgM and IL-2. These results are only seemingly discordant with our findings if we consider that the subset of activated cells (total PBMCs), and the stimulation employed (CpG, anti IgM and IL-2) was different from ours: naive B cells with CD40L and IL-21, which more effectively reproduce the *in-vivo* activation during the GC reaction and might generate a more powerful response [47].

In summary, having knowledge of the relevance of apoptosis in B cell survival, in this study we provide for the first time an analysis of Bcl-2 levels in various B cell subpopulations and anti-apoptotic mRNA expression assays after CD40L and IL-21 stimulation in naive B cells of CVID patients. Our data suggest a molecular mechanism for this tendency to apoptosis, with potentially lower NF-κB activation in the B cells of CVID patients (who might be unable to increase *BCL-XL* mRNA levels, which does not favor survival in the GC maturation process) and memory B cells from CVID patients expressing lower Bcl-2 protein levels (which could compromise their long-term survival). Alterations in the induction of anti-apoptotic proteins suggest defects in B cell signaling in CVID.

CVID is a complex disease with high variability in its clinical presentation and different penetrance, even when monogenic defects are present [21], which suggests that a polygenic heterogeneous predisposing genetic background [6], the involvement of epigenetic alterations [23,53] and multiple cumulative defects could lead to the B cell deregulation present in CVID patients.

## Acknowledgements

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## Disclosures

All authors state that they have no conflicts of interest.

## Author contributions

E. L. and L. P. conceived the project. E. L. II, L. P. and J. T. designed the experiments. L. P. performed the experiments and O. P. provided technical assistance. L. P. and E. L. wrote the manuscript.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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