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CD27+ B cells from a subgroup of common variable immunodeficiency patients are less sensitive to apoptosis rescue regardless of interleukin-21 signalling

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Summary

Common variable immunodeficiency (CVID) is a primary immunodeficiency characterized by hypogammaglobulinaemia and recurrent infections. Although the underlying cause is unknown, B cells from most CVID patients fail to differentiate to memory or plasma cells. We investigated if increased apoptosis could influence the fate of B cells. For this purpose we activated purified B lymphocytes of CVID patients with a surrogate T-dependent (anti-CD40) or T-independent [cytosine–phosphate–guanosine oligodeoxynucleotides (CpG-ODN) or anti-immunoglobulin (Ig)M)] stimulus with or without interleukin (IL)-21. We found that CD27+ B cells were more sensitive than CD27− B cells to spontaneous apoptosis and less sensitive to rescue from apoptosis. The addition of IL-21 down-modulated the protective effect of all the stimuli on CD27− B cells and the protective effect of CpG-ODN and anti-IgM on CD27+ B cells. In contrast, IL-21 rescued unstimulated CD27− B cells and improved the rescue of anti-CD40-stimulated CD27+ B cells. When we compared patients and controls, mainly CD27+ B cells from MB0 patients were less sensitive to rescue from apoptosis than those from MB1 patients and controls after activation, irrespective of the IL-21 effect. Increased apoptosis during an immune response could result in lower levels of immunoglobulin production in these patients.

Keywords: apoptosis, CD27+ B cells, CVID, IL-21, TRAIL

Introduction

Common variable immunodeficiency (CVID) is the most frequent symptomatic primary humoral immunodeficiency. It includes a heterogeneous group of disorders of unknown aetiology characterized by deficient antibody production, recurrent respiratory infections by encapsulated bacteria, mainly *Streptococcus pneumoniae* and *Haemophilus influenzae*, and poor response to vaccination. Patients benefit from immunoglobulin replacement therapy [1–4]. Several genetic mutations and polymorphisms [inducible T cell co-stimulator (ICOS), tumour necrosis factor receptor superfamily, member 13b (TNFRS13B/TACI), CD19, CD20, CD81, B cell-activating factor receptor (BAFF-R) and CD21] have been described in fewer than 10% of CVID patients, while the underlying molecular defect remains unknown for most of them [5–7]. A vast array of immunological defects have been described in CVID patients, of which the most outstanding is abnormal late B cell differentiation to CD27+ memory B cells, switched-memory B cells and plasma cells. Accordingly, patients have been classified depending on their number of naive, memory and switched-memory B cells [8,9]. Furthermore, a low percentage of memory B cells in CVID patients has been associated with a worse clinical presentation and poor response to vaccines [10–12]. Loss of memory B cells also occurs from the onset of acute HIV infection. Recently, low frequencies of CD27+ memory B cells and decreased production of antibodies have been described in successfully treated HIV patients in spite of drug-suppressed viraemia. Surface expression levels of TNF-related apoptosis-inducing ligand (TRAIL) on memory B cells correlated negatively with their peripheral blood frequency [13].

The generation of memory B cells and plasma cells is essential to establish efficient humoral immune responses. Co-operation of B cell receptor (BCR)-activated B cells with helper T cells is relevant and occurs through contact between T cell membrane molecules (CD40L, ICOS, etc.) and their corresponding B cell ligands [14]. The importance of several of these components of the immune system has

been exemplified by naturally occurring immunodeficiencies [15]. Furthermore, secretion of cytokines by T cells also instruct the differentiation of B cells, including interleukin (IL)-21 as one of the more potent cytokines for human B cell proliferation and differentiation [16–20]. Following antigenic stimulation, Toll-like receptor (TLR) can provide an additional signal for the differentiation of B cells and even substitute T cell-derived signals [21,22].

Apart from their effect on proliferation and differentiation, several of these stimuli also influence B cell survival. BCR activation has been shown to induce B cell apoptosis in the absence of survival signals such as that provided through CD40. Mainly produced by activated $CD4^+$ follicular T cells [19,23,24], IL-21 is a type I cytokine that belongs to a family that uses the common cytokine receptor γ-chain as a component of their receptors [25,26]. The stimulatory or inhibitory effect of IL-21 depends on the maturation and activation status of the B cell, the co-stimulatory accompanying signal and the presence of other cytokines. In humans, IL-21 is a potent inductor of plasma cell differentiation if combined with anti-CD40 [16], induces class-switch recombination and secretion of immunoglobulin (Ig)G and IgA in pre-switched IgM memory B cells [19,27] and is able to induce plasma cell differentiation and immunoglobulin production even by naive B cells [16]. However, IL-21 triggers B cell death when BCR is ligated [16,28]. A balance between apoptosis-inducing and survival signals must exist to preserve B cell homeostasis.

We have shown previously that, although acting through different pathways, neither cytosine–phosphate–guanosine oligodeoxynucleotides (CpG-ODN) nor anti-CD40 with IL-21 stimulation are able to induce B cells from CVID patients to produce normal levels of immunoglobulins, and this defect is not corrected by the addition of anti-IgM [29]. This could imply a signalling defect in both pathways or, alternatively, it could indicate that B cells from CVID patients die in culture after stimulation. In this study we evaluated the effect of IL-21 on spontaneous and TLR-9-, CD40- or BCR-induced apoptosis or proliferation of $CD27^-$ and $CD27^+$ B cells from CVID patients. The aim of the study was to ascertain if differences in response between controls and patients could determine a different fate of $CD27^-$ and $CD27^+$ B cells and explain the imbalanced B cell homeostasis and finally immune deficiency in CVID patients.

Methods

Patients

Twenty-two CVID patients were selected according to diagnostic criteria of the International Union for Immunological Societies scientific group for primary immunodeficiency diseases. Patients were classified into three groups according to Piqueras *et al.* [8]: (i) CVID patients with a low percentage of $CD27^+$ (memory phenotype) B cells or MB0; (ii) patients with normal IgD^+CD27^+ (non-switched-memory phenotype) and a low percentage of IgD^-CD27^+ (switched-memory phenotype) B cells or MB1; and (iii) patients with normal percentages of $CD27^+$ B cells or MB2. Patients received intravenous gamma globulin therapy every 21 days and did not suffer from infections at the time of the study. Peripheral blood samples were collected before gamma globulin replacement. Table 1 summarizes the patients' age, gender and percentages of B cell subpopulations. Twenty-two age- and sex-matched healthy blood donors were included as controls. The study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by CEIC (Balearic Islands Clinical Research Ethics Committee; IB 1564/11 PI). Informed consent was obtained from all subjects.

B lymphocyte purification, $CD27^-$and $CD27^+$ B cell sorting and cell culture

Peripheral blood mononuclear cells (PBMC) were isolated from 40 ml of heparinized blood by density gradient centrifugation. B lymphocytes were obtained from PBMC by negative selection using the Dynabeads Untouched™ human B cells separation kit (Dynal; Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. $CD27^-$ and $CD27^+$ B cells were sorted from 4×10^6 purified B cells using a Coulter Epics Altra HypersortTM system (Beckman Coulter, Brea, CA, USA). Purified B cells or sorted $CD27^-$ and $CD27^+$ B cells were resuspended in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), glutamine (2 mM) and antibiotics (penicillin and streptomycin). Purified B cells (1×10^6/ml) were labelled during 5 min at room temperature (RT) (25°C) with 1 μg carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen), following the manufacturer's instructions.

CFSE-free purified B cells, 5×10^4, or sorted $CD27^-$ and $CD27^+$ B cells and CFSE-labelled purified B cells were cultured in 96-well plates and stimulated with CpG-ODN (0·6 μg/ml; CpG-ODN type B; InvivoGen, San Diego, CA, USA), F(ab)2 goat anti-human IgM (5 μg/ml; Jackson ImmunoResearch, West Grove, PA, USA), anti-human CD40/TNFRSF5 antibody (1 μg/ml; R&D Systems, Abingdon, UK) without or with human recombinant IL-21 (100 ng/ml; Biosource, Cambridge, MA, USA). Cultures were maintained for 3 days at 37°C in a 5% CO_2 atmosphere.

Flow cytometry

B cell purity, apoptosis, proliferation and surface marker expression were analysed by flow cytometry using an Epics FC500 flow cytometer and the CXP software (Beckman Coulter).

Table 1. Age, gender and B lymphocyte subpopulations of common variable immunodeficiency (CVID) patients.

Patient	Age (years)	Gender (male/female)	CD19 (%)	CD19+ (%) Naive IgD+CD27−	Unswitched memory IgD+ CD27+	Switched memory IgD−CD27+	Group
1	43	M	5	94	5	<1	MB0
2	61	M	8	96	3	<1	MB0
3	29	M	15	95	<1	<1	MB0
4	29	F	9	87	6	2	MB0
5	61	F	19	84	5	5	MB0
6	60	F	5	95	1	1	MB0
7	60	M	2	92	3	<1	MB0
8	67	F	5	96	3	<1	MB0
9	34	F	16	70	2	3	MB0
10	38	M	4	90	5	1	MB0
11	23	M	14	91	4	<1	MB0
12	27	F	8	97	2	<1	MB0
13	73	F	27	72	27	<1	MB1
14	67	F	14	64	25	4	MB1
15	67	F	7	73	18	5	MB1
16	46	F	16	77	20	1	MB1
17	47	F	9	75	17	5	MB1
18	33	F	9	84	8	4	MB1
19	60	F	26	76	7	6	MB1
20	67	F	16	84	12	1	MB1
21	82	F	2	39	14	33	MB2
22	44	F	20	52	10	20	MB2

Current age (years), gender (M: male, F: female); percentage of peripheral blood B lymphocytes (CD19+) and percentages of naive [immunoglobulin (Ig)D+CD27−], memory unswitched (IgD+CD27+) and memory-switched (IgD−CD27+) B cell subpopulations (referred to total CD19+ B lymphocytes) and Piqueras classification of CVID patients.

Cell purity was assessed using the following monoclonal antibody combinations: anti-CD45 fluorescein isothiocyanate (FITC), anti-CD19 phycoerythrin cyanin 5 (PCy5) (both from Coulter Immunotech) and anti-CD3 phycoerythrin (PE) (Becton Dickinson, Franklin Lakes, NJ, USA) for purified B cells and anti-CD19 PCy7 plus anti-CD27 PCy5 (both from Coulter Immunotech) for sorted CD27− and CD27+ B cells. Purity was always superior to 95%.

Annexin V and propidium iodide staining protocol (Becton Dickinson) was performed to evaluate apoptosis of CSFE-free purified (Fig. 1a) and sorted CD27− and CD27+ B cells (Fig. 1b,c), following the manufacturer's instructions. Briefly, 1×10^5 cultured CFSE-free cells were harvested, stained with anti-CD19 PCy7 and anti-CD27 PCy5, washed with cold phosphate-buffered saline (PBS), resuspended in 100 µl binding buffer and stained with 5 µl of a 1·2 µg/ml solution of annexin V-FITC and 5 µl of a 50 µg/ml solution of propidium iodide. Cells were incubated for 15 min at RT (25°C) in the dark, resuspended in 400 µl of binding buffer and analysed. Propidium iodide positivity was used to exclude necrotic CD19+ cells and percentage of apoptotic cells (annexin V-FITC-positive) was calculated from the resulting population. Rescue from apoptosis was expressed as [(% baseline apoptosis − % post-stimulation apoptosis)/% baseline apoptosis] × 100, to indicate the decrease in apoptosis induced by each stimulus related to baseline apoptosis.

A CFSE dilution protocol was used to evaluate the proliferation of CFSE-labelled cultured purified B cells. Proliferation index was calculated on CD19+CD27− or CD19+CD27+ stained B cells attending to the number of divisions and the percentages of cells in each round of division, as described previously by Quah *et al.* [30].

TRAIL expression was evaluated in whole blood samples stained with anti-CD19 energy-coupled dye (ECD), anti-CD27 PCy7 (both from Coulter Immunotech) and anti-TRAIL-PE (Becton Dickinson)-conjugated monoclonal antibodies. TRAIL median fluorescence intensity (MFI) was measured in previously gated CD19+CD27− and CD19+CD27+ B cells.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4·0 software (San Diego, CA, USA). Data are expressed as median and 25th and 75th percentiles. The Mann–Whitney *U*-test was used to compare differences between B cells subpopulations. The Kruskal–Wallis test was used to compare differences between CVID patients

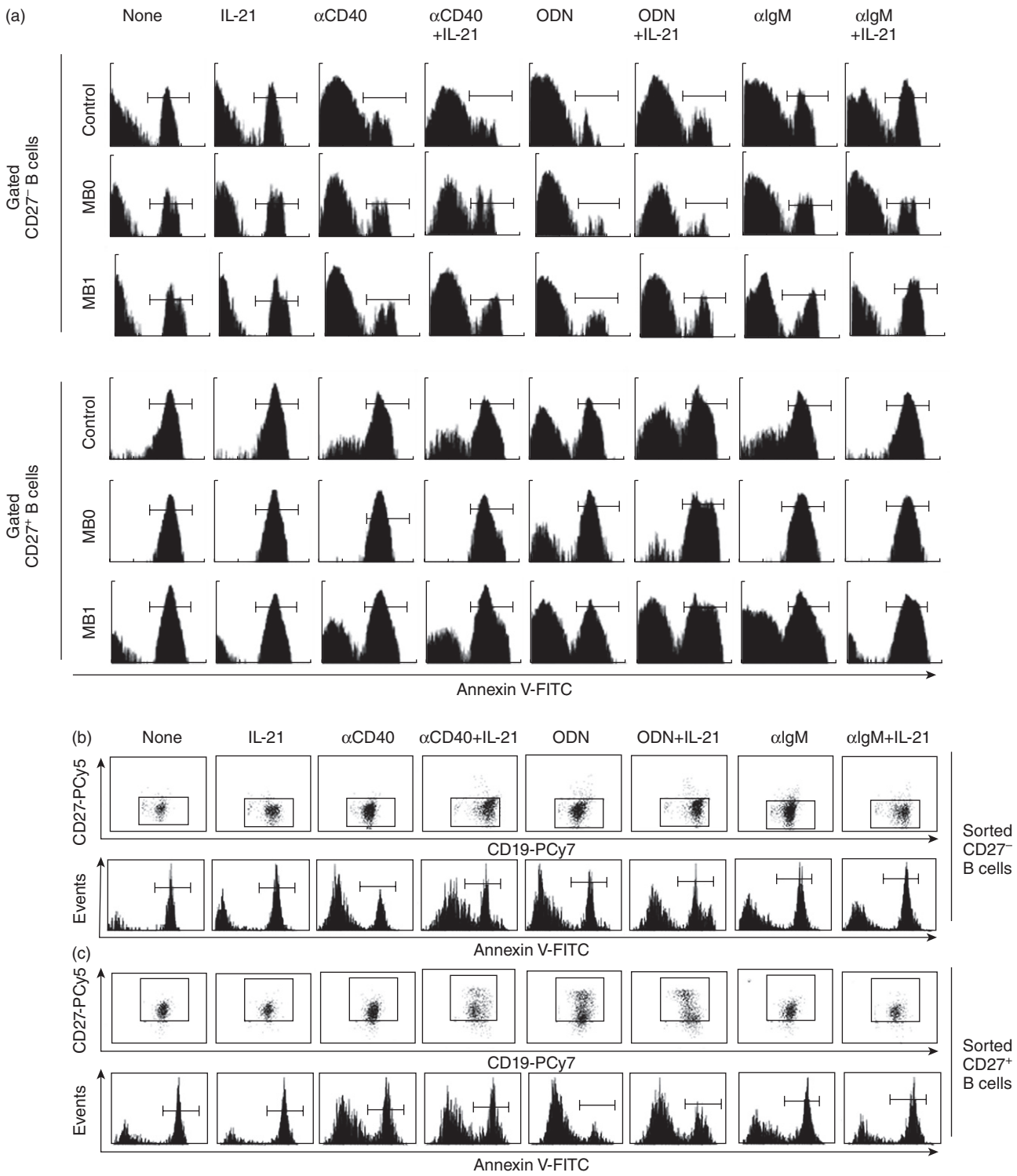
(a)
None
IL-21
αCD40
αCD40 +IL-21
ODN
ODN +IL-21
αIgM
αIgM +IL-21
Gated CD27⁻ B cells
Control
MB0
MB1
Gated CD27⁺ B cells
Control
MB0
MB1
Annexin V-FITC
(b)
None
IL-21
αCD40
αCD40+IL-21
ODN
ODN+IL-21
αIgM
αIgM+IL-21
CD27-PCy5
CD19-PCy7
Events
Annexin V-FITC
Sorted CD27⁻ B cells
(c)
CD27-PCy5
CD19-PCy7
Events
Annexin V-FITC
Sorted CD27⁺ B cells

Fig. 1. Representative experiments of spontaneous and post-activation apoptosis of CD27⁻ and CD27⁺ B cells. Purified B cells from common variable immunodeficiency (CVID) patients and controls or sorted CD27⁻ and CD27⁺ B cells from controls were stimulated with different combinations of CD40, Toll-like receptor (TLR)-9, B cell receptor (BCR) ligands or interleukin (IL)-21. (a) Purified B cells: markers in histograms differentiate apoptotic [annexin V-fluorescein isothiocyanate (FITC)-positive] from viable (annexin V-FITC-negative) cells on propidium iodide-free CD27⁻ (upper panel) or CD27⁺ (lower panel) gated cells. Histograms show a representative experiment from a healthy control (upper row) and CVID MB0 (middle row) and CVID MB1 (lower row) patients. (b–c) Sorted cells: CD27 expression (upper dot-plots rows) and apoptosis (lower histograms rows) of propidium iodide-free CD27⁻ (b) or CD27⁺ (c) sorted B cells. Dot plots and histograms are representative from three independent experiments performed on healthy controls.

groups and controls. The Wilcoxon test was used to compare differences between two paired groups of treatments (each stimulus with or without IL-21). A statistical test based on measures of central tendency comparison was not applicable to the particular case of anti-IgM combined with IL-21. A *P*-value less than 0·05 was considered statistically significant.

Results

CD27⁺ B cells are less sensitive to apoptosis rescue by single stimulus than CD27⁻ B cells

B cells die from apoptosis if maintained unstimulated in culture [31]. After 3 days, spontaneous apoptosis was higher in CD27⁺ than in CD27⁻ B cells (79·2 *versus* 57·6%, $P < 0{\cdot}001$) (Fig. 2a).

When B cells are stimulated, they are rescued from apoptosis. The effectiveness of the rescue depends upon both the kind of stimulus used and the subpopulation of B cells. For CD27⁻ B cells, the strongest rescue effect was induced by anti-CD40 followed by CpG-ODN and to a lesser extent by anti-IgM, whereas for CD27⁺ B cells, CpG-ODN appeared to be the strongest rescue stimulus (Fig. 2b). Nevertheless, all the stimuli evaluated were more efficient in the CD27⁻ than in the CD27⁺ population: anti-CD40 (77·9 *versus* 23·9%, $P < 0{\cdot}001$), CpG-ODN (71·4 *versus* 57·3%, $P < 0{\cdot}01$) and anti-IgM (52·7 *versus* 36·9%; $P < 0{\cdot}01$) (Fig. 2b).

Proliferation was evaluated simultaneously. Anti-CD40 and anti-IgM did not induce proliferation of either CD27⁻ or CD27⁺ B cells while CpG-ODN induced proliferation of both subpopulations (Table 2). Although CpG-ODN induced a lower level of proliferation on CD27⁻ than CD27⁺ B cells (PI = 0·1 *versus* PI = 1·8, respectively; $P < 0{\cdot}001$) (Table 2), it induced higher rescue from apoptosis in the CD27⁻ population (Fig. 2b). These aforementioned results suggest that proliferation and rescue from apoptosis are two independent processes.

CD27⁻ and CD27⁺ B cells from CVID MB0 patients are less sensitive to apoptosis rescue by single stimulus

CD27⁻ B cells from CVID MB0 patients were less sensitive to rescue from apoptosis when stimulated with a T-dependent stimulus (anti-CD40) than control subjects (65·4 *versus* 77·9%, $P < 0{\cdot}05$) (Fig. 3a). They were also less sensitive to rescue from apoptosis when stimulated with a T-independent stimulus (CpG-ODN) than control subjects or CVID MB1 patients, although differences did not reach statistical significance (58·8 *versus* 71·4 and 63·0%, respectively, $P = 0{\cdot}075$). CD27⁻ B cells from CVID MB1 patients were rescued from apoptosis similarly to controls, regardless of the stimulus used (Fig. 3a). After BCR engagement with anti-IgM CD27⁻ B cells from both CVID MB0 and MB1, patients were rescued equally from apoptosis than healthy controls.

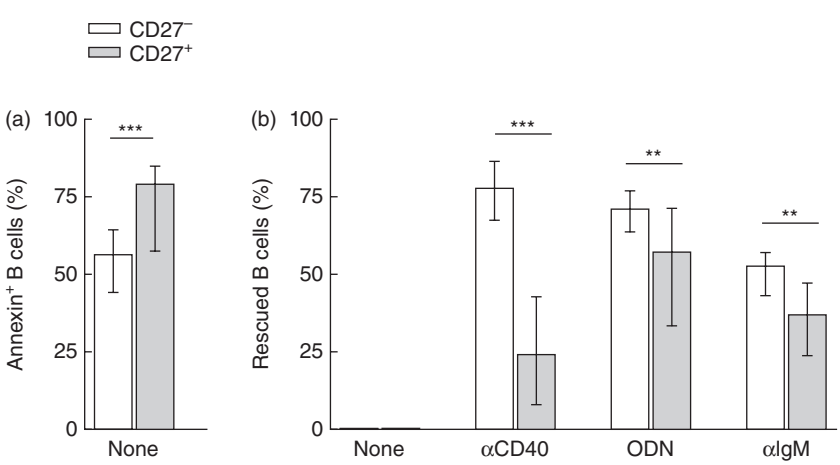

Fig. 2. Spontaneous apoptosis and anti-CD40, cytosine–phosphate–guanosine oligodeoxynucleotides (CpG-ODN) or anti-immunoglobulin (Ig)M activation-induced rescue from apoptosis of peripheral CD27⁻ and CD27⁺ B cells from healthy controls. (a) Percentage of apoptotic [annexin V-fluorescein isothiocyanate (FITC)-positive/propidium iodide (PI)-negative) CD27⁻ (white bars) and CD27⁺ (grey bars) unstimulated B cells. (b) Percentage of rescued CD27⁻ and CD27⁺ B cells upon stimulation with anti-CD40, CpG-ODN or anti-IgM. Data are given as median and 25th to 75th percentiles from 22 independent experiments (Mann–Whitney *U*-test *P*-values: $P < 0{\cdot}01^{**}$; $P < 0{\cdot}001^{***}$).

Table 2. Effect of interleukin (IL)-21 on CD27$^-$ and CD27$^+$ B cell proliferative responses.

	CD27$^-$ B cells			CD27$^+$ B cells			CD27$^-$ *versus* CD27$^+$ *P*-value	
	Lone	+ IL-21	*P*-value	Lone	+ IL-21	*P*-value	Lone	+ IL-21
Anti-IgM	0·0	0·0	n.s.	0·0	0·0	n.s.	n.s.	n.s.
Anti-CD40	0·0	0·4	***	0·0	1·0	***	n.s.	###
CpG-ODN	0·1	0·2	**	1·8	1·1	***	###	###

Proliferation index after anti-immunoglobulin (Ig)M, anti-CD40 or cytosine–phosphate–guanosine oligodeoxynucleotides (CpG-ODN) activation with or without IL-21 co-stimulation in CD27$^-$ and CD27$^+$ B cells from healthy controls ($n = 19$). Proliferation index was calculated as described in the flow cytometry section and takes into account both the number of divisions and the percentage of cells in each round of division. Wilcoxon's test (left grid) was applied to evaluate the effect of IL-21 addition to each stimulus on CD27$^-$ or CD27$^+$ B cells (*P* values: $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$; n.s.: not significant). Mann-Whitney test (right grid) was used to compare differences between B cell subpopulations proliferative responses to single or IL-21-combined stimuli (*P*-values: $^{\#\#\#}P < 0{\cdot}001$; n.s.: not significant).

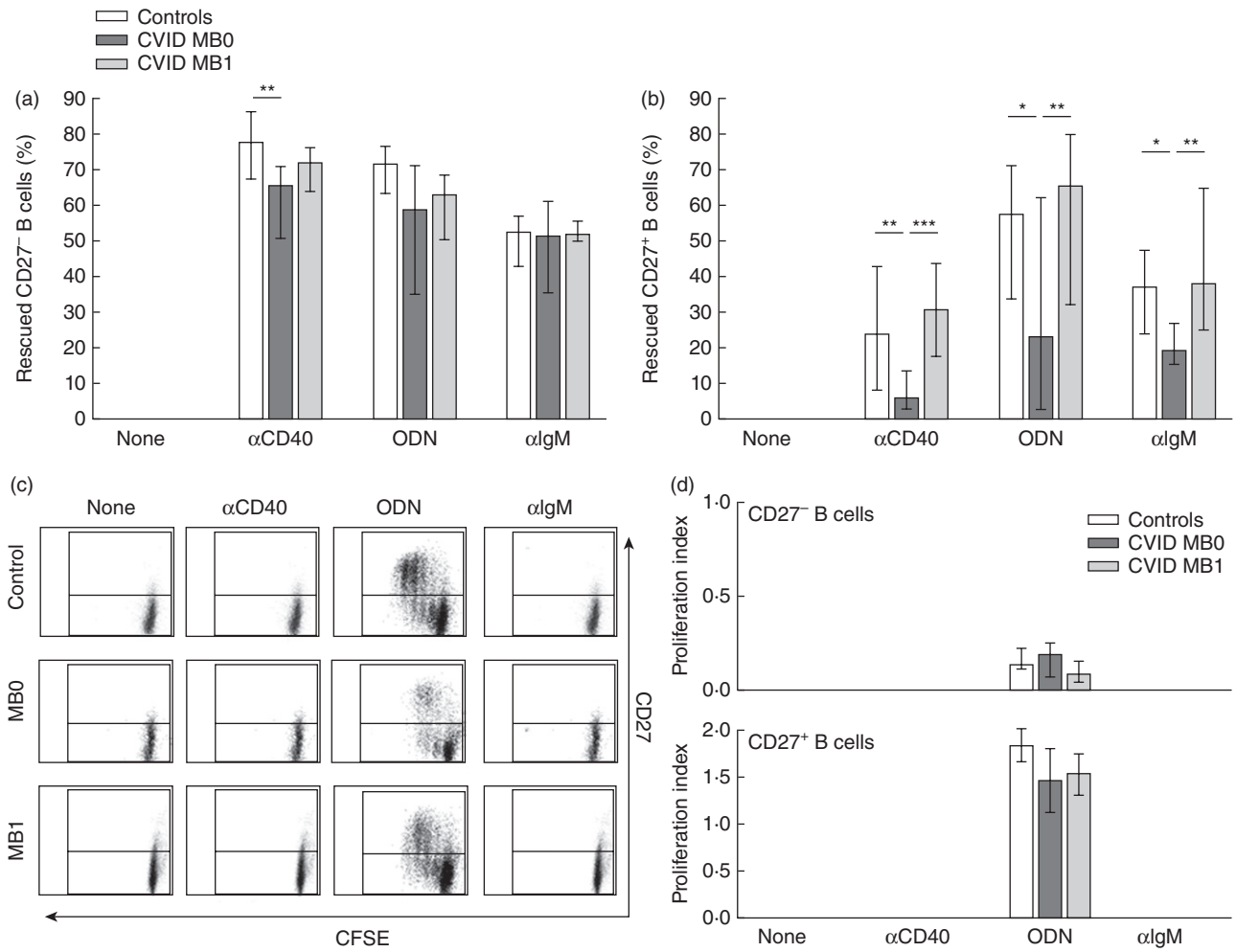

Fig. 3. Activation-induced rescue from apoptosis and proliferation of peripheral CD27$^-$ and CD27$^+$ B cells from common variable immunodeficiency (CVID) patients and healthy controls. (a) Percentage of rescued CD27$^-$ and (b) CD27$^+$ B cells upon stimulation with anti-CD40, cytosine–phosphate–guanosine oligodeoxynucleotides (CpG-ODN) or anti-immunoglobulin (Ig)M in healthy controls ($n = 22$; white bars), CVID MB0 patients ($n = 12$; dark grey bars) and CVID MB1 patients ($n = 8$; light grey bars). (c) Representative dot-plots with dividing and non-dividing CD19$^+$CD27$^-$carboxyfluorescein succinimidyl ester (CFSE)$^+$ (lower quadrants) and CD19$^+$CD27$^+$CFSE$^+$ (upper quadrants) B cells from a healthy control (upper row), one CVID MB0 patient (middle row) and one CVID MB1 patient (lower row) after stimulation with anti-CD40, CpG-ODN or anti-IgM. (d) Proliferation index after anti-CD40, CpG-ODN or anti-IgM activation of CFSE-labelled CD27$^-$ (upper panel) and CD27$^+$ (lower panel) B cells from healthy controls ($n = 19$; white bars), CVID MB0 patients ($n = 8$; dark grey bars) and CVID MB1 patients ($n = 6$; light grey bars). No proliferation was detected with anti-CD40 and anti-IgM. Data are given as median and 25th to 75th percentiles (Kruskal–Wallis test *P*-values: $P < 0{\cdot}05^*$; $P < 0{\cdot}01^{**}$; $P < 0{\cdot}001^{***}$).

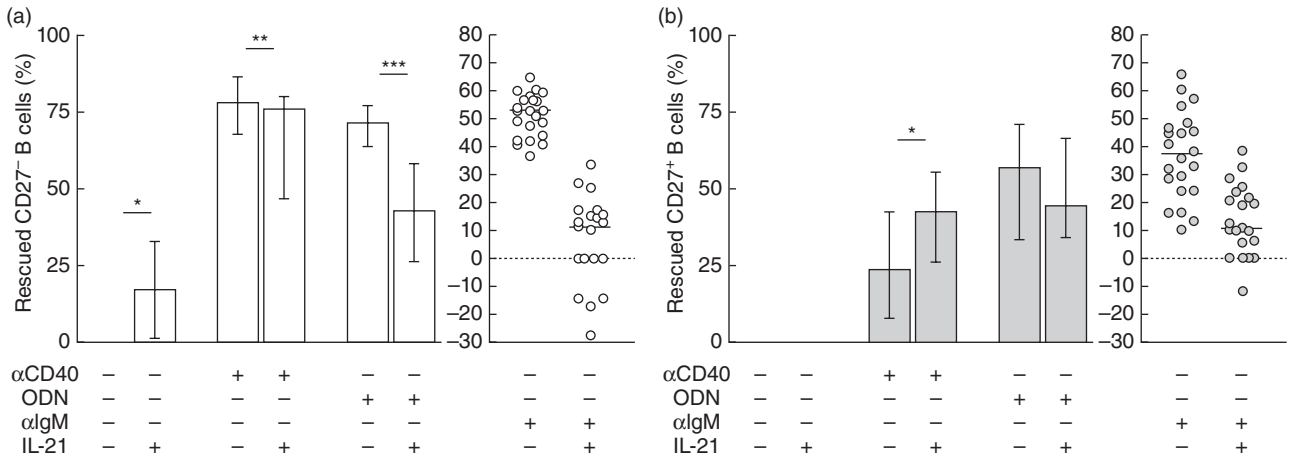

Fig. 4. Interleukin (IL)-21 effect on spontaneous apoptosis and activation-induced rescue from apoptosis of peripheral CD27$^-$ and CD27$^+$ B cells from healthy controls. (a) Percentage of rescued CD27$^-$ and (b) CD27$^+$ B cells upon stimulation with IL-21 alone or in combination with anti-CD40, cytosine–phosphate–guanosine oligodeoxynucleotides (CpG-ODN) or anti-immunoglobulin (Ig)M. Data are given as median and 25th to 75th percentiles from 22 independent experiments (Wilcoxon's test *P*-values: $P < 0{\cdot}05$*; $P < 0{\cdot}01$**; $P < 0{\cdot}001$***).

CD27$^+$ B cells from CVID MB0 patients, stimulated with either a T-dependent (anti-CD40) or a T-independent stimulus (CpG-ODN), were less sensitive to apoptosis rescue than control subjects (6·0 *versus* 23·9%, $P < 0{\cdot}01$; and 23·2 *versus* 57·3%, $P < 0{\cdot}05$, respectively) and CVID MB1 patients (6·0 *versus* 30·6%, $P < 0{\cdot}001$; and 23·2 *versus* 65·7%, $P < 0{\cdot}01$, respectively). They were also less sensitive to rescue from apoptosis after BCR engagement with anti-IgM than control subjects (19·2 *versus* 36·9%, $P < 0{\cdot}05$) or CVID MB1 patients (19·2 *versus* 38·2%, $P < 0{\cdot}01$) (Fig. 3b). With either stimulus CD27$^+$ B cells from CVID MB1 patients were rescued from apoptosis similarly to controls (Fig. 3b).

Thus, CD27$^+$ B cells from CVID MB0 patients appear to be resistant to apoptosis rescue irrespective of the stimulus. This was not linked to differences in proliferation because both CD27$^-$ and CD27$^+$ B cells from CVID MB0 patients proliferated similarly to controls and CVID MB1 patients (Fig. 3c,d).

IL-21 modulates apoptosis rescue of co-stimulated CD27$^-$ and CD27$^+$ B cells

IL-21 alone was able to rescue CD27$^-$ (16·9%) but not CD27$^+$ B cells from spontaneous apoptosis (Figs 1a and 4). In spite of this, the addition of IL-21 down-modulated the protective effect of anti-CD40 (77·9 *versus* 75·9%, $P < 0{\cdot}01$) and CpG-ODN (71·4 *versus* 42·7%, $P < 0{\cdot}001$) on CD27$^-$ B cells.

In CD27$^+$ B cells IL-21 tended to reduce the CpG-ODN rescue effect but increased the protective effect of anti-CD40 significantly (23·9 *versus* 42·8%, $P < 0{\cdot}05$) (Figs 1a and 4b).

IL-21 not only reverted the protective effect of anti-IgM on CD27$^-$ and CD27$^+$ B cells, but in some cases even increased apoptosis above spontaneous baseline values (Fig. 1a and scatter-plots in Fig. 4).

Similar results were obtained when we evaluated activation induced rescue from apoptosis on sorted CD27$^-$ and CD27$^+$ B lymphocytes stimulated with the same stimuli (histograms in Fig. 1b,c). Moreover, we did not find increased CD27 expression when we stimulated CD27$^-$ B cells with any of the stimuli (dot-plots in Fig. 1b), which validates the gating strategy when using purified total B cells.

Effect of IL-21 on apoptosis is not linked to proliferation

IL-21 modulates proliferation induced by co-stimulation on CD27$^-$ and CD27$^+$ B cells. This effect has to be taken into account when analysing the apoptosis rate.

Neither CD27$^-$ nor CD27$^+$ B cells proliferated in response to anti-IgM combined with IL-21 (Table 2). However, both subpopulations proliferated in response to IL-21 with anti-CD40, although the proliferation index was higher in CD27$^+$ B cells. Remarkably, IL-21 increased proliferation of CpG-ODN-activated CD27$^-$ B cells but decreased proliferation of CpG-ODN-activated CD27$^+$ B cells (Table 2).

In CD27$^+$ B cells, IL-21 reduction of CpG-ODN apoptosis rescue is accompanied by a reduction in the proliferative response. In contrast, the increase in anti-CD40 apoptosis rescue is accompanied by a proliferation enhancement (Fig. 4b and Table 2). However, IL-21 reduction in apoptosis rescue induced by anti-CD40 or CpG-ODN on CD27$^-$ B cells is not due to a negative effect on proliferation (Fig. 4a and Table 2). Furthermore, in spite of the higher proliferative response induced by IL-21 combined with anti-CD40 or CpG-ODN on CD27$^+$ *versus* CD27$^-$ B cells

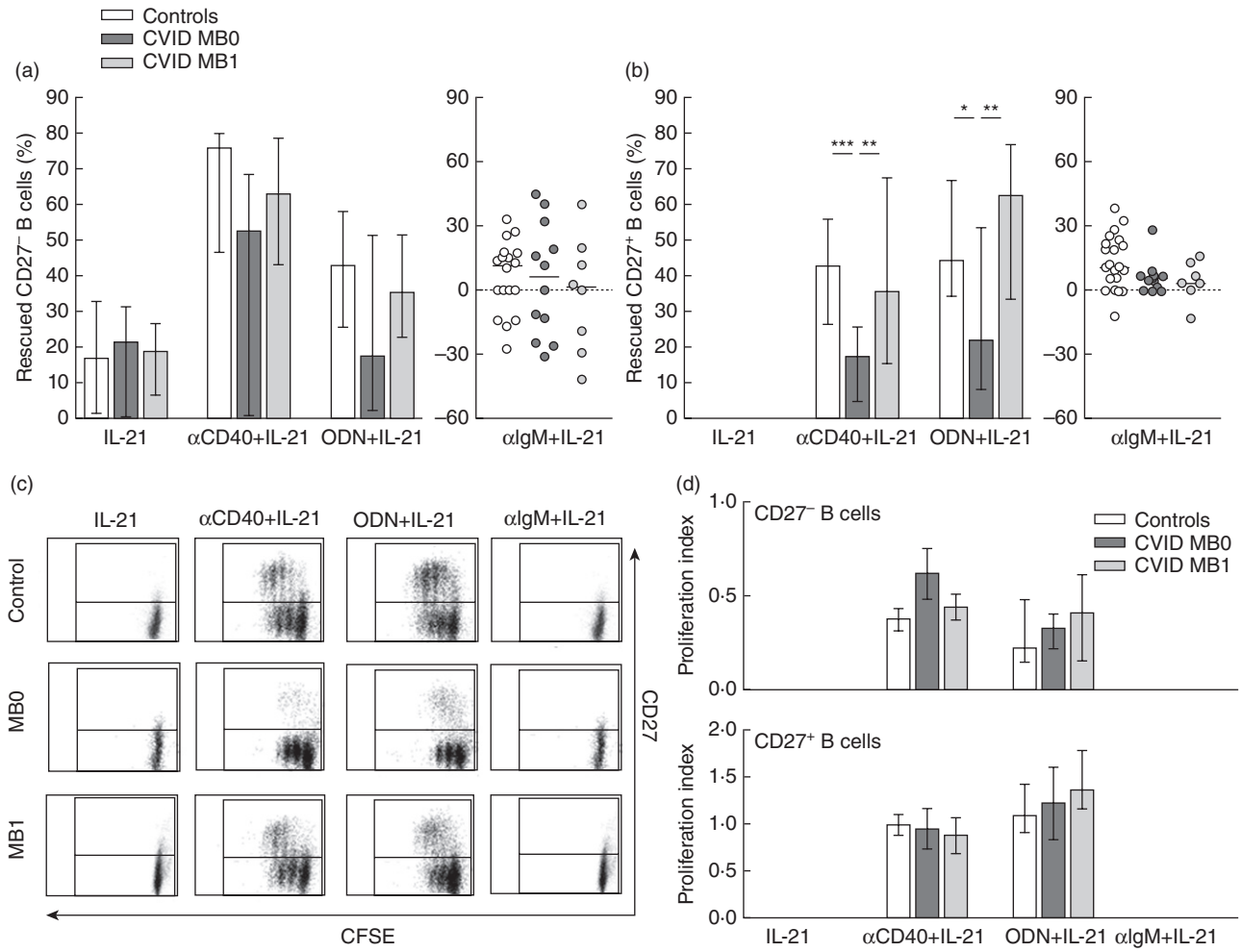

Fig. 5. Interleukin (IL)-21 effect on apoptosis rescue and proliferation of activated peripheral $CD27^-$ and $CD27^+$ B cells from common variable immunodeficiency (CVID) patients. (a) Percentage of rescued $CD27^-$ and (b) $CD27^+$ B cells upon stimulation with IL-21 alone or in combination with anti-CD40, cytosine–phosphate–guanosine oligodeoxynucleotides (CpG-ODN) or anti-immunoglobulin (Ig)M in healthy controls ($n = 22$; white bars/dots), CVID MB0 patients ($n = 12$; dark grey bars/dots) and CVID MB1 patients ($n = 8$; light grey bars/dots). (c) Representative dot-plots of dividing and non-dividing $CD19^+CD27^-$carboxyfluorescein succinimidyl ester $(CFSE)^+$ (lower quadrants) and $CD19^+CD27^+CFSE^+$ (upper quadrants) B cells from a healthy control (upper row), one CVID MB0 (middle row) and one MB1 (lower row) patient, after stimulation with IL-21 alone or in combination with anti-CD40, CpG-ODN or anti-IgM. (d) Proliferation index of CFSE-labelled $CD27^-$ (upper panel) and $CD27^+$ (lower panel) B cells from healthy controls ($n = 19$; white bars), CVID MB0 ($n = 8$; dark grey bars) and MB1 ($n = 6$; light grey bars) patients after stimulation with IL-21 alone or in combination with anti-CD40, CpG-ODN or anti-IgM. No proliferation was detected with IL-21 and anti-IgM + IL-21. Data are given as median and 25th to 75th percentiles (Kruskal–Wallis test *P*-values: $P < 0{\cdot}05$*; $P < 0{\cdot}01$**; $P < 0{\cdot}001$***).

(Table 2), the rescue from apoptosis is not higher in $CD27^+$ B cells for any of the stimulus (Fig. 4). Thus, although we cannot rule out that the effect of IL-21 on apoptosis is linked to proliferation, our results support the independence of these processes.

$CD27^-$ and $CD27^+$ B cells from CVID MB0 patients are less sensitive to apoptosis rescue by IL-21 combined with a single stimulus

IL-21 alone rescued both CVID MB0 and MB1 $CD27^-$ B cells similar to controls. $CD27^-$ B cells from CVID MB0 were less sensitive to apoptosis rescue by anti-CD40 plus IL-21 or CpG-ODN plus IL-21 than controls or CVID MB1 patients, although these differences were not statistically significant (Fig. 5a).

$CD27^+$ B cells from CVID MB0 patients were less sensitive to apoptosis rescue when stimulated with anti-CD40 and IL-21 or CpG-ODN and IL-21 than control subjects (17·6 *versus* 42·8%, $P < 0{\cdot}001$; and 21·9 *versus* 44·4%, $P < 0{\cdot}05$, respectively) and CVID MB1 patients (17·6 *versus* 35·8%, $P < 0{\cdot}01$; and 21·9 *versus* 62·5%, $P < 0{\cdot}01$, respectively).

$CD27^-$ and $CD27^+$ B cells from CVID MB1 (Fig. 5b) patients were rescued from apoptosis similarly to controls.

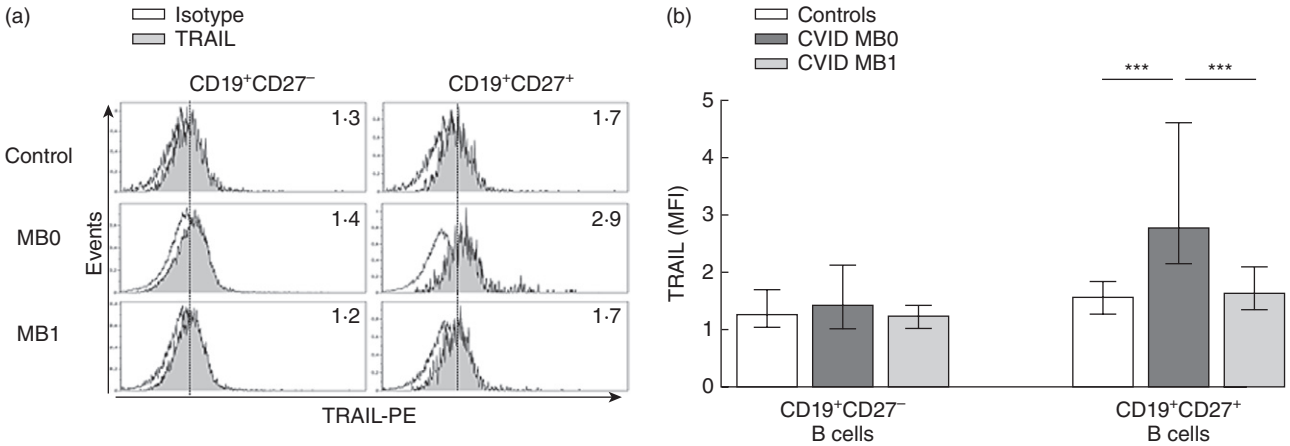

Fig. 6. Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) surface expression on peripheral blood B cells from common variable immunodeficiency (CVID) patients and healthy controls. (a) Representative histograms of TRAIL expression (grey) on $CD19^+CD27^-$ (left columns) and $CD19^+CD27^+$ (right columns) B cells from a healthy control (upper row), one CVID MB0 (middle row) and one CVID MB1 (lower row) patients. Numbers indicate TRAIL median fluorescence intensity (MFI) evaluated on a 3-decade log scale. (b) TRAIL MFI of $CD19^+CD27^-$ and $CD19^+CD27^+$ B cells from healthy controls ($n = 18$; white bars), CVID MB0 ($n = 9$; dark grey bars) and MB1 ($n = 6$; light grey bars) patients. Data are given as median and 25th to 75th percentiles (Kruskal–Wallis test *P*-values: $P < 0{\cdot}001$***).

IL-21 not only abrogated the protective effect induced by anti-IgM, but increased the percentage of apoptotic B cells both in controls and CVID patients irrespective of their group (Fig. 5a,b).

When we evaluated the proliferation index, we did not find differences between CVID patients and controls (Fig. 5c,d). Thus, again, differences of apoptosis rescue between $CD27^+$ B cells from CVID MB0 patients and controls cannot be attributed to differences on B cell proliferation (Fig. 5).

$CD27^+$ B cells from MB0 patients show higher TRAIL expression than controls and MB1 patients

Higher expression of TRAIL has been related to apoptosis and loss of peripheral memory B cells (identified as $CD27^+$) in successfully treated aviraemic HIV patients. We evaluated if differences in TRAIL expression on $CD27^+$ B cells from CVID MB0 patients could explain the observed resistance to apoptosis rescue. $CD27^-$ B cells from CVID MB0 and MB1 patients showed similar TRAIL expression than controls (Fig. 6). However, $CD27^+$ B cells from CVID MB0 patients showed higher TRAIL expression than controls (2·8 *versus* 1·6 MFI; $P < 0{\cdot}001$) or MB1 patients (2·8 *versus* 1·7 MFI, $P < 0{\cdot}001$). We did not find differences in $CD27^+$ B cells from CVID MB1 when compared to controls (Fig. 6).

Discussion

The B cell fate is determined by the nature of the antigen encountered and a combination of signals provided through membrane co-receptors or by secreted interleukins encountered in the lymphoid compartment. Unsuccessfully stimulated B cells die from apoptosis. Survival, growth and differentiation signals are required to maintain B cell homeostasis and to induce their differentiation into effector subsets.

In this study, we show that $CD27^+$ B cells are less sensitive to rescue from apoptosis than $CD27^-$ B cells, irrespective of the stimulus used. Although IL-21 rescues unstimulated $CD27^-$ B cells from spontaneous apoptosis and increases the protective effect of anti-CD40 in $CD27^+$ B cells, it reduces the protective effect of most stimuli used in both $CD27^-$ and $CD27^+$ B cells. When we evaluate CVID patients, we observe that $CD27^+$ B cells from MB0 patients are less sensitive to rescue from apoptosis than B cells from MB1 patients and normal controls after anti-CD40 or CpG-ODN stimulation. These differences are not restored by the addition of IL-21. This is in agreement with the higher TRAIL expression observed in CVID MB0 patients.

The effect of IL-21, one of the most important cytokines for B cell differentiation, depends upon the subset of B cell studied and accompanying co-stimulus [28]. Jin *et al.* [32] demonstrated that besides strain differences in mice, the context in which B cells were activated influenced their fate. IL-21-driven apoptosis and inhibition of proliferation were dominant when B cells were activated through TLR-4 and TLR-9. Co-stimulation and low apoptosis were observed in B cells stimulated with anti-IgM or anti-IgM plus anti-CD40, whereas both apoptosis and co-stimulation were detected when IL-21 acted on anti-CD40 previously activated B cells. This raised the possibility that different subsets of B cells responded differentially to IL-21. In our hands, although IL-21 rescues unstimulated $CD27^-$ B cells

from spontaneous apoptosis, it reduces the protective effect of most of the stimuli both in $CD27^-$ and $CD27^+$ B cells. On the contrary, IL-21 increases the protective effect of anti-CD40 in $CD27^+$ B cells. This suggests that IL-21 *per se* increases survival of $CD27^-$ (mostly naive and transitional) B cells, but this effect is lost after these cells are activated. However, $CD27^+$ B cells become sensitive to rescue from apoptosis if they are prestimulated with a surrogate T-dependent stimulus (anti-CD40). Stimulation through the BCR or with a T-independent stimulus (CpG-ODN) renders $CD27^+$ B cells insensitive to the protective effect of IL-21. IL-21 acts as a checkpoint for a productive B cell response. Only memory and marginal zone B cells (contained in the $CD27^+$ population) that receive cognate T cell help in the presence of IL-21 would be protected from apoptosis and directed to proliferation and eventually differentiation to antibody secreting cells. We also report that rescue from apoptosis is independent of proliferation. This is particularly evident with anti-CD40 that, although it does not induce proliferation, it rescues most $CD27^-$ B cells from apoptosis.

Our present results support that the inability of CVID B cells to produce normal levels of immunoglobulins *in vitro* (and *in vivo*) can be the consequence of an increased susceptibility to apoptosis upon stimulation. That would result in a reduced number of cells during an immune response. $CD27^-$, but particularly $CD27^+$ B cells, from our CVID MB0 patients are less sensitive to rescue from apoptosis than MB1 patients and controls.

Moreover, $CD27^+$ B cells from CVID MB0 patients showed significantly higher expression of TRAIL than controls or CVID MB1 patients. TRAIL is a member of the TNF superfamily of cytokines able to induce programmed cell death in tumour cells. Different subpopulations of B cells show distinct sensitivity to TRAIL-mediated apoptosis. BCR triggering sensitizes peripheral blood memory, but not naive human B cells, to TRAIL-mediated apoptosis [33] and TRAIL promotes death of normal plasma cells [34]. In agreement with our results, van Grevenynghe *et al.* [13] demonstrated that memory B cell survival was decreased significantly in aviraemic successfully treated (ST) HIV subjects compared with uninfected controls. Memory B cells (identified as $CD19^+CD27^+$) from ST subjects showed specifically higher expression levels of TRAIL that correlated negatively with the frequency of these cells. Production of immunoglobulins was lower in ST subjects as a result of reduced survival and not lower proliferation of B cells.

Increased apoptosis of B cells in the MB0 group can result in fewer cells developing into antibody-secreting cells upon stimulation, hypogammaglobulinaemia and poor humoral response to antigens. For CVID MB1 patients a different mechanism should be responsible, because their B cells behave like control B cells in their sensitivity to apoptosis. This holds true for the two evaluated CVID MB2 patients. Their B cell apoptosis rescue was similar to CVID MB1 patients and controls (data not shown). In a recent paper, Borte *et al.* [35] suggested that IL-21 restores immunoglobulin production in patients with CVID. Using purified B cells, they found that IL-21 reduced apoptosis from naive and memory B cells from 14 CVID patients. However, no CVID group distinction was made; stimulation with anti-CD40 and IL-21 also included IL-4, and they considered only the $CD27^-$ naive and $CD27^+$ IgD^- memory B cell populations (excluding $CD27^+IgD^+$). The proportion of MB1/MB2 to MB0 patients in their studied cohort might have influenced the final result and explain the apparently distinct conclusions.

We cannot exclude the possibility that the peripheral blood B cells with increased apoptosis found in CVID MB0 could be the result of incomplete activation by follicular $CD4^+$ T cells. In keeping with this, Hagn *et al.* [36] have demonstrated that human B cells co-cultured with incompletely activated CD4 T cells that secrete IL-21, but do not express CD40L, differentiate into granzyme B (GzmB)-secreting and potentially cytotoxic cells, able to induce slowly developing apoptosis of several cell lines. Activation of human B cells by IL-21 and BCR engagement in the absence of CD40 ligation results in their differentiation into GzmB-secreting cytotoxic cells rather than into plasma cells.

In summary, our findings reinforce the fact that (in humans) the net effect of different stimuli on B cells depends upon both the B cell subpopulation studied and the activation status of the B cell and underscore the relevance of these features in CVID physiopathology. We suggest that higher levels of apoptosis of CVID MB0 $CD27^+$ B cells during an immune response can result in lower levels of immunoglobulin production, irrespective of their proliferation. The results highlight the heterogeneity among CVID patients, where distinct molecular mechanisms underlie common clinical symptoms, and highlight the need to classify and study CVID patients separately when evaluating B cell responses.

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Disclosure

None of the authors has any potential financial conflict of interest related to this manuscript.

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