

Defective calcium signaling and disrupted CD20–B-cell receptor dissociation in patients with common variable immunodeficiency disorders

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Background: B cells of patients with common variable immunodeficiency (CVID) disorders display impairment in production of immunoglobulin class-switched antibodies, which is possibly contributed to by defects in early B-cell activation. On resting B cells, B-cell receptors (BCRs) are organized in oligomers that are signaling inactive. Their triggering by cognate antigen causes the lateral reorganization of BCRs and associated proteins into signalosomes, resulting in BCR-activated calcium entry. In resting cells the B-cell surface antigen CD20 is associated with the BCR but dissociates on signalosome formation.

Objective: We sought to determine whether CD20 dissociation from the BCR during early B-cell activation might contribute to the development of CVID disorders.

Methods: We evaluated BCR signalosome formation, internalization, and signaling in primary B cells of pediatric patients with CVID disorders and healthy control subjects.

Results: In many pediatric patients with CVID disorders, B cells exhibit significant deficits in BCR triggering-mediated calcium entry in the cytosol, which correlates with impaired plasmablast differentiation *in vitro*. These alterations did not originate from upregulation of CD22 or defects in calcium channels and did not involve gene mutations in phospholipase C γ 2 or Bruton tyrosine kinase. Instead, B cells from patients with CVID disorders exhibited reduced BCR dissociation from CD20. BCR or CD20 cross-linking induced less BCR internalization, and antibody-mediated CD20 triggering elicited less BCR downstream signaling, as measured based on secondary fluxes.

Conclusions: We propose that CD20 dissociation from the BCR signalosome is pivotal to BCR-mediated calcium mobilization in the cytosol. Defects in CD20/BCR signalosome conformation might predispose to the spectrum of CVID disorders. (*J Allergy Clin Immunol* 2011;■■■:■■■-■■■.)

Key words: B cells, common variable immunodeficiency, CD20, B-cell receptor

Common variable immunodeficiency (CVID) is a heterogeneous immunodeficiency characterized by defective antibody production.¹ Sporadic gene mutations have been described,² but the cause remains unknown for 90% of patients.³ Deficiency of CD81,⁴ CD21,⁵ and CD20⁶ led to CVID-like disorders with hypogammaglobulinemia and antibody synthesis deficits. Mutations in the B-cell receptor (BCR) coreceptor complex combined with abrogated B-cell effector functions suggested the possibility of defective B-cell activation in patients with CVID or CVID-like disorders.

B cells express up to 120,000 inactive BCRs.⁷ Ligation of cognate antigen induces the reorganization of signaling-active BCR oligomers, or signalosomes, that provoke increases in intracellular calcium ion concentrations.⁸ Binding of the coreceptor complex (CD19, CD21, CD81, and associated proteins) results in CD19 phosphorylation and recruitment of Src family tyrosine kinases and phosphoinositide 3 kinase. This results in activation of phospholipase C γ 2 (PLC γ 2), which, through generation of inositol 1,4,5-trisphosphate 3 (IP₃) and binding to IP₃ receptors at the endoplasmic reticulum (ER) membrane, facilitates calcium release from ER stores into the cytoplasm. Consequently, calcium enters the cell through store-operated calcium entry channels. We proposed that early defects in BCR-mediated calcium signaling might cause B-cell dysfunction in patients with CVID disorders. We investigated CD20, which is almost exclusively expressed on B cells.⁹ Although its role in B-cell function is not fully clear, CD20 is a direct regulator or component of a calcium channel.^{10,11} CD20 forms homo-oligomers that physically associate with the BCR,¹² and CD20 antibody treatment induces BCR-mediated signal transduction, including calcium flux, resulting in similar transcription patterns as seen with BCR triggering.^{13,14} After BCR ligation, CD20 oligomers dissociate and remain on the cell surface, whereas the BCR and its accessory molecules are endocytosed.^{12,15} CD20 deficiency causes CVID-like disease.⁶ Furthermore, B cells of a subgroup of patients with CVID exhibit defects in calcium influx induced by BCR signaling.⁵

We here report defective BCR-mediated calcium signaling in B cells of numerous pediatric patients with CVID disorders. Afflicted patients exhibited a reduction in cytosolic calcium mobilization on BCR cross-linking. Rates of calcium mobilization in fresh B cells from patients with CVID disorders correlated with B-cell differentiation efficiency *in vitro*. Although it had been known that B-cell activation induces plasma membrane

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Abbreviations used

AUC: Area under the curve
 BCR: B-cell receptor
 Btk: Bruton tyrosine kinase
 CVID: Common variable immunodeficiency
 ER: Endoplasmic reticulum
 IP₃: Inositol 1,4,5-trisphosphate 3
 LCL: Lymphoblastoid cell line
 NFAT: Nuclear factor of activated T cells
 PLCγ2: Phospholipase Cγ2

dissociation of the BCR from CD20 for induction of calcium flux, this phenomenon had not yet been associated with human disease. We show defects in BCR internalization and calcium mobilization in our cohort of patients with CVID disorders when induced by cross-linking of either CD20 or BCRs. Thus in selected patients CVID disorders might involve defects in dissociation of the BCR from surface-retained CD20 during BCR-mediated B-cell activation.

METHODS**Patients and healthy donors**

Forty-two pediatric patients (mean age, 12 years; range, 4–18 years) with CVID disorders, 14 healthy children (mean age, 9 years; range, 4–13 years), and 23 adult volunteers (mean age, 29 years; range, 23–40 years) were included; detailed information is available in the [Methods](#) section in this article's Online Repository at www.jacionline.org. This study was approved by the institutional review board, and informed consent was obtained.

Flow cytometry and functional assays

In addition to previously described phenotyping,^{16,17} PBMCs were stained with antibodies to CD19, CD20, CD21, CD22, CD27, CD38, CD45 (all from Becton Dickinson, Franklin Lakes, NJ), IgM F(ab')₂, and Igλ F(ab')₂ (Southern Biotechnology Associates, Birmingham, Ala). Samples were acquired on a FACS Canto II Flow Cytometer and analyzed with FACS Diva software (both from Becton Dickinson).

For calcium flux assays, one million fresh PBMCs were loaded directly with 4 μmol/L Fluo-3 and 10 μmol/L Fura Red (Invitrogen, Carlsbad, Calif), and later, CD19–allophycocyanin was added. Cells were washed twice and resuspended in Hank balanced salt solution or Ca²⁺-free PBS, both of which were supplemented with FCS. After measuring baseline cytosolic calcium levels, 20 μg/mL anti-IgM F(ab')₂ fragments (Jackson ImmunoResearch, West Grove, Pa) were added, and calcium influx was measured for 5 minutes. Subsequently, 2 μg/mL ionomycin (Calbiochem, San Diego, Calif) was added. PBMCs were incubated with 10 μg/mL anti-CD20 mAb rituximab (Roche Diagnostics, Mannheim, Germany)¹⁸ for 30 minutes to study CD20. EBV-transformed lymphoblastoid cell lines (LCLs) were generated.¹⁹ ER Ca²⁺ storage capacity was assessed with ionomycin (0.1 μg/mL) or thapsigargin (10 μmol/L; Santa Cruz Biotechnology, Santa Cruz, Calif) in Ca²⁺-free medium. B-lymphocyte differentiation assays were performed, as described previously.¹⁶

Confocal microscopy

LCLs from control subjects and patients with CVID disorder were sorted for high expression of IgM and CD20. Two million B cells were adhered on a cover slip coated with 1% Alcian blue (Klinipath bv, Geel, Belgium). Cells were stained at 0°C for IgM BCR and CD20 with 1:100 anti-human IgM-Cy3 F(ab')₂ fragments (Jackson ImmunoResearch) and 1:10 anti-human 2H7-Alexa Fluor 647 (BioLegend, San Diego, Calif). Cells were washed 3 times and resuspended in RPMI 1640 without phenol red containing 10% FCS and 10 mmol/L HEPES buffer. Cells were kept at 0°C and imaged on a 710

Laser Scanning Microscope Meta (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany). BCR stimulation was initiated by quickly increasing the temperature to 37°C and supplementing additional anti-IgM. Imaging was continued for 30 minutes at 37°C. By using ZEN 2009 software (Carl Zeiss MicroImaging), the percentage of colocalization was calculated, as described previously.¹⁵

Statistical analysis

Differences in nonparametric data were assessed by comparing the medians of groups by using Mann-Whitney *U* tests or Kruskal-Wallis tests, followed by *post hoc* Dunn comparisons. The effect of αCD20 was assessed with paired *t* tests, and correlations were calculated with Pearson correlation. Tests were performed 2-tailed. Data were analyzed with SPSS 15.0 software for Windows (SPSS, Inc, Chicago, Ill). Areas under the curve (AUCs) were calculated with GraphPad Prism 5.0 software (GraphPad Software, Inc, La Jolla, Calif).

RESULTS**Numbers of B cells from pediatric patients with CVID disorders are reduced in memory subsets but display a normal surface phenotype**

Children with CVID and CVID-like disorders had lower percentages of memory B cells than both control groups (CD27⁺IgM[−] immunoglobulin class-switched memory B cells [median ± interquartile range]: healthy adults, 14.6% ± 15.8%; healthy children, 7.1% ± 5.3%; patients with CVID disorders, 4.4% ± 4.5% [*P* = .01]; CD27⁺IgM⁺ memory cells: healthy adults, 25.5% ± 23.4; healthy children, 11.2% ± 13.3%; patients with CVID disorders, 7.9% ± 9.1% [*P* = .001]). No differences were observed in CD38[−]CD21^{low} B-cell populations between patients and control subjects, although in 2 patients with active autoimmune disease, we noticed expansion of CD21^{low} cells (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org). CD19 and IgM BCR expression on individual B-cell subpopulations were comparable between patients and control subjects (see [Fig E2, A](#), in this article's Online Repository at www.jacionline.org); IgM expression was slightly increased on CD27⁺IgM⁺ B cells in patients with CVID disorders compared with that seen in healthy children (see [Fig E2, B](#)). We observed no significant differences in CD20 and CD22 expression (see [Fig E2, C and D](#)). In line with previous studies, we found no significant differences between patients with CVID and those with CVID-like disorders.¹⁶ In conclusion, all subjects had a substantial IgM⁺ B-cell population that displayed a normal surface phenotype.

Impaired BCR cross-linking–induced calcium mobilization and defective B-cell differentiation *in vitro* in B cells from patients with CVID disorders

We analyzed the ability of fresh B cells to mobilize calcium on activation. Baseline calcium levels of B cells were similar in all groups. On αIgM F(ab')₂-mediated BCR cross-linking, B cells from both children with CVID and children with CVID-like disorders exhibited reduced calcium mobilization, as measured based on AUCs ([Fig 1, A](#); AUC [median ± interquartile range]: healthy adults, 200 ± 221; healthy children, 263 ± 120; patients with CVID, 124 ± 95; and patients with CVID-like disorders, 93 ± 69 [*P* < .001]). Initial peak influx was mainly decreased, whereas further kinetics patterns appeared unaffected. Indeed, calcium ratios (peak/baseline)⁵ were also decreased compared with those seen in control subjects (calcium ratio: healthy adults,

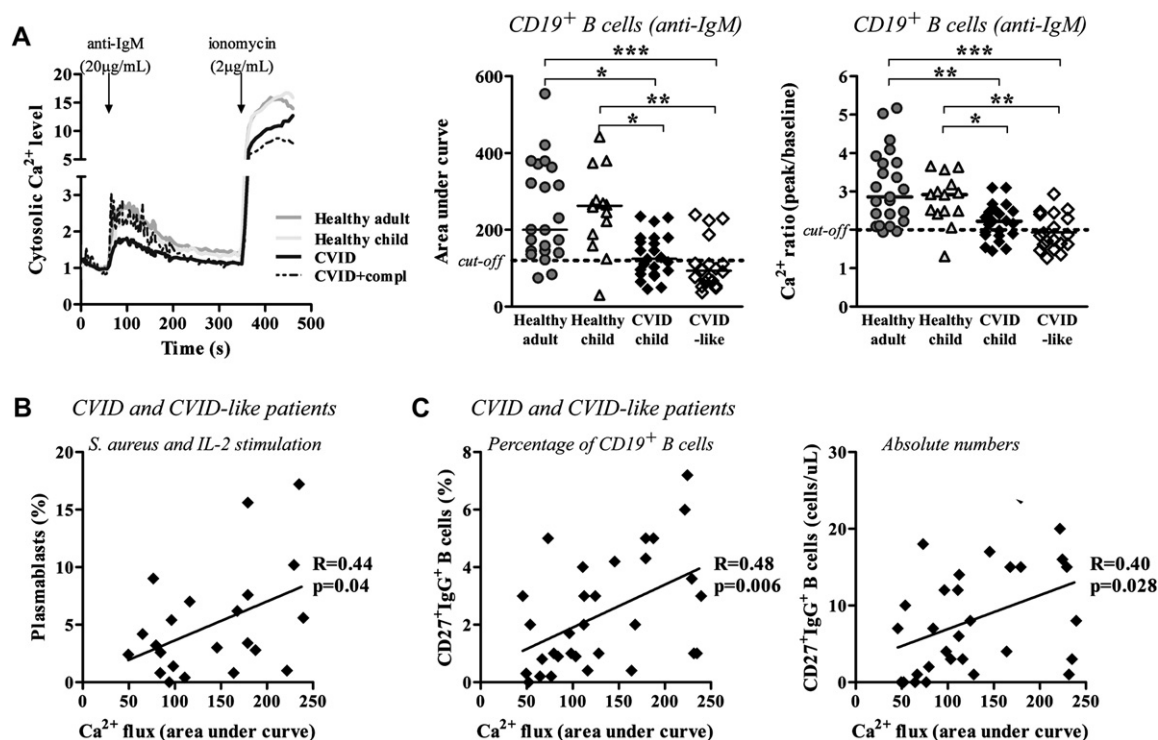


FIG 1. Decreased calcium mobilization in B lymphocytes from patients with CVID disorders. **A**, Ca²⁺ mobilization kinetics (left), AUCs (middle), and ratios (right). Cutoff levels are at a level above which 90% of the healthy control subjects have positive results. **B**, B-cell differentiation correlated with Ca²⁺ mobilization in patients with CVID disorders. **C**, Percentages (left) and absolute numbers (right) of IgG⁺ memory B cells correlated with BCR-mediated calcium influx in age-corrected CVID disorders (n = 30). Pearson correlations: *P < .05, **P < .01, and ***P < .001.

2.9 ± 1.5; healthy children 2.9 ± 0.8; patients with CVID, 2.2 ± 0.6; and patients with CVID-like disorders, 1.9 ± 0.8 [*P* < .001]). We could not classify patients with a decreased calcium flux into certain subgroups but noticed that patients with autoimmunity or lymphoproliferation had relatively high calcium mobilization (Fig 1, A, and see Fig E1, B). Because there were no differences between patients with CVID and patients with CVID-like disorders, they were combined in further experiments and referred to as having CVID disorders.

Restimulation of αIgM F(ab')₂-treated B cells with ionomycin, which acts directly on IP₃ receptors, induced suboptimal subsequent calcium mobilization in B cells from patients with CVID (calcium ratio: healthy adults, 17.2 ± 4.8; healthy children, 19.4 ± 6.6; patients with CVID, 11.5 ± 5.5; and patients with CVID-like disorders, 10.4 ± 10.0 [*P* < .001]). In contrast, CD19⁺ lymphocytes from patients with CVID-like disorders showed fluxes comparable with those seen in control CD19⁺ lymphocytes (data not shown).

We investigated whether decreased calcium mobilization could lead to subsequent B-cell defects. BCR-mediated calcium signals correlated significantly with *in vitro* differentiation into plasmablasts on stimulation with IL-2 and *Staphylococcus aureus* Cowan strain I antigen, which engages the BCR (among other receptors) and elicits a T-independent B-cell response (Fig 1, B),²⁰⁻²² and with proportions of IgG⁺ memory B cells (Fig 1, C, and see Table E1 in this article's Online Repository at www.jacionline.org). BCR-mediated upregulation of costimulatory molecules and nuclear factor of activated T cells (*NFAT*) gene

transcription²³ was comparable between patients and control subjects (see Fig E3 in this article's Online Repository at www.jacionline.org). Thus B cells from patients with CVID exhibit a selective deficit in BCR-mediated calcium mobilization, which positively correlates with decreased plasmablast differentiation *in vitro* and class-switched memory B-cell formation or maintenance *in vivo*, although these findings do not necessarily establish a causal relation.

Defective calcium mobilization is not caused by defects in plasma membrane channels, ER calcium storage release, and the BCR-signaling molecules Btk or PLCγ2

Plasma membrane calcium-channel function was evaluated. Lacking exogenous calcium influx, AUCs (and calcium ratios) remained significantly lower in patients with CVID disorders compared with those seen in control subjects (Fig 2, A; AUC: control subjects, 276 ± 124; patients with CVID disorders, 83 ± 223 [*P* = .02]). Calcium-depleted LCLs generated from patients with CVID disorders showed similar transmembrane calcium influx on CaCl₂ supplementation, as did control LCLs (Fig 2, B; AUC: control subjects, 37 ± 19; patients with CVID disorders, 47 ± 53 [*P* = .3]).

LCLs were treated with ionomycin or thapsigargin to investigate ER calcium release (see Fig E4 in this article's Online Repository at www.jacionline.org). Thapsigargin inhibits SERCA (sarco/endoplasmic reticulum Ca²⁺-ATPase) pumps, which

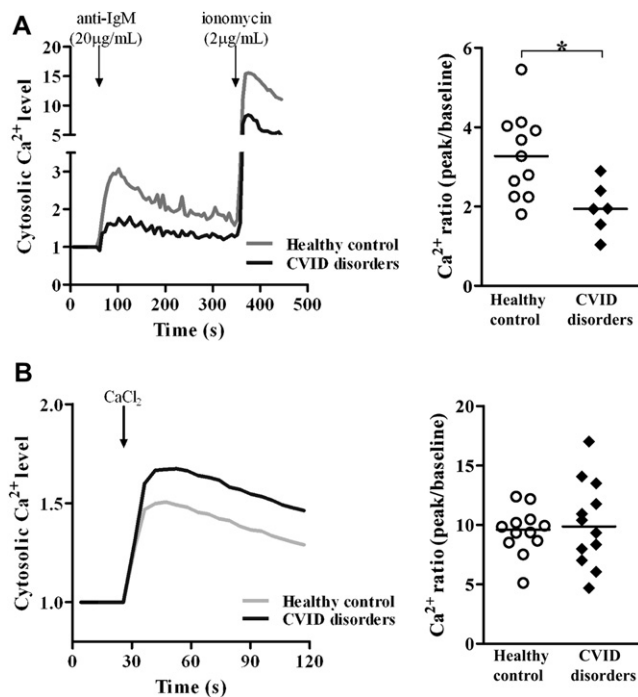


FIG 2. Plasma membrane calcium-channel function in B cells from patients with CVID disorders. **A**, ER Ca²⁺ release on anti-IgM in calcium-depleted medium: averaged kinetics plot (left) and Ca²⁺ ratios (right) in B cells of 11 control subjects and 6 patients with CVID disorders. **B**, CaCl₂ addition induces Ca²⁺ influx in LCLs from patients and control subjects (both n = 12). Lines indicate medians. Mann-Whitney U test: *P < .05.

maintain ER calcium stores.²⁴ Both treatments led to a similar pattern of calcium release into the cytosol between LCLs from patients and control subjects. Relevant tyrosine phosphorylation sites of BTK²⁵ and PLCG2^{26,27} were sequenced in 16 patients exhibiting reduced calcium ratios to survey the function of BCR-signaling cascade molecules, but no mutations were found (see the text in this article's Online Repository at www.jacionline.org).

Taken together, protein function of Btk and PLCγ2, as well as ER calcium homeostasis and plasma membrane channel function, appeared intact in our B cells from patients with CVID disorders. Henceforth, we focused on immediate BCR activation events in the plasma membrane.

Contribution of CD20 to activation-induced calcium mobilization defects in B cells from patients with CVID disorders

BCR and CD20 constitutively partly colocalize at the B-cell plasma membrane (Fig 3, A).¹⁵ CD20 mAb treatment specifically increased cytosolic calcium levels in B cells from healthy subjects and patients with CVID disorders after 30 minutes (Fig 3, B). Additionally, CD20-specific mAb ligation caused internalization of the BCR/coreceptor complex, as shown by a significant reduction in surface expression of Igλ light chains, CD21, and CD19 but not CD45 at 37°C but not at 4°C (Fig 3, C, and data not shown).

We next asked whether defective lateral dissociation of BCRs from CD20 could explain the defective calcium mobilization in patients with CVID disorders. B lymphocytes were pretreated with CD20-specific mAb for 30 minutes to induce CD20 cross-linking-induced B-cell activation, as shown previously and in Fig 4, B.^{13,28} The effectiveness in BCR/CD20 dissociation was

measured by inducing a secondary calcium flux on BCR stimulation. We found that B cells from healthy subjects but not patients were hyporesponsive to subsequent BCR triggering, reflecting that initial elicitation of the BCR-signaling pathway by anti-CD20 was productive in B cells from healthy subjects but not patients with CVID disorders (Fig 4, A; healthy control calcium ratio: only αIgM, 2.2 ± 1.1; αCD20 plus αIgM, 1.3 ± 0.35 [P = .004]; patients with CVID disorders: only αIgM, 1.8 ± 0.47; αCD20 plus αIgM, 1.4 ± 0.61 [P = .16]). Direct triggering of CD20 exhausted subsequent receptor-mediated calcium flux in B lymphocytes from healthy subjects but not patients. Furthermore, CD20 mAb decreased surface expression of CD19 in control subjects (CD19 mean fluorescence intensity × 10³: without αCD20, 4.78 ± 1.65; with αCD20, 2.00 ± 1.07 [P < .001]) but not patients (without αCD20, 2.87 ± 1.24; with αCD20, 1.68 ± 0.56 [P = .08]; Fig 4, B).

We hypothesized that CVID disorder pathogenesis might involve defects in the activation-induced disassembly of CD20 from the BCR signalosome¹² and visualized CD20 and BCR plasma membrane mobility on BCR cross-linking on IgM⁺ LCLs. On BCR cross-linking, dissociation and subsequent internalization of the BCR was observed, whereas CD20 remained at the cell surface.^{12,15} This process seemed less effective in the cells from patients with CVID disorders (Fig 4, C, and see Fig E5 in this article's Online Repository at www.jacionline.org).

In conclusion, we show that calcium signaling is disturbed in B cells from patients with CVID disorders and can be contributed to defective dissociation of the BCR from CD20 at the plasma membrane. Our findings suggest defective kinetics of the disassembly of BCR signalosomes to be a contributing factor in the development of CVID disorders.

DISCUSSION

We describe a selective defect in calcium mobilization in B lymphocytes observed in numerous pediatric patients with CVID disorders, which correlates with abrogated plasmablast development *in vitro* and decreased numbers of class-switched memory B cells *in vivo*. We show that CD20 dissociation from the BCR/coreceptor complex on B cells is required for activation-induced calcium signaling and propose that failure of this process might contribute to the development of CVID disorders.

Calcium flux is pivotal for lymphocyte function, and cytosolic calcium levels are tightly regulated. Calcium signaling influences cell-fate choices through differential regulation of transcription factor pathways, including NFAT and nuclear factor κB.^{23,29,30} We found that decreased calcium mobilization correlated with decreased B-cell differentiation into plasmablasts on BCR stimulation and with decreased fractions of IgG⁺ memory B cells in peripheral blood. To date, these findings cannot establish a causal relation, and other factors might be involved. Nevertheless, they suggest an important role for BCR-mediated calcium mobilization in the development or maintenance of end-stage B cells. Defective B-cell activation could explain the B-cell abnormalities characteristic of patients with CVID disorders. B-cell calcium mobilization was defective in a larger proportion of our pediatric cohort than was previously described in an adult population with CVID, and we did not find an increased expression of the inhibitory B-cell coreceptor CD22.⁵ Calcium fluxes were measured in fresh unfractionated CD19⁺ B cells. We asked whether decreased calcium fluxes in patients with CVID could be explained by

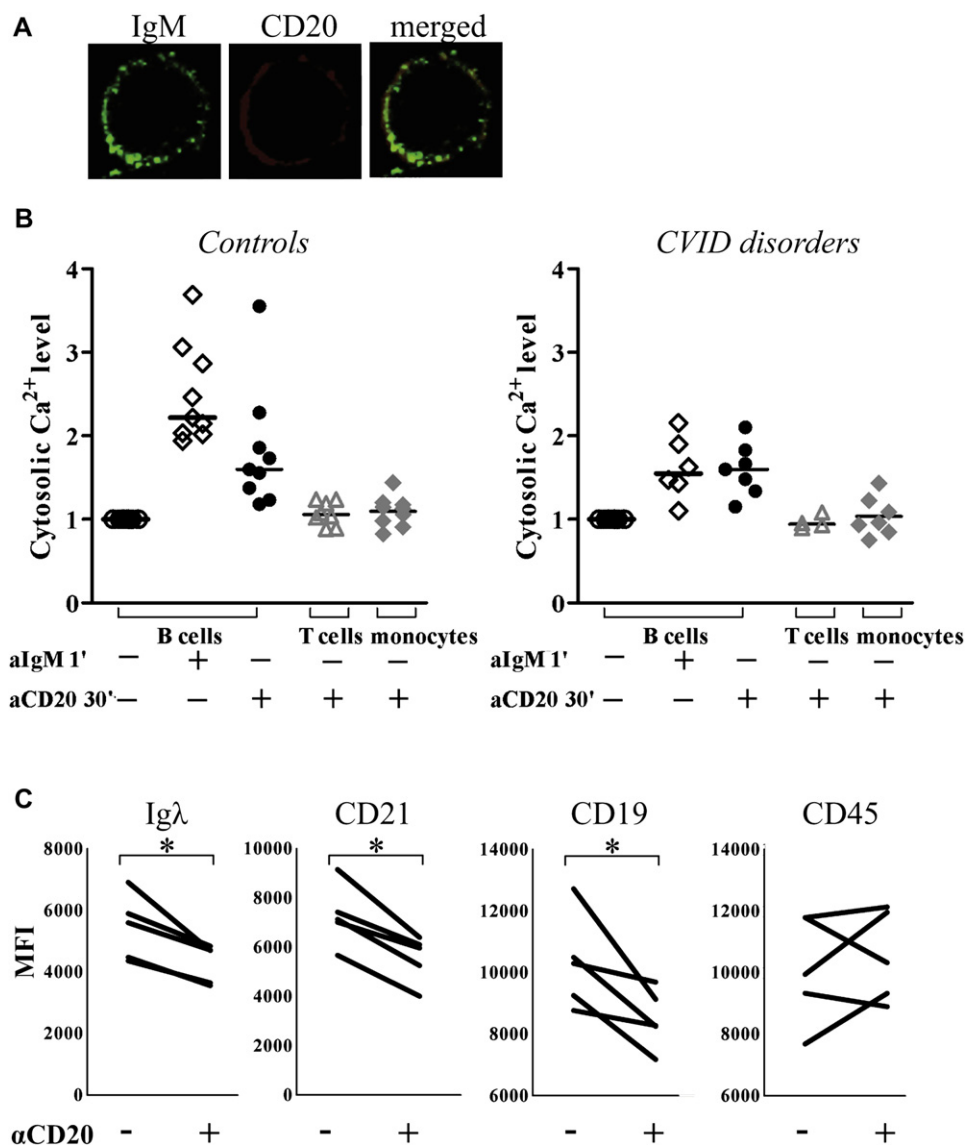


FIG 3. Anti-CD20 induces calcium influx and BCR/coreceptor internalization. **A**, Colocalization of IgM (green) and CD20 (red) in resting B cells. **B**, Anti-CD20 triggers a specific Ca^{2+} flux in B lymphocytes from healthy subjects (left) and patients with CVID disorders (right). **C**, Anti-CD20 treatment of healthy B cells leads to selective internalization of molecules clustered in the BCR/coreceptor complex. Paired t test: $*P < .05$.

differences in composition of the B-cell compartment but confirmed that patients with CVID have a relatively larger IgM⁺ B-cell compartment than healthy control subjects.^{16,17} Moreover, calcium mobilization was reduced in all posttransitional B-cell subpopulations in adults with CVID,⁵ and our decreased Ca^{2+} ratios did not correlate with percentages of IgM⁺ B cells (unpublished observations).

We tested plasma membrane calcium-channel and ER calcium storage capacity on cellular activation and found those in B cells of patients with CVID to be comparable with those in control subjects. These features are conserved between lymphocytes, and defects would likely cause a more severe combined immunodeficiency.^{31,32} Nevertheless, we cannot completely exclude the possibility that patients with sporadic CVID disorders have smaller defects in less pertinent or yet unidentified channels or in BCR-specific phosphotyrosine kinases. Because key residues

of PLCγ2 and Btk gene products were not mutated in calcium signaling-affected cells, it is unlikely that these will be causative factors in a considerable proportion of the population with CVID. Complete dysfunction of such molecules usually leads to negligible numbers of peripheral B cells and agammaglobulinemia,³³⁻³⁵ which was not seen in this cohort.

The plasma membrane undergoes dynamic remodeling during B-cell activation.^{15,36} We hypothesized this remodeling to be defective. CD20 homo-oligomers associate with the BCR in resting cells,^{15,28,37} but BCR stimulation causes dissociation of the BCR from CD20.¹² Direct triggering of CD20 induces calcium flux, which requires association of CD20 with the BCR and is mediated through BCR signaling.^{13,14} CD20 ligation on healthy B cells triggered a calcium flux that exhausted the BCR-signaling cascade; the ensuing BCR triggering scarcely induced subsequent signaling. These findings support recent work describing that

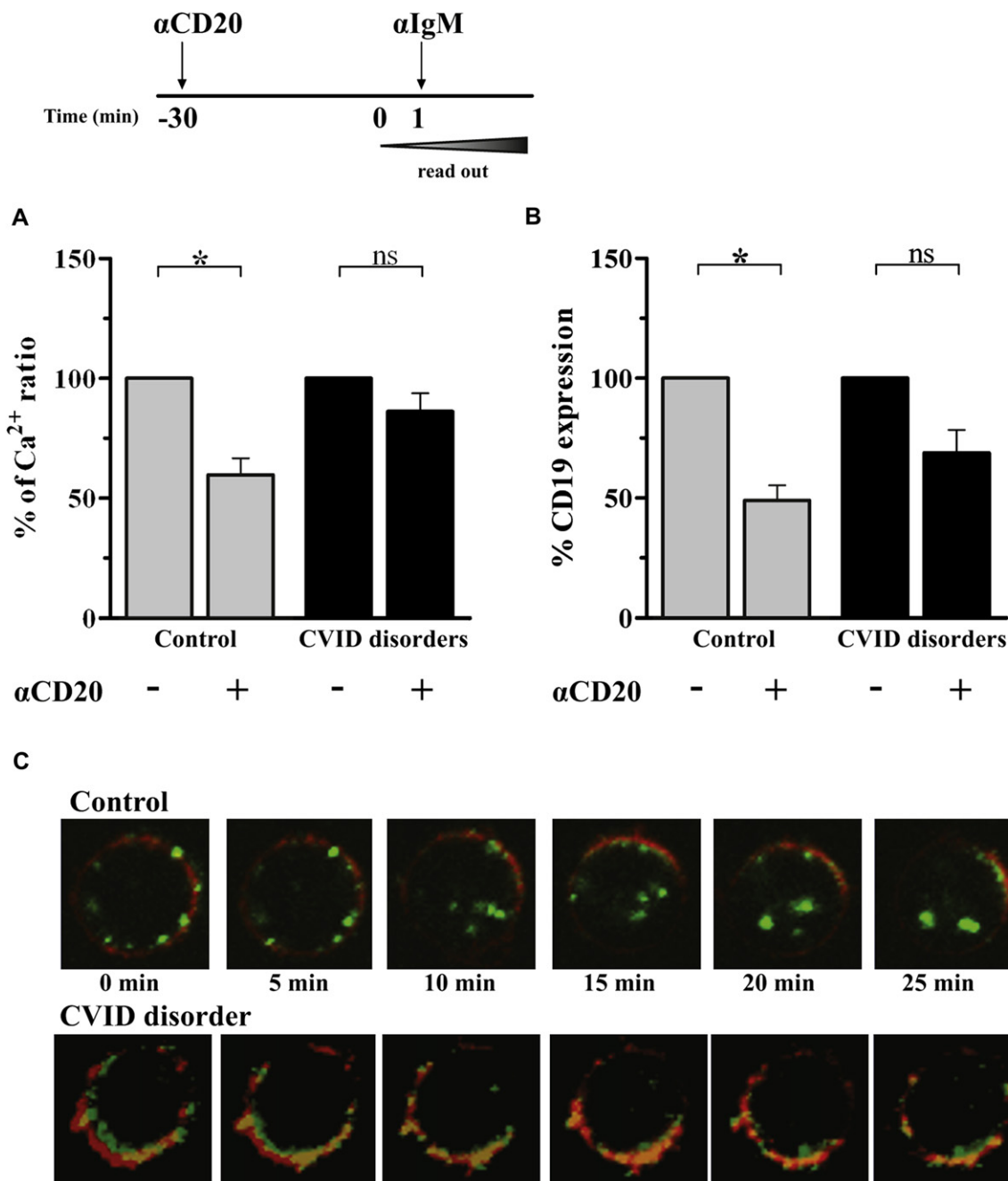


FIG 4. Anti-CD20 does not exhaust BCR-mediated calcium mobilization in patients with CVID disorders. **A** and **B**, Anti-CD20 pretreatment decreases Ca²⁺ mobilization (Fig 4, A) and CD19 surface expression (Fig 4, B) in primary B cells of 10 control subjects and 8 patients (means $\pm$ SEMs). Paired *t* test: **P* < .05. **C**, IgM BCR (green) and CD20 (red) dissociate on anti-IgM F(ab')₂ stimulation of LCLs from control subjects and patients with CVID disorders.

α CD20 pretreatment results in a time-dependent inhibition of the BCR-signaling cascade in healthy human B cells.¹⁸ In contrast, in B cells from patients with CVID disorders, α CD20 pretreatment triggers an initial calcium flux but does not yield complete BCR-mediated signaling because further residual calcium signaling can be elicited when BCRs are restimulated after 30 minutes.

We propose that on CD20 triggering, BCR triggering, or both, BCR-CD20 signalosomes induce downstream signaling, as described above and elsewhere, followed by rapid dissociation at the

cell surface, where CD20 remains while the BCR/coreceptor complex is endocytosed,^{12,15} triggering calcium signaling in the cytosol.^{13,18} We first stimulated with anti-CD20 and subsequently added anti-IgM. We chose this order because anti-CD20 treatment requires more time than anti-IgM treatment to elicit calcium fluxes, as measured in real time by means of flow cytometry. However, because both antibodies trigger the same signaling route, either order of (re)stimulation should yield similar results. Our data suggest this synergistic mechanism to be impaired in a subgroup

of patients with CVID disorders, rendering B cells less capable to resolve CD20/BCR oligomers, resulting in reduced BCR/coreceptor internalization and decreased calcium signaling. It remains to be identified which genes are involved in this process.

In conclusion, we found a calcium mobilization defect in B lymphocytes of children with CVID disorders, which we propose results from altered dissociation of the BCR from CD20. Other additional signalosome-associated proteins might be involved. Further studies are required to address the molecules involved in CD20-associated BCR signaling and the underlying genetic defects leading to the CVID disorder phenotypes for elucidation of possible intervention points for B-cell activation-targeted therapy.

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Key messages

- BCR-mediated calcium mobilization is impaired in numerous pediatric patients with CVID and related disorders.
- CD20-BCR dissociation is pivotal to BCR-mediated calcium signaling in human B cells.
- Defects in CD20-BCR dissociation might predispose to CVID and related disorders.

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METHODS

Patients

Of the 42 patients, 23 were given diagnoses of CVID according to the European Society for Immunodeficiencies criteria.^{E1} All but 2 patients with new diagnoses received immunoglobulin replacement therapy. Nineteen patients did not completely meet these CVID criteria and were therefore given a diagnosis of CVID-like disorder, which was defined as selective antibody deficiency (defined as the inability to produce protective titers of specific antibodies on vaccination to polysaccharide, to recall antigens, or both) combined with low IgA, low IgM, and/or low IgG subclass levels or a solitary decreased IgG level. These patients had recurrent infections and an inadequate response to prophylactic antibiotic treatment, which was defined by more than 4 breakthrough infections per year. Secondary immunodeficiencies (eg, iatrogenic or caused by enteral protein loss) were ruled out in all patients. Previous studies have shown that pediatric patients with CVID-like disorders, as described above, are clinically and immunologically (eg, B- and T-cell phenotype characterization) comparable with patients with definite CVID.^{E2,E3} Because the current flow cytometry-based CVID classifications are not applicable to children,^{E2} we did not attempt to classify them. However, 5 children already had disease-related complications, as described by Chapel et al.^{E4} These were enteropathy, autoimmune hemolytic anemia ($n = 3$), idiopathic thrombocytopenic purpura ($n = 3$), autoimmune nephritis, hepatosplenomegaly with extensive lymphadenopathy, lymphoid interstitial pneumonia, systemic lupus erythematosus, and vitiligo.

Ten healthy pediatric control subjects were asked to donate blood when elective surgery was performed. Leftover peripheral blood (1 mL) was used from 4 healthy pediatric control subjects from another approved study in our laboratory. All adult volunteers were healthy employees of the University Medical Center Utrecht.

Genetic analysis of *PLCG2* and *BTK*

Granulocytes were isolated from peripheral blood with the use of Ficoll gradient centrifugation, and subsequently, genomic DNA was isolated with the autopure kit (Qiagen, Hilden, Germany). Exon 22 of the *PLCG2* gene (NM_002661.2) and exon 18 of the *BTK* gene (NM_000061.2) were directly sequenced after PCR on an ABI 3100 automated sequencer (PE Applied Biosystems, Foster City, Calif) and a 3130XL genetic analyzer with the Applied Biosystems BigDye Terminator v1.1 cycle sequencing kit, according to the manufacturer's protocol. Analysis was done with SeqScape v2.5 software (Applied Biosystems) and Mutation Surveyor software (Softgenetics, LLC, State College, Pa). PCR conditions and primer sequences are available on request.

B-cell cultures

PBMCs were thawed quickly, washed twice, and resuspended in RPMI containing 10% FCS, 1% penicillin and streptavidin, and 1% glutamine in a 24-well plate for 24 or 48 hours with the following stimuli: phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (2 μ g/mL; Calbiochem, San Diego, Calif) as a positive control, anti-IgM F(ab')₂ fragments (20 μ g/mL; Jackson ImmunoResearch, West Grove, Pa), and anti-IgM F(ab')₂ fragments (10 μ g/mL) with IL-4 (200 U/mL; Immunotools, Friesoythe, Germany). After 24 hours, cold FACS buffer was added, and cells were washed twice. After Fc receptor blocking with murine serum, cells were stained for CD19, CD80, and CD86 (all from Becton Dickinson) for 30 minutes at 4°C. After washing twice, cells were acquired on a FACS Canto II Flow Cytometer and analyzed with FACS Diva software (both from Becton Dickinson). After 48 hours, cells were lysed, and total mRNA was isolated with Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions, and reversed with an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif). Primers were mixed with IQ SYBR green supermix (Bio-Rad Laboratories). The detection run started at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Assays were performed in triplicate on a C1000 Thermal Cycler (Bio-Rad Laboratories). Results were normalized to endogenous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA and the unstimulated sample (2^{- $\Delta\Delta$ CT}). The following primers were used: *GAPDH* forward, 5'-GTCGGAGTCAACGGATT-3'; *GAPDH* reverse, 5'-AAGCTTCCCGTTCTCAG-3'; *NFAT* forward, 5'-GCAGAGCACGGACAGCTATC-3'; and *NFAT* reverse, 5'-GGGCTTTCTCCACGAAAATGA-3' (all from Sigma-Aldrich, St Louis, Mo).

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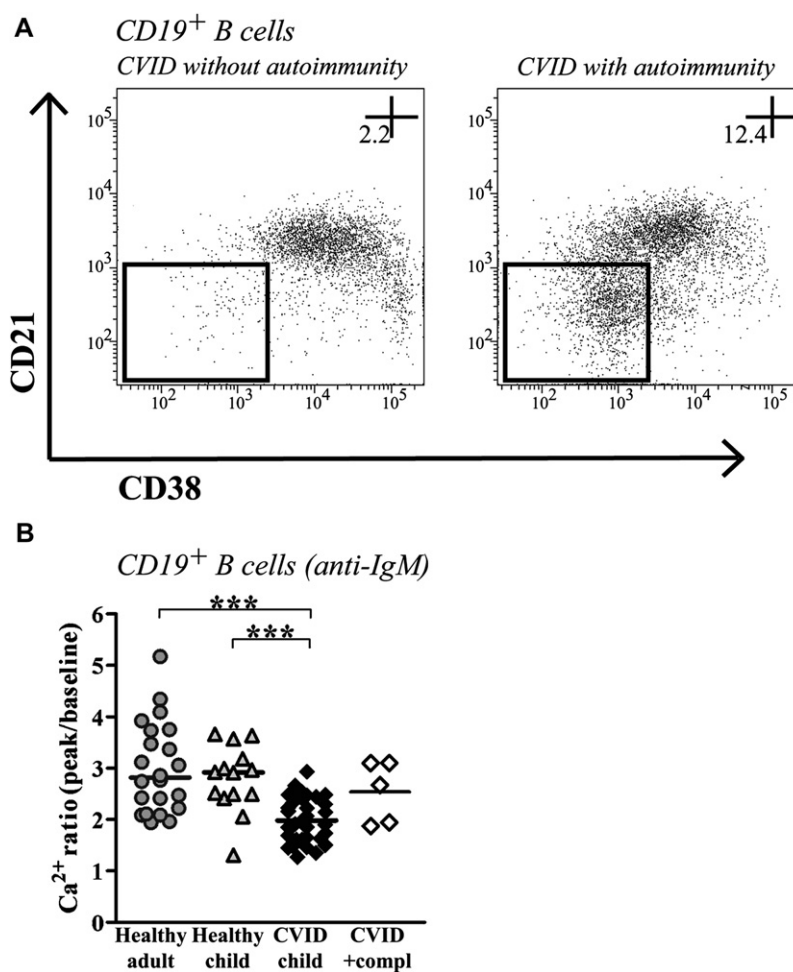


FIG E1. Characteristics of patients with CVID disorders with disease-related complications. **A**, Autoreactive CD21⁺CD38⁺ B-cell numbers are increased in patients with CVID with autoimmune complications (*right*) compared with numbers seen in patients without autoimmunity (*left*). **B**, Patients with CVID disorders with autoimmune or lymphoproliferative complications had greater calcium mobilization than patients without these complications. Kruskal-Wallis with *post hoc* Dunn comparisons: *** $P < .001$.

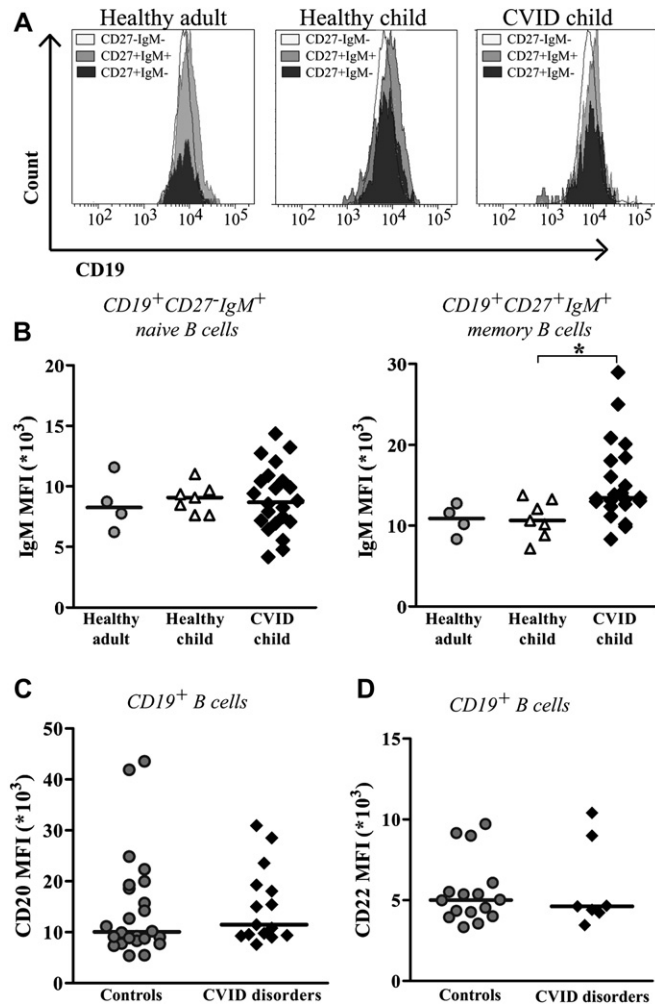


FIG E2. Expression of B-cell surface molecules in patients with CVID disorders. **A**, CD19 expression in 5 adults, 10 healthy children, and 34 children with CVID disorders. **B**, Mean fluorescence intensity (MFI) of IgM on naive B cells and memory B cells. **C** and **D**, CD20 (Fig E2, C) and CD22 (Fig E2, D) expression in patients and control subjects. Kruskal-Wallis with *post hoc* Dunn comparisons: * $P < .05$.

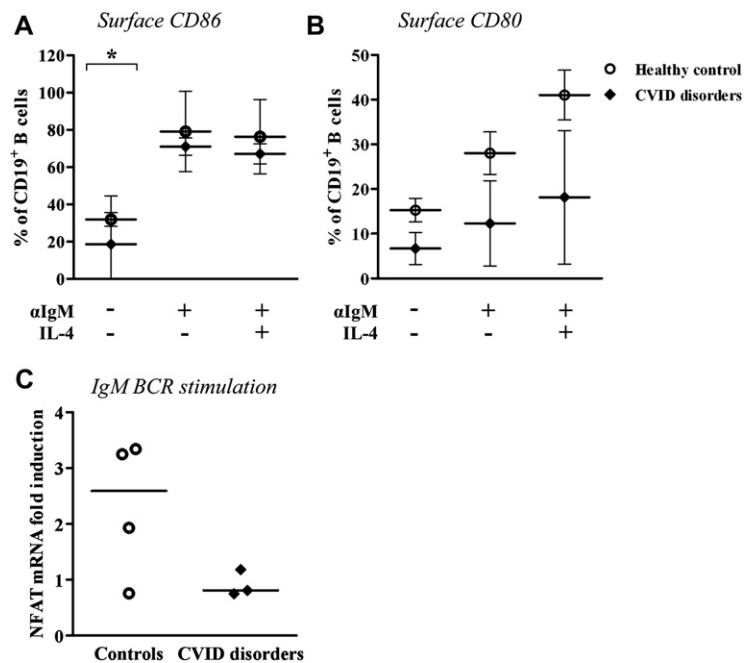


FIG E3. BCR-mediated upregulation of costimulatory molecules. Percentages of CD80⁺ (**A**) and CD86⁺ (**B**) B cells after 24 hours of culture. Means $\pm$ SEMs of 16 control subjects and 7 patients. Mann-Whitney *U* test: $*P < .05$. **C**, NFAT gene transcription in PBMCs of 4 control subjects and 3 patients after 48 hours of stimulation with anti-IgM F(ab')₂ fragments.

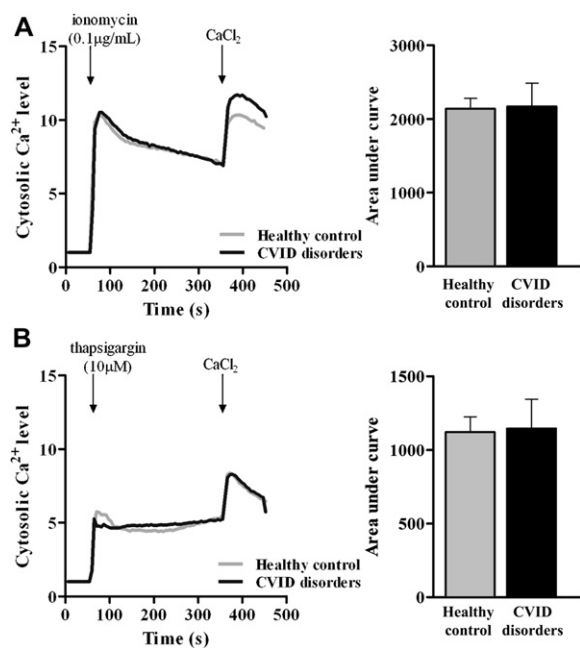


FIG E4. ER calcium mobilization and storage capacity in B lymphocytes from patients with CVID disorders. Kinetics of ER storage Ca^{2+} release after ionomycin stimulation (**A**) or thapsigargin depletion (**B**) in calcium-depleted medium, as demonstrated by an averaged kinetics plot (*left*) and AUC (*right*) in LCLs from 6 control subjects and 6 patients with CVID disorders. Bar graphs represent means $\pm$ SEMs.

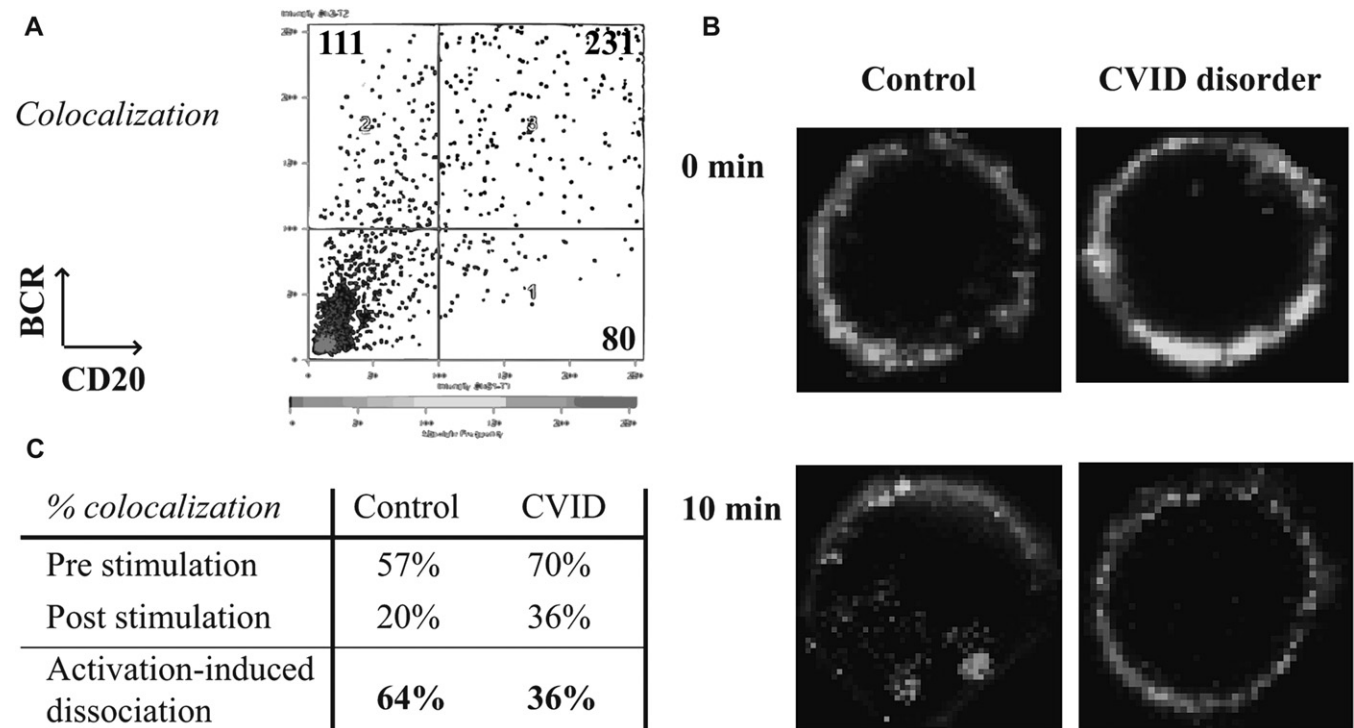


FIG E5. Impaired BCR-induced BCR/CD20 lateral dissociation in patients with CVID disorders. **A**, Colocalization was calculated by dividing double-positive pixels (BCR/CD20) by the positive pixels within the BCR image. **B**, LCLs from control subjects (*left*) and patients with CVID disorders (*right*) before (*upper panel*) and after (*lower panel*) BCR stimulation. **C**, Percentage of dissociation on BCR triggering based on greater than 25 cells per donor in 2 experiments.

TABLE E1. Distribution of B-cell subpopulations in patients with normal and decreased calcium mobilization

B-cell subpopulations	Patients with CVID disorders (n = 33; age, 7-18 y)			
	Normal calcium mobilization (n = 16 [49%])		Decreased calcium mobilization (n = 17 [51%])	
	Total B cells (%)	Absolute numbers (cells/ μ L)	Total B cells (%)	Absolute numbers (cells/ μ L)
CD19 ⁺ B cells	NA	338 (217)	NA	308 (272)
CD19 ⁺ IgD ⁺ CD10 ⁺ CD38 ⁺ recent bone marrow emigrants	12 (12)	40 (41)	15 (13)	55 (45)
CD19 ⁺ IgD ⁺ IgM ⁺ CD10 ⁻ CD27 ⁻ naive B cells	64 (23)	215 (218)	69 (16)	222 (641)
CD19 ⁺ IgD ⁺ IgM ⁺ CD27 ⁺ memory IgM B cells	6.0 (5.1)	17 (55)	2.5 (5.3)*	11 (24)
CD19 ⁺ IgG ⁺ CD27 ⁺ memory IgG B cells	3.3 (4.0)	15 (13)	1.0 (2.2)*	4 (11)*
CD19 ⁺ IgA ⁺ CD27 ⁺ memory IgA B cells	1.5 (1.3)	5 (7)	1.0 (1.3)	2 (5)

All values are median (interquartile range).

NA, Not assessed.

* $P < .05$, Mann-Whitney U test.