

# Disturbed canonical nuclear factor of $\kappa$ light chain signaling in B cells of patients with common variable immunodeficiency

Baerbel Keller, PhD,<sup>a</sup> Zoltan Cseresnyes, PhD,<sup>b</sup> Ina Stumpf,<sup>a</sup> Claudia Wehr, MD,<sup>a</sup> Manfred Fliegau, PhD,<sup>a</sup> Alla Bulashevskaya, PhD,<sup>a</sup> Susanne Usadel, MD,<sup>c</sup> Bodo Grimbacher, MD,<sup>a,d</sup> Marta Rizzi, MD,<sup>a</sup> Hermann Eibel, PhD,<sup>a</sup> Raluca Niesner, PhD,<sup>b</sup> and Klaus Warnatz, MD<sup>a</sup> *Freiburg and Berlin, Germany, and London, United Kingdom*

**Background:** Most patients with common variable immunodeficiency (CVID) present with severely reduced switched memory B-cell counts, and some display an increase of CD21<sup>low</sup> B-cell counts (CVID 21low), whereas others do not (CVID 21norm). Altered B-cell receptor (BCR) signaling might contribute to the defective memory formation observed in patients with CVID.

**Objective:** We sought to investigate canonical nuclear factor of  $\kappa$  light chain (NF- $\kappa$ B) signaling in B cells from patients with CVID as a central pathway in B-cell differentiation.

**Methods:** Degradation of inhibitor of  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ) and p65 phosphorylation, nuclear translocation of p65, and regulation of target genes and cell function were investigated after different modes of B-cell stimulation.

**Results:** BCR-mediated canonical NF- $\kappa$ B signaling was impaired in all mature naive CVID-derived B cells. This impairment was more profound in naive B cells from CVID 21low patients than CVID 21norm patients and most pronounced in CD21<sup>low</sup> B cells. The signaling defect translated into reduced induction of Bcl-xL and I $\kappa$ B $\alpha$ , 2 *bona fide* target

genes of the canonical NF- $\kappa$ B pathway. CD40 ligand- and Toll-like receptor 9-mediated signaling were less strongly altered. Signaling in CD21<sup>low</sup> B cells but not CD21<sup>+</sup> B cells of patients with HIV was similarly affected.

**Conclusion:** Combined with the previous description of disturbed Ca<sup>2+</sup> signaling, the discovery of NF- $\kappa$ B signaling defects, especially in CVID 21low patients, suggests a broad underlying signaling defect affecting especially BCR-derived signals. Given the immune phenotype of monogenic defects affecting Ca<sup>2+</sup> and NF- $\kappa$ B signaling, the latter is more likely to contribute to the humoral deficiency. The strongly disturbed BCR signaling of CD21<sup>low</sup> B cells is characteristic for this cell type and independent of the underlying disease. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

**Key words:** Common variable immunodeficiency, B cells, CD21<sup>low</sup> B cells, canonical nuclear factor of  $\kappa$  light chain, signaling, B-cell receptor, Toll-like receptor 9, CD40, HIV

Common variable immunodeficiency (CVID) is a highly variable antibody deficiency syndrome with a heterogeneous and often undefined genetic background.<sup>1,2</sup> Common to most patients is a severe reduction in circulating class-switched memory B-cell and plasma cell numbers in the bone marrow.<sup>3,4</sup> Immunoglobulin-secreting plasma cells develop in extrafollicular areas after antigen-induced activation in T-independent immune responses from B cells activated by pattern recognition receptors, such as Toll-like receptors (TLRs), or in T-dependent immune responses stimulated by T<sub>H</sub> cells activating CD40 and cytokine receptors in germinal centers (GCs). The GC reaction also leads to formation of high-affinity class-switched memory B cells that persist in human subjects for decades and develop rapidly into plasma cells secreting high-affinity antibodies after reactivation by antigen.<sup>5,6</sup>

Defects in patients with CVID underlying the humoral immunodeficiency frequently affect memory and plasma cell differentiation.<sup>7,8</sup> According to EUROClass,<sup>9</sup> a flow cytometry-based classification of CVID, one subgroup of patients with CVID is characterized by the increased presence of a rare subset of circulating B cells expressing only low levels of CD21 (>10% of CD21<sup>low</sup> B cells; CVID 21low) constituting more than 10% of peripheral B cells, which is rare in other patients with CVID (<10% of CD21<sup>low</sup> B cells; CVID 21norm) and healthy control subjects.

In addition to increased infection susceptibility, CVID 21low patients frequently have autoimmune and lymphoproliferative manifestations. A similar expansion of CD21<sup>low</sup> B cells has also been described in patients with chronic HIV infections or in patients with autoimmune disorders, such as systemic lupus

From <sup>a</sup>the Center for Chronic Immunodeficiency, University Medical Center and University of Freiburg; <sup>b</sup>JIMI imaging network and Biophysical Analytics, German Rheumatism Research Center, Leibniz Institute, Berlin; <sup>c</sup>Infektiologikum Freiburg, Division of Infectious Diseases, Department of Internal Medicine, University Medical Center Freiburg; and <sup>d</sup>the Institute of Immunity and Transplantation, University College London.

Supported by the German Federal Ministry of Education and Research (BMBF 01EO1303) and the Deutsche Forschungsgemeinschaft TRR130. M.R. was supported by "Margarete von Wrangell Habilitation Program" by the Ministry of Sciences, Research and Arts in Baden Württemberg and the European Social Fund.

Disclosure of potential conflict of interest: S. Usadel is a board member for Gilead, Merck Sharpe and Dohme, BMS, and AbbVie; has received payment for lectures from BMS, Janssen-Cilag, Merck Sharp & Dome, and ViiV; and has received travel support from Gilead, BMS, and Janssen Cilag. B. Grimbacher has received grants from BMBF, the European Union, Helmholtz, DFG, DLR, and DZIF; is employed by UCL and UKL-FR; and has received payment for lectures from CSL Behring, Baxalta, and Biotest. M. Rizzi has received grants from Novartis Stiftung. H. Eibel has received grants from BMBF and DFG. R. Niesner has received grants from DFG, TRR130, C01. K. Warnatz is a board member for Biotest, CSL Behring, and LFB Biomedicaments; has received grants from BMS, CSL Behring, and Biotest; has received payment for lectures from LFB Biomedicaments, Baxter, GlaxoSmithKline, CSL Behring, Pfizer, Biotest, Novartis Pharma, Roche, Meridian Health Comms, and Octapharma; and has received payment for manuscript preparation from UCB Pharma. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 1, 2015; revised April 3, 2016; accepted for publication April 19, 2016.

Corresponding author: Klaus Warnatz, MD, Center for Chronic Immunodeficiency, University Medical Center and University of Freiburg, Breisacherstr. 115, Freiburg 79106, Germany. E-mail: klaus.warnatz@uniklinik-freiburg.de.

0091-6749/\$36.00

© 2016 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2016.04.043>

**Abbreviations used**

|                           |                                                                    |
|---------------------------|--------------------------------------------------------------------|
| BCL10:                    | B-cell CLL/lymphoma 10                                             |
| BCR:                      | B-cell receptor                                                    |
| CARD11:                   | Caspase recruitment domain family member 11                        |
| CD40L:                    | CD40 ligand                                                        |
| CVID:                     | Common variable immunodeficiency                                   |
| CVID 21 <sup>low</sup> :  | Increase in CD21 <sup>low</sup> B-cell count                       |
| CVID 21 <sup>norm</sup> : | Normal CD21 <sup>low</sup> B-cell count                            |
| DIC:                      | Differential interference contrast                                 |
| GC:                       | Germinal center                                                    |
| HD:                       | Healthy donor                                                      |
| ICAM-1:                   | Intercellular adhesion molecule 1                                  |
| IKK:                      | Inhibitor of $\kappa$ B kinase                                     |
| MALT1:                    | Mucosa-associated lymphoid tissue lymphoma translocation protein 1 |
| MFI:                      | Mean fluorescence intensity                                        |
| NF- $\kappa$ B:           | Nuclear factor of $\kappa$ light chain                             |
| PE:                       | Phycoerythrin                                                      |
| PKC $\beta$ :             | Protein kinase C $\beta$                                           |
| PLC $\gamma$ 2:           | Phospholipase C $\gamma$ 2                                         |
| PMA:                      | Phorbol 12-myristate 13-acetate                                    |
| TLR:                      | Toll-like receptor                                                 |

erythematosus, rheumatoid arthritis, and others.<sup>10-12</sup> These CD21<sup>low</sup> B cells display features of activated B cells with a distinct RNA expression profile and changes in functional capacity.<sup>12-14</sup> This is associated with a reduced capacity of B-cell receptor (BCR)-mediated Ca<sup>2+</sup> mobilization in all B cells of CVID 21low patients, especially affecting the CD21<sup>low</sup> B-cell compartment.<sup>15</sup>

On antigen binding to the BCR, phosphorylation of phospholipase C $\gamma$ 2 (PLC $\gamma$ 2) leads to production of inositol trisphosphate (IP3), inducing Ca<sup>2+</sup> flux, and diacylglycerol (DAG), which activates protein kinase C $\beta$  (PKC $\beta$ ) and leads to activation of the canonical nuclear factor of  $\kappa$  light chain (NF- $\kappa$ B) pathway by phosphorylating the inhibitor of  $\kappa$ B kinase (IKK) complex. The inhibitor of  $\kappa$ B $\alpha$  (IkB $\alpha$ ) is phosphorylated, ubiquitinated, and degraded, and p65/p50 dimers are released and enter the nucleus to activate gene transcription. The canonical NF- $\kappa$ B pathway plays a central role in B-cell differentiation, maintenance and function in mice.<sup>16-20</sup>

Given the altered Ca<sup>2+</sup> activation in these patients, we analyzed the canonical NF- $\kappa$ B pathway in patients with CVID with reduced switched memory B cells. We found a significantly reduced NF- $\kappa$ B signal, with the strongest impairment in CVID 21low patients. The severely impaired canonical NF- $\kappa$ B signal in CD21<sup>low</sup> B cells was characteristic of this cell stage and not genetically determined because it was seen similarly in CD21<sup>low</sup> B cells of patients with HIV. However, the reduced BCR signal affecting both Ca<sup>2+</sup> and the canonical NF- $\kappa$ B signal in naive CD21<sup>+</sup> B cells was unique to the patients with CVID and might suggest an underlying genetic predisposition affecting B-cell activation.

**METHODS****Cell isolation and cultivation**

PBMCs were isolated from EDTA blood by using Ficoll density centrifugation and cultivated in RPMI 10% FCS.

**B-cell activation and survival**

IkB $\alpha$  degradation, phosphorylation of p65, phospho-IkB $\alpha$ , and phospho-PLC $\gamma$ 2 were determined by using intracellular flow cytometry, as described previously.<sup>21</sup> PBMCs were left untreated or stimulated with 15  $\mu$ g/mL F(ab)<sup>2</sup> goat anti-human IgM (SouthernBiotech, Birmingham, Ala), 5  $\mu$ g/mL CpG-ODN 2006 (InvivoGen, Toulouse, France), recombinant CD40 ligand (CD40L), or 200 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, Mo), as indicated. For some experiments, cells were pretreated with the proteasome inhibitor MG132 (10  $\mu$ mol/L) for 4 hours. Cells were fixed by means of addition of Cytofix and permeabilized with Perm Buffer III (both from BD Biosciences, San Jose, Calif), according to the manufacturers' instructions. After permeabilization, cells were stained with the appropriate antibodies and measured on an LSR Fortessa (BD Biosciences). For upregulation of IkB $\alpha$  and Bcl-xL, cells were stimulated for 36 hours and treated as described above. Activation markers were determined after cultivation of PBMCs with 10  $\mu$ g/mL anti-human IgM for 36 hours. Cells were washed and stained with the respective surface markers. For B-cell survival, naive B cells were isolated from PBMCs by using the MACS Naive B Cell Isolation Kit II (Miltenyi Biotech, Bergisch Gladbach, Germany). Cells were cultivated for 17 hours in the absence or presence of 10  $\mu$ g/mL anti-IgM, harvested, counted, and stained for flow cytometric assessment of apoptosis.

All flow cytometric stainings included CD38 to differentiate CD21<sup>low</sup>/CD38<sup>low</sup> B cells from CD21<sup>intermediate</sup>/CD38<sup>high</sup> transitional B cells.

**Confocal microscopy**

For nuclear translocation of p65, isolated B cells were left untreated or stimulated with 20  $\mu$ g/mL anti-human IgM for 2 hours and CD40L for 30 minutes. Cells were fixed with 4% paraformaldehyde, washed, and settled onto slides. After blocking (10% goat serum/2% immunoglobulin) and surface staining with CD21, permeabilization with 0.3% Triton was followed by anti-p65 and anti-IgM staining. Nuclei were stained with Hoechst33342. Images were taken on a Zeiss laser scanning microscope, LSM710 (Carl Zeiss, Jena, Germany) and evaluated and processed with Zeiss ZEN black software.

Additional information can be found in the [Methods](#) section and in [Figs E1](#) and [E2](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org).

**RESULTS****Reduced IkB $\alpha$  degradation in CVID-derived B cells after BCR stimulation**

BCR-induced NF- $\kappa$ B signaling was determined based on the degradation of IkB $\alpha$  comparing IgD<sup>+</sup>/CD27<sup>-</sup> naive B cells of 23 patients with CVID with a reduction in switched memory B-cell numbers and 28 healthy donors (HDs) after anti-IgM stimulation. Patients' characteristics are shown in [Table E1](#) in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org). Compared with HDs, we found impaired degradation of IkB $\alpha$  in patients' IgD<sup>+</sup>/CD27<sup>-</sup> B cells. The degree of impairment varied between patients: whereas in CVID 21norm patients (patients 17 and 22) it was near normal, IkB $\alpha$  degradation was severely reduced, especially in CVID 21low patients (patients 4, 5, and 10; [Fig 1, A](#)). The differentiation of CD21<sup>+</sup> and CD21<sup>low</sup> B cells revealed a comparable basal IkB $\alpha$  expression in unstimulated CD21<sup>+</sup> B cells of HDs and of CVID 21low and 21norm patients, but this was significantly decreased in patient-derived CD21<sup>low</sup> B cells ([Fig 1, B](#)). After BCR stimulation for 35 minutes, the mean fluorescence intensity (MFI) of IkB $\alpha$  decreased in CD21<sup>+</sup> B cells but remained significantly higher in B cells from patients with CVID compared with HDs. IkB $\alpha$  expression in CD21<sup>low</sup> B cells hardly decreased after stimulation ([Fig 1, C](#)), although the BCR is equally expressed on patients' CD21<sup>+</sup> B cells and even higher on CD21<sup>low</sup> B cells.<sup>13</sup> We determined the ratio of the MFI of IkB $\alpha$  after and

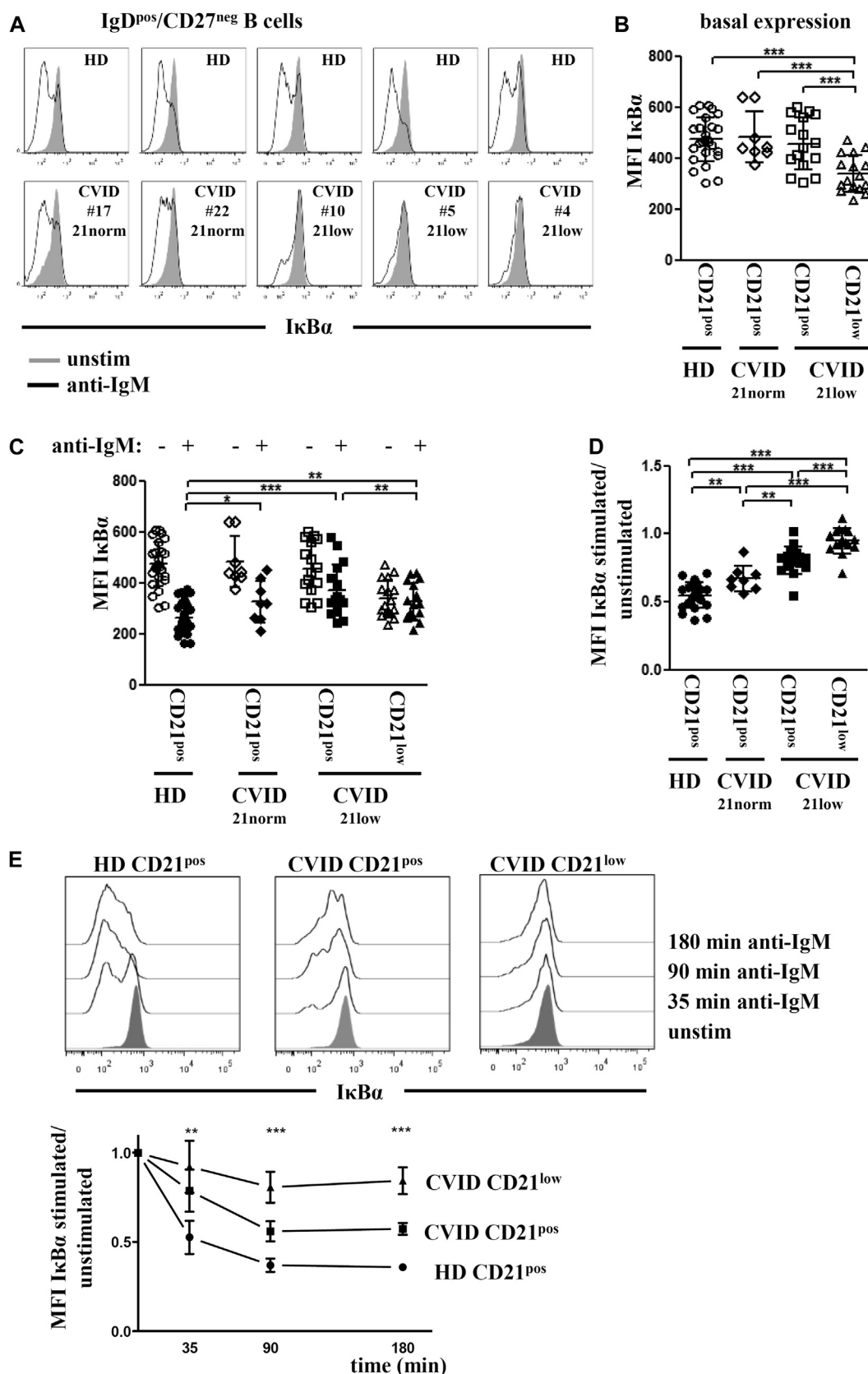


**FIG 1.** BCR-mediated  $\text{I}\kappa\text{B}\alpha$  degradation in B cells from patients with CVID. **A**,  $\text{I}\kappa\text{B}\alpha$  expression in B cells from representative HDs and CVID 21norm and 21low patients. **B**, Basal MFI of  $\text{I}\kappa\text{B}\alpha$ . **C**, MFI of  $\text{I}\kappa\text{B}\alpha$  with and without stimulation. **D**, Ratio of the MFI of  $\text{I}\kappa\text{B}\alpha$  after and without stimulation. **E**, Kinetics of  $\text{I}\kappa\text{B}\alpha$  degradation.  $P$  values ( $*P < .05$ ,  $**P < .01$ , and  $***P < .001$ ) derive from comparison of the 3 B-cell populations at each time point by using ANOVA.

before stimulation, reflecting the degree of induced I $\kappa$ B $\alpha$  degradation. This ratio was severely reduced in CD21<sup>+</sup> and especially CD21<sup>low</sup> B cells of CVID 21low patients compared with CVID 21norm patients and HDs (Fig 1, D).

Because of the more severe phenotype of B cells from CVID 21low patients, all further experiments were performed on B-cell subpopulations derived from these patients.

I $\kappa$ B $\alpha$  protein levels were examined up to 180 minutes after BCR stimulation to exclude that reduced I $\kappa$ B $\alpha$  degradation in B cells from patients with CVID was caused by delayed signaling. In all 3 examined B-cell subpopulations, the I $\kappa$ B $\alpha$  ratio was decreased after 35 minutes and further decreased after 90 minutes, which was more or less stable during the next 90 minutes. However, the pattern of defective NF- $\kappa$ B signaling was stable over time, with significantly reduced I $\kappa$ B $\alpha$  degradation in CD21<sup>+</sup> B cells compared with HDs and even lower responses in CD21<sup>low</sup> B cells at any analyzed time point (Fig 1, E).

CVID 21low patients with and without immunosuppressive therapy and patients with subcutaneous and intravenous immunoglobulin substitution (see Table E1) were compared to exclude a potential influence on NF- $\kappa$ B activation. We observed no significant differences between the different groups, implying that these medications did not cause the observed defect in NF- $\kappa$ B activation (data not shown).

### Failure of regular p65 phosphorylation and translocation after BCR stimulation

Next, BCR-mediated p65 activation was analyzed. p65 phosphorylation was significantly decreased in CD21<sup>+</sup> B cells of CVID 21low patients compared with CD21<sup>+</sup> B cells of HDs and not detectable in CD21<sup>low</sup> B cells, corroborating the results for I $\kappa$ B $\alpha$  degradation (Fig 2, A). After release from I $\kappa$ B $\alpha$ , p65/p50 heterodimers enter the nucleus to activate gene transcription. Thus nuclear translocation of p65 after BCR stimulation was determined by means of immunofluorescence. In the unstimulated state p65 is predominantly located in the cytoplasm (Fig 2, B). In about 40% of naive CD21<sup>+</sup> B cells from HDs, p65 translocated to the nucleus after anti-IgM stimulation, whereas in CD21<sup>+</sup> and CD21<sup>low</sup> B cells from CVID 21low patients, nuclear translocation of p65 was severely reduced, confirming the impairment in NF- $\kappa$ B activation downstream of the BCR.

### Activation of PKC $\beta$ is not generally affected

Although NF- $\kappa$ B and Ca<sup>2+</sup> activation (see Fig E3, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) was reduced, phosphorylation of PLC $\gamma$ 2 was intact in B cells of patients with CVID (see Fig E3, B), suggesting that the canonical NF- $\kappa$ B signal after BCR stimulation is affected downstream of PKC $\beta$ . Therefore cells were stimulated with PKC-activating PMA. Nearly complete I $\kappa$ B $\alpha$  degradation was induced in all B-cell subpopulations (see Fig E4, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The higher MFI of I $\kappa$ B $\alpha$  in CD21<sup>low</sup> B cells after stimulation originated from a small proportion of cells that did not properly degrade the inhibitory protein, but it was not due to a generally impaired signal downstream of PKC $\beta$ , as seen in CARD11-deficient B cells (see Fig E4, B). This implied an overall integrity of the canonical NF- $\kappa$ B

signaling pathway downstream of PKC $\beta$  in B cells of patients with CVID.

### Preactivation contributes to reduced I $\kappa$ B $\alpha$ basal levels in CD21<sup>low</sup> B cells

Reduced I $\kappa$ B $\alpha$  protein levels in CD21<sup>low</sup> B cells were associated with increased baseline phosphorylation of I $\kappa$ B $\alpha$  compared with CD21<sup>+</sup> B cells (Fig 3, A), implying that the preactivated state of these cells<sup>13</sup> might contribute to reduced I $\kappa$ B $\alpha$  expression through its degradation. Treatment with the proteasome inhibitor MG132 has little effect on total I $\kappa$ B $\alpha$  levels<sup>22</sup> in all B-cell populations (data not shown) but increased the detection of phosphorylated I $\kappa$ B $\alpha$  in unstimulated CD21<sup>+</sup> B cells of healthy control subjects and patients but not CD21<sup>low</sup> B cells (Fig 3, B). After IgM stimulation, I $\kappa$ B $\alpha$  phosphorylation increased further in the presence of the inhibitor. Also, this effect was reduced especially in CD21<sup>low</sup> B cells of the patients, corroborating the notion of impaired BCR-induced NF- $\kappa$ B signaling.

Analysis of *NFKBIA* (encoding I $\kappa$ B $\alpha$ ) RNA expression in sorted B-cell subpopulations did not reveal significant differences in CD21<sup>low</sup> B cells compared with CD21<sup>+</sup> B cells (data not shown), indicating that the increased I $\kappa$ B $\alpha$  phosphorylation and reduced basal I $\kappa$ B $\alpha$  protein levels in CD21<sup>low</sup> B cells did not translate into increased transcription of NF- $\kappa$ B target genes (see also below).

### CD40- and TLR9-induced NF- $\kappa$ B signaling is altered in B cells from patients with CVID

To test whether the NF- $\kappa$ B pathway downstream of other receptor pathways than the BCR was affected, we analyzed the degradation of I $\kappa$ B $\alpha$  after CD40 activation. CD40 triggers the canonical NF- $\kappa$ B pathway independent of PKC $\beta$ -caspase recruitment domain family member 11 (CARD11)/mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1)/B-cell CLL/lymphoma 1 (BCL10) by using different TNF receptor-associated factors (TRAFs), TAK1-binding protein (TAB), and TGF- $\beta$ -activated kinase 1 (TAK1).<sup>23</sup> Stimulation with trimeric CD40L at optimal concentrations (1:500) for 15 minutes induced a nearly complete degradation of I $\kappa$ B $\alpha$  in all CD21<sup>+</sup> and CD21<sup>low</sup> B cells from HDs and patients, although the MFI of I $\kappa$ B $\alpha$  after stimulation was still slightly higher in patients' CD21<sup>+</sup> B cells (Fig 4, A). In both patient-derived B-cell subpopulations, the I $\kappa$ B $\alpha$  ratio was significantly increased compared with that in HDs in CD21<sup>low</sup> B cells mainly because of the lower baseline expression of I $\kappa$ B $\alpha$  (Fig 4, A). When CD40L was titrated to suboptimal concentrations, the response was detectably reduced in CD21<sup>low</sup> B cells but not in CD21<sup>+</sup> B cells of patients with CVID (Fig 4, B). Nuclear translocation of p65 was strongly induced after CD40 stimulation in CD21<sup>+</sup> B cells of HDs but reduced in CD21<sup>+</sup> and CD21<sup>low</sup> B cells of patients (Fig 4, C). Thus the CD40-mediated canonical NF- $\kappa$ B activation was affected in B cells from patients with CVID, although to a lesser degree than BCR-mediated activation. CD40 expression was comparable in patients' CD21<sup>+</sup> B cells and slightly reduced in CD21<sup>low</sup> B cells (data not shown), which might contribute to the altered response in CD21<sup>low</sup> but not CD21<sup>+</sup> B cells.

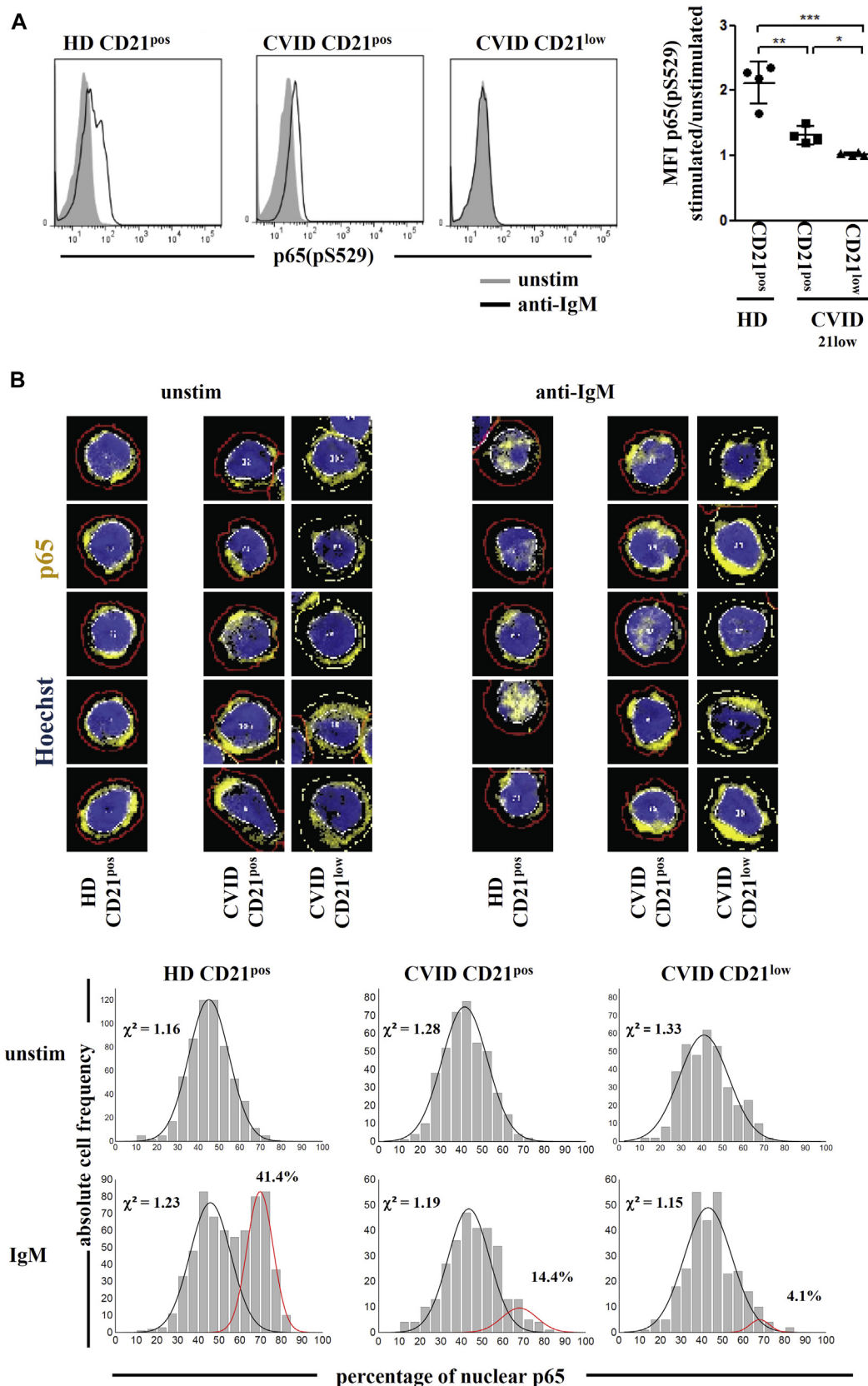


**FIG 2.** BCR-mediated activation of p65. **A**, Representative fluorescence-activated cell-sorting plots and statistical analysis of the MFI p65(pS529) ratio. **B**, Representative pictures and distribution of the percentage of p65 (yellow) nuclear translocation in CD21<sup>+</sup> B cells of HDs (n = 6) and CD21<sup>+</sup> (red) or CD21<sup>low</sup> B cells of CVID 21low patients (n = 6), as described in detail in this article's Online Repository. \*P < .05, \*\*P < .01, and \*\*\*P < .001.

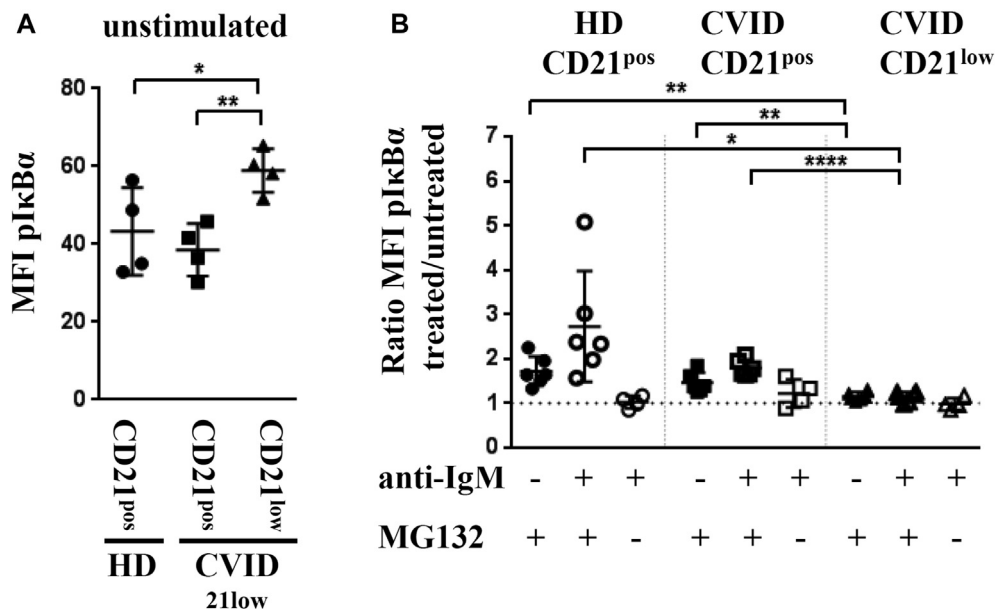


**FIG 3.** Phospho-IκBα levels in patients' B cells. **A**, Statistics of the MFI of phospho-IκBα in unstimulated B-cell subpopulations. **B**, Ratio of the MFI of phospho-IκBα after treatment with anti-IgM, MG132 and anti-IgM + MG132 to the MFI of untreated cells in B cells of HD CVID 21<sup>low</sup> patients. \* $P < .05$ , \*\* $P < .01$  and \*\*\*\* $P < .0001$ .

Also, the signaling cascade downstream of TLR9 involves the canonical NF-κB pathway. Therefore we determined TLR9-mediated IκBα degradation. Although IκBα was clearly degraded on CpG stimulation, the ratio of the MFI of IκBα revealed that IκBα degradation was significantly decreased in CD21<sup>+</sup> and especially CD21<sup>low</sup> B cells of CVID 21<sup>low</sup> patients compared with HDs (Fig 5). Similar to the results after CD40 stimulation, this reflected predominantly the reduced baseline expression of IκBα in CD21<sup>low</sup> B cells but also a partially impaired degradation after TLR stimulation.

### Reduced expression of NF-κB target genes and increased apoptosis after BCR stimulation

To determine the effect of the impaired activation of NF-κB signals after BCR stimulation, we examined the induction of the *bona fide* target genes *BCLXL* (*BCL2L1*) and IκBα (*NFKBIA*)<sup>19,20</sup> after anti-IgM stimulation for 36 hours. Induction of Bcl-xL was strongly reduced in patients' CD21<sup>+</sup> B cells compared with CD21<sup>+</sup> B cells of HDs and was absent in CD21<sup>low</sup> B cells (Fig 6, A). Upregulation of IκBα was absent in both CD21<sup>+</sup> and CD21<sup>low</sup> B cells of the patients, confirming impairment of the canonical NF-κB pathway downstream of the BCR (Fig 6, B).

Because Bcl-xL is an important NF-κB-induced antiapoptotic protein,<sup>24,25</sup> we were interested in regulation of apoptosis in patients' B-cell subpopulations. Annexin V staining was determined in isolated B cells after 17 hours of *in vitro* culture in the absence or presence of anti-IgM. CD21<sup>+</sup> B cells and especially CD21<sup>low</sup> B cells of the patients were more prone to undergo apoptosis, fitting a reduced prosurvival NF-κB signal (Fig 6, C).

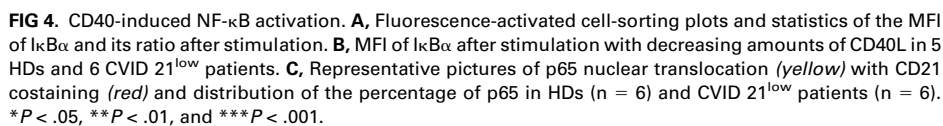
In addition, NF-κB signals contribute to the upregulation of activation markers, such as CD69, intercellular adhesion molecule 1 (ICAM-1), and CD25.<sup>26,27</sup> After gating on 4'-6-diamidino-2-phenylindole (DAPI)-negative cells, we found profoundly impaired upregulation of CD69, ICAM-1, and CD25 in CD21<sup>+</sup> and CD21<sup>low</sup> B cells of patients with CVID (Fig 6, D, and see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### Disturbed canonical NF-κB signaling is also seen in CD21<sup>low</sup> B cells derived from patients with HIV

To investigate whether the severely disturbed NF-κB signal in CD21<sup>low</sup> B cells is due to an underlying genetically determined immunodeficiency or a general nongenetic biochemical feature of CD21<sup>low</sup> B cells, we used CD21<sup>low</sup> B cells of 6 patients with HIV without antiretroviral treatment (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). In contrast to patients with CVID, in whom CD21<sup>low</sup> B cells usually resemble naive B cells according to the expression of CD27 and IgM, in patients with HIV, CD21<sup>low</sup> B cells can be assigned to different B-cell subpopulations depending on the expression of CD27 and the BCR isotype (and own unpublished observations).<sup>10</sup> To allow for direct comparison with naive-like CD21<sup>low</sup> B cells in patients with CVID, we focused on IgD<sup>+</sup>/CD27<sup>+</sup> CD21<sup>low</sup> B cells in patients with HIV. As observed for CVID-derived CD21<sup>low</sup> B cells, after short-term BCR stimulation for 35 minutes, IκBα degradation was significantly decreased compared with that seen in CD21<sup>+</sup> counterparts of HDs and patients (Fig 7, A). In line with our previous findings in patients with CVID, IκBα degradation was strongly induced after CD40 stimulation for 15 minutes, but at low concentrations of CD40L, the MFI of the residual IκBα expression was significantly higher in CD21<sup>low</sup> B cells compared with that in CD21<sup>+</sup> cells (Fig 7, B). Thus reduced IκBα degradation in CD21<sup>+</sup> cells was only seen in patients with CVID and might suggest a disease-associated impairment, whereas the severe deficiency in canonical NF-κB activation in CD21<sup>low</sup> B cells is a common feature of CD21<sup>low</sup> B cells.

### Patients with *NFKB1* haploinsufficiency present with the CVID smB– 21<sup>low</sup> phenotype

Last year, we described 3 familial cases of CVID with haploinsufficiency of p50 caused by heterozygous mutations in *NFKB1*,<sup>28</sup> and autosomal dominant mutations in *NFKB2* have been described in patients with a CVID-like syndrome.<sup>29,30</sup> Eight



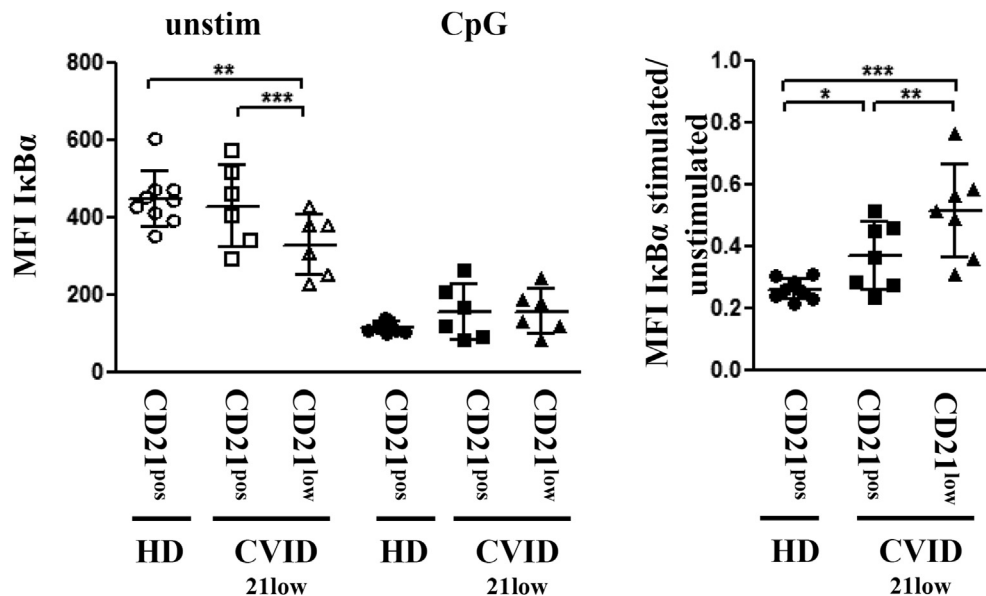


**FIG 5.** TLR9-mediated IκBα degradation. Statistics of the MFI of IκBα without and after CpG stimulation and the ratio thereof. \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001.

of 15 CVID 21low patients and 3 of 8 CVID 21norm patients have been analyzed by panel or whole-exome sequencing; no obvious disease-causing mutation/variant has been identified in *NFKB1*, *NFKB2*, *NFKBIA*, *IKBKB*, and *IKBKG*.

To corroborate a potential relation between the B-cell phenotype and disturbed NF-κB activation, we analyzed 2 published<sup>28</sup> and 3 additional adults with *NFKB1* mutations, presumably resulting in haploinsufficiency. All presented with a reduction of switched memory B-cell counts and a substantial accumulation of CD21<sup>low</sup> B cells in the peripheral blood, representing the CVID smB – 21low phenotype (Table I).

## DISCUSSION

Here we report impaired activation of the canonical NF-κB signaling pathway in B cells of a subgroup of patients with CVID with disturbed B-cell memory formation, as demonstrated by reduced degradation of IκBα, impaired phosphorylation and nuclear translocation of p65, and decreased upregulation of the NF-κB-induced proteins Bcl-xL and IκBα. Although in CD21<sup>+</sup> B cells of CVID 21low patients a residual NF-κB signal could be observed and seems to be associated with the underlying immunodeficiency, NF-κB activation in CD21<sup>low</sup> B cells was almost absent. This defect was linked to the specific B-cell stage because naive-like CD21<sup>low</sup> B cells in HIV-viremic patients demonstrated a similar impairment. Thus the signaling defect in CD21<sup>low</sup> B cells might be due to a regulatory response to chronic activation by an inflammatory environment, as in complex immune dysregulation in patients with CVID,<sup>9,31</sup> viremia in patients with HIV,<sup>32</sup> and patients with other chronic infections.<sup>33,34</sup>

The canonical NF-κB activation was mainly affected downstream of the BCR but also downstream of CD40 and less severely after CpG treatment. Thus there is a general defect in canonical NF-κB activation, which seems to correlate with the strength of the external signal and was overcome by a higher concentration of CD40L or stimulation by PMA, although not after BCR stimulation.

Cell stage specifically disturbed phosphorylation of IKK and degradation of IκBα in connection with normal PLCγ2 phosphorylation downstream of BCR activation, and a normal PMA-induced NF-κB signal has been described for murine B1a cells.<sup>35,36</sup> The authors proposed that decreased expression of Vav and increased expression of heat shock protein (hsp) 70 reduced the inhibition of phosphatases and resulted in defective NF-κB activation.<sup>36</sup> However, our preliminary results show that the general inhibition of phosphatases did not improve NF-κB signaling in CD21<sup>low</sup> B cells. Instead, increased IκBα phosphorylation at baseline and the lacking response to proteasome inhibition or BCR signals suggest that in CD21<sup>low</sup> B cells chronic activation can uncouple NF-κB pathway activation at this late stage from target gene transcription. Thus different mechanisms might be responsible for a similar pattern of altered signaling in CD21<sup>low</sup> B cells and B1a cells, which share activated status with CD21<sup>low</sup> B cells.<sup>13</sup> Although we could not identify the exact mechanism that failed during canonical NF-κB signaling, also the fact that BCR, CD40, and TLR signals are involved supports the idea that a late step during NF-κB activation is affected. On the other hand, it is important to note that B cells from CVID 21<sup>low</sup> patients show additional signaling alterations. In all mature B cells of CVID 21<sup>low</sup> patients, BCR-induced Ca<sup>2+</sup> mobilization was impaired, and as for NF-κB, the more severe impairment was observed in CD21<sup>low</sup> B cells.<sup>15</sup> Store-operated Ca<sup>2+</sup> entry was selectively affected,<sup>15</sup> but *vice versa*, this does not explain an impaired canonical NF-κB signal given the fact that ORAI1-deficient B cells show a normal canonical NF-κB signal after BCR stimulation (data not shown). These data suggest that the disturbed NF-κB signal we observed is only part of a broader signaling deficiency in B cells of CVID 21low patients, and consequently, we could not find mutations in known genes affecting specifically the activation of NF-κB. More extensive studies will be necessary to analyze the role of persistent inflammation in these patients on canonical NF-κB and also Ca<sup>2+</sup> signaling.

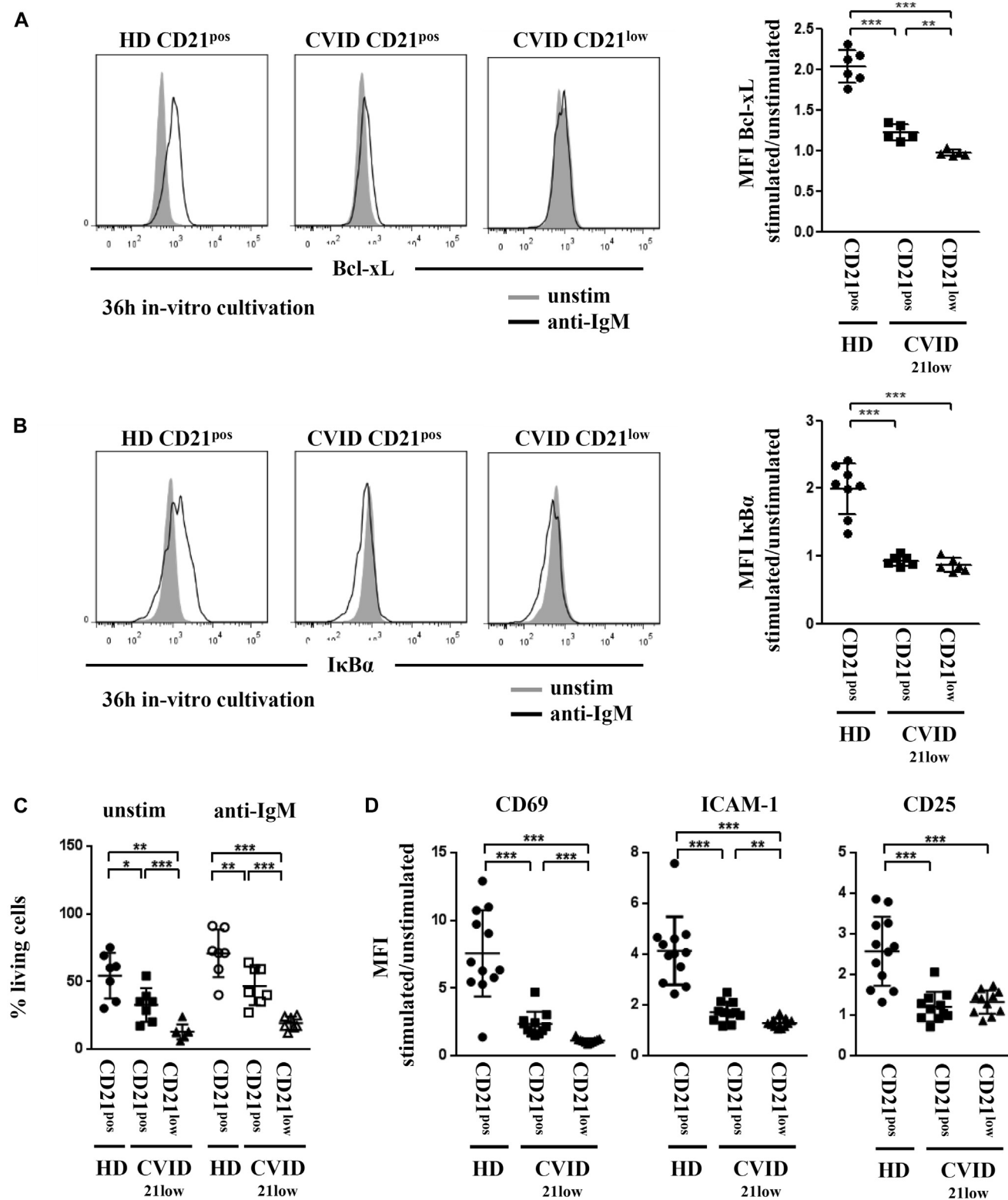


**FIG 6.** NF- $\kappa$ B target gene expression and survival in B cells of patients with CVID. **A** and **B**, Representative fluorescence-activated cell-sorting plots and statistics of the ratio of the MFI of Bcl-xL (Fig 6, A) and I $\kappa$ B $\alpha$  (Fig 6, B). **C**, Percentage of Annexin V<sup>+</sup>/4'-6-diamidino-2-phenylindole (DAPI)<sup>+</sup> B cells. **D**, Ratio of the MFI of CD69, ICAM-1, and CD25 with and without anti-IgM stimulation. \* $P$  < .05, \*\* $P$  < .01, and \*\*\* $P$  < .001.

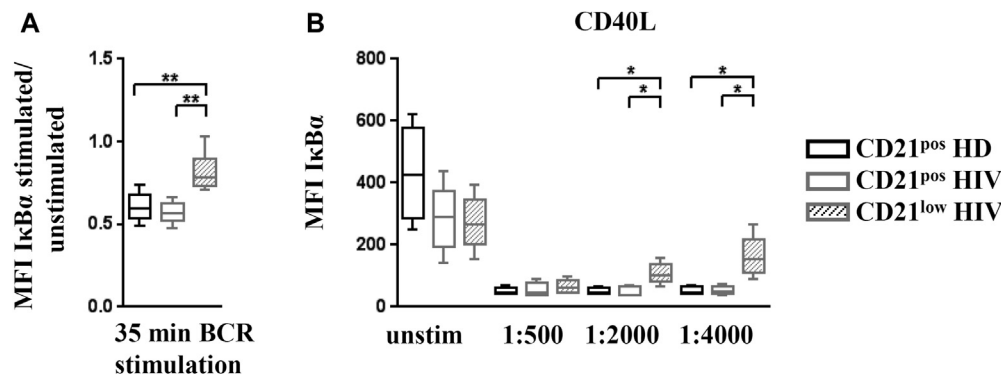


**FIG 7.** NF-κB signaling in HIV-derived CD21<sup>low</sup> B cells. **A**, Ratio of IκBα degradation in CD27<sup>+</sup>/IgD<sup>+</sup> CD21<sup>+</sup> or CD21<sup>low</sup> B cells after BCR stimulation in 6 HDs and 6 patients with HIV. **B**, IκBα degradation after stimulation with decreasing amounts of CD40L in 4 HDs and 5 patients with HIV. \**P* < .05 and \*\**P* < .01.

**TABLE I.** Patients with NFKB1 haploinsufficiency present with a CVID smB– 21<sup>low</sup> phenotype: Characteristics of patients with CVID with defined mutations of *NFKB1* resulting in NFKB1 haploinsufficiency

| No. | Sex | Age (y) | EUROClass classification | B-cell counts (cells/μL) | Switched memory B cells (%)* | CD21 <sup>low</sup> B cells (%)* | Previously reported |
|-----|-----|---------|--------------------------|--------------------------|------------------------------|----------------------------------|---------------------|
| 24  | M   | 70      | smB– 21 <sup>low</sup>   | 202                      | 1.9                          | 45.0                             | Fam089I.1           |
| 25  | F   | 37      | smB– 21 <sup>low</sup>   | 51                       | 0.9                          | 39.2                             | Fam089II.2          |
| 26  | F   | 40      | smB– 21 <sup>low</sup>   | 25                       | 0.3                          | 32.4                             | NP (Fam827_01)      |
| 27  | M   | 46      | smB– 21 <sup>low</sup>   | 367                      | 1.1                          | 35.9                             | NP (Fam830_01)      |
| 28  | F   | 40      | smB– 21 <sup>low</sup>   | 421                      | 2.3                          | 57.8                             | NP (Fam199_01)      |

NP, Not published.

\*Of B cells.

The role of specific signaling pathways in the differentiation of human memory B cells and plasma cells has not been fully elucidated. Severe deficiency in store-operated Ca<sup>2+</sup> entry because of ORAI1 or stromal interaction molecule 1 (STIM1) deficiency causes a combined immunodeficiency because of strong T-cell impairment<sup>37,38</sup> but did not affect immunoglobulin levels and memory B-cell formation (own unpublished data).<sup>37</sup> In contrast, in patients with homozygous defects in NF-κB signaling components, such as CARD11,<sup>21,39</sup> MALT1,<sup>40</sup> BCL10,<sup>41</sup> and IKKβ,<sup>42–44</sup> memory B-cell formation and antibody production are severely impaired, suggesting a more prominent role of NF-κB than Ca<sup>2+</sup> signaling in the differentiation of long-lived B-cell memory. This is supported by murine studies investigating the role of NF-κB and different NF-κB subunits in B-cell development and maturation and activation in general<sup>18,19,45</sup> and, more specifically, in plasma cell and memory B-cell formation.<sup>46</sup> Within the GC, only a small proportion of centrocytes is subjected to NF-κB signaling.<sup>47,48</sup> However, ablating c-Rel, one of the NF-κB transcription factors involved in the canonical pathway, in GC B cells disturbed GC maintenance and affinity maturation, and in the absence of p65, GCs formed, but plasma cell development was impaired.<sup>49</sup> Additionally, NF-κB is required for antigen-specific B-cell activation initiating the GC reaction.<sup>20</sup> Damdinsuren et al<sup>50</sup> have shown that a single round of BCR stimulation induces a first NF-κB signal that primes B cells to receive T-cell help. Thus it is tempting to speculate that impaired canonical NF-κB signaling in mature B cells, especially of CVID 21<sup>low</sup> patients, contributes to defective B-cell activation and survival and disturbs T-cell help and thus the outcome of the GC reaction, leading to

hypogammaglobulinemia and reduced class-switched memory B cells. The prominent role of this signaling pathway in the differentiation of B cells was recently underlined by the discovery of NFKB1-haploinsufficient patients with CVID.<sup>27</sup> Interestingly, all investigated patients not only presented with a defective class-switched memory B-cell compartment but also with a significant expansion of CD21<sup>low</sup> B cells, suggesting either an indirect link through the chronic inflammation observed in most of these patients or even a direct link of the disturbed canonical NF-κB activation and expansion of CD21<sup>low</sup> B cells.

In summary, we were able to demonstrate that the canonical NF-κB pathway is disturbed, especially in B cells of CVID 21<sup>low</sup> patients. This might result from a currently undefined genetic predisposition, which affects especially the BCR signal downstream of PLCγ2 but also the canonical NF-κB activation by TLR9 and CD40 signals. This disturbed canonical NF-κB pathway in CD21<sup>+</sup> B cells was associated with the underlying disease and might itself contribute to the immunodeficiency observed in the affected patients. CD21<sup>low</sup> B cells show an even more severe impairment of these signals independent of their origin. This observation suggests a cell stage-specific regulatory mechanism abrogating full activation of this population. An impaired NF-κB signal, as observed in the NFKB1-haploinsufficient patients, might itself contribute to the expansion of CD21<sup>low</sup> B cells. An improved understanding of these mechanisms might help the dissection of regulatory signaling properties during B-cell activation and their potential therapeutic application in patients with autoimmune disease, as well as immunodeficiency.

We thank our patients for their collaboration; Marion Klima, Monika Erler, and the team of the Infektiologikum Freiburg for patient care; Katrin Hübscher, Jessica Lineth Rojas Restrepo, and Pavla Mrovecova for excellent technical assistance; Dr Natalie Frede for NGS data analyses; and Philipp Scheuerlein for the collection of clinical data. We thank Dr Andreas Christ and Dr Marc Sultan, Novartis Institutes for BioMedical Research, Basel, Switzerland, for the exome sequencing of selected patients.

### Key messages

- B cells from CVID 21low patients display impaired canonical NF- $\kappa$ B signaling and target gene expression, especially after BCR stimulation.
- This signaling defect might contribute to the poor memory formation in these patients.
- CD21<sup>low</sup> B cells, independent of their origin, showed the strongest impairment, suggesting an additional cell stage-associated regulatory mechanism.

### REFERENCES

- Cunningham-Rundles C. The many faces of common variable immunodeficiency. *Hematology Am Soc Hematol Educ Program* 2012;2012:301-5.
- Jolles S. The variable in common variable immunodeficiency: a disease of complex phenotypes. *J Allergy Clin Immunol Pract* 2013;1:545-57.
- Warnatz K, Denz A, Dräger R, Braun M, Groth C, Wolff-Vorbeck G, et al. Severe deficiency of switched memory B cells (CD27(+)/IgM(-)/IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. *Blood* 2002;99:1544-51.
- Ochtrop ML, Goldacker S, May AM, Rizzi M, Draeger R, Hauschke D, et al. T and B lymphocyte abnormalities in bone marrow biopsies of common variable immunodeficiency. *Blood* 2011;118:309-18.
- Takemori T, Kaji T, Takahashi Y, Shimoda M, Rajewsky K. Generation of memory B cells inside and outside germinal centers. *Eur J Immunol* 2014;44:1258-64.
- Shlomchik MJ, Weisel F. Germinal center selection and the development of memory B and plasma cells. *Immunol Rev* 2012;247:52-63.
- Chinen J, Notarangelo LD, Shearer WT. Advances in basic and clinical immunology in 2014. *J Allergy Clin Immunol* 2015;135:1132-41.
- Salzer U, Warnatz K, Peter HH. Common variable immunodeficiency—an update. *Arthritis Res Ther* 2012;14:223.
- Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood* 2008;111:77-85.
- Moir S, Fauci AS. Insights into B cells and HIV-specific B-cell responses in HIV-infected individuals. *Immunol Rev* 2013;254:207-24.
- Wehr C, Eibel H, Masilamani M, Ilges H, Schlesier M, Peter HH, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. *Clin Immunol* 2004;113:161-71.
- Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B cells contain mostly autoreactive unresponsive clones. *Blood* 2010;115:5026-36.
- Rakhmanov M, Keller B, Gutenberger S, Foerster C, Hoenig M, Driessen G, et al. Circulating CD21low B cells in common variable immunodeficiency resemble tissue homing, innate-like B cells. *Proc Natl Acad Sci U S A* 2009;106:13451-6.
- Rakhmanov M, Gutenberger S, Keller B, Schlesier M, Peter HH, Warnatz K. CD21low B cells in common variable immunodeficiency do not show defects in receptor editing, but resemble tissue-like memory B cells. *Blood* 2010;116:3682-3.
- Foerster C, Voelxen N, Rakhmanov M, Keller B, Gutenberger S, Goldacker S, et al. B cell receptor-mediated calcium signaling is impaired in B lymphocytes of type Ia patients with common variable immunodeficiency. *J Immunol* 2010;184:7305-13.
- Bendall HH, Sikes ML, Ballard DW, Oltz EM. An intact NF-kappa B signaling pathway is required for maintenance of mature B cell subsets. *Mol Immunol* 1999;36:187-95.
- Gerondakis S, Siebenlist U. Roles of the NF-kappaB pathway in lymphocyte development and function. *Cold Spring Harb Perspect Biol* 2010;2:a000182.
- Pasparakis M, Schmidt-Supprian M, Rajewsky K. IkappaB kinase signaling is essential for maintenance of mature B cells. *J Exp Med* 2002;196:743-52.
- Gugasyan R, Grumont R, Grossmann M, Nakamura Y, Pohl T, Nestic D, et al. Rel/NF-kappaB transcription factors: key mediators of B-cell activation. *Immunol Rev* 2000;176:134-40.
- Kaileh M, Sen R. NF-kappaB function in B lymphocytes. *Immunol Rev* 2012;246:254-71.
- Stepensky P, Keller B, Buchta M, Kienzler AK, Elpeleg O, Somech R, et al. Deficiency of caspase recruitment domain family, member 11 (CARD11), causes profound combined immunodeficiency in human subjects. *J Allergy Clin Immunol* 2013;131:477-85.e1.
- Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, et al. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev* 1995;9:1586-97.
- Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-kappaB signaling pathways. *Nat Immunol* 2011;12:695-708.
- Grumont RJ, Rourke IJ, Gerondakis S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev* 1999;13:400-11.
- Grossmann M, O'Reilly LA, Gugasyan R, Strasser A, Adams JM, Gerondakis S. The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression. *EMBO J* 2000;19:6351-60.
- Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J* 1995;9:899-909.
- Cross SL, Halden NF, Lenardo MJ, Leonard WJ. Functionally distinct NF-kappa B binding sites in the immunoglobulin kappa and IL-2 receptor alpha chain genes. *Science* 1989;244:466-9.
- Fliegau M, Bryant VL, Frede N, Slade C, Woon ST, Lehnert K, et al. Haploinsufficiency of the NF-kappaB1 Subunit p50 in common variable immunodeficiency. *Am J Hum Genet* 2015;97:389-403.
- Chen K, Coonrod EM, Kumanovics A, Franks ZF, Durtsche JD, Margraf RL, et al. Germline mutations in NFKB2 implicate the noncanonical NF-kappaB pathway in the pathogenesis of common variable immunodeficiency. *Am J Hum Genet* 2013;93:812-24.
- Liu Y, Hanson S, Gurugama P, Jones A, Clark B, Ibrahim MA. Novel NFKB2 mutation in early-onset CVID. *J Clin Immunol* 2014;34:686-90.
- Mouillot G, Carmagnat M, Gerard L, Garnier JL, Fieschi C, Vince N, et al. B-cell and T-cell phenotypes in CVID patients correlate with the clinical phenotype of the disease. *J Clin Immunol* 2010;30:746-55.
- Moir S, Ho J, Malaspina A, Wang W, DiPoto AC, O'Shea MA, et al. Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J Exp Med* 2008;205:1797-805.
- Charles ED, Brunetti C, Marukian S, Ritola KD, Talal AH, Marks K, et al. Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B-cell subset. *Blood* 2011;117:5425-37.
- Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, Traore B, et al. Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. *J Immunol* 2009;183:2176-82.
- Morris DL, Rothstein TL. Abnormal transcription factor induction through the surface immunoglobulin M receptor of B-1 lymphocytes. *J Exp Med* 1993;177:857-61.
- Holodick NE, Rothstein TL. Atypical response of B-1 cells to BCR ligation: a speculative model. *Front Immunol* 2013;4:457.
- Feske S. Immunodeficiency due to defects in store-operated calcium entry. *Ann N Y Acad Sci* 2011;1238:74-90.
- Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, et al. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 2006;441:179-85.
- Greil J, Rausch T, Giese T, Bandapalli OR, Daniel V, Bekeredjian-Ding I, et al. Whole-exome sequencing links caspase recruitment domain 11 (CARD11) inactivation to severe combined immunodeficiency. *J Allergy Clin Immunol* 2013;131:1376-83.e3.
- Turvey SE, Durandy A, Fischer A, Fung SY, Geha RS, Gewies A, et al. The CARD11-BCL10-MALT1 (CBM) signalosome complex: stepping into the limelight of human primary immunodeficiency. *J Allergy Clin Immunol* 2014;134:276-84.
- Torres JM, Martinez-Barricarte R, Garcia-Gomez S, Mazariegos MS, Itan Y, Boisson B, et al. Inherited BCL10 deficiency impairs hematopoietic and nonhematopoietic immunity. *J Clin Invest* 2014;124:5239-48.
- Pannicke U, Baumann B, Fuchs S, Henneke P, Rensing-Ehl A, Rizzi M, et al. Deficiency of innate and acquired immunity caused by an IKBKB mutation. *N Engl J Med* 2013;369:2504-14.

43. Mousallem T, Yang J, Urban TJ, Wang H, Adeli M, Parrott RE, et al. A nonsense mutation in IKBKB causes combined immunodeficiency. *Blood* 2014;124:2046-50.
44. Burns SO, Plagnol V, Gutierrez BM, Al Zahrani D, Curtis J, Gaspar M, et al. Immunodeficiency and disseminated mycobacterial infection associated with homozygous nonsense mutation of IKKbeta. *J Allergy Clin Immunol* 2014;134:215-8.
45. Goetz CA, Baldwin AS. NF-kappaB pathways in the immune system: control of the germinal center reaction. *Immunol Res* 2008;41:233-47.
46. Klein U, Heise N. Unexpected functions of nuclear factor-kappaB during germinal center B-cell development: implications for lymphomagenesis. *Curr Opin Hematol* 2015;22:379-87.
47. Basso K, Dalla-Favera R. Germinal centres and B cell lymphomagenesis. *Nat Rev Immunol* 2015;15:172-84.
48. Shaffer AL, Rosenwald A, Hurt EM, Giltmane JM, Lam LT, Pickeral OK, et al. Signatures of the immune response. *Immunity* 2001;15:375-85.
49. Heise N, De Silva NS, Silva K, Carette A, Simonetti G, Pasparakis M, et al. Germinal center B cell maintenance and differentiation are controlled by distinct NF-kappaB transcription factor subunits. *J Exp Med* 2014;211:2103-18.
50. Damdinsuren B, Zhang Y, Khalil A, Wood WH 3rd, Becker KG, Shlomchik MJ, et al. Single round of antigen receptor signaling programs naive B cells to receive T cell help. *Immunity* 2010;32:355-66.

## METHODS

### Patients

All investigations were performed after patients had signed the informed consent form. The study was approved by the institutional review board (FR251/13 and 66/13) according to the declaration of Helsinki. The diagnosis of CVID was based on the 1999 European Society for Immunodeficiencies criteria as follows: a marked decrease in IgG levels ( $\geq 2$  SDs less than the mean for age) and a marked decrease in at least 1 of the isotypes IgM or IgA, as well as (1) onset of immunodeficiency at greater than 2 years of age, (2) absent isohemagglutinins and/or poor response to vaccines, and (3) exclusion of defined causes of hypogammaglobulinemia.

### Antibodies

Anti-CD19 allophycocyanin-Cy7, anti-CD19 Brilliant Violet 421, anti-CD21 phycoerythrin (PE)-Cy7, anti-CD25 peridinin-chlorophyll-protein complex-Cy5.5, anti-CD38 PerCp-Cy5.5, anti-CD86 allophycocyanin (BioLegend, San Diego, Calif), Annexin V PE, anti-CD27 Brilliant Violet 605, anti-CD69 fluorescein isothiocyanate, anti-ICAM-1 PE, anti-p65(pS256) Alexa Fluor 647, anti-IkBa PE, anti-PLC $\gamma$ 2 (pY759) (BD Biosciences, San Jose, Calif); anti-Bcl-xL Alexa Fluor 488, rabbit anti-human p65 XP (Cell Signaling Technology, Danvers, Mass); anti-IgD fluorescein isothiocyanate (SouthernBiotech); anti-IgM Alexa Fluor 488, anti-IgM Alexa Fluor 647 (Jackson ImmunoResearch Laboratories, Suffolk, United Kingdom); anti-human/mouse pIKBa (pS32/S36) eFluor 660 (eBioscience, San Diego, Calif); goat anti-human CD21 (GeneTex, Irvine, Calif); goat anti-mouse Alexa Fluor 647, goat anti-rabbit Alexa Fluor 546 (Life Technologies, Carlsbad, Calif); and Hoechst33342 (Sigma-Aldrich).

### Molecular biology

RNA of fluorescence-activated cell-sorted B-cell subpopulations was isolated by using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands), and cDNA was generated by use of Superscript II Reverse Transcriptase (Life Technologies, Carlsbad, Calif). Quantitative RT-PCR was performed with SBYR Green (Roche Diagnostics, Mannheim, Germany), and primer pairs were used as follows: IkBa, forward TGAAAGACGAGGAGTACGAGCAG and reverse CTCATGGATGATGGCCAAAGTGCA; RPLP0, forward CGGCCCCGAGAAGACCT and reverse TTGTACCCGTTGATGATAGAATGG (Apara Bioscience GmbH, Denzlingen, Germany).

### Statistics

Data were analyzed with GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif). Normal distribution was analyzed, and statistical differences were determined by using the paired *t* test, the unpaired *t* test, the Wilcoxon matched pairs test, or the Mann-Whitney test. Differences were considered statistically significant at *P* values of less than .05. Data are shown as means with SDs.

### Automatic scoring algorithm of p65 nuclear translocation in human B cells from confocal microscopy images

In the first step cell outlines were determined from the transmitted light differential interference contrast (DIC) images. Because a transmitted light

image usually lacks enough contrast to allow direct segmentation and intensity-based methods usually also fail to separate the cells from the background, we made use of the naturally appearing shadows around each cell that characterize a DIC image. We applied a shadow-casting algorithm from the ImageJ (National Institutes of Health, Bethesda, Md) toolkit to extend the endogenous shadows of the DIC images. We cast shadows from 8 directions equally distributed at 45° angles, thus creating 8 images of slightly rotated shadows around each cell. These 8 images were then combined, and the resulting image was segmented based on intensity values. The cells were filtered according to their diameter, which was set within the range of the mean cell size plus or minus the typical variance to identify cells that were forming clusters that the segmentation algorithm did not separate, as well as to exclude small-cell debris.

The nuclear outline was determined from the Hoechst fluorescence image by using the Otsu thresholding algorithm in ImageJ (water-shading algorithm). The segmented nuclei were submitted to the particle-finder algorithm of ImageJ, thus resulting in the outline of each nucleus after proper filtering for size (see the caption of Fig E4). Additionally, the cell clusters that were identified based on their large size were further segmented according to the number of contained nuclei.

The segmented cells received an ID for further tracking and were further analyzed regarding: (1) IgM (to identify B cells and to exclude non-B-cell contaminants) and (2) CD21 expression (to identify CD21<sup>+</sup> and CD21<sup>low</sup> B cells).

An automated algorithm selected only the fluorescence signal (both IgM and CD21) above a fourth of the maximum fluorescence intensity in an image that typically represented 2-fold of the background noise. Unspecific labeling because of use of secondary antibodies was excluded by appropriate control measurements because it felt less than the threshold. Because CD21 and IgM labeling is membrane bound, we additionally excluded cells showing a comparable nuclear and cytoplasmic fluorescence signal.

Using this strategy, we further analyzed only B cells in the image plane with a distinct nucleus that could be classified into CD21<sup>+</sup> or CD21<sup>low</sup> cells. The whole-cell and nuclear segmentation images were combined to calculate the whole-cell body area, as well as the nuclear area, and to generate a whole-cell mask and a nuclear area mask. The p65 distribution ratio was then calculated by multiplying the raw p65 fluorescence images with the mask of identified whole-cell bodies and with the mask of cell nuclei, respectively. The resulting p65 images specific for the identified cells and identified nuclei contain 2 values for each cell: a value of p65 contribution in the whole cell and a value of p65 contribution only to the nucleus. The percentage of nuclear p65 contribution was calculated for each identified cell in each image for each patient or HD for each condition. These values together with the corresponding cellular IDs were summarized in a table, and the frequencies per condition were plotted in a histogram (Fig E2). These histograms were fitted with the sum of 2 normal distributions (2-peak Gaussian functions), and the corresponding fitted parameters were calculated and used to automatically calculate the portion of cells showing p65 nuclear translocation per condition.

### REFERENCE

- E1. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. *Blood* 2008; 111:77-85.

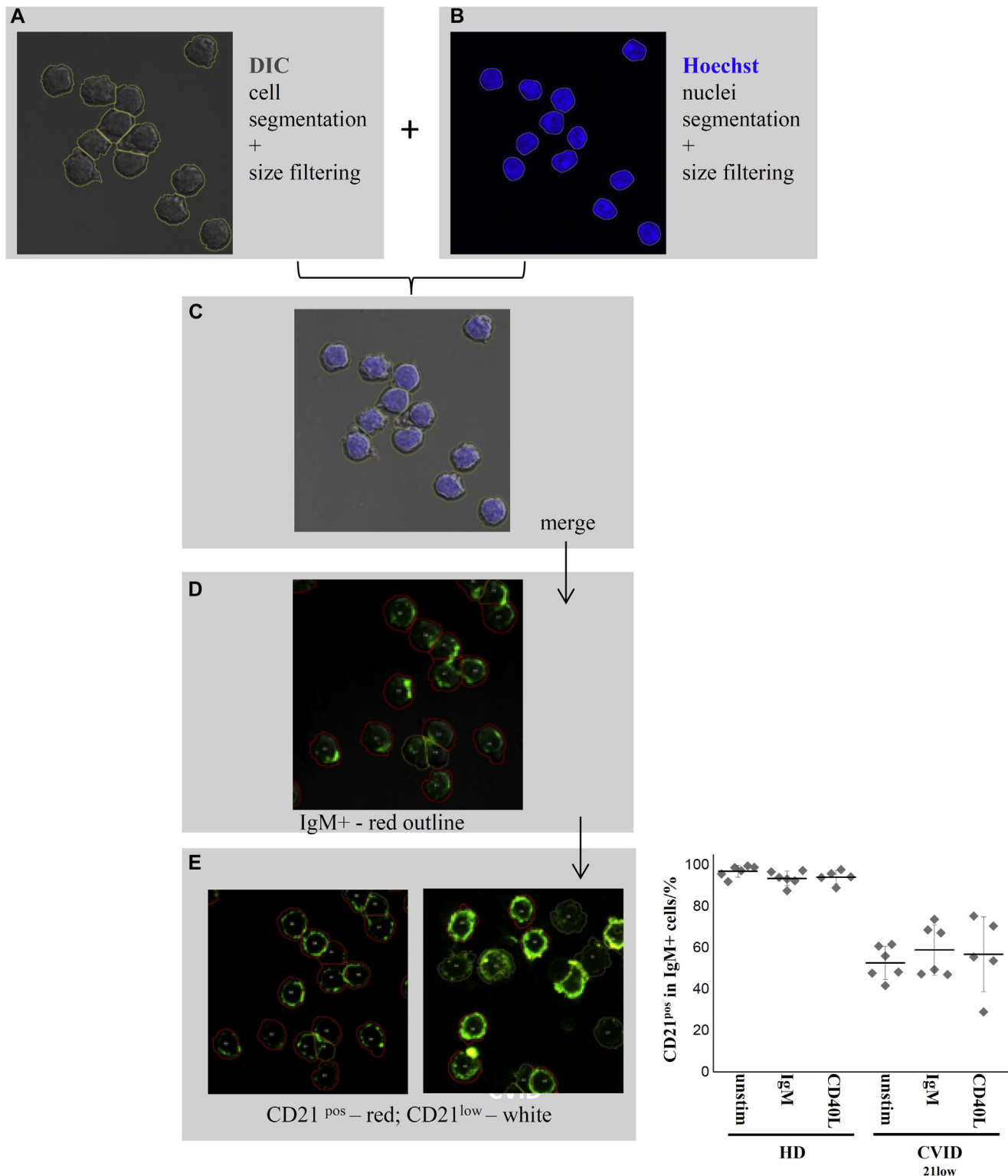


**FIG E1.** Automatic algorithm for the segmentation and classification of individual CD21<sup>+</sup> and CD21<sup>low</sup> B cells, respectively. **A**, DIC images were segmented after shadow casting them from 8 different angles. The objects were filtered by size to exclude cellular debris. The average size of a human B cell is known to be 80  $\mu\text{m}^2$ . We set the condition that objects of less than 20  $\mu\text{m}^2$  are debris. These objects have been excluded from further analysis. **B**, By using an intensity-thresholding algorithm, fluorescence images of the nuclear staining was segmented, and then the objects were filtered by size to exclude debris and other artifacts: only nuclei with a size of between 15 and 100  $\mu\text{m}^2$  were taken into account. Cell clusters from DIC images of a size of greater than 180  $\mu\text{m}^2$  were separated according to the number of contained cell nuclei. **C**, Results of object segmentation from the DIC images and Hoechst fluorescence images were merged

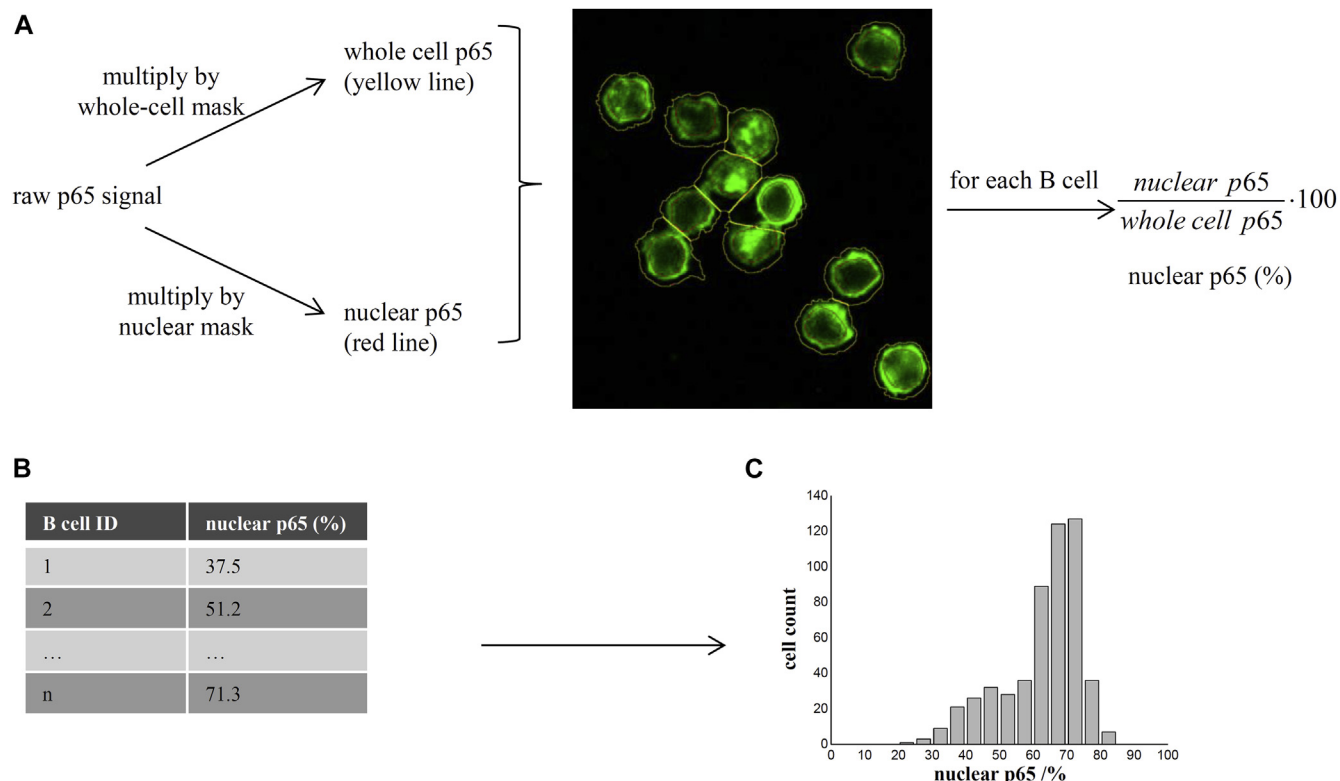


**FIG E2.** Automatic approach to evaluate the frequency of p65 nuclear distribution from confocal fluorescence images. **A**, Raw p65 fluorescence images were multiplied either by the mask of the whole-cell areas (from segmented DIC images) or by the nuclear mask (from segmented Hoechst images) to generate 2 images: 1 identifying the total p65 signal in the whole cell body and 1 identifying p65 signal only within the nucleus of each segmented B cell ( $\text{IgM}^+$ ). The 2 images are merged in 1 exemplary image of B cells of an HD after IgM treatment over 2 hours. The ratio between the 2 values for each cell defines the contribution of nuclear p65 to total p65 within a cell and thus is an indicator of p65 nuclear translocation. **B**, All these ratio values were summarized for each condition into a table that allows us to retrieve the source and characteristics of each cell ( $\text{CD21}^+$  or  $\text{CD21}^{\text{low}}$ ,  $\text{IgM}^+$  or  $\text{IgM}^-$ ) by using the cellular ID. **C**, From each table, we generated a graph showing the frequency of the nuclear p65 ratio values per condition. The background was not excluded, and we assumed it can vary strongly between different experiments. However, the bimodal nature of the distribution indicating cells not showing any p65 nuclear translocation and cells with p65 nuclear translocation is not influenced by any experimental parameter. Thus we approximated all distributions using 2-peak Gaussian functions to achieve minimum reduced  $\chi^2$  values (best approximation of the experimental data) to determine the percentage of B cells showing nuclear p65 translocation of the total number of B cells per condition.

together to further analyze only the objects having plausible B-cell size and only 1 nucleus of plausible size. Each cell received a cell ID for further tracking. **D**, By using simultaneous IgM fluorescence staining, the identified cells were verified to be B cells, whereas non-B-cell contaminants were excluded from further analysis. Non-B-cell contaminants represented  $4.4\% \pm 1.3\%$  of all imaged cells in patients with CVID and  $6.7\% \pm 1.5\%$  in HDs. Internal intensity references (ie, maximum fluorescence intensity, as well as background and background noise within an image) were used to counteract possible artifacts induced by means of variable acquisition and/or staining conditions typical for confocal microscopy. **E**, Only the  $\text{IgM}^+$  cells were evaluated as far as their CD21 signal is concerned. As previously described, the maximum fluorescence intensity, as well as background and background noise of CD21 staining within an image, were used as reference, to exclude artifacts caused by variable fluorescence signal between images. Only a signal greater than maximal intensity ( $I_{\text{max}}$ )/2 was considered to indicate  $\text{CD21}^+$  cells (corresponding to  $2 \times$  the background noise); at less than this value,  $\text{IgM}^+$  cells were considered to be  $\text{CD21}^{\text{low}}$  cells. For HDs, we could find only  $\text{CD21}^+$   $\text{IgM}^+$  cells, whereas for the patients with CVID, values varied between 20% and 40%. Thus our results are in accordance with results of previous studies.

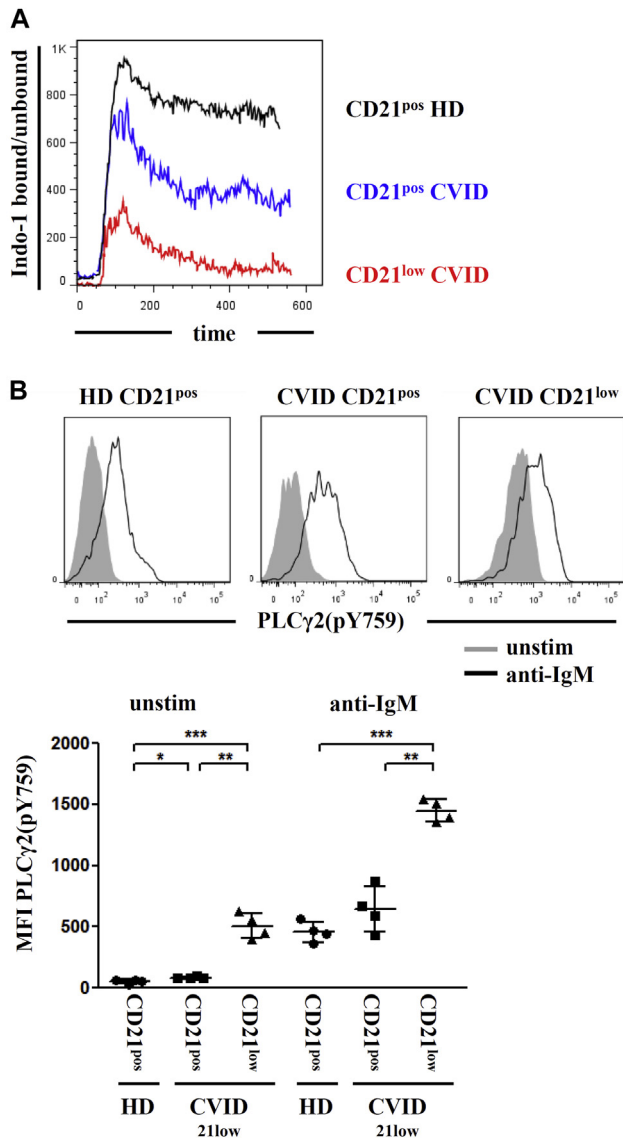


**FIG E3.** Impaired  $\text{Ca}^{2+}$  mobilization but intact phosphorylation of PLC $\gamma$ 2 in CD21<sup>+</sup> and CD21<sup>low</sup> B cells of patients with CVID. **A**, Representative fluorescence-activated cell-sorting plot of anti-IgM-induced  $\text{Ca}^{2+}$  mobilization in CD21<sup>+</sup> B cells of an HD and CD21<sup>+</sup> and CD21<sup>low</sup> B cells of a patient with CVID. **B**, Phosphorylation of PLC $\gamma$ 2 without and after stimulation with anti-IgM. \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .

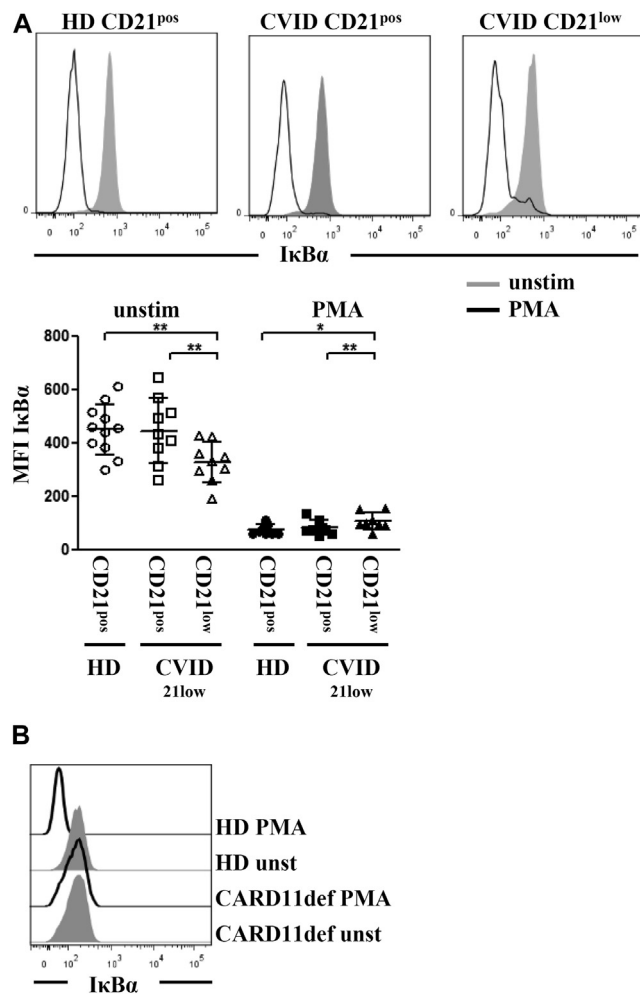


**FIG E4.** PMA-induced NF- $\kappa$ B activation is not affected in patients with CVID. **A**, Representative fluorescence-activated cell sorting plots and statistical analysis of the MFI of I $\kappa$ B $\alpha$  after PMA stimulation in B cells of HDs ( $n = 11$ ) and CVID 21<sup>low</sup> patients ( $n = 9$ ). **B**, Degradation of I $\kappa$ B $\alpha$  after PMA stimulation in naive B cells of a CARD11-deficient patient and 1 HD. \* $P < .05$  and \*\* $P < .01$ .

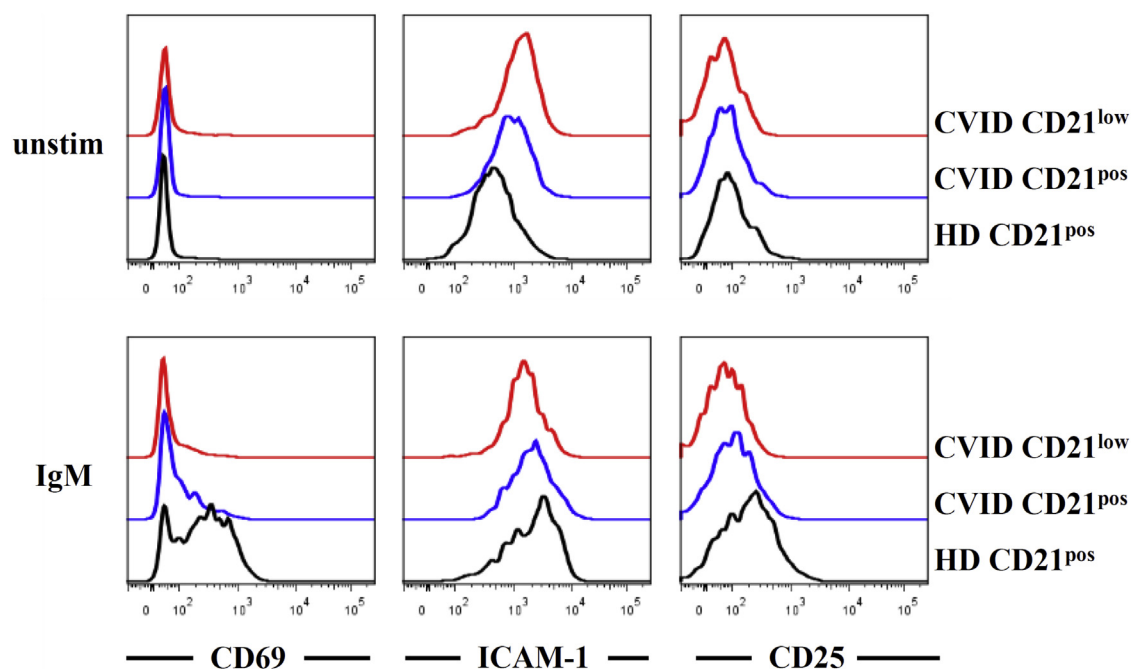


**FIG E5.** Reduced upregulation of B-cell activation markers in patients with CVID. Fluorescence-activated cell-sorting plots for CD69, ICAM-1, and CD25 expression without and after stimulation with anti-IgM in B-cell subpopulations of 1 representative HD and 1 CVID 21<sup>low</sup> patient.

**TABLE E1.** Characteristics of patients with CVID

| No. | Sex | Age (y) | Serum IgG* | EUROClass classification | Route of immunoglobulin substitution | Immunosuppression†   | Clinical manifestations       | B-cell counts (cells/ $\mu$ L) | Switched memory B cells (%)‡ | CD21 <sup>low</sup> B cells (%)‡ |
|-----|-----|---------|------------|--------------------------|--------------------------------------|----------------------|-------------------------------|--------------------------------|------------------------------|----------------------------------|
| 1   | F   | 44      | 13.4       | smB– 21 <sup>low</sup>   | IVIG                                 |                      | Spl, LA, ILD, AIE             | 255                            | 0.13                         | 58.1                             |
| 2   | F   | 64      | 8.35       | smB– 21 <sup>low</sup>   | SCIG                                 | Prednisolone, 5 mg/d | Spl, LA, NRH                  | 108                            | 0.7                          | 51.7                             |
| 3   | F   | 63      | 8.54       | smB– 21 <sup>low</sup>   | IVIG                                 |                      |                               | 77                             | 3.2                          | 45.3                             |
| 4   | F   | 46      | 4.3        | smB– 21 <sup>low</sup>   | SCIG                                 |                      | Spl, LA                       | 141                            | 1.6                          | 68.6                             |
| 5   | F   | 52      | 8.46       | smB– 21 <sup>low</sup>   | IVIG                                 | Prednisolone, 5 mg/d | Spl, LA, GD                   | 68                             | 4.4                          | 22.8                             |
| 6   | F   | 52      | 6.0        | smB– 21 <sup>low</sup>   | SCIG                                 | Prednisolone, 5 mg/d | ILD, AIC, arthritis           | 20                             | 4.4                          | 62.6                             |
| 7   | F   | 45      | 11.0       | smB– 21 <sup>low</sup>   | IVIG                                 |                      | Spl, LA, GD                   | 93                             | 0.3                          | 43.3                             |
| 8   | F   | 74      | 8.39       | smB– 21 <sup>low</sup>   | SCIG                                 | Prednisolone, 5 mg/d | Spl, LA, GD                   | 132                            | 1.0                          | 49.4                             |
| 9   | F   | 71      | 10.8       | smB– 21 <sup>low</sup>   | IVIG                                 |                      | Spl, LA, AIC                  | 82                             | 2.3                          | 24.3                             |
| 10  | F   | 39      | 11.2       | smB– 21 <sup>low</sup>   | IVIG                                 |                      | Spl, LA, GD                   | 270                            | 0.6                          | 23.8                             |
| 11  | F   | 60      | 12.7       | smB– 21 <sup>low</sup>   | IVIG                                 |                      | Spl, LA, AIC, AIE, GD         | 146                            | 0.81                         | 45.5                             |
| 12  | M   | 27      | 7.5        | smB– 21 <sup>low</sup>   | SCIG                                 | Prednisolone, 5 mg/d | Spl, LA, GD                   | 206                            | 1.7                          | 19.4                             |
| 13  | F   | 76      | 7.7        | smB– 21 <sup>low</sup>   | SCIG                                 |                      | LA, GD                        | 31                             | 0.5                          | 48.6                             |
| 14  | F   | 55      | 8.4        | smB– 21 <sup>low</sup>   | SCIG                                 | Prednisolone, 5 mg/d | Spl, LA, AIC, GD              | 192                            | 0.4                          | 37.4                             |
| 15  | F   | 39      | 11.4       | smB– 21 <sup>low</sup>   | SCIG                                 | Abatacept            | Spl, AIC, AIE, arthritis, NRH | 91                             | 1.6                          | 33.5                             |
| 16  | M   | 44      | 8.2        | smB– 21 <sup>norm</sup>  | IVIG                                 |                      | Spl, LA, AIE                  | 244                            | 1.3                          | 4.4                              |
| 17  | M   | 60      | 7.3        | smB– 21 <sup>norm</sup>  | IVIG                                 |                      |                               | 246                            | 4.5                          | 6.5                              |
| 18  | M   | 59      | 5.0        | smB– 21 <sup>norm</sup>  | IVIG                                 |                      | Spl, LA                       | 117                            | 1.1                          | 5.8                              |
| 19  | F   | 37      | 8.4        | smB– 21 <sup>norm</sup>  | IVIG                                 |                      | Spl, LA, GD                   | 436                            | 0.8                          | 3.9                              |
| 20  | F   | 27      | 9.7        | smB– 21 <sup>norm</sup>  | IVIG                                 |                      |                               | 376                            | 1.9                          | 3.0                              |
| 21  | M   | 31      | 9.8        | smB– 21 <sup>norm</sup>  | SCIG                                 |                      |                               | 100                            | 2.2                          | 5.6                              |
| 22  | F   | 40      | 11.8       | smB– 21 <sup>norm</sup>  | SCIG                                 |                      |                               | 144                            | 0.0                          | 4.4                              |
| 23  | F   | 27      | 8.5        | smB– 21 <sup>norm</sup>  | SCIG                                 |                      | Spl                           | 372                            | 1.8                          | 7.8                              |

Patients 1, 4, 5, 6, 10, 14, and 22 were submitted to whole-exome sequencing, and patients 12, 13, 19 and 23 were analyzed by using panel sequencing. EUROClass classification<sup>E1</sup> smB– refers to patients with CVID with less than 2% switched memory B cells of total B cells in the peripheral blood. 21<sup>low</sup> defines patients with more than 10% CD21<sup>low</sup> B cells of total B cells, and 21<sup>norm</sup> defines patients with less than 10%.

AIC, Autoimmune cytopenia; AIE, autoimmune enteropathy; GD, granulomatous disease; ILD, interstitial lung disease; IVIG, intravenous immunoglobulins; LA, lymphadenopathy; NRH, nodular regenerative hyperplasia; SCIG, subcutaneous immunoglobulins Spl, splenomegaly.

\*Serum IgG levels in grams per liter at the day of blood withdrawal.

†Immunosuppressive therapy at the time of investigation is listed.

‡Of B cells.

**TABLE E2.** Characteristics of patients with HIV

| No. | Plasma HIV RNA (copies/mL) | CD21 <sup>low</sup> B cells (%)* |
|-----|----------------------------|----------------------------------|
| 1   | 38                         | 47.2                             |
| 2   | 131,389                    | 20.7                             |
| 3   | 142,483                    | 16.9                             |
| 4   | 1,215                      | 4.8                              |
| 5   | 250                        | 13.9                             |
| 6   | 38,672                     | 10.8                             |

No patient had received antiretroviral therapy at the time point of analysis.

\*Of B cells.