

T-helper signals restore B-cell receptor signaling in autoreactive anergic B cells by upregulating CD45 phosphatase activity

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Background: We recently identified a human B-cell population that is naturally autoreactive and tolerized by functional anergy (B_{ND} cells).

Objective: We sought to identify the molecular mechanism of how anergic autoreactive B_{ND} cells escape functional anergy and whether this process is altered in patients with lupus.

Methods: Isolated peripheral blood naive and B_{ND} cells were cultured with various stimuli, and their activation status was determined by using an intracellular Ca²⁺ mobilization assay. Lyn kinase and Syk activities were assessed by using phospho-flow analysis. CD45 phosphatase activity was determined by using a novel flow-based assay, which takes advantage of the fluorogenic properties of phosphorylated coumaryl amino propionic acid, an analog of phosphotyrosine, which can be incorporated into peptides. Real-time quantitative PCR was used to quantitate *LYN*, *SYK*, and *CD45* mRNA.

Results: T-helper signals reversed the state of anergy, allowing B_{ND} cells to fully respond to antigenic stimulation by restoring signaling through the B-cell receptor (BCR). The mechanism was dependent on increased activity of the tyrosine phosphatase CD45 and CD45-dependent activation of Lyn and Syk. CD45 phosphatase activity was increased by T-cell help both in B_{ND} and naive B cells. Furthermore, we found that B_{ND} cells obtained from patients with systemic lupus erythematosus exhibited increased CD45 activity and BCR-signaling capacity, thus being less tolerized than B_{ND} cells from healthy control subjects.

Conclusion: Our findings suggest that CD45 is a key regulator of BCR-signaling thresholds mediated by T-cell help. This raises the possibility that B_{ND} cells could represent precursors of

autoantibody-secreting plasma cells and suggests a role for these autoreactive B cells in contributing to autoimmunity if not properly controlled. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: B-cell anergy, break of tolerance, T-cell help, CD45 phosphatase activity, systemic lupus erythematosus

Antibody diversity is a result of the near-stochastic process of V(D)J recombination, giving rise to B cells expressing B-cell receptors (BCRs) that are able to recognize an almost unlimited number of antigens. Because of the randomness of this process, B cells with self-reactive characteristics are produced and therefore need to be tolerized to avoid autoimmune reactions. Studies in human subjects have demonstrated that a substantial frequency of newly generated B cells was autoreactive.¹ Deficiency of proper B-cell tolerance contributes to the development of autoantibodies, leading to initiation and perpetuation of autoimmune processes. At least 3 main mechanisms accounting for silencing of self-reactive B cells are known: receptor editing, deletion, and anergy. The process of anergy is an important tolerance mechanism whereby cells are functionally inactivated.^{2,3} Anergic B cells are characterized by reduced ability to proliferate and secrete antibody on antigen encounter accompanied by reduced BCR-signaling responses, including intracellular calcium mobilization and tyrosine phosphorylation.²⁻⁴ In contrast, in the responding B cell BCR signaling is initiated by activation of Src family kinases, such as Lyn kinase, which are under the dual control of tyrosine phosphatase CD45 (activating) and C-terminal Src kinase (Csk; inactivating).⁵⁻⁷ Src family kinases phosphorylate the immunoreceptor tyrosine-based activation motif (ITAM) of the BCR Igα/β chains,⁸ which in turn recruit and activate Syk, propagating activation of downstream signaling kinases. Finally, this leads to assembly of the BCR signalosome and diversification of the BCR-signaling cascade.⁹⁻¹¹

Although the existence and identity of anergic B cells in animal models have been known for some time, little was known regarding the phenotype of the corresponding anergic human B-cell subset or subsets. We previously identified a human B-cell subset with autoreactive capacity that was tolerized by anergy in healthy subjects.¹² This population was naive, fully mature, and negative for surface IgM and expressed only IgD BCRs, hereafter referred to as B_{ND} cells (ie, a B-cell population that is naturally autoreactive and tolerized by functional anergy).¹² B_{ND} cells constitute 2.5% (range, 1% to 10%) of B cells in the peripheral blood of healthy subjects, have antibody variable region genes in germline configuration, and, by all current measures, are fully mature. By analyzing recombinant antibodies expressed from the variable genes of single cell-sorted B_{ND} cells, we demonstrated that the B_{ND} population was predominantly autoreactive, as

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Abbreviations used

APC:	Allophycocyanin
BCR:	B-cell receptor
B _{ND} cells:	B-cell population that is naturally autoreactive and tolerized by functional anergy
CD40L:	CD40 ligand
CFSE:	Carboxyfluorescein succinimidyl ester
Csk:	C-terminal Src kinase
FITC:	Fluorescein isothiocyanate
HEL:	Hen egg lysozyme
ITAM:	Immunoreceptor tyrosine-based activation motif
pCAP:	Phosphorylated coumaryl amino propionic acid
PE:	Phycoerythrin
PerCP:	Peridinin-chlorophyll-protein complex
pErk1/2:	Phosphorylated extracellular signal-regulated kinase 1/2
SLE:	Systemic lupus erythematosus
Treg:	Regulatory T

shown by binding to human HEp-2 cell antigens and double-stranded DNA.¹²

Anergic autoreactive B cells represent a reservoir of potentially harmful B cells that might drive pathogenesis if the state of anergy is revoked. The fact that nuclear autoantigen-reactive B cells become activated in patients with systemic lupus erythematosus (SLE)^{13,14} and that B_{ND} cells harbor reactivity toward these disease-relevant autoantigens highlights the importance of investigating the mechanisms of B-cell tolerance breaking in autoimmunity. T-cell help has been shown to effectively overcome B-cell anergy, leading to restoration of normal B-cell functions in murine models.^{2,15,16} Interestingly, by using regulatory T (Treg) cell-deficient mice, these cells were shown to be necessary and sufficient for the maintenance of B-cell anergy through a mechanism involving control of expansion of T_H cells.¹⁷⁻¹⁹ However, to date, this has not been addressed in human subjects, and therefore we asked whether T-helper signals could allow the anergic B_{ND} cells to escape functional anergy and sought a molecular mechanism.

Herein we report that stimulation with T-cell help forces anergic autoreactive B cells to respond to antigenic stimulation by restoring BCR signaling through upregulation of CD45 phosphatase activity. Additionally, T-cell help increased CD45 phosphatase activity in naive B cells, pinpointing CD45 as a key regulator of BCR-signaling thresholds mediated by T-cell help. Anergic B cells from patients with SLE exhibit higher BCR-signaling capacity and increased CD45 phosphatase activity, thus being less tolerized than B_{ND} cells from healthy subjects. This further implies that anergic B_{ND} cells might represent the precursors of autoantibody-secreting plasma cells in patients with B cell-driven autoimmune conditions and suggests these autoreactive B cells can contribute to autoimmune pathogenesis in patients with SLE if not properly controlled.

METHODS

Clinical samples

Blood samples were obtained from healthy adult blood donors and 17 patients with SLE (12 with active and 5 with inactive disease). Active SLE was defined as an SLE Disease Activity Index score of greater than 4, whereas inactive SLE was defined as an SLE Disease Activity Index score of less than 4 at the time of administration of the questionnaire.²⁰ All patients fulfilled the American College of Rheumatology 1997 revised criteria for SLE.²¹ Ethical

approval was obtained from the Regional Ethics Committee in South-Eastern Norway (2014/840/REK). Informed consent was obtained from all subjects.

Antinuclear antibodies were determined by means of HEp-2 staining with an indirect immunofluorescence technique. Titers of anti-double-stranded DNA were determined by using a fluoroenzyme immunoassay (EliA; Thermo Fischer Scientific, Waltham, Mass) and indirect immunofluorescence (Immuno Concepts, Sacramento, Calif). At sampling, all the patients were taking prednisolone (median, 7.5 mg; range, 2.5-12.5 mg) and hydroxychloroquine. Additional treatments included azathioprine (n = 3), mycophenolate (n = 2), and cyclosporine (n = 1). None of the patients had previously received B cell-depleting therapy.

Antibodies

CD27-fluorescein isothiocyanate (FITC; CLB-27/1), CD27-phycoerythrin (PE; CLB-27/1), CD19-PE/ALX610 (J3-119; Life Technologies, Grand Island, NY), IgD-PE (IA6-2), Annexin V-FITC, CD86-PE (V B070), CD80-FITC (L307.4), HLA-DR-peridinin-chlorophyll-protein complex (PerCP; L243), IgG-PE/Cy7 (G18-145), pan-CD45-allophycocyanin (APC)/Cy7 (2D1), CD3-PerCP (SK7; BD Biosciences, San Jose, Calif), IgM-APC (SA-DA4; SouthernBiotech, Birmingham, Ala), CD38-FITC (T16), CD19-ALX488 (HIB19), CD69-FITC (FN50; eBioscience, San Diego, Calif), CD95-FITC (UB2), CD3-PE/Cy7 (UCHT1; Beckman Coulter, Fullerton, Calif), and pan-CD45-PE/Cy7 (HI30) (BioLegend, San Diego, Calif) were used, according to the manufacturers' instructions. Nonspecific staining by Fc receptors was blocked with the Human FcR Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) before staining.

Cell preparations

Peripheral naive B cells were purified from buffy coats derived from healthy donors by means of negative selection of non-B cells and memory B cells with the Human Naïve Isolation Kit II (Miltenyi Biotec), according to the manufacturer's recommendations. Briefly, PBMCs were isolated by using a standard density gradient centrifugation technique (Lymphoprep, Axis-Shield), and red blood cells were removed by means of hypotonic lysis (ACK buffer containing 0.15 mol/L NH₄Cl, 10 mmol/L KHCO₃, and 0.1 mmol/L Na₂EDTA, pH 7.2), washed, counted, and subjected to naive B-cell isolation by incubating with Biotin Antibody Cocktail directed toward non-B-cell markers and CD27 (Miltenyi Biotec). Unwanted cells were depleted by means of incubation with Anti-Biotin Microbeads. Resultant naive B cells were routinely checked for purity by using flow cytometric staining and analysis for CD27-FITC, IgD-PE, CD19-PE/ALX610, IgG-PE/Cy7, and IgM-APC. Isolated naive B cells were greater than 98% pure and contained less than 0.5% CD27⁺ B cells.

Cell culture and stimulations

Purified naive B cells were cultured at 2.5×10^6 cells/mL in a volume of 200 μ L in RPMI 1640 plus GlutaMAX-1 (Life Technologies) supplemented with endotoxin and mycoplasma-tested 10% heat-inactivated FBS (Biochrom AG, Berlin, Germany) and penicillin/streptomycin. Recombinant human IL-4 (20 ng/mL to 0.5 μ g/mL; PeproTech, Rocky Hill, NJ) and soluble recombinant human CD40 ligand (CD40L; 100 ng/mL, MegaCD40L; Enzo Life Sciences, Farmingdale, NY) were used as T_H2 cell help factors that were provided to purified naive B cells for 24 to 36 hours before BCR ligation experiments. In some experiments recombinant human B-cell activating factor and a proliferation-inducing ligand (PeproTech) were used in naive B-cell cultures at a final concentration of 1 μ g/mL. CpG (Type B CpG oligonucleotide, ODN 2006; InvivoGen, San Diego, Calif) was used at 0.1 to 1 μ g/mL. The following cytokines were used at 10 ng/mL: IL-10, IL-17, IL-13, TNF- α (ImmunoTools, Friesoythe, Germany), and IFN- γ (PeproTech). IL-2 (Roche, Mannheim, Germany) was used at 20 U/mL, and IL-21 (PeproTech) was used at 50 ng/mL. Ionomycin/PMA (Cell Stimulation Cocktail, 500 $\times$; eBioscience) was included in experiments as a positive control for B-cell responsiveness. *De novo* protein synthesis was inhibited by cycloheximide

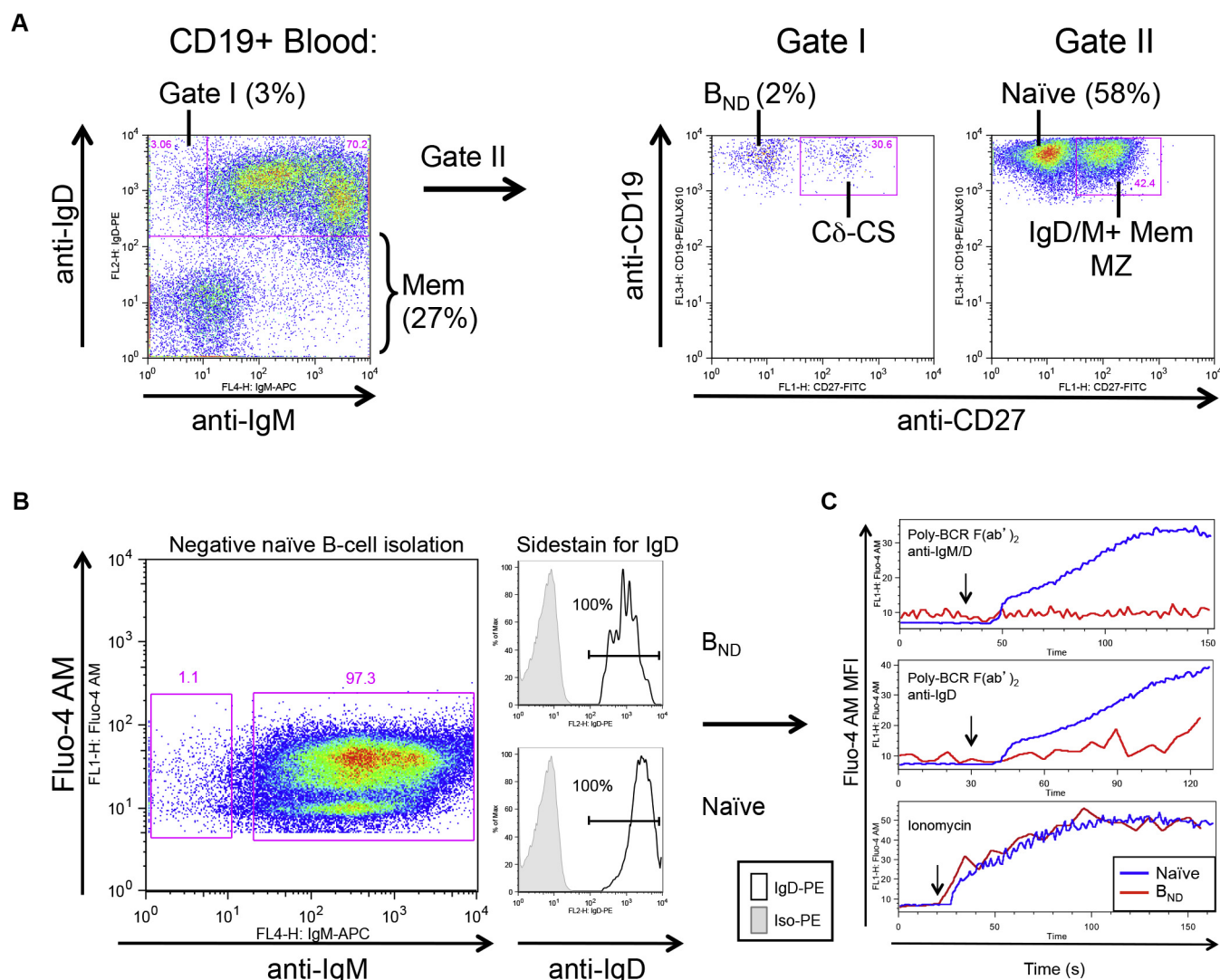


FIG 1. B_{ND} cells are gated as the CD19⁺IgD⁺IgM⁻CD27⁻ B-cell fraction and are anergic. **A**, Human peripheral blood B cells (CD19⁺) identified according to the expression of IgM and IgD can be further distinguished by CD27 expression. On average, 2% to 3% of all B cells are found in the IgD-only fraction (Gate I), where we find B_{ND} cells (2%), which are further separated from Cδ-CS cells (1%) by CD27 expression. Naïve B cells (58%) are found in the double-positive fraction (Gate II) and can be further separated from memory B cells by CD27 expression. MZ, Marginal zone. **B**, Left panel, Anergic B_{ND} cells are gated as IgM⁻ naïve B cells. Right panel, Side stains were performed in separate cell aliquots with anti-CD19, anti-IgD, and anti-IgG to test for purity. Isotype control is shown as the gray histogram, and IgD stain is shown as the open histogram. **C**, Ca²⁺ curves for B_{ND} cells (red lines) and naïve B cells (blue lines) stimulated with polyclonal F(ab')₂ BCR cross-linkers, anti-IgM/IgD (upper panel), anti-IgD (middle panel), and ionomycin stimulation as a control (lower panel).

(Sigma-Aldrich, St Louis, Mo) or vehicle (dimethyl sulfoxide), as indicated in the figure legends, at a final concentration of 1 to 5 μ g/mL. In some experiments cells were preincubated for 30 minutes at 37°C with 5 μ mol/L of the Syk inhibitor fostamatinib (R406; Selleckchem, Houston, Tex).

Flow cytometry

B-cell subsets were resolved as in Fig 1, A, and stained with anti-human mAbs (see above). For functional analyses, purified naïve B cells were isolated and resolved into B_{ND} or naïve B-cell subsets based on IgM expression. Cells (5×10^5) were harvested onto 96-well conical plates and washed in FACS wash buffer (PBS/2% FBS/0.1% NaN₃) before Fc receptors were blocked with the Human FcR Blocking Reagent (Miltenyi Biotec), according to the manufacturer's description. Cells were stained with anti-human mAbs on ice before being washed twice in FACS wash and once in PBS/0.1% NaN₃.

In some experiments cells were fixed by using 2% paraformaldehyde/PBS (wt/vol). Flow cytometric analyses were performed with either the FACSCalibur or FACSCanto II flow cytometers (BD Biosciences), and software analyses were performed with FlowJo Cytometric software (TreeStar, Ashland, Ore).

Ca²⁺ mobilization assay

The intracellular free [Ca²⁺]_i concentration in naïve B cells and B_{ND} cells was determined by using the Fluo-4 Direct Calcium Assay Kit (Life Technologies), according to the manufacturer's recommendations. Purified human naïve B cells were first harvested and resuspended in RPMI supplemented with 10% FCS (RPMI/FCS). B cells were warmed to 37°C before Fluo-4 Calcium Assay Reagent (Life Technologies) was added. Cells were incubated for 1.5 hours at 37°C, washed with PBS and stained for 30 minutes on ice with

anti-human IgM-APC, and washed and resuspended in 250 μ L of RPMI/FCSE supplemented with Probenecid at 0.2 mmol/L (Life Technologies). Alternatively, as a control, negatively isolated human naive B cells were divided into 2 fractions, where one fraction was stained with anti-IgM and gated on IgM⁺ cells to yield the calcium mobilization curve for B_{ND} cells. Cell fraction 2 was analyzed directly without IgM staining, where the majority of cells represented untouched naive B cells (data not shown). The 2 methods yielded comparable results. Cells were equilibrated to 37°C before analysis on a FACSCalibur flow cytometer (BD Biosciences). $[Ca^{2+}]_i$ levels were monitored over time on gated Fluo-4 AM–positive cells. After a baseline was established, cells were stimulated with 10 μ g/mL F(ab')₂ polyclonal mouse anti-human IgM and IgD or anti- κ/λ (SouthernBiotech) and acquired for 3 to 5 minutes. Kinetic curves were generated by using FlowJo software (TreeStar). For general phosphatase inhibition, hydrogen peroxide (Sigma-Aldrich) was included in Ca^{2+} flux experiments at the time of BCR stimulation at final concentrations ranging between 2.5 and 20 mmol/L, as indicated. For specific inhibition of CD45 phosphatase activity, CD45 inhibitor (PTP CD45 Inhibitor; Calbiochem, San Diego, Calif) was used at 5 to 20 μ mol/L during incubation with anti-IgM. Ionomycin stimulation was included as a positive control (Cell Stimulation Cocktail, eBioscience).

Phospho-flow analysis

Human naive B cells were isolated and cultured, as described above. Cells were equilibrated to 37°C and stained with anti-human IgM-APC at 37°C for 10 minutes. BCRs were cross-linked by adding a total of 10 μ g/mL each of F(ab')₂ mouse anti-human IgM and IgD or anti- κ/λ (SouthernBiotech) for the indicated time points (30 seconds to 10 minutes). Cells were immediately fixed with 2% paraformaldehyde/PBS at room temperature for 10 minutes and subsequently permeabilized by adding cold 100% methanol and incubating for 20 minutes at 4°C. Cells were washed twice and the phospho-specific antibodies rabbit polyclonal anti-phospho-Lyn (pTyr396)–ALX488 (dilution 1:50), anti-phospho-Lyn (pTyr507)–PE/Cy7 (dilution 1:50; Bioss, Woburn, Mass), and mouse anti-phospho-Syk (pTyr348)–PE (I120-722; BD Biosciences) or rabbit anti-phospho-Syk (Tyr525/526)–PE (C87C1; Cell Signaling, Danvers, Mass), phosphorylated extracellular signal-regulated kinase 1/2 (pErk1/2; T202/Y204), or PerCP-eFluor710 (MILAN8R; eBioscience) were added and incubated at room temperature for 1 hour. Washed cells were acquired on a FACSCalibur or FACSCanto II (BD Biosciences). Alternatively, untouched naive B cells were purified as described, half were left unstained (untouched naive B cells), and the other half were stained with anti-IgM; B_{ND} cells were gated as the IgM⁺ fraction. BCRs were cross-linked (see above), fixed, permeabilized, and stained with phospho-specific antibodies.

Flow cytometry–based CD45 phosphatase activity assay

A method to quantitate CD45 phosphatase activity at the single-cell level was recently described.²² The assay takes advantage of the fluorogenic properties of phosphorylated coumaryl amino propionic acid (pCAP), an analog of phosphotyrosine, which can be incorporated into peptides. Once delivered into cells, pCAP peptides are dephosphorylated by protein tyrosine phosphatases, and the resulting cell fluorescence can be monitored by using flow cytometry. A cell-permeable pCAP peptide specific for CD45 phosphatase activity was designed, taking advantage of peptide sequences of known CD45 substrates.²² For the cell-permeable pCAP peptide–based assay, naive human B cells were isolated, cultured, and stimulated, as described above, with IL-4, CD40L, or both for 24 to 36 hours. Cells were harvested, washed, and stained with CD19-ALX488, pan-CD45-PE/Cy7, and IgM-APC. Subsequently, cells were washed in RPMI medium (without phenol red), supplemented with 0.5% FBS, and equilibrated to 37°C before cells were incubated with CAP-SP1 (control peptide) or pCAP-SP1 (test peptide) at 2.5 μ mol/L for 15 minutes at 37°C. Cells were washed in FACS wash (PBS/2% FBS/0.1% Na₂S₂O₈) supplemented with 10 mmol/L sodium orthovanadate to inhibit tyrosine phosphatase activity and fixed in 2% paraformaldehyde (wt/vol) for 15 minutes at room temperature. CD45 phosphatase activity was measured as cell fluorescence

by using a FACSCanto II (BD Biosciences), with excitation with the violet laser at 405 nm and detection with a Pacific Blue emission filter.

Cell proliferation analysis and differentiation assay

Isolated B_{ND} and naive B cells were labeled with 2 μ mol/L carboxyfluorescein succinimidyl ester (CFSE; CellTrace CFSE Cell Proliferation Kit, Life Technologies) for 3 minutes at room temperature before labeling was quenched with complete RPMI on ice. Cells were washed 3 times in RPMI and cultured (0.5×10^6 cells/well) for 5 to 6 days in the presence of CD40L/IL-4 for proliferation. Cell proliferation was detected as dilution of CFSE compared with the unstimulated control. Unlabeled cells were cultured in the presence of CD40L/IL-4 for IgG class-switching (IgG⁺) or CD40L/IL-4/IL-21 for plasma cell differentiation (CD38⁺CD27⁺). Cells were gated on 7-aminoactinomycin D–negative cells for analysis of live cells only. All samples were stimulated with BCR cross-linkers.

Polyclonal human CD4⁺ T_H cells

B_{ND} and naive B cells (0.5×10^6) were cocultured with allogeneic CD4⁺ T_H cells at a 1:1 ratio. T_H cells were preactivated with CD3/CD28 Dynabeads (Invitrogen, Carlsbad, Calif) and rested before coculture with B cells for 24 to 36 hours. Cells were harvested, and functional analyses on B cells were undertaken (Ca^{2+} flux, phospho-flow, and CD45 activity measurement), as described above. B cells were gated as CD3⁺ cells.

Real-time quantitative PCR

Total RNA was isolated from cultured purified naive B cells by using TRIzol reagent (Life Technologies). Reverse transcription and quantitative PCR were performed with the TaqMan RNA-to-CT 1-Step Kit (Life Technologies) on the StepOnePlus System (Life Technologies): reverse transcription at 48°C for 15 minutes and then 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 1 minute at 60°C in 15- μ L reaction volumes with 5 ng of RNA as template per well. All PCR reactions were done in triplicates. Controls containing no template or no reverse transcriptase enzyme were included in each run. TaqMan primers and probes for human CD45, LYN, SYK, and POLR2A were chosen that did not amplify genomic DNA. Gene expression was quantified by using the comparative threshold cycle method and normalized to human POLR2A expression as a housekeeping gene. Values are expressed as means $\pm$ SEMs.

Statistical analyses

All statistical analyses were performed with Prism software (GraphPad Software, La Jolla, Calif). Where appropriate, data sets were analyzed with the D'Agostino-Pearson omnibus normality test or the Shapiro-Wilk normality test to evaluate distribution of data. For normally distributed data, significant differences between mean values were evaluated by using paired or unpaired 2-tailed Student *t* tests and 1-way ANOVA, followed by multiple comparison tests. For nonnormally distributed data, the Kruskal-Wallis test or Wilcoxon matched-pairs signed-rank test was used. A *P* value of less than .05 was considered statistically significant.

RESULTS

IgD⁺IgM⁺ naive B cells from healthy human subjects are anergic

In transgenic mouse models of tolerance, B cells that predominantly express IgD isotype BCRs with low levels of surface IgM are anergic.⁴ By analogy, we previously identified human CD19⁺IgD⁺IgM⁺CD27⁺ naive B cells as anergic (Fig 1, A and C) and predominantly autoreactive, underscoring their potential importance in autoimmunity.¹² A negative enrichment scheme was used to isolate B_{ND} and naive B-cell populations, followed by loading of Fluo-4 AM (see the Methods section and Fig 1, B). Baseline intracellular calcium levels were established for 20

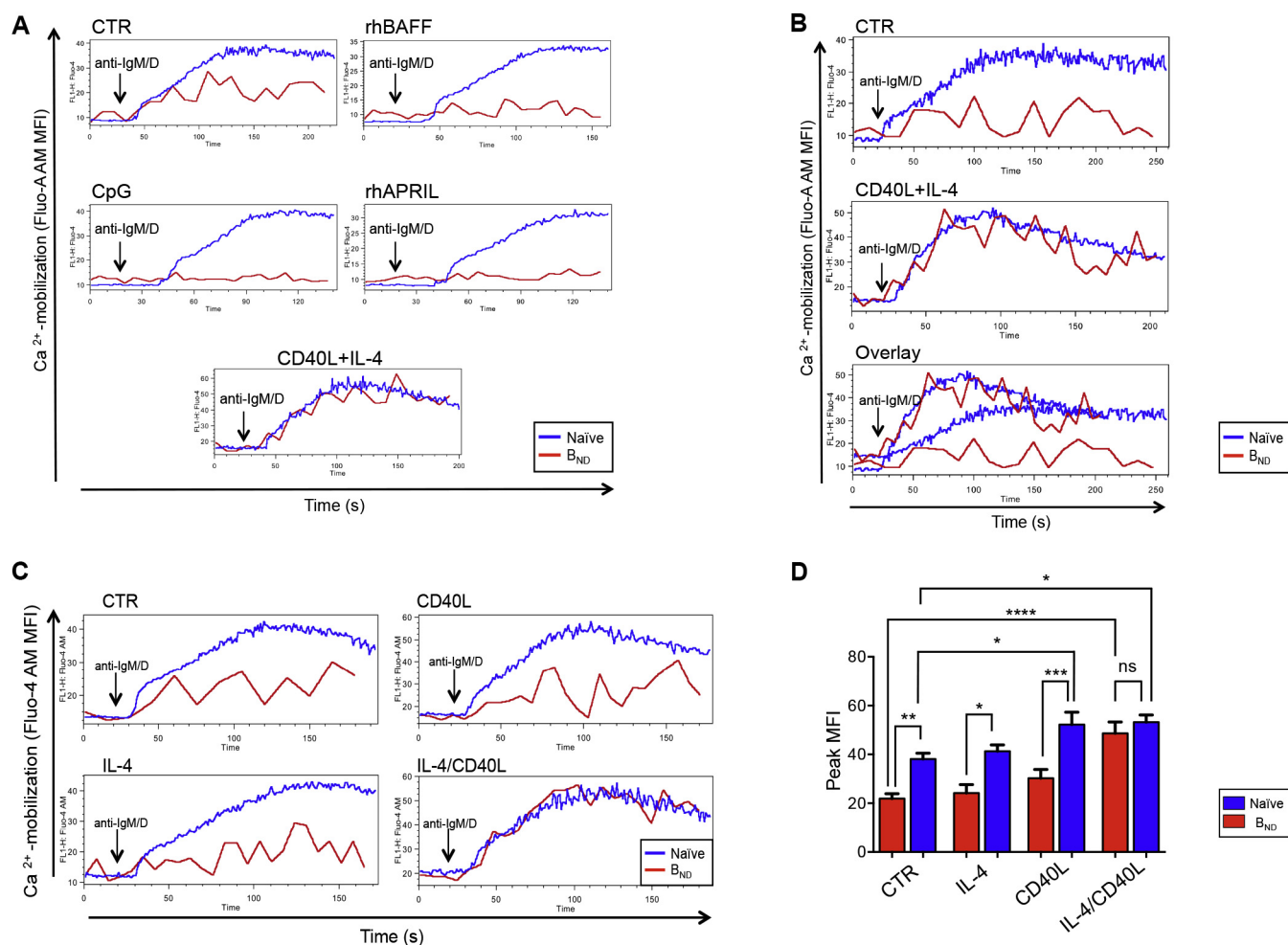


FIG 2. Anergic B_{ND} cells regain their capacity to mobilize intracellular calcium on receiving T_H2 signals. **A**, Ca^{2+} flux naive (blue line) and B_{ND} (red line) cells subjected to various stimuli during cell culture. Isolated naive B cells and B_{ND} cells were stimulated with recombinant human B-cell activating factor (rhBAFF), recombinant human a proliferation-inducing ligand (rhAPRIL), CpG, recombinant human IL-4 (rhIL-4), and CD40L, as described in the Methods section. **B**, T_H2 signals increase BCR-signaling capacity in naive B cells. Control cells (upper panel) of naive (blue line) versus B_{ND} (red line) cells and cells stimulated with T_H2 -mimicking signals (IL-4 and CD40L; middle panel) are shown. The lower panel shows an overlay of the control (CTR) and IL-4/CD40L-stimulated cells. **C**, Both T_H2 signals are required for regaining BCR signaling in B_{ND} cells. Blue line, naive cells; red line, B_{ND} cells. **D**, Graphs depict means $\pm$ SEMs of peak mean fluorescence intensity for B_{ND} cells (red bars) or naive B cells (blue bars) after BCR stimulation. P values were determined by using 1-way ANOVA, followed by the Sidak multiple comparison test. Data are representative of 14 independent experiments. * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$. ns, Not significant.

to 30 seconds, followed by BCR cross-linking. Reduced calcium response was observed in B_{ND} cells compared with naive B cells when cross-linking IgM and IgD BCRs (Fig 1, C, upper panel), confirming the anergic phenotype of B_{ND} cells.¹² When naive B cells and B_{ND} cells were stimulated with anti-IgD alone, an identical result was found, ruling out the contribution of reduced IgM levels on B_{ND} cells to the anergic phenotype (Fig 1, C, middle panel). Reduced Ca^{2+} flux in B_{ND} cells was also observed when various concentrations of BCR stimulant ranging from 2.5 to 20 μ g/mL were used (see Fig E1, A, in this article's Online Repository at www.jacionline.org) and with cross-linking with anti- κ/λ (data not shown). The differences observed between B_{ND} and naive B cells were not due to differential uptake of the calcium dye Fluo-4 AM or reduced viability because calcium peaks were equal when B_{ND} and naive B cells were treated with ionomycin (Fig 1, C, lower panel).

T_H2 signals restore BCR signaling in B_{ND} cells

Anergy as a tolerance mechanism does not remove autoreactive B cells from the repertoire, and therefore autoreactive B_{ND} cells could pose a threat if reactivated. We found that BCR signaling in B_{ND} cells was restored when these cells were prestimulated with the T_H cell factors IL-4 and CD40L overnight (Fig 2, A-D). These findings were confirmed by using polyclonally activated human T_H cell lines (see Fig E2, A, in this article's Online Repository at www.jacionline.org). In fact, Ca^{2+} flux in these cells was comparable with that observed in naive B cells, whereas no such effect was observed for the other B-cell stimulants (Fig 2, A and D, and see Fig E3 in this article's Online Repository at www.jacionline.org). Ionomycin stimulation was included as a control in every treatment regimen (see Fig E1, B). We observed that pretreatment with T_H2 signals also increased BCR-signaling capacity in naive B cells (Fig 2, B and D). Interestingly,

stimulation with CD40L alone was sufficient for enhancement of BCR signaling in naive B cells (Fig 2, D). These results suggested a general mechanism of T cell-mediated enhancement of BCR signaling.

The restored Ca^{2+} flux of B_{ND} cells was not due to spontaneous reversal of anergy during *ex vivo* cell culture because B_{ND} cells cultured without $\text{T}_{\text{H}2}$ cell factors for up to 4 days remained refractory to BCR stimulation (see Fig E1, C). Reduced viability did not explain the maintenance of anergy in cultured B_{ND} cells because calcium peaks were equal to those for naive B cells when stimulated with ionomycin (see Fig E1, D). The reversal of anergy in $\text{T}_{\text{H}2}$ factor-prestimulated B_{ND} cells was accompanied by expression of the activation markers CD80, CD86, CD69, and HLA-DR and the early apoptosis and activation marker CD95, a profile similar to that found in preactivated naive B cells (see Fig E4 in this article's Online Repository at www.jacionline.org). In summary, $\text{T}_{\text{H}2}$ -mediated signals restored BCR signaling in B_{ND} cells accompanied by identical regulation of activation markers and further underscoring the transformation of B_{ND} cells away from an anergic state.

Restoration of BCR signaling in anergic B_{ND} cells is mediated through the BCR-signaling kinase Syk

Inhibition of *de novo* protein synthesis significantly reversed enhancement of BCR signaling in both B_{ND} and naive B cells (see Fig E5 in this article's Online Repository at www.jacionline.org), and both cycloheximide-treated subsets responded when stimulated with ionomycin (see Fig E5, A, right panels). This prompted us to investigate signaling components proximal to the BCR. Syk is a key kinase in the BCR-signaling cascade crucial in the formation of the BCR signalosome. We found that the Syk inhibitor R406 (Fostamatinib) induced a robust downmodulation of Ca^{2+} flux in both $\text{T}_{\text{H}2}$ -cultured naive B cells and B_{ND} cells compared with vehicle-treated cells (Fig 3, A, left panels, and Fig 3, B). Response to ionomycin stimulation was not affected (Fig 3, A, right panels).

Subsequently, we investigated activation of Syk on $\text{T}_{\text{H}2}$ factor stimulation using phospho-flow detecting pSyk. Naive B cells and B_{ND} cells and their pSyk levels were resolved retrospectively through immunostaining and detection by means of flow cytometry (Fig 3, C and D). In agreement with the anergic nature of the B_{ND} cell subset, we observed increased activation of Syk in naive B cells compared with that in B_{ND} cells in the absence of $\text{T}_{\text{H}2}$ signals (Fig 3, C). However, on pretreatment of B_{ND} cells with both $\text{T}_{\text{H}2}$ -derived signals, a robust activation of Syk was evident (Fig 3, D upper panel, and Fig 3, E), and furthermore, a similar potentiation of Syk activation was evident also for naive B cells (Fig 3, D, lower panel, and Fig 3, F). These results were corroborated by coculturing B cells with polyclonally activated human T_{H} cell lines (see Fig E2, B). In accordance with Ca^{2+} mobilization, CD40L pretreatment alone also exerted a significant upregulation of pSyk levels in naive B cells. No significant upregulation of SYK mRNA was evident on stimulation with $\text{T}_{\text{H}2}$ factors (see Fig E6, A, in this article's Online Repository at www.jacionline.org). These findings showed the dependence of signaling through Syk in the $\text{T}_{\text{H}2}$ factor-mediated restoration of BCR-signaling capacity in B_{ND} cells.

Increased BCR signaling in anergic B_{ND} cells by $\text{T}_{\text{H}2}$ factors is accompanied by activation of Lyn kinase

Activation of Lyn kinase is responsible for phosphorylating ITAM tyrosines on the BCR-associated $\text{Ig}\alpha/\beta$ chains immediately

subsequent to antigen stimulation. This process is mandatory for Syk recruitment and activation and therefore initiates the BCR-signaling cascade.²³ Lyn kinase can be phosphorylated at different sites, instructing the activation status of this kinase.⁵⁻⁷ The activated form of Lyn kinase (pTyr396) is able to phosphorylate ITAM tyrosines; while phosphorylated on Tyr507, Lyn kinase is inactive. We investigated the activation status of Lyn kinase in B_{ND} and naive B cells and the effect of $\text{T}_{\text{H}2}$ factors on Lyn kinase activation. A decreased baseline level of activated Lyn-pTyr396 in B_{ND} cells compared with naive B cells on BCR stimulation was found, whereas a reversed pattern was detected for Lyn-pTyr507 (Fig 4, A). Thus B_{ND} cells exhibited a higher level of inactive Lyn kinase than naive B cells. However, on IL-4/CD40L stimulation, the activation of Lyn kinase was restored in the B_{ND} subset (Fig 4, B and C), whereas a nonsignificant trend toward decrease in inactive Lyn kinase levels was observed for B_{ND} cells (Fig 4, B and D). The active form of Lyn kinase was similarly increased on $\text{T}_{\text{H}2}$ signals in naive B cells in agreement with Ca^{2+} flux and pSyk data. No upregulation of LYN mRNA was evident on stimulation with $\text{T}_{\text{H}2}$ factors (see Fig E6, B). Therefore we conclude that inactivation of Lyn kinase is closely associated with anergy in B_{ND} cells and, conversely, that activation of Lyn kinase could be instrumental for breaking anergy.

Restoration of BCR signaling in anergic B_{ND} cells is dependent on CD45 phosphatase

The activation status of Lyn kinase is regulated by a balance between the activities of Csk and CD45 phosphatase.²⁴ Csk phosphorylates Lyn-Tyr507, promoting deactivation of Lyn kinase, and CD45 dephosphorylates Lyn-Tyr507, leading to autophosphorylation of Lyn-Tyr396 and activation. Therefore we hypothesized that restoration of BCR signaling in B_{ND} cells on IL-4/CD40L stimulation was dependent on phosphatases or kinases that are capable of regulating the activity of Lyn kinase. Blocking of general phosphatase activity in B_{ND} cells by hydrogen peroxide resulted in temporary inhibition of BCR signaling on stimulation with IL-4/CD40L (Fig 5, A), likely because of conflicting effects of globally inhibited phosphatases.

We next observed dose-dependent inhibition of Ca^{2+} flux on BCR stimulation in B_{ND} cells pretreated with IL-4/CD40L and the chemical inhibitor of CD45 phosphatase activity, PTP CD45, whereas B_{ND} cells stimulated with IL-4/CD40L and vehicle showed normal BCR-signaling capacity (Fig 5, B and C). Similarly, we also observed a downmodulatory effect of CD45 inhibitor in naive B cells, where inhibition of CD45 phosphatase brought the IL-4- and CD40L-mediated increase in BCR signaling down to control levels (Fig 5, B and C). The CD45 inhibitor did not interfere with ionomycin stimulation (Fig 5, D). These results clearly demonstrate a role for CD45 phosphatase in restoration of BCR signaling in B_{ND} cells.

$\text{T}_{\text{H}2}$ signals restore BCR signaling in B_{ND} cells by means of upregulation of CD45 phosphatase activity

We next investigated the expression of pan-CD45 surface protein in B_{ND} cells and naive B cells on stimulation with IL-4/CD40L and found no significant expression changes (Fig 6, C and D), although B_{ND} cells exhibited lower CD45 expression than naive B cells. The results were corroborated by means of

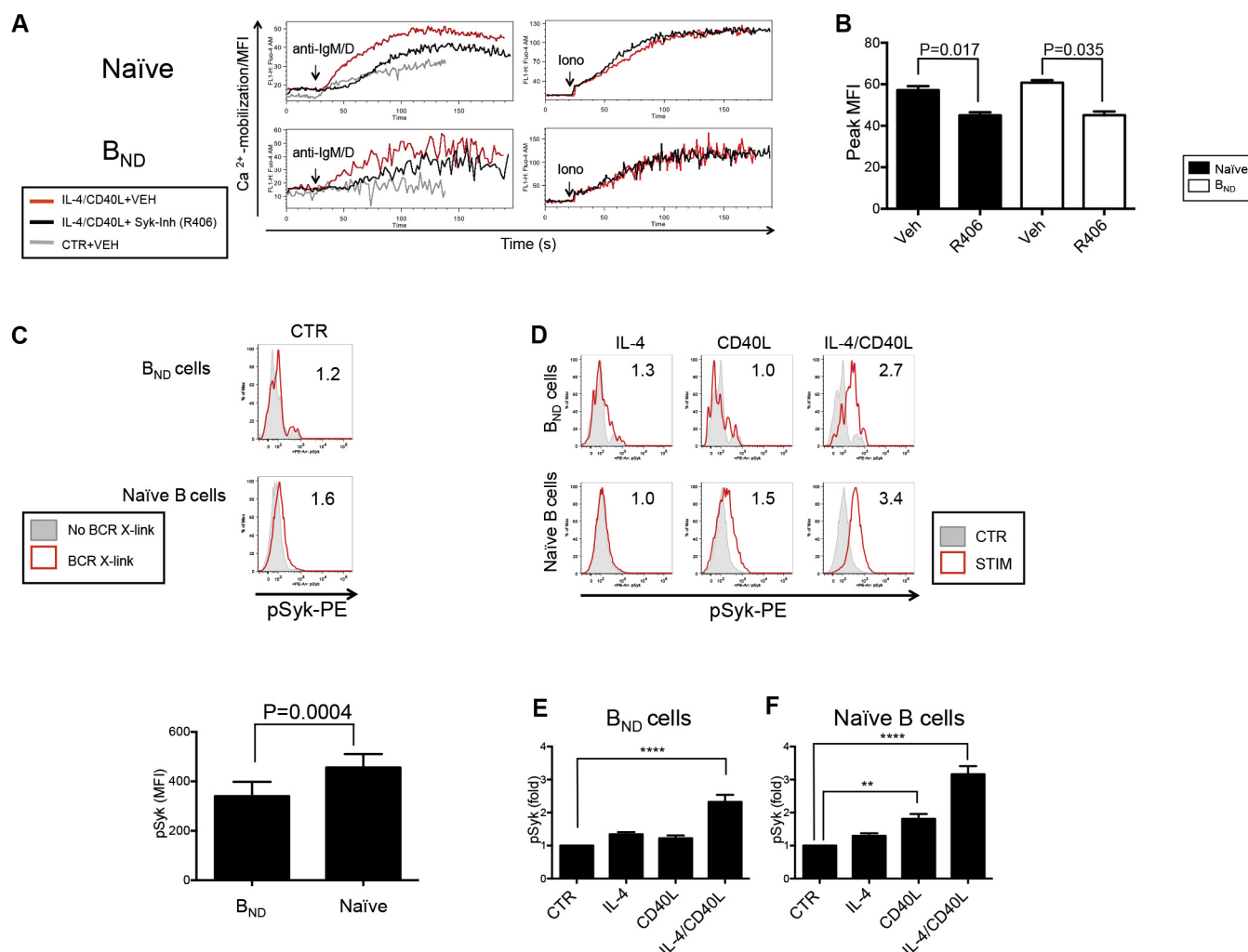


FIG 3. Restoration of BCR signaling in anergic B_{ND} cells on T_H2 factors is mediated through the BCR-signaling kinase Syk. **A**, The Syk inhibitor R406 downmodulated the IL-4/CD40L-induced increase in Ca^{2+} flux. *Left panels*, Syk inhibitor R406 (black line)– or vehicle (dimethyl sulfoxide, red line)–treated naive and B_{ND} cells. Unstimulated control/vehicle-treated cells are depicted as gray lines. *Right panels*, Ionomycin control. **B**, The graph shows means $\pm$ SEMs for peak mean fluorescence intensity values for IL-4/CD40L–stimulated naive (solid bars) and B_{ND} (open bars) cells for vehicle- or Syk inhibitor (R406)–treated cells. **C**, Phospho-flow analysis of pSyk in untreated B_{ND} cells (control [CTR], upper panel) and naive B cells (CTR, lower panel; gray histograms, no BCR cross-linking; red histograms, BCR cross-linking). Numbers represent the fold increase in pSyk MFI in BCR X-linked versus non-X-linked samples. **D**, T_H2 signals mediate the increase in pSyk levels in B_{ND} cells (upper panel) and naive B cells (lower panel). Stimulated samples are shown as red histograms, and control samples are shown as gray histograms. Numbers represent fold increase in pSyk MFI in stimulated versus nonstimulated samples. **E** and **F**, Graphs show means $\pm$ SEMs for fold increases in MFI of pSyk relative to CTR cells for B_{ND} cells (Fig 3, E) and naive (Fig 3, F) B cells. *P* values were determined by using the paired 2-tailed Student *t* test (Fig 3, B and C) and 1-way ANOVA, followed by the Dunnett multiple comparison test (Fig 3, E and F): ***P* < .01 and *****P* < .0001. Data are representative of 3 (Fig 3, A and B), 10 (Fig 3, C), and 5 (Fig 3, D and F) independent experiments.

quantitative RT-PCR analysis of mRNA levels (see Fig E6, C). This prompted us to further investigate regulation of CD45 phosphatase activity in B_{ND} cells and B_{ND} cells in which anergy is broken by IL-4/CD40L stimulation.

We used a single-cell assay to detect intracellular CD45 phosphatase activity, which takes advantage of the fluorogenic properties of pCAP, an analog of phosphotyrosine that can be incorporated into peptides (see the Methods section). A pCAP peptide, pCAP-SP1, that was both cell-permeable and a specific substrate for intracellular CD45 was engineered previously.²²

Using the pCAP-SP1 assay, we observed upregulation of CD45 phosphatase activity in both B_{ND} and naive B cells after provision of T_H2 signals (Fig 6, A and B). These findings were confirmed by using T_H cell lines (see Fig E2, C and D). Furthermore, in agreement with Ca^{2+} mobilization and phospho-Syk data, CD40L exerted an effect alone on naive B cells, whereas both IL-4 and CD40L was necessary for significant upregulation of CD45 activity in B_{ND} cells. In line with these results and the suggested CD45-dependent mechanism for reversing the deficit in BCR signaling, we also found lower CD45 phosphatase activity in untreated control

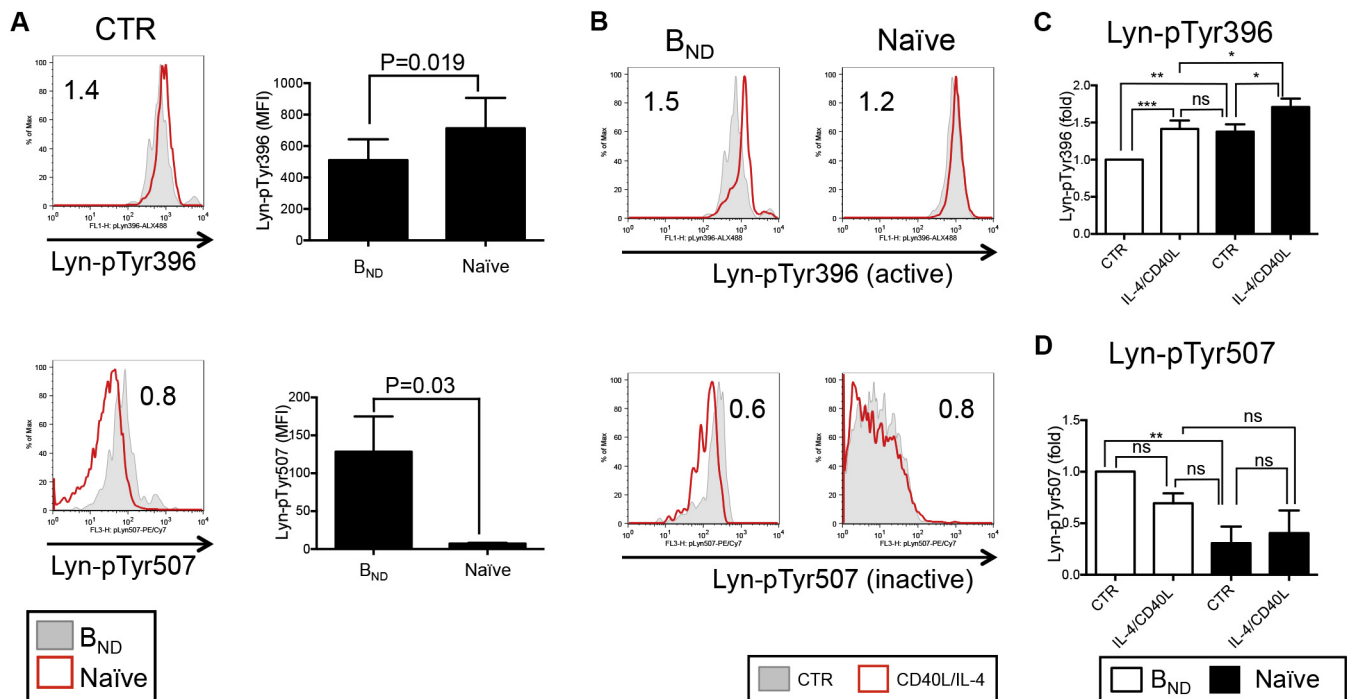


FIG 4. Restoration of BCR signaling in anergic B_{ND} cells on stimulation with T_H2 signals is accompanied by activation of Lyn kinase. **A**, B_{ND} cells express lower levels of the active form of Lyn kinase (Lyn-pTyr396, upper panel) and higher levels of the inactive form of Lyn kinase (Lyn-pTyr507, lower panel) than naïve B cells. Phospho-flow analysis of Lyn-pTyr396 and Lyn-pTyr507 in B_{ND} cells (gray histograms) and naïve B cells (red histograms) in control (CTR) samples. Right panels, Bar graphs show mean fluorescence intensity expressed as means $\pm$ SEMs for active and inactive Lyn. **B**, T_H2 factors (IL-4/CD40L) enhance the activation status of Lyn kinase. Red histograms, IL-4/CD40L-stimulated; gray histograms, CTR. **C** and **D**, The bar graph shows means $\pm$ SEMs for fold changes in the MFI for either Lyn-pTyr396 (Fig 4, C) or Lyn-pTyr507 (Fig 4, D) for B_{ND} cells (open bars) and naïve B cells (solid bars) relative to CTR cells. *P* values were determined by using the paired 2-tailed Student *t* test (Fig 4, A) and 1-way ANOVA, followed by the Sidak multiple comparison test (Fig 4, C and D). Data are representative of 10 (Fig 4, A-C), 5 (Fig 4, A), and 7 (Fig 4, D) independent experiments. **P* < .05, ***P* < .01, and ****P* < .001. ns, Not significant.

B_{ND} cells compared with that in naïve B cells (Fig 6, A, left panel). Specificity of the CD45 phosphatase assay was ensured by including CD45 phosphatase inhibitor during incubation with pCAP-SP1 peptide (see Fig E7, A, in this article's Online Repository at www.jacionline.org). CD45 inhibition resulted in a decrease in the active form of Lyn kinase (Lyn-pTyr396) and Syk, directly demonstrating an effect of CD45 phosphatase activity on the activation status of these kinases (see Fig E7, B and C). Furthermore, no effect of CD45 staining on CD45 activity was observed (data not shown). In summary, these results demonstrate a mechanism for reversal of anergy by T_H2 factors through upregulation of CD45 phosphatase activity, which allow activation of Lyn kinase and increased BCR signaling and reversal of anergy in B_{ND} cells.

B_{ND} cells have the ability to acquire features of fully activated B cells with antibody-producing capacity and are less tolerized in patients with SLE

We investigated the ability of B_{ND} cells to differentiate into plasma cells and undergo class-switching to IgG on CD40L/IL-4 stimulation. In the presence of IL-21, we observed that B_{ND} cells differentiated into plasma cells (CD38⁺⁺CD27⁺⁺) similarly to naïve B cells (see Fig E8, A, in this article's Online Repository at www.jacionline.org), whereas CD40L/IL-4 stimulation resulted in IgG class-switching (see Fig E8, B). Analysis of the

downstream BCR-signaling kinase pErk1/2 and proliferation capacity by CFSE dilution on CD40L/IL-4 stimulation further underscored the acquisition of an activated phenotype of B_{ND} cells on mimicked T-cell help, and importantly, this was CD45 dependent (see Fig E8, C and D).

We next investigated whether the autoreactive B_{ND} cell subset was tolerized in patients with the prototypical B cell-mediated systemic autoimmune disease SLE. We found that the B_{ND} cell population obtained from patients with SLE exhibited significantly increased intracellular Ca²⁺ flux on BCR stimulation compared with that seen in healthy control subjects (Fig 7, A). Strikingly, the increased BCR signaling potential in B_{ND} cells from patients with SLE was accompanied by increased CD45 phosphatase activity compared with that seen in healthy control subjects (Fig 7, B), whereas surface expression of CD45 was identical between patients and control subjects (Fig 7, C). When subdividing patients with SLE into those with active and those with inactive SLE (see the Methods section), no significant difference in CD45 phosphatase activity was detected between these patients (Fig 7, B), suggesting that the increased CD45 phosphatase activity might be an intrinsic feature of SLE. The increased Ca²⁺ flux and CD45 phosphatase activity was accompanied by increased activated Lyn kinase (Lyn-pTyr396) and Syk levels (Fig 7, D and E). Lastly, when B_{ND} cells from patients with SLE were pre-stimulated with T_H2 signals *in vitro*, CD40L stimulation alone was

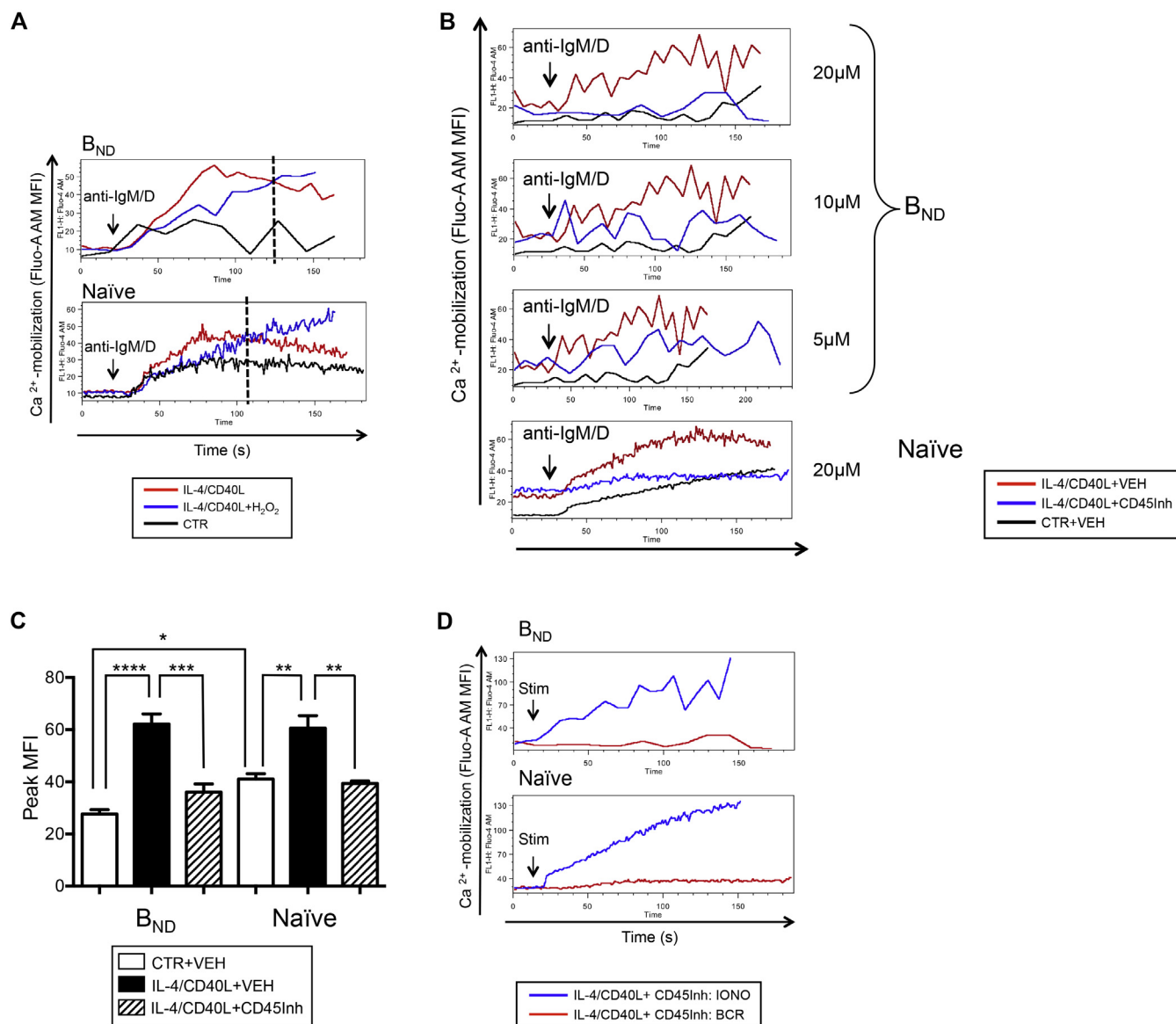


FIG 5. Inhibition of CD45 phosphatase suppresses restoration of BCR signaling in anergic B_{ND} cells on stimulation with T_H2 factors. **A**, Analysis of Ca²⁺ flux subsequent to stimulation with CD40L/IL-4 was performed in the presence (blue lines) or absence (red lines) of the general phosphatase inhibitor H₂O₂. Control cells are depicted as black lines. **B**, The CD45 inhibitor PTP CD45 Inhibitor prevents the restoration of BCR signaling on stimulation with T_H2 factors. Red lines, T_H2-stimulated/vehicle-treated cells; blue lines, T_H2-stimulated and CD45 inhibitor-treated cells; black lines, unstimulated/vehicle-treated cells. **C**, Graphs show means ± SEMs of peak mean fluorescence intensity of Ca²⁺ flux of B_{ND} cells and naive B cells. Open bars, Vehicle-treated control (CTR) cells; solid bars, IL-4/CD40L-stimulated, vehicle-treated cells; hatched bars, IL-4/CD40L-stimulated, CD45 inhibitor-treated cells. **D**, B_{ND} cells (upper panel) or naive B cells (lower panel) cultured with CD40L/IL-4 were stimulated with BCR cross-linking (red lines) or ionomycin (blue lines) as a control. *P* values were determined by using 1-way ANOVA, followed by the Sidak multiple comparison test: **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. Data are representative of 3 (Fig 5, A-C) independent experiments.

sufficient for achieving Ca²⁺ flux comparable with that seen in naive B cells (Fig 7, F). Taken together, this suggests that B_{ND} cells from patients with SLE were less tolerized than B_{ND} cells from healthy control subjects. Our results imply that anergic B_{ND} cells might represent precursors of autoantibody-secreting plasma cells in patients with B cell-driven autoimmune conditions and further opens up the possibility that these autoreactive B cells could contribute to autoimmune pathogenesis in patients with SLE if not properly controlled.

DISCUSSION

We report herein that B_{ND} cells could be rescued from anergy and that BCR signaling was restored on receiving T_H2 factors. In addition, the mechanism was mediated by increased activity of the protein tyrosine phosphatase CD45. Moreover, we found that patients with SLE had B_{ND} cells with a less pronounced signaling deficit and linked this finding to higher CD45 activity in B_{ND} cells from patients with SLE. The latter results suggest that B_{ND} cells in patients with SLE might have intrinsic defects

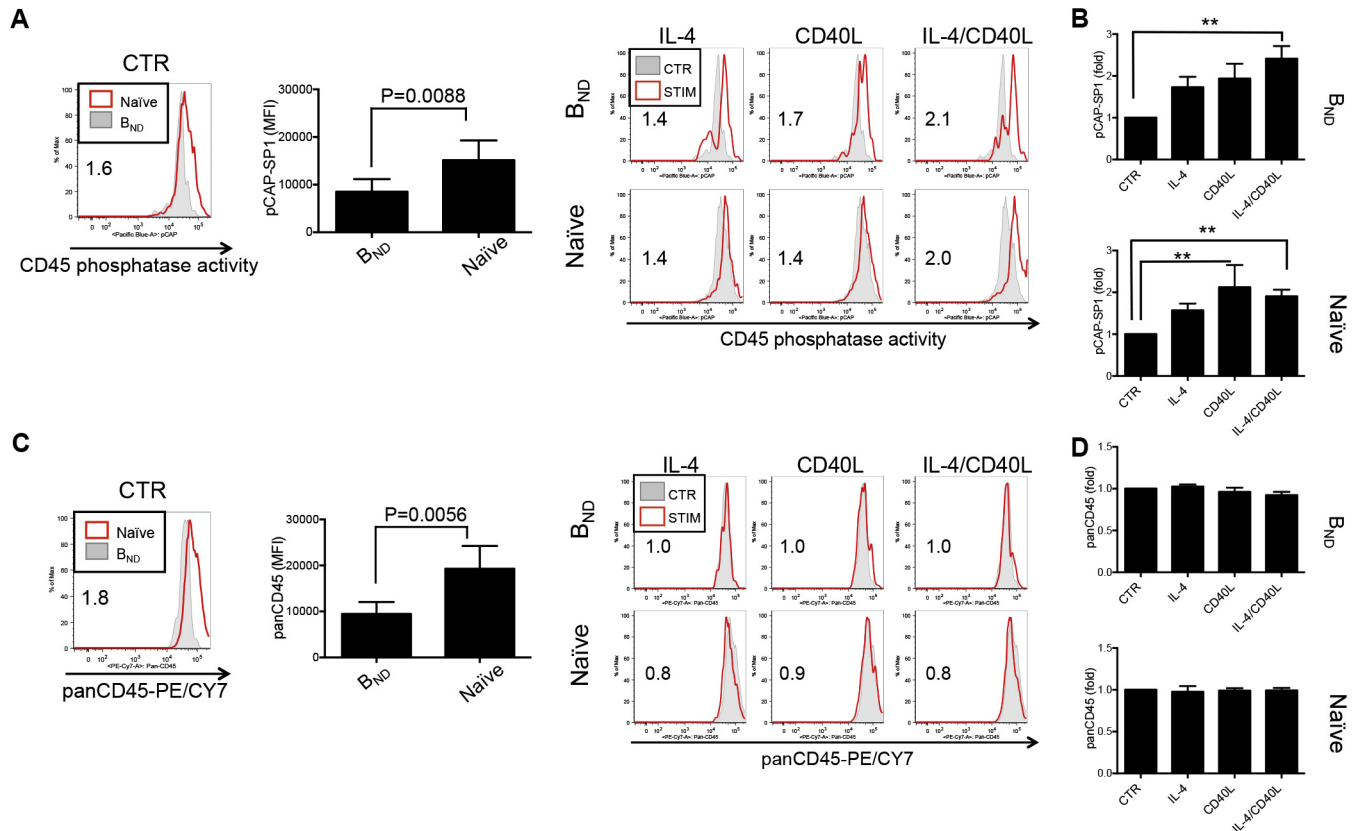


FIG 6. Restoration of BCR signaling in B_{ND} cells on T_H2 signaling is mediated through upregulation of CD45 phosphatase activity. **A**, CD45 phosphatase activity is upregulated by CD40L/IL-4. Left panel: gray histogram, CD45 phosphatase activity of B_{ND} cells; red histogram, naive B cells of control cells. Right panels: gray histograms, CD45 phosphatase activity in control (CTR); red histograms, T_H2-stimulated cells. **B**, Graphs show means $\pm$ SEMs of pCAP-SP1 mean fluorescence intensity relative to CTR in B_{ND} and naive B cells. **C**, Left panel: CD45 surface expression of B_{ND} cells (gray histogram) and naive B cells (red histogram) of untreated control cells. Right panels, Pan-CD45 surface expression in CTR (gray histograms) and T_H2 factor stimulated cells (red histograms). **D**, Graphs show means $\pm$ SEMs of pan-CD45 surface expression MFI relative to CTR in B_{ND} and naive B cells. *P* values were determined by using the 2-tailed Student paired *t* test (Fig 6, A and C) and 1-way ANOVA followed by the Dunnett multiple comparison test (Fig 6, B and D): ***P* < .01. Data are representative of 12 (Fig 6, A), 9 (Fig 6, C), and 4 (Fig 6, B and D) independent experiments.

leading to increased CD45 phosphatase activity or, alternatively, have received T_H factors and gained increased CD45 activity *in vivo*, contributing to the less tolerized state.

The hen egg lysozyme (HEL) transgenic model has been an effective system to investigate B-cell tolerance mechanisms.^{2,4,25} In this model B cells of mice that were double transgenic for an immunoglobulin receptor specific for HEL (HEL-Ig) and a soluble form of the autoantigen HEL are functionally anergic and unresponsive to stimulation through their BCR.^{2,4,25,26} Anergic B cells regained their ability to respond to antigens after T-cell help in this model.^{2,15,27,28} Extending these results to *bona fide* autoimmune responses, anergic double-stranded DNA-specific B cells obtained from VH3H9 heavy chain transgenic and anergic B cells obtained from a murine diabetes type 1 model also regained normal B-cell function on T-cell help^{16,29,30}; however, the mechanisms remained elusive. Interestingly, in Treg cell-deficient mice, helper T cells are sufficient for breaking B-cell anergy.¹⁷⁻¹⁹ The proposed function of Treg cells in this system is to prevent the activation and expansion of T_H and follicular helper T cells, which in turn facilitate breaking B-cell anergy and autoantibody production.^{17,18} Our findings support the idea

that effective T-cell help is instrumental in overcoming anergy and B-cell tolerance in autoreactive B cells in human subjects.

Restoration of BCR signaling in B_{ND} cells and the increase of BCR signaling in naive B cells was dependent on *de novo* protein synthesis, suggesting that the mechanism responsible could not be explained only by direct cross-talk between the BCR, IL-4, and CD40 signaling pathways. Furthermore, T-helper signals allowed enhanced activation of the BCR-proximal kinases Lyn and Syk. Autophosphorylation of Lyn-Tyr396 activates Lyn kinase, whereas phosphorylation of Lyn-Tyr507 inactivates the kinase.^{6,24} We found that in B_{ND} cells the inactive form of Lyn kinase could be detected in the majority of these cells; nevertheless, the inactive form was rarely detectable in resting naive B cells, corresponding to the activation state of these cells. However, on CD40L/IL-4 stimulation, downregulation of the inactive Lyn-pTyr507 kinase in B_{ND} cells was observed and accompanied by a simultaneous increase in levels of the active form, Lyn-pTyr396, allowing increased BCR signaling. However, Lyn kinase was also shown to exert a nonredundant downmodulatory effect on the BCR-signaling pathway in murine systems.³¹ Lyn kinase activates negative signaling effects

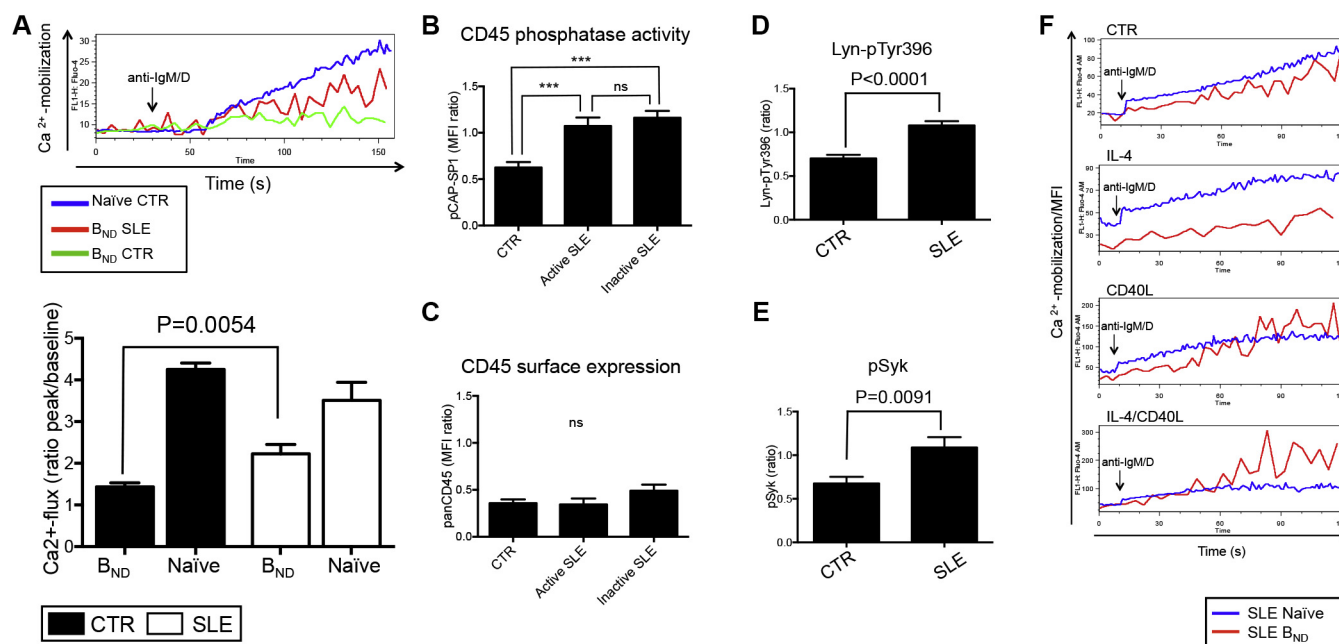


FIG 7. Increased BCR-signaling capacity in B_{ND} cells obtained from patients with SLE compared with healthy control subjects. **A**, Increased Ca^{2+} flux in B_{ND} cells obtained from patients with SLE compared with healthy subjects. A representative Ca^{2+} flux plot of B_{ND} cells obtained from patients with SLE (red line) and healthy control subjects (green line) compared with naive B cells (blue line) is shown. Lower panel, Naive and B_{ND} cells from patients with SLE (open bars) and healthy control subjects (solid bars). Bar graphs show the ratio of peak/baseline mean fluorescence intensity values plotted as means $\pm$ SEMs. **B** and **C**, CD45 phosphatase activity (Fig 7, B) and CD45 surface expression (Fig 7, C) in B_{ND} cells from patients with both active and inactive SLE compared with healthy subjects. Graphs show the fold change in mean $\pm$ SEM MFI for CD45 phosphatase activity (pCAP-SP1; Fig 7, B) or CD45 surface expression (Fig 7, C) in B_{ND} cells relative to that in naive B cells from patients with SLE or healthy control subjects. **D**, Representative Ca^{2+} flux of B_{ND} (red lines) and naive B (blue lines) cells from patients with SLE prestimulated with T-helper factors. **E** and **F**, Lyn-pTyr396 (Fig 7, E) and pSyk (Fig 7, F) levels in healthy donors and patients with SLE expressed as the ratio of means $\pm$ SEMs of B_{ND} cells versus naive B cells. *P* values were determined by using the unpaired 2-tailed Student *t* test (Fig 7, A, D, and E) and 1-way ANOVA with the Tukey multiple comparison test (Fig 7, B and C): ****P* < .001. ns, Not significant. Data are representative of 9 healthy donors and 9 patients with SLE (Fig 7, A); 15 healthy donors, 7 patients with active SLE, and 5 patients with inactive SLE (Fig 7, B and C); and 9 healthy donors and 7 patients with SLE (Fig 7, D and E). Depicted Ca^{2+} flux kinetic curves are representative of 4 experiments (Fig 7, F).

through phosphorylation of CD22 and Fc γ R2 and subsequent recruitment of SH2-containing protein tyrosine phosphatase and SH2-containing inositol 5-phosphatase, respectively. These negative regulatory phosphatases then dephosphorylate downstream targets, leading to reduced BCR signaling. From this, one might expect that an increase in Lyn kinase activity would result also in enhanced negative regulatory effects. Importantly, IL-4 was shown to enhance B-cell activation by relieving the negative regulatory effects of CD22 and Fc γ R2 and downregulating their expression.³² It is tempting to speculate that the observed requirement for both IL-4 and CD40L for restoration of BCR signaling in B_{ND} cells, with CD40L alone exerting an increase in signaling capacity in naive counterparts, could be explained by the observed effect of IL-4 in relieving negative BCR signaling. Altogether, our data suggest that T-cell help has the ability to potentiate activation of Lyn kinase, leading to Syk activation and eventually to restored BCR signaling in B_{ND} cells.

Regulation of Lyn kinase is carried out by the opposing effects of the protein tyrosine phosphatase CD45 and Csk, contributing to setting the important threshold of BCR signaling on antigen receptor stimulation in B cells.^{24,33,34} Inhibition of general

phosphatase activity resulted in a temporary decrease in restoration of Ca^{2+} flux in B_{ND} cells, suggesting the involvement of a phosphatase in this process. By using a pharmacologic inhibitor of CD45 phosphatase, a clear reduction in intracellular Ca^{2+} flux on BCR cross-linking both in naive and B_{ND} cells pretreated with T_H2 cell factors was observed, demonstrating that CD45 plays a crucial role in the transmission of T-cell help to B cells. The inactive Lyn-pTyr507 is progressively dephosphorylated, with increasing CD45 expression in murine B cells, whereas CD45 does not seem to dephosphorylate the active form of Lyn kinase (Lyn-pTyr396), a phenomenon that has been shown for T cells.^{24,35-37} Therefore CD45 plays a positive regulatory role during antigen receptor signaling in B cells, which is in line with our findings.

Further evidence that CD45 modulates BCR-signaling thresholds stems from the HEL transgenic system, where CD45-deficient HEL-specific B cells exhibited diminished signaling in response to the autoantigen HEL.³³ Furthermore, in experiments with supraphysiologic expression of CD45, anergy was transformed into deletion in the HEL-Ig/soluble HEL double-transgenic model.³⁵ Together, these studies provide evidence of the important role of CD45 in regulating antigen receptor–signaling thresholds in B cells.

We found increased activity but no change in expression levels of CD45 on stimulation with T_H2 factors. These findings might be related to the many alternatively spliced isoforms of the CD45 molecule, which vary in glycosylation, dimerization ability, and interaction with poorly characterized ligands.^{38,39} This increased activity was found by using a highly specific and robust assay that takes advantage of the fluorogenic properties of pCAP, an analog of phosphotyrosine that can be incorporated into peptides.^{22,40,41} Using the pCAP-SP1 peptide, we demonstrated that T_H2 cell factors significantly increased CD45 phosphatase activity in both B_{ND} and naive B cells. Moreover, on T-cell help, CD45 inhibition led to a decrease in levels of the active Lyn-pTyr396 form, further reinforcing the central role of CD45 in transmitting T-cell help to B cells, which is fully in line with data supporting a positive regulatory role for CD45 in B cells.³⁵ It has long been known that T-cell stimulatory factors enhance the activation and proliferation capacity of B cells in response to antigen. We have now revealed a novel connection between T-cell help and increased CD45 phosphatase activity, leading to decreased BCR-signaling thresholds in B cells receiving T-cell help.

The fact that nuclear autoantigen-reactive B cells become activated in patients with SLE^{13,14} and that B_{ND} cells harbor reactivity toward these disease-relevant autoantigens prompted us to investigate whether the autoreactive B_{ND} cell subset was tolerized in this prototypical B cell-mediated systemic autoimmune disease. We discovered that the B_{ND} cell population obtained from patients with SLE harbored increased BCR-signaling potential, and this was accompanied by increased CD45 phosphatase activity and, moreover, increased Lyn kinase and Syk activation. It is known that T_H cells from patients with SLE drive the production of antinuclear autoantibodies by cognate interaction with autoimmune B cells.^{42,43} Additionally, T cells from patients with SLE have increased and prolonged expression of CD40L on activation but also exhibit increased levels of baseline CD40L and even soluble CD40L.⁴⁴⁻⁴⁶ Interestingly, we found that B_{ND} cells from patients with SLE regained the ability to signal through the BCR similar to that of naive B cells by only receiving CD40L stimulation. Altogether, this suggests that B_{ND} cells from patients with SLE were less tolerized than B_{ND} cells from healthy control subjects and that B_{ND} cells from patients with SLE might have obtained a degree of T_H2-like factors.

Dysregulated and/or untolerized T cells can provide help in the form of CD40L (and IL-4) to autoreactive B_{ND} cells, leading to increased CD45 phosphatase activity and an activation state more permissive for BCR stimulation, eventually inducing the production of autoantibodies. Indeed, in support of this hypothesis, we observed that B_{ND} cells harbored the potential to differentiate into plasma cells, class-switch to IgG, obtain activation of downstream BCR-signaling kinases (pErk1/2), and proliferate.

Moreover, when investigating features of B_{ND} cells obtained from both patients with active and those with inactive SLE, we determined that CD45 phosphatase activity was equally increased compared with that in healthy subjects. These findings support the theory that CD45 phosphatase activity in patients with SLE is not dependent on disease severity and does not resolve as patients with SLE enter remission and, furthermore, that less tolerized B_{ND} cells might be an integral feature of SLE. These findings are in line with previous findings that patients with SLE in remission continue to produce increased numbers of self-reactive antibodies in the mature B-cell repertoire almost to the same extent as patients with untreated SLE with active disease and therefore

show continued defective early B-cell tolerance.^{14,47} Furthermore, previous studies indicate that the break of B-cell tolerance in patients with SLE is an important initiating feature of the disease and appears years before clinical manifestations develop.⁴⁸

In conclusion, this study pinpoints CD45 as a key regulator of BCR-signaling thresholds mediated by T-cell help. We propose that on T-cell help, the BCR-signaling threshold is decreased, thereby licensing B cells that have received T-cell help to more robustly respond to their cognate antigen. Our findings reveal a novel connection between T-cell help, increased CD45 phosphatase activity, and BCR signaling in human anergic and naive B cells. In line with this, we found that the B_{ND} cell population obtained from patients with SLE was significantly less tolerized than B_{ND} cells from healthy control subjects. This further opens up the possibility that anergic B_{ND} cells might represent the precursors of autoantibody-secreting plasma cells in patients with B cell-driven autoimmune conditions and suggests a possible role for these autoreactive B cells in contributing to autoimmunity in patients with SLE if not properly controlled. Thus B_{ND} cells can pose a danger in the development of systemic autoimmune diseases, especially if T-cell tolerance is also breached, allowing T-helper activation of these autoreactive cells. Our data provide new insight into the break of humoral immune tolerance with possible implications in patients with autoimmune diseases.

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Key messages

- T-cell helper factors restore BCR signaling in human anergic B_{ND} cells.
- CD45 phosphatase activity is upregulated by T-cell help in B cells.
- BCR signaling is regulated by T-cell help through CD45 phosphatase activity.
- B_{ND} cells from patients with SLE are less tolerized and harbor increased CD45 activity.

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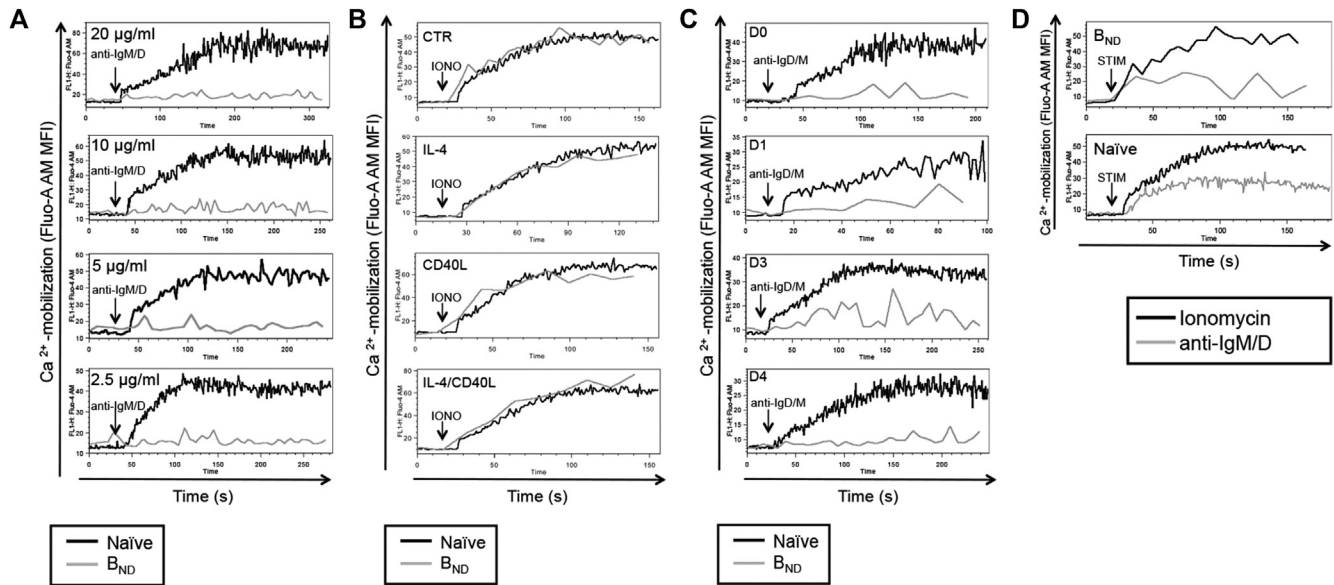


FIG E1. Stimulation characteristics and viability of naive cells and B_{ND} cells. **A**, Effect of concentration of cross-linking antibody for BCR stimulation of B_{ND} cells (gray lines) and naive B cells (black lines). **B**, Responses to ionomycin stimulation in T_H2 factor-treated naive B cells and B_{ND} cells. Negatively isolated B_{ND} cells (gray lines) or naive B cells (black lines) were cultured in the presence of different stimuli (IL-4 and CD40L). IONO, Stimulated with ionomycin. **C**, B_{ND} cells retain the anergic state during *ex vivo* culture. Negatively isolated B_{ND} cells (gray lines) and naive (black lines) B cells were cultured as described, and their calcium mobilization capacity on BCR cross-linking was assessed at the time points indicated. **D**, B_{ND} cells (upper panel) and naive B cells (lower panel) were treated with ionomycin (black lines) and compared with BCR cross-linking (gray lines).

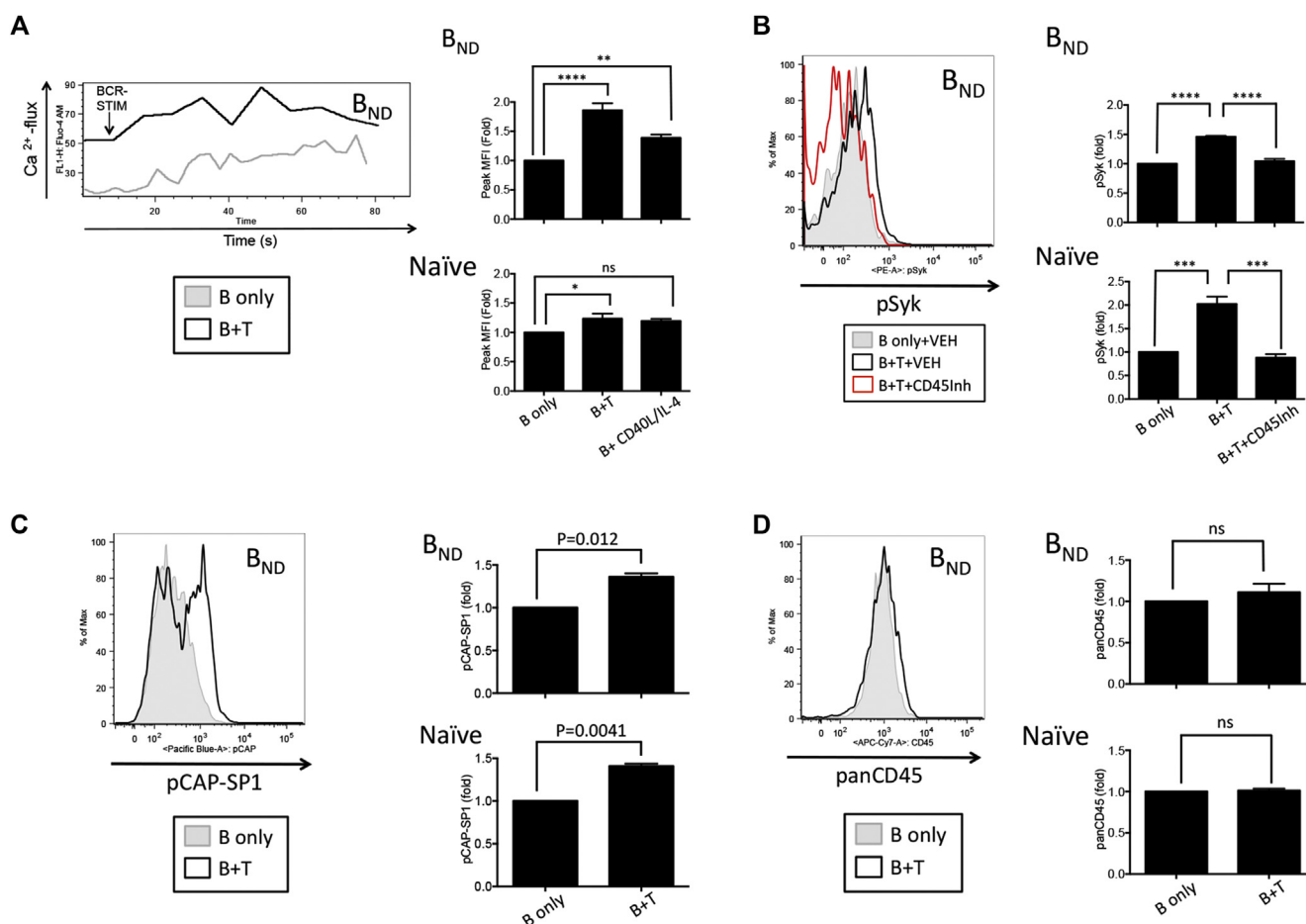


FIG E2. T cells have the ability to induce the key features of break of anergy in B_{ND} cells observed with mimicked T-helper signals. Polyclonal T-cell lines were used as a source of T-helper factors and cocultured with isolated B_{ND} cells and naïve B cells. T cells were gated as CD3⁺ cells and excluded from the functional analyses. **A**, Ca²⁺ flux in response to polyclonal IgM/IgD stimulation in the presence of T cells (black line) or B cells only (gray line). **B**, Phospho-flow analysis of pSyk (Tyr525) in vehicle-treated control B cells (gray histogram); B and T cell- cocultured, vehicle-treated cells (black histogram); and B and T cell-cocultured, CD45 inhibitor-treated cells (red histogram). **C**, B_{ND} cells and naïve B cells increase CD45 phosphatase activity (pCAP-SP1) on coculture with T cells (gray histogram, B cells only; black histogram: B- and T-cell coculture). **D**, CD45 surface expression does not increase on coculture with T cells (gray histogram, B cells only; black histogram: B- and T-cell coculture). Graphs depict means $\pm$ SEMs of mean fluorescence intensity values. *P* values were determined by using 1-way ANOVA and the Sidak multiple correction test (Fig E2, A and B) and paired *t* test (Fig E2, C and D): **P* < .05, ***P* < .01, ****P* < .001, and *****P* < .0001. *ns*, Not significant. Data are representative of 3 independent experiments.

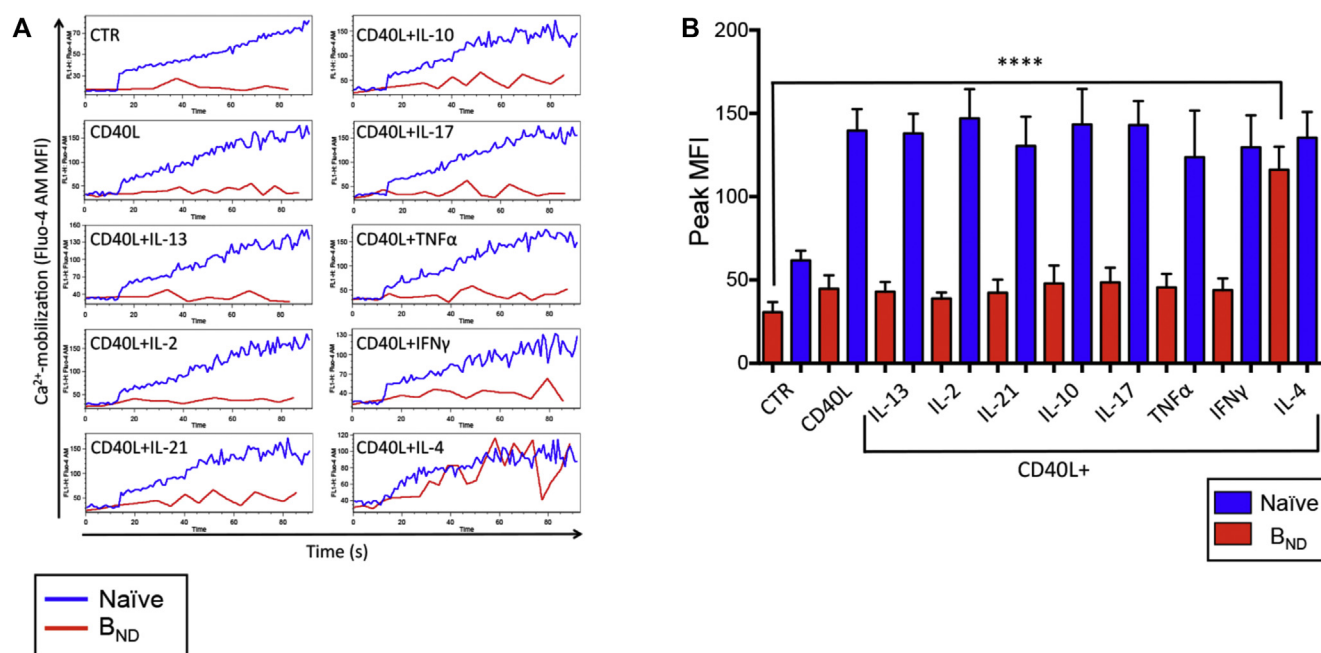


FIG E3. Anergic B_{ND} cells regain their capacity to mobilize intracellular calcium only on receiving CD40L and IL-4 (20 ng/mL) T-helper signals. **A**, Representative kinetic graphs of naïve (blue lines) and B_{ND} (red lines) cells subjected to cytokine stimulation during cell culture. **B**, Graphs depict means $\pm$ SEMs of peak mean fluorescence intensity for B_{ND} cells (red bars) or naïve B cells (blue bars) after BCR stimulation. *P* values were determined by using 1-way ANOVA, followed by the Dunnett multiple comparison test: *****P* < .0001. Data are representative of 3 independent experiments. CTR, Control.

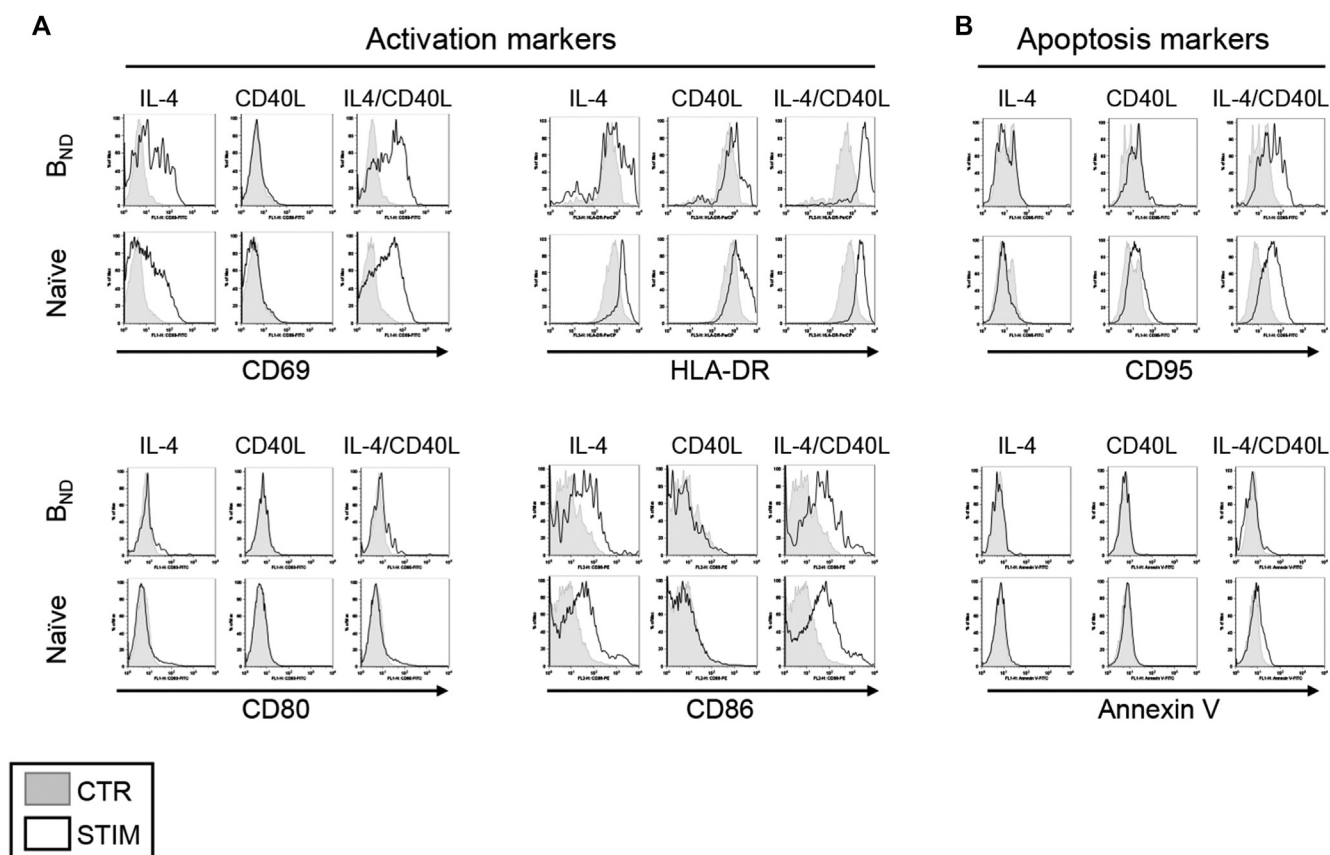


FIG E4. B_{ND} cells upregulate activation markers on stimulation with T_H2 factors. **A**, Activation markers expressed in naive B cells (lower panels) and B_{ND} cells (upper panels) in negatively isolated B cells stimulated with IL-4, CD40L, or both. **B**, Expression of the apoptosis markers CD95 and Annexin V in negatively isolated naive B cells (lower panels) and B_{ND} cells (upper panels) stimulated with IL-4, CD40L, or both. Gray histograms, Control-treated cells; black histograms, stimulated cells. Data are representative of 4 independent experiments. CTR, Control; STIM, stimulated.

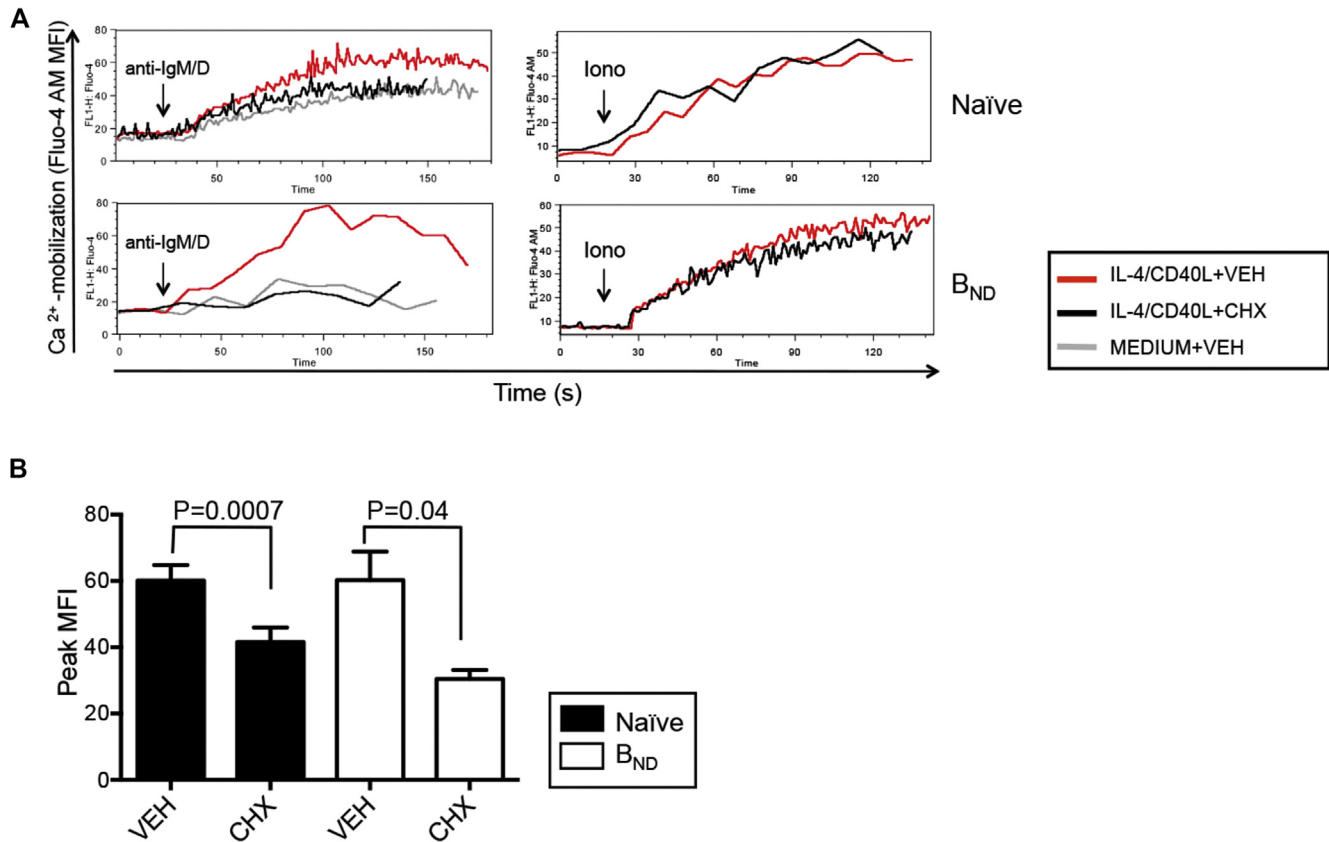


FIG E5. Restoration of BCR signaling in anergic B_{ND} cells on stimulation with T_H2 factors requires *de novo* protein synthesis. **A**, Effect of T_H2 signals (IL-4/CD40L) on BCR signaling is inhibited by addition of cycloheximide (CHX) in B_{ND} and naïve B cells. Representative kinetic calcium mobilization plots expressed as Fluo-4 AM mean fluorescence intensity over time in naïve B cells (*upper panel*) or B_{ND} cells (*lower panel*). CHX or vehicle was included in cell culture during stimulation with T_H2 factors. Cells were analyzed for Ca²⁺ flux subsequent to BCR cross-linking (*left panels*) or ionomycin as control (*right panels*). Red line, Vehicle (dimethyl sulfoxide)- and IL-4/CD40L-stimulation; black line, 5 μ g/mL CHX and IL-4/CD40L stimulation; gray line, unstimulated control and vehicle-treated cells. **B**, Bar graphs showing means $\pm$ SEMs of peak MFI values of naïve B cells (*solid bars*) or B_{ND} cells (*open bars*). *P* values were determined by using the paired 2-tailed Student *t* test (*n* = 4).

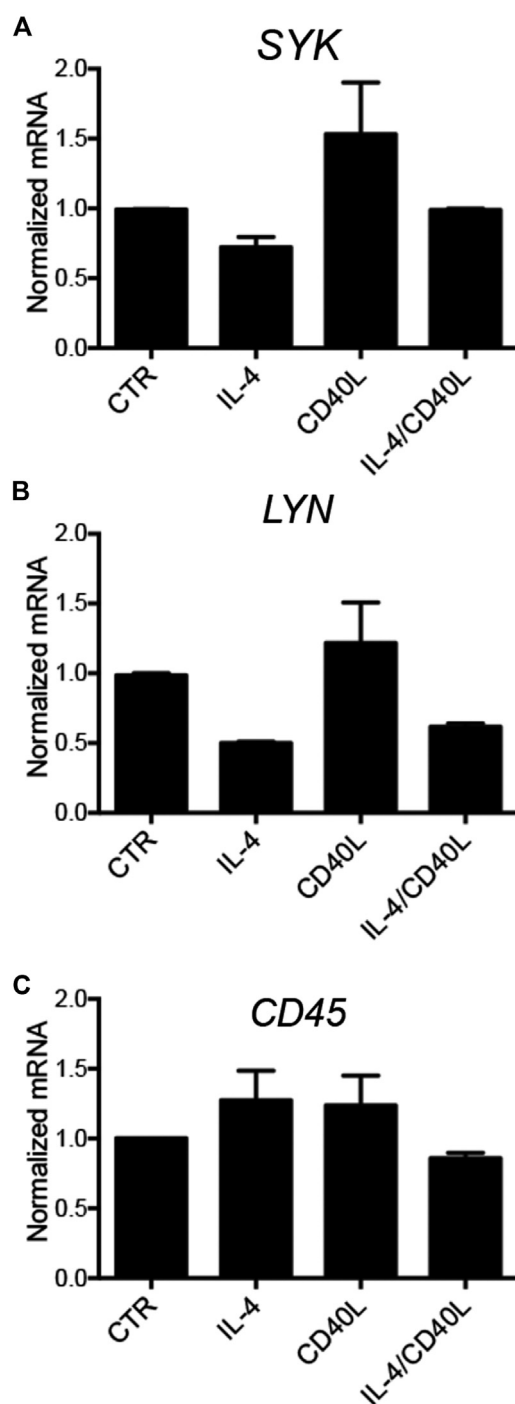


FIG E6. *SYK*, *LYN*, and *CD45* genes are not upregulated by stimulating naive B cells with T_H2 signals. Naive B cells were negatively isolated as described and cultured for 36 hours in the presence of IL-4, CD40L, or both. mRNA levels of *SYK* (A), *LYN* (B), and *CD45* (C) were determined by using quantitative real-time RT-PCR, as described in the Methods section. Data are expressed as the ratio of *SYK*, *LYN*, or *CD45* transcripts relative to expression of the housekeeping gene *POLR2A* as fold change compared with control (CTR). Data show mean $\pm$ SEM expression of 3 donors, all run in triplicates. *P* values were determined by using 1-way ANOVA and the Dunnett multiple comparison test.

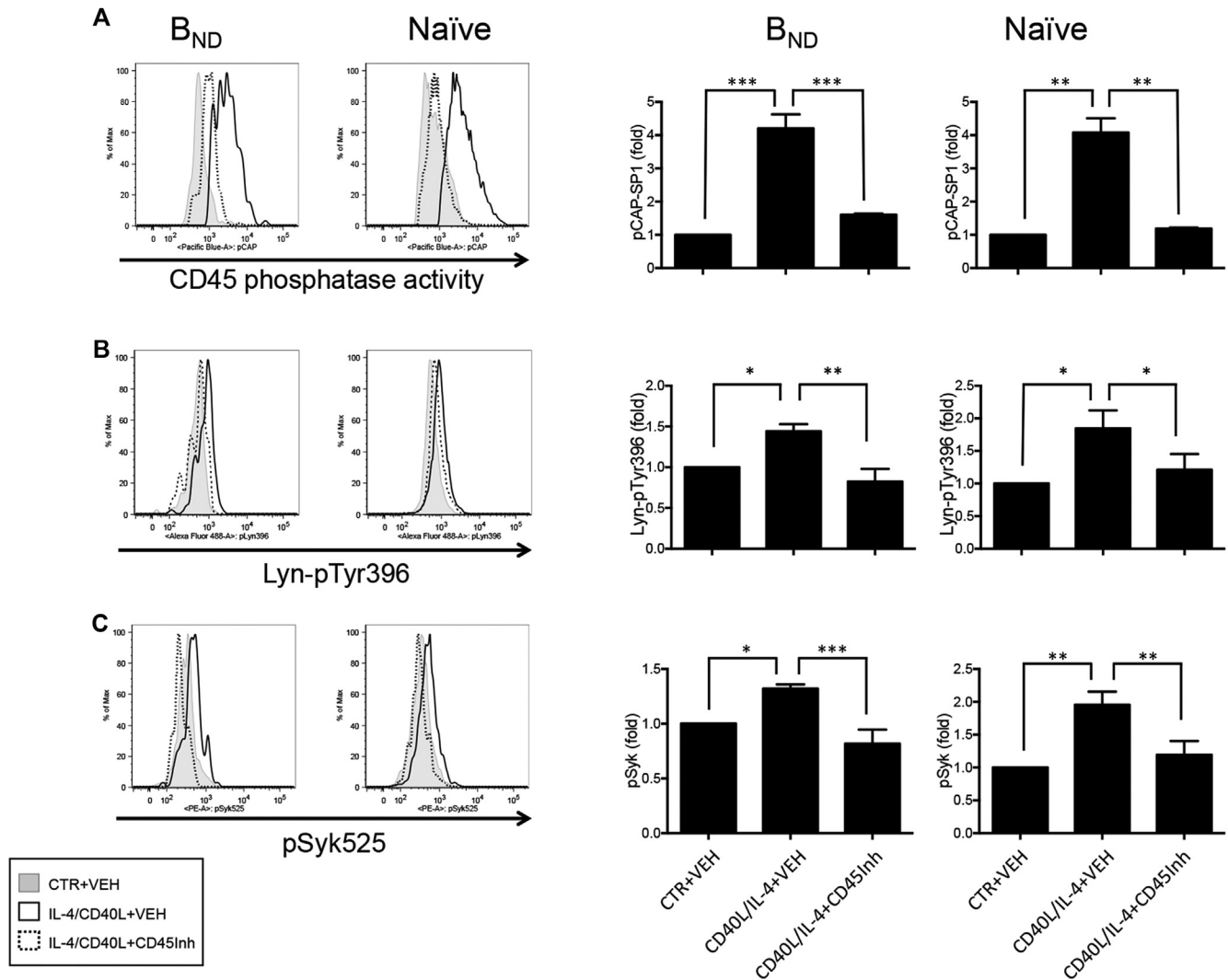


FIG E7. Effect of CD45 phosphatase inhibition on components of the BCR-signaling machinery. **A**, Specific inhibition of CD45 phosphatase activity in naïve B cells (*right panel*) and B_{ND} cells (*left panel*) by the PTP CD45 Inhibitor (Calbiochem) measured based on dephosphorylation of the CD45-specific substrate peptide pCAP-SP1. **B**, Effect of CD45 inhibitor on the activation status of Lyn kinase. Reduced levels of the active form of Lyn kinase (Lyn-pTyr396) in B_{ND} cells (*left panel*) and naïve B cells (*right panel*) were observed after treatment with CD45 inhibitor. **C**, Active Syk (pSyk525) is upregulated on CD40L/IL-4 pretreatment in a CD45-dependent manner both in B_{ND} cells (*left panel*) and naïve B cells (*right panel*). Control cells (gray histograms), IL-4/CD40L-cultured and vehicle-treated cells (black histograms), and IL-4/CD40L-cultured and CD45 inhibitor-treated cells (black dotted histograms) are shown. Bar graphs show means $\pm$ SEMs of mean fluorescence intensity values. *P* values were determined by using 1-way ANOVA with the Sidak multiple correction test: **P* < .05, ***P* < .01, and ****P* < .001. Data are representative of 4 (Fig E7, A and B) and 7 independent experiments (Fig E7, C). CTR, Control.

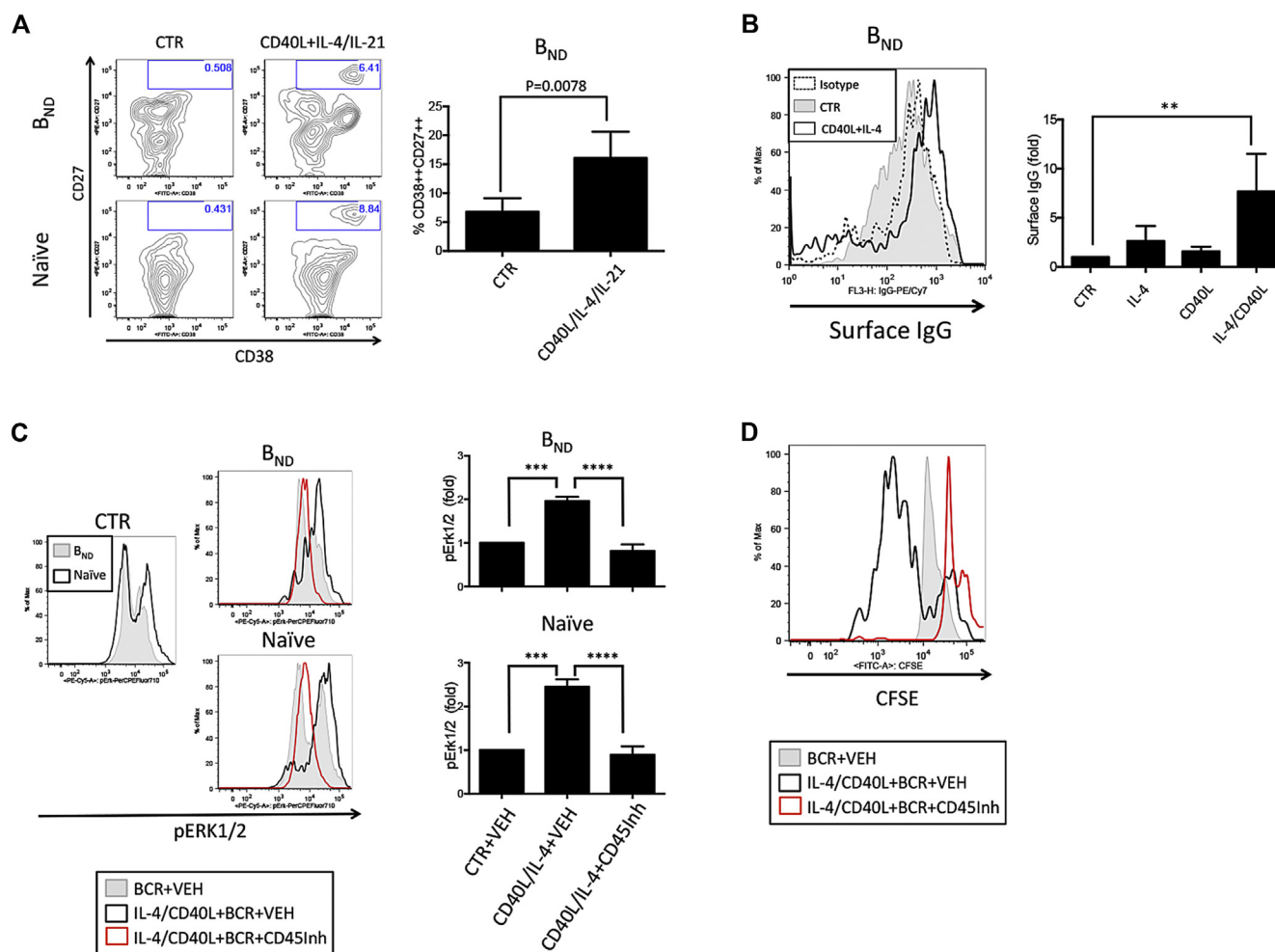


FIG E8. B_{ND} cells have the ability to acquire features of fully activated B cells with antibody-producing capacity. **A**, B_{ND} cells have the ability to differentiate to plasma cell precursors. Isolated B_{ND} cells (upper panel) and naïve B cells (lower panel) were cultured in the presence of IL-4/CD40L and IL-21 for plasma cell differentiation. All samples were stimulated with BCR cross-linkers. Right, Graph shows percentages of plasma cell precursors (CD38⁺CD27⁺). **B**, B_{ND} cells undergo class-switching to IgG on CD40L and IL-4 stimulation. Dotted histogram, Isotype control; gray histogram, control cells; black histogram, CD40L/IL-4-stimulated cells. All samples were stimulated with BCR cross-linkers. **C**, Activation of the downstream BCR-signaling kinase pErk1/2 on CD40L/IL-4 stimulation by using phospho-flow analysis. Gray histogram, Control, BCR- and vehicle-treated cells; black histogram, CD40L/IL-4-stimulated, BCR cross-linked, vehicle-treated cells; red histogram, CD40L/IL-4-stimulated and CD45 inhibitor-treated B_{ND} cells. **D**, Analysis of proliferation of B_{ND} cells on CD40L and IL-4 stimulation by means of CFSE dilution. Gray histogram, Control, BCR- and vehicle-treated cells; black histogram, CD40L/IL-4-stimulated, BCR cross-linked, vehicle-treated cell; red histogram, CD40L/IL-4-stimulated and CD45 inhibitor-treated B_{ND} cells. Graphs depict means $\pm$ SEMs of mean fluorescence intensity values (Fig E8, B and C). *P* values were determined by using the Wilcoxon matched-pairs signed-rank test (Fig E8, A), Kruskal-Wallis test (Fig E8, B), and 1-way ANOVA with the Sidak correction (Fig E8, C): ***P* < .01, ****P* < .001, and *****P* < .0001. Data are representative of 8 (Fig E8, A), 7 (Fig E8, B), and 4 (Fig E8, C and D) independent experiments, respectively.