CVID-associated B cell activating factor receptor variants change receptor oligomerization, ligand binding and signaling responses

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CRISPR/Cas9 site-directed mutagenesis

The custom-designed RNA 5'-AACCGGGTAAGGGGGACCCACGG used to target the TNFRSF13C gene was purchased from Integrated DNA Technologies (IDT).

The guide RNA (gRNA) was formed by combining equimolar amounts of 200µM crRNA and tracer RNA (tracrRNA, IDT) in IDTE duplex buffer to a final concentration of 44µM, followed by a 5min incubation at 95°C. For the formation of the ribonucleoprotein (RNP) complex the gRNA was mixed in a 1:1 ratio with 36µM Cas9 enzyme and incubated for 20min at RT. Prior to electroporation, 5 x 10^5 DG75 cells were washed twice with PBS (256xg; 5min; RT) and resuspended in 9µl Neon electroporation R buffer. The cell suspension was added to the RNP complex and mixed with 10.8µM electroporation enhancer (IDT). For each electroporation reaction, cells were loaded together into a 10µl neon tip and inserted in the neon tube containing electrolytic buffer. The Neon transfection system (Invitrogen) was set up for four different conditions (1350V, 30ms, 1 pulse; 1100V, 30ms, 2 pulses; 1700V, 20ms, 1 pulse; 1550V, 20ms, 1 pulse). Afterward, the cells recovered in RPMI medium supplemented with 20% FCS without antibiotics for 24-48h

Construction of BAFFR expression vectors

Before the subcloning of the BAFFR variants into the lentiviral expression vectors pNL-CEF-GS-GFP and pNL-CEF-GS-RFP, the vectors were cleaved with EcoRI and SalI restriction enzymes. The different PCR fragments encoding BAFFR constructs were amplified using the Q5 site-directed mutagenesis kit according to the manufacturer's protocol.

For the pNL-CEF expressing BAFFR variants (A52T, G64V and Dup92-95), the forward primer 5'-CCCAAGCTGGCTAGCGTTTGAATTCCACCATGAGGCGAGGGC and reverse primer 5'-GAGCTGTGGCATCAGAGATTCC were used. In the case of the

BAFFR variants (P21R, P146S and H159Y) encoded by retroviral MIGR-based expression vectors (1), the BAFFR-MIGR1 forward primer 5'-CAAGCTGGCTAGCGTTTGACCATGAGGCGAGGGC and the BAFFR-MIGR1 reversed primer 5'-GATCCGCTGCCTGGTCCGCCTTGTTGCTCAGGGCC were used.

After separation by agarose gel electrophoresis, the vectors and inserts were isolated using the NucleoSpin Gel and PCR clean-up kit. DNA fragments were ligated with the NEBuilder HiFi DNA Assembly kit (New England Biolabs) for 15min at 50°C, according to the manufacturer's instructions. STBL2 competent cells were inoculated in 2 ml LB containing 75µg/ml ampicillin overnight at 37°C. DNA was purified using the NucleoSpin Plasmid EasyPure kit (Macherey-Nagel) according to manufacturer protocol. The amino acid exchanges in BAFFR were confirmed by DNA sequencing with the aid of the Mix2 Sequencing kit (Eurofins Genomics) using the forward primer 5'-CACACTGAGTGGGTGGAGAC and the reverse primers 5'-GGATGCCCTGGGTGTGGTTGAT and 5'-CGGCGGCGGTCACGAACTCC.

Lentiviral gene transfer

3 x 10^5 293T cells were seeded one day before transfection into a 6-well plate in 2ml complete culture medium to obtain 60-80% confluence at the following day. The transfection was carried out using JetPEI reagent (Polyplus-transfection) according to the manufacturer's instructions, with 0.9µg packaging plasmid (pCD/NL-BH*), 0.9µg envelope plasmid (pVSV-G) and 1.5µg target DNA (encoding for the different BAFFR constructs) as described before (1, 2). After two days of cultivation, the virus particle-containing supernatant was collected, centrifuged (256xg; 7min, RT) filtered through 0.45µm syringe filter and pelleted (3600xg, 16h, 4°C). The next day after discarding

the supernatant, the concentrated virus was added to 3 x 10^4 DG75 BAFFR KO cells and incubated for 1h, followed spin infection (1111xg; 120min, 30°C).

PBMCs isolation

Peripheral blood mononuclear cells (PBMCs) from healthy controls and patients were isolated from whole blood by Ficoll density gradient centrifugation (1, 3). Blood samples were diluted 1:2 in sterile PBS supplemented with 2mM EDTA (PBS-EDTA) and layered over 20ml Ficoll (PanBiotech) in 50ml Falcon tubes. After centrifugation (1000xg; 15min; RT), PBMCs were isolated from the interphase and washed twice (585xg; 7min; RT) with PBS-EDTA. Cells were then counted and kept in culture for further analysis or were frozen at -80°C.

Flow cytometry

Flow cytometry was performed as described (1, 2). 1 x 10^6 cells/ml were resuspended in 200µl PBS supplemented with 5% FCS and 2mM EDTA ("FACS buffer"). Pellets were resuspended in 50µl of fluorochrome-conjugated antibodies diluted to the appropriate concentration with FACS buffer and incubated in the dark for 25 min at 4°C. The following antibodies were used: anti-CD19 APC-Cy7 (Biolegend), anti-CD27 PE-Cy7 (Biolegend), anti-IgD FITC (SouthernBiotech), anti-BAFFR APC (Biolegend). anti-pS6 PE (Cellsignal), and anti-CD79B APC (Biolegend). FACS buffer was added to 200 µl and cells were washed (256xg; 5min, 4°C). Cell pellets were resuspened in FACS buffer containing 100ng/ml 4′,6-Diamidino-2-phenylindole (DAPI, eBioscience) to discriminate living from dead cells. After a second washing step, cells were resuspended in 200 µl FACS buffer and analyzed using a FACS Canto II Flow cytometer and FACS Diva Software (BD Biosciences). The FCS files containing the data were analyzed with the FlowJo Software (BD Biosciences).

For intracellular flow cytometry, cells were first fixed with 4% paraformaldehyde for 20 min at room temperature in the dark. After pelleting the cells by centrifugation (256xg; 10 min, RT) and removal of the supernatant, cells were permeabilized by for 20 min in the dark in 0.1% saponin-PBS at RT.

Flow cytometry-based FRET

FRET was performed as described (1). DG75 BAFFR KO cells were co-transduced with lentiviral vectors carrying CD79B, BAFFR WT or the different variants fused to GFP or RFP at the C-terminal domain. Followed a 1h (BAFFR-BAFFR) or 6h (BAFFR-CD79B) stimulation with BAFF (0, 50,100ng/ml), cells were washed (256xg; 5min; RT) and resuspended in 100µl Fluorobrite DMEM medium (Thermo Fischer Scientific) containing 5% FCS and analyzed using a LSRII Fortessa flow cytometer (BD Biosciences). The donor fluorophore (GFP) signal was measured using the 488nm blue laser with a 505 long pass (LP) and 530/30 bandpass (BP) filters. RFP was generated by the 561nm yellow/green laser and detected using a 570 LP and 586/15 BP filters. The FRET signal (GFP-RFP) was the result of RFP excited by GFP and collected by the 595 LP and the 625/15nm BP filters. The single positive cells were used as controls to determine the spillover of GFP or RFP into the FRET channel. The data were analyzed with the FlowJo Software (BD Bioscience).

ELISA

ELISA was carried out as described previously (1, 2). Plates (Nunc flat-bottom Maxisorp) were coated at 4°C with 100µl per well of a 5µg/ml 2.81 rat IgG2b anti-human BAFF antibody diluted in bicarbonate buffer. After 16h, the ELISA-plate was blocked for 1h at RT with PBS supplemented with 1% BSA to reduce unspecific binding. After three washing steps with PBS 0.1% tween 20, the standard (BAFF) and the FLAG-BAFF ligand were pipetted in a dilution series and incubated for 2h at RT.

Followed by 3 more washing steps with PBS 0.1% tween 20, 100µl per well of a 1µg/ml 4.62 rat IgG2a biotinylated anti-human BAFF antibody diluted in PBS was added to the samples which were then incubated for 2h at RT. Signals were detected with streptavidin-conjugated alkaline phosphatase (SA-AP, Sigma Aldrich) at a 1:2500 dilution in TBS buffer, developed with 1mg/ml Para-Nitrophenylphosphat (PNPP) and recorded at a 405/492nm ELISA-Reader multiskan Fc (Thermo Fischer Scientific).

Ligand-binding assay

2x 10^5 cells of the different DG75 cell lines were stimulated with 50µl FLAG-BAFF in a serial dilution for 30min at 4°C. Cells were washed (256xg; 5min; 4°C) with FACS buffer and stained for 30min on ice with 50µl anti-FLAG antibody diluted in FACS buffer. After two washing steps (256xg; 5min; 4°C) samples were acquired on FACS Canto II Flow cytometer (BD Biosciences). The Bmax value was calculated with Prism 8 Software (GraphPad).

Western blot analysis

Whole-cell lysates of primary B cells or cell lines were prepared as described (2) and proteins were separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide at 80V for 3h. The transfer of proteins into a nitrocellulose membrane (0.2µM, Bio-Rad) was performed at 100 V for 1h. Immediately after, the membrane was blocked for 1h at RT in PBS containing 0.1% Tween (PBS-Tween) supplemented with 5% milk while shaking. The following primary antibodies were used to incubate the membranes overnight at 4°C: anti-BAFFR CT (Enzo), anti-TRAF3 (cell signaling), anti-NF-κB2 (Millipore, clone), anti-phospho-AKT (Ser 473, clone D9E, cell signaling), anti-phospho-p44/42 MAPK (Thr202/Tyr204, cell signaling) and anti-beta actin (clone AC15, Sigma Aldrich). The next day, membranes were washed with PBS-Tween and incubated for 1h while shaking with horseradish

peroxidase (HRPO)-coupled donkey anti-mouse IgG and donkey anti-rabbit IgG secondary antibodies (Jackson Immuno Research). After five washing steps with PBS-Tween, the chemiluminescent signal was detected with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fischer Scientific) or WesternBright Quantum HRP substrate (Advansta) using the Fusion Fx (Vilber Lourmat) and the Fusion Capt Advance Software (Vilber Lourmat). The differences in protein expression were quantified by densitometric analysis with the aid of the ImageJ software (NIH).

Co-immunoprecipitation

Co-immunoprecipitation was carried out as described before (2).

2 x 10^7 DG75 cells were stimulated with FLAG-BAFF in a time course (0, 15, 30, 60min). Cells were then washed once with ice-cold PBS and lysed for 15min in 500µl lysis buffer (0.2% NP-40, 20 mM Tris-HCl, 150mM NaCl, 10% glycerol, complete protease inhibitor cocktail). Cell lysates were centrifuged at 13000 rpm for 10 min at 4°C. 450µl of the supernatants were transferred into Eppendorf tubes containing 50µl anti-FLAG Agarose Beads (anti-Flag M2 Affinity gel, Thermo Fischer) that were previously washed and equilibrated with lysis buffer. After an ON incubation at 4°C while rotating, the samples were pipetted in a mini-column as described previously (49) and washed twice with 200µl lysis buffer. Next, the immunocomplexes were eluted with 40µl citrate buffer (pH 2.4) and neutralized with 10µl 1.5M Tris buffer (pH 9). For western blot detection, Laemmli sample buffer was added to the samples before they were vortex and heated at 95°C for 5min.

Fluorescence microscopy

3x 10^4 BAFFR-WT cells were resuspended in 100µl Fluorobrite DMEM medium (Thermo Fischer Scientific) containing 5% FCS and transfer to a collagen-coated 4 well microscopy dish (Greiner). Images were taken in a 63X magnification in GFP, RFP

and DAPI channels with a Axio Observer (Zeiss) inverted micrcoscope equipped with an incubation chamber at 6.5% CO_2 athmosphere. The data were analyzed with Zen software (Zeiss).

Structural models

All structural models of BAFFR were made with UCSF Chimera, UCSF Modeller (4-7) and Swiss model (8). They are based on the 3D model of BAFFR (https://www.alphafold.ebi.ac.uk/entry/Q96RJ3) generated by Alphafold (9).

Statistical analysis

Statistical analysis was performed with Prism 8.4.2 Software (GraphPad). For all statistical comparisons with more than two groups, the data were analyzed using the 2-way ANOVA with Šidák's multiple comparison test. Differences between groups were defined as significant when $p<0.05$ (ns $p>0.05$; *$p=0.01$ to 0.05; **$p=0.001$ to 0.01; ***$p=0.0001$ to 0.001; **** $p<0.0001$).

Supplementary Table S1 Allele frequencies and relative Risk of BAFFR varients in different ethnic populations										
				Allele Frequencies (1/n)				**Relative Risk European vs.**		
Variant	**rs ID**	**Reference Allele**	**Alternate Allele**	**African**	**East Asian**	**South Asian**	**European non-Finnish**	**African**	**East Easian**	**South Asian**
P21R	rs77874543	G	C	72	31	17	13	5.784	2.615	1.34
A52T	NA	G	A	NA	NA	NA	NA	NA	NA	NA
G64V	rs547352394	C	A	396	>6714	353	69	5.761	0.7611	5.084
A92-95DUP	rs752794297	C	CGCCAGGACCAGT	6754	>17558	>30196	>103636	NA	NA	NA
A92-95DUP	rs752794297	A	ACCAGGACCAGCG	>13684	>17584	>30196	104518	NA	NA	NA
P146S	rs777233252	G	A	>16210	>18380	>30614	113132	NA	NA	NA
H159Y	rs61756766	G	A	710	>19932	189	133	5.349	NA	1.419

Supplementary Table S2 Comparison of mammalian BAFFR sequences.

The amino acid sequences of 24 different mammalian BAFFR proteins were aligned to human BAFFR with MUSCLE (https://www.ebi.ac.uk/tools/msa/muscle/). The six different human BAFFR variants P21R, A52T, G64V, 92-05DUP, P146S, H159Y are shown in red and residues which are identical to the human variant are shown in bold letters. The transmembrane region is shown in italics. The numbering refers to the human BAFFR sequence and the evolutionary conservation of the residues corresponding to the wildtype residue of the different variants is shown above the numbering as % identity.

Cystein-rich domain

% IDENTITY TO HUMAN BAFFR	32
POSITION	1 10 20 30 40 45
Q96RJ3_HUMAN	-----MRRGPRSLRGRDAPAPTPCVPAECFDLLVRHCVACGLLRTPRPKP
H2QLT1_CHIMPANZEE	-----MRRGPRSLRGRDAPAPTPCVPAECFDLLVRHCVACGLLRTPRPKP
G3R8S3_GORLILLA	-----MRRGPRSLRGRDAPAPTPCVPAECFDLLVRHCVACGLLRTPRPKP
A0A5F7ZFG6_MACAQUE	-----MKRGPRSLRGRDAPAPTPCVPAECFDLLVRHCVACGLLRTPRPKP
H9KVT2_MARMOSET	-----MRRGPRSLRGRDAPVPTPCVPTECYDLLVRKCVDCRLLRRSPPKT
0A2K5SC14_WHITE_CAPUCHIN	-----MRRGPRSLRGREAPVPTPCVPTECYDLLVRKCVDCRLLRKSPPKT
A0A2K6GIX6_COQUERELS_SIFAKA	-----MRRGARRPRGRDGPPPTPCDPAQCFDLLVRHCVACKLLRTPEPRP
H0XRB7_OTELMUR_GARNETTI	-----MQRGARRPRGRDGPVPTSCYPAQCFDPLLRLCVDCELFRTPESPA
G3U9C5_AFRICAN_ELEPHANT	-----MGLGSRSMGGRDGSEPTLCGQAECFDLLLRVCVACRLFRTPEPSP
A0A6I9JZX1_CAPE_GOLDEN_MOLE	-----MARGARSMGGRDGPAPPQCGQGKCFDSLLRACVACRLFRTPEPSP
H0W812_GUINEA_PIG	-----MGPGSRSLKGMSGPAPTRCVQTECFDHLVRHCVSCRLLRTPD---
A0A6P3FQU0_COMMON_DEGU	-----MGQGARGLQGMNGPGPTHCVQAKCFDQLVRHCVDCKLLRTPEPRA
A0A6J2A960_CHEETAH	--MGSQRRGARGLRGREGAAPTKCVVDKCFDPLVRKCVDCRLLRTPEPGL
A0A6P6I3X8_PUMA	--MGSQRRGARSLRGREGSAPTKCVVDKCFDPLVRKCVDCRLLRTPEPGP
M3YY53_EUROPEAN_POLECAT	--MGSKRRGARNRRGRDSPAPTQCVQAQCFDPLVRNCVACNLFRTPEPRP
A0A3Q7UGN9_GRIZZLY_BEAR	--MGSKRRGARSLRGRDGPAPTQCVQAECFDPLVRNCVACKLFRTSEPRP
A0A2U3ZCW5_WALRUS	--MGSKRRGARSRRGRDGPAPTQCVQAECFDPLVRNCVACKLFRTPEPRP
A0A2Y9H1Y3_HAWAIIAN_MONK_SEAL	--MGSKRRGARSRPGRDGPAPTQCVQAECFDPLVRNCVACKLFRTPEPRP
A0A1S2ZRM0_EUROPEAN_HEDGEHOG	-----MRR--RGRRSRDGQAPSPCLEALCFDLLVRDCVACSFLHTSEPRP
F1MJD0_CATTLE	-----MQRGRRSLRGKDRPAPTQCLQTQCFDPLVRNCVACSLLRTTGPRL
A0A452EY37_GOAT	-----MPRGRRSLRGKDRPAPTQCLQTQCFDPLVRNCVACSLLRTTEPRL
A0A2Y9T0B2_SPERM_WHALE	MGFGRMRRGPRGLRGRDRPAAPQCLQTQCFDPLVRKCVACSLLRTTEPRP
A0A340WYL1_RIVER_DOLPHIN	-----MRRGARGLRGRDRPATPQCLQTQCFDPLVRKCVACSLLRT-EPRP
A0A6P3QWA8_FLYING FOX	-----MRRGARSPRGRDAPAPTQCVQAQCFDPLVRNCVACKLFRTPEPGP
A0A287AJ47_PIG	-----MRRRARSLRGRDGPVPTQCLQTQCFDLLVRNCVACSLLRTPDPGP

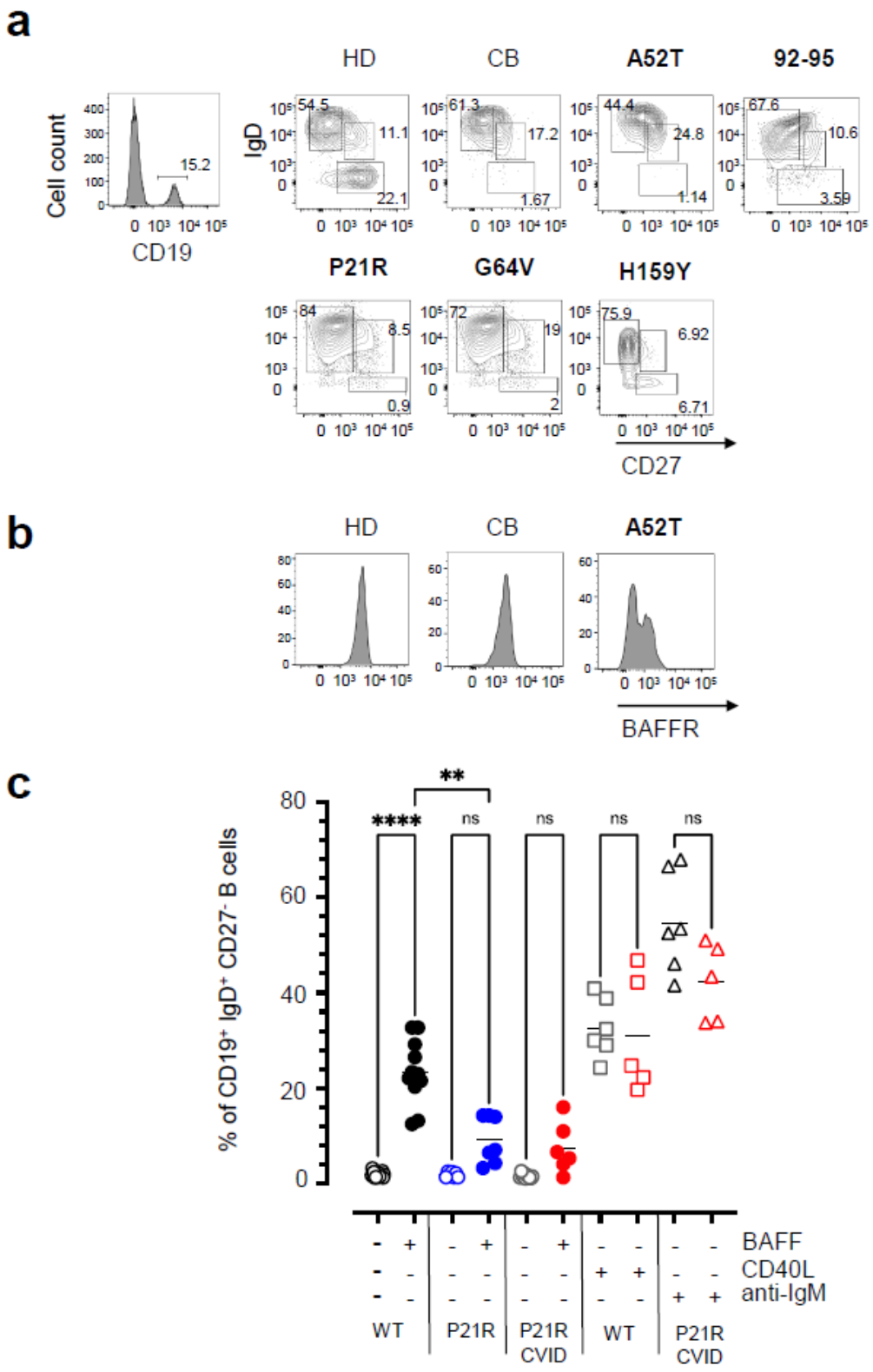
% IDENTITY TO HUMAN BAFFR		88 96
POSITION		46 50 60 70 80 87
		transmembrane domain
Q96RJ3_HUMAN		--AGA-SSPAPRTALQPQESVGAGAG----EAALPLPGLLFGAPALLGL
H2QLT1_CHIMPANZEE		--AGA-SSPAPRTALQPQESVGAGAG----EAALPLPGLLFGAPALLGL
G3R8S3_GORLILLA		------GKGDPRG---PQESVGAGAG----EAALPLPGLLFGAPALLGL
A0A5F7ZFG6_MACAQUE		--APA-SSPAPRTALQPQESVGAGAG----EAALSLPGLLFGAPALLGL
H9KVT2_MARMOSET		--AGA-SSPAPGTALQPQESVGPGAG----EVSLPVPRLLFGAPALLGL
0A2K5SC14_WHITE_CAPUCHIN		--AGA-SSPAPGTALQPQESVGAGAG----EVALPLPGLLFGAPALLGL
A0A2K6GIX6_COQUERELS_SIFAKA		--AGA-SSAAPGTALQPQESVGAGAGP---EAALPLPGLLFGAPALLGL
H0XRB7_OTELMUR_GARNETTI		PAAGL-SSVVPGTALQPQESVGAGGS----EAALPLSGLLLGAPALLGL
G3U9C5_AFRICAN_ELEPHANT		--AGA-SSPATGTALQPQKTVGAGAVP---QGALPLPGLLFGAPALLGL
A0A6I9JZX1_CAPE_GOLDEN_MOLE		A-AGA-SSSASGTAPQPQESVGADAGP---QGALPLPGLLFGAPALLGL
H0W812_GUINEA_PIG		-------SQAPGTVLQPQES-----------AMLPMPGLLFGAPVLLGL
A0A6P3FQU0_COMMON_DEGU		--AGA-SSQAPGTALQPQESVGAGTP----QAVLPLPGLLFGVPVLLGL
A0A6J2A960_CHEETAH		A-AGP-GSLAPGTALQPQESVGAGAAAETEAEAALPLPALLFGAPALLGL
A0A6P6I3X8_PUMA		A-AGP-GSLAPGTALQPQESVGAGAAAETEAEAALPLPALLFGAPALLGL
M3YY53_EUROPEAN_POLECAT		A-AGASSSPAPGTALQPQESVGPGGAA--EAEAALPLPALLFGAPALLGL
A0A3Q7UGN9_GRIZZLY_BEAR		A-AGA-SSLAPGTALQPQESVGPGTAA--EAEAALPLPALLFGAPALLGL
A0A2U3ZCW5_WALRUS		--ARA-SSLAPGTALQPQESVGPGAAA--EADTALPLPALLFGAPALLGL
A0A2Y9H1Y3_HAWAIIAN_MONK_SEAL		--AGA-SSLAPGTALQPQESVGPGAAA--EAEAALPLPALLFGAPALLGL
A0A1S2ZRM0_EUROPEAN_HEDGEHOG		--AGT-SSLEPGTALQPQESVGSGVA---PAEGALPLPALLVGAPAGLGL
F1MJD0_CATTLE		--AGDRSSLAPGTALQPQESAGPGTPA--EAEAALPLPALLFGAPALLGL
A0A452EY37_GOAT		A-GGP-SSLAPGTALQPQESAGPGTPA--EAEAALPLPALLFGAPALLGL
A0A2Y9T0B2_SPERM_WHALE		A-GGP-SSLAPGTALQPQESVGPGAAA--EAEAALPLPALLFGAPALLGL
A0A340WYL1_RIVER_DOLPHIN		GKNGP-SSLAPGTALQPQESVGPGATA---EAALPLPALLFGAPALLGL
A0A6P3QWA8_FLYING FOX		--AGP-SSLAPGTALQPQESVGAGTPA--HAEAALPLPALLFGAPALLGL
A0A287AJ47_PIG		A-GGP-SSLAPGTALQPQESVGPGAA---EAEAALPLPALLLGAPALLGL

% IDENTITY TO HUMAN BAFFR	**96**
POSITION	**90 100 110 120 130**
Q96RJ3_HUMAN	ALVLALVLVGLVSWRR---RQRRLRGASSAEAPD----GDKDAPEPLDKV
H2QLT1_CHIMPANZEE	ALVLALVLVGLVSWRR---RQRRLRGASSAEAPD----GDKDAPEPLDKV
G3R8S3_GORLILLA	ALVLALVLVGLVSWRR---RQRRLRGASSAEAPD----GDKEAPEPLDKV
A0A5F7ZFG6_MACAQUE	ALVLALVLVGLVSWRR---RQRRLRGASSAEAPD----GDKDKDEPLDKV
H9KVT2_MARMOSET	VLVLALVLVGLVSWRR---RQQRLRGAASSEAPDD---GDEAAPEPLDKL
0A2K5SC14_WHITE_CAPUCHIN	VLVLALGLVGLVSWRR---RQQRLRGTASTEATD----GDKDAPEPLDKV
A0A2K6GIX6_COQUERELS_SIFAKA	VLALALALLGLMSWRR-----RRLRGTASPEGPD----G-----EPLDSV
H0XRB7_OTELMUR_GARNETTI	ALALALALVGLVSWRY-----RRLRGAASPQAPN------EGQHEPLDNV
G3U9C5_AFRICAN_ELEPHANT	A--LAMGLVRLVRWRR------RRRGMAPPEASDRPDPPFPPLPESLDNV
A0A6I9JZX1_CAPE_GOLDEN_MOLE	A--LALVLVCLVHWRR------QRGGAAPPETPD------CDGNEPLDNV
H0W812_GUINEA_PIG	ALLLALVLVALVSWKW---RQRRTQ-------------------EDLSCV
A0A6P3FQU0_COMMON_DEGU	ALLLALVLVALVSWKW---RQRRTLEALERSQ-D----GESHVGKPPDNI
A0A6J2A960_CHEETAH	A--LALVLVGLLSWRW--RRRRRLRAAAPPGDPD----P--HPHEPLDDV
A0A6P6I3X8_PUMA	A--LALVLVGLLSWRWRRRRRRRLRAAAPPGDPD----P--HPHEPLDDV
M3YY53_EUROPEAN_POLECAT	A--LALVLVGLLGCRW---RRRRLRAGAPPGTPE----P--QPRETLDHV
A0A3Q7UGN9_GRIZZLY_BEAR	A--LALVLVGLLSWRR---RRRRLRAAAPPGAPD----PHAHPHEPLDNV
A0A2U3ZCW5_WALRUS	A--LALVLVGLLSWRW----RRRPHAAAPPRAPA------RHPHEALDNV
A0A2Y9H1Y3_HAWAIIAN_MONK_SEAL	A--LALVLVGLLSWRW-RRRRRRPHAAAPPGALA----P--HPHEALDNV
A0A1S2ZRM0_EUROPEAN_HEDGEHOG	A--LALLLLGLLSWRR-----RCGRGEARPEVA-----AAEPQEEPLDHV
F1MJD0_CATTLE	A--LALALLGLVTWKR---RRRQPGAAVVPEAPE-----AAEVPESLDVD
A0A452EY37_GOAT	A--LALALIGLVTWKR---RRRQPGAAVVPEAPE----------PLDVD
A0A2Y9T0B2_SPERM_WHALE	A--LALVLLGLVGWRR---QRRRPGAAAAPEALEA---RDGDQDEPLDSD
A0A340WYL1_RIVER_DOLPHIN	A--LALVLLGLVSWRR---RRRRPGAAAAPEALEAPD-GDQDAPEPLNSD
A0A6P3QWA8_FLYING FOX	A--LALVLVALVSWRQ-RRRRRRPPGPASPEARD----R--EQDEPLNSV
A0A287AJ47_PIG	A--LALVLLGLVSWRR---RRQRPRASAAPEAPEA---LDGDQDGPLDND

% IDENTITY TO HUMAN BAFFR	80 100 **TRAF3 BINDING**
POSITION	**131 140 150 160 170 179**
Q96RJ3_HUMAN	IILSPGISDATAPAW**P**PP-GEDPGTTPPG**H**SVPVPATELGSTELVTTKTA
H2QLT1_CHIMPANZEE	IILSPAISDATAPAW**P**PP-GEDPGTTPPG**H**SVPVPATELGSTELVTTKTA
G3R8S3_GORLILLA	IILSPGISDATAPAW**P**PP-GEDPGTTPPG**H**SVPVPATELGSTELVTTKTA
A0A5F7ZFG6_MACAQUE	IILSPGISDAAAPAW**P**PP-GEDPGTTPPG**H**SVPVPATELGSTELVTTKTA
H9KVT2_MARMOSET	IILSPGTTDATASAW**P**PP-GEDPGTTPPG**H**SVPVPATELGSTELVTTKTA
0A2K5SC14_WHITE_CAPUCHIN	IILSPGTTDATAPAW**P**PP-GEDPGTTPPG**H**SIPVPATELGSTELVTTKTA
A0A2K6GIX6_COQUERELS_SIFAKA	IILSPGTLDATAPVW**P**PPREEDPGTTPPG**H**SVPVPATELGSTELVTTKTA
H0XRB7_OTELMUR_GARNETTI	IVLSPGTIDTTAPVW**P**LPREEDPGTTPPG**H**SVPVPATELGSTELVTTKTA
G3U9C5_AFRICAN_ELEPHANT	IILSPGPIDATSPVW**S**PP--EDRATTLPG**H**SVPVPATELGSTELVTTKTT
A0A6I9JZX1_CAPE_GOLDEN_MOLE	IVLSPGSTDVTAPIW**P**PP--EDPATTPPG**H**SVPVPATELGSTELVTTKTM
H0W812_GUINEA_PIG	YILSSGTPDASAPDW**P**TP-REDPATTPSS**H**SVPVPATELGSTELVTTKTA
A0A6P3FQU0_COMMON_DEGU	YIPSSGTPDASAPVW**P**AP-REDPAATPSS**H**SVPVPATELGSTELVTTKTA
A0A6J2A960_CHEETAH	IIHPPKPLDATAPVW**P**PP-REDPANTPPA**H**SVPVPATELGSTELVTTKTA
A0A6P6I3X8_PUMA	IIHPPKPLDATAPVW**P**PP-REDPANTPPA**H**SVPVPATELGSTELVTTKTA
M3YY53_EUROPEAN_POLECAT	TILPPGPLDATAPIW**P**PT-REDTANTPPA**H**SVPVPATELGSTELVTTKTA
A0A3Q7UGN9_GRIZZLY_BEAR	IVLPPGPLDATAPVW**P**PT-GEDPANTPPA**H**SVPVPATELGSTELVTTKTA
A0A2U3ZCW5_WALRUS	IILPPGPLDATAPVW**P**PT-REDPANTPPA**H**SVPVPATELGSTELVTTKTA
A0A2Y9H1Y3_HAWAIIAN_MONK_SEAL	IILPPGPLDATTPVW**P**PT-REDPANTPPA**H**SVPVPATELGSTELVTTKTA
A0A1S2ZRM0_EUROPEAN_HEDGEHOG	IILPPEPLLATAPVW**P**PP-RDDPGATPTA**H**SVPVPATELGSTELVTTKTA
F1MJD0_CATTLE	TTLSPGTLDATAPIWLLP-SEDPAATPPT**H**SVPVPATELGSTELVTTKTA
A0A452EY37_GOAT	T--TPGTLDATAPIWLLP-SEDPAATPPT**H**SVPVPATELGSTELVTTKTT
A0A2Y9T0B2_SPERM_WHALE	NILSPGTLDATAPIWLPP-SEDPATTPPA**H**SVPVPATELGSTELVTTKTA
A0A340WYL1_RIVER_DOLPHIN	NVLSLGTLDATAPIWLPP-SEDPATTPPA**H**SVPVPATELGSTELVTTKTA
A0A6P3QWA8_FLYING FOX	-ILSPGPLDATAPIW**P**PP-TEDSATTPPA**H**SVPVPATELGSTELVTTKTA
A0A287AJ47_PIG	IVPSPGTLDATAPIWLPP-SEDSATTPPA**H**SVPVPATELGSTELVTTKTA

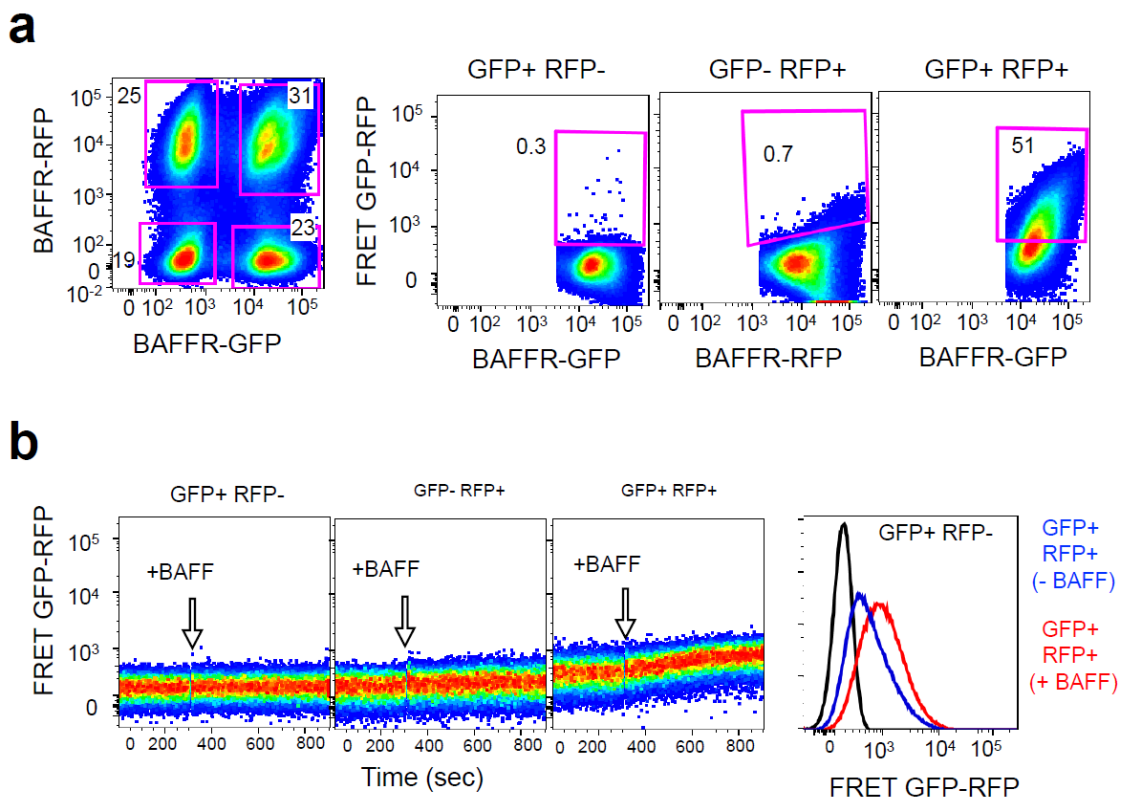
% IDENTITY TO HUMAN BAFFR

POSITION	**1**	**8**	**4**		
Q96RJ3_HUMAN	G	P	E	Q	Q
H2QLT1_CHIMPANZEE	G	P	E	Q	Q
G3R8S3_GORLILLA	G	P	E	Q	Q
A0A5F7ZFG6_MACAQUE	G	P	E	Q	Q
H9KVT2_MARMOSET	G	P	E	Q	-
0A2K5SC14_WHITE_CAPUCHIN	G	P	E	Q	-
A0A2K6GIX6_COQUERELS_SIFAKA	G	P	E	Q	Q
H0XRB7_OTELMUR_GARNETTI	G	L	E	Q	-
G3U9C5_AFRICAN_ELEPHANT	G	P	E	Q	-
A0A6I9JZX1_CAPE_GOLDEN_MOLE	G	P	E	Q	L
H0W812_GUINEA_PIG	G	P	E	Q	-
A0A6P3FQU0_COMMON_DEGU	G	P	E	Q	Q
A0A6J2A960_CHEETAH	G	P	E	Q	Q
A0A6P6I3X8_PUMA	G	P	E	Q	Q
M3YY53_EUROPEAN_POLECAT	G	P	E	Q	-
A0A3Q7UGN9_GRIZZLY_BEAR	G	P	E	Q	Q
A0A2U3ZCW5_WALRUS	G	P	E	Q	Q
A0A2Y9H1Y3_HAWAIIAN_MONK_SEAL	G	P	E	Q	Q
A0A1S2ZRM0_EUROPEAN_HEDGEHOG	G	P	E	Q	Q
F1MJD0_CATTLE	G	P	E	L	Q
A0A452EY37_GOAT	G	P	E	L	Q
A0A2Y9T0B2_SPERM_WHALE	G	P	E	Q	P
A0A340WYL1_RIVER_DOLPHIN	G	P	E	Q	P
A0A6P3QWA8_FLYING FOX	G	P	E	Q	Q
A0A287AJ47_PIG	G	P	E	Q	Q



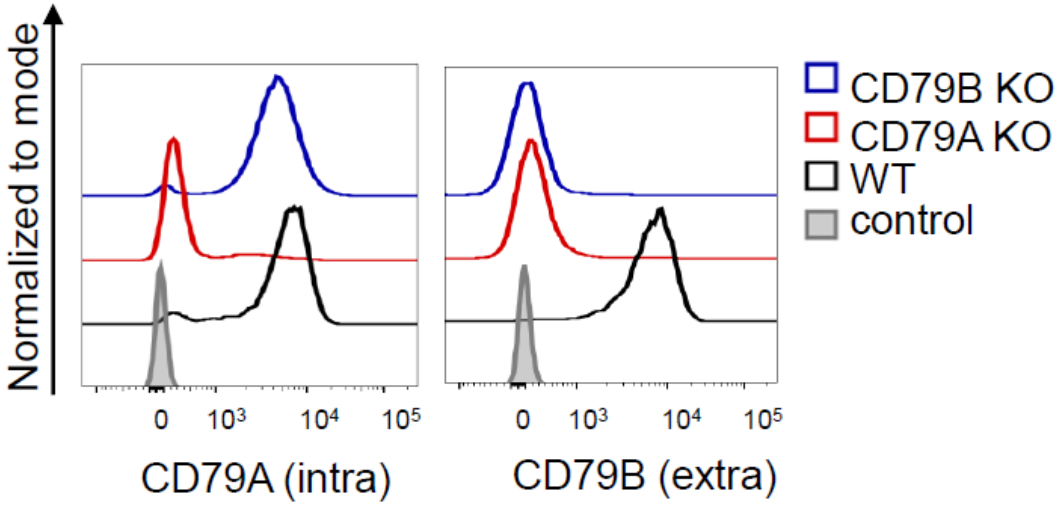
Supplemental Fig. S1 Analysis of PBMCs from CVID patients carrying different BAFFR variants

a CD19 positive B cells from CVID patients and healthy donors were gated into naïve (IgD+ CD27-), marginal zone (IgD+ CD27+) and memory B cells (IgD- CD27+). HD: healthy donor. CB: cord blood. **b** Different from other patients carrying heterozygous BAFFR variants, the patient with the homozygous BAFFR-A52T was treated for 1 year with rituximab and after >6m post recovery presented lower BAFFR on the cell surface. **c** Phosphorylation of the small ribosomal protein S6 in B cells of individuals with the P21R variant or with WT BAFFR. PBMC were activated for 1h with 20 ng/ml BAFF. Cells were stained first for the expression of CD19, IgD and CD27 on the cell surface followed by intracellular staining for pS6. The graph shows the percentages of pS6+ cells in the CD19+ IgD+ CD27- population representing naive B cells. The population was chosen as it showed the smallest difference between all individuals tested. Each data point represents one biological replicate. The statistically significant differences were calculated by the Brown-Forsythe and Welch ANVOA test ($P \geq 0.0333$; ns, 0.0021 - 0.0332 (*), 0.0002 - 0.02 (**), < 0.0001 (****)).



Supplemental Fig. S2 Gating strategy for flow-based FRET analysis and BAFF-induced changes of FRET signals. **a** Gating strategy of FRET analysis. DG-75 BAFFR KO cells were transduced with lentiviral vectors encoding for BAFFR-GFP and BAFFR-RFP and analyzed by flow cytometry using a LSR Fortessa II cytometer and FloJo software. The lentiviral transduction led to four different populations (GFP+, RFP-; GFP+, RFP+; GFP-, RFP- cells). FRET signals were detected as RFP fluorescence with a 625/15 nm bandpass filter excited by the 488 nm laser. These signals result from the fluorescence emission of the RFP acceptor fluorophore excited by the GFP fluorophore. After the appropriate compensation of the spillover signals from GFP and RFP emission into the channel detecting FRET signals, the gates for FRET+ cells were set using the single-positive GFP+ RFP- and GFP-RFP+ populations to determine background fluorescence. **b** Increase of FRET signals induced by BAFF binding to BAFFR. FRET signals were continuously recorded by flow cytometry from DG-75 cells

expressing BAFFR-GFP and BAFFR-RFP as described in (A). After 300 seconds (5 min), 50 ng/ml of BAFF were added and the recording was continued for another 10 min (900 sec timepoint). The plots display the FRET signals of the GFP+ RFP- GFP RFP+ and GFP+ RFP+ populations. The histogram overlay shows the increase of FRET signals 1h after adding 50 ng/ml BAFF to cells expressing BAFFR-GFP and BAFFR-RFP.



Supplemental Fig. S3 Inactivation of CD79A and CD79B in DG-75 cells

CD79A and CD79B were inactivated in DG-75 cells by CRISPR/CVas9 mutagenesis. The inactivation was confirmed by flow cytometry analyzing the intracellular (left histogram overlays) and the cell surface expression of CD79A and CD79B. Since CD79B surface expression requires CD79A, CD79A KO cells do not express CD79B on the cell surface.

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