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BOB.1 controls memory B-cell fate in the germinal center reaction

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ABSTRACT

During T cell-dependent (TD) germinal center (GC) responses, naïve B cells are instructed to differentiate towards GC B cells (GCBC), high-affinity long-lived plasma cells (LLPC) or memory B cells (Bmem). Alterations in the B cell-fate choice could contribute to immune dysregulation leading to the loss of self-tolerance and the initiation of autoimmune disease. Here we show that mRNA levels of the transcription regulator BOB.1 are increased in the lymph node compartment of patients with rheumatoid arthritis (RA), a prototypical autoimmune disease caused by the loss of immunological tolerance. Investigating to what extent levels of BOB.1 impact B cells during TD immune responses we found that BOB.1 has a crucial role in determining the B cell-fate decision. High BOB.1 levels promote the generation of cells with phenotypic and functional characteristics of Bmem. Mechanistically, overexpression of BOB.1 drives ABF1 and suppresses BCL6, favouring Bmem over LLPC or recycling GCBC. Low levels of BOB.1 are sufficient for LLPC but not for Bmem differentiation. Our findings demonstrate a novel role for BOB.1 in B cells during TD GC responses and suggest that its dysregulation may contribute to the pathogenesis of RA by disturbing the B cell-fate determination.

1. Introduction

Successfully established humoral memory is of central importance for the immune protection of the host against pathogens. It is mediated through sustained neutralizing antibodies produced by long-lived plasma cells (LLPC) and pathogen-experienced long-lived memory B cells (Bmem), which are rapidly reactivated upon re-encountering the immunizing antigens or pathogens [1]. Besides serving as a quantitative reservoir for antibody production, Bmem have a progressive increase in the quality of antibodies during recall responses in terms of antigen-binding affinity, as they express BCR repertoires that are more diverse and reactive to pathogen variants than oligoclonal LLPC do [2–4]. Therefore reactive memory plays an indispensable role for the protection of the host, particularly from viral escape mutants, against which the established repertoires of LLPC are no longer effective [5–8]. On the other hand, the breadth of BCR repertoire, the unique potential of Bmem to further diversify and mature the affinity of the antibody response together with a superior capacity to activate T cells may have detrimental consequences once tolerance is broken in the B cell compartment. Under these circumstances Bmem may serve as a reservoir of autoreactive cells that could be easily reactivated [9] in the presence of self- or transplantation antigens [10] thereby contributing to the breach

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of T-cell tolerance [11]. This could ultimately result in pathogenic immune responses that lead to overt autoimmune diseases or graft rejection. Therefore, the molecular mechanisms responsible for the generation and maintenance of Bmem have to be strictly controlled.

Long-lived isotype-switched high-affinity Bmem are made predominantly in germinal centres (GCs), transient microstructures that form within B-cell follicles of secondary lymphoid tissues, in contrast to low-affinity Bmem which can be generated primarily to T-independent antigens [12]. Within GCs, naïve B cells undergo clonal expansion, somatic hypermutation and class-switch recombination to enable immunoglobulin affinity maturation in response to T-cell–dependent antigens [13,14]. Following the activation with cognate antigen and upon receiving help from cognate T cells and signals from dendritic cells naive B cells have three potential fate choices: 1) form a GC within the B cell follicles; 2) differentiate into antibody-secreting LLPCs or 3) differentiate into Bmem [15–18]. At present, there is little insight into molecular and cellular mechanisms that control the cell-fate decision. It has been proposed that various factors, including the strength of the B-cell receptor-antigen interaction [3,19–22], competition for antigen and its presentation to cognate Th cells [23], costimulatory signals through complement receptors [24] and CD40 [25,26], cytokine-mediated signalling [27–29], transcriptional program [30] and even stochastic mechanisms [31,32] could influence the outcome of the GC reaction. However, despite the high relevance of Bmem for basic and translational immunology, the molecular mechanisms that contribute to the decision to differentiate into Bmem have been the least understood between these three cell fate decisions during GC reaction [13,18]. In contrast to the precursors of GC cells and LLPCs, where BCL6 and BLIMP1/XBP1 expression is associated with the commitment to the corresponding cell-fate decision [33–35], analysis of Bmem precursors has been complicated by a lack of a ‘master’ transcription factor. Yet, several transcription factors have been proposed recently to play an important role in instructing B cells to enter Bmem pool. Activated B cell factor-1, ABF1, was reported to be induced by T follicular helper cell-mediated signals and facilitate Bmem *in vitro* and *in vivo* by repressing BLIMP1 [36]. Another study identified the transcriptional repressor BACH2, a regulator of the cell-fate, to instruct GCBC to undergo differentiation into Bmem [37]. Accordingly, haploinsufficiency of BACH2 resulted in a reduced generation of Bmem, independently of the suppression of BLIMP1 [37]. In addition to BACH2, several other transcription factors are required to establish and maintain the identity and function of the GCBC, suggesting their major role in the GC during the immune response. This panel includes a lymphocyte-specific transcriptional regulator BOB.1 (B cell Oct binding factor 1), encoded by the *POU2AF1* (POU domain class 2-associating factor 1) gene. BOB.1, alternatively named as OCA-B (octamer coactivator from B cells) or OBF-1 (Oct-binding factor 1), interacts with the transcription factors Oct1 and Oct2 to enhance octamer-dependent transcription [38] and is indispensable for the GC formation and development of T cell-dependent immune responses [39–41].

Previously we have demonstrated that BOB.1 is highly expressed in centroblasts and centrocytes of normal secondary lymphoid tissues and in tertiary lymphoid structures of patients with autoimmune diseases (rheumatoid arthritis (RA) and Sjogren's syndrome) [42]. In addition, studies in mice lacking BOB.1 have indicated that this transcriptional regulator is a critical factor influencing resistance to an experimental model for rheumatoid arthritis, as BOB.1 deficiency in B cells abrogated GCBC formation, production of pathogenic antibodies and arthritis development in these animals [42].

In the present study, we propose that elevated expression of BOB.1 in B cells during T cell-dependent GC responses affect GC progeny that in case of self-antigens could induce or facilitate the progression from autoimmunity to overt autoimmune disease. To test this hypothesis, we analysed the expression of BOB.1 in draining lymph nodes of patients with RA and healthy controls. To further explore how different expression levels of BOB.1 affect B-cell responses during T cell-dependent GC reactions, we have examined the consequences of both overexpression and knockdown of BOB.1 in human primary B cells cultured with the combination of CD40 ligand and interleukin-21, mimicking two essential T follicular helper-derived signals.

2. Materials & methods

2.1. Patients and tissue sampling

The study included 29 patients with rheumatoid arthritis (RA) based on fulfilment of the American College of Rheumatology/European League Against Rheumatism 2010 criteria [43] and 31 otherwise healthy control subjects without any joint complaints, without elevated IgM-RF and/or ACPA levels, without an active viral infection, any history of autoimmunity or malignancy. Ultrasound-guided, core needle inguinal lymph node (LN) biopsies were obtained as previously described [44,45] and snap-frozen. The study was approved by the institutional medical ethical review board of the Academic Medical Center, and all study subjects gave written informed consent before inclusion. Human tonsils were obtained as leftover material after paediatric adeno-tonsillectomies. The study was approved by the Medical Ethical Committees of OLVG, St. Lucas or AMC, Amsterdam.

2.1.1. B cell isolation

Peripheral blood mononuclear cells (PBMC) were derived from buffy coats obtained from healthy donors provided by Sanquin Blood Supply Foundation, Amsterdam, through Ficoll-Paque gradient centrifugation on Lymphoprep (Nycomed). To obtain a single-cell suspension from tonsils, the tissue was cut into small pieces, mechanically disrupted using the Stomacher 80 Biomaster (Seward) and mononuclear cells were isolated with Ficoll-Paque gradient centrifugation. B cells were subsequently isolated by negative selection using B Cell Isolation Kit II (130-091-151, Miltenyi) on LD or LS magnetic separation columns (130-042-901 and 130-042-401, Miltenyi) according to the manufacturer's instructions. Tonsillar B cells were further divided into CD27$^+$ and CD27$^-$ cells using the Memory B Cell Isolation Kit (130-093-546, Miltenyi).

2.2. Cell culture

B cells (0.2–1*10^6 cells/ml) were cultured in IMDM (21980-032, Gibco) with 8% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (15140-122, Gibco), supplemented with 25 ng/ml recombinant mouse IL-21 (R&D systems) and co-cultured on 50 Gy irradiated murine L cell fibroblasts stably expressing CD40L (L cells, 10^5 cells/ml) at 37 °C in humidified air containing 5% CO$_2$.

2.3. Constructs and production of viruses

For the stable expression of a transgene into human lymphocytes we created a MMLV based retroviral expression vector system (LZRS) that employs the usage of an EMCV IRES sequence for the combined expression of a target gene and the EGFP or ΔNGFR reporter proteins. For maximal efficiency infection of lymphocyte cells, the virus was pseudotyped using the gibbon ape leukaemia virus (GALV) envelope and produced in the Phoenix virus production cell line (Nolan labs). The strict open reading frame of human BOB.1 (*POU2AF1*, Genbank NM_006235) was asymmetrically flanked with restriction sites, human codon-optimized synthesized at GeneArt (Thermo Fisher Scientific) and ligated into the LZRS transfer vector. Expression vectors without BOB.1 insert or BCL6-overexpressing constructs were used as controls. The retroviral vectors were transfected into the Phoenix-GALV cell line with X-tremeGENE™ 9 (06365809001, Roche). Two days later, transfected cells were selected by the addition of 2 μg/ml puromycin dihydrochloride (4089/50, R&D Systems). At day 6–10 after transfection, the cells were washed with PBS and cultured overnight in OPTIMEM

(31985-070, Thermo Fischer Scientific). Retroviral supernatant was collected the next day, centrifuged, filtered (SLHP033RS, Millipore), and frozen in aliquots at −80 °C.

Lentiviral constructs for silencing of BOB.1 (HSH013528-32-LVRU6MP, GeneCopoeia) and the scrambled control (CSHCTR001-LVRU6MP, GeneCopoeia) were transfected into the HEK293T/17 cell line together with the pMDL-RRE, pRSV-REV, pGC-FΔ30 and pCG HΔ24 packaging vectors with X-tremeGENE™ 9. Two days later lentiviral supernatant was collected twice, briefly centrifuged, filtered using (SLHP033RS, Millipore) and centrifuged overnight at 3000 g and 8 °C to concentrate the virus. The concentrate was subsequently frozen in aliquots at −80 °C.

2.4. Transduction

Non-tissue culture-treated plates were coated with 30 μg/ml human recombinant fibronectin (T100B, Takara) overnight at 4 °C. The fibronectin was removed, followed by a 60-min incubation at RT with 2% human serum albumin (H163NED, Sanquin) in PBS, followed by three washes with PBS. B cells were activated for 36 h on L cells and rmIL-21. Cells were then washed once in serum-free medium before resuspension in a viral supernatant and plated onto the coated wells. Cells were centrifuged at RT for 45 min at 360 g followed by a 7-h incubation at 37 °C. Afterwards, cells were transferred to the standard culture conditions described above.

2.5. Western blotting

Transduced B cells were FACS-sorted based on reporter expression, total protein lysates were reduced, denatured, separated by electrophoresis and transferred onto PVDF membranes. Membranes were washed in 0.05% Tween-20 pH 8.0 (Millipore), blocked with 2.5% milk, and incubated with primary antibodies: rabbit-*anti*-BOB.1 (1:2000 dilution) (SC-955, Santa Cruz Biotechnology), rabbit-*anti*-ABF1 (1:200 dilution) (sc-293482, Santa Cruz Biotechnology) or mouse *anti*-α-tubulin (1:3000 dilution) (T6199, Sigma-Aldrich) overnight at 4 °C in 2.5% milk/TBST. HRP-conjugated conjugated swine-*anti*-rabbit (P0399, Dako) and HRP-conjugated rabbit anti-mouse (P0260, Dako) (both 1:2000 dilution) were used for visualization of proteins with the SuperSignal West Femto Maximum Sensitivity Substrate (34094, Thermo Scientific) on the LAS4000 imaging system equipped with a CCD camera (GE Healthcare, Life Sciences).

2.6. Flow cytometry

The following antibodies against human proteins were used to analyse cells on the LSRFortessa (BD Biosciences): V450-conjugated *anti*-NGFR (562123), BV510-conjugated *anti*-NGFR (563451), APC-conjugated *anti*-IgG (550931), PE-conjugated *anti*-IgM (555783), PE-conjugated *anti*-IgD (555779), Pe-cy7-conjugated anti-CD38 (335825), APC-conjugated anti-CD27 (337169), APC-H7-conjugated anti-CD20 (641414), BUV395-conjugated anti-CD80 (565210), Alexa Fluor® 700-conjugated anti-CD86 (561124), BV711-conjugated *anti*-PDL-2 (564258), Alexa Fluor® 647-conjugated *anti*-HLA-DR/DP/DQ (563591), BV421-conjugated *anti*-ICOSL (564278), Alexa Fluor® 647-conjugated *anti*-CXCR5 (558113) all from BD Biosciences; BV650-conjugated anti-CD19 (302238), PE-cy7-conjugated anti-CD40 (334321), BV421-conjugated *anti*-BCL2 (658709) from BioLegend, APC-conjugated anti-CD25 (47-0259-41), Alexa Fluor® 647-conjugated Donkey anti-Rabbit IgG (A-31573), all from Thermo Fisher Scientific), Fitc-conjugated *anti*-IgA (130-093-071, Miltenyi), APC-conjugated Annexin-V (IQP-120A, IQProducts), PE-conjugated *anti*-BCL-XL (13835S, Bioké), eFluor™ 780-conjugated Fixable Viability Dye (65-0865-18, eBioscience), *anti*-MCL-1 (ab32087, Abcam), PE-conjugated *anti*-PDL-1 (FAB1561P, R&D Systems), Propidium Iodide (P4864, Sigma-Aldrich), FcR Blocking Reagent (130-059-901, Miltenyi). For intranuclear staining of BOB.1 cells were first stained with surface markers and then fixed/permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (00-5523; eBioscience) according to manufacturer's protocol and were stained with PE-conjugated anti-BOB.1 antibody (sc-23932, Santa Cruz Biotechnology). Data were analysed using Flow-Jo (Tree Star, Inc) software.

2.7. RNA extraction and qPCR

Total RNA was isolated from lymph node tissue biopsies using AllPrep DNA/RNA mini Kit 80204, Qiagen), including DNAse step (79254, Qiagen). (RNeasy Micro Kit (74004, Qiagen) was used to extract RNA from transduced B cells according to the manufacturer's instructions, including a DNAse step to remove genomic DNA (79254, Qiagen). RNA concentrations were determined using the Nanodrop (Nanodrop Technologies). Quantitative real-time PCR was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using TaqMan gene expression assays for *POU2AF1* (Hs01573371_m1), *BCL6* (Hs00153368_m1), *PAX5* (Hs00172003_m1), *PRMD1* (Hs00153357_m1), *XBP1* (Hs00231936_m1)*, IRF4* (Hs01056533_m1)*, REL* (Hs00968440_m1)*, MYC* (Hs00153408_m1), *BACH2* (Hs00222364_m1), *SPIB* (Hs00162150_m1), *ABF1* (Hs00231955_m1)*, CCL3* (Hs00234142_m1), *GAPDH* (4310884E) and *18S* (Hs99999901_s1) according to the manufacturer's protocol (Thermo Fisher Scientific). For custom codon-optimized *POU2AF1* assay following primes and probe were designed: Fw-CCGTGCTGCAGGATAT GGA, Rev- GTAGGCGTCGCTATCTTCCTCTT, FAM- CTCGGAGAGCC GCC-MGB.

2.8. Autologous B and T cell co-culture

T cells were isolated from human tonsils by negative selection using the Memory CD4 T cell isolation kit (130-091-893, Miltenyi) on LS magnetic separation columns (130-042-901 and 130-042-401, Miltenyi) according to the manufacturer's instructions and frozen in liquid nitrogen on the day of isolation. When indicated, CXCR5$^+$ T cells were sorted as positive for CXCR5 (Alexa Fluor® 647-conjugated *anti*-CXCR5, 558113, BD Biosciences) and negative CD19 (for FITC-conjugated anti-CD19, 302206, BioLegend). On the day preceding the co-culture, T cells were thawed, labelled using the CellTrace™ Violet Cell Proliferation Kit (C34557, Thermo Fisher Scientific) according to the manufacturer's instructions and rested overnight in the presence of 20 U/ml Recombinant Human IL-2 (202-IL-010, R&D systems). On day 6 after transduction, transduced B cells were FACS-sorted and co-cultured with autologous T cells in round-bottom 96 wells plates. For antigen-independent responses B and T cells were co-cultured for 2–5 days in a 1:2 stimulator:responder ratio with or without 1 μg/ml Staphylococcal enterotoxin B (SEB), a superantigen, which cross-links MHC class II α-chain on B cell surface and TCR β-chain on T cells (S4881, Sigma-Aldrich). T cells cultured in the presence of CD3/CD28 Dynabeads (11131D, Thermo Fisher Scientific) and 20 U/ml rhIL-2 were served as a positive control. The proliferation index was calculated as the sum of the cells in all generations divided by the estimated number of original parent cells. The estimated number of original parent cells was calculated by dividing the final number of cells in each generation by the squared generation number.

For antigen-specific responses autologous B and CD4-positive T cells were co-cultured for 6 days in the presence of 5 μg/ml of Tetanus Toxoid peptide (Statens Serum Institute, Denmark) in a 1:0,5 stimulator:responder ratio. B and T cells cultured without Tetanus Toxoid peptide and in the presence of CD3/CD28 Dynabeads or 1 μg/ml of SEB were applied as a negative and two positive controls correspondingly. To calculate the TT-specific proliferation rate, frequencies of T cells proliferating in the co-culture without TT peptide (background) were subtracted from T cells proliferating in the presence of TT peptide.

2.9. ELISA

Cytokine levels in supernatants were measured by multiplex ELISA (INFγ, IL-4, IL-5, IL-9, IL-10, IL-17A, IL-21, TNF) using a custom-made U-plex kit for electrochemiluminescent detection (Meso Scale Discovery (MSD), Rockville, MD, USA) according to the manufacturer's protocol. Data were analysed on a SECTOR instrument (MSD) using MSD's Discovery Workbench software. CXCL13 levels were assessed by ELISA (DCX130; R&D Systems) according to the manufacturer's protocol. Standards and samples were measured in duplicate and triplicate, respectively, in a 96-well flat bottom microplate (Nunc). For IgG ELISA EIA/RIA 96 wells plates (Costar) were coated with 5 μg/ml goat anti-human IgG or with 5 μg/ml goat anti-human IgM (Jackson) o/n at 4 °C. Plates were washed with PBS/Tween and blocked with PBS/4% milk (Protifar). Supernatants and standard (Human Serum Protein Calibrator, DAKO) were incubated for 1 h at RT, where after the wells were washed using PBS and incubated with g-a-h-IgG-HRP or -IgM-HRP (Jackson) for 1 h at RT. Subsequently, the wells were washed 5 times and bound IgG and IgM were visualized using TMB substrate buffer (BioSource).

2.10. Calcium flux

For calcium flux measurements, B cells were loaded with 2 μg/ml Indo-1 (I-1203, Thermo Fisher Scientific) for 30 min at 37 °C. Afterwards, cells were stained for extracellular markers at 4 °C and resuspended in HBSS (BE10-547F, Lonza) supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+} (CaCl$_2$ and MgCl$_2$). Cells were warmed to 37 °C before measurement and were stimulated with 12.5 μg/ml goat F (ab')2 *anti*-IgM (2022–01, Southern Biotech). Stimulation with 2 μg/ml of ionomycin was used as a positive control for maximum flux. Resultant variation in intracellular Ca^{2+} was measured by flow cytometry on the LSRFortessa (BD Biosciences) and analysed using Flow-Jo (Tree Star, Inc) software. For *CCL3* expression measurements, transduced B cells were plated 5*10^4 per well in a 96 round bottom plate and rested overnight without L cells and IL-21. Stimulation mixes were warmed to 37 °C before addition to the cells. Cells were stimulated with 12.5 μg/ml goat F (ab')2 *anti*-IgM (2022–01, Southern Biotech), 2.5 μg/ml AffiniPure Goat Anti-Human IgG (109-005-008, R&D Systems) or 3 mM EDTA control for 1 h at 37 °C before the cells were lysed in RLT buffer (RNeasy Micro Kit, 74004, Qiagen).

2.11. Antigen internalization

Memory B cells were isolated from influenza-vaccinated individuals, transduced with human BCL6 and BCL-XL as described in Refs. [46,47] and screened for HA binding. An influenza H1-specific B cell clone capable of binding and internalizing intact H1, but not H3 control protein were transduced with BOB.1-overexpressing or silencing constructs. H1 (A/California 07/2009) and H3 (H3N2 A/Victoria/361/2011) proteins (both from Protein Sciences) were fluorescently labelled using the Alexa Fluor™ 647 Protein Labelling Kit (A20173, Thermo Fisher Scientific) according to the manufacturer's instructions. Transduced B cells were incubated with Alexa Fluor® 647-labelled proteins on the ice at a concentration of 1 mg/ml in 1.5-ml microfuge tubes for 20 min and then washed. Cell pellets were resuspended in 0.5 ml culture medium and returned to ice or incubated at 37 °C for 1, 2, 5 and 10 min. Reactions were stopped by the addition of ice-cold PBS and fixation using the Fixation/Permeabilization Solution Kit (554714, BD Biosciences). Images were captured on ImageStream® (Amnis) and analysed with IDEAS® Analysis Software.

2.12. Animals

BOB.1-deficient mice were provided by Prof. R.G. Roeder (the Rockefeller University, USA) [39] and maintained on a C57BL/6NCrl background. MOG-specific TCR transgenic (2D2) C57BL/6J mice were provided by Prof. V. Kuchroo (Harvard Medical School, Boston, MA, USA) [48]. Spleens were collected from non-immunized mice, single-cell suspensions were obtained, erythrocytes removed. CD4$^+$ T cells were isolated from spleens of 2D2 mice and B220$^+$ B cells were isolated from spleens of WT or BOB.1-deficient mice with anti-CD4$^-$and anti-CD19 magnetic beads, correspondingly using LS columns (Miltenyi), according to manufacturer's instructions. To assess proliferative capacity, 2D2 T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE, 1 μg/ml, Molecular Probes) and subsequently co-cultured with WT or BOB.1-deficient B cells (1:1 ratio) in the presence or absence of MOG peptide (5 μg/ml, amino acid residues 35–55, sequence MEVGWYRSPFSRVVHLYRNGK, AnaSpec, Freemont CA, USA) or anti-CD3/CD28 antibodies for 72 h. Cells were then analysed for CFSE dilution by flow cytometry.

2.13. Statistical analysis

Differences between groups were analysed using Student's t-test, Mann-Whitney *U* test, and Dunn's nonparametric comparison for post hoc Kruskal-Wallis test, where appropriate. Values are expressed as mean and SD or median and IQR range, according to criteria for (non-) parametric analysis. GraphPad Prism software version 7 was used to perform the analyses. p-values < 0.05 were considered statistically significant.

3. Results

3.1. BOB.1 levels are aberrantly elevated in lymph nodes of patients with rheumatoid arthritis

Previously we demonstrated that expression of BOB.1 is aberrantly increased in rheumatoid arthritis (RA) synovitis and salivary glands of patients with Sjögren's syndrome [42]. Moreover, we observed a strong correlation between BOB.1 mRNA expression levels and the presence of ectopic GC-like structures in autoimmune disease target tissues [42]. In order to assess if these features point towards alterations in the autoimmune GC reaction or merely reflect an increase in tertiary lymphoid structures in the target tissue of patients with autoimmune diseases, we first investigated whether levels of BOB.1 are also elevated in the conventional secondary lymphoid organs of patients with systemic autoimmune diseases. qPCR analysis revealed that mRNA expression of *POU2AF1* encoding for BOB.1 gene was significantly higher in the

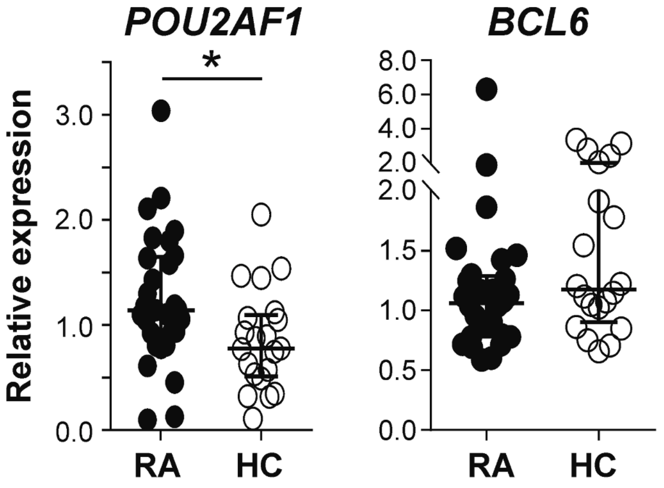

Fig. 1. **(a)** Expression of BOB.1 is aberrantly increased in lymph nodes (LNs) of patients with rheumatoid arthritis (RA) as compared to autoantibody-negative healthy individuals (HC) whereas no difference was detected in the expression of germinal center marker BCL6 (b). *$P < 0.05$.

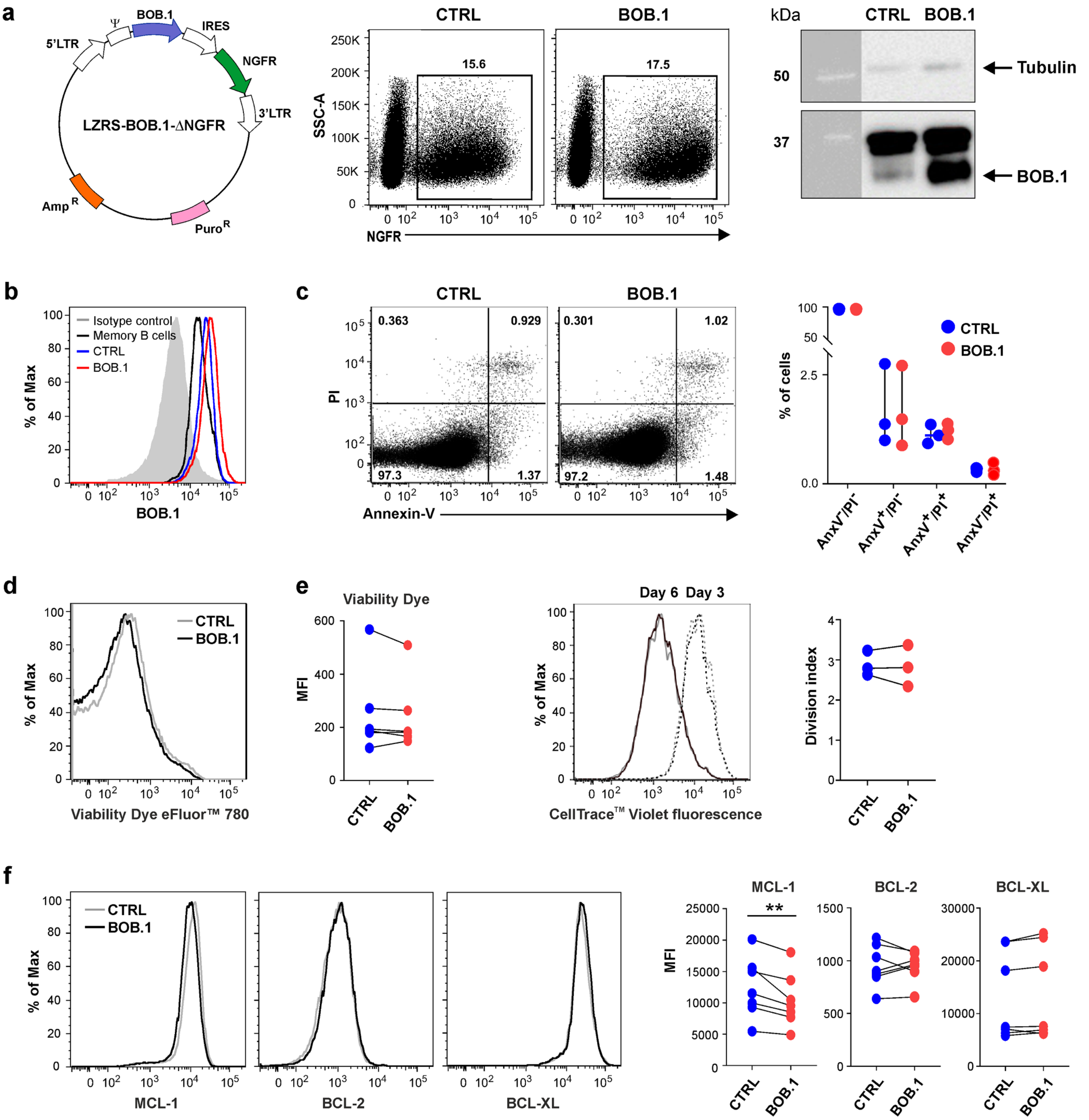

Fig. 2. The increased BOB.1 expression does not affect survival and proliferation of primary human B cells, except the expression of MCL-1. (a) [left panel] POU2AF1 cDNA was cloned into the LZRS retroviral vector upstream of IRES-ΔNGFR reporter and retrovirally overexpressed in primary human B cells, isolated from peripheral blood (BOB.1). [middle panel] B cells transduced with the vector, expressing fluorescent reporter without POU2AF1 cDNA were served as control cells (CTRL). [right panel] NGFR-positive cells were sorted and overexpression of BOB.1 was confirmed on the protein levels by Western blot analysis. (b) Overexpression of BOB.1 in tonsillar memory B cells was confirmed by flow cytometry. A representative plot of BOB.1 expression is shown before the transduction (black line) and after one week of culturing of cells transduced with BOB.1-overexpressing (red line) or control (blue line). Overexpression of BOB.1 did not affect death (bc), viability (d) or proliferation (e) of B cells cultured with CD40L and IL-21. However, expression of anti-apoptotic marker MCL-1, essential for survival plasma cells was significantly reduced in BOB.1-overexpressing cells as compared to control (f). $^{**}P < 0.01$.

lymph nodes (LNs) of patients with established RA as compared to healthy individuals (median (IQR) 1.42 (0.87–1.65) vs 0.78 (0.51–1.11), p = 0.01) (Fig. 1a), whereas mRNA levels of a GC marker BCL6 were not different between two groups (1.13 (0.87–1.35) in RA LNs vs 1.18 (0.90–2.01) in HC LNs, p = 0.36) (Fig. 1b).

3.2. BOB.1 overexpression suppresses MCL-1 without affecting the survival or proliferation of primary human B cells

Since our translational data suggest that an increase in the expression of BOB.1 in lymphoid tissues is associated with the presence of an autoimmune disorder, we set out to investigate how high levels of BOB.1 impact the phenotype and function of B cells by overexpressing

BOB.1 by retroviral-mediated gene transfer using the LZRS construct in which cDNA of human BOB.1 was inserted upstream of the IRES-ΔNGFR reporter (Fig. 2a left panel). After transduction, primary peripheral naive B cells were cultured on CD40L-L/IL-21 for 3 days, ΔNGFR-positive cells were FACS-sorted (Fig. 2a central panel) and overexpression of BOB.1 on the protein level was verified by Western blot analysis of B cell protein extracts probed with the anti-BOB.1 antibody (Fig. 2a right panel). Immunoblotting demonstrated an increase in expression of protein bands migrating at ∼34–35 kDa region that corresponds to two different BOB.1 protein isoforms p34 and p35 [49]. The signal at ∼37 kDa region is likely to be non-specific since it was detected in all cell types tested including stromal cells (data not shown). Overexpression of BOB.1 in tonsillar memory B cells was confirmed by flow cytometry analysis (Fig. 2b).

First, we analysed the effect of forced BOB.1 overexpression on survival, viability or proliferative capacities of B cells. Data analysis revealed no difference in apoptotic or necrotic cell death between BOB.1-overexpressing and control cells (Fig. 2c). Accordingly, viability dye staining revealed similar frequencies of viable cells between BOB.1-transduced and control cells (Fig. 2d). Furthermore, no impact of high BOB.1 expression was observed on cell proliferation as assessed by the dilution of the cell trace dye on day 3 or 6 after transduction (Fig. 2e). Analysing the expression of important anti-apoptotic factors, we observed no difference in the expression of BCL2 ($p = 0.93$) and BCL-XL ($p = 0.21$) proteins between BOB.1- and control vector-overexpressing B cells. However, we noticed a moderate yet significant decrease in the expression of MCL-1 in BOB.1-overexpressing B cells ($p = 0.01$) (Fig. 2f). Collectively, these data indicate that forced overexpression of BOB.1 in primary human naïve B cells does not affect cell death or proliferation of cells but decreases MCL-1 expression.

3.3. High levels of BOB.1 inhibit differentiation of B cells to plasmablasts

As MCL-1 is a critical factor for the maintenance and survival of plasma cells [50], we hypothesised that high levels of BOB.1 may interfere with plasma cell differentiation. To test our hypothesis, we examined the phenotype of transduced B cells isolated from peripheral blood after 6 days of culturing them under conditions mimicking T-cell help. Normal human B cells differentiate to antibody-producing plasma cells when cultured on CD40L-L cells in the presence of IL-21 [51,52], a process that is accompanied by a decrease in expression of CD20 and an increase in expression of CD38 and CD27. Flow cytometric data analysis revealed that in contrast to cells expressing control virus, BOB.1-overexpressing B cells expressed decreased levels of CD27 and CD38 proteins (Fig. 3a left panel). Accordingly, the percentage of IgD^{-}CD38^{++} plasmablasts was significantly lower in peripheral blood-derived B cells transduced with the BOB.1-overexpressing construct (mean ± SD of 6.1 ± 3.2% in BOB.1^{high} cells versus 16.8 ± 8.9% in CTRL, $p < 0.0001$). Correspondingly, B cells with overexpression of BOB.1 secreted less isotype-switched IgG (median 4567 pg/ml (IQR 4316–4666 pg/ml) in BOB.1^{high} versus 6113 pg/ml (5205–7301 pg/ml) in CTRL, $p = 0.04$), but not un-switched IgM (68361 pg/ml (67534–85427 pg/ml) in BOB.1^{high} versus 70573 pg/ml (47982–134240 pg/ml) in CTRL, $p = 0.9$), as compared to control cells (Fig. 3b), supporting the hypothesis that a high expression of BOB.1 in B cells inhibits their differentiation to plasmablasts during T cell-dependent B cells responses.

Since this assay was performed on B cells isolated from peripheral blood, comprising mainly naïve B cells that are not prone to differentiate into plasmablasts, we repeated similar experiments with CD27^{+}IgD^{-} memory B cells isolated from tonsils as they are more amenable to plasma cell differentiation [53]. Also in this set-up, the formation of CD20^{low}CD38^{++} plasmablasts was markedly decreased in cultures of memory B cells expressing high BOB.1 levels versus control after 6 days of stimulation with CD40L/IL-21 (9.3% ± 4.8 in BOB.1^{high} versus 19.4 ± 9.3 in CTRL, $p < 0.0001$) (Fig. 3c).

Together, these results demonstrate that elevated levels of BOB.1 inhibit the differentiation of both naïve and memory B cells towards plasmablasts in conditions mimicking a T cell-dependent GC response.

3.4. High levels of BOB.1 in B cells do not induce GC B cells but rather promote Bmem differentiation

As BOB.1 is preferentially expressed in GC B cells [42,54] and as the transcriptional repressor BCL6 proto-oncogene is a key regulator of GC B cell differentiation [55–57], we hypothesised that BOB.1 operates in the same pathways as BCL6 to promote the generation of GC B cells. Therefore, we assessed how overexpression of BOB.1 affects the expression of GC-related markers using as positive control B cells with forced overexpression of BCL6. Increase in the expression of BCL6 coincides with the increase in expression of CD10, CD38, CD95, and HLA-DR and in the decrease of CD44, CD27 and BCL2, discriminative markers of GC B-cells from other B-cell subsets [14,58]. Overexpression of BOB.1 did not impact the expression of CD10, CD95, HLA-DR, CD44 or BCL2 proteins (Figs. 2f and 3d). Consistent with phenotypic features, BCL6-overexpressing B cells upregulated expression of *PAX5*, *REL*, *MYC, SPIB* and *BACH2* mRNA and repressed BLIMP, XBP1 and IRF4, as revealed by qPCR analysis (Fig. 3e). In contrast, we did not observe any effect of BOB.1 overexpression on the expression of these transcription factors that are essential either for GC or for plasma cells differentiation, except for the decrease in expression of *AICDA* gene encoding activation-induced cytidine deaminase (AID), a molecule critically involved in somatic hypermutation and isotype switching (Fig. 3e).

Of interest, overexpression of BOB.1 and BCL6 mutually repressed each other's expression under these culture conditions (Fig. 3e), suggesting that these factors may antagonize each other in regulating cell-fate decision during the GC reaction.

Since BCL6 repression is necessary for plasma cells as well as for the memory B cells differentiation [59], and since we observed an increase in the expression of CD20 and a decrease in the expression of CD27 and CD38 in BOB.1-overexpressing cells (Fig. 3d), we hypothesised that BOB.1 overexpression in B cells promote their differentiation towards Bmem cells. Therefore, we analysed the effect of BOB.1 overexpression on the expression of BACH2 and ABF1, transcription factors previously suggested to play a role in the differentiation of B cells to Bmem. qPCR analysis revealed no difference in the expression of BACH2 between cells with high BOB.1 levels and the control cells. In contrast, B cells with forced overexpression of BOB.1 expressed significantly higher *ABF1* mRNA levels. The increase in the expression of ABF1 seen on the messenger level was confirmed on the protein level by Western blot analysis (Fig. 4f). Accordingly, flow cytometric analysis confirmed that frequencies of CD38^{-}CD27^{+}CD20^{+} memory B cells were significantly higher in cells with forced BOB.1 overexpression (13.4 ± 5.6% in BOB.1^{high} cells versus 7.7 ± 4.3% in CTRL, $p = 0.005$) (Fig. 3g).

Together these results suggest that high levels of BOB.1 in B cells do not contribute to the generation of GC B cells but rather promote Bmem differentiation during T-cell dependent immune responses.

3.5. Low expression levels of BOB.1 impair formation of Bmem but not plasmablasts

To further validate the hypothesis that BOB.1 expression controls Bmem cell fate, we examined whether suppression of BOB.1 expression would affect the formation of Bmem under these culture conditions. To do this, we suppressed BOB.1 expression with shRNA technology. By means of shRNA we were able to down-regulate BOB.1 mRNA and protein expression to approximately 10% of the levels as expressed by primary B cells transduced with the scrambled control shRNA (scRNA) and cultured with CD40L and IL-21 (Fig. 4a). Analysing cell phenotype on day 6 we observed a significant decrease in the frequencies of CD20^{+}CD27^{+} memory B cells upon BOB.1 knockdown (9.8 ± 4.8 in BOB.1^{low} versus 13.2% ± 6.4 in CTRL, $p = 0.02$). In contrast, the

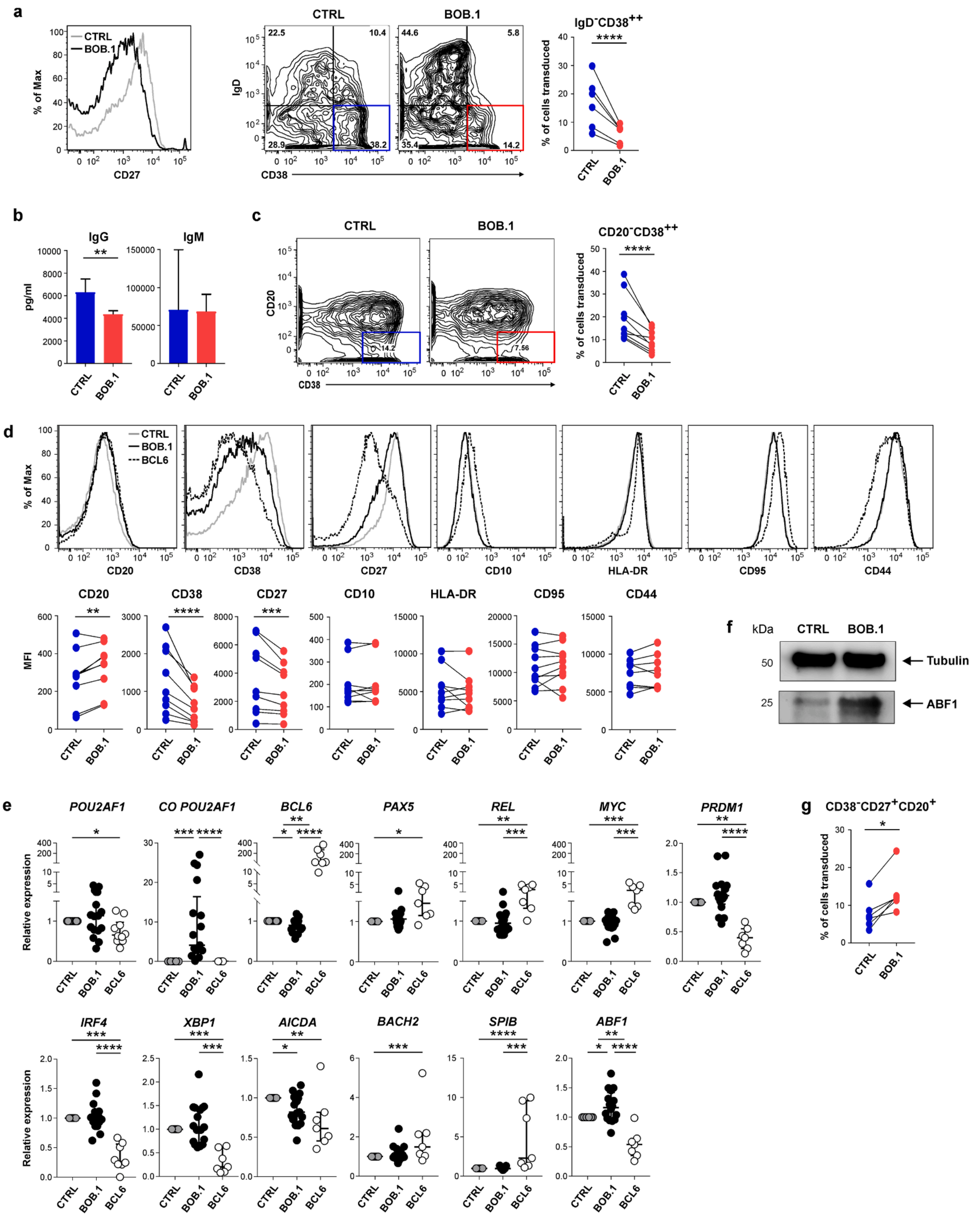

Fig. 3. High levels of BOB.1 in B cells during T cell-dependent immune responses inhibit differentiation of B cells to plasmablasts and GCBC promoting Bmem phenotype. (a) Flow cytometry analysis of BOB.1-transduced (BOB.1) or control-transduced (CTRL) naïve B cells (CD19 $^{+}$ CD27^{-}IgD +) cultured with CD40L and IL-21 for 6 after the transduction. BOB.1-overexpressing cells were characterized by reduced expression of CD27 [left panel] and CD38 [middle panel] as compared to CTRL cells. Accordingly, the percentage of IgD^{-}CD38^{+} plasmablasts was significantly decreased [right panel]; (b) BOB.1-overexpressing B cells secrete less IgG than CTRL cells; (c) overexpression of BOB.1 inhibited differentiation of CD19 $^{+}$ CD27^{+}IgD^{-} memory B cells towards CD20^{-}CD38^{++} plasmablasts; (d) in contrast to the overexpression of BCL6, overexpression of BOB.1 in B cells did not induce GCBC phenotype. Expression of CD20, CD38, CD27, CD10, HLA-DR4, CD95 and CD44 is GC is shown as flow cytometric histograms [upper panel] and quantified [lower panel]; (e) gene expression analysis confirmed that BOB.1 and BCL6 operate in an independent, mutually-exclusive pathways as overexpression of these molecules induced different transcription programs. High levels of BCL6 inhibited PC and Bmem differentiation by repressing PRMD1, XBP1, IRF4 and BOB.1 and ABF1 genes respectively and promoted GC cells differentiation via induction of PAX5, REL, MYC, SPIB and BACH2 genes. Overexpression of BOB.1 repressed BCL6 and induced expression of ABF1; (f) Immunoblot analysis demonstrating induced expression of ABF1 in BOB.1-overexpressing cells on the protein level; (g) the percentage of CD38^{-}CD27^{+}CD20^{+} cells consistent with Bmem phenotype was significantly increased in B cells expressing high levels of BOB.1 as compared to CTRL cells. CO-codon-optimized. Asterisks denote statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

formation of plasmablasts was similar in cells expressing low and high BOB.1 levels (4.8 ± 1.9 in BOB.1^{low} versus 5.8% ± 2.5 in CTRL, p = 0.98) (Fig. 4b and c).

3.6. B cells with high BOB.1 levels display phenotypic and functional characteristics of Bmem

As our data suggest that levels of BOB.1 in B cells stimulated with

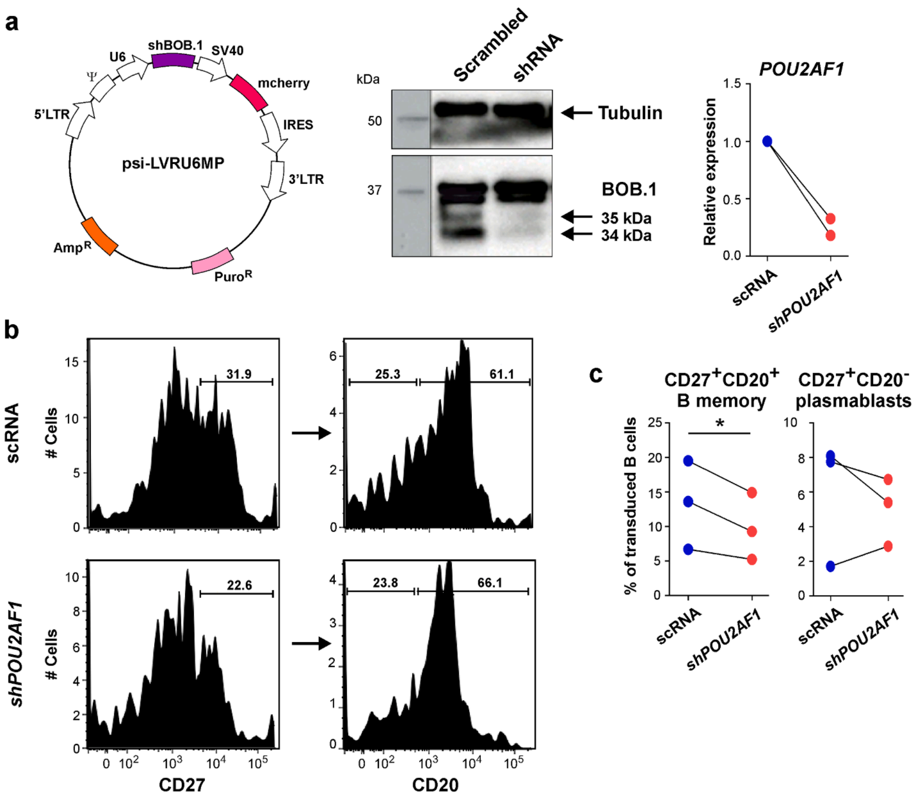

Fig. 4. Low expression levels of BOB.1 in B cells impair formation of Bmem but not plasmablasts. (a) BOB.1-targeting shRNA constructs were designed and cloned into lentiviral vector psiLvRU6MP [left panel], transduced B cells were lysed and protein extracts were analysed by Western blotting [middle panel] and qPCR [right panel]. Expression of BOB.1 is down-regulated more than 90% as compared to the levels normally expressed by primary B cells cultured with CD40L and IL-21. Flow cytometry analysis (b) and quantification (c) of cells demonstrated that generation of $CD27^+CD20^+$ Bmem cells, but not $CD27^+CD20^-$ plasmablasts was reduced in B cells transduced with shRNA targeting BOB.1 as compared to control cells transduced with scrambled RNA, $* < 0.05$.

CD40L/IL-21 affect a generation of Bmem cells we investigated next whether cells transduced with BOB.1 display phenotypic characteristics of Bmem, such as expression of high levels of co-stimulatory and activation molecules [60]. Flow cytometric analysis of membrane-bound molecules revealed a significant increase in the densities of CD40 and CD80 proteins (expressed as a mean fluorescent intensity) in BOB.1-overexpressing cells as compared to the control B cells ($p = 0.001$ and $p = 0.004$, respectively) (Fig. 5a). We also detected a trend towards an increase in expression of programmed death-ligand 1 (PD-L1, $p = 0.1$) and two (PD-L2, $p = 0.09$) in BOB.1-overexpressing cells. Levels of CD86, inducible co-stimulator ligand (ICOS-L), and MHC-II (human leucocyte antigen (HLA)-DP, -DQ and -DR) were not different between B cells with high BOB.1 levels and controls (Figs. 5a and 3d). Notably, BOB.1-overexpressing cells expressed higher levels of the high-affinity interleukin-2 (IL-2) receptor CD25 ($p = 0.002$), the marker of activated B cells [61] that have been shown to belong to memory subclass [58,62] (Fig. 5a). In contrast to the observations for overexpression, knockdown of BOB.1 did not affect expression of co-stimulatory molecules (data not shown), in agreement with above results indicating that amounts of BOB.1 protein remaining in cells after knockdown are still sufficient for the formation of plasmablasts and memory cells, although memory cells to a lesser extent than in control (Fig. 4bc).

In order to investigate whether higher expression of CD40, CD80 and CD25 molecules on BOB.1-overexpressing cells is functionally relevant we assessed the ability of BOB.1-transduced cells to interact with T cells using mixed lymphocyte reactions. To this aim we co-cultured BOB.1- and empty vector control-expressing B cells for 2–5 days with autologous $CXCR5^+$ memory T cells loaded with Cell Trace in the presence of SEB to induce an entangled mode of T-B cell interaction. Analysis at day 5 revealed that roughly 90% of T cells proliferated in the co-culture regardless of the presence of BOB.1-overexpressing construct (Fig. 5c), indicating that this time point might be inappropriate to detect differences in T cell responses. Indeed, data analysis at day 2 of co-culture demonstrated that T cells cultured with BOB.1-overexpressing B cells proliferate faster than those co-cultured with control vector-transduced B cells ($p = 0.004$) (Fig. 5b). In line with the increased proliferation, T cell co-cultured with BOB.1-overexpressing B cells produced higher levels of CXCL13 chemokine as well as IFNγ and IL-10 cytokines ($p = 0.02$, $p = 0.059$, $p = 0.04$, correspondingly) (Fig. 5b). Expression of other cytokines, such as IL-4, IL-5, IL-9, IL-17A and TNF were not different between co-cultures of T cells with BOB.1-transduced and control-transduced B cells (data not shown). As SEB exerts powerful T-cell proliferation bypassing the antigen-presenting stage, we assessed next whether elevated expression of CD80 and CD40 on BOB.1-overexpressing cells is translated into an increase in the antigen-specific T cell responses. To this aim, we evaluated proliferative response induced by the recall antigen tetanus toxoid in the co-culture of TT-peptide-pulsed BOB.1-overexpressing or control-overexpressing B cells with autologous T cells. Since we expected that T cells isolated from TT-vaccinated individuals harbour low frequencies of TT-specific cells we purified the $CD4^+$ memory pool to increase the relative frequency of specific T cells in the culture. Analysis of T cell proliferation at day 6 demonstrated a trend towards a higher frequency of TT-specific T cells during recall response in co-cultures with B cells expressing high BOB.1 levels as compared to those with control B cells (5.2% ± 6.5 versus 3.2% ± 5.2, $p = 0.069$, correspondingly). Using a more stringent antigen-specific model system to confirm this observation [48], B cells with genetic deletion of BOB.1 derived from BOB.1-knockout mice were less potent in antigen-presentation to T cells, as demonstrated by the decreased proliferation MOG-specific T cells in the co-culture with BOB.1-deficient B cells as compared to the similar co-culture with wild-type B cells (Fig. 5d). Taken together, these data indicate that levels of BOB.1 in B cells affect their phenotype and function such as the expression of co-stimulatory molecules and the propensity to activate antigen-specific T cells.

3.7. BOB.1 modulates B cell receptor functions

Previous studies demonstrated the importance of BCR affinity for the differential fate decisions as lower, intermediate, and higher BCR affinity predispose the activated B cells to differentiate into Bmem, GC, and plasma cells, respectively [19,63]. As BCR affinity is translated into

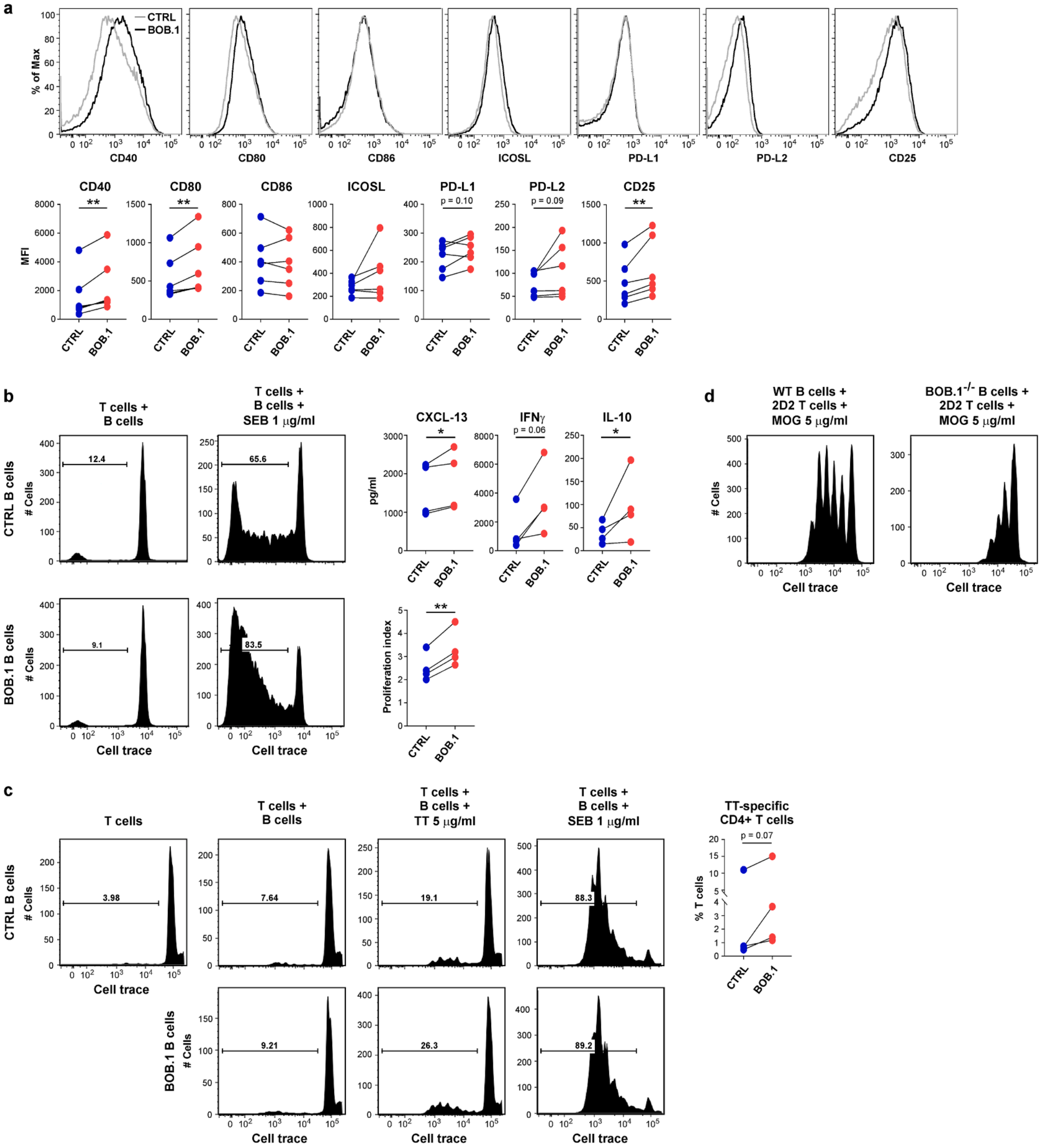

Fig. 5. B cells with high levels of BOB.1 express elevated levels of B cell activation markers CD40, CD80, CD25 and demonstrate increased cross-talk with autologous T cells. (a) Flow cytometric analysis [upper panel] and quantification [lower panel] of B cell activation markers in transduced $CD27^+$ B cells; (b) flow cytometric analysis of cell trace dilutions of autologous $CD4^+CD45RO^+CXCR5^{++}$ T cells co-cultured with SEB-pulsed $CD27^+$ B cells transduced with BOB.1 or control vector for 2 days [left panel], quantification of proliferation index [middle panel] and selected chemokines and cytokines in the co-culture [right panel]; (c) BOB.1-overexpressing B cells are capable to induce a higher rate of proliferation of autologous TT-specific $CD4^+$ $CD45RO^+$ memory T cells in the presence of TT peptide; (d) accordingly, B cells lacking function BOB.1 (isolated from spleens of BOB.1-KO animals) present MOG antigen less efficiently to MOG-specific 2D2 T cells compared to WT B cells. Asterisks indicate statistical significance: $*P < 0.05$, $**P < 0.01$.

the efficiency of B cells to internalize the antigen by endocytosis, we evaluated first whether high levels of BOB.1 would impact antigen internalization kinetics. To test this, we have transduced an influenza H1-specific B cell clone generated as previously described [46] with BOB.1- or control-overexpressing constructs. H1-specific B cells incubated with fluorescently-labelled H1 protein on ice retained staining at the cell surface, consistent with H1-binding to surface BCR, whereas incubation at 37 °C resulted in an accumulation of the staining inside of cells, consistent with internalization (Fig. 6a). H1-specific B cells expressing high levels of BOB.1 internalize the H1 antigen slower than their control counterparts, as measured by the differences in the frequencies of cells containing the labelled antigen in the cytoplasm (Fig. 6b left panel) as opposed to the one on the membrane (Fig. 6b right panel). In sharp contrast, silencing of BOB.1 expression with a specific short hairpin (sh)RNA resulted in the increased rate of the internalization of H1 protein as compared to the cells transfected with the scrambled shRNA (Fig. 6). As BCR signalling and internalization of antigen are mutually exclusive events [64], we assessed the impact of BOB.1 overexpression on calcium (Ca2+) flux upon BCR stimulation. Addition of IgM/IgG F (ab)2 antibodies to Indo-1-labelled B cells induced a rapid shift in the ratio of bound versus unbound Indo-1 in cells, indicating that BCR cross-linking induce Ca2+ flux. Comparing BOB.1 overexpressing B cells to the control, we observed a significant increase in Ca^{2+} responses in the cells expressing high levels of BOB.1 (2.4 ± 0.5, mean ± SD ratio of peak/baseline Ca^{2+} versus 1.7 ± 0.4, $p = 0.0013$) (Fig. 6d). However, in contrast, no differences in Ca^{2+} flux after BCR crosslinking were seen between B cells with BOB.1 knockdown and controls transfected with the scrambled RNA (data not shown), in line with the observation that suppression of BOB.1 does not affect the formation of plasmablasts (Fig. 4). Furthermore, the

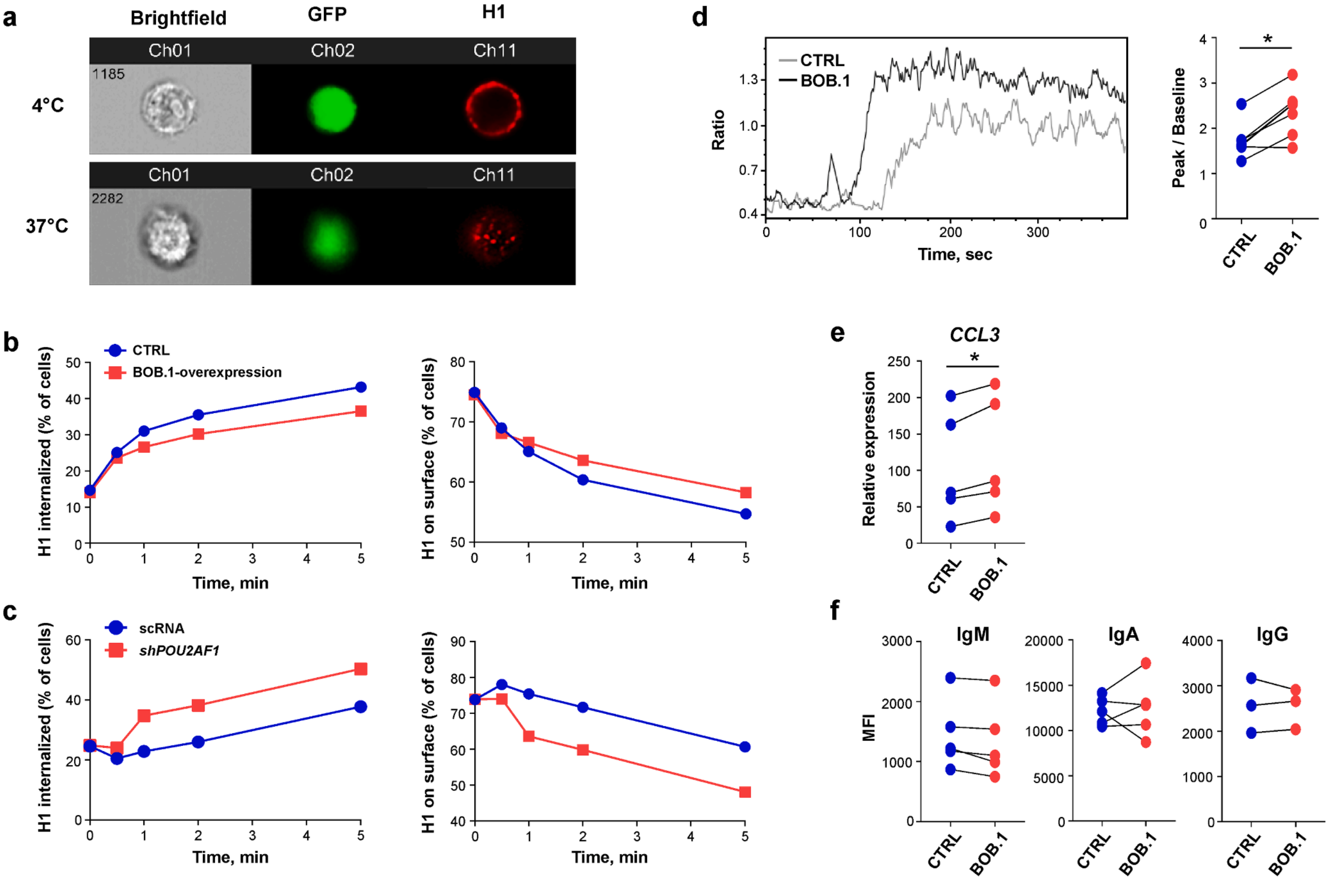

Fig. 6. BOB.1 expression levels modulate BCR internalization and strength. (a) Influenza H1-specific B cells capable of binding (at 4 °C) and internalizing (at 37 °C) intact AlexaFluor 594-conjugated H1 antigen transduced with BOB.1-overexpressing construct shows a decreased capacity to internalize the H1 antigen as compared to the control transduced cells (b). The same H1-specific B cell clone being transduced with BOB.1 shRNA is capable to internalize H1 antigen faster as compared to the scrambled control (c). (d) Ca^{2+} flux measured by FACS and the quantification of the height of the Ca^{2+} flux peak divided by the baseline signal demonstrated an increased Ca^{2+} flux in human $CD27^+$ B cells expressing high levels of BOB.1 as compared to CTRL cells; (e) an increased BCR-mediated calcium signalling in B cells expressing high levels of BOB.1 was mirrored with an increase in the expression of MIP-1 alpha as assessed by qPCR analysis; (f) overexpression of BOB1 does not affect surface expression of IgM, IgA and IgG immunoglobulins. $^*P < 0.05$.

increased BCR-mediated calcium signalling in B cells expressing high levels of BOB.1 was mirrored by an increase in the expression of MIP-1 alpha (*CCL3*) a chemokine, which is expressed in a calcium-dependent manner [65,66] (Fig. 6e). The observed increase of Ca^{2+} influx in BOB.1-overexpressed cells was not related to increased expression of the surface BCR in these cells (Fig. 6f). Altogether, these results indicate an important effect of BOB.1 expression level on the two crucial functions of BCR upon interaction with an antigen: antigen internalization and signalling.

4. Discussion

Whereas generation and maintenance of broadly-reactive Bmem are crucial for sustaining the capacity for comprehensive protection of the host, it remains poorly understood which molecular switches regulate this cell-fate decision in the GC reaction. In the present study we identified a novel role for BOB.1 in regulating the cell-fate outcomes during T cell-dependent (TD) antibody responses. So far, the major insights into the function of BOB.1 during immune responses came from a series of targeted gene-disruption studies in mice. Since central defects of BOB.1-deficient mice are a lack of GC formation in secondary lymphoid organs and a severely reduced immune response to TD antigens, accompanied by the profound reduction of secondary Ig isotypes [39–41], the function of BOB.1 was canonically associated with GC maturation and plasma cell differentiation. However, the interpretation of the BOB.1 knockout phenotype is rather complex since a complete absence of functional BOB.1 creates an artificial situation in these animals, which precludes the elucidation of the molecular function of BOB.1 during TD antibody responses. First of all, BOB.1 is expressed preferentially in GC B cells [54,67]. Therefore, complete loss of GCs, sites of cognate B cell:T cell interaction and expansion, upon BOB.1 deletion, is obviously responsible for the lack of high titres of switched antibody isotypes and memory responses in BOB.1-deficient animals. Furthermore, it has been demonstrated that BOB.1 is critical for the efficient transcription of immunoglobulin heavy-chain gene [68] and for V(D)J recombination of a subset of immunoglobulin kappa genes [69], thereby BOB.1 deletion impacts the IgVκ repertoire [70]. Moreover, albeit the first identified functions of BOB.1 were observed in the periphery, several lines of evidence suggest that this regulatory factor plays a significant role at early stages of B cell development in bone marrow [41,71–74]. Such B cell-intrinsic defects arising from the absence of BOB.1 might be accountable for the absence of GC formation and impaired production of switched *Ig* genes *in vivo* [39–41] and *in vitro* [75]. In addition, it has been shown that even though the loss of BOB.1 severely restricted the capacity of cells to differentiate to plasma cells, it was not absolutely required for the plasma cell differentiation as plasma cells were present even in the absence of BOB.1 and the plasma cell differentiation program of gene expression was normal in these cells [75]. Thus, overall, given the high expression levels in GC B cells [42,54,67], it remains unclear how exactly BOB.1 exerts its functions during GC reaction.

Over the past years we accumulated evidence that BOB.1 is an important molecular switch controlling pathogenic humoral responses in the RA joint. We have demonstrated that expression of BOB.1 is upregulated in ectopic "GC-like" structures in RA synovial tissue [42]. However, the question remained open as to whether such an increase arises from the differences in the cellular composition between RA and inflammation-matched non-RA control synovium or rather by different expression of BOB.1 by identical "GC"-like B cell subsets in RA and controls. Therefore, in the present study we evaluated whether the expression of BOB.1 is also different in the *bona fide* secondary lymphoid tissue between autoimmune and normal GC reaction by analysing the LN compartment of patients with RA and healthy controls. We demonstrated increased BOB.1 expression in the peripheral lymphoid tissue of patients with RA, although expression of GC-related marker BCL6 was comparable to control (Fig. 1). Since draining lymph nodes are a candidate compartment for the maintenance of peripheral tolerance and primary site for the generation of autoimmune responses [76], the increase in BOB.1 expression in LNs of patients with RA, further supports its role in the pathogenesis of this autoimmune disorder.

In the attempt to understand how aberrant expression of BOB.1 would affect B cell phenotype and function during TD immune responses we investigated the effect of its forced overexpression and knockdown in primary human B cells *in vitro* under conditions that mimic some key aspects of the GC reaction such as activation with CD40 ligand in the presence of IL-21. We found that the dynamics of expression of BOB.1 in activated B cells regulate cell-fate outcomes during TD responses: a) physiologic levels of BOB.1 in B cells drive plasmablasts differentiation; 2) high levels of BOB.1 promote Bmem differentiation; 3) low levels of BOB.1 are adequate for plasmablasts differentiation but not sufficient for formation of Bmem (Fig. 7). The concept that the expression magnitude of transcription factors orchestrates distinct cell-fate transition has been documented previously for IFR4 and BACH2 [37,77].

Several lines of observations support these findings. Firstly, human B cells differentiate into antibody-producing CD20^{low}CD38^{++} plasmablasts in response to IL-21/CD40L co-stimulation *in vitro* [51,52,78] as addition of IL-21 suppresses the differentiation of B cells towards Bmem and promotes their differentiation towards LLPCs [79]. This process is accompanied by an increase in endogenous BOB.1 expression in response to CD40-induced signalling (Fig. 2b) in accordance with previous report [67]. Secondly, albeit overexpression of BOB.1 does not impact survival, viability, or proliferative capacities of B cells, the IL-21/CD40L-induced formation of CD20^{low}CD38^{++} plasmablasts was inhibited, suggesting that high levels of BOB.1 interfere with this cell-fate. Accordingly, BOB.1-overexpressing cells secreted less isotype-switched immunoglobulin G but not un-switched immunoglobulin M, indicating that high levels of BOB.1 inhibit the formation of high-affinity, class-switched antibody-forming cells, consistent with reduced expression of AID and in line with previously published data that BOB.1 is crucial for TD but not for TI antibody production *in vitro* [75]. Since similar yet more pronounced inhibition of terminal B cell differentiation *in vitro* is observed during ectopic expression of BCL6 in B cells [56,57] and since both, BOB.1 and BCL6 are key regulators for GC initiation [80] and since BOB.1 has been demonstrated to regulate B-cell receptor (BCR)-mediated signals in marginal zone B cells [74], we initially hypothesised that BOB.1 and BCL6 factors operate in the same pathway driving GC B cell phenotype, therefore elevated expression of BOB.1 would skew B cells towards a "GC"-like cells. Somewhat unexpectedly, our data did not support this hypothesis as forced BOB.1 overexpression in B cells, in contrast to forced BCL6 overexpression, induced neither PAX5-mediated repression of BLIMP1 essential for the induction of GC B cell program nor the expression of GC-specific markers. Moreover, our results revealed that BOB.1 and BCL6 function to counter-regulate each other's expression, suggesting that high levels of BOB.1 in B cells during TD responses drive a cell fate which is distinct from GC B cell and plasma cell. Indeed, further analysis revealed that B cells expressing high levels of BOB.1 display phenotypic and functional

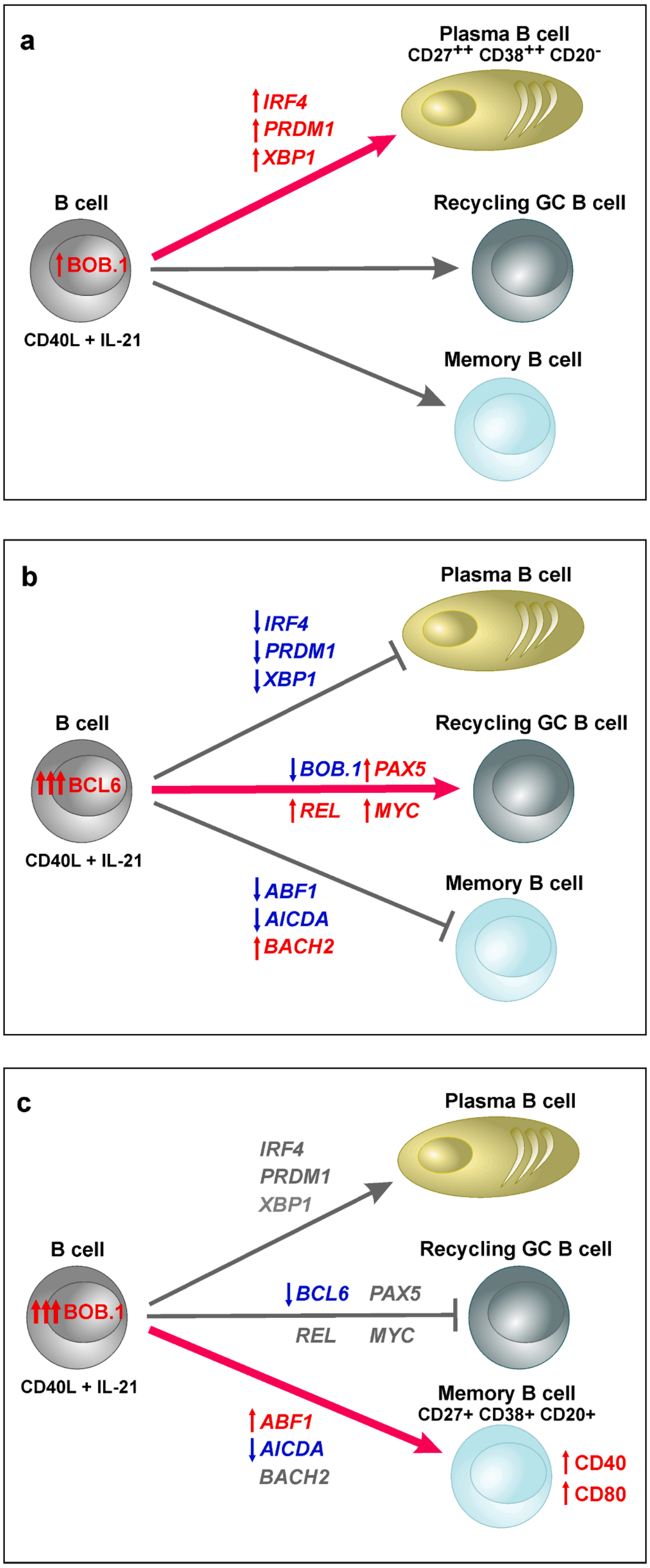

Fig. 7. A model for the impact of the BOB.1 protein levels on the cell-fate decision in GC during T-cell dependent immune responses. (a) B cells stimulated with CD40L and IL-21 upregulates expression of IRF4, PRDM1, XBP1 and BOB.1 resulting in PC differentiation; (b) overexpression of BCL6 induces GC-related transcription factors PAX5, REL and MYC resulting in the inhibition of PC differentiation and induction of GC differentiation; (c) aberrantly elevated BOB.1 protein levels suppress BCL6 and induce ABF1 leading to Bmem formation. Tick red line indicates a preferred B cell fate decision in each scenario.

properties of "memory"-like B cells compared with control (plasmablasts) cells when cultured in the presence of IL-21 and CD40L. These cells are categorized by a number of features, previously described as characteristics of Bmem: 1) an increased CD20 and a decreased CD27 and CD38 expression, resulting in $CD38^-CD27^+CD20^+$ phenotype consistent with Bmem; 2) the augmented expression of transcription factor ABF1, which has been shown to advance Bmem formation [36]; 3) an elevated expression of CD25, CD80 and CD40 molecules [58,62,81–86]; 4) an enhanced costimulatory capacities resulting in the induction of antigen-specific $CD4^+$ T cell proliferation in *vitro*; 5) decreased BCR affinity [4,19]; 6) an increased BCR signal strength [87]. The last point is of special interest as several studies have suggested that BCR signal strength is a major regulator of lineage decision [19,88,89]. Finally, and perhaps most importantly, the severely reduced expression of BOB.1 in B cells suppressed their capacity to differentiate to $CD20^+CD27^+$ memory B cells but permitted plasmablasts differentiation during TD responses. Interestingly, an aberrant overexpression of BOB.1 in B cells associated with repression in plasma cell differentiation and an increase in "memory"-like B cell pool is observed in patients with Waldenström's Macroglobulinemia [90].

This study has several limitations. First of all, as mentioned above, we noticed that CD40 ligation induces high expression of endogenous BOB.1 in human B cells (Fig. 2b), in accordance with data published earlier for mice B cells [54,67,91]. This has resulted in a narrow window for the comparison; however, we were still able to detect significant differences between BOB.1-overexpressing cells and control cells expressing high levels of endogenous BOB.1. Next, we observed that overexpression of BOB.1 in B cells suppresses BCL6 and induces expression of ABF1, yet we did not find any effects of aberrantly-induced BOB.1 on other key transcription regulators of GC or plasma cells differentiation, including SPI-B, which it has been reported to be a direct target of BOB.1 [92]. Particularly it is surprising that we do not see any effect on the Blimp1-encoding *PRDM1* gene expression despite a clear difference in their phenotype between BOB.1-overexpressing and control cells. Therefore, we propose that these selection events might be regulated via BCL6/ABF-1 expression ratio. Yet, further studies are needed to elucidate the exact molecular mechanisms underlying cell-fate choice made by B cells during TD GC reaction in response to different concentrations of BOB.1. In this study, we observed a trend towards increased expression of PD-L1 and PD-L2 on BOB.1-overexpressing cells (Fig. 5a). Although the function of both PD-1 ligands in cells expressing high BOB.1 levels remains to be addressed, the observed phenotype is consistent with the suppression of BCL6, a key negative regulator of PD-L1 and PD-L2 expression [93]. Lastly, in our study we mimicked TD immune responses by culturing B cells with CD40L-L cells and IL-21; however, we still lack the tools to create an *in vitro* system to precisely recapitulate genuine lymphoid structures, complex tissues comprising spatially-organized B and T cell areas, specialized populations of dendritic cells, well-differentiated stromal cells and high endothelial venules. Therefore rigorous *in vivo* or *ex vivo* systems are absolutely necessary for validation of the data obtained in this *in vitro* setup. For this aim, we have developed a mouse model with inducible overexpression of BOB.1 targeted specifically to B cells. Based on the current data we predict that cell-fate outcome in GCs of these mice will be skewed towards greater Bmem over LLPC formation after TD immunisation.

Importantly, we found that expression of BOB.1 is aberrantly increased in the lymph nodes of patients with RA. Considering the essential role of Bmem cells in the pathogenesis of RA it is tempting to speculate that BOB.1 dysregulation may contribute to the imbalance between Bmem and LLPC, leading to pathologic accumulation of Bmem, loss of self-tolerance and the development of autoimmune diseases in susceptible individuals. Further work is required to evaluate the causative link between increased levels of BOB.1, its role during TD GC responses and the pathogenesis of RA.

5. Conclusions

The aberrantly expressed in the lymph nodes of RA patients transcription regulator BOB.1 has a crucial role in determining the B cell-fate decision. High BOB.1 levels promote the generation of cells with phenotypic and functional characteristics of Bmem. Mechanistically, overexpression of BOB.1 drives ABF1 and suppresses BCL6, favouring Bmem over LLPC or recycling GCBC. Low levels of BOB.1 are sufficient for LLPC but not for Bmem differentiation.

Author contributions

M.J.L., D.L.P.B., N.G.Y. designed and analysed all the experiments, C.M.F., E.G.M.vB., N.O.P.vU., K.G., T.G.O'T., I.C.J.B., J.F.S., A.Q.B. were involved in performing and analyzing multiple experiments; H.S., S.B., A.T., M.K. gave consultation for experiment design; M.J.L., D.L.P.B., N.G.Y. wrote the paper with input from all co-authors.

Disclosure

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